

Engineering Blue Light Responses to Break the Carbon Water Trade Off and Approaches for Climate Resilient Plant Improvement

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Off and Approaches for Climate Resilient Plant Improvement

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Declaration

I hereby declare that this thesis, entitled " Engineering Blue Light Responses to Break the Carbon Water Trade Off and Approaches for Climate Resilient Plant Improvement" represents original research work I conducted. The original works included in this dissertation have not been previously submitted, except for chapter 2, 3 and 6, which have been published in peer reviewed journals with me as the first author. I acknowledge all sources of information, methodological approaches, and intellectual contributions from other researchers through systematic referencing, adhering to established academic conventions. Furthermore, I certify that this thesis does not contain any copyrighted material and accurately presents experimental findings.

Mengjie Fan

9th of August 2025

Acknowledgement

Where should I even begin? For a good proportion of my existence on this planet, I've taken comfort in the notion that nothing matters on the scale of the universe. I can't recall who coined that line, probably it was just me muttering in the lab. And yet, as I reach the end of this PhD journey, I feel an unexpectedly need to set my gratitude down in writing. This is not for the sake of academic convention, it is a sincere impulse of my desire. My first and foremost thanks go to Professor Tracy Lawson at Essex. The reasons are obvious, without your support and astute guidance, I would not now be crafting the final pages of this thesis. Also, thanks Elias who first introduced me to you. If one were to define the ideal PhD supervisor, patience, curiosity, rigour, knowledgeable, and a capacity for tolerant amusement at extremely minor catastrophes very occasionally, you embody the lot. Selecting elite lines from the vast gene pool of PhD mentors, you would certainly rank among the most distinguished. I also treasure the conferences, the discussions, the laughter, and let's not forget all the flavoured ethanol based liquid we've metabolised together over the past three and a half years. To my other supervisors, Mark, Andrew, and my board member Philippe, your collective guidance has been indispensable at every milestone. Harriet, my industry supervisor, I must confess that four years of quarterly NIAB meetings were not always occasions I awaited with bated breath. Yet you and everyone's warmth during those sessions and your tireless efforts to procure the materials my experiments demanded has transformed each meeting into something almost enjoyable and certainly invaluable.

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List of Abbreviations

Abbreviation	Definition
<i>A</i>	Net CO ₂ assimilation rate / Photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
<i>ABA</i>	Absciscic acid
<i>AHA</i>	Arabidopsis H ⁺ -ATPase (plasma membrane proton pump)
<i>A_{max}</i>	Light saturated photosynthetic rate ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
<i>ANOVA</i>	Analysis of variance
<i>ATP</i>	Adenosine triphosphate
<i>AZ</i>	Azygous (control plants lacking transgene)
<i>BHP</i>	Blue light signalling protein mediating stomatal opening
<i>BLUS1</i>	Blue light signalling 1 (guard cell specific kinase)
<i>C3</i>	C3 photosynthesis pathway (Calvin-Benson cycle)
<i>C_a</i>	Ambient CO ₂ concentration ($\mu\text{mol mol}^{-1}$)
<i>CBC1/CBC2</i>	Convergence of blue light and CO ₂ 1/2 (Raf-like kinases)
<i>cDNA</i>	Complementary DNA
<i>C_i</i>	Intercellular CO ₂ concentration ($\mu\text{mol mol}^{-1}$)

C_i/C_a	Ratio of intercellular to ambient CO ₂ concentration (dimensionless)
CO ₂	Carbon dioxide
COP1	Constitutive photomorphogenic 1 (E3 ubiquitin ligase)
CRY1	Cryptochrome 1 (blue light photoreceptor)
CRY2	Cryptochrome 2 (blue light photoreceptor)
CV	Coefficient of variation (%)
DAT	Days after treatment
DD	Drought deficit / drought conditions
DLI	Daily light integral (mol m ⁻² d ⁻¹)
DNA	Deoxyribonucleic acid
E	Transpiration rate (mmol H ₂ O m ⁻² s ⁻¹)
EFD	Energy flux density
EPF1	Epidermal patterning factor 1 (stomatal development regulator)
EPF2	Epidermal patterning factor 2
ETR	Electron transport rate (μmol e ⁻ m ⁻² s ⁻¹)
FAD	Flavin adenine dinucleotide
Fm	Maximum fluorescence (dark-adapted)
FMN	Flavin mononucleotide
Fo	Minimum fluorescence (dark-adapted)
Fv	Variable fluorescence (Fm - Fo)
Fv/Fm	Maximum quantum efficiency of PSII photochemistry

<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase
<i>g_{smax}</i>	Maximum stomatal conductance at high light (mol H ₂ O m ⁻² s ⁻¹)
<i>g_{smin}</i>	Minimum stomatal conductance at low light (mol H ₂ O m ⁻² s ⁻¹)
<i>g_{sw}</i>	Stomatal conductance to water vapor (mol H ₂ O m ⁻² s ⁻¹)
<i>g_{swmax}</i>	Anatomical maximum stomatal conductance (mol H ₂ O m ⁻² s ⁻¹)
<i>H⁺-ATPase</i>	Plasma membrane proton pump
<i>HB</i>	High blue light treatment (35% blue light)
<i>HSFA1</i>	Heat shock factor A1
<i>HT1</i>	High temperature 1 (protein kinase)
<i>HY5</i>	Elongated hypocotyl 5 (transcription factor)
<i>ICE1</i>	Inducer of CBF expression 1 (transcription factor)
<i>IDD16</i>	Indeterminate domain 16 (transcription factor)
<i>IQR</i>	Interquartile range
<i>IRGA</i>	Infrared gas analyser
<i>iWUE</i>	Intrinsic water use efficiency (A/gsw) (μmol CO ₂ mol ⁻¹ H ₂ O)
<i>J_{max}</i>	Maximum electron transport rate for RuBP regeneration (μmol e ⁻ m ⁻² s ⁻¹)
<i>J_{max}:V_{cmax}</i>	Ratio of electron transport to carboxylation capacity

<i>K</i>	Time constant for stomatal response (minutes)
<i>K_c</i>	Michaelis Menten constant for CO ₂ (μmol mol ⁻¹)
<i>K_o</i>	Michaelis Menten constant for O ₂ (mmol mol ⁻¹)
<i>KST1</i>	Potassium channel gene (potato guard cell-specific promoter)
<i>LB</i>	Low blue light treatment (5% blue light)
<i>LED</i>	Light emitting diode
<i>LOV</i>	Light-Oxygen-Voltage domain
<i>LSD</i>	Least significant difference (statistical test)
<i>LUE</i>	Light use efficiency (mmol CO ₂ mol ⁻¹ photons)
<i>MAP3K</i>	Mitogen-activated protein kinase
<i>MTHF</i>	5,10-methenyltetrahydrofolate
<i>MUTE</i>	MUTE transcription factor (stomatal development)
<i>NADPH</i>	Nicotinamide adenine dinucleotide phosphate (reduced)
<i>NPQ</i>	Non-photochemical quenching
<i>NPTII</i>	Neomycin phosphotransferase II (selection marker)
<i>O₂</i>	Oxygen
<i>OE</i>	Overexpression / overexpressor
<i>PAR</i>	Photosynthetically active radiation (400-700 nm)
<i>PCR</i>	Polymerase chain reaction

<i>PHR</i>	Photolyase-homologous region
<i>PHOT1</i>	Phototropin 1 (blue light photoreceptor)
<i>PHOT2</i>	Phototropin 2 (blue light photoreceptor)
<i>PPFD</i>	Photosynthetic photon flux density ($\mu\text{mol m}^{-2} \text{s}^{-1}$)
<i>PSI</i>	Photosystem I
<i>PSII</i>	Photosystem II
<i>qPCR</i>	Quantitative polymerase chain reaction
<i>R:FR</i>	Red to far-red light ratio
<i>RA</i>	Response amplitude
<i>Rd</i>	Dark respiration rate / mitochondrial respiration in light ($\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$)
<i>RNA</i>	Ribonucleic acid
<i>RT-PCR</i>	Reverse transcription polymerase chain reaction
<i>RuBP</i>	Ribulose-1,5-bisphosphate
<i>Rubisco</i>	Ribulose-1,5-bisphosphate carboxylase/oxygenase
<i>SCRM2</i>	SCREAM2 transcription factor
<i>SD</i>	Stomatal density (stomata mm^{-2})
<i>SE</i>	Standard error
<i>SLAC1</i>	Slow anion channel-associated 1
<i>SLAH3</i>	SLAC1 homolog 3
<i>Sl_{max}</i>	Maximum slope of stomatal opening ($\text{mol H}_2\text{O m}^{-2} \text{s}^{-1} \text{min}^{-1}$)

<i>SPA</i>	Suppressor of PHYA-105
<i>SPCH</i>	SPEECHLESS (master transcription factor for stomatal development)
<i>SPI</i>	Stomatal pore index
t_{90}	Time required to reach 90% of steady-state value (minutes)
T_{leaf}	Leaf temperature (°C)
<i>UV</i>	Ultraviolet radiation
V_{cmax}	Maximum carboxylation rate of Rubisco ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)
<i>VPD</i>	Vapor pressure deficit (kPa)
<i>WT</i>	Wild type
<i>WUE</i>	Water use efficiency ($\mu\text{mol CO}_2 \text{ mmol}^{-1} \text{ H}_2\text{O}$)
<i>WW</i>	Well-watered conditions
α	Quantum yield of CO_2 assimilation ($\text{mol CO}_2 \text{ mol}^{-1} \text{ photons}$) from modelling
Γ	CO_2 compensation point ($\mu\text{mol mol}^{-1}$)
η^2	Eta-squared (effect size in ANOVA)
ΦCO_2	Quantum yield of CO_2 assimilation (Measured)
τ	Time to reach 63% of total increase in stomatal conductance (minutes)
<i>R100</i>	$100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ red light
<i>R1000</i>	$1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$ red light
<i>R900B100</i>	$900 \mu\text{mol m}^{-2} \text{ s}^{-1}$ red + $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ blue light

<i>1OE3, 1OE4, 1OE5</i>	Specific transgenic lines overexpressing EPF1
<i>A31, A32</i>	Specific transgenic tobacco lines overexpressing H ⁺ -ATPase
<i>°Brix</i>	Measure of soluble solids content (sugar concentration)

Thesis Abstract

Plant productivity under climate change requires optimization of photosynthetic carbon assimilation while minimizing transpirational water loss, which is a fundamental physiological constraint governed by biophysical principles. This constraint arises because the steep leaf-air water vapor gradient, combined with the inherently faster diffusion coefficient of H₂O relative to CO₂, creates an inevitable trade off, whereby under typical condition, hundreds of water molecules are transpired for each CO₂ molecule assimilated during photosynthesis, particularly in C₃ species where the biochemical limitations of RuBisCO enzyme necessitate higher stomatal conductance to maintain adequate CO₂ supply for carboxylation reactions. This thesis investigates blue light signalling pathways as engineering targets for breaking this carbon-water tradeoff by examining regulatory mechanisms from photoreceptor-mediated signal transduction to whole plant level performance under dynamic environmental conditions. Novel large scale Imaging frameworks were developed to quantify guard cells patterning and its implications of spatial heterogeneity on the carbon water tradeoff which is previously inaccessible with conventional approaches (Chapter 3). The manipulation of spectral irradiance during development and induced hierarchical physiological modifications that enhance photosynthesis differentially across distinct temporal scales was explored in Chapter 4. A high throughput gas exchange protocol capable of collecting large amounts of data using a randomized unbiased algorithm was developed, and insights from this dataset led to the design of novel targeted spectral delivery strategies taking advantage of anatomical asymmetries to enhance photosynthetic efficiency, which resulted

successfully in increasing daily CO₂ assimilation by $8.2 \pm 1.3\%$ while minimizing energy input through a periodic blue light pulsed system (Chapter 5). Following this, we explored genetically modified plants and discovered blue light driven compensatory mechanisms responding to genetically induced anatomical constraints (Chapter 6). We then engineered molecular enhancement through guard cell specific H⁺-ATPase overexpression and demonstrated reduced photosynthetic transition penalties from 17.3% to 6.9% under a novel naturalistic fluctuating light program developed as another methodological contribution in this thesis (Chapter 7). These integrated findings and methods developed thus provide engineering pathways and validation methodologies for developing future crops with enhanced resource use efficiency under variable environments in future climate scenarios.

Chapter 1: Thesis Introduction

1.1 Photosynthetic engineering for food security and climate resilience

The convergence of global food security challenges and advancements in photosynthetic engineering presents one of the most critical intersections to address in our era. With projected food demand increases of 35-56% by 2050 and the need to feed 9-10 billion people within planetary boundaries, photosynthetic enhancement emerges as one of the primary remaining opportunities for sustainable yield improvements (Zhu *et al.*, 2010; van Dijk *et al.*, 2021; Falcon *et al.*, 2022; Chen *et al.*, 2024). The foundation framework for addressing these challenges was established by Godfray *et al.* (2010), who identifies multiple constraints including competition for land, water, energy, and climate change impacts. Building on this, Tilman *et al.* (2011) projected even higher demand increases of 100-110% from 2005 to 2050 based on income driven consumption patterns. Critically, environmental effects of the food system could increase by 50-90% by 2050 without intervention and will reach levels beyond planetary boundaries (Springmann *et al.*, 2018). However, Mueller *et al.* (2012) showed that closing yield gaps to 100% of attainable yields could increase global production by 45-70% for most crops through optimized nutrient and water management.

One of the first theoretical frameworks by Evans and Fischer (1999) identified photosynthetic efficiency as a critical component of yield potential below theoretical maximum for major crops. Recent breakthroughs have moved beyond theoretical possibilities to cross scale modelling connecting leaf photosynthesis to crop yield depending on photosynthetic enhancement targets (i.e., light harvesting, electron transfer bottlenecks) and environmental conditions (Zhu *et al.*, 2010; Wu *et al.*, 2019; Croce *et al.*, 2024). These theoretical frameworks have translated into many fields validated

achievements. For example, engineering faster non-photochemical quenching (NPQ) relaxation by overexpressing *Arabidopsis thaliana* violaxanthin de-epoxidase (AtVDE), Photosystem II subunit S (AtPsbS), and zeaxanthin epoxidase (AtZEP) increased tobacco biomass (plant dry matter productivity) by ~14–20% in multi-year field trials (Kromdijk *et al.*, 2016). This approach was successfully translated to food crops, with 24.5% seed yield increases in soybean without compromising protein or oil content (De Souza *et al.*, 2022). It has also been shown that photorespiratory bypass engineering achieved up to 40% biomass increases through synthetic glycolate metabolism pathways (South *et al.*, 2019), with parallel work in rice showing leaf level 18.4% increases in photosynthetic carbon assimilation (Shen *et al.*, 2019). Multi-gene stacking targeting electron transport and RuBP regeneration demonstrated 27% biomass increases while also enhancing water use efficiency (López-Calcano *et al.*, 2020). Rubisco engineering is also another emerging path with successful replacement of plant Rubisco with cyanobacterial variants achieving higher CO₂ fixation rates (Lin *et al.*, 2014), and recent overexpression in C₄ crops also yielded 15.5% biomass increases in field grown sorghum (Lin *et al.*, 2020).

While these photosynthetic enhancements demonstrate yield potential, their full realization also depends critically on optimizing water use efficiency (*WUE*) in an increasingly water limited world (Blatt *et al.*, 2014; Lawson, T & Blatt, M, 2014; Blatt *et al.*, 2021). The balance between carbon gain and water loss through stomatal pores represents a fundamental constraint that must be addressed alongside metabolic improvements. A meta-analysis by Liang *et al.* (2023) examining 5,352 observations across 444 species revealed that stomatal conductance decreases with elevated CO₂ (-

8.3% per 100 ppm) and warming (-1.5% per 1°C), thus highlighting the connections between climate change and plant water relations, as well as the necessity of engineering both photosynthetic capacity and water use efficiency in tandem.

Recent breakthroughs in stomatal regulation offer promising avenues for enhancing WUE without compromising yield. Vaidya *et al.* (2019) developed an ABA mimic with 7-fold increased receptor affinity and 10-fold greater *in vivo* activity, which was shown to successfully protect crops from water stress. Engineering approaches targeting stomatal density through EPF1 overexpression have shown particular promise in rice and barley, improving *WUE* without yield penalties (Hara *et al.*, 2007a; Casson & Gray, 2008). Most intriguingly, the discovery of regulatory kinases like CBC1/CBC2 by Hiyama *et al.* (2017a) revealed how blue light and CO₂ signals converge at the molecular level to optimize stomatal responses. Understanding these regulatory networks, particularly the unique role of blue light in rapidly modulating stomatal aperture and coordinating photosynthetic responses can be promising target for developing climate resilient crops that can maintain high productivity while efficiently managing water resources (see detailed discussion in 1.3.3).

1.2 Light as a Critical Environmental Signal in Plant Biology

Light serves as both the primary energy source driving photosynthesis and a sophisticated informational signal that influences plant development, metabolism, and environmental responses (Figure 1.1). Within the solar spectrum reaching the Earth's surface, blue light (400-500 nm) occupies a unique position in both energetic and regulatory functions. While representing approximately 25% of photosynthetically active

radiation (PAR) under clear sky conditions, blue wavelengths trigger disproportionately large effects on plant morphology, physiology, and fitness through specialized photosensory systems that have evolved over 450 million years of land plant evolution (Raven, 2009; Smith *et al.*, 2017).

The spectral quality of natural light environments varies dramatically across spatial and temporal scales. The majority of leaves experience light fluctuations throughout the day (Long *et al.*, 2022), with sun flecks providing up to 90% of the available light in lower layers of dense canopies (Slattery *et al.*, 2018). Under these fluctuating conditions, blue wavelengths act as rapidly signals adjusting stomatal aperture to optimize gas exchange. The fundamental properties of blue light in the context of plant interaction lies in its dual nature as both a photosynthetic driver and regulatory signal. McCree's action spectrum demonstrated that blue photons yield approximately 10-15% lower quantum efficiency for CO₂ fixation compared to red photons due to absorption by non-photosynthetic pigments and excess energy dissipation (McCree, 1972; Evans, 1987). Yet plants grown under blue deficient conditions can have severe developmental abnormalities including dysfunctional stomata, abnormal chloroplast ultrastructure, and impaired photosynthetic capacity, collectively termed "red light syndrome" (Hogewoning *et al.*, 2010; Trouwborst *et al.*, 2016; Kaiser *et al.*, 2019; Miao *et al.*, 2019). Blue light suppresses stem elongation primarily through cryptochrome signalling, whereas red light and far-red light regulate elongation via phytochromes. Additionally, blue light plays a crucial role in photosynthesis, photomorphogenesis and plant development and nutrient metabolism (Izzo *et al.*, 2020; Zhang *et al.*, 2022; Kong & Zheng, 2023; Chibani *et al.*, 2025b). This apparent contradiction resolves through recognition that blue light's primary value extends beyond

direct photosynthetic contribution to the coordination of developmental programs, metabolic regulation, and stress acclimation responses that ultimately determine photosynthetic performance under dynamic environmental conditions.

From an ecological perspective, plant blue light responses represent adaptive solutions to fundamental challenges facing photosynthetic organisms. Directional blue light perception enables phototropic growth toward optimal light environments with low sensitivity thresholds that enable seedlings to orient toward even minimal light gaps (Liscum *et al.*, 2014; Haga, K. & Sakai, T., 2023). Blue light mediated rapid stomatal opening synchronizes gas exchange with photosynthetic demand, while blue light induced chloroplast movements optimize light capture under low intensities and photoprotection under excess illumination (Kasahara *et al.*, 2004; Takemiya *et al.*, 2005; Lin *et al.*, 2019; Ishishita *et al.*, 2020). High light intensity can cause photodamage, reducing photosynthetic efficiency by leading to the formation of ROS and cellular damage, however a combination of blue and red light has been shown to significantly reduces ROS ($O_2^{\bullet-}$, H_2O_2) in alfalfa seedlings by regulating the activity of the antioxidant enzymes (Chibani *et al.*, 2025b; Khan *et al.*, 2025).

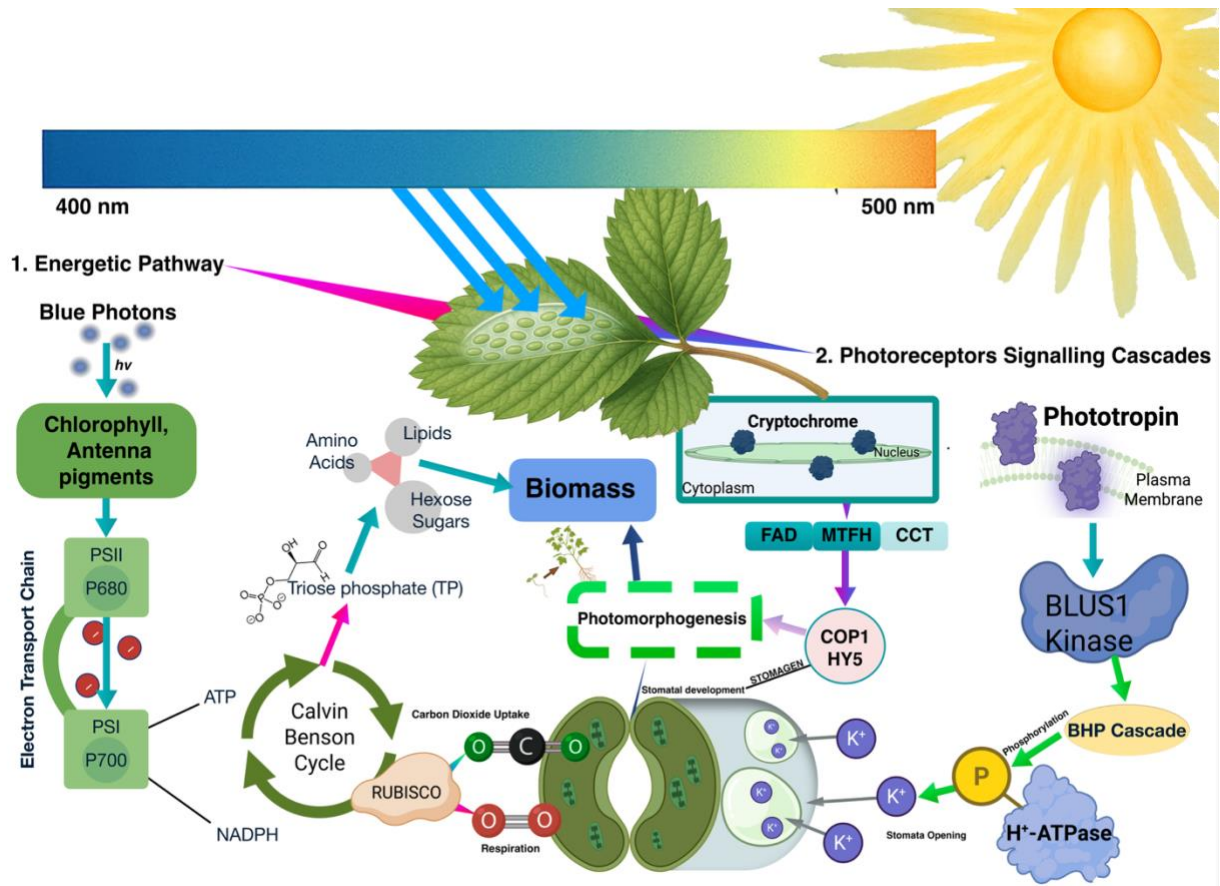


Figure 1.1. Simplified schematic of blue light's dual role as both energy and regulatory signal in plant leaves. 1. Blue photons excite chlorophyll pigments, driving electron transport through PSII and PSI to produce ATP and NADPH for the Calvin-Benson cycle. The resulting carbon assimilation generates sugars, amino acids, and lipids that contribute to biomass. 2. Blue light is perceived by phototropins and cryptochromes. Phototropins activate a BLUS1–BHP kinase cascade that phosphorylates plasma membrane H⁺-ATPases, driving K⁺ uptake and stomatal opening. Cryptochromes modulate transcription via COP1–HY5 signalling to promote photomorphogenesis and stomatal development. Together, these processes coordinate light harvesting with CO₂ uptake and growth.

Recent advances in understanding photosynthetic responses to fluctuating light reveal critical implications for crop productivity. It takes several minutes for photosynthesis to regain efficiency in both either sun-shade and shade-sun transitions, costing a calculated 10–40% of potential crop CO₂ assimilation (Long *et al.*, 2022). Most studies exploring the dynamic of photosynthesis have been performed at the leaf level and on plants grown under homogeneous light conditions which are then suddenly exposed to fluctuating light. It is well known that plants acclimate to both light intensity and dynamic regimes (Violet-Chabrand *et al.*, 2017; Matthews *et al.*, 2018) highlighting the need for canopy level investigations under natural conditions (Salvatori *et al.*, 2022). The integration of light not only serves as the final energy source of green plants metabolism, but also able to act as a signal to indirectly regulate plant growth and development through the activation of plant photoreceptors (Wu *et al.*, 2025).

The agricultural significance of understanding blue light responses has intensified with climate change and technological advances in controlled environment agriculture (CEA). Rising atmospheric CO₂ concentrations, increasing temperature variability, and shifting precipitation patterns demand crops capable of maintaining productivity under unprecedented environmental fluctuations (Leahey, 2009; Hofmann *et al.*, 2025). Concurrently, LED technology enables precise spectral manipulation in vertical farms and greenhouses, where blue light represents significant electricity costs yet remains poorly optimized (Kozai *et al.*, 1978; Xin *et al.*, 2019; Chan *et al.*, 2024; Trumpler *et al.*, 2024). Analysis of the FLUXNET2015 dataset provides observational evidence for widespread thermal acclimation of canopy scale photosynthesis and timescales across diverse biomes further emphasized the need to understand light-temperature interactions (Liu *et*

al., 2024). To situate this point, we note that quantifying blue light effects under realistic diurnal temperature cycles remains limited and improved joint light temperature protocols would sharpen inference at canopy scale. Evidence for specific blue light wavelengths in plant research is lacking, specifically, narrowband action spectra within 400-500 nm under diurnally fluctuating light are scarce, and many studies report only “%blue” without full spectral power distributions, with recent work showing that blue (423–595 nm) light treatment resulted in increased fresh and dry mass in both tomato and lettuce (Trumpler *et al.*, 2024). Quantifying the mechanistic basis of blue light responses, from photon capture through physiological integration to yield outcomes, therefore represents both a fundamental scientific challenge and practical necessity for sustainable intensification of food production systems in foreseeable future.

1.3 Blue Light Perception and Transduction

1.3.1 Molecular and Functional Specialization of Photoreceptor Families

The molecular basis of blue light perception in plants rests upon two major photoreceptor families that have evolved distinct structural features and signalling mechanisms. Phototropins, were first discovered through the pioneering work of Christie *et al.* (1998), who identified NPH1 (now known as phototropin1) as a flavoprotein mediating phototropic responses. These photoreceptors are plant specific serine/threonine kinases with unique molecular architecture. These proteins contain two N-terminal Light-Oxygen-Voltage (LOV) domains (LOV1 and LOV2) that bind flavin mononucleotide (FMN) as chromophores, coupled to a C-terminal kinase domain through a flexible linker region

(Christie *et al.*, 2014). The photochemical mechanism of phototropin activation involves formation of a covalent adduct between the FMN chromophore and a conserved cysteine residue within the LOV domain upon absorption of blue wavelengths. This photochemical reaction, occurring within microseconds, triggers conformational changes that propagate through the protein structure to activate the kinase domain (Crosson & Moffat, 2002; Harper *et al.*, 2003). Structural studies have revealed that LOV2 serves as the primary regulatory domain, while LOV1 modulates photosensitivity and dimerization properties (Halavaty & Moffat, 2007). The differential roles of these domains thus allow the fine tuning capability of light sensitivity across a broad dynamic range from 0.01 to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *Arabidopsis thaliana* possesses two phototropins genes, PHOT1 and PHOT2 which arose through gene duplication and subsequent functional divergence (Harada *et al.*, 2003; Christie *et al.*, 2015; Oide *et al.*, 2018). Phototropin 1 mediates responses under low light conditions with high sensitivity but slow recovery kinetics (half-life ~100 seconds), while phototropin 2 operates under high light with rapid cycling (half-life ~10 seconds) (Briggs & Christie, 2002; Takemiya *et al.*, 2005). It is important to remember that this functional specialization extends beyond simple sensitivity differences, as phot1 specifically controls root phototropism and leaf positioning under low light, while both phototropins redundantly mediate chloroplast accumulation, stomatal opening, and leaf expansion (Sakamoto & Briggs, 2002; Kinoshita *et al.*, 2003; Okada *et al.*, 2003; Emi *et al.*, 2005; Zhao *et al.*, 2018).

Cryptochromes constitute the second major class of plant blue light photoreceptors, which evolved from DNA photolyases but lost their DNA repair function while gaining light signalling capabilities (Ahmad & Cashmore, 1993; Ahmad & Cashmore, 1997; Ahmad *et*

et al., 1998; Lin & Shalitin, 2003; Ahmad, 2016). The Cryptochromes proteins contain an N-terminal photolyase-homologous region (PHR) that binds flavin adenine dinucleotide (FAD) as the primary chromophore and potentially a second chromophore, 5,10-methenyltetrahydrofolate (MTHF), although the role of the latter remains controversial (Malhotra *et al.*, 1995; Hoang *et al.*, 2008; Chaves *et al.*, 2011). The C-terminal extension while unique to cryptochromes and absent in photolyases, varies considerably between CRY1 (181 amino acids) and CRY2 (117 amino acids) which could suggest distinct regulatory properties and protein interactions (Yang *et al.*, 2000; Yu *et al.*, 2007). Numerous historical structural analyses over the past 2 decades have shaped our understanding of cryptochrome photoactivation. Upon blue light absorption, the FAD chromophore undergoes photoreduction from the oxidized state (FADox) through a semiquinone intermediate (FADH•) to the fully reduced form (FADH⁻) which is accompanied by electron transfer through a conserved tryptophan triad (Bouly *et al.*, 2003; Giovani *et al.*, 2003; Zeugner *et al.*, 2005). This photochemical change induces C-terminal domain displacement and oligomerization with CRY2 forming photoactivated tetramers that represent the active signalling state (Wang, Q *et al.*, 2016; Ma *et al.*, 2020). Recent discoveries have revealed additional layers of cryptochrome function beyond photochemical signalling. Chen *et al.* (2025) demonstrated that photoexcited CRY1 directly regulates stomatal development through physical interaction with the master transcription factor SPEECHLESS (SPCH). This blue light dependent interaction mediated through CRY1's N-terminal domain (CNT1), enhances SPCH transcriptional activity by stabilizing its binding to target gene promoters including MUTE, ICE1, and SCRM2. The functional consequence is remarkable with CRY1 overexpression increases

the stomatal index from approximately 30% to 38% producing characteristic stomatal clustering patterns (Cao *et al.*, 2021; Chen, L *et al.*, 2024). Conversely, genetic analysis reveals that CRY1 acts completely through SPCH as CRY1 overexpression in SPCH mutant backgrounds fails to produce any stomata (Chen, L *et al.*, 2024). This mechanism illustrates how cryptochromes can function as both photoreceptors and direct transcriptional coregulators, suggesting the possible avenue of enhancing functional performance (i.e., increase photosynthetic induction and enhanced water use efficacy) by manipulating light signals to precisely control the developmental outcomes.

1.3.2 Signal Transduction Pathways from Photon Capture to Cellular Response

The conversion of absorbed photons into biological responses requires sophisticated signal transduction machinery that amplifies and integrates photoreceptor activation. Calcium signalling represents one of the fastest responses to blue light, with phototropin activation triggering Ca^{2+} influx within seconds of illumination (Kagawa *et al.*, 2003; Spalding *et al.*, 2003; Hermanowicz *et al.*, 2018). Stoelzle *et al.* (2003) first demonstrated that blue light activates calcium permeable channels in *Arabidopsis* mesophyll cells specifically through the phototropins pathway and is independent of photosynthesis. These channels known as phototropins-activated calcium channels (PACCs) can generate transient cytosolic Ca^{2+} increases from 100 nM to 500-1000 nM that last 30-60 seconds (Harada *et al.*, 2003; Okada *et al.*, 2003; Zhao *et al.*, 2013). The spatial dynamics of calcium signalling have been proved critical for directional responses. For example, during phototropism, unilateral blue light creates asymmetric Ca^{2+} gradients across the

hypocotyl with higher concentrations on the shaded side correlating with enhanced growth (Zhao *et al.*, 2013; Haga, Ken & Sakai, Tatsuya, 2023; Haga *et al.*, 2023). These gradients require PHOTOTROPIN INTERACTING FACTOR 1 (PIF1) for maintenance and involve calcium binding proteins like CALCIUM DEPENDENT PROTEIN KINASE 23 (CPK23) that phosphorylate auxin transporters to establish growth-promoting hormone gradients (Sun *et al.*, 2013; Zhang, M *et al.*, 2021).

Phosphorylation cascades downstream of phototropins have been extensively characterized through phosphoproteomics approaches. Blue light triggers rapid autophosphorylation of phototropins at multiple serine residues with Ser-851 in the kinase activation loop being absolutely essential for all phototropin-mediated responses (Inoue *et al.*, 2008; Sullivan *et al.*, 2008). This autophosphorylation occurs within 30 seconds of blue light exposure and creates binding sites for 14-3-3 proteins that may regulate phototropins stability and subcellular localization (Kaiserli *et al.*, 2009; Tseng & Briggs, 2010). In guard cells for example, the pathway from phototropin to stomatal opening involves a series of phosphorylation events with specificity. Takemiya *et al.* (2013) identified BLUE LIGHT SIGNALING 1 (BLUS1) as a guard cell-specific kinase directly phosphorylated by phototropins at Ser-348. This phosphorylation relieves BLUS1 autoinhibition, enabling it to phosphorylate downstream targets. The discovery of BLUS1 resolved a long standing question about how phototropins, which mediate diverse responses in multiple tissues, achieve specificity for stomatal regulation. Interestingly, BLUS1 kinase activity also requires concurrent decreases in intercellular CO₂ concentration, even when BLUS1 is constitutively active, stomatal opening does not occur without a drop in CO₂, highlighting the need for both signals (Hosotani *et al.*, 2021).

Cryptochrome signal transduction on the other hand follows fundamentally different principles. It primarily relies on protein-protein interactions rather than enzymatic cascades. The key regulatory step involves light dependent interaction with the CONSTITUTIVE PHOTOMORPHOGENIC 1/SUPPRESSOR OF PHYA-105 (COP1/SPA) E3 ubiquitin ligase complex. In darkness, COP1/SPA targets positive regulators of photomorphogenesis like ELONGATED HYPOCOTYL 5 (HY5) for degradation. Blue light activated cryptochromes bind directly to SPA proteins through their C-terminal domains and in sequence disrupting the COP1/SPA complex and allowing accumulation of HY5 and other transcription factors (Wang *et al.*, 2001; Lian *et al.*, 2011; Liu *et al.*, 2011). Works from past decade have revealed additional interesting complexity in cryptochrome signalling through the discovery of BLUE LIGHT INHIBITOR OF CRYPTOCHROMES (BIC) proteins. These proteins are induced with activated cryptochromes upon blue light activation, but BIC1 and BIC2 's function is to interact with photoactivated cryptochromes to promote their degradation which effectively serve as a negative feedback regulator (Ma *et al.*, 2016; Wang, Q *et al.*, 2016; Lin *et al.*, 2017; Zhang, D *et al.*, 2020; Lin *et al.*, 2021). The natural balance between cryptochrome activation and BIC mediated inhibition might give plant the capability to precisely tune the blue light responses and prevents excessive photomorphogenic responses that could compromise plant fitness (Wang, Q *et al.*, 2016; Wang *et al.*, 2017).

Hormonal integration adds another layer of complexity to blue light signal transduction and although beyond the scope of this review, some of most interesting examples are during phototropism, phototropins regulate the subcellular localization and activity of PIN-FORMED (PIN) auxin efflux carriers. With blue light triggers rapid in internalization of

PIN3 from the plasma membrane on the illuminated side of the hypocotyl while maintaining membrane localization on the shaded side, this creates an auxin gradient that drives differential growth (Ding *et al.*, 2011; Haga *et al.*, 2015). Additionally, blue light and abscisic acid (ABA) signalling in guard cells demonstrates how environmental signals are hierarchically integrated. ABA completely suppresses blue light-induced stomatal opening through activation of OPEN STOMATA 1 (OST1/SnRK2.6) kinase, which phosphorylates and inactivates the H⁺-ATPase at sites distinct from those targeted by blue light signalling (Yin *et al.*, 2013; Zhang *et al.*, 2016). This antagonistic mechanism might have ensured that water conservation takes priority during drought stress and thus overriding the normal diurnal regulation of gas exchange.

1.3.3 Tissue-Specific Responses and Regulatory Mechanisms

The tissue specific nature of blue light responses reflects specialized expression patterns of signalling components and distinct cellular contexts that shape signal output. Guard cells represent the most specialized system for blue light perception, with unique features that distinguish their responses from mesophyll cells. This specialization makes guard cells a primary target of study and discussion on this thesis project. The extraordinary sensitivity of guard cells to blue light, with measurable responses at light intensity as low as 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$, has long puzzled plant biologists given the apparent simplicity of the phototropin-BLUS1-H⁺-ATPase signalling cascade. Recent breakthroughs have revealed that this remarkable sensitivity emerges from a complex network of signalling pathways, specialized metabolic architecture, and sophisticated amplification mechanisms that fundamentally distinguish guard cells from other plant cell types. The discovery of CBC1

and CBC2 (CONVERGENCE OF BLUE LIGHT AND CO₂) kinases represents a paradigm shift in understanding stomatal regulation, as these Raf-like proteins serve as the first identified molecular convergence point where blue light perception through phototropins directly integrates with CO₂ sensing pathways (Hiyama *et al.*, 2017a). This convergence occurs through direct phosphorylation of CBC kinases by activated phototropins, which subsequently inhibit S-type anion channels (SLAC1/SLAH3), thereby creating a molecular mechanism that coordinates photosynthetic demand with stomatal conductance. The identification of additional regulatory layers, including BHP kinase as an intermediate in H⁺-ATPase activation and CIPK23 as a parallel pathway activating K⁺ channels, demonstrates that blue light triggers multiple, partially redundant signalling cascades that ensure robust stomatal opening responses (Hayashi *et al.*, 2017; Inoue *et al.*, 2020). Perhaps most surprisingly, guard cells achieve their exceptional responsiveness despite possessing chloroplasts with only 1/16th the volume of mesophyll chloroplasts, a paradox resolved by Lim *et al.* (2022) who demonstrated that guard cell chloroplasts function primarily as ATP importers rather than photosynthetic powerhouses, utilizing specialized nucleotide transporters (NTT) to import cytosolic ATP. This metabolic specialization is further emphasized by stoichiometric modelling revealing that starch breakdown provides the most efficient osmolyte source during blue light-induced opening, with coordinated downregulation of glycolysis allowing rapid glucose accumulation (Sprent *et al.*, 2025). The application of single cell technologies has also crushed assumptions of guard cell uniformity, with scRNA-seq revealing continuous developmental trajectories and spatial transcriptomics demonstrating position dependent gene expression gradients that suggest functional specialization even within guard cell

populations (Lopez-Anido *et al.*, 2021; Xia *et al.*, 2022). Signal amplification occurs through multiple mechanisms, including the recently discovered dual phosphorylation of H⁺-ATPase at both Thr-881 and Thr-948, where sequential phosphorylation provides enhanced pumping activity compared to single modifications (Fuji *et al.*, 2024). The emergence of epigenetic regulation through H3K4 trimethylation provides an additional layer of control whereby environmental history influences current stomatal responses. H3K4 trimethylation is a histone modification that marks active chromatin regions, and in guard cells, this epigenetic mark can persist for weeks after environmental stimuli (i.e., long-day light, dehydration) which effectively creating a “molecular memory” that primes future stomatal responses (Aoki *et al.*, 2019; Huang *et al.*, 2019). Low-level phototropin activation initiates convergent signalling that engages specialized metabolic pathways and amplification modules. Together, these circuits generate rapid, high sensitivity responses that optimize photosynthesis while constraining water loss. This framework illuminates cellular specialization and pinpoints actionable targets for engineering climate resilient crops.

1.3.4 Circadian Regulation of blue light responses

Blue light entrains the circadian oscillator, and the clock reciprocally gates blue-light signaling, thereby poising photosynthetic and metabolic pathways for time-of-day–appropriate responsiveness to changing environmental inputs. Although not the primary focus of this thesis project, it’s worth mentioning and discussing here because it’s an undeniable link and function in the chapters discussing the photosynthetic and fruit yield

of strawberry plants. This temporal control system was first molecularly characterized through the circadian oscillation of CAB gene expression (Millar & Kay, 1991; Kay, 1993) which operates through photoreceptors and clock components that has effects on both plant fitness and agricultural productivity. The primary blue light photoreceptors cryptochromes and phototropins (as discussed above in 2.1) interact physically and functionally with core circadian clock components including CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and TIMING OF CAB EXPRESSION 1 (TOC1) to create a sophisticated regulatory system (Somers *et al.*, 1998; Más *et al.*, 2003). Typically blue light pulses administered during the subjective night phase advance the circadian clock by promoting CCA1 and LHY expression through cryptochrome mediated protein stabilization while TOC1 phosphorylation at serine 175 modulates its interactions with FHY3 and PIF5 which together act as a molecular timer that integrates light signals with circadian phase information (Millar, 2016; Oakenfull & Davis, 2017; Wang, X *et al.*, 2021). This bidirectional relationship ensures that blue light sensitivity itself oscillates throughout the day with stomatal conductance showing peak responsiveness during mid-morning hours coinciding with maximal expression of phototropins and H⁺-ATPase genes and has been found to result in up to 50% variations in guard cell sensitivity across the diurnal cycle (Dodd *et al.*, 2004; Kinoshita & Hayashi, 2011). However, the physiological consequences of circadian gated blue light responses extend far beyond stomatal regulation to carbon metabolism, photosynthetic efficiency, and stress resilience in ways that can profoundly impact plant productivity. Plants accumulate approximately 50% of daily photosynthate as transitory starch with degradation rates precisely calculated through circadian mechanisms to ensure carbon

availability until anticipated dawn independent of actual light conditions (Graf *et al.*, 2010; Scialdone *et al.*, 2013). This remarkable temporal arrangement involves linear starch breakdown controlled by circadian clocks that measure time since last dawn with clock mutants lacking functional LHY/CCA1 exhausting starch reserves prematurely according to their accelerated circadian period which often results in carbon starvation and 15-20% reductions in overall fitness (Dodd *et al.*, 2005; Graf *et al.*, 2010). Photosynthetic quantum yield also demonstrates robust circadian oscillations even under constant light conditions with its peak efficiency occurring at subjective midday through coordinated regulation of photosystem stoichiometry and electron transport components (Dodd *et al.*, 2015; Wang, X *et al.*, 2021). While blue light typically exhibits lower quantum yield for CO₂ fixation compared to red light, it is still critical at signalling functions to optimize PSI:PSII ratios and xanthophyll cycle dynamics and with longer term acclimation involving circadian control of photoprotective mechanisms that could anticipate daily light intensity patterns (Takahashi *et al.*, 2009; Cazzaniga *et al.*, 2013).

The translation of fundamental circadian to blue light interactions into agricultural applications has yielded transformative results in past decade particularly in controlled environment agriculture (CEA) where precise spectral and temporal manipulation enables unprecedented productivity gains. Commercial implementations in strawberry production exemplify this revolution, with facilities like Plenty's Richmond Farm achieving production densities of 40 kg/m² annually through AI optimized LED systems that analyse over 10 million data points daily to synchronize lighting with plant circadian rhythms and has reported reduction in land use compared to traditional farming (Shamshiri *et al.*, 2018; Kozai *et al.*, 2019). Blue light supplementation at 450-470nm wavelengths when properly

timed to circadian phase can increase strawberry inflorescence numbers by up to 300% under experimental conditions with commercial implementations demonstrating 18-24% increases in primary fruit fresh weight (Yoshida *et al.*, 2016; Nakayama & Nakazawa, 2023). Beyond horticultural crops, the principles of circadian-blue light optimization extend to diverse agricultural systems with tomato production benefiting from continuous lighting protocols that eliminate seasonal variations while achieving 47% energy reduction, and lettuce cultivars showing differential responses to continuous light with red-dominant spectra (80% red/20% blue) producing 40-60% heavier heads while minimizing physiological stress disorders (Velez-Ramirez *et al.*, 2011; Ohtake *et al.*, 2018). The disruption of circadian rhythms through mutation of clock genes or inappropriate lighting schedules reduces plant fitness primarily through misregulation of gas exchange and carbon metabolism with arrhythmic plants requiring 2-3 times more individuals to achieve equivalent competitive effects and showing 36-63% reduced photosynthetic efficiency compared to plants with properly synchronized clocks (Dodd *et al.*, 2005; Müller *et al.*, 2014). Through targeted modification of photoreceptor systems and circadian components, it is possible to enhance yield stability under fluctuating environmental conditions while reducing resource inputs in both controlled and field environments.

1.3.5 The Opportunity of Blue Light Priming Effects and Light memory in plants

Blue light priming can also induce persistent morphological and physiological adaptations that fundamentally alter plant architecture and photosynthetic performance (Wang, J *et al.*, 2016; Meinen *et al.*, 2019; Nie *et al.*, 2024). In the short term in *Arabidopsis thaliana*, phototropins activation triggers chloroplast repositioning movements that optimize light

capture under low intensities while providing photoprotection at high intensities ($>100 \mu\text{mol m}^{-2} \text{s}^{-1}$) which can reduce light absorption by up to 30% through strategic relocation to anticlinal cell walls (Suetsugu & Wada, 2012; Wada, 2013). These subcellular responses represent the fastest tier of blue light adaptation with PMI1 protein displacement occurring within 60 seconds and complete chloroplast repositioning achieved within 10-30 minutes (Suetsugu *et al.*, 2015). The cellular level transformations can also extend beyond organelle positioning, for instance, *Lactuca sativa* (lettuce) grown under blue enriched LED spectra (30% blue light) exhibits higher chloroplast density in palisade mesophyll cells compared to red light dominant conditions with these organelles displaying enhanced grana stacking and increased thylakoid membrane density that persists for 24-48 hours post treatment (Hogewoning, S. W. *et al.*, 2010; Muneer *et al.*, 2014).

Stomatal development is another particularly blue light sensitive process, with recent work revealing that cucumber seedlings grown under high blue light conditions (R:B ratio 1:9) show 45.75% higher stomatal densities compared to low blue light conditions (R:B ratio 9:1), mediated through the HY5-STOMAGEN signalling cascade where blue light induces the transcription factor HY5 to activate STOMAGEN expression in mesophyll cells (Kang *et al.*, 2009; Savvides *et al.*, 2012; Zhang, Yiting *et al.*, 2020). The discovery of CBC1/2 kinases is another merging points for blue light and CO_2 signals which functioning downstream of phototropins and has reveals sophisticated integration of environmental cues in stomatal regulation (Hiyama *et al.*, 2017b). Despite these structural enhancements, the effects on photosynthetic capacity prove more nuanced than often portrayed, rather than dramatic improvements, blue light's primary role appears to be

preventing photosynthetic dysfunction with as little as 7% blue light in growth spectra sufficient to maintain normal photosynthesis in *Solanum lycopersicum* (tomato) by preventing the "red light syndrome" characterized by dysfunctional stomata and abnormal chloroplast ultrastructure (Hogewoning, Sander W *et al.*, 2010; Kaiser, Elias *et al.*, 2019). Biochemical analyses across multiple species consistently show 10-43% increases in chlorophyll content under blue light treatments with *Brassica oleracea* (red cabbage) demonstrating the upper range of response, but also accompanied by 1.2-4.3 fold increases in carotenoid content that enhance photoprotective capacity (Li & Kubota, 2009; Hoffmann *et al.*, 2015). At the transcriptional level, approximately 20% of transcription factor genes in *Arabidopsis* seedlings respond to blue light within hours and often seen as upregulation of genes encoding PSII core proteins (PsbA, PsbD, PsbB), light-harvesting complex proteins (Lhcb1-6), and Calvin cycle enzymes including RbcS, FBPase, and GAPDH, though the magnitude of protein abundance changes remains modest compared to some claims in the literature (Ma *et al.*, 2001; Pooam *et al.*, 2021). The temporal persistence of these adaptations follows a clear hierarchy with molecular changes reverse within hours, chloroplast movements within 10-12 hours, cellular structural modifications persist for 24-72 hours, while anatomical changes at the tissue level can remain for weeks or become permanent in mature leaves, suggesting that plants maintain a "memory" of blue light exposure that keep responses poised for future high-light encounters (Davis *et al.*, 2011; Wada, 2013). Critical gaps in current understanding include the lack of comprehensive studies examining blue light priming effects across diverse crop species beyond model systems especially in agricultural and horticultural more relevant plants (i.e., wheat, rice, strawberry), limited investigation of how blue light

memory mechanisms interact with other environmental stresses such as drought or temperature extremes, insufficient data on the energetic costs versus benefits of maintaining blue light induced adaptations, absence of predictive models linking blue light exposure parameters to specific physiological outcomes across different developmental stages, and minimal research on how artificial selection in crops may have altered natural blue light response pathways (Huché-Thélier *et al.*, 2016; Battle *et al.*, 2020). These knowledge gaps become particularly relevant for controlled environment agriculture, where LED technology enables precise spectral control but where energy efficiency demands optimization of photon delivery (Ouzounis *et al.*, 2015). Whilst these questions remained, this emerging picture suggests that blue light acts not only as a driver of photosynthesis but also as an essential regulatory signal that coordinates plant form with function. It ensures photosynthetic machinery remains properly tuned to environmental conditions through a network of sensory proteins, signalling cascades, and persistent structural modifications. Thus, manipulating plant response through blue light priming might provide a viable path to optimize light capture, CO₂ fixation, and photoprotection in an ever changing light environment (Briggs & Huala, 1999; Smith *et al.*, 2017).

1.4 Methodological Advances in Studying Functional Blue Light Responses

The investigation of plant blue light responses has been modernized by technological advances that provide unprecedented spatial, temporal, and molecular resolution. These methodological innovations including those developed and used in this thesis project are critical for engineering climate resilient crops that can maintain productivity while optimizing water use efficiency under increasingly variable environmental conditions.

The Spectral Specificity in Gas Exchange

Modern day gas exchange systems have evolved beyond simple CO₂/H₂O analysers to become sophisticated platforms for dissecting wavelength specific photosynthetic responses. The integration of narrow bandwidth LED arrays (475 nm for Blue Light channel, 625 nm for Red Light in Licor's Li-6800 system for example) with traditional infrared gas analysers has enabled precise quantification of blue light specific stomatal responses while maintaining saturating photosynthetic conditions through supplemental red illumination (Barillot *et al.*, 2010; Matthews *et al.*, 2020; Violet-Chabrand *et al.*, 2021). Critical technical innovations include spectral isolation protocols using multichannel LED control systems, automated measurement sequences and stability control to capture both transient (dynamic) and steady state responses, and integration of PAM fluorometry for simultaneous quantum yield assessment (Busch *et al.*, 2024; Acevedo-Siaca & McAusland, 2025). These advances addressed longstanding challenges in separating stomatal from photosynthetic responses to blue light (Busch *et al.*, 2024). The methodological challenge of separating blue light stomatal responses from photosynthetic effects has been addressed through measurement protocols like pre-adaptation which

employ red light intensities of 600-800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 30-60 minutes to establish steady state photosynthesis before blue light application (Matthews et al., 2020). This approach reveals that blue light induces additional stomatal opening of 0.05-0.15 $\text{mol m}^{-2} \text{s}^{-1}$ beyond the red light baseline and with species specific kinetics ranging from 5-45 minutes to reach new steady states (Matthews *et al.*, 2018; Violet-Chabrand *et al.*, 2021). Temperature control emerges as a critical parameter, with modern systems maintaining leaf temperature within $\pm 0.1^\circ\text{C}$ through active Peltier based regulation. This precision is essential given the high temperature sensitivity of stomatal response (Peak & Mott, 2011; Urban *et al.*, 2017; Garen & Michaletz, 2024). Building on this foundation, Chapter 5 introduces a paradigm shift through randomized spectral programming. Rather than testing discrete light conditions, we developed Python-based background control systems that continuously vary both intensity (200-900 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and spectral composition (0-100% red:blue ratios and far-red additions) throughout diurnal cycles. This approach generates large datasets capturing thousands of unique light combinations and has revealed non-linear interactions between photon energy content and physiological responses that static measurements could miss entirely.

Large Scale Spatial quantification of stomatal patterning and functions

The recognition that stomatal responses vary spatially has led to the use of thermal imaging approaches for wide-area assessment. Infrared thermography has also evolved into a sophisticated tool for non-invasive assessment of blue light effects on stomatal function. Modern thermal cameras with microbolometer arrays achieve temperature resolution of 0.02°C at 30°C which is sufficient for the detection of subtle physiological

responses (Merlot *et al.*, 2002; Jones *et al.*, 2009; Vialet-Chabrand & Lawson, 2019; Pignon *et al.*, 2021; Fan *et al.*, 2024). Chapter 2 advances this field by establishing rigorous protocols for converting thermal maps into quantitative conductance indices. One of the key methods used here is the dynamic energy balance calculations that account for changing environmental conditions which makes real time tracking of stomatal behaviour across entire leaves during fluctuating light exposure possible.

However, thermal imaging alone cannot explain the mechanistic basis for spatial heterogeneity. Chapter 3 addresses this gap through a novel high-throughput confocal microscopy system capable of imaging stomatal distributions across 18 cm² areas which is orders of magnitude larger than traditional sampling approaches. By combining leaf chlorophyll autofluorescence with automated image analysis, we can now quantify not just density but spatial clustering patterns across large epidermal surface. This methodology also revealed critical insight that the relationship between anatomical features and physiological function depends fundamentally on sampling scale with correlations improving fivefold as sampling area increase, and that differential stomatal density between leaf surfaces can lead to compensatory physiological mechanisms that maintain or even enhance gas exchange despite reduced stomatal numbers (Chapter 3 and 6).

From Static to Dynamic: Mimicking Nature Light Environment

Natural light environments create complex fluctuation patterns spanning subsecond leaf flutter to minute scale cloud movements, fundamentally different from laboratory square wave protocols (Kaiser *et al.*, 2018; Slattery *et al.*, 2018). This complexity matters

because photosynthesis requires several minutes to adjust during light transitions which can cause steady state measurements to overestimate carbon gain by 10-40% (Slattery *et al.*, 2018). Despite sun flecks providing up to 90% of light in lower canopy layers, most photosynthetic research did not capture these dynamics. Recent studies using sinusoidal variations or recorded natural fluctuations remain limited in scope and standardization (Salvatori *et al.*, 2022). Current models bounded between full-light-integration and no-light-integration fail to capture the photosynthetic penalties inherent to fluctuating conditions (Graham *et al.*, 2017). This gap between artificial and natural conditions has hindered identification of targets for crop improvement under field environments. Chapter 7 addressed these limitations through the development of a biologically informed algorithm that creates a web based customizable stochastic light programs with tuneable coefficients of variation and high number of significant transitions per hour to match natural canopy dynamics. Critically, the program output identical light programs that can be used for growth light and for gas exchange system measurement. When applied to transgenic plants with guard cell specific H^+ -ATPase overexpression, these naturalistic conditions revealed 40% reduction in transition penalties and unexpected diminishment of blue light sensitivity compared to red light. Therefore, performance constraints invisible to conventional approaches have been revealed. This paradigm shifts from artificial to naturalistic light regimes fundamentally changes our ability to predict plant performance and identify improvement targets.

Needless to say, the true power of these methodological advances is through integration. By using these methods in combinations throughout this thesis project, we were able to ask and address fundamentally new questions regarding blue light responses. How does

spatial heterogeneity in stomatal distribution affect whole plant responses to fluctuating light? Do surface specific adaptations optimize performance under specific spectral environments? Can molecular enhancements overcome evolutionary constraints on response kinetics? Rather than viewing blue light responses through the narrow lens of individual techniques (i.e., gas exchange or transcriptomic quantification), we can now construct comprehensive models linking molecular manipulation to plant physiological mechanisms to ecological performance. This will allow us ultimately to translate fundamental discoveries into practical applications, whether optimizing controlled environment agriculture or engineering climate resilient crops (Bailey-Serres *et al.*, 2019; Eckardt *et al.*, 2022; Long *et al.*, 2022; Zhang *et al.*, 2025).

1.5 Thesis Objectives and Chapter Overview

This thesis project addresses critical knowledge gaps in our understanding of blue light responses in plants, with particular emphasis on developing novel methodologies and applying these to understand how blue light priming and spectral manipulation can enhance plant performance under variable environmental conditions. The overarching hypothesis tested in this thesis is that spatiotemporal manipulation of blue light via developmental priming and surface targeted pulsed delivery to the abaxial epidermis enhances diurnal carbon gain and resource use efficiency by accelerating guard cell signalling and reducing photosynthetic transition penalties under dynamic environments. By using high throughput methodologies and applying these to both model and crop

species, this work aims to provide actionable insights for optimizing plant photosynthetic performance in controlled environment agriculture and field conditions.

Chapter 2: Using Infrared Thermography for High Throughput Plant Phenotyping:

This chapter (Fan *et al.*, 2024) establishes the methodological foundation for non-invasive assessment of stomatal behaviour through thermal imaging. The work provides standardized protocols for using wet and dry reference materials to calculate stomatal conductance indices and addressed critical issues of emissivity calibration and boundary layer determination. An important application is for predicting stomatal conductance dynamics under fluctuating light conditions through resolution of the leaf energy balance. The analytical workflows presented enable researchers to extract physiological parameters from thermal time series, providing a scalable approach for phenotyping stomatal responses across diverse genotypes and environmental conditions. For instance, quantifying the rapid stomatal movements induced by blue light, which are challenging to capture at large spatial scales using gas exchange methods.

Chapter 3: High Throughput Quantification of Spatial Stomatal Patterning in Barley

This chapter presents a breakthrough methodology for analysing stomatal distribution patterns across large spatial scales. Using automated chlorophyll autofluorescence confocal microscopy and custom image analysis pipelines, over one million stomata were quantified across 18,205 mm² of leaf tissue. The work reveals that stomatal density varies spatially and that this heterogeneity has profound implications for gas exchange measurements. Monte Carlo simulations further demonstrated that traditional sampling approaches severely underestimate the sampling requirements for reliable stomatal

quantification. Analysis of EPF1 overexpressing lines reveals that genetic manipulation differentially impacts *SD* on the two leaf surfaces with implications for whole leaf gas exchange.

Chapter 4: Blue Light Priming Induces Modifications in Strawberry Leaf

Development and Stress Tolerance: This chapter investigates whether early exposure to elevated blue light percentages can prime strawberry plants for enhanced performance under subsequent stress conditions. Using controlled environment chambers with precise spectral control, plants were exposed to blue light gradients during early development before transfer to natural light conditions. The work reveals that high percentage of blue light priming can enhance photosynthesis, but the enhanced traits quickly normalized 30 days post priming, suggesting the high plasticity nature of leaf development to light.

Chapter 5: Improving Diurnal Carbon Gain with Pulsed Blue Light Illumination to

Abaxial Surface and Big Data Insights: This chapter used a novel experimental approach using randomized light programs to explore the full parameter space of intensity-spectrum combinations. Building on the insights from the large dataset collected, we identified key parameters contributing to the photosynthetic efficiency over the diurnal period. Modelling work on these datasets achieved $R^2 = 0.922$ in predicting CO_2 quantum yield from spectral and environmental parameters, which demonstrated the power of machine learning approaches for optimizing horticultural lighting strategies. This has inspired the design of a new blue light pulses system. For which, we demonstrated that periodic blue light pulses applied specifically to the abaxial leaf surface can enhance daily

carbon gain (> 8%) by modulating stomatal behaviour with reduced energy penalty of continuous blue light exposure.

Chapter 6: Surface Specific Reductions in Stomatal Density in EPIDERMAL PATTERNING FACTOR1 Wheat Differentially Affect Stomatal Red and Blue Light

Responses: This chapter examines how genetic reduction of stomatal density through TaEPF1 overexpression differentially affects blue light responses when illumination is provided to either the adaxial or abaxial leaf surface. Using bi-directional illumination protocols with standard gas exchange systems, the work measured whole leaf gas exchange while selectively illuminating either surface. The key finding revealed that transgenic lines with 11-20% reduced stomatal density (more pronounced on the abaxial surface) exhibit enhanced blue light sensitivity that compensates for anatomical limitations. When the abaxial surface was illuminated, EPF1 overexpressing lines showed dramatically enhanced stomatal responses to blue light up to 90% increase in conductance compared to 49% in wild type. This heightened sensitivity coincided with lower intercellular CO₂ concentrations under red only light, suggesting that diffusional constraints prime the stomatal signaling apparatus for enhanced blue light responses. These transgenic lines maintained 15-20% higher water use efficiency across all light conditions. The surface specific nature of these compensatory mechanisms reveals complex interactions between anatomical constraints, mesophyll signalling, and guard cell light perception that could be exploited for developing climate resilient crops.

Chapter 7: Engineering Faster Stomata Improves through Guard Cell Specific Overexpression of H⁺-ATPase Photosynthesis and Biomass Performance Under

Natural Fluctuating Light: This final data chapter tests whether molecular enhancement of stomatal machinery can overcome constraints on dynamic photosynthesis. Transgenic tobacco overexpressing Arabidopsis H⁺-ATPase (AHA2) specifically in guard cells was produced which resulted in constitutively higher stomatal conductance. Using a novel stochastic light program that mimics natural light dynamics, the work demonstrates that enhanced H⁺-ATPase expression reduces transition penalties by 40% and increases biomass accumulation under fluctuating light.

Collectively, this thesis project advances our understanding of light responses, with specific emphasis on blue light mechanisms, from molecular mechanisms to whole plant performance, while methodological development has enabled investigation at previously inaccessible scales. The findings have immediate applications in controlled environment industry, where spectral optimization could reduce energy costs while enhancing crop performance, and longer term implications for developing climate resilient crops through targeted manipulation of blue light response pathways and guard cell development.

Chapter 2: Using Infrared Thermography for High Throughput Plant Phenotyping

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Key words: plant thermal imaging, leaf temperature, thermal cameras, stomatal conductance, stomatal kinetics, high throughput phenotyping, infrared thermography, stress indices, crop water stress index, leaf boundary layer

Running Head: Thermal imaging of plants

Abstract (Chapter 2)

Infrared thermography offers a rapid, non-invasive method for measuring plant temperature, which provides a proxy for stomatal conductance and plant water status and can therefore be used as an index for plant stress. Thermal imaging can provide an efficient method for high-throughput screening of large numbers of plants. This chapter provides guidelines for using thermal imaging equipment and illustrative methodologies, coupled with essential considerations, to access plant physiological processes.

2.1 Introduction

Thermography, also known as infrared thermography or thermal imaging, is a non-invasive technique for measuring thermal radiation emitted by objects. The origins of using thermography for plant phenotyping date back to the 1950s (Scarth *et al.*, 1948; Tanner, 1963). Initially, various indices were developed for detecting crop stress, and they have been primarily used for scheduled irrigation application (Idso, 1982; Jackson, 1982). Over the past decade, thermography has garnered considerable interest in plant phenotyping due to its capacity to detect subtle temperature variations, which can indicate plant water status, and other phenotypic traits (Violet-Chabrand, SR *et al.*, 2017; Violet-Chabrand & Lawson, 2020; Pignon *et al.*, 2021). The recent development of field-portable thermal imaging systems has expanded the scope of analysis, leading to wider applications including screening for drought and heat-tolerant genotypes (Saint Pierre *et al.*, 2010; James & Sirault, 2012; Prashar & Jones, 2014) and mapping photosynthetic traits under dynamic and steady-state conditions (Salter *et al.*, 2020; Farella *et al.*, 2022). Remote UAV imaging systems have also been developed to screen for water status in different types of vegetation (Berni *et al.*, 2009; Zarco-Tejada *et al.*, 2012; Gonzalez-Dugo *et al.*, 2013).

One of the crucial mechanisms influencing leaf temperature is stomatal activity, which plays a vital role in modulating the balance between photosynthesis and transpiration (Matthews *et al.*, 2020). Building on this premise, researchers have developed indices to quantify leaf conductance and plant water loss using dry and wet reference materials (QIU *et al.*, 1996; Leinonen *et al.*, 2006; Blonquist Jr *et al.*, 2009). More advanced

methodologies employ energy balance calculations to derive diurnal estimates of transpiration and leaf conductance indices (Violet-Chabrand & Lawson, 2020). These approaches not only account for spatial variation in stomatal conductance and leaf energy balance (Lawson *et al.*, 1998) but also identify limiting factors under non-steady-state, thereby serving as essential components in the development of dynamic photosynthesis models across various species (Wang, Y *et al.*, 2021).

This technical workflow will provide guidance on using thermography for plant phenotyping, covering camera calibration and measurement environment setup to enhance the accuracy and sensitivity of infrared thermometry. Additionally, we provide step by step recommendations for employing thermography techniques to calculate stress indices and to estimate stomatal conductance using artificial reference materials such as filter paper or directly applying grease or water to the leaf for measurement in steady-state and dynamic light conditions (McAusland *et al.*, 2013; Violet-Chabrand & Lawson, 2019; Violet-Chabrand & Lawson, 2020).

2.2 Materials

2.2.1 Infrared (IR) Camera

An infrared camera is essential for thermography, as it records thermal radiation emitted by objects in the form of temperature data. When choosing an IR camera for plant phenotyping, factors such as resolution, sensitivity, wavelength range, frame rate, and temperature range should be considered (Sagan *et al.*, 2019). Although more costly, higher resolution cameras yield more detailed thermal images, enabling better detection of subtle temperature variations (Merlot *et al.*, 2002). Camera sensitivity, or noise

equivalent temperature difference (NETD), should be low (preferably less than 0.05 °C) for accurate detection of small temperature fluctuations in plants. Spectral sensitivity spans from 3 to 14 μm , with the most prevalent wavelengths for thermal imaging falling into two categories: 3-5 μm and 7-14 μm . The thermal sensitivity of the shorter 3-5 μm wavelengths is higher than that of the 7-14 μm wavelengths, as shorter wavelengths correspond to higher energy levels (Li *et al.*, 2014). Longer wavelengths are used for observing targets at great distances through extended atmospheric paths (Kaplan, 2007). Regardless of the camera used, before beginning measurements, users must ensure their camera is calibrated (see Note 1). To properly calibrate and measure, a Type K thermocouple is required for the determination of emissivity of references and sample plants, as the sensitivity of the thermocouple is greater than the sensitivity of the camera.

2.2.2 Environmental Control System

Investigating plant thermal responses to environmental changes can help to identify genotypes with enhanced adaptability and resilience to environmental challenges. To examine thermal responses to environmental changes, experimental design usually involves varying one or multiple environmental factors, e.g., temperature, humidity, or light intensity, over a specific duration, and this is best controlled by an environmental control system.

When performing measurements in a climate-controlled phenotyping platform or growth room, it is important to ensure appropriate air mixing to avoid temperature and humidity gradients within the measuring space (see Note 2). To help monitor the conditions, we

recommend incorporating a data logger into the environmental control environment that records air temperature and relative humidity. Additionally, we recommend placing quantum sensors at the height of the measurement plane to record radiation received at the leaf level and near reference materials to monitor the light conditions. These tools are not only useful for monitoring environmental parameters throughout the measurement process but are also essential for assessing leaf energy balance in dynamic environments. One can then derive the temporal response of stomatal conductance to assess plant responses to varying conditions (Violet-Chabrand & Lawson, 2019) (see Note 3).

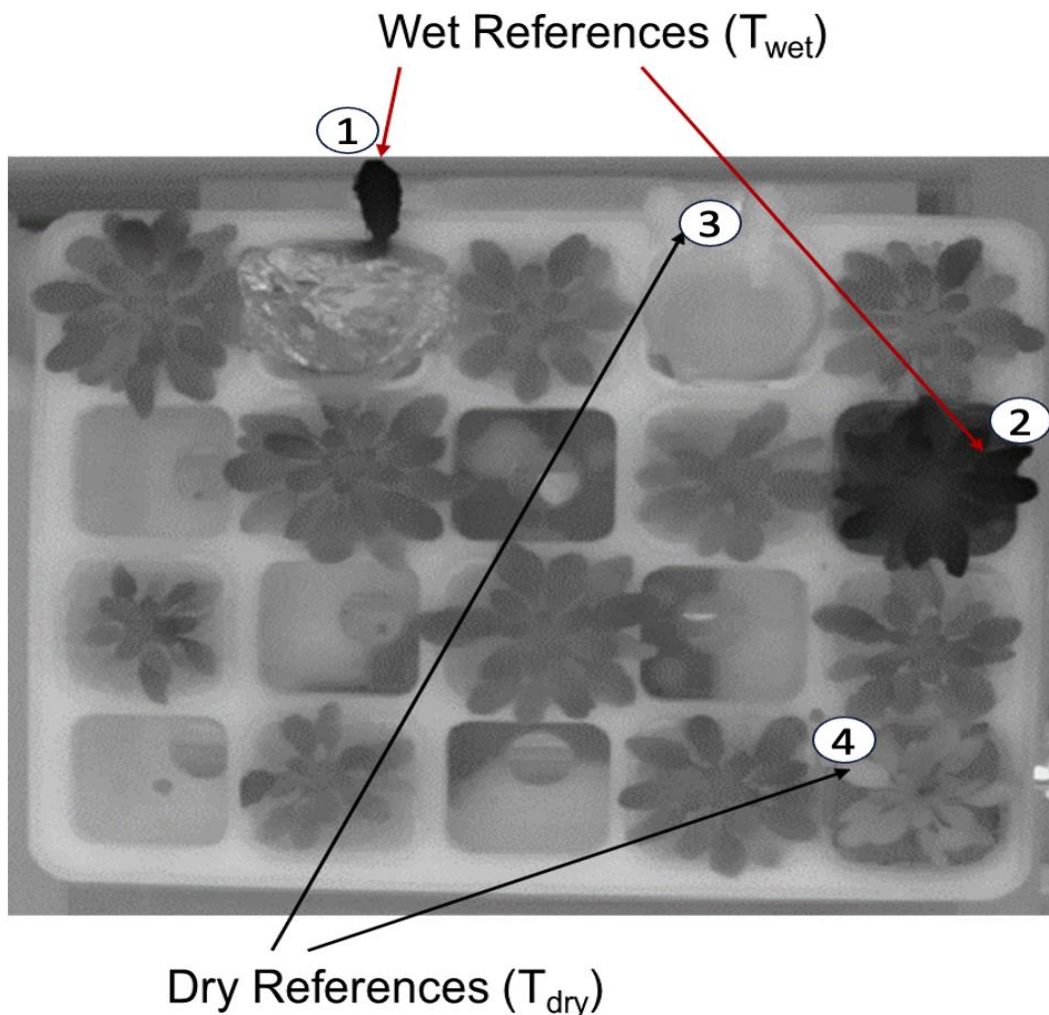


Figure 2.1. Greyscale thermal image of an *Arabidopsis thaliana* tray showing the four calibration targets used to bracket leaf temperature. 1: *Artificial wet reference* as a cotton wrapped metal leaf surrogate kept continuously moist by dipping its stem in a water reservoir that is shielded with aluminum foil to minimize solar heating. 2: *leaf wet reference* with an *Arabidopsis* leaf sprayed to saturation immediately before imaging. 3: An *artificial dry reference* using the same cotton/metal surrogate as in 1, but air-dry. 4: *Leaf dry reference* using an *Arabidopsis* leaf rendered non transpiring by coating both surfaces with petroleum jelly. Targets 1–2 represent the lower (T_{wet}) bound,

while targets 3 to 4 represent the upper (T_{dry}) bound of the leaf energy balance. Darker pixels correspond to cooler temperatures.

2.2.3 Reference objects

Reference objects, particularly wet and dry ones (Fig. 2.1), are pivotal for determining stress indices and estimating stomatal conductance (see Note 4). The use of these reference objects enables the normalization of thermal measurements, acting as benchmarks for leaf temperature under conditions of full transpiration (wet) and no transpiration (dry) (Leinonen *et al.*, 2006; Matsuda *et al.*, 2007; Bian *et al.*, 2019). When selecting materials for wet and dry references, it is crucial to take into consideration their thermal emissivity (the efficiency with which an object emits thermal energy), heat capacity, and surface roughness; those characteristics should ideally mirror those of the leaf. Similarly, the aerodynamic properties of the reference (size, thickness and shape) should be similar to the leaf to ensure a similar boundary layer.

Typically, water-saturated filter paper or fabric serve as effective wet references, and dry versions can act as dry references (Fig. 2.1). It is important to ensure that the chosen materials do not undergo significant property changes due to evaporation or heating during the course of the experiment. Designing the wet reference to maintain consistent evaporation rates (for example, by wicking water up from a reservoir) is beneficial for long-term monitoring. Alternatively, a leaf can be used as a wet reference by spraying the leaf with a fine mist of water. Ideally, this water should contain a small amount of detergent (i.e., Tween 20) to prevent large droplets of water from forming. However, if using this

method, then it is critical to ensure that the leaf remains wet with consistent evaporation rates throughout the experiment.

Similarly, you can create a dry reference using a leaf by applying a layer of grease or petroleum jelly to a leaf. This technique prevents evaporation of water from the leaf surface, effectively acting like a non-transpiring leaf. Given that the reference object is an actual leaf covered with grease, its physical properties closely mimic those of the leaves under investigation. However, as this method involves a destructive process, careful handling is required to avoid modifying the leaf optical and physical properties that influence the reference's interaction with the environment.

2.2.4 Imaging Analysis Software

Image analysis software is essential for processing, analysing, and interpreting thermographic data obtained from the IR camera. Various software options are available, such as FLIR Tools, MATLAB, and ImageJ, each offering a unique set of capabilities and features. When choosing image analysis software, consider factors like ease of use, compatibility with the IR camera file format, built-in tools for temperature calibration, region of interest (ROI) selection, and statistical analysis. Additionally, it is important to select software that enables batch processing of multiple images for large datasets. Custom scripts or plugins may be required for specific analyses, so ensure that the chosen software supports these features.

2.3 Camera setup and calibration

Before initiating the experiment, correctly setting up and calibrating the infrared camera is crucial to ensure accurate temperature measurements.

1. Position the camera on the tripod or mounting equipment, maintaining a consistent distance from the plants throughout the experiment. The distance should be adequate to capture the entire area of interest while maintaining a desirable level of detail.
2. Adjust the camera angle so it is perpendicular to the target surface, minimizing potential errors due to oblique viewing angles.

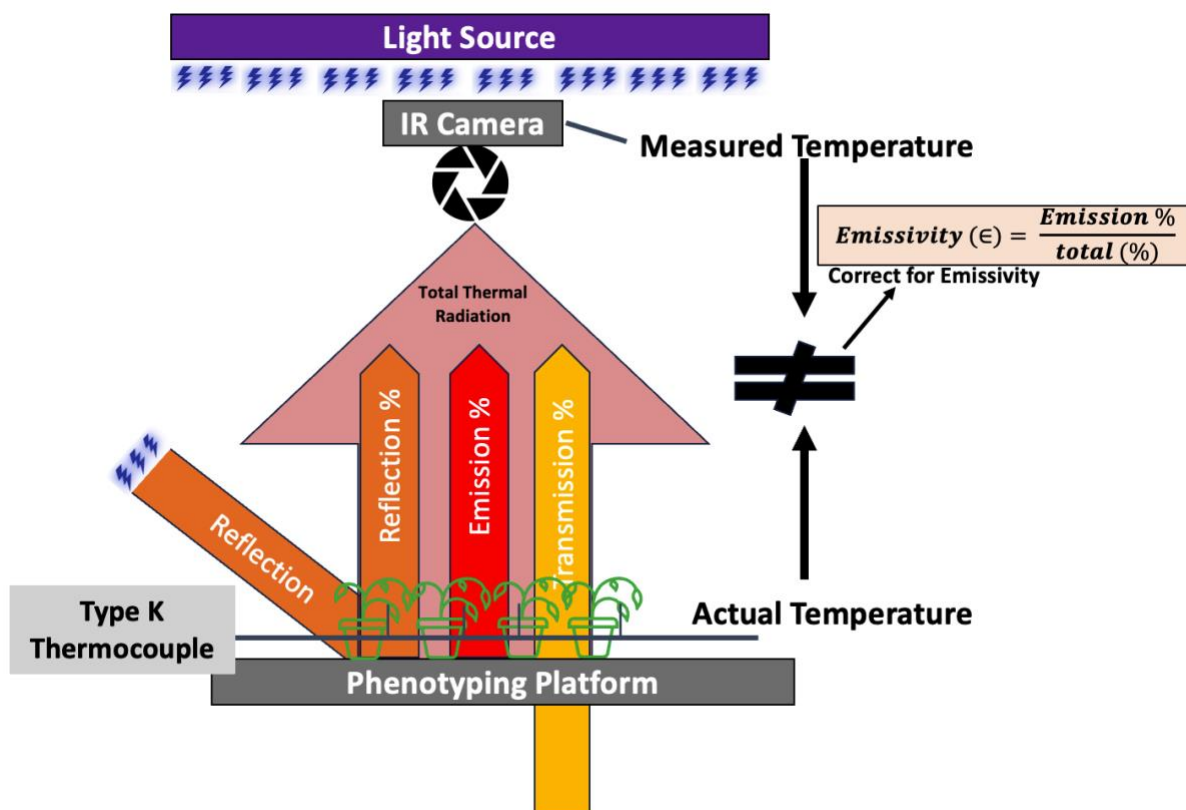


Figure 2.2. Schematic representation of light distribution within a plant phenotyping platform as interpreted by an infrared (IR) camera. This depiction underscores the potential discrepancy between the measured temperature as perceived by the IR camera and the actual temperature measured directly via a Type K thermocouple. Such variations can arise due to differences in emissivity,

necessitating careful calibration to ensure accurate temperature readings. Emissivity is calculated by comparing the emitted temperature as IR light against the actual temperature as measured by a thermocouple.

2.3.1 Determining Emissivity

Emissivity is a fundamental parameter in thermography, significantly affecting the accuracy of temperature measurements (Fig. 2.2). This term describes an object's efficiency in emitting thermal radiation compared to an ideal black body. Different materials have distinct emissivity values, which can influence the measurement results. For leaves, the typical emissivity ranges between 0.95 and 0.98, with 0.96 or 0.98 commonly employed.

However, to ensure accurate measurements, it may be necessary to determine the emissivity value specifically for the plant species or reference leaf of interest. The process of determining emissivity involves measuring the temperature of the object under the same conditions with both a thermal image (measured temperature) and a measurement with a thermocouple (known temperature). The measured temperature is then compared to the known temperature to confirm the accuracy of the camera settings. If inconsistencies are found, then adjustments should be made to the camera settings, and the test should be repeated until accurate temperature measurements are secured.

2.3.1.1 Emissivity test using a Type K thermocouple

1. Choose a representative region of interest (ROI) on the leaf or plant canopy that will be the focus of the thermographic measurements.
2. Attach a Type K thermocouple to the ROI, ensuring adequate contact between the thermocouple and the leaf surface.
3. Use the Type K thermocouple to measure the actual temperature of the ROI.
4. Position the thermal imaging camera so that it is facing perpendicular to the ROI and aimed at the ROI, making sure that the thermocouple is within the field of view.
5. Capture the image of the ROI and determine the apparent reflected temperature, which can be measured directly on the camera's onboard interface itself or using external software (e.g., FLIR Tools for FLIR camera systems), whichever provides suitable precision.
6. Adjust the emissivity setting of the thermal imaging camera to suit the ROI.
7. Compare the temperature reading from the thermal imaging camera with the actual temperature measured by the Type K thermocouple.
8. Repeat steps 6 and 7, adjusting the emissivity setting until the temperature reading from the thermal imaging camera matches the actual temperature measured by the Type K thermocouple.
9. Note down the final emissivity value for use in subsequent thermographic measurements of similar plant surfaces (see Note 5).

2.3.1.2 Emissivity test using a water bath

Alternatively, an emissivity test can be conducted by floating a leaf in a water bath at a known temperature and capturing an image of the leaf in the bath with the thermal camera. This method ensures that the leaf temperature equals the water bath temperature, providing another reliable approach for validating the camera settings.

2.3.2 Index of Stomatal Conductance

The Index of stomatal conductance (I_g) is a proxy for stomatal conductance, utilizing the temperatures of two reference materials (see Section 2.2.3). The first reference represents the temperature of a leaf with the theoretical minimum stomatal conductance, which can be a dry filter paper or a plant leaf covered with grease to prevent any evaporation of water. A second reference represents the temperature of a leaf with the theoretical maximum stomatal conductance and is represented by a wet filter paper or a leaf sprayed with water. This offers a quick and easy method to collect thermography data that can be used to measure relative differences in stomatal conductance and stomatal response between plants (see Notes 6 and 7)

1. Set up the thermal camera and reference objects according to the guidelines provided in the previous sections.
2. Ensure the dry reference object has similar physical properties to the leaf and is placed near the plant. Spray the wet reference object with water on both sides to maintain consistent wetness. Allow for one minute to pass to allow the wet reference to acclimate to the environment.
3. Position the thermal camera to capture clear images of the leaf, the dry reference, and the wet reference simultaneously. To account for spatial variabilities of

stomatal conductance on a leaf surface, it is beneficial to define multiple regions of interest (ROIs) within the imaging software.

4. Start the thermal imaging process and capture the temperature readings of the leaf (T_{leaf}), the dry reference (T_{dry}), and the wet reference (T_{wet}) objects.
5. Calculate the conductance index (Ig) using the following formula (Jones, 1999):

$$Ig = \frac{T_{dry} - T_{leaf}}{T_{leaf} - T_{wet}}$$

2.3.3 Field Crop Water Status Estimation

Crop Water Stress Index (CWSI) is used to assess the water status of plants. The CWSI provides a quantitative measure of plant water stress by integrating canopy temperature, air temperature, and the upper and lower limits of canopy temperature under non-transpiring and fully transpiring conditions, and thus is well-suited to field measurements.

1. Set up the IR camera and a visible light camera at an appropriate distance and angle, focusing on the target plants. Adjust the camera settings, including emissivity and calibration, following the guidelines provided in Section 3.2.
2. Capture simultaneous thermal and visible images of the target plants under the desired environmental conditions or treatments.
3. Calculate the crop water stress index (CWSI) using the following formula (Jones, 1999):

$$CWSI = \frac{T_c - T_a}{T_{c\_upper} - T_{c\_lower}}$$

where T_c is the canopy temperature, T_a is the air temperature, and T_{c_upper} and T_{c_lower} are the upper and lower limits of canopy temperature under non-transpiring and fully transpiring conditions, respectively.

4. Estimate the upper and lower limits of canopy temperature T_{c_upper} and T_{c_lower} using the thermal and visible imagery. This can be done by selecting well-watered and water-stressed plants from the visible imagery and determining their corresponding canopy temperatures from the thermal imagery.

2.3.4 Snap-shot measurement

This method is highly beneficial for rapid phenotyping and is particularly suited for field or large-scale applications. The snap-shot measurement approach can be employed using either a thermal imaging platform or a handheld thermal camera. The snapshot feature allows the user to capture a comprehensive thermal image from which measurements can be extracted at any pixel point. This flexibility facilitates comparisons across different samples captured under the same conditions.

1. Position the camera above the plant canopy or samples to capture an unobstructed view of the target area.
2. Adjust the camera height and focus to ensure a clear and sharp image of the observation area.
3. For deriving plant physiological indices, place wet and dry reference materials at the same height as the plant canopy. Allow at least 10 min for acclimation of the references, and an additional minute after wetting the wet reference.
4. Capture thermal images of the plant canopy or samples, making sure all relevant areas are included in the field of view.

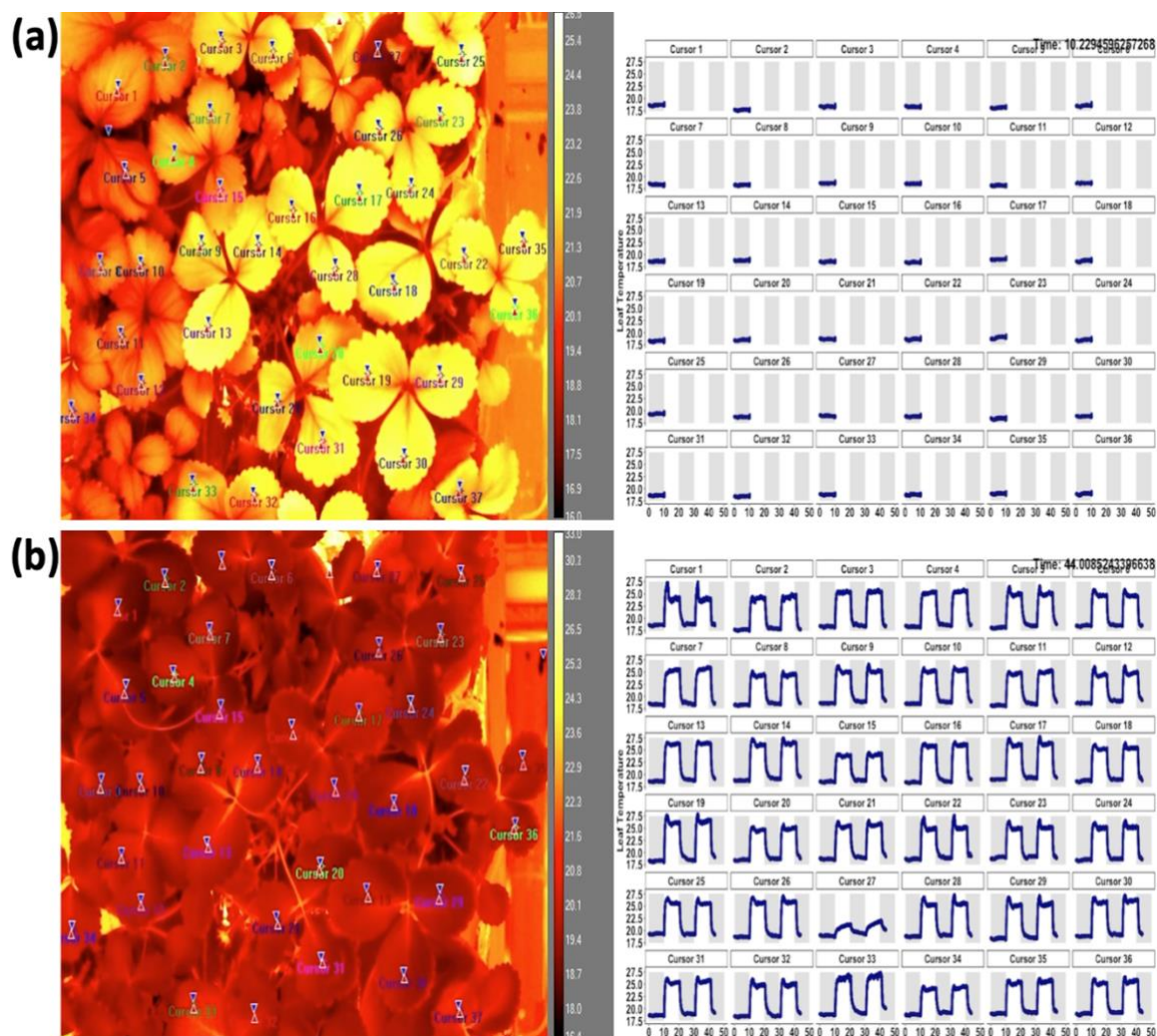


Figure 2.3. Illustration of time-series measurements using the thermal imaging technique for high-throughput leaf temperature screening in strawberry plants.

The plants were subjected to several rounds of step changes in light intensities, changing from 100 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, radiated by LED arrays. The grey-shaded regions in the plots denote periods of low light intensity, whereas white area denotes periods of high light intensity. Each cursor in the thermal images represents a 3x3 pixel area. Panel (a) corresponds to the transition from low to high light intensity, and (b) represents the transition from high to low light intensity. The full video of these

demonstration measurements can be accessed via the following link:

<https://vimeo.com/838953510>.

2.3.5 Time-series measurement

Time-series measurements can be particularly valuable in long-term monitoring of plant growth and development. By capturing thermal data over extended periods, we can derive the dynamics of plant responses and their implications for crop productivity, resilience, and resource use efficiency (see Note 8 for further considerations). This method is particularly useful for tracking plant responses to environmental fluctuations or treatments (see Fig. 2.3 and supplemental video for illustration). The following steps outline the process for conducting time-series measurements:

1. Set up the thermal camera or fixed thermal sensors in a suitable location, ensuring an unobstructed view of the plant canopy or samples.
2. Position the camera or sensors at an appropriate height and angle, adjusting the focus to ensure a clear and sharp image of the observation area.
3. Configure the thermal camera or sensors to capture images at predetermined intervals, depending on the desired time resolution of the study and storage capacity.
4. Place artificial wet and dry reference samples at the same height as the plant canopy to provide a reference for data analysis, allowing at least 10 min for acclimation of the references to the environment.

5. Begin capturing thermal images continuously, storing the data for subsequent analysis.

2.3.6 Boundary Layer Determination

There are a number of ways to determine leaf boundary layer. In a dynamic environment, such as one with continuously variable air speed, boundary layer can be calculated as:

$$g_{bh} = 0.00662(u/l)^{0.5}$$

where u is wind speed (m s^{-1}) and l is the characteristic dimension of the leaf (metres).

Boundary layer conductance (g_{bh}) can also be calculated using the black and white reference materials from Section 2.3 using the following formula:

$$g_{bh} = \frac{k_2 \frac{dT_2}{dt} - k_1 \frac{dT_1}{dt} - I_s(\alpha_2 - \alpha_1) + 2\sigma(\varepsilon_2 T_2^4 - \varepsilon_1 T_1^4)}{2\rho C_s(T_1 - T_2)}$$

where the variables K denote the energy amount (in $\text{J m}^{-2} \text{K}^{-1}$) necessary per unit area to alter the material's temperature by 1°K , α represents the absorbance of short-wave radiation (see Section 3.7 Step 5), σ is the Stefan–Boltzmann constant ($5.670374419 \times 10^{-8} \text{ W m}^{-2} \text{K}^{-4}$), ε is emissivity (see Section 3.2 for calculation), ρ is the air density (kg m^{-3}), C_s is specific heat capacity of humid air (i.e., $1800 \text{ J kg}^{-1} \text{K}^{-1}$), T_{air} is the air temperature (K), λ is the latent heat of water evaporation (i.e., $2.23 \times 10^6 \text{ J kg}^{-1}$), and E refers to the evaporative flux ($\text{kg m}^{-2} \text{s}^{-1}$). Subscript “1” pertains to parameters

associated with the leaf material, signifying the actual sample under study. Subscript “2” indicates parameters related to the reference material.

2.3.7 Predict stomatal conductance under dynamic light intensity

2.3.7.1 Guidelines for Thermographic Assessment of Stomatal Kinetics

The temporal dynamics of stomatal responses to variations in light intensity can be more accurately predicted through the resolution of the dynamic energy balance. This approach harnesses environmental parameters to overcome the limitations of using the simplistic temperature calculations of Section 3.3 in a fluctuating environment. In the following, we outline straightforward guidelines for predicting stomatal kinetics under dynamic conditions, as previously detailed by Vialet-Chabrand and Lawson (2019).

1. Incorporate climate sensors into the measurement to continuously log relative humidity and temperature of the air. Install a light energy sensor to record the light intensity (W m^{-2}) at the measurement height. If using a PAR quantum sensor, then convert the measured light intensity ($\mu\text{mol m}^{-2} \text{s}^{-1}$) to an energy unit (W m^{-2}) to facilitate subsequent calculations.
2. Place reference materials in the measurement environment that mirror the size, shape, thickness, and known thermal capacity of the leaf or the sample being investigated.
3. Secure a thermocouple to the underside of both the leaf material and the reference material (to avoid direct exposure to the light source radiation). This thermocouple will be used to corroborate the temperature readings obtained from the thermal camera (refer to Section 2.3.2).

4. Initiate the logging of temperature and environmental data as the programmed dynamic lighting sequence commences.
5. Determine α_1 and α_2 , which represent the absorbance of short-wave radiation on the leaf's top and bottom surfaces, respectively. Measure this absorbance using a light meter calibrated in (W m^{-2}). For α_1 , gauge the radiation directly absorbed by the leaf. For α_2 , measure the transmitted irradiance beneath the leaf.
6. Calculate leaf boundary layer conductance (g_{bh}) as described in Section 2.3.7 above
7. Predict the temperature kinetics of the transpiring leaf sample (T_1) using the known thermal properties of the reference material (T_2) with known thermal physical properties. Resolve the following equation to derive derivatives of sample and reference temperatures at any given time point (t), where subscript “1” pertains to parameters associated with the leaf material, signifying the actual sample under study, and subscript “2” indicates parameters related to the reference material:

$$\frac{dT_1}{dt} = \frac{k_2 \frac{dT_2}{dt} - I_s(\alpha_2 - \alpha_1) - (\varepsilon_2 - \varepsilon_1)L_d + 2\sigma(\varepsilon_2 T_2^4 - \varepsilon_1 T_1^4) + 2\rho C_s [g_{bw2}(T_2 - T_{air}) - g_{bw1}(T_1 - T_{air})] - \lambda E_1}{k_1}$$

where the variables K denote the energy amount (in $\text{J m}^{-2} \text{K}^{-1}$) necessary per unit area to alter the material's temperature by 1°K , α represents the absorbance of short-wave radiations encompassing both incident and diffuse light (I_s , W m^{-2}) (see Step 5), σ is the Stefan–Boltzmann constant ($5.670374419 \times 10^{-8} \text{ W m}^{-2} \text{K}^{-4}$), ε signifies the emissivity (see Section 3.2 for calculation), ρ is the air density (kg m^{-3}), C_s is the specific heat capacity of humid air (i.e., $1800 \text{ J kg}^{-1} \text{K}^{-1}$), T_{air} signifies

the air temperature (K), λ is the latent heat of water evaporation (i.e., 2.23×10^6 J kg^{-1}), E refers to the evaporative flux ($\text{kg m}^{-2} \text{s}^{-1}$), L_d is the long-wave radiation (W m^{-2}) received from the surrounding environment, and can be approximated by measuring the temperature of crumpled aluminium foil (which reflects infrared from the surrounding environment) using a thermal camera with emissivity set to 1.

8. Next, compute the total conductance to water vapor (g_{tw}) using the equation:

$$g_{tw} = \frac{k_2 \frac{dT_2}{dt} - k_1 \frac{dT_1}{dt} - I_s(\alpha_2 - \alpha_1) + 2\sigma(\varepsilon_2 T_2^4 - \varepsilon_1 T_1^4) + 2\rho C_s [g_{bh2}(T_2 - T_{air}) - g_{bh1}(T_1 - T_{air})]}{\lambda 0.018 \left(\frac{e_s - e_a}{P_{atm}} \right)}$$

9. Finally, estimate stomatal conductance (g_{sw} , $\text{mol m}^{-2} \text{s}^{-1}$) from g_{bw} and g_{tw} by applying the following:

$$g_{sw} = \frac{1}{\frac{1}{g_{tw}} - \frac{1}{g_{bw}}}$$

2.4 Notes

1. It is imperative to maintain calibrated thermal imaging equipment. This ensures that the results obtained are accurate and reliable. Keep in mind that over time, equipment can deviate from optimal standard performance, leading to potential inaccuracies in measurements. In most cases, calibration of thermal cameras must be performed by the manufacturer due to the need for specialized facilities. To check whether your camera

needs calibration, first find the camera accuracy range in the user manual. For example, a common method for checking a camera's calibration is to set the emissivity of your camera to 0.96 and measure the temperature of water that is boiling (i.e., significant bubbling over) and the temperature of ice that is melting. The temperatures should deviate less than 1 °C from 100 °C and 0 °C, respectively, if calibration is within the published range of your camera's accuracy (i.e., if camera accuracy is ± 1 °C, then your range is 101 °C to 99 °C for the boiling water and -1°C to 1°C for the melting ice).

2. Effective air mixing increases boundary layer conductance (g_b), facilitating greater heat and water vapor exchange between the leaf and surrounding environment. When constant airflow is maintained, g_b can be considered relatively consistent and is represented as a single value. In cases where the airflow around the leaf changes continuously (e.g., due to wind gusts), g_b needs to be taken into account (see Section 2.3.8) and can be calculated at the leaf scale using aerodynamic theory as reviewed by Schuepp (1993).

3. In appropriately controlled conditions, the thermal response of the plant can be assessed using an IR camera combined with analysis of the thermographic data to identify trends, patterns, or correlations between changing environmental conditions and plant thermal responses. This information can offer valuable insights into the underlying physiological processes and contribute to the development of more stress-tolerant plant varieties.

4. Choosing between an artificial reference, such as filter paper, and an actual leaf hinges on multiple factors. In setups like phenotyping platforms where uniformity is vital, artificial references might offer more consistent measurements. However, the innate properties of a leaf ensure a more representative thermal dynamic, making it a straightforward choice in many contexts. It's also crucial to note that the temperature ranges of some artificial references can overlap with those of the target leaves, complicating their use. Additionally, while natural leaves might change over time, artificial references often provide prolonged stability. Finally, sourcing or replicating consistent artificial references might be more feasible than obtaining uniform natural leaves, especially for specific species or leaf types.

5. Emissivity values, once established, are expected to be consistent for subsequent measurements. However, treatments that alter the optical properties of leaves -- like changes in water content or shifts in pigmentation -- can influence emissivity. In such cases, it's prudent to reassess and recalibrate emissivity values to ensure accurate thermal readings.

6. The I_g index can be used as a proxy for stomatal conductance, and if some measurements of actual stomatal conductance are made, then the I_g can be converted into values of stomatal conductance. However, the relationship between I_g and g_s may not be linear, and subject to considerable variation in response to environmental factors.

Thus, I_g is only suitable for relative comparisons between plants that were captured during the same measurement window.

7. An accurate measurement of g_s can be made with knowledge of the leaf boundary layer (Leinonen *et al.*, 2006; Guillioni *et al.*, 2008), however, the I_g can be taken further, to measure the leaf conductance to water vapour (g_{lw}). The g_{lw} can be used as an approximation for stomatal conductance, although it also includes other conductances such as water loss through the cuticle (Jones, 1999). g_{lw} is calculated as:

$$g_{lw} = I_g \times G$$

in which

$$G = g_{ref} \frac{T_{ref} - T_{wet}}{T_{dry} - T_{ref}}$$

where g_{ref} is another surface (e.g., leaf) with similar properties to the leaf being measured, with a known conductance to water vapour (that has been measured with a porometer or infra-red gas analyser), and with T_{ref} being the temperature of that surface.

8. Stomatal conductance varies not only across species, but also within the same plant under different environmental conditions, making it vital to conduct measurements under consistent and controlled conditions for meaningful comparisons (Lawson *et al.*, 1998; Lawson & Weyers, 1999). For example, stomatal conductance is known to fluctuate diurnally, responding to changes in light intensity, humidity, and temperature throughout the day. If measurements are taken at different times of the day without accounting for

difference driven by the surround microclimate, this could lead to erroneous interpretation of the physiological status of the plant. It is therefore crucial to conduct measurements at the same time of the day when comparing data points.

Chapter 3: Large scale quantification of stomatal patterning in Barley leaves over-expressing Epidermal patterning Factor 1 (EPF1) reveals differential stomatal density on the adaxial and abaxial surfaces and spatial heterogeneity which impacts stomatal function.

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Key words: stomatal density, abaxial, adaxial, heterogeneity, spatial, gas exchange, photosynthesis, water use, epidermal patterning factor, stomatal development, barley, imaging, chlorophyll autofluorescence, phenotyping

Running title: Large scale quantification of stomatal patterning reveals differential adaxial-abaxial regulation and spatial heterogeneity that impacts function

ABSTRACT (Chapter 3)

- Stomatal density varies spatially over the leaf surface and between abaxial and adaxial leaf surfaces, with distribution greatly influencing plant photosynthesis and water use. However, methodological limitations have prevented quantification of spatial heterogeneity and its consequences for gaseous exchange in monocot crops.
- Here we introduce a simple and rapid method to image and quantify stomatal patterning over large (18 cm²) leaf areas *in situ*. We used this approach to assess spatial variation across the adaxial and abaxial surfaces in barley (*Hordeum vulgare* sp.) wild type plants and mutants overexpressing the epidermal patterning factor 1 (EPF1).
- Analysing over a million stomata revealed significantly greater stomatal densities on the adaxial surface and towards the leaf tip in both genotypes. Over-expression of EPF1 however differentially reduced stomatal densities on the two surfaces, whilst also increasing spatial variability, particularly on the abaxial surface, compared to wildtype.
- The uneven stomatal distribution proved crucial to separate simultaneous gas exchange measurements on the two surfaces, with impacts on both photosynthetic carbon gain and water use efficiency. Knowledge of the relationship between stomatal patterning and gaseous function is critical for the development of future crops with improved performance.

3.1 INTRODUCTION

Stomata are microscopic formed by specialized guard cells on plant epidermal surfaces which regulate the fundamental trade-off between CO₂ uptake for photosynthesis and water loss through transpiration. This dual function positions stomata as critical determinants of plant productivity, water use efficiency, and climate resilience (Bertolino *et al.*, 2019; Lawson & Vialet-Chabrand, 2019; Papanatsiou, Maria *et al.*, 2019; Lawson & Jack, 2020; Pan *et al.*, 2024). The density and distribution of stomata across leaf surfaces significantly influence gas exchange capacity, water conservation, and ultimately plant performance under varying environmental conditions (Hetherington & Woodward, 2003; Lawson, T & Blatt, M, 2014). While stomatal patterning has been extensively studied in dicotyledonous plants, monocots which are globally important staple cereal crops have received comparatively less attention despite their agricultural significance and distinct developmental pathways (Croxdale, 1998).

Monocot stomatal patterning differs fundamentally from that of dicots in both structure and developmental trajectory. While eudicots often (but not always) exhibit what appears at as a ‘scattered’ stomatal arrangements of kidney shaped guard cells, grasses form parallel files of stomata with dumbbell shaped guard cells flanked by specialized subsidiary cells that enhance stomatal function (Raissig *et al.*, 2017; Rudall *et al.*, 2017). This unique four-celled stomatal complex in grasses enables more rapid and efficient stomatal responses to environmental fluctuations (Franks & Farquhar, 2007). Importantly, monocots tend to be amphistomatous, with stomata on both adaxial and abaxial surfaces

and often in different densities, which is a trait that thought to maximize photosynthetic capacity but may also increase vulnerability to water loss (Farber *et al.*, 2016; Watts *et al.*, 2024; Zhen *et al.*, 2025). These surface specific differences potentially allow differential regulation of gas exchange depending on environmental conditions, yet the functional consequences of such heterogeneity remain poorly understood in key cereal crops.

Stomatal development and patterning are regulated by a network of signalling peptides, with Epidermal Patterning Factors (EPFs) playing a central role as negative regulators. EPF1 and EPF2 are secreted by developing stomatal lineage cells, which bind to receptor complexes including ERECTA family receptor kinases and the co-receptor TOO MANY MOUTHS (TMMS), which triggers intracellular signalling cascades that inhibit stomatal development in neighbouring cells (Hara *et al.*, 2007b; Hara *et al.*, 2009; Hunt & Gray, 2009; Hunt *et al.*, 2010). This mechanism enforces the "one-cell spacing rule" that prevents adjacent stomatal formation and optimizes gas exchange efficiency (Hara *et al.*, 2007a; Dow *et al.*, 2014; Sack & Buckley, 2016). Manipulating EPF expression has emerged as a promising approach to alter stomatal density with significant physiological consequences. In particular, overexpression of EPF1 in various species consistently reduces stomatal density, enhances water use efficiency, and improves drought tolerance (Hughes *et al.*, 2017; Caine *et al.*, 2019; Dunn *et al.*, 2019) making it a promising and proved target for engineering climate resilient crops.

Spatial heterogeneity in stomatal distribution across leaf surfaces, along leaf axes, and within localized regions can significantly impacts plant physiological performance

(Weyers et al., 1996; Weyers & Lawson, 1997; Lawson & Weyers, 1999). Non-uniform stomatal patterns can create diffusion bottlenecks or enhance gas exchange efficiency depending on their specific arrangement (Dow *et al.*, 2014; Buckley, 2019). For instance, clustered stomata function less efficiently than evenly spaced ones due to competition for CO₂ diffusion pathways and ionic resources (Papanatsiou *et al.*, 2016). Understanding how genetic manipulations of stomatal density such as over expression of EPF1 affects not just overall stomatal density but also spatial distribution patterns is crucial for predicting the impact on whole plant performance. Yet such comprehensive spatial analyses have been challenging due to methodological limitations. Traditional methods for quantifying stomatal patterning have relied on extremely labour-intensive approaches such as epidermal peels or impressions to be examined under microscopes, typically sampling only minuscule leaf areas ($\leq 1 \text{ mm}^2$). These approaches cannot capture the full extent of spatial heterogeneity across leaf surfaces, nor can they efficiently analyse the thousands of stomata needed for statistically robust conclusions. More recent attempts using handheld microscopes remain constrained by small imaging fields, while automated approaches often require extensive training datasets that may not generalize well across species (Millstead *et al.*, 2020; Jayakody *et al.*, 2021; Gibbs & Burgess, 2024). These methodological limitations have particularly hindered detailed investigations in monocots, where the linear arrangement of stomata in files requires large-scale imaging to accurately assess patterning (Ferguson *et al.*, 2021; Xie *et al.*, 2021).

Here, we introduce a simple, rapid, and non-destructive technique that enables large-scale quantification of stomatal distribution using fluorescence microscopy of chlorophyll autofluorescence combined with automated image processing. This approach facilitates

analysis of over one million stomata across extensive leaf areas ($>18\text{ cm}^2$) without requiring staining, peeling, or other invasive sample preparations. We applied this methodology to investigate spatial variation in stomatal patterning across adaxial and abaxial surfaces in barley (*Hordeum vulgare*) wild-type plants and transgenic lines overexpressing EPF1 which exhibit reduced stomatal density.

Our present study using large scale image acquisition and spatial analyses revealed previously unrecognized heterogeneity in stomatal distribution between leaf surfaces *and* along the leaf axes, with distinct patterns between genotypes. We show that EPF1 overexpression not only reduced overall stomatal density but differentially affected the two leaf surfaces and altered spatial distribution patterns, particularly increasing variability on the abaxial surface. These spatial distribution patterns result in differential gas exchange measurements which affects both photosynthetic carbon gain and water loss. Our findings also establish direct links between genetic manipulation of stomatal development, resulting spatial patterning changes and physiological consequences. These insights could provide innovative targets for engineering improved water use efficiency in cereal crops facing increasingly erratic precipitation and rising temperatures in near future.

3.2 Materials and Methods

Plant material growth and preparation:

Transgenic barley (*Hordeum vulgare* L.) lines overexpressing the TaEPF1 gene under the rice actin promoter control (Dunn *et al.*, 2019) were grown along with azygous wild-type control lines. Seeds were surface-sterilized (70% ethanol for 2 min and rinsed with sterile RO water) and germinated in December 2023 and May 2024 under controlled conditions (20-22°C $\pm$ 0.5°C, 12-hour photoperiod, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD). Two weeks post-germination, seedlings were transplanted into 1L pots (peat-based compost) and grown for seven additional weeks in a CONVIRON ADAPTIS growth cabinet at the University of Essex (Colchester, UK) under the following conditions: 23°C, 65% RH, 14-hour photoperiod, 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at canopy level. Plants were watered regularly with Hoogland solution. Gas exchange measurements were conducted using multiple LI-6800 and LI-6400xt infrared photosynthesis systems (LI-COR Biosciences, Lincoln, NE, USA) between 08:00-14:00 on the third fully expanded leaf from the main tiller. The sampling area for the based was 40-42 mm from the base whilst the tip was 47-49.7 mm from the leaf tip, and sampling size was 40mm. The measurement protocol followed a systematic approach: gas exchange measurements were initially performed on intact leaves and the measurement marked for subsequent analyses. For imaging measurements, leaves remained attached to the plants and were carefully mounted between two microscope slides for the imaging process.

Gas Exchange Measurements

To examine how spatial variation in *SD* impacts dynamic gas exchange processes, we measured photosynthetic responses across anatomically distinct leaf regions (tip and

base) of the third fully expanded barley leaf using a custom designed split chamber system (Wall, Shellie *et al.*, 2022). This chamber allowed simultaneous, independent measurement of gas fluxes from adaxial and abaxial surfaces. Mixed gas with controlled CO₂ concentration at 400 ppm was supplied to the leaf chambers using two LI-6400XT portable photosynthesis systems (LI-COR Biosciences, Lincoln, NE, USA) at a fixed flow rate of 500 $\mu\text{mol s}^{-1}$. Humidity was regulated using two LI-610 dew point generators (LI-COR Biosciences) to maintain a vapour pressure deficit (VPD) of approximately 1.2 kPa. Leaf temperature was controlled at 22°C with a circulating water bath connected to two cooling pads affixed to the leaf chamber to minimize thermal gradients across the leaf surface.

Boundary Layer conductance (g_b) for each leaf surface in the split chamber was determined using water saturated filter paper to simulate a leaf with infinite stomatal conductance. Under controlled conditions as described above, g_b was calculated as 0.582 $\text{mol m}^{-2} \text{s}^{-1}$ per surface based on the relationship between transpiration rate and vapor pressure differential (Wall, Shellie *et al.*, 2022). This value was then incorporated into all subsequent conductance calculations.

Irradiance was provided to both leaf surfaces using two HelioSpectra DYNA LED lamps, with the white light channel set at a colour temperature of 5700K. The total photon flux density (PPFD) incident on both leaf surfaces was measured and calibrated using a quantum sensor (LI-250A, LI-COR Biosciences) positioned within the leaf chamber to ensure equal and accurate light intensity readings on both leaf surfaces. Leaves were first adapted to the chamber environment under 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 15 minutes to ensure acclimation. Subsequently, the light intensity was increased stepwise to 1000

$\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for a duration of 30 minutes. Gas exchange parameters, including net photosynthetic rate (A), stomatal conductance (g_{sw}), intercellular CO_2 concentration (C_i), were logged automatically every 10 seconds using the LI-6400XT systems. Leaf temperatures of both surfaces were measured using Type-K thermocouples (Omega Engineering), which were inserted through ports in the leaf chamber. Measurements were conducted at two positions on the leaf, one towards the base and one towards the tip, with each position measured twice.

Image acquisition

Image acquisition was designed to be rapid and conservative to avoid photobleaching and any potential photodamage (Laissue *et al.*, 2017) of the imaged leaf segment. Chlorophyll autofluorescence imaging was performed using a Nikon ECLIPSE Ti2-E Inverted Microscope with motorized XY and Z stage and 25mm field of view (Nikon Corporation, Tokyo, Japan). The microscope was equipped with a Crest X-Light V3 spinning disk (Crestoptics S.p.A., Rome, IT), but used in widefield fluorescence mode. The light source used was an LDI-7 Laser Diode Illuminator (89 North) with TTL triggering in a Ubob42 NIDAQ Ultimate Breakout Box to avoid illumination overhead (Kiepas *et al.*, 2020).

Images were acquired at 16-bit using a Teledyne Photometrics Kinetix camera (Tucson, AZ, USA). Image acquisition was controlled with NIS-Elements AR v 5.42.02 (Build 1801) on an HP Z4 workstation (Intel Xeon 3.9GHz, 128GB DDR4 RAM, NVIDIA Quadro RTX4000 GPU). Leaf segments were excited at 470 nm and a photon flux density at the

sample plane of 330 $\mu\text{mol}/\text{m}^2/\text{s}$, with 7ms exposure time for each image plane. For chlorophyll autofluorescence detection, we used a hard-coated interference filters set (Semrock Inc., IDEX corp., IL, USA) consisting of a chromatic reflector at 665 nm and a long-pass emission filter at 664 nm. Imaging was performed using a CFI Plan Apochromat Lambda D 4X objective combined with a 1.5X tube lens zoom, yielding a final resolution of 1.11 μm per image pixel side.

Large area imaging was accomplished using the 'Scan Large Image' command in the NIS-A 6D module. For each leaf segment, boundaries were defined and focused using 'focus surface' option to accommodate leaf curvature. Each field of view (2048 x 2048 pixels) was captured as a shallow z-stack (with 5 – 9 focal planes) and collapsed into a maximum intensity projection to capture all fluorescence in areas where the leaf segment was not planar. Adjacent fields were stitched with 3% overlap.

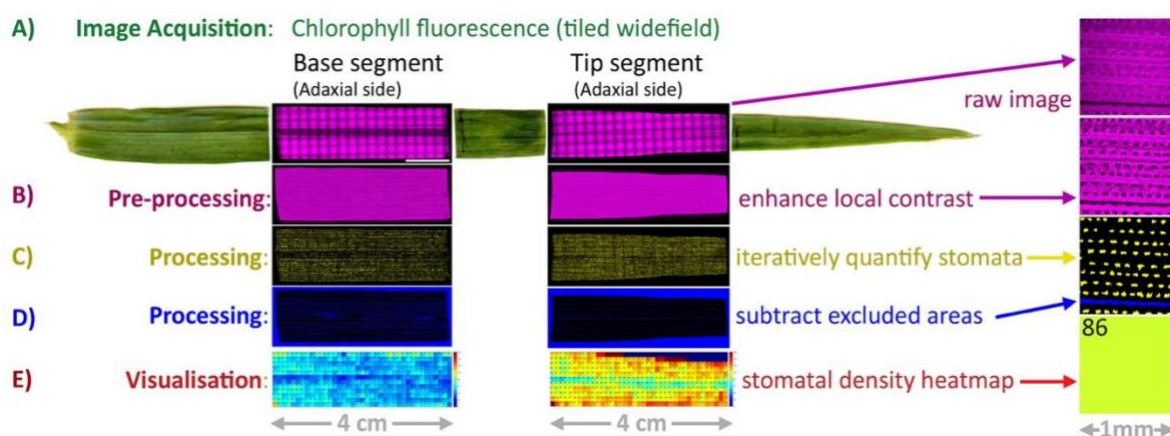


Figure 3.1. Image acquisition and image processing steps for stomatal quantification. (A) Barley flag leaf segments (at base and at tip) are imaged using tiled widefield and chlorophyll autofluorescence. Both sides (abaxial and adaxial) of each segment are imaged. (B) Pre-processing: Image contrast is enhanced locally. (C) Processing: The number and location of stomata (yellow) is determined for each side of each segment by iterative thresholding. (D) Processing: Areas that cannot harbour stomata, e.g. leaf edge, veins and degraded areas, are determined (blue) for each side of each segment. Stomatal density is then calculated as number of stomata (yellow) per area after subtracting excluded areas (blue). (E) Visualisation: The leaf segment is divided into uniform squares of 1mm² size. The stomatal density of each is visualised using a heatmap with spectral (rainbow) colour-coding. Numbers in heatmaps are indicated in the top left corner of each square.

Image processing and quantification

Stomata were quantified using Fiji v. 1.54f (Schindelin *et al.*, 2012). The complete workflow of image acquisition and processing is illustrated in Fig. 3.1. Raw images consist of large area (typically 38.0 ± 6.3 mm $\times$ 11.7 ± 1.4 mm), stitched chlorophyll autofluorescence micrographs for each leaf segment, with stomata appearing as distinctive non-fluorescent elliptical regions due to the reduced chloroplast density compared with the underlying mesophyll and the thick guard cell wall that is devoid of chloroplasts (Fig. 3.1A).

Images were pre-processed using contrast limited adaptive histogram equalization (CLAHE) (Zuiderveld, 1994) and median filtering (Fig. 3.1B). For CLAHE, starting values were a block size of 127, histogram binning of 256 and contrast stretching in the intensity transfer function between 8 and 14. Depending on the available leaf (and resulting image) quality, these values may have to be modified for optimal pre-processing. The fast-processing version of the CLAHE algorithm was used, which evaluates the intensity transfer function over the chosen block size, interpolating for locations in-between. This was followed by a median filter of size 8. This also can be modified to optimise pre-processing for a given dataset.

Stomata were quantified using iterative thresholding of the pre-processed images. Each thresholding was followed by the “Analyse particles...” command. *Moments* and *Li* thresholding had a size restriction of 800 – 4000 μm^2 , while *Minimum* used 800 – 3000 μm^2 . A circularity of 0.5 - 0.9 was used for each threshold. Areas and structures that did not harbour any stomata (such as leaf veins, borders and degraded leaf areas) were identified using *Percentile* thresholding of areas above 400'000 μm^2 . Stomatal density was then determined as number of stomata divided by available area (i.e. total area minus excluded (veins etc.) area). For the ‘heatmap’ representation, we divided each leaf into areas of 1 mm^2 . Leaf segment sizes had on average a width of 38.0 mm ($\pm$ 6.3 mm) and a height of 11.7 mm ($\pm$ 1.4 mm). The average processing time for a leaf segment, from pre-processing to stomatal quantification, was 5 min 14 s ($\pm$ 1 min 57 s).

Calculation of Global Spatial Variation and Local Spatial Autocorrelation

To quantify the spatial heterogeneity in stomatal density (SD) across the measured leaf areas, a global spatial deviation metric for each grid square within a measurement matrix was computed, where each grid square represents a 1 mm² area. We calculated the absolute deviation of the SD of each focal grid square from the overall mean SD of its respective genotype, leaf position, leaf side, and plant replicate group. For a cell at row r and column c with stomatal density $(SD)_{ij}$, the absolute global deviation was computed as:

$$D_{rc} = |SD_{rc} - \overline{SD}|$$

where $\overline{SD}$ is the mean stomatal density calculated over the entire measurement matrix for that group. To facilitate pattern comparisons across different SD magnitudes and genotypes, the deviation for each cell was normalized by the group mean to yield a unitless, relative global deviation:

$$D_{rc}^{rel} = \frac{D_{rc}}{\overline{SD}}$$

To assess whether stomatal densities were spatially clustered or randomly distributed across the measurement grid, we computed the global Moran's I statistic. Using a k-nearest neighbours' approach (with $k = 4$) to define spatial weights between grid squares, global Moran's I was calculated as:

$$I = \frac{n}{W} \frac{\sum_{i=1}^n \sum_{j=1}^n w_{ij} (SD_i - \overline{SD})(SD_j - \overline{SD})}{\sum_{i=1}^n (SD_i - \overline{SD})^2}$$

where SD_i and SD_j represent the stomatal density values of grid squares i and j , $\overline{SD}$ is the global mean stomatal density, w_{ij} is the spatial weight between cells i and j , n is the total number of grid squares, and W is the sum of all spatial weights $\sum_{i=1}^n \sum_{j=1}^n w_{ij}$. Spatial weights (w_{ij}) were derived using a k-nearest neighbours' approach ($k = 4$) with each cell (1mm^2) connected to its four nearest neighbours. Positive values of Moran's I indicated clustering of similar stomatal density values and negative values suggested dispersion and values near zero implied spatial randomness. Moran's I was calculated independently for each combination of genotype, leaf position (Tip and Base), leaf surfaces (Adaxial and Abaxial), and their biological replicate to identify global spatial patterns.

To identify localized patterns and to capture spatial clustering at finer scale, we computed local Moran's I (I_i) for each focal grid square (SD_i):

$$I_i = (SD_i - \overline{SD}) \sum_j w_{ij} (SD_j - \overline{SD})$$

where SD_i is the stomatal density of the grid square, SD_j represents stomatal densities of neighboring grid squares, $\overline{SD}$ is the global mean stomatal density, and w_{ij} is the spatial weight between grid squares i and j . The same k-nearest neighbour weights ($k = 4$) were applied. The magnitude of I_i was interpreted as the strength of local spatial autocorrelation, while the sign indicated the pattern type, with positive values indicated clustering of similar values, and negative values indicated local outliers. Heatmaps of local spatial autocorrelation were reconstructed by aggregated across replicates using median values at corresponding spatial positions.

Statistical Analysis

Statistical analyses were conducted using R (version 4.4.1; R Core Team, 2024). For each variable, normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) were assessed across genotype and leaf position and leaf surfaces groups. When both assumptions were met ($p > 0.05$), three-way ANOVA was performed with genotype and leaf position and leaf surfaces as fixed factors, followed by post-hoc analysis using estimated marginal means with Sidak's correction. If either assumption was violated, non-parametric Kruskal-Wallis tests were employed for genotype and leaf position and leaf surfaces separately, with Dunn's test (Bonferroni-corrected) used for post-hoc comparisons. Data were visualised using box plots overlay violin plot (with 95% compatibility intervals indicated by indentations) and a quasi-random distribution of data, along with displaying effect sizes. Corresponding p -values were produced using randomisation tests (Hooton, 1991; Nuzzo, 2017; Goedhart, 2019).

Stomatal sampling simulation analysis

To evaluate optimal sampling requirements for reliable stomatal density (SD) quantification, we implemented a Monte Carlo simulation framework using our selected SD matrix dataset ($406.7 \pm 74.3 \text{ mm}^2$ per leaf segment). For each experimental group (genotype $\times$ leaf position $\times$ leaf side $\times$ plant replicate), we performed 1000 random sampling iterations without replacement for every possible sample size (1 to n total cells) using parallel processing in R (version 4.4.1). Each iteration quantified: sample mean SD , standard deviation, absolute error, relative error, standard error, and 95% confidence intervals (using t -distribution). Minimum sampling requirements were defined as the

smallest sample size simultaneously achieving $\leq 10\%$ mean relative error and $\geq 95\%$ confidence interval coverage of the true population mean. To assess how sampling intensity influences structure-function relationships, we evaluated $SD - g_{sw}$ correlations across three distinct sampling regimes: small ($0.5-3 \text{ mm}^2$, $\leq 0.5\%$ of leaf area), medium ($10-30 \text{ mm}^2$, $\sim 5.1\%$), and large ($50+ \text{ mm}^2$, $\sim 34.8\%$) by linear regression analysis with significance testing of coefficients using Benjamini-Hochberg adjusted p-values.

3.3 RESULTS

3.3.1 Validation of the automated detection of stomata.

In order to demonstrate that our automated detection of stomata over a large area provided a good estimation of stomatal density, values were compared with manual standard counting methods. Fig. 3.2 provides an example of a large autofluorescence image (Fig. 3.2A) adjacent to a processed image (Fig. 3.2B) with manual counts overlaying stomata detected using automated detection. From this large-scale image 1668 stomata were manually detected compared with 1619 automatically detected, demonstrating 97.1%% of “ground truth” stomata were identified using our automated method. Additional validation of the automated method detection is provided in Suppl Fig. S3, in which ten randomly selected 1mm² squares were chosen from each leaf region, and stomatal counted both manually and using the automated method. Irrespective of the leaf area (base or tip) or which side of the leaf (abaxial or adaxial) was measured, detection rates were all greater than 90% (Supplementary Fig. S3.3), with an overall average detection accuracy of 95.2 % $\pm$ 3.6% standard deviation provided full validation of the approach.

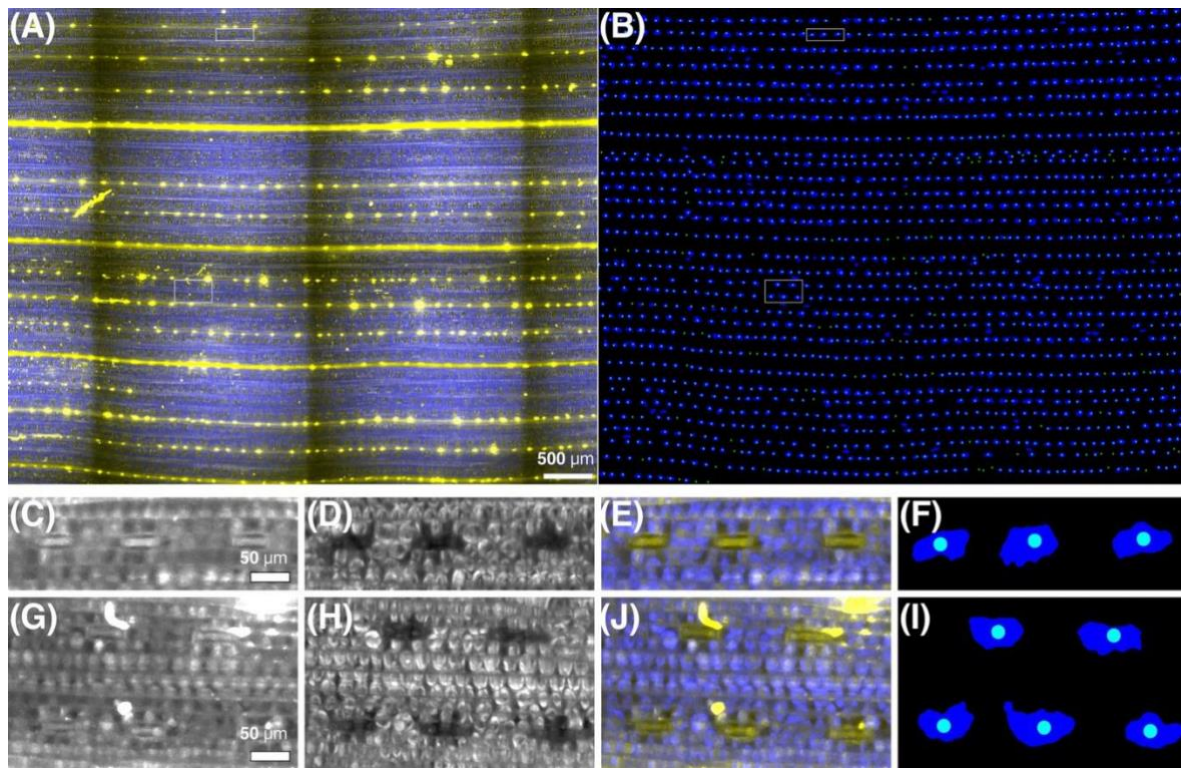


Figure 3.2. Validation of automatic stomatal detection. (A) Large image of a wildtype leaf (abaxial tip segment, 6.0 mm x 4.7 mm) showing chlorophyll autofluorescence (blue) with stomatal autofluorescence (yellow) overlaid. Stomatal autofluorescence further reveals leaf veins and other leaf features. (B) Manually identified stomata (green dots) overlaid with the outlines of the automatically identified stomata. The white rectangles indicate the location of the magnified insets shown in B). Scalebars - 500 μ m. (C-J) Magnified example of stomatal identification. (C & G) Stomatal autofluorescence highlights the closed stomatal guard cells as a line. (D & H) chlorophyll autofluorescence, stomatal locations are revealed by the absence of fluorescence. (E & I) Overlay shows that stomatal autofluorescence (yellow) colocalizes with the absence of chlorophyll autofluorescence (blue), (F & J) outline of automatically identified stomata (blue) colocalizing with manually identified

stomata (green dots). Scalebars 50 μm . A magnified example of non-specific signals (fibres and dust particles), as seen in the stomatal autofluorescence channel are not detected as stomatal using our approach, whilst stomata are still reliably identified by our automated method (G-J).

3.3.2 EPF1 overexpression results in distinct differential spatial stomatal patterning

The extensive spatial variation in stomatal patterning was quantified using our newly developed high-throughput imaging methodology that enabled analysis of over one million stomata across 18,205 mm^2 of leaf tissue and surfaces (Fig. 3.3A). We show the stomatal distribution across both adaxial and abaxial surfaces at two leaf positions (base and tip) and in a monocot species. Wildtype (WT) barley exhibited substantial spatial gradients in stomatal density (SD), with significantly higher SD at the leaf tip compared to the base region on both leaf surfaces (Fig. 3.3B). The tip to base ratio was remarkably consistent between surfaces (adaxial: 1.55, abaxial: 1.54), indicating a coordinated stomatal development. Interestingly, WT maintained nearly equivalent SD between adaxial and abaxial surfaces at both leaf positions (adaxial:abaxial ratio at base: 1.03, and tip: 1.04), which is similar to that observed in wheat (Wall, Shellie *et al.*, 2022) but contrasting with the typical abaxial dominant pattern seen in many dicot species (Muir *et al.*, 2014). EPF1 overexpression (1OE5) greatly altered stomatal patterning, with significant reductions in SD across all measured regions compared to WT (Fig. 3.3B). However, the magnitude of this effect varied substantially by both leaf position and surface. The most significant reductions can be seen on the abaxial surface, with decreases of

59.5% at the base and 64.9% at the tip. In contrast, the adaxial surface exhibited more moderate reductions of 19.9% at the base and 40.1% at the tip. This differential effect resulted in substantially altered adaxial:abaxial ratios in 10E5 plants (base: 2.04, tip: 1.77), potentially suggesting EPF1 overexpression disproportionately impacts abaxial stomatal development. While the positional gradient in *SD* (in which tip > base) was preserved in 10E5 plants, its magnitude was significantly diminished compared to WT, particularly on the adaxial surface (tip-to-base ratio at adaxial: 1.16, abaxial: 1.33) (Fig. 3.3B).

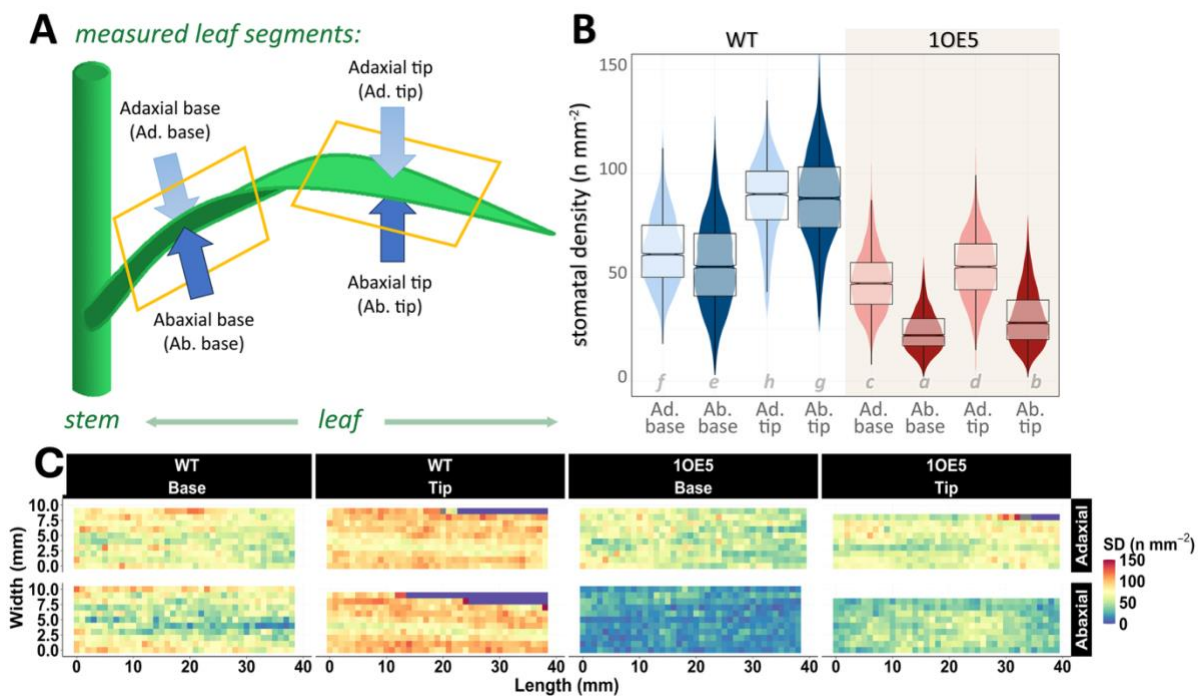


Figure 3.3. Stomatal density distribution and spatial patterns in barley. (A) Sampling layout showing base and tip segments imaged on both adaxial (Ad.) and abaxial (Ab.) leaf surfaces used in gas exchange and chlorophyll autofluorescence

imaging. (B) Box plots showing stomatal density distribution (stomata / mm²) in wild-type (WT, blue colours) and EPF1 overexpressed (1OE5, red colours) barley. Adaxial surfaces are light blue and light red, abaxial surfaces are dark blue and dark red. Within each box (interquartile range), the thick black line shows the median density of the segment, while indentations show the 95% compatibility interval. The data points for each box plot are underlaid in colour. (C) Representative heatmaps showing spatial distribution of stomatal density across standardized 39 × 10 mm leaf sections. Colour scale indicates stomatal density from 0 (blue) to 150 (red) stomata per mm².

Spatial heterogeneity analysis revealed that EPF1 overexpression not only reduced mean *SD* but also altered the pattern of stomatal distribution across the leaf surface. Representative heat maps of *SD* (Fig. 3.3C) demonstrated that 1OE5 plants exhibited more visible spatial variability, particularly on the abaxial surface, where the coefficient of variation reached 46.2% compared to 28.7-39.0% in WT. There was no significant difference in leaf width at either the base or tip between genotypes, although as expected the tip width was lower than the base (Supplementary Fig. S3.2).

3.3.3 Surface-specific gas exchange responses with overexpression of EPF1

Gas exchange measurements revealed significant interactions between genotype, leaf position, and leaf surface (Fig. 3.4). Stomatal conductance patterns closely mirrored the photosynthetic responses, with WT maintaining consistently higher values than 1OE5 across all measured regions (Fig. 3.4A). The genotype effect was particularly evident at the leaf tip, where WT adaxial g_{sw} exceeded 1OE5 by 79% (Supplementary Table S3.2). Notably, EPF1 overexpression had a more severe impact on abaxial conductance, with WT values being nearly three times higher than 1OE5 at the tip (0.20 vs 0.06 mol H_2O $m^{-2} s^{-1}$) and 2.8 times higher at the base (0.14 vs 0.05 mol H_2O $m^{-2} s^{-1}$). Under steady-state conditions, the impact of EPF1 overexpression on photosynthesis was most pronounced in the differential response between adaxial and abaxial surfaces (Fig. 3.4B). In 1OE5, adaxial photosynthetic rates exceeded abaxial values by 462% at the base and 463% at the tip. In contrast, WT exhibited more moderate adaxial-abaxial differences of 163% at the base and 91% at the tip. Despite these surface differences, both genotypes achieved similar maximum photosynthetic rates on the adaxial surface at the leaf tip (~ 15 $\mu\text{mol CO}_2$ $m^{-2} s^{-1}$, Fig. 3.4A).

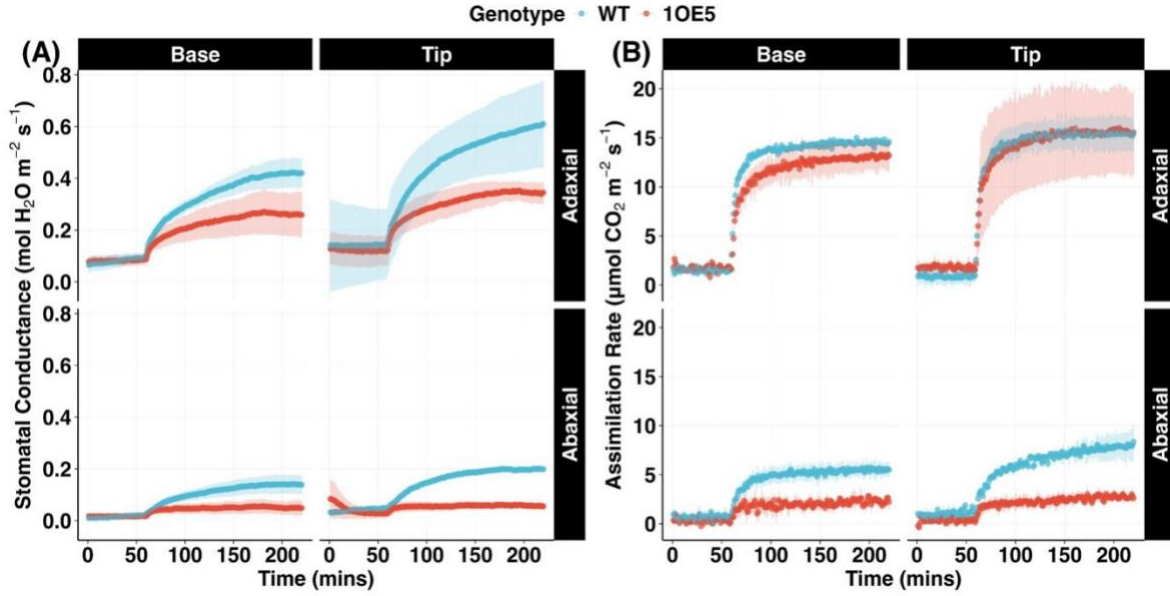


Figure 3.4. Gas exchange in WT (BLUE) and 10E5 (RED) barley lines.

Simultaneous and independent measurements of (A) stomatal conductance (g_{sw}) and (B) Net CO_2 assimilation rate (A), on the adaxial and abaxial surfaces in response to a step increase in light intensity (from 100 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD). Measurements were performed at the leaf base and tip using a custom-designed split-chamber system. Data points represent means $\pm$ SE ($n=5$).

3.3.4 Spatial heterogeneity of stomatal distribution differs between genotypes and leaf surfaces

To comprehensively analyse stomatal spatial patterning, we developed quantitative metrics assessing both global heterogeneity and local spatial autocorrelation across leaf surfaces (Fig. 3.5). Our approach used two complementary measures, with relative global

deviation (measuring overall heterogeneity by comparing SD in each cell relative to the global mean of the whole image) and local Moran's I statistic (quantifying the degree of spatial clustering by assessing similarity with neighbouring regions) (Fig. 3.5). The relative global deviation analysis revealed significant differences in stomatal distribution patterns between WT and 1OE5 plants ($p < 0.001$), with EPF1-overexpressing lines exhibiting substantially greater spatial heterogeneity across all measured leaf regions (Fig. 3.5B). This increased heterogeneity was most pronounced on the abaxial surface of the leaf base in 1OE5 plants (0.31 ± 0.003), representing a 55% increase compared to the corresponding region in WT plants (0.2 ± 0.003). Notably, we observed a significant interaction between genotype and leaf surface ($p < 0.001$), indicating that EPF1 overexpression differentially affected stomatal patterning on adaxial versus abaxial surfaces. While WT plants displayed relatively similar heterogeneity between leaf surfaces at the tip (adaxial: 0.107 ± 0.003 ; abaxial: 0.147 ± 0.003), 1OE5 plants showed greater disruption of spatial uniformity on the abaxial surface (adaxial: 0.178 ± 0.003 ; abaxial: 0.265 ± 0.003). This surface specific effect suggests distinct regulatory mechanisms controlling stomatal development on opposite leaf sides, with EPF1 disproportionately influencing the spatial coordination of stomatal initiation and differentiation more strongly on abaxial surfaces.

Local spatial autocorrelation analysis measured using Moran's I statistic provided additional insights into the degree to which neighbouring cells have similar stomatal densities (Fig. 3.5A, 3.5C). Mean local Moran's I values were generally low across all conditions ranging from 0.211 to 0.562, indicating predominantly random stomatal arrangements with localized regions of spatial structure. Interestingly, WT plants exhibited

higher spatial autocorrelation on abaxial surfaces (tip: 0.562 ± 1.28 ; base: 0.385 ± 0.88) compared to adaxial surfaces (tip: 0.268 ± 0.88 ; base: 0.213 ± 1.09), while 1OE5 plants displayed a reversed pattern with stronger autocorrelation on adaxial surfaces (tip: 0.474 ± 1.15 ; base: 0.389 ± 0.88) than abaxial surfaces (tip: 0.387 ± 0.89 ; base: 0.211 ± 0.68). These opposing patterns between genotypes suggest that EPF1 overexpression not only alters stomatal density but also disrupts the surface specific organization. The spatial autocorrelation heatmaps (Fig. 3.5A) revealed averaged complex patterning across leaf regions, with distinct clusters of positive spatial autocorrelation (red) indicating areas where similar stomatal densities aggregate, and regions of negative autocorrelation (blue) representing boundary areas between different density zones. These patterns were more pronounced and spatially coherent in WT plants, while 1OE5 plants displayed more fragmented and irregular spatial organization, particularly on abaxial surfaces.

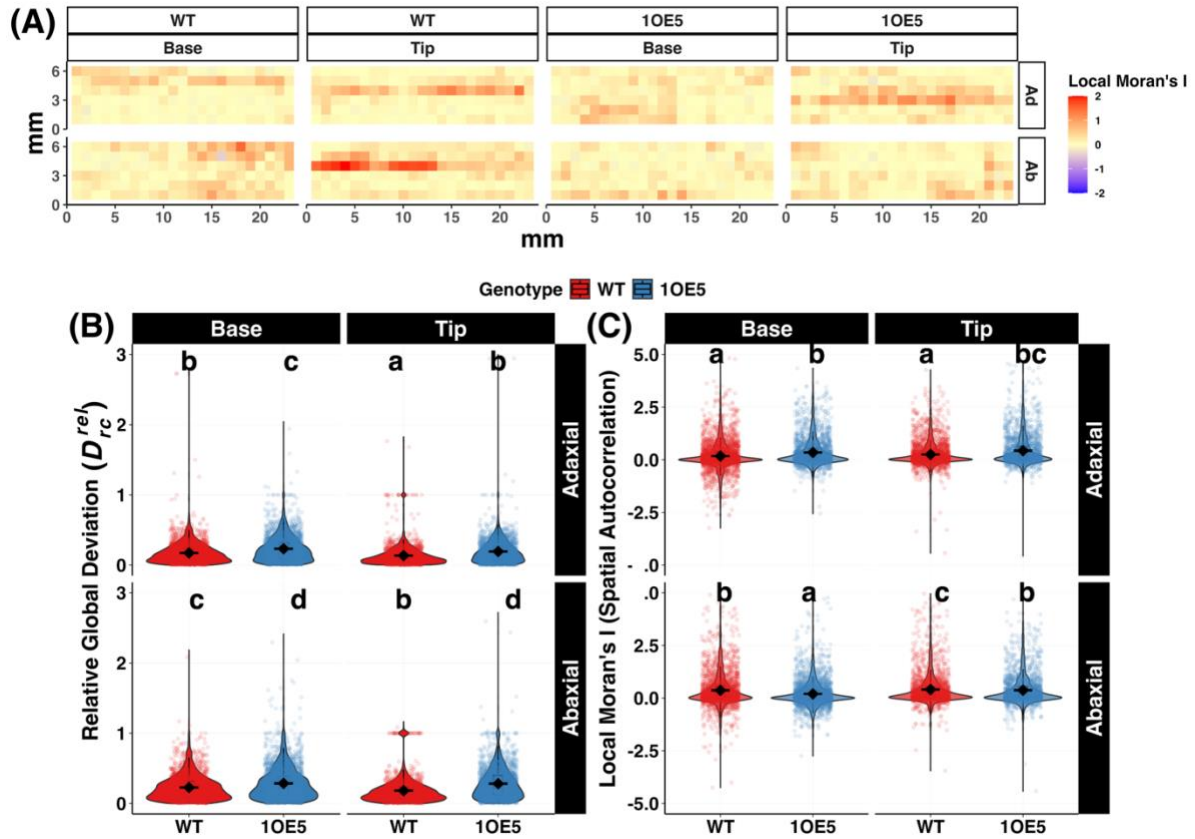


Figure 3.5. Spatial analysis of stomatal distribution patterns in wild-type and EPF1-overexpressing barley. (A) Heatmaps displaying median local Moran's I spatial autocorrelation values aggregated across 4-5 biological replicates at each spatial location. The colour scale ranges from red regions indicate positive spatial autocorrelation (clustering of similar values), through light yellow (zero) to, blue regions showing negative spatial autocorrelation (local outliers). Grid cells represent 1mm² areas. (B) Relative global deviation in stomatal density across genotypes (WT vs. 1OE5), leaf positions (base vs. tip), and leaf surfaces (adaxial vs. abaxial). (C) Local Moran's I spatial autocorrelation values (n = 1479-2775). Black diamonds and

error bars show mean $\pm$ SE. Letters denote significant differences between groups ($p < 0.05$).

3.3.5 Functional relationships between stomatal patterning and physiological performance

To understand the physiological implications of altered stomatal patterning, the relationship between stomatal density (*SD*), spatial distribution metrics, and gas exchange parameters were determined (Figure 3.6). As shown in Fig.3, EPF1

overexpressing plants (1OE5) exhibited overall significantly lower *SD* compared to WT which corresponded with differences in physiological performance. *SD* showed a strong positive correlation with photosynthetic rate in 1OE5 plants ($R^2 = 0.863$, $p < 0.001$), but this relationship was much weaker and not significant in WT plants ($R^2 = 0.176$, $p = 0.348$) (Figure 3.6A). The slope of this relationship was notably steeper in 1OE5 plants (0.424) compared to WT (0.160), suggesting that changes in stomatal density had a stronger impact on photosynthetic capacity in the transgenic line. Similarly, *SD* was strongly correlated with g_{sw} in 1OE5 plants ($R^2 = 0.756$, $p = 0.002$), but showed only a moderate, also non-significant relationship in WT plants ($R^2 = 0.358$, $p = 0.156$) (Figure 6B). Stomatal density showed no significant correlation with water use efficiency in either genotype (1OE5: $R^2 = 0.041$, $p = 0.599$; WT: $R^2 = 0.201$, $p = 0.313$) (Figure 5C). Despite the lack of correlation with *SD*, mean water use efficiency was 44% higher in 1OE5 plants ($53.07 \pm 17.75 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) compared to WT ($36.93 \pm 13.09 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) resulting in improved water conservation in the transgenic lines. The fundamental relationship between *A* and g_{sw} was strong in both genotypes (Figure 6D), with particularly high correlation in 1OE5 plants ($R^2 = 0.919$, $p < 0.001$) compared to WT ($R^2 = 0.782$, $p = 0.008$). Notably, the steeper slope in 1OE5 (44.35) compared to WT (19.68) indicates that for a given change in g_{sw} , *A* rates respond more sensitively in the EPF1 overexpression transgenic line, likely reflecting the reduced overall *SD* limiting gas exchange.

Spatial distribution metrics provided additional insights into stomatal function (Figure. 3.5), with no significant relationship between Local spatial autocorrelation with g_{sw} in either genotype (Figure 3.6E). In contrast, relative global deviation in stomatal patterning demonstrated significant negative correlations with g_{sw} in both 1OE5 ($R^2 = 0.587$, $p =$

0.016) and WT ($R^2 = 0.573$, $p = 0.049$) plants (Figure 3.6F). This suggests that the degree of global heterogeneity in stomatal distribution, rather than local clustering patterns, is a stronger determinant of functional difference in gas exchange.

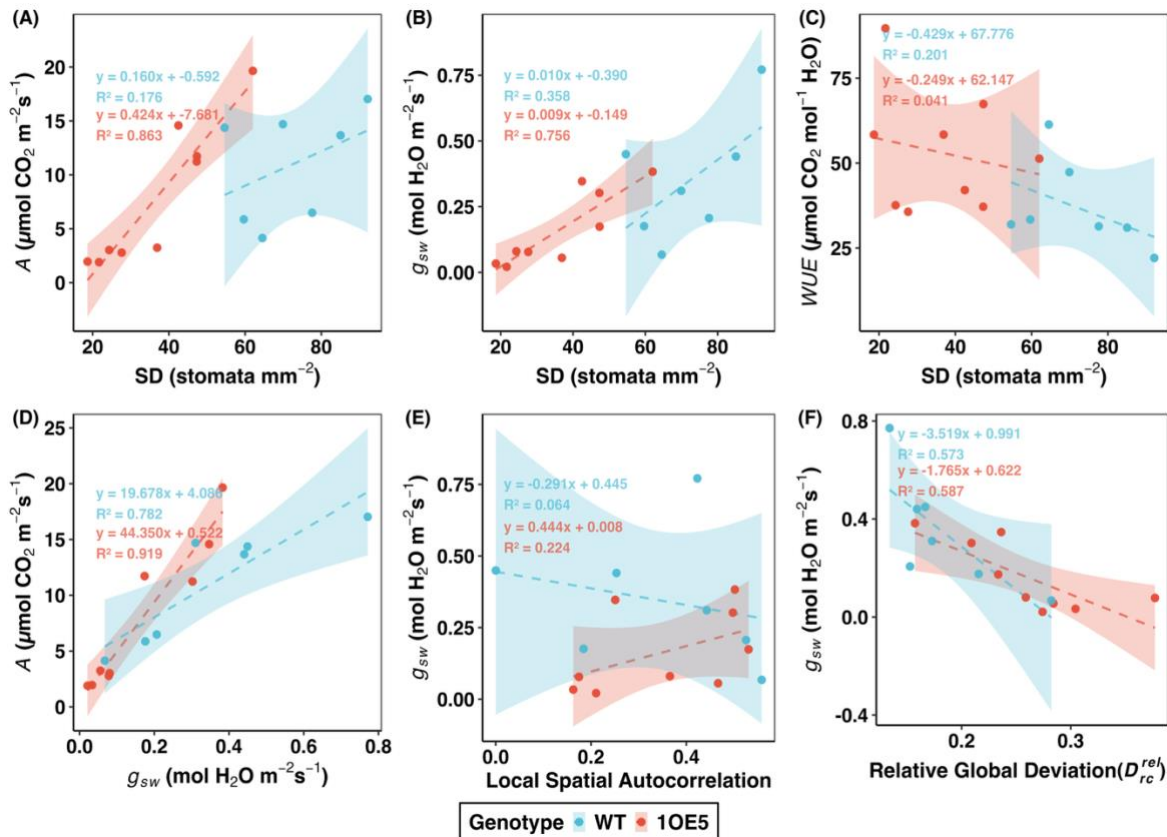


Figure 3.6. Linear regression relationships between stomatal characteristics and gas exchange parameters in wild-type (WT) and EPF1 overexpressing (10E5) barley. (A) stomatal density (SD) versus net photosynthetic rate (A); (B) SD versus stomatal conductance (g_{sw}); (C) SD versus water use efficiency (WUE); (D) A versus g_{sw} ; (E) local spatial autocorrelation versus g_{sw} ; and (F) relative global deviation versus g_{sw} . Blue points (WT, $n=7$) and red points (10E5, $n=9$) represent

individual measurements with genotype-specific regression lines and 95% confidence intervals (shaded areas). Regression equations and coefficients of determination (R^2) are provided for each genotype.

3.3.6 Large-scale stomatal sampling is essential for accurate density quantification and robust physiological correlations

To determine the minimum sampling requirements for accurately quantifying stomatal density (SD) across heterogeneous leaf surfaces, we conducted a comprehensive simulation analysis using our extensive matrix dataset (Fig. 3.7). Random sampling simulations (1000 iterations per sample size) revealed substantial variability in the area needed to obtain reliable SD estimates. While the minimum sampling requirement to achieve both acceptable accuracy ($\leq 10\%$ mean relative error) and statistical reliability ($\geq 95\%$ confidence interval coverage) ranged from as few as two 1mm^2 cells to as many as 57 1mm^2 cells (0.51-13.29% of total leaf area), our findings indicate that conventional small-scale sampling approaches are frequently insufficient (Fig. 3.7A-C). The simulation revealed critical sampling thresholds varied significantly between leaf surfaces and regions. Abaxial surfaces consistently required more intensive sampling (12.2 ± 8.5 cells, 3.03% of area) compared to adaxial surfaces (8.3 ± 12.5 cells, 2.02% of area). Notably, leaf tip regions, particularly in WT plants demonstrated substantially higher sampling requirements (10.9 ± 14.1 cells, 2.87% of area) than base regions (9.6 ± 5.9 cells, 2.17% of area). The most demanding sampling conditions were observed for WT adaxial tip regions (16.0 ± 27.3 cells, 3.83% of area), where the high standard deviation indicates

that some samples required substantially more cells to achieve reliability. To eliminate sampling-induced error across all experimental conditions, our data suggest that approximately 57 cells (13.29% of the total leaf area) would be necessary, which far exceeds typical sampling protocols often used to determine stomatal density.

Most critically, we demonstrated that sampling intensity fundamentally altered the strength of correlations between *SD* and physiological parameters (Fig. 3.7D). The relationship between *SD* and stomatal conductance (g_{sw}) improved dramatically with increased sampling area, with R^2 values strengthening from merely 0.17 ($p = 0.041$) with small sampling areas (0.5-3 mm², $\leq 0.5\%$ of total area) to 0.71 ($p < 0.001$) with medium sampling (10-30 mm², $\sim 5.1\%$ of area) and 0.87 ($p < 0.001$) with large sampling (50+ mm², $\sim 34.8\%$ of area). This represents a 415.9% improvement in explanatory power from smallest to largest sampling, with corresponding increases in the regression slope (from 0.0033 to 0.0088). Therefore, sampling scale critically affects the reliability of stomatal to physiology relationships, with small sampling area subjects to higher probability of producing biased and inconsistent correlations.

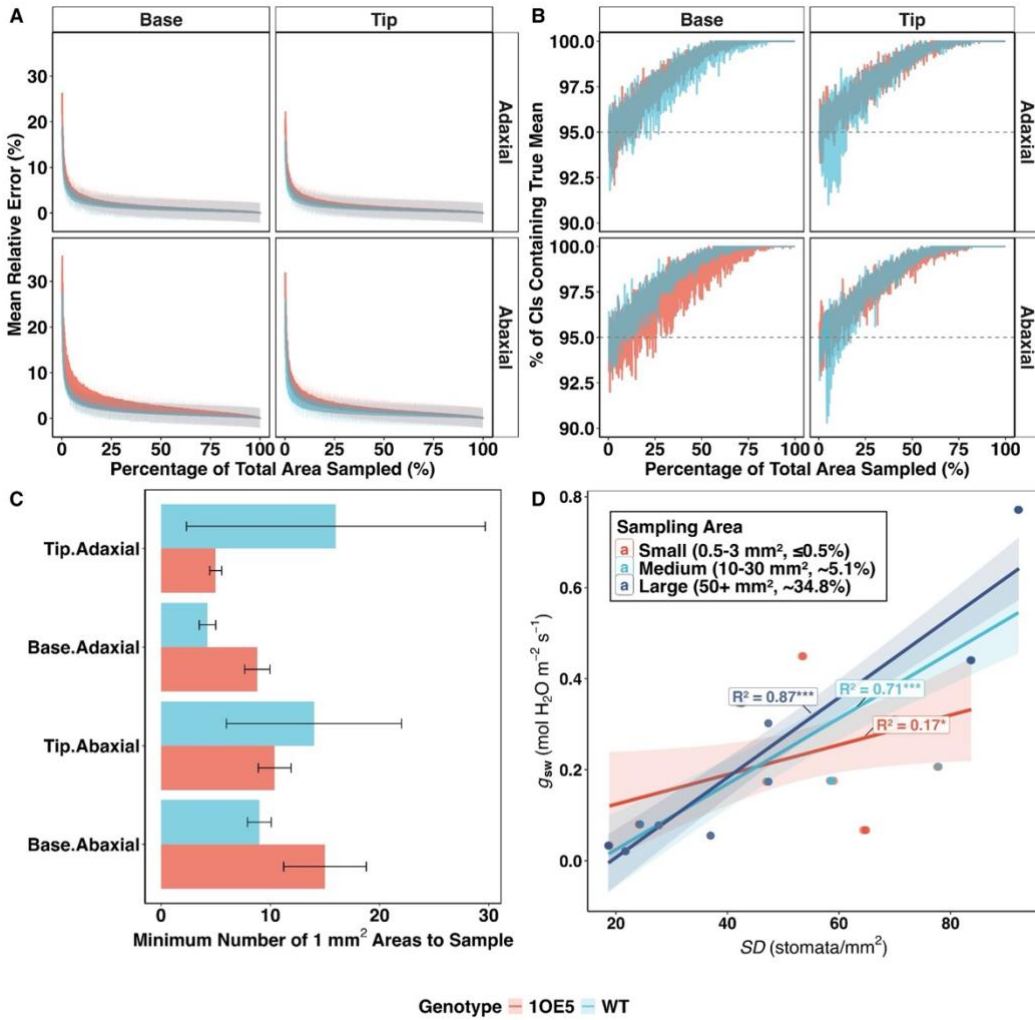


Figure 3.7. Larger scale stomatal sampling is essential for accurate density quantification and robust physiological correlations. (A) Mean relative error in stomatal density estimation as a function of sampling percentage in wild-type (WT, blue) and EPF1 overexpressing (1OE5, red) barley across different leaf surfaces (line types) and positions (panels). Ribbons indicate ± 1 SD of mean relative error. (B) Confidence interval coverage probability (percentage of 95% CIs containing true mean) as sampling percentage increases. Dashed line indicates 95% threshold for statistical reliability. (C) Minimum sampling requirements (number of 1 mm² areas)

needed to achieve $\leq 10\%$ mean relative error and $\geq 95\%$ CI coverage across different leaf position and surface combinations. Error bars represent $\pm \text{SE}$ ($n=4-5$ biological replicates per combination). (D) Relationship between stomatal density and stomatal conductance as a function of sampling area size. Points represent individual measurements with sampling area-specific regression lines and their corresponding R^2 values. Significance levels: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.4 Discussion

Manipulating stomatal density is a key target for altering g_{sw} to improve both photosynthesis and water use efficiency (WUE) (Long *et al.*, 2022) and the epidermal patterning factor family (EPF) of signalling peptides (which are negative regulators of stomatal development) are popular choices (Hara *et al.*, 2007a; Casson & Gray, 2008; Hunt *et al.*, 2010). Increased expression of EPF1 and EPF2 has been shown to decrease stomatal density, increase drought tolerance (Doheny-Adams *et al.*, 2012; Hepworth *et al.*, 2015) as well as enhance water use efficiency in several key crops (Hughes *et al.*, 2017; Bertolino *et al.*, 2019). Until recently the majority of studies examining changes in SD have focused on changes in density, usually in a single location of the leaf lamina and on the abaxial surface, as this is often the epidermis with the greatest stomatal density (Schlüter *et al.*, 2003; Yasmeen *et al.*, 2020; Bheemanahalli *et al.*, 2021; Sun *et al.*, 2021; Lei *et al.*, 2022). Although this is frequently the case for a large number of dicotyledonous plants, monocots, including many of our major crops such as barley, have equal SD on both surfaces or even greater numbers on the adaxial surface compared with the abaxial surface (Shellie *et al.*, 2022). Furthermore, few studies have examined spatial variation in stomatal patterning, and even fewer have investigated how differences in SD affect gas

exchange (Lawson *et al.*, 1998; 1999; 2002) and physiological performance (Harrison *et al.*, 2019; Lunn *et al.*, 2024; Pflüger *et al.*, 2024).

The lack of advancement in our understanding of such variation on physiological performance has been hampered by the lack of technology to assess both spatial variation in stomatal anatomical traits across large areas of the leaf, as well as methods to assess the impact on function within and between surface variation (Wall, Shellie *et al.*, 2022). Here we have demonstrated and validated a novel imaging method that overcomes these technological barriers which enabled comprehensive spatial analysis previously impossible with traditional sampling approaches. Traditional gas exchange approaches that use a cuvette to enclose a section of a leaf, typically measure gas exchange from both sides of the leaf simultaneously and although modifications to the kit can be made to restrict measurements to one side (Wall *et al.*, 2024), most systems are not capable of measuring both leaf surfaces independently and simultaneously (Wall, S. *et al.*, 2022; Wall *et al.*, 2024). Here we have demonstrated and applied a simple and novel imaging method using chlorophyll autofluorescence to quantify over one million stomata across 18 cm² of leaf surface area, which enabled high resolution spatial analysis previously impossible with traditional sampling methods. . The automated approach was validated by comparing with manual counts and demonstrated high accuracy (Fig. 3.2; Suppl Fig. S3.3). This approach combining automated image processing and spatial statistical analysis revealed detailed stomatal distribution patterns that conventional sampling methods might underestimate. Although this approach facilitates rapid high throughput detection of stomata it is currently limited to this single cell type, with no quantification of epidermal cell numbers or size. However, our acquired images provide

the necessary spatial resolution, contrast differentiation, and standardised imaging conditions that establish an ideal foundation for comprehensive epidermal characterisation through pixel based feature classifier models. Such measurement could be extremely valuable for resolving the under lying mechanisms relating to stomatal patterning, for example differences in spatial patterns may be due to epidermal expansion or cell differentiation (Lawson et al., 2002; Sacks & Buckley, 2016).

Using this approach we have demonstrated for the first time in barley (*Hordeum vulgare*) that altered expression of the EPF1 gene differentially affects *SD* on the two leaf surfaces. Taking into account spatial variation across the leaf we have demonstrated that, not only does altered expression of EPF1 (Hughes *et al.*, 2017) reduce *SD* on the abaxial epidermis more than the adaxial surface, the impact was greater at the tip than the leaf base. Several studies have demonstrated that regulation of *SD* using members of the EPF family have a greater effect on the abaxial epidermis (Jalakas *et al.*, 2024), however the majority of these studies have been restricted to model species such as *Arabidopsis*. Interestingly, it is not only alterations in expression of EPF genes that result in this differential patterning but disruption of other stomatal regulators have also been reported to have a greater impact on the abaxial surface (Dow *et al.*, 2014; Hronková *et al.*, 2015; Qi *et al.*, 2019), whilst changes in ERECTA-LIKE 2 (ERL2) increased adaxial but not abaxial stomatal index (Jalakas *et al.*, 2024). It is currently not known if stomatal patterning is regulated by the same pathways on both surfaces. A great many studies have investigated the regulation of stomatal development and spacing and have outlined many of the signal transduction pathways, and genes involved in the process (Bergmann, 2006; Pillitteri & Dong, 2013; Wei *et al.*, 2021), including the one cell spacing rule (Hara

et al., 2007a; Dow *et al.*, 2014; Sack & Buckley, 2016) that ensures that stomatal initiation must be one cell away from another to ensure appropriate stomatal function (Dow *et al.*, 2014; de Boer *et al.*, 2016). Once again, the majority of these studies have focused on abaxial surface patterning, with much less known about stomatal development and regulation of patterning on the adaxial surface (Watts *et al.*, 2024). Here the demonstration that overexpression of EPF1 has a much greater impact on SD on the abaxial surface, could be due to possible differences in the number or sensitivity of cell surface receptors on the abaxial surface, or that the EPF1 signal is not as strong at the adaxial surface. Alternatively, there are as yet unknown signalling components that influence surface specificity of stomatal development. Although there is little information available regarding the causes of differential stomatal development on the two surfaces, a recent study by Jalakas *et al.*, (2024) using a range of Arabidopsis mutants in stomatal patterning supported greater effects on the abaxial surface. The authors demonstrated in mutants such as *epf1/2* and *er*, stomatal precursors were typically found on the abaxial epidermis of fully expanded leaves. This asymmetric presence of arrested stomatal precursor cells between adaxial and abaxial surfaces further supports differential mechanisms controlling stomatal development on the two leaf surfaces.

Previous studies have also shown that the adaxial stomatal density and index are more responsive to environmental cues such as humidity and light (Hronková *et al.*, 2015; Devi & Reddy, 2018; Tulva *et al.*, 2024), supporting the idea of partly independent regulation (Shellie *et al.*, 2022; Jalakas *et al.*, 2024). Therefore, stomatal patterning on the two surfaces may depend on both underlying genetics as well as environmental cues.

Over expression of EPF1 not only resulted in differential differences in surface *SD* but also influenced spatial variation across the leaf lamina (Fig. 5), with a greater reduction at the tip than the base of the leaf. Our relative global deviation metric revealed a 55% increase in spatial heterogeneity on abaxial surfaces, with coefficients of variation reaching 46.2% in EPF1 overexpression lines compared to 28.7-39% in WT. Although significant spatial variation in *SD* within leaves is also well established in the literature (Smith *et al.*, 1989; Poole *et al.*, 2000), the majority of studies have focused on dicots, with monocots often considered to have more uniform stomatal distribution due to the ordered files (Nunes *et al.*, 2020). There are only a handful of studies that have explored the impact of spatial variation in stomatal characteristics within individual leaves on leaf function and these have shown that this spatial variation along with spatial differences in microclimate result in heterogeneous patterns of leaf gas exchange (Weyers *et al.*, 1997; Lawson *et al.*, 1998; Lawson & Weyers, 1999; Mott *et al.*, 2008; Kamakura *et al.*, 2011; Lawson *et al.*, 2018). Interestingly in our study *SD* was decreased to a greater extent at the tip than the base on both leaf surfaces. This maybe counterintuitive as it could be envisaged that the tip of the leaf is narrower due to reduced expansion of epidermal cells, which would result in higher *SD* (Lawson *et al.*, 2002). These findings indicate that the observed variation in density is due to cell differentiation rather than expansion. An alternative plausible explanation for these observations is that the tip of the leaf is also the oldest and therefore it could be that older parts of the leaf signals to newly differentiating cells, as has been shown between mature and young leaves (Lake *et al.*, 2001), or that as the leaf grows it becomes less sensitive to genetic regulation of *SD*, and/or that environment signals start to override genetic intervention.

As SD is a major determinant of functional behaviour, it is not surprising that larger decreases in g_{sw} were observed in the 1OE5 mutants (Fig.3.4), and that the magnitude of the decrease was strongly correlated with the decrease in numbers (Fig 3.6). The stronger SD and g_{sw} relationship observed in the 1OE5 mutants compared with WT (Fig.6), clearly demonstrates that stomatal numbers were not optimal in the WT and that density was actually higher than required for steady-state photosynthetic function. This is further exemplified by the fact that no significant differences in photosynthetic rates (A) were observed in the mutants on the adaxial surface, irrespective of leaf position (Fig. 3.4B). Overinvestment in stomatal and non-optimal patterning has been reported recently by Watts *et al.* (2024), in which the authors explored patterning of stomata on both leaf surfaces under different environmental conditions and concluded that stomata are not ideally dispersed on the two surfaces, and that patterning is surface independent as well as that any coordination between the patterning on the abaxial and adaxial would result in limited improvement in photosynthesis. The greater g_{sw} than required for A in the WT results in an erosion of water use efficiency (Lawson, T & Blatt, M, 2014; Franks *et al.*, 2015). On the other hand the substantially reduced abaxial g_{sw} in the mutants caused diffusional limitation on A (Violet-Chabrand *et al.*, 2021), highlighting the close coupling between A and g_{sw} (Wong *et al.*, 1979) at least on this surface. The greater decrease in WUE at the tip demonstrates the importance of taking into consideration spatial variation in gas exchange and water use efficiency, as cumulatively these differences could have significant impacts on whole plant water status (Martin & Stabler, 2002; Medrano *et al.*, 2015; Brillante *et al.*, 2017; Liu *et al.*, 2018), with important agricultural relevance. This within leaf surface variation also highlights the importance of adequate sampling protocols.

As demonstrated in our simulation analysis that conventional 1mm² samples substantially underestimate spatial heterogeneity and structural functional correlations (Fig. 3.7A-C). The simulation of 14.6 million sampling iterations showed that minimum sampling requirements range from 8-10 mm² to achieve both acceptable statistical reliability and accuracy. The local Moran's I analysis (Fig. 3.5A, 3.5C) also revealed distinct clustering patterns characterized by varying degrees of spatial autocorrelation. It is worth pointing out that this spatial organization has direct functional consequences, with relative global deviation showing significant negative correlations with stomatal conductance, revealing that uniform stomatal distribution enhances overall leaf-level gas exchange efficiency (Fig. 3.6F). These spatial patterns align with developmental mechanisms governing surface specific stomatal initiation. Our findings that EPF1 overexpression increases spatial heterogeneity (relative global deviation increased by 55% on abaxial surfaces) may reflect disrupted cell-cell signalling cascades that normally enforce ordered stomatal spacing, particularly in regions where stomatal precursors preferentially accumulate in the abaxial epidermis as shown recently by Jalakas *et al.* (2024).

To our knowledge, this is the first study that reported differential effects of EPF1 expression on stomatal density on the abaxial and adaxial leaf surface *and* measured the impacts on gaseous exchange of the two surfaces simultaneously. These findings highlight that stomatal patterning in monocots is differentially controlled in the adaxial and abaxial surfaces and raises questions regarding the signalling pathway or control of patterning on the adaxial surfaces. Furthermore, using a novel method we have shown that altered expression of EPF1 results in *SD* disparities over the leaf lamina, with consequences for both *A* and water use. Such spatial variation must be considered when

evaluating stomatal density in the context of genetic modifications or physiological performance, as it can significantly influence whole-plant water relations and affect the reliability of sampling protocols. Although we have also demonstrated an over investment in stomata on the adaxial surface, it is clear that the adaxial and not the abaxial surface supports greater gaseous diffusion and high photosynthetic rates (similar to wheat (Shellie *et al.*, 2022) despite equal *SD* on both surfaces. Understanding the underlying control of stomatal patterning on both surfaces relative to photosynthetic capacity will deliver new avenues for manipulating surfaces independently providing novel targets for producing plants with optimal gaseous exchange for photosynthetic performance, evaporative cooling and/or water use efficiency essential, for future crop resilience given the predicted changes to climate.

Chapter 4: Dynamic Plasticity of Photosynthetic Traits in Strawberry through Blue Light Modulation

Keywords: Blue light priming, *Fragaria × ananassa*, photosynthetic plasticity, drought resilience, spectral quality, stress memory, controlled environment

Running Title: Blue Light Priming Confers Persistent Drought Tolerance in Strawberry

Abstract (Chapter 4)

Spectral composition during plant development fundamentally shapes photosynthetic architecture and stress resilience. While blue light requirements for normal photosynthetic function are established, the persistence of spectral priming effects and their translation to stress tolerance remains poorly understood in horticultural crops. We conducted a two-phase experimental approach with *Fragaria* × *ananassa* cv. Malling Champion. Phase 1 characterized photosynthetic responses across a blue light gradient (5-45% of total PPFD). Phase 2 focused on temporal dynamics, comparing low (5%, LB) versus high (35%, HB) blue light priming for 4 weeks, followed by transfer to natural light and drought stress assessment. We quantified photosynthetic biochemistry, gas exchange kinetics, stomatal characteristics, optical properties, and yield performance. Blue light priming induced multilevel photosynthetic adaptations with distinct temporal signatures. HB treatment enhanced J_{max} (+35.4%) more than V_{cmax} (+15.9%) and increased stomatal density (+53.1%). While biochemical parameters normalized within 30 days post priming, structural modifications persisted. Critically, HB primed plants demonstrated superior drought resilience two months post priming, maintaining photosynthetic capacity (V_{cmax} : -2.0% vs -16.0% in LB) and yield stability (-1.4% vs -40.7% reduction). Blue light priming establishes a hierarchical stress memory system where transient biochemical modifications and persistent architectural changes collectively confer lasting drought tolerance. This discovery has potential for future targeted spectral manipulation during early development as a sustainable strategy for enhancing crop resilience in variable climates.

4.1 Introduction

Plant phenotypic adaptation to changing growth conditions represents a fundamental aspect of evolutionary biology (Larter *et al.*, 2018; Heyduk *et al.*, 2019). Light serves as the primary environmental signal regulating plant morphogenesis and metabolism. Plants perceive light quality through multiple photoreceptor families (see thesis introduction section 1.3.1 for details), and spectral composition during development ultimately shapes plant architecture and physiological adaptations (Devlin *et al.*, 2007; Jiao *et al.*, 2007; Hogewoning, S. W. *et al.*, 2010; Chen & Chory, 2011; Petroustos *et al.*, 2016).

While extensive research has characterized light mediated responses in natural environments, particularly in forest understory and field conditions where light quality varies unpredictably, controlled environment agriculture (CEA) can offer precision in manipulating spectral composition (Stamford *et al.*, 2023; Lanoue *et al.*, 2024; Ngcobo & Bertling, 2024; Paponov & Paponov, 2025). Unlike field studies where multiple environmental factors confound spectral effects, CEA systems also enable isolation of wavelength specific responses through programmable LED arrays, providing fundamental insights into photomorphogenic mechanisms while simultaneously optimizing production parameters (Kaiser, E. *et al.*, 2019; Zhang *et al.*, 2019; Lauria *et al.*, 2024). The advent of cost effective light-emitting diodes (LEDs) has revolutionized both research capabilities and commercial applications in protected cultivation. LED technology's narrow band emission spectra, minimal radiant heat output, and energy efficiency has enabled precise spectral recipes tailored to specific developmental stages or stress responses (Hogewoning *et al.*, 2010; Kaiser *et al.*, 2019; Stamford *et al.*, 2023; Lauria *et al.*, 2024; Hofmann *et al.*, 2025). These spectral manipulations induce

sophisticated acclimation mechanisms that prepare plants for future environmental challenges, with modifications extending beyond the duration of the initial stimulus (Crisp *et al.*, 2016; Lämke *et al.*, 2016; Jenkins, 2017). The phenotypic alterations driven by light environment are closely associated with modifications to photosynthetic efficiency that directly impact carbon assimilation (Foyer & Noctor, 2020; Luo & Keenan, 2020). The underlying mechanisms could involve coordinated adjustments in photosynthetic biochemistry, stomatal regulation, and leaf optical properties that collectively enhance plant performance under variable conditions (Hilker & Schmülling, 2019; Poorter *et al.*, 2019; Sakoda *et al.*, 2020).

Among the wavelengths within the photosynthetically active radiation (PAR) spectrum, blue light possesses unique attributes. Its shorter wavelength and higher energy content per quantum distinguish it from other wavelengths, like red light. While the role of blue light in photochemical quenching and as an initial energy source for CO₂ assimilation mirrors that of other wavelengths (Eberhard *et al.*, 2008), its perception by specific photoreceptors, such as phototropins and cryptochrome, is distinct (Jiao *et al.*, 2007). Biochemical studies have shown that blue light specifically modulates the photosynthetic apparatus through multiple pathways such as optimizing photosystem stoichiometry and promoting cyclic electron flow around PSI (Takahashi & Badger, 2011; Suorsa *et al.*, 2016). These photoreceptors mediate various blue light responses, including photomorphogenesis, stomatal opening, and photosynthetic apparatus functionality (McAusland *et al.*, 2016; MIAO *et al.*, 2016; Petroustos *et al.*, 2016). Importantly, blue light exposure triggers metabolic reprogramming that enhances photoprotective mechanisms, including xanthophyll cycle activity and antioxidant enzyme

expression which can provide cross protection against oxidative stress (Valle *et al.*, 2014; Matthews *et al.*, 2018; Roeber *et al.*, 2021; Chibani *et al.*, 2025).

Previous investigations predominantly compared monochromatic red light with red-blue combinations at fixed ratios, demonstrating that complete absence of blue wavelengths induces physiological disorders including excessive stem elongation, reduced photosynthetic capacity, and impaired stomatal function (Hogewoning *et al.*, 2010). Stomatal responses to blue light exhibit saturation kinetics, with maximal opening typically achieved at relatively low blue photon flux densities ($10\text{-}50\ \mu\text{mol m}^{-2}\text{ s}^{-1}$), whereas photosynthetic biochemical adaptations continue to respond to increasing blue light percentages well beyond stomatal saturation thresholds (Hogewoning *et al.*, 2010; Matthews *et al.*, 2020; Zhen & Bugbee, 2020; Ashikhmin *et al.*, 2024). The gradient responses to varying blue light intensities remain underexplored across many species. Notably, the augmentation of photosynthetic capacity with blue light is often nonlinear, with diminishing returns observed at blue light intensities exceeding 30-40% in spinach and cucumber (Matsuda *et al.*, 2007; Hogewoning, S. W. *et al.*, 2010). This nonlinearity reflects saturation of photoreceptor mediated responses and the energetic costs of maintaining enhanced photosynthetic machinery, suggesting an optimal range for blue light supplementation (Trouwborst *et al.*, 2016).

The phenomenon of physiological priming where prior environmental exposure enhances subsequent stress tolerance through metabolic and structural modifications can offers promising avenues for sustainable crop production (Savvides *et al.*, 2016). Light quality represents an ideal priming stimulus due to its fundamental role in regulating photosynthetic development and stress response pathways. Recent studies

demonstrated that spectral priming induces lasting anatomical modifications, including altered stomatal patterning and mesophyll structure, that persist throughout leaf development (Zhang *et al.*, 2019; Fanourakis *et al.*, 2020). These structural changes are accompanied by biochemical adaptations in carbon metabolism, photoprotective capacity, and hydraulic conductance that collectively enhance plant performance under stress conditions.

In perennial fruit crops such as strawberry (*Fragaria × ananassa*), the potential for using specific light wavelength combinations during early development to induce beneficial traits that persist through the production cycle aligns with commercial production practices and remain unexplored. As these plants experience distinct developmental phases from dormancy breaking through vegetative growth to reproductive development, and each presenting opportunities for environmental conditioning. The persistence of priming effects through these transitions could provide seasons long benefits for stress resilience and productivity. Furthermore, understanding the temporal dynamics of priming induced modifications is crucial for optimizing treatment protocols and predicting potential efficacy under fluctuating field conditions.

Therefore, this multiyear study aims to elucidate the temporal dynamics of photosynthetic adaptation and stress memory persistence by initially determining the optimal blue light percentage during the post dormancy vegetative growth phase (week 1-4 after transplanting) to foster desirable leaf traits conducive to enhanced photosynthesis. Subsequently, we examined the durability of these traits and their persistence under natural sunlight in 'light primed' plants. Perennial strawberries serve as an ideal subject, given their seasonal production cycle and vulnerability to water stress during fruit

development. This cycle provides a strategic opportunity to 'prime' plants following winter dormancy, optimally positioning them for the forthcoming growing season. Here, we test whether blue light priming during early vegetative development induces persistent enhancements in photosynthetic efficiency, stomatal regulation, and drought tolerance through integrated modifications in leaf physiology and biochemistry.

4.2 Material and Methods

Plant Material and Growth Conditions

Fragaria ananassa cv. Malling Champion (everbearing strawberry) plants were received as potted individuals in cold storage from RW Walpole (United Kingdom) and transplanted into 1.4-liter pots containing coconut coir moistened with Hoagland nutrient solution (electrical conductivity 1.7 mS/cm, pH 5.8 at 20°C). Following transplantation, plants were randomly allocated to compartmentalized sections within a controlled climate room (Aralab, Portugal) maintained at 20°C during the photoperiod and 16°C during the dark period, with relative humidity controlled between 60–70%.

Illumination during the priming phase was provided by HelioSpectra LED arrays (DYNA, HelioSpectra AB, Sweden). Throughout the vegetative phase, senescent leaves were removed weekly or biweekly depending on growth rate. After a 4 week priming period under controlled conditions, plants were re-potted into 4 L containers and transferred to a polytunnel for continued growth under natural light, simulating typical UK production settings. Substrate moisture content was continuously monitored using multiple soil

sensors (WET150, Delta-T Devices, Cambridge, UK). Soil moisture data were collected by a 12 channels GP2 datalogger (Delta-T Devices, Cambridge, UK) programmed to trigger irrigation events that maintained soil moisture at predetermined experimental levels. Nutrient solution was delivered to maintain the target electrical conductivity for the generative growth stage using three dosing pumps (D3WL, DOSATRON, NC, USA). During the flowering stage, plants received a modified nutrient solution with increased electrical conductivity (2.4 mS/cm) administered daily.

Experimental Design

To comprehensively assess blue light priming effects, we employed a two phase experimental approach: Phase 1 established dose response relationships across a blue light gradient, while Phase 2 focused on detailed temporal characterization of the most divergent treatments.

Experimental Phase 1: Leaf Response to Blue Light Percentage Gradient

The initial experimental phase (March–July 2022) assessed plant responses to varied percentage of blue light (420 and 450 nm) within the total spectral distribution using HelioSpectra DYNA LED arrays (HelioSpectra AB., Sweden) with all treatments receiving a total light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Five blue light treatments were evaluated: 5%, 15%, 25%, 35%, and 45%, with the remaining spectrum comprising red light (630 and 660 nm). Plants were grown under their respective light treatments for a 4 week priming period with a consistent 10 hour photoperiod. Spectral distribution for each treatment was

quantified at the average canopy height using a SpectraPen LM spectroradiometer (PSI, Czech Republic).

Experimental Phase 2: Focused Comparative Analysis and Post-Priming Responses

Based on results from Experimental Phase 1, the second phase (April–August 2023 and 2024, conducted with new plants in subsequent years from phase 1) focused on two light treatments: 5% blue light (here after LB for low blue) as the control and 35% blue light (here after HB for high blue) as the treatment condition. This refinement allowed for increased biological replication and a more comprehensive temporal assessment throughout the growth cycle. The experimental protocol involved a 4 week “light priming” phase under either LB or HB conditions, followed by subsequent relocation to the polytunnel for continued growth under natural sunlight. Measurements were conducted at five distinct time points: pre-priming (M1), 2 weeks into priming (M2), 4 weeks into priming (M3), 1 month post-priming (M4), and 2 months post-priming (M5). To assess the priming effect on stress tolerance, 1 month after the post-priming period, plants from both LB and HB treatments were subdivided into two irrigation regimes: well-watered (soil moisture content maintained at 60–65%) and water-limited (soil moisture content maintained at 30–35%).

Infra-red Gas Exchange Measurements

Newly developed, fully expanded leaves were used for all gas exchange measurements. Leaves were clamped inside the leaf chamber of infrared gas analyser systems (Li-6800, LI-COR Biosciences, NE, USA) equipped with 6 cm² cuvette and multiphase fluorometer

(6800-01A, LI-COR). The fluorometer light source provided peak wavelengths of 625 nm (red) and 475 nm (blue) for actinic illumination. Standard measurement conditions included a flow rate of 500 $\mu\text{mol s}^{-1}$, leaf temperature controlled at 20°C, vapor pressure deficit regulated at 1.2 kPa, and a reference CO_2 concentration fixed at 400 $\mu\text{mol mol}^{-1}$ (except during A/C_i response measurements).

Photosynthesis light response curve

Leaves were acclimated to a saturating light intensity of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for a minimum of 15 minutes. Subsequently, light intensity was sequentially decreased using the following PPFD levels: 1500, 1300, 1100, 900, 700, 550, 400, 250, 150, 100, 50, and 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$. A minimum stabilization period of 1 minute was allowed before logging each measurement point. Data were fitted to a non-rectangular hyperbola function following Von Caemmerer (2000):

$$A_n = \frac{\Phi \cdot I + A_{max} - \sqrt{((\Phi \cdot I + A_{max})^2 - 4k \cdot \Phi \cdot I \cdot A_{max})}}{2\Phi} - R_d \quad \text{Equation 1}$$

where A_n represents net CO_2 assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$), Φ denotes the quantum yield of CO_2 assimilation under light-limiting conditions ($\text{mol CO}_2 \text{ mol}^{-1} \text{ photons}$), I indicates incident PPFD ($\mu\text{mol m}^{-2} \text{s}^{-1}$), A_{max} denotes the light-saturated assimilation rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$), k represents the curve convexity (dimensionless), and R_d is the dark respiration rate ($\mu\text{mol m}^{-2} \text{s}^{-1}$).

Photosynthetic Induction Kinetics: Leaves were first dark-adapted overnight and then in the measurement chamber for another 20 min and minimum fluorescence was obtained,

before they were exposed to a saturating light pulse ($8000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 960 ms) to determine F_m and calculate the maximum quantum efficiency of PSII ($F_v/F_m = F_m - F_o/F_m$). Plants with F_v/F_m value higher than 0.78 were used for subsequent gas exchange measurements. Leaves were then acclimated to low light ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) for a minimum of 15 minutes. The induction protocol comprised further acclimation at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 15 minutes, followed by a step increase to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 60 minutes, and subsequently a step decrease to $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD. Gas exchange parameters were recorded at 2 second intervals throughout the measurement period. Stomatal conductance (g_{sw}) responses to changing light intensity were characterized using a modified exponential sigmoid function (McAusland *et al.*, 2016):

$$g_{sw} = (g_{smax} - g_{smin})e^{-e^{\left(\frac{\lambda - t}{K} + 1\right)}} + g_{smin}$$

where g_{smax} represents the steady-state stomatal conductance at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD ($\text{mol m}^{-2} \text{s}^{-1}$), g_{smin} denotes the minimum stomatal conductance during acclimation at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD ($\text{mol m}^{-2} \text{s}^{-1}$), λ indicates the initial lag time before stomatal response (minutes), K is the time constant for the stomatal response (minutes).

Estimation of Photosynthetic Biochemical Parameters

Dynamic CO_2 Responses (A/C_i) (Experimental Phase 1) was conducted following Salter *et al.* (2019). For selected treatments (5%, 25%, and 45% blue light), temporal changes in biochemical capacity were quantified by measuring photosynthetic induction across eight CO_2 concentrations. Leaves were exposed to a step increase in PPFD from 100 to

1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ measured at eight reference CO_2 concentrations (50, 150, 250, 400, 550, 700, 900, and 1200 $\mu\text{mol mol}^{-1}$) in randomized order to minimize hysteresis effects. A minimum one hour recovery period under low light conditions was used between successive measurements to ensure complete reequilibration of photosynthetic and stomatal processes. Net CO_2 assimilation rate (A), intercellular CO_2 concentration (C_i), and leaf temperature (T_{leaf}) were recorded at 2-second intervals. The resulting time-resolved A/C_i curves were used to estimate three biochemical parameters: maximum carboxylation rate of Rubisco (V_{cmax} $\mu\text{mol m}^{-2} \text{s}^{-1}$), maximum electron transport rate for RuBP regeneration (J_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), and mitochondrial respiration in the light (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$).

Parameter estimation was based on the Farquhar–von Caemmerer–Berry model for C_3 photosynthesis:

$$A = \min \left(\frac{V_{\text{cmax}}(C_i - \Gamma)}{C_i + K_c \left(1 + \frac{O}{K_o}\right)}, \frac{J(C_i - \Gamma)}{4.5C_i + 10.5\Gamma^*} \right) - R_d$$

Where K_c and K_o denote the Michaelis–Menten constants for CO_2 and O_2 , respectively ($\mu\text{mol mol}^{-1}$ and mmol mol^{-1}), and O indicates the ambient O_2 concentration (210 mmol mol^{-1}). Temperature dependencies of K_c and K_o were accounted for using Arrhenius functions (temperature dependent exponential relationships describing enzyme kinetics) with reference values at 25°C: $K_c = 336 \mu\text{mol mol}^{-1}$, $K_j = 262 \text{mmol mol}^{-1}$. Activation energies were 79.43 kJ mol^{-1} for K_c , 36.38 kJ mol^{-1} for K_o .

Model fitting used the Levenberg–Marquardt algorithm for non-linear least squares optimization in R, with initial parameter values were set to 60, 100, and 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for V_{cmax} , J_{max} , and R_d , respectively, with bound constraints for V_{cmax} , J_{max} in [0, 300], and R_d in [0, 5]. To analyze temporal dynamics, sequential A/C_i curves were fitted across the induction period. The resulting $V_{camx}(t)$ and $J_{max}(t)$ time series were segmented into an activation phase (0.5–15 minutes post-induction) and a stabilization phase (15.5–60 minutes), following Salter *et al.* (2020) for quantitative characterization of the dynamic activation processes for both carboxylation and electron transport capacities following increases in irradiance.

Steady-State A/C_i Responses (Experimental Phase 2) : For Experimental Phase 2, steady state A/C_i curves were measured under assumed saturating light (1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) using the following sequence of reference CO_2 concentrations: 400, 250, 150, 100, 50, 400, 550, 700, 850, 1000, 1200, 1500, and 400 $\mu\text{mol mol}^{-1}$. All other environmental conditions (temperature 20°C, VPD 1.2 kPa, flow rate 500 $\mu\text{mol s}^{-1}$) remained constant as described above.

Thermal Imaging Analysis (Chapter 2 Method)

Leaf temperature dynamics were assessed using thermal imaging at 30 days after treatment initiation (end of priming period, 30DAT), following gas exchange measurements. Measurements were conducted in a controlled environment room maintained at approximately 19°C. To minimize boundary layer resistance and enhance

uniformity of heat exchange, a fan was positioned at an appropriate distance from the measurement area. Thermal images were captured at 0.5 second intervals using a longwave infrared camera (FLIR a-SERIES, Teledyne FLIR, Oregon, USA; spectral range: 7.5–14 μm). A multilayer black cloth (emissivity 0.98) served as a dry reference, while wetted multilayer filter paper (emissivity 0.96) adjacent to the leaf provided a wet reference. A minimum of five regions of interest (ROIs), each comprising a 3×3-pixel square, were randomly selected per leaf sample to ensure representative sampling of the leaf surface temperature distribution. Leaf thermal kinetics were measured by first acclimation at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 20 minutes, followed by an increase to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 60 minutes, and subsequently a decrease to 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD for 10 minutes. Illumination was provided by two LED units (Elixia, HelioSpectra AB, Sweden) using blue (450 nm) and red (660 nm) channels at a ratio of 70% red and 30% blue. The stomatal conductance index was derived from leaf temperature measurements using the thermal references, where T_{dry} represented the temperature of a non-transpiring surface and T_{wet} represented the temperature of a fully transpiring surface.

Anatomical Measurements

Stomatal density was quantified using the impression method. Silicon-based dental material (Xantopren L Blue, Heraeus, Germany) was applied to the abaxial surface of hypostomatic strawberry leaves. After setting, a thin layer of clear nail polish was applied to these impressions and then transferred to microscope slides following the methodology

detailed by (Smith *et al.*, 1989). Eight replicate slides were prepared for each treatment group. The impressions were examined using a light microscope (Leica ATC 2000) at 40× magnification, with multiple fields of view assessed per impression to account for spatial heterogeneity in stomatal distribution.

Optical Properties and Pigment Analysis

Immediately following gas exchange measurements at each of the five measurement timepoints (1, 15, 30, 60, and 90 DAT) after priming treatment initiation, leaf optical properties were assessed on both adaxial and abaxial surfaces using a handheld spectral reflectance meter (PolyPen, PSI, Czech Republic) with the spectral range of 380–790 nm. This non-destructive approach was used to quantify leaf surface optical characteristics related to pigmentation, surface morphology, and internal leaf structure. Measurements were taken at three locations per leaf to account for spatial variability, with the average spectrum used for subsequent analysis of treatment effects on leaf optical properties.

Pigment Extraction and Quantification

At each measurement time point, leaf samples were collected for pigment analysis. Fresh leaf tissue (approximately 0.0324–0.0328 g per two leaf samples) was incubated in 10 mL of 100% acetone in darkness at 4°C to extract chlorophyll and carotenoid pigments. Absorbance of the extract was measured spectrophotometrically at 470, 649, 664, and 750 nm using a UV visible spectrophotometer calibrated with pure acetone as a blank reference. Samples were stored at 4°C overnight prior to final measurements to ensure complete extraction. Chlorophyll a, chlorophyll b, and total carotenoid concentrations

were calculated using the extinction coefficients and equations proposed by Lichtenthaler and Wellburn (1983), with absorbance at 750 nm used to correct for sample turbidity.

Growth and Yield Assessment

After the priming phase (30 DAT), morphological characteristics were quantified using a multispectral 3D scanner (PlantEye F600, Phenospex, Netherlands). This system employs a combination of laser triangulation and multispectral imaging to enable three-dimensional reconstruction of plant architecture with integrated spectral information. The scanner operates by projecting a laser line across the plant while simultaneously capturing the reflection with a camera, creating a point cloud with associated spectral reflectance data at each point. From this integrated dataset, key morphological parameters were derived, including plant height, leaf area index, projected leaf area, surface angle distribution, and an estimation of digital biomass based on volumetric reconstruction. These non-destructive measurements were used for the immediate assessment on the effects of light treatments on growth while preserving plants for subsequent yield measurements.

Fruit yield was assessed by harvesting and weighing Class 1 fruit (diameter ≥ 28 mm) throughout the production period. Fruit quality was evaluated by measuring soluble solids content ($^{\circ}\text{Brix}$) using refractometer. Additional fruit quality parameters including taste and flavour profiles were assessed at peak harvest by the lead author of this writing through systematic sensory tasting.

Statistical Analysis

All statistical analyses in this chapter were performed using R version 4.2.0 (R Core Team, 2023). Data normality was assessed using Shapiro-Wilk tests, and homogeneity of variance was verified using Levene's test. For normally distributed data, treatment effects were analysed using Student's t-tests (two treatments) or one-way ANOVA (multiple treatments) followed by Tukey's HSD post-hoc tests. Non-parametric alternatives (Wilcoxon rank-sum or Kruskal-Wallis tests) were employed when parametric assumptions were violated. Two-way ANOVA was used to assess treatment × time or treatment × drought interactions. Model parameters for photosynthetic light response curves were estimated using non-linear least squares regression (nlsLM function, minpack.lm package). Effect sizes were calculated using Cohen's d for pairwise comparisons. Statistical significance was set at $\alpha = 0.05$ unless otherwise noted. Data are presented as means $\pm$ standard error (SE) unless otherwise specified.

4.3 Results

4.3.1 Photosynthetic Responses to Blue Light Percentage Gradient (Experimental Phase 1)

During Experimental Phase 1, the adaptive responses of *Fragaria ananassa* cv. Malling Champion to varying proportions of blue light (5-45%) within the spectral composition provided during vegetative development were systematically evaluated (Figure 4.1). This initial gradient assessment was designed to characterize photosynthetic plasticity mechanisms and identify optimal spectral compositions for subsequent targeted experimental phase 2.

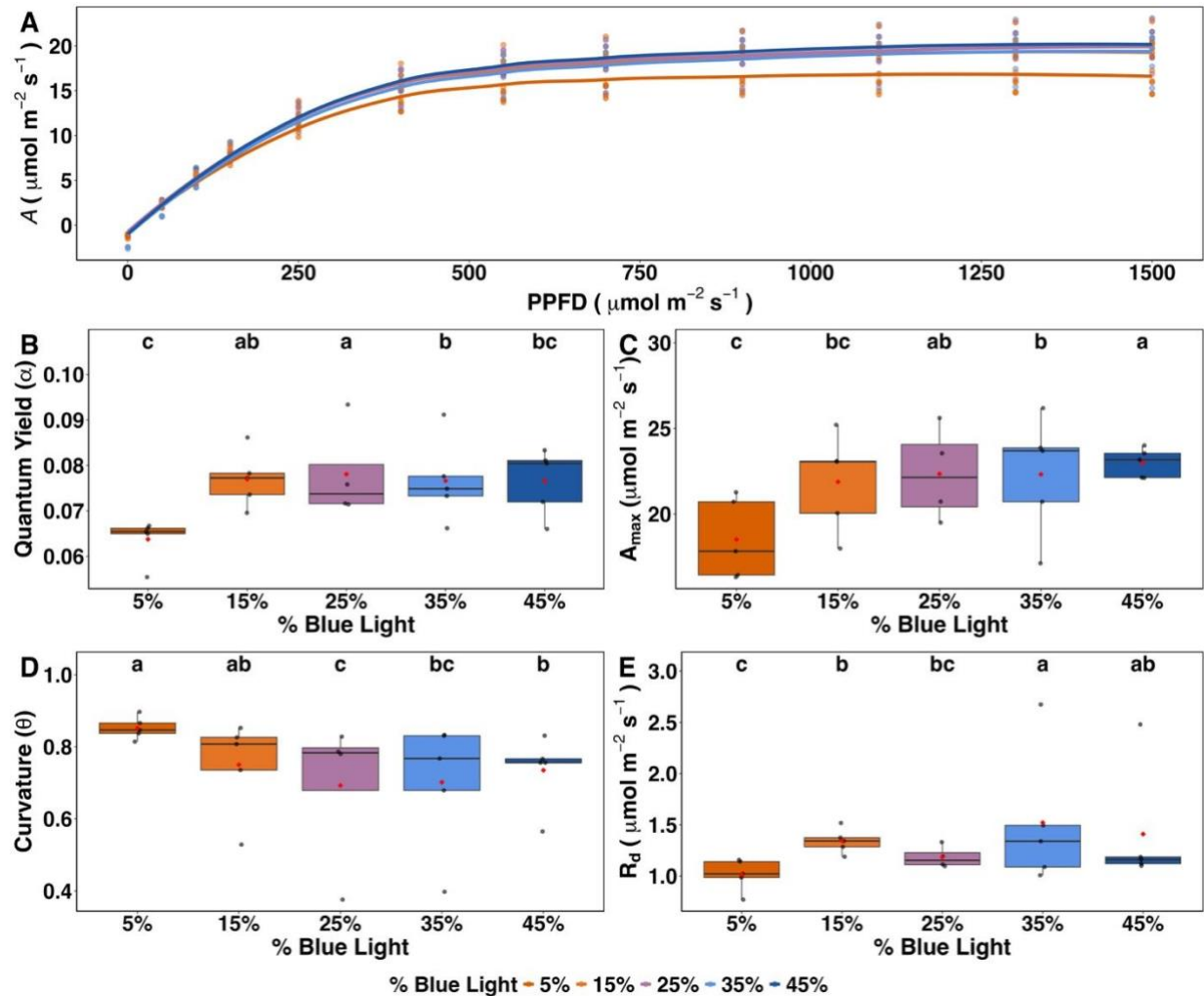


Figure 4.1. Photosynthetic responses of *Fragaria ananassa* cv. Malling Champion to varying blue light percentages during Experimental Phase 1. (A) Light response curves showing net CO₂ assimilation rate (A) as a function of photosynthetic photon flux density (PPFD) for plants grown under five blue light treatments (5%, 15%, 25%, 35%, and 45% of total irradiance, with remainder as red light). Points represent measured data with curves fitted using loess smoothing ($n = 4-5$). (B-E) Parameters derived from non-rectangular hyperbola model fitting: (B) quantum yield (α , mol CO₂ mol⁻¹ photons) (C) light-saturated CO₂ assimilation rate (D) curve convexity (θ ,

dimensionless), and (E) dark respiration rate (R_d $\mu\text{mol m}^{-2} \text{s}^{-1}$). Box plots show median, quartiles, and individual data points with means indicated by red diamonds. Different letters above bars indicate statistically significant differences between treatments ($n = 8$ plants per treatment).

Photosynthetic light response curves revealed significant spectral composition-dependent modifications in photosynthetic parameters (Figure 4.1A). The quantum yield (α) exhibited significant differences among blue light treatments ($F = 2.99$, $p = 0.045$), with a distinct nonlinear response pattern (Figure 4.1B). Plants developed under intermediate blue light percentages demonstrated superior quantum efficiency, with the 25% blue light treatment achieving the highest value ($\alpha = 0.078 \pm 0.005 \text{ mol CO}_2 \text{ mol}^{-1} \text{ photons}$), closely followed by 15% and 35-45% treatments ($\alpha = 0.077 \pm 0.003 \text{ mol CO}_2 \text{ mol}^{-1} \text{ photons}$). In contrast, plants grown under the 5% blue light regime exhibited markedly reduced photochemical efficiency ($\alpha = 0.064 \pm 0.002 \text{ mol CO}_2 \text{ mol}^{-1} \text{ photons}$) which representing a 20.1% reduction compared to the 45% blue light treatment. Light-saturated photosynthetic capacity (A_{max}) displayed a positive correlation with increasing blue light percentage (Figure 4.1C). The highest A_{max} values were observed in the 45% blue light treatment ($23.0 \pm 0.4 \mu\text{mol m}^{-2} \text{s}^{-1}$), with intermediate treatments (15-35% blue) showing comparable values (21.9 - $22.3 \mu\text{mol m}^{-2} \text{s}^{-1}$), while the 5% blue light treatment exhibited substantially reduced capacity ($18.5 \pm 1.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) which representing a 24.2% decrease relative to the 45% blue treatment. Curve convexity (θ) demonstrated an inverse relationship with blue light percentage, decreasing from 0.852 ± 0.014 at 5% blue light to 0.693 - 0.750 at higher blue light percentages ($p = 0.442$) as shown in Figure 4.1D. Similarly, dark respiration rates R_d exhibited a tendency to increase with elevated blue

light exposure (Figure 4.1E), with values ranging from $1.02 \pm 0.07 \mu\text{mol m}^{-2} \text{s}^{-1}$ (5% blue) to $1.41\text{-}1.52 \mu\text{mol m}^{-2} \text{s}^{-1}$ (35-45% blue), representing up to a 38.8% increase, though without reaching statistical significance ($p = 0.400$).

Analysis of assimilation rates across the full PPFD range revealed that optimal blue light percentage varied with irradiance level, with intermediate blue percentages (25%) maximized efficiency under limiting light, while higher percentages (45%) treatment enhanced performance under saturating conditions. Specifically, at PPFD values above $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, plants developed under 45% blue light consistently demonstrated superior assimilation rates, maintaining this advantage through to saturating light conditions ($1500 \mu\text{mol m}^{-2} \text{s}^{-1}$). The magnitude of treatment differences was most pronounced at high light intensities, with maximum divergence observed at $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (difference of $3.56 \mu\text{mol m}^{-2} \text{s}^{-1}$ between highest and lowest performing treatments). Therefore, these results from Experimental Phase 1 (Figure 4.1) demonstrate that spectral composition during vegetative development induces significant modifications in photosynthetic apparatus characteristics, with particularly notable effects on quantum yield and light-saturated photosynthetic capacity. The differential advantages observed under varying light intensities suggest blue light-induced adaptations in both light harvesting complexes and carbon fixation machinery.

4.3.2 Enhanced Dynamic Photosynthetic Biochemical Responses to Blue Light Treatments (Experimental Phase 1)

The temporal dynamics of underlying biochemical mechanisms were also examined through dynamic A/C_i response curves (Figure 4.2). Overall, all plants exposed to elevated blue light percentages exhibited substantially enhanced steady-state biochemical capacity, with significant treatment effects observed for both V_{cmax} ($F = 44.39$, $p < 0.001$) and J_{max} ($F = 27.32$, $p < 0.001$). During the initial activation phase (0-15 min), control plants (5% blue) exhibited numerically higher but not statistically significant baseline V_{cmax} values ($10.5 \pm 0.04 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared to 25% blue ($9.83 \pm 0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$) and 45% blue ($9.34 \pm 0.04 \mu\text{mol m}^{-2} \text{s}^{-1}$) treatments, while initial J_{max} values remained similar across treatments ($\sim 40 \mu\text{mol e}^{-} \text{m}^{-2} \text{s}^{-1}$) which can be thought reflecting comparable dark-adapted states (Figure 4.2A-B). The activation kinetics for V_{cmax} revealed treatment-specific patterns, with plants grown under 45% blue light demonstrating the most rapid activation rate ($0.0545 \mu\text{mol m}^{-2} \text{s}^{-1} \text{min}^{-1}$), approximately twice that of 25% blue ($0.0285 \mu\text{mol m}^{-2} \text{s}^{-1} \text{min}^{-1}$) and control treatments ($0.0259 \mu\text{mol m}^{-2} \text{s}^{-1} \text{min}^{-1}$), suggesting more responsive Rubisco activation under high blue light conditions. In contrast, J_{max} showed minimal changes during the initial activation phase, suggesting that electron transport capacity was not limiting during early induction (Figure 4.2A-B, inset panels).

During the stabilization phase (15-60 min), both elevated blue light treatments sustained significantly higher V_{cmax} (25% blue: $61.3 \pm 0.35 \mu\text{mol m}^{-2} \text{s}^{-1}$; 45% blue: $59.7 \pm 0.34 \mu\text{mol m}^{-2} \text{s}^{-1}$) compared to 5% Blue light control ($51.5 \pm 0.18 \mu\text{mol m}^{-2} \text{s}^{-1}$). A similar pattern

was observed for J_{max} , with 25% blue light inducing the highest electron transport capacity ($119 \pm 0.72 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), followed by 45% blue ($110 \pm 0.56 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$) and control ($107 \pm 0.40 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$). Analysis of steady-state values ($\geq 45 \text{ min}$) further emphasized the persistent nature of these photosynthetic modifications. Plants grown under 25% blue light maintained the highest final V_{cmax} ($68.3 \pm 0.05 \mu\text{mol m}^{-2} \text{s}^{-1}$) and J_{max} ($137 \pm 0.18 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$), representing increases of 25.6% and 19.1% over control plants, respectively. The 45% blue light treatment showed comparable final V_{cmax} ($66.6 \pm 0.08 \mu\text{mol m}^{-2} \text{s}^{-1}$) but lower J_{max} ($124 \pm 0.18 \mu\text{mol e}^- \text{m}^{-2} \text{s}^{-1}$) values, suggesting a possible upper threshold or stress response at the highest blue light percentage.

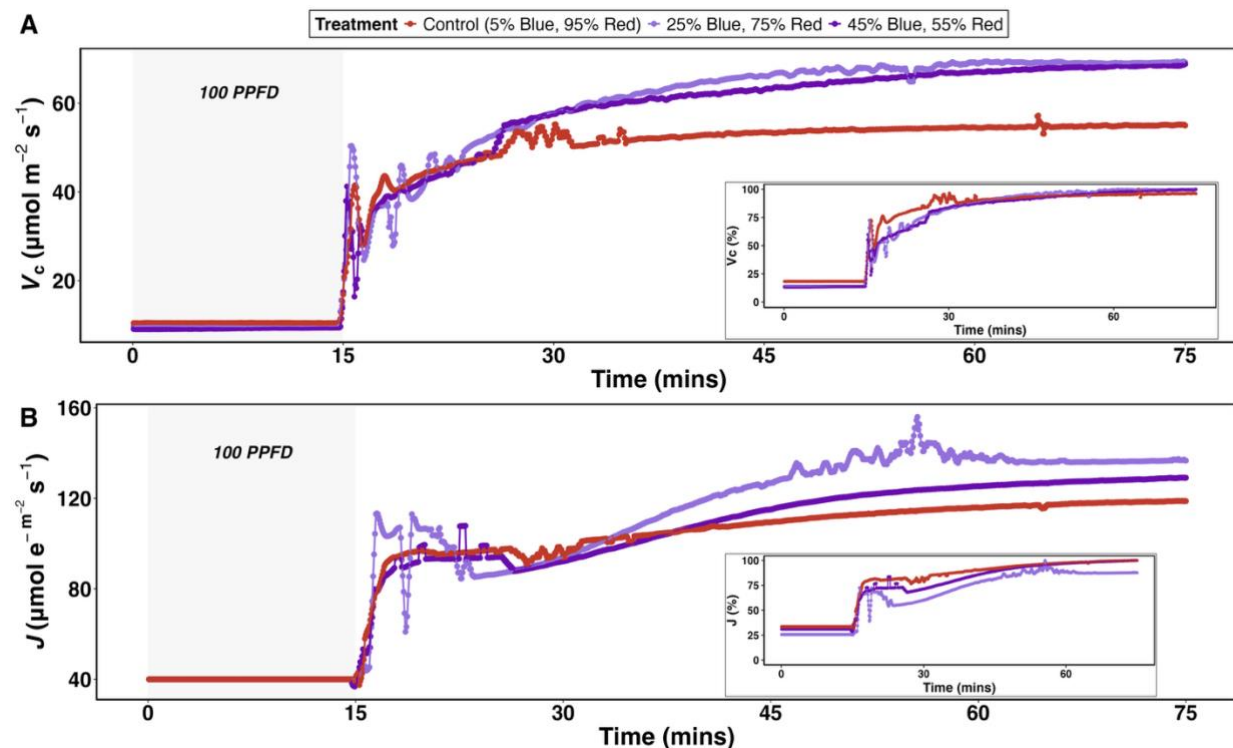


Figure 4.2. Dynamic photosynthetic biochemical responses following transition from low to high light in strawberry leaves developed under different blue light

percentages. Temporal changes in (A) maximum Rubisco carboxylation rate (V_{cmax}) and (B) maximum electron transport rate (J_{max}) following transition from low light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, gray shading) to high light (1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) for plants grown under three spectral compositions selected from experimental phase 1: control (5% blue, 95% red), 25% blue (75% red), and 45% blue (55% red). Points represent individual measurements. Inset panels show the relative values (% of maximum) for each treatment on differential activation kinetics.

4.3.3 Photosynthetic Adaptations During Priming and Persistence in Post-Priming Phase (Experimental Phase 2)

Based on findings from experimental phase 1 (Figure 4.1), the 5% blue light treatment (representing minimal blue light exposure) and the 35% blue light treatment (representing significant blue light exposure) were selected for the focused comparative analysis in Experimental Phase 2, which examined persistent photosynthetic adaptations and cross-tolerance to water limitation. Specifically, we examined photosynthetic adaptations induced during the 4-week blue light priming period (Days 1-30) and the persistence of this following transfer to natural light conditions (Days 30-90). At the beginning of the priming period (1 DAT), plants assigned to high blue (HB, 35%) and low blue (LB, 5%) treatments showed comparable photosynthetic performance with minimal differences in light response curves (Figure 4.3A). Assimilation rates at saturating light (PPFD ≥ 700 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were slightly lower in HB compared to LB plants (17.3 ± 0.49 vs. 17.8 ± 0.37 $\mu\text{mol m}^{-2} \text{s}^{-1}$, -2.5% difference). Fitted photosynthetic parameters then revealed

marginally higher quantum yield (α) in HB plants (0.0639 ± 0.0018 vs. 0.0606 ± 0.0014) with comparable light-saturated assimilation capacity (A_{max}) between treatments (21.4 ± 1.01 vs. $21.6 \pm 1.01 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure 4.3B-C). Halfway through the priming period (15 DAT), treatment-specific differences began to emerge in response to the differential spectral compositions. HB plants exhibited enhanced photosynthetic performance at saturating light intensities (17.8 ± 0.48 vs. $16.4 \pm 0.28 \mu\text{mol m}^{-2} \text{s}^{-1}$, 8.6% higher). Quantum yield was also trending higher in HB plants (0.0640 ± 0.0018 vs. 0.0596 ± 0.0014 ; $p = 0.072$) while A_{max} showed increasingly divergent values (20.9 ± 1.19 vs. $18.7 \pm 0.69 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure 4.3B-C). Dark respiration (R_d) was significantly higher in HB plants at this timepoint ($p = 0.008$), suggesting enhanced metabolic activity supporting photosynthetic acclimation (Figure 4.3E).

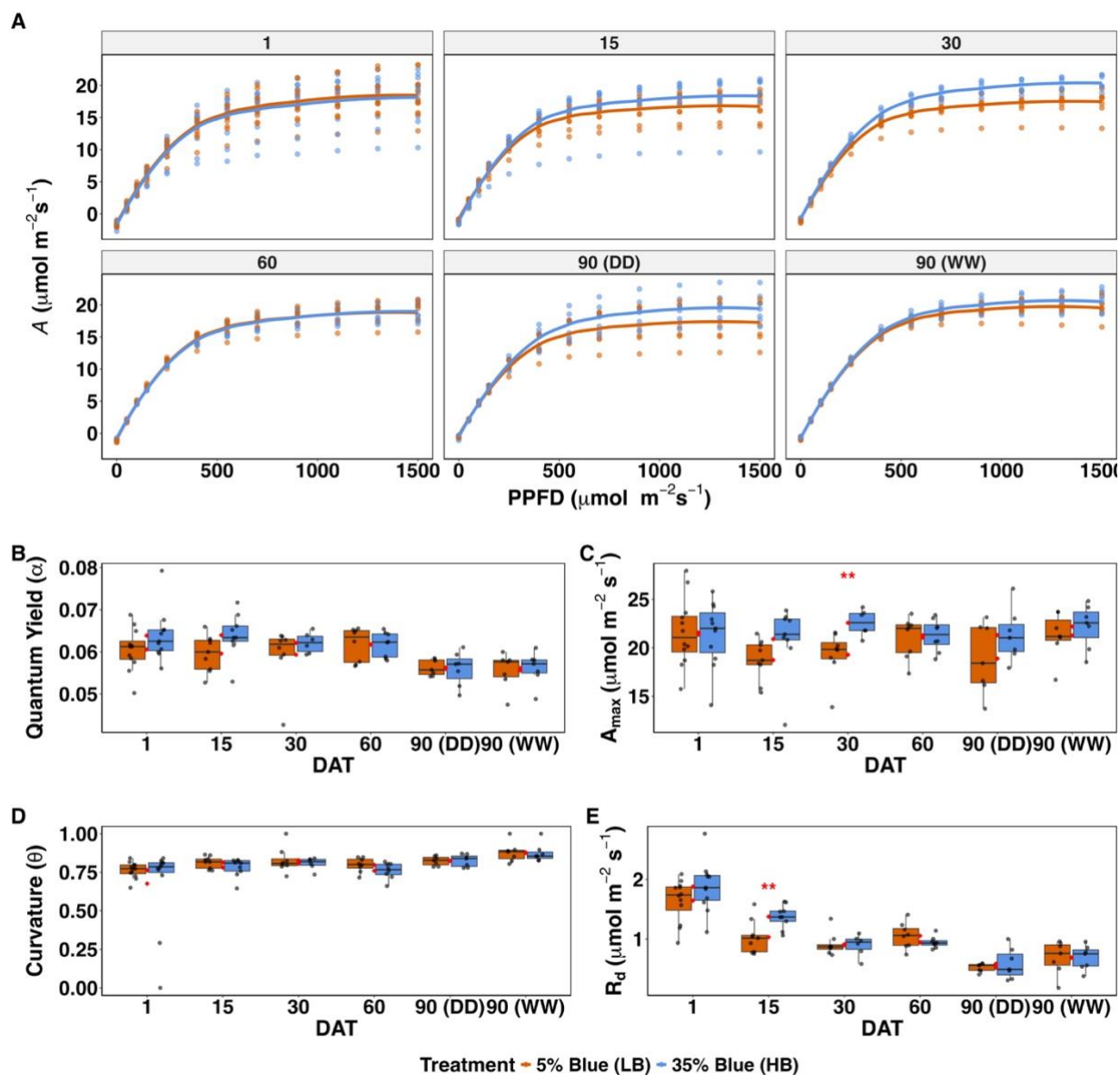


Figure 4.3. Photosynthetic adaptations during blue light priming and their persistence after transfer to natural light conditions. (A) Photosynthetic light response curves measured at five timepoints: during the 4-week priming period (1, 15, 30 DAT), and after transfer to natural light (60 DAT under normal irrigation, 90 DAT under both well-watered [WW] and drought [DD] conditions) under High blue (35%, blue

lines) and low blue (5%, orange lines) treatments. (B) quantum yield (α , mol CO₂ mol⁻¹ photons), (C) light-saturated CO₂ assimilation rate (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$), (D) curve convexity (θ , dimensionless), and (E) dark respiration rate (R_d , $\mu\text{mol m}^{-2} \text{s}^{-1}$). Data represent means $\pm$ SE (n = 12 plants per treatment). Boxplots show median, quartiles, and individual data points with means indicated by red diamonds. Statistical significance between treatments at each timepoint is indicated by asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001; t-test). Data represent means $\pm$ SE (n = 12 plants per treatment).

By the end of the 4-week priming period (30 DAT), just before transfer to natural light conditions in our commercial grade polytunnel, pronounced treatment differences were observed, with HB plants demonstrating substantially higher assimilation rates at saturating light intensities (19.7 ± 0.22 vs. $17.1 \pm 0.28 \mu\text{mol m}^{-2} \text{s}^{-1}$, 15.6% higher). At this light condition transition timepoint, A_{max} was significantly higher in HB plants (22.6 ± 0.55 vs. $19.3 \pm 0.86 \mu\text{mol m}^{-2} \text{s}^{-1}$; p = 0.008), with moderately elevated quantum yield (0.0621 ± 0.0010 vs. 0.0593 ± 0.0025) (Figure 4.3B-C). These differences were also evident at limiting light intensities (PPFD $\leq 250 \mu\text{mol m}^{-2} \text{s}^{-1}$), where HB plants maintained 6.6% higher assimilation rates (6.54 ± 0.72 vs. $6.14 \pm 0.59 \mu\text{mol m}^{-2} \text{s}^{-1}$).

However, one month after transfer to natural light conditions (60 DAT), the photosynthetic differences between treatments had largely dissipated, with nearly identical assimilation rates at both saturating (18.3 ± 0.22 vs. $18.2 \pm 0.25 \mu\text{mol m}^{-2} \text{s}^{-1}$, 0.2% difference) and limiting light intensities (6.19 ± 0.59 vs. $6.21 \pm 0.60 \mu\text{mol m}^{-2} \text{s}^{-1}$, -0.3% difference). Similarly, all photosynthetic parameters converged between treatments, with identical

quantum yield values (0.0617 ± 0.0011 vs. 0.0618 ± 0.0014) and A_{max} (21.3 ± 0.58 vs. $21.1 \pm 0.75 \mu\text{mol m}^{-2} \text{s}^{-1}$) as well as similar curve convexity (θ) and dark respiration rates (Figure 3B-E).

Interestingly, drought response at two months post-priming (90 DAT) revealed differential sensitivities between treatments despite both groups having experienced identical natural light conditions for two months. Under well-watered conditions (WW), both treatments maintained relatively similar photosynthetic parameters, with HB plants showing modestly higher assimilation rates at saturating light intensities (20.1 ± 0.23 vs. $19.2 \pm 0.23 \mu\text{mol m}^{-2} \text{s}^{-1}$, 4.4% higher) and comparable fitted parameters (Figure 4.3A). However, under drought (drying down) conditions (DD), plants previously exposed to high blue light demonstrated greater resilience by maintaining significantly higher assimilation rates (18.9 ± 0.38 vs. $16.9 \pm 0.48 \mu\text{mol m}^{-2} \text{s}^{-1}$, 11.9% higher) despite similar quantum yield values. The drought-induced reduction in overall assimilation was substantially smaller in previously HB-exposed plants (-5.1%) compared to LB plants (-10.2%), indicating enhanced drought tolerance in plants that experienced elevated blue light during the priming period. This differential response was particularly evident in A_{max} values, where drought caused minimal reduction in HB-primed plants (21.3 ± 1.02 vs. $22.2 \pm 0.86 \mu\text{mol m}^{-2} \text{s}^{-1}$ under well-watered conditions) while LB-primed plants exhibited more substantial impairment (18.9 ± 1.37 vs. $21.3 \pm 0.90 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure 4.3C).

4.3.4 Photosynthetic induction kinetics following light intensity step changes (Experimental Phase 2)

To investigate the temporal dynamics of photosynthetic responses to fluctuating light conditions, we subjected plants to step changes in irradiance and analysed photosynthetic induction and relaxation kinetics (Figure 4.4). Plants primed with contrasting blue light regimes (5% vs 35%) exhibited differential photosynthetic induction characteristics primarily at the end of the priming period (30 DAT), with these differences largely dissipating during subsequent measurement timepoints. At the end of the priming period (30 DAT), plants grown under 35% blue light demonstrated significantly enhanced photosynthetic induction capacity compared to those grown under 5% blue light (Figure 4.4A, C). The initial slope of CO₂ assimilation rate during induction was 36.2% higher in 35% blue light-treated plants (0.47 vs 0.35 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} \text{ min}^{-1}$; t-test, $p=0.018$, Cohen's $d=1.13$). Similarly, the amplitude of photosynthetic response ($A_{\text{amplitude}}$) was 45.9% greater in high blue light-treated plants (6.21 vs 4.25 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; t-test, $p=0.003$, Cohen's $d=1.62$), indicating enhanced photosynthetic capacity following the step increase in light intensity.

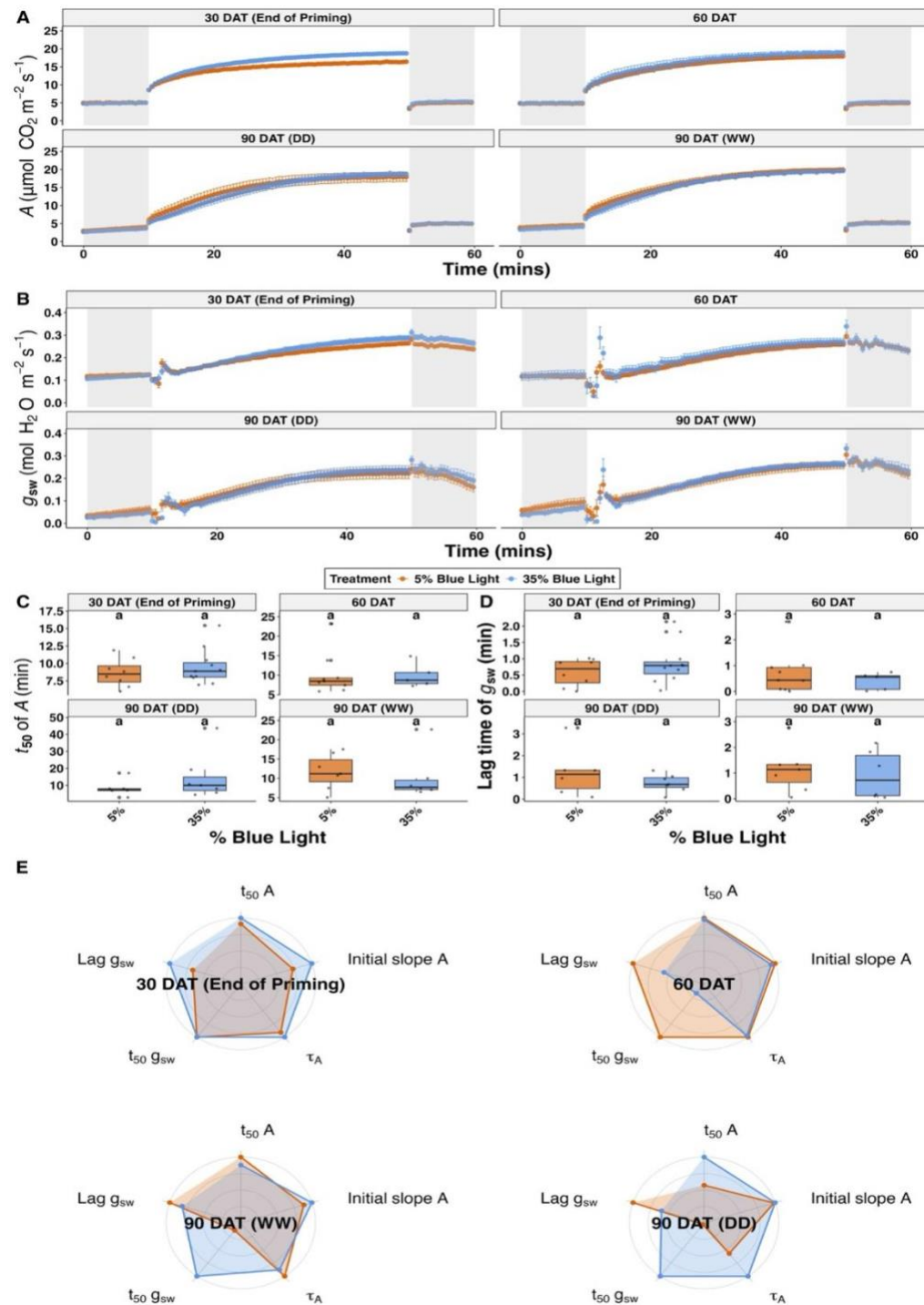


Figure 4.4. Photosynthetic and stomatal responses to step changes in light intensity (Experimental Phase 2). (A) CO_2 assimilation rate (A) and (B) stomatal

conductance (g_{sw}) in response to step changes in light intensity across four measurement timepoints: 30 DAT (end of priming), 60 DAT, 90 DAT well-watered (WW), and 90 DAT water-limited (DD). Grey shaded areas indicate periods of low light intensity ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$); unshaded areas represent high light intensity ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$). Data points represent means $\pm$ SE ($n= 8-11$). (C) Time to reach 50% of maximum assimilation rate (t_{50} of A) and (D) lag time of stomatal response across treatment groups and measurement timepoints, and statistical significance is indicated by different letters ($p<0.05$). (E) Radar plots displaying normalized kinetic parameters for both treatments at each measurement timepoint. Values are normalized as percentages of maximum values for each parameter.

Temporal analysis revealed that differences in photosynthetic induction parameters initially observed were not sustained at subsequent measurement timepoints (Figure 4.4C, E). At 60 DAT, 90 DAT under well-watered conditions, and 90 DAT under water limitation, no statistically significant differences in photosynthetic induction parameters were observed between the treatment groups, suggesting a transient nature of the priming effect.

Stomatal conductance (g_{sw}) kinetics characterised by parameters including lag time, time to 50% of maximum response (t_{50}), and response amplitude, showed no statistically significant differences between treatments at any measurement timepoint (Figure 4.4B, D). However, notable trends were observed, with 35% blue light-treated plants exhibiting 48.3% longer lag times at 30 DAT compared to 5% blue light-treated plants (0.86 min vs 0.58 min), although this difference was not statistically significant.

The response to water limitation (90 DAT) revealed pronounced treatment differences in the pattern of kinetic parameter changes (Figure 4.4E). Specifically, HB-primed plants unexpectedly enhanced induction kinetics under drought stress, while LB plants showed the typical drought-induced suppression of dynamic responses. While no within treatment comparisons between well-watered and water-limited conditions reached statistical significance, the magnitude and direction of response differed markedly between treatments. Plants primed with 35% blue light showed substantial increases in photosynthetic parameters under water limitation, with A_{t50} increasing by 41.7% and $A_{amplitude}$ by 41.1% compared to well-watered conditions. In contrast, 5% blue light-treated plants exhibited decreases in these parameters (-28.6% for A_{t50} and -14.8% for $A_{amplitude}$). This differential response pattern was even more obvious for stomatal parameters, with 35% blue light-treated plants showing 172.3% greater change in the time constant of stomatal response (τ_{ugsw}) compared to 5% blue light-treated plants when subjected to water limitation. The analysis of kinetic parameters as shown in radar plots (Figure 4.4E) further demonstrates the distinct "fingerprints" of induction responses across light treatment groups and measurement timepoints throughout the growing season. At 30 DAT, the 35% blue light treatment yielded enhanced photosynthetic induction capacity primarily through increased initial slope and amplitude, while differences in stomatal kinetics became more pronounced at later timepoints, particularly under water limitation.

4.3.5 Blue Light-Induced Modifications in Photosynthetic Biochemical Capacity (Experimental Phase 2)

To investigate the mechanistic basis of blue light-induced photosynthetic adaptations observed in the light response curves (Figure 4.3), *A/Ci* curve analysis to quantify carboxylation capacity (V_{cmax}) and electron transport rate (J_{max}) throughout the priming and post-priming periods were conducted (Figure 4.5). At the beginning of the priming period (1DAT), both high blue (HB, 35%) and low blue (LB, 5%) treatments exhibited similar biochemical parameters, with no significant differences in V_{cmax} (HB: 76.2 ± 4.7 vs. LB: $77.4 \pm 3.9 \mu\text{mol m}^{-2} \text{s}^{-1}$, $p = 0.843$) or J_{max} (HB: 176.6 ± 8.1 vs. LB: $171.2 \pm 9.4 \mu\text{mol m}^{-2} \text{s}^{-1}$, $p = 0.670$) (Figure 5A-B). The $J_{max}:V_{cmax}$ ratio that reflects the balance between RuBP regeneration and carboxylation capacities, was marginally higher in HB plants (2.37 ± 0.12 vs. 2.21 ± 0.05) but not statistically significant. By mid-priming (day 15), substantial biochemical differentiation was observed between treatments. While V_{cmax} showed a moderate increase in HB plants (80.2 ± 5.4 vs. $72.3 \pm 4.0 \mu\text{mol m}^{-2} \text{s}^{-1}$, 11.0% higher), J_{max} demonstrated a pronounced enhancement (165.3 ± 6.6 vs. $132.4 \pm 6.3 \mu\text{mol m}^{-2} \text{s}^{-1}$, 24.8% higher, $p < 0.01$). This differential stimulation of electron transport relative to carboxylation capacity resulted in a significantly higher $J_{max}: V_{cmax}$ ratio in HB plants (2.12 ± 0.12 vs. 1.84 ± 0.05 , $p < 0.10$), indicating a substantial shift in the balance between RuBP regeneration and carboxylation processes. The maximum biochemical divergence between treatments occurred at the end of the priming period (day 30), with HB plants exhibiting significantly higher V_{cmax} (87.6 ± 2.3 vs. $75.6 \pm 4.1 \mu\text{mol m}^{-2} \text{s}^{-1}$, 15.9% higher, $p < 0.05$) and markedly enhanced J_{max} (181.3 ± 4.5 vs. $133.9 \pm 6.7 \mu\text{mol m}^{-2} \text{s}^{-1}$, 35.4% higher, $p < 0.001$). ANOVA confirmed highly significant treatment effects at this timepoint

for both parameters (V_{cmax} : $F = 5.31$, $p = 0.040$; J_{max} : $F = 29.39$, $p < 0.001$). The J_{max} :
 V_{cmax} ratio remained significantly elevated in HB plants (2.07 ± 0.06 vs. 1.78 ± 0.05 , $p < 0.01$), suggesting a consistent blue light-induced reconfiguration of the photosynthetic apparatus favouring enhanced electron transport capacity.

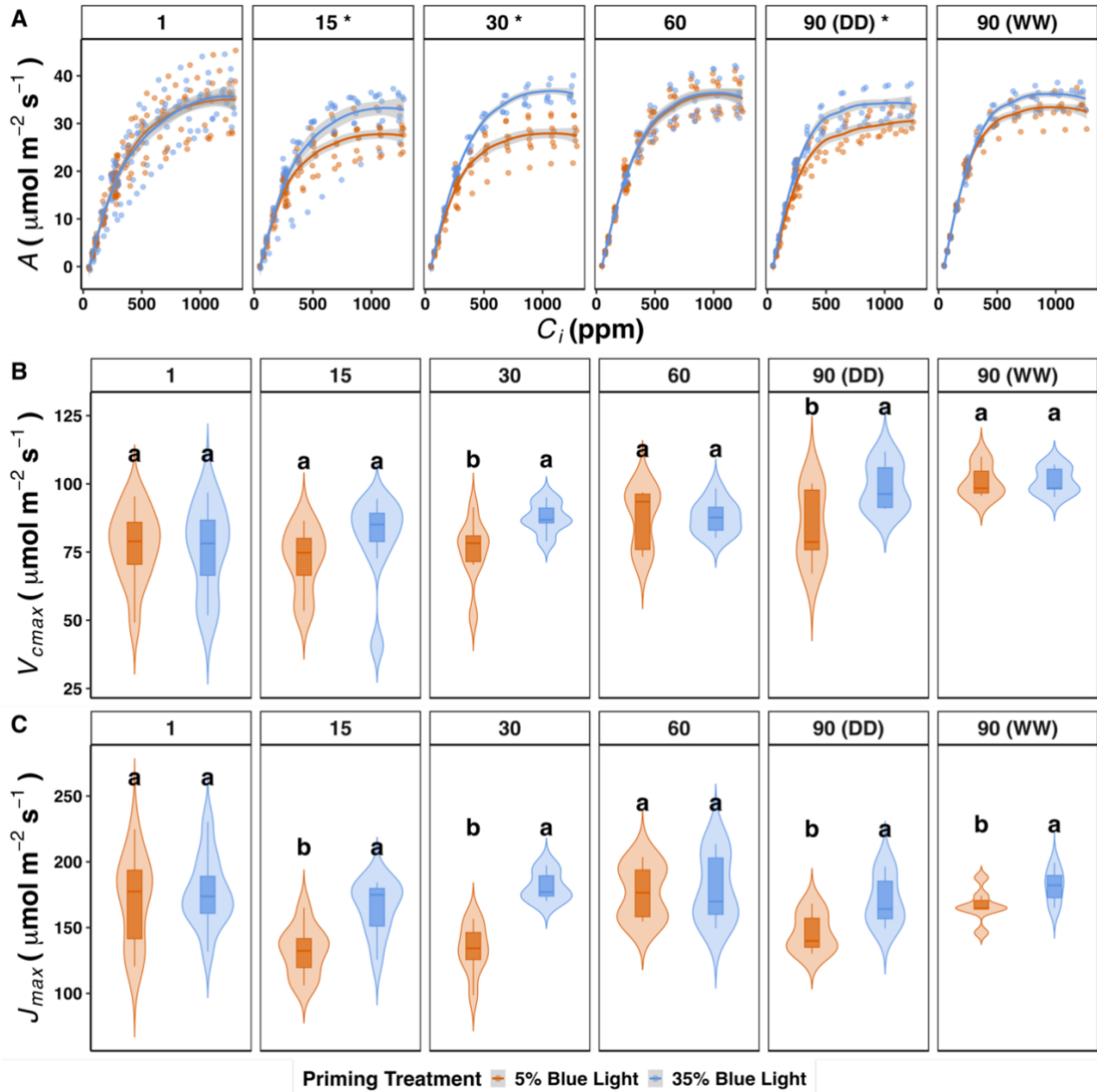


Figure 4.5. Blue light-induced modifications in photosynthetic biochemical capacity during priming and their persistence after transfer to natural light conditions. (A) maximum carboxylation rate (V_{cmax}) and (B) maximum electron transport rate (J_{max}) at six timepoints: during the 4-week priming period (days 1, 15, 30), after transfer to natural light conditions (day 60), and at day 90 under both well-watered (WW) and drought (DD) conditions. Statistical significance between treatments at each

timepoint is indicated by letters, with different letters denoting significant differences ($p < 0.05$). Data represent means $\pm$ SE ($n = 12$).

Following transfer to natural light conditions, biochemical differences between treatments rapidly diminished. By day 60, both V_{cmax} (HB: 87.8 ± 2.1 vs. LB: $87.7 \pm 3.8 \mu\text{mol m}^{-2} \text{s}^{-1}$, 0.1% difference) and J_{max} (HB: 179.3 ± 9.1 vs. LB: $176.8 \pm 7.3 \mu\text{mol m}^{-2} \text{s}^{-1}$, 1.5% difference) were virtually identical between treatments, with no statistical differences ($p = 0.983$ and $p = 0.828$, respectively). Likewise, the $J_{max}: V_{cmax}$ ratio converged to similar values (HB: 2.04 ± 0.06 vs. LB: 2.02 ± 0.05 , $p = 0.877$), indicating complete equalization of photosynthetic biochemistry after 30 days under identical light conditions. Surprisingly, the drought response assessment at day 90 revealed differential biochemical resilience between treatments. For example, under well-watered conditions (WW), both treatments maintained similar V_{cmax} values (HB: 101.1 ± 1.8 vs. LB: $100.9 \pm 2.2 \mu\text{mol m}^{-2} \text{s}^{-1}$, 0.2% difference, $p = 0.951$), though HB plants exhibited significantly higher J_{max} (181.6 ± 4.8 vs. $166.9 \pm 4.8 \mu\text{mol m}^{-2} \text{s}^{-1}$, 8.8% higher, $p < 0.05$). Under drought conditions (DD), however, HB plants maintained substantially higher V_{cmax} (99.0 ± 3.3 vs. $84.7 \pm 5.1 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16.9% higher, $p < 0.05$) and J_{max} (170.5 ± 7.2 vs. $146.1 \pm 5.6 \mu\text{mol m}^{-2} \text{s}^{-1}$, 16.7% higher, $p < 0.05$) compared to LB plants. These biochemical findings corroborate the light response curve data (Figure 4.3), confirming that blue light priming enhances drought resilience through maintenance of both carboxylation and electron transport capacities.

The drought-induced reduction in biochemical capacity was significantly different between treatments. HB plants exhibited minimal decreases in V_{cmax} (-2.0%) and moderate reductions in J_{max} (-6.1%), whereas LB plants showed significant decreases in

both parameters (V_{cmax} : -16.0%, J_{max} : -12.5%). ANOVA confirmed a significant treatment $\times$ drought interaction for V_{cmax} ($F = 4.45$, $p = 0.046$), indicating that prior blue light exposure fundamentally altered the plant biochemical response to water limitation. Although the interaction term for J_{max} was not statistically significant ($p = 0.398$), the consistent pattern across both parameters still suggests substantially enhanced drought resilience of photosynthetic biochemistry in plants previously exposed to elevated blue light.

4.3.6 Spectral Properties and Pigment Composition of Strawberry Leaves

The optical properties and pigment composition of strawberry leaves exhibited significant modulation in response to blue light treatment intensity during the priming phase (Figure 4.6). Spectrophotometric analysis revealed distinct absorbance patterns between plants grown under 5% versus 35% blue light regimes. At 1 DAT, minimal spectral differences were observed between treatments across the measured wavelength range (380-790 nm). However, by 30 DAT, substantial divergence in leaf absorbance characteristics appeared, with 15.8% of measured wavelengths exhibiting statistically significant differences

between treatments ($p < 0.05$). The most pronounced spectral differences at 30 DAT were localized in the near-UV (380-400 nm) and blue (400-500 nm) regions, where 35% blue light treated plants demonstrated higher absorbance values (mean p-values 0.0295 and 0.0403, respectively). This enhanced absorbance under high blue light extended across the visible spectrum, with exceptionally large treatment differences observed in the green region (254% higher in 35B vs. 5B), followed by substantial increases in the blue (70.8%), red (68.6%), and near-infrared edge (54.8%) regions. Notably, while both treatments exhibited peak absorbance at 432-433 nm wavelengths at 1 DAT, by 30 DAT the 35% blue treatment shifted toward longer wavelength peaks (661-663 nm), suggesting fundamental alterations in leaf optical properties.

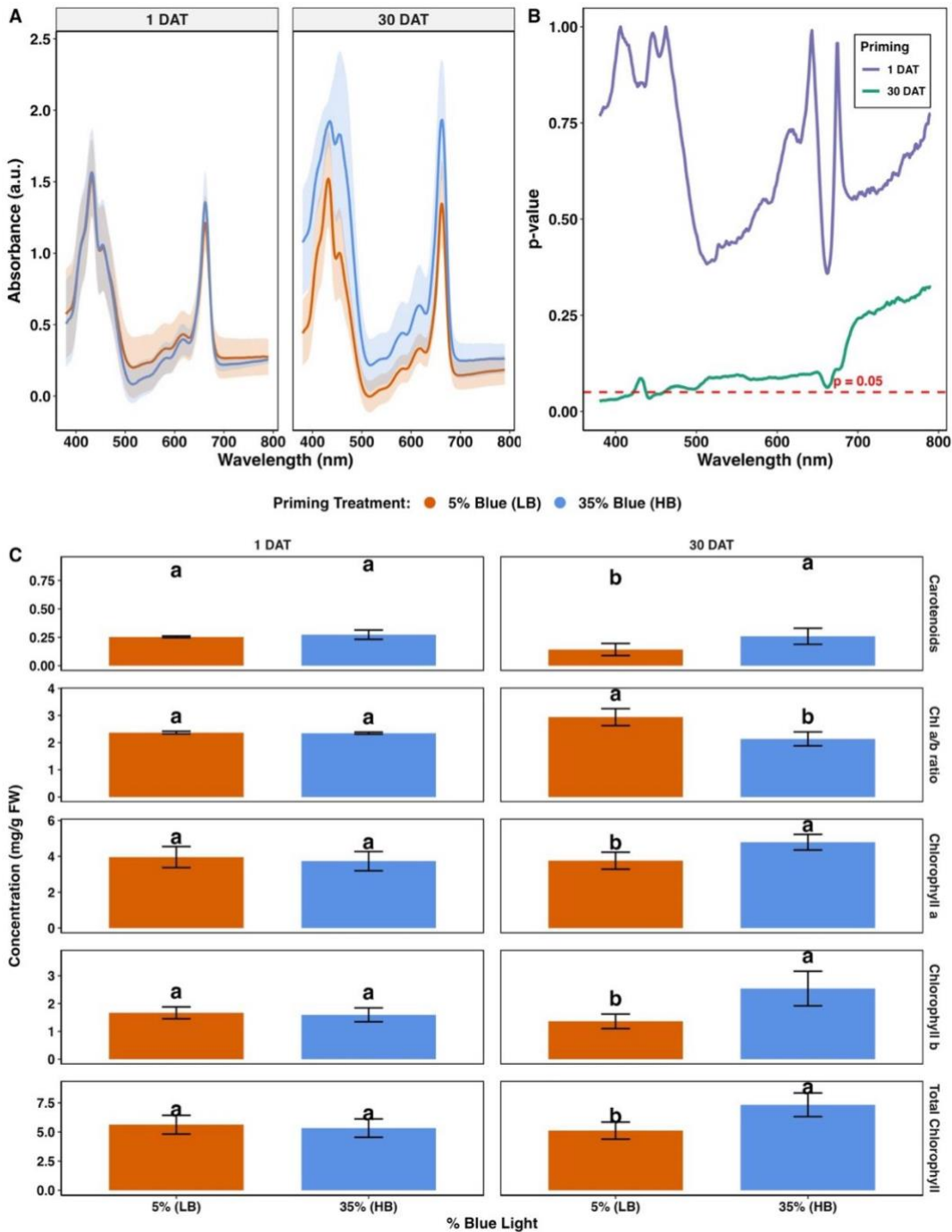


Figure 4.6. Spectral properties and pigment composition of strawberry leaves in response to blue light priming treatments. (A) Leaf absorbance spectra measured using a handheld spectral reflectance meter (Polypen) across the 380-790 nm range

for plants grown under 5% blue light (5B, orange) or 35% blue light (35B, blue) at 1 DAT and 30 DAT (end of priming). Lines represent means and shaded areas indicate ± 1 SD ($n = 4$). (B) Statistical significance (p -values) of treatment differences across the wavelength spectrum for both measurement timepoints. The red dashed line indicates $p = 0.05$. (C) Quantitative analysis of leaf pigment concentrations (Chlorophyll a, Chlorophyll b, Total Chlorophyll, Carotenoids, and Chlorophyll a/b ratio) in response to blue light treatments at 1 DAT and 30 DAT. Bars represent means $\pm$ SE ($n = 3-5$). Different letters above bars indicate statistically significant differences between treatments within each timepoint ($p < 0.05$).

These spectral modifications corresponded with concomitant changes in leaf pigment composition. At 1 DAT, pigment concentrations were comparable between treatments, with differences ranging from 0.8% to 7.5%. In contrast, by the conclusion of the priming period (30 DAT), all analysed pigments exhibited biologically meaningful differences ($>10\%$) between treatments. Plants exposed to 35% blue light developed substantially higher concentrations of chlorophyll b (86.6% increase, $p = 0.137$), carotenoids (81.4% increase, $p = 0.229$), total chlorophyll (43.2% increase, $p = 0.122$), and chlorophyll a (27.5% increase, $p = 0.156$) compared to the 5% blue light treatment. Interestingly, the chlorophyll a/b ratio decreased significantly in 35% blue light-treated plants (-27.3% , $p = 0.093$), indicating a differential enhancement of chlorophyll b relative to chlorophyll a. All pigment parameters exceeded our predetermined 10% biological significance threshold, despite not achieving conventional statistical significance due to limited replication ($n = 3-5$).

4.3.7 Stomatal Density Changes in Response to Blue light priming

Stomatal density (*SD*) showed noticeable changes in response to blue light priming treatments, with distinct patterns observed on both leaf surfaces over time (Figure 4.7). During the initial 4-week priming phase, both treatments showed an increase in stomatal density, however, with markedly different patterns. At the start of priming (1 DAT), plants assigned to high blue (HB, 35%) and low blue (LB, 5%) treatments had similar stomatal densities (124.0 ± 5.1 vs. 139.8 ± 4.5 stomata mm^{-2} , respectively). However, by the end of the priming period (30 DAT), HB plants reached the highest *SD* recorded throughout the entire experiment (190.0 ± 4.5 stomata mm^{-2}), representing a 53.1% increase from their starting values. This peak density was significantly higher than LB plants at the same timepoint (162.6 ± 4.5 stomata mm^{-2} $p < 0.001$) and was the largest difference observed between treatments (27.4 stomata mm^{-2} difference, 14.4% higher in HB plants). After transfer to natural light conditions, the two treatments showed contrasting patterns. LB plants interestingly continued to show an increase in stomatal density, reaching maximum values at 60 DAT (177.1 ± 4.5 stomata mm^{-2} , 26.7% above baseline). In contrast, HB plants declined from the 30 DAT peak and reached intermediate densities (161.3 ± 4.1 stomata mm^{-2} at 60 DAT, 30.0% above baseline). By 60 DAT, the treatment difference had actually reversed, with LB plants maintaining 9.8% higher densities than HB plants, though both remained well above their starting values. Water stress responses at 90 DAT also revealed different drought adaptation strategies between the priming treatments. Under drought conditions, LB plants achieved an absolute maximum *SD* (187.4 ± 10.1 stomata mm^{-2} , 34.1% above baseline), while showing only modest increases under well-watered conditions (146.2 ± 5.8 stomata mm^{-2} , 4.5% above baseline). HB plants showed

more consistent but moderate *SD* increases under both water conditions (173.4 ± 7.2 stomata mm^{-2} under drought, 163.6 ± 5.8 stomata mm^{-2} under well-watered conditions, representing 39.8% and 31.9% increases from baseline, respectively).

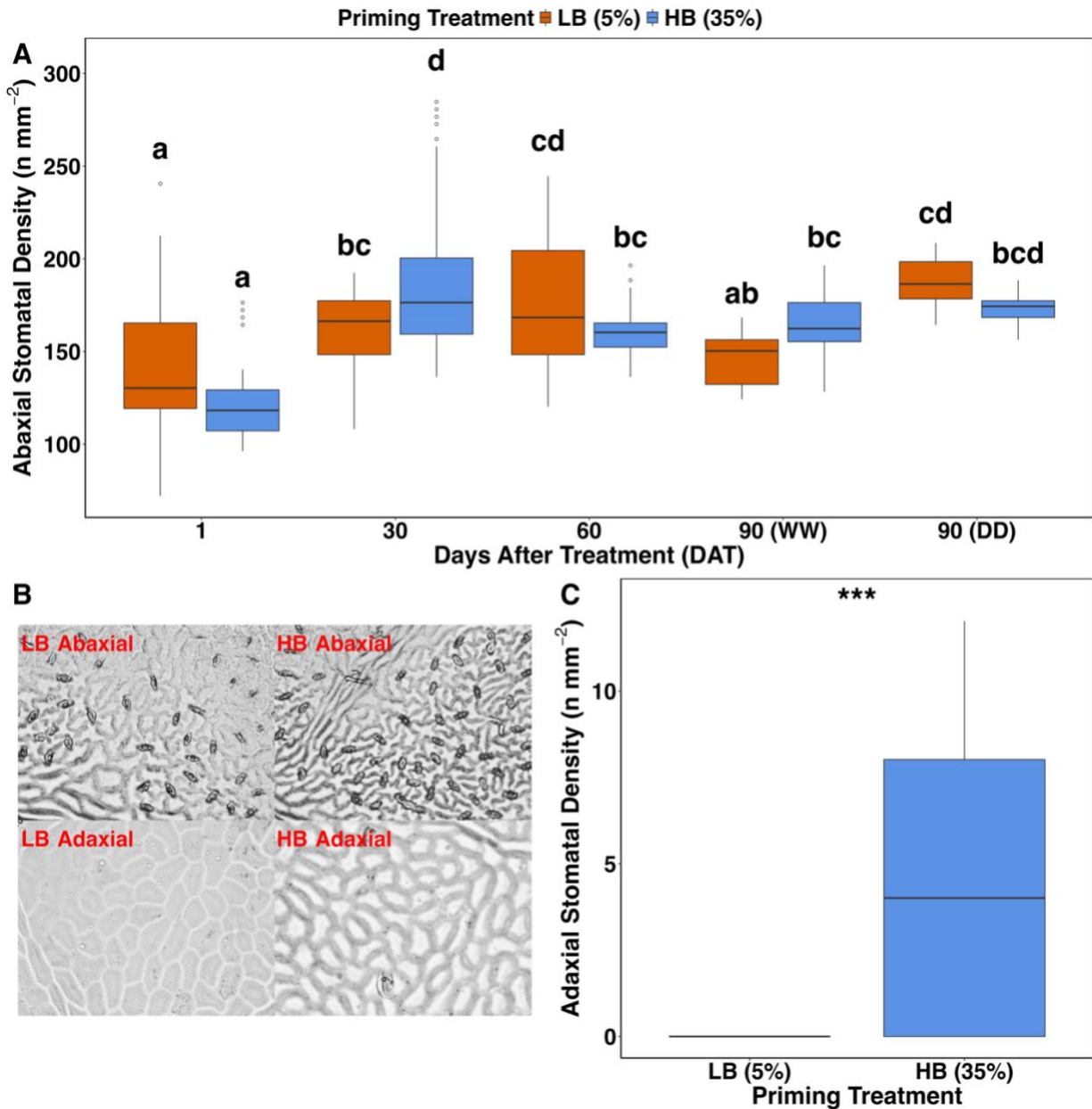


Figure 4.7. Blue light priming effects on stomatal density development in strawberry leaves. (A) Changes in lower surface (abaxial) stomatal density throughout the priming period (1-30 DAT) and after transfer to natural light (60-90 DAT) under low blue (LB, 5%, orange) and high blue (HB, 35%, blue) treatments. Plants were moved to natural light at 30 DAT, with different watering regimes applied at 90 DAT (WW =

well-watered, DD = drought). Letters above box plots indicate statistical groupings from post-hoc analysis ($p < 0.05$). (B) representative microscopy images showing stomatal distributions. (C) Upper surface (adaxial) stomatal density measured at 30 DAT, showing treatment-specific development of stomata on both leaf surfaces. Statistical significance indicated by asterisks (***) $p < 0.001$). Data show individual measurements with boxes indicating median, quartiles, and whiskers extending to 1.5× interquartile range ($n = 8-48$ per group).

Most surprisingly, the upper leaf surface (adaxial) at 30 DAT revealed striking differences in leaf architecture between treatments (Figure 4.7C). LB plants developed essentially no stomata on their upper surfaces (0.0 ± 0.0 stomata mm^{-2} , $n = 16$), maintaining the typical strawberry hypostomatous pattern with stomata only on the lower surface. However, HB plants developed a sparse but significant population of upper surface stomata (4.3 ± 1.0 stomata mm^{-2} , $n = 16$, $t = -4.259$, $p < 0.001$), suggesting a fundamental shift toward having stomata on both leaf surfaces. The ratio of lower-to-upper surface stomata in HB plants was 44.2:1, while LB plants maintained stomata exclusively on the lower surface.

4.3.8 Blue Light Priming Effects on Plant Architecture and Harvest Performance

Multispectral 3D scanning at the conclusion of the 4 week priming period (30 DAT) revealed treatment specific modifications in plant architecture and canopy structure (Figure 4.8). Plants exposed to high blue light (HB, 35%) developed larger 3D leaf areas compared to low blue light (LB, 5%) plants (207.7 ± 5.0 vs. 170.9 ± 20.5 cm², respectively) which representing a 21.5% increase with an estimated mean difference of 36.8 cm² (HB–LB; bootstrap 95% CI -6.9 to $+70.2$ cm²; $p = 0.131$; $n = 4$ per treatment; Figure 4.8B). Conversely, canopy light penetration depth showed the opposite pattern, with HB plants exhibiting reduced light penetration compared to LB plants (4.3 ± 0.3 vs. 5.1 ± 0.7 cm, respectively), consistent with denser canopy architecture with an estimated mean difference of -0.79 cm (HB–LB; bootstrap 95% CI -2.41 to $+0.50$ cm; $p = 0.355$; Figure 4.8C).

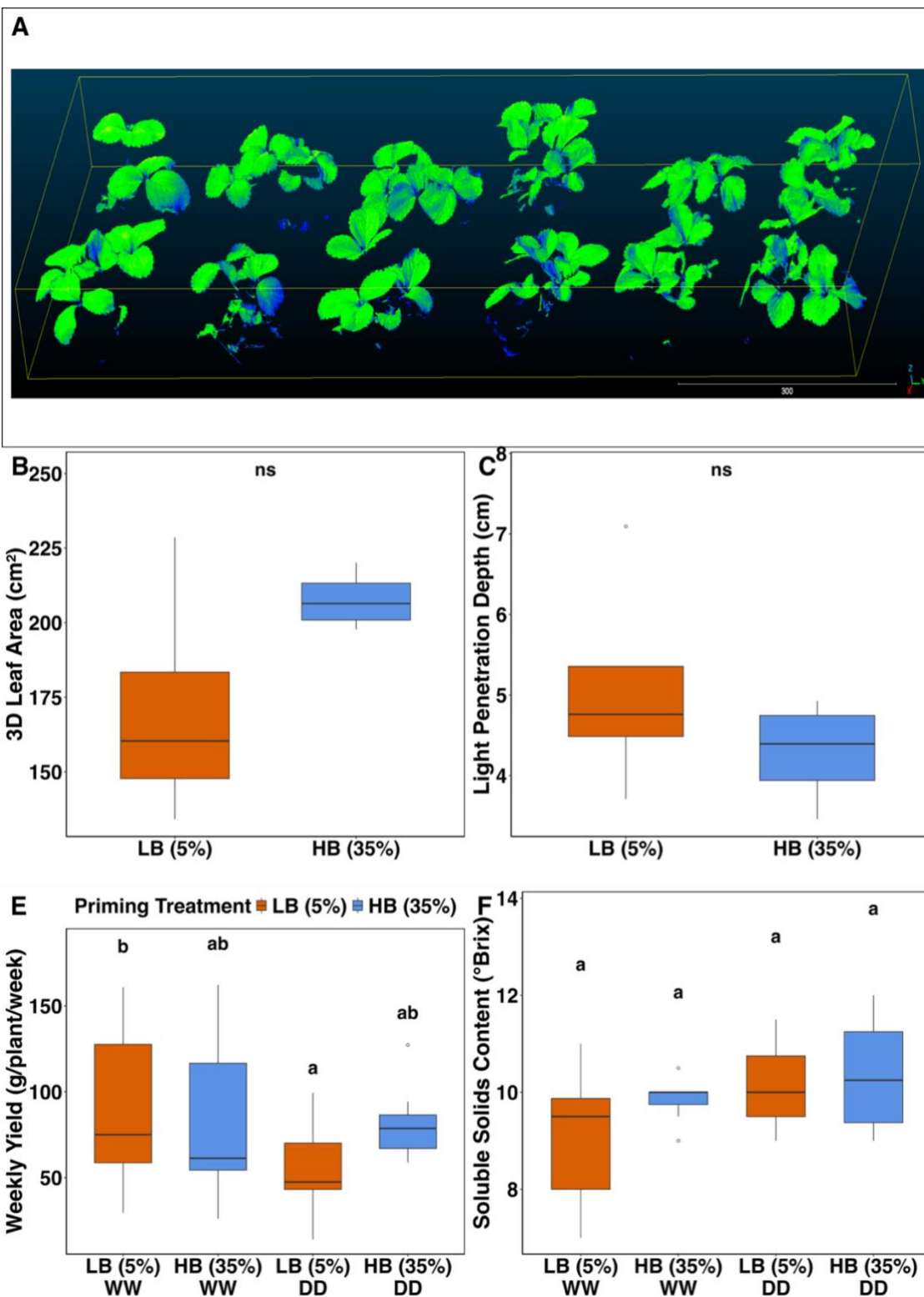


Figure 4.8. Blue light priming effects on plant architecture and harvest performance in strawberry plants. (A) Multispectral 3D scanning reconstruction (B) Three dimensional leaf area measured at 30 DAT using multispectral scanning system comparing low blue (LB, 5%, orange) and high blue (HB, 35%, blue) priming treatments. (C) Canopy light penetration depth at 30 DAT, indicating architectural modifications in canopy density and light interception characteristics. (E) Weekly fruit yield performance under factorial treatment combinations of light priming and irrigation regimes (WW = well-watered, DD = drought) on differential drought tolerance between priming treatments. (F) Soluble solids content (°Brix) as an indicator of fruit quality across treatment combinations. Data represent individual plant measurements with boxes showing median, quartiles, and whiskers extending to 1.5× interquartile range. For exploratory small sample analyses (n = 4–9 per combination) we report point estimates with two sided p-values and bootstrap 95% CIs to avoid binary “significant/non-significant” statements.

The translation of these morphological modifications to productivity showed distinct interactions between priming treatment and water availability (Figure 4.8E). Under well-watered conditions (WW), LB plants achieved higher weekly fruit yields than HB plants (91.1 ± 18.6 vs. 82.9 ± 14.8 g plant⁻¹ week⁻¹, respectively), indicating no clear advantage of HB when water is ample. However, water limitation exposed divergent treatment responses. While LB plants suffered a substantial 40.7% yield reduction under drought

stress (from 91.1 to 54.0 ± 9.3 g plant⁻¹ week⁻¹), HB plants maintained productivity with only a 1.4% yield decline (from 82.9 to 81.7 ± 8.8 g plant⁻¹ week⁻¹). This differential drought response resulted in HB plants achieving 51.3% higher yields than LB plants under water limited conditions (81.7 vs. 54.0 g plant⁻¹ week⁻¹), corresponding to a mean difference of $+27.7$ g plant⁻¹ week⁻¹ (HB–LB in drought; $p = 0.255$ at the whole-model level). Fruit quality assessment through soluble solids content showed consistent similar patterns across treatments and irrigation regimes (Figure 4.8F). Both treatments exhibited slight increases in sugar concentration under drought conditions, with values ranging from 9.1–9.9 °Brix under well-watered conditions to 10.2–10.4 °Brix under drought stress, with no clear treatment effect detected ($p = 0.351$). This suggests that blue light priming effects on drought tolerance operated primarily through yield maintenance rather than quality enhancement.

4.4 Discussion

4.4.1 High Percentage Blue Light Triggers Reprogramming of Photosynthetic Traits Through Distinct Signalling Cascades

Our multiyear experiments show that blue light priming induces multi-levels adaptations in strawberry across temporal and organizational scales. We showed in the experimental phase 1 that the nonlinear photosynthetic response with quantum yield plateauing at approximately 25% blue light while light saturated assimilation continuing through 45% blue (Figure 4.1), which aligns with the two-phase response model in *Arabidopsis* (Walters & Horton, 1995a; Walters & Horton, 1995b). These authors demonstrated that cryptochrome mediated signals control photosystem stoichiometry through distinct low fluence and high fluence responses and with the transition occurring at similar blue light fractions to our observed inflection point. The subsequent work by the same author revealed that these adjustments involve tetrapyrrole mediated retrograde signals from chloroplasts hence provided a mechanistic basis for the preferential enhancement of electron transport we observed (Walters *et al.*, 1999; Walters, 2005). Despite species specific differences in the magnitude of response, this photosynthetic remodelling pattern still shows consistency across many different species. Cucumber for example, exhibits optimal quantum efficiency at 7% blue with continued A_{max} increases up to 30% (Hogewoning *et al.*, 2010), while spinach shows peak performance at 24% blue (Matsuda *et al.*, 2007), and lettuce demonstrates maximum biomass at 20-30% blue (Cope *et al.*, 2014). Our observed strawberry's response (α peak at 25%, A_{max} continuing to 45%) positions it intermediate among these crops, likely reflecting differences in leaf optical properties and blue light attenuation through thicker palisade layers. The preferential

enhancement of J_{max} (+35.4%) over V_{cmax} (+15.9%) under 35% blue light might suggest targeted upregulation of photosynthetic electron transport components. Blue light specifically enhance electron transport capacity by elevating electron flow from Q_A to PSI and promoting photosystem activities (Li *et al.*, 2020), likely through increased expression of cytochrome b6f complexes and plastocyanin rather than proportional increases in all photosynthetic machinery (Mei, 2016; Liu & van Iersel, 2021; Jin *et al.*, 2024). It is also plausible that the greater electron transport capacity reflects an up regulation of cyclic electron flow (CEF) around PSI. Blue light has been shown to stimulate PGR5-dependent CEF in dicots such as *Arabidopsis* (Degen *et al.*, 2023; Degen & Johnson, 2024), and this pathway is indispensable for the rapid electron transport boost that rice exhibits under fluctuating high light (Yamori, 2016; Yamori & Shikanai, 2016; Yamori *et al.*, 2020).

4.4.2 Environmental Induction of Amphistomaty via High Percentage Blue Light priming

The appearance of adaxial stomata (4.3 ± 1.0 stomata mm^{-2}) exclusively in 35% blue light grown plants represents the first documented case of environmentally induced amphistomaty in strawberry. With an abaxial:adaxial ratio of 44:1, these upper surface stomata likely contribute less than 2.3% of total leaf conductance, which is below the 10% threshold Xiong and Flexas (2020) suggest is necessary for meaningful gas exchange enhancement. Further definitive functional assessment could consider the dual leaf chamber system (Wang *et al.*, 2008; Wall *et al.*, 2024) or selective surface occlusion techniques where petroleum jelly application to individual surfaces allows partitioning of adaxial versus abaxial gas exchange contributions (Carpenter *et al.*, 2007; Kane *et al.*,

2020; Zhang *et al.*, 2020). Nevertheless, the evolutionary context of induced amphistomaty requires consideration of environmental trade-offs. Recent modelling by Muir (2020) demonstrates that amphistomaty's benefits depend on vapor pressure deficit, for example, it is advantageous under high light with moderate VPD but potentially detrimental in humid greenhouse conditions where excessive conductance provides no photosynthetic benefit. It is also worth noting that our induced amphistomaty lacked the accompanying increase in vein density measurement typically seen in naturally amphistomatous species (Murphy *et al.*, 2014) which could potentially be limiting hydraulic support for enhanced gas exchange. At the molecular level, blue light responsive FaHY5 transcripts have been identified in strawberry leaves (Ye *et al.*, 2021; Liu *et al.*, 2023), which could support light mediated stomatal development hypothesis such as HY5-STOMAGEN signalling. Future work should consider performing qPCR to track FaEPFL9 (STOMAGEN orthologue) and FaSPCH expression dynamics during high percentage blue light exposure using low percentage blue light as control. The developmental significance of induced amphistomaty extends beyond immediate gas exchange contributions, as while direct conductance contribution remains minimal, these adaxial stomata may provide adaptive value during episodic stress events when rapid gas exchange modulation becomes critical.

4.4.3 Temporal Decoupling suggesting Multiple Memory Systems

The divergent persistence of priming effects reveals distinct memory systems operating through different mechanisms. Biochemical parameters (V_{max} , J_{max}) normalized within 30 days, consistent with typical protein turnover rates. However, this normalization may involve post-translational regulation beyond simple degradation. Modulation of PGR5/PGRL1-dependent cyclic electron flow can rapidly adjust apparent J_{max} independent of protein abundance (Suorsa *et al.*, 2016), though direct measurement of CEF in our system remains necessary. Additionally, retrograde signals from chloroplasts likely repress nuclear LHC gene expression once blue light fraction decreases which can further accelerating reversion to baseline capacity. On the other hand, structural features established during development remain relatively fixed explaining why *SD* remained 30-53% elevated even two months post-priming. The persistence of stress tolerance despite biochemical normalization suggests operation of an epigenetic or sustained redox memory system. While we lack direct chromatin immunoprecipitation data, the pattern still aligns with heat stress memory in *Arabidopsis* where H3K4me3 marks at loci like *HSFA2* and *APX2* enable faster stress response reactivation (Lämke *et al.*, 2016). Whether these marks are present in *Fragaria* remains untested. Future ChIP-seq analysis targeting these and other memory-associated loci (e.g., *ZAT10*, *ZAT12*) would test whether similar chromatin modifications underlie the observed high percentage blue light induced stress memory.

The blue light exposure also extends beyond simple pigment accumulation to fundamental alterations in leaf optical properties. The most spectral differences at 30 DAT

were in the near-UV (380-400 nm) and blue (400-500 nm) regions, where 35% blue light treated plants demonstrated significantly higher absorbance values (mean p-values 0.0295 and 0.0403, respectively). This enhanced absorption in high-energy wavelengths and coupled with the spectral absorbance shift from 432-433 nm peaks at 1 DAT to 661-663 nm peaks at 30 DAT, suggesting a significant reorganization of light harvesting architecture that fundamentally alters photon capture and energy distribution within the leaf.

This optical remodelling across multiple spectral regions has distinct functional implications. The 70.8% increased absorbance in the blue region suggests accumulation of UV-screening compounds beyond chlorophylls, likely including flavonoids and hydroxycinnamic acid derivatives that provide photoprotective filtering while potentially serving as antioxidants (Burchard *et al.*, 2000; Bilger *et al.*, 2001; Llansola-Portoles *et al.*, 2017). The exceptional 254% increase in green region absorption cannot be explained by chlorophyll alone and could well implicating for example, carotenoid protein interactions and potentially altered chloroplast ultrastructure affecting internal light scattering patterns (Moriwaki *et al.*, 2023). These modifications create a more optically dense leaf that captures photons more efficiently across the visible spectrum while simultaneously enhancing photoprotective capacity (Llorente *et al.*, 2020; Piccinini *et al.*, 2022). The 86.6% increase in chlorophyll b concentration drives specific optical consequences that extend beyond simple pigment accumulation. Chlorophyll b absorption peaks at 453 and 642 nm complement chlorophyll a peak at 430 and 662 nm, effectively broadening the photosynthetic action spectrum and enhancing light capture efficiency particularly in the blue-green regions where canopy light is often enriched

(Delegido *et al.*, 2011; Gao *et al.*, 2025). The reduced chlorophyll a/b ratio (−27.3%) indicates preferential assembly of peripheral LHCII complexes over core complexes which could create an optical antenna specifically optimized for blue-enriched environments. This targeted LHCII proliferation serves dual purposes, first by balancing excitation distribution between photosystems as demonstrated by Walters (2005), while simultaneously enhancing photoprotective capacity through increased binding sites for xanthophyll cycle pigments. Additionally, the 81.4% carotenoid increase provides critical photoprotective infrastructure with multiple optical functions, as these pigments absorb strongly in the 400-500 nm range (Solovchenko & Neverov, 2017). This can provide the first line defence against excess blue photons while their physical proximity to chlorophylls in light harvesting complexes enables efficient quenching of potentially damaging chlorophyll triplet states (Wang *et al.*, 2019; Marcolin *et al.*, 2024; Chibani *et al.*, 2025a). The expanded carotenoid pool, particularly violaxanthin and zeaxanthin, modulates energy dissipation through conformational changes in LHCII that alter excitation energy transfer pathways (Son *et al.*, 2019). This creates a dynamic photoprotective system capable of responding to rapid light fluctuations, which is a critical adaptation need for primed strawberry plants that will eventually face variable field or protected growth conditions under natural sunlight.

This coordinated pigment and optical changes create a light-harvesting system primed for environmental variability. Enhanced absorption across all wavelengths can increase photon capture efficiency under limiting conditions whilst the expanded photoprotective apparatus enables safe dissipation of excess energy under high irradiance. The 68.6% increase in red region absorption aligns with expanded LHCII complexes where

chlorophyll b enrichment specifically enhances absorption in the 640-660 nm range and could potentially improving photosynthetic efficiency under canopy shade where red light is depleted. Furthermore, the 54.8% increase in near infrared edge absorption suggests altered leaf internal structure affecting photon recycling and potentially enhanced light penetration to lower cell layers and this could create a more efficient three-dimensional light capture system (Knapp *et al.*, 1998; Slaton *et al.*, 2001; Egbert *et al.*, 2007; Karabourniotis *et al.*, 2021).

There has also been shown as evidence for ROS-mediated priming comes from parallel systems where blue light pre-treatment induced antioxidant enzyme expression. In barley for example, blue light exposure elevated ascorbate peroxidase and glutathione S-transferase levels and therefore inducing enhanced tolerance to subsequent photooxidative stress (Karpinski, 2013). The optical changes we observed, particularly the enhanced UV-blue absorption might had generated localized ROS production in chloroplasts and potentially triggering retrograde signalling cascades that establish antioxidant memory. Although we did not quantify ROS production or antioxidant enzyme activities in strawberry due to delayed lab safety paperwork, this mechanism can be tested by measuring APX activity, glutathione pools, and expression of singlet oxygen sensor genes in future work.

Lastly the metabolic consequences of these optical modifications extend beyond direct photoprotection. Enhanced photosynthesis under blue light would elevate leaf sucrose levels, potentially priming drought responses through trehalose-6-phosphate signalling that modulates ABA sensitivity and stomatal behaviour (Figueroa & Lunn, 2016). The

optical modifications could be ensuring efficient light capture under blue light enriched conditions would sustain this metabolic priming even as plants experience the fluctuating spectral conditions of natural sunlight, thus providing a biochemical bridge between the priming environment and subsequent stress challenges throughout the growth season.

4.4.4 Whole Plant architecture and yield dynamics under variable water conditions

Our whole plant level 3D quantification further demonstrated the potential of architectural modifications induced by high blue light priming (22% larger 3D leaf area with reduced light penetration), which created denser canopies that might impact yield differentially across water regimes. As shown in under well-watered conditions at 90 DAT, the 9.0% yield reduction in HB plants ($F = 1.43$, $p = 0.255$, $n = 4-9$ per treatment) suggests a trend warranting investigation in larger trials in commercial setup. The effect size (Cohen's $d = 0.52$, 95% CI: -0.41 to 1.45) indicates moderate practical importance despite wide confidence intervals. In contrast under drought treatment, the 51.3% yield advantage of HB plants represents a substantial effect ($d = 2.13$, 95% CI: 0.89 to 3.37). The mechanism of yield protection could integrate responses across scales. For example, higher *SD* with smaller apertures enables precise regulation on maintaining gas exchange when water is available while responding rapidly to hydraulic signals. Secondly, the compact architecture may reduce transpirational demand through mutual shading rather than boundary layer effects, as forced ventilation in polytunnels typically minimizes boundary layer resistance regardless of plant height (Katsoulas & Stanghellini, 2019). While our experimental setup with irrigation infrastructure closely mimicking UK commercial practice, the scale limitations and horizontal ventilation through side vents may not fully capture

microenvironmental complexities of large scale polytunnel systems where greater air movement would minimize boundary layer effects which could suggest water savings derive primarily from stomatal and biochemical adaptations rather than aerodynamic factors. From a climate engineering perspective, this trade-off between slight yield reduction under optimal conditions and substantial protection under stress represents valuable risk management for increasingly variable growing seasons, particularly when considering that enhanced drought tolerance could enable more aggressive deficit irrigation strategies that maintain 90-95% yield while increasing fruit quality parameters that maybe of high value consumer's interest. One potential proposed strategy with a four-week blue light priming protocol could offers a practical strategy for sustainable intensification, where 20-30% irrigation reduction without yield penalty would deliver substantial water conservation across global protected cultivation systems. Our light primed induced stress tolerance provides buffering capacity against various stress combinations inherent in climate change scenarios, though critical uncertainties regarding scalability and economic optimization remain. Therefore, necessitating systematic commercial scale trials across multiple production regions to determine minimum effective priming duration as well as potential for maintenance through periodic booster exposures (reinforcement priming) and cultivar specific responses that would enable transition from promising research finding to practical climate adaptation tool in commercial strawberry production systems.

Furthermore, our findings support transitioning from static to dynamic spectral management in strawberry production. Based on UK electricity prices (£0.106 kWh⁻¹, approximately \$0.134 kWh⁻¹), transitioning from 5% to 30% blue during the priming phase

increases costs modestly. For a 1000 m² greenhouse operating 16-hour photoperiods at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, the 8.5% increase in electricity consumption (assuming 2.7 $\mu\text{mol J}^{-1}$ for blue vs. 3.0 $\mu\text{mol J}^{-1}$ for red LEDs) represents an additional 340 kWh day⁻¹. Over a 4-week priming period, this adds £1,009 in electricity costs. Preventing a 10% yield loss during one drought event in a 50 ton ha⁻¹ crop valued at £2,400 ton⁻¹ would save £12,000 ha⁻¹, generating a return exceeding 11:1. Further work should focus on advanced control algorithms to enable environmental responsiveness. For example, blue pulse algorithms that adjust spectral output based on outside daily light integral, reducing supplemental blue when natural light provides adequate short wavelengths. Such systems could maintain priming benefits while minimizing energy expenditure. In parallel, one should seek for the integration with weather forecasting that could trigger prophylactic blue light pulses before anticipated stress events and thereby reinforcing memory responses when most needed.

Another avenue for future work should explore stress cross tolerance as inspired by the drought protection induced by blue light priming, whereby acclimation to one environmental cue fortifies plants against subsequent, distinct stresses (Hilker *et al.*, 2016; Hilker & Schmölling, 2019). For example, recent molecular studies have provided the mechanistic basis for such cross protection with the blue light photoreceptor CRYPTOCHROME 1 (CRY1) physically interacts with the master heat shock transcription factor HSFA1d in Arabidopsis, and thereby facilitating its nuclear translocation and accelerating heat shock protein accumulation, which subsequently enhanced thermotolerance at lethal temperatures (42°C) without prior heat exposure (Gao *et al.*, 2023; Degen & Johnson, 2024). This CRY1-HSFA1 regulatory module provides a

molecular explanation for how photomorphogenic signals can prime seemingly unrelated stress responses. While this specific interaction remains uncharacterized in strawberry, the conservation of both CRY1 and HSFA1 orthologs across eudicots suggests a plausible conserved mechanism underlying the multi stress resilience we observed, wherein blue light exposure during early development establishes a molecular framework for enhanced stress responsiveness that persists despite subsequent biochemical normalization.

Beyond immediate molecular priming, our findings also raise the possibility of transgenerational stress memory transmitted through strawberry's clonal propagation system which has been widely practiced in the UK. Recent work in potato demonstrated that mild drought stress imposed on mother plants induced specific DNA methylation changes in tubers that altered drought responsiveness in subsequent clonal generations, with effects varying predictably by cultivar genotype (Macko-Podgórní *et al.*, 2025). If analogous epigenetic mechanisms operate in strawberry runner formation, blue light priming of stock plants could generate nursery transplants carrying inheritable stress resilience without requiring repeated spectral treatments. Hence might effectively converting a temporary environmental signal into a stable agronomic trait. However, distinguishing genuine transgenerational epigenetic inheritance from simple metabolite or protein carry over through runner connections demands rigorous experimental design with parallel physiological assessments, whole genome sequencing across multiple clonal generations and reciprocal grafting experiments to separate systemic signals from cell autonomous epigenetic marks. Future studies should investigate molecular

mechanisms underlying stress memory, including potential epigenetic modifications and their transgenerational stability through clonal propagation.

Finally, there are several factors merit consideration in optimizing priming protocols used in this study. For example, circadian gating of light responses means morning versus evening blue light exposure could yield different outcomes due to temporal variation in HY5 stability and PIF activity (Nozue *et al.*, 2018). We applied blue light throughout the photoperiod, potentially missing opportunities for enhanced efficacy through targeted timing. Genotype against environment interactions likely also modulate priming responsiveness substantially. Factorial experiments testing multiple cultivars (June or everbearers) across VPD regimes would reveal whether certain genetic backgrounds show enhanced plasticity for induced amphistomaty or stress memory. Future follow up works should consider using better high throughput technologies such as hyperspectral imaging for real time stress detection alongside traditional physiological quantification (i.e., Gas Exchange) and machine learning for predictive spectral control and traits prediction at larger commercial scale. This should in theory enables personalize priming protocols to any specific cultivar environment combinations.

Chapter 5: Improving Diurnal Carbon Gain with Pulsed Blue Light Illumination to Abaxial Surface: high throughput gas exchange insights

Keywords: Photosynthesis, Blue Light, Abaxial Surface, Adaxial Surface, Stomatal Conductance, Spectral Reflectance, Diurnal Effect, carbon assimilation, Water Use Efficiency, Quantum yield optimization.

Running Title: Abaxial Blue Pulses Boost Carbon Gain

Abstract (Chapter 5)

Leaves present two anatomically and optically distinct surfaces, the adaxial (upper) and abaxial (lower), which can be illuminated independently, yet commercial lighting typically targets only the adaxial side. Optimizing photosynthesis in controlled growth environments remains constrained by conventional top down illumination strategies that typically miss the photosynthetic potential of abaxial leaf surfaces. Here we first present a data driven approach combining high throughput randomized light response phenotyping with machine learning to decode hierarchical photosynthetic regulation in strawberry (*Fragaria × ananassa*). Analysis of >2,000 unique light environment combinations revealed stomatal conductance as the dominant predictor of CO₂ quantum yield (68.4% feature importance), with blue light effectiveness exhibiting strong temporal dependencies. Surface specific measurements demonstrated that abaxial illumination maintains 77-79% of adaxial photosynthetic capacity despite receiving minimal attention in typical commercial lighting design. These computational insights guided development of a targeted intervention: a novel lighting system with periodic 10-minute blue light pulses (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) delivered to the stomata rich abaxial surface while maintaining constant total photon flux. This strategy enhanced diurnal carbon gain by 8% through sustained elevation of stomatal conductance (334% increase in conductance dynamics), with effects strengthening throughout the photoperiod (11.5% enhancement in final phase). Our findings establish a new paradigm for precision light management by delivering specific wavelengths to targeted leaf surfaces at optimal time intervals and thus demonstrate how big data driven computational discovery can drive practical innovations for improving resource use efficiency in future growth systems.

5.1 Introduction

Photosynthesis is the process in which plants convert solar radiation into chemical energy and powers nearly all terrestrial food systems, yet optimizing this fundamental process remains one of the most pressing challenges in the world with growing food demand. In vertical farming, the fastest growing sector of controlled environment agriculture with new startups emerging worldwide, electricity typically accounts for 25-30% of operational costs, with labour and energy totalling 60% of running expenses (Sheibani *et al.*, 2023). Despite improvements in LED efficiency reaching almost 90% over the last decade, the paradox persists as even the most efficient artificial lighting systems struggle to compete economically. This economic reality demands innovative approaches that maximize photosynthetic efficiency per unit of electrical energy invested.

The architecture and physiology of leaves have evolved to capture light coming from above, with the adaxial (upper) surface receiving the greatest irradiance under natural conditions (Falster & Westoby, 2003; Ruban, 2015; Truong *et al.*, 2015). However, a significant portion of highly efficient photon emissions from improved LEDs is wasted by natural beam spread beyond cropping areas in indoor farms, necessitating strategies that enhance crop canopy photon capture efficiency. While commercial practices have traditionally focused on optimizing top down illumination, emerging evidence suggests that the abaxial (lower) leaf surface plays an underappreciated yet potentially exploitable role in photosynthetic performance (Shimazaki, K-i *et al.*, 2007; Soares *et al.*, 2007; Paradiso & Marcelis, 2012; Paradiso *et al.*, 2020; Zhang, N *et al.*, 2021; Wall, Shellie *et al.*, 2022). Critical to understanding abaxial photosynthetic potential is the spectral

gradient that develops as light traverses leaf tissues. Blue and red light are absorbed more strongly by photosynthetic pigments than green light, predominantly by the top few cell layers, while green light can penetrate deeper into leaf tissues (Vogelmann & Evans, 2002; Liu & van Iersel, 2021). Blue photons, containing surplus energy not used for photosynthesis, are effectively absorbed by carotenoids in the shortwave PAR region (<520 nm), with this energy dissipated as heat (Kume, 2017). Consequently, most incident red and blue light is absorbed by chloroplasts in the adaxial mesophyll, with very little transmitted to abaxial cells (Evans, 1999; Pfündel *et al.*, 2018). This creates a unique light environment at the abaxial surface enriched in green and far-red wavelengths—precisely those wavelengths that guard cells can still perceive but which contribute less efficiently to photosynthetic electron transport.

Many plants including the strawberry (*Fragaria × ananassa*) are known as hypostomatous plants, with stomata are almost exclusively located on the abaxial epidermis (Jalakas *et al.*, 2024). These tiny but crucial pores regulate the flux of CO₂ into the leaf and water vapour out to the atmosphere by controlling aperture through changes in guard cell turgor in response to diverse signals, including light, CO₂, and vapor pressure deficit (VPD) (Lawson, 2009; Lawson, T & Blatt, MR, 2014). While red light has traditionally been emphasized for its high quantum efficiency in driving the photosynthetic electron transport chain, the regulatory effect of blue light on guard cells can profoundly modulate stomatal conductance and overall gas exchange performance (Kinoshita *et al.*, 2001; Wang *et al.*, 2014; Inoue, S-i & Kinoshita, T, 2017; Fuji *et al.*, 2024). The strong absorption of blue light by chlorophyll creates a large light gradient from top to bottom of leaves, resulting in upregulation of nonphotochemical quenching near the adaxial side while chloroplasts

near the bottom receive little blue excitation energy (Vogelmann & Evans, 2002; Liu & van Iersel, 2021). Consequently, abaxial illumination has the potential to bypass the filtering effect of upper tissues and directly stimulate guard cells.

The potential benefits of abaxial illumination extend beyond stomatal placement and spatial considerations. Importantly, optical properties also differ between adaxial and abaxial surfaces, with some species showing higher transmittance or distinct absorbance spectra on the lower side (Hughes & Smith, 2007; Falcioni *et al.*, 2024). These differences, combined with the predominance of abaxial stomata, suggest that wavelength specific delivery to the lower surface could optimise stomatal aperture independently of photosynthetic light requirements. Despite these theoretical advantages, empirical data under realistic diurnal conditions remain sparse, particularly regarding midday stomatal closure and dynamic light responses (Tenhunen *et al.*, 1984; Kamakura *et al.*, 2011). Conventional approaches using discrete light intensities or static light response curves, while valuable, miss important time dependent feedbacks and non-equilibrium states that characterize natural photosynthetic regulation.

We propose addressing these limitations through high throughput, randomised experimentation, which is a paradigm shift from traditional factorial design in growth environment optimization experiments. Rather than testing predetermined combinations of intensity and spectrum, programmable gas exchange instruments can vary light parameters on-the-fly and thereby automatically gathering large datasets that capture the full of environmental possibility space. By pairing randomised measurements with machine learning based analyses, we can elucidate the nuanced relationships among

photosynthesis, water use efficiency, spectral composition, and diurnal patterning that traditional methodologies cannot resolve at scale.

The present study used a systematic, data driven approach to elucidate surface specific photosynthetic responses in strawberry leaves. We first characterized the fundamental physiological and optical differences between adaxial and abaxial illumination through dynamic light response measurements, then implemented high throughput randomized light gas exchange protocols coupled with machine learning analyses to identify hierarchical control mechanisms governing photosynthetic efficiency across >2,000 unique environmental conditions. These computational insights, combined with spectral reflectance measurements revealed distinct optical properties between leaf surfaces and led to three critical findings: stomatal conductance emerged as the dominant predictor of quantum yield, blue light effectiveness exhibited strong temporal dependencies, and the illuminating on the abaxial surface maintained substantial photosynthetic capacity of conventional adaxial side illumination. This convergence of computational, optical, and physiological evidence led us to hypothesize that strategically timed blue light pulses delivered directly to the stomata rich abaxial surface could overcome the typical midday decline in photosynthesis by maintaining elevated stomatal conductance without proportional increases in energy input. To test this targeted intervention strategy, we designed a pulsed blue light system and conducted whole leaf gas exchange and have found that the surface specific spectral delivery could enhance diurnal carbon gain.

5.2 Material and Method

Plant Material and Growth Conditions

English commercially widely grown strawberry plants (*Fragaria × ananassa*, cv. Malling Champion) were used in this study. The plants were cultivated in outdoor conditions in 6L pots filled with peat compost soil, located in Colchester, UK from May 1, 2024, and continued until August 31, 2024. Prior and throughout to the commencement of measurements, soil pH was maintained at approximately 5.8, and electrical conductivity (EC) was kept around 2.2 by measuring the drained water on a weekly basis. To ensure consistency throughout the study, fully expanded mature leaves were selected and tagged with unique identification numbers.

Dynamic Photosynthetic Response to Illumination on different leaf surfaces

Photosynthetic responses to light were measured using eight LI-6800 infrared gas exchange systems (LI-COR Biosciences, Lincoln, NE, USA) with Li6800-01A fluorometer. To evaluate the effects of light intensity and light quality on both the adaxial and abaxial leaf surfaces. Plants were randomly divided into two groups. Measurements for Group A were conducted on the adaxial surface, while Group B's measurements were conducted on the abaxial surface. Initially, leaves were positioned in the 6 cm² leaf chamber of the LI-6800 gas exchange system. Baseline measurements were taken under low light conditions set at 50 PPFD (photosynthetic photon flux density) using 100% red light for a duration of 15 minutes. Following this, the light intensity was increased to 500 PPFD with 100% red light and maintained for 45 minutes. Subsequently, the light composition was

altered to 90% red and 10% blue at 500 PPFD, and measurements were taken for an additional 30 minutes. After completing these measurements, the plants were adapted to 50 PPFD for 2 hours before flipping the leaves and repeating the measurements on the opposite surfaces. Photosynthesis rates and stomatal conductance were recorded at each stage of light intensity and composition.

Randomized Diurnal Light Response

Gas exchange measurements were performed using eight LI-6800 Portable Photosynthesis Systems (LI-COR Biosciences, Lincoln, NE, USA). To ensure unbiased and randomized light conditions, the LI-6800 machines were programmed using a Python-based control program that randomized light intensity and spectral composition for each measurement event. The photosynthetic photon flux density (PPFD) was set to vary randomly between 200 and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$, while the fractions of red and blue light were randomized within a range of 0 to 100%. Due to the limitations of the LED light source in the LI-6800 fluorometer, the far-red light fraction was constrained to values between 0 and 5%. The Python script used the `random.randint()` function to generate unique parameter combinations for measurement points throughout the day.

Measurements were only logged after stability was achieved based on pre-defined criteria. Stability was determined by continuously monitoring the slope and standard deviation of the changes in CO_2 concentration (ΔCO_2) and H_2O concentration ($\Delta\text{H}_2\text{O}$). Stability was defined as $|\Delta\text{CO}_2| < 0.5 \mu\text{mol mol}^{-1} \text{min}^{-1}$ and

$|\Delta H_2O| < 0.5 \text{ mmol mol}^{-1} \text{ min}^{-1}$, with standard deviations of $< 0.1 \text{ } \mu\text{mol mol}^{-1}$ and $< 0.5 \text{ mmol mol}^{-1}$, respectively. Each machine waited a minimum of 30 seconds and a maximum of 300 seconds to achieve stability for any given parameter set. If stability was not achieved within this time window, the machine proceeded to the next randomized parameter set without recording a log point to thereby minimizing potential artifacts from unstable conditions. After logging a measurement, the program adjusted the chamber conditions to a new set of randomized light parameters for the next step. Environmental conditions were maintained consistently throughout the experiment, with leaf temperature set to 23°C, a reference CO₂ concentration of 400 $\mu\text{mol mol}^{-1}$, and a leaf-to-air vapour pressure deficit (VPD) of 1.2 kPa.

Diurnal Photosynthetic Measurements with Blue Light Pulses to the abaxial surface

To measure diurnal photosynthetic rates, a customized dual-chamber system connected to two LI-6400XT gas analysers (LI-COR Biosciences, Lincoln, NE, USA) was utilized for simultaneous measurement of both leaf surfaces. Plants were randomly assigned to either a red-light control treatment at 500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD or a treatment with periodic blue light pulses (100 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD) applied to the abaxial leaf surface. In the blue light pulse treatment, the adaxial surface was illuminated with red light at 500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD. Every 10 minutes, blue light at 100 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD was pulsed to the abaxial surface for a duration of 10 minutes. During the blue light pulse, the illumination on the adaxial surface was reduced by 100 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD to maintain a constant

total PPFD at the leaf surface. The red light only treatment served as a control, with treatments applied in random order (red first then blue, or vice versa). Measurements were conducted continuously over a 10-hour period, with photosynthetic rates and stomatal conductance data recorded every 10 seconds.

Spectral Reflectance Measurement

Spectral reflectance measurements were conducted using the PolyPen RP 410 instrument (Photon Systems Instruments, Drásov, Czech Republic), which measured reflectance across wavelengths from 380 to 1,050 nm. These measurements were performed on both the adaxial and abaxial surfaces of the leaves previously used for gas exchange measurements. The collected reflectance spectra were analysed to determine leaf optical properties, which were subsequently modelled against gas exchange-derived parameters to evaluate relationships between leaf optical properties and photosynthetic performance.

Feature Engineering and Predictive modelling

Raw data were processed to calculate additional spectral ratios and derived features. Red/blue (r/b), red/far-red (r/fr), and blue/far-red (b/fr) light ratios were calculated from the raw light intensity data (Q_{red} , Q_{blue} , $Q_{far-red}$). Extreme outliers were capped at the 95th percentile of each variable's distribution to minimize the influence of anomalies. Additional interaction and polynomial features were generated, including the product of blue light intensity and time of day ($Q_{blue} \times t$), squared blue light intensity (Q_{blue}^2), and squared time of day (t^2). A random forest regression model was trained to predict the quantum yield of CO_2 assimilation (Φ) as a function of gas exchange parameters and light spectral features. The model formula included gas exchange variables (g_{sw}), light intensities (Q_{red} , Q_{blue} ,

$Q_{\text{far-red}}$), spectral ratios (r/b , r/fr , b/fr), and derived features ($Q_{\text{blue}} \times t$, Q_{blue}^2 , t^2). The dataset was split into training (80%) and test sets (20%), and the model was trained with 100 trees. The mean squared error (MSE) and coefficient of determination (R^2) were calculated to evaluate model performance. Feature importance was determined based on the percentage increase in mean squared error (%IncMSE) and to identify key predictors of Φ .

Analysis of Photon Energy and Flux Density

Photon energy ($E\lambda$) for red ($\lambda = 625$ nm), blue ($\lambda = 475$ nm), and far-red ($\lambda = 735$ nm) light was calculated using Planck's equation ($E\lambda = hc/\lambda eV$) where eV is the energy conversion factor (1.602×10^{-19} J/eV). Energy flux density ($EFD\lambda$) for each wavelength was calculated as: $EFD\lambda = Q\lambda \times E\lambda \times 10^{-6}$, where $Q\lambda$ is the photon flux density ($\mu\text{mol m}^{-2} \text{s}^{-1}$).

Statistical Analysis

For carbon gain analysis, net CO_2 assimilation rates were integrated over each phase using numerical integration with values normalized to a per-hour basis to account for the measurement duration. To analyse stomatal conductance (g_{sw}) dynamics, we calculated rates of change over 10-minute intervals to match the experimental blue light pulse frequency. For each interval, the rate was computed as the difference in g_{sw} between the interval's end and start points and then divided by the time elapsed. Extreme outliers were removed using a z-score threshold of ± 3 . These rates were then aggregated by phase with the sum of absolute rate changes serving as a metric for stomatal dynamism. For both carbon gain and g_{sw} dynamics, statistical comparisons between light treatments were performed using paired Wilcoxon signed-rank tests (one-tailed testing for responses

under blue light pulses). Pairing was maintained at the leaf level ($n=5$) to account for individual leaf variation. Effect sizes were quantified as percentage differences between treatments. Statistical significance was set at $\alpha=0.05$. Temporal trends in g_{sw} rates were visualized using generalized additive models (GAM) with automated smoothing using the `mgcv` package (Wood, 2017).

5.3 Results

5.3.1 Surfaces specific photosynthetic induction to red and blue light

Photosynthesis (A) and stomatal conductance (g_{sw}) exhibited distinct responses to light intensity and quality depending on whether the adaxial or abaxial surfaces were illuminated (Figure 5.1). The dynamic responses during light transitions (Figure 5.1A,B) exhibited rapid acclimation kinetics, with both surfaces showing substantial increases in carbon assimilation upon exposure to increased red light intensity (578% and 571% increases for abaxial and adaxial surfaces, respectively, during R100 to R1000 transition) and more modest enhancements following blue light supplementation (6-8% increases during R1000 to R900B100 transition). Steady state conditions (Figure 5.1C,D), demonstrated that adaxial illumination maintained consistently higher photosynthetic rates across all light treatments, though the magnitude of this advantage varied with light conditions. While no significant difference emerged under low red light (R100, $p = 0.737$), adaxial illumination enhanced photosynthesis by 27% under saturating red light (R1000, $p = 0.024$) and by 30% under combined red-blue illumination (R900B100, $p = 0.011$) compared to abaxial side illumination. Nevertheless, abaxial illumination maintained

substantial photosynthetic capacity throughout all treatments, consistently achieving 77-79% of the carbon assimilation rates observed under adaxial illumination.

Stomatal conductance (g_{sw}) patterns revealed contrasting regulatory behaviour between leaf surfaces. Despite consistently higher median g_{sw} values under abaxial illumination across all light phases (52% higher under R100, 11% under R1000, and 16% under R900B100), these differences did not achieve statistical significance (all $p > 0.13$). The addition of blue light (R900B100 phase) elicited proportionally similar stomatal responses from both surfaces (20% and 18% increases for abaxial and adaxial, respectively), indicating conserved blue light signaling pathways despite the differential stomatal densities between leaf surfaces.

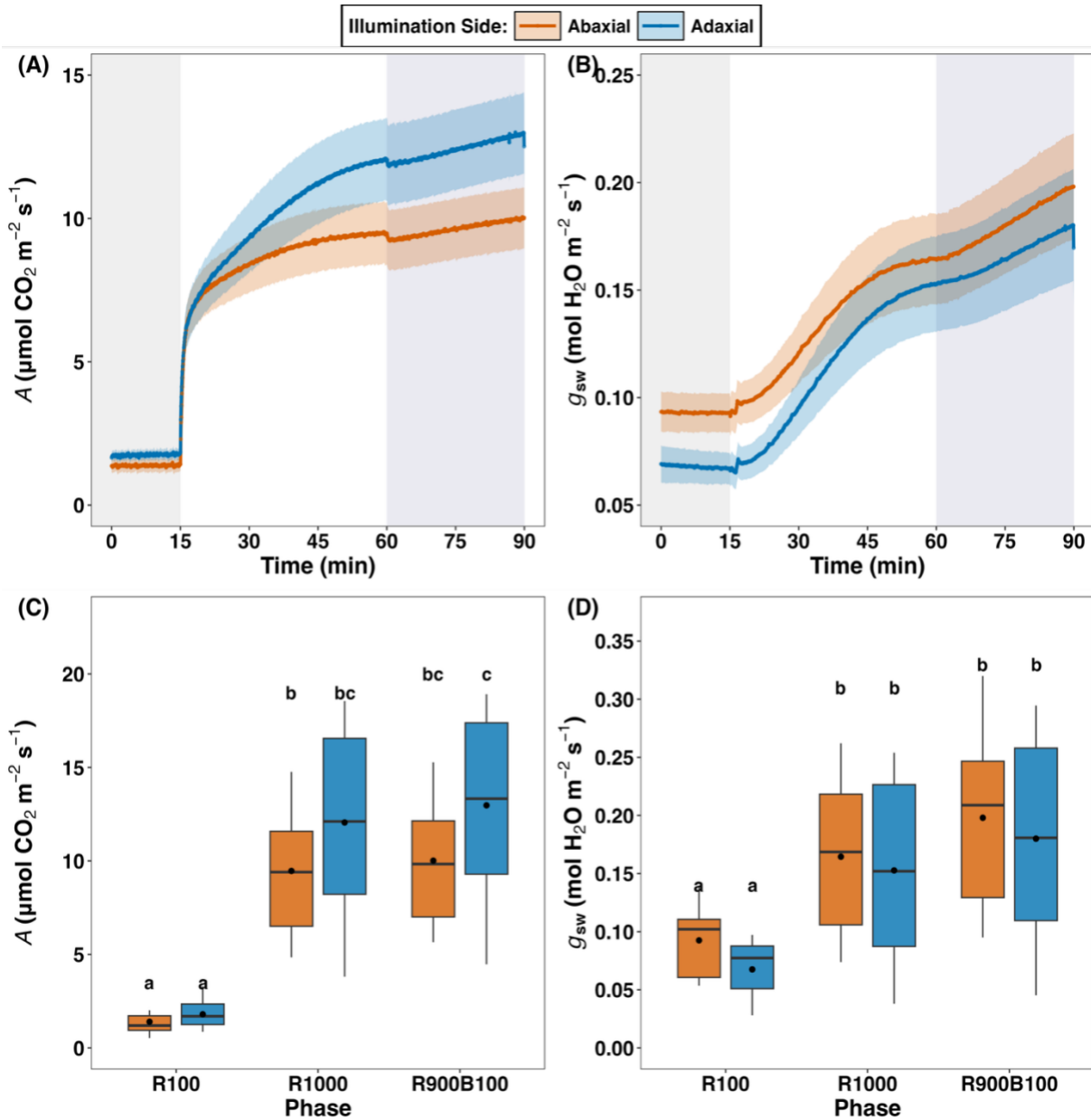


Figure 5.1. Dynamic and steady-state responses of photosynthesis and stomatal conductance to adaxial and abaxial illumination. (A, B) Time-course of net CO₂ assimilation rate (A) and stomatal conductance (g_{sw}) in response to sequential light treatments when illuminating either the adaxial (red) or abaxial (blue) leaf surfaces, with values representing means $\pm$ SE (n = 10). Plants were initially exposed to 100 $\mu\text{mol m}^{-2}$

s^{-1} of red light (R100), followed by transitions to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light (R1000) and then $900 \mu\text{mol m}^{-2} \text{s}^{-1}$ red + $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light (R900B100). (c, d) Steady-state values of A and gsw under each light treatment comparing adaxial (blue bars) and abaxial (orange bars) illumination (n=10). Different letters indicate significant differences ($p < 0.05$) based on post-hoc comparisons.

5.3.2 Differential Effects of Light Quality and Quantity on Photosynthetic Parameters

The relationships between light quality and gas exchange parameters were analysed using both photon flux density (PFD $\mu\text{mol m}^{-2} \text{s}^{-1}$) and energy flux density (EFD, $\text{eV} \cdot \text{mol m}^{-2} \text{s}^{-1}$), where EFD accounts for wavelength-specific photon energies calculated as $E = hc/\lambda$ (Figure 5.2). Blue photons (475 nm) carried 2.61 eV, red photons (625 nm) 1.99 eV, and far-red photons (735 nm) 1.69 eV, resulting in blue photons containing theoretically 31.6% more energy than red photons in our gas exchange light source. Quantum yield of CO_2 assimilation (ΦCO_2) displayed negative correlations with both total PFD ($r = -0.513$, $P < 0.001$) and total EFD ($r = -0.593$, $P < 0.001$) (Figure 5.2A,B). The correlation with EFD was 15.6% stronger than with PFD, indicating differential spectral effects on quantum efficiency. Analysis of wavelength contributions revealed opposing correlations with red light contributions positively correlated with ΦCO_2 (PFD basis: $r = 0.411$; EFD basis: $r = 0.407$), while blue light contributions showed negative correlations of similar magnitude (PFD basis: $r = -0.411$; EFD basis: $r = -0.408$). Stomatal conductance (g_{sw}) exhibited weak negative correlations with both total PFD ($r = -0.087$, $P < 9.3 \times 10^{-5}$) and total EFD ($r = -0.094$, $P < 2.3 \times 10^{-5}$) (Figure 5.2E,F). Wavelength specific contribution correlations remained minimal (all $|r| < 0.04$), with red contributions showing marginal positive correlations ($r = 0.037$) and blue contributions marginal negative correlations ($r = -0.035$). Far-red light, comprising $3.0\% \pm 1.4\%$ of total PFD and $2.3\% \pm 1.1\%$ of total EFD, showed distinct modulatory effects when analysed through spectral ratios. The red/far-red ratio positively correlated with ΦCO_2 ($r = 0.247$, $P < 0.001$) and A ($r = 0.250$, $P < 0.001$), while the blue/far-red ratio displayed negative correlations (ΦCO_2 : $r = -0.244$; A : $r = -0.198$,

both $P < 0.001$). Direct correlations between far-red intensity and physiological parameters were negative for ΦCO_2 ($r = -0.235$) but positive for A ($r = 0.164$).

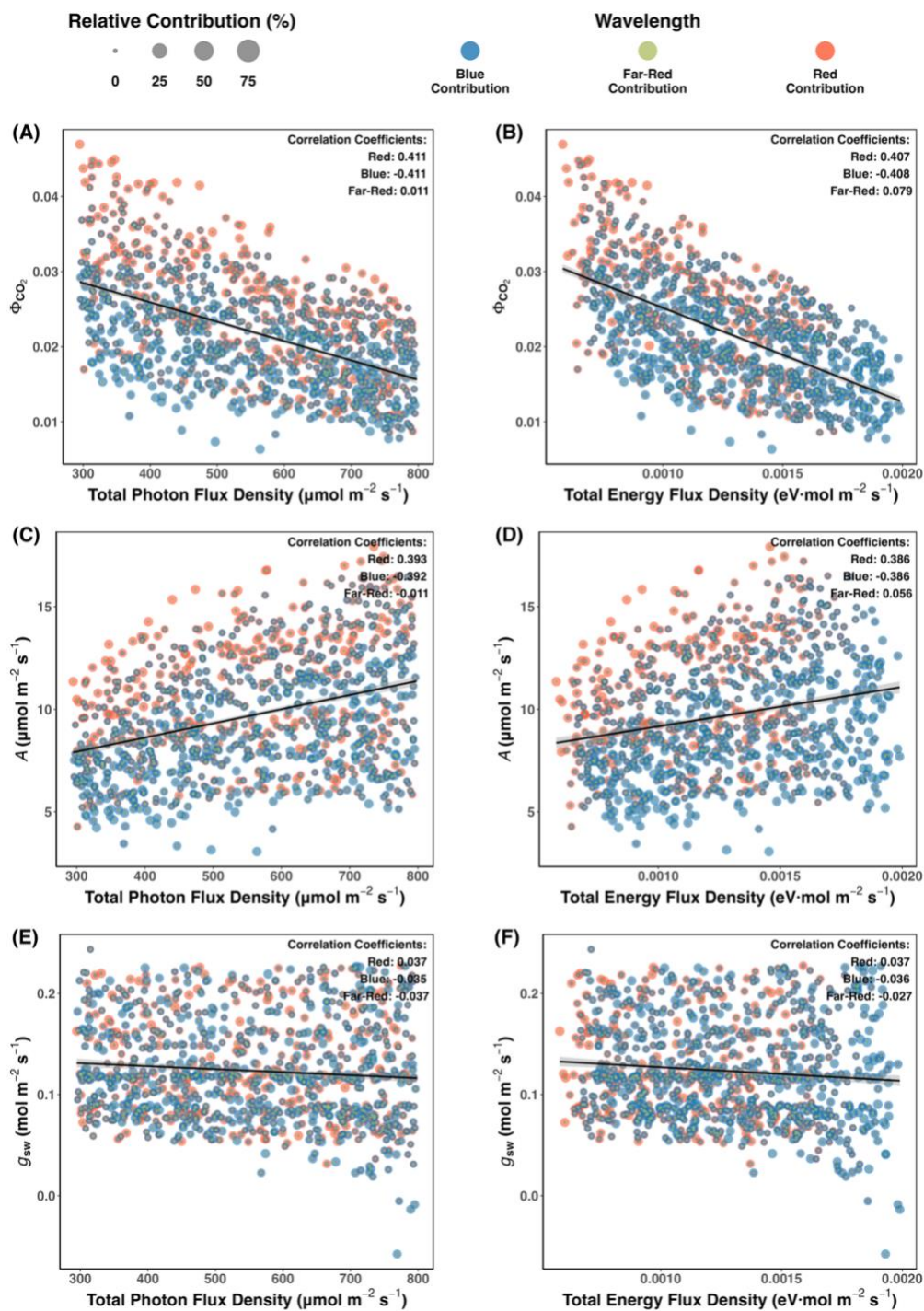


Figure 5.2. Relationships between total photon flux density (PFD) and energy flux density (EFD) with photosynthetic parameters. (A,B) Quantum yield of CO_2 assimilation (Φ_{CO_2}) versus total PFD and EFD. (C,D) CO_2 assimilation rate (A) versus

total PFD and EFD. (E,F) Stomatal conductance (g_{sw}) versus total PFD and EFD. Point sizes indicate the relative contribution of each wavelength (red, blue, and far-red light). Correlation coefficients for each wavelength's contribution are shown in the top right of each panel. Black lines represent linear regression fits with 95% confidence intervals.

5.3.3 Random Forest Model Reveals Hierarchical Controls on Quantum Yield of CO₂ Assimilation

To elucidate the hierarchical control mechanisms governing photosynthetic efficiency under dynamic light environments, a random forest regression model was trained and used to analyse 1,982 unique combinations of light quality, intensity, and temporal parameters (see Supplementary Materials SFig 3.1). The model demonstrated exceptional accuracy on independent test data ($R^2 = 0.922$, RMSE = 0.00209 mol mol⁻¹; Figure 5.3A), with minimal overfitting despite the R^2 difference of 0.061 between training (0.983) and test sets. The model was trained on 1,604 observations and validated on 401 independent test samples from the range of randomized experimental programs executed in the gas exchange system clusters.

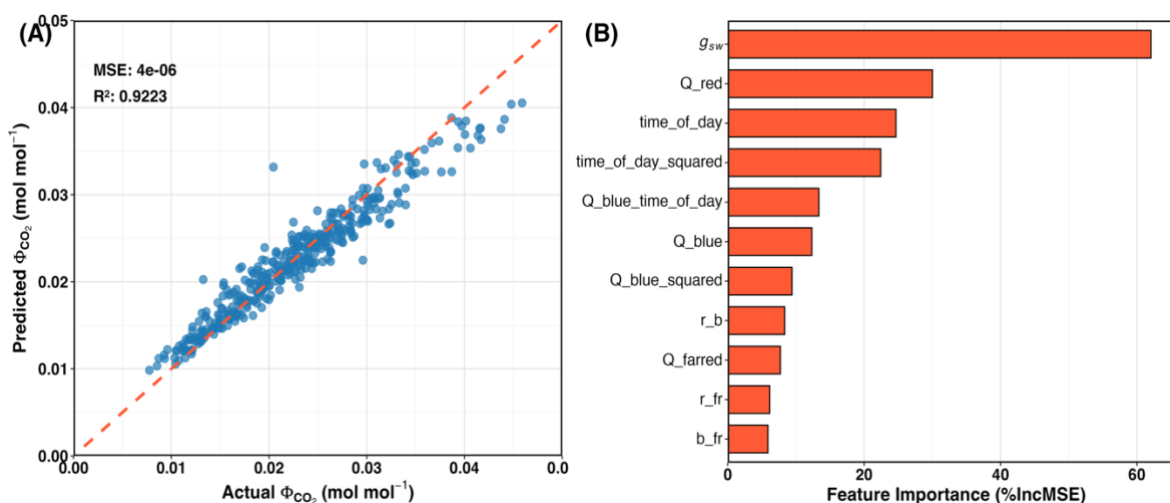


Figure 5.3. Random forest model performance and feature importance for predicting quantum yield of CO₂ assimilation (Φ_{CO_2}). (A) Relationship between predicted and observed Φ_{CO_2} values from 397 test samples with the dashed line indicating the 1:1 relationship. (B) Relative importance of predictive features ranked by percentage increase in mean squared error (%IncMSE) when each variable is permuted.

Feature importance analysis revealed that stomatal conductance (g_{sw}) was the most influential predictor, with a relative importance score of 62.1%, exceeding all other features by minimum 2-fold (Fig. 5.3B). Among the light-related parameters, red light intensity (Q_{Red}) was the second-highest importance factor (30.0%), followed by the quadratic term of time of day (23.3%). Interestingly, the interaction between blue light intensity and time of day, along with the quadratic term of blue light demonstrated moderate importance (12.7% and 10.6%, respectively). This temporal dependency of blue light effects is particularly noteworthy given the known lower quantum yield of blue photons compared to red photons, suggesting that blue light influence on Φ_{CO_2} may be

mediated through time-dependent effects on stomatal conductance rather than direct photochemical contributions. Spectral ratios (red/blue, red/far-red, and blue/far-red) showed relatively lower importance (7.6%, 6.2%, and 5.2%, respectively), indicating that absolute light intensities and temporal factors were more critical in determining ΦCO_2 than the proportional relationships between different wavelengths. This finding is particularly relevant given the randomized diurnal light response protocol employed, where light intensity and spectral composition were varied independently throughout the day while maintaining strict environmental controls for temperature and CO_2 concentration, and VPD.

5.3.4 Enhanced Carbon Gain and Sustained Stomatal Dynamics under Abaxial Blue Pulses

Diurnal patterns of photosynthesis and stomatal conductance were monitored using a dual-chamber system that enabled simultaneous measurement of both leaf surfaces while applying periodic blue light pulses ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) to the abaxial surface. Leaves exposed to blue light pulses maintained consistently higher rates of net CO_2 assimilation throughout the measurement period (Figure 5.4a). Mean photosynthetic rates in the blue pulse treatment ($17.0 \pm 0.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$) were significantly higher than in the control treatment receiving only red light to the adaxial leaf surface ($15.8 \pm 0.02 \mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$). The temporal pattern showed characteristic oscillations corresponding to the blue light pulse intervals with increases in photosynthetic rate typically coinciding with pulse periods (indicated by light blue shading in Figure 5.4A). These oscillations maintained amplitude throughout the measurement period, suggesting sustained sensitivity to the blue light stimulus. Stomatal conductance (g_{sw}) exhibited

dramatic responses to the treatments (Figure 5.4B), with consistently higher values under blue light pulses ($0.277 \pm 0.001 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) compared to the control treatment ($0.223 \pm 0.001 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$). The periodic nature of stomatal responses was evident in the blue pulse treatment where g_{sw} showing coordinated increases during pulse periods. The magnitude of these responses remained consistent throughout the measurement period, indicating maintained stomatal sensitivity to blue light.

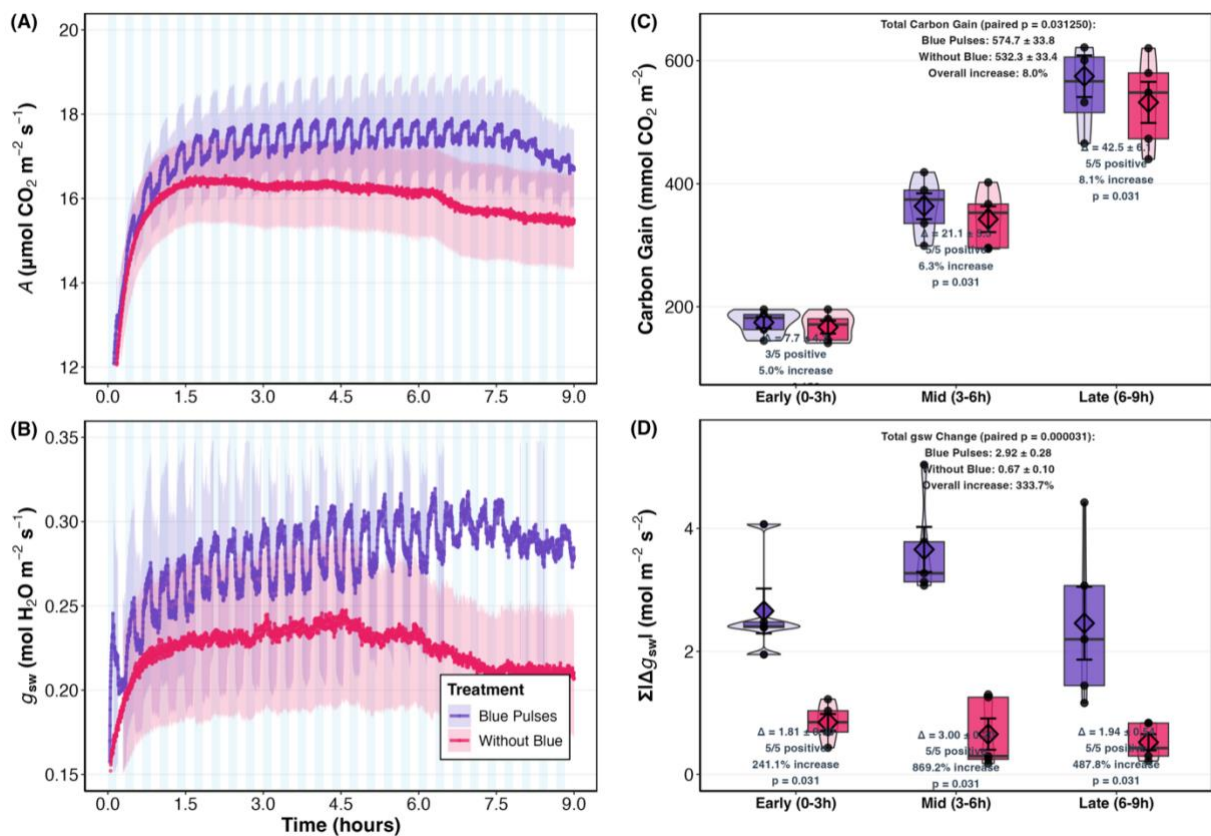


Figure 5.4. Diurnal patterns of photosynthetic parameters under abaxial blue light pulse treatment. Time courses of (A) net CO₂ assimilation rate (A) and (B) stomatal conductance (g_{sw}) in response to periodic blue light pulses or red light only control

treatment. Light blue shaded regions indicate periods of blue light application. Data points represent means $\pm$ SE ($n = 5$). (C) Total carbon gain analyzed in three phases (Early: 0-3h, Mid: 3-6h, Late: 6-9h). Box plots show the distribution of values with individual data points overlaid, diamonds represent means $\pm$ SE. Annotations show mean differences (Δ), percent increases, and significance levels for each phase. (D) Analysis of stomatal conductance (g_{sw}) rate changes calculated at 10-minute intervals. For all panels, purple represents my blue pulse treatment and pink represents control without blue light.

When analysed in three-hour phases, the enhancement of carbon gain by blue light pulses showed a progressive strengthening over time (Figure 5.4C). During the early phase (0-3h), carbon gain showed a modest increase of 4.98% (mean difference: $2.14 \pm 1.16 \text{ mol CO}_2 \text{ m}^{-2}$, $p = 0.156$). This effect became statistically significant during the mid-phase (3-6h), where blue light pulses enhanced carbon gain by 7.63% (mean difference: $3.73 \pm 0.588 \text{ mol CO}_2 \text{ m}^{-2}$, $p = 0.031$). The enhancement was most pronounced during the late phase (6-9h), reaching an 11.5% increase (mean difference: $5.93 \pm 0.678 \text{ mol CO}_2 \text{ m}^{-2}$, $p = 0.031$). The cumulative effect over the entire measurement period showed a significant 7.98% increase in total carbon gain under blue light pulses ($53.2 \pm 2.05 \text{ mol CO}_2 \text{ m}^{-2}$) compared to the control treatment ($49.3 \pm 1.85 \text{ mol CO}_2 \text{ m}^{-2}$; paired test, $p < 0.001$). The dynamic nature of stomatal responses was further characterized by analyzing the rate of change in stomatal conductance at 10-minute intervals (Figure 5.4D). The blue pulse treatment exhibited substantially larger fluctuations in g_{sw} rate changes, with maximum and minimum rates of 0.470 and $-0.538 \text{ mol m}^{-2} \text{ s}^{-2}$, respectively, compared to

more modest changes in the control treatment (maximum: $0.270 \text{ mol m}^{-2} \text{ s}^{-2}$, minimum: $-0.196 \text{ mol m}^{-2} \text{ s}^{-2}$). The enhanced stomatal responsiveness under blue light pulses was particularly evident when analysed by phase, showing significantly higher total g_{sw} changes of 241% ($p = 0.031$), 869% ($p = 0.031$), and 488% ($p = 0.031$) in the early, mid, and late phases, respectively. These phase-specific enhancements contributed to an overall 334% increase in total g_{sw} rate changes under blue light pulses ($2.92 \pm 0.280 \text{ mol m}^{-2} \text{ s}^{-2}$) compared to the control treatment ($0.674 \pm 0.104 \text{ mol m}^{-2} \text{ s}^{-2}$). The temporal patterns of both net CO_2 assimilation and stomatal conductance showed remarkable consistency in their response to blue light pulses throughout the mid and early measurement period. The mean response magnitude remained stable, and the enhancement of carbon gain actually strengthened over time, indicating no apparent desensitization to the periodic blue light stimulus.

5.4 Discussion

5.4.1 Differential photosynthetic responses to surface specific illumination

The optimization of photosynthesis in controlled environment represents a critical intersection of plant physiology and agricultural engineering, where even marginal improvements in light use efficiency can translate to substantial energy savings and productivity gains. Our investigation into surface specific illumination on photosynthetic responses in *Fragaria* × *ananassa* demonstrates that while adaxial illumination maintains physiological superiority, achieving 27-30% higher carbon assimilation rates under saturating light conditions (Figure 5.1C), the abaxial surface retains remarkable

photosynthetic competence at 77-79% of adaxial capacity, suggesting the functional importance of both leaf surfaces for whole leaf carbon gain under realistic light fields. This finding, along with multiple other emerging studies, challenges the conventional paradigm of exclusively top-down illumination strategies in controlled environment agriculture. (Paradiso & Marcelis, 2012; Paradiso *et al.*, 2020; Zhang *et al.*, 2021).

The differential photosynthetic capacity between leaf surfaces aligns with recent findings in wheat, where mesophyll cells associated with the adaxial surface demonstrated higher photosynthetic capacity than those associated with the abaxial surface (Wall *et al.*, 2022), though strawberry's hypostomatous architecture presents a contrasting stomatal distribution pattern. The influence of light orientation on dorsoventral regulation of photosynthesis results from differential sensitivity of stomatal opening to light orientation and fixed gradients of enzyme activation across the leaf (Soares *et al.*, 2007), suggesting that the 23% photosynthetic deficit under abaxial illumination in our study may arise from suboptimal light penetration patterns and enzyme distribution rather than fundamental physiological limitations. This distinction is particularly relevant for applied contexts as growers are increasingly adopting intercanopy lighting, where light could impinge directly on abaxial surfaces.

The maintained photosynthetic capacity under abaxial illumination (Figure 5.1A) can be partially explained by leaf optical properties. In dicot plants with strongly asymmetric dorsiventral orientation, the chloroplast-rich palisade parenchyma near the upper surface intercepts incoming light while spongy mesophyll and large air spaces on the abaxial side facilitate gas exchange (Ustin & Jacquemoud, 2020). Our spectral measurements

(Supplementary Figure S5.2) corroborate previous observations that light reflectance for adaxial leaves was lower than for abaxial ones, mainly due to an increase in the scattering coefficient for the lower leaf side (Cordón & Lagorio, 2007). This enhanced scattering at the abaxial surface will contribute to increased light path length within the mesophyll when illuminated from below, partially compensating for the less optimal tissue arrangement.

Stomatal conductance patterns observed under different illumination orientations (Figure 5.1B,D) reveal a fundamental asymmetry in guard cell responsiveness that cannot be explained by stomatal density alone. Recent single-cell RNA sequencing revealed that adaxial and abaxial guard cells rely on different K⁺ channels for stomatal opening, with distinct compositional differences in potassium ion channel expression between surfaces (Wei *et al.*, 2025). This molecular heterogeneity between guard cell populations provides a mechanistic basis for the differential conductance patterns we observed, particularly the enhanced responsiveness to blue light supplementation from the abaxial surface (20% increase versus 18% for adaxial). The rapid stomatal response to blue light addition in the R900B100 phase (Figure 5.1A, B) is a result of phototropin mediated signalling in surface specific responses. Blue light signals from phototropins activate plasma membrane H⁺-ATPase through CBC1/CBC2 kinases, which redundantly stimulate stomatal opening by inhibition of S-type anion channels (Hiyama *et al.*, 2017a). Given that strawberry stomata are predominantly abaxial, direct blue light exposure to this surface bypasses the optical filtering that occurs when light must traverse the adaxial epidermis and palisade mesophyll. The blue light signalling cascade, involving BLUS1 phosphorylation and subsequent H⁺-ATPase activation, occurs within minutes of phototropin activation (Inoue, S-i & Kinoshita, T, 2017), consistent with our observed rapid

responses during light transitions. From an evolutionary perspective, the retention of substantial photosynthetic capacity under abaxial illumination reflects adaptation to natural light environments where reflected and scattered light contribute significantly to carbon gain. Leaves typically experience alternating exposure of both surfaces to bright sunlight due to wind-induced movement, with this alternant irradiation affecting both net photosynthetic rate and transpiration (Zhang *et al.*, 2016). The 77% photosynthetic capacity we observed under abaxial illumination suggests that strawberry leaves have maintained the biochemical machinery to exploit diffuse and reflected light reaching the lower surface, particularly relevant in dense canopy situations or vertical farming systems with reflective surfaces. Furthermore, the differential surface responses have implications for crop modelling and phenotyping. Accurate assessment of intercellular CO₂ concentration and modelled photosynthetic CO₂ uptake requires accounting for cuticular conductance, particularly when stomata are largely closed under stress conditions (Márquez *et al.*, 2022).

5.4.2 Paradigm Shift Through High Throughput Randomized Phenotyping

The conventional approach to optimizing photosynthesis in controlled environments has relied on factorial experiments testing limited combinations of light intensity, quality, and duration. Our implementation of randomized light-response protocols (Fig. 5.2) across >2,000 unique combinations of light intensity, spectral quality, and temporal patterns represent a fundamental departure from this reductionist approach. Using eight parallel LI-6800 systems programmed to independently randomize parameters, our method can compressed months of sequential measurements under different experimental design into days of data collection while capturing transient states and non-

equilibrium responses invisible to conventional steady state analyses. Our results from randomized light program in gas exchange cluster (Fig. 5.2) showed that quantum yield of CO₂ assimilation (ΦCO_2) correlated negatively with total photon flux density (PFD, $r = -0.511$) and energy flux density (EFD, $r = -0.590$). The steeper slope with EFD highlights the elevated energy content of blue photons, which we known as fewer, higher energy photons can elicit comparable or even greater physiological responses than a larger number of lower energy photons due to specialized blue light photoreceptors (Hogewoning, Sander W *et al.*, 2010). By contrast, net assimilation rate (A) increased with both PFD and EFD (Fig. 5.2C and D) affirmed the classic quantum model of photosynthesis where greater photon availability generally drives higher electron transport (Alados *et al.*, 1996; Cook *et al.*, 2021). Meanwhile, stomatal conductance showed only a weak negative trend (around -0.086 to -0.092), implying steady stomatal conductance or potential partial stomatal closure.

To integrate this large multifactorial dataset, we trained a random forest model to predict ΦCO_2 . The result was a strong fit ($R^2 = 0.922$; $\text{MSE} = 4.0 \times 10^{-6}$), reflecting the power of combining extensive randomized measurements with machine learning approaches that has not been explored in depth in plant lighting research. Feature importance analysis singled out stomatal conductance (g_{sw}) as the most influential parameter (68.4%) which corroborated our hypothesis and common notion that stomatal regulation is central to assimilation efficiency (Fig. 5.3B). Red photon flux (Q_{Red}) ranked second (24.6%) which was consistent with red's known high quantum yield (Alados *et al.*, 1996). Interestingly, time of day (particularly its quadratic term, 23.3%) and blue light $\times$ time interactions (12.7%) also emerged prominently, this temporal dependence of blue light effects could be

explained by the circadian regulation of starch metabolism in guard cells, where blue light triggers a distinct degradation pathway that provides osmolytes for stomatal opening (Santelia & Lunn, 2017). Our observation that blue light $\times$ time interaction contributed 12.7% to model importance suggests that targeting illumination to coincide with peak guard cell responsiveness could optimize resource use efficiency.

These findings highlight that while red light remains a cornerstone for efficiently driving photosynthetic reactions, the timing and quantity of blue photons can modulate stomatal behaviour. A static lighting recipe risks missing crucial windows when stomata are most responsive. The convergence of our computational and physiological findings: stomatal conductance dominance in predictive models (68.4% feature importance, figure 5.3), temporal dependencies of blue light effectiveness (12.7% importance for blue $\times$ time interaction, figure 5.3), and sustained photosynthetic capacity when illuminated from abaxial surface (77% of adaxial, figure 5.1) pointed toward a targeted intervention strategy exploiting these synergistic mechanisms. It has been demonstrated in algal systems that pulsed lighting approaches can increase 3-10 $\times$ photon efficiency and 10-40% energy savings in vertical farms by exploiting temporal mismatches between light reactions (nanoseconds-milliseconds) and dark reactions (seconds-minutes) of photosynthesis (Zarmi *et al.*, 2020; Olvera-Gonzalez *et al.*, 2021; Diotto *et al.*, 2022; Chen *et al.*, 2023). These findings suggest that carefully timed blue light pulses targeting the stomata rich abaxial surface during periods of declining conductance could alleviate stomatal limitation without proportional increases in energy input.

5.4.3 Targeted Blue Light Pulses on the Abaxial Surface improved diurnal carbon gain

Guided by the above insights, particularly the emphasis on stomatal conductance and the abundance of guard cells on the abaxial surface of *Fragaria × ananassa*, we designed a pulsed blue light strategy (10 minute interval) to the leaf underside whilst balancing total PPFD by reducing red light through compensatory adaxial adjustments. Over the diurnal period, these pulses led to an ~8% increase in total carbon gain (Fig. 5.4C). Gains were moderate initially (4.98% in the first 3 hours) but peaked in the late phase (11.5% in hours 6–9), suggesting that periodic bursts of blue light help stave off the typical midday depression of photosynthesis (Fig. 5.4B,D). Stomatal conductance in the pulsed treatment soared at each blue interval (333.7% total increase in g_{sw} rate changes) and with especially dramatic spikes during the midday-phase (~869.2%) compared to top red light only treatment. This pulsatile approach likely prevented the gradual downregulation of gas exchange typically observed under continuous illumination (as also shown in our Fig. 5.4B). Direct abaxial illumination exposes guard cells to unattenuated blue photons which is contrasting to red light only top down lighting where blue photons must traverse pigmented adaxial tissues and mesophyll layers (Vogelmann & Evans, 2002; Cordon & Lagorio, 2007; Pathare *et al.*, 2019). While the response magnitude tapered somewhat in the final hours, possibly due to desensitization of photoreceptor or carbohydrate feedback, the mean g_{sw} and net carbon gain of blue pulses treatment remained significantly elevated throughout the final 3 hours photoperiod compared to the red only top lighting. One of the plausible explanation to this tapered response magnitude could lie in the phototropins internalization and subcellular relocation with phot1 being partially released from the

plasma membrane into the cytosol following extended blue light exposure (Paul & Pellny, 2003). Although the functional significance of phototropin relocalization remains debated, this cytosolic translocation may serve as a desensitization mechanism that attenuates responsiveness after prolonged activation (Winder *et al.*, 1998). Additionally, the progressive accumulation of photosynthates during the photoperiod can trigger metabolic feedback inhibition. For example, carbohydrate accumulation inhibits photosynthesis through regulatory networks typically involving sugar sensing and signalling, with excessive accumulation of phosphorylated intermediates restricting metabolic flow (Violet-Chabrand, S *et al.*, 2017; Drincovich & Maurino, 2022). The temporal dynamics observed in our blue pulsed experiment with peak enhancement during mid phase followed by partial attenuation aligned with established patterns of diurnal photosynthetic regulation (Ganzon-Fortes, 1997; Maxwell, 2002; Okamoto *et al.*, 2022). Phloem based feedback signals effectively regulate the balance between source and sink activities, with carbohydrate status acting as a metabolic signal that coordinates photosynthesis with growth demands (Quereix *et al.*, 2001; Chai *et al.*, 2025). Despite these regulatory constraints, the sustained elevation in both stomatal conductance and carbon gain throughout the final photoperiod demonstrates that targeted abaxial blue light pulses can partially overcome typical afternoon depression of photosynthesis, suggesting that spatial light targeting strategies can effectively modulate the source-sink balance and extend productive photosynthetic capacity deeper into the photoperiod. However, increased stomatal conductance could come at a cost. Transpiration rates may rise, potentially demanding more careful water management or adjustments to humidity and VPD, but genetically reducing stomatal density could mitigate this (Lawson, 2009; Franks *et al.*,

2015; Dunn *et al.*, 2019). Implementing underside lighting with pulsed control also requires additional investment in hardware and energy. A further cost-benefit analysis will elucidate its economic feasibility.

Looking beyond our immediate findings, these results reveal multiple avenues for advancing both research and global horticultural practice. By employing a randomized, diurnal light response framework with multiple programmable IRGAs, researchers can rapidly determine species-specific "optimal" lighting regimes in diverse vertical farm setups, thereby mitigating significant resources otherwise spent on guesswork or trial-and-error approaches. It is also possible to randomize not only light parameters but also CO₂ concentration, humidity, and boundary layer conditions, thereby pinpointing optimal dynamic environments for carbon gain of any species within a relatively short time period compared to other research method. This systematic method also helps to control for spatial heterogeneity in growth environments and intrinsic biological variation between individuals. Insights from these automated, randomized protocols have already guided our design of blue-light pulsing schemes that enhanced diurnal carbon gain, and further refinements, such as tuning pulse frequency, amplitude, or adding UV, green, or far red wavelengths could yield even greater improvements in photosynthesis or water-use efficiency. Additionally, coordinating these pulses with peaks in CO₂ supplementation or cooler daytime intervals may further boost resource-use efficiency.

Collectively, our study highlights that targeting the abaxial surface with timed pulses of blue light can overcome midday photosynthetic declines by sustaining stomatal conductance which has often been an overlooked bottleneck in traditional, single-sided

illumination strategies, including those for our model crop strawberry. Mechanistically, this synergy stems from guard-cell activation further showed the critical interactions with leaf anatomy, spectral composition, and diurnal timing. Equally important, our approach integrates a novel randomized protocol in which multiple programmable gas-exchange systems rapidly sample and predict optimal light, and possibly CO₂, and humidity conditions. By coupling this data intensive but automated method with practical pulsed light interventions, we have not only presented here an immediately actionable strategy to improve diurnal carbon gain and have also established a powerful research and development pipeline for boosting crop yield and resource efficiency in controlled environments cultivation system. As lighting technologies continue to evolve, adopting such targeted regimens by delivering the right wavelength at the right time to the right side of the leaf holds great promise for significantly enhancing biomass production.

Chapter 6: Surface-Specific Reductions in Stomatal Density in EPIDERMAL PATTERNING FACTOR1 Wheat Differentially Affect Stomatal Red and Blue Light Responses

(A version of this chapter has been accepted for publication as Fan, M., Dorussen, D., Gherli, H., & Lawson, T. (2025). Reduced stomatal density in wheat overexpressing EPIDERMAL PATTERNING FACTOR1 differentially affects red and blue light responses. *Plant Physiology*, 199(1), kiaf379. doi.org/10.1093/plphys/kiaf379)

Key words: Stomatal density, blue light, guard cell, bread wheat (*Triticum aestivum* L.), stomatal conductance (g_{sw}), net CO₂ assimilation rate (A), Epidermal Patterning Factor (EPF), water use efficiency (WUE), photosynthesis, mesophyll feedback, leaf surface polarity; Drought resilience.

Running Title: Blue Light Compensation for reduced Stomatal Density in Wheat

Abstract (Chapter 6)

Stomatal pores govern the trade off between CO₂ assimilation and water loss, and optimizing their performance is critical for crop resilience, particularly under dynamic field environments. Here, we show that overexpression of *Triticum aestivum* EPIDERMAL PATTERNING FACTOR1 (TaEPF1) in bread wheat (*Triticum aestivum*) reduces leaf stomatal density in a leaf surface-specific manner, with a greater decline on the abaxial surface than on the adaxial surface. TaEPF1 *overexpressors* exhibited substantially lower stomatal conductance than wild-type (WT) control plants, which resulted in diffusional constraints limiting photosynthesis when measured under monochromatic red light. However, upon partial substitution of red light with blue light, EPF1 *overexpressors* displayed an amplified and rapid stomatal opening response, particularly on the abaxial surface, where relative conductance increased by up to 90% versus 49% observed in the WT. Despite anatomical limitations in maximum conductance rate, this blue light sensitivity effectively compensated for the lower baseline gas exchange. The enhanced sensitivity to blue light was also concomitant with lower intercellular CO₂ levels under red light. When g_{sw} responses were normalized relative to stomatal density, stomatal sensitivity to red light was reduced at the pore level, whilst blue light sensitivity increased, which was particularly evident during abaxial surface illumination. Finally, the transgenic lines maintained a 15–20% higher water use efficiency across light regimes. These findings show a compensatory mechanism where genetically induced reduction in stomatal density is offset by heightened blue light sensitivity.

6.1 Introduction

Stomatal pores regulate the exchange of CO₂ and water vapor between the plant interior and the atmosphere, thus playing a critical role in balancing photosynthetic carbon gain and water loss (Caine *et al.*, 2019; Lawson & Matthews, 2020). Guard cells surrounding these pores exhibit rapid, dynamic responses to many environmental stimuli including light intensity, light spectra, vapor pressure deficit (VPD), and internal CO₂ concentration (C_i) that are essential for optimizing photosynthetic efficiency and maintaining water use efficiency (Drake *et al.*, 2013; Lawson & Vialet-Chabrand, 2019). In natural fluctuating environments, the speed and magnitude of stomatal adjustments are particularly important for sustaining photosynthesis while mitigating water loss under transient light conditions (Kaiser *et al.*, 2015; Buckley, 2019; Lawson & Vialet-Chabrand, 2019). Rapid opening to meet photosynthetic demands for CO₂ improves carbon assimilation (A), whilst rapid closure under conditions when A has decrease (e.g. low light intensity) improves intrinsic water use efficiency (W_i) (Faralli *et al.*, 2019; Papanatsiou, Maria *et al.*, 2019; Battle *et al.*, 2024).

Light quality as well as intensity, plays a pivotal role in modulating stomatal behavior. These light responses have been separated into the red or mesophyll responses and the specific blue light response (Matthews *et al.*, 2020). Traditionally red light has been linked directly with photosynthetic activity and has been associated with lowering intercellular CO₂ (C_i) through enhanced CO₂ uptake, which in turn promotes stomatal opening (Lawson *et al.*, 2014; Ando & Kinoshita, 2018; Taylor *et al.*, 2023; Taylor *et al.*, 2024). There is considerable evidence to suggest that C_i is not the only signal that mediates the red light responses and that C_i independent mechanisms exist (Messinger *et al.*, 2006;

Lawson, 2009; Matrosova *et al.*, 2015), although the exact mechanisms have not been fully elucidated (Mott *et al.*, 2008; Lawson *et al.*, 2018; Taylor *et al.*, 2024). In contrast, blue light elicits a distinct and guard cell specific response that operates effectively at low intensities ($< 50 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Violet-Chabrand *et al.*, 2021), is not linked to photosynthesis and reported to be up to 20 times more effective at inducing stomatal opening than red light (Doi *et al.*, 2015; Hiyama *et al.*, 2017a; Inoue, SI & Kinoshita, T, 2017; Matthews *et al.*, 2020). This blue light response is perceived by phototropins (PHOT1 and PHOT2) in the guard cells, which activate a signal transduction cascade involving the plasma membrane H^+ -ATPase, ion fluxes, and guard cell starch degradation to facilitate rapid stomatal opening (Inoue *et al.*, 2008; Horrer *et al.*, 2016; Inoue, SI & Kinoshita, T, 2017; Flütsch *et al.*, 2020). The stomatal blue light response can also aid leaf cooling under fluctuating light, which is critical to prevent heat stress (Zeiger & Field, 1982; Schwartz & Zeiger, 1984; Pearcy, 1990; Lawson, T & Blatt, MR, 2014). Such dual regulation highlights that stomatal dynamics are governed not only by mesophyll driven signals, but also by direct light signalling pathways that can adjust aperture independently of photosynthetic rates (Lawson, 2009). Notably, Taylor *et al.* (2024) observed significant suppression of C_i independent stomatal opening under high C_i conditions.

Improving water use efficiency by manipulating stomatal density using genetic approaches has emerged as a promising strategy, which is becoming increasingly desirable under conditions of water scarcity (Doheny-Adams *et al.*, 2012; Franks *et al.*, 2015; Hughes *et al.*, 2017). Overexpression of EPIDERMAL PATTERNING FACTOR1 (EPF1), which acts as a negative regulator of stomatal development by inhibiting the

formation of stomatal precursors, has been shown to decrease stomatal density and consequently, modulate gas exchange and photosynthetic performance (Hara *et al.*, 2007b; Hepworth *et al.*, 2015; Dunn *et al.*, 2019; Lu *et al.*, 2019). Another emerging yet underexplored aspect of stomatal density and the regulation of gaseous exchange is the differential patterning and behaviour in adaxial (upper) and abaxial (lower) leaf surfaces (Driscoll *et al.*, 2006; Wang *et al.*, 2008; Wall, Shellie *et al.*, 2022; Driesen *et al.*, 2023). Typically, the adaxial surface is exposed to higher light intensities, while the abaxial surface experiences more shade, leading to inherent differences in stomatal density *and* responsiveness. Unlike many dicots that show higher stomatal density on the abaxial surface, wheat exhibits amphistomatous leaves with relatively even distributions or even greater stomatal density on the adaxial surface, suggesting a distinct adaptation pattern that may influence gas exchange dynamics (Driscoll *et al.*, 2006; Wang *et al.*, 2008; Zhao *et al.*, 2020; Wall, Shellie *et al.*, 2022). Although these polarity cues and the environmental impact on these developmental pathways have been characterized in some species (Turner, 1970; Wang *et al.*, 2008; Sun *et al.*, 2021), there is limited study on the similar mechanisms operate in important monocots such as wheat. Differential regulation of stomatal development on these surfaces may have significant implications for gas exchange, especially when anatomical constraints, such as those with genetically reduced stomatal density infers limitations on CO₂ uptake for photosynthesis. For example, in wheat the majority of gas exchange takes place via the adaxial surface (Wall, Shellie *et al.*, 2022), whilst the abaxial surface has often been considered the major contributor to leaf gas exchange in other species (Fanourakis *et al.*, 2015; Richardson *et al.*, 2017; Mochizuki *et al.*, 2019), often due to reduced evaporative demand from this

surface as it is not in direct sunlight. Wall et al. (2022) also demonstrated that stomatal behaviour on one surface could not compensate for changes on the opposite side, suggesting independent functioning. The two surfaces (of wheat flag leaves) can receive direct illumination when growing erect, whilst when more horizontal, the two surfaces not only receive different light intensities but also difference spectra due to absorption of specific wavelengths by the mesophyll (Day *et al.*, 1994; Johnson *et al.*, 2005; Brodersen & Vogelmann, 2010; Th  roux-Rancourt & Gilbert, 2017) which has been shown to influence stomatal behaviour (Wang et al., 2016).

Despite the well established roles of red and blue light in driving stomatal dynamics, a critical gap remains in our understanding of how these responses may differ between the two surfaces and how engineered for reduced stomatal density may influence these responses. In particular, it remained underexplored how the adaxial and abaxial leaf surfaces respond to direct illumination and whether compensatory physiological mechanisms exist that might offset the diminished anatomical capacity for gas exchange from one surface by the other. Addressing these gaps is essential, as developing crops that maintain high photosynthetic performance while exhibiting improved water use efficiency under fluctuating environmental conditions are of great interest and surface specific manipulation of stomatal features could represent an exciting approach to potentially develop crops with improved water use efficiency.

In this study, we examined stomatal responses to red and blue light in wheat (*Triticum aestivum*) lines overexpressing TaEPF1 (TraesCS2B02G556200), which have reduced stomatal density (Hughes *et al.*, 2017; Dunn *et al.*, 2019). By integrating dynamic gas

exchange measurements with detailed anatomical and molecular analyses, we explore how EPF1 overexpression differentially modulates stomatal development and responsiveness when illumination was provided by to the adaxial or abaxial surfaces. Our work provides insights into the compensatory mechanisms, particularly the enhanced blue light induced stomatal opening that may enable plants with reduced stomatal density to optimize gas exchange and water use efficiency under complex, fluctuating light environments.

6.2 Materials and Methods

Plant Material and Growth Conditions

Triticum aestivum cv. Fielder transgenic lines overexpressing the *TaEPF1* gene (1OE3 and 1OE4), under the control of the rice actin promoter (Dunn *et al.* (2019) were germinated under controlled environmental conditions, with a 12-hour photoperiod at a stable temperature range of 21-22°C. Non transformed Fielder plants served as wild type control (WT). Plants were grown under these conditions for two weeks, after which seedlings were transplanted into 1L pots and grown for an additional seven weeks in a greenhouse at the University of Essex (Colchester, England, UK). The greenhouse conditions were as follows: average temperature of 20.9°C, relative humidity of 45-70%, and a 16-hour photoperiod (0600 to 2200 hours). Natural light was supplemented with high-pressure sodium lamps (HSE NXT 2 600W, HORTILUX SCHRÉDER, the Netherlands) to maintain minimum intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD at the canopy level throughout the photoperiod, as measured with SpectraPen (PSI, Czech Republic). The average daily light integral was approximately 16.668 $\text{mol m}^{-2} \text{d}^{-1}$, with light intensities

reaching up to $806 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD on clear days. Measurements were taken on flag leaves between Zadoks growth stage 45 (boots swollen) to stage 59 (ear emergence complete) (Zadoks *et al.*, 1974). Plants were grown using a randomized complete block design with genotypes randomly distributed across the greenhouse bench. Plants were regularly rotated within the greenhouse bench to minimize positional effects.

Leaf Gas Exchange Measurements and Dynamic Model Fitting of Stomatal Kinetics

Leaf gas exchange parameters were measured using Li-6800 infrared gas analysers with the 6800-01A fluorometer head (Licor Biosciences, NE, USA). The leaf chambers were set to maintain a CO_2 concentration of $400 \mu\text{mol mol}^{-1}$, a flow rate of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$, a leaf temperature of 23°C , and a vapor pressure deficit (VPD) of 1.2 kPa. To determine responses under varying illumination and across different leaf surfaces, measurements were conducted using bi-directionally illumination, illuminating either the adaxial or abaxial leaf surface. Measurements were conducted in a randomized order across multiple days to minimize systemic biases.

To measure assimilation rate (A) and stomatal conductance (g_{sw}), the flag leaf of the wheat plant was positioned in the 6 cm^2 chamber. Leaf area was determined by measuring leaf blade width at multiple points along the chamber length and calculating the effective area (average width $\times$ 3 cm). Each leaf surface was initially acclimated to an intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light (R100). Leaf gas exchange data were logged at 10-second intervals. After 10 minutes, the light intensity was increased to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ of red light (R1000) and maintained for 30 minutes. This was followed by a modification

in the light spectrum where 10% of the red light was substituted with blue light (475 nm), resulting in a combination of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of blue light and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of red light (R900B100), which was maintained for further 10 minutes. Throughout these light phases, leaf temperature was maintained at approx. 23°C. Water Use Efficiency (*WUE*) was calculated as the ratio of assimilation rate (*A*) to stomatal conductance (g_{sw}).

The dynamics of stomatal opening in response to the step increase in light intensity (R100 to R1000) and subsequent change in light quality (R1000 to R900B100) were quantified by fitting a sigmoidal to the temporal g_{sw} data, as described in McAusland *et al.* (2016):

$$g_s = (g_{smax} - g_{smin})e^{-e^{\left(\frac{\lambda-t}{k}+1\right)}} + g_{smin}$$

Where g_{smin} and g_{smax} represent the minimum and maximum steady state g_{sw} value under R100 and R1000 and R900B100 phases, λ is the initial response lag time, and k is the time constant of stomatal opening. The model was fitted using nonlinear least square regression and was further validated using a stringent root mean square error (RMSE) threshold of 0.1 for both light phases. Kinetic parameters were then derived from the fitted model, as the time to reach 63% of the total conductance (τ), and the maximum rate of conductance increase (Sl_{max}).

Leaf Epidermal Impressions and Measurements

Surface impressions of the abaxial and adaxial leaf surfaces excluding the mid-vein, were made using activated polysiloxane impression material (Xantopren L blue, Kulzer). After polysiloxane solidification, impressions were coated with clear nail varnish and transferred onto microscope slides for analysis. Stomatal density and guard cell lengths

were quantified using a Leica ATC2000 optical microscope with a SWIFT SC500 digital camera. ImageJ software was used to quantify stomatal density and guard cell dimensions from the captured images.

Estimation of Maximum Anatomical Stomatal Conductance

The maximum anatomical stomatal conductance to water vapor g_{smax} was calculated independently for adaxial and abaxial leaf surfaces across all genotypes using a modified equation from Franks and Farquhar (2007) adapted for dumbbell shaped stomatal morphology characteristic of greases following Nunes *et al.* (2022) .

$$g_{smax} = \frac{\frac{d_w}{v} \times SD \times 0.05148 \times GCL^2}{0.25 \times GCL + 0.128 \times GCL}$$

Where d_w is the diffusivity of water vapor in air at 23°C (gas exchange temperature), taken as 2.42×10^{-5} (m² s⁻¹). v is the molar volume of air at 23°C (0.024 m³ mol⁻¹). SD is the stomatal density and GCL is the guard cell length (μm). The coefficients 0.05148 and 0.128 are the grass specific pore geometry where pore length equals 0.44GCL and pore width equals 0.13GCL, with a 0.9 correction factor for the hexagonal pore shape, and pore depth approximates 0.25GCL based on the guard cell width at the stomatal centre in the dumbbell shaped complexes (Nunes *et al.*, 2022).

TaEPF1 Expression Analysis

Three leaf discs (diameter of 10 mm) were sampled from the flag leaf of glasshouse grown plants. To ensure RNA integrity, samples were immediately frozen in liquid nitrogen. The

frozen leaf tissues were mechanically homogenized using a Qiagen TissueLyser II, and total RNA was isolated from the resultant tissue powder using the NucleoSpin RNA/Protein Kit (Macherey-Nagel), following the manufacturer's instructions. Potential genomic DNA contamination was eliminated using DNase I treatment, followed by purification with the RNA Clean & Concentrator™-5 kit (Zymo Research). RNA quantity and quality were assessed using a NanoDrop One spectrophotometer (Thermo Scientific).

For cDNA synthesis, 1 µg of purified total RNA was combined with oligo(dT)12-18 primer (Invitrogen) and the RevertAid Reverse Transcriptase kit (Thermo Scientific), following the manufacturer's guidelines. The synthesized cDNA was subsequently diluted tenfold. Quantitative PCR (qPCR) was conducted on a Bio-Rad CFX384 Real-Time System using 2x qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd). Each 10 µl reaction mixture contained 5 µl of SyGreen mix, 3 µl of the diluted cDNA template, and 0.5 mM each of the forward and reverse primers. The thermal cycling program comprised an initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing/extension at 68°C for 15 s.

Gene expression was normalized using the reference gene *Ta2776* (RNase L inhibitor-like protein) (Paolacci *et al.*, 2009). The relative expression of the *TaEPF1* gene was calculated using the $2^{-\Delta\Delta C_t}$ method. All qPCRs were performed in triplicate to ensure technical reproducibility. The primer sequences used for the amplification were as follows:

Ta2776 Forward: 5'-CGATTCAGAGCAGCGTATTGTTG-3'

Ta2776 Reverse: 5'-AGTTGGTCGGGTCTCTTCTAAATG-3'

TaEPF1 Forward: 5'-ACCGTCCCATCAGGAGAAGCGG-3'

TaEPF1 Reverse: 5'-CGCACCGGAAGCTGACCATGAC-3'.

Statistical Analysis

Statistical analysis was conducted using R (version 4.3.0). Shapiro-Wilk test was used for normality. All gas exchange parameters along with their derived metrics (normalized A , normalized g_{sw} , WUE) were analysed through a multi-factorial analysis of variance (ANOVA) to access genotype effects (WT, 1OE3, 1OE4), illumination conditions (adaxial/abaxial), and light phase interactions (R1000, R900B100). Post-hoc evaluations used the least significant difference (LSD) test with significance groups ($\alpha = 0.05$) denoted by common letters. Anatomical traits and stomatal kinetic parameters derived from nonlinear modelling (τ , SI_{max}) underwent similar ANOVA assessment with subsequent LSD testing. All statistical tests maintained $\alpha = 0.05$ significance threshold with two-tailed distributions. Results are presented as means $\pm$ standard error or where appropriate, as median with interquartile range.

To determine if differences in SD were driven by genetic or environment parameters a variance components analyses was used to partition phenotypic variance into genetic (η^2) and residual environmental components for each trait (stomatal density: $N = 56$ plants per genotype, and all gas exchange derived traits: $N = 8$). Genotype explained 18.8–74.0 % of the total variance ($\eta^2 = 0.188$ – 0.740) which exceeded Cohen's threshold for a large effect ($\eta^2 \geq 0.14$) in every case according to conventional standards (Cohen, 2013; Lakens, 2013). Physiological traits showed the greatest genetic control ($\eta^2 \geq 0.590$ for all gas exchange variables) (Suppl Table S6.1). Measurement precision was high as within

genotype coefficients of variation averaged 12.0 % (range 8.4–21.3 %) and with comparable CV ranges for the WT and both OE lines (Table S6.1).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: TaEPF1 (TraesCS2B02G556200). Arabidopsis homologs referenced EPF1 (AT2G20875). Additional wheat gene accessions can be found in the Ensembl Plants database (https://plants.ensembl.org/Triticum_aestivum).

6.3 Results

6.3.1 Overexpression of EPF1 Reduces Stomatal Density and Maximum Anatomical Conductance differently on Adaxial and Abaxial Leaf Surfaces

TaEPF1 transcript abundance was significantly elevated in transgenic lines compared to WT (Fig. 6.1A). This increased *EPF1* expression was tightly associated with reduced stomatal density (*SD*) on both leaf surfaces (n=56). In WT plants, SDs were 119.5 ± 13.2 stomata mm^{-2} (adaxial) and 95.8 ± 15.5 mm^{-2} (abaxial). The transgenic lines exhibited significantly lower densities, however these differed on the two surfaces. On the adaxial surface, *SD* was significantly reduced to 107.2 ± 11.3 mm^{-2} (1OE3) and 105.5 ± 14.6 mm^{-2} (1OE4). Abaxial reductions were more pronounced, with decreases of 11.5% in 1OE3 (84.8 ± 8.9 mm^{-2}) and 19.9% in 1OE4 (76.7 ± 9.7 mm^{-2}) compared to WT (Fig. 1B). While the reductions in adaxial *SD* were similar between the two transgenic lines (~11–12%), the higher expressing 1OE4 line showed a more substantial decrease on the

abaxial surface (~20%), indicating differential effects of *EPF1* overexpression between leaf surfaces. Contrary to expectations, stomatal size (measured as guard cell length) was reduced in both transgenic lines compared to WT, again this decrease was greatest on the abaxial surface (Suppl. Fig.1). Maximum anatomical stomatal conductance (g_{smax}) calculated using stomatal density and guard cell dimensions (n = 56 per genotype per surface) was significantly reduced in transgenic lines (Fig. 1C). Adaxial g_{smax} decreased by 12.9% (1OE3) and 13.4% (1OE4) compared to WT ($p < 0.05$). Abaxial reductions were more substantial at 14.8% (1OE3) and 22.6% (1OE4) ($p < 0.05$). The adaxial and abaxial stomatal density ratio was affected by *EPF1* overexpression, with WT plants showing a ratio of 1.25 ± 0.0123 and 1OE3 maintaining a similar ratio (1.26 ± 0.0132). However, 1OE4 exhibited a significant 10.4% increase ($p = 0.0285$) to 1.38 ± 0.022 compared to WT.

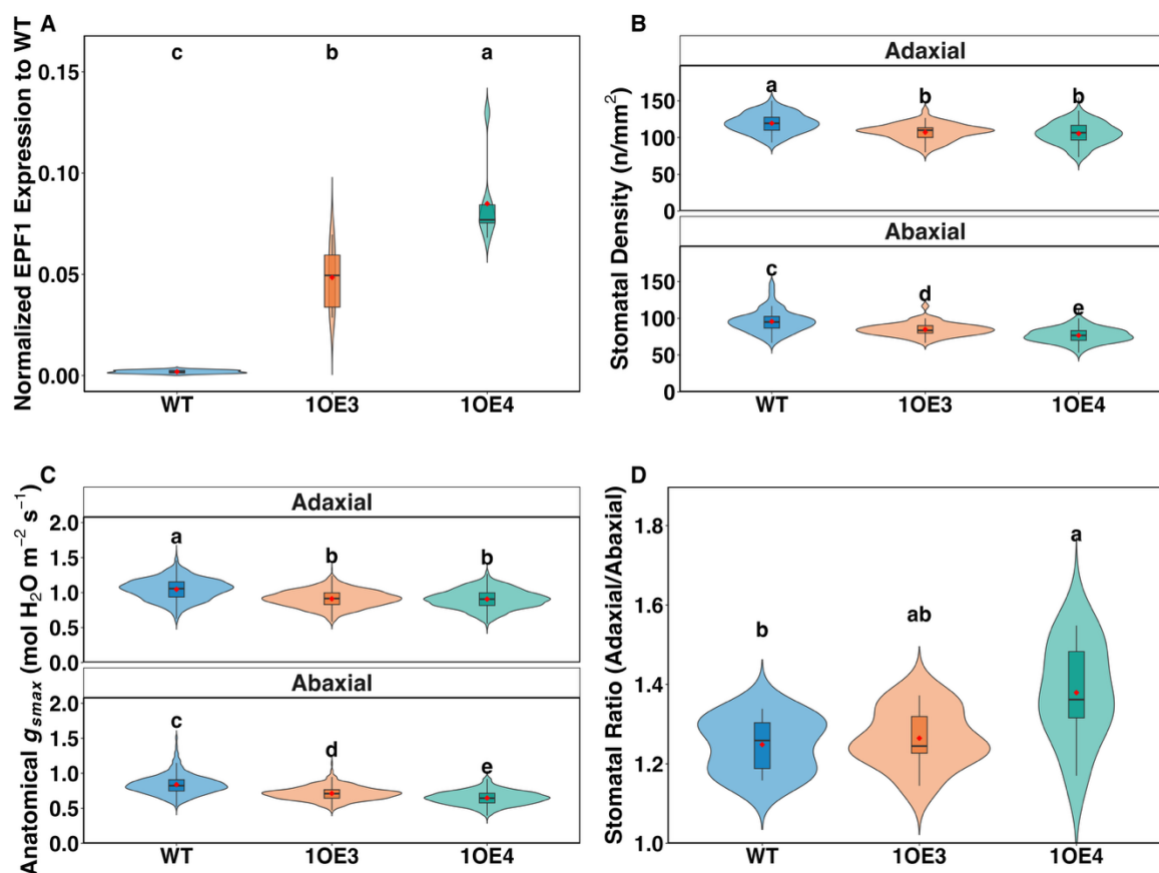


Fig.6.1 Impact of EPF1 overexpression on gene expression and stomatal traits in wheat. (a) EPF1 expression levels in WT (blue), 1OE3 (orange), and 1OE4 (teal) normalized to WT levels (n = 8). (b) Stomatal density measurements from adaxial and abaxial leaf surfaces (n = 56). (c) Calculated maximum anatomical stomatal conductance (g_{smax}) for both leaf surfaces based on stomatal density and guard cell length (n = 56). (d) Ratio of adaxial to abaxial stomatal density (n = 8). Violin plots display probability density distributions with embedded narrow box plots showing median (center line), interquartile range (box limits), and 1.5× IQR whisker extensions, while red diamonds indicate arithmetic means and outliers are

incorporated within the violin contours rather than displayed as separate points.

Note: Guard cell length ($n = 168$) is provided in supplementary material.

6.3.2 Reduced Stomatal Density Alters Photosynthetic Performance and Stomatal Responses to Blue light

Reduced stomatal density in TaEPF1 overexpressing lines (1OE3 and 1OE4) significantly affected photosynthetic performance and stomatal dynamics. Under low PPFD conditions of red only light (R100), both photosynthetic rate (A) and stomatal conductance (g_{sw}) were significantly higher in WT compared to transgenic lines, with these differences most pronounced on the abaxial surface and the 1OE4 line with the greatest EPF1 expression levels (Fig. 6.2A,B). Following a step increase in light to $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (R1000), g_{sw} increased at similar rates across genotypes. However, absolute values of both A and g_{sw} remained elevated in WT. On the abaxial surface, 1OE3 maintained higher A and g_{sw} compared to 1OE4 (Fig. 6.2A, 2B). The substitution of 10% red light with blue light (R900B100) induced rapid and substantial increases in g_{sw} across all lines relative to the steady R1000 phase. The kinetics of this g_{sw} response were notably faster than those observed during the initial red-light transition. While WT maintained higher absolute g_{sw} values on both surfaces (Fig. 6.2B), the transgenic lines exhibited significantly larger percentage increases in g_{sw} , particularly 1OE4 when the abaxial surface was illuminated (Fig. 6.2D). Photosynthetic rate (A), however, remained relatively unchanged in WT following blue light addition (Fig. 6.2C), meaning that enhanced g_{sw} did not translate to

increased carbon assimilation and that photosynthesis was not significantly limited by g_{sw} under steady state R1000. However, A increased by between 5-10% in transgenic lines under abaxial illumination indicating the removal of a diffusional constraint (Fig. 6.3).

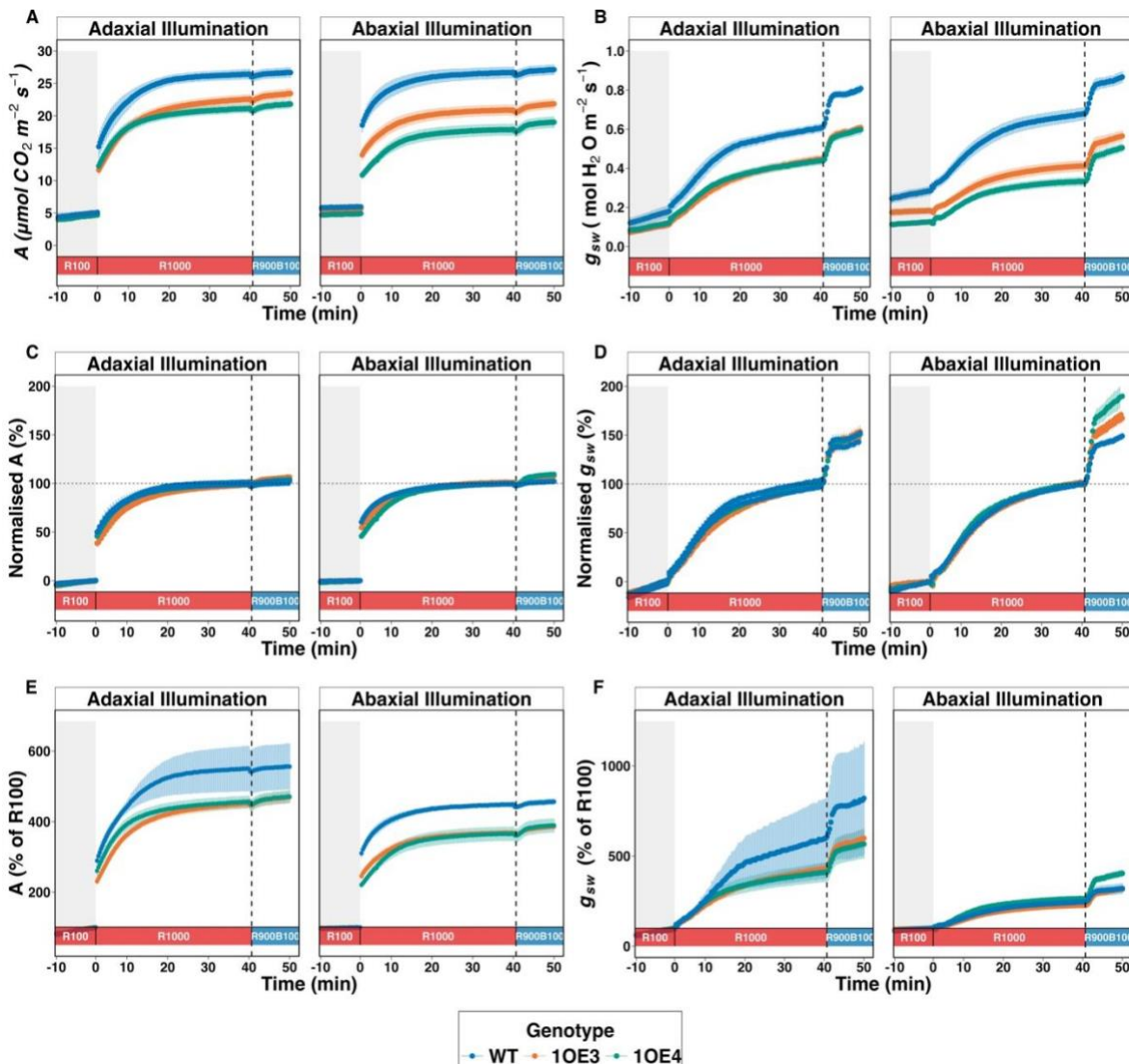


Fig.6.2 Dynamic Responses of Photosynthesis Rate (A) and Stomatal Conductance (g_{sw}) to step changes in PPFD and Light Quality. (A) Time course of photosynthesis rate (A) in response to a step increase in light intensity from 100 (R100) to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (R1000) under 100% red light, followed by exposure to

a combination of red and blue light (R900B100, 900 $\mu\text{mol l m}^{-2} \text{s}^{-1}$ red + 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue applied to either adaxial and abaxial leaf surfaces for an additional 10 minutes). (B) corresponding time course of stomatal conductance (g_{sw}) under the same light conditions. (C) and (D) are the normalised responses of A and g_{sw} , respectively, relative to the differential between the R100 and R1000 steady states, with the dashed horizontal line at 100% indicating the R1000 steady state. The vertical dashed line marks the transition from R1000 to R900B100. Data are presented as mean $\pm$ standard error ($n = 8-9$) for all genotypes. The grey shaded area in each panel indicates the period of low PPFD (R100) prior to the step increase to R1000

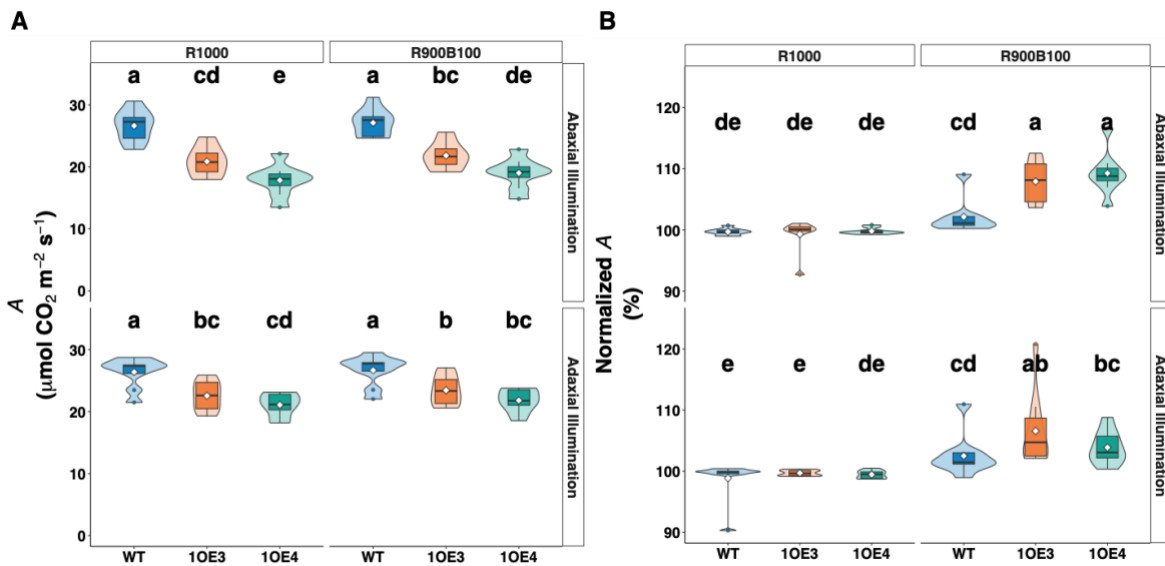


Fig. 6.3 Steady state net photosynthetic rates (A) in WT (blue) and EPF1 lines 1OE3 (orange) and 1OE4 (teal) under different light conditions. (A) Absolute A ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$). (B) Normalized A to steady state A at R100 and R1000 indicating

the relative performance under subsequent R900B100 light phase (n=8). Different letters denote statistically significant differences between groups ($p < 0.05$) based on LSD tests. Violin plots display probability density distributions with embedded narrow box plots showing median (centre line), interquartile range (box limits), and $1.5 \times$ IQR whisker extensions, while red diamonds indicate arithmetic means and outliers are incorporated within the violin contours rather than displayed as separate points.

6.3.3 Surface Specific Enhancement of Stomatal Blue Light Responses

Steady-state gas exchange values were derived from the final 10 measurement points of R1000 and R900B100 light phases under both adaxial and abaxial illumination. Stomatal conductance (g_{sw}) increased substantially in response to blue light (Fig. 6.4A). Under abaxial illumination, WT plants showed a significant increase in g_{sw} from 0.672 ± 0.096 to $0.867 \pm 0.086 \text{ mol m}^{-2} \text{ s}^{-1}$ ($p < 2 \times 10^{-16}$). Similar significant increases were observed in transgenic lines, as 1OE3 increased from 0.410 ± 0.081 to $0.565 \pm 0.080 \text{ mol m}^{-2} \text{ s}^{-1}$, while 1OE4 rose from 0.331 ± 0.065 to $0.505 \pm 0.067 \text{ mol m}^{-2} \text{ s}^{-1}$. However, no significant enhancements were observed under adaxial illumination across all genotypes. When normalized to R1000 baseline values (100%), the relative g_{sw} changes revealed distinct blue light sensitivity patterns across leaf surfaces and genotype (Fig. 6.4B). Under abaxial illumination, WT showed a modest increase of 49%, while transgenic lines demonstrated a statistically significant ($p > 0.0001$) enhanced responses, with g_{sw} in 1OE3 increased by 67% and 1OE4 showed the strongest response of 90%. Adaxial responses were more

moderate and similar across all plants, with increases by ca. 59% (WT), 57% (1OE3), and 53% (1OE4).

Intercellular CO₂ concentration (C_i) increased significantly following blue light exposure (Fig. 6.4C). Under abaxial illumination, C_i in 1OE3 rose from 289 ± 12 to 308 ± 8 $\mu\text{mol mol}^{-1}$ (6.6% increase), while 1OE4 showed an 8.6% increase from 290 to 315 $\mu\text{mol mol}^{-1}$. WT exhibited a smaller increase from 306 to 319 $\mu\text{mol mol}^{-1}$ (4.3%). It is worth noting that under steady-state R1000, transgenic lines maintained significantly lower C_i compared to WT, suggesting more constrained CO₂ influx diffusion under red only illumination. Additionally, C_i values were consistently lower under adaxial versus abaxial illumination across all genotypes.

Water use efficiency (WUE) under steady-state R1000 was significantly higher in transgenic lines compared to WT. Under abaxial illumination, transgenic lines exhibited 15-20% higher WUE than WT ($WUE \approx 39.6$). Blue light illumination (R900B100) decreased WUE across all genotypes due to mostly disproportionate increases in g_{sw} relative to photosynthetic rate (A). WUE decreased by approximately 21% in WT and 18-23% in transgenic lines. Notably, under abaxial illumination, transgenic lines maintained significantly higher WUE compared to WT under both light conditions, with 1OE4 maintaining 20-25% higher WUE even after blue light stimulation, indicating sustained water use advantages in *EPF1* overexpressing lines despite typical blue light induced reductions seen in the WT (Fig.4D).

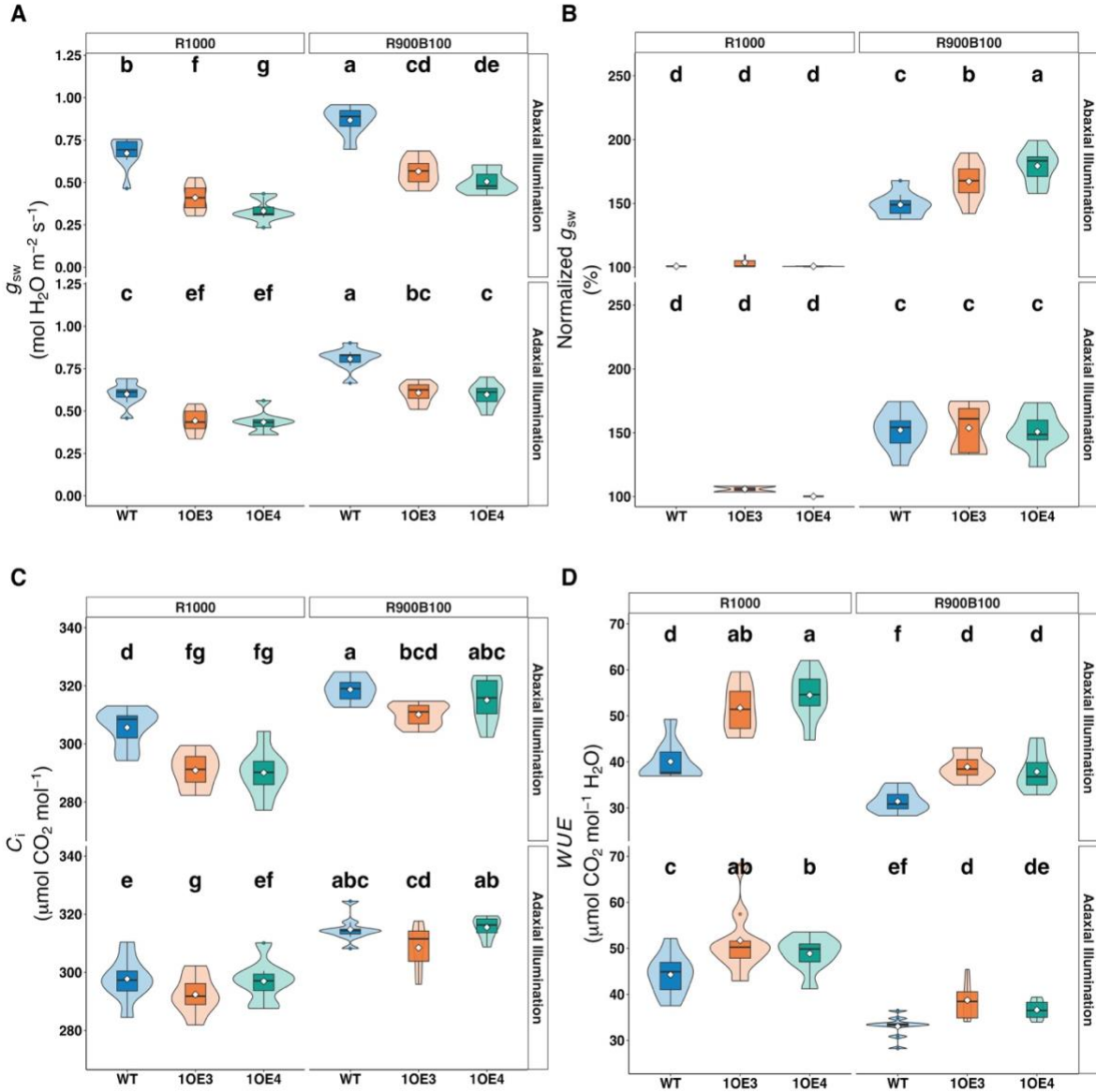


Fig.6.4 Steady-state gas exchange parameters in wild-type and EPF1-overexpressing wheat under different light conditions. (A) Absolute stomatal conductance (g_{sw}), (B) stomatal conductance normalized to R1000 values (set as 100%), showing relative increase under R900B100, (C) intercellular CO_2 concentration (C_i), and (D) water use efficiency (WUE , calculated as A/g_{sw}). Data are shown for WT (blue), 1OE3 (orange), and 1OE4 (teal) ($n = 8-9$ per genotype).

Different letters indicate statistically significant differences between groups ($p < 0.05$). Violin plots display probability density distributions with embedded narrow box plots showing median (center line), interquartile range (box limits), and $1.5 \times$ IQR whisker extensions, while red diamonds indicate arithmetic means and outliers are incorporated within the violin contours rather than displayed as separate points.

6.3.4 Abaxial surface specific effects of EPF1 overexpression on dynamic stomatal opening kinetics

Stomatal opening kinetics were quantified by fitting a sigmoidal model to gas exchange data under the red only light phase (R1000) and a subsequent mixed red/blue light phase (R900B100). This analysis yielded two key parameters: τ , the time required to achieve 63% of the total increase in stomatal conductance (g_{sw}), and Sl_{max} , the maximum slope of stomatal opening, which relates the effect on g_{sw} to the stomatal density. Under adaxial illumination during R1000, τ values were 6.43 ± 1.56 min in WT, 8.34 ± 1.48 min in line 1OE3, and 7.53 ± 1.62 min in line 1OE4 (Fig. 5a). Although the transgenic lines tended to exhibit slightly longer response times, these differences were not statistically significant ($p > 0.1$). Similarly, Sl_{max} under adaxial illumination during R1000 was not statistically different among genotypes. Additionally, no significant differences were observed for either parameter under R900B100 conditions on the adaxial surface ($p > 0.7$). In contrast to adaxial results, under abaxial illumination during the R1000 phase, τ values were similar across all genotypes. However, Sl_{max} differed significantly ($p = 0.000114$), with WT plants exhibiting an Sl_{max} of 0.496 ± 0.052 mol H₂O m⁻² s⁻¹ min⁻¹, whereas both transgenic

lines showed significantly reduced slopes (0.288 ± 0.041 in 1OE3 and 0.243 ± 0.102 in 1OE4), corresponding to reductions of approximately 42% and 51%, respectively, relative to WT. Under R900B100 on the abaxial surface, neither τ nor $S_{l_{max}}$ differed significantly among genotypes (Fig. 5B). While the overall timing of stomatal opening (τ) remains largely unaffected by EPF1 overexpression, the maximum slope of stomatal opening ($S_{l_{max}}$) is significantly affected in EPF1-overexpressing lines under abaxial illumination during high intensity red only light.

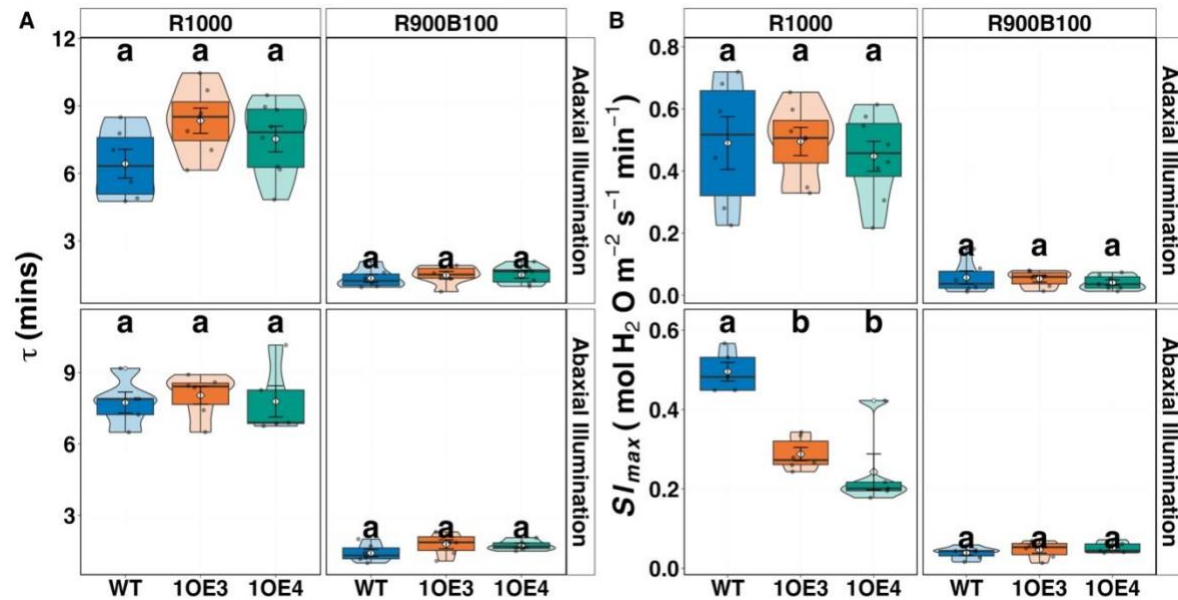


Fig 6.5. Surface specific dynamic Stomatal Opening Kinetics in WT (green), and EPF1 overexpressing lines 1OE3 (orange) and 1OE4 (teal) under R1000 or R900B100. (A) shows τ (τ), defined as the time to reach 63% of the total increase in stomatal conductance (g_{sw}), and (B) depicts $S_{l_{max}}$, the maximum rate of stomatal opening. Data are presented as violin plots overlaid with boxplots and mean $\pm$ SE. Different letters indicate statistically significant differences ($p < 0.05$).

6.3.5 Enhanced Stomatal Efficiency Under Blue Light Compensates For Reduced Density

To further assess whether individual stomata in EPF1-overexpressing lines exhibited altered functional capacity, we normalized g_{sw} responses (absolute value change as Δg_{sw}) by SD (Fig. 6.6). Under red light, stomatal efficiency ($\Delta g_{sw}/SD$) was significantly reduced in transgenic lines compared to WT. Adaxial illumination decreased efficiency by 7.4% in 1OE3 and 13.2% in 1OE4 relative to WT ($0.180 \pm 0.004 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1} \text{ mm}^{-2}$). The reduction was more evident when illumination was from the abaxial surface, where transgenic lines showed decreases of 16.4% (1OE3) and 22.0% (1OE4) compared to WT. Remarkably, blue light exposure revealed a dramatic compensatory response in transgenic lines. While WT plants showed modest increases in stomatal efficiency under blue light (0.00195 ± 0.0005 and $0.00438 \pm 0.001 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1} \text{ mm}^{-2}$ for adaxial and abaxial surfaces, respectively), transgenic lines exhibited surprising enhancements especially under abaxial illumination, with stomatal efficiency increased 165% in 1OE3 and 237% in 1OE4. The ratio of blue to red light responses within genotypes further highlighted this compensatory mechanism. While WT plants showed blue:red response ratios of 0.011 (adaxial) and 0.020 (abaxial), transgenic lines exhibited substantially higher ratios reaching 0.087 in 1OE4 under abaxial illumination which is a 4.3-fold increase compared to WT.

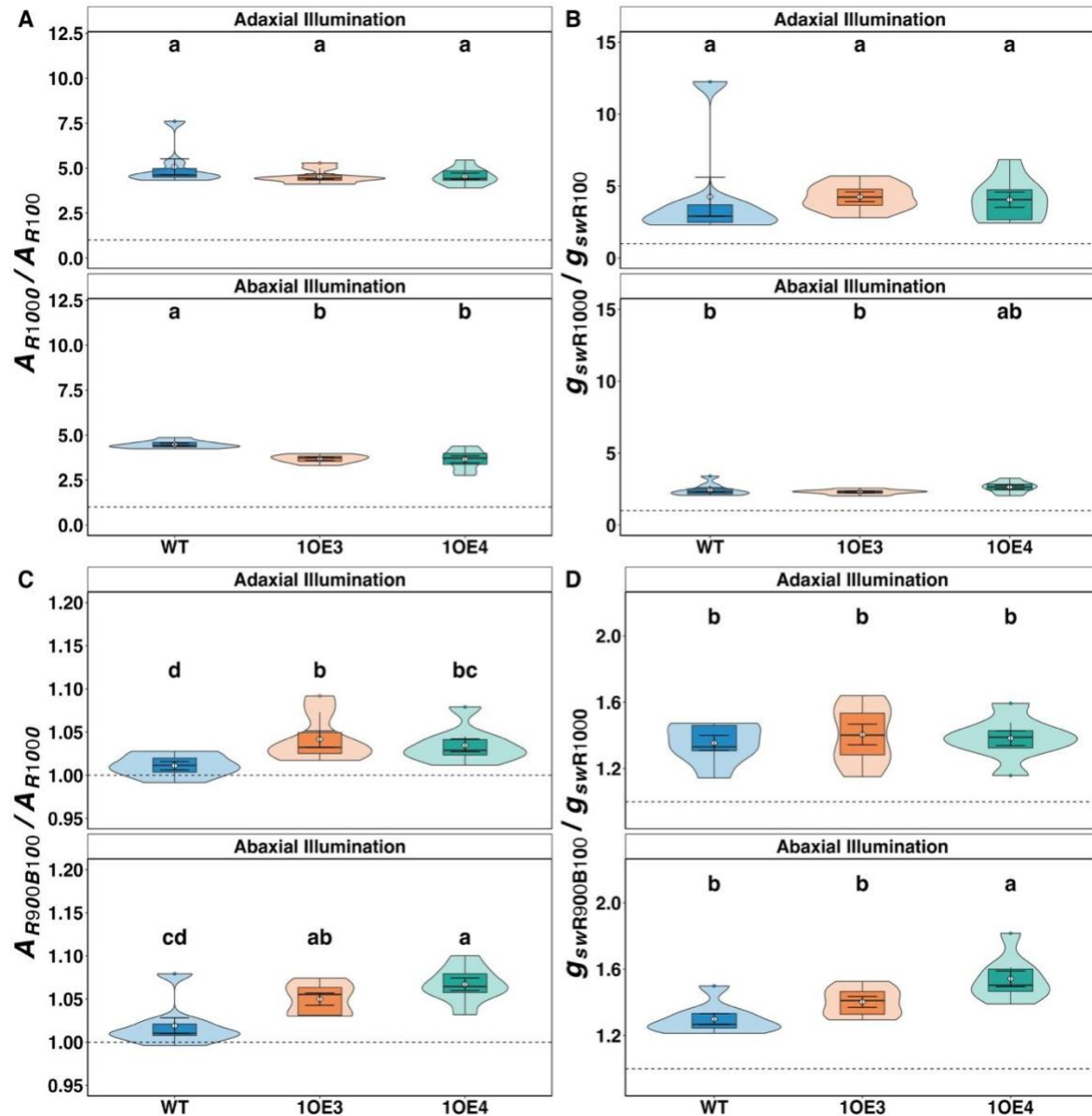


Fig 6.6. Light response ratios on differential red and blue light sensitivities.

(A) Photosynthetic rate ratio A_{R1000}/A_{R100} indicating red light response magnitude. **(B)** Stomatal conductance ratio g_{R1000}/g_{R100} . **(C)** Blue light modulation of photosynthesis ($A_{R900B100}/A_{R1000}$). **(D)** Blue light enhancement of stomatal conductance ($g_{R900B100}/g_{R1000}$). Violin plots display probability density distributions with embedded narrow box plots showing median (center line), interquartile range

(box limits), and 1.5× IQR whisker extensions, while red diamonds indicate arithmetic means and outliers are incorporated within the violin contours rather than displayed as separate points. Colors represent WT (blue), 1OE3 (orange), and 1OE4 (teal). Different lowercase letters indicate statistically significant differences ($p < 0.05$) determined by two-way ANOVA (genotype × surface) followed by Fisher's LSD post-hoc test ($n = 8-9$ per genotype).

6.4 Discussion

6.4.1 TaEPF1 overexpression differentially affects stomatal density on leaf surfaces

Our findings support the substantial evidence that TaEPF1 overexpression decreases stomatal density (*SD*), and the decrease was consistent with increased *EPF1* transcript levels in transgenic lines (Fig. 6.1A), aligning with earlier studies that have demonstrated EPF1 as key negative regulator of *SD* by inhibiting the formation of stomatal precursors (Hughes *et al.*, 2017; Caine *et al.*, 2019; Dunn *et al.*, 2019). Notably, here our study showed in wheat that overexpression of *EPF1* differentially influences *SD* on the abaxial and adaxial leaf surfaces, with a more pronounced decrease in *SD* on the abaxial surface (Fig. 6.1B). This differential response suggests either varied EPF1 expression or enhanced activity of inhibitory signal(s) and/or downstream effectors within this epidermal patterning domain. While distinct developmental pathways governing stomatal patterning across leaf surfaces are documented in dicots, including Arabidopsis Jalakas *et al.* (2024) less information is available for monocots. Recent research by Jalakas *et al.* (2024) further supports the notion that stomatal development is at least partly independently

regulated in the adaxial and abaxial epidermis and there is a need to identify key abaxial-specific regulators and key promoters (i.e., ICE Transcription factors, IDD16) (Lee *et al.*, 2017; Qi *et al.*, 2019; Zoulas *et al.*, 2021). This differential reduction in *SD* directly impacts anatomical maximum stomatal conductance (g_{swmax}), which provides a measure of the theoretical g_{sw} capacity based on anatomical features and assuming fully open pore apertures (Fig. 6.1C). Substantial reductions on the abaxial surface in the high expression line 1OE4 (Fig. 6.1C) demonstrates that reduced *SD* fundamentally constrains the maximum potential conductance for gas exchange with potential implications for physiology performance under variable environmental conditions (McElwain *et al.*, 2016; Pflüger *et al.*, 2024).

6.4.2 Consequence for gas exchange and photosynthetic performance

The reduction in *SD* observed in TaEPF1 overexpressing lines manifests directly in diminished g_{sw} and gas exchange. The reduced assimilation rate (*A*) and stomatal conductance (g_{sw}) under red light only condition (R1000), in the transgenic lines compared to WT (Fig. 6.2A&B), aligned with the well established relationships between *SD*, g_{sw} , and *A* (Franks & Beerling, 2009; Lawson, T & Blatt, MR, 2014) and is supported by the strong relationship between *A* and g_{sw} observed in both WT and transgenic lines, which demonstrates *A* dependence on g^{sw} (Suppl. Fig. 6.2). The anatomical constraints imposed by reduced *SD* and therefore g_{sw} on photosynthetic carbon gain are more apparent in the line with the greatest decrease in *SD* and when illumination was provided to the abaxial surface (Fig. 6.2). However, both transgenic lines also showed decreased

stomatal sensitivity to red light, and this was particularly evident when abaxial illuminated (Fig.6.6) suggesting both anatomical and functional limitations. Irrespective of the direction of illumination in the WT plants, A was near identical, demonstrating no difference in photosynthetic potential when light is received at either the abaxial or adaxial surface. Therefore, any decrease in A observed between the WT and transgenic lines is clearly driven by diffusional constraints as a result of low SD and reduced stomatal sensitivity resulting in reduced g_{sw} , confirming a fundamental constraint for gas exchange capacity (Suppl. Fig. 6.2). It is well established that low g_{sw} can limit CO_2 uptake for photosynthesis when consumption capacity is high (Wong *et al.*, 1979; Lawson *et al.*, 1998), reducing C_i and therefore photosynthesis (Morison & Lawson, 2007; Morison *et al.*, 2007).

Several studies have demonstrated that plants with altered SD maintain similar g_{sw} values to plants with no alterations in density, as changes in pore aperture compensated for any change in density (Bussis *et al.*, 2006; Lunn *et al.*, 2024). Rather than fully compensating for reduced numbers by increasing stomata aperture the transgenic lines maintain a more conservative g_{sw} and reduced sensitivity (Fig. 6.6). This more conservative stomatal operation resulted in the improved WUE observed in both EPF overexpression lines (Fig. 6.4D). These findings raise questions regarding the mechanism driving such responses. Previous studies have demonstrated that stomata on the abaxial surface are more responsive to light transmitted through the leaf (Wang *et al.*, 2008) impacting the overall g_{sw} response. The fact that much larger differences in g_{sw} were observed when illumination was provided from the abaxial surface suggests a mesophyll signal is involved in these responses (Lawson, T & Blatt, M, 2014). Numerous reports have

suggested that mesophyll signal(s) driven by mesophyll photosynthesis control stomatal opening (Lee & Bowling, 1992; Lee & Bowling, 1995; Messinger *et al.*, 2006; Fujita *et al.*, 2013) and are a key component to the red-light response (Lawson *et al.*, 2018). The importance of this unknown signal has been demonstrated comparing stomatal responses in epidermal peels to intact material (Fujita *et al.*, 2013), and in floating peels in solutions with illuminating mesophyll cells or chloroplasts (Lee & Bowling, 1992; Lee & Bowling, 1995), and epidermal-mesophyll transfer experiments (Mott *et al.*, 2008; Fujita *et al.*, 2013; Fujita *et al.*, 2019; Santos *et al.*, 2021). However, the same was not observed in WT plants suggesting some additional constraints in the EPF lines, driven by possible differences in stomatal density, size or stomatal sensitivity in the transgenics.

6.4.3 Enhanced Stomatal Blue Light responsiveness acts as a compensatory mechanism

Perhaps the most significant finding of this study is the reduced stomatal red-light responsiveness and enhanced responsiveness to blue light (R900B100) in EPF1 overexpressors particularly under abaxial illumination (Fig. 6.2D & 3B; Fig. 6.6). Under red light stomata efficiency ($\Delta g_{sw}/SD$) was greatly reduced in the transgenic lines compared with WT irrespective of surface illumination, although the extent of the decrease was greatest when abaxial illuminated, where the drawn down of C_i was greatest (Fig. 6.4C), confirming recent studies showing equal C_i dependent and C_i independent stomatal responses to red light (Taylor *et al.*, 2024). The larger percentage increase in g_{sw} observed in the transgenic lines with blue light was more than double (in

10E4) that of the increase observed in the WT. This heightened responsiveness also coincides with significantly lower C_i in both transgenic lines under steady state conditions (Fig. 6.4C), suggesting that enhanced blue light sensitivity may serve as a compensatory mechanism for photosynthetic limitations imposed by reduced SD.

Our findings align well with earlier observations that stomata on the abaxial epidermis tend to be inherently more light sensitive to specific wavelengths (Travis & Mansfield, 1981). However, this sensitivity appears to be species specific, with no difference in sensitivity between abaxial and adaxial stomata reported for *Vicia faba* (Yera *et al.*, 1986) and cotton (Lu *et al.*, 1993). Wang *et al.* (2008) using a lab-built gas exchange system capable of measuring light responses of two leaf surfaces independently, showed that the sensitivity to light, especially blue light, was higher in the abaxial stomata than the adaxial stomata in sunflower, supporting our observations. It has been proposed that surface-specific sensitivity particularly to blue light is due to a higher concentration of the blue light photoreceptors in abaxial than adaxial guard cells (Goh *et al.*, 1995), and/or that guard cells retain phot1 in the plasma membrane after exposure to blue light more than other cell types (Wana *et al.*, 2007; Wang *et al.*, 2008). Such observations likely underpin the rapid and robust blue light-induced stomatal opening observed here, especially on the abaxial surface, where EPF1 overexpression caused a more pronounced reduction in SD and hence a greater relative demand for compensatory opening (Büßis *et al.*, 2006). Interestingly, here we show reduced stomatal sensitivity to red light when SD is lower, contradicting those studies that have suggested function compensates for anatomical changes (Büßis *et al.*, 2006). These findings point towards a complex relationship between SD and function in such compensatory responses (Fig. 6.6).

It is well established that there is a close relationship between A and g_{sw} that is driven by mesophyll demands for CO_2 (Wong *et al.*, 1979; Xiong *et al.*, 2018; Sakoda *et al.*, 2020), driven by changes in C_i and other unknown C_i -independent signal(s) (Lawson *et al.*, 2014; Santelia & Lawson, 2016; Lawson *et al.*, 2018; Taylor *et al.*, 2024). Although this ‘mesophyll signal’ is associated with the red light, and the blue light response is considered independent, several studies have reported direct links between the two pathways. HIGH TEMPERATURE 1 (HT1) which is involved in both the red light responses (Matrosova *et al.*, 2015) and CO_2 signalling in guard cells (Pankasem *et al.*, 2024), has been shown to be involved in activation of the MAP3K Kinase CONVERGENCE OF BLUE LIGHT AND CO_2 1 and 2 (CBC1/CBC2) involved in blue light stomatal opening (Takahashi *et al.*, 2022). Hiyama *et al.* (2017a) further showed that CBC1/2 kinases integrate both blue light and CO_2 signals, by inhibiting S-type anion channels, facilitating stomatal opening by integrating signals from blue light and low C_i .

The consistently low C_i observed in the EPF1 overexpressing lines under red-only light (R1000) was insufficient to drive sufficient aperture changes to compensate for reduced density due to reduced sensitivity to red light (Fig. 6.6), when abaxially illuminated, however, subsequent blue induced stomatal opening, was able to compensate for this red light limitation imposed by lower SD and sensitivity (Fig. 6.6). This supports the integration of blue light and red light signalling in guard cells for regulating stomatal opening and that it is the integration of this signalling response that determines g_{sw} . Our observation that the reduction in red light sensitivity and magnitude of blue light response associated inversely with stomatal

density, with the greater differences observed in the 1OE4 lines with the lowest abaxial stomatal density supports an integrated signal.

Our dynamic analysis of stomatal opening kinetics further supports this. While the time required to achieve 63% of maximal conductance (τ) remained consistent across genotypes, the maximum increase in stomatal conductance (S_{max}) was reduced in transgenic lines, particularly during abaxial illumination with red only light (R1000). This kinetic trade-off reflects the inherent limitation imposed by both reduced number of stomatal units and reduced sensitivity (Fig. 6.6), while the speed of stomatal opening (τ) is maintained, the overall magnitude of change in conductance is constrained by fewer pores and lower aperture under red light. This is important in fluctuating light environments where both rapid and overall magnitudes of response are critical for maximizing CO₂ uptake (Shimazaki, K-i *et al.*, 2007; Violet-Chabrand & Lawson, 2019). However, the enhanced blue light response observed in the EPF1 overexpressors compensates for the reduced opening kinetics under red light, thereby supporting sustained photosynthetic performance. These findings highlight the importance of the stomatal blue light responses for maintain photosynthetic potential (Matthews *et al.*, 2019) and the complexity and potential co-ordination between stomatal red and blue light responses.

An alternative possible explanation for the differential responses between the two surfaces could also be the differences in the [CO₂] or C_i concentrations at those leaf surfaces. Recently Askanbayeva *et al.* (2024) used ¹³C abundance from epicuticular waxes from hypostomatous species to show lower [CO₂] at the adaxial surface compared

with the abaxial surface, whilst in amphistomatous species (such as wheat), $[CO_2]$ at each surface was dependant on SD . When illumination is directed at the abaxial surface as in our experimental protocol, this could create a particularly strong CO_2 limitation scenario in EPF1 overexpressing lines due to the substantially reduced abaxial SD (Fig. 6.1B) and altered ratio of adaxial to abaxial stomatal distribution (Fig. 6.1D). The resulting steeper CO_2 gradient in transgenic lines under abaxial illumination could trigger more robust mesophyll to guard cell signalling cascades specifically directly on this surface. Further research is needed to quantify these hypothesized CO_2 gradients across leaf tissues with altered AD/AB SD ratios (Márquez *et al.*, 2023).

6.4.4 Trade Off Between Carbon Assimilation and Water Conservation

Our findings also contribute substantial insight to the fundamental trade-off between carbon assimilation and water conservation (Fig. 6.4D). Whilst EPF1 overexpressors exhibited reduced assimilation rates due to constrained g_{sw} (Fig. 6.2A&C), these anatomical limitations were accompanied by improvements in water use efficiency (WUE). It is remarkable how the transgenic lines achieved a consistent 15–20% advantage in WUE compared to WT even following blue light application (Fig. 6.4D), which is typically associated with reduced WUE due to disproportionate increases in g_{sw} (Matthews *et al.*, 2020; Violet-Chabrand *et al.*, 2021). This trade-off holds particular relevance for drought tolerance mechanisms and illustrates the complexity of interactions between anatomical and functional traits in g_{sw} behaviour and the potential to exploit these for improved crop performance. Plants exhibiting reduced SD coupled with enhanced WUE likely

demonstrate superior adaptation to water limited environments, effectively minimizing transpirational water loss while maintaining adequate photosynthetic capacity during optimal light periods. Similar *WUE* enhancements following genetic manipulation of stomatal density have been documented across various crop species including wheat and rice plants (Franks *et al.*, 2015; Caine *et al.*, 2019; Dunn *et al.*, 2019). Importantly, our work extends these observations to differential surface specific patterning and impact on responses in wheat under different spectral compositions. While supporting that EPF1 overexpression represents a viable strategy for developing cultivars with enhanced drought resilience, we also show the possibility of developing future drought resistant or photosynthetically efficient cultivars by manipulating genes including but not limited to EPF family but doing so in a leaf surface specific manner.

Chapter 7: Engineering Faster Stomata through guard cell specific overexpression of H⁺-ATPase Improves Photosynthesis and Biomass Performance Under Natural Fluctuating Light

Keywords: Dynamic photosynthesis, Fluctuating Light, Stomata conductance, Guard cell, Tobacco, photosynthesis, Diurnal Carbon Assimilation, Light Spectral

Running Title: Enhanced stomata boost dynamic photosynthesis

Abstract (Chapter 7)

Natural light fluctuates stochastically with seconds scale dynamics while plant responses require minutes, constraining photosynthesis from the theoretical maximum capacity. Previous dynamic photosynthesis studies limited from fundamental artifacts by exposing constant light grown plants to fluctuating measurements. We developed a biologically informed stochastic light program (133 steps, 87.6% coefficient of variation, mimicking canopy dynamics) applied identically during both growth and gas exchange measurements to eliminating acclimation confounds. Using this ecologically relevant framework, we demonstrated that guard cell specific overexpression of Arabidopsis H⁺-ATPase (*AHA2*) in *Nicotiana tabacum* overcomes evolutionary constraints on stomatal-photosynthetic coordination by reducing transition performance deficits by 60% (17.3% to 6.9%) and accelerated photosynthetic responses by 36%, while maintaining higher intercellular CO₂ across all light intensities. Unexpectedly, enhanced H⁺-ATPase expression abolished blue light enhancement of photosynthetic induction which revealing saturation of wavelength dependent signalling pathways. These physiological improvements translated to biomass increases despite 22-33% higher transpiration. This combination of methodological innovation and targeted molecular enhancement provides a transformative approach for improving crop productivity under realistic light environments.

7.1 Introduction

Photosynthesis in nature operates at a mere 60-90% of its theoretical capacity, with fluctuating light environments imposing productivity penalties that cost global agriculture billions of dollars annually (Taylor & Long, 2017; Morales & Kaiser, 2020). This inefficiency emerges from a fundamental mismatch between environmental changes and physiological responses. While light intensity changes within seconds due to cloud movement, wind-induced canopy shifts, and solar angle variations, physiological responses including photosynthesis and stomatal conductance require minutes to hours for full adjustment (Lawson, T & Blatt, M, 2014; Kaiser *et al.*, 2015; Kromdijk, J. *et al.*, 2016; Muller *et al.*, 2018). The resulting temporal discord between environmental dynamics and plant adaptation represents one of the most tractable yet underexploited opportunities for enhancing crop productivity in a golden era of climate uncertainty and food security challenges.

The coordination between stomatal movements and photosynthetic biochemistry constitutes the primary constraint limiting plant performance under dynamic light conditions. Stomata typically require 5-30 minutes to reach new steady states following light changes, while photosynthetic electron transport responds within seconds and RuBisCO activation within minutes (Lawson, T & Blatt, M, 2014; McAusland *et al.*, 2016; Vialet-Chabrand, SR *et al.*, 2017; Lawson & Vialet-Chabrand, 2019; Osborne & Zhou, 2024). This non-synchronization creates cascading inefficiencies, for example, during periods of increasing light intensity, CO₂ supply limits photosynthesis until stomata open sufficiently, while during slow stomatal closure, excessive water loss continues until a new steady state is reached. Quantitative analyses reveal that these coordination failures

account for 10-40% reductions in daily carbon gain compared to theoretical steady state performance (Violet-Chabrand *et al.*, 2017). The complexity deepens when considering spectral quality, as blue light triggers rapid stomatal opening through phototropins mediated H^+ -ATPase phosphorylation within minutes, while red light drives slower responses through mesophyll derived signals and guard cell photosynthesis (Shimazaki, K *et al.*, 2007; Ando & Kinoshita, 2018; Matthews *et al.*, 2020; Bernardo *et al.*, 2023). Recent discoveries identified the plasma membrane H^+ -ATPase as one of the master regulators with dual phosphorylation at Thr-881 (responding to both blue and red light) and Thr-948 (specifically requiring blue light) creating a molecular coincidence detector for spectral integration (Fuji *et al.*, 2024; Hayashi *et al.*, 2024).

Critical knowledge gaps have prevented targeted engineering of stomatal and photosynthetic coordination, particularly regarding wavelength specific responses under fluctuating conditions. First, no previous studies have matched growth and measurement light regimes when evaluating dynamic photosynthetic performance which could creating artifacts that obscure true physiological constraints (Matthews *et al.*, 2018). Second, while recent breakthroughs achieved impressive results through optogenetic manipulation (2.2-fold biomass increase (Papanatsiou, M *et al.*, 2019)) or whole plant H^+ -ATPase overexpression (33% yield gain; (Zhang, M *et al.*, 2021)), these approaches either require synthetic biology tools incompatible with current crop transformation pipelines or cause pleiotropic effects from constitutive expression. Third, and most critically, the interaction between H^+ -ATPase expression levels and spectral sensitivity remains unexplored. Natural variation studies in C3 and C4 species revealed differential blue light responsiveness (Violet-Chabrand *et al.*, 2021) linked to evolutionary pressures (Bernardo

et al., 2023), yet how molecular enhancement of stomatal machinery affects spectral signal integration during dynamic light transitions remains unknown. This gap is particularly relevant given that agricultural lighting increasingly uses mixed spectra, and climate change might alter canopy light quality through shifting atmospheric conditions (Hofmann *et al.*, 2025).

Here, we test whether targeted overexpression of *Arabidopsis* H⁺-ATPase (AHA2) specifically in guard cells of *Nicotiana tabacum* can overcome evolutionary constraints on stomatal photosynthetic coordination under fluctuating light conditions while revealing how molecular enhancement affects spectral signal processing. Using a novel stochastic light program that mimics natural canopy dynamics during both plant growth and gas exchange measurements, we demonstrate that the enhancement of H⁺-ATPase transcript abundance not only accelerates response kinetics and reduces transition performance deficits but fundamentally alters wavelength dependent regulation.

7.2 Materials and Methods

Transgenic Plant Materials Generation

Transgenic *Nicotiana tabacum* L. lines were generated using Golden Gate cloning for modular assembly. The *Arabidopsis thaliana* H⁺-ATPase gene (AT4G30190) was codon optimized for *Nicotiana tabacum* and synthesized by Twist Bioscience (CA, United States), was assembled with the guard cell-specific potato *KST1* promoter and HSP18.2 terminator sequence (Plesch *et al.*, 2001; de Felippes *et al.*, 2022). This expression cassette was then cloned into the pAGM4723 binary vector containing the *NPTII* kanamycin resistance marker driven by the CaMV 35S promoter. Following *Agrobacterium tumefaciens* mediated transformation, transgene copy number was determined by quantitative PCR of the *NPTII* gene to identify single copy insertion events, and T1 plants exhibiting single copy insertions were self-pollinated and leaf discs submitted to iDNA Genomics (Norwich, UK) for segregation analysis using the same qPCR method on DNA for the *NPTII* marker. Plants identified as homozygous in the T2 generation (2 copies) were established as stable transgenic lines. From this population of homozygous lines, A31 and A32 were selected for detailed physiological characterization in this study. Azygous segregants (AZ) from the same transformation event served as genetically matched negative controls.

Functional expression of the H⁺-ATPase transgene was validated through quantitative RT-PCR analysis using a tissue specific normalization. Total RNA was extracted from whole leaf discs using the RNeasy Plant Mini Kit (Qiagen) with DNase I treatment, followed by integrity verification and purity assessment through spectrophotometric analysis (NanoDrop 2000, Thermo Scientific). First strand cDNA synthesized using the

UltraScript cDNA Synthesis Kit (PCR Biosystems) with integrated RNase inhibitor. Quantitative PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, United States) using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, UK), with thermal cycling parameters of 95°C for 2 minutes initial denaturation followed by 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. Critically, AHA gene specific primers AHA2-F (5'-ATGGCTGCTGAAGCTGAAGA-3') and AHA2-R (5'-TCACTTGGCAGCATCAGAGA-3') were designed to exclusively amplify the codon optimized *AtAHA2* transgene without cross amplification of endogenous tobacco *AHA* genes to ensure specificity for transgene derived transcripts. Expression normalization was achieved using *NtGORK* which encodes a guard cell-specific outward rectifying potassium channel, amplified with primers NtGORK-F (5'-TCCGTGTTTGTGAACTCTGC-3') and NtGORK-R (5'-ACTCCACTTGCTTCACACGA-3'), as this housekeeping gene choice enabled quantification of transgene expression relative to guard cell abundance within whole leaf samples. Melt curve analysis (65-95°C, 0.5°C increments) was used to confirm single product amplification for all primer pairs.

Seeds of selected lines were surface sterilized with 70% ethanol (1 min) followed by 10% sodium hypochlorite (10 min), then research grade potting media. After days post germination, uniform seedlings were transplanted to 96L pots and grown in walk-in controlled environment chambers (Aralab, Portugal) for four weeks. Environmental parameters were maintained at 25 °C (day/night), 60 ± 8% relative humidity and with no CO₂ concentration enrichment. Plants were grown under our generated natural fluctuating light program (described below) using HelioSpectra DYNA LED arrays with programmable spectral (5700k) and intensity control.

Natural Fluctuating Light Program Generator

We developed a simple biologically informed algorithm to generate naturalistic light fluctuation patterns while maintaining user determinable daily light integrals (DLI). The algorithm uses a temporal modulation function that mimics diurnal solar patterns with superimposed stochastic variation. We have also developed a web based interface for generating custom natural light programs and is available as [Natural Light Generator v3](#) hosted on Streamlit. The temporal modulation function $M(\tau)$ for normalized time $\tau \in [0, 1]$ was defined as:

$$M(\tau) = \begin{cases} 2\tau, & 0 \leq \tau < 0.5, \\ [4pt] 2(1 - \tau), & 0.5 \leq \tau \leq 1. \end{cases}$$

This function generates a symmetric triangular profile peaking at solar noon ($\tau = 0.5$) with $M(0.5) = 1.0$. Therefore, the instantaneous photosynthetic photon flux density (PPFD) values were generated stochastically:

$$\text{PPFD}_i \sim U[I_{\min}, I_{\max}M(\tau_i)]$$

where $U[a,b]$ denotes a uniform random distribution over interval $[a,b]$. For example, the light program generated and used for this study has $I_{\min} = 16 \mu\text{mol m}^{-2} \text{s}^{-1}$ (twilight conditions), $I_{\max} = 1384 \mu\text{mol m}^{-2} \text{s}^{-1}$ (peak sunlight), and $\tau_i = i/N$ represents the normalized time for step i out of N total steps.

To achieve the target DLI that is representative of typical species habitats (i.e., shade-tolerant, sun-like) and is user customizable, a normalization factor α was applied:

$$\alpha = \frac{DLI_{target}}{DLI_{initial}}, \text{ PPFD}_{final,i} = \min(\alpha \text{PPFD}_i, I_{max})$$

where $DLI_{initial}$ was calculated via trapezoidal integration with $\Delta t = 300$ s (5 minutes) set as default.

The generated light program used in this study consisted of 133 sequential PPFD steps (5 minutes each, 11.08-hour photoperiod) executed using HelioSpectra DYNA LED growth modules (Text file downloadable from our web based interface). Light uniformity across the growth area was verified by quantum sensor grid measurements. The light program used in this study has a PPFD range of 16-1,384 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and has a coefficient of variation of 87.6% with a total daily light integral of 17.1 $\text{mol m}^{-2} \text{d}^{-1}$. The kinetics of the light program has an average transition of 8.2 significant changes ($>100 \mu\text{mol m}^{-2} \text{s}^{-1}$) per hour.

Diurnal Gas Exchange Measurement

Leaf gas exchange was measured using 9 LI-6800 Portable Photosynthesis Systems (LI-COR Biosciences) equipped with a 6 cm^2 chamber head and multiphase flash fluorometer. Chamber conditions were controlled at 25°C leaf temperature, 400 $\mu\text{mol mol}^{-1} \text{CO}_2$, 70% relative humidity, and 500 $\mu\text{mol s}^{-1}$ flow rate. During diurnal measurements, the LI-6800 light source replicated the same intensity patterns from the growth fluctuating light program using compatible Light generator's exported JSON file in an "AutoGen-Loop" program. Each leaf was measured twice on separate days under identical PPFD sequences but different spectral compositions: pure red light (625 nm) or a red:blue mixture (90% red at 625 nm, 10% blue at 475 nm). Measurement order was randomized

across genotypes and days to minimize systematic bias. Data were logged at 5-second intervals throughout the photoperiod (7,990 data points per leaf measurement).

CO₂ Response Curves

Photosynthetic CO₂ response curves were measured under saturating light (1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 25°C. CO₂ concentrations were varied in the sequence: 400, 300, 200, 100, 50, 400, 600, 800, 1,000, 1,200, 1,500 $\mu\text{mol mol}^{-1}$. Steady state was achieved before recording. CO₂ response curves were analysed using the Farquhar-von Caemmerer-Berry model to derive V_{cmax} and J_{max} through the [Premium ACi Fitting Tool V1](#) .

Data Analysis

Raw data files (>313,000 observations) were processed first with the exclusion of the first 30 seconds following each light transitions to eliminate transient artifacts from H₂O related instabilities. Daily carbon gain and water loss were computed by numerically integrating the time course of A and E using the composite trapezoidal rule. The plant water economy was quantified as Intrinsic water use efficiency (A/g_{sw}). The degree of stomatal limitation was quantified using the C_i/C_a ratio framework established by Flexas *et al.* (2008). Light response curves were fitted to non-rectangular hyperbola equation to derive diurnal A_{max} (light-saturated rate).

Dynamic photosynthetic responses were analyzed for all step changes in PPFD exceeding 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For each such transition, the response time (t_{90}) was defined as the time required for A to reach 90 % of its new steady-state value. Response amplitude (RA) was calculated as:

$$RA = \frac{|\Delta A|}{|\Delta PPFD|} [(\text{molCO}_2\text{m}^{-2}\text{s}^{-1})/(\text{mol photons m}^{-2}\text{s}^{-1})]$$

where ΔA is the change in assimilation and $\Delta PPFD$ is the change in incident light.

Response speed (RS) was defined as $1/t_{90}$ (s^{-1})

Transition performance deficit (TPD) was quantified to assess carbon losses during dynamic light conditions. Theoretical steady state photosynthesis (A_{Steady}) was calculated using fitted non-rectangular hyperbola parameters for all observed light intensities. Transition periods were identified as measurements occurring within 5 minutes following any light change exceeding $50 \mu\text{mol m}^{-2} \text{s}^{-1}$. The instantaneous performance deficit was calculated as:

$$TPD = \frac{A_{Steady} - A_{Actual}}{A_{Steady}} \times 100 (\%)$$

where A_{Actual} represents measured photosynthesis during the transition period. Only positive deficits where $A_{Steady} > 0$ were included in the analysis. Mean TPD values were calculated across all valid transition periods to quantify the integrated carbon loss penalty associated with photosynthetic lag during fluctuating illumination.

Multispectral 3D Growth analysis

After gas exchange measurement, non-destructive 3D multispectral imaging was performed using a PlantEye F600 system (Phenospex, the Netherlands). Plants were scanned at harvest to generate 3D point clouds for plant architecture and spectral reflectance properties to measure parameters including total leaf area (mm^2), plant height

(mm), and NDVI (Normalized Difference Vegetation Index). Plants were harvested and dry weight were determined after oven drying at 80 °C for 96 hours. To provide a holistic assessment of plant performance at harvest, an integrated performance score (*IPS*) was calculated as the geometric mean of four normalized metrics, where each metric (biomass, leaf area, height, and NDVI) was normalized to the AZ control value (1.0). The fourth-root exponent was applied to the value to ensure that improvements in all parameters contribute equally to the overall score as follows:

$$IPS = (Biomass_{norm} \times Area_{norm} \times Height_{norm} \times NDVI_{norm})^{\frac{1}{4}}$$

Statistical Analysis

Analyses were performed in Python 3.13 using scipy, statsmodels, and scikit-learn. Linear mixed effects models were fitted using REML with genotype and treatment (spectral mix setting during gas exchange measurement) as fixed effects and plant ID as random effect. Post hoc comparisons were performed with Tukey's HSD test. When parametric assumptions were violated or sample sizes were small ($n < 15$), non-parametric Kruskal-Wallis tests were performed followed by Mann-Whitney U tests for pairwise comparisons. Multiple comparison corrections utilized the Holm-Bonferroni method. Principal component analysis was performed on z-score standardized physiological parameters using scikit-learn's PCA with StandardScaler preprocessing. Then the components with eigenvalues > 1 (Kaiser criterion) were retained for interpretation. Correlation network analysis was performed using Pearson coefficients with network edges included for $|r| > 0.15$ and $p < 0.05$ and visualized using networkx. Statistical significance was set at $\alpha = 0.05$ for all tests, with data presented as mean $\pm$ SEM unless otherwise stated.

7.3 Results

7.3.1 Natural Fluctuating Light Program for Field Light Dynamics

To establish a physiologically relevant experimental system, a stochastic light program comprising 133 discrete 5-minute steps over an 11.08-hour photoperiod was generated, with intensities ranging from twilight conditions ($16 \mu\text{mol m}^{-2} \text{s}^{-1}$) to peak sunlight ($1,384 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Figure 7.1A). The program's temporal structure followed a bimodal function with $M(\tau) = 2\tau$ for morning ($0 \leq \tau < 0.5$) and $M(\tau) = 2(1-\tau)$ for afternoon ($0.5 \leq \tau \leq 1$) to create naturalistic diurnal patterns with peak intensity occurring at 5.4 hours (step 65). The coefficient of variation (70.1%) substantially exceeded typical controlled environment studies while aligning with temperate forest edge to tropical understory conditions. Analysis of light intensity distribution revealed that plants in this study experienced low light conditions ($<200 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 25.6% of the photoperiod (170 minutes), medium intensities ($200\text{--}800 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 60.9% (405 minutes), and high light conditions ($\geq 800 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 13.5% (90 minutes) (Figure 7.1B). This distribution ensured substantial exposure to both light-limited and light-saturating conditions, with the majority of time spent in the transitional zone where stomatal regulation is most critical. This light program also generated 7.0 significant light transitions per hour ($|\Delta\text{PPFD}| > 100 \mu\text{mol m}^{-2} \text{s}^{-1}$), with 78 major transitions throughout the photoperiod. The largest recorded increase was $925 \mu\text{mol m}^{-2} \text{s}^{-1}$ and the largest decrease was $-1,113 \mu\text{mol m}^{-2} \text{s}^{-1}$, creating rapid fluctuations that challenge photosynthetic acclimation. The daily light integral totalled 17.1

mol m⁻² d⁻¹, representative of a moderately shaded to semi-open canopy environment (Figure 7.1C). Having established our fluctuating light program as both growth condition and measurement protocol, we next characterized the molecular and physiological outcomes of guard cell specific H⁺-ATPase overexpression

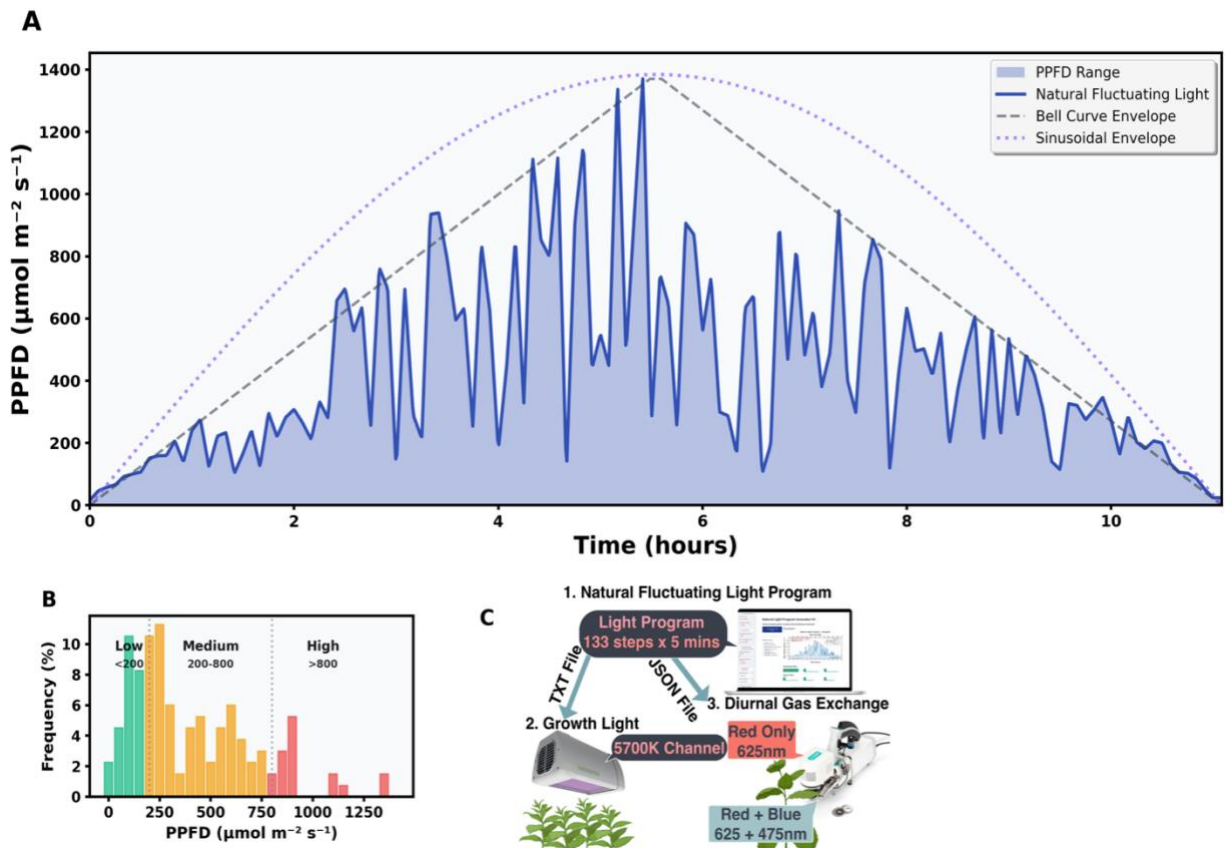


Figure 7.1. Natural fluctuating light program and experimental design. (A) Temporal profile of photosynthetic photon flux density (PPFD) throughout an 11.08-hour photoperiod. Solid line represents the programmed PPFD values, and dashed line indicates the theoretical bell curve envelope. **(B)** Frequency distribution histogram of PPFD values binned at 50 μmol m⁻² s⁻¹ intervals. Bars are color coded by light intensity zones as green (low, <200), orange (medium, 200-800), and red (high, >800 μmol m⁻²

s⁻¹). **(C)** Schematic representation of experimental implementation from growth light to gas exchange measurement using output from the Natural light generator.

7.3.2 Guard Cell-Specific H⁺-ATPase Overexpression Enhances Transcript Abundance Without Affecting Photosynthetic Biochemistry

Quantitative RT-PCR analysis of leaf disc epidermal preparations revealed substantial increases in AHA2 transcript abundance. Transgenic line A31 exhibited 151.0 ± 3.9 -fold higher expression relative to azygous controls, while A32 showed 172.4 ± 8.5 -fold enhancement ($F_{2,4} = 297.5$, $P = 3.6 \times 10^{-4}$; Figure 7.2A). Expression was normalized to the guard cell-specific GORK potassium channel reference gene to ensure tissue specific quantification. No significant difference existed between A31 and A32 expression levels ($P = 0.150$), suggesting comparable transgene activity despite independent insertion events.

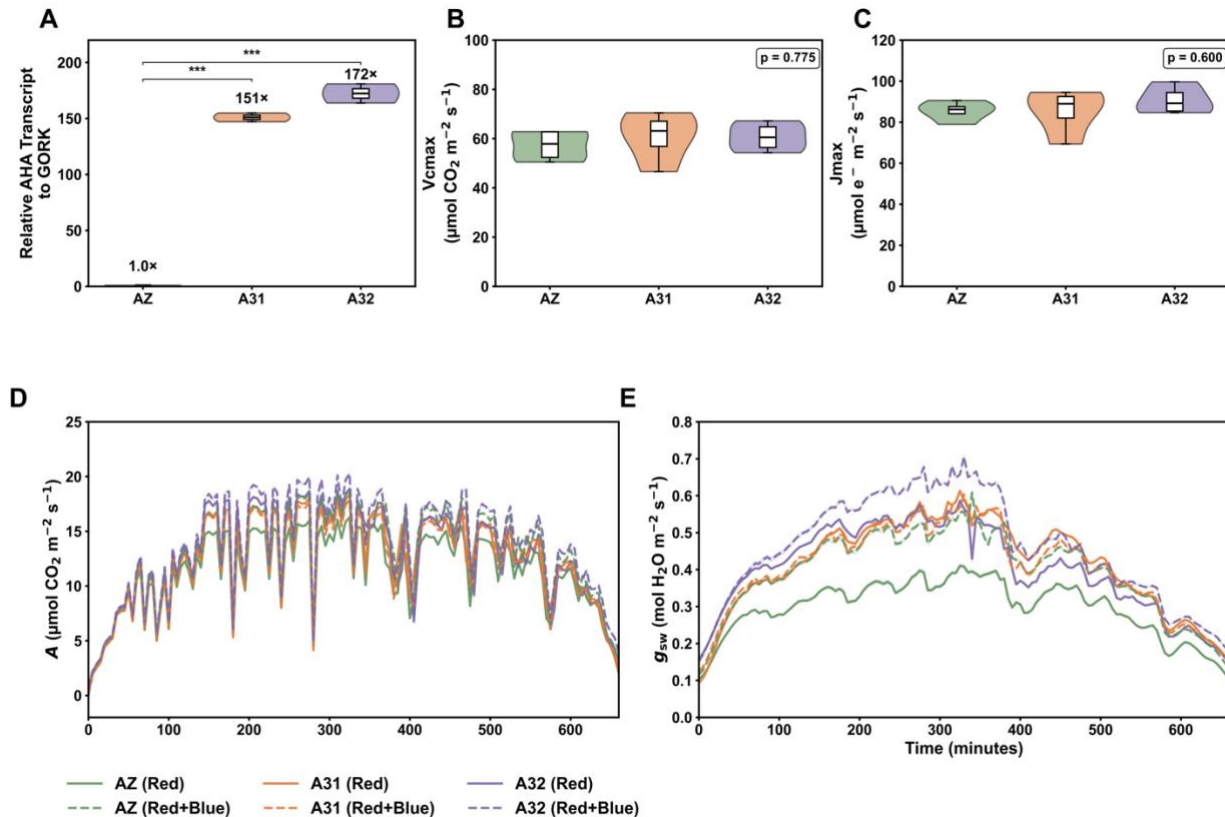


Figure 7.2. Guard cell specific H^+ -ATPase overexpression enhances transcript abundance and gas exchange performance without altering photosynthetic biochemical capacity. (A) Relative transcript abundance of H^+ -ATPase determined by quantitative RT-PCR normalized to *NtGORK* reference gene with azygous control set to 1.0. (B,C) Photosynthetic biochemical capacity parameters derived from CO_2 response curves in mean $\pm$ SEM. (D,E) Diurnal time series of gas exchange parameters measured under the fluctuating light program. Line colours: green (AZ), orange (A31), purple (A32). Solid lines represent red light treatment (625 nm) and dashed lines represent red+blue treatment (625+475 nm, 9:1 ratio). Each line represents 5 seconds resolution measurements averaged across 6-8 plants per genotype-treatment combination.

Biochemical characterization of the CO₂ response curves confirmed that enhanced H⁺-ATPase expression did not affect mesophyll photosynthetic capacity. Maximum carboxylation rates (V_{cmax}) determined from $A-C_i$ curves at saturating light (1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$) remained statistically identical across genotypes: $57.3 \pm 3.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ (AZ), 60.8 ± 5.2 (A31), and 60.6 ± 3.0 (A32) ($F_{2,9} = 0.26$, $P = 0.775$; Figure 2B). Similarly, maximum electron transport rates (J_{max}) showed no significant differences: 85.5 ± 2.4 (AZ), 85.5 ± 5.6 (A31), and $90.7 \pm 3.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ (A32) ($F_{2,9} = 0.54$, $P = 0.600$; Figure 2C). The $J_{max}:V_{cmax}$ ratio remained conserved at 1.41-1.50 across genotypes, indicating maintained stoichiometry between carboxylation and electron transport capacities.

Diurnal gas exchange measurements under the same fluctuating light program used for growth revealed consistently enhanced photosynthetic rates (A) and stomatal conductance (g_{sw}) in transgenic lines throughout the 11.08 hour photoperiod (Figure 7.2D & E). Peak photosynthetic rates occurred at 5.4 hours coinciding with maximum light intensity, reaching 18.4-18.9 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ under red light and 17.9-20.3 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ under red+blue illumination for transgenic lines, compared to 16.3 and 18.8 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ respectively for azygous controls. Peak stomatal conductance values demonstrated more pronounced enhancements with transgenic lines achieved 0.586-0.706 $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ versus 0.410-0.608 $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ in AZ, representing 20-40% increases depending on spectral conditions. All genotypes reached 50% of daily maximum photosynthesis within 50 minutes of illumination onset, indicating comparable induction kinetics at the whole-plant measurement scale.

7.3.3 Enhanced Stomatal Conductance Drives Complex Water Relations Under Fluctuating Light

Stomatal conductance (g_{sw}) across light intensity bins further revealed systematic genotype dependent responses with progressive differentiation at higher irradiances (Figure 7.3A). Under low light conditions ($0-200 \mu\text{mol m}^{-2} \text{s}^{-1}$), g_{sw} values showed moderate enhancement in transgenic lines: $0.283-0.294 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ (red light) compared to $0.216 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ in azygous controls. This differential expanded significantly at saturating intensities ($800-1,400 \mu\text{mol m}^{-2} \text{s}^{-1}$), where transgenic lines achieved in average $0.477-0.507$ (red) and $0.548-0.580$ (red+blue) $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ compared to 0.352 and $0.518 \text{ mol H}_2\text{O m}^{-2} \text{s}^{-1}$ in AZ which representing 36-44% and 6-

12% enhancements under red and red+blue illumination, respectively. All light intensity categories showed highly significant genotype effects ($F > 1373$, $P < 0.001$). Intrinsic water use efficiency (*iWUE*) exhibited unexpected diurnal patterns distinct from typical morning maxima (Figure 7.3B). Peak *iWUE* values occurred late in the photoperiod (hour 9), with azygous controls (AZ) under red light achieving the highest absolute values ($52.5 \pm 0.3 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$) compared to transgenic lines ($40.6\text{-}41.7 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$). Contrary to expected patterns, afternoon *iWUE* exceeded morning values by 11-32% across all genotype-treatment combinations, suggesting sustained photosynthetic performance despite prolonged stomatal opening. Blue light supplementation consistently reduced *iWUE*, particularly in azygous controls (19% reduction), while both transgenic lines showed minimal spectral sensitivity. Additionally, C_i/C_a ratios revealed enhanced CO_2 diffusion in transgenic lines across all light intensities (Figure 7.3C). At low light, all genotypes maintained high C_i/C_a values ($0.877\text{-}0.899$), indicating minimal stomatal constraint. As irradiance increased, C_i/C_a declined more steeply in AZ controls, stabilizing at 0.798 ± 0.001 under saturating conditions ($>1,000 \mu\text{mol m}^{-2} \text{ s}^{-1}$). Transgenic lines maintained significantly higher values of $0.829\text{-}0.830$ ($F_{2,4500} = 187.4$, $P < 0.001$), compared to the commonly reported 0.7 for C3 plants under steady-state conditions. This differential CO_2 supply optimization persisted across the entire light gradient, with transgenic advantages most seen at intermediate intensities ($400\text{-}800 \mu\text{mol m}^{-2} \text{ s}^{-1}$).

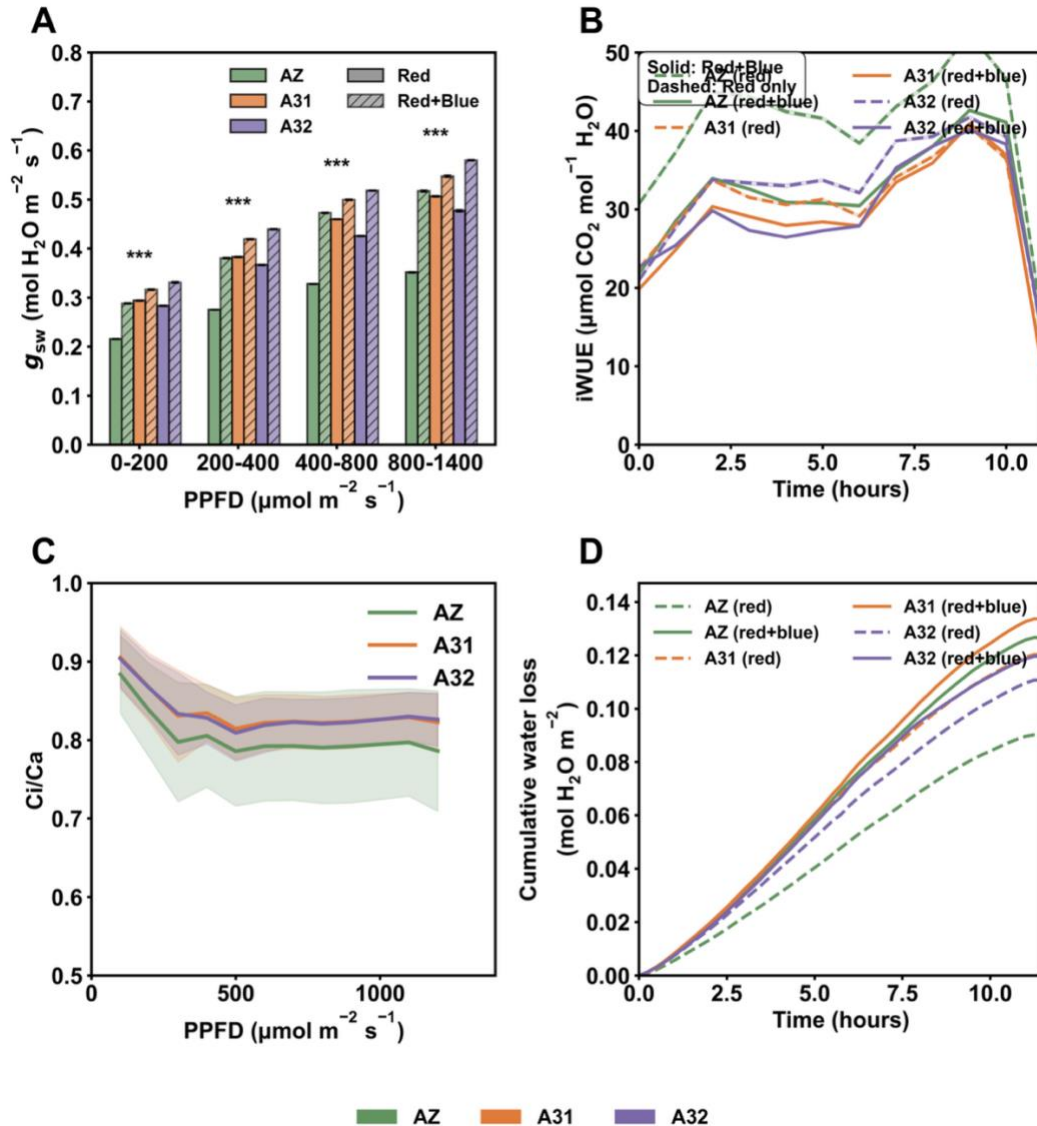


Figure 7.3. Stomatal dynamics and water relations throughout the diurnal cycle.

(A) Stomatal conductance responses binned by PPFD intensity categories. Bars show mean $\pm$ SEM for red (solid) and red+blue (hatched) light treatments. **(B)** Time course of intrinsic water use efficiency (A/g_{sw}) with 30 minute moving averages. **(C)** Relationship between intercellular to ambient CO_2 ratio (C_i/C_a) and incident PPFD. Lines represent means with 95% confidence intervals shown as shaded areas. **(D)** Cumulative transpirational water loss integrated over the photoperiod.

Cumulative water loss calculation further revealed significant differences in transpirational dynamics between genotypes (Figure 7.3D). Integration of transpiration rates over the photoperiod demonstrated that transgenic lines exhibited enhanced water flux, with A31 and A32 accumulating 0.120 ± 0.004 and 0.111 ± 0.007 mol H₂O m⁻² respectively under red illumination which represented 33.3% and 22.7% increases compared to azygous controls (0.090 ± 0.007 mol H₂O m⁻²; $F_{2,11} = 5.76$, $P = 0.019$). However, under combined red+blue illumination, transpirational differences were diminished with all genotypes converging to similar cumulative water loss values (0.120 - 0.134 mol H₂O m⁻²; $F_{2,11} = 0.55$, $P = 0.590$). Enhanced stomatal conductance observed in transgenic lines increased water consumption by 22-33% under red light. However, this water penalty disappeared under red + blue illumination, where all genotypes showed similar transpiration rates.

7.3.4 Light Response Optimization Yields Modest Integrated Performance Gains

Non-rectangular hyperbola modelling was performed over total 268,193 diurnal observations and have found increased light saturated photosynthesis (A_{max}) from 17.0 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ in AZ controls to 20.0 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (A31) and 19.8 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ (A32) under red illumination. Notably, with blue light in the measurement light (90%Red+10%Blue) elevated AZ control performance to 19.4 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, approaching transgenic capacity under red light alone. Transgenic line under red+blue mix had minimal in A31 (20.2 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and higher in A32 (21.4 $\mu\text{mol CO}_2 \text{ m}^{-2}$

s⁻¹). Apparent quantum yields remained remarkably conserved across genotypes and light ($\alpha = 0.056\text{-}0.060$ mol CO₂/mol photons) (Figure 7.4C), confirming that enhanced stomatal conductance did not compromise photochemical efficiency. Light compensation points exhibited substantial variation (7.6-16.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) without clear genotypic patterns, while dark respiration rates remained consistently low (0.45-0.94 $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$), indicating minimal metabolic costs (at the whole plant level) associated with constitutive H⁺-ATPase activity.

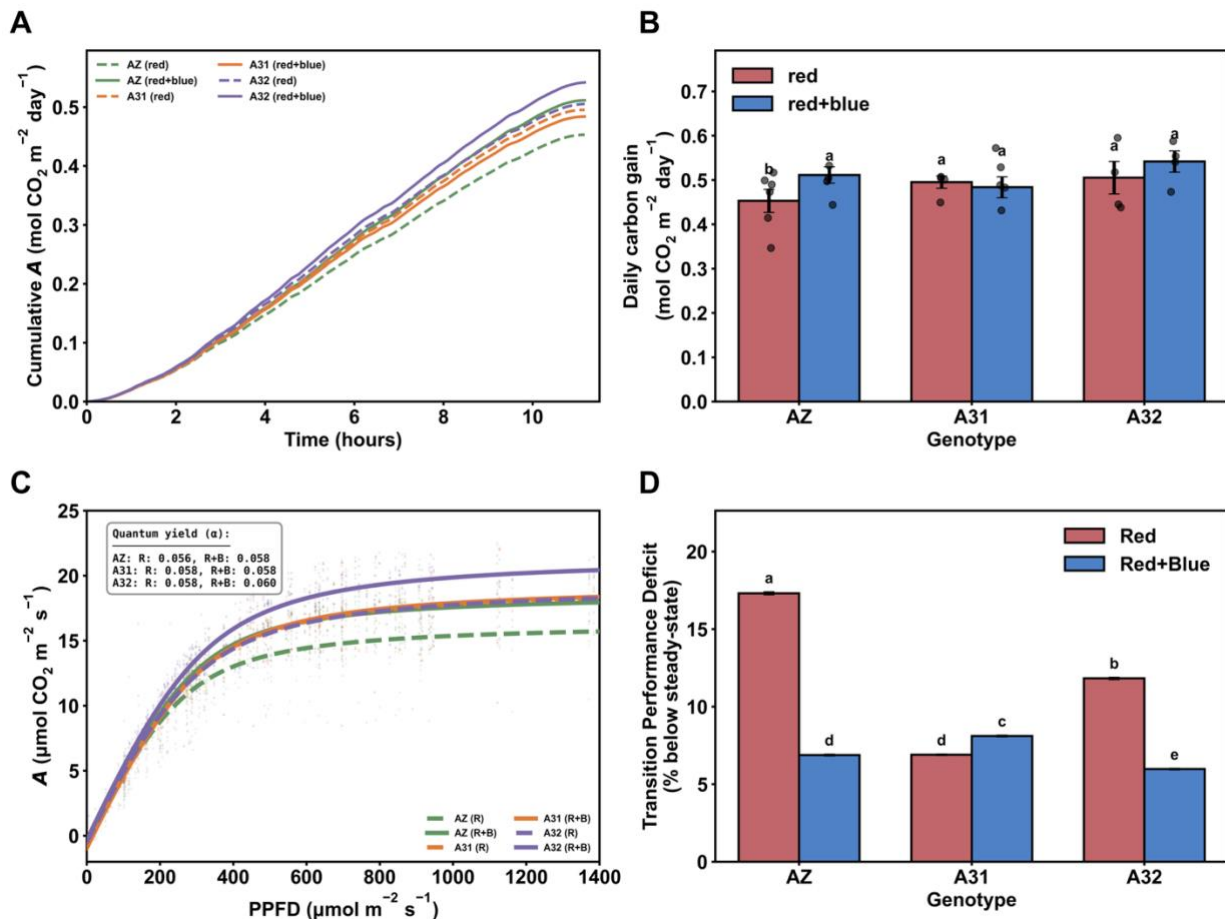


Figure 7.4. Integrated daily performance and photosynthetic light responses. (A) Cumulative carbon assimilation throughout the photoperiod. **(B)** Daily integrated carbon gain from individual plant measurements. Bars indicate means $\pm$ SEM **(C)** Light response curves fitted to non-rectangular hyperbola model. Scatter points represent 50-observation bins and lines show model fits. **(D)** Transition performance deficit (TPD) to quantify carbon loss during light transitions. Different letters indicate statistically distinct groups ($p < 0.05$).

Temporal carbon accumulation profiles revealed progressive divergence between genotypes throughout the photoperiod (Figure 7.4A). Initial rates (0-2 hours) showed minimal differentiation ($0.054\text{--}0.059 \text{ mol CO}_2 \text{ m}^{-2}$), with performance gaps emerging during sustained illumination. By midday photoperiod (6 hours into the diurnal gas exchange measurement), transgenic lines demonstrated 9.2-13.3% greater carbon accumulation relative to controls. Final integrated daily carbon gain ranged from $0.453 \text{ mol CO}_2 \text{ m}^{-2} \text{ d}^{-1}$ (AZ, red) to $0.542 \text{ mol CO}_2 \text{ m}^{-2} \text{ d}^{-1}$ (A32, red+blue) which represented a maximal enhancement of 19.6% from transgenic line. Transgenic lines under monochromatic red illumination achieved carbon gains of 0.491 ± 0.014 (A31) and $0.499 \pm 0.037 \text{ mol CO}_2 \text{ m}^{-2} \text{ d}^{-1}$ (A32), representing 7.4% and 9.3% improvements over AZ controls. These enhancements matched or exceeded the 8.3% improvement observed when azygous plants received blue light supplementation ($0.495 \pm 0.019 \text{ mol CO}_2 \text{ m}^{-2} \text{ d}^{-1}$). Critically, transgenic lines measured under red and blue mix yielded minimal further benefit ($p=0.398$).

Analysis of transition performance deficits (*TPD*) revealed pronounced genotypic differences in photosynthetic kinetics during diurnal long fluctuating light conditions. AZ controls under red illumination exhibited the highest *TPD* ($17.3 \pm 0.1\%$), indicating substantial carbon loss during light transitions. Guard cell-targeted H^+ -ATPase overexpression dramatically reduced these losses, with A31 demonstrating a 60% reduction in *TPD* ($6.9 \pm 0.1\%$, $p < 0.0001$) and A32 showing a 32% reduction ($11.8 \pm 0.1\%$, $p < 0.0001$) relative to AZ controls. Blue light supplementation consistently improved transition associated carbon losses across all genotypes, reducing AZ *TPD* to $6.9 \pm 0.1\%$ but less effective in transgenic plant (A31: $8.1 \pm 0.1\%$; A32: $7.3 \pm 0.1\%$).

7.3.5 Dynamic Photosynthetic and Stomatal Kinetics Under Fluctuating Light Reveal Accelerated Responses in H^+ -ATPase Overexpressing Lines

High resolution kinetic analysis of 3,967 light transitions from our diurnal measurement across 36 experimental runs revealed significant genotype dependent variations in photosynthetic and stomatal response dynamics (Figure 7.5). Time constants for 90% response achievement (t_{90}) following step increases in irradiance demonstrated accelerated photosynthetic induction in H^+ -ATPase overexpressing lines, with A32 achieving 35.8% faster responses than azygous controls under red illumination (2.48 ± 0.18 vs 3.37 ± 0.23 min; Kruskal-Wallis $H = 24.15$, $P = 0.0002$; Figure 7.5A). This acceleration was intermediate in A31 (2.74 ± 0.14 min) though not statistically different from AZ after Bonferroni correction ($P = 0.141$). Supplemental blue light paradoxically slowed photosynthetic induction in azygous controls by 27.6% (3.17 ± 0.16 min; $P = 0.002$) while exerting minimal effects on transgenic lines (A31: 2.44 ± 0.17 min; A32: 3.22 ± 0.20

min), suggesting altered blue light signal transduction in enhanced H⁺-ATPase backgrounds.

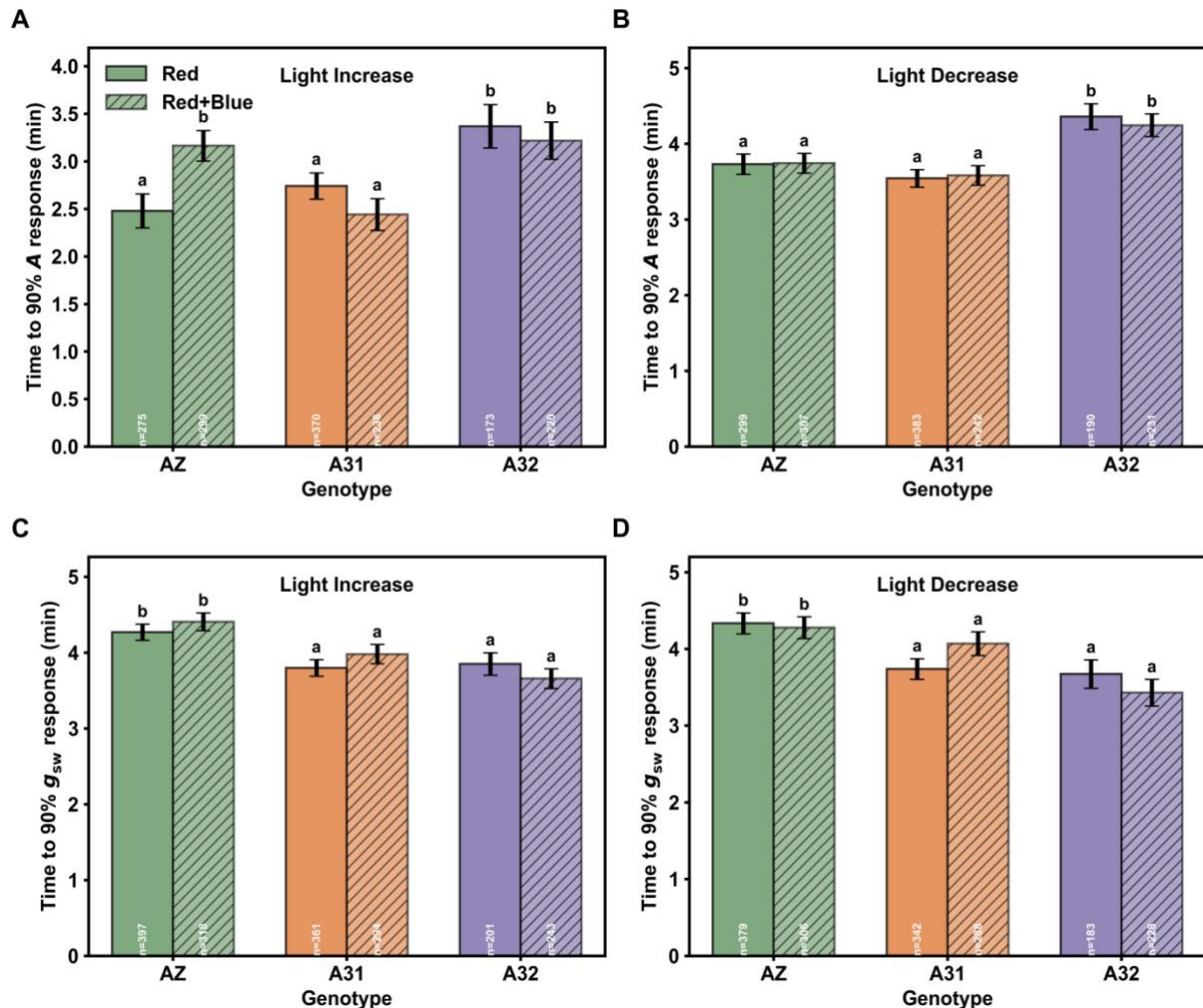


Figure 7.5. Photosynthetic and stomatal responses kinetics to dynamic light transitions. (A,B) Time constants (t_{90}) for photosynthetic responses to step increases and decreases in irradiance, respectively. Response times represent duration to achieve 90% of steady state change in CO₂ assimilation rate following transitions exceeding 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (C,D) Corresponding stomatal conductance response

times for light increases and decreases. Bar heights indicate population means $\pm$ SEM from kinetic analysis of 3,967 individual transitions across diurnal measurement runs (6 plants per genotype $\times$ 2 spectral treatments). Different letters denote significant differences within each panel.

Photosynthetic relaxation following step decreases in irradiance exhibited systematically slower kinetics across all genotypes, with t_{90} values ranging from 3.54 ± 0.12 min (A31, red) to 4.36 ± 0.17 min (A32, red), representing 1.3 to 1.5-fold longer response times compared to induction phases (Figure 7.5B). The asymmetry between induction and relaxation kinetics remained consistent across genotypes, indicating conserved deactivation mechanisms independent of H⁺-ATPase expression levels.

Stomatal conductance (g_{sw}) responses to increasing irradiance demonstrated enhanced kinetics in transgenic lines, with A31 and A32 achieving t_{90} values of 3.80 ± 0.11 and 3.85 ± 0.15 min respectively under red illumination, representing 11.0% and 9.8% accelerations relative to azygous controls (4.27 ± 0.11 min; Figure 7.5C). Unlike photosynthetic responses, blue light supplementation did not significantly modulate stomatal kinetics in any genotype ($P > 0.05$ for all within-genotype comparisons) suggesting wavelength independent regulation of dynamic stomatal responses. Stomatal relaxation following irradiance decreases showed similar acceleration patterns in transgenic lines (Figure 7.5D), with A32 demonstrating the fastest responses under combined red+blue illumination (3.43 ± 0.17 min).

7.3.6 Multi-dimensional Analysis Reveals Coordinated Physiological Enhancement with Complex Trade-offs

We calculated the Light use efficiency (*LUE*) or otherwise known as quantum yield to CO₂ assimilation and found the expected hyperbolic decline with increasing irradiance across all genotypes (Figure 7.6A). For example, under light limiting conditions (0-200 $\mu\text{mol m}^{-2} \text{s}^{-1}$), where quantum efficiency approaches theoretical maxima, transgenic lines showed variable responses with A31 maintained similar efficiency to AZ controls (44.2-45.5 vs 45.5-46.6 mmol CO₂/mol photons), while A32 exhibited enhanced efficiency under both spectral conditions (48.2-51.7 mmol CO₂/mol photons). This differential persisted across all light categories, with highly significant genotype effects ($F > 288$, $P < 10^{-267}$ for all bins). At saturating intensities ($>1200 \mu\text{mol m}^{-2} \text{s}^{-1}$), *LUE* converged to 11.6-14.6 mmol CO₂/mol photons across genotypes, with A32 maintaining the highest values. The preservation of enhanced quantum yield at low light intensities in A32 suggests improved light harvesting efficiency beyond stomatal effects alone. Temporal analysis of water use efficiency revealed unexpected diurnal patterns contradicting classical stomatal behaviour (Figure 6B). Rather than typical morning maxima followed by afternoon decline, all genotype treatment combinations showed increased *WUE* from morning to afternoon. Azygous controls exhibited 7.5% (red) to 20.5% (red+blue) increases, while transgenic responses varied with A31 showed 18.7% (red) and 9.5% (red+blue) increases, and A32 displayed rather intermediate responses (12.7% and 9.4%).

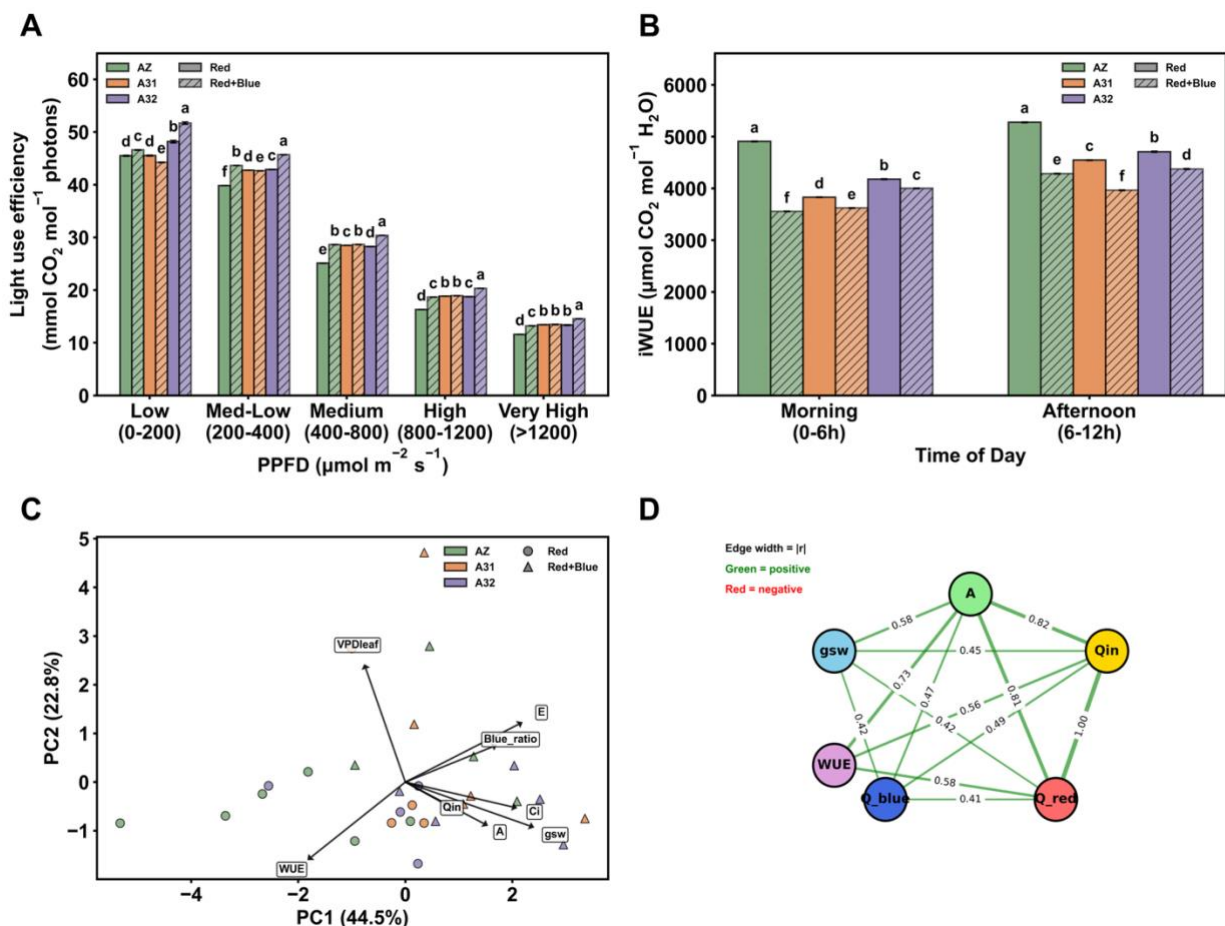


Figure 7.6. Multivariate physiological analysis reveals enhanced light use efficiency and altered stomatal optimization strategies in H⁺-ATPase overexpressors. (A) Light use efficiency (photosynthesis/PPFD × 1000) stratified by irradiance categories. Bars show population means ± SEM for each genotype-treatment combination. **(B)** Comparison of morning (0-6h) versus afternoon (6-11h) water use efficiency. **(C)** Principal component analysis biplot of eight physiological variables. Points represent individual plants and ellipses show 95% confidence intervals for

genotype clusters. Loading vectors scaled by explained variance. **(D)** Correlation network of physiological traits. Nodes sized by degree centrality with edge width proportional to Pearson correlation coefficient. Green edges, positive correlations; red edges, negative correlations. Only correlations with $|r| > 0.15$ and $P < 0.05$ shown.

Principal component analysis of eight physiological variables effectively segregated genotypes in multivariate trait space (Figure 7.6C). The first two components captured 67.3% of total variance (PC1: 44.5%, PC2: 22.8%), with PC1 showing high positive loadings for stomatal related parameters (gsw: 0.486, E: 0.442, Ci: 0.419) and negative loading for WUE (-0.368). Genotype separation along PC1 was strong as azygous controls clustered at -1.12, while transgenic lines occupied positive space (A31: 0.60, A32: 0.64), representing approximately 1.7 standard deviations of separation. Physiological trait network analysis revealed a highly interconnected system with 86.7% edge density at $|r| > 0.15$ threshold (Figure 7.6D). The correlation structure demonstrated tight coupling between photosynthesis and light parameters, with $A \times Q_{in}$ showing the strongest physiological relationship ($r = 0.822$) as expected. Photosynthesis and conductance coupling strengthened progressively from AZ controls ($r = 0.516$) through A31 (0.594) to A32 (0.632), suggesting enhanced coordination in transgenic lines. Notably, the typically negative gsw-WUE relationship observed in controls ($r = -0.169$) reversed to positive in A31 (0.208), suggesting fundamental alteration of stomatal optimization strategies. Network topology revealed no isolated nodes, with all traits connected through direct or indirect pathways which might confirm the systemic nature of H^+ -ATPase overexpression effects.

7.3.7 Physiological Enhancements Translate to Substantial but Statistically Marginal Biomass Gains

After four weeks of growth under fluctuating light conditions, comprehensive phenotyping revealed consistent numerical enhancements in transgenic lines (Figure 7.7A). Dry biomass accumulation reached 4.10 ± 0.36 g (A31) and 4.42 ± 0.36 g (A32) compared to 3.13 ± 0.33 g in azygous controls, representing 31.1% and 41.4% increases respectively (Figure 7B). While overall comparison showed marginal significance (Kruskal-Wallis $H = 4.65$, $P = 0.098$), focused hypothesis testing revealed A32 achieved significance using a one-tailed Mann-Whitney U test ($U = 1.0$, $P = 0.029$), consistent with the directional hypothesis of enhanced growth through improved stomatal function. 3D phenotypic analysis further revealed coordinated enhancement of plant architecture metrics. Total leaf area increased by 21.4% (A31: $72,420 \pm 8,439$ mm²) and 22.6% (A32: $73,091 \pm 773$ mm²) relative to controls ($59,634 \pm 4,511$ mm²), though high variability precluded statistical significance ($F_{2,9} = 1.87$, $P = 0.209$; Figure 7.7C). Plant height showed divergent responses between transgenic lines: A31 achieved 22.9% greater height (223 ± 14 vs 181 ± 12 mm), while A32 showed more modest 13.6% enhancement (206 ± 10 mm), approaching significance ($F_{2,9} = 3.04$, $P = 0.098$; Figure 7.7D). These architectural modifications suggest differential resource allocation strategies between transgenic lines despite similar AHA2 expression levels. Normalized Difference Vegetation Index (NDVI) values remained consistent across genotypes (0.658-0.667) with negligible differences ($F_{2,9} = 1.13$, $P = 0.364$; Figure 7.7E). This spectral uniformity suggest that enhanced growth resulted from improved gas exchange efficiency rather than altered chlorophyll content or canopy structure, consistent with guard cell-specific transgene expression

patterns. Interestingly, the relationship between mean photosynthetic rate during growth and final biomass yield demonstrated significant positive correlation ($r = 0.644$, $P = 0.024$), which validated gas exchange measurements as predictors of productivity (Figure 7.7F). Linear regression analysis ($\text{Biomass} = 1.129 \times A - 9.902$) explained 41.5% of biomass variance ($R^2 = 0.415$). Mean photosynthetic rates showed progressive enhancement from azygous controls ($11.6 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) through A31 (12.2) to A32 (12.8) which paralleled biomass accumulation patterns.

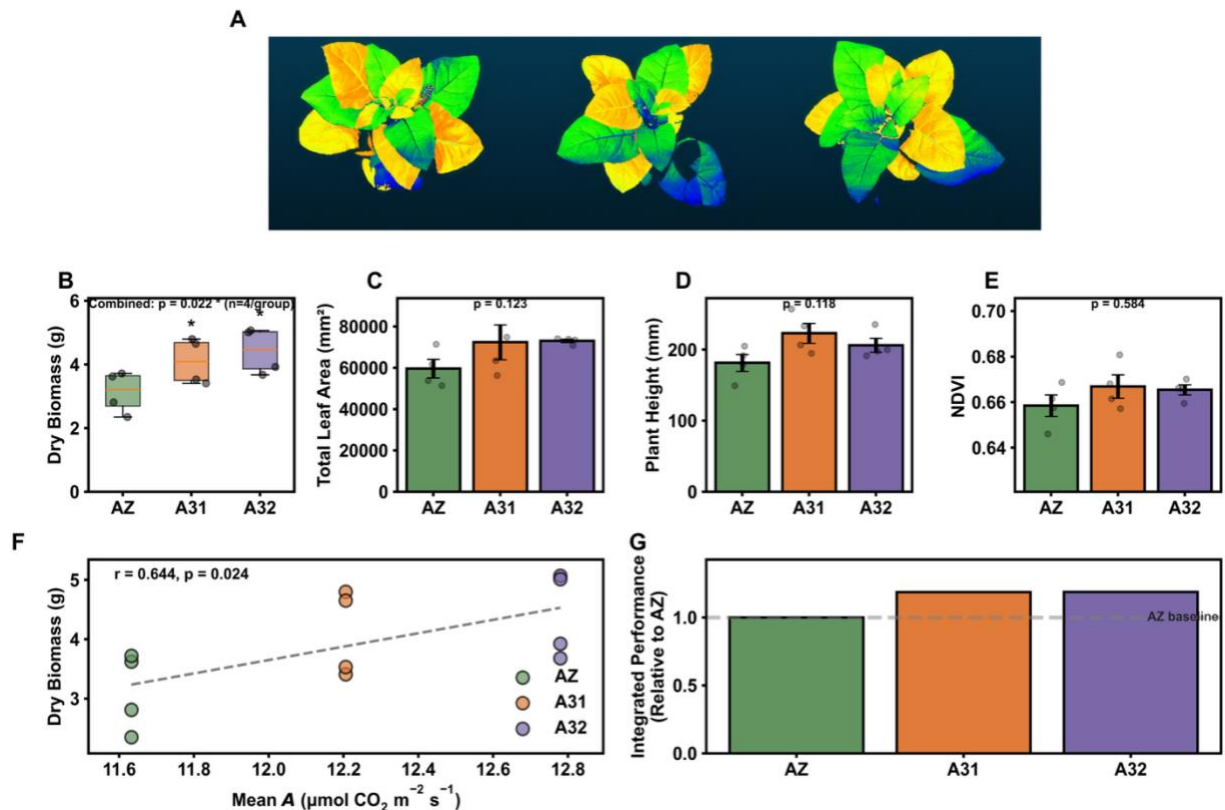


Figure 7.7. Harvest measurements and integrated growth performance. (A) Representative 3D plant reconstructions from multispectral imaging at harvest. **(B)** Dry biomass accumulation after 4 weeks growth. **(C)** Total projected leaf area from 3D

analysis. **(D)** Average plant height measurements. **(E)** Normalized Difference Vegetation Index (NDVI) **(F)** Relationship between photosynthetic rate and final biomass. Line shows linear regression with 95% confidence band. **(G)** Integrated performance index calculated as geometric mean of four normalized metrics relative to azygous baseline (horizontal line at 1.0). All measurements n = 4 plants per genotype.

Integrated performance assessment using geometric mean normalization of four phenotypic metrics (biomass, leaf area, height, NDVI) revealed nearly identical overall enhancement for both transgenic lines: 18.7% (A31) and 18.8% (A32) relative to azygous baseline (Figure 7.7G). This convergent performance despite divergent individual metrics suggests robustness of the H⁺-ATPase enhancement strategy across different developmental trajectories. The consistency between integrated scores and the magnitude of photosynthetic improvements suggests the translation of instantaneous physiological gains to longer term growth enhancement.

7.4 Discussion

7.4.1 Molecular Enhancement and Physiological Outcomes

The approximately 150-fold increase in AHA2 transcript abundance achieved through guard cell specific expression represents a fundamental shift in the stoichiometric constraints governing stomatal function. This dramatic enhancement contrasts with previous whole plant overexpression strategies that typically achieved 2-10 fold increases before encountering metabolic toxicity (Wang *et al.*, 2014; Zhang, M *et al.*, 2021). The tissue specific approach are essential, as wild-type guard cells already dedicate 10-20%

of plasma membrane protein to H⁺-ATPases (Jezek & Blatt, 2017) and our extreme overexpression likely saturates the available membrane space for additional pumps. Recent structural elucidation of the dual phosphorylation mechanism provides further molecular context for our physiological observations. The requirement for sequential phosphorylation at Thr948 followed by Thr881 creates a molecular coincidence detector that integrates multiple signals (Fuji *et al.*, 2024; Hayashi *et al.*, 2024). With vastly expanded H⁺-ATPase pools in the guard cells of our transgenic plants, even partial activation through either blue light induced BLUS1/PP1 signalling or red light mediated metabolic changes can generate substantial proton motive force, which would be expected to increase g_{sw} (Kinoshita & Shimazaki, 1999; Wang *et al.*, 2014; Inoue, SI & Kinoshita, T, 2017), which was observed across all measured light intensities (Figure 7.3A)

It is also worth noting that the preservation of WT V_{cmax} and J_{max} values (Figure 7.2B,C) from the CO₂ response curve measurements across genotypes despite dramatic changes in stomatal behavior is important. The maintained J_{max} : V_{cmax} ratio of 1.41-1.50 indicates balanced investment in light harvesting versus carbon fixation capacity, and most importantly suggesting that enhanced H⁺-ATPase expression does not trigger compensatory adjustments in photosynthetic biochemistry. This independence between stomatal and biochemical traits supports our genetic manipulation strategy of targeting and isolating diffusional limitations without disrupting the photosynthetic machinery. Although one might argue the energetic implications of sustained H⁺-ATPase activity, as each catalytic cycle consumes one ATP molecule, and with 150-fold more transcript abundance, the theoretical maximum ATP demand could exceed guard cell production capacity. The absence of growth penalties or metabolic stress symptoms indicates that

enhanced expression likely creates a larger reservoir of activatable pumps rather than constitutively active enzymes, though direct measurement of phosphorylation states would be required to confirm this hypothesis. This interpretation aligns with recent phosphoproteomics studies showing that H⁺-ATPase phosphorylation states, not total protein levels, primarily control activity (Nelson *et al.*, 2007; Gray *et al.*, 2015; Zhang *et al.*, 2018; Fuji *et al.*, 2024).

7.4.2 Improved transition performance under natural fluctuating light

The stochastically generated fluctuating light program revealed previously hidden constraints in photosynthetic performance that remain puzzling under conventional steady state or simple step changes dynamic measurements. The quantification of transition performance deficits (*TPD*) demonstrated that azygous controls lost 17.3% of potential carbon gain during dynamic illumination, which is a penalty that decreased by 60% in H⁺-ATPase overexpressing line A31. With correlation analysis confirming that stomatal conductance explained 67% of *TPD* variance (Figure 7.6D), while the remaining variance likely rose from biochemical limitations including Rubisco activation state and metabolite pool dynamics. This finding aligns with theoretical predictions that stomatal limitations could account for 10-40% of productivity losses under natural light fluctuations (Lawson, T & Blatt, MR, 2014; Taylor & Long, 2017), while extending beyond previous work by matching growth and measurement light regimes. Our methodological innovation of using identical 133 steps programs for both cultivation and gas exchange measurements eliminates the confounding effects reported in studies where plants adapted to constant light were suddenly exposed to fluctuations (Kaiser *et al.*, 2015; Violet-Chabrand, SR *et al.*, 2017). The coefficient of variation in our light program (87.6%)

substantially exceeded typical controlled environment studies while remaining within the range observed in temperate forest understories and crop canopies (Chazdon & Pearcy, 1991; Way & Pearcy, 2012). This ecological relevance are critical as plants grown under fluctuating conditions develop distinct phenotypes, including altered stomatal density, modified guard cell size, and enhanced metabolic flexibility (Matthews *et al.*, 2018). Our transgenic lines maintained a performance advantage despite these acclimation responses, suggesting that H⁺-ATPase enhancement confers benefits beyond simple increases in steady state conductance.

The mechanistic basis for reduced TPD likely involves multiple integrated processes. Enhanced stomatal conductance maintains higher chloroplast CO₂ concentrations during transitions which ensures Rubisco remains closer to CO₂ saturation when light intensity changes (Von Caemmerer & Farquhar, 1981). This buffering effect becomes particularly important during the critical first minutes following illumination increases, when photosynthetic biochemistry can respond more rapidly than stomatal mechanics. The 25-35% faster response times we observed in transgenic lines (Figure 7.5A) translate directly to captured photosynthetic potential that would otherwise be lost during the lag phase. Recent modelling work suggests that each 10% reduction in stomatal response time could yield 2-3% improvements in daily carbon gain under field conditions (Morales & Kaiser, 2020).

Furthermore, the maintenance of elevated C_i/C_a ratios throughout the light gradient (Figure 7.3C) indicates that our transgenic lines operate in a fundamentally different optimization space compared to wild type plants. Classical stomatal optimization theory developed from mostly steady state observations predict that plants should minimize

water loss per unit carbon gained (Cowan & Farquhar, 1977). However, under fluctuating conditions that are highly relevant to natural sunlight cultivation, the optimal strategy shifts toward maintaining higher baseline conductance to capture transient high light opportunities (Violet-Chabrand *et al.*, 2021). Our results empirically validate this theoretical framework while demonstrating that targeted molecular interventions can access this alternative optimization strategy.

7.4.3 Spectral quality effects on stomatal and carbon gain dynamics

The diminished blue light response in transgenic lines provides insights into signaling saturation. With H⁺-ATPase expression already enhanced 150-fold, the additional activation provided by blue light phototropin signaling may contribute minimally to overall pump activity. This interpretation is supported by the convergence of stomatal conductance values between red and red+blue treatments in transgenic lines (Figure 7.3A), contrasting with the maintained spectral sensitivity in azygous controls. Recent comparative studies across photosynthetic types provide evolutionary context for these spectral responses. C₄ plants show reduced blue light sensitivity compared to C₃ species, potentially due to their CO₂ concentrating mechanisms reducing the importance of rapid stomatal adjustments (Bernardo *et al.*, 2023). Our transgenic C₃ plants by maintaining elevated CO₂ delivery through enhanced conductance, may partially phenocopy this C₄-like state, though the enhanced red light responsiveness through expanded H⁺-ATPase pools likely contributes more than reduced blue sensitivity *per se*.

On the other hand, it is vital to reconciling performance gains with resource tradeoffs. The 22-33% increase in cumulative water loss under red light illumination (Figure 7.3D) represents the primary physiological cost of enhanced stomatal function. However, this

simplified accounting overlooks the other roles of transpiration in plant performance. Enhanced water flux drives proportional increases in nutrient delivery, with xylem sap carrying essential minerals from roots to shoots (Hawkins *et al.*, 2009; Thiry *et al.*, 2016). For example, the OSA1 rice field trials reported 33% yield increases alongside 46% improved nitrogen use efficiency (Kawai *et al.*, 2021; Zhang, M *et al.*, 2021) suggest that enhanced transpiration driven nutrient delivery may partially explain the growth benefits we observed. Furthermore, enhanced transpiration provides evaporative cooling that can maintain leaf temperatures below air temperature, potentially preserving photosynthetic efficiency during heat stress and potentially contribute to yield increases (Amani *et al.*, 1996; Richards *et al.*, 2010; Thapa *et al.*, 2018; Zhang *et al.*, 2023).

The unexpected afternoon increases in water use efficiency (Figure 7.3B) contradict classical diurnal patterns but align with emerging understanding of stomatal behaviour under fluctuating conditions. Traditional theory, developed from steady state measurements, predicts morning *WUE* maxima when vapor pressure deficits remain low. However, under dynamic light, the accumulated osmolytes and metabolites by afternoon may optimize turgor regulation and improve the photosynthesis-transpiration relationship (Buckley, 2019). This temporal shift in *WUE* optimization suggests that evaluation metrics for stomatal performance require reconsideration under realistic environmental conditions to minimize the variabilities caused by diurnal effects. Although our numerical but statistically marginal biomass increases (31-41%, $P = 0.098$) reflect the limited replication inherent to intensive physiological studies. Meta analyses of stomatal engineering experiments reveal that effect sizes of stomatal modifications typically diminish by 30-70% from greenhouse to field conditions due to environmental complexity (Bailey-Serres *et al.*,

2019). However, tissue specific modifications like the ones outlined here will likely show better field stability than constitutive approaches, as the correlation between instantaneous photosynthetic rates and final biomass after the gas exchange measurement ($r = 0.644$, Figure 7.7F) provides confidence that gas exchange improvements translate to growth enhancement, even if our sample size limited statistical power. The enhanced magnitude of stomata response observed in our study can enhance evaporative cooling which might suggest particular value for combination with heat sensitive biochemical enhancements. For example with accelerated Rubisco activation through thermostable activase variants addresses biochemical limitations (Scafaro *et al.*, 2019) while enhanced mesophyll conductance via aquaporin engineering could complement our stomatal improvements (Groszmann *et al.*, 2017).

Finally, our results contribute to mounting evidence that stomatal engineering represents a viable strategy for crop improvement under climate change. The successful examples now span multiple targets such as whole plant H^+ -ATPase overexpression achieving 33% yield gains in rice (Zhang, M *et al.*, 2021), optogenetic BLINK1 modifications yielded 2.2-fold biomass increases (Papanatsiou, M *et al.*, 2019), and potassium channel engineering enhanced drought tolerance while maintaining yields (Jezek & Blatt, 2017). Additionally, systems modelling predicts that optimizing multiple processes simultaneously could achieve 50-60% yield improvements under fluctuating field conditions (Wu *et al.*, 2019). Future optimization should also address the water use efficiency trade off through environmental response tuning. For example, creating phosphomimetic mutations to constitutively active H^+ -ATPases could be combined with enhanced ABA sensitivity to maintain drought responsiveness. Alternatively, optogenetic approaches allow precise

temporal control over stomatal aperture to potentially optimizing water use on hourly timescales. As climate variability predicted to increase, such dynamic optimization strategies may prove essential for maintaining productivity beyond just photosynthesis.

Conclusions

We demonstrated that guard cell targeted H^+ -ATPase overexpression substantially reduces photosynthetic transition penalties under ecologically relevant fluctuating light conditions. The combination of ~150-fold transcript enhancement with tissue specific expression created plants that operate in an alternative physiological space whilst maintaining higher baseline stomatal conductance while preserving environmental responsiveness. Our methodological innovation by matching growth and measurement natural fluctuating light regimes with stochastically generated fluctuations for the first time revealed performance constraints invisible to conventional approaches used in dynamic photosynthesis studies. This framework is easy to adapt as deployed as web application and should become standard practice for evaluating photosynthetic modifications intended for variable field environments. The unexpected spectral quality effects particularly blue light induced slowing of photosynthetic induction in wild type plants, highlight the complexity of wavelength interactions under dynamic conditions and suggest new avenues for spectral optimization in controlled environment agriculture. While water use increases represent a clear tradeoff, the potential enhancement of nutrient delivery and maintenance of productivity under current precipitation patterns suggest net benefits for many agricultural contexts. Field trials across multiple environments will ultimately determine the commercial viability of this approach. As we face the dual challenges of increasing food production and environmental sustainability, targeted molecular

interventions that enhance plant performance under realistic growth conditions (i.e., light dynamics) offer a promising research path forward.

Chapter 8: Thesis Conclusion

8.1 Thesis Findings

This thesis has demonstrated that blue light signalling serves as a multiscale regulatory mechanism in plants for controlling carbon and water balance. This mechanism operates through a hierarchical cascade beginning with photoreceptor activation and progressing through to overall plant performance. The integration of methodological innovations in imaging temperature response and anatomical characterization (Chapters 2 and 3), developmental plasticity analysis (Chapter 4), spectral delivery optimization (Chapter 5), the discovery of a blue light compensatory mechanism (Chapter 6), and molecular enhancement of natural fluctuating light response (Chapter 7) has yielded novel methods and insights into the mechanisms governing light perception and photosynthetic gas exchange coordination across multiple species (i.e., wheat, barley, tobacco, strawberry), with some critical to global food security.

The thermographic framework standardized in Chapter 2 standardised infrared imaging from a qualitative observation tool into a quantitative phenotyping platform at scale. The technical rigor established here aligns with recent advances showing that thermal imaging can detect genotypic variation in dynamic stomatal behaviour (McAusland *et al.*, 2014) with heritabilities comparable to destructive biochemical assays (Ferguson *et al.*, 2021; Pignon *et al.*, 2021). Our standardization protocols provide reproducible calibration methods, thereby establishing thermography as a legitimate high throughput phenotyping tool for stomatal traits. This method, along with the findings from the remaining chapters, will be highly useful in developing future climate resilient crops with altered gas exchange response patterns, such as optimized stomatal responses to blue light.

In chapter 3 the discovery of differential EPF1 effects between leaf surfaces represents more than anatomical curiosity, as it reveals fundamental constraints on how plants optimize gas exchange. The 59.5% reduction in abaxial stomatal density versus 19.9% adaxially in barley EPF1 overexpressors demonstrates that developmental signalling pathways exhibit surface specific sensitivities. The method innovation of using chlorophyll autofluorescence confocal microscopy has enabled, for the first time, large scale and high resolution quantification of over a million stomata, making spatial analysis relevant to physiological functions possible. The spatial heterogeneity analysis revealed 55% increased variance in stomatal distribution on EPF1 abaxial surfaces, which has important implications for water use efficiency under field conditions (Bertolino *et al.*, 2019).

Longitudinal tracking of blue light priming effect (Chapter 4) revealed that blue light operates through distinct temporal hierarchies of plant memory. The immediate biochemical responses with 35.4% J_{max} enhancement and 15.9% V_{cmax} increase under 35% blue light both dissipate within 30 days post priming. However, structural modifications persist; the 53.1% increase in stomatal density in high-blue-primed plants represents developmental commitments that might result in drought resilience months later. The drought protection mechanism appears multifaceted. While low blue primed plants showed 16% V_{cmax} reduction under water limitation, and high blue primed plants maintained capacity (2% reduction), suggesting persistent metabolic adaptation beyond visible structural changes. Furthermore, the preferential enhancement of J_{max} over V_{cmax} aligns with recent discoveries that blue light specifically upregulates cyclic electron flow components (Degen & Johnson, 2024; Woodford *et al.*, 2024), providing photoprotection

during stress episodes through enhanced ATP generation without accumulating excess NADPH (Ermakova *et al.*, 2024).

The finding within Chapter 5 demonstration that abaxial illumination maintains 77-79% of adaxial photosynthetic capacity represents a paradigm shift in our understanding of leaf optical properties and gas exchange optimization. The 8% enhancement in daily carbon assimilation through periodic 10 minute blue pulses to the abaxial surface, without increasing total photon input suggesting a fundamental advance in photon use efficiency. The temporal optimization of maximum benefit during mid to late photoperiod when baseline conductance naturally declines aligns with circadian regulation of guard cell metabolism. Our modelling analysis further revealed stomatal conductance as the dominant predictor of CO₂ quantum yield (68.4% feature importance) and thus provides the computational framework for predicting optimal pulse timing under diverse environmental conditions.

Chapter 6 resolves the critical question of whether water use efficiency gains from reduced stomatal density compromise dynamic light responsiveness particularly to red and blue light. The wheat EPF1 overexpression lines demonstrated that despite 20-30% lower baseline conductance under red light, blue light supplementation induced amplified responses with 90% relative conductance increases from steady state value under red light in EPF1 plants versus 49% in wild type on abaxial surfaces (Figure 6.2). It was postulated that this compensation mechanism operates through altered C_i to stomatal aperture relationships. The chronically lower intercellular CO₂ in reduced density leaves (180-200 $\mu\text{mol mol}^{-1}$ versus 240-260 $\mu\text{mol mol}^{-1}$ in wild type) creates a primed state for

blue light responsiveness. Recent discoveries that CBC1/CBC2 kinases link blue light and CO₂ signals in guard cells by inhibiting S-type anion channels provide the molecular framework support for this compensation (Hiyama *et al.*, 2017a), with lower baseline *C_i* enhances sensitivity to blue light triggered CBC signalling. The surface specific nature of these responses, with stronger compensation abaxially where EPF1 effects were greatest, demonstrates that developmental modifications and physiological responses are spatially coupled. This coupling ensures that genetic interventions targeting stomatal density need not sacrifice the dynamic control essential for fluctuating field conditions which can be a major concern in crop engineering work for water use efficiency (Hepworth *et al.*, 2015; Caine *et al.*, 2019).

The enhancement of H⁺-ATPase transcript in guard cells (Chapter 7) reduced transition penalties from 17.3% to 6.9% under fluctuating light addresses the fundamental constraint limiting photosynthetic efficiency in nature. The preservation of photosynthetic biochemistry (*V_{cm}*_{max}: 57-61 μmol m⁻² s⁻¹ across genotypes) while dramatically altering stomatal kinetics validates the strategy of targeting actuator capacity rather than sensor sensitivity. The unexpected abolishment of blue light enhancement during photosynthetic induction in AHA2 lines could suggest system level saturation, with vastly expanded H⁺-ATPase pools, even partial activation through red light driven metabolism generates sufficient proton motive force for rapid opening. This finding aligns with recent structural work showing sequential phosphorylation at Thr948 followed by Thr881 creates a molecular coincidence detector (Fuji *et al.*, 2024; Hayashi *et al.*, 2024), where excess AHA2 protein reduces the requirement for full dual phosphorylation. Furthermore, the matched growth and measurement conditions using our stochastic light program with 133

transitions over 11 hours proved critical for isolating engineered effects from acclimation artifacts. Previous studies conflating steady light growth with fluctuating measurements likely underestimated potential gains from enhanced stomatal kinetics.

8.2 Systemic Discussion, Limitations, and Future Directions

While controlled environment studies provide mechanistic clarity, translation to field conditions requires acknowledging additional layers of complexity. Natural canopies experience spectral dynamics beyond simple intensity fluctuations such as the red: far red ratio shifts with canopy position and solar angle, UV exposure varies with atmospheric conditions, and green light penetration creates vertical spectral gradients. Our finding that abaxial surfaces maintain high photosynthetic efficiency when illuminated directly (Chapter 5) becomes particularly relevant in dense canopies where reflected and transmitted light creates complex three dimensional light fields enriched in wavelengths that penetrate deeper into the canopy. Temperature interactions represent another critical knowledge gap. For example, guard cell metabolism exhibits strong temperature dependencies, with H⁺-ATPase activity showing Q₁₀ values of 2.0-2.5 (Gray *et al.*, 2015). Climate warming may therefore amplify blue light responses in some environments while potentially pushing the system beyond optimal operating temperatures in others. Recent work showing CONVERGENCE OF BLUE LIGHT AND CO₂ (CBC1/2) kinases integrate temperature and light signals suggests molecular integration points where breeding could optimize temperature to light response coupling (Hiyama *et al.*, 2017a). The drought resilience conferred by blue light priming (Chapter 4) may become increasingly valuable

as precipitation patterns become more variable, but whether priming effects persist under compound stress scenarios (heat + drought + high VPD) remains untested.

Scaling considerations present both challenges and opportunities. While stomatal responses show cell autonomy in signal perception, the emergence of patchiness and propagating waves of stomatal closure under stress indicates higher order coordination mechanisms. Our thermal imaging protocols can detect these spatial dynamics (Chapter 2), but understanding their functional significance requires integration with sap flow measurements, isotopic tracers, or whole plant hydraulic models. The 8% carbon gain from abaxial blue light pulses in strawberry may translate differently to field crops with different architectures, for example, erectophile cereals versus planophile legumes will exhibit distinct abaxial light exposure patterns requiring species specific optimization. This species specific optimization can be rapidly evaluated using our randomized gas exchange measuring method (Chapter 5) coupled with modelling approaches using environmental input beyond just light as presented in this thesis project.

One immediate application pathway of findings discovered in this thesis lies in controlled environment agriculture, where spectral control is already technically well adopted. Our demonstration that periodic 10 minute blue pulses enhance daily carbon gain (>8%) while minimizing energy input provides a software solution requiring minimal hardware modifications to existing LED systems. This can be immediately applied to high wire cultivation systems where growing benches are in rows and elevated, such that LED light strips can be easily installed on growing benches to provide pulsed blue light (i.e., commercial strawberry cultivation). In fact, for many commercial settings already with

inter-canopy lighting installed, implementation requires only reprogramming of spectral controllers, a zero capital improvement with potential returns of 8-10% daily carbon gain that will likely translate to biomass boost.

For field applications, genetic approaches offer more tractable near term implementation. The moderate stomatal density reductions (i.e., 20-30%) that maintain yield while improving water use efficiency represent achievable breeding targets, as demonstrated in recent field trials with EPF modified crops (Pitaloka *et al.*, 2022; Ferguson *et al.*, 2024). Modern genomic selection approaches could pyramid favourable alleles at EPF1, EPF2, and associated signalling components within 3-4 breeding cycles. The surface specific expression patterns we observed suggest that promoters driving preferential abaxial expression could maximize water savings while minimizing diffusional limitations. CRISPR based approaches targeting the endogenous EPF genes could achieve optimal density reductions without transgenic complications which potentially accelerate regulatory approval especially in Europe (Mohammed *et al.*, 2019).

8.3 Critical Limitations and Methodological Constraints

Several limitations constrain the interpretation and application of these findings within this thesis. Most fundamentally, our experiments examined single factor or limited factorial combinations of environmental variables. Real world scenarios involve simultaneous variation in light, temperature, humidity, CO₂, and nutrients interactions that may fundamentally alter blue light response dynamics. The VPD conditions in our growth chambers and gas exchange measurements (0.8-1.2 kPa) represent moderate evaporative demand, whereas under field conditions could exceeding 3-4 kPa, hydraulic

limitations may override biochemical signalling, thus can potentially negating blue light benefits.

The temporal dynamics of blue light priming remain incompletely characterized. While we documented both temporary and persistent effects at 30 and 60 days post priming, the molecular basis for this "memory" remains unclear (Jezek *et al.*, 2021). Without understanding the mechanistic basis on whether epigenetic modifications, metabolite accumulation, or structural preconditioning, we cannot predict memory duration or optimize reinforcement protocols. The energetic costs of maintaining primed states may become prohibitive under resource limitation, potentially explaining why such priming is not constitutively expressed under natural light conditions.

Lastly Genetic background effects likely influence the magnitude and even direction of responses. Our studies used single cultivars within each species, but preliminary data (not presented in this thesis) suggests substantial genotype by treatment interactions. Elite varieties selected for high harvest index may have inadvertently lost blue light responsiveness through linkage drag or pleiotropic effects. Although not presented in this thesis, one preliminary experiment conducted in first year examining quantum yield to CO₂ in response to blue light percentage gradients showed different magnitudes of response in everbearing versus June bearing strawberry cultivars. Therefore, systematic screening across diversity panels will be essential before implementing blue light optimization strategies in breeding programs. Simultaneously, this might lead to discovery of new alleles responsible for controlling these blue light sensitivity responses.

Future Directions

Future research directions in the short term can focus on developing moderate reduction EPF1 wheat lines across precipitation gradients to quantify genotype × environment interactions. This should include detailed phenotyping of root hydraulic properties, as reduced transpiration may affect root development and soil exploration. Additionally, trait stacking by combining moderate EPF1 expression (Chapter 6) with enhanced H⁺-ATPase activity (Chapter 7) and blue light hypersensitive phototropin variants should be tested for synergistic effects, or the removal of guard cell blue light signalling pathways for high water use efficiency in crops that are not usually limited by stomatal constraints during light transitions (i.e., monocot species). Furthermore, one should consider surface specific engineering, for example developing promoters that enable differential gene expression between adaxial and abaxial epidermis.

For spectral and temporal optimization, the ideal workflow is to use high throughput data collection methods outlined in Chapter 5 to derive large datasets of gas exchange responses to numerous random combinations of environmental inputs that one would like to optimize. With enough data collected, modelling approaches can more confidently predict optimal light settings, environmental controls (i.e., CO₂, VPD, temperature), duration, and diurnal effects across growth stages. Integration with weather forecasting could enable predictive spectral adjustments anticipating stress events. For any event inducing stress priming, it is important to elucidate the molecular mechanism of priming memory. This can be accomplished with ChIP-seq, ATAC-seq, or metabolomics to

identify epigenetic marks, chromatin accessibility changes, and metabolite signatures associated with persistent blue light priming effects.

Although beyond the author's current knowledge reserve and expertise for the moment, future research using synthetic biology approaches to design synthetic guard cell circuits incorporating environmental sensors (light quality, VPD, temperature) linked to actuator control represents a compelling frontier that builds directly upon the molecular insights gained from this thesis work. Future synthetic circuits could leverage the recently characterized convergence points in guard cell signalling networks, particularly the CBC1/CBC2 kinases that integrate phototropin mediated blue light signals with CO₂ sensing pathways (Hiyama *et al.*, 2017a). By designing synthetic promoters that respond to multiple environmental inputs (i.e., elements from heat shock proteins, ABA responsive elements), one could create guard cells with programmable response profiles. The ultimate goal would be engineering orthogonal signalling pathways that maintain homeostasis while enabling enhanced environmental responsiveness, particularly under the compound stresses anticipated with climate change. These synthetic systems could incorporate predictive elements, for example, using early morning temperature and humidity measurements to prime stomatal responses for anticipated midday stress, or implementing hysteresis mechanisms that prevent excessive stomatal oscillations during variable cloud cover. Such approaches would require sophisticated modelling frameworks that integrate molecular dynamics, tissue level gas diffusion, or whole plant water relations, all of which can benefit from the methodological innovations developed throughout this thesis project.

8.4 Broader Implications for Climate Adaptation

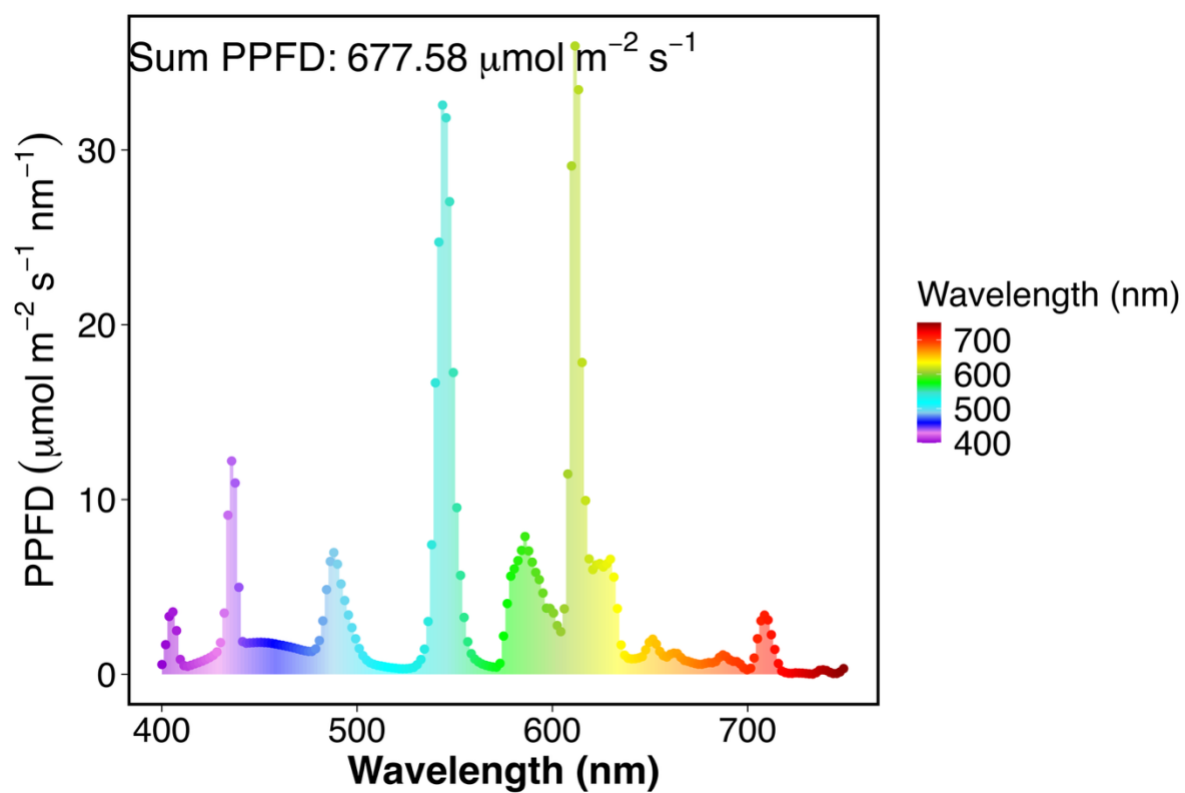
The convergence of water scarcity, temperature extremes, and atmospheric CO₂ enrichment creates an unprecedented challenge and opportunity for global food system. This thesis demonstrates that plants possess latent regulatory capacity that can be activated through targeted interventions, whether spectral, genetic, or molecular. The blue light system represents one of several underutilized regulatory networks that evolution has maintained but not maximized, providing biotechnological opportunities for rapid crop improvement. The success of relatively simple interventions presented in this thesis project, for example, periodic blue pulses, moderate density reductions, targeted protein overexpression, suggests that transformative improvements need not require wholesale redesign of plant metabolism. Instead, fine tuning existing regulatory networks to operate closer to their theoretical optima may provide substantial gains. This "regulatory optimization" paradigm contrasts with more ambitious proposals for introducing C4 photosynthesis into C3 crops or engineering photorespiration bypasses, thereby offering nearer term benefits with lower technical barriers.

Ultimately, this work demonstrates that the evolutionary constraints that shaped plant gas exchange regulation are not immutable barriers but rather starting points for informed engineering. By understanding how light signals coordinate development and physiology across multiple scales and species, we can design interventions that enhance crop performance while maintaining the robustness that enables plants to thrive under variable conditions. The path from fundamental discovery to commercial application remains challenging, but the potential benefits such as water savings with maintained yields, 10-

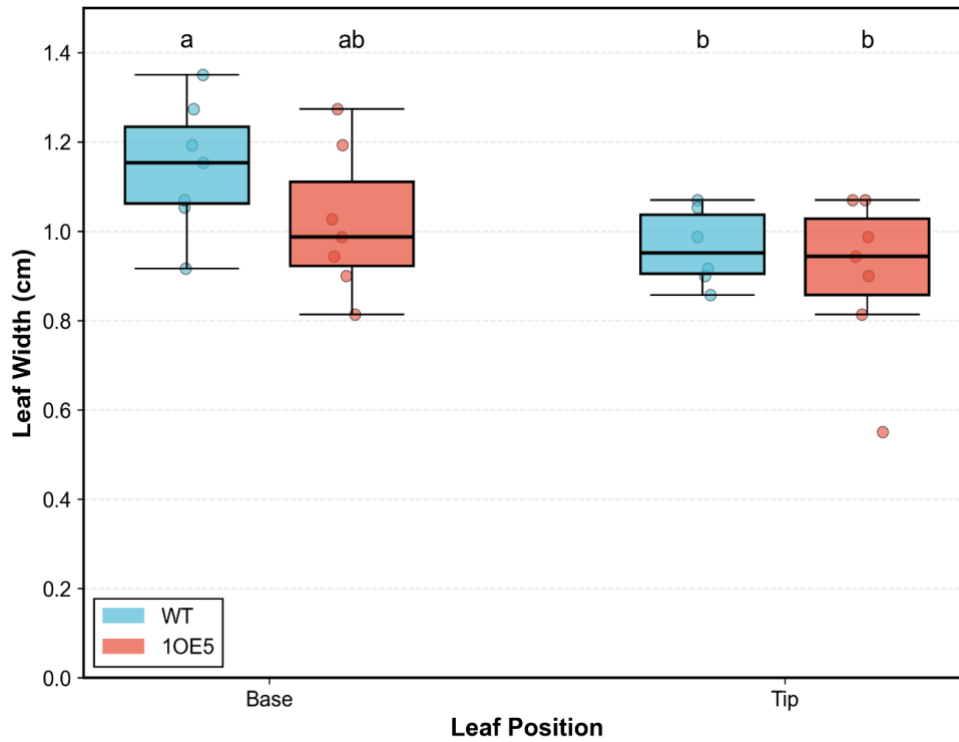
40% reduction in dynamic photosynthetic penalties to natural light will justify sustained investment in translating these findings from laboratory to field.

With each experimental chapter I concluded throughout the past four years, many questions have been answered. However, with each answer arise new questions about the molecular machinery, ecological significance, and agronomic potential. Yet the progress documented here provides both the conceptual framework and practical tools necessary to address these challenges systematically, offering hope for sustainable advancement of our future food system on this planet in an era of environmental change.

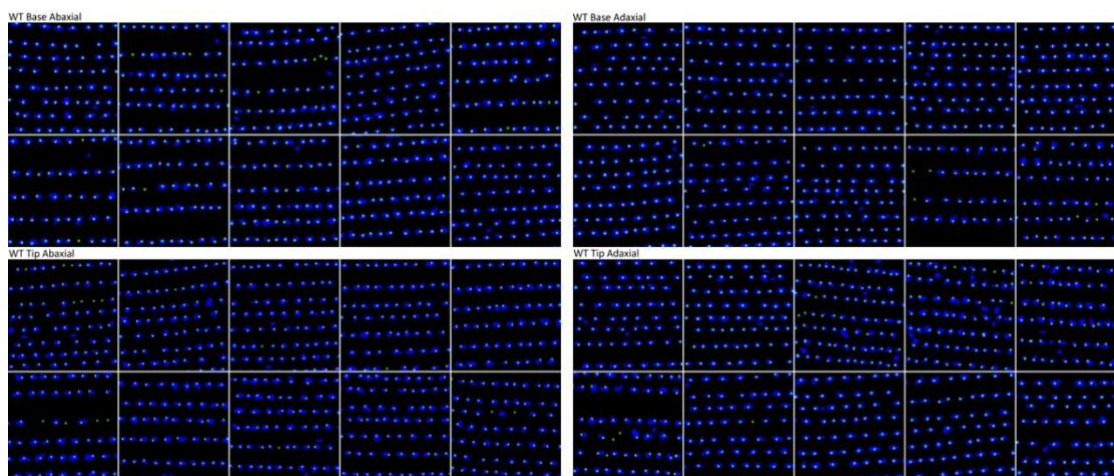
Supplementary Materials



Supplementary SFig 3.1. Representative growth light spectrum measured at flag leaf level.



Supplementary SFig 3.2. Leaf width variation between genotypes and positions along the barley leaf. Different letters indicate significant differences between groups (Tukey HSD test, $p < 0.05$).



Supplementary SFig 3.3. Manual validation of automated stomatal detection across 1mm² sampling areas. Ten randomly selected 1mm² squares were analysed for each leaf region: Base abaxial, base adaxial, tip abaxial, and tip adaxial. Overall mean detection accuracy was 95.2% ± 3.6% across all leaf regions.

Supplementary Table S3.1. Stomatal density and effect of *EPF1* overexpression

Location	WT	EPF1-OE	Reduction (%)	Effect Size (d)
Base Adaxial	58.93 ± 0.37 ^f	47.22 ± 0.33 ^f	19.9%	0.66
Base Abaxial	57.11 ± 0.47 ^e	23.14 ± 0.20 ^e	59.5%	2.01
Tip Adaxial	91.46 ± 0.43 ^h	54.75 ± 0.40 ^h	40.1%	1.85

Location	WT	EPF1-OE	Reduction (%)	Effect Size (d)
Tip Abaxial	87.83 ± 0.56 ^g	30.87 ± 0.33 ^g	64.9%	2.75

***Values represent mean ± SE (stomata/mm²).**

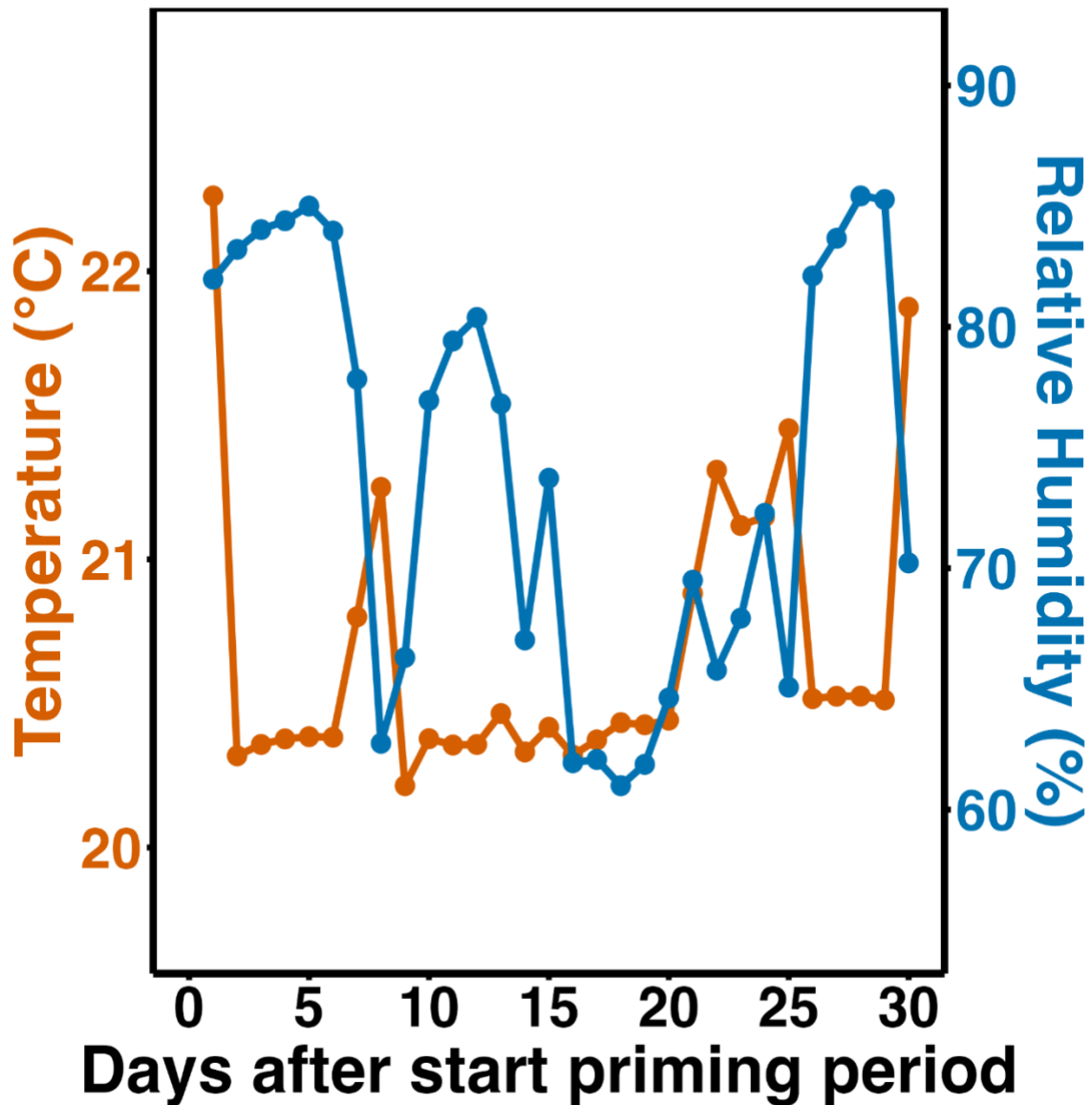
****Superscript letters indicate significant differences (Sidak, p<0.05)**

Supplementary Table S3.2. Coefficient of variation (%) in stomatal density

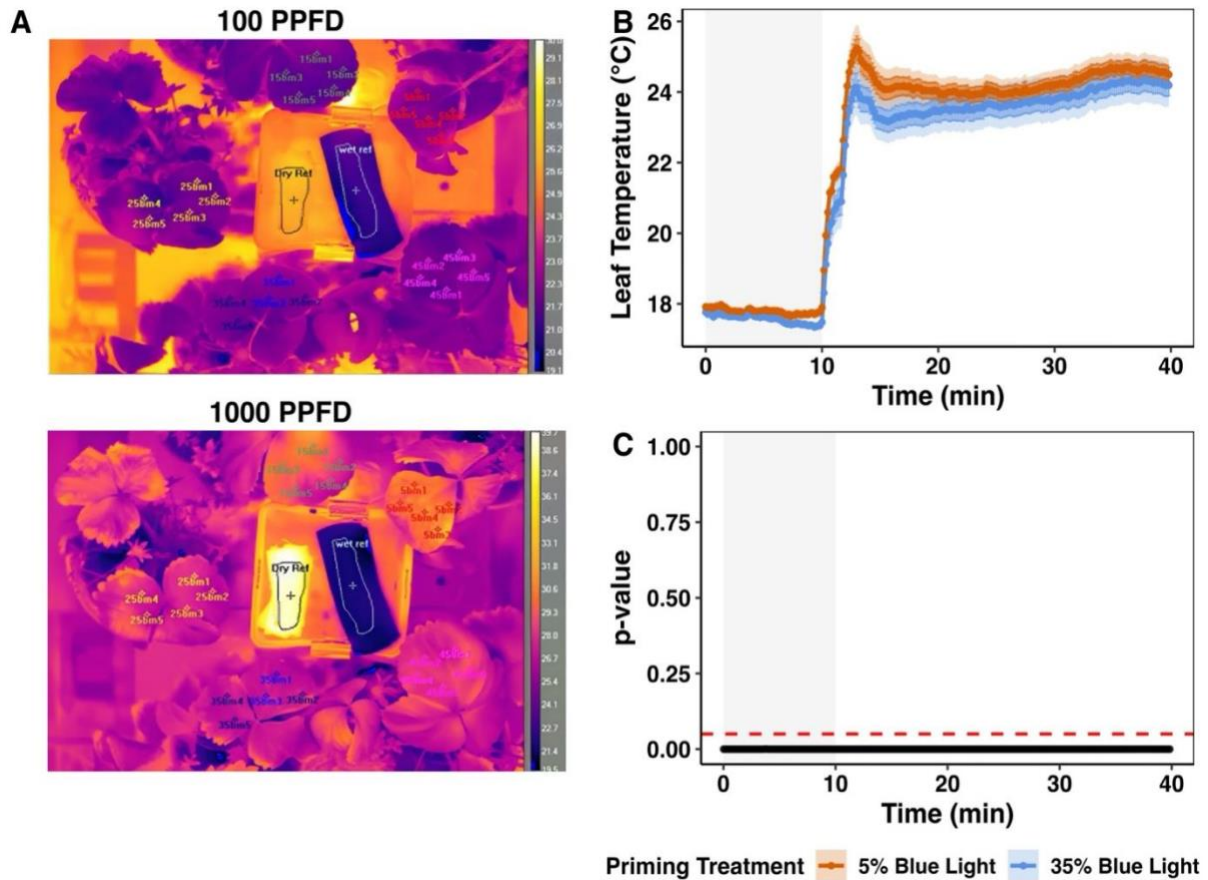
Location	WT CV (%)	EPF1-OE CV (%)	CV Change
Base Adaxial	33.00908	32.63391	-1.1%
Base Abaxial	38.98110	46.18026	+18.5%
Tip Adaxial	23.55720	31.51703	+33.8%
Tip Abaxial	28.68949	46.21185	+61.1%
*CV Change shows percent increase/decrease in spatial heterogeneity in EPF1-OE			

Supplementary Table S3.3. Stomatal distribution ratios

Ratio Type	Leaf Region	WT	1OE5
Adaxial:Abaxial	Base	1.03	2.04
Adaxial:Abaxial	Tip	1.04	1.77
Tip:Base	Adaxial	1.55	1.16
Tip:Base	Abaxial	1.54	1.33
*Values show relative stomatal density across leaf surfaces and positions			

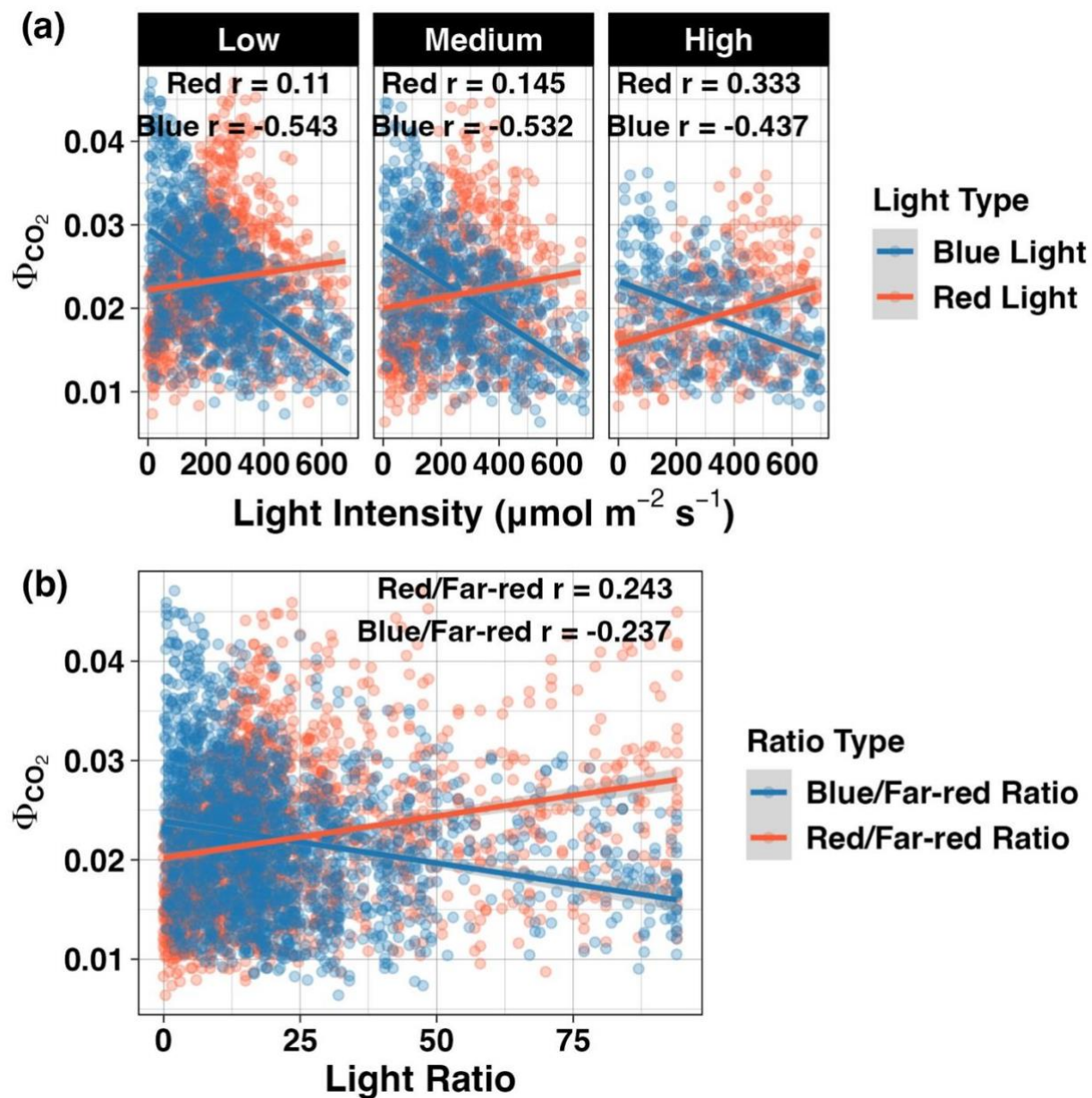


Supplementary Figure S4.1. Environmental conditions during the 30-day priming period. Daily average temperature (°C, orange line, left axis) and relative humidity (% , blue line, right axis) measured in the controlled climate room throughout the priming phase. Data points represent daily averages collected from continuous monitoring sensors.



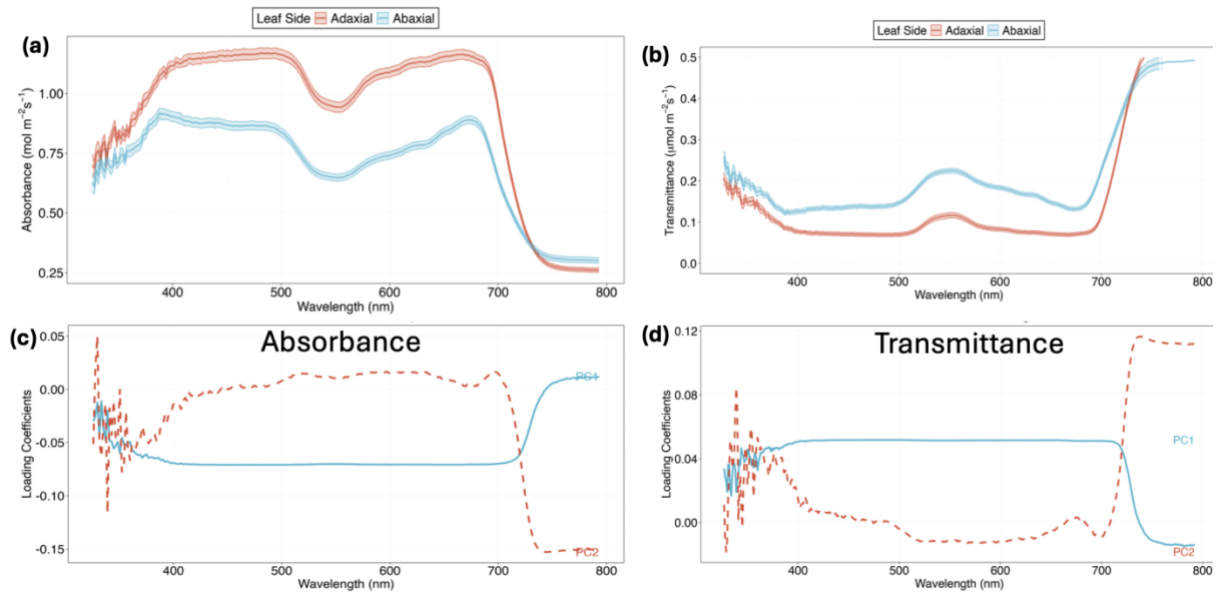
Supplementary Figure S4.2. Thermal dynamics during light-induced stomatal responses in blue light primed strawberry leaves. (A) Representative thermal images of differential temperature regulation between 5% and 35% blue light priming treatments during low light acclimation ($100 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD) and subsequent high light induction ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD). **(B)** Temporal dynamics of leaf temperature during photosynthetic induction ($n = 13$) Gray shading indicates the initial low light period (0-10 min), followed by step increase to saturating irradiance (10-40 min). **(C)** Statistical analysis of treatment-dependent temperature differences. ANOVA test p-

values calculated at 10-second intervals throughout the induction protocol. The horizontal dashed line denotes the significance threshold ($p = 0.05$).



Supplementary Figure S5.1. Interaction effects of far-red light with red and blue light on quantum yield of CO_2 assimilation (Φ_{CO_2}). (a) Relationships between light intensity and Φ_{CO_2} for red light (orange) and blue light (blue) under different far-red levels. Each panel represents data stratified into low, medium, and high far-red

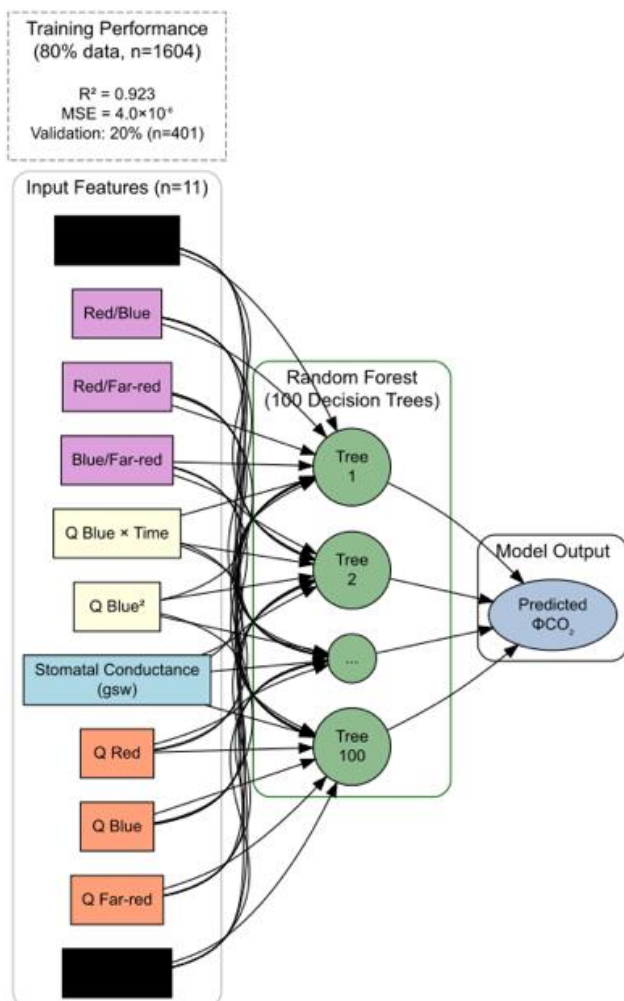
conditions, with regression lines showing 95% confidence intervals. (b) Relationships between spectral ratios (red/far-red and blue/far-red) and ΦCO_2



Supplementary Figure S5.2. Optical properties of adaxial and abaxial leaf surfaces. (a) Mean absorbance spectra. and (b) Mean transmittance spectra (c) PCA loadings against wavelength for absorbance measurements. (d) PCA loadings against wavelength for transmittance measurements. Data shown as means $\pm$ SE, n = 14 per leaf side.

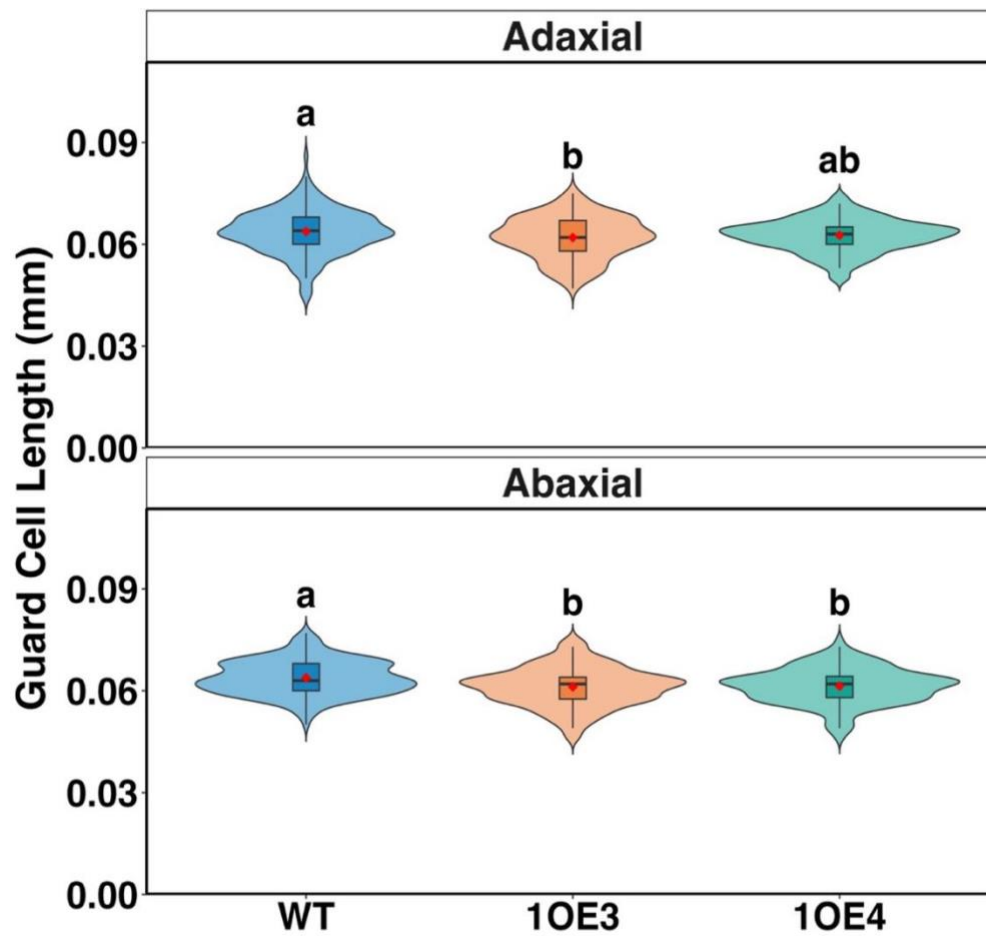
Principal component analysis (PCA) revealed distinct optical properties between adaxial and abaxial leaf surfaces. Mean spectral measurements showed clear differences between leaf sides for both absorbance (Supplementary Fig. 2a) and transmittance (Supplementary Fig. 2b) across the measured wavelength range (326-

700 nm). The PCA analysis showed clear separation between adaxial and abaxial surfaces for both absorbance (Supplementary Fig. 2c) and transmittance measurements (Supplementary Fig. 2d). The first two principal components explained 94.02% of the total variance in absorbance spectra (PC1: 77.37%, PC2: 16.65%) and 95.26% in transmittance spectra (PC1: 79.56%, PC2: 15.70%). Analysis of PCA loadings identified key wavelength regions driving these differences. In the green spectrum (520-525 nm), adaxial surfaces exhibited significantly higher absorbance (1.056 ± 0.042) compared to abaxial surfaces (0.710 ± 0.031 ; $t = 12.4$, $p < 0.001$). The most pronounced absorbance differences were observed in the yellow-orange region (575-586 nm), with peak separation at 579 nm where adaxial absorbance exceeded abaxial by 48.7%. Similarly, complementary patterns were observed in transmittance measurements, where adaxial surfaces showed significantly lower transmittance in the green spectrum (514-518 nm; 0.078 ± 0.003) compared to abaxial surfaces (0.176 ± 0.004 ; $t = -19.8$, $p < 0.001$). The largest transmittance difference occurred at 612 nm, with adaxial surfaces transmitting 55.3% less light than abaxial surfaces.

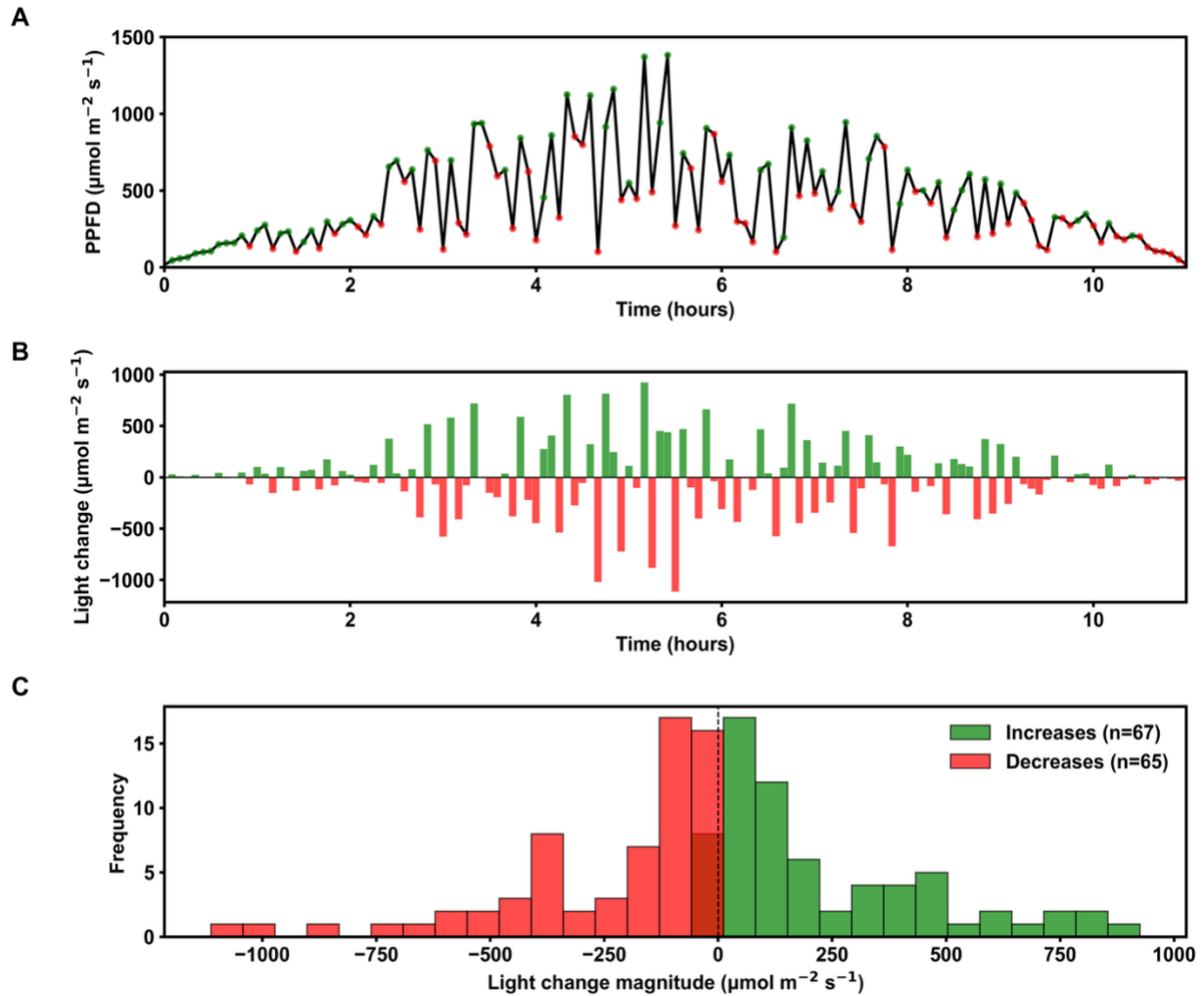


Supplementary Figure S5.3. Random forest model architecture for predicting quantum yield of CO₂ assimilation (ΦCO_2) from multidimensional light environment and physiological parameters. Input features (n = 11) are categorized into four functional groups: physiological parameters (stomatal conductance, g_{sw}), direct light quality components (photosynthetic photon flux densities for red, blue, and far-red wavelengths), temporal variables (time of day and its quadratic term capturing circadian influences), and spectral ratios (red/blue, red/far-red, blue/far-red) representing photomorphogenic signals. Two interaction terms (Q Blue $\times$ Time of Day, Q Blue²) capture non-linear relationships between blue light intensity and temporal

dynamics. Colour coding distinguishes feature categories as physiological (blue), light quantity (salmon), temporal (green), spectral ratios (purple), and interactions (yellow).



Supplementary Figure S6.1 Impact of EPF1 overexpression on guard cells length of two different leaf surfaces (n = 168) in WT (blue), 1OE3 (orange), and 1OE4 (teal).



Supplementary Figure S7.1. Analysis of light intensity transitions in the natural fluctuating light (NFL) program. (A) Time course of the complete NFL program showing PPFD values over 11.08 hours. Green dots indicate light intensity increases from the previous step, while red dots indicate decreases. (B) Magnitude of light intensity changes between consecutive 5-minute steps throughout the program. Green bars represent increases in PPFD, red bars represent decreases. (C) Frequency distribution of light change magnitudes showing 67 increase events (green) and 65 decrease events (red) during the program.

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