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INVESTIGATING THE EFFECTS OF UV-B AND TEMPERATURE ON SURVIVAL AND STOMATAL PHYSIOLOGY IN *ARABIDOPSIS THALIANA* SEEDLINGS

Gun Anna Mathilda Gustavsson

Abstract

Low fluence rate UV-B, perceived by the UV RESISTANCE LOCU 8 (UVR8) photoreceptor is an important environmental signal which affects plant development and growth via its effects on gene expression and plant physiology. Common plant responses to UV-B include inhibition of hypocotyl and petiole elongation, alterations to leaf size, the production of epidermal pigments and the generation of reactive oxygen species (ROS). This thesis investigates the effects of UV-B and temperature on survival and stomatal physiology in *Arabidopsis thaliana*. High and low temperatures are highly stressful to plants and cause great economic losses each year. There is some evidence that UV-B can protect plants against excessive heat and cold, but the response in Arabidopsis is poorly understood. In this thesis, no significant enhancement of Arabidopsis cold acclimation, basal thermotolerance or acquired thermotolerance was observed with UV-B supplementation. A potential indirect role for UV-B in cold acclimation was, however, identified via flavonoid production. This thesis further investigates the effects of low fluence UV-B on Radish Leaf development in a vertical aeroponic system in collaboration with the iCASE partner, LettUs Grow. Vertical farms use artificial light or a mixture of artificial and natural light. This means that growing plants are exposed to no, or very little UV-B. This thesis used the fast-growing microherb Radish Leaf to investigate the applicability of UV-B in a vertical farm system and its effects on plant development, pigment accumulation and antioxidant capacity. Low dose UV-B supplementation increase flavonol accumulation and antioxidant capacity, without negatively impacting plant growth and biomass. The effects of low fluence UV-B on stomatal apertures of Arabidopsis cotyledons were investigated. High fluence rate UV-B has been shown to close stomata in isolated epidermal peels of mature Arabidopsis, but the effect of UV-B on cotyledon stomata has not been established. This thesis shows that low dose UV-B, perceived by UVR8 opens stomata in a response requiring PHYTOCHROME INTERACTING FACTOR (PIF) transcription factors, auxin and ABA signalling.

Covid-19 impact statement

This project was laboratory-based. Lab access was removed for the first 3-month national lockdown in 2020, resulting in a delay in experiments I had planned. The planned work included growing plants for 3-4 weeks, meaning I lost several weeks' worth of work spent on plant maintenance before lockdown, in addition to lockdown time. Under the circumstances, there was no possibility to add a computer-based element to the project to compensate for lost lab-time. I therefore needed access to the lab space, equipment, and training on a regular basis to continue the project and ensure it was completed to the required standard. I was able to access the lab between mid-June 2020 and January 2021 for a limited time each week, but restrictions during this time on the number of people allowed in the same room meant that I was limited with the equipment I could use much of the time. This further slowed down the progress of the project significantly. It was also impossible to compensate for lost time by growing more plants as growth cabinet space is highly limited.

Interacting and problem-solving together with colleagues is a big part of a research project. This aspect was almost completely removed during these months due to the restriction on interaction between individuals.

Covid-19 delayed the start of my CASE placement with LettUs Grow, which was due to start in the autumn of 2020. Because of this, the placement was moved to the beginning of my final year, a critical time in this project.

The challenges faced were discussed with my supervisor. It was agreed I start writing up a data chapter during the first lockdown and continue writing the thesis introduction while lab access was still restricted. Weekly lab meetings were organised with my lab group to bring up any questions or challenges. However, this did not compensate fully the lack of face-to-face interaction possible in a lab without restrictions, and further slowed down the project.

Covid-19 did not only have a profound impact on my project during the lockdowns and restrictions but have had an effect on my learning process and the progression on this entire thesis.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: .... DATE: 23 January 2023

Dedication & Acknowledgements

Firstly, I want to extend my thanks to my supervisor, Professor Kerry Franklin, whose guidance, enthusiasm and kindness have been critical to my completion of this thesis. To everyone in the Franklin lab, past and present: Ioanna, Donald, Ashutosh, Bhavana, Ashley, Alvaro, Aline, Chris, Jess, and Nathan, thanks for making the lab immensely more fun. Thanks to my secondary supervisor Alistair Hetherington and the guard cell group for inviting me to your lab meetings. Various people of 324, too many to mention by name, have also contributed to making my time at Bristol very enjoyable. I would also like to thank my CASE partner, LettUs Grow, for taking me on with short notice and fully trusted my ideas, gave me autonomy and valued my input. To the people whose lives I have all but disappeared from for the past 4 years: my sister and brother, nephews and nieces, my Swedish girls, my friends in Bristol and elsewhere – I am truly lucky to count you as my friends. To Sam, whose encouragement and care got me through many a black hole, I cannot thank you enough. Finally, to my dad, for always believing, without a doubt, that I can do anything – thank you.

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Nomenclature

12L:12D 12 hour light : 12 hour dark photocycle

ABA   Abscisic acid

APB   Active phytochrome binding motif

AFPs   Anti-freeze proteins

ARE   Auxin response elements

ARF   AUXIN RESPONSE FACTOR

BES1   BRI1-EMS-SUPPRESSOR 1

bHLH   basic Helix-Loop-Helix

BIN2   BRASSINOSTEROID-INSENSITIVE 2

BL   Blue light

BR   Brassinosteroid

BZR1   BRASSINAZOLE-RESISTANT 1

CA   Cold acclimation

CBF   C-REPEAT BINDING FACTOR

CC1   CIRCADIAN CLOCK-ASSOCIATED 1

cDNA   complementary DNA

CDPK   Calcium-dependent protein kinase

CHS   CHALCONE SYNTHASE

COLD1 CHILLING TOLERANCE DIVERGENCE 1

COP1   CONSTITUTIVELY PHOTOMORPHOGENIC 1

COR   COLD REGULATED

CPD   Cyclobutene Pyrimidine Dimer

Cry   Cryptochrome

DREB   DEHYDRATION-RESPONSIVE ELEMENT-BINDING

EIL   Expansion-induced lysis

EtOH   Ethanol

FAD   FATTY ACID DESATURASE

FID   Freezing-induced dehydration

FR   Far-red light

F3'H   FLAVONOID 3' HYDROXYLASE

GC   Guard cell

GSK3   GLYCOGEN SYNTHASE KINASE 3

GUS   β-Glucuronidase

HOS1   HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1

HLH   Helix-Loop-Helix

HSF   Heat shock factor

HSP   Heat shock protein

HT   High temperature

HY5   ELONGATED HYPOCOTYL 5

HYH   HY5 HOMOLOGUE

IAA   Indole Acetic Acid

ICE1   INDUCED OF CBF EXPRESSION 1

LEA   LATE EMBRYOGENESIS ABUNDANT

LED   Light Emitting Diode

LOR   Loss of osmotic responsiveness

LT   Low temperature

MCA   MID1-COMPLEMENTING ACTIVITY

MPK   MITOGEN-ACTIVATED PROTEIN KINASE

OST1   OPEN STOMATA 1

PAM   Pule-Amplitude-Modulated

PAR   Photosynthetically Active Radiation

PBS   Phosphate Buffered Saline

phot   phototropin

phy   phytochrome

PIF   PHYTOCHROME INTERACTING FACTOR

PM   Plasma membrane

RCC1   CHROMATIN CONDENSATION 1

RL   Red light

ROS   Reactive oxygen species

RUP   REPRESSOR OF UV-B PHOTOMORPHOGENESIS

SAS   Shade avoidance syndrome

SFR   SENSITIVE TO FREEZING

SIG   SIGMA FACTOR

SLAC1 SLOW ANION CHANNEL-ASSOCIATED 1

SOD   Super Oxide Dismutase

SPA   SUPPRESSOR OF PHYA

TF   Transcription factor

Trp   Tryptophan

TT   TRANSPARENT TESTA

UV-B   Ultraviolet-B

UVR8   ULTRAVIOLET RESISTANCE LOCUS 8

WL   White light

WT   Wild type

WUE   Water use efficiency

ZL   Zeitlupe

CHAPTER 1 INTRODUCTION

Sunlight in the form of electromagnetic radiation is an important environmental signal regulating most stages of plant life, including during de-etiolation, leaf expansion, chlorophyll production and photosynthesis, phototropism, architecture, flowering, and senescence. Quantity, quality, duration, and incidence of light all influence the growth and development of plants. Plants can perceive and physiologically respond to wavelengths of the electromagnetic spectrum from Ultraviolet-B (UV-B; 280-315 nm) to far-red (FR; 725-735 nm). Within this range is photosynthetically active radiation (PAR; 400-700 nm), which contributes to photosynthesis but is also an important photomorphogenic signal providing plants with information about their surrounding environment.

Plants perceive light via specialised molecules called photoreceptors. These include the red light (RL) sensing phytochromes, the blue light (BL) and UV-A sensing cryptochromes, phototropins, and zeitlupes/LKP2/FKF1, and the UV-B sensing UV RESISTANCE LOCUS 8 (UVR8) (Borthwick *et al*., 1952; Butler *et al*., 1959; Koornneef *et al*., 1980; Gallagher *et al*., 1988; Huala *et al*., 1997; Somers *et al*., 2000; Mas *et al*., 2003; Kim *et al*., 2007; Sakai *et al*., 2001; Baudry *et al*., 2010; Rizzini *et al*., 2011). The photoreceptors link signal perception to downstream cellular signalling and physiological responses.

This introductory chapter will discuss plant perception, signalling pathways, and responses to UV-B light. It will also discuss temperature stress and the potential for light to ameliorate the negative effects of temperature stress. Finally, it will introduce the topic of stomatal movement and how stomatal movement is regulated.

1.1 UV-B

Ultraviolet-B light is a naturally occurring component of light emitted by the sun and part of the ultraviolet portion of the electromagnetic spectrum. The ultraviolet region (approx. 100-400 nm) is divided into three wavebands: UV-A (~315-400 nm), UV-B (280-315 nm) and UV-C (~100-280 nm). Stratospheric ozone absorbs all UV-C and part of the UV-B radiation emitted by the sun. The UV radiation reaching the Earth therefore contains predominantly UV-A, and some UV-B. Despite only comprising a small portion of the total sunlight, UV-B has a major impact on living organisms. UV-B can alter the structure of DNA and proteins, which can compromise cellular processes and result in death (Frohmeyer and Staiger, 2003). For example, UV-B can cause cyclobutene pyrimidine dimer (CPD) formation on nucleic acids which interfere with transcription (Britt, 2004). Plants have evolved mechanisms to protect against harmful UV-B radiation. These include the production of screening pigments that accumulate in the epidermal layers which absorb high energy photons, antioxidating compounds to counter oxidative effects of UV-B, leaf waxes and hairs to reflect UV-B, as well as enzymes which can repair DNA damage (Frohmeyer and Staiger, 2003; reviewed by Jansen *et al*., 1998 and Jenkins, 2009). UV-B research became increasingly important as a reduction in the atmospheric ozone layer was discovered towards the end of the 1980's and the beginning of the 1990's. More current

data suggest that reduced emissions of chlorofluorocarbons, among other chemicals, has helped recover stratospheric ozone levels (Strahan and Douglass, 2018). Nevertheless, UV-B remains an important environmental signal, and has since been investigated in a range of plant responses. Importantly, the UV-B research to date still leaves areas unexplored, which may possess potential avenues to improve plant growth and survival, something which is desirable in agriculture and horticulture. In addition to the harmful effects of high-dose UV-B, low-dose UV-B was found to elicit photomorphogenic responses (growth and development in response to light) in plants which was not the result of UV-B damage (Kim *et al*., 2002; Brown and Jenkins, 2008; Favory *et al*., 2009). For example, low fluence UV-B irradiation resulted in reduced hypocotyl elongation, cotyledon opening and altered root growth (Wellmann, 1976; Ballaré *et al*., 1995; Kim *et al*., 2002). These effects were not mediated by any known photoreceptors, and it was hypothesised that plants possess a specialised UV-B photoreceptor.

1.1.1 UVR8 discovery

In 2002, Kliebenstein and colleagues first identified the Arabidopsis mutant *uvr8-1* (Landsberg *erecta* background) which has a 15-nucleotide deletion in a gene similar to the human *REGULATOR OF CHROMATIN CONDENSATION 1* (*RCC1*). The mutant was hypersensitive to UV-B, displaying leaf necrosis and reduced growth, and had impaired expression of the gene encoding the flavonoid biosynthesis enzyme CHALCONE SYNTHASE (CHS), and increased expression of stress-related *PATHOGENESIS RELATED1 and 5*, after UV-B exposure. This suggested that the gene was fundamentally involved in UV-B protection and signalling. Later, the UVR8 protein was discovered to monomerise upon UV-B irradiation, and interact with a central regulator of light responses, CONSITITUTIVELY PHOTOMORPHOGENIC 1 (COP1), thus inducing signalling in response to UV-B, and was designated the UV-B photoreceptor (Favory *et al*., 2009; Rizzini *et al.*, 2011). Phylogenetic analysis revealed functional conservation of UVR8 from green algae to higher plants, highlighting UV-B protection as an important mechanism of early plant life (Fernández *et al*., 2016).

1.1.2 UVR8 structure and perception of UV-B

UVR8 is a 440-amino acids, seven-bladed β-propeller protein which, unlike other identified photoreceptors, does not have any extrinsic cofactors as the chromophore for light perception (Wu *et al.*, 2012; Christie *et al.*, 2012). As predicted by Rizzini *et al.* (2011), crystallographic and solution structure studies revealed that the inactive UVR8 protein exists as a homodimer held together by salt bridges between Arg/R and Asp/D residues across the dimer interface, with supporting cation-π interactions between the Trp aromatics (Christie *et al.*, 2012; Wu *et al.*, 2012). Indeed, Arg alignment and amino acid side chain charge appear highly important in maintaining the UVR8 dimer (Christie *et al.*, 2012; Wu *et al.*, 2012 Mathes *et al.*, 2015).

Each UVR8 protein has 14 naturally UV-B-absorbing Trp residues. Three of these, namely W233, W285 and W337, form a central pyramid at the dimer interface, essential for UV-B signalling *in vivo* (Christie *et al.*, 2012; Wu *et al.*, 2012; Zeng *et al.*, 2015). Christie *et al.* (2012) investigated the role of the Arabidopsis UVR8 Trp285 in UV-B perception. The authors created a GFP-tagged transgenic line with a Trp to Phe substitution at position 285 (UVR8^{W285F}) in a *uvr8-1* mutant, which did not restore UVR8-mediated expression of *ELONGATED HYPOCOTYL 5* (*HY5*), suggesting Trp285 is an essential part of the chromophore, with assistance from Trp233 and Trp337 (Christie *et al.*, 2012; Wu *et al.*, 2012; O'Hara and Jenkins 2012).

Although some questions remain around the mechanism for UVR8 activation and signalling initiation, computational modelling undertaken by Wu *et al.* (2014) support the suggestion made by Wu *et al.* (2012) that changes in charge of dimer interface residues destabilise the dimer and lead to monomerization. Mathes *et al.* (2015) subsequently reported proton-coupled electron transport involvement in UVR8 photoactivation. Some further clarification of the mechanism was provided by Zeng *et al.* (2015) who showed that spatial rotation of interface Trp residue side chains eject a water molecule from the epicenter during early signalling, weakening interactions at the dimer interface. Recent evidence from Li *et al.* (2022) shows that charge separation occurs between Trp residues, supporting the reports that charge differences are important for monomerization. The authors further show that, when excited, the energy of the Trp residue at the dimer interface triggers a charge separation

by production of a transient Trp anion. The electron can recombine with the Trp residue, or move to an adjacent Arg residue, which is then neutralised. The neutralisation of charge destroys a key salt bridge link between the dimers triggering further deconstruction of the links between the two monomers, resulting in full monomerization of the photoreceptor, and activation of signalling.

1.1.3 UVR8 signalling

In the absence of UV-B, UVR8 is located in the cytoplasm in the homodimeric form but is upon UV-B exposure rapidly monomerised and translocated to the nucleus where its function is focused (Brown *et al.*, 2005; Kaiserli and Jenkins, 2007). Structural similarities to human RCC1 encouraged research into whether plant UVR8 also interacts directly with chromatin. Indeed, UVR8 was found to associate with a small region of the chromatin containing the *HY5* gene, which is a central node in light signalling in Arabidopsis (Oyama *et al.*, 1997; Brown *et al.*, 2005; Cloix and Jenkins, 2008). However, several of the aspects of UV-B influence on *HY5* expression via UVR8-chromatin interaction remained unclear. Binkert *et al.* (2016) later suggested that chromatin interaction was not the main mode of action of UVR8 in UV-B response pathways as recombinant UVR8 did not bind chromatin *in vitro*.

After monomerization, UVR8 is capable of interacting with certain molecules to elicit UV-B signalling. Particularly, UVR8 interacts with COP1, an E3 ubiquitin ligase that targets molecules for degradation by the 26s proteasome (Österlund *et al.*, 2000). The UVR8-COP1 interaction occurs at two domains (Yin *et al.*, 2015) where at least one, the 27-amino acids of the C-terminus (C27) of UVR8, is essential and sufficient for interacting with the WD40 domain of COP1 (Cloix *et al.*, 2012). COP1 only interacts with the UVR8 monomer, as no interaction was seen in the constitutive dimer UVR8[W285F] (Rizzini *et al.*, 2011; O'Hara and Jenkins, 2012). Mutations in the *COP1* locus had previously revealed an important role for COP1 in plant growth and development in response to light as dark-grown seedlings displayed photomorphogenic responses in the absence of light (Deng *et al.*, 1991). von Arnim and Deng (1994) found that a GUS-COP1 reporter accumulated in the nucleus of Arabidopsis cells in the dark but not in the light, correlating with photomorphogenic responses. The role of COP1 in plant light responses has now been comprehensively investigated (Torii and Deng, 1997; Österlund and Deng, 1998; Yamamoto *et al.*, 1998; Österlund *et al.*, 2000). In visible light, COP1 is alone capable of Ub ligase

activity *in vitro*, but is *in vivo* reliant on SUPPRESSOR OF PHYA (SPA) proteins (Saijo *et al*., 2003;, Seo *et al*., 2003). Together, COP1 and SPAs form an active E3 Ub ligase that can suppress photomorphogenesis by targeting and degrading photomorphogenesis-inducing factors (Laubinger *et al*., 2004; Yang and Wang, 2006; Zhu *et al*., 2008; Ordoñez-Herrera *et al*., 2015). In the dark, reduced photoreceptor activity results in COP1-mediated repression of photomorphogenesis (fig. 1.1).

There are several ways that photoreceptors interact and suppress COP1-SPA activity in the light. In response to BL, cryptochromes bind to COP1/SPA and thereby suppress the Ub ligase activity of COP1/SPA (Holtkotte *et al*., 2017). The RL photoreceptor, phyB, hinders the interaction between COP1 and SPA1, resulting in low COP1 activity and photomorphogenesis (Lu *et al*., 2015). Phytochromes also suppress nuclear accumulation of COP1 in the light (von Arnim *et al*., 1997; Österlund and Deng, 1998; Subramanian *et al*., 2004) while both phytochromes and cryptochromes promote transfer of COP1 to the cytosol (von Arnim and Deng, 1994) and interact with SPA proteins to hinder COP1-SPA interaction (Fankhauser and Ulm, 2011; Liu *et al*., 2011; Lian *et al*., 2011; Zuo *et al*., 2011). These interactions and nuclear exclusions result in reduced E3 Ub ligase activity of the COP1/SPA complexes thus promoting photomorphogenesis. By contrast, under UV-B, COP1 promotes photomorphogenesis. Oravecz *et al*. (2006) showed that COP1 is required for the photomorphogenic UV-B response in Arabidopsis, and that SPA proteins were not essential. However, Huang *et al*. (2013) later found that UVR8 reorganises the COP1-SPA complexes, resulting in UVR8-COP1-SPA modules which positively regulate HY5. COP1 has been implicated in most responses to UV-B and UV-B-induced binding of UVR8 to COP1 is a crucial step in the main path for UVR8-dependent signalling in Arabidopsis that leads to photomorphogenesis and UV-B stress tolerance (Favory *et al*., 2009; Rizzini *et al*., 2011; Cloix *et al*., 2012; Oravecz *et al*., 2006). The UVR8-COP1 interaction is essential for nuclear accumulation of UVR8 and subsequent photomorphogenic responses to UV-B (fig. 1.1; Huang *et al*., 2014; Yin *et al*., 2016). Inhibition of hypocotyl elongation was not significantly reduced in a UV-B-exposed *cop1* mutant compared to WT (Kim *et al*., 2002). In addition, typical UV-B-induced gene expression changes and flavonoid accumulation were shown to be suppressed in *cop1* (Oravecz *et al*., 2006). Together,

these data suggested COP1 (together with UVR8) to be a positive regulator of photomorphogenesis under UV-B irradiation (Oravecz *et al*., 2006), contrasting its role under visible light.

A key component in light signalling is HY5 and its close homolog HY5 HOMOLOGUE (HYH). HY5/HYH belong to the basic Leu zipper (bZIP) family of transcription factors and are constitutively located to the nucleus. Brown and Jenkins (2008) presented evidence that HY5 and HYH act redundantly in many responses to UV-B, however HY5 appeared to be the more dominant. HY5 was shown to promote developmental responses in plants such as cell elongation and proliferation in response to light (Oyama *et al*., 1997). Following this discovery, over 3000 chromosomal sites were identified as potential HY5-binding sites (Lee *et al*., 2007). During early low-fluence rate UV-B responses, 100 up- and 7 down-regulated genes were identified by Ulm *et al*. (2004) using microarray analysis. Expression of several of these genes was impaired in a *hy5* mutant, suggesting HY5 is critically involved in UV-B-responsive gene expression. Indeed, UV-B irradiation enhanced UVR8-dependent binding of HY5 to promoters of UV-B-induced genes and HY5/HYH appeared to regulate the expression of most UVR8-dependent genes (Brown and Jenkins, 2008; Binkert *et al*., 2014).

Under visible light, COP1 negatively regulates HY5/HYH (Ang *et al*., 1998; Österlund *et al*., 2000; Hardtke *et al*., 2000; Saijo *et al*., 2003). In fact, HY5 and COP1 act antagonistically to regulate seedling development (Österlund *et al*., 2000). Hardtke *et al*. (2000) showed that phosphorylation at the COP1-binding domain of HY5 reduced interaction with COP1 and thereby degradation. However, unphosphosphorylated HY5 has higher physiological activity as it binds promoters with higher affinity than its phosphorylated state, providing another level of light-regulated control over HY5 activity. Conversely, during UV-B exposure, UVR8 associates with COP1 to form UVR8-COP1 complexes which stabilise HY5 and promote its activity (Huang *et al*. (2013). Interestingly, HY5 promotes its own expression as well as the expression of COP1, providing a positive feedback loop during UV-B exposure as well as a measure of control over its own activity (Binkert *et al*., 2014).

1.1.4 Control of UV-B signalling

Upon UV-B exposure, UVR8 monomerises and induces UV-B signalling. Active UVR8 monomers then revert to the inactive dimeric form, which effectively switches off UV-B signalling until a new UV-B signal is perceived (Wu *et al.*, 2012). Initially, reversal of the monomeric to dimeric form under conditions used for crystallisation prevented crystallographic analyses of the monomeric form (Christie *et al.*, 2012; Wu *et al.*, 2012). REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 (RUP1) and RUP2, two small WD40 proteins with sequence similarity to COP1 and SPA WD40 domains, interact with UVR8 and facilitate UVR8 ground state reversion through re-dimerization (fig. 1.1; Gruber *et al.*, 2010; Heijde and Ulm, 2012). *rup1* and *rup2* knock-out mutants are hypersensitive to UV-B, showing an increased photomorphogenic response, while the overexpression of RUP2 has a reduced photomorphogenic response via suppressed HY5 expression, indicating the RUP proteins importance in UVR8 signalling regulation (Gruber *et al.*, 2010). *RUP1* and *RUP2* transcripts are rapidly induced under UV-B (Gruber *et al.*, 2010). Similarly, they are also transcriptionally induced by cryptochromes in response to BL, and negatively regulate UV-B signalling, and provide cross-regulation between photoreceptors in a polychromatic light environment (Tissot and Ulm, 2020). This finding is important to consider for UV-B treatments. As BL suppressed UV-B signalling and responses, the composition of light in treatment chambers should be monitored.
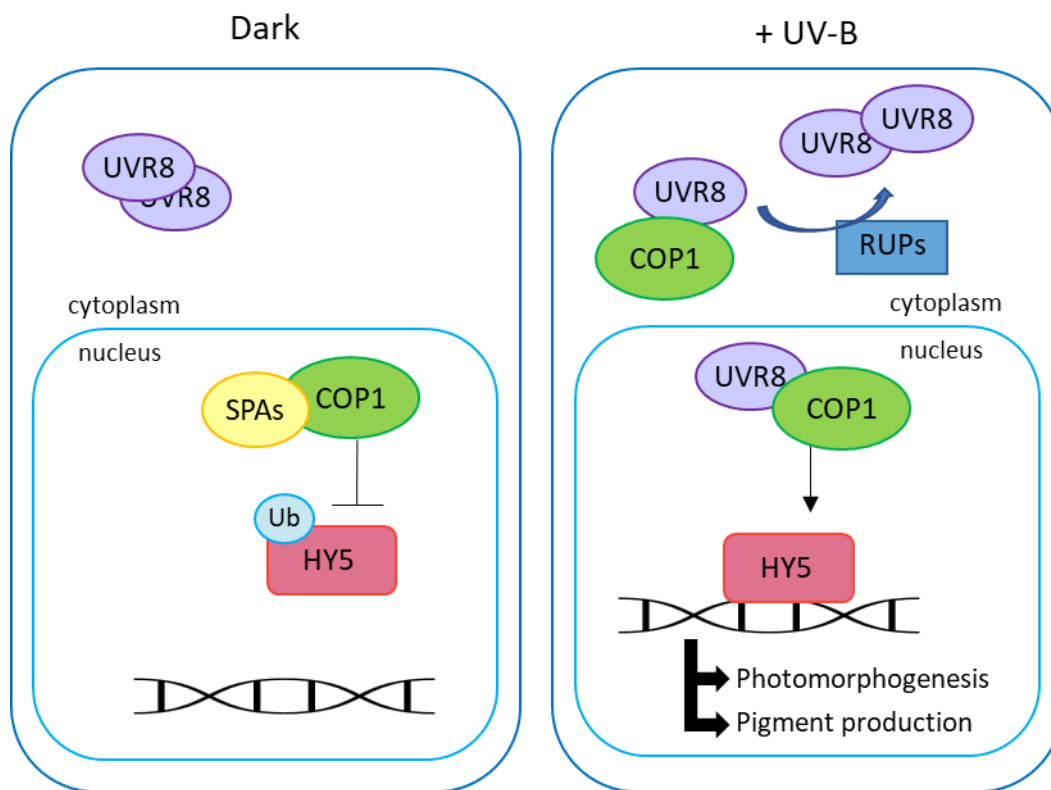
Figure 1.1 A simplified view of UV-B-induced gene expression. In the dark, UVR8 exists as dimers and will not interact with COP1. This allows COP1 to interact with SPA proteins, translocate to the nucleus and function as an E3 Ub ligase. COP1 ubiquitinates factors involved in promotion of photomorphogenesis, such as HY5, resulting in their degradation. Under darkness, HY5 does therefore not facilitate the expression of photomorphogenesis-related genes, and photomorphogenesis does not occur. Under UV-B, UVR8 monomerises and can bind to COP1, sequestering COP1 and preventing it from degrading HY5. This is necessary for UVR8 translocation to the nucleus where it carries out its function of promoting expression of photomorphogenesis-related genes and genes involved in typical UV-B responses, such as pigment production. This occurs, in part, through stabilisation of HY5. RUP1 and RUP2 promote UVR8 re-dimerisation, inactivating the UVR8 signalling pathway.

1.1.5 Physiological responses to UV-B

Regulation of photomorphogenesis is governed by complex networks linking photoreception with responses to light. When a seedling emerges from the soil, it encounters a range of wavelengths of light emitted by the sun, including UV-B.

Red, blue and UV-B light have strong inhibitory effects on hypocotyl elongation as part of the de-etiolation process. Control of hypocotyl elongation is also central in responses to changing environmental conditions in responses such as shade avoidance and thermomorphogenesis (Gray *et al*., 1998; Ballaré *et al*., 1990; Franklin and Whitelam, 2005). Downstream of the red, blue and UV-B photoreceptors, three groups of transcription factors are central to the control of hypocotyl elongation: the BRASSINAZOLE-RESISTANTs (BZRs), the AUXIN RESPONSE FACTORs (ARFs) and the PIFs (reviewed by Favero *et al*., 2021; PIFs will be further discussed in 1.1.6). These groups of regulators are affected by brassinosteroids, auxin, and regulated by light, respectively.

UV-B-mediated regulation of brassinosteroid signalling

Brassinosteroids (BRs) are a group of plant steroid hormones that affect many aspects of plant life, including growth and development (Nolan *et al*., 2019). When BRs are perceived by the co-receptor unit consisting of BRASSINOSTEROID INSENSITIVE (BRI1) and BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1), phosphorylation activity by BRASSINOSTEROID-INSENSITIVE 2 (BIN2) and GLYCOGEN SYNTHASE KINASE 3 (GSK3) enzymes is reduced. BIN2 and GSK3 target and phosphorylate the BR-responsive proteins BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR 1 (BES1). Phosphorylation of BZR1 and BES1 inhibits these factors from binding DNA, and promotes their exclusion from the nucleus (Favero *et al*., 2021). This means that reduced phosphorylation of BZR1 and BES1, when BR is perceived, allow BZR1 and BES1 DNA-binding and promotion of BR-induced gene expression of growth-related genes. UV-B inhibits BR signalling via UVR8 by suppressing the expression of BES1-targeted genes. UVR8 binds dephosphorylated BES1, inhibiting it from binding promoters of its target genes. However, UV-B does not regulate expression of genes involved in BR biosynthesis (Liang *et al*., 2018). UVR8-mediated

inhibition of BR signalling leads to alterations in cell elongation and plant growth responsible, in part, for the inhibition of hypocotyl elongation under UV-B (Liang *et al.*, 2018).

UV-B-mediated regulation of auxin signalling

Auxins are small indole growth regulators that regulate growth and development, particularly by their effect on cell elongation, division and differentiation. In short, regulation of auxin-induced gene expression is carried out by two groups of molecules: the Aux/IAA and the ARF proteins. ARFs bind auxin-responsive elements (TGTCTC; AREs) in promoters of auxin-responsive genes (Ulmasov *et al.*, 1999). Aux/IAA proteins bind to ARFs and inhibit their effect on gene transcription of auxin-induced genes (Teale *et al.*, 2006), creating competition between repression and promotion of gene expression in response to auxin. UV-B alters gene expression of genes connected to auxin signalling (Hectors *et al.*, 2007), often via HY5 (Cluis *et al.*, 2004; Hayes *et al.*, 2014) and will be further discussed below, in connection with the close link between auxin and PIF signalling.

UV-B-mediated regulation of PIF signalling

Phytochrome-interacting factors (PIFs) are basic helix-loop-helix (bHLH) transcription factors involved in many plant responses such as seed germination, photomorphogenesis and leaf senescence via regulation of gene expression. Structurally, PIFs possess a bHLH domain (a ~15 amino acid basic N terminal region which binds DNA, and a ~60 amino acid HLH region which governs dimerization) consisting of two α-helices connected by a region at the C-terminal which may form a loop (Castillon *et al.*, 2007). The remaining part of the protein is less conserved and can show great diversity in sequence. The PIF family of transcription factors are encoded by a subset of the bHLH superfamily (Toledo-Ortiz *et al.*, 2003). Eight different PIFs exist in Arabidopsis, named PIF1 to PIF8 (Pham *et al.*, 2018) of which PIF3 was the first to be described (Ni *et al*, 1998). PIFs were first discovered as factors that interact with phytochromes A and B. The N terminal regions of PIFs contain a conserved sequence motif, the active phytochrome binding (APB) motif, which binds phytochrome B (Khanna *et al.*, 2004). In addition to the APB motif, PIF1 and PIF3 also contain a phyA-binding APA motif (Pham *et al.*, 2018). Different PIFs show different affinities to different phytochromes, and to different regions within

phytochromes, suggesting PIFs help modulate the response to varying light conditions (Castillon *et al.*, 2007). phyB has been shown to bind all eight PIFs after its translocation to the nucleus following red light perception and its transformation into the inactive Pfr form (Ni *et al.*, 1998; Pham *et al.*, 2018). phyB can sequester PIFs and thereby inhibit DNA-binding (Pham *et al.*, 2018) and promote destabilization of PIFs via phosphorylation events followed by ubiquitination and subsequent degradation (Leivar and Quail, 2011). This prevents the PIFs from binding their target gene promoters and thus inhibits PIF-regulated gene expression (Park *et al.*, 2012). PIF3 accumulates in the nucleus during darkness, promoting skotomorphogensis (Bauer *et al.*, 2004). Ling *et al.* (2017) later showed that COP1 has a noncanonical function as part of a COP1-SPA complex in stabilising PIF3 in the dark via direct binding, and by supressing BIN2 phosphorylation of PIF3 which promotes its degradation by the 26S proteasome. Bauer *et al.* (2004) showed that PIF3 is rapidly degraded upon RL induction and is not detectable after 30 min, while Huq and Quail (2002) showed that PIF4 has the capacity to bind a G-box sequence motif in promoters of genes which govern light responses. The roles of PIFs are further evidenced by the responses of *pif* mutants. For example, the *pif4* mutant shows a hypersensitive response to red light, indicating a negative effect on photomorphogenesis by PIF4 (Huq and Quail, 2002). Furthermore, Toledo-Ortiz *et al.* (2003) showed that PIF3 and PIF4 can form homodimers and heterodimers, further increasing the variation of the gene regulation by PIFs.

UV-B has been shown to rapidly degrade PIF4 and PIF5 in a UVR8-mediated manner to induce typical UV-B responses such as inhibition of hypocotyl elongation in sunlight, inhibition of shade avoidance and inhibition of thermomorphogenesis (Hayes *et al.*, 2014; Hayes *et al.*, 2017; Sharma *et al.*, 2019; Tavridou *et al.*, 2020). UV-B also suppresses *PIF4* transcript accumulation, but this effect may be transient as decreases in *PIF4* and *PIF5* transcripts were seen after 3 h, but no difference from the control was seen at 6 h (Tavridou *et al.*, 2020). Hayes *et al.* (2017) also observed a decrease in *PIF4* transcript abundance after 4 h UV-B. *PIF4* transcript abundance has also been shown to vary on a diurnal cycle which may contribute to temporal variation observed during time course experiments (Nomoto *et al.*, 2012). PIFs have been found to regulate several auxin-related genes. For example, PIF5 G-box binding sites were found in genes of auxin biosynthesis and signalling components (Hornitschek

et al., 2012). In the same study, gene ontology analysis revealed that processes such as 'shade avoidance' and 'response to hormone stimulus' were enriched for genes that are regulated by PIF5, and supports the involvement of PIF5 regulation in auxin signalling. Furthermore, Sun *et al*. (2013) found that PIF4 upregulates auxin biosynthesis genes *IAA19* and *IAA29*. Hayes *et al*. (2014) found that UV-B antagonises shade avoidance by destabilising PIF4 and PIF5 proteins. Indeed, PIF4 and PIF5 destabilisation by UV-B also contributes to inhibition of hypocotyl elongation in non-shade conditions (Sharma *et al*., 2019; Tavridou *et al*., 2020). Hayes *et al.* (2014) also showed that *IAA29* transcript abundance was reduced in a UVR8-dependent manner in response to UV-B. This supports data from Hornitschek *et al*. (2012) who showed that a *pif4pif5* double mutant have much reduced auxin accumulation in response to a low R:FR ratio compared WT plants.

Interestingly, PIF7 was shown to be a critical regulator in thermomorphogenesis in shaded conditions (Burko *et al*., 2022), suggesting different PIFs are dominant in different responses to light and temperature. Gibberellic acid signalling is also closely involved in hypocotyl elongation and will be discussed in chapter 3.

UV-B-mediated regulation of cotyledon expansion

The process of cotyledon expansion promotes expansion and maturation of the first photosynthetic leaf tissues of a developing seedling during de-etiolation. It has long been known that phyB mediates red light-induced cotyledon expansion (Neff and Volkenburgh, 1994). Kim *et al.* (2002) showed that low fluence UV-B also contributes to cotyledon size, via modification of cotyledon cell expansion. Qian *et al*. (2020) later showed that the transcription factor MYB13, which is predominantly expressed in cotyledons, regulates several UV-B responses in Arabidopsis including cotyledon expansion. A UV-B light sensing mechanism was reported in root tissue of Arabidopsis seedlings (Tong *et al*., 2008), and later studies confirmed *UVR8* expression in all plant tissues, including roots (Rizzini *et al*., 2011). UVR8 expression in roots of other species has also been confirmed (Mao *et al*., 2015; Li *et al*., 2018; Li *et al*., 2018; Zhao *et al*., 2016). In Arabidopsis, UVR8 has been shown to interact with MYB73 and MYB77, to regulate root growth via the suppression of auxin-responsive genes (Yang *et al*., 2020). Interestingly, UV-B was shown to reduce root growth in soybean and barley, suggesting this may be a

conserved response (Zhang *et al.*, 2019; Ktitorova *et al.*, 2006). However, Kim *et al.* (2002) found no effect on low fluence UV-B on root growth in Arabidopsis, suggesting variation in experimental conditions could affect the efficacy of UV-B in this response.

UV-B-mediated regulation of protective responses

Many responses of plants to UV-B irradiation are preventative or protective to the UV-B itself. Under this category of responses falls the production of sun-screening pigments such as carotenoids and flavonoids. These accumulate in the epidermal cell layer of leaves and stems, protecting photosynthetic machinery from harmful UV-B rays (Chappell and Hahlbrock, 1984; Dangl *et al.*, 1987). Similarly, UV-B increases the production and accumulation of both reactive oxygen species (ROS) as well as upregulating the production and accumulation of ROS-scavenging enzymes and molecules, which neutralise ROS (Dai *et al.*, 1997). Flavonoids and ROS will be further discussed in chapter 3.

High fluence UV-B was shown to reduce stomatal aperture in mature *Arabidopsis thaliana* in a ROS-dependent manner (Tossi *et al.*, 2014). The role of UV-B in stomatal movement will be discussed in chapter 6.

1.1.6 UVR8-independent signalling

It is generally accepted that high fluence UV-B responses are mediated via stress signalling and not via UVR8, while low fluence UV-B responses are mediated in a UVR8-dependent manner (Brown and Jenkins, 2008). Despite this, there is evidence of low fluence UV-B responses not mediated by UVR8. For example, while leaf growth was affected in response to UV-B in WT plants but not in *uvr8* mutants, the inhibition of epidermal cell division was reduced in both WT and *uvr8* mutants, suggesting a UVR8-independent mechanism (Wargent *et al.*, 2009). More recent evidence from transcriptomics suggests that low fluence rate UV-B alter gene expression of a host of genes, independently of UVR8 (O'Hara *et al.*, 2019). The authors focused on the gene *ANAC13*, which was induced by as little as $0.1 \ \mu mol \ m^{-2} \ s^{-1}$ UV-B and did not involve signalling components such as COP1 or HY5/HYH; neither did it rely on activity of phototropins, phytochromes or cryptochromes (O'Hara *et al.*, 2019). It is not clear if other UV-B-specific photoreceptors exist, or what the UVR8-independent mechanism(s) may be.

1.2 Temperature stress in plants

Low temperature

Chilling and freezing temperatures are major environmental signals affecting plant growth and development, as well as limiting plant distribution across the globe (fig. 1.2). These limitations apply to natural biosystems and agriculture alike. While a cold treatment is often required to alleviate seed dormancy and promote germination, a sudden reduction in temperature (cold snap) can prove devastating to plant survival. For example, during the Eastern US spring freeze of 2007 where nightly temperatures between the 5th and 9th of April consistently reached below 0°C, the economic loss for agriculture in North America was estimated to $111.7 million (Gu *et al*., 2008). Such losses also affect food security, an ever-increasing problem in an increasingly populated world.

Plants sensitive to chilling (15°C-0°C) are generally native to tropical regions. In these areas, temperatures do not fall below 18°C (Kottek *et al*., 2006). Plants growing in tropical and subtropical regions (where temperatures can reach as low as 0°C but never below) are susceptible to chilling damage (Levitt, 1980). This is displayed through wilting, chlorosis and tissue death when exposed to temperatures of 0-15°C. Conversely, chilling-tolerant but freezing-sensitive plants are generally native to temperate climate areas. Here temperatures can fall below 0°C for extended periods of times (Kottek *et al*., 2006). Temperate plants growing in north-western Europe experience temperature differences up to 40°C across seasons, and as much as ~20°C over the diurnal cycle according to the Intergovernmental Panel on Climate Change (Mitchell and Jones, 2005). This thesis focused on the chilling-tolerant *Arabidopsis thaliana* which also has the capacity to acclimate to freezing temperatures. Chilling-sensitive plants will therefore not be discussed further.

1.2.1 Freezing injury

A major source of damage associated with freezing is freezing-induced dehydration (FID) following ice crystal formation as extracellular water freezes. This water has higher a freezing point than the intracellular water due to a lower solute concentration compared with the cytoplasm (Pearce, 2001). The ice formed lowers water potential (Ψ) of the apoplast (Ψ_a), and when Ψ_a falls below the Ψ of the

cytoplasm (Ψ_c), water moves by chemical osmotic force from the cytoplasm, resulting in a dehydrated intracellular environment. For example, approximately 90% of the intracellular osmotically active water moves into the extracellular space when temperatures reach -10°C (Thomashow, 1999). Dehydration is a major stress for cells, as it disrupts cellular biochemistry (Oliver *et al.*, 2010). Furthermore, FID can indirectly cause membrane damage through a mechanism called expansion-induced lysis (EIL; Gordon-Kamm and Steponkus, 1984; Thomashow, 1998). When the water in the apoplast thaws, increasing the water potential in this compartment, osmotic forces cause water to re-enter the cytoplasm. The cell, having undergone plasmolysis, now expands too rapidly for appropriate re-expansion of the plasma membrane (PM). The PM is damaged by this sudden increase in turgor, resulting in EIL.

Biological membranes are composed predominantly of phospholipids. These lipids are amphiphilic; two hydrophobic fatty acids connect to one hydrophilic head group containing a phosphate group. This bipolarity makes phospholipids excellent components of lipid bilayers. The hydrophilic head group remains in contact with the aqueous solutions of the cytoplasm or extracellular space, while the two hydrophobic fatty acid tails orientate themselves inwards, where they are sheltered from any contact with surrounding water. To function optimally, lipid membranes must maintain fluidity in order to allow diffusion and transport of proteins and other molecules in and across the membranes. The fluidity of a plant cell membrane is dictated, partly, by temperature (Levitt, 1980). LT promotes transitions of lipid membranes. For example, a lipid membrane can be destabilised via lamellar to hexagonal phase transition during freezing (Steponkus and Webb, 1992; Steponkus *et al.*, 1993). In fact, in isolated protoplasts, vesicles formed after osmotic contraction (Gordon-Kamm and Steponkus, 1984). Furthermore, cells can become unresponsive to further alterations in osmotic forces after a freeze-thaw cycle; this is referred to as loss of osmotic responsiveness (LOR) (Uemura *et al.*, 2003). Mutants in fatty acid desaturases have been shown to have altered tolerance to temperature changes (Penfield, 2008). For example, *fatty acid desaturase 2* (*fad2*) mutants that have a deficit in the activity of the endoplasmic reticulum 18:1 DESATURASE involved in the production of polyunsaturated lipids are severely sensitive to low temperatures. Moving from 22°C to 6°C resulted in the death of *fad2* mutants,

Figure 1.2 5-week-old *Arabidopsis thaliana* damaged by -6℃ displays extensive tissue damage and death as well as discolouration of still viable tissue.

whereas WT plants continued to develop, showing a chilling sensitive phenotype (Wallis and Browse, 2002). In addition to the osmotic forces created by ice crystals in the extracellular space, they can also physically damage membranes (Chen and Gusta, 1978; Hudson and Idle, 1962). Indeed, membrane stability appears to be crucial in determining plant health as deficiency in lipid polyunsaturation of developing chloroplast membranes resulted in leaf chlorosis at LT (Hugly and Somerville, 1992).

1.2.2 Cold acclimation

Some plants can protect themselves against the damaging effects of freezing temperatures. Cold acclimation (CA) is a collection of molecular and physiological responses to low, non-freezing, temperatures. These responses act to reduce the negative impact freezing temperatures have on plant health. CA is not an absolute, but rather depends on length and temperature of cold exposure (Gilmour *et al*., 1988; Guy *et al*., 1985; Kurkela *et al*., 1988; Kawamura *et al*., 2003). Plants as sessile organisms must have the capability to perceive, and appropriately respond to, surrounding temperature conditions in order to survive. Here, the perception of low temperature, cell signalling, and changes in gene expression are discussed in the context of cold acclimation.

1.2.3 Cold perception

While there is a large body of literature covering cell signalling and molecular events in plants exposed to varying temperatures, little is known of definitive cold sensing mechanisms (Markovskaya and Shibaeva, 2017). As highlighted by Franklin and Knight (2010) through pure thermodynamic effects, temperature affects various supramolecular and molecular structures encumbering research into *bona fide* cold sensors. Indeed, *Arabidopsis thaliana* appears to have several inter-connecting systems which may be responsible for cold sensing over a great range of temperatures (reviewed by McClung and Davies, 2010). One of the earliest events observed upon cold induction is a change in intracellular Ca^{2+} transients (Knight *et al*., 1991; Monroy and Dhindsa, 1995). Ca^{2+} signals are perceived by several different intracellular molecules which could be involved in the cold response signalling pathway (Whalley *et al*., 2011; Catalá *et al.,* 2003; Doherty *et al*., 2009). For example, calmodulin and calmodulin-like proteins are considered major sensors for changes in intracellular $[Ca^{2+}]$ (Reviewed by Roberts and Harmon, 1992). As calmodulin is a multifunctional protein, it has the potential to interact

with a host of cellular targets, affecting their activity in a Ca^{2+}-dependent manner (Hepler and Wayne, 1985; van der Luit *et al*., 1999). Calmodulin was shown to be a positive regulator for induction of cold acclimation genes in Alfalfa (Monroy and Dhindsa, 1995) and *kin* gene expression in Arabidopsis (Tähtiharju *et al*., 1997). Interestingly, overexpression of the CaM3 calmodulin isoform in Arabidopsis caused a reduction of gene expression of some cold-regulated genes (Townley and Knight, 2002). Ca^{2+} can also combine with Calcium-dependent protein kinases (CDPKs). CDPKs play a vast range of roles in plant growth and abiotic stress responses (Shi *et al*., 2018). Over-expression of one CDPK in rice maintained chlorophyll fluorescence (F_v/F_m) after a 4^oC treatment compared with non-transgenic plants (Saijo *et al*., 2000). Interestingly, upregulation of expression of genes encoding certain CDPKs occurred after cold acclimation (Monroy and Dhindsa, 1995), suggesting that Ca^{2+}-CDPK may act early in the cold response, but also after altered gene expression is induced. It has been suggested that PM fluidity and PM components, such as the rice (*Oryza sativa* L.) G-protein regulator CHILLING TOLERANCE DIVERGENCE 1 (COLD1; Ma *et al*., 2015) trigger Ca^{2+} channel activity. Activation of such PM components may be responsible for Ca^{2+} signatures (amplitude, duration and frequency of signal) produced by Ca^{2+}-permeable channels such as MCA1 and MCA2 (MID1-COMPLEMENTING ACTIVITY 1 and 2; Mori *et al*., 2018). LT was shown to induce cytosolic Ca^{2+} via MCA1 and 2. Other PM components may also be involved in acquired cold acclimation. It was found the quantity of 38 leaf PM proteins was altered during cold acclimation (Kawamura and Uemura, 2003). These included proteins known to be involved in membrane fusion. Liu *et al*. (2017) recently reported that a PM cold-responsive protein kinase 1 (CRPK1) phosphorylates 14-3-3 proteins. These phosphorylated 14-3-3 proteins can translocate from the cytosol to the nucleus of plant cells and destabilise proteins involved in freezing tolerance. Although fragments are known about LT perception, the mechanisms are complex and to date not clearly understood.

1.2.4 Cell signalling and gene expression during cold

Early studies suggested that levels of certain proteins were altered upon different temperature treatments. In Orchard grass (*Dactylis glomerata* L.), isolated plasma membrane fractions were analysed for differences in protein and lipid composition after a LT treatment (Yoshida and Uemura,

1984), while in Rapeseed (*Brassica napus*), newly synthesised proteins were labelled and separated, which revealed that some proteins specifically accumulate at LT (Meza-Basso *et al.*, 1986). This sparked further research into genetic changes during cold exposure and data have since shown both up- and down-regulation of genes during LT. Early on, Guy *et al.* (1985) showed increased expression of several mRNA transcripts in cold acclimated spinach (*Spinacia oleracea L.*) which correlated with increased freezing tolerance. Strand *et al.* (1997) found that transcript levels of genes encoding proteins involved in photosynthesis was lower in cold-treated plants compared with controls. Fowler and Thomashow (2002) investigated the transcriptome of plants exposed to 4°C over a time course of 0-168 h. The authors reported a total of 306 genes with altered expression during cold exposure. Of these, 218 were upregulated while 88 were downregulated. A different study collated data from several independent studies and presented a greater number of genes both upregulated and downregulated, with specific dependence on short, medium- or long-term cold exposure (Hannah *et al.*, 2005).

Among the genes with altered expression during cold exposure, *C-REPEAT BINDING FACTOR* (*CBF*) and *COLD REGULATED* (*COR*) genes play major roles in cold acclimation and plant survival during freezing. The *CBF* genes encode the CBF transcription factors which are part of the larger gene family (APETALA2/ETHYLENE-RESPONSIVE FACTOR-type transcription factors) and regulate expression of some *COR* genes (Jaglo-Ottosen *et al.*, 1998). In Arabidopsis, the CBF family of genes induced by LT exposure has three members, namely the paralogs *CBF1*, *CBF2*, and *CBF3* (also known as *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 1b*, *1c*, and *1a*, respectively). Increased expression is observed as early as 15 min after cold induction with accumulation of *CBF* transcripts after 2 h (Gilmour et al., 1998). Early studies showed that overexpression of *CBF1* and *CBF3* enhanced freezing tolerance in Arabidopsis (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). The phenotypes of *cbf* single and double mutants have been shown to be somewhat complex, yet *cbf* triple mutants confirm an important role for CBFs in cold acclimation (Jia *et al.*, 2016; Zhao and Zhu, 2016; Reviewed by Liu *et al.*, 2018). *CBF* homologs in several crops such as maize (*Zea mays* L.) and rice (*Oryza sativa* L.) were also identified, highlighting conservation of these genes (Qin *et al.*, 2004; Dubouzet *et al.*, 2003). The Arabidopsis genome contains further *CBF*-related genes, which are

expressed after dehydration stress and ABA treatment (*CBF4*; Haake *et al*., 2002) or high salinity (*DDF1*; Magome *et al*., 2004). Expression of the endogenous *CBF4* in grape (*Vitis vinifera*) and poplar (*Populus euphratica*) was enhanced during cold exposure (Xiao *et al*., 2008; Tian *et al.*, 2016). Interestingly, the grape *CBF4* was found not to be an orthologue to the *AtCBF4*, but had a 57% sequence similarity to *AtCBF1*. Constitutive expression of *AtCBF4* resulted in accumulation of certain *COR* genes in Arabidopsis (Haake *et al.,* 2002). Recently, Shi *et al.* (2016) investigated the precise regulation of *COR* gene expression by CBF1, 2 and 3, respectively, in Arabidopsis. The authors found that two thirds of *COR* genes that were regulated by CBFs were regulated by more than one CBF while the remaining third were regulated by single CBFs. Typically, it was CBF2 and 3 which predominantly coordinated regulation of *COR* gene expression, with CBF1 playing a smaller role. Conversely, Novillo *et al.* (2007) found that a *cbf2* mutant displayed greater freezing tolerance than WT controls, suggesting a negative feedback loop with control exerted by CBF2 over CBF1 and 3. The CBF transcription factors bind to *COR* gene promoters via recognition of a conserved CRT/DRE (C-REPEAT/DEHYDRATION RESPONSE ELEMENT) cis-element motif 5'-CCGAC-3' (Baker *et al*., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger *et al*., 1997) and thus regulate expression of some *COR* genes (Park *et al*., 2015).

CBF transcription has been shown to be governed by several regulators. These include, but are not limited to, INDUCER OF CBF EXPRESSION 1 and 2 (ICE1; Chinnusamy *et al*., 2003), CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR3 (CAMTA3; Doherty *et al*., 2009), MYB15 (Agarwal *et al*., 2006) CIRCADIAN CLOCK-ASSOCIATED 1 (CC1; Dong *et al*., 2011), and PIF3, PIF4 and PIF7 (Jiang *et al*., 2017; Lee and Thomashow, 2012). ICE1 is a bHLH TF and was first identified in Arabidopsis where expression patterns were also analysed. *ICE1* was constitutively expressed, with greater expression in stems and leaves than other plant tissues, and displayed only a minor upregulation during LT (Chinnusamy *et al*., 2003). ICE1 binds to a MYC recognition sequence in the *CBF* promoter activating expression during LT (Chinnusamy *et al*., 2003). The *ice1* mutant showed reduced expression of *CBF3* and other cold-inducible genes, as well as reduced chilling and freezing tolerance (Chinnusamy *et al*., 2003). ICE1 is itself regulated by HIGH EXPRESSION OF

OSMOTICALLY RESPONSIVE GENES1 (HOS1), an E3 ubiquitin ligase (Dong *et al*., 2006), required for ICE1 degradation. Previously, HOS1 had been shown to negatively affect freezing tolerance as a *hos1* mutant displayed enhanced expression of *CBF* gene transcripts and increased freezing tolerance (Lee *et al*., 2001). Interestingly, ICE1 was sumoylated by the SUMO E3 ligase SIZ1 and phosphorylated by OPEN STOMATA 1 (OST1) which may act to stabilise the protein and reduce its degradation by HOS1 (Miura *et al*., 2007; Ding *et al*., 2015). In addition to ubiquitylation and sumoylation, ICE1 was shown to be phosphorylated by MPK3 and MPK6 which alters the ICE1 TF activity (Li *et al.,* 2017; Zhao *et al*., 2017). *mitogen-activated protein kinase 3 (mpk3)* and *mpk6* loss of function mutants had higher freezing tolerance than WT plants, suggesting the MPK3/6 kinase cascade has a negative effect on the freezing tolerance response in Arabidopsis (Zhao *et al*., 2017). Fursova *et al*. (2009) identified *ICE2*, which provided enhanced freezing tolerance when overexpressed in a transgenic line. In plants with altered *ICE2* expression, expression of *CBF1* was also altered, suggesting certain control over FT is exerted by *ICE2* via *CBF1*. Interestingly, a recent study has shown that aberrant expression of *CBF* genes in the *ice1-1* mutant results from transgene-induced silencing and not ICE1 regulation (Kidokoro *et al*., 2020). This suggests that the control of *CBF* expression, and LT signalling during cold acclimation is still not fully understood. The brassinosteroid signalling pathway transcription factor BZR1 directly targets *CBF1* and *CBF2* and a gain of function *bzr1-1D* mutant displayed enhanced freezing tolerance (Li *et al*., 2017). This alludes to cold acclimation and freezing tolerance also being governed by hormonal signals. Negative regulators of *CBF* expression have also been identified. CAMTA3 is a calmodulin-binding transcription factor which positively regulates *CBF2* expression, and which may integrate cold-induced calcium signalling (as previously discussed) with LT responses (Doherty *et al*., 2009). The MYB15 TF was found to interact with *ICE1* and to negatively regulate *CBF* expression via recognition of and binding to Myb sequence in *CBF* promoters (Agarwal *et al*., 2006). The LT-induced expression of the Arabidopsis CBFs is also gated by the circadian clock (Harmer *et al*., 2000; Fowler *et al*., 2005) and photoperiod variations (Lee and Thomashow, 2012), representing further regulation of the LT response. In conclusion, many genes appear to be involved in cold acclimation and freezing tolerance and crosstalk between these, if it exists, is complex.

In addition to the CBF regulation of cold-induced genes, other regulatory mechanisms/networks have been described which may act together with, or independently from, the CBFs (Zhao *et al.*, 2017; Park et al., 2015). Indeed, Park *et al.* (2015) point out the complexity of the network of signals which govern freezing tolerance in Arabidopsis, and that cold acclimation is not entirely dependent on the CBF regulon alone. For example, nine LT-induced and 15 LT-repressed genes were assigned to a regulon controlled by the TF ZAT12 (Vogel *et al.*, 2004). ZAT12 reduced the transcript level of *CBF1, 2* and *3* in Arabidopsis, and constitutive expression of *ZAT12* reduced plant freezing tolerance, suggesting that ZAT12 controls a negative regulatory mechanism suppressing this process (Vogel *et al.*, 2004). Furthermore, the loss of function mutant *hos9-1* (high expression of osmotically responsive genes) had lower freezing tolerance than WT plants but the mutation had no effect on *CBF1*, *CBF2*, or *CBF3* expression (Zhu *et al.*, 2004). In contrast to HOS1, which acts as an E3 Ub ligase, *HOS9* encodes a member of the WUS clade of transcription factors genes. These data highlight two possible CBF-independent pathways.

Among the highly cold-induced genes in Arabidopsis are the CBF-targeted genes of the CBF regulon: the *COR* genes (Hajela *et al.* 1990; Gilmour et al., 1992; Lin and Thomashow, 1992). The *COR* genes of Arabidopsis comprise four gene families, *KIN2* (*COR6.6*; structurally similar to *KIN1*; Kurkela and Franck, 1990), *COR15*, *COR47* (RD17), and *COR78* (*DESICCATION-RESPONSIVE PROTEIN 29A, RD29A*, *LTI40*; Horvath *et al.*, 1993) with two members each (Wilhelm and Thomashow 1993; Nordin *et al.*, 1991; Yamaguchi-Shinozaki and Shinozaki 1993; Welin *et al.*, 1994). *KIN2* and *COR78* encode hydrophilic polypeptides which are LT- and desiccation-induced, respectively. *COR15* and *COR47* encode proteins with sequence similarity to LATE EMBRYOGENESIS ABUNDANT (LEA) proteins and are classed as dehydrins (Dure, 1993; Welin *et al.*, 1995; Close, 1997; Hundertmark *et al.*, 2008). LEA proteins were first identified in cotton by Baker *et al.* (1988) and are produced during late embryogenesis as a seed matures as well as during drought and LT stress (dehydration). In addition to the four main COR gene families, other genes typically involved in freezing tolerance are the *SENSITIVE TO FREEZING* (*SFR*) genes. McKown *et al.* (1996) investigated the effects of 7 mutations in the *SFR* genes and found alterations in anthocyanin, sucrose and glucose accumulation, as well as

FA composition seen during and after cold acclimation. The mutants *sfr3, sfr4, sfr6,* and *sfr7* had reduced anthocyanin during CA, while *sfr4* mutation prevented sucrose and glucose accumulation during cold exposure. *Sfr4* and *sfr7* mutations affected FA composition after CA, while *sfr1, sfr2* and *sfr5* did not differ from the WT in this study. A role for SFR2 in FT and specifically in chloroplast protection was identified as chloroplasts of *sfr2* mutants were severely damaged by freezing (Thorlby *et al*., 2004; Fourrier *et al*., 2008; Moellering *et al*., 2010). Interestingly, LT may also alter the expression of genes which do not play a major role in cold acclimation. For example, Jarillo *et al*. (1993) found that etiolated seedlings and mature leaves of plants exposed to LT had a higher level of *ALCOHOL DEHYDROGENASE* (*ADH*) mRNA, however an *adh* null mutant did not have altered capacity to cold acclimate.

1.2.5 Plant responses to cold

On a physiological level, the precise functions of many cold-induced gene products are not fully understood. However, evidence has been presented that COR15a translocates to the stromal compartment of chloroplasts (Lin and Thomashow, 1992; Nakayama *et al*., 2007) and displays cryoprotective activity (Lin and Thomashow, 1992; Artus *et al*., 1996; Steponkus *et al*., 1998; Nakayama *et al*., 2007). COR15a was also shown to prevent membrane fusion (Uemura *et al*., 1996) and lamellar-to-hexagonal II phase transition of freezing-induced dehydration of adjacent PM and chloroplast membranes, as well as lowering the freezing temperature at which such transitions occur (Steponkus *et al*., 1998). Membrane protection has long been considered one of the most vital aspects of protection against LT (Ristic and Ashworth, 1993; Steponkus *et al.,* 1993; Uemura and Steponkus, 1994). The constituents of membranes are important, as loss of function of sterol glycosyltransferase resulted in reduced freezing tolerance (Mishra *et al*., 2015). In general, dehydrins (a group of LEA proteins; Hundertmark *et al*., 2009) are intrinsically disordered proteins (IDPs) whose main function appears to be stabilisation of membranes, enzymes and nucleotides during abiotic stress (Graether and Boddington, 2014; Yu *et al*., 2018). Interestingly, the dehydrin COR47 was used as negative control in a study of membrane tolerance by Eriksson *et al*. (2011) because it does not bind vesicle membranes, yet expression was elevated after cold induction (Nylander *et al*., 2001; Seki *et al*., 2001) and enhanced

expression of *COR47* was shown to enhance freezing tolerance in Arabidopsis (Puhakainen *et al*., 2004). LEA proteins act as osmoprotectants, and COR47 is thought to have osmoprotective functions. The main mode of action of COR47 is, however, to date unclear. The Arabidopsis genome encodes two RD29 genes, RD29A and RD29B, of which RD29A is the most LT-responsive (Msanne *et al*., 2011). Whereas the function of RD29A in salt and ABA responses, and its role in root elongation during chronic LT in alpine species of Arabidopsis has been investigated, its precise function in the LT response remains unknown (Msanne *et al*., 2011; Lee *et al*., 2016; Lee *et al*., 2009). To counteract the negative physical effects of ice crystal formation, plants have antifreeze proteins (AFPs; also called ice-binding proteins) which adsorb to a forming ice crystal and prevent further crystal growth (Griffith *et al*., 1992; Bredow and Walker, 2017). Efforts to breed plants with enhanced freezing tolerance through upregulated AFPs have shown some promising results (Wallis *et al*, 1997) but have mostly been relatively fruitless (reviewed by Bredow and Walker, 2017).

1.2.6 Abiotic factors influencing cold tolerance in plants

Factors other than low temperatures can affect a plant's capacity to cold acclimate. Notably, other forms of osmotic stress, such as dehydration (Siminivotch and Cloutier, 1982; Lee and Chen, 1993; Mäntylä *et al*., 1995), and high salinity (Ryu *et al*., 1995) can prime the CA response. ABA treatment, which is a secondary effect of osmotic stress also influences CA (Chen and Gusta, 1983; Lee and Chen, 1993). Light may also indirectly affect LT tolerance capacity (Wanner and Junttila, 1999). The primary product of photosynthesis is sugars, and sugars can also act as cryoprotectants. For example, sucrose is a well-established contributor to cryoprotection of plant cells during freezing stress (Anchordoguy *et al*., 1987; Ristic and Ashworth, 1992). The *sfr4* mutant was shown to be impaired in sucrose and glucose accumulation and could not cold acclimate appropriately (McKown *et al*., 1996). Furthermore, the accumulation of Proline may also contribute to freezing tolerance. *CBF1, CBF2* and *CBF3-* overexpressing Arabidopsis plants had elevated levels of Proline and soluble sugars (Gilmour et al., 2000; Gilmour et al., 2004). However, results obtained by other groups were somewhat ambiguous when determining a direct effect of proline on freezing tolerance (Wanner and Junttila, 1999; Xin and

Browse, 1998; Nanjo *et al*,. 1999). Light quality, perceived by photoreceptors has great impacts on plant freezing tolerance and will be further discussed in chapter 3.

High temperature

Together with light, temperature cues are generally considered one of the most important environmental signals that affect plant growth and development (Kim *et al*., 2021). When plants experience temperatures outside of the range within which normal growth and development takes place, temperature stress occurs. Changes in plant distribution is already affected by climate change (Willis *et al*., 2008) and it is predicted that further changes will occur in the near future. It is therefore important to understand the responses of plants to elevations in temperature, and perhaps very important to understand the mechanisms of temperature signalling and thermotolerance.

1.2.7 Perception of high temperature

Similar to decreases in temperature, many cellular processes and components are affected by increases in temperature, and a definition of a thermosensor is therefore somewhat difficult to establish (Franklin *et al*., 2014). It is also well-known that the fluidity of biological membranes that are predominantly made up of lipids, are affected by changes in temperature. Changes can affect membrane-associated or membrane-bound molecules, which in turn trigger downstream signalling. However, as Penfield (2008) highlights, membranes are unlikely to be the main thermosensory system in plants as the signalling process is thought to be too unspecific. Though some mechanisms of thermosensing have been known for some time, in recent years several of these mechanisms have been refined. For example, phytochrome thermosensitivity has been known since the 1950's when phytochrome dark-reversion was shown to be sensitive to temperature (Borthwick *et al*., 1952). In this experiment it was found that germination in response to a RL pulse was decreased at higher temperature, due to the reversion of the phytochrome to the inactive form (Pr). The thermosensitive role of phytochromes was further explored and characterised more recently when it was shown that phytochromes, and particularly phyB, play a thermosensory role in Arabidopsis in both the dark and in the light (Jung *et al*., 2016; Legris *et al*., 2016). This was not due to total abundance of phyB, but on the proportion of phytochrome in the active Pfr state (Jung *et al.,* 2016). Furthermore, the early flowering phenotype of the *phyB* mutant is temperature sensitive, suggesting that phyB integrates light and temperature cues and regulates many important temperature-mediated, developmental processes in plants (Halliday *et al.*, 2003). It has also

been shown that cry1 modulates high temperature-mediated hypocotyl elongation, acting redundantly with phyB (Mazzella *et al.*, 2000; Ma *et al.*, 2016). Other phytochromes have also been linked to thermosensing (Jung *et al.*, 2016), as has ZEITLUPE (Miyazaki *et al.*, 2015). The reversion of monomer to dimer form of the UV-B photoreceptor, UVR8, is reduced at low temperatures, however the effects of this response on growth have not been established (Findlay and Jenkins, 2016). Collectively, these data suggest that receptors that were first considered to respond only to light, also respond to other environmental stimuli and the crosstalk between light and temperature signalling occurs during convergence of these environmental stimuli within a plant.

1.2.8 Responses to moderately high temperatures

Though relatively little is known about primary thermosensors in plants, more is known about signalling components within temperature signalling pathways and the gene expression underlying physiological responses. Perhaps the most conspicuous response to moderately elevated temperatures (~24-28°C) is thermomorphogenesis. The collection of physiological changes in response to high temperature (HT) include hypocotyl and petiole elongation, leaf hyponasty and early flowering (Gray *et al.*, 1998). These responses have been suggested to be aimed at alleviating HT stress via increased leaf cooling (Crawford *et al.*, 2012). Interestingly, Romero-Montepaone *et al.* (2021) found that high temperature also increases phototropism, which does not promote cooling, suggesting the elongation responses in response to HT also has other purposes. Furthermore, the authors also showed that the elongation response occurs during conditions when heat stress (≥35°C) is unlikely, supporting the purpose of HT-induced elongation growth may have other purposes than plant cooling. Recent evidence from Burko *et al.* (2022) shows that the elongation growth that occurs in thermomorphogenesis is enhanced under shaded conditions, providing another dimension for the response to elevated temperatures.

As discussed in 1.1.5, the hormone auxin and the PIF transcription factors are central to hypocotyl elongation growth. It is therefore unsurprising that both auxin and PIFs have been shown to be centrally involved in plant growth in response to elevated temperatures (Gray *et al.*, 1998; Koini *et al.*, 2009; Franklin *et al.*, 2011; Chung *et al.*, 2020). Much of the molecular signalling which underpins the HT responses involve signal integration between HT and light, and the temperature sensing capability of,

for example, phyB is reliant on irradiance levels (Legris *et al*., 2016). PIFs are often discussed as one of the strongest links between light and temperature signalling (Balcerowicz, 2020). For example, a study by Ma *et al*. (2015) found that the blue light photoreceptor, Cry1, represses the transcription factor activity of PIF4 leading to subsequent repression of auxin-related genes, which results in inhibition of hypocotyl elongation under elevated temperatures.

1.2.9 High temperature stress responses and acclimation to high temperatures

When temperatures rise beyond the levels at which a plant is adapted to live, much of cellular biochemistry cannot function. This results in accumulation of waste products, reduced photosynthesis, unbalanced water status and subsequent cell death. Often sites of necrosis or bleaching are seen on heat stressed leaves. Another challenge is changes to membrane fluidity due to the saturation status of membrane lipids and membrane composition. For example, the *fad6* mutant showed altered temperature tolerance in a study by Hugly *et al*. (1989), suggesting the control of membrane lipid composition is important to temperature adaptations.

Plants have an innate tolerance to high temperature called basal thermotolerance. In addition, some plants can also acclimate to higher temperatures. Plants that are exposed to high temperatures for even a short period of time (~15min) start to acclimate and obtain a tolerance to higher temperatures in a process called acquired thermotolerance (Penfield, 2008). Larkindale and Vierling (2008) found great changes in gene expression in response to heat stress with or without prior heat acclimation. Notably, the different acclimation treatments had somewhat different expression profiles, but core thermotolerance genes were upregulated in all treatments. Several genes have been implicated in acquired thermotolerance responses (reviewed by Song *et al*., 2012). Central to acquired thermotolerance is the accumulation of heat shock proteins (HSPs). Arabidopsis have 5 main groups of HSPs: the small HSPs (sHSPs), the chaperonins, the HSP70 family, the HSP90 family, and the HSP100 family. Many of these function as chaperones that prevent protein aggregation, aid in protein folding and refolding of denatured proteins under high temperatures (Song *et al*., 2012). Heat shock transcription factors (HSFs) are responsible for the induction of heat-responsive genes, such as the HSPs (Åkerfelt *et al*., 2010; Ohama *et al*., 2017).

The involvement of ROS production and signalling in HT stress and HT acclimation is complex. Traditionally, ROS were assumed to be predominantly damaging, but more recent research has shown ROS to be important signalling molecules that integrate environmental signals and activate signalling pathways within stress responses (Mittler *et al.*, 2022). In high temperature acclimation, ROS may even play a protective role. Volkov *et al.* (2006) found that treating plants with H_2O_2 enhanced the expression of HSP17.6 and HSP18.2, both indicating that gene expression of certain HSPs is ROS sensitive, and that ROS can promote protection against damaging high temperatures. The authors suggest that when plants experience high temperature and ROS accumulates, HSPs are produced, leading to thermotolerance. Physiologically, creeping bentgrass (*Agrostis stolonifera* var. *palustris*) pre-treated with H_2O_2 had higher quality scores than the controls after heat stress, but photosynthetic rates were decreased compared to the water control (Larkindale and Huang, 2003). Nevertheless, light was found to prime the ROS detoxification response in an HSF-mediated manner which leads to enhanced thermotolerance in Arabidopsis, supporting that a ROS scavenging system is critical for developing thermotolerance (Han *et al.*, 2019). Interestingly, the light priming response was mediated by phyB, giving this photoreceptor yet another role in temperature signalling. In contrast to this study however, Arico *et al.* (2019) showed that the *phyB* mutant had an increased thermotolerance and that this response is likely due to a change in PIF abundance and/or activity. This finding was supported by observations showing that shade cues (decreased R:FR) enhanced thermotolerance in WT plants, suggesting another role for PIFs. The involvement of UV-B in thermotolerance will be discussed in chapter 3.

1.3 Stomatal movement

Stomata are microscopic pores predominantly found on aerial parts of plants. Stomatal pores are flanked by two guard cells which can inflate or deflate to change the aperture of the pore. It is via stomatal pores that CO_2, oxygen and water vapour flow between the atmosphere and the inner plant tissues. Because stomata regulate gas exchange, they are not only important for photosynthesis and oxygen release, but also for the water status of plants and are integral to leaf turgor and whole plant water management (Lawson and Matthews, 2020). Transpiration through stomatal pores also plays an important role in heat regulation in plants, providing stomata with another important role (Crawford *et al.*, 2012). In addition to adjusting stomatal aperture, plants regulate gas exchange by the number and size of stomata produced and the patterning of stomata across the tissue (Hetherington and Woodward, 2003). In the context of this thesis, only stomatal aperture will be discussed.

1.3.1 Stomatal opening

The driving force behind stomatal opening are the fluxes of guard cell solutes. Early studies showed that when stomata are open, guard cells surrounding the pore contained a high concentration of K^+ and the addition of K^+ could open stomata in *Commelina* (Fujino, 1969). Further research found that several more solutes in addition to K^+ are responsible for the dynamic opening and closing of stomata. The current view is that malate^{2-}, Cl$^-$, NO3$^-$, and sucrose all play important roles (Allaway, 1973; Pearson, 1973). Changes in these solutes create osmotic imbalances between the outside and inside of the guard cells. These imbalances in turn drive water movements in and out of the guard cells. This results in turgor changes and when the guard cells inflate from an influx of water, they bend outwards creating a pore and thus control stomatal pore aperture. Mechanistically, stomatal opening also involves cell elongation where cell wall modulation plays a major role (Marom *et al.*, 2017).

Stomatal opening occur via activation of the plasma membrane H^+ ATPases and subsequent ATP-driven H^+ flux across the membrane. This creates plasma membrane hyperpolarisation and activates voltage-gated inward-rectifying K^+ channels. An influx of K^+ through channels KAT1 and KAT2/AKT1 as well as transport other ions (H^+, K^+, Cl$^-$ and malate^{2-}) into the guard cell cytoplasm via H^+ symport channels is followed by K^+ and Cl$^-$ movement into the vacuole (Jezek and Blatt, 2017). This in turn triggers water

uptake by the guard cells and turgor increases, which opens the stomatal pore (fig. 1.3; Jezek and Blatt, 2017; Inoue and Kinoshita, 2017). Energy sources used for stomatal opening include lipids (McLachlan *et al*., 2016) and starch (Horrer *et al*., 2016).

Stomata open in response to several environmental stimuli. In this thesis, stomatal movement will be discussed in the context of temperature in chapter 5, and light in chapter 6.

1.3.2 Stomatal closure

During stomatal closure, water leaves the guard cells due to an efflux of K^+ through the only known charge-activated plasma membrane K^+ efflux channel GORK, as the plasma membrane is depolarised due to the efflux of negatively charged ions from the guard cells (Ache *et al.,* 2000). It was shown that ROS were also involved in stomatal closure. H_2O_2 activates Ca^{2+} channels, allowing intracellular accumulation of Ca^{2+} which is otherwise maintained at a very low level due to its cytotoxic effect (White and Broadley, 2003; Sierla *et al*., 2016). Ca^{2+} activates calcium-dependent protein kinases which in turn phosphorylate SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1; Deng *et al*., 2021). SLAC1 regulates the ion movement across the guard cell plasma membrane which results in depolarisation and K^+ efflux. Ca^{2+} further activates anion channels responsible for anion efflux, and inhibit inwards-rectifying K^+ channels, preventing influx of K^+ ions. Ca^{2+}-dependent acidification of the vacuole also promote ion movement into the guard cell cytoplasm. These effects together result in stomatal closure (fig. 1.3).

1.3.3 Systems to investigate stomatal movement

Much physiological research has been conducted in isolated guard cells by peeling the epidermal layer of leaves and floating these on buffer while exposing them to specific conditions or treatments. This method is useful as it shows the guard cell specific responses without influence from underlying cell layers or systemic signalling in a plant. While useful in this context, it is not ecologically relevant or relevant to the physiology of a whole plant system. Significant bodies of research have also focused on leaf discs, where mature leaves are used to produce sections which are then treated in the same manner
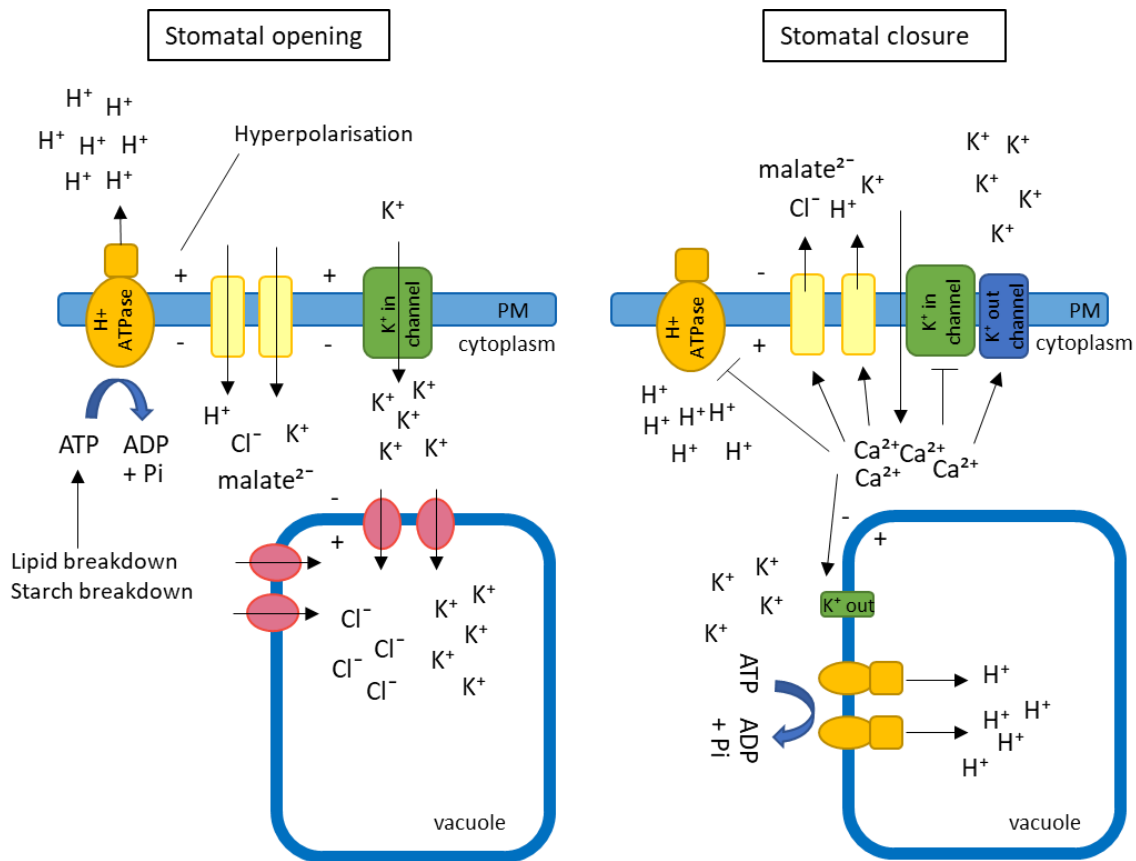
Figure 1.3 Ion movements during stomatal opening and closure. Hyperpolarisation of the PM driven by H⁺ efflux triggers activation of ion symporters and K⁺ channels. K⁺ accumulation as well as Cl⁻ and malate²⁻ accumulation in the cytoplasm and subsequent transport into the vacuole trigger water uptake by the guard cells and turgor increases, resulting in stomatal opening. Stomatal closure is driven by Ca²⁺ accumulation and inhibition of PM H⁺ ATPases, efflux of ions through symporters, inhibition of inwards-rectifying K⁺ channels and activation of K⁺ exporters and acidification of the vacuole. Together this leads to water efflux from the guard cells and stomatal closure.

as the epidermal peels. It is reasonable to suggest that some light signals may be dampened, or heightened, by the surrounding cell layers and pigments and thus decrease or increase the guard cell response. This is however, not widely highlighted in the literature but may be an important feature to be aware of when analysing data from leaf discs. In addition to epidermal peels and lead discs, the response of cotyledon stomata to environmental stimuli is also studied. The dicotyledonous nature of Arabidopsis allows two cotyledons, attached together by a small section of the hypocotyl and shoot meristematic tissue, to be detached together. Conveniently, these pairs of cotyledons are prone to float on buffer very uniformly, abaxial side down. Alternatively, the cotyledons can be treated while still growing, on plates or soil before detachment and analysis. Cotyledons provide a near whole plant system to work with in stomatal research. Cotyledons additionally represent a different developmental stage of plant tissue for use in stomatal assays than leaf discs and epidermal peels. Data from cotyledons may therefore differ significantly from data collected from leaf discs or epidermal peels and provide insight into stomatal behaviour during early plant development. In this thesis, a combination of epidermal peels, leaf discs and cotyledons were used to give an insight into the effects of UV-B and high temperature on stomata at different stages in development.

1.4 Aims of thesis

The aim of this thesis was to investigate the role of UV-B priming in plant abiotic stress responses. The roles of UV-B in freezing stress, heat stress and water use via stomatal regulation were investigated. This PhD project was funded by a BBSRC CASE studentship with agricultural engineers, LettUs Grow. As such, a further component of the thesis investigates the role of UV-B priming in enhancing the quality of aeroponically-grown horticultural produce.

CHAPTER 2 General Materials and Methods

2.1 Plant material

Arabidopsis thaliana were maintained in the Franklin lab seed stocks and the Hetherington lab at the University of Bristol. Wild type, mutant and transgenic lines used in this thesis have been described before and are outlined in table 2.1. *Raphanus sativus* seeds were provided by LettUs Grow. *Eruca vesicaria* and *Valerianella locusta* were obtained from Chiltern Seeds (Wallingford).

Table 2.1. *Arabidopsis thaliana* accessions used in this thesis.

Genotype	Citation	Description	Background
Columbia-0 (Col-0)		Wild type	
Wassilewskija (Ws)		Wild type	
Landsberg *erecta* (L. *er*)		Wild type	
uvr8-6	Favory *et al*., 2009	UVR8 null mutant, T-DNA insertion	Col-0
tt4	Koornneef *et al*., 1982; Li *et al*., 1993; Winkel-Shirley *et al.,* 1995	CHS deficient, EMS-induced mutation	Col-0
nced3-5	Frey *et al*., 2012	ABA biosynthesis impaired. T-DNA insertion in *NCED3* and *NCED5*	Col-0
q1124	Okamoto *et al*., 2013	Quadruple ABA receptor mutant, EMS-induced point mutation in *PYR1*, T-DNA insertion in *PYL1, PYL2,* and *PYL4*	Col-0
ost1-3	Ding *et al.,* 2015, SALK_008068	OST1 deficient, T-DNA insertion	Col-0
cop1-4	Deng and Quail, 1992		Col-0
pif4-101	de Lucas *et al.,* 2008, Koini *et al*., 2009	Loss of function mutant, T-DNA insertion	Col-0
pif1345 (*pifq*)	Leivar *et al*., 2008	PIF1,3,4,5 loss of function mutant, T-DNA insertions in *PIF1, PIF3, PIF4* and *PIF5*	Col-0
hy5KS50/hyh	Holm *et al*., 2001	HY5 and HYH deficient	Ws
DR5:GUS	Ulmasov *et al*., 1997	GUS under the control of the auxin responsive element, DR5	Col-0
pPIF3:GUS	Zhang *et al*., 2013	GUS under control of the native *PIF3* promoter	Col-0
pPIF4:GUS	Zhang *et al*., 2013	GUS under control of the native *PIF4* promoter	Col-0
pPIF5:GUS	Zhang *et al*., 2013	GUS under control of the native *PIF5* promoter	Col-0

phot1-5/2-1	Kinoshita *et al.*, 2001, Kagawa *et al.*, 2001; Huala *et al,*. 1997	Phot1 and phot2 deficient. Fast neutron irradiation-induced mutation in *NPH1* and T-DNA insertion in *NPL1*	Col-0
phyB-9	Reed *et al.*, 1993; Yoshida *et al,*. 2018	EMS-induced mutations in *PHYB* and *VEN4*	Col-0
OST1-GFP	Wang *et al.*, 2015	GFP under the control of the *OST1* native promoter	Col-0

2.2 Growth conditions

2.2.1 Seed treatment

Arabidopsis seeds were surface sterilised by a 10 min 70% (v/v) EtOH wash followed by further three washes with freshly autoclaved water. Seeds were planted individually directly onto compost or onto agar using ceramic forceps. Seeds were stratified at 4°C in darkness for 48-72 h. *tt4* and *tt7* seeds required 7 d stratification to induce germination. Seedlings for thermotolerance assays, including antioxidant capacity assays and transcript abundance, for chapter 4 were grown on agar plates for 7 or 11 d before being assayed. For all other experiments, seeds were grown entirely on soil.

2.2.2 Media

Compost media

Compost (SinClair all-purpose growth medium, William SinClair horticultural Ltd, Lincoln, UK) and sand (Horticultural Silver Sand, Melcourt Garden and Landscape, UK) was mixed to a ratio of 3:1 (v/v). All plants used for low temperature assays were sewn and germinated on soil.

Agar growth media

0.5 x Murashige and Skoog (Murashige and Skoog, 1962) medium was prepared by autoclaving 0.22% (w/v) MS basal salts (Duchefa Biochemie, Germany), 1% sucrose, and 0.6% (w/v) agar (Sigma) in distilled H_2O at pH 5.8.

MES media

10 mM MES media at pH 6.15 was used for stomatal bioassays as a holding buffer to not dehydrate epidermal peels or leaf discs during transfer.

10 mM/50 mM MES/KCl media at pH 6.15 was used for stomatal bioassays to supply epidermal peels and leaf discs with K^+ to facilitate stomatal opening.

2.2.3 Controlled climate growth chambers

After stratification, compost trays and agar plates were transferred to Microclima growth cabinets (Snijder Scientific, Wilburg, The Netherlands) and grown under 12 h (LT experiments) and 16 h (HT experiments, stomatal experiments) photocycles at 70% RH and 22°C. Compost trays were watered three times weekly with deionised water. White light at 60-70 µmol m^{-2} s^{-1} was supplied by cool white fluorescent tubes (SYLVANIA F36W/2084-T8 Brite-Gro or SYLVANIA LUXLINE PLUS FHO54W/T5/840, Munich, Germany). UV-B was supplied by narrowband UV-B light tubes (PHILIPS TL40W/01-RS, Hamburg, Germany) at a fluence rate of 1 µmol m^{-2} s^{-1} (fig. 1). Low fluence UV-B, which contributes to photomorphogenesis and promotes production of, for example, flavonoids, is considered between 0.1 – 1 µmol m^{-2} s^{-1} and high fluence UV-B which triggers stress responses is considered the be > 1 µmol m^{-2} s^{-1} (Tong *et al.*, 2008).

2.3 Total antioxidant capacity

Total antioxidant capacity of Arabidopsis and *Raphanus sativus* was analysed using the Trolox equivalent Total Antioxidant Capacity Kit (MAK187, Sigma-Aldrich). 50-100 mg of leaf tissue from the largest of the Radish cotyledons, and from whole Arabidopsis seedlings was frozen in liquid nitrogen and stored at -70°C. Samples were homogenised with stainless steel beads in a TissueLyser II (Qiagen). Samples were extracted in 0.5 – 1 mL of 1 X Phosphate Buffered Saline (PBS; pH 7.4) buffer and the supernatant was collected. The supernatant was diluted to bring values to within the Trolox standards. The absorbance of plant extracts was compared to a Trolox standard curve. Values were normalised to fresh tissue weight.

2.4 Plant pigment abundance analysis

Flavonol, anthocyanin and chlorophyll leaf abundances were measured using a Force-A Dualex meter (Force-A, France). Measurements are based on the difference in transmittance of a measuring beam which is absorbed by the pigment of interest, and a control beam which is not absorbed by the pigment. The measuring beam for flavonols is UV (~370-390 nm), and that for anthocyanins is green light (~500-510 nm) and the control beam is ~620 nm. Chlorophyll is measured using a near-infrared beam as

reference (~800 nm), and a FR measuring beam (~700-720 nm). The values for chlorophyll are given in µg cm $^{-2}$, while flavonols and anthocyanins are given in the relative unit of FLAV Units (Cerovic *et al.*, 2012).
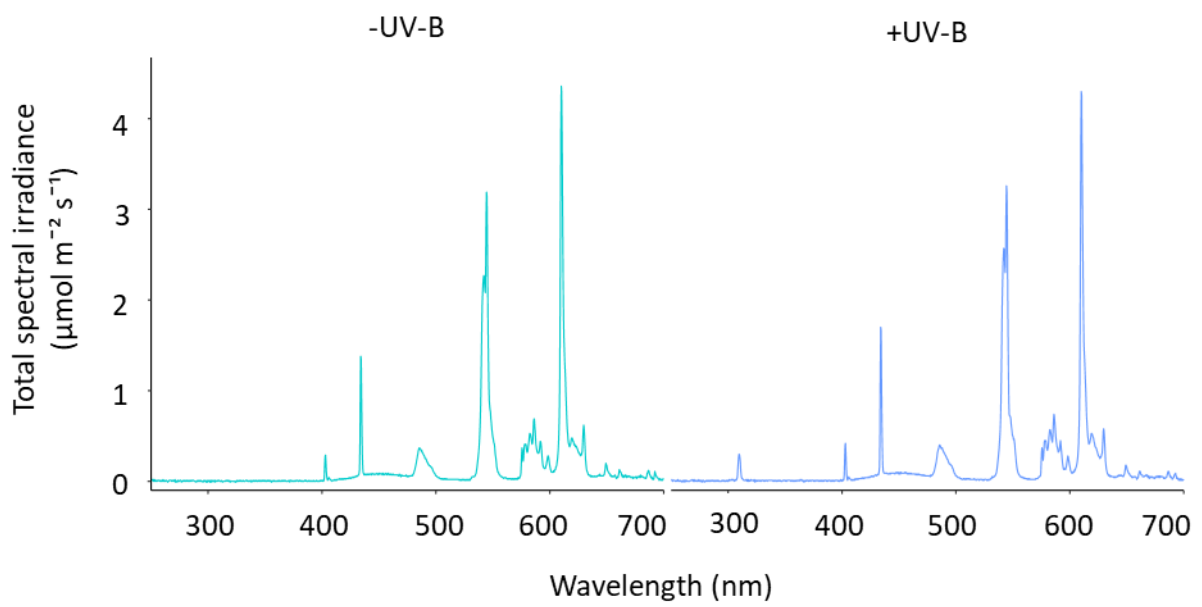
Figure 2.1Total spectral irradiance of controlled climate growth chambers. A peak can be seen at ~310nm in the UV-B treatment conditions.

2.5 Quantitative reverse transcription polymerase chain reaction

2.5.1 RNA extraction

RNA from *Arabidopsis thaliana* was extracted using the Spectrum™ Plant Total RNA Kit (STRN250, Sigma-Aldrich) and the manufacturer's protocol. Extracted RNA was treated with DNase1 to remove contaminating DNA (AMPD1, Sigma-Aldrich). Purity and yield of RNA was tested with a NanoPhotometer N60-Touch UV/Vis Spectrophotometer (Geneflow).

2.5.2 cDNA synthesis

cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit (4368814, Thermofisher Scientific) and RNase inhibitor (AM2696, Thermofisher Scientific). 1 µg RNA was used for each reaction.

2.5.3 Quantitative polymerase chain reaction

Quantitative PCR was performed with the Brilliant III SYBR Green qPCR kit according to the manufacturer's protocol (600882, Agilent Technologies). For primer sequences, refer to table 2.2. Threshold fluorescence was set to 0.05. Primer efficiency analyses were used to determine appropriate cDNA dilutions. Relative transcript abundance was calculated using the $\Delta\Delta$-CT method (Livak and Schmittgen, 2001) and normalised against *PP2A*, as specified in the specific figures. qRT-PCR primers were designed to amplify short amplicons (80-120 bp) and tested by the production of a standard curve. Acceptable primers had a standard curve $R^2 > 0.980$ and efficiency of 100% ±5%.

Table 2.2. qPCR primer sequences used in this thesis.

Primer name	Primer sequence 5'-3'
PP2A Forward	GTT CTC CAC AAC CGC TTG GT
PP2A Reverse	TAA CGT GGC CAA AAT GAT GC
Actin-2 Forward	TCAGATGCCCAGAAGTGTTGTTCC
Actin-2 Reverse	CCGTACAGATCCTTCCTGATATCC
COR15a Forward	CGTTGATCTACGCCGCT
COR15a Reverse	TCTCACCATCTGCTAATGCC
COR47 Forward	AGCTTCACCGATCCAACAGCTCTTC
COR47 Reverse	CGGGATGGTAGTGGAAACTGG
CHS Forward	ATC TTT GAG ATG GTG TCT GC
CHS Reverse	CGT CTA GTA TGA AGA GAA CG
HSP70-4 Forward	TTGTTGGACATTGACCTCTC
HSP70-4 Reverse	GGCAAACTTTTAATTTTATCCG
PIF4 Forward	GCCGATGGAGATGTTGAGAT
PIF4 Reverse	CCAACCTAGTGGTCCAAACG
PIF5 Forward	CAGATGGCTATGCAAAGTCAGATGC
PIF5 Reverse	AGATTTGGTTCTGTGCTTGGAGCTG
OST1 Forward	ATCAACCGGGCCAAA
OST1 Reverse	ATGTCCAAGCTTCCT
NCED3 Forward	CCGTGGTTTACGACA
NCED3 Reverse	CAGAAGCAATCTGGA
NCED5 Forward	CTTCCCGTTGGAACC
NCED5 Reverse	GTACCGGAGCGTAGTTACCG
IAA19 Forward	TGGCCACCGGTTTGTTCTTA
IAA19 Reverse	TCATAGCCTTGGCTCGAACC
IAA29 Forward	ATCACCATCATTGCC CGTAT
IAA29 Reverse	ATTGCCACACCATCCATCTT
YUC8 Forward	ATCAACCCTAAGTTCAACGAGTG
YU8 Reverse	CTCCCGTAGCCACCACAAG

2.6 Qualitative GUS analysis

The DR5 reporter system was used to investigate the presence of auxin and any differences in auxin between samples treated with UV-B. The reporter system using GUS expression driven by the *PIF3*, the *PIF4* and the *PIF5* promoters (*pPIF3::GUS*, *pPIF4::GUS* and *pPIF5::GUS*) was used to explore how UV-B and HT affected *PIF3, PIF4* and *PIF5* promoter activity and localisation. *DR5::GUS* and *pPIF3*::GUS, *pPIF4::GUS* and *pPIF5::GUS* seeds were obtained from NASC and seedlings were grown on soil and treated with 1 µmol m^{-2} s^{-1} UV-B for 6 h at 5 dag (days after germination). One sample was also collected pre-dawn. Approximately 20 seedlings per treatment were collected and rapidly transferred to 80 % (v/v) acetone for 30 minutes. Samples were then transferred to the assay buffer (100 mM Na$_3$POH, 100 mM NaP$_3$OH, 2 mM FeII, 2 mM FeIII, 0.2 % Triton-X, 2 mM 5-bromo-4-chloro-3-indolyl- glucoronide salt (X-Gluc, Thermofisher)) and incubated at 37°C in the dark for 18 hours (*pPIF3::GUS, pPIF4::GUS* and *pPIF5::GUS*) or 48 hours (*DR5::GUS*). Samples were then washed once in 100 % (v/v) EtOH and twice in 70 % (v/v) EtOH before being suspended in 50 % (v/v) EtOH for imaging by microscopy (VHX-S50 digital microscope, Keyence). Epidermal peels were fixed in 50 % (v/v) glycerol. GUS is very stable at high temperature (37°C) lending itself well to the high temperature assay to investigate effects of 35°C on PIF promoter activity (Gallagher, 1992).

2.7 Stomatal assays

Stomatal bioassays were carried out according to the protocol used by Pridgeon and Hetherington (2021). A total of 9 leaf discs, epidermal peels or cotyledon pairs detached from the hypocotyl were analysed over three independent experiments. Where epidermal peels were used, these were prepared on holding buffer (10 mM MES, pH 6.15) from a fully mature leaf of a 4-5 w old plant using the protocol by Kostaki *et al.* (2020). Leaf discs were prepared from 4-5 w old plants using a 4 mm biopsy punch, and where cotyledon pairs were used, these were detached from the hypocotyl of 4-5-day-old seedlings. Epidermal peels, leaf discs and cotyledons were transferred immediately post-preparation to 50 mm petri dishes containing 10 ml 10/50 buffer (10 mM MES, 50 mM KCl, pH adjusted to 6.15 with KOH) pre-warmed to 20°C. Experiments were carried out while floating all samples on opening buffer, or by detaching cotyledon pairs for immediate imaging. The petri plates were placed on the surface of water-

filled clear glass fish tanks with attached dip heater (Julabo, Stamford, UK) which maintained the water temperature. Cool water was circulated through tubes at one end of the tank to produce a current in the tank which promotes uniform heating of the water. Halophosphate white fluorescent light tubes (Crompton lamps, Bradford, UK) were positioned underneath the tanks and supplied the samples with 110-120 µmol m^{-2} s^{-1} white light (measured on the surface of the water). S4 shows light spectra for treatment tanks. Samples were treated for indicated times under specific light and temperature conditions detailed in the specific methods. After treatment, samples were transferred to microscope slides and immediately imaged using a Leica DM16000 B microscope fitted with a Leica DFC360FX monochrome camera. Three brightfield image stacks were taken, of which 10 stomata per plant sample were measured. The total stomata measured per plant and treatment was 90 (across three independent experiments). Stomatal apertures for each sample were averaged to give n=9 across three repeats.

2.8 Image processing

Microscopy and PAM fluorometer images were analysed with ImageJ (https://imagej.nih.gov/ij/). Photographs were processed with ImageJ and GIMP2 (Version 2.8.10).

2.9 Light measurements

Light spectra in growth cabinets and stomatal assay tank lighting rigs were captured with a FLAME spectroradiometer (Ocean Optics, USA) and recorded and analysed with OceanView software (version 2.0.8) and or Rstudio (version 1.4.1717.0). Light spectra were measured at the soil surface, or as close to the plant level as possible.

2.10 Data analysis

Rstudio (version 1.4.1717.0) with the package ggplot2 was used to analyse and plot quantitative data. Boxes in box plots represent the interquartile range (IQR), the line represents the median, and the whiskers represent the 25th and 75th percentiles. In these plots, all data points are represented by dots. Height of bars in bar charts represent the mean of the sample and the error bars the standard error of the mean (SE). Statistical analyses of data sets were carried out in RStudio. Where data was parametric, a

one, two or three way ANOVA was applied and a TukeyHSD post hoc test for multiple comparisons.

Where data was non-parametric, a Kruskal-Wallis test with a post hoc Dunn test was used.

CHAPTER 3 THE EFFECTS OF UV-B ON TEMPERATURE TOLERANCE IN *ARABIDOPSIS THALIANA*.

3.1 Introduction

Plants encounter and are adapted to withstand daily and seasonal fluctuations in temperature. However, when temperatures move outside the range within which a plant has adapted to thrive, stress occurs which can affect plant survival and reproduction (Wahid *et al*., 2007; Vierling, 1991). In recent years, many extreme weather events have occurred of which many are likely connected to an increase in global warming, and which put unprecedented pressure on the biosphere and survival of plants in both natural environments and in agriculture (Coumou and Rahmstorf, 2012).

Cold tolerance and UV-B

The direct interaction between light and freezing tolerance was reported first in the 1930's, where Dexter (1933) showed that illumination enhanced cold hardening in alfalfa. Later, Gray *et al.,* (1997) showed that the two factors have an additive effect on cold tolerance. Light is required for complete cold acclimation (CA) as Arabidopsis exposed to 1°C in the dark did not cold acclimate appropriately (Wanner and Junttila, 1997), and expression of genes previously ascribed as cold-induced were also light-dependent (Soitamo *et al.,* 2008). It has been shown that light affects both CBF-dependent and independent signalling pathways. R and FR light affected reporter gene expression under the control of C/DRE (Kim *et al.,* 2002), while low R:FR light also increased CBF expression in Arabidopsis at 16 and 22°C, however *COR* gene expression was only altered in the 16°C treatment group (Franklin and Whitelam, 2007). Effects of R and FR light on LT responses suggested that phytochrome play an intrinsic role in light and temperature crosstalk in plants. Indeed, Legris *et al.* (2016) later showed that phyB is involved in temperature perception in Arabidopsis via its temperature-dependent Pfr-Pr reversion, while Jung *et al.* (2016) described a role for PhyB in thermosensing and induction of high temperature-induced genes in the dark. In addition to phytochromes, evidence exists for crosstalk between blue light photoreceptors and cold signalling pathways. The barley *CBF14* gene showed strong induction in blue light compared with R and FR (Nóvak *et al.,* 2017), and the Arabidopsis *COR27* and *COR28* were also BL-responsive (Li *et al.,* 2016). PIFs have also been linked to the light-LT signalling network. Plants grown in short day photoperiods exhibited greater freezing tolerance than long day plants (Lee and Thomashow, 2012), which was suggested to be the result of PhyB, PIF4 and PIF7 activity. Interestingly, PIF3 was later found to be a negative regulator of CBF expression suggesting varying roles for PIFs in cold signalling (Jiang *et al.,* 2017). Toledo-Ortiz *et al.* (2014) described the existence of a regulatory module consisting of PIFs and the transcription factor and key photomorphogenesis regulator HY5. This module is responsive to both temperature and light signals and can integrate both to regulate gene transcription (Toledo-Ortiz *et al.,* 2014). Individually, HY5 was found to accumulate upon cold induction and regulate expression of cold-induced gene expression via the Z-box and cis-acting elements (Catalá *et al.,* 2011). A *hy5* mutant showed decreased freezing

tolerance in comparison to WT, and *HY5* expression was modulated by LT. However, *HY5* transcripts were not affected in a *cbf2* mutant or an antisense CBF1-AS3 mutant, suggesting HY5 may act upstream of CBFs (Catala *et al.,* 2011). Indeed, HY5 was later found to bind to the promoters of *CBF1, 2*, and *3* as well as *COR15a*, with higher affinity for *CBF3* (Norén *et al.,* 2016). Conversely, LT was found to regulate induction of genes normally regulated by light (Capel *et al.,* 1998), suggesting close integration of light and temperature signalling pathways.

There are several reasons to consider integration of UV-B light and LT signalling a possibility. Firstly, UV-B enhanced freezing tolerance in Rhododendron and wheat suggesting that, in some species, UV-B and LT signalling overlap (Dunning *et al.,* 1994; Chalker-Scott and Scott, 2004; Yang *et al.,* 2007). Secondly, phenotypic similarities exist: both UV-B and LT cause reduced growth and dwarfing in plants (Teramura and Sullivan, 1994; Gilmour et al., 2004). The underlying mechanisms for this common response are discussed below, as part of the third reason UV-B and LT signalling crosstalk is considered: that many signalling components have functional roles in both signalling pathways.

Reduced growth in both UV-B and LT involve the phytohormone gibberellic acid (GA). As well as promoting germination and flowering, GA also promotes hypocotyl elongation (Davies, 1995). LT and UV-B can reduce GA levels by upregulating GA 2-oxidase genes, which function in GA-inactivation (Achard *et al.,* 2008; Hayes *et al.,* 2014). UV-B irradiation also promoted a decrease in GA1, a precursor to bioactive GA (Fina *et al.,* 2018). When present, GA binds to GIBBERELLIC ACID INSENSITIVE DWARF1 receptors (GID1; Ueguchi-Tanaka *et al.*, 2005; Shimada *et al.*, 2008), triggering ubiquitination and degradation of DELLA proteins. A reduction in bioactive GA therefore stimulates an increase in DELLAs (Fu *et al.*, 2004). DELLAs, such as REPRESSOR-OF-ga1-3 (RGA) and GIBBERELLIC ACID INSENSITIVE (GAI), are repressors of GA-induced elongation growth (Peng *et al.,* 1997; Dill and Sun, 2001). DELLAs exert control on gene expression via negative regulation of the nuclear transcription factors PIF3 and PIF4 which positively control expression of genes involved in cell elongation, and by disrupting corticle microtubule arrangement (de Lucas *et al.,* 2008; Li *et al.,* 2016; Locascio *et al.*, 2013). LT appeared to modulate growth via a CBF-DELLA-dependent pathway in Arabidopsis (Achard *et al.,* 2008). The authors noted an increase in GFP-tagged DELLA proteins in

response to constitutive expression of *CBF1* which in turn was the result of an upregulation in GA-2-oxidases and a subsequent degradation of GA. Recent data also suggest that both LT and UV-B appear to affect expression of GROWTH REGULATING FACTORS (GRFs), transcription factors regulating expression of genes involved in cell expansion in leaves and cotyledons (Fina *et al.,* 2017; Lantzouni *et al*., 2020). Both UV-B and LT signalling involve the TF HY5 and its close homolog HYH. The role of HY5/HYH in light- and UV-B-induced photomorphogenesis is well-established (Ulm *et al*., 2004; Brown *et al.,* 2005; Brown and Jenkins, 2008). HY5 levels are also regulated by cold, and HY5 regulates the transcription of several cold-induced genes (Catalá *et al.,* 2011).

There is evidence that reactive oxygen species (ROS) are generated by both LT and UV-B in different species of plant (Dai *et al.,* 1997; Yang *et al.,* 2007; Kawarazaki *et al.,* 2013; Chen *et al.,* 2013; Takshak and Agrawal, 2014). Yang *et al*. (2007) found that the protective effect of UV-B on cold-exposed wheat likely involves ROS signalling and ROS scavenging activity. Indeed, such protective compounds constitute here the fourth reason for considering LT-UV-B crosstalk. Plant pigments are considered secondary metabolites that are not essential for growth, development, and reproduction but may confer certain other beneficial attributes. Two major plant pigment classes are the carotenoids and the flavonoids. In the context of UV-B and LT, only flavonoids will be discussed here.

Flavonoids are phenylpropanoids, with a basic polyphenolic skeleton comprising three C rings onto which further ketones or hydroxyl groups may attach and thereby change the activity of the compound (Panche *et al*., 2016). In plants, flavonoids are involved in many different processes such as plant defence, allelopathy, UV protection, and reducing oxidative stress by acting as antioxidants (Treutter, 2005; Weston and Mathesius, 2013; Li *et al.,* 1993; Nakabayashi *et al.,* 2013). Arabidopsis accumulate flavonoids in response to UV-B (Lois, 1994). This is generally considered an adaptive response to mitigate potential UV stress, as mutants deficient in different components of flavonoid biosynthesis are hypersensitive to UV-B irradiation (Li *et al*., 1993). Similarly, exposure to LT enhanced anthocyanin accumulation and expression of genes of phenylpropanoid metabolism in maize (Christie *et al.,* 1994). In Arabidopsis, PHENYLALANINE AMMONIA-LYASE and CHS were induced by LT in a light-dependent manner (Leyva *et al.,* 1995). Studies have since showed that LT exposure enhanced UV

screening in bean plants (Bilger *et al.,* 2007), and increased accumulation of flavonoids in lettuce (Becker *et al.,* 2014) and Arabidopsis (Korn *et al.,* 2008). Korn *et al.* (2008) also noted a positive correlation between flavonol accumulation and freezing tolerance, and it was later shown that certain Arabidopsis mutants deficient in flavonoid biosynthesis pathway enzymes exhibited reduced freezing tolerance (Schulz *et al.,* 2016). Light was required for flavonoid accumulation as cold treatment in the dark failed to increase transcript levels of *CHS, F3'H, F3H* and *FLS*, or accumulation of the flavonol aglycones kaempferol and quercetin (Bhatia *et al.,* 2018). Flavonoid accumulation was impaired in a *hy5* mutant, supporting the idea that HY5 may be a key component in light and LT crosstalk (Bhatia *et al.,* 2018). Interestingly, LT, gibberellins and HY5/HYH were all linked to anthocyanin accumulation in Arabidopsis (Zhang *et al.,* 2011; Zhang *et al.,* 2011). Chalker-Scott and Scott (2004) suggested the UV-B-induced freezing tolerance observed in Rhododendron may be the result of phenolic compound accumulation, however the involvement of UV-B in cold acclimation and freezing tolerance in Arabidopsis has to our knowledge yet to be explored.

Thermotolerance and UV-B

In the coming century, temperatures are predicted to rise, regardless of decreased anthropological pollution which contributes to global warming. The IPCC suggests that the scenario which predicts the lowest increase in temperature will increase global temperatures with 0.3-1.7°C (Collins *et al.*, 2013). An increase in regional heat waves was reported, with the greatest increase in heat wave frequency, based on the Berkley Earth temperature dataset (Perkins-Kirkpatrick and Lewis, 2020). With sudden fluctuations in temperature, plants rely on basal thermotolerance responses for protection, while a gradual increase in temperature can result in acquired thermotolerance in some species (Vierling, 1991). This is the result of a host of molecular and physiological adaptations that occur after a plant is exposed to moderately high temperatures. These responses are discussed in chapter 1.

Successful genetic engineering of heat tolerance in plants has been somewhat limited (Wahid *et al.*, 2007), and current EU laws prevent extensive use of genetically modified crops. Selective breeding has provided some success in cotton and wheat, but progress is slow (Azhar *et al.*, 2020; Ni *et al.*, 2019). Considering these limitations, other ways to enhance plant stress tolerance are desirable.

There has been some research into the interaction between UV-B and moderately (non-lethal) high temperature. UV-B and high temperature exposure produce Arabidopsis phenotypes which are visually very different (discussed in chapter 1). However, when applied simultaneously, UV-B was found to inhibit thermomorphogenesis, suppressing high temperature-induced elongation growth and leaf elevation by inhibiting auxin biosynthesis. This was achieved by suppressing *PIF4* transcript abundance leading to reduced PIF4 levels (Hayes *et al.*, 2017). In cucumber (*Cucumis sativus* L.), high temperature tolerance was investigated by exposing cotyledons and leaf discs to 50 °C (Caldwell, 1994). The study suggested that cucumber exposed to UV-B had an increased capacity to withstand elevated temperatures. Exposing rice (*Oryza sativa* L.) to 42°C for 24 h increased the tolerance to subsequent UV-B stress in a study by Murakami *et al.* (2004), suggesting there is potential crossover in UV-B and heat stress signalling pathways. In the same study, overexpression of the heat shock protein, sHSP17.7 in rice provided plants with both heat and UV-B protection, suggesting overlapping functions for heat signalling components in the UV-B and heat response. High fluence UV-B closed stomata in a study by Tossi *et al.* (2014), suggesting UV-B may be involved in maintaining cellular and plant water levels and turgor. To date, it is unknown whether this effect of UV-B on stomata provides any kind of protection against other stress responses.

Similar to cold stress, heat stress increases the synthesis of ROS (discussed in chapter 1; Heck *et al.*, 2003; Suzuki and Mittler, 2005). ROS production contributes to oxidative stress in plants and may be harmful if allowed to accumulate. In response, plants have evolved scavenging systems to neutralise ROS and minimise negative impacts (Suzuki and Mittler, 2005). UV-B irradiation promotes biosynthesis and accumulation of ROS-neutralising enzymes and molecules called antioxidants, suggesting that there may be a role for UV-B in regulating antioxidants during heat stress.

In addition to high and low temperature stress, this chapter explores some UV-B responses to water limitations. Comont *et al.* (2012) showed an interaction between UV-B and drought, while changes in water use efficiency and drought tolerance under UV-B supplementation were seen in some species (Robson *et al.*, 2015, Gitz III *et al.*, 2005). In a study by Guo *et al.* (2009), *UVR8* transcript abundance differed between drought-sensitive and drought tolerant barley. I therefore aimed to characterise UV-B

effects on water use efficiency (WUE), wilting and drought in *Arabidopsis thaliana*, alongside temperature stress responses.

Aims

The aims for this chapter were to investigate the effects of UV-B on temperature tolerance in Arabidopsis using temperature assays, PAM fluorescence and gene transcript abundance analysis to better understand the involvement of UV-B in the protection against temperature extremes. Based on the existing evidence from the literature that suggests crosstalk between light and both high and low temperature, it was hypothesised that UV-B would have a protective role. It was also hypothesised this mechanism would involve flavonoids and other antioxidants.

3.2 Specific methods

3.2.1 Cold acclimation treatment

Cold acclimation treatments were carried out in controlled climate chambers (JUMO dTRON 304, Snijder Scientific, Wilburg, The Netherlands) fitted with B and R LED lights, supplying ~60 µmol m^{-2} s^{-1}. The chambers were also fitted with supplementary UV-B bulbs providing 1 µmol m^{-2} s^{-1}. Light spectra for LT chambers can be seen in fig. S1.

3.2.2 Electrolyte leakage assay

A 20 L metal water bath (Clifton NE4-D series, Nickel-Electro LTD, Weston-super-Mare, UK) combined with an immersion dip cooler (Clifton DC1-300, Nickel-Electro LTD, Weston-super-Mare, UK) were used to cool ethylene glycol solution (Comma XstG40 AF/C Con, Moove Lubricants Ltd, Gravesend, UK) mixed with distilled de-ionised Milli-Q water (Millipore UK Ltd, Hertforshire, UK) to a 1:1 ratio. The protocol for electrolyte leakage assay was adapted from that previously described by Hemsley *et al*. (2014). 4 mm leaf discs from 3-week-old Arabidopsis were prepared using a cork borer and put in borosilicate glass tubes on ice. 5 mL de-ionised water was used to wash discs prior to cold treatment. Discs were equilibrated for 1 h at -2°C at which point small ice chips were added to each tube and a further 2 h of equilibration. Several studies show the LT50 for *Arabidopsis thaliana* around -4 to -8 °C, with cold acclimation lowering those temperatures to below -10 °C (Catalá *et al*., 2014; Zuther *et al*., 2015; Wanner and Junttila, 1999; Hannah *et al*., 2006). For this thesis, -2°C, -5°C and -8°C were selected to investigate freezing tolerance. Samples were collected after 30 min at the specific temperature and allowed to thaw overnight on ice at 4°C. The consecutive day, 5 mL of de-ionised water was added to each tube and placed on a shaker table (Luckham Rotatest Shaker, Luckham Ltd, Burgess Hill, UK) for 3 h. Discs were removed from the tubes and the electrolyte conductivity of the water solution from each tube was measured using a Primo Electrical Conductivity and Total Dissolved Solids tester (HANNA instruments Inc, Woonsocket, USA). Discs were frozen at -80°C for > 1 h and then returned to the water solutions. After subsequent 3 h shaking, total EL was measured. The initial reading was divided by the final reading and multiplied by 100 to provide percentage of total leakage. Electrolyte leakage was not used to assess thermotolerance as some stress signalling can open ion

channels of the plasma membrane resulting in an increase in conductance not attributed directly to membrane damage (Ilik *et al.,* 2018). Opening of the ion channels appears to be closely linked to reactive oxygen species signalling suggesting that at high temperature, where reactive oxygen species are highly active, electrolyte leakage may not be a suitable method. It is becoming clearer that oxidative stress is also involved in the cold response in plants, however it appears to be less fundamental than that under high temperature stress and is not fully understood (Costa-Broseta *et al.*, 2018; Distelbarth *et al.*, 2013; Suzuki and Mittler, 2005).

3.2.3 Thermotolerance assay

The protocol used for thermotolerance assays was adapted from Hong and Vierling (2000). 7 and 10-day-old seedlings developed on 0.5x MS media in 50 mm petri plates under 16 h photoperiods ± UV-B (1 µmol m^{-2} s^{-1}) were used for thermotolerance assays. Nine plates (approx. 30 seedlings per plate, equalling 270 seedlings total) per treatment across three independent experiments were analysed and percentage survival calculated by first calculating the percentage survival per plate. Seedlings with a green apical meristematic region were counted as survivors after 5 days recovery in a 16 h photoperiod at 20°C. Initially, an incubator oven was employed for heat treatments. However, temperatures were found to fluctuate throughout the oven at a magnitude of ± 1.5^{o}C (measured with external thermometers with probes). This was not considered to provide consistent experimental conditions and instead a heat bath with an immersion heater was used for all thermotolerance assays. The petri plates were placed on a plastic insert in the tank and the heated water covered 50-75 % of the petri plate walls. A temperature probe was used to ensure the insides of the petri dished heated to the desired temperature. A tube system cycling cold water was attached on one side of the bath to create a current which ensured uniform heating of the water in the bath. For acquired thermotolerance, plants were subjected to 38°C for 90 min and a subsequent 20°C for 1 h 30 min to develop thermotolerance. These acclimated plants were transferred, together with non-acclimated plants, to 45°C for 1 h 45 min. All heat treatments were carried out in the dark, unless otherwise stated, to ensure damage was the result of temperature increases and not photo-oxidative stress (Clarke *et al.*, 2009). Photographs were captured using a Nikon D3200 DSLR using a standard objective.

3.2.4 Chlorophyll fluorescence

Fluorescence measurements have been used to highlight differences in photosynthetic efficiency and stability at different temperatures in a range of Arabidopsis mutants in order to investigate the involvement of different genes in temperature responses (Falcone *et al.*, 2004). Here, chlorophyll fluorescence was used as a proxy for damage and recovery after heat stress in mature plants. Chlorophyll fluorescence was measured using a Pulse-Amplitude Modulated system (Walz, Germany) and data processed using the ImagingWin software (version 1.0). Fv/Fm readings reflecting PSII quantum efficiency were acquired from dark-adapted leaf tissue. Where time course data were collected, the samples were kept in the dark between measurements.

3.2.5 Drought assay

Watering was withheld from 3-week-old Arabidopsis developed without UV-B under normal watering conditions (watered three times weekly). During the withheld watering period, 50% of the plants were exposed to UV-B (1 µmol m^{-2} s^{-1}) in a background of white light (70 µmol m^{-2} s^{-1}). Pots containing soil but no plants were used as a control. Pots were weighed every day for 9 d. The average weight of the control pots was subtracted from the plant containing pots to obtain plant fresh weights. Water content was measured by weighing detached rosettes at the designated times and after oven-drying. The dry weight was subtracted from the fresh weights to get water weights.

3.2.6 WUE assay

The WUE assay was adapted from Simon *et al.* (2020). 50 mL falcon tubes were filled with soil and perlite (1:1) and topped up with 25 mL ionised water. The tops of the tubes were covered with cling film and a small hole was made using a needle. 5 seeds were deposited through the hole using a pipette. The tubes were wrapped in aluminium foil and placed in 4 °C for 48 h before transfer to a growth cabinet set to 70 % RH, 20 °C and 70 µmol m^{-2} s^{-1} PAR ±UV-B (1 µmol m^{-2} s^{-1}). After 1 week, all but one seedling per tube were removed. Initial tube weight was also recorded. After a further 3 weeks, the rosettes were removed, and final falcon tube weight was recorded. Rosettes were dried for 3 d in an incubator oven set to 60 °C before rosette dry weight was recorded. The soil water evaporation value was obtained from control tubes without plants, treated exactly the same as those with plants in. These

tubes were weighted at the beginning of the experiment and at the end and the values were averaged to get a mean value for soil water evaporation. This experiment was repeated twice.

WUE was calculated as:

WUE = rosette dry weight (mg) / (water used (mL) – soil water evaporation (g))

where

water used (ml) = initial falcon tube weight (g) – final falcon tube weight (g)

and

soil water evaporation (g) = 0.7165   Simon *et al.,* 2020

3.2.7 Wilting assay

Rosettes of 3-week-old Arabidopsis were detached from the hypocotyls and placed on wetted filter paper in petri dishes and kept at 20 °C and 70 % RH in a controlled climate growth chamber. Rosette weight was recorded every 40 min for 160 min, and again at 18 h. The UV-B treatments were as follows:

- \- \-   Developed without UV-B, no UV-B during wilting phase

- \- +   Developed without UV-B, UV-B during wilting phase

- + \-   Developed in UV-B, no UV-B during wilting phase

- + +   Developed in UV-B, UV-B during wilting phase

3.3 UV-B enhanced flavonol abundance but this does not result in enhanced cold acclimation or freezing tolerance in *Arabidopsis thaliana*.

Global changes in climate puts enormous stress on plant survival and health and therefore also on food production systems. Understanding signalling pathways that may protect plants from environmental stress is highly important in order to protect plants, and to develop plants with enhanced stress protection mechanisms. Here, it was investigated whether supplemental UV-B could promote protection against both low and high temperature, and whether UV-B plays a role in water status in *Arabidopsis thaliana*. It was hypothesised that UV-B exposure promotes protection against low and high temperature stress because of its positive effects on flavonoid production and antioxidant capacity. It was also hypothesised that UV-B may affect the water status of plants due to its effect on stomatal aperture.

3.3.1 UV-B and cold increased *CHS* transcript abundance and flavonol accumulation.

In line with published data, UV-B significantly increased the transcript abundance of *CHS* as well as flavonol abundance (F=, $p<0.05$, fig. 3.1a; F=, $p<0.05$, fig. 3.1b), (Shirley *et al.*, 1995; Jenkins *et al.*, 2001; Oravecz *et al.*, 2006; Tohge *et al.*, 2011). Plants not exposed to UV-B did not accumulate flavonols (data not shown). Cold treatment alone also failed to cause an accumulation of flavonols in the time frame investigated here (data not shown). Interestingly, the combination of cold and UV-B had a greater effect on both *CHS* transcript levels and flavonol accumulation than did UV-B alone, similar to published data (Schulz *et al.*, 2021). This suggests that CHS and flavonoids may be important for low temperature tolerance (Schulz *et al.*, 2016). Furthermore, UV-B enhanced the transcript abundance of *CHS* in the cold. It was hypothesised that this increase in flavonols could enhance cold acclimation, or have a positive effect on freezing tolerance in non-acclimated plants.

3.3.2 UV-B did not enhance cold tolerance in Arabidopsis

Consistent with previous reports (Gilmour *et* al., 1988), cold acclimation significantly reduced electrolyte leakage in wild-type plants at -2 and -5 °C but not at -8 °C (fig. 3.2a, F=52.691, $p<0.001$). Similarly, cold acclimation reduced EL in the *uvr8-6* mutant. Flavonoids have been shown to govern cold acclimation and promote freezing tolerance in Arabidopsis (Schulz *et al.*, 2016), while UV-B

strongly upregulates flavonoid biosynthesis (Lois, 1994). Despite the effects of UV-B on *CHS* transcription and flavonol accumulation seen in this study, the UV-B treatment used here did not elicit a protective effect in plants exposed to -2, -5 or -8 °C (fig. 3.2a). Increased transcript abundance of *CHS* and the accumulation of flavonoids in response to UV-B is UVR8-mediated (Jenkins *et al.,* 2001; Brown *et al.,* 2005; Favory *et al*., 2009). Here, in the *uvr8-6* mutant, prior UV-B exposure may be so damaging that freezing tolerance is reduced in non-acclimated plants (fig. 3.2a, green line). To investigate the effect of UV-B on the upregulation of *COR* genes, *COR15a* and *COR47* were selected as genes of interest. *COR15a* encodes a 15 kDa protein that protects plants against freezing by inhibiting inactivation of LACTATE DEHYDROGENASE (LDH) and RubisCO in chloroplasts, while *COR47* encodes a 47 kDa dehydrin protein that protect against dehydration events that can occur during freezing (Gilmour *et al*., 1992; Lin and Tomashow, 1992). As expected, the cold acclimation treatment enhanced transcript abundance of *COR15a* and *COR47* though this effect was not statistically significant due to variation in the data. However, UV-B did not have a further effect on these levels, suggesting UV-B may not affect the abundance of these COR proteins (fig. 3.2b).

3.3.3 Flavonoids may be involved in UV-B responses to freezing tolerance

The involvement of flavonoids in LT and UV-B crosstalk was investigated using a *tt4* and a *tt7* (also known as *f'3h*) mutant. The *tt4* mutant is deficient in CHS which catalyses the initial step of flavonoid biosynthesis (Tohge *et al*., 2009). The *tt7* mutant is deficient in F'3H, an enzyme which catalyses quercetin biosynthesis (Schoenbohm *et al.,* 2000). The *tt4* mutant had increased electrolyte leakage in -2 and -5 °C (F=13.47, p<0.001) suggesting that flavonoids may be required for freezing tolerance (fig. 3.3a). *tt4* plants that were exposed to UV-B prior to freezing treatment had lower electrolyte leakage than did the non-irradiated control, suggesting UV-B rescues this phenotype (F=13.47, p<0.001, fig. 3.3a). In contrast, electrolyte leakage in *tt7* was very similar to that in the WT control and was not statistically significant, suggesting that quercetin may not be the only critical flavonoid involved in the UV-B-induced reduction in electrolyte leakage in a flavonoid-deficient mutant such as *tt4* (fig. 3.3b).
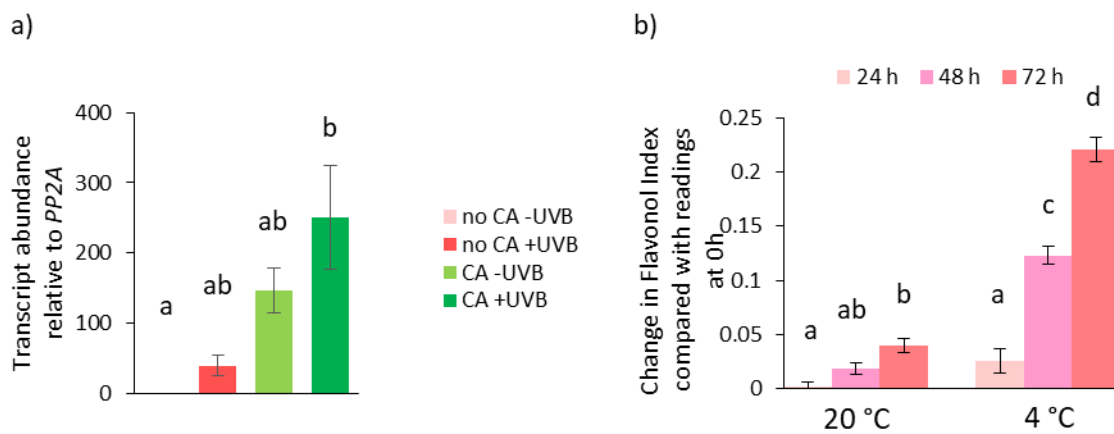
Figure 3.1 Effects of UV-B and cold on *CHS* transcript abundance and flavonol accumulation. 3-week-old Arabidopsis developed in a 12-hour photoperiod at 20°C in WL±UV-B (1 µmol m⁻² s⁻¹) were transferred to 20°C or 4°C ±UV-B. Bars represent means with error bars representing SE. **a)** *CHS* transcript abundance after 6 h UV-B and cold treatment. n=3. **b)** Flavonol accumulation after UV-B and cold. n=12. Data was analysed by 2-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.
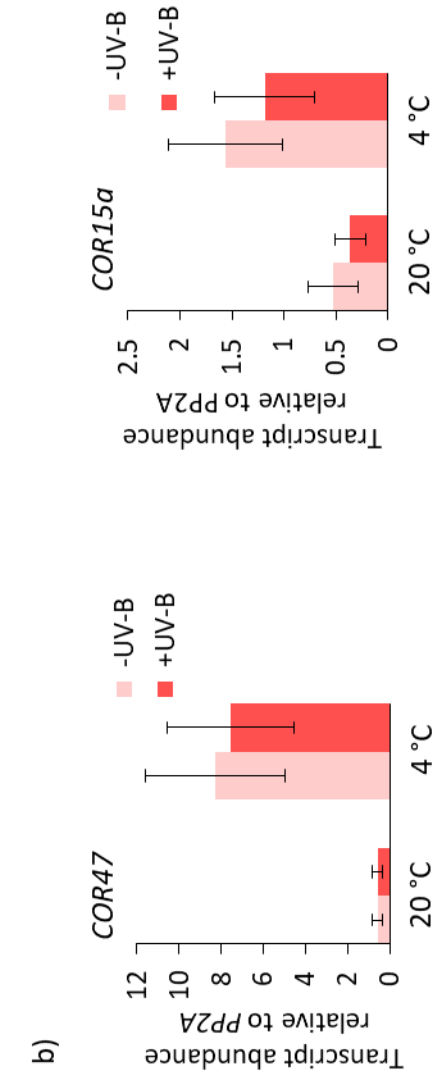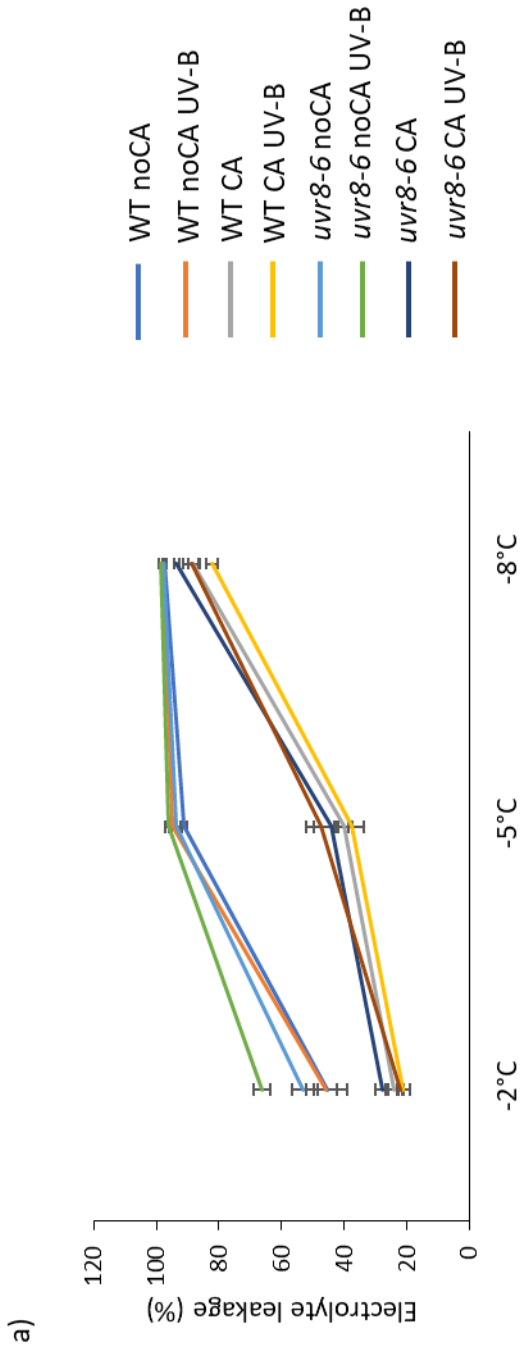
Figure 3.2 UV-B did not promote freezing tolerance in Arabidopsis. 3-week-old Arabidopsis developed in a 12 h photoperiod, 20°C and WL ±UV-B (1 μmol m^{-2} s^{-1}) were cold acclimated at 4°C before freezing or analysis. **a)** Electrolyte leakage of leaf discs cold acclimated for 12 h and exposed to freezing temperatures. Points represent means with error bars representing the SE. n=12. **b)** Effects of UV-B and 6 h cold acclimation on the transcript abundance of *COR15a* and *COR47*. Bars represent means with error bars representing the SE. n=3.
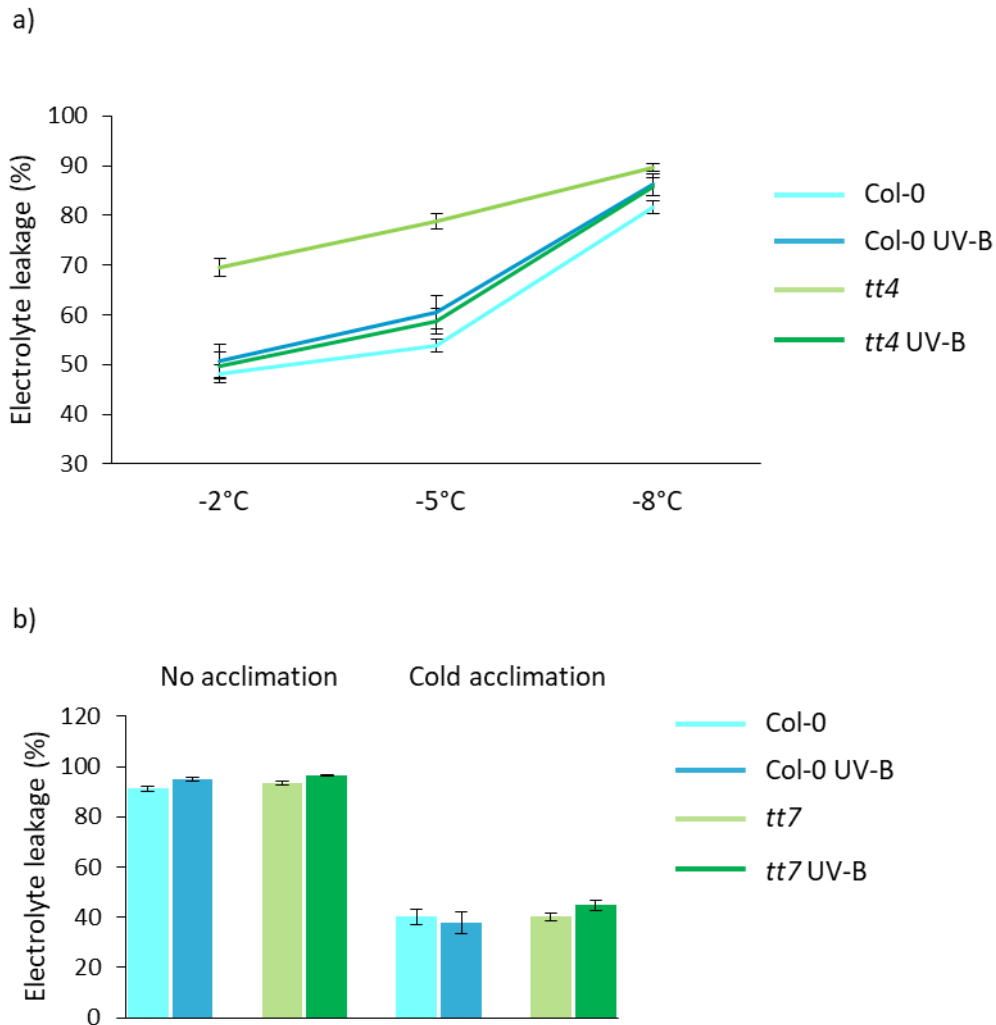
Figure 3.3 UV-B rescues the phenotype of *tt4*. Leaf discs from 3-week-old Arabidopsis grown in a 12 h photoperiod and 20°C in WL were exposed to UV-B (2.5 μmol m⁻² s⁻¹) for four days before cold acclimation at 4°C for 12 h without UV-B. Leaf discs were then exposed to freezing temperatures and electrolyte leakage was measured. **a)** Electrolyte leakage of *tt4* after -2, -5 and -8°C treatment. **b)** Electrolyte leakage of *tt7* after -5°C treatment.

3.4 UV-B enhanced antioxidant capacity but this did not result in enhanced thermotolerance in *Arabidopsis thaliana*.

3.4.1 UV-B enhanced antioxidant capacity of seedlings and upregulated heat responsive genes.

To investigate the effects of UV-B on antioxidant capacity, seedlings were exposed to different UV-B treatments. While all treatments enhanced antioxidant capacity (F=28.6, p<0.05, exposing seedlings throughout development to 1 µmol m⁻² s⁻¹ UV-B was most successful in increasing antioxidant capacity, suggesting the effect is cumulative (fig. 3.4). To investigate if there was any overlap between genes upregulated after UV-B exposure and high temperature, data sets from Ulm *et al*., 2004 and Li *et al*., 2019 were analysed. Seedlings in both experiments were 7d old. The length of exposure to three different UV-B treatments was 15 min, with 1 h recovery before harvest. High temperature exposure was 30 min at 37ºC. 72 genes were expressed during both UV-B and HT (fig. 3.5). Of these, 5 were identified as heat signalling components which may be involved in heat tolerance (fig. 3.5). In both data sets, genes with ≥2-fold expression compared to the control were included.

3.4.2 UV-B did not improve survival in Arabidopsis seedlings exposed to heat stress.

A high through-put assay adapted from Hong and Vierling (2000) was used to assess seedling thermotolerance. The lethal temperature for non-heat acclimated Arabidopsis was determined to be between 44-46 °C (fig. 3.6a). As many studies assessing thermotolerance in Arabidopsis seedlings employ a 45 °C treatment, this temperature was chosen (Hong and Vierling, 2000; Larkindale *et al*., 2005). No positive effect on survival was observed between three Arabidopsis WT accessions grown ± UV-B (fig. 3.6b), nor was there any difference in survival when Col-0 was exposed to different UV-B treatments prior to heat stress (fig. 3.6c). *HSP* gene transcripts have been used to assess heat induction (Kostaki *et al*., 2020). Heat stress significantly increased the transcript abundance of *HSP70-4* (At3g12580) in 7-day-old (F=7.266, p<0.05) and 11-day-old (F=11.9, p<0.01) plants, while UV-B had no additive effect. Interestingly, both heat and UV-B increased the transcript abundance of *CHS*, suggesting flavonoid biosynthesis could be important in heat stress tolerance (p<0.05, fig. 3.7a and b).

Furthermore, heat acclimation treatment had a greater impact on the increase in transcript abundance of *CHS* than did the 45 °C treatment, suggesting *CHS* may be involved in heat acclimation in Arabidopsis (fig. 3.7b). Despite the synergistic effect of UV-B on *CHS* transcript accumulation under heat acclimation, UV-B did not enhance survival in acclimated plants, but may instead have a negative effect as the survival was slightly reduced in the 7 d old UV-B-treated plants exposed to heat stress ($p<0.01$, fig. 3.7c).

To investigate whether the UV-B photoreceptor UVR8 plays a role in thermotolerance, *uvr8-6* mutants were assessed for basal thermotolerance in the same manner as the WT accessions. No difference in survival was seen between the *uvr8-6* mutants and Col-0 regardless of UV-B treatment (fig. 3.8), suggesting that the absence of UVR8 does not affect basal thermotolerance in the conditions used here.
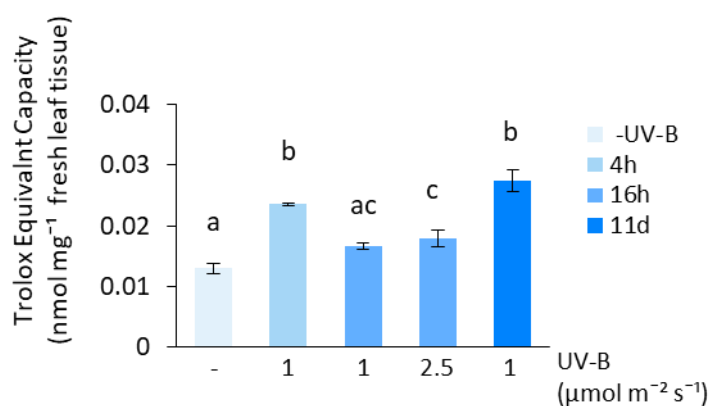
Figure 3.4 Antioxidant capacity after different UV-B treatments. 11-day-old Arabidopsis seedlings developed on ½ MS petri plates in WL ± UV-B (1 µmol m⁻² s⁻¹) were assessed for antioxidant capacity using a Trolox equivalent capacity assay kit. Bars represent the means with error bars representing SE. n=12. Different letters indicate statistically significant differences at p<0.05.
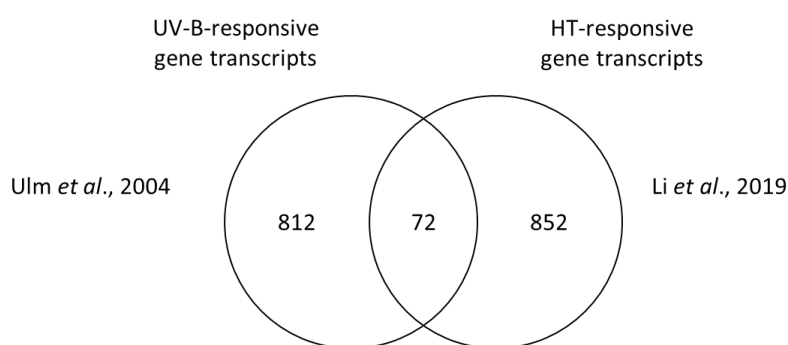
Submitted name	Gene name	Function
HEAT SHOCK TRANSCRIPTION FACTOR A3	AT5G03720	Expression reg by dreb2a, regs express of Hsps, involved in est thermotolerance
HSP20-like chaperones superfamily protein	AT5G51440	Pollen, flowering stage expression
HEAT SHOCK TRANSCRIPTION FACTOR B2A	AT5G62020	
HEAT SHOCK TRANSCRIPTION FACTOR A7A	AT3G51910	heat response, reg of protein metabolic process
Dehydration-responsive element-binding protein 2B	AT3G11020	Salinity, and dehydration induced transcription

Figure 3.5 Heat shock-related genes upregulated in Arabidopsis under both UV-B exposure and high temperature. Data sets from Ulm *et al*., 2004 and Li *et al*., 2019 were analysed. Several heat-regulated genes were upregulated under UV-B. Gene information was obtained from TAIR (The Arabidopsis Information Resource (https://www.arabidopsis.org/, 2021).
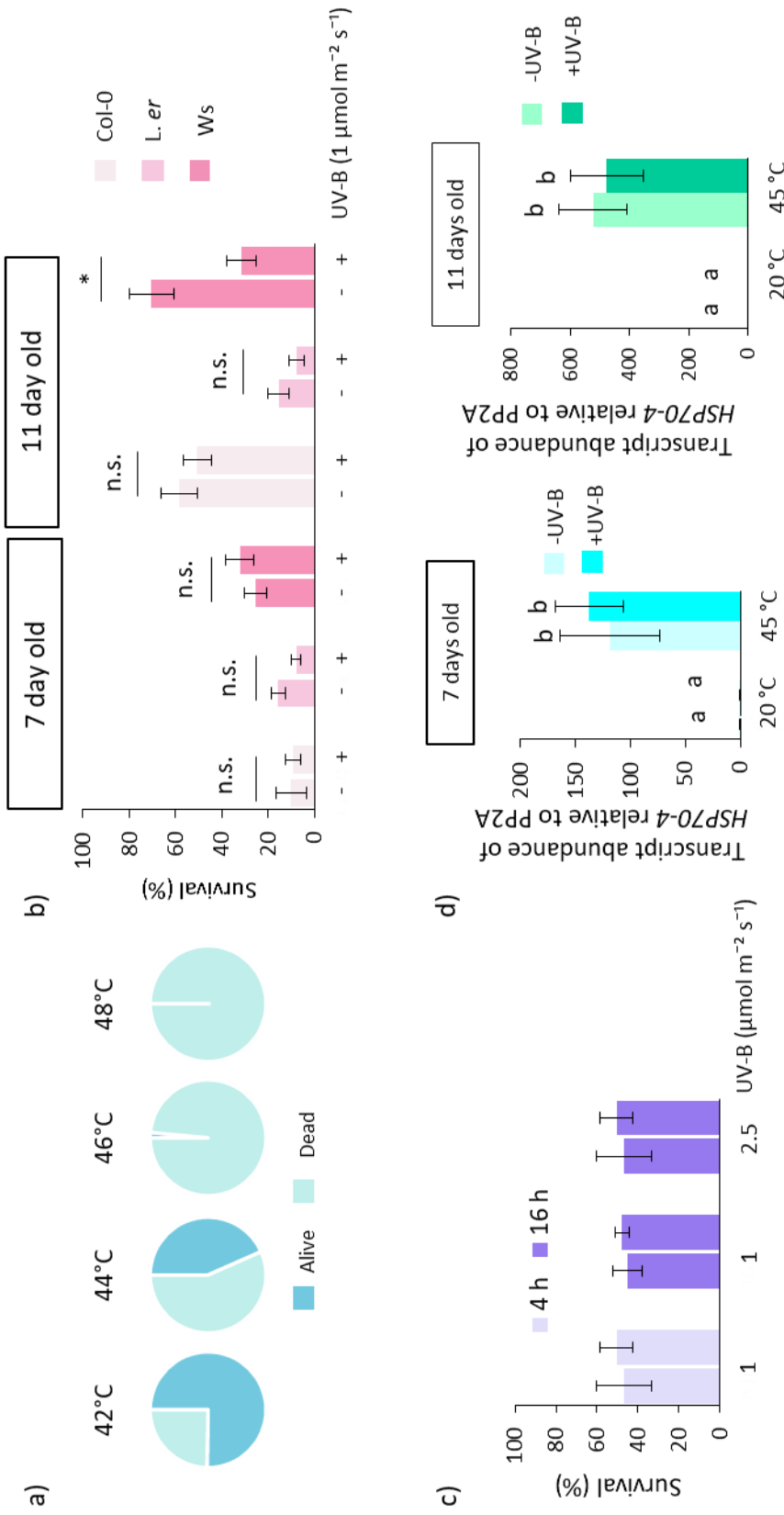
Figure 3.6 UV-B does not enhance survival after heat stress in Arabidopsis seedlings. 7 or 11-day-old seedlings developed in a 16 h photoperiod, 20°C and WL ±UV-B were exposed to UV-B and/or heat stress. **a**) The lethal temperature of 11-day-old Arabidopsis seedlings after 1 h 45 min heat stress. n=80-90. **b**) Survival of three Arabidopsis WT accessions after 45°C. n=90. **c**) Survival of Col-0 after short term UV-B treatments. n=80-90. **d**) *HSP70-4* transcript abundance after exposure to 45°C and UV-B. n=3. Bars represent the mean with error bars representing the SE. Data analysed by two-way ANOVA with Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.
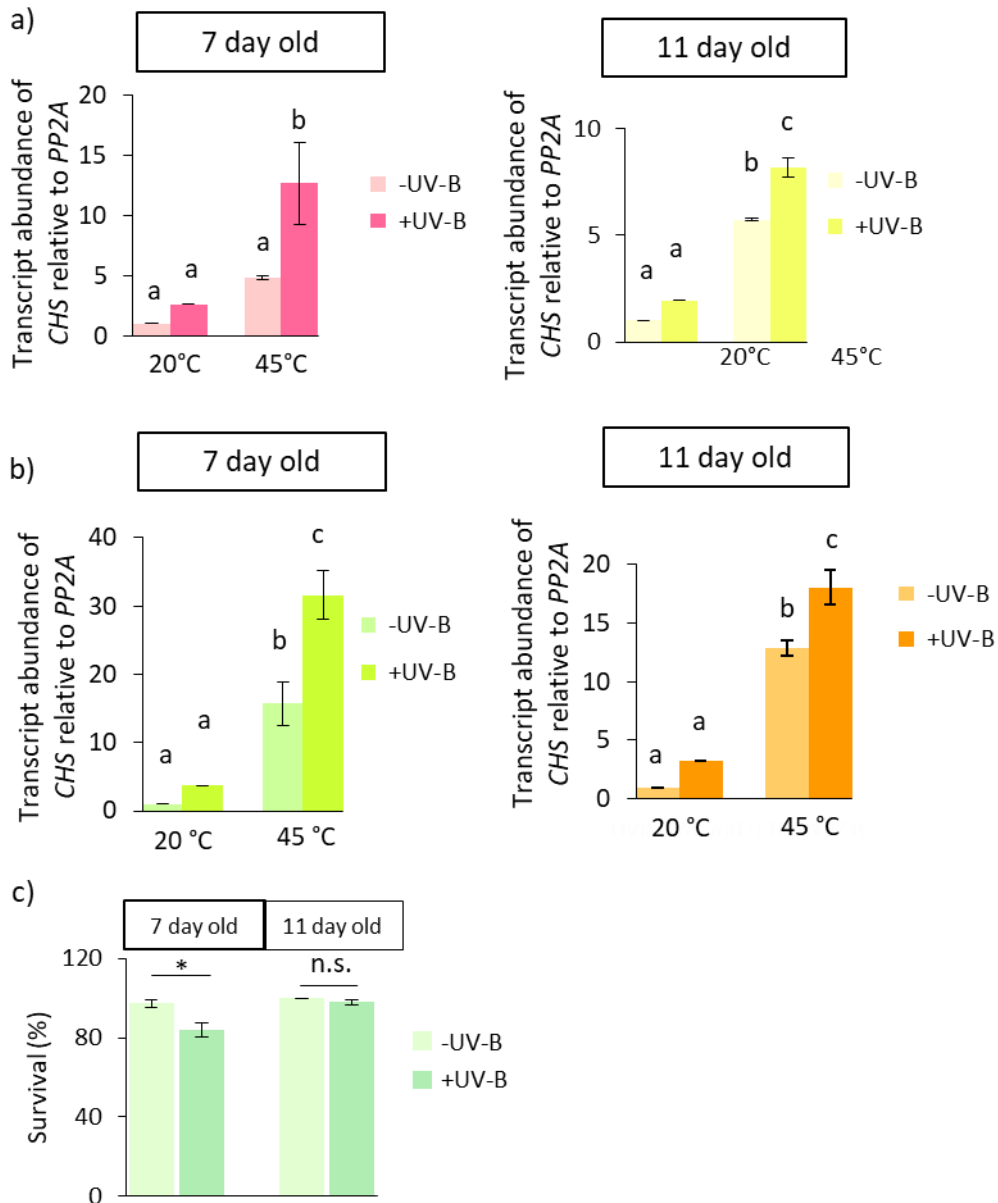
Figure 3.7 The effects of UV-B and heat stress on heat acclimation and *CHS* transcript abundance. 7 and 11-day-old Arabidopsis seedlings developed in a 16 h photoperiod, 20°C and WL ±UV-B (1 µmol m⁻² s⁻¹) were exposed to 20°C or 45°C. Seedlings that were heat acclimated were treated at 38°C for 1.5 h and allowed to recover for a further 1.5 h at 20°C prior to the 45°C heat stress treatment. **a)** *CHS* transcript abundance after heat stress. n=3. **b)** *CHS* transcript abundance after heat acclimation prior to heat stress. n=3. **c)** Survival of heat acclimated seedlings after heat stress. n=9. Bars represent means with error bars representing the SE. Data was analysed by 2-way ANOVA and Tukey post hoc test. Asterisks and different letters indicate statistically significant differences at p<0.05.
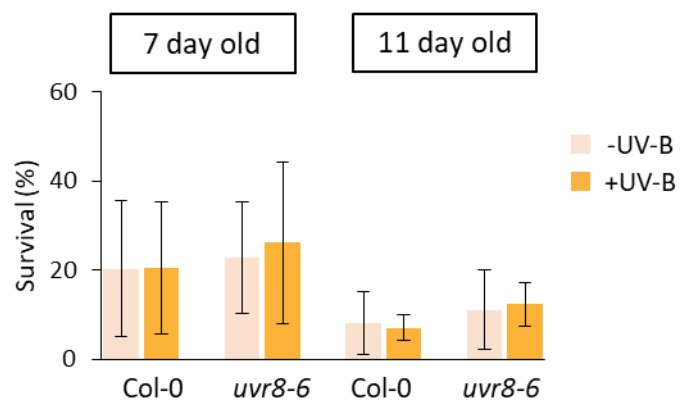
Figure 3.8 Thermotolerance of *uvr8-6* after UV-B and heat stress. 7 and 11-day-old WT and *uvr8-6* mutant seedlings developed in a 16 h photoperiod, 20°C and WL ±UV-B (1 μmol m^{-2} s^{-1}) were exposed to a 45°C heat stress treatment. Bars represent the means with error bars representing the SE. n=70-90. Data analysed with Two-way ANOVA. No statistically significant differences were found.

3.4.3 UV-B did not maintain photosynthetic capacity of plants exposed to heat stress.

Heat stress can have devastating effects on photosynthesis (Yan *et al.*, 2011). This is mainly due to the sensitivity of PSII to heat (Berry and Bjorkman, 1980). When leaves become damaged by heat they bleach, due to the destruction of chlorophyll (Larkindale *et al.*, 2005). The reduction in photosystem activity is measurable by chlorophyll fluorescence using a pulse-modulated system. Similar to other studies, chlorophyll fluorescence was here used as a proxy for leaf damage post stress (Ehlert and Hincha, 2008). We reasoned that if UV-B has a protective effect in heat stress, this will lead to maintenance of photosynthetic capacity under heat stress. Rosettes of 3-week-old Arabidopsis (Col-0) plants were grown in white light $\pm$ UV-B (1 µmol m^{-2} s^{-1}) at 20°C prior to being detached from the hypocotyl and placed on PBS buffer. Detached rosettes on buffer were used in order to fit into the heated water bath. Plants were exposed to a 45°C heat treatment for 30 min to assess Fv/Fm associated with basal thermotolerance or acclimated to heat by receiving a 38°C treatment for 45 min prior to the 45°C heat treatment. Fv/Fm was measured after 1, 2, and 24 hours. PSII quantum yield of unstressed plants is typically 0.83. Here, an average of 0.79-0.80 was measured for the control plants after 1 h, suggesting separation of the rosette from the roots caused some stress to the plants (fig. 3.9; 3.10). UV-B had a small negative effect on Fv/Fm at 20 °C, similar to the report from Badmus *et al.* (2022), though this was not a significant result in the conditions used here. Consistent with reports from other species and from Arabidopsis, heat treatment also reduced Fv/Fm (F=301.563, p<0.001, fig. 3.9) (Benkov *et al.*, 2019; Ribeiro *et al.*, 2008; Chen *et al.*, 2016). Heat acclimation resulted in a smaller reduction in Fv/Fm after 45°C heat stress (fig. 3.10). No significant effect of UV-B at any of the recovery times in non- and heat acclimated plants was observed, suggesting UV-B does not influence Fv/Fm in mature Arabidopsis plants. Interestingly, Fv/Fm was lower after heat acclimation than in the control plants (fig. 3.10), suggesting that while the acclimation process does protect plants from more severe high temperatures, the acclimation temperature is itself damaging, perhaps particularly to detached rosettes.
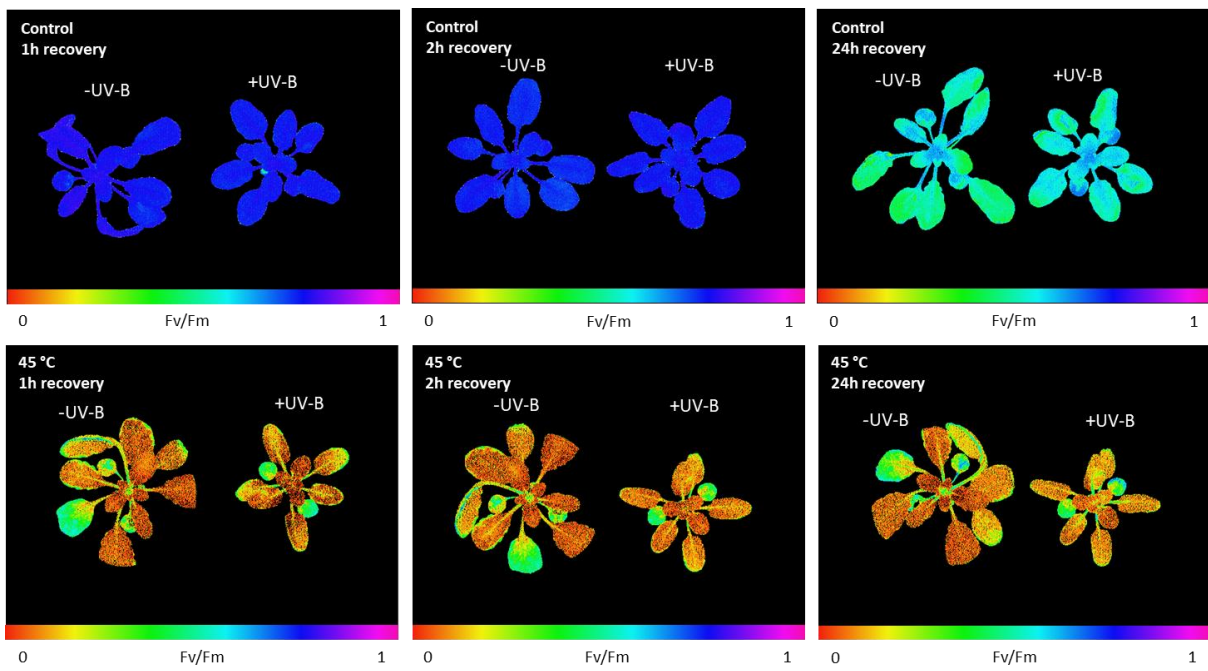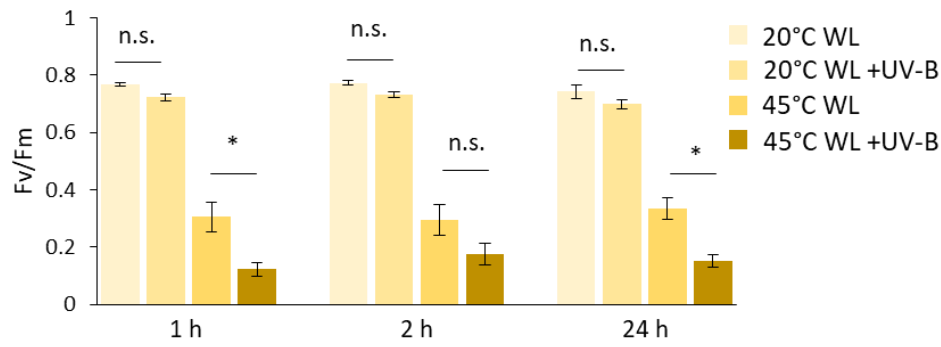
Figure 3.9 Photosynthetic capacity of Arabidopsis rosettes after UV-B and heat stress. Detached rosettes of 3-week-old Arabidopsis developed in a 16 h photoperiod, 20°C and WL ±UV-B (1 µmol m^{-2} s^{-1}) were placed in PBS buffer and exposed to a 45 °C heat stress treatment for 30 min in the dark and then allowed to recover at 20 °C. F_v/F_m measurements were taken at 1, 2 and 24 h post heat exposure. Bars represent means with error bars representing SE. Data were statistically analysed by 2-way ANOVA and Tukey post hoc test. Asterisks indicate statistically significant differences at p<0.05. n=12.

Figure 3.10 Photosynthetic capacity after UV-B, heat acclimation and heat stress.

Detached rosettes of 3-week-old Arabidopsis developed in a 16 h photoperiod, 20°C and WL ±UV-B (1 μmol m⁻² s⁻¹) were placed in PBS buffer and exposed to a 45 °C heat stress treatment for 30 min in the dark with or without prior heat acclimation, and then allowed to recover at 20 °C. F_v/F_m measurements were taken after heat acclimation and at 1, 2 and 24 h post heat stress. Bars represent means with error bars representing SE. Data were statistically analysed by 2-way ANOVA and Tukey post hoc test. Statistically significant differences are indicated by asterisks. n=12.
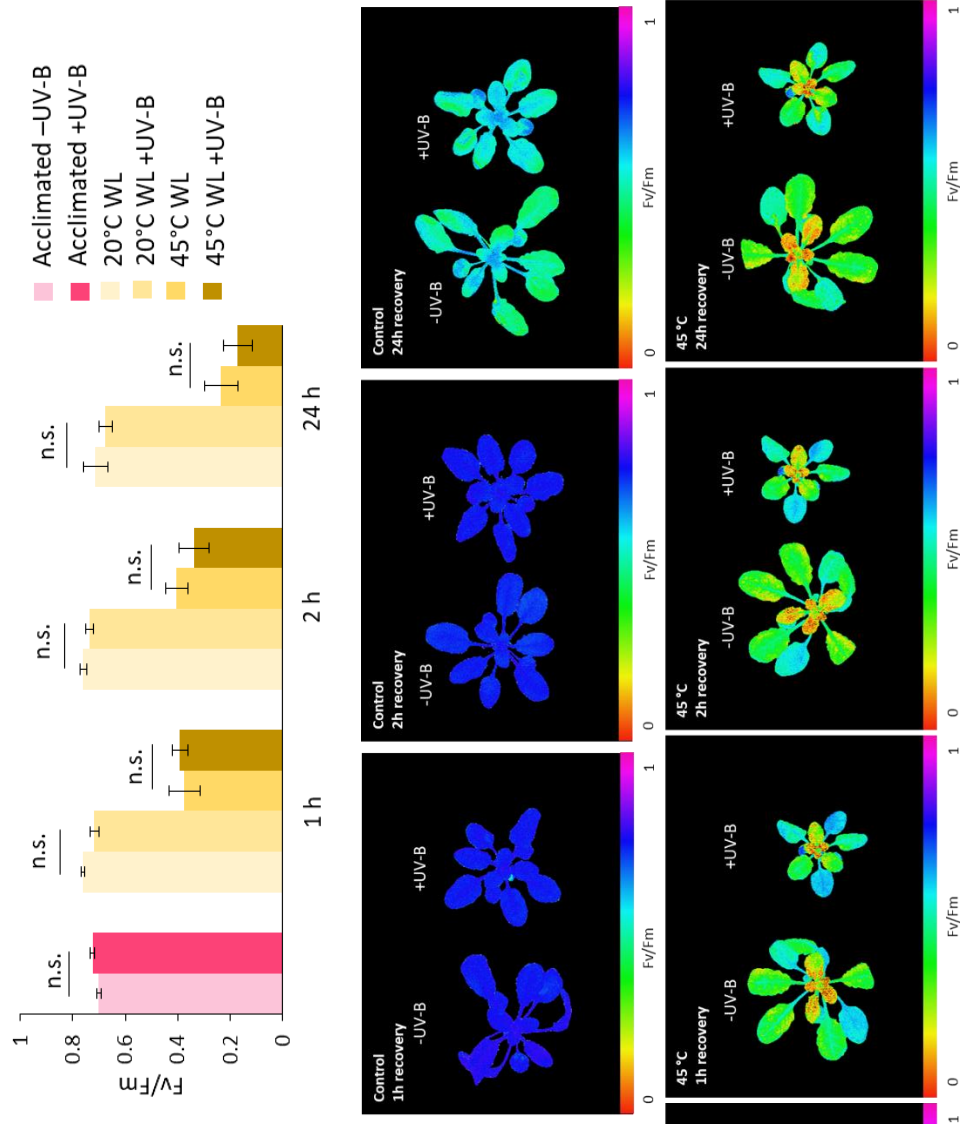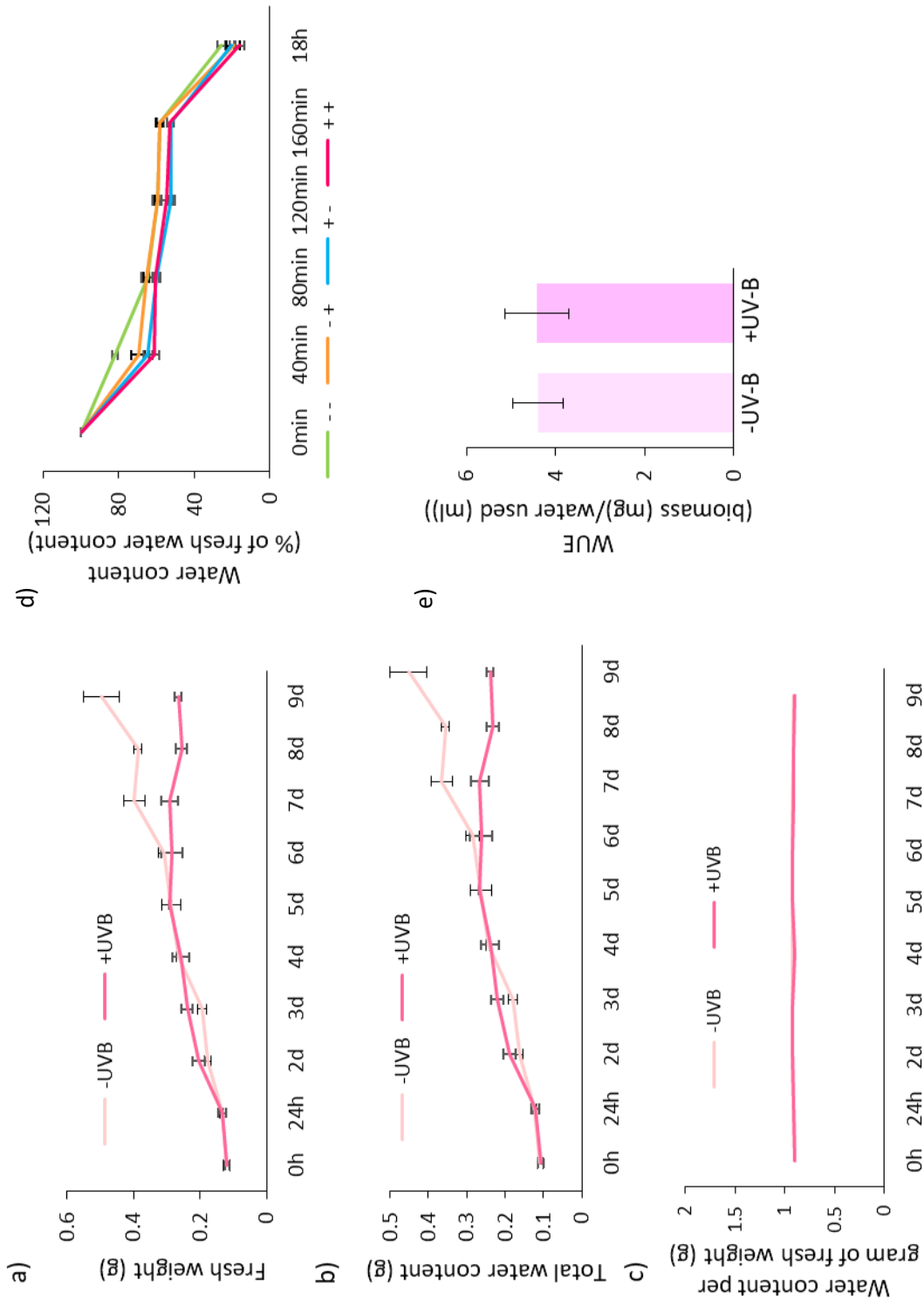
Figure 3.11 The effects of UV-B on drought, wilting and WUE. a) - c) Weight and water content of plants where watering was withheld. Plants were grown for 2 weeks in a 16 h photoperiod, 20°C and WL ±UV-B (1 μmol m⁻² s⁻¹) before watering was withheld. Points represent means each day with error bars representing SE. n=11. **d)** UV-B did not maintain water content in wilting seedlings. Bars represent means with error bars representing SE. n=12. **e)** WUE of plants grown ± UVB (μmol m⁻² s⁻¹). Bars represent means with error bars representing SE. n=12. Data were analysed by two-way ANOVA. No statistically significant differences were found between UV-B treated plants and control treatments.

3.5 UV-B did not alter tolerance to wilting or drought and had no effect on WUE in Arabidopsis.

Watering was withheld from growing 3-4-week-old Arabidopsis plants to assess the potential of UV-B to reduce water loss during drought. Plants were sampled each day for 9 days; fresh and dry weights were recorded to assess water content, by subtracting the dry weight from the fresh tissue weight. Detached rosettes exposed to four different UV-B treatments were used for the wilting experiments while plants grown in falcon tubes were used to assess WUE. Despite some evidence from the literature suggesting UV-B may improve drought tolerance, no such effect was observed here (fig. 3.11a-c). Furthermore, UV-B had minimal effect on wilting and WUE (fig. 3.11c and 3.11d).

3.6 Discussion

Cold tolerance

Based on reports in Rhododendron and wheat, it was hypothesised that UV-B enhances protection against cold in Arabidopsis via upregulation of flavonoid biosynthesis genes, and subsequent flavonoid accumulation (fig. 3.1; Dunning *et al.*, 1994; Chalker-Scott and Scott, 2004; Yang *et al.,* 2007). LT and UV-B produce similar phenotypes, with several mutual signalling components, suggesting there may be crosstalk between the signalling pathways, and cross-protection may occur (Teramura and Sullivan, 1994; Dunning *et al.,* 1994; Gilmour *et al.,* 2004). In the study by Chalker-Scott and Scott (2004), enhanced UV-B treatments over a period of 3-12 weeks produced the highest cross-protection against freezing temperatures in Rhododendron. Highest protection was achieved after 12 weeks. Here, Arabidopsis plants developed ±UV-B to investigate the effects on cross-protection to cold.

Electrolyte leakage has been used in various species and in response to different sources of damage and is an accepted method to assess cellular membrane damage (Ilik *et al.*, 2018; Sukumaran and Weiser, 1972; Gilmour *et al.,* 1988). Here, it was used in much the same manner as Hemsley *et al.* (2014) to gain an understanding of membrane integrity after freezing. Electrolyte leakage increased with each decrease in temperature (-2, -5, and -8°C, fig. 3.2a), providing a means to measure membrane damage after CA and UV-B treatments. Reports have shown that Arabidopsis can endure temperatures of around -3.5°C to -5.7°C without cold acclimation, meaning that these temperatures cause less than 50% electrolyte leakage (Ristic and Ashworth, 1993; Uemura *et al.*, 1995). >50% EL is considered lethal and often expressed as LT50 (Ristic and Ashworth, 1993; Uemura *et al.*, 1995; Hemsley *et al.*, 2014). Here, precise determination of LT50 for different treatments was not possible due to time constraints for experiments, but the EL data presented in this chapter are considered in context of a 50% leakage cut-off point.

Cold acclimated plants had lower EL than non-acclimated plants, suggesting cold acclimation was successful. Arabidopsis has been reported to survive -8 °C after 24 h cold acclimation at 1°C, -9.4 °C after 48 h CA, or -12 °C after a longer cold acclimation period (Ristic and Ashworth, 1993; Wanner

and Junttila, 1999). A positive effect on EL after 12 h CA was observed here. CA lowered the temperature of LT50 to around -5 °C. EL from CA plants was statistically significant from non-cold acclimated plants in all temperatures except for leaf discs exposed to -8°C (fig. 3.2a).

UV-B irradiation did not reduce EL in Col-0 exposed to -2, -5 or -8°C compared to the WT (fig. 3.2a). Neither did UV-B reduce EL in the *uvr8-6* mutant, which lacks the UV-B photoreceptor, UVR8, as it showed a wild-type response for electrolyte leakage at -5 and -8°C (fig. 3.2a). In fact, at -2 °C, *uvr8-6* had increased EL compared to the WT when grown in UV-B, suggesting this mutant may be damaged by UV-B exposure which then leads to reduced freezing tolerance. Here, a UV-B dosage of 1 µmol m^{-2} s^{-1} was given to plants throughout the growth period. This level of UV-B irradiation is generally considered non-stressful for lab-cultivated Arabidopsis (Tong *et al.,* 2008). However, without UVR8 signalling triggering accumulation of protective pigments and antioxidants, plants could experience stress to a higher extent. Relative transcript abundance of two key COR genes was investigated to elucidate whether UV-B affects COR gene expression during cold acclimation. Consistent with the literature, LT increased transcript abundance of both *COR15a* and *COR47* (fig. 3.2b and c; Thomashow, 1999; Wang and Hua, 2009; Hannah *et al.,* 2005), suggesting that low, non-freezing temperature treatments successfully induced cold acclimation, however no additive effect of UV-B was observed (fig. 3.2b and c). Together, these data suggest UV-B has little effect on electrolyte leakage and freezing tolerance. Alternatively, it may mean that a cold acclimation treatment can successfully mask any effects of UV-B as cold acclimation is very effective in protecting plants from freezing damage. By including non-cold acclimated plants, it was possible to deduce any effect UV-B had on freezing tolerance without the influence of CA. However, as discussed, no effects of UV-B were seen in non-CA plants.

The wild-type response of *uvr8-6* further suggests that UVR8 is likely not involved in temperature perception or modulation in the range of -5 °C to -8 °C. This is an interesting observation as other photoreceptors have been shown to possess thermosensory capabilities at higher temperatures (Legris *et al*., 2016; Hung *et al*., 2016, Qui *et al*., 2019). It would be interesting to monitor *COR* gene transcript abundance in a *uvr8-6* mutant to assess this further. Interestingly, the monomer/dimer equilibrium of

UVR8 in Arabidopsis is sensitive to temperature as a higher proportion of monomers compared to dimers exist at 8-10°C compared to at higher temperatures (Findlay and Jenkins, 2016), suggesting UVR8 signalling may be different at different temperatures.

It is possible that whole plants can withstand lower temperatures better than isolated leaf discs, as systemic signalling could prevent excessive membrane damage (Gorsuch *et al.,* 2010). It would be interesting to investigate the tolerance of whole plants to freezing damage. Unfortunately, this was not within the capacity of our lab to investigate due to technical issues with LT chambers (temperatures varied up to 2.5°C), and therefore whole plant freezing tolerance could not be assessed.

Only a small portion of the temperature ranges and UV-B fluence rates that plants are exposed to in nature were investigated here. It is possible that UV-B modulates expression of these genes at a different range of temperatures or influences the expression of other cold-regulated genes which could result in altered capacity to cold acclimate or withstand freezing (Kilian *et al.,* 2007). It would therefore be interesting to investigate the transcriptome of plants exposed to LT and UV-B at a broader range of temperatures than those explored here. However, because no effect of UV-B on EL was observed, this was not pursued in this study.

The involvement of flavonoids in LT and UV-B crosstalk was investigated using two flavonoid mutants, *tt4* and *tt7* (*f'3h*). In this study, the *tt4* mutant had an increased electrolyte leakage in the WL condition at -2 and -5 °C (fig. 3.3a), suggesting the lack of flavonoids may contribute to reduced freezing tolerance, similar to previous reports (Schulz *et al*., 2016). However, Schulz *et al.* (2016) found that the *f'3h* mutant (*tt7*) had enhanced freezing tolerance, something that was not seen in the present study where the *tt7* mutation had no effect on electrolyte leakage at -5 °C (fig. 3.3b). It is possible that quercetin does not contribute to freezing tolerance or that it may act redundantly with other flavonols (Schulz *et al.,* 2016). Interestingly, when *tt4* were exposed to UV-B prior to freezing treatments, the level of electrolyte leakage was similar to that of the WT (fig. 3.3a). It is possible that while flavonoids are not being produced under UV-B in the *tt4* mutant, UV-B contributes in some other way to improve freezing tolerance in these plants, rescuing the phenotype.

The flavonoid mutant data together with the transcript abundance analysis of *CHS* suggest flavonoids are important for freezing tolerance and that flavonoids may be important in a UV-B response during freezing or cold acclimation. However, as no improved freezing tolerance was seen in the WT with increased *CHS* transcript abundance and increased flavonol abundance, it is possible that the additional accumulation of *CHS* during LT and UV-B may not reflect CHS protein levels (Fortelny *et al.*, 2017). It is also possible that additional CHS is superfluous and will not lead to further freezing tolerance, or that an increase in flavonoid accumulation contributes to protection outside of the temperature range investigated here. It would be interesting to investigate CHS protein accumulation as well as a wider range of temperatures. It would also be interesting to investigate whether the increase in *CHS* transcripts is transient or persists for a longer period of time, and therefore may contribute to long-term freezing tolerance, compared to the relatively short-term response investigated here.

Despite no positive (or negative) effects of UV-B on freezing tolerance in Arabidopsis, investigating any potential avenues which may contribute to plant protection in an ever-changing climate is important. It is possible that the protective effects of UV-B on LT stress seen in Rhododendron and wheat are species-specific. Plants are genetically incredibly diverse, resulting in a multitude of ways that different environmental signals can be integrated, and responses produced.

Many UV-B responses are gated by the circadian clock (Fehér *et al.*, 2011; Takeuchi *et al.,* 2014) meaning that a UV-B response may be fluctuating across the day (Horak and Farré, 2015). Therefore, it would be interesting to investigate such fluctuations in UV-B responses on the freezing tolerance response. Alternatively, it is possible that UV-B is not involved in freezing tolerance in Arabidopsis. Although environmental signalling pathways often share signalling components and display extensive crosstalk, some pathways may not affect one another to the extent where a phenotype or a noticeable effect exists. Because only a small portion of the LT response in Arabidopsis was investigated, it is not possible to confidently say that UV-B, and its signalling pathway components, are exclusively not involved in LT sensing, signalling, or responses.

Thermotolerance

From data published by Ulm *et al*. (2004) and Li *et al*. (2019), it was elucidated that some genes that are upregulated during UV-B exposure in Arabidopsis are also upregulated during high temperature exposure. Gene information was collected from the Arabidopsis Information Resource centre (TAIR, 2021) and five out of the 72 genes identified were considered to be involved in high temperature tolerance as they code for heat shock proteins, dehydration factors or heat shock transcription factors (fig. 3.5). This suggested some crosstalk between UV-B and high temperature signalling exists which may confer cross-protection to plants.

To investigate the effects of different UV-B treatments have on antioxidant capacity, plants were exposed to 4 and 16 h or 11 d UV-B. The 16 h treatment was also divided into 1 and 2.5 μmol m^{-2} s^{-1} UV-B to elucidate whether a short, high fluence UV-B could further enhance antioxidant capacity. Antioxidant capacity was increased by UV-B in all treatments (fig. 3.4). These data showed 4 h is sufficient to increase antioxidant capacity significantly compared to the control, however the greatest effect was seen after plants developed under UV-B for 11 d, suggesting there is a cumulative effect of UV-B on antioxidants (fig. 3.4). Despite the increase in antioxidant capacity, no positive effect of UV-B was observed on survival after heat shock in Arabidopsis seedlings (fig. 3.6b). Three different WT accessions were investigated for basal and acquired thermotolerance, to account for any genetic variation based on ecotype origin (fig. 3.6b). In 11-day-old seedlings of the Ws accession, UV-B may have a small negative effect on thermotolerance. Research has shown that reactive molecules such as reactive oxygen species are important signalling molecules (Suzuki and Mittler, 2005) and disrupting their signalling during heat shock by scavenging ROS could instead be detrimental to plant thermotolerance.

There was no significant difference in survival between the UVR8-deficient mutant *uvr8-6* and the WT controls after UV-B and heat shock treatments (fig. 3.8), suggesting that inhibited perception of UV-B via UVR8 signalling pathways does not impact thermotolerance in Arabidopsis seedlings.

While it has been shown that UV-B can enhance thermotolerance in different species (Caldwell, 1994; Murakami *et al.*, 2004) it is possible that this response is species-specific. Therefore, species of high agricultural importance should be investigated specifically for the effects of UV-B on thermotolerance.

In accordance with published reports (Hong and Vierling, 2000; Larkindale *et al*., 2005), heat acclimation was highly effective at increasing Arabidopsis thermotolerance in the conditions used here. 11-day-old seedlings were transferred to a 38°C heating bath prior to heat stress treatment at 45°C. The pre-treatment resulted in close to 100% survival in both the UV-B-treated and the no UV-B control groups (fig. 3.7c). Further trials to find a treatment where effects of UV-B could be distinguished from the controls were not successful due to time restrictions and the unpredictable nature of working with lethal stress treatments. In terms of sudden heat shock, basal thermotolerance is of greatest interest, however improving acclimation to consistently higher temperatures in a warming climate is also highly important.

PSII quantum yield of dark-adapted detached rosettes was measured after the same heat treatments as for the basal and acquired thermotolerance assays. High temperature stress reduced chlorophyll fluorescence drastically in both acclimated and non-acclimated plants, with a more negative effect on plants not acclimated to heat (fig. 3.9, 3.10). These data are indicative of major stress caused by the heat treatment and support reports that acclimation to heat results in lower heat damage. No ameliorating effect of UV-B was observed at any time point after heat shock, suggesting UV-B does not provide protection to the photosynthetic machinery, and does not promote recovery of PSII efficiency 1, 2 or 24 h post heat shock compared to non-irradiated controls.

Interestingly, high temperature had a strong positive effect on *CHS* transcript abundance (fig. 3.7a and b) which contrasts with the study by Kim *et al*. (2017) who showed anthocyanin biosynthesis was repressed when plants were transferred from 17 to 28°C. However, the accumulation of *CHS* may be dependent on temperature treatment as plants were sampled after heat acclimation treatment (38 °C) and heat stress treatment (45°C) in this study. These data suggest a role for *CHS* in the heat stress response of Arabidopsis. Heat and UV-B exposure combined had the greatest effect on *CHS*

accumulation, suggesting that UV-B could enhance any effects CHS may have on the high temperature response, though no such effect could not be seen here.

UV-B had no effect on WUE, wilting or maintenance of water during drought in this study, suggesting that effects of UV-B on these responses may be species-specific (fig. 3.11).

CHAPTER 4 INVESTIGATING THE EFFECTS OF UV-B ON RADISH LEAF DEVELOPMENT IN A VERTICAL AEROPONIC FARMING SYSTEM

4.1 Introduction

An ever-increasing population has heightened the pressure on food production and will continue to do so in the future. Additionally, increased urbanisation has heightened the pressure on food transportation and storage from farm to consumer (Benke and Tomkins, 2017). With the potential to at least partially solve the issues to do with space and long transportation of food, vertical farming has become an increasingly popular method of locally growing quality produce in small indoor areas. Vertical farming constitutes growing produce in a stacked system often supplied with artificial light supplied from light bulbs, or part natural, part supplemented light (Eldridge *et al.*, 2020). Benefits of indoor vertical farming

include the potential to control growth environments, pests and maintain a steady production (Benke and Tomkins, 2017). Vertical farming often employs one of two irrigation methods: hydroponics or aeroponics. The work in this chapter was carried out during an CASE placement at the LettUs Grow vertical farm in St. Anne's, Bristol between September 2021 and December 2021. The LettUs Grow farm uses their own patented system of aeroponic irrigation under the control of their Ostara® software. Aeroponics is a soil-free system that utilises technology which atomizes water enriched with nutrients into minute aerosol droplets. This produces a mist which supplies the roots of the crops with necessary water and nutrients for growth (Lakhiar *et al.*, 2018; Eldridge *et al.*, 2020). Development of crop recipes optimized for specific crops in aeroponic systems is very important if this method of farming is to reach a level of efficiency to supplement or even replace conventional farming effectively. The application of UV-B in commercial growing facilities is a growing area of research. For example, Fraser *et al.* (2017) found that supplementing coriander plants with UV-B in dense canopies could reduce shade avoidance responses making plants more compact, while Sakalauskaitė *et al.* (2012) found that UV-B increased both antioxidants, flavonols and had a positive effect on growth parameters in Sweet Basil. To our knowledge, UV-B is virtually unexplored in the setting of indoor vertical aeroponic systems. An indoor farm with supplemental white light naturally excludes UV-B light as common commercial grow lights do not emit UV-B light. The reasons for not supplementing with UV-B are likely two-fold: growers are not aware of the subtle effects UV-B may have on crops, and secondly: UV-B is a health hazard to humans and farm staff would need to wear protective personal equipment to cover skin and protect the eyes, making work in a farm somewhat more cumbersome. In addition to this, the currently available UV-B light tubes are costly, although recent progress in UV-B LED development may mean affordable UV-B supplementation could be available in the near future (Kneissl *et al.*, 2019; Kahn *et al.*, 2022).

The aims for this chapter were to gain an understanding of the responses of Radish Leaf to UV-B in an aeroponic setting and evaluating these in context of the practicality of giving a UV-B treatment in a vertical farm setting. This work would then help to form a recommendation as to whether the UV-B treatments used would be a valuable addition to the crop recipe for Radish Leaf micro herbs.

4.2 Specific methods

This trial was conducted in one aeroponic grow bed in chamber R2 at the LettUs Grow site. A UV-B tube was attached underneath the above grow bed and Perspex sheets were used to block UV-B from half of the grow bed establishing a control treatment and provide personal protection to anyone attending the chamber (fig. 4.1).
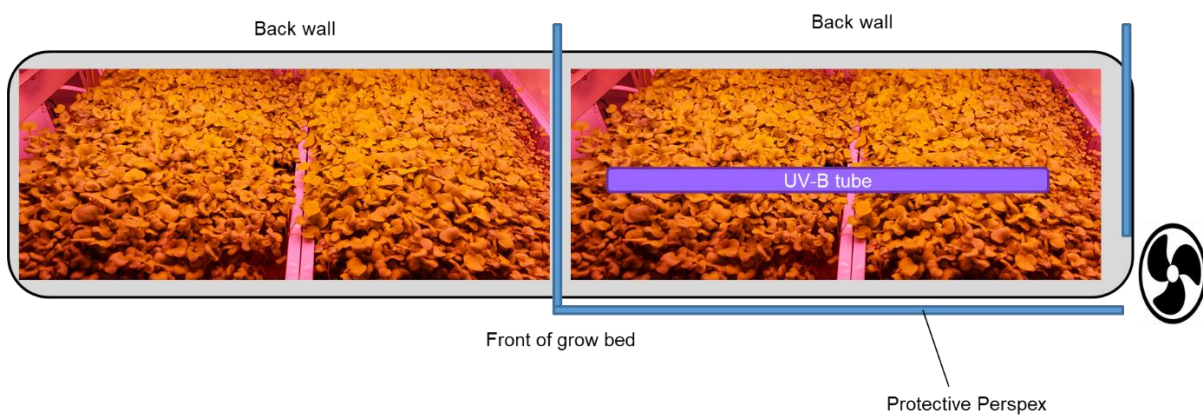
Figure 4.1 Grow bed set-up in the LettUs Grow stacked system. Perspex was attached to three sides of the grow bed and a fan was fitted to allow better air circulation to the UV-B irradiated plants. Two trays of Radish leaf were placed in each treatment per replicate.

Crop selection and growth cycle

Radish Leaf *var*. Sangria was selected for these trials as they are a microherb and grow fast, ensuring sufficient replicates could be collected within the 10 weeks for meaningful data analysis. Depending on timings, seeds were germinated for either three or four days and spent either four or five days on the grow bed before harvest. Radish Leaf were grown on a 16 h photocycle. Radish Leaf seeds were sown with a density of 45g/tray on jute BlueMat400 (HollandBioProducts, Nijmegen, The Netherlands) which were put flat in perforated confectionery trays. Mats were saturated with water prior to sowing, and seeds were misted immediately post-sowing to ensure imbibition for germination.

Environmental conditions

Light

All UV-B treatments were supplied by PHILIPS TL 20W/01 - RS narrowband UV-B tubes at a fluence rate of 1 μmol m^{-2} s^{-1}. The fluence rate was achieved by application of polyamide tape at regular intervals across the bulb. 1 μmol m^{-2} s^{-1} of UV-B has been shown to be sufficient to cause changes in growth, development and phenolic and antioxidant compound accumulation in many plants, and typically in the model plant *Arabidopsis thaliana* in which much UV-B research has been done (Jenkins, 2014). The UV-B tube used 18.7 W of power. To cover a whole bed with the same treatment given here, two tubes would be required meaning that 37.4 W of power would be used. All light was measured using an Ocean Optics FLAME spectroradiometer with OceanView software (version 2.0.8) and spectra were recorded for each treatment. Additionally, PAR and UV-B measurements were collected from eight places in the grow bed per treatment (total 16 places across the whole bed). Figure 4.2 displays the total irradiance for the two treatments as well as the distribution of the PAR and UV-B across the grow bed. Because inconsistency existed in the light levels across the beds, trays were rotated once daily, and where plants were used for individual measurements, such as hypocotyl length, these were collected from the middle of the grow beds. For total biomass and root mass, all tissue from the whole tray was collected.

Humidity and temperature

Humidity and air temperature for the growing period of the Radish Leaf UV-B trials are displayed in figure 4.3. On the 1st of October, there was a major issue with the HVAC system controlling the conditions in R2. This caused both the relative air humidity and the air temperature to fluctuate (fig. 4.3). No plants were kept on the grow beds at this time, so the experiment will not have been impacted by this issue.
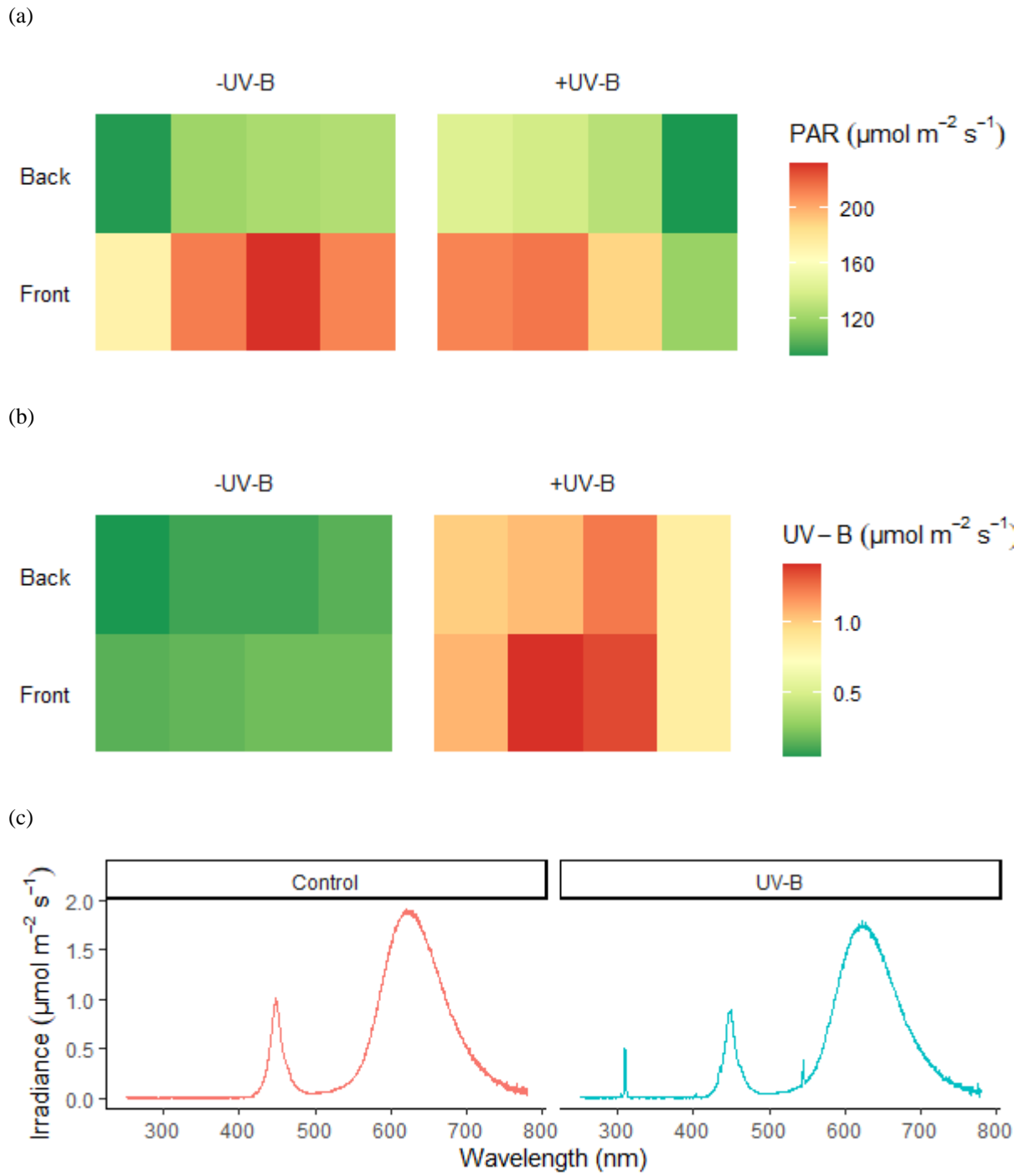
Figure 4.2 Light conditions across the grow bed. (a) PAR distribution across the grow bed. (b) UV-B distribution across the grow bed. (c) Total spectral irradiance for the control and UV-B treated areas of the grow bed.

Biomass yield

Foliage and root tissue was collected with scissors before foliage and root mass weights per tray were recorded.

Specific UV-B treatments

Two UV-B treatments were selected to analyse the effects of UV-B on growth characteristics, pigment production and antioxidant capacity.

- Daily (short, long-term) UV-B treatment

A daily UV-B treatment was applied at the photocycle noon as this would simulate when UV-B is naturally at its highest level in nature. This treatment was hypothesised to cause an accumulation of responses over the growth period. A short but long-term UV-B treatment may be desirable as this makes the chamber less suitable for work for only three hours per day during which it may be possible for personnel to not attend the farm to minimize UV-B exposure. It may also be possible that this treatment could cause effects predominantly on growth and development as it is applied from as early as four days after sowing (das). This treatment was applied to three batches of Radish Leaf, which were germinated for four days and then transferred to the aeroponic grow bed for a further four days before harvest.

- End of production (EoP, long, short-term) UV-B treatment

An EoP UV-B treatment was applied for the last two growing days for the duration of the white light (16 h per day) to investigate the effects of a long but short-term treatment of UV-B. This would allow the Radish cotyledons to develop fully before the UV-B was applied. This was hypothesized to have little effect on growth and development and possibly a greater effect on pigment and antioxidant accumulation. This treatment would perhaps be preferable to a daily UV-B treatment as it could be timed to a weekend or a time when the farm is less busy. This treatment was applied to three batches of Radish Leaf, which were germinated for three days and then transferred to the aeroponic grow bed for a further five days before harvest.
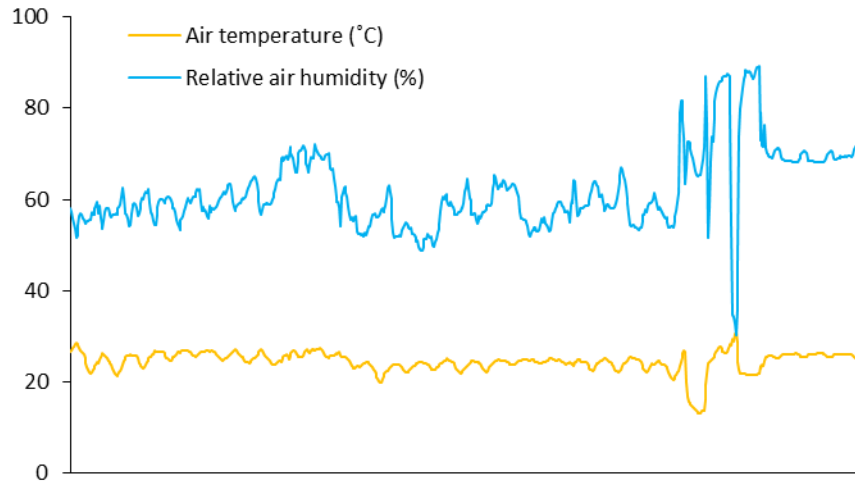
Figure 4.3 Humidity and air temperature in the LettUs Grow R2 chamber between the 9 October 2021 and 9 November 2021. The parameters were measured using the sensors in the LettUs Grow growth chambers, and processed via Ostara®.
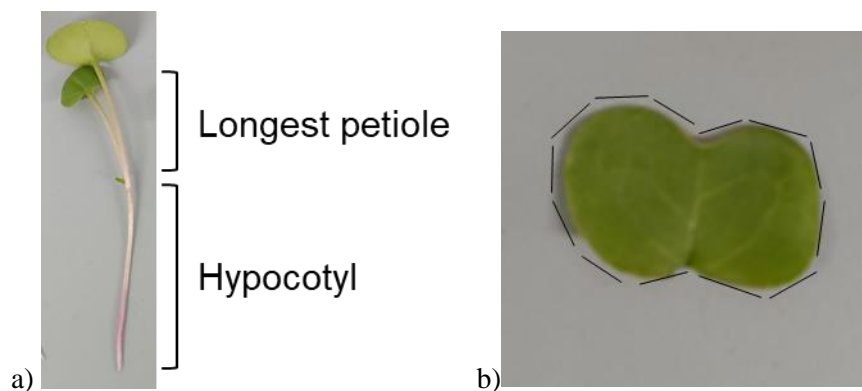
Figure 4.4 Parts measured for hypocotyl, petiole length and leaf area analysis. Plants were cut just above the roots and laid as flat as possible on a tray and photographed. **a)** Hypocotyl and petiole areas measured. **b)** The largest leaf of each cotyledon pair was cut and photographed. Black lines indicate leaf area measured. Hypocotyl and petiole lengths and leaf area were analysed using ImageJ.

Morphology-based characteristics

Hypocotyl and petiole lengths and leaf area (fig. 4.4) were captured from 30 plants per treatment and batch (total=90) using a Samsung A71 (64 MP PDAF) and analysed using ImageJ (https://imagej.nih.gov/ij/). Leaf weight was also recorded for the leaves whose areas were measured.

Compactness has been used as a measure of how compact plant architecture is after UV-B treatment. In coriander, UV-B increased compactness significantly (Fraser *et al.,* 2017). Compactness can affect the attractiveness of crops to consumers, and the potential of the plant to remain upright and not bend. Compactness can be calculated using the following formula:

(1)   Compactness = leaf area (mm²) / petiole length (mm)   (Fraser *et al*., 2019)

4.3 Results

Daily (short, long-term) UV-B treatment

Morphological characteristics

The effects of UV-B on Radish Leaf grown in an aeroponic system have not been extensively investigated. To achieve important improvements in pigment accumulation and antioxidant capacity, it is equally important not to reduce yield and affect plant morphology excessively. It is possible that UV-B could have a positive effect on growth parameters as in Sweet Basil, UV-B increased leaf area, fresh and dry biomass (Sakalauskaitė *et al.*, 2012). To investigate this, several important morphological traits as well as total yield was investigated in UV-B-treated Radish Leaf. Hypocotyl and petiole length, perhaps the two most crucial aspects of plant growth were not affected by the UV-B treatment (fig. 4.5a and b). However, leaf morphology appears to be more susceptible to UV-B irradiation as both the leaf area and the leaf weight differed between the UV-B treated plants and the control (F=4.576, $p<0.05$, one-way ANOVA, fig. 4.5c, and F=5.966, $p<0.05$, one-way ANOVA, fig. 4.5d). UV-B appeared to make plants somewhat more compact (fig. 4.6) however this was not a significant effect.

Yield

For a commercial grower, yield is perhaps the final decider for whether a treatment is useful or not. Due to the small sample size investigated here, only tentative weight can be put on the yield figures displayed. The control treatment appeared to have a wider spread of data suggesting quite substantial variability in the growth of Radish in the grow bed (fig. 4.7). For the UV-B treatment, the data was less varied and while neither foliage, nor root mass were significantly different from the control treatment, it appears that half of the data points for UV-B-treated trays lie over ~770 g per tray, while the number for the non-irradiated trays fall at around ~ 730 g (fig. 4.7a). Similarly, the root mass was slightly higher in the UV-B treatment, which may have important implications for nutrient and water uptake in the aeroponic system (fig. 4.7b).

Pigment analysis and antioxidant capacity

The importance of compounds such as flavonoids and various other antioxidants to human health has been the focus of much research (Lu *et al*., 2013; Chen *et al*., 2013). Leaf chlorophyll content did not vary between the control and the UV-B treatment (fig. 4.8a). In contrast, anthocyanin was marginally reduced in the UV-B-treated plants (p=0.029, Wilcoxon signed-rank test, fig. 4.8b), although it is worth pointing out that the spread in the data is very great, suggesting further replication is needed to establish such a relationship. The abundance of flavonols was significantly greater in the UV-B-irradiated plants compared to the control (p<0.001, Wilcoxon signed-rank test, fig. 4.8c). Interestingly, antioxidant capacity was not improved by the UV-B treatment (fig. 4.8d). There is great variation in this data, suggesting the treatment may not have been applied uniformly.

Water content analysis

Water preservation and optimizing the water use efficiency of vertical farming is important and many avenues should be investigated. To investigate whether UV-B increased water content of specific plants, fresh and dry weights were recorded and water weights calculated. There was no statistically significant effect of UV-B on fresh, dry or water weights of the sampled plants (fig. 4.9). To establish whether there is no true effect of UV-B on water content, a greater sample size, or bags of crop should be harvested, weighed, and dried to investigate this on a larger scale.

Figure 4.5 Morphological effects of the daily (short, long-term) UV-B treatment on Radish Leaf. a) Hypocotyl length. **b)** Petiole length. **c)** Leaf area. **d)** Leaf weight. Radish Leaf were grown in a 16 h photoperiod and exposed to 3 h UV-B (1 μmol m^{-2} s^{-1}) daily for 4 d. n=90 from 30 plants per treatment over three independent experiments. Plots show the median and the interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.

Figure 4.6 The effects of a daily (short, long-term) UV-B treatment on compactness of Radish Leaf. Radish Leaf were grown in a 16 h photoperiod and exposed to 3 h UV-B (1 µmol m⁻² s⁻¹) daily for 4 d. n=90 from 30 plants per treatment over three independent experiments. Plot shows the median and interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. Data statistically analysed by one-way ANOVA. No statistically significant difference was found.

Figure 4.7 The effects of a daily (short, long-term) UV-B treatment on Radish Leaf yield. a) Foliage biomass. **b)** Root biomass. Radish Leaf were grown in a 16 h photoperiod and exposed to 3 h UV-B (1 µmol m^{-2} s^{-1}) daily for 4 d. n=6 from 2 trays per treatment over three independent experiments. Plots show the median and interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.

Figure 4.8 The effects of a daily (short, long-term) UV-B treatment on pigment accumulation and antioxidant capacity in Radish Leaf. a) Chlorophyll abundance. **b)** Anthocyanin index. **c)** Flavonol index. **d)** Antioxidant capacity. Radish Leaf were grown in a 16 h photoperiod and exposed to 3 h UV-B (1 μmol m^{-2} s^{-1}) daily for 4 d. n=90 from 30 plants per treatment over three independent experiments for a), b) and c). n=12 from four plants per treatment over three independent experiments for d). Bars represent the means with error bars representing the SE. Data statistically analysed using one-way ANOVA, asterisks indicate significant differences from the control at $p < 0.05$.

Figure 4.9 The effects of a daily (short, long-term) UV-B treatment on water content of Radish Leaf. a) Fresh weight. **b)** Dry weight. **c)** Absolute water weight. **d)** Percentage water weight. Radish Leaf were grown in a 16 h photoperiod and exposed to 3 h UV-B (1 µmol m^{-2} s^{-1}) daily for 4 d. n=24 from 8 plants per treatment over three independent experiments. Bars represent the means with error bars representing the SE. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.

EoP (long, short-term) UV-B treatment

An EoP UV-B treatment was investigated as a contrast to the 3 h daily UV-B treatment to understand the differences between a short long-term treatment and a long short-term treatment and their differing effects on Radish Leaf. As mentioned, it was hypothesised that the daily (short, long-term) UV-B treatment has more effect on developmental traits, while the EoP (long, short-term) UV-B treatment could possibly provide a rapid boost to pigment and antioxidant accumulation.

Morphological characteristics

For the EoP treatment, none of the morphological characteristics were significantly altered by UV-B (fig. 4.10 and fig. 4.11) suggesting an EoP UV-B has little effect on morphology in Radish Leaf.

Yield

The lack of effects on morphological characteristics is supported by the finding that foliage mass and root mass did not change dependent on the UV-B treatment (fig. 4.12).

Pigment analysis and antioxidant capacity

Both chlorophyll (F=14.17, $p<0.05$, one-way ANOVA) and flavonol (Kruskal-Wallis X2=76.223, $p<0.05$) content were found to be increased after the EoP UV-B treatment compared to the control (fig. 4.13a and 4.13c), however anthocyanin (fig. 4.13b) was somewhat reduced (F=18.97, $p<0.05$, one-way ANOVA). This is interesting as the total antioxidant capacity was found to be increased by the UV-B treatment (F=5.016, $p<0.05$, one-way ANOVA, fig. 4.13d), suggesting that Radish Leaf may rely more on other antioxidants than anthocyanins for UV-B protection. As anthocyanins and flavonols are produced in the same biosynthetic pathway, more resources may be directed towards production in favour of other flavonoids as opposed to anthocyanins.

Water content analysis

Eight plants per treatment and batch were selected for water content analysis. This is a very small sample size and would likely benefit from a large-scale water content analysis of bags of harvested Radish leaf instead of individual plants. This kind of analysis was not performed here due to the lack of space. As can be seen in figure 4.14, fresh weight, dry weight or water weight did not vary between the treatment, suggesting at this very small scale, UV-B does not affect water content of Radish leaf.

Stomatal movement in Radish Leaf exposed to high temperatures

Stomata govern gas exchange between plants and the atmosphere, including transpiration. In the LettUs Grow grow bed system, water use is a very important factor. Here it was investigated whether stomata open significantly in increased temperatures, as increasing temperatures may be beneficial to increased plant growth. Stomata of Radish Leaf opened similarly to Arabidopsis observed by Kostaki *et al*., (2020) where a 30 °C treatment had no effect on aperture, but increasing the temperature to 35 °C saw a significant opening response. This is an important factor for growers to be aware of as an increase in temperature may lead to an increase in water use in Radish Leaf.
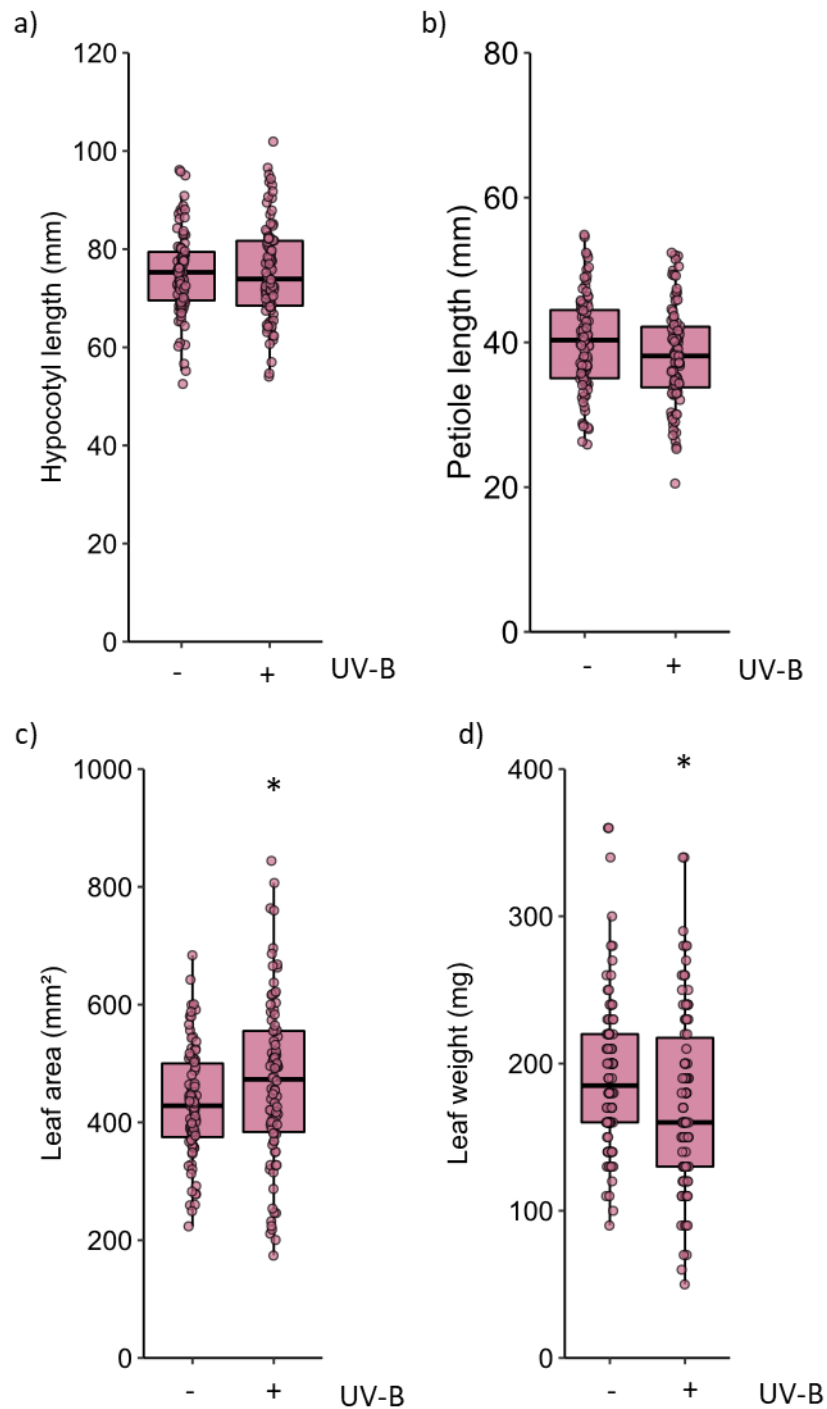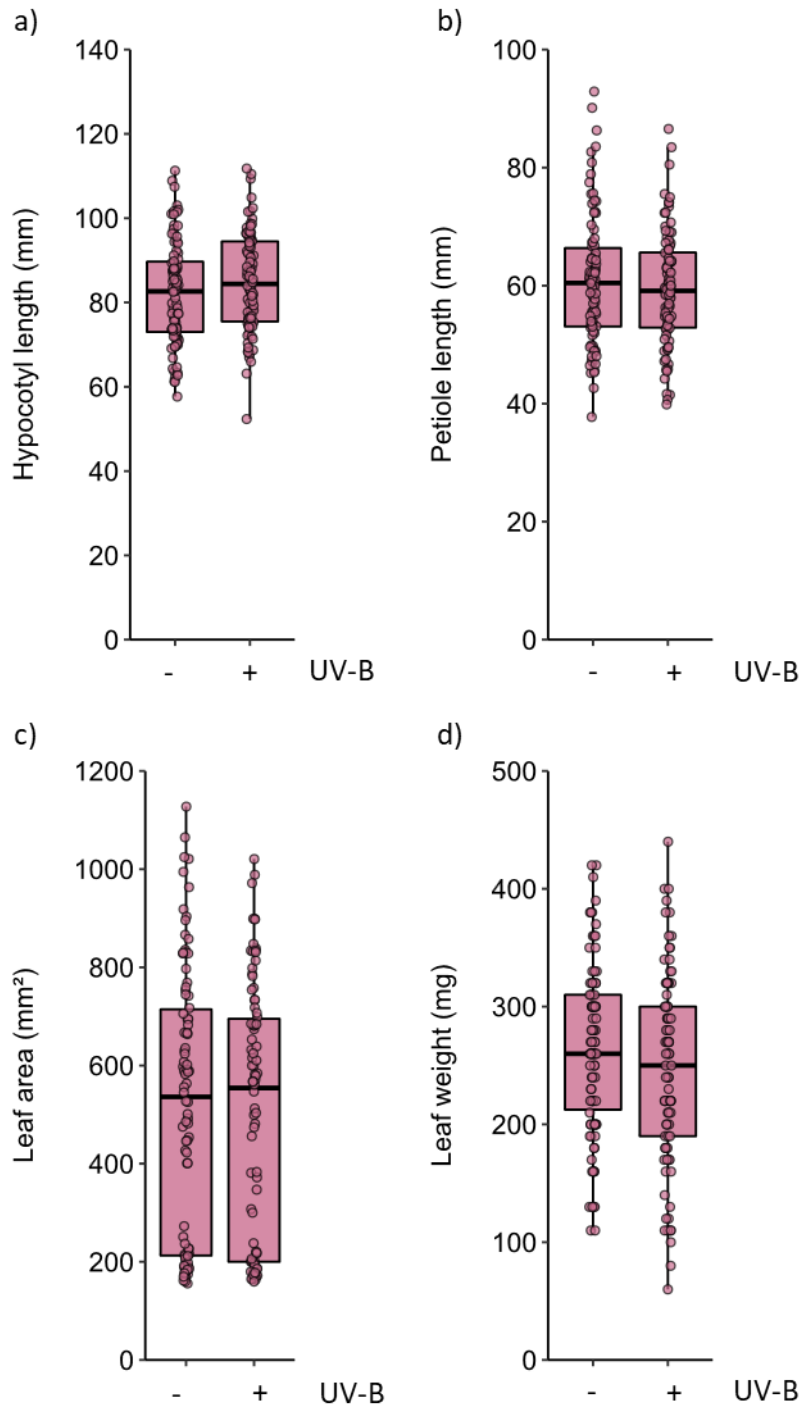
Figure 4.10 Morphological effects of an EoP (long, short-term) UV-B treatment on Radish Leaf. a) Hypocotyl length. **b)** Petiole length. **c)** Leaf area. **d)** Leaf weight. Radish Leaf were grown in a 16 h photoperiod and exposed to UV-B (1 µmol m^{-2} s^{-1}) for the last two growing days. n=90 from 30 plants per treatment over three independent experiments. Plots show the median and the interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.
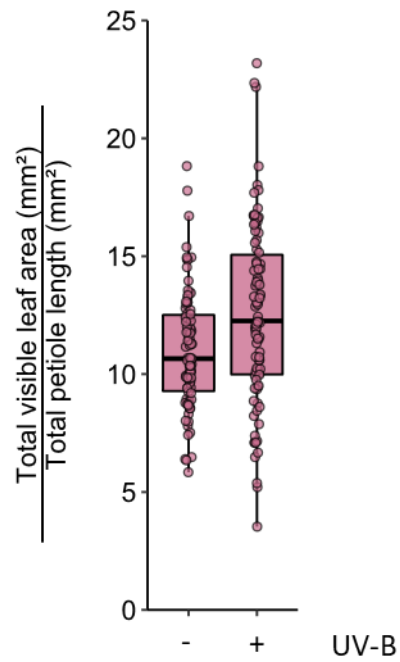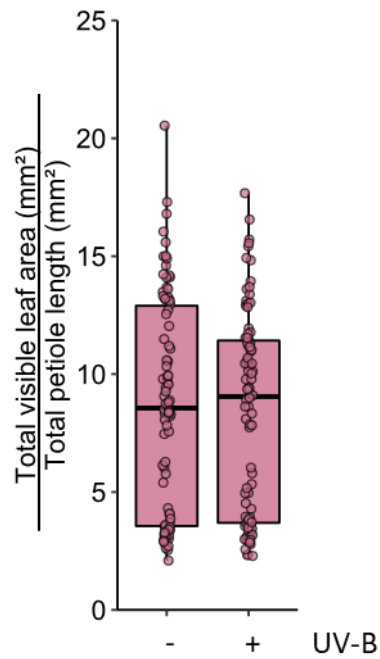
Figure 4.11 The effects of an EoP (long, short-term) UV-B treatment on compactness of Radish Leaf. Radish Leaf were grown in a 16 h photoperiod and exposed to UV-B (1 µmol m⁻² s⁻¹) for the last two growing days. n=90 from 30 plants per treatment over three independent experiments. Plot shows the median and interquartile range of each group. The upper and lower whiskers represent data within 1.5 * the interquartile range. All data values are represented by points. Data statistically analysed by one-way ANOVA. No statistically significant difference was found.
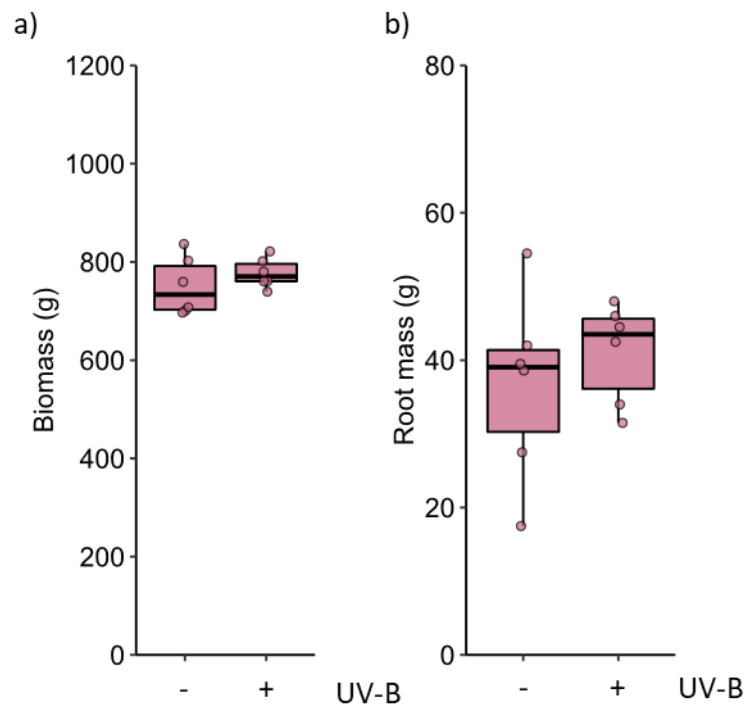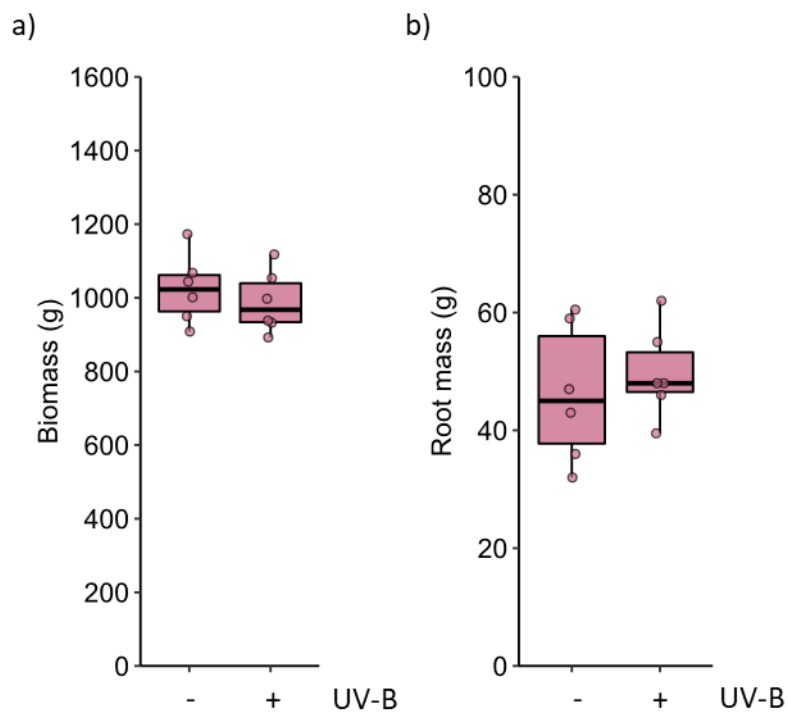
Figure 4.12 The effects of an EoP (long, short-term) UV-B treatment on Radish Leaf yield. a) Foliage biomass. **b)** Root biomass. Radish Leaf were grown in a 16 h photoperiod and exposed to UV-B (1 μmol m⁻² s⁻¹) for the last two growing days. n=6 from 2 trays per treatment over three independent experiments. Plots show the median and interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.
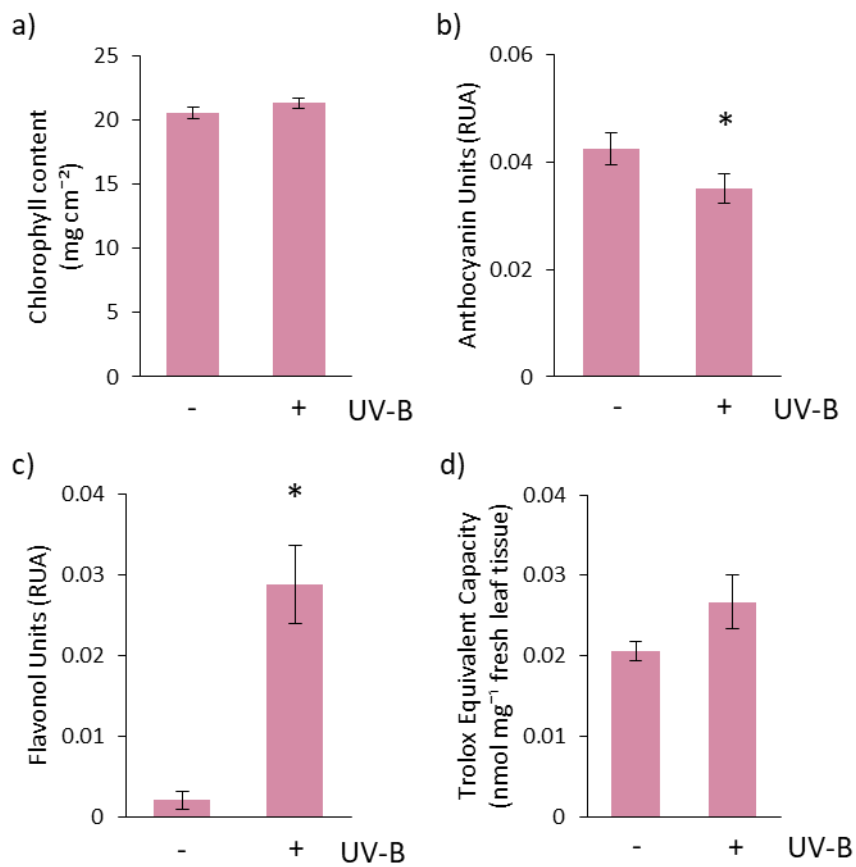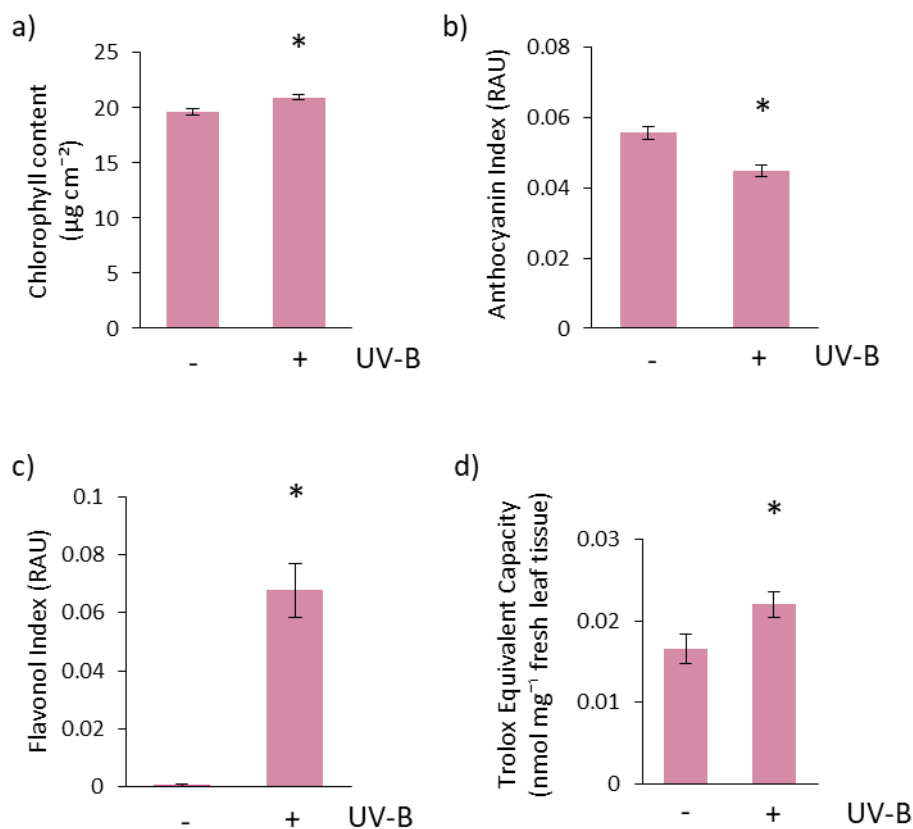
Figure 4.13 The effects of an EoP (long, short-term) UV-B treatment on pigment accumulation and antioxidant capacity in Radish Leaf. a) Chlorophyll abundance. **b)** Anthocyanin index. **c)** Flavonol index. **d)** Antioxidant capacity. Radish Leaf were grown in a 16 h photoperiod and exposed to UV-B (1 µmol m^{-2} s^{-1}) for the last two growing days. n=90 from 30 plants per treatment over three independent experiments for a), b) and c). n=12 from four plants per treatment over three independent experiments for d). Bars represent the means with error bars representing the SE. Data statistically analysed using one-way ANOVA, asterisks indicate significant differences from the control at $p < 0.05$.
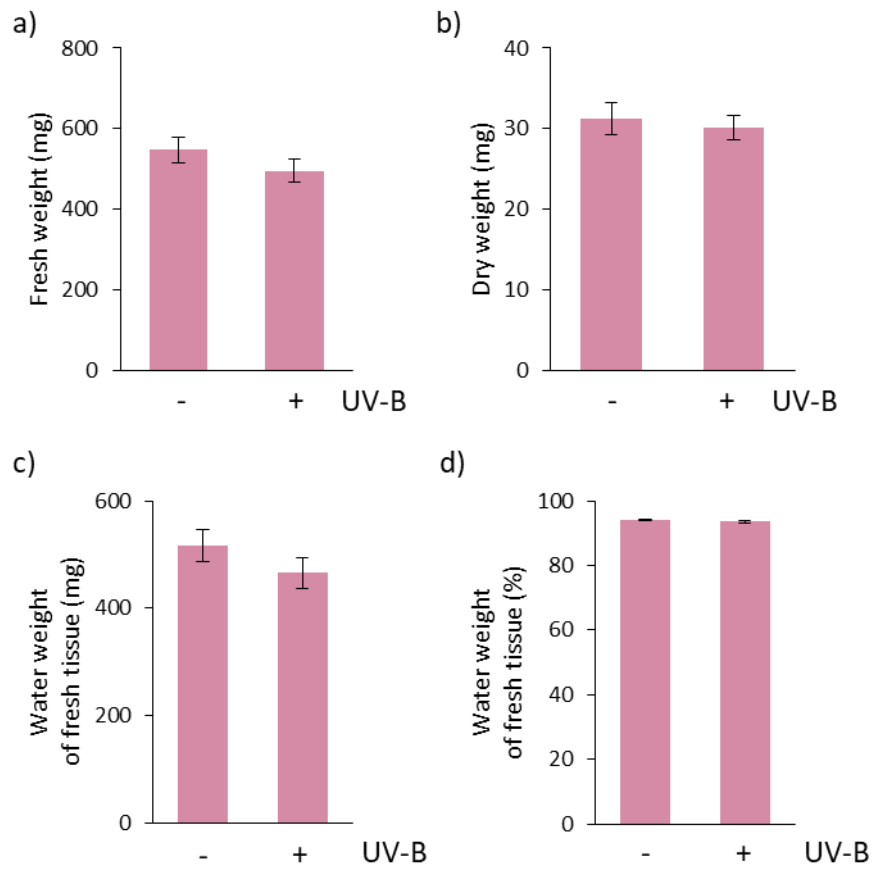
Figure 4.14 The effects of an EoP (long, short-term) UV-B treatment on water content of Radish Leaf. a) Fresh weight. **b)** Dry weight. **c)** Absolute water weight. **d)** Percentage water weight. Radish Leaf were grown in a 16 h photoperiod and exposed to UV-B (1 µmol m⁻² s⁻¹) for the last two growing days. n=24 from 8 plants per treatment over three independent experiments. Bars represent the means with error bars representing the SE. Data statistically analysed using one-way ANOVA. No statistically significant differences were found.
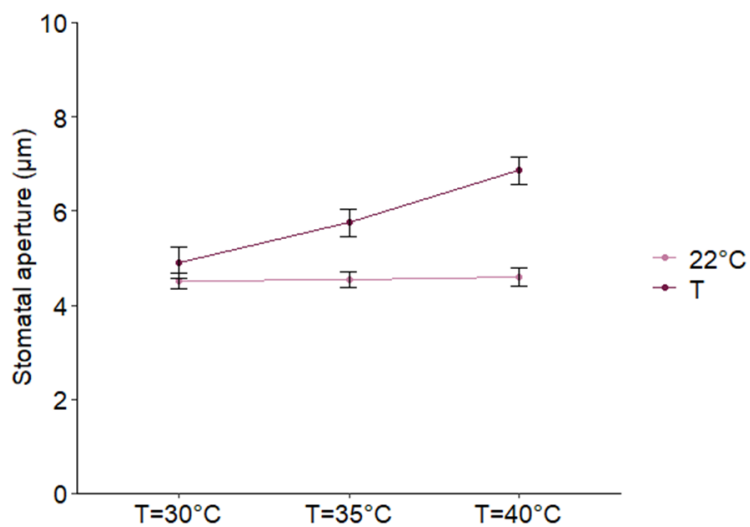
Figure 4.15 Stomatal aperture after exposure of Radish Leaf to high temperature. Leaf discs from Radish Leaf grown in a 16 h photoperiod for 4 d were placed in pre-heated 10/50 (10mM MES, 50mM KCl) buffer and allowed to acclimate at 22°C for 2h before transfer to fresh 10/50 and 30, 35 or 40°C. Stomatal aperture was measured after 2h. n=9, points represent means with error bars representing SE. Data statistically analysed using a one-way ANOVA and Tukey post hoc. Stomatal apertures were significantly more open in the 35°C and 40°C treatments compared to the 20°C control.

4.4 Discussion

The effects of two different UV-B treatments, EoP (long, short-term treatment) and daily (short, long-term treatment) treatments, on Radish Leaf morphology, yield, pigment accumulation, antioxidant capacity and water content were investigated.

The data suggest that the EoP treatment had lesser effects on plant morphology and yield, compared to the daily (short, long-term) UV-B treatment (fig. 4.5 - 4.7; fig. 4.10 – 4.12). This may be due to the fact that the EoP treatment was applied at the end of the growth period as opposed to throughout the developmental stage. Specifically, the daily treatment was found to affect leaf size and leaf weigh, while the EoP treatment did not (4.5c and d; 4.10c and d).

Similar to the response in sweet Basil (Sakalauskaitė *et al.,* 2013), leaf size in Radish increased after a 3 h daily UV-B treatment (fig. 4.5c). This contrasts with other crops, such as maize, lettuce and soybean where UV-B inhibited leaf growth as a result of reduced cell proliferation (Fina et al., 2017; Wargent *et al.*, 2009; Prasad *et al.*, 2005). However, a 3 h daily morning UV-B treatment increased leaf thickness of Basil leaves (Chang *et al.*, 2009). While leaf thickness was not specifically measured here, it can be inferred from the increased leaf area and reduced leaf weight from the daily UV-B treatment that overall thickness was reduced rather than increased in Radish Leaf (fig. 4.5c and d). Perhaps expectedly, the study in maize also found that UV-B reduced fresh and dry mass which was not seen in Radish (Fina *et al.,* 2017). In fact, though not statistically significant, UV-B appeared to increase both foliage biomass and root mass in the daily treatment (fig. 4.7). In transplanted lettuce plants, yield was higher after the UV-B treatment pre-transplantation (Wargent *et al.*, 2011). It is possible that due to the relatively low replicates and somewhat uneven UV-B distribution the effects on leaf area seen here could be an artefact of the experimental setup and further replicates under a more uniform UV-B treatment should be collected to confirm these data.

UV-B increases antioxidant capacity in several species of plant (Fraser *et al.*, 2017; Rao *et al.*, 1996; Prasad *et al.,* 2005). While the daily (short, long-term) UV-B treatment appeared to have produced higher levels of antioxidant as evidenced by the higher antioxidant capacity (fig. 4.8d), the difference

between the control and the UV-B treatment was only significant in the EoP treatment (fig. 4.13d). It is possible that the lack of significant differences between the control and the daily (short, long-term) UV-B treatment was an effect of UV-B dispersal as when the UV-B light is on for longer, as in the EoP treatment, even the plants towards the edges get a sufficient dose of UV-B to trigger antioxidant production. It is also possible that a 3 h treatment is insufficient to accumulate significantly higher antioxidant levels compared to the control, and other UV-B regimes should be investigated. For example, a 3 h treatment of a higher fluence rate, or a longer daily treatment are two possible alternatives. While the increase in antioxidant capacity was statistically significant, it is impossible to infer whether this increase is nutritionally significant. It is also possible that a UV-B treatment would be beneficial to plants that are grown in indoor nurseries and then transplanted outdoors, as plant such as lettuce have been shown to outperform no-UV-B control plants after transplantation (Wargent *et al.,* 2011).

Flavonol content appears to be more responsive to UV-B irradiation than total antioxidant capacity in Radish Leaf (fig. 4.8c and fig. 4.13c). In addition to being nutritionally important as part of the antioxidant system, flavonols could also alter flavour, suggesting that this may be an important variable to consider when producing commercially attractive crops. Flavonols have also been shown to be involved in responses such as freezing tolerance in Arabidopsis (*Schulz et al.*, 2016) and may also play other important roles in plants in addition to UV-B protection.

There were no significant effects of either UV-B treatments on water content in Radish Leaf plants (fig. 4.9 and fig. 4.14). This may be due to the difference being so small in individual plants that it is overlooked. To investigate this further, a larger sample size or collections of plants such as when bunched for packaging should be used.

It is well-known that high temperature exposure leads to open stomata in Arabidopsis (Kostaki *et al.,* 2020). In a system that is heavily reliant on irrigation such as aeroponics, water use is of vital importance. While many plants could be grown at higher temperatures to accelerate growth, this could also mean excessive water loss due to more open stomata. To establish the effects of elevated temperature on stomatal responses in Radish Leaf, stomatal apertures were measure after 30, 35 and

40°C. While 35 and 40 ˚C significantly opened stomata, 30 ˚C did not (fig. 4.15). This suggests that Radish Leaf could, similarly to Arabidopsis be grown in temperatures of up to at least 30°C without any significant effect on stomatal opening. High fluence UV-B has been shown to close stomata in mature Arabidopsis plants (Tossi *et al*., 2014). Unfortunately, due to space constraints, it was not possible to investigate the effects of elevated temperature and UV-B during different irrigation recipes. This would be interesting to look at as even a small effect on stomatal apertures could have a cumulative effect when affecting many plants.

In order for a UV-B treatment to be viable, costs of purchasing UV-B lighting and running costs must be minimised. The UV-B tube used in this experiment used 18.7 W of power. To cover a whole bed with the same treatment given here, two tubes would be required meaning that 37.4 W of power would be used. If a more uniform UV-B irradiation is to be achieved, multiple tubes would have to be installed (possibly four short tubes, or two long tubes in the LettUs Grow grow beds). This would increase the power usage unless tubes which are less powerful could be used. This could be a viable solution as much of the UV-B emitted by the tube used for this experiment was blocked by the polyamide tape meaning that the full output was not used. In contrast, the lights currently used in the LettUs Grow beds (Valoya) use 35W each (covering a whole bed). Typically, three Valoya bulbs are used per bed, equalling 105 W per bed. Unfortunately, UV-B tubes are still costly to purchase, however technology is moving rapidly forward, and it is possible that UV-B LEDs could be cheaper in the near future. Though positive effects of UV-B were seen on flavonol and antioxidant accumulation in Radish Leaf, a full evaluation of the effects of UV-B on multiple crops should be carried out in order to establish whether a UV-B treatment is a viable way to improve crop quality in the LettUs Grow aeroponic system. The sample size used for this study was relatively small. Taken together with the fact that environmental conditions in an aeroponic farm are less precise than those that can be achieved in a controlled climate growth chamber in a laboratory, a much greater sample size should be used in order to fully establish the effects of UV-B on crops grown in an indoor, vertical, aeroponic farm.

Conclusions

In this study, it was found that the EoP treatment had the greatest positive effect on pigment accumulation and antioxidant capacity without compromising yield or plant morphology, likely due to its application at the end of the growth period. The EoP treatment would therefore be the better treatment to improve these traits. Only two treatments were investigated here, and it is therefore possible that other UV-B treatments may have greater positive effects. UV-B treatments in combination with different irrigation schemes would be a good next step to investigate the effects of UV-B on water use in Radish Leaf.

CHAPTER 5 High temperature-mediated regulation of stomatal aperture in Arabidopsis seedlings

5.1 Introduction

An increase in the global temperature and increased frequency of heat waves are predicted within this century (Collins *et al*., 2013; Perkins-Kirkpatrick and Lewis, 2020). Stomata govern gas exchange and water status plants in response to the environment, making them closely interlinked with responses to a changing climate as well as more immediate responses to fluctuations in temperature. Understanding stomatal responses to high temperature will be very important for future manipulation of plant productivity and food production in order to feed a growing global population in the face of many climate uncertainties (Stevens *et al*., 2021). In response to high temperature, leaf transpiration is increased in order to increase evaporative cooling (Crawford *et al*., 2012; Bridge *et al*., 2013). It has long been known that high temperature (~30-45°C) promote stomatal opening in *Vicia faba* and *Zea*

mas L. (Rogers *et al*., 1979, Rogers *et al*., 1980; Rodriguez and Davies, 1982). More recent evidence suggests that this effect exists also in other species (Urban *et al*., 2017; Urban *et al*., 2017). In contrast, in subtropical tree species, stomatal conductance was decreased at high temperature, suggesting differential responses to elevated temperatures exist across the plant kingdom (Wu *et al*., 2018). In 2020, Kostaki *et al*. showed that a similar opening response exists in *Arabidopsis thaliana* and that this response requires the plasma membrane H^+ ATPase as well as full function of several 14-3-3 proteins. These components are also intrinsic to light-mediated stomatal opening (discussed in chapter 1 and 6). Blue light photoreceptors have been shown to mediate stomatal opening in response to blue light (Kinoshita *et al*., 2001; Mao *et al*., 2005; Shimazaki *et al*., 2007). Interestingly, high temperature-mediated stomatal opening was highly reduced in the *phot1-2/5-1* mutant and the *phot* mutant showed lower evapotranspiration and higher leaf temperature, suggesting the stomatal response is reliant on blue light signalling (Kostaki *et al*., 2020).

PIFs are a major hub for light and temperature signalling (Quint *et al*., 2016, Zhao and Bao, 2021). In particular, PIF4 and PIF7 have been shown to regulate thermomorphogenesis responses (discussed in chapter 1 and 3; Koini *et al*., 2009; Franklin *et al*., 2011; Lee *et al*., 2021; Fiorucci *et al*., 2020; Burko *et al*., 2022). While some reports show the involvement of PIFs in stomatal development (Casson *et al*., 2009; Lau *et al*., 2019), the role of PIFs in stomatal movement in Arabidopisis is not fully understood. Mutants deficient in both PIF3 and PIF4 show significantly greater stomatal apertures than the WT control under light conditions (Wang *et al*., 2010). Furthermore, the PIF family gene *OsPIL15* negatively regulates stomatal apertures in *Oryza sativa* (Li *et al*., 2022). While no difference in high temperature responses of stomata were seen in a mature Arabidopsis *pif4* mutant, suggesting PIF4 is not involved in stomatal responses to high temperature (Kostaki *et al*., 2020), this response has not been explored in seedlings.

Aims

The aims of this chapter were to establish the response of Arabidopsis cotyledon stomata to elevated temperature and investigate the involvement of PIFs in this response.

5.2 Specific methods

5.2.1 High temperature stomatal assay

Arabidopsis were grown as described in 2.2. 5-day-old Arabidopsis cotyledon pairs were detached and floated on 10/50 (10mM MES, 50mM KCl) buffer in a temperature controlled bath as described in 2.8, and allowed to acclimate in the dark for 2h before transferred to fresh buffer and 20, 28, or 35°C for a further 120 min (Kostaki *et al*., 2020). Three cotyledon pairs were analysed per treatment at 30, 60 and 120 min. Six cotyledon pairs were also heat treated in the dark for 120 min. 10 stomata per cotyledon pair were measured resulting in 30 stomatal measurements per replicate, as described in 2.8. The experiments were repeated three independent times.

5.3 High temperature opens stomata in Arabidopsis seedlings

It was hypothesised that high temperature open Arabidopsis seedling stomata and that this response involves PIFs. 28°C has been shown to be sufficient to trigger thermomorphogenesis (Quint *et al*., 2016; Casal and Balasubramanian, 2019). It has also been shown that growing plants in 28°C increases plant water loss and increases cooling capacity (Crawford *et al*., 2012). While stomatal opening capacity was not altered in the same study, the increased water loss suggests some impact of high temperature on stomatal behaviour. To investigate whether thermomorphogenesis-inducing temperatures open stomata in Arabidopsis cotyledons, plants were treated as described in section 5.2.1. Apertures were measured predawn, (before transfer to light and high temperature treatment), and after 2, 4 and 6h at both 20°C and 28°C. Here, the 28°C treatment failed to open stomata in Arabidopsis seedlings (fig. 5.1a). This suggests that the lack of stomatal opening seen by Kostaki *et al*. (2020) at 30°C may also occur in seedlings. (fig. 5.1a). In contrast, the same study found that a 35°C treatment significantly opened stomata in mature plants. Here, cotyledons were exposed to 35°C to investigate if this response is similar in early development. Stomatal apertures were significantly greater after heat treatment with the greatest effect seen after 120 min (F=127.2, p<0.05, fig. 5.1b). Interestingly, high temperature also opened stomata significantly in the dark. While Kostaki *et al*. (2020) also observed a significant increase in stomatal aperture at 35°C in the dark, the effect was smaller in mean aperture: ~0.5µm change versus

~1 µm seen here (fig. 5.1b). Chlorophyll fluorescence, here used as a proxy for viability, of leaf discs exposed to 35°C can be seen in fig. S2.

5.4 High temperature-induced stomatal opening in cotyledons involves PIFs

Because PIF4 is a key regulator of light and temperature-mediated responses in Arabidopsis seedlings, it was hypothesised that PIFs may regulate stomatal apertures in seedlings in response to high temperature. Indeed, the *pifq* mutant stomata did not open in response to high temperature while those of the WT did (F=4.3, p<0.05, fig. 5.2a), suggesting a regulatory role for PIFs in cotyledon stomatal movement. The *pif4* mutant displayed a reduced opening response to high temperature (p<0.05, fig. 5.2b), however this was not as strong as that of the *pifq* mutant, suggesting that PIF4 may not be the only PIF governing stomatal aperture in response to high temperature.
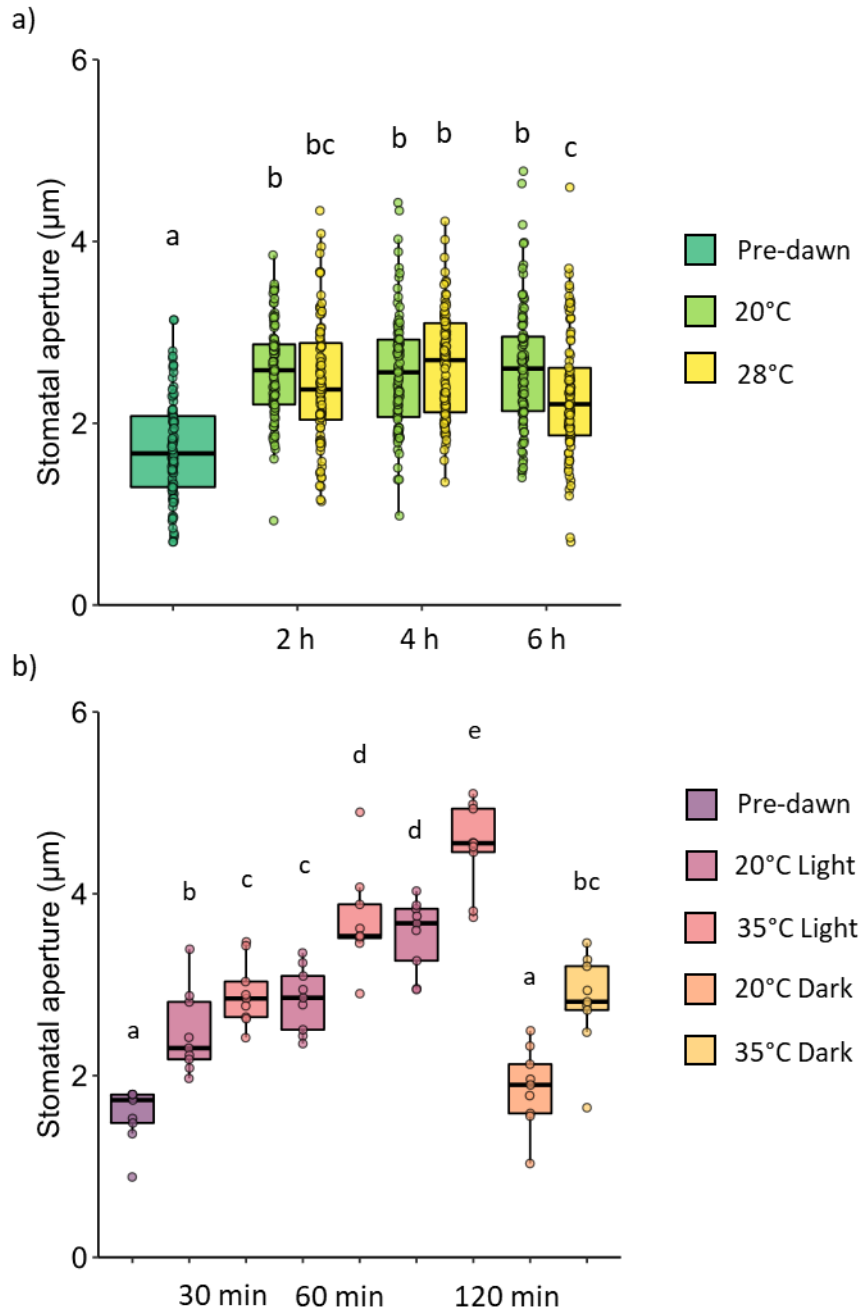
Figure 5.1 Stomatal aperture after exposure of Arabidopsis to high temperature. a) Stomatal aperture over 6 h at 28°C. Arabidopsis seedlings were grown in a 16 h photoperiod and transferred to 20 or 28°C on day 5 after germination. Stomatal apertures were measured predawn and after 2, 4 and 6 h treatment. n=9. **b)** Cotyledon pairs detached from the hypocotyl of Arabidopsis seedlings were floated on 10/50 buffer (10mM MES, 50mM KCl) for 2 h to acclimate and then transferred to 20°C or 35°C in the light or dark. n=9. Plots show the median and the interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. 90 stomata from 9 cotyledon pairs were measured over 3 individual experiments. Data were analysed by two-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.
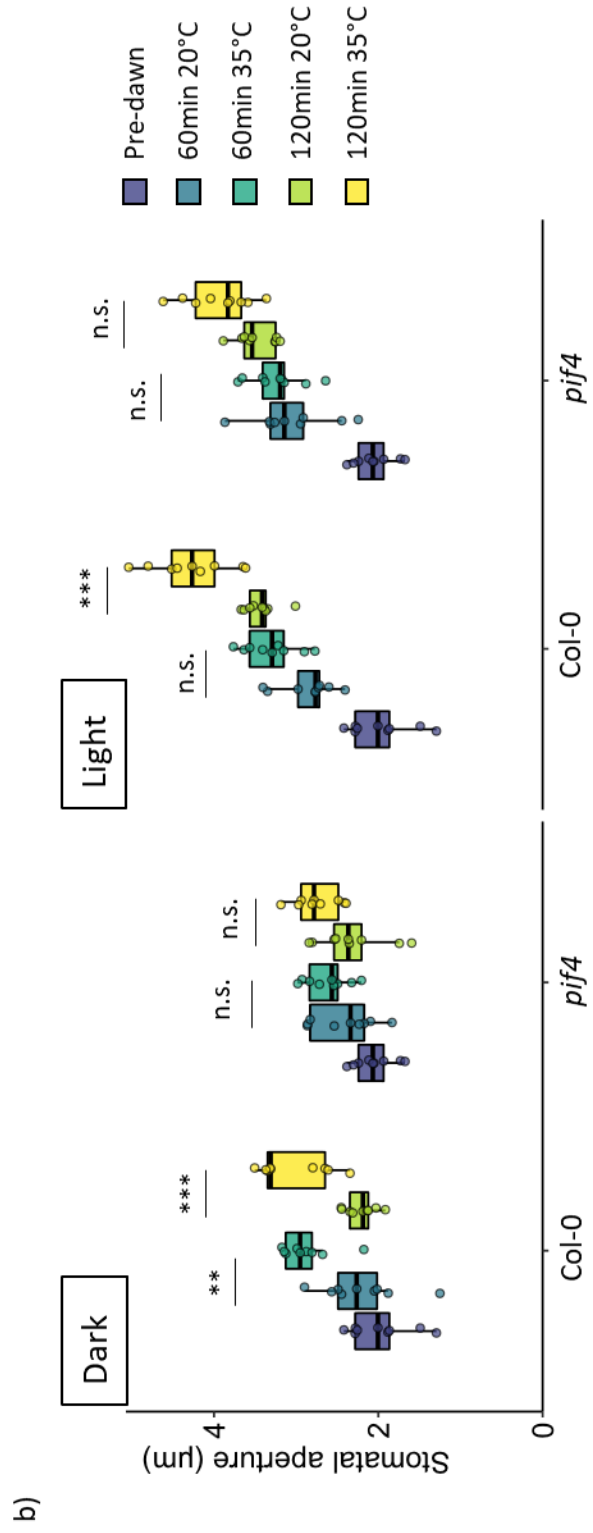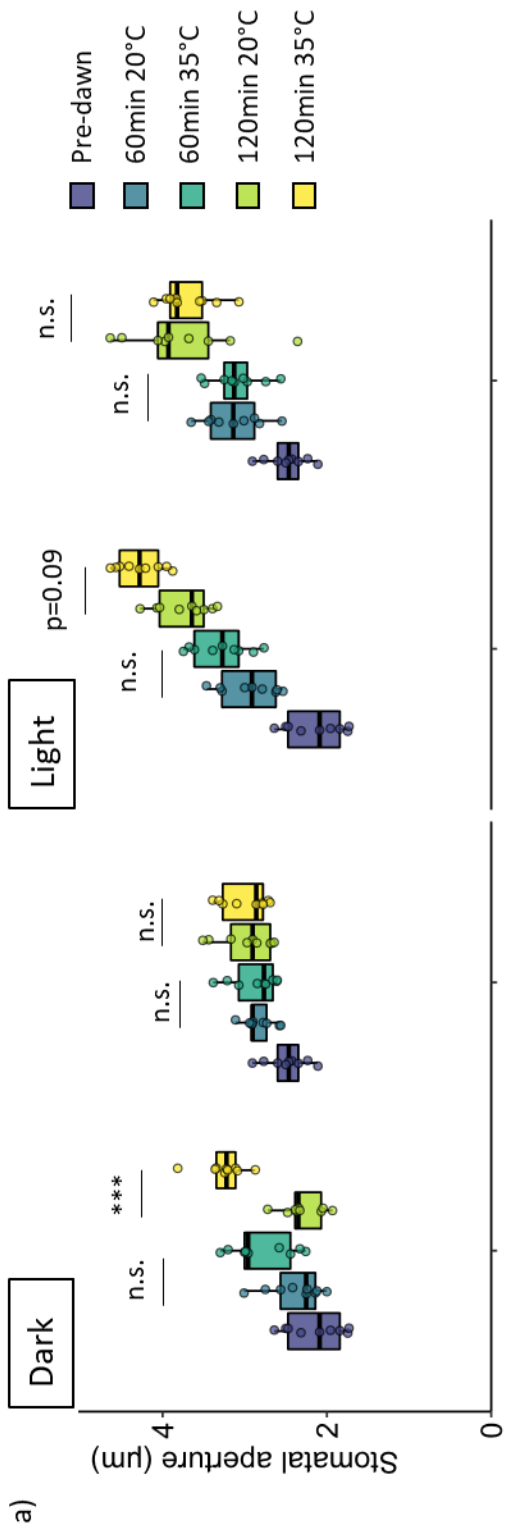
Figure 5.2 Stomatal aperture responses of pif mutants to elevated temperature. a) Stomatal aperture responses of WT and *pifq* mutants to 20°C and 35°C. **b)** Stomatal aperture responses of pif4 to 20°C and 35°C. Cotyledon pairs detached from the hypocotyl of Arabidopsis seedlings were floated on 10/50 buffer (10mM MES, 50mM KCl) for 2 h to acclimate at 20°C and then transferred to 20°C or 35°C in the dark or light. n=9. Plots show the median and the interquartile range of each group. The upper and lower whiskers represent data within 1.5 times the interquartile range. All data values are represented by points. 90 stomata from 9 cotyledon pairs were measured over 3 individual experiments. Data analysed by one-way ANOVA. Asterisks indicate statistically significant differences. **=$p < 0.01$, ***=$p < 0.001$.

5.5 Qualitative analysis of *PIF* promoter activity suggests no change in response to 35°C.

To investigate whether *PIF3, PIF4* and *PIF5* promoter activity is altered in the high temperature treatment used for stomatal aperture assays, a qualitative β-Glucuronidase (GUS) reporter assay was used (Lee and Schoffl, 1997). Transgenic lines, expressing GUS under the control of the native *PIF3, PIF4* and *PIF5* promoters (Zhang *et al.*, 2013) were grown and treated in the same conditions as for the high temperature stomatal assay described in 5.2. We hypothesised high temperature would increase PIF promoter activity as transcript abundance of *PIF3* and *PIF4* are both increased at elevated temperature (Koini *et al.*, 2009; Saini *et al.*, 2022). In the study by Saini *et al.* (2022), *PIF5* transcript abundance was not increased by the 28°C making this a useful comparison for the present study. Here, it appears that *PIF3* promoter activity was decreased by the 35 °C treatment, while *PIF4* and *PIF5* promoter activity was very similar to that at 20°C (fig. 5.3).
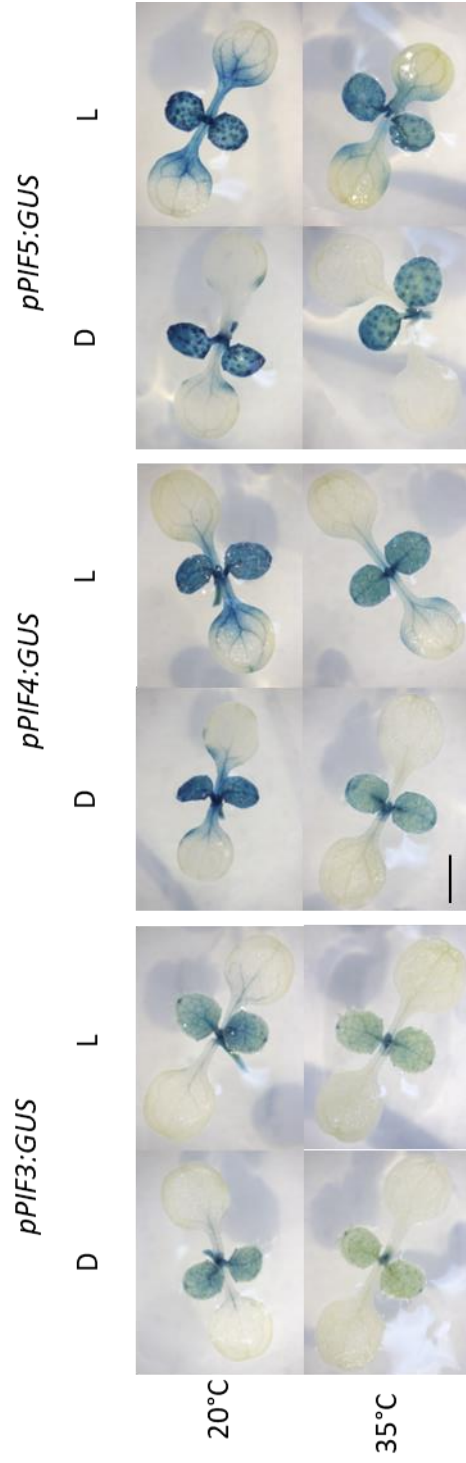
Figure 5.3 Promoter activity of PIF3, PIF4 and PIF5 in response to 35°C in the light and dark. 5-day-old Arabidopsis seedling cotyledon pairs were detached from the hypocotyls and floated on 10/50 buffer (10mM MES, 50mM KCl, pH 6.15) for 2 h to acclimate and then transferred to 20°C or 35°C for a further 2 h before immediately placed in 80% (v/v) acetone and then incubated in GUS staining buffer for 12 h. Cotyledons were washed once with 100% (v/v) EtOH, twice with 70% EtOH and then stored in 50% EtOH and imaged by microscopy. Approximately 12 plants were analysed per treatment. Photos show representative cotyledons.

5.6 Discussion

Work in this chapter showed stomatal opening responses to 35°C, similar to those observed by Kostaki *et al.* (2020)(fig. 5.1b). The greatest aperture was seen after the 120 min treatment (fig. 5.1). It also showed that cotyledon stomata readily respond to high temperature in the absence of light (fig. 5.1b). This suggests the high temperature-induced opening response may not rely on photoreceptor signalling such as that of phototropins (Kostaki *et al.*, 2020). These data suggest that very young seedlings already possess the capacity to regulate stomatal aperture in response to temperature, which may form a part of a greater suite of responses to a dynamic environment. It is possible that seedlings use elevated temperature as a signal to enhance evapotranspiration similar to that in mature plants (Crawford *et al.*, 2012; Kostaki *et al.*, 2020). A quadruple *pif* mutant was used to investigate the effects of several PIFs on the high temperature stomatal response. The *pifq* mutant did not respond to high temperature (fig. 5.2a) suggesting that this response is regulated by PIFs in seedlings. To investigate the involvement of PIF4, a single *pif4* mutant was investigated. While the response to high temperature was much reduced in the *pif4* mutant, these plants still showed some capacity of high temperature induced opening (fig. 5.2b). Taken together these data suggest PIFs may act in an orchestrated manner to regulate stomatal aperture. To investigate this further, single *pif1, pif3*, and *pif5* mutants should also be assayed. To further investigate PIF promoter activity, a GUS staining assay was used. Little difference in PIF promoter activity was observed suggesting that *PIF3*, *PIF4* and *PIF5* transcript abundances may not change in the high temperature conditions used here (fig. 5.3), however qPCR analysis of *GUS* transcript abundance was not possible due to time constraints. Other reports suggest *PIF4* transcript abundance quickly changes when plants are transferred to 28°C (Koini *et al.*, 2009). Lee *et al.* (2021) showed PIF1, 3, 4 and 5 promoted the thermomorphogenesis response by interaction with HECATES (HEC1 and HEC2), transcription factors which inhibit thermomorphogenesis, although there is no evidence transcript abundance of *PIF3* or *PIF5* is altered at high temperature (Hwang *et al.*, 2021). GUS is a very stable molecule and will accumulate over time and will often not correctly reflect the promoter activity (Gallagher, 1992). Alternatively, the 35°C may be damaging to the leaf tissue which interferes with the results. 35°C did not significantly affect photosynthetic efficiency in leaf discs of mature

Arabidopsis (S1), suggesting that 35°C does not cause damage to leaf tissue. However, due to time constraint no such analysis could be performed in cotyledons.

Conclusions

The data presented here show that Arabidopsis cotyledon stomata respond to a high temperature treatment and that this response is at least partially regulated by PIFs. It is not clear if any PIFs are dominant in this response, or whether PIF promoter activity follows similar patterns to those reported under slightly lower temperatures. To further investigate the role of PIFs in high temperature-induced stomatal opening, the response should be assessed in single and double mutants, as well as looking at GUS transcript abundance to elucidate effects not visible in the quantitative assay.

CHAPTER 6 UV-B-induced stomatal opening in *Arabidopsis thaliana* seedlings

Note: data from this chapter are currently in preparation for publication.

6.1 Introduction

The opening and closing of stomatal pores regulates gas exchange between a plant and the surrounding atmosphere, an event which is crucial for a plant to respond effectively to its environment. Stomatal movement is governed by two specialised guard cells flanking each pore. Changes in guard cell turgor allow the pores to open or close and this is driven by ion fluxes across the guard cell plasma membrane. Environmental cues such as light and temperature influence guard cell turgor and therefore stomatal movement. Stomata of several different plant species open in response to blue and red light (Holmes and Klein, 1985; Kinoshita *et al.*, 2001; Talbott *et al.*, 2002; Wang *et al.*, 2010; Inoue and Kinoshita,

2017). Early evidence showed phytochromes may be implicated in light responses of stomata (Habermann, 1973). Wang *et al*., (2010) found that a *phyB* mutant had a reduced stomatal response to red light while a transgenic line overexpressing PHYB was hypersensitive. Interestingly, the authors also found that *phyAphyB* double mutants had a more reduced response to red light than did the *phyB* single mutant, despite the *phyA* single mutant showing a WT response, suggesting phyA may be important in the absence of phyB (Wang *et al*., 2010). While guard-cell specific reduction in phyB did not result in lower stomatal conductance (g_s), the *phyB* mutant had reduced g_s suggesting the red light response of stomata is not guard cell specific (Weraduwage *et al*., 2022). The molecular and metabolic processes underlying red light-mediated stomatal movement are not fully understood. K^+ increased in guard cells of *V. faba* after red light illumination (Olsen *et al*., 2005), however Zhao *et al*. (2012) found that red light did not activate inward-rectifying K+ channels. Early studies showed that plasma membrane H^+ ATPase was activated by red light in *V. faba* guard cell protoplasts (Serrano *et al*., 1988) and when whole Arabidopsis leaves were irradiated with red light, plasma membrane H^+-ATPases were phosphorylated in a photosynthesis-mediated manner, and a 50 µmol m^{-2} s^{-1} dose of red light (considered to saturate photosynthesis) was sufficient to saturate H^+-ATPase phosphorylation (Ando and Kinoshita, 2019). Interestingly, previous data showed that photosynthesis-impaired mutants in tobacco were not compromised in stomatal conductance (Baroli *et al*., 2008). It is therefore still unclear how closely photosynthesis is linked with the red-light responses of stomata. An increased proportion of FR light also affects stomatal apertures. Tomato exposed to a low R:FR ratio had lower stomatal conductance, and stomata of *Commelina* had less open stomata when FR light was added to RL (Roth-Bajerano and Itai, 1981; Kalaitzoglou *et al*., 2019). In the fern *Adiantum capillus-veneris*, a decreased R:FR ratio instead increased stomatal conductance, suggesting the FR response may not be conserved throughout lineages (Doi and Shimazaki, 2008).

Red and blue light appear to synergistically enhance the stomatal response, eliciting stomatal pore apertures that are greater than when irradiated with monochromatic blue or red light (Suetsugu *et al*., 2014; Hosotani *et al*., 2021). Blue light is perceived by the blue light photoreceptor families of phototropins (phot1 and phot2) and cryptochromes (Cry1 and Cry2). These photoreceptors have all

been implicated in stomatal opening responses. While phototropins and cryptochromes act somewhat redundantly to mediate stomatal movement in response to blue light, phototropins appear dominant in mediating stomatal opening, particularly in low fluence blue light in Arabidopsis and other species (Kinoshita *et al*., 2001; Mao *et al*., 2005; Shimazaki *et al*., 2007; Wang *et al*., 2019; Boccalandro *et al*., 2012). When the Light, Oxygen and Voltage sensing (LOV) domains within the phototropins perceive blue light, the flavin chromophore binds to a Cys residue in the LOV domain leading to conformational changes of the photoreceptor and allow photoreceptor kinase activity (Christie, 2007). This results in both autophosphorylation of the phototropins themselves, and phosphorylation of BLUS1 at Ser-348, disrupting its self-inhibitory mechanism (Hosotani *et al*., 2021). BLUS1 in turn phosphorylates plasma membrane H^+ ATPase at the C-terminus and allow their association with 14-3-3 proteins, with the result of H^+-ATPase activation and initiation of a H^+ flux across the membrane (Kinoshita and Shimazaki, 1999). This hyperpolarisation of the plasma membrane subsequently triggers an influx of K^+ ions via voltage-gated inwards-rectifying K^+ channels (KAT1, KAT2, AKT1). An increased concentration of K^+ osmotically drives water uptake and subsequent turgor increases in the guard cells lead to stomatal opening (discussed in chapter 1). Interestingly, blue light-induced stomatal opening was reversed by a dose of green light in *V. faba* (Frechilla *et al*., 2000) and Arabidopsis (Talbott *et al*., 2006; Eisinger *et al*., 2013). The observed effects of different light environments suggest guard cells have the capacity to finely regulate stomatal aperture in dynamic light environments to modulate photosynthesis and transpiration.

While the mechanisms for red, and particularly, blue light-induced stomatal opening are well-studied, the effects of non-photosynthetic, short wavelength, light on stomatal movement are less clear. Some reports exist which implicate UV as a regulator of stomatal aperture (Isner *et al*., 2019; Nogues *et al*., 1999; Tossi *et al*., 2014; Williams *et al*., 2022). UV-A inhibited stomatal opening in a UVR8-independent manner in red and blue light but did not close stomata already opened by red and blue light (Isner *et al*., 2019). UV-B light is considered a "sun signal" meaning that plants perceiving UV-B launch responses typical for a plant in direct sunlight, but plants can also respond to UV-B levels far below those of sunlight (Brown and Jenkins, 2008). In sunlight, stomata are normally open to allow uptake of

CO_2 for photosynthesis (Inoue and Kinoshita, 2017). However, it has been shown that high fluence UV-B close stomata in a ROS-dependent manner in epidermal peels isolated from mature Arabidopsis plants (Tossi *et al*., 2014). The same study found that the UVR8 photoreceptor is involved, as well as the transcription factors HY5 and HYH, suggesting this is not purely a stress response like many other high fluence rate UV-B responses (Brown and Jenkins, 2008). In pea, *Commelina* and oilseed rape, high fluence rates of UV-B also decreased stomatal conductance, though the low UV-B dose had no effect (Nogues *et al*., 1999). Tossi *et al*. (2014) found that NO was a central mediator of stomatal closure in response to high fluence rate UV-B. NO targets inward-rectifying K^+ and Cl^- channels to prevent K^+ uptake and Cl^- exclusion, resulting in guard cell turgor changes and stomatal closure (Garcia-Mata, 2003). This response also requires Ca^{2+}, though NO did not affect Ca^{2+} plasma membrane channels despite an effect on total negative voltage charge on a whole-cell level, suggesting NO triggers intracellular Ca^{2+} release rather than an influx of Ca^{2+} (Garcia-Mata, 2003).

The effects of low fluence UV-B on stomatal movement in cotyledons are, to our knowledge, unexplored in Arabidopsis. In this chapter, the effects of UV-B on stomatal movement in young seedlings were investigated in order to begin to gain an understanding of how UV-B may be involved in seedling establishment and metabolism prior to maturity. With the help of mutants in common UV-B signalling components and ABA signalling, the underlying mechanisms for UV-B-induced stomatal opening were explored. A list of mutants used in this chapter can be found in table 2.1. The data presented in this chapter show that UV-B opens stomata over the course of the day in a UVR8-dependent manner. Evidence is presented of the involvement of specific signalling molecules in this response using mutant studies, and a role for auxin is suggested. This chapter concludes that UV-B can effectively open stomata and may be an important factor to consider for stomatal movement manipulation.

Aims

I aimed to establish the effects of low fluence UV-B on young seedling (cotyledon) stomatal aperture and elucidate the underlying signalling. This knowledge is important in order to understand seedling establishment in a natural environment where UV-B is present, and how fluctuations in UV-B exposure can influence stomatal movement and potentially gas exchange.

6.2 Specific methods

6.2.1 UV-B assay in darkness

A growth chamber set to 20°C and 70% RH, and fitted with a narrowband UV-B tube mounted on a free-standing rig was used for this experiment. Plants were grown as described in section 2.2. The night before the experiment, the cabinet was set to constant darkness. The next morning, pre-dawn samples were harvested right before the subjective dawn. At the time of dawn, the UV-B bulb was turned on to provide UV-B in a background of darkness. The UV-B bulb emits small amounts of light outside of the UV-B region, as shown in figure 6.1. This means that these data must be considered with caution as the light that the plants receive is strictly not only UV-B. Control plants were placed on a different shelf receiving no UV-B. No difference in stomatal aperture was seen in plants treated on different shelves (fig. 6.4). Seedlings were harvested and imaged as described in section 2.8.
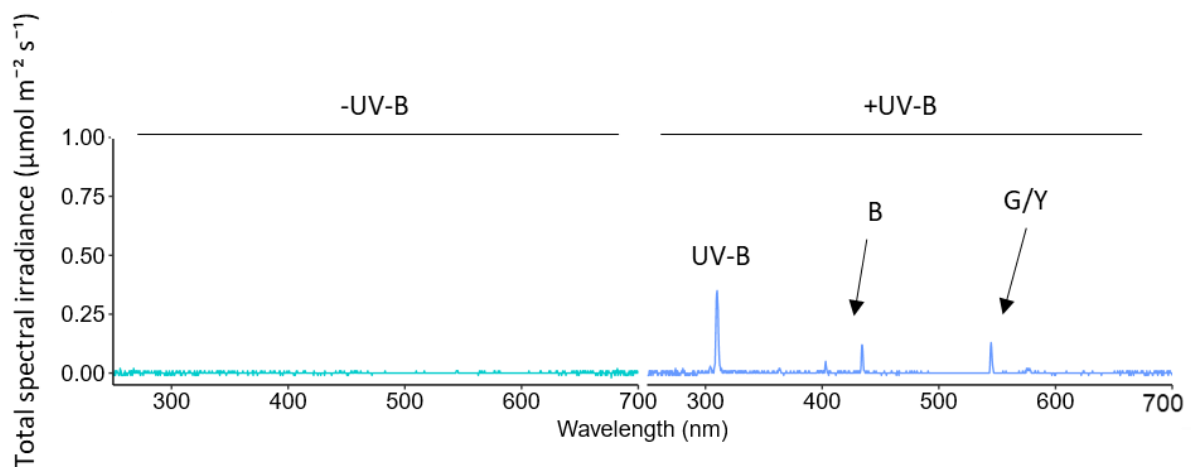
Figure 6.1 Total spectral irradiance for the UV-B in darkness experiment. Light was measured with an OceanOptics FLAME spectroradiometer and analysed with OceanView softwave (version 2.0.8).

6.2.2 Western blot

OST1-GFP Antibody

Tissue from 5-day-old transgenic Arabidopsis seedlings with GFP expression under the control of the native *OST1* promoter (SnRK2.6WT-GFP; Wang *et al.*, 2014) was harvested predawn (darkness) and after 6 h exposure to ±UV-B (1 µmol m⁻² s⁻¹) conditions and snap-frozen in liquid nitrogen. WT Col-0 were used as a negative control. Proteins were extracted in freshly prepared protein extraction buffer (50 mM Tris HCl pH 7.5, 0.5% Triton-X (v/v), 15 mM NaCl, 1% Sodium Deoxycholate, 1% Sigma protease inhibitor (v/v), 5 µM MG132, 1mM DTT). Samples were centrifuged at 4°C for 15 min at 14xg. The supernatant was transferred to fresh tubes and re-centrifuged in the same conditions. Total protein concentration was quantified from the supernatants using the RC DC™ protein assay (Bio-Rad). Laemmli buffer (250 mM Tris HCl pH 6.8, 2% SDS, 40% glycerol, 0.5% Bromophenol Blue, 20% β-mercaptoethanol) was used as loading buffer for SDS-PAGE. Laemmli buffer was added to samples at a ratio of 1:4 and samples were then heated to 95°C for 5 min. 50 µg protein samples were loaded onto a 10% polyacrylamide gel and run at 80V until samples left the wells and then increased to 100V until samples reached the bottom of the gel. x1 Tris/Glycine/SDS buffer (0.25M Tris, 1.92M Glycine, 35mM SDS) was used to run the SDS-PAGE. Transfer to a nitrocellulose membrane (ThermoFisher) membrane was performed using transfer buffer (20% EtOH, 10% Tris/Gly/SDS) at 100V for 1 h. The membrane was then stained with Ponceau stain for 15 min and then a H₂O wash before imaging. Blocking solution (5% skimmed milk powder in TBS-T (0.2M Tris base, 1.5M NaCl, 0.1% tween)) was added to the membrane for 15 min to reduce non-specific binding of the antibody. The membrane was then incubated with an Anti-GFP antibody (Sigma) at a dilution of 1:1000 in 3% milk in TBS-T at 4°C overnight. The next morning, the membrane was washed three times with TBS-T and then incubated with Anti-mouse IgG linked to Horseradish peroxidase at a concentration of 1:2000 in 3 % milk in TBS-T (w/v) for 1 h at room temperature. The membrane was washed again with TBS-T three times before visualisation with chemiluminescence. SuperSignal West Pierce and Femto Substrate (ThermoFisher Scientific) was used according to the manufacturer's instructions.

6.2.3 Chemical treatments

Stomatal bioassays were carried out as described in 2.8. Where 1-Naphthaleneacetic acid (NAA) and N-1-naphthylphthalamic acid (NPA) were used, these were mixed with 10/50 buffer and cotyledon pairs were treated in 50 mm petri dishes or in plastic multi-well plates (Sigma). 70% EtOH was added to the control only containing MES and no NAA. NPA was dissolved in DMSO and 70% EtOH and these were were used as the control for NPA experiments.

6.3 UV-B opens stomata over the course of the day in a UVR8-dependent manner.

High fluence rate UV-B was shown to close stomata in epidermal peels of mature Arabidopsis (Tossi *et al*., 2014), which was also observed in this study (S3). Here, a low fluence rate UV-B treatment opened stomata of Arabidopsis cotyledons significantly at six hours and maintained this response for the remainder of the timepoints investigated (F=10.03, p<0.05, fig. 6.2a). The greatest difference in aperture occurred at 8 h (fig. 6.2b). Considering the plants used for this experiment were grown in a 16 h photoperiod, the 8 h timepoint corresponds with "midday". This suggests that UV-B exposure may require cumulative responses over the course of the day to fully open stomata and maintain opening. It would be interesting to investigate the effects of UV-B across the whole day by measuring apertures at 10, 12, 14 and 16 h to gain an understanding of any circadian effects on the UV-B response. For convenience, the 6 h treatment was chosen for further experiments. In the *uvr8-6* mutant which lacks the UV-B photoreceptor, UVR8, the stomatal response to UV-B was completely abolished (fig. 6.2c), suggesting that this response is UVR8 mediated (fig. 6.2d). It is possible that the UV-B treatment is damaging for the mutant and this damage could affect the stomatal response. To eliminate this hypothesis, the response could be investigated in a *rup1/2* mutant, in which UVR8 is constitutively active due to the lack of re-dimerization by the RUP1 and RUP2 proteins. This mutant would be expected to have a stronger phenotype under UV-B showing more open stomata.

Next, we investigated whether two well-established UV-B signalling proteins are involved in the stomatal response to UV-B. Figures 6.3a and b show that a *cop1* mutant has much reduced stomatal apertures under UV-B compared to the WT control (F=21.11, p<0.05). It is important to note that the *cop1* mutant has very large apertures both in the dark and in the WL conditions, making comparisons difficult (F=89.09, p<0.05, fig. 6.3a). It is possible that the stomatal pores have reached maximum apertures and therefore any further response to UV-B is effectively masked. Considering the involvement of COP1 in UV-B-induced photomorphogenesis and stress responses (Favory *et al*., 2009), these data support a role for COP1 in UVB-induced stomatal responses as well.
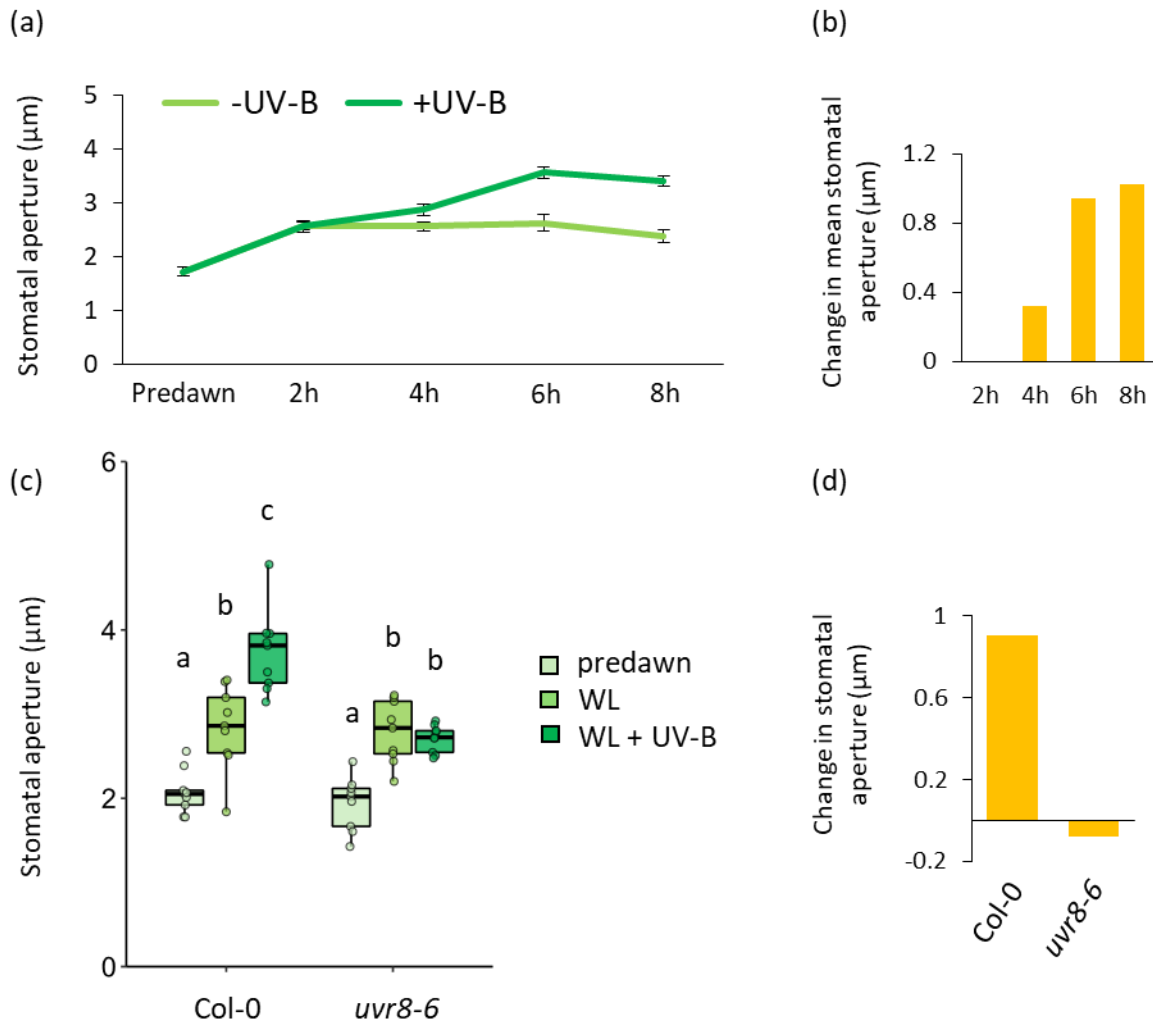
Figure 6.2 Stomatal aperture in response to low fluence UV-B. Arabidopsis developed in a 16 h photoperiod, 20°C and WL were transferred to WL ± 1 µmol m⁻² s⁻¹ UV-B 5 days after germination. Three cotyledon pairs were detached from the hypocotyls and rapidly analysed. **a)** Stomatal aperture over 8 h. Points represent mean apertures with error bars representing the SE. **b)** Change in stomatal aperture over 8 h. Bars represent (mean UV-B aperture) – (mean control aperture). **c)** Stomatal aperture in *uvr8-6* after 6 h. Bars represent the mean with error bars representing the SE. **d)** Change in stomatal aperture in *uvr8-6* after 6 h. Bars represent (mean UV-B aperture) – (mean control aperture). n=9. Data were analysed by 2-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.

A key promoter of photomorphogenesis responses is HY5 and its homologue, HYH (Oyama, *et al*., 1997). To investigate the possible involvement of HY5 in the stomatal response, a *hy5hyh* double mutant was exposed to 6 h UV-B. Despite the visibly slightly smaller, apertures of the *hy5hyh* mutant in WL compared to the WT control were not statistically significantly more closed (fig. 6.3c). The difference in aperture in *hy5hyh* between the WL and the UV-B supplemented condition was statistically significant and of the same magnitude as the WT (F=50.630, p<0.05, fig. 6.3d). This suggests HY5/HYH may not be involved in the stomatal UV-B response.

6.4 Stomata of Arabidopsis seedlings do not open under UV-B without white light

It has been shown that some photoreceptors other than UVR8 perceive UV-B or modulate the UV-B response (Hermanowicz *et al*., 2019; Rai *et al*., 2019; Tissot and Ulm, 2020). UVR8 perceives UV-A with the capacity to elicit signalling (Rai *et al*., 2020), suggesting evidence for complex crosstalk between light signalling pathways. Indeed, in this study, UV-B could not alone open stomata as when seedlings were exposed to just the UV-B irradiation, stomata did not open (fig. 6.4a). This effect was maintained through the 6 hours that stomatal apertures were measured (fig. 6.4b), suggesting that the UV-B-induced stomatal response is mediated by signalling involving additional photoreceptors to UVR8, or UVR8 stimulation by other wavelengths.

6.5 Involvement of other photoreceptors in UV-B-induced stomatal opening

Phototropins and cryptochromes govern the stomatal movement responses to blue and UV-A light (Kinoshita *et al*., 2001; Mao *et al*., 2005; Shimazaki *et al*., 2007; Wang *et al*., 2019; Boccalandro *et al*., 2012), while the involvement of phytochromes in stomatal opening is not fully understood (Holmes and Klein, 1985; Roth-Bejerano and Itai, 1987; Karlsson, 1988; Talbott *et al*., 2002, Wang *et al*., 2010). Here, the *phot1/2* and *phyB-9* mutants were used to investigate the role of these photoreceptors in the stomatal response to UV-B supplementation. The *phot1/2* mutant did not respond to the UV-B treatment suggesting phototropin involvement in UV-B signal perception and/or transduction in this response

(F=14.298, p<0.05, fig. 6.5a). It could also imply that the phototropin signalling pathway must be functional and that UVR8 may amplify phototropin signalling.
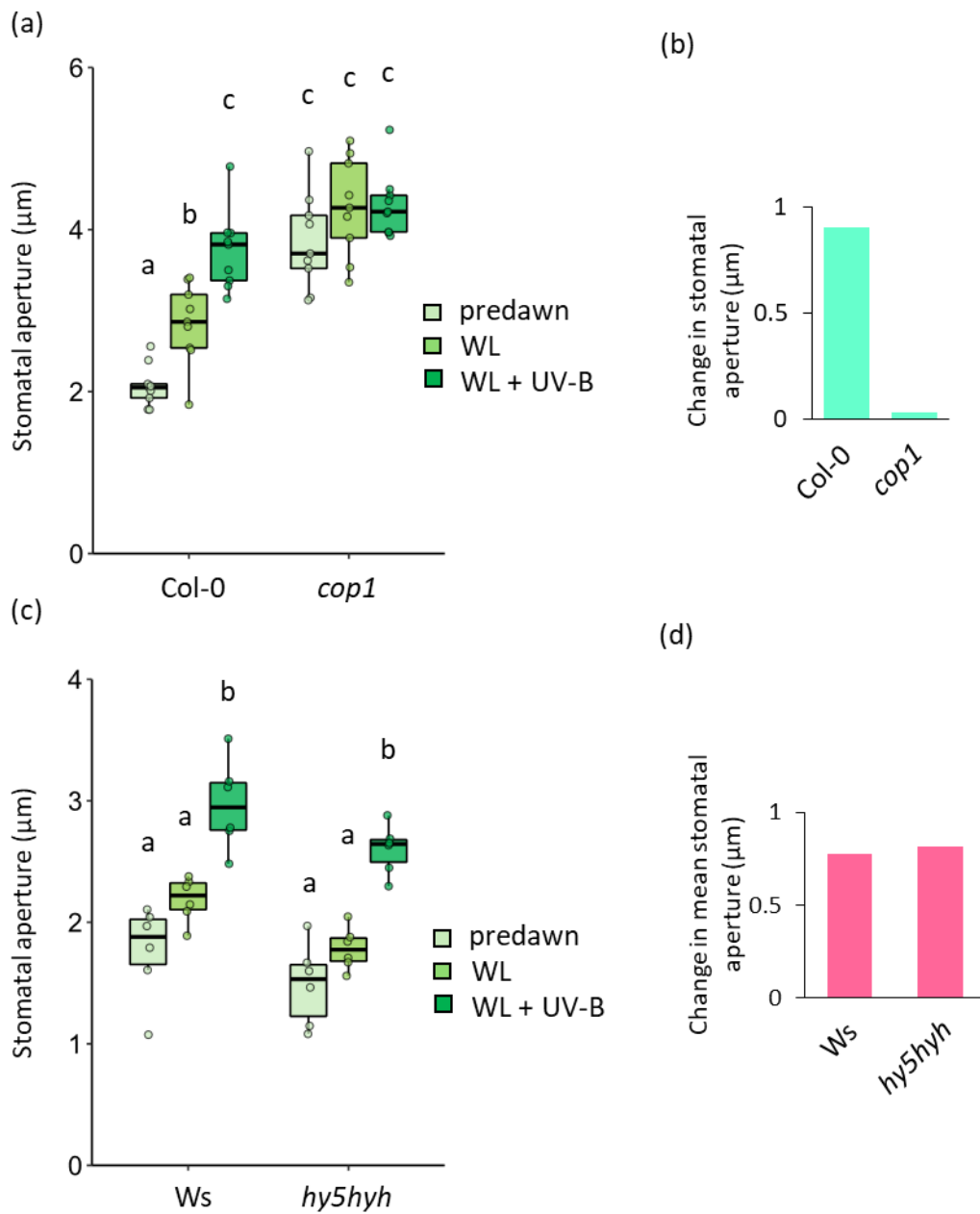
Figure 6.3 Stomatal apertures in *cop1-4* and *hy5hyh* in response to low fluence UV-B. Arabidopsis developed in a 16 h photoperiod, 20°C and WL were exposed to 6 h WL ± 1 µmol m⁻² s⁻¹ UV-B 5 days after germination. Three cotyledon pairs were detached from the hypocotyls and rapidly analysed. **a)** Stomatal response of *cop1-4* to UV-B. Bars represent mean apertures with error bars representing the SE. **b)** Difference in stomatal aperture in *cop1-4*. Bars represent (mean UV-B aperture) – (mean control aperture). **c)** Stomatal response of *hy5hyh* to UV-B. Bars represent mean apertures with error bars representing the SE. **d)** Difference in stomatal aperture in *hy5hyh*. Bars represent (mean UV-B aperture) – (mean control aperture). n=9. Different letters indicate statistically significant differences at p<0.05.
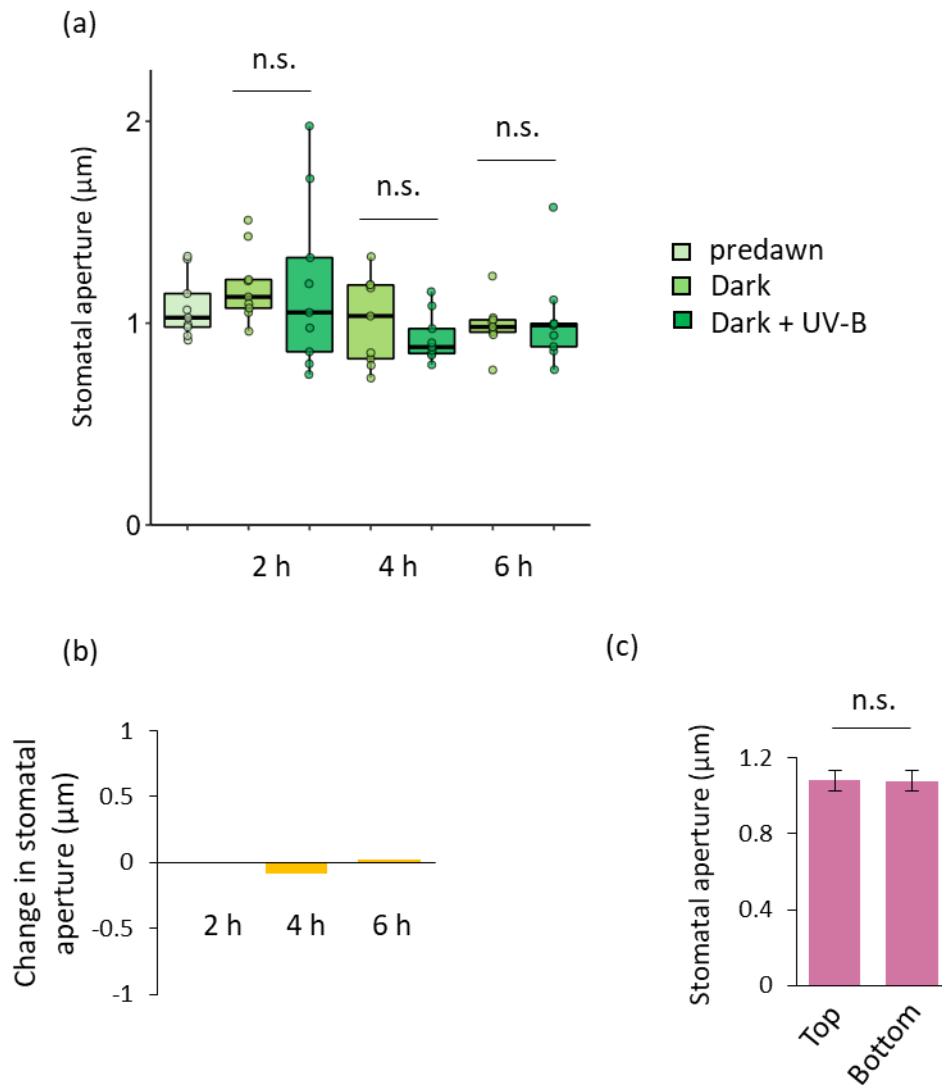
Figure 6.4 Stomatal responses to UV-B in the dark. Stomatal apertures of 5-day-old Arabidopsis seedlings over 6 h ± 1 µmol m⁻² s⁻¹ UV-B in a background of darkness. Three cotyledon pairs were detached from the hypocotyl and rapidly analysed every two hours for 6 h. **a)** Stomatal aperture ± UV-B in the dark. Bars represent mean apertures with error bars representing the SE. **b)** Change in apertures. Bars represent (mean UV-B aperture) – (mean control aperture). **c)** No difference in stomatal apertures were observed in the dark between different growth cabinet shelves. n=9. No significant differences were found between treatments.
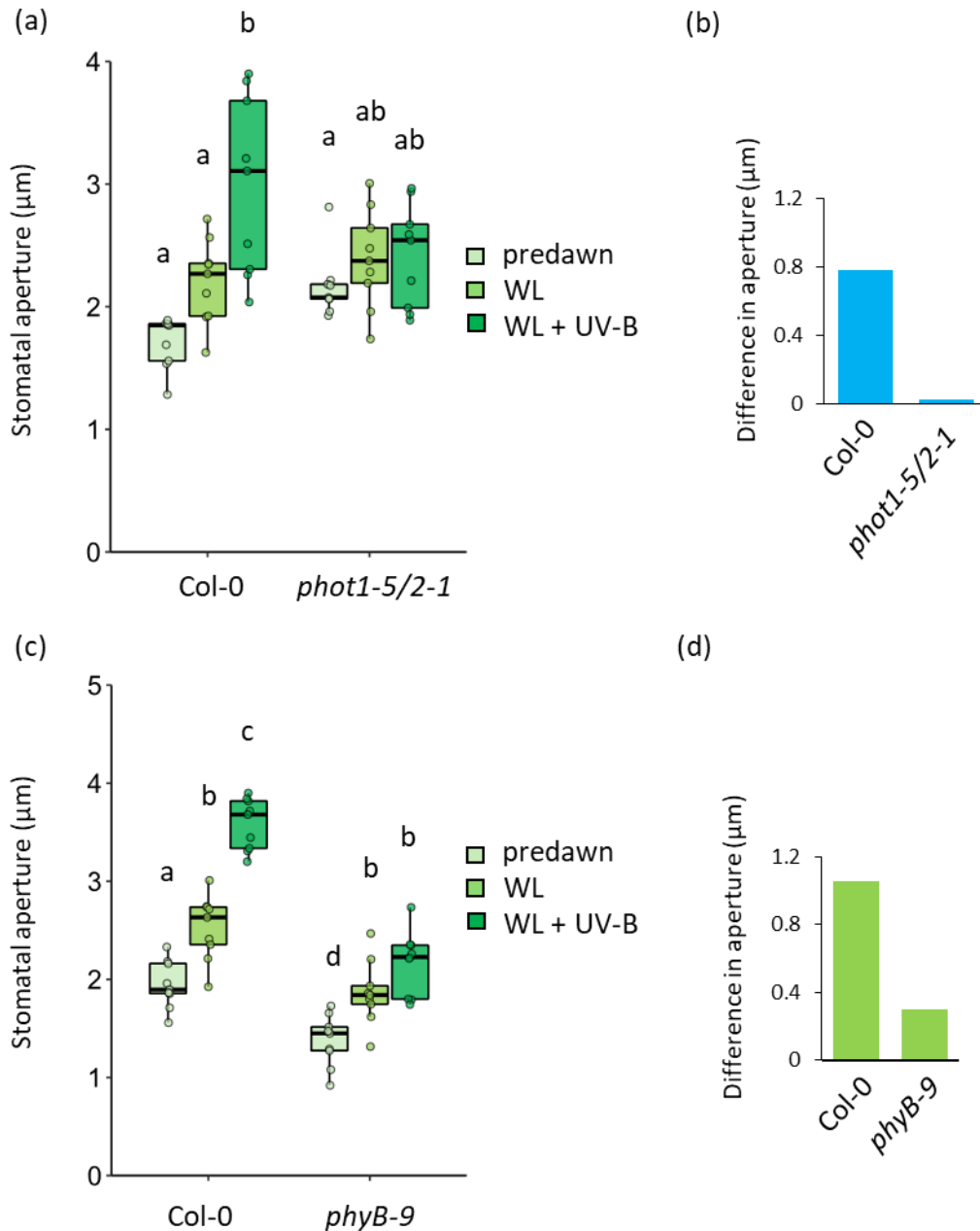
Figure 6.5 Stomatal apertures in phot1-5/2-1 and phyB-9 in response to low fluence UV-B. Arabidopsis developed in a 16 h photoperiod, 20°C and WL were exposed to 6 h WL ± 1 μmol m⁻² s⁻¹ UV-B 5 days after germination. Three cotyledon pairs were detached from the hypocotyls and rapidly analysed. **a)** Stomatal response of *phot1-5/2-1* to UV-B. Bars represent mean apertures with error bars representing the SE. **b)** Difference in stomatal aperture in *phot1-5/2-1*. Bars represent (mean UV-B aperture) – (mean control aperture). **c)** Stomatal response of *phyB-9* to UV-B. Bars represent mean apertures with error bars representing the SE. **d)** Difference in stomatal aperture in *phyB-9*. Bars represent (mean UV-B aperture) – (mean control aperture). n=9. Data were analysed by two-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.

Conversely, the *phyB-9* mutant responded to UV-B (fig. 6.5c), but this response was much reduced compared to the control (=11.23, p<0.05, fig. 6.5d). This may suggest some involvement of *phyB* in the UV-B response. It is important to note that the *phyB-9* mutant used in this study has a secondary mutation in the *VENOSA4* gene which affects chloroplast size, photosynthesis and leaf growth (Yoshida *et al*., 2018). To fully investigating the effects of the *phyB* mutation alone, T-DNA insertion lines such as those used by Seo *et al*. (2006) could be used.

6.6 PIFs regulate stomatal aperture in response to UV-B

Reports have shown PIFs act as negative regulators of stomatal aperture in response to red light (Wang *et al*., 2010; Li *et al*., 2022). UV-B inhibits hypocotyl elongation, a key photomorphogenic response, by inhibition of PIFs. This occurs independently of HY5 and HYH (Tavridou *et al*., 2020). As discussed in 6.3, UV-B-induced stomatal opening also appears to be independent of HY5 and HYH. Therefore, we hypothesised that PIFs may be involved in the stomatal response to UV-B light. Upon UV-B exposure, *PIF4* transcripts and PIF4 proteins are rapidly degraded (Hayes *et al*., 2014; Hayes *et al*., 2017; Tavridou *et al*., 2020). This is supported by the data presented here, where *PIF4* transcript levels were lower after the UV-B treatment, however only the difference at 4h was statistically significant (F=43.56, p<0.05, fig. 6.6b). This response was completely abolished in the *uvr8-6* mutant (fig. 6.6b). UV-B exposure also resulted in lower *PIF5* transcript levels (F=15.33, p<0.05, fig. 6.6c) supporting data presented by Sharma *et al*. (2019). For *PIF5*, however, the greatest difference was found at 2 h. The difference between the UV-B irradiated seedlings and the control then diminished at 4 and 6 h. To investigate the involvement of PIFs in the stomatal response to UV-B, the *pifq* mutant seedlings lacking PIF1, PIF3, PIF4 and PIF5 were exposed to WL ±UV-B for 6 h before apertures were measured. While UV-B opened stomata in the WT, it failed to open stomata of *pifq* (F=5.24, p<0.05, fig. 6.6a), suggesting a role for PIFs in stomatal opening. Similar to the *cop1* mutant, but not to the same extent, the *pifq* mutant has somewhat more open stomata predawn and in the WL condition, making the effect of UV-B difficult to determine. Single *pif* mutants should be assayed to assess the involvement of individual PIFs. However, redundancy between several PIFs exist in photomorphogenesis and it is possible they also act redundantly in the stomatal response to UV-B (Leivar and Monte, 2014).
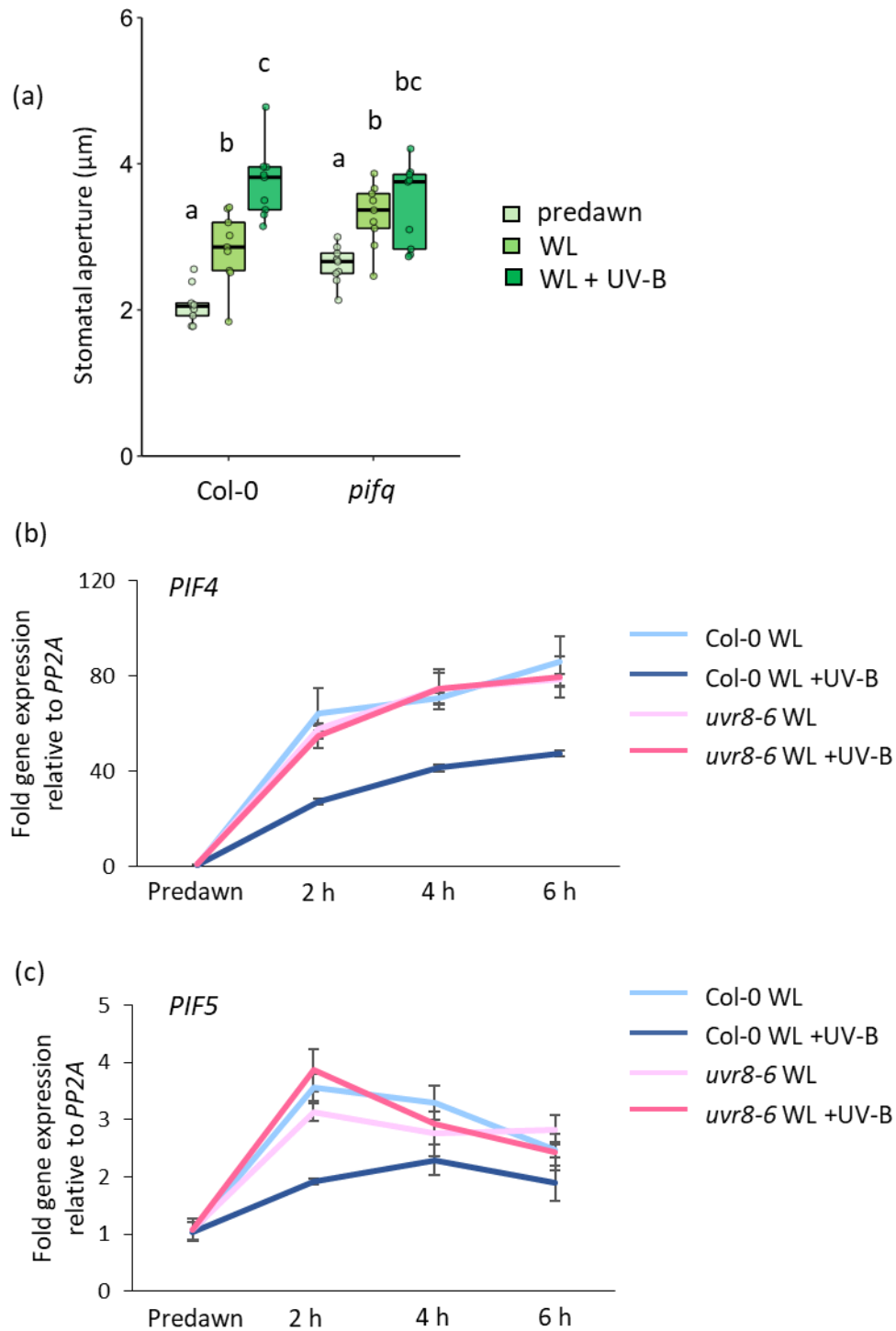
Figure 6.6 PIF responses to UV-B. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL for 5 days. **a)** Stomatal response of *pifq* cotyledons exposed to 6 h WL ± 1 μmol m⁻² s⁻¹ UV-B. Bars represent mean apertures with error bars representing the SE. n=9. **b)** *PIF4* transcript abundance in Col-0 and *uvr8-6* after 2, 4, and 6 h WL ± 1 μmol m⁻² s⁻¹ UV-B. n=3. **c)** *PIF5* transcript abundance in Col-0 and *uvr8-6* after 2, 4, and 6 h WL ± 1 μmol m⁻² s⁻¹ UV-B. n=3. Data were statistically analysed by two-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.

To investigate whether UV-B affects PIF promoter activity in the conditions used here, a transgenic Arabidopsis line expressing GUS under control of the native *PIF4* promoter was exposed to supplementary UV-B for 6 h (Zhang *et al.*, 2013). There was no visible difference between the treatments in intact cotyledons or in epidermal peels from mature plants (fig. 6.7). Measuring GUS transcript abundance could be used as a quantitative measure of *PIF* promoter activity; a GUS stain is quite stable and may not reflect dynamic changes (Franklin *et al.*, 2011). Alternatively, it is possible that the regulation of *PIF* transcripts occurs post-transcriptionally which could reflect the lack of difference in GUS activity in the conditions used here. It would also be possible to investigate the PIF4 protein abundance in these treatments.

6.7 Auxin as a negative regulator of stomatal opening in response to UV-B

In 6.6 it was found that PIFs are involved in the stomatal response to UV-B. This may suggest a role for auxin in stomatal aperture regulation as PIFs are closely linked to auxin signalling. For example, UV-B triggers the reduction in auxin biosynthesis via PIF4 to inhibit thermomorphogenesis (Hayes *et al.,* 2017). Arabidopsis cotyledon stomata did not respond to low dose of the synthetic auxin, NAA (fig. 6.8a). Stomata had a small but significant response to the highest concentration of the auxin inhibitor, NPA, which may suggest physiologically relevant concentrations of NPA have no effects on stomatal aperture (F=2.996, p<0.05, fig. 6.8b). To investigate the response to auxin under UV-B, cotyledons were floated on 10/50 MES/KCl buffer supplemented with auxin. The auxin treatment reduced the response to UV-B (F=6.72, p<0.05, fig. 6.9a) in a dose-dependent manner, though this was just above the alpha level (p=0.058, fig. 6.9b), possibly implicating auxin in the response to UV-B. To further investigate the UV-B effects on auxin in the conditions used here, the transcript abundances of auxin biosynthesis genes were analysed after 2, 4 and 6 h UV-B. UV-B reduced the transcript abundance of *IAA19, IAA29* and *YUC8*, however these differences were not statistically significant (fig. 6.9c, d and e). Interestingly, the pattern in transcript abundance for *IAA29* and *YU8* closely mimic that of the stomatal response to UV-B, shown in figure 6.2a. It was not possible to distinguish a difference in auxin activity between the treatments using the *DR5::GUS* reporter construct (fig. 6.10).

6.8 ABA signalling in UV-B-induced stomatal opening

Due to the well-studied effects of ABA on stomatal aperture, we investigated the UV-B-mediated stomatal responses in Arabidopsis mutants lacking important ABA signalling components. The ABA biosynthesis mutant, *nced3/5*, displayed a WT response to UV-B (fig. 6.11a) with a change in aperture almost identical to that of the WT control (fig. 6.11b). Conversely, the quadruple ABA receptor mutant, *q1124*, had a reduced yet statistically significant response to UV-B ($p<0.05$, fig. 6.12a). Together, these data suggest that ABA perception may be important in the UV-B response, while ABA biosynthesis could play a smaller role. OST1, a Snrk6.2 kinase, positively regulates guard cell responses to ABA (Acharya *et al*., 2013). The loss of function mutant *ost1-3* also had a reduced response to UV-B (fig. 6.12a) further supporting that ABA signalling may be involved in UV-B-mediated stomatal opening. Interestingly, *NCED3* and *NCED5* transcript abundance was not altered in the UV-B condition compared to the control (fig. 6.11c and d). The trend for *OST1* transcript abundance suggests UV-B may have a small positive effect on its expression in the WT, though this effects was not statistically significant here (fig. 6.12c). To further investigate the role of OST1 in the UV-B response, protein abundance was analysed by western blot. However, no difference in OST1 protein abundance was found in the conditions used here (fig. 6.13).

6.9 Deficiency in CHS impairs UV-B-induced stomatal opening

Together with signalling proteins, transporters, ion channels and pumps, reactive oxygen species (ROS) regulate stomatal movement (Sierla *et al*., 2016). In guard cells, ROS are generated by plasma membrane NADPH oxidases (Respiratory Burst Oxidase Homologs, RBOHs). Early events in stomatal closure involves accumulation of ROS in the apoplast and in the chloroplasts (Suzuki *et al*., 2011; Sierla *et al*., 2013). Tossi *et al*. (2014) found that stomatal closure in response to high fluence UV-B triggered an increase in H_2O_2 and NO with the same pattern as the stomata closure response, suggesting high fluence UV-B responses of stomata require ROS signalling. ROS are neutralised by enzymatic and non-enzymatic mechanisms. Flavonoids act as antioxidant agents, reducing the negative impact of ROS (Agati *et al*., 2012; Fini *et al*., 2011). A well-studied response to UV-B is the induction of *CHS* transcription and accumulation of CHS transcripts and flavonoids (Li *et al*., 1993; Brown et al., 2005).
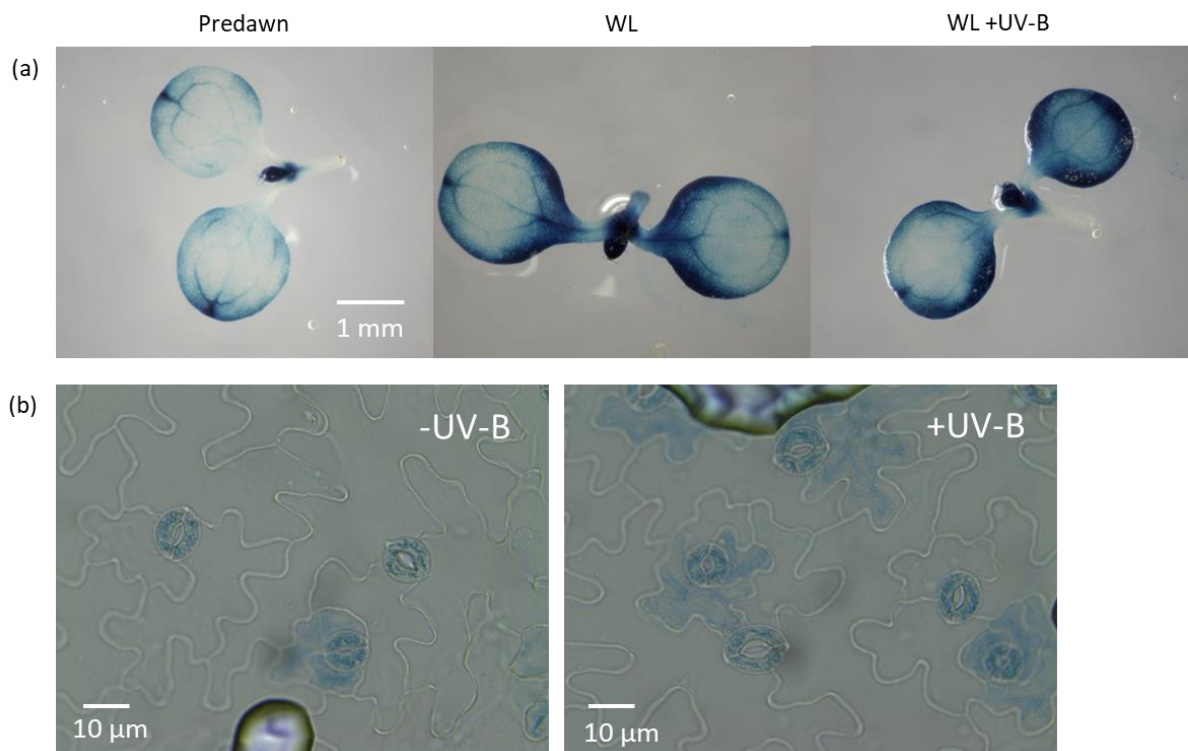
Figure 6.7 . GUS activity in *PIF4::GUS* transgenic lines treated with and without supplementary UV-B. In **a)** whole seedlings, n=20. **b)** epidermal peels, n=10. 5-day-old Arabidopsis cotyledons grown in a 16 h photoperiod, 20°C and WL were exposed to 6 h WL $\pm$ 1 µmol m^{-2} s^{-1} UV-B before an acetone wash and subsequent incubation with GUS staining buffer for 16 hours.
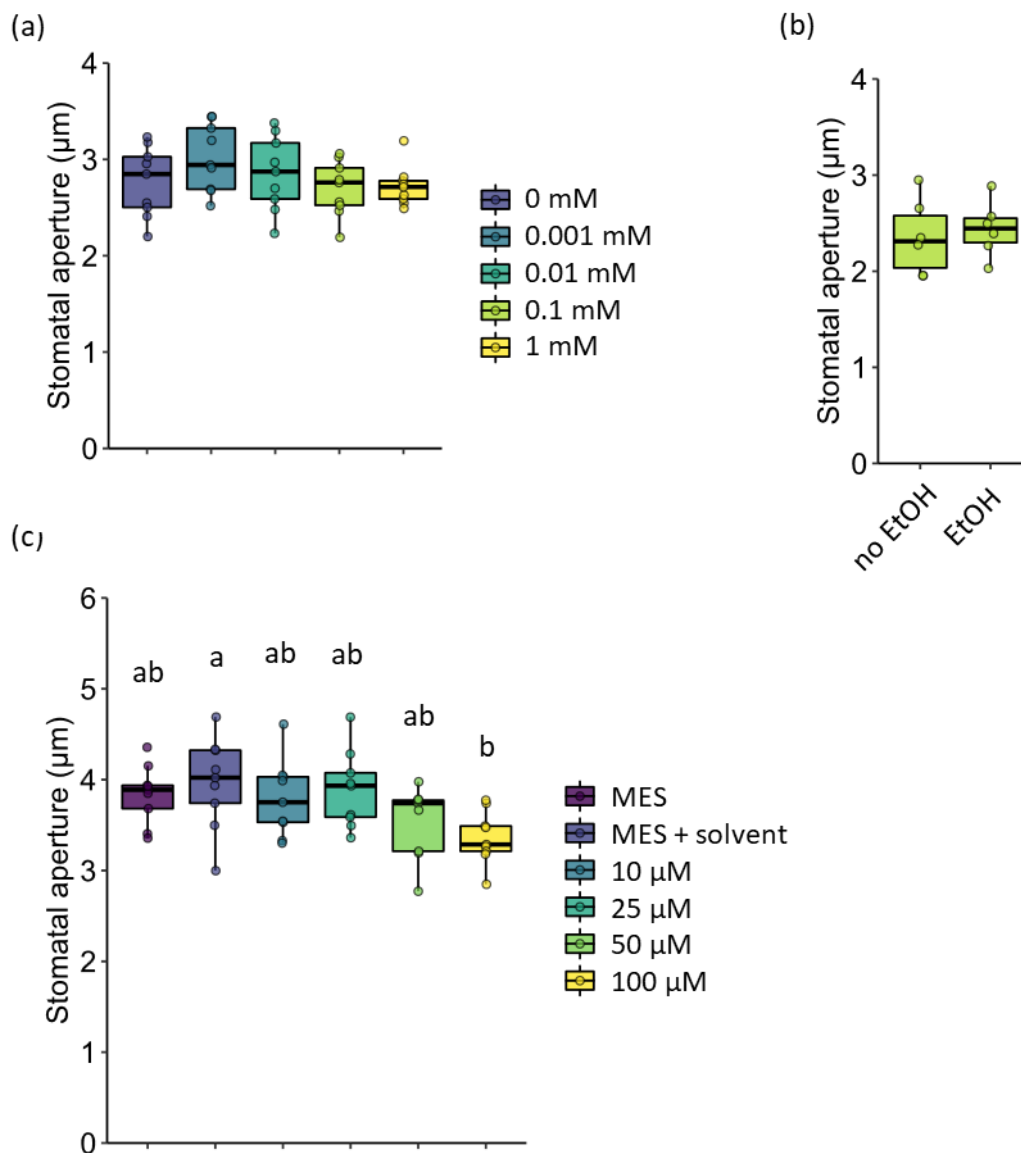
Figure 6.8 Stomatal responses to 1-Naphthaleneacetic acid (NAA) and N-1-naphthylphthalamic acid (NPA). Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-day-old cotyledons were floated on 10/50 (10 mM MES, 50 mM KCl) buffer with different NAA or NPA concentrations for 6 hours in the dark. **a)** NAA. **b)** 1 mM EtOH. **c)** NPA. Bars represent means with error bars representing the SE. n=9. Data were analysed by one-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at $p < 0.05$.
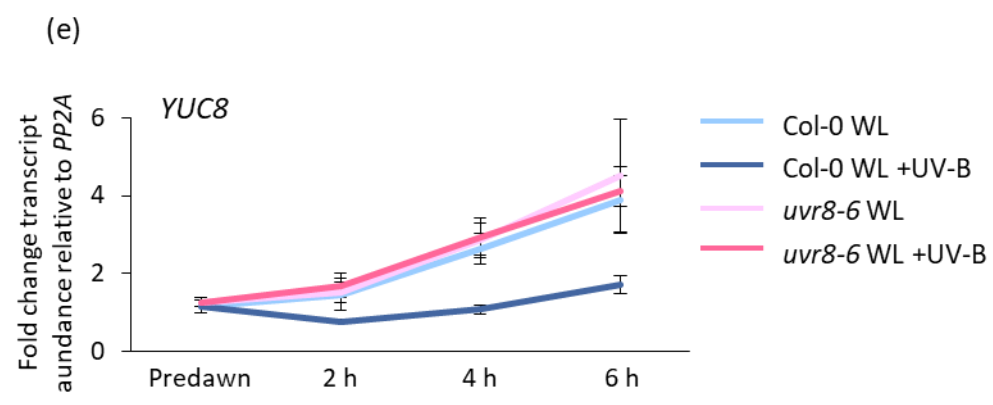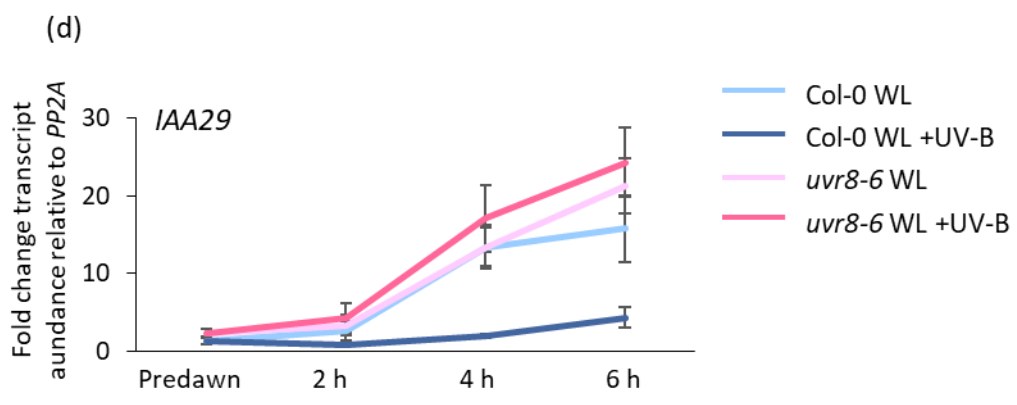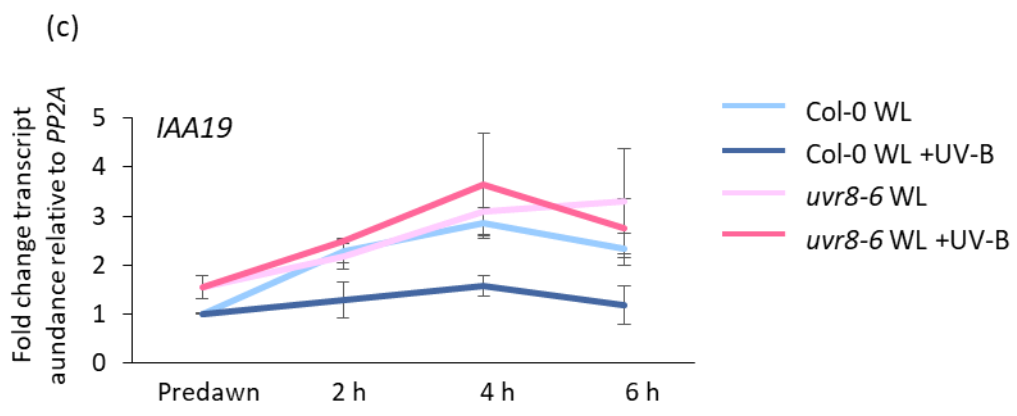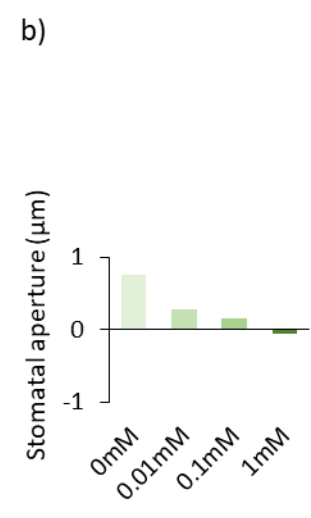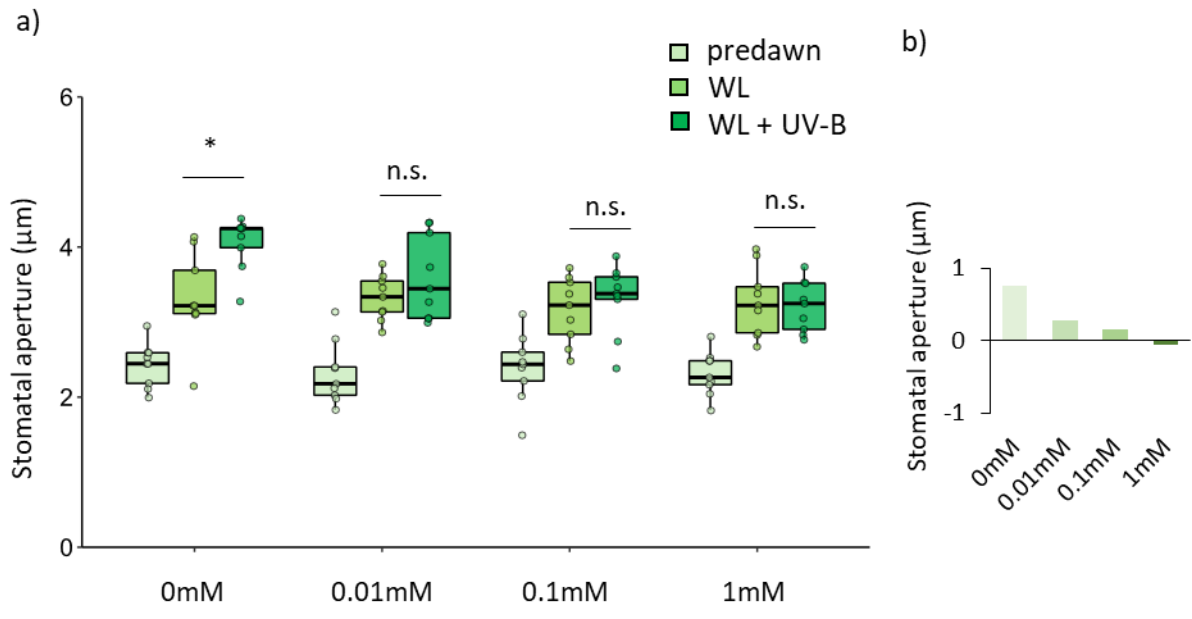
Figure 6.9 Stomatal responses to auxin under UV-B. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. **a)** Dose response to auxin under UV-B. 5-day-old cotyledons were floated on 10/50 (10 mM MES, 50 mM KCl) buffer with different NAA concentrations for 6 hours. Bars represent means with error bars representing the SE. n=9. **b)** Change in aperture in response to NAA. **c)** Relative transcript abundance of IAA19 over 6h WL ± 1 μmol m^{-2} s^{-1} UV-B. n=3. **d)** Relative transcript abundance of IAA29 over 6h WL ± 1 μmol m^{-2} s^{-1} UV-B. n=3. **(e)** Relative transcript abundance of YUC8 over 6h WL ± 1 μmol m^{-2} s^{-1} UV-B. n=3. Dots represent means at each timepoint with error bars representing the SE. Data were analysed by one-way ANOVA and Tukey post hoc test. Asterisk indicate statistically significant difference from the control at $p < 0.05$.
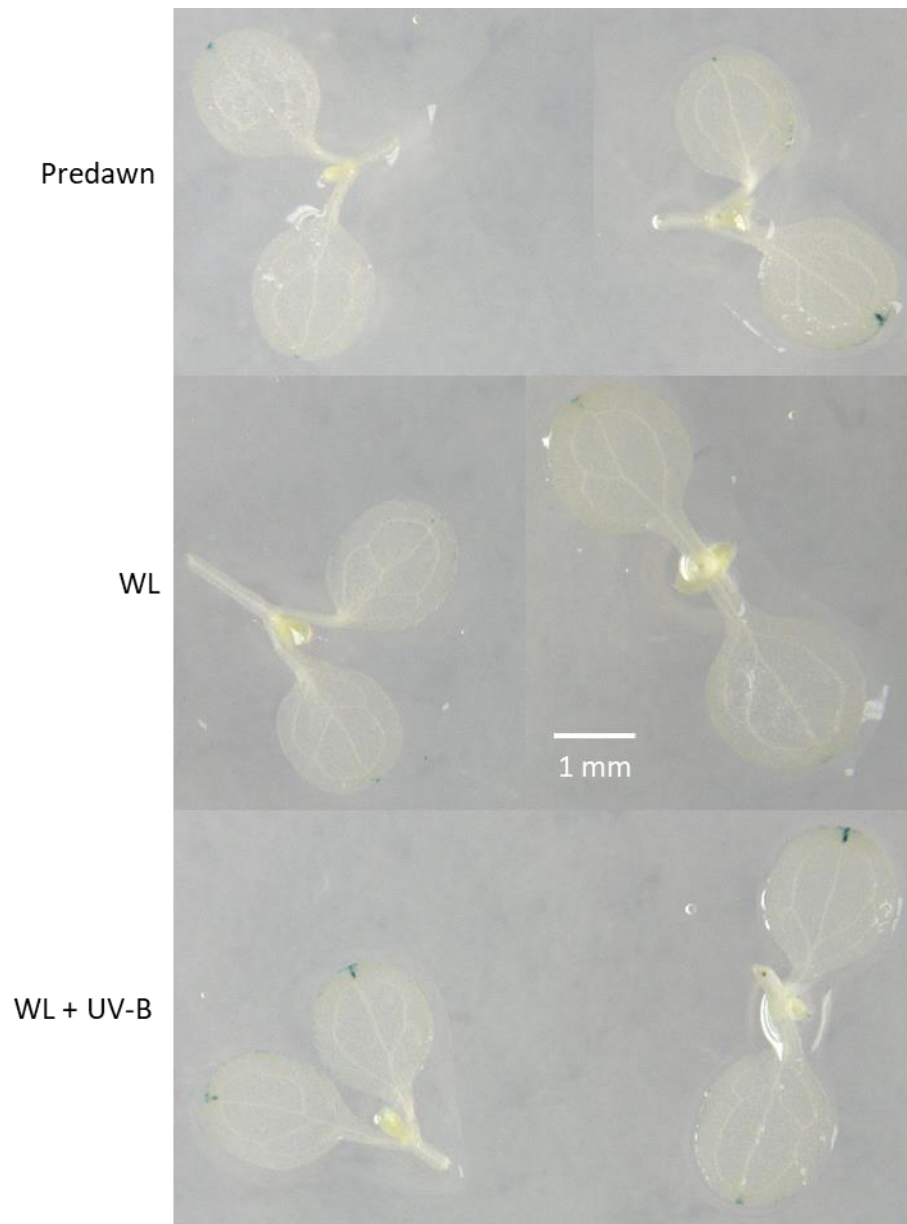
Figure 6.10 GUS activity in DR5:GUS transgenic lines treated with and without supplementary UV-B. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-day-old cotyledons were exposed to 6 h WL $\pm$ 1 μmol m^{-2} s^{-1} UV-B before an acetone wash and subsequent incubation with GUS staining buffer for 48 hours. n=20.
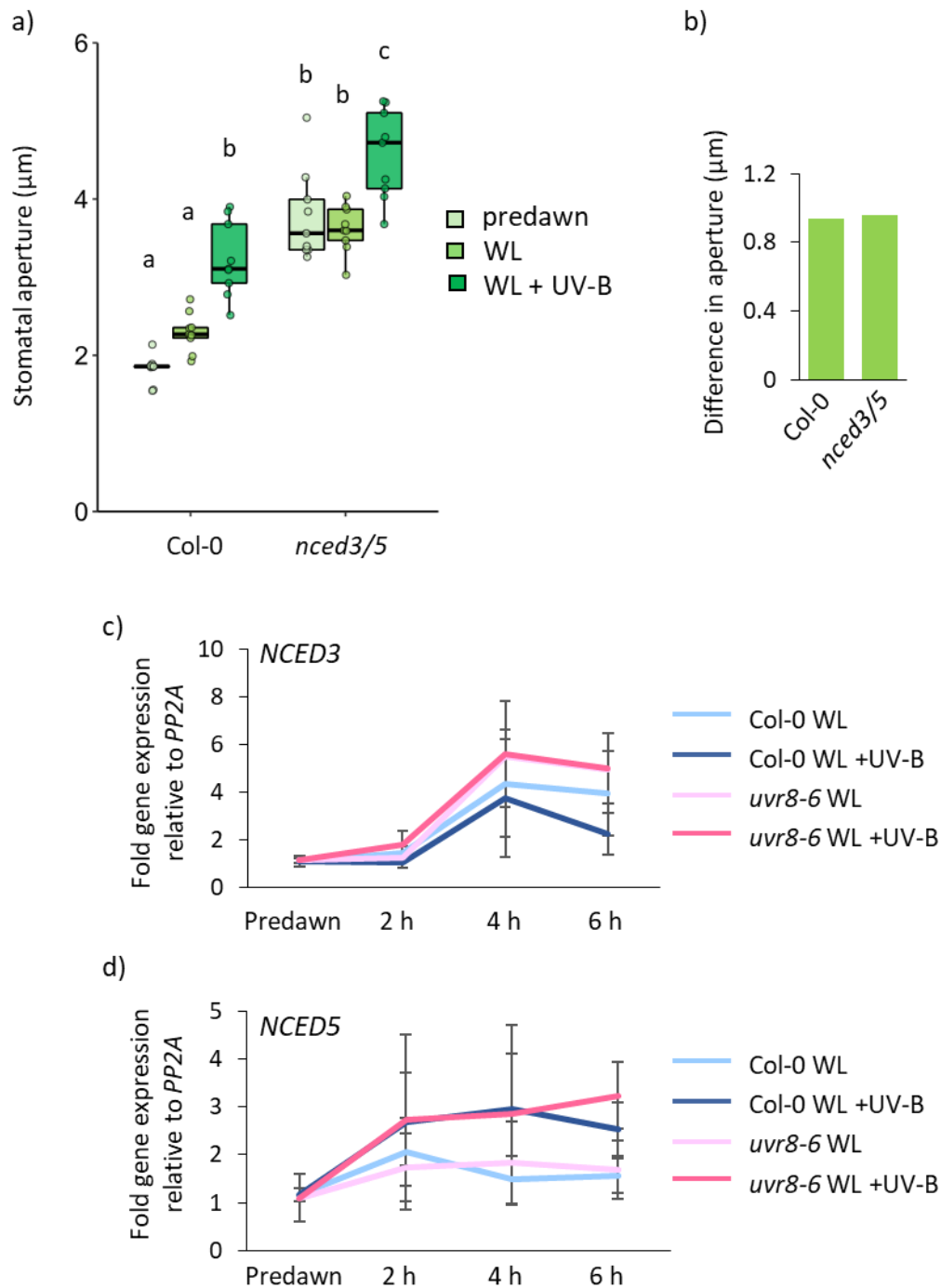
Figure 6.11 ABA biosynthesis in UV-B-induced stomatal opening. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-day-old cotyledons were exposed to 6 h WL $\pm$ 1 μmol m^{-2} s^{-1} UV-B before stomatal apertures were measured or tissue harvested for qPCR. **a)** Stomatal aperture of *nced3/5* after 6 h UV-B. Bars represent means with error bars representing the SE. n=9. **b)** Change in stomatal aperture of *nced3/5*. **c)** Relative transcript abundance of *NCED3* over 6h WL $\pm$ 1 μmol m^{-2} s^{-1} UV-B. n=3. **d)** Relative transcript abundance of *NCED5* over 6h WL $\pm$ 1 μmol m^{-2} s^{-1} UV-B. n=3. Dots represent means at each timepoint with error bars representing the SE of the mean. Different letters indicate statistically significant differences at p<0.05.
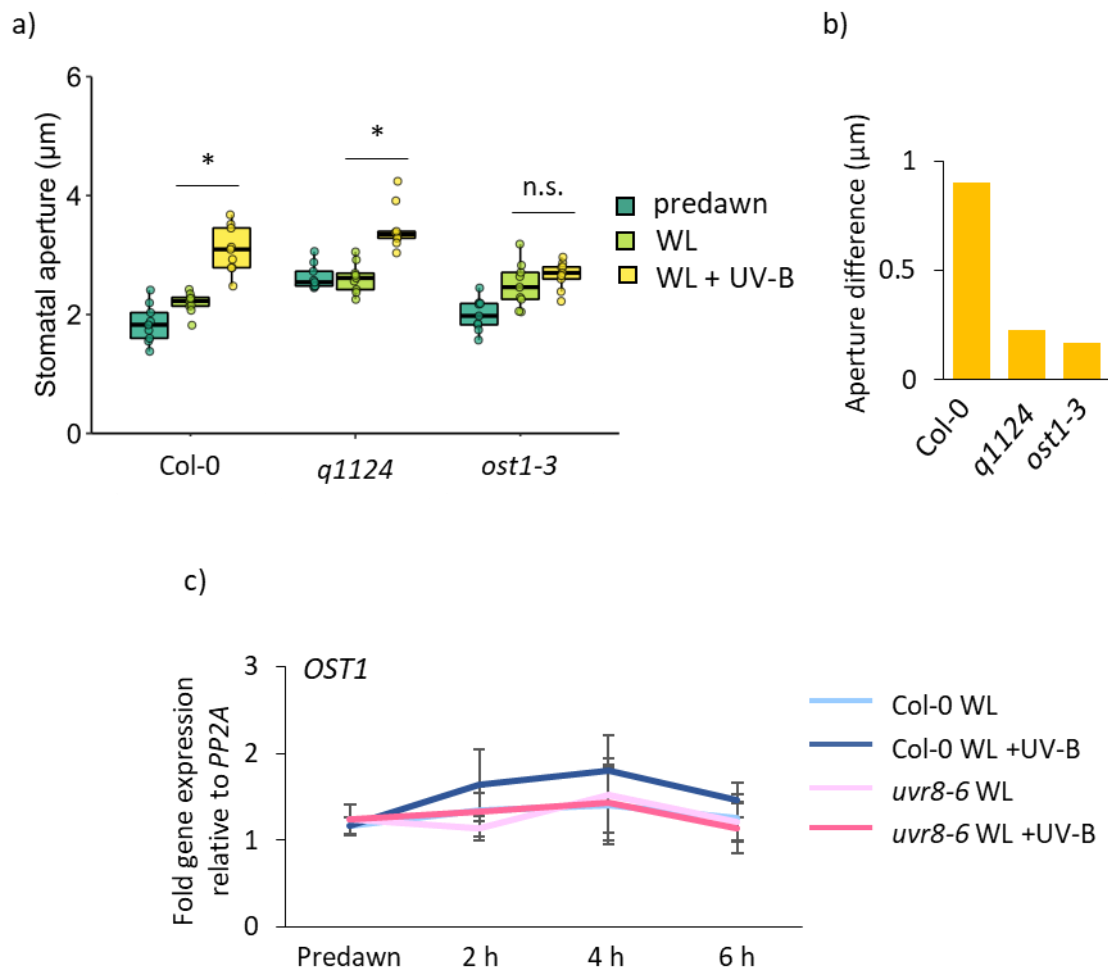
Figure 6.12 ABA signalling in UV-B-induced stomatal opening. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-day-old cotyledons were exposed to 6 h WL $\pm$ 1 µmol m^{-2} s^{-1} UV-B before stomatal apertures were measured or tissue harvested for qPCR. **a)** Stomatal aperture of *q1124* and *ost1-3* after 6 h UV-B. Bars represent means with error bars representing the SE. n=9. **b)** Change in stomatal aperture of *q1124* and *ost1-3*. **c)** Relative transcript abundance of *OST1* over 6 h WL $\pm$ 1 µmol m^{-2} s^{-1} UV-B. n=3. Data were analysed by two-way ANOVA and Tukey post hoc test. Asterisks indicate statistically significant differences from the control at p<0.001.
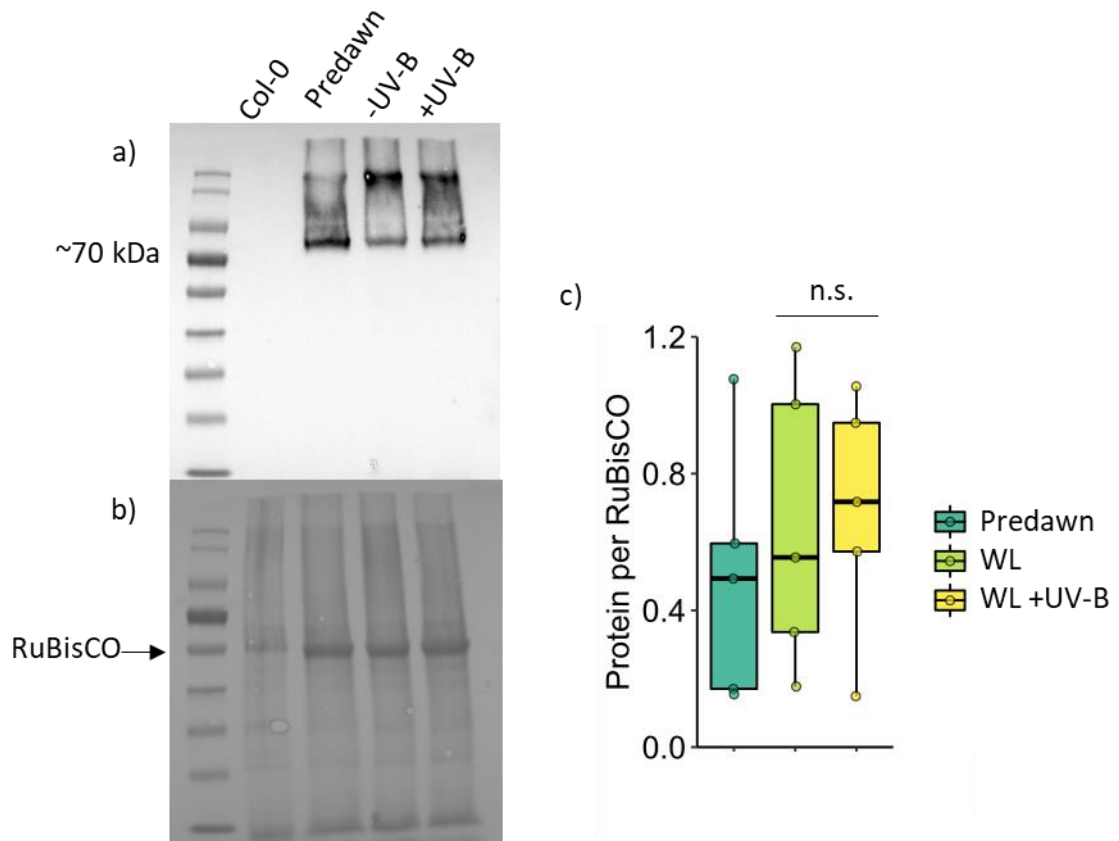
Figure 6.13 OST1 abundance as measured with GFP-tagged OST1 under the control of the native promoter in Arabidopsis thaliana seedlings. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-d-old cotyledons were exposed to 6 h WL $\pm$ 1 µmol m^{-2} s^{-1} UV-B. Proteins were extracted and diluted in Laemlli buffer. 50 µg protein sample was loaded onto the gel. **a)** OST1 abundance. **b)** Ponceau stain. RuBisCO band used for quantification is highlighted. **c)** OST1 quantification normalised to RuBisCO. n=4. Bars represent the mean with error bars representing SE.
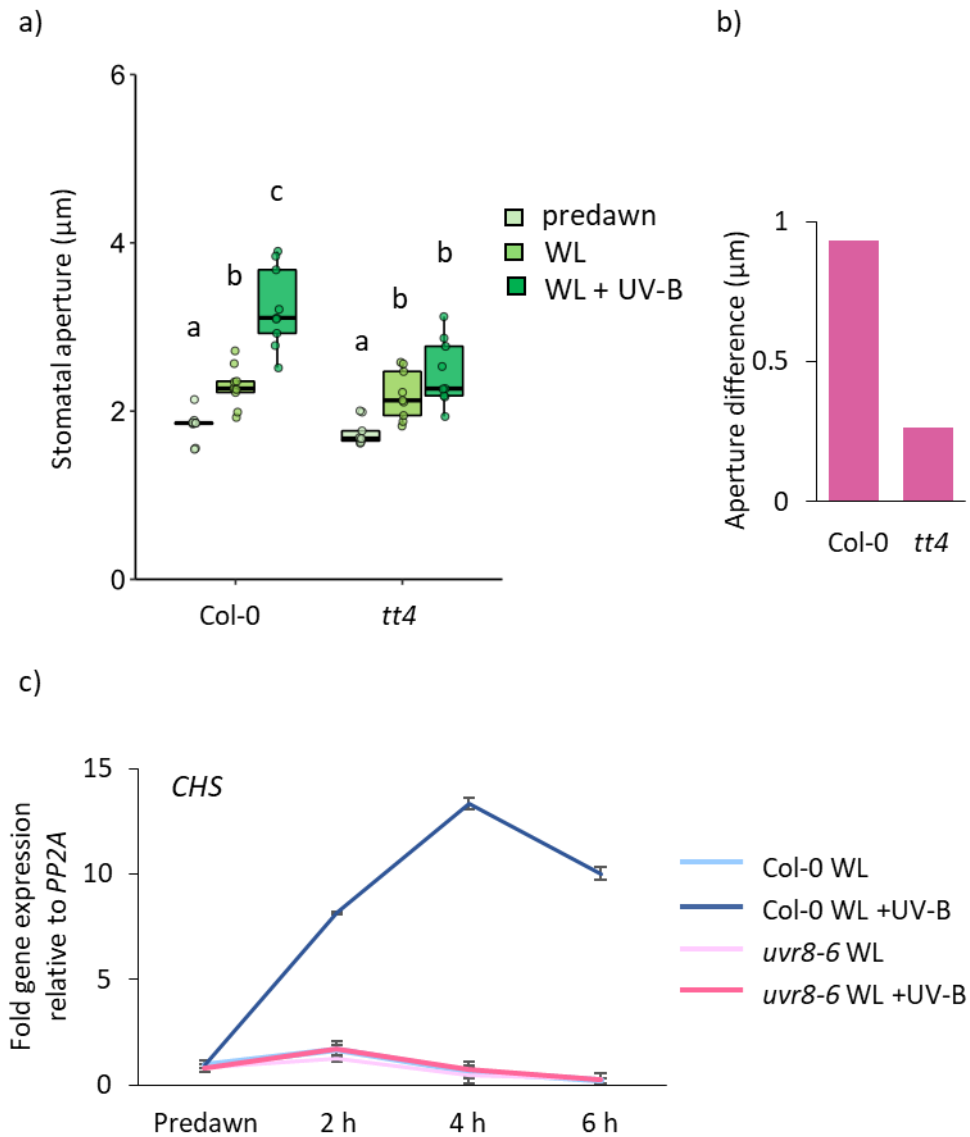
Figure 6.14 The role of CHS in UV-B-induced stomatal opening. Arabidopsis seedlings were grown in a 16 h photoperiod, 20°C and WL. 5-day-old cotyledons were exposed to $\pm$ 1 μmol m^{-2} s^{-1} UV-B before stomatal apertures were measured or tissue harvested for qPCR. **a)** Stomatal aperture of *tt4* after 6 h UV-B. Bars represent the means with error bars representing the SE. n=9. **b)** Change in stomatal aperture of *tt4*. **c)** *CHS* transcript abundance after 2, 4 and 6 h UV-B. n=3. Data were analysed by two-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.

In tomato, flavonoid content was increased significantly after 60 min of acute UV-B exposure (Shourie et al., 2014). In 11-day-old Arabidopsis seedlings, total antioxidant capacity was increased after 4 h low fluence UV-B (figure 3.4). To investigate whether a lack of CHS impaired UV-B-induced stomatal opening, *tt4* mutants were exposed to 6 h supplementary UV-B. In this study, *tt4* mutants had a much lower response to UV-B than the WT controls (F=14.06, p<0.05, fig. 6.14), suggesting a lack of antioxidants may affect stomatal movement. *CHS* transcript abundance was significantly upregulated by UV-B after 2 h and maintained for 6 h (F=27.7, p<0.05, fig. 6.14c).

6.10 Extending the findings to mature plants and other species

To assess the applicability of the findings from this chapter, the stomatal responses of Lamb's lettuce and Rocket leaf to UV-B were investigated. The response of mature Arabidopsis plants to UV-B was also assessed, using leaf discs and epidermal peels. The trend seen in leaf discs closely resembled that of the response in cotyledons (F=17.23, p<0.05, fig. 6.15a) suggesting the response is maintained throughout development and remains in mature plants. The response in epidermal peels was less than that of leaf discs and cotyledons, yet there was a significant increase in stomatal aperture in response to UV-B after 6 h (F=4.81, p<0.05, fig. 6.15c). Interestingly, Rocket leaf (F=11.21, p<0.05, fig. 6.16a) and Lamb's lettuce (F=7.06, p<0.05, fig. 6.16b) stomata also displayed enhanced opening to UV-B, suggesting that the findings presented in this chapter could have important impact on agricultural practices.
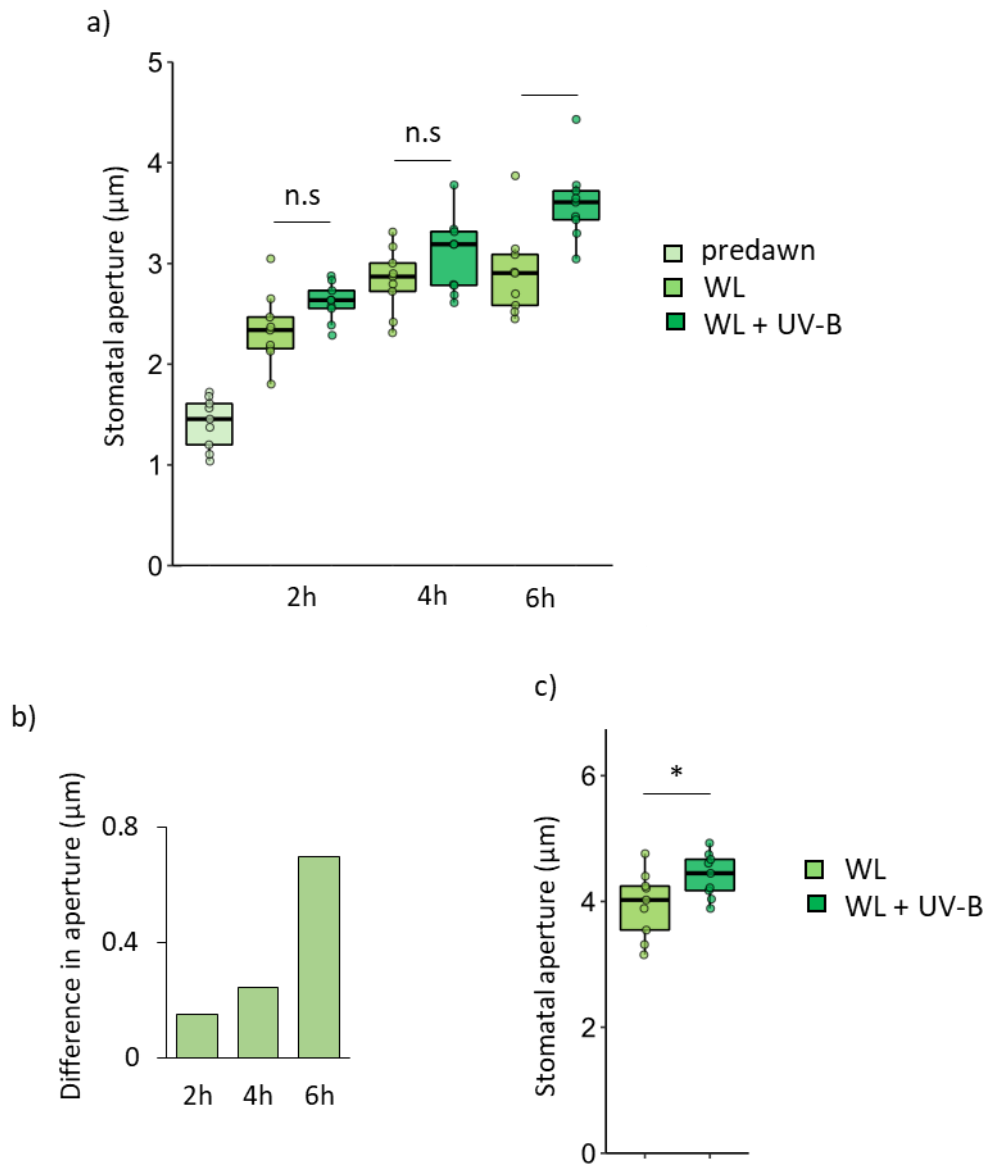
Figure 6.15 Stomatal responses of Arabidopsis leaf discs and epidermal peels to UV-B. Arabidopsis were grown in a 16 h photoperiod, 20°C and WL. 4-week-old Arabidopsis discs and peels were exposed to 6 h WL ± 1 µmol m^{-2} s^{-1} UV-B. **a)** Leaf disc stomatal apertures were measured after 2, 4 and 6 h. **b)** Change in stomatal aperture over 6 h. Bars represent (mean UV-B aperture) – (mean control aperture). **c)** Epidermal peel stomatal apertures were measured after 6 h. Bars represent means with error bars representing the SE. n=9. Data were analysed by two-way ANOVA and Tukey post hoc test. Asterisks and different letters indicate statistically significant differences at p<0.05. **=p<0.01.
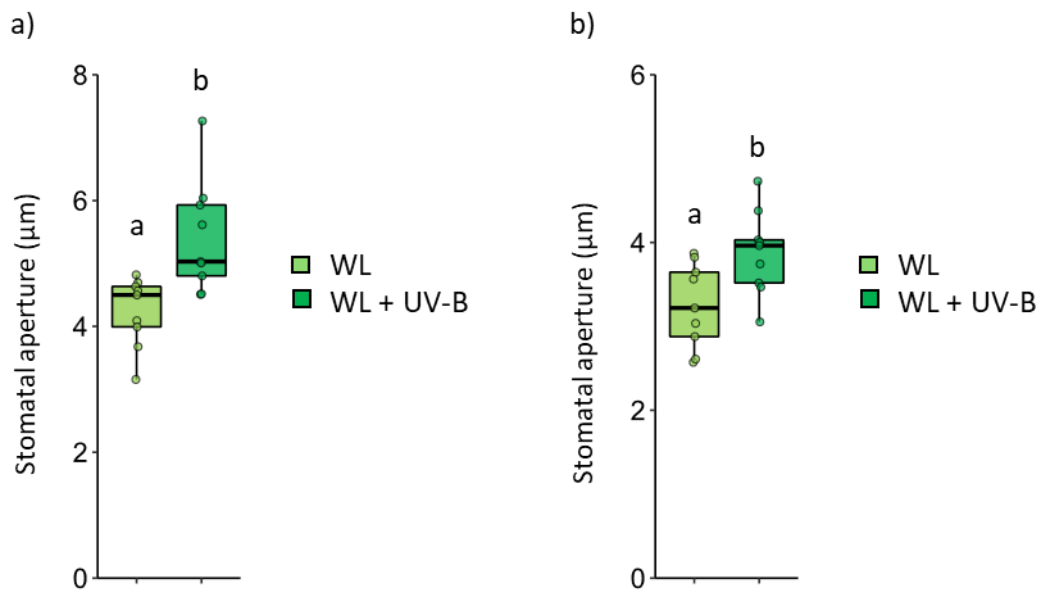
Figure 6.16 Stomatal responses of a) *Eruca vesicaria* **and b)** *Valerianella locusta* **to supplementary UV-B.**
Plants were grown in a 16 h photoperiod, 20°C and WL before exposed to 6 h WL $\pm$ 1 μmol m^{-2} s^{-1} UV-B. Bars represent means with error bars representing the SE. n=9. Data were analysed by two-way ANOVA and Tukey post hoc test. Different letters indicate statistically significant differences at p<0.05.

6.11 Discussion

Data presented in this chapter show that low fluence UV-B supplementation open stomata in a controlled environment. This response is UVR8-mediated, as the *uvr8-6* mutant did not respond to the UV-B treatment (fig. 6.2c). Recent data from Williams *et al*. (2022) suggest solar UV radiation increases leaf temperature while decreasing stomatal conductance in tomato. It is possible that a wider range of UV wavelengths can have differing effects on stomatal movement than those reported in this chapter. However, understanding the component responses are important to get a full view of the response of stomata to UV-B irradiation. For example, understanding how a short-term UV-B treatment could affect stomatal movement is important when dynamic UV-B treatments are used in an agricultural setting. Interestingly, the greatest effect of UV-B was seen after 8 h (fig. 6.2b), which may suggest cumulative effects lead to stomatal opening in response to UV-B. Many responses to UV-B involve gene transcription and protein accumulation, which may be the reason for the effect of UV-B not being visible at 2 h and then steadily increases until 8 h (fig. 6.2b). Tossi *et al*. (2014) found that high dose UV-B closes stomata in Arabidopsis, suggesting UV-B is a negative modulator of blue and red light-induced stomatal opening which may be important in order not to maintain widely open stomata and risk excess water loss in very bright sunlight (Yang *et al*., 2020). Here, a low fluence UV-B treatment opened stomata, suggesting stomatal responses are fluence rate-dependent. To investigate what signalling components may be involved in the UV-B response, several avenues were explored. UV-B did not open stomata in absence of other wavelengths of visible light (fig. 6.4). Phototropin mutants and the *phyB* mutant both had diminished responses to UV-B suggesting an integrated regulation of stomatal aperture in a dynamic light environment. Here, narrowband UV-B bulbs were used with the output wavelength at ~310 nm. Eisinger *et al*. (2000) reported that UV exposure had two major peaks for stomatal opening concentrated at 280 nm and 360 nm in broad bean, suggesting other UV-B wavelengths have the potential to open stomata. The authors suggest that this was mediated by blue light photoreceptors as supplementing blue light with UV light did not further open stomata as it did in a background of red light. Co-regulation of UV-B responses of stomata by other photoreceptors is important to note as seedlings experience a host of different light signals during early development and

an appropriate response is vital to ensure survival during a critical point in development. To investigate the influence of typical components in the UV-B signalling pathway on stomatal opening in response to UV-B, a *hy5hyh* double mutant and a *cop1* single mutant were used. The magnitude of the UV-B response in *hy5hyh* was very similar to that in the WT, while the response in *cop1* was much reduced (fig. 6.3). COP1 has been implicated in the response to water stress in Arabidopsis, where the water loss rate was much faster in a *cop1-4* mutant compared to WT, due to the failure to close stomata (Moazzam-Jazi *et al*., 2018). Interestingly, the authors further found that the lack of closure seen in the *cop1* mutant in the dark could be due to reduced MYB61 abundance, as MYB61 and MYB61 were previously shown to regulate stomatal aperture (Liang *et al*., 2005; Cominelli *et al*., 2005). Furthermore, COP1 was shown to promote ABA-induced stomatal closure by modulating the abundance of phosphatases and the phosphorylation status of, for example, OST1, a key component in ABA-induced stomatal closure (Chen *et al*., 2021). Here, the *cop1* mutant had significantly more open stomata in all conditions compared to the WT, and also failed to open significantly in response to UV-B. However, because of the magnitude of apertures, it must be noted that further opening in response to environmental factors may not be as prominent as in the WT. It would be interesting to investigate the involvement of COP1 in the UV-B response by using an induced knockdown or investigate the effect of reduced abundance of SPA proteins as these are often found in protein complexes with COP1 and will affect COP1 signalling if absent. PIFs have been implicated in stomatal aperture regulation (Wang *et al*., 2010; Li *et al*., 2022). Particularly, Li *et al.* (2022) report that PIFs have a negative role in stomatal opening in rice and maize, which is supported by data from Arabidopsis presented in this chapter (fig. 6.6). UV-B irradiation results in reduced *PIF4* and *PIF5* gene transcript and PIF4 and PIF5 protein abundance (Hayes *et al*., 2014; Hayes *et al*., 2017; Tavridou *et al*., 2020; Sharma *et al*., 2019). It may be that the pathway leading to UV-B-mediated PIF degradation also controls stomatal aperture under UV-B, however the link between PIFs and stomatal opening remained unclear. It was hypothesised that two possible effectors in the response could be ABA and auxin. While the role of ABA in stomatal closure is well-established, the role of auxin has long been contended in the literature with both opening and closure observed (Xiao-Ping and Xi-Gui, 2006; Snaith and Mansfield, 1984). Here, auxin treatment of cut cotyledon pairs resulted in reduced responses to UV-B in a dose-dependent manner, identifying

a novel role for auxin in UV-B-mediated stomatal regulation (fig. 6.9a). To support these data, qPCR of auxin biosynthesis genes was performed. As expected, UV-B reduced transcript abundance of *IAA19, IAA29,* and *YUC8* in WT controls while the response was abolished in the *uvr8-6* mutant. The greatest effect occurred at 6 h for *IAA29* and *YUC8* (fig. 6.9d and e). This trend follows the stomatal response of the WT and *uvr8-6* further supporting regulation of auxin biosynthesis transcripts may be important to UV-B-mediated regulation of stomatal aperture. Unfortunately, it was not possible to visually establish a difference in auxin activity as the cotyledons of the DR5-GUS seedlings did not stain (fig. 6.10). It is conceivable that auxin is present in such small quantities in cotyledons that it was impossible to see a difference (Gallagher, 1992) and more sensitive techniques such as liquid chromatography/mass spectrometry would need to be used.

To investigate the role of ABA in UV-B-induced stomatal opening, the ABA biosynthesis mutant *nced3/5* and the quadruple ABA receptor mutant *q1124* were used. The *nced3/5* mutant had a WT response to UV-B (fig. 6.11a and b), while the receptor mutant response was absent (fig. 6.12a and b). These data suggest that ABA perception is likely involved in the UV-B response. Reports have showed that ABA concentration is increased in response to UV-B (Rakitin *et al*., 2008), however little evidence exists connecting UV-B to ABA signalling. It may therefore be difficult to establish the true role for ABA in this response. It would be interesting to measure ABA in WT cotyledons during stomatal opening in response to UV-B to investigate the relationship between stomatal opening and ABA concentration. To further investigate ABA signalling, the ABA-insensitive *ost1-3* mutant was exposed to UV-B in the same manner, before stomatal aperture measurements. *ost1-3* also showed a small response to UV-B but it was much smaller than the WT response, suggesting downstream ABA signalling also to be involved (fig. 6.12a). Western blots of the OST1 protein expressed under the control of the native promoter showed no difference in protein abundance following UV-B treatment (fig. 6.13). A similar result was observed for transcript abundance of *OST1* (fig. 6.12c). These data suggest UV-B to have minor effects on transcript and protein abundance of OST1, yet when the protein is completely absent, the response to UV-B is impaired. It is possible that UV-B could regulate OST1 post-translationally and thereby regulate stomatal aperture. OST1 activity is controlled via regulation

of its phosphorylation state, and thereby the activity of phosphatases. For example, PP2C maintains OST1 dephosphorylation and inactivity. During ABA-induced closure, ABA inhibits PP2C, which contributes to reduced phosphorylation of OST1, and subsequent autophosphorylation and its enzymatic activity (Yunta *et al*., 2011). ABA-independent activation of OST1 has also been shown (Yunta *et al*., 2011). Furthermore, MAP3Kinase-dependent SnRK2-kinase activation is required for ABA signal transduction during osmotic stress (Takahashi *et al*., 2020). Interestingly, MAPK1 phosphatase, a key regulator of MAP kinases MAPK3 and MAPK6, accumulates in response to UV-B and regulates these under UV-B stress (Gonzalez Besteiro and Ulm, 2013). It is therefore possible that UV-B could regulate the phosphorylation status of OST1, which in turn could influence OST1 activity during stomatal opening in response to UV-B, however further experiments would need to be carried out to confirm this.

Stomatal aperture is regulated by changes in ROS (Pei *et al*., 2000). It was hypothesised that a lack of flavonoids which neutralise ROS, would cause stomata to respond differently to UV-B. Interestingly, no difference in aperture was found in the *tt4* mutant under WL conditions, suggesting flavonoids do not control stomatal aperture in this condition. Under UV-B however, stomata of *tt4* mutant seedlings did not respond to the same extent as the WT (fig. 6.14), assigning a previously unknown role to flavonoids. It is possible that an accumulation of ROS (which has a negative effect on stomatal opening) in the *tt4* mutant in UV-B maintains smaller stomatal apertures, while in the WT under UV-B where flavonoids are upregulated and able to remove ROS, this negative effect on aperture is not seen. It would be interesting to analyse ROS accumulation in the seedlings under low fluence UV-B to further investigate the role of ROS signalling in the UV-B response.

Tossi *et al*. (2014) showed that guard cells in epidermal peels of mature Arabidopsis close stomata in response to high fluence rate UV-B. Here, it was investigated whether a low fluence UV-B could open stomata in epidermal peels and in leaf discs of mature Arabidopsis in the conditions used here. Indeed, UV-B opened stomata in both discs (fig. 6.15a) and peels (fig. 6.15c), with epidermal peels responding slightly less to the treatment. In the WL condition, stomata of the peels were already greatly open, which may contribute the seemingly small response to UV-B. In leaf discs, the greatest difference between the

control and the UV-B treatment was found at 6 h (fig. 6.15b), following the observed trend in cotyledons (fig. 6.2a). These data suggest that low fluence UV-B can open stomata over the course of the day in mature plants as well as seedlings, providing a caveat to the generally accepted notion that UV-B closes stomata.

It was established in this chapter that Lamb's lettuce and Rocket stomata open in response to UV-B, suggesting conservation of the response and perhaps also of the opening mechanisms upon which UV-B acts (fig. 6.16a and b). This further suggests that there is the potential to manipulate stomatal aperture using UV-B in an agricultural setting as it is possible that relevant agricultural species have similar responses to UV-B. However, further research is required to fully understand how different species are affected by a low fluence UV-B treatment.

Low fluence rate UV-B is a regulatory signal of bright sunlight. In this chapter, it was shown that low fluence UV-B opens stomata. In contrast, high fluence rate UV-B is a stress signal and closes stomata in a ROS-dependent manner. Fluence rate is therefore an important factor to consider when supplementing with UV-B.

CHAPTER 7 General discussion

UV-B is a component of natural sunlight with many different effects on plant growth and development. Initially, UV-B was considered a stressor for plants, however it was soon found that low fluence rates of UV-B also has more subtle effects on plants without causing major stress responses (Brown and Jenkins, 2005). Most conspicuously, UV-B contributes to inhibition of hypocotyl and petiole elongation in several different plant species, alters leaf size and thickness, and level of hyponasty (Wellmann, 1976; Ballaré *et al*., 1995; Kim *et al*., 2002; Gray *et al*., 1998; Ballaré *et al*., 1990; Franklin and Whitelam, 2005; Hayes *et al*., 2014; Hayes *et al*., 2017; Sharma *et al*., 2019; Tavridou *et al*., 2020; Grammatikopoulos *et al*., 1998; Fina *et al*., 2017). UV-B also contributes to the accumulation of protective, sun-screening pigments, such as flavonols and anthocyanins in epidermises of plants (Frohmeyer and Staiger, 2003; Jansen *et al*., 1998; Jenkins, 2009). With such profound influence of

UV-B on plant growth, development and ability to produce protective molecules, the central question was whether UV-B has the possibility to protect plants from other environmental stresses as some stress signalling pathways may overlap with UV-B signalling. The focus was on temperature signalling as there is clear crosstalk between moderately high temperature and UV-B responses, as well as potential for UV-B-induced upregulation of flavonoid biosynthesis to promote freezing tolerance. The effects of UV-B on temperature stress and data from chapter 3 will be discussed in 7.1 below.

As part of an iCASE placement with the industrial partner LettUs Grow, the effects of UV-B on growth and development parameters of the microherb Radish Leaf were investigated. This was the first trial in the LettUs Grow vertical aeroponic farm in which UV-B was investigated, and provided LettUs Grow with information which they can take forward for future trials. The data from this placement and impact of the research will be discussed in 7.2.

Stomata are microscopic pores on aerial plant tissues through which gas exchange occurs. Stomata govern the uptake of CO_2 for photosynthesis, and the release of O_2 to the atmosphere, giving them a hugely important role in both plant and animal life (Woodward and Hetherington, 2003). Stomatal responses to visible light have been well-studied over the past century, however less is known about the effects of UV wavelengths on stomatal aperture responses. Tossi *et al*. (2014) showed that UV-B promotes stomatal closure in a ROS-mediated manner. This response may be attributable to stress as a high fluence rate was used. I therefore investigated the effects of low fluence rate UV-B in a growing-seedling system with the aim to gain an understanding of how UV-B affects stomatal apertures during early plant development. The effects of low fluence UV-B on stomatal opening will be discussed in 7.3.
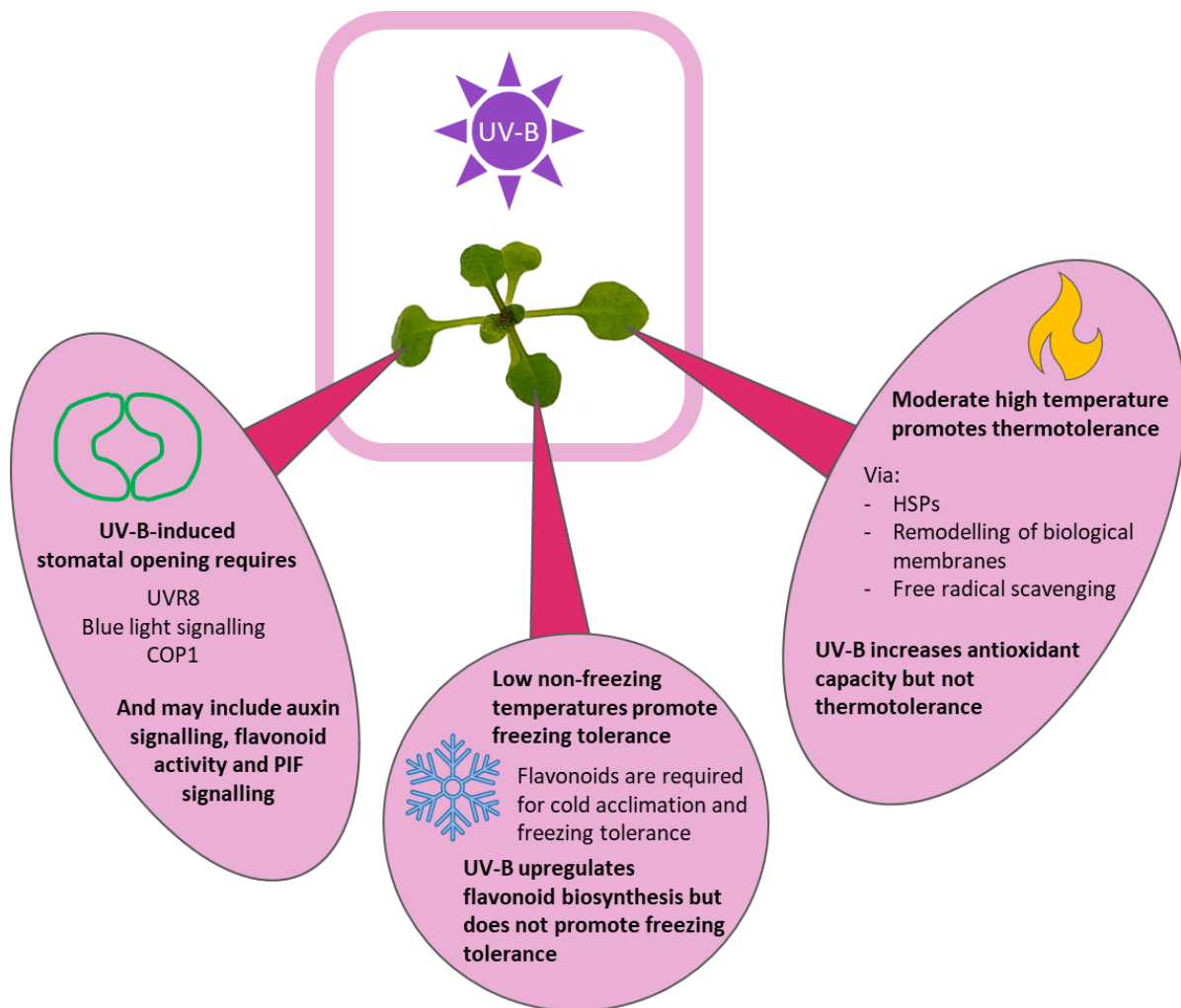
Figure 7.1 Proposed signalling involved in stomatal opening, freezing tolerance and thermotolerance and the influence of UV-B on these responses. Evidence was presented in this thesis that UV-B has no influence on freezing tolerance or thermotolerance in Arabidopsis, suggesting UV-B as a light signal does not affect how well a plant withstand these two major environmental stressors in the conditions used here. Low fluence rate was shown to increase stomatal apertures, opening stomata. This response was shown to require the UV-B photoreceptor, UVR8, the E3 Ub ligase, COP1, and could only occur in a background of white light, where the phototropin photoreceptors were shown to be necessary for opening. This thesis also discovered potential downstream signalling components and further research could elucidate the precise involvement of auxin and PIF signalling, as well as flavonoid activity in the UV-B-induced stomatal opening response.

7.1 The role of UV-B in temperature stress responses

Plants as sessile beings cannot move when environmental conditions become unfavourable. Instead, many plants can adapt to fluctuating conditions successfully. Despite this adaptability, when conditions move beyond what a plant is adapted to withstand, stress occurs. Stress can occur in response to many different signals. In this thesis, temperature stress was investigated, with the aim to characterise any ameliorating effects of UV-B on high and low temperature stress responses of *Arabidopsis thaliana*. It has been shown by electrolyte leakage assays that plants lacking components of the flavonoid biosynthesis signalling pathway have reduced freezing tolerance (Schulz *et al*., 2016). Flavonoids appear to be elements that are produced in response to stress, as it has been shown they are produced in response to both high and low temperature, as well as other abiotic stress conditions such as high salinity (Sharma *et al*., 2015; Schulz *et al*., 2016; Schulz *et al*., 2021; Li *et al*., 2017). In chapter 3, it was hypothesised that UV-B-induced flavonoid production could contribute to increased freezing tolerance. However, when plants developed in UV-B were exposed to freezing temperatures they showed similar electrolyte leakage as the leaf discs from plants that were never exposed to UV-B. This applied to both cold acclimated and non-cold acclimated plants. It must be noted that the cold acclimation treatment given was only 12 hours, yet contributed significantly to lowering electrolyte leakage at -2 and -5 °C. Because cold acclimation is so effective, additional effects of UV-B may be difficult to establish. However, as the cold acclimated plants that received the -8°C treatment had similar levels of electrolyte leakage to the non-acclimated plants, and UV-B had no effect here, it was concluded that UV-B does not contribute to freezing tolerance in the conditions used here (fig. 7.1). These data were supported by observations showing no effect of UV-B on the transcript abundance of two key *COR* genes, *COR15a* and *COR47*. It is possible that a shorter, higher fluence UV-B treatment could have a different effect on *COR* gene expression, but due to time constraints this was not investigated further. To further investigate the possible role of flavonoids in the UV-B response to cold, *tt4* mutants deficient in *CHS* (and therefore flavonoids) were given 4 days of UV-B treatment before electrolyte leakage measurement. As expected, *tt4* mutants in control conditions showed increased electrolyte leakage, similar to previous reports (Schulz *et al*., 2016). Interestingly, when *tt4* were exposed to UV-B prior to

freezing, the electrolyte leakage was significantly lower than the control, rescuing this phenotype. The precursor to flavonoids is 4-coumaryol-CoA which is also a precursor to many other natural products of plants. When a plant is deficient in CHS which catalyses the initial reaction of flavonoid biosynthesis, it is possible that the pool of 4-coumaryol-CoA is used for the production of other molecules, which may have protective effects on freezing tolerance. It would be interesting to investigate the pigment profile of these plants post UV-B using this layer chromatography or HPLC. Chapter 3 provides evidence that despite no obvious effect of UV-B on freezing tolerance in WT controls, UV-B may still have a role in freezing tolerance, especially when certain signalling pathways are impaired.

It is predicted that more frequent and severe high temperature events will occur as a result of global warming (Collins *et al.*, 2013). While plants are equipped to cope with relatively slow increases in temperature resulting in acclimation to high temperatures (acquired thermotolerance), more sudden onset of high temperature stress conditions can have devastating effects on plant growth and development. Many growing facilities such as glasshouses and polytunnels use materials which attenuate UV-B. It is not known whether this filtering of UV-B could have detrimental effects on thermotolerance in plants. UV-B upregulates both ROS and ROS-scavenging systems in Arabidopsis (Dai *et al.*, 1997; Treutter, 2005; Weston and Mathesius, 2013; Li *et al.,* 1993; Nakabayashi *et al.,* 2013). Similarly, high temperature stress has been shown to increase the ROS load leading to cellular stress. In chapter 3 we hypothesised that due to the effects of UV-B on ROS neutralisation, UV-B could protect plants from heat stress. First, it was shown that different UV-B treatments increases antioxidant capacity of Arabidopsis seedlings, with the most effective treatment being UV-B throughout development. However, this antioxidant capacity did not translate into increased thermotolerance as no positive effects of UV-B on basal thermotolerance were seen in three different accessions of Arabidopsis. Neither was there an effect of shorter or higher fluence rate UV-B treatments. Due to the efficacy of high temperature acclimation, the effects of UV-B on acquired thermotolerance were only briefly studied. In 7d old seedlings, UV-B may in fact have negative impact on acquired thermotolerance. This is important to note as young seedlings may be more susceptible to temperature stress while also exposed to UV-B. In some instances, UV-B is used as pest control (Meyer *et al.*, 2021)

and it may be that very young seedlings should be protected from UV-B, or given a very low dose (<1 µmol m⁻² s⁻¹) before a higher dose is applied, if a period of increased temperature is anticipated. The temperature treatment used in chapter 3 was very high (45°C). However, as was seen in the summer of 2022, temperatures in the UK reached 40.3°C, while similar temperatures were also recorded across Europe, making this research highly topical.

7.2 The effects of UV-B on Radish Leaf development in the LettUs Grow indoor, vertical, aeroponic farm

Indoor vertical farms bring many benefits to the farming industry. The possibility of utilising otherwise unused space for food production in controlled conditions, where environmental stress and pest exposure is reduced, is very attractive to growers. Particularly the use of old industrial buildings, or establishment of a vertical farm in areas where there is no land for farming, could lower food mileage and help the public to choose locally produced food. In addition to developing and optimising the technical components of an aeroponic system, optimising plant growing recipes is important. These recipes include information about sowing density, stratification times, growing times, watering regimes, nutrient requirements, and lighting conditions. UV-B had not previously been investigated in the LettUs Grow system for any species of plant. Due to the time constraint of the CASE placement, the fast-growing and relatively small Radish Leaf was chosen for the trials. Microgreens have become popular due to their potential to add important micronutrients and bioactive compounds to the human diet while also being visually attractive (Teng *et al.*, 2021). It was therefore interesting to LettUs Grow whether UV-B could increase nutritional values and/or aesthetics without major effects on plant growth. For this reason, two different UV-B treatments were chosen: a short, daily dose, and a longer end-of-production dose. It was hypothesised that the end-of-production treatment would have the greatest effect on pigment accumulation and antioxidant capacity while maintaining plant growth. Chapter 4 showed that while neither treatment had major impacts on plant growth, the daily UV-B treatment may have a small effect on leaf size while the end-of-production treatment did not. Additionally, the end-of-production treatment had a greater effect on flavonol accumulation, which may be considered valuable from a nutrition perspective. However, antioxidant capacity was somewhat higher for the daily UV-B treatment

(similar to the data presented in chapter 3), than for the end-of-production treatment, suggesting that different UV-B treatments may be beneficial depending on the desired effect. The effects of UV-B on water preservation were not investigated during this placement but would be an interesting avenue to explore in order to further improve the growth recipes of produce grown in controlled environment agriculture. At the present time, UV-B bulbs are expensive and for the small benefit they may bring to plant development, they are not currently viable as an option. The shift from standard fluorescent light bulbs to LEDs may bring more affordable options for UV-B supplementation. This means that developing plant recipes is a valuable way to stay ahead of competitors once inexpensive UV-B LEDs are available. Data presented in chapter 4 highlight that UV-B supplementation may be an important addition to growth recipes in vertical aeroponic systems and may add value to produce.

7.3 The effects of high temperature UV-B on stomatal opening in *Arabidopsis thaliana* seedlings

In order to acclimate to their surroundings, plants regulate stomatal apertures in order to control water status and gas exchange. High temperature (35°C) has been shown to open stomata in epidermal peels of mature Arabidopsis, and this response involves the H^+ ATPases and blue light receptors phototropins 1 and 2 (Kostaki *et al*., 2020). Despite performing a major role in the regulation of thermomorphogenesis responses to elevated temperature, the transcription factor PIF4 was not involved as a *pif4* mutant displayed a WT response in the HT conditions. PIFs are central to plant development and regulate many processes such as hypocotyl elongation in young seedlings (Hayes *et al*., 2014; Hayes *et al*., 2017; Sharma *et al*., 2019; Tavridou *et al*., 2020). Therefore, the effects of HT on stomatal apertures and whether PIFs play a role in stomatal aperture regulation in young seedlings were investigated. Chapter 5 showed that HT opens stomata in both the light and dark, and the effects are significant after 30 min of HT exposure. To compare this response to temperatures that initiate thermomorphogenesis, cotyledons were also exposed to 28°C for 2h. This temperature treatment failed to open stomata, suggesting that thermomorphogenesis responses may be separate from stomatal responses. Furthermore, both the quadruple *pif* mutant (*pif1345*) and the single *pif4* mutants did not open stomata in response to HT. This suggests that PIFs are involved in stomatal responses to HT in

cotyledons, and that developmental differences may exist in the regulation of stomatal aperture. It is not clear from these data if PIF4 is the dominant factor in this response, and further research would be required to elucidate the precise roles of PIFs in HT-induced stomatal opening in Arabidopsis seedlings.

In chapter 6, the effects of low fluence rate UV-B on Stomatal opening in Arabidopsis cotyledons were explored (fig. 7.1). In contrast to the effects of high fluence rate UV-B (Tossi *et al.*, 2014), low fluence effectively opened stomata in Arabidopsis cotyledons, and the effect was significant after 6 h. A small effect was seen at 4h, though this was not significant. Plants were grown in a 16h photoperiod. It appears that as midday approaches, UV-B has the greatest effect on stomatal opening, consistent with published data showing that UVR8 transcriptional responses are greatest at midday (Fehér *et al.*, 2011). Alternatively, it is possible that components that promote opening must accumulate before an effect is observed. Common UV-B signalling pathway components were shown to be required for the UV-B response. Both the *uvr8-6* and *cop1* mutants failed to open stomata in response to UV-B, suggesting that UV-B-induced stomatal opening occurs via UVR8 and COP1. COP1 has been shown to be a repressor of stomatal opening (Mao *et al.*, 2005). Similarly, here *cop1* stomatal apertures were wide open in the dark and control conditions. This further suggests that in response to UV-B, when there is no COP1, stomata cannot open any further. In contrast, the *hy5hyh* mutant showed a WT phenotype following UV-B exposure, suggesting that UV-B-induced stomatal opening does not involve HY5 and HYH-regulated gene expression. As HY5 regulates many genes involved in photomorphogenesis, it is possible that UV-B-induced stomatal opening is not linked to photomorphogenic growth responses. Interestingly, UV-B alone failed to open stomata as when UV-B was applied in a background of darkness, stomatal apertures did not change. This suggests that other photoreceptors than UVR8 are involved in UV-B-induced stomatal opening. Extensive crosstalk between photoreceptors in many responses to environmental stimuli has been observed (Fraser *et al.*, 2016; Franklin and Whitelam, 2004). It appears that functional UVR8, phot1, phot2 and phyB are required for the stomatal opening in UV-B as each of the single mutants failed to respond. Data presented in chapter 6 suggest that PIFs may be part of the signalling network. PIFs are implicated in many responses in plants, and as discussed in connection with stomatal responses to HT, may also play an important role in seedling stomatal

responses. PIFs regulate auxin biosynthesis and the role of auxin in plant growth is well-established (Favero *et al.*, 2021). The role of auxin in stomatal aperture regulation is, however, less clear. Here, no difference in apertures were seen in response to auxin in control conditions or darkness, but when UV-B was applied, plants exposed to auxin failed to open stomata. This suggests that auxin is a negative regulator of stomatal opening, and that UV-B may regulate auxin levels, which in turn regulates aperture. It is not clear, however, how auxin regulates guard cell specific ion fluxes which ultimately regulate aperture. There is evidence that auxin coordinates plant growth together with ABA (Emenecker and Strader, 2020). ABA biosynthesis appeared not to be involved in stomatal opening in response to UV-B. Instead, it is possible that ABA sequestering, release and perception play some role as the quadruple ABA receptor mutant (*q1124*) had impaired opening in response to UV-B, as did the ABA signalling network component mutant *ost1*. Despite this, UV-B does not appear to regulate *OST1* transcript abundance or OST1 protein levels. Further research is required to elucidate the involvement of ABA in UV-B-induced stomatal opening. Chapter 6 also showed that UV-B-induced stomatal opening may be a conserved response, as both rocket and lettuce stomata opened in response to low fluence UV-B. The novel discovery that low fluence rate UV-B can open stomata in young seedlings has considerable significance for understanding seedling establishment in agriculture, in addition to further advancing the field of stomatal biology.

Conclusions

This thesis explored the effects of UV-B and temperature on a range of plant metabolic, physiological, and developmental responses. Though no obvious effects of UV-B on freezing tolerance were observed, UV-B may play a role in low temperature stress tolerance due to its effects on flavonoid biosynthesis, as shown in chapter 3. Little evidence was found for a role of UV-B in thermotolerance, despite positive and cumulative effects on antioxidant capacity. No positive effects of UV-B were observed in the preliminary experiments into WUE, wilting and drought, suggesting the effects of UV-B on water status of Arabidopsis is minimal. Chapter 5 provided evidence for a PIF-mediated mechanism regulating stomatal aperture of young seedlings in response to high temperature. This is relevant as the roles of PIFs have, to our knowledge, not previously been explored in HT-induced stomatal opening and opens

a new avenue for PIF research. Evidence presented in chapter 6 show that levels of UV-B that cause physiological changes, but are not stressful, also induce stomatal opening. In this response, signalling components such as UVR8, COP1 and PIFs were shown to be involved. It is unclear what the effectors of the response are, but both auxin and ABA signalling appear to be involved. Future work in this field could, for example, include transcriptome analysis to identify genes of interest that are involved in stomatal movement and that are regulated by UV-B in young seedlings and in the conditions used in this study. This could allude to important signalling components in the response that are still unknown. Understanding the effects UV-B may have on temperature and stomatal responses in plants is important as a growing world population and global warming put an every-increasing pressure on food production.

CHAPTER 8 APPENDIX

Supplementary figures
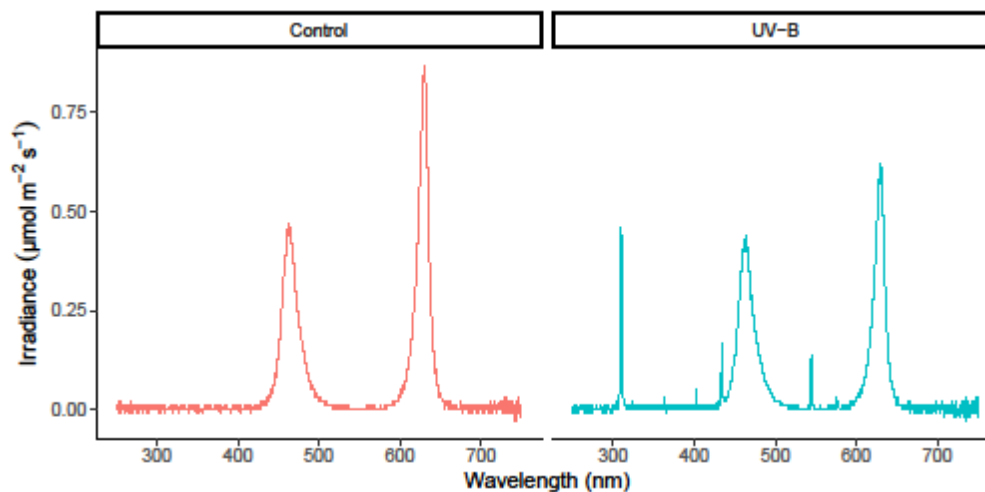
Figure S.1 Total spectral irradiance of cold treatment chambers. A peak can be seen at ~310 nm indicating the UV-B treatment in the right-hand panel. Measured with OceanOptics FLAME spectroradiometer and analysed with OceanView software (version 2.0.8).
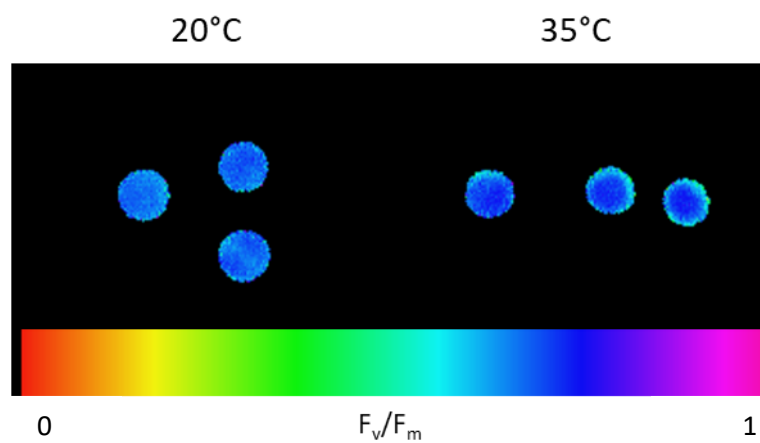
Figure S.2 Chlorophyll fluorescence after 35°C heat treatment in 3-week-old Arabidopsis leaf discs. Leaf discs from 4-week-old *Arabidopsis thaliana* grown in 20°C and a 16 h photoperiod were floated on 10/50 (10mM MES, 50mM KCl) buffer for 2 h to acclimate and then transferred to fresh 10/50 buffer and either 20°C or 35°C and dark adapted for 20 min prior to analysis. Chlorophyll fluorescence was measured with a WALZ MAXI-PAM as Fv/Fm and analysed with the ImagingWin (version 1.0) software.
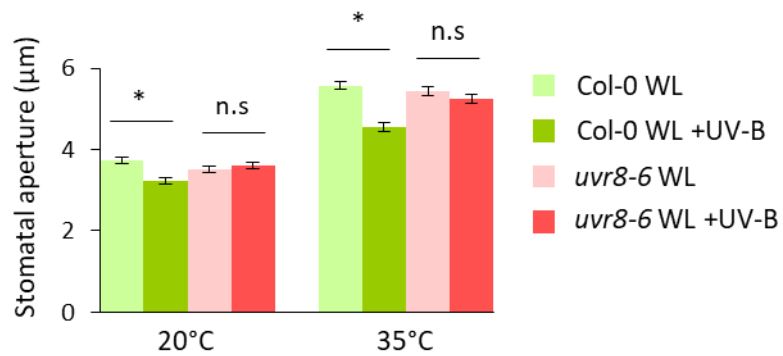
Figure S.3 Stomatal apertures after high fluence UV-B in 20 and 35°C. Leaf discs from 3-week-old Arabidopsis were grown in a 12 h photoperiod and 20°C before exposed to high fluence (2.7 µmol m^{-2} s^{-1}) UV-B and a 20 or 35°C treatment. A total of 90 stomatal apertures were measured from nine discs over three consecutive experiments. Bars represent the means with error bars representing the SE. Data analysed with 2-way ANOVA and Tukey post hoc test. Statistically significant differences from the control in the same genotype background or treatment are shown with asterisks. n=90.
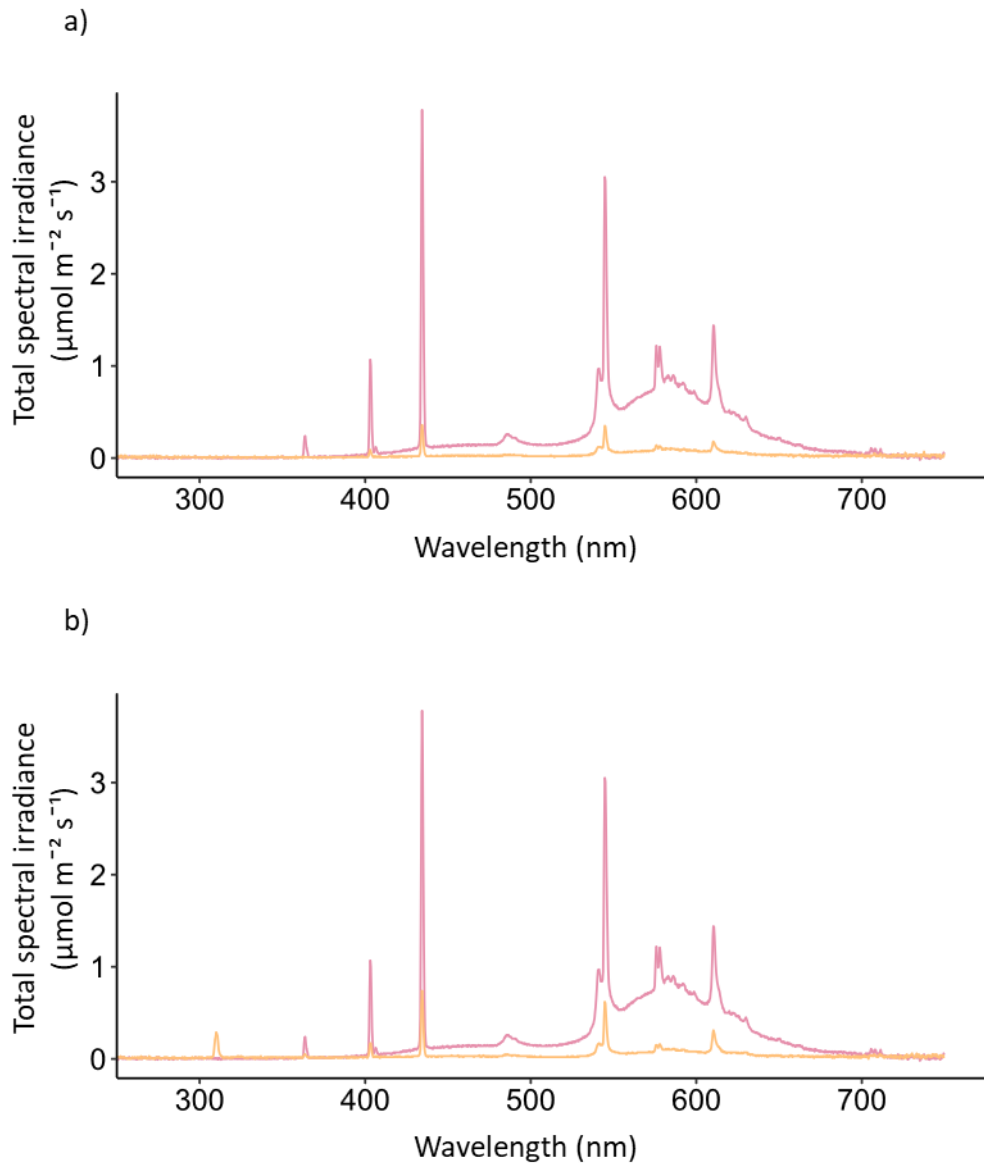
Figure S.4 Total spectral irradiance in tanks used for stomatal bioassays. a) Spectrum for the no UV-B treatment. **b)** Spectrum for the UV-B treatment. A peak can be seen at ~310 nm indicating the UV-B treatment. Measured with OceanOptics FLAME spectroradiometer and analysed with OceanView software (version 2.0.8).

Stomatal responses of Arabidopsis seedlings to dynamic environmental signals

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1. Introduction

Stomata are microscopic pores on aerial tissues of plants which regulate gas exchange between the atmosphere and inner plant tissues. Each pore is flanked by specialised **guard cells**, which adjust pore **aperture** in response to **abiotic and biotic cues,** such as **light and temperature**[1]. In abundant light stomata will open to take up CO_2 for carbon fixation and photosynthesis. Conversely, stomata close in the dark. **Ultraviolet-B (UV-B)** light is a component of natural sunlight. **High fluence** UV-B light closes stomata in epidermal peels of mature *Arabidopsis thaliana*[2]. Here, we investigate the effects of **low fluence UV-B** light on **seedling** (cotyledon) stomata. **High temperature (HT)** opens stomata in mature plants[3]. We investigate the effects of HT on cotyledon stomata. We also explore some underlying signalling components of these responses.

2. UV-B opens stomata in Arabidopsis seedlings over the course of the day

Pre-dawn control
Control
UV-B

UV-B-induced change in aperture

4-6d old **cotyledons** were transferred to ±UV-B (1 µmol m⁻² s⁻¹) in a background of 70 µmol m⁻² s⁻¹ PAR.

90 apertures per genotype per treatment were measured from 9 plants. Bars represent the mean from the 9 plants ± se

3. UV-B-induced stomatal opening is UVR8-dependent

Pre-dawn control
Control
UV-B

UV-B-induced change in aperture

Col-0
uvr8-6

uvr8-6 apertures were similar to wild type apertures pre-dawn and under WL alone, however were **not increased by UV-B**, suggesting UV-B-induced stomatal opening is **UVR8-dependent**.

Stomatal apertures were measured 6 h post-dawn. Pre-dawn apertures are shown as a comparative control.

UVR8
UV-B receptor

4. UV-B-induced stomatal opening is not mediated by HY5 and HYH.

Pre-dawn control
Control
UV-B

Ws
hy5hyh

UV-B-induced change in aperture

Ws
hy5hyh

HY5 and HYH
ELONGATED HYPOCOTYL 5 and HY5-homolog bZIP transcription factors involved in responses to UV-B

hy5hyh apertures were **slightly smaller than wild type** apertures pre-dawn, in WL and WL +UV-B, however the change in aperture in *hy5hyh* to UV-B was similar to the change in the WT. This suggests that UV-B-induced stomatal opening is **not dependent** on HY5 and HYH.

Stomatal apertures were measured 6 h post-dawn. Pre-dawn apertures are shown as a comparative control.

5. Heat stress opens stomata in Arabidopsis seedlings

Temperature treatment (mins)
30 60 120

Dark control (mins)
0 120

35° C increased stomatal apertures in Arabidopsis seedlings, suggesting seedlings can effectively respond to elevated temperatures. 28° C did not increase stomatal apertures (data not shown), suggesting that thermomorphogenic responses may not rely on changes in stomatal aperture.
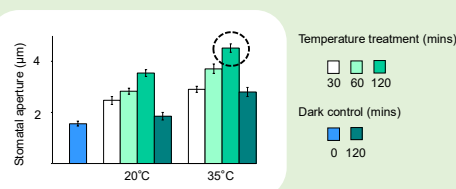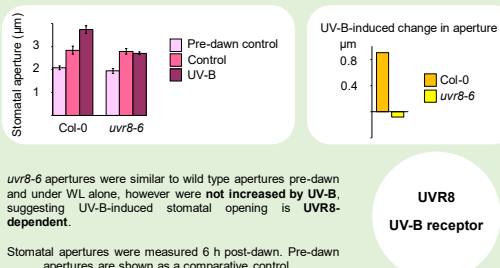
6. PIFs may be required for heat stress-induced stomatal opening

pre-dawn control
20°C
35°C

Col-0
pifq

PIFs
PHYTOCHROME INTERACTING FACTORS bHLH transcription factors. Govern many developmental light and temperature responses such as thermomorphogenesis and photomorphogenesis

5d old WT and *pPIF4:GUS* seedlings were floated on 10/50 mM MES/KCl buffer at 20° C for 2h before transfer to 20 or 35° C for another 2h. Seedlings were then washed in 90% acetone for 5 min before GUS staining solution was added at incubated at 37° C for 4h.

20°C   35°C

No visual difference in *PIF4* promoter activity between 20 and 35° C

7. Future work

Analyse stomatal apertures in several UV-B signalling mutants

Investigate transcript abundance of several UV-B signalling components

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