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Title:
The effects of ultraviolet B radiation on dark-induced foliar senescence in Arabidopsis thaliana.

The effects of ultraviolet B radiation on dark-induced foliar senescence in *Arabidopsis thaliana.*

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Submission date: 14/09/20

Word count: 10,930

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Science in the Faculty of Life Sciences.

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Abstract

Leaf senescence is an active and genetically controlled process characterised by leaf yellowing. It is essential for the remobilisation of cellular components and nutrient cycling and can occur naturally with developmental age or be induced by stressful abiotic conditions. Prolonged darkness is a particularly strong inducer of leaf senescence and this presents a problem within agriculture as many crops are stored in darkness following harvesting and during transportation. Leaf yellowing in crops degrades their nutritional quality and their cosmetic value, leading to increased waste in the food supply chain. High intensity UV-B irradiation has been previously shown to antagonise dark-induced senescence (DIS) in a UVR8-independent manner, but little work has been carried out to investigate the role of low intensity UV-B in DIS. Here, it has been found that a 4 h pre-harvest treatment with low intensity UV-B is sufficient to antagonise DIS in a UVR8 -dependent manner. This treatment did not increase leaf antioxidant content or reduce electrolyte leakage following dark incubation. UVR8-mediated antagonism of DIS is also not strongly dose dependent. This work indicates it may be possible to use short, low intensity UV-B treatments to antagonise DIS in an agricultural setting in order to reduce losses of harvested crops.

Covid-19 statement

The closure of the University of Bristol Life Sciences research facilities in March due to COVID-19 meant that all non-essential laboratory-based work was suspended. This meant that it was not possible to maintain a supply of plant material during this period, meaning any work continued once the laboratory re-opened at the end of June had to be delayed for 4-5 weeks whilst plant material was cultivated.

Prior to COVID-19, a series of qPCR experiments had been planned with the intention of looking into expression levels of senescence associated genes (SAGs) during dark incubation following treatment with UV-B light. These experiments were discussed at my APM and were due to begin at the start of April, to be completed by the end of May. This work would have required support from a senior member of the laboratory group (Dr Ashutosh Sharma) in order for training to be completed. It was not possible to complete these experiments due to both time constraints and restrictions in place on the number of people allowed in the laboratory at one time. These experiments would have contributed to the dissertation in terms of quantifying the progression of dark-induced senescence (DIS) on a finer scale and in terms of beginning to identify what elements of the DIS regulatory network were influenced by UV-B.

It was possible to finish the other research objectives laid out during my APM, though some experiments were carried out in a limited capacity, e.g. with fewer repeats, due to time constraints and availability of laboratory access. The experiments using *uvr8-6* Arabidopsis mutants were impacted by this and it was only possible to produce one full set of data. Completing these experiments in full, with the correct number of repeats, would have ensured the data was reproducible and so added weight to the conclusions drawn from it in the dissertation. It was not possible to delay my thesis submission, as I am registered to start a PhD in October 2020.

Acknowledgements

I undertook this project during a very strange year thanks to COVID-19, and it would not have been possible without the support of lots of wonderful people.

I would like to thank my supervisor Professor Keara Franklin for her invaluable support, encouragement and kindness throughout my project. I would also like to thank Dr Ashutosh Sharma for his technical knowledge and support with my laboratory work, and for providing me with Arabidopsis seeds. Thank you also to my lab group and various other members of lab 324 at the School of Life Sciences, Bristol, who welcomed me and often supported me with protocols and lab equipment.

Finally, I'd like to thank my mum, Jeannie Bowers, my nan, Gill Bowers, and my fiancé, Justin Sweetman for always believing in me.

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, is indicated as such. Any views expressed in the dissertation are those of the author.

Signed: Jessie Bowers-Martin Date: 14/09/20

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List of abbreviations

ABA	Abscisic acid
ABI5/EEL	ABA INSENSITIVE 5/ENHANCED EM LEVEL
bHLH	Basic-helix-loop-helix
Chl	Chlorophyll
Col-0	*Arabidopsis thaliana* Columbia-0 accession
CRY	Cryptochromes
Cu^{2+}	Copper (2+)/Cupric ion
DDI	Days of dark incubation
DI	Days of incubation
DIS	Dark-induced senescence
EIN3	ETHYLENE INSENSITIVE 3
FKF1	FLAVIN-BINDING KELCH REPEAT F-BOX 1
FR	Far-red light (700-800 nm)
GA	Gibberellic acid
GA2ox1	Gibberellin 2-beta-dioxygenase 1
GHG	Greenhouse gas
H_2O	Water
HY5	ELONGATED HYPOCOTYL5
HYH	HY5 HOMOLOG
JA	Jasmonic acid
LKP2	LOV KELCH PROTEIN 2
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
ORE1	*ORESARA1*
PBS	Phosphate buffered saline
PCD	Programmed cell death
PHOT	Phototropins
PHY	Phytochromes
PhyB	Phytochrome B
PIFs	PHYTOCHROME INTERACTING FACTORS
R	Red light (600-700 nm)

R:FR	Red light: far-red light ratio
ROS	Reactive oxygen species
RUP1	REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1
RUP2	REPRESSOR OF UV-B PHOTOMORPHOGENESIS 2
SAGs	Senescence-associated genes
SD	Standard deviation
SENs	Senescence-enhanced genes
Trp	Tryptophan
UV	Ultraviolet
UV-B	Ultraviolet B light (280-315 nm)
UVR8	ULTRAVIOLET B RESISTANCE LOCUS 8
uvr8-6	*ultraviolet B resistance locus 8* mutant (Col-0 background)
ZTL	Zeitlupes

Chapter 1 – Introduction

At present it is estimated that 2 billion people globally have experienced moderate to severe food insecurity, meaning that over 25% of the world's population either do not have regular access to sufficient nutritious food or are actively facing hunger and undernourishment due to reduction in the quantity of food available (FAO *et al.*, 2019). Moreover, the number of people experiencing hunger has generally increased since 2015 (FAO *et al.*, 2019) so this is clearly a growing problem.

Food insecurity is primarily driven by climate variability and extremes, conflicts and economic breakdown (FAO *et al.*, 2018) – all of which are complex, multifaceted problems that will not be easy to solve. Additionally, over the next 30 years the global population is predicted to increase by 2 billion people (United Nations, 2019), with 68% of these people living in urban areas (United Nations, 2018). A growing population, coupled with increasing urbanisation (Figure 1) and these other issues, presents a huge challenge to food security in terms of producing enough high quality, nutritious food. A key method of addressing food security will be to reduce food loss and waste (IPCC, 2019) and this

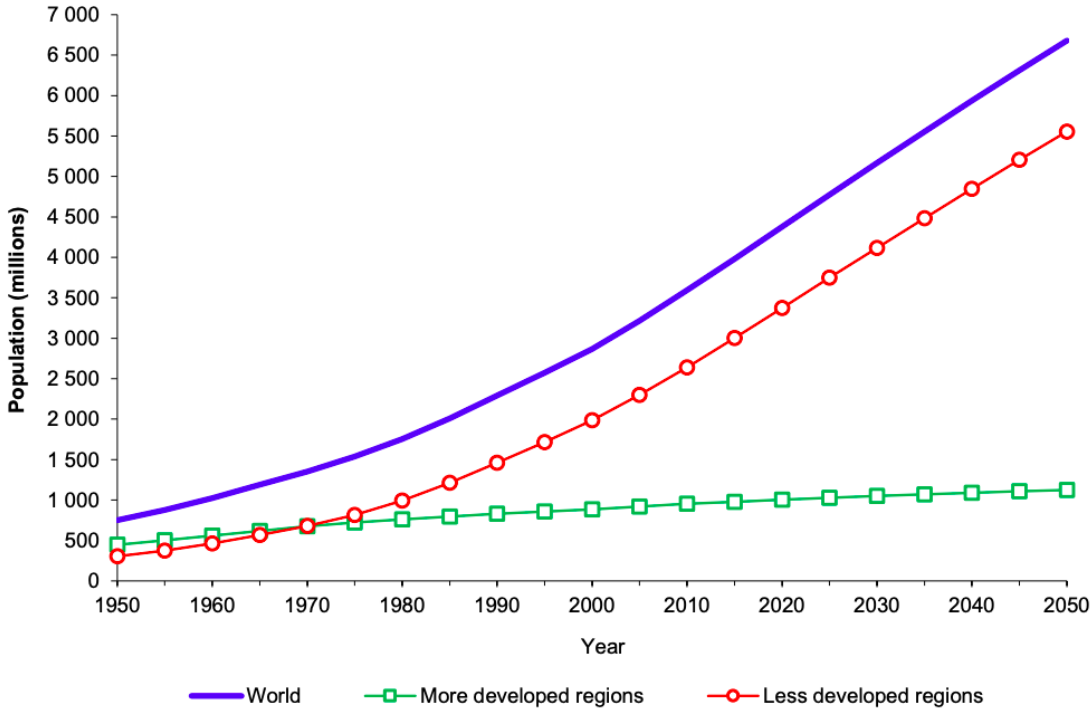

Figure 1. Estimated and projected urban populations of the world from 1950-2050. The urban population of less developed regions is growing faster than that of more developed regions, but the global trend is an increasingly urbanised population (Image from United Nations: World Population Prospects, 2018 revision).

has been identified as a key aim by the UK government as part of its Agriculture and Food Security Strategy Framework (BBSRC, 2017).

1.1 Waste in the food supply chain

Currently, 25-30% of the food produced globally is either lost or wasted at a cost of around $1 trillion (USD) annually (IPCC, 2019). This also accounts for around 8-10% of anthropogenic greenhouse gas (GHG) emissions (IPCC, 2019). The causes of food wastage differ between more and less developed countries (Porat *et al.*, 2018), but it is extremely important that these losses be reduced. In the UK, it is conservatively estimated that around 9% of perishable crops, such as fruits and vegetables, are lost between harvesting and consumption during the post-harvest stage of food production (Garnett, 2006). Furthermore, anecdotal evidence suggests 25-40% of perishable food that makes it to UK supermarkets is rejected for 'cosmetic' reasons (Stuart, 2009), though this has been difficult to quantify (Parfitt *et al.*, 2010). Cosmetic reasons for rejection include deviations in shape, size, weight or colour that do not contribute to the safety and quality of the product (de Hooge, van Dulm and van Trijp, 2018). Some cosmetic specifications are based on legislation, such as that set out by the European Union (European Union, 2011), but consumer opinion is also a strong factor and is largely responsible for the level of rejection by supermarkets (de Hooge *et al.*, 2018). Changing consumer opinion is one option for reducing food waste from this source, but this can be difficult to achieve, even when it is in the interest of consumer health (Kapur *et al.*, 2008). Another option would be to find ways of reducing the likelihood of cosmetic deviations, and other reductions in quality, occurring following harvesting of perishable crops. It is possible that increasing urbanisation (Figure 1) will exacerbate food waste from this source due to the challenges of transporting food from where it is produced, often in rural areas, over long distances to urban consumers without degrading the quality of the food.

1.2 Senescence

Senescence is an active and genetically controlled process which involves the dismantling of cellular components, including lipids, proteins and nucleic acids, and remobilisation of nutrients from senescing organs to younger parts of the plant or storage tissues. As such, senescence can be viewed as a "salvaging" process and is crucial for resource cycling and optimising plant fitness. The most striking example of senescence occurs in the autumn

when the leaves of deciduous trees change colour and eventually fall. Senescence is an agriculturally important process as it has a significant influence on yield when it is prematurely induced (Gregersen *et al.*, 2013). It can also significantly impact post-harvest quality.

Senescence can occur from a cellular level through to an organismal level at various points in a plant's lifespan; at an organismal level senescence precedes whole plant death, but it can also occur in individual cells, tissues or organs whilst the rest of the plant remains alive. Under optimal conditions senescence is initiated in an age-dependent manner (Schippers *et al.*, 2015), the timing of which can vary greatly between species and even ecotypes in Arabidopsis (Diaz *et al.*, 2005). This can be referred to as developmental senescence. However, senescence can also be prematurely induced by stressful environmental conditions such as nutrient deficiency, oxidative stress, extremes of temperature, drought and prolonged darkness (Lim *et* al., 2007; Jing *et al.*, 2002; Zimmermann and Zentgraf, 2005), and this type of senescence is referred to here as induced senescence.

Both developmental and induced senescence are highly controlled processes, with a variety of integrated endogenous and environmental cues feeding into their control. The existing knowledge about the initiation and regulation of senescence has been reviewed thoroughly (Schippers *et al.*, 2015; Fischer, 2012; Schippers *et al.*, 2007; Lim *et al.,* 2007; Quirino *et al.*, 2000; Kim *et al.,* 2016). However, many regulatory pathways, particularly those involved in induced senescence, have yet to be fully elucidated. Broadly ethylene, abscisic acid (ABA), reactive oxygen species (ROS), jasmonic acid (JA), darkness, dehydration, starvation and leaf detachment have been shown to promote senescence, whilst cytokinins, gibberellins, auxins and nitric oxide (NO) have been shown to slow senescence (Weaver *et al.*, 1998a; Buchanan-Wollaston *et al.*, 2005). Much of this current understanding has come from the discovery of a variety of genes whose transcripts are upregulated prior to and during senescence (Buchanan-Wollaston, 1997; Gepstein *et al.*, 2003), which are commonly referred to as senescence-associated genes (SAGs) and senescence-enhanced genes (SENs). It has been found that there is some overlap in the molecular mechanisms underpinning developmental and induced senescence (Weaver *et al.*, 1998; Van Der Graaff *et al.*, 2006), with many SAGs being similarly upregulated during

both processes. However, there are differences in the transcriptomic changes between developmental senescence and certain types of induced senescence (Buchanan-Wollaston *et al.*, 2005), particularly dark-induced senescence (DIS), suggesting there are also differential regulatory pathways.

1.2.1 Foliar senescence

Foliar senescence is an example of organ level senescence and is the last stage of development in leaves. It can be induced developmentally or by stress, and involves a set sequence of changes in gene expression, cell structure and metabolism within the leaf as part of the "senescence syndrome" (Jing *et al.*, 2002; Bleeckerl and Patterson, 1997). One of the most noticeable and earliest visible symptoms of foliar senescence is a change in leaf colour from green to yellow (leaf yellowing) which usually starts at the leaf margin and spreads inwards. Leaf yellowing during foliar senescence is caused by the semi-reversible breakdown and transition of chloroplasts into gerontoplasts which unmasks yellow pigments such as carotenoids. This process has been reviewed by Matile *et al.,* (1999) and Hörtensteiner (2006). Leaf yellowing reliably correlates with the onset of other changes involved in foliar senescence, including the induction of SAGs, making it a useful marker for studying the onset and progression of senescence (Lohman *et al.*, 1994; Hensel *et al.*, 1993; Weaver *et al.*, 1998). However, it has been found that certain senescence-inducing treatments decouple the degradation of chlorophyll from the progression of senescence. One example of this was shown in a study by Oh *et al.*, (1996) where it was found that treatment with N^6-benzyladenosine (a cytokinin) inhibited chlorophyll degradation during senescence but did not prevent a reduction in photochemical efficiency, indicating senescence was still occurring. It is also important to note that Arabidopsis leaves of different developmental stages can respond to senescence-inducing treatments differently (Weaver *et al.*, 1998). Therefore, it is prudent to use multiple indicators of senescence and only compare leaves of the same developmental stage in studies on foliar senescence.

Other symptoms of foliar senescence include a reduction in membrane integrity and photochemical efficiency, and an increase in expression of SAGs responsible for the breakdown of cellular components (Song *et al.*, 2014; Buchanan-Wollaston *et al.*, 2002). The symptoms of foliar senescence make it particularly important in an agricultural sense

as they can reduce the quality of harvested crops by causing leaf discolouration. Prolonged darkness has been shown to be a key inducer of premature foliar senescence (Oh *et al.*, 1996; Keech *et al.*, 2010), and this is termed dark-induced senescence (DIS). DIS highlights an important role for light in the regulation of senescence and presents a significant problem within agriculture as it is often necessary to transport crops over long distances in constant darkness following harvesting.

1.3 Light signalling

Given their photoautotrophic nature, light is an essential source of energy to plants, but it also acts as an important environmental cue and controls many aspects of plant development, including senescence. Plants have developed a range of highly sensitive, specialised suite of photoreceptors which allow them to perceive specific wavelengths (qualities), fluence rates (quantities) and directions of light (Figure 2). In combination with other environmental signals, different compositions of light can convey information to plants about prevailing environmental conditions such as shading by neighbouring plants, photoperiod and seasonality (Franklin *et al.,* 2005). Once light cues are perceived by the photoreceptors, an array of signalling cascades are initiated which ultimately produce tightly regulated responses that control plant growth and development. Light-regulated development in plants is termed photomorphogenesis and is vital for optimal growth and survival in a fluctuating environment.

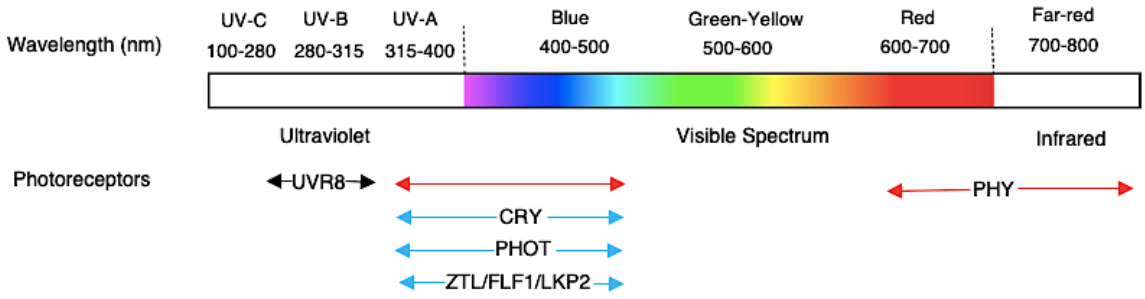

Figure 2. Photoreceptor-mediated perception of light by plants. Various photoreceptors have been identified that allow higher plants to perceive specific wavelengths of light (indicated by arrows) through from ultraviolet (UV) to infrared. The following photoreceptors have been shown to perceive a variety of wavelengths of light through from UV-A to far-red: Phytochromes (PHY), cryptochromes (CRY), phototropins (PHOT) and ZEITLUPE/FLAVIN-BINDING KELCH REPEAT F-BOX 1/LOV KELCH PROTEIN 2 (ZTL/FKF1/LKP2). However, only one photoreceptor has been shown to detect UV-B light: UV RESISTANCE LOCUS 8 (UVR8). As yet no green light-specific photoreceptor has been identified. (Diagram adapted from Huché-Thélier *et al.*, (2016)).

1.3.1 Light signalling and dark-induced senescence

Presently, multiple light-dependent mechanisms exhibiting considerable crosstalk have been identified that are involved in the regulation of senescence. Liebsch and Keech (2016) reviewed the role of light in senescence and identified the photoreceptor phytochrome B (phyB) and PHYTOCHROME INTERACTING FACTORS (PIFs) as key regulators of DIS. The roles of other photoreceptors in DIS are not so well understood. For example, one study has indicated a potential role for cryptochromes (CRY) in the control of senescence in soybean (*Glycine max*), with *cry* mutants exhibiting delayed senescence phenotypes (Meng *et al.*, 2013), whilst another study found that Arabidopsis *cry* mutants show no altered senescence phenotypes (Sakuraba *et al.*, 2014). It is possible that different plant species utilise different photoreceptor-mediated mechanisms for the control of senescence, but further work would be necessary to clarify this.

PIFs are a group of basic-helix-loop-helix (bHLH) transcription factors involved in the regulation of a wide variety of developmental processes, but they primarily negatively regulate photomorphogenic responses and are degraded by active phytochromes (for reviews see Jeong and Choi (2013) and Paik *et al.*, (2017)). PIF4 and PIF5 have been identified as important transcriptional activators in DIS (Sakuraba *et al.*, 2014). This study found that PIF4/5 act via both ethylene and ABA signalling pathways to activate *ORESARA1 (ORE1)*, a key senescence-promoting NAC transcription factor. Specifically, *ORE1* expression is upregulated through the PIF-mediated stabilisation and activation of *ETHYLENE INSENSITIVE 3 (EIN3)*, *ABSCISIC ACID INSENSITIVE 5 (ABI5)* and *ENHANCED EM LEVELS (EEL)* transcription factors (Liebsch and Keech, 2016). Through the collective action of PIFs, EIN3, AB15 and ORE1, multiple SAGs required for downstream senescence processes are promoted along with the genes *STAY-GREEN 1 (SGR1)* and *NON-YELLOW COLORING 1 (NYC1)*, which are required for chlorophyll degradation (Liebsch and Keech, 2016). Recently it has been found that PIF4/5 are degraded as a result of exposure to ultraviolet-B light (UV-B) via the UV RESISTANCE LOCUS 8 (UVR8) photoreceptor (Hayes *et al.*, 2014; Sharma *et al.*, 2019). These studies indicate a potential role for UV-B signalling in the regulation of DIS.

1.3.2 UV-B

Ultraviolet (UV) light is a fundamental element of solar radiation and is split into three categories: UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (100-280 nm). UV-C light has the shortest wavelength, and therefore the highest energy, but only wavelengths above ~295 nm reach the earth's surface without being absorbed by the atmosphere (Jenkins, 2014). However, the UV light that does reach the biosphere is still considered to be relatively high energy, is biologically active and is potentially damaging at high fluence rates. UV fluence rates are variable and impacted by a variety of factors including sun height, latitude, cloud cover, altitude, shading, atmospheric aerosol levels, ozone levels and ground reflection (Fioletov *et* al., 2010; WHO, 2019). Given this, plants are exposed to fluctuating levels of both UV-A and UV-B light in their natural environments and have developed mechanisms to perceive and respond to them.

UV-B makes up only ~5% of the UV radiation that reaches plants. Despite this, it can provide both informational cues and induce stress responses (Tilbrook *et al.*, 2013), depending on the intensity, wavelength and duration of UV-B exposure. The responses of plants to UV-B are varied and numerous and have been comprehensively reviewed (Jenkins, 2009; Tilbrook *et al.*, 2013; Zlatev *et* al., 2012; Robson *et al.*, 2015). Generally, responses to UV-B can be classified as either photomorphogenic or damaging in nature. Photomorphogenic responses are induced by UV-B-specific pathways and occur when the dose is low and non-stressful (0.1-1 μmol m^{-2} s^{-1}), whilst damaging responses are thought to be induced by non-specific signalling pathways when the dose is high and stressful (>1 μmol m^{-2} s^{-1}) (Ulm and Nagy, 2005; Gardner *et al.*, 2009). Low doses of UV-B can modify plant architecture and metabolism, promoting the synthesis of UV-protective compounds and antioxidants, whilst high doses of UV-B can cause damage to DNA, membrane lipids or proteins and can cause necrosis. Plants are more likely to experience damaging responses if they have not been acclimatised to UV-B prior to exposure.

1.3.3 UV-B signalling

UV-B light is perceived by the UVR8 photoreceptor (Rizzini *et al.*, 2011) (Figure 3), which was originally identified by Kliebenstein *et al.*, (2002). UVR8 has been shown to play a

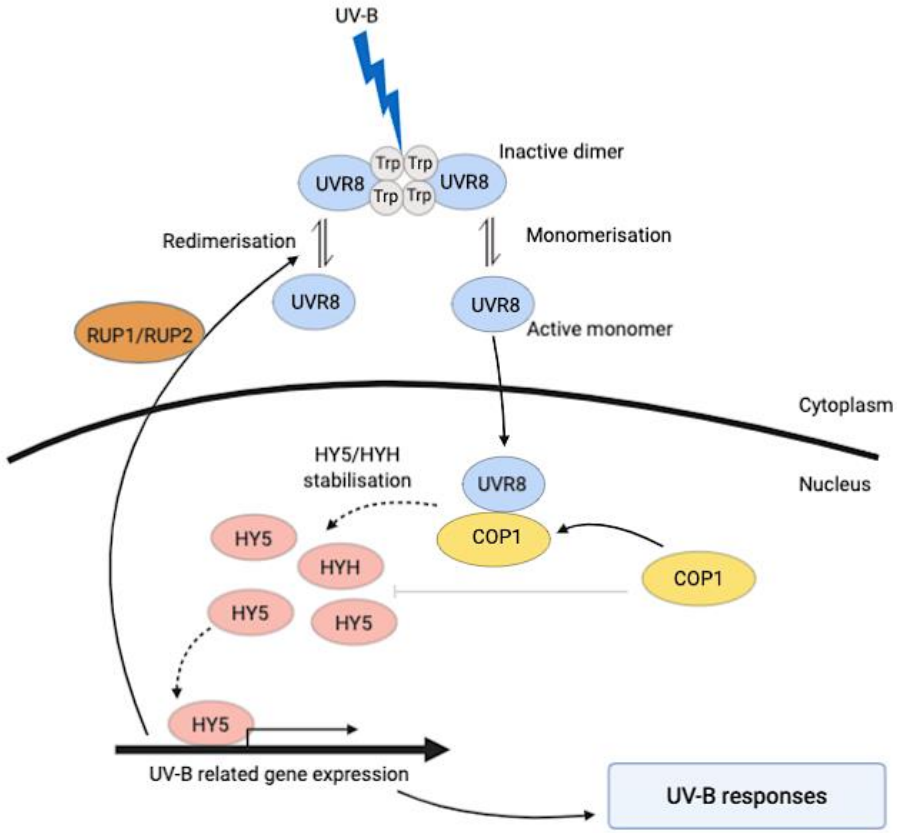

Figure 3. Perception of UV-B light by the UVR8 photoreceptor. Once ultraviolet B (UV-B) light is absorbed by the UV RESISTANCE LOCUS 8 (UVR8) photoreceptor, it initiates a cascade of molecular changes that lead to the expression of UV-B related genes which initiate UV-B responses. For full explanation of the mechanism, see chapter "1.3.3 *UV-B signalling*". Tryptophan (Trp), CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1), ELONGATED HYPOCOTYL5 (HY5), HY5 HOMOLOGUE (HYH), REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2 (RUP1 and RUP2). (Created with BioRender.com).

crucial role in UV-B mediated photomorphogenesis and in UV-B tolerance (Favory *et al.*, 2009). The role of UVR8 in UV-B signalling has been reviewed thoroughly (Heijde and Ulm, 2013; Tilbrook *et al.*, 2013). The UVR8 photoreceptor is a seven-bladed β-propeller protein which exists as a homodimer held together by a combination of salt bridges, cation–π interactions and hydrogen bonds when not in the presence of UV-B light (Di Wu *et al.*, 2012). UVR8 is unique among the known photoreceptors as it does not have an external cofactor acting as a chromophore, however, it is enriched with tryptophan (Trp) residues which naturally absorb UV-B light. Di Wu *et al.*, (2012) showed that two of these tryptophans, Trp285 and Trp233, function as the chromophore and are responsible for UV-B perception in UVR8.

Absorption of UV-B by Trp285 and Trp233 causes disruption of the bonds holding the UVR8 homodimer together and results in monomerization (Christie *et al.*, 2012).

Monomerization of UVR8 results in the transportation of the monomers from their original location in the cytosol into the nucleus and exposes a binding site for CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1). COP1 is an E3 ubiquitin ligase that targets molecules for proteasomal degradation via ubiquitination. It has been shown that COP1 is a master repressor of photomorphogenesis in darkness, but is also crucially important in UV-B signalling (Seo *et al.*, 2004, 2003; Deng *et al.*, 1992; Favory *et al.*, 2009; Rizzini *et al.*, 2011; Oravecz *et al.*, 2006). In the absence of UV-B, COP1 negatively regulates the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) and targets it for degradation within the nucleus (Osterlund *et al.*, 2000). In the presence of UV-B light, UVR8 monomers directly interact with COP1 and prevent it from degrading HY5 (Yin *et al.*, 2015; Favory *et al.*, 2009). It has been suggested that the stabilisation of HY5, and its homologue HY5 HOMOLOGUE (HYH), by COP1 and UVR8 is responsible for virtually all UVR8-mediated UV-B responses (Brown and Jenkins, 2008). UVR8-mediated signalling is essential for optimal survival as it results in UV protection for the plant, but it can also lead to impaired growth if left unchecked (Favory *et al.*, 2009). Balance in this signalling pathway is maintained by the activity of REPRESSOR OF UV-B PHOTOMORPHOGENESIS 1 and 2 (RUP1 and RUP2) proteins (Gruber *et al.*, 2010). RUP1 and RUP2 are largely homologous and act as part of a negative feedback loop. The negative feedback loop is initiated by COP1/UVR8 activity, in which they facilitate the redimerization of the UVR8 monomers to switch off the pathway.

Not all responses to UV-B are mediated by UVR8. Studies looking at genes upregulated in a UV-B dependent manner have been used to identify examples of UVR8-independent responses. When UV-B is applied at high fluence rates, many UVR8-indepdendent signalling pathways overlap with stress signalling pathways (Jenkins, 2009). For example, high doses of UV-B induce programmed cell death (PCD) via a mitogen-activated protein kinase (MAPK) cascade that is entirely independent of the UVR8 signalling pathway (Nawkar *et al.*, 2013). It has recently been found that UV-B applied at low fluence rates can induce UVR8-independent signalling pathways that are independent of known stress signalling pathways (O'Hara *et al.*, 2019), highlighting that UVR8-independent signalling is not specific to stress responses. It has been suggested that there is an as yet uncharacterised UV-B photoreceptor in addition to UVR8 (Takeda *et al.*, 2014) which may also control various responses to UV-B.

1.4 UV-B regulation of dark-induced senescence

This work focuses on the involvement of UV-B signalling in the regulation of DIS. It has been shown that PIFs are extremely important in the activation of DIS (Sakuraba *et al.*, 2014), and that relatively low dosage UV-B treatments can degrade PIFs (Hayes *et al.*, 2014; Sharma *et al.*, 2019). It has also been shown that treatment with high doses of UV-B can inhibit chlorophyll degradation, a key symptom of DIS, in Arabidopsis, lime (*Citrus latifolia* Tan.) and broccoli (*Brassica oleracea* L. Italica Group) (Sztatelman *et al.*, 2015; Srilaong *et al.*, 2011; Aiamla-or *et al.*, 2010). These studies indicate that treatment with UV-B inhibits DIS in some way. Sztatelman *et al.*, (2015) found that the inhibition of chlorophyll degradation by high doses of UV-B during DIS was not mediated by UVR8, whilst Sharma *et al.*, (2019) found that the degradation of PIFs by low doses UV-B was mediated by UVR8. It is, therefore, possible that there are different dose-dependent mechanisms underlying the role of UV-B in DIS, though this has not been specifically investigated at present. It has been posited that UV-B treatments can be used to enhance the post-harvest shelf life of economically important crop species (Aiamla-or *et al.*, 2010; Srilaong *et al.*, 2011). Given this, it is important that the role of UV-B in DIS is fully understood so that any UV-B treatments used in an agricultural setting may be used with maximal efficacy.

1.5 Aims and objectives

Despite the potential for a significant role of UV-B signalling in the regulation of DIS, there has been relatively little work published in this area. This work aims to investigate the effects of low intensity UV-B treatment on DIS in *Arabidopsis thaliana* within a laboratory setting, and probe the potential mechanisms by which any observed effects may occur.

Chapter 2: Materials and Methods

2.1 Seed stocks

Arabidopsis thaliana seeds of ecotypes Columbia (Col-0, *uvr8-6*) were obtained from Dr Ashutosh Sharma (School of Life Sciences, University of Bristol, Bristol, UK). Seeds of mutant Arabidopsis *uvr8-6* have been previously described (Favory *et al.*, 2009).

2.2 Growth conditions

Arabidopsis seeds (Col-0, *uvr8-6*) were sown directly onto a 3:1 ratio of compost (Sinclair All-purpose Growth Medium, William Sinclair Horticultural Ltd, Lincoln, UK) and sand (Horticultural Silver Sand, Melcourt Garden and Landscape, UK) then stratified in darkness at +5 °C for 48 h. Seeds were then transferred to a growth cabinet (Microclima 1600E, Snijder Scientific, The Netherlands) and watered three times per week. The cabinet was set to a 16 h photoperiod (16 h light: 8 h dark) and maintained at 20 °C and 70% relative humidity (RH). White light was provided by Philips Master TL-D36W/840 bulbs at 80 μmol m^{-2} s^{-1} (Appendix Figure S1). All light measurements were taken using an Ocean Optics Flame Spectrometer with OceanView software. Light conditions in the cabinet were checked weekly.

2.3 UV-B treatment

Prior to harvesting of leaf material for analysis, whole plants were either exposed to white light (- UV-B treatment), provided by Philips Master TL-D36W/840 bulbs at 80 μmol m^{-2} s^{-1}, or white light supplemented with 1 μmol m^{-2} s^{-1} UV-B (280-315nm) light (+ UV-B treatment) (Appendix Figure S1), provided by Philips Narrowband TL100W/01 UV-B bulbs, for either 4 or 6 h. This level of UV-B is equivalent to that measured on a cloudy day in Bristol, UK (Hayes, 2015) and is considered to be non-damaging. However, this dose of UV-B is still sufficient to induce metabolic and developmental changes (Brown and Jenkins, 2008), including changes in protein expression levels relevant to dark-induced senescence. For example, a 2 h treatment with ambient UV-B was shown to increase PIF degradation (Sharma *et al.*, 2019). Treatments of the same length and harvesting were done at the same respective time of day for all trials to avoid circadian effects.

2.4 Measuring chlorophyll content

Fully expanded, mature leaves (leaf 3 or 4) of Arabidopsis (Col-0, *uvr8-6*) were harvested for analysis following +/- UV-B treatment. 1 whole leaf was taken from 10 separate adult plants, resulting in 10 biological replicates per treatment group. This was repeated a minimum of 3 times for Col-0 experiments and once for *uvr8-6* experiments. Leaves were handled extremely gently in order to avoid any mechanical damage which could contribute to leaf yellowing. The leaves from each replicate were suspended in 8 mL 5 mM MES buffer (pH 5.8) in separate 50 mm petri dishes following harvesting, and then either incubated in the same light conditions they were grown in, or in complete darkness at 21 °C for 7 d. Incubating in light as well as darkness provided a control to confirm that changes to chlorophyll content were darkness-induced.

Chlorophyll content was determined using a chlorophyll meter (Force-A Dualex® 4 Scientific) which has been described by Cerovic *et al.,* (2012). This equipment allows for non-invasive measurements to be taken from the same leaves at different time points. Measurements were taken from 4 separate points on the adaxial leaf surface in order to mitigate for leaf heterogeneity and an average value was calculated. Measurements were taken at the same time of day in a randomised order to mitigate circadian effects as many chloroplast genes are under circadian control (Noordally *et al.*, 2013). The first measurement for each leaf was recorded immediately after harvesting. Subsequent measurements were recorded at different time points during incubation to produce time course data. The final measurement for each leaf was recorded following 7 d of incubation. Each sample was also photographed using a Nikon DSLR camera when chlorophyll measurements were recorded for visual analysis of leaf yellowing. Images were processed using Fiji 2.0.0.

2.5 Measuring total antioxidant capacity

Immediately following +/- UV-B treatment, ~50 mg of leaf material was harvested from mature leaves (leaf 3 or 4) on 10 separate adult Arabidopsis (Col-0) plants, resulting in 10 biological replicates per treatment. This was repeated a minimum of twice. The fresh weight of the leaf material was recorded before it was immediately frozen in liquid nitrogen and stored at -80 °C until analysis. To prepare samples for analysis, frozen material was homogenised using a TissueLyser II (Qiagen®) then extracted with 500 µl

ice cold 1X phosphate-buffered saline (PBS) buffer solution (pH 7.4). Prior to the addition of PBS buffer, care was taken to ensure samples remained frozen at all times. The samples were then centrifuged and 1 µl of the supernatant was collected for analysis.

Analysis was performed using a Total Antioxidant Capacity Assay Kit (Sigma-Aldrich). 0, 2, 4, 8, 16 and 20 nmol μl^{-1} Trolox standards were made up with 100 µl of Cu^{2+} working solution, as described by the manufacturer, and used to produce a standard curve for determining the total non-enzymatic antioxidant capacity of the samples (Figure S2). 1 µl of each sample was diluted x100 with sterile deionised H_2O prior to the addition of 100 µl of Cu^{2+} working solution. The samples and standards were incubated for 90 min in darkness at room temperature, then made up to 1 ml with sterile deionised H_2O. The absorbance (at 570 nm) of all samples and standards was then determined using a spectrophotometer (WPA Biowave II, Biochrom Ltd.). The absorbance readings for the Trolox standards were used to generate a standard curve (Figure S2), which was then used to calculate the antioxidant capacity of the samples. The antioxidant concentration in the samples (nmol) was normalised using the initial fresh weight (mg) of the sample to give the final Trolox equivalent antioxidant capacity (nmol mg^{-1}).

2.6 Measuring electrolyte leakage

Following +/- UV-B treatment, 3 whole leaves were harvested from 5 separate adult Arabidopsis (Col-0) plants, resulting in 5 biological replicates per treatment. This was repeated a minimum of three times. The leaves from each replicate were suspended in 10 mL 5 mM MES buffer (pH 5.8) in separate 50 mm petri dishes following harvesting, and then either incubated in the same light conditions they were grown in, or in complete darkness for 7 d.

After incubation, 3 leaf discs from each replicate were placed in a borosilicate tube containing 5 mL sterile deionised H_2O and shaken gently using a Rotatest Shaker (Model R100, Luckham Ltd.) for 3 h. Following shaking, the leaf material was removed from the tubes and electrolytes present in the liquid were quantified using a conductivity meter (Hanna Instruments pH Meter, PRIMO 2). The leaf discs were frozen at -80 °C for a minimum of 90 min then thawed to room temperature to cause cell lysis. The leaf discs were returned to their original liquid and shaken again for 3 h, then a final electrolyte

reading was taken. The initial electrolyte reading was divided by the final electrolyte reading to give the percentage of electrolyte leakage for each sample.

2.7 Statistical analysis

Chlorophyll content, electrolyte leakage and antioxidant capacity were analysed in GraphPad Prism Version 8.4.2 for macOS. All data was tested for normality and heterogeneity of variance. Where its assumptions were met by the data, parametric analysis of variance (one-way ANOVA) was carried out, followed by a Tukey-HSD post-hoc test to test for significant differences between specific groups. In the event that the assumptions for ANOVA were violated, the non-parametric equivalent test was carried out in the form of a Kruskal-Wallis test followed by Dunn's post hoc test.

2.8 Method development

2.8.1 *Whole leaf incubation conditions*

Whole leaves of Arabidopsis were used for measuring chlorophyll content and electrolyte leakage following incubation for 7 d. In the literature there are a variety of methods for harvesting and incubating whole leaves, including harvesting using forceps then floating them on buffer or placing them on damp towels. Initially both of these methods were tested. It was found that any type of mechanical damage caused during harvesting or incubation had a large impact on chlorophyll content, and general leaf integrity, at 7 d. After several trials it was found that using scissors to detach leaves at the base of the petiole and then transferring them extremely carefully by hand into buffer solution, adaxial surface up, was the best method for harvesting and incubating whole leaves.

2.8.2 *Measuring chlorophyll content*

In preliminary experiments it was found that leaf yellowing, as a result of chlorophyll degradation, during senescence does not occur evenly across the leaf surface. There is therefore variation in chlorophyll levels across the leaf surface. It was found that taking multiple measurements from 4 specific places (Figure 4) on the adaxial leaf surface was the best way to produce a representative measurement of chlorophyll content for the whole leaf.

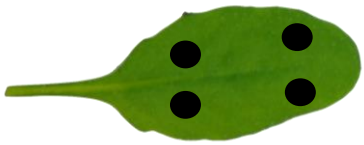

Figure 4. Dualex measurement diagram. Schematic representation of the locations on the adaxial leaf surface where measurements were taken in order to measure chlorophyll content. Black circles show where the chlorophyll meter was attached to the leaf.

Chapter 3 – Pre-harvest UV-B treatment reduces the progression of dark-induced leaf senescence in *Arabidopsis thaliana*

3.1 Introduction

Developmental senescence is a natural part of the plant life cycle and is essential for nutrient cycling. It optimises plant fitness by dismantling and relocating resources away from senescing parts of the plant to developing organs, ensuring resources are directed to where they are most needed (Buchanan-Wollaston, 1997). However, senescence can be prematurely induced by stressful environmental conditions such as drought, long periods of darkness and high temperatures. Prolonged darkness, coupled with leaf detachment, is a particularly strong inducer of premature senescence (Weaver *et al.*, 1998). DIS results in leaf yellowing via chlorophyll degradation and a reduction in membrane integrity (Song *et al.*, 2014), which presents an issue within agriculture as it can reduce the post-harvest quality of crops during transportation and reduce their shelf life. This issue may be particularly important for high value crop species such as chives and watercress which readily turn yellow during transport (Aharoni *et al.*, 1989). Refrigeration is one possible solution to the problem of DIS in agriculture as it can delay senescence (Hasperué *et al.*, 2015), but it is energetically costly to provide cold storage over long distances so if alternative methods which require less energy can be used, they could potentially be both economically beneficial and more environmentally friendly.

Recent work has indicated an important role for PIFs (bHLH transcription factors) in the regulation of DIS. It has been found that PIF4/5 are key transcriptional activators of DIS as they upregulate expression of *ORE1* (Zhang *et al.*, 2015; Sakuraba *et al.*, 2014), a key senescence-promoting NAC transcription factor. Furthermore, it has been found that short periods of irradiation with relatively low-fluence UV-B light can degrade PIFs (Sharma *et al.*, 2019; Hayes *et al.*, 2014). This presents a theoretical basis for a role of UV-B signalling in DIS. In the literature there are a few examples of high intensity UV-B treatments being used to successfully delay chlorophyll degradation during DIS in commercially important crop species (Aiamla-or *et al.*, 2010; Srilaong *et al.*, 2011). One study also found that a high dose of UV-B inhibits chlorophyll degradation during DIS in Arabidopsis (Sztatelman *et al.*, 2015). However, little work has been done to fully characterise this response, particularly using non-stressful doses of UV-B necessary to trigger the degradation of PIFs. Here, low intensity UV-B was applied prior to harvesting

and incubation in darkness to identify any effects of UV-B on DIS and the length of the dose was varied to investigate whether a dose-dependent relationship exists.

3.2 Results

3.2.1 *Pre-harvest UV-B treatment antagonises dark-induced senescence*

Prior to harvesting of leaf material, Arabidopsis Col-0 plants were treated with either white light (-UV-B treatment group) or white light supplemented with 1 μmol m^{-2} s^{-1} UV-B (+UV-B treatment group) for 4 h. This treatment length and photon flux density was chosen as an effective dose capable of influencing dark-induced senescence-specific pathways (Sharma *et al.*, 2019), whilst not causing damage (Brown and Jenkins, 2008). Leaves were then harvested and their chlorophyll content was measured prior to, and during, 7 d of incubation either in constant darkness (dark incubated) or in their pre-harvest growth conditions (light incubated). A decrease in chlorophyll content in dark-incubated samples was considered to indicate DIS.

In order to attribute any observed UV-B effects to regulation of DIS, it was necessary to compare the impact of the +/- UV-B treatments in both light and dark incubated leaves (Figure 5). Measurements of chlorophyll content were taken at 0, 2, 3, 6 and 7 days of incubation (DI). In the light-incubated leaves, there was no significant difference in chlorophyll content at any time point between the +/- UV-B treatments (one-way ANOVA, $p>0.05$). There was also no significant difference between the chlorophyll content measured at 0 DI and at 7 DI for each UV-B treatment (one-way ANOVA, $p>0.05$). This indicates that leaf detachment, per se, does not induce chlorophyll degradation and that the UV-B treatment had no effect on leaf chlorophyll content in these conditions. After 2 DI, there was a significant difference in chlorophyll content between the light- and dark-incubated leaves for both UV-B treatments (Tukey's HSD, $p<0.05$), with the light incubated leaves having significantly more chlorophyll than the dark incubated leaves at all time points. Together, these data suggest that dark incubation triggered chlorophyll degradation, and therefore DIS, and this process was measurable following 2 DI.

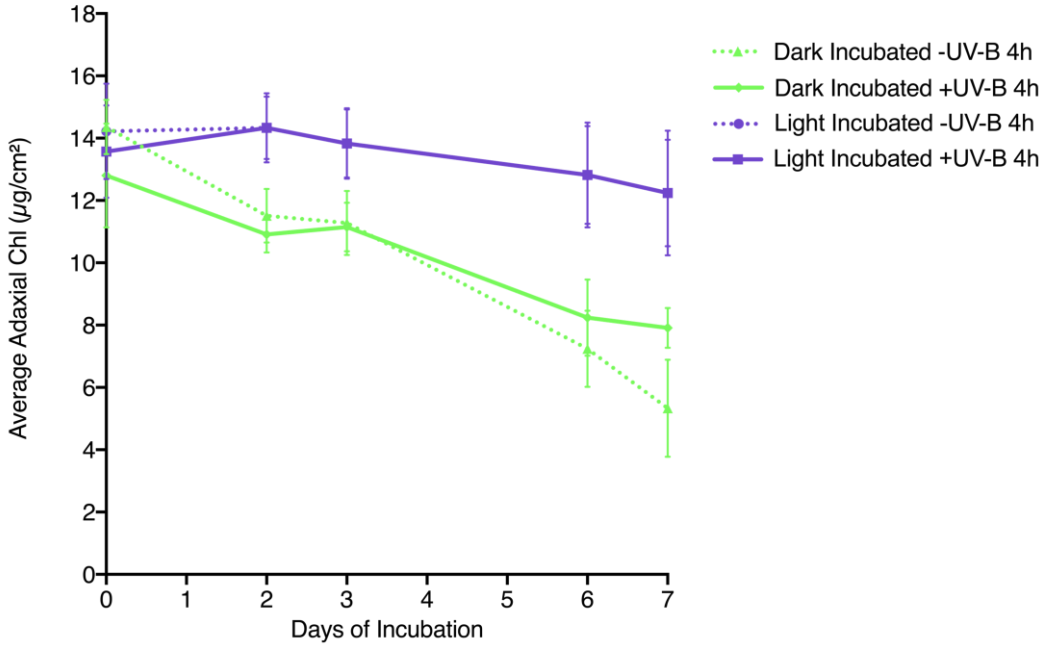

Figure 5. Dark incubation induces chlorophyll degradation which is reduced by pre-harvest UV-B treatment in Arabidopsis WT (Col-0). 4-week old Arabidopsis plants were treated with either white light (-UV-B treatment group, dotted lines) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (+UV-B treatment group, solid lines) for 4 h before leaf harvesting. Detached leaves were incubated in either their pre-harvest growth conditions (purple lines) or in constant darkness (green lines). Average adaxial chlorophyll (Chl) content (µg/cm^2) was calculated from 4 separate measurements for each leaf at different time points over the course of 7 days. 3 replicates were carried out, mean data from 1 representative replicate is shown (n= 7-10). Error bars represent SD.

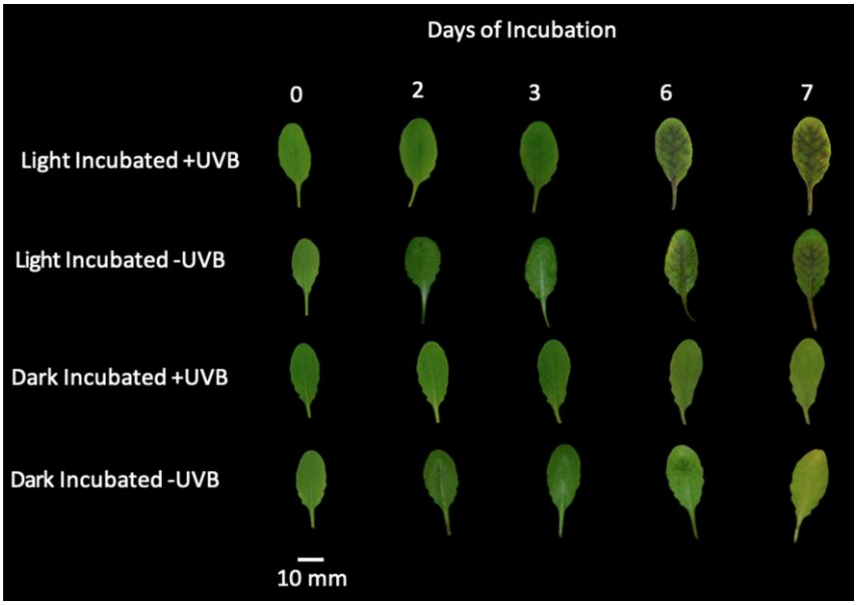

Figure 6. Treatment with UV-B visibly reduces leaf yellowing in dark-incubated leaves of Arabidopsis WT (Col-0). 4-week old Col-0 Arabidopsis plants were treated with either white light (-UV-B treatment group) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (+UV-B treatment group) for 4 h before leaf harvesting. Detached leaves were incubated in either their pre-harvest growth conditions (light incubated) or in constant darkness (dark incubated) for 7 d. Images were taken at 0, 2, 3, 6 and 7 days of incubation.

Despite both groups of dark incubated leaves showing a reduction in chlorophyll content over the course of 7 DI (Figure 5), the + UV-B treated group had significantly more chlorophyll at 7 DI than the – UV-B treated group (Tukey's HSD, $p<0.05$). Moreover, the + UV-B treated group appeared visibly less yellow after 7 DI (Figure 6). This observation was confirmed by the fact that the + UV-B treated group lost an average of 37% of their original chlorophyll content whilst the - UV-B treated group lost nearly twice as much as this, with an average loss of 63%. Overall, it can be inferred that both treatment groups began to undergo the process of DIS during dark incubation, but that this occurred to a lesser extent in the + UV-B treated group.

3.2.2 *The effect of pre-harvest UV-B treatment on dark-induced senescence may not be dose-dependent*

The possibility that a longer UV-B treatment may enhance the delay of dark-induced senescence was investigated. Prior to harvesting of leaf material, Arabidopsis Col-0 plants were treated with either white light (- UV-B treated) or white light supplemented with 1 μmol m^{-2} s^{-1} UV-B (+ UV-B treated) for either 4 or 6 h, with the – UV-B treated groups acting as controls. Leaves were then harvested, and their chlorophyll concentration was measured following 0 d and 7 d of incubation in constant darkness.

There was no significant difference in chlorophyll content at 0 d between any group (one-way ANOVA, $p>0.05$) (data not shown). Following 7 d of dark incubation, all treatment groups had begun to undergo dark-induced senescence and therefore had lost some of their original chlorophyll content. After 7 d, there was a significant difference in chlorophyll content between the 4 and 6 h + UV-B treated groups and the corresponding – UV-B treated groups (Tukey's HSD, $p<0.05$), but no significant difference between the 4 and 6 h + UV-B treated groups themselves (Tukey's HSD, $p>0.05$) (Figure 7). These data indicate that both the 4 and 6 h + UV-B treatments reduced the amount of chlorophyll lost compared to the – UV-B treatments, but that the 6 h + UV-B treatment was no more effective at doing so than the 4 h + UV-B treatment. It can therefore be concluded that the relationship between UV-B and dark-induced senescence is not highly sensitive to UV-B

dose. However, it would be necessary to investigate a wider variety of UV-B treatments over a longer time period to confirm this.

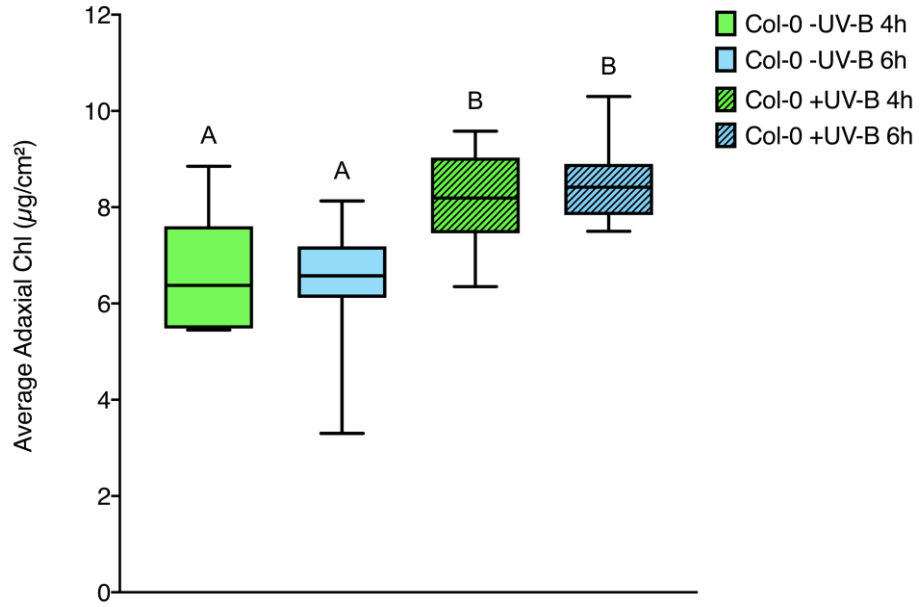

Figure 7. The effect of pre-harvest UV-B treatment on chlorophyll degradation in Arabidopsis WT (Col-0) leaves following 7 d of dark incubation is similar with 4 h and 6 h treatments. 4-week old Arabidopsis plants were treated with either white light (plain boxes) or white light supplemented with 1 μmol m^{-2} s^{-1} UV-B (striped boxes) for either 4 h (green boxes) or 6h (light blue boxes) before leaf harvesting. Detached leaves were incubated in constant darkness. Average adaxial chlorophyll (Chl) content (μg/cm^2) was calculated from 4 separate measurements for each leaf following 0 and 7 d of dark incubation. No significant differences were observed between any groups at 0 d (data not shown). Boxes represent the interquartile range, bars indicate the median and whiskers represent minimum and maximum values. Statistically significant differences (Tukey's HSD, $p<0.05$, $n=10$) between groups are denoted by differing upper case letters. 3 replicates were carried out, data from 1 representative replicate is shown.

3.2.2 *Pre-harvest UV-B treatment does not affect membrane integrity during dark-induced senescence*

Previous studies have suggested that dark-induced leaf senescence is accompanied by decreased membrane integrity, recorded as an increase in electrolyte leakage (Sakuraba *et al.*, 2014; Chen *et* al., 2016). As pre-harvest UV-B treatment delayed dark-induced chlorophyll degradation (Figures 5, 6), the possibility that it also affects electrolyte leakage was investigated. Arabidopsis Col-0 plants were treated with either white light (- UV-B treated) or white light supplemented with 1 μmol m^{-2} s^{-1} UV-B (+ UV-B treated) for either 4 or 6 h. Leaves were then harvested and % electrolyte leakage calculated following 7 d of dark incubation.

Electrolyte leakage levels at 7 DI were generally low (19-21%) in all treatment groups, but these data are comparable to levels reported by Zhang *et al.*, (2015) in a similar study. There was no significant difference in electrolyte leakage between any of the +/- UV-B treated groups (one-way ANOVA, $p>0.05$) (Figure 8). Together this suggests that relatively little membrane damage occurred during the 7 d of dark incubation and that treatment with UV-B had little influence on this. Due to time constraints, it was not possible to measure electrolyte leakage at 0 d for all treatment conditions. Replication with this control in place would be necessary to confirm these results. It is possible that if the leaves were left in darkness for more than 7 d that more membrane damage would occur, and differences may arise between the +/- UV-B treatments. Longer experiments would be necessary to investigate this.

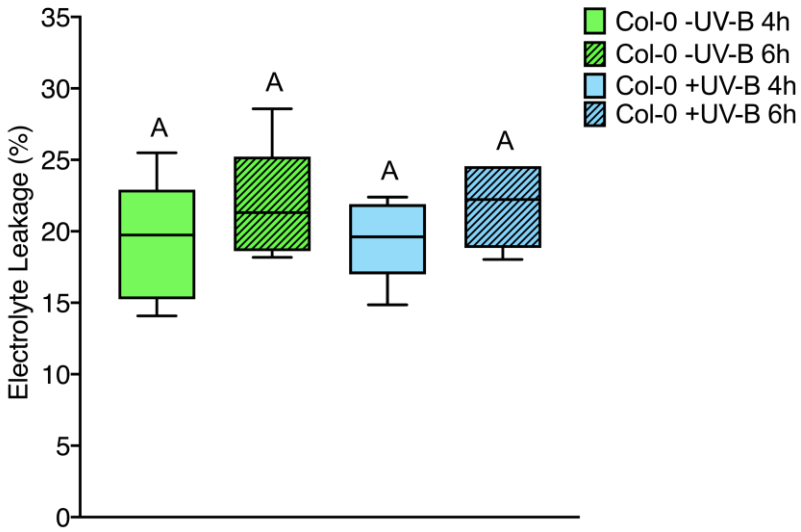

Figure 8. Treatment with UV-B does not affect electrolyte leakage in Arabidopsis WT (Col-0) following 7 d of dark incubation. 4-week old Arabidopsis plants were treated with either white light (plain bars) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (striped bars) for either 4 h (green bars) or 6h (blue bars) and then detached leaves were incubated in constant darkness. Electrolyte leakage (%) was calculated for each leaf following 7 d of dark incubation. Boxes represent the interquartile range, bars indicate the median and whiskers represent minimum and maximum values. Statistically significant differences (Tukey's HSD, $p<0.05$, $n=10$) between groups are denoted by differing upper case letters. 3 replicates were carried out, data from 1 representative replicate is shown .

3.3 Discussion

Dark-induced senescence (DIS) is triggered by prolonged exposure to darkness and causes leaf yellowing as a result of chlorophyll catabolism and a reduction in membrane integrity, among other physiological and biochemical changes (Song *et al.*, 2014). DIS can be considered an agriculturally relevant process as it can occur during the post-harvest

transportation of crops, making them less aesthetically pleasing and reducing their overall quality. It has previously been shown that various treatments with light can be used to delay DIS (Srilaong *et al.*, 2011; Aiamla-or *et al.*, 2010a; Sztatelman *et al.*, 2015). Here, the effect of pre-harvest treatment with low intensity UV-B on DIS was investigated by exposing whole Arabidopsis plants to +/- UV-B for various lengths of time, followed by harvesting and subjecting individual leaves to continual darkness. Given that chlorophyll degradation is considered to be a key marker of DIS and was used as a proxy measure for DIS. Electrolyte leakage can also indicate DIS and was also investigated.

Treatment with relatively high intensity UV-B inhibits dark-induced chlorophyll degradation in the commercially relevant crop species broccoli (*Brassica oleracea* L. Italica Group) and lime (*Citrus latifolia* Tan.) (Aiamla-or *et al.*, 2010a; Srilaong *et al.*, 2011), and in *Arabidopsis thaliana* ecotype Col-0 (Sztatelman *et al.*, 2015). There is little literature, however, on the effects of low intensity UV-B irradiation on dark-induced senescence. In this study, it was found that pre-harvest treatment with low intensity UV-B antagonises DIS in Arabidopsis Col-0 and that this response is not strongly dose-dependent.

Following 7 days of incubation (DI), dark-incubated leaves treated with UV-B were found to have significantly more chlorophyll and be visibly less yellow than those not treated with UV-B (Figures 5, 6). This effect was not seen prior to 7 DI, indicating that DIS was initiated in both +/- UV-B treated leaves but that treatment with + UV-B light antagonised its progression. In order to see if this effect becomes more pronounced over a longer time period, it would be useful to extend the period of incubation beyond 7 d in future studies. The low intensity UV-B treatment used in this study would potentially be safer and more economically viable to apply in an agricultural setting than a high intensity treatment, whilst also not being likely to cause other unwanted side effects. For example, Sztatelman *et al.*, (2015) found that the treatment with high intensity UV-B in their study retards chlorophyll degradation during DIS, but also resulted in cell death 3 d after the treatment. This could lead to a reduction in the overall quality of the plant material and therefore not be useful in an agricultural setting. For comparison, it would be useful to quantify cell death under the experimental conditions used in this study via trypan blue staining, or another similar method. The results from this work are useful for establishing

chlorophyll degradation as a reliable indicator of DIS following 7 DDI (days of dark incubation) as the chlorophyll loss seen was consistent across repeats. However, one drawback of the methods used here for quantifying chlorophyll content is that the Dualex chlorophyll meter can cause small amounts of mechanical damage to the leaf surface during measurements and this could have an impact on the chlorophyll readings. A possible solution to this would be to extract chlorophyll from leaf samples following 7 DDI using acetone and quantify the chlorophyll concentration with a spectrophotometer, though this method makes taking time-course data more difficult.

This study found no significant difference in electrolyte leakage following 7 DDI after treatment with either 4 or 6 h of UV-B (Figure 8), which suggests treatment with UV-B has little impact on membrane integrity during DIS. However, given that the levels of electrolyte leakage were generally low it may be possible that the period of dark incubation was not long enough to induce a severe reduction in membrane integrity and a role for UV-B may become evident if longer periods of incubation were investigated. In this instance, chlorophyll degradation appears to be more useful marker of the progression of senescence as it occurs reliably at an earlier timepoint under these experimental conditions.

The antagonism of DIS by low fluence UV-B appears to not be strongly dose-dependent as there was no significant difference in chlorophyll content following 7 DDI after treatment with either 4 or 6 h of UV-B (Figure 7). Conversely, a study by Sztatelman *et al.*, (2015) found that high fluence UV-B treatments influence chlorophyll degradation during DIS in a dose-dependent manner. The difference in these findings could be explained by a different mechanism underlying the response to high and low fluence treatments of UV-B. It has been found that treatment with a low dose of UV-B is sufficient to degrade PIF5 (Sharma *et al.*, 2019), which has been shown to be important in promoting senescence (Sakuraba *et al.*, 2014). If UV-B-mediated degradation of PIFs is the mechanism underlying the antagonism of DIS by UV-B, this may explain why the response isn't strongly dose-dependent – previous studies have indicated that treatment with UV-B for as little as 2 h is sufficient to degrade the PIFs present (Hayes *et al.*, 2014), so the effect on DIS would not increase with the length of the dose of UV-B after this point. Further experiments investigating the relationship between UV-B and PIFs in the context

of DIS would shed light on the mechanisms underlying this response and may explain help to explain these findings.

Chapter 4 – Understanding the mechanistic basis of UV-B-regulated antagonism of dark-induced leaf senescence

4.1 Introduction

UV-B radiation is an intrinsic element of natural sunlight. Though it only makes up a relatively small percentage of the total irradiation that reaches the earth's surface, it has been shown to be an important environmental signal for plants. The responses of plants to UV-B can be photomorphogenic or stress-induced in nature, and they have been comprehensively reviewed (Jenkins, 2009; Tilbrook *et al.*, 2013; Zlatev *et al.,* 2012; Robson *et al.*, 2015). These responses include morphological changes as well as molecular changes such as the upregulation of genes important in UV-protection, DNA repair mechanisms and antioxidant synthesis. The characterisation of both UVR8 as a UV-B specific photoreceptor and the mechanisms underlying UV-B absorption by UVR8 (Rizzini *et al.*, 2011; Di Wu *et al.*, 2012; Christie *et al.*, 2012) have led to a clearer understanding of plant UV-B perception. However, there is still much to be uncovered regarding the mechanisms underlying UV-B signalling.

Both the responses initiated by UV-B exposure and the mechanisms underlying the responses are influenced by the fluence rate of UV-B irradiation perceived. Generally, photomorphogenic responses are initiated by low intensity UV-B irradiation and mediated by the UVR8 photoreceptor (Favory *et al.*, 2009), whilst stress responses are initiated by high intensity UV-B irradiation and are mediated by alternative mechanisms. However, recent work has identified UVR8-independent pathways which are also independent of stress-response pathways initiated following the application of low fluence of UV-B (O'Hara *et al.*, 2019), indicating that UVR8-independent UV-B signalling is not solely specific to stress responses. A separate study has shown that UVR8 does not mediate UVB-induced inhibition of chlorophyll degradation during DIS when high intensity UV-B is applied (Sztatelman *et al.*, 2015), but the role of UVR8 in delaying DIS following treatment with low intensity UV-B has not been investigated. DIS is promoted and regulated by a complex network of factors. Given the broad variety of known responses to UV-B irradiation, identifying any crossover between the UV-B and DIS signalling networks will be necessary to begin to unravel the mechanistic basis of low intensity UV-B-regulated antagonism of DIS. Identifying whether the response is

mediated by UVR8 is the first step in doing this, but events downstream of UV-B perception will also require investigation.

Reactive oxygen species (ROS) play an important role in the promotion of senescence; the levels of ROS present in cells are increased during senescence as a result of both increased production through degradation of cell walls and chloroplasts, and through reduced antioxidant capacity (Zimmermann and Zentgraf, 2005). ROS can function as signalling molecules at low concentrations and are particularly important in stress acclimation, but it is widely acknowledged that an accumulation of ROS can be phytotoxic and cause oxidative damage to lipids, DNA, proteins and other biological molecules (see Dat *et al.*, (2000) for review). It is possible that a treatment which causes an increase in antioxidant capacity within cells could delay DIS via increased ROS scavenging. It has previously been shown that treatment with UV-B results in the enhancement of antioxidant capacity in mature tomato fruit, broccoli, soybean and sunflower (Liu *et al.*, 2011; Xu *et al.,* 2008; Costa *et al.,* 2002; Darré *et al.*, 2017). It was therefore questioned whether treatment with low intensity UV-B is sufficient to increase the antioxidant capacity of Arabidopsis prior to incubating in darkness, as this may function in the DIS regulatory network to antagonise the process. Here, both the function of the UVR8 photoreceptor during DIS and the impact of short, low intensity UV-B treatments on antioxidant capacity were investigated.

4.2 Results

4.2.1 *UV-B does not affect antioxidant activity during dark-induced senescence*

The possibility that preharvest UV-B treatment increases leaf antioxidant content during dark-induced senescence was investigated. Prior to harvesting of leaf material, Arabidopsis Col-0 plants were treated with either white light (- UV-B treatment group) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (+ UV-B treatment group) for either 4 h or 6 h. Leaf antioxidant activity (in Trolox equivalent capacity nmol mg^{-1}) was calculated following harvesting. No significant difference in antioxidant activity was found between the +/- UV-B treatments at this timepoint (Tukey's HSD, $p>0.05$), but a significant difference between the 4 and 6 h groups was observed (Tukey's HSD, $p<0.05$)

(Figure 9). These data suggest that the + UV-B treatments did not cause significant upregulation of antioxidant capacity.

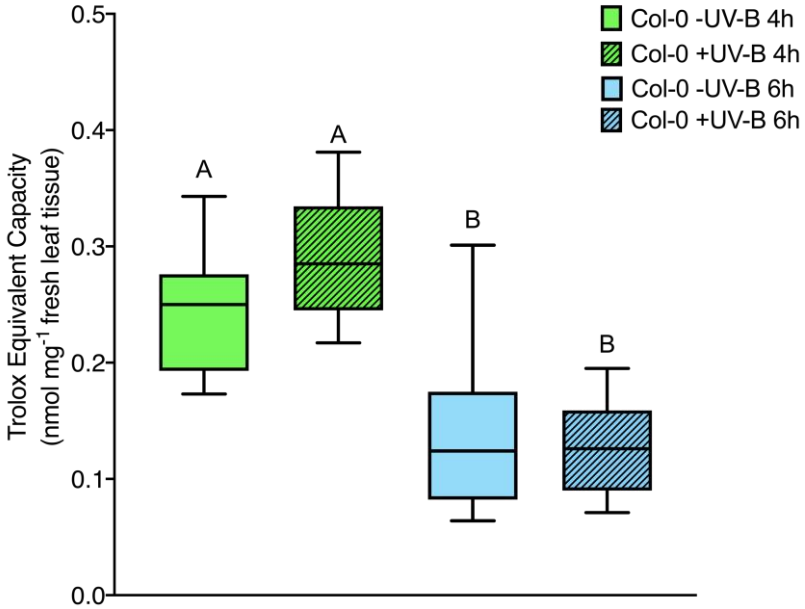

Figure 9. Treatment with 4 or 6 h UV-B does not influence the antioxidant activity of Arabidopsis WT (Col-0). 4-week old Arabidopsis plants were treated with either 4 or 6 h white light (plain boxes) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B for either 4h or 6h (striped boxes). Antioxidant activity (in Trolox equivalent capacity nmol mg^{-1}) was determined for individual detached leaves immediately following treatment. Boxes represent the interquartile range, bars indicate the median and whiskers represent minimum and maximum values. Statistically significant differences (Tukey's HSD, $p<0.05$, n=10) between groups are denoted by differing upper case letters. 3 replicates were carried out, data from 1 representative replicate is shown.

4.2.2 *The UVR8 photoreceptor mediates UV-B-regulated antagonism of dark-induced leaf senescence*

The possibility that the UV-B regulated antagonism of dark-induced leaf senescence seen in this study was mediated by the UVR8 photoreceptor was investigated. Prior to harvesting of leaf material, Arabidopsis plants (Col-0 and *uvr8-6)* were treated with either white light (- UV-B treatment group) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (+ UV-B treatment group) for 4 h. Leaves were then harvested, and their chlorophyll concentration was measured following 0 d and 7 d of incubation in constant darkness.

No significant difference in chlorophyll content was observed between any group at 0 d (one-way ANOVA, $p>0.05$) (data not shown). After 7 d there was no significant difference between the Col-0 – UV-B or *uvr8-6* -/+ UV-B groups (Tukey's HSD, $p>0.05$). The Col-0 +

UV-B group, however, had significantly more chlorophyll than the other three groups (Tukey's HSD, $p<0.05$) (Figures 10, 11). These data suggest that the UVR8 photoreceptor regulates UV-B-mediated inhibition of DIS. It should, however, be noted that this experiment was only repeated once due to the time restraints caused by COVID-19 and an issue with temperature control following a power cut. Further repeats would be therefore necessary to confirm these results.

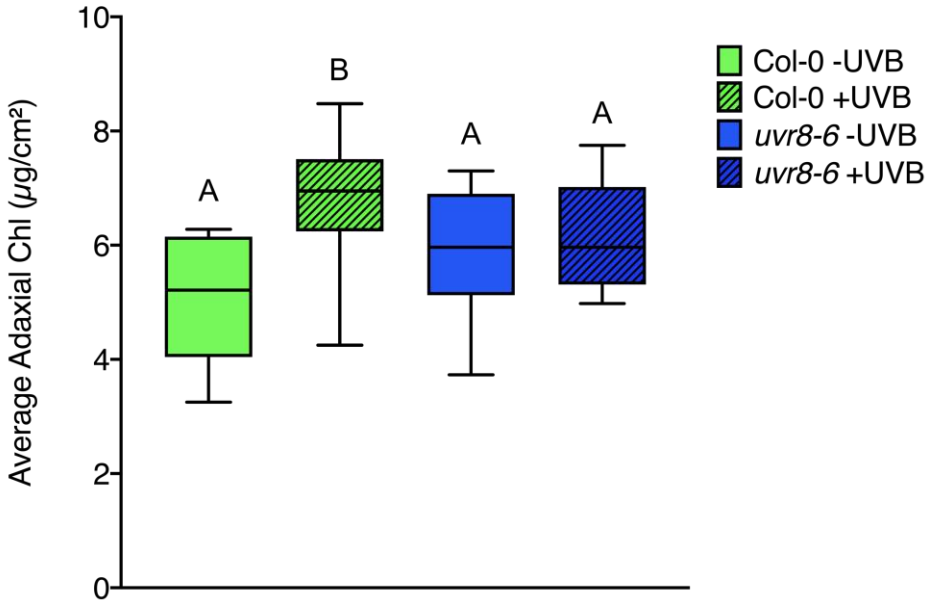

Figure 10. *uvr8-6* Arabidopsis mutants do not show reduced chlorophyll degradation following 7 d of dark incubation when treated with 4 h of UV-B. 4-week old Col-0 (green boxes) and *uvr8-6* (dark blue boxes) Arabidopsis plants were treated with 4 h white light (plain boxes) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (striped boxes) for 4 h. Detached leaves were incubated in constant darkness. Average adaxial chlorophyll (Chl) content (µg/cm^2) was calculated from 4 separate measurements for each leaf following 7 d of dark incubation. Boxes represent the interquartile range, bars indicate the median and whiskers represent minimum and maximum values. Statistically significant differences (Tukey's HSD, $p<0.05$, $n=10$) between groups are denoted by differing upper case letters. 1 replicate was carried out.

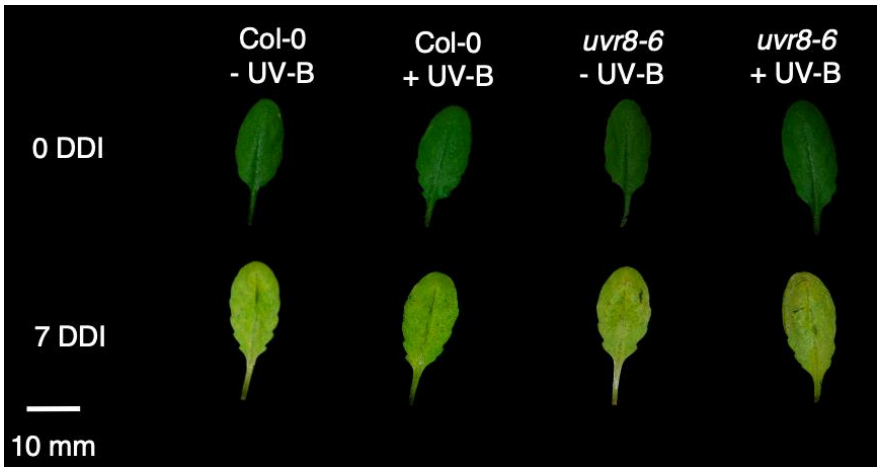

Figure 11. Treatment with UV-B does not reduce chlorophyll degradation or visible yellowing in *uvr8-6* Arabidopsis mutants. 4-week old Col-0 and *uvr8-6* Arabidopsis plants were treated with either white light (- UV-B treatment group) or white light supplemented with 1 µmol m^{-2} s^{-1} UV-B (+ UV-B treatment group) for 4 h before leaf harvesting. Detached leaves were incubated in constant darkness for 7 d. Images were taken at 0 and 7 days of dark incubation (DDI).

4.3 Discussion

The role of UVR8 as a UV-B photoreceptor responsible for mediating a wide suite of plant responses to UV-B light is well established. However, whether UVR8 mediates the low intensity UV-B induced antagonism of DIS has not been investigated. Previous work has found that high intensity UV-B induced antagonism of DIS is not mediated by UVR8 (Sztatelman *et al.*, 2015). During this study, *uvr8-6* mutants exhibited the same delayed senescence phenotype (inhibited chlorophyll degradation) as Col-0 plants following treatment with UV-B and dark incubation, indicating UVR8 was not responsible for regulating this response. The study by Sztatelman *et al.,* (2015) also ruled out involvement of the MAPK cascade. This cascade is responsible for UVR8-independent programmed cell death (PCD) following UV-B exposure (Nawkar *et al.*, 2013). As part of this work, a role for low intensity UV-B in the regulation of DIS has been established, with short treatments of UV-B antagonising DIS in mature Arabidopsis. Further establishing a role for UVR8 in this response would provide insight into the first steps in the mechanism and a basis for studying downstream elements of the regulatory network.

Experiments showed that UVR8 likely does mediate the inhibition of chlorophyll degradation during DIS. When compared with Col-0 plants under equivalent conditions, *uvr8-6* mutants did not show a reduction in chlorophyll degradation following treatment with low intensity UV-B (Figure 10), and therefore did not show a delayed senescence phenotype. This strongly indicates that the chlorophyll inhibition seen in Col-0 following

UV-B treatment is mediated, at least in part, by the UVR8 photoreceptor. These results also indicate the existence of different dose-dependent mechanisms underlying the role of UV-B in DIS. It is possible that high intensity UV-B initiates a UVR8-indepenedent stress-induced response which leads to delayed senescence, whilst low intensity UV-B activates an entirely separate signalling network which ultimately produces the same response. Given that these results came from only one data set, it would be necessary to repeat the experiments to confirm the findings.

This work also aimed to investigate whether UV-B-induced antioxidant upregulation was responsible for the delayed senescence phenotype seen in Col-0 plants treated with low intensity UV-B. ROS play an important role in the promotion of senescence, with an increased concentration of ROS leading to increased expression of SAGs (Navabpour *et al.*, 2003). Previous studies have shown that treatment with UV-B can cause an increase in antioxidant capacity in various horticultural species (Liu *et al.*, 2011; Xu, Natarajan and Sullivan, 2008; Costa *et al.,* 2002; Darré *et al.*, 2017), as well as in Arabidopsis Col-0 (Csepregi *et al.*, 2017), indicating a possibility that UV-B regulated antioxidant capacity may play a downstream role in the UV-B-dependent regulation of DIS. However, in this study it was found that the UV-B treatment used did not cause an increase in antioxidant capacity (Figure 9), with leaf material of plants treated with either 4 or 6 h of UV-B showing no increase in antioxidant concentration compared to untreated controls. There was a difference in the antioxidant capacity of those plants treated with 6 h of UV-B compared to 4 h, with the 6 h group having a significantly lower antioxidant capacity (Figure 9). This difference may have arisen due circadian regulated daily oscillation of antioxidant activity (Soengas *et al.*, 2018) as the 6 h treated plants were harvested 2 h later in the day prior to antioxidant quantification, compared to the 4 h treated plants. Given that this work previously identified no difference in the inhibition of chlorophyll degradation seen following 4 or 6 h of UV-B treatment (Figure 8) it is not likely that the difference in antioxidant capacity seen had an impact on this result.

Together, these results indicate that an increase in ROS scavenging caused by antioxidant upregulation does not play a role in the UV-B-induced antagonism of DIS identified in this work. It is possible that no increase in antioxidant capacity was seen here due to the singular, low intensity nature of the UV-B treatment used. In the study by Csepregi *et al.*,

(2017), Arabidopsis plants were exposed to 2 h of low intensity UV-B every day for 7 d, so it may be that repeated exposure to low intensity UV-B radiation is necessary to cause antioxidant upregulation. This work does not rule out the possibility of antioxidant upregulation enhancing UV-B-induced antagonism of DIS, but further work using UV-B treatments relevant to increasing antioxidant capacity would have to be carried out in order to investigate this.

Chapter 5 – Discussion

Leaf yellowing is one of the most visually striking elements of senescence in plants. It is caused by accelerated degradation of green chlorophyll pigments coupled with retention of yellow carotenoids (Lu *et al.*, 2001), resulting from either endogenous ageing cues or due to abiotic stresses. One such abiotic stress is prolonged darkness; this type of senescence is referred to as DIS. DIS presents as an issue in agriculture as it is common for crop plants to be stored in darkness following harvesting during transportation, and yellowing may reduce the quality and aesthetic value of the produce. Given the potential economic impact of premature senescence, as well as the pressing need to reduce waste in the food supply chain, it is essential that the regulatory networks controlling DIS are well understood and efforts are made to exploit this knowledge to limit premature senescence within agriculture.

Treatment with UV-B radiation has become relatively common in post-harvest technology as a method improving the longevity of harvested crops via its anti-microbial effects (Kasim and Kasim, 2018) and by reducing post-harvest yellowing (Srilaong *et al.*, 2011; Aiamla-or *et al.*, 2010). These studies on post-harvest yellowing indicate a role for UV-B in the regulation of DIS, though they have largely focused on relatively high intensity treatments. However, an interesting potential link between DIS regulation and low intensity UV-B radiation can be postulated following recent work which has found that PIFs play a key role in the promotion of DIS, and that these PIF4/5 can be degraded in a UVR8- dependent manner by short, low intensity doses of UV-B (Sakuraba *et al.*, 2014; Hayes *et al.*, 2014; Sharma *et al.*, 2019).

Here, it has been shown that low intensity UV-B light, perceived by the UVR8 photoreceptor, does play an important role in the regulation of DIS in Arabidopsis (Figures 5, 7, 10). Treatment with a 4 h dose of UV-B prior to harvesting leads to inhibition of chlorophyll degradation, and a visible reduction in leaf yellowing, during incubation in darkness (Figures 5, 6). This response is mediated by the UVR8 photoreceptor as the Arabidopsis null mutant *uvr8-6* does not show a delayed senescence phenotype in the form of inhibited chlorophyll degradation following treatment with UV-B (Figures 10, 11). This work also found the response is likely not a result of UV-B induced

upregulation of antioxidant capacity, as there was no significant difference in Arabidopsis plants treated with UV-B for either 4 or 6 h compared to untreated controls (Figure 9.)

A hypothetical regulatory network can be inferred in light of these results (Figure 12) in which the absorption of UV-B light by UVR8 induces multiple pathways leading to the inhibition and degradation of PIFs, which disrupts the PIF-mediated senescence-promoting feed-forward loops involving ABI5, EEL, EIN3 and ORE1 (Sakuraba *et al.*, 2014; Hayes *et al.*, 2014; Sharma *et al.*, 2019; Liebsch and Keech, 2016). One pathway is mediated by HY5/HYH and involves an increase in gibberellin 2-beta-dioxygenase 1 (GA2ox1) which leads to a reduction in GA synthesis, causing the destabilisation of DELLA proteins (a family of homologues which mediate GA signalling (Hussain and Peng, 2003))

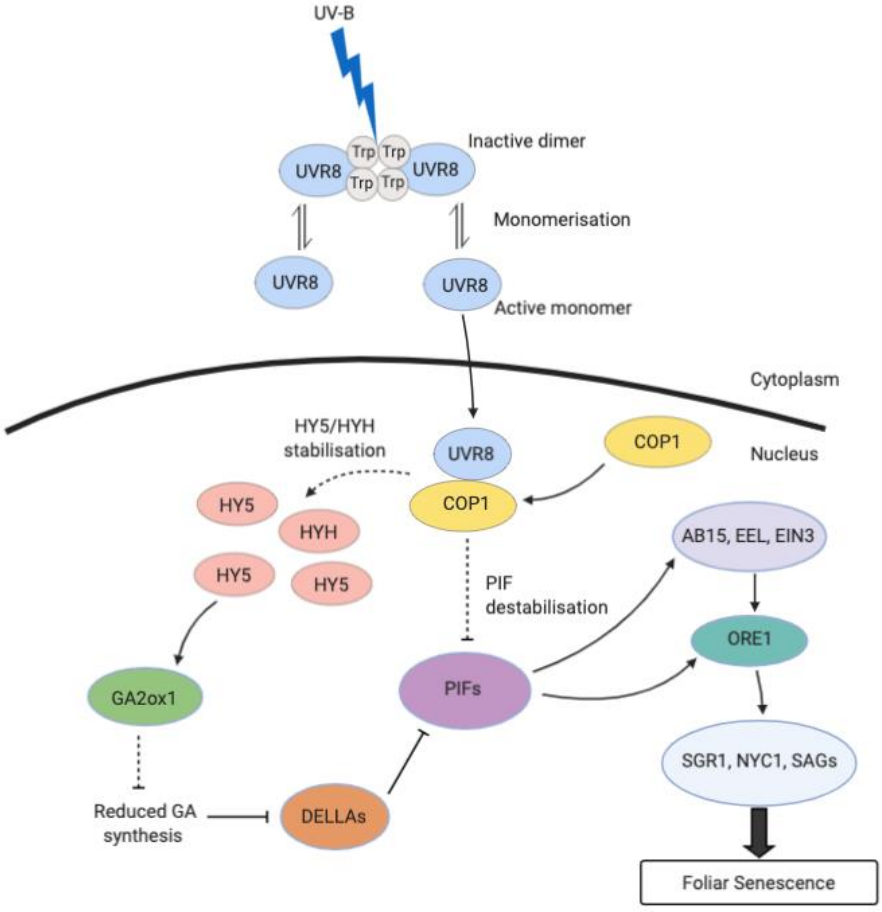

Figure 12. Hypothetical model depicting how UVR8 perception of UV-B inhibits PIF activity and antagonises senescence. When UV-B light is absorbed by UVR8, active UVR8 monomers interact with COP1. These has a dual impact leading to the inhibition and degrade PIFs. In one pathway, UVR8-COP1 complexes stabilise HY5/HYH which leads to an increase in gibberellic acid oxidases (GA2ox1) and a reduction in GA synthesis. This leads destabilisation of DELLA proteins and inhibition of PIFs. In another pathway, sequestration of COP1 by UVR8 destabilises and degrades PIFs. In the absence of UV-B, PIFs form multiple feed-forward loops with *AB15, EEL, EIN3, ORE1* which promote senescence via *SGR1, NYC1* and various *SAG*s. (Diagram adapted from Sakuraba *et al.,* (2014), Hayes *et al.,* (2014), Liebsch and Keech, (2016) and Sharma *et al.,* (2019). Created with BioRender.com).

and consequently inhibiting PIFs. The other pathway is independent of HY5/HYH, the sequestration of COP1 by the active UVR8 monomer destabilises and degrades PIFs. Previous work has also identified a UVR8-independent delayed senescence response in Arabidopsis following treatment with high intensity UV-B (Sztatelman *et al.*, 2015), suggesting there are potentially multiple mechanisms underlying this response which differ based on the intensity of UV-B perceived.

5.1 *Conclusions and future work*

This work has shown that treatment with low intensity UV-B antagonises DIS in a UVR8-dependent manner. It further suggests that this response is not mediated by UV-B-induced increases in antioxidant capacity or reductions in membrane permeability. It lays out the foundations for further investigation into how UV-B contributes to the regulation of DIS and highlights the importance of consistency in the fluence rates and lengths of treatments used in UV research, as there is clearly a distinction between the mechanisms underpinning the high and low intensity UV-B induced antagonism of DIS. The results seen here potentially have agricultural importance as low intensity treatments with UV-B are much safer to apply in an agricultural setting than high intensity UV-B. However, it would be necessary to further compare the efficacy of high and low intensity UV-B treatments in order to ascertain whether their application would reduce the impacts of premature senescence in an economically viable way. It would also be necessary to see if these results are replicable in a variety of crop species, particularly those strongly impacted by DIS such as herbs and microgreens.

Moving forwards, it would be useful to conduct follow up experiments quantifying the accumulation of SAGs under these treatment conditions in order to observe senescence on a finer scale. qPCR experiments had been planned as part of this work, but these were not completed due to the time restrictions and laboratory access limitations caused by COVID-19. *SAG13* can be used as an early marker of senescence as it shows very little basal expression in non-senescent leaves and is detectable around 48 h before the onset of leaf yellowing (Weaver *et al.*, 1998). Time course data of *SAG13* transcript abundance would be useful for tracking the initial onset and then progression of DIS on a molecular level. Other suitable genes for doing this include, but are not limited to, various other SAGs, *SEN1* and *ORE1* (Weaver *et al.*, 1998). It may also be useful to quantify the protein

expression levels of these genes using Western Blots to identify whether their transcript abundance directly translates to protein abundance or not. Given the theoretical relationship posited between UV-B induced antagonism of DIS and PIF4/5 activity, it would be of interest to quantify PIF4/5 protein abundance during DIS following UV-B treatment to confirm this link. Identifying other downstream targets of UV-B signalling that are relevant to senescence processes will be important for gaining a clearer understanding of the complex DIS regulatory network.

Finally, it may be interesting to investigate whether any crosstalk exists between temperature and UV-B signalling during DIS. In the assay described in Chapter 4 where the temperature exceeded the normal experimental conditions of 21 °C due to a power cut, senescence appeared to be accelerated with samples showing increased chlorophyll degradation and leaf deterioration. A recent publication found that leaf senescence is accelerated at high temperatures via PIF4/5 (Kim *et al.*, 2020). Given that UV-B degrades PIF4/5 (Sharma *et al.*, 2019), it may be interesting to conduct further experiments investigating whether any interaction exists between temperature and UV-B signalling during dark-induced senescence. If UV-B-induced degradation of PIF4 could be used to ameliorate PIF-mediated accelerated leaf senescence caused by a high ambient temperature, this may present an opportunity for reducing the need for refrigeration during the transport of harvested crops.

Chapter 6 – Appendix

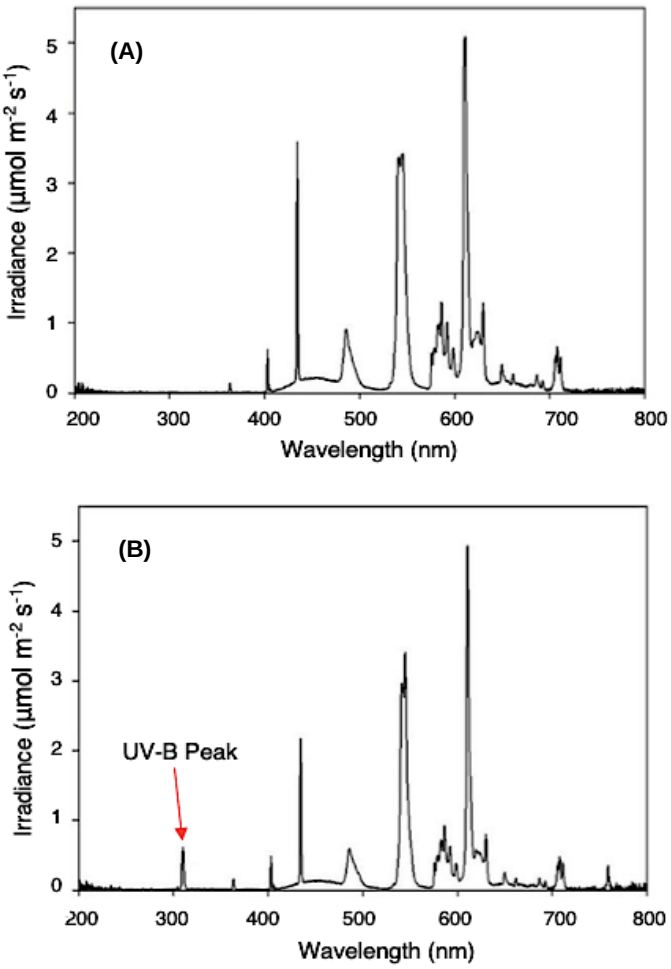

Figure S1. Growth cabinet light spectra. Measurements taken at sample height showing **(A)** white light at 80 µmol m^{-2} s^{-1} (- UV-B) and **(B)** white light supplemented with UV-B at 1 µmol m^{-2} s^{-1} (+ UV-B).

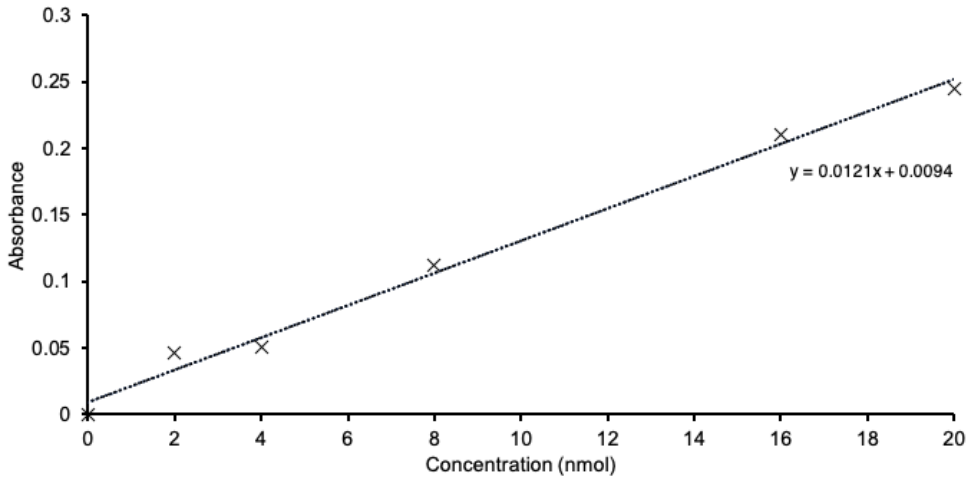

Figure S2. Example antioxidant assay standard curve. Absorbance of Trolox standards made up to 2, 4, 8, 16 and 20 nmol and their absorbance used to calculate the Trolox equivalent antioxidant capacity of samples.

Chapter 7 – References

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