THE ROLE OF ABA IN REGULATING THE MOVEMENT OF *CHLAMYDOMONAS REINHARDTII* IN RESPONSE TO STRESS

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A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy

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I. Author's Declaration

This thesis is submitted in fulfilment of the requirements of a Ph.D., and except where duly acknowledged or referenced, it is entirely my work. It has not been submitted, either in whole or part, for any other award at the University of the West of England or elsewhere.

Signed ..... Date 10/05/2019

II. Project Supervisors' Declarations

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III. Abstract

Plant hormones such as auxin, gibberellin, cytokinin, ethylene, and abscisic acid play key roles in growth and developmental processes in higher plants, for instance, *Arabidopsis thaliana*. However, the effect of these hormones on algal physiology is still unclear. Results suggest that these plant hormones do not affect the growth rate of *Chlamydomonas reinhardtii*. The effect of these hormones was examined in the movement of *C. reinhardtii. C.reinhardtii* cultures were transferred to a glass measuring cylinder then treated with 50µM of Abscisic Acid (ABA), 1 aminocyclopropane-1-carboxylic acid(ACC), Hydrogen peroxide (H_2O_2), 1-Naphthaleneacetic Acid (NAA) and combinations of ABA and ACC in either the light or dark. All these hormones except ABA did not alter the phototropic response of *C.reinhardtii*. In addition, the results showed that exogenous ABA significantly altered the HCO_3^- uptake of *C. reinhardtii* in a light-intensity-dependent manner. In high light ABA enhanced HCO_3^- uptake, while under low light uptake was diminished. Algae were sampled at different time points over 24h in a cyclic 16h photoperiod and were treated with, or without 50 µM ABA in either the light or dark and their position in the water column was monitored by measuring the A_{750} at different depths. The actual position attained by the algae in the water column correlated with the time at which they were sampled in the cycle; in general, ABA-induced upward movement of the algae. The algae also showed a differential, light-dependent directional taxis response to a fixed ABA source, moving horizontally towards the source in the light and away in the dark.

Plants possess glycine-rich RNA-binding proteins (class IV GRPs) that are involved in stress responses and are regulated by ABA. The sequence analysis revealed that *C. reinhardtii* appears to possess only a single class IV GRP gene, which we named *CrGRP1*; it encodes a flagellum-associated RNA-binding protein. The researcher shows that *CrGRP1* is expressed in circadian rhythm and in response to abiotic stresses. The expression of *CrGRP1* was also assessed during the algal movement experiments and appeared to decline to the greatest extent when ABA-induced the greatest upward movement of the algae. To determine the potential role of the *CrGRP1* gene in ABA and light-mediated movement, recombinant pChlamy4 constructs were made to overexpress and knock-down *CrGRP1*. Ongoing experiments suggest that *CrGRP1* negatively regulates the ABA-induced upward movement of the algae, presumably by binding mRNAs required for this response. The data suggest that the development of this response mechanism in motile algae may have been an essential step in the evolution of terrestrial plants and anticipate that these results will initiate novel algal research that may help to clarify the involvement of ABA in plant tropisms.

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With complete honour and satisfaction, I dedicate this Thesis to my parents and my daughter Hajar.

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VIII. Abbreviations

µg	microgram
µm	micrometer
µM	micromolar
µs	microsecond
A. thaliana	*Arabidopsis thaliana*
ABA	Abscisic Acid
ABFs	ABA-responsive element Binding Factors
ABRE	ABA-Responsive Elements
ACC	1-aminocyclopropane-1-carboxylic acid
***At*GRP**	*Arabidopsis thaliana*- Glycine-Rich Proteins
ATP	Adenosine Triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
C. reinhardtii	*Chlamydomonas reinhardtii*
C_2H_4	Ethylene
CaC_{l2}	Calcium chloride
Cdna	Complementary DNA
CIRP-A	Cold-inducible RNA-binding protein A
CrGRP1	*Chlamydomonas reinhardtii* - Glycine-Rich Protein1
CSD	Cold Shock Domain
CSPs	Cold Shock Proteins
DNA	Deoxyribonucleic acid
DNase I	DeoxyriboNuclease I
dNTP	Deoxynucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
et al.	*et al*ia
EtBr	Ethidium Bromide
FBS	Filner's Beijerincks Solution
GA3	Gibberellic Acid
gDNA	Genomic DNA
GRPs	Glycine-Rich Proteins
H_2O_2	Hydrogen peroxide
IAA	Indole-3-Acetic Acid
IPTG	Isoprophyl-ß-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton (s)
KIN	kinetin
L	Litre
LB	Luria-Bertani
LHCB	light-harvesting chlorophyll binding

M	Molar
mg	milligram
MgC_{l2}	Magnesium chloride
min	minute(s)
ml	millilitre
mM	milliMolar
NAA	1-Naphthaleneacetic Acid
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCBI	National Centre for Biotechnology Information
ng	nanogram
NH3	Ammonia
nm	nanometer
NOS	Nitric Oxide Synthases
OD	Optical Density
***Os*GRP**	*Oryza sativa* - Glycine-Rich Proteins
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PSII	photosystem II
RBM3	Mammalian RNA-binding protein 3
ROS	Reactive Oxygen Species
RRMs	RNA Recognition Motif
SDS	Sodium Dodecyl Sulfate
SDW	Sterilized De-ion Water
SE	Standard Error
Taq	DNA polymerase from Thermus aquaticus
TE	Tris, EDTA
Temp	Temperature
TRAF	Tumor necrosis Receptor Associate Factor
TRIA	1-Triacontanol
U	Unit(s) of enzyme activity
Unr	Upstream of N-ras
UV	Ultraviolet
V	Volts
v	volume
v/v	volume/volume
W	Weight
w/v	weight/volume
WRKY	transcription factors
WT	wildtype
xg	times gravity(s)
Z. mays	*Zea mays*bp -base pair
λ	lambda

1 Chapter One: Introduction

1.1 Chlamydomonas reinhardtii and biofuels

Approximately ten micrometres in diameter, C*hlamydomonas reinhardtii* (*C. reinhardtii*) belongs to a well-studied genus of unicellular green algae. It has two flagella, a large cup-shaped chloroplast, a cell wall made of hydroxyproline-rich glycoproteins, and a large pyrenoid in the chloroplast. It also has an eye-spot which is light-sensitive, allowing the cell to undergo phototaxis (Figure 1.1) allowing it to swim away from high light and towards low light (Bennett and Golestanian, 2015). Like many other algae, *Chlamydomonas* utilises a circadian clock system, and its potential for genetic analysis increases its utility as a model for this area of research (Merchant *et al*., 2007).

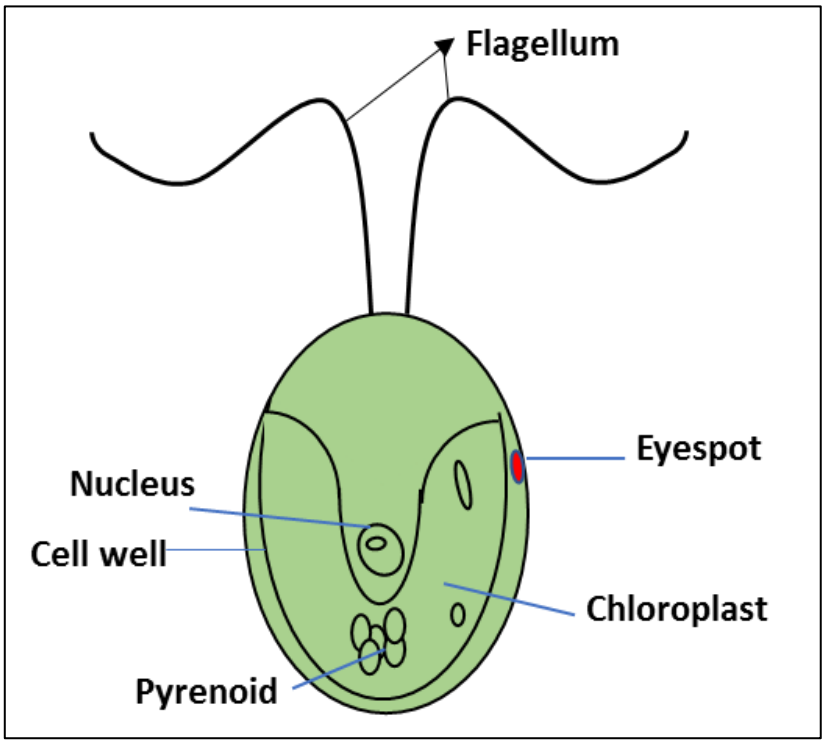


Figure 1.1: Chlamydomonas reinhardtii.
Chlamydomonas reinhardtii. A unicellular ovate green alga with a distinct cell wall, and a single chloroplast near the nucleus. The nucleus is typically located in the centre. There is an eyespot and one or several contractile vacuoles. The eyespot is typically located on the side. One or more pyrenoids are located within the chloroplast surrounded by starch bodies. Two anterior flagella are used for sensing and swimming (Adapted from Merchant *et al.*, 2007).

1.1.1 *Chlamydomonas reinhardtii* as a model organism

C. reinhardtii has been used as a model organism in molecular biology, especially relating to studies of flagellar motility, and of chloroplast dynamics, biogenesis and genetics (Rupprecht, 2009). It possesses many favourable features that have made it a useful model organism.

Firstly, it can be grown easily at a range of temperatures, can absorb a carbon source other than carbonate such as acetate, and can absorb complex metabolites from yeast extract. It has a doubling time of 24 hours (Rupprecht, 2009) similar to cells of higher plants, but its phenotype can often be observed almost immediately, while plants

often must be left to mature for many weeks or months to observe a phenotype. Secondly, mating experiments are readily carried out in *C. reinhardtii*, with subsequent benefits for classical genetics studies of linkage, dominance, and suppression (Goodenough *et al*., 1995). Only two mating types are present in this organism, plus and minus; in contrast, multiple mating types exist in some algae (Goodenough *et al*., 1995). Third, *C. reinhardtii* is normally haploid, and any mutations and their effects are seen immediately without the need for further crosses (Goodenough *et al*., 1995). Fourth, many known mutants of *C. reinhardtii* are available (Rupprecht, 2009). Fifth, like a plant cell, the cell of *C. reinhardtii* has a cell wall. Sixth, like animal sperm cells, *C. reinhardtii* has a flagellum, which makes phototaxis possible, moving towards or away from light to optimise the conditions for photosynthesis and to minimise photodamage when at risk. Thus, in some aspects, *C. reinhardtii* most closely models plant cells and in others, animal cells making it a powerful and versatile system for the study of a variety of molecular and cellular processes.

1.1.2 The life cycle of *C. reinhardtii*

There are two types of life cycles in *C. reinhardtii*: asexual and sexual life cycles. Usually, asexual reproduction occurs by fission so that the protoplast divides to form 4-8 zoospores almost the same size as the parent cell. In the sexual cycle, nitrogenous compound deprivation encourages the cells to create isogametes, which fuse in pairs, forming a fertilised egg. The fertilised egg loses its flagella and forms a thick wall which is resistant to adverse conditions. When conditions become favourable, the fertilised egg goes through meiosis to form four haploid zoospores (Figure 1.2).

In the sexual life cycle of *C. reinhardtii*, gametes are created from haploid vegetative cells, and they may merge to form diploid zygotes. After a period of

maturation, the zygotes are ready for germination and meiosis (Hoober, 1989). Light is required for gamete formation, the maintenance of mating competence in gametes, and zygote germination (Saito *et al*., 1998; Treier *et al*., 1989). There are two signals needed for gametogenesis, nitrogen starvation which is considered as the first signal, and exposure to blue light as a second signal. Incubating vegetative cells in the dark caused the formation of mating-incompetent pregametes; however, they can regain their mating ability when they are exposed to irradiation with blue light. In higher plants and several lower eukaryotes, the action spectrum of this response suggests that the photoreceptor involved has properties characteristic of blue-light receptors (Weissig and Beck, 1991). Although light encourages the conversion of pre-gametes to gametes, it is a slow operation that requires protein synthesis (Beck and Acker, 1992; Huang and Beck, 2003). Some separate signalling pathways appear to control these light responses. For example in the last step of the life cycle, zygote germination, initiating irradiation for ≈3h is sufficient for induction of meiosis. In the dark during the following 20h, subsequent processes such as meiosis and germination may happen (Huang and Beck, 2003). Analysis of mutants has indicated that at least one common gene product is involved in light signalling pathways that control pregamete to gamete conversion and zygote germination; the participation of the same photoreceptors in these response pathways has been predicted (Huang and Beck, 2003).

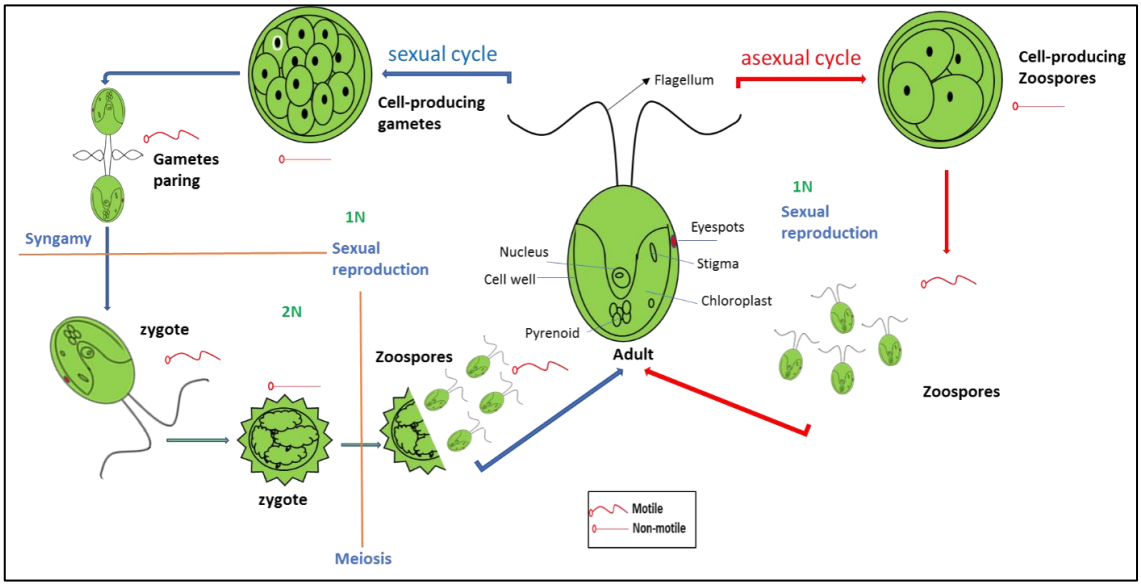


Figure 1.2: Life cycle of Chlamydomonas reinhardtii.
In the asexual cycle in *C. reinhardtii:* the zoospores are liberated from the parent cell or zoosporangium by gelatinisation or rupture of the cell wall. The zoospores are similar to the parent cell in structure but smaller in size. On the other hand, the sexual life cycle of *C. reinhardtii* consists primarily of four critical stages – gametogenesis, zygote formation, zygote maturation (zygospore formation), and meiosis (zygospore germination). When gametes of opposite mating types are mixed, flagellar adhesion triggers gamete activation that leads to the cell-cell fusion (zygote formation). Zygotes will develop into zygospores with a thick cell wall, which is a dormant stage in the life cycle. The cycle begins again when the appropriate environmental conditions stimulate the dormant zygote to undergo germination, in order to produce new haploid cells (adapted from Huang and Beck, 2003).

1.1.3 How algae move and why

Algae such as *C. reinhardtii* have two flagella and an eyespot which is light-sensitive. *C. reinhardtii* uses its flagella both for swimming and mating; it uses flagella for rotating its body to get to the correct position in the water. In addition, a study by Bennett and Golestanian (2015) reported that these organisms move towards or away from a light source in phototaxis. *C. reinhardtii* is a unicellular photosynthetic alga, and it has two types of phototaxis - positive and negative phototaxis (moving towards or away from a light source). Interestingly, the eyespot is placed on the side of the cell instead of in an anterior or posterior position. The equatorial position is advantageous because *C. reinhardtii* rotates its body during forward swimming, allowing the eyespot to scan the

incoming light from different directions. Then it can perceive the right position to protect itself from environmental stress such as strong light (Bennett and Golestanian, 2015).

1.1.3.1 *Regulation of flagellar movements by calcium signalling*

Flagella and cilia are well-conserved structures in eukaryotic cells. In many eukaryotes, Ca^{2+} is the critical intracellular factor affecting changes in the motility of cilia and flagella in response to extracellular environments (Inaba, 2015). Algae such as *Chlamydomonas* use rhodopsin-initiated Ca^{2+} currents to regulate flagella movements, and thus their orientation to light and phototactic responses (Harz and Hegemann, 1991). Light excitation of the eyespot triggers photocurrents causing an influx of Ca^{2+} ions to the flagella (Harz, and Hegemann, 1991). The response to the influx of Ca^{2+} ions is different in the cis-flagellum (closest to the eyespot) and trans-flagellum (furthest from the eyespot) depending on the movement of the body, the direction of light and amount of light received. Kamiya and Witman (1984) studied axonemes in reactivated demembranated models. After incubating the dememranated cells in the standard reactivation solution containing a different concentration of Ca^{2+}, they observed that increasing Ca^{2+} concentration decreases the beat amplitude of the cis-axoneme while decreasing Ca^{2+} concentration decreases the beat amplitude of the trans-flagellum (Kamiya and Witman, 1984).

1.1.3.2 *Pigments of green algae*

Chloroplast pigments in green algae are similar to those of higher plants. There are three pigments in green algae: chlorophyll a and b, carotenoids and phycobilins. Carotenoids include both carotenes (oxygen-free hydrocarbons) and xanthophylls (oxygen derivatives of carotenes). In all photosynthetic algae, the main pigment is chlorophyll a and no

species are known to lack this pigment. Chlorophyll b, β -carotene and lutein (the main carotenoid) are important general accessory pigments of the Chlorophyceae (Larkum and Howe, 1997). Some genera are found to have siphonoxanthin and its esters siphonein (Yoshida *et al*., 2003). Carotenoid pigments are also commonly present outside the chloroplasts, especially in resting cells and in the terrestrial *Trentepholia*, as well as in the eye-spots of the motile stages. These red or yellow coloured substances, known as *haematochromes* are made of two or more carotenoid pigments. In many cases, they are dissolved in fat globules. Accumulation of carotenoids happens under conditions of salinity, nitrogen deficiency or high light irradiance. A good example is in *Dunaliella*, where β- carotene accumulates between thylakoids in the chloroplast, and *Haematococcus*, where astaxanthin accumulates in lipid globules outside the chloroplast (Hagen *et al*., 2000; Wang *et al*., 2003). Accumulation of haematochromes colours the cells orange or red, with haematochrome accumulating up to 8–12 % of total cellular contents in *Dunaliella* (Orset and Young 1999). In contrast, animals cannot synthesise carotenoids, and they acquire the pigments through the food chain by consuming the primary producers. Haematochromes are responsible for colouring in some fish, crustaceans and birds (such as the pink flamingos) (Yoshida *et al*., 2003).

1.1.4 *Chlamydomonas reinhardtii* transgenesis

Many mutant strains of *C. reinhardtii* are publicly available at the *Chlamydomonas* Center (http://www.chlamy.org/). Previously, it was difficult to express foreign genes in *C. reinhardtii*, such as antibiotic resistance and reporter genes due to codon usage bias, because its chloroplast and nuclear genomes are highly GC and AT-rich, respectively (Matsuo and Ishiura, 2011). However, the development and widespread use of artificial gene synthesis have solved this issue; different codon adapted reporter genes, such as

green fluorescence proteins and luciferases, can be expressed in the chloroplasts and nuclei of *C. reinhardtii* (Franklin *et al*., 2002; Fuhrmann *et al*., 2004). The sequencing of the nuclear, mitochondrial and chloroplast genomes of *C. reinhardtii* made it one of the models for post-genomic research (Gray and Boer, 1988; Michaelis *et al*., 1990). Several studies have been carried out on the introduction of native or foreign genes into *C. reinhardtii*. The first successful DNA transformation of *C. reinhardtii* was achieved by Rochaix and van Dillewijn (1982) more than 30 years ago, a method which led to the development of many molecular tools for genetic manipulation (Walker *et al*., 2005). As an example, production of a given chemical compound within the chloroplast could be achieved via the expression of a specific gene from the chloroplast genome, expression from the nucleus (and potentially directing the protein into the chloroplast via targeting signals), or application of a knockdown/knockout strategy to attenuate respective antagonistic pathways (Walker *et al*., 2005).

Upon transformation, foreign DNA is inserted randomly in the *C. reinhardtii* genome. Polymerase chain reaction **(**PCR) and Southern blotting are standard DNA screening procedures that can detect the presence of recombinant DNA; RNA can be detected by RT-PCR or Northern blotting and proteins by Western blotting (Durmaz *et al*., 2015). Dominant and non-dominant markers are commonly used in the genetic manipulation of *C. reinhardtii*. The dominant marker "*ble*" gene confers resistance to the bleomycin antibiotic used for the nuclear transformation of *C. reinhardtii* (Lumbreras *et al*., 1998). Several techniques have been developed for the introduction of foreign DNA into *C. reinhardtii*. These include glass bead and vortexing techniques (Kindle, 1990), electroporation (Shimogawara *et al*., 1998) and *Agrobacterium*-mediated transformation

(Kumar *et al*., 2004). The expression of foreign genes needs to be driven by an appropriate promoter to work in *C. reinhardtii*. The most widely used constitutive promoter in *C. reinhardtii* is derived from the constitutively expressed *RbcS2* gene, encoding ribulose bisphosphate carboxylase oxygenase small subunit 2 (Stevens *et al*., 1996). The transcription level of genes driven by this promoter can be increased significantly if the promoter elements of the *C. reinhardtii* heat shock protein genes are fused upstream of the *RbcS2* promoter (Schroda *et al*., 2000).

1.1.5 B Biotechnological applications of *C. reinhardtii and* other algae

Algae are an important group of organisms for biotechnological exploitation, especially for food and pharmaceutical industries as well as in public health (Chu, 2012). For example, in Mexico, Spirulina is collected from Lake Texcoco and used for making a dry cake called tecuitlatl. Additionally, algae produce a different range of metabolites with various bioactivities that are yet to be fully exploited (Cardozo *et al* ., 2007). Microalgae such as Chlorella and Spirulina have been consumed as food supplements (nutraceuticals) by humans and also used as animal feeds. Some green algae are well recognised in skin care products such as anti-ageing creams, sun protection products and emollients. For example, an extract from *Chlorella vulgaris* (*Dermochlorella*, France) stimulates collagen synthesis in the skin, thus supporting tissue regeneration and wrinkle reduction. Moreover, microalgae have been exploited for wastewater treatment and used as a biological tool for assessment of environmental toxicants. Some of the marine microalgae are a potential source of long-chain polyunsaturated fatty acids (LC-PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which have been implicated to be beneficial in the prevention of cardiovascular disease. In humans, DHA

is significant for the proper development of the brain and eye in infants and has been shown to support cardiovascular health in adults (Ward and Singh., 2005).

Energy derived from living organisms, whether plant or animal, represents one of the most important sources of renewable energy (Chisti, 2007). One way to reduce the increasing amount of carbon dioxide in the atmosphere is by the use of biofuels to replace non-renewable energy sources. Therefore, according to Chisti (2007) biofuel research is now considering microalgae as a potential energy resource as fuels derived from other plants cannot meet even a fraction of the current energy demand throughout the world because it impinges on issues to do with land area and growth rate of plants (Chisti, 2007). Furthermore, the benefits of biofuels may include increased income from plant material and increased energy security in countries without oil reserves.

Table 1.1 lists the renewable energy resources derived from biomass (Amaro *et al* ., 2011; Balat, 2011). There are three types or generations of biofuels depending on the feedstock, as shown in Table 1.1 Rapeseed, soybeans, sunflower and palm, are considered as first-generation biofuel because they were the first crops to be used to produce biodiesel. However, it has faced several problems such as food markets and food security. After that, to reduce the dependency on edible oil, alternative biofuel sources, such as non-food feedstocks, have been developed to produce biodiesel. Energy crops form the second generation such as Jatropha, mahua, tobacco seed, and salmon oil. However, it faces a problem as well, as the products from the second generation may not be enough to replace first-generation feedstocks (Amaro *et al*., 2011; Balat, 2011). Therefore, the third generation, which is derived from microalgae, has emerged as one of the most promising alternative sources of lipid for use in biodiesel production because of their high photosynthetic efficiency to produce biomass and their higher growth rates and

productivity compared to conventional crops (Amar *et al* ., 2011). The third-generation biofuels derived from algal biomass have been considered as the best alternative bioresource that avoids the disadvantages of first and second-generation biofuels (Amar *et al* ., 2011; Balat, 2011).

TYPE	FEEDSTOCK
First generation	Rapeseed, soybeans, sunflower, palm,
Second generation	Jatropha, mahua, tobacco seed, salmon oil,
Third generation	Microalgae

Table 1-1: Biofuel types and their feedstock (from Amaro et al ., 2011; Balat, 2011).

Potentially, biofuels open up new avenues to increase competition with oil markets and offer a mechanism for the moderation of oil prices, in addition to ensuring a healthy supply of alternative energy sources. This will help in the fight against high gasoline prices and reduce dependence on fossil fuels, especially in the transport sector (Amaro *et al* ., 2011; Balat, 2011; Chisti, 2007). First and second-generation feedstocks have been researched for many years. Currently, much research is aimed at third generation biofuels, the production of fuels from algae. It was reported by researchers from the Netherlands that the production of biofuels from algae could become a reality in the next 10 to 15 years, because algae may potentially help to decrease global dependence on fossil fuels, especially in the transport sector which requires gas or liquid fuels (Amaro *et al* ., 2011; Balat, 2011).

1.1.6 *Chlamydomonas reinhardtii* as an ideal biofuel source

Algae are capable of producing ten times the amount of biofuel per unit area when compared with wheat and rapeseed (Chisti, 2007). Furthermore, algal production does

not directly compete with land for food crop production. In addition, unlike higher plants, algal biofuel production is considered carbon neutral as carbon dioxide is both utilised during algal growth and released during the subsequent use of algal-derived biofuel (Ahmad *et al*., 2011; Chisti, 2007). Table 1.2 gives information regarding the expected production of oil from algal farms compared with those from traditional agriculture. From this table it can be seen that the yield of biodiesel from microalgae is more than ten times that of palm oil, the largest oil crop source, yet uses only one-tenth of the land (Chisti, 2007), suggesting that its production per hectare could be a hundred times greater.

CROPS	OIL YIELD (L/ha) a	LAND AREA (M/ha)
Corn	172	1540
Soybean	446	594
Canola	1190	223
Jatropha	1892	140
Coconut	2689	99
Palm oil	5950	45
Microalgae b	136,900	2
Microalgae c	58,700	4.5

Table 1-2: Expected production of biofuels from different sources and their land area (from Chisti, 2007).

a - For meeting 50% of all transport fuel needs of the United States
b -70% oil in biomass by dry weight
c - 30% oil in biomass by dry weight

1.1.6.1 *Benefits of using algae as a source of biofuel*

There are some benefits to using microalgae as a source of biofuel, including the fact that firstly, microalgae are readily available in the environment and can be cultured under a range of climatic condition across the world, in contrast to higher plants (such as palm

oil), which are more climatically restricted. Algae are known to have the highest growth potential compared to other photosynthetic organisms (Hirano *et al*., 1997). Secondly, microalgae can grow in both fresh and brackish water. Growing them in brackish water will reduce the rates of freshwater use for agriculture (Hill *et al*., 2006). Thirdly, according to Leite (2013), algae can be considered a renewable source of fuels because they harvest sunlight energy to produce the organic compound as biomass. Fourth, the rate of algal growth is generally limited by carbon, in this case, carbon dioxide; rapid and large growth will, therefore, require large amounts of carbon dioxide. Carbon dioxide emitted in large quantities from the use of fossil fuels could be potentially diverted from increasing the atmospheric concentration into large-scale algal growth facilities (Amin, 2009; Demirbas, 2010). Finally, when comparing the productivity of microalgae to that of land plants, algae have a higher rate of biomass accumulation than plants. For example, the most common microalgae have oil levels in the range of 20 to 50% by their weight of dry biomass (Scott *et al*., 2010; Singh and Gu, 2010).

One area of microalgal research that has perhaps lagged behind higher plants is in the area of molecular biology. This area of microalgae research is limited in areas of understanding how microalgae can adapt and respond to environmental stress (biotic and abiotic). A specific area that needs development is understanding the molecular biology of plant hormones. Sequencing of the *C. reihardtii* genome has shown that this flagellate, motile, freshwater, photosynthetic green alga is also capable of synthesising and responding to abscisic acid (ABA) and other plant hormones (Wang, 2015). The function of the main stress hormone ABA will be described in the next section.

1.2 Abscisic acid (ABA) and the stress responses

Plant hormones are small organic molecules which influence many physiological functions at low concentrations; one of these is abscisic acid (ABA). Initially, there were two names for it; one group called it an 'abscisin II' because they thought it played an important role in abscission of fruits. Other groups at the same time called it a 'dormant' because they thought it played a role in bud dormancy. After that both groups agreed to call it an 'abscisic acid (ABA)', and they thought that in general ABA plays mostly inhibitory roles. However, it has many other roles (Arteca, 2013). It is also called a stress hormone because the production of ABA is stimulated by drought, water logging and other adverse environmental conditions.

ABA is a sesquiterpenoid ($C_{15}H_{20}O_4$) with a 15-carbon ring (Figure 1.3). The molecular structure of ABA has several significant features that facilitate its biological purposes. The side chain with the two double bonds and ABA's stereocenter are two such essential features. Exposure to UV light changes the confirmation from active to an inactive form (Fernando and Schroeder, 2016). ABA plays key roles in the growth and developmental process in higher plants. As a major plant hormone, it is involved in the regulation of essential physiological processes such as seed development and bud dormancy and the control of stomatal closure (Vishwakarma *et al.*, 2017).

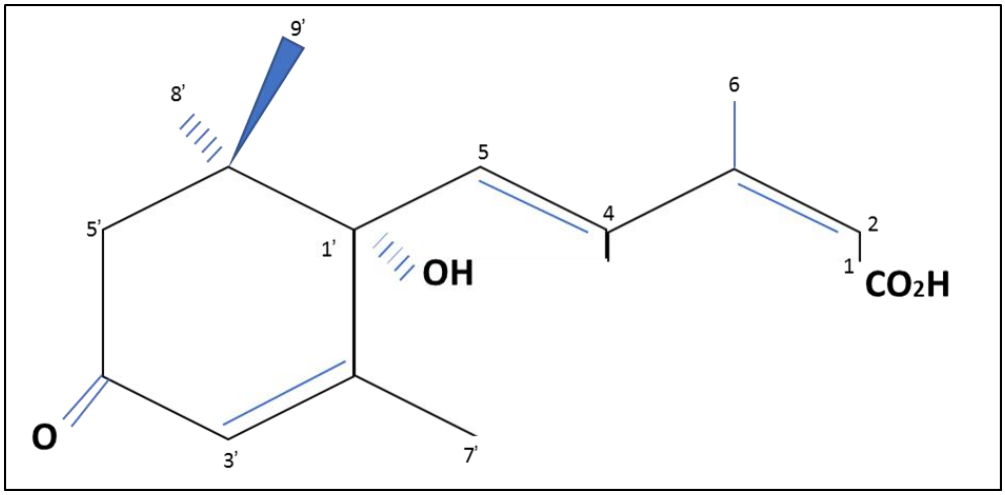


Figure 1.3: Structure of phytohormone abscisic acid (adapted from Cutler et al., 2010).

1.2.1 ABA and its biosynthesis in higher plants

In plants and other organisms, such as cyanobacteria, algae, and fungi, ABA levels increase with exposure to stress, suggesting a possible role of ABA in stress signal transduction (Lumba *et al*., 2014). There are two pathways for ABA biosynthesis in plants: direct and indirect, in which ABA is derived from the C15 compound farnesyl pyrophosphate and a C40 carotenoid, respectively (Zeevaart and Creelman, 1988; Zeevaart, 1999). The main pathway of ABA is the indirect pathway (Figure 1.4) that was reported by a study describing an ABA-deficient mutant that was completely different from other related genes (Fernando and Schroeder, 2016).

The biosynthetic pathway of carotenoids is well defined. Carotenoids, like other isoprenoids, are made from the C5 predictor, isopentenyl pyrophosphate (IPP). IPP is created from mevalonic acid in the cytosol, whereas in plastids where carotenoid combination occurs, it is produced via 1-deoxy-D-xylulose-5-phosphate (DXP) from pyruvate and glyceraldehyde-3-phosphate. DXP synthase is the enzyme that helps in the

synthesis of the first step of non-mevalonic acid IPP creation pathway (Eisenreich *et al*., 2001; Lichtenthaler, 1999). IPP is converted to a C20 product, geranylgeranyl pyrophosphate (GGPP). Transformation of GGPP to a C40 carotenoid phytoene produced by phytoene synthase (PSY) is the first committed and rate-limiting stage in the synthesis of carotenoids and is followed by phytoene being converted to ζ-carotene, lycopene, β-carotene and then to a xanthophyll, zeaxanthin. Phytoene desaturase (PDS) helps in the transformation of phytoene to β-carotene and is also one of the enzymes dedicated to carotenoid formation. (Cunningham and Gantt, 1998; Hirschberg, 2001). Zeaxanthin is then converted to xanthoxin and is exported to the cytosol where it is converted to abscisic aldehyde by a short-chain alcohol dehydrogenase/reductase (SDR), and then oxidised by the abscisic aldehyde oxidase (AAO) to form ABA (Nambara and Marion-Poll, 2005).

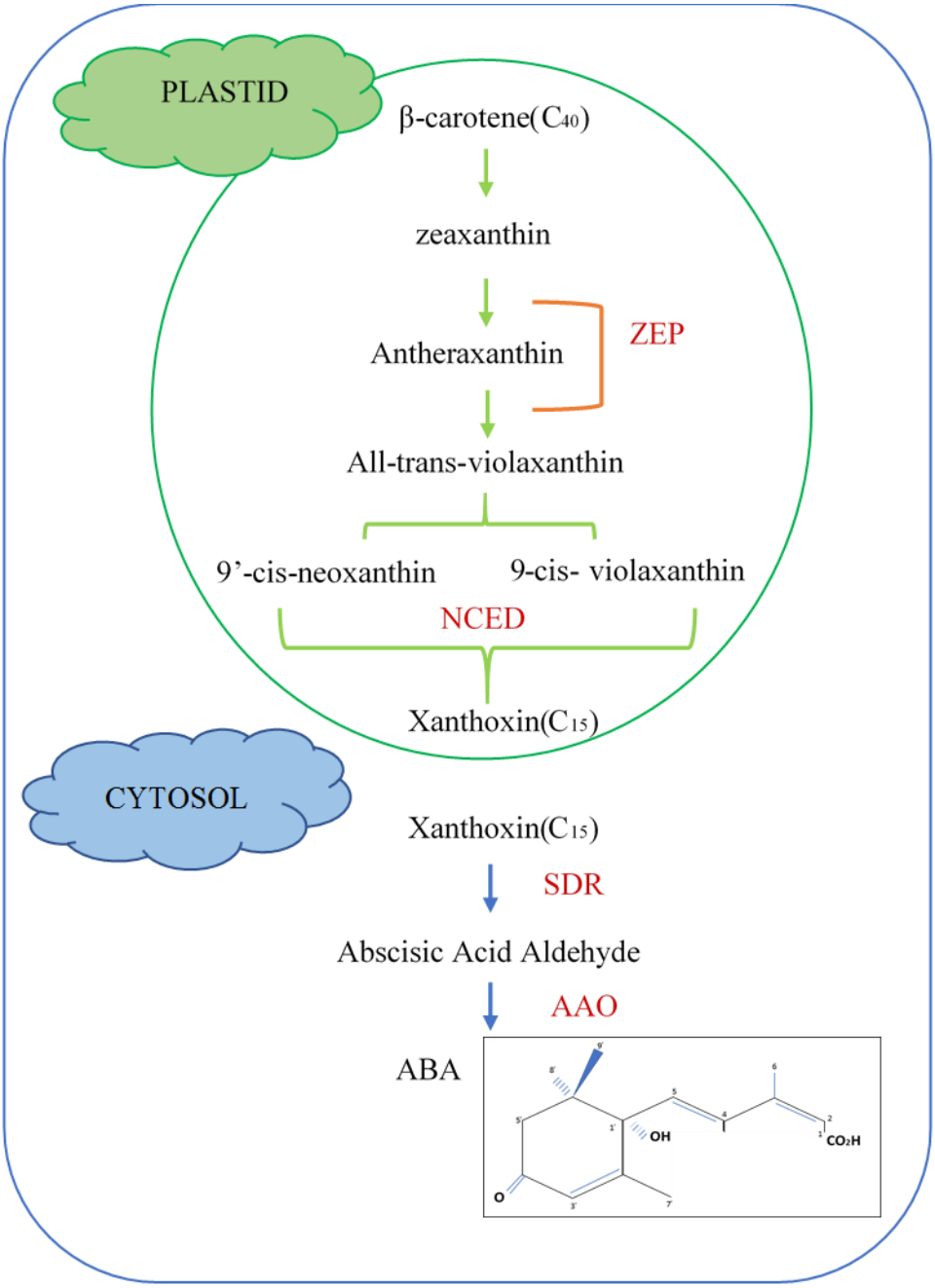


Figure 1.4: Indirect ABA biosynthesis pathway in higher plants

ABA comes from β-carotene (C40) through an oxidative cleavage reaction in plastids. The first stage of the ABA biosynthesis pathway is the conversion of zeaxanthin and antheraxanthin to all trans-violaxanthin, which will be catalysed by zeaxanthin epoxidase (ZEP). Antheraxanthin is the intermediate product. All–trans-violaxanthin is converted to 9-cis-violaxanthin or 9′-cis-neoxanthin by the 9-cis-epoxy carotenoid dioxygenase (NCED), which yields a C15 intermediate product called xanthoxin. Then the product xanthoxin is exported to the cytosol where xanthoxin is converted to ABA. Xanthoxin is then converted into ABA by two enzymatic reactions. Xanthoxin is first converted by the enzyme short-chain alcohol dehydrogenase/reductase (SDR), to an ABA aldehyde which is subsequently oxidised to ABA by the abscisic aldehyde oxidase (AAO) (adapted from Mehrotra *et al*., 2014).

1.2.2 Where and when does ABA biosynthesis occur?

ABA is produced in many parts of the plants but more abundantly inside the chloroplasts of green cells. For example, it is produced in response to environmental stress, such as heat stress, water stress, salt stress and synthesised in green fruits at the beginning of the winter period and in maturing seeds, establishing dormancy. The hormone is formed from mevalonic acid or xanthophyll, and it can be found everywhere in plant parts for example, in roots, flowers, leaves and stems. It is transported to all parts of the plant through diffusion as well as transport channels.

1.2.3 Role of ABA in plants

There were many physiological roles of ABA found after its discovery. Firstly, the ability of ABA to antagonise several gibberellins (GA) effects such as the promotion of seedling growth and α-amylase synthesis was reported by Wareing's group (Thomas *et al*., 1965). After that, other roles for ABA were reported, specifically, in guard cell responses and water retention. A good example for that is in the wilty tomato flacca mutant that was deficient in ABA where its phenotype could be rescued by exogenous ABA treatment (Imber and Tal., 1970, Tal *et al*., 1970), and in Xanthium stomatal closure after treating with ABA (Jones and Mansfield, 1970). In the early 1970s, a physiological model for ABA's critical role in guard cell regulation emerged, when ABA levels increase substantially after water deprivation (Jones and Mansfield, 1970). According to Sharp *et al*. (2004), the action of different hormonal signalling pathways and ABA help protect root growth during water deficit. ABA and other signalling pathways involve regulation of stress-responsive gene expression in cases where water uptake and water loss cannot be balanced by primary adaptive responses through different mechanisms that could be

exploited to avoid and/or tolerate dehydration (Zhu, 2002). ABA has also been found to affect pathogen responses (Ton *et al.*, 2009). Additionally, ABA regulates important processes of plant growth and development, like germination, embryo and seed development, vegetative development including heterophylly as well as general growth, promotion of seed desiccation tolerance and dormancy, seedling establishment, and reproduction. Severe ABA-deficiency or ABA-insensitive mutants display a lack of osmocompatible solutes: small molecules accumulated by cells to permit osmotic adjustment to a dehydrating environment without interfering with cellular function (Barrero *et al.*, 2005; Fujii and Zhu, 2009).

1.2.4 ABA signalling in plants

Although ABA has a wide range of biological functions in plant development and growth, the primary function is to regulate osmotic stress tolerance and plant water balance (Raghavendra *et al.*, 2010). Hence, understanding ABA signalling is important to improving crop performance. Genetic screens in *Arabidopsis thaliana* have revealed many downstream ABA signalling components. Recent findings in the area of ABA signalling reveal a unique hormone mechanism (Figure 1.5) where ABA binds to the ABA receptors Regulatory Components of ABA Receptor/Pyrabactin Resistance Protein1/PYR-like Proteins (RCAR/PYR1/PYLs). Proteins such as RCAR/PYR/PYL belong to the START-domain superfamily and have soluble ligand-binding properties. RCAR/PYR/PYL receptors are found in both cytoplasm and nucleus. ABA binding to RCAR/PYR/PYLs leads to inactivation of type 2C protein phosphatases (PP2Cs) like Abscisic Acid Insensitive 1 (ABI1) and its close homolog ABI2 (Nishimura *et al.*, 2010). The RCAR family of proteins that have 14 members, all of them bind to ABA and interact with PP2Cs. All the RCAR members are positive regulators of ABA signalling except

RCAR7/PYL13. In *Arabidopsis*, there are 80 PP2Cs identified, and six out of nine clades A PP2Cs act as negative controllers of ABA signalling (Nishimura *et al*., 2007). RCAR/PYR1/PYLs and these PP2Cs phosphatases function as co-receptors and form a high-affinity ABA-binding site. Sucrose non-fermenting Kinase-1-Related protein kinase 2s (SnRK2s), are necessary positive regulators of ABA signalling but lead to suppression of PP2C-mediated dephosphorylation when the PP2Cs are inactive. Therefore, ABA-dependent gene expression and ion channels are targetted by activated SnRK2s (Cutler *et al*., 2010; Raghavendra *et al*., 2010). Phosphorylated SnRK2s then phosphorylate ABA-responsive element Binding Factors (ABFs), which are essential leucine zipper transcription factors that bind to ABA-Responsive Elements (ABRE) (PyACGTGG/TC), the primary *cis*-element in the promoter region of downstream genes that are induced by ABA (Busk and Pagès, 1998; Lumba *et al*., 2014).

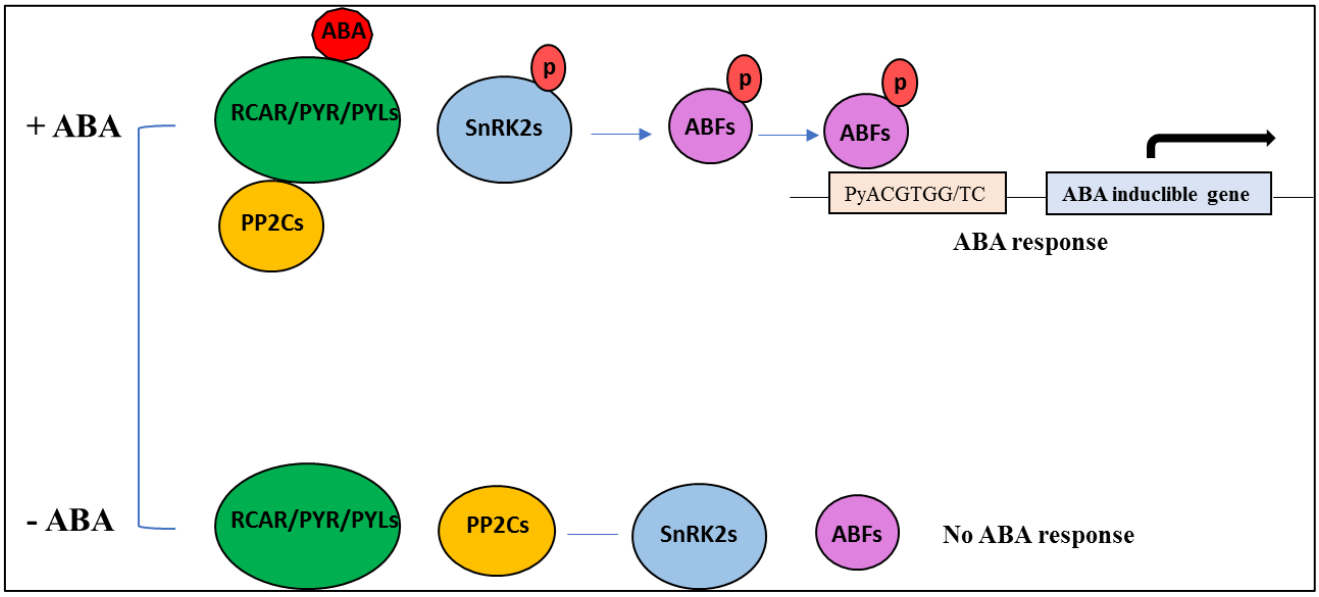


Figure 1.5: The main components in the core ABA signal transduction pathway (adapted from Fernando and Schroeder, 2016).
When ABA binds with RCAR/PYR/PYLs, it leads to inactivation of type 2C protein phosphatases (PP2Cs). Inactivation of PP2Cs causes suppression of PP2C-mediated dephosphorylation of sucrose nonfermenting kinase-1-associated protein kinase 2s (SnRK2s) that are advantageous critical regulators of ABA

signalling. Then, activated SnRK2 target ABA-based gene expression and ion channels. Phosphorylated SnRK2s ultimately phosphorylate ABA-responsive binding elements (ABFs), which are primary leucine zipper transcription factors that bind to ABA-Responsive Elements (ABRE) (PyACGTGG/TC), the predominant cis-element within the promoter region of downstream genes that are induced by ABA. On the other hand, when ABA is absent, the PP2Cs dephosphorylate SnRK2s, inhibiting kinase activity and thereby preventing downstream gene expression.

An ABA response eventually leads to changes in gene expression as with most signal transduction pathways, which may involve changes in transcription, stability and transcript processing. The specific changes depend on the developmental stage and cell type, such that there is no universal set of ABA-regulated genes, but they are generally thought to contribute to tolerance of dehydrating conditions. This dehydration may be developmentally imposed, as in seed or pollen maturation or response to environmental stresses such as drought, salinity, or low temperatures (Cutler *et al*., 2010).

A study by Fairley-Grenot and Assmann (1991) confirms that there is the involvement of a G-protein-coupled ABA signal transduction pathway in plants. A study by Wang *et al*. (2001) reported that loss-of-function alleles in the sole *A. thaliana* G-alpha subunit gene (GPA1) exhibit wild-type response to ABA-induced stomatal closure while they showed hypersensitivity to ABA at the level of germination and reduced guard cell sensitivity to ABA inhibition of stomatal opening. This and other research by Pandey *et al*. (2006) propose that a G-protein-coupled receptor (GPCR) might participate in ABA signal transduction. According to Colucci *et al*. (2002) the overexpression of GCR1, which is the sole classical GPCR encoded by the *A. thaliana* genome, reduces seed dormancy; however, genetic analyses of loss-of-function alleles did not involve GCR1 in direct ABA perception. The *gcr1* knockout mutants exhibit ABA hypersensitivity; while the data also point to pleiotropic roles for GCR1 in other signalling pathways (Chen *et al*., 2004).

1.2.5 ABA and abiotic stress signal*ling*

ABA is an important hormone in development and a key player in stress responses to environmental stimuli. Plants experience more than a few abiotic stresses which include accumulation of salt (saltiness), chilling or freezing temperatures, normal temperature (warmth) and water shortage (a dry spell or lack of hydration) (Fujita *et al*., 2011). Plants make use of ABA to adapt and remain unaffected by a stress situation and might change ABA levels continuously, based on changing physiological and environmental conditions relating to seed dormancy and development, initiation of growth, promoting stomatal closure, embryo morphogenesis, production of storage proteins and lipids, leaf senescence as well as resistance toward pathogens (Tuteja, 2007). Exposure to environmental stress such as drought can produce harmful effects on the development of plants. Abiotic stress is a damaging stress condition and stops crop production and yields even on watered land worldwide (Mahajan and Tuteja, 2005). Overall, stress is a many-sided event which happens throughout plant development. Plant responses differ depending on the degree of the stress and plant metabolic activity. In response to abiotic stresses, gene expression patterns change in drought, cold, high salt, or following ABA application; hence ABA metabolism and stress signals synergise to achieve cell homeostasis (Tuteja, 2007). It has been explored in a review by Rizwan *et al*. (2017) that plant tolerance against metal stress, increased plant growth and biomass, photosynthetic colours, and gas exchange features can be improved by application of endogenous and exogenous ABA.

1.2.5.1 *Water Stress*

Regarding crops, it is believed that lack of water is a chief limiting factor for plant growth under field conditions since plants are exposed to different levels of water stress daily. Water shortage destroys many plant abilities such as making food from light, evaporation of water from leaves, stomatal conductance, and metabolite accumulation (Sangtarash *et al*., 2009), and therefore results in a significant decrease in plant growth and productivity (Reddy, Chaitanya and Vivekanandan, 2004). Plant responses to drought incorporate changes in morphology and biochemistry, leading to acclimation in moderate conditions, and cause harm to plants and plant ingredients in extreme cases (Vishwakarma *et al*., 2017). Since water-stressed green plants have a higher concentration of ABA than well-hydrated crops, Sangtarash *et al*. (2009) suggested that the consequences of ABA application will improve the sufficiently-watered plants more than water-stressed plants.

It has been assumed for the past 25 years that an increasing concentration of ABA in drought-resistant plants also restricts the growth of the plant, mostly through stopping shoot growth (Trewavas and Jones, 1991). Some research has discussed the relationship between the ABA concentration of plant tissue or xylem sap and growth inhibition and recommended that the improved concentration of endogenous ABA in the drought-resistant plant was enough to support a part of plant growth and that not all growth inhibition results from water stress (Sangtarash *et al*., 2009).

It has also been reported that ABA stops shoot growth in appropriately watered plants. A series of reports has found that ABA deficiency in the plant under drought conditions causes improvement of shoot growth, which is also consistent with upregulation of endogenous ABA accumulation being responsible for plant growth inhibition. It was found that the rate of shoot elongation was high in ABA-deficient maize

seedlings (fluridone- treated or vp5 mutant) in comparison with the control (Sharp *et al*., 1994). A study by Bray (2002) illustrated that increased levels of ABA must inhibit increased ethylene production from tissues under water stress conditions. As a result, ABA accumulation in the course of drought could result in maintaining shoot progress as well as root development, instead of retarded growth, which was previously believed.

ABA is known to control the balance between intrinsic growth and surrounding conditions responses. *At*ABCG25 acts as a cell-membrane ABA transporter exporting ABA from cytoplasm to outside of the cells. Plants that over-express *At*ABCG25 show phenotypes of reduced evaporation of water from leaf without any growth retardation. A study by Kuromori *et al*. (2016) observed that *At*ABCG25 over-expression stimulated a local ABA response in guard cells. Furthermore, *At*ABCG25 overexpression increased drought tolerance, probably resulting from the maintenance of water content over the common threshold for survival after drought stress treatment (Kuromori *et al*., 2016).

Drought is one of the principal abiotic stresses that negatively influences the growth and crop yield of plants (Tripathi *et al*., 2016). Greater than half of the land area where crops can grow well can easily be harmed or influenced by drought (Kogan, 1997). Drought conditions create osmotic stress in organisms, which eventually cause desiccation and resistance to water uptake in plants. During osmotic stress conditions, ABA builds up and acts as a controller of stress response in plants (Nakashima and Yamaguchi-Shinozaki, 2013; Yamaguchi-Shinozaki and Shinozaki, 2006). It is known that ABA has positive effects on stress tolerance following exogenous application or through overexpressing the genes for its increased endogenous content in plants. A recent study by Li *et al*. (2016) reported that ABA could effectively improve drought-induced damage in creeping bentgrass (*Agrostis stolonifera*) by maintaining membrane stability

and leaf water status when a salicylic acid (SA), ABA, and γ-aminobutyric acid (GABA) are added exogenously. It was discovered that ABA, GABA, and SA had impacted the common metabolic pathways and furthermore caused differential changes in metabolite collection under drought stress (Li *et al*., 2016).

Work by Weiner *et al*. (2010) has provided evidence that a lack of water can have an impact on the expression of core ABA signalling parts, which includes the action of PYL /PYR/ RCAR ABA receptors, protein phosphatases 2C (PP2Cs), and subclass III SnRK2 protein kinases. Osmotic stresses such as cold, drought, and high salinity can cause cells not to have enough water at the time of seed improvement in vegetative development (Fujita *et al*., 2011). This results in hyperstimulation of active and effective plant ABA metabolism (Nambara and Marion-Poll, 2005) and transport (Kuromori *et al*., 2010). It was thought that drought-induced stress enforces high ABA levels in *Arabidopsis* leaves and safeguards the plant against disease connected with an avirulent strain of *Pseudomonas syringae pv* tomato (Mohr and Cahill, 2003). In growing and spreading tissues, the amount of ABA is 40-fold higher when under drought and salt stress (Zeevaart and Creelman, 1988). Mutants with non-functional ABA biosynthesis are adversely affected by surrounding conditions when compared to transgenic plants, which can elicit a greater hormonal response and have higher tolerance against biotic stress than the wild type (Iuchi *et al*., 2001; Qin and Zeevaart, 2002). A study by Huang *et al*. (2016) has shown that the ramie *BnbZIP3* gene was upregulated in the presence of drought, high salt water and ABA. The *BnbZIP3* gene belongs to the group of bZIP transcription factors. This might account for the increase of BnbZIP3 as it may contain

some cis-acting elements that are involved in ABA signalling and different stress responses.

Major ABA signalling components actively control both fast and slow ABA communication pathways to tackle not having enough water. Previous studies have found that there is a relationship between osmotic stress from high salt or drought and the two cellular pathways: one is ABA-direct, and the other is ABA-indirect. The cold stress response is achieved through changed gene expression via an ABA-indirect pathway. The ABA-direct pathway depends upon the availability of a cis-acting element called ABRE element (ABA-responsive element) (Tuteja, 2007). Gene-related studies point to a correlation between ABA-dependent and ABA-independent pathways and crosstalk or association of involved molecules in the signalling pathway. Calcium acts as a secondary messenger in response to stress and is a possible candidate to help cross communication. Extensive research has shown that ABA, drought, cold and high salt cause a sudden rise in calcium levels in plant cells (Mahajan and Tuteja, 2005; Tuteja, 2007).

1.2.5.2 *ABA regulation of seed germination and root growth*

Abscisic acid is one of the extremely important phytohormones that help in the regulation of developmental as well as physiological events which occur in plants (Cutler *et al*., 2010; Fujita *et al*., 2014, 2013, 2011). These events involve seed inactivity, seedling development and growth, and limiting the responses of many biotic stresses (Vishwakarma *et al*., 2017). Some studies have looked at genes involved in these processes. For example, a study by Fujii *et al*. (2007) confirmed that the ABA-insensitive mutants *abi*1 and *abi*2 interfere with some ABA responses, which involves inhibition of growth onset, the process of seedling growth, and support of stomatal closure. On the

other hand, other mutants such as abi3, *abi*4, and *abi*5 only display ABA insensitivity during seed germination and premature seedling development.

Another important process in signalling is protein phosphorylation. The importance of phosphorylation has been identified with the analysis of the triggering of ABA response elements; in other words, ABRE binding factors, also known as ABFs/ABREs. ABFs are transcription factors referred to as basic leucine zipper-type (bZIP), a transcription factor which is involved in ABA-mediated signalling. The proteins encoded by these ABA-responsive genes include proteins that recognise events that protect against attack, enzyme requirement for osmolyte production, or different transcription factors responsible for controlling other changes in gene expression (Bray, 2002; Zhu, 2002).

Although there are some known genes that are concerned with the signalling of ABA, certain significant components are still under study. The signalling process mediated by ABA is believed to be a very branched system. As discussed earlier, SnRK2.6 has been involved in the positive regulation of ABA signalling (section 1.2.4); however, it only completes its function in ABA responses of guard cells (Fujii *et al*., 2007; Mustilli *et al*., 2002). Also, ABI 1 and ABI 2 are negative regulators of the growth of seedlings, seed germination and closure of stomata (Fujii *et al*., 2007). This poses the question; what protein kinases might be present in the positive regulation of ABA signalling during the growth of seedling and beginning of the growth of the seed? *snrk2.2 snrk2.3* plants show insensitivity towards ABA during early seed growth and show that SnRK2.2 and SnRK2.3 are the protein kinases responsible for positively controlling the signalling at the beginning of seed growth. SnRK2.2 and SnRK2.3 are also connected to other roles in seed dormancy, stopping of seedling development, and gene expression

caused by ABA. Many of the ABA-induced genes showing decreased ABA responsiveness in snrk2.2 snrk2.3 mutants are thought to carry ABREs in their promoter area. Therefore, SnRK2.2 and SnRK2.3 are likely to influence the expression of those genes via phosphorylating one or more ABFs and, in that way, influence the binding of ABF to ABRE (Figure 1.6) (Fujii *et al*., 2007).

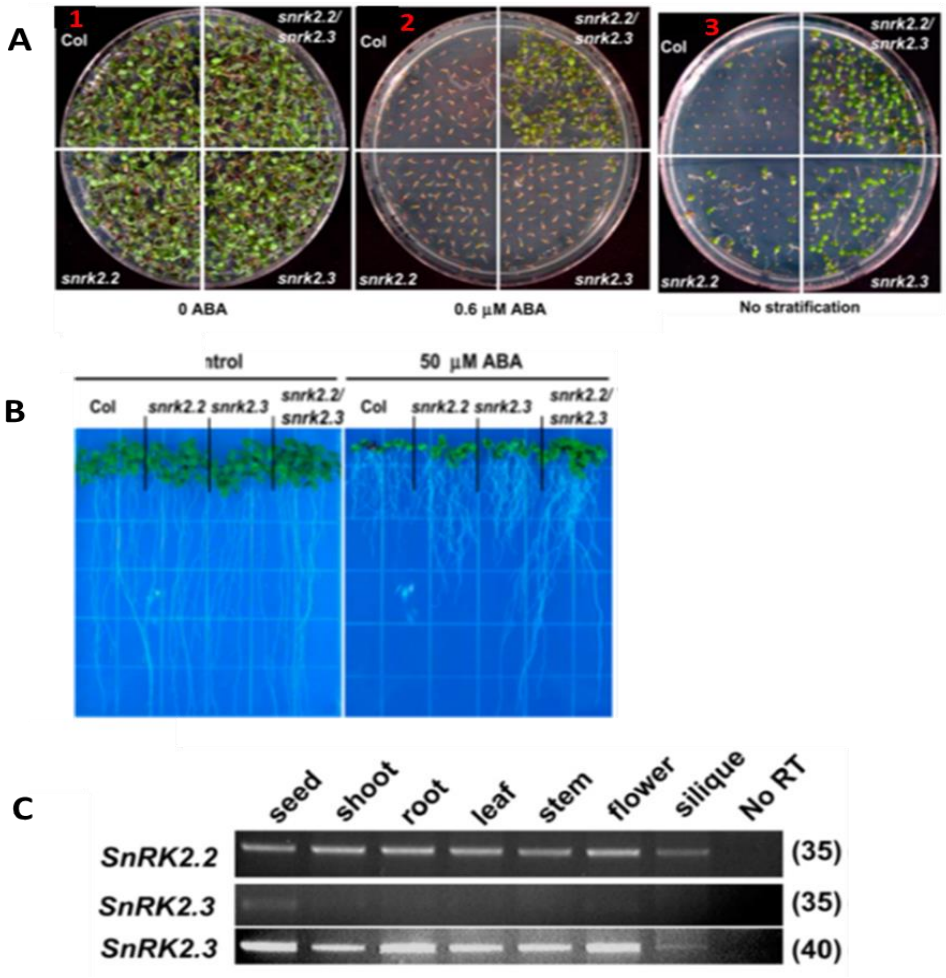


Figure 1.6: The relationship between SnRK2.2 and SnRK2.3 and ABA signalling (taken from Fujii et al., 2007).

Panel (A) Seed Germination and Dormancy Assays.(1) and (2) Photographs of Col-0, snrk2.2, snrk2.3, and snrk2.2 snrk2.3 seedlings on control (Murashige and Skoog [MS]) medium with 3% sucrose (1) or 0.6 mM ABA medium (2) at 9 d after the end of stratification. (3) Germination of nonstratified seeds at 7 d after sowing on MS medium with 3% sucrose. Panel (B)ABA Inhibition of Seedling Growth. Photographs of

seedlings at 14 d after transfer to control medium (MS medium with 3% sucrose) or medium containing 50 mM ABA. Seedlings were 4 d old at the time of transfer and had equal root lengths at that time. Panel (C) Expression of SnRK2.2 and SnRK2.3. RT-PCR analysis of tissue distribution of SnRK2.2 and SnRK2.3 expression. Numbers at right indicate the number of PCR cycles performed.

The continued cycle of reproduction critically depends on controlling the beginning of seed germination and seedling growth. There are some critical checkpoints at the stage of change transition from inactive state to the early growth stage and from the early growth stage until maturation (Kermode, 2005). It was reported that rare earth elements (REEs) show an adverse biological effect on the growth and yield of the plant (Jianrong *et al.*, 2014). However, the point at which these REEs are incorporated in phytohormone ABA signalling is not clear. The interaction of Lanthanum (La^{3+}) occurred with ABA signal in the growth of *Arabidopsis* root (Jianrong *et al*., 2014). Also, the amount of ABA that was used affected this process. For example, when 1µmol/L of ABA was added, the seed germination was stopped, and the root was subjected to elongation in *Arabidopsis*, but when 10 µmol/L of La^{3+} was introduced, the effects produced by ABA were rescued. In addition, root hair development was increased by ABA whereas the same was stopped by La^{3+}. Further, some studies showed that H_2O_2 formation that was caused by ABA was further inhibited by La^{3+} (Jianrong *et al*., 2014). Altogether, the interaction of La^{3+} with ABA may show a close relation with an H_2O_2 signal controlled by La^{3+} in root cells. La^{3+} may antagonistic ABA upstream of H_2O_2 formation.

Different genetic studies on the ABA-mediated regulation of seed germination as well as gene expression have recognised that different mutants of *Arabidopsis* have various sensitivities to ABA (Chen *et al*., 2008). A study by Lopez-Molina, Mongrand and Chua (2001) found that one of the ABA-insensitive mutants, *abi*5, was discovered for its ability to improve plant growth under high levels of exogenous ABA. *ABI*5 codes

for a bZIP transcription factor which, if accumulated, causes initial growth of seedling and inhibition of the seed germination. A study by Carles *et al.* (2002) suggested that ABI5 controls the synthesis of seed-specific ABA, and AtEM genes which code for the class I late embryogenesis (LEA) proteins that are extremely important for seed maturing during drought stress. ABA may also negatively regulate root gravitropism (Han *et al*., 2009) and root hydrotropism (Moriwaki *et al*., 2012), but the underlying signalling mechanisms remain unclear.

1.2.6 ABA and algae

Photosynthetic green algae evolved 1 to 1.5 billion years ago when a eukaryotic heterotroph encapsulated a cyanobacterium which ultimately formed a plastid (Leliaert *et al*., 2012). Many lineages diverged since (Yoon *et al*., 2004), including the chlorophytes and charophytes, the progenitors of terrestrial plants which appeared ≈500 million years ago (Delwiche and Cooper, 2015). The water to earth move must have posed a significant challenge to algae, and environmental differences required the adaptive evolution of protective mechanisms enabling them to become high-light and desiccation tolerant and sessile at the water surface. It is likely such mechanisms derived from those which had already evolved to ensure their survival. For example, Chlamydomonas sp alter their depth depending on light level and quality to attenuate photo-oxidative stress (Hegemann and Berthold, 2009). As protective mechanisms became more active, the depth to which algae needed to descend presumably reduced, enabling them to pass closer into the shoreline. Algal genomes encode primitive synthesis and signalling pathways for the majority of phytohormones, including ABA (Wang *et al*., 2015). In terrestrial plants, ABA plays a role in seed dormancy and germination, stomatal movements and in other responses to many stresses that alter their water status

(Vishwakarma *et al*., 2017). However, its algal role remains poorly defined with only a few papers suggesting its involvement in stress responses such as those resulting from desiccation (Holzinger and Becker, 2015) and salinity (Cowan and Rose, 1991) and in the initiation of the cell cycle (Kobayashi *et al*., 2016; Kobayashi and Tanaka, 2016). ABA treatment has also been shown to increase lipid accumulation in *Chlorella* (Wu *et al*., 2018).

A hundred species of algae have been examined for the presence of ABA, 96% of which contained ABA (Hartung and Gimmler, 1994; Hirsch, Hartung and Gimmler,1989; Jameson 1993). The investigations incorporated the following algal divisions: *Rhodophyta*, *Heterokontophyta*, *Haptophyta*, *Cryptophyta*, *Dinophyta, Euglenophyta, Chlorophyta and Streptophyta*. In the different cases in which ABA could not be distinguished, the negative outcome is likely to be the result of inadequacies in the analytical methods applied (*Porphyra*, Zhang, Yamane and Chapman, 1993; *Fritschiella tuberose*, Tietz and Kasprik 1986; *Caulerpa paspaloides*, Jacobs, 1986; *Enteromorpha compressa*, Niemann and Dörffling,1980). Studies by Hirsch, Hartung and Gimmler (1989) and Yokoya *et al*. (2009) reported that there were more than 12 Brazilian species with positive reports of ABA, including *Rhodophyta*, *Heterokontophyta*, *Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorophyta* and *Streptophyta*. ABA content in algal cells varied between 7 and 34 nmol ABA kg^{-1} FW. These numbers are similar to those of liverwort growing beneath the water (Hartung and Gimmler, 1994) but drastically lower than those of unstressed terrestrial plant life.

The effect of stress on algal ABA concentration has been examined. Salt stress has been applied to *Dunaliella* species (Cowan, Rose and Horne, 1992; Cowan and Rose,

1991; Hirsch, Hartung and Gimmler, 1989) and *Chlorella vulgaris* (Maršálek, Zahradníčková and Hronková, 1992). In all instances, salt stress elevated ABA levels. ABA production was stimulated by using nitrogen deficiency in *Dunaliella sp.* (Tominaga, Takahata and Tominaga, 1993), heat stress in *Chlorella vulgaris* (Bajguz, 2009), drought and acid stress in *Stichococcus bacillaris* and *Chlorella vulgaris* (Maršálek, Zahradníčková and Hronková, 1992), oxidative stress in *Haematococcus pluvialis* (Kobayashi *et al*., 1998,1997), light stress in *Chlorella minutissima* (Stirk *et al*., 2005), and alkaline shock in *Dunaliella acidophila* (Hirsch *et al*., 1989). Compared with cormophytes (10–20-fold increase), the stress-induced increase of ABA biosynthesis of cyanophytes and algae is much weaker (2–5-fold increase). In all instances, stress-induced ABA seems to be released across the plasma membrane to the surrounding medium, and frequently it has been analysed only in the latter. Because of the sturdy ABA efflux, cell ABA levels may not increase appreciably. Any impact of ABA that has been released from the algae to the surrounding environment on aquatic organisms inside the neighbourhood, as suggested by Hussain and Boney (1973), appears to be extraordinarily unlikely due to the fact that it is extremely diluted.

1.2.6.1 *ABA biosynthesis and metabolism in algae*

Incorporation of mevalonolacton into ABA of *Dunaliella parva* was observed by Cowan and Rose (1991); under salt stress condition the incorporation was increased. Moreover, they did inhibitor experiments with fluridone, norflurazon and AMO 1618; compounds which inhibit the formation of those carotenoids that are thought to be essential precursors of ABA. Due to a relatively weak reduction of ABA biosynthesis, they conclude that at least a significant part of ABA is not formed from carotenoids. On the

other hand, in green algae that contain chlorophyll a and b the 90-cis-neoxanthin was found, which is a suitable substrate for ABA synthesis. However, it was not found in other algal divisions such as heterokontophyta, rhodophyta and photoautotrophic prokaryotes, although all these organisms form ABA. Therefore, the latter may synthesise ABA through a direct synthesis pathway via farnesyl-diphosphate.

1.2.6.2 *Effect of ABA on algae*

There have been numerous attempts to elucidate the physiological function of ABA in algae by way of the treatment of algae with external ABA. Most of these experiments have been conducted in a situation of extremely poor understanding of the physiology of ABA in algae (e.g. prevalence, inner concentration, uptake and metabolism, permeabilities of membranes for ABA). Moreover, in most instances, extremely high external ABA concentration have been applied. This significantly excessive concentrations yield outcomes of marginal physiological relevance. Experiments do not deliver a consistent picture (Hartung and Gimmler 1994; Jameson 1993). For example, it has been proven that permeability of membranes to specific solutes became both stimulated in Chara (Ord *et al*., 1977; Wanless *et al*., 1973) or decreased in *Dunaliella* (Hirsch *et al*., 1989) while ion uptake was stimulated and inhibited, respectively. Photosynthesis was not affected in most cases. Stimulation of respiration was observed (Ullrich and Kunz, 1984), as well as its inhibition (Huang, 1991).

A study by Hartung (2010) reported that algal differentiation had been shown to be affected; gametogenesis in *Chlamydomonas* (Ishiura, 1976) and abscission of oospores in *Chara* (Driessche, Petiau-de Vries and Guisset, 1997). ABA promotes a growth inside the cyanobacteria Nostoc and Anacystis (Ahmad *et al*., 1978) and inhibits

growth in Coscinodiscus (Kentzer and Mazur, 1991; Hirsch *et al*., 1989). It was speculated that within algae, a high correlation might also exist between ABA content and the level of differentiation.

Moreover, a study by Tanaka, Ikeda and Miyasaka (2004) showed that halotolerant *Chlamydomonas* strains express LEA genes (cw80 Lea3), stimulated by salt and cold stresses, however not associated with ABA. This indicates that despite the fact that ABA is present and may be induced by stress, a clear physiological response (e.g. in gene expression patterns) could not be determined yet.

ABA was confirmed to have a protective effect against oxidative damage caused by salt and osmotic stress in the same species such as *Chlamydomonas reinhardtii* (Yoshida, 2005; Yoshida *et al*., 2004, 2003). When cells were treated with ABA, catalase and ascorbate peroxidase activities were shown to be significantly higher (Yoshida *et al*., 2003). ABA also protects *Haematococcus pluvialis* against oxidative stress, it may be necessary for the mitigation of oxidative damage in stressed algae (Kobayashi *et al*., 1998, 1997). Additional research is required in this field to address this issue fully.

ABA plays a role in the gravitropic/gravitaxis response of algae that is modulated by exposure to light. According to Fujii *et al*. (2009) there are no orthologues of the genes encoding the plant cytosol/nuclear localised PYR/PYL/RCAR receptor family in the *Chlamydomonas reinhardtii* genome. On the other hand, there are potential orthologues of genes encoding the G-protein coupled receptor (GPCR) protein, GCR2 (Liu *et al*., 2007) and GPCR-types, GTG1 and GTG2 (Pandey *et al*., 2009) in the genome of this alga. It is still unclear whether or not these proteins act as ABA receptors (Christmann and Grill, 2009; Guo *et al*., 2011; Risk *et al*., 2009). Thus, it will be important to determine the mechanisms through which algae such as *Chlamydomonas* perceive ABA

and mechanistically how its perception interacts with light signalling to determine both the orientation and direction of movement.

There is a connection between abscisic acid (ABA) and the expression and function of glycine-rich RNA-binding proteins (class IV GRPs) in plants such as *Arabidopsis thaliana.* Sequencing the *C. reihardtii* genome has suggested that there is only a single GRP gene, termed *Cr*GRP1. Now, the attention is focused on the GRPs.

1.3 The class IV glycine-rich RNA-binding proteins (GRPs)

1.3.1 Structural properties of class IV GRPs

In general, plants contain a high level of glycine (20 % up to 70 %). The reason is thought to be the presence of abundant glycine-rich proteins (GRPs) (Condit and Meaher, 1986). Currently, four classes of GRPs have been categorised. The first class of GRPs has a signal peptide followed by a glycine-rich domain, and it consists of (GIyGIyX) n repeats. Most of the GRPs in a signal peptide class have a cell-wall localisation, a structure-function (Cassab, 1998). The second class of GRPs has a glycine-rich domain which is followed by a C-terminal cysteine-rich region which involves (Gly Gly XXX Gly Gly) n repeats while the third class of GRPs has the lowest level of glycine when compared to other classes. They are characterised by the presence of (GlyXGlyx) repeats and by a higher degree of structural diversity. Both class II and III GRPs can also include a signal peptide, commonly at the amino terminus. In contrast, the defining feature of class IV GRPs is that they include nucleic acid binding domains (Figure 1.7; Kar, Nayak and Joshi, 2012).

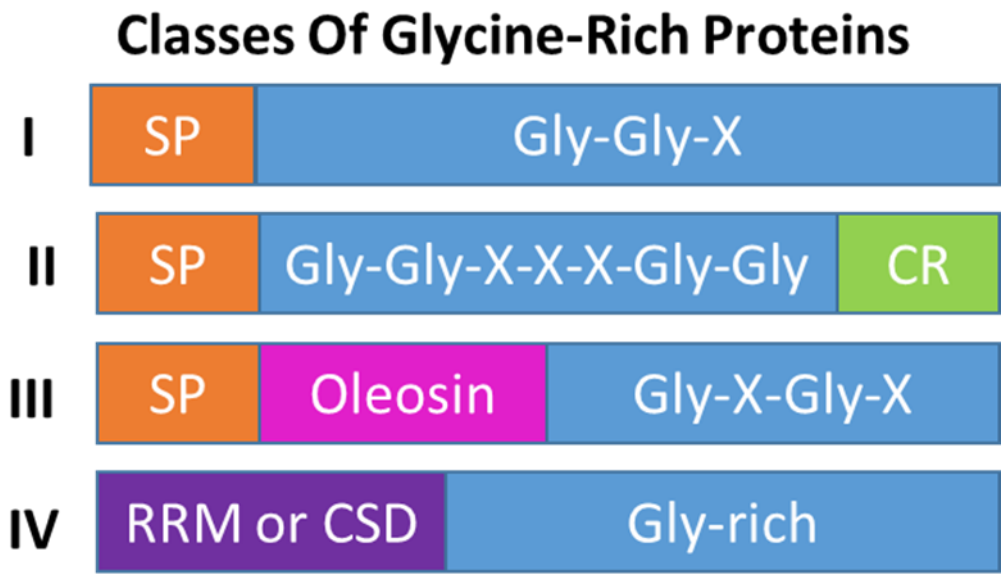


Figure 1.7: The structures for the four main classes of GRPs.
SP, signal peptide; CR, cysteine-rich; Gly, glycine; Oleosin, domain conserved in oleosin (adapted from Ciuzan *et al*., 2015).

RNA Recognition Motif (RRM) and Cold Shock Domain (CSD) are the two types of RNA-binding domains contained in class IV GRPs. A review by Ciuzan *et al*. (2015) describes these as type A and type B of class IV GRPs respectively. In *Arabidopsis thaliana*, the most studied GRPs, *At*GRP7 and *At*GRP8, are type A, whereas *At*GRP2 is type B. The class IV proteins are a distinct subgroup within the very heterogenous superfamily of glycine-rich proteins (GRPs), exemplified by the well-studied *Arabidopsis thaliana* protein *At*GRP7 (Ciuzan *et al*., 2015; Schmidt *et al*., 2010).

RRMs are found in a wide range of RNA-binding proteins including small nuclear ribonucleoprotein (snRNP) associated proteins, chloroplast RNA binding proteins, poly(A)-binding protein, heterogeneous RNPs and SR proteins (splice factors with serine–arginine-rich domains; Lunde *et al*., 2007). RNP-1 and RNP-2 are two of the conserved motifs in RRMs. RNP-1 contain eight amino acid residues with consensus

(K/R)G(F/Y)(G/A)FVX(F/Y). RNP-2 consists of a six amino acid consensus (L/I)(F/Y)(V/I)(G/K)(G/N)L. RNA-binding proteins that have RRMs are involved in pre-mRNA processing such as capping, polyadenylation and splicing (Hall, 2002). The cold shock domain (CSD) can bind both DNA and RNA. Cold-shock proteins (CSPs) is the name that derived from a conserved family of small prokaryotic cold-shock proteins. These proteins contain a CSD which is similar to an RRM and described as a beta-barrel structure with conserved protruding basic and aromatic residues required for RNA binding. A study by Sachs *et al*. (2012) reported that CSPs contain a single CSD and are thought to contribute to cold adaptation by working as RNA chaperones. Transcription factors contain a CSD in eukaryotes. A single CSD is present in the eukaryotic Y-box transcription factors. However up to five CSD have been documented in the protein UNR, an RNA-binding protein found in mRNA turnover (Doniger *et al*., 1992). The CSD in the Y-box proteins is required for their DNA-binding activity, and it also binds RNA and allows Y-box proteins to repress mRNA translation in both somatic cells and germline cells (Sommerville and Ladomery, 1996). In plants, the CSD is present in different proteins often in association with glycine-rich domains (Sasaki and Imai, 2012). According to Manival *et al*. (2001) both the RRM and the CSD bind single-stranded RNA.

1.3.2 Functional conservation of class IV GRPs

Research increasingly suggests that the class IV GRPs are involved in adapting to stress, mainly because of their increased expression after exposure to cold, abscisic acid (ABA) treatment, salicylic acid treatment, wounding or water stress. The following example illustrates this point. MhGR-RBP1 is a class IVa GRPs from *Malus hupehensis* (apple) with a high level of sequence identity with other GRPs such as from *Malus prunifolia*

(*Mp*RBP1 - 96%), *Oryza sativa* (*Os*GRP3 - 81%) and *A. thaliana* (*At*GRP7 - 80%; *At*GRP8 - 75%). MhGR-RBP1 is highly expressed in leaves compared with shoots and roots. Expression levels of MhGR-RBP1 increases when trees are subjected to drought, high salt, H_2O_2 and wounding stress. In contrast, when trees are treated with ABA for 24 hours the expression of MhGR-RBP1 decreases (Wang *et al*., 2012). Thus, the class IV GRPs may be helping apple trees adapt to surrounding or adverse environmental conditions.

At least six class IV GRPs are present in the rice genome (*Os*GRP1 to *Os*GRP6). Cold stress increases the expression of all six by more than double (except for *Os*GRP3). *Os*GRP1 and *Os*GRP6 are able to restore cold modification to a sensitive *Escherichia coli* mutant strain, confirming the conserved functional protection of these proteins (Kim *et al*., 2010). Kim *et al*. (2010) went on to examine the ability of *Os*GRPs to complement the cold sensitivity of an *Arabidopsis atgrp7* mutant, a class IVa GRP. They created plants that ectopically expressed *Os*GRP1, *Os*GRP4 and *Os*GRP6. *Os*GRP1 and *Os*GRP4 rescued the sensitivity to cold of the *atgrp7* mutant, whereas freezing tolerance was provided by OsGRP6 (Kim *et al*., 2010). Yang *et al*. (2014) created transgenic rice plants that express *At*GRP7 and *At*GRP2. The result was improved grain production under drought stress conditions, in addition to high preservation of plants, and also showed striking sequence homology between plant class IV GRPs and vertebrates.

Cold-inducible RNA-binding protein A (CIRP-A) is a class IV GRP found in *Xenopus laevis*. This protein has shown increased levels of expression when the liver and brain are exposed to lower temperatures. Its expression in the brain of *Xenopus* is controlled in a circadian fashion (Saito *et al*., 2000). This has become of interest since it is known that *At*GRP7 expression is also controlled in a similar manner (Carpenter *et al*.,

1994). CIRP-A plays a role in the migration and differentiation of cells of the pronephros during embryonic development; in this context, its functions are not restricted to cold adaptation (Peng *et al*., 2006).

Mammalian RNA-binding protein 3 (RBM3) is very much alike in sequence to CIRP-A and *At*GRP7. Its expression is caused by dangerously low body temperatures and low oxygen tension (Chip *et al*., 2011). RBM3 is highly expressed in the liver and muscles of the bear *Ursus americanus* during winter hibernation. Its expression increases up to 7.28-fold in several tissues. RBM3 is the most upregulated protein during hibernation over the winter (Fedorov *et al*., 2009). Therefore, RBM3, a mammalian protein, appears to be involved in cold adaptation, like the class IV plant GRPs is involved in plant cold adaptation.

RBM3 is involved in the regulation of protein translation in response to cold. It is not yet clear how this regulation occurs. One possible way could be through direct interaction with ribosomes (Dresios *et al*., 2005). Consistent with this suggestion, RBM3 associates with the 60S ribosomal subunit in an RNA-independent manner. Another possibility is that RBM3 interacts with microRNAs (small non-coding RNAs that can downregulate translation by binding target mRNAs). In favour of the microRNA interaction hypothesis, one study observed the levels of a small complex (<40S) that contain micro RNAs were reduced in cells that over-express RBM3 (Dresios *et al*., 2005). Because of the similarity of RBM3 and plant class IV GRPs, it is tempting to suggest that *At*GRP7 might also affect translation and microRNA function, but this remains to be examined.

RBM3 expression also increases in hypoxia. The upregulation of RBM3 is thought to happen independently of hypoxia-inducible factor 1 (HIF-1) (Wellmann *et al*., 2010). The mechanisms through which RBM3 helps cells adapt to hypoxic conditions are not yet known. RBM3 is required for cell growth and proliferation and protects cells from cell death caused by serum starvation (Wellmann *et al*., 2010), further suggesting that it might even be part of a new generation of RNA-binding proto-oncogenes (Lleonart, 2010).

1.3.3 Role of class IV GRPs in stress adaptation

In *A. thaliana*, *At*GRP7 appears to play a key role in the regulation of ABA and stress responses. In plants, ABA is involved in stress, water, cold and heat responses. In addition, ABA is involved in specific physiological events, such as stomatal opening and closing in the guard cells of *A. thaliana* (Kim *et al*., 2008). The expression of *At*GRP7 in *A. thaliana* was repressed when treated with ABA, high salt and mannitol concentrations (Cao *et al*., 2006). Furthermore, when comparing the transcript levels of two stress-induced genes *RD29A* and *RAB18* with a wild-type plant, the transcript levels were higher in the *atgrp7* knockout plants than with wild-type plants. Evidence suggests that AtGRP7 is involved in the regulation of ABA-induced and stress-induced genes (Cao *et al*., 2006). Kim *et al*. (2008) extended these findings, and they reported that after using another type of *At*GRP7 knock-out, the mutants were able to germinate and survive on both media with high concentrations of NaCl and with mannitol in comparison to either wild-type or over-expressed plants (Kim *et al*., 2008). Another study by Kim *et al*. (2007b) found that seed germination was affected by *At*GRP2 (a type B class IV GRP) under salt stress but not osmotic stress. Either the presence or absence of *At*GRP2 did not influence the seedling growth and plant development, thus suggesting that *At*GRP2 is

independent of the ABA pathway. The evidence indicated that *At*GRP2 and *At*GRP7 are involved in response to similar abiotic stresses, but their function may be antagonising each other (Figure1.8).

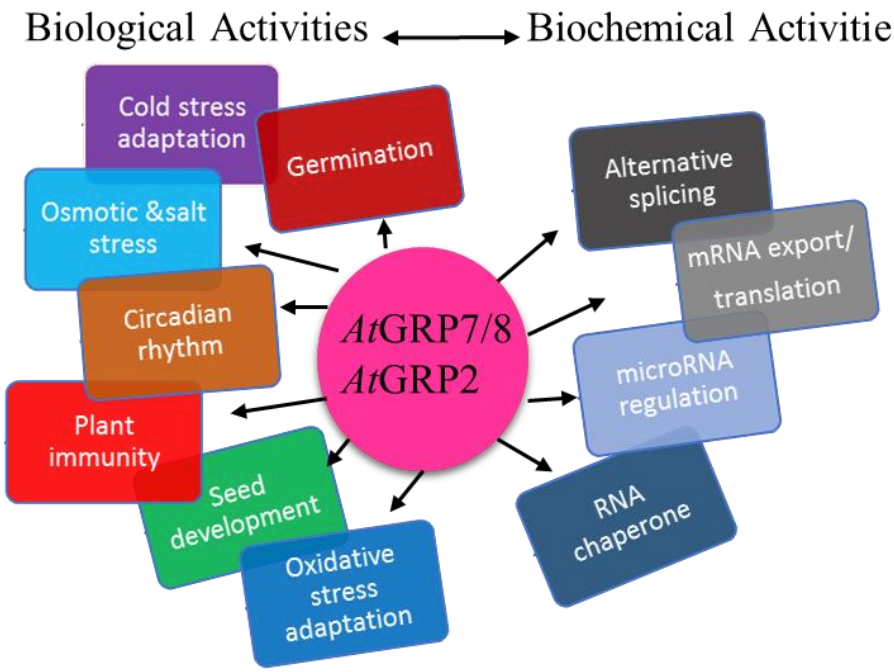


Figure 1.8: Biological activities of Arabidopsis thaliana GRPs (at GRP7 and GRP8 containing RRMs) and (at GRP2 containing a CSD) (adapted from Ciuzan et al., 2015).

Initial analysis of its sequenced genome suggests that there is only one member of the class IV GRPs in *C. reinhardtii*. It is called *CrGRP1*, and it will be described in detail in Chapter 4. It is hypothesised that class IV GRPs are equally important for the ability of *C. reinhardtii* to adapt to stress, intersecting with ABA-regulated pathways. The class IV glycine-rich proteins are likely to function by regulating gene expression at multiple levels on their mRNA targets by modifying alternative splicing, mRNA export, mRNA translation, and mRNA degradation.

1.4 Hypothesis and Aims

1.4.1 Hypothesis

ABA is one of the essential phytohormones required for the regulation of developmental as well as physiological events occurring in plants. Such events involve seed dormancy, seedling development and growth. In parallel, the class IV GRP proteins play critical roles in the adaptation to biotic and abiotic stresses that include pathogens, osmotic and salt stress, oxidative stress, and cold stress in high plants such as *Arabidopsis thaliana*. I hypothesized that ABA and GRPs also play a significant role in the adaptation to biotic and abiotic stresses in *C. reinhardtii*. Long term, it may be possible to manipulate GRPs to improve the use *of C. reinhardtii* in biotechnological applications, for example as a potential biofuel.

1.4.2 Aims

1- To investigate the role of plant hormones and particularly ABA in regulating the movement of *C. reinhardtii* in response to stress by pursuing the objectives:

- To explore the effect of plant hormones on the growth rate of *C. reinhardtii*.
- To explore the effect of plant hormones in the movement of *C. reinhardtii* in response to stress.
- To explore the effect of ABA in the movement of *C. reinhardtii* during circadian rhythm. Then, measuring ABA concertation through circadian rhythm as well.

2- To investigate the expression of the class IVa GRP *CrGRP1* in stress adaptation in *C. reinhardtii* by following the objectives:

- Characterise class IV in *C. reinhardtii*
- Determine the structure of *CrGRP1* gene

• Examine the expression of *CrGRP1* during the Circadian cycle with and without exposure to the phytohormone ABA. Then, examine the expression of *CrGRP1* in response to abiotic stresses such as H_2O_2-induced oxidative stress and changes to light conditions during the circadian rhythm.

• Examine the expression of *CrGRP1* following light-regulated movements of *C. reinhardtii* after treatment with ABA.

3- To investigate the expression and function of the class IVa GRP *CrGRP1* in stress adaptation in *C. reinhardtii* in order to:

• Determine the effect of overexpression and knock-down of *CrGRP1*.

• Characterise the phenotype and effect on target RNA.

• Examine the effect on the mobilisation of hormone responses.

2 Chapter Two: General Methodology

2.1 Sources of reagents

All reagents and analytical grade chemicals were obtained from Sigma-Aldrich Co. Limited. Agarose was supplied by Bio Line Limited. Agar for bacteria was obtained from Sigma and algal agar from Melford Laboratories Limited. Restriction enzymes and DNA-modifying enzymes were purchased from New England Bio Labs UK Limited. DNA molecular weight markers were obtained from Bio Line Limited. And Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.4cm gap, were purchased from Bio-Rad Laboratories Limited.

2.2 Bacterial strains, gene construct and plasmids

Escherichia coli is a non-photosynthetic bacterium that is commonly found in the lower intestine of warm-blooded animals and is widely used in genetic studies. Some strains possess flagella and are motile, whereas other strains lack flagella. TOP10 chemically competent *E. coli* cells (TOP10 cells; Invitrogen, Thermo Fisher Scientific) were purchased frozen and stored at -80°C to be used as hosts for most *E. coli* plasmids.

2.3 Algal strains, culture conditions, and stress treatments

C. reinhardtii wild - type strain CC1021 with cell wall (mt+) and CC849 without cell wall (mt) were obtained from Dr Man-Kim Cheung, the University of the West of England, Bristol. Wild - type strain 137 C with cell wall were bought from the *Chlamydomonas* Center (http://www.chlamy.org/). All *C. reinhardtii* strains used in this study were grown on TAP agar plates. For confocal experiments and RNA isolation,

algae were grown at 25 ºC, 16 h photoperiod, 133 rpm, 980 Lux in TAP media (2.42 g Tris base ,12.5 ml solution #1 (40 X FBS)), 5 ml solution #2 (phosphate (KPO_4), 5 ml solution #3 (trace mineral), then adjusted to pH 7.2 with diluted 1/10 glacial acetic acid to make up to 1 liter with distilled water and then autoclaved (see Appendix 8.1 to prepare the solutions #1, 2 and 3). Cultures were grown until A_{750} = 0.3 which is a mid-log phase. Growth was measured by following A_{750} nm. For protein and RNA extraction cells were collected by centrifugation at 4 °C for 10 min at 2500rpm (Beckman Coulter Allegra x 22r) before being stored at -80ºC.

For growth assays, *C. reinhardtii* cultures were grown as described above. Plant hormones such as Abscisic acid ABA (dissolving 2.64 mg of ABA (Sigma) in 1 ml of 100% (v/v) ethanol to give 10 mM-final concentration), 1-aminocyclopropane-1-carboxylic acid (ACC; dissolving 1.0 mg in 1 ml of water giving a 10 mM final concentration), indole-3-acetic acid (IAA; dissolving 1.75 mg in 1 ml of 95 % (v/v) ethanol, giving a 10mM final concentration) and 1-naphthaleneacetic acid (NAA; dissolving 1.86 mg (Sigma) in 1 ml of 1N NaOH giving a 10 mM final concentration) were added at various concentrations. The growth of the cultures was monitored over a 48h period by measuring A_{750} nm.

For stress treatments, *C. reinhardtii* wild - type strain CC1021 (mt+) algae were grown as described above and then cells were used for different stress treatments. For hydrogen peroxide (H_2O_2) stress treatment, the cells were directly cultured in TAP medium with H_2O_2 added in various concentrations (0.1µM, 1µM, 10µM, 100µM, 10mM, and 1mM). Cells were harvested and centrifuged at 4°C for 10 min at 2500 rpm. and then the cell pellets were stored at -80°C for RNA extraction.

Another stress was altering light conditions through the light/dark cycle. The culture was grown as described above and then the light condition was changed to 8h light and 16h night. Cells were harvested every two hours and centrifuged at 4°C for 10 min at 2500 rpm. Cell pellets were stored at -80°C for RNA extraction.

2.4 Isolation of RNA from C. reinhardtii culture

Total RNA was extracted from the mid-log phase of strain CC1021 of *C. reinhardtii*, using Trizol reagent (Sigma-Aldrich). Culture (25 ml) at 0.3 A_{750} were centrifuged at 4°C for 10 min at 2500 rpm, and the pellet was ground separately to a fine powder using a pestle and mortar and homogenised in 1ml of Trizol reagent. Samples were vortexed for five minutes and incubated at room temperature for five minutes. 0.2 ml of chloroform was added to the samples and tubes were shaken vigorously by hand for 15 seconds and incubated at room temperature for five minutes. Samples were centrifuged at 12000 rpm for 3-4 minutes (Fisher Scientific Centrifuge). The clear aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ml of isopropanol and incubated in liquid nitrogen for five minutes and then at room temperature for ten minutes. Samples were then centrifuged at 12000 rpm for 15-20 minutes (Fisher Scientific centrifuge). The supernatant was removed, and the pellet was washed with 200 µl of 80% ethanol. The tubes were centrifuged at 7500 rpm (a Fisher Scientific Centrifuge) for five minutes. The final RNA pellet was dried by leaving it at room temperature for five minutes and then it was dissolved in 20 µl of RNase- free water. The RNA concentration was then quantified using a Nanodrop (Thermo Scientific) and then stored at -80°C until further use.

2.5 Quantification of nucleic acids

Sample concentrations were determined by using a Nanodrop (Thermo Scientific). Samples (1.5 µl) were used for quantification. Concentrations were determined by measuring the absorbance at 260 nm as calculated based on the absorbance measured at 260nm, ×40 for RNA or ×50 for DNA. The samples were checked for purity by measuring the absorbance ratio at 260nm/280nm for any protein contamination and 260nm/230nm for any solvent and salt contamination.

2.6 Analysis of RNA samples by agarose gel electrophoresis

Agarose gels (1.2 & 2% w/v) were used to analyse the quality of the RNA or DNA samples (Sambrook *et al*., 1989). Agarose was mixed with 1X TAE buffer (10 mM Tris-Acetate, 0.5 mM EDTA, pH adjusted to 7.8.) and melted in a microwave oven. The mixture was cooled to 60°C, 3 µl EtBr (10 µg/ml) was added, and then it was mixed gently to avoid bubble formation. The mixture was poured into ae gel mould containing suitable well combs and was allowed to solidify. A 1X TAE buffer was poured into the gel buffer reservoir in the electrophoresis apparatus to about 2mm above the gel surface. RNA or DNA samples were mixed with a loading dye at a ratio of 5:1. The contents were loaded in respective gel slots and electrophoresis was carried out at 90 V for 45 minutes. After the run, the gel was visualised on a UV transilluminator (Alpha Innotech) with an Alpha Innotech camera.

2.7 DNase treatment of RNA

Removal of genomic DNA contamination from RNA samples was done by adding two units of DNase I and 5 µl of DNase I Reaction Buffer (10X) (New England Bio-labs) to

10 µg of RNA, making up a final volume of 50 µl which was incubated at 30 °C for 10 minutes. Finally, the DNAse was heat inactivated at 55 °C for five minutes. The RNA quality was checked by agarose gel electrophoresis, as described above (see Section 2.6).

2.8 Reverse transcription

First strand complementary DNA (cDNA) was synthesised from 5 µg of total RNA in a total reaction volume of 25 µl. RNA was mixed with 4 µl of 50 µM anchored oligo(dT) primer and spiked with 3 ng of human Tumour Necrosis Receptor Associating Factor 1 transcript (TRAF) in 3.25 µl of sterile distilled water and incubated at 70 °C for 10 minutes. Following that, it was kept on ice for a few seconds, then microfuged briefly and allowed to anneal at room temperature for five minutes. 2.5 µl of 10 X RT buffer, 1.25 µl of 20 mM dNTPs mix and 1µl of RNAse inhibitor enzyme were added and microfuged briefly. 1µl of Superscript III reverse transcriptase (200 U/µl, Invitrogen) was added to the reaction mixture, briefly mixed, and then incubated at 42 °C for two and half hours. The reverse transcriptase was heat inactivated at 65 °C for 10 minutes. Finally, the cDNA synthesis reaction was diluted ten times with sterile distilled water and used for RT-PCR. A negative control was included without RNA (template control) and RT (enzyme control).

2.9 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a PTC200 Peltier Thermocycler (MJ Research). Genomic DNA or cDNA was used as a template. The PCR products were analysed by agarose gel electrophoresis (see section 2.6).

2.9.1 Primer design

All primer pairs were aligned with the *C. reinhardtii* genome sequence from the NCBI database using the nucleotide BLAST search engine to determine their uniqueness. The chosen primers were synthesised by Eurofins Genomics, Germany (Table 2-1).

Primer Name	Forward primer (5' - 3')	Primer Name	Reverse primer (5' - 3')	Fragment size
E1F	CGGAACCATGTCGACCAAGC	E2R	CGAACTTGCTGAACAGGTTCG	119bp
E1F	CGGAACCATGTCGACCAAGC	E3R	GAGCAGGCGCTCTTGGCAGCG	224bp
E1F	CGGAACCATGTCGACCAAGC	E72R	TTACAGCCATAGTAGAGAGCCG	2500kb
E72F	CTGGAATTACGAATACGTGTGG	E72R	TTACAGCCATAGTAGAGAGCCG	226bp
CBLPF	ATGACCACCAACCCCATCATC	CBLPR	GGTCCCACAGCATGGCAATG	187bp
ROC15**	GCCGGTCCAGCAACAACAAC	ROC15	GTGTGCATGCGCCAGAAGAG	142bp
Zeocin_F1	GCCATGACCGAGATCGGCGAGCAGC	UTR_R1	CAAGGCTCAGATCAACGAGCGCCTC	
FMDV2A_ F1	GGTGAAGCAGACCCTGAACTTCGAC	UTR_R2	GATCAACGAGCGCCTCCATTTACAC	

Table 2-1: Oligonucleotide primers
***European Molecular Biology Laboratory (EMBL)*

2.9.2 Standard Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) mixture of 50µl contained: 1µl of diluted first strand cDNA solution, 1µl of forward primer, 1µl reverse primer (Eurofins MWG Operon, EBersberg, Germant) (see Table 2.1), 1.25µl of 20mM dNTP mix, 5µl of 10X PCR buffer, 2µl of 25mM $MgCl_2$, 0.5 Units of Taq DNA polymerase and 38.25µl of sterile distilled water. Samples were aspirated to mix and incubated under the following conditions in a thermocycler (A Bio-Rad PTC-200, Bio-Rad Laboratories Limited): 95°C initial denaturation for one minute, 50-60°C annealing for one minute, (exact temperatures were dependent on average melting temperature for each primer), 72°C extension for teo minutes repeated ×34-35, followed by a 72°C final extension for five minutes. During these incubation steps, samples were also heated above to prevent

evaporation. The amplified products were mixed with a loading dye at a ratio of 5:1 and 20µl of PCR amplified product was electrophoresed on 2% (w/v) agarose gel with 10 mg/ml ethidium bromide alongside DNA molecular markers and visualised under a transilluminator.

2.10 Extraction of DNA from agarose gels

The DNA fragment was cut from the agarose gel with a sharp, clean scalpel. The size of the gel slice was minimised by removing extra agarose. The gel slice was then weighed in an Eppendorf tube. Three volumes of Buffer QG (Qiagen) was added to one volume of gel (100 mg ~ 100 µl) and incubated at 50 °C for ten minutes (or until the gel slice had completely dissolved). After the gel slice had dissolved completely, one gel volume of isopropanol was added to the sample and mixed. Next, a QIAquick spin column was placed in a provided 2ml collection tube. Following that, 0.5 ml of Buffer QG was added to the QIAquick column and centrifuged at 20,000 ×g for one minute. For the wash step, 0.75 ml of Buffer PE was added to the QIAquick column and centrifuged at 20,000 ×g for one minute. The column was placed in a clean 1.5 ml microcentrifuge tube. Finally, to elute DNA, 50 µl of H_2O was added to the centre of the QIAquick membrane and the column was centrifuged for 1 min. The purification of DNA fragments was verified on a 1.5 % agarose gel by the addition of two volumes of dye into five volumes of purified DNA. Confirmation of extraction of the desired product was verified by sequencing both strands of the fragments using Eurofins MWG Operon sequencing service (Germany).

2.11 Plasmid DNA extraction from E. coli

Plasmid DNA was extracted using the alkaline lysis method with PEG purification. 200 ml of the culture of *E. coli* cells containing the plasmid (with or without a histidine tag and the antisense) in a suitable medium, such as LB containing the 70 % of ampicillin, was grown overnight at 37 °C. The following day, cultures were centrifuged (1000 xg, 4 °C, 15 min). The pellet was allowed to drain for a few minutes and the remaining drops of liquid removed with a pipette. Then, the cells were thoroughly swirled in 25 ml of ice-cold solution I (50 mM D [+]-glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA) to produce a cellular suspension. 50ml of freshly prepared, room temperature solution II (0.2 M NaOH, 1% (w/v) SDS) was added carefully by pouring it down the inside of the tube. Tightly capped, the tube was mixed with a mild end-to-end action to lyse the cells (N.B. not shaken or mixed vigorously but ensuring that all the inner surfaces of the tube came into contact with the solution). 7.5 ml of freshly prepared, ice-cold solution III (prepared by mixing 60ml of 5M potassium acetate with 11.5ml of glacial acetic acid and 28.5 ml of SDW) was added to the clear cell lysate. Tightly capped, the tube was remixed thoroughly, but gently with a similar end-over-end action and then placed on ice for 20 minutes. The suspensions were clarified by centrifugation (10,000 xg, 4°C, 15 min) and the clear supernatant filtered through a plug of glass wool in a filter funnel into a new 500ml tube/bucket.

After that, 250 ml of –20 °C ethanol was added, mixed well and then placed on ice for 15 min to allow efficient precipitation of material to occur. The precipitated material was pelleted by centrifugation (10,000 xg, 4 °C, and 30 min) and the supernatant

discarded. The inverted tube was allowed to drain for a few minutes, and the traces of ethanol were removed with a pipette. The pelleted material was then dissolved in exactly 10ml of SDW, and the solution was transferred to a 50 ml oak ridge centrifuge tube with a cap. Then, 2 grams of solid, A.R. grade ammonium acetate was added to the solution and dissolved by gently pipetting up and down (N.B. at this stage a fine precipitate of *E. coli* cell wall carbohydrates formed as the ammonium ions complex with these to form insoluble macromolecules). The suspensions were clarified by centrifugation (10,000 xg, 4 °C, and 5 min) and then the supernatant was transferred to a new 50 ml tube and 25 ml –20 °C ethanol was added, mixed well and placed on ice for 15 minutes to allow efficient precipitation of material to occur. Next, the precipitated material was pelleted by centrifugation (10,000 xg, 4 °C, 20 min), the supernatant was removed, and the pellet was completely dissolved in 4.5 ml of SDW with 0.5 ml of 5 M NaCl. 5 ml of phenol/chloroform (1:1) was added and then, tightly capped, the tube was shaken thoroughly, and the phases were separated by centrifugation (10,000 xg, 21 °C, 10 min).

The upper aqueous layer was transferred to a new 50 ml tube, and 12.5 ml of –20 °C ethanol was added, mixed well and placed on ice for 15 minutes. The precipitated nucleic acids were pelleted by centrifugation (10,000 xg, 4 °C, and 20 min) and the supernatant discarded. The inverted tube was allowed to drain for a few minutes and the last traces of ethanol were removed with a pipette. The pellets were dissolved in 200 µl of 10 µg ml-1 DNase-free, calf pancreatic RNase A solution and then transferred to a 1.5 ml Eppendorf tube. The tube was incubated at room temperature for 20 min for the RNA in the solution to be completely degraded. 15µl of 5M potassium acetate pH 5.5 and 800 µl of ethanol were added and mixed well for 10 min.

The pelleted plasmid DNA was completely dissolved in 140 µl of SDW and the solution was transferred to a new Eppendorf tube. 20 µl of 5 M NaCl and 40 µl of 50 % (w/v) PEG 8000 were added to the plasmid solution and mixed well by vortexing for a few seconds, before the tube was placed on ice for 15 minutes. The precipitated pellet of plasmid DNA was centrifuged for 10 min and then all the supernatant was removed carefully and the pellet was dissolved in 200 µl of SDW. The plasmid solution was sequentially extracted by shaking with a micro-centrifugation partition with TE pH 8.0 buffered phenol, phenol/chloroform (1:1) and, finally, chloroform.

The final upper aqueous phases were transferred to a new Eppendorf tube and the plasmid DNA precipitated by the addition of 15 µl of 5 M potassium acetate pH 5.5 and 1.0 ml of ethanol. The precipitated plasmid DNA was collected by microcentrifugation (13,000 rpm, room temp., and 10 min) and the supernatant was removed. The pellet was washed by adding 500 µl of 80 % (v/v) ethanol and microcentrifugation (13,000 rpm, room temp, 10 mins). The 80 % (v/v) ethanol was removed by washing and drying the plasmid DNA pellet under vacuum. Finally, the plasmid DNA pellet was dissolved in 50µl of SDW and stored at –20 ℃.

2.12 Restriction digests

Restriction digests were carried out in a total volume of 50 µL. The reaction mixture contained the appropriate reaction buffer (1X) and the restriction enzymes (10,000 U) to restrict up to 1 µg of DNA. The reaction mixture was incubated at 37˚C overnight. The following day, 50 µL of water was added. Then, 300 µL of Buffer QG (Qiagen) was added followed by 100 µl isopropanol. The solution was then mixed. Next, a QIAquick

spin column was placed in a 2 ml collection tube and centrifuged for one minute at (10,000 xg, 4 °C). 0.5 ml of Buffer QG was then added again to the QIAquick column and centrifuged for one minute at (10,000 xg, 4 °C). For the wash step, 0.75 ml of Buffer PE was added to the QIAquick column and centrifuged for one minute at (10,000 xg, 4 °C). The column was placed into a clean 1.5 ml microcentrifuge tube. Finally, to elute DNA, 20 µl of SDW was added to the centre of the QIAquick membrane and the column was centrifuged for one minute at maximum speed. The purification of DNA fragments was verified on 1.5 % agarose gel was performed by the addition of two volumes of dye into five volumes of purified DNA.

2.13 Movement assays

For the movement assays, the *C. reinhardtii* wild - type strain CC1021 (mt+) algae were grown as described above (see Section 2.3).

2.13.1 Measuring the motility of *C.reinhardtii* with different treatments

25 ml aliquots were transferred to a glass measuring cylinder and either 125 µl of ethanol (control) or 125 µl of (10mM H_2O_2, ACC and NAA) 100 % (v/v) ethanol was added. There were four tubes for each light and dark experiment; 25 ml of culture in the light (control), another 25 ml in the dark (control), 25 ml of culture treated with 50 µM of (H_2O_2, ACC and NAA) in the light, and the last 25ml of culture treated with 50 µM of (H_2O_2, ACC and NAA) in the dark. The cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the cylinders were photographed and then the A_{750} of the culture was measured by spectrophotometry at various depths from the surface by taking 1 ml of culture.

2.13.2 Measuring the motility of *C.reinhardtii* following ABA and ACC treatments

The following assay was with 50 µM ABA and a different concentration of ethylene (ACC) in (light-dark) experiments. 25 ml of mid-exponentially growing cells were harvested as described above. There were eight tubes for these experiments: 25 ml of culture in the light, another 25 ml in the dark, 25 ml of culture treated with 50 µM ABA plus 5 µM ACC, 10 µM ACC and 50 µM ACC in the light condition, and the remaining 25 ml of cultures were treated with 50 µM of ABA plus, 5µM ACC, 10 µM ACC and 50 µM ACC in the dark condition. The cylinders were then placed at 25 ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the A_{750} of the culture defined at various depths from the surface and the cylinders was photographed.

Finally, 50 µM ethylene (ACC) and different concentrations of ABA treatment in (light-dark)) experiments .25 ml of mid-exponentially growing cells were harvested as described above. There were eight tubes for these experiments: 25 ml of culture in the light, another 25 ml in the dark, 25 ml of culture treated with 50 µM of ACC plus 5 µM ABA 10 µM ABA and 50 µM ABA in the light, and the remaining 25 ml of cultures treated with 50 µM of ACC plus 5 µM ABA, 10 µM ABA and 50 µM ABA in the dark. The cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the cylinders were photographed and then the A_{750} of the culture defined was measured by spectrophotometry at various depths from the surface by taking 1 ml of culture.

2.13.3 Measuring the motility of *C. reinhardtii* with ABA treatment

25 ml aliquots were transferred to a glass measuring cylinders and either 125 µl of ethanol (control) or 12 5µl of 10 mM ABA in 100 %(v/v) ethanol was added. There were four

tubes for these experiments: 25 ml of culture in the light (control), another 25 ml in the dark (control), 25 ml of culture treated with 50 µM of ABA in the light, and the last 25ml of culture treated with 50 µM of ABA in the dark. The cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the cylinders photographed, and then the A_{750} of the culture defined was measured by spectrophotometry at various depths from the surface by taking 1 ml of culture. This experiment was monitored at different points of time during the culture; for example, the cells were harvested one hour in the photoperiod, eight hours in the photoperiod and 15 hours in the photoperiod, one hour in the dark period, four hours in the dark period and seven hours in the dark period. The experiment was repeated five times.

After that, the effect of ABA was measured on the horizontal movement of *C.reinhardt* cells. Cultures of *C.reinhardtii* were grown as described earlier. 25 ml aliquots of the cultures were transferred to a glass test tube (15cm in height and 1.5cm wide) in which 1 ml plugs of 1×TAP media,1.5 % (w/v) agar containing a different concentration of ABA had been set at the bottom. The tubes were then placed in the dark or under overhead illumination at 1570 Lux for two hours at 25ºC. After incubation, the tubes were photographed to illustrate the position attained by the of alga as described in (Figure 2.1). All experiments were repeated three times.

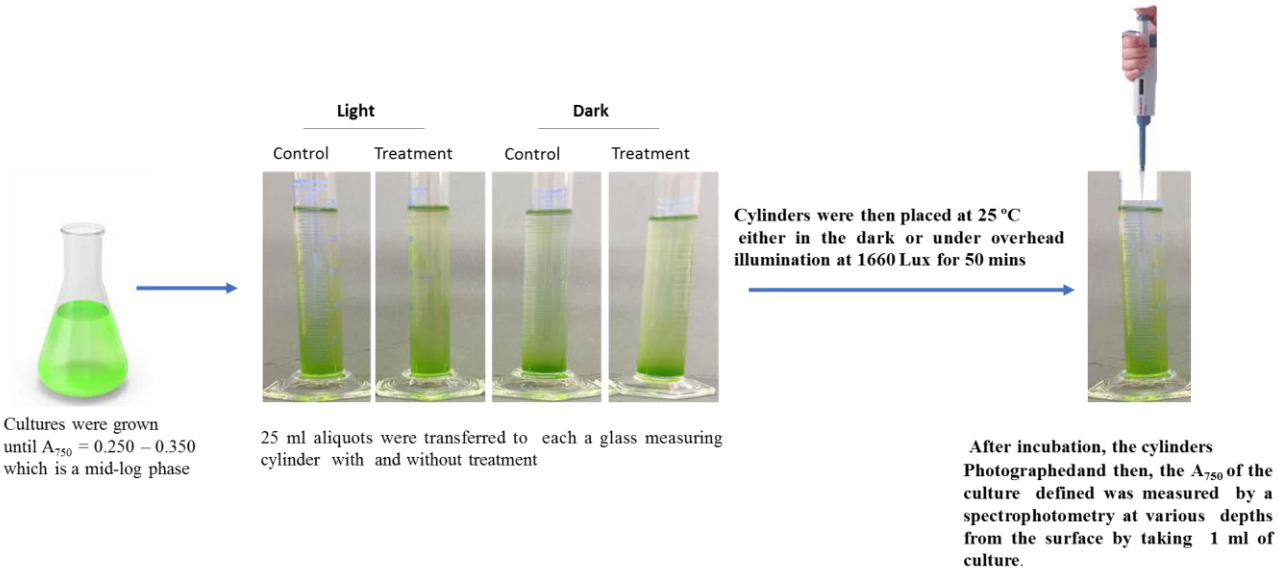


Figure 2.1: Described the method of how the experiment was done and how the movement of cells was measuring.

2.14 Circadian rhythm experiments

C. reinhardtii wild - type strain, CC1021 with cell wall, were grown at 25 ºC, in a 16-hour photoperiod, shaker 133 rpm, at 980 Lux in TAP media until A_{750} = 0.3, which is a mid-log phase. Growth was measured by following A_{750}nm. Cells were harvested every hour with and without 50 µM ABA. For RNA extraction, cells were collected by centrifugation at 4°C for 10 minutes at 2500 rpm before being stored at -80 ºC.

2.15 Measuring ABA levels during the circadian cycle

Freeze dried sample cells as finely ground material were added into a centrifuge tube containing 200 ml of sample extraction buffer (1x) (20 ml of sample extraction buffer concentrate (25 x)) were diluted into deionised or distilled water. Then samples were shaken overnight in the cold (4-5 °C) and dark. On the following day, solids were spun down and the supernatant was used directly. Standards were carried out according to the

plant hormone abscisic acid (ABA) ELISA Kit (My BioSource). The standard vial was centrifuged at 6000-10000 rpm for 30 seconds before opening. Then, the standard (10 x) was diluted with Sample Diluent (50 µl of standard (10 x) + 450 µl of Sample Diluent). This diluted standard (S7) served as the high standard (10 µg/ml). The standard was mixed for 15 minutes to ensure complete dilution with gentle agitation. 250 µl of Sample Diluent was pipetted into each tube (S0-S6) using the diluted standard (S7). Each tube was mixed thoroughly before the next transfer. Sample Diluent served as the zero standard (0 µg/ml). Next, a blank well was set without any solution. After that, 50 µl sample was added per well. Then, 50µl of antibody (1x) (10 µl of antibody + 990 µl of antibody diluent) was added to each well (but not to a blank well). After mixing, each well was incubated for 30 minutes at 37°C. Each well was aspirated and washed. This process was repeated twice for a total of three washes. 200 µl of Wash Buffer (1 x) (Dilute 20 ml of Wash Buffer Concentrate (25 x) into deionised or distilled water) was added to each well, which was left to stand for 10 seconds. Complete removal of liquid at each step was vital for a good assay performance. After the final wash, any remaining wash buffer was removed by aspiration or decanting. The plate was inverted and blotted on clean paper towels. 100 µl of Horseradish peroxidase enzyme (HRP-conjugate) (1x) (dilution 10 µl of HRP-conjugate + 990 µl of HRP-conjugate diluent) was added to each well (except to the blank well). Then, the well was mixed and incubated for 30 minutes at 37°C. An aspiration/wash process was repeated five times. 90 µl of TMB substrate (TMB is a chromogenic substrate used in staining procedures in immunohistochemistry) was added to each well, mixed and incubated for 20 minutes at 37°C. The plate was kept in the dark away from drafts and temperature fluctuations. Then, 50 µl of stop solution was added to each well and the plate was gently tapped to ensure thorough mixing. The

optical density of each well was determined within ten minutes by using a microplate reader set to 450 nm as described in Figure 2.2.

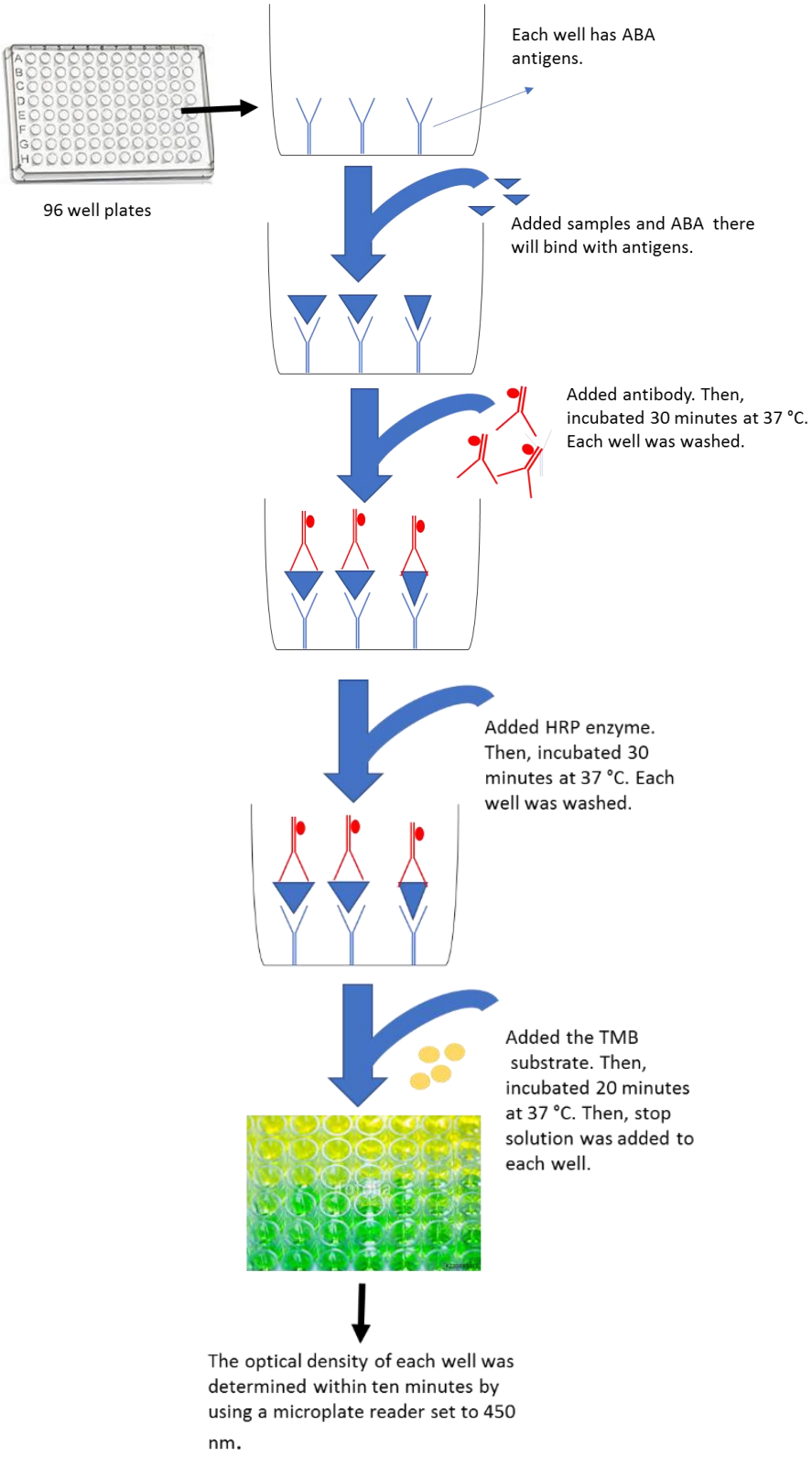


Figure 2.2: Description of the process of ABA ELISA Kit to measure ABA concentrations levels during the circadian cycle.

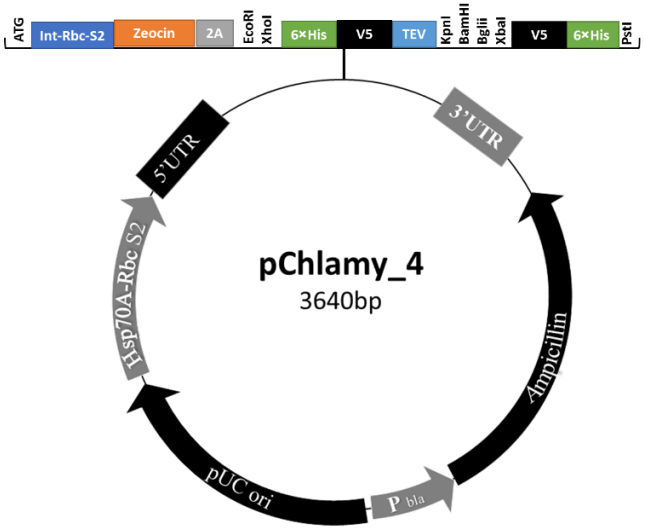
2.16 Effect of abscisic acid (ABA) on HCO^{3-} uptake by C. reinhardtii exposed to different light levels

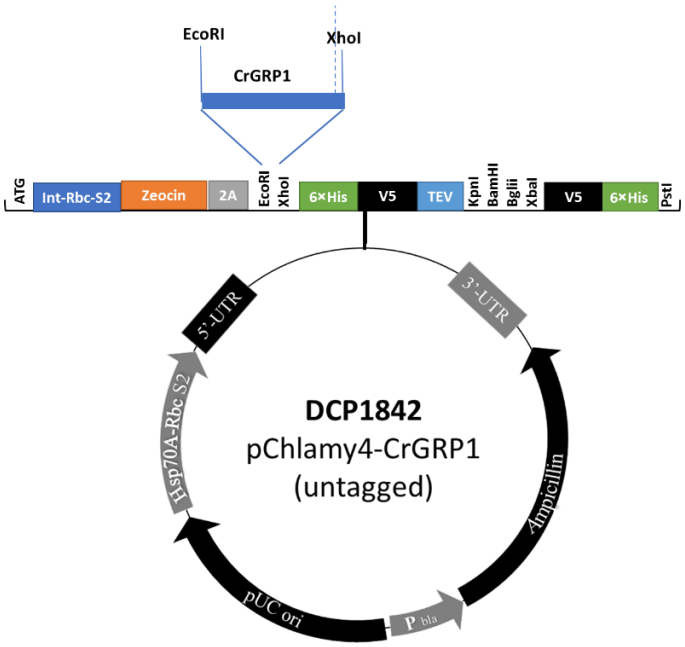
A 1.0 L culture of *C. reinhardtii* was grown until the mid-log phase (A750 = 0.3), and the cells were collected by centrifugation (200 x g, five minutes, at 23°C). The cell pellet was then gently resuspended in 50 mL of TAP media before mixing with 50 mL of 3 % (w/v) sodium alginate and the suspension was subdivided before the addition of ABA to get final concentrations of between zero and 50 µM. The alginate cell suspensions were immediately dropped through the nozzles of individual 25 mL syringes from a height of 40 cm into an excess volume of 0.18 M $CaCl_2$ to form spherical 2mm diameter gel beads of encapsulated algae +/- ABA. Replicates (n=5 for each combination of ABA concentration and light levels) with 1g aliquots of beads were then placed in sealed thin-walled glass vials containing 19.82 mL of TAP media with 180 µL of freshly prepared bicarbonate indicator buffer (0.47 mM thymol blue, 0.27 mM cresol red, 100 mM $NaHCO_3$, 1.1 M KCl) with no head space and were then immediately placed in a light tunnel at distance from a photosynthetic light source (minus UV) that exposed the samples to a range of light intensities of between 253 and 7440 Lux. The samples were incubated at 23 °C for 1 hour and the δA550 of the indicator buffer relative to time zero was recorded as an indication of the relative HCO_3- depletion of the media.

2.17 Preparing expression vectors to investigate the function of *CrGRP1* in *C. reinhardtii*

2.17.1 Design of pChlamy4 constructs

The expression vector pChlamy4 contains a *C. reinhardtii Hsp70A/RbcS2* chimeric promoter and a *C. reinhardtii RbcS2* terminator, both cloned into the polylinker of the *C. reinhardtii* vector pSP124S. To create the *CrGRP1* transformation construct pChlamy4-*CrGRP1*, the *CrGRP1* open reading frame was ligated into pChlamy4 at a different site between the *Hsp70A/RbcS2* promoter and *RbcS2* terminator, depending on the plasmid (see Figure 2.3).


ATG
Int-Rbc-S2
Zeocin
2A
EcoRI
XhoI
6×His
V5
TEV
KpnI
BamHI
BglII
XbaI
V5
6×His
PstI
5'UTR
3'UTR
pChlamy_4
3640bp
Hsp70A-Rbc S2
Ampicillin
pUC ori
P bla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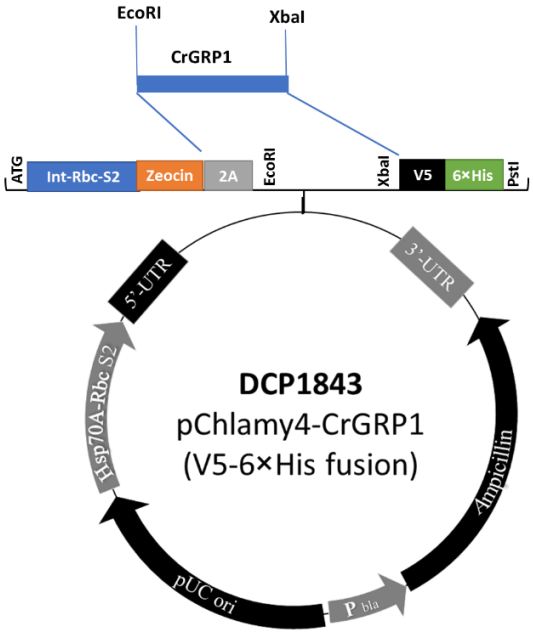
XhoI
CrGRP1
ATG
Int-Rbc-S2
Zeocin
2A
EcoRI
XhoI
6×His
V5
TEV
KpnI
BamHI
BglII
XbaI
V5
6×His
PstI
5'-UTR
3'-UTR
DCP1842
pChlamy4-CrGRP1
(untagged)
Hsp70A-Rbc S2
Ampicillin
pUC ori
P bla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XbaI
CrGRP1
ATG
Int-Rbc-S2
Zeocin
2A
EcoRI
XbaI
V5
6×His
PstI
5'-UTR
3'-UTR
DCP1843
pChlamy4-CrGRP1
(V5-6×His fusion)
Hsp70A-Rbc S2
Ampicillin
pUC ori
P bla

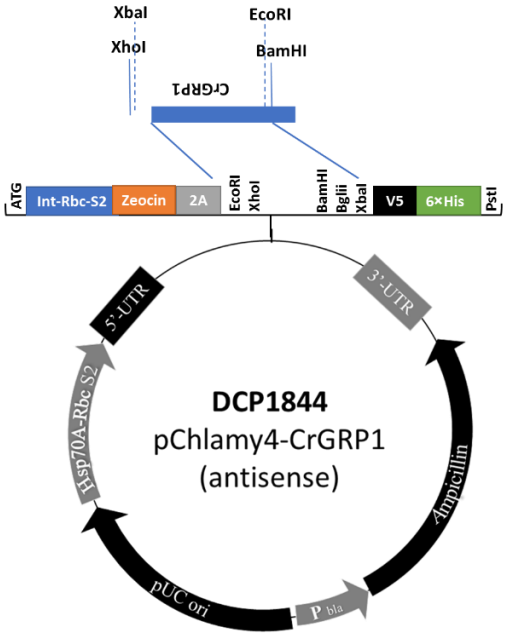

XbaI
EcoRI
XhoI
BamHI
CrGRP1
ATG
Int-Rbc-S2
Zeocin
2A
EcoRI
XhoI
BamHI
BglII
XbaI
V5
6×His
PstI
5'-UTR
3'-UTR
DCP1844
pChlamy4-CrGRP1
(antisense)
Hsp70A-Rbc S2
Ampicillin
pUC ori
P bla

Figure 2.3: Map of vector pChlamy_4 engineered to express CrGRP1 with or without a histidine tag, and the antisense construct designed to knockdown CrGRP1 expression.

2.17.2 Ligation into plasmid pChlamy4-TOPO

Ligation into pChlamy4-TOPO was carried out according to a GeneArt® Chlamydomonas Protein Expression Kit (Invitrogen). Briefly, each TOPO cloning reaction comprised: 4µL of the PCR product, 1µl of salt solution (provided by the manufacturers), and 1µl of TOPO vector/enzyme conjugate. The total volume of the reaction was 6µl and it was mixed gently and incubated for 5 minutes at room temperature before being placed on ice in readiness for transformation into *E. coli*. A control ligation of pUC19 TOPO (without any *CrGRP1* ORF inserted) was performed to provide a check for the pET101 *E. coli* plasmid size in subsequent steps. To give an indication of ligation efficiency, colony numbers in the control ligation/transformation vis-a-vis the pChlamy4 (GRP1) ligations/transformations were cengued. The control TOPO ligation reaction comprised: 4 µl of sterile milliQ water, 1µl of salt solution (provided by the manufacturers), and 1µl of TOPO vector/enzyme conjugate. The total volume of the reaction was 6µl and it was mixed gently and incubated for five minutes at room temperature before being placed on ice in readiness for transformation into *E. coli*.

2.17.3 Generating of transgenic organisms (pChlamy4 - *CrGRP1*- TOP10)

2.17.3.1 *Transforming pChlamy4 - CrGRP1 into E. coli Top10 cells*

An aliquot (3µl) of each TOPO cloning reaction was added to a phial each of One Shot TOP10 chemically competent *E. coli* cells (provided by Invitrogen). Each was mixed gently and incubated on ice for 15 minutes. Each combination of cells and DNA was then heated-shocked at 42 °C for 30 seconds and then transferred onto ice and immediately 250 µl of SOC medium (2% Tryptone, 0.5 % Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose-Invitrogen) was added and the mix was shaken at 37°C for one hour. The transformants were then plated onto

LB agar (Sigma) plates containing sterile filtered ampicillin (100 µg/mL final concentrations) and incubated overnight at 37°C. Colonies were screened the following day by plasmid DNA extraction and restriction enzyme digestion.

2.17.3.2 *DNA extraction and restriction digests*

Transformant colonies from each ligation were screened by streaking onto a fresh LB agar plate containing 100 µg/mL of ampicillin, then subculturing each of these onto a liquid LB containing 100 µg/mL of ampicillin, with growth overnight at 37°C. Plasmid DNA was extracted using the Alkaline Lysis Method with PEG Purification (see Section 2.11), before analysis by restriction digestion. The screening was accomplished with restriction enzymes (see Table 2.2) (New England Bio Labs, Ipswich, Massachusetts, USA). Restriction digestions were carried out in a total volume of 50µL. The reaction mixture contained the appropriate reaction buffer (1X), and the restriction enzymes (10,000U) used to cut 1µg of DNA. The reaction mixture was incubated at 37˚C overnight (as described in Section 2.12), according to the manufacturer's instructions. Restricted DNA was electrophoresed in a 1% agarose gel (1xTAE; 10 mM Tris-Acetate, 0.5 mM EDTA pH adjusted to 7.8) for visualisation.

Plasmid Name	**Plasmid Number**	**Restriction sites used**	**Calc Mol Weight**
pChlamy4-*CrGRP1*	DCP1842	*Eco*RI / *Xho*I	15783.8 Da
pChlamy4-*CrGRP1*-V5-6xHis	DCP1843	*Eco*RI / *Xba*I	18566.9 Da
pChlamy4-*CrGRP1* antisense	DCP1844	*Xho*I / *Bam*HI	N/A

Table 2-2: Restriction digestion enzyme sites used for Plasmid.

2.17.3.3 *Sequencing of recombinant plasmids*

Sequencing of the recombinant section of pChlamy4-*CrGRP1* was carried out using the primers (see Table 2.3) which had been designed for this purpose (see Appendix 8.3). Sequencing reactions were accomplished by adding only primers to purified plasmid and sent to Eurofins MWG Operon (Germany) to be sequenced.

Plasmid Name	Plasmid Number	Forward primer (5' -3')	Reverse primer (5' -3')
pChlamy4-*CrGRP1*	DCP1842	pChlamyFMDVfor 5' - GTGGCCGAGGAGCAGGACGCCC 3'	pChlamyUTRrev 5'- AGCGCCTCCATTTACACGGAGCGG 3'
pChlamy4-*CrGRP1*-V5-6xHis	DCP1843	pChlamyFMDVfor 5'- GTGGCCGAGGAGCAGGACGCCC 3'	pChlamyUTRrev 5'- AGCGCCTCCATTTACACGGAGCGG 3'
pChlamy4-*CrGRP1* antisense	DCP1844	pChlamyFMDVfor 5'- GTGGCCGAGGAGCAGGACGCCC 3'	pChlamyUTRrev 5'- AGCGCCTCCATTTACACGGAGCGG 3'

Table 2-3: Oligonucleotide primers for sequencing of transgenic organisms.

2.18 Transformation and verification of CrGRP1 constructs

2.18.1 Transformation of *C. reinhardtii*

2.18.1.1 *Algal culture*

C. reinhardtii cells, strain 173 C, were grown to early-phase in TAP medium. The culture was centrifuged in Falcon tubes for five minutes at 2500 rpm min^{-1} at 4˚C. The pellet was aseptically resuspended in 10mL of GeneArt® MAX Efficiency® Transformation Reagent and used for transformation.

2.18.1.2 *Transformation of constructs into C. reinhardtii by electroporation*

The transformation vector (pChlamy4-*GRP1*) was cut with *Sca*1 enzyme and prepared for transformation (see Section 2.12). *C. reinhardtii* cells were prepared for

transformation according to Section 2.17.3. The cell pellet was resuspended again in 10mL of GeneArt® MAX Efficiency® Transformation Reagent, and the cells centrifuged once more at 2500 rpm for five minutes. Cell pellets were resuspended in GeneArt® MAX Efficiency® Transformation Reagent at 2×10^8-3×10^8 cells/mL. Then, 2–4 µg of linearised DNA was added per 250µL of cell suspension and incubated at 2°C–8 °C for five minutes. Electroporation (the Gene Pulser® II-bio rad) was performed at 500 V, capacitance 50 µF, resistance 800 Ohms. Pulse time length was over 30 milliseconds for every electroporation. The cells were allowed to recover on the bench for 15 minutes and transferred to a 50mL Falcon tube containing 10 mL of TAP-40 mM sucrose solution (44 mL of 1 M sucrose to 1 L of TAP medium) at room temperature and then incubated at 26°C and 50 µE m–2 s–1 for 14–16 hours. The next day, the supernatant was discarded, and the cells were harvested by centrifugation at 2500 rpm for five minutes and then resuspended in 200 µL TAP medium at room temperature. An aliquot of the cells in TAP (200 µL) was spread-plated on TAP agar containing 5 µg/mL (final concentration in agar) of zeocin and incubated at 26°C in constant light for two to three weeks.

2.19 Statistical analysis

Where appropriate, groups of data were assessed for normality by Shapiro Wilk tests and for between-group homogeneity of variances around the means by Bartlett's tests. For HCO^{3-} depletion data, overall between data group comparisons of means were made by one- and two-way ANOVAs and *post hoc* pairwise mean comparisons by Tukey Kramer tests. For the algal movement data, comparisons of the relative distributions of the algae in response to the treatments were undertaken using $\chi 2$ tests of independence with *post*

hoc $\chi 2$ tests of goodness of fit. Endogenous ABA data were analysed by one-way ANOVA and selected pairwise comparisons by two sample t-tests. All tests were performed at the $\alpha=0.05$ level of confidence.

2.20 Bioinformatic analysis

2.20.1 Analysis of promoter elements

Promoter analysis was done by using Scan-pL programme to locate the ABA promoter element in the *CrGRP1* gene by comparing it with a GRPs plant genes promoter such as *A.thaliana.*

2.20.2 Alignments and Photogenetic tree analysis

NCBI software was used at protein levels to find how similar class IVa GRP1 to *A. thaliana* gens class IVa GRPs by using GRP1 sequencing, then searched for a similar class IVa GRP protein in a wide range of species and in different species of algae. After that, protein sequences were aligned using MUSCLE, and Neighbour-joining trees were drawn in Paup 4.0. To test the confidence of the clustering, bootstrap values were determined with 500 replicates.

3 Chapter Three: The effect of plant hormones on *C. reinhardtii* growth and movement

3.1 Introduction

Plant hormones stimulate processes that are necessary for them to survive. Phytohormones such as auxin, gibberellin, cytokinin, ethylene, and abscisic acid (ABA) play key roles in the growth and developmental processes of higher plants such as *Arabidopsis thaliana* and regulate responses to different abiotic stresses (Wani *et al.*, 2016). However, the role of these phytohormones is poorly understood in green algae. Algae evolved approximately one billion years ago, while terrestrial plants appeared later, approximately 400-600 million years ago (Delwiche and Cooper, 2015). Algae such as Chlamydomonas possess some, but not all, the phytohormone signalling pathways found in higher plants (Wang *et al.*, 2015) and those that are found in algae are either not as highly developed or as complex as in plants. Indeed, with such comparative single cell simplicity in mind, one could suggest, therefore, that the study of basic plant hormone signalling should have been examined first in algae. However, for obvious agronomic reasons researchers were more interested in understanding the signalling mechanisms in plants. Interestingly, while green algae seem to possess only rudimentary signalling pathways for auxin and ethylene, one of the more evolved phytohormone signalling pathways that they possess is that for ABA (Wang *et al.*, 2015).

In plants, the effect of the circadian clock has been recognised both in the gene expression and the regulation of physiological processes controlled by ABA (Dodd *et al.*, 2007; Finkelstein and Gibson, 2002; Zhu, 2002). A number of ABA metabolic enzymes are controlled by environmental signals including drought, salinity, temperature, and

light, as well as by other hormones and circadian rhythms (Nowak *et al*., 1988; Piotrowska-Niczyporuk and Bajguz, 2014;). ABA regulates plant responses to various abiotic stresses, and diurnal rhythms that alter their water status and both light and water are essential resources for plants. Therefore, plants have developed an internal regulator that allows them to detect and adapt to water availability and sunlight throughout the seasons (Fankhauser and Staiger, 2002). Plant physiological processes in a changing environment are regulated by the circadian clock in a 24-hour cycle (Staiger and Heintzen,1999). There are three gene components of the circadian clock in plants: input, oscillator and output genes. Output genes enable growth processes, leaf movements and flower opening (Staiger and Heintzen, 1999). The regulation of output genes by the circadian clock is mostly carried out at the transcriptional level through clock-response elements in promoters (Staiger, 2002), and up to 30% of the transcriptome may be regulated so. Genes such as *AtGRP7* and *AtGRP8* in Arabidopsis (Heintzen *et al*., 1997; Staiger and Green, 2011) may act as oscillators (Schmal, Reimann and Staiger, 2013), determining the timing of expression of numerous output genes under the overall control of the circadian pacemaker, a specialised oscillator that activates independently of other oscillators to drive balanced outputs and which is entrained by environmental cues.

The connection between ABA and clock signalling pathways is bidirectional as treatment with ABA lengthens the circadian period of gene promoter activity (Hanano *et al.*, 2006). A good example of this is stomatal opening and closing. ABA is less effective at closing the stomata in the morning than in the afternoon (Correia *et al.*,1995; Kim *et al*., 2008), when increased light intensity and higher temperatures may cause water-deficit conditions (Robertson *et al*., 2009).

In plants, ethylene often antagonises ABA responses, such as, for example, during germination. ABA promotes seed dormancy and inhibits germination, while ethylene promotes germination (Zhou *et al*., 1998). Green algae are known to synthesise ACC and make ethylene (Booker and DeLong, 2015) and possess many recognisable components of the ethylene signal transduction pathway found in terrestrial plants (Ju *et al*., 2015; Wang *et al*., 2015). Therefore, the effect on the vertical movement of *C. reinhardtii* of ACC combined with various concentrations of ABA and ABA with different concentrations of ACC was studied in either the light or dark. Also, the effect of the second messenger, hydrogen peroxide (H_2O_2), was also investigated because in plant stomata it is made in response to ABA and is required for ABA-induced stomatal closure to occur during the day (light) and night (dark) rhythm.

Since algae have phytohormone synthesis and signalling mechanisms, it is likely that they have a functional role in these microorganisms. A study by Iqbal *et al*. (2017), reported that phytohormones such as ethylene play an essential role in the growth and development of higher plants. For example, ripening usually starts in one region of climacteric fruit and spreads to the other regions that lag behind. This process is initiated, accelerated and integrated by ethylene, which diffuses from cell to cell throughout the fruit (Bleecker and Kende, 2000). Similarly, ethylene has been shown to be involved in cell differentiation and biochemical responses in algae (Plettner *et al*., 2005). A circadian rhythm of cell wall retention and release of ACC and its conversion to ethylene has been shown in the alga *Acetabularia. mediterranean* (Driessche *et al.*, 1988). Ethylene addition affects cap formation in this species and could be stimulatory or inhibitory depending on the timing of application (Driessche *et al*., 1988). Plettner, Steinke and Malin (2005) undertook experiments with the green alga, *U. intestinalis*, showing that

exogenous ethylene altered the concentration of chlorophyll a. A 3d incubation of *U. intestinalis* in 40 µM ethephon resulted in a 30% loss of chlorophyll in comparison to untreated controls. Such studies would suggest that ethylene may have physiological roles in algae and would indicate the presence of ethylene receptors and possible signal transduction pathways. In plants, ethylene is known to be involved in leaf senescence, which involves the loss of chlorophyll (Iqbal *et al*., 2017). A study by Plettner, Steinke and Malin (2005) suggested a similar role in algae. However, this begs the question as to why algae would want to undergo such a senescence-related process. One possible answer is that the algae may reduce chlorophyll levels under high light conditions to protect against photo-oxidation. Plants have organ-specific programmes of cell death and senescence coordinated by ethylene by which they lose leaves and recycle their mobilisable components under environmental conditions where either light levels are photosynthetically inefficient or the temperature is non-permissive, for example in the winter (Woo et al., 2013). Whether algae such as Chlamydomonas similarly use ethylene to coordinate such a programme of senescence during changes in their environment remains to be determined.

A role for auxin has not yet been conclusively found in algae (Kiseleva *et al*., 2012). Green algae have been shown to contain and metabolise auxins (Žižková *et al*., 2017) and exogenous applications of either indoleacetic acid (IAA) or 1-naphthaleneacetic acid (NAA) have been shown to improve their growth rate (Park et al., 2013) and to induce tolerance to salinity and temperature (Nowak *et al*., 1988; Piotrowska-Niczyporuk and Bajguz, 2014). However, more than a hundred species of algae have been examined for ABA, 96% of which contained this phytohormone (Wang *et al*., 2015). To date, the algal role of ABA appears to be one of involvement in stress

responses and increasing photosynthetic ability (Cowan and Rose, 1991; Holzinger and Becker, 2015). ABA treatment has also been shown to increase lipid accumulation in Chlorella (Wu *et al*., 2018). Thus, this study examined the effects of these hormones on the movement and growth rate of the green alga, *C. reihardtii*. To achieve this, the following was investigated:

1- The effect of plant hormones on *C. reinhardtii* growth rate by adding different concentrations of hormones such as ABA, NAA, IAA and ACC.

2- The effect of plant hormones on *C. reinhardtii* movement, either in the light or dark, with 50µM of either ABA, ACC, NAA or H_2O_2 and combinations of ABA and ACC.

3- The effect of 50µM ABA on the movement of *in C. reinhardtii* sampled at different time points during their day/night growth cycle.

4- The endogenous concentration of ABA in *C. reinhardtii* at different time points during their day/night growth cycle.

3.2 Results

3.2.1 Algal culture

Cultures of *C. reinhardtii* were grown at 25ºC with rotary shaking (133 rpm) with a 16 h photoperiod (980 Lux) in TAP media until A_{750} ~ 0.250 to 0.350 (mid-log phase – see Figure 3.1). The cell culture became light green in colour. At this point, the cell cultures were ready for use.

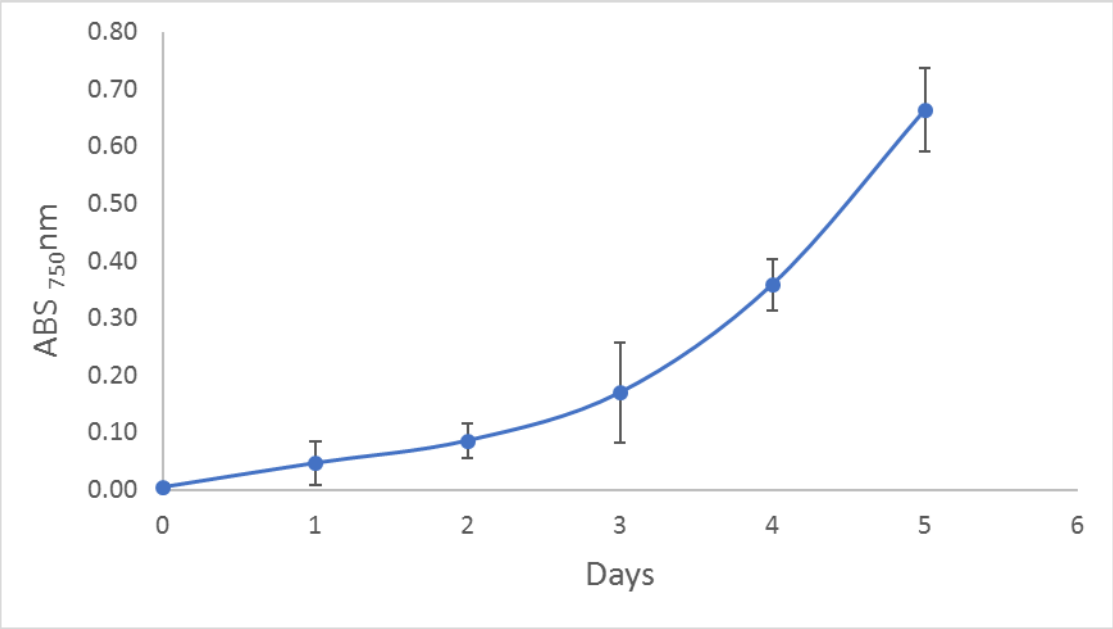


Figure 3.1: Growth curve of C. reinhardtii
The growth curve was used to determine the mid-log phase of *C. reinhardtii* strain CC1021. Cells were harvested at an absorbance of ~ 0.3. Growth was measured by measuring absorbance at A_{750} nm. Replicate (n=3).

3.2.2 Effect of phytohormones on *C. reinhardtii* growth rates

C. reinhardtii wild-type strain, CC1021 (mt+) algae, were grown as described in Chapter 2. At inoculation, 20ml cultures of *C. reinhardtii* were separately treated with either 0, 1, 10, 50 or 100µM concentrations of either abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA), or 1-naphthaleneacetic acid (NAA) and the growth of the algae was measured by following the A_{750} nm every 22 hours. Figure 3.2 shows that, compared to the control, ABA did not affect the growth rate of *C. reinhardtii* whereas NAA slowed algal growth at 100µM. IAA also did not affect the growth rate of *C. reinhardtii*. ACC marginally reduced growth when compared to the control. Overall, and bearing in mind that the experiment was only replicated once (n=2) (and therefore lacks statistical rigor), the results suggested that these plant hormones did not greatly affect the growth rate of *C. reinhardtii*.

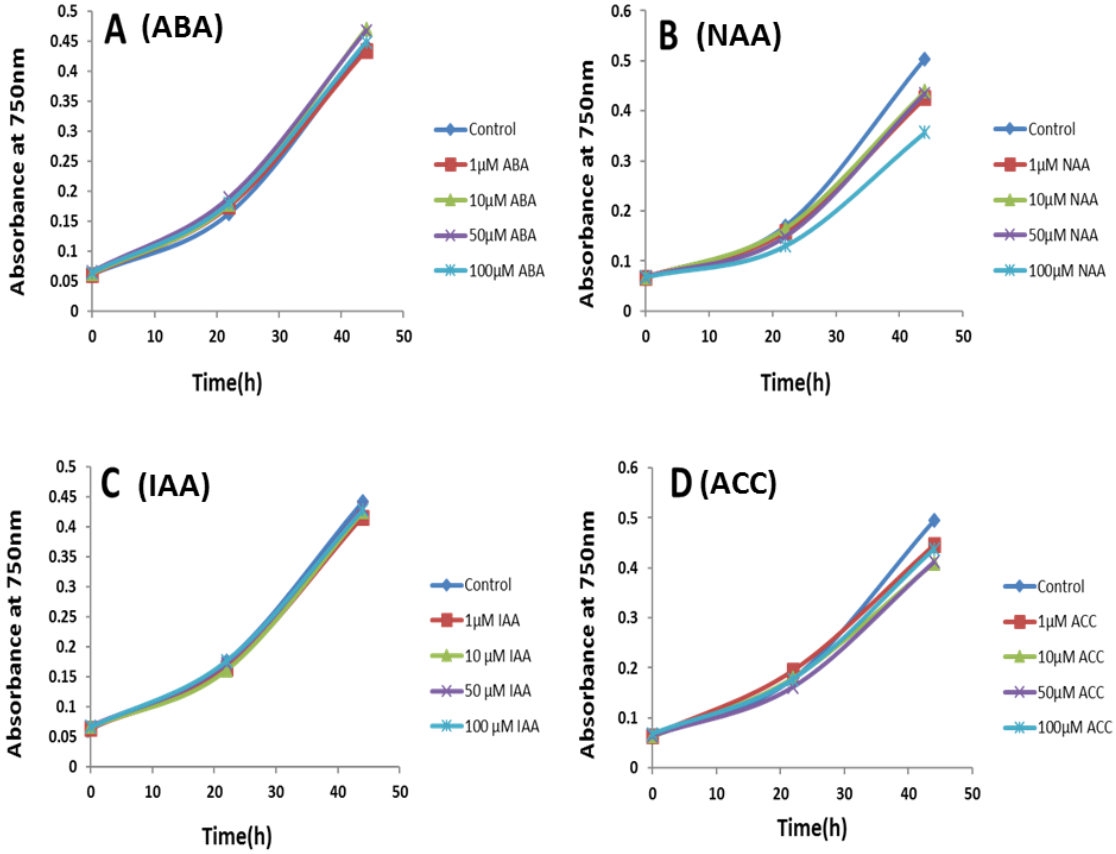


Figure 3.2: Plant hormones did not affect the growth rates of C. reinhardtii
C. reinhardtii cultures which were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders. ABA, ACC, IAA and NAA were added to the TAP growth medium in concentrations ranging from 0 to 100µM. The growth of cultures was monitored over a 48-hour period by measuring A_{750} nm. Values represent the means of n=2 experiments. (A) Abscisic acid (ABA), (B) 1-naphthaleneacetic acid (NAA), (C) indole-3-acetic acid (IAA) and (D) 1-aminocyclopropane-1-carboxylic acid (ACC).

3.2.3 Effect of plant hormones on algal movement

3.2.3.1 *ABA-induced a negative geotropic response in dark-incubated C. reinhardtii*

Cultures of *C. reinhardtii* were grown at 25ºC with rotary shaking (133 rpm) with a 16-hour photoperiod (980 Lux) in TAP media until A_{750} = 0.3. To study the effects of ABA on movement in both light and dark conditions, 25 ml aliquots were transferred to glass measuring cylinders and either 125µl of ethanol (control) or 125µl of 10mM ABA in 100% (v/v) ethanol was added. The cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the A_{750} of the cultures was determined by spectrophotometry at various depths from the surface and the relative cell densities calculated within each treatment (see Figure 3.3). In the light, the control algae retreated away from the source of illumination (see Figure 3.3B) such that a significantly ($P<0.05$ by χ^2 test of goodness of fit) increased proportion of the cells accumulated at increased depths with 3.4 fold more cells at a depth of 8cm than at 2cm from the surface (Figure 3.3A). In the light, compared to the control, 50µM ABA had no significant ($P>0.05$ by the χ^2 test of independence) effect on the distribution of the algae (see Figure 3.3A). In the dark, untreated algae generally remained more dispersed (see Figure 3.3B) but did settle downwards such that a significantly ($P<0.05$ by the χ^2 test of goodness of fit) increased proportion of the cells were at the 8cm depth from the surface than at the other depths sampled (see Figure 3.3A). In this case 1.7-fold more cells were at a depth of 8cm compared to 2cm from the surface. In the dark, treatment of the algae with 50µM ABA-induced a marked and significant ($P<0.05$ by the χ^2 test of independence) change in their distribution compared to the untreated cells (see Figure 3.3A). In this case, a significant ($P<0.05$ by the χ^2 test of goodness of fit) proportion of the algae banded at the 2cm depth from the surface with a much-reduced proportion of the cells remaining at the lower depths. Approximately 70% of the algae moved to the

2cm depth over the incubation period (see Figure 3.3A). Thus, preliminary investigations indicated that ABA induced a negative geotropic movement of the algae towards the surface of the water column.

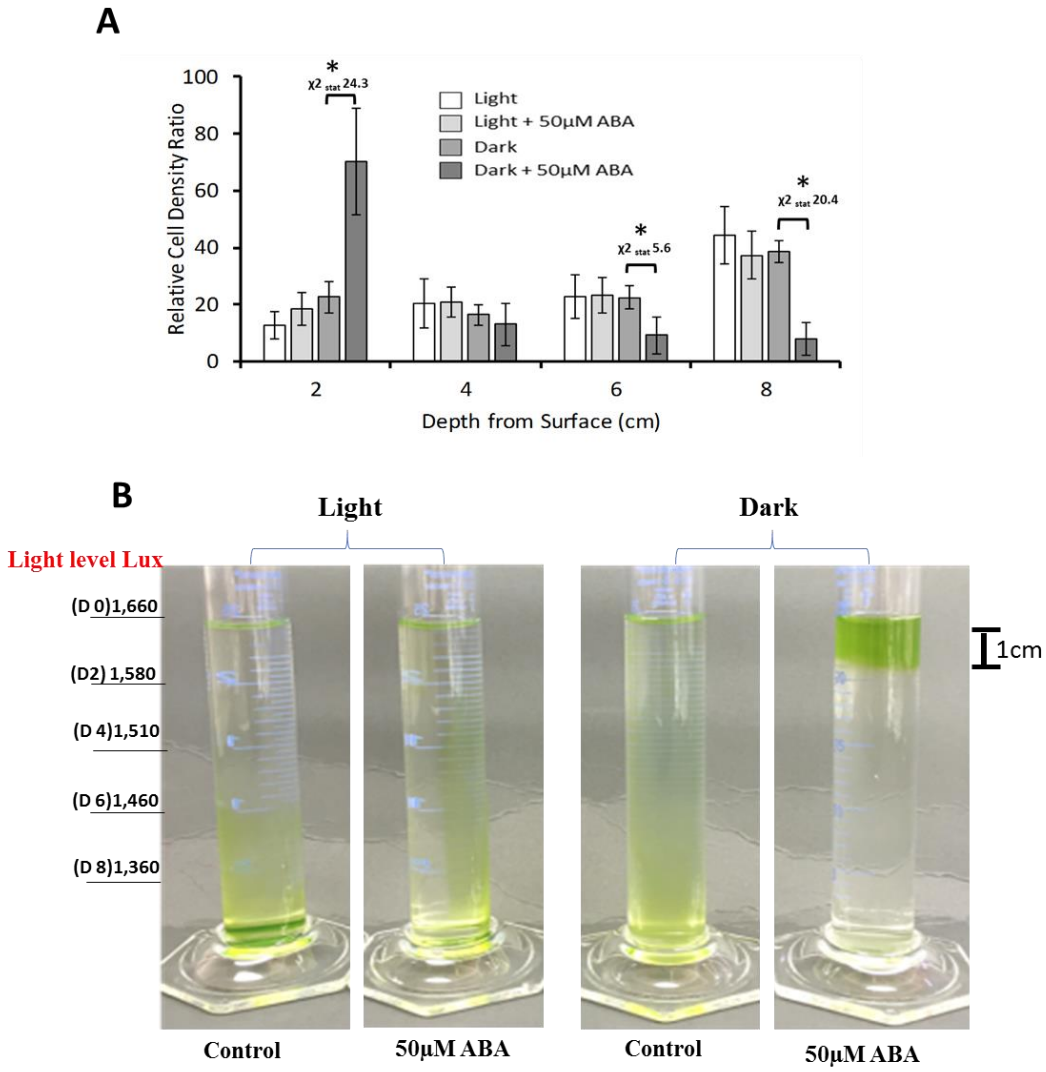


Figure 3.3: ABA altered the positioning of C. reinhardtii in the water column in the dark
C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the dark or the light for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=3) relative cell densities +/- the 95% confidence intervals around the means calculated in each case (A). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of the algae resulting from the ABA treatments at specific depths are indicated$^{(*)}$ along with the χ^2_{stat} values (larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled are shown in (B).

3.2.3.2 *The speed of algal movement in response to ABA treatment in the dark*

To study how quickly the motile algae moved up in the dark when treated with ABA, grown 25 ml aliquots of culture were transferred to glass measuring cylinders and 125µl of 10mM ABA in 100% (v/v) ethanol was added. The cylinders were then placed at 25ºC in the dark for a range of incubation times (5, 10, 20, 30 and 50 minutes). After incubation, the cylinders were immediately photographed to illustrate the positions attained by the algae in each case. After 30 minutes of incubation the algae had noticeably started to move upwards, while at 50 minutes approximately 80 % of algae were visually at a depth of up to 2cm from the surface of the media (see Figure 3.4).

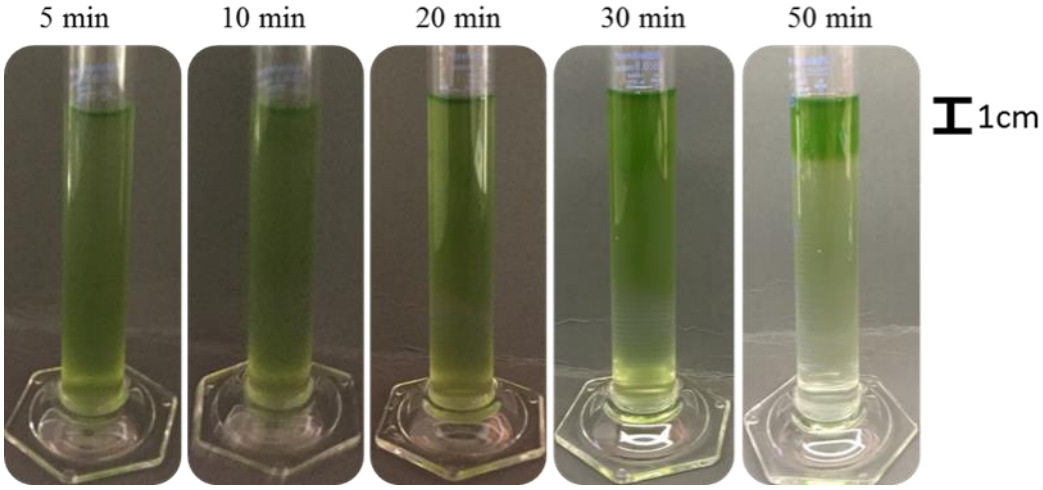


Figure 3.4: After 50 minutes in the dark ABA induces the C. reinhardtii to move upward
C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +50µM ABA with incubation in the dark for different incubation times of 5, 10, 20, 30, 50 and 70 minutes. Following incubation, the cylinders were photographed to illustrate how quickly the motile algae can move up in the dark when treated with ABA. Replicate (n=3).

3.2.3.3 *No effect of aminocyclopropane-1-carboxylic acid (ACC) on C. reinhardtii movement*

Cultures of *C. reinhardtii* were grown at 25ºC with rotary shaking (133 rpm) with a 16-hour photoperiod (980 Lux) in TAP media until A_{750} = 0.3. To study the effect of ACC on the movement of the algae in both light and dark conditions, 25 ml aliquots were transferred to glass measuring cylinders and either 125µl of water (control) or 125µl of 10mM ACC dissolved in water was added. Cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the A_{750} of the cultures was determined by spectrophotometry at various depths from the surface and the relative cell densities calculated within each treatment (see Figure 3.5). In the light, control algae retreated downwards away from the source of illumination as before, such that approximately a 2.6-fold higher cell density was recorded at a depth of 8cm compared to 2cm from the surface of the media. The addition of 50µM ACC had no significant ($P>0.05$ by the χ^2 test of independence) effect on the overall distribution in the light. In the dark the untreated algae similarly settled downwards and again the addition of ACC had no significant ($P>0.05$ by the χ^2 test of independence) effect on their distribution under these conditions. In summary, ACC treatment did not appear to affect the vertical positioning of the algae in the water column when they were incubated in either the light or the dark (see Figure 3.5A).

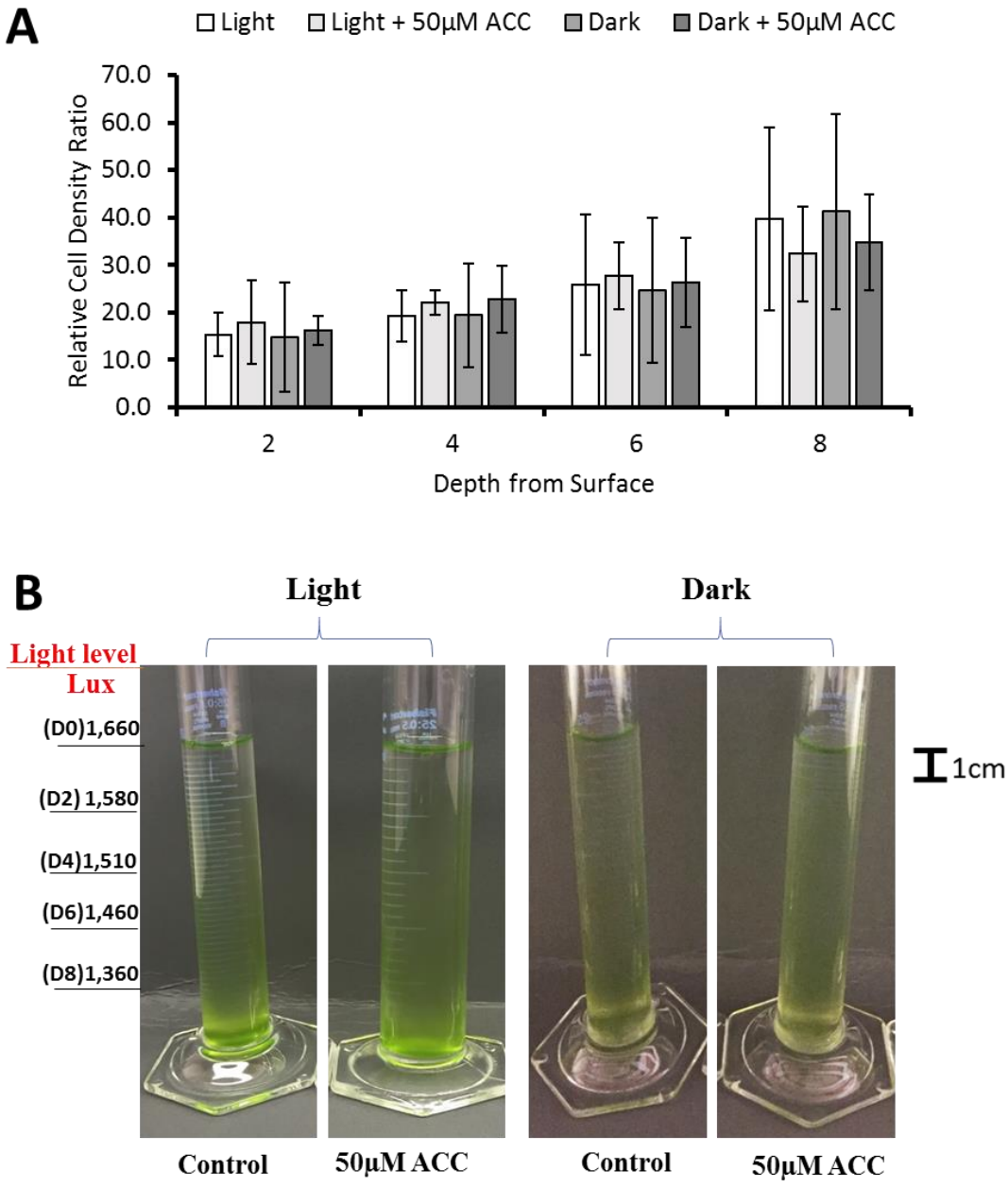


Figure 3.5: 1-aminocyclopropane-1-carboxylic acid (ACC) did not significantly affect the vertical positioning of C. reinhardtii in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +/- 50µM ACC with incubation in either the dark or the light for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=3) relative cell densities +/- the 95% confidence intervals around the means were calculated in each case (A). Within the light or dark incubations no significant ($P>0.05$ by χ^2 tests of goodness of fit) differences were apparent. A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled are shown in (B).

3.2.3.4 *No effect of hydrogen peroxide (H2O2) on C. reinhardtii movement in both light and dark condition*

In plants, hydrogen peroxide (H_2O_2) is a reactive molecule that integrates signalling networks in response to biotic and abiotic stress. The effect of H_2O_2 on the positioning of *C. reinhardtii* movement was examined in a similar way here (see Figure 3.6). As for the ACC treatment, the results showed that in comparison to the untreated control algae the 50µM H_2O_2 treatment had no significant ($P>0.05$ by the χ^2 test of independence) effect on the vertical positioning and distribution of the algae in the water column when they were incubated in either the light or the dark (see Figure 3.6A).

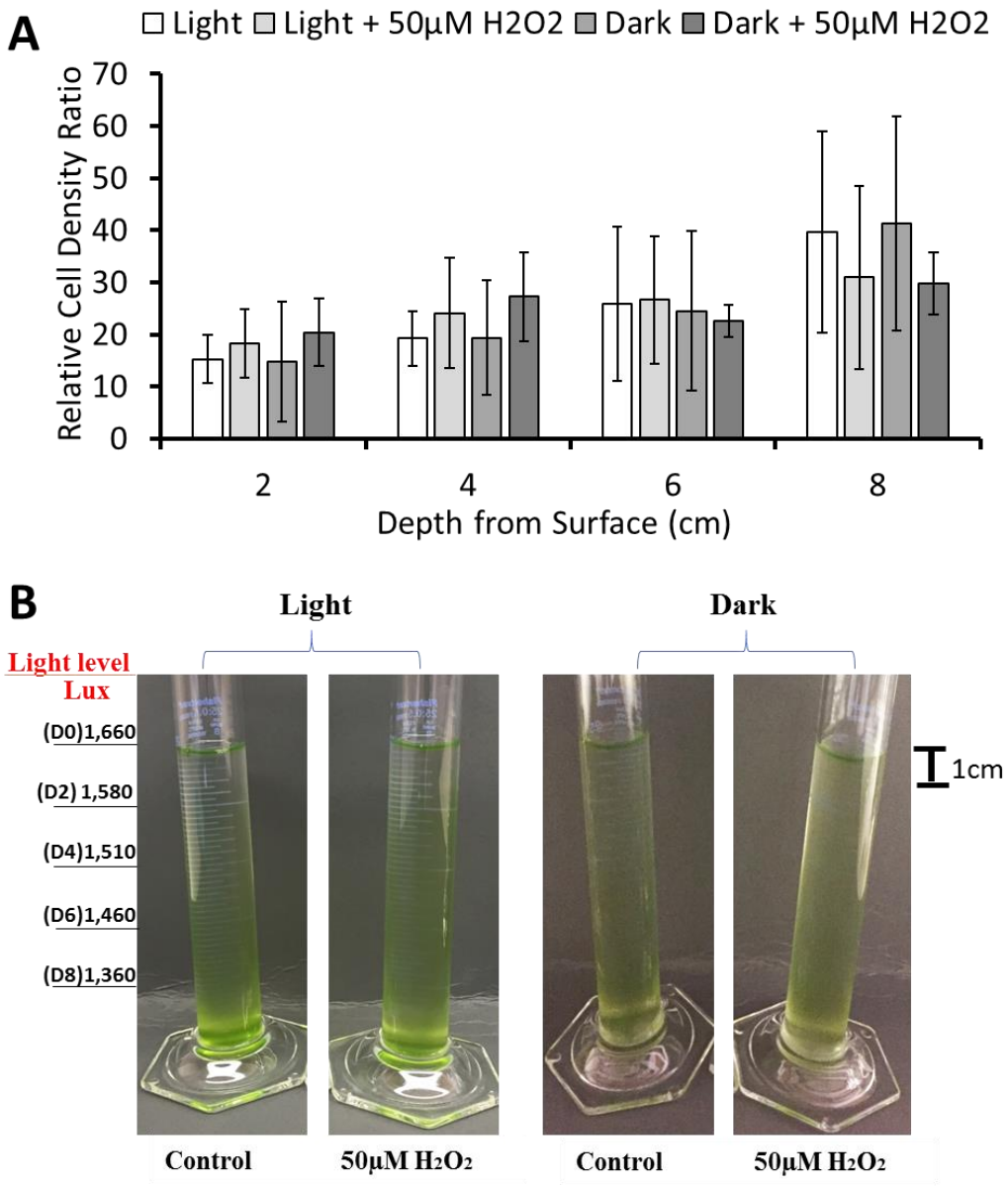


Figure 3.6: Hydrogen peroxide (H_2O_2) did not significantly affect the vertical positioning of C. reinhardtii in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +/- 50µM H_2O_2 with incubation in either the dark or the light for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=3) relative cell densities +/- the 95% confidence intervals around the means were calculated in each case (A). Within the light or dark incubations no significant ($P>0.05$ by χ^2 tests of goodness of fit) differences were apparent. A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled are shown in (B).

3.2.3.5 *No effect of naphthaleneacetic acid (NAA) on C. reinhardtii movement either in the dark or light*

Cultures of *C. reinhardtii* were grown as before and 25 ml aliquots were transferred to glass measuring cylinders and either 125µl of 1N NaOH (control) or 125µl of 10mM NAA dissolved in 1N NaOH was added. The cylinders were then placed at 25ºC, either in the dark or under overhead illumination at 1660 Lux for 50 minutes. After incubation, the A_{750} of the cultures was determined by spectrophotometry at various depths from the surface and the relative cell densities calculated within each treatment (see Figure 3.7). Compared to the untreated control algae, NAA at 50µM had no significant ($P>0.05$ by the χ^2 test of independence) effect on the vertical positioning of the algae in the water column when they were incubated in either the light or the dark. When the algae were incubated in the light there was some indication that NAA had a small effect and although the algae still generally retreated downwards away from the high-light, the proportion remaining at shallower depths was increased with the auxin treatment, but overall the distribution of the algae with and without NAA was not statistically significantly different (see Figure 3.7A). In summary, the 50µM NAA treatment did not alter either the phototropic response of *C. reinhardtii* or their vertical positioning in the water column in the dark.

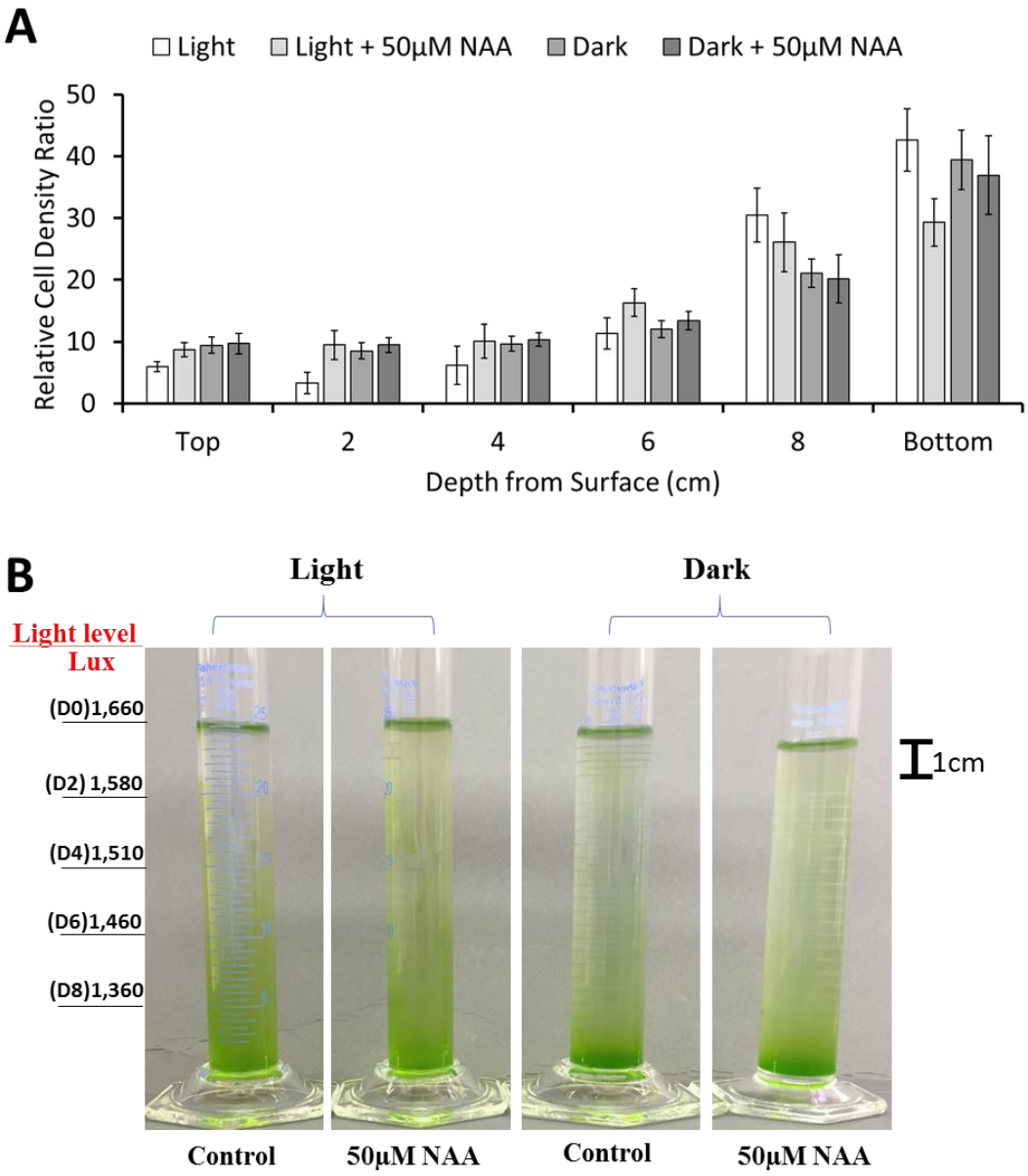


Figure 3.7: Exogenous 1-naphthaleneacetic acid (NAA) did not significantly affect the vertical positioning of C. reinhardtii in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +/- 50µM NAA with incubation in either the dark or the light for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) relative cell densities +/- the 95% confidence intervals around the means were calculated in each case (A). Within the light or dark incubations no significant ($P>0.05$ by χ^2 tests of goodness of fit) differences were apparent. A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled are shown in (B).

3.2.3.6 *Effect of combining ACC with ABA on C. reinhardtii movement*

In terrestrial plants, ethylene often works antagonistically to inhibit ABA responses. To examine the potential antagonistic effect of ACC on the observed effects of ABA on movement of the algae, 25 ml aliquots of similarly grown algae were transferred to glass measuring cylinders and either 125µl of ethanol (control) or 125µl of 10mM ACC was added with 125µl of 10mM ABA. The cylinders were then placed at 25ºC either in the dark or under overhead illumination at 1660 Lux for 50 minutes and the A_{750} of the cultures wase determined by spectrophotometry at various depths from the surface and the relative cell densities calculated within each treatment (see Figure 3.8A). As for the experiment shown in Figure 3.3, untreated algae moved downwards in the light and their overall distribution in the water column was not significantly ($P>0.05$ by χ^2 test of independence) altered by the combined addition of 50µM ABA and 50µM ACC in these conditions. In the dark, as with the treatment of the cells with 50µM ABA alone (see Figure 3.3), treatment of the cells with a combination of 50µM ABA and 50µM ACC induced a significant ($P<0.05$ by χ^2 test of independence) alteration in the distribution of the algae in the water column with a significant ($P<0.05$ by χ^2 test of goodness of fit) proportion of the cells banding at a 2cm depth from the surface compared to the untreated control cells. Thus, ACC treatment did not alter the ABA response observed previously.

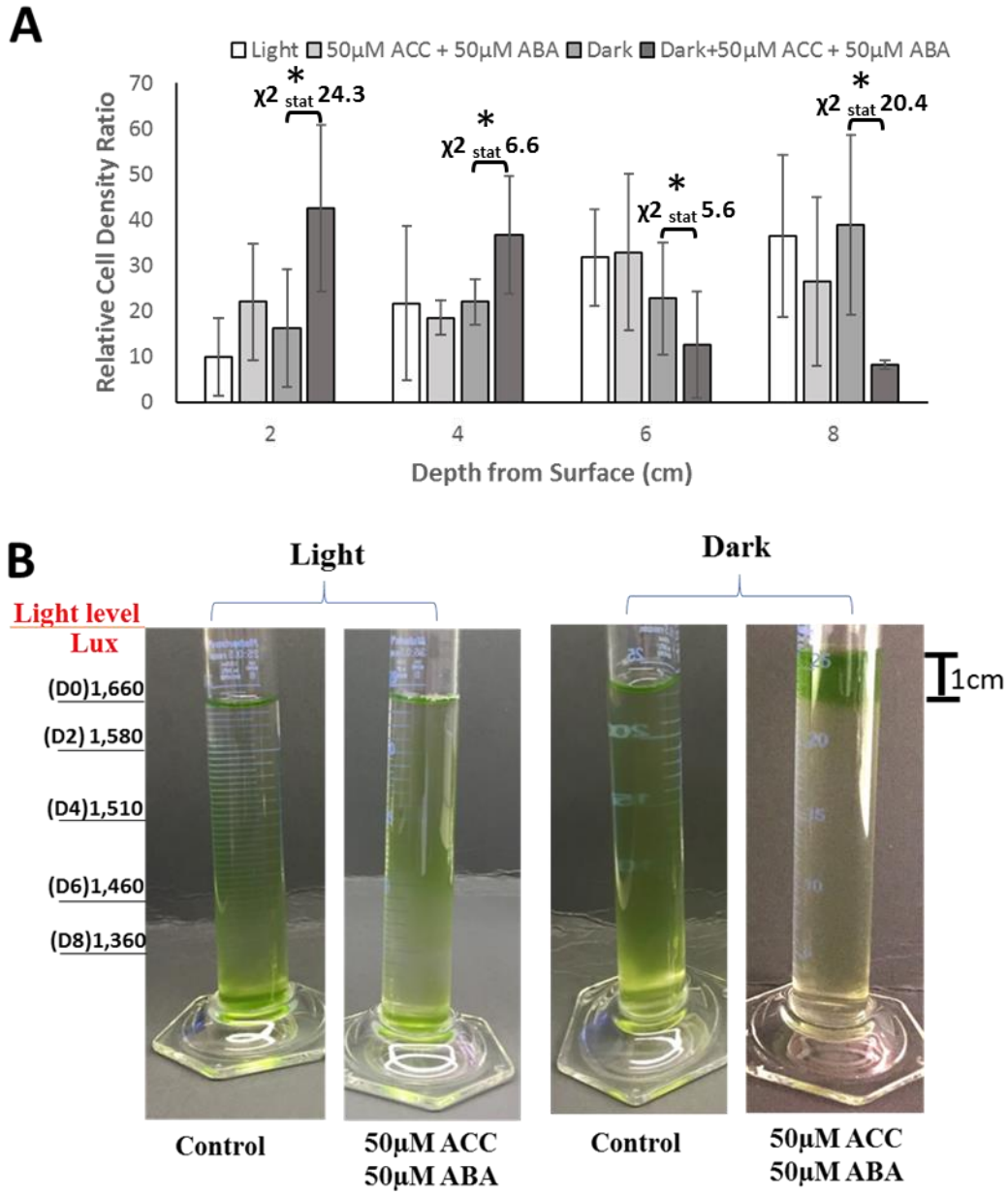


Figure 3.8: ACC did not alter the ABA-associated negative geotropic response of C. reinhardtii in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were divided between 25mL measuring cylinders and were treated +/- 50µM ACC + 50µM ABA with incubation in either the dark or the light for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=3) relative cell densities +/- the 95% confidence intervals around the means were calculated in each case (A). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of the algae resulting from the ABA treatments at specific depths are indicated$^{(*)}$ along with the χ^2_{stat} values (larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (B).

3.3 Responses of Chlamydomonas reinhardtii to the phytohormone abscisic acid (ABA) during the circadian cycle

Pphytohormone abscisic acid (ABA) regulates plant responses to various abiotic stresses and diurnal rhythms. Sequencing of the *C. reihardtii* genome has shown that this flagellate, motile, freshwater, photosynthetic green alga is also capable of synthesising and responding to ABA. Algae were sampled at different time-points during both a 16-hour photoperiod and a dark period of a 24-hour growth cycle (see Figure 3.9) and were treated +/- 50µM ABA in either the light or dark and their position in the water column was monitored by measuring the A_{750} at different depths.

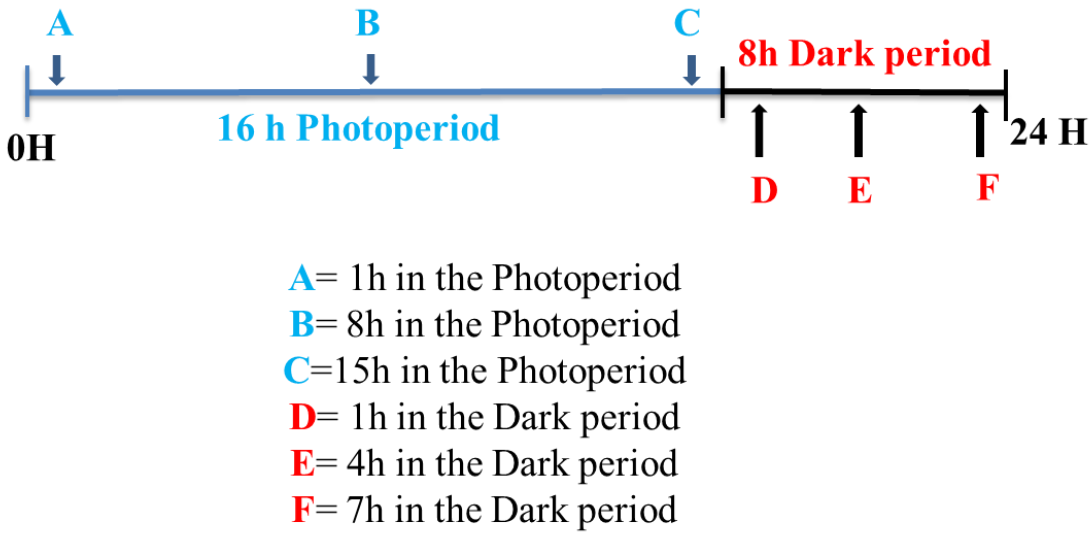


Figure 3.9: Sampling time points during the light/dark cycle
Cultures of *C. reinhardtii* were grown at 25ºC, 133rpm, over a 16-hour photoperiod/8-hour dark period at 980 Lux in TAP media until A_{750} = 0.3. 25. After that, cells were collected at a different time point during the circadian rhythm. Then, the effect of ABA on *C. reinhardtii* movement was observed.

3.3.1 Effect of ABA on movement one hour into the photoperiod

Similarly grown cultures of *C. reinhardtii* were sampled 1 hour after the start of the photoperiod and algae were again similarly assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation in either the light or dark (see Figure 3.10 C). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within each of the treatments were apparent. In the light, the algae retreated towards the bottom of the tube such that the A_{750} at a depth of 8cm was significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm, 4cm or 6cm depths (see Figure 3.10 A) with 3.4 fold more algae accumulating at a depth of 8cm than at 2cm. The addition of 50µM ABA in the light retarded this downward movement of the algae to a small extent such that while the A_{750} at the 8cm depth remained significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm depth, it was no longer significantly different from that at the 4cm and 6cm depths (Figure 3.10A). In the dark, a proportion of the algae remained near the surface with the majority settling downwards such that, overall, they remained more dispersed. The A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at 4cm, while that at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than that at all the other depths measured with 1.7 fold more algae at this depth compared to that at 2cm (see Figure 3.10A). The addition of 50µM ABA in the dark caused the algae to move upwards and accumulate near to the surface of the water column such that the A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at all the other depths measured, with approximately 70% reaching this position over the incubation period (see Figure 3.10A).

In order to compare the effect of ABA on the overall distribution of the algae

in either the light or the dark, relative cell densities were calculated from the A_{750} measurements. When comparing the distribution of the algae following their incubation either in the light or in the light with 50µM ABA, there was no overall significant ($P>0.05$ by χ^2 test of independence) difference observed (see Figure 3.10B). However, in the dark the addition of ABA caused a marked and significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column with those algae accumulating near to the surface at the 2cm depth being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (Figure 3.10 B). Significantly ($P<0.05$ by χ^2 test of goodness of fit) fewer algae were also apparent at the 6cm and 8cm depths in the dark +ABA treatment compared to when the algae were incubated in the dark alone. Thus, overall, when sampled at 1 hour into the photoperiod, ABA treatment induced the majority of the algae to swim upwards towards the surface when they were subsequently incubated in the dark.

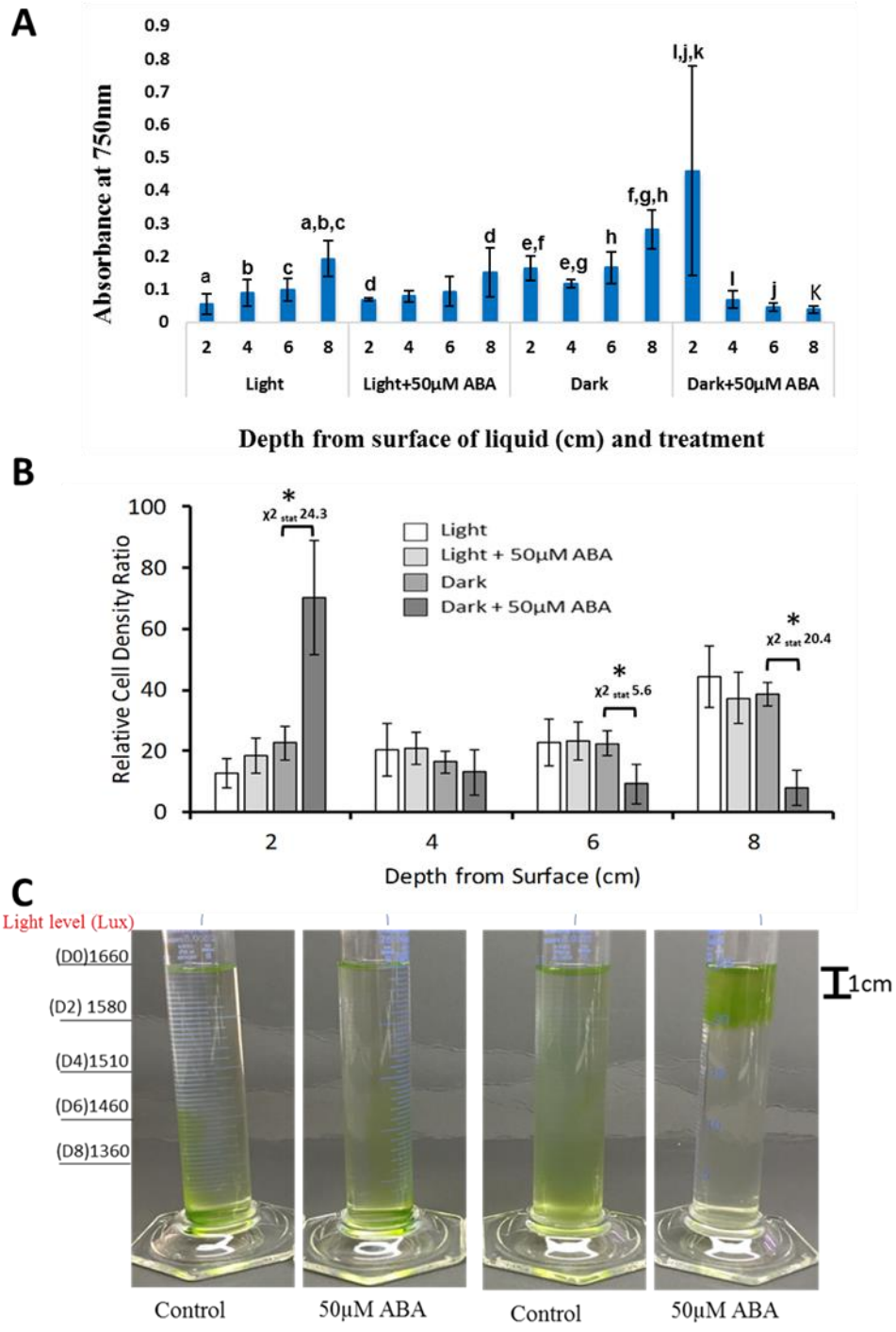


Figure 3.10: ABA altered the positioning of C. reinhardtii in the water column when sampled at one hour into the photoperiod and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 1 hour into the photoperiod and were divided between 25mL measuring cylinders and treated +/- 50µM ABA with incubation in either the light or dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise differences ($p<0.05$ by Mann-Whitney U-Test) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to k). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of

the algae resulting from the ABA treatments at specific depths are indicated(*) along with the χ^2_{stat} values (larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

3.3.2 Effect of ABA on movement eight hours into the photoperiod

C. reinhardtii cultures were similarly sampled 8 hours after the start of the photoperiod and algae were again assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation in either the light or dark (see Figure 3.11). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within each of the treatments were apparent. In the light, the algae retreated towards the bottom of the tube such that the A_{750} at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm, 4cm or 6cm depths (see Figure 3.11A) with 8.7 fold more algae accumulating at a depth of 8cm than at 2cm. The addition of 50µM ABA in the light retarded this downward movement of the algae to a small extent such that while the A_{750} at the 8cm depth remained significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm depth, only the 6cm depth was no longer significantly different from that at 8cm (see Figure 3.11A). In the dark, a proportion of the algae remained near the surface with the majority settling downwards such that, overall, they remained more dispersed. The A_{750} at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than that at all the other depths measured with 2.1-fold more algae at this depth compared to that at 2cm (see Figure 3.11A). The addition of 50µM ABA in the dark caused the algae to move upwards and accumulate near to the surface of the water column such that the A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at all the other depths measured, with approximately 55% reaching this position over the incubation period (see Figure 3.11A).

Again, in order to compare the effect of ABA on the overall distribution of the algae in either the light or the dark, the relative cell densities were calculated from the A_{750} measurements. When comparing the distribution of the algae following their incubation either in the light or in the light with 50µM ABA, there was no overall significant ($P>0.05$ by χ^2 test of independence) difference observed (see Figure 3.11B). However, in the dark the addition of ABA caused a marked and significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column, with those algae accumulating near to the surface at the 2cm depth being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (see Figure 3.11B). There was no significant difference in the algal distribution at the 4cm and 6cm depths when comparing dark alone with dark +ABA. Thus, overall, when sampled at 8 hours into the photoperiod, ABA treatment induced the majority of the algae to swim upwards towards the surface when they were subsequently incubated in the dark.

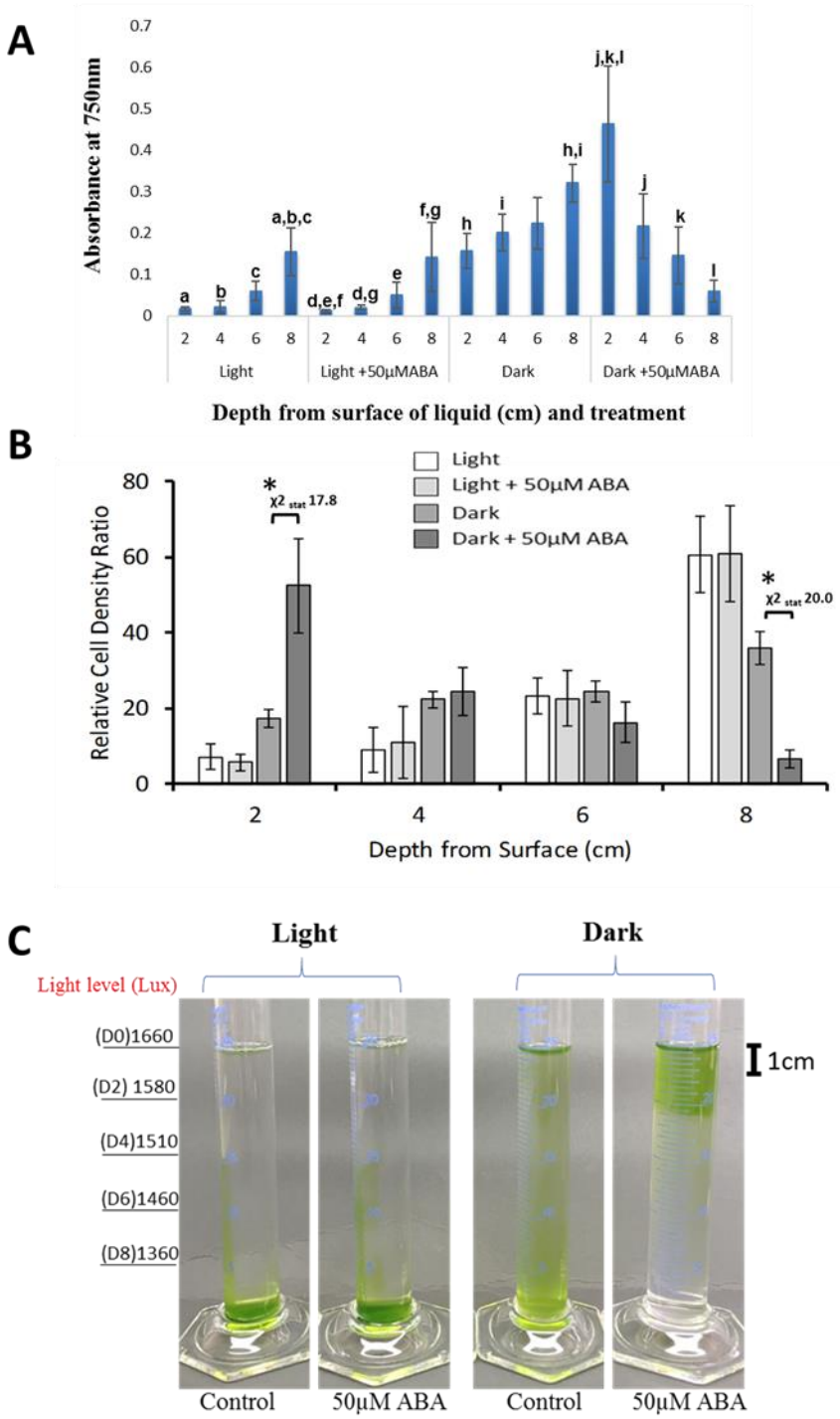


Figure 3.11: ABA alters the positioning of C. reinhardtii in the water column when sampled at eight hours into the photoperiod and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 8 hours into the photoperiod and were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the light or dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to m). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of the algae resulting from the ABA treatments at specific depths are indicated$^{(*)}$ along with the χ^2_{stat} values

(larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

3.3.3 Effect of ABA on movement fifteen hours into the photoperiod

C. reinhardtii were sampled 15 hours after the start of the photoperiod and algae were again similarly assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation, in either the light or dark (see Figure 3.12). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within some of the treatments were apparent, as at earlier sampling time points. However, in contrast to earlier sampling time points, when incubated in the light the algae showed less of a tendency to retreat towards the bottom of the tubes and remained more dispersed such that the A_{750} at the 8cm depth was not significantly ($P>0.05$ by Mann Whitney U test) different from that at the 2cm, 4cm or 6cm depths (see Figure 3.12 A). The addition of 50µM ABA in the light retarded this downward movement of the algae to a small extent such that while the A_{750} at the 8cm depth remained significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm and 4cm depths (Figure 3.12 A). In the dark, a proportion of the algae remained near the surface with the majority settling downwards such that, overall, they remained more dispersed. The A_{750} at the 8cm depth showed no significant ($P<0.05$ by Mann Whitney U test) difference than in the algal distribution at all the other depths (see Figure 3.12 A). The addition of 50µM ABA in the dark again caused the algae to move upwards and accumulate near to the surface of the water column such that the A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at all the other depths measured, with approximately 60% reaching this position over the incubation period (see Figure 3.12 A).

In order to compare the effect of ABA on the overall distribution of the algae in either the light or the dark, the relative cell densities were calculated from the A_{750} measurements. When comparing the distribution of the algae following their incubation either in the light or in the light with 50μM ABA, no overall significant ($P>0.05$ by χ^2 test of independence) difference was observed (see Figure 3.12B). However, in the dark the addition of ABA again caused a marked and significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column, with those algae accumulating near to the surface at the 2cm depth being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (see Figure 3.12 B). No significant difference in the algal distribution at the 4cm and 6cm depths when comparing dark alone with dark +ABA was observed. Thus, overall, when sampled at 15 hours into the photoperiod, ABA treatment induced the majority of the algae to swim upwards towards the surface when they were subsequently incubated in the dark.

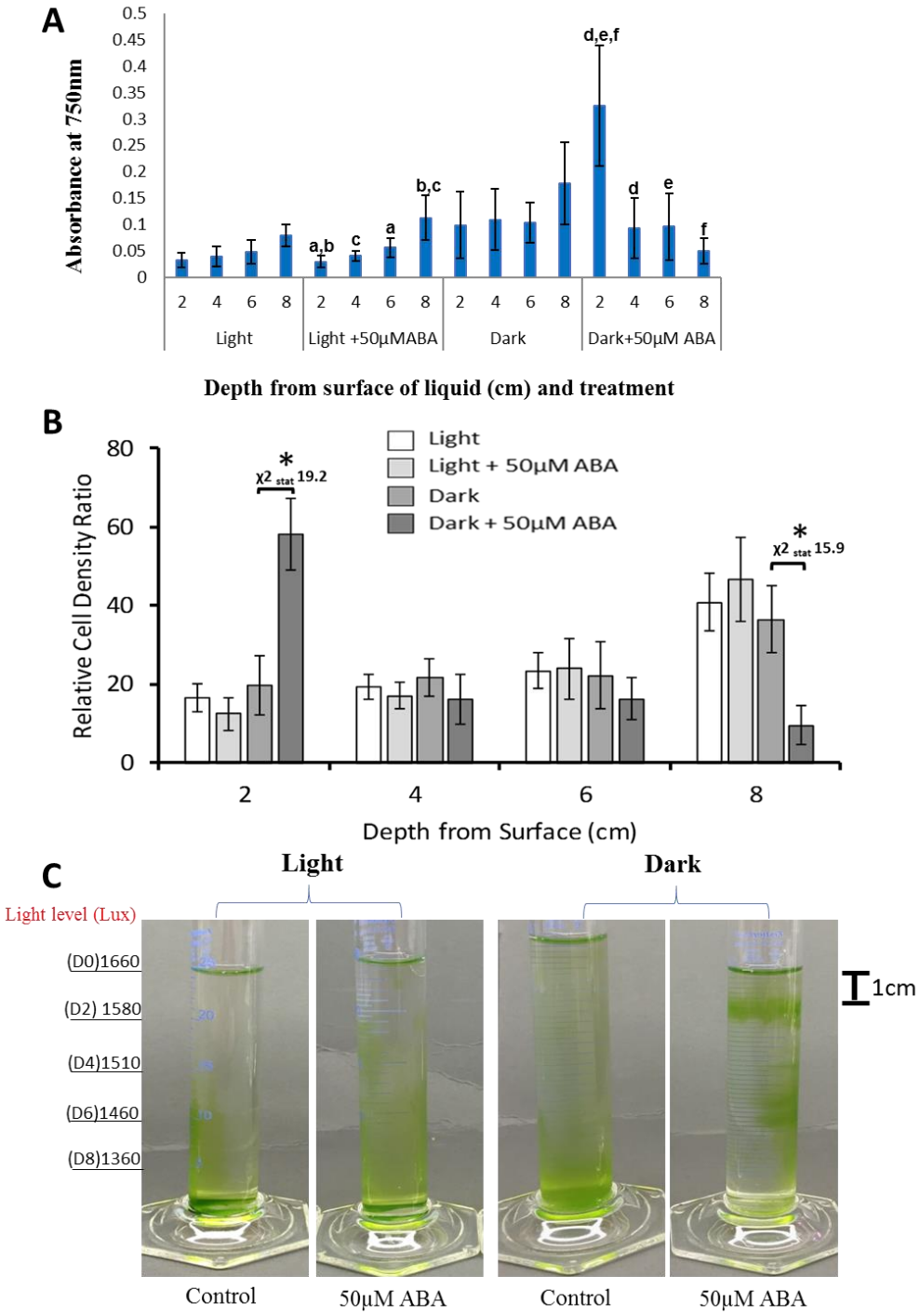


Figure 3.12: ABA alters the positioning of C. reinhardtii in the water column when sampled at fifteen hours into the photoperiod and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 15 hours into the photoperiod and were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the light or dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to f). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of the algae resulting from the ABA treatments at specific depths are indicated$^{(*)}$ along with the χ^2_{stat} values

(larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

3.3.4 Effect of ABA on movement one hour into the dark

Again, *C. reinhardtii* were similarly sampled 1 hour after the start of the dark period and algae were again assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation in either the light or dark (see Figure 3.13). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within some of the treatments were apparent. When incubated in the light, the algae moved downwards to a small extent but generally remained dispersed such that the A_{750} of the culture at any depth was not significantly ($P>0.05$ by Mann Whitney U test) different from that at any other depth measured (see Figure 3.13A). The addition of 50µM ABA in the light appeared to have no significant affect in this respect (see Figure 3.13A). In the dark the algae generally settled downwards such that the A_{750} at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than that at all the other depths measured, with 2.6 fold more algae at this depth compared to that at 2cm (see Figure 3.13A). The addition of 50µM ABA in the dark caused the algae to move upwards and accumulate near to the surface of the water column such that the A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at all the other depths measured, with approximately 30% reaching this position over the incubation period (Figure 3.13A).

In order to compare the effect of ABA on the overall distribution of the algae in either the light or the dark, the relative cell densities were again calculated from the A_{750} measurements. When comparing the distribution of the algae following their incubation either in the light or in the light with 50µM ABA, no overall significant ($P>0.05$ by χ^2 test of independence) difference was observed (see Figure 3.13B). However, in the dark

the addition of ABA again caused a significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column, with those algae accumulating near to the surface at the 2cm depth again being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (see Figure 3.13B). As before, the algae moved upwards significantly ($P<0.05$ by $\chi 2$ tests of goodness of fit) to form a band near to the surface, but at this sampling time point the proportion of the algae that did so was noticeably less than at earlier sampling time points (Figure 3.13 C). In this case, 2.1-fold more cells were at a depth of 2cm depth compared to 8cm from the surface. Thus, overall, when sampled at 1 hour into the dark period, ABA treatment induced the algae to swim upwards towards the surface when they were subsequently incubated in the dark, but to a lesser extent than when sampled at earlier time points during the photoperiod.

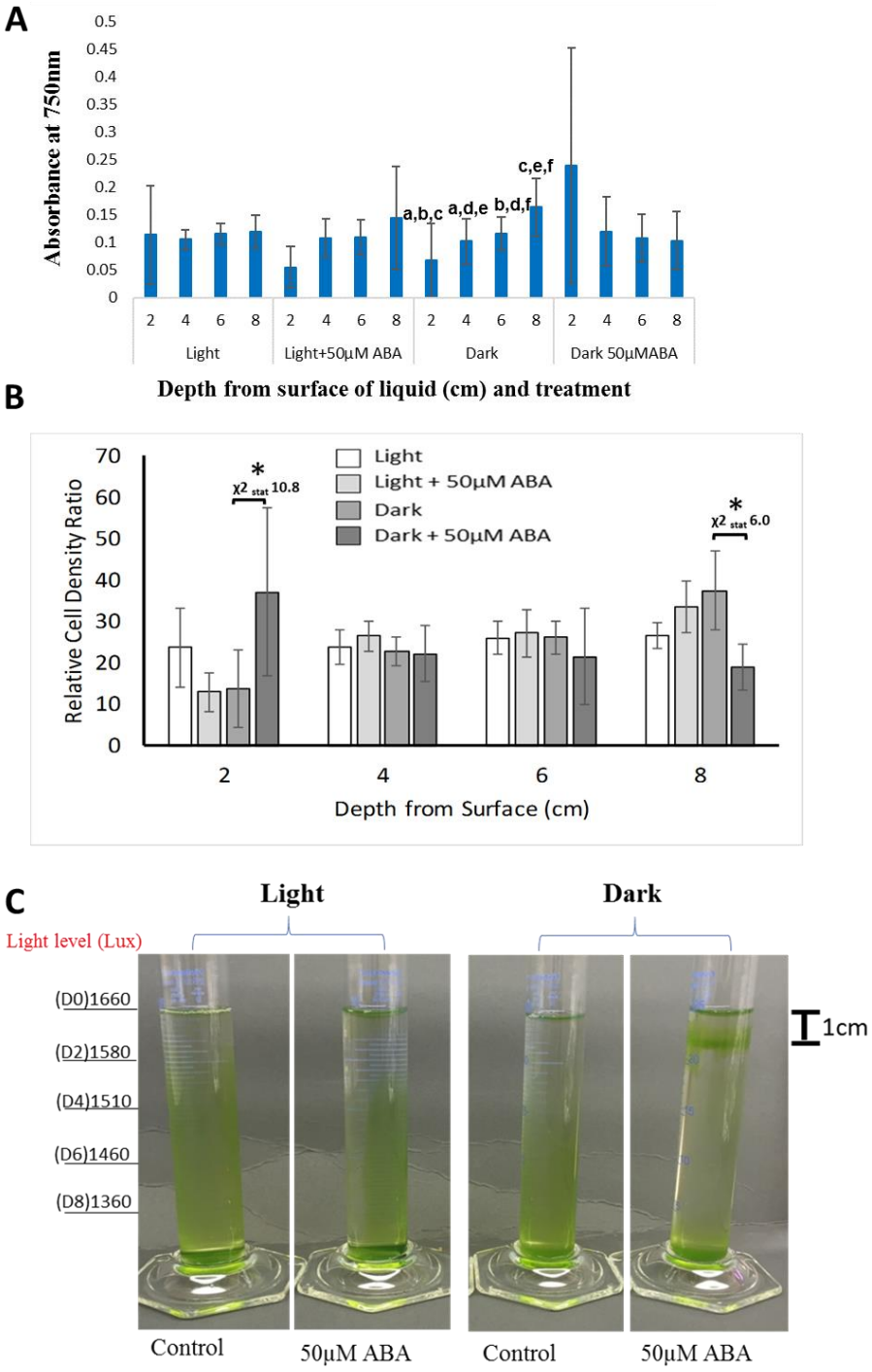


Figure 3.13: ABA alters the positioning of C. reinhardtii in the water column when sampled at one hour into a dark period and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 1 hour into the dark period and were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the light or dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to f). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of the algae resulting from the ABA treatments at specific depths are indicated$^{(*)}$ along with the χ^2_{stat} values

(larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

3.3.5 Effect of ABA on movement four hours into the dark

C. reinhardtii cultures were similarly sampled 4 hours after the start of the dark period and algae were again assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation, in either the light or dark (see Figure 3.14). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within each of the treatments were apparent. In the light, the algae retreated towards the bottom of the tube such that the A_{750} at 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm, 4cm or 6cm depths (see Figure 3.14A), with 2.5 fold more algae accumulating at a depth of 8cm than at 2cm. The addition of 50µM ABA in the light retarded this downward movement of the algae to a small extent such that while the A_{750} at the 8cm depth remained significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm depth, it was no longer significantly different from that at the 4cm and 6cm depths (Figure 3.14A). At this time point, the algae generally settled downwards in the dark. The A_{750} at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher from that at all the other depths measured with 2.5-fold more algae at this depth compared to that at 2cm (see Figure 3.14A). The addition of 50µM ABA in the dark caused the algae to move upwards and accumulate near to the surface of the water column such that the A_{750} at the 2cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher than at all the other depths (see Figure 3.14A).

In order to compare the effect of ABA on the overall distribution of the algae in either the light or the dark, the relative cell densities were calculated from the A_{750}

measurements. When comparing the distribution of the algae following their incubation either in the light or in the light with 50µM ABA, there was no overall significant ($P>0.05$ by χ^2 test of independence) difference observed (see Figure 3.14B). However, in the dark the addition of ABA caused a significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column with those algae accumulating near to the surface at the 2cm depth being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (see Figure 3.14B). The algae moved upwards significantly ($P<0.05$ by $\chi 2$ tests of goodness of fit) to form a discrete band at the 2cm depth, but again at this sampling time point the proportion of algae that did this was less than what occurred when the algae were sampled during the photoperiod (see Figure 3.14 C). Thus, overall, when sampled at 4 hours into the dark period, ABA treatment induced the algae to swim upwards towards the surface when they were subsequently incubated in the dark.

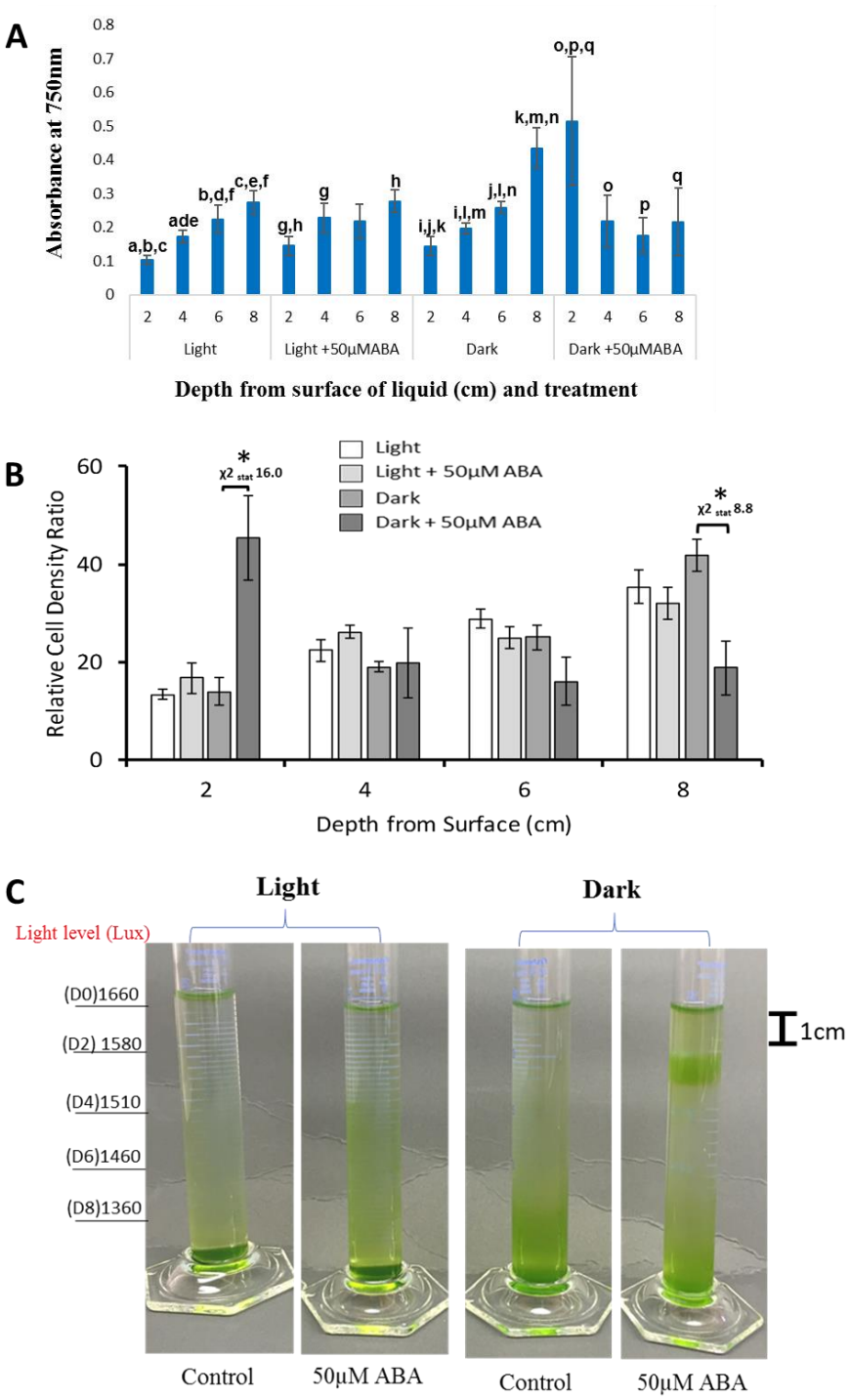


Figure 3.14: ABA alters the positioning of C. reinhardtii in the water column when sampled at four hours into a dark period and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 4 hours into the dark period and were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the light or dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to q). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of

the algae resulting from the ABA treatments at specific depths are indicated(*) along with the χ^2_{stat} values (larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

3.3.6 Effect of ABA on movement seven hours into the dark

Cultures of *C. reinhardtii* were sampled 7 hours after the start of the dark period and algae were again assayed for their vertical position in the water column 50 minutes after their treatment +/- 50µM ABA and incubation, in either the light or dark (see Figure 3.15). Following incubation, significant ($P<0.05$ by Kruskal Wallis) differences in the mean ranks of the A_{750} absorbances at different depths within each of the treatments were apparent. In the light, the algae retreated towards the bottom of the tube such that the A_{750} at the 6cm and 8cm depths were significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm depth (see Figure 3.15A), with 1.5 fold more algae accumulating at a depth of 8cm than at 2cm. The addition of 50µM ABA in the light retarded this downward movement of the algae. The A_{750} at the 8cm depth remained significantly ($P<0.05$ by Mann Whitney U test) higher than that at the 2cm and 4cm depths (see Figure 3.15A). In the dark, a proportion of the algae remained near the surface, with the majority settling downwards such that, overall, they remained more dispersed. The A_{750} at the 8cm depth was significantly ($P<0.05$ by Mann Whitney U test) higher from the 2cm and 4cm depths (see Figure 3.15A). The addition of 50µM ABA in the dark caused the algae to move upwards and accumulate near to the surface of the water column. However, there was no significant difference in A_{750} at the 2cm depth ($P<0.05$ by Mann Whitney U test) from the algal distribution at all the other depths (see Figure 3.15A).

In order to compare the effect of ABA on the overall distribution of the algae in either the light or the dark, the relative cell densities were calculated from the A_{750} measurements. When comparing the distribution of the algae following their incubation

either in the light or in the light with 50µM ABA, there was no overall significant ($P>0.05$ by χ^2 test of independence) difference observed (see Figure 3.15B). However, in the dark the addition of ABA caused a marked and significant ($P<0.05$ by χ^2 test of independence) change in the overall distribution of the algae in the water column with those algae accumulating near to the bottom at the 8cm depth being predominantly responsible for the significance ($P<0.05$ by χ^2 test of goodness of fit) of this observation (see Figure 3.15B). Significantly ($P<0.05$ by χ^2 test of goodness of fit) less algae were also apparent at the 8cm depths in the dark +ABA treatment compared to when the algae were incubated in the dark alone. Thus, overall, when sampled at 7 hours into the dark period, ABA treatment induced the majority of the algae to swim upwards towards the surface when they were subsequently incubated in the dark but less pronounced.

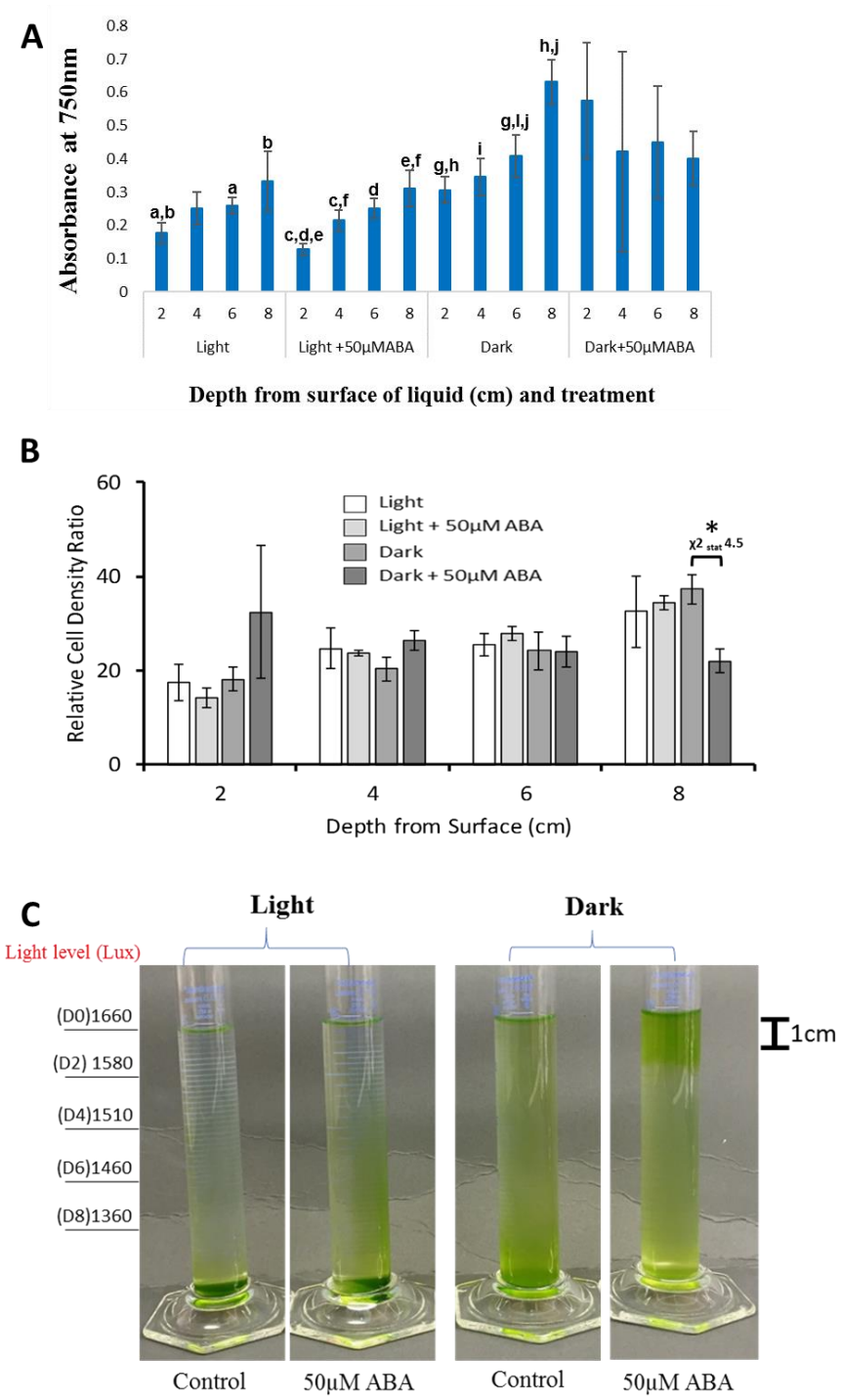


Figure 3.15: ABA alters the positioning of C. reinhardtii in the water column when sampled at seven hours into the dark period and incubated in the dark

C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3 were sampled 7 hours into the dark period and were divided between 25mL measuring cylinders and were treated +/- 50µM ABA with incubation in either the light or dark for 50 min. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated (A). Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to j). The relative cell densities +/- the 95% confidence intervals around the means are shown in (B). Within either the light or dark incubations, only significant ($P<0.05$ by χ^2 tests of goodness of fit) differences in the distribution of

the algae resulting from the ABA treatments at specific depths are indicated(*) along with the χ^2_{stat} values (larger χ^2_{stat} indicates a more significant difference). A representative image of the distribution of the algae in the measuring cylinders immediately following incubation and the light levels (Lux) at each of the depths sampled is shown in (C).

In summary, ABA led to the upward movement of algae when they were incubated in the dark to an extent dependent on their sampling time over the 24-hour cycle. For example, when sampled at the beginning of the photoperiod the response of the dark-incubated algae to the ABA treatment was very pronounced and after incubation 12.8-fold more algae accumulated at a depth of 2cm than at 8cm (see Figure 3.16). As the photoperiod progressed, the effect of the ABA on the dark-incubated algae was reduced, albeit insignificantly. However, at the end of the photoperiod and during the dark period, although ABA treatment still induced upwards movement of the algae in the dark, the effect was significantly ($P<0.05$ by Mann Whitney U test) less pronounced such that only approximately 2 fold more algae accumulated at 2cm depth than at 8cm from the surface.

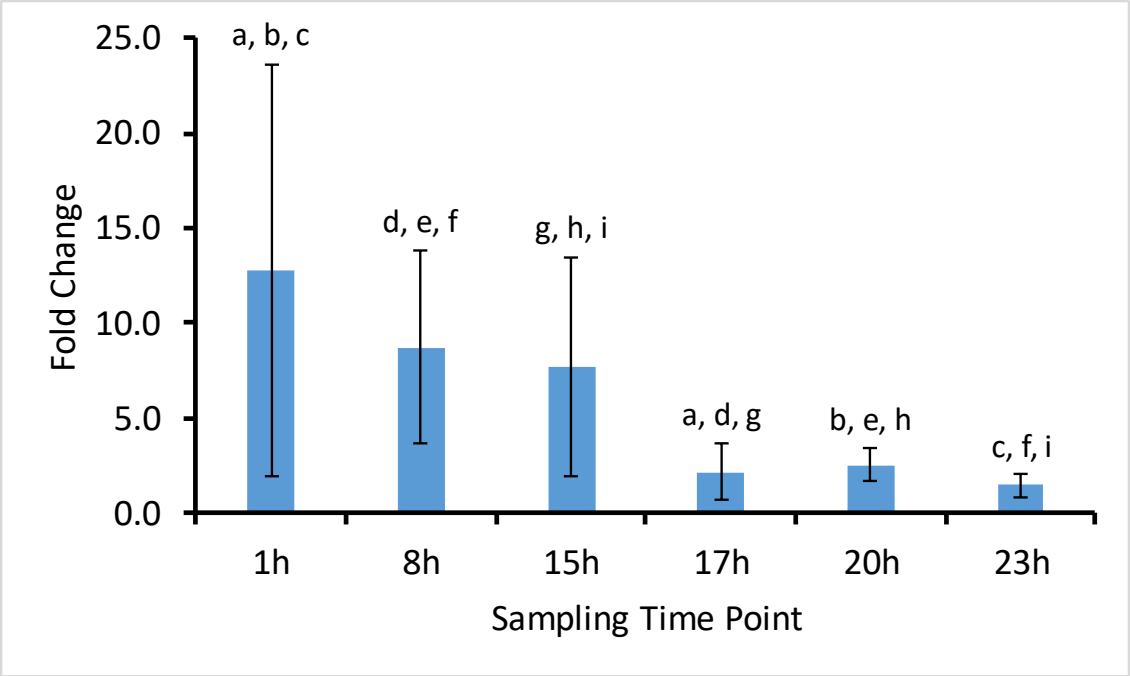


Figure 3.16: ABA alters the positioning of C. reinhardtii in the water column when sampled at different time points during the light/dark cycle and incubated in the dark
C. reinhardtii cultures were grown in TAP media until their A_{750} = 0.3, were sampled at different time points during the light/dark and were divided between 25mL measuring cylinders and were treated with 50µM ABA with incubation in the dark for 50 minutes. Following incubation, the A_{750} of the cultures in each of the cylinders was measured at the depths from the surface indicated and the mean (n=5) absorbances calculated. Significant pairwise difference ($p<0.05$ by Mann-Whitney U-Test)) in the mean ranks of the A_{750} measurements at different depths within treatment cylinders are indicated (a to i).

3.4 Horizontal movement of C. reinhardtii cells in response to ABA in the light and dark

Next, the effect of a fixed source of ABA on the movement of the algae in horizontal tubes was examined to separate the ABA effect from any gravity-dependent effects. After growing the cultures of *C. reinhardtii* in TAP media until A_{750} = 0.3, 25ml aliquots of the cultures were transferred to glass test tubes (15cm height and 1.5cm wide) in which 2ml plugs of 1×TAP media,1.5% (w/v) agar containing different concentrations of ABA had been placed at the bottom. The tubes were placed horizontally, either in the dark or under surrounding illumination, at 980 Lux for 2 hours at 25ºC. After incubation, they were photographed to illustrate the position attained by the motile algae (see Figure 3.17). In

the light, the algae moved towards the ABA gel plug while, conversely, in the dark they swam away (see Figure 3.17). Lower levels of ABA resulted in a similar, but intermediate response.

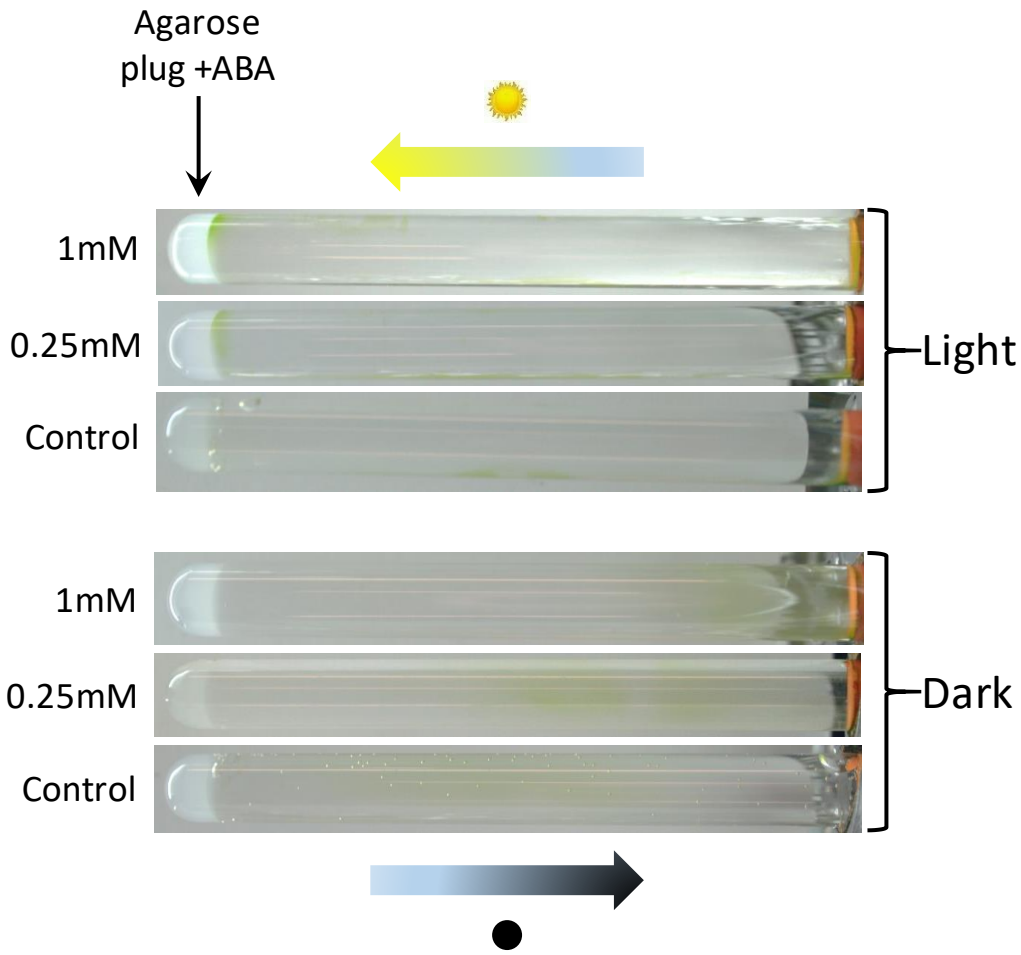


Figure 3.17: Opposing direction of travel of C. reinhardtii cells in response to ABA in the light and dark

Algal cultures were grown under a 16-hour photoperiod to the mid-log phase (A_{750} = 0.3) growth stage and were sampled mid photoperiod. Fully dispersed samples of the algal culture were placed in sealed tubes with a plug of agarose containing the concentrations of ABA indicated and were immediately placed horizontally in the light (980 Lux) or the dark for 50 minutes. Shown is a representative image indicating the positions attained by the algae immediately following the incubation period.

3.5 Measuring ABA levels in algae across the Circadian rhythm

The endogenous levels of ABA throughout a single diurnal growth cycle were determined by competitive ELISA. The endogenous ABA concentrations differed through the day and night cycle, suggesting an oscillatory pattern (see Figure 3.18). During the first 10 hours of the photoperiod, ABA levels increased significantly ($P<0.05$ by 2 sample t-test) by 1.8x to a maximum, and then declined significantly ($P<0.05$ by 2 sample t-test) to a minimum at 13 hours. After that, ABA levels significantly ($P<0.05$ by 2 sample t-test) increased again, reaching close to their maximum 2 hours into the dark period, before stabilising until the end of the 24-hour period. Interestingly, the time at which the level of ABA was lowest was that at which they began to form multiple bands when placed in the dark with ABA.

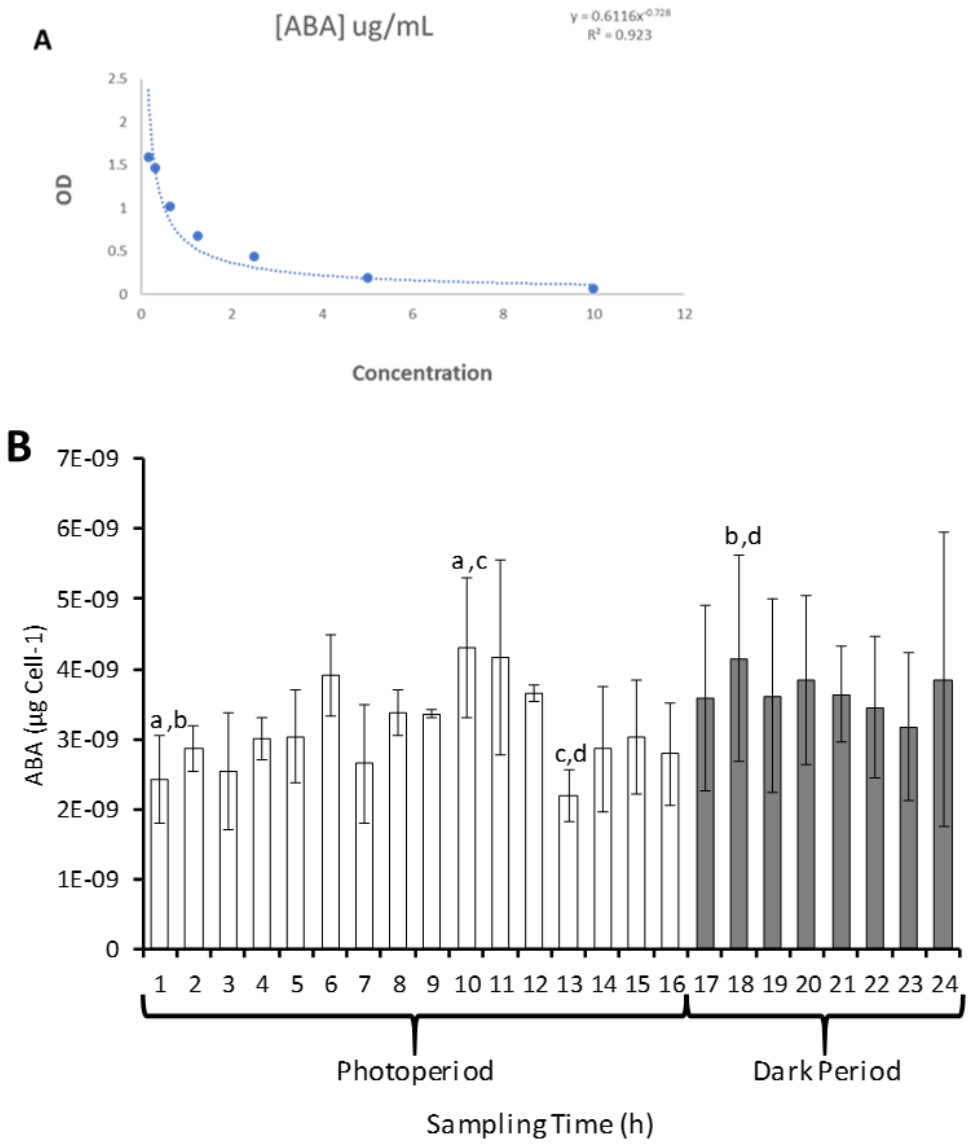


Figure 3.18: Endogenous abscisic acid (ABA) levels in C. reinhardtii
Algal cultures were grown under a 16-hour photoperiod to the mid-log phase (A_{750} = 0.3) growth stage and were sampled at the time points indicated over a subsequent 24-hour period, where the 16-hour photoperiod commenced at time = 0h. Algal cells were pelleted from n=3 replicate 25mL aliquots of culture. At each sampling point, the A750 of the cultures was determined to assess cell numbers. Pellets were extracted and assessed for ABA content by competitive ELISA (MyBioSource Inc.). The standard curve of ABA concentration are shown (A). Data are shown as the mean ABA content $cell^{-1}$ with error bars shown as +/- the 95% confidence interval around the mean in each case (B). Selected significant ($P<0.05$ by 2 sample t-test) pairwise differences in mean ABA levels are indicated (a to d).

3.6 ABA alters the photosynthetic efficiency of C. reinhardtii

Alginate-bead-encapsulated *C. reinhardtii* with different concentrations of ABA were incubated under various light levels in media containing a bicarbonate indicator. The mean rate of HCO^{3-} depletion of the medium by the algae generally increased with increasing light levels. Under comparatively high-light illumination (7440 Lux), 50µM ABA significantly ($P<0.05$ by Tukey Kramer's test) increased mean HCO^{3-} depletion of the media by the algae by ≈20% compared to the control algae under the same light levels. However, at low-light (253 Lux) 50µM ABA significantly reduced the mean HCO_3- depletion of the media by the algae by ≈14.5% (see Figure 8.3 in Appendix 8.7). Lower levels of ABA treatment had an an intermediate effect in this regard. At 1060 Lux ABA had no significant effect on the mean HCO^{3-} depletion of the media by the algae regardless of the treatment used (this experiment was performed by Adam Gregg).

3.7 Discussion

3.7.1 ABA alters photosynthesis, but not the growth of Chlamydomonas

Initially, this study examined the effect of various concentrations of abscisic acid (ABA), the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), indole-3-acetic acid (IAA) and 1-naphthaleneacetic acid (NAA) on the growth of *C. reihardtii*. The results indicated that the algal growth rate was not affected by ABA and IAA at all the concentrations tested (see Figure 3.1). When NAA was used, it slowed down the growth at a higher concentration of 100µM. ACC application also reduced growth a little bit when compared to the control of algae. A study by Park *et al.* (2013) indicated that, with the exception of ABA, plant hormones such as IAA, gibberellic acid (GA3), kinetin (KIN), and 1-triacontanol (TRIA) increased the growth rate of *C. reinhardtii*. Additionally, a study by Contreras-Pool *et al.* (2016) reported that the growth rate of *Chlorella saccharophila* was positively affected by ABA at various concentrations.

However, Contreras-Pool *et al.* (2016) investigated the effect of ABA by measuring cell volume and dry biomass accumulation. The weight increased because the cells got larger and became heavier when treated with ABA; the effect was not due to increased cell numbers. At higher light levels this study showed that ABA had the effect of increasing CO_2 uptake and perhaps this corresponded with the increased mass observed by Contreras-Pool *et al.* (2016); they grew their algae at 2970 Lux, a light intensity at which this study showed ABA increased HCO^{3-} depletion of the media by *C. reinhardtii*. Park *et al.* (2013) grew their algae at a light intensity of 6600 Lux. Perhaps, under these relatively high light conditions, auxin treatment does enhance *C. reinhardtii* growth. However, under the more ambient growth conditions used here (1485 Lux) this study could provide no evidence that ABA, IAA, NAA and ACC had any significant

effect on the growth rate of this alga. The question, then, is what ABA and the other phytohormones do in algae such as *Chlamydomonas*?

3.7.2 In the dark ABA-induced a negative geotropic response in Chlamydomonas

Since there was an indication that light levels had a bearing on the effect of the phytohormones, in particular ABA, on the growth of the alga, and since high light is likely a major source of abiotic stress to these microorganisms, this research next made a preliminary examination of the effect of NAA, ACC and ABA on the vertical movement of *C. reinhardtii* in response to high light and in the dark. Here, the results indicated that neither NAA, ACC nor H_2O_2 affected the vertical movement of *C. reihardtii* in the water column (see Figure 3.2) in either the light or dark. However, ABA significantly induced *C. reihardtii* to swim upwards in the dark (see Figure 3.3). *C. reinhardtii* are known to use their flagella to move to an appropriate environment, including a photosynthetically optimum distance from a light source (Foster and Smyth, 1980). Algal cells exposed to intense light move away from the light and stay there. However, here, when algae were treated with ABA, they retreated to a lesser extent from such a high light source. This suggests that the ABA affected the algal flagella movements in a way that led to a decrease in the extent of their avoidance movement in the light and an upward movement in the absence of light (see Figure 3.3). Thus, the data presented here suggests that ABA was involved in the algal movement in response to changes in the light levels in their environment.

ACC alone did not have any significant effect on the movement of the algae over the range of concentrations tested in this study or any significant antagonistic effect on the observed ABA responses in either the light or dark (see Figure 3.8). Thus, this study would suggest that, while the algae obviously respond to ABA, many of the

phytohormone signalling interactions and responses arose after the terrestrial transition and evolution of multicellular plants. However, it is clear that *Chlamydomonas* do respond to ABA. Recent sequencing of the *C. reinhardtii* genome has shown that it encodes most of the enzymes involved in ABA synthesis and metabolism and numerous genes with promoters containing ABA-responsive elements that potentially enable regulation by ABA in various contexts (Finkelstein, 2013). Thus, the question is how the alga perceives ABA. *Chlamydomonas* must have an ABA receptor. The alga does possess most of the ABA response and transport components, including ABFs, group A PP2C, SnRK2s, PDRs, ABCG25, and AITs (Wang *et al*., 2015). However, there are no orthologues of the genes encoding the plant cytosol/nuclear localised PYR/PYL/RCAR ABA receptor family (Fujii *et al*., 2009) in the *C. reinhardtii* genome (Wang *et al*., 2015). Potential orthologues of genes encoding the G-protein coupled receptor (GPCR) protein, GCR2 (Liu *et al*., 2007) and GPCR-types, GTG1 and GTG2, (Pandey *et al*., 2009) can be identified in the genome of this alga, but it is still unclear whether or not, as has been proposed for plants, these proteins really do act as ABA receptors (Christmann and Grill, 2009; Guo *et al*., 2011; Risk *et al*., 2009).

3.7.3 The ABA response in Chlamydomonas may be under light-mediated circadian control

Since ABA induced a response that appeared linked to ABA and light, it was determined whether that response also altered over a 24-hour period. Algae were sampled at different time-points over the 24-hour growth cycle (16-hour photoperiod), were treated with ABA, incubated in either the light or dark and their position in the water column monitored (see Fig 3.10). The result indicated that the actual resulting position of the algae in the water column depended on what time point they were sampled in the cycle.

For example, at the beginning of the photoperiod more 70 % of algae moved to the surface when treated with ABA in the dark (see Figure 3.10). In the middle of and later in the photoperiod, they also moved upwards in the dark when treated with ABA (see figures 3.11 and 3.12), but to a lower depth. During the dark period of the growth cycle, progressively less of the ABA-treated algae moved upwards in the dark and showed a more diffuse distribution in response to this hormone (see figures 3.13, 3.14 and 3.15). Such a differential response indicated the involvement of the circadian rhythm whereby the algae were only fully responsive to ABA in terms of their upward movement at the appropriate time, *i.e.* at dawn. This and other data suggest that just before sunrise and in the early morning algae respond to ABA and swim to the surface in order to photosynthesise. As the day progresses, they retreat from the increased light intensity and during the night swim down to repair their photosynthetic machinery and prepare for the next photoperiod. Early morning sunlight is less intense, and there is typically more red than blue light, meaning that the algae can tolerate being at the surface. In contrast, in the middle of the day, when there is a considerable increase in blue light intensity, remaining at the surface would likely result in photo-oxidative damage to their photosystems and so the algae retreat downwards (Fundamentals of Environmental-Measurements, 2014). There is also likely an evolutionary selective pressure for motile algae to remain at the bottom during the dark period. Many organisms swim up from the bottom during the night to feed and might well prey on algae that remained at the surface (Picapedra *et al*., 2018). Thus, those algae which retreated to the bottom at night likely had an increased chance of survival and thus instigated this rhythmic, ABA-driven response in the species.

In the dark, ABA-induced the algae to abandon their usual run and tumble manner of locomotion (Bennett & Golestanian, 2015) and to swim upwards. The question was,

then, how the algal cells use the ABA signal to do this? *Chlamydomonas* exhibit both positive and negative phototaxis depending on how much light the eyespot receives (Schaller *et al*., 1997). As the cell rotates about its body axis, the light intensity seen by the eyespot varies, and this produces differential movement of the two flagella, resulting in the algae swimming a helical path either towards or away from the light depending on its intensity. Here, one possibility was that the algal response to ABA in the dark was reversed by light. Thus, glass tubes, in which were placed at one end gel plugs of agarose containing various concentrations of ABA, were filled with well-dispersed suspensions of algae, immediately closed and placed horizontally, either with surrounding illumination or in the dark. Interestingly, in the light, the algae moved towards the ABA gel plug, while in the dark the algae swam away from the ABA source (see Figure 3.16). When lower levels of ABA were used in the gel plug the algae showed a similar, but an intermediate, response. Presumably, as the ABA diffused out of the gel plug, it formed a decreasing concentration gradient away from the agarose. Thus, the suggestion here is that either the algae orientated themselves using either an ABA concentration or perception gradient and that their direction of movement in response to the gradient depended on the presence or absence of light, or that light directly reversed the ABA response which occurred beyond a threshold concentration of the hormone. *Chlamydomonas* use rhodopsin-initiated Ca^{2+} currents to regulate flagella movements, and thus their orientation to light and phototactic responses (Harz and Hegemann, 1991). Ca^{2+} signalling is also known to regulate the gravitaxis of the alga, Euglena (Schwartzbach and Shigeoka, 2017). In higher plants, many ABA responses similarly involve Ca^{2+} signalling (Kudla *et al*., 2018). For example, the opening and closing of fully-hydrated *Arabidopsis* stomata in the light/dark cycle is an ABA-controlled process

(Ribeiro *et al*., 2009) involving downstream Ca^{2+} movement and signalling (Kudla *et al*., 2018). The case may be similar in motile algae such as *Chlamydomonas.*

Overall, the results suggest that ABA signals for upward movement of the algae and that this response is mediated by light levels and potentially the circadian rhythm. To better understand the potential involvement of the circadian rhythm underlying these observations, measurements were made of the endogenous ABA levels of the algae over a 24-hour period (see Figure 3.17). Through this incubation period the mean ABA levels varied between $2.2x10^{-9}$ to $4.3x10^{-9}$ µg $cell^{-1}$, which, assuming the mean volume of a *Chlamydomonas* cell to be 270 $µM^3$, equates to an endogenous concentration range of ≈30.8 to 60.3nM, which is similar to that described in other reports (Hartung, 2010). Interestingly, the time at which the minimum level of ABA was recorded is also that at which they began to band at multiple lower depths when placed in the dark with ABA treatment. Thus, although the change in ABA levels was considerably less than that observed in plants during stress (Hartung, 2010), there is evidence of a change during the daily cycle, and this does appear to coincide with the timing of altered responsiveness to the hormone.

3.7.4 How does this algal ABA response relate to plants?

Galvez-Valdivieso *et al.* (2009), reported that ABA is involved in plant responses to high-light, which reduces photo-oxidative damage to PSII. In maize leaves an increase in the production of ABA occurs following exposure to UV-B and is required for the associated synthesis of nitric oxide, which acts to ameliorate the damaging effects of such irradiation (Tossi *et al*., 2009). UV-B exposure similarly induces increased ABA synthesis in Arabidopsis plants (Rakitin *et al*., 2008). Other studies have also concluded that ABA improves the tolerance of grapevines to UV-B (Berli *et al*., 2010, 2011) and that the

increased levels of ABA that occur during drought stress reduce the sensitivity of several plant species to UV-B exposure (Berli and Bottini, 2013). ABA has also been shown to play a significant role in the regulation of expression of the light-harvesting chlorophyll a/b-binding protein (LHCB) under conditions of environmental stress. LHCB expression was inhibited when tomato leaves, *Arabidopsis* seedlings, *Lemma gibba* cells and developing seeds of soybean were treated with high concentrations of ABA (Bartholomew *et al.,* 1991; Staneloni *et al*., 2008; Weatherwax *et al*., 1996), while treatment with low levels of ABA increased LHCB1.2 transcripts in Arabidopsis seedlings and cab3 (GmLHCB) expression in soybean seeds (Chang and Walling, 1991; Voigt *et al*., 2010). Thus, physiological levels of ABA enhance LHCB expression (Liu *et al*., 2013), likely through the activity of the WRKY40 transcription factor, and may be required to adaptively fine-tune the photosynthetic capability of plants under conditions of environmental stress under varying light levels. Signalling *via* GCR1, GPA1, RPN1 and nuclear factor Y has been shown to integrate blue light and ABA signalling in order to regulate LHCB expression in etiolated Arabidopsis seedlings (Warpeha *et al*., 2007).

The results presented here suggest that an ABA signalling and high-light interaction increased algal photosynthetic efficiency in terms of HCO^{3-} uptake. Why ABA reduced algal HCO^{3-} uptake under low light is unclear. Wang *et al*. (2016) suggested that in terrestrial plants ABA initiates PSII repair mechanisms under low light conditions. Perhaps under low light conditions and in the dark ABA similarly initiates such processes in Chlamydomonas cells, which may explain their reduced photosynthetic efficiency. Previous studies by Saradhi *et al*. (2000) have reported that ABA may protect Chlamydomonas against photoinhibition and the results presented here would be consistent with such findings. What is interesting is the differential photosynthetic

response to ABA under various light levels and the fact that, at more ambient light intensities, ABA had no significant effect. Thus, the data suggest that ABA signalling and downstream responses in *Chlamydomonas*, as in plants, may be controlled by light and it may prove interesting to reassess this algal response under different light intensities and wavelengths.

The phytohormones control various aspects of plant growth and development and regulate responses to different abiotic stresses (Kim *et al*., 2008; Wani *et al*., 2016). ABA is involved in specific developmental events in plants, a good example being the light-dependent diurnal movements of guard cells that lead to stomatal opening and closing (Wani *et al*., 2016). Studies on seed germination also show that light plays a crucial role in regulating the expression of genes required for ABA metabolism and ABA levels. For example, in *Arabidopsis* and lettuce (*Lactuca sativa*) seeds, endogenous ABA levels are decreased under red light but increased under far-red light (Sawada *et al*., 2008; Seo *et al*., 2006; Toyomasu *et al*., 1994). High light levels induce stomatal closure in plants *via* an ABA-regulated process. As part of the stomatal closure process, calcium channels are activated by H_2O_2. The production of hydrogen peroxide by NADPH oxidases is induced by ABA (Pei *et al*., 2000). Loss of function mutation of the NADPH oxidases disrupts the activation of calcium channels, and the subsequent closure of stomata is impaired unless H_2O_2 is exogenously applied (Kwak *et al*., 2003). The growth and seed germination of such mutants are also less sensitive to ABA, the suggestion being that the function of H_2O_2 acts downstream of ABA in different physiological contexts. The *ABA insensitive 1* (*abi1*) and *abi2* mutants, defective in genes encoding protein phosphatase 2C-like enzymes, show reduced ABA induction of H_2O_2 and stomatal closure in response to H_2O_2 (Murata *et al*., 2001) and Meinhard and Grill, (2001) and Meinhard *et al*, (2002)

found that the activities of these protein phosphatases are negatively regulated by H_2O_2 *in vitro*. *Chlamydomonas* possess the phosphatases and operate calcium signalling mechanisms during the flagella movements that dictate their phototaxis responses, but here finds no evidence that H_2O_2 was involved could be found. Perhaps the involvement of reactive oxygen species signalling evolved as an additional stress signalling mechanism in sessile plants that are inevitable, and therefore exposed to an increased chance of photo-oxidative stress.

Such ABA responses may have evolved from the light stress responses of algae which dictate their movement up and down to reach optimal photosynthetic conditions. These responses probably evolved over millions of years, and when algae moved out of the water to become simple land-dwelling plants, they likely took this biochemical machinery with them (Delwiche and Cooper, 2015). During this evolutionary process, such cells may have retained their core ABA signalling mechanisms while expanding their downstream responses as they developed into multicellular species and simple plants with multiple organs and reproductive strategies. It is then interesting that the etiolation/de-etiolation response in plants appears to involve the expression of many ABA-related genes (Humplík *et al*., 2015) and that plants grow upwards more at night in the pre-dawn hours than they do during the day (Seluzicki *et al* .2017). Perhaps this suggests the retention in higher plants of the algal ABA response observed here.

3.7.5 Is ABA a gravitropic hormone?

When *Chlamydomonas* are placed in complete darkness together with ABA, the algae move upwards to the surface. This begs the question of how the cells determine which way is up. Dogma would dictate that the algae perceive gravity and that ABA-induced

the cells to swim agravitropically. But this then raises the further question as to how the cells may perceive gravity?

Much research has been conducted into the gravitropic mechanisms in plants, but the evidence remains inconclusive as to how they actually detect gravity and even less is known about how algae might achieve this. In plants, one proposal is the starch–statolith hypothesis, whereby the physical sedimentation of starch-filled amyloplasts (statoliths) in gravity-sensing cells (statocytes) triggers biochemical and physiological signals that are relayed to the responding tissues (Kiss, 2000). The transmitted signals induce differential cellular growth, resulting in the downward curvature of roots or upward curvature of shoots. The primary signal believed to trigger such differential growth is, of course, the plant hormone auxin (Band *et al*., 2012; Muday and Rahman, 2008), which accumulates to higher levels along one flank of the root or shoot, resulting in either differential growth inhibition or promotion and thus bending in the appropriate direction. Starch synthesis mutants are typically less gravitropic (Vitha *et al*., 2007), but this may be a result of the disruption of their energy balance and thus a general inability to be responsive to stimuli. *Chlamydomonas* do form starch granules in their chloroplast (Koo *et al*., 2017), but these would be almost non-existent at the end of the night, which is just when they are most responsive to ABA in terms of their upward movement. Thus, although it cannot be ruled out, it would seem unlikely that this alga uses such a starch granule sedimentation mechanism to sense gravity.

An alternative hypothesis is that the algae do not, in fact, perceive gravity at all, but are simply light responsive. Algae and the cyanobacteria from which they evolved are ancient organisms and evolved billions of years ago (Umen, 2014). Perhaps, then, in considering how these aquatic organisms orientate themselves, one must look even

further back in evolutionary history to the earliest forms of life. The current "best guess" is that very simple life forms with a genetic code based on RNA originated 3.8 billion years ago around alkaline thermal vents and used proton gradient mechanisms linked to a rudimentary ATP synthase to generate energy (Jheeta, 2017; Lodish, 2000). Such organisms may have moved up and down in the thermal incline using the change in pH across their outer membrane to drive internal energy production. The obvious question is why such very early, simple, single-cell life forms with no mechanisms of independent movement would have had any gravity sensing mechanisms. Presumably, floating around in the turbulence of the thermal vents, orientation with respect to gravity was not a selective evolutionary pressure in such cells. At some point, life escaped from the thermal vents and this required organisms to evolve alternative mechanisms for forming a proton gradient across a membrane. In the plant lineage, the mechanism that evolved was photosynthesis, which uses light to drive the movement of hydrogen ions across a membrane (Lodish, 2000). Photosynthetic cyanobacteria, which are already complex at a molecular level, may have evolved as early as 2.5 billion years ago (Schirrmeister *et al*. 2011). Of course, the prerequisite of the evolution of photosynthesis is that, first, an even more ancient organism must have evolved a protein/pigment based mechanism which enabled the perception of light (Leliaert, 2012). As soon as this occurred, such an organism would have been able to orientate itself towards the light. In other words, it would have been able to perceive which way was up and down without any inherent mechanism of sensing gravity *per se*. Presumably, photosynthesis evolved in organisms that lived at depth and were exposed to light levels considerably lower than that of terrestrial plants today. As such organisms evolved motility and came closer to the surface and encountered higher light intensities, they would have had to evolve

mechanisms to protect their photosystems. Thus, carotenoid biosynthesis and the inclusion of these photo-protective pigments in the photosynthetic machinery likely evolved at this time (Takaichi, 2011). They would also have needed to develop stress-related signalling mechanisms that enabled them to respond to diurnally increased light intensities, and of course ABA synthesis is intrinsically linked downstream to that of the carotenoids (Ruiz-Sola and Rodríguez-Concepción, 2012). The results of the experiments presented here would certainly support the hypothesis that such organisms used ABA in this context. The only intriguing molecular question remaining is how, in the absence of gravity sensing, such organisms maintain a perception of which way is up in the complete absence of light.

More interesting is the concept that, since land plants evolved from algae, ABA may thus be directly involved in their apparent gravitropic responses. There is increasing evidence that the terrestrial orientation of plants results as much from phototropism (Vandenbrink *et al*., 2014) and hydrotropism (Shkolnik and Fromm, 2016) as it does from gravitropism, and experiments conducted in the "microgravity" environment of the space station have done much to separate the contribution made by each of these tropisms in determining plant morphology (Poulet *et al*., 2016). The simple fact that seeds germinate normally on the space station and show normal meristem cell arrangement during this process would indicate that gravity is not required *per se* in the determination of plant morphology. Perhaps, as in the algal experiments described here, ABA may modulate the extent of the phototropic and hydrotropic responses exhibited by plants in a manner dependent on the environmental conditions. Further studies with *Chlamydomonas* may help to elucidate the interactive ABA signalling mechanisms that operate in algae, and

thus terrestrial plants, to optimise their biochemistry and physiology in a changing environment.

4 Chapter Four: Characterisation of the *CrGRP1* gene and analysis of its expression

4.1 Introduction

In plants such as *Arabidopsis thaliana*, at least eight genes encode GRPs (Kim *et al*., 2007). *AtGRP7* is regulated in response to the circadian rhythm, stress and ABA (Kim *et al*., 2008). There are strong indications that class IV GRP proteins play key *roles* in adaptation to biotic and abiotic stress which include pathogens, osmotic and salt stress, oxidative stress, and cold stress in plants such as *A. thaliana* (Fu *et al*., 2007; Kim *et al*., 2010, 2008, 2007; Schöning *et al*., 2008, 2007; Staiger and Green, 2011). *Chlamydomonas* appears to possess only a single class IV *GRP* which we have named *CrGRP1*. *C. reinhardtii* use flagella to move to an appropriate environment including the right distance from a light source. A study by Pazour *et al*. (2005), found that flagella contain more than 600 proteins. One of them is *CrGRP1* strongly suggesting that *CrGRP1* is involved in post-transcriptional control of its target mRNAs within flagella.

The class IV GRP proteins are a distinct subgroup within the heterogenous superfamily of glycine-rich proteins (GRPs), exemplified by the well-studied circadian *A. thaliana* protein *At*GRP7 (Schmidt *et al*., 2010). The class IV glycine-rich proteins are likely to work by regulating gene expression at multiple levels on their mRNA targets by modifying alternative splicing, mRNA export, mRNA translation, mRNA localisation and mRNA degradation (Kim *et al*., 2010).

The green alga *C. reinhardtii* is a well-studied unicellular flagellate alga. It grows rapidly and is amenable to biochemical analysis, cell biology, and classical genetics, as well as providing a useful tool for the study of basic biological processes. There is

considerable interest in developing it as a potential biofuel source in an economically viable bioreactor (Siaut *et al*., 2011). However, before this can be achieved, it is important to understand how the alga can adapt to biotic and abiotic stress.

It is hypothesised that the class IV GRPs are necessary for *C. reinhardtii* to adapt to stress; this question has not yet been addressed. Analysis of the alga's genome by BLAST suggested the presence of a single class IV GRP gene which named *CrGRP1*. To investigate the expression and function of *CrGRP1*, the following experiments were undertaken:

1- Examined the expression of *CrGRP1* in the Circadian cycle with and without exposure to the phytohormone ABA.

2- Examined the expression of *CrGRP1* in response to abiotic stresses such as H_2O_2. induced oxidative stress and changes to light conditions during the circadian rhythm.

3- Examined the expression of *CrGRP1* following light-regulated movements of *C. reinhardtii* after treatment with ABA.

4.2 The Sequence of CrGRP1

4.2.1 The *CrGRP1* promoter and transcribed sequence

In *A. thaliana* there are at least eight genes that encode class IV GRPs. At the start of this project, we wished to identify the class IV GRP gene sequences in algae. First, BLASTP was used to search in the *C. reinhardtii* genome for class IV GRP proteins, and found a single class IV GRP, which named *CrGRP1*. Figure 4.1 describes the *CrGRP1* gene, including its putative promoter, followed by its exons and introns. The promoter contains several potential binding sites for known transcription factors, some of which are linked to ABA signalling pathways. The size of the whole gene is ˜4801 bp including an upstream region that contain a promoter transcription factors binding site. However, the *Cr*GRP1 gene size with the seven introns and exons is 2821bp while it is 1743bp for mRNA after splicing.

```
   1 acctggtcaa tcaggggctg tggcaccacg cgatggggtt tgggtccgtg cctgacgcag
  61 agctccggca gctggcgagg agctgggggg cgttcgggcc ggcggcgcgg cgcggcaccg
 121 tgttcctgta ccggcccctg cttggcggag ctggcgaggc gcaggggcag gggcagcacg
 181 gcaagcggtg acgcaatgca gtagcctagt aggacagtcg gcagtagaca catctggatg
 241 ccccagggcg tgttccgcag ggtgtagtac cgaatgtggc gtagagctac tgcgttccgc
 301 ctacatgcaa cgccgaggga tggtcgggga gtcttgtaca atcccaagct gctaaatgag
 361 gggaagggtg caatccgggc ctctcgcgca ccctggatag gcgtggagta aggaatggag
 421 tatgactgaa ggagtaaggt cgccgtggca ggacgcggtg actcggcgcg gctgcgccat
 481 gtcaggtatg actgggaagt tccgccatgg tccgtggaaa gggtgcgatg aggcagtgtt
 541 aaggcaggtt ggtgaagtcc cgtagggtgc agtgattccc agaccccagc agatggcaaa
 601 caatttgcaa cggcagtgca ggagcgtgag gaatgcgcgt cagcaaaaca tcgacacaac
 661 ctgccagcta ccgtcggtac agtaacagcg cgcgaacgac gcaccaatgc cttgtccctt
 721 cttgctacgc atttatgtgg gcacgggtgg gagccacaca catcgggact acgctttcct
 781 gccccttgcc atgttccatc acgagcgggc agtgcaggag cgcgcggctg gtcgaacgcc
 841 ggagttactg tgccgacaag ttgctggctg gctggaagtt tggtccgtgg ggaagggtgc
 901 gatgggacag ggtgtgcgat gggacagggt gaggcgtagg tcggcattca cttggcatag
 961 tccctgaggt ccacgggaaa gggaggccag tgtatataac cgcaggatgt gtgggtgtgc
1021 atgtgccggt ctgtcggagc gaaagagagc caggcgtatg gacacacggc ttcacaggtg
                                                  DPBF
1081 cacttgcgac aggtgcccct gagacaagct gctggccatg accgctgagt aacagcaggt
1141 tgcatgccgg agggttcacc cggaggacga attgacggta ggttgccgag ggctgccatc
1201 aagcgagtac agggctgtca tccgacacagg gcagggccgc cagccaacag ctcaattcga
                           DRE/DPBF
1261 ccccgcctct ggcgctcagt tccacggcca actattgtaa cgcggtactg gtaacggggc
                                          ABRE/CAL
1321 agggcaaacg tttgcacacg cgcgccgttc tcccatattt gattgacgcc tgggttgagc
                       ABRE/CAL
1381 gtaacttgtt tctcggggct cagctgtatc agtgggacgc agccacgtgg ctgcaccaat
                                                  ABRE/GBOX
1441 ctgccccttg ccatgtccca tcacgtccat gtcgacacgg gcagggccaa ggggtgccct
                                        DRE/DPBF
1501 gctgtgacct gacggggaga cgaatcgacg gtgtggcctg ccgctcttgc agtcagctcg
                                DRE
1561 caatgtgcgc cactctccgt ccccgcagtt accgccatga ttctggtccc gaaactaagg
1621 tggggcaccg ctgtgttcac cagagttcgc aagccggcgt tgatcaagtc agaaagtcaa
1681 tgtcaaggga cgaccgcctc tcaacgagcg ctgagtgaca atggcgtgtg gtcagtgaaa
1741 gatggttcgt gtcgagcctg cggaggtgac catgccgcca agtttgacat ttccgtcagc
                                    ARE
1801 atttccgtta ggccagctcg cgtcagaccc gcgtggaggc tacccagcgt atgcggcaca
1861 catccggcac taccgatatc atcgcctaat gaaatacggg cgggaatcgg ctgacggccc
1921 atccggcaat acggcccatc cggcaatggg ccgcgttgtt gaaatactcc gagccgctca
1981 ggtctgatcc cacctgtact ctgtattcca atcaagtttc ctgtttggtt tcctggtttg
2041 agtatctagg cgtggtagac gccttttcct ttcagcaggc caatcacctg ctttctccgg
          E1F ------------------------------------------>
```

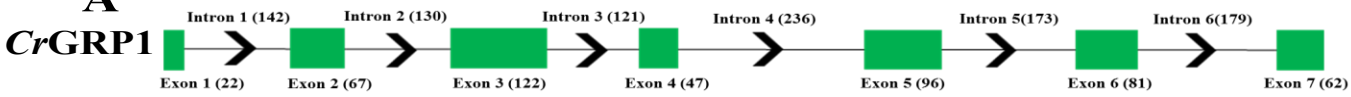

2101 ttcccttcct ctcggaaccA TGtcgaccaa gctctacgtc ggtgagcaat cataggcatg
M S T K L Y V
2161 caactgggct gcgcgaagtc gcgaaagctc gcgcgggaaa ggcggctggg gtcagccgcc
2221 tcaaaagcgc gcgctaaacc tctcggaacg gcctcgcacc tcctctcact cgctggccgc
E2F
E2R
2281 aggcaacctg tcgtgggaca cccgcgctga tgacctgacg aacctgttca gcaagttcgg
G N L S W D T R A D D L T N L F S K F G
2341 cgccgtcgag gtgagctttc gtctcgatgg gcccgaactg catctcggtt cagctggagg
A V A
2401 gccactctca gggcttgtcg cccggatccc cgaatatgca tgcactaatc gcaagtcttc
2461 atttgctttg ttccgcgcag gatgccttcg tcgccaccga ccgcgagact ggccggtcgc
D A F V A T D R E T G R S
E3F
E3R
2521 gcggctttgg cttcgtgact ctcgaggcca acgctgccaa gagcgcctgc tctgagtgcg
R G F G F V T L E A N A A K S A C S E C
2581 acggcaccga gttcatgggc cggtaagttt caacttatgt cggaaacgtt tcggtattgg
D G T E F M G R
2641 gcgccgcaga aagagatgca ccatagatgc cttccgacac tcgtgaatct catgtatttg
E4F
E4R
2701 ctttgtattt caacttcctg cagcaccatt cgcgtgaacg aggctactcc cctgggcgag
T I R V N E A T P L G E
2761 cgcccacctg gtgagttcaa ggctgccgag atcgcatgca actgtgtcta caaccccagg
R P P
2821 atacgctgat gcctggatac ctgttgtgga ggctttgtat tctgacgcat agtattgcat
2881 cactccaatc aatgacggct gctgcgggcg ttgggaagtt ccgaaatatc ctgctcgcca
2941 ggtatctccg tgcggcccgc cactgaccgc tttcctccct tgtcctcttc ccttgatccc
3001 gagcaggcgg ccgcggcggt ggccgtgggt acggtggtgg tggccgtggc cgtggcggtg
G G R G G G R G Y G G G G R G R G G
E5F
E5R
3061 actacggcgg cggccgtggc ggctacggtg gcggtggcta cggtgagcct tgcgagtgtc
D Y G G G R G G Y G G G G Y
3121 ctgttccaag caaaccacct gtgcgcccaa ggatcatctc gagtccgaca cttcggtgta
3181 gcgcttcaca aggcttccaa gggcatccaa gcagtgttgt gcgagtgcct tacagcgtgt
3241 gtttcggaat gctgacatgt tctgtttgtg tgtaggtggc ggcggctacg gtggcggccg
G G G G Y G G G R
E6F
E6R
3301 cggcggcggc ggctacggcg gcggtggcag cggcggctac ggcggcggcg gttacggtga
G G G G Y G G G G S G G Y G G G G Y
3361 ggccaacaca ggttcggccg attcttgcaa cgcgcgcttt gacgattggg tcacatctgc
3421 ttgatgttga gtcttgggtc gtgtgccaag ttgctgcaaa ggtttttttg ccaatgcctc

Figure 4.1: Structure sequences for C. reinhardtii CrGRP1 and its promoter sequences and structure.
The pink, gray, yellow, turquoise and dark yellow shaded sequences represent potential transcription binding sites in the promoter. DPBF=Dc3–promoter binding factor family, DRE=Dehydration-responsive element, ABRE/CAL= ABA responsive elements / CAULIFLOWER, ABRE/GBOX = ABA-responsive elements and ARE. The 5'UTR and 3'UTR are highlighted in orange text. The open reading frame is highlighted in green. Intron sequences are italicised in blue text. The light blue arrows denote primers which were designed across the various exons and the red highlight labels are potential apolyadenylation site.

4.2.2 Exon and intron structure of the *CrGRP1* transcript

The *CrGRP1* gene appears to include seven exons and introns; their sequences are presented in Figure 4.2 and the mapping of *CrGRP1* exons to the mRNA is shown in Figure 4.3. The splice sites are listed in Table 4.1

A



B

```
1    CTGTATTCCA ATCAAGTTTC CTGTTTGGTT TCCTGGTTTG AGTATCTAGG CGTGGTAGAC
61   GCCTTTTCCT TTCAGCAGGC CAATCACCTG CTTTCTCCGG TTCCCTTCCT CTCGGAACCA
121  TGTCGACCAA GCTCTACGTC Ggtgagcaat cataggcatg caactgggct gcgcgaagtc
181  gcgaaagctc gcgcgggaaa ggcggctggg gtcagccgcc tcaaaagcgc gcgctaaacc
241  tctcggaacg gcctcgcacc tcctctcact cgctggccgc agGCAACCTG TCGTGGGACA
301  CCCGCGCTGA TGACCTGACG AACCTGTTCA GCAAGTTCGG CGCCGTCGAG gtgagctttc
361  gtctcgatgg gcccgaactg catctcggtt cagctggagg gccactctca gggcttgtcg
421  cccggatccc cgaatatgca tgcactaatc gcaagtcttc atttgctttg ttccgcgcag
481  GATGCCTTCG TCGCCACCGA CCGCGAGACT GGCCGGTCGC GCGGCTTTGG CTTCGTGACT
541  CTCGAGGCCA ACGCTGCCAA GAGCGCCTGC T TGAGTGCG ACGGCACCGA GTTCATGGGC
601  CGgtaagttt caacttatgt cggaaacgtt tcggtattgg gcgccgcaga aagagatgca
661  ccatagatgc cttccgacac tcgtgaatct catgtatttg ctttgtattt caacttcctg
721  cagCACCATT CGCGTGAACG AGGCTACTCC CCTGGGCGAG CGCCCACCTG gtgagttcaa
781  ggctgccgag atcgcatgca actgtgtcta caaccccagg atacgctgat gcctggatac
841  ctgttgtgga ggctttgtat tctgacgcat agtattgcat cactccaatc aatgacggct
901  gctgcgggcg ttgggaagtt ccgaaatatc ctgctcgcca ggtatctccg tgcggcccgc
961  cactgaccgc tttcctccct tgtcctcttc ccttgatccc gagcagGCGG CCGCGGCGGT
1021 GGCCGTGGGT ACGGTGGTGG TGGCCGTGGC CGTGGCGGTG ACTACGGCGG CGGCCGTGGC
1081 GGCTACGGTG GCGGTGGCTA CGgtgagcct tgcgagtgtc ctgttccaag caaaccacct
1141 gtgcgcccaa ggatcatctc gagtccgaca cttcggtgta gcgcttcaca aggcttccaa
1201 gggcatccaa gcagtgttgt gcgagtgcct tacagcgtgt gtttcggaat gctgacatgt
1261 tctgtttgtg tgtagGTGGC GGCGGCTACG GTGGCGGCCG CGGCGGCGGC GGCTACGGCG
1321 GCGGTGGCAG CGGCGGCTAC GGCGGCGGCG GTTACGgtga ggccaacaca ggttcggccg
1381 attcttgcaa cgcgcgcttt gacgattggg tcacatctgc ttgatgttga gtcttgggtc
1441 gtgtgccaag ttgctgcaaa ggtttttttg ccaatgcctc tagtgttggt tgcttaaagt
1501 tgcctgacag gttttttcttc tgcctctctt tccagGCGGC AACGGTGGCG GCGGCTACGG
1621 TGCCGGTGGC GGCGGCTACG GTGGCGGCGG CTACTAAGCC CGCGGCTGAA GTCAGCGCAC
1681 GTCATGCCGC AGCACGCGGC ATGTTGAGGA GCCATCAAGG GAGCGTCAGA TTGGTGTAGG
1741 AGCAGTCTGC GAGACTTCTA AAGAGGCTTT GCAAGATCGT TGCCGAGGAC GCCGTGTACC
1801 GACGGGCGAT TTTCGCGTGC AAGCTTGAGT CATGAGACAA GGGCACGTTA GCGCGCGCCA
1861 GATGGTGCGT GCGGGCTCGG CGCGTTGGTA GTACAGGTGG CAAGCTTGCA CAACTTGTTT
1921 GGACGTCCAT CCACCGTATC AGTTCTACAA ACATAGCGTG GTGTGTTTCG GTCGAACGAA
1981 GAAACACATT ATGTGCTATG CATTGTTGTG CCGATTGCTG TGTGTGTACT GCGCATGTCT
2041 GCATGACAGA TGCAAAGCGC AAACTGCAGC CGGTGCTTTT GTGGTTCTGT GTATTGTGTG
2101 TTGGTTGATG AGGAAGTTGA GGGCAACAGG GGCGCAAGTC ACTCAGGCCC CGATGCCGAT
2161 TCCGTATCCT TTCCGGTGGT GGTTAGTGTT GCATACCGGT GTGTGTGTAG TTTGGTTTTG
2221 CGTTGGTTTG CACTTCTCAC ATGGTTTCTT CGTGTTTTCC AGTCGATGTT TGCCTGTATC
2281 CCCTATCCTC GAGCGCACCC TGGTCTGCAC GCCGTAAGCT GGGGCCCGGA AGCCTCTGCT
2341 GTGGCGGTGT GCGAGTCTCT TGGTTGCGTG CGGTTCCCGT AAGGCTTCCC GTGGTGGCGC
2401 AACACCGCGG CGACGTTTGC GTGGCGCGGG TGTGGTGAGG CTGCTGGATG GCGCCTCAAA
2461 CACTGGCGGC GCTTGGTGTT GTACCCTGTG CGCATCGGAT CCGAGTGTGT ATATGGCGAA
2521 TTACAGAACT ATGCCATATG CAACCACGTG CGGTGTACAT ACTCTGACCT TCTGCTTCGC
2581 ATGAGCGAGA GAGAGGACAC TGCCATGCTA CCCCAATTCA TCGGCTGGAA TTACGAATAC
2641 GTGTGGTGTT GCTTGATGCG TTACTCAAGT CCAATGGTGT TTCCAGTTCG GGTGCGAGAT
2701 ACGGGCGGCG CAAGTGGATT GACCCTTGCG TCTGCAAACG TACGAGCTAT CAGGTACTTG
2761 GTCCCCAGTC CGGAACTCAA TTGTCCGCTA CGGCTTCAAA ATACTGTCTC CCGGCTCTCT
2821 ACTATGGCTG TAAACTTAAA AGCGGAAA
```

Figure 4.2: Exons and introns of the CrGRP1 gene.
(A) Diagram showing the exon/intron structure of *CrGRP1*. (B) Capital letters in colours are the exon sequences (exon1- exon7), and small lowercase letters in italics correspond intron sequences. A potential poly(A) site is highlighted in yellow at the end of exon 7.

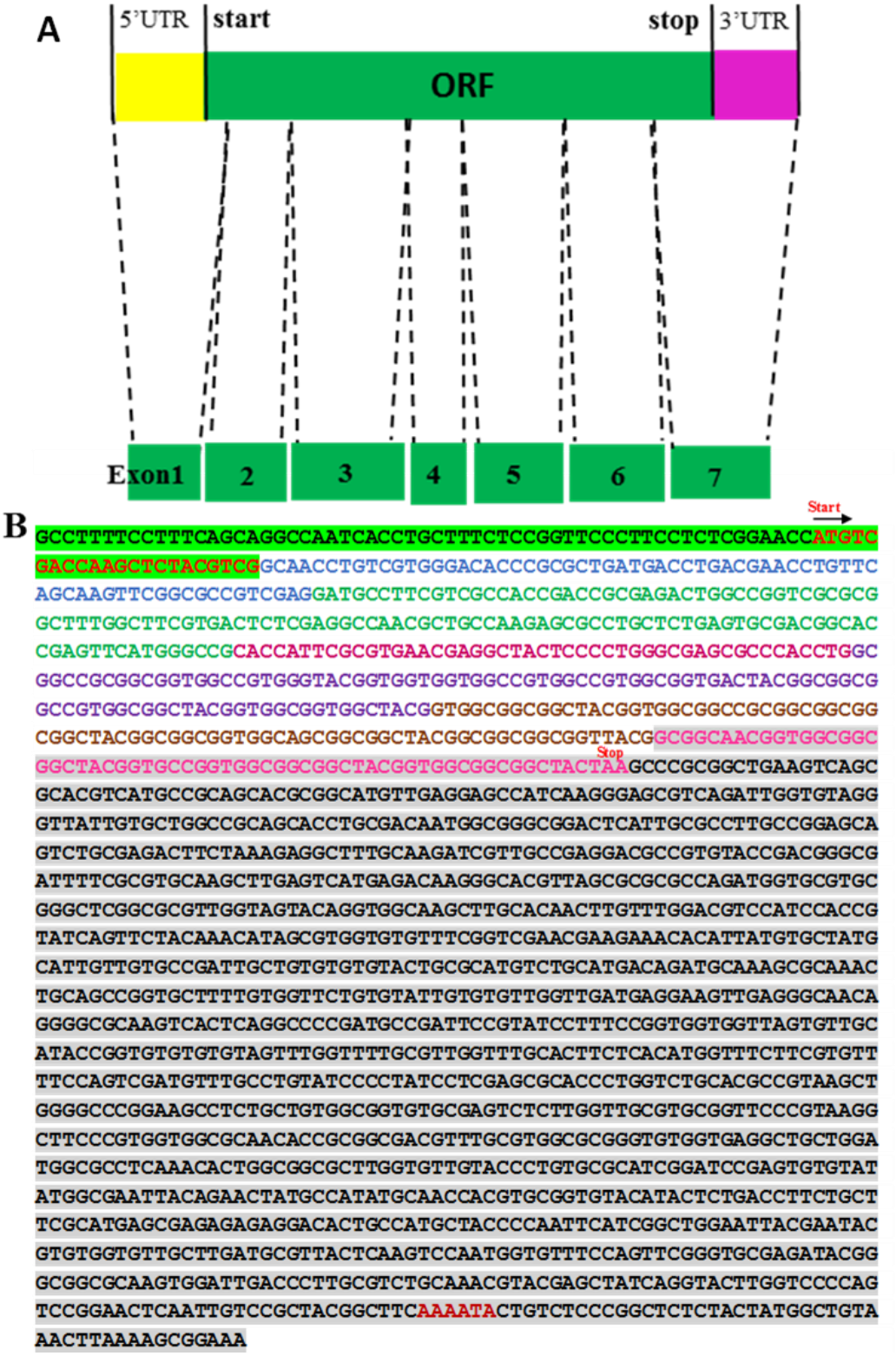


Figure 4.3: Mapping of exons of CrGRP1 to the mRNA.
(A) Mapping of exons of *CrGRP1* to the mRNA. (B) The *CrGRP1* mRNA sequence. Marked in green is the 5' UTR and in grey is the 3' UTR. The other colours are the exons in the mRNA (exon1- exon7); start and stop codons are also indicated, as well as the poly(A) signal.

	3'SS	**5'SS**
Exon 1	NA	TCGgtgagc
Exon 2	gcctcctctcactcgctggccgcagG	GAGgtgagc
Exon 3	tcttcatttgctttgttccgcgcagG	CCGgtaagt
Exon 4	tgctttgtatttcaacttcctgcagC	CTGgtgagt
Exon 5	gtcctcttcccttgatcccgagcagG	ACGgtgagc
Exon 6	gctgacatgttctgtttgtgtgtagG	ACGgtgagg
Exon 7	gtttttcttctgcctctctttccagG	NA

Table 4-1: Splice sites (SS) at the boundaries of CrGRP1 exons. The 3' splice sites include the putative pyrimidine tract. The exonic sequence is in capitals.

4.2.3 Features of *CrGRP1* amino-acid sequence and alignment with homologues across species

There are eight class IV GRP genes in *A. thaliana*. When comparing their sequences with *C. reinhardtii CrGRP1*, the most similar is *At*GRP7 (Figures 4.4 and 4.5). *At*GRP7 is regulated in response to circadian rhythm, stress and ABA (Cao *et al*, 2006). Searching for a similar class IVa GRP protein in a wide range of species revealed a very similar class IVa GRPs protein in different species such as *Oryza sativa Os*GRP2, *Xenopus laevis* CIRP-A (NP_001080069), *human RBM3* (NP_006734), *Camelina sativa Cs*GRP2a (AFK08576) and *Cs*GRP4 (XP_010512958.1) as shown in Figure 4.6). Class IVa GRP proteins contain an RNA regulating motif (RRM) in the N-terminus and a glycine-rich domain in the C-terminus. Moreover, similar class IVa GRPs were in other algal species such as *Volvox carteri f. nagariensis* (XP_002947747.1), *Polytomella sp. Pringsheim* (glycine-rich RNA binding protein - CAC86462.1) and *Monoraphidium neglectum* (RNP-1 like RNA-binding protein- XP_013904303.1), Figure 4.7.

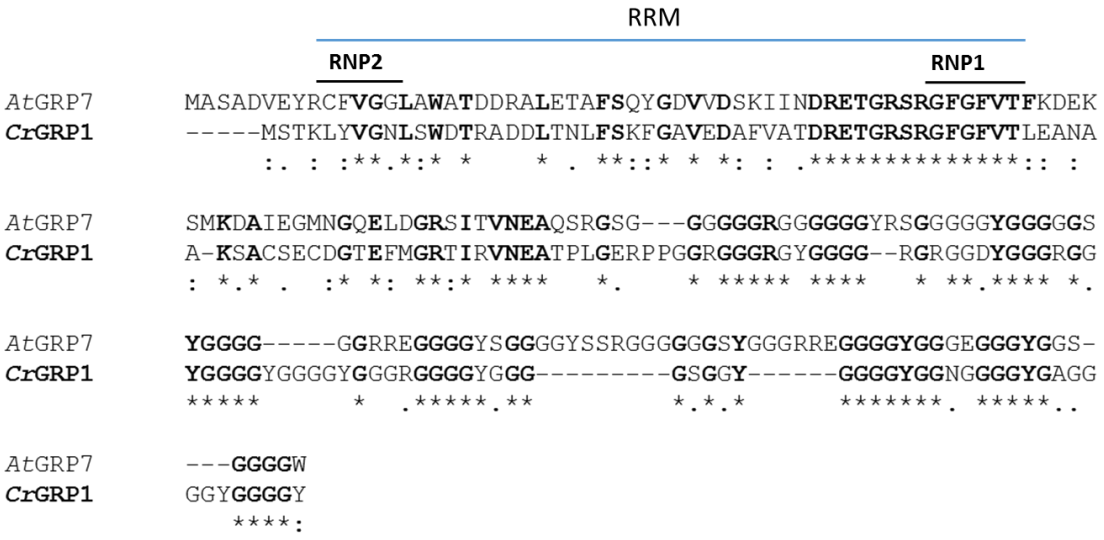


Figure 4.4: Amino-acid alignment of Arabidopsis thaliana AtGRP7 with C. reinhardtii CrGRP1.

The RNA Recognition Motif (RRM) with its characteristic RNP1 and RNP2 motifs is indicated; * denotes identity: denotes high conservation and weaker conservation.

A

```
                                                    RRM
                                                   RNP2
CrGRP1  ---------------------------------MSTKLYVGNLSWDTRADDLTNLFSK
AtGRP7  ----------------------------MASADVEYRCFVGGLAWATDDRALETAFSQ
AtGRP8  ----------------------------MSEVEYRCFVGGLAWATQDHDLERTFSQ
AtGRP4  ---MAFCNKLSGILRQGVSQSSNGPVTSMLGSLRYMSSKLFVGGLSWGTDDSSLKQAFTS
AtGRP6  MHYMGLFSRAGNIFRQPRAL--QASNAMLQGNLSLTPSKIFVGGLSPSTDVELLKEAFGS
                                                  : :**.*:  *    *  * .
                                        RNP1
CrGRP1  FGAVEDAFVATDRETGRSRGFGFVTLEANA-AKSACSECDGTEFMGRTIRVNEATPLGER
AtGRP7  YGDVVDSKIINDRETGRSRGFGFVTFKDEKSMKDAIEGMNGQELDGRSITVNEAQSRGSG
AtGRP8  FGDVLDSKIINDRETGRSRGFGFVTFKDEKAMRDAIEEMNGKELDGRTITVNEAQSRGSG
AtGRP4  FGEVTEATVIADRETGRSRGFGFVSFSCEDSANNAIKEMDGKELNGRQIRVNLATERSSA
AtGRP6  FGKIVDAVVVLDRESGLSRGFGFVTYDSIEVANNAMQAMQNKELDGRIIGVHPADSGGGG
        :* : :: :  ***:* *******: .     ..* .  :. *: ** * *: *   .

CrGRP1  PPGGRGGGRGYGGGGR--GRGGDYGGGRGGYGGGG-----YGGGGYGGGRGGGGYGGGGS
AtGRP7  G---GGGGRGGGGGGYRSGGGGGYGGGGGSYGGGGGGRR-EGGGGYSGGGGYSSRGGGGG
AtGRP8  G---G-GGRGGGGGGYRSGGGGGYGGGGGGYERRSGGYG-SG-------GGGGGR---GG
AtGRP4  PRSSF-------------GGGGGYGGGGGG--------------GY--------------
AtGRP6  GGGGF-------------ARRGGYGGGRGGYARGGFGRGGFGGGGYGFVR----------
                          .  *.**** *.

CrGRP1  GGYG-------GGGYGGNGGGGYGAGGGGYGGGGY     165
AtGRP7  GSY-GGGRREGGGGYGGGEGGGYGGS----GGGGW     176
AtGRP8  GGYGGGSGRREGGGYGGGDGGSYGGS----GGGGW     164
AtGRP4  ----------------------------------     136
AtGRP6  ----------------------------------     155
```

B

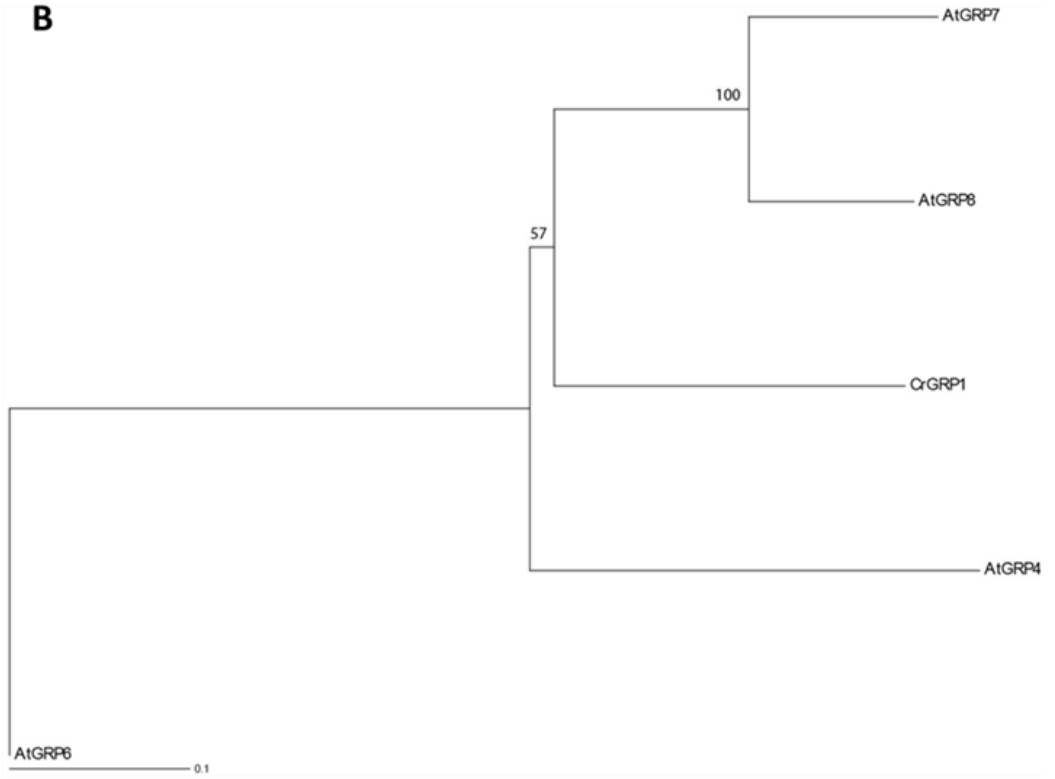


Figure 4.5: Alignment of CrGRP1 with similar class IVa GRPs proteins in Arabidopsis thaliana.

Class IVa proteins contain an RRM in the N-terminus and a glycine-rich domain in the C-terminus. (A) The alignment includes *A. thaliana* proteins *At*GRP2, *At*GRP4, *At*GRP6, *At*GRP7; *At*GRP8; and *C. reinhardtii CrGRP1;* * denotes identity: high conservation and weaker conservation. (B) the phylogenetic tree deduced from the alignment. The RNA Recognition Motif (RRM) with its characteristic RNP1 and RNP2 motifs is shown. Protein sequences were aligned using MUSCLE and Neighbour-joining trees were drawn in Paup 4.0. To test the confidence of the clustering, bootstrap values were determined with 500 replicates. The scale bar denotes number of amino acid substitutions per site.

A

```
                                                  RRM
                                                  RNP2
XlCIRP      ------------------------------MSDEGKLFIGGLNFETNEDCLEQAFTKY
HsRBM3      ------------------------------MSSEEGKLFVGGLNFNTDEQALEDHFSSF
CrGRP1      --------------------------------MSTKLYVGNLSWDTRADDLTNLFSKF
AtGRP7      -----------------------------MASADVEYRCFVGGLAWATDDRALETAFSQY
OsGRP2      MAAFNKLGSFLRHSGLTSSASAGSSPAMFNAARLMSTKLFVGGLSWNTNDDSLKEAFTSF
CsGRP4      MAFCNKLSGILRQG-VSH-SSNVPVTSMLGSLRYMSTKLFVGGLSWGTDDSSLKQAFSNF
CsGRP2a     MSFCNKLGGLLRQT-ITPSGGNVPVTSMLGSLRLMSTKLFVGGLSWGTDDQSLRDAFAHF
                                                 . : ::*.* : *    *   *: :
                                  RNP1
XlCIRP      GRISEVVVVKDRETKRSRGFGFVTFENVDDAKDAMMAMNGKSVDGRQIRVDQAGK---SS
HsRBM3      GPISEVVVVKDRETQRSRGFGFITFTNPEHASVAMRAMNGESLDGRQIRVDHAGK---SA
CrGRP1      GAVEDAFVATDRETGRSRGFGFVTLEA-NAAKSACSECDGTEFMGRTIRVNEATPLGERP
AtGRP7      GDVVDSKIINDRETGRSRGFGFVTFKDEKSMKDAIEGMNGQELDGRSITVNEAQS---RG
OsGRP2      GDVTEARVINDRESGRSRGFGFVSFANGDDAKSAMDAMDGKELEGRSIRVNFANE---RP
CsGRP4      GEVTEATVISDRETGRSRGFGFVSFSSEDSANTAVSEMDGKELNGRNIRVNLANE---RP
CsGRP2a     GEVVDAKVIVDRETGRSRGFGFVNFSDETAASAAISEMDGKDLNGRNIRVNQAND---RP
            * : :  :  ***: *******:.:      . *    :* .. ** * *: *

XlCIRP      GERRGGYRGGSSGGR-------------GFFRGGRGRGGGDRGYGSSRF-DNRSGGYGGS
HsRBM3      RGTRGGGFGAHGRGR-------------SYS-----RGGGDQGYGSGRYYDSRPGGYGYG
CrGRP1      PGGRGGGRGYGGGGR--GRGGDYGGGRGGYGGGGYG----GGGYGGGRGGGGYGGGGSGG
AtGRP7      SGGGGGGRGGGGGGYRSGGGGGYGGGGGSYGGGGGGRREGGGGYSGGGGYSSRGGGGGGS
OsGRP2      PGNR------GGGGY-GGGGGGYGNQGG-YGDGN-------RGYG-------------GQ
CsGRP4      SAPRSSF--GGGGGY-GGGGGG----GGGY-----------------------------
CsGRP2a     SAPRA-Y--GGGGGF-GGGGGSYGGGGGSYGGGGGSSFGGGGGDG------------GV
                     . *              :
```

B

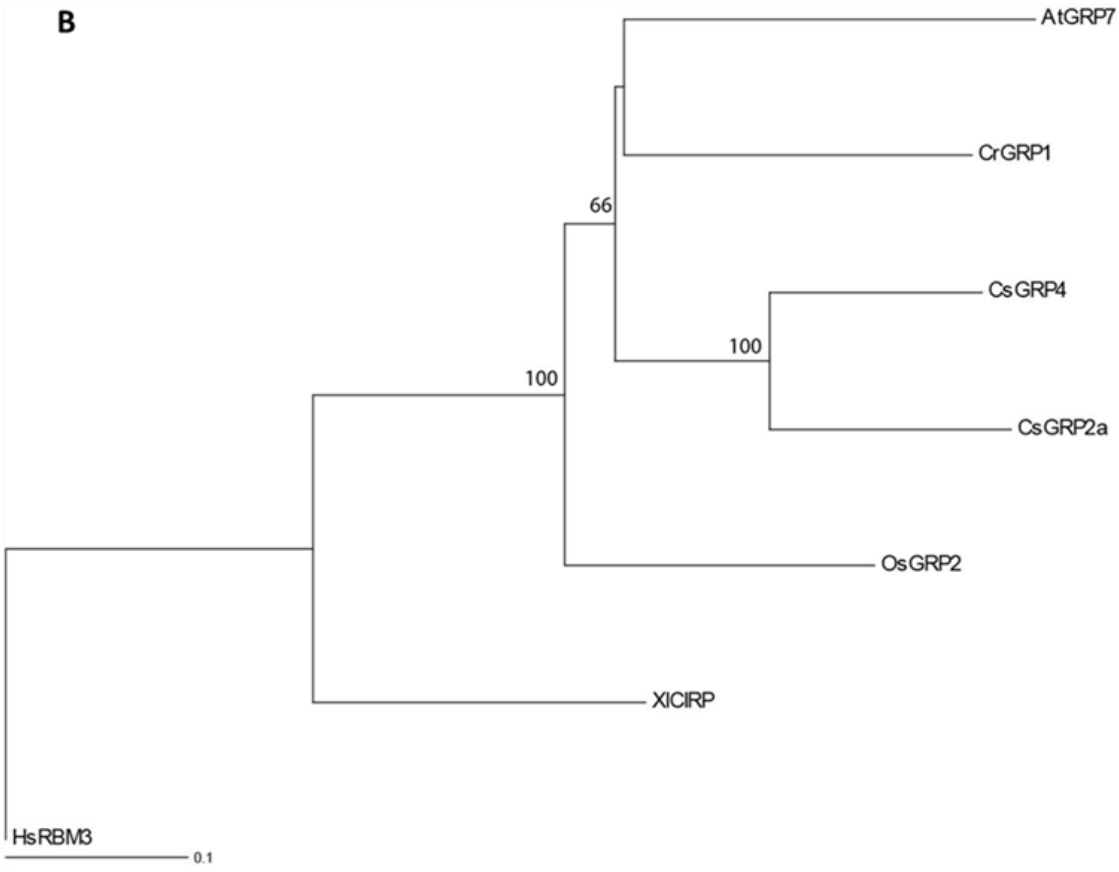
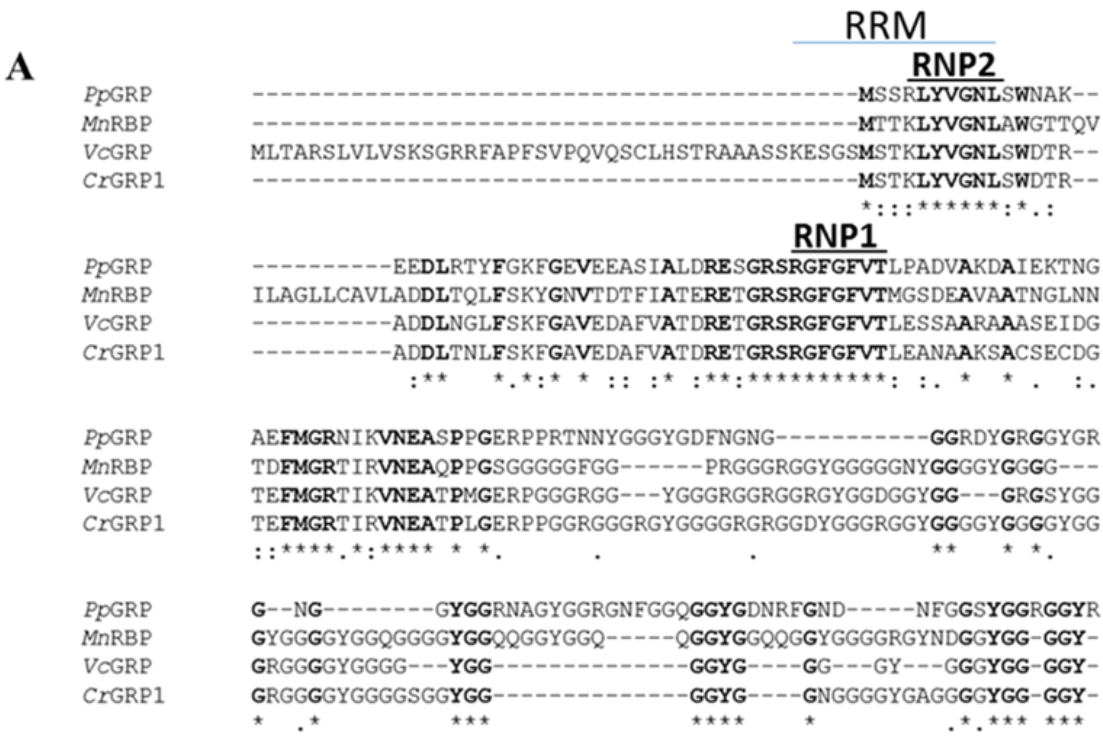


Figure 4.6: Alignment of similar class IVa GRPs protein present in a wide range of species across taxa.

Class IVa proteins contain an RRM in the N-terminus and a glycine-rich domain in the C-terminus. (A) The alignment includes *A.thaliana* proteins *At*GRP7, *Oryza sativa Os*GRP2; *Xenopus laevis* CIRP-A (NP_001080069); *human RBM3* (NP_006734); *Camelina sativa Cs*GRP2a(AFK08576); *Camelina sativa Cs*GRP4 (XP_010512958.1) and *C. reinhardtii CrGRP1,* * denotes identity, high conservation and weaker conservation. (B) The phylogenetic tree deduced from the alignment. The RNA Recognition Motif (RRM) with its characteristic RNP1 and RNP2 motifs is shown. Protein sequences were aligned using MUSCLE and Neighbour-joining trees were drawn in Paup 4.0. To test the confidence of the clustering, bootstrap values were determined with 500 replicates. The scale bar denotes number of amino acid substitutions per site.


A
RRM
RNP2
RNP1
PpGRP
MnRBP
VcGRP
CrGRP1

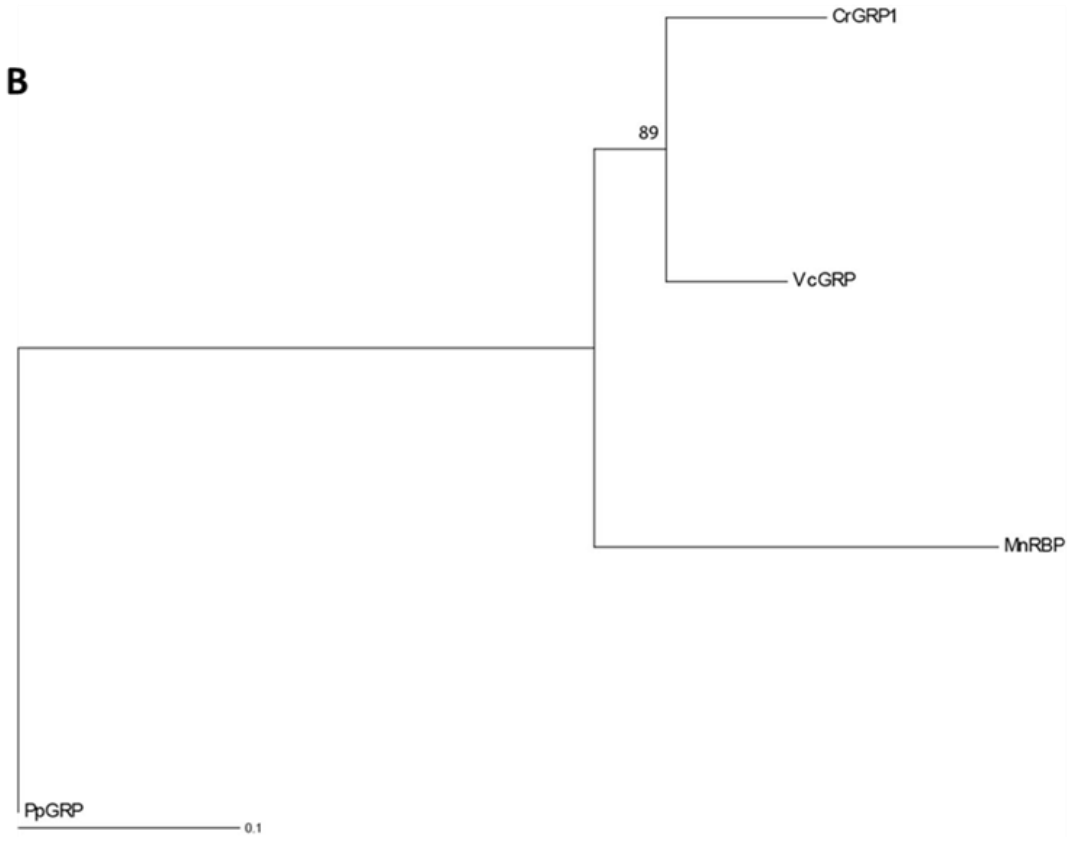

B
CrGRP1
89
VcGRP
MnRBP
PpGRP
0.1

Figure 4.7: Alignment of similar class IVa GRPs protein present in different algal species. Class IVa proteins contain an RRM in the N-terminus and a glycine-rich domain in the C-terminus. (A) The alignment includes *Volvox carteri f. nagariensi* s(XP_002947747.1), *Polytomella sp. Pringsheim* (glycine-rich RNA binding protein - CAC86462.1), *Monoraphidium neglectum* (RNP-1 like RNA-binding protein- XP_013904303.1) and *C. reinhardtii CrGRP:* * denotes identity: high conservation and weaker conservation. *(*B) The phylogenetic tree deduced from the alignment. The RNA Recognition Motif (RRM) with its characteristic RNP1 and RNP2 motifs is shown. . Protein sequences were aligned using MUSCLE and Neighbour-joining trees were drawn in Paup 4.0. To test the confidence of the clustering, bootstrap values were determined with 500 replicates. The scale bar denotes number of amino acid substitutions per site.

4.3 Expression of CrGRP1

4.3.1 Expression of *CrGRP1* during the Circadian cycle.

Cells of *C. reinhardtii* wild-type strain, CC1021 (mt +) were grown as described in Chapter 2. Cells were harvested every hour with and without 50µM ABA treatments and centrifuged at 4°C for ten minutes; cell pellets were stored at -80°C for RNA extraction. cDNA was synthesised from RNA extracted from mid-log growth phase and used for PCR amplification using *CrGRP1*-specific primers. Primers to *CrGRP1* were designed across several exons (Fig 4.1). Consistent amplification of the *Chlamydomonas* beta subunit-like polypeptide (*CBLP*) housekeeping gene confirmed that cDNA synthesis had worked, giving an amplicon of 187bp (Fig 4.8). Figure 4.1 B shows amplification of *ROC15*, a circadian gene, giving as expected an amplicon of 142bp (Mittag *et al*., 2014). The successful amplification of *CrGRP1* formally confirmed that the gene expresses a viable mRNA. Samples which were positive for the expression of *CrGRP1* using the primer combination 1F/2R yielded the expected amplicon size of 89bp (Fig 4.8) in samples that were treated with 50µM ABA, whereas in untreated samples the expression of *CrGRP1* was a bit lower during the daylight phase and started higher at the end of daylight and during the dark phase (Fig 4.8). These data suggested that *CrGRP1* is expressed in a circadian rhythm.

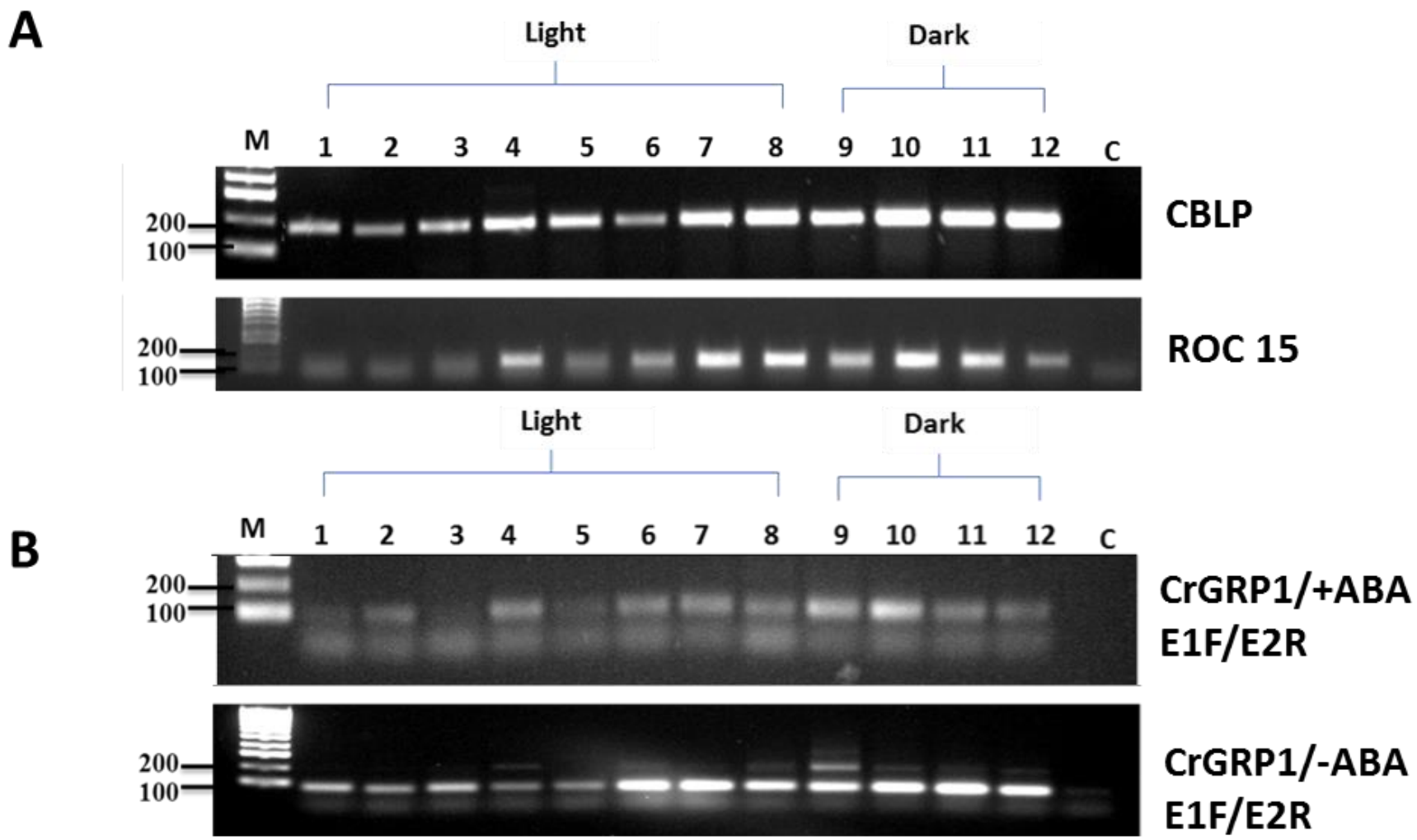

A
Light
Dark
M
1 2 3 4 5 6 7 8 9 10 11 12 C
200
100
CBLP
200
100
ROC 15
B
Light
Dark
M
1 2 3 4 5 6 7 8 9 10 11 12 C
200
100
CrGRP1/+ABA
E1F/E2R
200
100
CrGRP1/-ABA
E1F/E2R

Figure 4.8: Expression analysis of CrGRP1 in the Circadian rhythm in C. reinhardii.
PCR products from ABA treated (50 µM) and untreated culture were resolved on 1xTAE, 1.2% (w/v) agarose gel electrophoresis and visualised under UV light following EtBr staining. Molecular weight markers (Lane M – 100 bp DNA ladder) were used to confirm amplicon sizes, n=3 replicates. Lanes 1-8 contain the representative mRNA levels from a circadian experiment on the light condition at 2h, 4h, 6h, 8h,10h, 12h, 14h and 16h. Lane 9-12 represents the same as above from a circadian experiment on the dark condition at 18h , 20h , 22h and 24h. Lane C: control without cDNA added.

4.3.2 Expression of *CrGRP1* in response to abiotic stress

4.3.2.1 *Expression of CrGRP1 following hydrogen peroxide (H_2O_2)-induced oxidative stress*

Cultures of *C. . reinhardtii* wild-type strain, CC1021 (mt +) were grown and subjected to oxidative stress. For hydrogen peroxide (H_2O_2) stress treatment, cells were directly cultured in TAP medium with H_2O_2 at different concentrations (0.1 µM, 1 µM, 10 µM, 100 µM, 0.1 mM, and 1.0 mM). Cells were harvested from mid-log growth phase and centrifuged at 4 °C for 10 min for RNA extraction. First strand cDNA was synthesised from *C. reinhardtii* culture that had been treated with different concentrations of H_2O_2. To amplify *CrGRP1*, a range of primer combinations were tested including 1F-2R primers (89 bp amplicon), 1F-3R primers (190 bp) and 72F-R primers (224 bp); amplicon sizes were as expected (Fig 4.9 B, C, and D). Two bands were also produced in panel B. The band at 100 bp is the correct size for RNA in contrast to the other band of 250 bp which could be the RNA with intron included. Panel C shows three bands. The first one was at 190 bp confirms that the *CrGRP1* was expressed at the correct size consistent with spliced mRNA. The other band would be consistent with *CrGRP1* that was expressed as the mRNA retaining an intron as the splice was at size 500bp, but the identity of the 700 bp band is not known yet. However, it proved difficult to amplify the whole *CrGRP1* mRNA with primers exon 1F and 72R, with an expected amplicon size of 1743bp in treated cells (Fig 4.9, E), whereas the full-length sequence could be amplified in untreated cells (Lane 1, Fig 4.9, E). Both large amplicons, when sequenced separately, appeared to contain the exact same, full length *CrGRP1* amplicon, suggesting that the double band at this position is a gel running artefact (see Appendix 6). The larger amplicons in panels B and C, state result of the sequencing was also sequenced (see Appendix 6).

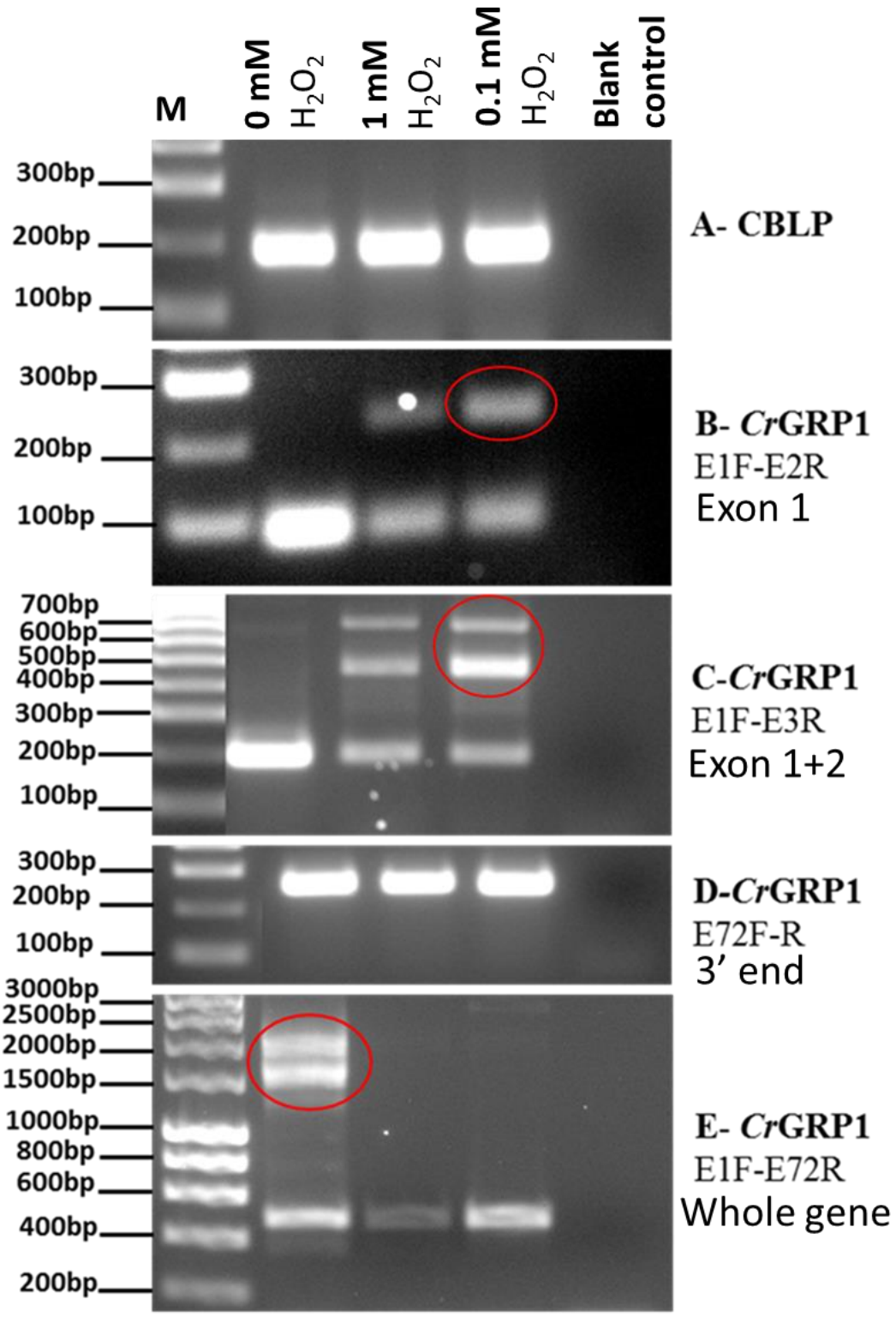


Figure 4.9: Expression of CrGRP1 in response to exposure to H_2O_2.
Total mRNA was extracted as earlier described. PCR output was performed with appropriate primers after cDNA synthesised. Amplicons were resolved in 1.2 %(w/v) agarose gel electrophoresis and viewed under UV light with ethidium bromide staining. n=3 replicates. Untreated cells express the full-length transcript (E) also, with all primers there was at least one band of the correct size. When cells were treated with

H_2O_2 the full-length transcript disappeared (red circle). The 3´ end of the transcript is still there (D). When H_2O_2 levels increased the result was a reduction in *CrGRP1* expression (B &C). A, B, C, D and E: Lane M, 100 bp DNA ladder; Lane 1, Untreated; Lane 2, treated with 1mM H_2O_2; Lane 3, 0.1mM H_2O_2; Lane 4, no template control.

4.3.2.2 *The expression of CrGRP1 in response to altering light conditions through the light/dark cycle.*

The light/dark cycle was modified to 8h light and 16 h dark simulating short days. Cells were harvested every 2 hours, RNA was isolated, and cDNA was synthesised. ROC15 is a circadian gene (Mittag *et al.*, 2014); it was amplified giving an expected amplicon of 142 bp (Fig 4.10 B); and as expected its expression peaked during light conditions (Mittag *et al.*, 2014). *CrGRP1* expression (190 bp amplicon) similarly peaked during the day. There was no apparent expression of the *CrGRP1* gene during the night (Fig 4.10 A). These data strongly suggest that *CrGRP1* is expressed in a circadian rhythm, comparable to its *A. thaliana* homologue *At*GRP7.

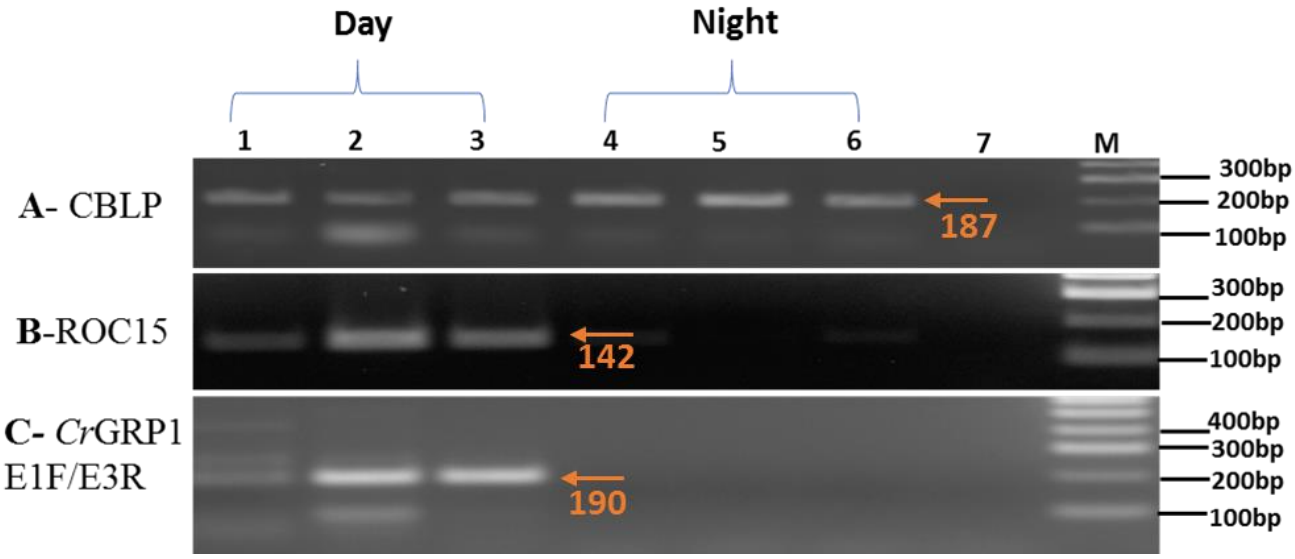


Figure 4.10: mRNA levels of CrGRP1 after changing the light conditions through the light/dark cycle.
A, B and C: Lanes 1-2-3 represent the cDNA from a circadian experiment on the light condition (day) at 9 h, 13 h, 15 h. Lanes 4-5-6 represent the cDNA from a circadian experiment on the dark condition (night) at 17 h, 19 h, 21 h. Lane 7, control without cDNA and Lane M, 100 bp DNA ladder. n=3 replicates.

4.3.3 Expression of *CrGRP1* in relation to ABA treatment and *C. reinhardtii* movement.

An aliquot of 25 ml (1×10^6-2×10^6 cells/mL) of cell suspension was transferred to glass measuring cylinder and treated with 50 µM of ABA. Cylinders were then placed at 25ºC either in the dark or under overhead illumination at 1660 Lux for 50 min. After an incubation period (50 min) the A_{750nm} of the culture obtained at various depths from the surface and the cylinders were photographed. This experiment was done at different time points throughout the culture period such that cells in the group labelled **A** were harvested after 1h in the photoperiod (Fig. 4.11). In **B** cells were harvested after 8h in the photoperiod representing the middle of the light phase; **C** after 15 h in the photoperiod; **D** harvested after 1h in the dark; **E** after 4 h in the dark representing the middle of dark phase and **F** after 7h in the dark. Samples were taken from each of these time points, and then treated for an hour with ABA either in dark or light conditions. RNA extraction was performed from cells in the logarithmic phase of growth and then frozen at -80 °C. This was followed by cDNA synthesis and PCR analysis of *CrGRP1* expression.

Results suggest an apparent decrease of *CrGRP1* expression in the ABA-treated group during the photoperiod compared to untreated samples (Fig 4.11). In contrast, *CrGRP1* levels appeared to be unaffected during the dark except the middle of the dark period when mRNA levels did appear decreased. When algae were taken from the middle of the dark phase and placed into the light phase, *CrGRP1* expression was restored. When ABA treated cells were placed either in the light or dark, *CrGRP1* expression decreased (Fig 4.11E). Similarly, when untreated cells grown in dark conditions, *CrGRP1* expression decreased as well. Together the data suggest that ABA reduces the expression of *CrGRP1* during the photoperiod and inhibits the expression of *CrGRP1* in the middle of the dark phase.

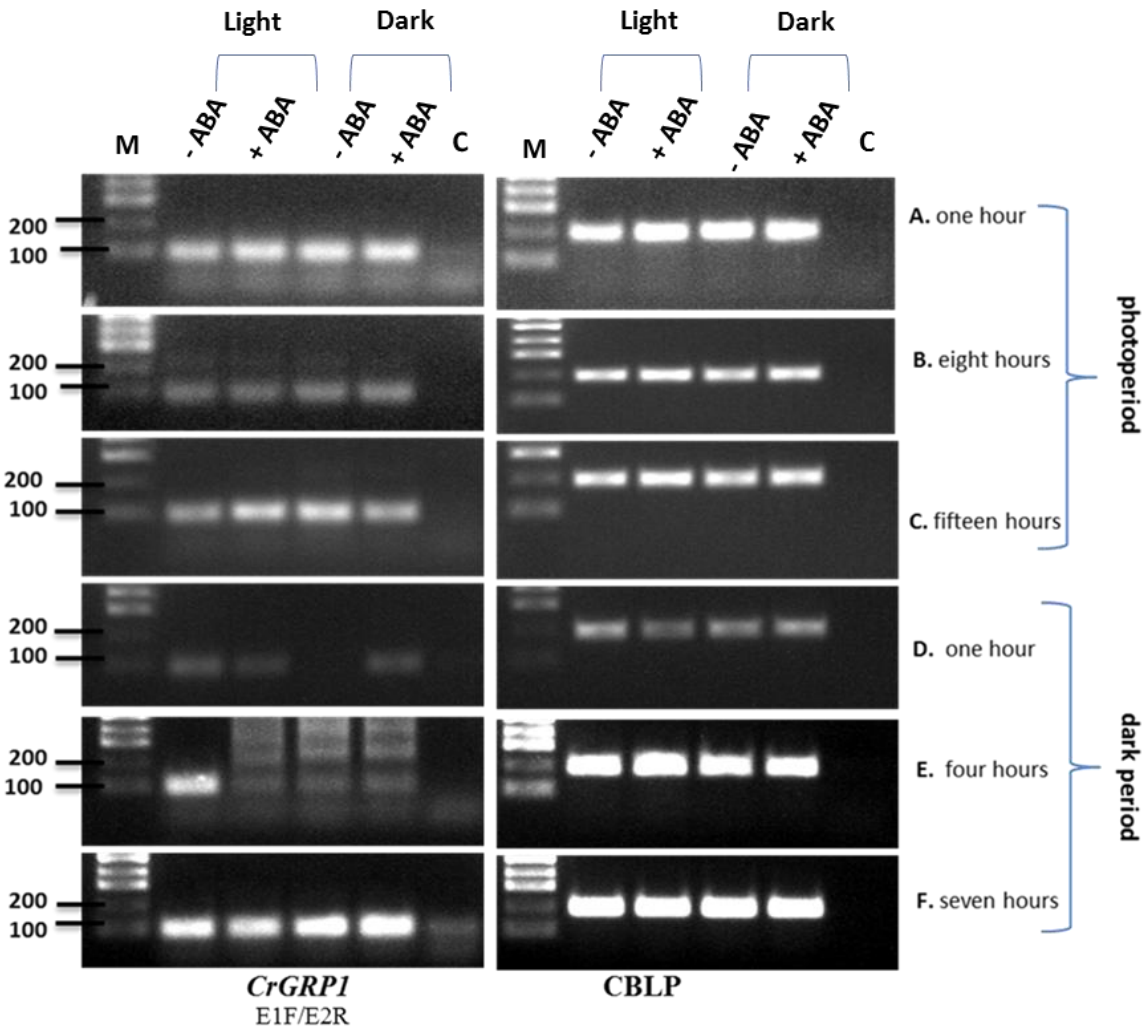


Figure 4.11: Expression of CrGRP1 following ABA treatment at different time points in the circadian cycle.

Samples were either treated with or without ABA. Amplicons were resolved in 1.2 %(w/v) agarose gel electrophoresis and viewed under UV light post ethidium bromide staining. n=3 replicates. A, B, C, D, E and F: Lane M, 100 bp DNA ladder; Lane 1, untreated in light; Lane 2, treated with ABA in light; Lane 3, untreated in the dark; Lane 4, treated with ABA in the dark and Lane 5, no template control.

4.4 Discussion

4.4.1 Analysis of the expression of *CrGRP1* during the Circadian cycle

The closest to *C. reinhardtii CrGRP1* sequence is that of *A. thaliana AtGRP7*. *AtGRP7* is regulated in response to circadian response, stress and ABA. Frequently, *C. reinhardtii* cells use flagella to move to an appropriate environment including the right distance from a light source. The expression of *CrGRP1* appears to be circadian regulated. *CrGRP1* showed lower expression during the day phase and highest expression during the dark phase (Fig 4.8), similar to the established circadian gene *ROC15*. In higher plants such as *A. thaliana At*GRP7 was the first gene encoding an RNA-binding protein to be considered circadian (Schmidt *et al*., 2010). The authors noted that the protein level was high during the day, but not during the night. In the present study, it was observed that the expression of *CrGRP1* was high during the day and not during the night.

Additionally, there is a functional relationship between ABA and GRPs in plants. When looking at the expression of *At*GRP7 and *At*GRP2 and their knockouts, both are expressed in contexts in which ABA plays important roles, for instance, plants do not germinate very well in an *At*GRP7 knockout (Kim *et al*., 2008 ; 2007). However, *At*GRP2 knockouts germinate very easily with ABA, and the phenotype looks like an ABA signalling pathway mutant (Kim *et al*., 2007). The evidence from *A.thaliana* suggests that *CrGRP1* may be involved in the ABA-mediated movement in response to environmental cues.

4.4.2 Analysis of the expression of *CrGRP1* in response to abiotic stress

4.4.2.1 *Expression of CrGRP1 following hydrogen peroxide (H_2O_2)-induced oxidative stress*

It was observed that H_2O_2 alters *CrGRP1*expression, presumably, by causing oxidative stress. *C. reinhardtii* cultures were treated with different concentrations of H_2O_2 (0 mM to 1.0 mM). The results show that the untreated cells express the full-length transcript (Fig 4.9). Also, using all primers, it obtained at least one band of the correct size. In algae treated with H_2O_2, the full-length transcript apparently decreased. However, the 3′ ends of the transcript were still detectable. When H_2O_2 concentration was increased, the result was a reduction in *CrGRP1* expression (Fig 4.9 B & C). Amplification of the housekeeping *CBLP* gene confirmed that cDNA synthesis had worked in each sample. Also, the use of –RT (no reverse transcription) controls, and the fact that CBLP primers are in adjoining exons, demonstrates that the signals were not due to contaminating genomic DNA. In the event of genomic DNA contamination, the band size of CBLP would have been bigger, 335 bp but instead, it was observed that the processed product was 187bp representing processed mRNA (Fig 4.9 C). Two bands were observed in Figure 4.8 B for *CrGRP1*. The band at 100 bp is the expected size for mRNA, in contrast to the other band measuring 250bp which may represent an mRNA with intron retention. Also, Figure 4.9 C shows three bands. The first one at 190 bp confirms that the *CrGRP1* was expressed at the correct size consistent with spliced mRNA. The second band was confirmed by sequencing to be an intron-containing *CrGRP1* mRNA with amplicon size of 500 bp, whereas the third band with amplicon size of 700 bp could not be sequenced (see Appendix 6 for sequence data). The 700 bp band could be a PCR artefact, or a non-specific band, or potentially an additional transcript expressed in response to H_2O_2 which would need further investigation. The data suggest that intron sequences are being

retained in *CrGRP1*mRNA; this might be a form of regulation of *CrGRP1* expression. Another possibility is that H_2O_2 causes degradation of *CrGRP1* transcript, perhaps through cleavage at a specific site. In summary, it appears that *CrGRP1* expression is regulated post transcriptionally, for example by regulating rates of intron retention.

4.4.2.2 *The expression of CrGRP1 in response to altering light conditions through the light/dark cycle.*

Next, the expression of *CrGRP1* was examined after changes in light conditions through the light /dark cycle. *CrGRP1* expression appeared clearly circadian on a diurnal basis. Amplification of *CrGRP1* gene mRNA from mid-log phase sample exposed to light/dark cycles shows that the *CrGRP1* gene is expressed in light conditions (day) and not as much in the dark (night) (Fig. 4.10 A). The *ROC15* gene (circadian gene) was also expressed in the light, not in the dark confirming that the *CrGRP1* was expressed in a similar circadian rhythm.

There are two circadian rhythms in *C. reinhardtii* through the night, the first one is called chemotaxis (to ammonium); it allows *C. reinhardtii* to find and store nutrients when solar energy is not available (Straley and Bruce, 1979). Another circadian rhythm in *C. reinhardtii* that peaks during the night is the ability of the cells to adhere to a glass surface (Mittag *et al*, (2005). ABA treatment significantly decreased the movement of *C. reinhardtii* whose flagella contain more than 600 proteins, and interestingly, one of which is *CrGRP1*. This suggests that ABA may be involved in the regulation of *CrGRP1* expression which then affects *C. reinhardtii* mobility in response to light stimuli. It is hypothesised that *CrGRP1* might be binding to key transcripts in the flagella, regulating their translation in response to changing environmental conditions (including day and night cycles).

4.4.3 Analysis the expression of *CrGRP1* in relation to ABA treatment *and C. reinhardtii* movement.

Finally, when the expression of *CrGRP1* was assessed during the algal movement experiments, it appeared to decline to the greatest extent when ABA-induced the greatest upward movement of the algae. Algal cells exposed to intense light move down the vessel and stay there. However, when algae were treated with ABA, algal cell movement was reduced. The suggests that ABA affects flagella movement which led to a decrease in the movement of *C. reinhardtii* cells and a possible decrease in *CrGRP1* mRNA levels (Fig 4.11) although, when looking at the expression of *CrGRP1* in the control samples , they all appeared similar (Fig 4.11A, B and C). Interestingly, in the middle of the dark phase, ABA appeared to inhibit the expression of *CrGRP1* only in the light. When the samples from the middle of the dark were returned to light, the expression was reversed to normal, but when treated with ABA and returned to light or dark condition, *CrGRP1* expression was inhibited by ABA.

An example that validates these findings is the expression of *Mh*GR-RBP1, which is a class IVa GRP in apple trees that was highly expressed in the leaves when compared with shoots and roots. When trees are treated with ABA for 24h, the expression of *Mh*GR-RBP1 decreases (Wang *et al*., 2012). In addition, in *A. thaliana At*GRP7 appears to play a key role in the regulation of ABA and stress response. The expression of *At*GRP7 in *Arabidopsis thaliana* was repressed when treated with ABA and NaCl or mannitol (Cao *et al*., 2006). Furthermore, when comparing the transcript levels in the mutants of *RD29A* and *RAB18* that have ABA-induced and stress-induced genes with a wild-type plant, the transcript levels were higher in the *At*GRP7 knockout plants than in wild-type plants (Cao *et al*., 2006). Kim *et al*. (2008) extended these findings and reported that after using another type of *At*GRP7 knock-out, the mutants were able to germinate and survive on

both media with high concentrations of NaCl or mannitol in comparison to either wild-type or overexpressed plant. Another study by Kim *et al*. (2007) found that the seed germination was affected by AtGRP2 under salt stress but not in the osmotic stress. Either in presence or absence of *At*GRP2, *AtGRP2* did not influence the growth of seedlings and plant development. This suggests that *At*GRP2, in contrast to *At*GRP7, is independent of the ABA pathway. The evidence indicated that *At*GRP2 and *At*GRP7 are involved in response to similar abiotic stresses, but their function may be antagonistic. Since *CrGRP1* is most closely related to *At*GRP7, it is hypothesised that in the middle of the dark period when *CrGRP1* levels drop, they release bound mRNAs. As a result, if *CrGRP1* disappears, then target mRNAs might be available for translation. If *CrGRP1* is not released, it would continue to repress the translation of the mRNAs it is bound to and ABA will be less effective, the light response will be more intense, and the algae will move down to the bottom of the growth vessel to avoid light stress. In summary the data presented here strongly suggest that ABA helps to regulate *CrGRP1* expression; future experiments will address the underlying mechanism.

5 Chapter Five: Functional analysis of *CrGRP1*

5.1 Introduction

In the previous chapters, data indicated that ABA regulates the movement of *C. reinhardtii* in the water column to position itself to optimise photosynthesis, and that *CrGRP1* may regulate the ABA-induced upward movement of the algae, presumably by binding mRNAs required for this response. Additionally, *CrGRP1* expression was observed to follow a Circadian rhythm. Together these findings suggest that there is a relationship between ABA signalling pathways and *CrGRP1* function. Therefore, in this chapter, we aimed to investigate the effect of overexpressing or silencing *CrGRP1* in transgenic lines on ABA-mediated movement responses.

There are several techniques that have been developed for the introduction of foreign DNA into *C. reinhardtii* such as glass beads and vortexing techniques (Kindle, 1990), electroporation (Shimogawara *et al.*, 1998) and *Agrobacterium*-mediated transformation (Kumar *et al.*, 2004). The electroporation technique was the method of choice here.

The aims of this chapter were:

1. To determine the effect of the overexpression with and without V5 6×His tag and knock-down of *CrGRP1* on overall phenotype. The V5-His tag allows detection and subsequent purification of the CrGRP1 protein.
2. To determine the effect of overexpression and knock-down of *CrGRP1* on the movement of *C. reinhardtii* in response to ABA.

5.2 Results

5.2.1 Generation of a pChlamy4 - *CrGRP1* construct

The restriction-cut *CrGRP1* open reading frame was cloned into pChlamy4 using T4 ligase. The cloning was carried out by Dundee Cell Products Ltd, James Lindsay Place, Dundee Technopole, Scotland U.K. The overexpression with and without V5 6×His tag and antisense constructs were transformed into TOP10 chemically competent *E. coli* cells. Transformations were plated onto LB plates containing 100 µg/mL of ampicillin and grown overnight at 37˚C. Colonies were then screened the following day by restriction digestion of their DNA using *Eco*RI and *Xba*I for overexpression, *Eco*RI and *Xho*I for overexpression plus a V5-6×His tag, *Bam*HI-Hf and *Xho*I for the antisense construct. Recombinant plasmids were selected for further work. Restriction cut recombinant plasmids were electrophoresed in a 1% agarose 1xTAE gel for visualisation (Figure 5.1). The band of ~3640 bp represents the expected length of the vector; the various constructs are shown in Figures 5.2 - 5.5. A band at ~480bp confirmed the presence of the *CrGRP1* open reading frame (5.1).

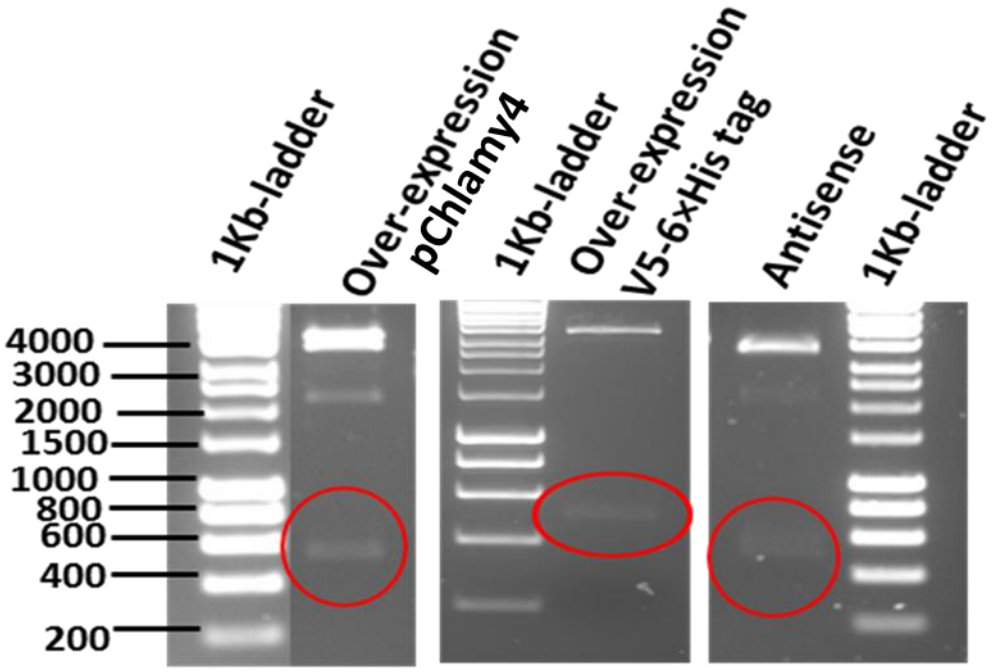


Figure 5.1: Restriction digest confirming the presence of the CrGRP1 ORF in the pChlamy4 vector.

Electrophoresis data obtained by digestion of recombinant *E. coli* plasmid pChlamy4 - *CrGRP1*. *Eco*RI and *Xba*I for overexpression, *Eco*RI and *Xho*I for overexpression V5-6×His tag, *Bam*HI-Hf and *Xho*I for the antisense. The *CrGRP1* ORF corresponds to a band of ~480bp (red circles). Markers (1 kb ladder) are shown on the left.

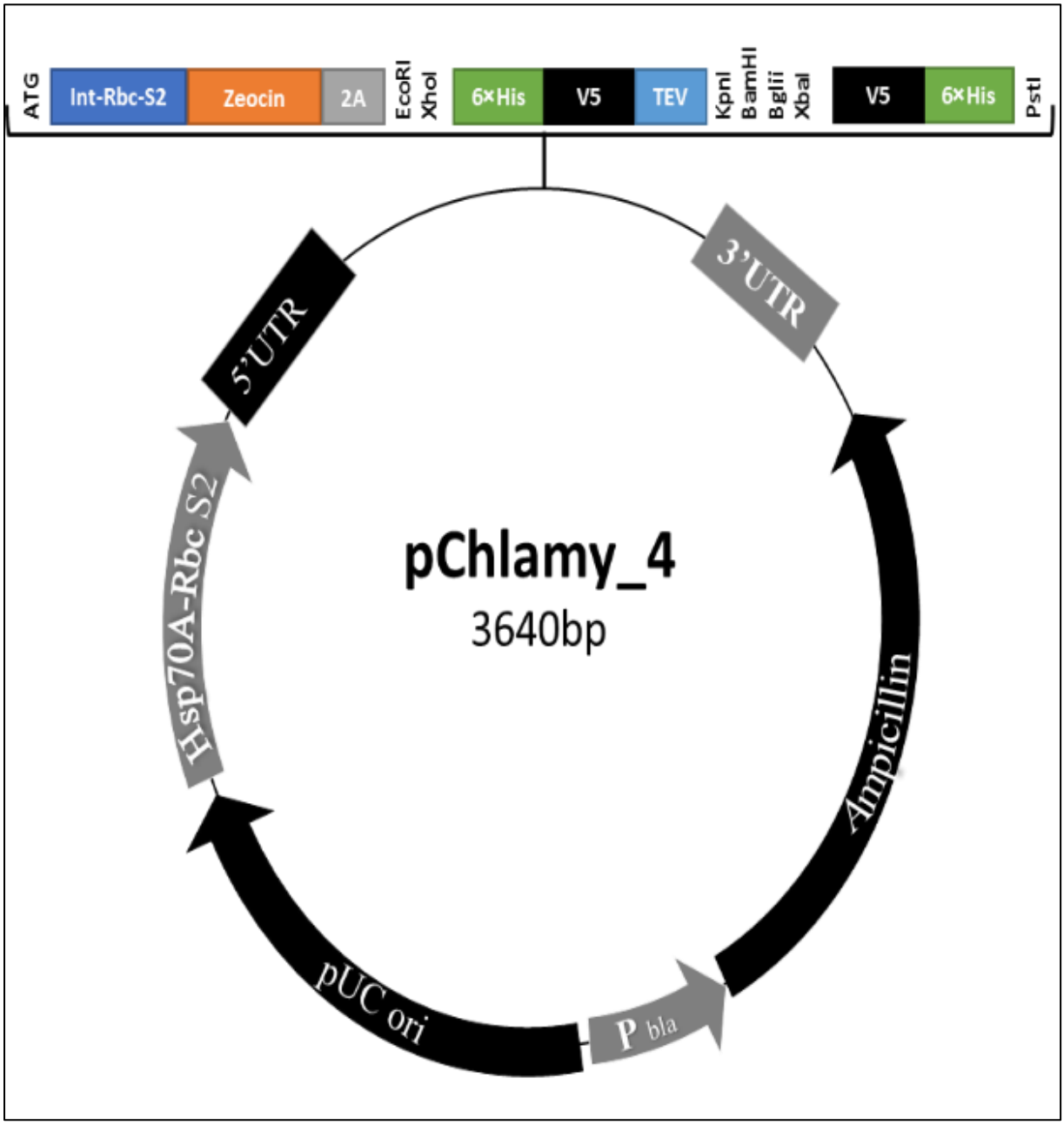


Figure 5.2: Map of vector pChlamy_4.
This vector pChlamy4 contains a *C. reinhardtii* Hsp70A/RbcS2 chimeric promoter and a *C. reinhardtii* RbcS2 terminator, both cloned into the polylinker of the *C. reinhardtii* vector pSP124S.

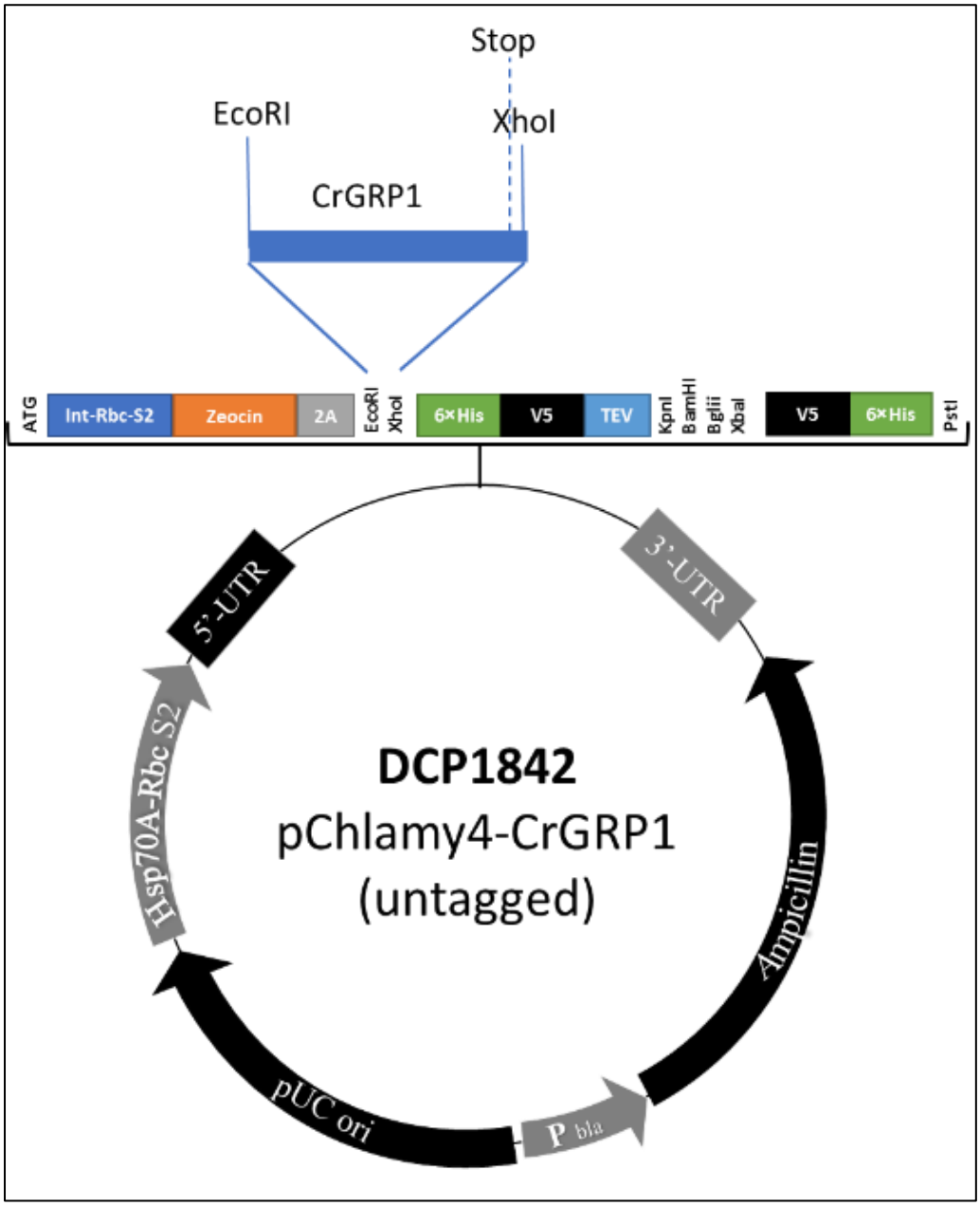


Figure 5.3: Over-expression vector construct.
The *CrGRP1* ORF was amplified from *C. reinhardtii* genomic DNA by PCR. The product was then purified, and adapters ligated to the ORF. This fragment was then ligated into the vector via *Eco*RI and *Xba*I restriction sites. The cloning was carried out by Dundee Cell Products Ltd, James Lindsay Place, Dundee Technopole, Scotland U.K.

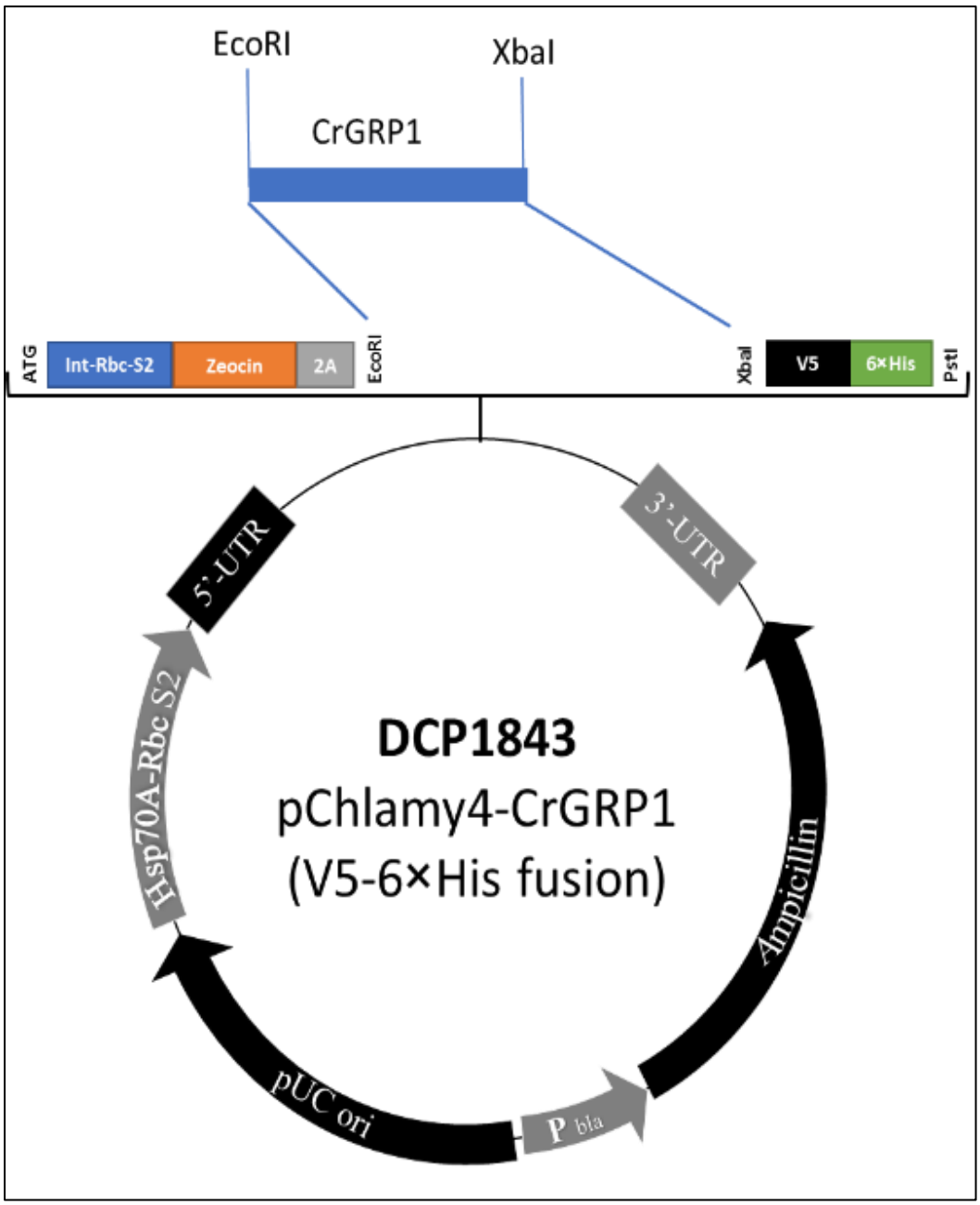


Figure 5.4: Over-expression with V5-6×His tag vector construct.
The *CrGRP1* ORF was amplified from *Chlamydomonas reinhardtii* genomic DNA by PCR. The product was then purified, and adapters ligated. The fragment was then ligated into the vector via *Eco*RI and *Xho*I restriction sites. The cloning was carried out by Dundee Cell Products Ltd, James Lindsay Place, Dundee Technopole, Scotland U.K.

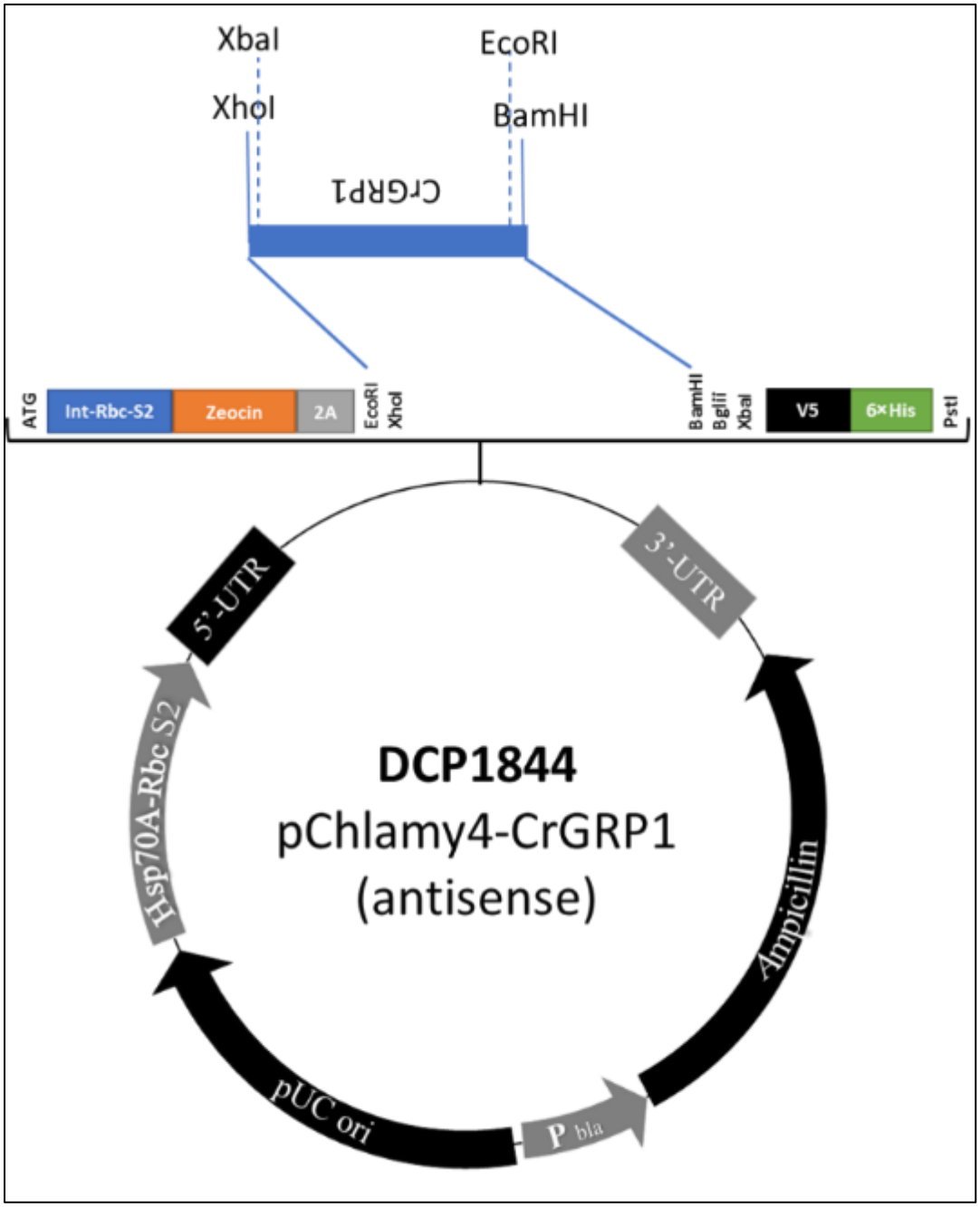


Figure 5.5: Antisense vector construct.
The *CrGRP1* ORF was amplified from *C. reinhardtii* genomic DNA by PCR. The product was then purified, and adapters ligated. The fragment was then cloned in the inverse orientation via *Bam*HI-Hf and *Xho*I restriction sites. The cloning was carried out by Dundee Cell Products Ltd, James Lindsay Place, Dundee Technopole, Scotland U.K.

To check the orientation in which the *CrGRP1* ORF was incorporated into the pChlamy4 vector, plasmids from this colony were cut with the *Sca*1 restriction enzyme.

The electrophoresis data (Figure 5.6) confirmed the correct orientation of the ORF; correctly oriented clones were chosen for transformation into *C. reinhardtii.*

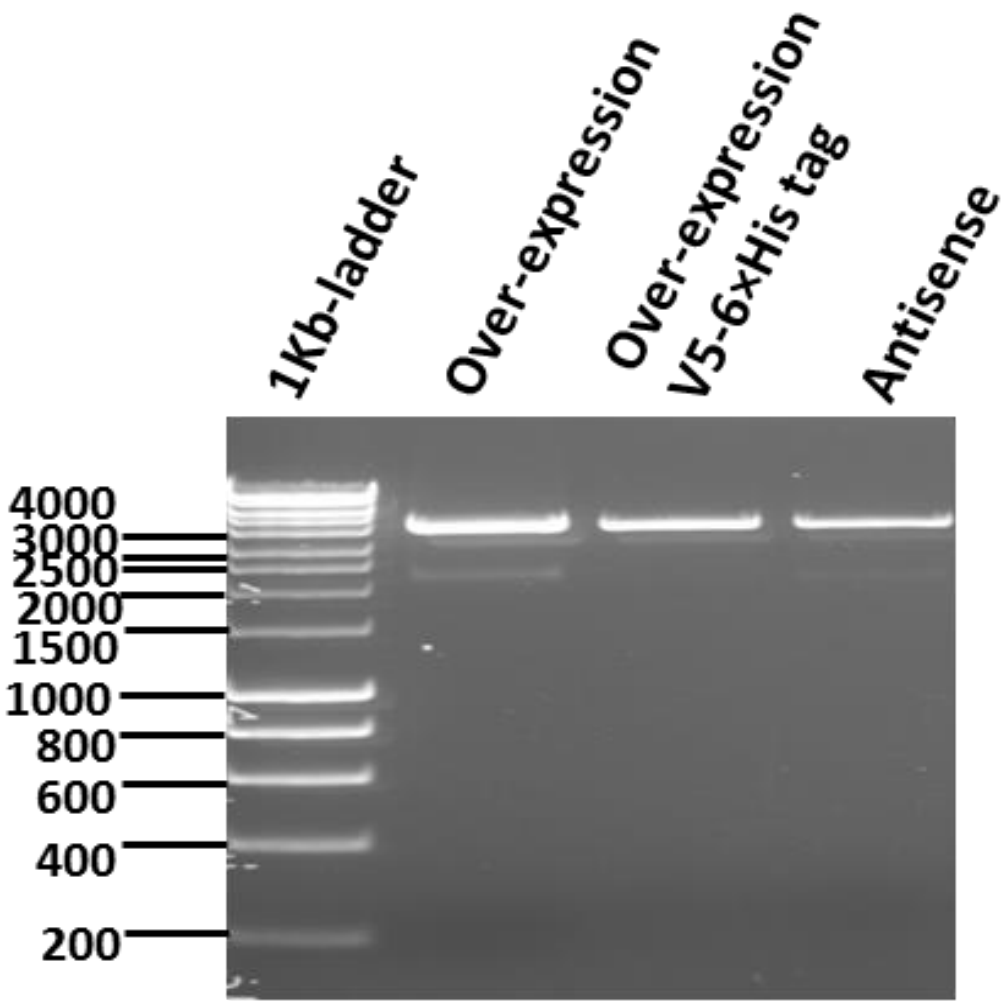


Figure 5.6: Restriction digest of CrGRP1 using Sca1 restriction enzyme.
Electrophoresis of recombinant *E. coli* plasmid (1842-43-44) following linearisation with *Sca*1 to check the orientation in which the *CrGRP1* ORF was incorporated into the pChlamy4 vector. *Sca*1 digestion of recombinant colony produced a band of ~4120 bp confirming that these recombinant plasmids carried the *CrGRP1* ORF in the correct orientation. Marker bands (1 kb ladder) are shown on the left.

5.2.2 Genetic transformation of *C. reinhardtii* with the pChlamy4 constructs

The expression construct pChlamy4 - *CrGRP1*, linearised with *Sca*1, was transformed into *C. reinhardtii* 137C cells by the electroporation method. *C. reinhardtii* strain 137C cultures were grown to early log phase in TAP medium. Cells were collected by

centrifugation and resuspended in transformation medium containing linearised pChlamy4 - *CrGRP*. Following electroporation (as described in 21.1.2), cells were incubated at 26°C in constant light for ~14–16 hours. Cells were plated on TAP agar containing 5µg/mL of zeocin. After 2-3 weeks of growth, zeocin resistant colonies appeared in each case (Figure 5.7).

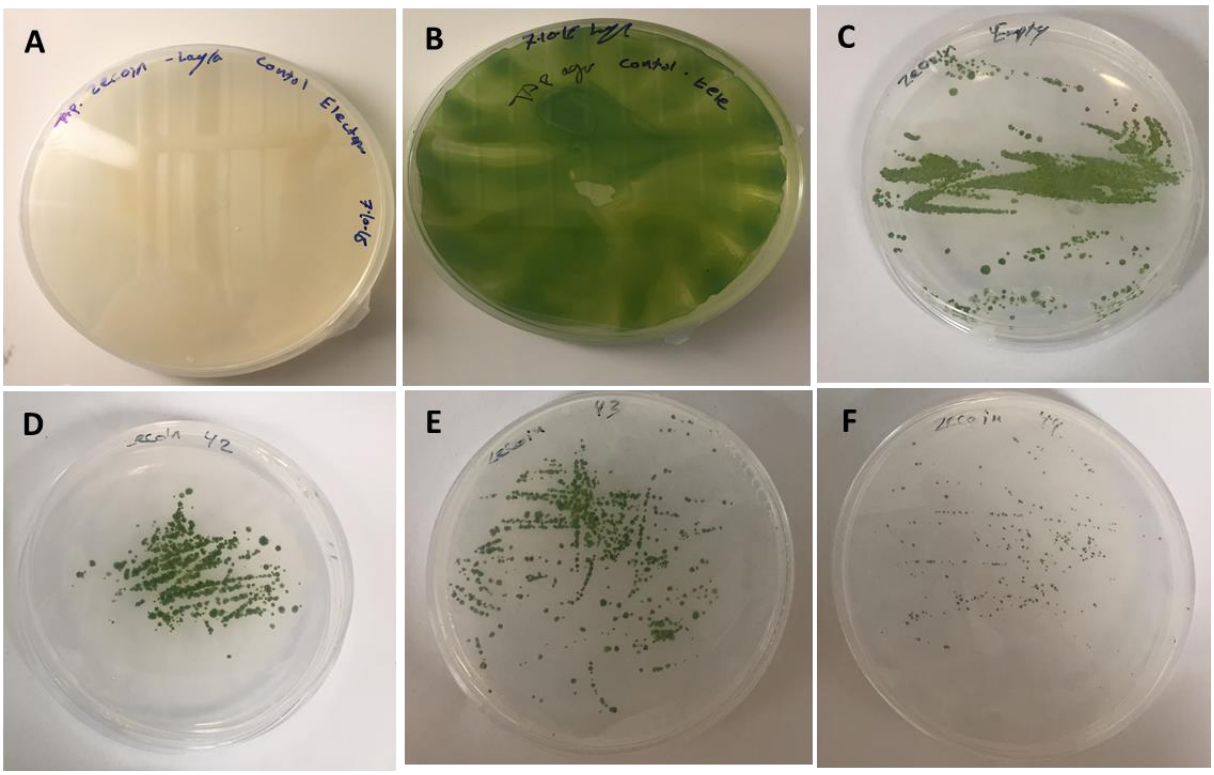


Figure 5.7: Transformed C. reinhardtii colonies after three weeks of growth.
Only cells which received and integrated the ble zeocin resistance gene grew to form colonies. A. No plasmid zeocin control. B. No plasmid control with no zeocin. C. Empty plasmid in the TAP- zeocin agar. D. Overexpression plasmid in the TAP- zeocin agar. E. Overexpression with V5 6-his tag plasmid in the TAP- zeocin agar. F. Antisense plasmid in the TAP- zeocin agar.

Transgenic algae were grown (at 25 ºC,16 h photoperiod,133 rpm, at 980 Lux) in TAP media with and without 2.5 µg/mL of zeocin until mid-log-phase (A_{750} = 0.3). Growth was measured by following A_{750} nm. Figure 5.8 shows that transgenic *C. reinhardtii* cells did not grow well in media that contained zeocin when compared with those carrying pChlamy4 only. However, they grew normally in TAP media without zeocin.

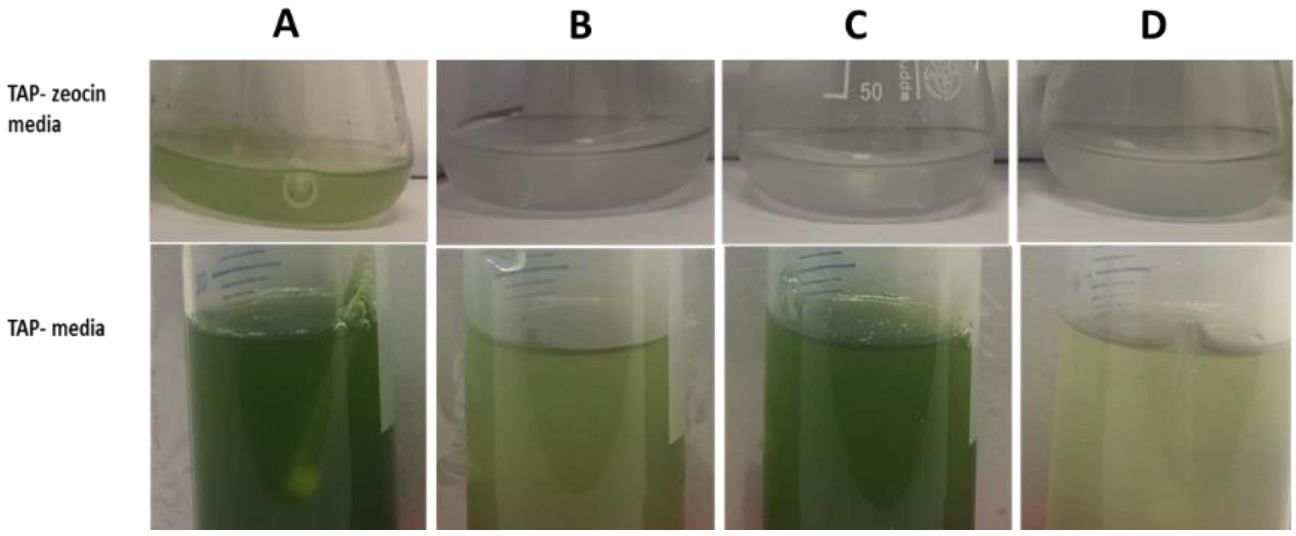


Figure 5.8: Growth of recombinant C. reinhardtii in TAP media with or without zeocin.
25 ml of *C. reinhardtii* culture at the stage of inoculation were grown in (25 ºC, 16 h photoperiod, 133 rpm, at 980 Lux) in TAP medium. Cultures were treated without and with 2.5 µg/mL of zeocin (final concentration in media). Growth was measured by following A750 nm. A. Non-recombinant plasmid. B. Overexpressed plasmid. C. Overexpression plasmid with V5 6-his tag. D. Antisense plasmid.

5.2.3 Effect of ABA on the movement of transgenic *C. reinhardtii*

Transgenic *C. reinhardti* were grown in standard conditions (25ºC, 133 rpm, 16 h photoperiod at 980 Lux) in TAP media until A_{750} = 0.3. The effects of ABA on movement in dark conditions were examined; 25 ml aliquots were transferred to a glass measuring cylinder and either 125 µl of ethanol (control) or 125 µl of 10 mM ABA in 100 %(v/v) ethanol was added. The cylinders were then placed at 25 ºC in the dark for 50 min. Most

of the cells moved up in control conditions. Algae containing overexpression plasmid (with and without V5 6 his tag) moved up, whereas they moved down in the case of algae carrying the antisense plasmid (Figure 5.9).

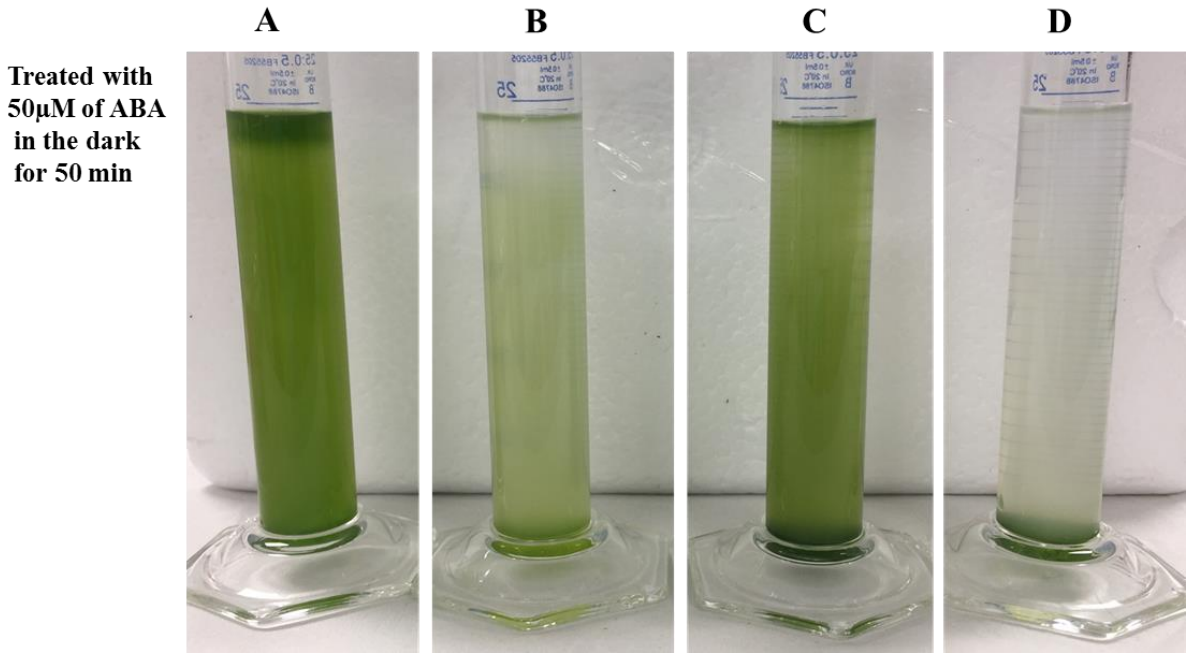


Figure 5.9: The movement of recombinant C. reinhardtii cells is differentially affected by ABA.
After growth in TAP media until A_{750} = 0.3, cultures were treated with 50 µM of ABA in the dark for 50min. Images of the cylinder were taken immediately after the 50 min incubation. A. Empty plasmid; B. Overexpression; C. Overexpression with the V5 6-his tag plasmid; D. Antisense plasmid.

5.3 Discussion

In order to determine the potential role of the *CrGRP1* gene in ABA and light mediated movement, recombinant pChlamy4 constructs were made. The non-recombinant, overexpression of *CrGRP1*, overexpression of *CrGRP1* with a V5 6-his tag and an antisense plasmid were transformed into algae. In addition, the expression construct pChlamy4 - *CrGRP1* was linearised with *Sca*1 and transformed into *C. reinhardtii* 137C cells by electroporation. Following electroporation, cells were incubated at 26°C in constant light for ~14–16 hours. Cells were plated on TAP agar containing 5µg/mL of zeocin. After 2-3 weeks of growth, zeocin resistant colonies were apparent for each plasmid (Figure 5.7). However, the number of colonies were different for each plasmid; the numbers of colonies carrying the overexpression plasmid were more than those transformed with the antisense construct. These observations suggest that the transformation of *C. reinhardtii* cells was successful. When the transformed algae were grown in standard conditions (at 25 ºC,16 h photoperiod,133 rpm, at 980 Lux) in TAP media with 2.5 µg/mL of zeocin, *C. reinhardtii* cells did not grow well, compared to cells growing in TAP media without zeocin (Fig 5.8). It is likely that transformed algae cells can not tolerate zeocin in growth media and it appeared that zeocin affected chlorophyll levels. Unfortunately, due to the lack of time, it was not possible to verify the transformations; however, work is currently in progress to address this.

The effects of ABA on movement in dark conditions were also examined. Most of the cells moved up in standard conditions. Transformants containing the overexpression plasmid, with and without V5 6 his tag, moved up, while all the algae moved down when transformed with the antisense plasmid (Figure 5.9). These data suggest that the presumed knock-down of *CrGRP1* prevents algae from moving up and instead they move

down. A study by Pazour *et al*. (2005) found that flagella contain more than 600 proteins; including *CrGRP1*.

In addition, Ciuzan *et al*. (2015) reported that in plants such as *A. thaliana*, the glycine-rich RNA-binding proteins are essential for plants' response to biotic and abiotic stresses. The phenotypic characteristics of *A. thaliana* knock-out mutants of genes that encode the glycine-rich proteins AtGRP2 and AtGRP7 were studied, comparing them to knock-out mutants of *ABI3*, *ABI4* and *ABI5*, genes linked to cell signalling through ABA. Results confirm that the abscisic acid insensitive (ABI) mutants developed faster than wild-type plants. In contrast, the glycine-rich protein knock-out mutants (*grp7-1* and *grp2*) did not grow as well, suggesting an involvement of these genes in key early developmental processes. Another study looked at the effect of overexpression of *At*GRP7 on seed germination, seedling growth, and stress tolerance under high salinity, drought, or cold stress conditions. It appears that *At*GRP7 affects the growth and stress tolerance of *A. thaliana*. It also appears to confer freezing tolerance, particularly via the regulation of stomatal closing and opening by the guard cells (Kim *et al*., 2008). As discussed in Chapter 3, the *CrGRP1* gene is closely related to *At*GRP7; therefore, it is reasonable to speculate that the *CrGRP1* gene in algae has similar roles in the response to biotic and abiotic stresses, including in processes regulated by ABA.

Although much remains to be done, including verification of the transgenic lines, and ideally the generation of a *CrGRP1* knock-out as well, and using a specific antibody to detect *CrGRP1*, work presented in this chapter has at least laid the foundations for a full functional characterisation of the *CrGRP1* gene.

6 Chapter Six: General Discussion and Conclusion

6.1 Summary of key findings and main conclusions

Currently, the culturing of microalgae has become a subject of interest as a result of shrinking supplies of fossil fuels and the limited amount of agricultural land that supplies the competing needs of food production and cultivation of plants for technical products such as biodiesel and ethanol (Douskova *et al*., 2009). Moreover, it could be used for other applications such as food and public health as well as in pharmaceutical industries Microalgae also offer very interesting possibilities for sewage and wastewater treatment and CO_2 sequestration (Douskova *et al*., 2009).

Before culturing algae on an industrial scale, understanding how algae adapt to biotic and abiotic stress is critical. In plants, some hormones play important roles in the many physiological processes, one of them is ABA (Kim *et al*., 2008). Moreover, GRP proteins play key roles in the adaptation to biotic and abiotic stresses in higher plants such as *A. thaliana.* Sequencing of the *C. reihardtii* genome has shown that this motile flagellate and freshwater photosynthetic green alga is also capable of synthesising and responding to ABA. Moreover, *C. reinhardtii* expresses at least one conserved member of the class IV GRPs which are involved in stress adaptation, and circadian rhythms *etc*. Therefore, the aims of this study were to investigate the role of plant hormones in *C. reihardtii* in response to stress and to investigate the expression and function of the class IV GRP RNA binding proteins in stress adaptation in *C. reinhardtii*.

This study confirmed that plant hormones, used in this study at different concentrations, did not affect the growth rate of *C. reinhardtii.* Additionally, these hormones did not affect the movement of *C. reinhardtii with the* exception of ABA. ABA

led to the upward movement of algae in the dark which was dependent on their sampling time over the 24h cycle. It was observed that algae cells move towards ABA in the light and away in the dark. This suggests that ABA may have a role in regulating the direction and orientation of movement in *C. reinhardtii*. Overall, ABA may modulate the extent of the phototropic and hydrotropic responses exhibited by plants dependent on the environmental conditions. More research with *C. reinhardtii* may help to explain the interactive ABA signalling mechanisms that operate in algae and, by implication, terrestrial plants to optimise their biochemistry in a changing environment.

In plants, ABA changes gene expression (Cutler *et al*., 2010). Consequently, this study explored the expression of *CrGRP1* in the circadian cycle with and without exposure to 50µM ABA. It also examined the expression of *CrGRP1* in response to abiotic stress such as H_2O_2 - induced oxidative stress and changes to light conditions during the circadian rhythm and examined the expression of *CrGRP1* following light-regulated movements of *C. reinhardtii* after treatment with 50µM ABA. Analysis of *CrGRP1* showed that it was expressed as a function of the circadian rhythm. From the bioinformatic analysis, data suggested that *CrGRP1* is closely related to *At*GRP7, itself an circadian regulated gene. *CrGRP1* expression and pre-mRNA processing was affected by H_2O_2 treatment; when cells were treated with H_2O_2, *CrGRP1* was expressed as normal. However, it was not possible to amplify the full-length transcript from the untreated control culture. Analysis of *CrGRP1* in response to altering light conditions through the light/dark cycle showed that the *CrGRP1* gene did not display any expression during the dark while it was expressed very well in the light. Furthermore, ABA reduced the expression of *CrGRP1* during the photoperiod and inhibited the expression of *CrGRP1* in the middle of the dark phase.

In brief, the results presented in this thesis suggest that: i) *CrGRP1* is a *bona fide* gene, it describes its sequence and can confirm that it expresses mRNA; ii) the transcript's processing (pre-mRNA splicing) might be regulated specifically through intron retention; and it describes its expression in the circadian rhythm and in response to ABA and abiotic stress; iii) for the first time it reports that ABA has a clear role in the regulation of algal movement particularly in response to light stimuli; and that iv) *CrGRP1* may negatively regulate the ABA-induced upward movement of the algae, presumably by binding mRNAs in the flagellum and associated structures required for this response.

Flagella contain more than 600 proteins, one of them is *CrGRP1* (Pazour *et al.*, 2005). The responses to ABA may involve regulation of *CrGRP1* expression. Therefore, the effect of overexpressing or silencing *CrGRP1* in transgenic lines on ABA responses was investigated. The initial, preliminary results showed that most of the cells moved up in both overexpression vector transformants, with and without V5 6 his tag, whereas in contrast, all algae moved down when transformed with the antisense. These results are only preliminary due to time constraints; however, they are beginning to confirm that *CrGRP1* is involved in ABA-regulated responses specifically movement. These findings have interesting evolutionary implications. In the long term, it may be possible to manipulate class IVa GRPs to improve the use *of C. reinhardtii* as a potential biofuel.

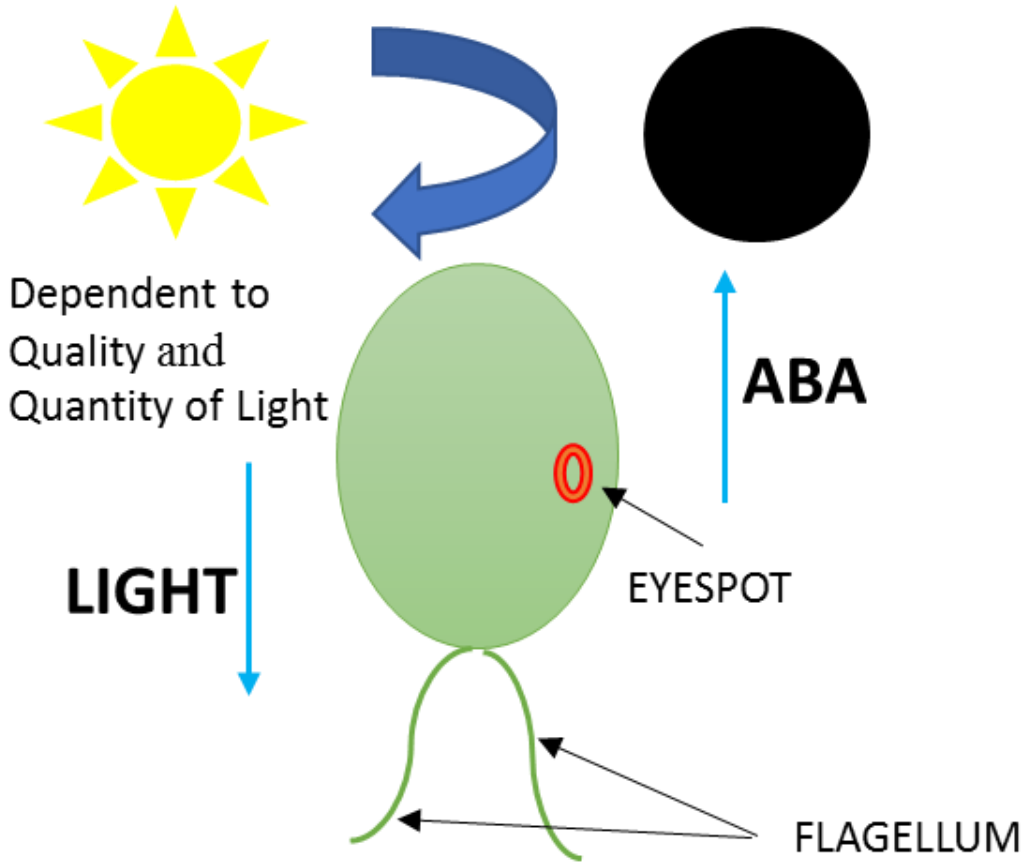


Figure 6.1: The model of the relationship between circadian clock, light and ABA on the movement of Chlamydomonas reinhardtii and arrows are the direction of movement in light and dark.
The movement of algae was affected by the quality and quantity of the light during the day; they move from the surface to the bottom to avoid stress. However, algae move upward of surface in the dark when treated with ABA but also, their movement level was different from the beginning of the dark until dawn. So, there is a relationship between circadian clock, light and ABA on the movement of *C. reinhardtii*.

Here we propose a model for interaction between circadian clock, light and ABA on the movement of *C. reinhardtii* (Figure 6.1). Since ABA treatment differentially altered the photosynthetic efficiency of the algae in a light dependent manner, thus indicating an ABA/light signalling interaction, it was reasoned that ABA signalling could be involved in determining their vertical position in the water column. *C. reinhardtii* cells move upwards in the dark while they move downwards in the day when they treated with ABA. However, the movement away from the light during the day depends on light quality and quantity. Additionally, the movement of the cells in the dark depends on the

time of the cycle as shown in experiment in the algae were treated with ABA throughout the circadian rhythm (Fig 6.2). *C. reinhardtii* cells use flagella and eyespots to protect themselves from the stress. A presumed knockdown of *CrGRP1*, a class IVa GRP protein found in flagella, causes cells to move down (preliminary findings). In summary, ABA regulates the movement of *C. reinhardtii* in the water column to position itself to optimise photosynthesis and that *CrGRP1* negatively regulates the ABA-induced upward movement of the algae.

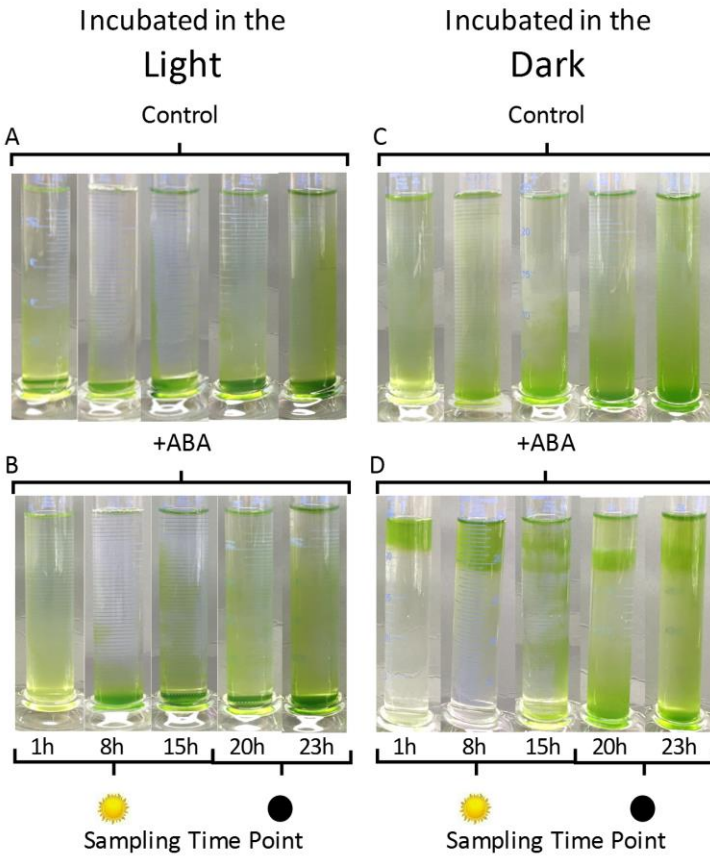


Figure 6.2: Exogenous abscisic acid (ABA) induced a negative geotropic movement response in C. reinhardtii.
Algal samples were placed either illuminated from above (A and B) or placed in the dark (C and D). Shown is a representative image of the positions attained by the algae immediately following the period of incubation in either the light or dark.

6.2 Future work

Light is the crucial thing that affects the plant's growth and their development (Stagnari *et al*., 2018). However, there are three factors regarding light that affect this process. The first factor is that intensity of light, for example, how bright the light is. Duration is considered as a second factor; how long the plant receives the light which varies depending on the seasons. The last factor is that both red and blue light are essential for plants to flourish at different stages of growth (Stagnari *et al*., 2018). Therefore, further work on the effect of ABA on movement could focus on the light /dark cycle, but with modified parameters. For example, in this study, the cultures were grown in the 16 light/ 8 h dark. Experiments could be repeated using 12h light /12 h dark, 8h light /16 h dark, 10h light/14h dark, and so on. In this study, it used only the white filter while a range of colour combinations could be deployed. In plants, different light colours help plants achieve different goals (Kami *et al*., 2010). For example, fluorescent light is optimal for cultivating plants indoors. Vegetative leaf growth is encouraged by blue light while red light, when combined with blue, helps plants flower. It would be of interest to examine the expression of *CrGRP1* in these different conditions. Furthermore, work on the effect of plant hormones such as ABA on *C. reinhardtii* in different light conditions could be performed in space, to eliminate the effects of gravity.

Further research is immediately needed to verify and fully characterise the phenotype of pChlamy4 transformants, thus building up a clearer understanding of the function of *CrGRP1*. The use of His- and V5-tagged *CrGRP1* will facilitate the purification of *CrGRP1* and associated proteins and mRNAs using a nickel column. The class IV glycine-rich proteins are likely to work by regulating gene expression at multiple levels on their mRNA targets by modifying alternative mRNA splicing, export,

translation, and degradation. Once a suitable antibody is generated, pull-down assays could also be used to investigate the molecular partners and RNA targets of *CrGRP1*. In terms of intracellular localisation, it will be of interest to confirm the localisation of CrGRP1 in flagella (and potentially in other cellular locations).

There is also a need to examine the expression of *CrGRP1* in different environmental conditions, in the circadian rhythm, and in response to ABA, more quantitatively using qPCR. It will also be of interest to determine how *CrGRP1* expression might be regulated post-transcriptionally, for example via intron retention, given the results presented in this thesis.

7 References

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8 Appendices

8.1 Buffers and Solutions

TAE buffer (50X)
Tris: 242 g+ Glacial Acetic acid: 57.1 ml +EDTA: 100 ml from 0.5 M stock + Distilled water: make up to 1L.
Loading dye (6X)
Bromophenol blue: 0.25 per cent + Glycerol: 30 per cent in water
Ethidium bromide
10 mg of sodium salt of Ethidium bromide (Et Br) was dissolved in 1ml of sterile water and stored at room temperature in amber vials.
Western Buffers
10x running buffer (1L)
30g Tris (250mM) +140g glycine (1.9M) + 10g SDS made up to 1L with D.I. water. pH 8.3
1x running buffer (1L)
70mL 10x running buffer + 630mL D.I. water
10x transfer buffer (1L)
30.29g Tris (250mM) + 142.63g glycine (1.9M) made up to 1L with D.I. water
1x transfer buffer (1L)
100mL 10x transfer buffer + 200mL methanol + 700mL H_2O
10x TBS (1L)
Dissolve 60.6g Tris + 87.6g NaCl in 600mL D.I. water. Add 100mL of dilute HCl (1N). pH to 7.6 with HCl. Make up to 1L with D.I. water.

Staining solution
Coomassie Brilliant Blue (R250)
25mg in 100ml of destaining solution

Destaining solution: (for 100ml)
Double distilled water - 50ml
Methanol - 40 ml
Glacial acetic acid - 10 ml

TAP medium

Tris-Acetate-Phosphate (TAP) growth medium for *Chlamydomonas reinhardtii* was prepared as following described

1. FBS (Filner's Beijerincks Solution) 40 X Strength.

0.6M NH_4Cl 32g/L NH_4Cl

32Mm $MgSO_4$. $7H_2O$ 8g/L $MgSO_4$

27Mm $CaCl_2$. $2H_2O$ 4g/L $CaCl_2$

Make up in 1L distilled water Autoclave. Store at 4˚C.

2. Phosphate solution (KPO4) 200 X strength.

62ml 1M K_2HPO_4 179.18g

41ml 1M KH_2PO_4 136.09g

397ml distilled water

Final volume = 500ml. autoclave. Store at 4˚C.

3. Modified trace mineral Solution-200 X strength.

27mM EDTA 5g Na_2 EDTA. $2H_2O$
Or 5.6g Na_4 EDTA. $2H_2O$
Dissolve in 400 ml distilled water by heating and stirring. pH to 6.5 with 5 M NaOH
The $FeSO_4$ should be prepared last to avoid oxidation.
Add the following in order – dissolve each fully before adding next component:
3.6 mM$FeSO_4$ 0.5g $FeSO_4 \cdot 7H_2O$
15mM$ZnSO_4$ 2.2g $ZnSO_4 \cdot 7H_2O$
37mM$H3BO_3$ 1.14g $H3BO_3$
5.2mM$MnCl_2$ 0.51g $MnCl_2 \cdot 4H_2O$
1.3 mM$CuCl_2$ 0.11g $CoCl_2 \cdot 2H_2O$
1.1mM$MH2MoO_4$ 0.10g $MH_2MoO_4.H_2O$ or0.13 g $Na_2MoO_4. 2H_2O$
1.3 mM $CoCl_2$ 0.16g $CoCl_2 \cdot 6H_2O$
Make up to 500ml with distilled water. Autoclave. Store at 4˚C.
The solution should be clear and pale green but will turn purple upon storage.

To make the final liquid TAP medium, mix the following:
For 1liter:
To 900ml de-ionised water add:
2.42g Tris base
12.5ml solution #1 (40 **X** FBS)
5ml solution #2 (phosphate (KPO_4)
5ml solution #3 (trace mineral)
Adjust the pH 7.2 with diluted 1/10 glacial acetic acid
Make up 1 liter with distilled water.
pH should be 7.2. Autoclave.

For solid TAP medium, add 15g/L agar. Autoclave.

8.2 Sequence of CrGRP1

Gene ID: 5720095; exons in yellow, introns in italics

```
1     CTGTATTCCA ATCAAGTTTC CTGTTTGGTT TCCTGGTTTG AGTATCTAGG CGTGGTAGAC
61    GCCTTTTCCT TTCAGCAGGC CAATCACCTG CTTTCTCCGG TTCCCTTCCT CTCGGAACCA E1F
121   TGTCGACCAA GCTCTACGTC GGTGAGCAAT CATAGGCATG CAACTGGGCT GCGCGAAGTC
181   GCGAAAGCTC GCGCGGGAAA GGCGGCTGGG GTCAGCCGCC TCAAAAGCGC GCGCTAAACC
241   TCTCGGAACG GCCTCGCACC TCCTCTCACT CGCTGGCCGC AGGCAACCTG TCGTGGGACA
301   CCCGCGCTGA TGACCTGACG AACCTGTTCA GCAAGTTCG GCGCCGTCGAG GTGAGCTTTC E2F/E2R
361   GTCTCGATGG GCCCGAACTG CATCTCGGTT CAGCTGGAGG GCCACTCTCA GGGCTTGTCG
421   CCCGGATCCC CGAATATGCA TGCACTAATC GCAAGTCTTC ATTTGCTTTG TTCCGCGCAG
481   GATGCCTTCG TCGCCACCGA CCGCGAGACT GGCCGGTCGC GCGGCTTTGG CTTCGTGACT E3F
541   CTCGAGGCCA ACGCTGCCAA GAGCGCCTGC T TGAGTGCG ACGGCACCGA GTTCATGGGC E3R
601   CGGTAAGTTT CAACTTATGT CGGAAACGTT TCGGTATTGG GCGCCGCAGA AAGAGATGCA
661   CCATAGATGC CTTCCGACAC TCGTGAATCT CATGTATTTG CTTTGTATTT CAACTTCCTG
721   CAGCACCATT CGCGTGAACG AGGCTACTCC CCTGGGCGAG CGCCCACCTG GTGAGTTCAA E4F/E4R
781   GGCTGCCGAG ATCGCATGCA ACTGTGTCTA CAACCCCAGG ATACGCTGAT GCCTGGATAC
841   CTGTTGTGGA GGCTTTGTAT TCTGACGCAT AGTATTGCAT CACTCCAATC AATGACGGCT
901   GCTGCGGGCG TTGGGAAGTT CCGAAATATC CTGCTCGCCA GGTATCTCCG TGCGGCCCGC
961   CACTGACCGC TTTCCTCCCT TGTCCTCTTC CCTTGATCCC GAGCAGGCGG CCGCGGCGGT
1021  GGCCGTGGGT ACGGTGGTGG TGGCCGTGGC CGTGGCGGTG ACTACGGCGG CGGCCGTGGC
1081  GGCTACGGTG GCGGTGGCTA CGGTGAGCCT TGCGAGTGTC CTGTTCCAAG CAAACCACCT E5F/E5R
1141  GTGCGCCCAA GGATCATCTC GAGTCCGACA CTTCGGTGTA GCGCTTCACA AGGCTTCCAA
1201  GGGCATCCAA GCAGTGTTGT GCGAGTGCCT TACAGCGTGT GTTTCGGAAT GCTGACATGT
1261  TCTGTTTGTG TGTAGGTGGC GGCGGCTACG GTGGCGGCCG CGGCGGCGGC GGCTACGGCG
1321  GCGGTGGCAG CGGCGGCTAC GGCGGCGGCG GTTACGGTGA GGCCAACACA GGTTCGGCCG E6F/E6R
1381  ATTCTTGCAA CGCGCGCTTT GACGATTGGG TCACATCTGC TTGATGTTGA GTCTTGGGTC
1441  GTGTGCCAAG TTGCTGCAAA GGTTTTTTTG CCAATGCCTC TAGTGTTGGT TGCTTAAAGT
1501  TGCCTGACAG GTTTTTCTTC TGCCTCTCTT TCCAGGCGGC AACGGTGGCG GCGGCTACGG
1561  TGCCGGTGGC GGCGGCTACG GTGGCGGCGG CTACTAAGCC CGCGGCTGAA GTCAGCGCAC
1621  GTCATGCCGC AGCACGCGGC ATGTTGAGGA GCCATCAAGG GAGCGTCAGA TTGGTGTAGG E71F/E71R
1681  GTTATTGTGC TGGCCGCAGC ACCTGCGACA ATGGCGGGCG GACTCATTGC GCCTTGCCGG
1741  AGCAGTCTGC GAGACTTCTA AAGAGGCTTT GCAAGATCGT TGCCGAGGAC GCCGTGTACC
1801  GACGGGCGAT TTTCGCGTGC AAGCTTGAGT CATGAGACAA GGGCACGTTA GCGCGCGCCA
1861  GATGGTGCGT GCGGGCTCGG CGCGTTGGTA GTACAGGTGG CAAGCTTGCA CAACTTGTTT
1921  GGACGTCCAT CCACCGTATC AGTTCTACAA ACATAGCGTG GTGTGTTTCG GTCGAACGAA
1981  GAAACACATT ATGTGCTATG CATTGTTGTG CCGATTGCTG TGTGTGTACT GCGCATGTCT
2041  GCATGACAGA TGCAAAGCGC AAACTGCAGC CGGTGCTTTT GTGGTTCTGT GTATTGTGTG
2101  TTGGTTGATG AGGAAGTTGA GGGCAACAGG GGCGCAAGTC ACTCAGGCCC CGATGCCGAT
2161  TCCGTATCCT TTCCGGTGGT GGTTAGTGTT GCATACCGGT GTGTGTGTAG TTTGGTTTTG
2221  CGTTGGTTTG CACTTCTCAC ATGGTTTCTT CGTGTTTTCC AGTCGATGTT TGCCTGTATC
2281  CCCTATCCTC GAGCGCACCC TGGTCTGCAC GCCGTAAGCT GGGGCCCGGA AGCCTCTGCT
2341  GTGGCGGTGT GCGAGTCTCT TGGTTGCGTG CGGTTCCCGT AAGGCTTCCC GTGGTGGCGC
2401  AACACCGCGG CGACGTTTGC GTGGCGCGGG TGTGGTGAGG CTGCTGGATG GCGCCTCAAA
2461  CACTGGCGGC GCTTGGTGTT GTACCCTGTG CGCATCGGAT CCGAGTGTGT ATATGGCGAA
2521  TTACAGAACT ATGCCATATG CAACCACGTG CGGTGTACAT ACTCTGACCT TCTGCTTCGC
2581  ATGAGCGAGA GAGAGGACAC TGCCATGCTA CCCCAATTCA TCGGCTGGAA TTACGAATAC E72F
2641  GTGTGGTGTT GCTTGATGCG TTACTCAAGT CCAATGGTGT TTCCAGTTCG GGTGCGAGAT
2701  ACGGGCGGCG CAAGTGGATT GACCCTTGCG TCTGCAAACG TACGAGCTAT CAGGTACTTG
2761  GTCCCCAGTC CGGAACTCAA TTGTCCGCTA CGGCTTCAAA ATACTGTCTC CCGGCTCTCT E72R
2821  ACTATGGCTG TAAACTTAAA AGCGGAAA
```

Green: forward primers, and where shown, the complement is the reverse primer too.
Blue: reverse primer alone
Purple: overlap (applies to exon 2 primers only).

Messenger RNA:

```
   1  ctgtattcca atcaagtttc ctgtttggtt tcctggtttg agtatctagg cgtggtagac
  61 gccttttcct ttcagcaggc caatcacctg ctttctccgg ttcccttcct ctcggaacca
 121 tgtcgaccaa gctctacgtc ggcaacctgt cgtgggacac ccgcgctgat gacctgacga
 181 acctgttcag caagttcggc gccgtcgagg atgccttcgt cgccaccgac cgcgagactg
 241 gccggtcgcg cggctttggc ttcgtgactc tcgaggccaa cgctgccaag agcgcctgct
 301 ctgagtgcga cggcaccgag ttcatgggcc gcaccattcg cgtgaacgag gctactcccc
 361 tgggcgagcg cccacctggc ggccgcggcg gtggccgtgg gtacggtggt ggtggccgtg
 421 gccgtggcgg tgactacggc ggcggccgtg gcggctacgg tggcggtggc tacggtggcg
 481 gcggctacgg tggcggccgc ggcggcggcg gctacggcgg cggtggcagc ggcggctacg
 541 gcggcggcgg ttacggcggc aacggtggcg gcggctacgg tgccggtggc ggcggctac
 601 gtggcggcgg ctactaagcc cgcggctgaa gtcagcgcac gtcatgccgc agcacgcggc
 661 atgttgagga gccatcaagg gagcgtcaga ttggtgtagg gttattgtgc tggccgcagc
 721 acctgcgaca atggcgggcg gactcattgc gccttgccgg agcagtctgc gagacttcta
 781 aagaggcttt gcaagatcgt tgccgaggac gccgtgtacc gacgggcgat tttcgcgtgc
 841 aagcttgagt catgagacaa gggcacgtta gcgcgcgcca gatggtgcgt gcgggctcgg
 901 cgcgttggta gtacaggtgg caagcttgca caacttgttt ggacgtccat ccaccgtatc
 961 agttctacaa acatagcgtg gtgtgtttcg gtcgaacgaa gaaacacatt atgtgctatg
1021 cattgttgtg ccgattgctg tgtgtgtact gcgcatgtct gcatgacaga tgcaaagcgc
1081 aaactgcagc cggtgctttt gtggttctgt gtattgtgtg ttggttgatg aggaagttga
1141 gggcaacagg ggcgcaagtc actcaggccc cgatgccgat tccgtatcct ttccggtggt
1201 ggttagtgtt gcataccggt gtgtgtgtag tttggttttg cgttggtttg cacttctcac
1261 atggtttctt cgtgttttcc agtcgatgtt tgcctgtatc ccctatcctc gagcgcaccc
1321 tggtctgcac gccgtaagct ggggcccgga agcctctgct gtggcggtgt gcgagtctct
1381 tggttgcgtg cggttcccgt aaggcttccc gtggtggcgc aacaccgcgg cgacgtttgc
1441 gtggcgcggg tgtggtgagg ctgctggatg gcgcctcaaa cactggcggc gcttggtgtt
1501 gtaccctgtg cgcatcggat ccgagtgtgt atatggcgaa ttacagaact atgccatatg
1561 caaccacgtg cggtgtacat actctgacct tctgcttcgc atgagcgaga gagaggacac
1621 tgccatgcta ccccaattca tcggctggaa ttacgaatac gtgtggtgtt gcttgatgcg
1681 ttactcaagt ccaatggtgt ttccagttcg ggtgcgagat acgggcggcg caagtggatt
1741 gacccttgcg tctgcaaacg tacgagctat caggtacttg gtccccagtc cggaactcaa
1801 ttgtccgcta cggcttcaaa atactgtctc ccggctctct actatggctg taaacttaaa
1861 agcggaaa
```

Protein:

```
   1 mstklyvgnl swdtraddlt nlfskfgave dafvatdret grsrgfgfvt leanaaksac
  61 secdgtefmg rtirvneatp lgerppggrg ggrgyggggr grggdygggr ggyggggygg
 121 ggygggrggg gyggggsggy ggggyggngg ggygaggggy ggggy
```

8.3 PChlamy_4 vector sequence

TCGCTGAGGCTTGACATGATTGGTGCGTATGTTTGTATGAAGCTACAGGACTGATTTGGCGGGCTATGAG
GGCGGGGGAAGCTCTGGAAGGGCCGCGATGGGGCGCGCGGCGTCCAGAAGGCGCCATACGGCCCGCTGGC
GGCACCCATCCGGTATAAAAGCCCGCGACCCCGAACGGTGACCTCCACTTTCAGCGACAAACGAGCACTT
ATACATACGCGACTATTCTGCCGCTATACATAACCACTCAGCTAGCTTAAGATCCCATCAAGCTTGCATG
CCGGGCGCGCCAGAAGGAGCGCAGCCAAACCAGGATGATGTTTGATGGGGTATTTGAGCACTTGCAACCC
TTATCCGGAAGCCCCCTGGCCCACAAAGGCTAGGCGCCAATGCAAGCAGTTCGCATGCAGCCCCTGGAGC
GGTGCCCTCCTGATAAACCGGCCAGGGGGCCTATGTTCTTTACTTTTTTACAAGAGAAGTCACTCAACAT
CTTAAAATGGCCAGGTGAGTCGACGAGCAAGCCCGGCGGATCAGGCAGCGTGCTTGCAGATTTGACTTGC
AACGCCCGCATTGTGTCGACGAAGGCTTTTGGCTCCTCTGTCGCTGTCTCAAGCAGCATCTAACCCTGCG
TCGCCGTTTCCATTTGCAGGATGGCCATGCAT

ATGGCCAAGCTGACCAGCGCCGTTCCGGTGCTCACCGC
GCGCGACGTCGCCGGAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGAGGAC
GACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCGGTCCAGGACCAGGTGAGTCGAC
GAGCAAGCCCGGCGGATCAGGCAGCGTGCTTGCAGATTTGACTTGCAACGCCCGCATTGTGTCGACGAAG
GCTTTTGGCTCCTCTGTCGCTGTCTCAAGCAGCATCTAACCCTGCGTCGCCGTTTCCATTTGCAGGACCA
GGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCGGCCTGGACGAGCTGTACGCCGAGTGGTCG
GAGGTCGTGTCCACGAACTTCCGGGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGG
GGCGGGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAGGAGCAGGACGCCCC
GGTGAAGCAGACCCTGAACTTCGACCTGCTGAAGCTGGCGGGCGACGTGGAGAGCAACCCGGGCCCCGAA
TTCCTCGAGCACCACCACCACCACCACGGCAAGCCCATCCCCAACCCCCTGCTGGGCCTGGACAGCACCG
AGAACCTGTACTTCCAGGGGGTACCAGGATCCGGAAGATCTGGTCTAGAGGGCAAGCCCATCCCCAACCC
CCTGCTGGGCCTGGACAGCACCCGTACCGGTCACCACCACCACCACCACTAACTGCAGCCGCTCCGTGTA
AATGGAGGCGCTCGTTGATCTGAGCCTTGCCCCCTGACGAACGGCGGT

GGATGGAAGATACTGCTCTCAA
GTGCTGAAGCGGTAGCTTAGCTCCCCGTTTCGTGCTGATCAGTCTTTTTCAACACGTAAAAAGCGGAGGA
GTTTTGCAATTTTGTTGGTTGTAACGATCCTCCGTTGATTTTGGCCTCTTTCTCCATGGGCGGGCTGGGC
GTATTTGAAGCGGCGGTATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTA
TTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGATTATCAAAAAGGATCTTCACCTA
GATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGT
TACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGAC
TCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCG
AGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGT
GGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC
CAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTAT
GGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCG
GTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGG
CAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAAC
CAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACC
GCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGA
TCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC
TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACA
CGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCA
TGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATC
TTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTG
GTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATAC
CAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATA
CCTCGCTCTGCTAATCCTGTTACCAGTGGCTGTTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGAC
TCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCT
TGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGA
AGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCA
GGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGT
GATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTT
TTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCC

TTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGG

Zeocin_F1 5′-GCCATGACCGAGATCGGCGAGCAGC-3′
FMDV2A_F1 5′-GGTGAAGCAGACCCTGAACTTCGAC-3′

UTR_R1 5′-CAAGGCTCAGATCAACGAGCGCCTC-3′
UTR_R2 5′-GATCAACGAGCGCCTCCATTTACAC-3′

FMDV 2A
MCS1
6×His
V5 epitope
TEV
MCS2
V5 epitope
6×His
MCS3
3UTR

8.4 Diagram illustrating the primers designed

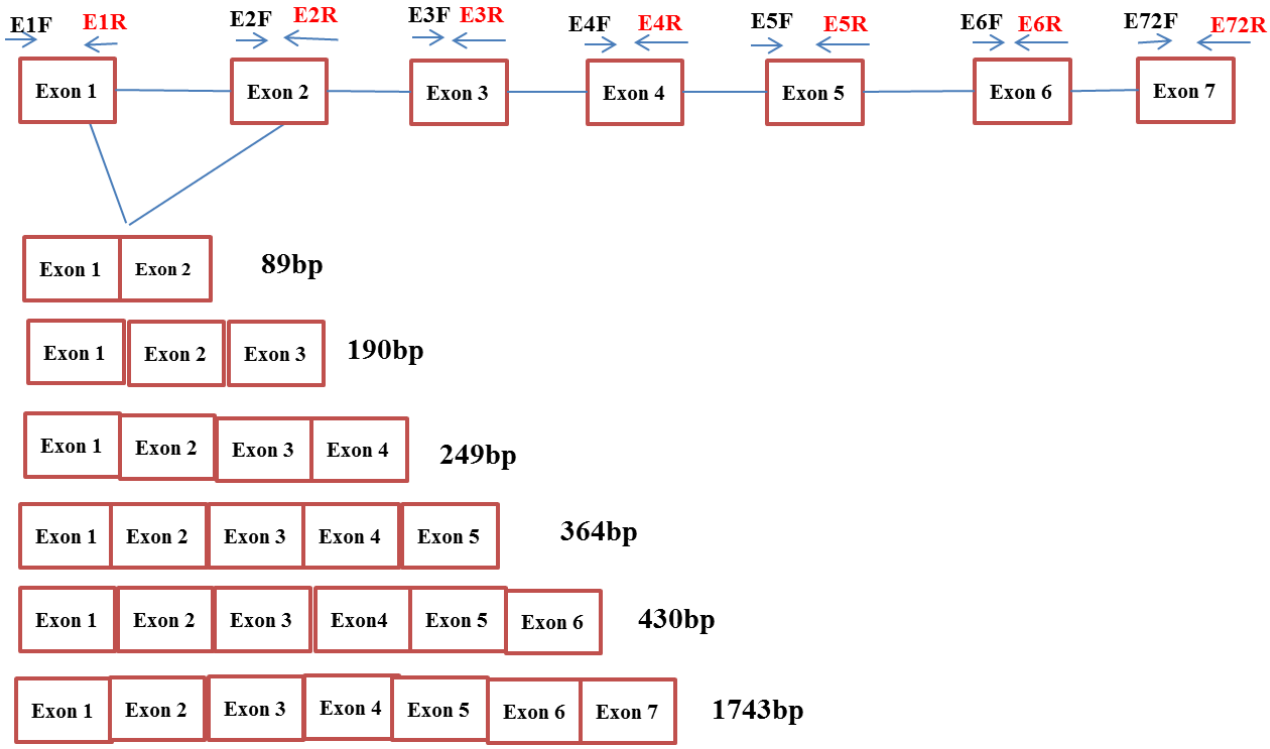


Figure 8.1:Diagram illustrating the primers designed and used in this study in relation to CrGRP1 exons.

Primer name	Expected band from RNA only	Expected band from RNA and intron
E1F+E2R	89bp	230bp
E1F+E3R	190bp	471bp
E1F+E72R	1,743bp	2,728bp

Table 8-1:The primer name and the expected size of band

8.5 Relative Cell Density Data Analysis

(a) 1h into Photoperiod (t=1h) For Figure 3.11

Incubation (Light/Dark)	**Light**	**Light**	**Light**	**Light**		**Light**	**Light**	**Light**	**Light**		**Dark**	**Dark**	**Dark**	**Dark**		**Dark**	**Dark**	**Dark**	Dark
Treatment	Control	Control	Control	Control		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	13	20	23	44		19	21	23	37		23	16	22	39		70	13	9	8
Standard Deviation	4	7	6	8		5	4	5	7		4	3	3	3		15	6	5	5
Standard Error	2	3	3	4		2	2	2	3		2	1	1	1		7	3	2	2
95% Confidence Interval	5	9	8	10		6	5	6	9		5	4	4	4		19	7	6	6

Chi- Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			*chi-sq*	*p-value*	*x-crit*	*sig*	*Cramer V*
Light	13	20	23	44		Pearson's	1.7	0.6	7.8	no	0.1
Light + ABA	19	21	23	37		Max likelihood	1.7	0.6	7.8	no	0.1
						CHI-SQUARE					
Dark	23	16	22	39			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	70	13	9	8		Pearson's	50.7	0.0	7.8	yes	0.5
						Max likelihood	53.9	0.0	7.8	yes	0.5

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	23	16	22	39
Dark + ABA	70	13	9	8
P value	0.00	0.53	0.02	0.00
Significant (Y/N)	Y	N	Y	Y
Chi^2 Stat	24.3	0.4	5.6	20.4
Effect Rank	1	4	3	2

(b) 8h into Photoperiod (t=8h) For Figure 3.12

Incubation (Light/Dark)	**Light**	**Light**	**Light**	**Light**		**Light**	**Light**	**Light**	**Light**		**Dark**	**Dark**	**Dark**	**Dark**		**Dark**	**Dark**	**Dark**	**Dark**
Treatment	**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	7	9	23	61		6	11	23	61		17	22	25	36		53	24	16	7
Standard Deviation	3	5	4	8		2	8	6	10		2	2	2	4		10	5	4	2
Standard Error	1	2	2	4		1	3	3	5		1	1	1	2		5	2	2	1
95% Confidence Interval	3	6	5	10		2	10	7	13		2	2	3	4		13	6	5	2

Chi-Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light	7	9	23	61		Pearson's	0.4	0.9	7.8	no	0.0
Light + ABA	6	11	23	61		Max likelihood	0.4	0.9	7.8	no	0.0
						CHI-SQUARE					
Dark	17	22	25	36			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	53	24	16	7		Pearson's	39.5	0.0	7.8	yes	0.4
						Max likelihood	42.3	0.0	7.8	yes	0.5

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	17	22	25	36
Dark + ABA	53	24	16	7
P value	0.00	0.75	0.20	0.00
Significant (Y/N)	Y	N	N	Y
Chi^2 Stat	17.8	0.1	1.7	20.0
Effect Rank	2	4	3	1

(c) 15h into Photoperiod (t=15h) For Figure 3.13

Incubation (Light/Dark)	**Light**	**Light**	**Light**	**Light**		**Light**	**Light**	**Light**	**Light**		**Dark**	**Dark**	**Dark**	**Dark**		**Dark**	**Dark**	**Dark**	**Dark**
Treatment	**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	17	19	23	41		12	17	24	47		20	22	22	36		58	16	16	9
Standard Deviation	3	3	4	6		3	3	6	9		6	4	7	7		7	5	4	4
Standard Error	1	1	2	3		2	1	3	4		3	2	3	3		3	2	2	2
95% Confidence Interval	4	3	5	7		4	3	8	11		7	5	8	8		9	6	5	5

Chi-Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light	17	19	23	41		Pearson's	1.2	0.8	7.8	no	0.1
Light + ABA	12	17	24	47		Max likelihood	1.2	0.8	7.8	no	0.1
						CHI-SQUARE					
Dark	20	22	22	36			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	58	16	16	9		Pearson's	36.8	0.0	7.8	yes	0.4
						Max likelihood	38.8	0.0	7.8	yes	0.4

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	20	22	22	36
Dark + ABA	58	16	16	9
P value	0.00	0.35	0.34	0.00
Significant (Y/N)	Y	N	N	Y
Chi^2 Stat	19.2	0.9	0.9	15.9
Effect Rank	1	4	3	2

(d) 1h into Dark Period (t=17h) For Figure 3.14

Incubation (Light/Dark)	**Light**	**Light**	**Light**	**Light**		**Light**	**Light**	**Light**	**Light**		**Dark**	**Dark**	**Dark**	**Dark**		**Dark**	**Dark**	**Dark**	**Dark**
Treatment	**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	24	24	26	26		13	26	27	33		14	23	26	37		37	22	22	19
Standard Deviation	8	3	3	3		4	3	5	5		7	3	3	8		16	6	9	4
Standard Error	3	1	1	1		2	1	2	2		3	1	1	3		7	2	4	2
95% Confidence Interval	9	4	4	3		5	4	6	6		9	4	4	9		20	7	12	6

Chi-Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light	24	24	26	26		Pearson's	4.1	0.2	7.8	no	0.1
Light + ABA	13	26	27	33		Max likelihood	4.2	0.2	7.8	no	0.1
						CHI-SQUARE					
Dark	14	23	26	37			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	37	22	22	19		Pearson's	17.3	0.0	7.8	yes	0.3
						Max likelihood	17.8	0.0	7.8	yes	0.3

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	14	23	26	37
Dark + ABA	37	22	22	19
P value	0.00	0.95	0.50	0.01
Significant (Y/N)	Y	N	N	Y
Chi^2 Stat	10.8	0.0	0.5	6.0
Effect Rank	1	4	3	2

(e) 4h into Dark Period (t=20h) For Figure 3.15

Incubation (Light/Dark)	**Light**	**Light**	**Light**	**Light**		**Light**	**Light**	**Light**	**Light**		**Dark**	**Dark**	**Dark**	**Dark**		**Dark**	**Dark**	**Dark**	**Dark**
Treatment	**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	13	22	29	35		17	26	25	32		14	19	25	42		45	20	16	19
Standard Deviation	1	2	2	3		2	1	2	3		2	1	2	3		7	6	4	4
Standard Error	0	1	1	1		1	0	1	1		1	0	1	1		3	3	2	2
95% Confidence Interval	1	2	2	3		3	1	2	3		3	1	3	3		9	7	5	5

Chi-Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light	13	22	29	35		Pearson's	1.1	0.8	7.8	no	0.1
Light + ABA	17	26	25	32		Max likelihood	1.1	0.8	7.8	no	0.1
						CHI-SQUARE					
Dark	14	19	25	42			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	45	20	16	19		Pearson's	27.5	0.0	7.8	yes	0.4
						Max likelihood	28.7	0.0	7.8	yes	0.4

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	14	19	25	42
Dark + ABA	45	20	16	19
P value	0.00	0.90	0.16	0.00
Significant (Y/N)	Y	N	N	Y
Chi^2 Stat	16.7	0.0	2.0	8.8
Effect Rank	1	4	3	2

(f) 7h into Dark Period (t=23h) For Figure 3.16

Incubation (Light/Dark)	Light	Light	Light	Light		Light	Light	Light	Light		Dark	Dark	Dark	Dark		Dark	Dark	Dark	Dark
Treatment	**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**		**Control**	**Control**	**Control**	**Control**		**50µM ABA**	**50µM ABA**	**50µM ABA**	**50µM ABA**
Depth from Surface (cm)	2	4	6	8		2	4	6	8		2	4	6	8		2	4	6	8
Mean Relative Cell Density Ratio	17	25	25	32		14	24	28	34		18	20	24	37		30	25	23	21
Standard Deviation	3	3	2	6		2	1	1	1		2	2	3	3		7	3	4	2
Standard Error	1	2	1	3		1	0	1	1		1	1	1	1		3	1	2	1
95% Confidence Interval	4	4	2	8		2	1	1	1		3	3	4	3		9	3	5	3

Chi-Squared test of Independence

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light	17	25	25	32		Pearson's	0.5	0.9	7.8	no	0.1
Light + ABA	14	24	28	34		Max likelihood	0.5	0.9	7.8	no	0.1
						CHI-SQUARE					
Dark	18	20	24	37			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA	30	25	23	21		Pearson's	8.1	0.0	7.8	yes	0.2
						Max likelihood	8.2	0.0	7.8	yes	0.2

Chi-Squared test of Goodness of Fit

	Depth from Surface (cm)			
	2	**4**	**6**	**8**
Dark	18	20	24	37
Dark + ABA	30	25	23	21
P value	0.08	0.46	0.90	0.03
Significant (Y/N)	N	N	N	Y
Chi^2 Stat	3.1	0.5	0.0	4.5
Effect Rank	2	3	4	1

Effect of Sampling Time

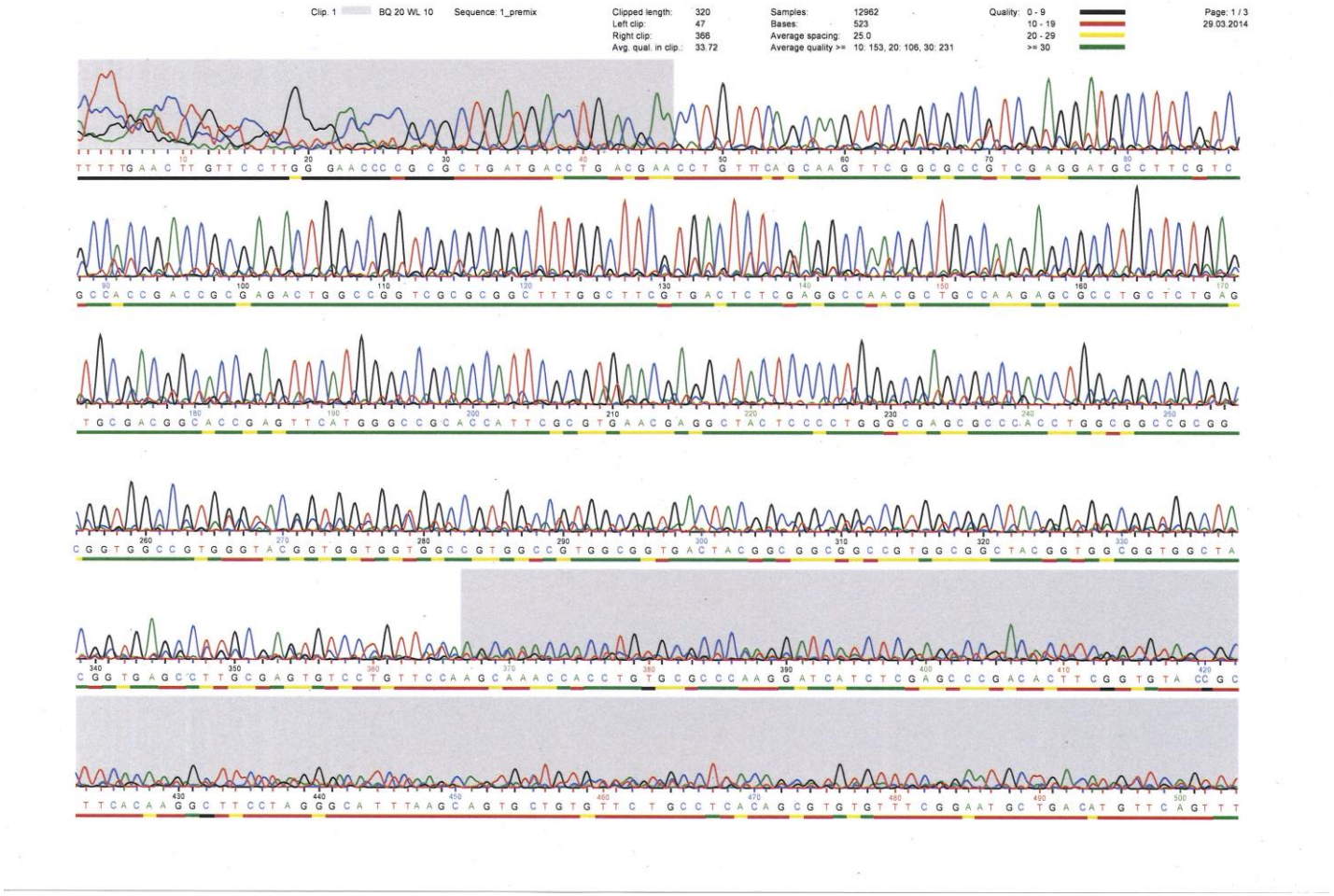
	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light 1h into photoperiod	13	20	23	44		Pearson's	25.0	0.0	21.0	yes	0.1
Light 8h into photoperiod	7	9	23	61		Max likelihood	26.0	0.0	21.0	yes	0.1
Light 15h into photoperiod	17	19	23	41							
Light 4h into dark period	13	22	29	35							
Light 7h into dark period	17	25	25	32							
P value	0.29	0.11	0.91	0.02							
Significant (Y/N)	N	N	N	Y							
Chi^2 Stat	4.9	7.6	1.0	11.4							
Effect Rank	3	2	4	1							

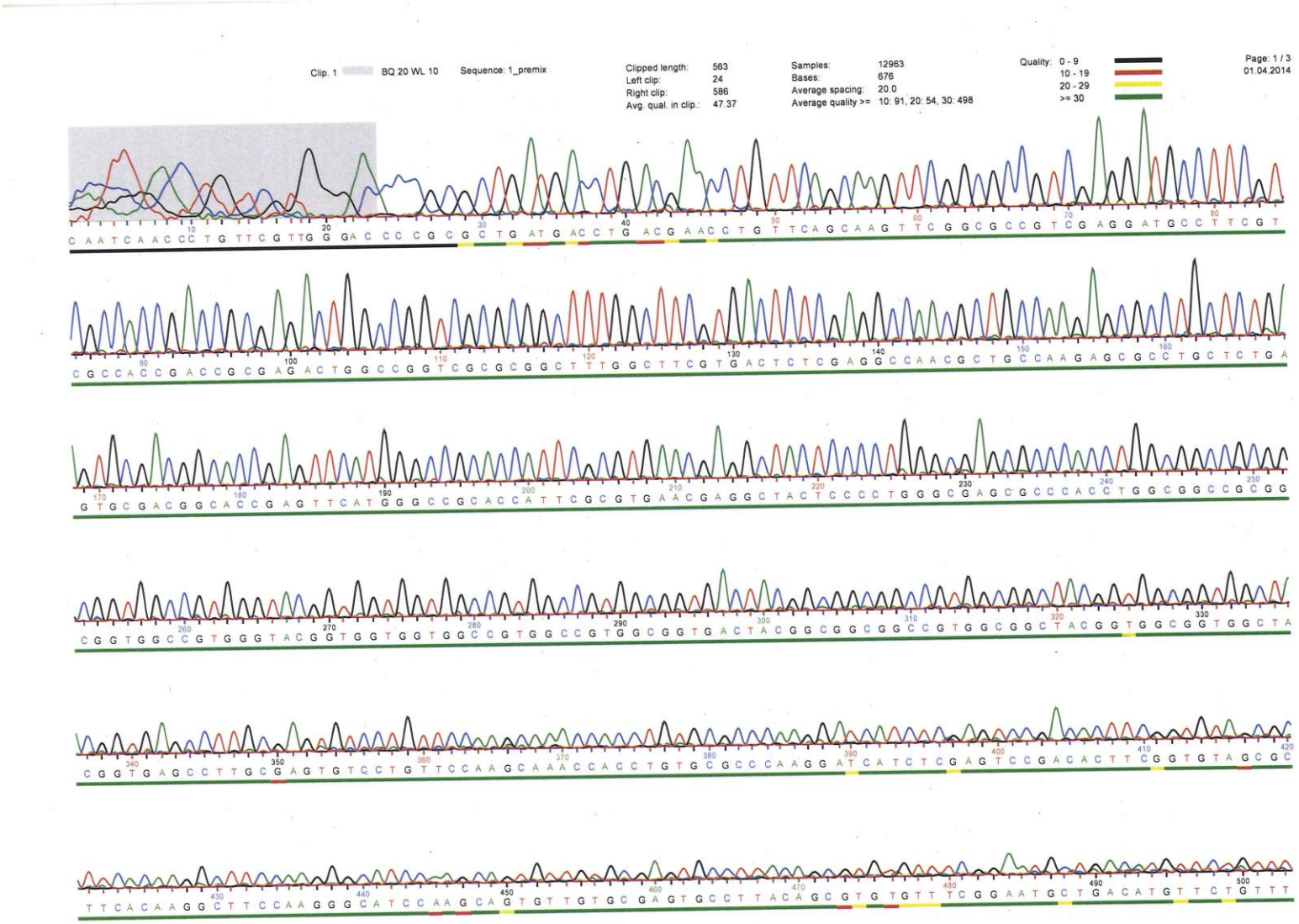
	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Light + ABA 1h into photoperiod	19	21	23	37		Pearson's	28.5	0.0	21.0	yes	0.1
Light + ABA 8h into photoperiod	6	11	23	61		Max likelihood	29.4	0.0	21.0	yes	0.1
Light + ABA 15h into photoperiod	12	17	24	47							
Light + ABA 4h into dark period	17	26	25	32							
Light + ABA 7h into dark period	14	24	28	34							
P value	0.11	0.12	0.96	0.01							
Significant (Y/N)	N	N	N	Y							
Chi^2 Stat	7.4	7.3	0.7	13.2							
Effect Rank	2	3	4	1							

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Dark 1h into photoperiod	23	16	22	39		Pearson's	4.2	1.0	21.0	no	0.1
Dark 8h into photoperiod	17	22	25	36		Max likelihood	4.3	1.0	21.0	no	0.1
Dark 15h into photoperiod	20	22	22	36							
Dark 4h into dark period	14	19	25	42							
Dark 7h into dark period	18	20	24	37							
P value	N/A	N/A	N/A	N/A							
Significant (Y/N)	N/A	N/A	N/A	N/A							
Chi^2 Stat	N/A	N/A	N/A	N/A							
Effect Rank	N/A	N/A	N/A	N/A							

	Depth from Surface (cm)					CHI-SQUARE					
	2	**4**	**6**	**8**			chi-sq	p-value	x-crit	sig	Cramer V
Dark + ABA 1h into photoperiod	70	13	9	8		Pearson's	42.9	0.0	21.0	yes	0.2
Dark + ABA 8h into photoperiod	53	24	16	7		Max likelihood	43.8	0.0	21.0	yes	0.2
Dark + ABA 15h into photoperiod	58	16	16	9							
Dark + ABA 4h into dark period	45	20	16	19							
Dark + ABA 7h into dark period	30	25	23	21							
P value	0.00	0.22	0.19	0.01							
Significant (Y/N)	Y	N	N	Y							
Chi^2 Stat	17.1	5.7	6.1	13.9							
Effect Rank	1	4	3	2							

8.6 Sequence Data

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20 - 29
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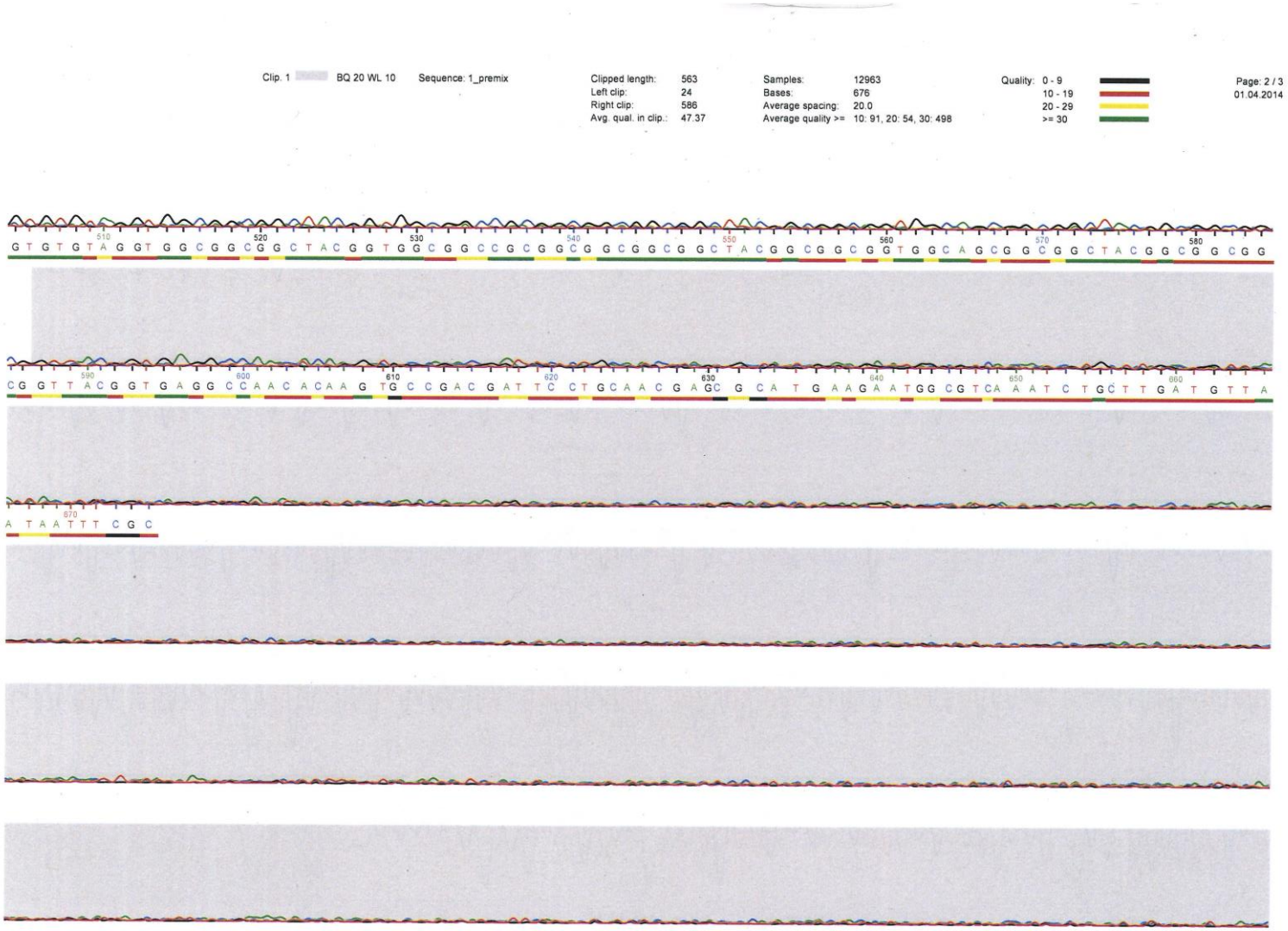
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10 - 19
20 - 29
>= 30
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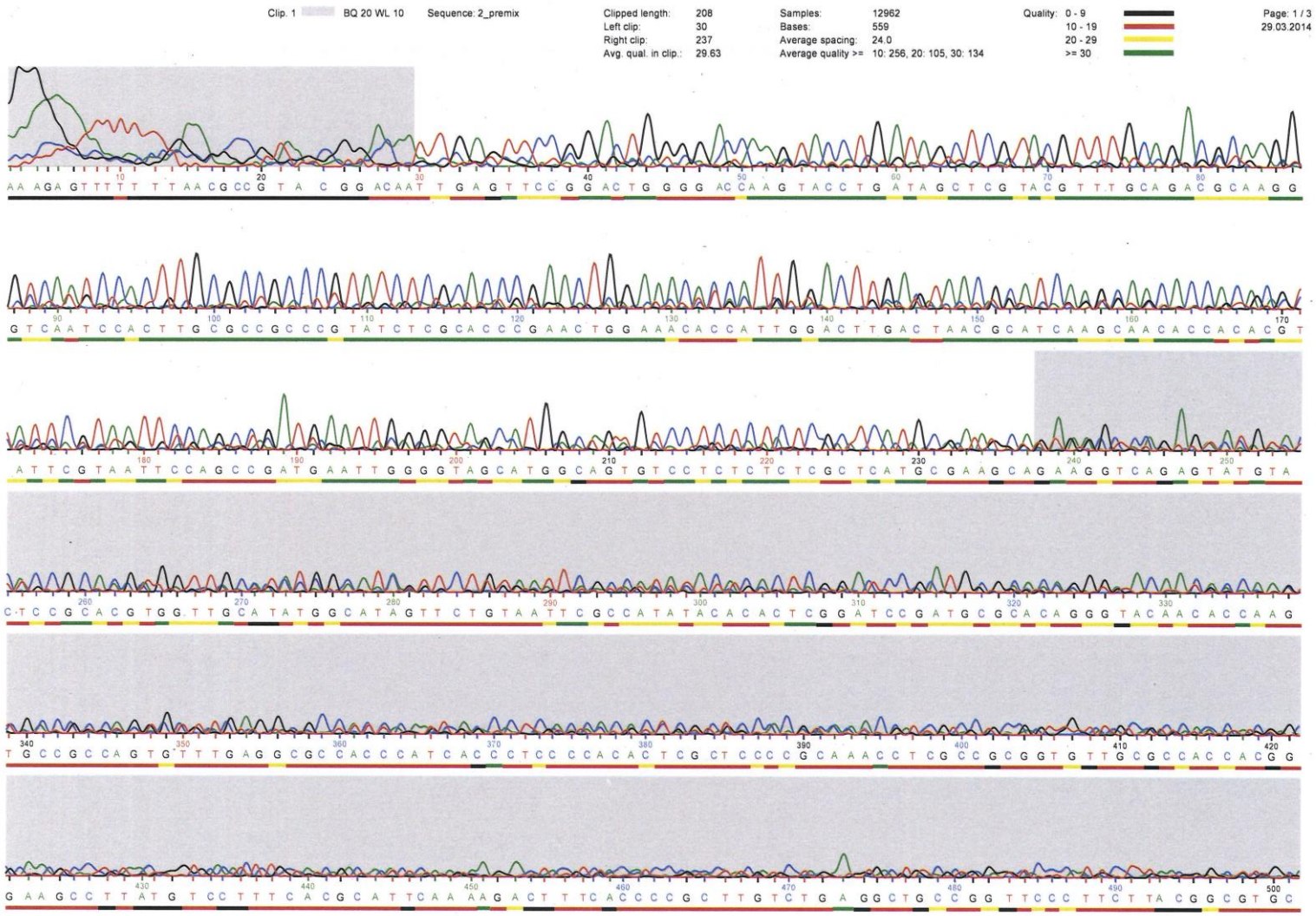
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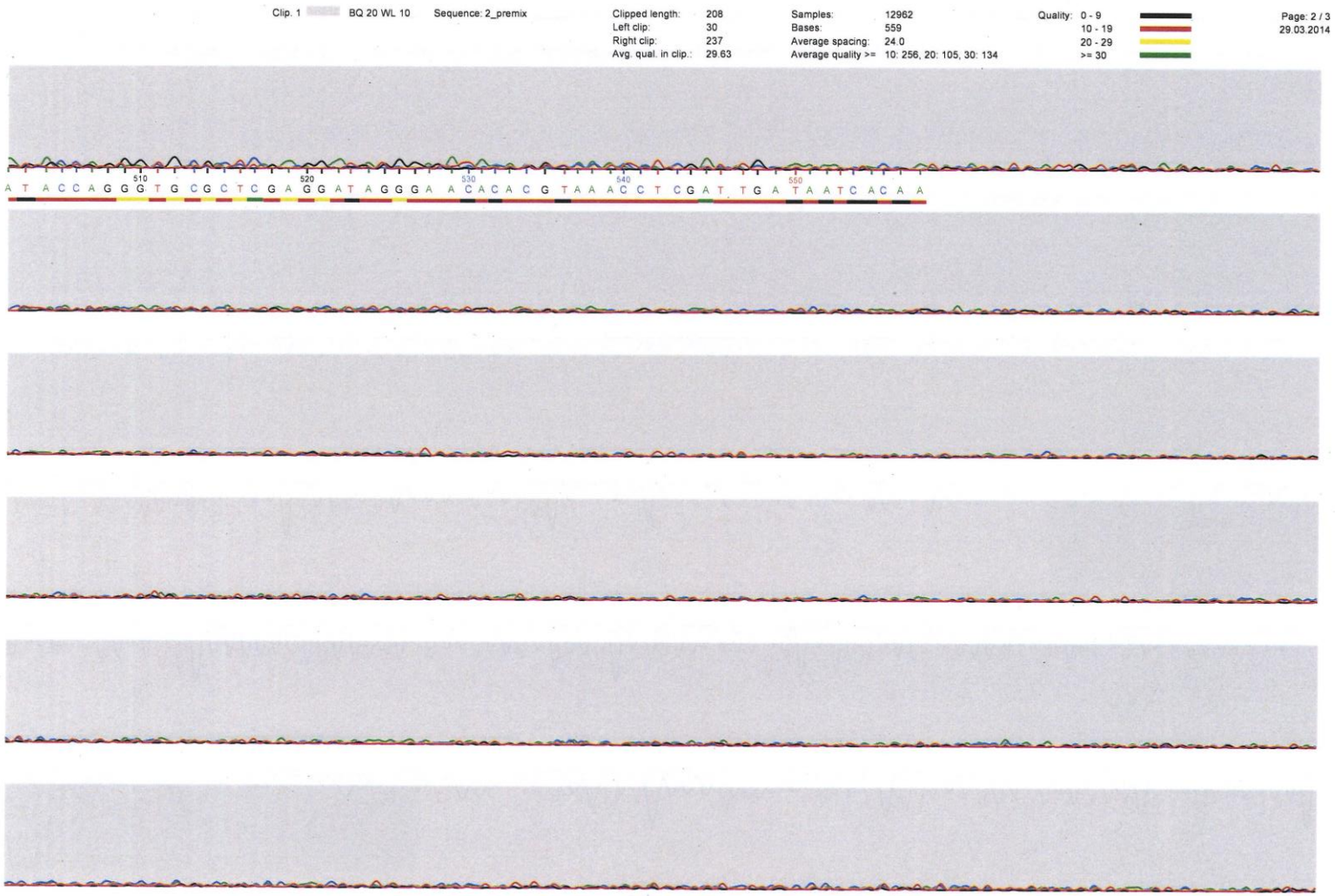
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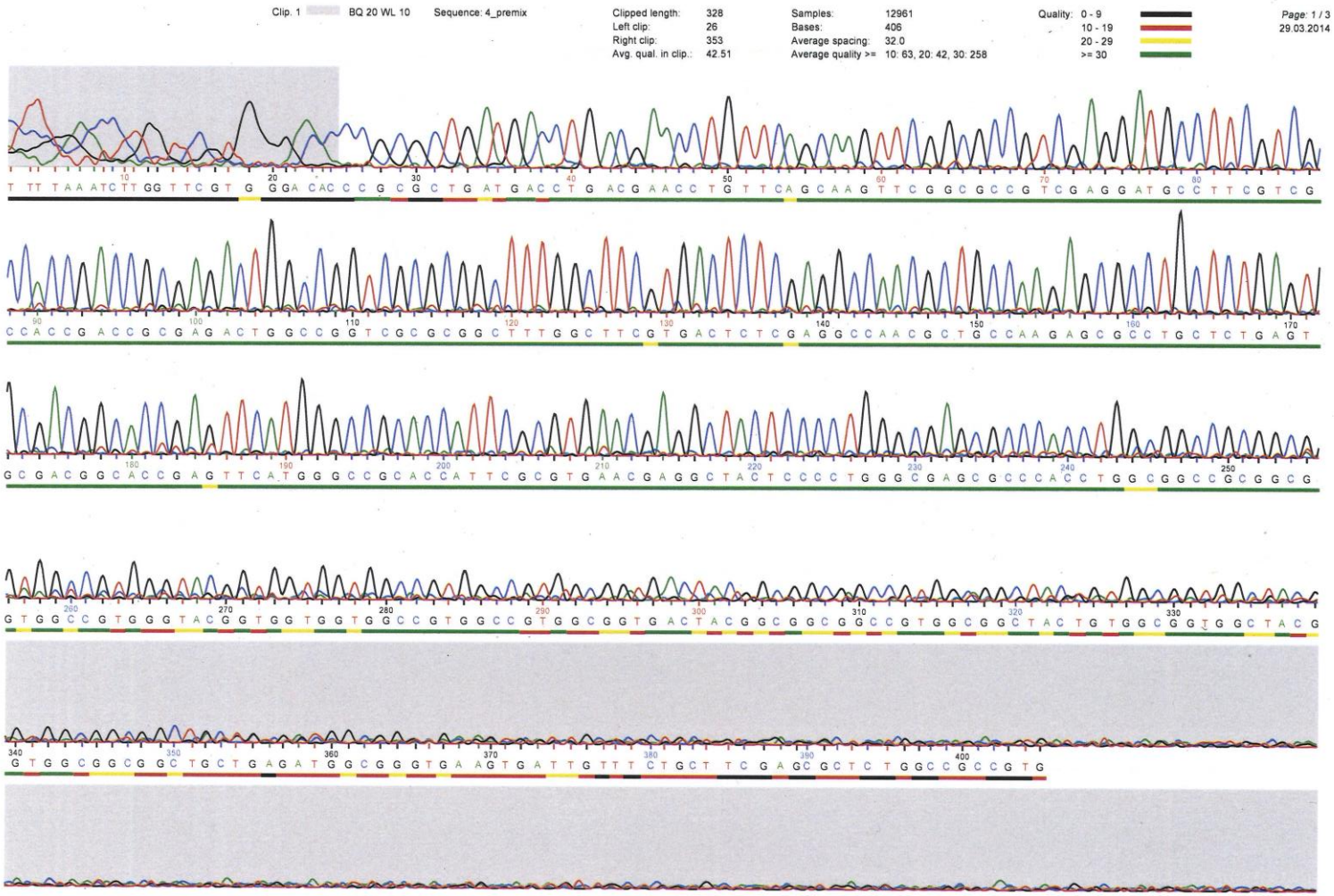
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20 - 29
>= 30
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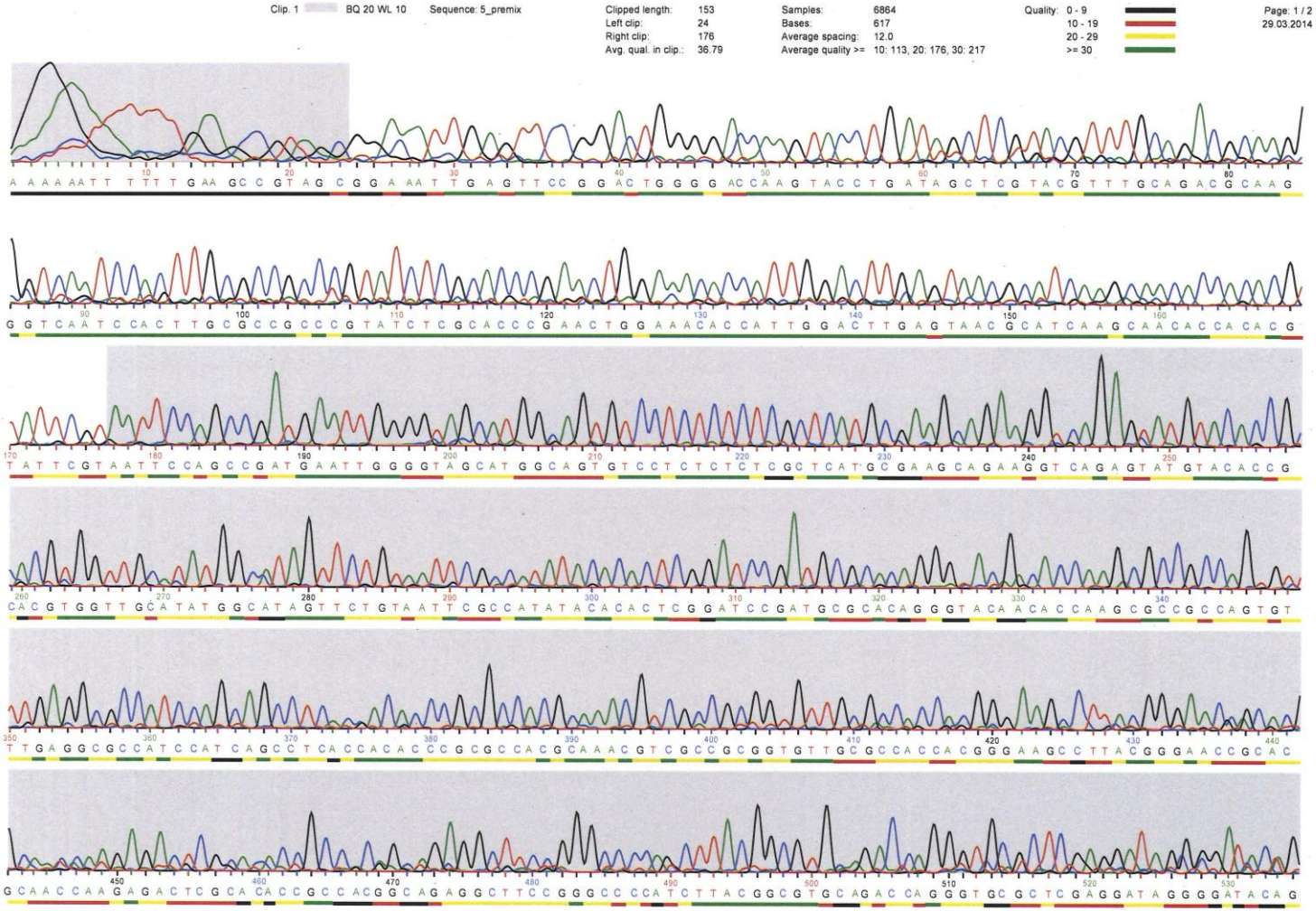
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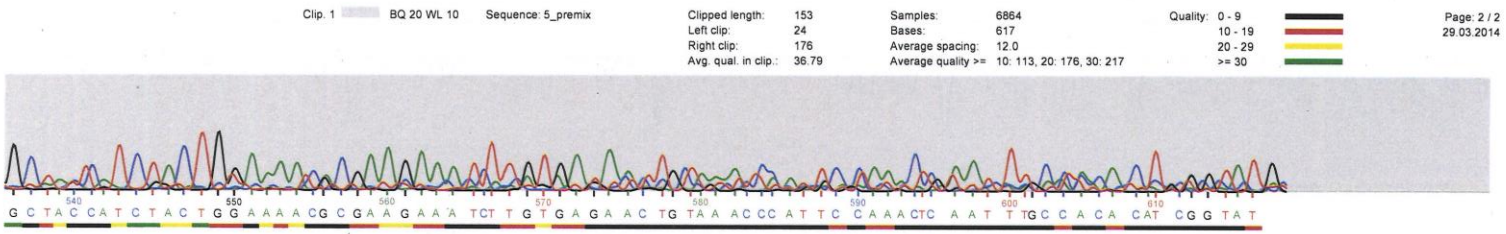
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Samples: 6864
Bases: 617
Average spacing: 12.0
Average quality >= 10: 113, 20: 176, 30: 217
Quality: 0 - 9
10 - 19
20 - 29
>= 30
Page: 2 / 2
29.03.2014
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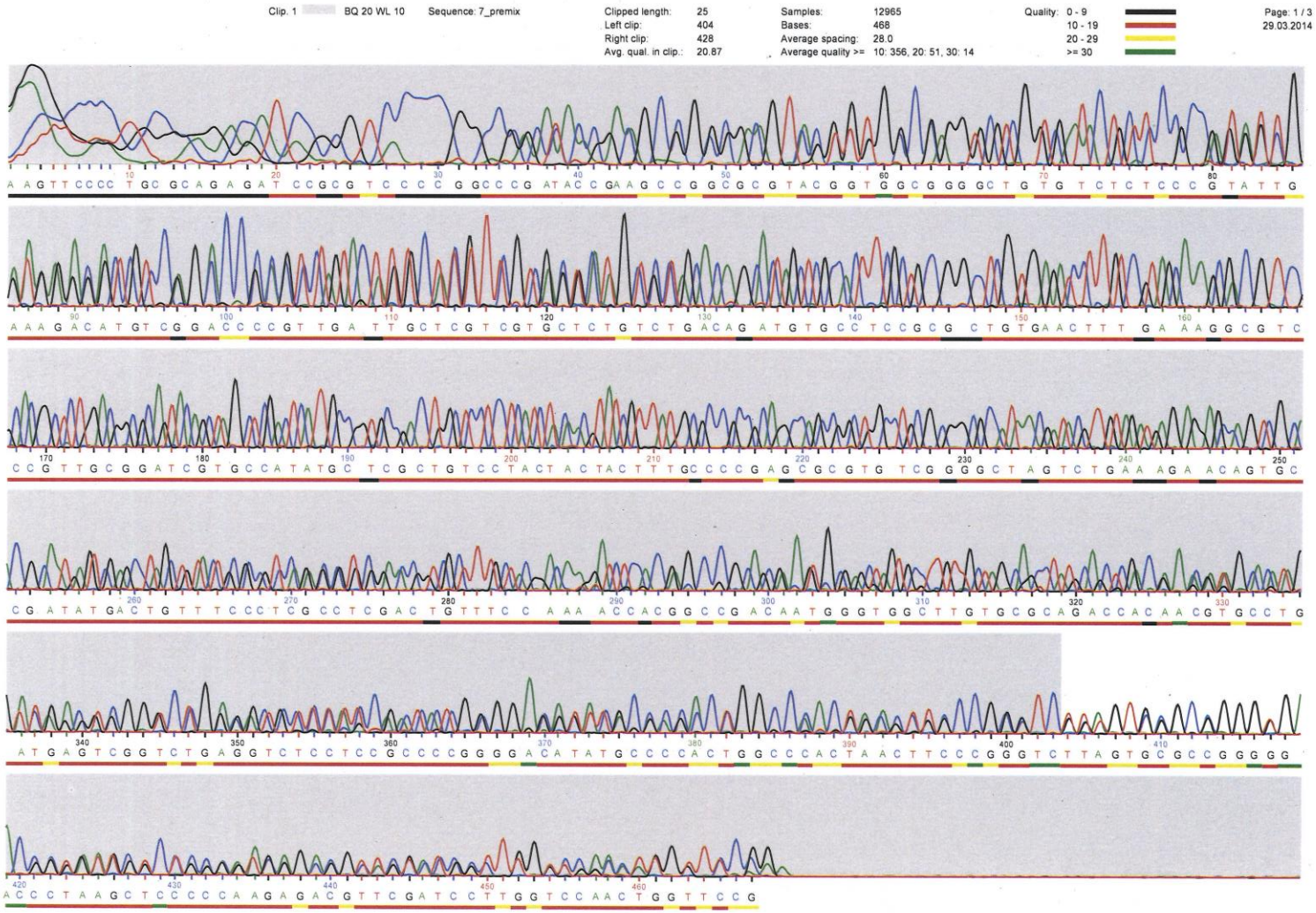
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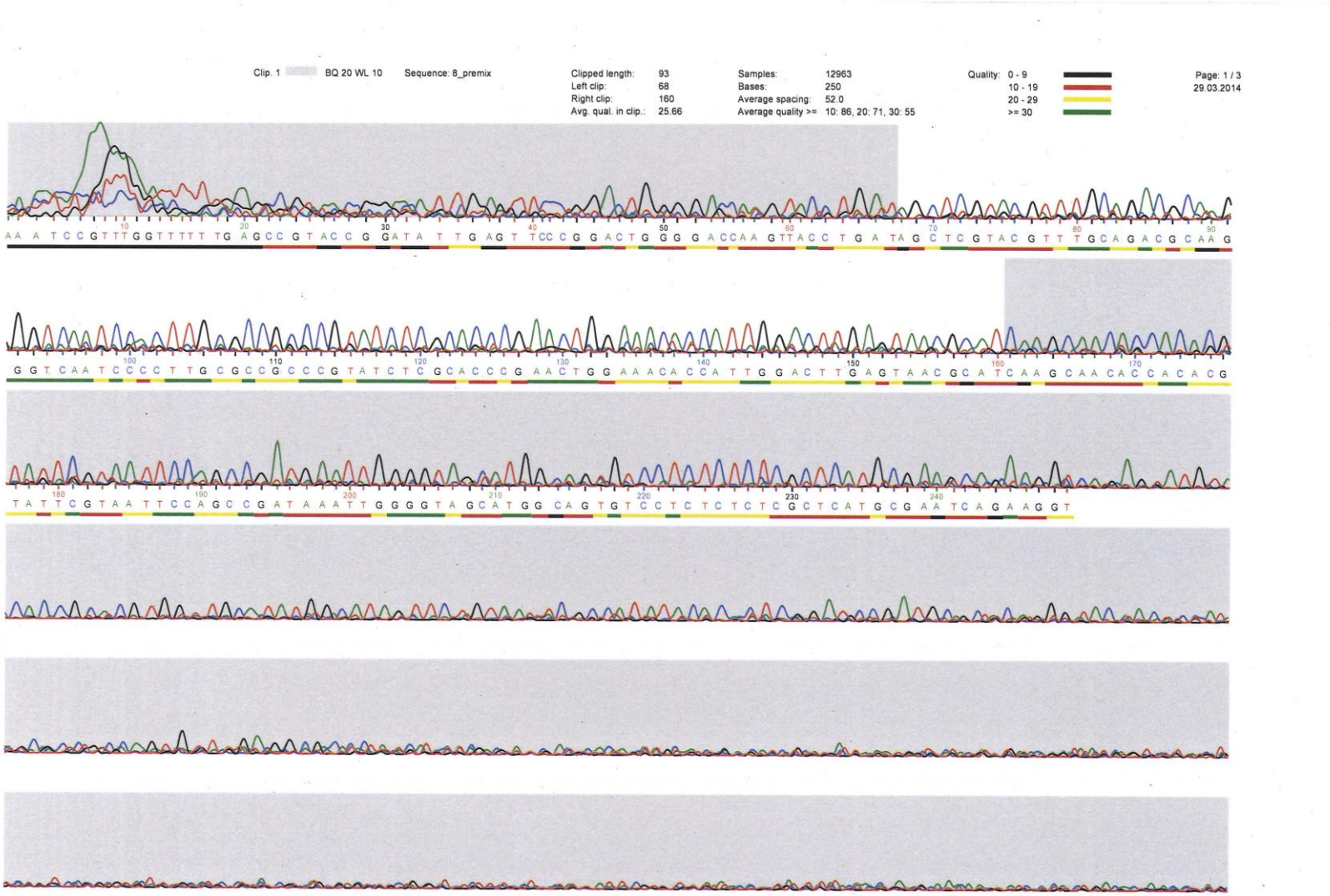
20 - 29
>= 30
Page: 1 / 3
29.03.2014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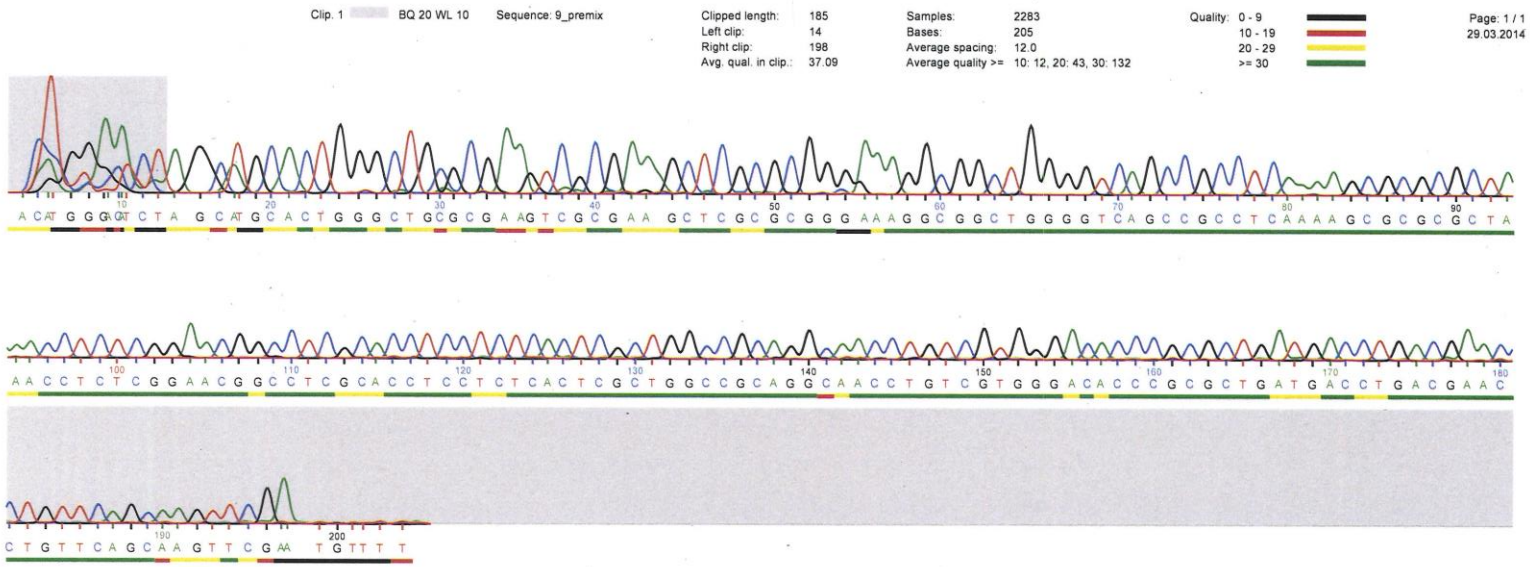
29.03.2014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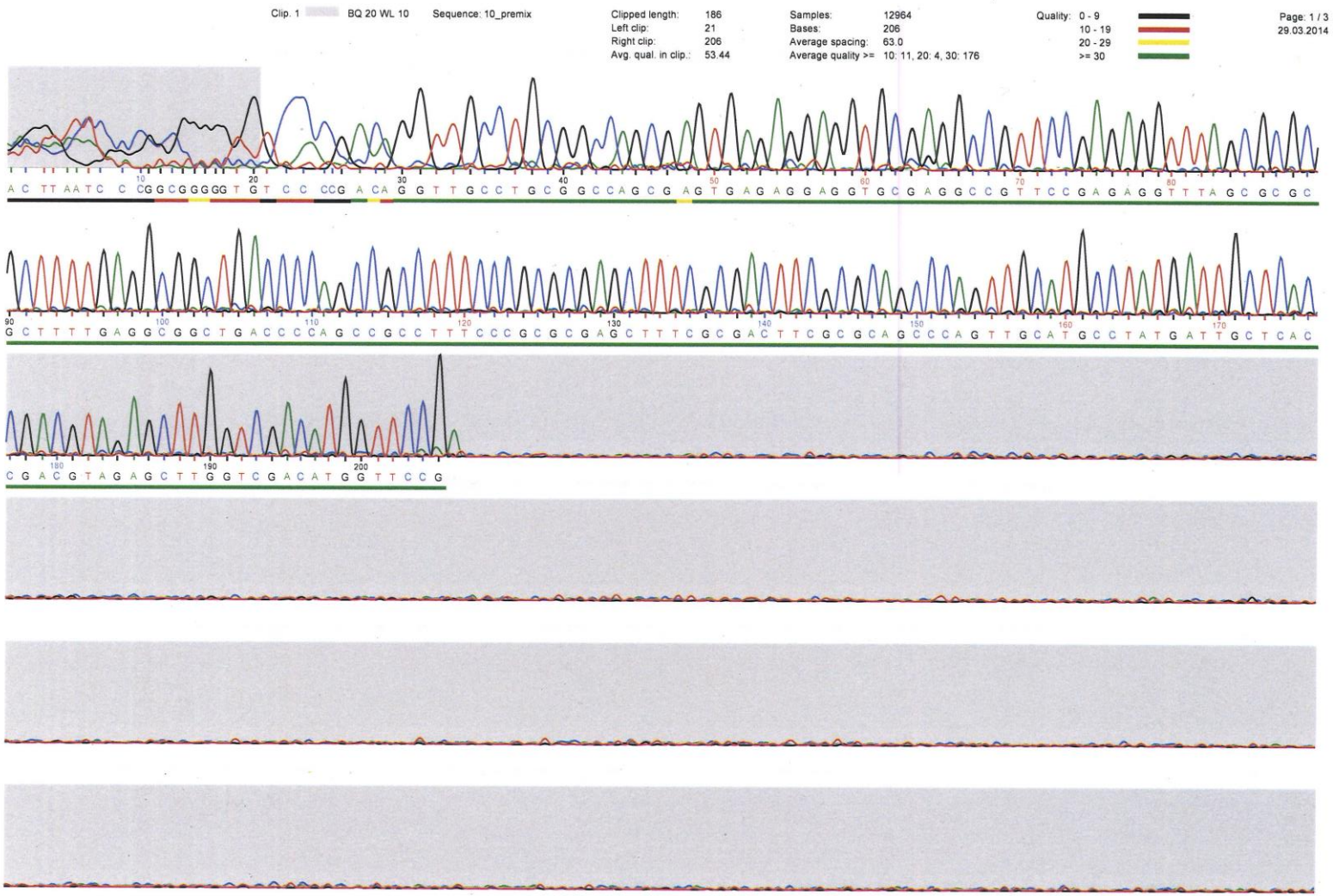
Page: 1 / 1
29.03.2014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>= 30
Page: 1 / 3
29.03.2014
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G C T T T T G A G G C G G C T G A C C C C A G C C G C C T T T C C C G C G C G A G C T T T C G C G A C T T C G C G C A G C C C A G T T G C A T G C C T A T G A T T G C T C A C
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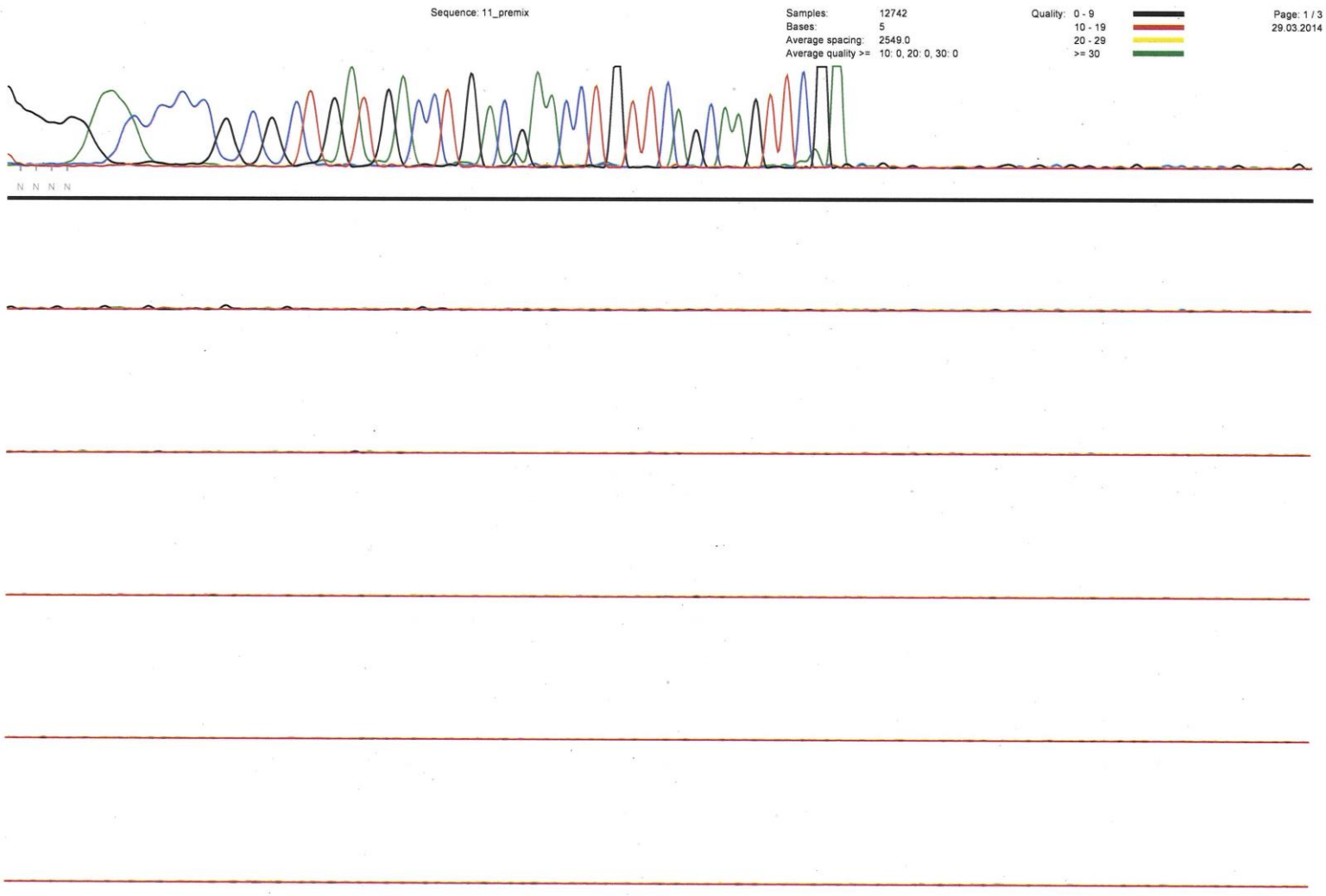
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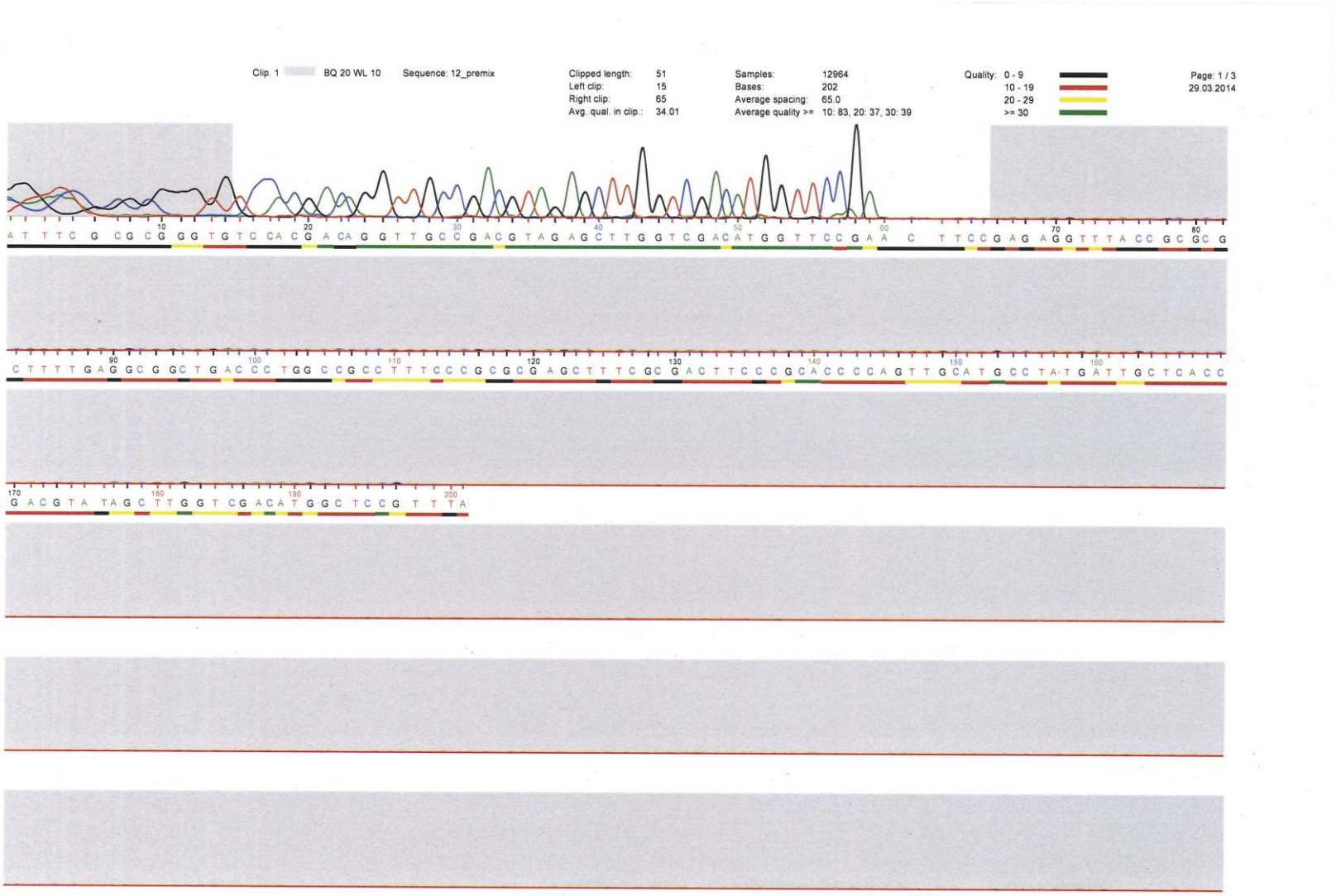
>= 30
Page: 1 / 3
29.03.2014
N N N N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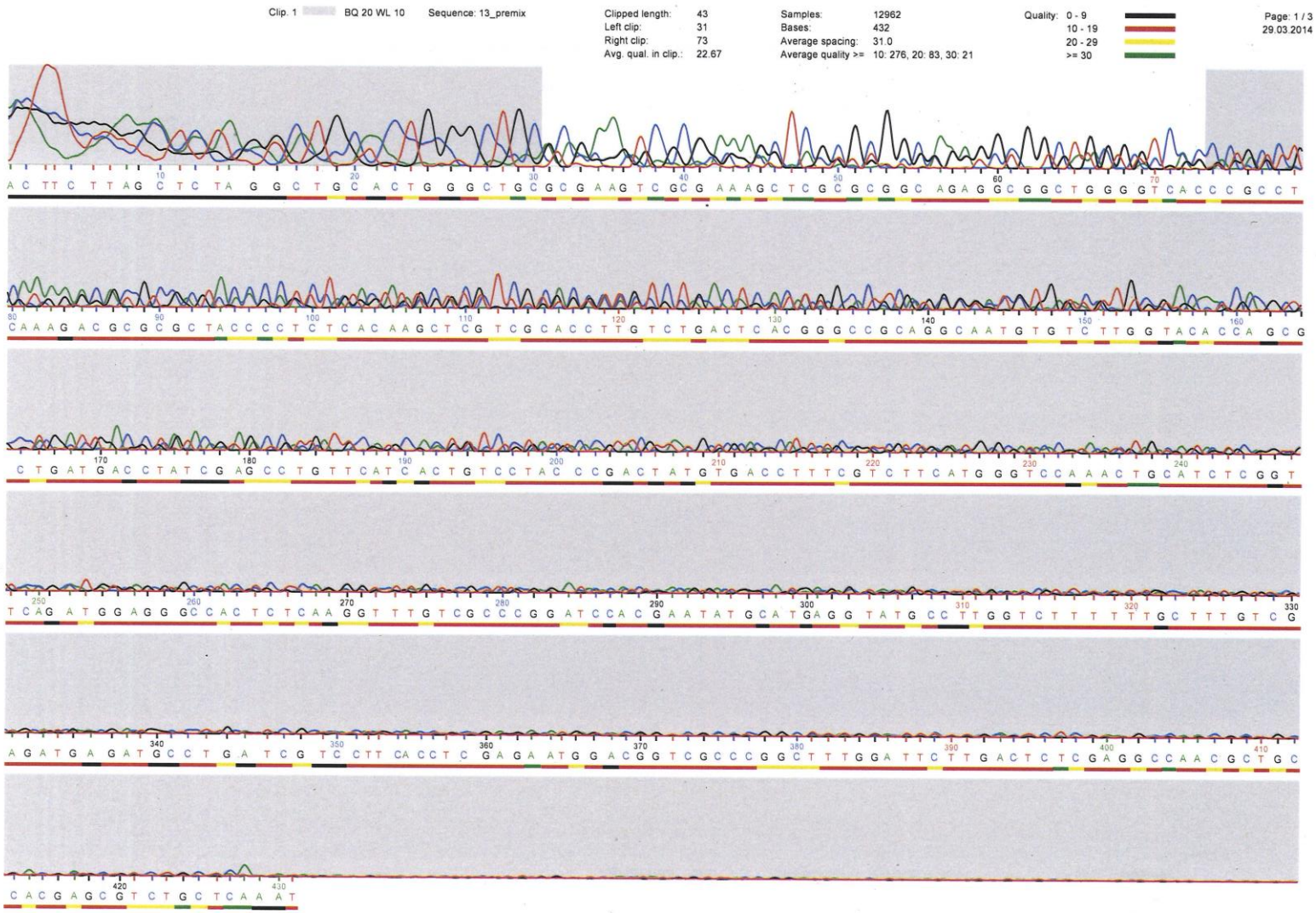
Page: 1 / 3
29.03.2014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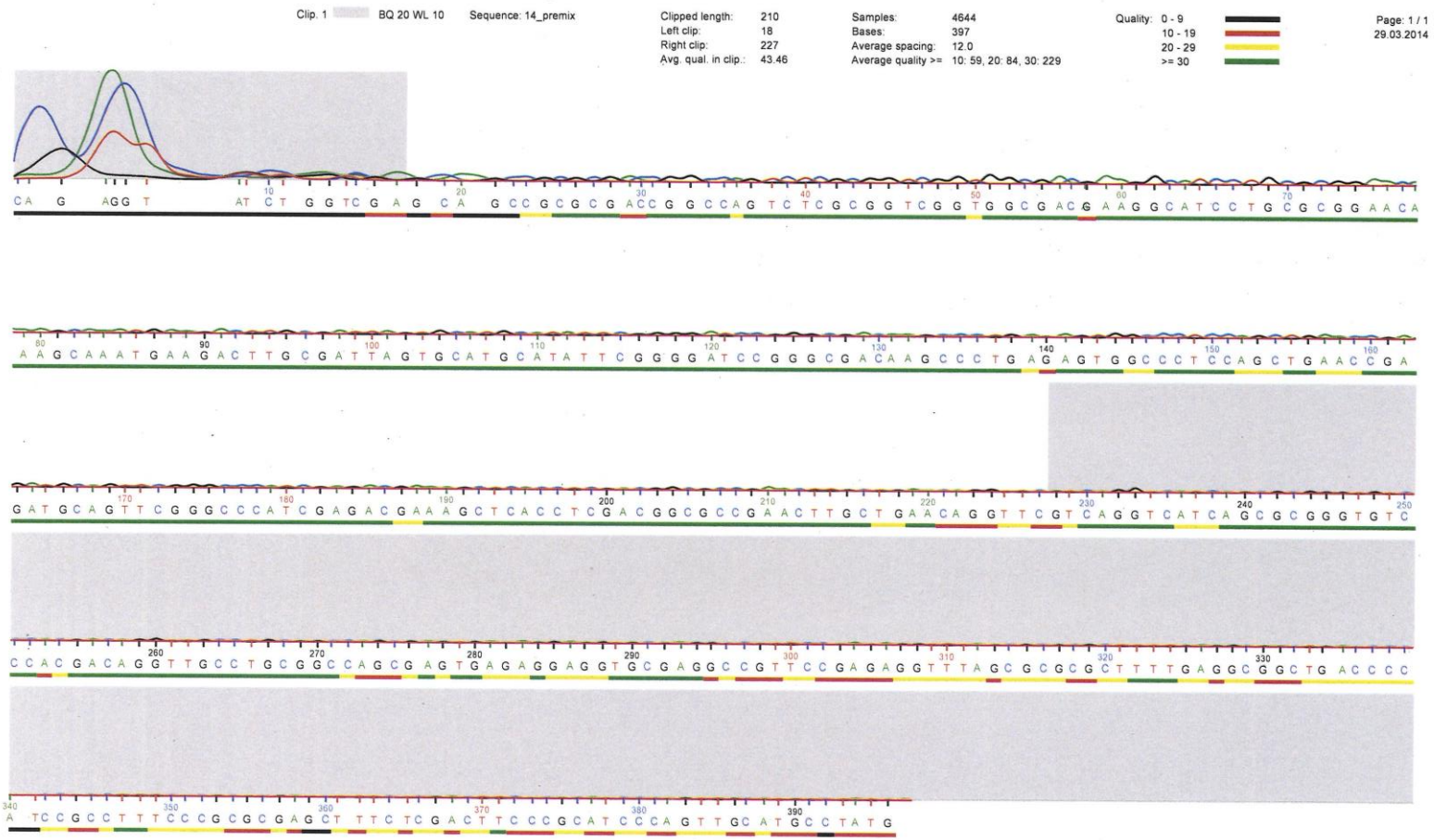
Page: 1 / 3
29.03.2014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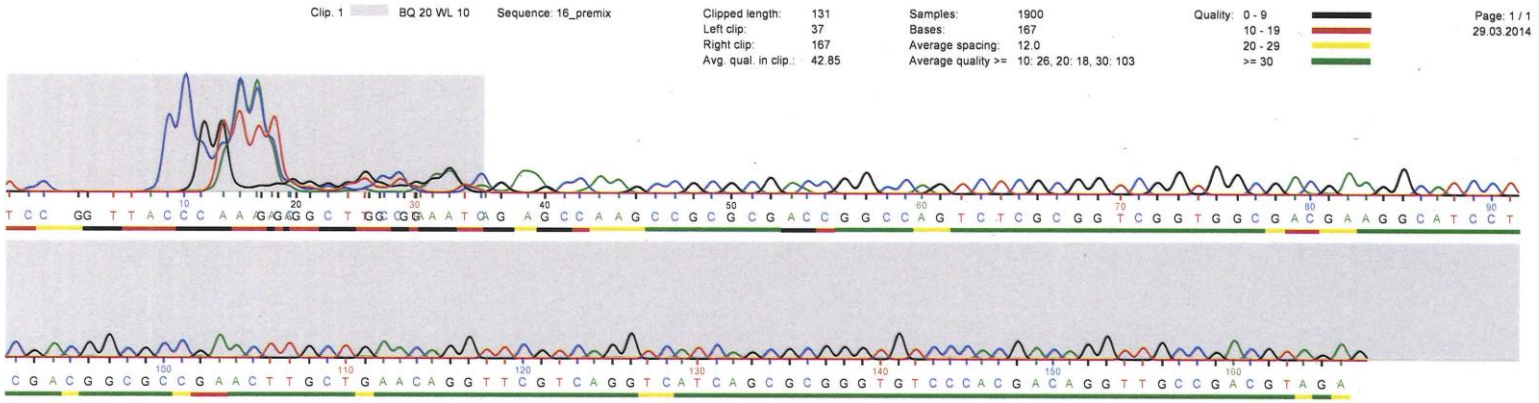
Page: 1 / 1
29.03.2014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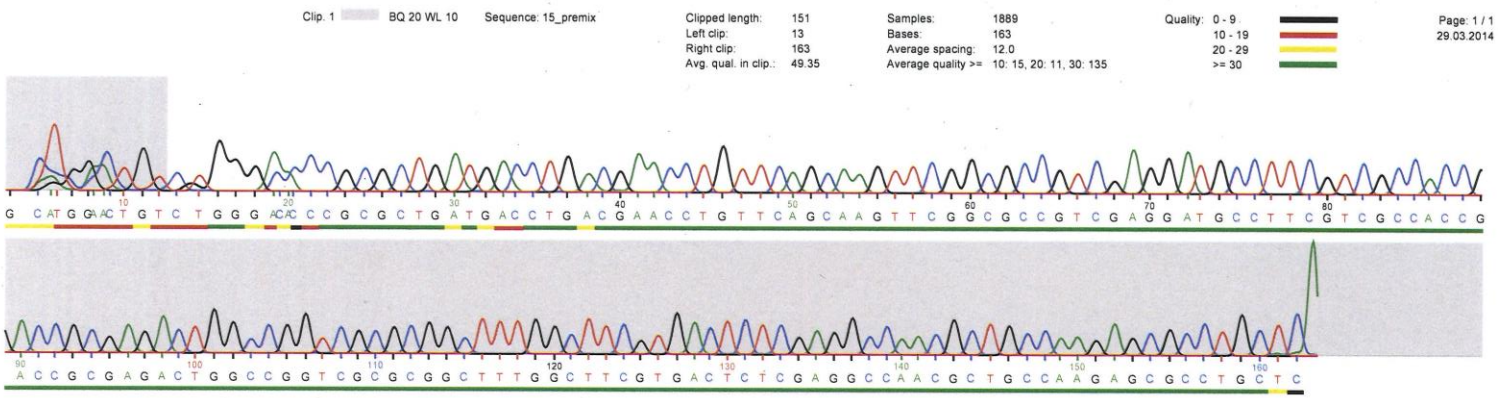
Page: 1 / 1
29.03.2014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20 - 29
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Page: 1 / 1
29.03.2014
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8.7 More figure for some experiments

- Related to chapter 3

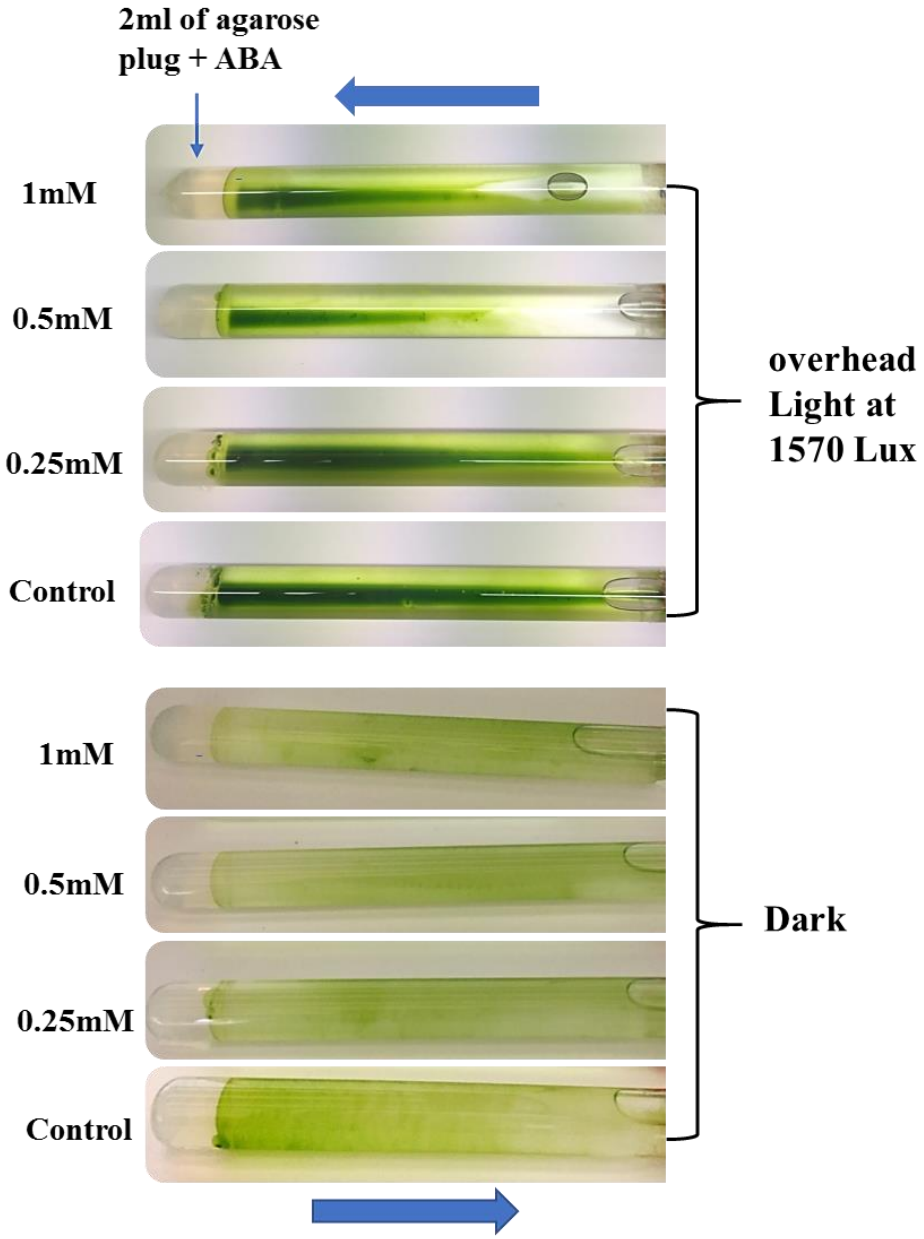

Figure 8.2: Opposing direction of travel of C. reinhardtii cells in response to ABA in the light and dark.
Algal cultures were grown under a 16h photoperiod to the mid-log phase (A_{750} = 0.3) growth stage and were sampled mid photoperiod. Fully dispersed samples of the algal culture were placed in sealed tubes with a plug of agarose containing the concentrations of ABA indicated and were immediately placed horizontally in the light (at 980 Lux) or the dark for 50 min. Shown is a representative image indicating the positions attained by the algae immediately following the incubation period.

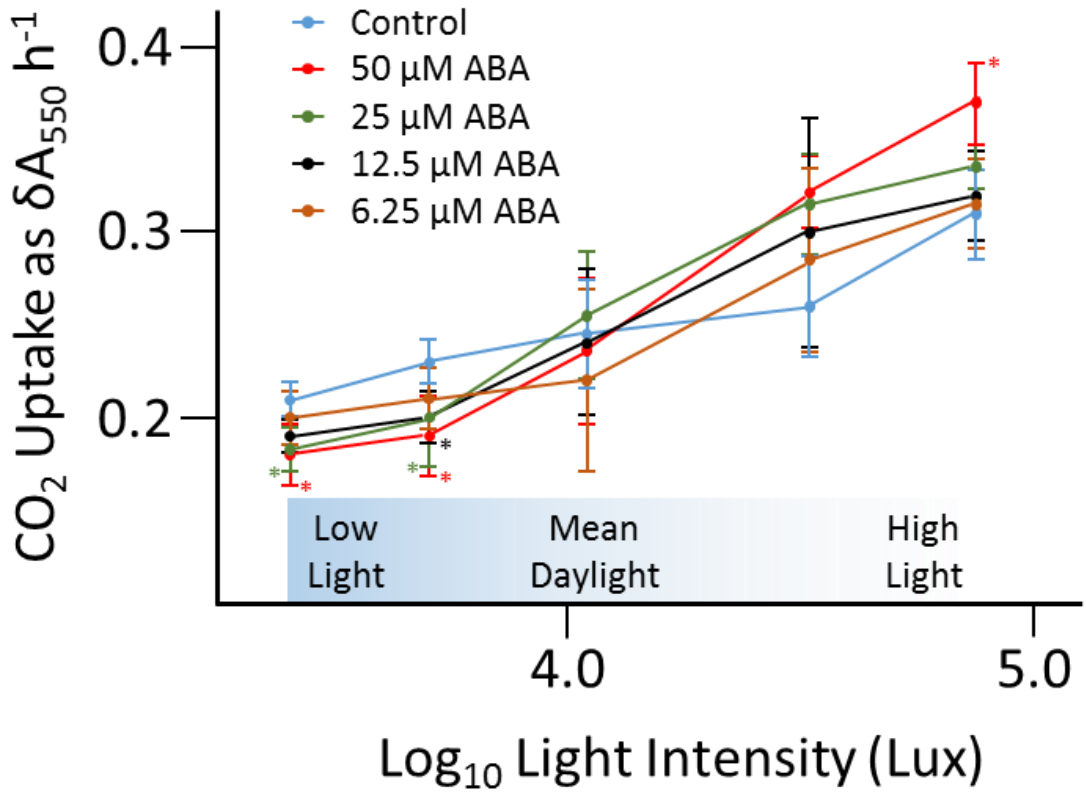

Figure 8.3: Exogenous abscisic (ABA) acid differentially altered HCO^{3-} uptake by C. reinhardtii in a light-dependent manner.

Mid-log phase (A_{750} = 0.3) algal cultures were immobilised in alginate gel beads containing the concentrations of ABA indicated and were incubated for 1 hour under different light intensities with bicarbonate indicator buffer in TAP media. Depletion of HCO^{3-} in the media was monitored as the change in absorbance in the indicator buffer at 550nm after the incubation period. Shown are the mean δA550 h-1 of n=5 replicates with errors as the 95% confidence intervals around the means in each case.

- Related to chapter 4

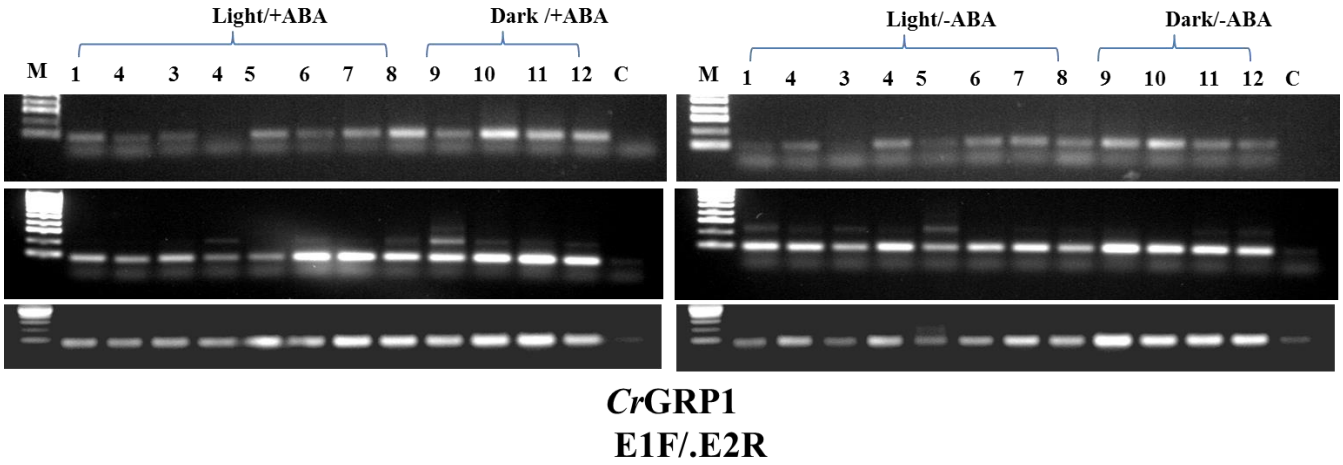

Figure 8.4: Expression analysis of CrGRP1 in the Circadian rhythm in C. reinhardii.
After treating cells without and with 50µM ABA PCR products were resolved on 1xTAE, 1.2% (w/v) agarose gel electrophoresis and visualised under UV light following EtBr staining. Molecular weight markers (Lane M – 100 bp DNA ladder) were used to confirm amplicon sizes, n=3 replicates. Lane 1-8 represents the representative mRNA levels from a circadian experiment on the light condition at 2h, 4h, 6h, 8h,10h, 12h, 14h and 16h. Lane 9-12 represents the same as above from a circadian experiment on the dark condition at 18h , 20h , 22h and 24h. Lane C: control without cDNA added.

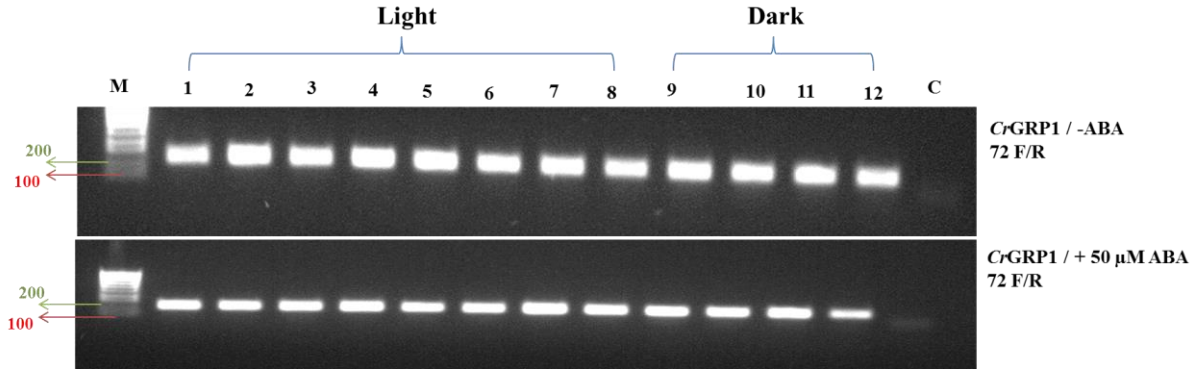

Figure 8.5: Expression analysis of CrGRP1 in the Circadian rhythm in C. reinhardii.
After treating cells without and with 50µM ABAm PCR products were resolved on 1xTAE, 1.2% (w/v) agarose gel electrophoresis and visualised under UV light following EtBr staining. Molecular weight markers (Lane M – 100 bp DNA ladder) were used to confirm amplicon sizes, n=3 replicates. Lane 1-8 represents the representative mRNA levels from a circadian experiment on the light condition at 2h, 4h, 6h, 8h,10h, 12h, 14h and 16h. Lane 9-12 represents the same as above from a circadian experiment on the dark condition at 18h , 20h , 22h and 24h. Lane C: control without cDNA added.

- Related to chapter 5

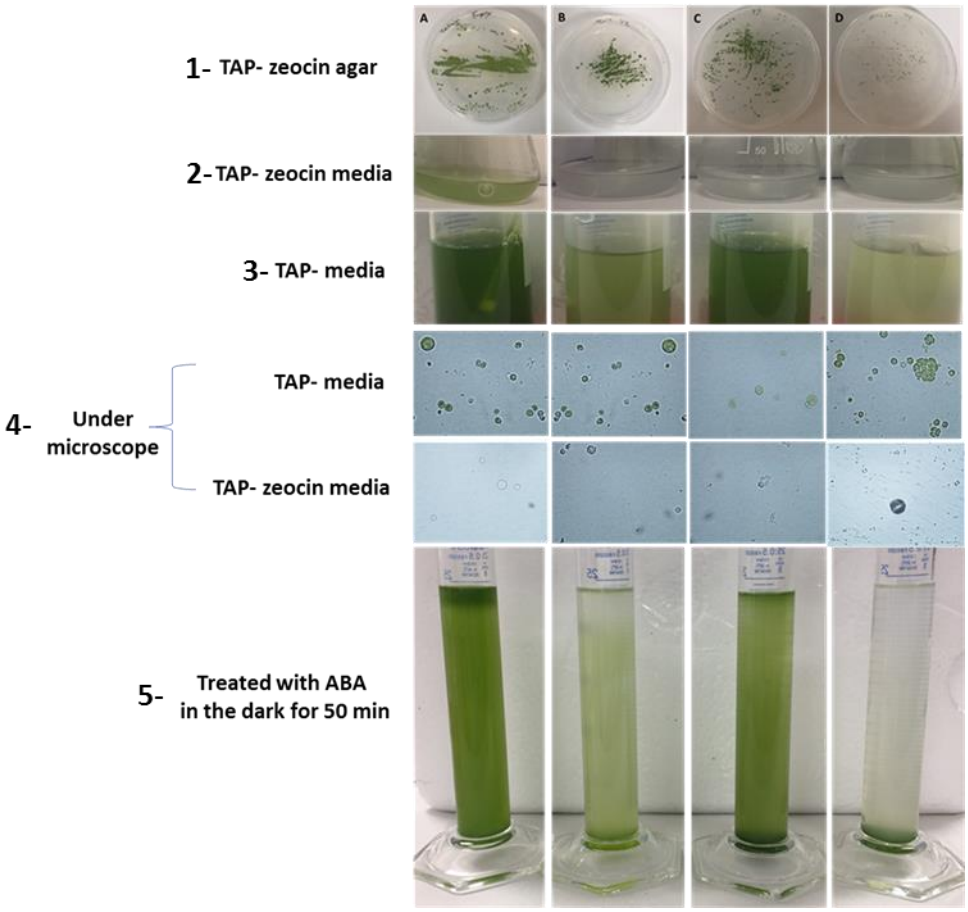

Figure 8.6:Transformed C. reinhardtii colonies after three weeks of growth and the movement of recombinant C. reinhardtii cells is differentially affected by ABA

Panel 1, only cells which received and integrated the ble zeocin resistance gene grew to form colonies. A. Empty plasmid in the TAP- zeocin agar; B. Overexpression plasmid; C. Overexpression with V5 6-his tag plasmid; D. Antisense plasmid in the TAP- zeocin agar. **Panel 2**, some colonies were growing in the TAP- zeocin media but the transgenic cells were white colour. **Panel 3**, then growth some colonies with TAP media without zeocin and they were growth very well. **Panel 4**, cells were seeing under microscope. **Panel 5**, After growth in TAP media until A_{750} = 0.3, cultures were treated with 50 µM of ABA in the dark for 50min. Images of the cylinder were taken immediately after the 50 min incubation. A. Empty plasmid; B. Overexpression; C. Overexpression with the V5 6-his tag plasmid; D. Antisense plasmid.

8.8 Abstract of a paper submitted to Scientific Reports

Abscisic acid induced a negative geotropic response in dark-incubated *Chlamydomonas reinhardtii*

Layla Al-Hijab, Adam Gregg, Rhiannon Davies, Heather Macdonald, Michael Ladomery[#] and Ian Wilson*[#]

University of the West of England, Bristol, Dept. Applied Sciences, HAS, Frenchay Campus, Coldharbour Lane, Bristol BS16 1QY, UK.

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[#]Joint senior authors

Abstract

The phytohormone abscisic acid (ABA) plays a role in stresses that alter plant water status and may also regulate root gravitropism and hydrotropism. ABA also exists in the aquatic algal progenitors of land plants, but other than its involvement in stress responses, its physiological role in these microorganisms remains elusive. We show that exogenous ABA significantly altered the HCO3 - uptake of Chlamydomonas reinhardtii in a light-intensity dependent manner. In high light ABA enhanced HCO3 - uptake, while under low light uptake was diminished. In the dark, ABA induced a negative geotropic movement of the algae to an extent dependent on the time of sampling during the light/dark cycle. The algae also showed a differential, light-dependent directional taxis response to a fixed ABA source, moving horizontally towards the source in the light and away in the dark. We conclude that light and ABA signal competitively for algae to position themselves in the water column to minimise photo-oxidative stress and optimise photosynthetic efficiency. We suggest that the development of this response mechanism in motile algae may have been an important step in the evolution of terrestrial plants and that its retention therein strongly implicates ABA in the regulation of their relevant tropisms.

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- Related to chapter 1 for Figure 1.6

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