THE INFLUENCE OF THE CIRCADIAN CLOCK ON STOMATAL CONDUCTANCE AND CARBON ASSIMILATION IN *ARABIDOPSIS THALIANA*

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Summary

Plants have evolved an internal feedback system of gene expression, the circadian clock, to allow for adaptations to changes in diurnal conditions to maximise their ability to utilise the environment for nutrients and growth. The circadian clock aligns the plants processes with fixed external signals, such as the light intensities at dawn and dusk. This study investigated the influence of the circadian clock on stomatal conductance and carbon assimilation and the decoupling of these processes.

Timing of Cab (TOC1) and Circadian Clock Associated 1 (CCA1) genes were overexpressed and tested to investigate the impact of altered clock rhythms. Evidence showed of significant increases in carbon assimilation for the TOC1 overexpressed genotype compared to the CCA1 overexpressed genotypes. Data also showed the TOC1 overexpressed genotype had a lower Water Use Efficiency (WUE) than any of the other genotypes, signifying a potential weakening of stomatal sensitivity to internal water levels and allowing for increased photosynthesis at the detriment of water conservation.

Changes to light conditions were investigated to look at any corresponding impact on the decoupling of processes when the plant would not be able to correctly align itself to dawn. Changes in light regimes were shown to increase the extent of the decoupling between stomatal conductance with carbon assimilation. Shifting the timings of dawn 2 hours behind “natural dawn” were shown to impact the size but not the timing of the decoupling in relation to the initial increase in light intensity.

Overall, this study suggests that the circadian clock is not the cause of the decoupling between these processes but does have an impact on the size of the difference between these process that were thought closely linked.

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Introduction

In 2011 the human population grew to 7 billion, and it is estimated to reach 8 billion by 2025, and nearly 10 billion by 2050 (Cohen, 2003). The required increase in urbanisation to house the growing population, the increasing demand for food supplies along with the changes in diet (particularly of many eastern countries) to a more meat-based diet (Liu, Yang and Savenije, 2008) is putting increasing pressure on plant scientists and breeders to improve plant productivity, one of the largest issues facing mankind. It has been predicted that the current food production needs to double by 2050 to be able to support the population (Crist, Mora and Engelman, 2017) and this must be achieved with less land and less inputs such as water. Therefore, any increase in water retention and water use efficiency will be vital in helping agriculture, as well as increasing the amount of yield produced per plant.

In 2000 it was estimated that approximately 75% of the water used by humans was for agriculture (Wallace, 2000). Therefore, investigating and implementing changes to agricultural practices will be a resourceful method of increasing water efficiency to allow for more surplus to maintain the growing population. Even small decreases in this number will enable more water to be allocated for agricultural and commercial uses of the growing population, or to be used in drier regions or for more water intensive crops such as rice. It is estimated that 7% of the global population currently live with water scarcity and that figure will increase to approximately 67% by 2050 due to climate change and the increase in populations in regions such as China, North Africa, Sub-Sahara Africa and South-central Asia (Wallace, 2000).

Wallace (2000) showed that of the water used in the watering of irrigated agricultural fields, 30% of the water is lost in the storage and transport of water to the fields, 37% of the water is lost to evaporation, and 63% of the remaining water is lost as runoff or draining. This means 44% of the total water used was lost with no gain to the crop, so aiding the efficiency of the water used and lost as transpiration by the plant would help drastically. It would help in reducing the amount percentage that is “wasted” by not reaching the plant, if the crops were able to more efficiently utilise water in transpiration and stomatal conductance, and to reduce the amount of water input used in irrigation and watering. It would also be possible to reduce the amount of water put in as irrigation or watering if less was needed to maintain the same levels of crop production in very water-scarce areas, but to be able to study and alter water efficiency in new ways, the mechanisms and responses much be understood.

Changes to transpiration and water requirements occur throughout the day, with fluctuations caused by changes in the plants body clock, known as the circadian clock. Adapting to changes in the environment throughout the day, such as light intensity and humidity, help plants to maintain an effective and sustainable rate of photosynthesis and transpiration, so looking into the circadian clock and how it functions opens new avenues for investigation into water retention.

Transpiration and Plant Water Loss Mechanisms

Transpiration through stomata is ~~one of~~ the main mechanisms of water loss from a plant. Approximately 95% of the gaseous water lost to the atmosphere is through these stomata (Matthews, Vialet-Chabrand and Lawson, 2018). The rate of transpiration in plants changes throughout the day and is variable

dependent on many abiotic and biotic factors, such as the temperature, soil water concentration and leaf solute concentration. With reduced soil water concentration, and thus an increase in soil salt concentrations, transpiration was found to be reduced and in extreme drought the rate of root uptake as well, so that the plant would not lose too much water and start dying (Razzaghi et al., 2011).

One result of transpiration is thermoregulation. Leaf cooling is a necessity due to a plants sessile nature and inability to move from hot environments and the gain of heat energy from the absorption of light energy, photosynthesis and metabolite production. While plants have a few methods of reducing heat, transpiration from the leaves is one of the main mechanisms to reduce surface temperatures (Crawford et al., 2012). The high heat capacity of water allows for a lot of heat and energy to be lost from the mesophyll and stomata when water evaporates and leaves the plant via the stomata (Crawford et al., 2012). Without this thermal regulation, leaves can become temporarily damaged and in extreme cases permanently, with chloroplast photosystems and other metabolic processes being disrupted and reducing the rate and efficiency of photosynthesis (Drake et al., 2018). With global temperatures estimated to increase in the coming decades more water loss by transpiration is likely to occur, intentionally to both cool down the plant and unintentionally by a higher rate of evaporation. This will exacerbate the issue of needing more water to grow the increased amount of crops, and also reduce the amount of land available to grow crops on as desertification affects semi-arid regions (Sivakumar, 2007). The increase in temperature will reduce the amount of crop biomass produced, with Peng et al's (2004) study showing a 10% reduction in

grain yield for each 1°C increase in minimum growing temperature in their samples, and further predictions of approximately 16% reduction in maize, wheat and sorghum in the central United States (Peng et al., 2004).

Stomata and Guard Cells

The sites of transpiration in the leaf are the stomatal pores, openings in the leaf surface which allow for diffusion of gases between the leaf interior and external atmosphere. The leaf surface is otherwise covered in an impermeable waxy cuticle layer to reduce water loss, which despite covering 95-99% of the leaf surface contributes only ~5% of the gas exchange (Matthews, Vialet-Chabrand and Lawson, 2018).

Stomatal aperture is controlled by two specialised epidermal guard cells which respond to internal and external signalling to open and close the stomatal pore. Signals include environmental changes like light intensity, metabolic changes in the guard cells and surrounding tissue, and the water potential uptake from the soil to the roots (Farquar and Sharkey, 1983).

Stomatal aperture is determined by the amount of turgidity of the guard cells, which increases and decreases the size of the stomatal pore and the flow of gases into and out of the leaf. This occurs due to the accumulation of potassium ions and sugars in the guard cells, decreasing the water potential and drawing water in via osmosis (Shimazaki et al., 2007). This increase in volume increases the cells turgor which due to a thickened inner cell wall causes the cells to curve and create the stomatal opening (Figure 1). The increase in volume is attributed to the movement of ions from the cytosol into the vacuoles

of cells, and the binding of these vacuoles together, and the closure of stomata is the reverse of this process (Shimazaki et al., 2007).

The opening and closing of stomata are in response to changes in red and blue light wavelengths interacting with the guard cells. Red light has a lesser effect on stomatal opening than blue light, but still drives photosynthesis, decreasing the intercellular CO_2 concentration (Zeiger et al., 2002).

Although blue light responses require a background of red light to occur, it does cause different reactions in the plant. Photoreceptors are responsible for detecting and initiating light responses in the plant, Zeaxanthin absorbs blue light, and Phytochromes absorb red light. In guard cells this and signals potassium (K^+) and chloride (Cl^-) uptake into the cell through potassium-gated channel proteins from the activation of plasma membrane H+ ATP-ase polarising the cell membrane (Shimazaki et al., 2007). Blue light also stimulates the production of malate^{2-} and the uptake of Cl^- and NO_3^- to respond to the increase in positive charge of the cell. Malate production occurs from starch which is stored in the guard cells being hydrolysed and transformed (Shimazaki et al., 2007).

Guard cells can swiftly react to change in light intensity, with reactions in *Arabidopsis thaliana* being recorded within 15 minutes of change (Lawson and Blatt, 2014), arising from cloud cover or interference from the leaf canopy.

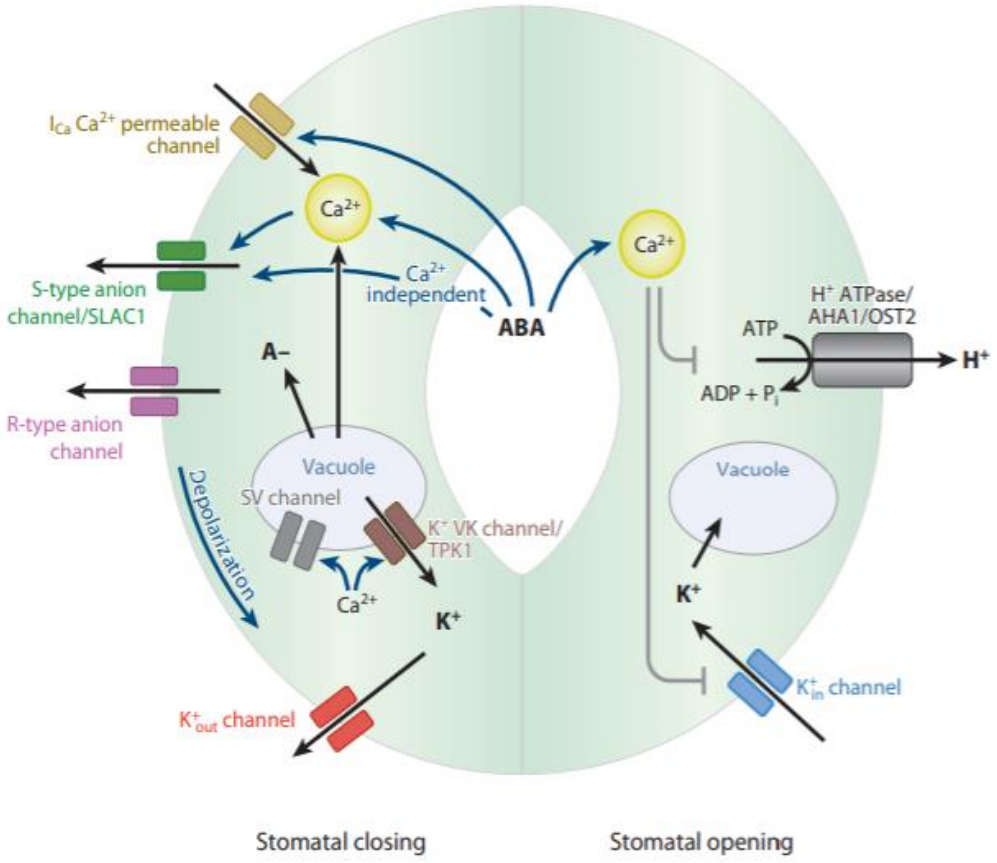

Figure 1: Diagram model of guard cell signalling and ion regulation in response to ABA causing changed in cell turgidity. Edited from Kim et al., 2010.

Stomatal Size and Abundance

The maximum amount of stomatal conductance (g_s) and thus water loss from a leaf is directly dependent on the size and number of stomata, which itself in heavily directed by environmental factors (Franks and Farquhar, 2006). One factor is the amount of atmospheric CO_2, with increases being directly linked to a reduction in the number of stomata found on leaves (Gray et al., 2000). Increasing atmospheric CO_2 from climate change will lead to reduced number of stomata, reducing the amount of water vapour lost from the plant, however

it will also allow even more increases in water use efficiency if stomata are not needed to be open as wide or as long to gain the same amount of CO_2.

Investigating the behaviours of guard cells under different conditions and times of the day will allow for a greater understanding on how this may be implemented in the future. It has been shown that in plants grown with a reduced number of stomata, the aperture is increased which increases the surface area of diffusion, taking in more CO_2 and losing more water, to counteract the loss of stomatal pores (Lawson and Blatt, 2014). While changes to stomata are known to have an influence on the plants ability to transpire, changes to stomatal density have been shown to be countered by changes to the stomatal aperture (Büssis, von Groll, Fisahn and Altmann, 2006). When grown under natural light conditions, mutants with increased or decreased numbers of stomata were shown to have similar rates of stomatal conductance and carbon assimilation to wild type plants, however when tested under high light conditions were shown to have reduced levels of photosynthesis (Büssis, von Groll, Fisahn and Altmann, 2006). This implies that stomata have a direct influence on the water use efficiency of a plant and are able to adapt with morphological plasticity. The number of stomata on a leaf is also variable to the water availability of the plant, with an increased number in water scare plants but a decrease in droughted conditions and stomatal aperture reduced in both cases (Xu and Zhou, 2008).

Photosynthetic Carbon Fixation

Stomatal conductance is strongly correlated to the rate of photosynthesis (Wong et al., 1979). Both respond to changes in light intensity, with photosynthesis being markedly faster than stomatal responses (Lawson, von Caemmerer, Baroli, 2010; Lawson and Blatt, 2014; McAusland et al., 2016).

The coupling of conductance and photosynthesis is due to the diffusion of CO_2 and osmosis of water in out of the stomata pores. The CO_2 is required to fuel the Calvin Cycle in the photosynthetic pathway and is converted into 3-phosphoglycerate and into sugars used by the plant for growth, maintaining metabolism and converted into the seeds and edible tissue required by farmers.

The amount of carbon fixation, and therefore photosynthesis, can be limited due to factors such as light intensity, stomatal conductance, and Calvin Cycle throughput. The conversion of light energy into metabolites by photosynthesis is influenced by the amount of light intensity reaching the photosystems of the chloroplasts in the plant. Higher amounts of light intensity can increase the rate of photosynthesis until the incoming energy becomes too great and starts damaging the photosystems, temporarily reducing the ability of the plant to capture light and slowing the rate of carbon assimilation (Kulheim, Agren and Jansson, 2002). Photosynthesis may also be limited by the amount of CO_2 available. Closed stomata stop any gaseous transfer from the plant's interior to the atmosphere and can slow and stop photosynthesis if CO_2 is blocked from entering the leaf for an extended period. Therefore, plants have to balance the need for photosynthetic products and energy with the amount of water lost via transpiration to ensure that it does not cause damage or death.

Chlorophyll Fluorescence and Photosynthetic Efficiency

Photosynthesis is the main metabolic process in any plant, creating the energy and carbon-based products used to fuel all other processes and to build the physical structure of new growth. However, measuring the direct rate *in vivo* has difficulties, controlling and measuring the gaseous input and output of leaves in a field environment would require management and be easily disrupted by non-airtight seals around the leaves and cause damage to the delicate tissue. Another method of measuring photosynthesis has been to investigate the emission of chlorophyll fluorescence, one of the three outputs of energy once light activates Photosystem II (PSII) in the chloroplast. Light activation of PSII releases energy to the electron transfer chain releases energy as activated electrons (photochemical quenching) heat (nonphotochemical quenching), and as light fluorescence, which can be measured and used to calculate the amount of photochemical quenching occurring at specific light levels and used as a stand in for the rate of photosynthesis (Baker, 2008).

The reduction of the primary quinone accepted of PSII by electron acceptance can is driven by exposing the leaf to a large increase in light intensity and is used to determine the percentage of energy utilised by photochemical quenching and fluorescence emission (Baker, 2008). The use of light radiation has allowed for a non-damaging method of measuring the quantum yield of a plant both in the lab and the field and can be used to easily create light response curves and as a basic indicator of plant stress.

Decoupling of Stomatal Conductance and Carbon Assimilation

Matthews et al (2018) examining the responses of stomata in different light regimes uncovered a decoupling between the rate of carbon fixation and the stomatal conductance in *A. thaliana* over the course of a day. The experiments were ran with plants grown under one of three light conditions, fluctuating intensity with a daily repeated pattern of light, fluctuating intensity with a random pattern of light or a constant intensity square wave of light. All regimes had the same amount of total daily intensity. The paper showed that acclimatisation to the type of light influences the responses of stomatal conductance at different times of the day, and as such the rates of photosynthesis and water use efficiency. The results found that square wave conditions had a higher rate of carbon assimilation consistently than the randomised pattern of light and was significantly higher than the fluctuating pattern of light (Figure 2. A). It would be expected that the stomatal conductance would follow the same trend due to their tight coupling, however Matthews' (2018) experiment found that it did not. A decoupling of stomatal conductance and carbon assimilation was discovered in the diurnal tests (Figure 2. B). This decoupling led to a loss of water via transpiration with no increase in carbon assimilation, negatively affecting the plants water use efficiency. This decoupling was responsible for up to 25% of the daily water loss from the plant, so a quarter of the entire transpiration loss was unnecessary and brought no beneficial increase in CO_2 to the plant. That the shape of the element acclimatised to different growing light conditions, it was hypothesised that it may be due to circadian elements being mis-aligned with

other plant processes as they are not activating under natural light at the correct time.

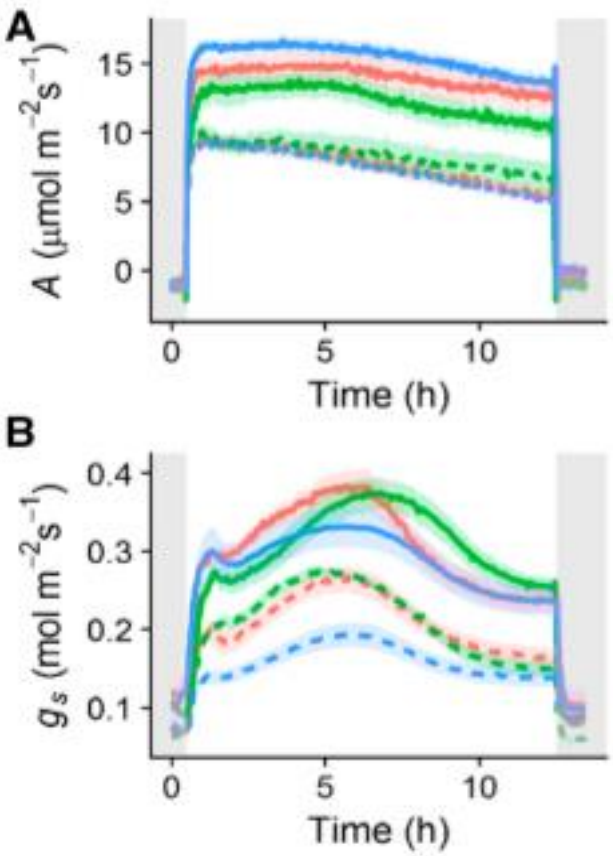

Figure 2: Diurnal measurements of Carbon Assimilation (A) and Stomatal Conductance (B) for *Arabidopsis thaliana*. Blue line represents plants grown under a Square Wave of light. Red represents plants grown under a fluctuating wave in a fixed pattern of light. Green represents plants grown under a fluctuating wave of light with a random pattern. Solid lines represent tests taken under high light, dashed lines represent tests taken under a low light. Edited from Matthews' et al., 2018.

The Circadian Clock

To regulate an internal metabolism throughout different diurnal environmental changes, most organisms on the planet evolved a set of timing mechanisms called the circadian clock. These mechanisms help the organisms adapt to maximise their ability to survive and thrive throughout the vastly different environments of the day and night. While in animals these changes may relate to visual aid or sleep cycles, in plants they help regulate carbon fixation and stomatal conductance, to maximise the profit while minimising the water and

energy lost in these processes (Gorton, Williams and Assmann, 1993). Irregularities can cause disunity between the plant cycles and the diurnal cycle and which can be assumed to detract from optimal plant health.

The circadian cycles are not conserved over different genetic phyla and can have wide differences between organisms; however they are heritable and progeny grown under different diurnal timings to parents are able to adapt to their parents' natural diurnal clock when exposed to those conditions (Harmer, 2009). In the plant *Arabidopsis thaliana*, it is maintained by a series of genes which self-regulate in a series of negative feedback loops, known as its central oscillator and is the main area of expression (Jones, 2009). There are also input and output pathways, which affect and are affected by external plant systems however the main circadian clock is thought to be the gene cycles (Harmer, 2009).

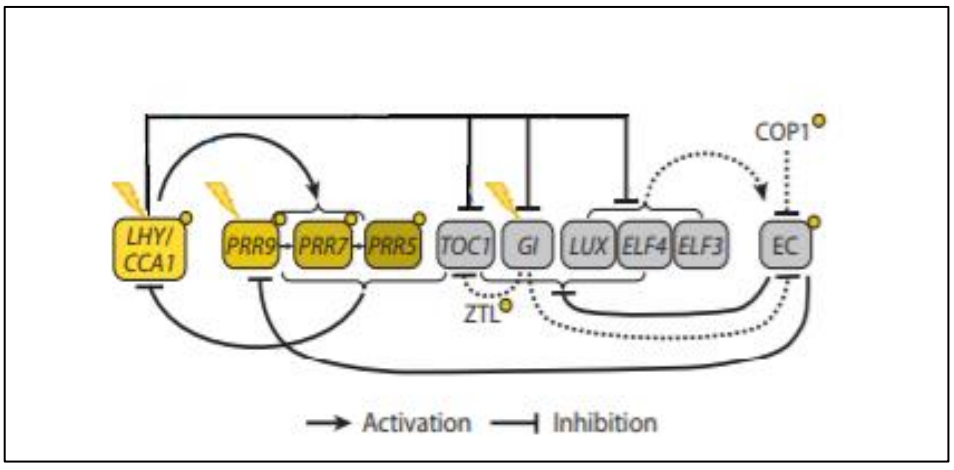

Figure 3: Diagram of the *Arabidopsis thaliana* circadian clock
Edited from Millar, 2016.

The 'beginning' of the circadian clock is the activation of the morning phase genes, mainly **C**ircadian **C**lock Associated 1 (*CCA1*) and Late Elongated Hypocotyl (*LHY*). The level of gene activation rises from the middle of the night,

peaks around dawn causing a similar peak in protein levels following a small delay (Millar, 2016). These genes inhibit the expression of genes activated in the evening such as Timing of Cab Expression (*TOC1*). *CCA1* and *LHY* also activate *PRR* genes, which themselves inhibit the production of *CCA1* and *LHY*, reducing their expression and allowing for the evening complex genes such as *TOC1* and *LUX* to increase in gene expression and to start transcribing proteins (Millar, 2016). These evening genes self-inhibit each other, with *EC* reducing expression of genes such as *TOC1* and *LUX*, and reducing levels of *PRR* which will allow for the increase in activation of *LHY* and *CCA1* and the circadian Clock restarts, as shown in Figure 3. There is some redundancy in the circadian system, with silencing genes not causing the clock to halt. Many experiments have been undertaken silencing and overexpressing genes, and the majority of them cause a change in the period for the clock to 'reset'. For example, silencing *CCA1* causes a short clock period, while overexpressing causes the period to become arrhythmic, the same effect is found for *LHY* and *TOC1* (Hsu and Harmer, 2014; Litthauer, 2017; Millar, 2016).

Table 1: Arabidopsis genes and functions pertaining to the circadian clock (Taken from McClung, 2006).

			Circadian Clock Phenotype	
Gene	Locus ID	Function	Loss of Function	Overexpression
CCA1	At2g46830	Single Myb domain transcription factor	Short period	Arrhythmic
CKB3	At3g60250	Casein kinase II regulatory subunit	Not known (gene family)	Short period
CRY1	At4g08920	Blue light photoreceptor	Long period in blue light	Short period in blue light
CRY2	At1g04400	Blue light photoreceptor	Long period in blue light	Short period in blue light
DET1	At4g10180	Repressor of photomorphogenesis	Short period	Not known
ELF3	At2g25930	Unknown	Arrhythmic in continuous light	Long period
ELF4	At2g40080	Unknown	Arrhythmic	Not known
GI	At1g22770	Unknown	Short period, low amplitude	Short period, low amplitude
LHY	At1g01060	Single Myb domain transcription factor	Short period	Arrhythmic
LUX	At3g46640	Myb transcription factor	Arrhythmic	Arrhythmic
PHYA	At1g09570	Red light photoreceptor	Long period in far-red light	Short period in far-red light
PHYB	At2g18790	Red light photoreceptor	Long period in red light, leading phase in white light	Short period in red light, lagging phase in white light
PIF3	At1g09530	Basic helix-loop-helix transcription factor	Wild type	Wild type
PRR3	At5g60100	Pseudo-response regulator	Short period	Wild type
PRR5	At5g24470	Pseudo-response regulator	Short period	Low amplitude, long period
PRR7	At5g02810	Pseudo-response regulator	Long period	Not known
PRR9	At2g46790	Pseudo-response regulator	Long period	Short period
SRR1	At5g59560	Unknown	Leading phase, low amplitude	Not known
TIC		Gene not yet identified	Short period, low amplitude	Not known
TOC1	At5g61380	Pseudo-response regulator	Short period	Arrhythmic
ZTL	At5g57360	F-box protein	Long period	Arrhythmic

The circadian genes expression and the proteins transcribed cause a variety of changes in the plant. In *A. thaliana* there is a rhythmic cotyledon and leaf movements driven by increases and decreases in the plant pulvinus, a rhythm in the expansion of abaxial and adaxial cells of leaf stems and a rhythm in the elongation of the inflorescence stems, and hypocotyl stems in seedlings, arising from the circadian clock (McClung, 2006). The changes induced by the circadian clock can mimic the rhythm of activation of the subsequent genes. Hypocotyl growth have been shown to be greatest in the evening and lowest in the morning in both seeds and adult plants (Yakir et al., 2006). 23 genes coding for enzymes involved in the creation of photoprotective pigments peak in coordination in the early morning before sunrise, to create and distribute those pigments before the dawn light starts to damage the photoreceptors, absorbing light in the visible and ultraviolet range (Harmer et al., 2000). Photosynthetic genes coding for the creation of CAB and Rubisco are also highly expressed in

the morning in the anticipation of dawn to be able to function at maximum efficiency (Schaffer et al., 2001). There has been shown to be a link between the production of the hormone abscisic acid (ABA) and the circadian TOC1 gene, which is notable for ABAs involvement in plant water management and drought responses (Legnaiolio, Cuevas and Mas, 2009). Legnaiolio et al's 2009 study showed that the levels of ABA were impacted by the over and under expression of TOC1, with TOC1 overexpressed Arabidopsis plants being significantly less likely to survive droughted conditions than WT plants, and TOC1 under expressed plants being significantly more likely to survive. These results show a direct impact of the circadian clock on the plants water efficiency and ability to sufficiently regulate water expenditure.

Model Plant – Arabidopsis thaliana

A model plant to test any changes and effects regarding many biological processes would be Arabidopsis thaliana. It is used globally in experiments and was first presented as a model species for plant genetic experiments in 1907. Since then it has risen in popularity, due to several physiological and genetic advantages that make it ideal for working in laboratories. The plant itself is relatively small and quick to grow, with a life cycle of around 8 weeks, therefore it can be grown and run with multiple generations in a small period of time, instead of waiting for months or years with larger plant species. A. thaliana is able to self-fertilise and produce a large number of seeds, allowing for it to be easily used to create large numbers of individuals with chosen gene expressions for experimentation, rather than having genetic editing occurring on financial crops which may take much longer and more money to discover whether any effect is even present.

The *Arabidopsis* genome is one of the smallest angiosperm genomes. This allows for an ease in gene isolation to see whether a foreign gene was successfully transplanted, or to find out the effect of certain genes activating under conditions. This has allowed *Arabidopsis* to be extensively used with Agrobacterium Mediated Gene Transfer and other genetic engineering methods to create and confirm some of the adaptations which have gone on to create vitally important changes in other species (Somerville and Koornneef, 2002). Although *Arabidopsis* is not a crop or medical plant and some responses may not be able to be assumed to be similar in plant species with different carbon concentrating mechanisms or guard cell shapes, its use and ability to easily be reproduced make it a model species for testing gene alterations in the circadian pathway.

Research Project Direction

The cause of the decoupling found between stomatal conductance and carbon fixation in the altered light conditions in Matthews' (2014) study is an important issue, and one not well understood. With so much water being lost from the plant with no photosynthetic gain, it is a large issue, and one that needs to be understood and remedied. While not previously found in plants grown under natural fluctuating conditions, it was hypothesised that the decoupling effect may be due to alterations in the circadian clock arising from the square wave light. Without the gradient of light changes from nocturnal dark to diurnal light in its regular 24-hour cycle, and eventually the circadian clock was thought to have been temporally altered or caused changes in the plant which led to the decoupling. In order to investigate the effect, the circadian clock of the plant

would need to be altered to try and recreate the decoupling element on plants grown in more ‘normal’ light conditions.

Whether the source of the de-coupling is due to the circadian clock rhythm or as a response to changes in light could be investigated by interfering with a natural light cycle and discovering the size, shape and presence of the de-coupling. Interfering with the circadian clock by altering genes could help with studying any impact on the decoupling, giving an idea of what is triggering the decoupling and help to understand where the decoupling occurs in the plants systems. Growing multiple generations of plants under the fluctuating and square light conditions and assessing their efficiency against “normal” light conditions could also be investigated to understand whether these changes are acclimatisation to the change in growth light regime or if they become encoded in the plant genome and are a heritable adaptation.

A better understanding of the way plants adapt to different light conditions may not have a large effect on the majority of crop fields grown outside but may have an increasing effect on the rising amount of plants grown indoors and in greenhouses. Artificial greenhouse cultivation may be a method of increasing crop yield without having to fight against natural forces to change some of the inputs. Without access to pests, rainwater or lack of, or direct sunlight, those conditions can be artificially created to produce more optimal growing conditions.

The Impact of the Circadian Clock on Carbon Assimilation and Stomatal Conductance in *Arabidopsis thaliana*

Introduction

Plants, much like animals, have an internal mechanism that adapts to changes in the time of day to alter metabolic processes to continue functioning at peak efficiency (Jones, 2009). This mechanism is controlled by highly conserved genes that have been shown to alter plant processes and adapt to diurnal changes and control the activation of up to 40% of other genes in *Arabidopsis thaliana (Jones, 2009)*. These genes are called circadian genes and cause changes including adjustments to plant hormone production, flowering times and hypocotyl growth (Jones, 2009). For *A. thaliana*, there is thought to be three main circadian feedback cycles (Jones, 2009), one of which was investigated in this paper. The circadian genes investigated were Circadian Clock Associated 1 (CCA1), Timing Of Cab Expression (TOC1) and Late Elongated Hypocotyl (LHY) which function in a three-way interlocking set of feedback loops, activating and silencing morning and evening genes to adapt to increases and decreases in factors such as light intensity and air humidity (Jones, 2009).

Understanding how these circadian genes impact plant processes such as stomatal conductance (g_s) and carbon assimilation (A) could be vital in helping to improve our knowledge of plant metabolic systems and investigations into improving the fitness of important economic and food crop varieties. Low g_s restricts the plants ability to take in CO_2 to utilise for photosynthesis, reducing

carbon assimilation, whilst higher conductance causes an increase in water loss via transpiration. Understanding how these mechanics can be influenced by circadian genes throughout the day could be useful in understanding new pathways to maximise growth and crop production and how the plants' resource requirements change.

Previous studies have shown a decoupling of stomatal conductance with carbon assimilation in Arabidopsis plants (Matthews et al., 2018), with g_s increasing without any change to *A*. This decoupling causes a large loss in water via transpiration without gain in photosynthesis. The cause is unknown but is hypothesised to be linked to the circadian clock due to alterations in g_s and *A* from change to light conditions (Matthews et al, 2018). Therefore, changes to the circadian clock are hypothesised to have an impact on the difference in g_s and *A* after decoupling, and the timing of and peak of decoupling.

Methods and Materials

A. thaliana mutants were grown with either the TOC1 or CCA1 genes overexpressed specifically in guard cells, under controlled conditions to assess for differences in photosynthetic capacity and stomatal behaviour. Two lines of the gene edited plants were grown (TOC1-1, TOC1-2, CCA1-1, CCA1-2), along with a non-edited Wild Type (WT) line. All experiments were run under 400 μmol^{-1} CO_2, 21ºC and a flow rate of 300 μmol^{-1}.

Temporal Changes to Stomatal Conductance and Carbon Assimilation

To investigate the impact of alterations in guard cell clock on stomatal responses, g_s was monitored following a step change in light intensity. Leaves were placed into a Li-Cor 6400XT chamber and left to adapt to a light intensity of 100 µmol m^{-2} s^{-1}. Once conductance had reached steady state, light intensity was increased to 1000 µmol m^{-2} s^{-1} for 1 hour, then reduced back to 100 µmol m^{-2} s^{-1}. *A* and g_s were recorded throughout the experiment. Measurements were taken for the time of stomatal response from smallest to largest stomatal aperture (τ_i), the maximum rate of stomatal conductance (G_i), and the time taken to reach maximum stomatal conductance (ΔG_i).To investigate any differences in stomatal behaviour over the diurnal period, g_s and *A* were recorded during a step change in light intensity. Leaves were placed inside an ADC LCpro T Infra-red Gas Analyser cuvette for 30 minutes with a light intensity of 0 µmol m^{-2} s^{-1} to dark-adapt. Light intensity was increased to 150 µmol m^{-2} s^{-1} for 8 hours then reduced back to 0 µmol m^{-2} s^{-1} for an hour. Assimilation and stomatal conductance were recorded every 60 seconds.

Stomatal conductance is calculated by the Li-6400XT by the equation:

$$g_s = \frac{1}{\frac{1}{g_{tw}} - \frac{k_f}{g_{bw}}}$$

Where g_s is stomatal conductance (mol H_2O $m^{-2}s^{-1}$)., g_{tw} is the total conductance of the leaf (mol H_2O $m^{-2}s^{-1}$) measured by water vapour concentration, leaf temperature and atmospheric pressure (Appendix i), g_{bw} is the boundary layer conductance to water vapour (mol H_2O $m^{-2}s^{-1}$) (Appendix ii) and k_f is a factor based on the stomatal ratio of the leaf (Appendix iii).

To investigate any differences in stomatal behaviour over a prolonged diurnal period, g_s and assimilation were recorded from measurements taken using full plant chambers. The plants were set in an air-tight chamber and left to adjust for approximately 24 hours, then exposed to a light intensity of 1000 µmol m^{-2} s^{-1} for 72 hours.

Photosynthetic Efficiency

To investigate the differences in photosynthetic efficiency over the day, measurements of photosynthetic efficiency were taken using a Technologica Chlorophyll Fluorescence Imager within 3 timepoints, 9:00-10:30, 12:00-13:30 and 16:30-18:00. Plants were dark-adapted for at least 20 minutes prior to measurements to allow so light receptors were available. A 6354 µmol m^{-2} s^{-1} pulse of light was shot at the plants for the first recording, then the ambient light was increased to 1500 µmol m^{-2} s^{-1} for 5 minutes before applying the pulse again. This was repeated for 1250, 1000, 500, 250, 150 and 50 µmol m^{-2} s^{-1} of light to create a light response curve.

Total Leaf Surface Area

To discover whether there was an effect on the growth rates of the different genotypes, leaf area measurements were taken on a Technologica Chlorophyll Fluorescence Imager every Monday, Wednesday and Friday from approximately 2 weeks after planting until half of an experimental group had entered flowering stage. The repeats taken per day differed throughout the experiment as samples were taken and used for the full plant chamber diurnals. The images were altered using ImageJ to gain surface area measurements which were compared between genotypes.

Stomatal Pore Counts

To investigate whether changes in stomatal conductance or assimilation would occur due to metabolic or morphological differences, stomatal imprints were taken with Xantopren Blue moulds and studied under a microscope for numeric counts of stomata.

Statistical Analysis

All statistical analyses were run on R studio, Microsoft Excel or SPSS v.25 computer programs, including One-Way ANOVA tests, Repeated Measures ANOVA tests, and Tukey posthoc tests.

Results

Photosynthetic Efficiency

There was no significant difference in the quantum efficiency between any of the treatment types when tested in the morning experiment ($F(4,13) = 2.236$, $p=0.121$), midday experiment ($F(4,13) = 0.243$, $p=0.909$), or evening experiment ($F(4,16) = 0.272$, $p=0.892$), as tested by a repeated measures ANOVA.

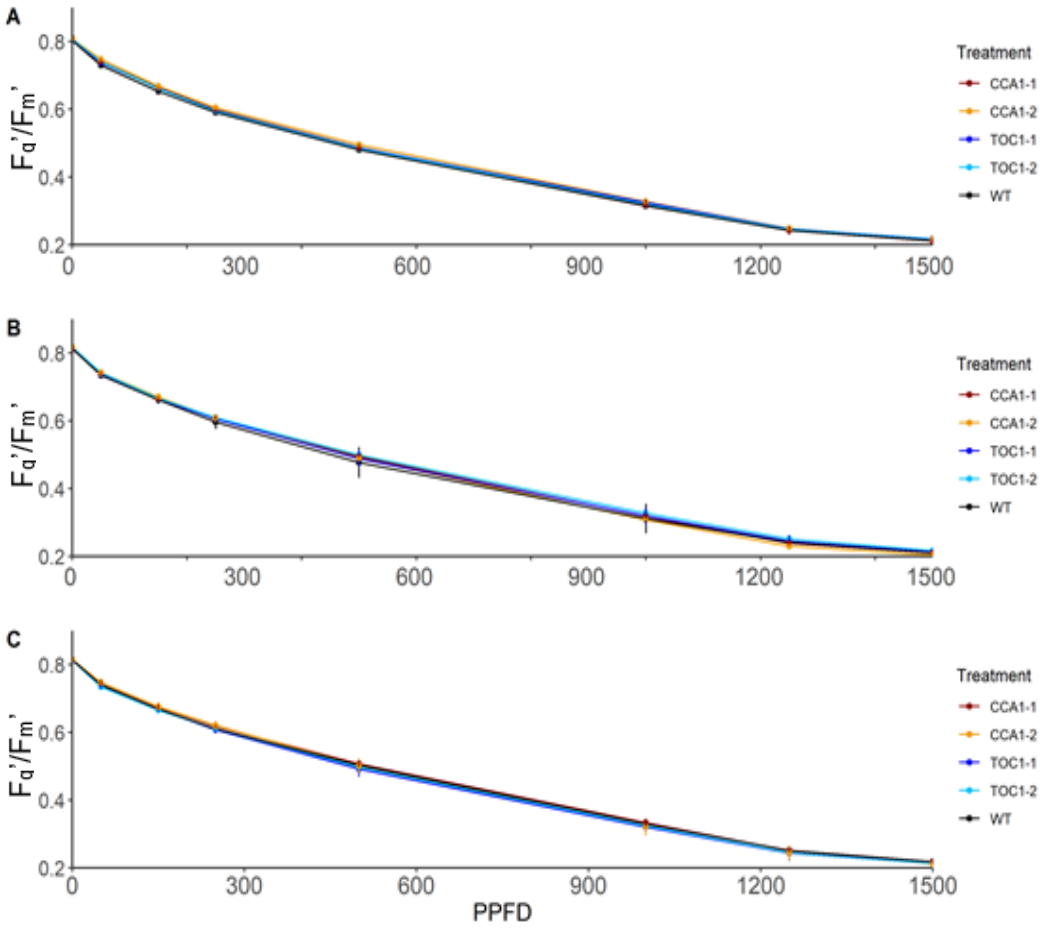

Figure 4: Mean values of maximum quantum yield of PSII for each genotype of *A. thaliana* for a range of PPFD values, taken at 9:00-10:30am (A), 12:30-14:00 (B) and 16:00-17:30 (C). Error bars represent Standard Deviation.

Carbon Fixation Responses to Changes in Ambient CO2 Concentrations (Curve)

The results of the *A/Ci* curve, showing the change in photosynthesis as the amount of CO_2 increases, showed a significant difference in between the treatments ($F(4,23) = 6.367$, $p<0.001$) with a repeated measures ANOVA. This significance was found to show that the TOC1-2 treatment had a significantly more efficient carbon fixation than the TOC1-1 ($p=0.013$), CCA1-1 ($p=0.036$) and CCA1-2 ($p=0.003$) treatments.

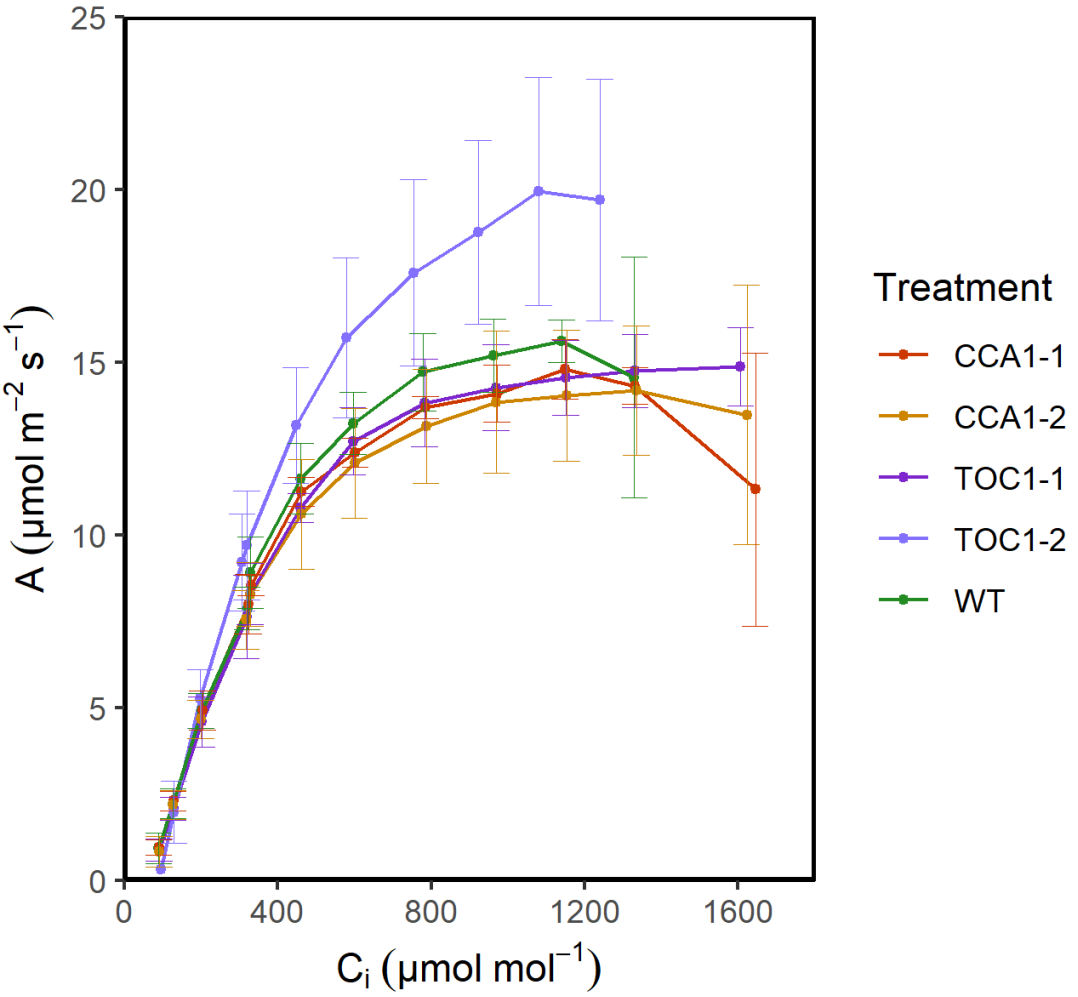

Figure 5: Response of carbon assimilation to increases in CO_2 for each treatment of *A. thaliana*. Error bars represent Standard Deviation.

Stomatal Changes in Response to Step Increases in Light Intensity

Figure 6 shows the response of stomata to a step change in light intensity from 100 to 1000 µmol m^{-2} s^{-2}. The WT treatment had the slowest rate of stomatal response to the increase in light, but the highest end stomatal conductance and greatest difference in conductance before and after the change in light. There was a significant difference in the rate of response, the TOC1-2 treatment responded significantly quicker than any of the other treatments, however the change in stomatal conductance was not significantly larger.

There was no difference in the maximum stomatal conductance between any of the treatments.

Whilst there was no large difference in the change in stomatal conductance after the change in light intensity, there was a trend in which the WT treatment had the largest change, followed by the TOC1-1 and TOC1-2 treatments, then the CCA1-2 and CCA1-1 treatments.

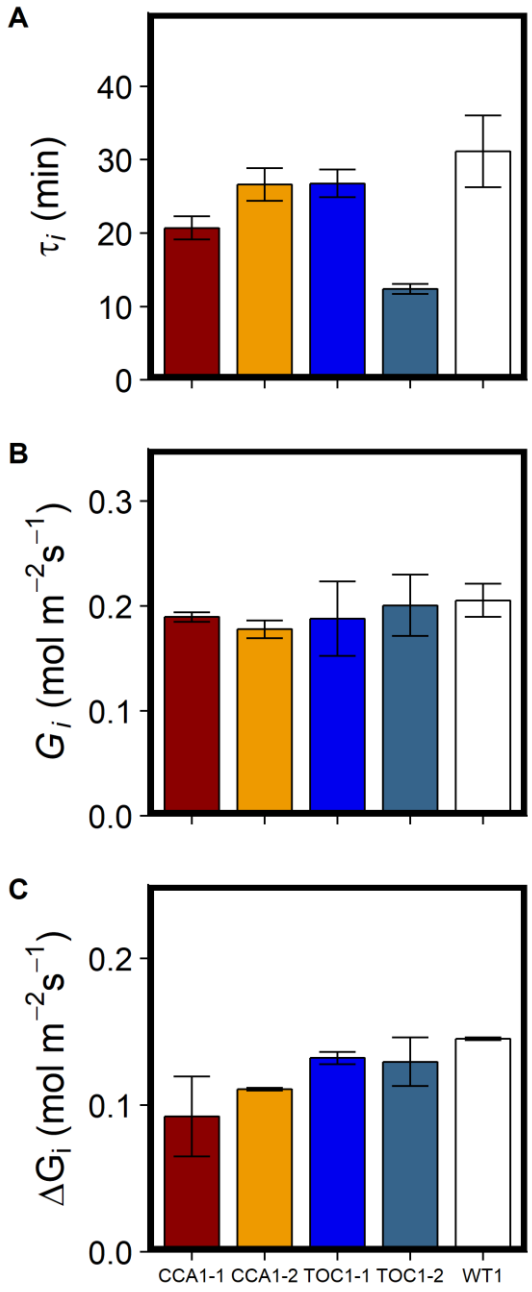

Figure 6: The speed of change in stomatal conductance (A), maximum stomatal conductance (B), and the amount of change of stomatal conductance (C) in response to a step increase of light intensity from 100 to 1000 µmol m^{-2} s^{-1} for an hour for each treatment. Error bars represent 95% confidence interval.

Diurnal Fluctuations of Carbon Assimilation and Stomatal Conductance

All treatments had an initial rise and fall in both measurements tested, before adjusting to the light intensity increase and *A* remains relatively stable throughout the experiment (Figure 7). TOC1-1, TOC1-2, CCA1-1 and WT had relatively similar levels of maximum *A*, whilst CCA1-2 had a lower rate of carbon fixation throughout the day. TOC1-1 and TOC1-2 had the highest rates of maximum g_s, and so the largest decoupling of the processes, followed by WT, CCA1-1 then CCA1-2 which did not show much of a decoupling.

Stomatal conductance exhibited the decoupling with carbon assimilation in all treatments to varying degrees. Whilst the size of the decoupling changed between treatments, the timing and maximum size was the same for all the treatments.

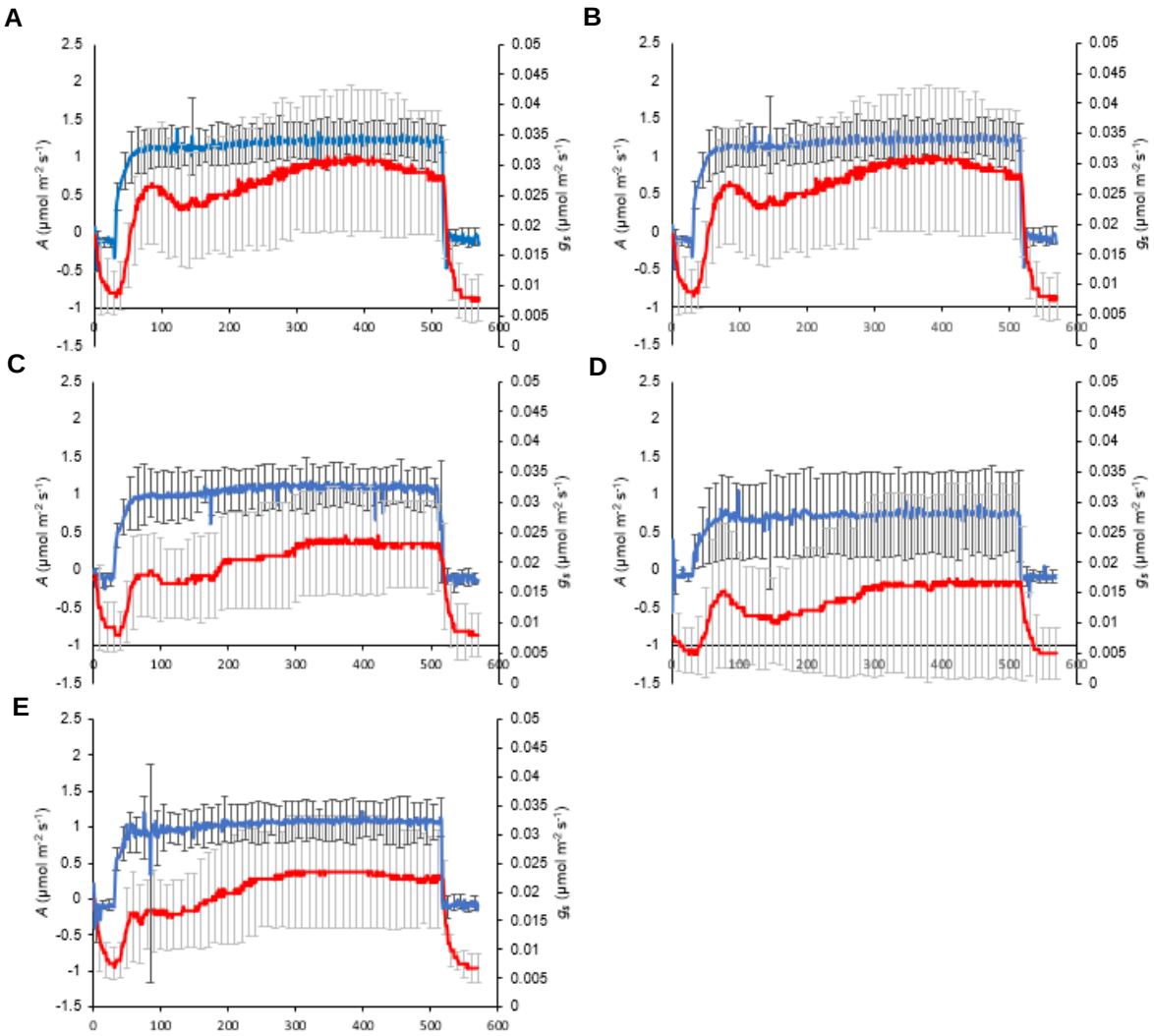

Figure 7: Mean rates of stomatal conductance (red) and carbon assimilation (blue) over a diurnal period measured between 8:00-17:30 every minute under 150µmol m^{-2} s^{-1} for *A. thaliana* for each treatment, TOC1-1 (A), TOC1-2 (B), CCA1-1 (C), CCA1-2 (D), and WT (E). Error bars represent Standard Error.

There was no significant difference between treatments for diurnal carbon assimilation (Fig. 8A), however there was a trend for TOC1 plants being more efficient and CCA1 plants being less efficient than WT plants.

The stomatal conductance for TOC1-1 was significantly higher than for CCA1-2, ~80 mol m^{-2} day^{-1} comparted to 40 mol m^{-2} day^{-1} (Fig. 8B). The data showed a similar trend to *A*, with TOC1-1 and TOC1-2 having a higher rate of gas exchange than the WT, and CCA1-1 and CCA1-2 having a lower rate than WT.

There was no significant difference in the WUE between any treatments (Fig. 8C), however there was the same trend as in Figure 8A and 8B, with TOC1 treatments losing more water for carbon assimilated so having a lower WUE than the WT treatment, and the CCA1 plants gaining more biomass for water lost.

Figure 8D shows that the atmospheric CO_2 conditions that the diurnals were run under were the same for all the treatments, as any difference would have an effect on the other data collected.

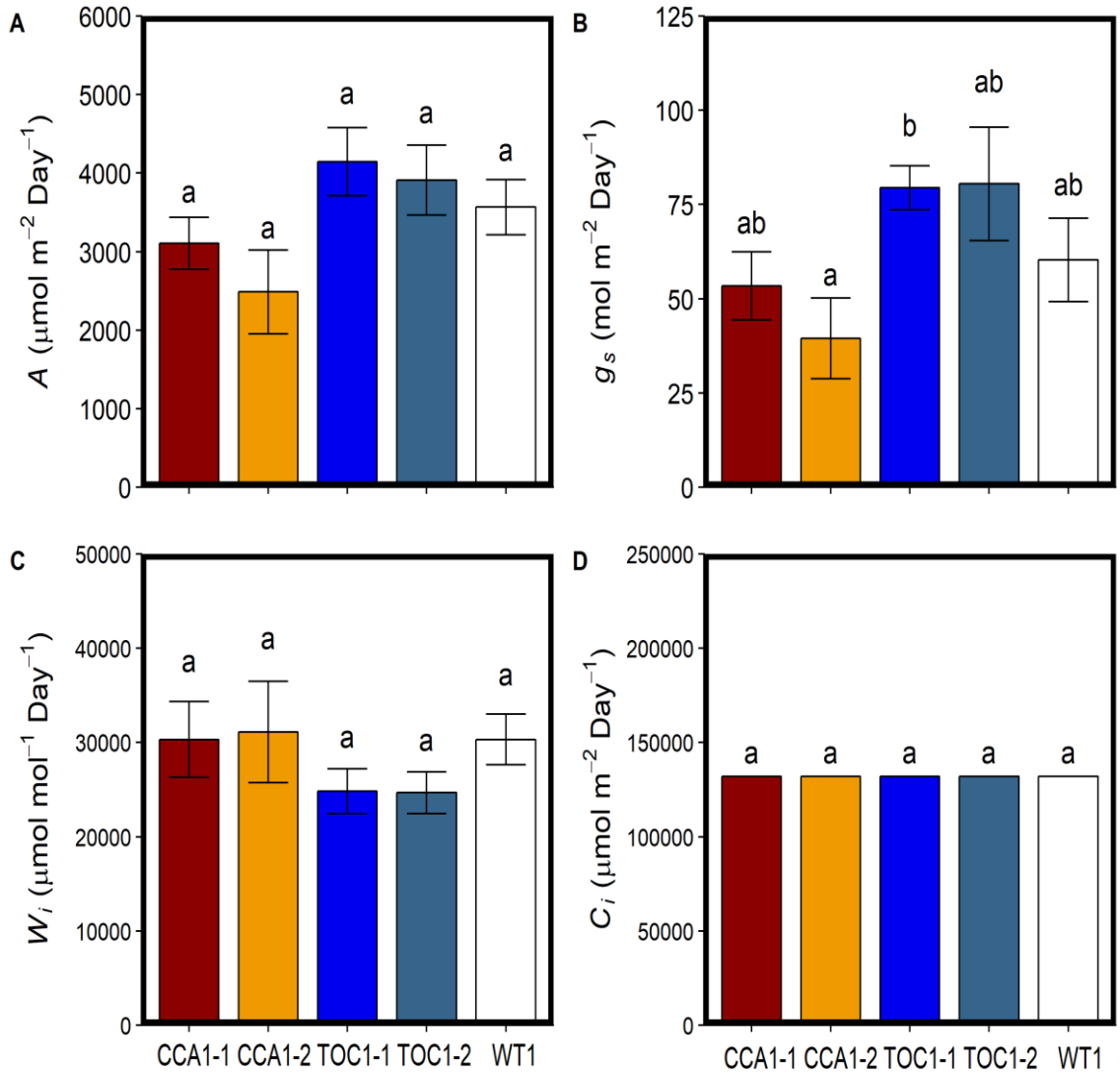

Figure 8: Diurnal measurements of treatments when exposed to 150 µmol m^{-2} s^{-1} of constant light intensity for 8 hours, showing (A) total rate of carbon assimilation, (B) total stomatal conductance, (C) daily Water Use Efficiency and (D) ambient CO_2. Error bars represent 95% confidence interval, letters represent results of Tukey's posthoc test.

Plant Growth Rates

Figure 9 shows the growth of the treatments over time, as measured by imaging leaf areas for 6 individuals for each treatment 3 times per week. The graph shows that the WT treatment grew slower at the beginning than any of the treatments until the 22nd May where it grew to a size similar the other treatments. CCA1-2 had the largest leaf area at the conclusion of the experiment, followed by TOC1-2, TOC1-1, CCA1-1, and the WT treatment.

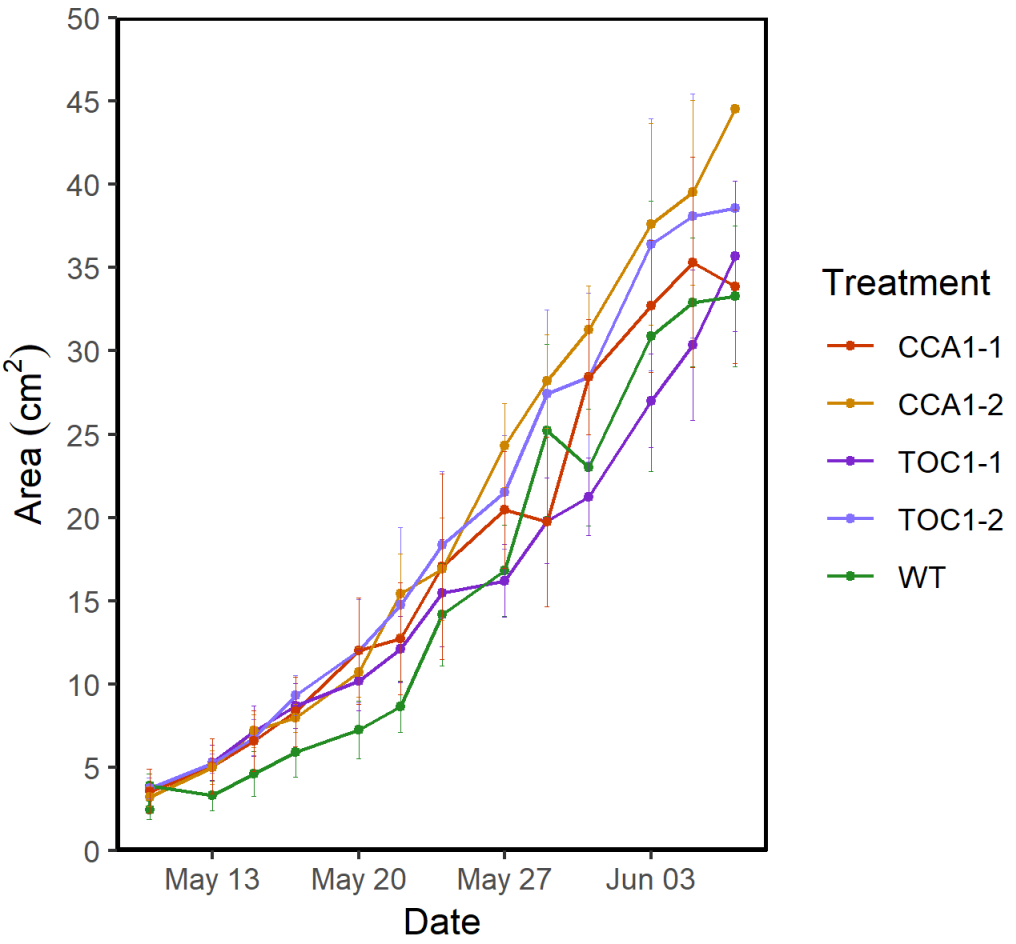

Figure 9: Mean leaf area for 6 plants of each treatment for 4 weeks, from 2 weeks after planting to flowering growth stage taken from surface area measurement from photographs. Error bars represent Standard Deviation.

Stomatal Pore Count

There was no significant difference between the number of stomatal pores between any of the treatments ($F(4,19) = 2.258$, $p = 0.101$).

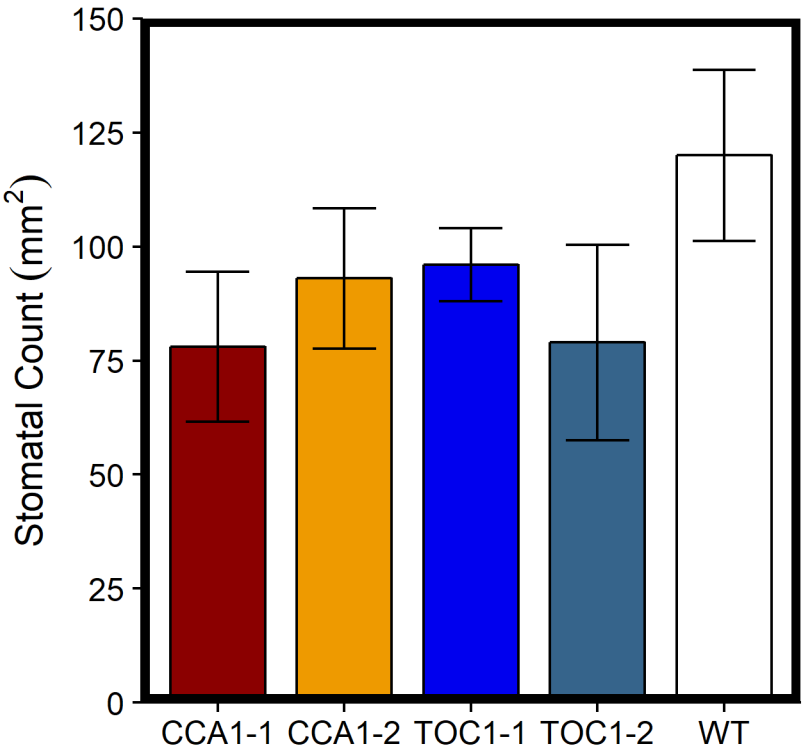

Figure 10: Mean stomatal density of both adaxial and abaxial sides of leaves for each treatment. Error bars represent Standard Deviation.

Discussion

Photosynthetic Efficiency

Overexpressing the TOC1 and CCA1 genes was shown to not have a significant effect on the quantum efficiency of Arabidopsis (Figure 4), with the small error bars confirming for multiple individuals. However, the overexpressing of the genes occurred in the guard cells of the plants and not the mesophyll or palisade of leaf tissue, the main locations of chloroplasts and photosynthesis. Therefore, any impact on the maximum rate of photosynthesis for the plants could have been too small and obscured by the photosynthesis of the other leaf tissues, which had not been gene edited. The similarities between the plants may also be due to the Calvin Cycle functioning in the presence of CO_2 or O_2 as either photosynthesis or photorespiration, to produce ATP energy for the plant. Either process released the fluorescence that is measured, so the behaviour of the guard cells and the concentration of internal CO_2 may not have caused any noticeable change in the amount of fluorescence produced.

Photosynthetic Response to Increases in Carbon Dioxide Concentrations

With no difference in the quantum efficiency between treatments, tests were undertaken to gather the efficiency of the plants to photosynthesise against increasing levels of CO_2. TOC1-2 performing significantly more efficiently than TOC1-1, CCA1-1 and CCA1-2 may be due to changes in the production or efficiency of Calvin Cycle enzymes, altering the rate at which the plants can fix CO_2, due to the plants having no significant difference in the maximum rate of photosynthesis (Figure 4), or number of stomatal pores (Figure 10) impacting

the amount of CO_2 able to be up taken at once. The levels of Calvin-Cycle enzyme ribulose-1,5-bisphosphate has been shown to fluctuate with circadian patterns of photosynthesis, and higher concentrations would allow for more throughput of converting CO_2 into usable photosynthetic products (Fredeen, Hennessey and Field, 1991).

TOC1-2 performing differently to TOC1-1 may be due to differences in the location of the insertion of the plasmid genes during the process of gene editing. The difference may have caused a discrepancy in either the strength of expression or caused a change in another plant process by interfering with other DNA loci. Therefore, it cannot be conclusively stated that TOC1 overexpressed treatments have a higher limit of CO_2 before other limiting factor affect photosynthesis, or any of the other differences between the two lines. The use of two separate lines of each gene altered plant is to spot possible gene insertion issues such as this.

Stomatal Response to Step Increase in Light Intensity

The main location of gas exchange between the atmosphere and the intracellular membranes of the leaf is via the stomata, so its ability to adapt and change to internal and external factors is a large part of this investigation. The overexpressing of TOC1 genes cannot be reasonably attributed to having sped up the rate of stomatal response to increases in light intensity as whilst TOC1-2 was shown to have a quicker rate of stomatal response than any of the other treatments (Figure 6.A), this quick response was not shown for both TOC1 treatments, as TOC1-1 showed similar speeds to CCA1-2, and was slower than CCA1-1. Whilst TOC1-2 responded the fastest to changes in light, the change

did not lead to a greater rate of carbon assimilation compared to the other treatments and the increase was not significantly larger, therefore the rates of stomatal conductance before and after the light increase can be assumed to be similar. This disagrees with the results from Figure 8.B, which states that TOC1-1 has a significantly higher rate of stomatal conductance over the day than CCA1-2. This effect may be due to the longer experimental time giving the plants more opportunity to acclimatise and react to the increases in light, and for different periods of the circadian cycle to come into effect and the reaction to overexpressed genes to influence g_s.

The arrhythmic periods of the circadian clock caused by overexpression of CCA1 and TOC1 genes may have caused a desync between the production of photosynthetic pigments and the optimal daily time for photosynthesis. Photosynthetic pigments accumulate before dawn to protect the plant as it “wakes” and starts photosynthesising as the light intensity increases (Yakir et al., 2016), so in the TOC1 overexpressed plants there may have been a higher concentration of the pigments and higher protection of the photosystem antennae. This protection may have allowed for the TOC1 overexpressed plants to be able to function more efficiently as the light increases than the Wild Type or CCA1 overexpressed plants, causing an increased need for CO_2 to fuel the photosynthesis and so cause a more rapid response in stomata, as shown in Figure 6.

Diurnal Fluctuations of Carbon Assimilation and Stomatal Conductance

The results agreed with Matthews et al. (2018) showing the presence of the decoupling of stomatal conductance with carbon assimilation for all treatment groups.

Diurnal measurements of each of the treatments under constant light showed the decoupling of stomatal conductance and carbon assimilation that was found in Matthews et al's investigation (2018). They all showed the variance in stomatal conductance, with an increase and decrease without any corresponding effect on carbon assimilation, which did not fluctuate throughout the day. The impact of the decoupling was largest in the TOC1-1 and TOC1-2 treatments, and was much higher than the CCA1-1, CCA1-2 and WT treatments, which may suggest that the overexpression of the evening TOC1 genes may increase the amount of gaseous transfer with the environment or inhibit mechanisms to prevent water loss. The CCA1 plants may have had a lower rate of stomatal conductance due to an inability of CCA1 guard cell overexpressed plants to accurately predict the changes in light intensity at dawn and so be out of tune to the light regimes grown and tested under (Hassidim et al., 2017).

There was no significant difference in the rate of carbon fixation over the day (Figure 8.A), however there was a trend that agrees with the diurnal time graphs with TOC1-1 and TOC1-2 having a higher rate than the WT, CCA1-1 then CCA1-2 treatments. This trend is also found for the stomatal conductance, with the TOC1 treatments having higher rates of daily g_s than the WT and CCA1 treatments. The presence of the trend in both measurements may suggest that there is a biological effect being played out on the plant's metabolism, however not to such a large extent as to become statistically significant. As shown in

Figure 8.C, TOC1 having higher rates of *A* and g_s led to a reduced water use efficiency, the amount of biomass gained divided by the water lost via transpiration for this increase, per day. This suggests that although the TOC1 individuals were able to photosynthesise more and had a higher stomatal conductance, they were less water efficient and more susceptible to damage in droughted conditions.

TOC1 overexpressed plants being more effective at photosynthesising than the CCA1 overexpressed, or WT plants shows evidence that agrees with the assumption than due to increased levels of CAB and Rubisco gene expression before dawn, the morning would be the most efficient time for photosynthesis (Schaffer et al., 2001). Having gene expression peak before dawn implies that the start of the signal arises from the evening Circadian genes and follows their levels of activation after a short delay. This preparation for the production of Calvin Cycle enzymes ensures that there is no bottleneck in the photosynthetic pathway and that Rubisco active sites are not the limiting factor of energy production.

In regular Arabidopsis plants, enzymes encoding mRNA for seven photosystem I and three photosystem II reaction centres have been shown to peak around midday (Harmer et al., 2000). An increase in this peak and as such an increase in the rate of photosynthesis may be assumed by the overexpression of the morning CCA1 gene, however Figure 8 shows an opposite effect, with TOC1 plants having a higher rate of carbon assimilation. This discrepancy may be due to the change in the periods of the circadian clock to an irregular pattern caused by the overexpression of both genes, desyncing

the timing of maximum light intensity with the plants ability to produce and maintain photosystems and be able to utilise the resource.

It can be implied that when not grown in droughted or semi-arid conditions, the TOC1 overexpressed line of plant would be more efficient and potentially able to grow quicker and create a larger yield, with the higher resources of photosynthetic products that would be available to the plant. But when water is or could potentially be a limiting factor, the CCA1 overexpressed treatment may be better suited to that environment, being able to conserve and utilise the water to a better extent than the TOC1 or WT plants and be able to successfully grow to maturity, flower and create a usable yield instead of becoming stunted or dying from dehydration.

Previous studies have shown that overexpressing CCA1 and TOC1 genes in the Arabidopsis circadian clock causes an arrhythmia in the periods of gene expression. With the genes only being overexpressed in the guard cells, this change in the timing of the circadian cycle may explain the presence of the decoupling between stomatal conductance and carbon fixation. The photosynthetic tissue of the leaf mesophyll being out of sync with the guard cells could have caused the difference in the behaviour of g_s and A, with the photosynthetic pathway and stomatal behaviour predicting and preparing for different environmental conditions. Guard cell circadian cycles have been shown to be different to other plant tissue cycles, so changes caused by the overexpression could have had knocked the different cycles, which must have been able to coordinate circadian gating, out of sync (Hassidim et al., 2017)

The changes in g_s and *A* between treatments can be concluded to be due to the overexpression of the corresponding genes in the guard cells, as during the diurnal all plants were exposed to the same amount of internal CO_2 (Figure 8D), and there was no significant difference in the amount of stomata in the leaves. Having a differing availability of CO_2 to the primary photosynthetic tissue of the mesophyll and palisade cells would have a large effect on the rate of photosynthesis as less photorespiration would occur due to the Rubisco enzymes duel functionality, and to stomatal conductance as the stomata would need to be open less and for shorter periods of time to take in CO_2, conserving water by reducing transpiration. Therefore, having no differences in the number of stomata would ensure than any changes between treatments are due to the investigation.

The Impact of Light Regimes on the Circadian Clocks control of Stomatal Conductance and Carbon Assimilation

Introduction

Circadian rhythms can influence a plants response to external stimuli, through changes to gene activation and silencing. These changes result in different protein production that can aid in plant metabolism and growth due to the timing of their activity and when they would be most efficient (Jones, 2009).

The intensity of the responses to these external stimuli can change depending on the time of day, a response to the circadian oscillation of gene activation known as circadian gating (Hotta et al., 2007). The gating is thought to change the sensitivity of the plant to the stimuli to encourage reactions when it is most beneficial and lessen reactions when it is less beneficial (Hotta et al., 2007). Examples of gating are light intensity, stomatal movement, and temperature, increasing or decreasing the output of circadian gene pathways allowing it to be more advantageous to the external changes (Hotta et al., 2007), so changes to the light regimes a plant is grown under can be expected to have an impact on the decoupling between stomatal conductance and carbon assimilation if they are not as closely linked as previously thought.

Shifts to the timing of ‘dawn’ and light intensity at dawn is expected to have an effect on the rate of photosynthesis. Plants that have been grown with circadian clock cycles matching the growth environment have been shown to have more chlorophyll, a higher rate of photosynthesis and grow faster than plants under non-natural conditions (Dodd et al., 2005). Evidence was found in plants grown and tested with variations of 10 hour light-10 hour dark, 12 hour light-12 hour

dark, and 14 hour light-14 hour dark conditions (Dodd et al., 2005). Therefore, growth conditions of a plant have been shown to have a great effect on the rate of photosynthesis, and thus the amount of nutrients available to grow and produce crops to be harvested.

Understanding how much the cycle can increase the rates of photosynthesis is needed to understand the impact the cycle has on overall plant growth and function. Knowledge of how the Circadian cycle functions under temporally changed light conditions can help demonstrate the changes that the clock makes throughout its daily cycle, by showing the potential adjustments to maximise the efficiency of gene activation and protein production.

Previous studies have shown that fluctuations in stomatal aperture throughout the day are under the influence of the Circadian cycle, and that these changes are reliant on regular light or temperature signals to ensure that they remain aligned to the conditions most suited for photosynthesis at varying times of the day (Hassidim et al., 2017). Knowledge of how the circadian clock genes impact the rates of photosynthesis and stomatal conductance and the close relationship between these two processes (Matthews et al., 2018) could aid with understanding the underlying mechanisms involved in the ordination between *gs* and *A* and the genes involved. Exploitation of such knowledge could result in novel targets to increase plant carbon assimilation, water use efficiency and plant growth.

Methods and Materials

A. thaliana seeds collected from plants grown under fluctuating and square wave light regimes by Matthews' et al (2018) were germinated and grown under controlled conditions of 150 µmol m^{-2} s^{-1} PPFD for a period of 2-3 weeks, before being placed under Heliospectra LX601 lights with either a square wave of light pattern of 460 µmol m^{-2} s^{-1} or a fluctuating light with an equal daily integral light intensity.

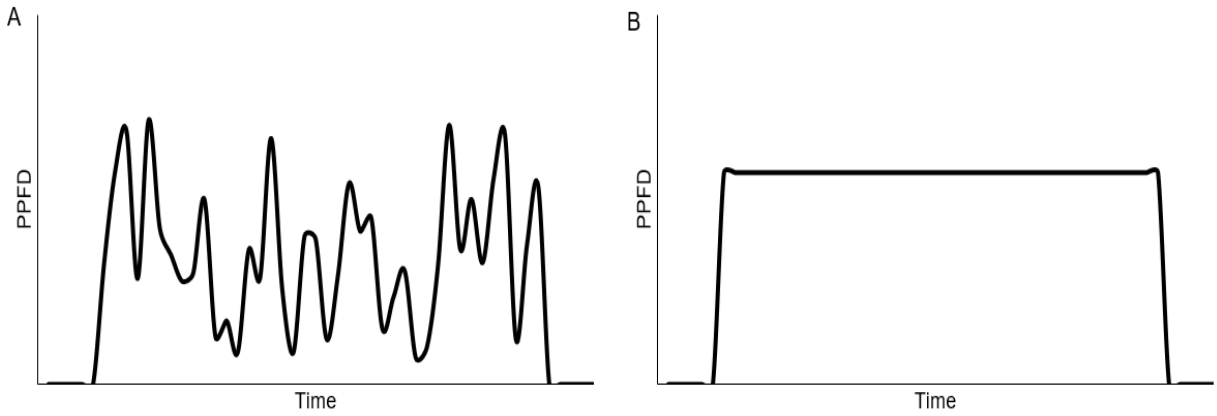

Figure 11: Examples of fluctuating (A) and square (B) waves of light used in plant growth regimes. For fluctuating light after the experimental start light intensity increases and decreases mirroring natural conditions. For square light after the initial experimental start light intensity remains constant throughout the experiment. Not representative of actual regimes used.

Plant treatment nomenclature was as follows; square light seed grown under square light treatment SQsq, plants grown from square light seed under fluctuating light treatment as SQfl, plants grown from fluctuating light seed under square light treatment as FLsq, and plants grown from fluctuating light seed under fluctuating light treatment as FLfl.

Photosynthetic Efficiency

To investigate differences in photosynthetic efficiency over the diurnal period, measurements of photosynthetic efficiency were taken using a Fluroimager chlorophyll fluorescence system at 3 time points, 9:00-10:30 ('morning'), 12:00-

13:30 (‘midday’) and 16:30-18:00 (‘evening’). Plants were first dark-adapted for at least 20 minutes prior to measurements to allow for light receptors to become available. After pulse of 6354 µmol m^{-2} s^{-1} light was shot at the plants for the first recording, then the ambient light was be increased to 1500 µmol m^{-2} s^{-1} for 5 minutes before applying the pulse again. This was be repeated for 1250, 1000, 500, 250, 150 and 50 µmol m^{-2} s^{-1} of light to create a light response curve.

Temporal Changes to Stomal Conductance and Carbon Assimilation

To investigate any differences in stomatal behaviour over the diurnal period, stomatal conductance (g_s) and carbon assimilation (A) were measured using an IRGA (ADC LCpro T) during a step increase in light intensity. The IRGA experiments were run under approximately 400 µmol^{-1} CO_2, 21ºC and a flow rate of 300 µmol^{-1}. Leaves were placed inside a leaf cuvette for 30 minutes with 0 µmol m^{-2} s^{-1}. Light intensity was increased to 150 µmol m^{-2} s^{-1} for 8 hours then reduced back to 0 µmol m^{-2} s^{-1} for 60 minutes. Assimilation and gs were recorded every 60 seconds. Measurements were taken starting at 8am representing a “natural dawn” and a 2-hour shift starting at 10am.

Stomatal Pore Counts

To investigate whether any changes in stomatal conductance or assimilation would be due to differences in number or morphology of stomata, leaf imprints were taken with Xantopren Blue moulds and studied under a microscope. (Dwelle et al., 1983)

Statistical Analysis

All statistical analyses were run on R studio, SPSS 25 or Microsoft excel computer programs. Figures were created on R studio or Microsoft excel computer programs.

Results

Photosynthetic Efficiency

Chlorophyll fluorescence was measured using 8 different light intensities to gather a light response curve detailing the quantum efficiency of each treatment. Significant interactions between the treatments and the maximum quantum yields were found in the morning experiment ($F(20,3) = 5.004$, $p = 0.009$), midday experiment ($F(16,3) = 7,380$, $p = 0.003$) and evening experiments ($F(20,3) = 6.039$, $p = 0.004$) as shown by a repeated measures ANOVA with a Greenhouse-Geisser correction.

In the morning experiment, the quantum efficiency significantly decreased from the SQfl treatment to the FLfl ($p = 0.039$) and FLsq ($p = 0.035$) treatments, as shown using a Bonferroni post hoc

In the midday experiment, SQsq had a significantly higher quantum efficiency than FLfl ($p = 0.004$) and FLsq ($p = 0.034$), as did SQfl to FLfl ($p = 0.040$).

In the evening experiment, the quantum efficiency of SQsq was significantly higher than FLfl ($p = 0.048$) and FLsq ($p = 0.038$), and SQfl had significantly higher results than FLfl ($p = 0.045$) and FLsq ($p = 0.036$).

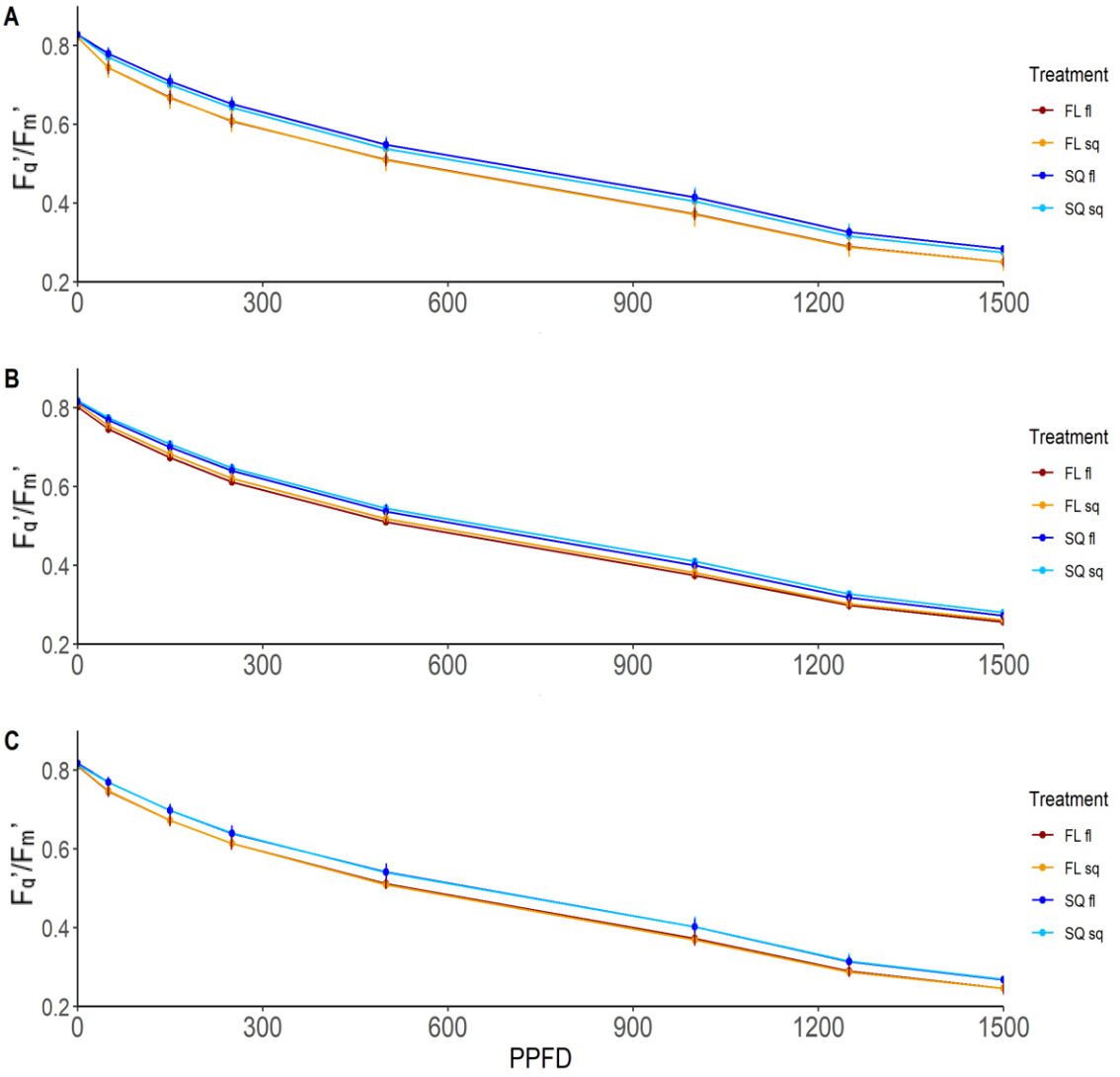

Figure 12: Mean values of maximum quantum yield for PSII of *A. thaliana* for a range of light intensities, taken between 9:00-10:30 (A), 13:30-1400 (B) and 16:00-17:30 (C). Error bars represent Standard Deviation.

Diurnal Fluctuations of Carbon Assimilation and Stomatal Conductance

All treatments had an initial rise and fall in both measurements tested, before adjusting to the light intensity increase and *A* remains relatively stable throughout the experiment (Figure 13). Fluctuating light conditions (FLfl and SQfl) caused higher rates of carbon assimilation and stomatal conductance than the square light conditions (FLsq and SQsq). Under fluctuating light conditions, the fluctuating seed performed better than the square seed, with the opposite being found in square wave light conditions where the square seed had higher rates of *A* and g_s. Stomatal conductance exhibited the decoupling with carbon assimilation in all treatments to varying degrees.

When the timing of the measurements was shifted, the square wave light conditions had higher rates of carbon assimilation and stomatal conductance than the fluctuating light conditions (Figure 14). This is in contrast to the fluctuating seeds performing better than the square wave seeds. The FLfl treatment had the highest levels of stomatal conductance and carbon assimilation is the 8:00-17:30 time period had the lowest once the timings had been shifted.

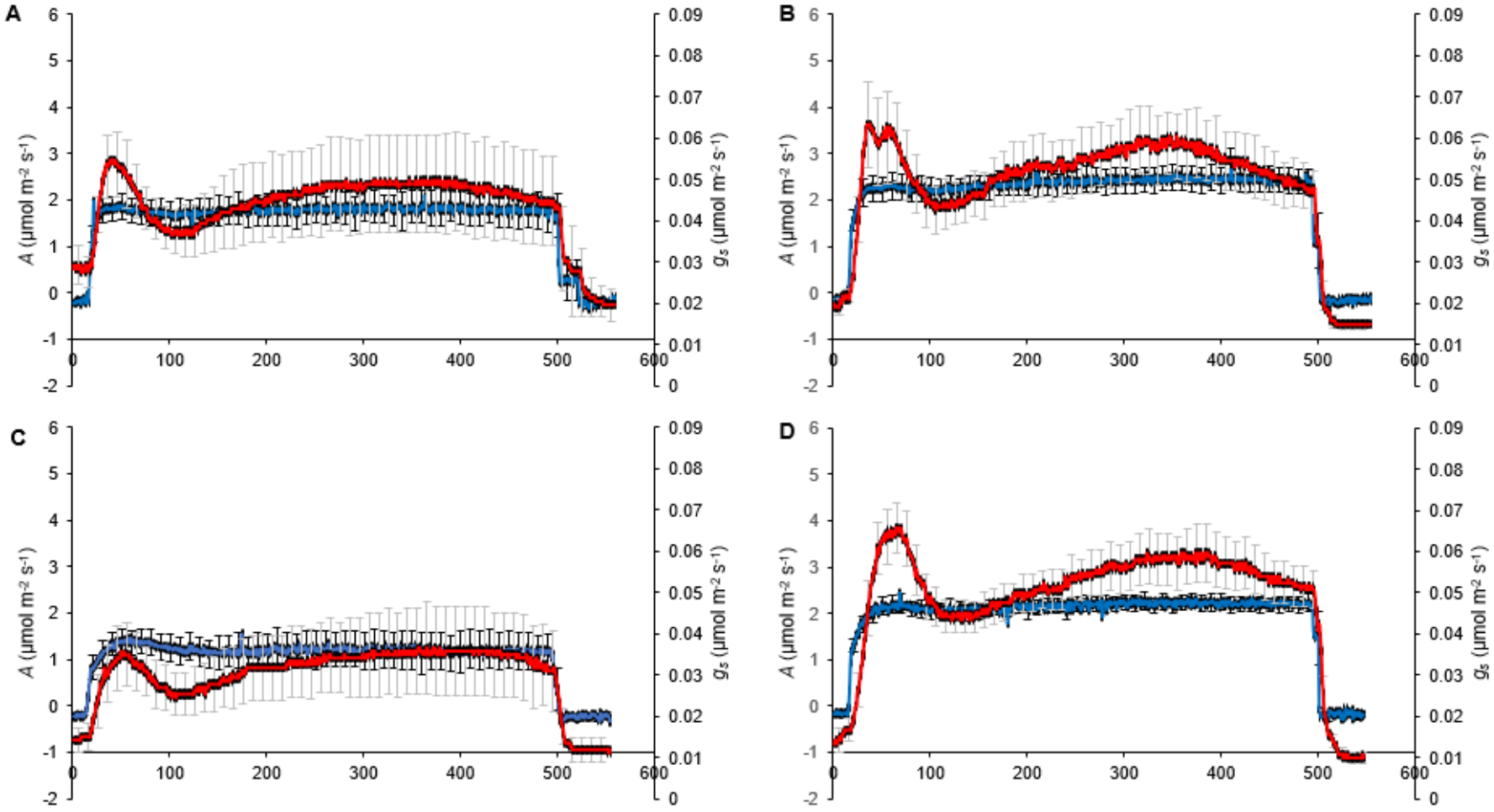

Figure 13: Mean rates of stomatal conductance (blue) and carbon assimilation (red) over a diurnal period measured between 8:00-17:30 every minute for *A. thaliana* for each treatment, SQsq (A), SQfl(B), FLsq (C), FLfl (D). Error bars represent Standard Erro

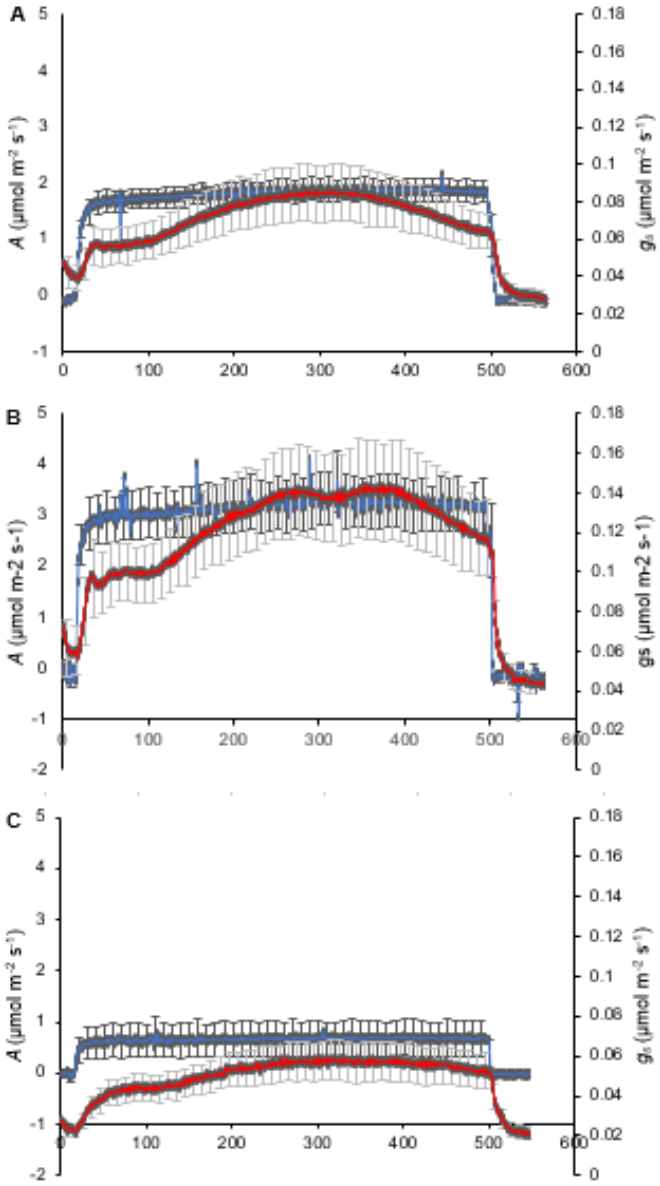

Figure 14: Mean rates of stomatal conductance (blue) and carbon assimilation (red) over a diurnal period measured between 10:00-19:30 every minute for *A. thaliana* for each treatment, SQsq (A), FLsq (B), FLfl (C). Error bars represent Standard Error.

Rates of carbon assimilation varied significantly between treatments, with the SQsq treatment having a significantly lower rate than the FLfl (P=0.006) and SQfl (P=0.03) treatments (Figure 15.A.). The fluctuating light conditions had higher levels then the square light conditions for both seed types tested. The

fluctuating seed had higher *A* than the square seed in both light conditions tested.

Stomatal conductance followed the trend shown by carbon assimilation. The fluctuating light conditions had higher rates of g_s than the square light treatments, with FLfl having significantly higher rates of conductance than SQsq (P=0.009) (Figure 15.B.). The fluctuating light seed also had higher rates of g_s than the square light in both light conditions tested.

There was no significant difference between any of the treatments for water use efficiency (Figure 15.C.)

Rates of ambient CO_2 in which the experiments were undertakes varied, with SQsq having a significantly higher amount than the SQfl and FLfl treatments (Figure 15.D.)

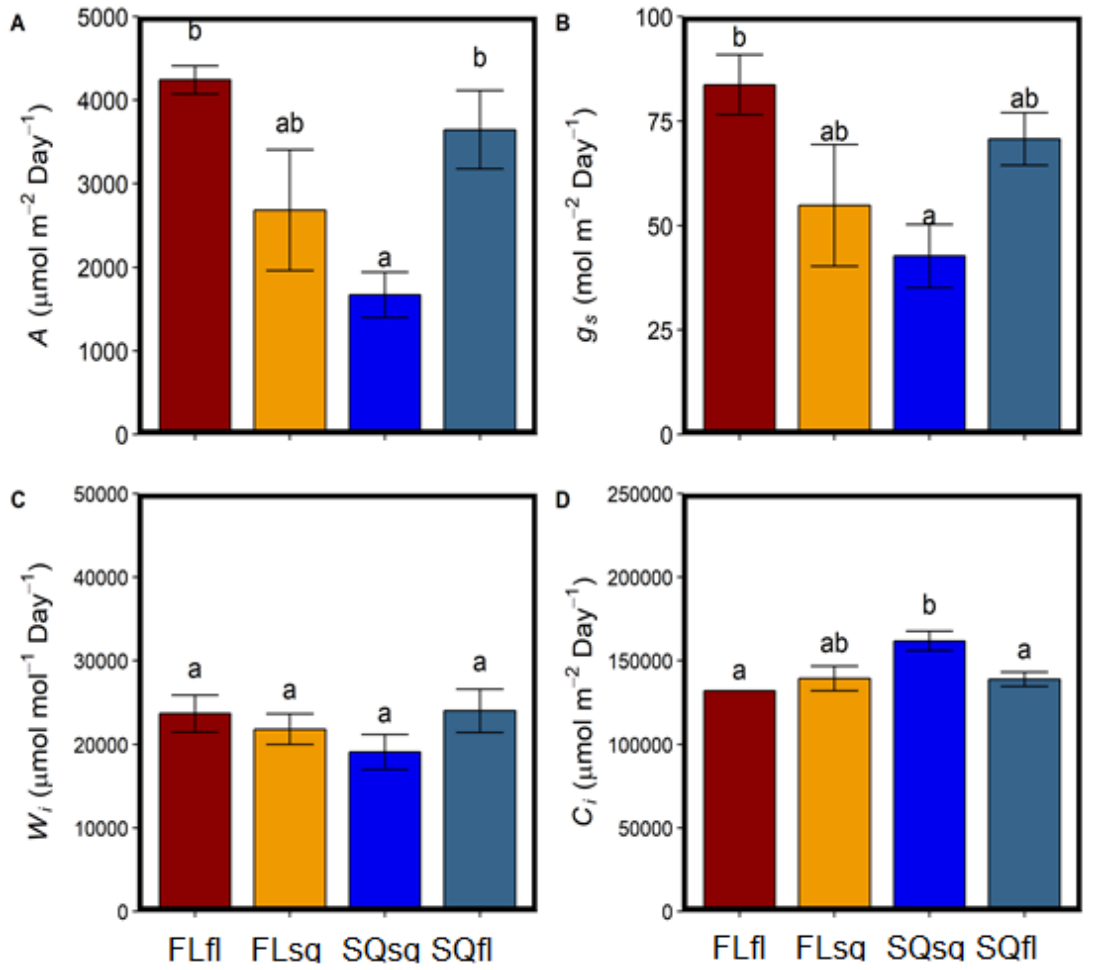

Figure 15 Diurnal measurements of treatments when exposed to 150 µmol m^{-2} s^{-1} of constant light intensity for 8 hours between 8:00-17:30, showing (A) total rate of carbon assimilation, (B) total stomatal conductance, (C) daily water use efficiency and (D) ambient CO_2. Error bars represent 95% confidence interval, letters represent significance as results of Tukey's posthoc test.

There was no significant difference between the three treatments SQsq, FLsq or FLfl in total carbon assimilation (Figure 16.A), stomatal conductance (Figure 16.B) or water use efficiency (Figure 16.C) for the plants grown in the experimental period of 10:00-19:30. There was a significantly higher internal [-CO_2-] (C_i) under which the experimental conditions were run for the FLsq treatment than the SQsq treatment (Figure 16.D).

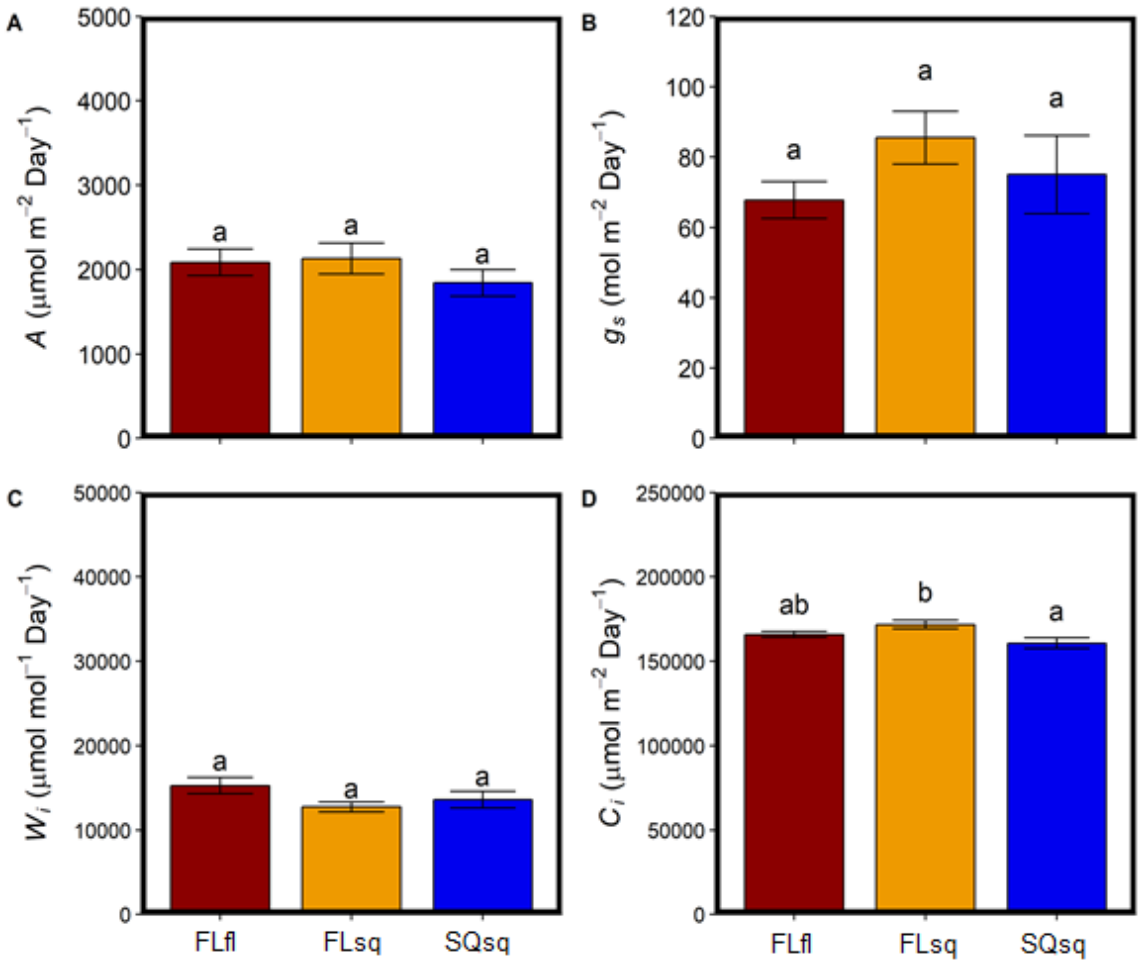

Figure 16: Diurnal measurements of treatments when exposed to 150 µmol m^{-2} s^{-1} of constant light intensity for 8 hours between 10:00-19:30, showing (A) total rate of carbon assimilation, (B) total stomatal conductance, (C) daily water use efficiency and (D) ambient CO_2. Error bars represent 95% confidence interval, letters represent significance as results of Tukey's posthoc test.

Stomatal Pore Counts

There was no significant difference between the number of stomatal pores on the leaves of plants grown under the different lighting regimes (P=0.058).

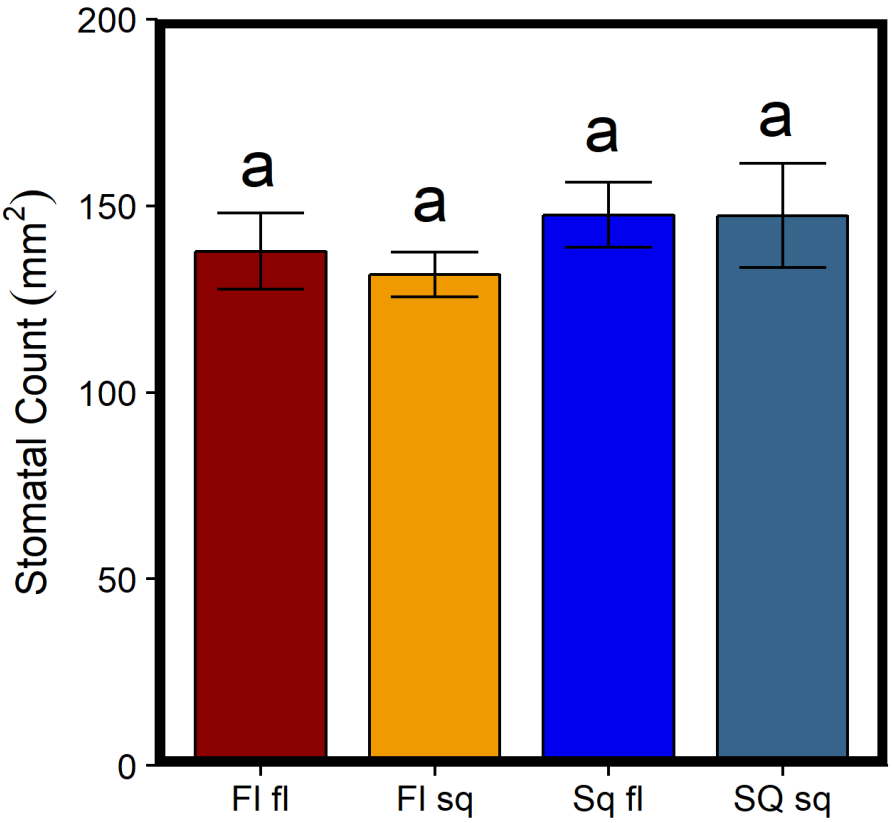

Figure 17: Mean stomatal density of both adaxial and abaxial sides of leaves for each treatment of *A. thaliana*. Error bars represent Standard Deviation.

Discussion

Photosynthetic Efficiency

The light regime the plants were grown under was shown to have an impact on the quantum efficiency throughout the day by the decrease shown from SQfl to FLfl in the morning, midday, and evening treatments. SQsq also had significantly higher quantum efficiency than FLfl and FLsq in the morning and evening, which shows that the square seed plants performed better than the fluctuating seed which hints at a genotypic effect of the light growth conditions on the samples tested. This effect may be due to the square light plants adapting to having one step increase in light intensity, and so requiring Calvin Cycle enzymes and photosystem II antennae to be able to prepare for the sharp increase in light, rather than the slower “natural” dawn of fluctuating light. The square light seed plants having consistently higher maximum quantum efficiency suggests that their adaptation to the change in light regime has allowed them to be able to absorb more light energy than the fluctuating seed plants. However, as shown in Figures 15 and 16, the maximum rates of carbon fixation were reduced in the square light seed plants. Therefore, the adaptation may have allowed them to be more efficient at collecting light for a short period, but not be able to continue that high level over a longer period of a day, overall inhibiting the rate of photosynthesis.

Conductance and Assimilation Fluctuations to Diurnal Changes

The results agreed with Matthews et al. (2018) showing the presence of the decoupling of stomatal conductance with carbon assimilation for all treatment groups.

Carbon assimilation remained relatively stable throughout the day and stomatal conductance increased, peaked, and decreased without any corresponding changes for all treatments (12). This impact of the decoupling was greatest in the FLfl treatment, with greater changes in stomatal conductance throughout the day, followed by FLsq, SQsq then SQfl.

This effect shows a change in size of this decoupling in the diurnal rate of g_s was affected by the light growth, and a possible genotypic effect was shown with the fluctuating seed offspring plants having a greater rate of stomatal conductance and as such a greater loss of water through transpiration than the square wave seed offspring plants for both time periods tested.

The size of the decoupling changes between light regimes and seed types. When tested at the 8am dawn period, the fluctuating light growth conditional plants had a higher rate of stomatal conductance, and so lost more water without any corresponding gain in photosynthesis than the plants grown under square light. This shows that the growth condition has more of an effect than the conditions of previous generations and that the genes causing this decoupling are not hereditary.

Figure 15 A. and B. show the carbon assimilation and stomatal conductance daily rates for the four treatments, and with FLfl having the highest results followed by SQfl, FLsq then SQsq. FLfl and SQfl had significantly higher rates of carbon assimilation than SQsq, showing that whatever seed type was used the fluctuating conditions closely resembling natural light enabled the plants to more efficiently photosynthesise, and thus grow faster and larger than the plants grown under square wave of light which would be nutrient limited.

Constant levels of light found in the square light treatment may have also damaged the photosynthetic pigments in the PSI and PSII antennae, in a process called photoinhibition, reducing the amount of light energy able to be absorbed and used to assimilate CO_2 (Powles, 1984). Increases and decreased in light intensity allow for any photosystems damaged to be repaired and to continue efficiently capturing light energy, whilst a square wave of light does not allow for damaged photoreceptors to be repaired or replaced.

Stomatal conductance followed the trend set by carbon assimilation, with FLfl plants having significantly higher rates of gaseous transfer than the SQsq treatment. While FLsq had a higher rate, it was not significant like in the original light conditions experiment. The mirroring of these two processes is due to the strong links between intaking carbon dioxide, and the rate of photosynthesis in the chloroplasts of the plant. If there is a lowered need for CO_2, then the stomatal aperture can be closed to reduce the intake of gas and the loss of water, while when more in needed the aperture can increase, allowing more CO_2 to enter the plat and be assimilated and more water lost via transpiration (Wong et al., 1979).

With no significant difference in the number of stomatal pores between treatments (Figure 17), the changes in stomatal conductance are therefore unable to be due to changes in the available sites of conductance, and rather in stomatal pore aperture and behaviour.

Once the light conditions had been shifted by 2 hours, there was no significant difference in the carbon assimilation or stomatal conductance between the

three treatments (Figure 16). The rates of carbon assimilation were much lower than before the shift and more uniform between the treatments. This implies that the change in timing of initial light activation has a large effect on the rate of photosynthesis and therefore ability of the plant to thrive, and that this effect is larger than the type of light conditions that a plant is grown under. This is due to the different seed types having similar rates of carbon assimilation, as well as the types of light condition.

This decrease in carbon assimilation may be due to a misalignment of the circadian clock to the external conditions the plants are grown under. Genes activated by the clock changes throughout the day to best adapt to changes in light intensity and intrinsic water concentrations. Genes coding for the creation of the CAB protein and Rubisco which is used in photosynthesis are highly expressed in the morning in anticipation of the light from dawn which will be captured and converted into photosynthetic products (Schaffer et al., 2001). The change of as little as 2 hours has had an impact, with the genes and enzymes being produced for periods and conditions that are different to the actual growth conditions.

While the water use efficiency did not significantly differ between treatments tested at the same time, there was a large decrease when comparing from the 8:00 data to the 10:00 data for each treatment. This could be due to the increase in stomatal conductance losing more water through transpiration and a reduction in carbon assimilation leading to more water ‘wasted’ by the plant unable or unwilling to reduce stomatal aperture. The cause of this effect could be due to the plants circadian rhythm expecting lower light and cooler conditions than the environment it was currently exposed to. If the plants cycle

was expecting conditions resembling earlier in the day, the ‘normal’ conditions, then light intensity would have been lower and expected to rise later in the day so Calvin Cycle enzymes would not necessarily be at maximum concentration, and temperatures would have been lower so less water loss through transpiration and evaporation requiring a smaller stomatal aperture.

Changes in the growth light conditions of plants was shown to have an effect on the decoupling of stomatal conductance and carbon assimilation, with plants grown under “normal” fluctuating conditions losing more water needlessly to this effect than those grown under a square wave of light. However, this effect may be due to increased rates of stomatal conductance therefore causing more water to be lost, with further research needed to decisively conclude this impact.

Shifts to the timing of light activation away from natural dawn was also shown to have an impact on the height but not on the overall timing of this decoupling. The decoupling shifts with the change in light and was not fixed to the same timings as when grown under “natural dawn” conditions, therefore implying that circadian clock rhythms play a large part in the creation of this large, needless loss in water with no photosynthetic gain. More time periods would be needed to be tested, both shifting before and further after “dawn”, to help determine the relationship between the circadian genes and the decoupling of the two important processes.

Conclusion

The aim of this research was to investigate the source of the decoupling between carbon assimilation and stomatal conductance, and the factors which may affect it. Based on experimental data altering the circadian clock of plants, and growth light intensity regimes of plants, it can be concluded that while these variables do not cause the decoupling of these processes, they have an impact on the size of and the amount of water lost via transpiration. The size of the decoupling was shown to fluctuate between the wild type "normal" *Arabidopsis thaliana* and the gene overexpressed individuals in Chapter 2, and between the fluctuating seed fluctuating light plants (natural conditions) and the altered seed and light regimes in Chapter 3. The start and peak of the decoupling did not change between the overexpressed and non-overexpressed wild type circadian plants, or between light regimes, signifying that the circadian clock and growth conditions has an impact on the decoupling response but is not the proximal cause.

This implies that the initial increase in carbon assimilation and stomatal conductance in the morning when the plant starts photosynthesising in response to the increasing light energy may not be as strongly linked as previously thought. The processes may have similar initial causes, increases in light intensity causing stomata to open and photosynthesis to occur, yet to not have a direct feedback link. This would allow for other inputs to affect one of the processes but not the other, shown by the increase and decrease in stomatal conductance with no corresponding change in carbon assimilation. Further study is needed to investigate the underlying cause of the decoupling to better understand and mitigate the large loss in daily water, or to lead to

increases in carbon assimilation to match the changes in stomatal conductance.

The research showed evidence of the decoupling between stomatal conductance and carbon assimilation at the low light levels used in the experiments. The reduction in both carbon assimilation and stomatal conductance for the CCA1 overexpressed plants in chapter 2 and the square light growth conditions of plants in chapter 3 could be attributed to the light levels used in the experiments. Further experimentation can be recommended by repeating the diurnal measurements at higher light intensities, to test whether the decoupling occurs earlier when under light stress and whether the size is influenced.

The use of diurnal measurements were to gather data on one day's change in stomatal conductance and carbon assimilation. However the use of ADC and Li-Cor machines limited the length of time available to test the plants under, as they were unsuitable for prolonged use. Longer experiments, looking at multiple days data would provide better insight into the results, to ensure that the data collected was not due to stress or if the plants would be able to adapt to the new conditions

Overexpression of the clock genes was undertaken in order to impact the circadian cycle and alter its rhythm, investigating any further influence on the decoupling of vital plant processes. The data showed that there was an influence caused by the circadian clock, but it is not thought to be the initial cause of the discrepancy. Due to the clocks cyclical feedback mechanisms, multiple genes function to inhibit and promote other genes, so although TOC1

and CCA1 were overexpressed, their impact in the cycle was not. Other genes, such as PRR and LHY for CCA1 function in similar ways inhibiting TOC1, Gibberellin and LUX evening genes. So any impact on overexpressing the morning or evening genes in their entirety might require overexpressing multiple genes to better represent the influences these genes have on stomatal conductance. The overexpression of the genes in these experiments were focused on the guard cells, not the rest of the plant tissue. Therefore, any impact that the alterations had may have been muted by the continuation of the circadian clock in the carbon fixating mesophyll tissue. Future experiments could investigate whether the changes in stomatal conductance was due only to the changes in the guard cell behaviour leading to little change in carbon assimilation, or if the clock was altered would changes occur in the daily rate of carbon assimilation.

The results of this thesis can conclude that the decoupling of stomatal conductance and carbon assimilation was shown to be found in the *Arabidopsis thaliana* at varying sizes for all the conditions tested in the experiments, confirming that there is a need for more information on the causes of this phenomenon. As mentioned in Chapter 1, water conservation is an increasingly important issue regarding plant and crop production, and one method of helping to alleviate this issue may be to remove the cause of this decoupling or to ensure that the processes remain linked an increase the rate of photosynthesis in line with stomatal conductance.

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Appendix

i) Equation of total conductance of the leaf;

$$g_{tw} = \frac{E\left(1000 - \frac{W_l + W_s}{2}\right)}{W_l - W_s}$$

where *E* is the rate of transpiration, W_l is the molar concentration of water vapour in the leaf (mmol H_2O (mol air)$^{-1}$) and W_s is the sample air water mole fraction (mmol H_2O (mol air)$^{-1}$).

ii) Equation of the boundary layers conductance to water vapour (mol H_2O $m^{-2}s^{-1}$);

$$g_{bw} = sg_1 + g_0$$

where *s* is the leaf area and g_1 and g_0 are the slope and offset of boundary layer as a function of leaf area.

iii) Equation of the ratio of stomatal conductance from one side of the leaf to the other;

$$k_f = \frac{k^2 + 1}{(k + 1)^2}$$

where *k* is the estimate of the ratio of stomatal density on one side of the leaf compared to the other.