Molecular physiological responses and acclimation of the seagrass species *Z. muelleri* to light limitation

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The Thesis presented meets the standards and requirements set out by the University of Technology Sydney

Certificate of original ownership

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the doctoral degree and/or fully acknowledged within the text. I also certify that this thesis has been written by me (Peter A. Davey). Any help that I have received in my research work and the preparation of this thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in this thesis.

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Preface

The Chapters within this PhD thesis have been written with the intention of submission to scientific journals. The chapters are therefore presented in a typical journal format, ready for submission. Chapter 1 has already been published in a scientific journal (Functional and Integrative Genomics; IF = 2.265) as a critical literature review. Chapters 4 and 5 will be submitted in the near future to scientific journals as original research articles. Scientific work, which I have contributed to, is listed in Appendix 4, one of these pieces of work has been published in another journal (Frontiers in Plant Science; IF =4.495), whilst the other piece of work (a book chapter) is in preparation. Given that this thesis is presented as a series of ready to submit manuscripts, there is an element of repetition in the introduction of some of the chapters.

A foreword

"Look deep into nature, and then you will understand everything better" - Albert Einstein

Personally for me, the above quote sums up my PhD journey over the past three and a half years. Only through bioinformatics analyses, did true meaning come from the observations I made in the laboratory at UTS. To unravel the complexity of one organism over three and a half years has been a huge accomplishment for me, one that I have immenseley enjoyed; however, with the satisfaction came the challenge, one that I found testing at times. By undertaking this PhD, I feel that I have come a long way, learning about myself, and seagrasses in many ways. A journey, which gave me appreciation for how complex nature can be. As the saying goes - "There's more than meets the eye."

General Abbreviations A to Z

2D-IEF	Two Dimensional- Isoelectric Focusing
ABA	Abscisic Acid
AFLP	Amplified Fragment Length Polymorphism
AL-fact	Actinic Light Factor
AMSA	Australian Marine Sciences Association
ANU	Australian National University
ATP	Adenosine 5'-triphosphate
AU	Arbitrary Units
BBH	Bidirectional Best BLAST Hit
BCV	Biological Coefficient of Variation
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BP	Biological Process
BUSCO	Benchmarking Universal Single-Copy Orthologs
BWA	Burrows-Wheeler Aligner
C3	C3 photosynthesis / Climate Change Cluster
C4	C4 photosynthesis
CA	Carbonic Anhydrase
CAP3	Contig Assembly Program 3
CC	Cellular Component
CCM	Carbon Concentrating Mechanism
cDNA	complementary Deoxyribonucleic Acid
CEGMA	Core Eukaryotic Genes Mapping Approach
CHIP-Seq	Chromatin Immunoprecipitation-Sequencing
chl	Chlorophyll
Ci	Inorganic Carbon
CPM	Counts Per Million
CRBB	Conditional Reciprocal Best BLAST
Ct	Cycle threshold
Cyt.b6f	Cytochrome b6f
ddRAD	double digest Restriction Associated DNA
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DOGMA	Dual Organellar GenoMe Annotator
DVC A	Divinyl chlorophyllide A
EBI	European Bioinformatics Institute
EMBL	European Molecular Biology Laboratory
EST	Expressed Sequence Tag
ETR	Electron Transport Rate
FC	Fold Change
FDM-SSR	Functional Domain Marker - Simple Sequence Repeat
FDR	False Discovery Rate

FPKM	Fragments Per Kilobase of transcript per Million mapped reads
Fv/Fm	Maximum quantum yield of photosystem II
G3P	Glyceraldehyde 3-phosphate
GABA	Gamma-aminobutyric acid
GBS	Genotype By Sequencing
GC-MS	Gas Chromatography Mass Spectrometry
gDNA	genomic Deoxyribonucleic Acid
GGPP	Geranylgeranyl diphosphate
GLM	Generalized Linear Model
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HPC	High Performance Computing
HPLC	High Performance Liquid Chromatography
Ik	Half-saturation constant
ISBW	International Seagrass Biology Workshop
KAAS	KEGG Automatic Annotation Server
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
LHC	Light Harvesting Complex
LHCI / LHCA	Photosystem I Light Harvesting Complex
LHCII/ LHCB	Photosystem II Light Harvesting Complex
LL	Light Limited
MDS	Multi-Dimensional Scaling
MF	Molecular Function
Meas. Int	Measuring Intensity
MEME	Multiple EM for Motif Elicitation
MEP	2-C-methyl-D-erythritol 4-phosphate
MIQE	Minimum Information for Publication of Quantitative. Real-Time PCR Experiments
miRNAs	micro Ribonucleic Acid
MISA	MIcroSAtellite identification tool
mRNA-Seq	Messenger RNA-Sequencing
MTDB	Mangrove Transcriptome Database
mya	million years ago
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH / NADP(H)	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
ncRNAs	non-coding Ribonucleic Acids
NGS	Next Generation Sequencing
No-RT	No Reverse Transcriptase
NPQ	Non-Photochemical Quenching
NRQ	Normalised Relative Quantity
NSW	New South Wales

NZGL	New Zealand Genomics Limited
ORF	Open Reading Frame
P-I	Photosynthesis - Irradiance
PAM	Pulse Amplitude Modulated
PC	Plastocyanin
PCR	Polymerase Chain Reaction
PDA	Photodiode Array Detector
PEPC	Phosphoenolpyruvate carboxylase
PhiX	Enterobacteria Phage PhiX174
polyA	Polyadenylated
P(MC)	Probability (Multiple Correction)
PPOs	Polyphenol Oxidases
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
psu	Practical salinity units
PTFE	Polytetrafluoroethylene
qPCR	Quantitative Polymerase Chain Reaction
QC	Quality Check
QTL	Quantitative Trait Loci
RAPD	Random Amplification of Polymorphic DNA
RBH	Reciprocal Best BLAST Hit
rETRmax	relative maximum Electron Transport Rate
RFLP	Random Fragment Length Polymorphism
RIN	RNA Integrity Number
RLC	Rapid Light Curve
RNA	Ribonucleic Acid
RNA-Seq	RNA-Sequencing
ROI	Reactive Oxygen Intermediate
ROS	Reactive Oxygen Species
RPKM	Reads Per Kilobase of transcript per Million mapped reads
RSEM	RNA-Seq by Expectation-Maximization
RT-qPCR	Real Time-quantiative Polymerase Chain Reaction
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RubP	Ribulose-1,5-bisphosphate
SAM	Sequence Alignment Map
Sat. Width	Saturation Width
Sat.Int	Saturation Intensity
SNP	Single Nucleotide Polymorphism
sRNA	small Ribonucleic Acid
SRP	Signal Recognition Particle
SSL	Super Saturating Light
SSR	Simple Sequenc Repeat

STAR	Spliced Transcripts Alignment to a Reference
TAIR	The Arabidopsis Information Resource
TBAA	tert-Butyl acetoacetate
TCA	Tricarboxylic Acid
TF	Transcription Factor
Tm	Melting temperature
TMM	Trimmed Mean of M-Values
TPM	Transcripts Per Million
UNIX	UNIX programming environment
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet
Yi	Initial quantum yield of photosystem II

List of figures

List of tables **Pg.**

CONTENTS

Thesis summary

Understanding how a keystone marine species responds to its extrinsic environment is important to ensure adequate conservation measures are in place, especially with increasing reports of climate change and anthropogenic disturbance events. For the Southern Hemisphere seagrass, *Zostera muelleri*, this scenario is no different. This keystone species is native to Australia and New Zealand, providing many socio-economic benefits to the coastal zone. Over the past few decades, a reduction in water quality (light limitation) has led to numerous reports of *Z. muelleri* meadow loss in Australia and New Zealand. Although seagrass biologists have a firm understanding of the physiological, morphological and ecological changes within light limited *Z. muelleri* meadows, no current knowledge exists on how *Z. muelleri* responds to light limitation at the transcriptional level. By investigating transcriptional regulation, new knowledge was obtained on how this species responds to light limitation, allowing for more appropriate conservation measures. Encompassing the advances in RNA-Seq, this project has examined how *Z. muelleri* responds to light limitation over a 14-day period, through transcriptional regulation, photobiology and physiology, both at the nuclear and chloroplastic level. Main findings indicate that important regulational shifts occur in genes associated with photosynthesis, photo-pigments, carbon metabolism, reactive oxygen species (ROS) homeostasis and secondary defence metabolism. Both nuclear and chloroplast encoded genes involved in photosynthetic processes have been shown to be correlated with downstream changes in photophysiology, and thus are both crucial for the response as well as the acclimation to light limitation. This research also compared genome-guided transcriptome assembly versus *de novo* assembly, indicating the superiority of genome-guided protocols when a genome is available. Whilst this PhD thesis offers a new level of knowledge to seagrass biologists, it also provides candidate molecular markers, which can be used in future monitoring efforts and population genetic studies.

PhD thesis aims and objectives

The overall aim of this thesis is to investigate how *Zostera muelleri* responds to light limitation using a multi-disciplinary approach. By combining mRNA-seq and RT-qPCR protocols with already established photobiology and pigment profiling techniques, we will not only obtain a new level of understanding on how this seagrass species responds at the transcriptional level, but also how transcriptional regulation is linked to downstream changes in photophysiology. Such work is timely, given that seagrasses are increasingly threatened by light limitation within the coastal environment.

Objectives

- To examine and provide a critical literature review on the current state of molecular profiling and omics techniques in seagrass biology, whilst identifying key knowledge gaps in previous and current research.

- To address the background knowledge associated with light perception in higher plants, seagrasses and *Zostera muelleri*: fundamental knowledge and further direction for research will be discussed.

- To complete *in silico* characterisation and data mining of the *Z. muelleri de novo* transcriptome, based on whole plant tissue.

- To characterise leaf tissue-specific responses of *Z. muelleri* to light limitation; to establish links between transcriptional regulation of nuclear-encoded genes and downstream photophysiology.

- To investigate the expression of chloroplast-encoded photosynthetic genes in *Z. muelleri* in response to light limitation. To designate suitable reference genes and link chloroplastic-encoded gene expression with downstream photobiology.

CHAPTER 1

The emergence of molecular profiling and omics techniques in seagrass biology; furthering our understanding of seagrasses

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Abstract

Seagrass meadows are disappearing at alarming rates as a result of increasing coastal development and climate change. The emergence of omics and molecular profiling techniques in seagrass research is timely, providing a new opportunity to address such global issues. Whilst these applications have transformed terrestrial plant research, they have only emerged in seagrass research within the past decade; we have observed a significant increase in the number of publications in this nascent field, and as of this year the first genome of a seagrass species has been sequenced. In this review, we focus on the development of omics and molecular profiling and the utilisation of molecular markers in the field of seagrass biology. We highlight the advances, merits and pitfalls associated with such technology, and importantly we identify and address the knowledge gaps, which to this day prevent us from understanding seagrasses in a holistic manner. By utilising the powers of omics and molecular profiling technologies in integrated strategies, we will gain a better understanding of how these unique plants function at the molecular level and how they respond to on-going disturbance and climate change events.

1.1 Introduction

Seagrass meadows play major roles in the promotion and protection of coastal biodiversity, as well as nutrient recycling and coastal protection (Orth et al., 2006; Cristianen et al., 2013). Equally important, their carbon sequestration capacity dwarfs that of boreal, temperate and tropical forests (McLeod et al., 2011). It has been estimated that the total productivity of seagrass meadows is approximately $29,000 US dollars per hectare per year, which is considerably more than that of terrestrial forests, grasslands and open ocean productivity (Costanza et al., 2014).

A recent meta-analysis has suggested that we are loosing a staggering 7% of global seagrass meadow coverage per year (Waycott et al., 2009), a figure that is likely to increase in future due to mounting anthropogenic and climate change pressures (Orth et al., 2006; Ralph et al., 2007; Björk et al., 2008; Waycott et al., 2009). Given the wide range of threats that have been identified for seagrass meadows (Orth et al., 2006; Björk et al., 2008; Waycott et al., 2009), it is of major concern that we still lack fundamental knowledge about the molecular biology of these plants and how they will respond to future climates. In comparison to our molecular knowledge of terrestrial plants, our understanding of seagrass molecular biology is somewhat in its infancy.

Sequencing technologies in plants have rapidly developed since the genome sequencing of the model plant *Arabidopsis thaliana* (Kaul et al., 2000). As of 2013, genomes had been sequenced for 49 plant species (Michael and Jackson, 2013). Whilst many important crop species have already had their genome sequenced (Yu et al., 2002; Jallion et al., 2007; Paterson et al., 2009; Schnable et al., 2009; Schmutz et al., 2010; PGSC, 2011; Chalhoub et al., 2014; Mayer et al., 2014), it has only been of this year that the first genome of a seagrass (*Zostera marina)* has been completely sequenced (Olsen et al., 2016); it is therefore expected that we will observe increased research activity in this niche area. Whilst deciphering of the genome is invaluable, the insights offered by *de novo* transcriptomics, proteomics and metabolomics are also of high value. The *1k plant transcriptome project* by the iPlant Collaborative is one such example which has taken advantage of transcriptome sequencing. For seagrasses, several studies have made use of transcriptomics to date (Gu et al., 2012; Franssen et al., 2014; Kong et al., 2014; Olsen et al., 2016). The importance of molecular profiling and omics in plant science not only offers opportunities for bio-prospecting (Annadurai et al., 2012), but also for exploring the fundamental genetic mechanisms of plants

(Mochida and Shinozaki, 2011) and projecting how species will respond to disturbance and climate change events (Ahuja et al., 2010). In this review, we discuss the current role of omics, molecular profiling and the use of genetic markers in the field of seagrass biology and how they have and will further help us to understand seagrasses in a more holistic manner. Furthermore, such information will help us to understand how seagrasses will respond to future climatic and disturbance events. We also highlight the merits and pitfalls of such techniques, and the knowledge gaps, which currently exist in seagrass biology.

1.2 Seagrasses: A unique group of plants

Seagrasses are a polyphyletic group of marine plants belonging to the monocotolydonous lineage of the angiosperms. Seventy-two species are classified within 6 families; Cymodoceaceae, Hydrocharitaceae, Posidonia, Ruppiaceae, Zannichelliaceae and Zosteraceae (Short et al., 2011). It should be noted that much debate is still associated with seagrass phylogenetics and taxonomy (Papenbrock, 2012). Seagrasses evolved ca. 100 million years ago (mya) during the Cretaceous period (den Hartog, 1970); recent evolutionary analysis for *Z. marina* indicates this species underwent a whole genome duplication event approximately 72-64 mya, but diverged from the monocot genera, Spirodela approximately 135-107 mya (Olsen et al., 2016). The seagrasses have feasibly experienced the most extreme evolutionary events witnessed in the angiosperm lineage (Olsen et al., 2016); they have evolved unique features to cope with survival in a saline, CO_2-limited and dynamically changing marine environment due to tidal oscillations which change light availability, water flow and temperature. Fig. 1 highlights the common specialised adaptive traits of seagrasses. For more detail on such specialisations please refer to available literature (Ackerman, 2006; den Hartog and Kuo, 2006; Larkum, 2006; Marbà et al., 2006; Touchette, 2007).

1.3 The current status of omics and molecular profiling in seagrass biology

Omic and molecular profiling studies have provided seagrass biologists a revolutionary approach to how seagrasses can be studied. The emergence of these approaches in seagrass biology has been relatively slow in comparison to terrestrial plants. To the best of our knowledge 31 research-based studies (excluding reviews and

editorial notes) have been published since 2006, which integrate such approaches (Table 1, Fig. 2). In such a short period of time, these studies have presented us with novel information on evolution, stress response, resilience and variation within and between the species studied. Studies have given us an insight into how seagrasses and land plants are similar but also dissimilar at the molecular level. Such advances are; however, majorly limited to only two species, *Z. marina* and *P. oceanica* (Table 1). These two species are geographically distributed in the Northern Hemisphere, and from a critical perspective, a wider range of global seagrass species need sequenced, especially now that technology cost has depreciated and technology has become readily accessible. Much of the current focus has been on thermal response, whilst some attention has been emphasised on light response; as such a broader approach is needed in seagrass omics, taking other important anthropogenic and climatic stressors into account. Noteworthy, in this respect we have recently observed the examination of seagrass species including the Southern Hemisphere species, *Zostera muelleri,* and the species *Cymodocea nodosa* (Table 1).

Transcriptome studies which have been completed in seagrasses to date (Table 1) have provided us with snapshots of gene expression at given times under specific conditions in species. The majority of these studies have focussed on short-term response, rather than recovery and resilience over longer periods of time. Franssen et al. (2014); however, provide a good example of an environmental response and recovery study. Transcriptomics is of course highly valuable, but without doubt deep genomic sequencing can provide more information on coding sequences as well as non-coding sequences. Such information is important for the advances of understanding genomic structure, function and evolution. Least to say, epigenetics is one area of seagrass omics that has failed to receive much attention to date (Table 1). Transposable elements, micro-RNAs (Lorenzetti et al., 2016), sRNAs, ncRNAs and other non-coding genic elements can help us to understand how coding regions of the genome are controlled and expressed under different environments, as previously shown in grape vine (Singh et al., 2012) and rice (Zhang et al., 2016). The genome of *Z. marina* and genome-wide analysis of *P. oceanica* provide details of non-coding regions and miRNAs within seagrass genomes (Barghini et al., 2015; Olsen et al., 2016); and as such this information will be most valuable for future epigenetic research in seagrass. It goes to mention, CHIP-Seq has yet to emerge in seagrass research; with the design of suitable

antibodies and utilisation of suitable methodology, epigenetic regulation such as histone modification can be effectively studied (Shin et al., 2012). In terms of genome complexity, the size of the *Zostera muelleri* genome has been estimated to be ~900 Mbp (Golicz et al., 2015), whilst the *Zostera marina* genome is 202.3 Mbp (Olsen et al., 2016). *P. oceanica* is suggested to exhibit a genome size that is 5 times larger than *Z. marina* (Barghini et al., 2015). Such information reveals the variation between seagrass species at the molecular level, and without doubt makes them an interesting group of plants to study, given that they are all functionally adapted to the marine coastal environment. Examination of the literature; however, reveals that several key knowledge gaps exist.

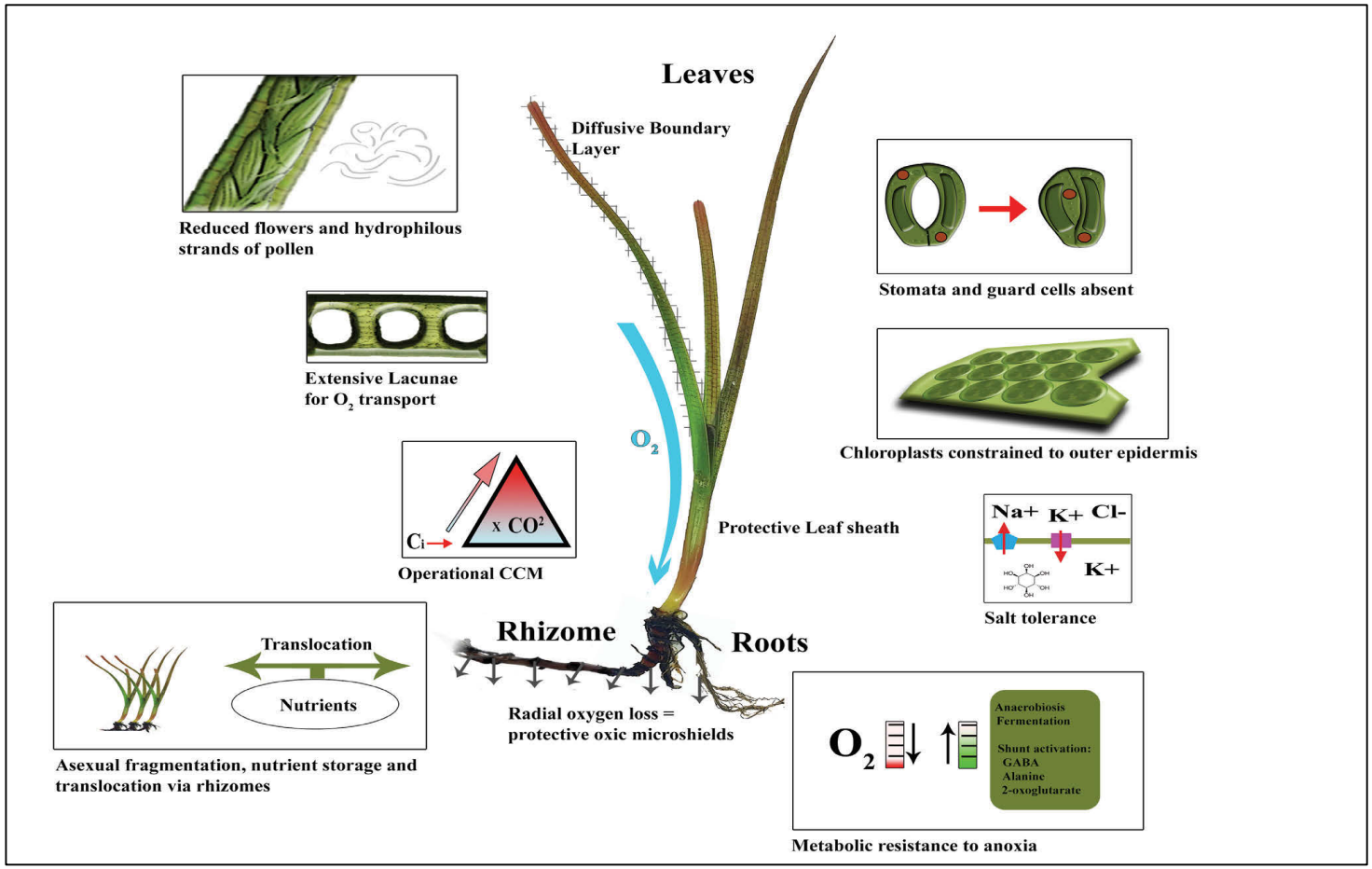

Figure 1: Illustrative diagram collectively showing the specialised traits of seagrasses, which allow for seagrasses to live a submerged life in the coastal marine environment. Information sources: Marbà et al., 2002; Ackerman, 2006; Larkum et al., 2006; Touchette, 2007; Broderson et al., 2015; Hasler-Sheetal et al., 2015; Olsen et al., 2016. Illustrative model concept is based on the *Zostera* genus.

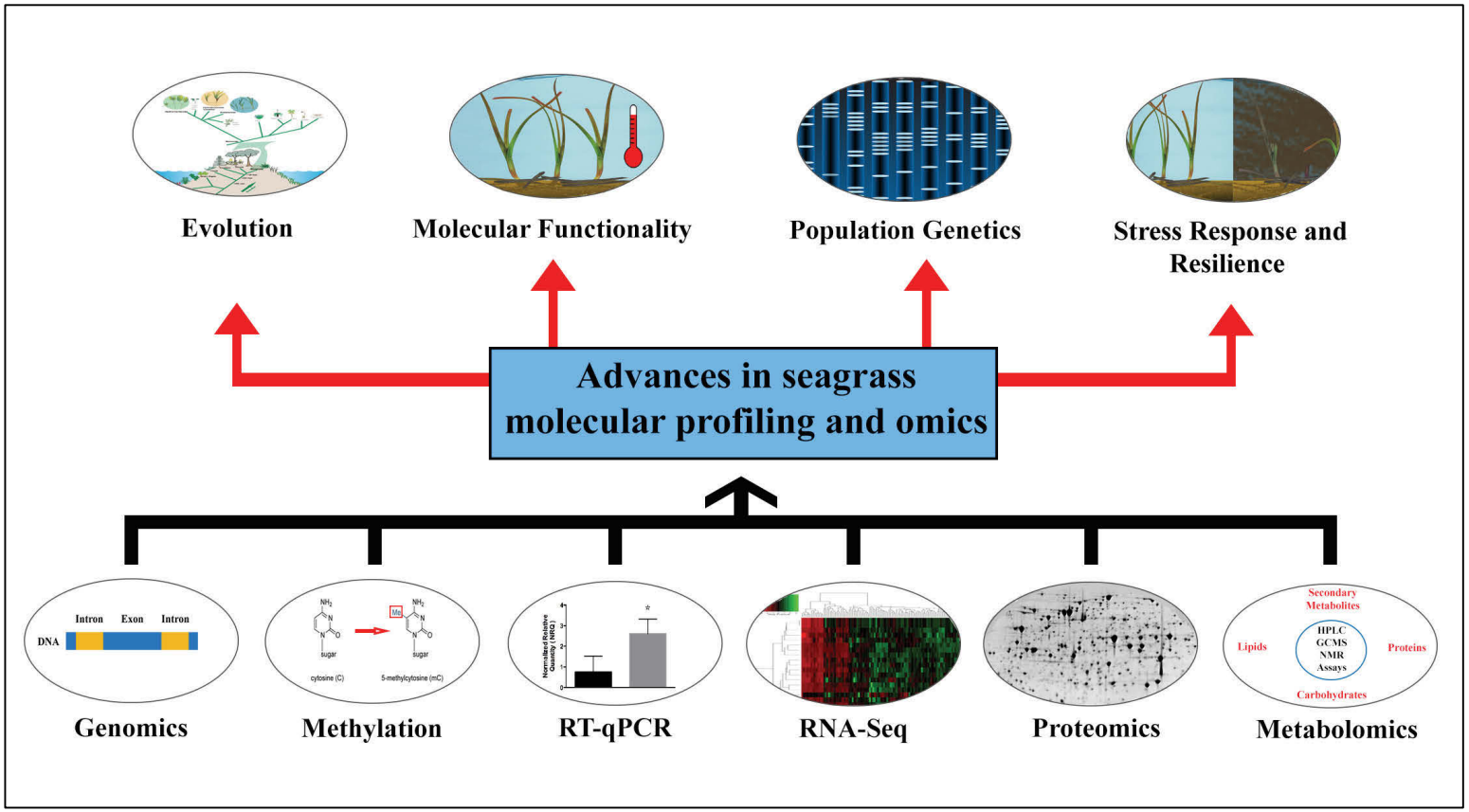

Figure 2: The advances in seagrass molecular profiling and omics to date. The technologies that have been utilised are shown along the bottom of the illustration, whilst types of study conducted to date are shown at the top of the illustration.

1.4 Seagrass light perception and response at the molecular level

Perhaps the biggest threat known to seagrass ecosystems is direct and indirect light limitation (Ralph et al., 2007). In the past, large areas of seagrass die-off have been attributed to light limitation as a result of poor water quality (Ralph et al., 2007). Such threats are predicted to increase with increasing anthropogenic disturbance and climate change. Photo-physiology methods utilising Pulse Amplitude Modulated (PAM) chlorophyll fluorometry have served as the most effective tools for understanding how seagrasses respond and acclimatise to varying light. PAM technology provides us with quantitative measurements (Ralph, 2002; Dattolo et al., 2014) and therefore comprehensive estimations of plant health. To date, only three species of seagrass; *Zostera marina* (Kong et al., 2014), *Zostera muelleri* (Pernice et al., 2015; Schliep et al., 2015) and *Posidonia oceanica* (Mazzuca et al., 2009; Greco et al., 2013; Dattolo et al., 2013, 2014), have been characterised using molecular datasets in relation to varying irradiance. Such studies have long been awaited, as they allow us to characterise how seagrasses use environmental light cues to control regulation and metabolism. The genome has also provided valuable insight into light perception (Olsen et al., 2016).

Dattolo et al's. (2013) *in situ* study on the acclimation of *P. oceanica* to different water depths (i.e light levels) has identified several regulatory networks and pathways involved in response to different depth gradients and thus has provided a host of eco-genomic resources for future studies. Additionally, seagrass plasticity at the functional molecular level is evident in response to varying light. For *P. oceania* such studies are important, given that this species is rapidly disappearing in the Mediterranean (Dattolo et al., 2013). Changes in photosynthesis, cellular energetic metabolism, protein turnover and stress response were most widely observed at the transcript and proteomic level. Indeed, proteolysis and protein turnover have also previously been shown to up-regulate in *P. oceanica* under chronic low light in previous proteomic experiments (Mazzuca et al., 2009). Dattolo et al., (2013) also noted differences in the chlorophyll binding proteins between plants occurring at different depths, suggesting photosystem complexes may re-arrange to cope with the different levels of light irradiance as similarly observed in land plants (Masuda et al., 2003). Additional work by Dattolo et al. (2014) has shown that distinct light associated gene expression is linked to depth distribution. Furthermore, the photosynthetic light harvesting complex B (*LHCB*) genes

Table 1: Known to us, the molecular profiling and omic studies published since the establishment of the seagrass NGS era in 2006. A comprehensive search was conducted on 'Web of science' for the topics 'seagrass sequencing', 'seagrass gene expression', 'seagrass proteomics', 'seagrass metabolomics'. Additional studies based on personal knowledge are also provided. Last accessed: June, 2016.

Species	Study/ Stress Type	Molecular / NGS Application	Author	Year
Z. marina	Thermal	RT-qPCR	Ransbotyn and Reusch.	2006
Z. marina	Thermal	EST	Reusch et al.	2008
P. oceanica	Light	Proteomics	Mazzuca et al.	2009
P. oceanica, Z. marina	Online database repository	Online repository creation	Wissler et al.	2009
Z. marina	Thermal	Genotyping and RT-qPCR	Bergmann et al.	2010
Z. marina	Detection of *L. zosterae* pathogen	qPCR	Bergmann et al.	2011
Z. marina	Thermal	EST, *de novo* assembly, RT-qPCR	Franssen et al.	2011
Z. noltii	Thermal	EST	Massa et al.	2011
Z. marina	Thermal	RT-qPCR	Winters et al.	2011
P. oceanica, Z. marina	Evolution	EST	Wissler et al.	2011
P. oceanica	Cadmium toxicity	DNA methylation	Greco et al.	2012
P. oceanica	Thermal	Transcriptomics and metabolomics	Gu et al.	2012
P. oceanica	Reference genes	RT-qPCR	Serra et al.	2012
P. oceanica	Depth gradient / Light	SSH-EST and Proteomics	Dattolo et al.	2013
P. oceanica	Light	DNA Methylation	Greco et al.	2013
Z. marina	Salinity	EST	Kong et al.	2013
Z. marina	Defence gene modulation	RT-qPCR	Brakel et al.	2014
P. oceanica	Depth gradient / Light	Micro-array and RT-qPCR	Datollo et al.	2014
Z. marina, Z. noltii	Thermal	Genome-wide transcriptome analysis	Franssen et al.	2014
Z. marina	Thermal / Light / Salinity	RNA-Seq *de novo* assembly	Kong et al.	2014
P. oceanica	Analysis of repetitive genome	Genomics approach	Barghini et al.	2015
Z. muelleri	Genome wide characterization	Comparative genomics approach	Golicz et al.	2015
Z. marina	Anoxia	Metabolomics	Hasler-Sheetal et al.	2015
P. oceanica	Reference genes / Volcanic vents	RT-qPCR	Lauritano et al.	2015
Z. muelleri	Dredging stress	RT-qPCR	Pernice et al.	2015
C. nodosa	Salinity	Proteomics	Piro et al.	2015a
P. oceanica	Chloroplast proteomic methods	Proteomics	Piro et al.	2015b
Z. marina	Light	Genotyping and RT-qPCR	Salo et al.	2015
Z. muelleri	Reference genes	RT-qPCR	Schliep et al.	2015
Z. marina	Characterization of *LHC* genes	EST-library and RT-qPCR	Kong et al.	2016
Z. marina	Whole genome characterization	Genome assembly	Olsen et al.	2016

have been found to be more abundant in *Z. marina* than in terrestrial counterparts, thereby presumably enhancing photosynthetic performance at lower irradiances in the water column (Olsen et al., 2016). Kong et al. (2016) have also recently identified light harvesting complex (*LHC*) genes in *Z. marina* suggesting that *LHC* genes are conserved across marine plants and land plants.

The photoreceptor and light-mediated transcription factors in *Z. marina* (Kong et al., 2014; Olsen et al., 2016) have been identified. The most significant difference in *Z. marina* compared to land plants is that only 2 phytochromes (PHYA and PHYB) have been identified, this may suggest that PHYC is absent in seagrasses perhaps due to a submerged lifestyle, given that this receptor has less of a role in red-light detection (Franklin et al., 2003). Additionally, it has also been suggested that *PHYC* plays a role in flowering, which is of course reduced at the genic level in seagrasses (Woods et al., 2014; Olsen et al., 2016) and may therefore be associated with such functional reductions. Similarly, UV light protective *UVR8* genes have been lost completely (Olsen et al., 2016). In *P. oceanica* photoreceptors have also been reported for blue and red wavelengths (Greco et al., 2013); suggesting the importance of these genes in perception of light quality within the water column. Additionally, Kong et al. (2016) have also validated changes in expression of light harvesting complexes in response to spectral shifts. Whilst cited research (Olsen et al., 2016) gives us an idea that seagrasses may rely less on far red: red light, we suggest that further research should investigate how shallow and deep dwelling seagrass species utilise wavelengths of light differently, as key evolutionary differences may exist. Transcripts associated with chlorophyll production, pigment synthesis, binding, and the photo-protective xanthophyll cycles have also been identified (Datollo et al., 2013; Datollo et al., 2014; Kong et al., 2014; Olsen et al., 2016) suggesting that adaptation, acclimation and photo-protection are all logically regulated at the molecular level in seagrasses, and lead to changes observed at the physiological level (Ralph et al., 2002; Sharon et al., 2009). The sequencing of the chloroplast genome of *Zostera marina* (Olsen et al., 2016) will become a valuable resource for understanding light responses. Given the realistic threat of meadow decline in relation to low light, low light related senescence needs to be examined in detail. In recent work (Grandellis et al., 2016) molecular profiling has identified several mechanisms, which play a role in the process of senescence during light starvation in the potato crop. The roles of brassinosteroids in low light response are of interest, as

brassinosteroids have been shown to promote resistance to low light stress in tomato (Cui et al., 2016). These hormones are indeed conserved in seagrasses (Olsen et al., 2016).

The application of gene expression profiling technology, such as RT-qPCR has also played an important role in shaping the molecular research of seagrasses. Pernice et al. (2015) have recently utilised a molecular tool kit to detect dredging-associated stress (light-starvation through increased turbidity) in *Z. muelleri* in the port of Gladstone in Queensland, Australia. It is possible that tool kits like this one can provide a model for the implementation of further molecular-based monitoring efforts. These approaches should; however, be designed carefully and treated with caution as gene expression has been found to be highly variable between genotypes of *Z. marina* in shading and recovery experiments (Salo et al., 2015). As a result, we therefore suggest that ecological consultancy and marine scientists use a combination of chlorophyll fluorometry, physiology and molecular techniques until a further understanding of molecular light responses and the genetic variation within regional meadows is acquired.

1.5 Carbon fixation in seagrasses – challenging old beliefs with new technology

It has long been accepted that seagrasses contain a carbon concentrating mechanism (CCM) to support carbon sequestration. A detailed conceptual diagram of the suggested seagrass CCM is clearly explained and illustrated by Larkum et al. (2006). CCM's are a common adaptation in many autotrophic organisms (Badger and Price, 2003; Raven et al., 2008) with CO_2-limited environments often observed as the driving force behind such selectivity (Raven et al., 2008). Despite comprehensive reviews on seagrass carbon fixation and metabolism (Touchette and Burkholder, 2000; Beer et al., 2002; Larkum et al., 2006), our knowledge of carbon fixation in seagrasses at the molecular level is still poor. Given the emergence of omics, interest seems revived, now that we possess higher resolution capability. In respect to photosynthetic systematics; to classify seagrasses as C3 or C4 photosynthetic autotrophs remains a challenge in its own right; past studies have observed C3 and C4 carbon signatures present across a range of seagrasses (Andrews and Abel, 1979; Benedict and Scott, 1979; Beer et al., 1980). Of course such conflicting reports are perplexing given that we

know seagrasses lack true Kranz anatomy and bundle sheath cells. A recent analysis of an EST-derived dataset may of course provide subtle clues of evolutionary based pressure occurring within photosynthetic and carbon metabolism pathways in *P. oceanica* and *Z. marina* (Wissler et al., 2011); however, given the size of the dataset, more effort is needed to validate such findings.

Z. marina carbonic anhydrase and boron HCO_3^- transporter genes have also been identified, perhaps providing evidence of the CCM operation (Olsen et al., 2016). RubisCO sub-units have also been shown to be negatively regulated within *P. oceanica* in response to lower levels of light (Mazzuca et al., 2009; Dattolo et al., 2014) while methylation activity of Phosphoenolpyruvate carboxylase (PEPC) is altered during changes in irradiance level (Greco et al., 2013). The previous theory of C3-C4 intermediate photosynthesis existing in seagrass species (Touchette and Burkholder, 2000) remains plausible; however, C4 related enzymes are also known to play roles in anaplerotic reactions within plants (Doubnerová and Ryšlavá, 2011). It is possible C4-type photosynthesis within seagrasses, could operate independently of true Kranz anatomy; however, this is supported by a theory of single-cell C4 photosynthesis (Edwards et al., 2004), which has been shown to operate in the aquatic plant, *Hydrilla verticillata*, a close relative of the *Halophila* genus of seagrass (Bowes et al., 2002; Bowes et al., 2011). We therefore suggest that a range of carbon fixation pathways may exist across the seagrass group until further work elucidates the exact carbon fixation pathways. We believe that omics alone will not unlock the carbon fixation pathway of seagrasses, but perhaps an integrated approach involving omics, microscopy and immuno-localisation techniques is necessary. Such work will allow us to accurately determine seagrass response to predicted CO_2 fluctuations in the future.

1.6 Are stress and environmental response signatures between land and marine plants different?

An obvious difference between land plants and seagrasses is that seagrasses are fully submerged. Our understanding of how plants respond to their environment is growing steadily, but the differences between land plants and seagrasses remain largely elusive. In seagrasses, oxidative stress protective genes have commonly been identified and associated with light response (Dattolo et al., 2013; Salo et al., 2015), immune modulation (Brakel et al. 2014), thermal stress (Franssen et al. 2011; Winters et al.

2011; Franssen et al. 2014), extreme environments (Lauritano et al., 2015). Whilst typical eukaryote Reactive Oxygen Species (ROS) and antioxidant activity has been observed in seagrasses, the catalase gene has been reduced to a single copy in *Z. marina* (possibly due to reduced xylem characteristics of submerged plants), whilst all 3 types of superoxide dismutase remain (Olsen et al., 2016). Another interesting observation is that increased ROS activity has been observed in *P. oceanica* meadows (Dattolo et al., 2013) under low light. Typically, increased activity is characteristic of high irradiance exposure in land plants; however, Dattolo et al., (2013) suggested that seagrasses under low light may be more vulnerable to the extrinsic environment, given that immune functioning is an energy costly process.

Heat Shock Proteins, a group of molecular chaperones involved in stabilising proteins (Rocheta et al. 2014) have also been identified (Reusch et al., 2008; Bergmann et al., 2010; Franssen et al., 2011; Franssen et al., 2014; Lauritano et al., 2015; Massa et al., 2011; Piro et al., 2015a; Salo et al., 2015), whilst molecular chaperones, detoxifying cytochromes and metallothionein-type molecules have been found to play important roles in seagrass adaptability and resilience (Bergmann et al., 2010; Kong et al., 2013; Kong et al., 2014; Lauritano et al., 2015). One metallothionein gene in particular, *MT2L* is found to be highly expressed in *Z. marina* (Olsen et al., 2016). Molecular chaperones aid in promoting correct protein folding, stabilisation and preventing proteins from aggregating during stressful conditions (Hartl et al., 2011), whilst cytochromes and metallothioneins aid in scavenging and detoxification processes (Gautam et al., 2012). Glutathione-related transcripts have been isolated and profiled in *P. oceanica, Z. muelleri* and *Z. marina* (Lauritano et al., 2015; Massa et al., 2011; Pernice et al., 2015; Olsen et al., 2016). Glutathione is an essential molecule used for signalling, detoxification of ROS and Reactive Oxygen Intermediates (ROIs) as well as normal development in plants (Noctor et al., 2011). Transcription factors (TFs) which bind to cis-regulatory elements in the genome and which help regulate transcriptional processes are known to play a wide role in plant development, growth and stress response within plants, in seagrasses transcription factors are present (Kong et al., 2013; Kong et al., 2014; Golicz et al., 2015; Olsen et al., 2016). Additionally, Olsen et al. (2016) have identified and characterised many miRNA families present in *Z. marina,* including those miRNA families that appear to be lost through evolution.

Molecular studies (Dattolo et al., 2013; Kong et al., 2014; Golicz et al., 2015; Olsen et al., 2016) have provided novel insight into the role of hormones and associated transcription regulators in seagrasses. Of particular interest, it has been suggested that the ethylene-signalling pathway has been partially or wholly lost in Z. *muelleri* (Golicz et al., 2015). This study suggested that the loss of the ethylene pathway may be an adaptation to a fully submerged lifestyle, and in additional analysis the authors also failed to detect similar transcripts in EST databases of *Z. noltii* and *Z. marina* species of the Northern Hemisphere, indicating a general phenomenon across seagrass species. Given that ethylene is a volatile gaseous hormone and that seagrasses lack stomata, the partial loss of this hormone pathway in seagrasses is likely to reflect adaptation to a fully submerged life in the marine environment. The *Z. marina* genome has indeed (as of this year) backed such hypothesis and findings, showing that the ethylene pathway is largely reduced in *Zosteraceae* species (Olsen et al., 2016). If silencing of the ethylene pathway has occurred, seagrasses must therefore possess alternative signalling pathways, which are involved in germination, root hair growth and senescence that work independently of ethylene. As a result, there is a need to clarify such findings with further experimentation.

In respect to other hormones, Z. *marina* has lost the ability to produce volatiles. Key terpenoid pathways involved in producing volatiles are absent, whilst terpenoid pathways involved in primary metabolism remain (Olsen et al., 2016). The presence of non-volatile phytohormones, the cytokinins, abscisic acid and gibberillic acid pathways have also been identified (Olsen et al., 2016). Suggestively, future work should utilise the power of deep sequencing technology in combination with analytical chemistry, e.g. metabolomics and HPLC/UPLC to verify the presence and function of phyto-hormones within seagrasses. Studies on higher plants could of course be used as sound examples of how we may accomplish such feats (Jia et al., 2016; Cui et al., 2016). Whilst the cross-talk between metabolic pathways including ROS homeostasis, hormone signalling, and downstream signalling cascades remains largely elusive in model plants, it will arguably be a long time before such complexity is understood in seagrasses.

1.7 Osmoregulation at the molecular level

Perhaps the most intriguing characteristic of seagrasses is their superior ability to live in highly saline environments compared to terrestrial plants. Whilst in depth

reviews on seagrass osmo-regulation and salt tolerance are available (Touchette, 2007), our focus lies with the molecular realm of seagrasses. To our knowledge, the first EST dataset came from Kong et al. (2013). In this study the authors identified 163 genes, which were suggested to play a role in hyper-saline response; photosynthesis, metabolism and energy pathways. Metallothionein, metallothionein-like, transporter proteins, stress proteins, ROS scavengers and carbohydrates played significant roles, supporting earlier beliefs that carbohydrates act as osmolites and regulate cellular osmolality (Touchette, 2007). Furthermore, it has recently been confirmed that the cell wall composure of *Z. marina* has been found to contain many celluloses, pectins and algal-like polysaccharides, which help to adjust osmolarity during low tides. Even more interestingly, seagrasses have regained their ability to produce sulphated polysaccharides, further promoting the success of osmoregulation in these marine plants (Olsen et al., 2016).

Characterisation of the *de novo Z. marina* transcriptome (Kong et al., 2014) has identified K^+ channel and transporter transcripts, as well as *SOS*, *NHX*, *CLC* and Na^+/H^+ pump associated transcripts which all play a role in osmoregulation. Olsen et al. (2016) have made the discovery of an H^+-ATPase gene, which is highly expressed in vegetative tissue of *Z. marina* and encodes for a salt tolerant H^+-ATPase. *AHA* genes, which are unique to the alismatids, have also been identified by Olsen et al. (2016). Such discoveries have indicated compartmentalisation and detoxification of sodium and chloride ions are important molecular mechanisms operating within seagrasses to counteract high external salinities, which would otherwise be toxic to vascular plants (Kong et al., 2014); as Na^+ is one of the most disruptive ions to cellular processes. Whilst seagrasses are extremely salt tolerant compared to most other plants, a very recent combination of semi-quantitative proteomics and physiology methodologies have been used to study the response of the seagrass *Cymodocea nodosa* to hyper-salinity (43 psu) over 30 days. From protein expression, they found the metabolism of the leaf changes through over-expression of cytochrome b559 subunits and the down-regulation of structural PSII, PSI proteins and RuBisCo, with a shift in carbon metabolism (Piro et al., 2015a). Cytochrome b559 is considered a prerequisite for PSII assembly, suggesting assembly and repair of photosystems are enhanced during hyper-saline stress. Interestingly, Beer et al. (1980) found an increase in the carbon fixing enzyme PEPC in *C. nodosa* in response to hyper-salinity, possibly signifying that the decrease in

RubisCO is compensated for by PEPC, to maintain the rate of carbon fixation (Piro et al., 2015a). PEPC may also be playing an anaplerotic role in providing intermediates for the TCA cycle or serving a role in stress response (Doubnerová and Ryšlavá, 2011). Piro et al. (2015a) also found an increase in cellular respiration, hardening of cell walls and evidence of sodium compartmentalisation in the vacuole of *C. nodosa*. It can be stated though, that such findings by Piro et al. (2015a) are congruent with previous studies (Muramatsu et al., 2002; Kong et al., 2014) indicating that seagrasses exhibit a decrease in photosynthetic activity under hyper-salinity, but an increase in detoxification and osmoregulation mechanisms.

Attention should also be directed towards hypo-salinity. No current studies in the omics frontier have investigated hypo-salinity at this time; however, Collier et al. (2014) have identified a signature stress response in seagrasses whereby significant shoot proliferation occurs before mortality in response to hypo-salinity. Whilst the study was rigorous, we do need to study such responses at the molecular level as increased rainfall has recently been attributed to seagrass decline in parts of the world, including Cairns, Northern Australia (McKenna et al., 2015). On an evolutionary note, Tyerman et al's (1984) theory of new leaf growth in *Posidonia australis* (via gradual osmotic exposure) should be re-investigated. Such a phenomenon may hold vital clues of how seagrasses evolved and populated the marine environment. Future research efforts on seagrass osmoregulation are important for numerous reasons, i) Such research allows us to understand the complexity of osmoregulation in seagrasses, ii) it helps to explain why some seagrass species are more tolerant to brackish / hyper-saline waters than others, iii) it allows us to determine how seagrasses will survive in future climates where it is expected that the frequency and extent of rainfall events will change, and iv) such research may also play a role in enhancing salt tolerance in agricultural crops. To date, omics has provided us with much knowledge on the mechanisms of salt tolerance in seagrasses, but it can be said that this area of study holds further promise for the future and has the potential to expand significantly.

1.8 Tolerance to anoxia and phytotoxic sediment

Seagrasses are regularly exposed to anoxia/hypoxia in the marine environment (Borum et al., 2006; Brodersen et al., 2015; Hasler-Sheetal et al., 2015). Possibly the most ingenious adaptation of the seagrasses is the presence of a well-developed

aerenchyma (lacunae) system, which provides (i) an efficient sulphide detoxification system, (ii) channels oxygen to the below ground biomass to form protective oxic microshields and (iii) the ability to self-induce efficient anaerobic respiration in the below-ground biomass without damaging tissues caused by excess acidosis and ethanol toxicity during anaerobiosis (Hasler-Sheetal., 2015). It must be stated that terrestrial plants do not normally possess aerenchyma as terrestrial soils are abundant with oxygen; however, like most biological phenomena, there are exceptions such as rice and sorghum which are regularly subjected to water-logging. Aquaphytes also possess aerenchyma, therefore it is assumed that such anatomy is likely to have been an early evolutionary adaptation in aquatic plants.

Although recent work has helped to establish the interactions of sediment sulphides with *Z. muelleri* (Broderson et al., 2015) and *Z. marina* (Hasler-Sheetal and Holmer, 2015), a more recent non-targeted metabolomics approach utilising GC-MS and metabolite enrichment analysis has uncovered critical metabolic systems which are responsible for anoxia tolerance in seagrasses (Hasler-Shetal et al., 2015). *Z. marina* has been found to possess metabolic measures which prevent harmful lactate and pyruvate from accumulating in the tissues via the alanine, GABA and 2-oxoglutarate shunts (Hasler-Sheetal et al., 2015). It has been hypothesised that a regulatory 'low oxygen-sensing' pathway exists, which induces fermentative respiration in the belowground biomass (Hasler-Sheetal et al., 2015). In land plants such a pathway has already been identified in *Arabidopsis* (Gibbs et al. 2011). Given that several seagrass die-off events have been attributed to anoxia and sulphide intrusion in the past (Zieman et al., 1999; Koch et al., 2007), it is critical that we uncover the genetic networks involved, in the hope of implementing suitable monitoring efforts.

1.9 Other areas needing investigation and development

Reviews on seagrass sexual reproduction and seed dispersal exist (den Hartog, 1970; Ackerman, 2006, Kendrick et al., 2012); however, only two studies (Golicz et al., 2015; Olsen et al., 2016) have provided molecular evidence that *Z. muelleri* and *Z. marina* have lost functional genes associated with pollen development and inflorescence morphology; MADS-box transcription factors associated with floral development have been shown to be reduced in *Z. marina* (Olsen et al., 2016). Given that sexual reproduction has been shown to significantly increase in response to disturbance events

(Cabaço and Santos, 2012), it is vital that we understand what triggers sexual reproduction and how it is regulated at the molecular level. Ultimately the ability to out-cross ensures the maintenance of genetic diversity (Ackerman, 2006) and meadow resilience.

It terms of invasive seagrass species it is surprising that no omics work has yet been initiated on *Halophila stipulacea*. This species that is native to the western Indian Ocean and Red Sea has shown rapid geographical dispersion since the 1800's, spreading to the Mediterranean and the Caribbean (Willette et al., 2014). It rapidly colonises, has the ability to survive in a wide range of environmental conditions and possesses great resilience to disturbance events. The photo-physiology of this species alone suggests a high degree of plasticity in response to extreme light environments (Sharon et al., 2009; Sharon et al., 2011). As such its tolerance and resilience make it an excellent candidate species to examine at the molecular level, allowing us to understand what makes this species more tolerant and resilient to environmental change than the majority of seagrasses. Furthermore, such work conducted using artificial mesocosm environments will also allow us to predict its geographical boundary limits.

Further investigation of the rhizosphere is also necessary. The only rhizome and root tissue specific omics research to date is metabolomics by Hasler-Sheetal et al., (2015) investigating anoxia in *Z. marina*. We therefore encourage the targeting of such plant tissue as previously conducted in crop species (Zhang et al., 2014; DuanMu et al., 2015). Additionally, the use of meta-genomics in seagrass biology is an area, which can undergo expansion. Understandably, the utilisation of metagenomics is time-consuming and often difficult; however, with the observed improvement in resources and computational processing (Land et al., 2015), biologists should be expanding their research interests towards the rhizosphere. This will allow us to learn more about seagrass-sediment interaction, seagrass-microbe interaction and the fundamentals of growth and development, which seem to be under-represented in the previous literature (Wetzel and Penhale, 1979; Hansen et al., 2000). Given that pathogenicity has caused seagrass meadow loss in the past (Brakel et al., 2014), we must understand and learn more about the concepts of microbes and seagrass interactions in the rhizosphere, and also in the canopy. Recently, progress has been made by studying microbiomes of *H. stipulacea* (Mejia et al., 2016; Rotini et al., 2016). In these studies, differences were observed in the bacteriome composition associated with this species under varying

environmental conditions, highlighting functional plant-microbe interactions. This work provides a new platform where by microbial communities can be used to assess the ecological condition of seagrass meadows.

In terms of omics and experimental protocols there is a lot to consider with aquatic plants. For plants, it can be challenging to conduct accurate sequencing based experiments in general; however, given that seagrasses grow in an aqueous environment, a further level of difficulty presents itself; uniformity in non-investigative parameters is critical for a successful experiment. Initial considerations should focus on replicate number, depth of sequencing, and the time frame in which gene expression occurs between treatments; as such, running pilot experiments can help. Gene expression is known to be stochastistic; such variation may arise from differences due to environmental factors, multi-genotype presence and the influence of genetic mosaics within experimental populations (Becheler et al., 2010; Reusch and Boström, 2011; Sherman et al., 2016). Moreover, given that variations in ploidy have been detected across seagrass species, polyploidy can also promote transcriptome and proteomic downstream re-arrangement within a time frame of as little as one or two generations (Leitch and Leitch, 2008); as a result, it is important to work with plants of minimal genetic variation in order to maximise the resolution of detecting accurate changes in gene expression. Researchers should also be aware of contamination from other marine organisms including unicellular algae and filamentous epiphytes. Genotyping before conducting such experiments may therefore be of benefit to the researcher to minimise genic variation. Additionally, screening for contaminating sequences should also be considered to improve assemblies. Batch affects arising from the use of a multiple aquaria set-up, non-uniform RNA processing, different tissues, and sequencing cell lane bias must also be accounted for. In relation to genic variation, no transcriptomic studies on seagrasses to date have essentially provided records of variation between biological replicates of treatments when conducting differential expression analysis. We therefore encourage that future efforts do so; to give researchers an idea of how uniform gene expression is between samples.

As for the majority of non-model organisms, the bioinformatic resources for seagrasses are limited. The necessity of online database portals specific to seagrass research is arguably one area, which can undergo further development. To date, the only publically available database, to our knowledge is the EST database, *Dr. Zompo*

(Wissler et al., 2009) that contains ESTs for *P. oceanica* and *Z. marina*. Whilst the sequence reads of many experiments in Table 2 are available in sequence archives, there is a need for user-friendly and interactive web portals, whereby the user can perform annotation, mine for candidate genes of interest and genetic markers. As such sequencing of many more seagrass species is necessary. Higher plant specific databases such as TAIR (Swarbreck et al., 2008), PLAZA (Proost et al., 2009) and the Mangrove Transcriptome Database (MTDB) (Dassanayake et al., 2010) are just some examples of what can be achieved for seagrass-specific bioinformatics portals.

1.10 Genetic marker utilisation in seagrass biology

The advent of molecular technologies over the past 20 years has undoubtedly paved the way to a better understanding of seagrass genetics and their adaptability to environmental stress and climate change (Reusch et al., 2001; Procaccini et al., 2007). For the first time, these studies have provided (i) insight into the genetic diversity within and between meadows (ii) clearer understanding of seagrass phylogenetics, (iii) improved systematic nomenclature) and (iv) observation of meadow resilience in the wake of disturbance events (Procaccini et al., 1999; Reusch et al., 2002; Waycott et al., 2006). These studies of course have relied on the availability of validated molecular markers. The need for such markers is a requisite given the amounting pressures which seagrass meadows are faced with.

1.11 Development and advancement of molecular markers

Molecular markers were introduced into seagrass biology in the 1980's; initial markers included simplistic phylogenetic evolutionary markers and allozymes (Les, 1988; Triest, 1991a; Waycott et al., 2006). With the increasing popularity of PCR (Polymerase Chain Reaction) protocols and development of molecular biology techniques, molecular markers for genotyping became established. Development of RFLP (Random Fragment Length Polymorphism) and RAPD (Random Amplified Length Polymorphic DNA) markers occurred, alongside the development of AFLP (Amplified Fragment Length Polymorphism) markers. RAPD markers quickly gained the reputation for being problematic as they possessed low-resolution capacity (failing to detect levels of polymorphism) and were associated with poor reproducibility (Reusch, 2001; Procaccini et al., 2007). Earlier work utilising these methods revealed little to no polymorphism in seagrasses as such systems were based on dominant

markers rather than co-dominant markers. With improvements in procedures in the mid 90's (Alberte et al., 1994; Procaccini and Mazella, 1996), higher levels of polymorphism were detected in seagrass populations (Reusch, 2001; Waycott et al., 2006). It wasn't until the introduction of high-resolution SSR markers (Simple Sequence Repeats; Litt and Luty, 1989) that detection rate of variability and polymorphism increased significantly within seagrass species (Procaccini and Mazella, 1998; Reusch, 1999b; Reusch, 1999c). Additionally, nuclear and plastid encoded markers such as *ITS*, *rbcL*, and *matK* have had impact in population genetics work, especially in determining taxonomy of seagrass species (Lucas et al., 2012; Coyer et al., 2013).

1.12 SSR markers: High-resolution popularity

SSR markers occur at frequent intervals, are easily identifiable, and have co-dominant characteristics. As a result, SSRs are the current choice of high-resolution genetic marker in seagrass population genetics due to their ability to identify polymoprhisms (Table 2). *Thalassia hemprechii* and *Zostera marina* are perhaps the most widely exploited seagrasses for SSRs to date (Table 2). Until recently SSR marker validation and usage in tropical species was somewhat lagging behind. Only in the past 5 years have SSR markers been validated for select *Cymodocea, Enhalus, Syringodium* and *Thalassia* species (Gao et al., 2012; Nakajima et al., 2012; Matsuki et al., 2012; Matsuki et al., 2013; Wainwright et al., 2013a, 2013b; Arriesgado et al., 2014, 2015). It could also be argued that the majority of SSR work has arisen from the Americas, Europe and Asia to date (Table 2). Recent efforts in Australia have; however, identified SSRs for Australian seagrass species *Z. muelleri, Z. nigricalis, Posidonia australis* and *Posidonia sinuosa* (Sinclair et al., 2009; Sherman et al., 2012; Smith et al., 2013). More work needs to be conducted in the Indo-Pacific, a hot spot for seagrass diversity. Additionally, the numbers of polymorphic loci remain limited for *Halophila*. In seagrasses, validated SSRs have shown the capacity to cross-amplify in other closely related species of seagrass (Reusch, 2000; Sinclair et al., 2009; Smith et al., 2013); however, cross-amplification of markers has not been a major focus in seagrass biology as compared to crop plants. More recently, novel methods have been developed to detect genic-SSRs in seagrasses. Genic-SSRs have so far been identified in the seagrasses *Zostera marina* and *Posidonia oceanica* (Oatjen and Reusch, 2007; Reusch

et al., 2008; Massa et al., 2011). Such methodologies reduce time and cost by scanning assembled transcriptomes or EST libraries for SSR's using software. The main benefit of genic-SSRs is that they can be associated with functionally annotated transcripts and functional protein domains, thus serving as FDM-SSRs (Functional Domain Marker-SSRs); however, compared to genomic SSRs, the rate of polymorphism discovery can be lower.

1.13 Is there a need for further advances in the field of genetic markers?

Whilst Single Nucleotide Polymorphisms (SNPs) are the most widely abundant polymorphism in organisms (Appleby et al., 2009), their use in seagrasses has been limited. SNPs have desired characteristics including low error rates, high frequencies of occurrence and a simple mutation mechanism; however, they are often bi-allelic (Appleby et al., 2009) and as a result are not as desirable as SSRs. Thirty-seven SNPs have been identified in *Zostera marina* (Ferber et al., 2008) and utilised in population genetics (Oetjen et al., 2010). Whilst the availability of sequenced genomes allows for SNPs to be detected easily, novel and cost effective methods such as ddRAD (double digest Restriction Associated DNA) sequencing and GBS (Genotyping By Sequencing) can make use of reduced genome complexity and therefore offer cheaper alternatives than whole genome sequencing (Elshire et al., 2011; Peterson et al., 2012). Such methods have yet to be implemented in seagrass studies. GBS and ddRAD can detect SNPs, microsatellites, deletions and insertions. The approach provides a higher resolution than microsatellite screening. Additionally, it can provide information on QTL (Quantitive Trait Loci) and associate mapping, giving us the ability to identify regions of the genome that may offer greater levels of phenotypic fitness, as previously conducted in higher plants (Zeng et al., 2006; Båga et al., 2007). Fine scale genetic diversity, mosaics and somatic mutations are also worth further investigation in seagrasses (Reusch and Boström, 2011; Sherman et al., 2016), in order to determine how their presence can affect meadow resilience and the success of using SNPs whilst minimising error rates. The final challenge (although not extensively discussed in this review) is the ability to discriminate between taxa. It is our belief that many seagrass taxonomists still encounter trouble whilst determining the phylogenetics of seagrass species. As such, advancements in molecular markers and high-throughout genomic

Table 2: The number of polymorphic SSR loci and SNP loci validated in seagrass species to date. Organised by number of polymorphic loci per species. A search was conducted using 'Web of Science' with the search terms 'microsatellite loci seagrass', 'microsatellite markers seagrass', 'polymorphic microsatellite seagrass' and 'polymorphic loci seagrass'. Last accessed: June, 2016.

Species	**Source(s) of information**	**# Polymorphic SSR Loci**
Thalassia hemprichii	Matsuki et al. 2012; Wainwright et al. 2013b; Van Dijk et al. 2014	49
Zostera marina	Reusch et al. 1999b; Reusch 2000; Oetjen and Reusch. 2007; Peng et al. 2012	45
Zostera japonica	Coyer et al. 2004; Jiang et al. 2011a; Zhang et al., 2015	34
Cymodocea rotundata	Arriesgado et al. 2014	29
Syringodium isoetifolium	Matsuki et al. 2013; Wainwright et al. 2013a	27
Cymodocea nodosa	Alberto et al. 2003b; Ruggiero et al. 2004	22
Zostera muelleri	Sherman et al. 2012	20
Syringodium filiforme	Bijak et al. 2014	17
Enhalus acoroides	Gao et al. 2012; Nakajima et al. 2012	16
Cymodocea serrulata	Arriesgado et al. 2015	15
Posidonia oceanica	Procaccini and Waycott. 1998; Alberto et al. 2003a	14
Thalassia testudinum	Van Dijk et al. 2007	14
Zostera nigricaulis	Sherman et al., 2012; Smith et al. 2013	14
Posidonia australis	Sinclair et al. 2009	10
Halophila ovalis	Xu et al. 2010	10
Ruppia cirrhosa	Martinez-Garrido et al. 2014	10
Ruppia maritima	Yu et al. 2009	10
Zostera noltii	Coyer et al. 2004	9
Halodule wrightii	Larkin et al, 2012	8
Posidonia sinuosa	Sinclair et al. 2009	6
Halophila Beccarii	Jiang et al. 2011b	6
Halophila minor	Xu et al. 2010	6
Zostera caespitosa	Peng et al. 2012	2
Species	**Source(s) of information**	**# Polymorphic SNP loci**
Zostera marina	Ferber et al. 2008	37

sequencing can surely aid in such research and provide clearer relationships between species.

1.14 Concluding remarks

Seagrass biologists have undoubtedly made up for lost time by uncovering a wealth of molecular knowledge over the past decade. Perhaps the most significant contribution has been the genome sequencing of *Z. marina* and various transcriptome studies. Such knowledge has allowed us (i) to obtain a clearer idea of evolutionary traits in seagrasses and (ii) to elucidate the role that molecular processes play in the systematic regulation of seagrasses in response to their environments. We envision that omics and profiling techniques will become much more common in the seagrass biology field, as observed in the higher plant field. As such seagrass biologists should seek out higher plant studies as suitable models for deciphering the molecular biology of seagrasses. The development of future technology, sequencing of more omic datasets and implementation of epigenetic research will no doubt aid in increasing the accuracy and knowledge gained from such research. Whilst SSR markers remain the preferred choice of marker, the expansion and utilisation of ddRAD and GBS sequencing approaches in the future can help overcome the limitations in that approximately only one third of seagrass species have suitable genetic markers. On a final note, omics and molecular profiling approaches should be considered as complimentary to physiological and ecological approaches and not as the sole answer to all biologically relevant questions. As such integrated studies will help to play a more influential role in our understanding of seagrasses, especially in regard to future climate change and disturbance events.

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CHAPTER 2

Light perception in higher plants, seagrasses and *Zostera muelleri*: Fundamental knowledge and the need for further research

Abstract

In this chapter, current knowledge of light and photosynthesis is discussed with respect to physical and biological differences observed between terrestrial and marine environments. Plant organelles associated with light absorption, and photosynthesis are described in detail along with associated reactions. The photosystem light harvesting complexes, light absorbing pigments, photo-protective pigments, light reception and light signaling mechanisms of plants are discussed in detail at the molecular level. Comparisons are drawn between terrestrial plants and seagrasses throughout this chapter. The most crucial part of this chapter discusses common low light responses in plants including seagrasses. This chapter concludes with a discussion detailing our current knowledge on *Z. muelleri's* response to light, and the further knowledge gaps which need to be addressed in order to understand light limitation response in this species.

2.1 Light and photosynthesis: terrestrial vs. marine environments

Phototrophic organisms require light for growth, development and survival (Chen et al., 2004). They convert inorganic carbon to organic carbon and oxygen by a process known as photosynthesis, which is light dependent. This generalised photosynthetic reaction can be defined as:

$$6CO_2 + 12H_2O \xrightarrow{\text{Light}} C_6H_{12}O_6 + 6O_2$$

Land plants obtain a full spectrum of light (UV to far-red) with limited resistance unless cloud cover or spatial competition with other plants for light exists. In the coastal euphotic zone (Alcoverro et al., 2001) light is the dominant regulator of primary productivity. In coastal sub-tidal marine environments, the path of light can be affected by additional abiotic and biotic parameters, which would not normally affect terrestrial plants. For instance, significant amounts of light are attenuated through scattering processes when photons come into contact with particles in the water. This is made worse by the oscillatory motion of waves, as waves re-suspend sediment and refract light. As the depth of the water column increases, the quantity and quality of light also decreases; as such the principle of the Beer-Lambert equation applies in this situation, whereby the coefficient of light absorption is significantly greater (Larkum et al., 2006) than that of the terrestrial biosphere.

Not only does the quantity of light decrease with water depth, but the spectral quality also diminishes due to absorption and photon scattering. Within the first few meters of the water column, far-red and red light are attenuated and thus fail to penetrate deeper waters, green light is next to disappear, whilst blue light disappears last. Light quality and quantity in the water column is further reduced by poor water quality (Dennison et al., 1993). Water quality tends to deteriorate during episodes of excess turbidity, severe weather, anthropogenic run-off, coastal development and algal bloom events (Ralph et al., 2007).

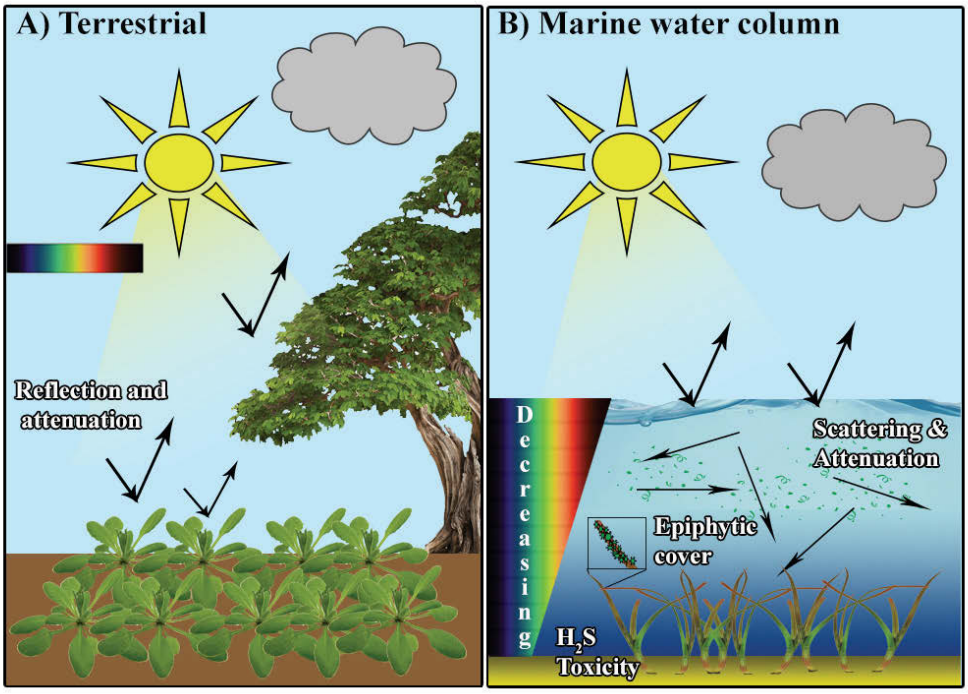

Figure 3: The physical light environment of A. terrestrial plants and, B. sub-tidal marine plants. Black arrows represent light scattering and attenuation processes. Spectral light availability is represented by a wavelength spectrum bar.

In the case of seagrass, for light to penetrate to the leaf surface, light must also bypass an often-present layer of epiphytic growth on the leaves. Shading is also a common phenomenon in seagrass meadows; the removal of a dominant species in a mixed seagrass meadow community allows for other less dominant species to exploit resources including light and nutrients (Williams, 1987). These factors are problematic for seagrasses, as they require approximately 20-25% more irradiance than their counterpart terrestrial angiosperms, due to the physical nature of how light travels in water (Dennison et al., 1993). Seagrasses need to counter-balance the threat of hydrogen sulphide toxicity from the surrounding sediments by producing adequate supplies of oxygen from photosynthesis. This oxygen in turn migrates to the roots and detoxifies hydrogen sulphide (Brodersen et al., 2015). Seagrasses must also maintain a positive carbon balance so that net primary production proceeds. Fig. 3 shows the differences that distinguish terrestrial and aquatic environments with respect to their ambient light environment.

2.2 Organelles responsible for light absorption in higher plants

Chloroplasts, the light harvesting centers in plants, are plastid organelles, with uniparental inherited genomes (Birky, 1995); Chloroplast-encoded genes are transcribed and translated independently from the nuclear genome in plants (Cortleven et al., 2009). As a result, both nuclear and chloroplast-derived genes are essential for the regulation of light response and photosynthesis within the chloroplast. Chloroplast gene expression, confirmation and stoichiometry are regulated by variations in light quality and quantity (Pfannschmidt et al., 1999). In plants, chloroplasts are typically between 5-10 μm in size (Wise and Hoober, 2007), and are present across the leaf epidermis and mesophyll tissues. In the seagrasses, the chloroplasts are exclusively in the epidermis (Larkum et al., 2006). This trait is perhaps an evolutionary adaptation to a submerged low-light niche. Chloroplasts have three membranes; the outer and inner chloroplastic membranes; and the thylakoid membrane. The thylakoid membranes can be found in stacked arrangements known as grana. Embedded in the thylakoid membranes are proteins that form photosystems I and II, cytochrome b6/f, the NADPH complex and ATP synthase complex (Dekker and Boekema, 2005).

2.3 Light-dependent reaction of photosynthesis

Light harvesting antennae complexes capture light in plants in the form of photons. This energy is transferred to the photosystem II (P680) complex. P680 refers to the 680 nm wavelength absorption of PSII. The oxygen-evolving complex of PSII splits water via oxidation into hydrogen and oxygen, and transfers higher-state electrons from PSII to PSI via the electron transport chain reducing enzymes plastoquinone, cytochrome b6/f and plastocyanin. This process is termed the "Z-scheme" (Fig. 4). In the PSI (P700) protein complex, electrons once again become excited by light, which provides power to reduce NADP to NADPH, via the enzyme ferrodoxin-NADP+ reductase. The NADPH produced at the end of the Z-scheme is then used in the light-independent reactions of photosynthesis together with ATP, ribulose-1, 5-bisphosphate (RubP) and carbon dioxide to form two glyceraldehyde 3-phosphate (G3P) molecules, whilst RubP is recycled. G3P is then converted into simple carbohydrates such as starch, sucrose and cellulose depending on the plant's metabolic requirements.

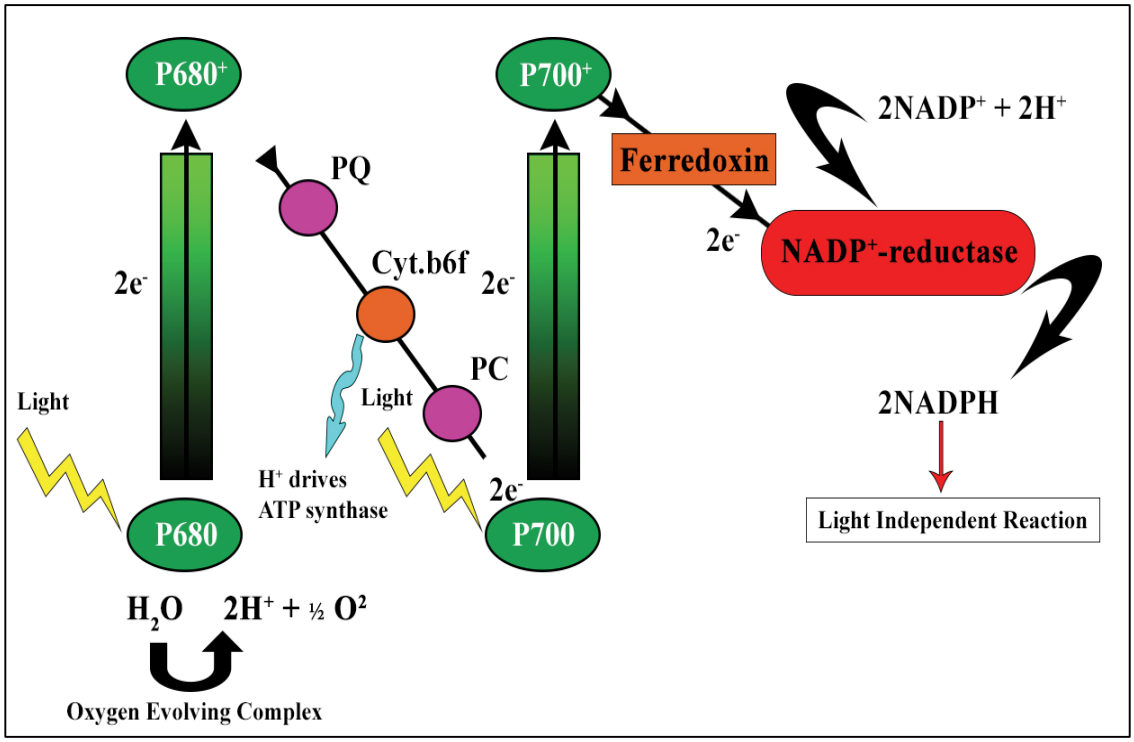

Figure 4: The light dependent reaction of photosynthesis (Z-scheme) in higher plants. PQ = Plastoquinone; Cyt.b6f = Cytochrome b6/f complex; PC = Plastocyanin. P680 refers to photosystem II and P700 refers to photosystem I. Positive (+) characters refer to excited state of the photosystems.

2.4 Photosystem-light harvesting complexes

In land plants, the photosystem and light harvesting complexes (LHCs) have been studied intensively, particularly the PSII-LHC complex. Changes in light can take place on many different time scales (Tikkanen and Aro, 2012) and as such, regulation of the photosystem-LHC complexes can be complex in nature. It has been documented that over 40 proteins are associated with PSII, either as stable or transient forms (Järvi et al., 2015). The core proteins which form PSII are D1 and D2 proteins; CP43; CP47; and the oxygen enhancer protein complex. The D1 (psbA) and D2 (psbD) proteins form a heterodimer complex and form the reaction centre of PSII, whilst CP43 and CP47 bind to chlorophylls (Komenda et al., 2012). The role of psbA in photo-damage repair is critical for the continued regeneration of PSII and functioning of photosynthesis (Tikkanen et al., 2012). FtsH protease proteins (membrane-bound ATP-dependent metalloproteases) are also crucial for removing light damaged proteins from the PSII complex (Komenda et al., 2012). PSII-LHC proteins undergo phosphorylation and dephosphorylation by protein kinases and phosphatases respectively, which control

rates of photosynthesis. These elements are tightly controlled via redox state and stroma regulation, which are influenced by environmental conditions and cellular feedback loops (Aro and Ohad, 2003).

Throughout evolution, the LHC antennae proteins of PSII (LHCBs) have been under most selection pressure due to the efficiency of PSII (~0.8) compared to PSI (~1.0), the LHCB proteins serve multiple roles as light harvesting antennae, in pigment binding, quenching and photoprotection (Ballottari et al. 2012). LHCB proteins are tightly coupled with the xanthophyll cycle, and as a result, coupling of such elements is essential for photoprotection to occur (Dall'Osto et al., 2010). In higher plants, four light harvesting complex (LHCAs) are associated with photosystem I (Croce et al., 2002). These polypeptides have shown light dependent regulative responses (Bailey et al., 2001) and are suggested to play similar roles as LHCBs, although their spectroscopic properties differ substantially (Wientjes et al., 2009). Chlorophyll binding proteins are essential for the binding of chlorophyll molecules to the core photosystems and accessory antennae complexes in order to capture sufficient amounts of light (Wang and Grimm, 2015). Similarily, accessory antennae proteins including the early light-induced proteins (ELIPs) (Hutin, 2003) and helix proteins (Andersson et al., 2003) also play significant roles in light capture. Pigment composition and adjustment is therefore critical for maintaining optimal photosynthesis and preventing damage to the light harvesting complexes. Photo-pigments can be divided into three main groups: the chlorophylls; carotenoids (including xanthophylls); and the anthocyannins.

2.5 Chlorophyll pigments

Chlorophylls (Chl*a* –*f*) are an essential group of molecules necessary for photosynthesis. They are found across a range of phototrophic clades including cyanobacteria, algae and plants. Chl*a* and Chl*b* are two of the most highly abundant chlorophylls in plants (Barber and Archer, 2001). They absorb blue and red light wavelengths (Lichtenthaler et al., 1987) and are contained within the chloroplast thylakoid membranes, more specifically; the majority of chlorophyll pigments are associated with the light harvesting complexes. Their arrangement and stoichiometry around the light harvesting complexes is highly specific (Paulsen, 1997). Chl*a* is a component of the peripheral antennae and core photosystem complexes; chl*b* is only integrated within the peripheral antennae complexes (Jia et al., 2016). In regard to light

availability, photosynthetic pigments adjust in relative composition to enhance light capture and photosynthesis (Lichtenthaler et al., 1987). The adjustments in chl*a*/*b* ratios bring about a change in antennae size. Further, the activity of the enzyme chlorophyllide *a* oxygenase (*CAO*) is also involved in adjusting the size of antennae (Tanaka et al., 2001). Plants that are grown in shaded environments typically possess larger antennae complex and more chlorophyll compared to those grown in high irradiances (Anderson et al., 1988), this has been demonstrated in *Arabidopsis thaliana* and *Nicotiana tabacum* (Tanaka et al., 2001; Pattanayak et al., 2005). In seagrasses, decreased Chl*a*/*b* ratios have previously been documented in response to low light availability (Collier et al. 2012; Genazzio and Durako, 2015; Park et al., 2016) suggesting size enhancement of the antennae complexes.

2.6 Carotenoids and the xanthophyll pigments

All light harvesting chl*a*/*b* complexes contain carotenoid pigments (Hobe et al., 2000). Carotenoids are known for their roles in photosystem complex formation, stabilisation, light absorption and photo-protection. They are localised primarily in the thylakoids, bound to the light harvesting complexes. They absorb light in the most intense spectral range of sunlight, transferring energy to the PS reaction centers (Domonkos et al., 2013) and partake in reactive oxygen scavenging, including singlet oxygen produced when chlorophyll is in the triplet energy state (Triantaphylidès and Havaux, 2009). Carotenoids are formed from the basis of isoprenoid substrate generation in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway of plastids.

From the product of the MEP pathway; geranylgeranyl diphosphate (GGPP), phytoene is produced via the enzyme Phytoene synthase (PSY). Phytoene is metabolised into trans-lycopene by a series of desaturation and isomerisation reactions involving the activity of Phytoene desaturase, Zeta-carotene desaturase and Carotene isomerase enzymes (Nisar et al., 2015). Lycopene is converted to either α- or β-carotene by β-hydroxylase enzymes. Lutein, the main xanthophyll pigment found in green plants and seagrasses (Casazza et al., 2002), formed from α- carotene. β-carotene is metabolised into other xanthophyll pigments (violaxanthin, antheraxanthin, zeaxanthin) through β-hydroxylase enzymes and a series of epoxidation reaction steps. Neoxanthin, another xanthophyll pigment is formed through the activity of neoxanthin synthase from violaxanthin. Both violaxanthin and neoxanthin undergo cleavage by 9-

cis-epoxycarotenoid dioxygenase to form absisic acid, an important plant hormone involved in plant growth, development and response to environmental perturbations. In seagrasses α- carotene, β-carotene, violaxanthin, antheraxanthin, zeaxanthin, lutein and neoxanthin are all present (Ralph et al., 2002; Casazza and Mazzella, 2002). Casazza and Mazzella (2002) have also detected the presence of other pigments in *Posidonia oceanica* and *Halophila stipulacea*; however, these still need further validation. The xanthophylls are conserved in seagrasses, as they are in land plants and are important for playing a role in non-photochemical quenching and adaptation to high irradiance (Ralph et al., 2002; Collier et al., 2008; Schubert et al., 2015).

2.7 The xanthophyll cycle and non-photochemical quenching

When exposed to high irradiance, the electron transport chain of photosynthesis can become saturated in plants. After saturation occurs, photoinhibition is triggered where excited electrons associated with PSII can follow one of three transfer paths. 1.) Electrons can be passed to adjacent chlorophyll molecules around PSII whereby energy is gradually passed through the Z-scheme. 2.) The excited state electron can return to the ground state re-emitting energy or 3.) The excited molecule can return to the ground state whereby the excess energy is dissipated as heat energy; such a phenomenon is termed non-photochemical quenching (NPQ). At room temperature chlorophyll fluorescence mainly originates from PSII and is quite low, plants maintain a steady state of chlorophyll fluorescence. The yield of triplet state chlorophyll and fluorescence vary in relation to the average lifetime of single state excitated chlorophyll. Triplet state chlorophyll can lead to the production of singlet oxygen by transferring energy to ground-state O_2 (Müller et al., 2001).

In non-photochemical quenching (Fig. 5), the photosystem II associated psbS protein brings about conformational changes so that accessory photo-pigments capture the excess energy to prevent photo-damage (Niyogi et al., 2004). The pigments associated with this process are the xanthophyll pigments; violaxanthin, antheraxanthin and zeaxanthin. Violaxanthin is de-epoxidated to antheraxanthin, which undergoes further de-epoxidation into zeaxthanin under increasing irradiance. These reactions are caused by a low pH level generated within the chloroplast lumen (Hirschberg, 2001). These reactions are known to occur in a biphasic mode, which is characteristic of diurnal shifts observed in higher plants (Adams and Demmig-Adams, 1992; Ralph et

al., 2002). Consensus advocates that accumulation of carotenoids is a light dependant process (Demmig-Adams and Adams, 1992; Logan et al., 1996; Matsubara et al., 2009). In various plants including bryophytes (Marschall and Proctor, 2004; Bonnett et al., 2010) and seagrasses (Silva et al., 2013; Tuya et al., 2016) exposure to low light environments can lead to an increase in non-photo protective carotenoid pigment concentrations such as lutein, β-carotene and neoxanthin. These pigments have been found to increase inversely in response to light; it has been suggested that such regulation enhances the light absorption and photosynthetic capacity of certain plants in shaded environments (Silva et al., 2013). Lutein and neoxanthin have been found to be associated with the LHC protein complex (Thayer and BjÖrkman, 1990).

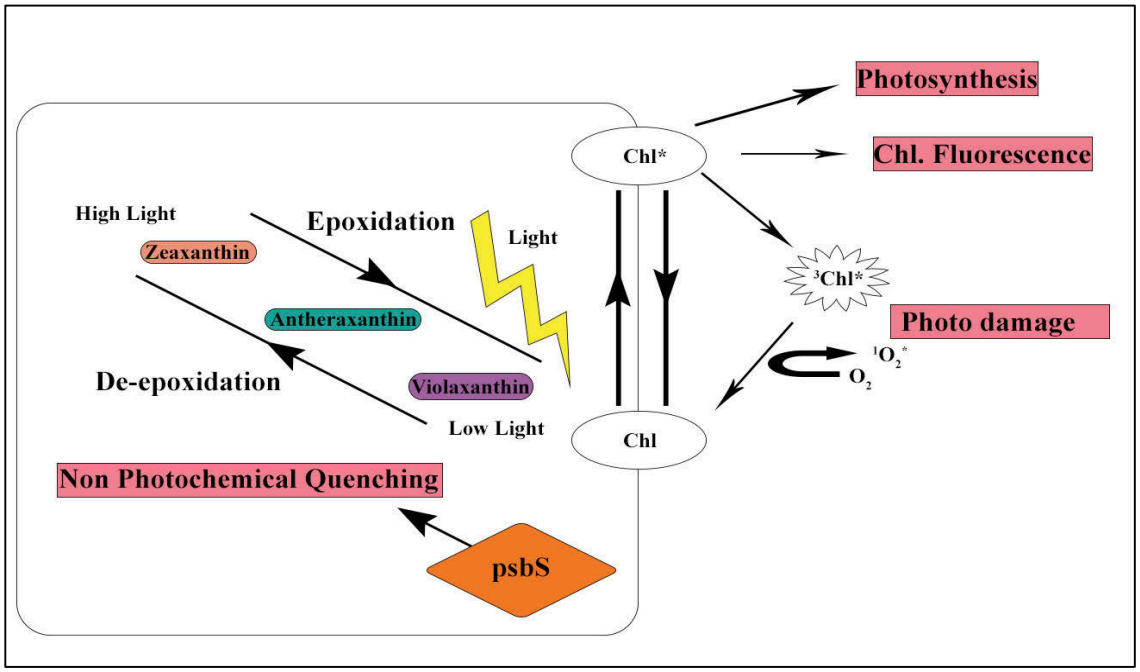

Figure 5: Schematic diagram of chlorophyll excitation in response to high light, the various fates of the excited chlorophyll molecule are represented. In association with NPQ, the role of the psbS protein and the xanthophyll cycle are shown.

2.8 Anthocyannins

Anthocyannins are most commonly known for providing inflorescence-associated tissues with their blue and red-purple appearance; however, from a photophysiological perspective anthocyannins and their counter-part flavonoids are known for their role in UV-B protection (Harborne and Williams, 2000). In both land plants and seagrasses, the reddening of leaves has often been associated with the

accumulation of anthocyannins (Novak and Short, 2010), especially in seagrasses that are distributed in shallow inter-tidal environments, which generally experience higher irradiances.

2.9 Sensing quality and quantity of environmental light

Plants detect differences in the quantity and quality of light in order to control biological processes through the regulation of genetic networks and key metabolic pathways (Chen et al., 2004). Many vital processes are regulated by light, including growth and development, flowering, daily circadian rhythm, shade avoidance, chloroplast movement, elongation and seed germination (Li et al., 2011). As a result, plants have developed 'photoreception', a means of sensing such changes. Photoreception in plants occurs mainly through phytochrome, cryptochrome and phototropin apoproteins (Chen et al., 2004). In the model plant *Arabidopsis*, five types of phytochrome exist, PHYA–PHYE (Clack, 1994). Generally, in angiosperms three main phytochromes exist; PHYA, PHYB and PHYC. The two extra phytochromes in *Arabidopsis* (PHYD and PHYE) are present only in dicotyledonous plants and are thought to have evolved in recent genome duplication events (Mathews and Sharrock, 1996). Phytochromes are known for their perception of far-red and red light and their conformation is determined by the ratio between the two wavelengths (FR: R). PHYB is the most prominent receptor involved in perception of low red: far-red ratios (Franklin et al., 2005). Further downstream signaling involves genic regulatory and hormonal networks. *PHYA* and *PHYB* genes are both present in the seagrass *Z. marina* and may be important for day / night cycle related circadian rythym; however, no current work on Zosteraceae has profiled these genes in response to light. The absence of the *PHYC* gene in *Z. marina* is discussed in Chapter 1, section 1.4.

Arabidopsis has 3 cryptochromes, CRY1-CRY3. Only CRY1 and CRY2 are known to be involved in shade response. The activity of cryptochromes increases as blue light increases (Casal, 2012). The presence of cryptochrome genes has indeed been detected in *Z. marina*; however, as previously discussed (See Chapter 1, section 1.4), the *UVR8* gene that allows for UV light detection (Rizzini et al., 2011) is entirely absent. Phototropin 1 and phototropin 2, significantly important receptors found in higher plants are also essential for optimisation of photosynthesis, phototropism, chloroplast movement and stomatal opening (Chen et al. 2004) The *Phot1* gene plays a

key role in low blue light fluence and *Phot2* plays a role in high light response (Briggs & Christie, 2002). Research has suggested that phototropins promote plant growth in response to blue light in low light environments (Takemiya et al., 2005). In *Z. marina* these genes are present, and most probably play an important role in light perception in marine monocots, given the absence or lack of red light in the marine environment (Olsen et al., 2016).

2.10 Common low light responses in plants

Low light responses in plants are not as well studied as high light responses. In most circumstances, terrestrial plants receive adequate light apart from when instances of bad weather occur (cloud cover) or when competition is evident from neighboring plants, for example in the under canopy of forests. Such competition for limited resources leads to plastic developmental responses; being sessile, plants must adapt their growth and development around the ambient light environment (Franklin and Whitelam, 2005). A reduction in the ratio of red to far-red light acts as a warning of competition and this triggers a response (Sessa et al., 2005). There are two separate response mechanisms to light limitation in plants. Plants can either be classified as 'shade tolerant' or 'shade avoidant' (Gommers, 2013). Shade tolerance can be attributed to the minimum light levels that plants require to survive, as such the length of the growing season, and environmental stress can have a large impact (Valladares and Niinemets, 2008). Shade avoiding plants generally respond by stem elongation, petiole elongation, hyponasty and reduced branching (Gommers, 2013). The shade avoidance response is widespread in the angiosperm lineage of plants (Sessa et al., 2005). If the plant grows successfully towards light, the shade avoidance response is rapidly reverted through phytochrome photoconversion (Morelli and Ruberti, 2002).

Just like shade-avoidance plants, shade tolerant plants increase photosynthetic quantum efficiency, total chlorophyll, lutein and defense compounds; however, they typically suppress all shade-avoidance mechanisms (Gommers et al., 2013). They decrease their respiration rates, chlorophyll *a*/*b* ratios, RubisCO and xanthophyll content (Valladares and Niinemets, 2008). Defense is known to increase in response to low light via the production of secondary metabolites including polyphenylpropanoids, antioxidants, ROS scavengers and polyphenol oxidases (Ibrahim & Jafaar, 2012; Zhan et al., 2014). Although phenotypic plasticity such as elongation is low in shade-tolerant

species, plasticity for certain traits, especially those optimising light capture, can be high (Valladares and Niinemets, 2008). Hormonal signaling and genetic feedback loops are essential to effectively respond to shading. Light limitation can cause significant shifts in photosynthetic efficiency and inorganic carbon fixation, in turn disrupting carbon source-sink-storage equilibrium (Brouquisse et al., 1998). Indeed, with higher plants and seagrasses the rate of photosynthesis and carbon fixation can decline in response to light limitation, whilst utilisation of nutrient stores and morphological changes are also observed (McMahon et al., 2013). Some seagrasses such as Cymodoceaceae and Hydrocharitaceae have slower responses to light limitation than Zosteraceae (Collier et al., 2012); however generally speaking, lateral branching of the above ground material of seagrasses seems to impacted in low irradiances (Enríquez et al., 2005; Peralta et al., 2002). Such observations are in line with shade avoidance response. As such, the responses that seagrasses elicit in response to low light seem to correspond to a mixture of shade avoidance and tolerance.

The characteristics of light absorption and perception in higher plants have been discussed with special reference to seagrasses and shading responses. Howver, chapter 1, section 1.4 has highlighted the importance of understanding light response in seagrasss at the molecular level, and how shading can ultimately lead to the collapse of seagrass meadows. In more recent times various studies have delved into the molecular physiology of select European seagrasses. Procaccini et al., (2017) carried out a study in which it was demonstrated that depth-specific fluctuations of gene expression and protein abundance modulate the photophysiology of *Posidonia oceanica*. The photochemical pathway of energy use was more efficient in shallow plants due to higher ligh availability; however, these plants needed more photo-protection and photo-repair requiring higher translation and protein synthesis than deeper plants.

2.11 Our knowledge of light response in *Z. muelleri* to date

Z. muelleri is found in inter-tidal and sub-tidal waters usually less than ~ ten meters deep, the depth distribution of this species being strongly determined by light availability (Dennison, 1993). *Z. muelleri* populations are perennial. The plants typically have 3-5 leaves, although this number can vary depending on life stage. Leaves are typically 30cm in length and 0.5cm wide; however, size can vary. This species can be found along the East and South coast of Australia, as well as the islands

of New Zealand. For *Z. muelleri*, it has been suggested that photosynthetic saturating light is approximately 195 - 242 μmol photons m^{-2} s^{-1} (Schwarz, 2004) in temperate regions. Recent fieldwork by Bulmer et al. (2016) record saturating irradiances of 180 μmol photons m^{-2} s^{-1} in winter months and 425 μmol photons m^{-2} s^{-1} during summer months in the North Island of New Zealand. The location of this study site had similar latitude to Sydney, Australia; where most of this PhD project was conducted. In lower lattitudes such as Gladstone, Queensland, *Z. muelleri* can be subjected to daily irradiance of over 1000 μmol photons m^{-2} s^{-1} (Petrou et al., 2013). Losses of *Z. muelleri* in Australia have previously been attributed to reductions in light availability (Kirkman, 1978; Bulthius, 1983; King and Hodgson, 1986; Walker and McComb, 1992; Dennison et al., 1993; Preen et al., 1995; Short and Wyllie-Echeverria, 1996; Campbell and McKenzie, 2004; Ralph et al., 2007).

PAM (Pulse Amplitude Modulated) fluorometry has given us the greatest insight of how *Z. muelleri* responds to variation in irradiance (Schwarz et al., 2004; Petrou et al., 2013; York et al., 2013; Maxwell et al., 2014), with decreased photosynthetic rates observed in lower irradiance. Analytical chemistry methods including spectrophotometry and HPLC have allowed us to detect pigment changes and xanthophyll cycle shifts with respect to changing light environments (Abal et al., 1994; Petrou et al., 2013; York et al., 2013). At the morphological and physiological level, detailed literature is available for the many changes observed in *Zosteraceae* in response to low light (McMahon et al., 2013); however, fine scale knowledge at the molecular level is currently lacking. No molecular studies to date have examined how light limitation effects the transcriptional regulation of *Z. muelleri* using high throughput omic datasets. To date there is only one RT-qPCR based study including expression profiling of select genes in *Z. muelleri* in response to dredging stress (Pernice et al., 2015; as discussed in chapter 1, section 1.4). By using next generation sequencing and high throughput bioinformatics analysis, in conjunction with photophysiology and RT-qPCR, a new tier of knowledge can be obtained, as to how *Z. muelleri* responds and acclimates in response to light limitation.

2.12 References

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CHAPTER 3

De novo transcriptome assembly and *in silico* characterisation of the seagrass, *Zostera muelleri* with respect to shifts in irradiance

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Abstract

Zostera muelleri is an ecologically and economically important seagrass species found in Australian and New Zealand coastal waters. The survival of this euryhaline species depends on light availability. In this present study, the development of the *de novo Z. muelleri* transcriptome is reported. Transcript reconstruction produced a total of 52,616 annotated unigenes and 40,312 complete open reading frames (ORFs). Unigenes were identified which were represented by photo- and light-associated gene ontology (GO) terminologies. The expression of chlorophyll binding protein orthologs was profiled, suggesting light-responsive expression similar to that of land plants. Regulatory elements including transcription factors and a total of 33,726 microsatellites were identified and characterised. This data provides the foundation for new understandings into how this keystone species perceives and responds to light, and provides valuable ecogenomic resources for further research efforts.

3.1 Introduction

Zostera muelleri is an important species of seagrass distributed across Australian coastlines, as well as New Zealand coastlines (Jones et al., 2008; Golicz et al., 2015). As a euryhaline species it is found in estuarine and lagoon environments (den Hartog, 1970), providing a wide array of ecological and economic services (Waycott et al., 2009). *Z. muelleri* meadows are biodiversity hotspots and play important roles in marine ecosystem health such as: nutrient recycling; supporting the sustainability of fish stocks; trapping sediment; sequestering substantial amounts of 'blue' carbon stocks and providing coastal protection (Björk et al., 2008). Seagrasses have evolved and adapted to live in dynamic marine environments; *Z. muelleri* for example has developed several physiological and genetic modifications (Golicz et al., 2015) to cope with tidal oscillations, sediment re-suspension, salinity fluctuations, increased nutrient loads and light fluctuations. Despite their importance, seagrass meadows including those of the *Zosteraceae* family are disappearing (Kirkman, 1997; Campbell and McKenzie, 2004). Consequently, blue carbon stocks and ecosystem services are diminishing. According to estimates, up to 7% of seagrass meadows have been lost each year since the 1980's (Waycott et al., 2009).

Among the environmental factors that govern the survival and environmental fitness of plants, light plays a critical role in aquatic plant adaptation, determining the depth distribution of plants, especially at the maximum depth limit (Dennison et al., 1993; Ralph et al., 2007). Due to the fact that *Zostera* species dominate shallow sub-tidal waters, they frequently encounter fluctuations in light availability as a result of changes in water quality (Ralph et al., 2007). Light limitation is an ever-increasing threat to the functionality and survival of seagrasses (Ralph et al., 2007; McMahon et al., 2013). It plays a key role in the regulation of photosynthetic electron transport rate, metabolic efficiency and carbon assimilation. Qualitative and quantitative differences in light regulate important plant physiological and developmental processes (Chen et al., 2004; Jiao and Deng, 2007). Consequently, optimal levels of light are required for normal plant functioning and development. Previous studies have shown that light variation is a significant stressor for seagrasses (Ralph et al., 1995, Ralph et al., 2007; McMahon et al., 2013). However, several investigations have demonstrated that seagrasses possess high plasticity to respond and acclimate to changes in light (Major and Dunton, 2002; Sharon et al., 2009; Dattolo et al., 2014).

Transcriptomics (mRNA-seq) has become the preferred method to profile RNA based regulation of many model and non-model species to delineate the genetic basis of adaptation in plants. Till now, profiling transcriptional changes in plants in response to light perception and response has largely focused on land plants (Tyagi and Gaur, 2003). The *Zostera marina* genome has been recently sequenced (Olsen et al., 2016) and to an extent, the characterisation of certain light responsive transcripts has been completed in *Z. marina* (Kong et al., 2016). Despite few reports describing photosynthetic estimates such as maximum quantum yield of PSII (Fv/Fm) to profile the kinetics of the light-mediated responses in seagrasses (Peralta et al., 2002; Major and Dunton, 2002; Ralph et al., 2007; McMahon et al., 2013), none of the previous studies have focused on characterising genes associated with light perception and regulation using mRNA-Seq technology. This study therefore aims to provide insights into the characterisation of the light-associated transcriptome of *Z. muelleri,* while simultaneously mining genetic resources for future studies. This is imperative as increased climate change and disturbance events have been linked to poor water quality, and loss of seagrass meadows through light limitation (Ralph et al., 2007).

3.2 Materials and methods

3.2.1 Sample collection, experimental layout and sample preparation

To characterise and understand the transcriptomic response of *Z. muelleri* in relation to varying light intensity, over 100 plants with 10 cm of intact sediment were collected from Pelican Banks, Gladstone Harbour (Queensland, Australia) in November, 2011.Plants were collected in close proximity of one another to maximise the chances of working with only one genotype. These plants were then acclimatised at the University of Technology Sydney (UTS; Australia) for 2 months in an artificial open-air greenhouse mesocosm facility. Acclimatised plants were kept at 24°C by using heater-chillers; salinity was kept between 32-36 units (Lewis, 1980) and adjusted using deionised water /seawater when necessary. Mesocosms were aerated by free-flowing air stones, additionally Elite mini pumps (Hagen, Canada) were used to circulate the water and minimise the diffusion boundary layer (Larkum et al., 2006). Water was changed on a fortnightly basis to minimise epiphyte growth. After the initial 2 month acclimatisation period, shading cloth of different thickness was used to induce a variation of light exposures. Approximately 40 plants were used for each of the three

light conditions. Shading cloth was placed over select areas of the open flow aquarium tank (400L). Shade cloth covers were constructed to impose the following light intensities: 100% solar irradiance – no shade (~1,000 μmol photons m^{-2} s^{-1}); 25% (~250 μmol photons m^{-2} s^{-1}) and 1% solar irradiance (~10 μmol photons m^{-2} s^{-1}) (Fig. 6). A calibrated light meter (LiCor 250A) and an underwater 2-pi irradiance sensor were used to measure irradiance levels at the top of the seagrass canopy.

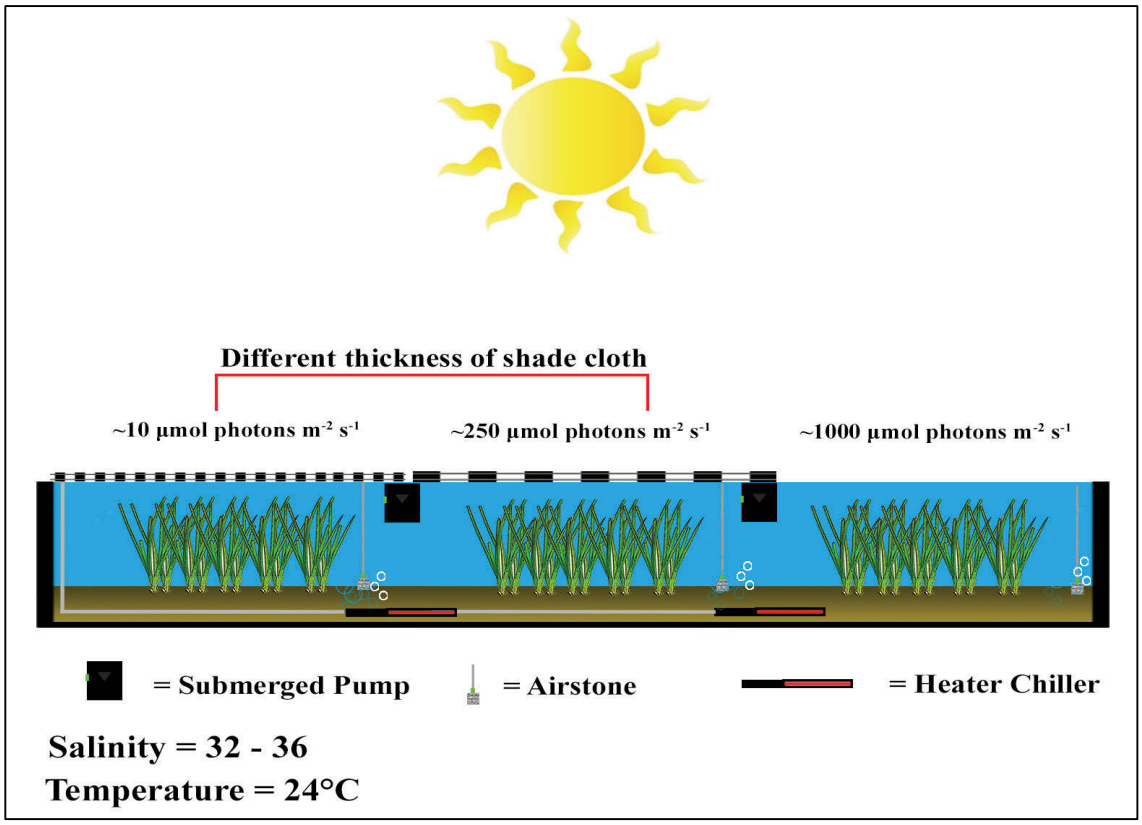

Figure 6: Experimental set-up of the artificial mesocosm study with varying light intensities: ~1,000 µmol photons m^{-2} s^{-1}; ~250 µmol photons m^{-2} s^{-1}; and ~10 µmol photons m^{-2} s^{-1}. Aquarium related equipment is labelled in the legend above. Temperature was kept at 24°C and salinity between 32 and 36 units.

For transcriptome profiling, whole plants were used as samples. These were collected on Days 9, 12 and 14 of the experiment. Plant biomass was hand dried and epiphytes were gently removed using thumb and forefinger. Plants were then snap-frozen in liquid nitrogen and stored at -80°C prior to processing; RNA extraction was performed as per the manufacturer's protocol (PureLink ™ RNA Mini Kit; Life Technologies) with the addition of On-column PureLink ™ DNase (Life Technologies) to remove contaminating genomic DNA. RNA quantity and quality checks were

performed using Qubit 2.0 Fluorometric Quantification (Life Technologies) and Nanochip technology (Agilent 2100 Bioanalyzer) respectively. High-quality RNA (integrity number >7) was sequenced at the New Zealand Genomics Limited (NZGL) sequencing facility (Otago, New Zealand) using Illumina Hiseq2000 paired-end sequencing technology. A total of 9 libraries (9 individual plants) were sequenced.

3.2.2 Quality checks and transcriptome assembly

Quality of the raw reads was assessed using FastQC software. Reads were trimmed and cleaned with Trimmomatic (Version 2.35; Bolger et al. 2014), with the following parameters: ILLUMINACLIP: 2:30:10 LEADING:5 TRAILING:5 SLIDINGWINDOW:4:5 MINLEN:25. In lieu of the fact that a *Z. muelleri* genome did not exist at the time of analysis and writing; *and Z. marina* and *Z. muelleri* are diverged species, we decided to implement a *de novo* transcriptome assembly approach. *De novo* assembly was performed on *Z. muelleri* using Trinity software (Version 2.2.1; Haas et al., 2013). Settings previously used for assembling angiosperm species were used (Chen et al., 2015), these were as follows: --normalize_reads --min_kmer_cov 3 --min_glue 3 --group_pairs_distance 250 --path_reinforcement_distance 85. Contigs over 200 bp were retained and a default K-mer size (K=25) was implemented. Following the production of an original assembly, CD-HIT-EST version 4.6.6 (Li and Godzik, 2006) was used to remove redundant transcripts. A word size of nine and identity threshold of 0.95 was applied. Non-redundant transcripts were then re-assembled using the overlap-layout consensus algorithm implemented in the Contig Assembly Program (CAP3) (Huang and Madan, 1999). The following settings were used: identity cut-off threshold of 95%; overlap length cut-off of 50; specific clipping range N >50; specific gap penalty factor of 3 and a max number of 1000 word occurrences. These parameters where chosen to maximise contig length, minimise spurious reads and to limit the reporting of contigs with highly variable read coverage. Fig. 7 provides an overview of the pipeline implemented.

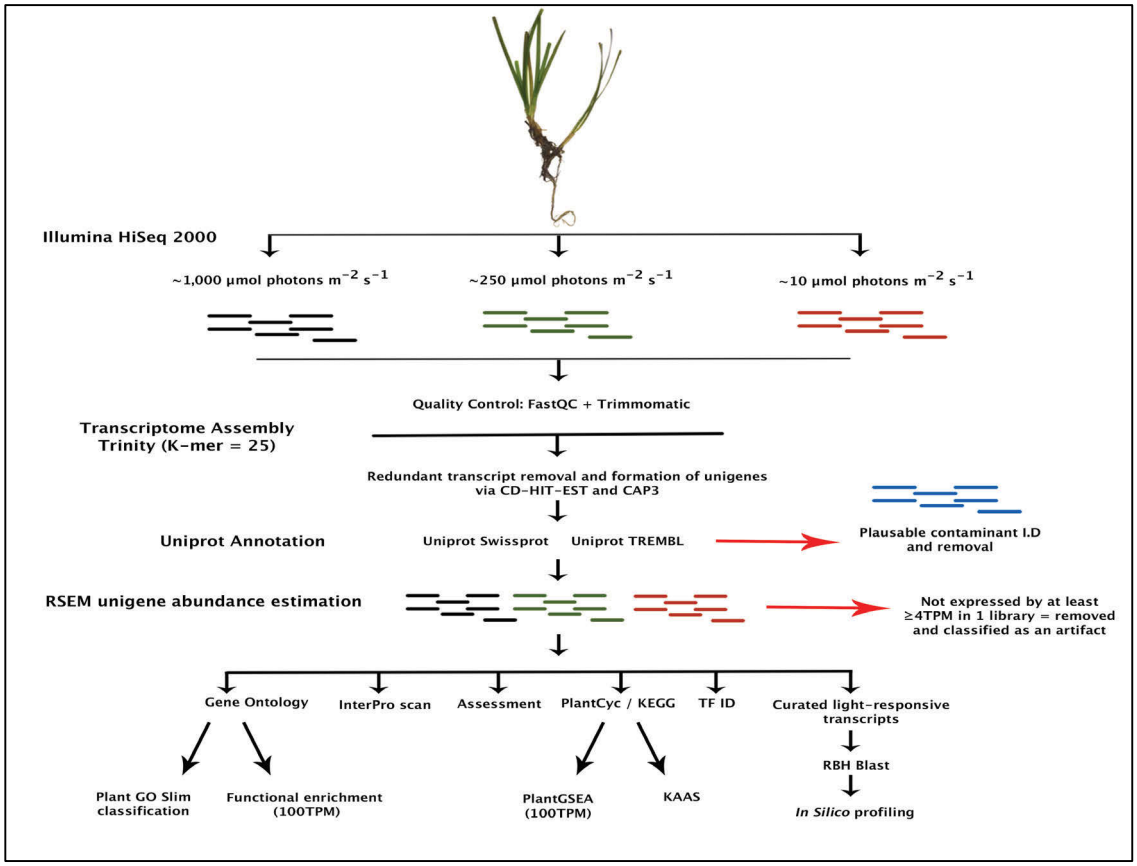

Figure 7: A schematic flow diagram representing the methodology used to assemble, characterise and profile the *Z. muelleri* transcriptome.

3.2.3 Annotation and filtering of unigenes

Unigenes were functionally annotated with the Uniprot Swissprot and Uniprot TREMBL databases (http://www.uniprot.org) using DIAMOND protein alignment software (Buchfink et al., 2015); an E cut-off value of 1.0 x 10^{-5} was used to assign functional annotations. The best hit for each unigene was retained on the basis of percentage identity, bitscore and e-value. The best hits were then carefully examined for signs of contamination with other water-borne organisms, given that seagrass meadows contain a high diversity of free-living organisms. Although the seagrass plants used for sequencing were cleaned before harvesting, the high-resolution capacity of RNA sequencing can also detect contaminating transcripts. Unigenes that were not annotated as genes of plant origin were discarded. The aim of this approach was to minimise the number of false positive unigenes in the final assembly and downstream analysis. Such approaches have previously been conducted on plants and marine organisms (Li et al., 2012; Richardson and Sherman, 2015; Olsen et al., 2016). After removal of

contaminating unigenes, expression estimates were tabulated in a matrix for all of the individual mRNA libraries using the software, RSEM (Version 1.2.28; Li and Dewey, 2011).

After TMM-normalisation of the libraries, a threshold of four transcripts per million (TPM) in at least 1 or more libraries was used to remove likely artifacts and transcripts with low coverage. The threshold used to determine 'expressed' genes is the more conservative of two thresholds described within Wagner et al. (2013). TPM as a measure of gene expression offers a superior approach to compare gene expression between libraries as opposed to previously described RPKM/FPKM approaches (Wagner et al., 2012).

3.2.4 Evaluation of the transcriptome assembly

To assess the quality of the assembled transcriptome, cleaned reads were mapped back to the final transcriptome assembly using Bowtie software (Version 1.1.2) to assess the percentage of proper-paired input reads used in the assembly process. *Z. muelleri* unigenes were also compared to the BUSCO early plant release database (Simão et al., 2015), which encompasses evolutionary informed expectations of gene content from near-universal single-copy orthologs. Conditional Reciprocal Best BLAST (CRBB) analysis (Aubry et al. 2014) applied through Transrate (Smith-Unna et al., 2015) was used to assess the orthology of the *Z. muelleri* transcriptome against the proteome of *Physcomitrella patens* (a bryophyte ancestor), five flowering land plant proteomes, *Z. marina,* and the proteome of the most simplistic marine monocot, *Spirodela polyrhiza*.

3.2.5 Functional classification and detection of genetic elements

Transdecoder (Release v3.0.1; Haas et al., 2013) was used to detect open reading frame (ORF) coding regions of unigenes. This software identifies ORFs based on a minimum length (at least 100 amino acids long) and a log-likelihood scoring system. An optional homology search was conducted within Transdecoder to retain ORF regions which had a positive homology hit (BLASTP; E-value: 1.0×10^{-5}) to the *Arabidopsis* 'TAIR10_pep_20101214 _updated' peptide database (TAIR10). Transcription factors and genetic regulators were identified via PlantTFcat (http://plantgrn.noble.org/PlantTFcat/). Microsatellite SSRs (Simple Sequence Repeats) were mined from the transcriptome using EST Trimmer and MISA (http://pgrc.ipk-

gatersleben.de/misa/); MISA SSR definitions were set as follows: 1-10,2-6,3-4,4-3,5-3,6-3 with 100 nucleotides as the maximum distance between two SSRs. For classification of gene ontology (GO) terminologies represented in the *Z. muelleri* transcriptome, plant GO-slimming was implemented in Blast2GO (Basic version).

3.2.6 Enrichment analysis and *in silico* profiling of light responsive orthologs

To identify the enriched GOs across light treatments, a threshold of ≥ 100 TPM was implemented on the TMM normalised gene counts. GO categories within light treatments (≥ 100 TPM) were used as "foreground" datasets; these were compared to the entire GO dataset of the assembled transcriptome as previously described (De Paolo et al., 2014; Marinov et al., 2014) using Goatools (https://github.com/tanghaibao/Goatools). Fishers exact test with Benjamani and Hochberg (Benjamani and Hochberg, 1995) correction for multiple comparison was applied with an FDR threshold value of 0.05. KEGG Automatic Annotation Server (KAAS) was used to obtain KEGG ontologies and pathways for the transcriptome (http://www.genome.jp/kegg/kaas/). All 21 plants in the KAAS database were selected for annotation purposes in conjunction with the nucleotide BBH (bi-directional best hit) BLAST algorithm. For the detection of enriched metabolic pathways, the transcriptome was annotated specifically to *Arabidopsis*, using an e-value of 1×10^{-5}. PlantGSEA (Yi et al., 2013) enrichment analysis was then implemented against the *A. thaliana* specific PlantCyc and KEGG databases using hypergeometric testing. A Benjamini and Hochberg 0.05 FDR correction was applied (Benjamini and Hochberg, 1995). To identify chlorophyll a-b binding proteins from *Zostera marina* in *Z. muelleri*, RBH (Reciprocal Best Hit) BLAST searches were performed with default parameters of >70% sequence identity and sequence coverage of >50%.

3.3 Results

3.3.1 Transcriptome annotation and assessment

The original transcriptome assembly was produced using a total of 9 libraries (A.1; Table 1), a total of 527,987 transcripts were classified into 337,754 components by the Trinity software. For this assembly, an N50 value of 1,607 bp was obtained. Following redundancy removal (CD-HIT-EST and CAP3), best-hit results for unigenes were obtained from DIAMOND protein alignment searches; these were then screened to

determine presence of contaminating unigenes. A final annotated transcriptome assembly was then produced, which consisted of 52,616 unigenes, exhibiting an N50 value of 2,159 bp. The total GC content was 41.40% for the final assembly. Table 3 shows the summary statistics of the final *de novo* transcriptome reconstruction.

Table 3: Summary of statistics of the original and final *Z. muelleri de novo* assemblies.

Trinity Assembly	Original Assembly	Final Assembly
Trinity Assemblage size (K=25)	482,825,825 bp	86,497,417 bp
Total Trinity transcripts	527,987	-
Total Trinity components	337,754	-
Number of unigenes	-	52,616
Unigene N25 stats	2,624 bp	3,225 bp
Unigene N50 stats	1,607 bp	2,159 bp
Unigene N75 stats	772 bp	1,427 bp
Total GC count	199,040,715 bp	35,813,763 bp
GC%	41.22 %	41.40 %

Table 4: Coverage distribution of the *Z. muelleri* transcriptome across related plant species. Proteome = number of genome predicted proteins, *Z. muelleri* unigenes =52,616. CRBB = Conditional Reciprocal Best BLAST. Reference proteomes were downloaded from ftp://ftp.ensemblgenomes.org/pub/plants/ release-32. The *Spirodela polyrhiza* proteome was downloaded from http://spirodelagenome.org, *Zostera marina* from http://uniprot.org.

Plant species	Proteome	CRBB unigenes	CRBB ref	cov50	cov95
Zostera marina	20,559	37,575	12,718	12,049	10,125
Musa acuminata	36,519	33,699	12,609	10,193	4,785
Vitis vinifera	29,927	33,466	10,852	9,341	4,702
Oryza sativa	42,132	32,797	11,541	9,499	3,891
Arabidopsis thaliana	35,386	32,653	11,802	9,667	4,540
Spirodela polyrhiza	18,888	30,178	9,303	7,479	2,743
Physcomitrella patens	38,354	27,233	9,293	6,841	2,325
Amborella trichopoda	27,313	31,810	9,623	8,451	4,059
Zea mays	149,669	34,686	16,464	12,323	4,452

To quantify the accuracy of the final transcriptome assembly, CRBB (Conditional Reciprocal Best BLAST) analysis was conducted (Aubry et al., 2014) against the proteome of the bryophyte ancestor *Physomitrella patens*, five flowering land plants, *Z. marina* and the *Spirodela polyrhiza* proteome through transrate software (Smith-Unna et al., 2015). This analysis revealed high coverage of the final assembled transcriptome against the species used as references (Table 4). Most unigenes had CRBB hits to *Z. marina* (37,575), *Z. mays* (34,686) and *M. acuminata* (33,699). Further

validation of the transcriptome was completed using orthology comparison analysis against the BUSCO plant specific database (Simão et al., 2015); 846 out of 956 core BUSCOs (88.49%) were identified in *Z. muelleri*; 615 out of 846 BUSCOs were duplicated (72.70%), additionally 27 partial BUSCOs (2.82%) were recovered, 83 BUSCOs (8.68%) were not identified. Re-alignment and mapping of the reads to the transcriptome indicated that 73.09% of proper-paired reads were used in the assembly. Functional coverage distribution hit analysis demonstrated 83.04% of functional hits were assigned to *Zostera marina* (A.1; Table 2). The second species with most functional assigned annotation hits was *Musa acuminata* (2.15%). The top five species (A.1; Table 2) with most functional hits were represented by dicot and monocot plants.

3.3.2 Functional classification and enrichment analysis

Gene Ontology (GO) terms were assigned to a total of 38,925 (73.98%) unigenes, and Interproscan protein domains to 48,089 (91.40%) unigenes in the final assembly. 40,312 complete open reading frames (ORFs) were identified within the final *Z. muelleri* transcriptome assembly. Plant-specific GO-slimmed terms are documented (A.1; Fig. 1-3) providing a comprehensive catalogue of the molecular functions, biological processes, and cellular components within *Z. muelleri*. Table 5 highlights the many GO terminologies identified in the transcriptome, which are associated with photosynthesis, light perception and light-driven processes. We found 151 unigenes associated with the GO term 'Photosynthesis – GO:0015979'; 85 with the GO term 'Chlorophyll binding – GO:0016168'; 82 with the GO term 'Photosystem I – GO0009522', and 19 under 'Photosystem II – GO:0009523' (Table 5). Additionally, GO terms associated with red and blue wavelengths of light were identified. GO enrichment analysis (Fig. 8) for light treatments (FDR < 0.05) revealed 285 GO terms were enriched across all treatments. Terms enriched and shared between both ~250 μmol photons m^{-2} s^{-1} and ~10 μmol photons m^{-2} s^{-1} conditions, consisted in total of 36 GO terms. Various enriched terms were associated with both the light-dependent and independent reactions of photosynthesis, the chloroplast, chlorophyll binding and pigment synthesis (A.1; Table 3). An enrichment of 'Photosystem 1 - GO:0009522' was observed in line with 'chlorophyll binding - GO:0016168', 'photosynthesis, light harvesting - GO:0009765', 'Photosystem – GO:0009521', 'Photosynthesis – GO:0015979' and 'protochlorophyllide reductase activity - GO:0016630'. Enrichment of 'photo-respiration - GO:0009853',

'ribulose-bisphosphate carboxylase activity – GO:0016984', 'hydrogen peroxide metabolic process - GO:0042743' and 'hydrogen peroxide catabolic process - GO:0042744' was observed along with enrichment of various antioxidant-associated terms (A.1; Table 3).

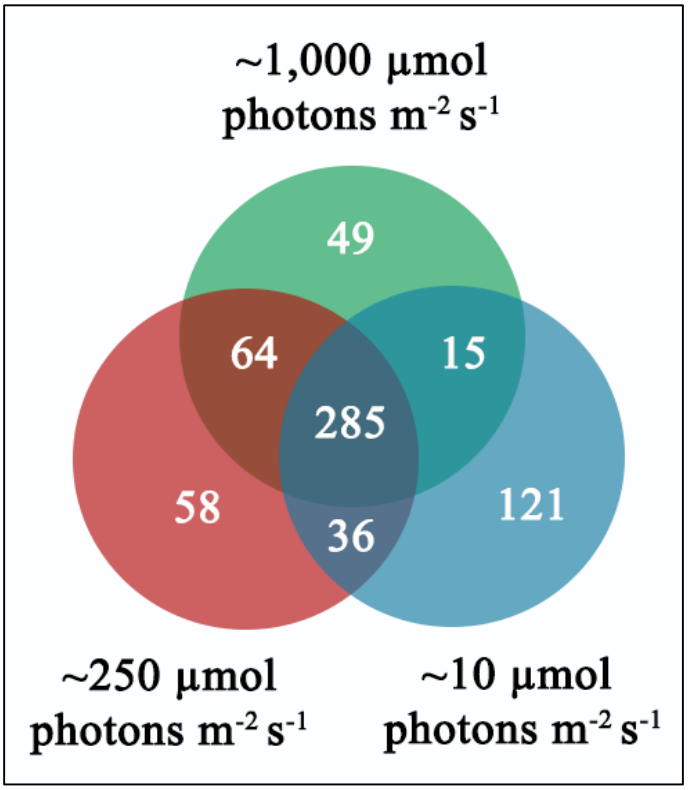

Figure 8: Venn diagrams representative of the GO terms enriched in each of the light conditions. Labelling units are in µmol photons m^{-2} s^{-1}.

In ~10 μmol photons m^{-2} s^{-1}, 121 GO terms were enriched (A.1; Table 4). Many terms were associated with ROS activity, anti-oxidant homeostasis, fatty acid oxidation and secondary defense metabolism terms. Select terms were strongly associated with chorismate metabolism, the shikimate pathway, catechol oxidase activity, L-phenylalanine metabolism and oxylipin metabolism. In ~250 μmol photons m^{-2} s^{-1}, 58 GO terms were enriched (A.1; Table 5), they largely constituted terms associated with binding, ribosomal processes, energy metabolism, protein regulation and turnover. In ~1,000 μmol photons m^{-2} s^{-1} of light, a total of 49 GO terms (A.1; Table 6) were enriched; the terms 'starch biosynthetic process - GO:0019252'; 'starch metabolic process - GO:0005982'; 'amyloplast - GO:0009501'; 'glycogen (starch) synthase activity - GO:0004373' were enriched.

Table 5: Number of unigenes distributed across GO categories putatively involved in light associated processes and light harvesting.

GO Accession	GO terminology	# Unigenes
GO:0015979	Photosynthesis	151
GO:0016168	Chlorophyll binding	85
GO:0009522	Photosystem I	82
GO:0009765	Photosynthesis, light harvesting	70
GO:0009523	Photosystem II	19
GO:2000028	Regulation of photoperiodism, flowering	13
GO:0048573	Photoperiodism, flowering	12
GO:0009584	Detection of visible light	11
GO:0009881	Photoreceptor activity	11
GO:0009585	Red, far-red light phototransduction	9
GO:0009416	Response to light stimulus	9
GO:0009640	Photomorphogenesis	4
GO:0009882	Blue light photoreceptor activity	3
GO:0009638	Phototropism	3
GO:0009637	Response to blue light	2
GO:0009644	Response to high light intensity	1
GO:0010304	PSII associated light-harvesting complex II catabolic process	1

3.3.3 Metabolic pathway enrichment across light irradiances

KEGG mapping of the assembled unigenes using KAAS revealed a total of 11,686 KAAS annotations. 25 of these were annotated under 'carbon fixation in photosynthetic organisms (ko00710)', 86 under 'carbon metabolism (ko01200)', 34 under 'photosynthesis (ko00195)' and 12 under 'photosynthesis - antenna proteins (ko00196)'. To look for enriched metabolic pathways in each of the three light regimes, plantGSEA analysis was performed. Analysis revealed the enrichment of 31 pathways in the presence of ~1,000 μmol photons m^{-2} s^{-1} (A.1; Table 7). 'Starch and sucrose metabolism' (FDR=4.20E-4) and 'Starch biosynthesis' (FDR=3.84E-3) were enriched in ~1,000 μmol photons m^{-2} s^{-1} when compared to ~250 and ~10 μmol photons m^{-2} s^{-1}. With respect to higher irradiance, the enrichment of 'xanthophyll cycle' (FDR=0.049) and 'antheraxanthin and violaxanthin biosynthesis' (FDR=0.049) was observed. In ~10 μmol photons m^{-2} s^{-1}, 50 pathways were found to be enriched (A.1; Table 9), amongst these 'Photosynthesis – antennae proteins' (FDR = 4.15E-4), the PlantCyc pathway 'Sucrose degradation III' (FDR = 9.74E-4), 'glycolysis/ gluconeogenesis (FDR =9.74E-

4)' and 'UDP-D-Xylose biosynthesis (FDR = 3.78E-4)' were all enriched. Additionally, in ~10 μmol photons m^{-2} s^{-1}, terms associated with nitrogen metabolism and plant secondary metabolism were enriched, including the pathway 'Plant-pathogen interaction (FDR = 5.23E-4)'.

3.3.4 Antennae complex genes involved in light capture and response

To identify the functional orthologs of *Zostera marina* chlorophyll a-b binding proteins in *Z. muelleri,* RBH (Reciprocal Best Hit) analysis was conducted between the *Z. muelleri* transcriptome and the annotated *Zostera marina* protein sequences. A total of 8 unigenes were identified which provided an RBH hit to *Zostera marina,* these unigenes were regulated negatively in a light-independent manner (more abundant in low light than high light). The unigenes were further queried against the NCBI non-redundant protein database using BLASTP (e-value 1.0 $x10^{-5}$); given that the *Z. marina* uniprot entries lacked detailed description. The chlorophyll a-b binding unigene, which changed the most in abundance across light treatments, was *Zosmul42810* – the light harvesting complex II (LHCB type 1-like) gene. In ~1,000 μmol photons m^{-2} s^{-1} it was expressed at 1,853.51 TPM, compared to 6,907.43 TPM in 250 μmol photons m^{-2} s^{-1} and 12,300.89 at 10 μmol photons m^{-2} s^{-1} of light. *Zosmul34312* - Chlorophyll binding protein 151 underwent the second largest change between ~1,000 μmol photons m^{-2} s^{-1} (697.63) and ~10 μmol photons m^{-2} s^{-1} (10,937.39). Similarly, in ~10 μmol photons m^{-2} s^{-1} an upregulation of chlorophyll a-b binding proteins associated with both photosystems II and I was observed: *Zosmul10406* – LHCA2*1; *Zosmul12429* – CP24 10A; *Zosmul34314* – Chlorophyll binding protein 13; *Zosmul38563* – CP26; *Zosmul47324* – Chlorophyll binding protein 7 and *Zosmul912* – CP29.

3.3.5 Identification of transcriptional regulators and microsatellites

Profiling of the transcription factors in *Z. muelleri* revealed a total of 6,971 transcriptional regulators (Fig. 9). C_2H_2 type transcription factors (1,039), WD40-like (894) and MYB-HB-like (490) were the most abundant transcription factors. 274 BHLH (Basic Helix-Loop-Helix) and 205 HAP3/NF-YB transcription factors were identified in *Z. muelleri*. Moreover, 35 FAR transcriptional regulators were identified. Transcription factor analysis revealed 40 ARF (Auxin Response Factor) transcription factors and 72 Auxin-Indole-3-Acetic Acid (Auxin-IAA) transcription factors.

A total of 20,731 unigenes contained SSRs. In total 33,726 SSRs were identified;

7,988 unigenes contained more than 1 SSR, 3,651 SSRs were found in compound formation. The most common class of SSRs identified (14,326 occurences) were tri-nucleotide repeats, followed by tetra-nucleotides (9,086 occurences) (Fig. 10). The number of iterations ranged from 3 to 29 repeats, with 4 iterations being most common, followed by 3, 5 and 10 iterations. SSRs with more than 12 iterations were rare (less than 1%). Among repetitive motifs, AAG/CTT repeats (4,392; 13.02%) were most common, followed by A/T (3,168; 9.39%) and AGG/CCT (2,428; 7.20%) repeats. The top 10 repetitive motifs are given (A.1; Table 10). SSR information and primers for validation can be found at the following URL: https://www.dropbox.com/s/ 3x3pjoxuplfo4p7/ SSRs_and_primers_Zmuelleri.xlsx?dl=0.

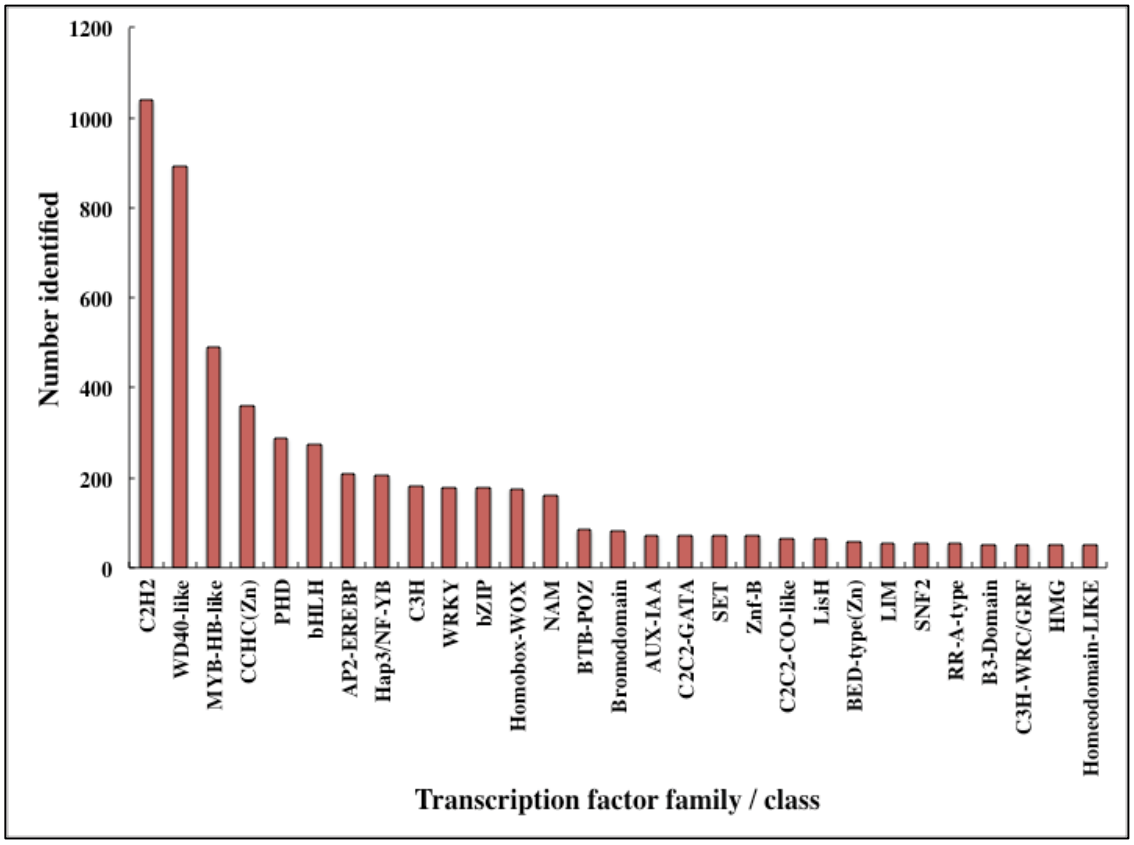

Figure 9: The transcription factors family/types (represented by a minimum of 50 occurrences) identified in the *Z. muelleri* transcriptome.

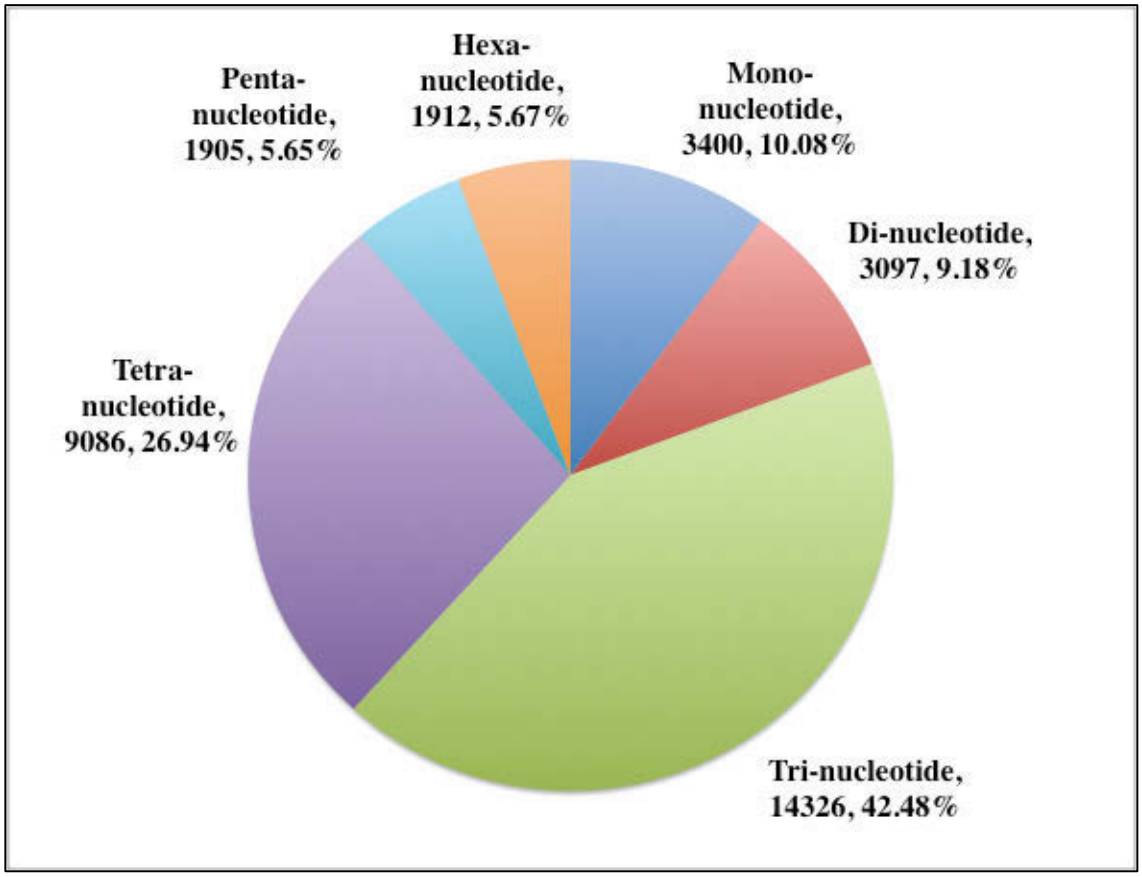

Figure 10: Pie chart showing the composition (in %) of SSR sequence types in *Z. muelleri*.

3.4. Discussion

De novo transcript re-construction of non-model plant species is a challenging task compared to genome-guided transcriptome assembly (Martin and Wang, 2011). Several approaches such as single K-mer and multi K-mer have previously been described and compared for optimal construction of transcriptomes (Sablok et al., 2014). In this study, Trinity (Haas et al., 2013) was used, which is a de-Bruijn assembler. It accurately constructs transcripts taking sensitivity and computational resource scalability into account (Haas et al., 2013). Despite such benefits offered by de-Bruijn assemblers, *de novo* transcript reconstruction remains challenging as many redundant transcripts are produced (Zhao et al., 2011). By utilising a redundancy removal protocol, manual contamination screening and a gene expression threshold of 4TPM (Wagner et al., 2013), a reduced set of unigenes was obtained which were well supported by read coverage and higher plant functional annotations.

To quantify the accuracy of the transcriptome, several approaches including CEGMA (Parra et al., 2007), orthology-based functional conservation (Mundry et al.,

2012) and functional homology assignments have been used previously. BUSCO analysis reported a high percentage of single-conserved orthologs that were duplicated in the final transcriptome; it has been reported that *Z. marina* (Olsen et al., 2016) underwent a whole genome duplication. Although the *Z. muelleri* draft genome was not available during the analysis of this chapter (Lee et al., 2016), it was recently published and highlights that *Z. muelleri* did undergo a possible further whole genome duplication event separate from *Z. marina*. The increase observed in N50 size between the original assembly and the final assembly indicates the reporting of larger, more complete transcripts in our final assembly. A high percentage of proper-paired reads used to assemble the transcriptome were succesfully re-aligned to the final assembly. The GC% content of the final *Z. muelleri* transcriptome (Table 3) assembled in this study coincides with previous reports for land plants and the marine angiosperm *Z. marina* (Garg et al., 2011; Huang et al., 2014; Kong et al., 2014; Sablok et al., 2014).

CRBB analysis (Table 4) demonstrated high coverage of the final transcriptome against flowering and ancestral plants. Most CRBB unigenes were assigned to *Zostera marina*, *Zea Mays* and *Musa acuminata*, indicating the relatedness of *Z. muelleri* to other flowering plants and ancestral plants. Functional distribution hit analysis results demonstrated that a large proportion of unigenes were assigned to *Z. marina*, followed by *Musa acuminata* and *Vitis vinifera* gene annotations. The large proportion of functional hits to *Z. marina* annotations highlights the similarities between these two species within the *Zostera* genus. All assembly assessment procedures conducted in this study indicated that the final transcriptome was of sufficient quality and appropriate for mining genes of interest.

GO terms and InterProscan protein domain terms were assigned to a large proportion of the final transcriptome; moreover, a high number of complete ORFs were detected, highlighting the high degree of annotation and functionality within the transcriptome. The identification of unigenes associated with the GO terms 'Chlorophyll binding – GO:0016168' and 'Photosynthesis, light harvesting – GO:0009765' highlight the importance of chlorophyll binding and photosystem adjustments associated with light absorption in *Z. muelleri*. One way that plants can adapt to changing light regime is through the regulation of chlorophyll binding proteins associated with both photosystems II and I, which help regulate light absorption in plants. These are highly conserved components of the photosynthetic apparatus and are

partially responsible for the regulation of energy flow (Barros and Kühlbrandt, 2009). The identification of 'Red, far-red light phototransduction', 'Response to blue light' and 'Blue light photoreceptor activity' suggest *Z. muelleri* can detect varying wavelengths of light at the transcriptional level, as described in *Z. marina* and other higher plants (Olsen et al., 2016). Sessile marine animals such as *Acropora millepora* corals also have the ability to detect blue light through crypotchromes, suggesting blue light is an important environmental factor for functioning and development in marine organisms (Levy et al., 2007).

Enrichment of the terms associated with photosynthesis in ~250 and ~10 μmol photons m^{-2} s^{-1} suggest responses and changes in photosynthesis and light harvesting function associated with lower irradiances. It is common for plants to adjust their photosynthetic apparatus based on the amount of light that is available. The enrichment of 'photo-respiration - GO:0009853' and 'ribulose-bisphosphate carboxylase activity – GO:0016984' along with 'hydrogen peroxide metabolic process - GO:0042743' and 'hydrogen peroxide catabolic process - GO:0042744' may indicate a stress response in *Z. muelleri* in response to lower irradiance. Previous research has highlighted that photorespiration occurs within green plant tissues in response to ROS accumulation (Voss et al., 2013) during stress. The enrichment of 'protochlorophyllide reductase activity - GO:0016630' was observed in the lower irradiances. In several plant species the light independent (dark) chlorophyll-producing enzyme is found to cause greening in response to reduced environmental light (Fujita and Bauer, 2000). In *Z. muelleri* previously at a physiological level, this enzyme has been shown to increase in darkness, allowing for light-independent synthesis of chloroplasts and chlorophyll (Adamson et al., 1985). In shade tolerant plants including seagrasses, an increase in chlorophyll *a* and *b* occur during light limitation (Abal et al., 1994; Silva et al., 2013) in order to enhance light absorption for photosynthesis. This enzyme thus serves a purpose in maximising light absoption in *Z. muelleri* in low light environments.

When GO enrichment analysis was conducted on ~10 μmol photons m^{-2} s^{-1} plants, a strong response in plant defence and secondary metabolism was evident. In the seagrass, *Posidonia oceanica*, enrichment of antioxidant terms have also been documented (Dattolo et al., 2013); the authors have suggested plants are more vulnerable in low light environments due to the reduced energy budget as a result of decreased photosynthetic activity. Energy is essential to support an efficient immune

system. Analysis also demonstrated enrichment of oxylipin metabolism in 10 μmol photons m^{-2} s^{-1}, this process is an important aspect of stress signaling and innate immunity in plants (Howe and Schillmiller, 2002). The production of phenylpropanoids and polyphenol oxidases (PPOs) also observed at 10 μmol photons m^{-2} s^{-1} have previously been documented to accumulate in plants exposed to low irradiance (Mohr et al., 1979; Ibrahim and Jafaar, 2012; Zhan et al., 2014). The data suggests that under low light, *Z. muelleri* plants may have compromised immunity to the environment and as such increase their defense metabolism pathways. In ~250 μmol photons m^{-2} s^{-1} important processes were enriched including processes necessary for energy metabolism, protein turnover, functioning and development.

The enrichment of 'starch biosynthetic process - GO:0019252', 'starch metabolic process - GO:0005982', 'amyloplast - GO:0009501' and 'glycogen (starch) synthase activity - GO:0004373' in ~1,000 μmol photons m^{-2} s^{-1} of light indicate increased starch production in higher irradiances, which is generally associated with increased photosynthetic rates (Huber and Huber, 1992). Amyloplasts are the compartments in which starch is processed and stored within (Bechtel and Wilson, 2003); enrichment of such cellular compartments additionally supports evidence in this study, that starch is being produced in increasing amounts under ~1,000 μmol photons m^{-2} s^{-1} compared to the lower irradiances.

KAAS analysis highlighted the presence of various genes associated with carbon fixation and photosynthesis in *Z. muelleri*. The 25 sequences involved in 'carbon fixation in photosynthetic organisms (Ko00710)' included enzymes that are typically associated with C4 metabolism. Whilst the systematics of the photosynthetic pathway of the *Zostera* genus remains undefined, there have been previous suggestions that *Zostera* possess C3-C4 intermediate pathways (Larkum, 2006). It must be noted that the existence of C4 enzymes are also common in C3 plants and can have anapleurotic roles such as replenishing intermediates of the TCA cycle (Aubry et al., 2011). PlantGSEA analysis (A.1; Table 7) results indicated enrichment of 'Starch and sucrose metabolism' and 'Starch biosynthesis' in ~1,000 μmol photons m^{-2} s^{-1}. The enrichment of these specific pathways is in agreement with the GO enrichment analysis results for *Z. muelleri* plants in ~1,000 μmol photons m^{-2} s^{-1}, providing a strong signature of CO_2 fixation into starch and the production of sucrose in high irradiance. The enrichment of 'xanthophyll cycle' and 'antheraxanthin and violaxanthin biosynthesis' at ~1,000 μmol

photons m^{-2} s^{-1} suggests the protective xanthophyll cycle is preventing photo-oxidative damage from occuring under high irradiances (Jahns and Holzwarth, 2012). This has previously been observed at the physiological level in *Z. muelleri* (Petrou et al., 2013). In ~10 μmol photons m^{-2} s^{-1}, the enrichment of 'Photosynthesis – antennae proteins' suggests a conformational change in antennae, presumably allowing for efficient light capture in light limited environments. The enrichment of 'Sucrose degradation III', 'glycolysis/ gluconeogenesis' and 'UDP-D-Xylose biosynthesis' point towards modification of sink to source carbon ratios within the plants. In parallel with carbon metabolism, the enrichment of terms associated with nitrogen metabolism were observed, plants are known to change their carbon and nitrogen ratios in close association with changes in irradiance and photosynthesis (Paul and Pellny, 2002). 'Plant-pathogen interaction' observed in plantGSEA analysis further compliments GO enrichment results, suggesting a compromise in *Z. muelleri* immunity under ~10 μmol photons m^{-2} s^{-1}.

In *Arabidopsis*, LHCII is found to be expressed in lower quantities in intense light (Wientjes et al., 2013) and the LHCII complex undergoes proteolysis in higher irradiances (Yang et al., 2001). In this study, lower quantities of the LHCII unigenes were found in ~1,000 μmol photons m^{-2} s^{-1} compared to ~10 μmol photons m^{-2} s^{-1}, suggesting the LHCII chlorophyll binding proteins serve an important role in enhancing light absorption for photosynthesis. In *Z. marina* (Kong et al., 2016), LHCII genes were generally found to be down-regulated under high irradiances. Lower levels of LHCI unigenes were found in *Z. muelleri* under ~1,000 μmol photons m^{-2} s^{-1} as opposed to ~10μmol photons m^{-2} s^{-1}. This was also observed in *Z. marina* (Kong et al., 2016). The LHCI complex is also regulated in a light-dependant manner (Durnford et al., 2003). In Barley, the CP29 protein and LHCI are found to increase in low light conditions (Humbeck and Krupinska, 2003). In *Z. marina*, the LHCII antennae complex protein family has undergone expansion during evolution, presumably due to lower irradiances in the water column compared to terrestrial environments of higher plants (Olsen et al., 2016).

Transcription factors regulate transcription by binding to the cis-regulatory elements of genes, in turn regulating gene expression in response to a wide variety of stimuli including light (Naika et al., 2013). In the transcriptome assembly, C_2H_2 transcription factors were most abundant and are known to play important roles in

physiological processes, hormonal signal transduction and response to environmental stimuli in plants (Jiang and Pan, 2012; Kielbowicz-Matuk, 2012). The second most abundant transcription factor family identified was 'WD40-like'; these are known to be involved in a variety of cellular processes within eukaryotes. They are a diverse super-family of regulatory genes and a select few WD40-like domains such as *COP1* and *SPA1* are found also to be associated with light signaling and photomorphogenesis (van Nocker and Ludwig, 2003). BHLH (Basic Helix-Loop-Helix) and HAP3/NF-YB transcription factors were also found, these have been associated with development, light-mediated signaling and phytochrome regulation (Miyoshi et al., 2003; Duek and Fankhauser, 2005). FAR transcription factors also identified have previously been associated with phytochrome A and regulative feedback mechanisms (Hudson et al. 2003). The identification of ARF (Auxin Response Factor) transcription factors and Auxin-Indole-3-Acetic Acid (Auxin-IAA) transcription factors highlight the important role that play in maintaining plant function and development in varying light conditions (Hoecker et al., 2004; Halliday et al., 2009; Keuskamp and Pierek, 2013).

3.5 Conclusion

The *de novo* transcriptome assembly reported in this study reveals specific gene groups involved in light responses of *Z. muelleri*. In this study, gene groups associated with photosynthesis, light responses and modification of light harvesting complexes were described. Closer investigation of the expression profiles of chlorophyll a-b binding genes shows distinct changes with respect to light limitation. Results indicate these genes generally follow similar regulation to light as seen in in *Z. marina* and terrestrial plants. The additional ecogenomic resources mined from the transcriptome provide access to data in order to design functional molecular markers for *Z. muelleri*. Such information comes at a time when a lack of genic resources exists for Australian seagrasses.

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CHAPTER 4

Changes in photophysiology and gene expression in *Zostera muelleri* in response to light limited environments

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Abstract

Despite documented loss of *Zostera muelleri* meadows in Australia and New Zealand in response to light limitation, the coordinated molecular and physiological mechanisms driving the response of this keystone species to light limitation remains largely unexplored. In order to understand the molecular mechanisms of the photo-physiological response, a molecular physiology approach was used, which incorporated mRNA-Seq analysis, chlorophyll fluorometry and pigment profiling using HPLC to study the effect of light limitation on *Z. meulleri* over six days. 1,593 (7.51%) genes of a leaf-tissue specific transcriptome (21,225 total genes) were found to be differentially expressed on Day 2, and 1,481 (6.98%) genes were differentially expressed on Day 6. On both days, genes associated with photosynthesis, pigments and light response were differentially expressed. Gene regulation was correlated with significant decreases in $rETR_{max}$ and I_k, and an increase in Yi. The down-regulation of photo-protective pigments and the accumulation of photosystem antennae enhancing pigments also occurred in response to light limitation. A switch from photosynthesis to carbohydrate stores for energy requirements was observed on Day 6. Interestingly, significant changes in the expression transcripts encoding putative abscisic acid (ABA) related functions were observed on both Days 2 and 6, suggesting a potential role of ABA in seagrasses in response to light limitation.

4.1 Introduction

Zostera muelleri provides habitat, promotes marine biodiversity, prevents coastal erosion and accounts for significant blue carbon stocks (Björk et al., 2008, Mcleod et al., 2011) in Australia and New Zealand. This species is found in estuarine and sheltered sub-tidal waters, and its depth distribution is strongly influenced by light availability in the water column (Dennison et al., 1993), where light is critical for optimum functioning, growth and survival of seagrasses (Ralph et al., 2007). Lack of light in the marine water column has been strongly correlated with *Zostera muelleri* meadow decline in the past (Preen et al., 1995; Kirkman, 1997; Campbell and McKenzie, 2004; Ralph et al., 2007). Further loss of *Z. muelleri* meadows is a realistic scenario due to forecasted climate change and coastal development (Ralph et al., 2007). To date, various morphological and physiological changes have been documented in seagrasses in response to light (McMahon et al., 2013). Chlorophyll fluorometry based methods have been used to study the responses of *Zostera* seagrass to changes in environmental light (Ralph et al., 2002; Schwarz et al., 2004; Ralph and Gademann, 2005; Turner et al., 2006; Bité et al., 2007; Silva et al., 2013; McMahon et al., 2013). Chlorophyll fluorometry provides estimates of the efficiency of photosystem II and the rate of photosynthesis in seagrasses, and the latter can be directly measured via electron transport rate through rapid light curves (RLCs) (Ralph and Gademann, 2005). HPLC profiling of changes in pigment concentration provides an additional means to assess the photophysiological adaption of *Zostera muelleri* to changing light (Ralph et al., 2002; Petrou et al., 2013; York et al., 2013; Maxwell et al., 2014). Together, both approaches have shown that *Zostera* responds to changing light levels through adjustment of photosynthetic electron transport rate and shifts in pigment composition. However, the gene regulatory processes by which *Z. muelleri* responds and adjusts to changing light levels are not yet known.

Recent investigations of seagrasses through Next Generation Sequencing (NGS) have begun to describe the molecular and genetic details of seagrass biology in detail (Davey et al., 2016), including the draft genome of *Z. muelleri* (Lee et al., 2016). Transcriptomics through NGS sequencing of mRNA (RNA-seq) allows for the broad quantification of the expression of all transcribed genes in an organism at a given time and condition. This technology has become popular for understanding the molecular regulation of plants in response to environmental change (Egan et al., 2012) and can

provide us with information in early transcriptional responses in seagrasses (Franssen et al., 2014; Kong et al., 2014; Jueterbock et al., 2016).

In this study, using a molecular physiology approach, combining pigment profiling, photobiology measurements, and transcriptomics, these techniques were used to assess the biological response of *Z. muelleri* in detail. This approach allowed for the characterisation of how *Z. muelleri* responds to light limitation over a time period of 6 days, including time-resolved changes in transcriptional regulation and photophysiology. This study is an important step in further understanding how this species responds and acclimates to light limited environments due to anticipated meadow decline in the future.

4.2 Materials and methods

4.2.1 Field analyses and sample transplantation

The field site chosen for this study was an enclosed marine lagoon – Narrabeen Lakes situated in Narrabeen, New South Wales, Australia. For field based analyses and sample collection, an area of seagrass meadow was chosen as highlighted (Fig. 11). All fieldwork was conducted in the winter period of August, 2015. *Z. muelleri* plants with intact sediment were collected. In order to mimic the conditions of Narrabeen Lakes at the University of Technology Sydney (UTS) aquarium facility, salinity and temperature of the water were measured in the field. The ambient salinity was 28 units (Lewis, 1980) and the water temperature was 20°C. Rapid light curves (RLCs) of *Z. muelleri* were conducted roughly at mid-day to determine suitable light conditions for the experiment (A.2; Fig. 1). Seagrass plants were transported to the aquarium facility at UTS in darkened containers with seawater to prevent desiccation and to minimise stress.

4.2.2 Aquarium set-up and acclimation period

Excess sediment and epiphytes were gently removed at UTS and the plants were gently washed using saline water (28 units). Individual plants including below ground tissue were then separated from ramets and introduced into four separate aquaria at random. Approximately 30-40 individual plants were transplanted into each aquarium. Below ground tissue was buried in the sediment and rhizomes were kept horizontal. Each aquarium contained approximately 4-5 cm of sediment (40% natural sediment: 60% washed sand; commercially available from Bunnings Warehouse, Australia).

Filtered seawater was filled to 4 cm below the top of each aquarium, and maintained at this level for the duration of the experiment. Water flow and air were supplied to the aquaria in the form of submerged Elite mini pumps (Hagen, Canada) and airstones in order to maintain efficient gaseous diffusion across the leaf boundary layers of the leaves (Larkum et al., 2006).

Figure 11: Scaled topographical map of Narrabeen Lakes. Sampling site is indicated by a blue star. North arrow and scale represented. Map created using ArcGIS online (esri).

A 28 x 30 cm area in the middle of each aquarium was chosen for transplantation; to control for variation in light intensity ranges across each aquaria. Light was provided by aquaria lighting systems (Aqua Illumination Hydra 52 LED; Iowa, USA) hanging 35 cm above the water surface; one system was placed over each aquarium. Acclimation of all tanks lasted 18 days. Light acclimation consisted of a 12 hour dark: 12 hour light cycle in the form of a ramping light regime whereby mid-day maximum irradiance was ~200 μmol photons $m^{-2} s^{-1}$ at canopy level to mimic the diurnal cycle at Narrabeen Lakes. A calibrated light meter (Licor 250A) and 2-pi underwater irradiance sensor were used at canopy height to determine irradiance levels prior to

acclimation. Throughout the entire experiment, ambient water temperature was maintained at 19±1°C; salinity was kept at 28 ± 1 units and adjusted when necessary using deionised water /seawater to mimic environmental conditions at Narrabeen Lakes. Odyssey light loggers (Dataflow Systems Limited, New Zealand) were programmed, calibrated and inserted into tanks at canopy level to record ambient irradiance (every 5 minutes) over the duration of the experiment (A.2; Fig. 2). The shade treatment aquariums were switched to shading light regimes (20 µmol photons m^{-2} s^{-1} mid-day maximum irradiance) pre-dawn on experiment Day 1, in order not to disrupt the experiment (Fig. 12).

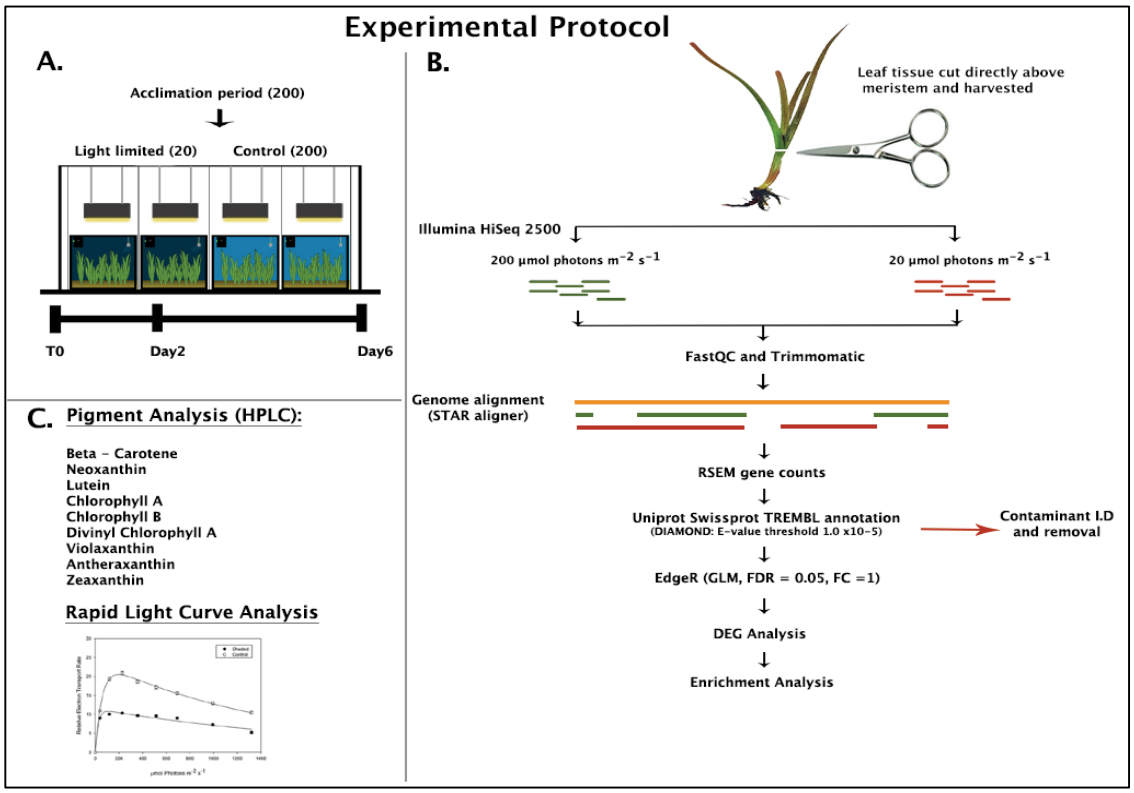

Figure 12: Experimental protocol and layout. A. Mesocosm layout detailing acclimation period, experimental treatments and sample collection time points. Light units are µmol photons m^{-2} s^{-1}. B. mRNA-Seq genome guided pipeline to determine transcriptome and differential gene expression, green reads = 200 µmol photons m^{-2} s^{-1}; red = 20 µmol photons m^{-2} s^{-1} and orange = genome. C. Physiological and photobiology measurements recorded including the pigments analysed. Plants for RNA-sequencing, pigment analysis and rapid light curve analysis were all independent of one another.

4.2.3 Photo-biology measurements and rapid light curve analysis

Chlorophyll fluorometry measurements were conducted using a Walz dive PAM unit, diving F-probe and attachable leaf clip. For all PAM measurements, the bottom of leaf number two was used in order to make comparisons across treatments. Leaf number two has been used for direct measurements of seagrass chlorophyll fluorometry previously (Ralph et al., 2002; Petrou et al., 2013). Once the clip was attached to the leaf, a five second period of darkness followed in order for an initial PSII quantum yield (Yi) measurement to be made (Ralph and Gademann, 2005). For all measurements the following settings were used: Measuring Intensity = 8; Saturation Intensity = 8; Out-Gain = 2; Saturation width = 0.8s. For RLC analysis, eight actinic light intensities were used to form Photosynthesis-Irradiance (P-I) curves: 38, 118, 227, 358, 515, 691, 993 and 1322 μmol photons m^{-2} s^{-1}. Actinic light steps lasted for 10 seconds each. RLC analysis was done before the start of the experimental (to ensure acclimation was successful), Day 2 and Day 6; all measurements were conducted at mid-day (n = 5, unless stated). All measurements were conducted on the same plants for each treatment throughout the experiment.

4.2.4 Leaf tissue sampling and processing

Samples for RNA extraction and pigment analyses were taken at mid-day on Day 2 and Day 6 for control and light limited treatments. Plants were randomly sampled across both aquaria for each treatment. Entire leaf tissue from above the basal meristem (all of the plant leaves) of individual plants (n = 3) was harvested, cleaned with saline water to remove as much contaminating matter as possible, and snap frozen in liquid nitrogen. For pigment samples, the same protocol applied (n = 3). Samples were stored at -80ºC until processing.

For RNA extraction; sterilised, pre-chilled pestle and mortars were used to grind tissue into fine powder in liquid nitrogen; between 80-130mg of fresh weight tissue was used in conjunction with an Ambion PureLink mini RNA extraction kit and Purelink On-Column DNAse digest kit. Nanodrop 2000 technology and Agilent Bioanalyzer 2100 with nanochip technology was used to measure and assess the quality of the extracted RNA. All RIN numbers were above 7, providing reliable, high quality material for sequencing. A TruSeq mRNA stranded kit, with an oligo-dT mRNA

enrichment protocol was used in combination with the Illumina Hiseq2500 paired end sequencing platform by ANU sequencing facility, Canberra, Australia.

Finely ground seagrass samples were extracted in the absence of light and added to 1.5 ml of chilled HPLC grade acetone in amber glass vials for High Performance Liquid Chromatography (HPLC) analysis of the pigments. Samples were vortexed for 30 seconds (x 3 times) and stored at -20°C overnight. Pigment extracts were then filtered through 0.2 μM PTFE 13mm syringe filters and stored at -80 °C until analysis. An Agilent 1290 HPLC system equipped with a binary pump with integrated vacuum degasser, thermostatic column compartment modules, Infinity 1290 autosampler and PDA detector were used for the analysis. Column separation of pigments was performed using Agilent's Zorbax Eclipse XDB C8 HPLC 4.6 mm × 150 mm and guard column using a gradient of tetrabutylalkylammonium acetate (TBAA) methanol mix (30:70) (solvent A) and methanol (Solvent B) as follows: 0–22 min, from 5 to 95% B; 22–29 min, 95% B; 29-31 min, 5% B; 31-40 min, column equilibration with 5%B. Column temperature was maintained at 55°C. A sandwich injection approach was set using the auto injector program, where the TBAA buffer and samples were drawn alternatively in the sequence, 310:30:300:30:230 (μL) and then mixed in the loop and injected. A complete pigment spectrum from 270 to 700 nm was recorded using a PDA detector with 3.4 nm bandwidth. Calibration was performed using individual pigment standards (DHI, Denmark).

4.2.5 Read processing, transcriptome assembly and annotation

Raw read quality was visualised using FastQC. For quality trimming, Trimmomatic (Version 0.2.35; Bolger et al., 2014) was used with the following settings: ILLUMINACLIP: TruSeq3-PE-2.fa:2:30:10 LEADING:5; TRAILING:5; SLIDING WINDOW:4:5; MINLEN:25. Quality checks were performed a second time via FastQC to ensure that the removal of poor quality reads was successful. For alignment of the reads to the *Z. muelleri* genome, STAR ultrafast aligner (version 2.5.2b; Dobin et al., 2013) was used. An index of the genome was created with the options sjdbGTFtagExonParentTranscript Parent and sjdbOverhang = 100. The options alignIntronMax = 25,000 (Li et al., 2015) and quantMode were used in conjunction with RSEM (Li and Dewey, 2011) for alignment and read counts. Uniprot Swissprot and Uniprot TREMBL databases were downloaded from the Uniprot FTP server (August,

2016) for annotation of genes. DIAMOND BLASTX protein aligner (Buchfink et al., 2015) was used for protein alignment and annotation. An e-value threshold of 1.0 x 10^{-5} was used. In order to retain the best BLAST hit for the genome sequences (as complete gene annotations were previously unavailable; Lee et al., 2016), sequence hits were filtered based on 3 criteria; bitscore; e-value; and % identity. Manual contamination screening was implemented for the best BLAST hits.

4.2.6 Differential gene expression analysis and gene ontology enrichment

Genes that were not plausible contaminants and provided best hits to higher plant species were taken forward for analysis in EdgeR (Robinson et al., 2010). Differential expression analysis was conducted using a GLM fit model. Filtering was utilised as suggested in the EdgeR vignette to remove genes that were not expressed in the transcriptome by at least 1 CPM (counts per million) in at least 3 out of 12 samples (3 being the group size for each condition); a recommended practice to remove possible artifacts or non-expressed genes (Chen et al., 2014). TMM-normalisation of libraries was applied. The biological coefficient of variance (BCV) was plotted to determine variability between biological replicates. For identification of differentially expressed genes, contrasts were completed using a p-value of 0.05 and an FDR multiple correction threshold of 0.05 (Benjamani and Hochberg, 1995). One log fold change (FC) was set as an additional threshold to identify significant differential expression. Hierarchical clustering was then performed on the differentially expressed genes for Days 2 and 6 by using the R package – heatmap.2; LogCPM values were z-scaled and centered. Ten-thousand-fold bootstrapped hierarchical clustering (Ashworth et al., 2016, Suzuki and Shimodaira, 2006) was additionally used to identify tightly correlated subsets of transcripts representing co-responsive and potentially co-regulated genes. First, 400 clusters were created by height-based tree cutting, a common practice for clustering which heuristically pre-supposes the expected number of clusters. Bootstrapping was then performed to test the robustness and significance of gene-level co-expression. The search for DNA sequence motifs in upstream regions (-350 to +50 bp relative to gene starts) of co-expressed genes was performed using MEME (Bailey and Elkan, 1994). GO enrichment analysis was performed using GOATOOLS (https://github.com /tanghaibao/goatools) with an FDR threshold of 0.05 (Benjamani and Hochberg, 1995),

foreground datasets consisted of up and down-regulated genes, the entire transcriptome was used as the background dataset.

4.2.7 Statistical testing of photobiology and pigment data

Sigmaplot (Version 12.5) was used to fit an empirical equation previously used for rapid light curve fitting in phytoplankton, seagrass and coral (Platt et al., 1981; Ralph and Gademann, 2005; Wangpraseurt et al., 2014). $rETR_{max}$ (relative maximum electron transport rate) Maximum, I_k (half saturation constant) and Yi (initial effective quantum yield of PSII) values were obtained from the fitted curve plots. Non-parametric methods were chosen based on the characteristics of the data for statistical analysis. Independent Mann-Whitney U statistical tests were performed to determine if light limitation had any significant effect on *Z. muelleri* photosynthetic capacity compared to the control treatment ($P<0.05$). Independent student statistical t-tests were performed on the pigment data, because the data was parametric in nature ($P<0.05$). All statistical testing was performed in IBM SPSS Statistics 21.

4.3 Results

4.3.1 Photobiology

During pre-treatment, no significant difference was observed in $rETR_{max}$ between control and LL plants (Fig. 13H; Table 6; $p = 0.690$, $z = 0.522$, $U = 15.000$), I_k ($p = 0.151$, $z = 1.567$, $U = 20.000$) or Yi ($p = 0.151$, $z = 1.471$, $U = 19.500$). Significant decreases were observed in LL compared to control plants on Day 2. The median $rETR_{max}$ in control plants (17.44) was significantly higher ($p = 0.032$, $z = -2.205$, $U = 1.00$) than the median $rETR_{max}$ in LL plants (10.95). Significant differences were observed in I_k ($p = 0.016$, $z = -2.449$, $U = <0.0005$), with a decrease in I_k between control (37.98) and LL plants (18.07). A significant increase was observed in Yi between control (0.765) and LL (0.788) plants ($p = 0.032$, $z = 2.205$, $U = 19.00$). On Day 6, significant differences were observed again in $rETR_{max}$ between control (20.58) and LL (10.77) plants ($p = 0.008$, $z = -2.611$, $U = < 0.0005$). I_k on Day 6 was also significantly different between control (49.68) and LL (23.40) plants ($p = 0.008$, $z = -2.611$ $U = < 0.0005$).

4.3.2 Pigment shifts observed through HPLC profiling

Significant changes in pigment composition were recorded on Days 2 and 6 (Fig. 13A-13G; A.2; Tables 1-2). On Day 2, a significant increase was observed in chlorophyll a (p= 0.008) and b (p = 0.007), total chlorophyll (p = 0.07) and lutein (p = 0.045) (Fig. 13). On Day 6 a significant increase was observed in all pigment profiles, apart from divinyl chlorophyll a. The violaxanthin and neoxanthin pool in LL plants increased on both days compared to control, but was only statistically significant different on Day 6 (neoxanthin, p =0.025; violoxanthin, p = 0.026). Neither antheraxanthin nor zeaxanthin pigments of the photo-protective xanthophyll cycle pool were detected during the experiment (A.2; Fig. 3).

Table 6: Statistical comparisons between control and light limited plants on Days 2 and 6. Median, p-values, U values and Z-test scores are reported for Mann-Whitney-U statistical tests. Significant (P<0.05) results are indicated by an asterix (*). The number of biological replicates was n = 5, except for Day 2 when n = 4 for light limited plants (loss of one replicate sample).

Parameter	Statistic Values	Pre-treatment	Day 2	Day 6
$rETR_{max}$	Control median	17.334	17.442	20.581
	Light limited median	18.564	10.949	10.771
	P-value	0.690	0.032*	0.008*
	U	15.000	1.000	<0.0005
	Z	0.522	-2.205	-2.611
I_k	Control median	40.858	37.977	49.677
	Light limited median	43.123	18.074	23.397
	P-value	0.151	0.016*	0.008*
	U	20.00000	<0.0005	<0.0005
	Z	1.567	-2.449	-2.611
Yi	Control median	0.765	0.765	0.766
	Light limited median	0.779	0.788	0.775
	P-value	0.151	0.032*	0.421
	U	19.500	19.000	17.00000
	Z	1.471	2.205	0.946

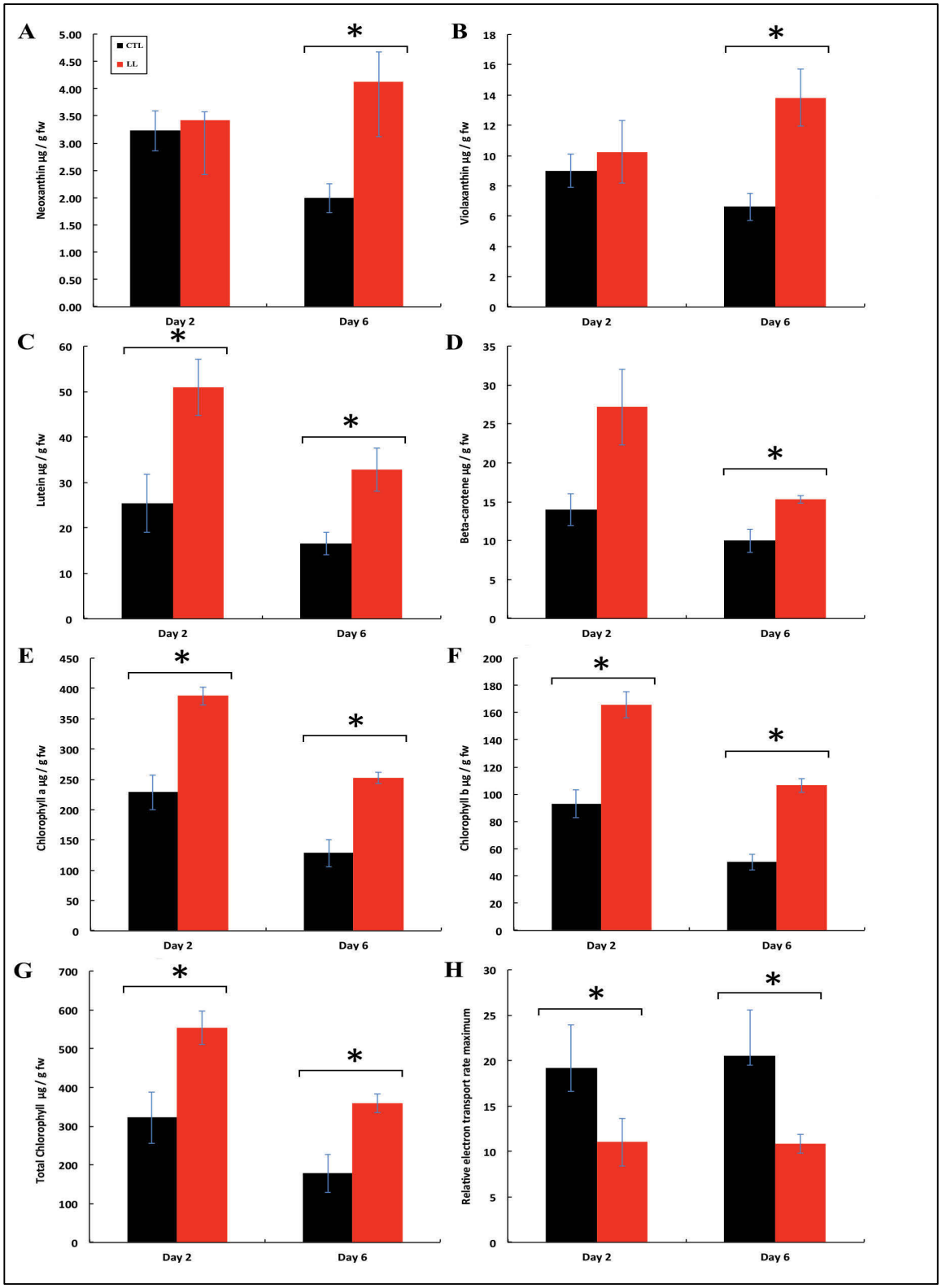

Figure 13: A – G. Pigment concentrations (µg/ gram fresh weight in control and light limited seagrass plants; n = 3). Significance is represented by an asterix (*). H. Relative electron transport rate maximum of control and light limited plants across Days 2 and 6 (n = 5). All error bars are representative of standard deviation of mean. Ctl = Control, LL = Light limited.

4.3.3 Transcriptome assembly

In total 12 libraries were used to assemble the transcriptome (A.2; Table 3). Screening of the gene annotations suggested that 647 genes previously defined in the draft genome (Lee et al., 2016) came from alternative organisms other than plants; these were declared as contaminating sequences. Most contamination came from gamma-proteobacteria (245 hits), whilst other traces of contamination came from viral, prokaryotic and other eukaryotic sources. In total 21,225 functionally annotated genes were expressed in the leaf transcriptome (59.16% of the reported genome; Lee et al., 2016). Any gene, which did not obtain an annotation from the uniprot database, was excluded from the final transcriptome assembly due to uncertainties of source. 19,074 genes (89.87%) of the leaf transcriptome obtained a match to *Z. marina*, 374 genes (1.76%) had a functional hit to the monocot species *Musa malaccensis* (banana) and 168 genes (0.88%) to *Vitus vinifera* (grapevine) (A.2; Table 4).

4.3.4 Differential expression analysis

On fitting a generalised linear model (GLM) in EdgeR, a biological coefficient of variation (BCV) of 0.237 was obtained (A.2; Fig. 4; Common dispersion = 0.056). In the differential gene analysis, multiple genes received the same annotation. Fine-grained bootstrapped heirarchial clustering and a BLASTN of the gene models from the genome (All vs. all) which were present in the transcriptome identified highly similar genes with correlated expression (A.2; Fig. 5).

Plotting logFC dimensions 1 and 2 via MDSplot (A.2; Fig. 6) indicated that there was variation between and within replicate groups. A larger spread was more evident in the light limited (LL) plants compared to control plants. 1,593 (7.51%) differentially expressed genes were identified on Day 2, of which 530 were found to be significantly up-regulated in LL plants and 1,063 genes to be significantly down-regulated in LL plants. 1,481 (6.98%) differentially expressed genes were identified on Day 6, of which 610 genes were significantly up-regulated in LL plants and 871 were significantly down-regulated in LL plants (Fig. 14). From here on in, down-regulation refers to down-regulation of genes in LL plants compared to control plants on Days 2 and 6, whilst up-regulation refers to up-regulation in LL plants compared to control plants. Fine-grained bootstrapped hierarchical clustering (Fig. 15) additionally identified examples of transcript clusters that were significantly coordinated and down-

regulated in expression in LL plants on Day 2 and more highly expressed in LL plants on Day 6 (Fig. 15b). No conclusive trends were drawn from the specifc differentially regulated genes identified in the clusters; however, common DNA sequence motifs in the putative upstream promoter regions of these genes were detected using MEME (Bailey and Elkan, 1994; A.2. Fig. 7).

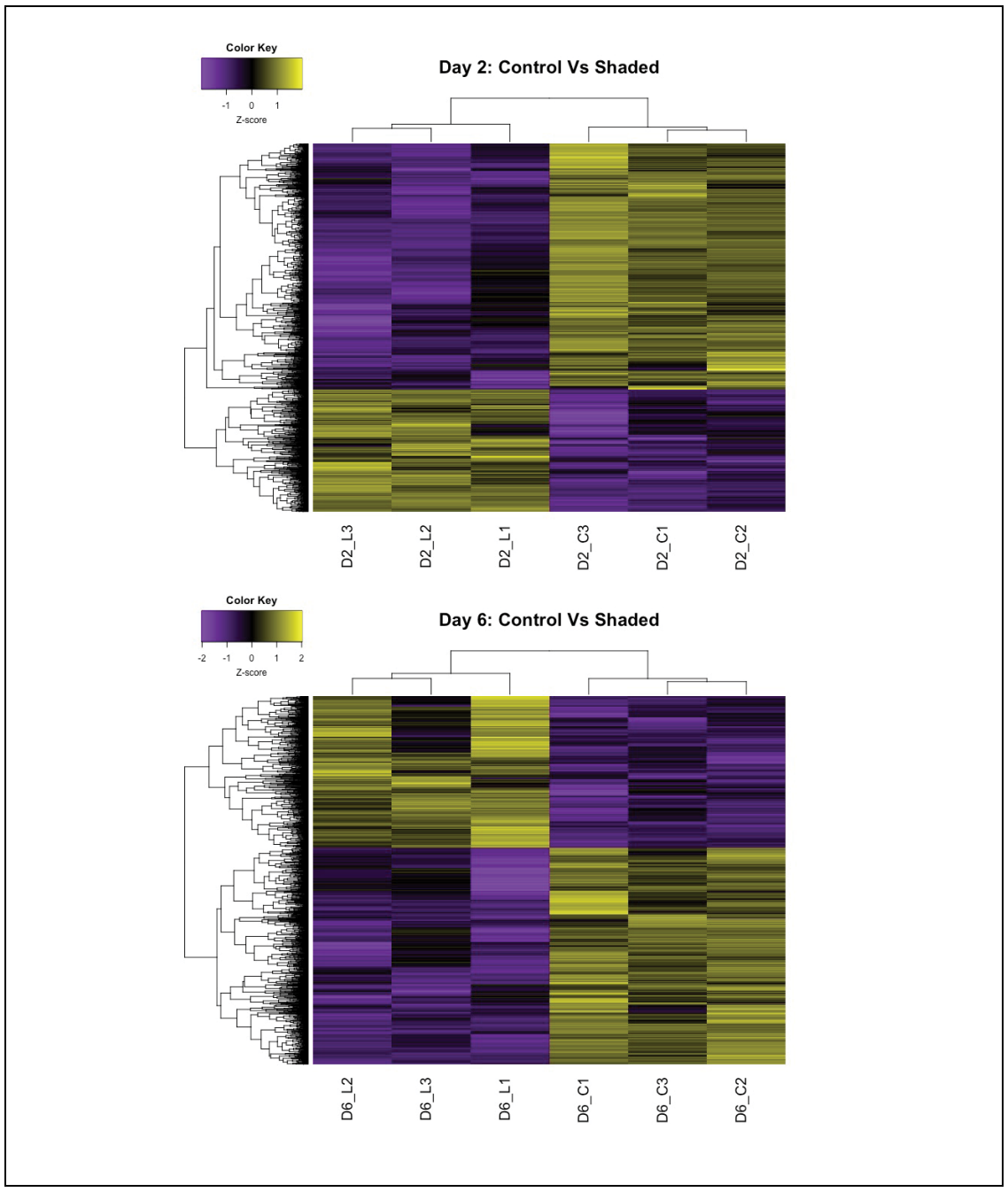

Figure 14: Differential gene expression represented by hierarchical clustered heatmaps. Normalised LogCPM gene counts are centered and z-scaled. D2 = Day 2, D6 = Day 6. Light limited replicates (L1-3; 20 µmol photons m^{-2} s^{-1}) and control plant replicates (C1-3; 200 µmol photons m^{-2} s^{-1}) are indicated.

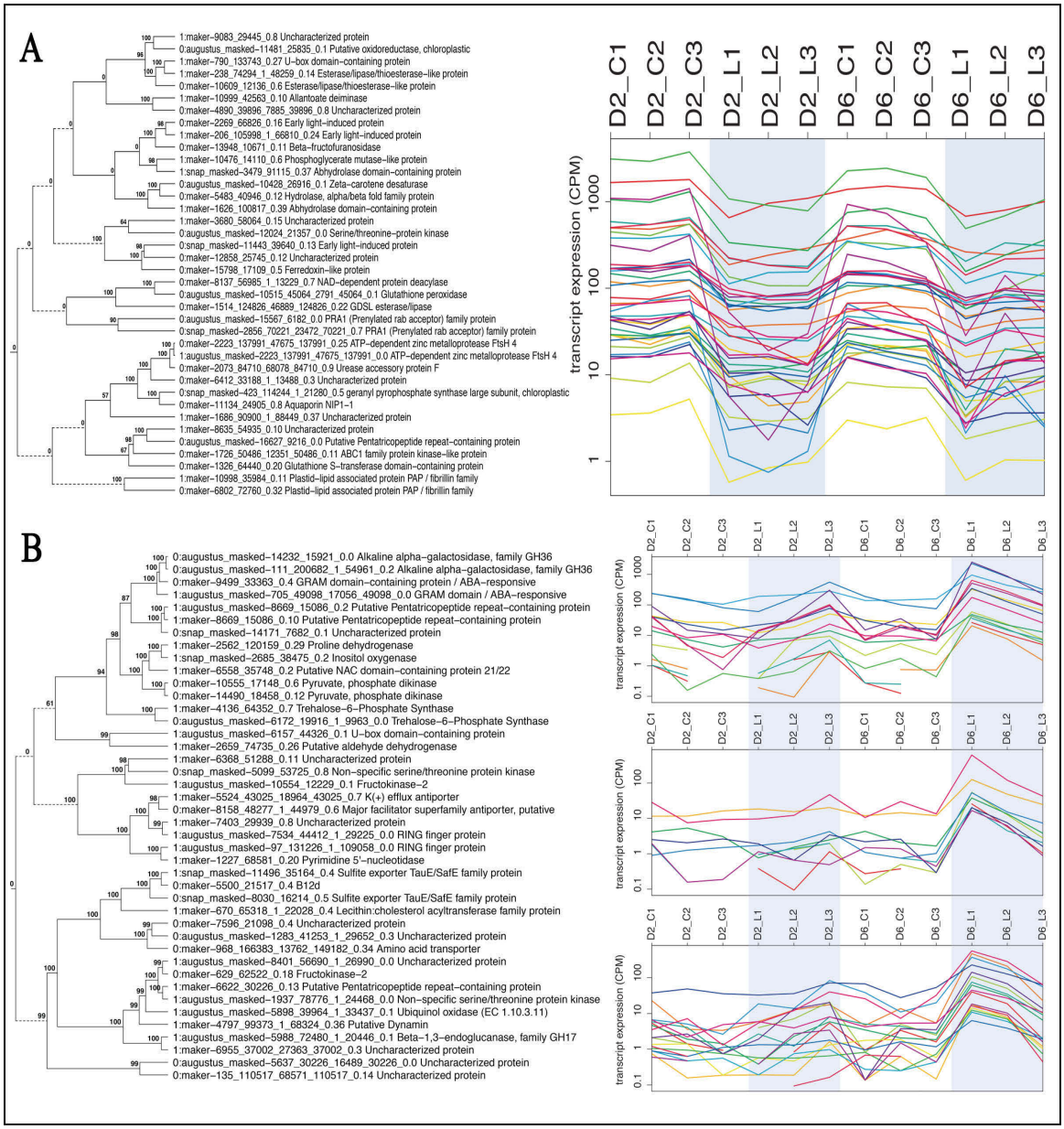

Figure 15: Bootstrapped hierarchical clustering of correlated transcripts. A. A cluster of correlated transcripts that decreased in expression under light limitation on Day 2. B. Three closely related clusters of transcripts that increased in expression under light limitation on Day 6.

4.3.5 Regulation of genes involved in photosynthesis and carbon fixation

All but 3 of the 33 genes identified associated with photosynthesis and primary carbon fixation (A.2; Table 5) were down-regulated on Day 2. A schematic diagram represents regulation of genes associated with these processes (Fig. 16). These genes were 'ATP-dependent zinc metalloprotease FtsH 1', chlorophyll a-b binding protein, chloroplastic' and 'NAD(P)H-quinone oxidoreductase subunit M, chloroplastic' gene (*ndhM*; Fig. 16). One chlorophyll a-b binding protein was down-regulated, along with four early light induced protein genes (*ELIPs*) that were down-regulated by between 5.21-6.12 fold. A down-regulation in the gene *LTD* - 'Protein LHCP TRANSLOCATION DEFECT' was also observed. Down-regulation of One Helix Protein - *OHP1* and *OHP2* genes, the *Psb28* PSII reaction centre gene, 1 *PsbW*, 2 *PsbS*, 1 *PsbQ*-like and 3 *PsbP* photosystem II sub-unit genes occurred in LL plants. The down-regulation of the *psbQ*-like gene was in corollary with the down-regulation of *psbP*. The down-regulation of a *ycf48* PSII assembly factor gene and 'Photosystem II stability/assembly factor HCF136' gene were also observed.

In respect to the photosynthetic electron transport rate of light limited *Z. muelleri* plants, the down-regulation of 6 key genes was observed; 2 Cytochrome b6/f complex subunit genes, a 'Cytochrome C biogenesis CSS1' gene, 'NDH-dependent cyclic electron flow 1' gene and 2 chloroplastic proton gradient regulation 5-like genes. In respect to inorganic carbon fixation, 1 gene encoding for carbonate dehydratase was down-regulated in LL plants of Day 2 along with the down-regulation of genes associated with RubisCO regeneration; 'Ribose-5-phosphate isomerase' and 'Phosphoribulokinase'.

On Day 6 similar down-regulation of key photosynthetic genes was observed (A.2; Table 6); however, key photosynthetic genes appeared less in number. 3 chlorophyll a-b binding protein genes were down-regulated. Again *FtsH2*, *Psb28*, *PsbP* and *PsbS* genes were all down-regulated. The down-regulation of 2 'Post-illumination chlorophyll fluorescence increase protein (*PIF*)' genes occurred on Day 6, which were not down-regulated on Day 2.

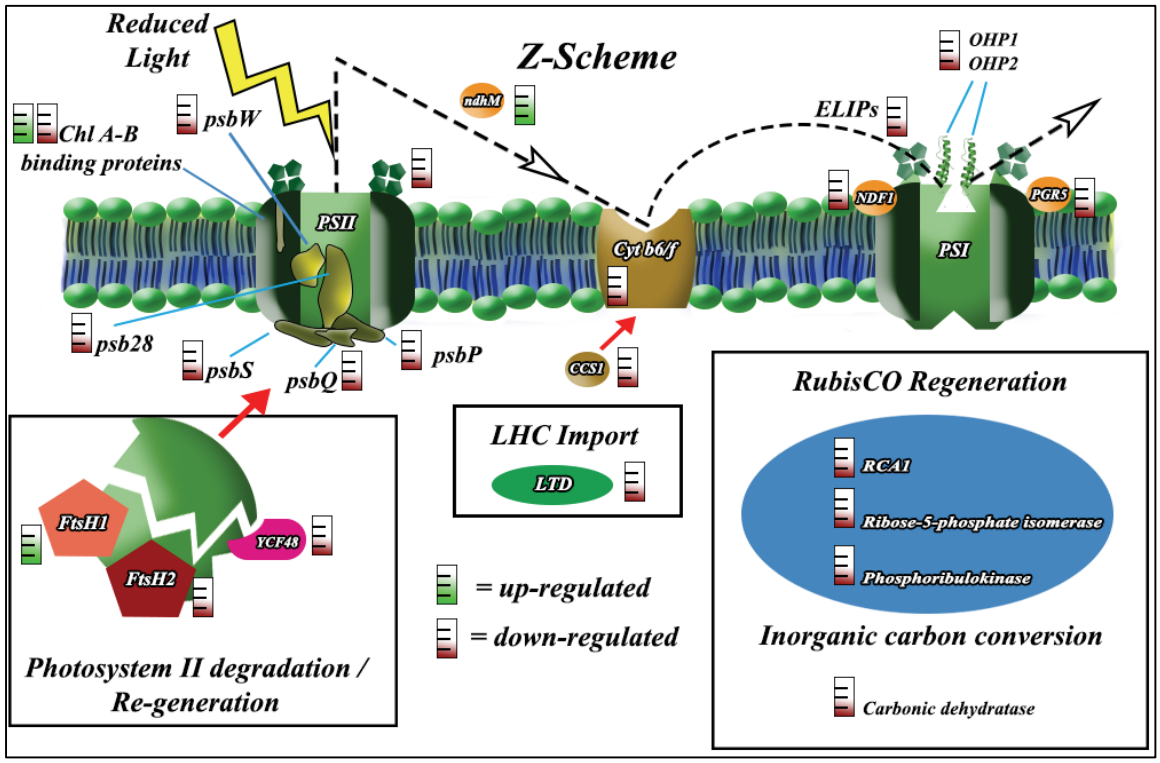

Figure 16: Significant photosynthetic gene regulation on Day 2 (FDR threshold = 0.05; fold change of 1). Significant down-regulation of many genes can be observed. *CCS1* = Cytochrome C biogenises protein; *NDF1* = NDH-DEPENDENT CYCLIC ELECTRON FLOW 1; *Cyt b6/f* = Cytochrome b6/f sub-unit; *PGR5* = Proton Gradient Regulation 5; *LTD* = Light harvesting complex translocation defective protein; *OHP* = One helix proteins; *FtsH* = ATP-dependent Zinc metalloproteases; *YCF* = hypothetical chloroplast open reading frame genes; *psb* = photosystem two sub units; *ELIPs* = Early light induced protein genes.

4.3.6 Regulation of genes associated with pigment pathways

Among the genes differentially expressed on Day 2 (Fig. 17), a general down-regulation (A.2; Table 7) was observed in genes associated with pre-cursor terpenoid metabolism pathways. Significant up-regulation in 2 genes encoding for protochlorophyllide reductase (POR) occurred (FC: 1.72, 1.39). On Day 6 (A.2; Table 8) these genes were again up-regulated (FC: 2.86, 2.51). 'Chlorophyll(Ide) b reductase NOL, chloroplastic' genes were also down-regulated on both days in LL plants; the regulation of these genes is in line with the pigment quantities obtained via HPLC analysis for Days 2 and 6. A down-regulation of chalcone synthase and anthocyanin specific genes was also observed in LL plants compared to control on both Days. Down-regulation of 2 tocopherol related genes was also observed on both days.

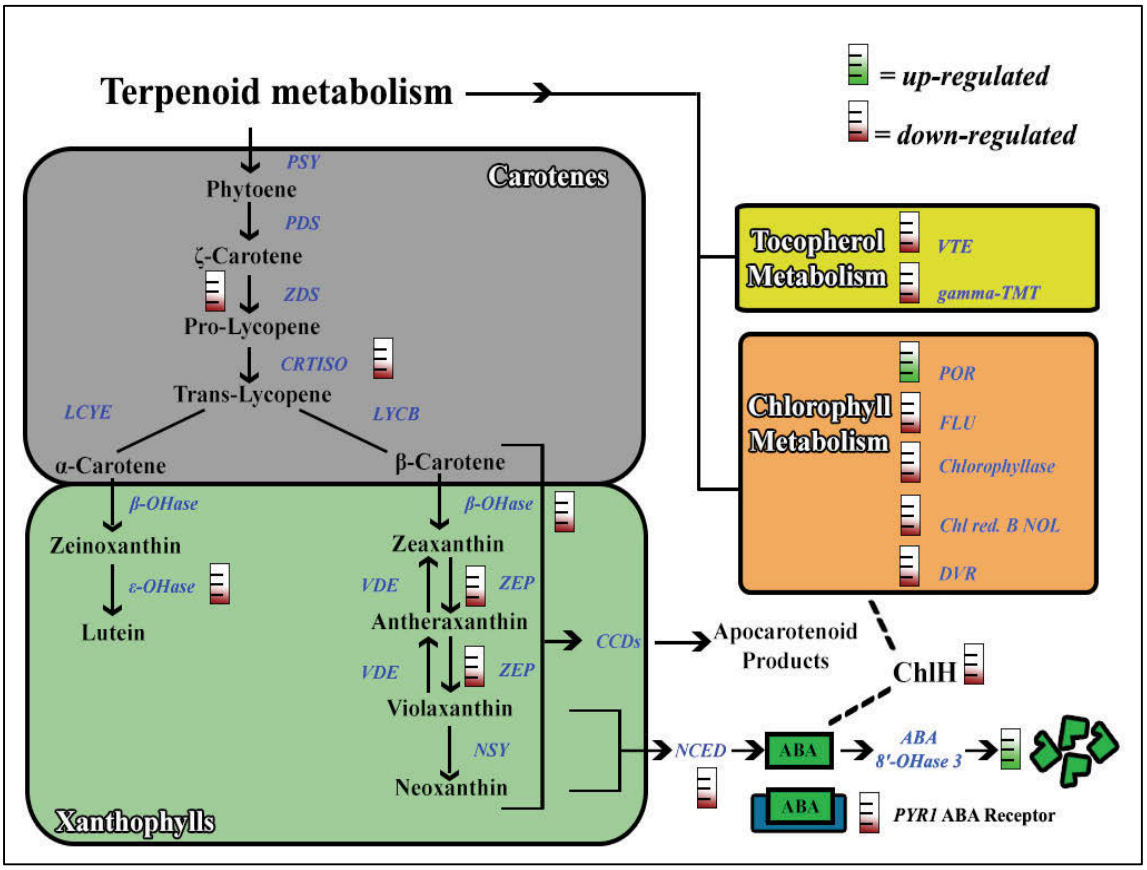

Figure 17: Significant gene regulation (FDR = 0.05; fold change of 1) associated with the carotenoid, xanthophyll, tocopherol, chlorophyll and ABA synthesis pathways on Day 2 of light limitation. *PSY* = Phytoene synthase; *PDS* = Phytoene desaturase; *ZDS* = Zeta carotene desaturase; *CRTISO* = Carotenoid isomerase / Prolycopene isomerase; *LYCB* = Lycopene beta-cyclase; *β-OHase* = *β*-carotene hydroxylase; ε-*OHase* = Epilson hydroxylase; *ZEP* = Zeaxanthin epoxidase; *VDE* = Violaxanthin de-epoxidase; *CCD*s = Carotenoid cleavage dioxygenases; *NCED* = Nine cis-epoxycarotenoid dioxygenase; *POR* = Protochlorophyllide; *FLU* = Blue fluorescent light; *Chl.red B NOL* = Chlorophyllide B reductase NOL; *ChlH* = Mg chelatase sub-unit H; *DVR* = Divinyl chlorophyllide A 8-vinyl-reductase; *VTE* = Tocopherol cyclase; *gamma-TMT* = Tocopherol O-methyltransferase; *ABA 8'-OHase 3* = Absicic acid 8' hydroxylase 3; *NSY* = Neoxanthin synthase.

On examination of the genes, which were significantly regulated and associated with carotenoid and xanthophyll synthesis, a down-regulation was observed in β-carotene hydroxylase 1, which converts beta-carotene into xanthophyll pigments. The down-regulation of two genes encoding for Zeaxanthin epoxidase (*ZEP*) also falls into line with the down-regulation of *psbS* genes reported. On Days 2 and 6, lutein increased in LL plants compared to control plants. In terms of gene expression, there was a significant down-regulation of 'Carotene epsilon-monooxygenase, chloroplastic'. On Day 2 and 6, a down-regulation in 9-cis-epoxycarotenoid dioxygenase 4 (*NCED4*), the

PYR1 and Mg-chelatase H Abscisic acid receptors was observed, whilst an up-regulation in Abscisic acid 8'-hydroxylase 3 in LL plants was observed. On Day 6, 3 'GRAM domain-containing protein / ABA-responsive' genes were up-regulated in LL plants.

4.3.7 Regulation of genes involved in light perception and signaling

On Day 2, 31 genes (A.2; Table 9) involved in photoreception and light response were identified as differentially expressed including phytochromes (undefined sub-types), phytochrome A-associated F-box protein gene, two phototropin-2 genes, a BLUEPAS/LOV protein B gene and *COP1*. On Day 6, 15 key light-regulated genes were identified (A.2; Table 10). A significant down-regulation of two 'phototropic-responsive NPH3 proteins' was observed on Day 6. On both days a range of CONSTANS-LIKE genes and response regulators were differentially expressed.

4.3.8 Gene Ontology (GO) enrichment analysis

A total of 20 significantly enriched GO terms (FDR < 0.05) were associated with genes up-regulated in Day 2, while a total of 58 significantly enriched GO terms were represented by the down-regulated genes in Day 2. On Day 6, 35 enriched GO terms were associated with the up-regulated gene set and 35 in the down-regulated gene set (A.2; Tables 11-14).

4.3.8.1 Photosynthesis and Sugar Enriched GO terms

On Day 2, 'trehalose metabolic process - GO:0005991' and 'trehalose biosynthetic process - GO:0005992' were enriched in the up-regulated gene set (A.2; Table 11). In the down-regulated gene set of Day 2, the GO terms 'photosynthesis - GO:0015979' and 'photosystem II oxygen evolving complex - GO:0009654' were observed (A.2; Table 12). On day 6, GO terms enriched in the up-regulated gene set included both trehalose terms reported for day 2 (A.2; Table 13). On Day 6, many terms associated with carbohydrate catabolism were represented in the up-regulated gene subset including; 'carbohydrate catabolic process - GO:0016052' , 'amylase activity - GO:0016160', 'beta-amylase activity - GO:0016161', 'polysaccharide catabolic process - GO:0000272', 'hydrolase activity, hydrolyzing O-glycosyl compounds - GO:0004553' and 'cellular carbohydrate catabolic process - GO:0044275'. GO terms associated with down-regulated genes on day 6 included ' amyloplast - GO:0009501', 'starch

biosynthetic process - GO:0019252' , 'starch metabolic process - GO:0005982' 'glycogen (starch) synthase activity - GO:0004373'. The 'tricarboxylic acid cycle - GO:0006099' and 'phosphoenolpyruvate carboxykinase activity - GO:0004611' were also enriched in the up-regulated gene sub-set on Day 6.

4.3.8.2 Light and pigment enriched GO terms

No enrichment of pigment related GO terms was evident in the up-regulated gene set of Day 2 (A.2; Table 11); however, in the down-regulated gene set of Day 2, 'chalcone isomerase acitivity - GO:0045430' was represented (A.2; Table 12). When the down-regulated sub-set of genes on Day 6 were examined (A.2; Table 14), isoprenoid and tetraterpenoid terms were enriched along with 'carotenoid biosynthetic process - GO:0016117' and 'carotenoid metabolic process - GO:0016116'. When LL plants on Day 6 were compared to LL plants on Day 2, there was an under-representation of GO terms; however 'cytokinin metabollic process - GO:0009690' was significantly down-regulated in LL plants on Day 6 (A.2; Table 15).

4.3.8.3 Secondary metabolism and ROS enriched GO terms

On Day 2, there was enrichment of GO terms asociated with secondary metabolism and ROS scavenging in the down-regulated genes of LL plants (A.2; Table 12). For ROS homeostasis; 'Catalytic activity - GO:0003824' , 'oxidoreductase activity - GO:0016491', 'glutathione peroxidase activity - .GO:0004602', 'flavonoid metabollic process - GO:0009812', 'flavanoid biosynthetic process - GO:0009813' and 'oxidoreductase complex - GO:1990204' were represented. For secondary metabolism; 'cinnamic acid biosynthetic process - GO:0009800' , phenylpropanoid biosynthetic process - GO:0009699' , 'cinnamic acid metabolic process - GO:0009803', 'phenylalanine ammonia-lyase activity - GO:0045548' , 'phenylpropanoid metabolic process - GO:0009698', 'L-phenylalanine catabolic process - GO:0006559', and 'secondary metabolite biosynthetic process - GO:0044550' were represented. On Day 6 (A.2; Table 14) less ROS terms were represented in the down-regulated genes of LL plants, whilst several secondary metabolism terms represented on Day 2 were still enriched.

4.4 Discussion

In summary, the results obtained, indicate that molecular gene expression is linked to downstream phenotypic changes observed at the physiological level. The response of *Z. muelleri* to low light is one which is systematic.

In relation to the contamination screening conducted in this study, similar protocols have previously been applied to *Zostera marina* assemblies to remove contaminating transcripts (Olsen et al., 2016; Jueterbock et al., 2016). Recent scientific research (Hassenrück et al., 2015; Govers et al., 2016) has highlighted the diverse assemblages of life forms associated with seagrasses. It is now commonly accepted that seagrass plants are holobiont organisms (Hassenrück et al., 2015) – whereby various other life forms exist on and within the plant tissues. Given that we found such plausible contaminants, it is common that draft genomes are improved over time with additional read coverage and improved annotation (Yu et al., 2002). The large number of genes in our leaf-specific transcriptome which received functional annotation hits to *Z. marina* highlight the similarities between the two *Zostera* species, despite the fact that at least one extra whole genome duplication event occurred in *Z. muelleri* (Lee et al., 2016).

The BCV value (0.237) for the experiment is a typical value of a well-conducted experiment (McCarthy et al., 2012). BCV provides the user with a measure of how much variability there is within an RNA-Seq experiment; having a low BCV is essential for realistic and accurate determination of differential expression (Chen et al., 2014). It has been stated that genetically identical organisms obtain a BCV of around 0.10 (McCarthy et al., 2012). The larger spread seen in LL RNA library samples in the MDSplot (A.2; Fig. 6) in contrast to control library samples could be due to the fact that LL treatment plants were exposed to environmental change (i.e. light limitation – 20 μmol photons m^{-2} s^{-1}), and control plants were maintained under conditions more similar their natural environment (i.e. ~200 μmol photons m^{-2} s^{-1}). It is common for individuals of a heterogenous population to elicit individual fitness to environmental change. From the number of genes reported as differentially expressed on Day 2 and Day 6, slightly more genes were differentially expressed on Day 2. The identification of clusters of transcripts that were significantly coordinated in expression and their associated upstream promoter sequences suggest that these gene promoters could potentially relate to the regulation of these genes. Currently, little is known or has been measured in regard to the molecular regulation of gene expression in seagrasses.

The decrease in $rETR_{max}$, I_k and the increase in Yi across Days 2 and 6 imply a modification of the photosynthetic apparatus of *Z. muelleri* plants in response to LL. A higher initial photosynthetic quantum efficiency (Yi) in conjunction with a decrease in $rETR_{max}$ and I_k has been reported previously in *Zostera* species subjected to lower irradiances (Bité et al., 2007; Petrou et al., 2013; Park et al., 2016). When the differentially expressed genes associated with photosynthesis and carbon fixation between control and LL plants were examined, 3 genes were found to be up-regulated, whilst 30 genes were down-regulated. *FtsH1,* one gene which was up-regulated, is a thylakoid membrane bound proteolysis gene, FtsH proteins form complexes with other FtSH proteins in the thylakoid membrane and are involved in Photosystem II D1 degradation (Wagner et al., 2012). The regulation change observed in *FtSH1*, and the down-regulation of 2 *FtSH2* encoding genes, suggest modification of proteolysis activity in the chloroplast associated with PSII repair. This could be due to lower photo-oxidative damage rates occuring in LL plants. In *Synechocystis*, the deletion of the *ndhM* gene led to the inhibition of CO_2 uptake and cyclic electron flow around photosystem 1 (He et al., 2016). In higher plants the absence of *ndhM* has previously been associated with a dysfunctional NADPH complex (Rumeau et al., 2005). This NADP(H) supercomplex gene may be transcribed in *Z. muelleri* to greater amounts in LL, to maintain the stability of the complex, allowing for photosynthesis to continue under LL conditions.

The down-regulation of four *ELIP* genes are congruent with previous studies; transcription of ELIP proteins is typically induced by high irradiances (Hutin et al., 2003). ELIP proteins are known to bind to carotenoids and chlorophyll within the light-harvesting complexes and therefore serve a role in light response and protection (Hutin et al., 2003). The corallory down-regulation of the *LTD* gene in *Z. muelleri* is also in line with the down-regulation of *ELIP* genes. This gene is known to encode a protein, which is necessary for the translocation of nuclear-encoded light harvesting complexes into the chloroplast via the chloroplast signal response particle (SRP) pathway (Ouyang et al., 2011). The down-regulation of *OHP1* and *OHP2* in LL conditions mirrors previous reports of expression profiles in *Arabidopsis* with respect to irradiance; *OHP* genes are associated with the light harvesting complexes of PSI and are up-regulated in response to high light intensities (Andersson et al., 2003).

With respect to the photosystem sub-unit genes, the down-regulation of 2 *psbS* genes and 1 *psbQ*-like gene was observed. The psbS protein is involved in inducing photoprotection of photosystem II and I, and the photo-protective xanthophyll cycle (Roach and Krieger-Liszkay, 2012). The psbQ protein is necessary for the binding of psbS to photosystem II; both are essential for stablilisation of the Photosystem II supercomplex (LHCII) (Ifuku et al., 2011). The down-regulation of the psbP protein was also observed at the proteomic level in *Z. muelleri* (Kumar et al., 2016), this sub-unit is again important for stabilisation of the photosystem (Ifuku et al., 2014). The down-regulation of the core PSII *psb28* gene, *ycf48* PSII assembly factor gene and 'Photosystem II stability/assembly factor HCF136' suggest PSII turnover rate is decreased in LL. In high light, regeneration of PSII increases due to photo-oxidative damage (Lindahl et al., 2000; Bailey et al., 2001; Kato et al., 2009; Flügge and Dietzel, 2016). The *ycf48* and *HCF136* assembly factors are important for the stabilisation of PSII (Plücken et al., 2002). Results indicated a down-regulation of 6 key genes associated with the electron transport rate in LL plants on Day 2. A down-regulation in cytochrome b6/f and cyclic electron transport has previously been documented in plants subjected to lower irradiances (Laisk et al., 2005), Cytochrome b6/f controls electron flow between both photosystems. The Cytochrome C biogenesis CSS gene that was reported as down-regulated, is associated with complex b6/f formation (Nakamoto et al., 2000). The gene regulation associated with light-harvesting complexes, photosystems and electron transport flow are the most likely cause of the changes in photobiology observed in this study.

A carbon concentrating mechanism (CCM) is believed to operate in select seagrass species including *Zostera* species, as seawater contains low CO_2 concentrations (Larkum et al., 2006). The down-regulation of carbonate dehydratase, the inorganic carbon converting enzyme; 'Ribose-5-phosphate isomerase' and 'Phosphoribulokinase' involved in RubisCO regeneration on Day 2 point to a decrease in inorganic carbon uptake / conversion. As such, regulation of these genes is in line with the significant decreases observed in $rETR_{max}$ and I_k in LL plants, suggesting decreased inorganic carbon fixation. Down-regulation of Phosphoribulokinase also occurred on Day 6, suggesting lower rates of RubisCO regeneration were still occuring. At the proteomic level, RubisCO has been reported to be significantly down-regulated in *Z. muelleri* in light limiting scenarios (Kumar et al., 2016). The down-regulation of 2 'Post-

illumination chlorophyll fluorescence increase protein (*PIF*)' genes on Day 6 have previously been documented to be induced by *PGR5*-like genes involved in plastaquinone reduction (Ifuku et al., 2011; Gotoh et al., 2010). In LL plants on Day 2, these *PGR5*-like genes were down-regulated. *PIF* is also involved in NDH-mediated non-photochemical reduction of the plastoquinone pool (Wang and Portis, 2007), suggesting the rate of plastaquinone pool reduction decreases in LL. On Day 6, a *psaB* RNA binding gene was down-regulated. This gene is required for the translation of *PsaB* mRNA into psaB protein (Dauvillée et al., 2003), which is one of the main PSI core proteins.

On both Days 2 and 6, a statistically significant difference was observed in chlorophyll *a* and *b* content (Fig. 13E and F). Previous studies have also indicated a significant shift in chlorophyll *a* and *b* in *Zostera* species under changing light regimes (Abal et al., 1994; Collier et al., 2012; Kohlmeier et al., 2016). A significant increase in total chlorophyll on both Days 2 and 6 was observed (Fig. 13G). These shifts allow for more efficient light capture in light limited environments (Boardman, 1977; Franklin, 2008). Increases were observed in neoxanthin, lutein and β-carotene levels on Day 6 in LL plants, our results are in line with previous studies on *Zostera marina* (Silva et al., 2013) and *Posidonia sinuosa* (Collier et al., 2008); highlighting that these 3 specific pigments are enhancing the antenna size and light capturing capabilities of PSII in low light environments (Gruszecki et al., 2010; Silva et al., 2013) In profiling the protective xanthophyll pigments, violaxanthin was detected; antheraxanthin and zeaxanthin were not. This could be explained by the fact that (i) antheraxanthin and zeaxanthin are only measurable once *Zostera* photosystems become saturated (Ralph et al., 2002), (ii) photo-protection was unnecessary for these plants as we acclimatised them at 200 μmol photons m^{-2} s^{-1} for 18 days, and in our experiment, light was reduced, not increased. On the contrary, it is common for plants, which experience increases in irradiance to maintain elevated levels of zeaxanthin and antheraxanthin (Demmig-Adams et al., 1989; Ralph et al., 2002).

In relation to genes associated with pigment pathways, the up-regulation of protochlorophyllide genes occurred. Protochlorophyllide accumulation has been observed at the physiological level in *Z. muelleri* in response to light reduction (Adamson et al., 1985). The down-regulation of chlorophyllase and 'Chlorophyll(Ide) b reductase NOL, chloroplastic' genes in LL plants are in line with the HPLC results in

this study indicating the down-regulation of these genes in LL play an important role in the increased chlorophyll levels we observed in LL plants. These genes are responsible for the degradation of chlorophyll (Gupta et al., 2012). In shade tolerant plants including seagrasses (Ralph et al., 2007; Sharon et al., 2011), increased chl*a* and chl*b* content has previously been observed and enhances light capture for photosynthesis (Beneragama and Goto, 2011). The down-regulation of chalcone synthase and anthocyanin-specific genes can be compared to previous reports, where by anthocyanin content is known to increase in higher irradiances within *Arabidopsis* (Zhang et al., 2012). Reddening of the leaves, which is associated with anthocyannins, has previously been reported in *Z. muelleri* in respect to high UV levels and irradiances (Abal et al., 1994; Fyfe, 2004). In LL scenarios and from the data in this chapter, it is evident that anthocyanin pigments are transcribed less in LL, confirming previous reports (Abal et al., 1994; Novak and Short, 2010; Zhang et al., 2012). Additionally, the down-regulation of tocopherol genes is in line with reports that suggest tocopherol acts as a photo-protectant in the PSII reaction centre during high irradiances (Krieger-Liszkay and Trebst, 2006). In the seagrass *Posidonia oceanica*, tocopherol genes were found to be regulated in a light-dependant manner (Dattolo et al., 2014).

In relation to the genes which were significantly regulated and associated with carotenoid and xanthophyll synthesis, such regulation could be due to the fact that photo-protection xanthophyll pigments play less of a role in low-light environments, the down-regulation of the β-carotene hydroxylase 1 gene on Day 2 leads to an accumulation of β-carotene in the plant leaves, as conversion of β-carotene to zeaxanthin is reduced. An increased β-carotene pool within *Z. muelleri* agrees with the recorded behavior of *Z. marina* in low light – with β-carotene acting as a light capturing pigment (Silva et al., 2013). In the seagrass *P. oceanica,* a decrease in *ZEP* and *psbS* was observed in deeper plants compared to shallow plants (Dattolo et al., 2014). The build up of violaxanthin and neoxanthin (whilst not significant on Day 2) can possibly be explained by further downstream reactions involving Abscisic acid biosynthesis via the xanthophyll pigments (Barickman et al,. 2014). Abscisic acid is metabolised directly from violaxanthin and neoxanthin by 9-cis-epoxycarotenoid dioxygenase (*NCED*). The significant down-regulation of 'Carotene epsilon-monooxygenase, chloroplastic', which is responsible for the hydroxylation of α-carotene leading to lutein production, was observed on Day 2 and 6. Whilst gene expression may not explain the increase in the

lutein pool, one possible reason for lutein accumulation in low-light environments could be that lutein is photo-degraded in a light-dependent manner (Shi and Chen, 1997); lutein can therefore accumulate in greater amounts in low light.

The down-regulation observed in 9-cis-epoxycarotenoid dioxygenase 4 (*NCED4*), combined with an up-regulation in Abscisic acid 8'-hydroxylase 3 in LL plants provides new insight into ABA (Abscisic acid) regulation in seagrasses at the transcriptional level. These two enzymes are involved in ABA biosynthesis and catabolism respectively, with the regulation of both these genes combined, a down-regulation in ABA homeostasis and a net reduction in ABA levels is evident. Furthermore, a down-regulation in expression of the Abscicic acid *PYR1* and Mg-chelatase H receptors (Shen et al., 2006) on both days suggests that there is a significant change in ABA hormone mediated signaling under LL conditions. Interestingly, ABA biosynthesis itself is derived from violaxanthin and neoxathin pools (Nambara and Marion-Poll, 2005). In land plants, ABA is well known for its role in regulating stress responses. ABA regulates stomatal conductance, which affects photosynthesis and sugar metabolism (Finkelstein, 2013; Rolland et al,. 2005; Yu et al,. 2015). Seagrasses do not have stomata, but responses of *NCED4* and ABA synthesis to low light conditions are in line with what is observed in land plants. In land plants ABA synthesis decreases during periods of extended darkness (Thompson et al,. 2000). The down-regulation of ABA homeostasis suggests that seagrasses in LL conditions increase their sink strength and sugar demand, as a result of changes in photosynthetic productivity. Such expression seems opposite to land plants (Yu et al., 2015), in which sink strength and source activity increase in response to endogenous ABA levels. Given the fact that hormones were not profiled in this study, such a statement is purely speculative and merits further work.

Previous reports have shown that increasing ABA levels and dormancy in Barley is promoted by blue light (Gubler et al., 2008); whilst *NCED* is inhibited by darkness and causes dormancy-release. Indeed, our data show down-regulation of phototropin 2 and a BLUEPAS/LOV domain protein genes on both days, indicating a decrease in blue light levels; as such we can speculate that blue light may also drive ABA synthesis in seagrasses. Currently no literature exists on the role of ABA in seagrasses. Given the important role that ABA plays in stress responses and plant-environment interactions in land plants, continued research into the role and function of

this hormone in seagrasses is important. Further, in congruence with the loss of volatile hormones and parts of the eythlene pathway previously documented in *Zostera* species (Golicz et al., 2015; Olsen et al., 2016; Lee et al., 2016), the ABA pathway could also respond differently to land plants. ABA in land plants is associated with salinity and drought stress; as such, the highly saline environment in which seagrasses live could impose adjusted mechanisms of ABA regulation.

The differential regulation of phytochromes, phototropins and other key light-responsive genes provide evidence that seagrasses and terrestrial plants are alike with respect to photoreception, perceiving and responding to both quality and quantity of light. Two SPA1-RELATED3 genes were down-regulated on Day 2. Additionally, the constitutively photomorphogenic 1 (*COP1*) central regulator gene of light reception and signalling was significantly regulated (Yi et al., 2005). These genes are regulated by the phytochromes (Shikata et al., 2014) and are known to be repressors of photomorphogenesis (Laubinger and Hoecker, 2003). Such regulation suggests *Z. muelleri* may activate photomorphogenises in shaded environments as previously hypothesised by Rose and Durako (1994). Given that the Phototropin 2 (which senses directional blue light) and BluePAS/LOV protein B gene were significantly down-regulated in LL plants, we suspect such regulation mirrors what can be seen in land plants under lower irradiances (Pedmale and Liscum, 2007). A number of CONSTANS-like genes were identified which are generally associated with photoperiod and flowering (Lagercrantz and Axelsson, 2000). Response regulators associated with circadian rythym, light and environmental change (Salomé and McClung, 2005) were also identified, demonstrating the complexity of the light-regulated transcriptome of *Z. muelleri*. These results further indicate that molecular responses of light limitation in *Z. muelleri* should therefore be examined in closer detail.

On Day 2, the enrichment of GO terms associated with 'trehalose metabolic process - GO:0005991' and 'trehalose biosynthetic process - GO:0005992' coincide with previous studies, in which trehalose levels accumulate within plants in response to stress and to provide increased resistance to stressors (Fernandez et al., 2010; Garg et al., 2002). The enrichment of 'photosynthesis - GO:0015979' and 'photosystem II oxygen evolving complex - GO:0009654' in LL plants on Day 2 suggested down-regulation of photosynthesis, in response to reduced light. This has previously been observed at the physiological level in *Zostera muelleri* (York et al., 2013; Petrou et al.,

2013). On Day 6, enrichment associated with trehalose metabolism was again observed, suggesting the continuation of a stress response in relation to light limitation in LL plants. Furthermore, the enrichment of GO terms on Day 6 associated with carbohydrate catabolism, amylase and hydrolase activity suggest the breakdown of carbohydrates was occuring. β-amylase is involved in the breakdown of starch within the amloplasts of plant chloroplasts. In line with these observations, GO terms associated with 'amyloplast - GO:0009501', 'starch biosynthetic process - GO:0019252', 'starch metabollic process - GO:0005982' and 'glycogen (starch) synthase activity - GO:0004373' were enriched in the down-regulated subset of genes on Day 6. Such processes have been associated with carbon balance, previously observed within plants subjected to light limiting irradiances (Zeeman et al., 2004). At night, plants are known to initiate starch breakdown to fuel the plant with energy and carbon in the absence of photosynthesis (Santelia et al., 2015). Additionally, on Day 6 the enrichment of 'tricarboxylic acid cycle - GO:0006099' and 'phosphoenolpyruvate carboxykinase activity - GO:0004611' GO terms were observed, highlighting that replenishment of energy in the TCA cycle (O'Leary et al., 2011) is important in low light conditions. For *Z. muelleri*, recent proteomic evidence has emerged, which demonstrates similar results and suggests insufficient energy production occurs under light limited conditions (Kumar et al., 2016). Between Days 2 and 6, there are apparent signatures of a switch from source (photosynthesis) to sink (carbohydrate stores), in order to replenish cellular energy reserves. The lower rates of photosynthesis observed both at the transcriptional level and physiological level in this study support the hypothesis that under light limited conditions, photosynthesis may not provide sufficient amounts of energy to sustain the correct functioning and development of *Z. muelleri* plants.

The enrichment of GO terms in the down-regulated subset of genes in LL plants on Day 2 included 'chalcone isomerase acitivity - GO:0045430'. This enzyme is associated with the production of anthocyannin pigments in plants (Holton and Cornish, 1995). Under high irradiances and UV light, it has previously been demonstrated that reddening of *Z. muelleri* leaves occurs as a result of increased anthocyannin pigment content (Abal et al., 1994). Given that this GO term was enriched in the down-regulated subset of genes in LL plants on Day 2, our results are in agreement with previous physiological observations (Abal et al., 1994; Novak and short, 2010). On Day 6 there was substantial evidence for shifts in pigment precursor pathways and the carotenoid

pigment pathway. This has previously been observed at the physiological level within *Z. muelleri*, in relation to changes in ambient light levels (Abal et al., 1994; York et al., 2013; Kohlmeier et al., 2016). When comparing Day 6 LL plants to Day 2 LL plants, an enrichment of cytokinin associated GO terms were present in the down-regulated set of genes. in Day 6 LL plants. Cytokinins are associated with controlling certain aspects of photosynthetic gene regulation (Cortleven et al., 2009), this demonstrates that changes associated with the regulation of cytokinins and photosynthesis occurs more on Day 2 than Day 6 in *Z. muelleri*.

On Day 2, the enrichment of ROS scavenging, ROS homeostasis and secondary metabolism terms were present in the down-regulated sub-set of genes in LL plants. Results highlight that these gene groups are down-regulated during initial light limitation. Recent work has shown that ROS homeostasis activity increases in *Z. muelleri* at the proteomic level under higher irradiances (Kumar et al., 2016). This may be due to less photo-oxidative damage occurring in lower irradiances (Ibrahim et al., 2012). There have also been reports that high irradiances typically induce the formation of secondary metabolites in plants (Akula and Ravishankar, 2011). It can be speculated that remobilisation of starch stores occurs some time between Days 2 and 6, ROS homeostasis is no longer down-regulated; however, results indicate that secondary metabolism is still widely represented in the down-regulated subset of genes in LL plants on Day 6. This suggests secondary metabolism is costly and energy is shunted to vital primary metabolic pathways. Such observations may suggest *Z. muelleri* is conforming to a compromise between growth and defense, which is commonly seen in many plants (Lavinsky et al., 2015).

4.5 Conclusion

A systematic profiling approach of *Z. muelleri* in response to light limitation has provided evidence allowing for the linking of gene regulation to downstream phenotypic changes observed in photobiology and photosynthetic pigments. Previously such information has remained elusive or fragmented at best in *Zostera* species with respect to light limitation; however, the release of the *Zostera muelleri* genome along with advancements in seagrass bioinformatic resources have provided better platforms for such studies to be conducted. This research has opened up further questions, which are associated with the regulation of ABA in seagrasses in response to changes in light.

The gene mining and detection of significant differential gene changes provides a good platform for further work in *Zostera* species.

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CHAPTER 5

Effect of light limitation on chloroplast-encoded photosynthetic gene expression and photobiology in *Zostera muelleri*

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Abstract

While there is evidence in land plants that the first organelles responding to light reduction are the chloroplasts, the molecular mechanisms that drive the responses of these organelles to light limitation in seagrass remains poorly understood. In the present study, we profiled the level of expression of 15 chloroplast-encoded photosynthetic genes in response to light limitation using Reverse Transcription – quantitative PCR within the Southern Hemisphere seagrass, *Zostera muelleri*. The significant changes in gene expression were found to be correlated with changes in downstream photobiology using chlorophyll PAM-fluorometry techniques. Nuclear-encoded reference genes offered superior normalisation when compared to chloroplast-encoded reference genes. Six of fifteen photosynthetic genes associated with photosystems I and II, cyclic electron flow, the NADH supercomplex and the proton driven ATPase complex were found to be significantly down-regulated in response to light limitation. This research delivers new insights into the photosynthetic electron transport of *Z. muelleri*, to better understand how this marine angiosperm regulates its photosynthetic machinery in response to light limitation, a common and frequently occurring threat to seagrass meadows in Australia.

5.1 Introduction

Since 2006 Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) has been used to study transcriptional processes in seagrasses (Ransboytn and Reusch, 2006). RT-qPCR offers an accessible and rapid approach to profiling the expression of genes in an organism, especially in response to environmental changes (Bergmann et al., 2010; Winters et al., 2011; Serra et al., 2012; Brakel et al., 2014; Dattolo et al., 2014; Pernice et al., 2015; Pernice et al., 2016). The use of multiple reference genes is now commonly accepted for normalisation of RT-qPCR targeted gene expression in plants (Volkov et al., 2003; Nicot et al., 2005; Remans et al., 2008; Cortleven et al., 2009), including seagrasses (Serra et al., 2012; Dattolo et al., 2014; Lauritano et al., 2015; Pernice et al., 2015; Schliep et al., 2015). For the Southern Hemisphere seagrass, *Zostera muelleri*, RT-qPCR has only been applied to monitor reduced light (dredging impact) and nutrient loading stress in seagrass plants from the port of Gladstone, Queensland, Australia (Pernice et al., 2015; Pernice et al., 2016). In these studies, designated nuclear-encoded reference genes were used for normalisation (Schliep et al., 2015).

Light limitation within the marine water column has frequently been documented in Australia as a problem for seagrass meadows – a consequence of anthropogenic and climatic disturbance events (Ralph et al., 2007). There have been many occurrences whereby light limitation has led to the decline of *Z. muelleri* meadows in Australia (Walker and Mccomb, 1992; Preen et al., 1995; Kirkman, 1997; Campbell and McKenzie, 2004). As such it is important to understand the molecular response of this native keystone seagrass to light limitation.

The first organelles of phototrophs which respond to light reduction are the light harvesting centres themselves – the chloroplasts. Therefore, it is important to understand chloroplast photosynthetic gene regulation in *Z. muelleri* and how such genes influence the rate of electron transport and the rate of inorganic carbon fixation. Whilst mRNA sequencing approaches offer scientists the benefit of profiling thousands of genes at any given time, mRNA enrichment protocols used within these approaches frequently make use of oligodT polyadenylated (polyA) tail enrichment to isolate mRNA. Unlike nuclear gene transcription, polyA tails are used in the degradation of chloroplast transcripts (Rorbach et al., 2014; Castandet et al., 2016). As such, mRNA enrichment approaches are not ideal for accurately determing chloroplastic encoded

gene expression (Castandet et al., 2016). An RT-qPCR approach making use of hexamer primers was therefore chosen in this study to accurately profile gene expression.

In order to apply RT-qPCR to study chloroplast-encoded gene expression, previous research on *Nicotium tobacum* has suggested that chloroplast-encoded reference genes provide superior normalisation compared to nuclear-encoded reference genes (Cortleven et al., 2009); The authors took chloroplast uniparental inheritance into consideration – whereby the chloroplast is inherited from only one parent (Birky, 1995). Angiosperms such as *Zostera* inherit their chloroplast genomes typically from their maternal parent (Hoober, 1984; Provan et al., 2008); chloroplasts thus contain independent transcriptional and translational machinery separate from the nuclear genome (Raven, 2015).

Previously scientists have used, and are still using nuclear-encoded reference genes to profile chloroplast-encoded target genes (Puthiyaveetil et al., 2008; Hotto et al., 2010; McGinley et al., 2013; Qiao et al., 2013; Štefanić et al., 2013; Kremnev and Strand, 2014; Powikrowska et al., 2014), this includes seagrass based studies (Dattolo et al., 2014; Marín-Guirao et al., 2016). Although the main aim of this research was to profile chloroplastic-encoded photosynthetic gene expression and then correlate expressional changes with downstream photobiology traits, chloroplastic-encoded reference genes were also compared against nuclear-encoded reference genes to determine which methodology and therefore which set of reference genes provided superior normalisation.

5.2 Materials and methods

5.2.1 Sample transplantation and experimental set-up

Seagrass plants were collected in August 2015 from Narrabeen Lakes, an enclosed lagoon situated north of Sydney, New South Wales, Australia. Samples were harvested from a single meadow to limit genetic heterogeneity. Plants were removed as intact sediment turfs to prevent damage to the below ground tissues. They were then transported to the UTS: Climate Change Cluster (C3) seagrass mesocosm facility in plastic containers filled with seawater. At UTS, individual plants were separated from ramets, and epiphytes and marine fauna were removed. Seagrasses (30-40 plants) were randomly planted into four aquaria. Below ground tissues were buried in the sediment

so that rhizomes were placed horizontally. The sediment consisted of a mixture of 40% natural sediment and 60% washed sand. Water was maintained at 28 salinity units (Lewis, 1980). An aquarium Elite mini pump (Hagen, Canada) and airstone were used to agitate the water in each aquarium. LED aquaria lighting systems (Aqua Illumination Hydra 52, USA*)* provided illumination, and one system was placed over each aquarium. Growth irradiance was 200 µmol photons m^{-2} s^{-1} at mid-day, integrated over a diel ramp up, ramp down daily cycles (12 hrs light: 12 hrs darkness). Acclimation lasted 18 days. Temperature and salinity were maintained at 19 ± 1ºC and 28 ± 1 units (as measured at Narrabeen Lakes). A calibrated 2-pi underwater irradiance sensor (Licor 250A) was used to establish a light limited (20 µmol photons m^{-2} s^{-1}) treatment. On initiation of the experiment, two of the four tanks had their lights set to the pre-determined light limited light conditions – 20 µmol photons m^{-2} s^{-1} mid-day irradiance. Control aquaria were maintained at 200 µmol photons m^{-2} s^{-1} mid-day irradiance.

5.2.2 Rapid light curve assessment and photobiology

Rapid light curves were conducted using a Diving PAM fluorometer (Walz, Germany), Diving F-Probe and Walz leaf clip before the start of the experiment (mid-day on the day before induction of the experiment) and again on Day 4 of the experiment (mid-day). The leaf clip was attached 2 cm above the meristem of leaf number two (Ralph and Short, 2002). Once attached, the leaf clip was closed for 5 seconds then re-opened before a light curve was initiated to ensure that the measurements were of the current light acclimation state. The following PAM settings were used: AL-fact 1.00; Sat.Int: 8, Sat. width: 0.8 seconds; Meas. Int 8; and Out Gain 2. The following eight actinic light levels were used for 10 seconds each; 38, 118, 227, 358, 515, 691, 993 and 1322 µmol photons m^{-2} s^{-1}. An exponential curve fitting protocol previously used for phytoplankton, seagrasses and corals (Platt et al., 1981; Ralph et al., 2005; Wangpraseurt et al., 2014) was fitted to the raw rapid light curve data using Sigmaplot 12.5 software. The relative maximum electron transport rate - $rETR_{max}$ was calculated along with I_k (minimum saturation value of photosynthesis) and Yi – initial effective quantum yield of photosystem II. IBM SPSS statistics (Version 21) was used for statistical testing.

Table 7: Chloroplast / nuclear-encoded reference genes, and chloroplast-encoded target genes validated and profiled in this study. Gene names are provided (abbreviated and in full), along with primer melting temperatures (Tm, °C), oligo sequences, amplicon size (AS; bp), amplification efficiencies (E; %) and standard curve co-efficient values (R^2).

Gene	Full Name	Tm	Forward primer sequence	AS	E %	R2
Chloroplast-encoded reference genes						
rps2	chloroplast ribosomal protein S2	67.2	GCAGGAGTTCATTTTGGCCA	163	105	0.98
		65.3	GTTTTCCTCCACTTGCTGCA			
rps11	chloroplast ribosomal protein S11	65.8	TGGTTTCTTGGTCCTCTGCA	163	103	0.99
		64.9	GCATCTCTTCCTAGACCCGG			
rpoc2	chloroplast RNA polymerase C2	60.5	GAATTAGGGGAAGCCGTAGG	109	89	0.99
		60.0	CAGTACCGCCCGTGAATACT			
ndhC	NADH dehydrogenase subunit C	67.3	TTTGGGGATGCTTGGGTACA	196	95	0.98
		68.7	CCCCTTTTCGCCATGCATAA			
ndhI	NADH dehydrogenase subunit I	65.4	ATCGGTCAGGGTTTCATGGT	188	90	0.99
		67.7	CGCCAATCAACAACGGGTAA			
Nuclear-encodeed reference genes						
GDPH	Glyceraldehyde 3-phos. dehydrogenase	56.4	TTGAGGGTTTGATGACCACA	101	95	1.00
		60.5	GAATCCTGCAGCTCTTCCAC			
PP2Aa	Protein phosphatase 2A subunit a	58.4	ATGTTGGCGGAGTGGAATAC	107	99	0.98
		58.4	CCGACACAATGAATCCACAG			
Target genes						
ndhB	NADH dehydrogenase subunit B	64.2	ATCTCCCACTCCAGTCGTTG	226	92	0.98
		63.4	ATATCCGATTTGACCGATGG			
atpB	ATPase beta-subunit	63.6	TACGGAAATGGGTTCTTTGC	238	91	0.97
		63.9	CTCACCAACGATCCAAGGTT			
ndhJ	NADH dehydrogenase subunit J	63.9	GGGACTCCATTGCTGTCATT	215	91	0.99
		63.9	GGCACTTTTCCAAATCCAGA			
atpE	ATPase epilson-subunit	64.2	AAATACGCCTCAACGACCAA	235	92	0.99
		63.9	GATTCGACTCGCGTTCTAGC			
atpA	ATPase alpha-subunit	64.0	GACCCTCTTACGGTCGATGA	180	91	0.99
		63.9	CGCTGCATCAGTGAATGTTT			
petA	Cytochrome F	58.4	CCCGATCGTATTTCTCCTGA	103	93	0.98
		60.1	CAGGAGCGGGACCTATAACA			
psbD	Photosystem II D2	58.4	CTCAACTTGCGTGCCTATGA	118	90	0.99
		58.4	CCATCCAAGCACGAATACCT			
psbE	Cytochrome B559 subunit alpha	56.4	CTATTCATTGCGGGTTGGTT	103	90	0.99
		59.5	GAATCCCTTGTCGGCTCTC			
PsaA	PSI P700 apoprotein A1	60.1	GTGATGGACCTGGAAGAGGA	139	94	0.99
		58.4	GTACCCCAAACATCCGATTG			
psbN	Photosystem II reaction center N	58.4	CGCCATCTCCATATCTGGTT	104	96	0.98
		58.4	CGTGTTCCTCGAATGGATCT			
psbA	Photosystem II D1	63.8	GCAGCTTGGCCTGTAGTAGG	155	91	0.99
		63.8	CCAAGGTTAGCACGGTTGAT			
atpF	ATPase F-subunit	63.6	CCGGGAGTTTTGGGTTTAAT	235	100	1.00

		63.7	GCCCCTCTCCGTAATTCTTC			
psbC	Photosystem II CP43 chl a/b binding	63.8	AGGTATTCGTGCTTGGATGG	191	90	0.99
		63.8	CCCGAGTAGTTTACCGGACA			
ndhA	NADH dehydrogenase subunit A	63.4	AGCAGGCATACAACAGCGTA	151	95	1.00
		63.0	TGCTATAGTGGGTCCAAGACTG			
rbcl	Rubisco large sub-unit	63.8	CAGGGGGTATTCATGTTTGG	172	99	0.98
		63.1	ATCACGCCCTTCATTACGA			

5.2.3 RNA sampling, extraction and quality checks

RNA samples were harvested at mid-day on Day 4 from plants where the leaf was excised directly above the basal meristem. Three biological replicates were randomly sampled from each treatment, across each of the two aquaria per treatment. Epiphytes were removed, samples were hand dried, wrapped in labeled foil and snap frozen in liquid nitrogen. Samples were kept at -80°C to maintain RNA integrity. Total RNA was extracted by grinding leaf tissue into a fine powder using pre-chilled pestle and mortars. Ethanol (70%) and RNAse spray (Thermofisher) were used to clean equipment before and between sample grinds to eradicate RNAse and prevent cross-contamination of samples. Tissue powder (80-130 mg) was used in conjunction with an Ambion PureLink mini RNA extraction kit (50 preps) and Purelink On-Column DNAse digest. Using Nanodrop 2000 technology, the quantity and absorbance ratios were checked to ensure good quality RNA had been extracted. Due to initial problems with genomic DNA amplification in the No-RT (No-Reverse Transcriptase) negative control during optimisation, a second round of gDNA removal was completed using the turbo DNAase treatment kit (Ambion).

5.2.4 Prediction of chloroplast genes and primer design

PhiX contamination and TruSeq adaptors were removed from Illumina raw reads of the *Zostera muelleri* genome (Lee et al. 2016). All plastid sequences were downloaded (January, 2016) from NCBI RefSeq plastids repository (ftp://ftp.ncbi.nlm.nih.gov /refseq/ release/plastid/). Genomic reads were mapped to the chloroplast genomes using the BWA aligner software (Li and Durbin, 2009) with a length seed of 32, maximum seed difference of 2 and error rate of 0.02%. Following alignment, SAM formatted files were sorted, indexed and the aligned reads were extracted (SAM flag; -f4). Aligned reads were then assembled and a total of 140 scaffolds were obtained, scaffolds smaller than 100 bp were discarded given that they

may represent assembly artifacts. Genes were predicted with DOGMA – a tool used for annotating plant chloroplast genomes (Wyman et al., 2004). For nuclear reference gene candidates, previously published reference genes were tested for efficiency (Schliep et al., 2015). For additional nuclear reference genes, the *Zostera muelleri* genome (Lee et al., 2016) was annotated using NCBI BLASTX (e-value threshold of 1.0×10^{-5}) against the Uniprot Swissprot and TREMBL Viridiaeplantae specific databases; the best hits based on hit score, e-value and similarity identity were maintained for each gene model. Suitable candidates then underwent a reciprocal best hit (RBH) BLAST against *Z. marina* to identify conserved orthologs. Primer design for genes (Table 7) was conducted using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers were additionally designed around conserved gene functional domains.

5.2.5 Reverse transcription and qPCR protocol

For reverse transcription, a high capacity cDNA reverse transcription kit (200 reactions, Thermo-Fisher Scientific) was used with 200 ng of RNA per sample, 10x RT buffer, 25x dNTP mix (100 mM), 10x Random Primers and Multiscribe™. Reverse transcriptase was carried out in a total reaction volume of 20 µL. The PCR reaction thermal cycle consisted of 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C with a final infinite step at 4°C. A 1:3 dilution series was used to create a standard curve for primer efficiency validation. For qPCR, a 10 µL reaction volume was added per qPCR plate well. 5 µL of SYBR green master mix (Thermo fisher), 0.4 µL of 2 µM forward and reverse primers (80nM concentration each), 0.2 µL of sterile water and 4 µL of cDNA template were used. For each of the two treatments, three biological replicates and three technical replicates per biological replicate were used. 96 well qPCR 0.1 mL plates and optical adhesive covers (Life technologies) were used. The thermal qPCR cycle consisted of a denaturing step at 94°C for 10 minutes, then 40 cycles of 94°C, 60°C, 68°C for 30 seconds each followed by 68°C for 5 minutes.

5.2.6 RT-qPCR data analysis and normalisation

The amplification efficiencies of primers were calculated using the following equation within StepOne™ qPCR software:

$$Efficiency = \left({}_{10}\left[-\frac{1}{slope} \right]_{-1} \right) \text{ X } 100$$

MIQE (Minimum Information for publication of quantitative Real Time PCR Experiments) guidelines suggest primer efficiency ranges should typically be within 90-110% (Taylor et al., 2010). However, primer efficiencies as low as 85% have been reported previously (Fernandez et al., 2011; Schliep et al., 2015). We conformed to a threshold limit of at least 5 Ct cycles difference between sample and the No-RT control (Nolan et al., 2006). All R^2 coefficient values reported were above 0.95 for standard curves. Ct values for technical replicates were checked for high standard deviation; typically, 0.5 Ct cycles difference being the upper limit. The fluoresence threshold was set to 0.03 for all genes profiled. Using SLqPCR (Kohl, 2007), the GeNorm approach was used to determine the best reference genes (Vandesompele et al., 2002), from chloroplast-encoded reference genes only, from nuclear-encoded reference genes only, then from chloroplast and nuclear-encoded reference genes combined. Target gene expression was then normalised to reference genes to determine which set of reference genes provided superior normalisation. A Primer 6 and PERMANOVA+ (Primer-e) permutation statistical testing approach (Bray-Curtis similarity, 4999 permutations and Monte-Carlo simulation) was used to determine the presence of significant changes in gene expression (Normalised Relative Quantity - NRQ).

5.3 Results

5.3.1 Photobiology

To determine if there were significant differences in $rETR_{max}$, I_k and Yi between control and light limited plants, Mann-Whitney statistical tests were conducted as the data was non-parametric in nature (Table 8). $rETR_{max}$ during pre-treatment were not significantly different between control and light limited plants ($U = 15.00$, $Z = .522$, $p = 0.690$. On Day 4 there were significant differences (Fig. 18) in $rETR_{max}$ between control

and light limited plants (U =0.00, Z = -2.611, p = .008). For I_k, again no significant difference was observed during pre-treatment between control and light limited plants (U = 20.00, Z = 1.567, p = .151). On Day 4, median I_k values were significantly different between control and light limited plants (U =0.00, Z = -2.611, p = .008). No significant difference was observed in Yi in pre-treatment; however, on Day 4 of the experiment a significant difference was observed between control and light limited plants (U = 25.00, Z = 25.00, p = 0.08). After 4 days of treatment, a significantly lower $rETR_{max}$ and a lower I_k was observed in light limited plants. In order for such photo-physiological changes to occur, transcriptional regulation was presumed to be playing a significant role in downstream photo-physiological changes. We therefore profiled the expression of 15 photosynthetic chloroplast-encoded genes after 4 days of treatment.

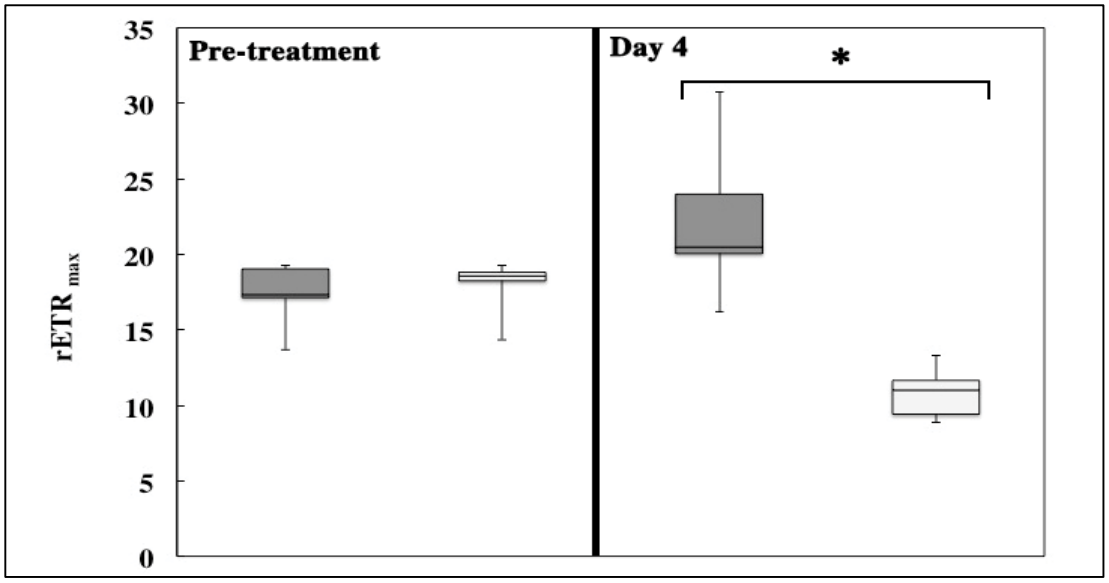

Figure 18: Relative ETR_{max} of *Z. muelleri* plants during pre-treatment and Day 4 in control (dark grey) and light limited treatments (white). Asterix (*) = statistical significance observed ($p<0.05$) between treatments.

Table 8: Statistical comparisons between control and light limited plants on Day 4. Median, p-values, U values and Z-test scores are reported for Mann-Whitney-U statistical tests. Significant results are indicated by an asterix (*), n = 5.

Parameter	Statistic Values	Pre-treatment	Day 4
$rETR_{max}$	Control light median	17.334	20.48
	Light limited median	18.564	11.00
	P-value	0.69	0.008*
	U	15	<0.0005
	Z	0.522	-2.611
I_k	Control light median	40.858	46.27
	Light limited median	43.123	22.93
	P-value	0.151	0.008*
	U	20	<0.0005
	Z	1.567	-2.611
Yi	Control light median	0.765	0.771
	Light limited median	0.779	0.790
	P-value	0.151	0.008*
	U	19.5	25.00
	Z	1.471	2.611

5.3.2 Selection of reference genes

The amplification efficiencies of primer sets specific to 5 chloroplast reference genes, 2 nuclear reference genes and 15 chloroplast-encoded target genes were successfully validated (Table 7). All gene amplification efficiencies were within the range previously published for RT-qPCR studies (Fernandez et al., 2007; Schliep et al., 2015), the chloroplast-encoded reference candidate gene *rpoC2* –RNA polymerase C2 having the lowest efficiency of 89%, whilst the highest efficiency was 105%, obtained by *rps2 –Chloroplast ribosomal protein S2*. All primer sets displayed single peak melt curves, with no presence of multiple peaks. Correlation co-efficient values (R^2) for efficiency based standard curves were all above 0.95.

Among the chloroplast-encoded reference genes, the most stable were: *ndhI* and *ndhC* – 0.115 (joint 1st), *rpoC2* -0.235, *rps11* – 0.335 and lastly *rps2* – 0.437 (Fig. 19). For all 7 reference genes – nuclear and chloroplast –encoded combined, the order in which genes were ranked was (most stable first); *ndhI* and *ndhC* – 0.115 (joint 1st), *rpoC2* – 0.235, *rps11* – 0.335, *rps2* – 0.437, *PP2Aa* – 0.585 and *GDPH* – 0.674 (Fig. 20). For Vplots, the Vandesompele GeNorm procedure recommended a minimum of

two reference genes to normalise against, with no added benefit of using 3 or more (below the typical 0.15 GeNorm V cut-off threshold; not shown).

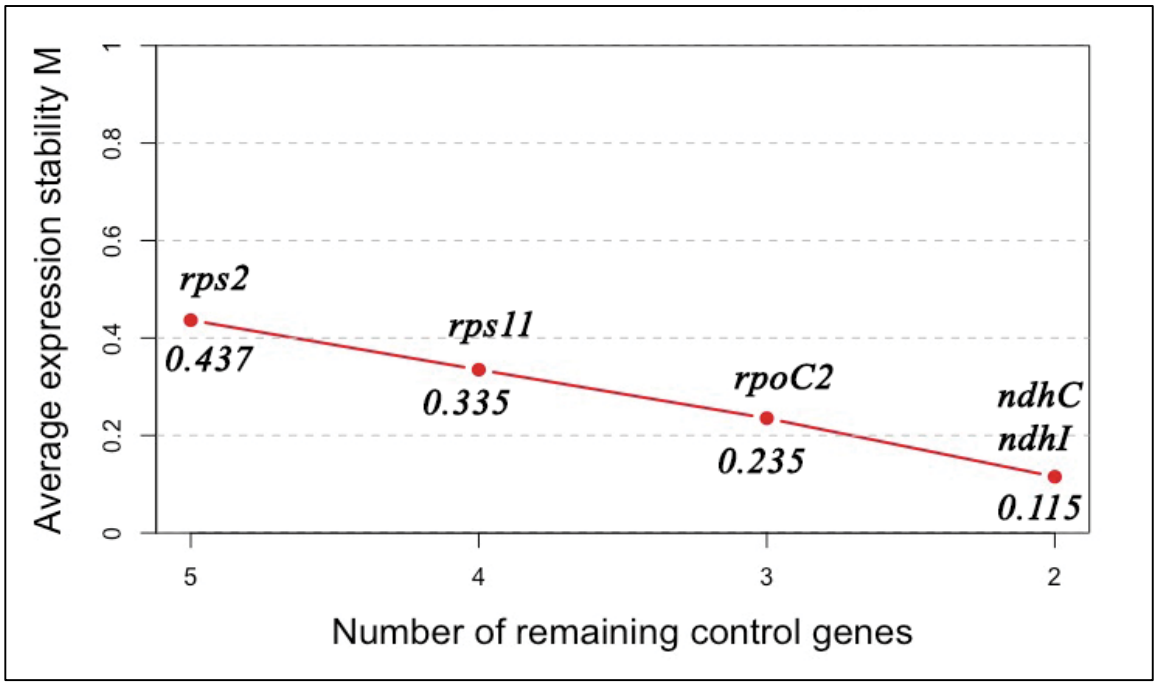

Figure 19: Gene stability measure of chloroplast-encoded reference genes only. Names and average M-stability values are shown on the plot.

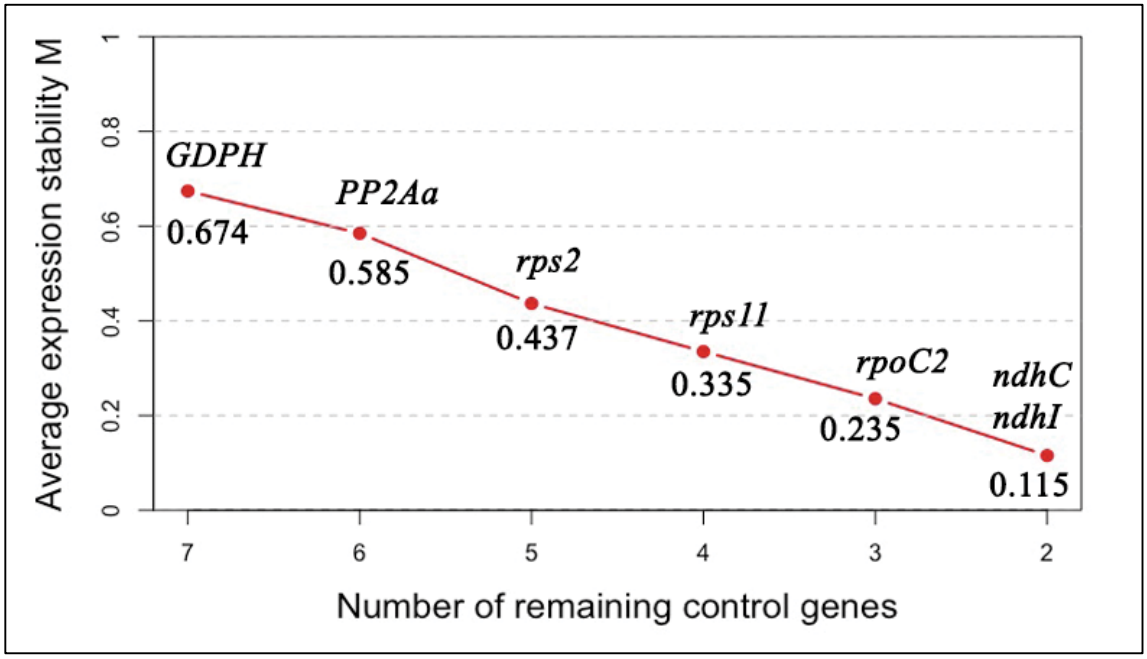

Figure 20: Gene stability measure of chloroplast-encoded and nuclear-encoded reference genes combined. Names and average M-stability values are shown on the plot. *ndhC*, *rpoC2*, *rps11* and *rps2* are chloroplast-encoded genes. *GDPH* and *PP2Aa* are nuclear-encoded genes.

5.3.3 Target gene expression profiling to nuclear-encoded reference genes

When normalised to nuclear-encoded reference genes, all of the target genes decreased in expression in light limited plants compared to control plants (Fig. 21A) and 6 genes showed a statistically significant ($p<0.05$) down-regulation in expression in light limited plants (Table 9; Fig. 21A). When target gene expression was normalised using chloroplast-encoded reference genes, the expression of *psbA*, *psbC* and *ndhA* was significantly different ($P <0.05$; Fig 21B).

Table 9: Significance of gene expression change between control and light limited plants when normalised to chloroplast-encoded (cp) reference genes and nuclear-encoded (nc) reference genes. P (MC) = P-value (multiple correction), T = pairwise t-test value. Statistical significance ($p<0.05$) is represented by an asterix (*).

Normalised to cp-encoded			**Normalised to nc-encoded**		
Gene	T	P(MC)	**Gene**	T	P(MC)
ndhB	1.519	0.196	*ndhB*	0.875	0.454
atpB	1.734	0.135	*atpB*	1.508	0.185
ndhJ	2.009	0.116	*ndhJ*	2.315	0.068
psbA	2.606	0.047*	*psbA*	0.623	0.581
atpE	1.005	0.356	*atpE*	2.402	0.042*
atpA	1.586	0.186	*atpA*	2.655	0.026*
petA	0.377	0.73	*petA*	2.054	0.072
psbD	1.321	0.259	*psbD*	4.222	0.010*
psbE	1.505	0.218	*psbE*	2.004	0.099
PsaA	0.431	0.71	*PsaA*	2.876	0.027*
psbN	1.21	0.283	*psbN*	4.068	0.011*
atpF	0.378	0.78	*atpF*	1.202	0.289
psbC	3.106	0.033*	*psbC*	2.208	0.069
ndhA	3.269	0.026*	*ndhA*	2.892	0.019*
rbcl	0.802	0.486	*rbcl*	1.934	0.089

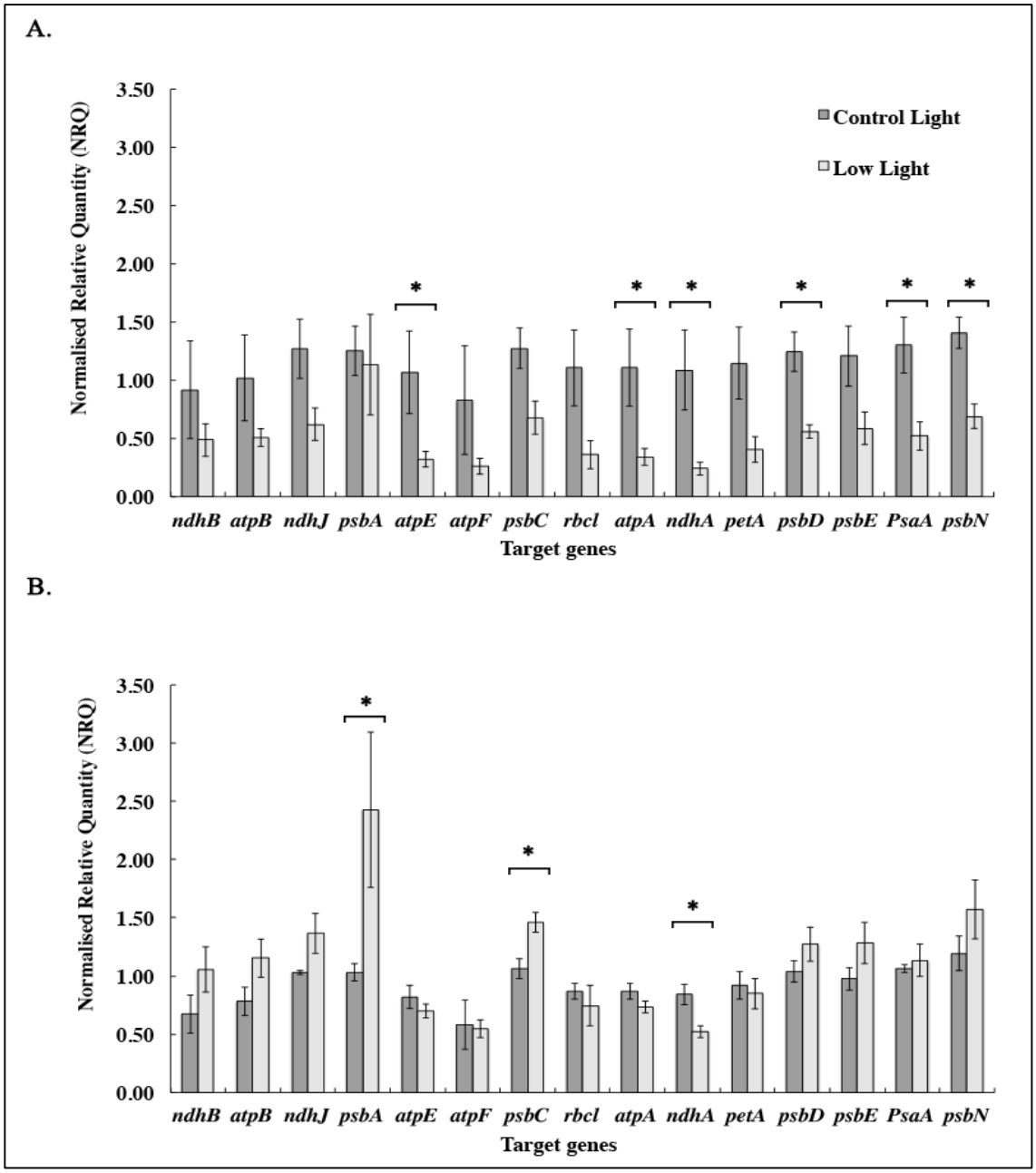

Figure 21: A. Target genes normalised to nuclear-encoded reference genes (Normalised Relative Quantity – NRQ). B: Target genes normalised to chloroplast-encoded reference genes. Asterix (*) and brackets indicate statistically significant difference between control and light limited (low light) *Z. muelleri* plants. n = 3.

5.4 Discussion

The non-significant difference observed during pre-treatment between control and light limited plants suggested successful acclimation of all aquarium plants to 200 μmol photons m^{-2} s^{-1} during the acclimation stage. On Day 4, the statistically significant difference observed in $rETR_{max}$, I_K and Yi indicated a decrease in photosynthetic rate and the minimum saturating irradiance of photosynthesis in *Z. muelleri*, along with an

increase in initial effective quantum yield of photosystem II. These statistically significant differences were observed in response to a ~90% light reduction between control light (200 μmol photons m^{-2} s^{-1}) and the light limitation treatments (20 μmol photons m^{-2} s^{-1}). Photosynthetic electron transport rate of *Z. muelleri* has previously been documented to decrease by 40% when light was reduced by 90% (Bité et al., 2007). Given that light limited plants showed a decrease from a median of 20.48 to 11.00, over 50% reduction in $rETR_{max}$ and about a 50% reduction in I_k were observed. The decrease observed in $rETR_{max}$, I_k and the increase in Yi are in agreement with previous reports (Campbell et al., 2003, Bité et al., 2007; Ochieng et al., 2010; Petrou et al., 2013). These significant changes are characteristic of a seagrass shade-adaptation response (Campbell et al., 2008).

Presence of single melt curves in RT-qPCR work indicated that primer sets chosen for gene profiling were specific to amplifying the genes of interest. Whilst primers were successfully validated for 5 chloroplast-encoded reference candidate genes, only 2 nuclear-encoded reference genes were validated. Initially an attempt was made to optimise and validate the reference genes previously reported for *Z. muelleri* light stress based RT-qPCR experiments (Schliep et al., 2015); the same protocol that was used for the validation of all other reference genes in this experiment was used on several occassions to validate the previously reported reference genes (Schliep et al., 2015); however, validation proved unsuccessful as amplification was largely inconsistent with poor efficiencies and coefficient values. One explanation for the failure of these nuclear reference genes to perform as acceptable standards in this study could be due to genetic differences between *Z. muelleri* seagrass populations. Given that a few thousand km's in distance separate the Gladstone, Queensland population (Schliep et al., 2015) and from the population in which plants originated from in this study (Narrabeen Lakes, Sydney, NSW), it may be possible that the two populations are isolated from one another, exhibiting differences in their genetic profiles. In New South Wales alone, restricted gene flow was documented between north and south state meadows (Sherman et al., 2016). Presumably the extra distance between northern NSW and Gladstone, Queensland adds to the increased likelihood of isolation by distance. Another plausable reason that previous reference genes (Schliep et al., 2015) did not amplify could be due to the fact that previous reference genes were designed around *Z. marina* genes and not *Z. muelleri* genes (Schliep et al., 2015) It is fundamentally

acknowledged in RT-qPCR studies that universal reference genes are rare in occurrence, due to the constant and variable pressures put upon organisms in different environments and locations (Kozera and Rapacz, 2013). Results therefore suggest that future studies involving design of reference genes should be specifically completed for each region. Given the limitations of only having 2 designated reference genes, more nuclear-encoded candidates may increase the likelihood of more stable normalisation (Fig. 20). In contrast to the lower M-stability values obtained by chloroplast-encoded reference genes, the M-stability values of *PP2Aa* (0.585) and *GDPH* (0.674) nuclear-encoded reference genes were both below the 1.5M stability threshold for the designation of suitable reference genes previously reported in RT-qPCR studies (Ling and Salvaterra, 2011; Zhu et al., 2013), making them ideal reference genes for normalisation.

All of the genes chosen for profiling in this study represent important molecular components of photosystem II, photosystem I, the electron transport chain, and the proton driven ATPase complex. In line with the photobiology reported in this study, normalisation of target genes to nuclear-encoded reference genes provided results that can be associated with the changes observed in ETR_{max}, I_k and Yi between control and light limited plants (Fig. 21A).

When normalised to chloroplast-encoded reference genes; *psbA*, *psbC* and *ndhA* were significantly different (Table 9; Fig. 21A) between control and light limited *Z. muelleri* plants. However, *psbA* and *psbC* showed contradicting behavior to previous reports, in that gene expression was up-regulated in light limited plants, as opposed to down-regulated in light limited plants. *psbA* levels have also shown to increase in response to higher irradiance in plants (Trebitsh et al., 2000), the regulation of *psbC* is also positively controlled in a light-dependant manner. *psbC* transcripts that are synthesised in dark grown Barley become more stable under illumination. Light-dependant regulation of *psbC* can also be observed in Cyanobacterium; *psbC* transcripts become more abundant under illumination than in darkness (Mullet et al., 1990; Colón-lópez et al., 1998). While chloroplast-encoded reference genes were more stable than nuclear-encoded reference genes in this experiment, the results obtained are counterintuitive to what has been described previously for photosynthetic gene expression in plants in response to decreases in light.

Cortleven et al. (2009) measured endogenous cytokinin content in association with gene profiling in *N. tobacum* to assert correct gene expression, given the beliefs that cytokinin levels were solely related to controlling the regulation of photosynthetic plastid proteins. Whilst such inference can be assumed, the relative quantification of electron transport can be directly measured through the use of chlorophyll fluorometry, this approach is superior for two reasons. Firstly, whilst it is acknowledged that cytokinins may serve an important purpose in photosynthesis, the regulation of endogenous cytokinin can also be influenced by abiotic, biotic stresses and plant development signaling cues (Ha et al., 2012). These parameters may or may not be controlled effectively or accounted for variation in experimental set-ups. Secondly, chlorophyll fluorometry has long been used as a routine and effective tool for monitoring photosynthetic performance such as electron transport rate in plants, including *Zostera* species (Ralph et al., 2002, Ralph and Gademann, 2005; Petrou et al., 2013). Whilst these results obtained for chloroplast-encoded normalised target genes are contrasting with previous reports (Mullet et al., 1990; Colón-lópez et al., 1998; Trebitsh et al., 2000), the results obtained for nuclear-encoded normalised target genes are more consistent with previous literature and should also be discussed.

The *psaA* gene encodes for one of the main Photosystem I reaction centre apoproteins. In *Oryza sativa*, *psaA* mRNA was found at lower levels in dark-grown seedlings (Kapoor et al., 1994*). psaA* transcript expression was also found to be significantly greater in *Arabidopsis* plants under 250 μmol photons m^{-2} s^{-1} of light when compared to plants in 40 μmol photons m^{-2} s^{-1} of light (Kawabata and Takeda, 2014). In *Brassica rapa*, protein degradation of *psaA* occurs under higher irradiances (Jiao et al., 2004). The rate of psaA protein regeneration is greater in higher irradiances compared to lower irradiance, this explains why this transcript is significantly decreased in light limited *Z. muelleri* plants in this study compared to control plants when normalised to nuclear-encoded reference genes. The *psbN* gene associated with photosystem II assembly is located in the stroma lamallae (Torabi et al., 2014). It has been demonstrated that the absence of this gene in *Nicotiana tabacum* mutants impairs photosystem production and results in phenotypic trait changes including low pigment accumulation, pale green leaves, reduced growth and photo-sensitivity. The authors additionally noted a reduction in electron transport rate (Krech et al., 2013). Another study on *N. tobacum* highlighted that the *psbN* gene was important for photosystem II

re-generation and recovery from photo-inhibition. In this study, the significantly lower rate of photosynthetic electron transport rate can be correlated with the significant down-regulation in *psbN* expression in *Z. muelleri*.

When normalised to nuclear-encoded reference genes, a significant down-regulation was observed in the *Z. muelleri psbD* gene in response to light limitation. This gene encodes the D2 subunit of the photosystem II reaction centre (Suorsa et al., 2014), it is important for the assembly of PSII (Komenda et al., 2004). The gene is activated by the blue light photoreceptors Cryptochrome 1 and 2, as well as phytochrome A (Thum et al., 2001). In *Chlamydomonas reinhardtii* and in the cyanobacterium, *Acaryochloris marina, psbD* transcripts increase during exposure to high irradiance and UV light (Schwarz et al., 2012; Kiss et al., 2012). PSII regeneration increases under higher irradiances due to the rate of photo-damage caused to the photosystems (Vass, 2012). The regulation of *psbD* in *Z. muelleri* follows the same light-responsive regulation as observed in many phototrophic organisms mentioned, and most probably serves the same conserved role.

The NADH dehydrogenase subunit A (*ndhA)* gene associated with the formation of the NADH dehydrogenase complex (Suorsa et al., 2009) was significantly down-regulated (Table 9) in light limited *Z. muelleri* plants compared to control plants. This gene is specifically involved in the electron transport chain of photosynthesis and helps to control the rate of electron flow and light harvesting capacity during photosynthesis (Suorsa et al., 2009; Finazzi et al., 2016). In Barley, the expression of *ndhA* is increased in response to higher irradiance and photo-oxidative conditions (Martín et al., 1996). In accordance, *atpA and atpE*, the beta and eplison subunits of the ATP-synthase complex were down-regulated. This ATP-synthase complex is driven by the thylakoid proton gradient during photosynthesis. In turn, the ATP produced is used during photosynthetic CO_2 fixation for reducing power (Rochaix and Ramundo, 2015). The significant down-regulation of these select genes possibly suggests the balance of NADH: ATP ratios are maintained despite the decrease in relative electron transport rate (Johnson, 2011).

Given that all of the target genes profiled were down-regulated in light limited *Z. muelleri* plants (6 significantly; Fig. 21A), there is strong evidence from this study to suggest that the regulation of these chloroplast-encoded target genes is important for controlling the rate of photosynthesis and electron transport in *Z. muelleri*. Such results

suggest the transcriptional regulation of the photosynthetic electron transport rate in *Zostera muelleri* is similar to other phototrophs (Kapoor et al., 1994; Thum et al., 2001; Jiao et al., 2004; Suorsa et al., 2009; Kiss et al., 2012; Schwarz et al., 2012; Vass, 2012; Krech et al., 2013; Kawabata and Takeda, 2014; Torabi et al., 2014; Finazzi et al., 2016).

5.5 Conclusion

This study highlights the need for specific reference gene design, and the choice of suitable reference genes for each new *Z. muelleri* population investigated in the future. In order to profile the expression of chloroplast-encoded photosynthetic genes in *Z. muelleri*, nuclear-encoded genes should be used based on (i) the gene expression data presented within this study (normalised to nuclear-encoded reference genes), (ii) previous profiling of chloroplast photosynthetic genes in other phototrophs and higher plants reported in this sudy; (iii) the correlations of photo-physiology and gene expression reported in this study and (v) the large knowledge gaps which still exist surrounding chloroplast regulation. The gene expression data reported here (normalised to nuclear-encoded reference genes) suggests that the molecular mechanisms driving changes in photosynthetic electron transport rate of *Z. muelleri* in response to light limitation are similar to other higher plants.

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SYNTHESIS, OUTLOOK AND CONCLUSIONS

6.1 Overview

Before the commencement of this PhD project, many studies have been conducted, which provided valuable knowledge on how *Z. muelleri* responds to changes in light (Adamson et al., 1985; Abal et al., 1994; Collier et al., 2012; York et al., 2013; Petrou et al., 2013); however, these were limited to physiological findings. Given that omics and next generation sequencing techniques have been used in plant biology for the past two decades, since the sequencing of the *Arabidopsis* genome (Michael and Jackson, 2013), there was a clear need to integrate the transcriptional regulation of *Z. muelleri* in response to varying light. The focus of this PhD thesis was therefore set on one of the most credible stressors of *Z. muelleri* – light limitation. When this PhD thesis was commenced in 2013, literature on Zosteraceae omics did not exist. Only a few studies up until now have focused on light response in seagrasses at the transcriptional level (Chapter 1). Within the last year, the *Z. marina* genome (Olsen et al., 2016) and *Z. muelleri* draft genome (Lee et al., 2016) have been published, providing superior resources and foundation for further research to be conducted. The most closely related study which has existed up until now, and which was based on transcriptional responses of seagrasses to varying light regimes, was completed on *Z. muelleri*'s sister species, *Zostera marina,* using Illumina next generation sequencing in 2014 (Kong et al., 2014). No differential gene expression or enrichment analysis was conducted in this study, leaving this niche field open to further work.

This PhD project has examined the acute response and acclimation of *Z. muelleri* to light reduction over a period of 14 days. Chapter 3 focussed on the later stages (i.e. days 9 to 14) of *Z. muelleri* response to light limitation, whilst Chapter 4 and 5 focussed on the earlier stages (i.e. the first 6 and 4 days of response, respectively). Chapter 5 being specifically focussed on chloroplast-encoded photosynthetic gene regulation. In this PhD thesis, results indicate that several important functions were occuring at the transcriptional level in *Z. muelleri* in response to light limitation, providing a better understanding of how transcriptional regulation governs phenotypic level changes in photophysiology (Adamson et al., 1985; Abal et al., 1994; Collier et al., 2012; York et al., 2013; Petrou et al., 2013; Silva et al., 2013; Kohlmeier et al., 2016). Therefore, new knowledge on how *Z. muelleri* responds to light limitation has been gained.

6.2 Photo-physiological acclimation occurs early in *Z. muelleri*

Upon analysis of the rapid light curves described in Chapter 4, $rETR_{max}$ and I_k both decreased significantly by Day 2 in light limited plants (20 μmol photons m^{-2} s^{-1}) whilst Yi increased significantly. *Z. muelleri* remained in this state on Day 6. In Chapter 5, $rETR_{max}$ and I_k were significantly lower on Day 4 in light limited plants than control plants (200 μmol photons m^{-2} s^{-1}), while Yi was significantly increased in low light plants on Day 4. On day 6, Yi was not statistically significant (Chapter 4), suggesting acclimation was occurring slowly. In general, the results indicate that *Z. muelleri* responds and starts to acclimate to light limitation by Day 2. On examination of literature recently made available, Kohlmeier et al. (2016) reported that photo-physiology changes occured in *Z. muelleri* within a time frame of several hours, in response to diel tidal cycles. This suggests that *Z. muelleri* plants start to change their photo-physiology much quicker than anticipated and possess high plasticity to changes in light within their environment. In Chapter 4, significant differences were observed in photosynthetic pigment composition as early as Days 2 and 6. Results indicated that lutein, β-carotene and chlorophyll content all increased significantly under limited light. A possible reason for these findings is that these pigments offer enhanced light absorption properties to the *Z. muelleri* photosystems (Silva et al., 2013). The findings of this thesis with respect to photo-physiological responses confirm *Z. muelleri* has high plasticity to changing light. Such attributes are potentially critical to the success of this plant in colonising the inter-tidal and sub-tidal coastline of Australia.

6.3 Both nuclear and chloroplast-encoded genes play important roles in *Z. muelleri*, in response to light limitation

1,593 genes were found to be differentially expressed on Day 2 in light limited plants (20 μmol photons m^{-2} s^{-1}) compared to control plants (200 μmol photons m^{-2} s^{-1}) (Chapter 3). These included genes associated with photosystems II and I, photosystem accessory components; cyclic electron transport; carbon fixation; pigments and light perception; and signaling. Observed shifts in gene expression correlate with the downstream photo-physiology, the decreased rates we observed in $rETR_{max}$ and I_k and increase in Yi can be attributed to this gene expression. An open mind must be maintained as regulation at other molecular levels are also important aspects to consider i.e. post-translational and epigenetic. Chapters 3 and 4 characterised and profiled high-

throughput data in the form of mRNA transcriptomes. These chapters made use of high throughput sequencing approaches consisting of mRNA enrichment protocols (polyA enrichment). As previously stated in the introduction to Chapter 5, under polyadenylation, chloroplast-encoded transcripts become instable and undergo accelerated degradation (Rorbach et al., 2014; Castandet et al., 2016). Chloroplast transcripts therefore cannot be quantified accurately using such protocols (Castandet et al., 2016). Instead hexamer based random primers were used in Chapter 5 for RT-qPCR chloroplast gene profiling. In this Chapter, 6 key chloroplast-encoded genes, which were associated with photosystems II and I, cyclic electron flow, cytochrome b6/f and the proton driven ATPase complex were significantly down-regulated in light limited plants compared to control light plants. Significant concomitant reductions were also observed in $rETR_{max}$, I_k, with a significant increase observed in Yi. Taking Chapters 3, 4 and 5 into consideration, combined results indicate that transcriptional regulation of both nuclear and chloroplast-encoded genes are vital for the response and acclimation of *Z. muelleri* to light limitation.

6.4 Trends observed in *Z. muelleri* over 14 days in response to light limitation

A photosynthetic and photophysiological response was osberved in *Z. muelleri* to light limitation as early as Day 2. A change in photosynthetic pigments and associated genes was also observed on Days 2 and 6, pointing to a reduction in the need for photo-protection, and a shift towards enhancing the light capturing capabilities of the photosystems (Fig. 22). As early as Day 6, an emerging trend of rebalancing the carbon metabolism was evident (Fig. 22), which extended further into Days 9, 12 and 14 (Chapter 3). In *Z. muelleri*, it has previously been observed that leaf size, shoot numbers and plant biomass all decrease during extended exposures to light limitation in *Zostera muelleri* (Collier et al., 2012; McMahon et al., 2013; York et al., 2013). The decrease in carbon fixation, due to reduced rates of photosynthesis in light limited plants (Chapters 3,4 and 5) is inadequate in supplying the plants with sufficient amounts of fixed carbon. This response has also been observed at the proteomic level in *Z. muelleri* to light limitation (Kumar et al., 2016). The signatures of change in carbon metabolism suggest that plants are using their carbohydrate stores to supply the energy demand of the TCA cycle. The use of these carbohydrate stores can ultimately lead to

changes in leaf morphology; shoot loss and biomass loss (Collier et al., 2012; McMahon et al., 2013; York et al., 2013). In the marine environment, synergistic stressors should now be closely examined, including low oxygen content and sulphide intrusion in *Z. muelleri* (Broderson et al., 2015), as multiple stressors are often present in light limited marine environments.

Another observation in Chapter 4, was the down-regulation of ROS homeostasis, secondary plant defence metabolic genes and GO groups on Days 2 and 6 of light limitation. At the proteomic level, Kumar et al. (2016) noticed increased ROS activity in *Z. muelleri* subjected to higher irradiances. In Days 9, 12 and 14, the contrary was observed: secondary plant metabolism, ROS related homeostasis and scavenging was increased under light limitation (Chapter 3; Fig. 22). These results suggested that *Z. muelleri* could conserve energy during the early days of light limitation (Days 2 and 6), shunting energy to only the vital primary metabolic pathways. During Days 9, 12 and 14; however, the increase in secondary metabolism and ROS activity may be attributable to a compromised immunity. Cross-talk is known to occur between ROS and secondary metabolic pathways (Jacobo-Velázquez et al., 2015). This compromised immunity could be due to the lack of photosynthetically fixed carbon, and the gradual utilisation of carbon stores from as early as Day 6. Having a fully functional immunity is costly to a plant and as such, this relies heavily on light availability. Similar observations have been made in *P. oceanica* (Dattolo et al., 2013), in which the authors suggested this theory originally. Given that previous studies have documented shoot loss, leaf loss and plant death in response to light limitation, this scenario is very likely.

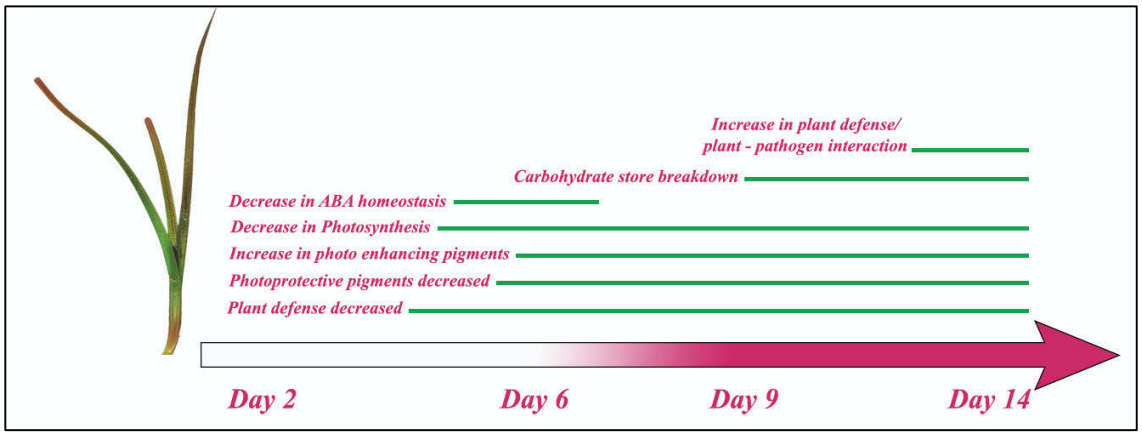

Figure 22: Schematic diagram highlighting the trends of how *Z. muelleri* responds to light limitation over a period of 14 days.

6.5 Genome-guided assembly versus *de novo* transcriptome assembly; Research challenges and implications for the future

During the initial stages of this project (Chapter 3), a *de novo* transcriptomic approach was used to characterise and profile whole *Z. muelleri* plants at the transcriptional level in response to light limitation. *De novo* assembly has long been associated with difficulties, especially when used for profiling and elucidating plant transcriptomes due to the large number of paralogs and isoforms that they can contain (Duan et al., 2012; Ward et al., 2012; Nakasugi et al., 2014). Such difficulties were also encountered in this thesis (Chapter 3) when using this approach. Consequently, a genome-guided transcriptome assembly approach was used in Chapter 4 as the *Z. muelleri* genome became available; this approach made use of already predicted gene models for gene counts (Lee et al., 2016). Between the two approaches used in this thesis (*de novo* and genome-guided assembly), and in line with previous publications (Schneeberger et al., 2011; Liu et al., 2016; Steijger et al., 2013), we found that genome-guided assembly was superior to *de novo* transcriptome assembly for numerous reasons.

(i) Spurious transcripts with low read coverage

Two *de novo* assembly software programs were used in this PhD thesis (Haas et al., 2013; Peng et al., 2013). For Chapter 3, Trinity; a short read *de novo* assembler, which makes use of a de bruijn graph algoritihm for constructing transcripts from short RNA-seq reads, was used (Haas et al., 2013). Whilst a genome-guided approach was used in Chapter 4 making use of STAR alignment software (Dobin et al., 2013) and RSEM count software (Li and Dewey, 2011), an additional piece of work was conducted to compare genome-guided and *de novo* assembly for the data and analysis of Chapter 4 (Appendix 3). In this instance both Trinity and IDBA-tran de bruijn assembly software programs (Peng et al., 2013) were used to assemble 8 transcriptomes in total, which were then combined into a 'super assembly'. The resulting super assembly was then processed using Evidentialgene (Gilbert, 2002), a software pipeline to remove redundancy and retain the most likely coding sequence per gene locus (Appendix 3). This additional approach was taken as *de novo* assemblies do offer the advantage of detecting novel genes (Birol et al., 2009; Grabherr et al., 2011). This can be useful for when genomes are incomplete, as in the instance for the *Z. muelleri* genome (Lee et al.,

2016). Although Trinity and IDBA-tran are widely used assemblers, in the present work we found that *de novo* assembly produced large numbers of spurious transcripts, with little supporting read coverage (Chapter 3 and Appendix 3). This is one major problem associated with *de novo* transcriptome assembly (Steijger et al., 2013), as the algorithms they are based upon, each have their own design limits and flaws. Given that eukaryotic genomes are highly complex and encompass alternatively spliced gene variants (Liu et al., 2016), caution should be exercised when using *de novo* transcriptome assembly, as these two factors can typically lead to assembly errors including the reporting of incorrect isoforms, incomplete exon coverage and transcript prediction (Steijger et al., 2013).

(ii) Over inflation of gene numbers and redundancy

In Chapter 3, after redundancy removal; utilizing CD-HIT-EST (Li and Godzek., 2006) and CAP3 (Huang and Madan, 1999), with 'expressed gene' thresholds based on 4 TPM (transcripts per million; Wagner et al., 2013) and contamination filtering, we recovered a large number of unigenes that matched the same best hit gene annotation in *Zostera marina*. Whilst it has been established that *Z. muelleri* underwent a further single / multiple whole genome duplication event(s) separate from *Z. marina* (Lee et al., 2016), and thus several copies of the same gene may be expected; in several instances we found that over 100 unigenes mapped to the same *Z. marina* gene annotation using DIAMOND BLASTX protein alignment (Buchfink et al., 2015; Chapter 3). We also found the same problem in the additional *de novo* 'super' assembly used for comparison of Chapter 4 data (Appendix 3). This raised concerns on how effective and accurate current redundancy removal procedures actually are in *de novo* methods. Gene numbers for both Chapters 2 and Appendix 3 were both relatively inflated to the predicted number of genes in the *Z. muelleri* genome (Lee et al., 2016). Again such results bring into question how one confidentially determines an isoform from a gene, simply based on redundancy procedures.

(iii) The detection of contaminating transcripts

In the past year with the sequencing of the *Z. marina* genome (Olsen et al., 2016), the release of a *Z. marina* transcriptome (Jueterbock et al., 2016) and the work conducted in Chapters 3, 4 and Appendix 3 of this thesis, there is substantial evidence

accumulating that seagrasses are holobiont organisms just like corals (Bourne et al., 2009), many other animal and plant species (Zilber-Rosenberg and Rosenberg, 2008). At the International Seagrass Biology Workshop (ISBW) 12 Conference held last year in Wales, UK; Professor Jeanine Olsen discussed such theories, which sparked interest in relation to the work conducted in this PhD thesis. Given the paradigm of a seagrass holobiont, the question raised here is; how one goes about correctly annotating and determining which genes are transcribed by the host and what genes are transcribed by other organisms associated with the seagrass plant? In this thesis, this scenario provided initial confusion and difficulty as to how one should best separate plausable contaminants from the data. It was decided, that it was best to complete a manual contamination screening approach for each dataset, whereby transcripts which belonged to non-plant taxa were removed, mirroring the efforts of other seagrass high-throughput studies (Olsen et al., 2016; Jueterbock et al., 2016). Initial work in this thesis did at first entail annotating the transcriptomes to viridiplantae specific Uniprot databases; however, upon re-thinking the analyses, it was decided to use the complete Uniprot Swissprot (curated) and Uniprot TREMBL (uncurated) databases to obtain higher numbers of functionally annotated genes, but also higher resolution to identify plausible contaminants that are not present in the Viridiplantae specific taxanomic clades. In essence, one must be mindful that, despite efforts in cleaning the seagrass leaves before sequencing without damaging them, the sequencing reads obtained may more realistically resemble that of a meta-transcriptome dataset. Until further advances are made in seagrass bioinformatic resources, this problem will remain a challenge and should be considered by all seagrass biologists to obtain sound analysis. Such an approach as used in this thesis will allow the reporting of minimum false positive results.

(iv) Increased biological variation in *de novo* assemblies

When the genome-guided assembly (Chapter 4) was compared to the 'super' *de novo* assembly (Appendix 3), an increase in BCV (Biological Co-efficient of Variance) from 0.237 to 0.587 (Appendix 3) was observed. A low BCV is crucial for accurately estimating differential gene expression (McCarthy et al., 2012), it was therefore decided based on the assemblies and statistics, that genome-guided assembly was superior. Whilst *de novo* assembly provided a slight increase in the number of differentially

expressed genes, redundancy was evidently present; for example, multiple copies of genes existed in the differentially expressed gene sub-sets for Days 2 and 6. Such increases in variance from the *de novo* assembly have previously been attributed to expression of alternatively spliced variants, poor expression estimates of similar transcripts, incomplete transcripts and artifacts in the *de novo* assembly (Steijger et al., 2013; Appendix 3).

6.6 Future research direction from this thesis

6.6.1 The seagrass holobiont paradigm and consequences for *Z. muelleri* under light limited environments

Despite the use of aseptic technique in this thesis, sequence transcripts from sources other than plant origin (Chapter 3, Appendix 3) were still obtained. Even when the draft genome of *Z. muelleri* was annotated (Chapter 4; Lee et al., 2016) with the complete Uniprot Swissprot and TREMBL databases (no annotations provided with the original publication – Lee et al., 2016), 647 gene models were potentially detected from other organisms including prokaryotes and other marine eukaryotes. The most abundant contaminating organisms in the leaf-specific transcriptome of Chapter 4 were gamma-proteobacteria. Many assorted fungi and protist genes were particularly prominent. During the later sampling points of this thesis (Days 9, 12 and 14 of Chapter 3), significant enrichment of metabolic pathways involved in 'Plant-pathogen interaction' were observed, as well as significant enrichment of secondary plant metabolism GO terms and pathways. It is therefore possible that light limitation leads to a decreased energy budget and increased pathogen load on the plant, due to a compromised immune system.

It has been documented that the pathogenicity of *Labyrinthula sp*. protists, which cause the eelgrass (*Z. marina*) 'wasting disease' are correlated with a reduction in light (Trevathan-Tackett et al., 2013). Whilst *Labyrinthula zosteraceae* has been the most documented seagrass pathogen to date, recent work (Govers et al., 2016) has isolated *Phytophora* species associated with *Zostera marina*, which is known to cause rot in various plant clades. In this thesis, we also found traces of several *Phytophora* species in the annotation best hits before contamination filtering. As such further metagenomic approaches and characterisation of disease susceptibility should be

investigated in *Z. muelleri* under light limitation, especially during pro-longed periods of chronic exposure.

6.6.2 Further investigation of light response and acclimation in *Z. muelleri*

This thesis covers time points taken throughout 14 days of *Z. muelleri* response and acclimation to light limitation. The next step is to now confirm what transcriptional-wide changes occur over longer periods of time in *Z. muelleri* in response to light limitation. Seagrass mortality occurs over longer exposures to light limitation (Chartrand et al., 2012; McMahon et al., 2013; York et al., 2013) with Chartrand et al., 2012 suggesting seagrass decline after 2 weeks. One other aspect of light response and acclimation, which needs to be addressed in *Z. muelleri*, is also the transcriptional changes associated with high light response and photo-oxidative stress. During summer months, seagrasses that exist in shallow inter-tidal areas of coastal zones are prone to leaf bleaching, desiccation and mortality (Unsworth et al., 2012).

6.6.3 Design and implementation of molecular markers for reactive monitoring of light-related stress through targeted gene expression assay

Current seagrass monitoring and assessment protocols reveal changes in seagrass communities through morphological change and loss of populations, but these approaches are severely limited by their inability to diagnose seagrass health in real-time for effective intervention and management. There is, therefore, a critical need to develop new diagnostic tools with fast turn-around times, based on cutting-edge technology, to monitor seagrass and preserve existing meadows. In Chapters 3, 4 and 5 selected gene groups were targeted for expression profiling. The work conducted in Chapters 4 and 5 provided more in-depth expression profiling of genes involved in photosystem light capture, the photosynthetic electron transport chain and carbon fixation at both nuclear and chloroplastic level. In both Chapters 3 and 4, key GO groups were discussed, which provide candidate gene groups for the design of a molecular tool kit to monitor light-related stress in seagrass. Chapter 3 additionally identified over 30,000 simple sequence repeat (SSR) motifs, which can be used in future genotyping, population genetic and gene flow studies.

Transcriptome assembly and bioinformatics analyses are perhaps the most challenging and labour-intensive steps in the workflow of identifying molecular marker genes. The work conducted in this thesis paves the way for designation of molecular

markers, which can be used in future laboratory and field experiments based on *Z. muelleri*. The high similarity between *Z. marina* and *Z. muelleri* genes (Chapters 3, 4 and 5) means that the work conducted in this thesis can and may also be applied to the wider *Zostera* genus. In this respect, a new gene expression profiling technique known as nanostring (nanostring technologies®) has been made available for gene expression profiling and out-competes RT-qPCR approaches (used in Chapter 5 of this thesis). Nanostring technology makes use of small oligo hybridization probes (typically 50bp in size), nCounter® barcoding technology and cartridges. This technology allows for a rapid and cost-effective means to multiplex and profile up to 800 genes at once. It is already used as a clinical diagnostic tool to assess a patient's risk of recurrence of breast cancer in order to develop the appropriate medical treatment (Veldman-Jones et al, 2015).

Like micro-array technology, plates can be custom designed to contain gene probes of interest, making monitoring projects more viable, reducing cost, time and labour. Such approaches would indeed be suitable for long-term monitoring efforts of seagrasses. Unlike RT-qPCR, which takes considerable time to test primer efficiency; run quantifiable plates and then analyse the data, nanostring technology encompasses all of these steps into one protocol, which takes less than 5 hours from loading of the plates to analysed results. Global normalisation can be conducted; as well as geometric normalisation of reference genes. Plate design is the longest part of the process. Whilst this protocol provides an attractive alternative to RNA-seq, it is limited to only 800 genes at one given time. The genes and gene groups, which are of interest in this PhD thesis, can therefore be put forward for future *Z. muelleri* monitoring efforts associated with light limitation. Such approaches will not only allow rapid detection of light limitation in *Z. muelleri* meadows, but it will also have the capability to deliver fast and reliable results without the difficulty of utilizing bioinformatics approaches, which are highly time-consuming and difficult to learn.

6.7 Conclusions

i.) A substantial literature review based on the molecular biology of seagrasses has identified areas, which have undergone substantial development in the past few decades, more specifically in the last ten years. The review has also highlighted the need to address all seagrass species and not just model

species. This thesis has therefore provided new insights and knowledge on how *Z. muelleri* responds to light limitation. This thesis described the first characterisation of a Southern Hemisphere seagrass in response to an environmental perturbation using next generation sequencing.

ii.) A 14-day window was chosen to study transcriptome-wide changes of *Z. muelleri* have been examined in response to acute light limitation. Key differences in the transcriptional regulation of genes associated with photosynthesis, pigment composition, primary metabolism and secondary metabolism were investigated. These gene regulation shifts correlated with downstream photophysiology in *Z. muelleri*.

iii.) The data indicate that chloroplast-encoded photosynthetic genes play an important role in the response to light limitation. Regulational shifts in these genes can be correlated with downstream changes in $rETR_{max}$, I_k and Yi. Additionally, the use of nuclear-encoded reference genes seemed to be superior to chloroplast-encoded reference genes for normalisation of target genes using RT-qPCR.

iv.) Sequencing of the *Z. muelleri* and *Z. marina* genomes have been important advances in seagrass molecular biology in 2016. In this PhD thesis, comparisons between *de novo* based assemblies and genome-guided assemblies highlight that genome-guided assemblies are superior to *de novo* assembly. However, with time, the draft genome assembly of *Z. muelleri* needs to undergo further improvement and completion by increasing read coverage.

v.) Completion of this PhD thesis has identified new considerations for future research, which were previously not identified in the critical literature review. These new considerations which require further investigation include (i) the paradigm of the *Z. muelleri* holobiont, (ii) the use of new targeted gene expression assays such as nanostring technology in monitoring projects, (iii) chronic exposure of *Z. muelleri* to light limitation, (iv) response and acclimation of *Z. muelleri* to high light stress and (v) the use of other omics technologies to further complement our knowledge of *Z. muelleri* response and resilience to light limitation.

6.8 References

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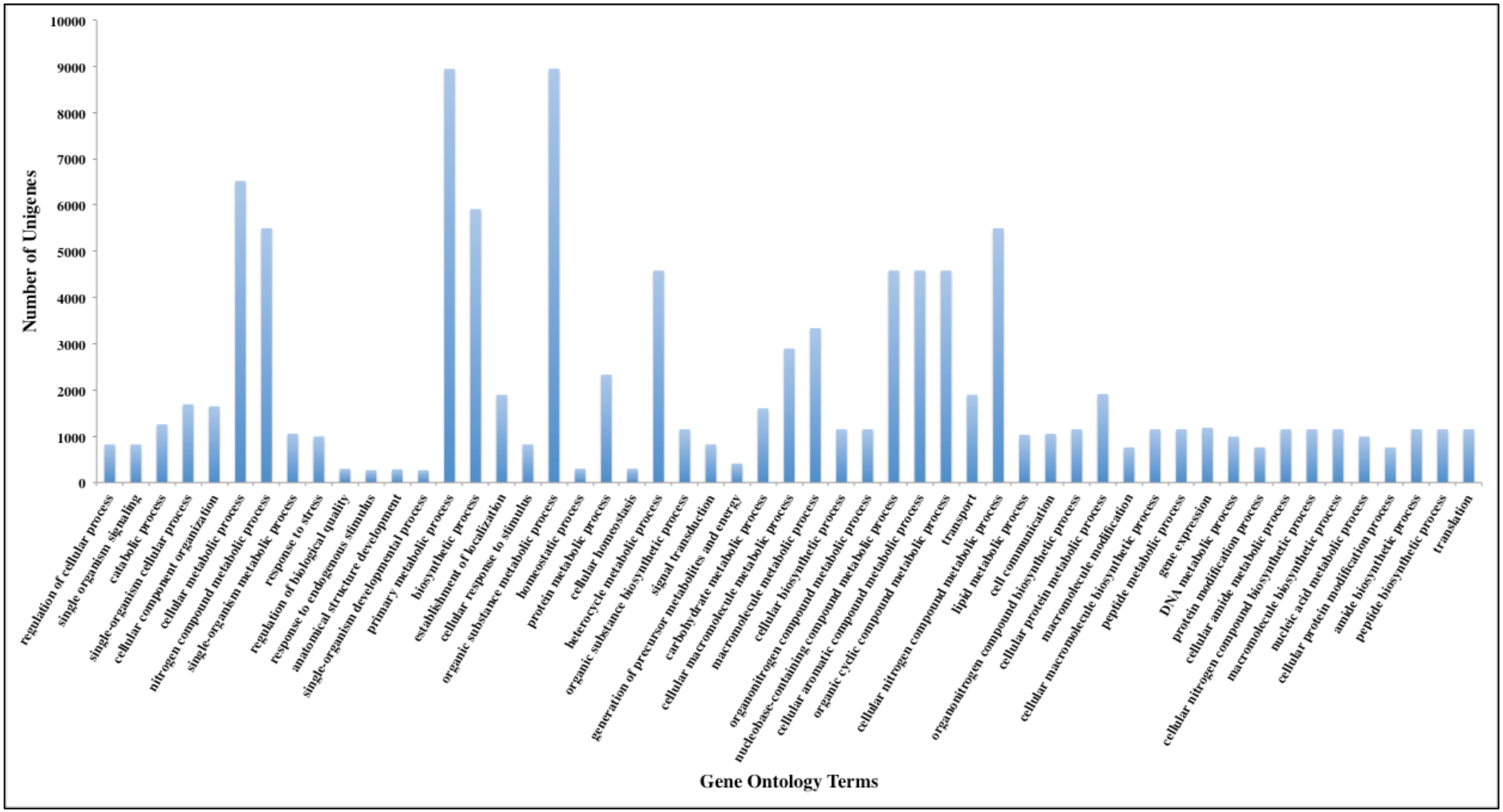
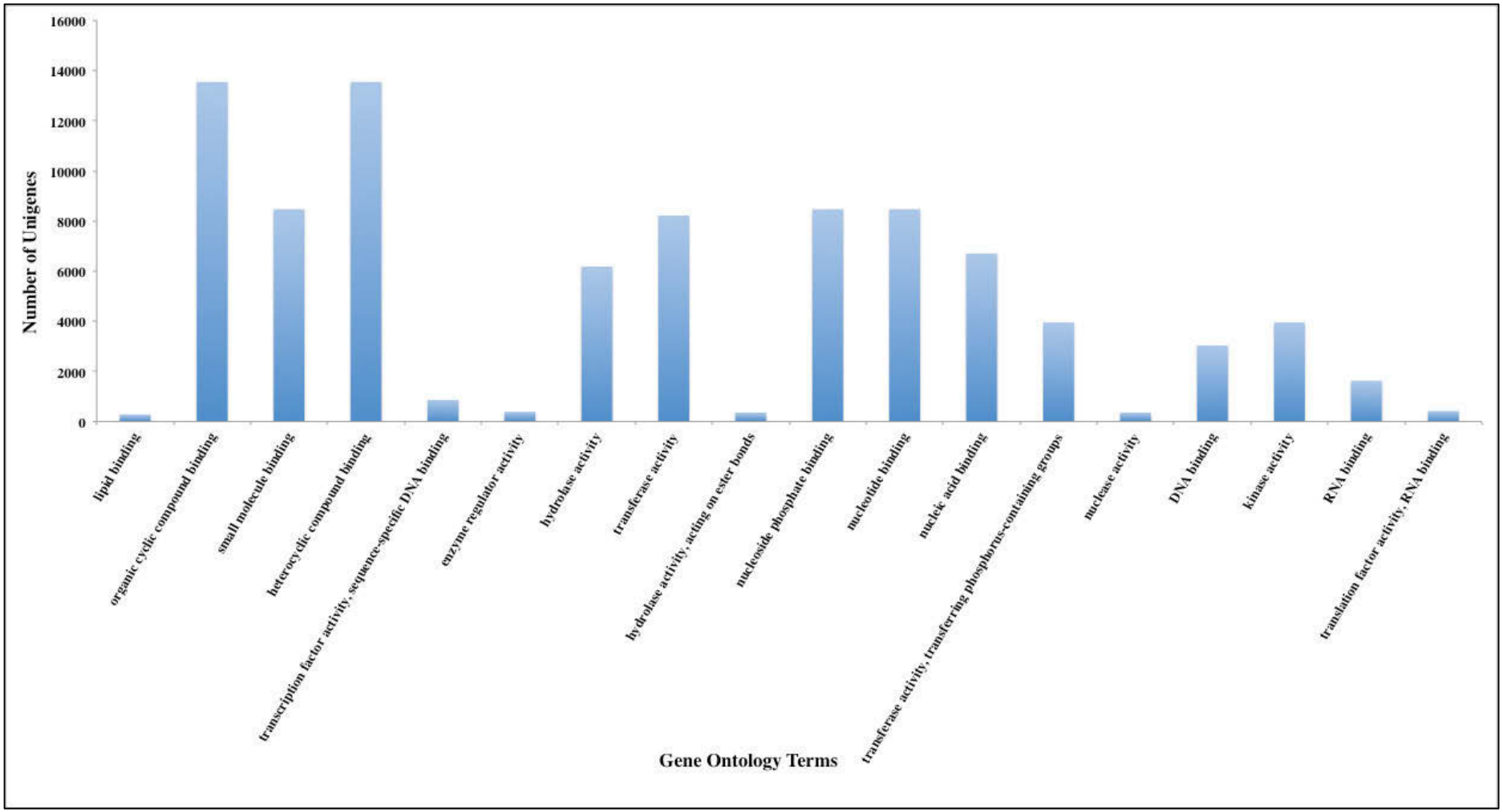
APPENDIX 1

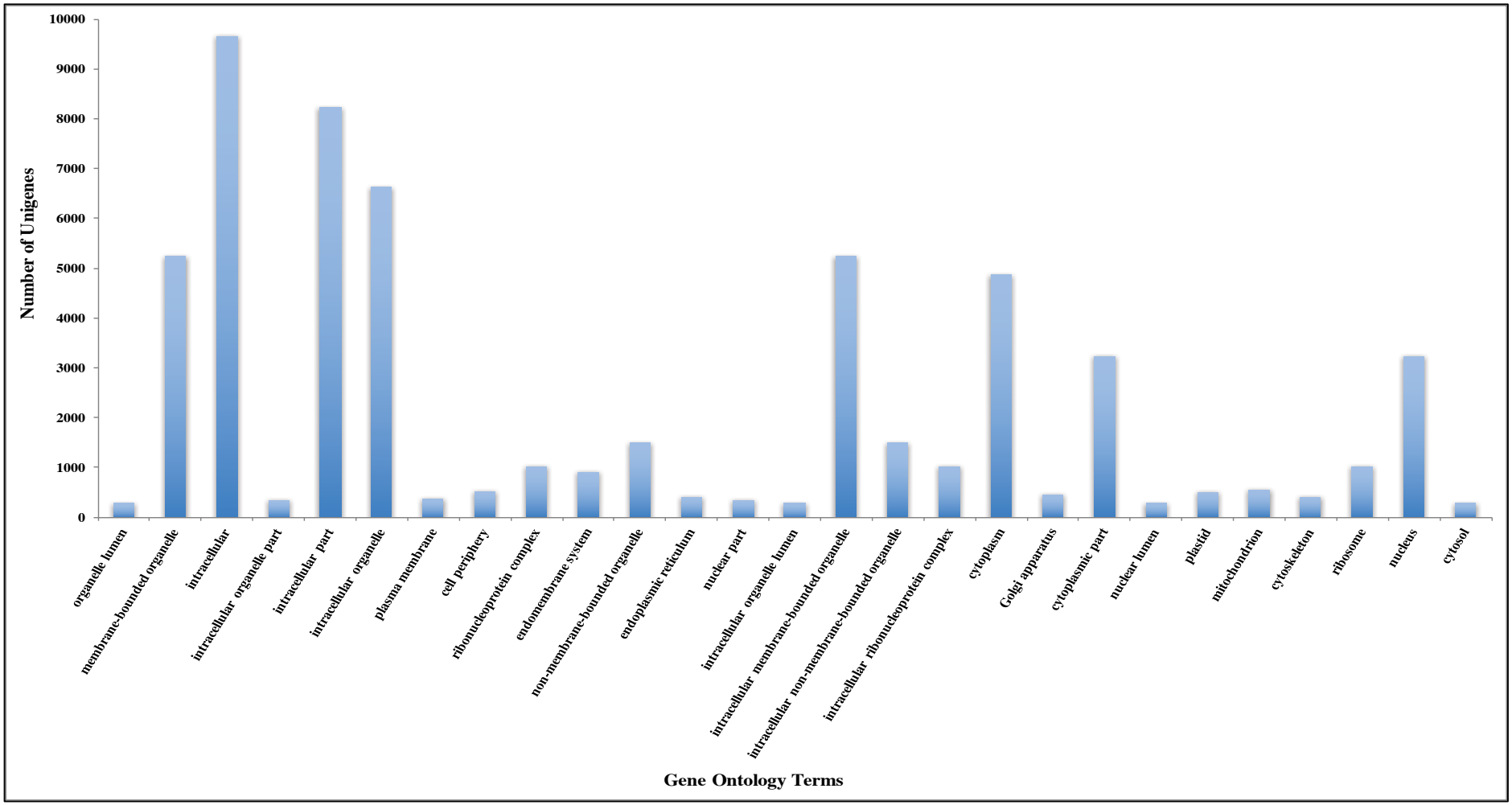
Chapter 3 supplementary information



A.1; Figure 1: Plant GO-Slim characterisation of the *Z. muelleri* transcriptome 'Biological Processes'. A threshold of 250 or more unigenes per GO term was applied. Level 3 and below (Blast2GO software) are only shown.



A.1; Figure 2: Plant GO-Slim characterisation of the *Z. muelleri* transcriptome 'Molecular Functions'. A threshold of 250 or more unigenes per GO term was applied. Level 3 and below (Blast2GO software) are only shown.



A.1; Figure 3: Plant GO-Slim characterisation of the *Z. muelleri* transcriptome 'Cellular Compartments'. A threshold of 250 or more unigenes per GO term was applied. Level 3 and below (Blast2GO software) are only shown.

A.1; Table 1: Number of paired end reads (bp) in each library used to assemble the *Z. muelleri* transcriptome.

Library	Number of paired end reads (bp)
1,000 µmol photons m^{-2} s^{-1} Day 9	20,942,646
1,000 µmol photons m^{-2} s^{-1} Day 12	21,219,189
1,000 µmol photons m^{-2} s^{-1} Day 14	17,673,140
250 µmol photons m^{-2} s^{-1} Day 9	25,875,223
250 µmol photons m^{-2} s^{-1} Day 12	33,049,017
250 µmol photons m^{-2} s^{-1} Day 14	26,482,775
10 µmol photons m^{-2} s^{-1} Day 9	16,069,314
10 µmol photons m^{-2} s^{-1} Day 12	18,411,131
10 µmol photons m^{-2} s^{-1} Day 14	23,738,879

A.1; Table 2: Functional distribution hit analysis showing top 5 most represented plant species in DIAMOND protein alignment searches (E-value of 1.0×10^{-5}.); 52,616 = total number of best hits retained.

Species	# Functional Hits	Percent (%)	Plant Type
Zostera marina	43,692	83.04	Monocot
Musa acuminata subsp. malaccensis	1,131	2.15	Monocot
Vitis vinifera	770	1.46	Dicot
Cajanus cajan	497	0.94	Dicot
Theobroma cocao	297	0.56	Dicot

A.1: Table 3: Enriched GO terms in ~10 µmol photons $m^{-2} s^{-1}$ and ~250 µmol photons $m^{-2} s^{-1}$ of light combined. GO = Gene Ontology, NS = Classification, Name = Name of GO term, FDR threshold = 0.05. BP = Biological Process; CC = Cellular Compartment and MF = Molecular Function.

GO	NS	Name	FDR
GO:0046906	MF	tetrapyrrole binding	5.52E-08
GO:0009522	CC	photosystem I	2.11E-07
GO:0016168	MF	chlorophyll binding	2.11E-07
GO:0009521	CC	photosystem	4.35E-07
GO:0009765	BP	photosynthesis, light harvesting	2.16E-06
GO:0044436	CC	thylakoid part	2.16E-06
GO:0018298	BP	protein-chromophore linkage	5.87E-06
GO:0009535	CC	chloroplast thylakoid membrane	9.97E-06
GO:0055035	CC	plastid thylakoid membrane	9.97E-06
GO:0034357	CC	photosynthetic membrane	1.16E-05
GO:0042651	CC	thylakoid membrane	1.16E-05
GO:0042744	BP	hydrogen peroxide catabolic process	1.23E-05
GO:0042743	BP	hydrogen peroxide metabolic process	1.23E-05
GO:0098796	CC	membrane protein complex	1.91E-05
GO:0072593	BP	reactive oxygen species metabolic process	2.66E-05
GO:0044435	CC	plastid part	3.60E-05
GO:0044434	CC	chloroplast part	3.60E-05
GO:0006091	BP	generation of precursor metabolites and energy	0.000142
GO:0006979	BP	response to oxidative stress	0.000321
GO:0016491	MF	oxidoreductase activity	0.000326
GO:0004601	MF	peroxidase activity	0.000326
GO:0020037	MF	heme binding	0.000326

GO:0016684	MF	oxidoreductase activity, acting on peroxide as acceptor	0.000361
GO:0008152	BP	metabolic process	0.000503
GO:0016209	MF	antioxidant activity	0.00169
GO:0005576	CC	extracellular region	0.00187
GO:0044237	BP	cellular metabolic process	0.00483
GO:0016984	MF	ribulose-bisphosphate carboxylase activity	0.0158
GO:0016630	MF	protochlorophyllide reductase activity	0.0158
GO:0015979	BP	photosynthesis	0.0202
GO:0016679	MF	oxidoreductase activity, acting on diphenols and related substances as donors	0.0243
GO:0009853	BP	photorespiration	0.0261
GO:0032991	CC	macromolecular complex	0.0318
GO:0043234	CC	protein complex	0.0399
GO:0044444	CC	cytoplasmic part	0.0433
GO:0044248	BP	cellular catabolic process	0.0435

A.1; Table 4: Enriched GO terms in ~10 μmol photons $m^{-2} s^{-1}$ of light. GO = Gene Ontology, NS = Classification, Name = Name of GO term, FDR threshold = 0.05. BP = Biological Process; CC = Cellular Compartment and MF = Molecular Function.

GO	NS	Name	FDR
GO:0009423	BP	chorismate biosynthetic process	1.71E-08
GO:0009073	BP	aromatic amino acid family biosynthetic process	2.16E-08
GO:0009072	BP	aromatic amino acid family metabolic process	2.16E-08
GO:0043650	BP	dicarboxylic acid biosynthetic process	2.29E-08
GO:0005506	MF	iron ion binding	2.29E-08
GO:0016705	MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	2.29E-08
GO:0046906	MF	tetrapyrrole binding	2.29E-08
GO:0043436	BP	oxoacid metabolic process	2.48E-08
GO:0016053	BP	organic acid biosynthetic process	2.58E-08
GO:0044283	BP	small molecule biosynthetic process	2.75E-08
GO:0048037	MF	cofactor binding	2.75E-08
GO:0016614	MF	oxidoreductase activity, acting on CH-OH group of donors	2.75E-08
GO:0019752	BP	carboxylic acid metabolic process	2.80E-08
GO:0004497	MF	monooxygenase activity	2.85E-08
GO:0046394	BP	carboxylic acid biosynthetic process	3.12E-08
GO:0006082	BP	organic acid metabolic process	3.12E-08
GO:0020037	MF	heme binding	3.12E-08
GO:0044710	BP	single-organism metabolic process	3.22E-08
GO:0044281	BP	small molecule metabolic process	3.22E-08
GO:0016491	MF	oxidoreductase activity	3.22E-08
GO:0044699	BP	single-organism process	4.59E-08
GO:0044763	BP	single-organism cellular process	4.76E-08
GO:0003824	MF	catalytic activity	6.25E-08

GO:0031408	BP	oxylipin biosynthetic process	9.40E-08
GO:0031407	BP	oxylipin metabolic process	9.40E-08
GO:0046417	BP	chorismate metabolic process	1.66E-07
GO:0003885	MF	D-arabinono-1,4-lactone oxidase activity	1.66E-07
GO:0043167	MF	ion binding	3.08E-07
GO:0050662	MF	coenzyme binding	9.40E-07
GO:0009064	BP	glutamine family amino acid metabolic process	9.96E-07
GO:0015116	MF	sulfate transmembrane transporter activity	3.08E-06
GO:0008271	MF	secondary active sulfate transmembrane transporter activity	3.08E-06
GO:1901682	MF	sulfur compound transmembrane transporter activity	3.08E-06
GO:0016899	MF	oxidoreductase activity, acting on the CH-OH group of donors, oxygen as acceptor	5.31E-06
GO:0003866	MF	3-phosphoshikimate 1-carboxyvinyltransferase activity	5.49E-06
GO:0043648	BP	dicarboxylic acid metabolic process	8.57E-06
GO:0006541	BP	glutamine metabolic process	1.12E-05
GO:0046872	MF	metal ion binding	1.90E-05
GO:0043169	MF	cation binding	1.90E-05
GO:0055114	BP	oxidation-reduction process	1.96E-05
GO:0051213	MF	dioxygenase activity	2.64E-05
GO:0016616	MF	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	2.97E-05
GO:0044711	BP	single-organism biosynthetic process	4.90E-05
GO:0004664	MF	prephenate dehydratase activity	4.90E-05
GO:0016679	MF	oxidoreductase activity, acting on diphenols and related substances as donors	5.09E-05
GO:0009094	BP	L-phenylalanine biosynthetic process	5.86E-05
GO:0008037	BP	cell recognition	6.65E-05
GO:0048544	BP	recognition of pollen	6.65E-05
GO:0016838	MF	carbon-oxygen lyase activity, acting on phosphates	8.76E-05

GO:0019419	BP	sulfate reduction	9.10E-05
GO:0016702	MF	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	0.000147
GO:1901605	BP	alpha-amino acid metabolic process	0.000152
GO:0003849	MF	3-deoxy-7-phosphoheptulonate synthase activity	0.00026
GO:0016835	MF	carbon-oxygen lyase activity	0.000271
GO:0016682	MF	oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	0.000335
GO:0003674	MF	molecular_function	0.000337
GO:0016765	MF	transferase activity, transferring alkyl or aryl (other than methyl) groups	0.000369
GO:0044702	BP	single organism reproductive process	0.000497
GO:0009095	BP	aromatic amino acid family biosynthetic process, prephenate pathway	0.000577
GO:0006558	BP	L-phenylalanine metabolic process	0.000655
GO:0051156	BP	glucose 6-phosphate metabolic process	0.000741
GO:0006098	BP	pentose-phosphate shunt	0.000741
GO:0016021	CC	integral component of membrane	0.000741
GO:0016701	MF	oxidoreductase activity, acting on single donors with incorporation of molecular oxygen	0.00085
GO:0031224	CC	intrinsic component of membrane	0.000899
GO:0016653	MF	oxidoreductase activity, acting on NAD(P)H, heme protein as acceptor	0.00133
GO:0022414	BP	reproductive process	0.00158
GO:1901564	BP	organonitrogen compound metabolic process	0.0016
GO:0004457	MF	lactate dehydrogenase activity	0.0016
GO:0004459	MF	L-lactate dehydrogenase activity	0.0016
GO:0004107	MF	chorismate synthase activity	0.0016
GO:0015103	MF	inorganic anion transmembrane transporter activity	0.00212
GO:0006739	BP	NADP metabolic process	0.00217
GO:0019682	BP	glyceraldehyde-3-phosphate metabolic process	0.00217
GO:0006520	BP	cellular amino acid metabolic process	0.00217

GO:0016671	MF	oxidoreductase activity, acting on a sulfur group of donors, disulfide as acceptor	0.00376
GO:0044425	CC	membrane part	0.00386
GO:0004345	MF	glucose-6-phosphate dehydrogenase activity	0.0044
GO:0005509	MF	calcium ion binding	0.00494
GO:0008171	MF	O-methyltransferase activity	0.00512
GO:0006081	BP	cellular aldehyde metabolic process	0.0058
GO:0016651	MF	oxidoreductase activity, acting on NAD(P)H	0.0058
GO:0006493	BP	protein O-linked glycosylation	0.00595
GO:0047769	MF	arogenate dehydratase activity	0.00595
GO:0030247	MF	polysaccharide binding	0.00625
GO:0001871	MF	pattern binding	0.00625
GO:0006635	BP	fatty acid beta-oxidation	0.00735
GO:0008509	MF	anion transmembrane transporter activity	0.00738
GO:0009062	BP	fatty acid catabolic process	0.00795
GO:0070469	CC	respiratory chain	0.0117
GO:0003857	MF	3-hydroxyacyl-CoA dehydrogenase activity	0.0119
GO:0019395	BP	fatty acid oxidation	0.0145
GO:0008652	BP	cellular amino acid biosynthetic process	0.0145
GO:0005996	BP	monosaccharide metabolic process	0.0153
GO:0016405	MF	CoA-ligase activity	0.0153
GO:0015291	MF	secondary active transmembrane transporter activity	0.0156
GO:0034440	BP	lipid oxidation	0.0167
GO:0072329	BP	monocarboxylic acid catabolic process	0.0178
GO:0009916	MF	alternative oxidase activity	0.018
GO:0016878	MF	acid-thiol ligase activity	0.018
GO:0004575	MF	sucrose alpha-glucosidase activity	0.018

GO:0004564	MF	beta-fructofuranosidase activity	0.018
GO:0090599	MF	alpha-glucosidase activity	0.018
GO:0016836	MF	hydro-lyase activity	0.0183
GO:0006006	BP	glucose metabolic process	0.0251
GO:0004601	MF	peroxidase activity	0.0253
GO:0072524	BP	pyridine-containing compound metabolic process	0.0266
GO:0050660	MF	flavin adenine dinucleotide binding	0.0299
GO:0016684	MF	oxidoreductase activity, acting on peroxide as acceptor	0.0305
GO:0008152	BP	metabolic process	0.0334
GO:0006732	BP	coenzyme metabolic process	0.0344
GO:0004128	MF	cytochrome-b5 reductase activity, acting on NAD(P)H	0.0344
GO:0044242	BP	cellular lipid catabolic process	0.0358
GO:0016829	MF	lyase activity	0.0358
GO:0009507	CC	chloroplast	0.0368
GO:0006730	BP	one-carbon metabolic process	0.0386
GO:0005777	CC	peroxisome	0.0386
GO:0004097	MF	catechol oxidase activity	0.0386
GO:0046395	BP	carboxylic acid catabolic process	0.0421
GO:0009536	CC	plastid	0.0454
GO:0016054	BP	organic acid catabolic process	0.0468

A.1; Table 5: Enriched GO terms in ~250 μmol photons m^{-2} s^{-1} of light. GO = Gene Ontology, NS = Classification, Name = Name of GO term, FDR threshold = 0.05. BP = Biological Process; CC = Cellular Compartment and MF = Molecular Function.

GO	NS	Name	FDR
GO:0043043	BP	peptide biosynthetic process	1.65E-08
GO:0043604	BP	amide biosynthetic process	1.65E-08
GO:0043603	BP	cellular amide metabolic process	1.65E-08
GO:0009059	BP	macromolecule biosynthetic process	1.65E-08
GO:0044267	BP	cellular protein metabolic process	1.65E-08
GO:0044391	CC	ribosomal subunit	1.65E-08
GO:0043232	CC	intracellular non-membrane-bounded organelle	1.65E-08
GO:0043228	CC	non-membrane-bounded organelle	1.65E-08
GO:0005840	CC	ribosome	1.65E-08
GO:0030529	CC	intracellular ribonucleoprotein complex	1.65E-08
GO:1990904	CC	ribonucleoprotein complex	1.65E-08
GO:0006518	BP	peptide metabolic process	1.82E-08
GO:1901566	BP	organonitrogen compound biosynthetic process	1.82E-08
GO:0044249	BP	cellular biosynthetic process	1.82E-08
GO:0003735	MF	structural constituent of ribosome	1.82E-08
GO:0006412	BP	translation	2.02E-08
GO:0009058	BP	biosynthetic process	2.02E-08
GO:1901576	BP	organic substance biosynthetic process	2.02E-08
GO:1901564	BP	organonitrogen compound metabolic process	2.02E-08
GO:0034641	BP	cellular nitrogen compound metabolic process	2.02E-08
GO:0043170	BP	macromolecule metabolic process	2.02E-08
GO:0044260	BP	cellular macromolecule metabolic process	2.02E-08
GO:0034645	BP	cellular macromolecule biosynthetic process	2.02E-08

GO:0019538	BP	protein metabolic process	2.02E-08
GO:0044271	BP	cellular nitrogen compound biosynthetic process	2.02E-08
GO:0043229	CC	intracellular organelle	2.02E-08
GO:0043226	CC	organelle	2.02E-08
GO:0044444	CC	cytoplasmic part	2.02E-08
GO:0005198	MF	structural molecule activity	2.02E-08
GO:0032991	CC	macromolecular complex	2.04E-08
GO:0044237	BP	cellular metabolic process	2.10E-08
GO:0044464	CC	cell part	2.10E-08
GO:0044424	CC	intracellular part	2.35E-08
GO:0006807	BP	nitrogen compound metabolic process	2.62E-08
GO:0009987	BP	cellular process	2.66E-08
GO:0071704	BP	organic substance metabolic process	2.78E-08
GO:0044238	BP	primary metabolic process	3.25E-08
GO:0015935	CC	small ribosomal subunit	5.85E-08
GO:0005575	CC	cellular_component	2.17E-07
GO:0008150	BP	biological_process	2.85E-07
GO:0008152	BP	metabolic process	6.31E-07
GO:0005853	CC	eukaryotic translation elongation factor 1 complex	0.00107
GO:0003746	MF	translation elongation factor activity	0.00119
GO:0006457	BP	protein folding	0.00125
GO:0044422	CC	organelle part	0.00306
GO:0044446	CC	intracellular organelle part	0.00306
GO:0005200	MF	structural constituent of cytoskeleton	0.0042
GO:0000028	BP	ribosomal small subunit assembly	0.00468
GO:0005525	MF	GTP binding	0.00802

GO:0019001	MF	guanyl nucleotide binding	0.00802
GO:0032561	MF	guanyl ribonucleotide binding	0.00802
GO:0003924	MF	GTPase activity	0.00995
GO:0019464	BP	glycine decarboxylation via glycine cleavage system	0.0262
GO:0015934	CC	large ribosomal subunit	0.0262
GO:0005960	CC	glycine cleavage complex	0.0262
GO:0019843	MF	rRNA binding	0.0267
GO:0044769	MF	ATPase activity, coupled to transmembrane movement of ions, rotational mechanism	0.0328
GO:0042026	BP	protein refolding	0.0344

A.1; Table 6: Enriched GO terms in ~1,000 μmol photons m^{-2} s^{-1} of light. GO = Gene Ontology, NS = Classification, Name = Name of GO term, FDR = 0.05. BP = Biological Process; CC = Cellular Compartment and MF = Molecular Function.

GO	NS	Name	FDR
GO:0019252	BP	starch biosynthetic process	6.44E-08
GO:0005982	BP	starch metabolic process	8.59E-08
GO:0009501	CC	amyloplast	7.86E-06
GO:0008184	MF	glycogen phosphorylase activity	7.86E-06
GO:0004373	MF	glycogen (starch) synthase activity	1.19E-05
GO:0034637	BP	cellular carbohydrate biosynthetic process	2.40E-05
GO:0009250	BP	glucan biosynthetic process	2.51E-05
GO:0033692	BP	cellular polysaccharide biosynthetic process	4.20E-05
GO:0009507	CC	chloroplast	6.38E-05
GO:0009536	CC	plastid	7.61E-05
GO:0016051	BP	carbohydrate biosynthetic process	0.000222
GO:0006073	BP	cellular glucan metabolic process	0.000237
GO:0044042	BP	glucan metabolic process	0.000237
GO:0000271	BP	polysaccharide biosynthetic process	0.000256
GO:0004645	MF	phosphorylase activity	0.000256
GO:0044264	BP	cellular polysaccharide metabolic process	0.000298
GO:0044262	BP	cellular carbohydrate metabolic process	0.000649
GO:2000028	BP	regulation of photoperiodism, flowering	0.000877
GO:0030170	MF	pyridoxal phosphate binding	0.00364
GO:0016758	MF	transferase activity, transferring hexosyl groups	0.0041
GO:2000241	BP	regulation of reproductive process	0.00411
GO:0006112	BP	energy reserve metabolic process	0.00461
GO:0005977	BP	glycogen metabolic process	0.00461

GO:0005978	BP	glycogen biosynthetic process	0.00461
GO:2000026	BP	regulation of multicellular organismal development	0.00478
GO:0048580	BP	regulation of post-embryonic development	0.00478
GO:0005976	BP	polysaccharide metabolic process	0.00492
GO:0005975	BP	carbohydrate metabolic process	0.00496
GO:0015980	BP	energy derivation by oxidation of organic compounds	0.00655
GO:0044711	BP	single-organism biosynthetic process	0.0112
GO:0051239	BP	regulation of multicellular organismal process	0.0112
GO:0044723	BP	single-organism carbohydrate metabolic process	0.0112
GO:0008150	BP	biological_process	0.0153
GO:0009058	BP	biosynthetic process	0.0153
GO:0006950	BP	response to stress	0.021
GO:1901606	BP	alpha-amino acid catabolic process	0.0265
GO:0043168	MF	anion binding	0.0307
GO:0004222	MF	metalloendopeptidase activity	0.0307
GO:0044710	BP	single-organism metabolic process	0.0316
GO:0042724	BP	thiamine-containing compound biosynthetic process	0.0361
GO:0009228	BP	thiamine biosynthetic process	0.0361
GO:0035251	MF	UDP-glucosyltransferase activity	0.0361
GO:0046527	MF	glucosyltransferase activity	0.0361
GO:0019199	MF	transmembrane receptor protein kinase activity	0.0361
GO:0016757	MF	transferase activity, transferring glycosyl groups	0.0394
GO:0004375	MF	glycine dehydrogenase (decarboxylating) activity	0.0394
GO:0016642	MF	oxidoreductase activity, acting on the CH-NH2 group of donors, disulfide as acceptor	0.0394
GO:0008152	BP	metabolic process	0.0447
GO:0050896	BP	response to stimulus	0.048

A.1: Table 7: PlantGSEA metabolic pathway enrichment of terms in ~1,000 μmol photons m^{-2} s^{-1} of light. FDR threshold = 0.05.

Description	Category	NO. Genes in Overlap (k)	FDR
Metabolic pathways	KEGG	13	5.47E-05
Starch and sucrose metabolism	KEGG	4	4.20E-04
Circadian rhythm - plant	KEGG	3	7.38E-04
Stilbenoid, diarylheptanoid and gingerol biosynthesis	KEGG	3	8.60E-04
Biosynthesis of phenylpropanoids	KEGG	5	8.60E-04
Limonene and pinene degradation	KEGG	3	8.60E-04
starch biosynthesis	PlantCyc	3	3.84E-03
Ubiquinone and other terpenoid-quinone biosynthesis	KEGG	2	0.0117
thiamine biosynthesis II	PlantCyc	2	0.0117
simple coumarins biosynthesis	PlantCyc	2	0.0117
phenylpropanoid biosynthesis	PlantCyc	2	0.0159
Inositol phosphate metabolism	KEGG	2	0.0162
Biosynthesis of alkaloids derived from shikimate pathway	KEGG	3	0.0256
Phenylalanine metabolism	KEGG	2	0.0256
Phenylpropanoid biosynthesis	KEGG	2	0.0278
flavonoid biosynthesis	PlantCyc	2	0.0289
scopoletin biosynthesis	PlantCyc	1	0.049
xanthophyll cycle	PlantCyc	1	0.049
IAA biosynthesis I	PlantCyc	1	0.049
glycolysis IV (plant cytosol)	PlantCyc	2	0.049
glycolysis I (plastidic)	PlantCyc	2	0.049
myo-inositol biosynthesis	PlantCyc	1	0.049
4-hydroxyphenylpyruvate biosynthesis	PlantCyc	1	0.049
camalexin biosynthesis	PlantCyc	1	0.049

antheraxanthin and violaxanthin biosynthesis	PlantCyc	1	0.049
tyrosine degradation I	PlantCyc	1	0.049
1D-myo-inositol hexakisphosphate biosynthesis III (Spirodela polyrrhiza)	PlantCyc	1	0.049
phenylalanine degradation III	PlantCyc	1	0.049
glycine cleavage complex	PlantCyc	1	0.049
gluconeogenesis from PlantCyc	PlantCyc	2	0.049
proline degradation II	PlantCyc	1	0.049

A.1: Table 8: PlantGSEA metabolic pathway enrichment of terms in ~250 μmol photons m^{-2} s^{-1} of light. FDR threshold = 0.05.

Description	Category	NO. Genes in Overlap (k)	FDR
Ribosome	KEGG	34	2.86E-35
Metabolic pathways	KEGG	21	5.76E-05
Oxidative phosphorylation	KEGG	7	7.26E-05
Flavonoid biosynthesis	KEGG	3	1.34E-03
Biosynthesis of phenylpropanoids	KEGG	7	1.99E-03
Proteasome	KEGG	4	4.40E-03
Glucosinolate biosynthesis	KEGG	2	7.85E-03
Fructose and mannose metabolism	KEGG	3	0.0116
Methane metabolism	KEGG	3	0.013
Amino sugar and nucleotide sugar metabolism	KEGG	3	0.0371
flavonoid biosynthesis	PlantCyc	3	0.0383
glucosinolate biosynthesis from phenylalanine	PlantCyc	2	0.0383
glucosinolate biosynthesis from tryptophan	PlantCyc	2	0.0383
cellulose biosynthesis	PlantCyc	4	0.0383
Glyoxylate and dicarboxylate metabolism	KEGG	2	0.0384
glucosinolate biosynthesis from dihomomethionine	PlantCyc	2	0.0441
glucosinolate biosynthesis from hexahomomethionine	PlantCyc	2	0.0441
glucosinolate biosynthesis from homomethionine	PlantCyc	2	0.0441
glucosinolate biosynthesis from trihomomethionine	PlantCyc	2	0.0441
glucosinolate biosynthesis from pentahomomethionine	PlantCyc	2	0.0441
glucosinolate biosynthesis from tetrahomomethionine	PlantCyc	2	0.0441

A.1: Table 9: PlantGSEA metabolic pathway enrichment of terms in ~10 μmol photons m^{-2} s^{-1} of light. FDR threshold = 0.05.

Description	Category	NO. Genes in Overlap (k)	FDR
Metabolic pathways	KEGG	68	1.11E-30
Biosynthesis of plant hormones	KEGG	22	8.72E-12
Biosynthesis of phenylpropanoids	KEGG	19	4.58E-11
alpha-Linolenic acid metabolism	KEGG	10	1.23E-10
Biosynthesis of alkaloids derived from shikimate pathway	KEGG	15	5.32E-09
Stilbenoid, diarylheptanoid and gingerol biosynthesis	KEGG	7	2.48E-06
Phenylalanine, tyrosine and tryptophan biosynthesis	KEGG	7	2.65E-06
Limonene and pinene degradation	KEGG	7	4.28E-06
jasmonic acid biosynthesis	PlantCyc	8	6.56E-06
Starch and sucrose metabolism	KEGG	8	7.23E-06
Flavonoid biosynthesis	KEGG	5	1.08E-05
Nitrogen metabolism	KEGG	6	2.70E-05
chorismate biosynthesis	PlantCyc	6	5.96E-05
Amino sugar and nucleotide sugar metabolism	KEGG	7	1.15E-04
Oxidative phosphorylation	KEGG	8	1.41E-04
Phenylpropanoid biosynthesis	KEGG	6	2.24E-04
UDP-D-xylose biosynthesis	PlantCyc	6	3.78E-04
Pyruvate metabolism	KEGG	6	4.06E-04
Propanoate metabolism	KEGG	4	5.23E-04
Plant-pathogen interaction	KEGG	7	5.23E-04
Methane metabolism	KEGG	5	7.47E-04
Alanine, aspartate and glutamate metabolism	KEGG	5	7.47E-04
Carbon fixation in photosynthetic organisms	KEGG	6	9.19E-04
Phenylalanine metabolism	KEGG	5	9.48E-04

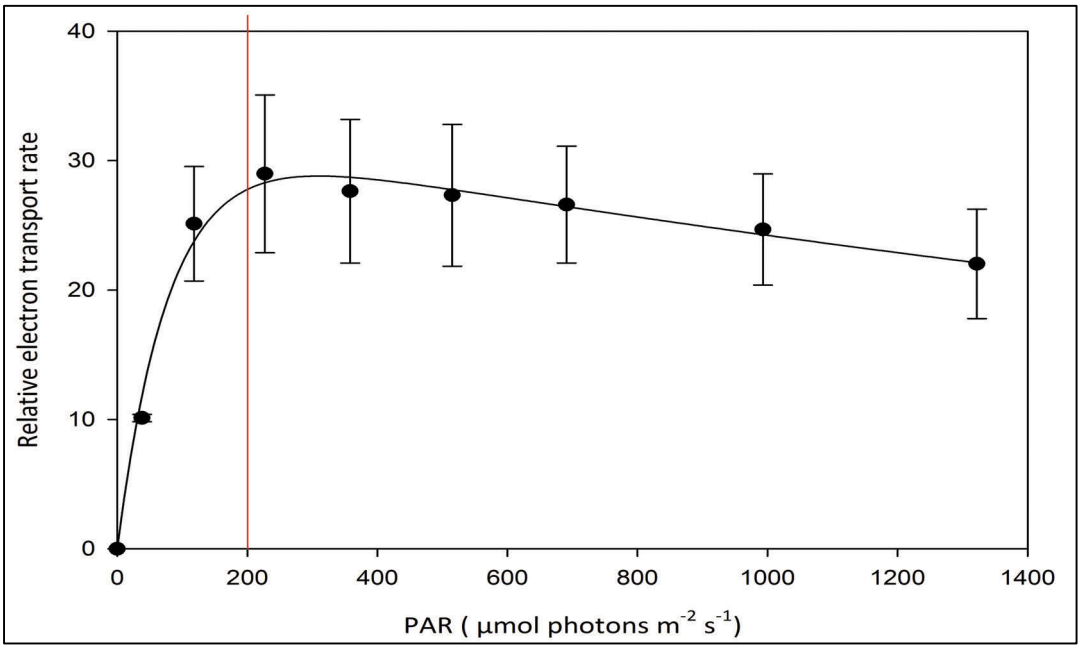
Ascorbate and aldarate metabolism	KEGG	4	9.53E-04
Biosynthesis of alkaloids derived from histidine and purine	KEGG	8	9.53E-04
sucrose degradation III	PlantCyc	7	9.74E-04
Glycolysis / Gluconeogenesis	KEGG	6	9.74E-04
13-LOX and 13-HPL pathway	PlantCyc	4	1.01E-03
Fatty acid metabolism	KEGG	4	2.04E-03
Fructose and mannose metabolism	KEGG	4	3.73E-03
Pentose and glucuronate interconversions	KEGG	3	3.73E-03
Photosynthesis - antenna proteins	KEGG	3	4.15E-03
Flavone and flavonol biosynthesis	KEGG	2	4.23E-03
Glutathione metabolism	KEGG	4	4.51E-03
Biosynthesis of alkaloids derived from ornithine, lysine and nicotinic acid	KEGG	7	4.56E-03
Pentose phosphate pathway	KEGG	4	5.53E-03
Ubiquinone and other terpenoid-quinone biosynthesis	KEGG	3	8.24E-03
Galactose metabolism	KEGG	3	0.0112
Cyanoamino acid metabolism	KEGG	2	0.0183
Biosynthesis of terpenoids and steroids	KEGG	6	0.019
suberin biosynthesis	PlantCyc	4	0.021
pentose phosphate pathway (oxidative branch)	PlantCyc	3	0.0225
simple coumarins biosynthesis	PlantCyc	3	0.0225
One carbon pool by folate	KEGG	2	0.0241
ascorbate glutathione cycle	PlantCyc	4	0.0243
ammonia assimilation cycle II	PlantCyc	3	0.0243
Biosynthesis of alkaloids derived from terpenoid and polyketide	KEGG	5	0.0337
beta-Alanine metabolism	KEGG	2	0.0386
Arginine and proline metabolism	KEGG	3	0.0436

A.1; Table 10: The top ten most common SSR repeat motifs found within the *Z. muelleri* transcriptome.

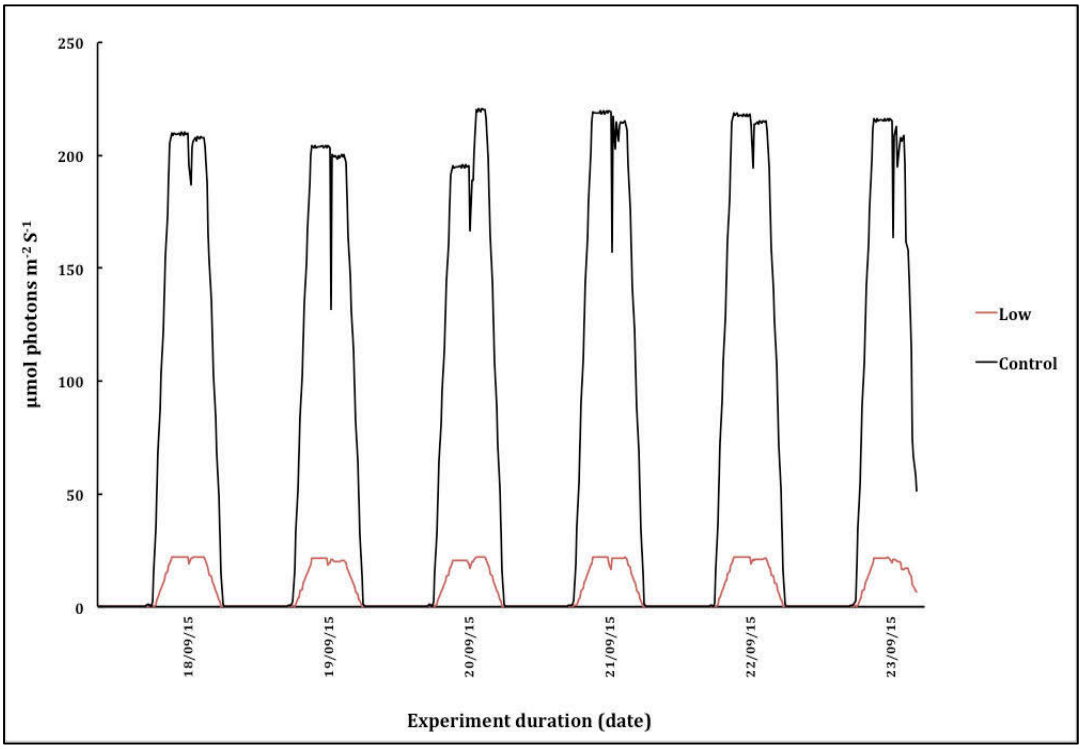
Motif	Total	Percent (%)
AAG/CTT	4,392	13.02
A/T	3,168	9.39
AGG/CCT	2,428	7.20
ATC/ATG	1,764	5.23
AG/CT	1,690	5.01
ACC/GGT	1,412	4.19
AGC/CTG	1,268	3.76
AAAT/ATTT	1,218	3.61
AAAG/CTTT	1,178	3.49
AT/AT	941	2.79

APPENDIX 2

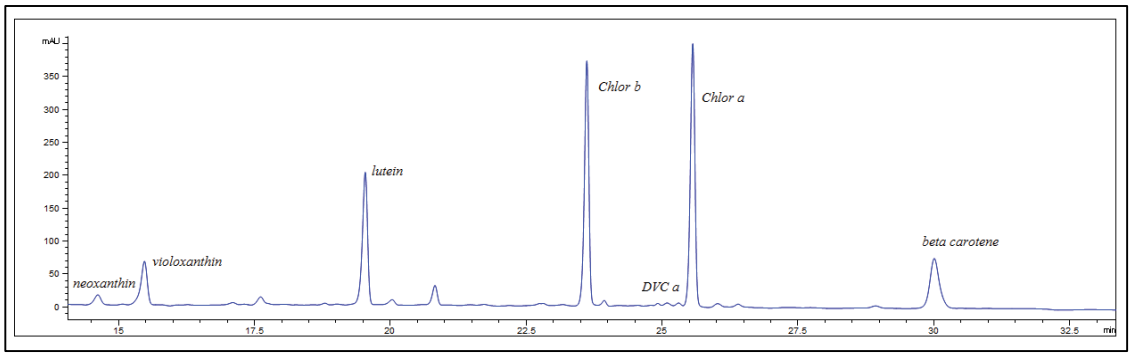
Chapter 4 supplementary information



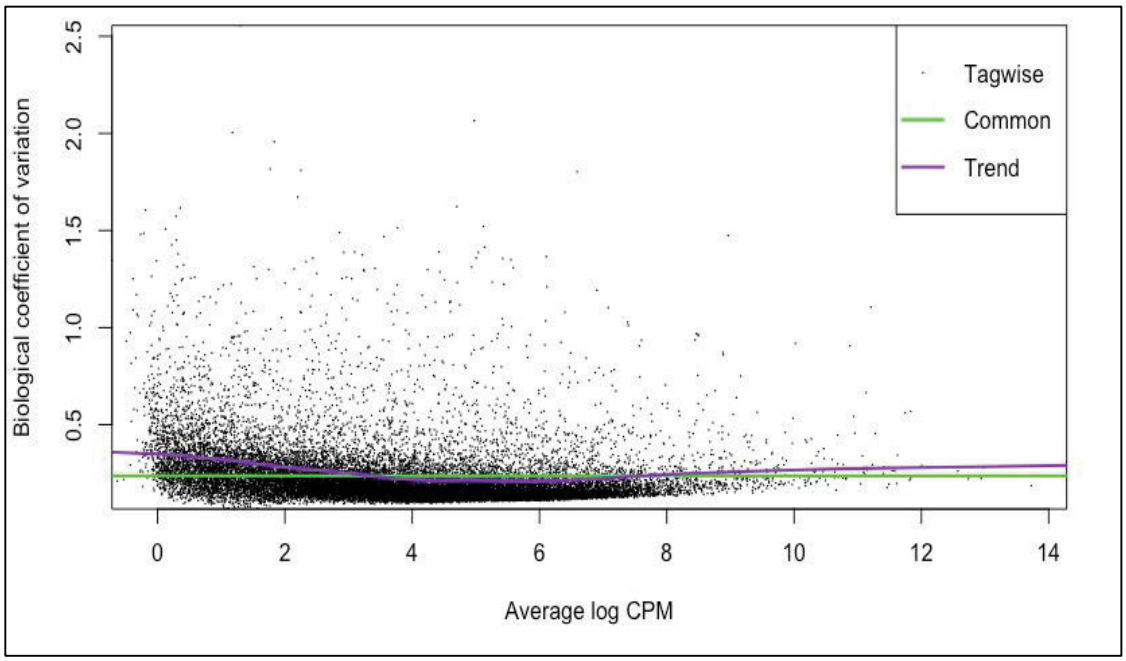
A.2; Figure 1: RLC analysis of *Z. muelleri* plants from Narrabeen Lakes (n = 6) to determine control irradiance for the experiment during Australian winter, 2015. Standard deviation represented by error bars.



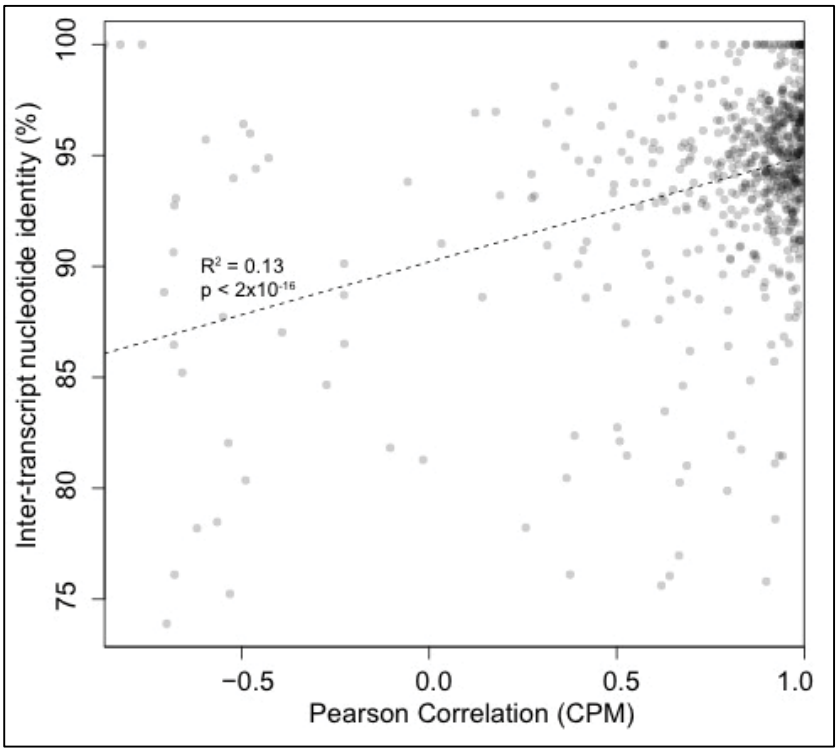
A.2; Figure 2: Odyssey light logger data from the experiment across 6 days: 18/09/15 to the 23/09/15. Mid-day spikes are caused by single time point sampling effort disturbance.



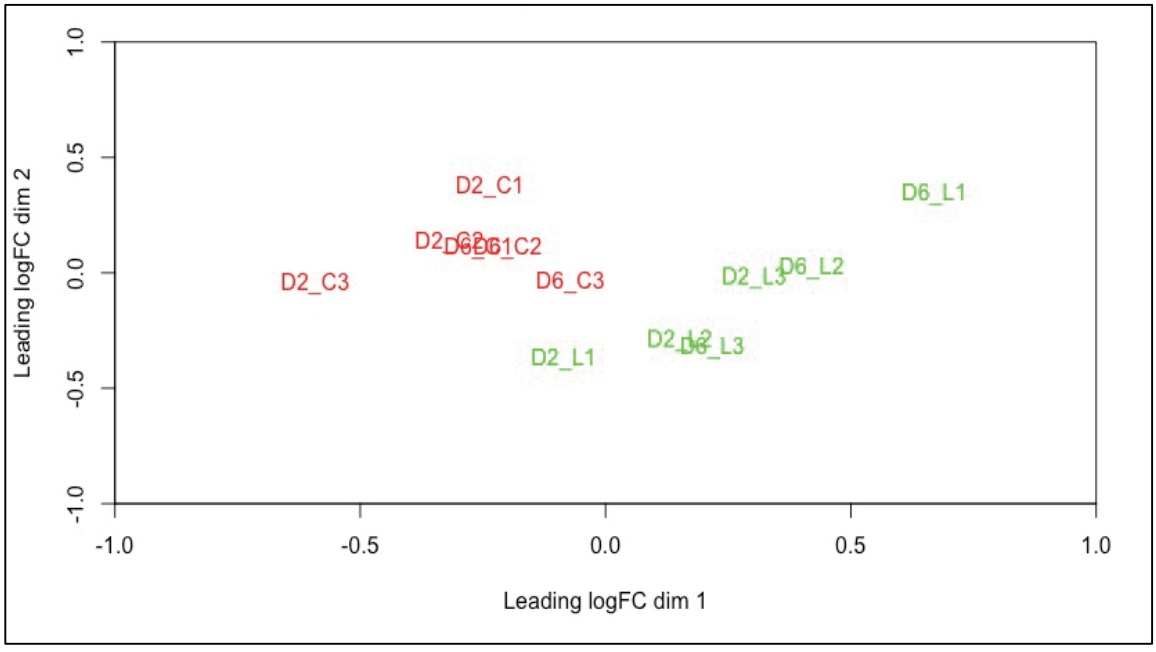
A.2; Figure 3: Chromatogram of the pigment peaks identified in HPLC analysis. Retention time (minutes) shown on X-axis. Intensity (AU) shown on Y-axis.



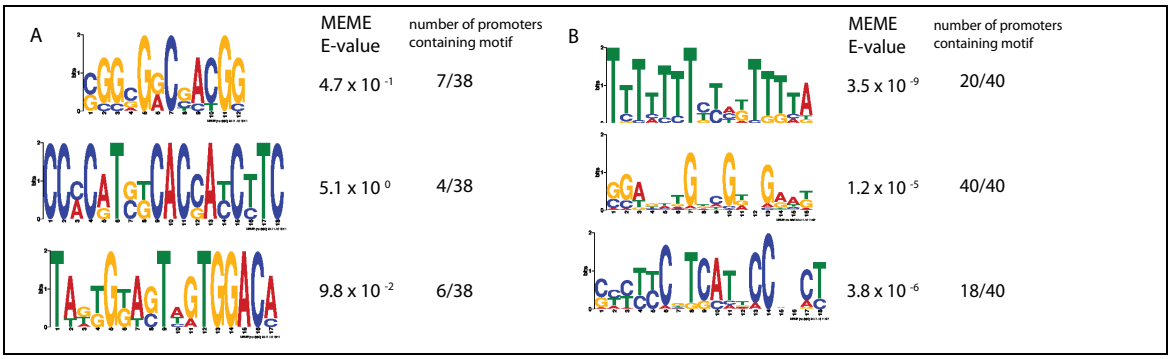
A.2; Figure 4: Biological coefficient of variation between RNA-Seq samples within the experiment. Common dispersion is represented in green. A BCV value of 0.24 was obtained based on the common dispersion. Trended and tagwise dispersions are also represented; however, common dispersion is used to gauge variation within EdgeR (Mccarthy et al. 2012).



A.2; Figure 5: Redundant or duplicated transcripts that are present in the draft genome of *Z. muelleri* (Lee et al. 2016) and are correlated in expression. The correlation of expression is plotted for 1,051 differentially expressed transcript models in this study versus the percent nucleotide identity against redundant, similar or duplicated transcript models in the draft genome.



A.2; Figure 6: Multi-dimensional scaling (MDS) plot showing similarities between RNA samples (Leading LogFC dimension 1 Vs Leading logFC dimension 2). D2 = Day 2, D6 = Day 6, L1-L3 = LL plants 1 – 3, C1-C3 = Control plants 1-3.



A.2; Figure 7: DNA sequence motifs detected by MEME in the putative upstream promoter regions of A. Genes down-regulated under light limitation by Day 2 and, B. Genes up-regulated under light limitation by Day 6.

A.2; Table 1: Xanthophyll and carotenoid pigments profiles on days 2 and 6 (n = 3). Significant results are highlighted with an asterix (*) character (P<0.05). Independent student t-tests were conducted.

Parameter	Neoxanthin	Violaxanthin	Lutein	B- Carotene
Control Day 2 Mean	3.23 ± 0.37	9.01 ±1.11	25.38 ± 6.36	14.01 ± 2.07
Light limitation Day 2 Mean	3.42 ± 0.16	10.24 ±2.06	50.94 ±6.20	27.16 ± 4.87
Significance	**0.659**	**0.627**	**0.045***	**0.068**
Control Day 6 Mean	1.99 ± 0.27	6.61 ± 0.91	16.49 ± 2.45	10.00 ± 1.51
Light limitation Day 6 Mean	4.12 ± 0.55	13.83 ± 1.88	32.79 ± 4.72	15.32 ± 0.52
Significance	**0.025***	**0.026***	**0.037***	**0.029***

A.2; Table 2: Chlorophyll pigments profiles on days 2 and 6 (n = 3). Significant results are highlighted with an asterix (*) character (P<0.05). Independent student t-tests were conducted.

Parameter	Chl. a	Chl. b	Total Chl.	Chla/b	DVC A
Control Day 2 Mean	228.79 ± 28.77	93.11 ± 10.25	321.9 ± 66.94	2.45 ± 0.15	0.99 ± 0.17
Light limitation Day 2 Mean	387.72 ± 15.19	165.58 ± 9.53	553.31 ± 42.49	2.35 ± 0.09	1.11 ± 0.11
Significance	**0.008***	**0.007***	**0.07***	**0.366**	**0.579**
Control Day 6 Mean	128.36 ± 22.87	50.08 ± 5.62	178.44 ± 49.24	2.53 ± 0.28	0.72 ± 0.18
Light limitation Day 6 Mean	251.83 ± 9.32	106.47 ± 4.96	358.30 ± 24.09	2.37 ± 0.10	0.65 ± 0.19
Significance	**0.007***	**0.002***	**0.05***	**0.408**	**0.800**

A.2; Table 3: Number of paired end reads (bp) in each library used for the assembly of the genome-guided transcriptome.

Library	Number of paired end reads (bp)
Day 2 Control 1	29,703,072
Day 2 Control 2	26,528,619
Day 2 Control 3	22,769,311
Day 2 LL 1	21,495,576
Day 2 LL 2	49,607,961
Day 2 LL 3	30,852,035
Day 6 Control 1	30,133,818
Day 6 Control 2	37,928,946
Day 6 Control 3	29,211,116
Day 6 LL 1	18,502,789
Day 6 LL 2	33,415,092
Day 6 LL 3	34,128,144

A.2; Table 4: Top 10 species/ genus groups with functional best hits to the *Z. muelleri* transcriptome.

Species	**No. of Functional Hits**	**% Functional Hits**
Zostera marina	19,074	89.87
Musa malaccensis	374	1.76
Vitis vinifera	168	0.79
Oryzae sp	90	0.42
Gossypium raimondii	82	0.39
Theobroma cocoa	66	0.31
Amborella trichopoda	65	0.31
Populus trichocarpa	65	0.31
Rosales sp.	58	0.27
Gossypium arboreum	56	0.26

A.2; Table 5: Differentially regulated genes associated with photosynthesis and carbon fixation on Day 2. FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-3914_28672--0.4-mRNA-1	1.62	7.17E-07	ATP-dependent zinc metalloprotease FtsH 1
1:maker-2782_81305--0.21-mRNA-1	1.34	6.59E-06	NAD(P)H-quinone oxidoreductase subunit M, chloroplastic
0:maker-395_77190--0.24-mRNA-1	1.11	9.47E-03	Chlorophyll a-b binding protein, chloroplastic
0:maker-15487_9847--0.7-mRNA-1	-1.08	1.29E-03	One-helix-protein 1
0:maker-2793_182425_49376_182425--0.20-mRNA-1	-1.10	3.96E-05	One-helix-protein 2
0:maker-9031_36205--0.16-mRNA-1	-1.10	4.36E-02	Protein LHCP TRANSLOCATION DEFECT
0:maker-191_91516--0.36-mRNA-1	-1.12	9.56E-03	Protein PROTON GRADIENT REGULATION 5, chloroplastic
1:maker-1404_39015--0.10-mRNA-1	-1.14	2.76E-03	PsbP-like protein 1
0:maker-1095_69318--0.23-mRNA-1	-1.15	1.68E-04	NDH-dependent cyclic electron flow 1
1:augustus_masked-1745_99153_24418_92867--0.4-mRNA-1	-1.16	4.81E-06	Ribose-5-phosphate isomerase
1:augustus_masked-2545_67960_58274_67960--0.0-mRNA-1	-1.17	2.16E-02	Carbonate dehydratase
1:augustus_masked-12458_30159--0.0-mRNA-1	-1.19	1.68E-02	Cytochrome b6f complex subunit, putative, expressed
1:maker-4283_116817--0.24-mRNA-1	-1.20	6.24E-04	Chlorophyll a-b binding protein, chloroplastic
0:maker-46_154371_128735_154371--0.12-mRNA-1	-1.21	1.64E-07	Cytochrome c biogenesis protein CCS1, chloroplastic
0:maker-671_122366--0.32-mRNA-1	-1.23	3.59E-02	ATP-dependent zinc metalloprotease FtsH 2
1:maker-8437_15512--0.6-mRNA-1	-1.28	3.72E-02	Photosystem II reaction center W protein, chloroplastic
0:maker-11443_39640--0.16-mRNA-1	-1.29	2.38E-07	PGRL1A transmembrane protein
0:augustus_masked-5431_21660_11321_21660--0.0-mRNA-1	-1.46	6.07E-04	Cytochrome b6f complex subunit, putative, expressed
1:maker-235_135763_59916_135763--0.16-mRNA-1	-1.52	2.36E-06	PsbP domain-containing protein
0:maker-3141_85102--0.18-mRNA-1	-1.54	9.67E-03	PsbQ-like 1
0:maker-3925_34724--0.15-mRNA-1	-1.83	3.15E-11	ATP-dependent zinc metalloprotease FtsH 2
1:maker-10959_11664--0.3-mRNA-1	-1.91	7.18E-06	Phosphoribulokinase

1:snap_masked-3126_87528--0.6-mRNA-1	-2.00	5.84E-04	Photosystem II reaction center Psb28 protein
1:maker-2783_154383_67051_154383--0.26-mRNA-1	-2.02	8.02E-09	Photosystem II stability/assembly factor HCF136
0:maker-488_99413_8928_99413--0.7-mRNA-1	-2.14	6.64E-12	Ycf48-like protein
0:maker-2243_61579--0.19-mRNA-1	-2.31	2.95E-10	Photosystem II subunit S
1:maker-8777_31386--0.8-mRNA-1	-2.34	8.23E-08	Photosystem II subunit S
0:maker-6777_25331_3304_25331--0.6-mRNA-1	-2.78	4.90E-04	PsbP domain-containing protein 1
0:maker-7779_20186--0.3-mRNA-1	-3.85	4.15E-02	Carbonate dehydratase
0:maker-2269_66826--0.16-mRNA-1	-5.21	1.69E-13	Early light-induced protein
1:snap_masked-8483_21486--0.1-mRNA-1	-6.05	4.90E-05	Early light-induced protein
1:maker-206_105998_1_66810--0.24-mRNA-1	-6.07	8.81E-13	Early light-induced protein
0:snap_masked-11443_39640--0.13-mRNA-1	-6.12	1.89E-24	Early light-induced protein

A.2; Table 6: Differentially regulated genes associated with photosynthesis and carbon fixation on Day 6. FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-104_74335_26975_48195--0.9-mRNA-1	-1.03	1.21E-02	Chlorophyll a-b binding protein, chloroplastic
1:maker-2084_84948--0.26-mRNA-1	-1.13	1.90E-04	PsaB RNA binding protein
0:maker-20112_6378--0.6-mRNA-1	-1.20	1.07E-02	Chlorophyll a-b binding protein, chloroplastic
1:maker-235_135763_59916_135763--0.16-mRNA-1	-1.22	4.26E-04	PsbP domain-containing protein
0:augustus_masked-3850_43878_1_37733--0.1-mRNA-1	-1.29	6.58E-06	Post-illumination chlorophyll fluorescence increase protein
1:maker-4283_116817--0.24-mRNA-1	-1.30	3.55E-04	Chlorophyll a-b binding protein, chloroplastic
1:snap_masked-3126_87528--0.6-mRNA-1	-1.38	3.78E-02	Photosystem II reaction center Psb28 protein
0:maker-12548_20554--0.5-mRNA-1	-1.43	1.32E-04	Post-illumination chlorophyll fluorescence increase protein
0:maker-2243_61579--0.19-mRNA-1	-1.60	3.41E-05	Photosystem II subunit S
1:maker-8777_31386--0.8-mRNA-1	-1.60	5.36E-04	Photosystem II subunit S
0:maker-235_135763_59916_135763--0.21-mRNA-1	-1.66	1.06E-02	PsbP domain-containing protein
1:maker-10959_11664--0.3-mRNA-1	-1.69	1.39E-04	Phosphoribulokinase
0:maker-5204_59108--0.17-mRNA-1	-1.90	2.34E-02	ATP-dependent zinc metalloprotease FtsH 2
1:maker-206_105998_1_66810--0.24-mRNA-1	-3.06	1.52E-04	Early light-induced protein
0:maker-2269_66826--0.16-mRNA-1	-3.23	2.67E-06	Early light-induced protein
0:snap_masked-11443_39640--0.13-mRNA-1	-3.31	1.03E-09	Early light-induced protein

A.2; Table 7: Differentially regulated genes associated with photosynthetic pigments and ABA (absicic acid) on Day 2. FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-1402_73274_1_21391--0.5-mRNA-1	1.81	5.45E-04	Abscisic acid 8'-hydroxylase 3
0:maker-1161_47387_9729_45100--0.6-mRNA-1	1.72	9.37E-03	Protochlorophyllide reductase
0:maker-11131_58707--0.17-mRNA-1	1.45	9.21E-04	Phosphomevalonate kinase
1:maker-9736_34395--0.15-mRNA-1	1.39	3.48E-02	Protochlorophyllide reductase
0:maker-1165_70192_17631_68114--0.18-mRNA-1	-1.04	1.55E-04	Solanesyl diphosphate synthase 1, chloroplastic
0:maker-4479_24389--0.3-mRNA-1	-1.07	2.73E-03	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
1:maker-6476_54803--0.15-mRNA-1	-1.10	4.12E-02	Carotene epsilon-monooxygenase, chloroplastic
0:augustus_masked-15881_6497--0.0-mRNA-1	-1.17	3.00E-03	Magnesium chelatase subunit chlH
1:maker-6690_51232_16891_51232--0.18-mRNA-1	-1.22	6.66E-08	Carotenoid isomerase 1
1:maker-8501_32418--0.3-mRNA-1	-1.23	1.79E-02	Beta-carotene hydroxylase 1
0:snap_masked-6594_55942--0.7-mRNA-1	-1.29	2.92E-03	Tocopherol cyclase
0:maker-5_137424--0.16-mRNA-1	-1.30	8.87E-04	Chalcone-flavonone isomerase family protein
0:maker-117_92563_1_40758--0.23-mRNA-1	-1.35	1.33E-05	Chlorophyll(Ide) b reductase NOL, chloroplastic
1:snap_masked-5123_61931_18394_61931--0.5-mRNA-1	-1.36	7.75E-04	Divinyl chlorophyllide a 8-vinyl-reductase, chloroplastic
0:augustus_masked-14067_24824--0.2-mRNA-1	-1.42	1.21E-07	Chlorophyll(Ide) b reductase NOL, chloroplastic
1:snap_masked-2649_154349--0.12-mRNA-1	-1.42	6.78E-07	Zeaxanthin epoxidase, chloroplastic
1:augustus_masked-7071_55472_1_28049--0.0-mRNA-1	-1.49	3.05E-04	Magnesium chelatase subunit chlH
0:maker-4568_54193--0.12-mRNA-1	-1.57	4.81E-09	Zeaxanthin epoxidase, chloroplastic
1:maker-2469_35827--0.17-mRNA-1	-1.62	1.08E-11	ATP-dependent zinc metalloprotease FtsH 2
0:augustus_masked-8102_16114--0.1-mRNA-1	-1.64	7.51E-04	ATP-dependent zinc metalloprotease FtsH 2
0:maker-15214_6557--0.5-mRNA-1	-1.79	2.26E-03	Protein FLUORESCENT IN BLUE LIGHT, chloroplastic
0:snap_masked-423_114244_1_21280--0.5-mRNA-1	-1.83	2.13E-08	Heterodimeric geranyl(Geranyl) pyrophosphate synthase large subunit 1, chloroplastic
1:maker-2118_97639--0.32-mRNA-1	-1.86	9.73E-07	Tocopherol O-methyltransferase

0:augustus_masked-1711_78151--0.1-mRNA-1	-1.89	1.56E-08	Solanesyl-diphosphate synthase 2, chloroplastic
0:maker-1238_66824_1_56791--0.27-mRNA-1	-1.91	5.73E-11	Zeta-carotene desaturase
0:maker-15603_18549--0.2-mRNA-1	-1.99	3.89E-04	Chalcone-flavonone isomerase family protein
0:augustus_masked-10428_26916--0.1-mRNA-1	-2.02	8.01E-09	Zeta-carotene desaturase
1:augustus_masked-7350_18842--0.0-mRNA-1	-2.18	1.51E-07	Chlorophyllase
0:augustus_masked-5192_28799--0.0-mRNA-1	-2.21	1.12E-02	Abscisic acid receptor PYR1
1:maker-10561_30732--0.11-mRNA-1	-2.42	3.11E-20	Solanesyl-diphosphate synthase 2, chloroplastic
0:augustus_masked-17242_7076--0.0-mRNA-1	-2.47	2.62E-04	Anthocyanidin synthase
0:maker-7730_53428_3192_53428--0.7-mRNA-1	-2.67	1.00E-05	Naringenin-chalcone synthase
1:augustus_masked-7730_53428_3192_53428--0.1-mRNA-1	-2.80	2.41E-04	Naringenin-chalcone synthase
1:maker-12400_12659--0.5-mRNA-1	-2.95	2.82E-03	Chlorophyll synthase
0:augustus_masked-3522_68758--0.2-mRNA-1	-3.11	2.22E-03	Nine-cis-epoxycarotenoid dioxygenase 4
1:maker-3550_85912--0.32-mRNA-1	-3.16	3.42E-02	Chalcone-flavonone isomerase family protein
0:maker-3476_70335--0.29-mRNA-1	-3.32	3.55E-11	Tocopherol O-methyltransferase

A.2; Table 8: Differentially regulated genes associated with photosynthetic pigments and ABA (absicic acid) on Day 6**.** FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-9499_33363--0.4-mRNA-1	6.68	4.90E-06	GRAM domain-containing protein / ABA-responsive
1:augustus_masked-705_49098_17056_49098--0.0-mRNA-1	6.57	4.45E-05	GRAM domain-containing protein / ABA-responsive
1:maker-794_82340_1_5844--0.4-mRNA-1	3.06	2.96E-03	Chalcone synthase 2
0:maker-1161_47387_9729_45100--0.6-mRNA-1	2.86	7.45E-06	Protochlorophyllide reductase
1:maker-9736_34395--0.15-mRNA-1	2.51	3.01E-05	Protochlorophyllide reductase
0:maker-1402_73274_1_21391--0.5-mRNA-1	1.72	2.94E-03	Abscisic acid 8'-hydroxylase 3
1:maker-13275_13993_1_11379--0.7-mRNA-1	1.26	3.84E-02	1-deoxy-D-xylulose-5-phosphate synthase, Transketolase
1:maker-4014_83423_1_75173--0.17-mRNA-1	1.25	2.47E-05	GRAM domain-containing protein / ABA-responsive
1:augustus_masked-6068_67721--0.9-mRNA-1	-1.00	4.38E-03	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
0:maker-117_92563_1_40758--0.23-mRNA-1	-1.02	2.90E-03	Chlorophyll(Ide) b reductase NOL, chloroplastic
0:maker-3925_34724--0.15-mRNA-1	-1.02	6.36E-04	ATP-dependent zinc metalloprotease FtsH 2
1:snap_masked-13403_25142--0.6-mRNA-1	-1.03	4.19E-05	Carotenoid isomerase 1
0:maker-6595_42371_6340_42371--0.12-mRNA-1	-1.04	8.17E-03	Prolycopene isomerase
0:augustus_masked-14067_24824--0.2-mRNA-1	-1.10	2.16E-04	Chlorophyll(Ide) b reductase NOL, chloroplastic
0:maker-3810_50053_1_13347--0.5-mRNA-1	-1.12	5.05E-04	Phytoene desaturase
1:maker-2118_97639--0.32-mRNA-1	-1.13	1.18E-02	Tocopherol O-methyltransferase
1:maker-6690_51232_16891_51232--0.18-mRNA-1	-1.16	8.79E-07	Carotenoid isomerase 1
0:maker-1238_66824_1_56791--0.27-mRNA-1	-1.24	8.53E-05	Zeta-carotene desaturase
0:maker-1165_70192_17631_68114--0.18-mRNA-1	-1.25	5.94E-06	Solanesyl diphosphate synthase 1, chloroplastic
1:augustus_masked-2855_91590--0.6-mRNA-1	-1.53	4.86E-03	Phytoene desaturase
0:snap_masked-423_114244_1_21280--0.5-mRNA-1	-1.61	1.86E-05	Heterodimeric geranyl(Geranyl) pyrophosphate synthase large subunit 1, chloroplastic
1:maker-10561_30732--0.11-mRNA-1	-1.67	6.62E-10	Solanesyl-diphosphate synthase 2, chloroplastic

0:augustus_masked-1711_78151--0.1-mRNA-1	-1.70	8.09E-07	Solanesyl-diphosphate synthase 2, chloroplastic
0:maker-3476_70335--0.29-mRNA-1	-1.90	9.75E-04	Tocopherol O-methyltransferase
0:augustus_masked-10428_26916--0.1-mRNA-1	-1.92	8.61E-07	Zeta-carotene desaturase
1:augustus_masked-7730_53428_3192_53428--0.1-mRNA-1	-2.17	9.70E-03	Naringenin-chalcone synthase
0:augustus_masked-3522_68758--0.2-mRNA-1	-2.45	3.10E-02	Nine-cis-epoxycarotenoid dioxygenase 4
0:augustus_masked-17242_7076--0.0-mRNA-1	-2.57	1.97E-04	Anthocyanidin synthase
0:augustus_masked-14649_13519--0.0-mRNA-1	-2.81	1.33E-02	Naringenin-chalcone synthase
0:augustus_masked-5192_28799--0.0-mRNA-1	-2.96	7.24E-04	Abscisic acid receptor PYR1
0:maker-7730_53428_3192_53428--0.7-mRNA-1	-3.01	1.11E-06	Naringenin-chalcone synthase

A.2; Table 9: Differentially regulated genes associated with light reception and signaling on Day 2. FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-11888_10393--0.5-mRNA-1	1.74	3.37E-04	Putative Light-regulated protein
0:snap_masked-1961_63114--0.11-mRNA-1	1.59	1.66E-02	Zinc finger protein CONSTANS-LIKE 16
1:maker-7436_67493--0.14-mRNA-1	1.28	3.07E-05	CONSTANS-like protein
0:maker-1961_63114--0.24-mRNA-1	1.28	4.92E-03	Zinc finger protein CONSTANS-LIKE 16
0:maker-9999_31034--0.5-mRNA-1	1.23	1.56E-08	Phytochrome
0:maker-4811_65630--0.38-mRNA-1	1.07	2.63E-04	Putative LOV domain-containing protein
0:augustus_masked-13933_15206--0.1-mRNA-1	1.06	2.44E-03	CONSTANS-like protein
0:maker-6975_45238--0.9-mRNA-1	-1.06	4.39E-03	BluePAS/LOV protein B
0:maker-6413_62067--0.15-mRNA-1	-1.14	1.37E-06	Phytochrome A-associated F-box protein
1:maker-6544_19094--0.5-mRNA-1	-1.15	2.17E-06	Constitutively photomorphogenic 1
0:maker-4500_24313--0.10-mRNA-1	-1.20	4.34E-04	DNA photolyase
0:maker-8649_15121--0.4-mRNA-1	-1.21	4.85E-02	Protein ACCUMULATION AND REPLICATION OF CHLOROPLASTS 6, chloroplastic
1:maker-10559_19089--0.11-mRNA-1	-1.27	8.78E-05	Zinc finger protein CONSTANS-like protein
0:augustus_masked-7586_35622--0.1-mRNA-1	-1.29	1.90E-03	DNA photolyase
1:maker-3906_30754--0.7-mRNA-1	-1.37	1.48E-05	Acclimation of photosynthesis to environment
1:maker-7851_59105--0.5-mRNA-1	-1.42	1.66E-03	Protein SPA1-RELATED 3
0:maker-106_87687_1_75123--0.30-mRNA-1	-1.45	9.85E-05	Two-component response regulator-like PRR73
0:augustus_masked-3758_120039--0.0-mRNA-1	-1.52	1.19E-08	Zinc finger protein CONSTANS-like protein
0:maker-10890_35840--0.11-mRNA-1	-1.60	7.56E-07	Putative LOV domain-containing protein
1:maker-9094_14423--0.7-mRNA-1	-1.65	1.97E-06	Phototropin-2
1:maker-2416_59808--0.22-mRNA-1	-1.69	8.48E-03	MADS-box transcription factor 3
0:maker-717_47633--0.11-mRNA-1	-1.73	9.80E-07	Protein SPA1-RELATED 3
1:maker-542_76290--0.40-mRNA-1	-1.74	7.09E-11	DNA photolyase, putative isoform 1

0:maker-4291_41003--0.12-mRNA-1	-1.79	4.61E-02	Zinc finger protein CONSTANS-like protein
1:maker-363_185605_1_27475--0.8-mRNA-1	-1.85	2.85E-05	Phytochrome
1:maker-2569_115832--0.35-mRNA-1	-1.93	4.00E-03	MADS-box transcription factor 3
1:maker-3491_42360_10117_42360--0.9-mRNA-1	-2.03	6.99E-03	Response regulator 7
1:maker-8272_15790--0.7-mRNA-1	-2.22	9.65E-09	Phototropin-2
1:augustus_masked-12782_19212--0.1-mRNA-1	-2.52	9.31E-03	Phytochrome
0:maker-2450_75455--0.17-mRNA-1	-2.74	1.28E-09	Phytochrome E
1:augustus_masked-835_56565_1_39681--0.2-mRNA-1	-4.16	1.47E-07	Zinc finger protein CONSTANS-like protein

A.2; Table 10: Differentially regulated genes associated with light reception and signaling on Day 6. FC = Fold change, FDR = 0.05.

Genome Accession	FC	FDR	Annotation
0:maker-4811_65630--0.38-mRNA-1	1.19	4.45E-05	Putative LOV domain-containing protein
0:maker-4239_52844_13802_52844--0.11-mRNA-1	1.13	1.53E-05	Phytochrome
0:maker-9999_31034--0.5-mRNA-1	1.10	9.21E-07	Phytochrome
1:maker-363_185605_1_27475--0.8-mRNA-1	-1.11	2.19E-02	Phytochrome
0:maker-6975_45238--0.9-mRNA-1	-1.25	8.25E-04	BluePAS/LOV protein B
0:maker-8649_15121--0.4-mRNA-1	-1.25	4.85E-02	Protein ACCUMULATION AND REPLICATION OF CHLOROPLASTS 6, chloroplastic
0:maker-106_87687_1_75123--0.30-mRNA-1	-1.35	5.25E-04	Two-component response regulator-like PRR73
1:maker-8272_15790--0.7-mRNA-1	-1.37	1.03E-03	Phototropin-2
1:maker-8783_65131--0.15-mRNA-1	-1.39	2.12E-06	Phototropic-responsive NPH3 family protein
0:augustus_masked-3758_120039--0.0-mRNA-1	-1.44	3.14E-07	Zinc finger protein CONSTANS-like protein
0:maker-2579_46448_8995_46448--0.6-mRNA-1	-1.56	2.70E-02	Response regulator
1:augustus_masked-835_56565_1_39681--0.2-mRNA-1	-1.73	1.82E-02	Zinc finger protein CONSTANS-like protein
1:maker-10559_19089--0.11-mRNA-1	-1.79	4.38E-07	Zinc finger protein CONSTANS-like protein
0:maker-1168_147898_72688_147898--0.16-mRNA-1	-3.03	9.61E-03	Phototropic-responsive NPH3-like protein
0:maker-4291_41003--0.12-mRNA-1	-4.79	1.03E-02	Zinc finger protein CONSTANS-like protein

A.2; Table 11: Enriched GO terms in the up-regulated sub-set of genes in LL plants on Day 2. FDR = 0.05. NS = Classification; MF = Molecular Function; BP = Biological Process; CC = Cellular Compartment.

GO	NS	Name	FDR
GO:0003700	MF	transcription factor activity, sequence-specific DNA binding	2.36E-07
GO:0001071	MF	nucleic acid binding transcription factor activity	2.36E-07
GO:0043565	MF	sequence-specific DNA binding	0.00169
GO:0004866	MF	endopeptidase inhibitor activity	0.00169
GO:0030414	MF	peptidase inhibitor activity	0.00169
GO:0061135	MF	endopeptidase regulator activity	0.00169
GO:0061134	MF	peptidase regulator activity	0.00169
GO:0006351	BP	transcription, DNA-templated	0.0075
GO:0097659	BP	nucleic acid-templated transcription	0.0075
GO:0003677	MF	DNA binding	0.0075
GO:0009311	BP	oligosaccharide metabolic process	0.00828
GO:0016301	MF	kinase activity	0.00833
GO:0005984	BP	disaccharide metabolic process	0.0159
GO:0004497	MF	monooxygenase activity	0.0193
GO:0004869	MF	cysteine-type endopeptidase inhibitor activity	0.0218
GO:0005992	BP	trehalose biosynthetic process	0.0229
GO:0005991	BP	trehalose metabolic process	0.0276
GO:0046351	BP	disaccharide biosynthetic process	0.0329
GO:0016773	MF	phosphotransferase activity, alcohol group as acceptor	0.0334
GO:0032774	BP	RNA biosynthetic process	0.0484

A.2; Table 12: Enriched GO terms in the down-regulated sub-set of genes in LL plants on Day 2. FDR = 0.05. NS = Classification; MF = Molecular Function; BP = Biological Process; CC = Cellular Compartment.

GO	NS	Name	FDR
GO:0003824	MF	catalytic activity	6.20E-06
GO:0016491	MF	oxidoreductase activity	6.20E-06
GO:0044710	BP	single-organism metabolic process	0.000117
GO:0009063	BP	cellular amino acid catabolic process	0.00013
GO:1901565	BP	organonitrogen compound catabolic process	0.000301
GO:0044699	BP	single-organism process	0.00197
GO:0046395	BP	carboxylic acid catabolic process	0.00267
GO:1901606	BP	alpha-amino acid catabolic process	0.00284
GO:0016054	BP	organic acid catabolic process	0.00321
GO:0016717	MF	oxidoreductase activity, acting on paired donors, with oxidation of a pair of donors resulting in the reduction of molecular oxygen to two molecules of water	0.00405
GO:0016036	BP	cellular response to phosphate starvation	0.00503
GO:0009071	BP	serine family amino acid catabolic process	0.00526
GO:0006546	BP	glycine catabolic process	0.00526
GO:0016872	MF	intramolecular lyase activity	0.00526
GO:0004602	MF	glutathione peroxidase activity	0.00526
GO:0072330	BP	monocarboxylic acid biosynthetic process	0.00545
GO:0004673	MF	protein histidine kinase activity	0.00677
GO:0016775	MF	phosphotransferase activity, nitrogenous group as acceptor	0.00677
GO:0000155	MF	phosphorelay sensor kinase activity	0.00677
GO:0043436	BP	oxoacid metabolic process	0.00698
GO:1901617	BP	organic hydroxy compound biosynthetic process	0.00739

GO:0009267	BP	cellular response to starvation	0.00855
GO:0009800	BP	cinnamic acid biosynthetic process	0.00855
GO:0009803	BP	cinnamic acid metabolic process	0.00855
GO:0042594	BP	response to starvation	0.00855
GO:0045548	MF	phenylalanine ammonia-lyase activity	0.00855
GO:0006082	BP	organic acid metabolic process	0.00922
GO:0000786	CC	nucleosome	0.0101
GO:0044815	CC	DNA packaging complex	0.0121
GO:0006979	BP	response to oxidative stress	0.0122
GO:0044282	BP	small molecule catabolic process	0.014
GO:0044281	BP	small molecule metabolic process	0.0146
GO:0044763	BP	single-organism cellular process	0.0146
GO:0019752	BP	carboxylic acid metabolic process	0.015
GO:0044283	BP	small molecule biosynthetic process	0.015
GO:0009699	BP	phenylpropanoid biosynthetic process	0.0154
GO:0032993	CC	protein-DNA complex	0.0194
GO:0006544	BP	glycine metabolic process	0.0199
GO:0015979	BP	photosynthesis	0.0199
GO:0006559	BP	L-phenylalanine catabolic process	0.025
GO:0016841	MF	ammonia-lyase activity	0.025
GO:0005984	BP	disaccharide metabolic process	0.0281
GO:0009812	BP	flavonoid metabolic process	0.0281
GO:0009813	BP	flavonoid biosynthetic process	0.0281
GO:0006520	BP	cellular amino acid metabolic process	0.0281
GO:0045430	MF	chalcone isomerase activity	0.0281
GO:1990204	CC	oxidoreductase complex	0.0299

GO:0009605	BP	response to external stimulus	0.0301
GO:0050896	BP	response to stimulus	0.0309
GO:0009536	CC	plastid	0.0336
GO:0009654	CC	photosystem II oxygen evolving complex	0.0347
GO:0009698	BP	phenylpropanoid metabolic process	0.0351
GO:0044550	BP	secondary metabolite biosynthetic process	0.0351
GO:0009941	CC	chloroplast envelope	0.0351
GO:0009526	CC	plastid envelope	0.0351
GO:0044712	BP	single-organism catabolic process	0.0482
GO:0005215	MF	transporter activity	0.0482
GO:0016462	MF	pyrophosphatase activity	0.0482

A.2; Table 13: Enriched GO terms in the up-regulated sub-set of genes in LL plants on Day 6. FDR = 0.05. NS = Classification; MF = Molecular Function; BP = Biological Process; CC = Cellular Compartment.

GO	NS	Name	FDR
GO:0009311	BP	oligosaccharide metabolic process	3.78E-05
GO:0016052	BP	carbohydrate catabolic process	3.78E-05
GO:0005984	BP	disaccharide metabolic process	3.78E-05
GO:0005992	BP	trehalose biosynthetic process	7.37E-05
GO:0005991	BP	trehalose metabolic process	9.18E-05
GO:0046351	BP	disaccharide biosynthetic process	0.000106
GO:0044275	BP	cellular carbohydrate catabolic process	0.000106
GO:0044262	BP	cellular carbohydrate metabolic process	0.000149
GO:0009312	BP	oligosaccharide biosynthetic process	0.000308
GO:0005975	BP	carbohydrate metabolic process	0.000333
GO:0003700	MF	transcription factor activity, sequence-specific DNA binding	0.00039
GO:0001071	MF	nucleic acid binding transcription factor activity	0.00039
GO:0016160	MF	amylase activity	0.000521
GO:0004553	MF	hydrolase activity, hydrolyzing O-glycosyl compounds	0.00081
GO:0016798	MF	hydrolase activity, acting on glycosyl bonds	0.000938
GO:0004866	MF	endopeptidase inhibitor activity	0.00162
GO:0061134	MF	peptidase regulator activity	0.00162
GO:0030414	MF	peptidase inhibitor activity	0.00162
GO:0061135	MF	endopeptidase regulator activity	0.00162
GO:0016769	MF	transferase activity, transferring nitrogenous groups	0.0044
GO:0008483	MF	transaminase activity	0.0044
GO:0043565	MF	sequence-specific DNA binding	0.0064

GO:0044723	BP	single-organism carbohydrate metabolic process	0.00692
GO:0006099	BP	tricarboxylic acid cycle	0.00692
GO:0044724	BP	single-organism carbohydrate catabolic process	0.00782
GO:0000272	BP	polysaccharide catabolic process	0.0229
GO:0016161	MF	beta-amylase activity	0.0236
GO:0043168	MF	anion binding	0.0248
GO:0016051	BP	carbohydrate biosynthetic process	0.0266
GO:0004869	MF	cysteine-type endopeptidase inhibitor activity	0.0266
GO:0030170	MF	pyridoxal phosphate binding	0.0357
GO:0034637	BP	cellular carbohydrate biosynthetic process	0.0362
GO:0009733	BP	response to auxin	0.0399
GO:0004611	MF	phosphoenolpyruvate carboxykinase activity	0.0399
GO:0020037	MF	heme binding	0.0455

A.2; Table 14: Enriched GO terms in the down-regulated sub-set of genes in LL plants on Day 6. FDR = 0.05. NS = Classification; MF = Molecular Function; BP = Biological Process; CC = Cellular Compartment.

GO	NS	Name	FDR
GO:0003824	MF	catalytic activity	8.20E-05
GO:0009501	CC	amyloplast	0.000273
GO:0019252	BP	starch biosynthetic process	0.000578
GO:0005982	BP	starch metabolic process	0.00214
GO:0004373	MF	glycogen (starch) synthase activity	0.00214
GO:0016491	MF	oxidoreductase activity	0.00265
GO:0008299	BP	isoprenoid biosynthetic process	0.0044
GO:0006720	BP	isoprenoid metabolic process	0.00482
GO:0044711	BP	single-organism biosynthetic process	0.0102
GO:0009800	BP	cinnamic acid biosynthetic process	0.0102
GO:0009803	BP	cinnamic acid metabolic process	0.0102
GO:0016108	BP	tetraterpenoid metabolic process	0.0102
GO:0016109	BP	tetraterpenoid biosynthetic process	0.0102
GO:0016117	BP	carotenoid biosynthetic process	0.0102
GO:0016116	BP	carotenoid metabolic process	0.0102
GO:0045548	MF	phenylalanine ammonia-lyase activity	0.0102
GO:0009536	CC	plastid	0.0114
GO:0004638	MF	phosphoribosylaminoimidazole carboxylase activity	0.0127
GO:0009699	BP	phenylpropanoid biosynthetic process	0.0151
GO:0044282	BP	small molecule catabolic process	0.0158
GO:0009063	BP	cellular amino acid catabolic process	0.0181
GO:0044710	BP	single-organism metabolic process	0.0224

GO:0009507	CC	chloroplast	0.0224
GO:0006559	BP	L-phenylalanine catabolic process	0.0225
GO:0016841	MF	ammonia-lyase activity	0.0225
GO:0046395	BP	carboxylic acid catabolic process	0.0284
GO:0070569	MF	uridylyltransferase activity	0.0302
GO:1901565	BP	organonitrogen compound catabolic process	0.0307
GO:0009698	BP	phenylpropanoid metabolic process	0.033
GO:0044550	BP	secondary metabolite biosynthetic process	0.033
GO:0016054	BP	organic acid catabolic process	0.033
GO:0042221	BP	response to chemical	0.033
GO:0042445	BP	hormone metabolic process	0.0351
GO:0044712	BP	single-organism catabolic process	0.0428
GO:0048037	MF	cofactor binding	0.0483

A.2; Table 15: Enriched GO terms in the down-regulated sub-set of genes in LL plants on Day 6 compared to LL plants on Day 2. FDR = 0.05. NS = Classification; MF = Molecular Function; BP = Biological Process; CC = Cellular Compartment.

GO	NS	Name	FDR
GO:0009690	BP	cytokinin metabolic process	0.00116
GO:0034754	BP	cellular hormone metabolic process	0.00116
GO:0042445	BP	hormone metabolic process	0.00139
GO:0010817	BP	regulation of hormone levels	0.00174
GO:0009308	BP	amine metabolic process	0.0383

APPENDIX 3

Alternative *de novo* protocol for chapter 4

Refer to Chapter 4; the protocol below was utilised for producing an altnerative *de novo* assembly for data analysis in Chapter 4. This was conducted in order to compare the differences between *de novo* assembly and genome-guided assembly (Chapter 4).

Materials and methods for *de novo* assembly

Assembly of a super transcriptome, annotation and redundancy removal

Given the challenges of *de novo* assembly in Chapter 3, a 'super assembly' was assembled using two *de novo* assembly software programs. Trinity was the first software program that was used (Haas et al., 2013). Cleaned reads (A.2; Table 3) were digitally normalised within trinity and a default k-mer size of 25 was chosen. A second assembler, IDBA-tran (Peng et al., 2013) was then used to produce seven assemblies in total: 31; 41; 51; 61; 71; 81 and 91 k-mer sized words were chosen. This protocol was chosen, as no one k-mer size is optimal for producing the best assembly (Peng et al., 2013). Assemblies were then merged and the tr2aacds software pipeline – evidential gene package (Gilbert, 2002) was implemented to remove redundancy and provide the most evidential gene per loci. Transcripts were annotated using DIAMOND BLASTX protein alignment software (Buchfink et al., 2015) with an e-value of 1×10^{-5}. Best hits were retained for each species based on bit score, e-value, and % similarity. Best hits were then filtered for contaminants manually. RSEM software (Li and dewey, 2011) was then used to estimate gene abundances. The final transcriptome used for differential expression analysis consisted of those genes, which passed EdgeR filters (See Chapter 4).

Comparison of datatsets and differential expression analysis

A.3; Table 1: Comparison of genome-guided assembly and *de novo* super assembly statistics.

Assembly attributes	Genome-guided	*De novo*
Number of total genes	21,225	44,574
BCV (Biological co-variance)	0.237	0.587
Up-regulated on day2 / day6	530 / 610	943/ 851
Down-regulated on day2 / day 6	1,063 / 871	1,295/ 1,271

*** All citations in this Appendix can be found in the Chapter 4 reference list.**

APPENDIX 4

Contributions, grant success and training courses

Proteome analysis reveals extensive light stress-response reprogramming for the seagrass *Zostera muelleri* (Alismatales, Zosteraceae) metabolism
KUMAR, M., PADULA, M., **DAVEY, P**, PERNICE, M., ZIJAN, J., SABLOK, G., PORCIA, L. & RALPH, P. 2016. *Frontiers in Plant Science,* 7.

Abstract

Seagrasses are marine ecosystem engineers that are currently declining in abundance at an alarming rate due to both natural and anthropogenic disturbances in ecological niches. Despite reports on the morphological and physiological adaptations of seagrasses to extreme environments, little is known of the molecular mechanisms underlying photo-acclimation and/or tolerance in these marine plants. This study applies the two-dimensional isoelectric focusing (2D-IEF) proteomics approach to identify photo-acclimation/tolerance proteins in the marine seagrass *Zostera muelleri*. For this, *Z. muelleri* was exposed for 10 days in laboratory mesocosms to saturation (control, 200 μmol photons m-2 s-1), supersaturation (SSL, 600 μmol photons m-2 s-1), and light limitation (LL, 20 μmol photons m-2 s-1). Using LC-MS/MS analysis, 93 and 40 protein spots were differentially regulated under SSL and LL conditions, respectively, when compared to the control. In contrast to the LL condition, *Z. muelleri* robustly tolerated super-saturation light than control conditions, evidenced by their higher relative electron transport rate and half-saturation of photosynthetic rate values. Proteomic analyses revealed up-regulation and/or appearances of proteins belonging to the Calvin-Benson and Krebs cycle, glycolysis, the glycine cleavage system of photorespiration, and the antioxidant system. These proteins, together with those from the inter-connected glutamate-proline-GABA pathway, shaped *Z. muelleri* photosynthesis and growth under super-saturation light conditions. In contrast, the LL condition negatively impacted the metabolic activities of *Z. muelleri* by down-regulating key metabolic enzymes for photosynthesis and the metabolism of carbohydrates and amino acids, which is consistent with the observation with lower photosynthetic performance under LL condition. This study provides novel insights into the underlying molecular photo-acclimation mechanisms in *Z. muelleri*, in addition to identifying protein-based biomarkers that could be used as early indicators to detect acute/chronic light stress in seagrasses to monitor seagrass health.

Seagrasses of Australia - Chapter 16: Photosynthesis and metabolism of seagrasses
Larkum, AWD., Sablok, G., Pernice, M., Schliep, M., **Davey, P. A**, Szabo, M., Raven, J. A., Lichtenberg, M., Elgetti Brodersen, K., Ralph, P. *In Review*.

Previous reviews (see e.g. Larkum et al., 2006) have dealt extensively with several topics and for this reason this chapter has dealt on a narrower range of topics. These are:

a) the uptake of inorganic carbon by the leaves of seagrasses,
b) the biochemistry of carbon fixation in photosynthesis,
c) the influence of anatomy on photosynthesis and gaseous transport to the rhizome and roots, and,
d) the effect of epiphytes on photosynthesis.

Of these four topics the one most extensively treated here is the first, the uptake of Ci from the ambient seawater. This is because there are large lacunae in our knowledge of these processes, which are so profoundly important to our understanding of how seagrasses have become so successful in our seas over the last 100 million years or so and how today they are so important for the production of "blue carbon". The second topic (b) is noteworthy because modern "omics" and direct profiling of genes and their products promises to throw light on a topic that has defined research efforts over a long period. Nonetheless, if we were to mention some of the most important findings in seagrass photobiology since the previous review, they should include:

(i) that active HCO_3^- uptake does not seem to occur across the plasmalemma.

(ii) that seagrasses utilise extracellular conversion of HCO_3^- to CO_2, for enhanced CO_2 uptake and utilisation in photosynthesis, e.g., through extracellular carbonic anhydrase (CA) activity.

(iii) the potential occurence of a Carbon Concentrating Mechanism (CCM) in at least some seagrasses.

(iv) confirmation of shade-adaptation, and thus operation of the xanthophyll cycle and other dissipative mechanisms in photoprotection under excess light intensity.

(v) that leaf epiphytes can alter the spectral light composition towards a greener light-field, which is not as effectively absorbed by the main light harvesting pigments.

However, we will have to wait to see whether a C_4-type of photosynthetic metabolism is eventually shown in seagrasses.

Grants

EMBL Australian travel grant, 2014 - $3,500

This grant was award for travel to EMBL and EBI institutes in Europe to undertake laboratory placement and attend taught courses. At EMBL in Heidelberg, Germany, I completed a lab placement with the Heisler plant development group. During my stay I learned how to apply transformation techniques to *Arabidopsis* in order to complete research based on Auxin signalling in meristem tissue. I also completed the course 'Introduction to statistics for bioinformaticians' at EMBL, Heidelberg. At the EBI Institute in Hinxton, England, I completed a course in 'Introduction to next-generation sequencing platforms'.

UTS School of Life Sciences travel grant, 2014 -Undisclosed

This grant was used for travelling to ISBW (International Seagrass Biology Workshop) in Sanya, China.

Courses

Intersect Australia Intermediate UNIX workshop
University of Technology Sydney, Australia - 2013

NZGL advanced transcriptomics workshop
Auckland University campus, New Zealand - 2013

Software carpentry course
University of Queensland, Australia - 2014

EMBL Introduction to statistics for bioinformaticians
EMBL, Heidelberg, Germany – 2014

EMBL Introduction to next-generation sequencing platforms
EBI, Hinxton, Cambridge, England – 2014

Conferences

ISBW 11, Sanya, China - 2014
Poster presentation
Title: 'Exploring the functional landscape of the *Z. muelleri* transcriptome: implications for light adaptation'

AMSA Seagrass workshop – 2015
Geelong, Victoria, Australia.