UVB radiation; a specific regulator of the growth and development of *Arabidopsis thaliana*.

Aoife Marie Coffey B.Sc.(Hons)

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School of Biological, Earth and Environmental Sciences

Head of School: Professor Sarah Culloty

Research Supervisor:

Marcel Jansen

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Table of contents

Declaration

This work has not been previously accepted in substance for any degree and has not been concurrently submitted in candidature for any degree. This thesis is the result of my own independent work/investigation, except where otherwise stated.

Aoife Coffey

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Abstract

Research into the impacts of UVB radiation on plants and ecosystems began in the 1970's in answer to concerns about the degradation of the stratospheric ozone layer. Early research focused solely on UVB as an agent of plant stress but recently the thinking surrounding UVB has undergone a paradigm shift, now it is seen as a key regulator of plant growth and development. The "UVB response" encompasses a multiplicity of changes in gene expression, metabolism and morphology. A thorough description of the range, complexities and interconnectedness of this response has only begun. The overall aim of this thesis was to explore the functional role the UVB response pathway and attempt to clarify some of the mechanisms behind these. This was achieved using *Arabidopsis thaliana* as a model system in both indoor and outdoor experiments. It was found that a plants ability to up regulate total soluble phenolics in response to a low dose UVB is potentially more important for UV-protection than accumulation of quercetins and kaempferols that are specifically glycosylated at C-7. Interestingly, the flavonoid glycosylation pattern affected plant morphology. Yet, one of the primary findings of this study was that the UVB induced changes in morphology were transitory. This study also demonstrated the role of UVB radiation and the UVB photoreceptor on morphology and biochemical make-up under changeable, complex, outdoor conditions. It was concluded that a functional UVB photoreceptor is required for optimized plant growth under natural UVB. Evidence of potential practical applications of UVB radiation within the protected cropping industry were also investigated using *Lactuca sativa*. Based on the findings it is proposed that key plant responses to UVB radiation may be exploitable in the context of improved crop quality and nutritional value.

Chapter 1

Introduction

Ultraviolet denotes electromagnetic rays that are beyond the violet and visible parts of the light spectrum. As radiant energy from the sun it can be broken down into three parts UVC (200-280nm), UVB (280-315nm) and UVA (315-400nm). UVC radiation is completely blocked by the Earth's atmosphere and does not reach ground level. UVB and UVA on the other hand make up approximately 7% of the light spectrum that reaches ground level (Frohnmeyer & Staiger, 2003). The primary focus of this thesis is the effects of UVB radiation on plants. UVB is heavily absorbed by the stratospheric ozone layer so the portion which reaches ground level is relatively small (Madronich *et al.,* 1998). However, with a short, highly energetic wavelength its influence is significant even at less than 1% of the total light spectrum (Caldwell & Flint, 1997). The actual UVB dose experienced at ground level can vary considerably depending on a number of factors. The position on the Earth surface, physical geography, local climatic conditions, season and pollution can all either reduce or enhance UVB at ground level (Madronich *et al.,* 1998; Kakani *et al.,* 2003; Calbó *et al.,* 2005; Liley & McKenzie, 2006; McKenzie *et al.,* 2009).

In the 1970's scientists Rowland and Molina proposed that chlorofluorocarbons (CFC's), which were routinely being released into the atmosphere in large quantities, could cause chemical degradation and permanent damage to the ozone layer (Molina & Rowland, 1974). Almost a decade later another group with the British Antarctic Survey found compelling evidence that CFC's were already significantly degrading the stratospheric ozone layer (Farman *et al.,* 1985). Abnormally low stratospheric ozone concentrations were discovered above Halley Bay near the South Pole (Farman *et al.,* 1985). While the description of this thinning as a hole in the ozone layer is metaphorical, the evidence presented was no less shocking and it prompted international action. The implications of increasing UVB levels at ground level due to

the thinning of the stratospheric ozone layer could have been far reaching, as human health, crop production and natural ecosystems are all vulnerable to high levels of UVB (Nolan & Amanatidis, 1995). The Montreal Protocol on Substances that Deplete the Ozone Layer was agreed in September 1987 and since then it has undergone several revisions. However, its success is undeniable. It is the first international treaty to address a global environmental threat and has been described by Kofi Annan as "perhaps the single most successful international agreement to date". Decreases in the atmospheric burden of ozone depleting substances may have brought about the stabilization of the stratospheric ozone but due to natural variation in the stratospheric ozone layer, definitive evidence of a recovery is not yet detectable (Ravishankara *et al*., 2009; McKenzie *et al.*, 2014). Furthermore, lack of action in response to climate change and emissions of damaging compounds such as nitrous oxides, have the potential to undo the strides made towards the recovery of the ozone layer (Ravishankara *et al*., 2009; McKenzie *et al.*, 2014). So, while the health of the ozone layer is not an urgent concern right now, due to its vulnerability it requires continued monitoring.

Much of the research into plant UVB interactions originated in the 70's and 80's when the focus of many research projects was on elucidating the biological effects of higher than normal levels of UVB radiation. This research identified that UVB radiation is highly effective at eliciting a reaction in plants. It is absorbed by vital proteins and nucleic acids and damage to said can result in DNA-damage, the production of ROS and impairment of cell processes such as photosynthesis (Jansen *et al.,* 1998; Hollósy, 2002). Exposure to very high levels of UVB radiation can ultimately lead to severe retardation of plant growth (Rozema *et al.,* 1997). In the context of this thesis, high UVB is defined as levels known to cause stress, whereby UVB exposure is sufficient

to cause massive development of ROS, over-riding the antioxidant capacity regulated by non-specific stress pathways and contributing to both signalling and gene expression (Hideg *et al*., 2013). As research has progressed from the early stages several shortcomings in UV exposure methodologyhave been identified, in particular the unrealistically high levels of UVB used and the lack of attenuation by realistic background levels of PAR and UVA (Rozema *et al.,* 1997). This resulted in the damaging effects of UVB being exaggerated (Rozema *et al.,* 1997). Serious reductions in primary production, predicted by lab-based studies, were not reproduced under more natural conditions (Rozema *et al.*, 1997; Ballaré *et al.*, 2011). Further research has shown that the mechanisms behind acclimation, damage prevention and amelioration in plants in response to UVB are sophisticated and well-developed (Jansen *et al.,* 1998). It should also be remembered that during the evolution of land plants the ozone layer was likely much thinner and the levels of UVB experienced at ground level much higher (Rozema *et al.,* 1997). Building on the early discoveries there has been a shift in focus towards understanding the intricacies of the UVB response pathway. The realisation that UVB stress is rare (Ballaré *et al.,* 2011) raises new questions in relation to the UVB response. Indeed, unpicking the complex responses to low more natural doses of UVB radiation has proven to be a fascinating and dynamic area of research. For example, plant herbivore interactions can be affected directly and indirectly by UVB. Increased resistance to herbivores has been linked to upregulation of UV induced protective compounds (Ballare, 2014) and species-specific responses to direct UVB exposure such as a herbivores vision, and target plant acquisition can be affected by changes in the UV spectrum (Paul and Gwynn-Jones, 2003). Rather than an agent of plant stress, it is increasingly recognised that UVB acts as an environmental regulator and potentially as a proxy

measure of co-occurring environmental conditions (Jansen *et al.,* 2012). A well-documented response to UVB radiation is evident in plant biochemistry (Jansen *et al.,* 2008). Secondary metabolites are versatile compounds that can mediate multiple interactions between plants and their environment (Schreiner *et al*., 2014). Changes in the metabolite profile of plants enhance resistance to biotic stress such as necrotrophic pathogens and herbivores and abiotic stressors such as drought (Jenkins, 2014). UVB triggers the accumulation of secondary metabolites such as phenolics, carotenoids and glucosinolates (Schreiner *et al.,* 2014) even at low doses that do not induce stress. In Arabidopsis, the UV-induced flavonoids are primarily quercetins and kaempferols rhamnosylated at the seven position (Hectors *et al.,* 2014). Accumulation of specific, glycosylated flavonoids has also been observed in other UV-exposed species for example kale (Neugart *et al.,* 2012). The function of the specific glycosylation pattern is not clear (Hectors *et al.,* 2014). Irrespective of glycosylation, the up-regulated flavonoids act as sunscreen and antioxidants preventing any potential damage caused by UVB exposure (Agati & Tattini, 2010).

The benefits of UVB induced accumulation of secondary metabolites are not only felt by the plants themselves. Consumption of plant polyphenols such as flavonoids other plant secondary metabolites have been strongly associated with the maintenance of a healthy diet and a milieu of health benefits including protective functions against a range of chronic diseases (Vinson *et al.,* 1998). Evidence from a long-term dietary study has found that plant secondary metabolites may help with weight control, an important finding in light of the recent obesity epidemic (Bertoia *et al.,* 2016). Quercetin and kaempferol, both of which are up regulated by UVB exposure, have antibacterial, antiviral and anti-inflammatory properties (Dillard & Bruce German, 2000). Secondary metabolites such as flavonoids and anthocyanins are also known as

bioactive components of food (de Pascual-Teresa & Sanchez-Ballesta, 2008). Flavonoids and anthocyanins have been associated with free radical scavenging and it is suggested they act as a protective element against the development of cancer, cardiovascular disease and other chronic ailments in human consumers (de Pascual-Teresa & Sanchez-Ballesta, 2008). Accumulation of secondary metabolites also change the flavour and colouration of some food crops, and these sensorial characteristics potentially affect a foodstuffs acceptability to the consumer (Spence, 2015). Consequently, by enhancing the concentrations of plants secondary metabolite through UVB exposure, the nutritional value and attractiveness of a crop may be improved.

Exposure to UVB mediates changes in plant morphology; this phenomenon is widely reported across a range of species. The response is characterised primarily by shorter, thicker leaves, shorter petioles, leaf curling, inhibited development of the hypocotyl and stem and changes in the root/shoot ratio (Hollósy 2002; Jansen, 2002; Wargent *et al.*, 2009 (a); Wargent *et al.* 2009 (b); Hectors *et al.,* 2012). The "UVB phenotype" commonly refers to a plant with dwarf morphology. While the change in plant architecture is often described as a response to UVB, the functional and ecological relevance of this response remains elusive (Robson *et al.*, 2014). The dividing line between stress induced reduction in growth (SIMR) and the development of true UVB phenotype remains blurred (Robson *et al.*, 2014). It is also unclear if concurrent changes in concentrations of secondary metabolites are linked to the changes in morphology or if they are simply parallel phenomenon (Robson *et al.,* 2014).

UVB induced morphogenesis in response to low ecologically relevant levels of UVB seldom adds up to a reduction in biomass but more commonly equals a redistribution of growth (Rozema *et al.*, 1997). For a grower the ability to produce a more compact

and robust dwarf plant may actually be a desirable outcome (Wargent *et al.*, 2011). Such plants may tolerate harvest, packaging and transportation better, ultimately yielding more harvestable, commercially valuable biomass (Wargent & Jordan, 2011). The horticultural industry already makes extensive use of protected environments. These cropping systems provide the opportunity for manipulation of plant responses using both, ambient or artificial environmental stimuli to produce a more valuable or tailored product. Manipulating the light environment to improve crop quality has become a practical option with the advent of new wavelength specific transmitting plastics and more affordable LED lighting systems (Paul *et al.*, 2005). However, actual reductions in leaf area and shoot mass which could lead to reduced biomass have also been reported (Robson *et al.*, 2014). Any UV response which reduces the marketable biomass of a crop plant would not be a positive outcome. Thus, the exact conditions required to allow morphological and metabolic manipulation without any negative impacts on biomass need to be investigated so that the potential of UVB as low input tool within the horticulture industry can be fully realised.

For some time a description of the physiological UV-responses existed without the knowledge of the photoreceptor which orchestrated them. However, in recent years said photoreceptor has been identified through a genetic approach which detected *UV RESISTANCE LOCUS8* (Kliebenstein *et al.*, 2002). Further research has confirmed UVR8 as the UVB photoreceptor (Rizzini *et al*., 2011). Plants which contain the mutated, inactive form of UVR8 were found to be hypersensitive to UVB radiation (Kliebenstein *et al*., 2002). The full extent of the influence of UVR8 on plant physiology has not yet been elucidated, but ongoing research is uncovering that UVR8 is integrated with other photoreceptor pathways (Jenkins, 2014). A shared signalling network has been proposed to regulate plant responses to shade involving

cryptochrome, phytochrome and UVR8 (Fraser *et al.,* 2016). Many of the studies conducted on UVR8 have taken place indoors but interestingly its influence has been demonstrated under outdoor conditions as well (Morales *et al.,* 2012). Morales *et al*., (2012) found that UVR8 is also important for gene expression and biochemical composition in natural sunlight. UVR8 has been conserved throughout the plant kingdom, and amino acid sequences like those found in Arabidopsis have also been found in mosses and green algae (Jenkins, 2014; Rizzini *et al.,* 2011). However much remains to be discovered about this photoreceptor in plant species apart from Arabidopsis and its integration with other signalling pathways (Jenkins, 2014).

Aims

1. To investigate the effects of a low chronic dose of UVB on the morphology and flavonoid-profile of *Arabidopsis thaliana.* Attention will be paid to the consequences of leaf age and development for morphological and biochemical response to UVB radiation.
2. To investigate whether UVB responses are local and/or or transmitted systemically throughout the Arabidopsis plant. The plasticity of plant responses to UVB radiation will be examined by selectively exposing individual leaves of an Arabidopsis rosette to UVB radiation.
3. To investigate the importance of a specific flavonoid glycosylation pattern on the development of the UVB phenotype, UV acclimation and protection of Arabidopsis plants under a low chronic dose of UVB. This experiment will be facilitated by the use of transgenic plants that lack the ability to catalyse glycosylation at the C-7 position (*ugt89c1*).
4. To investigate adaptation to local or prevailing light conditions, by comparing the UVB responses of the local Arabidopsis accession, Bur-0 with those of

Col-0 and Ler. A series of outdoor experiments will be conducted with the intention of exploring the effects of natural UVB radiation within the context of an oceanic climate, close attention will be paid to the influence of season on the outcomes.

5. To investigate the functional role of the photoreceptor UVR8 under natural light conditions. In this outdoor experiment it is aimed to assess the importance of UVR8 for growth development and UV protection of *Arabidopsis thaliana* exposed to natural levels of UVB radiation through the use of the *uvr8-1* mutant
6. To determine the effects of natural levels UVB on the bronze lettuce Cos 'Dixter'. Of particular interest is whether the UVB response in an oceanic climate is measurable and strong enough to affect a commercial crop.

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Chapter 2

An investigation into the effects of low chronic UVB exposure on the morphology and flavonoids of *Arabidopsis thaliana*.

Abstract

As sunlight is of primary importance for plant growth and development exposure to UVB radiation is unavoidable. As a short highly energetic wavelength, it can cause damage to vital cell processes and organs often resulting in a reduction in primary production. However, it has been realised that under natural UVB levels plant stress and damage are rare. As sessile organisms, plants have developed a range of strategies to ameliorate the damaging effects of UVB. The focus of this study was to investigate the effects of low chronic doses of UVB on morphology and flavonoids of *Arabidopsis thaliana* rosettes. The impact of leaf age and developmental stage on UVB specific quercetin and kaempferols was also investigated. An investigation into the systemic versus local nature of UVB response was carried out to examine the ability of plants to tailor its response to UVB. The effects of relatively low dose UVB on morphology appears to be transitory, however the effects of UVB on phenolics are more persistent over time. There was significant up-regulation of specific quercetins and kaempferols in the presence of UVB. Evidence of both systemic and local effects of UVB was found in the morphology and total soluble phenolics.

Introduction

As a source of energy and information, sunlight is of primary importance for plant growth and development. Consequently, exposure to UVB radiation is unavoidable for plants and despite making up only a small fraction of the light spectrum its impact can be significant. For the most part UVB is absorbed by the stratospheric ozone layer but even the percentage that does reach ground level can cause damage to vital cell processes and organs due to its highly energetic nature (Jansen *et al*., 1998; Hollósy, 2002). The possibility of increasing UVB levels at ground level was once a major concern for natural environments, crop systems and human health. The success of the Montreal Protocol (1987) largely assuaged these concerns. Furthermore, several studies have shown that the evidence for UVB damage under natural conditions is rare (Searles *et al*., 2001).

Through the early research, it was realized that as sessile organisms plants have developed a range of mechanisms to deal with the damaging effects of UVB (Jansen *et al*., 1998). It is now the mechanism and adaptive relevance behind the UVB response that is of most interest. The UVB photoreceptor has only recently been described, the UVB response is mediated by *UVB RESISTANCE LOCUS8* (UVR8), a dedicated UVB photoreceptor (Kliebenstein *et al*., 2002; Rizzini *et al*., 2011). This photoreceptor is expressed throughout the plant and allows for a rapid response to UVB exposure (Rizzini *et al*., 2011). The photomorphogenic response orchestrated by UVR8 acts at a genetic level to change plant morphology, biochemistry, photosynthetic competence and defences in response to UVB (Jenkins, 2014).

UVB through the UVR8 photoreceptor induces numerous changes in plant morphology including shorter, thicker leaves, shorter petioles, leaf curling, inhibited

development of the hypocotyl and stem and changes in the root/shoot ratio (Jansen, 2002; Hectors *et al*., 2012; Wargent *et al*., 2009 (a); Wargent *et al*., 2009 (b); Hollósy, 2002). The results of these changes in plant architecture is the development of a stockier more compact plant described as dwarf. A similar phenotype is induced by a broad range of stressors and is collectively described as Stress-induced Morphogenic Responses (SIMR) (Potters *et al*., 2007). While once, the UVB phenotype was purported to be a consequence of damage or stress there is now evidence that through UVR8 it can be produced under very low, non-stress inducing levels of UVB. This UVR8 mediated response perhaps allows for the refinement of a plants physiology and biochemistry in response to their immediate surroundings and environment (Robson *et al*., 2014). There is also evidence that in some cases the changes seen in morphology are transitory (Hectors *et al*., 2010). It has been speculated this is due to the redirection of resources to allow for the up regulation of protective mechanism such as ROS scavenging, UV screening and DNA repair capacities (Robson *et al*., 2014). However, a satisfactory explanation of why exposure to UVB induces changes in morphology has not yet been described and major questions remain about the adaptive relevance of such a response (Robson *et al*., 2014).

Changes in flavonoids have also been extensively documented in the study of UVB responses (Jenkins, 2014). Quercetin and kaempferol glycosides are up regulated in response to UVB even at relatively low doses (Hectors *et al*., 2014; Kolb *et al*., 2001; Ryan *et al*., 1998). Flavonoids are most commonly found as glycosolated derivatives and can vary with leaf age and developmental stage (Jordan *et al*., 1998; Hectors *et al*., 2012). They are protective compounds against biotic and abiotic stressors fulfilling the role of antioxidants and UVB screens (Agati & Tattini, 2010). The metabolic changes induced by UVB can also enhance a plants ability to cope with other

potentially stressful environmental conditions (Jenkins, 2014). This suggests that plants may be utilising UVB as proxy to generally upregulate stress tolerance to concurrent environmental and climatic conditions (Jansen & Bornman, 2012). Up-regulation of flavonoids is often reported to parallel changes in morphology but it has not yet been established if these reponses are functionally linked or simply co-occuring (Robson *et al*., 2014).

The changeable nature of the natural environment means that plants need to be super adaptors, optimising their phenotype to maximise their tolerance to a broad range of conditions. Changes to morphology and biochemistry can be metabolically costly and can affect fitness. To ensure the changes made are appropriate; information is key. Cryptochrome, phytochrome and the relatively newly identified UVR8 gather information about the light environment, its spectral quality and quantity. The isolation and identification of UVR8 moved our understanding of plant responses to the UVB portion of the light spectrum significantly (Kliebenstein *et al*., 2002; Brown *et al*., 2005; Rizzini *et al*., 2011). Perception of UVB by UVR8 triggers a signalling cascade which switches on or off a range of responses (Brown *et al*., 2005; Jenkins, 2014). Through the study of UVR8, it has been realized that even at very low levels of UVB the impact on the physiology of a plant can be significant. Evidence has been found of an interaction between phytochrome and UVR8, in the mediation of the shade avoidance response (Hayes *et al*., 2014). Another study which looked at light competition between Arabidopsis rosettes found that shade avoidance was a localised response which worked on a leaf by leaf basis (Mullen *et al*., 2006). On the other hand, responses to high light stress have been found to be systemic, defences against ROS caused by high light are up-regulated in distal leaves (Karpinski *et al*., 1999; Mullineaux *et al*., 2000). The latter response is also thought to be mediated by the

red:far red photoreceptor phytochrome. There is also evidence that some responses to high levels of UVB are systemic with flavonoids and other signalling compounds being transported around the plant (Tossi *et al*., 2012; Liu *et al*., 2015). While some evidence of a systemic response to high levels of UVB exists, it is not clear if the induced changes to low, more environmentally relevant doses are also systemic. Potentially systemic responses to high levels of UVB represent an induction of more generic stress acclimation or tolerance pathways. It remains to be seen whether low levels of UVB produce a similar systemic response or rather a local one.

This experiment was undertaken to investigate the effects of low chronic doses of UVB on morphology and flavonoids of *Arabidopsis thaliana* rosettes. It is hypothesised that even at relatively low doses UVB exposure will result in altered morphology and plant biochemistry. Three time points were selected to investigate the effect of leaf age and developmental stage on the UVB induced accumulation in total phenolics and morphology. The impact of UVB on specific quercetin and kaempferols was also investigated with particular attention paid to the effects of leaf age and developmental stage. To investigate the ability of a plant to tailor its response to UVB, an investigation into the systemic versus local nature of UVB induced changes in morphology and total soluble phenolics was subsequently carried out.

Materials and Methods

Plant Material

Seeds of *Arabidopsis thaliana* Columbia-0 were cold treated at 4^0C before sowing into flats containing sieved John Innes No.2 compost (J. Arthur Bowers, William Sinclair Horticulture Ltd., Firth Rd., Lincoln, LN6 7AH). The flats were covered with cling film and placed in a temperature controlled growth room on a 16 hour light / 8 hour

dark photoperiod. They received only PAR in the growth room, no UV-A or UV-B, at an intensity of 40-60µmol m^{-2} s^{-1}. Once the seeds had germinated, the cling film was removed. At the cotyledon stage, the seedlings were transplanted into 200ml individual pots containing John Innes No. 2 compost. The seedlings were placed back into the growth room and covered with cling film for a further 2 days until they re-established. They were allowed to reach the 1.04 growth stage (Boyes *et al*., 2001) before beginning the experiment.

Experimental Set-up

Experiments were conducted in a self-contained light box, fitted with PAR (36W Philips Master TLD Reflex Tube, BLT Direct), UV-A (Fluorescent Blacklight Blue 36W, 1200mm) and UV-B (TL12, Phillips, Eindhoven, The Netherlands) fluorescent tubes. Temperature within the box was 22^0C +/- 2 degrees and a relative humidity of 30%. The intensity of the PAR was 60-80 µmol m^{-2} s^{-1} and the UV-A was 0.16mWcm^{-2}. A dimmable ballast (Sylvania-Biosystems, Wageningen, The Netherlands) was used to regulate the intensity of the TL12 tubes without changing the UV-B spectrum (verified with Ocean Optics Spectroradiometer (USB2000+RAD) (Ocean Optics, Dunedin, FL, USA). The output of the UV-B tubes was set to generate 0.6W/m^2 +/- 0.04 Watt/m^2. Plants grown +uvb were exposed for 4, 7 and 10 days for two hours each day at noon this translates to a biological effective dose of 0.6648kJ m^{-2}/day (Flint and Caldwell, 2003). The UV-C component that is generated by the TL12 tubes was blocked using a filter of cellulose acetate (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany). Control plants (-uvb) were grown under UV-B blocking filter (125µm thickness, Polyester film, Tocana Ltd., Elizabeth's Cross, Ballymount Cross Ind. Est., Ballymount, Dublin 24). Both filters were placed 5cm above the plants on opaque frames. Both filters were changed after 20 hours of UV-B exposure. The

photoperiod in the light box was the same as in the growth room, 16 hour light/ 8 hour dark sequence. The plants were acclimated in the light box for a minimum of 24 hours before switching on the UV-B lights.

EXP 1: Morphological analysis

Leaf and rosette morphology was analysed after 4, 7 and 10 days of UV-B exposure. Whole rosettes were photographed for rosette diameter measurements. Rosettes were then dissected and leaves were arranged in developmental order, with L1 (Leaf 1) being the oldest leaf and L9 (Leaf 9) being the youngest. Following this, leaves were photographed for processing with ImageJ software (Abràmoff *et al.*, 2004). Parameters measured included total leaf area, length, width, petiole length, leaf blade length, width, and area. Leaves with petioles less than 2mm were not included in analysis.

Photosynthetic Efficiencies

Chlorophyll *a* fluorescence (F_v/F_m) was determined using an Imaging PAM (Waltz, Germany) as a proxy measure of the maximal quantum yield of photosystem (PS) II efficiency. F_v/F_m values were determined after plants had grown for 4, 7 and 10 days under +/- UVB radiation. Whole rosettes were dark adapted for a minimum of 20 minutes before F_v/F_m was determined. Three measurements were taken at random from each rosette and pooled per rosette.

Total soluble phenolics

Total soluble phenolics were extracted using acidified methanol (1%HCL, 20%H_2O, 79%CH_3OH) (Biswas & Jansen, 2012). The whole leaves were placed in micro-tubes with 1ml acidified methanol and incubated in the dark at 4^0 C for 4 days. The

supernatant was drawn off using a pipette and placed in quartz glass curvette. Absorbance was recorded at 330nm on a spectroradiometer (Shimadzu – UV visible spectrophotometer – 160A). Absorbance was normalized per leaf using total leaf area and this measurement was referred to as Total Soluble Phenolics.

Individual flavonoid compounds were analysed following Hectors *et al.,* (2012). L4, L5 and L6 were identified and separated from the rest of the rosette. Leaves from at least 5 plants were pooled to provide enough biomass for analysis from each treatment. L4, L5 and L6 were all analysed separately. This was repeated independently 3 times. Samples were then analysed in the University of Antwerp as follows. Arabidopsis leaves were frozen using liquid nitrogen and ground in a Magna Lyser (Roche, Basel, Switserland). To extract flavonoids, leaves were homogenized in acidified methanol (5µl 62.5% (v/v) methanol acidified with 0.125% (v/v) formic acid per milligram fresh weight) and sonicated in an ultrasonic bath for 30min followed by filtration [(True Nylon Syringe filter, 0.2 µm), Grace Davison Discovery Science, Deerfield, IL]. Kaempferol-3-rhamnosidoglucoside (10^{-2}M final concentration; Carl Roth GmbH, Karlsruhe, Germany) was used as internal tracer to take into account recovery losses and ionization efficiency.

Flavonoid compounds were analysed using an ACQUITY UPLC chromatography system combined with an ACQUITY TQD (Waters, Milford, MA) mass spectrometer. Samples were injected on a VanGuard pre-column (BEH C18, 1.7 µm, 2.1×5mm2; Waters) coupled to a reversed phase column (HSS C18, 1.8 µm, 2.1×100 mm2; Waters). The solvents used were water, 0.1% formic acid (C) and acetonitrile, 0.1% formic acid (D). TQD analysis was performed in ESI(+)-MRM mode. Samples were eluted during a 4-min run using a constant flow rate of 600 µlmin^{-1} and a column temperature of 40^0 C. Solvent gradient started at 13.5% D, slowly increasing to 16.7%

D in 1.5 min and further increasing to 51%D in 2.5 min. The column was rinsed for 1 min at 86% D and equilibrated at 13.5% D between samples. TQD analysis was performed in ESI(+)-MRM mode using the following parameters: capillary voltage 3 kV, cone voltage 20 V, source temperature 150^0 C, desolvation temperature 350^0 C and collision energy 30 V. Chromatograms obtained were processed using QUANLYNX v4.1 (Waters). Concentrations were calculated using the reference compound Kaempferol-3-rhamnosidoglucoside with retention time 2.54 min and fragmentation pattern 595 > 287.

EXP 2: Investigation of the systemic vs local nature of the UVB response.

To asses if the UVB response in morphology and phenolics is a systemic or local effect a UVB filtration method was adopted. Seeds were sown, transplanted and grown on as described above. This consisted of two treatments:

Treatment 1; L4-uv

Treatment 1 involved the identification and selection of Leaf 4 of Arabidopsis rosettes at the Boyes 1.04 stage (Boyes *et al*., 2001). Leaf 4(L4) was covered using a strip of Mylar suspended not more than 3mm above it using a supporting pin. The strip was cut to fit each individual rosette (Fig. 2.1).The Mylar strips were changed on each plant as the treatment progressed to allow the rosettes and L4 to expand normally while continuing to block L4 from receiving direct UVB and to prevent encroachment of the strip onto other leaves (Fig 2.1). The Mylar strip allowed the rest of the rosette to receive direct UVB while L4 did not. This treatment is referred to as L4-uv (Fig 2.1).

Treatment 2; L4+uv

Treatment 2 involved the identification and selection of Leaf 4 of Arabidopsis rosettes at the Boyes 1.04 stage (Boyes *et al.*, 2001). The whole rosette was covered using a piece of Mylar suspended not more than 3mm above it on pins. A wedge was cut above Leaf 4, this allowed L4 to received direct UVB while the rest of the rosette was shielded. The piece of Mylar was cut to fit each individual rosette (Fig. 2.1). It was changed each day for each plant as the treatment progressed to allow the rosettes and L4 to expand normally while L4 continued to receive direct UVB but blocking it from the rest of the rosette. This treatment is referred to as L4+uv (Fig2.1).

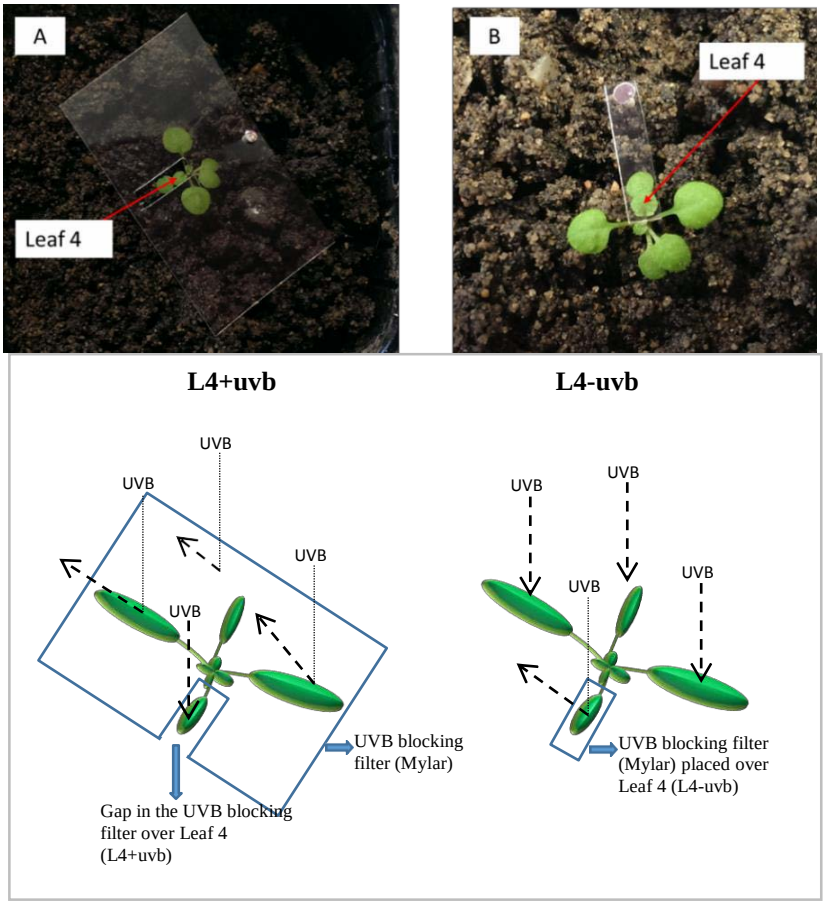

Fig. 2.1 Photographs of Arabidopsis rosettes, which describe the experimental setup on day 1 of the treatment L4+uv, panel A and L4-uv, panel B. This figure also includes a schematic diagram describing the UVB exposure experienced by each treatment during the experiment. A cellulose acetate covered both of the treatments for the duration of the experiment.

L4-uv and L4+uv plants were placed under cellulose acetate filters grown for 7 days in a growth box with PAR, UVA and UVB. Included in this experiment as controls were fully exposed (+uvb) and unexposed (-uvb) rosettes grown in the same growth box.

Plant growth analysis

After 7 days, growth rosettes were dissected and leaves were arranged in developmental order and photographed for processing with ImageJ software. Parameters measured included rosette diameter, total leaf area and petiole length. Leaves with petioles less than 2mm were not included in analysis.

Chlorophyll *a* fluorescence and total soluble phenolics analysis were carried out as in Experiment 1.

Statistical analysis

Analysis was carried out using IBM SPSS Statistics 21. Prior to any analysis, all datasets were assessed for normality. All were found to be normal apart from the specific flavonoid data, this data was LG10 transformed. In Experiment 1 rosette diameter, F_v/F_m, biomass, petiole length, blade length, total leaf area, blade width and total soluble were analysed using t-tests. Analysis flavonoids extracted by UPLC-MS was carried out using a Two-Way ANOVA. Experiment 1 was repeated independently 5 times for day 4 and 7 treatments and 4 times for day 10 treatments. Experiment 2 analysis were carried out using a one way ANOVA for the rosette diameters and T-tests for the morphological and total soluble phenolic data. For the morphological data, standard error bars represent the error from the mean of 12 individual plants; standard

error for the total soluble phenolics was from 5 individual plants. Experiment 2 was repeated independently 3 times.

Results

EXP 1: *Arabidopsis thaliana* plants grown +/-UVB for 4, 7 and 10 days.

Arabidopsis rosettes were grown +/- a low dose of UVB radiation for 4, 7 or 10 days. At the beginning of the experiment plant had reached the Boyes 1.04 stage developmentally, leaves 1-4 were visible but leaves 5 and 6 were not (Boyes *et al.*, 2001).

Morphology

Rosette diameter was reduced under UVB treatment at each time-point by between 15 and 22% although this difference was only significant at day 4 (p=0.03) and day 7(p=0.02) (Fig. 2.2).

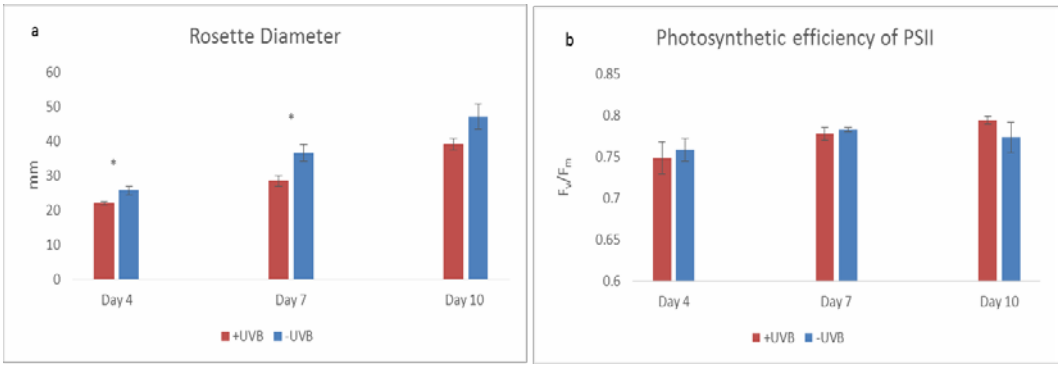

Fig.2.2 Panel (a), Rosette diameter (mm) and panel (b) the maximal photosynthetic efficiency of PSII measured as F_v/F_m of plants grown +/- a low dose of UVB for 4, 7 and 10 days. Error bars represent the standard error from the mean of 4 replicates for day 4 and day 10 and 5 replicates for day 7 for rosette diameter. Error bars represent the standard error from the mean of 4 replicates for F_v/F_m.

Biomass of individual UVB treated leaves was significantly reduced only at the day 7 time point in leaves L3 (p=0.04) and L4 (p=0.0009) (Fig. 2.3). Overall, the petioles of plants treated with UVB were shorter than those that were grown without UVB. The significant differences ranged between 18-29% (Fig. 2.3). The petioles of +uvb plants

measured after 4 days UVB exposure were significantly shortened in L1 (p=0.003), L2 (p=0.001), L3 (p=0.01) and L4 (p=0.008). After 7 days UVB exposure again petioles of L1 (p=0.003), L2 (p=0.0001), L3 (p=0.004) and L4 (p=0.002) were significantly shorter than those of unexposed plants were. At the 10 day time point only the petiole of L5 was significantly shorter (p=0.03). The change observed in petiole length also affected the total leaf length, which was between 3 and 26% longer in untreated leaves than UVB treated (Fig. 2.3). These differences were significant in L1 (p=0.01), L2 (p=0.02), L3 (p=0.03) and L4 (p=0.04) after 4 days UVB treatment and L1 (p=0.008), L2 (p=0.01), L3 (p=0.02) and L4 (p=0.01) after 7 days UVB treatment had reduced total leaf length in comparison with untreated leaves. Total leaf length of plants grown for 10 days was not significantly changed (Fig. 2.3).

The width and length of the leaf blades of plants grown +/- UVB radiation were not significantly different from each other at any of the time points (Fig. 2.3).

Photosynthetic Efficiency

The maximal Photosynthetic efficiency of PSII measured as F_v/F_m, was tested per rosette at each time points but no difference was found between the treatments (Fig 2.2).

Total Soluble Phenolics

Total soluble phenolics were extracted from individual leaves, absorbance peak was read at 330nm and normalised using leaf area (mm^2). There was an increase in total soluble phenolics in UVB treated plants at all time-points but this increase was only significantly different at certain time points (Fig. 2.3). There was also an increase in

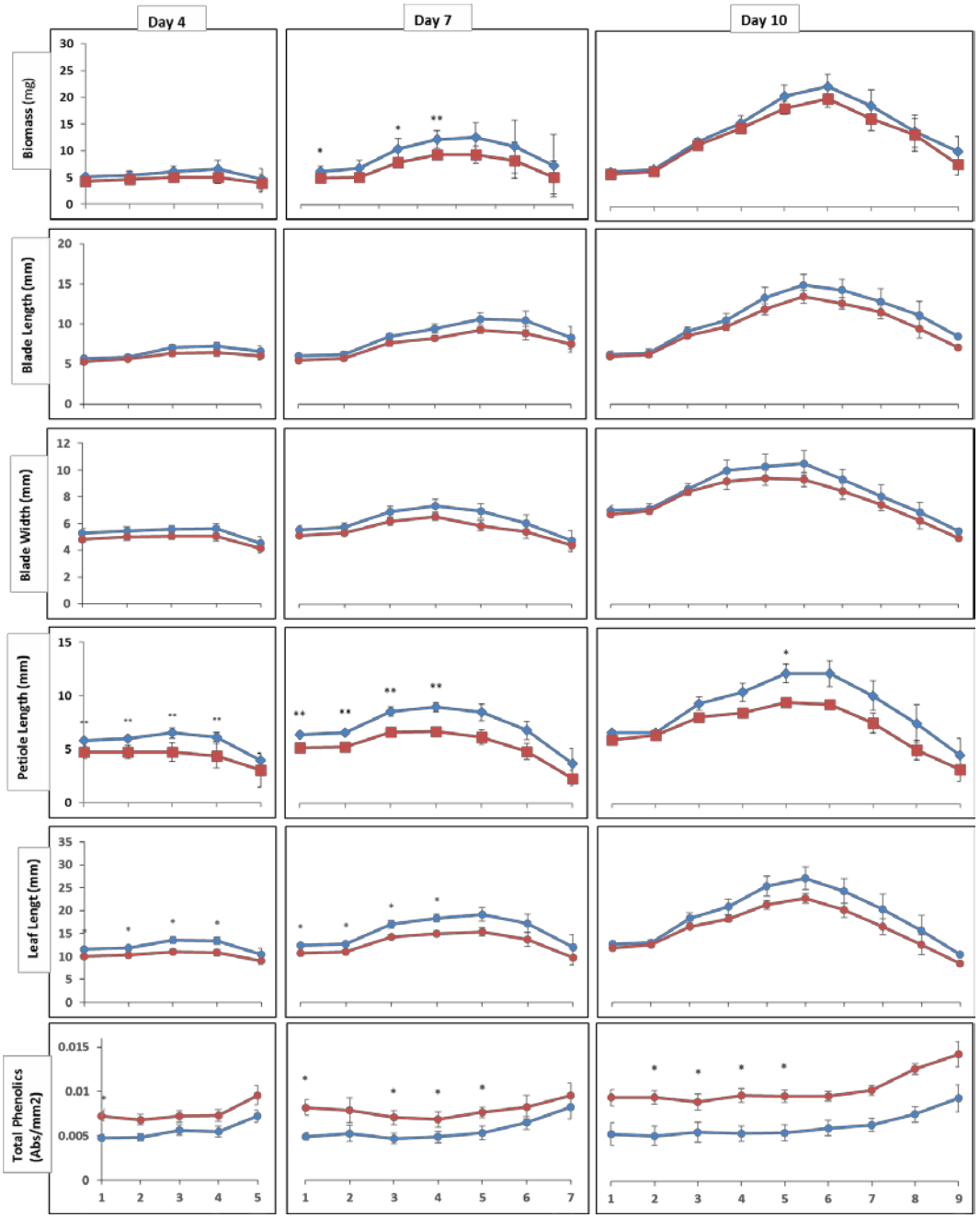

Fig. 2.3 The (a-c)biomass (mg), (d-f) blade length (mm), (g-i) blade width (mm), (j-l) petiole length (mm), (m-o) leaf area (mm^2) and (p-r) total soluble phenolics for Arabidopsis rosettes treated for 4, 7 or 10 days +/- UVB. Leaf numbers are on the X axis, with 1 being the oldest true leaf of a rosette and 9 being the youngest. The red line is the +uvb treatment and the blue line is the –uvb treatment. Error bars represent the standard error form the mean of 5 replicates for day 4 and 7 and 4 replicates for day 10. Asterisks (*) represent significant difference between the +/- uvb treatment for that leaf . T-tests were used for analysis

total soluble phenolics concentration with decreasing leaf age, this was evident in both the + and – UVB treatments (Fig. 2.3). L1 after 4 days +uvb treatment had 35% higher total soluble phenolics content than plants grown without UVB (p=0.02) (Fig. 2.3). After 7 days +uvb L1 (p=0.02), L3 (p=0.02), L4 (p=0.04) and L5 (p=0.05) had a

between 32- 44% increase in total soluble phenolics in comparison to plants grown without UVB radiation (Fig. 2.3). The UVB induced change in total soluble phenolics was also found after 10 days +uvb L2 (p=0.05), L3 (p=0.05), L4 (p=0.03) and L5 (p=0.05) had between 37 and 45% higher total soluble phenolics content than –uvb plants (Fig. 2.3).

Table 2.1. Summary of a two-way ANOVA on the effects of UVB radiation and leaf age on specific glycosylated quercetins (pmol/g FW), and kaempferols (pmol/g FW), isolated and identified using UPLC-TQD mass spectrometry.

			Quercetin				Kaempferols			
Main Effects			**Q-3-[R-G]-7-R**	**Q-3-[G-G]-7-R**	**Q-3-G-7-R**	**Q-3-R-7-R**	**K-3-[R-G]-7-R**	**K-3-[G-G]-7-R**	**K-3-G-7-R**	**K-3-R-7-R**
Leaf Number	L4		1669 a	27.2 a	1958.7 a	1708.9 a	38634.7a	472 a	37612.8 a	86866.7a
	L5		3135.8 a	23.9 a	3289.8 a	4432.7 a	72245.5a	450.6 a	70085.6 a	152119.8ab
	L6		2669 a	22.9 a	2830 a	4073.5 a	86927a	449 a	85063.2 a	179035.6 b
Treatment	+ uvb		4426.6 b	36.3 b	4974.9 a	5989.8 b	101348b	736.9 b	101254.4 b	198330.2 b
	- uvb		556.6 a	13 a	410.7 a	820.2 a	65935.7a	177.5 a	27253.3 a	80373.3 a
		df		ANOVA						
F value Leaf No.		**2**	0.64	0.80	0.53	0.75	2.49	0.04	3.10	3.75
Sig			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	*
F value Treatment		**1**	12.23	4.14	10.06	9.83	13.05	19.91	18.04	14.39
Sig			**	*	**	**	**	***	***	**
Leaf No. x Treatment		**2**	0.18	2.76	0.12	0.27	0.14	0.06	0.17	0.23
Sig			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s

n.s.= not significant, * = p ≤0.05, **=p≤0.001, *** = p≤ 0.0001, according to two-way ANOVA.
Comparisons to be made within columns Means in the same column and same main effect with the same letter are not significantly different, p>0.05 according to Tukey' range tests.

To investigate the increase in total soluble phenolics and to establish a relationship between UVB and individual phenolic compounds, extraction and identification was carried out using UPLC-MS. A 7 day treatment was selected as it was the time point where the maximum difference was achieved between + and – UVB treatments in

morphology and total soluble phenolics. L4 to L6 were selected as they gave a developmental gradient from a fully mature leaf (L4) to a still developing leaf (L6) at the time of harvest. A two-way ANOVA was used to assess the effect of treatment and leaf age.

Extraction and isolation of individual flavonoids identified eight compounds as being differentially accumulated four quercetins glycosides and four kaempferols glycosides (Fig. 2.4). The most abundant phenolics in both irradiated an un-irradiated leaves were kaempferol glycosides. Overall, there is a strong induction of all eight compounds under the UVB treatment although ratios vary between compound and leaf number. Quercetins were on average between 51 to 90% higher in UVB treated leaves versus untreated leaves. Kampferols were on average between 48 to 80% higher in UVB treated plants. In all of the compounds the differences between the UVB treated and untreated was significant (Table 2.1). In 3 out of 4 kampferols the highest concentration was found in the youngest leaf, this was evident in both UVB treated and untreated samples (Fig. 2.4). Quercetins were more variable but again in both treated and untreated plants the highest concentration was found in the youngest leaf for 3 out 4 compounds (Fig. 2.4). However, the developmental trend was only significant in one of the compounds, K-3-R-7-R ($p=0.038$) in this kaempferol glycoside the lowest concentration was found in L4 the oldest leaf and the highest in L6 the youngest leaf (Fig. 2.4 & Table 2.1).

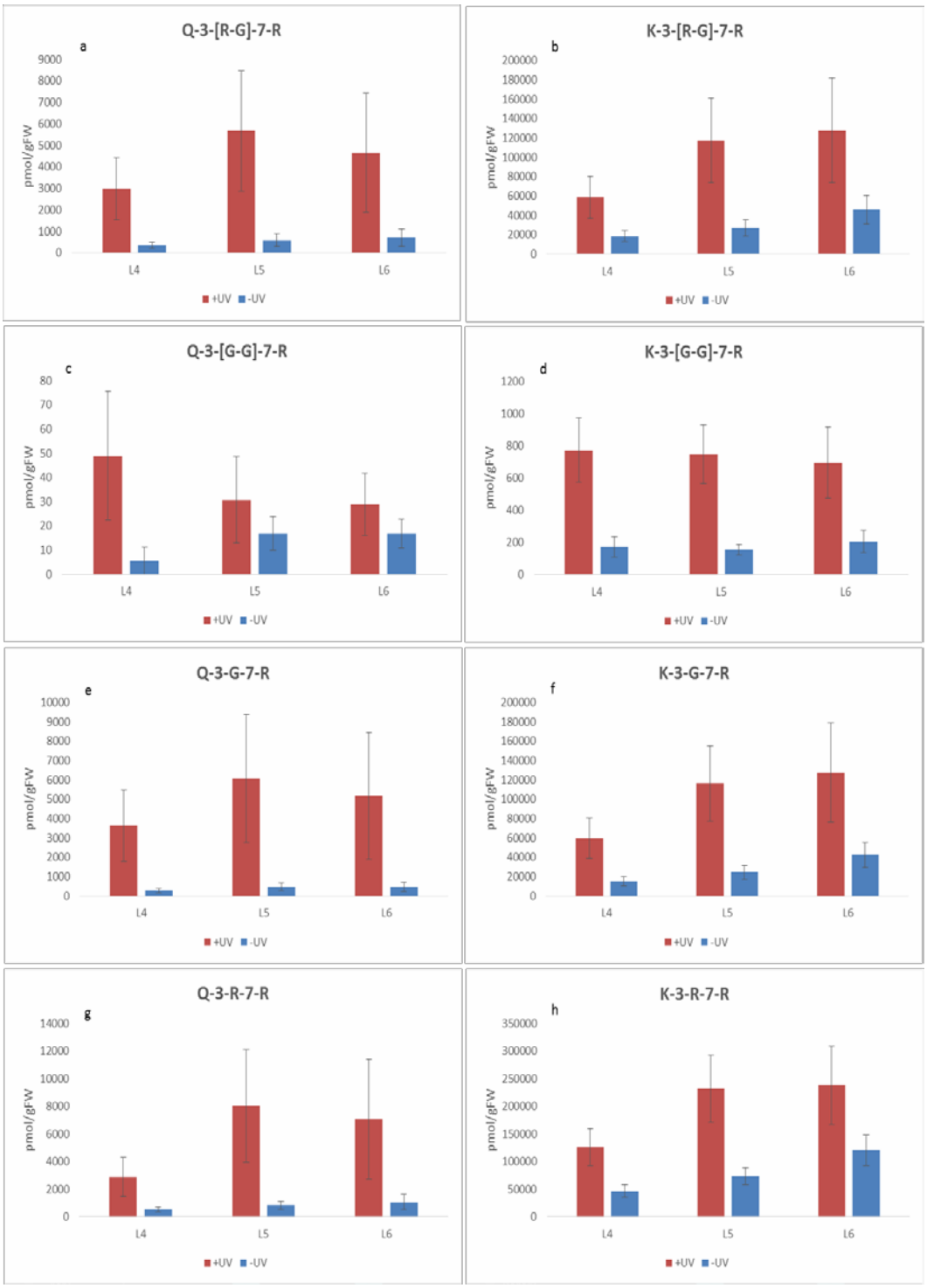

Figure 2.4 Quercetin and kaempferol concentrations in Col-0 grown with or without UVB for 7 days. Levels of quercetin and kaempferol derivatives were quantified using UPLC-TQD mass spectrometry. Error bars represent the standard error from the mean of five replicates for Col-0. Panels a,c,e,g are quercetin derivatives and panels b,d,f,h are kaempferol derivatives:

(a) Quercetin 3-O-rhamnosyl-glucoside 7-O-rhamnoside (Q-3[R-G]-7-R)
(b) Kaempferol 3-O-rhamnosyl-glucoside 7-O-rhamnoside (K-3[R-G]-7-R)
(c) Quercetin 3-O-glucosyl-glucoside 7-O-rhamnoside (Q-3[G-G]-7-R)
(d) Kaempferol 3-O-glucosyl-glucoside 7-O-rhamnoside (K-3[G-G]-7-R)
(e) Quercetin 3-O-glucoside 7-O-rhamnoside (Q-3-G-7-R)
(f) Kaempferol 3-O-glucoside 7-O-rhamnoside (K-3-G-7-R)
(g) Quercetin 3-O-rhamnoside 7-O- rhamnoside (Q-3-R-7-R)
(h) Kaempferol 3-O-rhamnoside 7-O- rhamnoside (K-3-R-7-R)

EXP 2: Investigation of the systemic nature of the UVB response.

Plants were selected at the Boyes 1.04 stage (Boyes *et al*., 2001). Leaf 4 was identified and either blocked from receiving direct UVB radiation for 7 days while the rest of the rosette was exposed or Leaf 4 was exposed to direct UVB while the rest of the rosette was blocked from receiving direct UVB. These treatments are subsequently identified as L4-uv or L4+uv. A +UVB and –UVB control were also included in this experiment.

Plant growth analysis

Rosette diameter, petiole length, total leaf area and biomass were recorded for all plants. The rosette diameters of –uvb plants were 16% larger than those of +uvb and L4-uv plants and 8% larger the L4+uv plants. One-way ANOVA found that the differences between untreated plants and the L4-uv and UVB treated plants were significant ($p=0.012$) (Fig. 2.5).

Petiole length:

Overall L4-uv plants had shortened petioles, up to 21% shorter in comparison to plants grown –uvb, and were similar in length to the +uvb plants (Fig 2.6). This was observed across the rosette and no difference was seen in leaf 4 despite being protected from direct UVB by a UVB blocking Mylar strip. Petiole lengths of L4+uv plants were of a similar length to the untreated plants except for L4. The petiole of L4 of the L4+uv was shorter than L4 of the -uvb plants, T-test found that this difference from –uvb treatment was significant (Fig 2.6).

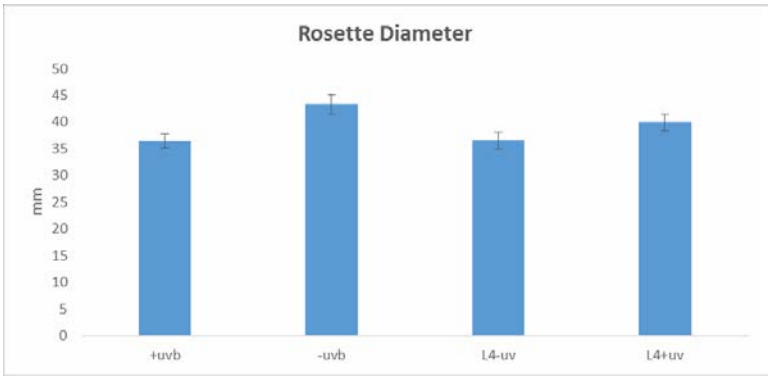

Figure 2.5 Rosette diameters (mm) of Col-0 plants grown +/- UVB, L4-uv and L4+ uv for 7 days. Error bars represent the standard error from the mean of 12 replicates.

Leaf area:

The leaf areas of the L4-uv plants are similar to those of plants under the +uvb treatment, both of which are smaller than the –uvb treatment (Fig. 2.6). L4 of the L4-uv treatment did not deviate from the tendency despite being protected from receiving direct UVB. In L4+uv plants, despite the majority of the rosette being protected from direct UVB radiation, the total leaf area across the rosette was between 4-22% smaller than the –uvb treated plants (Fig. 2.6). However, the differences in leaf area between L4+uv and the –uvb treated plants were only significant at L4. L4 of the L4+uv treatment received direct UVB radiation was significantly smaller than L4 of the –uvb treated plants ($p=0.007$).

Biomass:

The biomass of L4-uv leaves was less than –uvb and + uvb treated plants. Leaves of the L4-uv plants have biomass on average 8% smaller than the +uvb plants but the difference is not significant. There is no evidence of L4 having increased biomass as a result of being shielded from direct UVB. Some differences in biomass are evident from Fig. 2.6 between L4+uv leaves and the –uvb treatment. The biomass of L4+uv

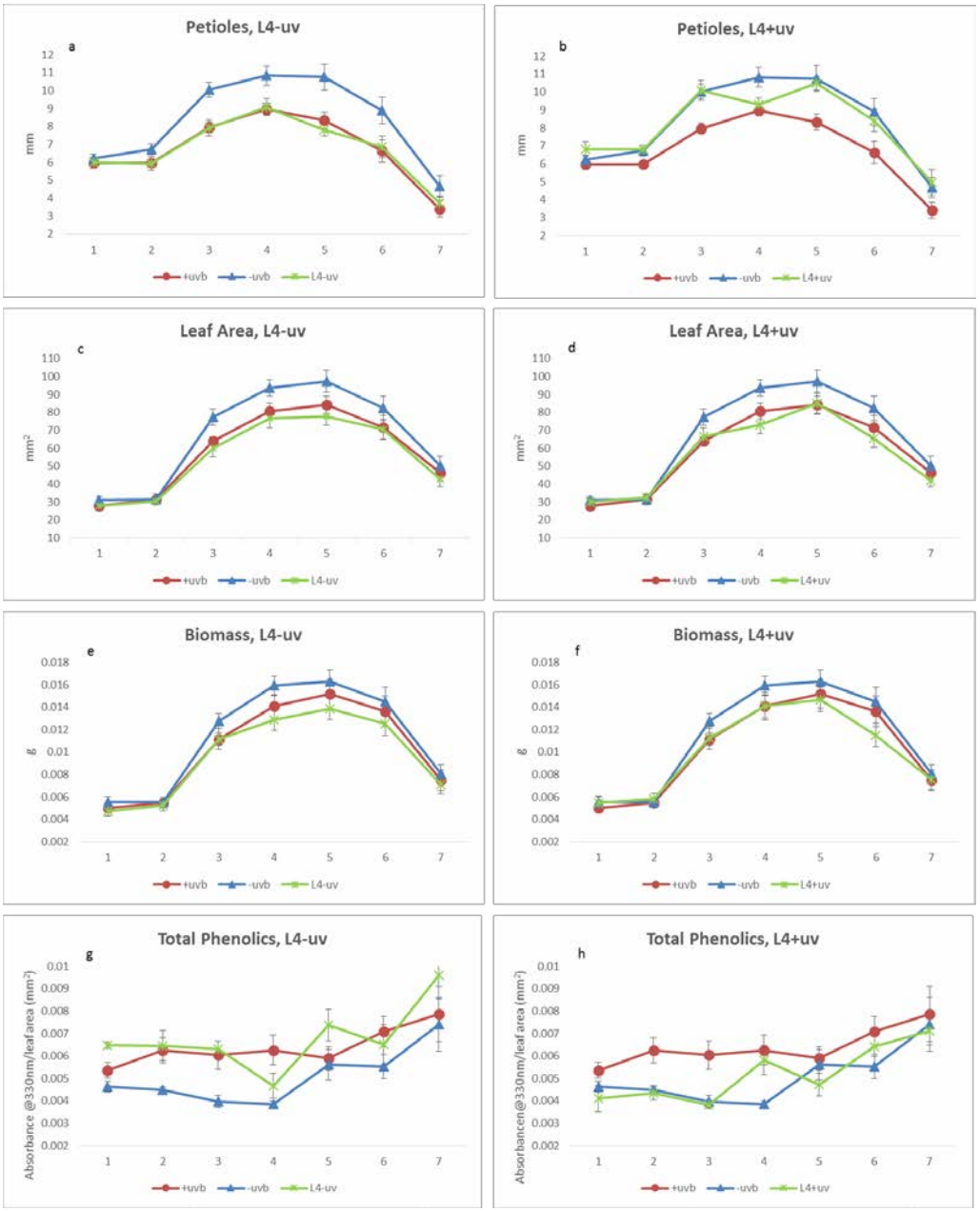

Figure 2.6 (a-b) Petiole lengths (mm), (c-d) leaf area (mm^2), (e-f) biomass(g) and (g-h) total soluble phenolics (A330nm/mm2) for plants grown +/- UVB, L4-uv, panels a,c,e,g and L4+uv, panels b,d,f,h for 7 days. Error bars represent the standard error from the mean of 12 replicates.

leaves was between 7 and 20% smaller than that of –uvb plants but none of the differences were statistically significant. There was no evidence of L4 of the L4+uv plants having further reduced biomass as a result of receiving direct UVB.

Total soluble phenolics:

The L4-uv treatment produced plants that had total phenolic levels similar to those found in +uvb plants. Total soluble phenolics in L4-uv plants were between 12 and 37% higher than found in –uvb plants across the rosette (Fig. 2.6). L4 of the L4-uv treatment which was covered with UVB blocking Mylar had slightly lower phenolics than the +uvb plants although this difference was not significant (Fig. 2.6). In the L4+uv plants, the total soluble phenolics were found to be at similar levels to the –uvb plants, levels were between 5 and 37% lower than +uvb treated plants (Fig. 2.6). L4 of the L4+uv treatment which was exposed to direct UVB had total soluble phenolics closer to those found in the +uvb plants (Fig 2.6). There was 39% more phenolics than found in the –uvb treatment. T-test found that the increase in phenolics in comparison to the –uvb treatment was significant, $p=0.02$.

Discussion

Despite a significant body of work focused on elucidating the mechanism and adaptive relevance behind the UVB response, significant questions remain. This study aimed to investigate the response of *Arabidopsis thaliana* rosettes to a low dose of UVB with a focus on the leaf age and developmental stage. Plants have the ability to respond systemically or locally to a range of stimuli, on this basis the response to a low dose UVB was further investigated.

The key findings from this study are that (1) the effects of UVB on morphology appear transitory, (2) the effects of UVB on phenolics were persistent, (3) there was an up-regulation of specific quercetins and kaempferols in the presence of UVB, (4) the effect of UVB on petioles and total phenolics was local, (5) however the effect of UVB on leaf area and leaf biomass did appear to be systemic.

In this study, it was found that a relatively low level of UVB radiation given with a background of PAR and UVA, changes plant morphology and increases total soluble phenolics without any reduction in the efficiency of PSII measured as F_v/F_m. Rosette diameter, petiole length and total leaf length were significantly decreased in UVB treated plants grown. The observed changes in morphology were not persistent, and had diminished and were no longer significant at the day 10 time-point. This suggests that overtime there is acclimation of plants to the low dose of UVB radiation or an outgrowing of the morphological effect. In the case of annual species evidence of a delay in the onset of flowering with increasing UVB dose has been found (Llorens *et al.,* 2015). Transient disruption in leaf development has also been observed in the study of birch tree responses to UVB (Robson & Aphalo, 2012). Hectors *et al*. (2010) also found that the changes in blade length/width ratio induced by UVB were transitory. Here the effects are largely seen in the petiole length, as rosette diameter and total leaf length are a function of this. It has been hypothesised that the difference between the + and – UVB treatments is due to the re-allocation of resources during the up-regulation of UVB defences such as flavonoids (Robson *et al*., 2014). However, evidence of a relationship between resource allocation and a slow down or cessation of growth has not yet been identified (Robson & Aphalo, 2012; Kotilainen *et al*., 2009). It could also be due to the activation of a stress response SIMR (Potters *et al.*, 2007) due to the initial shock of UVB exposure. SIMR is a generic stress response which produces a dwarf phenotype similar to the UVB phenotype (Potters *et al.,* 2007). The morphological response to UVB in this instance is observed in the older leaves. Older leaves have previously been reported to be more sensitive to UVB than younger ones (Jordan, 1998). Potentially this supports the hypothesis that the changes in petiole length are due to some initial UVB stress or shock, which is ameliorated as

the experiment progresses. As an argument against stress, little other evidence of it was found and the F_v/F_m results remained high across the rosettes for the duration of the treatment.

Changes in accumulated total soluble phenolics are also a very commonly reported effect of UVB exposure. In this experiment, we find that total soluble phenolics are significantly up regulated, as are eight specific quercetin and kaempferol glycosides. Plants grown under UVB had up to 45% higher total phenolic content than –uvb plants (Fig. 4). The increase in total phenolics is evident for the duration of the experiment. So despite the transitory effect on morphology the increase in phenolics is maintained. This potentially suggests that the reduction in petiole length is occurring independently of the increase in phenolics rather than one influencing the other. More in depth analysis of the specific flavonoids accumulated was carried out at the day 7 time point. Here we also found significant increases in eight out of the eight flavonoids identified in the UVB treated plants with quercetins at up to 90% higher and kaempferols up to 80% higher in UVB treated plants. In agreement with several studies it was observed that kampferols were the most abundant flavonoid detected (Hectors *et al.*, 2014). It was observed in both quercetins and kaempferols that the highest concentrations were in the youngest leaves although this was only significant for one of the kaempferols. There is also evidence of developmental differences in total soluble phenolics, younger leaves had more phenolics than older leaves, again this was not found to be significant. Jordan (1998) also found that younger inner leaves of an Arabidopsis rosette increased levels of UV-absorbing pigments faster and to higher levels than older outer leaves when exposed to UVB. This phenomenon has also been identified in petunia plants, the effect of UV induced phenolics decreased with leaf age (Ryan *et al.*, 1998). Potentially this is because of extra protection

required by younger leaves as they develop. An intriguing question is whether older leaves co-regulate flavonoid accumulation in younger leaves, particularly when the latter are in the metabolic sink stage.

To investigate whether the UVB response is systemic or local two treatments were devised L4+uv and L4-uv. Evidence was found of both a local response and of a systemic one. Plants have a range of inducible defence mechanism that act as an immune system in response to biotic and abiotic stresses in the environment. In clonal plants exposed to UVB, increases in secondary metabolites were also found in the un-exposed ramets (Liu *et al*., 2015). Well documented examples of systemic responses in plants to stress include Systemic Acquired Resistance which is most commonly associated with biotic stressors but once activated can up-regulate tolerance to abiotic stress also (Kuć, 2001). Systemic Acquired Acclimation was identified in relation to high light stress and utilised H_2O_2 as a communication device to up-regulate a plant's capacity to deal with ROS even in distal and emergent leaves removed from direct contact with the stressor (Karpinski *et al*., 1999; Mullineaux *et al*., 2000). Nitric oxide has been identified as potential communication device in response to high levels of UVB (Tossi *et al*., 2012). Through a systemic response to NO non-irradiated maize leaves are also protected from secondary exposure to a high dose of UVB (Tossi *et al*., 2012).

Tossi *et al*. (2012) also speculated that flavonoids had a part to play in information transfer between different organs and as they remained high for 96 hours post UVB exposure acted as a memory of the stress event. It has long been known that flavonoids can act as antioxidants and screens to protect vital organs from UVB stress (Agati & Tattini, 2010) but as they have also been found to be highly mobile and could act as communication device to switch on systemic defences (Buer *et al*., 2007). Tossi *et al*.,

(2012) found that NO is required for the up-regulation of flavonoids in response to UVB. In this case, leaf biomass and leaf area responded systemically to UVB. The leaf biomass and leaf area of L4+uv was smaller than unexposed plants despite only one leaf, L4 receiving direct UVB. This is potentially due to the transfer and communication of the UVB signal around the rosette.

Previously in this chapter there has been evidence of differential responses depending on the age of the leaf. Younger leaves appear to accumulate more flavonoids and older leaves are more susceptible to morphological change. All leaves undergo a transition from a carbon sink to a source as they develop; the emergent leaf is supported by carbohydrate imported from other parts of the plant, most likely a fully mature leaf (Turgeon, 1989). It is possible that as an emergent leaf L4 received information as well as carbon. Examples of signal transfer throughout plants are well documented (Mullineaux *et al*., 2000; Kuć, 2001; Gordon *et al*., 2012; Tossi *et al*., 2012; Liu *et al*., 2015). This suggests that as L4 matured it would have been influenced not only by the environment it was experiencing directly but also by signals from older more mature leaves. Given the progression and development of the rosette during the study it is also likely that L4 became a source of carbon and information as time passed. The metabolic switch between a sink and a source could result in a rosette with traits of being UVB treated and untreated at the same time. This can be seen in the L4+uv plants which have petioles the same length as unexposed plants except for L4 but its leaf biomass and leaf area are reduced.

Due to the natural behaviour of light waves, selectively blocking UVB radiation from parts of a rosette using a filter is not going to 100% eliminate it. Stray UVB light waves could explain why L4-uv and L4+uv have a mixture of both a systemic and a local responses. However, the high degree of specificity i.e. the shorter petiole of L4 in the

L4 +uv plants, and the increase in total soluble phenolics in the same leaf suggests that the experimental setup was targeted enough to provide valuable information. It is also possible that the addition of a filter so close to the rosette could have increased the ambient temperature experienced by the rosette or part of the rosette. Yet, the L4+uv plants were nearly entirely covered in a piece of Mylar and no stress related decrease in F_v/F_m was observed. Total soluble phenolics, which have previously been shown to be sensitive to changes in temperature, were also not affected.

As mentioned, evidence of a localized response specific to one leaf within a rosette was also found, total soluble phenolics content increased significantly when L4 only of a rosette was exposed to UVB. It was also found that the petiole length of an exposed leaf in an un-irradiated rosette was shortened. Interestingly the opposite did not happen when one leaf of a rosette was blocked form receiving direct UVB there was no increase in petiole length. In this case, there was some decrease in total phenolics but it was not significant. There is evidence that the inhibition of growth under UVB is linked to a Shade Avoidance Syndrome regulated by the red:far red ratio (Hayes *et al.*, 2014). Through the UVB photoreceptor, UVR8, auxin biosynthesis is inhibited, preventing elongation even if a strong lower R:FR ratio is present (Hayes *et al.*, 2014). Although, evidence has also shown that prevention of elongation mediated by UVR8 can be overridden in dense stands of plants before shading has become an issue (Ballaré *et al*., 1987; Ballaré *et al*., 1990). In full sunlight, before the quality of PAR has been reduced plants had detected their neighbours and begun to elongate (Ballaré *et al*., 1987; Ballaré *et al*., 1990). This suggests a degree of precision control, a relationship between Red; Far red ratio, UVB and PAR and the ability for plastic adjustment to subtle changes in the light spectrum (Ballaré *et al*., 1990). Overall, the L4-uv rosette received a higher UVB dose; only one leaf was blocked from receiving

direct UVB. The strongest signal being received was that of full artificial sunlight as UVB was being detected by the majority of the rosette. There was no local response in the one shaded leaf (L4) suggesting that the signal received by just one leaf is not sufficient to change morphology or total phenolics significantly. On the other hand, L4+uv is elongating in the absence of UVB inhibition of growth, perception of UVB by one leaf results in a cessation of elongation. The effect is localized, as perception of UVB in one leaf is not strong enough signal to induce a systemic response. The rest of the rosette is still elongating in the absence of UVB as a proxy for full sunlight. A study which investigated shade avoidance in neighbouring Arabidopsis rosettes found that leaves moved to a more vertical orientation if there was a danger of shading by another rosette, this was achieved through differential growth in the petioles (Mullen *et al*., 2006). Interestingly, it was found that this response was localized to the specific leaf being shaded (Mullen *et al*., 2006). Mullen *et al*. (2006) also found a role for a photoreceptor other than phytochrome in this response but did not identify it, the speculated upon photoreceptor in this case could be UVR8. This ability for precise and localized photo-morphogenesis shows how plants can gather information, and generate appropriate responses to very small changes in their physical and climatic environment, showing the value of responding to a local stimulus.

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Chapter 3

The importance of flavonoids glycosylated at the C-7 position for the development of the UVB phenotype and UV protection and acclimation in *Arabidopsis thaliana.*

Abstract

Flavonoids have been commonly identified to offer photo-protection, by acting as antioxidants and sunscreens. In *Arabidopsis thaliana*, this concerns especially kaempferol and quercetin di- and triglycosides, rhamnosylated at position seven. It has also been identified that (family-1 glycosyltransferase gene) UGT89C1 catalyses rhamnosylation at the C-7 position of some flavonols. Given the association between flavonol 7-O-rhamnosides and UV-exposure, it can be hypothesised that any plant lacking the ability to catalyse the rhamnosylation of C-7 flavonoids is more susceptible to UVB stress. To investigate the importance of C-7 flavonoids for the development of the UVB phenotype and for protection and acclimation to UVB radiation, the UV-responses of *ugt89c1* knockouts were studied. It was found that knockout *ugt89c1* line contained substantially lower concentrations of 7-out-of-8 7-rhamnosylated flavonoids in both control and UV-exposed plants. Interestingly, there was no plant stress in the knockout line as evidenced by the lack of effect on photosynthetic efficiency of PSII of UVB treated *ugt89c1* plants. The total soluble phenolics in the *ugt89c1* knockout were increased under the UVB treatment, implying that other flavonoids were acting as UVB protectants in place of the lacking 7-rhamnosylated quercetins and kaempferols. The morphological response of UVB treated *ugt89c1* knockouts displayed shortened petioles and leaf lengths when UVB exposed, but the leaf biomass remained unchanged. This shows that some aspects of morphology are affected by the flavonoid glycosylation pattern.

Introduction

UVB radiation is made up of short high-energy wavelengths that, in high doses, can cause damage to vital cell structures and impact on plant fitness. Exposure to potentially harmful UVB is unavoidable for plants but they have developed a complex system of checks balances to mitigate any potential damage. UVB stress under natural conditions is considered rare (Rozema *et al.,* 1997). Instead of causing damage, UV-induces a number of specific plant responses. The photomorphogenic response to UVB includes changes to plant morphology, biochemical make-up, photosynthetic competence and plant defences (Jenkins, 2014). Plant architecture is altered resulting in a more dwarfed phenotype. However, the range of responses elicited by UVB exposure is not yet fully understood and research is ongoing. Of particular interest are UV-induced flavonoids and their relationship with the development of the UVB phenotype. Flavonoids are a large class of secondary metabolites encompassing more than 10,000 structures (Agati *et al.,* 2012). They are present in a wide array of cell and subcellular structures and carry out a multiplicity of roles (Agati *et al.,* 2012). Flavonoids and their derivatives are upregulated and act in response to an array of environmental conditions or stressors making them versatile compounds. One of the commonly identified roles of flavonoids is that of photo-protection, acting as antioxidants in response to high light and UV (Agati & Tattini, 2010). A further role as sunscreens has also been identified evidenced by their presence in external appendices such as trichomes (Agati & Tattini, 2010).

The majority of flavonoids in plant cells are glycosylated because as aglycones they are toxic (Offen *et al.,* 2006). In their glycosylated form, the hydroxyl group is protected from degradation and flavonoid solubility and transport into the vacuoles is enhanced (Offen *et al.,* 2006). Hectors *et al.,* (2014) identified that kaempferol and

quercetin di- and triglycosides, all specifically rhamnosylated at position seven, were accumulated in response to UVB exposure. A further study on Kale also found that UVB induced only specific glycosylated flavonoids (Neugart *et al.,* 2012). A study by Yonekura-Sakakibara *et al.* (2007) identified a gene that was prominent in determining the flavonoid glycosylation pattern of Arabidopsis. It was found that the family-1 glycosyltransferase gene (UGT), UGT89C1, was highly correlated with flavonoid biosynthesis pathways. Specifically, it was identified that UGT89C1 catalyses rhamnosylation at the C-7 position of some flavonols (Yonekura-Sakakibara *et al.,* 2007). To confirm the physiological function of UGT89C1 in Arabidopsis two T-DNA insertion lines were created *ugt89c1-1* and *ugt89c1-2.* The flavonoid profiles of these two lines were analysed and it was found that three major kaempferol 7-O-rhamnosides essentially disappeared, and new kaempferol derivative peaks were detected. Likewise, the levels of the corresponding quercetin 7-O-rhamnosides were also significantly reduced in comparison to the wildtype. This indicated that UGT89C1 encodes an UDP-rhamnose:flavonol7-O-rhamnosyltransferase (Yonekura-Sakakibara *et al.,* 2007). As such, the UGT89C1 knockouts are lacking the transferase enzyme that enables the downstream creation of quercetin and kaempferols rhamnosylated at position seven. There is further evidence linking UGT89C1 to the UVB response, as its expression is enhanced by UVB exposure (Oravecz *et al*., 2006). UV-mediated upregulation of UGT89C1 is signalled through the UVB photoreceptor UVR8, as its expression was impaired in *uvr8-1* (Brown *et al.,* 2005).

Thus, UV-B specifically induces expression of the UDP-rhamnose:flavonol7-O-rhamnosyltransferase, and this results in accumulation of flavonol 7-O-rhamnosides. Given the association between flavonol 7-O-rhamnosides and UV-exposure, it might be hypothesised that any plant lacking said flavonoids may suffer some form of injury

even under relatively low levels of UVB. This study aimed to investigate the morphological response, efficiency of PSII, accumulation of total soluble phenolics and of specific glycosylated flavonoids of *ugt89c1* knockouts that were exposed to a low dose of UVB with a background of PAR and UVA over seven days. This experiment was undertaken to assess the importance of quercetins and kaempferols glycosylated at the seven position for the development of the UVB phenotype and for protection and acclimation to UVB radiation.

Materials and Methods

Plant Material

Seeds of the Arabidopsis transferase knock-outs *ugt89c1-1* and *ugt89c1-2* were obtained from RIKEN BioResourse Center, 3-1-1 Koyadai, Tuskuba, Ibaraki 305-0074, Japan, and were originally produced using T-DNA-insertion technology. The T-DNA was inserted in the UGT89-gene of Col-0, which was used as a control in the experiments. Seeds of *Arabidopsis thaliana* transferase mutant *ugt89c1-1* and *ugt89c1-2* were cold treated at 4^0C before sowing into flats containing sieved John Innes No.2 compost (J. Arthur Bowers, William Sinclair Horticulture Ltd., Firth Rd., Lincoln, LN6 7AH). The flats were covered with cling film and placed in a temperature controlled growth room on a 16 hour light/ 8 hour dark photoperiod. They received only PAR in the growth room, no UV-A or UV-B, at an intensity of 40-60µmol m^{-2} s^{-1}. Once the seeds had germinated, the cling film was removed. At the two-cotyledon stage, the seedlings were transplanted into individual 200ml pots containing John Innes No. 2 compost. The seedlings were placed back into the growth room and covered with cling film for a further 2 days until they re-established. They

were allowed to reach the 1.04 growth stage (Boyes *et al*., 2001) before beginning the experiment.

Experimental Set-up

Experiments were conducted in a self-contained light box, fitted with PAR (36W Philips Master TLD Reflex Tube, BLT Direct), UV-A (Fluorescent Blacklight Blue 36W, 1200mm) and UV-B (TL12, Phillips, Eindhoven, The Netherlands) fluorescent tubes. Temperature within the box was 22^0C +/- 2 degrees and a relative humidity of 30%. The intensity of the PAR was 60-80 µmol m^{-2} s^{-1} and the UV-A was 0.16mWcm^{-2} A dimmable ballast (Sylvania-Biosystems, Wageningen, The Netherlands) was used to regulate the intensity of the TL12 tubes without changing the UV-B spectrum (verified with Ocean Optics Spectroradiometer (USB2000+RAD) (Ocean Optics, Dunedin, FL, USA). The output of the UV-B tubes was set to generate 0.6W/m^2 +/- 0.4 Watt/m^2. Plants were exposed for 4, 7 and 10 days for two hours each day at noon. This translates to a biological effective dose of 0.6648kJ m^{-2}/day (Flint and Caldwell, 2003). The UV-C component that is generated by the TL12 tubes was blocked using a filter of cellulose acetate (95um thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany). Control plants (-uvb) were grown under UV-B blocking filter (125um thickness, Polyester film, Tocana Ltd., Elizabeth's Cross, Ballymount Cross Ind. Est., Ballymount, Dublin 24). Both filters were placed 5cm above the plants on opaque frames. Both filters were changed after 20 hours of UV-B exposure. The photoperiod in the light box was the same as the growth room, 16 hour light/ 8 hour dark sequence. The plants were acclimated in the light box for a minimum of 24 hours before switching on the UV-B lights.

Morphological analysis

Leaf morphology was analysed after 7 days of UV-B exposure. Leaves were arranged in developmental order, with L1 (Leaf 1) being the oldest leaf and L9 (Leaf 9) being the youngest. Following this, leaves were photographed for processing with ImageJ software (Abràmoff *et al.*, 2004). Parameters measured included biomass, petiole length, leaf length, and leaf area. Leaves with petioles less than 2mm were not included in analysis.

Photosynthetic Efficiency

Chlorophyll *a* fluorescence (F_v/F_m) was determined using an Imaging PAM (Waltz, Germany) as a proxy measure of the maximal quantum yield of photosystem (PS) II efficiency. F_v/F_m values were determined after plants had grown for 7 days under +/- UVB radiation. Whole rosettes were dark adapted for a minimum of 20 minutes before F_v/F_m was determined. Three measurements were taken at random from each rosette and pooled per rosette.

Total soluble phenolics

Total soluble phenolics were extracted using acidified methanol (1%HCL, 20%H_2O, 79%CH_3OH) (Biswas & Jansen 2012). The whole leaves were placed in micro-tubes with 1ml acidified methanol and incubated in the dark at 4^0 for 4 days. The supernatant was drawn off using a pipette and placed in quartz glass curvette. Absorbance was recorded at 330nm on a spectroradiometer (Shimadzu-UV visible spectrophotmeter - 160A).

L4 was identified and separated from the rest of the rosette. Leaves from at least 5 plants were pooled to provide enough biomass for analysis from each treatment. This

was repeated independently twice for *ugt89c1* and 5 times for Col-0. Samples were then analysed in the University of Antwerp. Individual flavonoid compounds were analysed from L4 following Hectors *et al.* (2012). Arabidopsis leaves (L4) were frozen using liquid nitrogen and ground in a Magna Lyser (Roche, Basel, Switserland). To extract flavonoids, leaves were homogenized in acidified methanol (5µl 62.5% (v/v) methanol acidified with 0.125% (v/v) formic acid per milligram fresh weight) and sonicated in an ultrasonic bath for 30min followed by filtration [(True Nylon Syringe filter, 0.2 µm), Grace Davison Discovery Science, Deerfield, IL]. Kaempferol-3-rhamnosidoglucoside (10^{-2}M final concentration; Carl Roth GmbH, Karlsruhe, Germany) was used as internal tracer to take into account recovery losses and ionization efficiency.

Flavonoid compounds were analysed using an ACQUITY UPLC chromatography system combined with and ACQUITY TQD (Waters, Milford, MA) mass spectrometer. Samples were injected on a VanGuard pre-column (BEH C18, 1.7 µm, 2.1×5mm2; Waters) coupled to a reversed phase column (HSS C18, 1.8 µm, 2.1×100 mm2; Waters). The solvents used were water, 0.1% formic acid (C) and acetonitrile, 0.1% formic acid (D). TQD analysis was performed in ESI(+)-MRM mode. Samples were eluted during a 4-min run using a constant flow rate of 600 µlmin^{-1} and a column temperature of 40 ^{0}C. Solvent gradient started at 13.5% D, slowly increasing to 16.7% D in 1.5 min and further increasing to 51%D in 2.5 min. The column was rinsed for 1 min at 86% D and equilibrated at 13.5% D between samples. TQD analysis was performed in ESI(+)-MRM mode using the following parameters: capillary voltage 3 kV, cone voltage 20 V, source temperature 150 ^{0}C, desolvation temperature 350 ^{0}C and collision energy 30 V. Chromatograms obtained were processed using QUANLYNX v4.1 (Waters). Concentrations were calculated using the reference

compound Kaempferol-3-rhamnosidoglucoside with retention time 2.54 min and fragmentation pattern 595 > 287.

Statistical Analysis

Investigation of *ugt89c1-1* and *ugt89c1-2* found no significant differences between them so they were treated as one, now referred to as *ugt89c1*. Comparisons +/- UVB were made on a leaf-by-leaf basis using T-Test. Comparisons between Col-0 and *ugt89c1* were also made on a leaf-by leaf basis within in treatments using T-tests. All data were checked for normality and homogeneity of variance and found to be normal and homogenous.

Results

The transgenic knockouts *ugt89c1-1* and *ugt89c1-2*, together with a Col-0 control, were treated with a low dose of UVB radiation for 7 days to assess the importance of flavonoids rhamnosylated at position 7 for UVB acclimation and protection. Morphological parameters, total soluble phenolic data, efficiency of PSII andspecific flavonoid data were recorded at the end of the 7 day exposure. Investigation of *ugt89c1-1* and *ugt89c1-2* found no significant differences between the two knock out lines so they were treated as one, now referred to as *ugt89c1*.

The biomass of Col-0 leaves treated with UVB was less than that of the untreated Col-0, this difference was significant for L3 ($p=0.04$) and L4 ($p=0.0009$). In contrast, there were no significant differences in leaf biomass between the UVB treated and untreated *ugt89c1* plants. It was found that the leaves of the +uvb *ugt89c1* plants were not significantly different from the leaves of the +uvb Col-0 plants. However, the biomass of all leaves of the untreated *ugt89c1* plants was significantly smaller than that of untreated Col-0 plants (Fig 3.1). Petioles of the +uvb *ugt89c1* were between 20

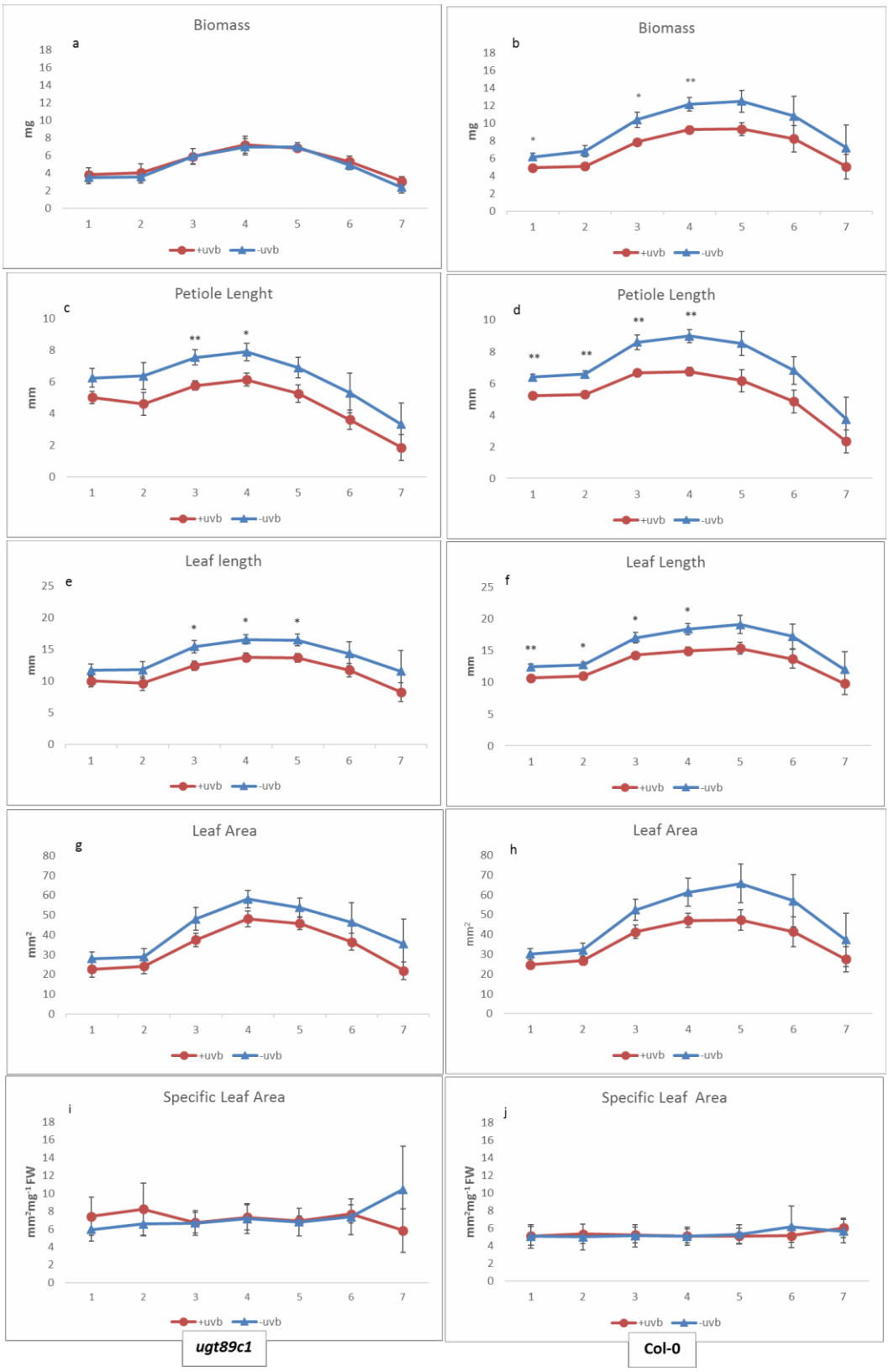

Figure 3.1 Panels a,c,e,g,i refer to *ugt89c1* and b,d,f,h,j to Col-0. Parameters measured were (a+b) biomass (mg), (c+d) petiole length(mm), (e+f) leaf length(mm), (g+h) leaf area (mm^2) and (i+j)specific leaf area ($mm^2mg^{-1}FW$). Both sets of plants were grown +/-UVB for 7 days. Data is analysed per leaf, with 1 being the oldest leaf and 7 the youngest . Error bars represent the standard error from the mean of 8 replicates for ugt89c1 and 5 for Col-0. T-tests were used to compare +/- UVB treatments. Asterisk (*) refer to comparisons made UVB treated and untreated plants within each panel and not to comparisons made between Col-0 and *ugt89c1*.

and 45 % shorter than those of untreated plants, this difference was found to be significant for L3 ($p \leq 0.001$) and L4 ($p \leq 0.05$). The leaf length of the +uvb *ugt89c1* plants was also found to be reduced by between 15 and 28% in comparison to the –uvb plants. The reduction in leaf length was significant for L3, L4 and L5. Both of these parameters were also compared with Col-0 plants grown +/- UVB. It was found that the plants that neither the +uvb Col-0 and *ugt89c1* nor the -uvb Col-0 and *ugt89c1* were significantly different from each other (Fig 3.1). The area per leaf was also measured but no significant differences were found between +/- UVB *ugt89c1* plants (Fig. 3.1). There was also no significant difference between the +/- UVB Col-0 plants. The SLA (Specific leaf area) of *ugt89c1* plants and Col-0 was not changed with UVB treatment (Fig. 3.1).

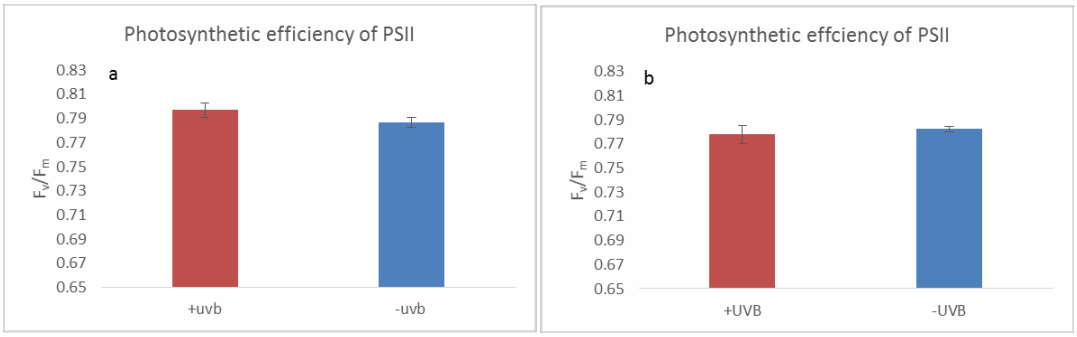

Figure 3.2 The maximal quantum yield of photosystem II measured as F_v/F_m of (a) *ugt89c1* knockout line and (b) Col-0 grown for 7 days +/- UVB Error bars represent the standard error from the mean of 8 replicates for *ugt89c1*.

The maximal photosynthetic efficiency of PSII of *ugt89c1* plants grown +/- UVB was measured as F_v/F_m. It was tested per rosette after 7 days UVB exposure but no difference was found between the treatments (Fig. 3.2). There was also no significant difference found between *ugt89c1* plants and Col-0.

Total soluble phenolics were quantified per leaf across the rosette. There was an increase of between 7 and 50% in total soluble phenolics in the UVB treated *ugt89c1*

plants, this difference was only significant in L3 (Fig. 3.3). However, in Col-0 plants grown +/- UVB significant difference in total soluble phenolics were found between L1 (p=0.02), L3 (p=0.02), L4 (P0.04) and L5 (p=0.05) (Fig. 6.3). Comparisons between Col-0 and *ugt89c1* found that there were no significant differences between the responses to the +UVB and–UVB treatments.

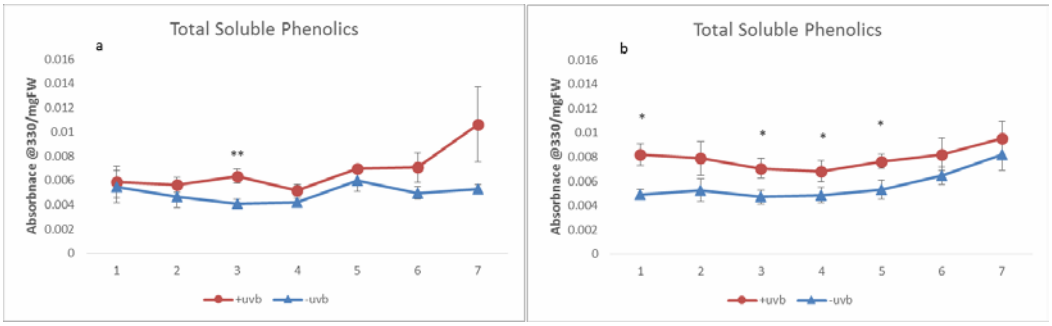

Figure 3.3 Total soluble phenolics extracted with a 1% acidified methanol solution and normalized versus leaf area. Panel a, *ugt89c1* knockout line, panel b the wild-type Col-0 grown for 7 days +/- UVB. Data is analysed per leaf, with 1 being the oldest leaf and 7 the youngest. Error bars represent the standard error from the mean of 6 replicates for ugt89c1 and 5 for Col-0. T-tests were used to compare +/- UVB Asterisk (*) refer to comparisons made between UVB treated and untreated plants within each panel and not to comparisons made between Col-0 and *ugt89c1*

Flavonoids were isolated and extracted from L4 of the *ugt89c1* plants +/- UVB and Col-0 +/- UVB after 7 days. In UVB treated Col-0 plants eight compounds were identified as being up regulated under UVB exposure, 4 quercetins and 4 kaempferols all rhamnosylated at the seven position (Fig 3.4). The most abundant phenolics in both irradiated an un-irradiated leaves were kaempferol glycosides. Concentrations of quercetins in Col-0 were on average between 51 to 90% higher in

UVB treated leaves versus untreated leaves. Concentrations of kampferols in Col-0 were on average between 48 to 80% higher in UVB treated plants (Fig. 3.4). The differences in flavonoid concentration between the UVB treated and untreated Col-0 leaves was significant for all of the eight compounds (Q-3-[R-G]-7-R ($p \leq 0.001$), K-3-[R-G]-7-R ($p \leq 0.001$), Q-3-[G-G]-7-R ($p \leq 0.05$), Q-3-G-7-R ($p \leq 0.001$), K-3-[G-G]-7-R ($p \leq 0.0001$) K-3-G-7-R ($p \leq 0.0001$), Q-3-R-7-R ($p \leq 0.001$), K-3-R-7-R ($p \leq 0.001$)). However, these same compounds were at extremely low levels or completely absent

in both the UVB treated and untreated *ugt89c1* (Fig. 3.4). Out of the 8 compounds analysed 4 of them were found in higher concentrations in the untreated *ugt89c1* plants, (K-3-[R-G]-7-R, Q-3-G-7-R, K-3-[G-G]-7-R and K-3-R-7-R). For one

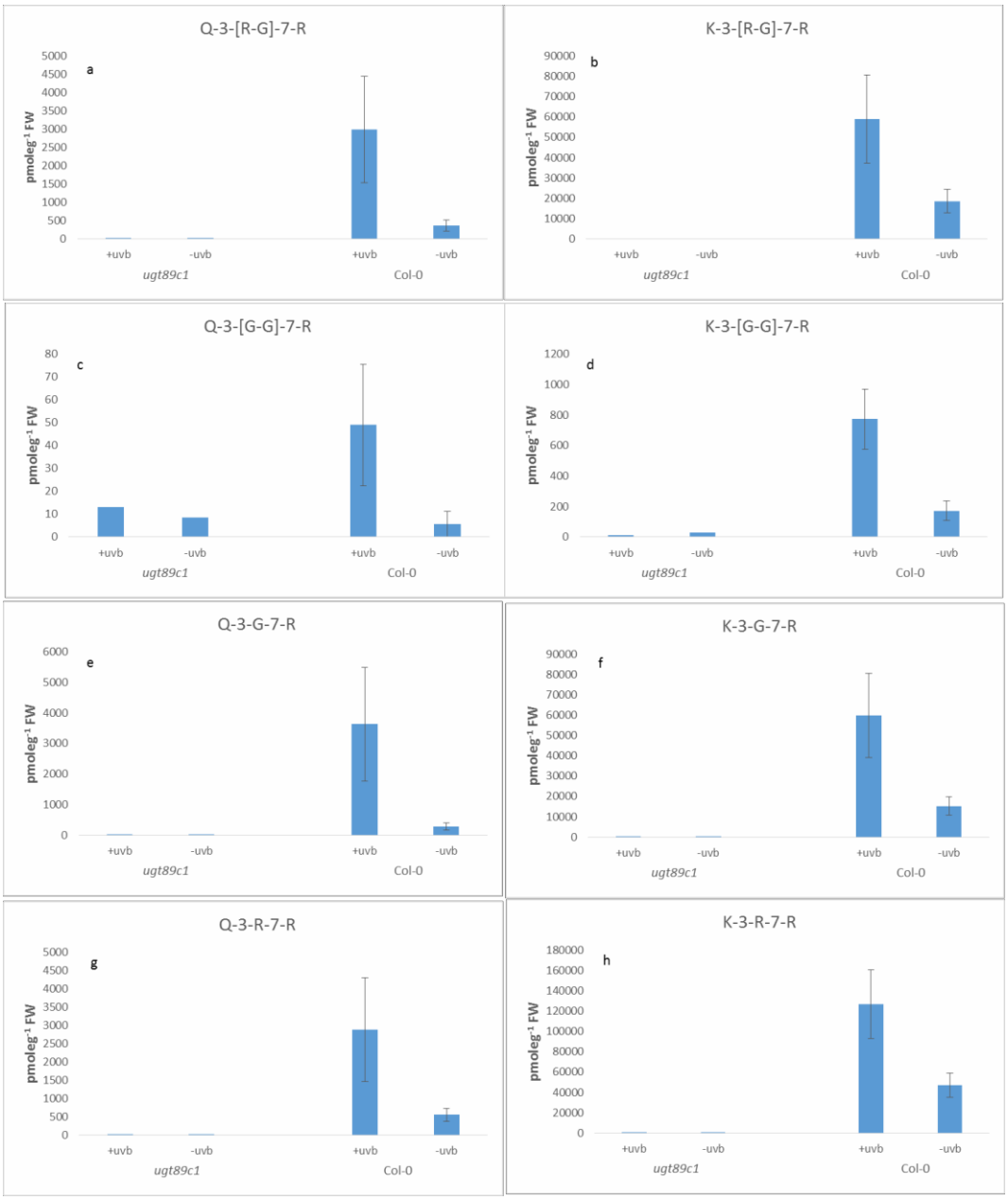

Figure 3.4 Quercetin and kaempferol concentrations in *ugt89c1* knockout line and Col-0 grown with or without UVB for 7 days. Levels of quercetin and kaempferol derivatives were quantified using UPLC-TQD mass spectrometry. Error bars represent the standard error from the mean of five replicates for Col-0. There are no error bars for *ugt89c1* as this is preliminary data. Panels a,c,e,g are quercetin derivatives and panels b,d,f,h are kaempferol derivatives:

(a) Quercetin 3-O-rhamnosyl-glucoside 7-O-rhamnoside (Q-3[R-G]-7-R)
(b) Kaempferol 3-O-rhamnosyl-glucoside 7-O-rhamnoside (K-3[R-G]-7-R)
(c) Quercetin 3-O-glucosyl-glucoside 7-O-rhamnoside (Q-3[G-G]-7-R)
(d) Kaempferol 3-O-glucosyl-glucoside 7-O-rhamnoside (K-3[G-G]-7-R)
(e) Quercetin 3-O-glucoside 7-O-rhamnoside (Q-3-G-7-R)
(f) Kaempferol 3-O-glucoside 7-O-rhamnoside (K-3-G-7-R)
(g) Quercetin 3-O-rhamnoside 7-O- rhamnoside (Q-3-R-7-R)
(h) Kaempferol 3-O-rhamnoside 7-O- rhamnoside (K-3-R-7-R)

compound, Q-3-[R-G]-7-R, the concentration was the same in both treated and untreated, in the remaining 3, Q-3-[G-G]-7-R, K-3-G-7-R and K-3-R-7-R, higher levels were found in the UVB treated *ugt89c1* plants. Only in one compound was there a comparable concentration with Col-0, Q-3[G-G]-7-R was found to be 73% higher in UVB treated Col-0 than UVB treated *ugt89c1*. For the other 7 flavonoids, concentrations in UVB treated *ugt89c1* were <1% of those measured in UVB treated Col-0, and were outside the accuracy range of the quantification method.

Discussion

Flavonoids are widely accepted to be a central component of the UVB response (Agati & Tattini 2010). Here we investigated the functional role of the 7-rhamnosylation process for UV protection under a relatively low chronic UV dose. In parallel, we also investigated the link between increasing flavonoid levels and decreasing plant size through the use of a transferase mutant *ugt89c1*.

In this study, it was found that: (1) knockout *ugt89c1* contained substantially lower concentrations of 7-out-of-8 7-rhamnosylated flavonoids in both control and UV-exposed plants (2) total soluble phenolics in the *ugt89c1* knockout were increased under the +uvb treatment (3) photosynthetic efficiency of PSII was unaffected by UVB in both Col-0 and *ugt89c1* plants (4) UVB treated *ugt89c1* knockouts had shortened petioles and leaf lengths but the biomass of the +UVB plants remained unchanged.

The *ugt89c1* transferase mutant is a knock out line, which is lacking the transferase enzyme required to produce quercetins and kaempferols rhamnosylated at the seven position. These quercetins and kaempferols are specifically identified as upregulated in response to UVB exposure and it has been suggested that 7-O-rhamnosylation has

an important function in the UVB acclimation process (Hectors *et al.,* 2014). In this study it was found that *ugt89c1* knockout line did not accumulate quercetins and kaempferols glycosylated at the seven position in response to UVB exposure nor were these compounds present at significant concentrations in un-irradiated plants. These data are consistent with those of Yonekura-Sakakibara *et al.* (2007) who reported the lack of 7-rhamnosylation under visible light. This confirms the importance of UGT89C1 gene for the specific pattern of flavonoid-rhamnosylation observed in *Arabidopsis thaliana* (Yonekura-Sakakibara *et al.,* 2007). As this gene has been observed responding to UVB radiation through UVR8 (Brown *et al.*, 2005; Oravecz *et al.*, 2006), question arise concerning the adaptive role of 7-rhamnosylation during UV-acclimation.

In this study, it was found that despite being unable to accumulate flavonoids rhamnosylated at position 7 in response to UVB, the *ugt89c1* knockout plants did not appear to be particularly sensitive to UVB injury. An increase in the total soluble phenolic levels of the *ugt89c1* knockouts was observed under UVB radiation and preliminary UPLC-TQD mass spectrometry investigation identified several novel compounds in the knockout line. This is in line with the findings of

Yonekura-Sakakibara *et al.*, (2007) who also found new flavonoid derivative peaks in the knockout line. The newly identified compounds were also found in Col-0 (Fig 3.5). Some of the newly identified compounds are present in comparable concentrations in both Col-0 and *ugt89c1* plants, but for others the levels in Col-0 are much lower than the *ugt89c1* plants, suggesting a re-direction of substrates following the lack of 7-rhamnosylation in the *ugt89c1* plants (Fig. 3.5). Indeed, some of these new compounds have tentatively been identified as the precursors of the seven rhamnosylated flavonoids, while others are yet unidentified. Out of the 14 new

compounds identified 10 of them are up regulated in UVB exposed *ugt89c1* plants (Fig. 3.5). It is possible that these newly identified compounds are contributing to the increase in total soluble phenolics in the *ugt89c1* lines, and act as UVB protection in place of the missing 7-rhamnosylated quercetins and kaempferols. Indeed the *ugt89c1* knockout line shows little indication of plant stress as evidenced by the comparable values for F_v/F_m found in both +/- UVB treated plants. Interestingly, studies involving *Arabidopsis thaliana* tt mutants which have constitutively lower levels of flavonoids than wild types have shown that these are highly sensitive to UVB radiation (Li *et al.*, 1993). Implying that, it is a plants ability to up-regulate flavonoids in general in response to UVB, which is most important for UVB protection and acclimation. On the other hand, in an outdoor study using *uvr8-1* it was found that flavonoids that had accumulated in response to low temperatures did not provide any UVB protection (chapter 4, this thesis). This suggests that there is a difference between the inability to perceive UVB and the inability to activate an appropriate response pathway. There is also the possibility that the protective capacity of alternative antioxidants has been up regulated in *ugt89c1* in response to UVB. Tocopherols and polyamines are known to be accumulated in response to UVB (Hectors *et al.*, 2014), contributing to the overall antioxidant capacity of the plant and therefore stress tolerance. These findings bring into question the relative importance of these specific glycosylated flavonoids under relatively low doses of UVB radiation and suggest that alternative mechanisms and pathways can protect the plant in this scenario.

There is evidence that flavonoids can influence plant architecture, potentially directing morphological changes in response to prevailing climatic and environmental conditions. Flavonoids are known to modulate auxin transport therefore can affect elongation and tropic responses such as photo and gravi-tropism (Brown *et al.*, 2001;

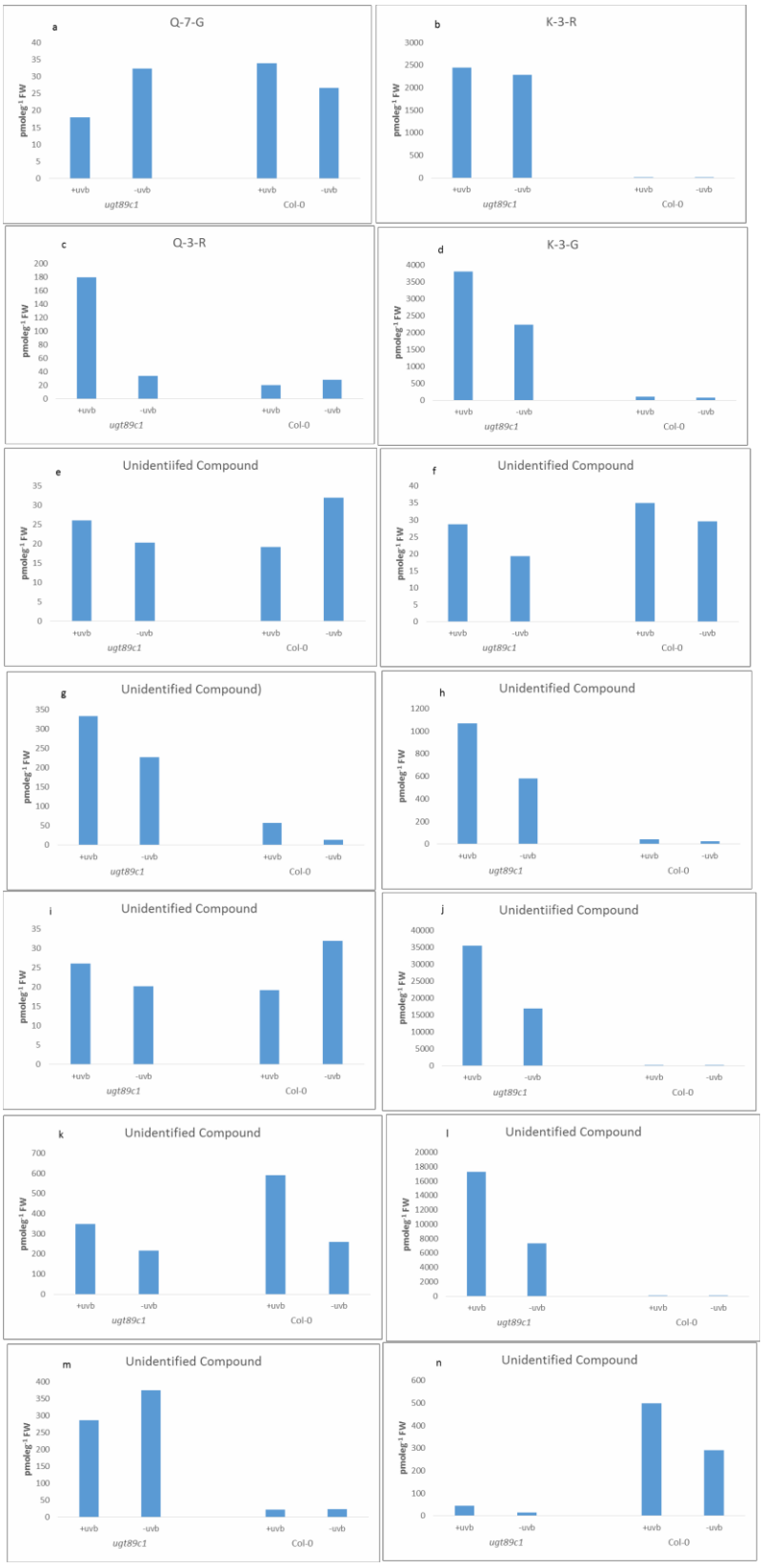

Figure 3.5. Preliminary data of possible precursors to position 7-rhamnosylated quercetin and kaempferols, panel (a) quercetin 7-rhamnoside panel (b) kaempferol 3-O-rhamnoside (c) quercetin 3-O-rhamnoside and (d) kaempferol 3-O-glucoside are tentatively identified and panels (e-n) are as yet unidentified. The *ugt89c1* knockout line and Col-0 were grown for 7 days +/-UVB. Levels were quantified using UPLC-TQD mass spectrometry.

Peer & Murphy, 2007). Exposure to UVB radiation often results in dwarfism and while this phenomenon is widely reported it is not yet clear why being smaller is advantageous in the presence of UVB. An increase in flavonoids and a decrease in size often parallel each other but functionally linking the two has remained elusive (Robson *et al.*, 2014). It is also not clear if this relationship is linked to specific flavonoids such as the quercetins and kaempferols rhamnosylated at position seven up regulated by UVB or if the general upregulation in flavonoids is linked with a reduction in plants size. As part of a shade avoidance strategy mediated through the UVB photoreceptor UVR8 and phytochrome a reduction or increase in elongation is a desirable outcome however the mechanism behind it has not been described(Hayes *et al.*, 2014). Another hypothesis suggests that there is a redirection of resources to the production of flavonoids resulting in carbon deficit and a slowdown in growth although in practice there is little evidence to support such a hypothesis (Kotilainen *et al.* 2009; Robson & Aphalo, 2012).

Here we find that in the presence of UVB, the *ugt89c1* transferase mutant has shorter petioles and shorter leaves typical of the UVB response, despite lacking the specific glycosylated flavonoids commonly accumulated in response to UVB. This would seem to decouple the morphological response from the biochemical one. Yet, unlike in Col-0, the biomass of *ugt89c1* leaves was not affected by UVB exposure. In Col-0 leaf proportions remain unchanged, as decreases in petioles and leaf length are matched by decreases in biomass. In contrast, these proportions change in the *ugt89c1* knockout upon UV-exposure. The leaves of *ugt89c1* plants are relatively light in the absence of UV-radiation. Thus, while some aspects of the morphological response to UVB, like petiole length, are unaffected by the flavonoid glycosylation pattern, other aspects are affected, like biomass per leaf. The divergence in biomass responses

between Col-0 and *ugt89c1* would suggest that there are subtleties within the morphological response to UVB that are not yet understood. Thus rather than decoupling morphological changes from the biochemical one it has been observed that flavonoid glycosylation patterns does affect certain aspects of leaf development.

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Chapter 4

Seasonal effects of UVB radiation on three Arabidopsis accessions under natural solar conditions.

Abstract

Despite making up only a small percentage of the solar spectrum, UVB wavelengths have come under significant scrutiny largely due to their energetic nature. There are significant variations in UVB experienced at ground level based on a number of factors including latitude, altitude, climate and season. This study aimed to find evidence of UVB effects on plant morphology and phenolics within the context of an oceanic climate and to assess any evidence of seasonality in the UVB response. Genotypic differences in the adaptive response to UVB were assessed by comparing *Arabidopsis thaliana* Ler and Col-0 lines to a local accession, Bur-0. Plants were grown outdoors using filters to change the natural light spectrum at seven timepoints over a 12 month period. Evidence from this study finds a strong seasonal effect on morphology and total phenolics across the three accessions. A clear UVB effect on morphology was found during the summer. However, there was no specific adaptive responses based on genotype to UVB radiation.

Introduction

UVB radiation is a natural part of the solar spectrum. It ranges from 280-315nm and is largely prevented from reaching the surface of the Earth by the stratospheric ozone layer (Frohnmeyer & Staiger, 2003). Approximately 3% of the energy from the sun that reaches ground level is UV, and only about 5% of this is UVB radiation. Depending on latitude and altitude, there are significant variations in the concentration of UVB experienced at ground level (Liley & Mckenzie, 2006). The annual dose of UVB is higher over tropical regions because of the angle of the sun and the thickness of the ozone layer (Kakani *et al.,* 2003). The UVB dose is lower in temperate areas as the ozone layer is thicker and the Sun's rays are reaching the Earth's surface at an acute angle. Geographic features are not the only factor that can affect UVB reaching the surface, climate such as cloud cover, albedo, changing seasons and air pollution can also influence UVB at ground level (Madronich *et al*., 1998; Calbó *et al*., 2005; McKenzie *et al.,* 2009). Ireland's climate is temperate and characterised by a high degree of cloud cover year round. The effects of clouds can be contradictory and range from small enhancements of UV at ground level to total blocking of UV penetration (Calbó *et al*., 2005). It is suggested that a combination of refraction and scattering of direct and diffuse sunlight can result in UVB enhancements of up to 8% in comparison to clear-sky days (Sabburg and Wong, 2000). Intuitively it might be considered that UVB would not play significant role at ground level in an oceanic climate due to the prevailing weather conditions but until now, this has not been fully investigated.

Despite making up only a small percentage of the solar spectrum, UVB wavelengths have come under significant scrutiny, largely due to their energetic nature. In the past concern over a depleted stratospheric ozone layer has focused research on the negative effects of UVB (Rozema *et al.,* 1997). The dosages used in supplemental UVB studies

were high to stimulate ozone depletion scenarios (Rozema *et al.*, 1997). To living organisms' high levels of UVB radiation can be harmful, it is absorbed by vital proteins and nucleic acids and damage to said can result in the production of ROS and impairment of cell processes such as photosynthesis (Jansen *et al.,* 1998; Hollósy, 2002). More recently, strict regulation of ozone depleting emissions, laid out in the Montreal Protocol (1987) have helped to stabilize the ozone layer assuaging concerns over the impact of increasing UVB for now (Ravishankara *et al.,* 2009, McKenzie *et al*., 2014). However research is continuing, as it has been realised that ambient levels of UVB are rarely a source of plant stress but rather a positive source of information (Jansen *et al.*, 2012). A dedicated UVB photoreceptor has recently been discovered. Since then it has been shown that in the absence of damage, UVB through UVR8 can induced specific changes in gene-expression and physiology (Brown *et al.,* 2005). This UVR8 pathway is activated and responsive to low, ecologically relevant fluence rates of UVB (Brown & Jenkins, 2008).

Commonly reported UVB responses include increases in secondary metabolites and changes in plant architecture (Hectors *et al.,* 2014; Robson *et al.,* 2014). Flavonoids, particularly quercetin and kampferol, are upregulated in Arabidopsis in response to UVB exposure. Changes in the metabolite profile induced by UVB can increase cross-tolerance to biotic stress such as necrotrophic pathogens and herbivores and abiotic stress such as drought (Jenkins, 2014). This implies that UVB could be a proxy measure for other environmental or climatic parameters such as shade, high light or drought (Jansen *et al*., 2012). Thus, while UVB enhanced secondary metabolites are providing protection or cross-tolerance in plants they can also be beneficial to human health acting in a protective manner against chronic diseases and obesity (Vinson *et al*., 2001; Bertoia *et al*., 2016).

Changes in morphology are also a well-documented UVB response. The UVB phenotype is typically characterised by dwarf morphology in the form of shorter, thicker leaves, shorter petioles, leaf curling, inhibited development of the hypocotyl and stem and changes in the root/shoot (Jansen, 2002; Hectors *et al.*, 2012; Wargent *et al.*, 2009 (a); Wargent *et al*., 2009 (b); Hollósy, 2002). While a smaller plant can sometimes be an indication of a stress response, under natural levels of UVB the dwarf morphology has often been observed without any reduction in biomass or indication of stress (Robson *et al.*, 2014). The adaptive relevance of a change in growth form is still under investigation and major questions remain (Robson *et al.*, 2014). That said a dwarf morphology is not necessarily a negative outcome, in many cases a more compact and robust plant may be desirable and ultimately produce more harvestable or marketable biomass (Wargent *et al.*, 2011). Indeed, natural levels of UVB are now more frequently regarded as a regulator rather than an agent of plant stress, a regulator which can prime defences, promote more compact studier plants and produce a more nutritionally beneficial crops (Jansen *et al.*, 2012; Wargent & Jordan, 2011).

Much of what is known about UVB comes from lab-based studies using artificially high doses of UVB. Outdoors studies are limited and few look at the seasonal variation in UVB effects. Lab-based studies fail to capture the variation in plant responses to climate and seasonality. This gap between the lab and natural conditions make it difficult to compare and extrapolate data between the two. Frequently, it is found that results from indoor and outdoor assays differ significantly from each other.

The ability of plants to adapt to local geographical and climatic conditions is an important selective force which has lead to a range of within species genetic variation (Shindo *et al*., 2007). Local acessions have overtime developed phenotypic adaptations to local conditions, this leads to phenotypes which are ecologically

specialized and can optimise performace in a given region. Given the wide distribution of *Arabidopsis thaliana* the phenotypic variation which exists in accessions can help to inform us of the ecological signifcance of specific adaptations (Koornneef *et al.*, 2004). Variations in the Arabidopsis species included resistance to stressors such as salt, drought, temperature extremes, disease and enhanced capacity to deal with ROS (Koornneef *et al.*, 2004).

Landsberg erecta (Ler) and Columbia-0 (Col-0) are both commonly used in lab studies as model systems in a range of mechanistic and regulatory studies. While significant understanding of plants relationship with UVB has been gained from such studies there is dearth of information about the behavior of such accesions under ambient UVB and natural growing conditions. Burren-0 is an Irish accession which possibly has adaptations to local environmental conditions. The physiological variations in these accession may help to understand the relationship and functional role of plant responses to ambient levels of UV raditaion.

Based on the literature, it is hypothesised that even with low levels of direct sunlight evidence of a UVB effects will be found under Irish growing conditions. This study aimed to find evidence of UVB effects on plant morphology and phenolics within the context of an oceanic climate and to assess any evidence of seasonality in the UVB response. Ler and Col-0 lines were compared to a local accession, Bur-0 to attempt to identify any adaptations to natural UVB. Also the experiment was set over the an entire year to observe the consequences of growth within the natural growing season and outside of it.

Materials and Methods

Plant Material

Seeds of *Arabidopsis thaliana* accessions Landsberg erecta (Ler), Columbia-0 (Col-0) and Burren-0 (Bur-0) were cold-treated for a minimum of seven days before being sown into flats of sieved John Innes No.2 compost (J. Arthur Bowers, William Sinclair Horticulture Ltd., Firth Rd., Lincoln, LN6 7AH). The flats were covered in cling film and placed in a temperature controlled growth room on a 16 hour light/ 8 hour dark photoperiod, under 60-80µmol m^{-2} s^{-1} PAR (Fig 4.1). Once the seeds had germinated the cling film was removed. At the cotyledon stage the seedlings were transplanted into 200ml individual pots containing John Innes No. 2 compost (Fig. 4.1). The seedlings were then placed back into the growth room and covered with cling film for a further two days until established. Once the seedlings had reached the 1.02 stage (Boyes *et al*., 2001) they were transferred to the greenhouse and subsequently to a cold frame to acclimate to outdoor conditions (Fig 4.1). Time spent in the greenhouse and cold-frame varied depending on the time of year and speed of growth, plants were ready to use at the Boyes 1.04 growth stage. This process was repeated seven times over the year during January, February, May, July, September, October and November.

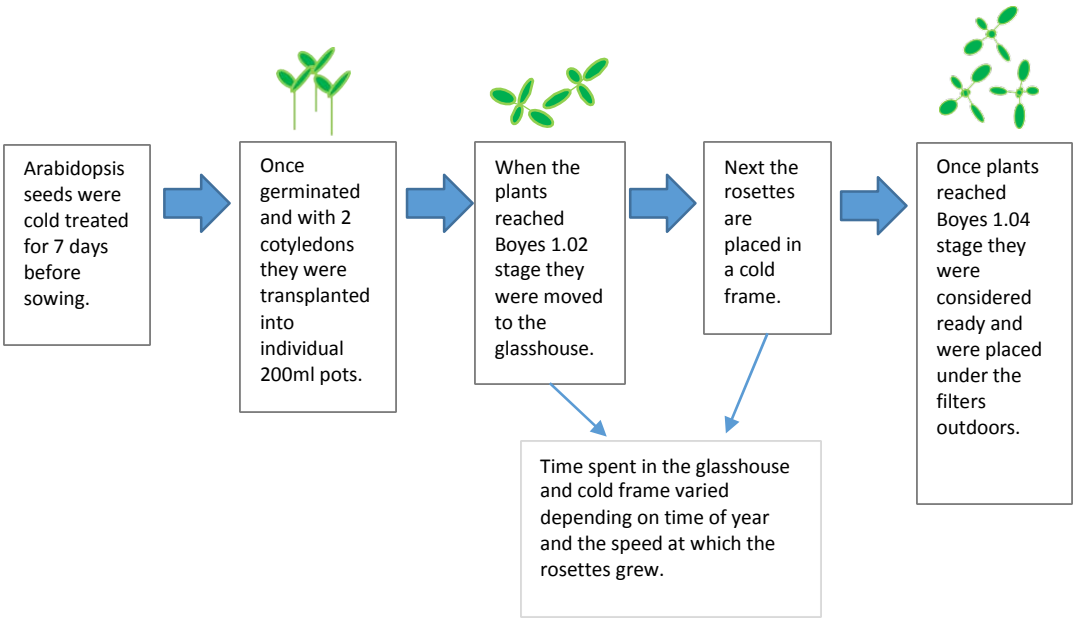

Figure 4.1 Description of the preparation of plant material used in Chapter 4 and 5.

UV-exposure Conditions

A UV-filtration approach with ambient solar light was used for this experiment. A total of three treatments were used; UV transparent cellulose acetate filter, uv-a/b (visible + UVA and UVB) (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany), UV-B blocking mylar filter, uv-a (visible + UVA) (125µm thickness, Polyester film, Tocana Ltd., Ballymount, Dublin, Ireland) and a UV opaque filter, uv-0 (visible), (poly-tunnel plastic, BPI Visqueen, Stevenston, U.K.). The cellulose acetate and Mylar were changed after 20 days exposure to solar light to prevent the changing of the light spectrum caused by degradation of the plastic. The transmission of the filters was measured using a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A) (Fig. 4.2b).

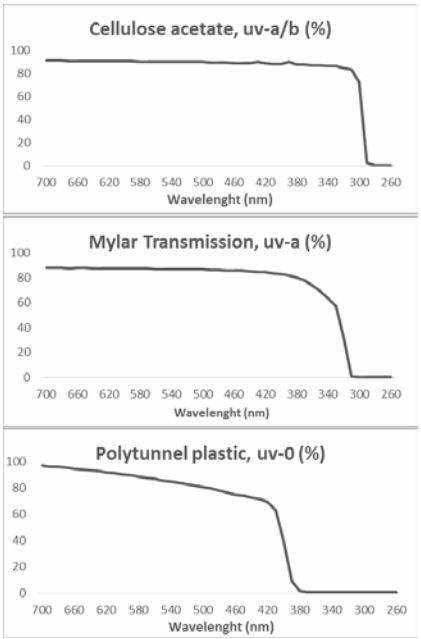

Figure 4.2(a) Outdoor exprimental setup 50x50cm corri-board frames, were used to mount the filters. These were tilted slightly at the northern edge

Figure 4.2(b) The transmission properties of UV transparent cellulose acetate filter, uv-a/b (visible + UVA and UVB) (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany), UV-B blocking polyester filter "Mylar", uv-a (visible + UVA) (125µm thickness, Polyester film, Tocana Ltd., Ballymount, Dublin, Ireland) and a UV opaque filter, uv-0 (visible), (200 µm thickness, poly-tunnel plastic, BPI Visqueen, Stevenston, U.K.).

Frames measuring 50cm x 50cm were constructed using opaque corri-board (Fig. 4.2a). These frames supported the filters that were suspended above the plants. There was four replicates of each treatment. The frames were randomly set out at a non-shaded site in Cork, South West Ireland (51°53'58"N 8° 29'14"W). The frames were tilted slightly to allow for air circulation with the northern edge of the frame raised off the ground (Fig. 4.3). Four plants of each genotype were placed under each frame.

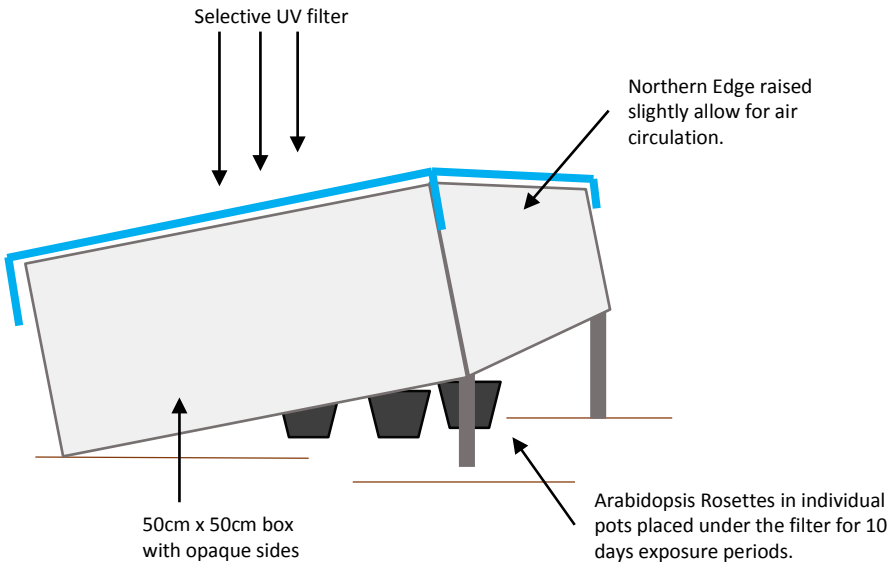

Figure 4.3 Diagram of the frames which supported the UV selective filters used outdoors in Chapter 4, 5 and 6.

Morphological Parameters

Leaf and rosette morphology were analysed after ten days of growth under the filters outdoors. Rosettes were dissected and then photographed for processing using ImageJ software (Abramoff *et al.*, 2004). Various morphological parameters, including rosette diameter (mm), biomass (mg) and leaf area (mm^2) were measured. The smallest leaves (defined as having a petiole of less than 2mm) were not included in analysis.

Biochemical Analysis

After ten days of growth, total phenolics were extracted from all leaves that had a petiole measuring longer than two millimetres. Whole leaves, including the petioles were placed in micro-tubes with 1ml acidified methanol (1%HCL, 20%H_2O, 79% CH_3OH) and incubated in the dark at 4°C for four days. Absorbance was recorded at 330nm on a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A). Absorbance was normalized per leaf using total leaf area.

Photosynthetic Efficiency

Chlorophyll fluorescence (F_v/F_m) was determined using an Imaging PAM (Waltz, Germany) as a proxy measure of the maximal quantum yield of photosystem (PS) II efficiency. F_v/F_m values were determined after plants had grown for ten days under outdoor conditions. Whole rosettes were dark adapted for a minimum of 20 minutes before F_v/F_m was determined. Three measurements were taken at random from each rosette and pooled per rosette. To test UVB protection capacity, plants were subsequently placed in a UVB box overnight and challenged with 2.4 ± 0.3 W/m^2 UVB for 13 hours. A second F_v/F_m value was then measured.

Statistical Analysis

Relationships between environmental factors influencing growth and biochemistry such as temperature, hours of sunshine and global solar radiation and UV treatment were tested using multiple regression analysis with IBM SPSS Statistics 21. Prior to regression analysis it was confirmed that all data sets were suitable for regression analysis and that there was no violation of the assumption of linear multicollinearity and homoscedasticity. Meteorological data were obtained from Met Eireann (65/67 Glasnevin Hill, Dublin 9, D09 Y921). It was found that there was a high degree of correlation between the independent variables; temperature, hours of sunshine and global solar radiation. For this reason, they were analysed in separate regressions.

For a complete understanding of the influence of UV treatment under varying weather conditions (seasonality) January and July were chosen as case studies and analysed in more detail. Prior to any analysis, all data sets were assessed for normality; in the case of non-normal data the square root transformation was applied. If it was not possible to obtain normal data an ANOVA was carried out on the ranked data and a Kruskal-Wallis post hoc test was used. Normal data was analysed statistically using parametric interaction ANOVAs, with multiple comparison test being carried out using Tukey's range test.

Results

A full set of meteorological data were obtained to cover the same period as the growth trials. Temperatures during the trial period ranged between 3.1 and 15.6°C (Fig. 4.4).

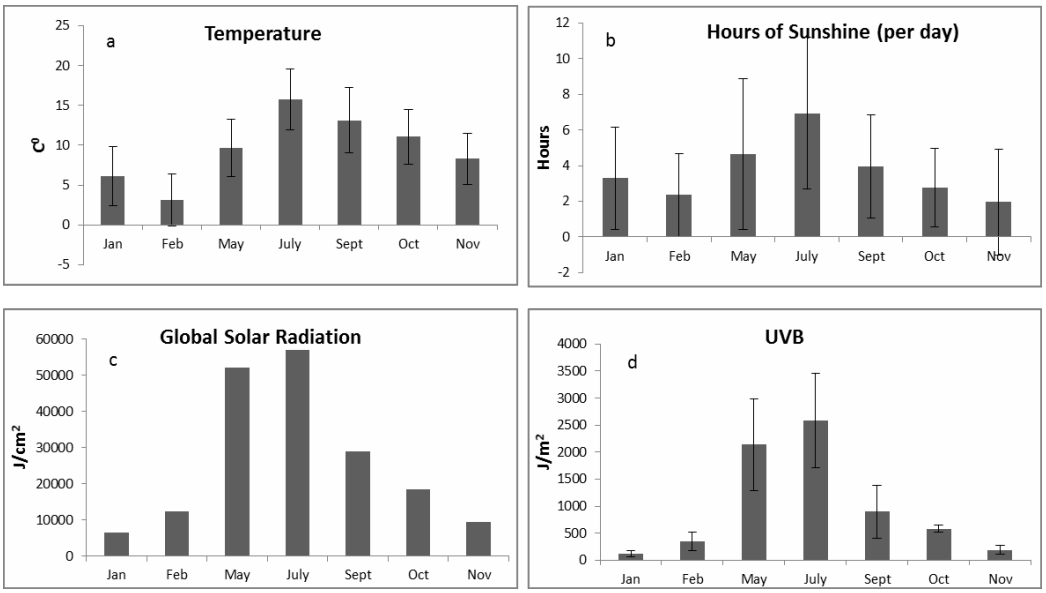

Figure 4.4. Weather data supplied by Met Eireann. Temperature (a) and Hours of Sunshine (b) were collected at Cork Airport, Co. Cork. Global Solar Radiation (c) and UVB (d) was collected at the Valentia Observatory, Co Kerry. Temperature, Hours of Sunshine and UVB are the mean values for each 10 day exposure period. Global Solar Radiation is a mean monthly figure calculated by Met Eireann.

Total hours of sunshine and UVB dose ranged between 3 to 7 hours and 118 to 2583 J/cm^2 per day, respectively (Fig 4.4). Monthly means of global solar radiation during the trial ranged between 6,464 and 56,973 J/m^2 (Fig. 4.4).

Initial data analysis identified a number of candidate environmental parameters that could potentially account for the overall trends in rosette diameter, leaf area, biomass, total phenolic content and F_v/F_m. These environmental parameters were temperature, global solar radiation, hours of sunshine and UVB irradiance.

The meteorological parameters temperature, global solar radiation, hours of sunshine and UVB irradiance were all significantly correlated with each other. The correlation between the meteorological parameters mean that they lack independence and have to be analysed in separate multiple regressions. Regression analysis of separate meteorological parameters with the measured biological responses identified several significant correlations (R^2 values) (Table 4.1). Using temperature as the independent variable produced the highest R^2 values indicating that temperature is the strongest

determinant of plant size and total UV absorbing pigment content (Table 4.1). Temperature accounted for between 49 and 74% of the variation in rosette diameter, leaf area, total UV absorbing pigments and F_v/F_m for Ler, Col-0 and Burren-0 (Table 4.1). Hours of sunshine accounted for between 7 and 41% of the variation in biological responses, while global solar radiation and UVB irradiance contributed 15 – 49% and 9 - 37%, respectively (Table 4.1).

Table 4.1. R^2 values from the multiple regression model, using data from all seven months, Dependent variable = Constant+ (B_1 xTemp) + (B_2 x uv-a/b) + (B_3 x uv-a), asterisks are used to indicate the significance of the R^2 value (* = p ≤0.05, **=p≤0.001, *** = p≤ 0.0001).

	Temperature			*Hours of sunshine*			*Global solar radiation*			*UV-B*		
	Ler	Col-0	Bur-0	Ler	Col-0	Bur-0	Ler	Col-0	Bur-0	Ler	Col-0	Bur-0
Rosette	0.73 ***	0.61 ***	0.74 ***	0.30 ***	0.30 ***	0.3 ***	0.36 ***	0.39 ***	0.39 ***	0.22 ***	0.31 ***	0.26 ***
Leaf Area	0.74 ***	0.65 ***	0.63 ***	0.36* **	0.41 ***	0.31 ***	0.43 ***	0.49 ***	0.43 ***	0.28 ***	0.37 ***	0.34 ***
Total Phenolics	0.7 ***	0.72* **	0.7 ***	0.17 **	0.21 ***	0.27* **	0.2 ***	0.22 ***	0.27 ***	0.09	0.11 *	0.16 *
Fv/Fm	0.49 ***	0.55 ***	0.61 ***	0.07	0.12 **	0.16 **	0.15 ***	0.26 ***	0.26 ***	0.09	0.18 *	0.17 *

Further exploration of correlation between temperature and the biological responses included the UV filters as independent variables. The uv-a/b and uv-a filters were compared to the uv-0 filter which acted as a control. This approach allowed for identification of the impact that the filters had on the fit of the regression, within the context of the seasonal trend, which was largely dominated by temperature. From the Part No. Squared it is evident that temperature accounts for a large part of the R^2 value for all biological responses, but there is also evidence that uv-a/b filter contributes significantly to the regression (Table 4.1 & Table 4.2). Leaf area and rosette diameter are significantly affected by the uv-a/b treatment, in Ler, Col-0 and Bur-0 (Table 4.2). The negative slope-values suggest that uv-a/b filter is associated with a decrease in leaf area and rosette diameter (Fig. 4.7 & Table 4.2).

Table 4.2. Slopes and there significance and the Part Nos. Squared from a multiple linear regression model (Dependent variable = Constant+ (B_1 xTemp) + (B_2 x uv-a/b) + (B_3 x uv-a)) including temperature and the 3 filters as independent variables. The slope informs if a particular variable is making a statistically significant and unique contribution to the equation. The Part No. Squared describes the unique contribution that independent variable makes to the total R^2 and thus to the variation in the dependent variable.

		Ler			*Col-0*			*Bur-0*		
		Slope	Sig	Part No. Squared	Slope	Sig	Part No. Squared	Slope	Sig	Part No. Squared
Leaf area	Temp	0.82	***	0.67	0.78	***	0.6	0.77	***	0.6
	uv-a/b	-0.30	***	0.07	-0.26	**	-0.05	-0.21	**	0.03
	uv-a	-0.17	*	0.02	-0.10	ns	-7.7x10-3	-0.07	ns	4.23x10-3
Rosette Diameter	Temp	0.82	***	0.67	0.73	***	0.53	0.82	***	0.69
	uv-a/b	-0.27	***	0.06	-0.33	***	0.08	-0.24	**	0.04
	uv-a	-0.13	ns	0.01	-0.16	ns	0.02	-0.04	ns	1.37x10-3
Fv/Fm	Temp	0.69	***	0.47	0.738	***	0.54	0.77	***	0.6
	uv-a/b	-0.10	ns	8.1x10-3	0.001	ns	1x10-6	-0.10	ns	6.89x10-3
	uv-a	-0.14	ns	0.01	-0.07	ns	3.14x10-3	-0.16	ns	0.02
Total Phenolic	Temp	-0.84	***	0.72	-0.84	***	-0.72	-0.84	***	0.7
	uv-a/b	-0.03	ns	5.29x10-4	-0.08	ns	-4.6x10-3	-0.01	ns	8.1x10-5
	uv-a	0.07	ns	3.6x10-3	0.003	ns	-9x10-6	-0.67	ns	3.36x10-3

For all three accessions, there was no significant filter effect on photosynthetic efficiency measured as F_v/F_m across the yearlong study (Fig. 4.4 & Table 4.2). A significant relationship of F_v/F_m with temperature was found, as temperature increases so did the efficiency of PSII (Fig. 4.4 & Table 4.2). To test the acquired UVB tolerance of these plants they were subjected to an artificially high dose of UVB over 13 hours. Here we also found no significant difference between the three UV treatments used for growing the plants.

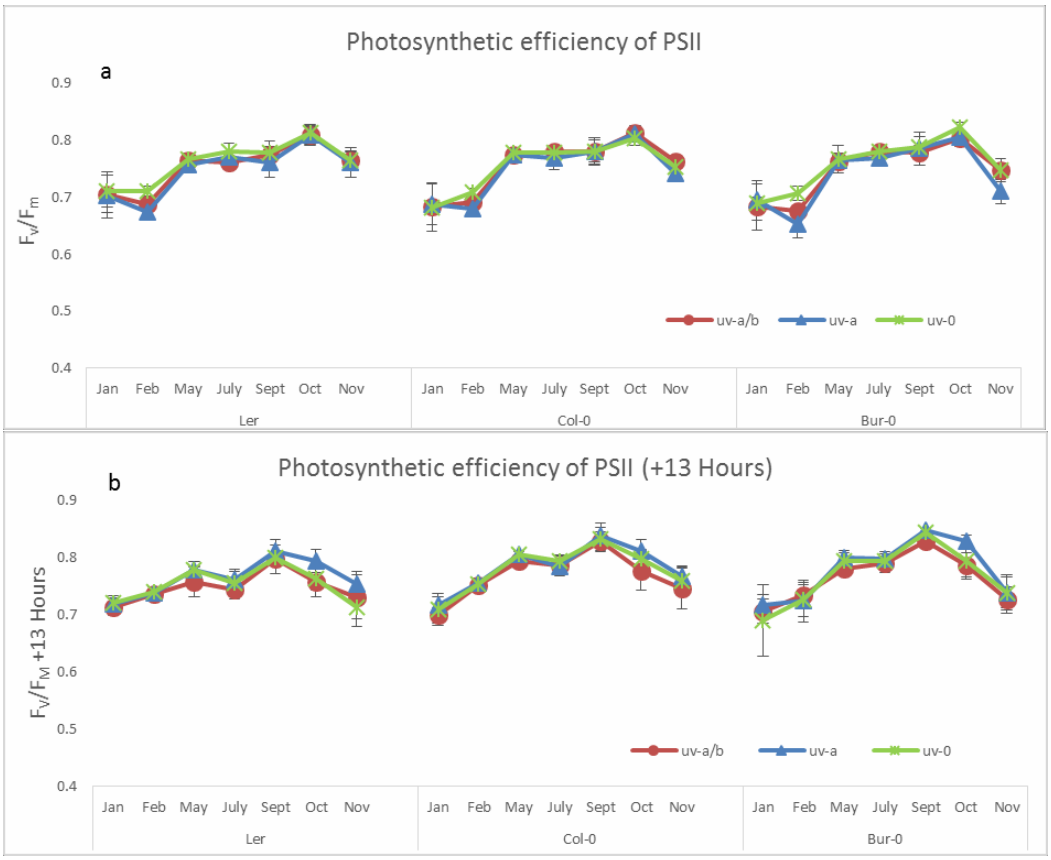

Figure 4.5 Panel (a) is the Quantum yield of photosystem II measured as F_v/F_m of Landsberg erecta (Ler), Columbia-0 (Col-0) and Burren-0 grown for 10 days outdoors over 7 months and panel (b) is the F_v/F_m value of the same plants retested after 13 hours high intensity UVB treatment (2.4 ± 0.3 W/m^2). Data was measured non-destructively from whole rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible**).**

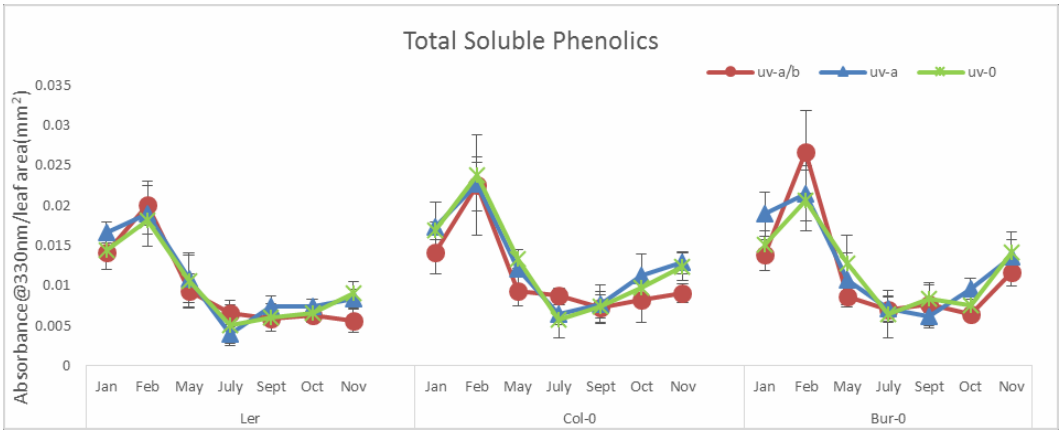

Figure 4.6 Total soluble phenolics extracted with a 1% acidified methanol solution and normalized using leaf area from Landsberg erecta (Ler), Columbia-0 (Col-0) and Burren-0 grown for 10 days outdoors over 7 months. Data was taken from leaf 4 of the rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible).

UV-absorbing pigments were not affected by filter type in the 3 accessions (Fig. 4.5 & Table 4.3). UV-absorbing pigments increased during the winter months and decreased during the summer months, this trend was the reverse of the trend observed in growth parameters (Fig. 4.6).

January and July responses

To explore the dataset, two months were chosen as case study. January and July were chosen as representative of the months with the highest and the lowest incidents of UVB. In January, there was no significant effect of UV treatment on the morphology of accessions (Table 4.3). There were however significant differences between the accessions in rosette diameter. Potentially this is due to differences between the accessions ($F(2, 27) = 22.613$. $p=0.0001$) (Table 4.3). Both Col-0 and Bur-0 had larger rosette diameters than Ler, 21% and 16% respectively (Table 4.3). The only significant UV effect was on phenolics, UVA treated plants had higher total phenolic levels than the UVB treated plants and while this difference was significant the actual difference between the treatments was small($F(2, 27) = 0.772$, $p=0.001$) (Table 4.3).

In July, there was clear evidence of a UV effect on the biomass, rosette diameter and leaf area of exposed plants. The biomass of plants grown under the UV transmitting filters was between 42 and 52% less than that of plants grown under the uv-0 filter (Table 4.3 & Fig. 4.7). Rosette diameters were between 18 and 37% less and leaf area was 33 and 48% less than plants grown under the uv-0 filter (Table 4.3 & Fig. 4.7).

Table 4.3 Summary of two-way ANOVAs on the effects of accession and filter type on biomass (mg), rosette diameter(mm), leaf area (mm^2), total soluble phenolics and F_v/F_m grown outdoors for 10 days in January 2013 and July 2013

Main Effects			January					July				
			Biomass (mg)	Rosette Diameter(mm)	Leaf Area(mm^2)	Total Phenolics	F_v/F_m	Biomass (mg)	Rosette Diameter(mm)	Leaf Area (mm^2)	Total Phenolics	F_v/F_m
Accession	Ler		11.26 a	12.06 a	8.75 a	0.0152 a	0.7028 a	137.54 a	31.05 a	73.01 a	0.0054 a	0.7706 a
	Col-0		11.39 a	15.23 b	10.10 a	0.0161 a	0.6933 a	142.66 a	31.40 a	78.31 a	0.0068 a	0.7756 a
	Bur-0		12. 18 a	14.29 b	11.56 a	0.0160 a	0.6883 a	150.77 a	37.69 b	78.55 a	0.0068 a	0.7763 a
Filter	uv-a/b		11.14 a	13.21 a	9.0824 a	0.014 a	0.6869 a	105.21 a	25.90 a	54.28 a	0.0058 a	0.7738 a
	uv-a		11.06 a	13.92 a	9.8979 a	0.018 b	0.6942 a	126.60 a	33.38 b	68.89 a	0.0058 a	0.7677 a
	uv-0		12.63 a	14.46 a	11.4301 a	0.016 ab	0.6939 a	217.71b	40.85 c	102.77 b	0.0073 a	0.7799 a
		df	ANOVA									
F value Ecotype		**2**	0.556	22.613	2.736	0.772	0.902	0.213	167.933	0.226	2.903	0.851
	Sig		ns	***	ns	ns	ns	ns	*	ns	ns	ns
F value	Filter	**2**	1.761	2.856	1.961	9.505	0.201	12.137	670.466	19.929	2.837	2.73
	Sig		ns	ns	ns	**	ns	***	***	***	ns	ns
Genotype x Filter		**4**	0.482	0.406	0.558	0.83	0.094	0.187	57.349	0.956	0.863	1.228
	Sig		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	Total Jan	**27**										
	July	**18**										

ns= not significant, * = p ≤0.05, **=p≤0.001, *** = p≤ 0.0001, according to two-way ANOVA. Comparisons to be made within columns Means in the same column and same main effect with the same letter are not significantly different, p>0.05 according to Tukey tests

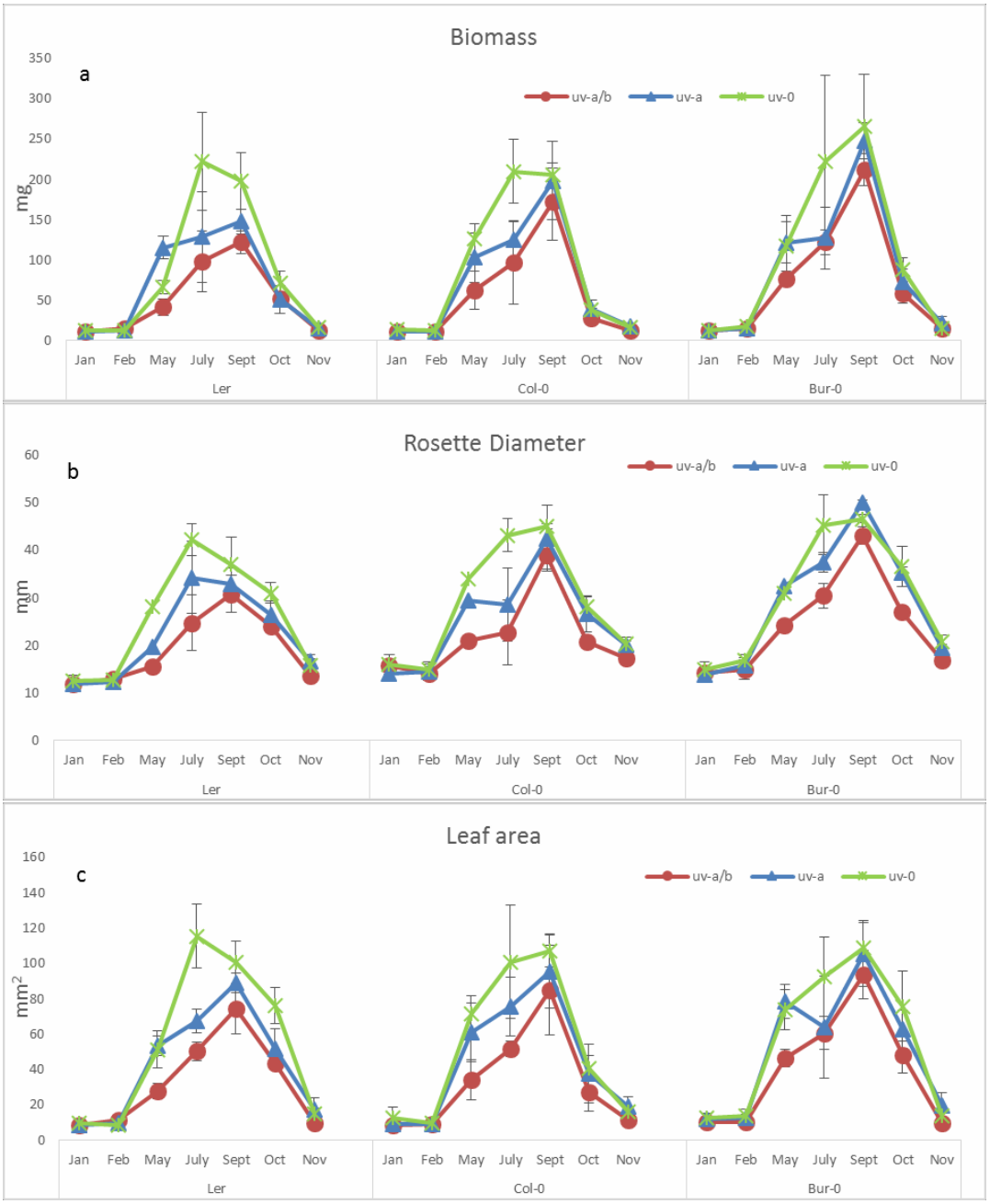

Figure 4.7. The biomass (mg), panel (a), rosette diameter (mm) panel (b) and leaf area (mm^2) panel (c) of Arabidopsis ecotypes Landsberg erecta (Ler), Columbia-0 (Col-0) and Burren -0 grown for 10 days outdoors over 7 months. Biomass and leaf area data represented above is from leaf 4 of the rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Starting rosette diameter, biomass and leaf area of the whole rosettes was 9 mm or less, no more than 5 mg and less than 50 mm^2 respectively. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible).

The reduction in size and weight of plants grown under the uv-a/b and uv-a filter was significant for biomass ($F(2, 18)=12.137$, $p=0.0001$), rosette diameter ($F(2, 18)=$

670.466, p=0.0001) and leaf area (F(2,18)=19.929, p=0.0001) (Table 4.3 & Fig. 4.7). There was also a significant difference between the rosette diameters of the three accessions, Bur-0 was on average 18% larger than Ler and 17% larger than Col-0 (F(2, 18)=167.933, p=0.05) (Table 4.3). No accession or filter effect was found on F_v/F_m or Total UV-absorbing compounds (Table 4.3).

Discussion

The Irish climate is described as oceanic and is characterised by high levels of rainfall, relatively low hours of direct sunshine, and a lack of temperature extremes. This study aimed to investigate the impact of UVB on plant growth in Ireland and how the changing seasons affected this.

The key findings of this study are; (1) significant variations in plant growth and total soluble phenolics were found throughout the year; (2) analysis of this variation found that it was linked to changes in temperature and season; (3) within the seasonally induced changes in morphology, there were UV mediated changes clearly evident during the summer months; (4) however, the observed morphological changes were not paralleled by UVB induced changes in total soluble phenolics; (5) also there was no evidence in differential responses to UVB between the three accessions.

Plant growth and development is entrained by the seasonal cycles. This study was set over the course of a year to observe any seasonal pattern in plant responses to UVB. It was found that the primary driver behind the observed patterns in morphology, UV-absorbing pigments and F_v/F_m values was seasonal changes in temperature. Biomass, rosette diameter and leaf area all increased with higher temperatures and decreased again as temperatures dropped. This growth pattern was evident across the three filter treatments. However, there were significant UV mediated differences found between

the treatments in the summer months. *Arabidopsis thaliana* accessions Ler, Col-0 and Bur-0 exhibited a more dwarfed phenotype when grown under the uv-a/b and uv-a filters in the months of May and July. A dwarf plant is considered a typical morphological response to UVB exposure but it can also be an indication of plant stress. Yet, we find no evidence of a stress response in F_v/F_m. Stress-induced Morphogenic Responses (SIMR) can also produce a dwarf phenotype in response to a range of conditions (Potters *et al*., 2007). Plants used in this experiment came from a greenhouse and potentially they could have experienced an initial UVB shock when placed outdoors. A UVB induced phenotype does not typically have a reduced biomass, it is characterised by a re-direction of growth as opposed to a cessation or slow in growth (Robson *et al.*, 2014). Here we do find a reduction in biomass in parallel to reduced leaf area and rosette diameter potentially suggesting that the phenotype is caused by stress rather than UVB exposure. However, as mentioned before there was no evidence of a UVB effect on F_v/F_m. Additionally, it was observed that plants grown under UVA treatment also had had a dwarf morphology; levels of UVA in natural sunlight would not normally be identified as a cause of plant stress. A study carried out using the same experimental conditions using the UVR8 mutant *uvr8-1* found that under natural sunlight the mutant plant had a dwarf phenotype but also showed indications of plant stress (Chapter 5). When exposed to UVB as part of a natural spectrum the uvr8-1 mutant, which is impaired in UVB perception, had reduced F_v/F_m values and lower total phenolic content. Similar symptoms of plant stress were not identified in Ler, Col-0 or Bur-0. This would suggest that the reduction in biomass, and leaf area were not caused by SIMR and were due to UVB exposure.

An increase in UV-absorbing pigments is often reported as a UVB-mediated response but here no evidence of said was found. Despite morphological changes during the

summer months, the total phenolic content was comparable under all three filter throughout the seasons. Temperature was identified as the primary driver behind the seasonal changes in total phenolic concentration, potentially this is masking changes induced by UVB (Bilger *et al*., 2007; Leyva *et al.,* 1995). Outdoor studies of lichens and mosses reported that the complex responses to natural environmental conditions elicited much larger changes in UV-absorbing pigments than UVB and the seasonal variations were likely to conceal any UVB effects (Bjerke *et al.,* 2005; Gehrke, 1999). This suggests that under outdoor conditions the UVB mediated accumulation of UV-absorbing pigments are not evident here, although it is one of the most commonly reported UVB effects (Rozema *et al*., 1997; Jansen *et al*., 1998). It should be considered that a lack of response in this instance does not mean that the composition of the total phenolics pool has not been altered. Studies using supplemental UVB have shown that there are significant increases in total phenolics and the ratios between specific quercetins and kampferols have also been changed (Hectors *et al*., 2014). Outdoor studies on birch trees have also shown that individual compounds change in response to UVB radiation rather than total phenolics (Kotilainen *et al*., 2009; Morales *et al.,* 2010).

Genotypic differences between accession can be significant and have the potential to enhance our understanding of the ecological role of specific adaptations. Cooley *et al.,* (2001) compared the responses of seven accessions exposed to supplementary UVA and UVA+B under outdoor conditions from May to June. Several parameters were measured and compared, responses ranged from insensitive, promotive to inhibitory and it was found that results varied with treatment, accession and the parameter measured (Cooley *et al*., 2001). Ler and Col-4 which were included responded to supplemental UVA/B with a background of natural sunlight by reducing leaf area,

width and length and petiole length significantly, interestingly these same parameters were insensitive to UVA (Cooley *et al*., 2001). This is in contrast to the results from this study (July data) where it was observed that the plants under the uv-a filter had reduced biomass, rosette diameter and leaf area. Biswas & Jansen (2012) also investigated genotypic differences in UV responses to supplemental UVA/B and UVA in a laboratory based study and found that growth was stimulated in Bur-0 under PAR+UV-A. This apparent contradiction between the results could be a product of natural sunlight versus artificial or supplemented light. Between the accessions, a significant difference was found was in rosette diameter, this was observed in both January and July. In January, Col-0 and Bur-0 were larger than Ler and in July Bur-0 was larger than both Col-0 and Ler suggesting that there was some difference in the growth rates between the accessions. However, there was no evidence of genotypic differences in the response of the three accession to UV radiation. This begs the question how important is being smaller if Bur-0 consistently has a larger rosette but suffers no added damage under UVB. Cooley *et al.* (2001) found a great degree of diversity when comparing the responses of different accessions but the ecological relevance is limited as the study was only carried out during May-June. The fixed time point failed to take into consideration variations in accession response to changing climatic conditions. Given the complexity of the outdoor environment, differences such as increases in secondary metabolites that would be noted in more controlled conditions between the accessions are likely being masked by larger trends in this dataset.

Arabidopsis is widely used for mechanistic studies of plant responses but is seldom grown outdoors. This study highlights the potential conflicts between findings in controlled conditions and the outdoors. The morphology of Arabidopsis rosettes

grown outdoors showed evidence of significant UV mediated changes but another commonly reported UV response was not observed i.e. increases in total phenolics. This study emphasises the importance of a holistic approach to the investigation of environmental stimuli and their effects on plant growth. Irish climatic conditions might suggest that low UVB would not be a factor in plant growth. Evidence from this assay finds a clear UVB induced morphological effect, though only in the summer. This suggests that UVB is only a factor during the summer when levels are at their highest. A caveat should to be considered in light of this finding; under natural conditions, Arabidopsis does actively grow in Ireland during the late summer months. Arabidopsis is a short lived early spring-summer annual or autumn-winter annual, plants set seed from May to June or from September-October (Thompson, 1994; Koornneef *et al*., 2004). Suggesting that while a significant UVB effect was found it may not be evident or an issue for natural populations of Arabidopsis as they would be senescing and going to seed when UVB levels are at their highest in Ireland. As the UVB effect on morphology is seen in a plant growing outside of its natural season (i.e. July) further investigation is required in to the ecological relevance of the dwarfing response under natural conditions. For a more comprehensive understanding of the UVB effects in an oceanic climate a study of plant species that are actively growing or developing during the summer months needs to be undertaken. However, this does not detract from the findings of this study, as it is the first to look at the UVB response in an Irish context and it is important in furthering our knowledge of the seasonal effects of UVB.

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Chapter 5

A functional UVR8 pathway is required for optimized plant growth year round under natural light conditions.

Abstract

UVB wavelengths are biologically active; in plants, they can induce a range of molecular, biochemical, morphological and developmental responses. Although much progress has been made in elucidating UVB perception and signalling pathways under controlled laboratory conditions, understanding of the adaptive, ecological role of UVB responses is still very limited. In this study, we analysed the functional role of UVR8 under natural light conditions or outdoors, by studying growth, photosynthetic competence and accumulation of UV absorbing pigments in a mutant lacking functional UVR8 protein. It was found that the influence of UVB on morphology is restricted to the summer, and is independent of UVR8. In contrast, UVB had an effect on the content of UV-absorbing pigments and the maximal efficiency of photosystem II of photosynthesis in the *uvr8-1* mutant, and throughout the year. It is concluded that the UVR8 photoreceptor plays an adaptive role throughout the year, in the temperate climate zone, even when UVB levels are relatively low.

Introduction

Impacts of UVB radiation on plants were first systematically investigated in the late 1970s and early 80s, because of increasing awareness of the thinning of the stratospheric ozone layer due to anthropogenic activities (Farman *et al*. 1985). Concerns over the state of the ozone layer have recently been somewhat assuaged and the United Nations Environment Programme has concluded in its latest report that concentrations of ozone depleting compounds within the atmosphere are decreasing (McKenzie *et al.,* 2014). The changes in emissions policy initiated by the Montreal Protocol(1987) may have brought about the stabilization of the stratospheric ozone but due to natural variation, any recovery of the ozone layer is not yet detectable (Ravishankara *et al*., 2009; McKenzie *et al.,* 2014). It should also be considered that continuing changes in our climate, including emissions of nitrous oxides, have the potential to undo the recent strides made towards the recovery of the ozone layer (Ravishankara *et al.,* 2009; McKenzie *et al.,* 2014).

UVB wavelengths are biologically active; in plants, they can induce a range of molecular, biochemical, morphological and developmental responses (Agati & Tattini, 2010; Heijde & Ulm, 2012; Robson *et al*., 2014). Stimulated by concern over the deterioration of the ozone layer, much of the early plant based research focused on the possible impacts of higher-than-normal UVB levels on plants (Rozema *et al.,* 1997; Kakani *et al*., 2003). UVB can potentially have a significant and damaging impact on living organisms due to its absorbance by important structures such as proteins and nucleic acids (Jansen *et al*., 1998; Hollósy, 2002), and consequently has commonly been considered a stressor. However, photosynthetic organisms such as plants have evolved a complex set of checks and balances allowing them to proliferate while managing the potential for UVB-mediated DNA damage and oxidative stress

through highly complex and effective defence responses (Rozema *et al*., 1997, Jansen *et al.*, 1998). Current research indicates that UVB-mediated plant stress is the exception rather than the rule (Ballaré *et al*., 2011). Conversely, UVB is increasingly recognised as an environmental regulator, acting via a specific photoreceptor to control plant growth and development.

Our understanding of the molecular mechanisms underlying UVB perception and signalling has rapidly increased in the last few years. A pivotal step was the identification of the *UV RESISTANCE LOCUS8* (UVR8) gene-product as the UVB photoreceptor (Rizzini *et al*., 2011). UVR8 was identified through screening for plants which exhibited a hypersensitive response to UVB exposure (Kliebenstein *et al*., 2002). Since then, it has been recognised that UVR8 plays a key role in UVB-mediated control of hundreds of genes, including several important for flavonoid induction (Brown *et al.*, 2005). Brown *et al.*, (2005) showed that UVR8 acts in a UVB-specific manner and mutants were unaffected by other stimuli. Yet, it is becoming increasingly clear that overlaps exist in gene transcription and up-regulation between UVA, blue light responses and what is thought of as a classic "UVB response" (Morales *et al*., 2012). It is possible that, through cross-talk, UVR8 influences other pathways and co-regulates non-UVB related responses. The potential also exists for the opposite to occur and UVR8-mediated responses may be stimulated by other environmental factors (Jenkins, 2014)

Well documented, regulatory UVB responses include the adjustment of the plant metabolic profile, including increases and decreases in levels of specific glycosylated flavonoids, primarily quercetins and kampferols (Ryan *et al.*, 1998; Kolb *et al.*, 2001; Hectors *et al*., 2014). Some of these changes, in metabolite profile are associated with enhanced resistance to biotic stress, such as necrotrophic pathogens and herbivores

and abiotic stress, such as drought (Jenkins, 2014). This suggests that plants exploit UVB as a proxy measure for other environmental or climatic parameters such as shade, high light or drought (Jansen *et al*., 2012). Another example of a UV-response is the UVB-mediated change in plant morphology. This response is widely reported across a range of species, and is characterised primarily by shorter, thicker leaves, shorter petioles, leaf curling, inhibited development of the hypocotyl and stem and changes in the root/shoot ratio (Hollósy 2002; Jansen, 2002; Wargent *et al*., 2009 (a); Wargent *et al.,* 2009 (b); Hectors *et al*., 2012). As in the case of UVB-mediated changes in metabolite profile, major questions remain about the adaptive relevance of such a response (Robson *et al.,* 2014).

Although much progress has been made in elucidating UVB perception and signalling pathways under controlled laboratory conditions, understanding of the adaptive, ecological role of UVB responses is still very limited. Thus, despite evidence of the presence of UVR8 in a range of photosynthetic organisms (Jenkins, 2014), the functionality of this photoreceptor in a complex environment remains to be established. In this study, we analysed the functional role of UVR8 under natural light conditions outdoors, by studying growth, photosynthetic competence and accumulation of UV absorbing pigments in a mutant lacking functional UVR8 protein (Brown *et al.*, 2005). It is hypothesised that plants lacking a functional UVR8 pathway will be insensitive to UVB in the natural environment. Thus will not exhibit the typical UVB response but may be stressed by the natural light spectrum. In parallel, the relative importance of UVB radiation and seasonal variation in UVB under temperate climatic conditions was also assessed. The overall aim of this study was to establish the adaptive role of UVR8 for growth and development of *Arabidopsis thaliana.*

Materials and Methods

Seeds of *Arabidopsis thaliana* accession Landsberg erecta (LER) and a *uvr8-1* mutant (Kliebenstein *et al.,* 2002) in the same genetic background (kindly donated by Prof. Gareth Jenkins) were stratified for a minimum of seven days before being sown into flats of sieved John Innes No.2 compost (J. Arthur Bowers, William Sinclair Horticulture Ltd., Firth Rd., Lincoln, LN6 7AH). The flats were covered in cling film and placed in a temperature controlled growth room on a 16h light/8h dark cycle, under 60-80 µmol m^{-2} s^{-1} PAR. Once the seeds had germinated the cling film was removed. At the cotyledon stage the seedlings were transplanted into individual 200ml pots containing John Innes No. 2 compost. The seedlings were then placed back into the growth room and covered with cling film for a further two days until established. Once the seedlings had reached the 1.02 stage (Boyes *et al.,* 2001) they were transferred to the greenhouse and subsequently to a cold frame to acclimate to outdoor conditions (Chapter 4, Fig 4.1). Time spent in the greenhouse and cold-frame varied depending on the time of year and speed of growth, plants were ready to be transferred to outdoor conditions at the Boyes 1.04 growth stage (Boyes *et al*., 2001) (Chapter 4, Fig 4.1). This process was repeated seven times over the year during January, February, May, July, September, October and November.

UV-exposure Conditions

A UV-filtration approach with ambient solar light was used for this experiment. A total of three treatments were used; UV transparent cellulose acetate filter, uv-a/b (visible + UVA and UVB) (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany); UV-B blocking polyester filter (Mylar), uv-a (visible + UVA) (125µm thickness, Polyester film, Tocana Ltd., Ballymount, Dublin, Ireland); and a

UV opaque filter, uv-0 (visible), (200 µm thickness, poly-tunnel plastic, BPI Visqueen, Stevenston, U.K.). The transmission of the filters was measured using a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A) (Fig 4.2b Chapter 4).

Frames measuring 50cm x 50cm were constructed using opaque corri-board. These frames supported the filters that were suspended above the plants (Chapter 4. Fig.4.3). There were four replicates of each treatment. The frames were randomly set out at a non-shaded site in Cork, South West Ireland (51°53'58"N, 8°29'14"W). The frames were tilted slightly to allow for air circulation with the northern edge of the frame raised off the ground (Chapter 4, Fig. 4.3). Four plants of each genotype were placed under each frame for 10 days.

Morphological Parameters

Leaf and rosette morphology was analysed after ten days of growth under the filters outdoors. Rosettes were dissected and then photographed for processing using ImageJ software (Abràmoff *et al.*, 2004). Various morphological parameters, including rosette diameter (mm), biomass (mg) and leaf area (mm^2) were measured. The smallest leaves (defined as having a petiole of less than 2 mm) were not included in analysis.

Biochemical Analysis

After ten days of outdoor growth, total UV absorbing pigments were extracted from all leaves that had a petiole measuring longer than two millimetres. Whole leaves, including the petioles were placed in micro-tubes with 1ml acidified methanol (1% HCL, 20% H_2O, 79% CH_3OH) and incubated in the dark at 4^0C for four days. Absorbance was recorded at 330nm on a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A). Absorbance was normalized per leaf using total leaf area.

Photosynthetic Efficiency

Chlorophyll fluorescence (F_v/F_m) was determined using an Imaging PAM (Waltz, Germany) as a proxy measure of the maximal quantum yield of photosystem (PS) II. F_v/F_m values were determined after plants had grown for ten days under outdoor conditions. Whole rosettes were dark adapted for a minimum of 20 minutes before F_v/F_m was determined. Three measurements were taken at random from each rosette and pooled per rosette. To test UVB protection capacity, plants were subsequently placed in a UVB box overnight and challenged with 2.4 ± 0.3 W/m^2 UVB for 13 hours. A second F_v/F_m value was then measured.

Statistical Analysis

The seven months of data (Jan., Feb., May., July., Sept., Oct., Nov.,) for rosette diameter, biomass, leaf area, F_v/F_m and total phenolics were analysed using non-parametric three-way ANOVAs (IBM SPSS Statistics 21) due to lack of homogeneity of variance within/between samples.

To understand the influence of UV treatment under varying weather conditions (seasonality), January and July were chosen as case studies and analysed in more detail. Prior to any analysis, all data sets were assessed for normality, in the case of non-normal data the square root transformation was applied. If it was not possible to obtain normal data an ANOVA was carried out on the ranked data and a Kruskal-Wallis post hoc test was used. For all normal data, a parametric two-way ANOVA was carried out followed by a Tukeys Range Test.

Results

The *Arabidopsis thaliana* accession Landsberg erecta (Ler) and a UVR8 mutant (*uvr8-1*) in the same genetic background, were grown outdoors in a series of ten-day assays

spread over a period of seven months. These assays were conducted to investigate the impact of natural levels of UVB radiation within the context of temperate climatic conditions. Specifically, seasonal variations in UVB responses were assessed by exploiting mutants of the UVB photoreceptor UVR8.

Plant growth analysis.

The data of rosette diameter (mm), rosette biomass (mg) and leaf area (mm^2) showed clear seasonal growth patterns (Fig. 5.1 &Table 5.1)). A non-parametric three way ANOVA found significant differences between months in biomass ($F(6,124) = 193.2$, $p \leq 0.0001$) rosette diameter ($F(6,120) = 218.6$, $p \leq 0.0001$) and leaf area ($F(6,122) = 178.1$. $p \leq 0.0001$) (Fig. 5.1 & Table 5.1). Rosette diameter, biomass and leaf area were respectively, 3, 12, and 8 fold larger during the summer and autumn compared to the winter months (Fig 5.1 & Table 5.1). This pattern was observed across the three filter treatments, and in both Ler and the *uvr8-1* mutant (Fig. 5.1 & Table 5.1).

Analysis maximum quantum yield of photosystem II.

The maximum quantum yield of photosystem II (F_v/F_m) was less responsive to changes in season than plant growth (Fig. 5.1 and 5.2). In particular, the lower rosette diameter, rosette biomass and leaf area observed in October and November were not matched by a similarly strong decrease in F_v/F_m values (Fig. 5.1 and 5.2). However, F_v/F_m values were lower in January and February than in May to November ($F(6,126) = 72.8$, $p \leq 0.0001$) (Fig 5.2 & Table 5.1).

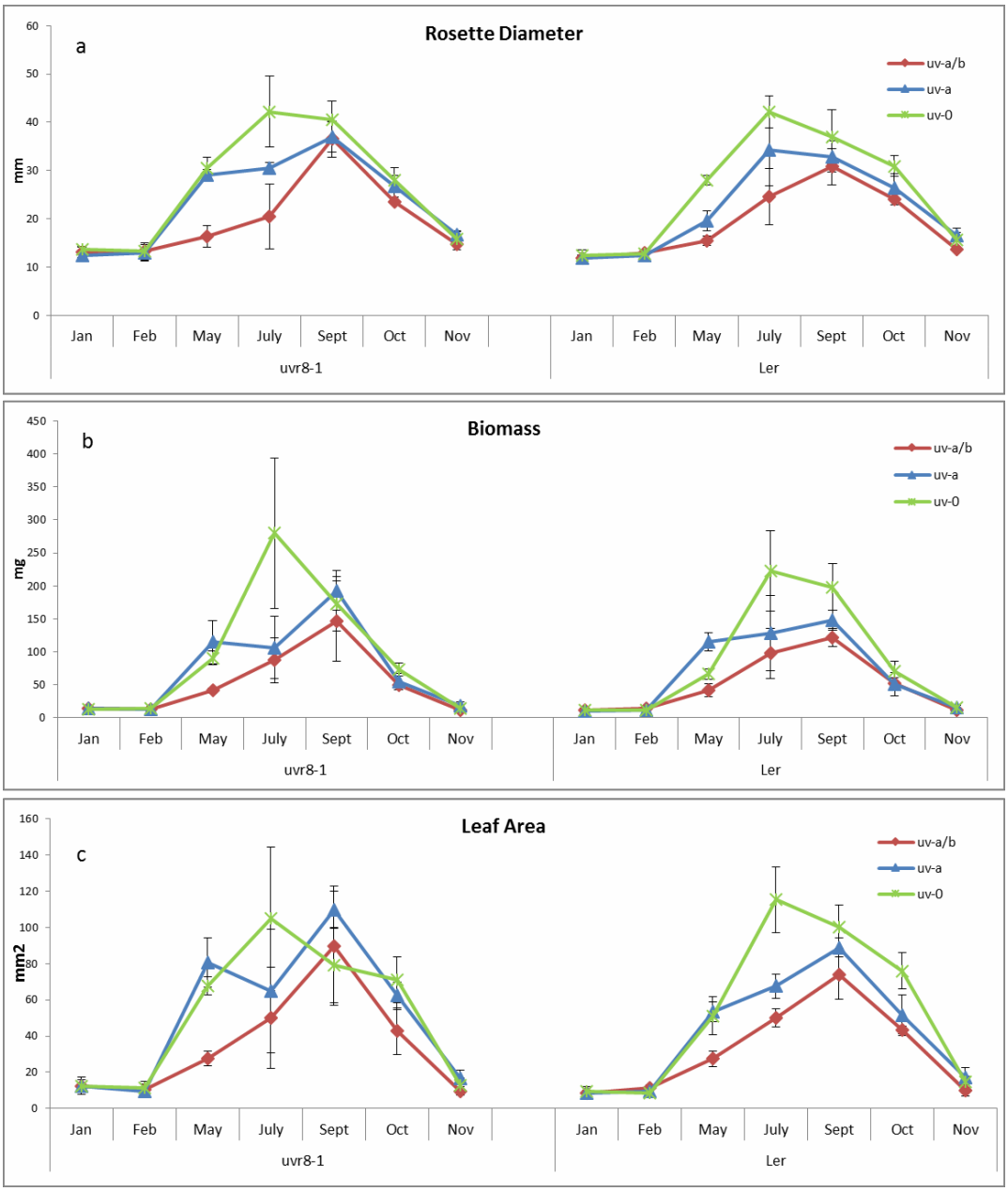

Figure 5.1 Rosette diameter (mm) (Panel a), leaf area (mm^2)(Panel b) and biomass (mg) (Panel c), of Landsberg erecta and the *uvr8-1* mutant grown for 10 days outdoors over 7 months. Biomass and leaf area represented above is from leaf 4 of the rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Starting rosette diameter, biomass and leaf area of the whole rosettes was 9 mm or less, no more than 5 mg and less than 50 mm^2 respectively. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible).

To further test UV-protection, a sub-group of plants were treated with 13 hours of high intensity UVB following the end of the ten day outdoor growing period. After this treatment the maximal quantum yield of PSII was measured to assess the plants resilience and acquired UVB tolerance. It was found that Ler was unaffected by the

Table 5.1 Summary of three-way non parametric ANOVA on ranked data, on the effects of accession type, filter and month on biomass, diameter, leaf area, Fv/Fm Fv/Fm+13 and Total phenolics

Main Effects	Biomass (mg)			Diameter (mm)			Leaf Area (mm^2)			Fv/Fm			Fv/Fm +13			Total Phenolics (ABS 330nm/ mm^2)		
	df	F	Sig	df	F	Sig	df	F	Sig	df	F	Sig	df	F	Sig	df	F	Sig
Ecotype	1	2.9	ns	1	7.3	**	1	5.2	*	1	24.3	***	1	185.7	***	1	13.8	***
Treatment	2	15	***	2	42.3	***	2	24.2	***	2	20.4	***	2	66.4	***	2	29.2	***
Month	6	193.6	***	6	218.6	***	6	178.1	***	6	72.8	***	6	46.9	***	6	116	***
Eco x Treat	2	0.9	ns	2	2	ns	2	1.5	ns	2	10.5	***	2	26.2	***	2	11.7	***
Eco x Month	6	1.9	ns	6	2.6	ns	6	2.6	*	6	0.7	ns	6	1.5	ns	6	2.5	*
Treat x Month	12	4.1	***	12	4.8	***	12	4.9	***	12	1.1	ns	12	3.6	***	12	5.7	***
Eco x Treat x Month	12	0.6	ns	12	1.6	ns	12	1	ns	12	0.7	ns	12	2.4	***	12	1.1	ns
Error	**124**			**120**			**122**			**126**			**126**			**122**		

ns =not significant, * = p≤ 0.05, ** = p ≤ 0.001, *** = p ≤ 0.0001, according to three-way non-parametric ANOVA. Posthoc analysis were not carried out as the ANOVA was run on ranked data and post hoc analysis is not considered appropriate this type of data.

high dose of UVB and in some months the F_v/F_m values even increased slightly (Fig. 5.2, Panel b). In contrast, in the case of the *uvr8-1* mutant grown under uv-a/b filter there were clear indications of further UVB stress following exposure to a high dose; i.e. its F_v/F_m values decreased further (Fig. 5.2, Panel b). Analysis of F_v/F_m values after the high UVB dose found significant differences between the wild-type Ler and the mutant *uvr8-1* ($F(1,126) = 185.7$, $p \leq 0.0001$) (Fig.5.2, Panel b).

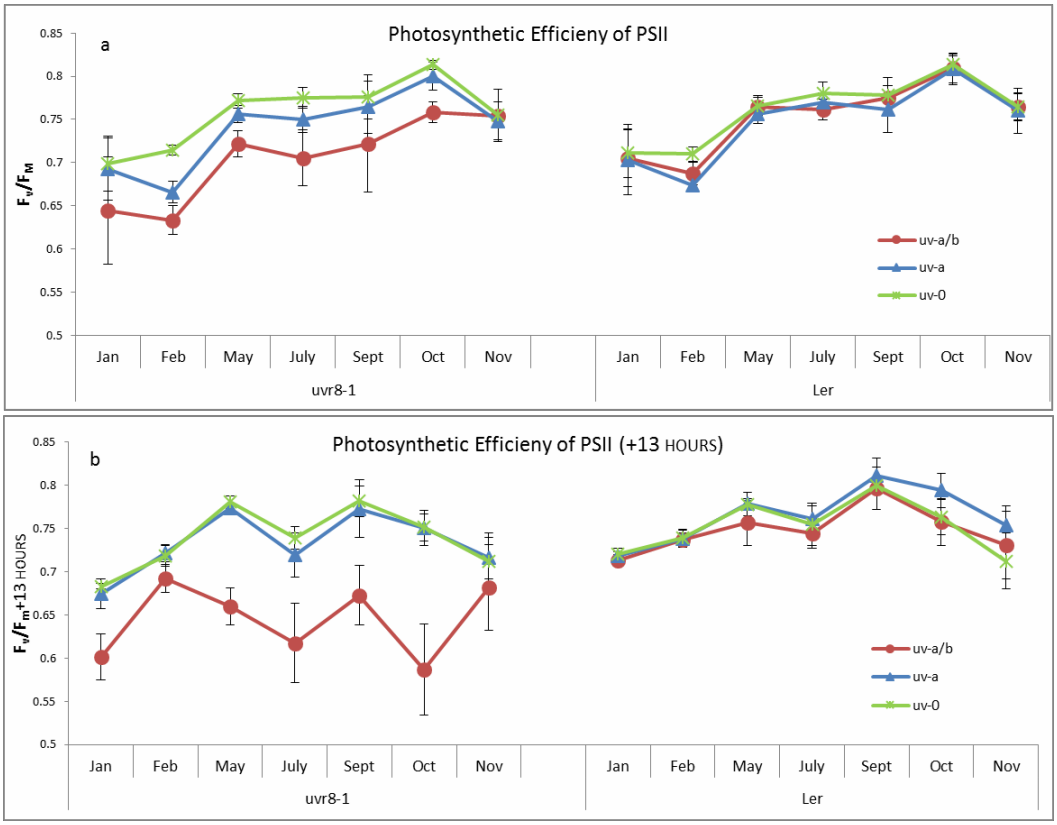

Figure 5.2 Quantum yield of photosystem II measured as F_v/F_m of Landsberg erecta and the *uvr8-1* mutant grown for 10 days outdoors over 7 months (Panel a) and the F_v/F_m value of the same plants retested after 13 hours high intensity UVB treatment(2.4 ± 0.3 W/m^2)(Panel b). Data was measured non-destructively from whole rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible).

Accumulation of Total Soluble Phenolics

The trend in total soluble phenolic content of leaves was the opposite of that seen for morphological parameters (Fig. 5.1); i.e. lower total phenolics during the summer months and higher levels during the autumn/winter ($F(2, 124) = 116$, $p \leq 0.0001$) (Fig. 5.3 & Table 5.1).

Clearly, changes in overall content of total soluble phenolics in wild-type Ler were not associated with changes in morphology.

However, levels of total soluble phenolics in UVB treated *uvr8-1* mutants were reduced, the difference found between the *uvr8-1* mutant and the wild-type Ler were significant ($F(1,122) = 13.8$, $p \leq 0.0001$) (Fig. 5.3 & Table 5.1).

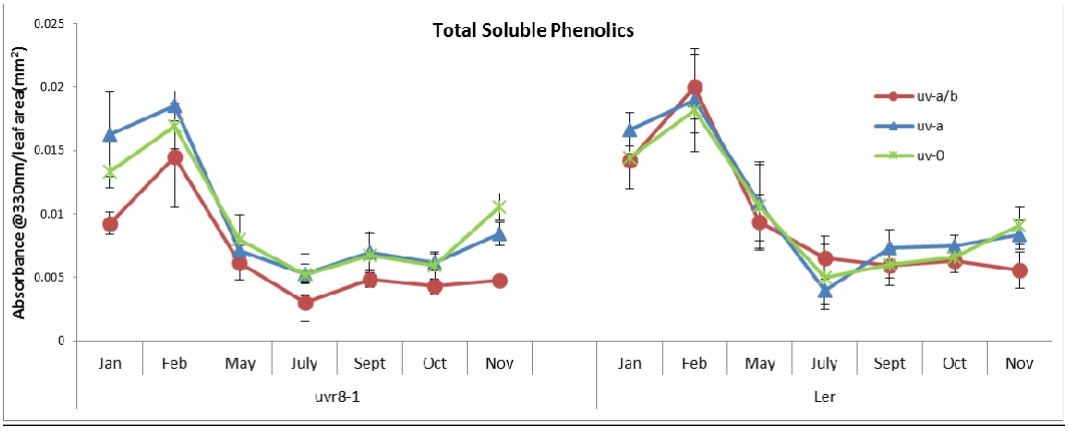

Figure 5.3 Total Soluble phenolics extracted with a 1% acidified methanol solution and normalized using leaf area From the *uvr8-1* mutant and the wild-type Landsberg erecta grown for 10 days outdoors over 7 months. Data was taken from leaf 4 of the rosettes. Error bars represent the standard deviation from the mean of 4 replicates. Filter specifications: uv-a/b (visible + UVA and UVB), uv-a (visible + UVA) and uv-0 (visible).

<u>January and July responses</u>

January and July plant-response datasets were examined in more detail in order to analyse the effects of relatively low winter levels of UVB, compared to the much higher summer levels. For each month, separate two-way ANOVAs were used to explore the impact of filters and genotype on biomass, rosette diameter, total UV absorbing pigments and F_v/F_m following 10 days of outdoor growth under natural light conditions.

In January, biomass ($F(1, 18) = 8.85$, $p=0.008$) and rosette diameter ($F(1, 18) = 9.728$, $p=0.006$) of the *uvr8-1* mutant were larger than of the wild-type Ler, and although the difference was small it was found to be significant (Table 5.2). There were no significant differences caused by the different filter treatments on biomass ($F(2, 18) = 0.07$, $P=0.933$), rosette diameter ($F(2, 18) = 3.146$, $p=0.067$) or F_v/F_m ($F(2, 18) = 0.95$, $p = 0.405$) (Table 5.2).

However, it was found that there was a significant filter, genotype and interaction effect (F (2, 18) =4.665, p=0.020) on total soluble phenolics (Table 5.2).

Table 5.2 Summary of a two-way ANOVAs on the effects of genotype and filter on biomass (mg), rosette diameter (mm), total phenolics (A330/mm^2) and F_v/F_m grown outdoors for 10 days in January and July.

		January				July			
Main Effects		Biomass (mg)	Rosette Diameter-mm	Total Phenolic	F_v/F_m	Biomass (mg)	Rosette Diameter-mm	Total Phenolic	F_v/F_m
Genotype	*uvr8-1*	14.1a	13.1a	0.011a	0.68a	146.7a	31a	0.005a	0.76a
	Ler	11.3b	12.1b	0.017b	0.70a	144.7a	31a	0.005a	0.77b
Filter	uv-a/b	12.9a	12.1a	0.014a	0.67a	92.4a	22.6a	0.005a	0.75a
	uv-a	12.7a	12.5a	0.013b	0.70a	117.3a	32.4b	0.005a	0.77a
	uv-0	12.4a	13.1a	0.015b	0.69a	251b	38.2b	0.005a	0.78b
	df	ANOVA							
F value Genotype	1	8.85	9.728	11.863	1.452	0.109	0	1.339	12.58
Sig		**	**	**	ns	ns	ns	ns	**
F value Filter	2	0.07	3.146	16.39	0.95	13.273	12.128	0.292	10.31
Sig		ns	ns	***	ns	***	***	ns	**
Genotype x Filter	2	0.933	0.459	4.665	0.041	0.829	2.304	5.103	1.365
Sig		ns	ns	*	ns	ns	ns	*	ns

ns= not significant, * = p ≤0.05, **=p≤0.001, *** = p≤ 0.0001, according to two-way ANOVA. Means in the same column and same main effect with the same letter are not significantly different, p>0.05 according to Tukey tests.

A one-way ANOVA was used to identify the source of the significance. It was found that *uvr8-1* mutant grown under the uv-a/b filter had a significantly lower level of UV absorbing pigments than the *uvr8-1* mutants grown under the uv-a filter or the uv-0 filter, as well as all of the wild-type plants under all treatments (F(5,18)= 10.795, p≤0.0001) (Table 5.2).

In July, there was a significant filter effect on biomass (F (2, 16) =13.273, p≤ 0.0001) and rosette diameter (F (2,18) = 12.128, p≤0.0001) (Table 5.2). Post hoc Tukey tests showed that the biomass of uv-a/b and uv-a treated plants was significantly smaller than that of plants grown under the uv-0 filter (Table 5.2). The rosette diameters of the uv-a/b treated *uvr8-1* mutant and the wild-type Ler were also significantly smaller than those of plants grown under the uv-a and uv-0 filters (Table 5.2). There was no genotype effect observed in the morphological parameters. F_v/F_m values for *uvr8-1* grown under the uv-a/b filter were lower than those for all other treatments and the wild-type Ler (Table 5.2), these data were found to be non-normal. A non-parametric two-way ANOVA found significant differences between the

genotypes F (1,18)=12.578, p≤0.001 and also found significance between the filters F(2,18)=10.313, p≤0.001(Table 5.2). Further analysis of F_v/F_m data using a Kruskal-Wallis non-parametric post hoc analysis found that *uvr8-1* plants grown under the uv-a/b and uv-a filters were significantly different from those grown under the uv-0 filter as well as the wild-type Ler. In July, it was also found that *uvr8-1* had a lower content of UV absorbing pigments than Ler when grown under the uv-a/b filter, similar to the result found in January (Table 5.2).

Discussion

Understanding the ecological role of UVB perception through the UVR8 photoreceptor is currently in its infancy, and very few studies have analysed the functional role of UVR8 under outdoor conditions (Davey *et al*., 2012; Morales *et al*., 2012), and none have done so in a natural vegetation. Fully elucidating the complexities and range of UVR8 mediated responses is only recently underway. This study aimed to investigate the functional role of the UVB photoreceptor, UVR8, by using the *uvr8-1* mutant, under temperate climatic conditions. This climate is characterised by abundant rainfall, lack of temperature extremes and relatively low hours of direct sunshine (3 - 7 hours per day). Seasonality is an important factor when considering the timing of outdoor studies, especially when the aim is to investigate UV and the functionality of its photoreceptor, UVR8. By setting this study over the course of 12 months it was aimed to investigate the temporal nature and periodic fluctuation of the response to natural UVB.

The findings of this study show that (1) plant morphology is only affected by UV in May and July, for both Ler and the *uvr8-1* mutant; (2) there was no evidence of changes in phenolics in the wild-type Ler when grown under different UV-filters; (3) there was no evidence of UVB impaired photosynthesis in the wild-type Ler grown under natural sunlight conditions; (4) the *uvr8-1* mutant suffered impaired PSII activity during all months except for November when

grown with UV (5); the *uvr8-1* mutant had reduced levels of UV absorbing pigments during all months when grown with UV.

Environmental conditions other than UV have the strongest influence on the seasonal trends in morphology, total soluble phenolics and F_v/F_m values of the wild-type Ler in this study. This is likely due to prevailing climatic conditions, including low incidents of direct sunlight and therefore, relatively low UV.

Under outdoor conditions, UVB radiation did not cause measurable photosynthetic stress to the *Arabidopsis thaliana* Ler accession as illustrated by the consistent values for F_v/F_m measured on plants raised under uv-a/b (visible light + UVA and UVB), uv-a (visible light + UVA) and uv-0 (visible light) filters, and throughout the various seasons. Additionally, wild-type plants that were propagated under outdoor conditions were not sensitive to an artificially high dose of UVB radiation. Nevertheless, the Ler accession did exhibit the typical, more dwarfed phenotype when grown under the uv-a/b-filter in the summer months of May and July. Thus, the distinct UVB phenotype (Jansen, 2002; Robson *et al*., 2014) developed under outdoor conditions in the summer only. In May and July there was also some evidence of a dwarfing response under the uv-a filter suggesting that there is some activation of the pathways controlling the dwarfing mechanism by UVA. This is consistent with the findings of study by Morales *et al.* (2012) who studied the transcriptome and metabolite pathways of Arabidopsis plants in natural sunlight and noted similar effects of UVA and UVB, but this does contradict indoor UVA supplementation studies, which demonstrated that UVA increased elongation (Biswas and Jansen, 2012). The UVB mediated morphological response is not visible in autumn or winter months. It can be speculated that this is due to (1) UVB levels being too low to elicit the response (2) UV effects being masked by responses to other, unfavourable weather conditions or (3) plant growth being too slow for the dwarf morphology to become evident. A measureable UVB response was induced in the *uvr8-1* mutant (Figs. 5.2&5.3) despite the

fluence rates of UVB being relatively low making the first and second option unlikely. Rosette diameters of the wild-type Ler and *uvr8-1* mutant grew by an average of 28% during January and February across treatments indicating relative good growth, and making the third option also unlikely. It can, however, be argued that the UV-doses required to induce a morphological response are higher than those required to induce flavonoid accumulation, i.e. a decoupling of two key UV-responses, or alternatively that morphological responses are more readily masked by environmental factors than biochemical responses.

There is no clear evidence for a UVB-mediated increase in UV absorbing pigments in the wild-type Ler. Total phenolic contents are comparable under all three filters year round despite filter-dependant changes in leaf area, rosette diameter and biomass during the summer months. The change in total phenolic concentrations throughout the year appears to be primarily mediated by seasonal changes, and this seems to mask any additional UVB effect (Leyva *et al*., 1995; Bilger *et al*., 2007). Thus, although the UVB mediated accumulation of UV-absorbing pigments is one of the most widely reported UV-responses (Rozema *et al.,* 1997; Jansen *et al*., 1998) this effect is not necessarily clear under natural conditions. Similar conclusions have been reached in outdoors studies of lichens and mosses. These studies reported that seasonal variations in total soluble phenolics were larger than those between UV-treatments suggesting that the effects of UVB are minor and are concealed in the complex response triggered by natural environmental conditions (Gehrke, 1999; Bjerke *et al*., 2005). Yet, the lack of response in the total concentration of UV absorbing pigments does not mean that the composition of the pool of pigments has not been altered. Growth room studies using artificial UVB have shown that not only are the total phenolics increased with UVB exposure but the composition and ratios between the quercetins and kampferols, and their glycosides are also changed (Hectors *et al.,* 2014).

This study suggests that the greatest impact of UVB on Ler occurs during the summer months. Thus, it might be speculated that the functional role of UVR8 is temporal and only important when UVB levels are high. Yet, our results using the *uvr8-1* mutant show otherwise.

In this study, we find that the morphology of the *uvr8-1* mutant, like that of the wild-type Ler, was altered due to UV-exposure during May and July. The rosette diameter, biomass and leaf area of plants grown under the uv-a/b and uv-a filters were smaller than those grown without any UV (Fig. 5.1). The mutant plants lack the ability to perceive UVB through the UVR8 photoreceptor and thus, in theory, should lack the typical UVB mediated dwarfing response, which has been associated with this photoreceptor (Favory *et al*., 2009). Here we find that, the growth responses of the mutant plants match those of the wild-type (Fig. 5.1). Thus, based on the data presented in this chapter, it may be concluded that UVR8 plays no substantial role in the control of plant morphology. This conclusion contradicts a substantial body of evidence on the role of this photoreceptor in plant UV response (Favory *et al*., 2009; Jenkins, 2014; Robson *et al*., 2014). However, the similar morphological response of Ler and the *uvr8-1* mutant, plus their occurrence in the season with highest UV levels, suggest a stress mediated response (Jansen *et al*., 2012). Alternatively, it should be recognised that UVB may impact on plant morphology through various other (simultaneously operating) mechanisms that do not directly involve UVR8 (Jansen *et al*., 2012). There is also a possibility of crossover responses mediated by UVA as *uvr8-1* mutant plants and wild-type grown under uv-a filters both exhibit a more dwarfed morphology, suggesting a morphogenic-role for cryptochrome under natural light conditions (Morales *et al*., 2013, Jenkins, 2014). Thus, the observed changes in morphology in this study suggest that under field conditions UVR8 is potentially not the only driver in the development of a dwarf phenotype.

Seasonal patterns in the levels of accumulated total soluble phenolics were evident in both the wild-type Ler and *uvr8-1* mutant. In this study, we found that the *uvr8-1* mutant, when exposed

to UVB had significantly lower levels of total soluble phenolics than the wild-type (Fig. 5.3). These data can be interpreted as an indication of the role of the UVR8 photoreceptor. Several studies have shown that the lower levels of phenolics in UVR8 mutants grown in the presence of UVB are due to a lack of induction (Demkura & Ballare, 2012, Morales *et al*. 2012). In this instance however, it seems that there is a reduction in total UV absorbing capacity in the *uvr8-1* mutant grown under UVB. The levels of UV absorbing pigments measured in uv-a and uv-0 treated plants are higher than those seen in the uv-a/b treated *uvr8-1* mutants. A possible explanation for this is a self-perpetuating negative loop. The UV-B photoreceptor controls, amongst others photo-repair activities (Brown *et al.*, 2005), and anti-oxidant defences (Hideg *et al*., 2013), lack of which may impair gene transcription, and secondary metabolism, respectively. This, in turn may negatively affect synthesis of UVB specific flavonoids. As a consequence, of non-induction of biosynthesis of these specific flavonoids there is increased cellular damage. In turn, cellular damage may further impede flavonoid biosynthesis, or limit supply of photosynthetic carbon for phenolic synthesis, resulting in a further cellular damage (Koricheva *et al*., 1998; Lavola *et al*., 2000; Sumbele *et al*., 2012).

In support of this theory, we find that plants lacking functional UVR8 had lower F_v/F_m values throughout the year when grown under full sunlight, even when fluence rates of UVB are relatively low in January and February. The measured F_v/F_m values are significantly lower than values recorded for wild-type plants, which were grown under the same conditions (Fig. 5.2). Thus, the *uvr8-1* mutant is more susceptible to UVB damage due to the consistently lower levels of UVB absorbing pigments that were measured throughout all seasons. Similar conclusions on the importance of UV absorbing pigments for UV protection were drawn in various studies, Arabidopsis tt4 and tt5 flavonoid mutants were found to be significantly more sensitive to UVB than the wild-type (Li *et al*., 1993; Landry *et al*., 1995). An alternative explanation for lower F_v/F_m values is based on the role of UVR8 in controlling expression of

several chloroplast protein genes (Davey *et al.*, 2012). Although this phenomenon was attributed to high levels of UVB (Davey *et al*., 2012), evidence from this study may suggests that UVR8 is required not only to maintain photosynthetic efficiency during times of high fluence rates but also when UVB levels are relatively low under natural light conditions.

The *uvr8-1* plants suffered further when exposed to an artificially high dose of UVB in agreement with lab based studies (Kliebenstein *et al*., 2002). This indicates that even high levels of UV absorbing pigments, induced by natural light conditions, variations of temperature and other features of growth outdoors, have not afforded the *UVR8* mutants any cross tolerance to elevated UVB. On the other hand, the wild-type Ler is seemingly unaffected by the high dose of UVB. The idea of low level UV, perceived by UVR8, providing cross-tolerance to high UVB and other abiotic and biotic stresses is greatly discussed (Ballaré *et al*., 2011, Hideg *et al., 2*013). For example, Arabidopsis plants grown with UVB have been found to have a higher tolerance of drought stress (Poulson *et al*., 2006). It has also been found that UVB exposed Arabidopsis showed resistance to the necrotrophic fungal pathogen *Botrytis cinerea*, an effect which was reduced in UVR8 impaired plants (Demkura & Ballaré, 2012). Conversely, we have found strong accumulation of total phenolics in a *uvr8-1* mutant, especially in the winter months of January and February. Yet, winter grown *uvr8-1* plants still display relatively low F_v/F_m values when grown under natural UVB, or when treated with a high dose of UVB radiation. This shows that cold induced phenolics are specific in their functionality, and do not protect against either natural or artificial UVB.

In this study, we show that the UVR8 photoreceptor plays an adaptive role throughout the year even when UVB levels are relatively low. Impaired PSII function and a reduction in UV screening pigments in the *uvr8-1* mutant lead us to conclude that a functional UVR8 pathway is necessary for optimized plant growth year round under natural light conditions.

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Chapter 6

The effects of UV radiation on the bronze lettuce *Lactuca sativa* L. (cv Cos 'Dixter') and its potential as a tool for precision manipulation of crop quality.

Abstract

UV wavelengths are a natural part of the solar spectrum and are associated with the accumulation of various plant polyphenols such as flavonoids and anthocyanins, as well as changes in plant architecture. Studies have shown strong association between the consumption of plant polyphenols and a milieu of human-health benefits including protective functions against chronic diseases and obesity. The primary aim of this study was to investigate the effects of natural UV radiation on the bronze lettuce Cos 'Dixter' with a focus on potential increases in the nutritional and monetary value of the end-product. It was found that it is possible to utilise ambient UV radiation in Ireland to increase secondary metabolites in the bronze lettuce Cos. More specifically UVA radiation increased anthocyanin concentration after just 72 hours, it was also found that UVA treated plants retained higher levels of anthocyanin following the removal of the plants from the outdoor growing conditions to a UV-free environment. These findings suggest a short pre-harvest treatment with natural UVA radiation can improve quality and nutritional composition of salad crops grown in Ireland.

Introduction

UVB wavelengths are a natural part of the solar spectrum, and are defined as ranging from 280 to 315 nm. As a short, high-energy wavelength, UVB radiation can induce a range of molecular, biochemical, morphological and developmental responses in plants and its effects have become the focus of much research (Agati & Tattini, 2010; Heijde & Ulm, 2012; Robson *et al.*, 2014). Thinning of the ozone layer followed by the discovery of a hole in the UV-screening ozone layer over the Antarctic led scientists to consider the possible consequences of increased UVB at ground level (Rozema *et al.*, 1997; Kakani *et al.*, 2003). Damage caused by UVB to living organisms can be significant, as vital proteins and nucleic acids absorb in the UV spectrum (Jansen *et al*., 1998; Hollósy, 2002). Strict regulation of ozone depleting emissions, laid out in the Montreal Protocol (1987), have helped to stabilize the ozone layer (Ravishankara *et al.*, 2009; McKenzie *et al.*, 2014). However, this does not imply that biological effects of UVB radiation are now considered irrelevant. On the contrary, there is an increasing realisation that even relatively low natural levels can have a significant impact on plant growth. The discovery of the UVB photoreceptor UVR8 has demonstrated that low intensities of UVB radiation are perceived by the plant, and can induce specific changes in gene-expression and physiology, in the absence of cellular damage (Brown *et al.*, 2005). Conversely, high levels of UV-B may cause plant stress and inhibition of growth due to impairment of cellular processes (Jansen *et al.*, 1998).

Low, natural doses of UVB radiation can influence the accumulation of various plant secondary metabolites, as well as change plant architecture (Hectors *et al.*, 2014; Robson *et al*., 2014). Changes in the composition and concentration of a range of plant secondary metabolites or plant polyphenols, many with a protective role, are a well-documented UVB response (Ryan *et al.*, 1998; Kolb *et al.*, 2001; Agati & Tattini, 2010; Hectors *et al.*, 2014). Increasingly people are being encouraged to eat a diet rich in plant polyphenols, these include flavonoids and

anthocyanins that are present in fruit and vegetables. Studies have shown strong associations between the importance of a diet high in plant polyphenols and human health (Vinson *et al.,* 2001, Bertoia *et al.,* 2016). The health benefits of such a diet include protective functions against a range of chronic diseases (Vinson *et al.,* 2001). A recent long-term dietary study has shown that a diet enriched in flavonoids and anthocyanin may help with weight control an important finding in light of the recent obesity epidemic (Bertoia *et al.,* 2016).

Flavonoids are a class of the most common plant polyphenols. The accumulation of specific flavonoids is strongly stimulated by exposure to UV light (Ryan et al. 1998; Hectors et al. 2014; Kolb et al. 2001). In plants, flavonoids have been associated with UV-screening, but especially antioxidant defences (Agati & Tattini 2010). These compounds have been associated with a range of potential health benefits for human consumers. For example, quercetin and kaempferol, the accumulation of both of which is stimulated by UV, have antibacterial, antiviral and anti-inflammatory properties (Dillard & Bruce German, 2000). Another group of plant polyphenols are anthocyanins. Anthocyanins are natural pigments that are responsible for the colouration of many plant species. They are also known as a bioactive component of food, helping to scavenge free radicals and potentially act as a protective element against the development of cancer, cardiovascular disease and other chronic ailments in human consumers (de Pascual-Teresa & Sanchez-Ballesta 2008). Concentrations of anthocyanins can also affect the sensorial characteristics of food crops. The colour of food can strongly influence its acceptability to a consumer as it is often the first trait that registers (Ryan *et al.,* 1998; Spence 2015). Enhancing the concentrations of both flavonoids and anthocyanins using UVB may increase both the attractiveness of the crop and its nutritional value for human consumers.

In parallel with changes in the levels of plant metabolites, plant morphology across a range of species is also altered by UVB radiation. The UVB phenotype is primarily characterised by shorter, thicker leaves, shorter petioles, leaf curling, inhibited development of the hypocotyl

and stem and changes in the root/shoot ratio (Hollósy 2002; Jansen 2002; Wargent *et al*., 2009 (a); Wargent *et al*,. 2009 (b); Hectors *et al.*, 2012;). The functional role of these UV-induced morphological changes has been suggested to UV-avoidance (i.e. self-shading), but conclusive evidence remains lacking (Robson *et al*., 2014). Morphological changes constitute a redistribution of growth, and therefore do not lead to a decrease of plant biomass per sé. Thus, some of the UV-induced changes in plant morphology may be commercially desirable. For example, a more compact and robust plant may better tolerate harvest, packaging and transportation, ultimately yielding more harvestable, commercially valuable biomass. Yet, alterations in morphology such as reduced leaf area, or reduced shoot-mass may ultimately lead to decreased biomass accumulation in some field crops such as pea, oats, rice and beans (Kakani *et al.* 2003, Robson *et al*., 2014). Similarly, stress caused by relatively high UV-doses may also decrease biomass accumulation. Such reductions in vegetative growth will not be a desirable outcome in the context of commercial cropping. Thus, although UVB has potential for use as a low input precision tool within the Irish horticulture industry, UV-exposure conditions need to be carefully calibrated to generate the advantages, without the disadvantages. Further investigation are needed to identify the exact, presumably crop-specific, conditions required to allow morphological and metabolic manipulation without any negative impacts on biomass.

The horticultural industry makes extensive use of various structures for the protected growing of crops. Protected environments provide the opportunity for manipulation of plant responses using both ambient or artificial environmental stimuli. In turn, this enables growers to produce a tailored crop with a potentially higher value (Wargent & Jordan, 2011). Most traditional polytunnel and greenhouse covers exclude all, or most, of the UVB and a portion of the UVA as well (Krizek, 2004). The advent of new materials such as specific wavelength transmitting plastics and new technologies such as LED lighting systems have made manipulation of the

crop light environment a viable option (Paul *et al.,* 2005). Developments in this area have the potential for use in large-scale commercial cropping systems but can also be utilised in low-tech small-scale crop production.

In Ireland, crops grown in protected environments contributed a value of 85.3m Euro to the Irish Horticulture Sector in 2014 (DAFM, 2014). In Ireland, lettuce is a major protected crop species. In 2011, lettuce alone was grown on 113.4 hectares at a value of 7.9m Euro (DAFM 2015). The impact of natural levels of UV on crop plants has not yet been assessed in the context of the Irish climate. Understanding in more detail the actual impact of UV in Ireland is important to be able to exploit the full potential for precise and tailored plant manipulation, producing nutritionally enhanced and physically robust crops without the need for supplemental UV. The hypothesis underpinning this is that ambient UVB has potential to enhance the nutritional composition of a commercial crop without reducing biomass. To this end the effects of UVB radiation on biomass and secondary metabolites of the bronze lettuce Cos 'Dixter' were investigated. To achieve this a series of experiments were undertaken utilising a UV filtration system to manipulate ambient UV radiation levels.

Materials and Methods

Cos 'Dixter' lettuce seeds (*Lactuca sativa* L.) were sown into plug trays and kept in the greenhouse until, they had two true leaves and the roots were well established. Seedlings were then transplanted into individual 9cm diameter pots using Bord na Mona potting compost mix (N:P:K ratio,1:3:1). The seedlings were returned to the greenhouse until they had 4 true leaves when they were considered ready for use.

UV-exposure Conditions

A UV-filtration approach was used for this experiment. Plants were grown outdoors, in frames covered by UV blocking or UV-transmitting filters. A total of three treatments were used; (1)

a UV transparent cellulose acetate filter referred to as uv-a/b (visible + UVA and UVB transmitted) (95µm thickness; Kunststoff-Folien-Vertrieb GmbH, Hamburg, Germany); (2) a UV-B blocking 'mylar' filter, referred to as uv-a (visible + UVA) (125µm thickness, Polyester film, Tocana Ltd., Ballymount, Dublin, Ireland) and (3) a UV opaque filter referred to as uv-0 (visible), (polytunnel plastic, BPI Visqueen, Stevenston, U.K.). The cellulose acetate and Mylar were changed after each 20 days of exposure to solar light to prevent the changing of the transmission spectrum caused by degradation of the plastic. The transmission of the filters was measured using a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A) (Chapter 4, Fig. 4.2b).

Frames measuring 50 cm x 50 cm were constructed using opaque corriboard. These frames supported the filters that were suspended above the plants (Chapter 4, Fig 4.3). The sides of the frames were closed. There were four replicate frames for each treatment. The frames were randomly set out at a non-shaded south facing site in Cork, South West Ireland (51^{o}53'58"N 8^{o} 29'14"W). The frames were tilted slightly to allow for air circulation with the northern edge of the frame raised off the ground (Chapter 4, Fig 4.3). For each experiment, four individual lettuce plants were place under each frame. The plants were watered daily as needed.

Climate

All experiments took place between 01/05/2015 and 31/07/2015. During this time period the temperature range was between 8.7 ^{0}C and 15.8 ^{0}C with an average of 12.3 ^{0}C. The average number of hours of sunshine was 5.3 per day with a min of 0 and a max of 14.8 hours per day. Meteological data were obtained from Met Eireann, Cork Airport Weather station which is located 5.8km from the field site.

Experiment 1: Impact of UV under Irish growing conditions at three times points over 21 days.

This experiment assessed the impact of UV over of 21 days on plant growth and the accumulation of secondary metabolites. Lettuce plants were grown from seed to the four true leaf stage in a greenhouse before being considered ready for use. They were then placed outdoors under frames supporting UV filters, of which there were three; uv-a/b, uv-a and uv-0. There were four independent replicates of each treatment. Four sunlight exposure time-points were taken, T0 (at the beginning), T1 (7 days exposure), T2 (14 days exposure), T3 (21 days exposure). At each time point, a plant was selected at random from the replicate plants within each treatment. Firstly, biomass was taken from the lettuce rosette leaves. The rosettes were then photographed and leaf discs were taken for biochemical analysis. At each time point, the largest, most fully expanded leaf and the youngest leaf of not less than 4 cm in length were selected. Leaf discs were taken from the tip of the largest leaf, the base of the largest leaf and from the youngest leaf (of not less than 4cm in length); these sections represent a developmental and an exposure gradient.

Experiment 2: Short-term UV exposure

This experiment was designed to test how rapidly secondary metabolites accumulated in response to UV. Lettuce plants were prepared and placed under the UV filters as per Experiment 1. There were four independent replicates of each treatment. Three sunlight exposure time-points were selected, 24 hours, 48 hours and 72 hours. At each time-point, leaf discs were taken for biochemical analysis as detailed in Experiment 1.

Experiment 3: Persistence of secondary metabolites

This assay was undertaken to assess the persistence of UV induced secondary metabolites after the removal of the UV stimulus. Lettuce plants were prepared and placed under the UV filters

as per Experiment 1. There were four independent replicates of each treatment. Three time-points were selected, T7O (7 days growth outdoors under uv-a/b, uv-a or uv-0 filters), T24G (7 days growth outdoors under uv-a/b, uv-a or uv-0 filters, followed by 24 hours under photosynthetic active radiation (PAR) only in a growth room) and T96G (7 days growth outdoors under uv-a/b, uv-a or uv-0 filters, followed by 96 hours under PAR only in a growth room). At each time-point, leaf discs were taken for biochemical analysis as detailed in Experiment 1.

Biochemical Analysis

Total soluble phenolics as well as anthocyanins were extracted from leaf discs, as detailed for each experiment (Biswas & Jansen, 2012). The leaf discs were placed in micro-tubes with 1ml acidified methanol (1% HCL, 20% H_2O, and 79% CH_3OH) and incubated in the dark at 4 ^0C for four days. Peaks were identified at 330nm for total flavonoids and 530nm for anthocyanins using a spectrophotometer (Shimadzu – UV visible spectrophotometer- 160A).

Growth Analysis

Above ground biomass was measured using a Scout Pro SPU402, Ohaus balance. Biomass from 5 plants was taken at the beginning of experiment 1. Biomass was also taken from one plant from each replicate of all treatments at each time point in Experiment 1. Photographs were also taken for visual comparison of the plants

Statistical analysis

All analysis was carried out using IBM SPSS Statistics 21. Prior to any analysis, all data sets were assessed for normality. In the case of non-normal data transformation was applied. Data were analysed statistically using parametric interaction ANOVAs with multiple comparison tests being carried out using Tukey's Range Test. All means and standard deviations are from back-transformed data. Standard deviations are calculated from the mean of four independent

replicates. Each filter frame was considered an independent replicate, there were three filters (uv-a/b, uv-a and uv-0) and 4 replicates frames of each treatment.

Results

Experiment 1: Impact of UV under Irish growing conditions at three time points over 21 days.

This study was undertaken to determine the impact of 7 (T1), 14 (T2) or 21 (T3) day exposure of the bronze lettuce Cos 'Dixter' to UV under Irish weather conditions. Plants were grown under either (partially) UV transmitting filters (uv-a/b or uv-a) or a UV blocking filter (uv-0).

Table 6.1 Summary of two-way ANOVAs on the effects of time and filter type on biomass (g), total soluble phenolics (A330nm) in the leaf tip, base and youngest leaf, and total anthocyanins (A530nm) content in the leaf tip, base and youngest leaf form plants grown outdoors under uv-a/b, uv-a and uv-0 filters for 7 (T1). 14(T2) and 21 (T3) days.

Main Effects		Biomas (g)	Flavonoid Leaf Tip	Flavonoid Leaf base	Flavonoid Young leaf	Anthocyanin Leaf Tip	Anthocyanin Leaf Base	Anthocyanin Young leaf
Time	T0	0.56 a	1.11b	0.77a	0.69 a	0.14 b	0.09 b	0.05 b
	T1	1.65 b	0.90a	0.65 a	0.51 a	0.05 a	0.17 a	0.01 a
	T2	8.78 c	1.32 b	1.14 b	0.48 a	0.20 b	0.22 c	0.05 b
	T3	13.96 d	2.92 c	2.24 c	1.62 b	0.42 c	0.09 d	0.14 c
Filter	T0T0	0.56 a	1.11 a	0.77 a	0.69 a	0.14 ab	0.09 ab	0.05 a
	uv-a/b	5.27 b	1.45 ab	1.16 b	0.88 a	0.22 b	0.03 bc	0.08 a
	ıv-a	8.82 c	1.90 c	1.39 b	0.90 a	0.22 c	0.18 c	0.08 a
	ι v-0	10.29 c	1.78 bc	1.33 b	0.83 a	0.13 a	0.27 a	0.04 a
	df					ANOVA		
F value Time	2	264.4	160	133.9	44.9	136.6	101.9	113.6
Sig		***	***	***	***	***	***	***
F value Filter	2	27.8	14.3	5.9	0.3	26.4	16.4	1.5
Sig		***	***	**	n.s.	***	***	n.s.
Time x Filter	4	0.9	1	0.6	1.7	0.5	1.3	1.1
Sig		n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

ns= not significant, * = $p \leq 0.05$, **=$p \leq 0.001$, *** = $p \leq 0.0001$, according to two-way ANOVA. Comparisons to be made within columns Means in the same column and same main effect with the same letter are not significantly different, $p>0.05$ according to Tukey's Range Test

Anthocyanin concentrations across the lettuce rosette and treatments increased over time (Fig. 6.1). Particularly in leaf tips anthocyanin concentrations increased by up to 66% over the

duration of the 21-day growth period. The effect of time on anthocyanin concentration in leaf tips ($F(2,41) = 136.678$. $P \leq 0.0001$), leaf base ($F(2,41) = 101.95$, $p \leq 0.0001$) and youngest leaves ($F(2,41) = 113.61$, $p \leq 0.0001$) was statistically significant for all three leaf sections (Table 6.1).

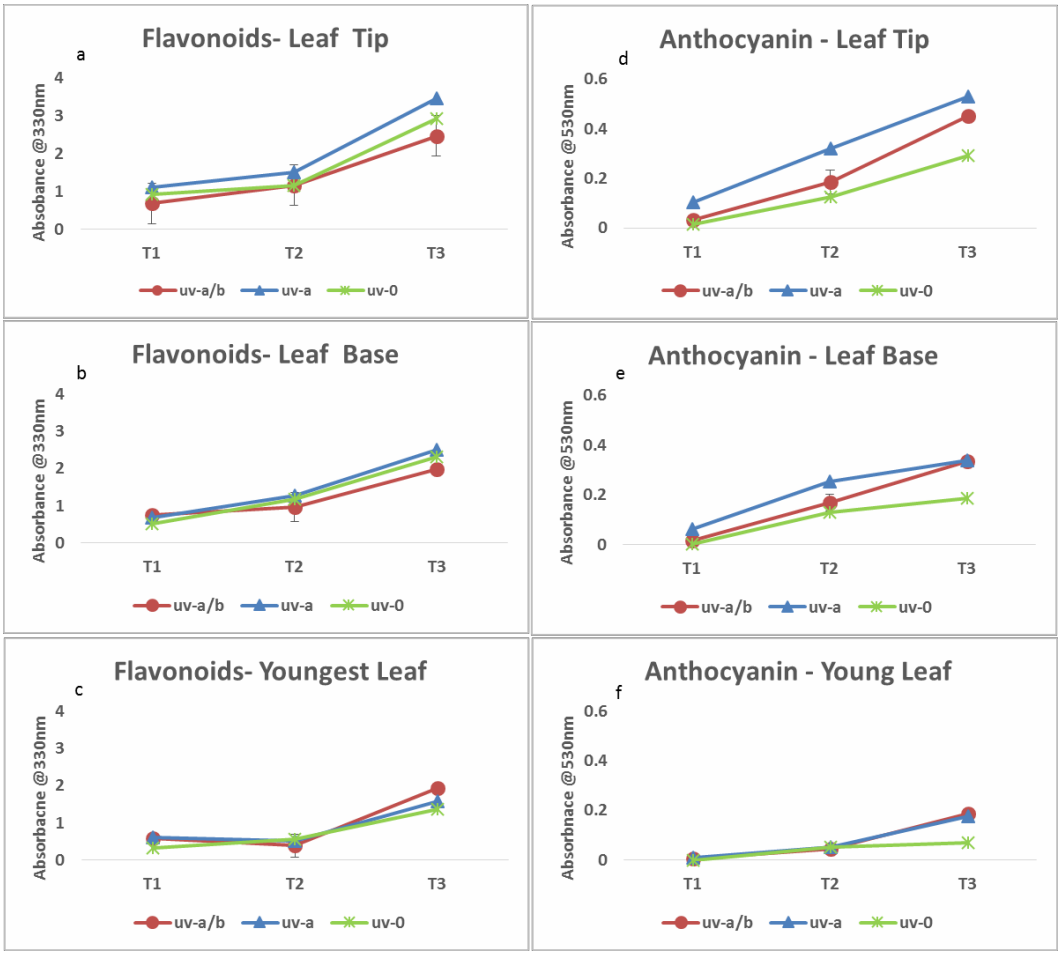

Figure 6.1 Flavonoids, panels a-c and anthocyanins, panels d-f extracted with 1ml acidified methanol from leaf discs taken from the leaf tip of the most mature leaf a+d the base of the most mature leaf b+e and the youngest leaf c+f. After growth outdoors for 7 days (T1), 14 days (T2) and 21 days (T3). Error bars represent the standard error from the mean of 4 replicates. Filter specifications: uv-a/b (visible +UVA and UVB), uv-a (visible +UVA) and uv-0 (visible). T0 readings for total soluble phenolics A330nm,,leaf tip = 1.1165 (se 0.08), leaf base = 0.7739 (se 0.37), youngest leaf= 0.6928 (se 0.20). T0 readings for anthocyanins A530nm; leaf tip = 0.1451 (se 0.04), leaf base= 0.09 (se 0.04) and youngest leaf = 0.06 (se 0.02).

When anthocyanins were measured in the leaf tip, all three filter treatments resulted in significantly different anthocyanin concentrations from each other. The highest concentration of anthocyanins was found in the plants grown under the uv-a filter ($F(2, 41) = 26.482$, $p \leq 0.0001$). Anthocyanin concentration in the leaf base differed between treatments the highest concentration was found in the plants grown under the uv-a and the uv-a/b filter and the lowest

was found in plants grown under the uv-0 and plants tested at the beginning of the experiment. Though the actual difference was small it was significant ($F(2,41)= 16.457$, $p\leq 0.0001$) (Table 6.1 & Fig 6.1). In the youngest leaves there was no difference between the filter treatments (Table 6.1 &Fig. 6.1).

Analysis of the extracted UV-absorbing compounds revealed that after 21 days (T3) growth outdoors the total concentration of flavonoids across treatments was between 57 and 65% higher than in T0 plants (Table 6.1 & Fig 6.2). There was a statistically significant increase in UV-absorbing pigments over time in the leaf tip ($F(2,41)= 159.998$, $p\leq 0.0001$), base ($F(2,41)=133.898$, $p\leq 0.0001$) and the youngest leaves ($F(2, 41)= 44.898$, $p\leq 0.0001$) (Table 6.1 & Fig. 6.2).

Figure 6.2 Experiment 1, after 14 days growth outdoors, it was noted that plants grown with UVA &B radiation had longer, thinner leaf blades in comparison to those grown under just UVA and visible and visible light only. This could be an indicator of UVB induced morphogenesis, although further parameters, which would provide evidence for this, were not measured in this instance.

Overall filter effects on flavonoids were modest, plants grown under the uv-a filter had up to 24% more flavonoids than those grown under the uv-a/b filter but this

difference between the filters was only found to be significant in the leaf tip. In the leaf base the filter effect on flavonoids in the leaf base was also significant, but the actual difference was found to be between plants grown in the greenhouse (T0) and those grown under the filters.

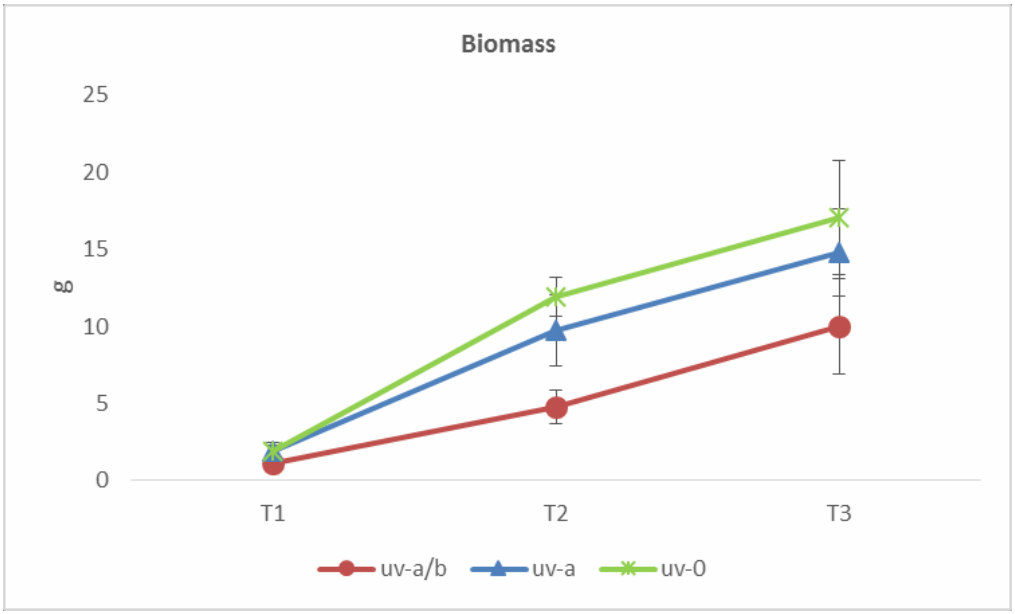

Figure 6.3 Biomass (fw) (g) of the whole rosette of Cos 'Dixter' grown outdoors under filters for T1 (7days), T2(14 days) and T3 (21 days). Starting biomass (0.56g). Error bars represent the standard error from the mean of 4 replicates. Filter specifications: uv-a/b (visible +UVA and UVB), uv-a (visible +UVA) and uv-0 (visible)

Biomass increased with time for all filter treatments (Figure 6.3). While plants weighed on average less than 1g at the start of the experiment, after 3 weeks weight ranged up to 17g per plant. The effect of time was statistically significant ($F(2, 41)=264.358$, $p\leq0.0001$) and across all filter treatments there was an increase in weight over the duration of the experiment. There was also a significant difference in the weight of plants exposed to different filter treatments ($F(2,41)= 27.792$, $p\leq0.0001$). (Table 6.1& Fig. 6.3). The biomass of the plants under the uv-a or uv-0 filters reached in both cases around 15g after 21 days of growth. There was no

significant difference between these two filter treatments in terms of accumulated biomass. However, lettuce plants raised under the uv-a/b filter accumulated 50% less biomass than that of plants grown under uv-0 filters (Table 6.1 & Fig. 6.3), and this effect was statistically significant ($p \leq 0.0001$).

Experiment 2: Short-term exposure to UV.

The purpose of this experiment was to determine how rapidly UV-absorbing pigments and anthocyanins accumulated in lettuce plants grown under each of the different UV-transmitting filters. Lettuce plants were placed under uv-a/b, uv-a and uv-0 filters for 24, 48 and 72 hours.

Anthocyanin content increased over time in all leaf sections when plants were kept under the uv-a/b filter or the uv-a filter (Fig. 6.4). Under both filters, levels increased by up to 60% after 72 hours UV exposure.

Table 6.2 Summary of two-way ANOVAs on the effects of time and filter type on Flavonoids(A330nm) in the leaf tip, base and youngest leaf, and total anthocyanins (A530nm) content in the leaf tip, base and youngest leaf form plants grown outdoors under uv-a/b, uv-a and uv-0 filters for 24 Hrs, 48 Hrs and 72 Hrs

Main effects		Flavonoid leaf tip	Flavonoid leaf base	Flavonoid young leaf	Anthocyanin leaf tip	Anthocyanin leaf base	Anthocyanin young leaf
Time	24 Hrs	0.73	0.75 a	0.74 a	0.08	0.08 a	0.07 a
	48 Hrs	0.90	0.82 a	0.61 a	0.16	0.13 b	0.07 a
	72 Hrs	0.97	0.94 a	0.73 a	0.19	0.14 b	0.09 a
Filter	uv-a/b	0.91	0.85 a	0.79 b	0.18	0.14 b	0.13 c
	uv-a	0.97	0.93 a	0.74 b	0.17	0.14 b	0.07 b
	uv-0	0.72	0.73 a	0.55 a	0.08	0.07 a	0.03 a
	df	ANOVA					
F Value Time	2	4.488	2.283	2.127	26.264	6.3	2.415
Sig		*	n.s	n.s	***	**	n.s.
F Value Filter	2	4.995	2.881	6.782	20.951	12.167	19.151
Sig		*	n.s.	**	***	***	***
Time x Filter	4	3.182	0.65	1.797	7.557	1.836	1.847
Sig		*	n.s	n.s	***	n.s.	n.s.

ns= not significant, * = $p \leq 0.05$, **=$p \leq 0.001$, *** = $p \leq 0.0001$, according to two-way ANOVA. Comparisons to be made within columns Means in the same column and same main effect with the same letter are not significantly different, $p > 0.05$ according to Tukey's Range Tests

No substantial increases in anthocyanin content were observed in plants kept under the uv-0 filter. Analysis of the anthocyanin content of the leaf tip found that there were significant main effects for time, filter as well as an interaction effect. One-way ANOVA was used to discover the source of the interaction effect. In the leaf tip there were no statistically significant differences in anthocyanin concentration after 24 Hrs ($p= 0.927$) but there were after 48 Hrs($p= 0.007$) and 72 Hrs ($p\leq 0.001$) (Table 6.2). Post hoc testing found that at 48 Hrs plants grown under the uv-a/b filter had higher anthocyanin levels than those kept under the uv-0 filter. Similarly, after 72 Hrs plants under both the uv-a and uv-a/b filters had higher anthocyanin levels than those under the uv-0 filter. Analysis of the anthocyanin content in the leaf base samples found significant main effects for both time ($F (2,36)=6.3$, $p=0.006$) and filter ($F(2,36)=12.167$, $p\leq0.0001$). The leaf base of plants kept under uv-a and uv-a/b filters for 48 and 72 hours contained higher levels of anthocyanin than equivalent leaf bases of plants grown under uv-0 filters (Table 6.2). Anthocyanin concentration in the youngest leaves were not significantly different over time ($P=0.108$). They were however, significant differences between the filters ($p\leq0.0001$), all three filter treatments were significantly different from each other uv-a/b had the highest and uv-0 had the lowest anthocyanin content (Table 6.2).

There were increases in flavonoids over the duration of the experiment. However, effects were relatively modest compared to the more substantial increases in anthocyanins observed in the same plants. Nevertheless, there were significant changes in flavonoids over time and between treatments (Fig. 6.4 & Table 6.2). In the leaf tip, both time and filter had significant effects on flavonoid levels, additionally there was also a significant interaction effect $F (4, 36)= 3.182$, $p= 0.029$ (Table 6.2). Further investigation using a one-way ANOVA found that after 48 Hrs ($p\leq0.001$), plants grown under the uv-a/b and uv-a filters had higher flavonoid content those grown under the uv-0 filter.

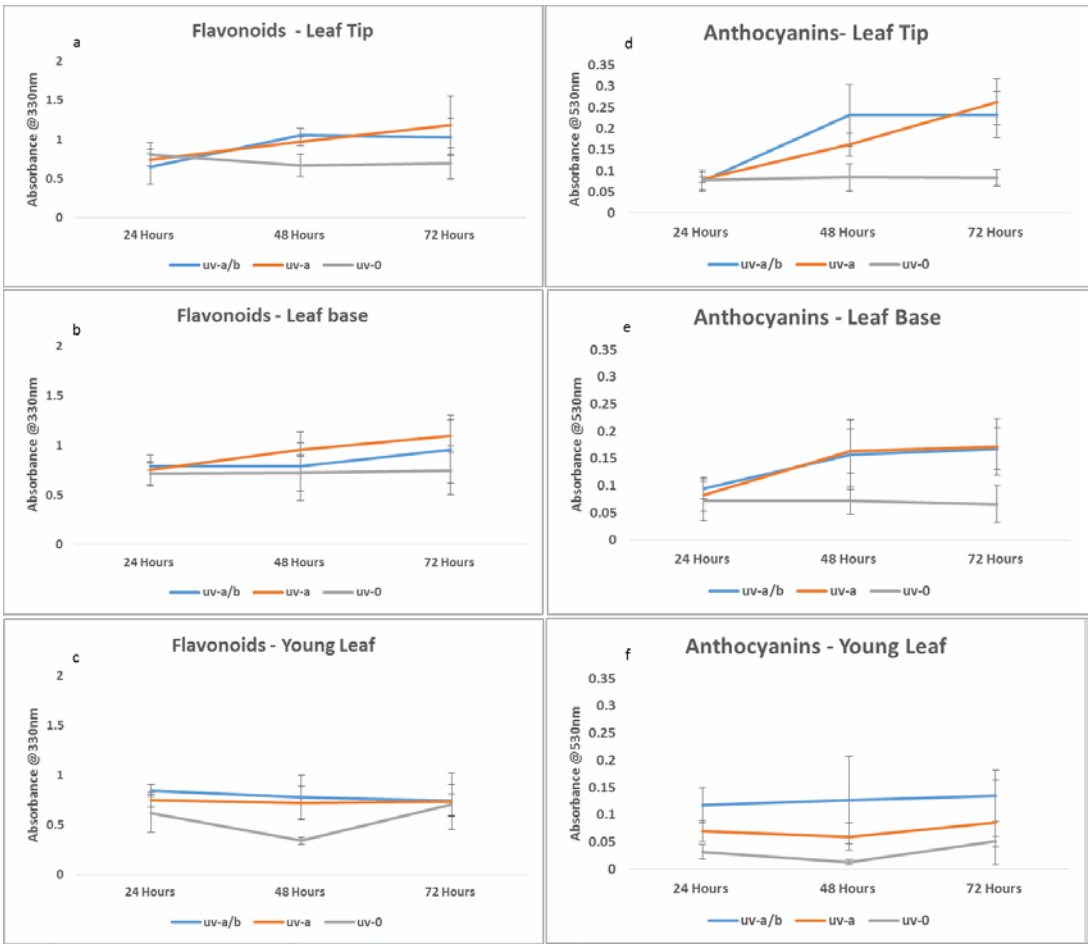

Figure 6.4 Flavonoids, panels a-c and anthocyanins, panels d-f extracted with 1ml acidified methanol from leaf discs taken from the leaf tip of the most mature leaf (a+d) the base of the most mature leaf (b+e) and the youngest leaf (c+f). After growth outdoors for 24 hours, 48 hours and 72 hours. Error bars represent the standard error from the mean of 4 replicates. Filter specifications: uv-a/b (visible +UVA and UVB), uv-a (visible +UVA) and uv-0 (visible)

There was no significant difference between treatments after 24 Hrs ($p=0.408$) or 72 Hrs ($p=0.09$) (Table 6.2). There were also significant changes in the flavonoid levels of the youngest leaf, in this case only the filter main effect is statistically significant $F(2, 36)= 6.782$, $p= 0.004$). Post-hoc Tukey test found that the youngest leaf of plants grown under the uv-a/b and uv-a filters have higher levels of flavonoids than the youngest leaves of plants grown under the uv-0 filter. There was no statistically significant difference found at any of the time points for the leaf base samples.

Experiment 3: Persistence of the secondary metabolites.

This study looked at the persistence of the UV induced anthocyanin and flavonoids in growing plants after the removal of the UV stimulus. Plants were grown outdoors under filters for seven days and then moved to a PAR only growth room for either 24 or 96 hours. Leaf discs were taken from four plants from each treatment at T7O (7 days outdoor under uv- filters), T24G (7 days outdoor under uv filters followed by 24 hours in the growth room under PAR only lights) and T96G (7 days outdoor under uv- filters followed by 96 hours in the growth room under PAR only lights). In the case of T96G the youngest leaf tested would have formed after the removal of the plants to a UV free growth room.

Figure 6.6 Experiment 3 plants grown outdoors under uv-a/b, uv-a and uv-0 for 7 day followed by 96 (T96G). hours in a PAR only growth room

Anthocyanin concentrations decreased across the rosettes after plants were moved to a PAR only growth room. Following 96 hours in the growth room, anthocyanin concentrations were reduced by 58% in the leaf tip up to 90% in youngest leaves (Fig. 6). In the leaf tip, leaf base and the youngest leaf under each treatment anthocyanin concentrations decrease significantly over time time, leaf tip $F(2, 36)= 59.158$, $p\leq0.0001$, leaf base $F(2, 36) = 24.685$, $p\leq0.0001$, and youngest leaf $F(2, 36) = 46.916$, $p\leq0.0001$ (Table 6.3 & Fig 6.5). Post-hoc analysis found that leaf base anthocyanin levels were significantly higher at T7O than at the other 2 time-points,

but there was no significant difference in anthocyanin levels when the T24G and T96G time points were compared. In the youngest leaf, anthocyanin levels across all 3 time-points were significantly different, with T7O having the highest levels and T96G having the lowest anthocyanin content.

Table 6.3 Summary of two-way ANOVAs on the effects of time and filter type on Flavonoids (A330nm) in the leaf tip, base and youngest leaf, and total anthocyanins (A530nm) content in the leaf tip, base and youngest leaf from plants grown outdoors for 7 days under uv-a/b, uv-a and uv-0 filters and then moved to a PAR only growth room for for 24 Hrs and 96 Hrs.

Main effects		Flavonoid leaf tip	Flavonoid leaf base	Flavonoid young leaf	Anthocyanin leaf tip	Anthocyanin leaf base	Anthocyanins young leaf
Time	T7O	1.26 c	1.2c	0.69c	0.26	0.20 b	0.05c
	T24G	0.98 b	0.86b	0.44b	0.16	0.11 a	0.02 b
	T96G	0.77 a	0.67a	0.11a	0.11	0.07a	0.01a
Filter	uv-a/b	0.99a	0.88a	0.45a	0.19	0.14 b	0.03 a
	uv-a	1.07a	0.95 a	0.32a	0.20	0.15 ab	0.02 a
	uv-0	0.96a	0.92 a	0.47 a	0.13	0.10 a	0.03 a

	df	ANOVA					
F Value Time	2	54.271	42.763	70.72	59.158	24.685	46.916
Sig		***	***	***	***	***	***
F Value Filter	2	2.612	0.685	1.971	15.237	5.089	1.32
Sig		n.s	n.s.	n.s.	***	*	n.s
Time x Filter	4	2.616	0.647	2.081	4.296	2.002	2.105
Sig		n.s	n.s	n.s.	***	n.s.	n.s

ns= not significant, * = p ≤0.05, **=p≤0.001, *** = p≤ 0.0001, according to two-way ANOVA. Comparisons to be made within columns Means in the same column and same main effect with the same letter are not significantly different, p>0.05 according to Tukey's Range Tests

In the leaf base there were significant differences between the filters ($F(2, 36) = 5.089$, $p=0.05$), it was found that uv-a treated plants had significantly higher levels of anthocyanin than uv-0 but not uv-a/b treated plants however this effect was small (Table 6.3). There was also a significant difference between the filters and an interaction effect in the leaf tip ($F(4, 36) = 4.296$, $p=0.001$) (Table 6.3). One-way ANOVA to investigate the interaction found that pre-treatment had a significant effect at T24G ($p= 0.006$) plants grown under the uv-a/b and uv-a filters had up to 46% higher levels of anthocyanin than plants grown under the uv-0 filters. At T96G ($p= 0.001$) plants grown under the uv-a filter still had anthocyanin levels 46% higher than plants grown under the uv-0 filter but now also have anthocyanin levels 47% higher than those grown under the uv-a/b filter.

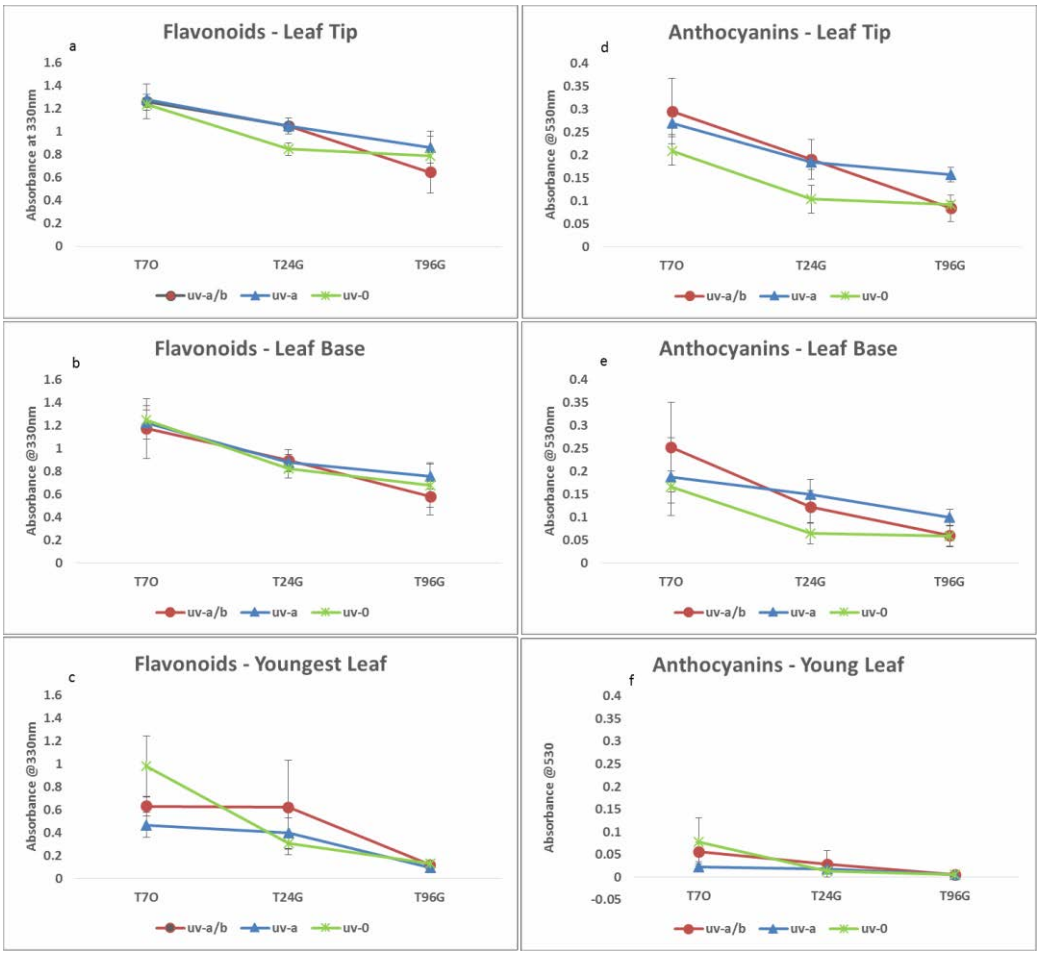

Figure 6.5 Flavonoids, panels a-c and anthocyanins, panels d-f extracted with 1ml acidified methanol from leaf discs taken from the leaf tip of the most mature leaf a+d the base of the most mature leaf b+e and the youngest leaf c+f. After growth outdoors from plants grown outdoors for 7 days under uv-a/b, uv-a and uv-0 filters and then moved to a PAR only growth room for for 24Hrs and 96Hrs Error bars represent the standard error from the mean of 4 replicates. Filter specifications: uv-a/b (visible +UVA and UVB), uv-a (visible +UVA) and uv-0 (visible)

Over time flavonoid concentrations decreased in the leaf tip, base and youngest leaves by up 40, 47 and 84% respectively over the 96 hours that plants were kept in the UV free growth room (Fig. 6.5). The decreases in flavonoid content over time were significant in all leaf sections (leaf tip $F(2, 27) = 54.271$, $p \leq 0.0001$, leaf base $F(2, 27) = 42.763$, $p \leq 0.0001$, youngest leaf $F(2, 27) = 70.72$, $p \leq 0.0001$) (Table 6.3).The highest levels of flavonoids were found in the T7O plants and the lowest in the T96G (Table 6.3 &Fig. 6.5).

There were no significant differences between flavonoid concentrations in plants that were pre-treated with different filter treatments. This applied across all leaf sections (leaf tip ($F(2, 27)$

= 0.092, p= 0.092), leaf base (F(2,27)= 0.685, p=0.513) and youngest leaf (F(2,27)=1.971, p=0.159).

Discussion

Increased environmental awareness and a focus on health are influencing and changing consumer preferences, consequently this is leading too developments and innovations in food production and marketing (Schreiner *et al.*, 2013). Furthermore, socio-economic changes in western society mean people have less time available for food preparation so they looking for good quality, nutrient dense food stuffs that are quick to prepare but are also produced using sustainable and low impact methods (Schreiner *et al.*, 2013). In parallel, government bodies throughout the western world put heavy emphasis on the importance of fruits and vegetable as part of a healthy diet and to aid in the prevention of some chronic diseases. An environmental element with the potential to contribute to the demand for more nutritious food is UV radiation. As an environmental factor, UV has been largely overlooked in Irish growing systems. To address this, a series of outdoor experiments were undertaken to assess the impact solar UVB has on vegetative growth and accumulation of secondary metabolites in the bronze lettuce Cos 'Dixter' under Irish weather conditions.

In this study, clear changes in levels of plant secondary metabolites were noted in response to UV exposure during growth. Interestingly it was UVA rather than UVA & B radiation, which elicited the most significant increases in anthocyanins. Voipio and Autio (1995) also observed higher anthocyanin content in lettuce in response to UVA radiation although their study used supplementary UVA. Here we revealed the role of low, natural levels of UVA in increasing anthocyanin in lettuce. Tsormpatsidis *et al.*, (2008) found that UV transparent filters increased anthocyanin content of the red lettuce Lollo rosso more than UVB blocking filters or UV opaque filters under outdoor conditions. This was attributed to the UVB portion of the light

spectrum (Tsormpatsidis *et al.,* 2008). Krizek *et al.,* (1998) showed that excluding both UVA and UVB significantly reduced anthocyanin content of red lettuce (*Lactuca sativa* L.(cv. New Red Fire). UV-radiation is not the only factor influencing anthocyanin accumulation. Batavia lettuce, and some berries increase anthocyanin content in response to increasing temperature and radiation (Wang & Zheng 2001, Zheng *et al.,* 2012). Whereas pomegranate and red oak lettuce up-regulated anthocyanin biosynthesis in response to low temperatures (Borochov-Neori *et al.,* 2011; Marin *et al.,* 2015). In this study plants were not exposed to low temperatures (lowest recorded temperature was 8.7 ^{0}C), but rather UVA has been identified as the primary driver behind the observed increases in anthocyanin content, with smaller effects caused by UV-B exposure.

Accumulation of flavonoids is one of the most widely reported UV-responses (Rozema *et al.,* 1997; Jansen *et al.,* 1998; Agati & Tattini, 2010). In this study some increases were observed, after 48 hours growth under filters plants under the uv-a/b filter and the uv-a filter had higher levels of flavonoids than those grown under the uv-0 filter. However, there was no clear evidence of flavonoid accumulation being directly associated with UVB exposure. This contradicts Tsormpatsidis *et al.* (2008) García-Macías *et al.* (2007) and Krizek *et al.,* (1998) who all found a strong induction of flavonoid compounds in lettuce plants in response to UVB exposure. In more controlled conditions it has been shown that UVB exposure not only increased concentrations of flavonoids but also altered the composition and ratios of specific flavonoid groups (Hectors *et al.,* 2014). While strong evidence exists for the link between UVB and flavonoids it has also been observed that in complex outdoor conditions other factors such as temperature can conceal the effects of UV on flavonoids (Bjerke *et al.,* 2005; Gehrke, 1999, this thesis, chapter 3). For example, Bjerke *et al.,* (2005) found that concentrations of UV-B-absorbing phenolics in lichens do not show a simple relationship to UV-B dose as differences between treatments were overshadowed by seasonal differences.

In the absence of solar UV exposure, lettuce biomass is up to 2.5 times higher than in plants grown under the uv-a/b filters. This reduction in lettuce biomass under ambient levels of both UVA and UVB is consistent with previous studies conducted using similar experimental setups (Krizek *et al.,* 1998; Tsormpatsidis *et al.*, 2008). For example, Tsormpatsidis *et al*. (2008) found that plant grown under a UV blocking filter had between 40 and 122% higher dry weight than those grown under a UV transmitting filter. Nevertheless, UVB induced reductions in biomass are a relatively rare phenomenon under natural light conditions (Ballaré *et al.,* 2011). Alternatively, Wargent *et al*., (2011) found that morphology in lettuce leaves changed under UV transmitting filters, leaf area, length and width reduced and thickness increased but the fresh weight remained the same as those grown under UV blocking filters. The reason for the observed reduction in biomass under filters transmitting both UVA and UVB is unclear. Tsormpatsidis *et al.* (2010) suggests a metabolic cost related to the production of secondary metabolites. On the other hand, high levels of UVB are known to cause stress, whereby "high" is defined as UV-B levels sufficient to lead to a massive development of ROS, over-riding the antioxidant capacity regulated by non-specific stress pathways and contributing to both signalling and gene expression (Hideg *et al*., 2013). Although it should be noted that even during the summer months, UV-B levels are relatively low in Ireland (the maximum UVB irradiance during the course of the experiment was 216 W/m^2). Perhaps the most realistic scenario is that a combination of UV exposure with and additional stressor was responsible for the observed decrease in biomass, as has been argued by Bornman *et al*. (2015). Consistent with this scenario, Lau *et al.* (2006) found that maize grown on a nutrient deficient medium was more susceptible to damage when exposed to ambient UVB, than maize under optimal nutritional conditions. The lettuce plants in this experiment were grown in 6 cm pots and it is highly likely that they experienced a degree of nutrient deficiency as the trial progressed.

Nutrient stress combined with UVB could have caused photo-inhibition resulting in reduced carbon synthesis and ultimately reduced biomass due to the additive stresses.

The reduction in biomass may not be a desirable outcome in a commercial context. To explore if anthocyanin accumulation can occur in the absence of decreased biomass, we assessed two separate scenarios. Firstly, plants were exposed to UVA, in the absence of UVB. Under these conditions it was found that plants developed significantly higher levels of secondary metabolites without the decrease in biomass seen in the UVB exposed plants.

Secondly, we analysed the minimum UV exposure time required for anthocyanin and UV-absorbing pigments to accumulate within the lettuce rosettes. In the case of both anthocyanin and flavonoids, there was a significant increase in concentration after just 48 hours exposure to UVA and UVB (Table 6.2 & Fig. 6.4). Interestingly, after 72 hours, lettuce plants grown with just UVA radiation had the greater increase in anthocyanins over plants grown under UV-0 or UV- A& B radiation (Fig. 6.4). Tsormpatsidis *et al.* (2010) also investigated the potential of short-term UV exposure, with the same objective to reduce yield loss. These authors found that transferring plants to a UVA & B (in combination with natural sunlight) six days before harvest increased secondary metabolites as well as dry weight of lettuce plants. Thus, an improvement in the colouration and nutritional content of the crop can be achieved in a relatively short period of time, revealing the potential of using UV as a tool in horticulture.

A further consideration is the persistence of the UV-induced phytochemicals post-harvest or on removal from the UV stimulus. Salad crops are normally on supermarket shelves within 24 hours after harvest, but it is unknown how stable and persistent UV induced secondary metabolites in lettuce are once the UV stimulus is removed. After just 24 hours in the PAR only growth room, levels of both flavonoids and anthocyanins had already decreased. There was no difference between UV treatments in the reduction of flavonoids. While levels of

anthocyanins declined in all treatments, there were differences between treatments in the anthocyanin content (Table 6.3 & Fig. 6.5). UVA treated plants retained a higher anthocyanin content than those grown under the UVA&B and the UV-0 filters after 96 hours (Table 6.3 & Fig. 6.5). In agreement with these findings Ferrara *et al.* (1997) also reported a reduction in anthocyanins but found flavonoid levels were maintained postharvest although this assay was conducted on cut lettuce leaves stored at 5^0C. Alternatively, several studies have reported increases in secondary metabolites during the post-harvest period as ripening progressed though these studies were undertaken on fruit rather than salad crops and under a variety of storage conditions (Connor *et al.*, 2002, Goncalves *et al.*, 2004, Kalt *et al.*, 1999). It has been previously reported that the UV transmittance of leaves changes throughout the day in response to the strength of the UV signal they are receiving (Barnes *et al*., 2008). This would suggest that the increase in secondary metabolites is temporal and dependant on a continuous signal to maintain high levels. These findings imply that post-harvest treatment of lettuce plants have consequences for the nutritional value of the product.

Nutriceuticals and super-foods are increasingly becoming buzzwords. Consumers' response to these makes the ability to grow a premium product by utilising a natural resource an attractive choice. Ambient UV has been largely ignored as a tool for plant manipulation in Ireland. This study illustrates that precision manipulation using natural UV radiation is possible. Both UVA and UVB radiation had a positive effect on secondary metabolites but UVA may be the more preferable treatment as it did not reduce biomass significantly. In addition, anthocyanin levels increase significantly under UVA radiation after just 72 hours exposure. Additionally, while both anthocyanins and flavonoids decreased during storage it was found that UVA treated plants retained higher levels of anthocyanin following the removal of the plants from the outdoor growing conditions to a UV-free environment. These findings

suggest a short pre-harvest treatment with natural UVA radiation could improve quality and nutritional composition of salad crops grown in Ireland.

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Chapter 7

General Discussion

"Plants exist in the weather and light rays that surround them – waving in the wind and shimmering in the sun. I am always puzzling over how to draw such things" – Hayao Miyazaki.

The puzzle in this case was not how to draw plants but how to further the understanding of the relationship plants have with the sunlight, in which they shimmer. As sessile organisms, which are completely dependent on sunlight for their existence, it is hardly surprising that the means to mediate responses to UVB are highly sophisticated and developed. Research into the UVB response pathway has been extensive and is ongoing but plants are loath to relinquish all their secrets. The overarching aim of this thesis was to examine the functional role UVB responses and provide a more detailed understanding of the mechanisms behind these. UVB morphogenesis, changes in biochemistry, influence of seasonality and the functional roles of UVR8 were all investigated with the aim advancing understanding of plant UVB responses. As an agent of plant manipulation UVB radiation and its associated response have potential for use in passive and supplemental systems. However, utilization of plant responses to UVB radiation can only be fully realised with a better understanding of the fundamental rules that govern it.

What are the effects of a low chronic dose of UVB on the morphology and flavonoids profile of *Arabidopsis thaliana*?

A key finding of this study was that the effects seen on morphology were transitory. Petioles were found to be shortened and leaf biomass was reduced, however, as the experiment progressed these differences diminished. There have been several studies, involving a range of species, which also noted this phenomenon (Hectors *et al.*, 2010; Robson & Aphalo, 2012; Llorens *et al.*, 2015). However, a satisfactory explanation has not yet been proposed. One theory suggests that the regulatory response activated by UVB diverts resources during leaf expansion but this is followed by compensation and by maintaining the maximum growth rate

for longer (Robson & Aphalo, 2012). The reallocated resources are potentially used in the up regulation of UV protection mechanism such as ROS and DNA repair (Robson *et al*., 2014). Yet, the relationship between a reallocation of resources a slow down or cessation of growth has not been shown yet (Kotilainen *et al.*, 2009; Robson & Aphalo, 2012). Alternatively, it has been proposed that transient effects on rosette growth are caused by a slow down or cessation in growth due to stress, i.e. SMIR (Potters *et al.*, 2007). The effect on morphology in this study is largely evident in older leaves, which have been found by Jordan *et al.* (1998) to be more sensitive to UVB exposure. However, with no evidence of plants stress in this study, further investigation is required into the transitory nature of low dose UVB effects on plant morphology.

The effects on total soluble phenolics were more persistent. UVB treated plants contained up to 45% more total soluble phenolics than untreated ones. The up-regulation of total soluble phenolics was evident throughout the experiment. This change was further investigated using UPLC-TDQ mass spectrometry. In response to UVB exposure, eight quercetin and kaempferol derivatives were identified as being significantly up regulated some by up to 90%. Interestingly, the levels of total soluble phenolics, quercetins and kaempferols were higher in younger than in older leaves. This begs the question, are resources (e.g.flavonoids) being reallocated from older leaves to younger leaves while the latter are in the carbon sink stage. Further investigation is required into the differing response of older and younger leaves to develop this hypothesis. The findings in this thesis also bring into question the theory that the purpose of a dwarf morphology was to decrease overall UVB exposure by increasing self-shading (Jansen, 2002). If the morphological response to UVB is transitory then this would suggest that any role in UV protection is temporary. Ultimately implying, that the functional role and ecological relevance of UVB morphogenesis is not to reduce UVB through self-shading and has yet to be determined.

Is the UVB effect Systemic of Local?

Evidence of both a local and a systemic effect was found in response to UVB. The UV-effects on leaf area and leaf biomass were systemic suggesting that even if only one leaf of a rosette was receiving a UVB signal there was systemic communication throughout the rosette. In contrast, the UVB effect on petiole length and total soluble phenolics was local, i.e. when only one leaf was exposed to UVB, only that leaf displayed a UVB response. Signal transmission and communication throughout entire plants is well demonstrated in response to a variety of signals (Mullineaux *et al.*, 2000; Kuć, 2001; Tossi *et al.*, 2012; Gordon *et al.*, 2012; Liu *et al.*, 2015). Although, there is also evidence that plants have the ability to produce a local response (Mullen *et al.*, 2006). The findings of this study suggest that there is a role for both local and systemic signalling in the UVB response. This tailored response allows a high degree of plasticity in response to dynamic environmental conditions.

Seasonal changes and local adaptations to UVB radiation

A local accession, Bur-0, was used to examine the possibility of specific adaptions to prevailing weather conditions. In this assay the dominant effect was that of seasonality. Plant growth parameters and total soluble phenolics changed throughout year, and analysis of the variation showed that changes were closely linked with changes in temperature. As temperature increased so did rosette diameter, leaf biomass and leaf area. On the other hand, levels of total soluble phenolics decreased with increasing temperature. Previous studies have also found that seasonal effects are larger and can overshadow the more subtle effects of UVB exposure under natural conditions (Gehrke, 1999; Bjerke *et al.*, 2005;). Yet, evidence of a UVB effect on morphology was found during the summer months. Rosette diameter, leaf area and biomass all decreased under the UVB treatment. Interestingly, this effect was also found in the UVA treated plants suggesting that there is a degree of cross-over or potentially cross-talk between

responses to UVA and UVB. While the observed decrease in rosette diameter and leaf area was significant, it should be noted that Arabidopsis does not normally grow in Ireland during the summer months. This raises the question whether a similar result could be found in a plant that naturally grows during the summer. No differences was found between the three Arabidopsis accessions in their responses to UVB throughout the year. Although there have been several studies which have found differential responses to UVB in different accessions under both outdoor and indoor conditions (Cooley *et al.,* 2001; Biswas & Jansen, 2012). However, given the changeable nature of outdoor environment, divergent traits in response to UVB that would be noted in more controlled conditions between the accessions are likely being masked by larger trends in this dataset. It would be interesting to focus future research on the differential response in a wider group of accessions, using reciprocal planting, over a longer time period to encompass seasonal changes in climate.

The role of UVR8 in ambient sunlight.

The investigation of the ecophysiological role of the UVR8 photoreceptor is still at the early stages and studies in natural sunlight are rare. The *UV RESISTANCE LOCUS8* (UVR8) gene-product was identified as the UVB photoreceptor (Rizzini *et al.,* 2011). Since its identification, it has been recognised that UVR8 acts in a UVB-specific manner and plays a key role in UVB-mediated control of hundreds of genes, including several important for flavonoid induction (Brown *et al.,* 2005). To encompass the influence of seasons on the UVB response the study was set over 12 months. It was found that the UVB effect on the morphology of the *uvr8-1* mutant was only seen during the summer months. However there was a reduction in total soluble phenolics and F_v/F_m in UVB treated *uvr8-1* plants throughout the year. The UVR8 mutant *uvr8-1* was found to exhibit a similar morphological response to the wild type Ler, in that a reduction in plant size was evident in the summer months. Potentially this finding contradicts a large body of lab-based studies, which find that a functional UVR8 pathway is

required for a typical UVB "dwarfing" response to be observed. However, it is possible that there are divergent mechanisms at play. It can be hypothesised that the wild type Ler is responding to perceived UVB and develops a typical UVB phenotype. On the other hand, the *uvr8-1* mutant might be experiencing stress resulting in a dwarf phenotype. Stress might be an indirect result of the lack of UVB perception, as the latter might have prevented a lack of induction of protective responses. In this study, it was also found that the mutant plants had reduced total soluble phenolics levels and F_v/F_m values throughout the year unlike the wild type. Outwardly, the product is the same but the route taken by both plants is different. Alternatively, these findings could de-couple the morphological response to UVB from the direction of the UVR8 photoreceptor. There was evidence of dwarf morphology in the UVA treated plants too, suggesting that there could be some cross-over in response between crytochrome and UVR8. Indeed a recent study has also proposed a link between phytochrome and UVR8 in the shade avoidance mechanism although the interaction has not yet been fully detailed (Fraser et al., 2015). These findings affirm the importance of the UVR8 photoreceptor and the necessity of a functional UVR8 pathway for optimized plant growth throughout the year.

Potential for the use of ambient UVB in commercial cropping

The practical applications of UVB have been largely over looked in an Irish context. The potential of utilising ambient levels of UVB to enhance the nutritional content and end value of the Bronze lettuce Cos 'Dixter' was investigated. The up regulation of secondary metabolites, in this case flavonoids and anthocyanins, is commonly reported in response to UVB. High dietary levels of these compounds have been associated with an array of potential health benefits (Vinson *et al.*, 2001; Schreiner *et al.*, 2013 de Pascual-Teresa & Sanchez-Ballesta, 2008; Bertoia *et al.*, 2016). Flavonoids and anthocyanins are also associated with an increased degree of tolerance to biotic and abiotic stressors in plants. The parallel phenomenon

to an increase in secondary metabolites is often the development of a “dwarf” phenotype. In the context of commercial cropping a dwarf plant may be more tolerant of harvesting, packaging and transport. However, an actual reduction in vegetative growth would not be a desirable outcome. For this reason, a better understanding of crop plant responses to natural UVB is required. In the course of this study, it was found that UVA/B, as well as UVA on its own, could enhance the accumulation of flavonoids and anthocyanins under natural sunlight in a relatively short period (72 hours). It was also measured that plants that received UVA without UVB did not display a significant reduction in leaf biomass, but still had enhanced flavonoid and anthocyanin levels. The plants that received the UVA treatment also retained their anthocyanin content post-harvest for longer periods than those that received the combined UV-A/B treatment. This study illustrates the potential of UV within the protected cropping industry; UV exposure could result in benefits for growers and consumers. To fully ensure the efficacy and potential applications of UV radiation field scale trials should be undertaken using a variety of crops grown within protective systems with a view to providing structured recommendations.

Are flavonoids glycosylated at the C-7 position important for the development of the UVB phenotype, UV acclimation and protection?

In Chapter three more of the mechanistic aspects of the UVB response were explored. A transferase knock out mutant which is unable to produced quercetins and kaempferols glycosylated at the C-7 position was used to assess the importance of said flavonoids for the development of the UVB phenotype and for protection and acclimation to UVB. It was discovered that despite having dramatically lower levels of flavonoids specifically rhamnosylated at the 7 position under UVB treatment, the *ugt89c1* plants were not measurably injured by UVB exposure. There was an increase in total soluble phenolics and preliminary investigation identified an array of new flavonoids compounds, which could account for the

lack of sensitivity to UVB. Some of the newly identified compounds were tentatively identified as the precursors to the C-7 rhamnosylated flavonoids. However, further investigation is required to confirm these finding and to examine the ability of newly induced compounds to protect against potential damage caused by UVB exposure. These findings suggest that it is more important to be able to generally up regulate total phenolic content than produce specific flavonoids. It was also found that some aspects of the UVB induced morphogenesis were apparent in the *ugt89c1* plants while others were not. The knockout line had shortened petioles in response to UVB but the biomass remained unchanged. This would seem to suggest that various aspects of the UVB morphological response are differentially mediated. Additional inquiries into the relationship between the accumulation of flavonoids and UVB induced changes in morphology is required.

Key messages:

1. Findings in laboratory-based studies are important for detailed understanding of the mechanism behind the UVB response. Due to competing stimuli and the complexity of the outdoor environment, responses evident in the laboratory are not always clear outdoors. However, both are required for a holistic understanding of the UVB response pathway and its functional role.
2. A functional UVR8 photoreceptor is required for optimized plant growth under natural sunlight year round.
3. Natural levels of UVB could be utilised to enhance the nutritional quality and harvestable biomass of protected cropping systems.
4. From the evidence of potential crossover between UVB and UVA responses, it could be considered that the UVB response is linked to other photo reactive pathways. Thus, it can be hypothesised that UVB is used to gather information and inform an appropriate response to dynamic light conditions.

5. The ability of a plant to generally up regulate total soluble phenolics in response to relatively low level UVB is potentially more important than specific upregulation of quercetins and kaempferols glycosylated at C-7.

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