



University of
Nottingham

UK | CHINA | MALAYSIA

Warning flashing lights: Investigating the impact of stomatal density on photosynthesis in dynamic light environments

Thesis submitted to the University of Nottingham for the
degree of Master of Research
September 2025

Olivia Clapp
Student ID: 20711130

Supervisors: Alexandra Gibbs and Erik Murchie

School of biosciences, Faculty of sciences

University of Nottingham

Word count: 27,700

Acknowledgements

I would like to thank my supervisors Alex Gibbs and Erik Murchie, as well as the whole of the Murchie lab for their support and direction on this project. Additionally, I would like to thank Ute Voss for her guidance on how to conduct the root architecture experiment and for the use of the Voss lab's root imaging equipment. Thanks, should also go to July Gray at The University of Sheffield who supplied the mutants used in this study and to the British geographical survey who carried out the carbon isotope analysis on my behalf. Lastly, I would like to thank my friends and family who have supported me through the undertaking of this degree.

Abstract

Light availability in a crop canopy is not constant. When light is available, delays in photosynthetic induction can limit crop yields. Low availability of carbon dioxide (CO₂), due to slow stomatal opening, can restrict the ability of a plant to photosynthesise in dynamic light environments. Plants with mutations in their epidermal patterning factor genes, regulators of stomatal development, display variations in their stomatal density and stomatal clustering. This study tests the impact of low and high stomatal density on plant ability to photosynthesise under different fluctuating light treatments. An *EPF2* overexpressor (*EPF2OE*), *epf1epf2* loss of function mutant and Col-0 wild type *Arabidopsis thaliana* were grown under three different light treatments. A short fluctuating light treatment, a long fluctuating light treatment, and a constant light treatment were used. These plants were evaluated for carbon assimilation, stomatal conductance, water use efficiency, chlorophyll content, root architecture, and growth. *epf1epf2* plants displayed higher stomatal conductance, photosynthetic induction rates and water use efficiency. They also showed greater growth under the short fluctuating treatment compared to the two other genotypes tested. This suggests that having a higher stomatal density and conductance allows for more photosynthesis when grown under highly fluctuating light environments compared to other genotypes. Having a faster stomatal response to changes in light intensity had a greater positive impact on water use efficiency than low stomatal density, showing that the increased photosynthetic induction rate in high stomatal density plants offsets any increase in water loss. The *EPF2OE* plants displayed a higher adaxial stomatal density and conductivity than expected. This suggests that low stomatal density plants can compensate for lower abaxial stomatal density by increasing their adaxial stomatal density. It also raises questions about the role of the *EPF2* gene in the adaxial stomatal development pathway. The results of this study call into question the usefulness of low stomatal density genotypes in improving crop drought stress.

Table of contents

1. Introduction	9
1.1 Increasing yields	9
1.2 Climate change and the impact on agriculture.....	10
1.3 Improving photosynthesis	11
1.4 Role of stomata	15
1.5 Stomata at night	17
1.6 Stomatal kinetics	18
1.7 Stomatal development	19
1.8 <i>EPF</i> mutant studies	22
1.9 Dynamic light environments	23
1.10 Gaps in current knowledge	25
1.11 Aims and hypotheses	26
2. Methods	27
2.1 Plant material and plant growth	27
2.2 Light treatments	28
2.3 Plant area	29
2.4 Chlorophyll fluorescence	29
2.5 Chlorophyll assay	30
2.6 Gas exchange	31
2.7 Stomatal density	31
2.8 Carbon isotope analysis	32
2.9 Root architecture analysis	32
2.10 Statistical analysis	33
3. Results	34
3.1 Plant rosette growth was greatest under the constant light treatment	34
3.2 Light treatment and genotype had an impact	34
on plant growth	
3.3 Stomatal density was greater in <i>epf1epf2</i> plants compared to Col-0	37
and <i>EPF2OE</i> plants	
3.4 The difference between abaxial stomatal density and adaxial stomatal	37
density is smaller in the <i>EPF</i> mutant plants compared to wild type	
3.5 <i>epf1epf2</i> mutants display a higher WUEi than Col-0 and <i>EPF2OE</i>	38
3.6 Plants under constant light display greater WUEi.....	38
3.7 Abaxial stomatal conductivity was higher than adaxial stomatal	39
conductivity	
3.8 There was not a difference between daytime and	39
nighttime stomatal conductivity	
3.9 The <i>epf1epf2</i> genotype displays the highest stomatal conductivity	40
3.10 Genotype, but not light treatment, has a significant effect on	41
daytime photosynthetic induction	
3.11 Mean CO ₂ assimilation was lower in plants grown under the	43
short fluctuating light treatment	
3.12 Daytime carbon assimilation is higher than nighttime	44
carbon assimilation under low light	

3.13	Plants grown under the constant light treatment show lower levels.....	45
	of NPQ induction under light stress	
3.14	Differences between light treatments were significant under one	46
	second light fluctuations	
3.15	The constant light treatment displays lower a chlorophyll a/b ratio	47
	in the <i>EPF2OE</i> genotype	
3.16	Total root length differed between plants grown in the daytime and	48
	plants grown in the nighttime	
4.	Discussion	50
4.1	The genotype of a plant did not impact its' growth.....	50
4.2	Constant light led to the greatest plant growth.....	51
4.3	The <i>epf1epf2</i> genotype has the greatest stomatal density.....	52
4.4	<i>EPF</i> genes have different roles in abaxial and adaxial stomatal.....	53
	development	
4.5	High stomatal density gives a greater plant water usage.....	54
4.6	Plants have greater water use efficiency under constant light.....	55
4.7	A greater stomatal density increases stomatal conductivity.....	55
4.8	Stomatal conductivity was not different between the	56
	daytime and the nighttime	
4.9	Daytime photosynthetic induction was highest in the <i>epf1epf2</i>	58
	genotype	
4.10	Photosynthetic induction differs in the nighttime compared to the	59
	daytime	
4.11	Fluctuating light induced light stress in plants	60
4.12	The <i>EPF2OE</i> genotype adjusts its' chlorophyll content to acclimate	61
	to dynamic light conditions	
4.13	Differences in WUE were not due to differences in root growth.....	62
4.14	Future studies	63
4.15	Implications of these findings for crop plants	64
5.	Conclusion	67
6.	Bibliography	69
7.	Appendix	81
7.1	Timeline	81
7.2	Statistical models	81

List of abbreviations

Y_p – Yield potential
 ε_i – Efficiency of light capture
 ε_c - Efficiency of conversion of intercepted light into biomass
 η - Harvest index
RUE - Radiation use efficiency
FACE – Free air carbon dioxide enrichment
ET – Evapotranspiration
CO₂ – Carbon dioxide
WUE – Water use efficiency
COP21 – 21st Conference of parties (2015 UN climate change conference)
 g_s – Stomatal conductance
CBB – Calvin Benson Bassham
SBPase – Sedoheptulose-1,7-bisphosphatase
FBPase – Fructose-1,6-bisphosphate
2PG – 2-phosphoglycerate
C4 – 4 carbon acid
C3 – 3 carbon acid
NPQ – Non-photochemical quenching
qE – Energy dependent quenching
ROS – Reactive oxygen species
 Φ – Quantum yield efficiency of photosynthesis
GROK – Outward rectifying potassium
VPD – Vapour pressure deficit
 g_n – Nighttime stomatal conductance
MMC – Meristemoid mother cell
SLGC – Stomatal lineage guard cell
GMC – Guard mother cell
SPCH – SPEACHLESS
ICE1 – INDUCER OF CBF EXPRESSION 1
SCRM2 – SCREAM2
EPF2 – Epidermal patterning factor 2
EPFL – EPF/EPF-LIKE
ER – ERECTA
MAP – Mitogen activated protein
 $g_{w(op)}$ – Stomatal conductance to water vapour
arp – Actin related protein
WUEinst – Instantaneous water use efficiency
PAR – Photosynthetically active radiation
NPQ_L_n – Non-photochemical quenching during light adaptation
NPQ_L_{ss} – Steady state non-photochemical quenching
EA – Elemental analyser
IRMS – Isotope ratio mass spectrometer
WUEi – Intrinsic water use efficiency
MS – Murashige and skoog
ANOVA – Analysis of variance
LHCB – Chlorophyll a/b binding protein

List of figures

- Figure 1: A diagram showing the processes where energy is lost from the sun through to the accumulation of biomass in C3 and C4 crops
- Figure 2: A diagram of the Calvin Bassham Benson cycle
- Figure 3: A schematic showing the rate of NPQ relaxation after a sunfleck has passed
- Figure 4: A schematic showing the rate of induction of CO₂ assimilation and water loss in stomata with different rates of opening and closing
- Figure 5: A diagram of ion movement in and out of guard cells and the guard cell vacuole during the opening and closing of stomata
- Figure 6: A diagram showing the conditions under which stomata are induced to open and close
- Figure 7: Diagrams of stomata in the *Poaceae* family and the non- *Poaceae* family
- Figure 8: A diagram of the stomatal development pathway
- Figure 9: A diagram showing the stomatal density in each of the genotypes used in this study
- Figure 10: Line diagrams showing the total light intensity in each of the light treatments
- Figure 11: Scatter plots showing the growth in rosette area
- Figure 12: Boxplots showing the plant rosette area growth at 3 weeks after transfer to individual pots
- Figure 13: A Boxplot showing the stomatal density per mm² for the abaxial and adaxial side of the leaf for each genotype in each light treatment
- Figure 14: A bar graph showing the mean Δ value for each genotype in each light treatment
- Figure 15: A boxplot comparing the stomatal conductivity values between the abaxial and the adaxial side of the leaf in each genotype
- Figure 16: A boxplot comparing the stomatal conductivity values between the daytime and the nighttime in each genotype
- Figure 17: A bar graph showing the daytime and nighttime photosynthetic induction rate in each genotype in repeat two

- Figure 18: A bar graph showing the daytime and nighttime photosynthetic induction rate in each genotype in repeat three
- Figure 19: Graphs showing the carbon assimilation measured in $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity and $15 \mu\text{mol m}^{-2}\text{s}^{-1}$ light intensity against time
- Figure 20: Bar graphs showing the average daytime and nighttime carbon assimilation under the high ($150 \mu\text{mol m}^{-2}\text{s}^{-1}$) and low ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) light intensities
- Figure 21: A graph showing the average NPQ values over time
- Figure 22: Graphs showing average NPQ values over time under one second and one minute fluctuations in light intensity
- Figure 23: Boxplots showing the chlorophyll content and the chlorophyll a/b ratio
- Figure 24: Bar graphs showing the total root length, the total number of lateral roots, and the total lateral root length

1. Introduction

1.1 Increasing yields

The global population is increasing, and it is expected to reach 9.8 billion by 2050. In order to meet the demands of this larger population, food production will need to increase by at least 70% (Islam and Karim, 2019). One method to increase food production, is to increase the area of land under cultivation. Cropland and pastureland occupy around 39% of the land's ice-free surface (Ellis et al., 2010). Historic changes to land use practices, including a 70% increase in irrigated cropland area, have enabled global grain harvests to double in the four decades prior to 1999 (Gleick, 2003) (Kanianska, 2016). However, these changes to land use are accompanied by negative environmental effects. To avoid these negative consequences, whilst still increasing food supply, the yield potential (Y_P) of crops must increase in the coming decades.

Yield potential (Y_P) is defined as the maximum attainable yield per unit land area (Foulkes et al., 2022). This is determined by the efficiency of light capture (ε_i), the efficiency of conversion of intercepted light into biomass (ε_c) and the harvest index (η) (Koester et al., 2014) (Equation 1). Harvest indices and efficient light capture have been brought close to their theoretical maxima by plant breeding, but little improvement has been made to the efficiency of conversion of intercepted light (Long et al., 2006). Photosynthesis is the primary mechanism by which light energy is converted into biomass.

$$Y_P = \varepsilon_i + \varepsilon_c + \eta \quad (1)$$

The theoretical maximum conversion efficiency of solar energy into biomass is 4.6% and 6%, for C3 and C4 crops respectively (Zhu et al., 2008). The actual rate of solar energy conversion efficiency is lower than the theoretical maximum rate, with the highest reported rate around 2.4% and 3.7%, for C3 and C4 crops respectively (Beadle and Long, 1985, Monteith et al., 1977). Attempts to improve radiation use efficiency (RUE), of which photosynthetic efficiency is a major component, through crop breeding, has led to an increase in genetic gain of $0.0049 \text{ g MJ}^{-1} \text{ year}^{-1}$ in maize (Messina et al., 2022). This accounts for 32% of the documented increase in yield in the US corn belt. Thus, photosynthetic improvement is possible through crop breeding and represents a desirable target for crop improvement. Additionally, there are multiple processes contributing to the energy loss (Figure 1) that could be targeted for improvements to increase RUE.

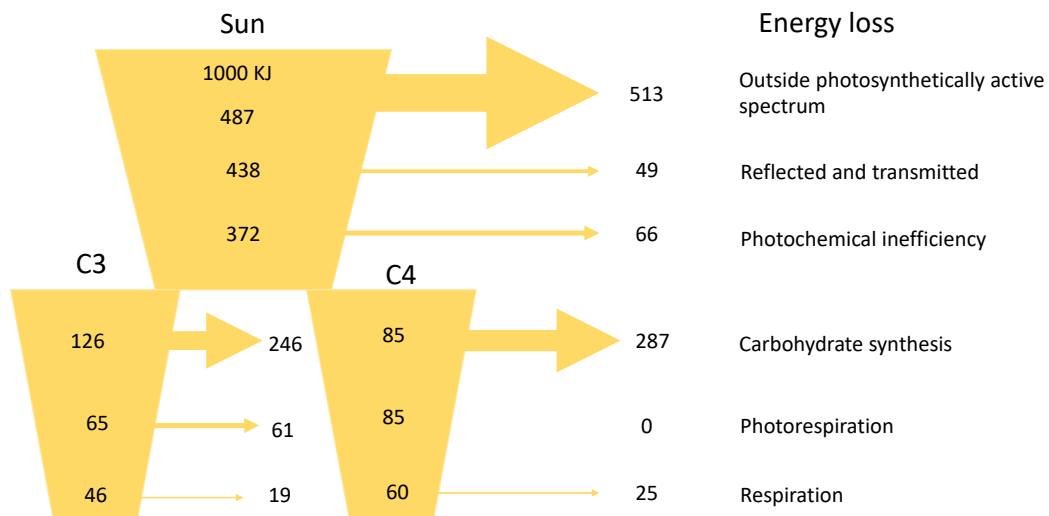


Figure 1: A diagram showing the processes where energy is lost from the sun through to the accumulation of biomass in C3 and C4 crops. The size of the arrow reflects the amount of energy lost in KJ to scale. Redrawn from (Zhu et al., 2008).

1.2 Climate change and the impact on agriculture

Under elevated carbon dioxide (CO_2) levels light-saturated carbon uptake, nighttime carbon assimilation, growth, and above-ground production increase. Whilst, specific leaf area and stomatal conductance decrease (Ainsworth and Long, 2005). Higher atmospheric CO_2 levels increase the concentration of CO_2 around RuBisCo, thus reducing photorespiration rates. A metanalysis of Free-air CO_2 enrichment (FACE) studies found an increase in photosynthesis and production with an increase in atmospheric CO_2 levels (Ainsworth and Long, 2005). However, there were species specific differences observed, with trees being more responsive to increased CO_2 than herbaceous species. The Ainsworth and Long (2005) study also found that across the FACE studies they looked at, species that carried out C4 photosynthesis were less responsive to an increase in atmospheric CO_2 concentration than species that carried out C3 photosynthesis. Increased atmospheric CO_2 levels, as a result of climate change, have caused partial closure of stomata in temperate and boreal forests. This partial closure has led to an increased CO_2 uptake, reduced evapotranspiration (ET) and a higher forest water use efficiency (WUE) (Keenan et al., 2013). If global average temperature increases are kept below 3°C , the reduced agricultural production in the tropics, due to climate change, will be offset by production increases in the temperate zone (Easterling et al., 2007).

Despite this increase in temperate production, climate change will increase the frequency of heatwaves, droughts, and floods. Models have predicted that the global average chance of a major heatwave increases from 5%, which was the risk from 1981-2010, to 28% at 1.5°C warming and 92% at 4°C warming (Arnell et al., 2019). In addition to rising temperatures, climate change also increases the risk of agricultural drought from 9%, under pre-industrial temperatures, to 24% at 1.5°C warming and 61% at 4°C warming. On top of this, the likelihood that a year will contain a flood, greater than that of the reference period

50 year flood, increases from 2%, under pre-industrial temperatures, to 2.4 % at 1.5 °C warming and 5.4% at 4 °C warming (Arnell et al., 2019). The January – September 2024 global surface temperature was recorded to be 1.28°C above the 20th century average (NOAA National Centers for Environmental Information, 2024). This is a 175-year record.

The Paris agreement is an international agreement introduced at the UN Climate Change Conference (COP21) in Paris; it was signed in 2015. The 196 signing parties agreed to pursue efforts to limit the global temperature increase to 1.5 °C above pre-industrial levels (The Lancet Planetary, 2018). Exceeding 1.5 °C could result in changes known as climatic tipping points (Armstrong McKay et al., 2022). These events include the abrupt thawing of boreal permafrost, the breakdown of major ocean circulation systems and the collapse of tropical coral reef systems. These changes are self-perpetuating and irreversible. Climatic tipping points can have severe regional impacts, such as, higher temperatures and higher frequency of droughts, which will impact agriculture (OECD, 2022).

Changes to global temperature and precipitation will impact soil moisture and soil temperature. This can alter the length of a growing season. Reduced growing seasons will reduce the maximum crop yield (Calzadilla et al., 2013). This presents a challenge for attempts to increase food production.

Climate change has also been predicated to alter the frequency and magnitude of droughts (FAO, 2019). In the decade 2010 to 2020 droughts were calculated to be responsible for crop losses totalling \$30 billion (Gupta et al., 2020). Thus, droughts represent a major threat to future crop yield potential. Despite this risk, global water usage is increasing, it has been predicted that the world will face a 40% global water deficit by 2030 (United Nations, 2021). This will increase pressure on plant breeders to produce more water efficient crop varieties.

Stomatal conductance (g_s) is a measure of the rate of CO₂ uptake and water loss, it is an estimate of the rate of gas exchange through a leaf (Pietragalla and Pask, 2012). g_s has a large influence on global fluxes of water, as 35×10^{18} g of water pass through the stomata of leaves annually (Hetherington and Woodward, 2003). Changes to the area, density or conductance of stomata all have an impact on the transpiration rate and the WUE of a plant. This makes stomata a key target for attempts to increase drought tolerance in crops.

1.3 Improving photosynthesis

Proposed methods for improving photosynthesis include the optimisation of the expression of Calvin Benson Bassham (CBB) cycle (Figure 2) enzymes. The natural distribution of enzymes in the CBB cycle is suboptimal and could limit photosynthesis (Zhu et al., 2007). Under elevated CO₂ conditions, carbon fixation becomes limited by RuBP regeneration not the rate of RuBisCo (Masle et al., 1993, Rosenthal et al., 2011). Hence, it has been predicted that higher levels of sedoheptulose-1,7-bisphosphatase (SBPase) and fructose-1,6-bisphosphate (FBPase) could support higher productivity (Miyagawa et al., 2001, Lefebvre et al., 2005).

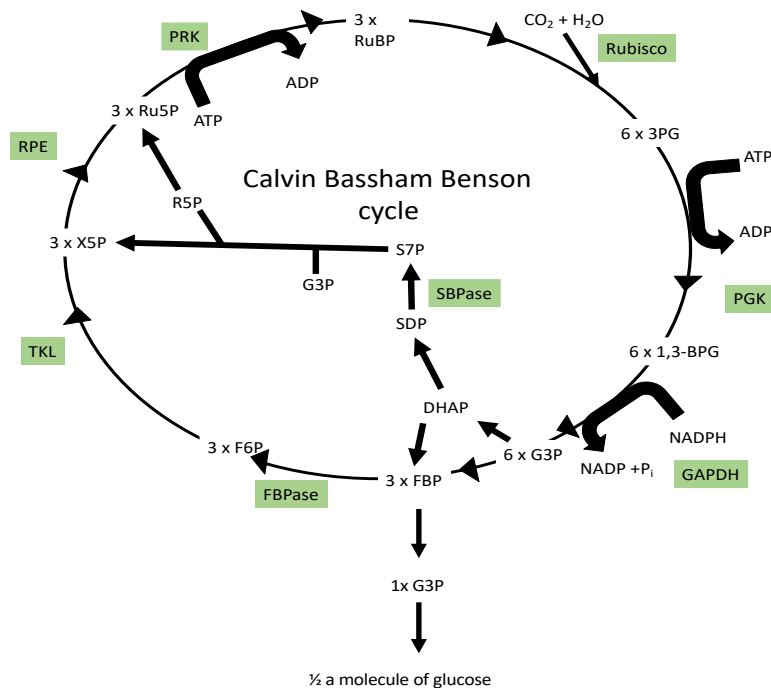


Figure 2: The Calvin Bassham Benson cycle depicting the fixation of carbon to produce glucose. The cycle must be completed twice, fixing six molecules of CO₂, to produce one molecule of glucose. All enzymes used in the Calvin Bassham Benson cycle are in green.

Another approach that has been suggested to improve the efficiency of photosynthesis is engineering RuBisCo to increase the enzyme's specificity to CO₂. This would help to reduce the energy lost due to photorespiration (Kubis and Bar-Even, 2019). Photorespiration occurs when RuBisCo fixes molecular oxygen instead of CO₂. This produces 2-phosphoglycerate (2PG), which is toxic to plants and must be recycled. Photorespiration occurs due to the dual carboxylase and oxygenase activity possessed by RuBisCo (Eckardt, 2005). The photorespiration pathway could also be rewired to reduce the inefficiencies associated with the 2PG recycling. Three different photorespiratory bypasses have been created by South et al. (2019). One of these bypasses uses five *E.coli* genes to convert 2PG into glycerate. The other two pathways both convert 2PG into glyoxylate, which is used in the glyoxylate cycle to produce malate from acetyl-CoA. These two pathways achieve this using different genes. The productivity of tobacco (*Nicotiana*) plants grown in the field increased up to 39% when one of these photorespiratory bypasses was introduced (South et al., 2019).

C4 photosynthesis can reduce or eliminate instances of photorespiration depending on the species, this is achieved through a carbon concentrating mechanism that increases the CO₂ concentration around rubisco (Jenkins et al., 1989). C4 plants possess Kranz anatomy, this is formed of two concentric layers of chlorenchyma cells, with bundle sheath cells surrounded by a layer of mesophyll cells (Lundgren et al., 2014). CO₂ diffuses into the mesophyll cells where it is incorporated into a four carbon (C4) acid, this acid is then transported to the bundle sheath cells. The C4 acid is converted back into a three carbon (C3) acid and the resultant molecule of CO₂ is released close to the RuBisCo enzyme

(Kellogg, 2013). This increases the concentration of CO₂ around the RuBisCo enzyme reducing the instances of O₂ fixation by the enzyme. Hence, C4 plants show an increased efficiency of photosynthesis relative to C3 plants of 1.4%.

Major food crops, such as rice (*Oryza*) and wheat (*Triticum*), use C3 photosynthesis. Attempts have been made to engineer C4 photosynthesis into these crops to increase their yields, however, this has yet to be achieved (Schuler et al., 2016). Engineering C4 photosynthesis requires the engineering of Kranz anatomy into C3 plants and editing of the location and level of expression of multiple genes involved in C4 photosynthesis. In addition to the complexity of this process, the frequency of photorespiration decreases as atmospheric CO₂ increases. So C4 engineering, if achieved, would show a decreased benefit as atmospheric CO₂ continues to increase.

Altering the kinetics of non-photochemical quenching (NPQ) is another proposed method to increase photosynthetic efficiency. NPQ is induced in plants as a response to high light stress (Müller et al., 2001). NPQ dissipates excess energy as heat. It consists of several processes with energy dependent quenching (qE), the rapidly reversible component, making up the largest of these (Ruban, 2016). NPQ is activated under high light stress to prevent oxidative damage through the dissipation of light energy as heat. This is caused as, under high light, the rate of linear electron transport along the thylakoid membrane can exceed the capacity of the CBB cycle to produce reducing agents. When this occurs, there is a build-up of electrons at photosystem II, these excess electrons can be transferred to molecular oxygen creating reactive oxygen species (ROS), peroxides and radicals. These molecules cause oxidative stress and damage the D1 subunit of photosystem II, this causes a reduction in quantum yield efficiency of photosynthesis (Φ) at low light. In severe cases damage to the D1 subunit can cause a permanent reduction in plant photosynthetic capacity, which is termed photoinhibition (Sharma et al., 2023).

Plants exist in dynamic light environments and will often experience periods of high light that can lead to the induction of NPQ. Upon movement to low light plants will relax the NPQ response, however, this relaxation can be slow to occur. This causes a period of unnecessary, transient depression of photosynthetic efficiency due to slow NPQ relaxation (Figure 3) (Kromdijk et al., 2016).

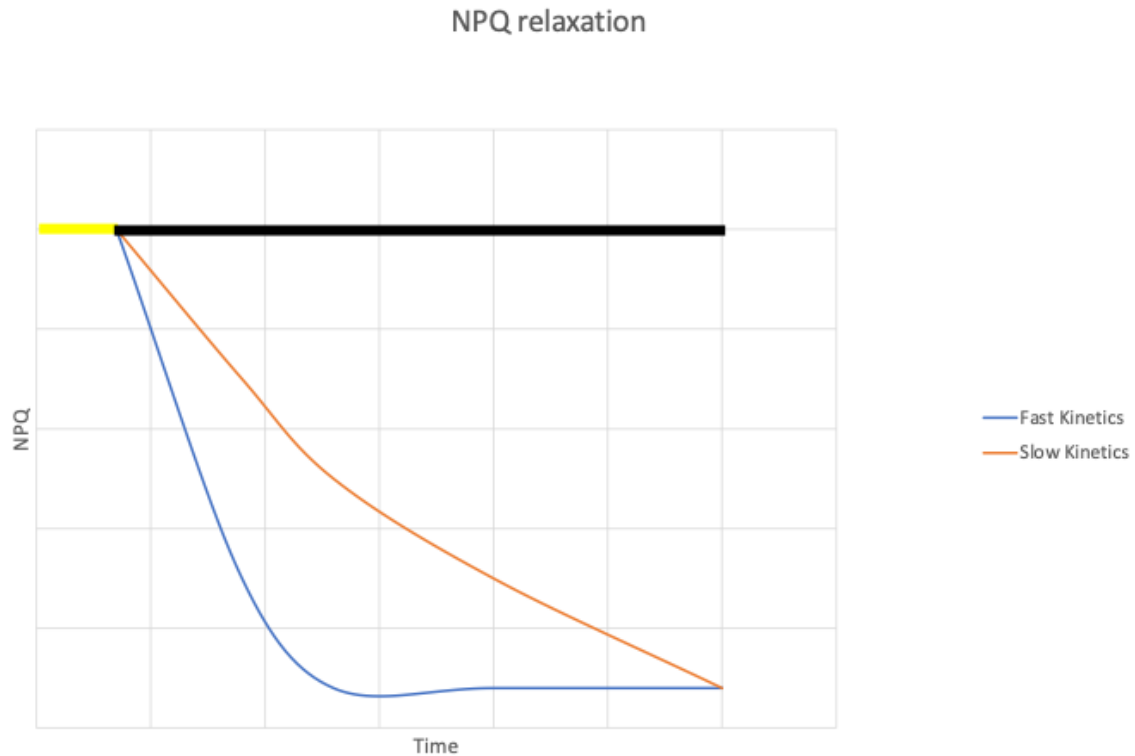


Figure 3: A schematic showing the rate of NPQ relaxation after a sunfleck has passed in plants with slow NPQ kinetics and fast NPQ kinetics. The yellow and black bars at the top of the graph represents the presence and absence of a sunfleck respectively. This schematic is illustrative only and is not representative of real data.

Speeding up the relaxation kinetics of NPQ has been suggested as a method to improve photosynthetic efficiency, particularly in dynamic light environments. VPZ plants, which over express PsbS, violaxanthin de-epoxidase, and zeaxanthin epoxidase, have been produced. PsbS is a thylakoid lumen protein responsible for sensing high luminal pH, a by-product of high light stress, and inducing NPQ (McAusland et al., 2019). While violaxanthin de-epoxidase and zeaxanthin epoxidase are enzymes involved in xanthophyll cycle (Zeppel et al., Garcia-Molina and Leister, 2020). This cycle converts the pigment violaxanthin to zeaxanthin, zeaxanthin is more effective at dissipating light energy as heat than violaxanthin. *Arabidopsis thaliana* VPZ plants showed improved NPQ kinetics, but reduced growth under fluctuating light (Garcia-Molina and Leister, 2020). A similar result was also found in VPZ potato (*Solanum tuberosum*), where NPQ kinetics were improved compared to wild type, but tuber growth was reduced (Lehretz et al., 2022). However, VPZ tobacco (*Nicotiana*) plants have been found to have increased CO₂ uptake and increased dry matter by up to 15% when grown in fluctuating light (Kromdijk et al., 2016). VPZ soybean (*Glycine max*) plants showed a 20% yield increase under field conditions (De Souza et al., 2022). It has been suggested that the differences seen in growth and yield between these different studies may be due to different levels of overexpression. Therefore, there is a need to optimize the expression level of the VPZ construct to the selected species (Khan et al., 2024).

Increasing stomatal kinetics has also been suggested as a method of improving photosynthetic efficiency particularly in dynamic light environments. Stomatal responses are an order of magnitude slower than photosynthetic responses (McAusland et al., 2016). Thus, slow stomatal opening upon entering a period of high light can delay photosynthetic induction (Figure 4). Species with elliptical shaped guard cells, which have slower opening kinetics, take a longer time to reach 95% CO₂ assimilation than species with dumbbell shaped stomata. (McAusland et al., 2016).

Plants that show clustering of their outward rectifying potassium (GORK) channel display faster stomatal kinetics. These plants had improved water efficiency and CO₂ assimilation (Horaruang et al., 2022). Plants with reduced stomatal density have been engineered these plants have reduced stomatal conductance and exhibit less water loss. Under water scarce conditions these plants are less adversely affected than wild type plants (Doheny-Adams et al., 2012).

1.4 Role of stomata

Stomata are pores on the surface of leaves surrounded by a pair of guard cells. Guard cells control the opening of stomata through the movement of ions (Figure 5) (Dietrich et al., 2001). When ions enter guard cells; some of these ions are moved to the vacuole. Water will move into the vacuole with these ions. The movement of water into vacuole makes the guard cell turgid, this opens the pore. Open stomatal pores allow for the exchange of gases with the atmosphere. Stomatal opening is essential for the acquisition of CO₂, used during photosynthesis in the CBB cycle. Stomata are driven to open under high light, low CO₂, high temperatures and high humidity (low VPD) (Figure 6) (Pankasem et al., 2024). While stomata are driven to close under low light, high CO₂, biotic stress and low humidity (high VPD) (Figure 6) (Pankasem et al., 2024).

In addition to CO₂ entering the leaf through open stomatal pores, water also leaves the leaf. Despite stomatal pores occupying only 3% of the leaf's surface area, 98% of all CO₂ taken up and all water loss occurs through these pores (Lawson and Blatt, 2014). ET is responsible for recycling approximately 60% of land precipitation back to the atmosphere (Oki and Kanae, 2006). Plant mediated transpiration accounts for 65% of this ET (Good et al., 2015). So, water loss through stomata has a key ecological function. However, plants have to carefully control water loss to prevent drought stress. This is done by integrating information on soil moisture and vapour pressure deficit (VPD) (Trugman et al., 2018). Vapour pressure deficit is a measure of air moisture, it is the difference between the amount of moisture in the air and the amount of moisture that the air can hold (Sulman et al., 2016).

Stomata close when VPD is high, as the reduced humidity in the environment creates a larger osmotic potential. This would increase the rate of transpiration if stomata were left open. Closing stomata under these conditions allows plants to conserve water (Grossiord et al., 2020). This means that plants have to balance the need to conserve water with the need to open their stomata to take in CO₂ for photosynthesis. VPD has risen, as a result of climate change. The increase in VPD between the years 1997-2020 was ten times greater than the increase in VPD between the years 1981-1996 (Liu et al., 2024). This has resulted in an increase in ET and it has been suggested as the reason behind ecosystem WUE being stable since 2001 (Flanagan and Farquhar, 2014). Despite the fact that an increase in CO₂ atmospheric concentration was expected to increase WUE.

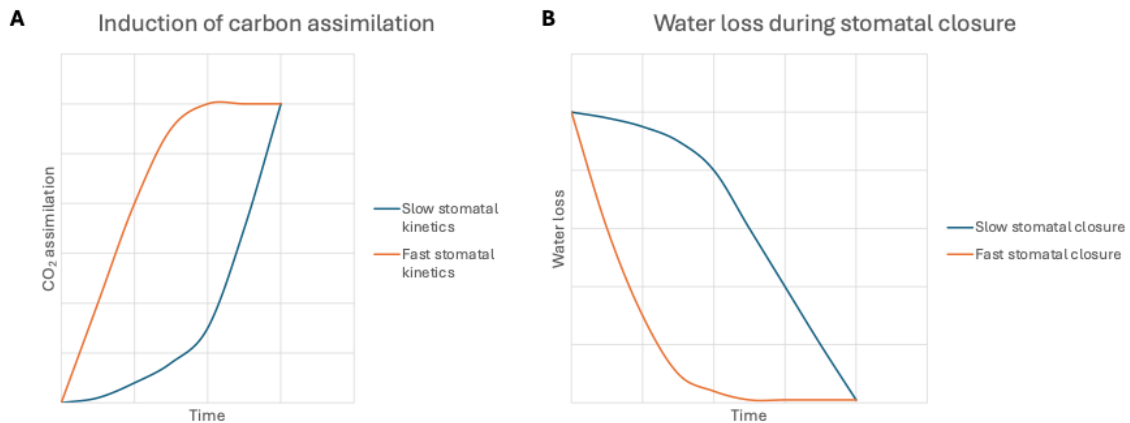


Figure 4: A schematic showing the rate of (A) induction of CO₂ assimilation upon illumination and (B) a schematic showing the rate of water loss through open stomata during stomatal closure. These schematics are illustrative only and values are not representative of real data.

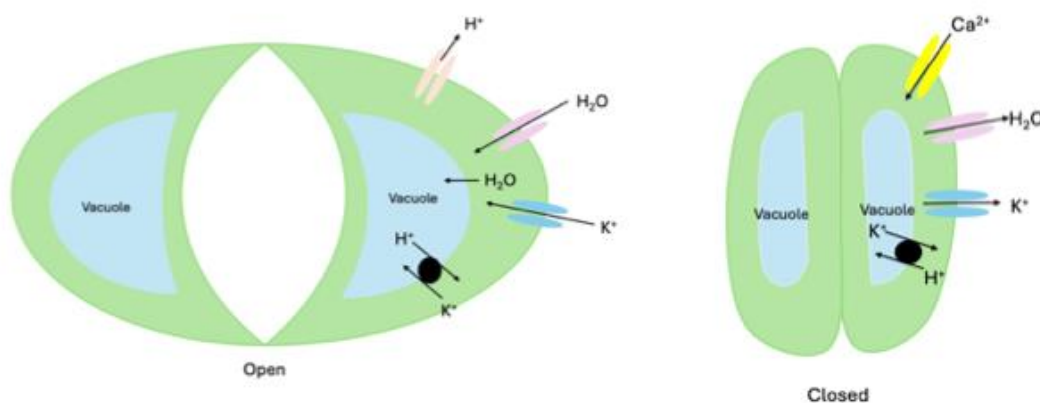


Figure 5: A diagram of ion movement in and out of guard cells and the guard cell vacuole during the opening and closing of stomata.

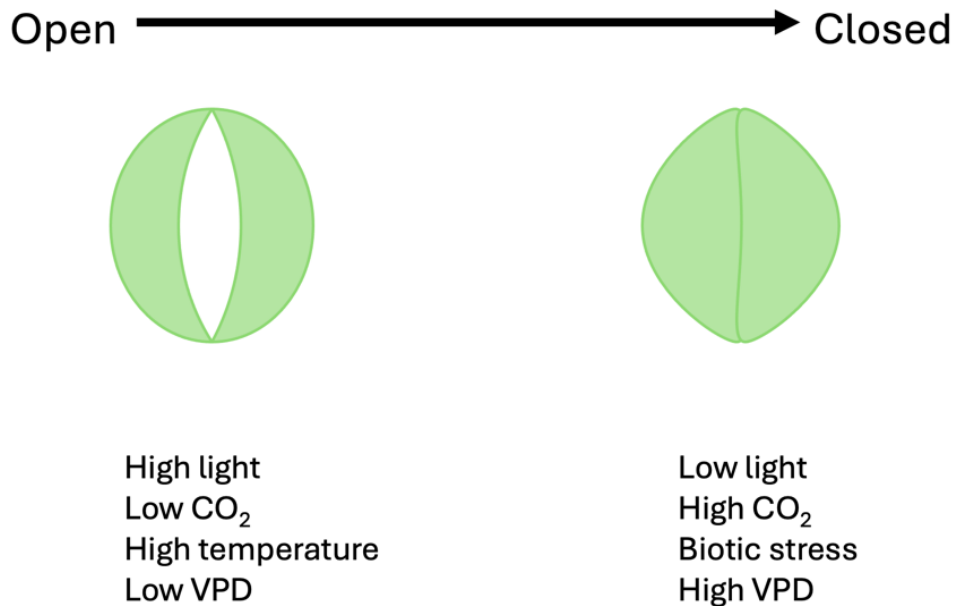


Figure 6: A diagram showing the conditions under which stomata are induced to open and close.

1.5 Stomata at night

Nocturnal stomatal conductance (g_n) has been found in a range of both C3 and C4 species, although the role of g_n is not fully understood. Some of these species include important crops such as, wheat (*Triticum aestivum*), sunflower (*Helianthus annuus*), and cotton (*Gossypium hirsutum* L.) (Snyder et al., 2003, McAusland et al., 2023, Caird et al., 2007). As well as, the model plant species *Arabidopsis thaliana* (Christman et al., 2009). Nighttime stomatal conductance can be a large contributor to water loss. In wheat, g_n results in 5 to 18% of water loss occurring (McAusland et al., 2021).

Some drivers of g_s are similar between daytime and nighttime. In the day, high VPD has a negative effect on g_s , this is typically characterised by a negative log relationship between VPD and g_s (Oren et al., 1999). However, at night the relationship between g_s and VPD appears to be species dependent. A negative effect of VPD on g_n has been found in *Populus angustifoli*, but this effect was not seen in *P. balsamifera*, despite these species belonging to the same genus (Howard and Donovan, 2009). Some species display positive effects of VPD on g_n , such as species within the genus *Acer* (Dawson et al., 2007). While in many tree species VPD has been found to have no effect on g_n (Barbour et al., 2006). Although, overall a negative effect of VPD on g_n has been observed, this is similar to daytime responses. This was found through a metaanalysis looking at the response of stomata to VPD across all studies and species available (Chowdhury et al., 2022).

During the day, guard cells respond to elevated CO₂ conditions by closing stomatal pores (Koolmeister et al., 2024). Studies report differing responses of g_n to high atmospheric

CO₂ concentrations. In *Eucalyptus sideroxylon* increases in CO₂ concentration have a positive effect on g_n (Zeppel et al., 2012). Whilst studies in *Arabidopsis thaliana* found a negative effect of increased CO₂ concentration on g_n , similar to the response of stomata in the daytime (Costa et al., 2015). The differences between these studies are thought to be due to species specific differences.

g_n has been found to increase throughout the night. A metanalysis of 81 studies in 63 different species found g_n increased between 8 PM and 3 AM (Chowdhury et al., 2022). This trend was consistent across environments and was found in a range of different tree and crop species. Although the authors of this metanalysis partially attribute the change in g_n to a decrease in VPD, commonly observed at night.

1.6 Stomatal kinetics

Slow stomatal opening and closing is proposed to lead to less efficient CO₂ uptake and unnecessary water loss (Figure 4). Under conditions favourable to stomatal opening and photosynthesis, slow stomatal opening increases the time taken for the plants commence photosynthesis. In dynamic light environments the ability to rapidly initiate photosynthesis has a large impact on the daily amount of photosynthesis the plant is able to carry out. Additionally, when conditions become unfavourable for photosynthesis or under conditions that increase ET, slow stomatal closing increases the amount of time stomata are open and therefore increases water loss (Figure 4). This makes plants less water efficient.

Stomatal kinetics can differ between plant families. Faster stomatal kinetics have been observed in the *Poaceae* family as these plants have a unique stomatal morphology. Most species have two kidney shaped guard cells, however *Poaceae* have dumbbell shaped guard cells, that are flanked by subsidiary cells (Figure 7) (Nunes et al., 2020). Dumbbell shaped stomata only require a small change to cell volume to induce stomatal opening. Additionally, the subsidiary cells provide ions to the guard cells, this increases the rate of osmotic flux and allows for faster stomatal movement (Charrier, 2022). Stomatal aperture is generally considered to have a greater effect on g_s than stomata morphology, except for when the differences in stomatal morphology are great (Bertolino et al., 2019).

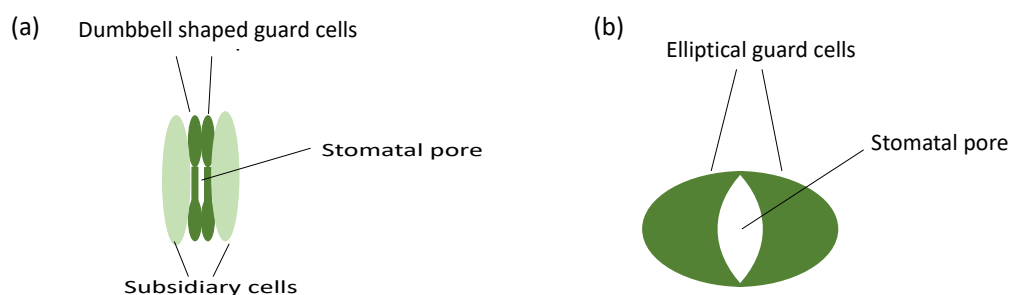


Figure 7: (a) A diagram of stomata in the *Poaceae* family, showing the dumbbell shape and the flanking subsidiary cells. (b) A diagram showing the elliptical guard cells found in plants outside the *Poaceae* family. These elliptical shaped stomata lack subsidiary cells.

In addition to stomatal morphology, stomatal kinetics has also been linked to stomatal density. Stomatal density refers to the number of stomata found on the surface of leaves; it is thought to be negatively correlated with stomatal size (Hetherington and Woodward, 2003). This inverse relationship may be due to geometrical constraints, as it is structurally possible to have higher densities of smaller stomata, but not to have high densities of large stomata (Haworth et al., 2023). However, this inverse relationship could also have a functional purpose as stomatal density is altered by atmospheric CO₂ concentration. Leaves developed under high CO₂ concentrations have a higher stomatal density and smaller stomatal pores (de Boer et al., 2011). Analysis of herbarium and fossilized plant remains found that stomatal density and stomatal size have changed throughout history, in response to changes in atmospheric CO₂ concentration (Franks and Beerling, 2009).

A high stomatal density and small stomatal size has been associated with faster stomatal kinetics during opening and greater g_s (Drake et al., 2013). Guard cell turgor pressure, which regulates stomatal open and closing, is controlled by ion flux and the subsequent osmotic adjustments. The speed of stomatal kinetics is determined by the capacity and speed of solute transport. Which in turn, is controlled by the density and activity of guard cell membrane transporters. Smaller stomata, have smaller guard cells, with a larger surface area to volume ratio, this increases the number of ion transporters in a given volume (Franks and Farquhar, 2007). As these ion transporters are responsible for ion flux, an increased number of ion transporters has been suggested as an underlying cause of smaller stomata having faster kinetics. Similarly to the subsidiary cells in the *Poaceae* family, this enables a quick increase of solute concentration in guard cells (Hetherington and Woodward, 2003). This increased rate of stomatal movements in plants with a higher stomatal density is a thought to help maximise WUE in dynamic light environments (Bertolino et al., 2019).

1.7 Stomatal development

The stomatal development pathway (Figure 8) starts with an asymmetric division that produces a meristemoid mother cell (MMC) (Geisler et al., 2000). The MMC will divide asymmetrically several times to produce a meristemoid cell and a larger sister cell, called a stomatal lineage guard cell (SLGC). The SLGC can terminally differentiate into a pavement cell. Alternatively, SLGCs can undergo an asymmetric spacing division (Bergmann and Sack, 2007, Nadeau and Sack, 2002). This asymmetric division produces a satellite meristemoid cell that is orientated away from the existing stomatal precursor (Geisler et al., 2000). The orientation of these divisions ensures that stomata develop at least one cell apart, this is referred to as the one-cell spacing rule (Hara et al., 2007).

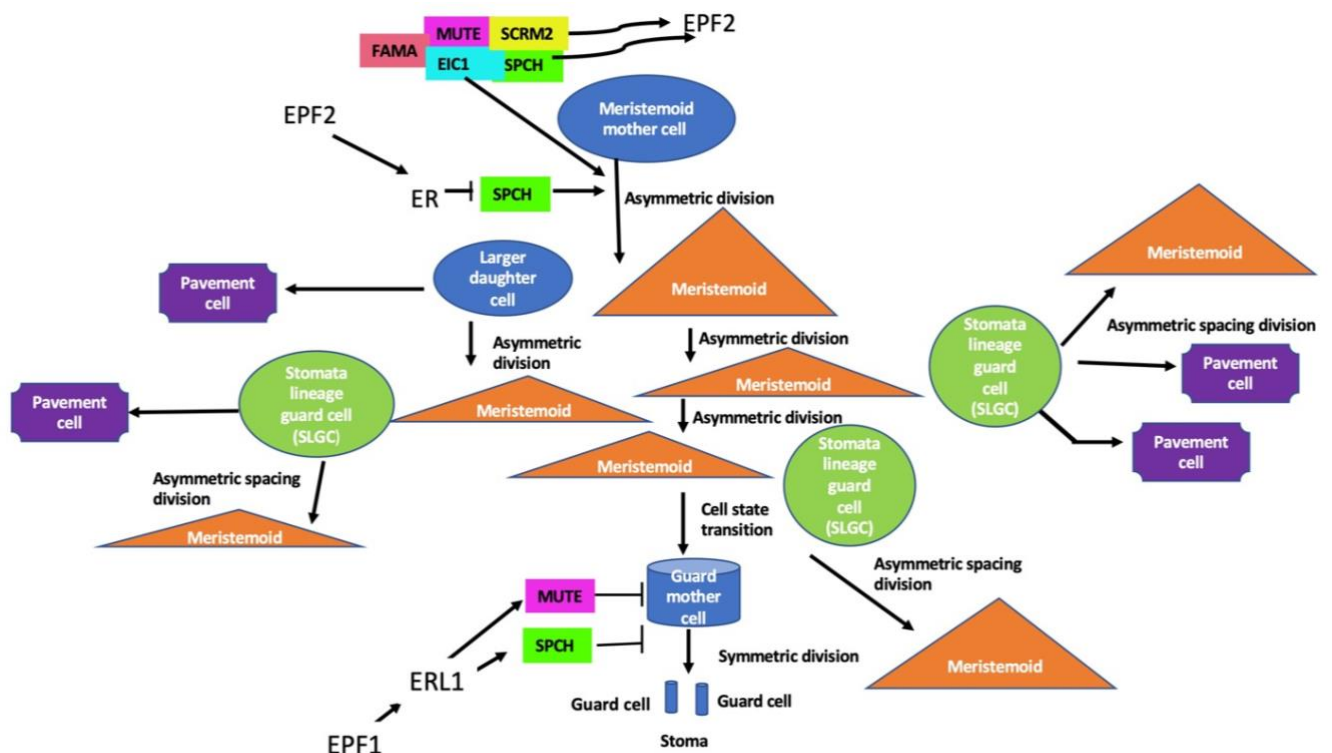


Figure 8: A diagram of the stomatal development pathway, showing cell types and regulators involved in the pathway.

The meristemoid can undergo amplifying divisions, these are asymmetric divisions that produce a SLGC and regenerate the meristemoid. These amplifying divisions increase the total number of epidermal cells (Hara et al., 2009). After a number of amplifying divisions, the meristemoid loses its ability to undergo additional amplifying divisions. The meristemoid then undergoes a cell state transition to produce the guard mother cell (GMC). The GMC divides once, symmetrically, to produce two daughter cells. These daughter cells undergo a cell state transition to form two guard cells (Ohashi-Ito and Bergmann, 2006).

The asymmetric division of the MMC is triggered by the heterodimerization of the transcription factors *SPEECHLESS* (*SPCH*), *MUTE*, *FAMA*, *INDUCER OF CBF EXPRESSION 1* (*ICE1*), and *SCREAM2* (*SCRM2*). *SPCH* and *SCRM2* activate *EPIDERMAL PATTERNING FACTOR 2* (*EPF2*) signalling. *EPF2* is a putative peptide ligand in the *EPF/EPF-LIKE* (*EPFL*) gene family. This gene family encodes cysteine rich peptides. *EPF2* is recognised by the receptor kinase *ERECTA* (*ER*), this triggers a downstream mitogen-activated protein (MAP) kinase pathway. *SPCH* is phosphorylated by this MAP kinase pathway, resulting in its inhibition. This negatively impacts the heterodimerization of the five required transcription factors, thus inhibiting asymmetric division of the MMC. So, *EPF2* plays a crucial role in the enforcement of the one-cell spacing rule.

epf2 loss of function mutants struggle to enforce the one-cell spacing rule, thus these mutant plants have higher numbers of stomata (Figure 9). *epf2* loss of function mutants also show clustering of stomata, this occurs when two or more stomata make direct contact. Stomatal clustering mutants show increased interaction between stomatal

pores (Brown and Escombe, 1901). This increases diffusive resistance by up to 15%, resulting in decreased CO₂ assimilation rates (Lehmann and Or, 2015). Additionally, *epf2* mutants fail to open under exposure to opening stimuli and show attenuated responses to closing stimuli (Papanatsiou et al., 2016). Small arrested stomatal lineage cells have been observed in *epf2* mutants (Doheny-Adams et al., 2012).

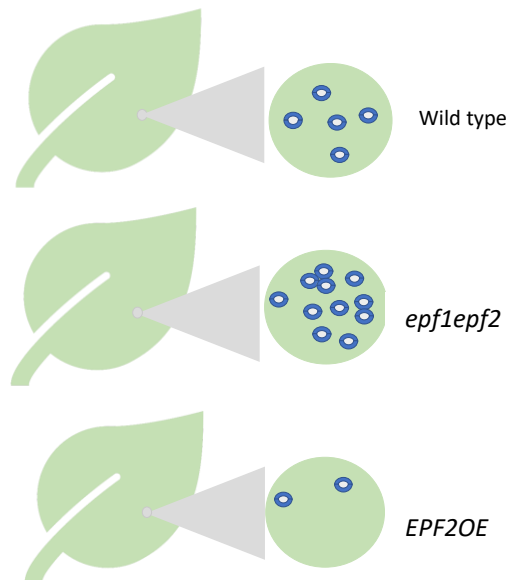


Figure 9: A diagram showing the stomatal density and clustering found in wild type, *epf1epf2* plants and EPF2OE plants.

Overexpression of *EPF2* reduces the number of stomatal lineage divisions, these plants have a lower stomatal density and a lower stomatal index than wild type plants (Hunt and Gray, 2009). The stomatal index is a ratio of stomatal pores to epidermal cells in the plant epidermis. Plants with a low stomatal index have more pavement cells relative to stomatal pores than plants with a higher stomatal index (Woodward, 1987).

EPF1 is also an epidermal patterning factor in the *EPF/EPFL* gene family. Similar to *EPF2*, *EPF1* also plays a crucial role in enforcing the one-cell spacing rule. *EPF1* acts as a cell-to-cell signal to prevent the development of stomata in neighbouring cells (Hara et al., 2007). *EPF1* is expressed in late-stage stomatal precursors, such as GMCs and late meristemoids. *EPF1* may act by activating *ERL1* to repress guard cell differentiation. *ERL1* activates inhibitors of *MUTE* and *SPCH*, antagonising the development of new guard cells (Herrmann and Torii, 2021).

epf1 loss of function mutants also show an increase in stomatal density alongside stomatal clustering, similarly to *epf2* loss of function mutants. *epf1epf2* loss of function double mutant plants have been created, these show an additive phenotype, with increased stomatal densities and clustering (Doheny-Adams et al., 2012). The increased stomatal densities observed are accompanied by a decrease in stomatal size.

1.8 EPF mutant studies

1.8.1 Stomatal conductance

Stomatal conductance is a major determinant of photosynthetic rates and yields (Faralli et al., 2019). Increased stomatal density has been linked to increased g_s . g_s measurements are done over a set area and are typically given per unit squared. If stomatal density in the measured area is high, then there are more stomata in this area to release water and take in CO_2 . Thus, plants with a higher stomatal density typically display higher g_s values than plants with lower stomatal density. *epf1epf2* loss of function mutants, which have an increased stomatal density, show increased operating stomatal conductance to water vapour ($g_{w(op)}$). While *EPF2* overexpressors, which show a decreased stomatal density, show a decreased $g_{w(op)}$ (Franks et al., 2015).

In addition to anatomical features, such as stomatal size and density, there are also biochemical and structural determinants of g_s . Cell structural movements can change the shape and volume of guard cells. As turgor pressure controls stomatal opening, these changes in cell shape alter stomatal kinetics and g_s . Actin related protein 2 (*arp2*) and *arp3* mutants show slower stomatal opening than wild type controls (Jiang et al., 2012). Showing the crucial role for the guard cell cytoskeleton in stomatal kinetics.

At higher temperatures VPD decreases and there is a corresponding increase in g_s (Urban et al., 2017). Mechanisms for regulating g_s by temperature are at least partly independent from mechanisms linked to water status and carbon metabolism (Urban et al., 2017). Heat induced increases in g_s can lead to drought induced mortality in water stressed plants (Marchin et al., 2022). This creates a precarious situation for the future, as temperature rises, expected due to climate change, could exacerbate the impact of expected water shortages on plants through increasing g_s .

A study by Xiong et al. (2022b) looked at the impact of stomatal density on photosynthesis in fluctuating light, it used natural variation amongst rice (*Oryza*). It found that plants with a high density of small stomata display a greater g_s . *Arabidopsis thaliana* *EPF1* loss of function plants have also been studied under fluctuating light, these *EPF1* plants had a lower stomatal density and showed a faster stomatal conductance and a greater cumulative CO_2 assimilation compared to wild type (Sakoda et al., 2020).

1.8.2 Water use efficiency

EPF2OE plants show an increased WUE and a similar potential rate of photosynthesis when compared to wild type controls (Franks et al., 2015, Ferguson et al., 2024). However in C3 *EPF2OE* plants, a lower operating CO_2 assimilation rate was observed (Franks et al., 2015). In C4 crops, *EPF2OE* plants show increased g_s , WUE, and drought tolerance, similar to C3 crops. However, in contrast to C3 *EPF2OE* plants, C4 plants do not show the same reduction in CO_2 assimilation rate (Ferguson et al., 2024). This increase in WUE in *EPF2OE* plants is due to reduced g_s . When plants have lower g_s , they show lower rates of transpiration (Schlüter et al., 2003). If photosynthesis remains similar in these *EPF2OE* plants compared to wild type plants, then the *EPF2OE* plants can fix more CO_2 per unit of water lost due to transpiration. This gives *EPF2OE* plants a higher instantaneous WUE (WUE_{inst}), as WUE_{inst} is defined as the net photosynthetic rate divided by the net transpiration rate (Hatfield and Dold, 2019).

EPF1 overexpression plants in rice (*Oryza*) and wheat (*Triticum*) also show a reduced stomatal density, improved WUE and improved drought tolerance (Caine et al., 2019, Dunn et al., 2019). *epf1epf2* loss of function mutants display a lower WUE than wild type, whilst operating CO_2 assimilation rate was unchanged.

As *EPF2OE* plants show an increased WUE, suggestions have been made that decreasing stomatal density could be used as a tool to improve drought efficiency in crops (Franks et al., 2015). However, there are concerns that single gene modifications, such as those made to the *EPF2* gene, could have negative pleiotropic effects on other metabolic and developmental processes (Flexas et al., 2021, Ferguson et al., 2024). The reduced CO₂ assimilation rates in C3 *EPF2OE* plants should also raise concerns over using *EPF2OE* mutation as a drought resistance strategy. Additionally, WUE in *EPF2OE* plants has not been looked at under fluctuating light conditions. It is known that dynamic light reduces WUE, and that this is exacerbated by slow stomatal closing (Eyland et al., 2021). As, stomatal conductivity has been found to be lower in *EPF2OE* plants than wild type, fluctuating light is likely to have a greater impact on *EPF2OE* plants than wild type plants (Franks et al., 2015). It is important to assess the impact of fluctuating light on WUE in *EPF2OE* plants if reducing stomatal density through *EPF2OE* mutation is being looked at as a method of improving crop drought tolerance.

1.8.3 Root architecture

A study of *EPF2OE* and *epf2-1* plants found no differences in root system architecture in *Arabidopsis thaliana* (Mawodza et al., 2022), suggesting that there is not a link between stomatal density and root system architecture. However another study, also carried out in *Arabidopsis thaliana*, found that plants with a higher stomatal density and greater g_s had a larger rooting area (Hepworth et al., 2016). The Hepworth et al. (2016) study suggested that the larger root area allowed plants with a greater stomatal density to offset the increased transpiration that it found in *epf1epf2* mutant plants, through increasing water uptake by the plant. This could help to improve WUE in high stomatal density plants. The differences found in root system architecture between these two studies are thought to be due to differences in the age of the plants when studied and the type of soil used.

In rice (*Oryza*), *EPF1* overexpression has been found to increase root cortical aerenchyma formation (Mohammed et al., 2019). Aerenchyma is tissue that contains air spaces, it develops in the plant root due to stressful conditions, such as, hypoxia, drought or nutrient deficiency (He et al., 1996, Yang et al., 2012, Abiko and Obara, 2014). Under low O₂ conditions the increased air spaces aid in O₂ diffusion from the shoot to the root. However, *EPF1* overexpression did not impact root growth, root elongation or root length when compared to wild type. This supports the Mawodza et al. (2022) study suggesting that *EPF* mutation does not impact root system architecture, although it does impact root tissue composition.

1.9 Dynamic light environments

Most plants, including crops, exist as part of a canopy. Canopy architecture can generate a patchy light environment with patches of sun, partial shade, and full shade. The level of light dynamicity in a canopy varies with canopy architecture. More erectophile (upright) canopies have a higher light canopy transmission, compared to more planophile (horizontal) canopies (Durand and Robson, 2023). In canopies, plants experience high levels of shading from neighbouring plants. Wind can cause rapid leaf movements that can quickly shade or quickly remove shade from plants. This creates a dynamic light environment for the plants in the canopy.

Rapid leaf movements produced as a result of wind can create short periods of high light. This is caused by the removal of shade on a plant and are known as windflecks

(Burgess et al., 2021). Windflecks typically act on the second or sub-second scale. Longer periods of high light, called sunflecks, occur when the sun passes a gap in the canopy. These last minutes to hours (Evans, 1956). A brief period of reduced light intensity experienced by a leaf is called a shade-fleck (Kaiser et al., 2018). For many parts of the canopy shade-flecks and sun/windflecks are both brief deviations from the baseline level of irradiance, not the two binary states under which plants exist. However, at the top of a canopy these are the two binary states in which a plant can exist (Retkute et al., 2018), plants at the top of the canopy do not have a baseline level of irradiance. This means that sun/windflecks will have a differing impact on photosynthesis based on position of the leaf in the canopy. Lower in the canopy the baseline irradiance will be lower, so during a sun/windfleck it would be expected that the change in magnitude of light intensity from the baseline would be greater.

Sunflecks make an important contribution to plant photosynthesis, with 10-90% of daily photosynthesis occurring during sunflecks (Durand et al., 2021, Leakey et al., 2004). Sunflecks are longer and more intense in crop canopies than tree canopies. Thus, crop canopies experience a higher photosynthetically active radiation (PAR) increase per sunfleck than tree canopies (Durand and Robson, 2023). As many crops exist in these dynamic environments, their ability to maintain a high photosynthetic rate under such conditions is important for crop yields.

High light stress is one challenge that plants face in dynamic light environments. When plants experience a sunfleck, they experience a strong increase in PAR (Caine et al., 2019), and they are often unable to use the additional light. This induces the high light stress response in plants (Alter et al., 2012). Slow NPQ kinetics reduces a plant's photosynthetic efficiency in dynamic light environments, as it takes these plants longer to relax NPQ once the sunfleck has passed (Figure 3). The light stress induced by fluctuating light can cause photodamage, this is a permanent reduction in photosynthetic efficiency. Chloroplast movement has a key role in protecting plants in fluctuating light environments. Chloroplasts accumulate in areas with low light intensity to more efficiently capture available light (Wada and Kong, 2011). Then in periods of high light chloroplasts move perpendicular to the cell wall to avoid absorbing excess light (Cazzaniga et al., 2013).

The rate of CO₂ assimilation in dynamic light environments can be reduced by slow stomatal kinetics (Eyland et al., 2021). When light intensity, evaporative demand and demand for CO₂ are low, plants tend to decrease their stomatal aperture (Kaiser et al., 2018). A plant's ability to photosynthesise is reduced in low light intensities, so the benefit of having open stomata is reduced. When stomata are open, ET will still occur under shaded conditions. So, it is beneficial for plants to reduce their stomatal aperture in low light intensities to avoid unnecessary water loss. When a shade-fleck has passed and the PAR incident on the leaf is greater, plants with slow stomata kinetics will take a while to increase their stomatal aperture. It will take longer for plants with slow stomatal kinetics to take in CO₂ for fixation in photosynthesis (Figure 4). So, photosynthesis is not able to start instantaneously when a shade-fleck passes. The slower the rate of stomatal opening, the longer the delay in starting photosynthesis.

1.9.1 Stomatal kinetics and dynamic light

Compared to plants grown in constant light, plants grown under fluctuating light conditions display reduced carbon gain and lower productivity (Slattery et al., 2017). Slattery et al.

(2017) suggest that improving stomatal closure kinetics in response to fluctuating light could improve canopy WUE and yield.

Higher stomatal densities have been linked to increased photosynthetic performance under fluctuating light intensities. In highly dynamic light environments, there is typically a delay in photosynthetic response, this can take tens of minutes and this period is referred to as photosynthetic induction (Taylor and Long, 2017, Adachi et al., 2019). Photosynthetic induction in dynamic light environments is mainly limited by stomatal kinetics, with some additional limitation due to the electron transport rate and the activation of CBB cycle enzymes (Percy et al., 1990).

Smaller stomata, which are correlated with higher stomatal densities, display faster stomatal kinetics (Franks and Farquhar, 2007). Under fluctuating light conditions a higher density of smaller stomata display a faster stomatal response when compared to plants with lower stomatal densities (Lawson and Blatt, 2014). Stomatal size has only been found to correlate with stomatal opening rate not with stomatal closing rate (Elliott-Kingston et al., 2016). So, changes to stomatal size and density can only represent half of the picture when it comes to speeding up stomatal kinetics.

Additionally, there is some uncertainty over the role that small stomata play in photosynthetic induction. Plants with lower stomatal density and a larger stomatal size may promote g_s under low light (Doheny-Adams et al., 2012). Thus, decreasing stomatal delay during the initial phase, after light intensity increases. This uncertainty necessitates further study into the role that stomatal morphology plays in speeding up stomatal kinetics for dynamic light environments.

Dynamic light environments are known to decrease plant WUE; this is due to slow stomatal closure after a sunfleck has passed. Under these conditions the plant will not be photosynthesising but stomata will still be closing and water will be lost through these partially open stomata (Figure 4) (Eyland et al., 2021). This reduces the so called “crop per drop” and results in a lower plant WUE.

A previous study in rice (*Oryza*) has found that plants with a high stomatal density display faster stomatal responses under fluctuating light (Xiong et al., 2022b). The faster stomatal responses led to faster photosynthetic induction rates and higher biomass accumulation. However, the plants with a greater stomata density display lower intrinsic WUE. The Xiong et al. (2022b) study looked at natural variation in rice plants, not at *EPF* mutant plants, the impact of fluctuating light on photosynthetic induction in these plants is unknown.

1.10 Gaps in current knowledge

Previous studies looking at stomatal density in the *EPF* mutant plants and have consistently found that the *EPF2OE* genotype has a lower abaxial stomatal density than the Col-0 and *epf1epf2* genotype. An increased abaxial stomatal density relative to wild type is how *epf1epf2* plants are identified. However, these studies do not look at the adaxial stomatal density in these genotypes and the impact of *EPF* genes on adaxial stomatal development is unknown. The stomatal development pathways for abaxial and adaxial stomatal development are different and it is not known if the *EPF* genes act in the adaxial stomatal development pathway. As, adaxial and abaxial guard cells use different potassium ion channels in stomatal closing, it is possible that stomatal conductivity differs between the two types of stomata (Wei et al., 2020). Despite this, studies measuring stomatal

conductivity in *EPF* plants look only at abaxial stomatal conductivity and little is known about the impact of *EPF* gene mutation on adaxial stomatal conductivity.

Previous studies on *EPF* plants have not looked at this group under fluctuating light. Whilst studies on the impact of fluctuating light on stomatal density have not used *EPF* mutant plants, so the impact of *EPF* gene mutations in plants grown under fluctuating light is unknown. *EPF* overexpression is being considered as a mechanism to increase crop drought tolerance, so it is important to understand how fluctuating light impacts these mutant plants, as light in crop canopies is dynamic.

While stomatal conductivity has been studied in *EPF* mutant plants, it is not known how increased stomatal conductivity in the *epf1epf2* mutants impacts photosynthesis in fluctuating light. A study by Xiong et al. (2022b) found that plants with a high density of small stomata display faster photosynthetic induction rates under fluctuating light. However, Xiong et al. (2022b) study looks at natural variation not *EPF* mutants, it is therefore important to address whether increased stomatal density through the *epf1epf2* loss of function gene mutation also shows an increase in photosynthetic induction under fluctuating light. A study by Sakoda et al. (2020) found that stomata conductance and photosynthetic induction were higher in *Arabidopsis thaliana epf1* plants compared to wild type under fluctuating light. The Sakoda et al. (2020) study did not look at *epf1epf2* mutants. The impact of the additive *epf1epf2* loss of function phenotype on CO₂ assimilation in fluctuating light is unknown. As there has been previous study on the *epf1* loss of function mutants studying the *epf1epf2* plants under fluctuating light would allow for comparison of the impact of mutating both the *epf1* and *epf2* genes compared to just the *epf1* gene.

Most studies on WUE in *EPF* plants grow the plants under constant light, but fluctuating light is known to impact WUE. What is not known is if the reduction in WUE, normally observed under fluctuating light conditions, can be offset in *epf1epf2* plants due to their higher abaxial stomatal density increasing photosynthetic induction. If WUE can be offset, then increasing stomatal density could be a method to increase WUE. This would contrast current thinking in the field which supports decreasing stomatal density to increase WUE. *EPF2* over expression is being looked at as a mechanism to improve crop drought tolerance, as fields are dynamic light environments, it is important to understand how fluctuating light will impact WUE in *EPF2OE* plants.

Previous studies have come to different conclusions on the impact of *EPF* genes on root architecture and this is not a settled question, necessitating more study. Understanding the root architecture in *EPF* plants is needed to better understand the role of water uptake in drought tolerance in *EPF* plants.

1.11 Aims and hypotheses

In this study *EPF2OE* and *epf1epf2* plants, which have mutant *EPF* genes, along with wild type Col-0 *Arabidopsis thaliana* were evaluated for their photosynthetic performance under three different light treatments. This study aimed to (i) explore the impact of stomata density on CO₂ assimilation in dynamic light environments and (ii) explore the impact of stomatal density on intrinsic WUE.

The hypotheses I'm testing in this study are (i) if plants have a higher stomatal density, then the plants will have greater stomatal conductance (g_s), (ii) if plants have a higher stomatal density, then the plants will display faster photosynthetic induction, and (iii) if plants have a higher stomatal density, then the plants will have lower WUE.

2. Methods

A timeline of measurements taken in each repeat of the experiment can be found in the appendix.

2.1 Plant material and plant growth

To assess the impact of stomatal density on a plant's ability to cope with fluctuating light both low- and high-density stomatal mutant plants were studied. The plants used for this study had genetic alterations to their *EPF* genes; these are common mutants that have been studied before. However, the *EPF* mutant plants used in this study have not been used in fluctuating light studies previously. As such, the results of this study present novel information regarding the impact of fluctuating light on this commonly studied set of mutant plants.

Arabidopsis thaliana epf1epf2 and *EPF2OE* seeds were obtained from the Grey lab at The University of Sheffield and have been described by Hunt and Gray (2009) and by Hara et al. (2009). *EPF2OE* mutants were produced by Hunt and Gray (2009) by transforming a p35S:EPF2-TAP construct into agrobacterium. *Arabidopsis thaliana* plants were then transformed by floral dipping. The *epf1epf2* double loss of function mutants were produced by Hara et al. (2009) by crossing homozygous *epf1* mutants with homozygous *epf2* mutants and the offspring were selfed. The *epf1* and *epf2* mutants used by Hara et al. (2009) were produced through T-DNA insertion.

The *EPF2* over expressor was chosen over the *EPF1* overexpressor as the *EPF2* gene acts higher up in the stomatal development pathway than the *EPF1* gene. Overexpressing the *EPF1* gene results in leaves with stomatal precursors. These precursors form when cells start the stomatal development pathway but cannot continue the pathway due to repression from *EPF1*.

The mutant plants and their background ecotype Col-0 were stratified by suspending them in 1 mL of H₂O in 1.5 mL Eppendorf tubes for 48 hours at 5°C. Plants were grown in a growth room (The University of Nottingham) at 20°C/ 18°C in the daytime and nighttime respectively, in ambient CO₂ (450 ppm) under well-watered conditions. Plants were grown with a 16 h day length (06:00 to 22:00). Half the plants (45 individuals) in the second and third repeats of this experiment were grown under shifted daytime conditions still with a 16h day length (18:00 to 10:00), this allowed for nighttime measurements to be taken on these plants during the day.

Plants were germinated under the constant light treatment in nursery pots. After two weeks these plants were transferred into individual 9cm pots (Soil: BP Substrate 2, pH: 6.0 Fert g/l: 1.5) and moved into their respective light treatments. Five plants of each genotype were assigned to each light treatment, 90 plants were used in repeat one and 180 plants were used in repeat two and three, due to additional nocturnal light treatments being added. Within each light treatment, the placement of plants was randomly assigned, this ensured that any variation in the availability of light within a light treatment was randomly distributed. In the first repeat of this experiment, plants spent the first two weeks in a different growth room to the one used for the rest of this experiment. This growth room (The University of Nottingham) had a 16h day length (06:00 to 22:00), at 20°C/ 18°C in day and night respectively, in ambient CO₂ (450 ppm). Plants in this growth room were also grown under well-watered conditions. After two weeks of growth plants were moved from this growth room to the other growth room, where they were placed under their respective light treatments.

2.2 Light treatments

Plants were grown under one of three different light treatments; this allowed for the impact of growth under fluctuating light to be assessed. Plants were subjected to one of three different light treatments (figure 10) provide by a Heliospectra DYNA light (Heliospectra, Sweden) set to white light at 100. Using artificial light allowed for the timing of fluctuations and the intensity of the light available to be controlled. It also allowed for the plants to be grown in a growth room, where temperature can also be controlled. However, regularly spaced artificial light fluctuations are a less accurate representation of field conditions, compared to growth in a green house or under artificial light recreating a natural light fluctuation pattern that has been previously measured in a crop canopy.

All treatments provided a 30 minute ramp up phase and a 30 minute ramp down phase at the beginning and end of each day. This ramp up/down phase consisted of step wise increases/decreases in light intensity until the final light intensity was reached for the ramp up phase and until the light was turned off for the ramp down phase. For all daytime light treatments the ramp up phase started at 5:30 AM and the light intensity reached its maximal value at 6:00 AM. While the ramp down phase started at 21:30 PM and the light intensity reached zero at 22:00 PM. In the nighttime light treatments, the ramp up phase started at 17:30 PM and the light reached its maximal light intensity at 18:00 PM. While the ramp down phase started at 9:30 AM and the light intensity reached zero at 10:00 AM. Outside of the ramp up/down phase, the constant light treatment does not turn the light off or change the light intensity. The short fluctuating treatment turned the light on and off every 10 minutes. Whilst the long fluctuating treatment turned the light on and off every 30 minutes. It was not possible to study light fluctuations on a shorter scale than 10 minutes using the Heliospectra DYNA lights, as this equipment limits the number of times the light can be turned on or off.

Total light intensity across the day was the same for all light treatments. In order to do this, light intensity had to be lower in the constant light treatment as the light is on for a longer period of time over the course of the day. The total daily photon dose in all treatments was $124,620 \mu\text{mol m}^{-2}$, and the average photosynthetic photon flux density (PPFD) was $8.65 \times 10^{-3} \mu\text{mol cm}^{-2} \text{s}^{-1}$. The light intensity under the constant, short and long treatment was on average $134 \mu\text{mol m}^{-2} \text{s}^{-1}$, $275 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $275 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively when the light was switched on. In repeat two and repeat three of the experiment plants were grown under clear covers to reduce thrip damage. The clear covers also reduced the light intensity to an average of $27 \mu\text{mol m}^{-2} \text{s}^{-1}$, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$, and $40 \mu\text{mol m}^{-2} \text{s}^{-1}$, for the constant, short, and long light intensity respectively.

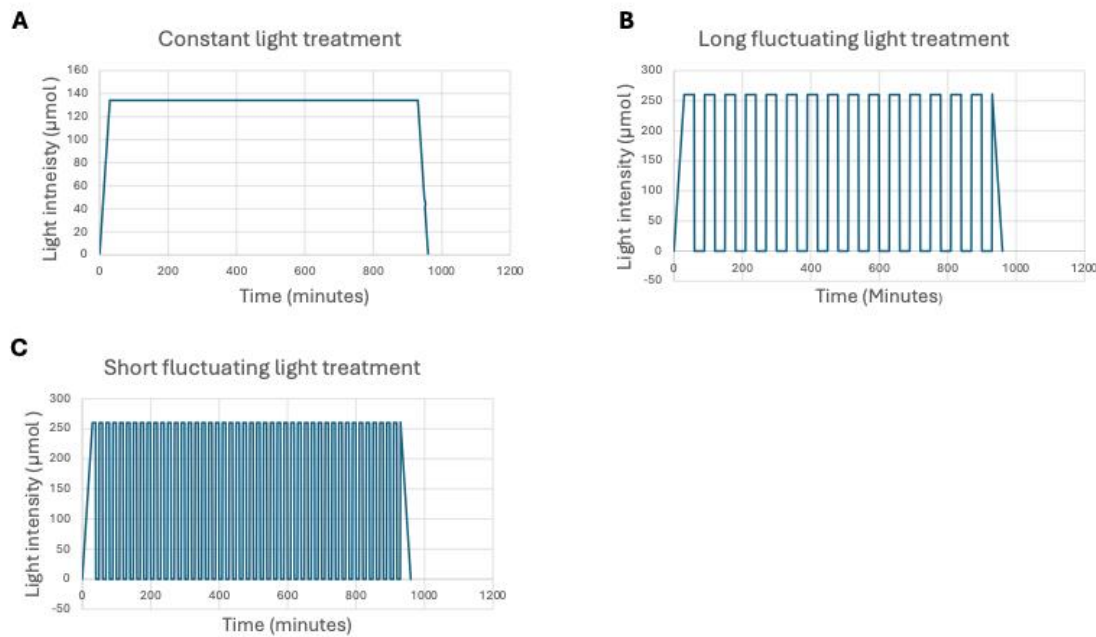


Figure 10: Line diagrams showing the total light intensity in each of the light treatments. The total integrated light received by plants in each light treatment was kept constant across the day.

2.3 Plant area

Plant area was measured to look at how light fluctuations and stomatal density impact plant growth. Increased photosynthetic performance is often reflected in an increased plant biomass and plant growth (Bengoa Luoni Sa and Driever Sm, 2025). Thus, measurements of plant growth can inform on the ability of plants to cope with light fluctuations. In repeat one of the experiment, after seedlings were transferred to individual pots, plants were photographed once every two weeks. Measuring the area changes only once every two weeks made it difficult to observe patterns in growth and changes in growth rate. Thus, the frequency of measurements was increased in repeat two and three of the experiment to twice a week. ImageJ was used to measure the area of the plant rosette in these photographs (Schneider et al., 2012). This was done by using the ruler in the photograph to set the scale, followed by changing the colour threshold to isolate the leaves from the background. The area of the isolated leaves was then measured using the measuring function to give the plant rosette area. Plant growth from the day of seedling transfer was then calculated by subtracting the measured area from the area of the plant on the day that it was transferred to the light treatment (two weeks).

2.4 Chlorophyll fluorescence

Chlorophyll fluorescence was measured at four weeks old (two weeks after transfer to the light treatments), using a FluorCam, model: FC800-222 (Photon systems instruments, Czech Republic). Plants were dark adapted for 40 minutes prior to measurements in the FluorCam. The protocol used provided a saturating pulse of light every minute for 50 minutes, this saturating light causes reaction centres to close preventing photochemistry from occurring. As photochemistry cannot occur, this saturating light is either dissipated through NPQ or is

used to fluoresce chlorophyll. Due to this, measurements of chlorophyll fluorescence can be used to calculate the NPQ during light adaptation (NPQ_{L_n}). An actinic light intensity of 2,000 $\mu\text{mol m}^{-2}\text{s}^{-1}$ was used in the FlourCam protocol, with periods of light, interspersed with periods of no light. This showed both NPQ induction and relaxation. NPQ is induced during light stress by the plant, looking at the levels of NPQ induced and at the rate at which induction and relaxation of NPQ occurs informs on the ability of the plant to withstand light stress. If fluctuating light has a priming effect on plants, then differences in NPQ between the light treatments would be expected.

Chlorophyll fluorescence was also measured using a Dual Pam, model: Dual-DB (Walz, Germany). Compared to the FlourCam, the Dual Pam has a faster switching time, enabling chlorophyll fluorescence to be measured under quickly fluctuating light. This allows for plants to be assessed for their ability to cope with rapid light fluctuations similar to wind flecks. Two different protocols, A and B, were used at three weeks and four weeks after transfer to light treatment respectively. In both protocols actinic light was provided by arrays of 635 nm light-emitting diodes at intensities of 272 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for the high light and 15 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for the low light. Saturating pulses of light at an intensity of 7044 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ were delivered every minute in both protocols, this saturating light was the measuring light that determined chlorophyll fluorescence. Protocol A had a five minute warm up phase, after this the protocol delivered pulses of actinic light switching between the low and high light intensity every minute. Protocol B had a five minute warm up phase, after this the protocol delivered pulses of actinic light switching between the low and high light intensity every second. Protocol B is simulating wind flecks where light intensity can rapidly fluctuate due to leaf movements. While protocol A is simulating longer periods of high light intensity that may be found in field conditions, these could be caused by gaps in cloud cover, movements in the position of the sun, or by gaps in the canopy cover.

2.5 Chlorophyll assay

Chlorophyll content was measured to check if differences seen in photosynthesis and NPQ between the plants were due to differences in chlorophyll content. Leaves were sampled at five weeks old and frozen with liquid nitrogen. The samples were weighed and 1ml of 80% acetone and silica beads were added to samples prior to grinding in a FastPrep-24™ (MP Biomedicals, USA). Samples were centrifuged, model: HERAEUS PICO17 Centrifuge (Thermo scientific, USA) for five minutes at 3,000 rpm. 2ml of 80% acetone and 1 ml of sample were added to a glass cuvette. The sample was measured in a spectrophotometer, model: Jenway 6305 Spectrophotometer (Jenway, UK) at absorbances A645nm and A663nm. Chlorophyll a and b content, as well as total chlorophyll content and chlorophyll a/b ratio were calculated using equation 2, 3, 4, and 5 respectively.

$$\text{Chlorophyll } a = (12.7 \times A663 - 2.69 \times A645) \times \frac{3}{100} \times \text{mass of leaf (g)} \quad (2)$$

$$\text{Chlorophyll } b = (22.9 \times A645 - 4.86 \times A663) \times \frac{3}{1000} \times \text{mass of leaf (g)} \quad (3)$$

$$\text{Chlorophyll } a + b \left(\frac{\text{mg}}{\text{g}} \right) = [8.02 \times A663 + 20.20 \times A645] \times 3/1000 \times \text{mass of leaf (g)} \quad (4)$$

$$\text{Chlorophyll } a/b \text{ ratio} = \frac{[12.7 \times A_{663} - 2.69 \times A_{645}] \times \frac{3}{1000} \times \text{mass of leaf (g)}}{[22.9 \times A_{645} - 4.86 \times A_{663}] \times \frac{3}{1000} \times \text{mass of leaf (g)}} \quad (5)$$

2.6 Gas exchange

Stomatal conductivity was measured on the abaxial and adaxial sides of leaves using a porometer, model: LI-600 (LI-COR, USA). The porometer was clamped onto one leaf per plant, with the side of the leaf that was being measured facing downwards. The porometer compares flow rate and water vapour that enters and leaves the chamber to calculate the leaf's stomatal conductance to water. Measurements were taken under ambient light, which was measured by the quantum sensor on the porometer. All daytime measurements were conducted between 9 AM and 12 PM and all nighttime measurements were conducted between 13 PM and 15 PM at five weeks after the plants were transfer to the light treatments.

CO₂ assimilation measurements were taken as these measurements inform on the light independent stage of photosynthesis; this stage was chosen as it is directly impacted by gas exchange and stomatal conductivity. So, if photosynthesis was limited due to low stomatal conductivity, the impact of this would be observed in the light independent stage through CO₂ assimilation measurements. Conversely if light availability was the limiting factor for photosynthesis, this would be observed in the light dependent stage, which can be measured by measuring oxygen production (Holloway-Phillips, 2018).

CO₂ assimilation and the rate of stomatal induction were measured using a gas exchange analyser, model: LI-6800 (LI-COR, USA). The gas exchange analyser chamber was clamped onto 1 leaf per plant. 2mm gaskets were used on this chamber due to the small size of the leaves, using a smaller gasket size allowed for more of the leaf chamber to be filled, which increases the reliability of the results. This chamber contains a detector that can detect the difference between the CO₂ and H₂O in the air stream before and after this air is passed through the sample chamber. These differences are used to calculate CO₂ assimilation, along with evaporation and stomatal conductance. The protocol measured plants at 15 µmol m⁻²s⁻¹ light intensity for 15 minutes, followed by 15 minutes at 150 µmol m⁻²s⁻¹. Plants were measured at 25°C, humidity 60%, and 400 ppm CO₂ at five and six weeks after transfer to light treatments. Photosynthetic induction was calculated using equation 5. Photosynthetic induction was only looked at in repeat two and repeat three of the experiment, this was because the leaves on plants in repeat one of the experiment were too small to be placed in the gas exchange analyser leaf chamber. The photosynthetic induction data from repeat two and three of the experiment were separated as there were large differences in the data collected between the repeats.

$$\text{Photosynthetic induction} = \frac{\text{Increase in CO}_2 \text{ assimilation}}{\text{Time taken to increase CO}_2 \text{ assimilation}} \quad (5)$$

2.7 Stomatal density

Stomatal density was measured when the plants were seven weeks old. Dental resin (Coltene Whaledent, Switzerland) was applied to the abaxial and adaxial sides of fully expanded leaves. Nail varnish peels were taken from the set resin, using clear tape which was placed onto a microscope slide. One stomatal impression was taken per plant. Microscope slides were analysed using a Leica 2 light microscope (Leica microsystems,

United Kingdom) and three images were taken per slide. The number of stomata in each image was manually counted. The number of stomata counted was divided by the area from which the count was taken in mm², to give the stomatal density per mm².

2.8 Carbon isotope analysis

Leaf samples were taken eight weeks after transfer to the light treatments. Samples were weighed and dried for 45 hours at 70°C. 2 mm silica beads were added to 1.5 mL tubes along with the plant samples. The tubes were placed in a FastPrep-24™ tissue homogenizer (MP Biomedicals, USA) for milling. 2mg of each sample was weighed into ultra clean pressed tin capsules. Carbon and nitrogen isotope analysis was carried out using an Elementar vario ISOTOPE cube elemental analyser (EA) coupled to an isoprime precisION isotope ratio mass spectrometer (IRMS) with an onboard centrION continuous flow interface system. Carbon isotope values were used to calculate Δ values using equation 6, where $\delta^{13}C_a$ is the variation in carbon isotope composition in the atmosphere and $\delta^{13}C_p$ is variation in the carbon isotope composition in the sample (Ma et al., 2022). A value of -8 was used for $\delta^{13}C_a$. Δ values are used to estimate intrinsic water use efficiency (WUEi), plants with lower Δ values have a greater WUEi.

WUEi was looked at in this study over WUEinst, which looks at the ratio between transpiration rate and the rate of carbon assimilation. WUEinst is calculated by dividing the operating CO₂ assimilation rate by the evaporation rate, both of these values can be measured using a gas exchange analyser. WUEinst looks at WUE under certain environmental conditions, e.g humidity. On the other hand, WUEi looks at the ratio between carbon assimilation and stomatal conductance, this looks at the plants' inherent capacity for carbon gain relative to water loss through stomata. WUEi was chosen for this study as it only looks at water loss through stomata not whole plant transpiration.

$$\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \delta^{13}C_p} \quad (6)$$

2.9 Root architecture analysis

Root architecture was analysed to see if differences in WUE between genotypes were due to differences in water uptake through roots as opposed to differences in evaporation through stomata. Seeds were sterilised using 50% bleach solution following the protocol outlined in Lindsey et al. (2017). 10 seeds per genotype were sown on square petri dishes (120x120mm) containing 0.5 Murashige and Skoog (MS) medium. The medium was prepared following the protocol outlined in Duncombe et al. (2020) with 10 µl of Triton added. This is a non-ionic surfactant, that helps seeds absorb water more effectively.

Plates were left at 5°C for 24 hours before being moved into the growth room. The growth room had 16-hour day length (06:00 to 22:00) or (18:00 to 10:00), at 20°C/ 18°C in ambient CO₂ (450 ppm). Plates were left to germinate for six days under the constant growth treatment, before being moved into their respective light treatments, where they were left for a further 13 days. Plates were then imaged using an inhouse built semi-automatic imaging setup and software as described in Bagley et al. (2020). Root system architecture was quantified using EZ-Root-VIS (Shahzad et al., 2018). Total root length, the number of lateral roots, and the total length of lateral roots were studied, and all values

were given as the combined total for the 10 *Arabidopsis thaliana* plants on each plate. This was done due to the difficulties in separating out the roots belonging to different plants.

2.10 Statistical analysis

All statistical tests were carried out using the R programming language available from the Comprehensive R Archive Network family of internet sites. A two-way analysis of variance (ANOVA) was calculated for comparisons between more than two groups, when the residuals were normally distributed. If residuals were not normally distributed the Kruskal-Wallis test was used instead as it is a non-parametric alternative. The normality of residuals was looked at using a Shapiro-Wilk test, where the residuals were considered normally distributed if $p > 0.05$. A list of the models analysed can be found in the appendix. A p value of 0.05 or less was considered significant for all statistical tests ran. Tukey's Honestly Significant Difference test was used for post-Hoc tests and the Student's t-Test was used for pairwise comparisons between light treatments and genotypes for data with normally distributed residuals. Where residuals were not normally distributed, a Wilcoxon test was used for pairwise comparisons, as it is a non-parametric test.

For analysis on rosette plant growth, the midway point (three weeks) was used for statistical analysis. This was because some plants died in later stages of the experiment and destructive measurements reduced the rosette area in other plants.

3.0 Results

3.1 Plant rosette growth was greatest under the constant light treatment

Plants grown under the constant light treatment grew the largest in repeats two and three of the experiment (figure 11). In repeat one, plants grown under the long light treatment grew both the largest and the smallest. Whilst, in repeat three plants grown under the long light treatment displayed the smallest growth. In repeat two, plants grown under the short light treatment grew the smallest.

Plant rosette area in the first week after seedling transfer shows a smaller range compared to plant rosette area at five weeks across all genotypes and light treatments. In repeat two of the experiment the rosette area rapidly increases around day 18, this isn't seen in repeat three of the experiment where plants show a more linear growth rate. The growth rate cannot be observed from repeat one as measurements were only taken once every other week, so there are not enough data points.

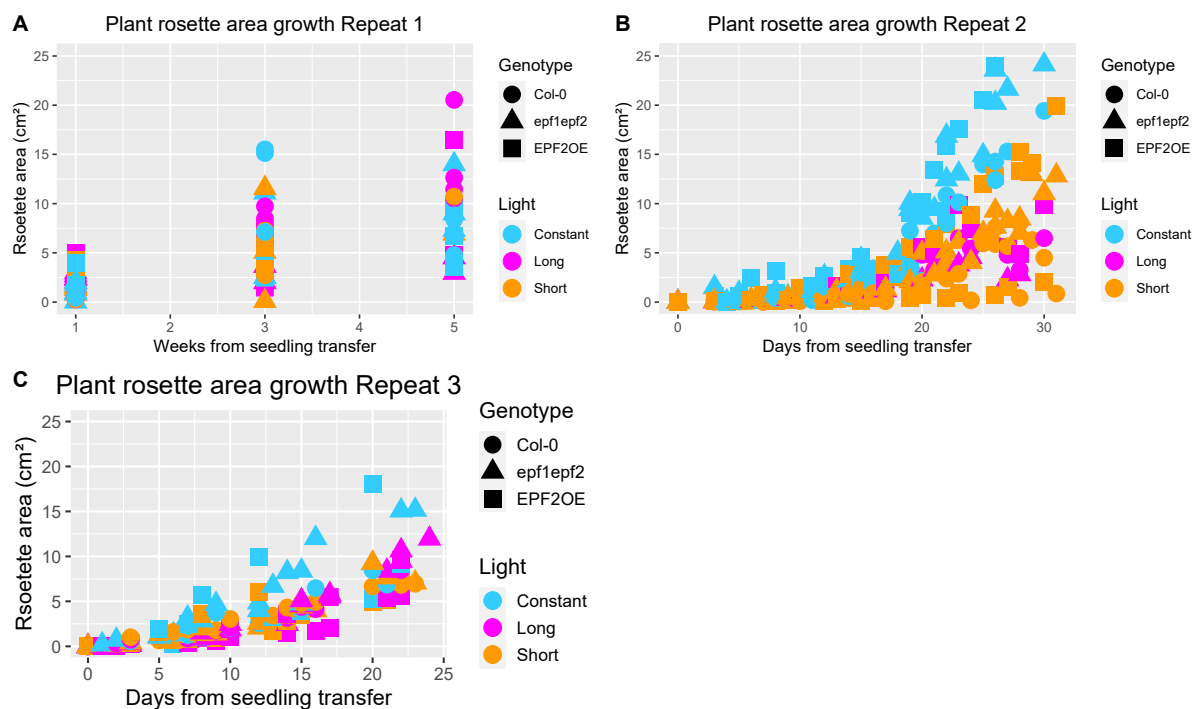


Figure 11: Scatter plots showing the growth in rosette area from the size on the day of seedling transfer against time measured in weeks for repeat one (A) and in days (B) (C) for repeat two and three. All values depicted are represented as the mean. Repeat one had five replicates per genotype per light treatment, with 45 plants total. Repeat two and three had 10 replicates used for each genotype in each light treatment, with 90 plants studied in total.

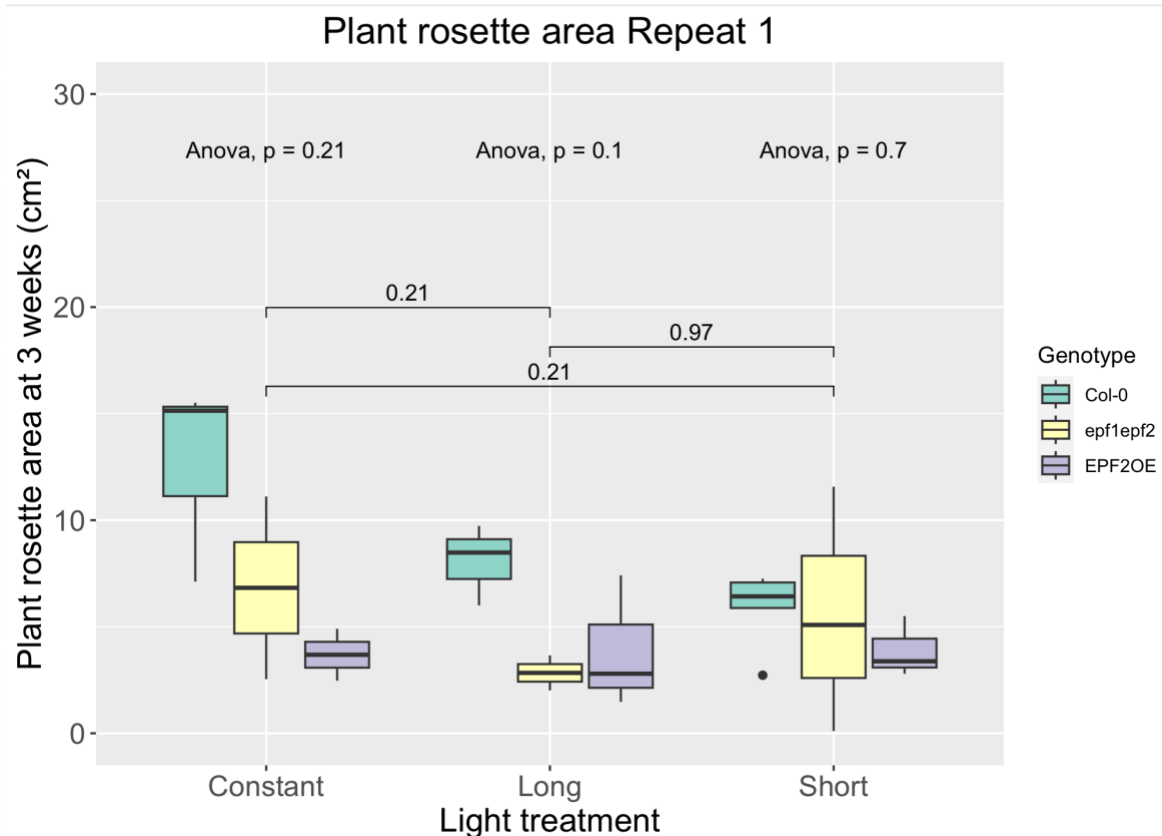
3.2 Light treatment and genotype had an impact on plant growth

The rosette plant growth at the midway point (three weeks after seedling transfer) was used for statistical analysis. Area data from repeat one was normally distributed, whilst data from repeats two and three were not normally distributed. The light treatment a plant was grown under had a significant impact on the plant rosette area ($p < 0.05$) in repeat two, but not in repeat one or repeat three. A Wilcoxon test showed that there was significant difference between plant growth in the constant light compared to both fluctuating

treatments. However, there was not a significant difference in rosette growth between the plants in the two fluctuating light treatments. Genotype was not found to have a significant impact on the rosette area growth of the plants in repeat two and three. In repeat one, genotype was found to have a significant impact on plant rosette growth ($p < 0.05$).

At the mid-way point (three weeks after seedling transfer) in repeat one of the experiment, Col-0 plants showed the greatest average plant rosette growth (Figure 12). Growth in Col-0 plants was significantly greater than growth in the *EPF2OE* plants ($p < 0.05$). There was no significant difference between growth in the Col-0 plants and the *epf1epf2* plants. *EPF2OE* plants displayed the smallest plant rosette growth, but this growth was not significantly smaller than the growth in the *epf1epf2* plants. In repeats two and three, plants grown under the constant light treatment displayed greatest rosette growth. In repeat two the growth in the constant light treatment was significantly greater than the growth in the long and the short fluctuating light treatments ($p < 0.05$). Plants grown under the two fluctuating light treatments show similar levels of growth at the mid-way point in both repeat two and repeat three of the experiment.

A



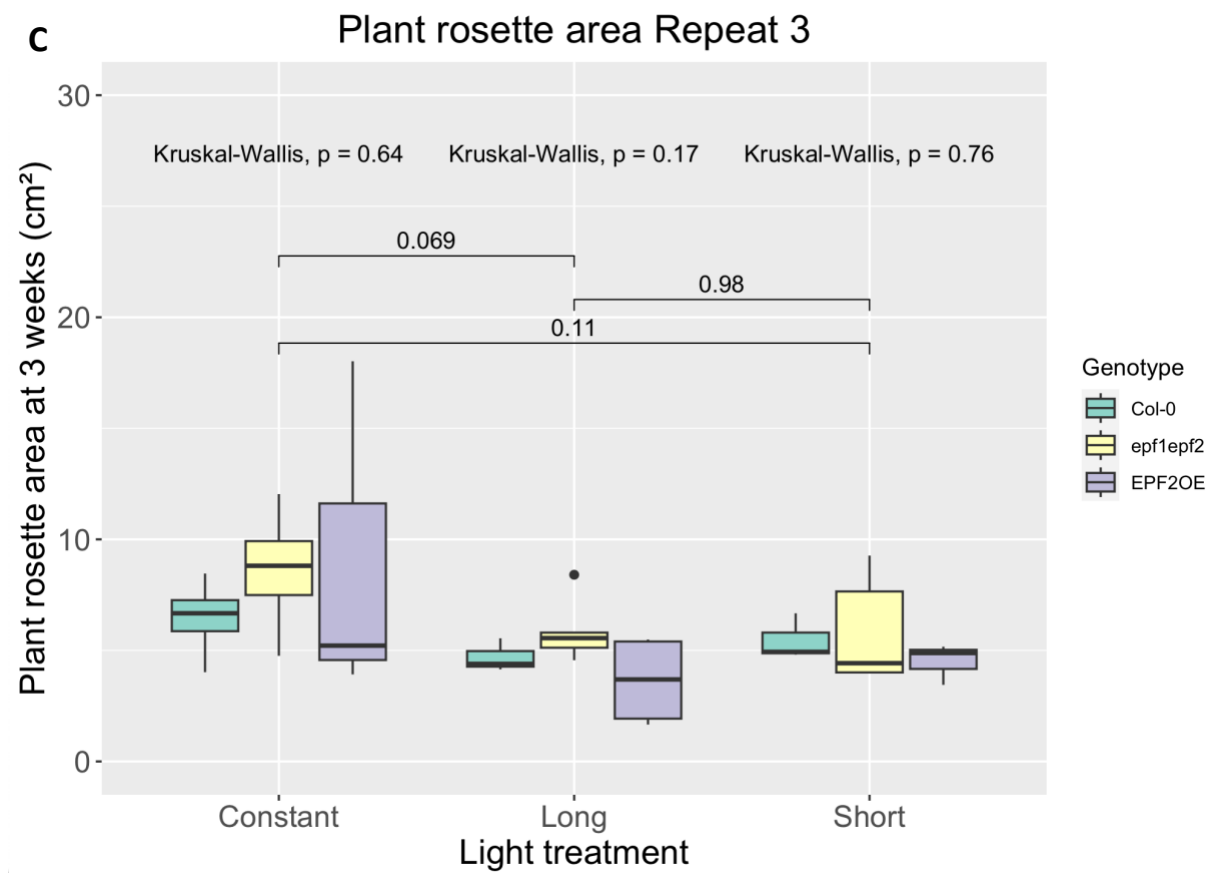
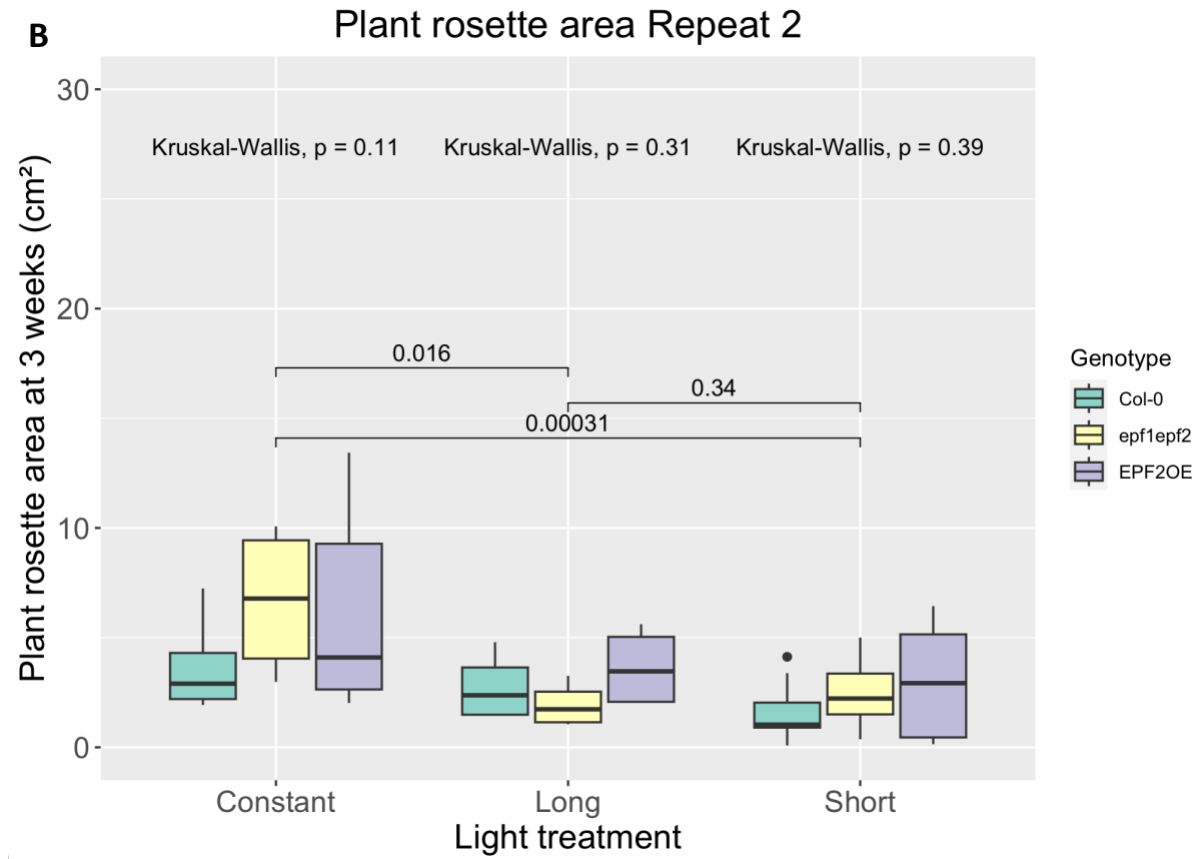


Figure 12: Boxplots comparing the plant rosette area growth at three weeks after the transfer of seedlings to individual pots. (A), (B) and (C) compare plant rosette growth in repeat one, two, and three of the experiment respectively. Pairwise comparisons between the light treatments in (B) and (C) were calculated using a Wilcox test, these are shown above the boxplots. Kruskal Wallis tests comparing growth between genotypes are displayed at the top of the plot in (B) and (C). In (A) pairwise comparisons between light treatments were calculated using a Students' T-test and an ANOVA was used to compared growth between the genotypes. p values from the ANOVA are displayed at the top of the graph in (A). Different tests were used in (A) compared to (B) and (C) because the data in repeat one is normally distributed whilst the data in repeat two and three of the experiment was not normally distributed. Repeat one had five replicates per genotype per light treatment, with 45 plants total. Repeat two and three had 10 replicates used for each genotype in each light treatment, with 90 plants studied in total.

3.3 Stomatal density was greater in *epf1epf2* plants compared to Col-0 and *EPF2OE* plants

In all light treatments the stomatal density was significantly higher in the *epf1epf2* genotype when compared to both Col-0 and *EPF2OE* genotypes ($p < 0.5$) (Figure 13). In all three light treatments the Col-0 and *EPF2OE* genotypes did not display significantly different stomatal densities.

3.4 The difference between abaxial stomatal density and adaxial stomatal density is smaller in the *EPF* mutant plants compared to wild type

There is a significant difference in stomatal density between the abaxial and adaxial sides of the leaf ($p < 0.05$). The *EPF2OE* and *epf1epf2* mutants show a smaller difference between the stomatal density in the abaxial and adaxial sides of the leaf in all light treatments. In the short light treatment only the Col-0 genotype shows a significant difference in the stomatal density between the abaxial and adaxial sides of the leaf. In the long fluctuating light treatment, all genotypes show a significant difference in stomatal density between the abaxial and adaxial sides of the leaf. While in the constant light treatment, all genotypes show no significant difference between the stomatal density on the abaxial and adaxial sides of the leaf.

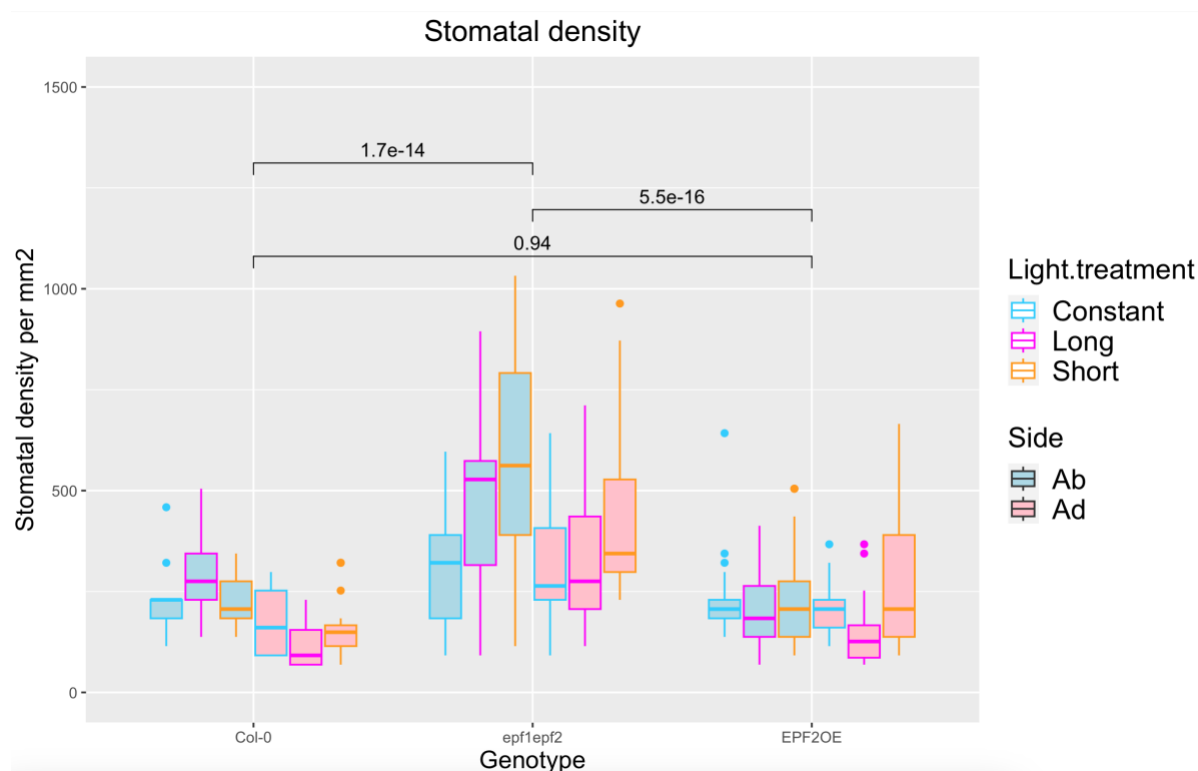


Figure 13: A Boxplot showing the stomatal density per mm² for the abaxial and adaxial side of the leaf for each genotype in each light treatment. The p values above the boxplots are from a Wilcox test comparing the stomatal densities between genotypes. There were 13-24 replicates used for each genotype in each light treatment, with 168 plants studied in total.

3.5 *epf1epf2* mutants display a higher WUEi than Col-0 and *EPF2OE*

In the constant light treatment and the long fluctuating light treatment, *epf1epf2* displays a significantly lower stable carbon isotope discrimination value compared to Col-0 and *EPF2OE* ($p < 0.05$) (Figure 14). Under the short fluctuating light treatment *epf1epf2* displays a significantly lower stable carbon isotope discrimination value than *EPF2OE* ($p < 0.05$), but not Col-0. Stable carbon isotope discrimination is a proxy for intrinsic WUE, with a lower stable carbon isotope discrimination value (more negative) indicating a greater WUEi.

3.6 Plants under constant light display greater WUEi

Plants grown under constant light have a significantly lower mean stable carbon isotope discrimination value ($p < 0.05$), compared to plants grown under the short and long fluctuating light treatments. Plants grown under long fluctuating light display a lower mean stable carbon isotope discrimination value compared to the short light treatment, -0.776 and -0.775 respectively, although this difference isn't significant.

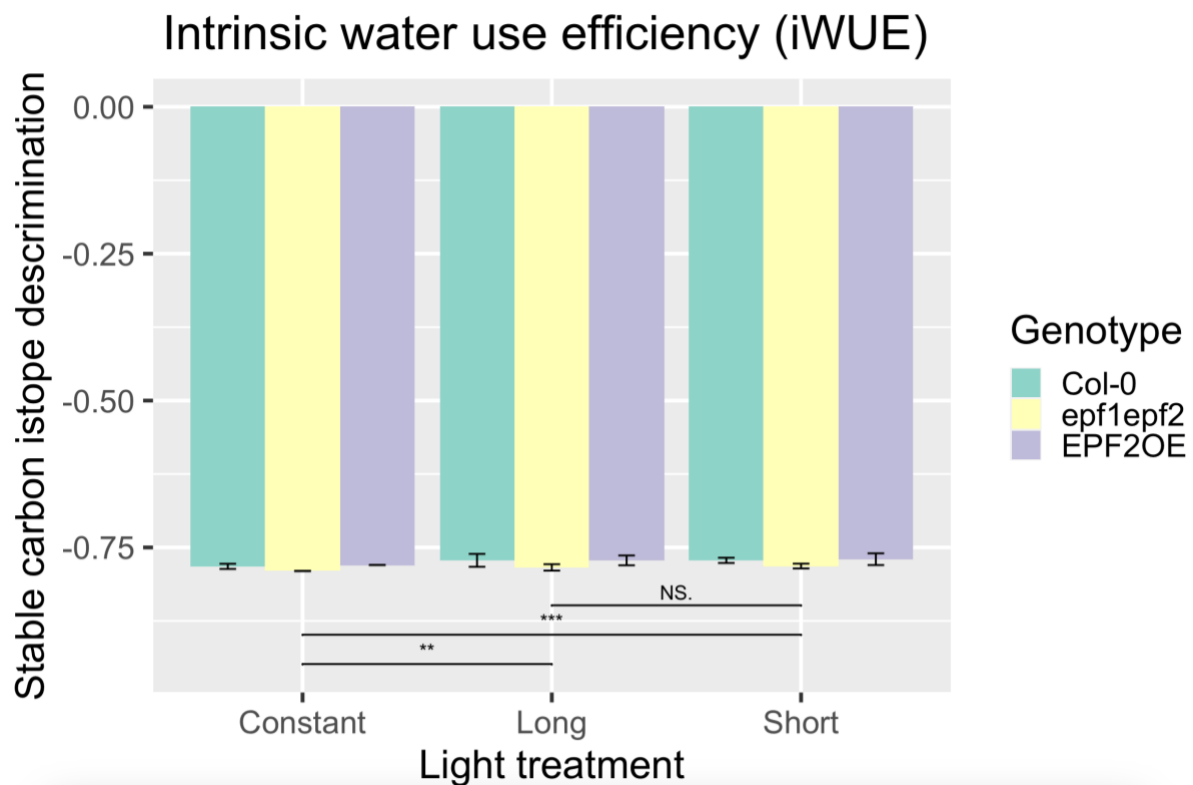


Figure 14: A bar graph showing the mean stable carbon isotope discrimination value for each genotype in each light treatment. Error bars represent the standard deviation from the mean. Asterisks below the bar graphs indicate the results from a pairwise t-test between light treatments, where * denotes a p value of 0.05 or less, ** denotes a p value of 0.01 or less, and *** denotes a p value of 0.001 or less. There were 5-9 replicates used for each genotype in each light treatment, with 57 plants studied in total.

3.7 Abaxial stomatal conductivity was higher than adaxial stomatal conductivity

The side of the leaf measured had a significant effect on stomatal conductivity ($p < 0.05$), with abaxial stomatal conductivity being significantly greater than adaxial stomatal conductivity in all three genotypes (Figure 15). The difference in stomatal conductivity between the abaxial and the adaxial sides of the leaf was greatest in the *epf1epf2* mutant.

3.8 There was not a difference between daytime and nighttime stomatal conductivity

The time of day does not have a significant effect on the stomatal conductivity displayed. Although average daytime stomatal conductivity was found to be higher than average nighttime stomatal conductivity (Figure 16). The difference between daytime and nighttime stomatal conductivity, is most pronounced in the *EPF2OE* genotype, and least pronounced in the Col-0 genotype.

3.9 The *epf1epf2* genotype displays the highest stomatal conductivity

On the abaxial side of the leaf the *epf1epf2* genotype displays a significantly higher stomatal conductivity compared to the Col-0 and *EPF2OE* genotypes ($p < 0.05$). There was no significant difference in stomata conductivity between the Col-0 and *EPF2OE* genotypes. There was also not a significant effect of genotype on adaxial stomata conductivity. The light treatment a plant was in also did not have a significant impact on the stomatal conductivity displayed by the plant.

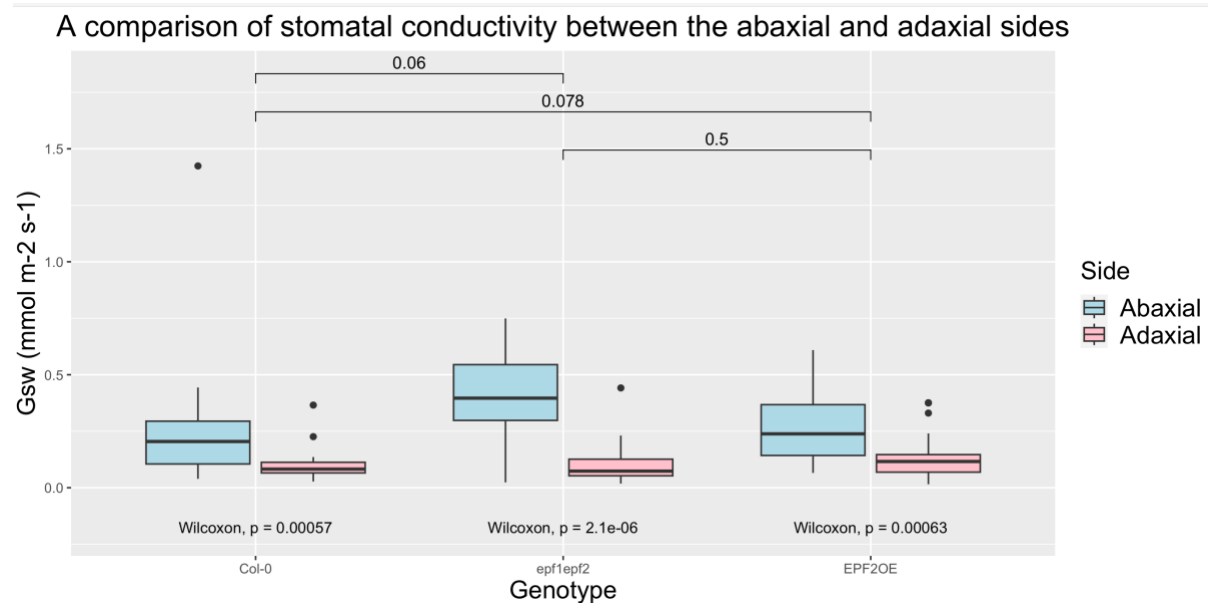


Figure 15: A boxplot comparing the stomatal conductivity values between the abaxial and the adaxial side of the leaf in each genotype. p values from a pairwise Wilcoxon test between genotypes are displayed above the box plots. p values from a Wilcoxon test between the abaxial and adaxial values within each genotype are displayed beneath the boxplots. There were 5-11 replicates used for each genotype in each light treatment, with 75 plants studied in total.

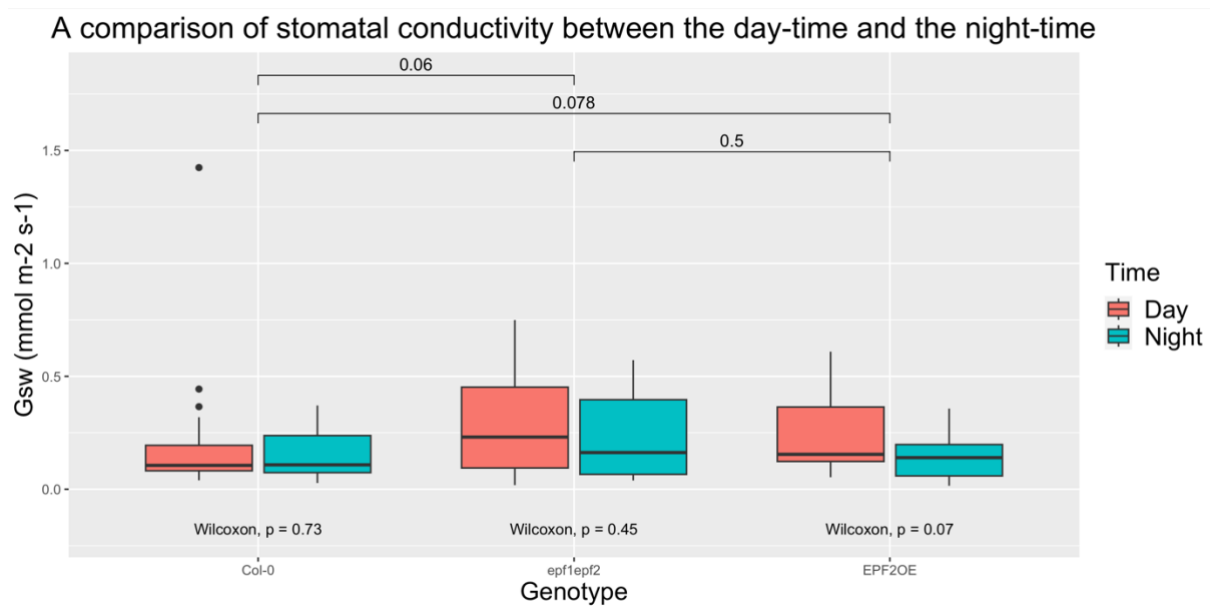


Figure 16: A boxplot comparing the stomatal conductivity values between the daytime and the nighttime in each genotype. p values from a pairwise Wilcoxon test between genotypes are displayed above the box plots. p values from a Wilcoxon test between the daytime and nighttime stomatal conductivity values within each genotype are displayed beneath the boxplots. There were 24-26 replicates used for each genotype, with 26 plants studied during the daytime and 44 plants studied during the nighttime, 75 plants in total were studied.

3.10 Genotype, but not light treatment, has a significant effect on daytime photosynthetic induction

In repeat three of the experiment, genotype had a significant effect on photosynthetic induction ($p < 0.05$). The Col-0 genotype shows a lower daytime photosynthetic induction rate compared to the daytime photosynthetic induction rate in the *epf1epf2* genotype and the *EPF2OE* genotype in the constant and long light treatments (Figure 18). However, the *EPF2OE* plants displayed a lower daytime photosynthetic induction rate than Col-0 in the short light treatment. The *epf1epf2* genotype has the highest daytime photosynthetic induction rate of the three genotypes in each light treatment. The light treatment a plant was grown under did not have a significant effect on daytime photosynthetic induction in repeat three. Additionally, neither light treatment nor genotype had a significant effect on the nighttime photosynthetic induction rate in repeat three.

In repeat two neither light treatment nor genotype had a significant effect on daytime or nighttime photosynthetic induction (Figure 17). Despite not being significant, the *epf1epf2* genotype had the highest daytime photosynthetic induction rate in each of the three light treatments in repeat two. The Col-0 genotype had the lowest daytime photosynthetic induction rate in the constant light treatment in repeat two, with the *EPF2OE* genotype having the lowest daytime photosynthetic induction rate in the long and short fluctuating light treatments. The *epf1epf2* genotype had the lowest nighttime photosynthetic induction rate in all three light treatments in repeat two. The Col-0 genotype

had the highest nighttime photosynthetic rate in the constant light treatment in repeat two. Whilst, the *EPF2OE* genotype had the highest nighttime photosynthetic induction rate in the long and short fluctuating light treatments. Large error bars for the photosynthetic measurements show high variability between measured photosynthetic induction between plants of the same genotype in the same light treatment.

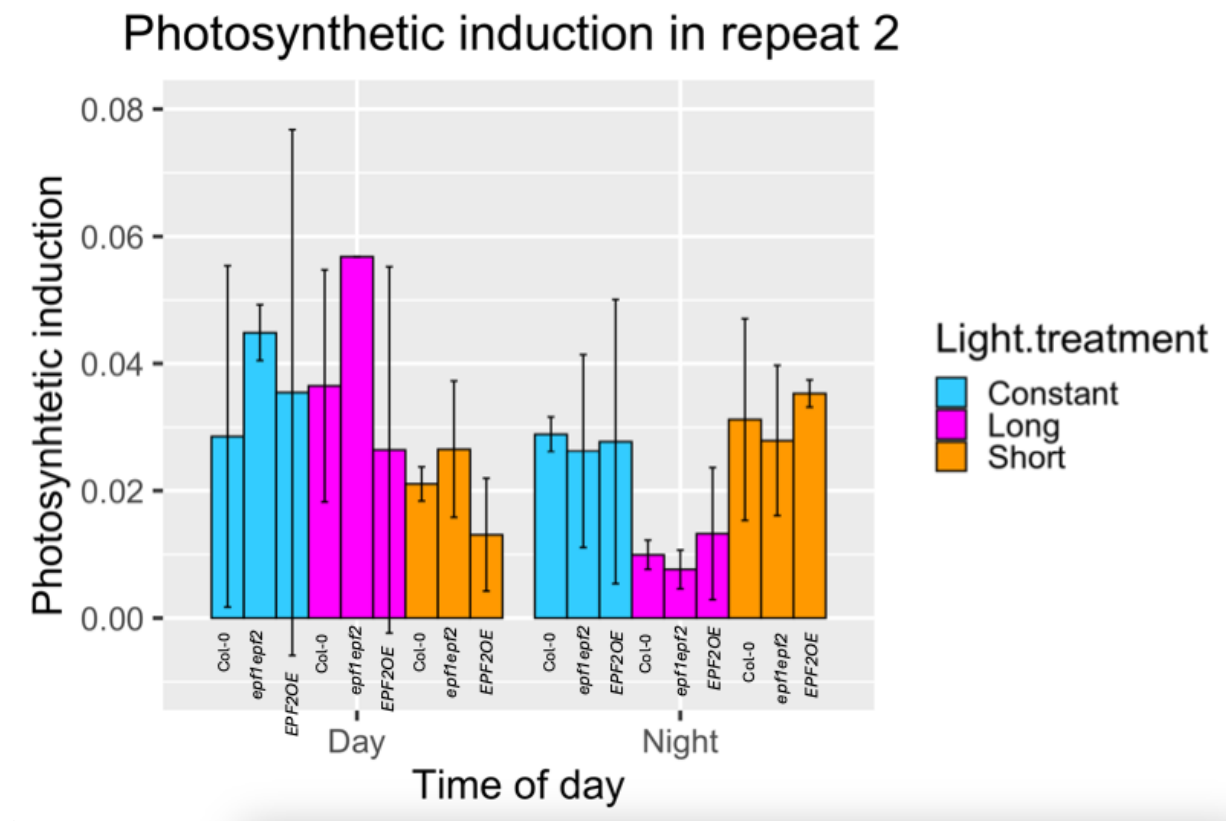


Figure 17: A bar graph showing the daytime and nighttime photosynthetic induction rate in each light treatment for each genotype in repeat two of the experiment. The genotype for each bar is written below the bar. There were 1-3 replicates used for each genotype, in each light treatment with 18 plants studied during the daytime and 19 plants studied during the nighttime, 37 plants in total were studied.

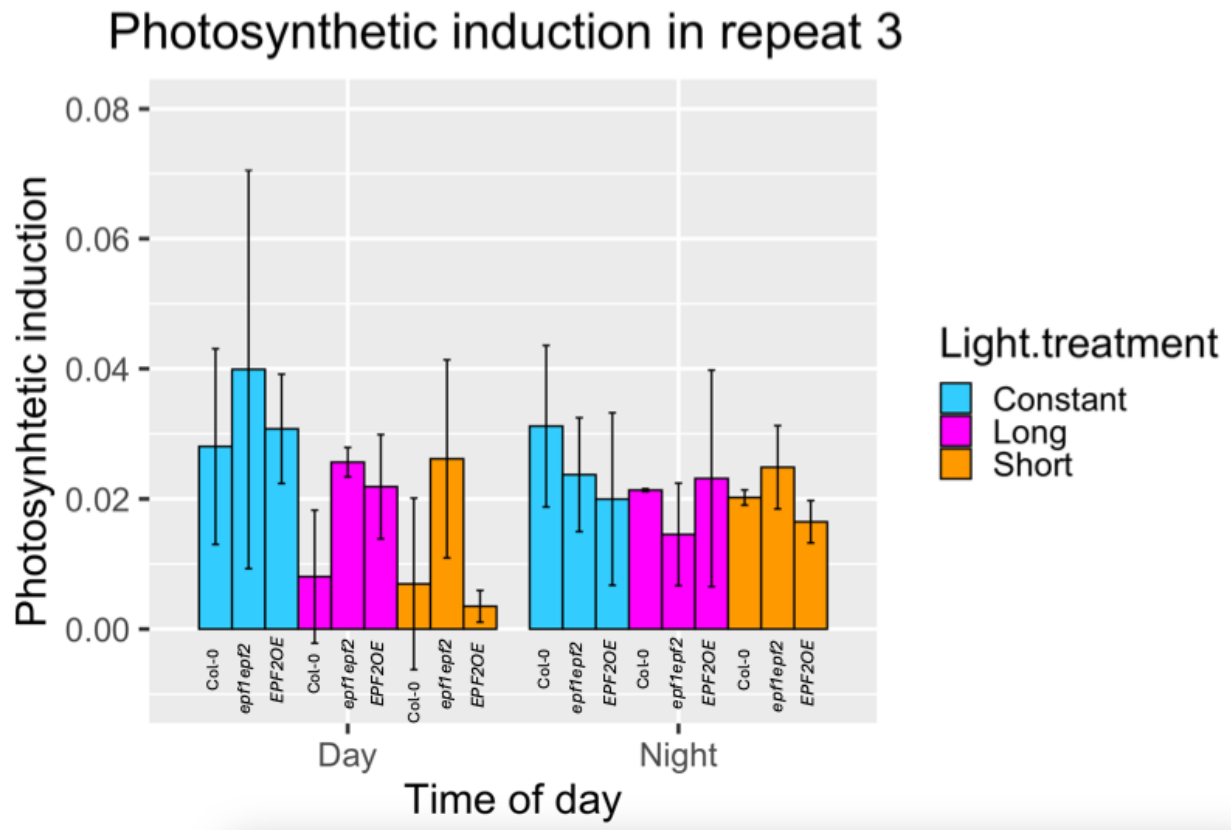


Figure 18: A bar graph showing the daytime and nighttime photosynthetic induction rate in each light treatment for each genotype in repeat three of the experiment. The genotype for each bar is written below the bar. There were 2-5 replicates used for each genotype, in each light treatment with 27 plants studied during the daytime and 32 plants studied during the nighttime, 59 plants in total were studied.

3.11 Mean CO₂ assimilation was lower in plants grown under the short fluctuating light treatment

Plants grown under the short fluctuating light treatment displayed the lowest average CO₂ assimilation rates in their respective genotypes, with the exception of nighttime carbon assimilation in repeat three (Figure 19). Although light treatment did not have a significant effect on mean CO₂ assimilation at either of the light intensities measured.

Plants with the *epf1epf2* genotype, grown under constant light treatment had the highest mean CO₂ assimilation in repeat two, in both the daytime and the nighttime. Genotype was also found to not have a significant effect on mean CO₂ assimilation, so this greater carbon assimilation seen in the *epf1epf2* genotype is also not significant. The *EPF2OE* plants grown under constant light had the highest mean CO₂ assimilation in repeat three in both the daytime and the nighttime, although again this was not significant.

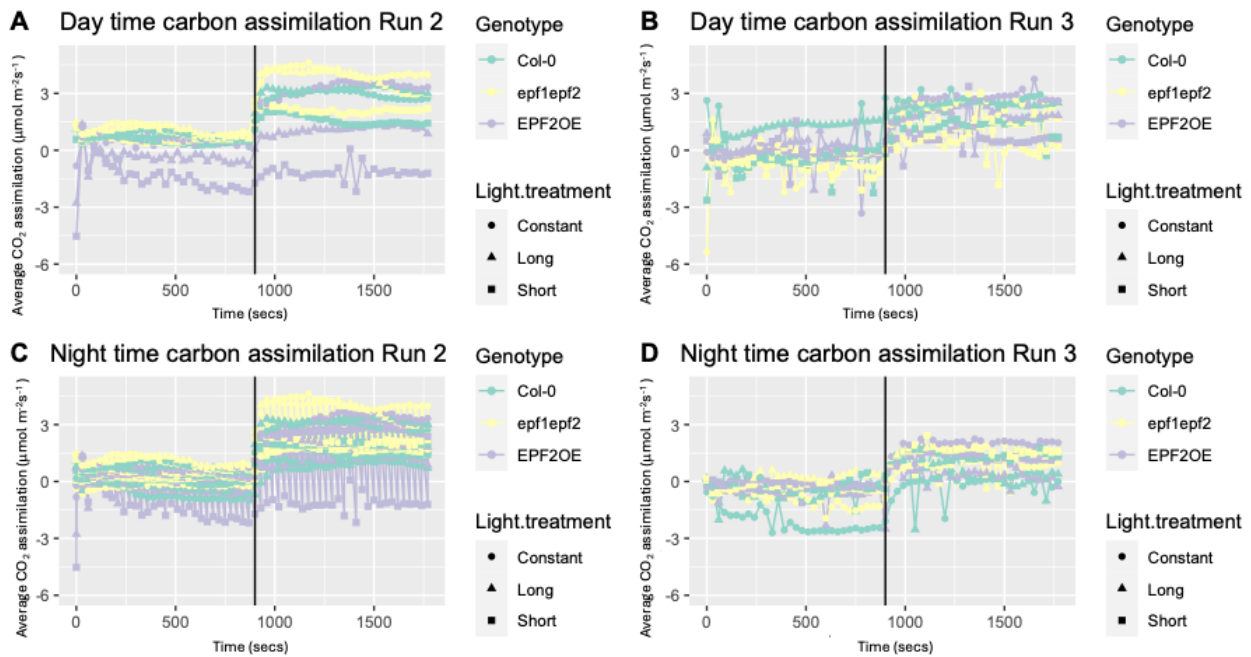


Figure 19: Graphs showing the average daytime (A and B) and nighttime (C and D) carbon assimilation against time for both repeat two (A and C) and repeat three (B and D). The black line at 900 seconds shows the time point when the light intensity in the LI-COR chamber was increased from $15 \mu\text{mol m}^{-2}\text{s}^{-1}$ to $150 \mu\text{mol m}^{-2}\text{s}^{-1}$. In repeat two (A and C) there were 1-3 replicates used for each genotype, with 37 plants studied in total. In repeat three (B and D) there were 2-5 replicates used for each genotype, in each light treatment with 59 plants studied in total.

3.12 Daytime carbon assimilation is higher than nighttime carbon assimilation under low light

The time of day (daytime vs nighttime) had a significant effect on the average carbon assimilation in $15 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light ($p < 0.05$), but not in $150 \mu\text{mol m}^{-2}\text{s}^{-1}$ of light (Figure 20). When measured under low light ($15 \mu\text{mol m}^{-2}\text{s}^{-1}$) daytime carbon assimilation is higher than nighttime carbon assimilation in all light treatments in the Col-0 genotype, but not the *epf1epf2* and the *EPF2OE* genotype. Under high light ($150 \mu\text{mol}$) nighttime carbon assimilation was lower in all genotypes in the long fluctuating and constant light treatments, but nighttime carbon assimilation was higher in the short light treatment. The exceptions to this are the *epf1epf2* genotype in repeat two, where daytime carbon assimilation is higher than nighttime in the short fluctuating light treatment. As well as the *EPF2OE* genotype in the long fluctuating light treatment in repeat two, where nighttime carbon assimilation is higher than daytime carbon assimilation.

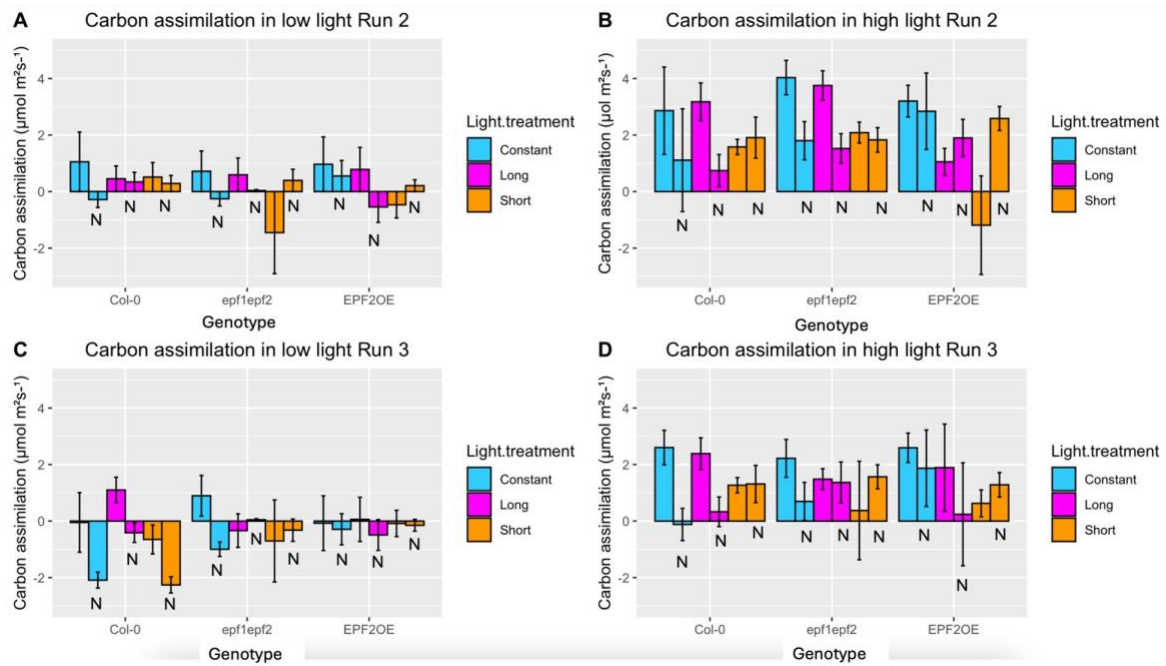


Figure 20: Bar graphs showing the carbon assimilation measured in 15 μmol m⁻²s⁻¹ light intensity (A and C) and 150 μmol m⁻²s⁻¹ light intensity (B and D) in repeat two (A and B) and repeat three (C and D). Nighttime measurements are denoted with an N. In repeat two (A and B) there were 1-3 replicates used for each genotype, in each light treatment, with 37 plants studied in total. In repeat three (C and D) there were 2-5 replicates used for each genotype, in each light treatment with 59 plants studied in total.

3.13 Plants grown under the constant light treatment show lower levels of NPQ induction under light stress

The light treatment a plant is grown under has a significant effect on the NPQ level induced during light stress ($p < 0.05$) (Figure 21). Plants grown under the long light treatment display the highest levels of NPQ, followed by plants grown under the short light treatment. Plants grown under the constant light treatment display the lowest levels of NPQ. A post hoc analysis showed that there was a significant difference between the fluctuating light treatments and the constant light treatment, but that there was not a significant difference the two fluctuating light treatments.

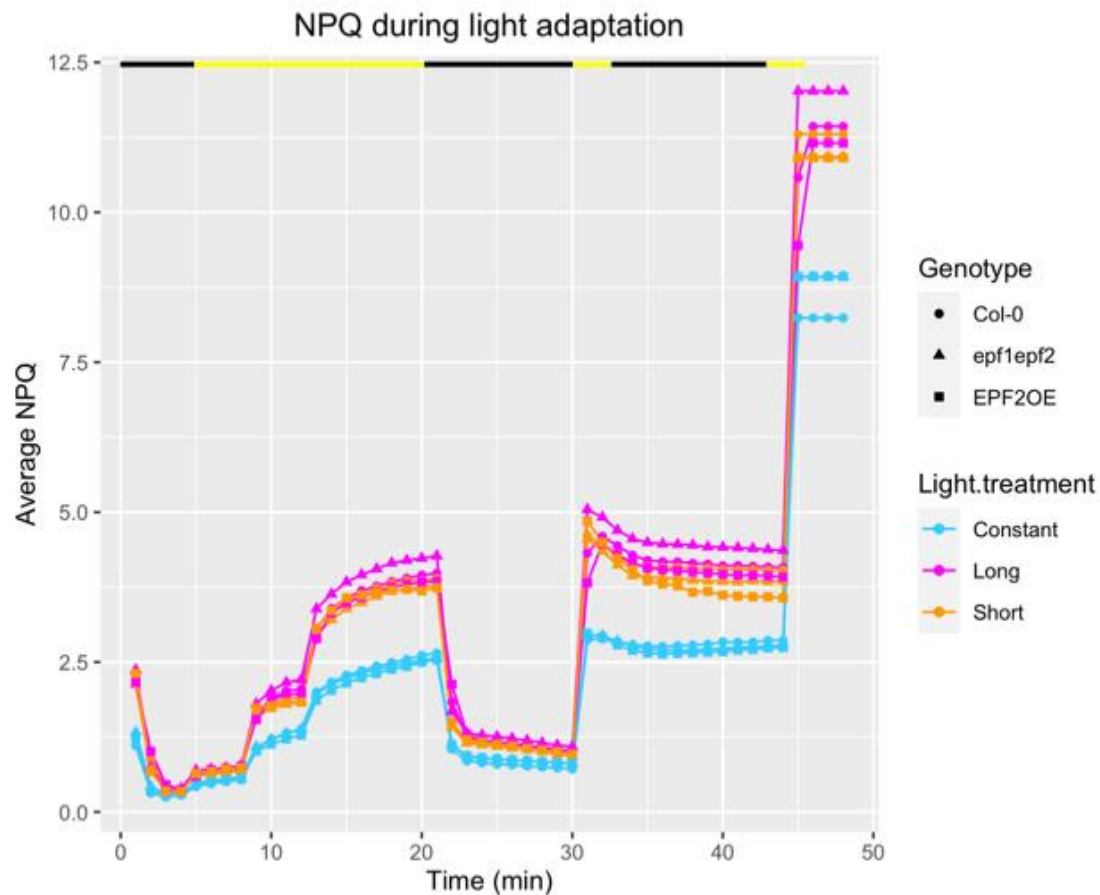


Figure 21: A graph showing the average NPQ value over time during light adaptation. The yellow and black bars above the graph represent the periods of light and no light respectively. An actinic light intensity of $2,000 \mu\text{mol m}^{-2}\text{s}^{-1}$ was used. Five replicates were used for each genotype in each light treatment, with 45 plants studied in total.

3.14 Differences between light treatments were significant under one second light fluctuations

When measured with the Dual pam under the one second light fluctuations the light treatment the plant was grown under had a significant effect on the NPQ value ($p < 0.05$), whilst the genotype of the plant did not have a significant effect on the NPQ value. There was a significant difference between the NPQ value induced in plants grown under the constant and long light treatments and between the long and short light treatments. However, there was not a significant difference between the NPQ values induced in plants grown under the constant and short light treatments. Under the one second fluctuations, plants grown under the long light treatment had the highest level of NPQ induction, followed by plants grown under the short light treatment. Plants grown under the constant light treatment displayed the lowest level of NPQ induction under one second light fluctuations. Under the one minute fluctuations neither the genotype of the plant nor the light treatment it was grown under had a significant effect on the average NPQ value measured (Figure 22). Under one minute light fluctuations most plants showed very similar

rates of NPQ induction and relaxation and similar NPQ levels regardless of genotype or the light treatment they were grown under. Col-0 plants grown under the short light treatment are the exception to this, these plants reach lower maximal levels of NPQ and higher minimal levels of NPQ. These plants also have a slower rate for NPQ induction and relaxation.

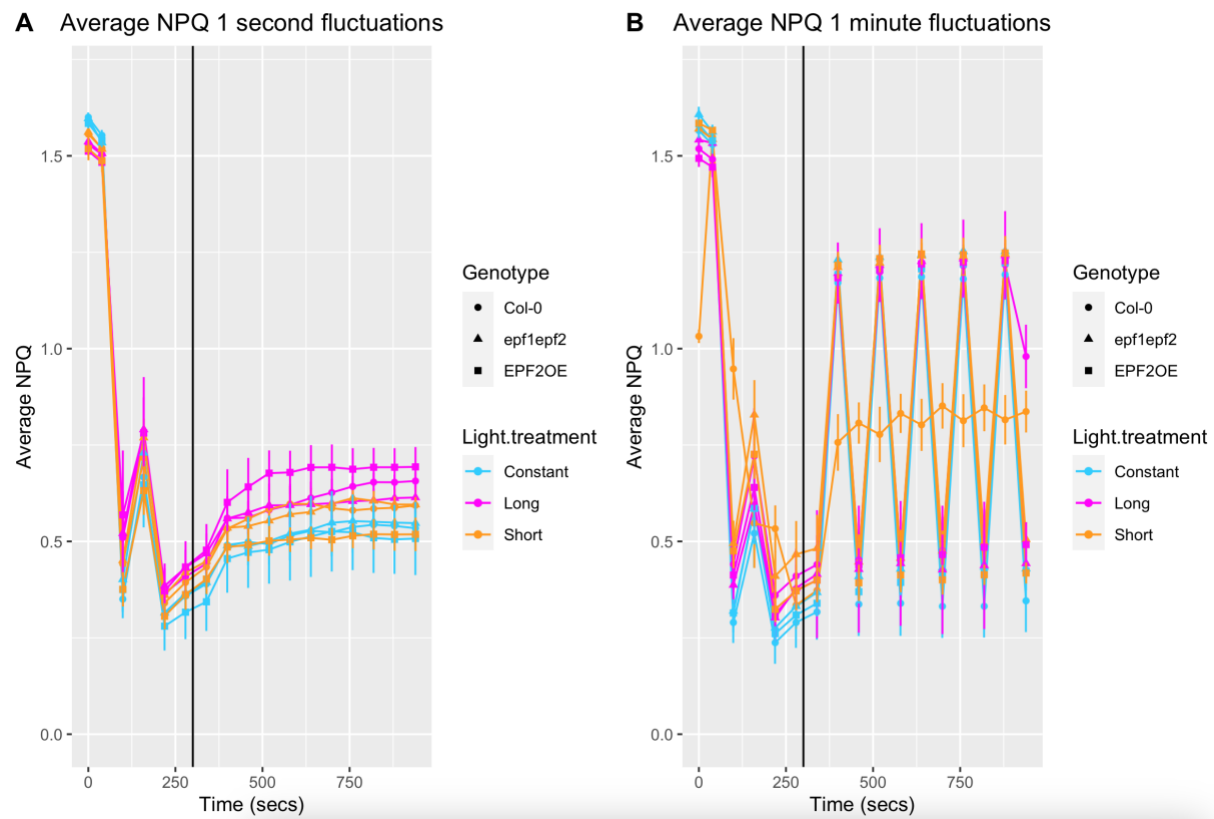


Figure 22: Graphs showing average NPQ values over time under one second fluctuations in light intensity (A) and one minute fluctuations in light intensity (B). The black line at 300 seconds represents the end of the five minute warm up phase and the beginning of the actinic light fluctuation program. For the one second light fluctuation program (A) 4-10 replicates were used for each genotype in each light treatment and 64 plants in total were studied. For the one minute light fluctuation program (B) 3-9 replicates were used for each genotype in each light treatment and 48 plants in total were studied.

3.15 The constant light treatment displays lower a chlorophyll a/b ratio in the *EPF2OE* genotype

Light treatment had a significant effect on the chlorophyll a/b ratio ($p < 0.05$) (Figure 23), but only in the *EPF2OE* genotype. *EPF2OE* plants grown in the constant light treatment had a significantly lower chlorophyll a/b ratio than plants grown in the long fluctuating light treatment. A pairwise student's t-test showed that there was not a significant difference in chlorophyll a/b ratio between plants grown in the short fluctuating and constant light treatments, in the *EPF2OE* genotype. The *EPF2OE* genotype grown in the constant light treatment did not have significantly different chlorophyll b content, chlorophyll a content or

chlorophyll a and b content combined compared to plants in the fluctuating light treatments.

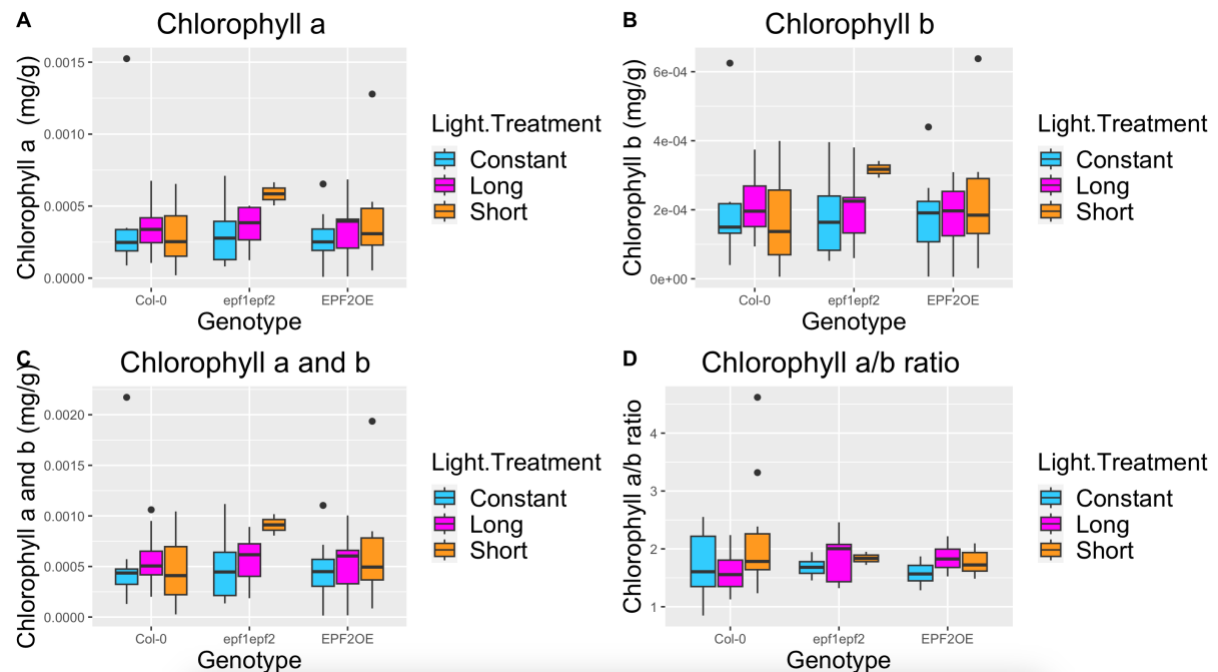


Figure 23: Boxplots showing the chlorophyll a (A), chlorophyll b (B), and chlorophyll a and b (C) content and the ratio of chlorophyll a to chlorophyll b content (D). There were 2-12 replicates used for each genotype and 78 plants were studied in total.

3.16 Total root length differed between plants grown in the daytime and plants grown in the nighttime

There was a significant difference in total root length between the plants grown during the day and the plants grown during the night under shifted daytime conditions (18:00 to 10:00) ($p < 0.05$). However, there was not a significant difference in the number of lateral roots or the total lateral root length between plants grown in the night and plants grown during the day. The total root length was higher in plants grown during the night for all plants except the Col-0 and *epf1epf2* genotype grown under the constant light. Differences between light treatments and between genotypes were not significant.

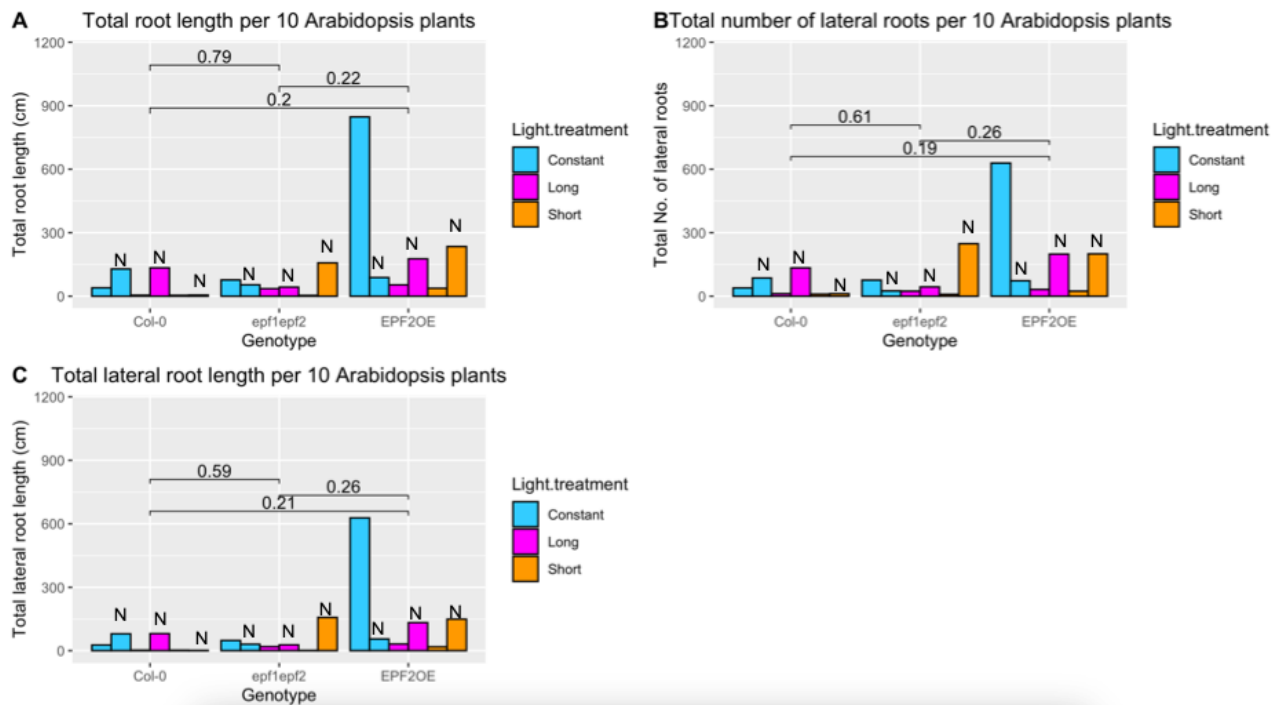


Figure 24: Bar graphs showing the total root length (A), the total number of lateral roots (B), and the total lateral root length (C) for each genotype in each light treatment. The p values above the bars are from a pairwise student's t-test between the genotypes. Plants grown in shifted light conditions are denoted with an N. 10 plants of each genotype were sown per plate; all values are given as the total value across all 10 plants on each plate. 180 plants in total were studied.

4. Discussion

This study aimed to look at the impact that stomatal density has on photosynthesis in fluctuating light conditions. It sought to test the hypothesis that having a greater stomatal density would allow for greater stomatal kinetics and a reduced stomatal response time to changing light conditions. The high stomatal density *epf1epf2* mutant, was tested against wild type Col-0 plants and the *EPF2OE* low stomatal density mutant. The WUE of these genotypes was also examined to test the hypothesis that having a lower stomatal density would lead to plants with an improved WUE. This hypothesis was tested as it has previously been asserted that decreasing plant stomatal density, through mutations to genes like *EPF2*, could be used to increase plant WUE and improve drought tolerance.

The findings of this study indicate that the *epf1epf2* mutants have a greater stomatal conductivity and have greater rates of daytime photosynthetic induction. The *epf1epf2* mutants also displays the highest photosynthetic induction under the short fluctuating light treatment. Thus, the *epf1epf2* mutants are able to better use the available light when grown under short light fluctuations. Additionally, the data suggest that the *epf1epf2* mutants display the greatest WUEi. This disagrees with the hypothesis that plants with a lower stomatal density will display a greater WUEi. It also challenges current ideas around improving drought resistance through reducing a plant's stomatal density.

4.1 The genotype of a plant did not impact its' growth

The genotype of a plant did not have a significant effect on the growth of the plant in repeat two or repeat three of the experiment. Additionally, in all of the repeats of the experiment plants grew best under constant light regardless of genotype, as shown by the plant rosette area at the three-week mark (Figure 12). In repeat one genotype did impact plant growth, but in this repeat, measurements were taken less frequently, and the plants sustained the greatest amount of thrip damage. In repeat one plants were moved between growth rooms at two weeks old, during the move these plants were subjected to cold stress. Plants subjected to cold stress have been found to show reduced growth (Ding et al., 2024). Additionally, cold stress has been shown to induce stomatal closure (Agurla et al., 2018). This suggests that the exposure to cold stress in repeat one not only decreased overall plant growth relative to plant growth in repeat two and three, but that it also had a genotype specific effect. Cold temperatures can cause an imbalance in the water status of a plant, closing stomata upon exposure to cold stress can protect plants from cold induced water loss. These differences in cold stress induced stomatal closure may explain differences observed in growth between the genotypes in repeat one, as only plants in repeat one of the experiment were exposed to cold stress. This makes the area measurements from repeat one of the experiment unreliable and it is likely that genotype does not impact growth when plants are not exposed to thrip damage or cold stress, as was found in repeat two and repeat three.

A study by Doheny-Adams et al. (2012) compared rosette area in Col-0, *EPF2OE*, and *epf1epf2 Arabidopsis thaliana* plants, they found that growth was largest in the *EPF2OE* plants and smallest in the *epf1epf2* plants. Which is at odds with the results from this study which found that there was no significant impact of genotype on plant rosette area growth. A key difference between these two studies are the conditions that the plants were grown under. The Doheny-Adams et al. (2012) study was looking at drought tolerance while this study was looking at growth under light fluctuations. In the Doheny-Adams et al. (2012) study plants were grown at either 30% soil water conditions or 70% soil water conditions,

whereas in this study, plants were grown under well-watered conditions. The genotypes tested had genetic alterations to genes involved in the control of stomatal density and stomata play a role in transpiration. Thus, it is expected that differences in soil water concentration will affect plant growth. This likely explains why the conclusions between these two studies differ.

Results differ between different repeats of the experiment; thrip infestations in repeat one and three of the experiment likely had an impact on this. These infestations caused substantial damage to plants. Plant herbivory by these thrips reduced the plant rosette area, thus distorting plant growth measurements. Repeat one was the most impact by thrip damage, if thrips target plants with genome specificity, this could explain the impact of genotype on plant rosette area that was found in repeat one of the experiment but not in repeat two or repeat three of the experiment. Thrip infestation has been linked to a reduction in stomatal conductivity in damaged leaves however, no previous studies have linked thrip herbivory rates with stomatal density or the *EPF* genes (Ellsworth et al., 1995).

Additionally, in repeat one of this experiment growth measurements were taken every other week, however, in repeat two and three of this experiment growth measurements were taken twice a week. This makes the results between repeats of the experiments difficult to compare. The light intensity the plants were grown under also varied between repeats of the experiment. Covers were added to reduce thrip damage in repeats two and three. These covers reduced the light intensity for repeat two and repeat three from $134 \mu\text{mol m}^{-2}\text{s}^{-1}$ and $275 \mu\text{mol m}^{-2}\text{s}^{-1}$ for the constant and fluctuating light treatments to $27 \mu\text{mol m}^{-2}\text{s}^{-1}$ and $40 \mu\text{mol m}^{-2}\text{s}^{-1}$ respectively. The differences in light intensity between repeats of the experiment could also explain the differences in growth between the experiments. Due to the large differences seen, repeats of the experiment have been analysed separately.

4.2 Constant light led to the greatest plant growth

The light treatment a plant was grown under did have a significant effect on plant growth, with plants grown under constant light displaying the greatest rosette area in repeat two and repeat three of the experiment. Differences between plant growth at the three-week point were more pronounced between the fluctuating light treatments and the constant light than between the two fluctuating light treatments. This finding agrees with previous studies on fluctuating light and plant growth in *Arabidopsis thaliana*, which found that plant growth under fluctuating light is lower than plant growth under constant light, even when the light intensity in the constant light treatment is lower than the light intensity in the fluctuating light treatment (Violet-Chabrand et al., 2017).

Plants grown under constant light are likely to display greater rosette area growth than plants grown under fluctuating light. As under constant light, stomata will remain open, unless a factor other than fluctuating light triggers them to close, and photosynthesis will remain on. While under fluctuating light, plants will turn off photosynthesis and close stomata during the dark period, photosynthesis is then induced, and stomata are opened during the light period. So, under fluctuating light some of the available PAR will be unutilised, as photosynthesis will still be inducing and stomata still opening for a period of time while light is available. This decrease in PAR utilisation, in fluctuating light, is likely responsible for the decreased growth seen in the fluctuating light treatments.

The difference in plant growth between the three light treatments increased over the course of the experiment. With the difference in rosette area being larger towards the

end of the experiment, than at the beginning of the experiment. This differs from the Violet-Chabrand et al. (2017) study which found that the greatest difference in rosette area between light treatments was in the middle of the study, with the rosette areas converging across light treatments at the beginning and at the end of the experiment. The differing results between these studies could be due to the thrip infestation in this experiment reducing the rosette area in some plants and thus distorting the differences in plant growth between the light treatments. The rosette area in the constant light treatment, may be lower due to increased thrip infection in this light treatment. The light intensity was lower in the constant light treatment; this has been linked to reduced induction of type VI glandular trichomes (Escobar-Bravo et al., 2018). These trichomes play an important role in defence against thrips.

The differences between these studies could also have been due to different light intensities being used. The highest light intensity used in this study was $40 \mu\text{mol m}^{-2}\text{s}^{-1}$ in repeat two and repeat three, and $275 \mu\text{mol m}^{-2}\text{s}^{-1}$ in repeat one. While the highest light intensity used in the Violet-Chabrand et al. (2017) study was $460 \mu\text{mol m}^{-2}\text{s}^{-1}$, this large difference in light intensity likely contributed the differences seen in plant growth between the studies. Plants in this study were all germinated under the constant light treatment, while the plants in the Violet-Chabrand et al. (2017) study were germinated under both constant and fluctuating light treatments. This could have also contributed to the differences seen in plant growth between the studies, as light availability plays a role in regulating seed germination (Yang et al., 2020).

4.3 The *epf1epf2* genotype has the greatest stomatal density

This study found that the *epf1epf2* genotype had a greater stomatal density than the Col-0 and the *EPF2OE* genotype on both the abaxial and the adaxial sides of the leaf in all the light treatments. This agrees with many previous studies that have also found that the *epf1epf2* genotypes show a greater stomatal density than the Col-0 genotype and confirms the genotype (Franks et al., 2015, Hunt and Gray, 2009, Dow et al., 2014). This result was expected, due to the role of *EPF* genes as negative regulators of stomatal development. Additionally, it verifies that creating *epf1epf2* loss of function mutants does lead to an increased stomatal density.

However, this study found that the *EPF2OE* genotype does not have a significantly lower stomatal density than the Col-0 genotype on either the abaxial or the adaxial side of the leaf. This was unexpected as the *EPF2* gene is a negative regulator of stomatal development, over expressing the *EPF2* gene has previously been found to decrease stomatal density. Stomatal density has been previously linked to water availability, with stomatal density being lower under water scarce conditions (Xu and Zhou, 2008). Large differences between stomatal density in the Col-0 and *EPF2OE* genotype found in previous studies may be exacerbated by water stress, while plants in this study were grown under well-watered conditions.

Light is known to play a role in the regulation of stomatal development, with light exposure being shown to significantly increase the number of stomata per unit area (Wei et al., 2020). All the light treatments used in this study had the same daily photon dose, so there should not be a difference in the amount of light received by plants regardless of the light treatment they experienced. This likely explains why there was not a significant effect of light treatment on stomatal density. However, it is possible that the stress caused by light fluctuations negatively impacted light absorption, through the induction of NPQ (Niu et al.,

2023). This could result in lower light absorbance in the short light treatment, causing a lower number of stomata per unit area. The Col-0 and *EPF2OE* genotype display a lower abaxial stomatal density in the short light treatment compared to the constant light treatment, this could be a result of the impact of short fluctuating light on stomatal development. This is not seen in the *epf1epf2* genotype, but this may be due to an increased photosynthetic rate, allowing for more of the available light to be used and reducing the dissipation of light through NPQ.

Whilst displaying a lower abaxial stomatal density, the *EPF2OE* mutant seems to be able to offset this by increasing its adaxial stomatal density. This suggests that abaxial and adaxial surfaces of the leaf are not independent of each other, which agrees with a study by Mott and Peak (2018) which found that artificially reducing gas exchange on the adaxial surface led to abaxial stomata opening. However, this finding disagrees with a study by Wall et al. (2022) that suggested that there was no link between the two sides of the leaf. The Wall et al. (2022) study suggested this as they found that blocking the abaxial stomata did not result in compensation by the adaxial stomata. The authors of the Wall et al. (2022) study suggest that the difference seen between their results and the results of the Mott and Peak (2018) were species specific. The Wall et al. (2022) study was carried out on *Triticum aestivum* (Wheat), while the Mott and Peak (2018) study was carried out on *Xanthium strumarium* (Cocklebur). Wheat is a member of the *Poaceae* family and as such will have different stomatal morphology to cocklebur plants. The difference in stomatal morphology could also explain why the findings of this study agree with the Mott and Peak (2018) study as *Arabidopsis thaliana* is also not a member of the *Poaceae* family. This suggests that the two sides of the leaf are able to communicate with each other, and that this ability has been lost in the *Poaceae* family. Further study will be needed to verify this.

4.4 *EPF* genes have different roles in abaxial and adaxial stomatal development

Mean adaxial *EPF2OE* stomatal conductance is higher than mean adaxial Col-0 stomatal conductance. The *EPF2OE* genotype also shows a smaller difference between the abaxial and adaxial stomatal densities compared to the other genotypes. Adaxial *EPF2OE* stomatal density was expected to be lower than *epf1epf2* and Col-0 adaxial stomatal density, due to the role that the *EPF2* gene plays as a negative regulator in the stomatal development pathway. The deviation from this expectation indicates that the *EPF2* gene plays a different role in the abaxial and adaxial stomatal development pathways. There are clearly differences in the ways that the *EPF2* gene acts in the abaxial and adaxial stomatal development pathways, and differing regulation in these pathways has been previously observed (Jalakas et al., 2024, Hronková et al., 2015). Overexpressing the *EPF2* gene gives different results on the two sides of the leaf, highlighting the need for more studies to look at both sides of the leaf, as there are clearly differences in how the abaxial and adaxial stomata develop.

This reduced difference between the abaxial and adaxial stomatal densities suggests that the *EPF2OE* genotype may be overcompensating for a reduced abaxial stomatal density by increasing their adaxial stomatal density. Thus, minimising the reduction in total stomatal density caused by *EPF2* gene overexpression.

Reduced stomatal density through over expression of the *EPF2* gene has previously been suggested as method of reducing water loss and improving plant WUE. As transpiration occurs through stomata, it has been suggested that reducing stomatal density

will reduce transpiration and improve plant water usage (Franks et al., 2015, Liu et al., 2021, Robertson et al., 2023). However, if *EPF2OE* plants can overcompensate for reduced abaxial stomatal density by increasing adaxial stomatal density then they cannot be looked at as a target for increasing plant WUE.

4.5 High stomatal density gives greater plant water usage

Despite previous findings that the low stomatal density mutant *EPF2OE* has an improved WUE (Franks et al., 2015). This study found that the WUE_i was greatest in the high stomatal density mutant *epf1epf2*, while *EPF2OE* had the lowest WUE_i in the constant and short light treatment. *EPF2OE* and Col-0 had the same WUE_i in the long fluctuating light treatment. Thus, the results of this study find the opposite to the Franks et al. (2015) study, which suggested that a lower stomatal density improves WUE. The differences between these studies are likely due to different methods being used to look at WUE. In this study WUE_i was looked at using Δ values as a proxy, the Δ values are calculated using carbon isotope discrimination. During photosynthesis, plants discriminate against ^{13}C in favour of ^{12}C carbon. The variation in ^{13}C composition between the photosynthetic products and the atmosphere can act as a proxy for the number of carbon atoms fixed per molecule of water lost. With lower (more negative) Δ values indicating a greater WUE_i. The Franks et al. (2015) study measured WUE_{inst}, this was done by dividing the photosynthetic rate by the transpiration rate (Hatfield and Dold, 2019). The WUE_i method allows for comparison between the genotypes on the basis of only stomatal density, while WUE_{inst} also includes other factors that influence water loss, for example humidity (Ye et al., 2020). These differences between the two measurements likely explain the different conclusions of the two studies.

The results of this study also disagree with a study by Xiong et al. (2022b) which found that under fluctuating light, plants with a greater stomatal density display a lower WUE. The Xiong et al. (2022b) study grew rice (*Oryza*) plants outside under natural irradiance. The differences seen in water use efficiency between the Xiong et al. (2022b) study and this study are likely due to differences in the type of fluctuating light used. This study used artificial light with regularly timed periods where the light was off, whilst the Xiong et al. (2022b) study used natural light with any fluctuations in light being due to chance. Thus, the light will have fluctuated more in this study than the Xiong et al. (2022b) study, exacerbating the impact of stomatal kinetics on WUE in this study.

Smaller stomata are often associated with having faster stomatal kinetics, due to having a greater guard cell transporter to cell volume ratio. Stomatal size and density are inversely proportional, so high stomatal density genotypes like *epf1epf2* are expected to have faster stomatal kinetics. Faster stomatal kinetics will allow plants to close their stomata faster when light is not available, to reduce transpiration when photosynthesis cannot occur (Figure 4). Hence, plants with a greater stomatal density and faster stomatal kinetics in fluctuating light are expected to have a greater WUE than plants with a lower stomatal density and slower stomatal kinetics. This could explain why the *epf1epf2* genotype has the greatest WUE_i in the fluctuating light treatments. Although it does not explain why the *epf1epf2* genotype also has the greatest WUE_i in the constant light treatment. Plants in this experiment were grown under well-watered conditions. Under these conditions, stomata would not be induced to close due to low water potential and are likely to be open. The *epf1epf2* genotype would have more open stomata and this is

expected to facilitate more CO₂ uptake which could increase photosynthetic rates, boosting WUEi in this genotype under the constant light treatment.

4.6 Plants have greater water use efficiency under constant light

Plants grown under constant light are significantly more water use efficient than plants grown under either of the fluctuating light treatments. During the dark periods in the fluctuating light treatment, photosynthesis cannot occur as there is no available light. Stomata respond to changing light intensity, but there is a period between the light turning off and stomata closing where stomata will be open and water will be lost through them (Figure 4), but where no photosynthesis is occurring. This will reduce the amount of carbon atoms fixed per molecule of water lost, thus fluctuating light reduces the WUE of plants compared to constant light. Most studies looking at WUEi in fluctuating light environments do not compare this to the WUEi in constant light environments. Instead, it has been assumed plants grown under constant light have a greater WUE than plants grown under fluctuating light, which this study has shown.

However, a study by Kimura et al. (2020) compared WUEi in plants grown under short light fluctuations and plants grown under long light fluctuations. The Kimura et al. (2020) study found that WUEi in wild type *Arabidopsis thaliana* was greater in the short light fluctuations than under the long light fluctuations. Suggesting that long light fluctuations have a greater impact on WUEi than short light fluctuations. This disagrees with what was found in this study. In this study there was not a significant difference in the WUEi between plants in the long and short fluctuating light treatment, differences in WUEi were only observed between plants in the constant light and plants in fluctuating light treatments. The light fluctuations used in the Kimura et al. (2020) study were recreations of measured light fluctuations in a rice field for the long fluctuations. While the light fluctuations in the short light treatment were recreations of measured light fluctuations on a forest floor. As these are recreations of measured light fluctuations, they will not occur at regular times or occur for set periods of time, unlike the fluctuations used in this study. The light intensities used, and the total daily irradiance received by plants also differed between the studies. These differences in light regime are likely responsible for the differences seen in WUEi in fluctuating light between this study and the Kimura et al. (2020) study.

4.7 A greater stomatal density increases stomatal conductivity

The high stomatal density mutant *epf1epf2* displayed the greatest abaxial stomatal conductivity. This was expected as stomatal conductivity, a measure of the rate at which gasses pass through stomata, is measured per unit area. Hence, it is expected that plants with more stomata per unit area will have a greater rate of gas exchange per unit area. This finding agrees with other studies that have also found that the *epf1epf2* high stomatal density genotype displays a greater stomatal conductance compared to wild type (Hepworth et al., 2015). This increase in stomatal conductivity is not exclusive to the *epf1epf2* genotype, with the low root hair, high stomatal density mutant *rs14 rhd6 rsl1* also displaying a greater stomatal conductivity compared to wild type *Arabidopsis thaliana* (Hepworth et al., 2016). This indicates that the increased stomatal conductivity is not due to pleiotropic effects in the *epf1epf2* mutant but is instead due to the increased stomatal density caused by mutations to the *EPF1* and *EPF2* genes.

However, the low stomatal density mutant *EPF2OE* did not display a significantly lower abaxial stomatal conductivity than wild type. The wild type displayed the lowest

stomatal conductivity, which was not expected. However, previous studies have found that under water stress the *EPF2OE* genotype displays a greater stomatal conductivity than expected (Hepworth et al., 2015). While plants in this study were well-watered, the exact amount of water given to plants throughout this study was not controlled. If plants were water stressed, this may have contributed to the higher-than-expected stomatal conductivity exhibited by the *EPF2OE* genotype. Although water stress would not explain why the Col-0 genotype displayed the lowest stomatal conductance. As under water stress conditions the *epf1epf2* genotype would be expected to display the lowest stomatal conductance, and under non-water stressed conditions *EPF2OE* would be expected to show the lowest stomatal conductance.

The *EPF2OE* genotype displays a greater adaxial stomatal conductivity than Col-0. This is likely due to the increased adaxial stomatal density seen in the *EPF2OE* genotype. These additional adaxial stomata that were observed in the *EPF2OE* genotype could have a function in gas exchange, increasing the total *EPF2OE* stomatal conductivity. However, the cause of the higher abaxial stomatal conductance in the *EPF2OE* genotype compared to the Col-0 genotype is unclear.

Across all three genotypes the adaxial side of the leaf displayed lower stomatal conductivity than the abaxial side of the leaf. This was expected as for all the genotypes, except the *EPF2OE* plants grown under the long light treatment, as the abaxial stomatal density was greater than the adaxial stomatal density in the majority of plants. The difference between mean abaxial and mean adaxial stomatal conductivity was smallest in the *EPF2OE* genotype. This is expected due to the higher adaxial stomatal density in the *EPF2OE* genotype. This means that the additional adaxial stomata in the *EPF2OE* genotype are functional, and they have a role in gas exchange. Similarly to adaxial stomatal density, adaxial stomatal conductance is rarely studied. This again highlights the need for more research into adaxial stomata and their function. One study did compare adaxial and abaxial stomatal density in *Arabidopsis thaliana*, it found that abaxial stomatal conductance was higher than adaxial stomatal conductance (Driesen et al., 2023). This supports what was seen in this study, where abaxial stomatal conductance was also higher than adaxial stomatal conductance. However, the Driesen et al (2023) study did not look at the *EPF2OE* or the *epf1epf2* mutants.

The genotype of the plant was not found to have a significant effect on adaxial stomata conductivity. This is despite there being significant differences in the adaxial stomatal density between genotypes. This was unexpected, as a higher stomatal density per mm² should result in higher gas exchange through stomata per mm². However, this does support the idea that adaxial and abaxial stomata have different functional roles. This theory has been previously supported by Wang et al. (1998) which found that abaxial and adaxial stomata had differing responses to abscisic acid and calcium ions, both of which are common stimuli for stomatal closure.

4.8 Stomatal conductivity was not different between the daytime and nighttime

Across all three genotypes there was not a significant difference between the stomatal conductivity during the daytime and during the nighttime. Although daytime stomatal conductivity was higher than nighttime stomatal conductivity in all three genotypes. The fact that daytime and nighttime stomatal conductivity did not significantly differ was unexpected, as stomata are predicted to close at night. This stomatal closure is thought to

limit transpiration and save water, to improve plant fitness (Caird et al., 2007). Thus, it was expected that stomatal conductivity would be higher in the day than in the night, while this was observed it was not significant. This result was likely seen as daytime and nighttime measurements were not taken on the same plants. Differences in watering and subsequent differences in water potential between the plants measured in the day and the plants measured in the night likely explains why differences were not seen between the daytime and nighttime measurements. The temperature in the growth room was reduced at night; mimicking the natural decrease in temperature observed at nighttime. Thus the higher than expected nighttime stomatal conductivity was unlikely to be due to a need for stomatal cooling (Gommers, 2020). Under water scarce conditions, plants reduce their stomatal conductivity to limit water loss. So, if plants were water stressed when measured they would display a lower stomata conductivity than seen under well-watered conditions. Plants in this study were meant to be well-watered, but the water given to plants wasn't measured and so the water level plants received will have varied between light compartments. Thus, water stress will have differed between the plants measured for nighttime and daytime stomatal conductivity, this could explain the differences seen in stomatal conductivity between these plants.

Previously, it was thought that stomata close fully at night. However, total nighttime stomatal closure would result in no nighttime stomatal conductivity, as gasses would not be able to move through these closed stomata. Therefore, there must be some degree of stomatal openness at nighttime to explain this nighttime stomatal conductivity. Nighttime stomatal conductivity has also been found in other studies, looking at a range of different species (Resco de Dios et al., 2019) (Yu et al., 2019). A number of different theories have been developed to explain why plants display this nighttime stomatal openness. One of these theories is partial stomatal closure, so called "leaky stomata" (Barbour et al., 2006). The "leaky stomata" theory states that fully closing stomata can be energetically expensive for plants, so plants will only fully close their stomata when under water stress conditions (Wang et al., 2021). It has also been hypothesized that partial nighttime stomatal openness occurs due to the need for transpiration. This hypothesis states that transpiration is needed to deliver nutrients and oxygen to cells in the plant (Dawson et al., 2007). Transpiration is also used to lower leaf temperature, in a process known as leaf cooling (Coupel-Ledru et al., 2016). Plants in this study will not have been heat stressed, and thus leaf cooling is unlikely to be the reason for the nighttime stomatal conductance observed in this study. Another theory states that having partially open stomata at night could help to prevent a toxic build-up of CO₂, which could interfere with plant metabolism (Marks and Lechowicz, 2007). Lastly, it has been hypothesised that plants open their stomata before sunrise to facilitate photosynthesis earlier in the day (Resco de Dios et al., 2016). However, this is unlikely to be the reason for the nighttime stomatal conductance observed in this study, as nighttime measurements in this study were taken towards the beginning of the night, not towards the end of the night.

The stomatal conductivity in the daytime and nighttime follows the same patterns. In both the daytime and the nighttime, the stomatal conductivity was highest in the *epf1epf2* genotype and lowest in the Col-0 genotype. This aligns with findings from a study by Chieppa et al. (2021) where a higher nighttime stomatal conductivity was associated with a higher daytime stomatal conductivity. The Chieppa et al. (2021) study also linked a higher stomatal density with a higher daytime and nighttime stomatal conductivity, which agrees with the findings of this study.

4.9 Daytime photosynthetic induction was highest in the *epf1epf2* genotype

The *epf1epf2* genotype displayed the fastest daytime induction rate in each light treatment. This agrees with findings by Sakoda et al. (2020), which found that *epf1* mutants displayed faster rates of photosynthetic induction. These findings were expected as photosynthetic induction rate was predicted to be faster in plants with faster stomatal kinetics. As, these plants can quickly increase their stomatal aperture in response to the light intensity increasing. An increased stomatal aperture is linked to an increase in gas exchange. Quickly increasing gas exchange in plants where CO₂ concentration is the limiting factor for photosynthetic rate can increase the photosynthetic rate (Kaiser, 2009). The *epf1epf2* genotype has a greater abaxial stomatal density and stomatal density is inversely proportional to stomatal size (Hetherington and Woodward, 2003). So, whilst not measured in this study, previous studies have found that the *epf1epf2* genotype has smaller stomata (Franks et al., 2015). Leaves with smaller stomata have been found to display higher rates of gas exchange (Drake et al., 2013). These past findings suggest that the high density, small stomata mutant *epf1epf2* would be expected to display greater stomata kinetics, and thus higher rates of photosynthetic induction. This was observed in this study for the daytime photosynthetic induction.

The *EPF2OE* genotype was expected to show the lowest photosynthetic induction rate, but this was only seen in the fluctuating light treatments, not in the constant light treatment. Previous studies have shown that genotypes with a lower stomatal density display slower stomatal kinetics when grown under constant light (Xiong et al., 2022b). Photosynthetic induction rates are impacted by light intensity, but also by VPD and CO₂ concentration. The daytime photosynthetic induction rate in Col-0 was lower than the *EPF2OE* mutants in constant light in repeat two of the experiment, which was unexpected. While it isn't fully clear why this result was observed, it may be due to a high CO₂ concentration, high VPD or drought stress during measurements. As any of these environmental conditions would promote stomata closure, this would act against the increased light intensity stimulating the stomata to open (Araújo et al., 2011).

Most studies investigating the impact of stomatal density on photosynthetic induction in fluctuating light intensities have only looked at plants grown under constant light and measured under fluctuating light. They have not looked at plants grown and measured under fluctuating light, as this study does. The method used in other studies means that any impact of growing under fluctuating light is missed. These impacts on photosynthetic induction could be positive, for example a priming response, or negative, for example a light induced stress response (Marler, 2020, Violet-Chabrand et al., 2017). The daytime photosynthetic induction rate is lowest in the short fluctuating light treatment. This suggests that growing under short fluctuating light reduces a plant's ability to induce photosynthesis upon an increase in light intensity. So instead of priming plants to quickly induce photosynthesis upon an increased light intensity, growing under the short fluctuating light treatment actually stresses the plant. This light stress reduces the plant's ability to respond to increases in light intensity and delays photosynthetic induction. The daytime photosynthetic induction rate in the short light treatment is highest in the *epf1epf2* genotype. This suggests that the *epf1epf2* genotype is best able cope with the short fluctuating light treatment, as the photosynthetic induction rate is least impacted by short fluctuating light in the *epf1epf2* genotype. However, the differences in photosynthetic induction rates between light treatments are not significant.

A study by Soleh et al. (2016) found that differences in photosynthetic induction between different soybean genotypes were mostly due to the activation speed of the carboxylation process. The carboxylation process involves the incorporation of CO₂ to RuBP, this is catalysed by RuBisCo (Soleh et al., 2016). The activation speed of the carboxylation process will be impacted by both the carboxylation capacity of the rubisco enzyme, and by the availability of CO₂. This supports the findings of this study that having a greater stomatal density, and hence a greater abaxial stomatal conductance, which increases the ability of the plant to take in CO₂, will increase the plant photosynthetic induction rate.

4.10 Photosynthetic induction differs in the nighttime compared to the daytime

Nighttime photosynthetic induction is poorly understood. In the dark photosynthesis cannot occur, so photosynthesis occurring during the night is due to artificial light being given to the plant. Photosynthetic induction rates during the nighttime, do not just reflect the ability of a plant to respond to increases in light intensity. This is due to the impact of the circadian clock on the expression of genes required for photosynthesis. Genes involved in light harvesting, such as the chlorophyll a/b binding protein (LHCB), cycle with a circadian period (Kloppstech, 1985). This means that during the day there are higher levels of LHCB available, and plants can better use the available light, this should theoretically increase the photosynthetic induction rate in the daytime relative to the nighttime. This was not what was observed in this study, with some rates of photosynthetic induction being higher during the day and some being lower during the day. In repeat two of the experiment the nighttime photosynthetic rate was lower in the long fluctuating and constant light treatment, with the exception of Col-0 plants grown under the constant light treatment. A reduced nighttime photosynthetic rate would be expected if photosynthetic induction was influenced by the circadian clock. However, the nighttime photosynthetic induction rate was higher than the daytime photosynthetic induction rate for plants in the short fluctuating light treatment across all genotypes in repeat two and repeat three. If the short fluctuating light treatment induces stress in the plants, that reduces the photosynthetic induction rate, a period without this stress during the night, when the light is off, will induce less stress in the plant than is seen during the daytime. This “low stress” period during the night likely allows plants grown in the short fluctuating light treatment, which causes the highest amount of light stress, to induce photosynthesis faster during the nighttime.

The effect of this “low stress” period is also seen with the average carbon assimilation results, with the daytime carbon assimilation being higher than the nighttime carbon assimilation in the constant and long fluctuation light treatment. Whilst the nighttime carbon assimilation for plants grown in the short fluctuating light treatment, is higher than the daytime carbon assimilation. This again is likely due to there being less light stress at nighttime compared to daytime for plant in the short fluctuating light treatment. This means that during the nighttime plants have had time to recover from the light stress before measurements of carbon assimilation are taken.

The differences in nighttime photosynthetic induction rates between the genotypes do not follow an expected pattern. As, the *EPF2OE* genotype displays the highest nighttime photosynthetic induction rate in the long fluctuating light treatment in repeat two and three, when it would be expected to show the lowest photosynthetic induction rate. The Col-0 genotype displays the highest nighttime photosynthetic induction rate in the constant light treatment in both repeats. The *epf1epf2* genotype displays the lowest nighttime

photosynthetic induction rate in the long fluctuating light treatment, but the highest in the short fluctuating light treatment in repeat two.

4.11 Fluctuating light induced light stress in plants

Average daytime carbon assimilation was lowest in plants grown under the short fluctuating light treatment. This reduction in carbon assimilation is likely seen due to the short fluctuating light treatment causing light stress within the plant. Plants grown under fluctuating light conditions, in previous studies, have been found to have a slow growth rate in early development, this was hypothesised to be due to these plants not being able to fully utilise the light energy absorbed for carbon fixation (Violet-Chabrand et al., 2017). The plants with the highest average daytime carbon assimilation were grown under the constant light treatment, with the exception of the Col-0 genotype under low light in repeat three, this supports the idea that fluctuating light induces light stress which impacts the carbon assimilation in these plants.

The NPQ levels induced during one second light fluctuations were lower in plants grown under the constant light treatments, than in plants grown under the fluctuating light treatment. This supports the idea that growth under light fluctuations induces light stress in the plant and negatively impacts the plant's ability to cope with light fluctuations, as opposed to priming the plant to better cope with light fluctuations. Plants grown under the constant light treatment also displayed lower NPQ induction under one minute light fluctuations in the FlourCam, suggesting that these plants are best able to handle the light stress. This supports the idea that growing plants under light fluctuations negatively impacts a plants ability to tolerate fluctuating light, instead of priming plants for future fluctuations.

This is at odds with a previous study by Marler (2020), which found that light flecks increased the amount of photosynthesis a plant was able to carry out during a subsequent light fleck. So, the Marler (2020) study supports a priming effect of fluctuating light during plant growth on a plant's ability to photosynthesise and cope with light stress in subsequent periods of transient high light. This study disagrees with Marler (2020), as a priming effect of the fluctuating light treatments has not been observed in this study. In the Marler (2020) study, natural light was used with artificial shading to create varying levels of shade. The study was conducted in a greenhouse, with the light intensity and the spectral quality of light varying depending on the weather. However, in this study all the light used was artificial and so the intensity and length of high periods of light could be controlled. The priming light flecks experienced by plants in the Marler (2020) study were more frequent and shorter in duration than the periods of light used in fluctuating light treatments in this study. These differences in the intensity, duration, and frequency of light fluctuations likely explains the differences found in the priming capabilities of fluctuating light between the Marler (2020) study and this study. The Marler (2020) study also uses periods of high light interspersed with periods of low light, while this study uses periods of light interspersed with periods of no light. The transition from no light to light, may induce more stress within a plant than the transition from low light to high light. Thus, the light regime use in this study may have induced more stress than the light regime used in the Marler (2020) study, preventing the priming effect from being observed.

Fluctuating light has been shown to induce long term acclimation responses in plants within a few hours (Terashima et al., 2005). These acclimation responses include cell elongation and shoot elongation; these are shade avoidance responses that aim to move the leaf out of the shaded region (Terashima et al., 2005). However, shade avoidance

responses will not improve plant performance under the fluctuating light used in this study as there is not an unshaded region into which the plant can grow. This lack of an unshaded region in the light treatments, rendering one type of acclimation not possible, could explain why priming by fluctuating light was not seen in this study. As if long-term acclimation to shade did occur but as there was no higher light intensity that the plant could grow into the positive effects of this acclimation to fluctuating light would be lost. This shows the importance of testing plants under field conditions as it is not always possible to accurately replicate the dynamic light environments found in crop canopies in a growth room.

The light treatments only had a significant impact on NPQ in this study when measured with a FlourCam and the Dual Pam with one second fluctuations, but not when measured with a Dual Pam with one minute fluctuations. The different results observed between the different methods are unlikely due to differences in the duration of fluctuations between the Dual Pam protocols. As at the end of the warmup phase before the light fluctuation programs began the genotypes with the highest and lowest NPQ levels differed between the two Dual Pam protocols. This suggests that differences seen during fluctuations were not due to differences in the fluctuations themselves as they were present before the actinic light fluctuation programs began.

4.12 The *EPF2OE* genotype adjusts its' chlorophyll content to acclimate to dynamic light conditions

When grown under the long fluctuating light treatment the *EPF2OE* genotype has a higher chlorophyll a/b ratio than it does when grown under the other light treatments. However, levels of chlorophyll a and b in the *EPF2OE* genotype are not affected by the light treatments. Chlorophyll a is the primary light harvesting pigment used by *Arabidopsis thaliana*, while chlorophyll b is an accessory pigment. Chlorophyll b primarily absorbs light energy in the blue region of the light spectrum; chlorophyll b then transfers this light energy to the chlorophyll a pigments (Tanaka and Tanaka, 2011). While chlorophyll a pigments absorb in the blue-violet and red regions of the light spectrum. The chlorophyll a pigment is essential to photochemistry and is common to nearly all oxygenic photosynthetic organisms (Tanaka and Tanaka, 2011). Chlorophyll a can be converted into chlorophyll b through the chlorophyll cycle, thus the ratio of chlorophyll a to chlorophyll b can be informative into how this cycle is running in different plants. The chlorophyll a/b ratio was higher in the long light treatment for the *EPF2OE* genotype. This may be due to chlorophyll b pigments being converted to chlorophyll a pigments via the chlorophyll cycle (Ito et al., 1996). As despite the chlorophyll a and chlorophyll b content not being significantly different between the constant and long light treatment in the *EPF2OE* genotype, the average chlorophyll a content is higher in the long light treatment compared to the constant light treatment. Little is understood about what regulates the forward reaction (conversion of chlorophyll a to chlorophyll b) of the chlorophyll cycle, but high chlorophyll a/b ratios have been linked to plant acclimation to high light stress (Tanaka and Tanaka, 2011, Kitajima and Hogan, 2003). The low chlorophyll a/b ratio seen in the constant light in the *EPF2OE* genotype could be due to the low light intensity used for this light treatment, as chlorophyll b synthesis is enhanced under low light intensities (Kume et al., 2018). Chlorophyll b is more efficient at absorbing light energy at low light intensities, so in the constant light treatment the *EPF2OE* genotype is able to acclimate to a low light intensity by reducing the chlorophyll a content relative to the chlorophyll b content. Low chlorophyll b content has been linked to an increased sensitivity to photoinhibition in plants (Leverenz et al., 1992). The *EPF2OE* plants

grown in constant light display high chlorophyll b content relative to chlorophyll a content, so would be expected to show a reduced sensitivity to photoinhibition.

A previous study found that silencing of the *EPF1* gene does not have a significant effect on chlorophyll content (He et al., 2025). This agrees with the findings of this study, as total chlorophyll content was not significantly different in the *epf1epf2* genotype. There are no previous studies linking *EPF* genes to chlorophyll a/b ratios or the chlorophyll cycle, so the reason why the chlorophyll a/b ratios acclimate to the light treatment in some genotypes but not in other genotypes is unknown. As acclimation is only observed in the mutant genotypes, there may have been unknown off target or pleiotropic effects of mutations to the *EPF* genes.

4.13 Differences in WUE were not due to differences in root growth

The genotype and the light treatment the plants were grown under were found not to have a significant impact on root growth or architecture. Root growth can impact WUE in plants by altering the plant water uptake depth. Increased water uptake depth is linked to a reduced WUE (Li et al., 2025). As, there are no significant differences in root growth amongst the different genotypes or light treatments, differences in root growth between plants are not responsible for differences in WUE seen between the genotypes and the light treatments in this study. This means that differences seen in WUE are likely due to factors affecting the stomata and stomatal kinetics. Studies in other species have also found that plant performance under drought conditions was best explained by anatomical traits of stomata, xylem, and veins (Jhaveri et al., 2024). The Jhaveri et al. (2024) study found investing in below ground tissue did not aid in plant growth during drought, indicating that differences in root growth did not improve plant WUE.

Previous studies have disagreed with each other about the impact of the *EPF* genes on root growth. This study agrees with the Mohammed et al. (2019) study and the Mawodza et al. (2022) study that the *EPF2OE* and the *epf1epf2* genotypes does not display significantly different root growth or root architecture. However it disagrees with the Hepworth et al. (2016) study which found that plants with a higher stomatal density displayed a larger rooting area. The Hepworth et al. (2016) study suggests that the *epf1epf2* genotype should show greater root growth, which was not what was found in this study. The Hepworth et al. (2016) study and this study used similar methods to study root architecture. Both studies used *Arabidopsis thaliana* grown on Murashige and Skoog medium. However the Hepworth et al. (2016) study used shorter day conditions (9 hours compared to 16 hours), the seeds in the Hepworth et al. (2016) study were also stratified for a greater length of time. Day length is known to impact root growth, with plants growing under longer day conditions displaying greater root systems (Xu et al., 2023). The longer day length in this study compared to the Hepworth et al. (2016) study could explain why differing results were found. The impact of stratification time on the root architecture and growth is unclear.

Total root growth differed significantly between the plants grown during the day and the plants grown during the night. The circadian clock is known to play a role in regulating root elongation, with root elongation peaking at dawn (Yazdanbakhsh et al., 2011). Growing plants in the night could have a negative effect on the circadian regulation of genes involved in root elongation, this is because exogenous cues such as temperature can be used as entrainment signals for the circadian clock (Bustillo-Avedaño et al., 2022). The lower temperatures in the night in the growth room could be counteracting the light signals giving

the plant conflicting messages about the time of day. This could have a negative effect on the circadian control of root elongation in the nighttime plants, causing differences in root length between the daytime and the nighttime plants. Lateral root development has previously been found to be controlled by the circadian clock (Voß et al., 2015). Thus, it would be expected for there to also be a difference in the number of lateral roots between the daytime and the nighttime plants, which was not observed.

4.14 Future studies

To better understand the implications of these results, future studies could address the role of adaxial stomata in the low stomatal density plants. This study has shown that adaxial stomata in the *EPF2OE* mutant display a greater stomatal conductivity compared to adaxial stomata in the other genotypes. In the *EPF2OE* genotype adaxial stomata behave in a manner similar to that of abaxial stomata. Future studies could use a range of low stomatal density plants to check if the adaxial stomata display this increased stomatal conductivity only in *EPF2OE* plants or if this is seen in all low stomatal density plants. Investigating low stomatal density mutants could also aid in the discovery of genes involved in the adaxial stomatal development pathway, as the regulation of this pathway is believed to be controlled by different genes to those that regulate the abaxial stomatal development pathway (Jalakas et al., 2024). If increased adaxial stomatal conductivity is only seen in the *EPF2OE* plants, then this could elucidate a role for the *EPF2* gene in the response of adaxial stomata to opening and closing stimuli. If the increased stomatal conductivity is seen in all low stomatal density mutants, then the higher adaxial stomatal conductivity is more likely to represent a compensatory increase in adaxial stomatal conductivity to offset a lower abaxial stomatal conductivity in low stomatal density mutants. Additionally, this study found that the *EPF1* and *EPF2* genes play a different role in the abaxial stomatal development pathway than they do in the adaxial stomatal development pathway. *EPF* genes are known to play a regulatory role in the abaxial stomatal development pathway, but their role in the adaxial stomatal pathway is unclear. Further research on this is needed, especially as it seems that adaxial stomata can compensate in plants with a reduced abaxial stomata density. Thus, understanding what controls development of adaxial stomata could help in the discovery of any internal signals between the abaxial and adaxial stomatal development pathways.

The low stomatal density mutant, *EPF2OE*, was found to have a low chlorophyll a/b ratio under constant light. This suggests that *EPF2* favours the forward reaction of the chlorophyll cycle lowering the chlorophyll a/b ratio. As increasing *EPF2* expression under constant light results in less chlorophyll a relative to chlorophyll b, as chlorophyll a is used in the forward reaction to produce chlorophyll b. However, the *EPF2* gene does not have a known role in the chlorophyll cycle. Further research is needed to see if there is any interaction between *EPF2* and known components of the chlorophyll cycle that could promote the forward reaction of the chlorophyll cycle. This would show whether the *EPF2OE* plants show a lower chlorophyll a/b ratio due to a direct role of *EPF2* in the chlorophyll cycle or whether the low chlorophyll a/b ratio is due to another cause, such as pleiotropic effects.

This study differed from other studies looking at dynamic light environments, as the light fluctuations used went from no light to light. Other studies, such as the Marler (2020) study, fluctuated light intensities from low to high, as opposed to on to off. This study found a differing priming effect of growing under fluctuating light treatments than the Marler (2020) study. Studies investigating the differences between plants growing in on/off light

fluctuations compared to low/high light fluctuations would be useful in determining whether standardisation of light fluctuations used in dynamic light research is necessary. Currently studies vary in whether they use on/off or low/high light fluctuations making results difficult to compare as the impact of this difference is currently unknown. Knowing the impact of this difference is also important as many tools used for measuring the impact of dynamic light use low/high light fluctuations, not on/off fluctuations. If there is a large difference between the impact on a plant of on/off light fluctuations compared low/high light fluctuations, measuring tools will also need to be standardised to allow for better comparison of results taken using different measuring tools.

Nighttime photosynthetic rates were found in this study to differ from daytime photosynthetic rates. Little is known about the regulation of nighttime photosynthesis. The role of the circadian clock in controlling photosynthetic induction rates during the night is not fully understood. There are a number of important genes involved in photosynthesis that are controlled by the circadian clock. To test the impact of circadian clock genes on nighttime photosynthesis, future studies could look at daytime and nighttime photosynthesis in circadian clock mutants. This would allow the impact of these circadian genes on photosynthetic regulation to be assessed. Transcription assays looking at the level of gene transcripts for key photosynthetic genes at night would help show which circadian clock gated photosynthesis genes were responsible for the regulation of photosynthesis at nighttime. This would help to identify candidate photosynthetic circadian clock genes for mutation studies. In the short light treatment, the nighttime photosynthetic rate exceeded that of the daytime photosynthetic rate. Carrying out these transcription assays under different light treatments would allow for testing of the causes of higher nighttime photosynthetic rate under the short light treatment. As, this could either be due to differences in the expression of circadian clock genes, or it could be due to reduced light stress at nighttime.

4.15 Implications of these findings for crop plants

The key problem that this research aimed to address was does increasing stomatal density improve a plant's photosynthetic performance under dynamic light conditions. This is an important question to address as plants grown in the field have to deal with fluctuating light environments. Light is critical to photosynthesis, so it is important to understand how rapidly changing light intensity impacts photosynthesis. Crops in some parts of the world are increasingly at risk of drought, due to climate change causing rising temperatures. In response to this, it has been suggested that stomatal density in crops could be reduced to improve WUE (Franks et al., 2015). It is important to understand how reducing stomatal density would impact photosynthesis under the fluctuating light conditions that would be found in the field. This study has shown that reducing stomatal density does not improve WUE under fluctuating light conditions. This calls into question the usefulness of reducing stomatal density to improve WUE, if this increased efficiency is not observed under the fluctuating light and is unlikely to be observed in field conditions. It is likely that under field conditions drought tolerance in plants with a lower stomatal density would be increased, but this would be at the expense of yield due to a lower photosynthetic induction rate. This study also found that high stomatal density plants were best able to cope with the short fluctuating light treatment, although all genotypes fared best under the constant light treatment. This suggests that reducing stomatal density in order to cope with drought, as

suggested in the Franks et al. (2015) paper, could have a negative impact on photosynthesis under field conditions, which would negatively impact yield.

A study in rice (*Oryza*) plants by Xiong et al. (2022b) looked at the impact of stomatal density on photosynthetic induction under fluctuating light. The Xiong et al. (2022b) study found that plants with a higher stomatal density had faster rates of stomatal responses to changes in light intensity and faster rates of photosynthetic induction. The plants with greater stomatal densities also exhibited greater WUE. This supports what was found in this study that the high stomatal density *epf1epf2* mutants displayed a greater photosynthetic induction rate and a greater WUE under fluctuating light compared to wild type and low stomatal density mutants. The Xiong et al. (2022b) study looked at 12 rice (*Oryza*) genotypes that displayed natural variation in stomatal density, it did not look at *EPF* gene mutants. The fact that the results in this study and the results in the Xiong et al. (2022b) study agree with each other suggests that these findings are likely to be consistent across different species, with different types of stomatal morphology and across both natural variation in stomatal density and variation due to mutations in genes involved in the stomatal development pathway. Whilst the impact of stomatal density on photosynthesis in fluctuating light environments needs to be tested in other crop species to see if the same conclusions are drawn, the agreement between this study and the Xiong et al. (2022b) study suggests that the conclusions drawn from this study are likely to be applicable to a range of different crop species and to both natural and mutation caused variation in stomatal density.

Most previous studies using *EPF* mutant plants, have used *Arabidopsis thaliana*, as this study also does. *Arabidopsis thaliana* plants are amphistomatous, this means that they have stomata present on both their abaxial and their adaxial surface (Jalakas et al., 2024). However, most plants are hypostomatous, meaning that they only have stomata present on their abaxial surface or that they only have few stomata present on their adaxial surface (Drake et al., 2019). As little is known about adaxial stomata and it is still unclear whether there is signalling between abaxial and adaxial stomatal development, with studies coming to different conclusions, studies need to be carried out in amphistomatous and hypostomatous crop species. This is because we do not fully understand how the presence of adaxial stomata in *Arabidopsis thaliana* is impacting the stomatal density and kinetics of abaxial stomata.

Studies conducted in rice (*Oryza*) *EPF* mutant plants, looking at *OsEPF* loss of function mutations have found that *OsEPF* genes have a role in the stomatal development pathway with *OsEPF* loss of function mutants displaying a greater stomatal density (Xiong et al., 2022a). However, these *OsEPF* loss of function mutants also displayed a role in the development of the awn. The awn is an organ found in some grass species that is thought to have a role in herbivory defence and seed dispersal (Suganami et al., 2024). Hence, when using *EPF* gene mutations to alter stomatal density in crop plants the role of *EPF* genes in other developmental pathways also needs to be considered.

Most studies looking at the role *EPF* genes in crop plants have focused on members of the *Poaceae* family, this is because the *Poaceae* family contains economically important crops, such as, wheat (*Triticum*), barley (*Hordeum*), and rice (*Oryza*) (Yoshioka et al., 2017) (Hughes et al., 2017) (Caine et al., 2019). These studies in *Poaceae* crops have found that over expressing *EPF* genes to reduce stomatal density improves drought tolerance (Hughes et al., 2017) (Caine et al., 2019). This is at odds with what was observed in this study, where plants with the highest stomatal density displayed the greatest WUE. However, plants in this study were grown under fluctuating light, whilst the plants in the Hughes et al. (2017) and

the Caine et al. (2019) study were grown under constant light in a growth chamber, this is not reflective of field conditions. The results from this study suggest that fluctuating light reduces the WUE in plants with a low stomatal density, due to a slow photosynthetic induction rate in these plants. This highlights the importance of studying plants under field conditions, where the light environment is dynamic.

There have been few studies on *EPF* mutant plants in crops outside of the *Poaceae* family. It is important to study these crops as they will have a different stomatal morphology to plants in the *Poaceae* family. Studies in crop plants from non-*Poaceae* families will be important to verify that *EPF* genes behave in the same manner in these crops as they do in *Arabidopsis thaliana*.

A study of the impact of fluctuating light on drought tolerance in rice (*Oryza*) found a link between the response of stomata to fluctuating light and the WUE of these plants in the field (Qu et al., 2016). The Qu et al. (2016) study found that plants with a faster stomatal closing response relative to the stomatal opening response under fluctuating light displayed a greater WUE. The Qu et al. (2016) study demonstrated the trade-off for plants between maximising photosynthetic light use efficiency which is greatest with faster stomatal opening and reducing water loss which is greatest with faster stomatal closing. Additionally, the Qu et al. (2016) study raises the important point that stomatal opening and closing times can be different from each other. This study looked at stomatal response to increasing light intensity through measurements of CO₂ assimilation, thus it only looked at stomatal opening not stomatal closure. As stomatal opening and closure responses can be different, they should both be looked at in future studies. The Qu et al. (2016) study looks at rice plants, the authors make the point that rice plants are typically grown in paddy fields, which are wet environments. These conditions favour optimisation of stomatal responses for photosynthetic light use efficiency over WUE in rice plants. As such the growth environment of crops needs to be considered when attempts to improve drought tolerance through altering stomata are made.

In summary, the primary implication of these findings for crops is that any research done on the WUE of crop plants with a reduced stomatal density needs to be done under field conditions and needs to take into account the normal growth environment of the crop species used. The current view in the field that stomatal density can be reduced to improve WUE and drought tolerance ignores the negative impact that low stomatal density has on photosynthetic induction under fluctuating light conditions.

This prompts the need to find alternative methods to increase crop drought tolerance due to the risk of drought in the future as a result of climate change. As it is unlikely that reducing stomatal density will improve drought tolerance when tested in the field without negatively impacting yield due to reduced photosynthetic induction rates in low stomatal density plants. A potentially more promising path to increasing crop drought tolerance could be through increasing stomatal kinetics. There are a number of different methods through which this could be done, one of which is to express light gated potassium ion channels in guard cells. A study by Papanatsiou et al. (2019) did this in *Arabidopsis thaliana*, the plants possessing light gated potassium ion channels displayed a greater biomass under fluctuating light without impacting the plants WUE. So, changing the ion channels in guard cells does improve plant drought tolerance under fluctuating light, but it also required gene editing, which is a controversial technique that is not allowed in many countries.

5. Conclusion

This study aimed to address two main questions: what is the impact of stomatal density on CO₂ assimilation in dynamic light environments and what is the impact of stomatal density on intrinsic WUE? The results of this study indicate that plants with an increased stomatal density are better able to cope with dynamic light environments. As, the *epf1epf2* genotype displayed the greatest CO₂ assimilation and daytime photosynthetic induction in the short light treatment. This agrees with hypothesis two of this study that if plants have a higher stomatal density, then they will display faster rate of photosynthetic induction.

The *epf1epf2* genotype displayed a greater abaxial stomatal conductivity, this partially agrees with hypothesis one of this study that if plants have a higher stomatal density, then they will have greater g_s . However, genotype was not found to cause a difference in stomatal conductivity on the adaxial side of the leaf. This increased abaxial stomatal conductivity allows high stomatal density plants to maintain a high intercellular CO₂ concentration. So, when photosynthesis is able to occur, during light periods, the CO₂ required for photosynthesis is already inside the plant.

Whilst the *epf1epf2* genotype does the best in the short light treatment compared to other genotypes, all genotypes fair best in the constant light treatment. This is because growth in dynamic light environments does not prime plants for future light fluctuations, instead it causes light stress within the plants. This light stress reduces the plants' photosynthetic capabilities and increases NPQ in subsequent light fluctuations.

In addition to displaying greater resilience to dynamic light environments, plants with an increased stomatal density also display greater WUE. This is at odds with findings of previous studies, which have suggested that reducing stomatal density could be used as a strategy to increase drought tolerance in plants by increasing the plants WUE. It is also at odds with hypothesis three of this study, which is that if plants have a higher stomatal density, then the plants will have lower WUE. As, the *epf1epf2* genotype displays the highest photosynthetic induction rate, the higher WUE can be accounted for by an increased carbon fixation efficiency. This increases the molecules of CO₂ that can be fixed per molecule of water lost. Root growth and architecture do not differ between the genotype or the light treatments studied so differences in WUE can be confidently attributed to differences in shoot side process, such as differences in stomatal conductivity.

This increase in adaxial stomatal density in the *EPF2OE* genotype suggests that overexpressing the *EPF2* gene is not the most effective way to reduce stomatal density. The findings of this study highlight the importance of studying the adaxial stomata, the adaxial stomatal density and conductivity are rarely looked at in studies of *EPF* mutant plants. The adaxial stomatal development pathway is also poorly understood, although it is known to be different to the abaxial stomatal development pathway (Jalakas et al., 2024). Due to this, we do not know the role that *EPF* genes play in the adaxial stomatal development pathway.

This study grew plants under fluctuating light, as well as measuring plants under fluctuating light. This method showed that instead of having a priming effect on plants, growth under dynamic light induces a greater degree of light stress on plants compared to plants grown under constant light. Many studies looking at photosynthesis under dynamic light only measure under dynamic light they do not grow the plants under dynamic light. These studies miss the impact of acclimation to fluctuating light.

Carbon assimilation was chosen as the method by which photosynthetic performance was assessed, thus focusing on the light independent aspect of photosynthesis. This allowed for gas exchange through stomata to be analysed, but it missed any changes in light capture

due to changes in chlorophyll content. The carbon assimilation method was chosen as differences in gas exchange were expected due to the differing stomata densities between the genotypes. Whilst differences in chlorophyll content were not expected. As, the mutated *EPF* genes have a known role in the abaxial stomatal development pathway but are seemingly unconnected to the chlorophyll cycle.

The findings of this study have indicated that low stomatal density plants may be able to compensate for a lower abaxial stomatal density by increasing their adaxial stomatal density. This is important as it calls into question our ability to create plants with fewer stomata. It also raises important questions about the role of *EPF* genes in adaxial stomatal development. Adaxial stomata are rarely studied, and little is known about what regulates their development. This study has repeatedly showed the importance of measuring stomatal conductivity and density on the abaxial and adaxial sides of the leaf in *EPF* mutant plants. As the impact of *EPF* gene alteration on plant stomatal density differs between the abaxial and adaxial side of the leaf. Adaxial stomatal conductivity is frequently ignored in other studies as it is lower than abaxial stomatal conductivity, so it is thought of as negligible. Despite being lower, adaxial stomatal conductivity can still give useful insights into gas exchange in plants. This was shown in this study where, although adaxial stomatal conductivity was lower than abaxial stomatal conductivity, the gap between adaxial and abaxial stomatal conductivity between genotypes was informative. Hence this study has shown that there are key differences between the abaxial and adaxial sides of the stomata, and that going forward studies need to be carried out on both sides of the leaf.

This study also finds that the *epf1epf2* high stomatal density mutants are better able to cope with dynamic light environments compared to wildtype plants. *epf1epf2* plants also show greater WUE. This suggests that increasing stomatal density may represent a method to improve plant photosynthetic performance in field conditions and to boost drought tolerance. Few studies have previously looked at the impact of fluctuating light on WUE and the results of this study suggest that high stomatal density improves WUE in fluctuating light. This challenges previous assertions that reducing stomatal density improves WUE and highlights the importance of studying plants in dynamic light environments.

6. Bibliography

- ABIKO, T. & OBARA, M. 2014. Enhancement of porosity and aerenchyma formation in nitrogen-deficient rice roots. *Plant Science*, 215, 76-83.
- ADACHI, S., TANAKA, Y., MIYAGI, A., KASHIMA, M., TEZUKA, A., TOYA, Y., KOBAYASHI, S., OHKUBO, S., SHIMIZU, H. & KAWAI-YAMADA, M. 2019. High-yielding rice Takanari has superior photosynthetic response to a commercial rice Koshihikari under fluctuating light. *Journal of Experimental Botany*, 70, 5287-5297.
- AGURLA, S., GAHIR, S., MUNEMASA, S., MURATA, Y. & RAGHAVENDRA, A. S. 2018. Mechanism of Stomatal Closure in Plants Exposed to Drought and Cold Stress. *Adv Exp Med Biol*, 1081, 215-232.
- AINSWORTH, E. A. & LONG, S. P. 2005. What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO₂. *New Phytologist*, 165, 351-372.
- ALTER, P., DREISSEN, A., LUO, F.-L. & MATSUBARA, S. 2012. Acclimatory responses of Arabidopsis to fluctuating light environment: comparison of different sunfleck regimes and accessions. *Photosynthesis Research*, 113, 221-237.
- ARAÚJO, W. L., FERNIE, A. R. & NUNES-NESI, A. 2011. Control of stomatal aperture: a renaissance of the old guard. *Plant Signal Behav*, 6, 1305-11.
- ARMSTRONG MCKAY, D. I., STAAL, A., ABRAMS, J. F., WINKELMANN, R., SAKSCHEWSKI, B., LORIANI, S., FETZER, I., CORNELL, S. E., ROCKSTRÖM, J. & LENTON, T. M. 2022. Exceeding 1.5°C global warming could trigger multiple climate tipping points. *Science*, 377, eabn7950.
- ARNELL, N. W., LOWE, J. A., CHALLINOR, A. J. & OSBORN, T. J. 2019. Global and regional impacts of climate change at different levels of global temperature increase. *Climatic Change*, 155, 377-391.
- BAGLEY, M. C., STEPANOVA, A. N., EKELOF, M., ALONSO, J. M. & MUDDIMAN, D. C. 2020. Development of a relative quantification method for IR-MALDESI mass spectrometry imaging of Arabidopsis root seedlings. *Rapid communications in mass spectrometry: RCM*, 34, e8616.
- BARBOUR, M. M., CERNUSAK, L. A., WHITEHEAD, D., GRIFFIN, K. L., TURNBULL, M. H., TISSUE, D. T. & FARQUHAR, G. D. 2006. Nocturnal stomatal conductance and implications for modelling $\delta(18)\text{O}$ of leaf-respired CO₂ in temperate tree species. *Funct Plant Biol*, 32, 1107-1121.
- BEADLE, C. L. & LONG, S. P. 1985. Photosynthesis — is it limiting to biomass production? *Biomass*, 8, 119-168.
- BENGOA LUONI SA & DRIEVER SM 2025. Adding Time Dimension to Photosynthesis Studies: A Path Toward Linking Biomass to Photosynthesis.
- BERGMANN, D. C. & SACK, F. D. 2007. Stomatal Development. *Annual Review of Plant Biology*, 58, 163-181.
- BERTOLINO, L. T., CAINE, R. S. & GRAY, J. E. 2019. Impact of Stomatal Density and Morphology on Water-Use Efficiency in a Changing World. *Frontiers in Plant Science*, 10.
- BROWN, H. T. & ESCOMBE, F. 1901. Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Proceedings of the Royal Society of London*, 67, 124-128.

- BURGESS, A. J., DURAND, M., GIBBS, J. A., RETKUTE, R., ROBSON, T. M. & MURCHIE, E. H. 2021. The effect of canopy architecture on the patterning of “windflecks” within a wheat canopy. *Plant, Cell & Environment*, 44, 3524-3537.
- BUSTILLO-AVENDAÑO, E., SERRANO-RON, L. & MORENO-RISUENO, M. A. 2022. The Root Clock as a Signal Integrator System: Ensuring Balance for Survival. *Frontiers in Plant Science*, Volume 13 - 2022.
- CAINE, R. S., YIN, X., SLOAN, J., HARRISON, E. L., MOHAMMED, U., FULTON, T., BISWAL, A. K., DIONORA, J., CHATER, C. C., COE, R. A., BANDYOPADHYAY, A., MURCHIE, E. H., SWARUP, R., QUICK, W. P. & GRAY, J. E. 2019. Rice with reduced stomatal density conserves water and has improved drought tolerance under future climate conditions. *New Phytologist*, 221, 371-384.
- CAIRD, M. A., RICHARDS, J. H. & DONOVAN, L. A. 2007. Nighttime stomatal conductance and transpiration in C3 and C4 plants. *Plant physiology*, 143, 4-10.
- CALZADILLA, A., REHDANZ, K., BETTS, R., FALLOON, P., WILTSHIRE, A. & TOL, R. S. J. 2013. Climate change impacts on global agriculture. *Climatic Change*, 120, 357-374.
- CAZZANIGA, S., DALL' OSTO, L., KONG, S.-G., WADA, M. & BASSI, R. 2013. Interaction between avoidance of photon absorption, excess energy dissipation and zeaxanthin synthesis against photooxidative stress in Arabidopsis. *The Plant Journal*, 76, 568-579.
- CHARRIER, G. 2022. The hare and the tortoise: stomatal kinetics under light change in Poaceae crops. *Plant Physiol*, 189, 7-9.
- CHIEPPA, J., BROWN, T., GIRESI, P., JUENGER, T. E., RESCO DE DIOS, V., TISSUE, D. T. & ASPINWALL, M. J. 2021. Climate and stomatal traits drive covariation in nighttime stomatal conductance and daytime gas exchange rates in a widespread C(4) grass. *New Phytol*, 229, 2020-2034.
- CHOWDHURY, F. I., ARTEAGA, C., ALAM, M. S., ALAM, I. & RESCO DE DIOS, V. 2022. Drivers of nocturnal stomatal conductance in C3 and C4 plants. *Science of The Total Environment*, 814, 151952.
- CHRISTMAN, M. A., DONOVAN, L. A. & RICHARDS, J. H. 2009. Magnitude of nighttime transpiration does not affect plant growth or nutrition in well-watered Arabidopsis. *Physiologia Plantarum*, 136, 264-273.
- COSTA, J. M., MONNET, F., JANNAUD, D., LEONHARDT, N., KSAS, B., REITER, I. M., PANTIN, F. & GENTY, B. 2015. Open All Night Long: the dark side of stomatal control. *Plant Physiol*, 167, 289-94.
- COUPEL-LEDRU, A., LEBON, E., CHRISTOPHE, A., GALLO, A., GAGO, P., PANTIN, F., DOLIGEZ, A. & SIMONNEAU, T. 2016. Reduced nighttime transpiration is a relevant breeding target for high water-use efficiency in grapevine. *Proceedings of the National Academy of Sciences*, 113, 8963-8968.
- DAWSON, T. E., BURGESS, S. S. O., TU, K. P., OLIVEIRA, R. S., SANTIAGO, L. S., FISHER, J. B., SIMONIN, K. A. & AMBROSE, A. R. 2007. Nighttime transpiration in woody plants from contrasting ecosystems. *Tree Physiology*, 27, 561-575.
- DE BOER, H. J., LAMMERTSMA, E. I., WAGNER-CREMER, F., DILCHER, D. L., WASSEN, M. J. & DEKKER, S. C. 2011. Climate forcing due to optimization of maximal leaf conductance in subtropical vegetation under rising CO₂. *Proceedings of the National Academy of Sciences*, 108, 4041-4046.
- DE SOUZA, A. P., BURGESS, S. J., DORAN, L., HANSEN, J., MANUKYAN, L., MARYN, N., GOTARKAR, D., LEONELLI, L., NIYOGI, K. K. & LONG, S. P. 2022. Soybean

- photosynthesis and crop yield are improved by accelerating recovery from photoprotection. *Science*, 377, 851-854.
- DIETRICH, P., SANDERS, D. & HEDRICH, R. 2001. The role of ion channels in light-dependent stomatal opening. *Journal of Experimental Botany*, 52, 1959-1967.
- DING, Y., SHI, Y. & YANG, S. 2024. Regulatory Networks Underlying Plant Responses and Adaptation to Cold Stress. *Annual Review of Genetics*, 58, 43-65.
- DOHENY-ADAMS, T., HUNT, L., FRANKS, P. J., BEERLING, D. J. & GRAY, J. E. 2012. Genetic manipulation of stomatal density influences stomatal size, plant growth and tolerance to restricted water supply across a growth carbon dioxide gradient. *Philos Trans R Soc Lond B Biol Sci*, 367, 547-55.
- DOW, G. J., BERGMANN, D. C. & BERRY, J. A. 2014. An integrated model of stomatal development and leaf physiology. *New Phytologist*, 201, 1218-1226.
- DRAKE, P. L., DE BOER, H. J., SCHYMANSKI, S. J. & VENEKLAAS, E. J. 2019. Two sides to every leaf: water and CO₂ transport in hypostomatous and amphistomatous leaves. *New Phytologist*, 222, 1179-1187.
- DRAKE, P. L., FROEND, R. H. & FRANKS, P. J. 2013. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. *Journal of experimental botany*, 64, 495-505.
- DRIESEN, E., DE PROFT, M. & SAEYS, W. 2023. Drought stress triggers alterations of adaxial and abaxial stomatal development in basil leaves increasing water-use efficiency. *Hortic Res*, 10, uhad075.
- DUNCOMBE, S. G., BARNES, W. J. & ANDERSON, C. T. 2020. Chapter 11 - Imaging the delivery and behavior of cellulose synthases in *Arabidopsis thaliana* using confocal microscopy. In: ANDERSON, C. T., HASWELL, E. S. & DIXIT, R. (eds.) *Methods in Cell Biology*. Academic Press.
- DUNN, J., HUNT, L., AFSHARINAFAR, M., MESELMANI, M. A., MITCHELL, A., HOWELLS, R., WALLINGTON, E., FLEMING, A. J. & GRAY, J. E. 2019. Reduced stomatal density in bread wheat leads to increased water-use efficiency. *Journal of Experimental Botany*, 70, 4737-4748.
- DURAND, M., MATULE, B., BURGESS, A. J. & ROBSON, T. M. 2021. Sunfleck properties from time series of fluctuating light. *Agricultural and Forest Meteorology*, 308-309, 108554.
- DURAND, M. & ROBSON, T. M. 2023. Fields of a thousand shimmers: canopy architecture determines high-frequency light fluctuations. *New Phytologist*, 238, 2000-2015.
- EASTERLING, W. E., AGGARWAL, P. K., BATIMA, P., BRANDER, K. M., ERDA, L., HOWDEN, S. M., KIRILENKO, A., MORTON, J., SOUSSANA, J.-F. & SCHMIDHUBER, J. 2007. Food, fibre and forest products. *Climate change*, 2007, 273-313.
- ECKARDT, N. A. 2005. Photorespiration Revisited. *Plant Cell*, 17, 2139-41.
- ELLIOTT-KINGSTON, C., HAWORTH, M., YEARSLEY, J. M., BATKE, S. P., LAWSON, T. & MCELWAIN, J. C. 2016. Does size matter? Atmospheric CO₂ may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO₂. *Frontiers in Plant Science*, 7, 1253.
- ELLIS, E. C., KLEIN GOLDEWIJK, K., SIEBERT, S., LIGHTMAN, D. & RAMANKUTTY, N. 2010. Anthropogenic transformation of the biomes, 1700 to 2000. *Global Ecology and Biogeography*, 19, 589-606.
- ELLSWORTH, D. S., TYREE, M. T., PARKER, B. L. & SKINNER, M. 1995. Impact of Pear Thrips Damage on Sugar Maple Physiology: A Whole-Tree Experiment. In: PARKER, B. L.,

- SKINNER, M. & LEWIS, T. (eds.) *Thrips Biology and Management*. Boston, MA: Springer US.
- ESCOBAR-BRAVO, R., RUIJGROK, J., KIM, H. K., GROSSER, K., VAN DAM, N. M., KLINKHAMER, P. G. L. & LEISS, K. A. 2018. Light Intensity-Mediated Induction of Trichome-Associated Allelochemicals Increases Resistance Against Thrips in Tomato. *Plant Cell Physiol*, 59, 2462-2475.
- EVANS, G. C. 1956. An Area Survey Method of Investigating the Distribution of Light Intensity in Woodlands, with Particular Reference to Sunflecks. *Journal of Ecology*, 44, 391-428.
- EYLAND, D., VAN WESEMAEL, J., LAWSON, T. & CARPENTIER, S. 2021. The impact of slow stomatal kinetics on photosynthesis and water use efficiency under fluctuating light. *Plant Physiology*, 186, 998-1012.
- FAO 2019. Proactive Approaches to Drought Preparedness—where are we now and where do we go from here? : Food and Agriculture Organization of the United Nations Rome, Italy.
- FARALLI, M., MATTHEWS, J. & LAWSON, T. 2019. Exploiting natural variation and genetic manipulation of stomatal conductance for crop improvement. *Current Opinion in Plant Biology*, 49, 1-7.
- FERGUSON, J. N., SCHMUKER, P., DMITRIEVA, A., QUACH, T., ZHANG, T., GE, Z., NERSESIAN, N., SATO, S., CLEMENTE, T. & LEAKEY, A. 2024. Reducing stomatal density by expression of a synthetic EPF increases leaf intrinsic water use efficiency and reduces plant water use in a C4 crop. *bioRxiv*, 2024.02. 01.578512.
- FLANAGAN, L. B. & FARQUHAR, G. D. 2014. Variation in the carbon and oxygen isotope composition of plant biomass and its relationship to water-use efficiency at the leaf- and ecosystem-scales in a northern Great Plains grassland. *Plant, Cell & Environment*, 37, 425-438.
- FLEXAS, J., CLEMENTE-MORENO, M. J., BOTA, J., BRODRIBB, T. J., GAGO, J., MIZOKAMI, Y., NADAL, M., PERERA-CASTRO, A. V., ROIG-OLIVER, M., SUGIURA, D., XIONG, D. & CARRIQUÍ, M. 2021. Cell wall thickness and composition are involved in photosynthetic limitation. *J Exp Bot*, 72, 3971-3986.
- FOULKES, M. J., MOLERO, G., GRIFFITHS, S., SLAFER, G. A. & REYNOLDS, M. P. 2022. Yield Potential. In: REYNOLDS, M. P. & BRAUN, H.-J. (eds.) *Wheat Improvement: Food Security in a Changing Climate*. Cham: Springer International Publishing.
- FRANKS, P. J. & BEERLING, D. J. 2009. Maximum leaf conductance driven by CO₂ effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences*, 106, 10343-10347.
- FRANKS, P. J. & FARQUHAR, G. D. 2007. The mechanical diversity of stomata and its significance in gas-exchange control. *Plant physiology*, 143, 78-87.
- FRANKS, P. J., T, W. D.-A., BRITTON-HARPER, Z. J. & GRAY, J. E. 2015. Increasing water-use efficiency directly through genetic manipulation of stomatal density. *New Phytol*, 207, 188-195.
- GARCIA-MOLINA, A. & LEISTER, D. 2020. Accelerated relaxation of photoprotection impairs biomass accumulation in Arabidopsis. *Nature Plants*, 6, 9-12.
- GEISLER, M., NADEAU, J. & SACK, F. D. 2000. Oriented asymmetric divisions that generate the stomatal spacing pattern in arabidopsis are disrupted by the too many mouths mutation. *Plant Cell*, 12, 2075-86.
- GLEICK, P. H. 2003. Water Use. *Annual Review of Environment and Resources*, 28, 275-314.

- GOMMERS, C. 2020. Keep Cool and Open Up: Temperature-Induced Stomatal Opening. *Plant Physiol*, 182, 1188-1189.
- GOOD, S. P., NOONE, D. & BOWEN, G. 2015. Hydrologic connectivity constrains partitioning of global terrestrial water fluxes. *Science*, 349, 175-177.
- GROSSIORD, C., BUCKLEY, T. N., CERNUSAK, L. A., NOVICK, K. A., POULTER, B., SIEGWOLF, R. T. W., SPERRY, J. S. & MCDOWELL, N. G. 2020. Plant responses to rising vapor pressure deficit. *New Phytologist*, 226, 1550-1566.
- GUPTA, A., RICO-MEDINA, A. & CAÑO-DELGADO, A. I. 2020. The physiology of plant responses to drought. *Science*, 368, 266-269.
- HARA, K., KAJITA, R., TORII, K. U., BERGMANN, D. C. & KAKIMOTO, T. 2007. The secretory peptide gene EPF1 enforces the stomatal one-cell-spacing rule. *Genes & development*, 21, 1720-1725.
- HARA, K., YOKOO, T., KAJITA, R., ONISHI, T., YAHATA, S., PETERSON, K. M., TORII, K. U. & KAKIMOTO, T. 2009. Epidermal cell density is autoregulated via a secretory peptide, EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis leaves. *Plant Cell Physiol*, 50, 1019-31.
- HATFIELD, J. L. & DOLD, C. 2019. Water-Use Efficiency: Advances and Challenges in a Changing Climate. *Frontiers in Plant Science*, Volume 10 - 2019.
- HAWORTH, M., MARINO, G., MATERASSI, A., RASCHI, A., SCUTT, C. P. & CENTRITTO, M. 2023. The functional significance of the stomatal size to density relationship: Interaction with atmospheric [CO₂] and role in plant physiological behaviour. *Science of The Total Environment*, 863, 160908.
- HE, C. J., MORGAN, P. W. & DREW, M. C. 1996. Transduction of an Ethylene Signal Is Required for Cell Death and Lysis in the Root Cortex of Maize during Aerenchyma Formation Induced by Hypoxia. *Plant Physiology*, 112, 463-472.
- HE, S., SUN, H., CHEN, Q., YANG, Y., ZHOU, Z., CHANG, S., LU, S., LIANG, Z., YANG, J. & FEI, X. 2025. Comprehensive identification of cotton EPF/EPFL receptors and functional characterization of the GhEPFL1-1-GhER1 module in drought tolerance. *BMC Plant Biol*, 25, 901.
- HEPWORTH, C., DOHENY-ADAMS, T., HUNT, L., CAMERON, D. D. & GRAY, J. E. 2015. Manipulating stomatal density enhances drought tolerance without deleterious effect on nutrient uptake. *New Phytologist*, 208, 336-341.
- HEPWORTH, C., TURNER, C., LANDIM, M. G., CAMERON, D. & GRAY, J. E. 2016. Balancing Water Uptake and Loss through the Coordinated Regulation of Stomatal and Root Development. *PLOS ONE*, 11, e0156930.
- HERRMANN, A. & TORII, K. U. 2021. Shouting out loud: signaling modules in the regulation of stomatal development. *Plant Physiol*, 185, 765-780.
- HETHERINGTON, A. M. & WOODWARD, F. I. 2003. The role of stomata in sensing and driving environmental change. *Nature*, 424, 901-908.
- HOLLOWAY-PHILLIPS, M. 2018. Photosynthetic Oxygen Production: New Method Brings to Light Forgotten Flux. *Plant Physiol*, 177, 7-9.
- HORARUANG, W., KLEJCHOVÁ, M., CARROLL, W., SILVA-ALVIM, F. A. L., WAGHMARE, S., PAPANATSIU, M., AMTMANN, A., HILLS, A., ALVIM, J. C., BLATT, M. R. & ZHANG, B. 2022. Engineering a K⁺ channel 'sensory antenna' enhances stomatal kinetics, water use efficiency and photosynthesis. *Nature Plants*, 8, 1262-1274.
- HOWARD, A. R. & DONOVAN, L. A. 2009. Soil nitrogen limitation does not impact nighttime water loss in Populus. *Tree Physiology*, 30, 23-31.

- HRONKOVÁ, M., WIESNEROVÁ, D., ŠIMKOVÁ, M., SKŮPA, P., DEWITTE, W., VRÁBLOVÁ, M., ZAŽÍMALOVÁ, E. & ŠANTRŮČEK, J. 2015. Light-induced STOMAGEN-mediated stomatal development in Arabidopsis leaves. *Journal of Experimental Botany*, 66, 4621-4630.
- HUGHES, J., HEPWORTH, C., DUTTON, C., DUNN, J. A., HUNT, L., STEPHENS, J., WAUGH, R., CAMERON, D. D. & GRAY, J. E. 2017. Reducing Stomatal Density in Barley Improves Drought Tolerance without Impacting on Yield. *Plant Physiol*, 174, 776-787.
- HUNT, L. & GRAY, J. E. 2009. The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. *Curr Biol*, 19, 864-9.
- ISLAM, S. M. F. & KARIM, Z. 2019. World's demand for food and water: The consequences of climate change. *Desalination-challenges and opportunities*, 2019.
- ITO, H., OHTSUKA, T. & TANAKA, A. 1996. Conversion of chlorophyll b to chlorophyll a via 7-hydroxymethyl chlorophyll. *J Biol Chem*, 271, 1475-9.
- JALAKAS, P., TULVA, I., BĒRZIŅA, N. M. & HÖRAK, H. 2024. Stomatal patterning is differently regulated in adaxial and abaxial epidermis in Arabidopsis. *J Exp Bot*, 75, 6476-6488.
- JENKINS, C. L. D., FURBANK, R. T. & HATCH, M. D. 1989. Mechanism of C4 Photosynthesis: A Model Describing the Inorganic Carbon Pool in Bundle Sheath Cells. *Plant Physiology*, 91, 1372-1381.
- JHAVERI, R., CANNANBILLA, L., BHAT, K. S. A., SANKARAN, M. & KRISHNADAS, M. 2024. Anatomical traits explain drought response of seedlings from wet tropical forests. *Ecol Evol*, 14, e70155.
- JIANG, K., SOREFAN, K., DEEKS, M. J., BEVAN, M. W., HUSSEY, P. J. & HETHERINGTON, A. M. 2012. The ARP2/3 complex mediates guard cell actin reorganization and stomatal movement in Arabidopsis. *The Plant Cell*, 24, 2031-2040.
- KAISER, E., MORALES, A. & HARBINSON, J. 2018. Fluctuating Light Takes Crop Photosynthesis on a Rollercoaster Ride. *Plant Physiol*, 176, 977-989.
- KAISER, H. 2009. The relation between stomatal aperture and gas exchange under consideration of pore geometry and diffusional resistance in the mesophyll. *Plant, Cell & Environment*, 32, 1091-1098.
- KANIANSKA, R. 2016. Agriculture and Its Impact on Land-Use, Environment, and Ecosystem Services. In: ALMUSAED, A. (ed.) *Landscape Ecology - The Influences of Land Use and Anthropogenic Impacts of Landscape Creation*. London: IntechOpen.
- KEENAN, T. F., HOLLINGER, D. Y., BOHRER, G., DRAGONI, D., MUNGER, J. W., SCHMID, H. P. & RICHARDSON, A. D. 2013. Increase in forest water-use efficiency as atmospheric carbon dioxide concentrations rise. *Nature*, 499, 324-327.
- KELLOGG, E. A. 2013. C4 photosynthesis. *Curr Biol*, 23, R594-9.
- KHAN, N., CHOI, S.-H., LEE, C.-H., QU, M. & JEON, J.-S. 2024. Photosynthesis: Genetic Strategies Adopted to Gain Higher Efficiency. *International Journal of Molecular Sciences*, 25, 8933.
- KIMURA, H., HASHIMOTO-SUGIMOTO, M., IBA, K., TERASHIMA, I. & YAMORI, W. 2020. Improved stomatal opening enhances photosynthetic rate and biomass production in fluctuating light. *Journal of Experimental Botany*, 71, 2339-2350.
- KITAJIMA, K. & HOGAN, K. P. 2003. Increases of chlorophyll a/b ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. *Plant Cell Environ*, 26, 857-865.
- KLOPPSTECH, K. 1985. Diurnal and circadian rhythmicity in the expression of light-induced plant nuclear messenger RNAs. *Planta*, 165, 502-506.

- KOESTER, R. P., SKONECZKA, J. A., CARY, T. R., DIERS, B. W. & AINSWORTH, E. A. 2014. Historical gains in soybean (*Glycine max* Merr.) seed yield are driven by linear increases in light interception, energy conversion, and partitioning efficiencies. *J Exp Bot*, 65, 3311-21.
- KOOLMEISTER, K., MERILO, E., HÖRAK, H. & KOLLIST, H. 2024. Stomatal CO₂ responses at sub- and above-ambient CO₂ levels employ different pathways in *Arabidopsis*. *Plant Physiology*, 196, 608-620.
- KROMDIJK, J., GŁOWACKA, K., LEONELLI, L., GABILLY, S. T., IWAI, M., NIYOGI, K. K. & LONG, S. P. 2016. Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science*, 354, 857-861.
- KUBIS, A. & BAR-EVEN, A. 2019. Synthetic biology approaches for improving photosynthesis. *Journal of Experimental Botany*, 70, 1425-1433.
- KUME, A., AKITSU, T. & NASAHARA, K. N. 2018. Why is chlorophyll b only used in light-harvesting systems? *Journal of Plant Research*, 131, 961-972.
- LAWSON, T. & BLATT, M. R. 2014. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiol*, 164, 1556-70.
- LEAKEY, A. D. B., SCHOLLES, J. D. & PRESS, M. C. 2004. Physiological and ecological significance of sunflecks for dipterocarp seedlings. *Journal of Experimental Botany*, 56, 469-482.
- LEFEBVRE, S., LAWSON, T., ZAKHLENIUK, O. V., LLOYD, J. C., RAINES, C. A. & FRYER, M. 2005. Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiol*, 138, 451-60.
- LEHMANN, P. & OR, D. 2015. Effects of stomata clustering on leaf gas exchange. *New Phytol*, 207, 1015-25.
- LEHRETZ, G. G., SCHNEIDER, A., LEISTER, D. & SONNEWALD, U. 2022. High non-photochemical quenching of VPZ transgenic potato plants limits CO₂ assimilation under high light conditions and reduces tuber yield under fluctuating light. *Journal of Integrative Plant Biology*, 64, 1821-1832.
- LEVERENZ, J. W., ÖQUIST, G. & WINGSLE, G. 1992. Photosynthesis and photoinhibition in leaves of chlorophyll b-less barley in relation to absorbed light. *Physiologia Plantarum*, 85, 495-502.
- LI, L., REN, W. & TIAN, L. 2025. Temporal patterns in root water uptake and intrinsic water-use efficiency of overstory and understory tree species in a subtropical humid pine forest. *Agricultural and Forest Meteorology*, 371, 110626.
- LINDSEY, B. E., 3RD, RIVERO, L., CALHOUN, C. S., GROTEWOLD, E. & BRKLJACIC, J. 2017. Standardized Method for High-throughput Sterilization of *Arabidopsis* Seeds. *J Vis Exp*.
- LIU, M., YANG, G., YUAN, W., LI, Z., GAO, M., YANG, Y., LONG, H., MENG, Y., LI, C., HU, H., LI, H. & YUAN, Z. 2024. Overridingly increasing vegetation sensitivity to vapor pressure deficit over the recent two decades in China. *Ecological Indicators*, 161, 111977.
- LIU, Q., WANG, Z., YU, S., LI, W., ZHANG, M., YANG, J., LI, D., YANG, J. & LI, C. 2021. Pu-miR172d regulates stomatal density and water-use efficiency via targeting PuGTL1 in poplar. *J Exp Bot*, 72, 1370-1383.
- LONG, S. P., ZHU, X.-G., NAIDU, S. L. & ORT, D. R. 2006. Can improvement in photosynthesis increase crop yields? *Plant, Cell & Environment*, 29, 315-330.

- LUNDGREN, M. R., OSBORNE, C. P. & CHRISTIN, P.-A. 2014. Deconstructing Kranz anatomy to understand C4 evolution. *Journal of Experimental Botany*, 65, 3357-3369.
- MA, W. T., YU, Y. Z., WANG, X. & GONG, X. Y. 2022. Estimation of intrinsic water-use efficiency from $\delta(13)C$ signature of C(3) leaves: Assumptions and uncertainty. *Front Plant Sci*, 13, 1037972.
- MARCHIN, R. M., BACKES, D., OSSOLA, A., LEISHMAN, M. R., TJOELKER, M. G. & ELLSWORTH, D. S. 2022. Extreme heat increases stomatal conductance and drought-induced mortality risk in vulnerable plant species. *Glob Chang Biol*, 28, 1133-1146.
- MARKS, C. O. & LECHOWICZ, M. J. 2007. The ecological and functional correlates of nocturnal transpiration. *Tree Physiol*, 27, 577-84.
- MARLER, T. E. 2020. Artifleck: The Study of Artifactual Responses to Light Flecks with Inappropriate Leaves. *Plants*, 9, 905.
- MASLE, J., HUDSON, G. S. & BADGER, M. R. 1993. Effects of Ambient CO₂ Concentration on Growth and Nitrogen Use in Tobacco (*Nicotiana tabacum*) Plants Transformed with an Antisense Gene to the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase. *Plant Physiology*, 103, 1075-1088.
- MAWODZA, T., MENON, M., MURINGAI, N., MAGDYSYUK, O. V., BURCA, G. & CASSON, S. 2022. Investigating root architectural differences in lines of *Arabidopsis thaliana*. L. with altered stomatal density using high resolution X-Ray synchrotron imaging. *Plant and Soil*, 481, 607-619.
- MCAUSLAND, L., ACEVEDO-SIACA, L. G., PINTO, R. S., PINTO, F., MOLERO, G., GARATUZA-PAYAN, J., REYNOLDS, M. P., MURCHIE, E. H. & YEPEZ, E. A. 2023. Night-time warming in the field reduces nocturnal stomatal conductance and grain yield but does not alter daytime physiological responses. *New Phytologist*, 239, 1622-1636.
- MCAUSLAND, L., ATKINSON, J. A., LAWSON, T. & MURCHIE, E. H. 2019. High throughput procedure utilising chlorophyll fluorescence imaging to phenotype dynamic photosynthesis and photoprotection in leaves under controlled gaseous conditions. *Plant Methods*, 15, 109.
- MCAUSLAND, L., SMITH, K. E., WILLIAMS, A., MOLERO, G. & MURCHIE, E. H. 2021. Nocturnal stomatal conductance in wheat is growth-stage specific and shows genotypic variation. *New Phytol*, 232, 162-175.
- MCAUSLAND, L., VIALET-CHABRAND, S., DAVEY, P., BAKER, N. R., BRENDDEL, O. & LAWSON, T. 2016. Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologist*, 211, 1209-1220.
- MESSINA, C. D., ROTUNDO, J., HAMMER, G. L., GHOSH, C., REYES, A., FANG, Y., VAN OOSTEROM, E., BORRAS, L. & COOPER, M. 2022. Radiation use efficiency increased over a century of maize (*Zea mays* L.) breeding in the US corn belt. *J Exp Bot*, 73, 5503-5513.
- MIYAGAWA, Y., TAMOI, M. & SHIGEOKA, S. 2001. Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1, 7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nature Biotechnology*, 19, 965-969.
- MOHAMMED, U., CAINE, R. S., ATKINSON, J. A., HARRISON, E. L., WELLS, D., CHATER, C. C., GRAY, J. E., SWARUP, R. & MURCHIE, E. H. 2019. Rice plants overexpressing OsEPF1 show reduced stomatal density and increased root cortical aerenchyma formation. *Sci Rep*, 9, 5584.

- MONTEITH, J. L., MOSS, C. J., COOKE, G. W., PIRIE, N. W. & BELL, G. D. H. 1977. Climate and the efficiency of crop production in Britain. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 281, 277-294.
- MOTT, K. A. & PEAK, D. 2018. Effects of the mesophyll on stomatal responses in amphistomatous leaves. *Plant Cell Environ*, 41, 2835-2843.
- MÜLLER, P., LI, X.-P. & NIYOGI, K. K. 2001. Non-Photochemical Quenching. A Response to Excess Light Energy1. *Plant Physiology*, 125, 1558-1566.
- NADEAU, J. A. & SACK, F. D. 2002. Control of Stomatal Distribution on the *Arabidopsis* Leaf Surface. *Science*, 296, 1697-1700.
- NIU, Y., LAZÁR, D., HOLZWARTH, A. R., KRAMER, D. M., MATSUBARA, S., FIORANI, F., POORTER, H., SCHREY, S. D. & NEDBAL, L. 2023. Plants cope with fluctuating light by frequency-dependent nonphotochemical quenching and cyclic electron transport. *New Phytologist*, 239, 1869-1886.
- NOAA NATIONAL CENTERS FOR ENVIRONMENTAL INFORMATION 2024. Monthly Global Climate Report for September 2024. Published online
- NUNES, T. D. G., ZHANG, D. & RAISSIG, M. T. 2020. Form, development and function of grass stomata. *The Plant Journal*, 101, 780-799.
- OECD 2022. Climate Tipping Points: Insights for Effective Policy Action.
- OHASHI-ITO, K. & BERGMANN, D. C. 2006. *Arabidopsis* FAMA Controls the Final Proliferation/Differentiation Switch during Stomatal Development. *The Plant Cell*, 18, 2493-2505.
- OKI, T. & KANAE, S. 2006. Global hydrological cycles and world water resources. *science*, 313, 1068-1072.
- OREN, R., SPERRY, J., KATUL, G., PATAKI, D., EWERS, B., PHILLIPS, N. & SCHAFER, K. 1999. Survey and synthesis of intra- and interspecific variation in stomatal sensitivity to vapour pressure deficit. *Plant, Cell and Environment*, 22, 1515-1526.
- PANKASEM, N., HSU, P.-K., LOPEZ, B. N. K., FRANKS, P. J. & SCHROEDER, J. I. 2024. Warming triggers stomatal opening by enhancement of photosynthesis and ensuing guard cell CO₂ sensing, whereas higher temperatures induce a photosynthesis-uncoupled response. *New Phytologist*, 244, 1847-1863.
- PAPANATSIU, M., AMTMANN, A. & BLATT, M. R. 2016. Stomatal Spacing Safeguards Stomatal Dynamics by Facilitating Guard Cell Ion Transport Independent of the Epidermal Solute Reservoir. *Plant Physiol*, 172, 254-63.
- PAPANATSIU, M., PETERSEN, J., HENDERSON, L., WANG, Y., CHRISTIE, J. M. & BLATT, M. R. 2019. Optogenetic manipulation of stomatal kinetics improves carbon assimilation, water use, and growth. *Science*, 363, 1456-1459.
- PEARCY, R. W., RODEN, J. S. & GAMON, J. A. 1990. Sunfleck dynamics in relation to canopy structure in a soybean (*Glycine max* (L.) Merr.) canopy. *Agricultural and Forest Meteorology*, 52, 359-372.
- PIETRAGALLA, J. & PASK, A. 2012. Stomatal conductance. *Physiological breeding II: a field guide to wheat phenotyping*. A. Pask, J. Pietragalla, D. Mullan & M. Reynolds (eds.). *México, CIMMYT*, 15-17.
- QU, M., HAMDANI, S., LI, W., WANG, S., TANG, J., CHEN, Z., SONG, Q., LI, M., ZHAO, H., CHANG, T., CHU, C. & ZHU, X. 2016. Rapid stomatal response to fluctuating light: an under-explored mechanism to improve drought tolerance in rice. *Funct Plant Biol*, 43, 727-738.

- RESCO DE DIOS, V., CHOWDHURY, F. I., GRANDA, E., YAO, Y. & TISSUE, D. T. 2019. Assessing the potential functions of nocturnal stomatal conductance in C3 and C4 plants. *New Phytologist*, 223, 1696-1706.
- RESCO DE DIOS, V., LOIK, M. E., SMITH, R., ASPINWALL, M. J. & TISSUE, D. T. 2016. Genetic variation in circadian regulation of nocturnal stomatal conductance enhances carbon assimilation and growth. *Plant, Cell & Environment*, 39, 3-11.
- RETKUTE, R., TOWNSEND, A. J., MURCHIE, E. H., JENSEN, O. E. & PRESTON, S. P. 2018. Three-dimensional plant architecture and sunlit–shaded patterns: a stochastic model of light dynamics in canopies. *Annals of Botany*, 122, 291-302.
- ROBERTSON, B. C., HAN, Y. & LI, C. 2023. A Comparison of Different Stomatal Density Phenotypes of *Hordeum vulgare* under Varied Watering Regimes Reveals Superior Genotypes with Enhanced Drought Tolerance. *Plants (Basel)*, 12.
- ROSENTHAL, D. M., LOCKE, A. M., KHOZAEI, M., RAINES, C. A., LONG, S. P. & ORT, D. R. 2011. Over-expressing the C3 photosynthesis cycle enzyme Sedoheptulose-1-7 Bisphosphatase improves photosynthetic carbon gain and yield under fully open air CO₂ fumigation (FACE). *BMC Plant Biology*, 11, 123.
- RUBAN, A. V. 2016. Nonphotochemical Chlorophyll Fluorescence Quenching: Mechanism and Effectiveness in Protecting Plants from Photodamage. *Plant Physiol*, 170, 1903-16.
- SAKODA, K., YAMORI, W., SHIMADA, T., SUGANO, S. S., HARA-NISHIMURA, I. & TANAKA, Y. 2020. Higher Stomatal Density Improves Photosynthetic Induction and Biomass Production in *Arabidopsis* Under Fluctuating Light. *Frontiers in Plant Science*, 11.
- SCHLÜTER, U., MUSCHAK, M., BERGER, D. & ALTMANN, T. 2003. Photosynthetic performance of an *Arabidopsis* mutant with elevated stomatal density (*sdd1-1*) under different light regimes. *Journal of Experimental Botany*, 54, 867-874.
- SCHNEIDER, C. A., RASBAND, W. S. & ELICEIRI, K. W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, 9, 671-675.
- SCHULER, M. L., MANTEGAZZA, O. & WEBER, A. P. M. 2016. Engineering C4 photosynthesis into C3 chassis in the synthetic biology age. *The Plant Journal*, 87, 51-65.
- SHAHZAD, Z., KELLERMEIER, F., ARMSTRONG, E. M., ROGERS, S., LOBET, G., AMTMANN, A. & HILLS, A. 2018. EZ-Root-VIS: a software pipeline for the rapid analysis and visual reconstruction of root system architecture. *Plant physiology*, 177, 1368-1381.
- SHARMA, N., NAGAR, S., THAKUR, M., SURIYAKUMAR, P., KATARIA, S., SHANKER, A. K., LANDI, M. & ANAND, A. 2023. Photosystems under high light stress: throwing light on mechanism and adaptation. *Photosynthetica*, 61, 250-263.
- SLATTERY, R. A., WALKER, B. J., WEBER, A. P. M. & ORT, D. R. 2017. The Impacts of Fluctuating Light on Crop Performance. *Plant Physiology*, 176, 990-1003.
- SNYDER, K. A., RICHARDS, J. H. & DONOVAN, L. A. 2003. Night-time conductance in C3 and C4 species: do plants lose water at night? *Journal of Experimental Botany*, 54, 861-865.
- SOLEH, M. A., TANAKA, Y., NOMOTO, Y., IWAHASHI, Y., NAKASHIMA, K., FUKUDA, Y., LONG, S. P. & SHIRAIWA, T. 2016. Factors underlying genotypic differences in the induction of photosynthesis in soybean [*Glycine max* (L.) Merr.]. *Plant, Cell & Environment*, 39, 685-693.
- SOUTH, P. F., CAVANAGH, A. P., LIU, H. W. & ORT, D. R. 2019. Synthetic glycolate metabolism pathways stimulate crop growth and productivity in the field. *Science*, 363, eaat9077.

- SUGANAMI, M., YOSHIDA, H., YOSHIDA, S., KAWAMURA, M., KOKETSU, E., MATSUOKA, M. & KOJIMA, S. 2024. Redefining awn development in rice through the breeding history of Japanese awn reduction. *Frontiers in Plant Science*, Volume 15 - 2024.
- SULMAN, B. N., ROMAN, D. T., YI, K., WANG, L., PHILLIPS, R. P. & NOVICK, K. A. 2016. High atmospheric demand for water can limit forest carbon uptake and transpiration as severely as dry soil. *Geophysical Research Letters*, 43, 9686-9695.
- TANAKA, R. & TANAKA, A. 2011. Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807, 968-976.
- TAYLOR, S. H. & LONG, S. P. 2017. Slow induction of photosynthesis on shade to sun transitions in wheat may cost at least 21% of productivity. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372, 20160543.
- TERASHIMA, I., HANBA, Y. T., TAZOE, Y., VYAS, P. & YANO, S. 2005. Irradiance and phenotype: comparative eco-development of sun and shade leaves in relation to photosynthetic CO₂ diffusion. *Journal of Experimental Botany*, 57, 343-354.
- THE LANCET PLANETARY, H. 2018. Can the Paris Agreement save us from a climate catastrophe? *The Lancet Planetary Health*, 2, e140.
- TRUGMAN, A., MEDVIGY, D., MANKIN, J. & ANDEREGG, W. 2018. Soil moisture stress as a major driver of carbon cycle uncertainty. *Geophysical Research Letters*, 45, 6495-6503.
- UNITED NATIONS 2021. The United Nations world water development report 2021: Valuing water.
- URBAN, J., MILES, I., ANNE, M. M. & AND TESKEY, R. O. 2017. Stomatal conductance increases with rising temperature. *Plant Signaling & Behavior*, 12, e1356534.
- VIALET-CHABRAND, S., MATTHEWS, J. S., SIMKIN, A. J., RAINES, C. A. & LAWSON, T. 2017. Importance of Fluctuations in Light on Plant Photosynthetic Acclimation. *Plant Physiol*, 173, 2163-2179.
- VOS, U., WILSON, M. H., KENOBI, K., GOULD, P. D., ROBERTSON, F. C., PEER, W. A., LUCAS, M., SWARUP, K., CASIMIRO, I., HOLMAN, T. J., WELLS, D. M., PÉRET, B., GOH, T., FUKAKI, H., HODGMAN, T. C., LAPLAZE, L., HALLIDAY, K. J., LJUNG, K., MURPHY, A. S., HALL, A. J., WEBB, A. A. & BENNETT, M. J. 2015. The circadian clock rephases during lateral root organ initiation in *Arabidopsis thaliana*. *Nat Commun*, 6, 7641.
- WADA, M. & KONG, S.-G. 2011. Analysis of Chloroplast Movement and Relocation in *Arabidopsis*. In: JARVIS, R. P. (ed.) *Chloroplast Research in Arabidopsis: Methods and Protocols, Volume I*. Totowa, NJ: Humana Press.
- WALL, S., VIALET-CHABRAND, S., DAVEY, P., VAN RIE, J., GALLE, A., COCKRAM, J. & LAWSON, T. 2022. Stomata on the abaxial and adaxial leaf surfaces contribute differently to leaf gas exchange and photosynthesis in wheat. *New Phytologist*, 235, 1743-1756.
- WANG, X. Q., WU, W. H. & ASSMANN, S. M. 1998. Differential responses of abaxial and adaxial guard cells of broad bean to abscisic acid and calcium. *Plant Physiol*, 118, 1421-9.
- WANG, Y., ANDEREGG, W. R. L., VENTURAS, M. D., TRUGMAN, A. T., YU, K. & FRANKENBERG, C. 2021. Optimization theory explains nighttime stomatal responses. *New Phytologist*, 230, 1550-1561.
- WEI, H., KONG, D., YANG, J. & WANG, H. 2020. Light Regulation of Stomatal Development and Patterning: Shifting the Paradigm from *Arabidopsis* to Grasses. *Plant Communications*, 1.

- WOODWARD, F. I. 1987. Stomatal numbers are sensitive to increases in CO₂ from pre-industrial levels. *Nature*, 327, 617-618.
- XIONG, L., HUANG, Y., LIU, Z., LI, C., YU, H., SHAHID, M. Q., LIN, Y., QIAO, X., XIAO, J., GRAY, J. E. & JIN, J. 2022a. Small EPIDERMAL PATTERNING FACTOR-LIKE2 peptides regulate awn development in rice. *Plant Physiol*, 190, 516-531.
- XIONG, Z., DUN, Z., WANG, Y., YANG, D., XIONG, D., CUI, K., PENG, S. & HUANG, J. 2022b. Effect of Stomatal Morphology on Leaf Photosynthetic Induction Under Fluctuating Light in Rice. *Frontiers in Plant Science*, Volume 12 - 2021.
- XU, Y., KOROMA, A. A., WEISE, S. E., FU, X., SHARKEY, T. D. & SHACHAR-HILL, Y. 2023. Daylength variation affects growth, photosynthesis, leaf metabolism, partitioning, and metabolic fluxes. *Plant Physiol*, 194, 475-490.
- XU, Z. & ZHOU, G. 2008. Responses of leaf stomatal density to water status and its relationship with photosynthesis in a grass. *J Exp Bot*, 59, 3317-25.
- YANG, L., LIU, S. & LIN, R. 2020. The role of light in regulating seed dormancy and germination. *J Integr Plant Biol*, 62, 1310-1326.
- YANG, X., LI, Y., REN, B., DING, L., GAO, C., SHEN, Q. & GUO, S. 2012. Drought-induced root aerenchyma formation restricts water uptake in rice seedlings supplied with nitrate. *Plant and Cell Physiology*, 53, 495-504.
- YAZDANBAKHS, N., SULPICE, R., GRAF, A., STITT, M. & FISAHN, J. 2011. Circadian control of root elongation and C partitioning in *Arabidopsis thaliana*. *Plant Cell Environ*, 34, 877-894.
- YE, Z. P., LING, Y., YU, Q., DUAN, H. L., KANG, H. J., HUANG, G. M., DUAN, S. H., CHEN, X. M., LIU, Y. G. & ZHOU, S. X. 2020. Quantifying Light Response of Leaf-Scale Water-Use Efficiency and Its Interrelationships With Photosynthesis and Stomatal Conductance in C(3) and C(4) Species. *Front Plant Sci*, 11, 374.
- YOSHIOKA, M., IEHISA, J. C. M., OHNO, R., KIMURA, T., ENOKI, H., NISHIMURA, S., NASUDA, S. & TAKUMI, S. 2017. Three dominant awnless genes in common wheat: Fine mapping, interaction and contribution to diversity in awn shape and length. *PLoS One*, 12, e0176148.
- YU, K., GOLDSMITH, G. R., WANG, Y. & ANDEREGG, W. R. L. 2019. Phylogenetic and biogeographic controls of plant nighttime stomatal conductance. *New Phytologist*, 222, 1778-1788.
- ZEPEL, M. J. B., LEWIS, J. D., CHASZAR, B., SMITH, R. A., MEDLYN, B. E., HUXMAN, T. E. & TISSUE, D. T. 2012. Nocturnal stomatal conductance responses to rising [CO₂], temperature and drought. *New Phytologist*, 193, 929-938.
- ZHU, X.-G., DE STURLER, E. & LONG, S. P. 2007. Optimizing the Distribution of Resources between Enzymes of Carbon Metabolism Can Dramatically Increase Photosynthetic Rate: A Numerical Simulation Using an Evolutionary Algorithm. *Plant Physiology*, 145, 513-526.
- ZHU, X.-G., LONG, S. P. & ORT, D. R. 2008. What is the maximum efficiency with which photosynthesis can convert solar energy into biomass? *Current Opinion in Biotechnology*, 19, 153-159.

7. Appendix

7.1 Timeline

Time	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
	Plant seeds		Transfer seeds	FlourCam measurements	Sample for chlorophyll assay	Dual Pam protocol A	Stomatal density measurements	Carbon isotope sampling
			Growth measurements	Growth measurements	Stomatal conductivity measurements	CO ₂ assimilation measurements	Dual Pam protocol B	
					CO ₂ assimilation measurements	Growth measurements		
					Growth measurements			

Figure 20: A timeline of measurements taken in each repeat of the experiment.

7.2 Statistical models

The following models were analysed by ANOVA:

Water use efficiency ~ Light+ Genotype

Photosynthetic induction ~ Genotype + Light treatment + Time of day

CO₂ assimilation at high light intensity ~ Genotype + Light treatment + Time of day

Chlorophyll a content ~ Genotype + Light treatment

Chlorophyll b content ~ Genotype + Light treatment

Chlorophyll a+ b content ~ Genotype + Light treatment

Chlorophyll a/b ratio ~ Genotype + Light treatment

The following groups were compared using a Kruskal-Wallis test as the data was not normally distributed:

Plant rosette area ~ Genotype

Plant rosette area ~ Light treatment

Stomata per mm² ~ Genotype

Stomata per mm² ~ Light treatment

Stomata per mm² ~ Side of leaf

Stomatal conductivity ~ Genotype

Stomatal conductivity ~ Light treatment

Stomatal conductivity ~ Side of leaf

CO₂ assimilation ~ Genotype

CO₂ assimilation ~ Light treatment

CO₂ assimilation ~ Time of day

CO₂ assimilation ~ Light intensity

CO₂ assimilation at low light intensity ~ Genotype

CO₂ assimilation at low light intensity ~ Light treatment

CO₂ assimilation at low light intensity ~ Time of day

NPQ (measured in FlourCam) ~ Time of day

NPQ (measured in FlourCam) ~ Genotype

NPQ (measured in FlourCam) ~ Light treatment

NPQ (Dual Pam, one second fluctuations) ~ Genotype

NPQ (Dual Pam, one second fluctuations) ~ Light treatment

NPQ (Dual Pam, one minute fluctuations) ~ Genotype

NPQ (Dual Pam, one minute fluctuations) ~ Light treatment

Total root length ~ Genotype

Total root length ~ Light treatment

Number of lateral roots ~ Genotype
Number of lateral roots ~ Light treatment
Total lateral root length ~ Genotype
Total lateral root length ~ Light treatment