RESPONSES OF *ARABIDOPSIS THALIANA* TO UV-C RADIATION AND *HYALOPERONOSpora ARABIDOPSIDIS*

by

Sharl Mintoff BSc (Hons)

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<tbody>
<tr>
<td>avr</td>
<td>Avirulence</td>
</tr>
<tr>
<td>ATR</td>
<td>Arabidopsis thaliana recognised</td>
</tr>
<tr>
<td>BABA</td>
<td>β-aminobutyric acid</td>
</tr>
<tr>
<td>CFCs</td>
<td>Chloro-fluorocarbons</td>
</tr>
<tr>
<td>CPD</td>
<td>Cyclobutane pyrimidine dimer</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3 diaminobenzidine</td>
</tr>
<tr>
<td>DDG</td>
<td>2-deoxy-D-glucose</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>Et</td>
<td>Ethylene</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector triggered immunity</td>
</tr>
<tr>
<td>ETS</td>
<td>Effector triggered susceptibility</td>
</tr>
<tr>
<td>FW</td>
<td>Fresh weight</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post inoculation</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic Acid</td>
</tr>
<tr>
<td>LTB</td>
<td>Lactophenol trypan blue</td>
</tr>
<tr>
<td>NER</td>
<td>Nucleotide excision repair</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP triggered immunity</td>
</tr>
<tr>
<td>R (gene/protein)</td>
<td>Resistance gene/protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPP</td>
<td>Resistance to Peronospora parasitica</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>sdH$_2$O</td>
<td>Sterile distilled H$_2$O</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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PRESENTATIONS AND PUBLICATIONS ARISING FROM THIS RESEARCH

Conference presentations:


Mintoff, SJL and Cahill, DM (2011) Priming for resistance against pathogens: UV-C radiation as an agent of pathogen resistance, 18th Biennial Australasian Plant Pathology Society Conference, Darwin, NT

Mintoff, SJL and Cahill, DM (2012) Sub-lethal UV irradiation of Arabidopsis thaliana primes resistance to Hyaloperonospora arabidopsidis, American Phytopathological Society Annual Meeting, Rhode Island, USA

PUBLICATIONS IN PROGRESS

Mintoff, SJL, Rookes, JE and Cahill, DM (2014) Analysis of the roles of callose, lignin and ROS in the model system of A. thaliana and H. arabidopsis

Mintoff, SJL, Rookes, JE and Cahill, DM (2014) UV-C irradiation promotes cross-tolerance in Arabidopsis to infection by Hyaloperonospora arabidopsidis
ABSTRACT

Plants need to respond to a plethora of abiotic and biotic stresses in their environments. Yet, in order to cope with such an array of different stressors, common mechanisms are involved in the acclimatisation and repair responses, which enable plants to become more tolerant to a different secondary stressor. This thesis examines key components of the cross-tolerance response in Arabidopsis when pre-treated with low levels of the abiotic stressor UV-C radiation. Following exposure plants become more resistant to infection by virulent isolates of the biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis*. To characterise the factors involved in the response to UV-C irradiation, pathogen attack and combinations of both, plants were examined at morphological, biochemical and molecular levels.

Plants exposed to sub-lethal dosages UV-C radiation (250, 500 or 1000 Jm\(^{-2}\)) displayed leaf curling and bronzing at the higher dosages used and dose-dependent changes in leaf expansion. When the irradiated tissue was examined microscopically, it was clear that UV-C, even at ‘sub-lethal’ dosages, caused cell death and callose deposition. Hydrogen peroxide concentrations in leaves increased soon after irradiation at the higher dosages, and an induction of the salicylic acid marker gene *PR1* and jasmonic acid and ethylene marker gene *PDF1.2* was observed. In response to infection by *H. arabidopsidis*, a similar induction of defence-related responses was found in both the compatible and incompatible interactions, yet this occurred at different times and at different levels across the leaf. Deposition of lignin and callose occurred in the compatible and incompatible interactions, with the deposition of both occurring in direct association with pathogen growth in the compatible interaction or localised within or surrounding the hypersensitive response (HR) in the incompatible interaction. Changes in H\(_2\)O\(_2\) concentrations in response to the initial infection displayed
similarities in both the compatible and incompatible interactions with an increase in H$_2$O$_2$ accumulation at the early time points followed by a drop to basal levels. However, a second increase of H$_2$O$_2$, present at 72 hpi only, occurred in the incompatible interaction, and is likely associated with the formation of HR. Increased expression of the ROS marker gene GST1 was aligned with the heightened levels of H$_2$O$_2$ further demonstrating that a second oxidative burst is essential for successful resistance in this system which was found to be coupled to increased expression of PR1.

Plants that were exposed to UV-C radiation and then infected with a virulent isolate of *H. arabidopsidis* 24 h later, showed up-regulation of the defence markers, *PR1*, *GST1* and increased H$_2$O$_2$ accumulation. When examined in detail, it was apparent that a reduction in pathogen colonisation had occurred and the appearance of HR-like lesions within the irradiated tissue in response to the pathogen. This change in host susceptibility was directly correlated with the pre-activation of H$_2$O$_2$ generation leading to increased *PR1* expression, prior to and during the early infection stages.

This research has increased our understanding of the responses of Arabidopsis to exposures of low levels of the abiotic stressor UV-C radiation and provided new insights into the model Arabidopsis- *H. arabidopsidis* patho-system. This work has also identified key mechanisms that are likely involved in the cross-tolerance response of Arabidopsis caused by UV-C irradiation that increases resistance towards *H. arabidopsidis*. 
Chapter 1: Introduction and literature review

Terrestrial plants are constantly exposed to abiotic stressors such as temperature, drought, salinity and ultraviolet (UV) radiation, as well as biotic stresses caused by herbivorous animals, insects and infection by pathogenic microorganisms. To survive, plants have had to adapt and acclimatise to this plethora of environmental stresses. In order to limit the damaging effects of UV radiation, plants possess DNA repair mechanisms such as photo-reactivation, UV shielding compounds such as flavonoids, anthocyanin and anti-oxidant systems (Li et al., 1993, Vonarx et al., 1998, Britt, 1999, Bashandy et al., 2009). These adaptations suggest that UV radiation was a key selection pressure for the evolution of photosynthetic organisms for the colonisation of land (Björn et al., 2002). In response to biotic stresses, such as plant pathogens, plants have developed defensive mechanisms to overcome infection. Due to the absence of mobile immune cells, and must rely on the innate immunity of each cell to resist infection. These defence mechanisms involve both preformed physical structures and inducible molecular interactions (Nürnberger et al., 2004, Jones et al., 2006). This literature review will examine the adaptations and mechanisms involved in the survival and resistance toward two seemingly unrelated stressors, ultraviolet radiation and pathogen infection. It will also focus on the way in which UV irradiation of plants can influence the response of plants to pathogens in a laboratory environment.

1.1. Effects and responses of plants caused by ultraviolet radiation

The ultraviolet spectrum is divided into three spectra based on their wavelength, UV-A (320-390 nm), UV-B (280-320 nm) and UV-C (< 280 nm). In reality, less than 7% of the total solar radiation that reaches the surface consists of UV wavelengths, the majority of UV radiation that plants and animals are exposed to is UV-A and the higher
wavelengths of UV-B, as atmospheric gases and ozone effectively filter out wavelengths below 290nm (Kunz et al., 2006, Prado et al., 2012). Research concerning the effects of UV radiation on plants received considerable attention with the discovery of ozone depletion caused by the usage and release of anthropogenic chloro-fluorocarbons (CFCs) (Farman et al., 1985). Extensive knowledge has been gained since then concerning the detrimental effects of high dosages of UV radiation (in particular UV-B), and the mechanism and adaptations that allowed for the repair and survival of plant tissue. The discovery of the UVR8 protein, for example, increased the understanding of the mechanisms that plants use to perceive UV-B wavelengths and specifically respond to UV-B radiation in nature (Jenkins, 2009, Christie et al., 2012).

Although UV exposure was traditionally thought to be detrimental to plant development, it is becoming increasingly clear that exposing plants to UV can be beneficial to enhance defences to both abiotic and biotic stressors. Exposure to UV-B or UV-C radiation can be used as a means to increase plant protection to a variety of stresses in an agricultural setting, such as augmenting defences and post-harvest protection against pathogens or other abiotic stressors (Ballare et al., 1996, Vicente et al., 2005, Charles et al., 2008a). However, it should be noted that some exposure studies performed in laboratory environments tend to use higher dosages of UV radiation compared to natural environment.

1.1.1. Detrimental effects of ultraviolet radiation on plants

Ultraviolet radiation can have detrimental effects on cellular physiology at high irradiances. Changes in plant growth and morphology have been observed in plants exposed to high dosages of UV-B or relatively low dosages of UV-C and generally lead to reduced biomass in a variety of plants (Cen et al., 1990, Wang et al., 2007). Symptoms caused from UV over exposure in leaves can result in: reduced leaf
expansion, reduced photosynthetic performance, and leaf ‘bronzing’ or browning that is believed to be due to the oxidation of phenolic compounds (Cline et al., 1966, Mackerness et al., 1999, Wang et al., 2007). Epidermal thickening, leaf glazing and upward leaf curling are also symptoms of UV over exposure and have been suggested to limit or attenuate the effects UV exposure (Jansen et al., 1998, Wang et al., 2007, Wargent et al., 2009). However, various wavelengths of UV-B and UV-C can cause direct or indirect damage to cellular components which can lead to direct excitation of molecules, such as DNA, or oxidative damage through the formation of reactive oxygen species (ROS), and includes damage to membranes, proteins, nucleic acids and reductions in photosynthetic output (Landry et al., 1995, Zacchini et al., 2004).

Plants are not defenceless when exposed to UV radiation, as they possess prevention and repair mechanisms that can limit UV mediated damage. Flavonoids are generally found in leaf epidermal cells and considered the plants UV ‘sunscreen’ as these compounds have the ability to absorb UV wavelengths, and are produced in response to UV exposure (Lois, 1994). The role of flavonoids and sinapates esters in UV protection has been demonstrated with the examination of mutants with reduced flavonoid production in Arabidopsis, tt4 (transparent testa 4; chalcone synthase) and tt5 (transparent testa 5; chalcone isomerase) and fah1 (flerulic acid hydroxylase 1) exhibit hypersensitivity towards UV-B exposure with an increased instances of reduced biomass, upward leaf curling and bleaching, when compared to wild type plants (Li et al., 1993, Landry et al., 1995). Whereas Arabidopsis mutants that contained increased levels of flavonoids demonstrated an increase in tolerance towards UV-C exposure (Bashandy et al., 2009). Although flavonoids are vital in UV protection, on their own, they are not able to prevent damage, therefore other mechanisms are employed to manage and repair this damage.
1.1.2. DNA damage and repair

UV-B exposure can directly excite nucleotides and indirectly damage DNA through oxidative stress. Both UV-B and UV-C can directly cause similar DNA damage products, resulting in the formation of cyclobutane pyrimidine dimers (CPD), and pyrimidine (6, 4) pyrimidone photoproducts (6-4 photoproducts) (Kunz et al., 2006). UV-C wavelengths lie in the same range as the absorption maxima of DNA and can thus cause lesions in DNA more efficiently in comparison to UV-A or UV-B (Stapleton, 1992). This damage can inhibit transcription and DNA replication, by causing the DNA and RNA polymerases to stall at these points (Donahue et al., 1994, Britt, 1995, Dany et al., 2001). A unique adaptation of plants and photosynthetic organisms to combat DNA damage caused by UV-exposure is that they can use light-dependant photo-reactivation to repair DNA damage. This ability uses the enzyme photolyase to harness the energy of blue and UV-A wavelengths (350 nm-450 nm) in order to reverse the UV induced damage caused by CPD and 6-4 photoproducts (Chen et al., 1994, Britt, 1995, Kunz et al., 2006). Nucleotide excision repair (NER) is not light dependant; and removes any mutations in the DNA. Repair complexes recognise sites of damage and generate ‘nicks’ at specific distances away from the damaged region and this segment of DNA is removed, the remaining gap is filled in by DNA polymerase and the nicks are sealed by DNA ligase (Britt, 1995, Britt, 1996, Sinha et al., 2002).

1.1.3. Reactive oxygen species damage and antioxidant systems

Reactive oxygen species (ROS) have been shown to be vital signalling molecules and are involved in a multitude of cellular processes. High levels of ROS accumulation can damage cellular components, such as proteins, lipids and nucleic acids. Increases in H₂O₂ in particular have been shown to be induced in plants exposed to either UV-B or C radiation (Murphy et al., 1990, Green et al., 1995, Zacchini et al.,
ROS generation has been observed in UV-B exposed plants, yet the processes involved in ROS generation in plants exposed to UV are not fully understood. Metabolic processes, such as photosynthesis and respiration, have been implicated as potential sources of ROS (Jenkins, 2009), and ROS generated in response to UV-B may involve the contribution of NADPH oxidases (Kalbina et al., 2006). High levels of ROS can lead to oxidation of cellular components and damage tissue exposed to higher than normal UV dosages. The cell membrane is a major target of high ROS production and lipid peroxidation alters its fluidity and selectivity of substances that can cross the membrane (Gill et al., 2010). ROS also causes irreversible damage to proteins (Gill et al., 2010). DNA can also be a target of ROS mediated damage, which can result in modifications to the nucleotide bases, strand breaks and DNA–protein crosslinks (Roldán-Arjona et al., 2009, Sharma et al., 2012).

In an attempt to maintain steady state of ROS levels, the plant activates antioxidant systems and antioxidant enzymes, such as superoxide dismutase (catalyses $\text{O}_2^-$ to $\text{H}_2\text{O}_2$ and $\text{O}_2$), catalase (catalyses $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$) and ascorbate peroxidase (also involved in scavenging $\text{H}_2\text{O}_2$) (Asada, 1999, Mittler, 2002). Many other antioxidant molecules also exist within sites of potential oxidant generation. Ascorbic acid and glutathione are considered crucial in the protection against oxidative damage (Gechev et al., 2006, Gill et al., 2010). Carotenoids and flavonoids play a role in ROS suppression. Carotenoids have a variety of biological roles such as light capture and can also function to scavenge singlet molecular oxygen and other ROS species formed through photosynthesis, and although flavonoids are referred as the plants ‘sunscreen’ they also have been shown to possess antioxidant properties and are thought to scavenge ROS to limit oxidative damage within the cell (Stahl et al., 2003, Gill et al., 2010).
1.1.4. Beneficial effects of UV radiation on plants

Although high dosages of UV radiation have detrimental effects on plants, lower dosages can have beneficial effects through the stimulation of a ‘eustress’ or ‘cross-tolerance’ response. This response generally involves the exposure to low levels of a normally harmful stress, which allows the affected cells to become tolerant to a second unrelated stress (Lichtenthaler, 1996). This type of induction demonstrates a cross over in mechanisms that deal with abiotic and biotic stressors. This type of cross-tolerance appears to play a role in nature in regards to UV-B exposure, where field or greenhouse grown crop plants exposed to ambient levels of UV-B were shown to be more resistant to insect herbivory compared to plants grown in the absence of UV-B exposure (Ballare et al., 1996, Mazza et al., 2013). This response does not only appear to be restricted to plants, this type of cross-tolerance response has also been demonstrated in the cells of Caenorhabditis elegans. DNA damage induced by UV-B or ionising radiation appeared to be involved in increasing the survivability of C. elegans cells which were exposed to heat stress or pathogen infection, and it is thought that DNA damage repair is involved in this process (Ermolaeva et al., 2013).

Research in plant-UV interactions has uncovered that UV-B and UV-C exposure can increase the activity of genes involved in the activation of the pathogen defence pathways. For example, induction of the salicylic acid (SA) pathway was observed by the enhanced expression of EDS5 (Enhanced disease susceptibility 5) in Arabidopsis thaliana and PRI (Pathogenesis-related 1) in Nicotiana tabacum in response to UV exposure (Green et al., 1995, Shapiro et al., 2001, Nawrath et al., 2002). UV exposure also resulted in an increase in SA levels which led to increased resistance toward the hemi-biotrophic bacterial pathogen Pseudomonas syringae pv. tomato DC3000 in Arabidopsis (Yalpani et al., 1994, Shapiro et al., 2001, Nawrath et al., 2002). Kunz et
al., 2008, demonstrated that when Arabidopsis Col-0 and Ler ecotypes irradiated with sub-lethal doses of UV-C radiation (100-500 Jm$^{-2}$), doses where the whole plant survives after UV-C exposure, then infected 24 hours later with the obligate biotroph oomycete *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*), increasing resistance to the pathogen occurred in a dosage dependant manner. It was also demonstrated in the same system that DNA damage and repair mechanisms were involved in the interaction and that there was enhanced resistance to the pathogen in DNA repair deficient mutants.

In response to UV exposure, expression of genes involved in the jasmonic acid (JA) defence pathway, marked by expression of *PDF1.2* (Plant defensin 1.2), as well as production of protease inhibitors had been noted in *A. thaliana* and tomato plants, respectively (Conconi *et al.*, 1996, Mackerness *et al.*, 1999). However, resistance to pathogens was also shown in plants exposed to high doses of UV-C radiation and demonstrated increased resistance towards the necrotrophic pathogen *Botrytis cinerea* which was shown to be associated with camalexin production (Stefanato *et al.*, 2009). More recently, plants exposed to UV-B radiation also showed increased resistance to *B. cinerea* with the involvement of *UVR8* mediated sinapate biosynthesis, however, camalexin or JA did not appear to play a role in this system (Demkura *et al.*, 2012). Although there has been examination of some aspects of this phenomenon, it is clear that this response is not fully understood as different systems can yield different responses.
Figure 1.1. Detrimental and beneficial responses of plants to high or ambient/sub-lethal dosages of UV radiation. Left side of image represents the effects and damage caused by high levels of UV exposures, right side of image represents ambient to sub-lethal exposures and the resulting changes that are induced within the plant.
**1.2. Plant pathogen interactions**

In order to combat the invasion of potentially pathogenic microorganisms, plants have developed an arsenal of defences, such as the cuticle, cell wall fortifications, antimicrobial compounds and direct responses induced by pathogen recognition (Dangl *et al.*, 2001, Hématy *et al.*, 2009, Ahuja *et al.*, 2012). Pathogenic microorganisms have also developed ways to counter host plant defences either by suppressing host immunity or through different infection strategies. Generally, plant pathogens can be classified into three main groups based on their infection strategy, and termed as biotrophic, necrotrophic or hemi-biotrophic. Biotrophic pathogens require living host cells to gain its nutrients, whereas, necrotrophic pathogens kill cells and destroy tissue to gain nutrients (Glazebrook, 2005). Hemi-biotrophs rely on a combination of both the biotrophic and necrotrophic infection strategies, where initial infection relies on the biotrophic stage and can switch to a necrotrophic stage after the cell/tissue dies (Perfect *et al.*, 2001). In order to respond to such a diverse infection strategies, key signalling hormones such as salicylic acid (SA) and jasmonic acid (JA) and ethylene (Et) are used to influence which defence strategy needs to be employed. These pathways are usually mediated through the antagonism of the competing pathways, where the activation of one leads to the repression of the other (Glazebrook, 2005, Jones *et al.*, 2006).

**1.2.1. Plant defences against pathogens**

Preformed defences against pathogens consist of physical barriers and antimicrobial compounds. These defences include the cuticular wax layers, plant cell walls, secondary metabolites and antimicrobial proteins to prevent infection from potentially pathogenic organism (Thordal-Christensen, 2003, Liu *et al.*, 2007, Ferreira *et al.*, 2007). These attributes allow the plant to take preventative measures against any potential pathogen before infection can occur. If the invading organism eludes the
physical barriers, more active defence mechanisms are activated following perception of non-self-signals by the plant. Inducible defences consist of the triggering of physiological and biochemical changes that occurs when a pathogen is perceived by cellular receptors (Nürnberger et al., 2004). The gene-for-gene model, originally proposed by Flor, 1942, suggested that plants produce R proteins that correspond to the invading pathogens avr protein. However, this model does not seem to explain all R protein interactions, where R proteins do not directly interact with the corresponding avr proteins but instead detect the changes that the avr proteins induce, hence termed the guard hypothesis (Dangl et al., 2001, Mazzotta et al., 2011). The current generalised model of inducible resistance involves the recognition of the pathogen leading to the activation of defences, though pathogen associated molecular patterns (PAMP) triggered immunity and effector triggered immunity (Jones et al., 2006, Spoel et al., 2012).

1.2.2. PAMP triggered immunity

The first line of inducible defence is the recognition of PAMPs at the cell surface, hence the term PAMP triggered immunity (PTI) (Jones et al., 2006, Thomma et al., 2011). Recognition of pathogenic molecular patterns are perceived by transmembrane receptors that contain receptor like protein (RLP) or a receptor like kinase (RLK) motif that recognise highly conserved surface components of potential pathogens (Chisholm et al., 2006, Altenbach et al., 2007). Recognition of a PAMP through the transmembrane receptors leads to a signal cascade that involves map kinase signalling, increased expression of defence related genes, an oxidative burst, callose deposition and cell wall fortification (Figure 1.2a) (Felix et al., 1999, Nürnberger et al., 2004, Schwessinger et al., 2008). In response to this, adapted pathogens have developed mechanisms to overcome PTI with the use of various strategies to deliver effector
Chapter 1: Introduction and literature review

R protein recognition

Defence activation

RESISTANCE

PTI

Effector release

Defence suppression

SUSCEPTIBILITY

ETS

R protein recognition

Defence activation

RESISTANCE

ETI

Trans-membrane receptor

Cell wall

Pathogen effector

Cell membrane

R protein

Pathogen haustoria
**Figure 1.2.** Model of inducible immunity in response to pathogen attack. 

a) PTI, Recognition of PAMPs on the surface of the pathogenic structure by trans-membrane receptors in the cell membrane leads to defence activation and pathogen resistance. 
b) ETS, release of effectors by the pathogen that suppress the host basal defences leads to susceptibility. 
c) ETI, release of effector by the pathogen is detected by resistance proteins, which activate defences and leads to resistance.
proteins that interact with components of PTI and result in the suppression of the defence response, leading to effector triggered susceptibility (ETS) (Figure 1.2b) (Jones et al., 2006, Chisholm et al., 2006, Block et al., 2010).

1.2.3. Effector triggered immunity

Effector-triggered immunity (ETI) takes place after the pathogen has evaded PTI defences and secretes or introduces effector proteins into the cell (Jones et al., 2006). In this case, the effector proteins themselves become the targets when resistance proteins (R proteins) recognise them directly or through changes to the effector targets. Most R proteins contain conserved domains, which include nucleotide-binding sites (NB) and leucine rich repeats (LRR) (Van Der Biezen et al., 1998, Collier et al., 2009). Detection of an effector (avirulence) protein (avr protein) by an R protein will result in an effective and rapid defence response involving the generation of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxides and the triggering of the hypersensitive response (HR) (Figure 1.2c) (Dangl et al., 2006, Kachroo et al., 2007). Activation of an R protein by an avr protein results in a signalling cascade and the recruitment of chaperone proteins SGT1b, RAR1 and HSP90 to regulate R protein activity, which can lead to the activation of the salicylic acid pathway in order to prevent pathogen spread (Holt et al., 2005, Azevedo et al., 2006).

1.2.4. The salicylic acid pathway

Activation of the salicylic acid pathway following pathogen challenge initiates defence response that targets pathogens with a biotrophic lifestyle, and usually results in cells undergoing HR. This response takes place after ETI R protein perception of avr proteins, which often leads to the generation of ROS within the cell and initiates rapid programmed cell death (Thomma et al., 2001, Glazebrook, 2005, Kachroo et al., 2007,
Activation of this pathway occurs in response to the recognition of the pathogen avr proteins. This involves signalling through the activation of \textit{EDS1} (Enhanced disease susceptibility 1) and \textit{PAD4} (Phytoalexin deficient 4) and/or \textit{NDRI} (Non race-specific disease resistance 1) (Aarts \textit{et al.}, 1998, Shapiro \textit{et al.}, 2001). These genes are essential in R gene mediated defences, and lead to enhanced expression of \textit{EDS5} (Enhanced disease susceptibility 5), which is believed to be a positive regulator of SA biosynthesis in a defence response (Nawrath \textit{et al.}, 2002). The NPR1 (Non expresser of PR genes 1) protein exists as an oligomer in the cytoplasm and is believed to also be a regulator of the SA pathway and has been shown to be essential in \textit{PRI} gene expression and SA biosynthesis (Ahn \textit{et al.}, 2007, Zhang \textit{et al.}, 2010). A change in the cellular redox state causes the bonds between the NPR1 oligomers to break and the monomers are transported into the nucleus where they interact with TGA transcription factors to activate defence genes such as \textit{PRI} (Mou \textit{et al.}, 2003, Kachroo \textit{et al.}, 2007). The activation of the SA pathway may effectively act against biotrophic pathogens, such as \textit{Hyaloperonospora arabidopsidis} and the hemi-biotroph \textit{Pseudomonas syringae}. However some necrotrophs such as \textit{Botrytis cinerea} which can deliberately activate this response in order to aid them in their infection process (Nawrath \textit{et al.}, 1999, El Oirdi \textit{et al.}, 2007).

\subsection*{1.2.5. The jasmonic acid and ethylene pathways}

The JA and Et pathways are commonly associated with wounding of plant tissue, defence against most necrotrophic pathogens and wounding caused by herbivores (Glazebrook \textit{et al.}, 2005). These pathways induce genes involved in wound healing due to mechanical damage and the generation of phyto-chemicals and protease inhibitors used to deter or kill feeding insects (Howe, 2004). Activation of the JA pathway in responses to wounding or pathogen attack has been shown to induce the production of
an array of secondary metabolites such as alkaloids, terpenoids and phenylpropanoids (Pauwels et al., 2009). The effectiveness of JA pathway has been demonstrated, where the suppression of the JA pathway leads to susceptibility to *Alternaria brassicicola* in the normally resistant Arabidopsis (Penninckx et al., 1998, Flors et al., 2008). More recently activation of the genes associated with the JA/Et pathway was shown to be induced in plants resistant to the necrotrophic *Phytophthora cinnamomi* (Allardyce et al., 2013).

Activation of the JA defence pathway involves signalling through the JAR1 (Jasmonic acid resistant 1) mediated conjugation of jasmonic acid to isoleucine, where it acts as a signal for the induction COI1 (Coronatine insensitive 1) activity that results in the degradation of the JA repressor JAZ (Jasmonate-zim domain) proteins (Suza et al., 2008, Bari et al., 2009, Kazan et al., 2012, Wang et al., 2013). Both JAR1 and COI1 appear to be essential in JA signalling, as mutation of either gene will lead to a jasmonic acid insensitive phenotype, which causes loss of resistance to necrotrophic pathogens (Thomma et al., 1998). This ultimately leads to expression of *PDF 1.2*, which has been shown to be expressed in the resistance response to necrotrophs (Stintzi et al., 2001). However, ethylene biosynthesis is also required for effective defences against infection and acts synergistically with the JA pathway, as suppression of the ethylene pathway leads to the loss of expression of *PDF 1.2* in response to *A. brassicicola* (Penninckx et al., 1998). Manipulation of the JA/Et pathways has also been found to be an effective strategy for the infection of pathogens, as the bacterial pathogen *P. syringae* has been shown to produce a coronatine which can activate JA defences in order to suppress the SA pathway which is effective in suppressing this pathogen (Zhao et al., 2003).
1.2.6. Cell wall fortification

Cell wall fortification by polysaccharides such as callose or phenylpropanoid-based polymers such as lignin may be a central component of effective defence. Callose is a polysaccharide comprised of repeating $\beta$-1, 3-glucans with occasional $\beta$-1,6-branches (Albersheim et al., 2011). Its synthesis requires the substrate UDP-glucose and cell membrane bound callose synthases and callose is deposited between the cell membrane and the cell wall (Stass et al., 2009). Callose deposition has been documented during normal plant growth (such as cell division, plasmodesmata regulation and pollen development), but also as a response to wounding, chemical stress and pathogen defence (Parker et al., 1993, Chang et al., 1999, Jacobs et al., 2003, Rookes et al., 2008, Chen et al., 2009). The regulation and production of callose has also been associated with calcium signalling and cell membrane integrity (Köhle et al., 1985, Wissemeier et al., 1987). However, the exact role of callose in plant pathogen interactions remains an ambiguous one.

Callose induction has been observed in both susceptible and resistant plant pathogen interactions. Callose-containing papilla first appear under the sites of cell wall penetration by the invading pathogen and does appear to have some role in penetration resistance of potential invading microorganisms, as its presence is thought to stop or slow down the pathogens ingress into host tissue or cell (Stone et al., 1992, Zeyen et al., 2002a). At the epidermis of host leaves, callose has been implicated to play a role in penetration resistance against non-adapted pathogens. This role in penetration resistance was demonstrated through callose inhibition studies in wheat, barley and oat, where plants impaired in callose production displayed increased penetration of non-adapted pathogens in normally resistant plants (Zeyen et al., 2002b). This role for callose was further demonstrated in Arabidopsis plants treated with $\beta$-aminobutyric acid (BABA),
which induced rapid and increased levels of callose-containing papillae in leaves in response to the virulent downy mildew *H. arabidopsidis*, leading to the prevention of pathogen penetration of the host’s tissue (Zimmerli *et al.*, 2000). This characteristic was also found in a recent study, where early increases in callose production in Arabidopsis in a *PMR4* over-expresser mutant, prevented the penetration of the virulent powdery mildew *Golovinomyces cichoracearum* (formerly *Erysiphe cichoracearum*) (Ellinger *et al.*, 2013).

Overcoming the first barrier of defence leads to colonisation of the host tissue by adapted virulent pathogens, and colonisation of the mesophyll can still result in callose deposition, which usually surrounds pathogenic structures such as haustoria (Donofrio *et al.*, 2001, Jacobs *et al.*, 2003, Dong *et al.*, 2008). Although the encasement of haustoria of virulent pathogens takes place, questions remain about its ability to perturb nutrient/effector transfer and its defensive capabilities. In response to virulent necrotrophs, callose does appear to have a role in basal resistance. In Arabidopsis, application of the callose inhibitor 2-deoxy-D-glucose (DDG) to BABA primed plants removed the induced resistance effects towards *A. brassicicola* and returned the pathogen induced lesion sizes to that of un-primed plants (Ton *et al.*, 2004). Similar effects were also observed in tomato leaves treated with DDG, which displayed increased susceptibility to the virulent necrotroph *B. cinerea* (Asselbergh *et al.*, 2007).

Lignin too, is an important molecule in plant development and is responsible for thickening and strengthening cell walls and waterproofing xylem cells to allow water transport (Vanholme *et al.*, 2008). This phenylpropanoid-based polymer is composed of: hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units derived from the monolignol subunits p-coumaryl, coniferyl and sinapyl alcohols, respectively (Goujon *et al.*, 2003).
The deposition of lignin takes place once the synthesised monolingols are transported to the cell wall and are oxidised by peroxidases and laccases for polymerisation (Goujon et al., 2003, Vanholme et al., 2008). Lignin production has been observed in wound healing and induced by pathogen infection (Ke et al., 1989, Soylu, 2006). Lignification in response to pathogen attack is thought to strengthen cell walls by increasing their resistance to digestion by enzymes released by an invading pathogen (Moura et al., 2010). Lignin deposition in cell wall appositions has been shown to play a role in penetration resistance in both virulent and avirulent pathogens. When lignin genes were silenced, increased susceptibility was found in both susceptible and resistant interactions in infected wheat plants (Bhuiyan et al., 2009a, Bhuiyan et al., 2009b).

1.3. The model pathogen *Hyaloperonospora arabidopsidis*

The oomycete *Hyaloperonospora arabidopsidis* (formerly *Peronospora parasitica*, formerly *Hyaloperonospora parasitica*) belongs to the kingdom Stramenopila and phylum oomycota. It is an obligate biotrophic pathogen which causes downy mildew of *Arabidopsis thaliana* (Coates et al., 2010). As this pathogen is an obligate biotroph, it is unable to complete its lifecycle outside the living host’s tissue. *H. arabidopsidis* is a natural pathogen of Arabidopsis, therefore an ideal model pathogen for the study plant pathogen interactions of biotrophic pathogens and much progress has been made since the initial studies into this pathogen’s lifecycle in the 1990s. Advancements in the knowledge of plant pathogen interactions brought about by studying this oomycete range from pathogen infection and development, identification of R genes across various Arabidopsis ecotypes, characterisation of fundamental stages of the involvement of the SA pathway in pathogen defence and to the more recent publication of the *H. arabidopsidis* genome (Koch et al., 1990, Parker et al., 1993,
Initial infection commonly takes place after the conidiophore (Figure 1.3a) releases conidiospores (Figure 1.3b) which come into contact with leaf tissue, as the conidiospores lack motile structures and have to be dispersed either by wind, water or dispersion by the conidiophores twisting violently (Slusarenko et al., 2003). Once in contact with plant tissue the conidia will germinate and form a germ tube and an appressorium (Figure 1.3c i), where a penetration hypha forms underneath the appressorium and penetrates the epidermal layer by forcing its way between the cell junctions (Koch et al., 1990). As the hypha penetrates the tissue, feeding structures (haustoria) invade adjacent cells with the formation of the primary haustorium (Figure 1.3c ii), and the oomycete will continue with prolific hyphal growth and infect new cells with haustoria throughout the leaf (Figure 1.3d) (Donofrio et al., 2001). As the pathogen proliferates through the host tissue, the pathogen will begin its reproductive stages either asexually or sexually. Days after infection the hyphae will begin to form asexual reproductive structures, which grow out of the stomata first as a conidiophore initial, before forming a mature conidiophore bearing conidiospores, eliciting further infection with the release of conidiospores (Koch et al., 1990, Mauch-Mani et al., 1994, Slusarenko et al., 2003). The sexual stage of *H. arabidopsidis* takes place alongside the asexual stage of development and involves the production of the oogonia (Figure 1.3e) and fertilisation of female oogonia by the male antheridium to produce oospores (Figure 1.3f) (Dangl et al., 1992, Slusarenko et al., 2003). These oospores are released into the soil from degraded leaf material and have the ability to infect the root systems of Arabidopsis plants and is one of the main sources of infection in nature during spring (Figure 1.3g) (Slusarenko et al., 2003).
Figure 1.3. Life cycle *Hyaloperonospora arabidopsidis*. Infection of Arabidopsis takes place through asexually or sexually produced spores. Asexually produced infection a-c, sexually produced infection e-g. a) Asexual reproductive structure, conidiophore. b) Conidiospores produced from conidiophores. c) Initial infection of the leaf, i) germinating conidiospore on the epidermal surface of the leaf with germ tube, ii) formation of the primary haustorium consisting of penetration hyphae and haustoria in surrounding cells. d) As infection matures the hyphae proliferates throughout the leaf mesophyll infecting new cells. Sexual reproduction begins with the formation of e) oogonia, once fertilised become f) oospores, which are used for long-term survival. g) Oospores infect the root systems of Arabidopsis seedling when conditions are favourable. Bars represent 20 μm.
1.4. Thesis objectives

This study aims to dissect the mechanisms of the ‘cross tolerance’ response caused by UV-C irradiation and its role in increasing the resistance of *A. thaliana* to the virulent isolates of *H. arabidopsidis*. This would allow for further insight into the commonalities between two completely different stressors and the relationships between the repair and defensive mechanisms involved. This work will examine what changes are induced by UV-C exposure alone, determining the differences in the compatible and incompatible interactions due to pathogen infection alone. Then examine the changes to the typical compatible interaction in plants treated with UV-C radiation and inoculated with the virulent isolate of *H. arabidopsidis* 24 hours later, in an attempt to identify key components of this response that have not been previously identified to occur at sub-lethal dosages of UV-C (Figure 1.4).

Chapter 2: In order to determine how irradiation of a plant leads to increased resistance to plant pathogens, this chapter will examine the changes induced by exposing Arabidopsis to sub-lethal dosages of UV-C radiation. Examination of changes in morphology and growth of the plant following irradiation will determine the extent of damage and H$_2$O$_2$ production caused by using ‘sub-lethal’ dosages of UV-C radiation. The examination of commonly used pathogen defence markers such as callose, lignin, salicylic marker gene *PRI* and jasmonic acid/ethylene marker *PDF1.2*, will allow for the identification of pathogen defence associated mechanisms induced by UV-C radiation, which could potentially be involved in the induced resistance.

Chapter 3: Although defence markers may be induced in response to UV-C exposure, it is important to set a baseline for the ‘normal’ induction of defence mechanisms in response to *H. arabidopsidis* infection and examine these responses in
both the compatible and incompatible interactions. Examination of key pathogen defence markers such as \( \text{H}_2\text{O}_2 \) activity, callose and lignin deposition and pathogen defence marker genes will allow for characterisation of differences of the deposition and timing of the hosts responses to virulent and avirulent isolates of \textit{H. arabidopsidis}.

\textbf{Chapter 4:} Using the knowledge gained from the effects of UV-C exposure alone and the effects of pathogen susceptibility and resistance alone, this chapter will examine the role that the UV-C induced changes has on the normally compatible interactions in two Arabidopsis ecotypes, in attempt to identify the changes in the plants response to the pathogen that leads it from a susceptible interaction to a resistant interaction. This will be achieved by identifying the changes to the normal compatible interaction observed between Arabidopsis and \textit{H. arabidopsidis} and examine the changes to callose and lignin formations, \( \text{H}_2\text{O}_2 \) activity, defence gene expression and pathogen growth in response to the induced resistance.
Figure 1.4. Proposed regulatory elements of the responses of *A. thaliana* to UV-C irradiation and subsequent infection by a biotrophic pathogen. This model represents what is currently known to occur in *A. thaliana* plants exposed to sub-lethal dosages of UV-C radiation and how it relates to increased pathogen resistance to biotrophic pathogens.
Chapter 2: Morphological, cellular and molecular responses of *Arabidopsis thaliana* to UV-C radiation

Abstract

Arabidopsis plants were exposed to 250, 500 or 1000 Jm\(^{-2}\) of UV-C radiation and the responses were examined at a physiological, molecular and biochemical level. Examination of exposed plants revealed reduced leaf areas over time with the severity of reduction increasing with dosage. Symptoms of UV over exposure were found to occur in the 1000 Jm\(^{-2}\) dosage with leaf glazing, bronzing and curling. At a microscopic level, extensive damage to the mesophyll was observed within the cellular layers of exposed leaf tissue, with cell death occurring in both a dose and time dependant manner. Also observed was the deposition of callose along the walls of irradiated cells. As time progressed, callose concentration continued increasing up to 72 h after initial irradiation. No change in lignin deposition was observed within the irradiated tissue 168 h following irradiation. The activity of reactive oxygen species were examined, an increase in GST1 expression occurred 3-6 hours after irradiation and increases in hydrogen peroxide concentrations at 24 hours were also found following irradiation in plants exposed to 500 and 1000 Jm\(^{-2}\). The increase in H\(_2\)O\(_2\) may also account for the increases in the activity of salicylic acid and jasmonic acid/ethylene marker genes, which was seen in the rapid and sustained induction *PR1* and *PDF1.2* in plants treated with the 500 and 1000 Jm\(^{-2}\) dosages.
Chapter 2: Morphological, cellular and molecular responses of *Arabidopsis thaliana* to UV-C radiation

2.1. Introduction

Many studies have examined the effects of increased levels of UV-B radiation on plants due to stratospheric ozone depletion and most studies show that increases in UV-B radiation can have detrimental impacts on plants. Meta-analysis performed on a number of previous studies examined the effects on plants grown under field conditions that were exposed to ambient or supplemented UV-B exposures generally resulted in a reduction in plant biomass, however this reduction usually did not usually exceed 20% (Newsham *et al.*, 2009, Ballare *et al.*, 2011). It is also becoming increasingly apparent that exposure of plants to UV radiation can have beneficial roles in plant development and in responding to other stresses. For example, studies of plants that were grown under natural ambient UV-B in field conditions, showed less insect herbivory when compared to plants grown under attenuated UV-B conditions (Ballare *et al.*, 1996, Mazza *et al.*, 2013).

In laboratory experiments, exposure of plants to increased levels of UV-B radiation have been shown to induce upward cotyledon and leaf curling, increased reflectivity and leaf bronzing and reductions in both biomass and leaf expansion (Li *et al.*, 1993, Green *et al.*, 1995, Wargent *et al.*, 2009). It appears that some of these photomorphogenic changes only occur at wavelength lower than UV-A region. An example of this is shown with the upward leaf curling responses. In *Brassica napus* cotyledons that were exposed to UV-A or B, UV-A treated plants did differ from control plants, yet only UV-B treated cotyledons curled upward (Wilson *et al.*, 1993). Symptoms of UV over exposure do not appear to be exclusive to UV-B wavelengths, as similar responses have also been reported to occur in a variety of plants exposed to UV-C radiation, also showing symptoms of leaf curling, bronzing and reduced biomass (Cline *et al.*, 1966, Tiburcio *et al.*, 1985, Tang *et al.*, 2010).
UV-C is also known for its ability in causing DNA damage (about 200 × more efficient at causing DNA damage then UV-B) and extensive cellular damage and is widely used as a means to study DNA damage and repair as the wavelength most commonly used is 254 nm, which is remarkably close to the absorption maximum of the DNA molecule (Kunz et al., 2006). Geno-toxic stress can be induced by UV-C at a variety of dosages ranging from 0 to 2 kJm$^{-2}$ which can result in the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Ulm et al., 2002, Kunz et al., 2008). However, at much higher dosages UV-C (5-50 kJm$^{-2}$), exposure leads to increases in reactive oxygen species, lipid peroxidation and cell death of protoplasts and cells within calli and leaf tissue (Bornman et al., 1983, Danon et al., 1998, Zacchini et al., 2004).

The stress caused by UV exposure appears to facilitate the activation of a ‘cross tolerance’ response in which the recovery of the plant can result in the activation of pathways commonly associated with pathogen defence and wounding. This has been observed as an increase in pathogen defence signalling, such as the activation of the salicylic acid and the jasmonic acid/ethylene pathways in response to UV-B or C (Green et al., 1995, Mackerness et al., 1999, Nawrath et al., 2002). This type of response has been shown to increase the resistance of plants to pathogens. UV-C in particular, was used to induce systemic acquired resistance in Arabidopsis, which were then infected with the hemi-biotrophic pathogen Pseudomonas syringae. Irradiated plants showed decreases in bacterial numbers over time (Shapiro et al., 2001). Kunz et al., 2008, demonstrated that UV-C pre-treatments, up to 500 Jm$^{-2}$, can cause increases in resistance in Arabidopsis towards the biotrophic pathogen H. arabidopsidis in a dose dependant manner, and demonstrated that DNA damage repair plays a role in that interaction. However, little else had been explored in terms of the induction of pathogen
defence mechanism induced by UV-C irradiation. A year later, Stefanato et al., 2009, demonstrated similar findings with leaves pre-treated with UV-C displaying increased resistance to the necrotrophic pathogen Botrytis cinerea with reductions in lesions size, however in this study, this resistance was attributed to the increases in camalexin concentrations within irradiated tissue.

Due to the damaging aspects of UV-C irradiation, questions remain concerning the damage and changes within the plant induced by exposures of ‘sub-lethal’ dosages. If DNA damage and repair is involved in the UV-C induced resistance to pathogens, then how does this translate to downstream defences that act to halt or slow pathogen attack? Although some aspects of this interaction appear to been uncovered, there are still gaps in the information regarding the activation and timing of the mechanisms that are induced by dosages of sub-lethal UV-C radiation that may contribute to overall pathogen resistance to both necrotrophic and biotrophic pathogens. Questions remain whether common pathogen defence markers are activated, such as cell wall fortifications, ROS generation and the activation of defensive genes at dosages much lower than previously reported. This study examines in detail, the UV photomorphological effects of sub-lethal exposures that occur between 250 and 1000 Jm⁻², in order to determine the physiological changes that occur over time in Arabidopsis. In addition, examine the timing and activation of key pathogen defence markers, such as callose and lignin production, induction of SA and JA/Et marker genes and ROS activity caused by UV-C irradiation.
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2.2. Materials and methods

2.2.1. Growth conditions of experimental plants

*Arabidopsis thaliana* (Col-0) seeds (LEHLE seeds, Texas, USA), were surface sterilised (45% (v/v) ethanol, 5% (v/v) of 30% H₂O₂) for 5 minutes, washed 3 times in sdH₂O, and suspended in sterile 0.15% (w/v) agar in dH₂O. The suspended seeds were placed onto Murashige and Skoog medium plates (0.44% (w/v) Murashige and Skoog basal medium, 3% (w/v) sucrose, 0.8% (w/v) bacteriological agar and adjusted to pH 5.7 with 1M KH₂PO₄ or K₂HPO₄), with a sterile pipette tip inside a Laminar Flow workstation. The plates were sealed and placed at 4°C for 2 days for stratification, then transferred into growth chambers (Thermoline scientific, Australia) with 12h day/night cycle at 21°C, and a light intensity of 100 μmol m⁻² s⁻¹ under cool white fluorescent lights. After 19 days the seedlings were transferred to soil (Debco® Premium potting mix, Debco, Victoria, Australia), with 3 plants per 6 cm diameter pot, then returned to the above growth conditions for 7 days, in transparent 10 L plastic containers with the lids on to maintain humidity, and the lids removed after 2 days. All plants used were 26 days old at the start of the experiments (table 2.1).

<table>
<thead>
<tr>
<th>Time points (h)</th>
<th>Plant age (days)</th>
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<tbody>
<tr>
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<td>33</td>
<td>33</td>
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<td>34</td>
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*Table 2.1.* Experimental time points used in this study following UV-C irradiation in relation to plant age. The age of the plants used in all experiments is represented in the top row with age in days. Experimental time points following irradiation used in all experiments are represented in the bottom row expressed in hours.
2.2.2. UV-C irradiation of Arabidopsis plants

Col-0 plants were exposed to UV-C radiation at doses of 250, 500 or 1000 Jm$^{-2}$ (which equated to 30, 60 and 120 seconds of exposure, respectively), with an 80 cm germicidal (non-ozone producing) UV-C lamp with a peak emission of 254 nm (Australian Ultra Violet, Victoria, Australia) housed in a light proof chamber (Dimensions; 120 × 50 × 60 cm) at a distance of 34 cm above the leaves. UV-C emission was measured at the leaf surface by a UVX digital radiometer with a UVX-25 sensor (Ultra-Violet Products Ltd, Cambridge, UK). To determine the desired UV-C dose plants were exposed to UV-C for increased amounts of time and calculated by dividing desired dosage (Jm$^{-2}$) by UV-C emission (Jm$^{-2}$/s). Plants were irradiated in an otherwise dark environment; following treatment, irradiated plants were then returned to normal growth conditions.

2.2.3. Measurement of leaf area of irradiated leaves

Each experiment consisted of three pots per control and per treatment, with 3 plants per pot, plants were exposed to UV-C radiation as described in 2.2.2, photos were taken at 0, 24, 48, 72, 120 and 168 hours (h) after irradiation. All photos included a scale by using a 30 cm ruler placed adjacent to the plants foliage. Measurements were taken from 4 flat leaves fully exposed to UV-C treatment between the 4th-10th rosette leaves, as leaves at this stage were expanded and received the full dosage of UV-C radiation. Leaf areas were calculated with the image analysis program ImageJ® 1.44 (Schneider et al., 2012). Data shown represents two independent experiments of average leaf area per pot.
2.2.4. Visualisation and quantification of cell death within irradiated leaves

Irradiated leaves were excised from a minimum of three plants; only leaves fully exposed to light were selected. Excised leaves were placed in 80% (v/v) ethanol at 0, 24, 48 and 72 h with daily changes of ethanol until the leaves were cleared of pigments. Staining to visualise cell death was based on the method of Koch and Slusarenko, 1990, with the following modifications; the cleared tissue was placed in lactophenol-trypan blue stain (LTB stain) (10 ml lactic acid, 10 ml glycerol, 10 ml phenol, 10 mg trypan blue and 10 ml dH₂O), and vacuum infiltrated twice for 5 minutes and left over night. Stained leaves were then destained in chloral hydrate (5 g/ml) overnight, and then mounted on a glass microscope slide with 50% glycerol. Stained leaves were viewed with a light microscope (Axioskop 2 mot plus, Zeiss, Göttingen, Germany); dead cells were observed as dark blue stained collapsed cells. Cell death was quantified by counting stained dead cells present in images of irradiated tissue within a field of view at 100× magnification consisting of an area of 7.6 mm², using the cell count feature of ImageJ® 1.44. Data presented represents mean cell death present in 4 randomly selected leaves and was replicated with three independent experiments.

2.2.5. Visualisation and quantification of callose deposition

Leaves were removed from experimental plants and cleared with daily changes of 80% (v/v) ethanol. The decolourised tissue was stained overnight with aniline blue stain (0.5% (w/v) aniline blue in 0.15M K₂HPO₄), then rinsed in dH₂O and mounted onto a slide with 50% glycerol and viewed with a fluorescence microscope (Axioskop 2 mot plus, Zeiss, Göttingen, Germany) with a UV filter. Callose concentrations were quantified using the method of Kohler et al., 2000. Leaf tissue, 50 mg fresh weight (FW), was collected and cleared of pigments in 80% (v/v) ethanol with daily changes until completely cleared. Leaves were then placed in screw top 1.5ml microfuge tubes
containing 100 μl of 80% (v/v) ethanol. Callose was extracted by macerating tissue and the extract was centrifuged for 20 minutes at 12,000 g to pellet the homogenised plant material. The ethanol was removed, 300 μl of DMSO was added and the sample was vortexed to dislodge the pellet, and then boiled for 30 minutes. After boiling, the samples were left to cool for 15 minutes, and centrifuged for 5 minutes at 12,000 g. Once centrifuged, 100 μl of the supernatant was added to 200 μl 1M NaOH and 1.2 ml of the loading mixture (400 μl 0.1% (w/v) aniline blue, 590 μl 1M glycine/NaOH buffer, 210 μl 1M HCl) was also added. Each sample was run with a parallel auto-fluorescence blank. Tubes were vortexed and placed at 50°C for 20 minutes and left to cool to room temperature for 30 minutes. Fluorescence was measured with a single channel fluorometer (Cary eclipse fluorescence spectrophotometer, Varian Instruments, Mulgrave, Victoria, 3170) with an excitation wavelength of 393 nm and an emission wavelength of 479 nm. Callose contents were compared to standards created with β 1, 3 glucans from Euglena gracilis (Sigma-Aldrich, MO, USA) and callose contents were expressed as ng β 1, 3 glucans equivalents mg⁻¹ FW. For individual experiments, samples underwent a single measurement. Data presented represents the mean of four independent experiments.

2.2.6. Lignin staining and visualisation

To visualise lignin deposition the Weisner reaction was used, which allows phloroglucinol to react with aldehyde groups of lignin to produce a red/pink coloured reaction (Albersheim et al., 2011). Leaves cleared of pigments were stained in 1% phloroglucinol (w/v) in 70% (v/v) ethanol and left overnight in the dark. Tissue was then placed onto microscope slides and hydrochloric acid (10.5M) drops were added to cover the tissue for 5 minutes, then removed and rinsed with sdH₂O. Samples were visualised within 20 minutes of preparation and viewed with a light microscope. Images
are representative of three independent experiments.

2.2.7. Hydrogen peroxide quantification

Hydrogen peroxide was extracted from 50 mg of leaf tissue frozen with liquid nitrogen, as described in Estavillo et al. 2011 and Xing et al. 2007 with some modifications. Frozen tissue was ground into a fine powder and 500 μl of 40mM potassium phosphate buffer (pH 6.5) was added. Samples were vortexed and centrifuged for 15 minutes at 13,000g at 4°C. \( \text{H}_2\text{O}_2 \) quantification was achieved by using a commercial kit (Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit, Invitrogen, Mulgrave, Victoria, 3170). The amplex red reaction mixture (100 μl) was placed into wells within a 96 well fluorescence plate (Microplate 96 well, Greiner Bio-One), mixed with 100 μl of the \( \text{H}_2\text{O}_2 \) containing samples and incubated in the dark for 30 minutes. Fluorescence of the samples was measured using a fluorescence micro-plate reader (FLUOstar Omega, BMG labtech Pty. Ltd. Mornington, Victoria) and results were expressed as \( \mu \text{M H}_2\text{O}_2 \text{ mg}^{-1} \) (FW). For individual experiments, each sample contained three technical replicates. Data presented represents the mean of three independent experiments.

2.2.8. Tissue sampling and RNA extraction

Aerial tissue of irradiated plants was removed from the root systems and a minimum of 80 mg tissue (pooled together from of a minimum of three plants) was frozen in liquid nitrogen and stored at -80°C until required. Tissue samples were taken at 0, 3, 6, 24, 48 and 72 h and total RNA was extracted with a commercially available RNA extraction kit, (SV Total RNA isolation system, Promega, Alexandria, New South Wales). RNA was quantified with a spectrophotometer (Nanodrop ND-1000, NanoDrop, DE, USA), with 1 μl of the RNA sample used to quantify total RNA and the
concentration given as ng/μl and the purity of the sample was assessed by the ratio absorbance of 260/280. The quality was assessed by running RNA on a 1.5% agarose gel in 0.5× TBE buffer.

2.2.9. cDNA synthesis

cDNA was derived from 1 μg of RNA, which was transferred to a sterile RNase free PCR tube, and 200 ng of random hexamer primers (Bioline, Alexandria, New South Wales) was added to the tube and the solution adjusted to a final volume of 12 μl with DEPC treated sdH2O. Tubes were incubated at 70°C for 5 minutes and allowed to cool on ice, and 8 μl of a master mix (1 μl 10mM dNTP , 4 μl 5× reaction buffer, 0.25 μl Tetro Reverse Transcriptase (Bioline, Alexandria, New South Wales,) and 2.75 μl DEPC treated sdH2O was added. Samples were incubated in a thermo-cycler (PCT-100, MJ research Inc, MA, USA) at 42°C for 60 minutes and the reaction was terminated by heating to 70°C for 10 min then cooled to 4°C.

2.2.10. Gene expression analysis using semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription PCR was conducted with a commercially available master mix (GoTaq® Green Master mix, Promega, Alexandria, New South Wales). Primers were created for the genes GST1 (AT1G02930), PRI (AT2G14610), PDF1.2 (AT5G44420) and UBQ5 (AT3G62250) was used as an internal standard as it showed the most stable expression in response to UV-C treatments. PCR amplification took place in a 20 μl reaction (8 μl GoTaq, 10 μl sdH2O, 0.5 μl F primer, 0.5 μl R Primer and 1 μl cDNA). Primers were optimized by determining annealing temperature and the appropriate cycle to ensure the PCR within the exponential phase. PCR conditions of all primer sets contained an initiation step of 94°C for 2 minutes, a denaturing step of 94°C for 30 seconds at the beginning of every cycle and an extension
step of 72°C at 30 seconds. The reaction was terminated by incubating the sample at 4°C. The annealing temperatures and cycles varied with primer sets (Table 2.2). The resulting PCR was run on a 1.5% agarose gel in 0.5× TBE. Images are representative of two independent experiments.

### 2.2.11. Statistical analysis

Statistical analysis of was performed using Sigma plot 11 (Systat Software, San Jose, CA, USA). Statistical analysis of the data of the leaf area, cell death and callose quantifications were subjected to a two-way ANOVA with a Tukey post hoc test, whereas data of the H₂O₂ quantification was subject to a one-way ANOVA with a Tukey post hoc test.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>PCR conditions</th>
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<tbody>
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<td>GST1 F</td>
<td>TGATGTCAGCAACCCAAGCA</td>
<td>Annealing: 54°C Cycles: 26 cycles Fragment size: 497bp</td>
</tr>
<tr>
<td>GST1 R</td>
<td>TGTCGAGCTCAAAGATGGTGAA</td>
<td></td>
</tr>
<tr>
<td>PR1 F</td>
<td>TCGTTCACATAATTCCCACGA</td>
<td>Annealing 54°C Cycles: 31 cycles Fragment size: 462bp</td>
</tr>
<tr>
<td>PR1 R</td>
<td>TGGCTATTTCTCGATTTTTAATCGTC</td>
<td></td>
</tr>
<tr>
<td>PDF1.2 F</td>
<td>TGTAACAAACAACGGGAAATAAAAAC</td>
<td>Annealing 54°C Cycles: 33 cycles Fragment size: 369bp</td>
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<tr>
<td>PDF1.2 R</td>
<td>CATGGCTAAGTTTGCTCCATCA</td>
<td></td>
</tr>
<tr>
<td>UBQ5 F</td>
<td>AAACCCTAACGGGGAAGACCA</td>
<td>Annealing 52°C Cycles: 34 cycles Fragment size: 458bp</td>
</tr>
<tr>
<td>UBQ5 R</td>
<td>TCAAGCTTCAACTCCTTCTTCTTG</td>
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</table>

Table 2.2. Primer sequences of genes of interest used in gene expression analysis and corresponding optimised PCR steps with expected fragment size. Annealing temperatures were chosen for specific primer sets based on a temperature gradient PCR that ranged between 50 and 60°C. PCR cycle numbers were determined by band intensity of PCR reaction every two cycles between 20 and 36 cycles.
2.3. Results

2.3.1. Changes in Arabidopsis morphology following exposure to UV-C radiation

Arabidopsis Col-0 plants exposed to 250, 500 or 1000 Jm\(^{-2}\) of UV-C radiation were assessed to identify morphological symptoms of exposure at each dosage and observations were made at 0, 72, 120 and 168 h. Arabidopsis plants in the control group continually grew, with green healthy foliage and fully expanding leaves across all time points (Figure 2.1a-d). The 250 Jm\(^{-2}\) treatment group showed no observable difference compared to the control group (Figure 2.1e-h). Plants exposed to the 500 Jm\(^{-2}\) treatment showed a slight reduction in the size of plants, this was first noted at 72 h (Figure 2.1j), and growth was further reduced at 120 and 168 h (Figure 2.1k & l), in comparison to 0 Jm\(^{-2}\) control plants. Changes were also observed in the 1000 Jm\(^{-2}\) treated plants, with the stunting of growth first observed at 72 h, when compared to other dosages at the same time point (Figure 2.1n). This consisted of leaf bronzing and chlorosis, increased reflection towards light and slight upward curling of leaves at 120 h (Figure 2.1o). These symptoms were more prominent by 168 h, with leaves showing chlorosis and stunted growth (Figure 2.1p).

2.3.2. Changes in leaf expansion of UV-C exposed leaves

Measurements of leaf area revealed the true extent of the changes in growth of UV-C irradiated leaves. Measurement of the 4\(^{\text{th}}\) - 10\(^{\text{th}}\) rosette leaves permitted the tracking and measurement of leaf expansion of directly irradiated leaves over time. No significant difference was observed at the 0 and 24 hour time-points across any dosage. However, significant reduction in leaf area occurred in plants irradiated with 250 Jm\(^{-2}\) at 120 and 168 h (P<0.05). Leaves exposed to 500 Jm\(^{-2}\) showed significant reductions at 72 h and remained reduced at 120 and 168 h (P<0.05). However, leaves exposed to 1000 Jm\(^{-2}\) demonstrated the earliest reduction at 48 h (P<0.05), and leaf areas remained significantly
Figure 2.1. Responses of Arabidopsis to the different doses of UV-C radiation over 168 h. a-d) 0 Jm$^{-2}$ control plants, e-h) Plants treated with 250 Jm$^{-2}$, i-l) plants irradiated with 500 Jm$^{-2}$, m-p) plants irradiated with 1000 Jm$^{-2}$ showing reduced growth, leaf bronzing (white arrows) and curling (blue arrows) occurring at o) 120 h and p) 168 h, inset: p i) leaf bronzing, p ii) leaf curling. Bars represent 1 cm, inset 1 mm.
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reduced when compared to the control at 72, 120 and 168 h (P<0.05) (Figure 2.2). Also noted from this same data set, was a delay in leaf expansion in UV treated plants when compared to the control plants. When comparisons of individual treatments were made to their initial 0 h time points, the appearance of the first significant increases in leaf area occurred later in the UV treatments (Figure 2.2 seen as +). The earliest significant increase in leaf area occurred at 48 h in the 0 Jm⁻² control, and significant increases in leaf area occurred all time all remained time points when compared to the 0 h in the control group (P<0.05). Whereas, the earliest significant increase in leaf area when compared to the respective 0 h time points in leaves exposed to: 250 Jm⁻² occurred at 72 h, 500 Jm⁻² at 120 h (P<0.05), and no significant increase occurred 1000 Jm⁻² treatment was seen at any time point.

### 2.3.3. Assessment of leaf tissue damage

In leaf tissue stained with LTB, cell death was evident in all UV-C irradiated tissues. No cell death was observed in the 0 Jm⁻² control leaves at any time point (Figure 2.3a-d). Leaves exposed to the 250 and 500 Jm⁻² treatments did not show the severe morphological symptoms that were observed 1000 Jm⁻² treatment. However, UV-C induced cell death was apparent in all UV treated leaves. No cell death was observed in leaves in the first 48 h following irradiation in the 250 Jm⁻² treatment (Figure 2.3e-g). Cell death first occurred in leaves exposed 250 Jm⁻² at 72 h (Figure 2.3h). Leaves exposed to the 500 Jm⁻² treatment did not show any signs of cell death at 0 or 24 h (Figure 2.3i & j), and cell death first appeared at 48 h (Figure 2.3k) and present at 72 h (Figure 2.3l). No cell death was observed at 0 h in leaves treated with the 1000 Jm⁻² treatment (Figure 2.3m), but cell death was apparent by 24 h (Figure 2.3n) and the number of stained cells continued to increase at 48 and 72 h (Figure 2.3o & p). Cell death was only located in the mesophyll layers of the leaf tissue with no cell death occurring in the adaxial epidermal layer.
Figure 2.2. Leaf area measurement of Arabidopsis plants irradiated with UV-C over 168 h. Changes in leaf areas over time in the 0 Jm$^{-2}$ (bars with diagonal lines), 250 Jm$^{-2}$ (white bar), 500 Jm$^{-2}$ (grey bar) and 1000 Jm$^{-2}$ (black bar). * represents significant differences compared to 0 Jm$^{-2}$ control at each corresponding time point. + represents significant difference of growth of individual treatments when compared to their corresponding 0 h time point. Error bars represent ± SE.
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Figure 2.3. Examination of the extent of cell death caused by UV-C exposures in Arabidopsis leaf tissue occurring over 72 h. Cell death is seen as dark stained collapsed cells. a-d) 0 Jm$^{-2}$, e-h) 250 Jm$^{-2}$, cell death occurred at h) 72 h, i-l) 500 Jm$^{-2}$, Cell death observed at k) 120 and l) 168 h, m-n) 1000 Jm$^{-2}$, cell death observed at n) 72 h, o) 120 h and p) 168 h. Bar represent 100 μm.
2.3.4. Quantification of cell death in UV-C treated leaves

A significant amount of cell death was present in the 1000 Jm\(^{-2}\) treatment when compared to all other treatment groups at 24 h (P<0.001), significant increases in cell death also occurred in leaves treated with 500 and 1000 Jm\(^{-2}\) at 48 h when compared to the control and 250 Jm\(^{-2}\) (P<0.001). This trend continued at 72 h with the exception that significant cell death was also present in leaves exposed to 250 Jm\(^{-2}\) as well as 500 and 1000 Jm\(^{-2}\) (P<0.001) (Figure 2.4). Quantification of cell death within leaf tissue did not show cell death in the 0 h time point in any treatment (data not shown) nor was it evident in the non-treated 0 Jm\(^{-2}\) control across any time point.

2.3.5. Induction of callose deposition following UV-C treatments

Leaves were stained with aniline blue to reveal if callose deposition had occurred along the cell wall. Deposition of callose occurred in the epidermal and mesophyll cell layers and comparisons of treatments demonstrate clear dose dependency. No observable change was noted in the 0 Jm\(^{-2}\) control at any time point (Figure 2.5a-d) or at 0 h in any treatment (Figure 2.5 e, i, m). The 250 Jm\(^{-2}\) treatment showed the least amount of callose producing cells when compared to the plants exposed to the 500 and 1000 Jm\(^{-2}\) treatments, with callose deposits appearing as small individual clusters of spots on the cell wall at 24 and 48 h (Figure 2.5f & g). This pattern was also seen at 72 h along with callose sheets lining portions of the cell wall (Figure 2.5h). The 500 Jm\(^{-2}\) group demonstrated a similar trend, but with spots appearing in the 24 and 48 h with areas of concentrated callose deposits (Figure 2.5j & k), and partial encasements of the affected cell at 72 h (Figure 2.5l). The 1000 Jm\(^{-2}\) contained the most callose producing cells, with concentrated area of callose as small clusters present on the cell walls at 24 and 48 h (Figure 2.5n & o). At 72h, partial encasements of cells were observed along the wall of surviving cells (Figure 2.5p).
Figure 2.4. Quantification of cell death in irradiated Arabidopsis leaf tissue. Counts represent cell death present in microscopic fields of view, at 100× magnification in the 0 Jm$^{-2}$ (bars with diagonal lines), 250 Jm$^{-2}$ (white bar), 500 Jm$^{-2}$ (grey bar) and 1000 Jm$^{-2}$ (black bar) treatments. Asterisks represent significant difference from the 0 Jm$^{-2}$ control (P <0.001), error bars represent ± SE.
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**Figure 2.5.** Callose deposition in response to UV-C irradiation in Arabidopsis leaf tissue over 72 h. Callose deposition is seen as bright blue fluorescence, initially appearing as spots along the cell wall before forming into sheets. a-d) 0 Jm$^{-2}$ control leaves, (e-f) 250 Jm$^{-2}$, callose observed at f) 24 h, g) 48 h and h) 72 h. i-l) 500 Jm$^{-2}$, callose observed at j) 24 h, k) 48 h and l) 72 h. m-p) 1000 Jm$^{-2}$, callose observed at n) 24 h, o) 48 h and p) 72 h. Bar represents 100 μm.
2.3.6. Changes in callose contents in UV-C treated leaves

To further examine the presence of callose, β-1, 3 glucans were extracted and quantified and demonstrated a dose dependent relationship within UV treated leaf tissue. A significant increase in β-1, 3 glucans concentrations occurred at 24 h in the 500 and 1000 Jm⁻² treated leaves when compared to the control group (P<0.05). Dose dependence was noted at 24 h; with twice the amount of β-glucans in the 1000 Jm⁻², when compared to the 500 Jm⁻² treatment (95.3 ng/mg and 47.8 ng/mg, respectively) (Figure 2.6). At 48 h, β-1, 3 glucan concentration in leaves treated with 500 and 1000 Jm⁻² remained significantly higher than the control (P<0.05) and reached a maximum concentration of 64.3 ng/mg in the 500 Jm⁻² and 216.8 ng/mg in the 1000 Jm⁻². The trend observed at 48 h remained at 72 h with significant differences seen in the 500 and 1000 Jm⁻² groups (P<0.05). However, the concentrations of β-1, 3 glucans at these dosages did not exceed that seen at 48 h with 64.2 ng/mg in the 500 Jm⁻² and 199.2 ng/mg in the 1000 Jm⁻². However, no significant difference was seen in the 250 Jm⁻² across any time (Figure 2.6).

2.3.7. Presence of lignin in UV treated tissue

Phloroglucinol was used to test for the presence of lignin, where pink staining confirms lignin presence (not shown). Pink staining was only observed in lignified vascular tissue in stained leaves, with no staining occurring in cell walls of epidermal or mesophyll cells of 0 Jm⁻² controls at 0 or 168 h (Figure 2.7a & b) or the 0 h of UV-C treated leaves (Figure 2.7 c). The presence of lignin was not observed in surviving cells in UV treated leaves; however, some pale orange staining was noted in the cell walls of dead cells. This staining may indicate the presence of a lignin-like or precursor lignin molecule produced by severely damaged cells prior to cell death (Figure 2.7d).
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**Figure 2.6.** Total amount of extracted β-1, 3 glucans from UV-C treated *Arabidopsis* leaves. Callose concentrations expressed as β-1, 3 glucans equivalents in 0 Jm$^{-2}$ (bars with diagonal lines), 250 Jm$^{-2}$ (white bar), 500 Jm$^{-2}$ (grey bar) and 1000 Jm$^{-2}$ (black bar). Asterisks represent statistical difference from the 0 Jm$^{-2}$ control within each time point (P <0.05). Error bars represent ± SE.
Figure 2.7. Phloroglucinol test to determine the presence of lignin in irradiated leaves. 0 Jm\(^{-2}\) control mesophyll cells at a) 0 h and b) 168 h. Mesophyll cells treated with 1000 Jm\(^{-2}\) at c) 0h and d) 168 h with pale orange staining present in dead cells (black arrows), no staining was observed in surviving cells. Bar represents 50 μm.
2.3.8. **Hydrogen peroxide quantification in UV-C irradiated leaf tissue**

Quantification of hydrogen peroxide in UV-C treated leaves demonstrated increases in hydrogen peroxide content over time. No significant difference was observed between control and UV-C treated tissue at 0 h. Variability in concentration of H$_2$O$_2$ at 6 h made it difficult to determine the extent of H$_2$O$_2$ concentrations at this time point. At 24 h a trend emerged, with significant increases of H$_2$O$_2$ concentrations in the plants exposed to 500 and 1000 Jm$^{-2}$ (P<0.05) when compared to the 0 Jm$^{-2}$ control plants. By 48 h, H$_2$O$_2$ concentrations in plants exposed to 1000 Jm$^{-2}$ remained elevated compared to the control and other treatments, but no significant differences was found for any treatment (Figure 2.8).

2.3.9. **Defence gene expression following UV-C exposure**

The expression of the key pathogen defence related marker genes GST1 (ROS marker), PR1 (SA defence pathway marker) and PDF1.2 (JA defence pathway marker) were examined. GST1 showed increased expression at 3 and 6 h across all UV-C treatments, indicating an early oxidative burst following irradiation (Figure 2.9a). PR1 demonstrated a slight increase in expression at 6 and 48 h in the 250 Jm$^{-2}$ treatment when compared to the control, but the 500 and 1000 Jm$^{-2}$ treatments showed a considerable increase in PR1 expression by 6 h and this expression remained elevated for all the subsequent time-points (Figure 2.9b). Expression of PDF 1.2 in the 0 Jm$^{-2}$ control tissue increased at 3 and 72 h. A similar pattern was also seen in plants exposed to 250 Jm$^{-2}$ with high expression at 3 h, decreasing at 6 and 24 h then an increase at 72 h. In both the in the 500 and 1000 Jm$^{-2}$ treated plants increased expression was seen between 6-48 h followed a decrease in expression at 72 h (Figure 2.9c). UBQ5 was used as an internal standard and showed stable expression across each treatment, slight reductions were noted in the 250 Jm$^{-2}$ treatment at 48 and 72 h.
Chapter 2: Morphological, cellular and molecular responses of *Arabidopsis thaliana* to UV-C radiation

**Figure 2.8.** Hydrogen peroxide concentration within UV-C Arabidopsis leaves over 48 h. $\text{H}_2\text{O}_2$ concentration present with the 0 Jm$^{-2}$ (diagonal lines bar), 250 Jm$^{-2}$ (white bar), 500 Jm$^{-2}$ (grey bar) and 1000 Jm$^{-2}$ (black bar) treatments. Asterisks represent significant difference compared to 0 Jm$^{-2}$ control within each time point (P $<$ 0.05). Error bars represent ± SE.
Figure 2.9. Gene expression profile of UV irradiated plants over a 72 hours. a) ROS marker gene GST1, b) salicylic acid defence pathway marker gene PRI, c) jasmonic acid defence/ethylene pathways marker PDF1.2, d) UBQ5 was used as an internal standard (housekeeping gene).
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2.4. Discussion

This study unravelled some of the roles that UV-C irradiation has in the activation of mechanisms of pathogen defence, and examined the changes in the growth of *Arabidopsis* due to the exposure to sub-lethal doses of UV-C. The time points presented in these experiments were crucial for determining what effect UV-C treatments have on activating *Arabidopsis* defences before pathogen infection. The data presented reveals that UV-C exposure results in cell death, \( \text{H}_2\text{O}_2 \) production and callose synthesis within the first 24 h following irradiation. More importantly, the increases in expression of pathogen defence related genes *PR1* and *PDF1.2* following high exposures, indicates the activation of the SA and the JA/Et defence pathways.

2.4.1. Sub-lethal dosages of UV-C radiation caused changes to the growth and morphology of *Arabidopsis*

When compared to controls, plants exposed to the low and mid-level dosages of UV-C radiation did not show any observable signs of severe stress, yet plants exposed to the highest treatment clearly showed morphological changes. In previous studies, upward leaf curling, leaf bronzing and glazing have been observed in plants exposed to UV-B and UV-C radiation (Wilson *et al.*, 1993, Yalpani *et al.*, 1994, Landry *et al.*, 1995, Mackerness *et al.*, 1999, Nara *et al.*, 2002). Leaf curling has been suggested to be a developmental response in order to limit the amount leaf area exposed to low levels of UV-B radiation (Jansen *et al.*, 1998), however it is likely in this case that the relatively high dosages of UV-C caused curling due to the presence of cell death that may be caused by oxidative damage (Tang *et al.*, 2010). Leaf bronzing has been suggested to involve the oxidation of phenolics in damaged cells (Cline *et al.*, 1966), and although this has been widely reported in many UV irradiation studies, very little work has been undertaken to identify what phenolics have been oxidised as a result of irradiation. Leaf
glazing (increased reflectivity of the upper leaf surface), may be attributed to increases in the epicuticular wax layer, which increase light reflection to limit light penetration (Kakani et al., 2003, Wang et al., 2007).

The most severe morphological symptoms, such as leaf curling and bronzing, were observed in Arabidopsis plants exposed to 1000 Jm\(^{-2}\) of UV-C radiation, and little change was noted in the lower dosages of UV-C exposure, these symptoms illustrate the similarities in UV overexposure symptoms in both UV-B and C irradiation and that these symptoms are not exclusive to UV-B radiation. This also demonstrates that the 1000 Jm\(^{-2}\) dosage causes significant damage to the plant and exposures above this dosage may not be suitable for studying the sub-lethal effects of UV-C radiation.

Reduction in leaf areas of UV-C exposed plants revealed subtle differences in the timing and the amount of expansion across all treatment groups. The trend observed showed that the timing of decrease in leaf expansion was associated with the increase in UV-C dosages. This was seen in the 250 Jm\(^{-2}\) dosage where significant reductions did not occur until 120 h, whereas the reductions in the 500 Jm\(^{-2}\) appeared earlier at 72 h, and the most severe reductions in the 1000 Jm\(^{-2}\) starting at 48 h when compared to the 0 Jm\(^{-2}\) control. This trend is not surprising, as reduction in leaf area and biomass has been reported in a variety of plant species that were exposed to increased supplemental UV-B dosages (Cen et al., 1990, Wargent et al., 2009). When individual groups were compared to their 0 h time points, delayed leaf expansion was observed in the 250 and 500 Jm\(^{-2}\). The first significant increase in leaf expansion occurs at 48 h in the 0 Jm\(^{-2}\) control plants, yet this increase in leaf expansions is delayed to 72 and 120h in 250 and 500 Jm\(^{-2}\) exposed plants, respectively. No increase in leaf area was seen in the 1000 Jm\(^{-2}\) at any time point during this experiment. The delay in growth seen in the 250 and 500
Jm$^{-2}$ exposed leaves could be the result of the time required for the completion of cellular repair, an indicator of this is DNA damage repair, with less DNA damage occurring in the plants exposed 250 Jm$^{-2}$ and is repaired faster compared to the 500 Jm$^{-2}$ treatment group (Kunz et al., 2008). However, the lack of growth seen in the 1000 Jm$^{-2}$, could be the result of more serious damage to the leaf tissue, such as cell death.

The appearance of cell death in the mesophyll caused by UV-C treatments did provide some explanation of the change in growth of affected leaves. Plants treated with 1000 Jm$^{-2}$ displayed the most extensive cell death as a result UV-C irradiation, this is not surprising as UV-C is a potent inducer of cell death at much higher dosages (Gao et al., 2008, He et al., 2008). Cell death present in irradiated tissue does also follow a dose dependent manner when quantified. Also noted was the randomised distribution of the UV-C induced cell death, this type of randomised pattern of cell death has also been observed in UV-C studies in various plant species where dead cells are surrounded by relatively healthy surviving cells (Bornman et al., 1983, Stefanato et al., 2009).

2.4.2. Callose deposition and alterations to the cell wall

Callose deposition is induced by mechanical and chemical stressors and by pathogens (Galway et al., 1987, Wissemeier et al., 1987, Jacobs et al., 2003). Surprisingly, surviving cells in UV-C treated tissue deposited callose along cell walls. This was unusual considering no mechanical, chemical or biotic stress was applied to these plants that would normally induce callose deposition and to my knowledge callose deposition has not been reported to occur in response to UV irradiation. Callose production caused by chemical stresses or elicitors has been thought to be linked with electrolyte leakage due to the disruption or lipid peroxidation of the cell membrane (Köhle et al., 1985, Kauss et al., 1986, Piršelová et al., 2013). It seems likely that the
UV-C exposures used in this study were sufficient to directly or indirectly (possibly through the generation of reactive oxygen species) alter integrity of the cell membrane, which may have led to the deposition of callose, however the exact chain of events that leads to production of callose is still yet to be established. Callose first appeared in affected cells as small spots along the cell wall, similar deposition was observed in response to ultrasound and wounding experiments where callose was initially being synthesised around pit fields at earlier time points (Currier et al., 1964, Galway et al., 1987), before forming sheets of callose fortifying sections of the cell wall. However, it is unclear if the callose deposition observed in response to UV-C irradiation is also being synthesised surrounding pit fields. When quantified, it became evident that callose production in both the 500 and 1000 Jm$^{-2}$ treated leaves, had reached its maximum at 48 h and callose concentrations plateau at 72 h, this indicates that callose synthesis has stopped or slowed. This suggests that rapid callose deposition in response to UV-C occurs within the first 48 h after exposure within affected cells. What is unclear at this stage is if the halting or slowing of callose deposition at the 72 h time point is due to the repair of the stressed cell and is no longer produced or the involvement of callose degradation.

The presence of lignin was tested with phloroglucinol, lignin appears pink/red when stained with phloroglucinol and reacted with HCl (Albersheim et al., 2011). Staining did not show a positive colour change for lignin deposition of cell walls of surviving cells. However, partial staining was observed in the remnant cell wall of the dead cells, which appear pale orange when stained. This may indicate the presence of a precursor lignin like material that was deposited onto the cell wall before cell death, similar staining was also noted in cells which had undergone HR in response to pathogen attack which also contained a lignin like material (Soylu, 2006).
composition of this material is not known, and may warrant further investigation to determine the exact nature of this compound.

2.4.3. Changes in hydrogen peroxide activity

Generation of reactive oxygen species in particular hydrogen peroxide is known to be induced by UV-C radiation at high dosages and with repeat exposures (Mahdavian et al., 2008, Tang et al., 2010). However, according to the data gained from this study it appears that relatively low dosages of UV-C can stimulate H$_2$O$_2$ generation. Quantification of hydrogen peroxide contents in irradiated tissue provided evidence for the activity of ROS accumulation. The clearest increase occurred at 24 h, with a significant increase occurring in plants exposed to 500 and 1000 Jm$^{-2}$, with no significant increase occurring in the 250 Jm$^{-2}$ at this time point. Although an increase is noted at 24 h, this information does not provide enough information to decipher the events following irradiation and further analysis was needed. This was addressed by examining ROS inducible gene activity. The increase of reactive oxygen species following UV-C irradiation becomes clearer when examining the expression of the oxidative burst marker gene GST1. Expression of GST1 is associated with increases in reactive oxygen species in both biotic and abiotic stresses as the phi class of the GST family is involved in protecting cells from oxidative stress, and is used as a marker of ROS activity (Sharma et al., 1994, Marrs, 1996, Sweetlove et al., 2002, Love et al., 2005, Rookes et al., 2008, Sappl et al., 2009). Increase expression at 3 and 6 h indicates an oxidative burst occurring after irradiation in all UV-C treated tissues. This initial oxidative burst is consistent with a previous study, where rose cell suspensions irradiated with 558 Jm$^{-2}$ UV-C demonstrated a peak in H$_2$O$_2$ concentrations at 60-90 minutes after exposure (Murphy et al., 1990). This early increase in GST1 caused by increased ROS levels may explain the subsequent increase of PRI, as increased levels
of \( \text{H}_2\text{O}_2 \) has been associated with increased levels of SA accumulation and \( \text{PRI} \) induction (Leon et al., 1995, Mukherjee et al., 2010). However, the 250 Jm\(^{-2}\) treatment did clearly demonstrate early \( \text{GST1} \) activation, yet very little change was seen at later timer points in relation to \( \text{PRI} \) expression, when compared to the higher dosages. This indicates that even though \( \text{GST1} \) expression occurs in the 250 Jm\(^{-2}\), the oxidative burst and/or the damage caused by UV-C that had occurred was not enough to induce a sustained expression of \( \text{PRI} \).

### 2.4.4. Increases in defence gene expression

Expression of \( \text{PRI} \) showed the most significant increases in the 500 and the 1000 Jm\(^{-2}\) treatments that occurred at 6 h and expression remained increased for the remainder of the time course. This demonstrates the involvement of the salicylic acid pathway following UV-C exposure, and this result is consistent with other studies that showed the activation of \( \text{PRI} \) and SA accumulation in plants exposed to supplemental UV-B or a single dose of UV-C irradiation in Arabidopsis (Mackerness et al., 1999, Shapiro et al., 2001, Nawrath et al., 2002, Fragnière et al., 2011). The expression of \( \text{PDF1.2} \) in response to treatments of 500 and 1000 Jm\(^{-2}\) demonstrates that UV-C irradiation can stimulate jasmonic acid/ethylene pathway normally associated with wounding and defence against necrotrophic pathogens (Penninckx et al., 1998, Bari et al., 2009). Expression of \( \text{PDF1.2} \) has also been shown to be induced by UV-B radiation in Arabidopsis, with increases in JA and Et concentrations preceding its expression (Mackerness et al., 1999). In tomato plants, UV-C exposure is thought to induce a wounding-like response mediated by the octadecanoid signalling pathway (Conconi et al., 1996). If UV-B radiation can induce JA and ET, and UV-C can mimic a wound response in tomato then it is possible that the UV-C dosages used in this study were causing similar effects that would account for the induction of \( \text{PDF1.2} \).
All UV-C exposures induced changes within the leaf tissue, the most severe UV-C exposure symptoms and consisted of reductions in leaf growth and significant amounts of damage to the leaf tissue, and occurred in plants treated with the 1000 Jm⁻², which is lower than most dosages that have been previously reported. The most puzzling outcome of this work was the production of callose in response to UV-C irradiation. Although this work hypothesises that the alteration of cell membrane integrity, possibly through the generation of H₂O₂, might lead to callose formation, further work needs to be undertaken to determine if this is the cause of callose production in response to UV-C radiation. The presence of callose alone raises questions about the potential role it plays in this interaction and whether other wavelengths of UV or even high light could stimulate its production. Expression of PRI had previously been shown to be induced by UV radiation; this work shows that PRI induction can be induced by UV exposures lower than 1000 Jm⁻². The expression of PDF1.2 may also signify that the UV-C exposures are sufficient to induce a wound-like state that activates the jasmonic acid/ethylene pathways.
Chapter 3: New insights into the responses of Arabidopsis to infection by the biotrophic oomycete *Hyaloperonospora arabidopsidis*

**Abstract**

*Hyaloperonospora arabidopsidis* is a naturally occurring pathogen of *Arabidopsis thaliana*. The Arabidopsis ecotype Col-0 was infected with the virulent (Noks1) and avirulent (Cala2) isolates of *H. arabidopsidis*, in order to track the growth of the pathogen over 168 hours and observe the host responses. In response to pathogen attack callose, lignin and H$_2$O$_2$ production occurred in both the compatible and incompatible interactions. In the compatible interaction callose deposits first formed collars around the neck of the haustoria at 48 hpi and progressed to fully encase the haustoria in response to infection by the Noks1 isolate. In the incompatible interaction callose deposition occurred in cells surrounding the resulting hypersensitive lesion caused by the recognition of the Cala2 isolate also at 48 hpi. When quantified, significantly more callose was present in the compatible interaction when compared to incompatible interaction, and this increase is most likely associated with the prolific growth of the compatible Noks1 isolate. Lignin was also detected in both interactions; however it was only detected after 72 hpi with complete lignification of infected cells only in the compatible interaction, lignin was also present within hypersensitive cells in the incompatible interaction. Activity of *GST1* in infected plants demonstrated an induction of an early ROS burst at 3-6 hpi in both the compatible and incompatible interactions, yet a second ROS burst was present at 72 hpi only in the incompatible interaction in response to Cala2 infection which corresponded with the prominent appearance of the hypersensitive response and increased *PR1* induction when compared to infection by the compatible isolate.
3.1. Introduction

Hyaloperonospora arabidopsidis (formerly Peronospora parasitica and Hyaloperonospora parasitica) is an oomycete plant pathogen that causes downy mildew in Arabidopsis thaliana. Although this particular pathogen is not responsible for considerable losses in agriculture it has proven to be an extremely valuable model patho-system for understanding the interactions between plants and biotrophic pathogens. This interaction has been the subject of reviews, spanning a wealth of information that has been gained from researching this system (Slusarenko et al., 2003, Coates et al., 2010). However, comprehensive comparisons between the responses of the host to infection by the virulent and avirulent isolates of H. arabidopsidis in relation to the pathogens growth, timing of a wide range of key defensive markers and the extent of defence activation between the compatible and incompatible interactions has not been fully explored.

The differences in compatibility (virulent pathogen/susceptible plant) and incompatibility (avirulent pathogen/resistant plant) have been studied in a variety of host-pathogen interactions, and depending on the pathogens lifestyle can elicit differing responses in the host. Plant pathogens can be classed based on the infection strategy and the subsequent damage cause to the host due to the pathogen life cycle. Generally, most pathogens can be classed as necrotrophic, hemi-biotrophic or biotrophic pathogens. Necrotrophic pathogens often kill host cells and tissue in-order to feed on the released nutrients, whereas, hemi–biotrophic pathogens initially infect and feed on living cells before the necrotrophic stage beings, and biotrophic pathogens require living tissue in order to gain nutrients (Perfect et al., 2001, Glazebrook, 2005). Due to the different infection styles of plant pathogens, plants can activate different defence pathways to
Biotrophic pathogens, such as *Hyaloperonospora arabidopsidis* require living cells to gain nutrients, however, evasion and suppression of the host defences is essential for the successful infection in order for it to gain nutrients and complete its lifecycle (Panstruga, 2003). Much work had been undertaken to understand the interaction within the Arabidopsis and *H. arabidopsidis* patho-system. In *H. arabidopsidis* infection, the initial stages of infection with both compatible and incompatible interactions are very similar; with both interactions requiring the penetration of the host epidermis and infection of host cells (Koch *et al.*, 1990, Parker *et al.*, 1996). In a compatible interaction, it is essential for the pathogen to gain nutrients from the host while remaining undetected by the host. Much like many other biotrophs, *H. arabidopsidis* gains nutrients from host cells by penetrating the cell wall with specialised feeding structures known as haustoria (Panstruga, 2003). The haustoria has also been shown to be involved in host defence suppression with the release of effectors from the haustoria of rust fungi entering the host cells of *Uromyces vignae* and *Uromyces fabae* (Voegele *et al.*, 2003, Kemen *et al.*, 2005). Although infiltration of the cell requires penetration of the cell wall, it does not however, result in the penetration of the host’s plasma membrane. Instead, the invading haustorium causes the invagination
of the host’s cell membrane and produces an extra haustorial matrix that encases and separates the haustoria and the host cell membrane (Mims et al., 2004).

However, in an incompatible reaction recognition of pathogen effectors by host R proteins results in a rapid activation of defences and ultimately leads to the activation of salicylic acid defence pathway and the hypersensitive response which results in cell death of infected cells. An example of pathogen recognition occurs with the Arabidopsis Col-0 ecotype, where the two RPP2 R proteins recognise the \textit{H. arabidopsidis} effector protein ATR2 present in the \textit{H. arabidopsidis} Cala2 isolate (Sinapidou et al., 2004). This recognition leads to the rapid accumulation of salicylic acid, which leads to the restriction of the pathogen by activation of HR (Parker et al., 1996, Sinapidou et al., 2004).

Although the recognition and activation of the R gene mediated defences stops pathogen invasion, plants involved in the compatible interaction are not defenceless and activation of basal defences can occur. Both the compatible and incompatible interactions induce similar defensive mechanisms such as: ROS bursts, callose deposition, lignification and increases in pathogen defence genes. However, the timing and distribution can vary between both interactions. An initial ROS burst has been reported in a variety of plants in response to both virulent and avirulent pathogens, a second much larger ROS burst occurs in response to avirulent pathogens is believed to be associated the activation of defences (Able et al., 2000, Torres et al., 2006). The deposition of callose is observed in both the compatible and incompatible interactions appearing at the sites of infection. In the compatible interaction, callose surrounds the haustoria of virulent isolates of \textit{H. arabidopsidis} (Parker et al., 1993, Donofrio et al., 2001, Dong et al., 2008). In an incompatible interaction callose was observed in cells
surrounding the HR lesion caused by the avirulent pathogens *Pseudomonas syringae* and *Phytophthora infestans* in the leaves of Arabidopsis and potato respectively (Ahn *et al.*, 2007, Vleeshouwers *et al.*, 2000). Although the role of callose deposition in plant pathogen defence is not fully understood, it is thought that its presence can act as physical barrier to slow pathogen infection (Stone *et al.*, 1992). In some cases lignification of cell walls was also observed in hypersensitive cells in plants infected with an avirulent strain of *Pseudomonas syringae* and avirulent *H. Arabidopsidis*, yet little else is known concerning the role of lignification in response to virulent isolates of *H. arabidopsidis* (Mauch-Mani *et al.*, 1996, Mohr *et al.*, 2007).

The aim of this study is to determine the responses and timing of key events that lead to susceptibility or resistance toward the biotrophic pathogen *H. arabidopsidis* and to set a baseline for a pathogen susceptibility/resistance profile, which could be used to characterise the level of susceptibility or resistance caused by further treatments. What is not currently known is if the biphasic H$_2$O$_2$ peak that has been shown previously in other patho-systems in response to an avirulent pathogen, could occur in response to the incompatible interaction between *H. arabidopsidis* and Arabidopsis. Although callose has been observed in many studies of *H. arabidopsidis* and other pathogens no attempt has been made to extract and quantify the callose contents that are present in the compatible and incompatible interactions in order to further examine its role in plant pathogen interactions. Examining the growth stages of the pathogen during its colonisation of the host, and correlating those to certain host responses will allow for the tracking of infection and the host responses within a defined time course.
3.2 Materials and Methods

3.2.1. Reactivation of *H. arabidopsidis*

*H. arabidopsidis* isolates were obtained from dried infected plant material containing oospores of Noks1 or Cala2, and was kindly provided by Dr. Mary Coates and Professor James Beynon (University of Warwick, UK). Ground dried leaf tissue was scattered onto moistened soil that was sprayed with dH$_2$O; Arabidopsis seeds (Ws-eds1) originally obtained from Professor Eric Holub (University of Warwick, UK), were sprinkled on top of the dried material and then misted dH$_2$O. The pots were sealed in transparent plastic containers and placed into growth chambers at 16°C with a 10/14 h photoperiod and seedlings were misted with dH$_2$O every 24 hours. After 7 days the seedlings were examined under stereo-microscope for the appearance of conidiophores over a period of 2-3 weeks (Koncz *et al.*, 1992). When sufficient conidiophores were present on multiple plants, the pathogen was transferred to new Ws-eds1 plants that were between 2-4 weeks old. Prior to transfer of pathogen, new uninfected plants were misted with dH$_2$O, and leaves that contained spore-producing conidiophores were excised and rubbed onto the surface of the new uninfected leaves.

3.2.2. Maintenance and inoculum preparation of *H. arabidopsidis*

Both isolates were maintained on 2-4 week old Ws-eds1 plants in separate sealed transparent containers and new plants infected every week, conidiospores were transferred to new plants using either the rub inoculation method, or by creating an inoculum (Koch *et al.*, 1990). *H. arabidopsidis* conidiospore inoculum was derived from heavily sporulating leaves that were excised and placed into 6 x 1.5 ml microfuge tubes, with at least three leaves per tube, and filled with 1 ml of sterile dH$_2$O. Each tube was vortexed and the leaf material removed and the tubes centrifuged in a bench top
centrifuge at 6,000 rpm for five minutes. The supernatant was removed from each tube (0.5 ml each) the conidiospores were resuspended, and the suspensions were condensed into two tubes and centrifuged at 6000 rpm for five minutes. The supernatant was removed and the conidiospores were resuspended in 150 μl of sdH2O and condensed into single tube, and the concentration of the conidiospores was counted using a haemocytometer with the inoculum adjusted to a final concentration of 1×10^5 spores/ml.

3.2.3. Pathogen inoculation and growth conditions of experimental plants

Arabidopsis Col-0 plants (27 days old) were inoculated with *H. arabidopsidis* spores. Four leaves per plant were inoculated with a 2 μl drop of the inoculum (1×10^5 spores/ml) of Noks1 (virulent) or Cala2 (avirulent) isolate or mock inoculated with sterile dH2O (sdH2O). Infected plants were placed into sealable transparent containers and returned to growth conditions as described in section 3.2.1.

<table>
<thead>
<tr>
<th>Plant age (days)</th>
<th>26</th>
<th>27</th>
<th>28</th>
<th>29</th>
<th>30</th>
<th>31</th>
<th>32</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Time points (hpi)</td>
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<td>24</td>
<td>48</td>
<td>72</td>
<td>120</td>
<td>168</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Table 3.1.** Experimental time points used in this study following pathogen inoculation in relation to plant age. The age of the plants used in all experiments is represented in the top row. Experimental time points following inoculation used during the experiment represented in the bottom row expressed as hours post inoculation (hpi).
3.2.4 Visualisation of pathogenic structures and cell death

Macroscopic observations of pathogen structures were conducted 168 hours post inoculation (hpi), and images acquired under a stereo microscope (Zeiss 2000-C Stemi, Zeiss, NSW, Australia). Infected leaf tissue was excised at 0, 24, 48, 72, 120 and 168 hpi, and placed into 80% (v/v) ethanol, with daily changes for 7 days to remove photosynthetic pigments. Tissue was then stained with LTB and viewed under light microscopy as described in section 2.2.4., images presented are representative from three independent experiments. Measurement of pathogen hyphal length was based on the method of Soylu et al., 2004, with minor modifications. Pathogen growth was quantified by counting the amount of cells parallel to the hyphal length at 200× magnification, in an area of 1.5 mm², and the hyphal length was counted to a maximum of 20 cells due to the prolific growth of the pathogen. The sizes of the HR lesions were quantified by counting the number of dead cells present within a lesion. Both hyphal length and HR cell death was counted from at least three infection sites within a representative leaf, and replicated with three independent experiments.

3.2.5 Callose visualisation and quantification

Infected leaves were excised at 0, 24, 48, 72, 120 and 168 hpi, placed in 80% (v/v) ethanol which was changed daily, and the tissue stained with aniline blue as described in section 2.2.5. The formation of callose collars and encasements was quantified from aniline blue stained images at 200 × magnification over the course of the infection at 48, 72, 120 and 168 hpi. All haustoria present in the image were counted and the percentages of collared and encased haustoria calculated. Data shown was expressed as percentage callose formation per field of view, data obtained from a representative leaf from three independent experiments. Callose concentrations were
Chapter 3: New insights into the responses of Arabidopsis to the biotrophic oomycete *H. arabidopsidis*

quantified using the Kohler *et al.* 2000 method as stated in section 2.2.5. Data presented represent the mean of four independent experiments.

### 3.2.6. Lignin staining and visualisation

Lignin localisation was visualised by phloroglucinol staining of infected leaves. Excised leaves were placed in 80% (v/v) ethanol at 0, 24, 48, 72, 120 and 168 hpi to remove photosynthetic pigments. Once cleared, the leaves were stained with 1% phloroglucinol (w/v) in 70% (v/v) ethanol as described in section 2.2.6. Images presented are representative of three independent experiments.

### 3.2.7. Visualisation and quantification of hydrogen peroxide

To visualize hydrogen peroxide production in cells, infected leaves were excised from plants at 0, 24, 48 and 72 hpi and stained with 3, 3 diaminobenzidine (DAB). DAB stained tissue was stained according to the method of Thordal-Christensen *et al.* 1997, with minor modifications. Leaves were excised and floated in 1 mg/ml 3, 3 diaminobenzidine (Sigma-Aldrich Pty. Ltd, Sydney, Australia), at pH 3.8 and returned to normal growth conditions described in section 3.2.1 for one hour. To remove photosynthetic pigments leaves were then placed in 80% (v/v) ethanol with daily changes until the tissue was clear. The decolourised tissue was mounted on a microscope slide with 50% glycerol and viewed under a light microscope; H$_2$O$_2$ was viewed as a red-brown precipitate. Images presented are representative of 3 independent experiments. Hydrogen peroxide was extracted from 50 mg of infected leaf tissue and collected and frozen in liquid nitrogen at 0, 3, 6, 24, 48 and 72 hpi and quantified as described in section 2.2.7. The quantification of H$_2$O$_2$ represents the mean of three independent experiments.
3.2.8. Tissue sampling and RNA extraction

Aerial tissue of inoculated plants was removed from the root systems and minimum of 80 mg (pooled together from each of a minimum of three plants) was collected, snap frozen in liquid nitrogen and stored at -80°C. Samples were taken at 0, 3, 6, 24, 48 and 72 hpi and total RNA was extracted and quantified as described in section 2.2.8.

3.2.9. Gene expression analysis using semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription PCR was performed with 1 μl of cDNA derived from 1 μg total RNA that was synthesised as described in section 2.2.9, using a commercially available master mix as described in section 2.2.10. The appropriate numbers of cycles used in the PCR were re-optimised in order to compensate for the change in the expression levels of GST1, PRI and PDF1.2 that were caused by the changes in growth conditions and pathogen infection (table 3.2). The reactions were run on a 1.5% agarose gel in 0.5× TBE. Images shown are representative of three independent replicates.

3.2.10. Statistical analysis

Statistical analysis was performed using Sigma plot 11. The standard error of the means (SEM) was calculated and the statistical analysis of the data concerning quantification of callose collar and encasement and the quantification of callose concentrations subjected to a two-way ANOVA with a Tukey post hoc test. The H2O2 concentration quantification data was to a two-way ANOVA with a Student-Newman-Keuls method post hoc test.
Table 3.2. Primer sequences of genes of interest used in gene expression analysis and corresponding optimised PCR steps with expected fragment size.
3.3. Results

3.3.1. Susceptibility and resistance of Arabidopsis to *H. arabidopsidis* isolates Noks1 and Cala2

The responses of Arabidopsis plants to infection by *H. arabidopsidis* isolates Noks1 and Cala2, was visually assessed at 168 hpi. Leaves that were mock inoculated (sdH2O) did not display any observable change, and remained green and healthy (Figure 3.1a); no changes were observed on the epidermal surface of the inoculation site (Figure 3.1b). Plants inoculated with the virulent *H. arabidopsidis* Noks1 isolate demonstrated a typical compatible interaction, with conidiophores on infected leaves (Figure 3.1c). Closer examination of the Noks1 infected leaf surface revealed branched conidiophores producing conidiospores, protruding from the epidermis (Figure 3.1d). Plants inoculated with the avirulent *H. arabidopsidis* Cala2 isolate did not demonstrate visible disease symptoms (Figure 3.1e), but small hypersensitive lesions were present at the site of inoculation on the leaf surface, where infection had failed, which demonstrated an incompatible interaction (Figure 3.1f).

3.3.2. Comparison of the host-pathogen relationship in the compatible and incompatible interactions between Arabidopsis and *H. arabidopsidis*

Germination of the conidiospores resulted in germ tube formation and penetration of the epidermis between the anticlinal cell walls of epidermal cells, in both the compatible and incompatible isolates (Figure 3.2b). Upon infiltration of the host, the Noks1 isolate demonstrated compatibility by successfully colonising host tissue, with extracellular hyphae growing between cells of the leaf tissue, and penetration of host cell wall with haustoria along the hyphae (Figure 3.2c). In contrast, infiltration of host tissue by the Cala2, led to rapid hypersensitive cell death (Figure 3.2d).
Figure 3.1. Arabidopsis Col-0 leaves infected with *H. Arabidopsidis* at 168 hpi. a) Mock treated leaf inoculated with sdH2O, b) surface of mock treated leaf showing undisturbed epidermis. c) Noks1 inoculated leaf displays typical compatible interaction with conidiophores present on the leaf surface, d) magnified image of Noks1 inoculated leaf with conidiophores growing out of the leaf and conidiospores on the leaf surface. e) Cala2 infected tissue displaying the incompatible reaction with the presence of HR (red circle), f) magnified area of the Cala2 inoculated leaf with HR regions seen as small areas of brown patches (arrows). Scale bars represent 0.5 cm (a, c, e) and 1 mm (b, d & f).
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No pathogen growth or cell death was observed in mock-inoculated samples at any time points (Figure 3.3a, d, g, j, m &p), or the pathogen inoculated samples at 0 hpi (Figure 3.3b & c). At 24 hpi, the Noks1 isolate displayed initial signs of infection with the presence of penetration hyphae and primary haustoria development (Figure 3.3e). However, no change was noted in the Cala2 infected tissue at 24 hpi with un-germinated spores on the leaf surface (Figure 3.3f). At 48 hpi, tissue infected with Noks1 showed formation of hyphae, growing away from the initial infection producing haustoria (Figure 3.3h). The hypersensitive response in Cala2 infected tissue with the first appearance of localised cell death (Figure 3.3i). At 72 hpi, Noks1 hyphal growth became more prolific and haustoria formation was visible inside plant cells along the hyphae (Figure 3.3k). In contrast, sites of infection by Cala2 only contained hypersensitive cell death caused by Cala2 recognition (Figure 3.3l). This trend continues at 120 hpi with prolific hyphal growth of the Noks1 isolate (Figure 3.3n), and HR sites present in the Cala2 infected leaves (Figure 3.3o). However, at 168 hpi the Noks1 produced oospores in infected leaves (Figure 3.3q) and in Cala2 infected tissue only areas of cells death where HR had occurred remained at this time point (Figure 3.3r).

Growth of the pathogen and the hosts responses were tracked in both the compatible and incompatible interactions (table 3.3). In the compatible Noks1 infected tissue, infiltration and development of primary haustorium occurred at 24 hpi, however hyphal growth was not observed until 48 hpi with hyphae spanning 5.29 cell lengths. Hyphal length had doubled at 72 hpi as hyphae lengths span 13.5 cell lengths as the infection became more prolific. Hyphal growth at 120 and 168 hpi became extensive with hyphae greater than 20 cell lengths, and production of reproductive structures such
Figure 3.2. Pathogen growth and the host response to infection by *H. arabidopsidis* compatible Noks1 and incompatible Cala2 isolates. a) Mock treated tissue 48 hpi. b) Germinating Cala2 conidiospore (CS) at 48 hpi, with germ tube (GT) infiltration of the epidermal layer and formation of penetration hypha (PH). c) *H. arabidopsidis* compatible Noks1 isolate infecting cells at 168 hpi, with hyphae (hy) growing between cells of the mesophyll layer and haustoria (ha) penetrating the cell wall (cw) of individual cells (inset). d) *H. arabidopsidis* incompatible Cala2 inoculated tissue at 168 hpi with cell death resulting from HR (arrowhead), occurring where the Cala2 infection had been contained. Bars represent 50 μm, inset 10 μm.
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Figure 3.3. Pathogen growth in both the compatible (Noks1) and incompatible (Cala2) interactions over 168 hpi. No changes were observed at 0 hpi in the a) mock, b) Noks1 or c) Cala2 inoculated leaves. d) At 24hpi healthy mesophyll cells present in the mock treated tissue, e) infiltration and primary haustorium (inset) formation became present in the Noks1 infected tissue, f) no change in Cala2 with un-germinated spores remaining on the epidermis. At 48hpi no change was noted in the g) mock treated control, h) extension of hyphae with formation of haustoria (ha) in cells in the Noks1 infected tissue (inset), i) appearance of HR in response to Cala2 infiltration. Infection at 72hpi, j) mock treated tissue, k) hyphal growth extending across mesophyll cells infected cells adjacent to the hyphae (inset) and l) hyper-sensitive cells where infection had failed. At 120 hpi, m) mock treated tissue, n) prolific hyphal growth of Noks1 within the leaf tissue and o) HR present in Cala2 infected tissue. At 168 hpi, p) no change seen in the mock treated tissue, q) sexual reproduction of the pathogen occurs in the Noks1 isolate with the presence of oospores, r) HR lesions showing the site of failed infection by Cala2. Bars represent 50μm.
Table 3.3. Pathogen growth and activation of resistance to *H. arabidopsidis* isolates over time in Col-0.

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Hyphal length (average # cells)</th>
<th>Pathogen structures</th>
<th><em>Growth stage</em></th>
<th>HR present</th>
<th># cells in HR</th>
<th>Location of HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>No change in tissue</td>
<td>0</td>
<td>No</td>
<td>0</td>
<td>No change in tissue</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
<td>Infiltration/ primary haustoria present</td>
<td>2-3</td>
<td>No</td>
<td>0</td>
<td>Spores Present NO HR</td>
</tr>
<tr>
<td>48</td>
<td>5.29±0.58</td>
<td>Hyphal proliferation</td>
<td>4</td>
<td>Yes</td>
<td>2.40±1.79</td>
<td>Spores present, HR in mesophyll</td>
</tr>
<tr>
<td>72</td>
<td>13.5±1.74</td>
<td>Hyphal proliferation</td>
<td>5</td>
<td>Yes</td>
<td>2.75±0.74</td>
<td>Mostly mesophyll some epidermal cells</td>
</tr>
<tr>
<td>120</td>
<td>&gt;20</td>
<td>Extensive hyphal growth and conidiophore production</td>
<td>5-9</td>
<td>Yes</td>
<td>5.09±1.01</td>
<td>Mostly mesophyll some epidermal cells</td>
</tr>
<tr>
<td>168</td>
<td>&gt;20</td>
<td>Extensive hyphal growth, conidiophore/oospore/oogonia production</td>
<td>6-9</td>
<td>Yes</td>
<td>3.73±1.00</td>
<td>Mostly mesophyll some epidermal cells</td>
</tr>
</tbody>
</table>

* Growth stage described in Donofrio *et al*. 2001, which in summary states: stage 1: conidiospore germination, stage 2-3: penetration of the epidermis and formation of the primary haustoria, stage 4: formation of hyphae and haustoria, stage 5: prolific hyphal colonisation, stage 6-8: formation of asexual reproductive structures, stage 9: conidiophore formation.
with hyphae greater than 20 cell lengths, and production of reproductive structures such as conidiospores and oospore formation. In contrast, plants infected with Cala2 spores clearly demonstrated incompatibility with HR development occurring at the sites of infection, effectively halting pathogen growth. However, HR only became apparent at 48 hpi, occurring in mesophyll cells that consisted of an average of 2.4 hypersensitive cells. This trend continues at 72, 120 and 168 hpi with the number of dead cells present in the HR lesion averaging 2.75, 5.09 and 3.73 respectively, with no pathogen growth seen at any of these time points.

3.3.3. Callose deposition in response to pathogen infection

In response to pathogen attack, callose production was observed in both the compatible and incompatible interactions. It was found surrounding pathogen structures or fortifying cell walls that were in proximity to the HR. No staining of callose was observed in mock treated tissue (Figure 3.4a). Callose production was observed in the anticlinal epidermal cell walls, where the appressoria and penetration hypha had been forced through the epidermal layer (Figure 3.4b). In the compatible interaction callose formation first occurred as a collar that surrounded the neck of the haustoria along the cell wall, which is followed by the complete encasement of the haustoria (Figure 3.4c). In the incompatible interaction, fortification of cell walls occurred at the sites of failed infection and were localised where HR had occurred (Figure 3.4d).

Callose formation occurred in response to pathogen infection across the life cycle of the pathogen in the Noks1 isolate and only located in association to the pathogenic structures. In contrast, callose deposition in the Cala2 infected tissue was observed in cells surrounding HR lesions. No callose was observed in the mock inoculated control tissue at any time point (Figure 3.5a, d, g, j, m, p) or at 0 hpi in
Figure 3.4. Callose formation in response to *H. arabidopsidis* infection. a) Mock treated tissue 48 hpi with no callose produced. b) Penetration by Cala2 of the epidermal layers causes callose production in anticlinal cells along the penetration hyphae (arrows heads); insets show combination of fluorescent and light microscopy images of the same area, at 48 hpi. c) Compatible interaction of the Noks1 isolate 48 hpi, callose deposition seen as encasements of haustoria (EC), and collars of callose (arrow) surrounding the neck of the haustoria (ha). d) Callose formation along cell wall of an epidermal cell at 72 hpi in Cala2 infected tissue, deposition of callose occurred in cells associated with HR and incompatibility. Bars represent 50μm.
Noks1 and Cala2 infected tissues (Figure 3.5b & c). At 24 hpi, no callose was observed in the Noks1 infected tissue (Figure 3.5e); however, callose deposition was located along the epidermal cell walls in contact with the penetration hyphae (Figure 3.5f). At 48 hpi, callose production followed Noks1 growth, with callose encasement of haustoria involved in the initial infection and callose collars surrounding the necks of haustoria in newly infected cells (Figure 3.5h). In response to Cala2 infection, callose deposition occurred in cells surrounding hypersensitive cell death (Figure 3.5i). At 72 hpi, as Noks1 infection spread, more haustoria become encased in callose and hyphal growth was more prolific (Figure 3.5k). Tissue inoculated with Cala2 formed small amounts of callose lining the walls of cells surrounding sites of HR (Figure 3.5l). This trend continued at 120 hpi, with prolific Noks1 growth, resulting in callose encasements of older haustoria and callose collars surrounding newer haustoria (Figure 3.5n). In contrast to the Cala2 infected tissue, callose was observed in cells close to the HR sites (Figure 5.3o). In the Noks1 infected tissues at 168h, haustoria became encased by callose as infection matured (Figure 3.5q). In Cala2 infected tissue at 168 hpi, callose was restricted to sites where HR had occurred (Figure 3.5r).

Although callose production occurred in both the compatible (Noks1) and incompatible (Cala2) interactions, the deposition in both interactions differed in both distribution and cell layer (table 3.4). The compatible interaction, demonstrated callose deposition at 48 hpi, and only appeared in cells infected with the Noks1 isolate within the mesophyll. The distribution of callose at this time point appeared as collars, which appeared as a ring of callose surrounding the neck of the haustoria, or which eventually led to the entire encasement of the haustoria itself. This pattern of callose deposition remained for the entire time course following pathogen spread. In contrast, the callose deposition in response to the incompatible interaction and was detected at 24 hpi along...
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Figure 3.5. Callose deposition in response to the compatible (Noks1) and incompatible (Cala2) interactions. No callose production at 0 hpi in a) Mock, b) Noks1 or c) Cala2 inoculated tissue. At 24 hpi no callose was observed in the d) mock or e) Noks1 inoculated tissue. f) Callose was detected at 24 hpi in the epidermal layer of Cala2 treated leaves. At 48hpi, g) Mock inoculated tissue, h) callose present in Noks1 infected tissue surrounding haustoria in infected cells i) callose present in mesophyll cells surrounding HR site. At 72 hpi, j) no change seen in mock inoculated leaves, k) callose deposited around haustoria of the as infection becomes more prolific, l) callose localised to sites where HR had occurred. At 120 hpi, m) no callose in the mock inoculated leaves, n) callose deposition around haustoria following Noks1 infection of cells and o) localised deposition along the cell walls of cells in proximity of the HR site in Cala2 infected tissue. This trend continues at 168 hpi, p) no callose observed in mock inoculated tissue, q) callose production surrounding and encasing haustoria in leaves infected with Noks1, and r) callose only present in areas where HR occurred in response to Cala2 infection. Bar represents 50μm.
the cell walls of epidermal cells where initial penetration occurred. At 48 hpi, callose was produced at the cell wall at the sites of pathogen penetration; these epidermal cells also produce spots where callose is produced before forming sheets along the cell wall. At 72 hpi epidermal cells involved in the pathogen penetration had been fully fortified and callose was produced in the mesophyll cells surrounding the site of HR. Production of callose was limited to the sites of HR, which was also seen at the 120 and 168 hpi.

By examining the Noks1 compatible interaction it was clear that the formation of callose collars and encasements surrounding haustoria changed as infection matured (Figure 3.6). Infection by Noks1 isolate at 48 hpi demonstrated a significant difference in the amount of haustoria surrounded by collars or encasements, 70.27% and 28.60% respectively (P<0.05), with 1.14% of haustoria showing no callose deposition. The distribution of callose collar and encasements only slightly changed at 72 hpi, with 76.42% and 24.77% respectively (P<0.05). At 120 hpi a decrease in collars and an increase in encasements occurred with 63.93% and 34.15%, respectively (P<0.05) and was observed with 1.16% haustoria showing no callose. However, at 168 hpi no significant difference was noted between collars and encasement due to a dramatic decrease in collars to 38.48% and an increase in encased haustoria to 58.60% with 2.94% of haustoria showing no callose.

Based on observation from microscopy alone it is evident more callose is present in the Noks1 compatible interaction when compared to the Cala2 incompatible interaction. This was further demonstrated when callose was extracted and quantified (Figure 3.7). Preliminary callose quantification experiments revealed that changes in callose concentrations were detected at 168 hpi and not at earlier time points (data not
Table 3.4. Characterisation and timing of callose production during the compatible and incompatible interaction in Col-0

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Compatibility (Noks1)</th>
<th>Incompatibility (Cala2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Location</td>
</tr>
<tr>
<td>0</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>24</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>48</td>
<td>Yes</td>
<td>Mesophyll</td>
</tr>
<tr>
<td>72</td>
<td>Yes</td>
<td>Mesophyll</td>
</tr>
<tr>
<td>120</td>
<td>Yes</td>
<td>Mesophyll</td>
</tr>
<tr>
<td>168</td>
<td>Yes</td>
<td>Mesophyll</td>
</tr>
</tbody>
</table>
Figure 3.6. Percent of differing callose formations that surrounded haustoria over time in the Noks1 infected Col-0 plants. Callose formations observed surrounding haustoria were collars (diamonds), encasements (square) or no callose (circle). Asterisks represents significant differences between collars and encasements with each individual time points (P<0.05), error bars represent ± SEM.
Figure 3.7. Total amount of extracted β-1, 3 glucans extracted from Col-0 leaves infected with *H. arabidopsidis* at 0 and 168 hpi. Leaves were either inoculated with mock (dH₂O) (diagonal line bar), compatible Noksl isolate (white bar) or incompatible Cala2 isolate (grey bar). Asterisk represent significant difference from mock treatment at corresponding time point (P<0.001), error bars represents ± SEM.
shown); as a result two time points (0 and 168hpi) were chosen to represent these interactions. When total callose concentrations were quantified, no differences were noted between the mock, compatible and incompatible interactions at 0 hpi. However, at 168 hpi a significant increase in callose concentrations was observed in the compatible interaction when compared to the control (P<0.001), and no difference was observed when comparisons were made between the control and the incompatible interaction.

3.3.4. Lignin deposition in response to pathogen infection

The appearance of lignin in response to pathogen infection became evident with phloroglucinol staining. Lignin was not detected in unstressed mesophyll cells of control tissue (Figure 3.8a). Examination of epidermal cells revealed that lignin was deposited in areas of the cell wall where *H. arabidopsidis* conidiospores had originally penetrated the epidermal layer (Figure 3.8b). Lignification of cells also occurred in tissue infected with the Noks1 isolate and was localised in cells infected by *H. arabidopsidis* (Figure 3.8c). In contrast tissue infected with Cala2 demonstrated staining only in dead cells were HR occurred with no lignification of cell walls in living cells that surrounding the site of attempted infection (Figure 3.8d).

The presence or the absence of lignin in the compatible and incompatible interactions was recorded at 0, 24, 48, 72, 120 and 168 hpi (table 3.5). No change in lignin distribution occurred at 0, 24 or 48 hpi. However, staining occurred at 72 hpi in the Noks1 and Cala2 infected tissue, with pink staining in cells infected with the Noks1 isolate and red/pink staining in hypersensitive cells in the Cala2 infected leaves. The Noks1 infected leaves at 120 and 168 hpi contained more lignin-positive cells within the mesophyll over time. In contrast, staining in Cala2 infected leaves was limited to hypersensitive cells at the remaining 120 and 168 hpi time-points.
Figure 3.8. Lignin production in response to pathogen infection. a) mock treated mesophyll tissue, no lignification observed 168 hpi. b) Sites of Noks1 penetration between adjacent epidermal cells at 168 hpi (black arrowheads), sealed with lignified cell walls, seen as pink staining (inset). c) Lignification of mesophyll cell walls in Noks1 infected tissue at 168 hpi (black arrows). d) Red – pink staining of the hypersensitive cells within the mesophyll that occurred under the epidermal penetration sites at 120 hpi with the Cala2 isolate. Bars represent 50 μm. ha = haustoria hy = hyphae.
Table 3.5. Deposition of lignin in the compatible and incompatible interactions

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Staining</th>
<th>Location</th>
<th>Staining</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No</td>
<td>Not observed</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>24</td>
<td>No</td>
<td>Not observed</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>48</td>
<td>No</td>
<td>Not observed</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>72</td>
<td>Pink</td>
<td>Lignified infected mesophyll cells</td>
<td>Red/pink</td>
<td>Mesophyll HR/dead cells</td>
</tr>
<tr>
<td>120</td>
<td>Pink</td>
<td>Lignified infected mesophyll cells</td>
<td>Red/pink</td>
<td>Mesophyll HR/dead cells</td>
</tr>
<tr>
<td>168</td>
<td>Pink</td>
<td>Lignified infected mesophyll cells</td>
<td>Red/pink</td>
<td>Mesophyll HR/dead cells</td>
</tr>
</tbody>
</table>
Figure 3.9. Lignification of cell walls in response to pathogen infection. No lignin detected at 0 hpi in the a) mock, b) Noks1 or c) Cala2 inoculated tissue. At 72 hpi no change observed in the d) mock, e) lignified cells were present in Noks1 infected leaves (black arrowhead/inset), and f) staining present in hypersensitive cells caused by Cala2 (black arrowheads). This trend continued at 120 hpi, no increase in lignin in the g) mock inoculated, h) more intense lignin staining in the Noks1 infected leaves and i) staining localised to hypersensitive cells. At 168, no lignin observed in the j) mock, k) lignin present in Noks1 infected cells and l) staining only localised in the hypersensitive cells. Bar represent 50 μm.
Lignin staining was not observed in the mock treated control at any time point (Figure 3.9a, d, g & j) or within the pathogen infected tissue at 0 hpi (Figure 3.9b-c), 24 hpi or 48 hpi (data not shown). At 72 hpi, lignification occurred in mesophyll cells directly infected with the Noks1 isolate (Figure 3.9e), with the complete lignification of the cell wall. In contrast, staining only occurred in hypersensitive mesophyll cells in response to Cala2 infection at 72 hpi (Figure 3.9f). At 120 and 168 hpi, lignin producing cells increased as Noks1 infection become more prolific and infection spread (Figure 3.9h & k). However, in Cala2 infected tissue lignification was only detected within the walls of hypersensitive cells at the 120 and 168 hpi time points (Figure 3.9i &l).

3.3.5. Changes in hydrogen peroxide contents

Hydrogen peroxide was visualised in infected tissue with DAB staining which was seen as red – brown precipitate. No substantial staining was observed in mock treated tissue (Figure 3.10a). Intense DAB staining was observed along the cell walls of epidermal cells where penetration of the epidermis through the anticlinal space between the cell walls had occurred (Figure 3.10b). In the Noks1 compatible interaction, entire cells which had been infiltrated by haustoria stained positive for H$_2$O$_2$ (Figure 3.10c), with the distribution of H$_2$O$_2$ positive cells following the growth of the pathogen. Hydrogen peroxide accumulation was also observed in the incompatible interaction in response to Cala2 infection, with staining occurring in hypersensitive cells (Figure 3.10d).

Over the course of pathogen infection, H$_2$O$_2$ appeared in both compatible and incompatible interactions. However, no staining was observed in the mock inoculated treatments (Figure 3.11a, d, g & j), or at 0 hpi in the Noks1 or Cala2 treated tissue (Figure 3.11b & c). At 24hpi, germination of the conidiospores and the initial infiltration
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**Figure 3.10.** Hydrogen peroxide localisation in response to pathogen infection 120 hours post inoculation. a) Mesophyll cells of mock inoculated tissue, no staining present. b) Localisation of \( \text{H}_2\text{O}_2 \) along the cell walls surrounding the site of initial penetration by Noks1. c) Localisation of \( \text{H}_2\text{O}_2 \) in cytoplasm in cells infected with Noks1. d) Staining of HR region in Cala2 infected tissue, \( \text{H}_2\text{O}_2 \) localised within dead cells with prominent staining. Bars represent 50µm.
Figure 3.11. Localisation of H$_2$O$_2$ in Col-0 leaves infected with the virulent or avirulent
*H. arabidopsidis* isolates. No staining present in the a) mock, b) Noks1 or c) Cala2 treated
tissue at 0 hpi. At 24 hpi, no staining observed in the d) mock treatment, e) DAB staining
was observed in Noks1 inoculated tissue where spore germination had occurred (black
arrowhead and inset). f) Cala2 infected tissue displayed staining in an epidermal cell. At
48 hpi, g) no staining present in the mock, h) staining was localised to the cell wall in
contact with the penetration hyphae of Noks1 (arrows and inset) and i) staining of
epidermal and hypertensive mesophyll cells (inset) in response to Cala2 infection. At
72hpi, j) no staining observed in the mock, k) staining localised only in Noks1 infected
cells, l) staining of hypersensitive cells in Cala2 infected tissue, staining also observed in
chloroplast in surrounding cells (inset). Bars represent 50μm.
of the epidermal layer showed cells staining positive for H$_2$O$_2$ in both the Noks1 (Figure 3.11e) and Cala2 (Figure 3.11f). While intense staining was not observed in Noks1 infected at 48 hpi, some staining occurred in the epidermal layers surrounding areas of the cell walls where the penetration hyphae had infiltrated the tissue (Figure 3.11h). In Cala2 infected tissue, strong staining was observed in epidermal cells and the mesophyll cells below it (Figure 3.11i). Staining was observed at 72 hpi in infected cells with Noks1 (Figure 3.11k). In contrast staining in Cala2 infected tissue occurred only in areas of HR, located within dead cells and the chloroplast of surrounding cells (Figure 3.11l).

The initial appearance of H$_2$O$_2$ accumulation occurred quickly in direct response to the pathogen infection (table 3.6). In both the compatible and incompatible interaction, H$_2$O$_2$ was localised in epidermis cells in contact with germinating spores penetrating the epidermal layer at 24 hpi. At 48 hpi, H$_2$O$_2$ accumulation in response to the Noks1 isolate only occurred in the areas of the cell wall in direct contact with the penetration hyphae. In the Cala2 infected tissue H$_2$O$_2$, occurred in epidermal cells and hypersensitive mesophyll cells directly below. The most dramatic changes in H$_2$O$_2$ accumulation occurred at 72 hpi, with clear staining of Noks1 infected cells in the compatible interaction, where entire cells displayed staining. The incompatible interaction, showed the presence of H$_2$O$_2$ accumulation in hypersensitive cells and cells surrounding this HR region triggered by the Cala2 isolate.

Quantification of hydrogen peroxide in infected leaves showed differing trends in the Noks1 and Cala2 treatments. Although no significant differences were found at 0, 3, 6, 24 and 48 hpi, a general increase in H$_2$O$_2$ was observed at 6 hpi in response to both the Noks1 and Cala2 isolates. From 6 hpi, tissue infected with the Noks1 isolate showed
Table 3.6. Summary of the presence and location of H$_2$O$_2$ in the compatible and incompatible interactions

<table>
<thead>
<tr>
<th>Time (hpi)</th>
<th>Compatible (Noks1)</th>
<th>Incompatible (Cala2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_2$O$_2$</td>
<td>Location</td>
</tr>
<tr>
<td>0</td>
<td>No</td>
<td>Not observed</td>
</tr>
<tr>
<td>24</td>
<td>Yes</td>
<td>Epidermal cells affected by pathogen penetration</td>
</tr>
<tr>
<td>48</td>
<td>Yes</td>
<td>Epidermal cells, cell wall in contact with penetration hypha</td>
</tr>
<tr>
<td>72</td>
<td>Yes</td>
<td>Infected mesophyll cells</td>
</tr>
</tbody>
</table>
Figure 3.12. Hydrogen peroxide concentrations present in Col-0 leaves infected with the compatible and incompatible isolates of *H. arabidopsidis*. Leaves were inoculated with either sdH₂O (mock) (diagonal line bars), the Noks1 isolate (white bars) or the Cala2 isolate (grey bars). Asterisk represent significant difference from mock treatment at corresponding time point (P<0.05), error bars represents ± SEM.
a steady decrease in H$_2$O$_2$ concentrations for the remaining time points. A similar trend was also seen in the Cala2 infected tissue, however a significant increase in hydrogen peroxide was observed at 72 hpi (P<0.05) (Figure 3.12).

3.3.6. Changes in defence gene expression

Examination of defence gene expression in infected tissue demonstrated up-regulation of defence related genes in both the compatible and incompatible interactions (Figure 3.13). Expression of $GST1$ in the compatible (Noks1 infected) interaction showed an early increase of expression at 3 and 6 hpi with a return to baseline levels for the remainder of the time course. The incompatible (Cala2 infected) interaction also showed an early increase in $GST1$ expression at 3 and 6 hpi followed by a decrease in expression, however a second phase of increased expression was observed at 48 and 72 hpi.

Levels of the SA marker gene $PR1$ were increased with gene induction in both the compatible and incompatible interactions. Expression increased at 24 hpi and remained expressed at 48 and 72 hpi in the compatible interaction. In the incompatible interaction, $PR1$ displayed an up regulation in expression much earlier at 0, 3, 24 and 48 hpi, and a prominent increase was observed at 72 hpi. Expression of the JA marker gene $PDF1.2$ demonstrated a gradual up-regulation over time in the mock inoculated, Noks1 and Cala2 treatments with no discernible change in expression being observed between these treatments. UBQ5 was used as an internal standard and showed even expression; however, slight reductions were noted in the Noks1 6 hpi and Cala2 72 hpi samples.
Figure 3.13. Gene expression profile of Col-0 plants infected with compatible and incompatible *H. arabidopsidis* isolates over a 72 hours. Expression of ROS marker gene GST1, salicylic acid defence pathway marker gene *PR1*, jasmonic acid/ethylene defence pathway marker *PDF1.2* and *UBQ5* was used as an internal standard (housekeeping gene).
3.4. Discussion

This work examined the growth of the pathogen in both the virulent and avirulent isolates of *H. arabidopsidis* and the response caused within the host’s tissue within a defined time course. Defence responses occurs in both the compatible and incompatible interaction with the deposition of callose, lignin, H$_2$O$_2$ generation and expression of *PRI*, yet the timing, localisation and expression varied between the two different interactions. The compatible interaction demonstrated callose and lignin deposition, yet only in cells directly affected with the pathogen, and this was the case for the localisation of H$_2$O$_2$. Expression of *PRI* occurred, however an increase in expression occurred at a slower rate. The incompatible interaction demonstrated complete resistance to the Cala2 isolate with formation of HR and complete suppression of the pathogen, deposition of callose and localisation of H$_2$O$_2$ occurred in cells surrounding HR, whereas lignin was only present within hypersensitive cells and *PRI* expression occurred earlier.

3.4.1. Pathogen development and resistance to *H. arabidopsidis*

The early stages of *H. arabidopsidis* infection have been shown to be similar between compatible and incompatible isolates; with germination of conidiospores, formation of germ tubes, penetration of anticlinal spaces between epidermal cell walls by the appressoria and penetration hyphae and formation of the primary haustorium (Koch *et al.*, 1990, Donofrio *et al.*, 2001, Slusarenko *et al.*, 2003). Compatibility in the interaction of the Noks1 isolate and Col-0, demonstrated the active infiltration and establishment within the host’s tissue by hyphae growth from the primary haustorium and establishment of haustoria within cells. As time progressed, infection became more
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prolific until the pathogen reached the reproductive stage of its life cycle (both sexual and asexual).

Incompatibility resulted in rapid and localised cell death in response to Cala2 infection that first appeared at 48 hpi and became more prominent at 72 hpi, the resulting HR lesions present on the leaf surface appeared as small patches of flecking necrosis (Holub *et al.*, 1994, Holub, 2008). This resistance to the Cala2 isolate relies on effector triggered immunity and penetration of host cells in-order to enable recognition of the pathogen by the R proteins RPP2A and RPP2B (Sinapidou *et al.*, 2004). Upon examination, only a small number of hypersensitive cells were triggered in the mesophyll in response to Cala2, this illustrates that effective resistance does not require large amounts of cell death to halt pathogen infection in this system. However, this observation should not be used to generalise resistance to other *H. arabidopsidis* isolates or in other ecotypes of Arabidopsis, as the hypersensitive response in those situations can vary both in size and form (Holub *et al.*, 1994, Mert-Turk *et al.*, 2003).

3.4.2. Callose and lignin are produced in response to pathogen attack

In the early stages of infection, callose deposition was observed in adjacent epidermal cells along portions of the cell wall affected by the penetration peg. As infection progressed, the distribution of callose differed greatly between the two isolates. The compatible interaction showed callose distribution in relation to direct penetration of the cell wall by the haustoria of the Noks1 isolate. In infected cells, callose was deposited as a collar that surrounds the neck of the haustoria before the entire haustoria is encased as the infection matures. This pattern of callose deposition surrounding haustoria had been widely reported to occur in Arabidopsis infected with *H. arabidopsidis* and other plant pathogen systems (Parker *et al.*, 1993, Donofrio *et al.*, 1993).
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2001, Soylu *et al.*, 2004, Dong *et al.*, 2008, Romero *et al.*, 2008). Interestingly, the deposition of callose as collars or encasements may serve as a useful guide to determine the direction of the leading end of the hyphae as it proliferates through the tissue. It may also serve as a useful tool to determine the relative age of *H. arabidopsidis* infection in host leaves, as collars first occur in relatively newly infected cells and encasements are present in cells where infection has matured. In the incompatible response, callose only appeared along the portions of the cell wall closest to the HR region, with deposition occurring along the walls of adjacent mesophyll and epidermal cells. This type of deposition of callose surrounding the HR lesions has also been noted in other avirulent biotrophic and hemi-biotrophic pathogens in Arabidopsis (Roetschi *et al.*, 2001, Ahn *et al.*, 2007, Romero *et al.*, 2008).

When quantified, it became clear callose was more abundant in the compatible interaction when compared to the incompatible interaction at 168 hpi. This stark increase in callose deposition in the compatible interaction must be due to prolific growth of the pathogen, as the pathogen grows, more cells are penetrated and more callose is produced. In this interaction, this assay may be useful to measure virulence of the pathogen, as callose production seems to be dependent on pathogen growth. However, the lack of callose seen in the incompatible interaction is more likely due to the sensitivity of this assay, as callose production is clearly shown in the microscopy. Callose deposition in the incompatible interaction was only restricted to limited number of cells affected by the pathogen and resulting HR, in contrast to the compatible interaction where callose contents increase because of proliferating infection and the increasing amount of infected cells. This illustrates the minimal amount of callose deposited along walls of cell surrounding HR, this type of deposition appears to be a resistance mechanisms that surrounds HR caused by multiple pathogens (Dietrich *et al.*, 2001, Soylu *et al.*, 2004, Dong *et al.*, 2008, Romero *et al.*, 2008).
1994, Ahn et al., 2007). The presence of callose surrounding the HR lesion poses questions; is the presence of callose caused by pathogen recognition and deposited as a preventative measure, and would this interaction occur in the same way if callose was inhibited?

Lignin production also occurred within infected leaves, with the earliest detection of lignin occurring at 72 hpi. In the compatible interaction, cells infected with the Noks1 isolate displayed staining of the entire cell wall. The presence of lignin within the wall of infected cells may not have a major contribution to overall basal resistance to this pathogen during the initial stages of infection, as its deposition only occurred after infection had been fully established within a cell and does not appear in unaffected cells surrounding infection as a preventative measure. It was also noted that lignin positive staining was present in cells which had undergone HR in the incompatible interaction. The presence of lignin positive staining with hypersensitive cells is similar to previous work where phloroglucinol staining revealed lignin deposition in dead cells within trailing necrosis caused by H. arabidopsidis and hypersensitive cells triggered by Pseudomonas syringae (Mauch-Mani et al., 1996, Soylu, 2006). This would suggest that the lignin biosynthesis had to have been occurring before cell death in the HR lesion was triggered.

3.4.3. Accumulation of hydrogen peroxide in response to pathogen infection

Changes in hydrogen peroxide contents in both the compatible and incompatible interactions was initially localised in epidermal cells where conidiospores had germinated and infiltrated the epidermal layer. H$_2$O$_2$ was localised at the cell walls surrounding the penetration hyphae, which is consistent with previous work examining H$_2$O$_2$ localisation of initial pathogen penetration (Slusarenko et al., 2003). DAB
staining was present in the compatible interaction as infection took hold and appeared to be localised to specific cells containing haustoria. As time progressed, DAB staining only appeared within infected cells and appeared to be occurring throughout the entire cell, this distribution is similar to the distribution of lignin deposition in infected cells across the entire cell wall. This localised presence of H$_2$O$_2$ may explain why lignification only occurred specifically in *H. arabidopsidis* infected cells and not in the cells surrounding them. H$_2$O$_2$ has been shown to be involved in cell wall cross-linking and lignin polymerisation, and this may account for the similarities in distribution between H$_2$O$_2$ and lignin staining (Thordal-Christensen *et al.*, 1997, Christensen *et al.*, 2000, Denness *et al.*, 2011). The incompatible interaction also displayed similar staining to the compatible interaction at 24 hpi, where penetration of the epidermal layer had occurred. However, intense staining was detected within cells that had undergone HR and within the chloroplast of cells surrounding HR lesions. This intense staining may be the result or the remnant of the oxidative burst which occurred from the recognition of the pathogen effectors which lead to the triggering of the hypersensitive response (Ahn *et al.*, 2007, Hwang *et al.*, 2011).

When quantified, an increase H$_2$O$_2$ was noted at 6 hpi in both the compatible and incompatible interaction, although not statistically significant, this may actually be the result of spore germination that has been reported to occur at this time point (Slusarenko *et al.*, 2003). The similarities between the compatible and incompatible interaction ends at 72 hpi where a clear increase was seen in the incompatible Cala2 interaction which may be associated with the hypersensitive response which is shown to predominantly occur at this time point. This pattern of H$_2$O$_2$ increases between the two isolates is further demonstrated in the expression of the ROS marker gene *GST1*, which showed early gene activation in both the compatible and incompatible interaction and
later activation of \( GST1 \) in the incompatible interaction. This would suggest that an biphasic increase in an incompatible response which leads to the triggering of the hypersensitive response, this biphasic nature of ROS has been shown in other patho-systems in response to avirulent pathogens (Lamb et al., 1997, Able et al., 2000, Mur et al., 2005, Tsuda et al., 2010). The timing of the second increase of \( H_2O_2 \) in the incompatible reaction occurred much later than in previous studies, this delay corresponds to the prominent appearance of the hypersensitive lesions at 72 hpi, and is most likely attributed to the infection process of \( H. arubidopsidis \) itself.

### 3.4.4. Defence gene activation in response to \( H. arubidopsidis \) infection

Expression of the oxidative burst marker gene \( GST1 \) has been demonstrated to show differing expression between compatible and incompatible interactions, as a result of infection by \( H. arubidopsidis \) and other pathogens (Rairdan et al., 2001, Rustérucci et al., 2007, Hwang et al., 2011). This system demonstrated the difference in expression of \( GST1 \) between the compatible and incompatible interactions of \( H. arubidopsidis \), with early expression in both interactions and a later burst that only occurred in the incompatible response. Salicylic acid accumulation is known to induce resistance to biotrophic and hemi-biotrophic pathogens, and applications of SA can suppress normally virulent pathogens (Nawrath et al., 1999, Glazebrook, 2005, Lovelock et al., 2013). However, \( PR1 \) expression can be observed in response to virulent and avirulent pathogens, this has been demonstrated with various biotrophic and hemi-biotrophic pathogens in Arabidopsis. The activation of this \( PR1 \) in a compatible interaction occurs much slower and at a lower expression level when compared to the incompatible interaction (Reuber et al., 1998, Ahn et al., 2007, Hwang et al., 2011). Col-0 plants expressed \( PR1 \) in response to both the compatible and incompatible interactions and although \( PR1 \) was expressed in Noks1 infected tissue, expression of \( PR1 \) in Cala2
infected tissue occurred earlier with higher expression at the later time points. It should be noted that early expression of *PR1* at 0 hpi in the Cala2 treated tissue should be interpreted carefully as it seems unlikely to be purely caused by Cala2 infection. Expression of the JA/Et marker gene *PDF1.2* did not change when compared to the mock inoculated control, although the expression of *PDF1.2* was observed to increase over the time course. It is not clear at this stage whether this expression pattern is due change in growth condition from 21°C to 16°C, or an effect of the inoculation process. This type of expression pattern was not observed in the mock treatment of the *GST1*, *PR1* or *UBQ5* PCRs. However, the lack of change in expression levels is to be expected as activation of the jasmonic acid/ethylene pathways is commonly associated with defence against necrotrophic pathogens (Thomma *et al.*, 1998, Glazebrook, 2005, Allardyce *et al.*, 2013).

These results demonstrate that callose deposition was significantly higher in the compatible interaction when compared to the incompatible interaction, and this large difference in callose deposition is associated with pathogen growth of the compatible Noks1 isolate. Although, lignin was present in both interactions, it only was present in cells directly infected by the pathogen, where lignin only occurred in cells that contained haustoria after the infection had been fully established, or cells that had undergone HR. The generation of hydrogen peroxide occurred in response to both compatible and incompatible isolates in the early time points and a second ROS burst was detected in the incompatible interaction, which corresponded with the formation of HR and restriction of the pathogen and increased expression of *PR1*. This study examined the ‘normal’ interaction of pathogen attack and host defences between *H. arabidopsidis* and Arabidopsis; this will allow comparisons to be made to the normal progression of disease when other stressors are applied.
Chapter 4: UV-C irradiation increases resistance of Arabidopsis to virulent isolates of *Hyaloperonospora arabidopsidis*

Abstract

Plants exposed to UV-C dosages greater than 250 Jm$^{-2}$, 24 h before pathogen inoculations, showed a decrease in pathogenic structures such as conidiophores and hyphae within host leaves. Examination of the UV-C induced resistance to normally virulent isolate of *H. arabidopsidis* at a cellular level revealed reduced hyphal growth and the appearance of highly localised cell death resembling the hypersensitive response, which was present in the 500 and 1000 Jm$^{-2}$ treatments. Formation of HR-like lesions resembled HR that is mediated by R protein recognition in its distribution of cell death, presence of lignin, callose that surrounded the lesion and appeared to be triggered by pathogen infection and not UV-C alone. Callose contents were significantly increased at early time points plants treated with 500 and 1000 Jm$^{-2}$ and remained elevated though out the time course. However, more callose was produced due to pathogen infection at 168 hpi in the 0 and 250 Jm$^{-2}$ when compared to the higher dosages at this time point. Further examination of the induction of H$_2$O$_2$ and *PRI* expression revealed both were elevated at early time points before pathogen infection. This pre-activation of defences caused by the initial UV-C exposure is the most likely source for the presence of the hypersensitive like lesions present in Col-0 and Ler ecotypes of Arabidopsis that were infected with the virulent isolates Noks1 or Cala2 respectively.
4.1. Introduction

Plants are exposed to a wide variety of stressors in the environment and it is becoming increasingly apparent that the responses to each of these stresses share common mechanisms responsible for cellular repair and acclimation, for example, where one type of stress can alleviate the effects of an entirely different future stress. It is this property that causes a eustress/hormic response (activation of beneficial attributes caused by low levels of a normally harmful stress) which can be a useful tool to alter the response of the plants for a specific need (Stevens et al., 1996). Plants exposed to UV radiation have been shown to induce similar responses to other stresses and as a result, this activates a ‘cross-tolerance’ response, which increases tolerances to other stresses. This cross tolerance is also observed in nature, where studies that examined herbivory in field grown plants under natural and attenuated UV-B conditions showed that plants grown under natural UV-B conditions were shown to be more resistant to insect herbivory when compared to plants grown in attenuated conditions (Ballare et al., 1996, Mazza et al., 2013). Exposure to UV-C had been shown to decrease chilling injury caused to peppers (Capsicum annum), when stored at 0°C (Vicente et al., 2005). Other stressors such as heat shock have been shown to induce resistance to other stressors such as temperature, drought and salinity in maize seedlings (Gong et al., 2001).

Many studies have examined the use of UV irradiation in a horticultural setting, where various fruit and vegetables are exposed to wavelength of UV-C radiation. In a post-harvest situation a treatment of UV-C has been found to be useful, as it can delay ripening by delaying the activity of cell wall degrading enzymes, and reduce spoilage due to pathogen attack by increasing resistance (Charles et al., 2009, Bu et al., 2013). In postharvest situations, ‘hormic’ doses are commonly used and have been shown to
reduce the disease symptoms of pathogens in a variety of hosts. UV-C pre-treated sweet potato demonstrated increased resistance to *Fusarium solani*, with decreased lesion sizes and increases in phenylalanine ammonia-lyase (PAL) activity (Stevens *et al.*, 1999). In tomato fruit, resistance to *Botrytis cinerea* was achieved in pre-treated fruit exposed to the hormic dose of 3.7 kJm\(^{-2}\) of UV-C by limiting the spread of the pathogen by the formation of structural barriers and phytoalexin accumulation (Charles *et al.*, 2008). Microarray analysis also showed that tomato fruit exposed to a dose of 4 kJm\(^{-2}\) UV-C irradiation caused the induction of a wide range of genes including those involved in pathogen defence such as ethylene biosynthesis and expression of an array of PR genes (Liu *et al.*, 2011). This overlap in gene expression may explain the increase in resistance to pathogens.

In plants, exposure to UV (B or C) radiation has been shown to induce activation of pathogen defence pathways that involve the hormones salicylic acid and jasmonic acid (Mackerness *et al.*, 1999, Shapiro *et al.*, 2001). However, only a few studies have been undertaken to determine if similar responses can be induced within a whole plant, to that seen in post-harvest studies. In Arabidopsis, UV-C pre-treatment of plants have been shown to have increased resistance to the necrotrophic pathogen *Botrytis cinerea*, was associated with UV-C induced camalexin production (Stefanato *et al.*, 2009). Increased resistance to *B. cinerea* was also observed in Arabidopsis plants pre-treated with UV-B and was attributed to *UVR8* mediated sinapate biosynthesis (Demkura *et al.*, 2012). Reductions were also seen in bacterial numbers of the hemi-biotrophic pathogen *Pseudomonas syringae* in Arabidopsis leaves pre-treated with UV-C. Also demonstrated was the induction of genes involved in SA pathway *EDS5* and *PRI* which is involved in defence against hemi-biotrophic and biotrophic pathogens (Shapiro *et al.*, 2001, Nawrath *et al.*, 2002, Glazebrook, 2005). In addition, Kunz *et al.*, 2008, showed
increased resistance to the obligate biotroph *H. arabidopsidis*, with the formation of hypersensitive lesions and reduction in pathogen growth, and demonstrated the involvement of DNA repair mechanisms, through the use of mutants deficient in UV absorbing pigments (tt5) or DNA repair (such as uvr2, uvr3 and ercc1-1). Although some progress has been made in studying the effects of UV pre-treatments in plant pathogen interactions, significant gaps in knowledge remain.

Although it is now known that UV radiation can stimulate SA and SA inducible gene that results in *PR1* induction, this may explain the increased resistance towards hemi-biotrophs and biotrophs (Mackerness *et al.*, 1999, Nawrath *et al.*, 2002, Kunz *et al.*, 2008). However, information is lacking regarding the transition of the plants between susceptible and induced resistance interaction at a cellular level. This study examines in detail the effects UV-C pre-treatments caused to the ‘normal’ compatible interaction between the Arabidopsis Col-0 and the *H. arabidopsidis* isolate Noks1, and will also determine if similar responses of UV-C induced resistance to *H. arabidopsidis* can be observed in a second Arabidopsis ecotype Lansberg erecta (Ler) in response to infection by the virulent Cala2 isolate. This will be achieved by examining pathogen associated markers such as callose, lignin, H$_2$O$_2$ accumulation and pathogen defence pathway activation and will determine if this induced response can mimic that of the typical incompatible interaction.
Chapter 4: UV-C irradiation increases resistance of Arabidopsis to virulent isolates of *H. arabidopsidis*

### 4.2. Materials and methods

#### 4.2.1. Growth conditions, UV-C treatments and pathogen infection of experimental plants

*Arabidopsis thaliana* seeds of the Col-0 or Ler ecotypes were surface sterilised for 5 minutes, washed with sdH2O then suspended in sterile 0.15% water agar and placed onto MS medium plates. The MS medium plates were stratified for two days at 4°C, and then placed in growth chambers at 21°C with a 12h day/night cycle for 19 days. After 19 days, the seedlings were transferred to soil as described in section 2.2.1. Arabidopsis plants (Col-0 or Ler) at 26 days old were exposed to UV-C radiation at doses of 250, 500 or 1000 Jm⁻² as in section 2.2.2, and were returned to normal growth conditions for 24 h. At 27 days, plants were inoculated with the spore suspension of the appropriate virulent *H. arabidopsidis* isolate, 2 μl drops on 4 leaves per plant (Col-0 with Noks1 or Ler with Cala2), and plants were then placed into growth chambers set at 16°C. Pathogen growth conditions, spore suspension preparation and inoculations conducted as in section 3.2.1, 3.2.2 and 3.2.3.

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<th>Plant age (days)</th>
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**Table 4.1.** Experimental time points used in this study in relation to the age of the plant. The top row represents the age of the plants used in all experiments. The bottom row represents the experimental time points following inoculation. Plants were 26 days old when exposed to UV-C radiation, and then inoculated 24 h later at a plant age of 27 days, which is designated the 0 hpi time point.
4.2.2. Examination and quantification of *H. arabidopsidis* disease progression

Irradiated and infected plants were examined 168 hpi, as in previous experiments it was determined that this is the time point in which the pathogen had completed its life-cycle and changes in pathogen growth within suitable host tissue could be observed. Macroscopic observations of pathogenic structures were made at 168 hpi, and images acquired under a stereo microscope. Conidiophores present on infected Col-0 and Ler leaves were quantified on both the adaxial and abaxial surfaces of inoculated leaves at 168 hpi, and counted to a maximum of 20 conidiophores (Kunz *et al.*, 2008). For quantification of conidiophores, all groups (control and treatments) consisted of 9 plants, with 4 leaves inoculated on each plant. Data presented for conidiophore counts represents the average conidiophore per leaf, from a minimum of 5 independent experimental replicates. To examine the growth of the pathogen in irradiated leaves at 168 hpi, leaves were excised, cleared in 80% (v/v) ethanol and stained with LTB as in section 2.2.4. The changes to pathogen infection caused by UV-C pre-treatments were assessed in the LTB stained leaves and placed into 1 of 5 categories; Category 1: Pathogen infection only (pathogen = Noks1 or Cala2), Category 2: Pathogen & HR-like lesion, Category 3: Pathogen & cell death (UV-C induced), Category 4: HR-like lesion only and Category 5: Cell death only (UV-C induced). Percentages of each interaction were calculated for each treatment group, the data presented represents the mean percentage of three independent experiments.

4.2.3. Visualisation of callose and lignin within irradiated and infected leaves

For visualisation of callose and lignin deposition, irradiated and infected leaves were excised from the plants at 168 hpi, and leaves placed in 80% (v/v) ethanol, with daily changes to remove pigments. Once cleared, leaves were then stained with either aniline blue stain (0.5% (w/v) aniline blue in 0.15M K₂HPO₄) to visualise callose or...
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with 1% (w/v) Phloroglucinol in 70% (v/v) ethanol to visualise lignin as in sections, 2.2.5 and 2.2.6 respectively. Images presented are representative of at least two independent experiments.

4.2.4. Quantification of callose within experimental leaves

Based on results discussed in previous chapters, selected time points were chosen to represent both UV-C irradiation (48 hpi = 72 h following irradiation) and pathogen infection (168hpi). Col-0 plants were irradiated and four leaves were inoculated with the Noks1 isolate 24 hours later. Samples were taken at 0, 48 and 168 hpi, with the 0 and 48 hpi representing 24 and 72 h following UV-C treatment. Four infected leaves were excised per plant to a minimum of 50 mg of leaf material and cleared of pigments in 80% (v/v) ethanol, callose was extracted and quantified using the method of Kohler *et al.*, 2000 as in section 2.2.5. Data represents the mean of four independent experiments.

4.2.5. Hydrogen peroxide quantification in experimental leaves

Hydrogen peroxide was quantified in irradiated Col-0 leaves inoculated with the Noks1 isolate. Samples were taken a 0, 3, 6, 24, 48 and 72 hpi, in order to determine if any changes had occurred to the typical compatible (Col-0/Noks1) H$_2$O$_2$ accumulation due to UV-C pre-treatments. Irradiated and infected leaf tissue (50 mg), was collected and frozen in liquid nitrogen and stored at -80°C. H$_2$O$_2$ was extracted and quantified as stated in section 2.2.7. Quantification data represents the mean of three independent experiments.
4.2.6 Tissue sampling and RNA extraction

A minimum of 80 mg of aerial tissue (pooled together from of a minimum of three plants) of irradiated Col-0 plants inoculated with the Noks1 isolate was removed from the root systems and snap frozen in liquid nitrogen and stored at -80°C until needed. Samples were collected after pathogen inoculation a 0, 3, 6, 24, 48 and 72 hpi. RNA was extracted with a commercially available kit as in section 2.2.8.

4.2.7. Gene expression analysis using semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription PCR was performed with 1 μl cDNA that was derived from 1 μg of total RNA described in section 2.2.9, using a commercially available master mix as in section 2.2.10. The PCR conditions used for PRI, PDF1.2 and UBQ5 gene expression analysis was that used in chapter 3 (table 3.2), as it was suited to pathogen infection. However, the GST1 reaction was run for 26 cycles as in chapter 2 (table 2.2), due to the large increase in expression caused by UV-C irradiation.

4.2.8. Statistical analysis

Statistical analysis was performed using Sigma plot 11. Conidiophore quantification data was analysed with a one-way ANOVA with a Tukey test and percent of infected leaves displaying different interactions was analysed with a one-way ANOVA on ranks, with Dunn’s methods pairwise comparison. Callose quantification was analysed with a one-way ANOVA with a Tukey test, and quantification of hydrogen peroxide was analysed with a two way ANOVA with a Tukey test.
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4.3. Results

4.3.1. Changes to disease symptoms resulting from UV-C pre-treatment.

Col-0 plants were irradiated with 250, 500 or 1000 J m\(^{-2}\) and were infected with the Noks1 isolate of *H. arabiopsidis* 24 h later. Subsequent infection of the plants by the pathogen varied as UV-C dosage increased. In the 0 J m\(^{-2}\) control plants, Noks1 inoculation resulted in typical compatible interaction that occurs in this system, with healthy-looking green leaves showing full disease symptoms such as conidiophore production (Figure 4.1a & b). Plants exposed to 250 J m\(^{-2}\) (Figure 4.1c & d) and 500 J m\(^{-2}\) (Figure 4.1e & f) showed a lower frequency of conidiophore proliferation on infected leaves, and leaves displayed mild chlorosis. The biggest reduction of conidiophores occurred in the 1000 J m\(^{-2}\) group, with little or no conidiophores present in infected leaves and leaves displayed leaf bronzing and glazing (Figure 4.1g & h).

When Ler plants were exposed to the same dosages of UV-C, then infected with the Cala2 isolate of *H. arabiopsidis* 24 h later, a similar reduction in conidiophores was found to that of the Col-0/Noks1 interaction. For the 0 J m\(^{-2}\) control plants, compatibility was shown by the presence of numerous conidiophores protruding from healthy-looking infected leaves (Figure 4.2a & b). Despite the appearance of hypersensitive-like lesions surrounding the inoculation site in the 250 J m\(^{-2}\) treatment group, conidiophores were also present (Figure 4.2 c & d). The 500 J m\(^{-2}\) displayed reduced amounts of conidiophores accompanied by hypersensitive-like lesions that surrounded the initial inoculation sites (Figure 4.2e & f). Plants treated with 1000 J m\(^{-2}\) had little to no conidiophore production within inoculated leaves and treated plants showed signs of leaf curling (Figure 4.2g & h).
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**Figure 4.1.** UV-C irradiated Col-0 plants infected with the virulent *H. arabidopsidis* isolate Noks1 and examined at 168 hpi. a) 0 Jm\(^{-2}\) control plants infected with Noks1, conidiophores present in inoculated leaves, b) conidiophores extending from control leaf (inset). c) Plants irradiated with 250 Jm\(^{-2}\) UV-C radiation, d) leaf irradiated with 250 Jm\(^{-2}\) displaying conidiophores extending from the leaf. e) Plants irradiated with 500 Jm\(^{-2}\) with fewer conidiophores. f) Leaf irradiated at 500 Jm\(^{-2}\), then infected with Noks1. g) Plants treated with 1000 Jm\(^{-2}\) displaying little conidiophore production on infected leaves and signs of leaf bronzing and glazing (inset), h) leaf irradiated with 1000 Jm\(^{-2}\) UV-C and infected with Noks1. Bars in a, c, e & g represent 1 cm, and bars in b, d, f & h represent 0.5 cm and bars in inset b) 1 mm, g) 0.5cm.
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Figure 4.2. UV-C irradiated Ler plants infected with the virulent *H. arabidopsidis* isolate Cala2 and examined at 168 hpi. a) 0 Jm$^{-2}$ control plants infected with Cala2, b) conidiophores extending from control leaf (inset). c) Plants irradiated with 250 Jm$^{-2}$, d) leaf irradiated with 250 Jm$^{-2}$ showing conidiophores and HR (black arrowhead) along inoculation site. e) Plants irradiated with 500 Jm$^{-2}$ with less prolific conidiophores growth on inoculated leaves and prominent areas of HR, f) leaf irradiated with 500 Jm$^{-2}$ UV-C, displaying HR lesions within initial inoculation site (inset). g) Plants treated with 1000 Jm$^{-2}$ display little conidiophore production on infected leaves and also displaying UV induced leaf curling (white arrowhead) h) leaf irradiated with 1000 Jm$^{-2}$ UV-C and infected with Cala2. Bars in a, c, e &g represent 1 cm, and bars in b, d, f &h represent 0.5 cm, bars in insets 1 mm.
4.3.2. Changes in *H. arabidopsidis* conidiophore production

The numbers of conidiophores present on control and UV-C treated leaves was used as a quantifiable marker of the infection progression of Noks1 and Cala2 in the Col-0 and Ler ecotypes respectively. Both Col-0 and Ler plants were irradiated with dosages of 250, 500 or 1000 Jm\(^{-2}\), then inoculated with their respective virulent isolates of *H. Arabidopsidis*. Col-0 plants inoculated with the Noks1 isolate displayed an average of 16 conidiophores per leaf in the 0 Jm\(^{-2}\) control group. No significant difference in the conidiophore production was observed in plants in the 250 Jm\(^{-2}\) treatment group, which contained an average of 13 conidiophores per leaf. Significant reductions were observed in plants exposed to the 500 and 1000 Jm\(^{-2}\) dosages compared to the 0 Jm\(^{-2}\) control, with averages of 10 (P< 0.05) and 1 (P<0.001) conidiophores per leaf respectively (Figure 4.3a).

Ler plants exposed to UV-C radiation and inoculated 24 h later with the Cala2 isolate, showed a similar trend as infected Col-0 plants, with decreased conidiophore production correlating with increased UV-C dosage (Figure 4.3b). Ler plants in the 0 Jm\(^{-2}\) control group contained an average of 17 conidiophores per leaf. No significant difference in conidiophore production was observed in plants treated with 250 Jm\(^{-2}\), compared to the infected control plants, with an average of 15 conidiophores per leaf. Significant reductions in average conidiophores per leaf were found in plants treated to 500 and 1000 Jm\(^{-2}\), with 11 (P<0.05) and 2 (P<0.001) conidiophores per leaf respectively.
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Figure 4.3. Average number conidiophores present on leaves exposed to UV-C radiation prior to infection. a) Col-0 plants exposed to UV-C and then infected with the Noks1 isolate, b) Ler plants exposed to UV-C and then infected with the Cala2 isolate. Asterisk represents significant differences compared to 0 Jm$^{-2}$ control, one asterisk represent (P<0.05), two asterisk represent (P<0.001), error bars represent ± SEM.
4.3.3. **Examination of pathogen colonisation within UV-C pre-treated leaves**

Examination of hyphal colonisation within infected tissue demonstrated UV-C induced changes to pathogen infection at 168 hpi. *H. arabidopsidis* (Noks1) growth in Col-0 displayed a typical compatible interaction, with the completion of its life cycle where prolific hyphae growth and conidiophore production had occurred in the 0 Jm$^{-2}$ (Figure 4.4a). Similar prolific hyphal colonisation was also observed in the 250 Jm$^{-2}$, with conidiophore production, and no observable decrease in pathogenic colonisation of the mesophyll (Figure 4.4b). Pathogen growth was observed in the 500 Jm$^{-2}$ treatment group at a lesser extent when compared to the control leaves. The appearance to hypersensitive-like lesions near the sites of pathogen penetration was also observed (Figure 4.4c). In the 1000 Jm$^{-2}$ treatment, extensive randomised cell death was present with in the mesophyll and little to no pathogen growth was observed in irradiated leaves (Figure 4.4d).

*H. arabidopsidis* (Cala2) colonisation of the Ler ecotype also displayed changes to infection due to the exposure to UV-C radiation. Prolific hyphal colonisation was observed in the Ler leaves in the 0 Jm$^{-2}$ control plants (Figure 4.4e). Although a similar amount of pathogen growth was observed in the 250 Jm$^{-2}$ treatments, cell death occurred in hypersensitive-like lesions in association to pathogenic growth. Their presence did not significantly reduce pathogen growth and colonisation of tissue (Figure 4.4f). Prominent hypersensitive-like lesions were present in leaves irradiated with 500 Jm$^{-2}$, accompanied by reduced hyphal colonisation of the pathogen within the host tissue (Figure 4.4g). Extensive randomly distributed cell death was present within the mesophyll of leaves; however, HR-like lesions were also noted on leaves irradiated with 1000 Jm$^{-2}$, and resulted in limited pathogen growth in the irradiated tissue (Figure 4.4h).
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<table>
<thead>
<tr>
<th>UV-C Irradiation (Jm^-2)</th>
<th>Col-0/Noks1</th>
<th>Ler/Cala2</th>
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Figure 4.4. Growth of *H. arabidopsidis* in UV-C irradiated leaves at 168 hpi. a-d) Col-0 plants irradiated with UV-C and inoculated with the Noks1 isolate. a) 0 Jm$^{-2}$, conidiophore protruding out of stomata and producing conidiospores. b) 250 Jm$^{-2}$, hyphal colonisation of mesophyll cells infected with haustoria (white arrowhead). c) 500 Jm$^{-2}$, HR-like area within the mesophyll of infected tissue (black arrowhead). d) 1000 Jm$^{-2}$, UV-C induced cell death present within the mesophyll of affected leaves (red arrowhead). e-h) Ler irradiated with UV-C and inoculated with Cala2 isolate e) 0 Jm$^{-2}$, pathogen hyphae colonising mesophyll and haustoria infecting cells (white arrowhead). f) 250 Jm$^{-2}$, hyphae colonising mesophyll (white arrowhead) and areas of cell death (red arrowhead). g) 500 Jm$^{-2}$, HR-like cell death in the mesophyll of infected leaves (black arrowhead). h) 1000 Jm$^{-2}$, HR-like lesion surrounded by cell death (black arrowhead). Bars represent 50 μm.
When host responses were examined at a microscopic level, it became apparent that different interactions within the host tissue had occurred that were induced by UV-C irradiation, and resulted in four distinct interactions in the Col-0/Noks1 and five in the Ler/Cala2 systems. The 0 Jm$^{-2}$ control only showed one category, which was Noks1 infection alone of inoculated leaves (100%). In the 250 Jm$^{-2}$ treatment group three different interactions were found in inoculated leaves: Noks1 infection alone (70%), Noks1 infection accompanied with HR-like lesions (21.6%) and HR-like lesions with no pathogen growth (8.3%). The 500 Jm$^{-2}$ displayed 4 different interactions and consisted of: Noks1 infection only which was significantly reduced (33.3%) when compared to the control (P<0.01), Noks1 infection accompanied by HR-like lesions (13.3%), HR-like lesions with no Noks1 growth (33.3%) and only cell death (20%). The 1000 Jm$^{-2}$ displayed two different interactions within inoculated leaves, leaves that contained Noks1 infection only was significantly reduced (6.6%) (P<0.01) and leaves that only contained cell death with no Noks1 infection was significantly increased (93.3%) (P<0.01) (Figure 4.5).

A similar trend was also observed in the Ler/Cala2 system as UV-C dosage increased. The 0 Jm$^{-2}$ contained only one interaction within inoculated leaves, which was Cala2 infection only (100%). In the 250 Jm$^{-2}$ treatment four interactions were observed, Cala2 infection only (50%), Cala2 growth accompanied with HR-like lesions (25%), Cala2 infection in the presence of UV-induced cell death (8.3%) and leaves only containing HR-like lesions (16.6%). The 500 Jm$^{-2}$ also displayed four interactions, Cala2 infection only was significantly reduced (15%) (P<0.05), Cala2 infection in the presence of cell death (13.3%), HR only with no pathogen present showed a significantly increase (65%) (P<0.05) and leaves only containing UV-C induced cell death (6.6%). The 1000 Jm$^{-2}$ showed four different interactions, and it should be noted a
Figure 4.5. Percentage of Col-0 leaves displaying different UV-C induced interactions to the *H. arabidopsidis* isolate Noks1. Irradiated leaves infected with Noks1 displaying induced changes to the compatible interaction. Asterisks represent significant difference compared to the 0 Jm$^{-2}$ control (P<0.01).
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Figure 4.6. Percentage of Ler leaves displaying different UV-C induced interactions to the *H. arabidopsidis* isolate Cala2. Irradiated leaves infected with Cala2 displaying induced changes to the compatible interaction. Asterisks represent significant difference compared to the 0 Jm$^{-2}$ control (P<0.05).
significant decrease had occurred with no leaves displaying Cala2 infection only (0%) (P<0.05), Cala2 infection accompanied by HR (6.6%), Cala2 infection in the presence of cell death (6.6%), leaves only containing HR-like lesions with no pathogen present significantly increased (40%) (P<0.05) and leaves only containing cell death was significantly increased (46.6%) (P<0.05)(Figure 4.6).

4.3.4. Changes to callose distribution in response to UV-C irradiation and pathogen infection

Callose deposition in UV-C treated plants was examined at 168 hpi to determine if callose distribution in response to pathogen infection was altered. In the Col-0 ecotype, the Noks1 isolate demonstrated typical compatibility with prolific hyphal growth and callose formations that encased the haustoria within infected cells, which primarily occurred in the mesophyll of the 0 Jm⁻² control (Figure 4.7a). In the 250 Jm⁻² treatment, callose deposition surrounded haustoria within infected cells; however, callose had also been deposited in localised areas where pathogen growth was absent, which occurred along the walls of epidermal cells and underlying mesophyll cells (Figure 4.7b). The 500 Jm⁻² treatment displayed callose encased haustoria in infected cells and deposition of callose along cell walls of epidermal cells of uninfected cells (Figure 4.7c). In the 1000 Jm⁻² treatment, substantial callose deposition occurred along the entirety of the cell walls in the absence of pathogen growth (Figure 4.7d).

In the Ler ecotype, the Cala2 isolate also demonstrated compatibility in the 0 Jm⁻² control, with callose deposition that had encased haustoria within infected cells (Figure 4.7e). In the 250 Jm⁻² treatment, callose encased haustoria, and deposition also occurred along walls of epidermal and mesophyll cells in HR-like areas where pathogen growth had occurred (Figure 4.7f). Although pathogen growth was observed in the 500 Jm⁻²...
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Figure 4.7. Callose deposition in response to UV-C radiation and pathogen infection examined at 168 hpi. a-d) Col-0 plants infected with the Noks1 isolate. a) Callose deposition localised around haustoria of infected cells. b) 250 Jm$^{-2}$, callose deposition associated with haustoria and along the cell walls in highly localised areas (white arrowhead). c) 500 Jm$^{-2}$, callose deposition in association with haustoria and along the cell walls of epidermal cells. d) 1000 Jm$^{-2}$, extensive deposition of callose lining the entirety of cell walls (inset). e-h) Ler plants irradiated and infected with the Cala2 isolate. e) 0 Jm$^{-2}$, callose deposition that occurred in association with the haustoria. f) Callose deposition in association with haustoria also lining cell walls of epidermal and within localised patches of mesophyll cells. g) 500 Jm$^{-2}$, large HR-like areas containing large amounts of callose (white arrowhead). h) 1000 Jm$^{-2}$, callose deposition that completely fortified the cells walls in highly localised areas (white arrowhead). Bar represents 200 μm, inset 50μm.
Figure 4.8. Total amount of extracted β-1, 3 glucans from Col-0 leaves irradiated with UV-C and infected with the Noks1 isolate. Callose concentrations expressed as β-1, 3 glucan present within the 0 Jm$^{-2}$ (bars with diagonal lines), 250 Jm$^{-2}$ (white bars), 500 Jm$^{-2}$ (grey bars) and 1000 Jm$^{-2}$ (black bars). Asterisks represent a significant difference compared to the 0 Jm$^{-2}$ control at corresponding time points (P<0.05), error bars represent ± SEM.
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treatment, prominent HR-like lesions were found along the initial inoculation site that contained large amounts of callose deposition along the affected cell walls (Figure 4.7g). The 1000 Jm⁻², showed little pathogen growth, callose deposition occurred in epidermal cells, and in highly localised areas of mesophyll cells within and surrounding the HR like lesions (Figure 4.7h).

Quantification of callose in the Col-0 ecotype, revealed increases that were due to both UV-C irradiation and eventual increases due to Noks1 infection (Figure 4.8). β-1, 3 glucan concentrations were increased within tissue irradiated with 500 and 1000 Jm⁻² (38.63 ng/mg and 132.13 ng/mg respectively) at 0 hpi, however only the 1000 Jm⁻² showed a statistically significant increase when compared to the control (P<0.05). At 48 hpi, β-1, 3 glucan concentration in the 500 and 1000 Jm⁻² treatments (23.51 ng/mg and 184.85 ng/mg respectively) remained significantly higher than the 0 Jm⁻² control (P<0.05). However, at 168 hpi, large increases in β-1, 3 glucans was found in the 0 and 250 Jm⁻² treatments (215.14 ng/mg and 203.63 ng/mg respectively). Significantly less β-1, 3 glucans were present in the 500 and 1000 Jm⁻² UV-C treatment groups (138.57 ng/mg and 131.96 ng/mg respectively)(P <0.05) when compared to the 0 and 250 Jm⁻² treatments that occurred at 168 hpi.

4.3.5. Changes in the distribution of lignin caused by UV-C pre-treatment in infected plants

The presence of lignin was examined by staining leaf tissue with phloroglucinol to determine if lignification in response to the pathogen is altered due to the UV-C pre-treatment. In the Col-0 ecotype, positive staining for lignification occurred in cell walls of directly penetrated by the Noks1 isolate in the 0 Jm⁻² control plants (Figure 4.9a). No change in the distribution of lignin was observed in the 250 Jm⁻² treated plants, as
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Figure 4.9. Lignification of cell walls in response to UV-C pre-treatments and pathogen inoculation and examined at 168 hpi. a-d) Col-0 leaves infected with the Noks1 isolate. a) 0 Jm$^{-2}$, lignification of cell walls that have penetrated by the pathogen (black arrowhead) (inset). b) 250 Jm$^{-2}$, lignified cells responding to pathogen invasion (black arrowhead). c) 500 Jm$^{-2}$ HR-like cell death staining positive for lignin. d) 1000 Jm$^{-2}$, UV-induced cell death, with pale orange staining with in dead cells (inset, grey arrowhead). e-h) Ler leaves infected with the Cala2 isolate. e) 0 Jm$^{-2}$, lignified cells penetrated by *H. arabidopsidis* (inset). f) 250 Jm$^{-2}$, localised areas of cells with lignified walls infected with *H. arabidopsidis* (black arrowheads). g) 500 Jm$^{-2}$, HR-like lesions staining positive for lignin (black arrowheads). h) 1000 Jm$^{-2}$, lignin positive staining occurring within HR-like lesion. Bars represent 100 μm, inset 50 μm.
lignification occurred in cells affected by the pathogen (Figure 4.9b). In leaves treated with 500 Jm\(^{-2}\), intense staining was observed in hypersensitive like cells, indicating lignification had occurred in these cells prior cell death (Figure 4.9c). In leaves treated with 1000 Jm\(^{-2}\), staining was observed as pale orange staining within in dead cells that were scattered throughout the mesophyll (Figure 4.9d).

In the Ler ecotype, plants exposed to UV-C radiation demonstrated similar staining when inoculated with the Cala2 isolate. Cell walls stained positive for lignin that had been penetrated by Cala2, in the 0 Jm\(^{-2}\) (Figure 4.9e). Similar staining was also observed in the 250 Jm\(^{-2}\) treatments, where staining only occurred in cells infected by the pathogen (Figure 4.9f). In the 500 Jm\(^{-2}\), lignification had occurred within hypersensitive-like cell death (Figure 4.9g). At 1000 Jm\(^{-2}\), hypersensitive like lesions became apparent with pink/red staining of walls of dead cells, these lesions also appeared amongst the UV-C induced cell death in affected tissue that stained a pale orange colour (Figure 4.9h).

### 4.3.6. Changes in H\(_2\)O\(_2\) generation in response to the compatible Noks1 isolate induced by UV-C pre-treatments

Hydrogen peroxide was quantified in the Col-0 ecotype that was irradiated then infected the Noks1 isolate. The levels of H\(_2\)O\(_2\) in the 0 Jm\(^{-2}\) control groups demonstrated the baseline levels of H\(_2\)O\(_2\) in response to pathogen colonisation, with a gradual decrease in concentration over time. The 250 Jm\(^{-2}\) did not show any change with no significant differences found when compared to the control. Increases were seen in the 500 Jm\(^{-2}\) across various time points, yet no significant differences were found. The 1000 Jm\(^{-2}\) displayed significant increases in H\(_2\)O\(_2\) concentrations at 6, 48 and 72 hpi (P<0.05) (Figure 4.10).
4.3.7. UV-C induced changes to pathogen defence gene expression in Col-0 plants infected with the compatible Noks1 isolate

Gene expression analysis was conducted in Col-0 plants pre-treated with UV-C radiation and then infected with the compatible Noks1 isolate, in order to determine changes that were induced by the UV pre-treatments. The ROS marker gene GST1 displayed little change in the 0 Jm$^{-2}$, apart from an increase in expression at 72 hpi. An increase in expression level of GST1 occurred in the 250 Jm$^{-2}$ treatment, with highest expression occurring at 3-6 hpi followed by a decrease and a final increase at 72 hpi. Both the 500 and 1000 Jm$^{-2}$ displayed high levels of expression that occurred from the 0 to 24 hpi followed by a gradual decrease in expression (Figure 4.11a).

The SA marker gene PRI also showed altered expression in plants infected with Noks1. PRI expression did not display any change in activity between 0-48 hpi, however in increase was observed at 72 hpi. The 250 Jm$^{-2}$ did not demonstrate a significant change in expression levels when compared to the 0 Jm$^{-2}$. However, PRI expression remained increased across all time points in the 500 and 1000 Jm$^{-2}$ treatments (Figure 4.11b). Expression of the JA/Et marker gene PDF1.2, did not display any change in expression in the 0 Jm$^{-2}$ control. Increased expression was observed at 24 and 72 hpi in plants treated with 250 Jm$^{-2}$, and at 3, 24 and 72 hpi in plants treated with 500 Jm$^{-2}$. The 1000 Jm$^{-2}$ also displayed an increase in PDF1.2 expression levels from 0 to 6 hpi with a further increase at 24 to 72 hpi (Figure 4.11c). UBQ5 was used as an internal standard and displayed stable expression across the experiment, however slight reductions in expression were noted in the 0 Jm$^{-2}$ 24 hpi and 500 Jm$^{-2}$ 0 hpi samples.
Figure 4.10. $H_2O_2$ generation in the Col-0 ecotype irradiated with UV-C and then infected with the Noks1 isolate over 72 h. $H_2O_2$ concentrations present with 0 Jm$^{-2}$ (bars with diagonal lines), 250 Jm$^{-2}$ (white bars), 500 Jm$^{-2}$ (grey bars) and 1000 Jm$^{-2}$ (black bars) treatments. Asterisk represents significant differences with the corresponding 0 Jm$^{-2}$ control at each time point ($P<0.05$), error bars represent $\pm$ SEM.
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Figure 4.11. Gene expression profile of UV irradiated Col-0 plants infected with Noks1 over 72 hours; a) ROS marker gene *GST1*, b) salicylic acid defence pathway marker gene *PR1*, c) jasmonic acid/ethylene defence pathway marker *PDF1.2*, d) *UBQ5* was used as an internal standard (housekeeping gene).
4.4 Discussion

This study examined the changes to the typical host response in the compatible interactions that was induced by UV-C irradiation, which rendered Arabidopsis ecotypes Col-0 and Ler more resistant to the normally virulent isolates of *H. arabidopsidis*. By examining the activated defence responses that was caused by UV-C irradiation this work further elucidates the mechanisms that are involved in the transition from susceptibility to induced resistance. Accumulation of H$_2$O$_2$ and *PRI* induction due to UV-C irradiation and prior pathogen infection must play a role in the formation of the HR-like lesions triggered in response to pathogen attack. The distribution of callose and lignin in response to the pathogen changed from the typical compatible distribution with lignin and callose occurring in cells infected with haustoria, to a distribution that was observed in a typical incompatible interaction with lignification of hypersensitive cells and callose that deposition surrounded these regions.

4.4.1. UV-C exposure increases resistance to *H. arabidopsidis* infection

Col-0 and Ler plants exposed to UV-C radiation demonstrated a reduction in disease symptoms caused by *H. arabidopsidis*, seen as a reduction in hyphal colonisation and conidiophore production. Hypersensitive-like lesions occurred in irradiated tissue at the site of inoculation. The absence of these lesions in UV-C irradiated tissue only indicates that these HR-like regions were triggered by pathogen infection as demonstrated in chapter 2. These lesions may have contributed to the reduced pathogen colonisation by halting infection or possibly attenuating the complete colonisation of the host. As the UV-C dosage increased, disease severity and the quantity of conidiophores decreased. This illustrates a clear, dose dependence that was
found in both the Col-0 and Ler ecotypes of Arabidopsis. These results agree with Kunz et al., 2008, which also showed a dose dependent reduction in *H. arabidopsidis* conidiophores with increasing sub lethal doses of UV-C.

Microscopic examination of pathogen infected leaves revealed a reduction in pathogen colonisation and the highly localised HR-like cell death, which is similar to the HR cell death caused by incompatibility as demonstrated in chapter 3. This HR-like cell death indicates a specific cellular response to the invading pathogen, which ultimately leads to the death of affected cells and halt pathogen growth. The reduction in pathogen growth and the appearance of HR-like cell death has been reported in plants infected with normally virulent pathogens, where resistance has been induced by priming agents (Ahn et al., 2007, Slaughter et al., 2012). A notable difference between the two ecotypes of Arabidopsis occurred in the 1000 Jm$^{-2}$ group, where most of the Col-0 leaves infected with Noks1 displayed extensive cell death caused by the high UV-C dosage. However, Ler leaves infected with Cala2 displayed both distinct HR-like cell death and UV-C induced cell death. This illustrates that variations seen between the Col-0 and Ler ecotypes may be the result of difference in tolerances towards UV-C exposure.

**4.4.2. UV-C exposure induced changes to callose and lignin deposition**

In a compatible interaction, callose has been shown to encase the haustoria of *H. arabidposidis*, and more importantly, callose is deposited in association with the pathogen (Donofrio et al., 2001). This is seen in both the Noks1/Col-0 and Cala2/Ler systems at 0 Jm$^{-2}$. The ‘normal’ compatible distribution of callose began to change as dosages of UV-C were applied, and callose deposition occurred in areas free of hyphal growth. These areas contained HR-like cell death that was triggered by pathogen
penetration, and was surrounded by cells producing callose along their walls, this type of distribution is similar to that seen in an incompatible interaction (chapter 3). Col-0 leaves irradiated with 1000 Jm$^{-2}$ displayed mesophyll cells walls that were completely encased with callose; this interaction is mostly due to the high dosage of UV-C irradiation and not in response to the pathogen itself. Whereas, the deposition of callose in Ler plants treated with 1000 Jm$^{-2}$ was localised within cells surrounding the HR-like regions.

When callose contents were quantified in irradiated Col-0 leaves infected with Noks1, the concentrations present across specific time points appeared as a hybrid of the callose content profiles shown to occur in response to UV-C irradiation alone (chapter 2) and $H.\ arabidopsidis$ infection (chapter 3). Callose contents in the 0 and 250 Jm$^{-2}$ treated leaves, did not display a large extent of callose in the early time points, however a large increase in callose concentration was found at 168 hpi in both the control and the 250 Jm$^{-2}$ treatment groups. This increase is associated with pathogen growth, and as more cells are infected more callose is produced, and this pattern is clearly shown to occur in the compatible interaction of Noks1 and Col-0 (chapter 3). Callose was present prior to pathogen inoculation within leaves treated with 500 and 1000 Jm$^{-2}$, the deposition seen at 0 and 48 hpi is consistent with the profile observed in plants exposed with UV-C radiation alone at these time points, as these time points equate to the 24 and 72 hours after irradiation (shown in chapter 2). At the 168 hpi time point the similar amount of callose was found in the 500 and 1000 Jm$^{-2}$, yet contained lower concentrations of callose when compared to the 0 and 250 Jm$^{-2}$ treatments. Due to the pathogen growth observed in the 500 Jm$^{-2}$ treatment, the callose quantified may be a combination of UV-C and pathogen induced callose. However, because of the
limited pathogen growth observed in the 1000 Jm\(^{-2}\) treatment it is highly likely that the callose present in this treatment is due to the exposure to UV-C radiation.

Ellinger et al. 2013, successfully suppressed the normally virulent powdery mildew *Golovinomyces cichoracearum* by inhibiting penetration of the epidermis with increased callose deposition at early time points. Although callose production is clearly shown to be induced by UV-C radiation at 0 hpi, the role of callose in the early interactions and its involvement in limiting with *H. arabidopsidis* penetration has not yet been fully explored. However, callose produced by UV-C exposure may not completely impede penetration of the epidermis; the presence of HR-like lesions in the mesophyll layer indicates that germination of the conidiospores led to the penetration of the epidermis and infection of the mesophyll cells before the induction of cell death. This indicates that the callose that was induced by UV-C irradiation before infection was not sufficient to completely halt pathogen penetration on its own.

Lignin was detected within the walls of infected cells in the control and 250 Jm\(^{-2}\) treatments where pathogen growth remained prolific. As the dosages increased, lignification also occurred within the walls of cells which had undergone cell death in HR-like areas where similar formations of lignin was also observed in the hypersensitive cells caused by the incompatible interaction observed in chapter 3 and other plant patho-systems (Mauch-Mani et al., 1996, Mohr et al., 2007). This makes the HR-like cell death distinct with positive pink/red staining, when compared to the randomised cell death caused by UV-C radiation alone, which stained a pale orange. This differential staining clearly demonstrates that the UV-C induced cell death and the localised HR-like cell death are not the same response as lignification in this system.
only occurred in cells directly affected by the pathogen and lignin was produced prior to HR-like cell death.

### 4.4.3. Changes in hydrogen peroxide generation caused by UV-C irradiation may alter the plants susceptibility

UV exposure has been shown to induce increases of $H_2O_2$ in exposed Arabidopsis plants as shown in chapter 2, and in a variety of other plant systems (Mahdavian et al., 2008, Tang et al., 2010). It became clear that UV-C pre-treatment of plants had considerably altered the $H_2O_2$ concentration profile of the typical Nok1/Col-0 compatible interaction with elevated levels of $H_2O_2$ in the 500 and 1000 Jm$^{-2}$ treatments in both $H_2O_2$ concentration and $GST1$ expression. Increased levels of $H_2O_2$ within plants prior and during pathogen infection have been shown to result in increased resistance and reductions in the growth *Pseudomonas syringae* (Mukherjee et al., 2010). The elevated levels of $H_2O_2$ may be a vital signal in the activation of UV-C induced resistance to *H. arabidopsis*, whereby UV-C induction of ROS in irradiated cells can lead to an augmented defence response when ROS is again induced in response to initial pathogen infection leading to the activation of defence responses.

The explanation given may be true for the formation of HR-like cell death, however, this does not explain the lack of HR-like cell death in Col-0 leaves treated with 1000 Jm$^{-2}$, as leaves free of pathogen growth contained extensive UV induced cell death yet contained significant increases in $H_2O_2$. The increased $H_2O_2$ in this treatment may also reflect the occurrence of UV-C induced cell death within the irradiated leaf tissue.
4.4.4. UV-C irradiation induced changes to defence gene expression that contributed to resistance

The strong induction of PR1 observed in the 500 and 1000 Jm$^{-2}$ treatments suggests that the salicylic acid defence pathway was active in response to UV-C exposure prior and during pathogen infection of the Noks1 isolate. UV-C exposure has previously been shown to induce SA accumulation and PR1 expression in Arabidopsis (Shapiro et al., 2001, Nawrath et al., 2002). The fact that the PR1 expression is occurring before and during pathogen infection at these dosages may considerably hamper *H. arabidopsis* efforts to colonise the host’s tissue. This type of ‘augmented’ PR1 expression has been shown previously in Arabidopsis plants that have been primed by various inducers, which resulted in these plants displaying high levels of PR1 expression between 0-6 hours following inoculations, which commonly led to activation of HR and a decreases in *P. syringae* growth (Ahn et al., 2007, Zhang et al., 2009). If SA accumulation and PR1 induction is occurring before infection and during initial penetration, it could explain the rapid defence responses and HR-like formation that occurs in normally susceptible leaves towards *H. arabidopsis* colonisation. Expression of the JA/Et marker gene PDF1.2 is increased in the 500 and 1000 Jm$^{-2}$ dosages of UV-C treatments when compared to the 0 Jm$^{-2}$ controls is most likely due to the UV-C exposure of the plants and the resulting damage that may mimic wounding (chapter 2), as the jasmonic acid/ethylene pathways are associated with wounding and defences to necrotrophic pathogens (Penninckx et al., 1998, Bari et al., 2009).

UV-C irradiation increases resistance of *H. arabidopsis* by causing a decrease in pathogen proliferation and activation of HR-like responses at the sites of pathogen infection, which involves cell death, callose and lignin production in the affected cells.
Chapter 4: UV-C irradiation increases resistance of Arabidopsis to virulent isolates of *H. arabidopsis*

This type of cell death arrangement strongly resembles the resulting HR lesion that occurs with an incompatible interaction. The distribution of callose and lignin with cell death, accompanying with the induced HR-like lesions, is strong evidence that this is a result of pathogen interactions and not caused by UV-C irradiation alone. The clear increase to H$_2$O$_2$ concentrations caused by UV-C exposure in the higher dosages most likely leads to the activation of the SA defence pathway and results in increases in *PR1* expression. This in turn causes the rapid activation of defence responses similar to that seen in a typical incompatible interaction caused by an avirulent isolate of *H. arabidopsis*. Although this study further characterised the responses of UV-C induced resistance and filled in some gaps in the knowledge of this response, new questions have emerged, in particular the formation of the HR-like region, if early induction of H$_2$O$_2$ and *PR1* induced by UV-C radiation, would this mean that these lesions would form sooner when compared to the HR caused R gene interaction with a avirulent pathogen? This question alone would warrant further investigation.
Chapter 5: General discussion

5.1. Summary

This thesis examined the response of Arabidopsis to the abiotic stressor, ultraviolet C radiation and the biotic stressor *Hyaloperonospora arabidopsidis* alone and in combination. Examination of the impact of these stressors at a morphological, cellular and molecular level allowed for the identification of similarities and differences between the two types of stress. This work provides new insights to the current knowledge of the responses of Arabidopsis when exposed to UV-C radiation, and further elucidated the extent of damage caused and the potential mechanisms that may contribute to the cross-tolerance response that could induce resistance to pathogen attack (chapter 2). Tracking the host responses caused by *H. arabidopsidis* at various stages of the pathogen life cycle allowed for further characterisation of the plant-pathogen interactions within this system in response to both the compatible and incompatible isolates (chapter 3). The characterisation of the compatible interaction within the Col-0 ecotype set a base line for the ‘normal’ interaction and became useful in determining the changes to the compatible interaction in plants pre-treated with UV-C radiation at a cellular and molecular level. When plants were pre-treated with UV-C radiation then infected the virulent isolates of *H. arabidopsidis*, a dose-dependent relationship occurred where increases in UV-C dosages led to the decrease in pathogen infection (chapter 4). This research advances our understanding of the effects of sub-lethal dosages of UV-C radiation on Arabidopsis, identified potential mechanisms that are induced that could play a role in the increase in resistance towards pathogens, and provided new insights into the responses of Arabidopsis to infection by the compatible or incompatible isolates of *H. arabidopsidis.*
5.2. Exposure to sub-lethal doses of UV-C radiation induces changes in growth, callose deposition, $\text{H}_2\text{O}_2$ production and pathogen defence-related genes in Arabidopsis

A dose-dependent change in growth of UV treated Arabidopsis plants were observed in all UV-C dosages. Irradiances below 1000 Jm$^{-2}$ showed decreased leaf area compared to the control, however, the subsequent increases in leaf expansion seen at later time points and the lack of observable exposure symptoms demonstrate the plants recovery to this stress. The most severe changes occurred at the highest dosage (1000Jm$^{-2}$), where leaf area of affected leaves did not increase in any of the time points tested. The plants appeared visibly stressed, with leaf bronzing and curling. These symptoms were not observed until 72 h after irradiation. At the microscopic level, cell death was observed in the mesophyll layer in all UV-C treatments. The extent of tissue damage increased with dosage with the 1000 Jm$^{-2}$ displaying the most extensive cell death damage to the leaf tissue, which is not surprising as UV-C has been shown to be a potent inducer of plant cell death but at much higher levels (Danon et al., 1998, Gao et al., 2008).

The highest dosage used in this experiment, is still technically a sub-lethal dosage irradiation, as the irradiated plants survived after treatment. The severe damage to the photosynthetic tissue, through cellular damage and cell death illustrates that dosages above 1000 Jm$^{-2}$ may not be an appropriate treatment for the study of the effects of sub-lethal UV-C radiation on Arabidopsis. Also noted were the significant changes, such as leaf curling and bronzing, were not observed until 72 h after irradiation. Generally, studies exposing plants to relatively high levels of UV-C radiation end their experiments at 48 or 72 h after irradiation, and may not observe these severe symptoms and the true extent of damage as the experiment had ended (Nawrath et al., 2002, Stefanato et al., 2009).
A major finding of this research showed that callose deposition occurred following UV-C exposure and was produced by surviving cells. This is the first demonstration of callose deposition to my knowledge in response to UV radiation, and has not been reported in plants irradiated with UV (A, B or C), or light in general. Furthermore, changes in light intensity appear to alter the extent of callose deposition when stimulated by elicitors, and its synthesis appears to be associated with H$_2$O$_2$ generation (Luna et al., 2010). Deposition of callose in response to heavy metals or elicitors had been shown to involve disruption of the cell membrane and calcium signalling (Köhle et al., 1985, Wissemeier et al., 1987). Considering no chemical or physical stress was applied to the UV-C treated plants, and as UV-C treatments induced an increase H$_2$O$_2$ with in the first 24 h following irradiation, it would seem likely that the H$_2$O$_2$ generated would have been sufficient to alter the integrity of the cell membrane and possibly lead to callose synthesis. Although callose was present in irradiated tissue, no change in lignin contents was observed within treated tissue.

ROS generation has been shown to be closely linked with the activation of the salicylic acid defence pathway (Leon et al., 1995, Mukherjee et al., 2010). The induction of the SA pathway was observed in the form of increased expression of the SA marker gene $PRI$ in response to UV-C irradiation (chapter 2). Also observed was the increase in expression $PDF 1.2$, following irradiation which signifies the induction of the jasmonic acid/ethylene pathways (Penninckx et al., 1998, Zimmerli et al., 2004). Although the induction of $PDF 1.2$ might not significantly contribute to resistance to biotrophic pathogens (Thomma et al., 1998), this suggests that the damage caused by the higher doses of UV-C radiation induced a wound-like state that would be sufficient in the activation of these pathways.
Figure 5.1. Proposed model of events within an Arabidopsis cell following irradiation with 500 Jm\(^{-2}\) of UV-C. Incoming UV-C radiation causes DNA damage, which is perceived by the cell and initiate DNA repair mechanisms and possibly perpetuates a stress signal, an increase in \(\text{H}_2\text{O}_2\) generation leads to the induction of \(\text{GST1}\), and reductions in \(\text{H}_2\text{O}_2\) levels. The changes in \(\text{H}_2\text{O}_2\) levels induce production of salicylic acid and \(\text{PRI}\) expression, but may also cause damage to the cell membrane through lipid peroxidation causing the release of linolenic acid which induces the jasmonic acid/ethylene pathways and \(\text{PDF 1.2}\) expression. Text in bold represents the events shown in this work, upward arrows represents increases in concentration or expression, dashed lines represents proposed events based on previous work and numbers correspond to the studies which demonstrated these responses; 1: Kunz et al., 2008, 2: Green & Fluhr, 1995 and 3: Conconi et al., 1996.
5.3. Induction of key pathogen resistance mechanisms during infection does not always lead to resistance

Both the incompatible and compatible *H. arabidopsidis* isolates showed similar pathogen development in the early infection stages, from initial epidermal penetration to primary haustorium development within Col-0. Successful infection leads to the prolific colonisation of Col-0 and conidiophore production of the compatible (Noks1) isolate. Whereas, detection of the ATR2 effector protein by the RPP2 R protein leads to the activation of the hypersensitive response in the host and containment of the *H. arabidopsidis* Cala2 isolate (Sinapidou et al., 2004). In the compatible and incompatible interactions, several defence markers appeared in both interactions, yet the timing and distribution varied. Callose was observed surrounding the neck of the haustoria before it was completely encased. There is very good evidence to suggests that callose containing papillae act as a mechanical barrier to slow pathogen invasion and expose the invading organism to antimicrobial compounds (Stone et al., 1992, Hématy et al., 2009, Voigt et al., 2009, Zimmerli et al., 2000, Zeyen et al., 2002b). However, its effectiveness in the compatible interaction with a biotrophic pathogen and its capacity to perturb pathogen growth by surrounding the haustoria is still not fully understood, particularly in response to *H. arabidopsidis*. Callose was also present during incompatibility, at a much lower extent, in cells surrounding the resulting HR lesion (chapter 3). When quantified it was found that a significant increase in callose deposition occurred within the compatible interaction when compared to the incompatible interaction. This increase was clearly due to the prolific growth of *H. arabidopsidis* Noks1 isolate with callose deposition surrounding the haustoria.

Lignification was detected only within the walls of cells directly infected with *H. arabidopsidis*. In the compatible interaction, lignification occurred within the entire...
wall of cells infected with the *H. arabidopsidis* Noks1 isolate. In the incompatible interaction, when Col-0 plants were infected with the *H. arabidopsidis* Cala2 isolates, positive staining for lignin was detected within the walls of hypersensitive cells within the HR lesions. Lignification only occurred within the walls of cells directly infected by the pathogen, and was first observed at 72 hpi after infection had been established, this may indicate that the presence of lignin in this system may not play a major role in basal resistance during the initial stages of infection and colonisation.

Hydrogen peroxide was observed within epidermal cells at early time points as the germinating conidiospores penetrated the epidermal layer, which was observed in response to both isolates of *H. arabidopsidis*. At later time points, H$_2$O$_2$ localisation displayed a similar pattern of distribution to that seen in the lignification of cell walls, with H$_2$O$_2$ localisation only occurring within cells directly infected with the compatible Noks1 isolate and intense staining within the hypersensitive cells caused by the incompatible Cala2 isolate. The similar distribution of hydrogen peroxide may explain the distribution of lignified cells, as H$_2$O$_2$ has been shown to be involved in cell wall crosslinking and lignin polymerisation (Thordal-Christensen *et al.*, 1997, Denness *et al.*, 2011). It also became clear that an oxidative burst occurred much earlier than staining could identify, and was observed in both *GST1* expression and H$_2$O$_2$ quantification. The first oxidative burst occurred in the early time points in both compatible and incompatible isolates and may coincide with the germination of the conidiospores (Slusarenko *et al.*, 2003). The second burst only occurred within the incompatible interaction at the 72 hpi time point and coincided with the activation of the HR, which prominently occurred at this time point (chapter 3). Previous studies have also shown that a biphasic peak of H$_2$O$_2$ contents occurred in response to avirulent pathogens in tobacco cell cultures and *in planta* (Able *et al.*, 2000, Mur *et al.*, 2005).
Chapter 5: General discussion

Pathogen growth

**SUSCEPTIBILITY**

**RESISTANCE**

- Cell wall
- Lignified cell wall
- Callose deposition
- Cell membrane
- DAB ($H_2O_2$) staining

0-6 hpi

- $H_2O_2$
- GST1
- PR1

24 hpi

- PR1

48 hpi

- PR1

72 hpi

- PR1
- HR

Pathogen growth

HR activation
Figure 5.2. Representative model of events in Arabidopsis Col-0 within 72 hours following inoculation with a *H. arabidopsidis* isolate resulting in a compatible or incompatible interaction. Cells on the left represent a single cell responding to infection by the compatible isolate of *H. arabidopsidis* at different time points. In the compatible interaction, penetration of the epidermal cell layer leads initial \( \text{H}_2\text{O}_2 \) accumulation and *GST1* expression and slight *PR1* expression leading to gradual increases over time, as the pathogen infects the cell with the haustoria. DAB staining was observed and callose deposited in collar a formation before the haustoria is encased and the cell wall is lignified. The cells on the right represent a single cell responding to infection by the incompatible isolate of *H. arabidopsidis*. In the incompatible interaction, penetration of the epidermal cell layer and led to an initial \( \text{H}_2\text{O}_2 \) accumulation with increases *GST1* and *PRI* expression. As pathogen penetration of cells occurs, DAB staining was observed, increases in *PRI* expression, a secondary increase in *GST1* expression and \( \text{H}_2\text{O}_2 \) accumulation resulting in the activation of the HR and restriction of the pathogen. Text size represents the relative amount of activity of \( \text{H}_2\text{O}_2 \) and gene expression.
Expression of the salicylic acid marker gene *PRI* is commonly associated with resistance to biotrophic pathogens. Expression of the *PRI* has been observed in interactions to virulent pathogens and occurs at a much later time point and at a lower level when compared to the expression within plants infected with an avirulent pathogen (Tao *et al.*, 2003, Ahn *et al.*, 2007). In the Arabidopsis – *H. arabidopsidis*, system *PRI* gene expression was examined and it was evident that increased expression occurred in both the compatible and incompatible interactions. Expression was increased at 72 hpi in the incompatible (Cala2) interaction when compared to the compatible (Noks1) interaction; this up-regulation coincides with the activation of an HR (chapter 3). When tested, no significant changes in the expression of the JA/Et marker gene *PDF1.2* was observed in any treatment at any time. The activation of the jasmonic acid and ethylene pathways is commonly associated with defence against necrotrophic pathogens and wounding and is not normally involved in the resistance to biotrophic pathogens (Thomma *et al.*, 1998, Nawrath *et al.*, 1999).

5.4. UV-C irradiation caused increased resistance to *H. arabidopsidis*

Although the doses used were termed sub lethal, significant changes were found at the cellular level. The presence of cell death within the mesophyll of leaves only treated with UV radiation illustrated the efficiency of UV-C irradiation to induce cell death. This UV-C induced cell death may contribute (likely a small contribution) to the suppression of *H. arabidopsidis*, as the UV-C induced cell death may limit the number of competent cells available for the pathogen to successfully infect in order to gain nutrients. When disease severity was quantified a reduction in conidiophore numbers was clearly shown in the leaves treated with 500 and 1000 Jm⁻², and no significant reduction was observed in the 250 Jm⁻² treatment group. When pathogen infection was examined further it became apparent that, as UV-C dosages increased, the changes to
the typical host responses towards the compatible interaction caused by the virulent *H. arabidopsidis* isolates resembled that of an incompatible interaction. This change was observed by the presence of HR-like cell death that occurred in highly localised areas, specific to pathogen infected tissue, and not normally found in a typical compatible interaction. Also noted was the apparent difference of the number of leaves containing HR-like lesions induced by UV-C irradiation, within the Col-0 and the Ler ecotypes, the Ler ecotype displayed more HR-like lesions in response to pathogen infection when compared to the Col-0 ecotype. This demonstrates variation within the two ecotypes when they receive similar treatments.

When examining the reduction in disease symptoms and pathogen growth caused by UV-C treatments, intermediate responses in resistance were found which ranged between reductions in susceptibility to hybrid interactions displaying both resistance characteristics and UV-C induced damage characteristics as dosages increased. One of the most notable UV-C induced characteristic was observed mostly in Col-0 leaves irradiated with the 1000 Jm$^{-2}$ dosage, where leaves displayed an abundance of UV-C induced cell death in the absence of pathogen growth. Although the 1000 Jm$^{-2}$ clearly demonstrated the best increases in resistance to *H. arabidopsidis*, it should also be noted that it was also the dosage that displayed the most severe UV over exposure symptoms.

5.4.1. Role of induced callose induction and the presence of lignin

The role of callose in plant-pathogen interactions is still an ambiguous one and although callose deposition is a common feature of such interactions, deposition has been reported to occur in both the compatible and incompatible interactions. In the present study, callose deposition initially occurred in response to UV-C irradiation and
lined the walls of surviving cells. In response to pathogen infection, callose deposits surrounded the haustoria of the virulent isolates of *H. arabidopsisidis* in infected cells. As the UV-C dosages increased, callose was also observed surrounding the resulting HR-like lesions similar to the distributions seen in the incompatible interactions (chapter 3). The synthesis of UV-C induced callose prior to pathogen infection raises questions about the role of callose in the subsequent infection of the pathogen 24 hours after the plants were irradiated. Callose production in the 500 and 1000 Jm⁻² is significantly increased at 48 and 72 h after irradiation (chapter 2), which equated to 24 and 48 hpi. Its ability to slow pathogen penetration cannot be ruled out, as a significant amount of callose was already present before and during initial pathogen infection.

Although a recent study demonstrated that elevated levels of callose at early time points can prevent the infection of the adapted biotrophic pathogen *Golovinomyces cichoracearum* through increased penetration resistance, it is unlikely that this is occurring within UV-C treated plants (Ellinger *et al.*, 2013). As HR-like lesions were present within mesophyll of UV-C treated leaves, this suggests that penetration of the epidermis and infiltration of the mesophyll cell layer had occurred prior to activation of HR-like cell death. When the presence of lignin was tested, it became clear that collapsed dead cells present within the HR-like lesions, mimicked the lignin deposition seen in the incompatible interaction observed in this work (chapter 3) and other pathosystems (Southerton *et al.*, 1990, Mohr *et al.*, 2007). This further illustrates the distinct differences between UV-C induced cell death and the highly pathogen specific hypersensitive-like cell death induced by *H. arabidopsisidis* infection within UV-C pre-treated leaves.
5.4.2. UV-C induced generation of ROS plays a key role in induced immunity

Generation of H₂O₂ in response to UV-C irradiation may play a key role in the alteration and augmentation of Arabidopsis’s defences to the normally virulent isolates of *H. arabidopsidis*. Exposure to UV-C radiation alone was sufficient to induce an oxidative burst, which was observed in leaves treated with 500 and 1000 Jm⁻² at 24 h after irradiation (chapter 2). This induction of UV-C induced H₂O₂ occurs at the same time point in which plants are inoculated with *H. arabidopsidis*, yet in these experiments this time point is designated 0 hpi. The subsequent germination of conidiospores and penetration of the tissue during this heightened H₂O₂ state may induce a secondary oxidative burst, which likely leads to the rapid activation of defence responses.

As demonstrated in chapter 3, the increased expression of the oxidative burst marker *GST1* corresponded with the formation of HR, at 48-72 hpi in the incompatible interaction of *H. arabidopsidis* Cala2 isolate and Col-0. Yet, the expression of *GST1* in UV-C pre-treated Col-0 plants does not share similarities to that of a R protein mediated interaction, as expression of *GST1* at 500 and 1000 Jm⁻², was at a maximum at the 24 hpi time-point and decreased thereafter. This difference in gene expression may be the result of two possibilities; 1) the extent of HR-lesions was not sufficient within the Col-0 ecotype to demonstrate clear activation of *GST1* and a more appropriate experiment would test *GST1* expression of induced resistance within the Ler ecotype, as the extent of HR-like lesions was more prominent. Alternatively, 2) the already induced defence state led to a much more rapid formation of HR-like lesions at earlier time points than that observed in an incompatible interaction.
5.4.3. Pre-activation of *PR1* leads to increased resistance to the virulent isolate of *H. arabidopsidis*

Expression of the SA defence pathway marker gene *PR1*, was low in the Arabidopsis Col-0 plants exposed to 0 and 250 Jm⁻² of UV-C radiation, and infected with *H. arabidopsidis* Noks1 isolate, however increased expression of *PR1* was observed in the 500 and 1000 Jm⁻² UV-C treatments. The slow and weak induction of *PR1* at the lower levels was consistent with expression in response to the virulent isolate when compared to avirulent isolate of *H. arabidopsidis* (chapter 3) and also observed in plants infected with virulent and avirulent strains of *P. syringae* (Tao *et al.*, 2003, Ahn *et al.*, 2007). The increased expression found for the 500 and 1000 Jm⁻² UV-C treatments is consistent with *PR1* expression induced by UV-C exposure alone, as demonstrated in chapter 2.

Previous work, clearly demonstrated salicylic acid accumulation in *N. tabacum* and *A. thaliana* plants that were irradiated with UV-C radiation (Yalpani *et al.*, 1994, Nawrath *et al.*, 2002). The enhanced *PR1* expression observed for the higher dosages of UV-C in this research suggests an increase in the activity of the SA defence pathway, which occurred prior and during initial pathogen infection. Enhanced activation of *PR1* during the early stages of pathogen infection caused by thiamine priming, has also been shown to decrease pathogen growth of a virulent strain of *P. syringae* (Ahn *et al.*, 2007). The early induction of *PR1* and the involvement of the salicylic acid pathway would explain the increased resistance toward *H. arabidopsidis* in UV-C irradiated plants.

A small to moderate increase in the jasmonic acid/ethylene defence marker gene *PDF1.2* was observed in tissue treated with UV-C radiation and infected with a virulent
Chapter 5: General discussion

Cell wall
Callose deposition
Cell membrane

0 Jm$^{-2}$

H$_2$O$_2$
GST1
PR1

24 hpi

PR1

GST1
PR1

48-72 hpi

H$_2$O$_2$
GST1
PDF1.2
PR1

Pathogen growth
SUSCEPTIBILITY

HR-like cell death
RESISTANCE

0-6 hpi

H$_2$O$_2$
GST1
PR1

>500 Jm$^{-2}$

H$_2$O$_2$
GST1
PR1
**Figure 5.3.** Representative model comparing the events which lead to increased resistance to the compatible isolate of *H. arabidopsidis*. Model presented is based on data gathered from chapter 4 and data gathered from chapter 2 and 3 and compares the interactions seen at 0 and dosages greater than 500 Jm$^{-2}$. Cells on the left represent an individual untreated cell infected with *H. arabidopsidis*, typical compatibility occurs, where pathogen penetration and infection leads to a slow induction of *GST1* and *PR1*. Cells on the right represent UV-C pre-treated cells infected with *H. arabidopsidis*, where UV-C treatments caused a pre-activation of defences including callose deposition, H$_2$O$_2$ accumulation, *GST1* and *PR1* expression prior to pathogen infection and remain elevated during pathogen infection leading an earlier activation of hypersensitive-like cell death and restricts the pathogens growth. Text size presents relative amount of H$_2$O$_2$ and defence gene expression.
isolate of *H. arabidopsidis*. Its activation is likely to be due to the extent of tissue damage caused by the irradiation process and the stimulation of a wound-like response and is not likely to play a significant role in resistance to *H. arabidopsidis*. The relatively low change in expression of *PDF 1.2* may also reflect the antagonism between the SA and JA/Et pathways. As the potential increase in SA activity in response to UV-C irradiation and pathogen infection may lead to the repression of the JA/Et pathways (Glazebrook, 2005, Bari *et al.*, 2009), however further analysis is required to determine the true extent of defence hormone signalling in this system.

5.5. Final conclusion

The dosages of UV-C used in this thesis are lower than previously reported in plants exposed to a single dose of UV-C irradiation (Yalpani *et al.*, 1994, Shapiro *et al.*, 2001, Stefanato *et al.*, 2009), it still caused changes to growth, stimulation of H$_2$O$_2$ and a sustained expression of *PR1* in dosages greater than 250 Jm$^{-2}$. A major finding of this work is the synthesis of callose in response to UV-C irradiation within cells which survived treatment. This work also provides insights into the stimulation and deposition of callose as no chemical or mechanical stress was provided and may involve changes at the cell membrane as discussed in chapter 2. This does raise questions for the role of callose in ‘high light’ protection, and future work could examine the effects of other UV wavelengths to determine if this deposition is a more general response and whether plants deficient in callose synthesis would display a change in the amount of damage caused by dosages lower than 1000 Jm$^{-2}$.

This study also examined in depth, the interaction between Arabidopsis and *H. arabidopsidis* by tracking the host responses to infection in relation to the pathogen life cycle with in the host. Also identified was a second oxidative burst (via *GST1*
expression levels) that only occurred in the incompatible interaction that corresponded
to the formation of HR. Also brought into question is the role of callose within the
compatible interaction, where significantly more callose is present in a compatible
interaction when compared to an incompatible interaction. Due to the abundance of
callose in the compatible interaction, does the callose that surrounds the haustoria
sufficiently hinder nutrient/effector transfer between the haustoria and the cell? In
response to UV-C pre-treatments Arabidopsis exhibits increased resistance to the
virulent isolates of *H. arabidopsidis* with the suppression of pathogen growth and the
formation of HR-like lesions. Even though it was shown that DNA damage/repair is
involved (Kunz *et al.*, 2006, Kunz *et al.*, 2008), it appears that the stimulation of H$_2$O$_2$
and *PRI* also plays a role. This raises questions about the involvement of H$_2$O$_2$ and
whether its production is a result of DNA damage or other off-target damage caused to
other cellular components. In addition, future work could examine whether similar
increases in pathogen resistance could occur in other patho-systems.

This study has further characterised the responses and mechanisms involved in
the increased resistance of Arabidopsis caused by the exposure to the abiotic stressor of
UV-C radiation to the biotic stressor of *H. arabidopsidis* infection. Examination of this
cross-tolerance response provides new insights in responses of Arabidopsis to these
stresses at a microscopic, biochemical and molecular level. Furthermore, this
information will aid in future research, dealing with ‘cross-tolerance’ mechanisms in
order to improve plant resistance towards plant pathogens or other stressors.
Chapter 5: General discussion

Sub-lethal UV-C radiation

A. thaliana

DNA damage/ repair

Signal initiation

Biochemical responses

Cellular responses

Morphological responses

? ? ? ?

SA, JA/Et ROS Callose

INCREASED RESISTANCE

Pathogen infection

Figure 5.4. Proposed regulatory elements of the responses of A. thaliana to sub-lethal doses of UV-C radiation and infection by a biotrophic pathogen. This model represents what known to occur in A. thaliana plants exposed sub-lethal dosages of UV-C radiation with the addition of the results from this research as indicated in orange text and solid arrows.


References


