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The regulation and role of Oxidative Signal-Inducible1 protein kinase in *Arabidopsis thaliana*

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LIST OF ABBREVIATIONS

AOS	active oxygen species
Asc	ascorbate
Avr	avirulence
bp	base pairs
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
CDPK	calcium-dependent protein kinase
cfu	colony forming units
CHX	cyclohexamide
$[Ca^{2+}]_c$	cytosolic free calcium
DAB	3'3'-diaminobenzidine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DPI	diphenylene iodinium
DTT	1,4-dithio-DL-threitol
EDTA	ethylenediamine tetraacetic acid
EMS	ethyl methanesulfonate
ET	ethylene
X *g*	times gravity
GFP	green fluorescent protein
GSH	glutathione
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
HR	hypersensitive response
JA	jasmonic acid
kb	kilobase
KB	King's broth
kD	kilodalton
LB	Luria Bertani media
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase

$MgCl_2$	magnesium chloride
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog basal media
MW	molecular weight
Na-SA	sodium salicylate
OD	Optical Density
OXI1	Oxidative Signal-Inducible1
p	probability
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
p*I*	isoelectric point
PR	Pathogenesis related proteins
pv.	pathovar
R protein	resistance protein
RLK	receptor-like protein kinase
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
SE	standard error
T-DNA	transfer DNA
TE	Tris-EDTA
TMV	Tobacco Mosaic Virus
Ub/26S	Ubiquitin/26S
VIGS	virus-induced gene silencing
YFP	yellow fluorescent protein

The regulation and role of Oxidative Signal-Inducible1 protein kinase in *Arabidopsis thaliana*

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Oxidative Signal-Inducible1 (OXI1) protein kinase induced by active oxygen species (AOS) has previously been isolated in *Arabidopsis thaliana* and shown to be required for two separate H_2O_2-mediated processes, namely root hair development and basal defence against the fungal pathogen *Hyaloperonospora parasitica*. This study attempted to further characterise OXI1 protein kinase. Confocal microscopy and subcellular fractionation studies revealed a cytosolic localisation pattern for OXI1. Employment of a bioinformatics approach confirmed the induction of *OXI1* gene expression in response to a range of AOS generating stimuli. However, the transcriptional increase of *OXI1* in response to salinity and heat appears to be of no biological significance since the *oxi1* mutant did not display altered tolerance to these two stresses in comparison to wild type. Interestingly, increases in *OXI1* transcript levels are not matched by increases at the translational level, even in transgenic lines overexpressing *OXI1*. Therefore OXI1 protein levels appear to be under tight control and one mechanism of regulation is the short half life of OXI1. In transgenic lines constitutively expressing *OXI1*, OXI1 protein levels are differentially regulated in response to different stimuli. OXI1 is degraded upon cellulase treatment which mimics wounding whereas OXI1 protein levels remain the same in response to pathogen infection. However, *OXI1* gene expression is induced in wild type Arabidopsis by the bacterial pathogen *Pseudomonas syringae*. Mutational analysis as well as pharmacological evidence points to a role for NADPH oxidase as being partly responsible for the induction of *OXI1* during pathogenesis. The *oxi1* mutant is more susceptible to both virulent and avirulent *P. syringae* infection. Therefore OXI1 is required for basal defence mechanisms and gene-for-gene resistance against *P. syringae*. It is proposed that rather than preventing the initial stages of bacterial infection OXI1 functions to slow the progress of subsequent bacterial growth. Surprisingly, transgenic lines overexpressing *OXI1* also display enhanced susceptibility to *P. syringae* and *H. parasitica* infection suggesting that control of OXI1 protein levels, either positively or negatively, appears to be key for the correct modulation of disease resistance responses and further illustrate the complexity surrounding the regulation of OXI1 protein kinase.

CHAPTER 1

Introduction

CHAPTER 1: Introduction

1.1 Signal transduction in plants

Plants rely on the co-ordinated regulation of complex signalling networks to survive, reproduce and successfully colonise the environment they inhabit. The plasticity and flexibility of plant metabolism allows plants to maintain cellular integrity when challenged with stress. Signal transduction pathways initiate at perception of the stimulus which often leads to the activation or production of second messengers triggering a phosphorylation cascade that terminates in the activation of transcription factors to direct the expression of the appropriate genes (Figure 1; Trewavas and Malhó (1997) and Xiong *et al.* (2002)). However, this simplified version of signal transduction pathways has since been extended to encompass many other facets and becomes more complex as research into plant signal transduction networks progresses.

An extensive range of signalling molecules are known in plants to date and include cytosolic free calcium ($[Ca^{2+}]_c$), active oxygen species (AOS), nitric oxide (NO), cyclic nucleotide monophosphates (cNMP), phospholipids, protein kinases and hormones. Given this variety of signalling molecules plants may be capable of activating many signalling networks using various combinations of these signalling molecules. However, most processes or stress responses elucidated thus far appear to make use of the same signalling components though some degree of specificity has to be present in order to initiate distinct end responses. For example, many physiological processes and stress conditions cause changes in the concentration of $[Ca^{2+}]_c$ such as pollen tube growth (Holdaway-Clarke *et al.*, 1997), touch (Knight *et al.*, 1991), salt, drought, cold (Knight *et al.*, 1996; Kiegle *et al.*, 2000) and pathogen infection (Grant *et al.*, 2000b). The specificity of a $[Ca^{2+}]_c$ transient to elicit a defined end response is thought to reside in the physiological condition as well as the type of cell perceiving the stress, spatio-temporal kinetics of the $[Ca^{2+}]_c$ increase, the calcium store mobilised and downstream targets sensing the change in $[Ca^{2+}]_c$ (McAinsh and Hetherington, 1998; Sanders *et al.*, 1999). It was also demonstrated that overexpression of a single calcium dependent protein kinase from rice (*OsCDPK7*) conferred plants with enhanced tolerance to cold, salinity and drought (Saijo *et al.*, 2000). This finding further illustrates that multiple stresses can rely on the same signalling components acting as convergence points for

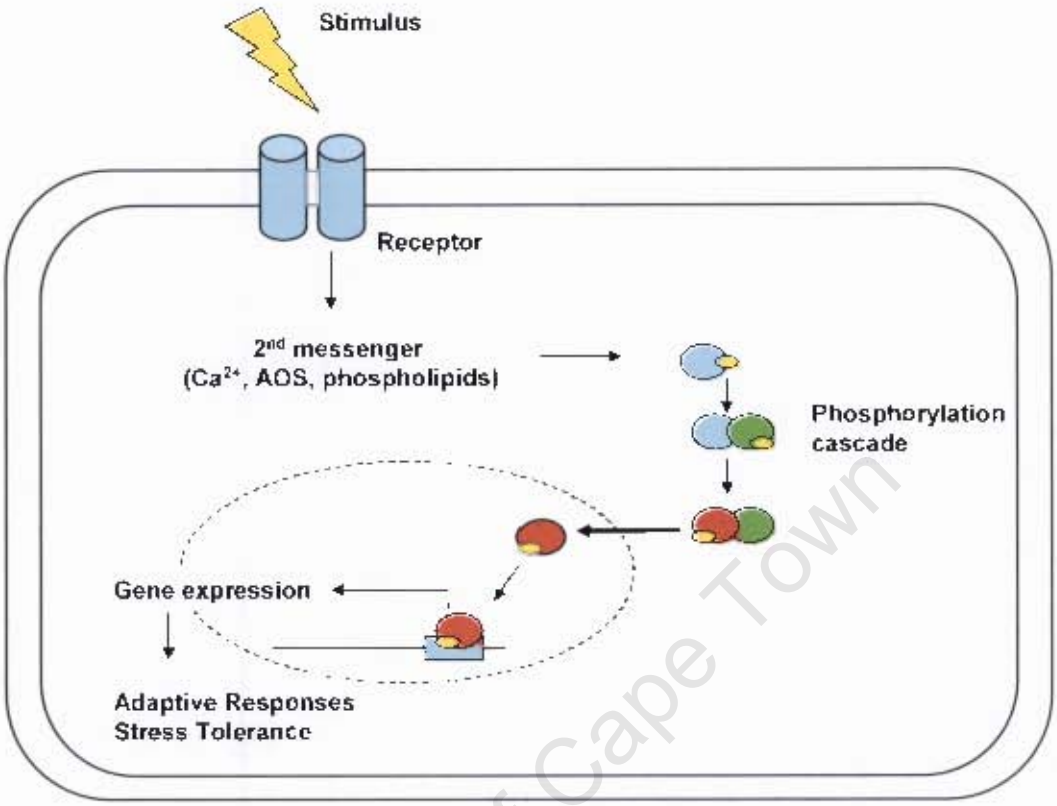


Figure 1.1 General overview of a signal transduction pathway

The external stimulus is perceived by a receptor, which may or may not be membrane bound, and results in the production of second messengers which relays the signal by activating a phosphorylation cascade. The transfer of the phosphate group (yellow circle) terminates by activating a transcription factor (red circle) which translocates to the nucleus to direct expression of the appropriate genes that would facilitate stress tolerance. It should be noted that this is an example of a signal transduction pathway. The activation of a receptor may not necessarily cause production of second messengers (but for example result in the biosynthesis of hormones such as auxin) nor may the signal transduction pathway culminate in the phosphorylation of transcription factors to direct gene expression for adaptive responses.

different signalling pathways to elicit enhanced stress tolerance. On the other hand, a single stress can also trigger multiple signalling pathways that may differ spatially and temporarily and be responsible for different end response. Considering the effects of salt stress, salt imposes both an ionic and osmotic stress component in plants. Salt stress activates the Salt-Overly-Sensitive (SOS) pathway that functions to specifically restore ion homeostasis (reviewed in Chinnusamy *et al.* (2004)). Salt treatment induces an increase in $[Ca^{2+}]_c$ that activates the calcium binding protein SOS3 which forms a complex with SOS2, a serine/threonine protein kinase, at the plasma membrane (Knight *et al.*, 1997; Halfter *et al.*, 2000; Ishitani *et al.*, 2000; Liu *et al.*, 2000). The SOS3-SOS2 complex induces the expression as well as the activity of the Na^+/H^+ antiporter SOS1, which directs the removal of excess Na^+ from the cytoplasm (Qui *et al.*, 2002). Concurrently, the SOS3-SOS2 complex may prevent Na^+ entry into the cytosol by inhibiting Na^+ transporters such as HTK1 (Rus *et al.*, 2001) thereby restoring ion homeostasis and facilitating salt stress tolerance (Zhu, 2002).

Osmotic stress imposed by either drought or high salinity, activates the expression of several stress responsive genes via either an abscisic acid (ABA) - dependent or independent pathway (Ishitani *et al.*, 1997; Koorneef *et al.*, 1998). Stress responsive genes contain *cis*-acting elements such as the ABA response element (ABRE), the dehydration response element (DRE) and the MYB/MYC recognition sequences in their promoters (MYBRS/MYCRS) (Zhu, 2002). The ABA-dependent pathway regulates gene expression by activating transcription factors such as AREBs, MYB2 and MYC2 which bind to ABRE, MYBRS and MYCRS respectively, whereas the ABA-independent pathway activates DREB transcription factors which bind to the DRE *cis*-acting element (Xiong *et al.*, 2002; Chinnusamy *et al.*, 2004). ABA-dependent gene expression during osmotic stress is also mediated in part via phospholipid inositol 1,4,5-triphosphate (IP_3) and $[Ca^{2+}]_c$ (DeWald *et al.*, 2001; Sanchez and Chua, 2001).

Additionally, it has been reported that salt and osmotic stress caused an increase in 3',5'-cyclic guanosine monophosphate (cGMP) levels in Arabidopsis seedlings (Donaldson *et al.*, 2004). The authors suggested the existence of two cGMP dependent pathways in response to salt stress, one which was Ca^{2+}-dependent (ionic) and the other independent of changes in $[Ca^{2+}]_c$ (osmotic). It remains to be tested whether cGMP plays a role in activation of the SOS pathway during salt stress. Finally, both salt and

cold stress induces the same mitogen-activated protein kinase (MAPK) cascade that is responsible for the induction of several genes involved in processes such as transcription, cellular defence, signalling and metabolism and the MAPK cascade is also essential for salt and cold stress tolerance (Tiege *et al.*, 2004). The upstream activators of the MAPK cascade during cold and salt stress are currently unknown. Taken together the above data clearly illustrates that a single stress can activate multiple signalling pathways and the sharing of signalling components between different stresses add to the complexity of plant signal transduction pathways (Figure 1.2).

Another factor contributing to the complexity of signal transduction pathways is the existence of positive and negative feedback loops, which serve to reiterate or repress the activated signal transduction pathway respectively. Transcriptional increases and subsequent translation of the actual components involved in a specific signal transduction cascade is proposed to increase the availability of components required for that signal transduction cascade and perhaps ensure its prolonged activation (Yamamoto *et al.*, 1998). For example, two components, the enhanced disease susceptibility1 (EDS1) and phytoalexin deficient4 (PAD4), are required for disease resistance in response to biotrophic pathogens acting upstream of salicylic acid (SA) signalling. It has been demonstrated that the nonexpresser of pathogenesis-related genes protein (NPR1) and SA are needed for positive feedback for the induction of *EDS1* and *PAD4* expression (Falk *et al.*, 1999; Jirage *et al.*, 1999). Negative regulation of a wound-induced MAPK cascade occurs during wounding through the transient expression of a phosphoprotein phosphatase type 2C protein *MP2C* (Meskiene *et al.*, 1998). MP2C is required to inactivate the wound-induced MAPK cascade and the termination of this pathway prevents further expression of *MP2C* (Meskiene *et al.*, 1998). It is important to note that not only are the signalling components themselves important but so too are those molecules which modify, deliver or assemble the signalling components to a particular signal transduction cascade (Xiong *et al.*, 2002; Chinnusamy *et al.*, 2004).

The above discussion briefly highlights the complexity of signal transduction pathways. It is also appreciated that signal transduction pathways or signalling networks are still much more complex owing to the fact that plants can be subjected to multiple stresses at any given time e.g. high salinity and pathogen challenge. Therefore different signalling

pathways are simultaneously activated, which can interact either in an antagonistic or co-operative manner and the plant needs to discriminate between these different pathways to elicit the appropriate response. Deciphering the complexity of plant signal transduction pathways will be greatly aided with the existence of large collections of T-DNA knockouts of Arabidopsis, complete sequencing of plant genomes, RNAi-silenced transgenic plants, the use of microarray to study global expression patterns as well as advances made in the field of proteomics.

This study focuses on characterisation of a protein kinase that is induced in response to AOS, Oxidative Signal-Inducible1 (OXI1). Therefore to understand how OXI1 may fit into what is known about signalling processes mediated by AOS and protein kinases, the following sections will emphasise the role of these two common signalling components in plant signal transduction pathways.

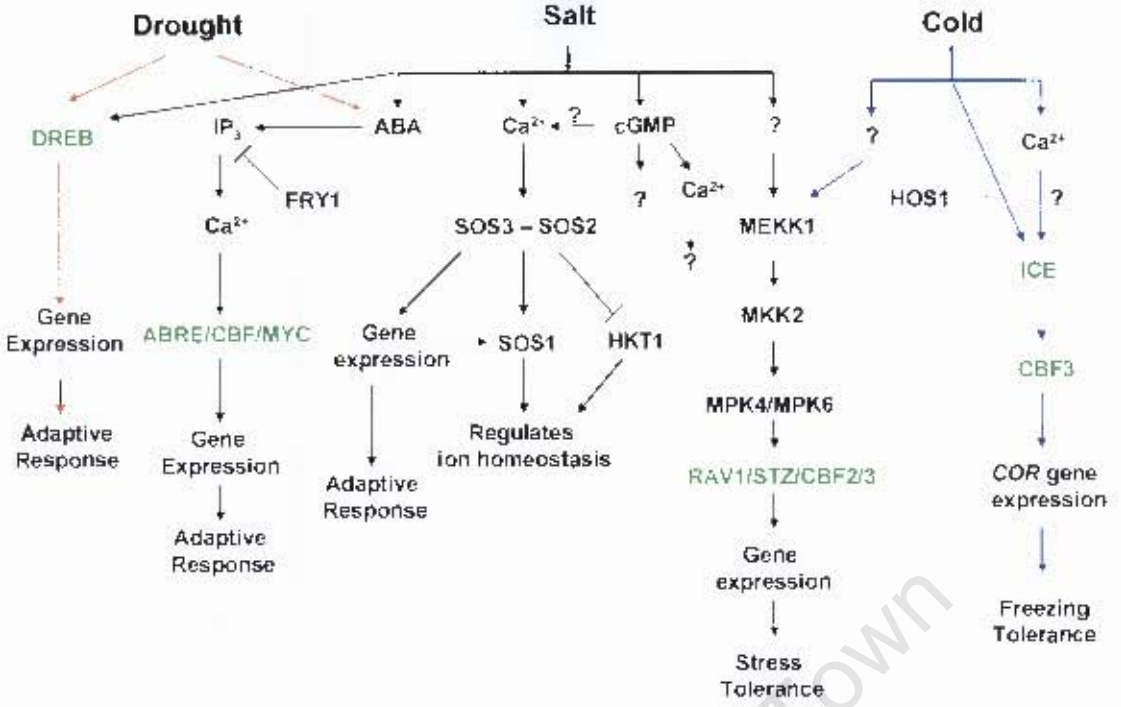


Figure 1.2 Signal transduction pathways activated during abiotic stress

Salt, drought and cold stress induces multiple signal transduction pathways to initiate stress tolerance. These stresses can share signalling components to direct stress tolerance such as the induction of the MAPK (MEKK1-MKK2-MPK4/6) cascade in response to cold and salt stress and the induction of an ABA-dependent and independent pathway during osmotic (drought and salt) stress. Other signalling pathways are unique to the specific stress e.g. the SOS pathway which restores ion homeostasis during salt stress (through activation of SOS1 and inhibition of the Na^+ transporter HKT1) and the ICE pathway activated during cold stress which specifically directs the expression of the *cold-stress-responsive* (*COR*) genes (Chinnusamy *et al.*, 2003). Components have also been identified that serve to negatively regulate stress tolerance, for example FRY1 encoding a bifunctional enzyme inhibits the accumulation of IP_3 and HOS1, a putative E3 ubiquitin-conjugating enzyme, presumably targets an as yet unidentified positive regulator (as indicated by ?) of the ICE pathway for degradation. The signal transduction pathways induced by the different stresses are colour coded black, brown and blue for salt, drought and cold stress respectively, known transcription factors are labelled green and the question mark (?) indicates unknown components or interactions. It should be noted that the above diagram is merely used as an example to illustrate the complexity of signal transduction pathways and is by no means a complete representation of all the known components interacting during abiotic stress signalling, for example cold stress also induces stress responsive gene expression via ABA-dependent and independent signalling pathways (for review see Xiong *et al.* (2002) and Chinnusamy *et al.* (2004)).

1.2 Active Oxygen Species

The reduction of O_2 to H_2O is required for the efficient production of energy during aerobic metabolism. However, O_2 can also be partially reduced giving rise to AOS which are highly reactive molecules that can interact with a variety of biological molecules to perturb their function thus leading to cellular damage. Moreover, evidence for a role for AOS in signal transduction pathways is ever increasing but the identification of the direct mechanisms by which AOS achieves this lags behind (Van Breusegem *et al.*, 2001; Vranová *et al.*, 2002).

1.2.1 Generation of AOS

AOS are most commonly produced as byproducts of normal cellular metabolism and in plants this includes the processes of photosynthesis and respiration. There are a range of AOS produced in plants with differing physical characteristics or chemical properties. The first AOS produced is the superoxide anion (O_2^-), which is the addition of a single electron to molecular O_2 (Equation 1). The production of O_2^- occurs during a range of redox reactions. In the chloroplast, the majority of O_2^- is produced upon reduction of O_2 by photosystem I (PSI) at the level of ferredoxin-$NADP^+$ reductase (Mehler, 1951; Asada, 1999), in PSII at the level of P_{680}, pheophytin and protein Q_A (Navari-Izzo *et al.*, 1999) as well as in the plastoquinone pool during the reaction of O_2 with plastosemiquinone (Ivanov and Khorobrykh, 2003). Peroxisomes produce O_2^- through the oxidation of xanthine to uric acid by xanthine oxidase (Sandalio *et al.*, 1988; del Río *et al.*, 1998) and a small electron transport chain that is NAD(P)H-dependent resides in the peroxisome membrane and generates cytosolic O_2^- (López-Huertas *et al.*, 1999). The contribution of plant mitochondria in AOS production has not been the focus of much research but during conditions of stress over-reduction of the electron transport chain of complex I and III and the ubiquinone zone may produce excessive O_2^- (Purvis, 1997; Turrens, 1997; Maxwell *et al.*, 1999). Furthermore, cytochrome P_{450} enzymes in the cytoplasm and endoplasmic reticulum can produce O_2^- during detoxification reactions where electron leakage to O_2 occurs (Urban *et al.*, 1997) and plasma membrane NADPH oxidases also generate O_2^- under conditions of stress (Bolwell and Wojtaszek, 1997; Mittler, 2002; Torres *et al.*, 2002). O_2^- is moderately reactive, has a half-life of 2-4 µs and at low pH it can be protonated to produce the perhydroxyl radical ($^{\bullet}HO_2$) (Equation 2). Unlike O_2^-,

$^{\cdot}HO_2$ can cross biological membranes and initiates lipid auto-oxidation by removing H^+ from polyunsaturated fatty acids and lipid hydroperoxides (Halliwell and Gutteridge, 1989; Vranová *et al.*, 2002).

$$O_2 + e^- \longrightarrow O_2^- \quad \textbf{1}$$

$$H^+ + O_2^- \longleftrightarrow {}^{\cdot}HO_2 \quad \textbf{2}$$

$$^{\cdot}HO_2 + {}^{\cdot}HO_2 \longrightarrow H_2O_2 + O_2 \quad \textbf{3}$$

$$^{\cdot}HO_2 + O_2^- + H_2O \longrightarrow H_2O_2 + O_2 + OH^- \quad \textbf{4}$$

Harber-Weiss / Fenton reaction

$$O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad \textbf{5}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \quad \textbf{6}$$

Alternatively O_2^- rapidly dismutates to the more stable and less reactive AOS hydrogen peroxide (H_2O_2) which has a half-life of 1 ms (Van Breusegem *et al.*, 2001). $^{\cdot}HO_2$ can either spontaneously react with itself or O_2^- to produce H_2O_2 (Equation 3 and 4). However, metal containing superoxide dismutase (SOD) can catalyze the conversion of O_2^- and $^{\cdot}HO_2$ to H_2O_2 approximately 10^{10} times faster than the spontaneous rate (Lamb and Dixon, 1997). H_2O_2 can also be formed at the plasma membrane via NADPH oxidase and cell wall bound peroxidases and in the apoplast through gemin-like oxalate oxidases and amine oxidases (Bolwell and Wojtaszek, 1997; Vranová *et al.*, 2002). In peroxisomes H_2O_2 is formed as a result of the glycolate oxidase reaction during photorespiration, β-oxidation of fatty acids, enzymatic reaction of flavin oxidases and during the oxidation of uric acid to allantoin (del Río, 1992; Corpas *et al.*, 2001).

The most reactive AOS is the hydroxyl radical (OH^-) which is formed by the interaction of O_2^- and H_2O_2 in conjunction with reduced transition metals such as iron and copper in a reaction known as the Haber-Weiss or Fenton reaction (Equation 5 and 6). OH^- is a strong oxidant and has a half life of less than 1 μs and it can react with any biological

molecule such as lipids, nucleic acids and proteins thereby causing severe cellular damage (Van Breusegem *et al.*, 2001).

During conditions of excess light, PSII may be completely reduced thereby resulting in the formation of triplet chlorophyll molecules. Here the primary electron donor P_{680} and pheophytin the primary electron acceptor recombine to produce triplet P_{680} since the P_{680}^{+}/$Pheo^{-}$ radical pair cannot transfer excited electrons to the pool of already reduced plastoquinone (van Mieghem *et al.*, 1989; Laloi *et al.*, 2006). Singlet oxygen ($^1O_2$) is produced during the transfer of the excited energy from triplet chlorophyll onto triplet ground state oxygen (Durrant *et al.*, 1990). The half life of $^1O_2$ is 4 µs in H_2O and 100 µs in non-polar solutions and $^1O_2$ either transfers its excitation energy to or reacts with biological molecules (Vranová *et al.*, 2002; Ivanov and Khorobrykh, 2003).

1.2.2 Antioxidant scavenging prevents AOS accumulation

In light of the cytotoxic nature of AOS, plants are equipped with extensive antioxidant scavenging mechanisms to alleviate or prevent oxidative damage. AOS can accumulate within intracellular compartments, the cytosol and in the apoplast and different antioxidant mechanisms are present in different subcellular locations. The OH^{-} radical is highly reactive and cannot be directly controlled therefore antioxidant processes serve to detoxify the precursor AOS, O_2^{-} and H_2O_2, and in addition the sequestering of metal ions prevents or limits OH^{-} formation (Van Breusegem *et al.*, 2001). The major enzymatic AOS scavenging mechanisms include the actions of SOD to scavenge O_2^{-} and catalase (CAT) and ascorbate peroxidase (APX) to regulate H_2O_2 accumulation.

There are different isozymes of SOD present in plants and not only is their activity developmentally and environmentally controlled but they are found to reside in the chloroplast, cytoplasm, mitochondria, peroxisomes and apoplast illustrating the importance of SOD in control of O_2^{-} concentrations (Van Camp *et al.*, 1997; Van Breusegem *et al.*, 2001; Mittler, 2002). CAT located in peroxisomes and glyoxisomes catalyses the conversion of H_2O_2 to H_2O (Equation 7) and has an affinity for H_2O_2 in the mM range hence functions to remove excess H_2O_2 (Willekens *et al.*, 1997).

$$H_2O_2 + H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \qquad \textbf{7}$$

There are 3 classes of catalases in plants: class 1 catalases are found in photosynthetic tissue and scavenges H_2O_2 produced during photorespiration, class 2 catalases occur in vascular tissue and are proposed to play a role in lignification and finally class 3 catalases are present in seeds and young plants and detoxify H_2O_2 produced during fatty acid degradation in glyoxisomes (Willekens *et al.*, 1994). Members of each class have been found in tobacco, Arabiodopsis, rice and maize (Willekens *et al.*, 1994; McClung, 1997; Frugoli *et al.*, 1998) and catalases appear to be the primary H_2O_2 scavenging enzymes. Furthermore, suppression of specific CAT isozyme activities in tobacco rendered transgenic tobacco plants more sensitive to oxidative damage in response to ozone and salt stress demonstrating the importance of CAT activity for scavenging H_2O_2 under conditions of stress (Willekens *et al.*, 1997). Although, CAT is only present in peroxisomes it has been demonstrated that oxidative stress induces the production of peroxisomes and since H_2O_2 is able to diffuse from the cytosol to peroxisomes CAT is efficient in scavenging high levels of H_2O_2 (Lopez-Huertas *et al.*, 2000; Mittler, 2002).

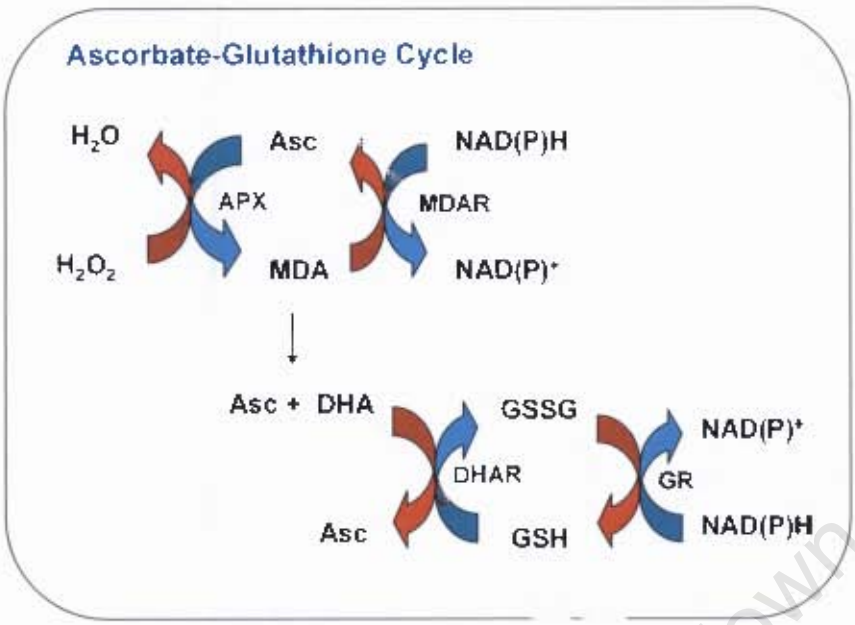
The ascorbate-glutathione cycle is found in all cellular components and detoxifies H_2O_2 through the recycling of ascorbate (Asc) and glutathione (GSH) to their reduced forms with the aid of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHR) and glutathione reductase (GR) (Dalton *et al.*, 1986; Noctor and Foyer, 1998; Mittler, 2002). APX converts H_2O_2 to H_2O using Asc as the electron donor forming monodehydroascorbate (MDA) which spontaneously forms dehydroascorbate (DHA). Asc is then regenerated either by MDAR utilising NADPH as the reductant or in a GSH-dependent reaction catalysed by DHAR. During the latter reaction GSH is oxidised to its disulfide form (GSSH) and GSH regeneration is catalysed by GR utilising the reducing power of NADPH (Figure 1.3 a). APX has a higher affinity than CAT for H_2O_2 operating in the μM range and consequently is proposed to function in modulating the signalling role for H_2O_2 rather than detoxification *per se*, however given the different subcellular localisations for APX its detoxification function cannot be excluded (Van Breusegem *et al.*, 2001; Mittler, 2002). The water-water cycle in chloroplasts is another way in which antioxidants modulate the production of ROS protecting the photosynthetic apparatus from both oxidative damage when CO_2 fixation is limited or in the absence of stress (Asada, 1999; Rizhsky *et al.*, 2003). Electrons produced during the splitting of H_2O molecules are transferred to PSII and PSI transfers these electrons to O_2 forming $O_2^{\cdot-}$, which through the action of membrane bound

copper/zinc SOD dismutates O_2^- to H_2O_2. A thylakoid-APX (tAPX) converts H_2O_2 to H_2O using Asc as the reductant and the resultant MDA is reduced back to Asc with addition of electrons from PSI by ferredoxin (Figure 1.3 b). The action of cytosolic glutathione peroxidase (GPX) serves to scavenge H_2O_2 in the cytosol using GSH as the reductant and the oxidised GSSH is recycled to GSH through the reducing power of NADP by glutathione reductase (GR) (Figure 1.3 c and (Dixon *et al.*, 1998)).

Additional components of the plant AOS scavenging network include the non-enzymatic low molecular weight molecules such as Asc, GSH and carotenoids. Asc can react with H_2O_2 in a similar reaction to that catalysed by APX to yield MDA (Noctor and Foyer, 1998; Rizhsky *et al.*, 2002). Carotenoids not only deactivate excited sensitizer molecules involved in the production of $^1O_2$ but they also interact with $^1O_2$ to produce triplet excited carotene and ground state O_2 and the triplet excited carotene dissipates its energy to the surrounding solvent returning to ground state carotenoid (Stahl and Sies, 2003). Carotenoids can also efficiently scavenge $^{\bullet}HO_2$ and are likely to play a role in the protection of cellular membranes and lipoproteins against oxidative damage (Stahl and Sies, 2003).

It should be noted that although different types of antioxidant mechanisms can scavenge the same AOS, their occurrence in different subcellular locations, induction by different stresses and possible interaction with each other creates a complex network of AOS production and scavenging.

A



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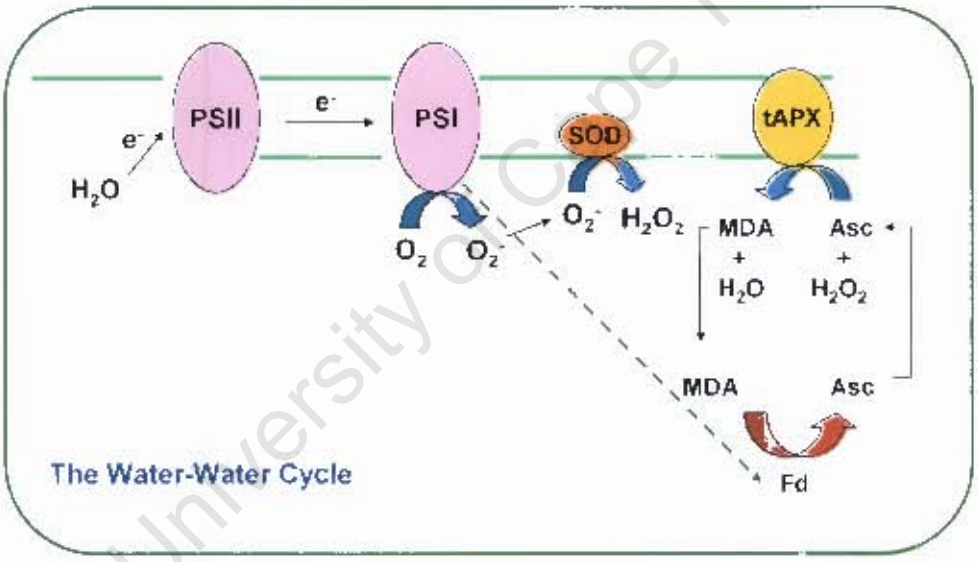

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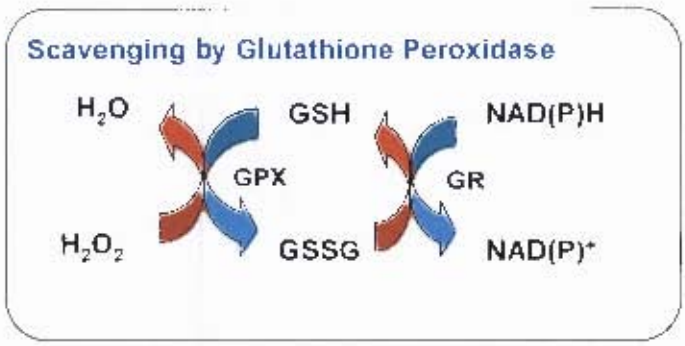


Figure 1.3 Antioxidant scavenging mechanisms (Adapted from Mittler (2002))

(A) The ascorbate-glutathione cycle occurs in all cellular compartments and uses ascorbate (Asc) to reduce H_2O_2 to H_2O and is itself oxidised to monodehydroascorbate (MDA) via ascorbate peroxidase (APX). Glutathione (GSH) and NADPH are used to reduce dehydroascorbare (DHA) and MDA respectively, catalysed by DHA reductase (DHAR) or MDA reductase (MDAR) to regenerate Asc. GSH is oxidised to its disulphide form GSSG and regenerated via glutathione reductase (GR) in an NADPH-dependent manner. (B) The water-water cycle occurs in the chloroplast. During this process H_2O is split and electron (e^-) transfer occurs via PSII and PSI which oxidises O_2 to O_2^- that is dismutated to H_2O_2 by SOD. Thylakoid APX (tAPX) catalyses the reduction of H_2O_2 to generate H_2O again, using Asc as the reductant. MDA is reduced to Asc by ferredoxin using e^- from PSI as indicated by the dashed green arrow. (C) Finally glutathione peroxidase which occurs in the cytosol catalyses the conversion of H_2O_2 to H_2O using GSH as the reductant. GSH is regenerated by GR in an NADPH-dependent manner. (A-C) The red arrow indicates the reduction while the blue arrow indicates an oxidation reaction.

1.2.3 Direct functions of AOS

1.2.3.1 Destructive nature of AOS accumulation

Oxidative stress is characterised by the accumulation of AOS resulting from a severe imbalance between AOS generating and AOS scavenging mechanisms and thereby disrupting the normal cellular redox state (Moller, 2001; Rentel and Knight, 2004). Oxidative stress leads to cellular damage or even plant cell death that is caused by the ability of different AOS, given their difference in chemical properties, to react to varying degrees with different biological molecules. This interaction alters the function and/or stability of these biological molecules. For example, O_2^- can oxidise the amino acids histidine, methionine and tryptophan therefore altering protein conformation, H_2O_2 has the ability to oxidise SH groups and OH^- can react with all biological molecules given its highly reactive nature and most importantly it also reacts with DNA and can cause mutations in the DNA sequence (Moller, 2006). Taken together with the fact that simultaneous generation of different AOS can occur in response to a single stimulus, unregulated AOS accumulation can be cytotoxic.

Lipid peroxidation also results from oxidative stress and is caused primarily by $^{\bullet}HO_2$ and the OH^- radicals (Equation 8 a-d) which removes H^+ from polyunsaturated fatty acids ($-CH_2-$) converting fatty acids to toxic lipid peroxides ($-CHO_2H-$) (Halliwell and Gutteridge, 1989; Vranová *et al.*, 2002). Therefore lipid peroxidation causes damage to biological membranes which is attributed to the breakage and shortening of polyunsaturated fatty acids increasing membrane fluidity and permeability (Grant and Loake, 2000; Moller, 2006). Lipid peroxides can also affect mitochondrial processes, for example, 4-hydroxy-2-nonenal (HNE) a byproduct of lipid peroxidation binds to and inactivates lipolic acid, an essential cofactor for decarboxylating dehydrogenases (Millar and Leaver, 2000) and HNE also inhibits the activity of alternative oxidase, an enzyme that provides an alternative pathway for electron transport during respiration and photosynthesis (Maxwell *et al.*, 1999; Rizhsky *et al.*, 2002; Winger *et al.*, 2005).

$$-CH_2- + OH^- \longrightarrow -C^{\bullet}H- + H_2O \qquad \textbf{8 a}$$

$$-CH_2- + {}^{\bullet}HO_2 \longrightarrow -C^{\bullet}H- + H_2O_2 \qquad \textbf{8 b}$$

$$-C^{\bullet}H- + O_2 \longrightarrow -C^{\bullet}HO_2- \qquad \textbf{8 c}$$

$$-C^{\bullet}HO_2- + -CH_2- \longrightarrow -CHO_2H- + -C^{\bullet}H- \qquad \textbf{8 d}$$

1.2.3.2 Involvement of AOS during abiotic stress

Various abiotic stresses such as extremes of temperature, salt, drought and ozone induce the accumulation of AOS (Conklin and Last, 1995; Larkindale and Knight, 2002; Xiong *et al.*, 2002). The generation of AOS in response to abiotic stress is detrimental to plant survival, presumably through the destructive nature of AOS as highlighted above. Evidence for this stems from the observation that during abiotic stress, AOS accumulation induces the activity of AOS scavenging mechanisms such as APX and CAT (Mittler, 2002) and transgenic plants which overexpress AOS scavengers display increased tolerance to environmental stress (Bonhert and Sheveleva, 1998; Nuccio *et al.*, 1999). Furthermore, transgenic tobacco plants which lack APX and/or CAT are more sensitive than wild type to oxidative stress (Rizhsky *et al.*, 2002). It was also demonstrated that *NahG* Arabidopsis, deficient in SA accumulation, were more tolerant to salt and osmotic stress and it was suggested that the mechanism of enhanced tolerance could be due to the lack of SA-induced AOS accumulation (Borsani *et al.*, 2001).

It has been suggested that AOS may also have a beneficial role in response to abiotic stresses. For example, ABA induction in response to abiotic stresses is proposed to play a role in stress tolerance (Xiong *et al.*, 2002) and ABA is reported to induce H_2O_2 (Kwak *et al.*, 2003) hence H_2O_2 could act downstream of ABA to initiate stress tolerance but this remains to be tested. ABA was also shown to be required for protection against oxidative damage in response to heat stress in Arabidopsis (Larkindale and Knight, 2002) therefore the interaction between ABA and AOS in response to abiotic stresses may be complex.

1.2.3.3 Antimicrobial activity of AOS

Although AOS toxicity is detrimental to plant cells, it is also toxic to invading microbial pathogens thereby playing a beneficial role in disease resistance by limiting pathogen spread (Lamb and Dixon, 1997). It was demonstrated that H_2O_2 production inhibited germination of spores of fungal pathogens (Peng and Kuc, 1992) while removing O_2^- by addition of O_2^- scavengers in tobacco cells challenged with *Pseudomonas syringae* pv. *tabaci* yielded higher bacterial titres than cells not harbouring additional scavengers

(Keppler *et al.*, 1989). Furthermore, transgenic potato plants expressing a glucose oxidase gene (which catalyses the reaction between glucose and O_2 to produce gluconate and H_2O_2) from *Aspergillus niger* displayed enhanced resistance to *Erwinia caratovora* infection (Wu *et al.*, 1995). This enhanced disease resistance is attributed not only to the several-fold increase in H_2O_2 levels which directs cell death through AOS toxicity but also to the signalling roles of H_2O_2 (addressed in Section 1.2.4.1).

1.2.3.4 Oxidative cross-linking of cell walls

AOS also serve to contain microbial invaders by reinforcing the cell wall through oxidative cross-linking of cell wall structural proteins during pathogen attack (Lamb and Dixon, 1997; Grant and Loake, 2000). Elicitor-treated or exogenously applied H_2O_2 caused insolubilisation of two cell wall proteins in soybean and bean cells and one of these proteins was rich in tyrosine (Tyr) and proline (Bradley *et al.*, 1992). Although, the nature of these cross-links is unclear it has been proposed that a tetrameric derivative of Tyr, di-isodityrosine may represent the intermolecular linkage (Brady and Fry, 1997) particularly since protease digestion of cell walls only identified intrapolypeptide loops of isoditryosine, an oxidatively coupled dimer of tyrosine (Grant and Loake, 2000). Additionally, it has been observed that fungal elicitors and wounding induces the expression of tyrosine-rich cell wall structural proteins and inhibits the expression of those with low tyrosine content (Sheng *et al.*, 1991; Lamb and Dixon, 1997) providing further evidence that oxidative cross-linking of cell wall structural proteins is an important protection mechanism in defence. Oxidative cross-linking of cell wall bound phenolic compounds and polysaccharides can also strengthen the cell wall. H_2O_2 accumulation has been proposed to facilitate oxidative coupling of polysaccharide-bound feruloyl residues (Fry *et al.*, 2000) and consequently may promote cross-linking of polysaccharide chains within the cell wall since polysaccharides with ester-linked feruloyl groups are abundant in the plant cell wall (Grant and Loake, 2000).

1.2.4 Signalling role of AOS

The most intriguing function of AOS is their ability to act as signalling molecules. The most extensively studied signalling role for AOS is their involvement in disease resistance. AOS also plays a signalling role in physiological processes such as stomatal closure and root hair development.

1.2.4.1 AOS accumulation mediates biotic stress tolerance

There are two different forms of disease resistance pathways namely nonhost resistance and gene-for-gene resistance, with the former encompassing basal defence mechanisms. Nonhost resistance is effective against most microorganisms rendering them non-pathogenic to the plant and thus they are unable to cause disease (Mysore and Ryu, 2004). The first line of defence during nonhost resistance is that of the preformed barriers such as the waxy cuticle and the cell wall (as reviewed in Thordal-Christensen (2003)). If the potential pathogen overcomes these barriers, recognition by the plant of characteristic pathogen-associated molecular patterns (PAMPs) such as bacterial flagella, lipopolysaccharides, elongation factor Tu, or fungal chitin and β-glucans, sets off a series of inducible basal defence mechanisms including ethylene (ET) production, callose deposition and induction of defence-related genes (Ingle *et al.*, 2006). To overcome nonhost resistance pathogens have evolved effector proteins known as avirulence (Avr) gene products that are thought to suppress basal defence mechanisms and facilitate successful colonisation of the pathogen and disease ensues (Dangl and Jones, 2001). Concomitantly, the plant has evolved specific resistance (R) genes that would recognise the activity or presence of its cognate pathogen Avr protein and during this gene-for-gene resistance response a similar set of defence responses such as that of nonhost resistance are induced and culminate in the prevention of disease (Somssich and Hahlbrok, 1998; Dangl and Jones, 2001; Nürnberger and Scheel, 2001). It should be noted that in most cases recognition of the Avr protein by the R protein is not a direct interaction. For example, the bacterial Avr protein AvrRpm1 induces the phosphorylation of the Arabidopsis RPM1-interacting protein4 (RIN4), a negative regulator of basal defence systems, to further suppress basal defence mechanisms (Mackey *et al.*, 2002). However, the R protein RPM1 recognises or detects the phosphorylation of RIN4 and

activates defence responses leading to disease resistance (Mackey *et al.*, 2002). This Avr-R protein reaction is classified as an incompatible interaction and the pathogen is termed avirulent. If either the plant or the pathogen does not harbour the R or Avr protein respectively then the pathogen is able to overcome the basal defence system and disease ensues. The pathogen is therefore virulent on the plant and the interaction is defined as compatible (Somssich and Hahlbrok, 1998). It is important to note that the different disease resistance pathways induce similar and overlapping defence mechanisms and the outcome of disease or resistance is determined by the ability of the pathogen to overcome these defence mechanisms and by the plant's ability to recognise this suppression.

One of these overlapping defence mechanisms is the production of an oxidative burst characterised by the accumulation of AOS in response to pathogen infection. It was observed that inoculation of soybean cells with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* induced a biphasic increase in AOS accumulation, as measured by H_2O_2 production (Levine *et al.*, 1994). The initial phase was rapid but weak and transient while the second phase of H_2O_2 production was much more pronounced and prolonged occurring between 3 and 6 hours after inoculation (Levine *et al.*, 1994). Generation of O_2^- and H_2O_2 has been reported in many plant systems infected with either avirulent fungal, bacterial or viral pathogens (Orlandi *et al.*, 1992; Lamb and Dixon, 1997; Grant and Loake, 2000). Therefore it is established that during gene-for-gene resistance, avirulent pathogen infection generates a biphasic increase of AOS at the infection site (Lamb and Dixon, 1997; Grant and Loake, 2000). During nonhost resistance similar two-phase kinetics of AOS accumulation have been reported in tobacco cells inoculated with *P. syringae* pv. *syringae*, which is not pathogenic on tobacco (Kepler *et al.*, 1989). Furthermore, the wheat powdery mildew fungus which is a nonhost in barley induced H_2O_2 production at the sites of attempted penetration and failed to cause disease therefore associating nonhost resistance in barley with the oxidative burst (Huckelhoven *et al.*, 2001). In response to virulent pathogen infection only the weak transient increase in AOS is observed and the sustained second phase is absent and disease progresses (Levine *et al.*, 1994; Lamb and Dixon, 1997; Grant and Loake, 2000). This observation provides evidence that it is the second phase of the oxidative burst that is responsible for the induction of defence responses such as the hypersensitive response (HR) characterised as rapid localised

cell death at the infection site as well as the induction of defence related genes (Lamb and Dixon, 1997; Grant *et al.*, 2000b). Initiation of the HR together with the direct functions of AOS, as mentioned earlier, such as AOS toxicity (Peng and Kuc, 1992) and oxidative cross-linking of cell walls (Bradley *et al.*, 1992; Levine *et al.*, 1994) serve to the contain the pathogen and limit the spread of infection.

There are several possible sources of AOS production during pathogen infection. Pharmacological studies, as well as genetic evidence, point to a role for NADPH oxidase as the major source of AOS during pathogen challenge. Co-infiltration of diphenylene iodinium (DPI), the chemical inhibitor of NADPH oxidases, and the avirulent *P. syringae avrRpt2* isolate into Arabidopsis leaves prevented H_2O_2 accumulation at the infection site, reduced the HR and inhibited the expression of the defence gene *glutathione-S-transferase* (*GST1*) (Alvarez *et al.*, 1998). Similarly, Arabidopsis mutants lacking either or both of the respiratory burst oxidase genes *AtrbohD* or *AtrbohF*, which encode the catalytic subunits of NADPH oxidase, displayed a reduction in both H_2O_2 accumulation and cell death in response to *P. syringae* pv. tomato *DC*3000 *avrRpm1* infection in comparison to wild type Arabidopsis (Torres *et al.*, 2002). These authors also demonstrated that although these genes work together to control AOS production and initiation of the HR, *AtrbohD* is responsible for the majority of the AOS produced while *AtrbohF* markedly affects cell death during the incompatible interaction. Alternative and/or additional mechanisms for AOS production in response to pathogen challenge include cell-wall bound peroxidases and oxalate oxidase since the activity of these enzymes were shown to increase in onion epidermis and barley, respectively, during fungal infection (Zhang *et al.*, 1995; Zhou *et al.*, 1998a; McLusky *et al.*, 1999).

Early events acting upstream of the oxidative burst during disease resistance responses include pathogen-induced ion fluxes which initiate H_2O_2 production. Recognition of the activity of the avirulent AvrRpm1 gene product of *P. syringae* by the Arabidopsis RPM1 resistance protein triggers an increase in $[Ca^{2+}]_c$ which is essential for and precedes H_2O_2 accumulation and the HR during the incompatible interaction (Grant *et al.*, 2000b). Similarly, the plant-derived cell wall elicitor, oligogalacturonic acid (OGA), induces an increase in $[Ca^{2+}]_c$ followed by H_2O_2 accumulation in Arabidopsis seedlings (Hu *et al.*, 2004). Inhibition of the OGA-induced $[Ca^{2+}]_c$ increase by calcium channel blockers and calcium chelators also abolished the oxidative burst whereas inhibition of NADPH

oxidase activity only prevented H_2O_2 accumulation and the $[Ca^{2+}]_c$ transient was unaffected (Hu *et al.*, 2004). This is indicative that the OGA-induced $[Ca^{2+}]_c$ increase is required for the oxidative burst. Additionally expression of defence related gene proteins chalcone synthase (CHS), phenylalanine ammonia lyase (PAL), GST and Pathogenesis-Related protein-1 (PR-1) requires both the increase in $[Ca^{2+}]_c$ and H_2O_2 since defence gene expression is suppressed by pharmacological agents inhibiting either of these processes (Hu *et al.*, 2004).

Although there are various forms of AOS (see Section 1.2.1), AOS generated during pathogen attack are comprised mainly of H_2O_2 and O_2^- (Scheel, 1998; Grant and Loake, 2000). The OH^- and $^1O_2$ radicals are thought to play minor roles in AOS signalling pathways due to their short half lives and destructive nature however they have also not been the focus of much research on the signalling role of AOS during pathogenesis (Laloi *et al.*, 2004; Laloi *et al.*, 2006). O_2^- is the first AOS generated through the action of NADPH oxidase as well as peroxidases and rapidly converted to H_2O_2 and O_2 through the activity of SOD (Jabs *et al.*, 1997; Corpas *et al.*, 2001). These two forms of AOS (H_2O_2 and O_2^-) have been shown to play parallel as well as different roles in pathogen defence.

H_2O_2 is moderately reactive and has the ability to cross biological membranes therefore it is proposed to be the chief AOS signalling molecule activating plant defence responses (Vranová *et al.*, 2002). Numerous reports provide evidence that modulating H_2O_2 levels (decreasing H_2O_2 through inhibition of enzymes required for production or increasing H_2O_2 through the suppression of antioxidant scavenging enzymes) during pathogen attack or elicitor treatment affects defence related processes. For example, transgenic tobacco plants deficient in peroxisomal catalase activity exposed to high light intensities exhibit elevated H_2O_2 levels, SA accumulation and induction of PR proteins and display enhanced resistance to pathogen challenge in comparison to wild type plants (Chamnongpol *et al.*, 1998). Similarly transgenic potato plants expressing glucose oxidase have constitutively elevated sub lethal levels of H_2O_2, which induce SA accumulation and expression of defence related genes, increasing resistance of these transgenic plants to a broad range of pathogens (Wu *et al.*, 1997). In soybean cells, the accumulation of H_2O_2 during avirulent pathogen infection causes the induction of the defence genes *GST* and *glutathione peroxidase* (*GPx*) (Levine *et al.*, 1994). It was

recently illustrated that Arabidopsis ascorbate-deficient mutants exhibited microlesions and constitutive *PR* gene expression and although they lacked induction of H_2O_2 sensitive genes these mutants displayed increased resistance to *P. syringae* infection (Pavet *et al.*, 2005) indicative that modulation of AOS levels affects disease resistance responses.

A few studies have implicated a separate role for the short-lived O_2^- radical from H_2O_2 in plant defence responses. DPI and SOD, but not catalase which converts H_2O_2 to H_2O, prevents phytoalexin synthesis during elicitor treatment of parsley cell cultures demonstrating that generation of O_2^-, and not H_2O_2, is required for the induction of phytoalexins (Jabs *et al.*, 1997). Although H_2O_2 is capable of inducing the expression of *PR-1*, lesion formation as well as the accumulation of *PR-1* mRNA in the Arabidopsis *lesion-simulating disease response* (*lsd1*) mutant requires extracellular O_2^- (Jabs *et al.*, 1996). These authors showed that O_2^- accumulation not only correlated to the region where cell death occurred but also initiated the runaway cell death phenotype of the *lsd1* mutant grown under long days. Additionally, a cytosolic *ascorbate peroxidase* gene in rice is induced by paraquat treatment through H_2O_2 but not by O_2^- (Morita *et al.*, 1999).

Apart from the apparent involvement of H_2O_2 in local defence responses, H_2O_2 also appears to play an integral part in the establishment of systemic acquired resistance (SAR). SAR is characterised as a systemic, long-lasting and broad-spectrum plant defence mechanism that confers resistance in the plant to subsequent pathogen attack by either the primary infecting pathogen or different pathogens (Dempsey *et al.*, 1999). Challenge of Arabidopsis with avirulent *P. syringae* induced a primary oxidative burst at the site of infection as well as secondary oxidative bursts in discrete cells in distal tissue resulting in low frequency micro-HRs (Alvarez *et al.*, 1998). Both the primary and secondary oxidative bursts are essential for resistance to subsequent pathogen attack and expression of the defence genes *GST* and *PR-2* in systemic tissue (Alvarez *et al.*, 1998). It is suggested that rather than H_2O_2 diffusing from the primary source, H_2O_2 accumulation at the initial infection site reiterates further H_2O_2-generating microbursts, possibly through activation of NADPH oxidase (Alvarez *et al.*, 1998). SA may also aid H_2O_2 signal amplification as SA accumulation, which is necessary for SAR, is not only preceded by and induced by the oxidative burst but SA itself is able to stimulate H_2O_2 production (Rao *et al.*, 1997; Alvarez *et al.*, 1998; Van Camp *et al.*, 1998; Vranová *et al.*,

2002). It has also been proposed that redox signalling mediates SAR with the observation that the active monomeric NPR1, induced by SA-mediated reduction of conserved cysteine residues, and *PR-1* gene expression occurred in distal tissue following infection with an avirulent pathogen (Mou *et al.*, 2003). Furthermore it was shown that SA-mediated changes in redox conditions regulated the interaction between NPR1 and the bZIP (basic leucine zipper) transcription factor TGA1 to direct binding to promoter elements and activate gene expression (Deprés *et al.*, 2003). However, the enzymes responsible for reduction of cysteine residues of NPR1 and TGA1 are currently unknown but it is proposed that thioredoxins and glutaredoxins are possible candidates given their abundant role in controlling cellular redox status (Rouhier *et al.*, 2004; Fobert and Després, 2005; Gelhaye *et al.*, 2005).

The above discussion illustrates the importance of AOS in the establishment of defence responses however the mechanisms by which AOS signalling progresses to initiate disease resistance are currently unknown. The identification of downstream components of AOS will greatly aid our understanding of how AOS are able to act in a signalling capacity.

1.2.4.2 Physiological processes

Apart from stress adaptation, AOS also plays a signalling role in mediation of physiological processes. For example, the plant hormones auxin and abscisic acid (ABA) have been shown to induce the accumulation of AOS, which is required for processes such as root development and stomatal closure.

1.2.4.2.1 Root development

Several lines of evidence implicate a signalling role for AOS in processes affecting either root or root hair development. Auxin-induced AOS accumulation was shown to be an essential signalling component for root gravitropism. AOS accumulated in the convex endodermis in response to either gravistimulation or application of auxin to vertical roots (Joo *et al.*, 2001). Root bending occurred following application of exogenous H_2O_2 to vertical roots pre-treated with auxin transport inhibitors and root gravitropism was inhibited by scavenging AOS with antioxidants (Joo *et al.*, 2001). AOS accumulation also

mediates root elongation. The NADPH oxidase mutant *atrbohF* and the double mutant *atrbohD/F* displayed shorter root lengths in comparison to wild type and were also insensitive to ABA-inhibition of root elongation, further implicating a role for AOS in ABA-mediated signal transduction pathways (Kwak *et al.*, 2003). The level of AOS production in the root of the *atrbohD/F* mutant in response to ABA was not determined in the aforementioned study. However, the authors had demonstrated that ABA-induced AOS accumulation was dramatically impaired in guard cells of the *atrbohD/F* mutant in comparison to wild type and therefore a similar lack of AOS in response to ABA can be comprehended in the root tissue of the mutant (Kwak *et al.*, 2003). Additionally the direct quenching of AOS produced in plant roots restricted root elongation (Demidchik *et al.*, 2003; Laloi *et al.*, 2004).

Genetic evidence for a role for AOS in root hair development stems from the isolation of the Arabidopsis *root hair defective2* (*rhd2*) mutant which has a mutation in the gene encoding the NADPH oxidase C catalytic subunit. The *rhd2* mutant contains reduced levels of AOS in growing root hairs which leads to disruption of both Ca^{2+} uptake and root cell expansion and consequently *rhd2* mutants have shorter root hairs (Forman *et al.*, 2003). It was recently shown that a RhoGTPase GDP dissociation inhibitor spatially regulates root hair growth through the activation of RHD2 (Carol *et al.*, 2005). This is in accordance with mammalian cells where GTPase Rac regulates NADPH oxidase and AOS production (Pitzschke and Hirt, 2006). AOS can also function to mediate cellular responses in the root when plants are challenged with stress. For example, H_2O_2 mediates plant root cell responses including gene expression and changes in the kinetics of potassium (K^+) uptake when plants are grown under conditions of K^+ deprivation (Shin and Schachtman, 2004). The expression of genes normally induced during K^+ deprivation was prevented in the *rhd2* mutant but application of exogenous H_2O_2 to *rhd2* not only rescued the expression of genes induced during K^+ deficiency but also induced high-affinity K^+ transport activity in roots exposed to sufficient K^+ conditions (Shin and Schachtman, 2004). Although it is clear that AOS mediates development and responses in both roots and root hairs, the downstream signalling components have yet to be elucidated but may involve protein kinase cascades which will be discussed in later sections.

1.2.4.2.2 Stomatal closure

A signalling role for AOS in the adaptive response of stomatal closure under conditions of stress stems from the observation that abscisic acid (ABA) requires H_2O_2 to activate plasma membrane calcium channels which induce stomatal closure (Pei *et al.*, 2000). Furthermore it was shown that the *atrbohD/F* double mutant failed to induce AOS accumulation in its guard cells in response to ABA and exogenous application of H_2O_2 rescued stomatal closing in the *atrbohD/F* mutant (Kwak *et al.*, 2003). These observations provide genetic evidence that the NADPH oxidases AtrbohD and AtrbohF are responsible for the production of ABA-induced AOS accumulation during stomatal closure and that AOS plays a role in guard cell signalling (Kwak *et al.*, 2003). The ABA signalling cascade mediating stomatal closure was further dissected with the isolation of the *open stomata1* (*ost1*) protein kinase mutant. OST1 functions downstream of ABA and upstream of AOS since the *ost1* mutant is still able to close its stomata in response to H_2O_2 and the *ABA-insensitive1-1* (*abi1-1*) mutant cannot activate OST1 kinase in an ABA-dependent manner (Murata *et al.*, 2001; Mustilli *et al.*, 2002). This data suggests that OST1 mediated protein phosphorylation functions between ABI1 and AOS generation in ABA-induced stomatal closure and presents a link between kinase activity and AOS accumulation in regulating a physiological process (Laloi *et al.*, 2004). However, it has yet to be determined whether OST1 regulates NADPH oxidase.

1.2.5 Downstream sensors of AOS

Although it is clear that the presence of AOS is crucial to various cellular processes and the plant defence, very little is known about the components that directly sense the change in AOS production to transduce specific signals leading to defined end responses. It is established that the presence of H_2O_2 leads to the activation of a number of protein kinases particularly those belonging to the mitogen activated protein kinases (MAPKs) (Kovtun *et al.*, 2000; Desikan *et al.*, 2001b; Rentel *et al.*, 2004) and H_2O_2 can also inactivate protein phosphatases (Meinhard and Grill, 2001). For example the protein tyrosine phosphatase AtPIP1 which also regulates the activity of MAPKs has been suggested to play a role in H_2O_2-mediated signalling, thus linking AOS to phosphorylation/dephosphorylation signalling cascades (Gupta and Luan, 2003; Hancock *et al.*, 2006). However, it has not been shown that H_2O_2 oxidises any of these

proteins directly therefore the need to identify H_2O_2 sensing components up-stream of kinase activation that directs activation of specific signalling pathways remains.

Factors and components likely to be involved in the direct sensing of H_2O_2 were recently reviewed (Hancock *et al.*, 2006). Thiol (-SH) modification of cysteine residues by H_2O_2 i.e. oxidation of -SH group to sulphenic (-SOH), sulphinic ($-SO_2H$) or sulphonic ($-SO_3H$) acid or formation of a disulphide bridge if two cysteine residues are present, is a key event affecting the activity and structure of proteins (Vranová *et al.*, 2002; Hancock *et al.*, 2006). Additionally H_2O_2 can also oxidise other amino acids such as Tyr, Trp and His (Drögre, 2002) which implicates numerous proteins as targets for H_2O_2. AOS sensors, however, must likely have specific characteristics to enable mediation of signalling pathways, for example the oxidation status should be able to be reversed so as to switch off or regulate the signal. Methionine may be oxidised to yield MetSO and can be rapidly reduced back to Met by the protein MetSO reductase capable of using thioredoxin as a reductant (Sadanandom *et al.*, 2000; Romero *et al.*, 2004). The ability of a given protein to be oxidised by H_2O_2 would depend on the availability of the location of the amino acid to be oxidised within the protein as well as the cellular redox status (Hancock *et al.*, 2006). H_2O_2 signal transduction may not propagate in a very reduced environment since under these conditions the antioxidant scavenging systems will be at their full potential having high concentrations of Asc and GSH to control excess H_2O_2 production. A few proteins regulated by H_2O_2 or cellular redox status have recently been established. These include the ethylene histidine receptor kinase (ETR1), NPR1 and TGA1 and TGA4, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), alcohol dehydrogenase, S-adenosyl methionine and glutamine synthetase (Deprés *et al.*, 2003; Mou *et al.*, 2003; Desikan *et al.*, 2005; Hancock *et al.*, 2005).

ETR1 is required for H_2O_2 mediation of stomatal closure since the Arabidopsis *etr1-1* mutant that contains a Cys65Tyr mutation displays reduced stomatal closure in response to H_2O_2 (Desikan *et al.*, 2005). However it has yet to be determined whether the oxidation of Cys65 (Desikan *et al.*, 2005) is a direct effect of H_2O_2 or requires another H_2O_2 sensing protein. GAPDH is an important enzyme involved in the glycolytic pathway therefore its involvement in H_2O_2 signalling is unexpected. H_2O_2 was shown to directly modify GAPDH based on an approach using the 5'-iodoacetamide fluorescein tag (IAF) which competes with H_2O_2 for covalent modification of thiol groups (Hancock *et al.*,

2005). Therefore if a protein experienced a reduction in its binding capacity to IAF due to H_2O_2 pre-treatment then that protein was presumably oxidised by H_2O_2. GAPDH from cytosolic Arabidopsis extracts is inhibited by H_2O_2 in a concentration dependent manner while GAPDH activity is restored by reduced glutathione or the reductant dithiothreitol indicative that inhibition is reversible (Hancock *et al.*, 2005). Additionally GAPDH has been found in locations other than that expected if it was only involved in glycolysis, for example in Arabidopsis plant cells walls (Chivasa *et al.*, 2002) and GAPDH has been shown to become associated with mitochondria during oxidative stress (Sweetlove *et al.*, 2002). It has been shown in mammalian cells that oxidation of a Cys residue within GAPDH inactivates its normal function but allows interaction with and causes activation of the enzyme phospholipase D thereby propagating signalling events associated with phospholipase D (Kim *et al.*, 2003). Similarly, GAPDH may play a role in H_2O_2 mediated signalling in plant cells particularly since oxidation is reversible albeit at low H_2O_2 concentrations (Hancock *et al.*, 2005; Hancock *et al.*, 2006).

A microarray experiment involving transcriptome analysis of Arabidopsis treated with exogenous H_2O_2 first identified the induction of *ETR1* gene expression in response to H_2O_2 treatment (Desikan *et al.*, 2001a). Subsequent analysis, owing to the fact that histidine kinase activity is involved in tolerance to oxidative stress in yeast (Singh, 2000), illustrated a role for ETR1 in H_2O_2 mediated stomatal closure (Desikan *et al.*, 2005). This illustrates that global gene expression profiling may be useful in identifying components that are regulated by or sense AOS accumulation. Expression profiles of tobacco treated with H_2O_2 has revealed differential regulation of a number of genes that are involved in other hormone and/or stress responsive pathways (Vandenabeele *et al.*, 2003). Additionally, microarray analysis of a catalase-deficient Arabidopsis line exposed to high light demonstrated differential regulation of a transcriptional cluster involved in anthocyanin biosynthesis in response to H_2O_2 treatment (Vanderauwera *et al.*, 2005). However, whether any of these genes function as H_2O_2 sensing proteins would require further investigation through mutational and/or biochemical analysis and would greatly aid the understanding of H_2O_2 signalling.

1.2.6 In brief: AOS and Nitric oxide

NO is emerging as an important signalling component of various cellular processes and responses and interacts with SA and jasmonic acid (JA) signalling pathways (Wendehenne *et al.*, 2004). The production of NO often correlates spatially and temporarily with AOS generation (Neill *et al.*, 2003). S-nitrosylation occurs when NO reacts with thiol groups to yield a –S-NO group and hence NO may compete with H_2O_2 to covalently modify thiol groups (Hancock *et al.*, 2006). Many proteins that has been shown to be modified by NO were also potentially oxidised by H_2O_2 (Lindermayr *et al.*, 2005) therefore the activation of a particular signalling pathway might be dependent on the type of thiol modification.

Under some conditions NO and AOS may be able to function co-operatively to mediate signal transduction pathways. For example, NO has also been shown to regulate plant cell death together with H_2O_2 during the incompatible interaction since chemical inhibition of NO production reduced the HR in Arabidopsis challenged with avirulent *P. syringae* (Delledonne *et al.*, 1998). Additionally, NO is able to react with O_2^- to produce peroxynitrite, a strong oxidant, which in turn can convert xanthanine dehydrogenase to the O_2^- producing enzyme xanthanine oxidase (Corpas *et al.*, 2001). Therefore NO may stimulate or reiterate O_2^- -mediated signalling pathways. NO inhibits respiratory cytochrome *c* oxidase of plant mitochondrial and consequently may stimulate the production of O_2^- and H_2O_2 since inhibition of cytochrome c oxidase increases electron flow from ubiquinone (Millar and Day, 1997; Van Camp *et al.*, 1998). Conversely, NO also functions to protect cells from oxidative damage since it induces the expression of the antioxidant genes catalase, SOD and GST (Durner *et al.*, 1998; Polverari *et al.*, 2003). Therefore NO plays both antagonistic and co-operative roles with AOS and further research is required fully elucidate the interaction between these two signalling molecules during different cellular conditions.

1.2.7 Summary

AOS are generated by a number of different mechanisms and the plant has evolved an elaborate antioxidant defence system to protect the cell from the damaging effects of AOS. The signalling capacity of AOS is important in establishment of disease resistance

and also has a role in physiological processes. Although AOS accumulation has been shown to be detrimental to abiotic stress, AOS may also potentially trigger signalling cascades to facilitate stress tolerance. For instance, the activation of antioxidant defence mechanisms or the change in cellular redox may affect the activity of transcription factors to induce the expression of abiotic stress responsive genes. To understand how AOS signals are transduced it is important to identify proteins that directly sense or are differentially regulated by AOS. Therefore employment of studies to understand the role of a kinase induced by AOS such as OXI1 may shed light on AOS signal transduction pathways.

1.3 Protein Kinases

Protein kinases act to phophosphorylate target proteins (termed phosphoproteins) and this modification changes the protein structure which ultimately affects the activation, localisation, half-life and/or protein-protein interactions of the phosphoprotein (Huber and Hardin, 2004). Phosphorylation, and equally dephosphorylation, (carried out by phosphatases) events are crucial to normal metabolic processes as well as signalling cascades during conditions of stress. The Arabidopsis genome encodes over 1000 protein kinase genes (Initiative, 2000) further demonstrating the importance of these components. There are many different classes of protein kinases and the following sections will only focus on the main types, their current signalling perspectives and where known, their regulation.

1.3.1 Different types of protein kinases

1.3.1.1 Receptor-like protein kinases (RLKs)

The RLKs are by far the largest family of protein kinases boasting more than 600 members in Arabidopsis (Initiative, 2000). RLK are serine/threonine protein kinases and generally consist of an extracellular domain, a single transmembrane domain and a cytoplasmic kinase domain (Johnson and Ingram, 2005). There is great variability in their extracellular domain which is most likely due to the large amount of different ligands that can bind RLKs (Shiu and Bleecker, 2003). Putative ligands of different RLKs include carbohydrates, steroids, polypeptides and cell wall components of microbes, however

relatively few cognate ligands have actually been identified (Morris and Walker, 2003; Johnson and Ingram, 2005).

1.3.1.2 Calcium-dependent protein kinases (CDPKs)

Calcium is a well established second messenger in all eukaryotic organisms. A range of stimuli trigger changes in the $[Ca^{2+}]_c$ concentration, which are sensed by various Ca^{2+}-sensitive proteins that may then go on to transduce the signalling cascade to launch the appropriate end response (Knight, 2000). One such group of Ca^{2+}-sensitive proteins are the plant and protozoan specific Ca^{2+}-dependent protein kinases (CDPK) of which there are 34 members in Arabidopsis (Cheng *et al.*, 2002). Although there is numerous biochemical and expression data across various plant species to indicate that CDPKs are involved in range of cellular and metabolic processes, the significance of their role *in vivo* as well as the signal transduction pathways they mediate is sorely lacking.

1.3.1.3 The AGC Kinase Family

The AGC kinase family is so called because this family includes cAMP-dependent protein kinase A, cGMP-dependent protein kinase G and the phospholipid-dependent protein kinase C (Bögre *et al.*, 2003). In both animals and yeast these kinases regulate critical cellular functions such as protein synthesis, cell growth and gene transcription since they are downstream effectors of key intracellular second messengers including cAMP, cGMP, phospholipids and calcium (Peterson and Schreiber, 1999). The Arabidopsis AGC kinase family consists of 39 members based on searching the Arabidopsis database for the signature motifs consisting of a kinase domain, the hydrophobic pleckstrin homology (PH) domain which is the putative lipid-binding site and the activation T-loop (Bögre *et al.*, 2003). Only a few members of the Arabidopsis AGC kinase family have been characterised and were shown to play roles in processes such as auxin signalling, root hair development, pathogenesis and perception of blue light/UVA (Christie *et al.*, 1998; Benjamins *et al.*, 2001; Jarillo *et al.*, 2001; Oyama *et al.*, 2002; Rentel *et al.*, 2004) .

1.3.1.4 Mitogen-activated protein kinases (MAPKs)

The MAPKs are among the most conserved proteins in yeast, animals and plants (Zhang and Klessig, 2001). A MAPK cascade encompasses minimally of three components: a MAP kinase kinase kinase (MAPKKK) which phosphorylates a MAP kinase kinase (MAPK) that in turn phosphorylates a MAP kinase (MAPK). It is well documented in animals and yeast that MAPK cascades relay the perception of an external stimulus by a receptor into intracellular responses (Widmann *et al.*, 1999; Chang and Karin, 2001). In Arabidopsis there are 80 MAPKKK, 10 MAPKK and 20 MAPK (Initiative, 2000) and by sequence and signature motif analogy putative orthologues for the different MAPKKK, MAPKK and MAPK exist in alfalfa, tobacco and rice (Nakagami *et al.*, 2005). Extensive research has been undertaken in a number of plant systems to elucidate the individual components involved in plant MAPK cascades and the processes that they regulate.

The MAPKKK are serine/threonine protein kinases and are activated either directly by the receptor perceiving the stimulus, through intermediate bridging proteins or perhaps interlinking MAPKKKK (Jonak *et al.*, 2002). Although there are 10 MAPKKKK genes in Arabidopsis that are related to the yeast sterile20 or mammalian p21-activated protein kinase MAPKKKK, there is no evidence to show that these kinases regulate MAPK cascades in plants (Jonak *et al.*, 2002). The MAPKKK are divided into two main subgroups, those that are related to the animal MEKKKs and yeast MAPKKKs and the other subgroup consists of those related to the Raf-like protein kinases. The Raf-like subgroup of plant MAPKKK contain the signature motif GT-X_2(W/Y)MAPE (where X_2 represents any two amino acids) while members of the other subgroup are either related to MEKK-like kinases containing the signature sequence G(T/S)P-X-(W/Y/F)MAPEV or to the ZR1-interacting kinases which have GTPEFMAPE(L/V)Y. Despite the large number of putative plant MAPKKK only a few members have been shown to phosphorylate or activate a MAPKK. There is also evidence for autophosphorylation within the kinase domain for some of these MAPKKK such as the Constitutive Triple Response 1 protein kinase (Huang *et al.*, 2003), which illustrates that these MAPKKK may be regulated through autophosphorylation.

MAPKK have a conserved S/T-X_{3-5}-S/T motif (where X_{3-5} represents a stretch of any 3 to 5 amino acids) and are activated by phosphorylation of these two serine/threonine

residues by MAPKKK (Jonak *et al.*, 2002). The dual specificity MAPKK phosphorylates MAPK on threonine and tyrosine amino acids in the T-X-Y motif in the active site of the MAPK. MAPKs are also serine/threonine protein kinases and they have the ability to phosphorylate a range of molecular components including transcription factors and other protein kinases thereby directing downstream end responses (Nakagami *et al.*, 2005; Pitzschke and Hirt, 2006).

MAPK signalling is extremely complex, given that the same MAPK components are induced by different stimuli to initiate distinct end responses (Asai *et al.*, 2002; Ahfors *et al.*, 2004). Therefore these MAPK components may act as points for cross talk between different signal transduction pathways. Furthermore, a single MAPKKK can also phosphorylate different MAPKK thereby allowing MAPKKKs to act as convergence points for different signal transduction pathways (Mizoguchi *et al.*, 1996; Ichimura *et al.*, 1998; Asai *et al.*, 2002). Different types of protein kinases may also act upstream or downstream of MAPK signalling cascades to initiate the cascade or to direct particular end responses, respectively (Jonak *et al.*, 2002; Pitzschke and Hirt, 2006). Therefore different protein kinases may be involved in response to a particular stress and form an intricate web of signal transduction networks to initiate stress tolerance and such is the case for the plant response to pathogen attack.

1.3.2 The role of protein kinases in disease resistance

1.3.2.1 Recognition of the invading pathogen

The ability of a plant to activate the appropriate defence responses following challenge with a potential pathogen relies on recognition by the plant of the invading microorganism. The RLKs are paramount in this regard. It has been demonstrated that the Arabidopsis Leucine-rich-repeat-RLK (LRR-RLK) FLAGELLIN INSENSITIVE2 (FLS2) binds bacterial flg22 (Gomez-Gomez and Boller, 2000; Chinchilla *et al.*, 2006) and activates a MAPK cascade which leads to induction of plant defence genes and disease resistance (Asai *et al.*, 2002). Furthermore, Arabidopsis protoplasts isolated from *fls2* mutants were unable to induce the expression of the defence genes *WRKY29*, *FLG22-INDUCED RECEPTOR-LIKE KINASE1* and *GST1* in response to flg22 treatment (Asai *et al.*, 2002). Additionally, the *fls2* mutant was as susceptible as wild type when leaves

were infiltrated with virulent *P. syringae* but *fls2* was more susceptible than wild type when *P. syringae* was sprayed on its leaves (Zipfel *et al.*, 2004). Taken together, these results indicate that FLS2 is required for mediation of basal defence responses and also plays a minor role in restricting virulent bacterial invasion, presumably at an early step before delivery of bacterial virulent effector proteins.

Another RLK involved in early recognition of PAMPs is the elongation factor Tu (EF-Tu) receptor (EFR). The importance of bacterial EF-Tu recognition by EFR in defence was recently demonstrated since the transformation efficiency by *Agrobacterium tumefaciens* in the Arabidopsis *efr* mutant was increased in comparison to wild type (Zipfel *et al.*, 2006). However, the downstream components activated after EF-Tu perception are still unknown. The LRR-RLK ERECTA plays a role in resistance to bacterial wilt, a severe bacterial disease of plants caused by *Ralstonia solanacearum*, since transgenic Landsberg *erecta* Arabidopsis expressing the *ERECTA* gene exhibit increased resistance to *R. solanacearum* infection (Godiard *et al.*, 2003). It is not known whether ERECTA is involved in recognition of the pathogen or activated as a consequence of other signalling events induced during the defence response.

During gene-for-gene resistance, for example, the tomato R gene product Pto (which encodes a serine/threonine protein kinase) recognises the Avr proteins, AvrPto and AvrPtoB, expressed by *P. syringae* and induces an HR (Martin *et al.*, 1993). It was shown using yeast-two-hybrid assays that Pto strongly and specifically binds AvrPto and that Pto kinase activity is required for HR induction (Tang *et al.*, 1996; Rathjen *et al.*, 1999). Pto-mediated resistance requires the activity of several genes including MAPKs and the transcription factors NPR1 and TGA since virus-induced silencing (VIGS) of these genes in tomato severely compromises resistance (Ekengren *et al.*, 2003). Therefore these examples demonstrate that protein kinase activity plays an early role in pathogen recognition during both nonhost and gene-for-gene resistance.

1.3.2.2 Downstream kinase activity positively regulates disease resistance

After recognition of PAMPs or effector proteins in many cases via protein kinases as outlined above, protein kinase activity is also required for downstream events or signalling. The first complete MAPK cascade module mediating nonhost resistance or

basal defence responses was identified in response to bacterial flagellin using an Arabidopsis protoplast transient expression system (Asai *et al.*, 2002). The MAPK module triggered either directly or indirectly by FLS2 consists of the MAPKKK AtMEKK1, which activates the MAPKKs AtMKK4 and AtMKK5 that in turn phosphorylates the MAPKs AtMPK3 and AtMPK6 (Asai *et al.*, 2002). This phosphorylation cascade led to the expression of the WRKY transcription factors *WRKY22* and *WRKY29* and defence related genes such as *GST1* (Asai *et al.*, 2002; Gomez-Gomez and Boller, 2002). Furthermore, transient overexpression of either the MEKK1 kinase domain or constitutively active MKK4 or MKK5 conferred Arabidopsis leaves with enhanced resistance to the bacterial pathogen *P. syringae* as well as the fungal pathogen *Botrytis cinerea* indicative that defence responses activated by this MAPK cascade are effective against both bacterial and fungal pathogens (Asai *et al.*, 2002). Additionally, the bacterial elicitor harpin was shown to induce the activity of MPK4 and MPK6 indicating that MAPK components are involved in more than one PAMP signalling cascade required for nonhost resistance responses (Desikan *et al.*, 2001b). Moreover, silencing of the wound- (WIPK) or SA-induced protein kinase (SIPK; orthologues of Arabidopsis AtMPK3 and AtMPK6 respectively) in tobacco rendered these transgenic plants susceptible to *P. cichorii* infection, for which wild type tobacco is not a host plant (Sharma *et al.*, 2003). Therefore protein kinase activity, particularly MAPK components, is essential for mediating downstream events leading to nonhost resistance responses.

Protein kinase activity also mediates downstream signalling events during gene-for-gene resistance. Several MAPK cascades are activated during the Pto/AvrPto incompatible interaction in both tobacco and tomato and each of these cascades are capable of inducing resistance (Figure 1.4). In tobacco the MAPKKKs NPK1 and MAPKKKα phosphorylates the MAPKKs MEK1 and MEK2 respectively which in turn activate the MAPKs NTF6 and WIPK or SIPK (Ekengren *et al.*, 2003). Although both WIPK and SIPK are activated by MEK2, MAPKKKα only activates SIPK indicating that a separate MAPKKK induces the activity of WIPK via MEK2 (del Pozo *et al.*, 2004). Therefore WIPK and SIPK are likely not to have redundant functions during the Pto/AvrPto interaction. Similarly in tomato, Pto/AvrPto induces the activity of MAPKKKα which activates MKK4 and MKK2 and these phosphorylate the MAPKs MPK2 and MPK3, the orthologues of SIPK and WIPK respectively (Pedley and Martin, 2004). It is not yet determined whether

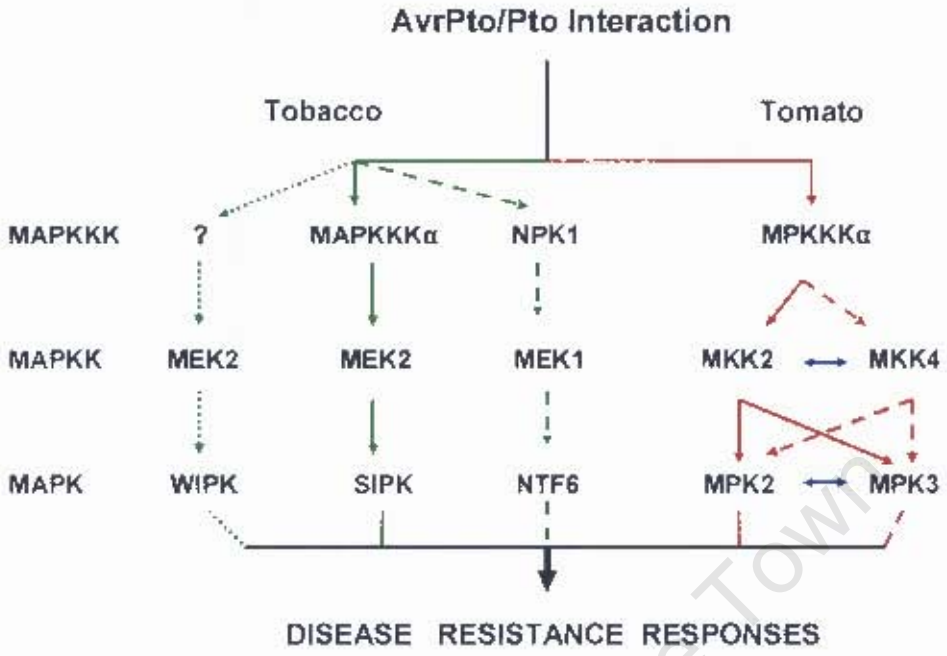


Figure 1.4 Activation of different MAPK signalling pathways during the AvrPto/Pto incompatible interaction in tobacco and tomato

Following infection with avirulent *P. syringae* expressing the avirulence gene AvrPto, tomato and tobacco plants expressing the Pto resistance gene initiate the activation of MAPK cascades. In tobacco there are 3 possible pathways (green arrows) whereas in tomato there is either one or two such pathways (red arrows) depending on whether MKK2 and MKK4 as well as MPK2 and MPK3 have redundant functions, as indicated by the blue double arrow. The red and green solid and dashed arrows each represent independent pathways. The upstream activator of the MEK2-WIPK cascade has not been identified (?). The exact processes that these pathways may regulate to facilitate disease resistance are currently unknown.

MKK4 and MKK2 or MPK2 and MPK3 have redundant functions therefore at least two possible MAPK cascades can be activated during the Pto/AvrPto interaction in tomato (Figure 1.4). Additionally, the MAPKKs MEK1 and MEK2 as well as the MAPKs WIPK, SIPK and NTF6 are required for establishment of resistance gene *N*-mediated defence responses, such as the HR, in tobacco plants challenged with tobacco mosaic virus (Zhang and Klessig, 1998; Jin *et al.*, 2003; Liu *et al.*, 2003). In transgenic tobacco plants expressing the tomato Cf-9 R protein both WIPK and SIPK are activated upon treatment with the corresponding Avr9 peptide from *Cladosporium fulvum* (Romeis *et al.*, 1999). Therefore different R/Avr incompatible interactions make use of the same MAPK components to elicit disease resistance responses.

CDPKs also mediate disease resistance responses during gene-for-gene resistance. Considering the Cf-9/Avr9 incompatible interaction, NtCDPK2 and NtCDPK3 were shown to be essential for the induction of the HR (Romeis *et al.*, 2001). Transgenic tobacco plants expressing the tomato Cf-9 protein and in which VIGS of *NtCDPK2* and *NtCDPK3* genes has occurred, showed a significantly reduced and delayed HR in response to infection with *C. fulvum* carrying the Avr9 peptide (Romeis *et al.*, 2001). The activation of these CDPKs may serve to activate MAPK cascades to induce HR since WIPK and SIPK are also activated during the Cf-9/Avr9 interaction (Romeis *et al.*, 1999). It is also possible these CDPKs could be downstream effectors of a MAPK signalling cascade since it has been shown that Ca^{2+} and phosphorylation are both required for the full activation of NtCDPK2 (Romeis *et al.*, 2000), however the identity of the kinase responsible for phosphorylating NtCDPK2 is unknown. Additionally, increases in $[Ca^{2+}]_c$ which precede the accumulation of AOS are among the early responses during incompatible interactions (Grant *et al.*, 2000b). It was shown that the Arabidopsis calmodulin-like domain protein kinase 1 (AtCPK1) enhanced NADPH oxidase activity both in a cell-free system and tomato protoplasts (Xing *et al.*, 2001). Taken together these data suggest that CDPKs mediate the HR through regulation of the oxidative burst and play a role in disease resistance signalling (Cheng *et al.*, 2002).

1.3.2.3 The role of protein kinases in crosstalk during disease resistance

The aforementioned discussion clearly places protein kinases as crucial components mediating disease resistance responses. Additionally protein kinases provide overlap between gene-for-gene and nonhost resistance since similar protein kinases are activated during both resistance responses, e.g. WIPK and SIPK (Ekengren *et al.*, 2003; Sharma *et al.*, 2003). Mutational analysis provided evidence to support this hypothesis. For example, silencing of Arabidopsis *MPK6* renders *mpk6* mutant plants more susceptible to both virulent and avirulent isolates of *P. syringae* as well as to avirulent *Hyaloperonospora parasitica* (Menke *et al.*, 2004).

It is generally accepted that SA-dependent signalling is largely responsible for mediating disease resistance to biotrophic pathogens whereas JA/ET signal transduction pathways control resistance responses to necrotrophic pathogens and/or insects (Glazebrook, 2005). There is a large body of evidence which suggests that SA and JA have antagonistic effects on each other (Thomma *et al.*, 1998; Petersen *et al.*, 2000; Thomma *et al.*, 2001; Glazebrook, 2005). Protein kinase activity may mediate the balance between SA and JA responses. For example, the *mpk4* mutant shows elevated SA levels, enhanced resistance to virulent *P. syringae* and constitutive induction of *PR* gene expression suggesting that this MAPK negatively regulates plant defence (Petersen *et al.*, 2000). However, *mpk4* lacks responsiveness to JA and it has recently been reported that the *mpk4* mutant is also compromised in defence gene activation in response to ET and more susceptible to the necrotrophic pathogen *Alternaria brassicicola* (Brodersen *et al.*, 2006). Therefore MPK4 positively regulates defence responses against necrotrophic pathogens. Furthermore, MPK4 kinase activity appears to mediate the balance between SA and JA responses by suppressing both activators of SA and repressors of JA/ET defence responses through regulating the activity of EDS1 and PAD4 (Brodersen *et al.*, 2006).

Despite the numerous reports whereby protein kinases positively regulate different disease resistance responses, protein kinase activity may also negatively regulate disease resistance. For example, the Enhanced Disease Resistance1 (EDR1), which encodes a MAPKKK, negatively regulates plant defence responses since the *edr1* mutant exhibits enhanced resistance to virulent pathogens however these defence

responses are not constitutively active but inducible and activated via SA (Frye *et al.*, 2001). Additionally *edr1* mediated resistance was not suppressed in the *ein2/edr1* double mutant infected with *Erysiphe cichoracearum* indicating that JA and ET signalling pathways were not involved in *edr1* resistance since the *ein2* mutation blocks ET- and JA-mediated responses (Frye *et al.*, 2001).

Although most of the evidence for the involvement of protein kinases in disease resistance responses stems from studies investigating components of MAPK cascades, it is clear that protein kinase activity is imperative for the effective establishment of disease resistance. Additionally, identification of the upstream activators and downstream transducers of MAPK signalling cascades may reveal novel roles for other types of protein kinases in disease resistance responses.

1.3.3 The involvement of protein kinases during abiotic stress

Various protein kinases are transcriptionally upregulated and their enzymatic activity increased in response to different abiotic stresses (Cheng *et al.*, 2002; Pitzschke and Hirt, 2006). It is interesting to note that components of MAPK cascades involved in biotic stress tolerance are also activated during abiotic stress. For example, MPK4 and MPK6 are activated in response to cold, salt, drought, wounding and touch (Ichimura *et al.*, 2000) while osmotic stress induces the activity of MPK3 and MPK6 (Droillard *et al.*, 2002). Therefore protein kinases can function as convergent points between abiotic and biotic signal transduction pathways.

Modulation of protein kinase activity can also enhance or decrease stress tolerance. Overexpression of OsCDPK7 in rice resulted in the enhanced expression of some stress responsive genes such as *rab16A*, *salT* and *wsi18*, which encode late embryogenesis abundant proteins, and conferred transgenic plants with increased tolerance to cold, salt and drought stress (Saijo *et al.*, 2000). Additionally the Arabidopsis *mkk2* null mutant was significantly compromised in cold and salt stress tolerance in comparison to wild type and overexpression of *MKK2* yielded transgenic plants with increased resistance to cold and salt stress (Tiege *et al.*, 2004). These results also show that plants make use of similar components (such as a single protein kinase) or signal transduction cascades to promote tolerance to a diversity of abiotic stresses. Further research is required to

elucidate how a single component is able to mediate tolerance to different stresses i.e. where specificity arises.

1.3.4 Interaction between protein kinases and AOS

There is evidence to suggest that the generation of AOS acts both upstream and downstream of protein kinase activity. Both MPK3 and MPK6 in Arabidopsis and SIPK and WIPK in tobacco become activated in response to AOS (Kovtun *et al.*, 2000; Kumar and Klessig, 2000) as well as ozone treatment (Samuel *et al.*, 2000; Ahfors *et al.*, 2004). Although AOS production during abiotic stress has been shown to be detrimental to plant survival, AOS activation of MPK3 and MPK6 in response to ozone treatment activates adaptive responses since RNAi silenced MPK3 and MPK6 transgenic lines displayed increased sensitivity to ozone (Miles *et al.*, 2005). These findings implicate a positive role for AOS production in response to abiotic stress tolerance.

Evidence for protein kinase activity acting upstream of AOS accumulation stems from studies involving programmed cell death. It has been demonstrated that overexpression of a constitutive active form of MEK2 (MEK^{DD}) in tobacco induced an HR-like cell death that was dependent on the presence of the respiratory burst oxidase gene *NbrbohB* (Yoshioka *et al.*, 2003). *NbrbohB* is responsible for generating AOS during the resistance responses to *P. infestans* and other biotrophic pathogens (Yoshioka *et al.*, 2003). Furthermore, MEK^{DD} induced the expression of *NbrbohB* indicating that the MEK2 pathway might be part of an amplification cascade to generate AOS (Yoshioka *et al.*, 2003; Nakagami *et al.*, 2005). Consistent with the aforementioned findings, constitutively active Arabidopsis MKK4 and MKK5, the orthologues of tobacco MEK2, induced an HR-like cell death and H_2O_2 accumulation (Ren *et al.*, 2002). Recently, transgenic potato plants harbouring a constitutively active form of a MAPKK (StMEK1) driven by a pathogen-inducible promoter were shown to display increased resistance to virulent *Phytophthora infestans* through AOS accumulation and an HR-like phenotype (Yamamizo *et al.*, 2006). Surprisingly, these transgenic plants were also more resistant to infection with the necrotroph *Alternaria solani* (Yamamizo *et al.*, 2006) while H_2O_2 accumulation has previously been shown to aid infection by necrotrophs (Govrin and Levine, 2000). Nonetheless activation of a MAPK cascade in potato plays a role in resistance to both biotrophic and necrotrophic pathogens and this data also places a

putative MAPK signalling pathway upstream of H_2O_2. As mentioned earlier AtCPK1 enhanced NADPH oxidase activity both in a cell-free system and tomato protoplasts (Xing *et al.*, 2001), further implicating kinase activity upstream of AOS signalling.

It is proposed that MAPK cascades control the generation of AOS through regulation of redox-sensitive proteins particularly during pathogen defence (Pitzschke and Hirt, 2006). For example, recently the identification of putative substrates of MPK4 and MPK6 included two thioredoxins and an ascorbate oxidase-like protein (Feilner *et al.*, 2005). As mentioned earlier (Section 1.2.4.1), modulation of AOS levels through altering the activity of antioxidant systems enhanced disease resistance under conditions which favoured AOS accumulation i.e. inhibition of CAT or APX. Additionally, redox changes regulates the activity of NPR1 particularly during SAR and it is proposed that thioredoxin may be the enzyme responsible for the reduction of NPR1 (Deprés *et al.*, 2003; Fobert and Després, 2005). Therefore if redox sensitive proteins are indeed substrates of MAPKs then MAPK cascades may indirectly enhance AOS accumulation during pathogen attack by inactivating various antioxidant scavenging mechanisms thereby promoting disease resistance responses.

Under different cellular conditions phosphorylation events may precede or follow the accumulation of AOS and therefore AOS and protein kinases could possibly act in signal amplification loops. Although AOS has been shown to induce protein kinase activity (Kovtun *et al.*, 2000; Rentel *et al.*, 2004), it has yet to be whether AOS modulates protein kinase activity directly or through AOS sensor proteins.

1.3.5 Protein kinases involved in root hair development and hormone signalling

In addition to playing a role in plant responses to stress, protein kinases also mediate processes of normal cellular development.

Members of the Arabidopsis AGC kinases have been implicated in root hair development (Bögre *et al.*, 2003). The *incomplete root hair* (*IRE*) gene encoding a serine/threonine protein kinase is proposed to mediate tip growth in plants cells since root hairs of the *ire* mutant are 40% shorter than that of wild type Arabidopsis seedlings and *IRE* expression was strongest in elongating root hair cells (Oyama *et al.*, 2002). However the precise

signalling mechanisms remain to be elucidated. Two independent reports identified the AGC2-1 kinase or OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1) protein kinase as being required for root hair development since as with the *ire* mutant, mutants of this gene display shorter root hairs under conditions of mild stress (Anthony *et al.*, 2004; Rentel *et al.*, 2004). OXI1 is the subject under investigation in this study therefore its role in plant signalling processes identified thus far and further characterisation is discussed in Section 1.4.

The serine/threonine protein kinase PINOID (PID, another member of the AGC kinases) positively regulates polar auxin transport, which directs developmental and tropic plant responses (Benjamins *et al.*, 2001). PID regulates polar auxin transport by controlling the asymmetrical distribution of the PINFORMED protein family which are transporter-like membrane proteins functioning as efflux carriers in polar auxin transport (Benjamins *et al.*, 2001; Friml *et al.*, 2004) . The activity of PID also links calcium to auxin-regulated plant development since it was shown that two calcium-binding proteins, TOUCH3 (TCH3) and the calmodulin-related PID-BINDING PROTEIN 1 (PBP1), interact with PID in a calcium-dependent manner and all three of these genes are induced in response to auxin (Benjamins *et al.*, 2003).

The plant hormone ABA is involved in a variety of responses including stomatal closure. A role for CDPKs in ABA signalling was illustrated using a transient expression protoplast system. The promoter of the *HVA1* gene was transcriptionally fused to the GFP reporter gene and it was observed that the expression of *HVA1* in response to ABA was significantly enhanced by the expression of the Arabidopsis *CDPK1* (Sheen, 1996). Therefore CDPK activity may be required to mediate various ABA regulated processes.

1.3.6 Regulation of protein kinase activity

There are a variety of mechanisms by which protein kinases are regulated. The most common form of regulation of protein kinase activity is that of dephosphorylation by phosphatases. The removal of the phosphate group from an activated protein kinase will essentially inactivate the kinase blocking its signalling capability. For example, in response to wounding the *MP2C* gene, encoding a phosphoprotein phosphatase type 2C (PP2C), in alfalfa is transiently expressed and has been shown to negatively regulate

the wound induced MAPK pathway (Meskiene *et al.*, 1998). Further studies demonstrated that MP2C directly inactivates the MAPK SIMK but not SAMK, which are both induced by wounding (Meskiene *et al.*, 2003). Additionally, the dephosphorylation of SIMK was specific to MP2C since two other PP2Cs with similar properties to MP2C were unable to dephosphorylate and inactivate SIMK (Meskiene *et al.*, 2003). Hence MP2C specifically negatively regulates the SIMK signalling pathways in response to wounding, particularly since the expression of *MP2C* also correlates with the time of SIMK inactivation (Meskiene *et al.*, 2003). Another study demonstrating the regulation of a MAPK pathway by protein phosphatases resulted from the identification of the *mitogen-activated protein kinase phosphatase1* (*mkp1*) Arabidopsis mutant that is hypersensitive to genotoxic stress (Ulm *et al.*, 2001). MKP1 encodes a dual specific threonine/tyrosine phosphatase and it was shown to negatively regulate MAPK activity with the use of an in-gel kinase assay. Plants extracts obtained from genotoxic treated wild type, *mkp1* mutant and *MKP1* overexpressing seedlings displayed intermediate, high and very low levels of kinase activity respectively (Ulm *et al.*, 2001). Additional studies revealed that the *mkp1* mutant was more tolerant to salt stress and that MKP1 interacts with MPK6 as well as MPK3 and MPK4 although to a lesser degree with the latter two MAPK (Ulm *et al.*, 2002). Therefore MKP1 acts to suppress the activity of MAPK (e.g. MPK6) in response to abiotic stress to presumably modulate the adaptive response. This data also suggests that unlike MP2C from alfalfa, a single MKP is capable of regulating several MAPK in Arabidopsis and hence affecting the activity of multiple signalling pathways.

Other types of protein kinases are also regulated by dephosphorylation events. *In vitro* analysis has demonstrated that the plant kinase-associated phosphatase KAPP interacts with and is thought to negatively regulate the signalling of several RLKs including FLS2 (Gomez-Gomez *et al.*, 2001; Johnson and Ingram, 2005). It has also been observed that some CDPKs undergo autophosphorylation and in the case of a groundnut CDPK this activates the kinase (Chaudhuri *et al.*, 1999) whereas a CDPK in winged bean is inhibited by autophosphorylation (Saha and Singh, 1995). Dephosphorylation of the winged bean CDPK1 by a soluble phosphor-Ser-phosphatase serves to activate the kinase (Ganguly and Singh, 1999). Thus depending on the nature of the protein kinase dephosphorylation events may also positively regulate signal transduction cascades. There are numerous genes in the plant genome encoding putative phosphatases, for example there are 69 *PP2C* genes in Arabidopsis (Meskiene *et al.*, 2003), but the

function and targets for most of these are unknown which limits our understanding of the regulation of signalling pathways by dephosphorylation events.

Phospholipids are important signalling components in animals, yeast and plants mediating processes such as cell growth and division and apoptosis (Vanhaesebroeck *et al.*, 2001; Bögre *et al.*, 2003). There is evidence to suggest that CDPK activity is regulated by phospholipids. In carrot *in vitro* DcCPK1 activity is enhanced 2 to 30 times more in the presence of phospholipids and Ca^{2+} than Ca^{2+} alone (Farmer and Choi, 1999). Furthermore, phosphatidylinositol increases both substrate phosphorylation and enhances autophosphorylation of AtCPK1 (Binder *et al.*, 1994). However, it is not known whether phospholipids regulate CDPK activity *in vivo*. The AGC kinase 3-phosphoinositide-dependent protein kinase (PDK1) is a central regulator of processes affected by lipid-derived signals in both animals and plants (Belham *et al.*, 1999; Bögre *et al.*, 2003). Although evidence is lacking to show how and if lipids regulate the intrinsic activity of PDK1, it has been shown that the Arabidopsis PDK1 is the only AGC kinase to have a lipid binding domain (Bögre *et al.*, 2003) and that PDK1 binds to a range of signalling lipids (Deak *et al.*, 1999). It has been suggested that lipid specificity may direct PDK1 to a particular subcellular localisation where it can target specific downstream components to elicit defined end responses (Bögre *et al.*, 2003).

It has been previously mentioned that the accumulation of AOS can result in the activation of protein kinases (Section 1.3.4). However, it has also been demonstrated that a change in cellular redox as brought about by AOS generation can negatively regulate the activity of RLKs. For example, in Arabidopsis thioredoxin inhibits the S-locus receptor kinase and this inactivation is necessary for full pollen acceptance since transgenic plants with reduced levels of the thioredoxin proteins, TH1 and TH2, produce fewer seeds (Haffani *et al.*, 2004). Furthermore the Cf9 R protein of tomato, a LRR-containing receptor-like protein that lacks the cytoplasmic kinase domain, was shown to interact in its cytoplasmic domain with a tomato thioredoxin CITRX (Rivas *et al.*, 2004). This interaction negatively regulated the signalling responses of Cf9 such as cell death (Rivas *et al.*, 2004). Therefore it appears that RLKs can be regulated by redox sensitive proteins perhaps serving as negative feedback loops, for example if RLK signalling initiates an increase in AOS which changes the redox status of the cell and activates

redox sensitive proteins which then goes on to inhibit the RLK and block the signalling cascade.

The ubiquitin/26S (Ub/26S) protein degradation pathway is emerging as an important process in regulating signal transduction pathways (Vierstra, 2003). It has been shown that animal RLKs are regulated by degradation through the Ub/26S proteasome pathway (Dupre *et al.*, 2004) and a similar mechanism may exist in plants. It was recently demonstrated that FLS2 is subject to ligand-mediated receptor endocytosis (Robatzek *et al.*, 2006). Binding of flg22 directs FLS2 accumulation in mobile intracellular vesicles which facilitates degradation of FLS2 via lysosomal and/or proteosomal degradation pathways (Robatzek *et al.*, 2006). It is most likely that other protein kinases will also be shown to be regulated by the Ub/26S pathway as research into this field progresses.

It has been illustrated, particularly in the case of MAPK cascades, that a given protein kinase can be induced by a variety of stimuli. It has been proposed that the use of the same MAPK components in different cellular responses is regulated by the activity of scaffold proteins (Pitzschke and Hirt, 2006). Scaffold proteins bind several signalling molecules of a particular signalling pathway to create multienzyme complexes. In regulation of yeast and animal MAPK pathways scaffold proteins not only facilitate MAPK activation but also protect the bound MAPK components against activation by irrelevant stimuli (Whitmarsh and Davis, 1998). Although none have been identified in plants thus far, their isolation may shed light on the complexity of plant MAPK signal transduction pathways. How specificity is achieved in other plant kinases activated by multiple stimuli is not known.

1.3.7 Summary

In conclusion all the different types of kinases discussed in this report are involved in an array of cellular processes ranging from stomatal closure and development to pathogen responses. The different types of protein kinases are also able to activate each other either directly or indirectly to bring about defined cellular responses, for example FLS2 activation of a MAPK cascade in response to the bacterial elicitor flg22 required for resistance responses (Asai *et al.*, 2002). Additionally, different protein kinases commonly interact with other signalling molecules or components such as $[Ca^{2+}]_c$, H_2O_2 and ET etc.

further illustrating their role as important signalling components. Single protein kinases, albeit in large families, are able to be activated by a variety of different stimuli adding to the complexity of different signal transduction pathways. A major topic for current research is the issue of specificity as well as regulation for all the different groups of protein kinases. Identification of upstream activators, downstream targets and negative regulators will help reconcile how a single kinase can function in different signalling pathways leading to distinct cellular responses. Therefore not only the identification but importantly functional characterisation of different protein kinases will greatly aid the understanding of the complex web of plant signal transduction pathways.

1.4 Aim of present work and thesis structure

The OXIDATIVE SIGNAL-INDUCIBLE1 protein kinase, a member of the AGC kinase family, was previously identified as being responsive to H_2O_2 treatment and its activity was required for full activation of MPK3 and MPK6 in response to H_2O_2 and cellulase treatment which mimics a wound response (Rentel *et al.*, 2004). It was also observed that OXI1 is transcriptionally upregulated in response to a wide variety of stimuli that cause the induction of AOS (Rentel, 2002), suggesting a signalling role for OXI1 as a downstream effector of H_2O_2-mediated processes.

In Chapter 3 of this study, conditions that caused transcriptional increases in *OXI1* gene expression and the regulation of the OXI1 protein are addressed. Firstly, using a microarray approach to determine whether only AOS-generating stimuli regulate *OXI1* transcription as well as to identify genes that are co-expressed with *OXI1* to provide insights into possible signal transduction pathways OXI1 may regulate. The biological significance of *OXI1* induction in response to conditions of stress was analysed using mutational analysis. Investigating the regulation of OXI1 entailed determining its subcellular localisation and its protein expression profile under conditions of stress. OXI1 possesses putative N-myristoylation sites and thus potentially could be recruited to the membrane. The subcellular localisation of OXI1 was demonstrated using two techniques that of confocal microscopy and subcellular fractionation obtained through differential centrifugation steps. It has previously been reported that OXI1 is either a relatively unstable protein or that OXI1 protein levels are tightly controlled since detecting OXI1 protein in plant extracts proved extremely difficult (Rentel, 2002). Extensive western

analysis was undertaken in conjunction with the use of chemical inhibitors for the processes of translation and protein degradation in an attempt to reconcile the regulation of OXI1 protein. Finally, to identify potential downstream targets of OXI1 a 2-Dimensional SDS PAGE approach was employed.

OXI1 was demonstrated to be involved in basal defence mechanisms in response to virulent *H. parasitica* since the *oxi1* mutant was more susceptible than wild type in response to this pathogen (Rentel *et al.*, 2004). In Chapter 4 an in depth analysis for the role of OXI1 in resistance responses to another biotrophic pathogen *P. syringae* and to the necrotrophic pathogen *B. cinerea* was performed. This pathogenesis study was aided by the generation (described in Chapter 3) of various transgenic lines including *oxi1* knockouts in different genetic backgrounds, transcriptional and translational fusions of OXI1 to reporter genes and lines overexpressing OXI1.

CHAPTER 2

Materials and Methods

CHAPTER 2: Materials and Methods

2.1 Chemicals, antibiotics, enzymes and kits

All chemicals, antibiotics, enzymes and kits were purchased through one of the following companies:

Bioline Ltd., London, UK

Duchefa Biochemie BV, Haarlem, The Netherlands

Fermentas International Inc., Ontario, Canada

GibcoBRL Life Technologies Ltd., Paisley, UK

Merck & Co. Inc., Whitehouse Station, USA

New England Biolabs, Hitchin, UK

Promega Corporation, Madison, USA

ProLume, Woburn, MA, USA

Qiagen, Crawley, UK

Roche Molecular Biochemicals, Basel, Switzerland

Santa Cruz Biotechnology Inc., Santa Cruz, California, USA

Sigma-Aldrich Company Ltd., Ltd., Crawley, UK (Sigma, UK)

TaKaRa Shuzo Co. Ltd, Shiga, Japan

2.2 Plant Material and Growth Conditions

Arabidopsis thaliana seeds acquired from Lehle Seeds (Lehle, Texas, USA) of the Col-0 or Ws-2 ecotypes were used in most experiments. A comprehensive list of all transgenic and mutant seeds used in this study is contained in Table 2.1.

Table 2.1 List of transgenic and mutant lines used in this study

Transgenic Line	Ecotype	Resistance Phenotype	Source
oxi1 knockout	Ws-2	Kanamycin	(Rentel, 2002)
oxi1 knockout	Col-0	Kanamycin	SALK T-DNA collection (Alonso *et al.*, 2003)
35S::OXI1	Ws-2	Kanamycin	Generated in this study
35S::OXI1-YFP	Ws-2	Kanamycin	Generated in this study
OXI1::GUS	Col-0	Kanamycin	(Rentel, 2002)
oxi1 + OXI1::*OXI1* Complement	Ws-2	BASTA	(Rentel *et al.*, 2004)
OXI1::OXI1-YFP	Ws-2	Kanamycin	(Rentel *et al.*, 2004)
atrboh D	Col-0	BASTA	(Torres *et al.*, 2002)
npr1	Col-0	Kanamycin	(Cao *et al.*, 1994)

2.2.1 Sterilization of Arabidopsis seeds

The desired amount of seeds were counted and placed in a 1.5 mL microfuge tube. Seeds were incubated in 70% (v/v) ethanol for 5 min with vigorous shaking. After aspiration of the ethanol, seeds were incubated in a solution of bleach (10% (v/v) bleach and 0.02% (v/v) Triton-X) for 15 min shaking vigorously. Thereafter seeds were washed 5 times with sterile H_2O and resuspended in the appropriate volume of 0.1% (w/v) agar (approximately 200 µl per 100 seeds). Sterilized seed was subsequently stratified for 2-4 days at 4°C in the dark prior to plating on nutrient medium.

2.2.2 Growth on Nutrient Media

Stratified sterilized seed were plated on either nutrient media (5 mM KNO_3, 2 mM $MgSO_4.7H_2O$, 2 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM Fe.EDTA, 1 X Micronutrients and 0.75% (w/v) agar; (Haugh and Sommerville, 1986) or 1 X MS (Murashige and Skoog, 1962) media with macro and micro nutrients (Duchefa, Haarlem, The Netherlands) by single seeding with a sterile pasteur pipette in a laminar flow hood. Plates were then transferred to a controlled growth chamber at 21°C and grown under fluorescent light (80 - 100 µmol photon/sec/m^2) with a 16 hr light and 8 hr dark cycle.

2.2.3 Arabidopsis root culture

Transgenic Arabidopsis seedlings containing 35S::OXI1-YFP construct were grown on nutrient media for 7 days post germination. Approximately 15 – 20 seedlings were then transferred to sterile conical flasks containing 50 mL liquid 1 X MS with macro and micro nutrients supplemented with 3% (w/v) sucrose, 0.5 µg/mL 1-Naphthylacetic acid and 0.05 µg/mL Kinetin (final concentrations). Flasks were shaken at 80 – 100 rpm at room temperature in the dark for 1 week. Seedlings were then transferred to fresh media and grown for another week in the dark until an extensive root system had developed.

2.2.4 Soil grown Arabidopsis

Seeds were hydrated in 0.1% (w/v) agar and stratified at 4°C for 1 - 3 days in the dark. Seeds that had been collected from plants dipped with *Agrobacterium* were surface sterilized as described in Section 2.2.1. For germination on soil, seeds were planted either on peat plugs (Jiffy Products, International AS, Norway) or a mixture of soil composed of peat plugs and vermiculite in a 1:1 ratio. The pots were covered with clingfilm to ensure 100% humidity. Seedlings were grown in a controlled environment room at 21°C under fluorescent light (80-100 µmol photon/sec/m^2) with a 16 hr light and 8 hr dark cycle. The clingfilm was removed after 1 week.

2.3 DNA Techniques

2.3.1 DNA Extraction

The superquick DNA extraction method (Edwards *et al.*, 1991) was employed. A four week old leaf or a single whole seedling was homogenized in 500 µl of superquick extraction buffer (200 mM Tris pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% (w/v) SDS) and heated at 60°C for 10 min. An equal volume of chloroform:isoamylalcohol (24:1 (v/v)) was added and mixed well by vortexing. The sample was centrifuged at 10 000 X *g* for 10 min at 4°C. The DNA was precipitated with 0.7 X volume of cold isopropanol and 0.1 X volume of 3 M NaOAc pH 5.6. The DNA pellet was obtained by centrifugation at 10 000 X *g* for 10 min at 4°C and the pellet washed in 70% (v/v) ethanol. DNA was resuspended in 50-100 µl TE containing RNase (2 µg/mL).

2.3.2 Quantification of DNA

DNA concentrations were determined by reading the optical density of samples at a wavelength of 260 nm either using a UV spectrophotometer (Beckman Coulter, Fullerton, USA) or spotting 1 µl of undiluted DNA directly onto the Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmigton, USA). An alternative method involved comparing the fluorescence intensities of commercial DNA ladders (New England Biolabs, Hitchin, UK and GibcoBRL, UK) of known concentrations to the DNA samples electrophoresed on an agarose gel.

2.3.3 Restriction Digests

Plasmid DNA or PCR products were digested with 5 – 10 U of restriction enzyme supplied by NEB (Hitchin, UK) at 37°C for 2-4 hrs, except where the enzyme required a lower temperature for optimal digestion. In general, 1 U of enzyme per µg of DNA was used and the enzyme volume never exceeded 10% of the total reaction volume. In the case of double digests, the digest was performed in the restriction enzyme buffer in which both enzymes function at their maximum activity. Sequential digests were performed if the restriction enzymes in the double digest required different buffers for their optimal activity. In this case the enzyme with the lower salt buffer was incubated

first and after digestion the restriction buffer was adjusted to the higher salt concentration and the second restriction enzyme was added.

2.3.4 Electrophoresis of DNA

DNA samples in 1 X loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanole FF, 40% (w/v) sucrose) were loaded onto a 1% (w/v) agarose gel together with the appropriate DNA size ladder (1 kb, 100 bp or low mass ladders). The gels were electrophoresed at 100 V in 1 X TAE buffer (40 mM Tris, 1 mM EDTA, 0.11% (v/v) glacial acetic acid and 0.160 µg/mL ethidium bromide) for approximately 1 hr. DNA was visualised on a short or long wavelength (254 nm or 365 nm) UV transilluminator (UVP Inc, San Gabriel, USA).

2.3.5 Gel Extraction of DNA

DNA bands were visualised on a long wavelength (365 nm) UV transilluminator to prevent DNA damage whilst being excised from the gel using a razor blade. DNA was extracted from the gel slice using the Wizard SV Gel and PCR Clean up system (Promega Corporation, Madison, USA) as per manufacturer's instructions.

2.3.6 Generation of DNA probes for northern analysis

2.3.6.1 Amplification of DNA

Primers were designed to specific regions within the gene of interest and designed to have at least a 40% GC content. Table 2.2 provides a detailed list of all the primers used for the generation of probes in this study. PCR reactions were carried out in a 96 well Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA). Thermo stable DNA Taq polymerase from either Bioline (London, UK) or Fermentas (Ontario, Canada) was used. A final $MgCl_2$ concentration of 1.5 mM proved to be optimal in all PCR reactions. Genomic DNA was used as the template in most of the reactions, apart from *GST1* where a colony PCR was performed by inoculating a single colony of *Escherichia coli* strain DH5α containing the pBluescript SK^- plasmid harbouring the full length cDNA *GST1* (Grant *et al.*, 2000a) into the PCR reaction mix. A standard PCR programme was

employed for generation of all probes, apart from varying the annealing temperature specific to the primer set. A denaturing step at 94°C for 5 min allowed complete denaturing of the DNA and was followed by 35 cycles consisting of denaturing at 94°C for 1 min, annealing for 1 min at the appropriate temperature and finally an extension at 72°C for 1 min. After completion of this cycling, a final extension at 72°C for 10 min was performed. The PCR products were electrophoresed on a 1% (w/v) agarose gel and the desired bands were cut from the gel and purified with the Wizard SV Gel and PCR Clean up system.

Table 2.2 Primer sets used for the generation of probes

Gene	Primer Sequence (T_m °C)	T°C*	Size (kb) of product	Reference
ESC1 (At1g31580)	Forward (ECS1_for): ATGGCATCTTCTATAGTCTC (56) Reverse (ECS1_rev): TGACTTGGTGAGTTTTTTGG (56)	55	0.409	(Rentel, 2002)
PR1 (At2g14610)	Forward (PR1 F): GCTCTTGTTCTTCCCTCG (62) Reverse (PR1 R): GTGTAGTGACCACAAACTCCA (56)	51	0.307	(Denby *et al.*, 2005)
VSP1 (At5g24780)	Forward (VSP1_for): CGGCATCCGTTCCAGCCGTC (68) Reverse (VSP1_rev): CTAGAGAGGAGAGTGTCGTC (62)	60	0.331	(Menke *et al.*, 2004)
GST1 (At2g29450)	Forward: T7 Reverse T3	56	0.9	(Murray, 2001)

* Represents the annealing temperature for the primer set used in the PCR reaction

2.3.6.2 *PDF1.2* and *OXI1* probes

Both *PDF1.2* (At5g44420) and *OXI1* (At3g25250) DNA probes were generated through restriction digests. A 400 bp *PDF1.2* DNA fragment was excised from pZL1 plasmid by digestion with *SalI* and *NotI* (Denby *et al.*, 2005). A full length 1.4 kb *OXI1* DNA fragment inserted into pUC2X35S plasmid, generated in this study, was excised with *PstI* and *BamHI*.

2.3.7 Sequencing of plasmids or PCR products

DNA to be sequenced was purified using the Qiagen MinElute gel extraction kit (Qiagen, Crawley, UK) according to manufacturer's instructions. DNA was sequenced using the Big-Dye sequencing system. Primers designed for sequencing are listed in Table 2.4. Reactions consisting of 4 µl Big-Dye reaction mix, 0.5 X buffer (40 mM Tris, 1 mM $MgCl_2$, pH 9), 4 pmol desired primer, 250 ng template DNA and made up to 20 µl with H_2O were used for sequencing. The PCR cycle conditions were 96°C for 1 min followed by 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min. The DNA was precipitated with 50 µl 95% (v/v) ethanol and 2 µl 3 M NaOAc pH 5.2 at room temperature for no longer than 15 min. The sample was centrifuged at 10 000 X *g* for 20 min at room temperature. The supernatant was discarded immediately and the pellet was washed with 70% (v/v) ethanol and air dried. The sequencing was performed in an automated sequencer (Applied Biosystems automatic capillary action).

Table 2.4 Primers designed for sequencing

Primer Name	Sequence (5' → 3')
ox1-seq_1	CCCTCTAGCATAATGTCGAC
ox1-seq_2	CCGATGACTTTATCGGTAGC
ox1-seq_3	TTAAGGAAGCCATCGAGAAG
ox1-seq_4	ATTTTACGCGGCGGAGCTTG
ox1-seq_5	CGTCGGAACAGAGGAATACG
ox1-seq_6	TGGTGATAAGGGAACCGATG
ox1-seq_7	GTCTATGGACTATGGTGTAC
OX1-YFP-rev	CGCCCTCGCCGGACACGCTG
35S:OX1-YFP_1	GAAGTTCATCTGCACCACCG
35S:OX1-YFP_2	CATCCTGGGGCACAAGCTGG
35S:OX1-YFP_3	GATCACTCTCGGCATGGACG
35S:OX1-YFP_4	GCATGACGTTATTTATGAGATG
oxi1_k/o 123	GCATGCAAGCTTGGCACTGG

2.3.8 Cloning Techniques

2.3.8.1 PCR Amplification and plasmids

The *OXI1* coding region with its intron and *OXI1-YFP-cmyc* were amplified from their respective plasmids (pUC18 containing *OXI1* and pBluescript SK^- harbouring *OXI1-YFP-cmyc* (Rentel, 2002)) with Pyrobest DNA polyermase (Takara Shuzo co., Japan), a high fidelity Taq. DNA template ranging from 2 – 10 ng was used in 50 µl reaction volumes and low cycle numbers (15-25) were utilized to minimise misreading

and introduction of errors. The PCR mixture and cycle conditions were performed as per manufacturer's protocol with annealing temperatures for different primer sets provided in Table 2.5.

Table 2.5 Details of primer sets used to generate overexpressing *OXI1* constructs

Primer set	Sequence 5' to 3' (T_m °C) and Restriction Site	Annealing Temp
OXI1		
ox1_forward (forward)	GCGC ***CTGCAG*** GTCGACATTATGCTAGAGGG (60)	58°C
	PstI	
ox1_rev (reverse)	GCGC ***GGATCC*** GTACACCATAGTCCATAGAC (58)	
	BamHI	
OXI1-YFP-cmyc		
OX1-YFP_for (forward)	GCGC ***GGATCC*** GTCGACATTATGCTAGAGGG (60)	60°C
	BamHI	
OX1-YFP_rev (reverse)	GCGC ***CCCGGG*** CAAGACCGGCAACAGGATTC (62)	
	XmaI	

The PCR products were cloned into pUC2X35S a high copy number plasmid containing two *35S CaMV* promoters, using the restriction enzymes indicated in Table 2.5 followed by subcloning into the binary vector pBINPLUS through the unique restriction sites *AscI* and *PacI*. Both vectors were a gift from Malcolm Campbell, Department of Botany, University of Toronto, Canada.

Table 2.6 Vectors used for cloning of overexpressing *OXI1* constructs

Vector	Resistance	Blue/White Screening (*lacZ*)
pUC2X35S	Ampicillin	No
pBINPLUS	Kanamycin	Yes

2.3.8.2 Ligation

A 3:1 (insert:vector) molar ratio was used in ligation reactions for most experiments, alternatively if not enough insert was produced a 1:1 ratio had to be utilised. The amount of insert to be utilised in the ligation reaction was calculated according to the following equation:

$$\frac{\text{Amount of vector (ng)} \times \text{size of insert (kb)}}{\text{Size of vector (kb)}} \times \text{insert:vector molar ratio} = \text{Amount of insert (ng)}$$

The ligation mix consisted of insert and vector DNA in T4 ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 µg/µl BSA) with 200 units of T4 DNA ligase and the reaction volume did not exceed 10 µl. The ligation reaction was incubated overnight at 16°C. The T4 enzyme was inactivated by heating at 65°C for 10 min. The ligation reaction, usually 5-8 µl, was transformed into *E.coli*.

2.3.8.3 Blunt ending

Approximately 4 µg of PCR products were blunt ended using 5 Units of Klenow (NEB, Hitchin, UK) in NEB Buffer and 33 µM of each dNTP. The reaction mix was incubated at room temperature for 15 min and the reaction stopped by the addition of EDTA to a final concentration of 10 mM. The enzyme was heated inactivated at 75°C for 10 min.

2.3.8.4 Transformation of Competent Cells

2.3.8.4.1 *Escherichia coli* (*E. coli*)

Competent cells of *E. coli* strain DH5α (GibcoBRL, UK), in aliquots of 100 µl, were thawed on ice and 50 -100 ng DNA was added. Cells were left on ice for at least 30 min before being heat shocked for 2 min at 42°C and snap cooled on ice for 1 min. 0.5 -1 mL LB was added to each tube which were shaken horizontally at 37°C for 1 hr. Cells were then plated onto pre-warmed LB agar plates with appropriate antibiotics and incubated overnight at 37°C.

2.3.8.4.2 *Agrobacterium tumefaciens* (*A. tumefaciens*)

Approximately 1 µg of plasmid DNA was added immediately to -80°C frozen competent cells of *A. tumefaciens* strain C58C1 (Deblaere *et al.*, 1985). Cells were heat shocked for 5 min at 37°C. The total volume of DNA added did not exceed 25% of the total volume of cells. Cells were then placed on ice for 1 min and 1 mL of LB was added. In order to allow for better aeration this step was performed in a McCartney bottle rather than a microfuge tube. Cells were incubated at 28°C for 2-4 hrs with shaking and plated on pre-warmed LB plates with the appropriate antibiotics. The plates were then incubated at 28°C for 2-3 days.

2.3.8.5 Plasmid mini and maxi preps

High copy number plasmid DNA was isolated from small culture volumes using the Qiagen mini-prep kit (Quiagen, UK) whereas low copy number plasmid DNA was purified utilising the Qiagen maxi-prep (Quiagen, UK) from large culture volumes. Both mini- and maxi-prep kits were used as per manufacturer's instructions.

Alternatively, alkaline lysis mini-preps were used to isolate plasmid DNA. A single colony harbouring the plasmid of interest was grown in 5 mL culture volume overnight at 37°C. The bacterial cells were harvested by several centrifugations at 10 000 X *g* for 30 sec. The pelleted cells were thoroughly resuspended in 200 µl of solution 1 (50 mM glucose, 25 mM Tris pH 8 and 10 mM EDTA) and followed by the addition of freshly prepared solution 2 (0.2 M NaOH and 1% (w/v) SDS). The tube was inverted several times to mix and kept at room temperature until the solution cleared but not longer than 5 min. A volume of 300 µl of solution 3 (60% (v/v) 5 M potassium acetate and 11.5% (v/v) acetic acid) was added and the tube shaken vigorously without vortexing to mix and incubated on ice for 3-5 min. The lysed cells were centrifuged at 10 000 X *g* for 5 min at 4°C and the supernatant was transferred to a clean tube. To precipitate the DNA, an equal volume of isopropanol was added to the supernatant and incubated at room temperature for less than 5 min. The DNA was pelleted by centrifugation at 10 000 X *g* for 5 min at 4°C. The pellet was washed with 70% (v/v) ethanol and dried for 5-10 min. The DNA was resuspended in 50 µl of TE containing 20 µg/mL RNase.

2.4 Plant Transformation

2.4.1 Plant preparation

The primary bolts were removed so that only the rosette leaves remained, which encouraged secondary bolt formation. Plants could be clipped several times before transformation with *A. tumefaciens* and were fertilized regularly with Phosphrogen (Bayer CropScience Group, Hertfordshire, UK) to maintain the health of the plant. Plants were transformed with *A. tumefaciens* harbouring the appropriate construct 8 days after the plants were clipped.

2.4.2 Preparation of transformed *A. tumefaciens*

A single colony of transformed *A. tumfaciens* streaked from a fresh plate was inoculated into 5 mL LB with appropriate antibiotics and grown for 2-3 days at 28°C. The night before transformation, 5 mL of this culture was used to inoculate a 500 mL LB (with selective antibiotics) in a 2 L flask and grown for 24 hrs at 28°C. The bacterial culture was centrifuged at 3 500 X *g* for 15 min at room temperature to pellet the cells. The cells were resuspended in 0.5 – 1 X the original culture volume in a solution of 5% (w/v) sucrose with 0.05% (v/v) silwet L-77 surfactant added just prior to use.

2.4.3 Dipping of Arabidopsis

The aerial parts of plants were dipped into the resuspended *Agrobacterium* for a couple of seconds and rested on their sides in a tray lined with tissue paper. The tray was covered with clingfilm and returned to the growth room overnight. The next day the plants were placed upright and subsequently watered from below to ensure that the *Agrobacterium* were not washed off. The dipped plants were dipped again with the same transformed *Agrobacterium* to increase the efficiency of transformation.

2.4.4 Isolation of transformed homozygous lines

T_0 seed was collected from the dipped plants and surface sterilized. Seedlings were grown under standard conditions on 1 X MS media containing the appropriate antibiotic to select for transformants. The T_1 seedlings that survived were heterozygous and were transplanted onto soil and grown in the growth room to maturity. Seeds were harvested from each plant separately. Homozygous or heterozygous T_2 seedlings were grown on plates and lines showing 100% survival in the presence of antibiotics were considered to be homozygous for the transgene.

A homozygous *oxi1* knockout from the SALK T-DNA Collection (San Diego, California, USA) was confirmed by northern analysis of *OXI1* as described in Section 2.5.6 and genotyping individual seedlings for the presence of the T-DNA insert. The T-DNA was inserted in the opposite direction to the gene and the primers used for genotyping were Lba1 5'-GGTTCACGTAGTGGGCCATCG-3', Left genomic primer (LGP) 5'-GCAATTTTTGGGCCATTGGGC-3' and the Right genomic primer (RGP) 5'-GCTAATACAAGCTCCGCCGCG-3'. A standard PCR reaction was performed with the primer sets and an annealing temperature of 58°C with 1.5 mM $MgCl_2$ proved to be optimal.

2.5 RNA Techniques

2.5.1 RNA Extraction

Two protocols were used to extract RNA from either seedlings or leaves (100-300 mg). The first was using the RNeasy Plant Total RNA kit (Qiagen, UK) and performed as per manufacturer's instructions. Alternatively, total RNA was isolated using a modified version of the guanidinium thiocyanate-phenol-chloroform protocol (Chomczynski and Sacchi, 1987). Plant material was homogenized in 500 µl solution D (4 M Guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarkosyl, 0.7% (v/v) β-mercaptoethanol). 500 µl H_2O-saturated phenol pH 4, 50 µl 3 M sodium acetate pH 4 and 150 µl chloroform:isoamylalcohol (49:1(v/v)) was added to the homogenate and the mixture incubated on ice for 15 min. The samples were then centrifuged at 10 000 X *g* for 20 min at 4°C. The top phase was transferred to a clean microfuge tube and the RNA

precipated with 500 µl isopropanol by incubating on ice for 10 min followed by centrifugation at 10 000 X *g* for 20 min at 4°C. The supernatant was discarded and the pellet washed with 500 µl of 75% (v/v) cold ethanol. The pellet was air dried for approximately 10 min and resuspended in DEPC H_2O (for cDNA synthesis) or formamide (for northern analysis).

2.5.2 Quantification of RNA

The RNA concentration was determined using either a UV spectrophotometer (Beckman Coulter, Fullerton, USA) and diluting the RNA sample 1:100 or by spotting 1 µl of undiluted RNA sample directly onto the Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmigton, USA). The optical density of the RNA samples was read at a wavelength of 260 nm where an OD_{260} of 1 is equal to 40 µg RNA/mL.

2.5.3 cDNA synthesis

A maximum of 2.5 µg RNA was added to 0.5 µg oligo dT (GibcoBRL, UK) in a total volume of 12 µl, mixed by vortexing and immediately heated at 70°C for 10 min to denature the RNA. The tube was transferred to ice for at least 2 min and 8 µl reaction cocktail (1 X First strand buffer, 10 mM DTT, 0.5 mM dNTP (RNase-free) and 1 µl (200 U) Superscript II enzyme (Invitrogen, UK)) was added. The samples were incubated at 42°C for at least 50 min for cDNA synthesis followed by incubation at 72°C for 15 min to inactivate the enzyme.

2.5.4 Electrophoresis of RNA

RNA sample application buffer (2 X MOPS pH 7 (0.4 M MOPS, 0.1 M sodium acetate, 10 mM EDTA), 2.32% (v/v) formaldehyde, 70% (v/v) foramide and 0.1 mg/mL EtBr), 0.75 X the volume of RNA (10-20 µg), was added to the RNA sample, heated at 65°C for at least 2.5 min to denature the RNA and snap cooled on ice. The samples were then loaded onto a formaldehyde-agarose gel (1.3% (w/v) agarose, 1 X MOPS, 3.75% (v/v) formaldehyde) and electrophoresed at 80 – 100 V for 2-3 hrs in 1 X MOPS buffer. The ethidium bromide stained gel was viewed using a short wavelength UV transilluminator and photographed to be used as a loading control between RNA samples.

2.5.5 Transfer of RNA

RNA was transferred onto either Hybond N or Hybond N^+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) by capillary action. A glass plate was placed over a reservoir of 20 X SSC and a wick made from 3 MM Whatman paper. The gel was placed on the wick and surrounded with parafilm to ensure capillary action occurs only through the gel. The membrane (pre-wet with 2 X SSC) was placed over the gel, ensuring no air bubbles form, followed by 3 pieces of Whatman paper wet with 2 X SSC, tissues (100 sheets), a glass plate and a weight. The transfer was left to occur overnight and the next day the RNA was cross-linked onto the membrane with a UV illuminator (Amersham, UK) at 700 joules.

Prior to Northern analysis, membranes were incubated in Methylene Blue Stain (0.5 M sodium acetate pH 5.2 and 0.04% (w/v) methylene blue) with gentle agitation at room temperature. The membranes were washed with deionised H_2O to remove background staining. The methylene blue stained membranes were scanned and used to visualise whether RNA loading in the various samples were equal.

2.5.6 Northern Analysis

DNA or cDNA probes (50-100 ng) were labelled through random priming with α-[^{32}P]dCTP using the Megaprime DNA labelling kit (Amersham, UK) as per manufacturer's instructions. Radiolabelled probes were purified using the Sigmaspin post reaction clean up columns (Sigma, St Louis, USA) as per manufacturer's instructions and the entire volume of purified probe added to hybridisation buffer for northern analysis.

Membranes were pre-hybridised in hybridisation buffer containing 5 X SSC, 50% (v/v) formamide, 0.5% (v/v) SDS, 5 X Denhardt's solution (0.1% (w/v) Ficoll; 0.1% (w/v) polyvinylpyrrolidone (PVP); 0.1% (w/v) bovine serum albumin) and 100 µg/mL denatured salmon sperm DNA, at 42°C for at least 2 hrs rotating in a mini rotary hybridisation oven (ThermoHybaid, UK). The purified radio-labelled probe was denatured at 95°C for 5 min, snap cooled on ice and added to the hybridisation buffer. The membranes were incubated overnight rotating at 42°C. The following day the membranes were rinsed

with Wash solution 1 (2 X SSC and 0.1% (w/v) SDS) followed by 2 X 15 min washes at 42°C in each of wash solution 1, 2 (1 X SSC and 0.1% (w/v) SDS) and 3 (0.1 X SSC, 0.1% (w/v) SDS). The membranes were sealed in plastic bags to contain moisture and prevent drying.

For Northern analysis of *PR1* the Church and Gilbert protocol was followed (Church and Gilbert, 1984). Membranes were incubated in pre-hybridisation buffer (0.5% (w/v) skim milk, 0.5 M PB stock buffer (1 M $Na_2HPO_4.2H_2O$ and 0.34% (v/v) H_3PO_4) 1 mM EDTA and 7% (w/v) SDS) at 65°C for at least 1 hr. The membranes were incubated in the least volume of hybridisation buffer (0.5 M PB, 1 mM EDTA, 7% (w/v) SDS and the radiolabelled probe) required to cover the membrane, overnight at 60°C. The following day the membranes were washed at least twice for 15 min in Wash buffer A (5% (w/v) SDS, 40 mM PB, 1 mM EDTA) at 60°C. The membranes were sealed in plastic bags.

To obtain a signal from the northern blots, the sealed membrane was placed in an autoradiography cassette with intensifying screens and exposed onto a sheet of Biomax ML film (Kodak, USA) at -70°C for at least a week. The exposure time is dependent on the total counts per second recorded, using a Geiger counter, on the blot upon completion of the final wash. The film was developed manually under safe red light in a dark room by placing the film for 2 – 3 min in developer solution, rinsed in 3% (v/v) acetic acid for 1 min, the film was fixed in fixer solution for 2 min and finally rinsed with H_2O and allowed to dry. X-rays were scanned using a HP scanner.

2.5.7 Differential Display

To identify protein kinases involved in SA signalling, RNA was extracted from 3 week old Arabidopsis leaves treated with either 1 mM sodium salicylate (Na-SA) or 1 mM 4-hydroxybenzoic acid (4-HBA) using the RNeasy Plant Total RNA kit (Qiagen, UK) as per manufacturer's instructions. cDNA synthesis was carried out as described in Section 2.5.3 and a 1:10 dilution of cDNA was used as the template in subsequent PCR amplification with degenerate kinase specific primers. The sequence of the primers is as follows: forward primer VIB 5'-CCGTCGACGAYYTNAARCCNGANAA-3' and the reverse primer 5'-GCGAATTCYTCNGGNGCNARRTAYTC-3' where Y represents C or T, R represents A or G and N represents any nucleotide. A standard 40 cycle PCR was

performed with an annealing temperature of 55°C and an annealing time of 1 min. 10 µl of each PCR sample was electrophoresed on a 6.7% TBE polyacrylamide gel (6.7% acrylamide:bisacrylamide, 0.05% (w/v) ammonium persulphate, 0.175% (v/v) TEMED and 1 X TBE) to allow for higher resolution of bands at 180 V, Amps not limiting, for approximately 4 hours.

The gel was stained with SYBR Green I Nucleic Acid gel stain (Molecular Probes, Eugene, OR, USA) made at a 1:10 000 dilution in 1 X TBE from the 10 000 X concentrate in DMSO. The gel was left shaking in the dark in staining solution for approximately an hour at 60-80 rpm. Bands were visualised on a long wavelength UV transilluminator and the differentially expressed bands were excised, cloned and sequenced as described in Sections 2.3.7 and 2.3.8.

2.6 Protein Techniques

2.6.1 Protein Isolation for Western Analysis

Prior to protein extraction, plant material was kept in liquid N_2. For the standard protocol, approximately 250 mg plant tissue, either whole seedlings or leaves, was homogenized in 300 µl 1 X TBS protein extraction buffer (2 mM Phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamide (BAM), 10 mM ε amino caproic acid (ACA) and 1 mM EDTA). The protein extraction buffer for investigating OXI1-YFP protein expression was composed of 25 mM Tris pH 8, 15 mM EDTA, 75 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 1 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM PMSF, 2 mM BAM, 10 mM ACA and 0.1% (v/v) Tween 20. The homogenate was centrifuged at 10 000 rpm for 10 min at 4°C and the supernatant was collected. Protein concentrations were determined using the Bradford assay (Bradford, 1976), using BSA as a standard curve. Protein samples for electrophoresis were prepared by adding an equal volume of 2 X SDS sample application buffer (125 mM Tris pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.0025% (w/v) Bromophenol Blue and 2% (v/v) β-mercaptoethanol) to the supernatant.

2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A 10 or 12% (v/v) polyacrylamide gel from a 40% (w/v) acrylamide/bisacrylamide (Bio-Rad Laboratories, Inc. Hercules, USA) stock solution was prepared using the Bio-Rad mini protean cell system. The separating gel consisted of 10 or 12% (v/v) polyacrylamide, 375 mM Tris pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate (APS) and 0.05% (v/v) Temed and was over-layed with a stacking gel of 5% (v/v) acrylamide/bisacrylamide, 125 mM Tris pH 6.8, 0.1% (w/v) SDS, 0.09% (w/v) APS and 0.1% (v/v) Temed. Protein samples (15 – 30 µg of total protein) were electrophoresed at 100 V in running buffer (25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS) for approximately 2 hrs or until the dye front had run off the gel. Proteins were transferred overnight onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany) at 30 V in transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) Methanol).

2.6.3 Western Analysis

The membranes onto which the protein was transferred were stained with Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid) for 10 min at room temperature and thereafter washed with deionised water until the background was clear. Images of the stained membranes were obtained using a standard HP scanner prior to western blotting. The membranes were incubated in 10% Blotto with sodium azide (10% (w/v) skim milk powder, 0.02% (w/v) NaN_3 in 1 X TBST pH 7.5 (50 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween 20)) either for at least an hour or overnight, depending on which antibody was to be used. The protein blots were subsequently incubated in primary antisera (Table 2.7) for at least 1 hr at 37°C and washed with 10% blotto without NaN_3 three times for 10 min each. The blots were incubated in horseradish peroxidase-conjugated goat anti-rabbit (Chemicon International, Temecula, USA) secondary antibody at a 1:5000 dilution in 10% blotto for 1.5 hrs at 37°C followed by three washes at room temperature with 1 X TBST for 5 min each. Chemiluminescent detection was performed as previously described (Durrant and Fowler, 1994).

Table 2.7 Primary antisera

Antisera	Dilution	Blocking time	Incubation time	Source
anti-GST1	1:1000	1 hr	2 hrs	(Denby et al., 2005)
anti-PR1	1:125	1 hr	2 hrs	(Denby et al., 2005)
anti-GFP	1:125	overnight	1 hr	Santa Cruz Biotechnology (USA)
anti-PIP1	1:3000	1 hr	2 hrs	A gift from Helen Collett, MCB Department, UCT
anti-PIP1	1:5000	1 hr	overnight	A gift from Helen Collett, MCB Department, UCT

2.6.4 Subcellular Fractionation

Arabidopsis root culture prepared as described in section 2.1.3 was ground with a mortar and pestle in liquid N_2 and the protein was extracted by addition of 8 mL of protein extraction buffer (50 mM Tris pH 8.3, 0.3 M sucrose, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 2 mM PMSF and 1 Complete protease inhibitor cocktail tablet (Roche Diagnostics, Randburg, South Africa) per 50 mL protein extraction buffer). The homogenate was filtered through sterile cheese cloth to clarify the homogenate. The clarified homogenate was centrifuged at 10 000 X *g* for 10 min at 4°C. To obtain a soluble cytosolic fraction the supernatant (approximately 5 mL) was centrifuged at 113 000 X *g* for 30 min between 5°C and 20°C in a Beckman LS-65 Ultracentrifuge (Beckman, Palo Alto, USA) using the SW 65Ti rotor (Volotovski *et al.*, 2003). The pellet consisting of the membrane fraction was resuspended in protein extraction buffer used for OXI-YFP detailed in Section 2.6.1 and the supernatant comprised the cytosolic fraction. The protein concentrations were determined using a Bradford assay and the protein samples were used for Western analysis.

2.7 2-D Gel Analysis

2.7.1 Protein isolation for 2-D gel analysis

Plant tissue (250-300 mg) was homogenised in 500 µl protein extraction buffer (0.5 M Tris pH 7.5, 10 mM EDTA, 1% (v/v) Triton X-100, 2% (v/v) β-mercaptoethanol, 1 mM NaF, 0.5 mM Na_3VO_4 and 0.5 mM PMSF) and centrifuged at 10 000 X *g* for 5 min at 4°C. The supernatant was transferred to a fresh 2 mL tube and protein concentrations were determined using the Bradford assay. 250 µg of protein from each sample was used for subsequent steps. An equal volume of ice cold phenol pH 8 was added to the supernatant and the mixture was vortexed for 10 sec followed by centrifugation at 10 000 X *g* for 1 min. This allowed the phases to separate and the proteins are seen at the milky white phase interface. The phase interface should not be disturbed. 80% of the upper aqueous phase was discarded, that volume replaced by the addition of extraction buffer and the mixture vortexed for 10 sec and centrifuged at 10 000 X *g* for 1 min at 4°C. The upper aqueous phase (80%) was again removed and discarded. To precipitate the proteins, 5 X volume (1.5 mL) of 0.1 M ammonium acetate in methanol was added and incubated overnight at -20°C. The next day the samples were centrifuged at 10 000 X *g* for 5 min at 4°C and the supernatant was discarded. The pellet was washed with 0.1 M ammonium acetate in methanol followed by a wash with 80% (v/v) acetone. The pellet was air dried for at least 30 min in the fume hood and resuspended in 155 µl IPG sample buffer (6 M urea, 2 M thiourea, 2% (w/v) CHAPS. 2% (v/v) IPG buffer, 0.3% (w/v) DTT and trace Bromophenol Blue) and kept at room temperature before being used to hydrate the ReadyStrip™ IPG strips pH 4-7 (Bio-Rad Laboratories, Inc. Hercules, USA).

2.7.2 Rehydration of IPG strips

A 7 cm IPG narrow range strip pH 4-7 was inserted gel side up into a ZOOM® IPGRunner™ Cassette (Invitrogen Life Technologies, Paisley, UK). The protein sample was carefully loaded into the cassette and onto the gel ensuring that no air bubbles formed, as per manufacturer's instructions. The cassette was incubated at room temperature on a flat surface overnight.

2.7.3 Isoelectric focusing

The cassette was assembled into the ZOOM® IPGRunner™ Mini-Cell as per manufacturer's instructions. Deionized water was used as the running buffer. The isoelectric focusing was performed using a step voltage protocol of 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 1 hr 45 min. The IPG strips were immediately placed into 15 mL of equilibration buffer 1 (6 M urea, 0.375 M Tris pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol and 2% (w/v) DTT) and left gently shaking for 10 min at room temperature. The strips were subsequently incubated in 15 mL of equilibration buffer 2 (6 M urea, 0.375 M Tris pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) Iodoacetamide and trace bromophenol blue) gently shaking at room temperature for 10 min and immediately loaded onto the gel for the SDS-PAGE.

2.7.4 2-D SDS-PAGE

A 12% polyacrylamide gel was set up as detailed in section 2.7.2 in a 20 X 10 cm gel system (PeQlab biotechnology GmbH, Germany), during the isoelectric focusing. Upon equilibration of the IPG strips, 0.5% (w/v) agarose in 1 X running buffer (25 mM Tris pH 8.3, 192 mM glycine and 0.1% (w/v) SDS) was pipetted onto the stacking gel and the equilibrated strips were loaded in the agarose and the agarose was allowed to set. The second dimension was electrophoresed slowly at 80 V for 1 hr and then increased to 120 V for 2-4 hrs until the dye front ran off the bottom of the gel. Each mini gel was fixed by incubating in 100 mL fix solution (50% (v/v) Methanol and 10% (v/v) acetic acid) by gentle agitation for 30 min followed by an overnight incubation in fresh fix solution. The next day, the gels were washed three times with 100 mL sterile distilled H_2O and either stained immediately or stored in H_2O at 4°C.

2.7.5 Staining for proteins

In order to detect phosphorylated proteins, the Pro-Q® Diamond Phosphoprotein Gel Stain (Invitrogen Life Technologies, Paisley, UK) was used. Each mini gel was incubated in 60 mL phosphoprotein stain with gentle agitation for 60-90 min in the dark at room temperature. To reduce the background staining, the gels were incubated three times in 80-100 mL per mini gel of destain solution (20% (v/v) acetonitrile and 50 mM sodium

acetate pH 4) for 30 min each time with gentle shaking at room temperature in the dark. Still protected from light, the gels were washed twice with ultrapure H_2O gently shaking at room temperature for 5 min each.

To determine the amount of total protein, gels were stained with colloidal coomassie (1% (v/v) Coomassie G250 stock, 3% (v/v) orthophosphoric acid and 6% (w/v) ammonium sulphate) for 24-48 hrs at room temperature. The gels were destained with 25% (v/v) methanol for no longer than 5 min and rinsed with sterile H_2O and stored at 4°C in H_2O.

The Coomassie G250 stock was made by dissolving 30 g coomassie (Gerva Blue G) in 750 mL 7.5% (v/v) acetic acid at 70°C and ammonium sulphate was added slowly while stirring until the solution turned clear. The solution was allowed to cool to room temperature and the supernatant was discarded while the precipitate was dissolved in 300 mL 50% (v/v) ethanol and 10% (v/v) acetic acid and stored at room temperature.

2.7.6 Imaging and Analysis of 2-D Gels

The stained gels were scanned with a PharosFX Molecular Imager System (Bio-Rad). The images obtained were analysed using the Bio-Rad PDQuest 2-D Analysis software. Individual gels were normalised against the total density of spots on the gel and the intensity of a phosphorylated protein spot was expressed as the density of that spot obtained with the Phosphoprotein stain relative to the intensity of that spot obtained with the Colloidal Coomassie stain.

2.8 Abiotic Stress Treatments

2.8.1 Cellulase, osmotic shock and high iron concentrations

Seedlings (10 – 14 days old) were transferred from nutrient media agar plates into 2 mL of sterile H_2O in a 6 well microtitre plate and left overnight in the growth room to recover. The next day, seedlings were treated with 2 mL of cellulase (Duchfe, Haarlem, The Netherlands), NaCl, mannitol or Fe.EDTA at double the desired final concentration for the appropriate amount of time. Treated seedlings were harvested by flash freezing in liquid nitrogen and stored at -70°C.

For protein analysis studies using the inhibitors cyclohexamide (CHX) or MG132, seedlings were first incubated in a final concentration of 50 µM CHX or 100 µM MG132 for 30 min following overnight recovery. Seedlings were then treated with 0.1% (w/v) cellulase (final concentration) and harvested at the indicated times.

2.8.2 Heat

The heat tolerance assays described in the following sections were adapted from Hong and Vierling (2000).

2.8.2.1 Hypocotyl elongation phenotype

Seeds were plated on 1 X MS plates and exposed to light for 4 hrs in a growth chamber to allow germination and subsequently covered in foil. Seedlings were grown in the dark for 2.5 days at 22°C. Seedlings were incubated in a hybridisation oven for 2 hrs at either 38°C, 38°C for 2 hrs followed by a second incubation at 45°C for 2 hrs or 45°C only. The seedlings were returned to the growth chamber and hypocotyls were measured 2.5 days later. The control seedlings were left in the growth chamber, in the dark, for the entire duration of the experiment.

2.8.2.2 Survival of young seedlings

3 day old seedlings on 1 X MS agar plates grown under standard conditions were incubated in a hybridisation oven at 47°C for 30, 45 and 120 min. The seedlings were returned to the growth room after treatment and the survival of seedlings were analysed 7 days post treatment. Control seedlings were kept at 22°C.

2.8.2.3 Heat acclimation

10 day old seedlings were incubated in a hybridisation oven at 38°C for 90 min, 38°C for 90 min followed by 22°C for 2 hrs and thereafter 45°C for 2 hrs or 45°C only for 15, 30, 45, 60 and 120 min. The seedlings were returned to the growth room and 5 days later analysed for survival.

2.9 Biotic Stress Treatments

2.9.1 *Hyaloperonospora parasitica*

2.9.1.1 *H. parasitca* isolates and growth conditions

Virulent *H. parasitica Emco5* and *Emoy2* as well as the avirulent isolate *Maks9* were a gift from Anne Rehmany, Warwick, HRI, UK. All isolates were maintained on soil grown *eds1* mutant seedlings and grown in a closed transparent container to ensure high humidity under an 8 hr light 16 hr dark cycle at 16°C.

2.9.1.2 Plant inoculation and disease assay

Approximately 250-300 mg *Arabidopsis* shoots infected with sporulating *P. parasitica* ranging from 7-10 days old were vortexed in 5 mL sterile distilled H_2O to release the spores. The spore concentration was determined using a haemocytometer and the spore suspension was adjusted to 5-6 X 10^4 spores/mL in sterile distilled H_2O. Soil grown 7 day old *Arabidopsis* seedlings, grown under standard growth conditions, were sprayed evenly with the spore suspension using an airbrush power back (Humbrol Ltd., Hull, UK). Infected seedlings were placed back in a transparent closed container and transferred to a growth chamber at 16°C with an 8 hr light and16 hr dark cycle.

Approximately 7 days post infection, infected tissue was weighed and spores released as described above by vortexing in H_2O. The spore concentration was calculated using a haemocytometer and expressed as spores/mL per mg tissue.

2.9.2 *Pseudomonas syringae*

2.9.2.1 *P. syringae* isolates and growth conditions

The virulent *Pseudomonas syringae DC 3000 pv tomato (Pst)* and both avirulent *Pst avrB* (a gift from Gail Preston, Department of Plant Sciences, Oxford University, UK) *and Pst avrRpt2* (a gift from Barbara Kunkel, Department of Biology, Washington University, USA) strains were grown on King's broth (King *et al.*, 1954) media (KB)

supplemented with the appropriate antibiotics at 28°C for 1-2 days. To select for *Pst*, KB media contained 50 µg/mL Rifampicin and both avirulent strains were selected on media supplemented with 50 µg/mL each of Rifampicin and Kanamycin.

2.9.2.2 Plant inoculation and disease assay

A single colony was inoculated into 5 mL liquid KB with appropriate antibiotics and incubated shaking overnight at 28°C. The cells were pelleted by centrifugation at 10 000 X *g* for 30 sec and washed once with 10 mM $MgCl_2$ before final resuspension in 10 mM $MgCl_2$ to obtain an OD_{600nm}=0.1 which is approximately 5 X 10^7 colony forming units (cfu)/mL (Katagiri *et al.*, 2002). Three to four week old plants were pressure infiltrated with an inoculum ranging from 5 X 10^5-10^6 cfu/mL using a 1 mL syringe on the abaxial surface of leaves. Either the entire surface of the leaf or a section approximately the size of the head of the syringe was infiltrated depending on the nature of the experiment. Successful infiltration was indicated by water soaking of the leaves. Infected plants were then covered with clingfilm and leaves were harvested for subsequent analysis at given time points. Control plants were infiltrated with 10 mM $MgCl_2$.

To determine the severity of disease, bacterial growth within the plant was assessed over a period of three days. For each time point, all leaves from three plants per *Arabidopsis* line per strain of *Pst* were infected and a leaf disc of 0.5 cm^2 in diameter was harvested from leaves on each plant and pooled to represent one sample. Three samples were therefore obtained for each *Arabidopsis* line per strain of *Pst* and the leaf discs were homogenized in 10 mM $MgCl_2$ and a serial dilution performed. 10 µl of each dilution series was spotted onto KB media supplemented with the selective antibiotics and the plates were incubated for 2 days at 28°C. The number of bacterial colonies that developed were counted and expressed as cfu/unit area over time (where 1 unit area represents 1 leaf disc of 0.5 cm^2 in diameter.

2.9.2.3 Systemic Acquired Resistance

In order to induce SAR, the entire abaxial surface of leaves from 3-4 week old plants was pressure infiltrated with 1 mM SA and the plants were covered with clingfilm. The same leaves were challenged 2 days later with a secondary infection of *Pst* at an

inoculation density of 5 X 10^5 cfu/mL. Control plants were infiltrated with 0.5% (v/v) ethanol for the primary challenge and then infected with *Pst*. Bacterial growth within the plant was assessed 2 days after the secondary infection as described in Section 2.9.2.2.

2.9.2.4 Root pathogenicity assay

Single *Arabidopsis* seeds were germinated on individual peat plugs and grown under standard growth conditions for 3-4 weeks. Each peat plug was flooded with 10 mL virulent *P. syringae* at an OD_{600} = 0.2 – 0.4 in KB media which is an inoculum of approximately 1-5 X 10^8 cfu/g soil (Bais *et al.*, 2004). Infected plants were then covered with clingfilm to ensure high humidity and transferred to a 30°C room with a 16hr light and 8 hr dark cycle. Plants were assessed every day for seven consecutive days for the appearance of disease symptoms. Ten plants per line were used for each experiment. Control plants were infected with 10 mL KB containing 50 µg/mL Rifampicin and transferred to 30°C room. Photographs were taken with a Sony cybershot 5.0 megapixel camera.

2.9.3 *Botrytis cinerea*

2.9.3.1 *B. cinerea isolates* and maintenance

The *Botrytis cinerea* isolates GLUK-1 (Kliebenstein *et al.*, 2005), grape (Denby *et al.*, 2004) and *Brassica oleracea* (Ferrari *et al.*, 2003) were maintained on sugar free apricot halves at room temperature. Every 2- 3 weeks isolates were subcultured onto fresh apricots.

2.9.3.2 Plant inoculations and disease assay

Spores aged between 10-14 days were harvested by adding 3 mL sterile H_2O onto infected apricots and gently rubbing spores with a sterile glass rod until the H_2O is slightly cloudy. The spore concentration was determined using a haemocytometer and adjusted to a suspension of 5 X 10^4 spores per mL in 50% (v/v) grape juice. Leaves of 3-4 week old plants were excised and placed onto 0.8% (w/v) agar in a large petri dish. The upper surface of each leaf was drop inoculated with 4 µl of spore suspension and

the petri dish was covered and infected leaves returned to the growth room. Control plants were infected with 4 µl of 50% (v/v) grape juice. 10 leaves from 10 different plants per *Arabidopsis* line were used for each isolate. Photographs of the developing lesion were taken 2-5 days post infection and the lesion size was measured using ImageJ 1.34n software. For the GUS and DAB assays, leaves were treated as above and harvested at day 1-3 post infection for staining (see below).

2.10 Transient protein expression in tobacco

A single colony of *A. tumefaciens* harbouring the construct of interest was inoculated into a 2 mL LB culture with the appropriate antibiotics and incubated at 28°C shaking at 200 X *g* overnight. The following day the entire culture was harvested by centrifugation at 10 000 X *g* for 1 min and the pellet was resuspended in 1 mL 1 X infiltration buffer pH 5.6 (50 mM MES, 25 mM glucose, 20 mM $Na_3PO_4.12H_2O$). The cells were pelleted by centrifugation and resuspended in 1 X infiltration buffer with 100 µM acetosyringone. This step was repeated. A 1:5 dilution of cells was used to measure the optical density at 600 nm. 1 mL of cells in 1 X infiltration buffer with 100 µM acetosyringone was prepared at the OD_{600nm} values of 0.01, 0.03 and 0.1 from the 1:5 stock dilution. The resultant dilutions of cells were pressure infiltrated into the lower leaf epidermis of young tobacco plants (before flowering) using a 1 mL disposable syringe. The leaf area inoculated was marked with a felt tip pen. The tobacco plant was incubated under constant light at 20°C for 2 days to allow for the expression of the protein.

2.11 Confocal Microscopy

Confocal images of the 35S::OXI1-YFP transgenic lines and transient expression in tobacco were performed with a Zeiss LSM 510 Confocal Microscope, using either a Plan Neofluor 10X/0.3 or a 25X/0.8 Imm corr DIC lens (Zeiss, Welwyn, Garden City, UK). Arabidopsis leaves, whole seedlings or 0.75 cm^2 tobacco leaf sections cut from tobacco leaves, transiently expressing the 35S::OXI1-YFP construct, with a sharp razor blade were mounted on a glass slide in water and imaged under the microscope.

2.12 *In vivo* Histochemical GUS staining

GUS activity was determined by incubating leaves in a GUS staining solution (100 mM $NaPO_4$ pH 7.0, 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.1% (v/v) Triton X-100 and 0.5 mg/mL 5-Bromo-6-Chloro-3-Indolyl-β-D-Glucuronide (X-gluc)) in the dark shaking gently at 37°C overnight or until blue staining could be visualised. The leaves were de-stained with 80% (v/v) ethanol. Pictures were obtained by scanning the leaves with a Canonscan 8400F Scanner (Canon, Lake Success, New York, USA).

2.13 *In vivo* staining for H_2O_2

To detect the presence of H_2O_2, leaves were incubated in a solution of 1 mg/mL 3,3'-diaminobenzidine (DAB) gently shaking at room temperature for approximately 2-4 hrs until a reddish-brown precipitate could be seen. The leaves were de-stained with 80% (v/v) ethanol and pictures were obtained by scanning the leaves with a Canonscan 8400F Scanner (Canon, Lake Success, New York, USA).

2.14 *In vivo* reconstitution of aequorin and $[Ca^{2+}]_c$ measurements

Reconstitution of aequorin was performed essentially as described in Knight *et al.* (1991). Approximately 5-10 seven day old transgenic seedlings expressing apoaequorin were incubated in microtitre plates containing a 2 mL solution of the luminophore coelentrazine (ProLume, Woburn, MA, USA) at a final concentration of 5 µM overnight in the dark at 21°C. Following reconstitution, seedlings were placed individually in a plastic cuvette (Sarstedt Ltd., Leicester, UK) in 0.5 ml H_2O for approximately 10 min and transferred to the light tight luminometer chamber (A. J. Neuroinstruments, Abingdon, Oxon, UK) where SA, 4-HBA or Na-SA was delivered by injection with a syringe through the injection port.

Arabidopsis cell cultures expressing apoaequorin were first washed with a solution of 3% (w/v) sucrose since the MS media in which cells were cultured contained calcium. Approximately 50 mL of cells were centrifuged at 1000 rpm for 5 min at room temperature in a Beckman CS-6KR centrifuge (Beckman, Palo Alto, USA). The supernatant was discarded and the cells were resuspended in 50 mL 3% (w/v) sucrose.

This wash step was repeated 3 times and after the final wash, the required amount of cells was incubated in 3% (w/v) sucrose containing coelentrazine at a final concentration of 5 µM. These cells were left shaking in the dark overnight at 21°C at approximately 100 rpm. 0.5 mL of 0.8% (w/v) agar was added to the plastic cuvettes and allowed to set. 0.5 mL of cultured cells were added to these cuvettes and transferred to the luminometer chamber where cells were treated with SA or Na-SA delivered by injection. For the aequorin consumption experiments, cell cultures were treated in the plastic cuvettes with the solutions indicated for the amount of time stated and then transferred to the luminometer for discharge of the remaining aequorin.

For both the seedlings and cells cultures, bioluminescence counts were recorded over intervals of 1 sec, 5 seconds after the addition of stimulus or solution, with a digital chemiluminometer with discriminator (Thorn EMI electron tubes) fitted with a cooling unit for the photomultiplier tube (Knight *et al.*, 1991; Knight *et al.*, 1996). At the end of each experiment the remaining aequorin was discharged by addition of 1 mL 2 M $CaCl_2$ in 20% (v/v) ethanol. Calibration of $[Ca^{2+}]_c$ was performed as described by Knight *et al.* (1996) using the calibration equation pCa=0.332588(-logk) + 5.5593, where k is a rate constant equal to luminescence counts per second divided by the total remaining counts.

CHAPTER 3

Investigating the regulation of *Oxidative Signal-Inducible1* Protein Kinase

CHAPTER 3: Investigating the regulation of *Oxidative Signal-Inducible1* protein kinase

3.1 Rationale

The Arabidopsis *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*) gene encodes a serine/threonine protein kinase that is induced by a variety of AOS generating stimuli (Rentel, 2002; Rentel *et al.*, 2004). As discussed earlier, plants make use of AOS accumulation as an important component of different signalling events to transduce the appropriate end responses during cellular development and conditions of stress. The *oxi1* null mutant displayed shorter root hairs than wild type under conditions of mild stress and showed enhanced susceptibility when challenged with virulent *H. parasitica* (Rentel *et al.*, 2004). A role for OXI1 is therefore implicated in these two diverse AOS-mediated processes namely root hair development and basal resistance to a virulent fungal pathogen. If OXI1 is involved in these and a number of other environmental conditions that lead to AOS production then it is important to address how OXI1 may be able to discriminate between the different AOS signals to activate specific end responses. In an attempt to provide insight into the function of OXI1 protein kinase various transgenic lines of *OXI1* were generated, including *oxi1* null mutants in different genetic backgrounds, overexpression of *OXI1* and an OXI1-YFP protein fusion to address localisation of OXI1.

A similar approach helped in determining the function of the Arabidopsis mitogen-activated protein kinase kinase 2 (MKK2). MKK2 is activated by both salt and cold stress and directly phosphorylates MPK4 and MPK6 in both yeast and Arabidopsis protoplasts (Tiege *et al.*, 2004). This biochemical data was supported not only by the isolation of an *mkk2* null mutant but also through the generation of transgenic Arabidopsis overexpressing *MKK2*. In comparison to wild type Arabidopsis the *mkk2* mutant was defective in MPK4 and MPK6 activation and hypersensitive to both salt and cold stress, whereas transgenic plants overexpressing *MKK2* displayed constitutive activation of MPK4 and MPK6 with heightened tolerance to salt and cold stress (Tiege *et al.*, 2004). Therefore mutational and genetic analysis demonstrated *in vivo* a function for a MKK2 mediated pathway in salt and cold stress signalling in Arabidopsis. Similarly, a single member of the Hsp100 family *Heat Shock Protein 101* (*HSP101*) was shown to be

critical in the process of both basal and acquired thermotolerance to heat stress (Queitsch *et al.*, 2000). Modulation of expression of the *HSP101* gene through antisense regulation or overexpression resulted in reduced and enhanced tolerance to heat stress respectively (Queitsch *et al.*, 2000). Similarly, *oxi1* null mutants and transgenic lines overexpressing *OXI1* are useful in determining whether transcriptional up regulation of the *OXI1* gene by a given stimulus is of biological significance in response to that stimulus.

The expression pattern and localisation of genes using reporter gene fusion constructs has been extensively used to gain greater insight into the regulation of genes. For example, fusion of the promoter from the gene of interest to the reporter gene β-glucuronidase (GUS) allows the identification of spatial transcriptional changes of the gene of interest *in situ* therefore proving advantageous over northern analysis. As previously mentioned, the *IRE* gene encodes a serine/threonine protein kinase that modulates root hair growth in Arabidopsis (Oyama *et al.*, 2002). The *IRE* promoter-*GUS* gene fusion demonstrated that *IRE* expression occurred in the elongating root hair cells as well as in pollen grains, which develop into pollen tubes by tip growth, indicative that *IRE* has a common role in tip growth of plant cells (Oyama *et al.*, 2002). Similarly, a 1.2 kb promoter region of the Arabidopsis WRKY transcription factor 6 (*AtWRKY6*) driving expression of the GUS gene reveals a complex developmental, organ specific and spatial expression pattern for the *AtWRKY6* gene (Robatzek and Somssich, 2001). Furthermore, 5' deletions of the *AtWRKY6* promoter allowed identification of various cis-acting elements or regions of the promoter that were responsible for a specific expression pattern of *AtWRKY6*. For example a 218 bp promoter was sufficient for GUS activity in root tissue and senescing leaves whereas an additional 127 bp fragment was required for the pathogen inducible GUS activity of *AtWRKY6* (Robatzek and Somssich, 2001).

The translational fusion of a gene of interest to a reporter gene such as green or yellow fluorescent protein (G/YFP) provides insight into the subcellular localisation of the fusion protein. Such approaches are useful for determining where in the cell a given protein resides providing clues to its function. For example, whether a putative membrane protein does reside in the membrane or if a transcription factor resides in or is able to translocate to the nucleus upon stimulus. The duplicated reporter gene GFP (2XGFP)

displays a cytosolic localisation, however when fused to the C-terminal of the transcription factor AtWRKY6, the fusion protein AtWRKY6::2XGFP exhibits a constitutive nuclear localisation pattern indicating that AtWRKY6 resides and functions in the cell nucleus (Robatzek and Somssich, 2001).

In addition to mutational analysis of individual genes the ever growing database of microarray experiments is allowing researchers to analyse the expression of a particular gene under a variety of experimental conditions gaining insight into the processes the gene of interest may regulate. Furthermore, a range of freely available tools such as the Arabidopsis co-expression tool (ACT) allows genes that display similar expression profiles to a gene of interest across a large number of experiments as chosen by the researcher to be identified (Jen *et al.*, 2006). These computational analyses are based on transcriptional changes within the genome and in most cases an increase in transcription often reflects involvement in a given process. For example, the zinc finger *RESPONSIVE TO HIGH LIGHT* (*RHL41*) gene is rapidly induced in response to high light and overexpression of *RHL41* conferred transgenic Arabidopsis with increased tolerance to high light intensities (Iida *et al.*, 2000). However, transcriptional increases may not always reflect a *bona fide* involvement of a gene in a particular cellular process or biological response. Additional evidence such as biochemical and/or mutational analysis would be required to support gene expression data, such as approaches used to illustrate the involvement of *MKK2* in tolerance to salt and cold stress.

3.2 Isolation of an *oxi1* knockout line from the SALK T-DNA insertion facility

Natural variation between different Arabidopsis ecotypes in susceptibility to pathogen attack, light and abiotic stress has been reported and is attributed to genetic diversity amongst the ecotypes (Breyne *et al.*, 1999; Chevalier *et al.*, 2004). Despite a 98% sequence similarity of OXI1 protein between Ws-2 and Col-0 ecotypes, subtle differences exist in the absolute level of induction of the *OXI1* transcript upon cellulase treatment between different Arabidopsis ecotypes (Rentel, 2002). The isolation of *oxi1* null mutants in different genetic backgrounds may reveal additional functions for *OXI1* if, for example, due to natural variation OXI1 protein kinase is involved in a particular signal transduction pathway in a certain ecotype but not in another. Most Arabidopsis mutants

that have been isolated are in the Col-0 genetic background therefore an *oxi1* mutant in this ecotype will facilitate genetic analysis with potential interacting partners. The involvement of OXI1 in particular AOS mediated processes will be further strengthened if two *oxi1* null mutants obtained from independent knockout populations share the same mutant phenotype.

The SALK insertion line *SALK_135617.28.90.X* identified through a blast search of the SALK T-DNA insertion collection (Col-0 background) contains a single insertion 243 bp upstream of the predicted ATG start codon of *OXI1* (Figure 3.1). Segregating T_3 seed was obtained from the Arabidopsis Biological Resource Centre. Ten individual T_3 plants were tested for the presence of the *OXI1* transcript. One of these lines, line 5, failed to induce *OXI1* gene expression upon cellulase treatment and was considered to be an *oxi1* null mutant (Figure 3.2). Homozygosity for the T-DNA insertion was confirmed through genotyping of twelve T_4 progeny of the *oxi1* null mutant (Figure 3.3). A PCR product of 600 bp using a genomic primer specific for *OXI1* and a primer specific to the left border of the T-DNA indicates the presence of the T-DNA. The absence of a 1 kb PCR product using genomic primers specific for *OXI1* designed across the T-DNA insertion confirmed disruption of the *OXI1* gene and homozygosity of the *oxi1* null mutant.

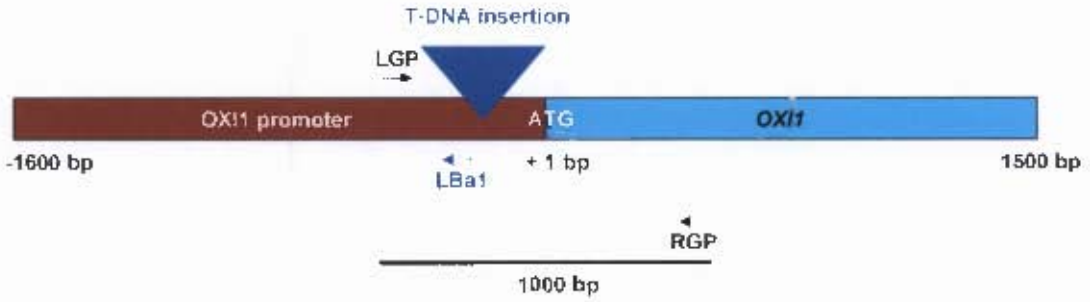


Figure 3.1 SALK _135617.28.90.X insertion line producing an *oxi1* null mutant in Col

The SALK _135617.28.90.X insertion line contains a T-DNA insert (indicated by the blue triangle) 243 bp upstream of the predicted ATG start codon of *OXI1*. The left and right genomic primers (LGP and RGP respectively) produce a 1 kb PCR product in the absence of the T-DNA insert. The left border T-DNA primer LBa1 together with the LGP results in a 600 bp PCR product when the T-DNA is present.

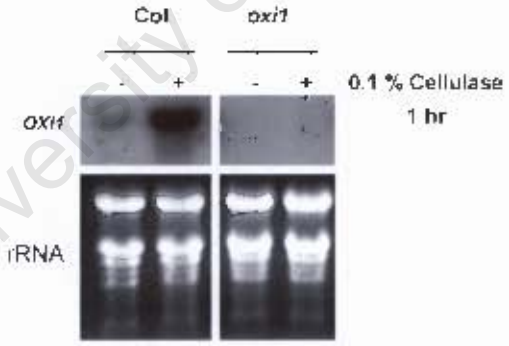
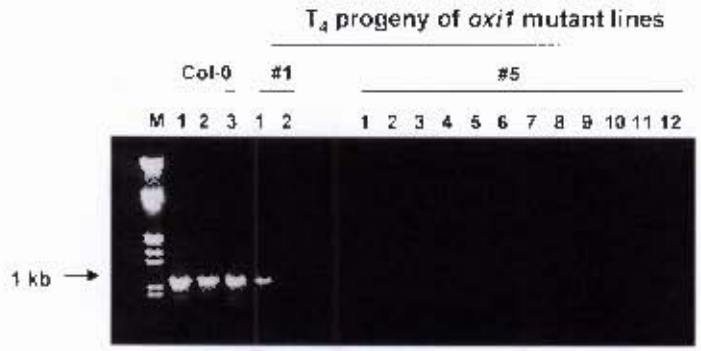


Figure 3.2 SALK_135617 insertion line 5 does not induce *OXI1* transcript

Ten day old seedlings of Col-0 and *oxi1* knockout isolated from SALK_135617 insertion line 5 incubated in H_2O (-) or treated with 0.1% cellulase (+) for 1 hour. The *oxi1* mutant does not induce *OXI1* transcript or show basal levels and is thus a null mutant. The ethidium bromide stained rRNA was used as a loading control.

A



B

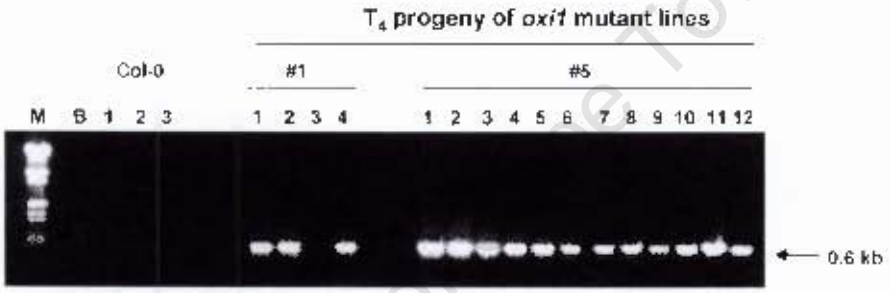


Figure 3.3 Genotyping of T_4 progeny of the *oxi1* null mutant

PCR analysis performed on individual T_4 progeny from T_3 lines either heterozygous (#1) or homozygous (#5) for the T-DNA insertion into the *OXI1* gene. A 1 kb PCR product using genomic primers specific for *OXI1* indicates a functional copy of the OXI1 gene present in the wild type controls (Col-0) and heterozygous line 1-1 (A). All progeny of line 5 lack the wild type 1 kb product but harbour the T-DNA insertion (B) as indicated by the presence of the 0.6 kb PCR product using T-DNA specific primer and the left genomic primer of *OXI1*. λ DNA digested with *HindIII* is used as a marker (M) and B in B represents a blank PCR reaction containing no DNA.

3.3 Isolation of transgenic Arabidopsis homozygous for *OXI1::GUS* reporter gene and *OXI1* complementation constructs

The *OXI1::GUS* reporter gene construct consists of a 1.61 kb genomic DNA fragment upstream of the *OXI1* start codon and first two nucleotides of its coding region fused to the GUS reporter gene (Rentel *et al.*, 2004). The *OXI1::GUS* reporter gene was shown to respond in the same manner as the endogenous *OXI1* transcript after wounding and cellulase treatment (Rentel, 2002). Transgenic plants heterozygous for the OXI1::GUS construct were grown on MS plates containing the antibiotic kanamycin. Kanamycin resistant individuals were self fertilised and their progeny screened for 100% resistance to kanamycin which suggests homozygosity. The presence of the *OXI1::GUS* transgene was confirmed by detecting wound-induced GUS activity. A line whose progeny all showed complete resistance to kanamycin and GUS activity was considered to be homozygous for the *OXI1::GUS* construct (data not shown).

In response to stimuli that cause the generation of AOS the *oxi1* null mutant, isolated from the Wisconsin knockout facility in the Ws-2 background, induces a larger *OXI1* transcript in comparison to the wild type *OXI1* gene (Figure 3.4 and Rental (2002)). To sequence this *oxi1* mutant transcript a 350 bp PCR product was amplified from cDNA prepared from cellulase treated *oxi1* mutant seedlings, using an OXI1 gene specific primer (oxi1_forward) and a primer within the right border of the T-DNA (oxi1k/o_123). Sequencing data revealed that the T-DNA is inserted 67 bp downstream of the predicted start codon and encodes a non functional protein comprised of 23 amino acids (Figure 3.5). The *oxi1* mutant (Ws-2) was complemented by transformation with a 4.707 kb DNA fragment consisting of the wild type *OXI1*, 2.148 kb sequence upstream of the *OXI1* transcription start site and 1.173 kb of the 3' untranslated region (Rentel *et al.*, 2004). T_1 progeny resistant to the herbicide BASTA and hence heterozygous for the transgene were treated with either 0.1% cellulase or 250 µM sodium salicylate and northern analysis revealed complementation in at least 4 independent lines due to the presence of the wild type *OXI1* transcript (Figure 3.4). T_2 individuals were self fertilized and the progeny tested for 100% resistance to BASTA to obtain a homozygous line.

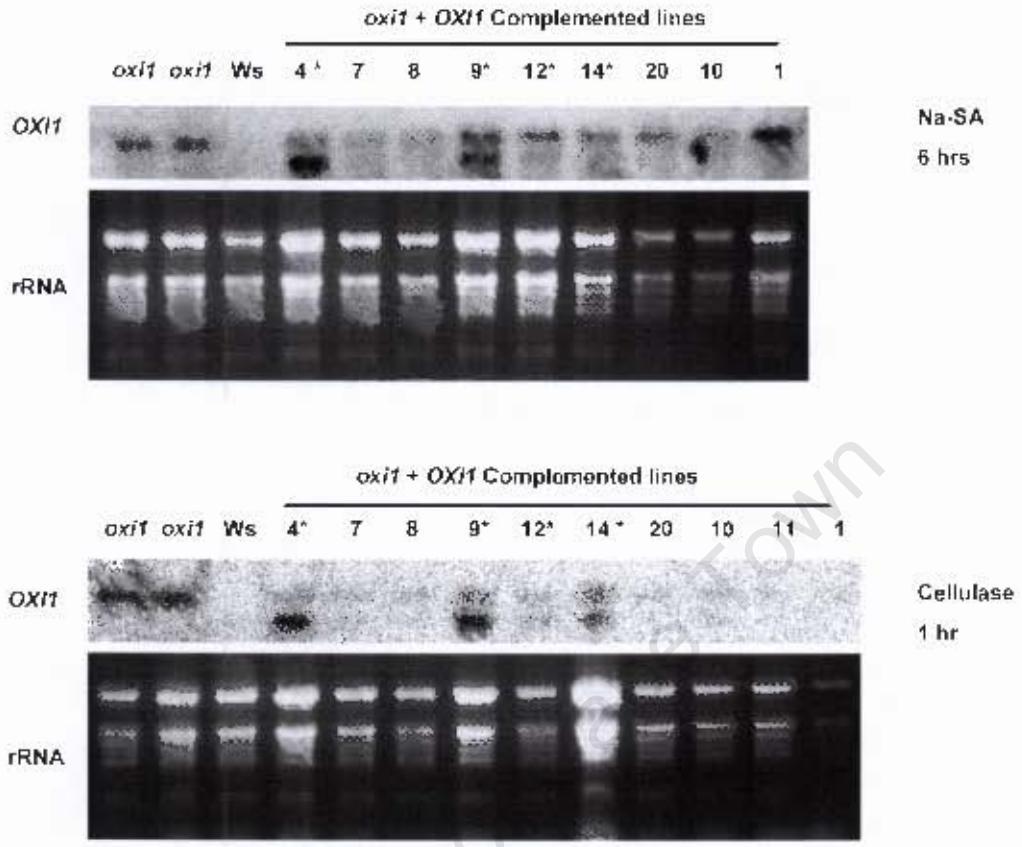
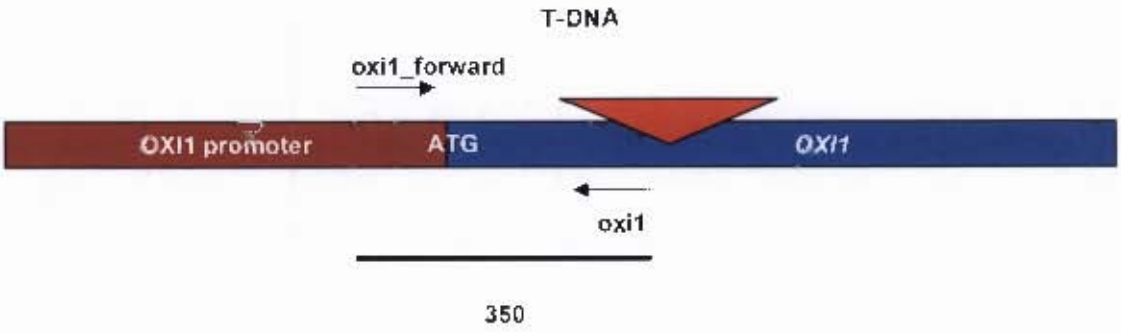


Figure 3.4 Complementation of the *oxi1* null mutant by wild type *OXI1*

T_1 progeny of independently transformed *oxi1* + *OXI1* transgenic lines as well as the *oxi1* null mutant and wild type control (Ws-2) were treated with either 250 µM Sodium salicylate (Na-SA) for 6 hrs or 0.1% Cellulase for 1 hour to induce *OXI1* gene expression. The presence of the smaller *OXI1* wild type transcript in the transgenic lines marked with * indicates successful complementation of these Arabidopsis lines. A full length 1.4 kb *OXI1* DNA fragment was used as a probe. The ethidium bromide stained rRNA is used as a loading control. It is noted that *OXI1* expression is absent in the Ws-2 samples treated with both Na-SA and cellulase but this is due to an insufficient amount of RNA loaded for these sample.

A



B

```
1          ATGCTAGAGGGAGATGAGAAACAGAGCCGAGCTCTGGATTTTAATCGACTCGAGGTTTTG
1           M  L  E  G  D  E  K  Q  S  R  A  L  D  F  N  R  L  E  V  F

61         TCATTACAGTAGCATCATCAGACCAAAAGTTACCCCCCAATAGTTTGAAACTAGAAAGCT
21          S  L  Q  *  E  H  E  T  K  S  *  A  R  T  V  *  N  *  K  A

121        CGCAATTGAGGTCTCATTCGGCATTCAGGCTCCGCAACCCTTGGCAAGGGGCATCGGTGC
41          R  N  *  G  L  I  R  H  S  G  C  A  T  V  G  K  G  D  R  C

181        GGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTT
61          G  P  I  R  Y  Y  A  S  W  R  K  G  D  V  L  Q  G  D  *  V

241        GGGTAACTGCCAGGGTTTTCCCAGTCACTGACGTTGTAAAACGACGGCCAGTGCCAAGCT
81          G  *  L  P  G  F  S  Q  S  L  T  L  *  N  D  G  Q  C  Q  A

301        TGCATGC
101         C  M
```

Figure 3.5 Sequencing of *oxi1* mutant transcript induced in the *oxi1* null mutant isolated from the Wisconsin knockout facility

(A) The 350 bp PCR product amplified using gene specific *OXI1* primers (oxi1_forward) and a primer within the right border of the T-DNA (oxi1 k/o_123) to sequence the *oxi1* mutant transcript. The primer sequences for ox1_forward and oxi1 k/o_123 can be found in the Materials and Methods. (B) Sequence data revealing the *OXI1* DNA sequence (in bold) that aligns with the database and the remaining sequence is that of the right border of the T-DNA insert. The T-DNA is inserted 67bp downstream of the predicted ATG of *OXI1*. Conceptual translation of the DNA sequence shows that it encodes a truncated non functional protein of 23 amino acids.

3.4 Generation of transgenic Arabidopsis overexpressing OXI1 and an OXI1-YFP fusion protein

An important question to address is whether OXI1 protein levels mirror the accumulation of the *OXI1* transcript in response to stimuli that induces *OXI1* mRNA. Immunoblot analysis using an antibody raised to a peptide sequence specific to OXI1 failed to produce detectable levels of OXI1 protein in plant extracts, even under conditions that result in a large increase in the amount of mRNA transcript (Rentel, 2002). Similarly, transgenic Arabidopsis expressing an OXI1-YFP fusion protein under control of the native OXI1 promoter failed to produce detectable levels of the fusion protein using either the purified OXI1 antibody or a commercial full length GFP antibody (Rentel, 2002). This data suggests that OXI1 protein is present at very low levels in the plant even under stress conditions. Hence, production of transgenic Arabidopsis constitutively overexpressing OXI1 may yield detectable levels of the protein and provide insights into its regulation. Furthermore, determining the subcellular localisation of OXI1 in Arabidopsis could be aided by the generation of the OXI1-YFP fusion protein under control of the constitutive 35S Cauliflower Mosaic Virus (CaMV) promoter since overexpressing the fusion protein may allow for visualisation of OXI1-YFP using confocal microscopy. Analysis of the subcellular localisation of OXI1-YFP may provide clues as to whether OXI1 is a mobile signalling component or localised to the membrane upon stimulus.

Arabidopsis ecotype Ws-2 plants were transformed using *Agrobacterium tumefaciens*-mediated transformation with a construct containing either a 1.4 kb DNA fragment including the entire *OXI1* coding region or a 2.5 kb OXI1-YFP protein fusion (1.4 kb DNA fragment containing the entire OXI1 coding region and 1.1 kb DNA fragment containing the YFP coding region) with a c-myc epitope tag. Both constructs were under control of the 35S CaMV promoter (Figure 3.6). To generate the 35S::OXI1 construct, the *OXI1* coding region was amplified from genomic DNA from the Ws-2 ecotype whereas the *OXI1* coding region, amplified from a plasmid containing the OXI1-YFP-cmyc fusion, was from the Col-0 ecotype and present in the 35S::OXI1-YFP-cmyc construct. Prior to transformation into Arabidopsis, the constructs were sequenced to determine whether any alterations in the DNA sequence had been

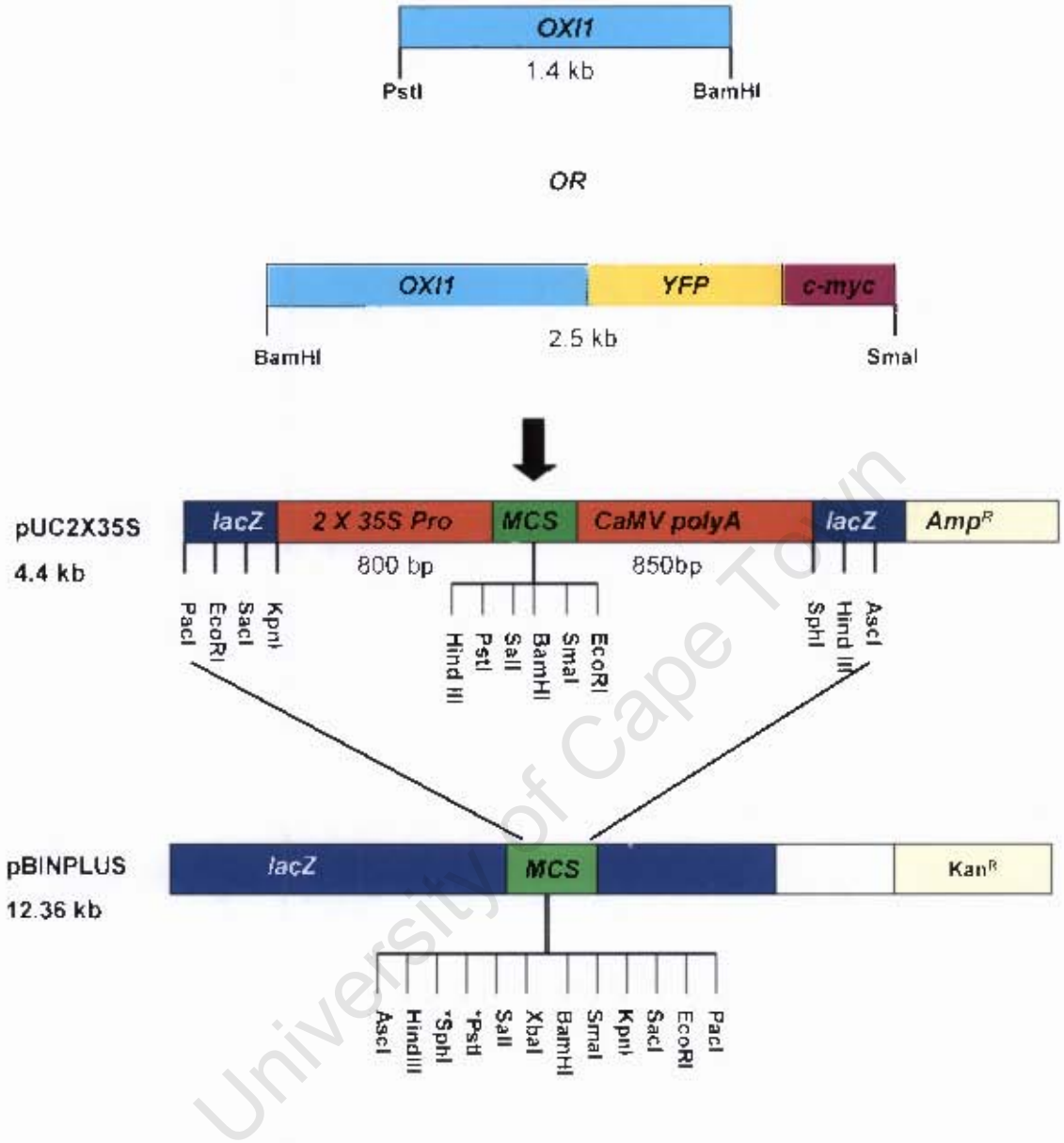


Figure 3.6 Transformation cassette used to produce 35S::OXI1 and 35S::OXI1-YFP transgenic plants

The coding region of *OXI1* containing the intron as well as 70 bp 3' UTR and the *OXI1* gene fused in frame to YFP with a c-myc epitope tag was cloned into pUC2X35S vector with the restriction sites indicated. The pUC2X35S vector is ampicillin resistant (Amp^R) and does not have blue/white selection since the *lacZ* gene is disrupted. The resultant constructs were subcloned into the binary vector pBINPLUS through the unique restriction sites of PacI and AscI. pBINPLUS is kanamycin resistant (Kan^R) and has blue/white selection. The multiple cloning site (MCS) for each vector is indicated. *PstI and SphI sites in pBINPLUS vector are not unique.

introduced during amplification of *OXI1* and to confirm that the fusion protein was in frame (Figure 3.7). Seven amino acid changes were found in OXI1 protein from the Ws-2 ecotype compared to that from Col-0, although it is difficult to distinguish changes due to amplification errors from genuine polymorphisms between the ecotypes. However, none of these amino acid changes occurred in the protein kinase active site signature nor did these changes alter the overall charge of the protein (Figure 3.7 B). The constructs contained the kanamycin resistant gene therefore transgenic seedlings were selected on the basis of kanamycin resistance as described in Section 2.4.4.

T_2 plants that exhibit 100% kanamycin resistance in their T_3 progeny were considered to be homozygous for the transformation cassettes. Northern analysis of *OXI1* expression before and after cellulase treatment revealed that at least four homozygous T_2 individuals for each construct were constitutively expressing *OXI1* or *OXI1-YFP* (Figure 3.8). Transgenic homozygous lines 1-5-3 and 1-20 that constitutively expressed *OXI1* and *OXI1-YFP* respectively were used for further analysis and characterisation of OXI1 protein kinase. These lines were chosen because they exhibited a high basal expression level of *OXI1* and the variability of OXI1 expression between the water control and cellulase treated samples were not as great as compared to the other transgenic lines, taking rRNA loading into account. Only one homozygous line was selected for each construct since originally transformed T_0 Arabidopsis seed were collected and pooled hence the T_2 kanamycin resistant individuals could have arisen from the same transformation event and could possibly be siblings. However, the variability of *OXI1* and *OXI1-YFP* gene expression between the different overexpressing lines suggests that these individuals represent independently transformed lines and possess different expression profiles due to the position effect of the inserted transgene. Initial comparative studies using two different T_2 lines for both 35S::OXI1 and 35S::OXI1-YFP transgenic lines yielded similar results.


A
OXI1
35S::OXI1

B

Figure 3.7 DNA and protein sequence alignment of the 35S::OXI1 construct

The *OXI1* coding region amplified from genomic DNA of the Ws-2 Arabidopsis ecotype displays 17 base pair changes compared to the sequence of *OXI1* from the Col-0 ecotype. The base pair changes are highlighted in turquoise (A). The protein alignment is shown in B with the amino acid changes found in the 35S::OXI1 construct compared to OXI1 protein from Col-0 ecotype. The protein kinase active site signature is highlighted in blue and the amino acids highlighted in grey represents changes of amino acids with similar properties while those in yellow represents changes in the charge of the amino acid.

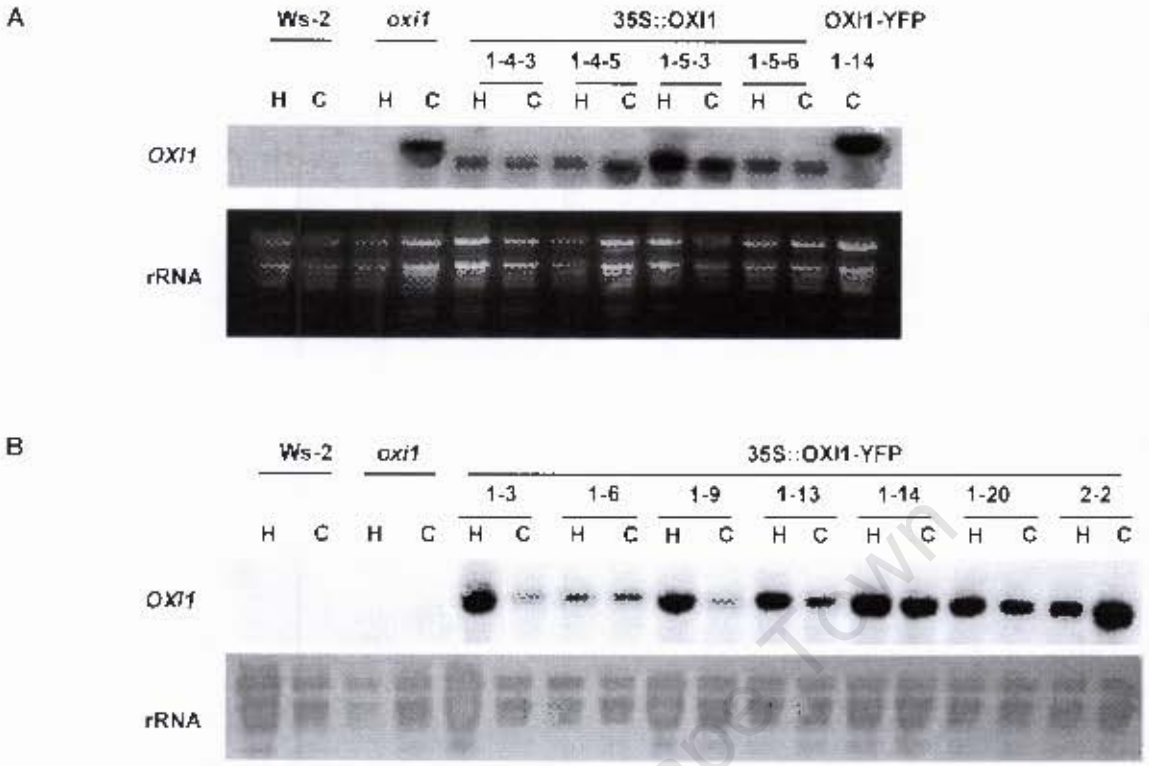


Figure 3.8 Transgenic Arabidopsis overexpressing *OXI1* and *OXI1-YFP*

Northern analysis demonstrated that transgenic Arabidopsis were constitutively expressing either *OXI1* (A) or *OXI1-YFP* (B). Ws-2, the *oxi1* knockout (Ws-2 background) and individual T_2 lines homozygous for the transgene were treated either with H_2O (H) or 0.1% Cellulase (C) for 1 hour to induce the *OXI1* gene. Blots were probed with a full length 1.4 kb *OXI1* DNA fragment that would recognise the wild type *OXI1*, the mutant *oxi1* and *OXI1-YFP* transcripts. In the 35S::OXI1 and 35S::OXI1-YFP, both *OXI1* and *OXI1-YFP* transcripts were expressed at a high basal level and were not induced upon stimulus, taking rRNA loading into account. The *OXI1-YFP* transcript would be considerably larger than the *OXI1* transcript as demonstrated in A, indicative that *OXI1* was overexpressed since the *OXI1* probe hybridised to the *OXI1* region of the *OXI1-YFP* transcript. Ws-2 treated with 0.1% cellulase did not produce detectable levels of *OXI1* (A and B) indicative that native *OXI1* was either not induced or induced to very low levels under these experimental conditions therefore the expression of *OXI1* in wild type seedlings was masked by the high levels of expression within the 35S::OXI1 or 35S::OXI1-YFP transgenic lines. Similarly cellulase failed to induce or produce detectable expression levels of the *oxi1* mutant transcript under these experimental conditions (B). The ethidium bromide (A) or methylene blue stained (B) rRNA were used as loading controls.

3.5 Microarray analysis of the *OXI1* gene

A two-fold bioinformatics approach was performed to ascertain the possible signal transduction pathways that OXI1 protein kinase could be involved in as well as the identification of genes that are co-regulated with *OXI1*. In both approaches the NASC dataset downloaded from http://arabidopsis.info/, which at the time consisted of 1877 microarray experiments was utilised. These microarray experiments consisted mostly of Affymetrix DNA microarray data comprising approximately 22 000 genes of the Arabidopsis genome as well as data from cDNA printed arrays (Craigon *et al.*, 2004).

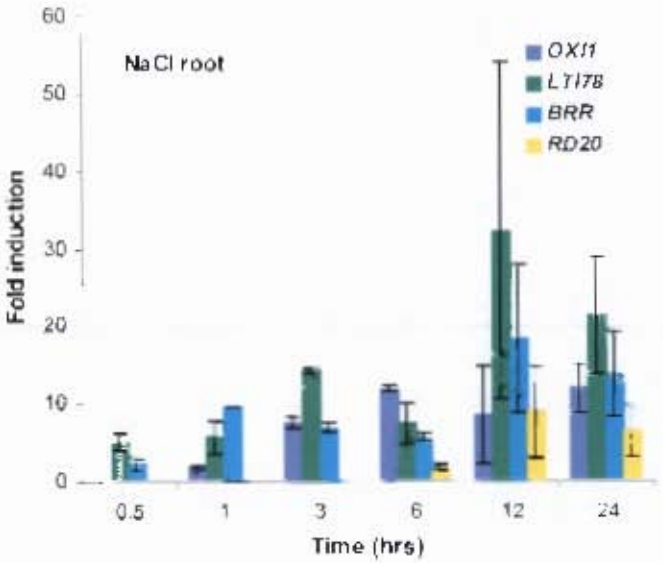
In the first instance the signal intensity of *OXI1* expression was compared to all the other genes within each individual array and slide of every experiment in the NASC dataset and experiments were ranked, in descending order, according to those in which *OXI1* expression was one of the highest expressed genes (kindly performed by Prof. Cathal Seoighe, NBN Bioinformatics Node, University of Cape Town). For example, even though cyclohexamide (CHX) and salt stress induced *OXI1* to an absolute signal intensity of approximately 27000 and 6000 respectively, salt stress was more highly ranked because *OXI1* expression was more highly expressed in comparison to all the other genes in the salt stress array than in the CHX array. Therefore this analysis allows for comparison of individual genes across different microarray experiments which could differ greatly in absolute signal intensities due to different hybridisation or experimental conditions. The top 100 slides in which *OXI1* expression was highly ranked was used as the cut off for this analysis. Although numerous experiments were identified, in most of these experiments replicate arrays were not closely ranked. This means that the ranking of *OXI1* was not reproducible and hence these experiments were excluded from the analysis. Those experiments where the induction of *OXI1* was reproducible amongst replicate arrays and significantly higher than *OXI1* expression in the control samples for any given experiment are represented below. For each experiment, the total number of genes present among the different slides was comparable and *OXI1* expression was presented as the level of induction in the treated sample relative to the control. A two-fold or more induction of *OXI1* gene expression was considered to be significant.

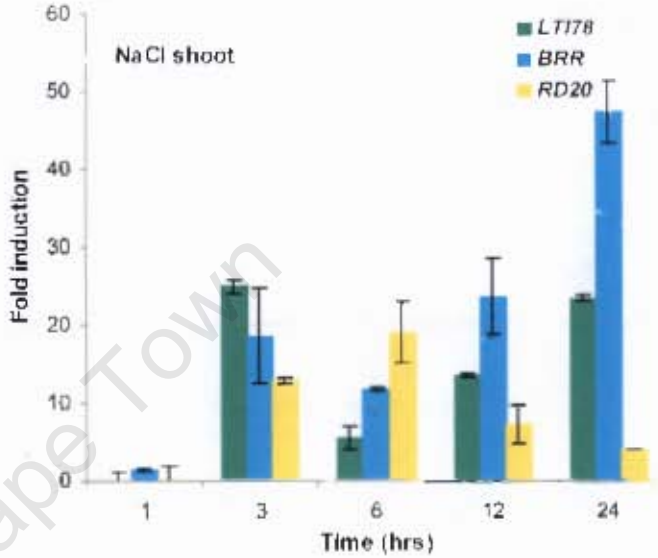
Salt stress was the highest ranked experiment for *OXI1* gene expression and expression occurred in the root but not shoot tissue of 16 day old Arabidopsis seedlings

(Figure 3.9 A). To confirm that the lack of OXI1 expression in the shoot tissue was a real event and not due to the nature of the experiment, the expression profiles of other NaCl-induced genes were determined. The *Low temperature-induced protein 78* (*LTI78*), *benzodiazepine receptor-related protein* (*BRR*) and *calcium binding RD20 protein* (*RD20*) were amongst the highest induced genes in response to 100 mM NaCl treatment in another microarray experiment comparing transcriptome changes induced during salt, osmotic and cold stress (Kreps *et al.*, 2002). Although different induction profiles occurred amongst the two tissue types for each gene, *LTI78*, *BRR* and *RD20* were induced in both root and shoot tissue validating conditions of the experiment (Figure 3.9 A). Surprisingly, oxidative stress induced by 10 µM methyl viologen as well as osmotic and drought stress all of which employed similar growth conditions and treatment as the salt stress microarray, since these experiments all originate from the AtGenExpress Consortium, did not cause induction of *OXI1* gene expression in either root or shoot tissue (data not shown). However, other abiotic stresses such as genotoxic stress causing DNA damage, cold, heat and UV-B all triggered early induction of *OXI1* in Arabidopsis (Table 3.1). This early induction of *OXI1* in response to abiotic stress suggests that OXI1 protein kinase may be involved in the early signalling events perhaps mediating perception of the stimulus and early responsiveness of the plant.

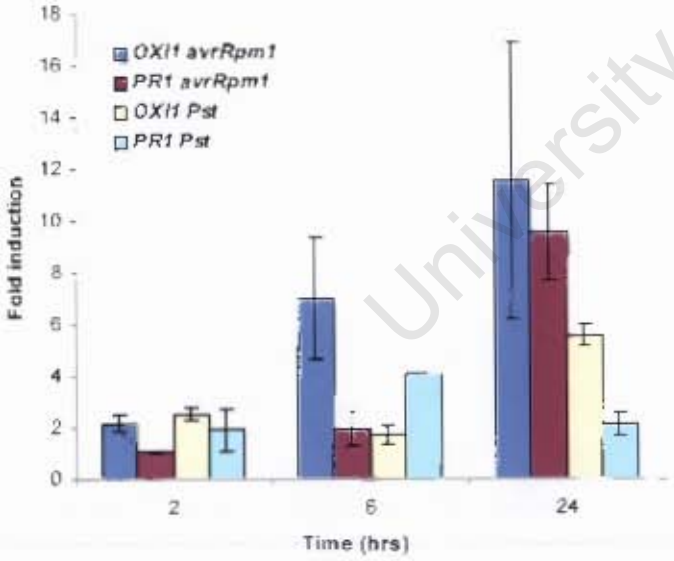
Auxin is a well characterised plant hormone that acts as a central regulator of plant development (Leyser, 1998; Benjamins *et al.*, 2003). Strikingly, treatment of seven day old Arabidopsis seedlings with the auxin transport inhibitor 2-3-5-triioldobenzoic acid (TIBA) results in a 17 fold increase in *OXI1* expression within 3 hours (Table 3.1). This finding suggests that auxin signalling may suppress *OXI1* expression. However, the employment of other inhibitors of auxin in the same microarray experiment such as naphthylphthalamic acid and p-cholraphenoxybutryric acid did not cause induction of *OXI1* (data not shown). There is a likelihood that *OXI1* expression is linked to auxin signalling given the requirement of OXI1 for normal root hair development under conditions of mild stress but *OXI1* induction by TIBA may also be due to the actual nature of the chemical itself.

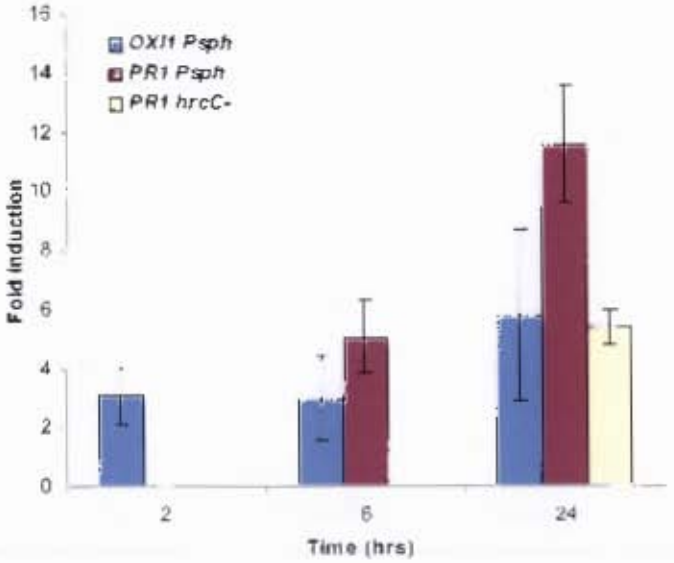
A





B





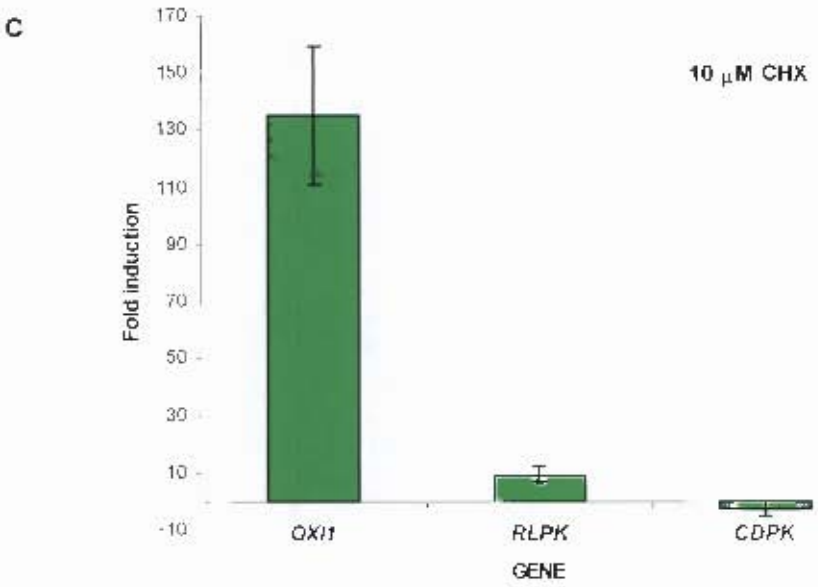


Figure 3.9 Analysis of *OXI1* gene expression from publicly available AtgenExpress microarray experiments

The induction of *OXI1* gene expression was analysed in response to 150 mM NaCl (A), pathogen challenge (B) and 10 µM CHX (C). Each data point represents the average fold induction across at least 2 Affymetrix microarray slides, as calculated as the signal intensity obtained from the treatment samples versus the signal intensity of the control samples (A, B and C). The standard error for each data point is shown. A: Three week old Arabidopsis seedlings Col-0 ecotype were treated with 150 mM NaCl or H_2O (control samples) and the root and shoot tissue was separated to determine whether the different tissue types underwent similar transcriptional changes. *OXI1* was induced in the root in response to salt stress but absent in the shoots. The NaCl-induced *Low temperature-induced protein 78* (*LTI78*), the *benzodiazepine receptor-related* (*BRR*) *protein* and the *calcium binding RD20 protein* (*RD20*) were used as control genes for this experiment. B: Five week old Arabidopsis plants were challenged at a concentration of 10^8 cfu/ml with either virulent *Pseudomonas syringae* pv. *tomato Pst*DC3000 (*Pst*), an avirulent *Pst*DC3000 *avrRpm1* (*avrRpm1*) strain, the dysfunctional type III secretion system strain *hrcC*$^-$ or the non-host pathogen *P. syringae* pv. *phaseolicola* (*Psph*). Arabidopsis plants inoculated with 10 mM $MgCl_2$ were used as the control samples. *OXI1* was induced by pathogen challenge and the induction of *Pathogenesis related protein1* (*PR1*) validated experimental conditions. C: Three week old Arabidopsis seedlings were treated with 10 µM CHX or H_2O (control samples) for 3 hrs. The expression of three different protein kinases: *OXI1*, *Receptor-like protein kinase* (*RLPK*) and the *calcium dependent protein kinase* (*CDPK*) exhibited different induction profiles with *OXI1* being induced by more than 100 fold while *CDPK* expression was reduced.

Table 3.1 The induction of *OXI1* gene expression by abiotic stresses

Experimental Conditions	Experimenter	Tissue	Time (hrs)[a] 1st induced	Fold induction [b]
Cold (4°C)	AtGenExpress Consortium	Root	6 (24)	2 (2.1)
Genotoxic	AtGenExpress Consortium	Root	3 (24)	2.3 (3.1)
Genotoxic	AtGenExpress Consortium	Shoot	6 (12)	2.3 (5.3)
UV-B stress	AtGenExpress Consortium	Root	0.5	2.8
UV-B stress	AtGenExpress Consortium	Shoot	0.5 (3)	3.7 (15.1)
Heat (55°C)	Jodi Swidzinski	Cell Culture	10 min	7.5
Auxin transport inhibitor	AtGenExpress Consortium	Whole Seedlings	3	17.1

[a] and [b] : the number in brackets represent the time point and level of *OXI1* expression exhibiting maximal induction respectively.

Biotic stress treatments were also among the highest ranked experiments for *OXI1* expression. Arabidopsis plants challenged with both virulent and avirulent strains of the biotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC3000 induced the expression of *OXI1* (Figure 3.9 B). *Pathogenesis related protein 1* gene (*PR-1*) is induced by different isolates of *P. syringae* and associated with resistance to this bacterial pathogen (Uknes *et al.*, 1992; Dempsey *et al.*, 1999). Therefore the induction of *PR-1* gene expression was used as the control gene for this microarray experiment. *OXI1* gene expression exhibited a similar fold induction as *PR-1* in response to virulent and avirulent *P. syringae* as well as the non-host pathogen *P. syringae* pv. *phaseolicola* (Figure 3.9 B). Therefore this data points to a role for OXI1 in mediating disease resistance against this bacterial pathogen. However the *P. syringae* DC3000 *hrcC* mutant (*hrcC*$^-$), which lacks a functional type III secretion system and is unable to cause disease on Arabidopsis (Roine *et al.*, 1997), induces *PR1* and not *OXI1* gene expression. Both the *hrcC*$^-$ mutant and the *P. syringae* pv. *phaseolicola* do not produce an HR (Roine *et al.*, 1997; Lu *et al.*, 2001) indicative that early or preformed defences are sufficient for resistance to these organisms. The *hcrC*$^-$ mutant also fails to initiate an oxidative burst whereas an oxidative burst presumably occurs in Arabidopsis infected with *P. syringae* pv. *phaseolicola* since *GST1* expression is induced during the latter interaction (Lamb and Dixon, 1997; Lu *et al.*, 2001). Therefore perhaps the oxidative burst is responsible for *OXI1* induction in response to *P. syringae* pv. *phaseolicola* infection and the lack of OXI1 expression in response to the *hrcC*$^-$ mutant may be due to the absence of AOS.

Interestingly, the greatest fold induction of *OXI1* gene expression resulted from treatment of Arabidopsis plants with the chemical inhibitor of translation CHX. *OXI1* was induced more than 100 fold following a 3 hr CHX treatment (Figure 3.9 C). A receptor like protein kinase (RLPK) was induced 10 fold whereas a calcium dependent protein kinase (CDPK) was down regulated in response to CHX treatment (Figure 3.9 C). This data illustrates that the induction of *OXI1* by CHX is not due to a general effect of CHX on all proteins or protein kinases. It also suggests that OXI1 transcription may be actively repressed by a protein with a fairly short half-life.

Microarray analysis has further revealed that *OXI1* gene expression is induced by both biotic and abiotic stresses therefore OXI1 is likely to be involved either directly or indirectly in numerous signal transduction cascades. The next step would be to use the *OXI1* transgenic lines to establish whether OXI1 does have a non-redundant role in these stresses, for example test whether the *oxi1* null mutant is compromised in salt stress tolerance in comparison to wild type. To further delineate these putative *OXI1* mediated signal transduction cascades the identification of other proteins or components that either interact with OXI1 or together with OXI1 regulates these processes would be invaluable.

The second bioinformatics approach consisted of finding genes whose expression correlates with *OXI1* expression across all the microarray experiments contained in the NASC dataset. To perform co-expression analysis a Spearman correlation between the *OXI1* gene and every other gene in the dataset was used (kindly performed by Prof. Cathal Seoighe, NBN Bioinformatics Node, University of Cape Town). The correlation coefficient provides a measure of how closely the expression profile of any given gene matches the expression profile of *OXI1* with a maximum value of +1 (i.e. *OXI1* with itself) indicative of perfect correlation (Jen *et al.*, 2006). Genes that show strong correlation with each other, where one or both genes are implicated in a particular biological process, suggest that these correlated genes may regulate that defined biological process in concert. For example, recently a linear regression approach was used successfully to identify genes involved in secondary cell wall synthesis and determine other biological processes which these co-expressed genes regulated (Persson *et al.*, 2005). In the aforementioned example *cellulose synthase genes* (*CESA*) *4*, *7* and *8* involved in secondary cell wall formation were used as the driver genes to perform correlation across 408 Affymetrix Arabidopsis microarray data sets and 64 of the highest ranked 100 genes for each driver overlapped (Persson *et al.*, 2005). Consequently, the *Irregular or Collapsed Xylem 8* and *13* (*IRX8* and *IRX13*) genes were identified and mutational analysis supported the involvement of *IRX8* and *IRX13* in cell wall development since mutants displayed severe abnormalities in xylem cell morphology and reduced cellulose content (Persson *et al.*, 2005). Additionally it is postulated that *CESA* genes are involved in other biological pathways such as glucose catabolism and brassinosteroid biosynthesis since *CESA* genes highly correlates with genes involved in these biological processes (Persson *et al.*, 2005).

A list of the 50 highest ranked genes that correlate with *OXI1* expression is provided in Table 3.2, however these genes have relatively low correlation values. It was observed that most of these co-expressed genes have not been characterised experimentally and annotation of these genes are based on sequence similarities. Therefore it is difficult to predict which biological processes OXI1 might be involved. However, a few genes associated with defence related processes such as *WRKY6*, *CRR2* and *GST* have been identified further implicating a role for OXI1 in disease resistance pathways (Table 3.2). In an attempt to find a set of stronger correlated genes with *OXI1* the Arabidopsis co-expression tool which contains a smaller data set of 322 ATH1 array hybridisations was utilised (Jen *et al.*, 2006). Higher correlation values were obtained and surprisingly only 3 genes; a zinc finger protein, an ABC transporter and a phosphoglycerate mutase family protein, were common between the two data sets (Table 3.3). Changing the data set to comprise of arrays in which *OXI1* transcription was shown to be induced did not yield any significantly stronger correlated genes nor did it produce a distinct set of genes involved in a particular process (Table 3.4). Therefore OXI1 may be involved in a host of signal transduction networks since the assortment of genes co-expressed with *OXI1* are involved in a wide variety of biological processes (Tables 3.2 - 3.4). It has been suggested that genes regulated by different or multiple signalling cascades would produce poorly correlated sets of genes (Jen *et al.*, 2006). For example, each signalling cascade that activates the driver gene potentially induces a different subset of correlated genes hence combination of these signalling cascades and gene subsets reduces the overall correlation to the gene of interest (Jen *et al.*, 2006). Alternatively, it is possible that the lack of strong correlation simply means that transcription of *OXI1* is not of biological significance.

Table 3.2 Genes from the NASC dataset best-correlated with *OXI1* using the Spearman correlation coefficient

Gene	Description/Similarity	Correlation Coefficient	Process	Reference
At4g24160	hydrolase, alpha/beta fold family protein	0.693		
At2g15480	UDP-glucoronosyl/UDP-glucosyl transferase family protein	0.680		
At4g20860	FAD-binding domain-containing protein (electron carrier / oxidoreductase)	0.672		
At5g14730	expressed protein	0.650		
At4g36990	heat shock factor protein 4 (HSF4)	0.646	Heat Stress	(Czarnecka-Verner *et al.*, 2000)
At4g21390	S-locus lectin protein kinase family protein	0.642		
At3g47780	ABC transporter family protein transport protein ABC-C	0.640		
At1g62300	WRKY family transcription factor (WRKY6)	0.635	Senescence/Defence	(Robatzek and Somssich, 2001)
At1g80820	cinmoyl-CoA reductase, putative identical to CCR2	0.633	Defence	(Lauvergeat *et al.*, 2001)
At4g20830	FAD-binding domain-containing protein (electron carrier)	0.632		
At4g39670	expressed protein	0.629		
*At1g65690	harpin-induced protein-related / HIN1-related / harpin-responsive protein	0.624	Defence	(Gopalan *et al.*, 1996)
At5g59820	zinc finger (C2H2 type) family protein (ZAT12)	0.623		
*At2g17290	calcium-dependent protein kinase isoform 6 (CPK6 / CDPK6)	0.621	Defence	(Romeis *et al.*, 2001)
At3g50260	AP2 domain-containing transcription factor	0.619		
*At3g52450	U-box domain-containing protein similar to immediate-early fungal elicitor protein CMPG1	0.618	Defence	(González-Lamothe *et al.*, 2006)
At5g63790	no apical meristem (M) family protein	0.616		
At5g12340	expressed protein	0.612		
At1g19020	expressed protein	0.608		
At2g46500	phosphatidylinositol 3- and 4-kise family protein / ubiquitin family protein	0.607	Protein degradation	
At5g25930	leucine-rich repeat family protein / protein kinase family protein	0.605		
At3g10500	no apical meristem (M) family protein	0.604		
At4g13180	short-chain dehydrogese/reductase (SDR) family protein	0.602		
At1g71697	choline kinase	0.600		
At3g53150	UDP-glucoronosyl/UDP-glucosyl transferase family protein	0.599		

* Process postulated due to published data of either similar protein members in Arabidopsis or other plant species

Table 3.2 Genes from the NASC dataset best-correlated with *OXI1* using the Spearman correlation coefficient *continued*

Gene	Description/Similarity	Correlation Coefficient	Process	Reference
At2g39660	protein kinase, putative	0.599		
*At1g17170	glutathione S-transferase, putative	0.595	Defence	(Murray, 2001)
At2g37430	zinc finger (C2H2 type) family protein (ZAT11)	0.595		
At4g26470	calcium-binding EF hand family protein	0.595		
At5g05140	transcription elongation factor-related	0.593		
At3g25610	haloacid dehalogese-like hydrolase family protein	0.591		
At3g46620	zinc finger (C3HC4-type RING finger) family protein	0.588		
At1g30700	FAD-binding domain-containing protein (electron carrier)	0.588		
At1g05575	expressed protein	0.587		
At1g08940	phosphoglycerate/bisphosphoglycerate mutase family protein	0.586		
At2g41380	embryo-abundant protein-related	0.585		
At1g02400	gibberellin 2-oxidase, putative / GA2-oxidase, putative	0.583		
At2g32020	GCN5-related N-acetyltransferase (GT) family protein	0.582		
At3g52400	syntaxin, putative (SYP122)	0.581	Cell wall deposition	(Assaad *et al.*, 2004)
At3g44190	pyridine nucleotide-disulphide oxidoreductase family protein	0.580		
At5g54840	GTP-binding family protein	0.579		
At3g28210	zinc finger (AN1-like) family protein	0.579		
At1g09970	leucine-rich repeat transmembrane protein kinase	0.578		
At5g03380	heavy-metal-associated domain-containing protein	0.577		
At5g12880	proline-rich family protein similar to hydroxyproline-rich glycoprotein	0.577		
*At2g29490	glutathione S-transferase, putative similar to glutathione S-transferase 103-1A	0.577	Defence	(Murray, 2001)
At2g27660	DC1 domain-containing protein	0.576		
At3g05360	disease resistance family protein / LRR family protein contains leucine rich-repeat	0.574		
At3g59700	lectin protein kinase, putative similar to receptor lectin kinase 3 [Arabidopsis thaliana]	0.574		
At3g46930	protein kinase family protein	0.573		

* Process postulated due to published data of either similar protein members in Arabidopsis or other plant species

Table 3.3 25 highest ranked genes co-expressed with *OXI1* using ACT

Gene	Description/Similarity	Correlation Coefficient
AT5G59820	**zinc finger (C2H2 type) family protein (ZAT12)**	**0.854**
AT5G57510	hypothetical protein	0.830
AT3G62260	protein phosphatase 2C	0.820
AT3G61190	BON1-associated protein 1 (BAP1)	0.804
AT1G61340	F-box family protein; similar to late embryogenesis abundant protein	0.799
AT4G19460	glycosyl transferase family 1 protein	0.795
AT1G66400	calmodulin-related protein, putative similar to calmodulin-related protein 2, touch-induced	0.787
AT5G17350	expressed protein	0.783
AT1G48860	3-phosphoshikimate 1-carboxyvinyltransferase	0.783
AT5G52020	AP2 domain-containing protein low similarity to DREB1B	0.774
AT2G27080	harpin-induced protein-related / HIN1-related / harpin-responsive protein-related	0.769
AT4G33920	protein phosphatase 2C family protein similar to Ser/Thr protein phosphatase 2C	0.769
AT4G30430	senescence-associated family protein similar to senescence-associated protein 5	0.767
AT5G46590	no apical meristem (NAM) family protein	0.766
AT3G47780	**ABC transporter family protein transport protein ABC-C**	**0.765**
AT5G15640	mitochondrial substrate carrier family protein	0.761
AT5G54490	calcium-binding EF-hand protein, putative similar to EF-hand Ca2+-binding protein CCD1	0.756
AT3G48850	mitochondrial phosphate transporter	0.754
AT4G33985	expressed protein	0.748
AT2G39650	expressed protein	0.747
AT4G21865	expressed protein	0.745
AT2G41640	expressed protein contains Pfam domain	0.743
AT1G08940	**phosphoglycerate/bisphosphoglycerate mutase family protein**	**0.741**
AT5G65300	expressed protein	0.738
AT2G47550	pectinesterase family protein	0.738

* Genes in bold text represent those genes that are common to both the 1877 and 322 array data sets

Table 3.4 25 highest ranked genes co-expressed with *OXI1* in response to cold, heat, oxidative stress and pathogen infection using ACT

Gene	Description/Similarity	Correlation Coefficient
AT4G37370	cytochrome P450, putative similar to Cytochrome P450 91A1	0.816
AT2G30400	ovate family protein 57% similar to ovate protein	0.803
AT4G17500	ethylene-responsive element-binding protein 1 (ERF1) / EREBP-2 protein	0.802
AT4G30390	expressed protein	0.793
AT1G59500	auxin-responsive GH3 family protein similar to auxin-responsive GH3 product	0.777
AT3G10500	no apical meristem (NAM) family protein similar to to NAC2	0.770
AT1G05575	expressed protein	0.769
AT5G66890	putative disease resistance protein (CC-NBS-LRR class)	0.767
AT5G03610	GDSL-motif lipase/hydrolase family protein	0.766
AT3G48450	nitrate-responsive NOI protein, putative similar to nitrate-induced NOI protein	0.764
AT2G34500	cytochrome P450 family protein similar to Cytochrome P450 61	0.762
AT2G15480	UDP-glucoronosyl/UDP-glucosyl transferase family protein	0.757
AT2G23270	expressed protein	0.755
AT5G64300	riboflavin biosynthesis protein, putative (RIBA)	0.755
AT1G63720	expressed protein	0.753
AT5G42380	calmodulin-related protein	0.753
AT4G24160	hydrolase, alpha/beta fold family protein	0.751
AT1G77450	no apical meristem (NAM) family protein	0.750
AT1G30860	expressed protein	0.750
AT1G15520	ABC transporter family protein similar to ABC1 protein	0.748
AT1G72900	putative disease resistance protein (TIR-NBS class)	0.748
AT5G27380	glutathione synthetase (GSH2)	0.747
AT1G74080	myb family transcription factor (MYB122)	0.746
AT4G34135	UDP-glucoronosyl/UDP-glucosyl transferase family protein	0.746
AT3G14050	RelA/SpoT protein, putative (RSH2)	0.745

3.6 *oxi1* mutants do not show an abiotic stress phenotype

Salt and heat stress caused maximal induction of *OXI1* gene expression of 12 and 7.5 fold respectively (Figure 3.8 A and Table 3.1). The induction of *OXI1* by these stresses was confirmed through northern analysis (Figure 3.10). Interestingly osmotic stress imposed by treatment of Arabidopsis seedlings with 300 mM mannitol did not result in the induction of *OXI1* (Figure 3.10 A). Furthermore *OXI1* expression was not induced in response to osmotic stress in the AtGenExpress microarray experiment (data not shown). This indicates that the induction of *OXI1* by salt stress is more likely due to the ionic component of NaCl rather than the osmotic stress it is causing since 150 mM NaCl and 300 mM Mannitol impose the same osmolarity effect. During the Fenton reaction, Fe^{2+} catalyses the reaction between superoxide and H_2O_2 producing the highly reactive hydroxyl radical (OH^-) (Grant and Loake, 2000). Therefore treatment of Arabidopsis seedlings with high iron concentrations should result in oxidative stress caused by the accumulation of OH^-. However treatment of seedlings with double the concentration of Fe.EDTA (Fourcroy *et al.*, 2004) required for normal development, over a period of 24 hours, did not result in the induction of *OXI1* gene expression (Figure 3.10 A). This suggests that OXI1 protein kinase may not be responsive to all forms of AOS initiating oxidative damage.

Given the expression of *OXI1* after salt and heat stress, it was investigated whether the *oxi1* null mutant harboured any visually discernable morphological phenotypic defect or enhancement in response to NaCl or heat stress in comparison to wild type. Identification of such phenotypes would be useful for large scale screening of gain of function mutants in the *oxi1* background to help dissect the signalling pathway of this oxidative stress inducible kinase. It would also help to establish a biological role for OXI1 protein kinase. However, no such difference was found between the *oxi1* mutant and wild type seedlings subjected to various NaCl concentrations with respect to survival of seedlings (Figure 3.11). Although salt stress induced *OXI1* expression in the root tissue of Arabidopsis (Figure 3.9 A), no difference was observed between the root length of the *oxi1* mutant and wild type seedlings grown on MS media containing 50 and 100 mM NaCl (data not shown). This data suggests that although *OXI1* expression is induced by salt stress, this kinase is not playing a pivotal role in aiding the plant to cope with salt stress.

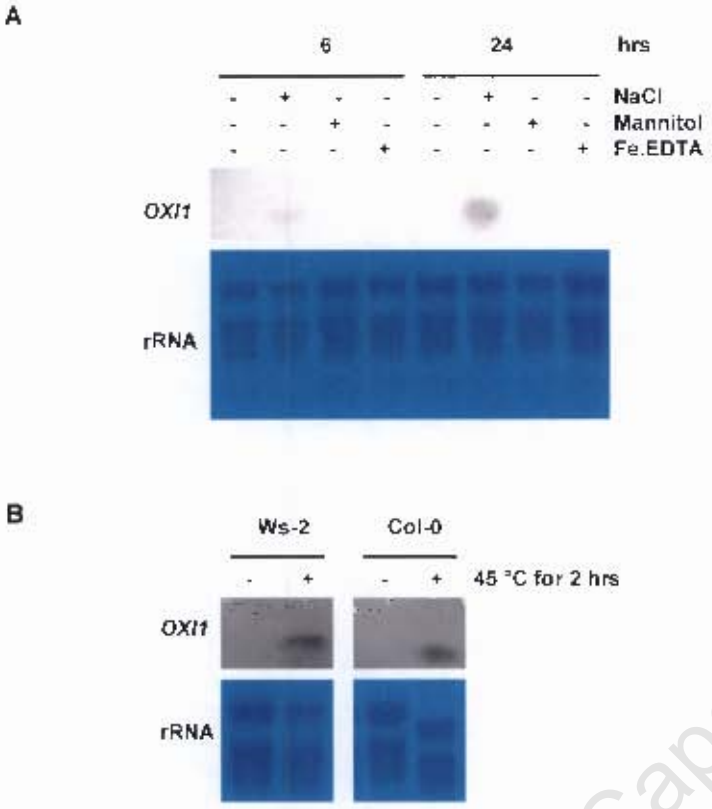
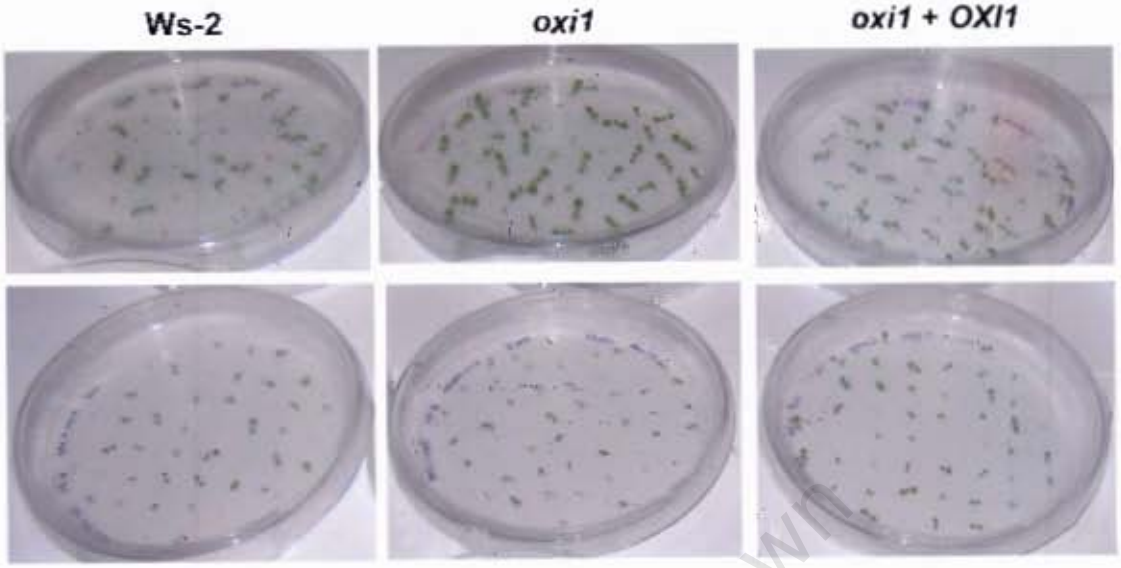


Figure 3.10 Northern analysis confirms induction of *OXI1* by NaCl and Heat stress

Ten day old Ws-2 seedlings were treated either with 150 mM NaCl, 300 mM Mannitol, 0.1 mM Fe.EDTA or H_2O (control) for 6 and 24 hrs (A). Ten day old seedlings were incubated either at 45°C (+) or left in the growth room at 22°C (-) for 2 hours (B). A full length *OXI1* genomic DNA probe was used to determine the level of *OXI1* induction. The methylene blue stained rRNA is used as a loading control.

A



B

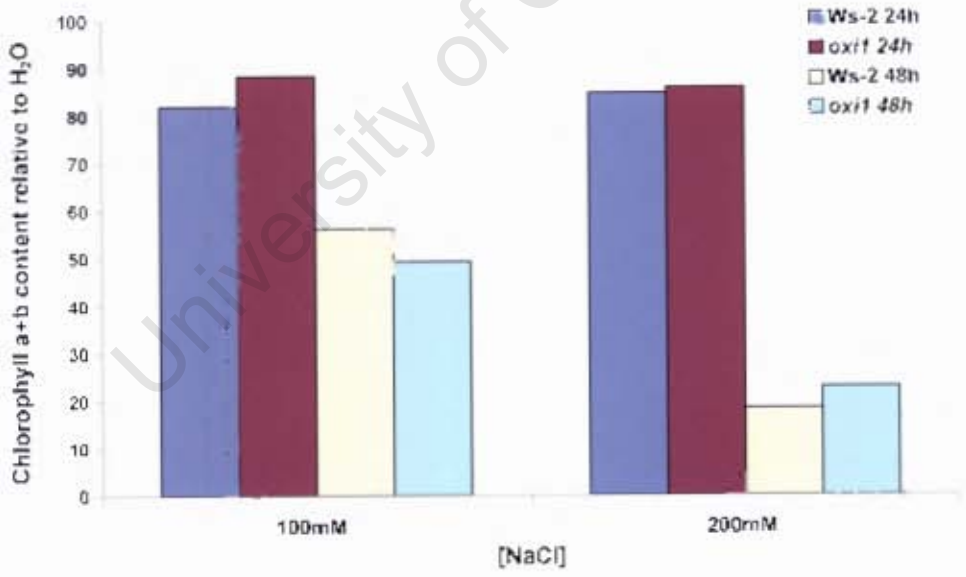


Figure 3.11 The *oxi1* null mutant exhibits wild type responses during salt stress

Seven day old Arabidopsis seedlings of genotypes Ws-2, *oxi1* and *oxi1* + *OXI1* complemented line displayed stunted growth and low germination rates to a similar extent when grown on MS media supplemented with 100 mM NaCl in comparison to those seedlings grown on MS media only (A). Five leaf discs from 4 week old Arabidopsis leaves of genotypes Ws-2 and *oxi1* were treated with H_2O, 100 mM or 200 mM NaCl and extent of chlorophyll bleaching over 24 and 48 hrs was determined. The amount of chlorophyll a and b present in treated samples was expressed as a percentage of the amount present in the H_2O control sample (B).

Acquired thermotolerance is the phenomenon whereby Arabidopsis seedlings pre-exposed to an increased temperature which has no negative effect on growth, for example 38°C, are able to survive a normally lethal subsequent heat stress (Hong and Vierling, 2000; Larkindale *et al.*, 2005). Since *OXI1* gene expression is induced by heat stress it was investigated whether the *oxi1* mutant is less able than wild type to withstand heat stress or compromised in process of acquired thermotolerance. Additionally, *Heat Shock Transcription Factor 4* (*HSF4/HsfB1*) is among the top genes identified from the microarray analysis to possibly be co-regulated with *OXI1* (Table 3.2). The *oxi1* mutant subjected to a heat stress of 45°C or 47°C at different stages of development, displayed wild type responses and developed normal acquired thermotolerance (Figure 3.12). Therefore OXI1 protein kinase is probably not required for signal transduction pathways mediating plant responses to heat stress. Consequently, OXI1 provides an example of whereby the induction of a gene does not necessarily correlate with a crucial involvement in the particular process by which it is induced.

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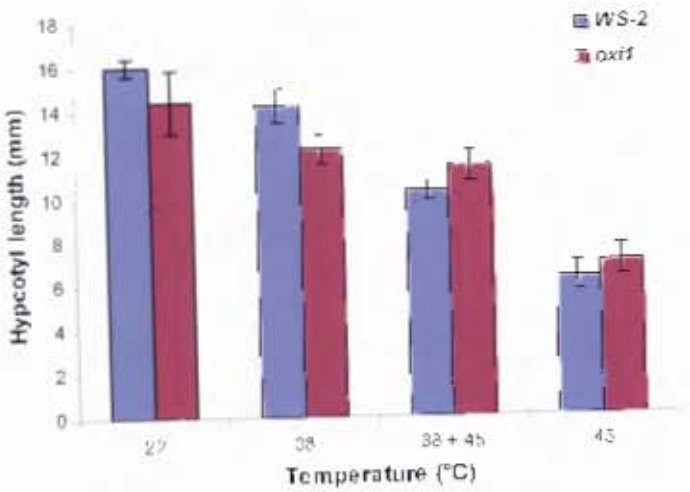

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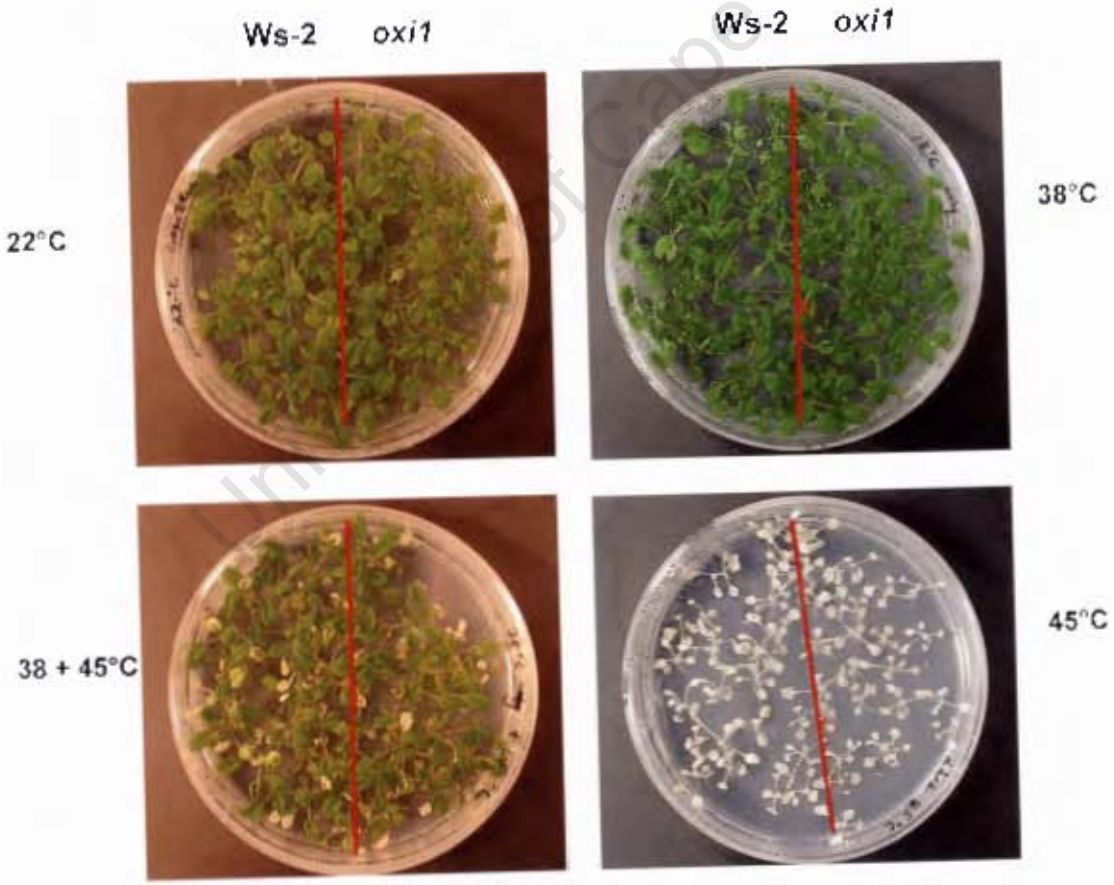

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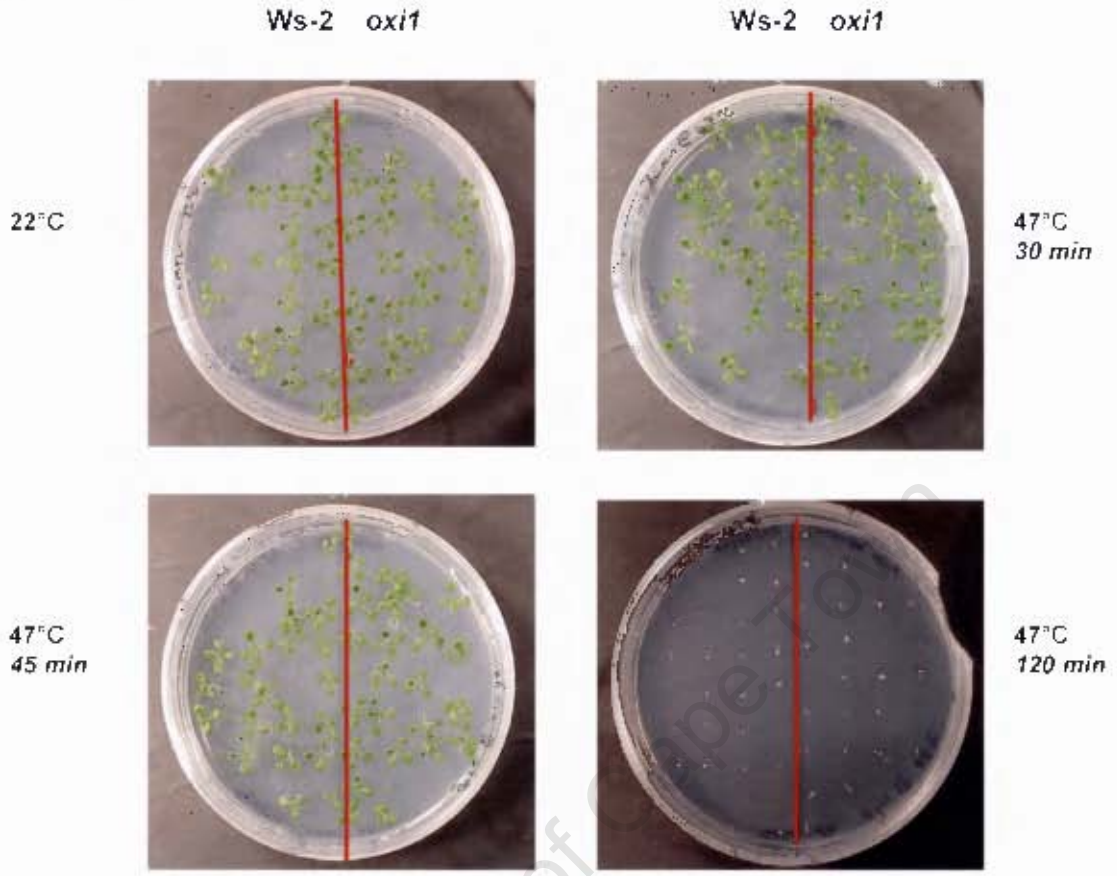

Figure 3.12 The *oxi1* mutant displays wild type responses to heat stress and is not compromised in the establishment of acquired thermotolerance

Hypocotyl elongation of 5 day old dark grown Arabidopsis seedlings of genotypes Ws-2 and *oxi1* subjected for a period of 2 hrs (at 2.5 days old) to 38°C. 38°C followed by 45°C (acclimation) or 45°C heat stress was measured (A). The control seedlings were grown in the dark at 22°C for five days. Ten day old Ws-2 or *oxi1* seedlings were subjected to either 38°C for 90 min, 38°C for 90 min followed by 22°C for 2 hrs and thereafter 45°C for 2 hrs (38 + 45°C) or 45°C only heat stress and returned to the growth chamber at 22°C (control temperature) where survival was assessed after 5 days (B). The *oxi1* mutant developed acquired thermotolerance efficiently since both hypocotyl length and survival increased in seedlings pre-exposed to 38°C compared to 45°C only heat stress (A and B). Three day old Ws-2 and *oxi1* seedlings were subjected to 47°C for the duration indicated and returned to the growth chamber at 22°C (control temperature) and survival was assessed after seven days with a 2 hr heat stress proving to be the critical time point for both genotypes (C). Photographs were taken with a Nikon Coolpix 990 digital camera.

3.7 Localisation of OXI1

OXI1 protein kinase is thought to act as a signalling molecule perceiving the changes in AOS and activating downstream effectors to facilitate the desired end responses. In order to interact with other signalling components within a particular signal transduction cascade, OXI1 either has to reside in the same vicinity as or have the ability to translocate to its interacting molecules. OXI1 lacks conserved signal sequences and possesses putative N-myristoylation sites through which proteins are anchored to membranes suggesting both a cytosolic and membrane localisation (Rentel, 2002). The cellular localisation of OXI1 was investigated using the 35S::OXI1-YFP construct since previous attempts to visualise the fluorescent OXI-YFP fusion protein under control of the native OXI1 promoter proved unsuccessful.

Transient expression of fluorescent OXI1-YFP protein in young tobacco leaves was used to preliminary analyse its localisation pattern with the aid of confocal microscopy. A construct containing a cytosolic YFP (a gift from Dr Ian Moore, Department of Plant Sciences, University of Oxford) was also transformed into tobacco and used as a control for the confocal data. The expression of OXI1-YFP appeared to be cytosolic in location due to it being present in transvacuolar strands and excluded from the nucleus (Figure 3.13). It was observed that the expression level of OXI1-YFP was significantly lower than that of the cytosolic YFP protein since the latter produced a much stronger signal under the same settings for the confocal microscope. Additionally the rate of infection of tobacco cells with OXI1-YFP was significantly lower than that of the cytosolic YFP protein since only a few cells were detected that expressed the OXI1-YFP protein in comparison to the control YFP (Figure 3.13). This was another indication that OXI1 protein kinase might be expressed at a low level and subjected to tight control.

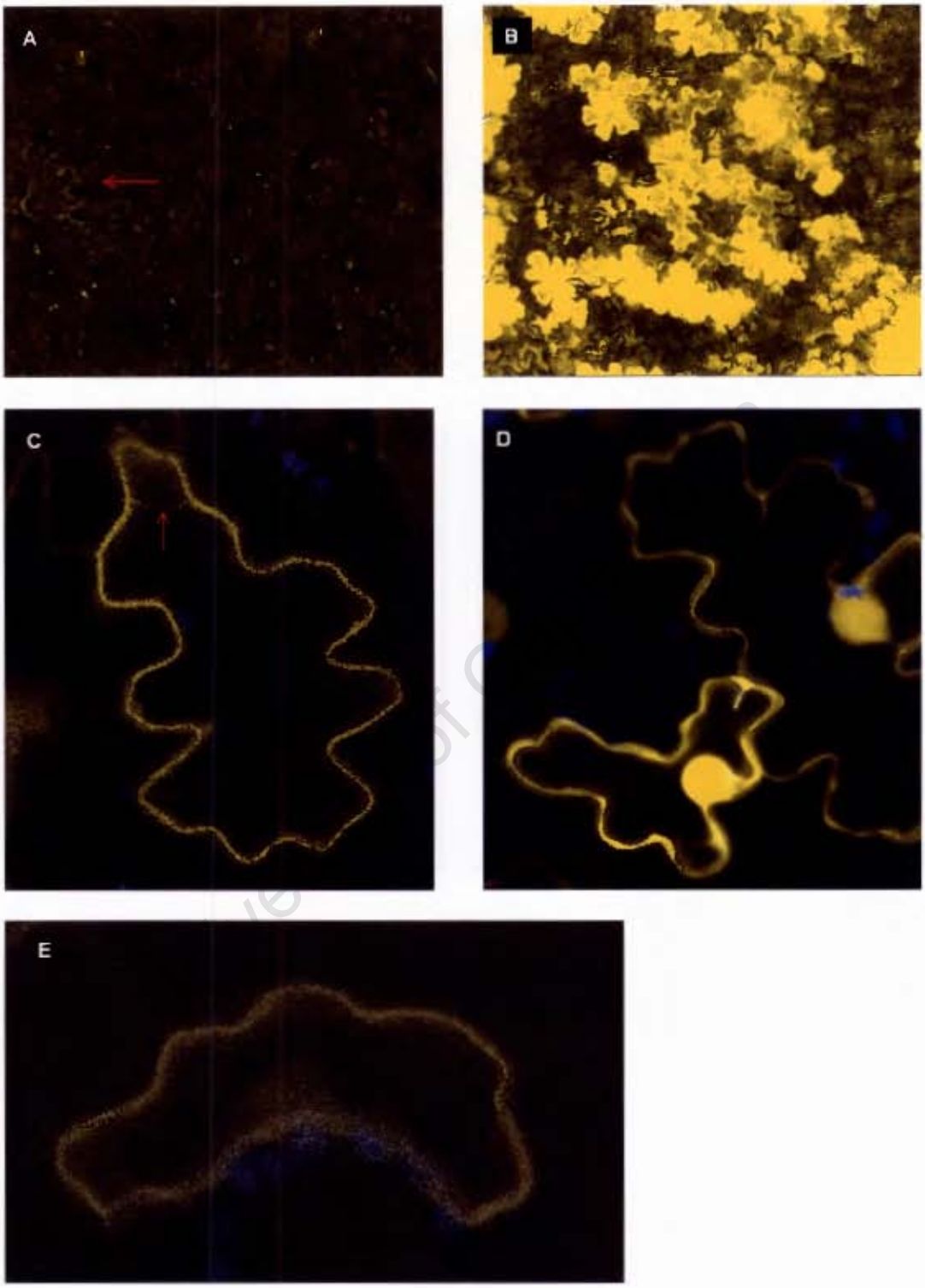
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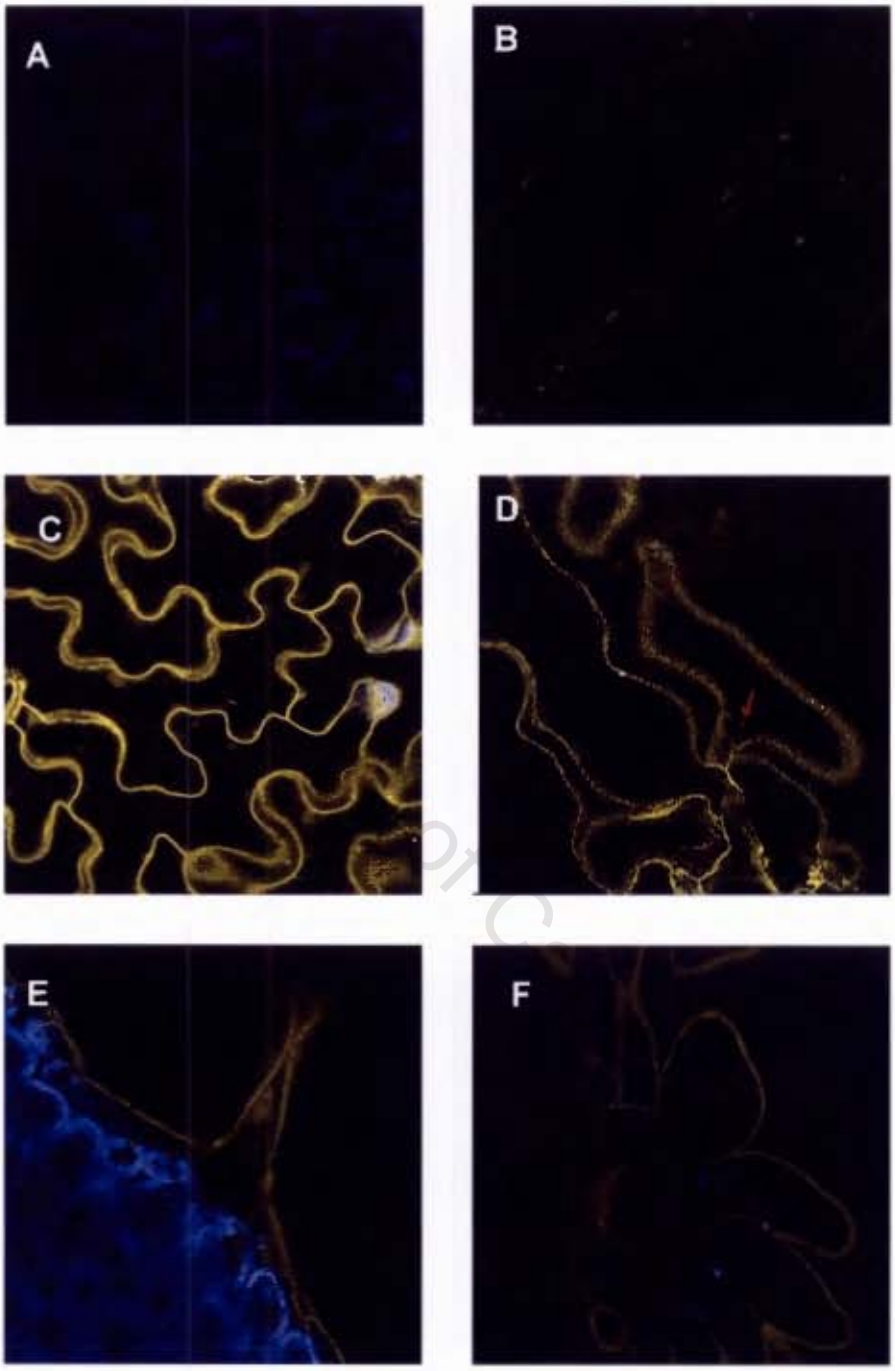
Figure 3.13 Transient expression of OXI1-YFP in tobacco reveals cytosolic localisation

Laser confocal microscopy images of epidermal cells of tobacco leaves transiently expressing the OXI1-YFP protein fusion (A, C and E) or a cytosolic localised YFP (B and D). Images were scanned using a Plan-Neofluar 10x (A and B) or C-Apochromat 40x (C-E) objective at an excitation wavelength of 514 nm and emission window of 527-563 nm. The number and fluorescence intensity of epidermal cells expressing the OXI1-YFP protein fusion (red arrow in A) is significantly lower than those expressing cytosolic YFP (A and B). The higher magnification of single epidermal cells showed that the fusion protein under control of the 35S CaMV promoter was weakly expressed in the cytosol due to the presence of transvacuolar strands (red arrow in C) and excluded from the nucleus (C and E) in comparison to the cytosolic YFP control (D).

Confocal analysis of stably transformed 35S::OXI1-YFP Arabidopsis revealed a definitive cytosolic localisation due to the presence of transvacuolar strands (Figure 3.14 D). Although the expression of OXI1-YFP was not uniform within the plant, expression did occur within the epidermis of the shoots as well as in root tissue (Figure 3.14). The expression level of OXI1-YFP was still relatively low and was not age dependent since similar expression profiles were observed in older leaves compared to young seedlings. Treatment of 35S::OXI1-YFP seedlings with cellulase did not exhibit a differential pattern of expression of OXI1-YFP compared to untreated seedlings nor did it cause the protein to move (data not shown). The localisation of OXI1-YFP to the plasma membrane could not be discerned under these experimental conditions since it is difficult to distinguish between the cytoplasm and the plasma membrane. Plasmolysis of 35S::OXI1-YFP with 500 mM mannitol to cause shrinkage of the cytoplasm was attempted but did not provide any clear pattern (data not shown).

A subcellular fractionation approach separating membrane from cytosolic fractions was employed to confirm the confocal data on the localisation of OXI1-YFP (Volotovski *et al.*, 2003). Protein extract from two week old 35S::OXI1-YFP transgenic seedlings was subjected to ultracentrifugation to obtain crude cytosolic and membrane fractions (Volotovski *et al.*, 2003). In order to establish the purity of the subcellular fractions, antibodies specific for cytosolic or membrane localised proteins were utilised. The antioxidant Glutathione-S-transferase 1 (GST1) protein was only present in the cytosol (Figure 3.15 A). Similarly, the aquaporin plasma membrane intrinsic proteins 1 and 2 (PIP1 and PIP2), which are expressed in the Arabidopsis root and involved in water transport (Boursiac *et al.*, 2005), were exclusive to the membrane fraction (Figure 3.15 B and C). These results indicate that pure membrane and cytosolic fractions had successfully been obtained. Western analysis using anti-GFP serum revealed that the OXI1-YFP protein was present at a low level in the cytosol and undetectable in the membrane fraction (Figure 3.15 D). Furthermore, subcellular fractionation of Arabidopsis root cultures of 35S::OXI1-YFP to enrich for OXI1-YFP, since the *OXI1* gene expression was found to be high in the roots when seedlings were grown on plant nutrient media containing ordinary agar (Rentel *et al.*, 2004), produced similar results (Figure 3.15 E). Therefore the data presented here strongly suggest that OXI1-YFP is localised in the cytoplasm and not anchored to the membrane.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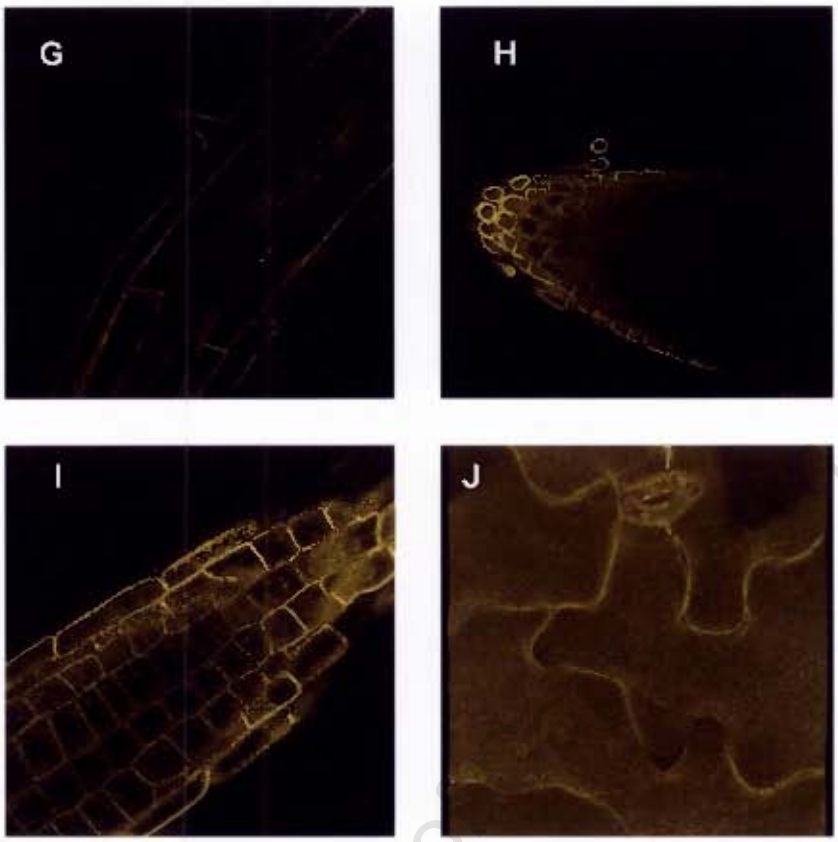

Figure 3.14 Localisation of OXI1-YFP is mainly cytosolic and expression occurs throughout 35S::OXI1-YFP transgenic Arabidopsis seedlings.

Laser confocal microscopy images of Ws-2 (A and B) and stably transformed 35S::OXI1-YFP (C-J) 5 day old Arabidopsis seedlings. Faint autofluorescence is visualised in Ws-2 roots (B) but absent in the cotyledons (A) using the same settings on the confocal microscope to detect the OXI1-YFP fluorescent protein. Weak expression of OXI1-YFP is detectable in the cotyledons (C, D and J), trichomes (E and F) and roots (G-I) of 35S::OXI1-YFP transgenic seedlings. The presence of transvacuolar strands (red arrow in D) indicates a cytosolic localisation. J represents a 3-D projection of cotyledons expressing OXI1-YFP in the cytosol. Exclusion of OXI1-YFP from the nucleus may suggest a plasma membrane localisation as well (C and D). All images were obtained using the C-Apochromat 40x objective at an excitation wavelength of 514 nm and emission window of 527-563 nm.

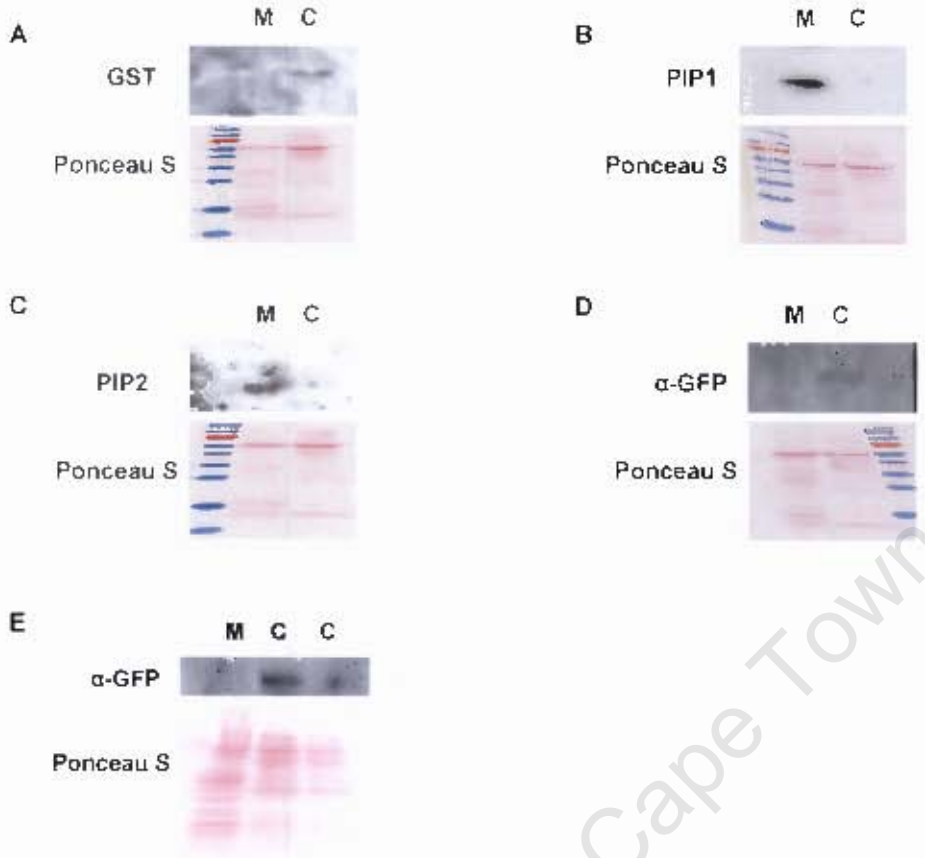

Figure 3.15 Subcellular fractionation illustrates cytosolic OXI1-YFP localisation

35S::OXI1-YFP transgenic Arabidopsis seedlings (A-D) or Arabidopsis root cultures (E) were separated into membrane (M) and cytosolic (C) fractions through Ultracentrifugation. Immunoblot analysis was performed to determine purity of fractions and in which fraction OXI1-YFP was being expressed. Anti-GST1 (A) was used as the cytosolic protein control, anti-PIP1 (B) and anti-PIP2 (C) detected the membrane proteins PIP1 and PIP2, and anti-GFP serum that recognised OXI1-YFP (D and E) were used at dilutions of 1:1000, 1:5000, 1:3000 and 1:125 respectively. Pure fractions were obtained since cytosolic and membrane proteins were only detected in the cytosolic or membrane fractions respectively. The Ponceau S stained membranes were used as loading controls.

3.8 Does OXI1 negatively regulate its own promoter?

It has been observed that the absolute level of induction of the *oxi1* mutant transcript is comparatively higher than that of the wild type *OXI1* gene in response to stimulus (Figure 3.4 and Rentel (2002)) therefore the lack of OXI1 protein results in increased OXI1 transcript. Additionally, microarray data analysis revealed that treatment of Arabidopsis seedlings for 3 hrs with 10 µM CHX, which inhibits translation, induced *OXI1* gene expression by more than 100 fold (Figure 3.8 C) suggesting that a protein repressor (with a short half life) of *OXI1* may exist and possibly requires OXI1 protein to be activated. Therefore the consequent lack of OXI1 protein in the *oxi1* mutant and during treatment with CHX may possibly be responsible for the heightened *OXI1* transcription that is if OXI1 exerts negative regulation either directly or indirectly on its own promoter.

To address this theory, the level of induction of both the *oxi1* mutant and *OXI1* wild type mRNA transcripts in response to cellulase treatment were monitored in *oxi1* mutant lines complemented with the wild type *OXI1* gene. Presumably if OXI1 protein kinase negatively regulates its own promoter either directly or indirectly through activation of repressor proteins, then the level of induction of the *oxi1* mutant transcript in response to stress treatment should be reduced in the *oxi1* complemented line in comparison to the *oxi1* knockout. This reduction of the *oxi1* mutant transcript would be attributed to the expression of wild type OXI1 protein now present in the *oxi1* complemented line. A considerable reduction in the amount of *oxi1* mutant transcript was observed in ten day old seedlings of two independent *oxi1* complemented lines in response to cellulase treatment compared to those levels obtained in the *oxi1* null mutant (Figure 3.16). However, it was found that this reduction was not consistent in the complemented lines between various experiments (data not shown). Furthermore the wild type *OXI1* gene, which contains more than 2 kb of sequence upstream of the predicted ATG, was strongly induced in both *oxi1* complemented lines upon cellulase treatment compared to the wild type Ws-2 control. It should be noted that weak induction of the *OXI1* gene had occurred in the Ws-2 sample (Figure 3.16) but it could only be detected at exposure times which resulted in over exposure of the *oxi1* mutant and complemented *OXI1* transcripts in the other transgenic lines (data not shown). Therefore it is more likely that OXI1 protein kinase does not negatively regulate its own promoter and the increased

expression of the complemented *OXI1* gene may be due to position effect of the inserted construct. Additionally the increased expression of the *oxi1* mutant transcript might be due to increased transcript stability

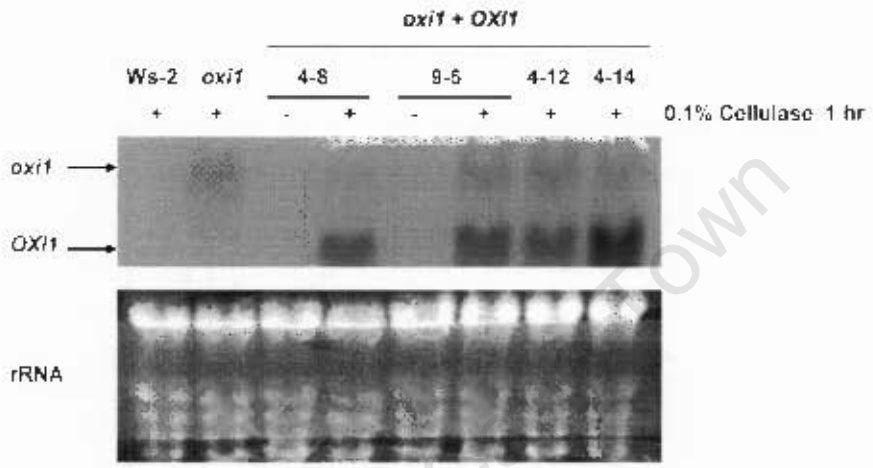

Figure 3.16 Does OXI1 protein kinase negatively regulate its own promoter?

Ten day old seedlings of genotypes Ws-2 (wild type), *oxi1* null mutant and the homozygous *oxi1* complemented lines (*oxi1* + *OXI1*) 4-8, 9-5, 4-12 and 4-14 treated with 0.1% cellulase (+) or H_2O (-) for 1 hour. A full length *OXI1* genomic DNA probe was used to determine the level of induction of the *oxi1* mutant and the *OXI1* wild type transcript. The ethidium stained rRNA was used as a loading control.

3.9 OXI1 protein has a short half-life

Post transcriptional and post translational processes both serve to control changes in gene expression thus an increase at the transcriptional level does not necessarily reflect increased protein synthesis. It is important to establish whether induction of *OXI1* gene expression results in increased protein production or activity. Previously it has been demonstrated that OXI1 protein kinase is activated by H_2O_2 and cellulase treatment within 5 and 15 min respectively, while no induction of the OXI1 protein using an affinity purified OXI1 antibody was detected in that time frame (Rentel *et al.*, 2004). Numerous attempts to utilise the anti-OXI1 serum to further study protein regulation of OXI1 both in wild type plants and transgenic Arabidopsis overexpressing OXI1 has been met with little success. This is again indicative that OXI1 protein may be present at very low levels in plant extracts and subject to tight control since OXI1 protein cannot be consistently detected even in the 35S::OXI1 transgenic lines which has a relatively high amount of *OXI1* transcript constitutively present (Figure 3.8 A).

Employing a slightly different approach, a commercial full length GFP antibody was utilised to detect the OXI1-YFP protein fusion expressed in stably transformed 35S::OXI1-YFP transgenic lines. Transgenic seedlings constitutively expressing a 56 kD YFP-aequorin protein fusion, targeted to specific cell types of the Arabidopsis root, (Kiegle *et al.*, 2000) and Ws-2 seedlings were used as positive and negative controls for the GFP antibody respectively. Western analysis illustrated that the 35S::OXI1-YFP transgenic lines expressed an intact OXI1-YFP protein fusion of the expected size of 77 kD which is absent in the Ws-2 control samples (Figure 3.17 A). This initial western analysis revealed that in Arabidopsis seedlings OXI1-YFP protein is present at a low steady state level and is induced by cellulase within 10 min of treatment and still detectable 1 hour post treatment (Figure 3.17 A). However, several subsequent western analyses with appropriate water controls failed to repeat this observation that cellulase induces expression of OXI1-YFP protein. Instead in most cases a marginal rise in OXI1-YFP protein from a low basal level is observed in the water controls at all time points tested (Figure 3.17 B and C). Interestingly, cellulase appeared to be causing the degradation of OXI1-YFP protein compared to the water controls as early as 15 min post treatment and this degradation was consistent amongst several experiments (Figure 3.17 B and C). Perhaps low levels of OXI1 are sufficient to transduce the

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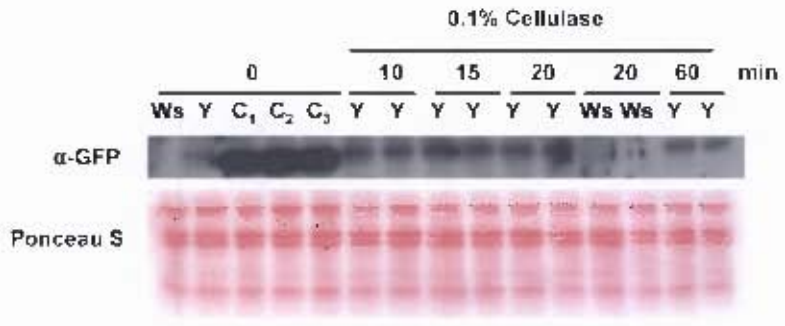

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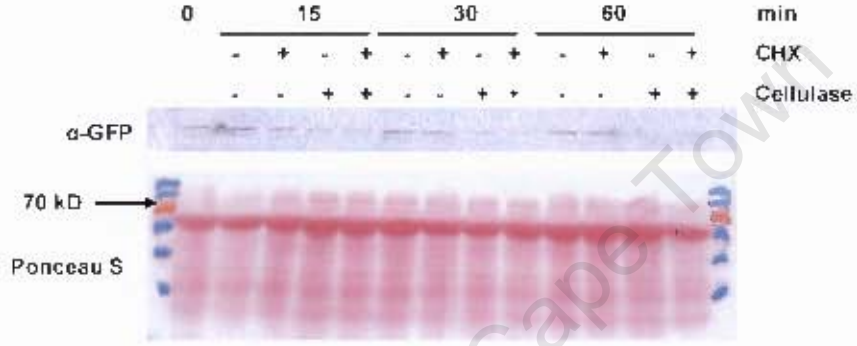

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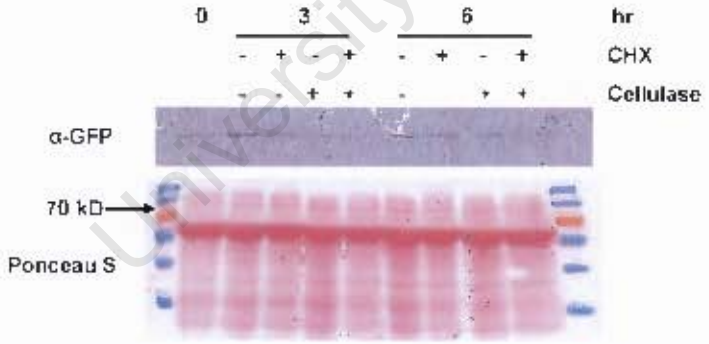

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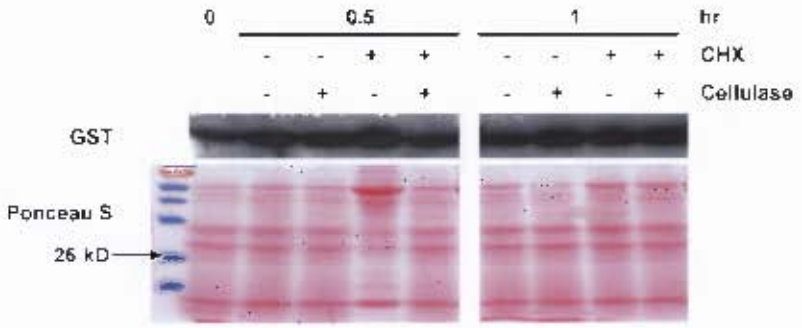

Figure 3.17 OXI1 protein targeted for degradation

(A) Immunoblot analysis of ten day old seedlings of the genotypes Ws-2 (Ws), 35S::OXI1-YFP (Y) and transgenic seedlings expressing a YFP-aequorin protein fusion targeted to the epidermis (C_1), elongation zone cortex and epidermis (C_2) and pericycle (C_3) of the Arabidopsis root showing induction of OXI1-YFP. Seedlings were harvested at Time 0 hrs (0) or treated with 0.1% cellulase for the time indicated. (B & C) Immunoblot analysis of ten day old 35S::OXI1-YFP seedlings illustrating degradation of OXI1-YFP protein by cellulase treatment. (D) Immunoblot analysis of ten day old 35S::OXI1-YFP seedlings indicating that GST exhibits constitutive protein expression and that GST protein expression is unaffected by CHX pre-treatment. (B, C and D) Seedlings were left in water (-/-), treated with 50 µM CHX or 0.1% cellulase and harvested at the times indicated. In the case of double treatments, seedlings were subjected to 50 µM CHX for 30 min prior to 0.1% cellulase treatment. A pre-stained protein molecular weight marker was loaded onto each gel to determine correct size of OXI1-YFP and GST, which should be detected around the 70 kD (B and C) and 26 kD (D) bands respectively as indicated by the arrow for each gel. The GFP and GST antibodies were used at a 1:125 (A-C) and 1:1000 dilution (D) respectively. The Ponceau S stained membranes were used as loading controls.

signalling cascades elicited by cellulase treatment and degradation of OXI1 is required to turn off these pathways.

In order to provide insight into the regulation of OXI1-YFP protein in terms of rate of synthesis and/or protein stability, chemical inhibition of protein synthesis was employed. CHX is an inhibitor of translation and has been used successfully in Arabidopsis to prevent protein synthesis (Navarro *et al.*, 2004). If OXI1-YFP is subject to a high or fast rate of synthesis, it would be expected that the control protein levels (i.e. H_2O samples) would decrease in the presence of CHX. Alternatively, if the reduction of OXI1-YFP in response to cellulase is dependent on the expression or activity of another protein and OXI1-YFP itself has a low rate of synthesis, then OXI1-YFP might accumulate if CHX prevents expression of this putative negative regulator. Here it was observed that OXI1-YFP protein was detectable up to 6 hours in the presence of 50 µM CHX to a level which was comparable to that of the H_2O controls (Figure 3.17 C). This result suggests that OXI1-YFP possibly has a low rate of synthesis. Furthermore, CHX pre-treatment did not affect the reduction of OXI1-YFP in response to cellulase treatment indicative that *de novo* protein synthesis of another protein was not required to target OXI1-YFP for degradation during cellulase treatment (Figure 3.17 B and C). The expression profile of the antioxidant protein Glutathione-S-transferase 1 (GST1) was investigated to determine whether the conditions employed resulted in inhibition of protein synthesis. GST1 was chosen since it has been suggested that cellulase treatment mimics a wound response because it breaks down the cell wall and *GST1* mRNA accumulated within 1 hour in response to wounding (Rentel, 2002). GST1 was constitutively expressed at a very high level in the 35S::OXI1-YFP transgenic line and its expression was unaffected in response to both cellulase and CHX treatment (Figure 3.17 D). This result was consistent between two independent experiments. Inhibition of protein synthesis has not been illustrated by GST1 expression presumably because GST1 is a stable protein and *de novo* protein synthesis is not required, during the time course tested, to maintain the high level of expression. However, the lack of change in GST1 protein levels in response to cellulase treatment does illustrate that the degradation of OXI1-YFP by cellulase treatment is specific to the regulation of OXI1-YFP protein and not a general phenomenon of proteins in response to cellulase treatment. It should also be noted that, unlike GST1, the results obtained with CHX treatment in relation to OXI1-YFP protein were extremely variable amongst different experiments. In some instances no OXI1-YFP

protein was detected in the H_2O or CHX samples and in other cases CHX treatment both with and without cellulase displayed a higher level of OXI1-YFP expression in comparison to H_2O and cellulase treatment alone (Figure 3.18 A). This latter observation disputes the previous finding that cellulase treatment reduces OXI1-YFP independent of CHX treatment and suggests that there may indeed be a negative regulator of OXI1-YFP protein following cellulase treatment. The variability between different CHX experiments could possibly be due to the low level of OXI1-YFP expression, which is near the limit of detection together with the fact that OXI1 may be subject to different types of regulation to maintain this low expression level. Therefore it is difficult to draw any meaningful conclusions about the rate of OXI1 protein synthesis or the influence of other proteins on OXI1-YFP expression based on these experiments with CHX, particularly since inhibition of protein synthesis under these experimental conditions could not be demonstrated.

The Ub/26S proteasome pathway has emerged as an important process causing the degradation of constituent proteins and is vital for maintaining the cellular integrity of the plant cell (Vierstra, 2003). The proteasome inhibitor MG132 has been used successfully to prevent protein degradation in Arabidopsis (Planchais *et al.*, 2004). It was investigated whether the proteasome was responsible for the degradation of OXI1-YFP by cellulase treatment. Pre-treatment of 35S::OXI1-YFP seedlings with MG132 resulted in a marked increase in the amount of OXI1-YFP protein, within one hour, not only in the cellulase treated sample but also with MG132 alone in comparison to the water control (Figure 3.18 A). The latter observation demonstrated for the first time that OXI1-YFP protein has a short half-life. Additionally, the proteasome degradation pathway might be partially responsible for the degradation of OXI1-YFP by cellulase treatment since the level of OXI1-YFP expression protein (in the cellulase and MG132 treated sample) was still reduced compared to that of MG132 treatment alone (Figure 3.18 A).

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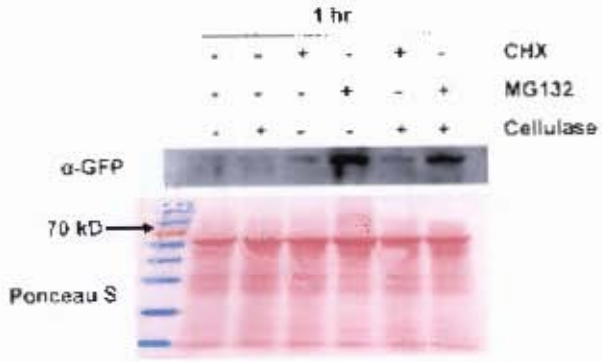

35S::OXI1-YFP

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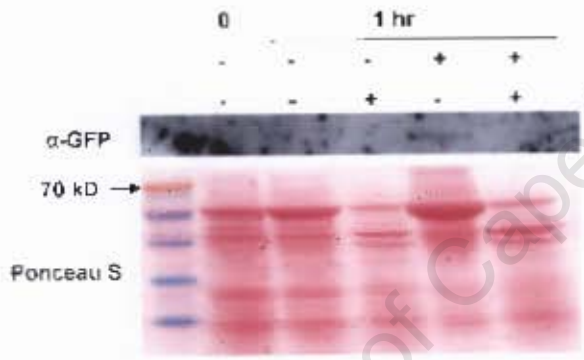

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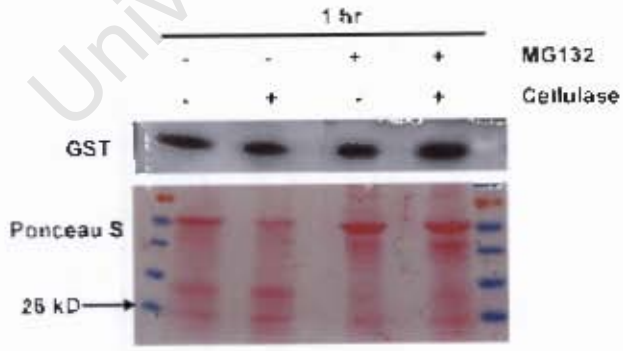

35S::OXI1-YFP

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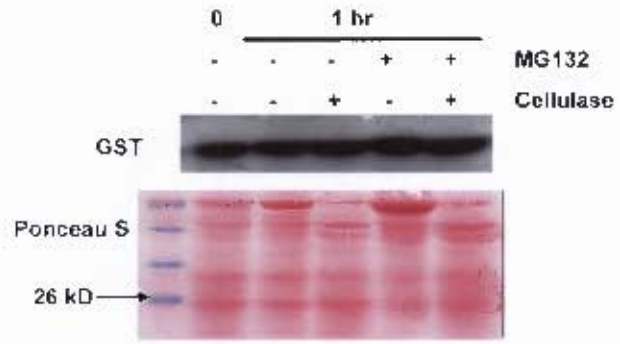


OXI1::OXI1-YFP

Figure 3.18 OXI1-YFP protein under control of either the native OXI1 promoter or the 35S CaMV promoter has a fast degradation rate

Immonublot analysis of ten day old 35S::OXI1-YFP and OXI1::OXI1-YFP seedlings illustrating that OXI1-YFP protein has a short half life since OXI1-YFP protein levels are increased within 1 hour, following inhibition of the proteasome by MG132 (A and B). Seedlings were left in water (-/-/-), treated with 100 µM MG132, 50 µM CHX (A only) or 0.1% cellulase and harvested after 1 hour (A-D). In the case of double treatments, seedlings were subjected to either 50 µM CHX or 100 µM MG132 for 1 hour prior to 0.1% cellulase treatment. A pre-stained protein molecular weight marker was loaded onto each gel to determine the correct bands corresponding to OXI1-YFP (70 kD) and GST (26 kD). GST has a slow degradation rate since MG132 treatment does not significantly increase GST protein levels (C and D). The GFP and GST antibodies were used at a 1:125 dilution (A and B) and 1:1000 dilution (C and D) respectively. The Ponceau S stained membranes were used as controls for loading. This experiment was performed twice with similar results.

As mentioned earlier, previous attempts to detect OXI1-YFP protein under the control of a 1.6 kb fragment of the OXI1 promoter in plant extracts proved unsuccessful (Rentel, 2002). This is not surprising given that overexpression of OXI1-YFP using the 35S CaMV promoter did not yield, as usually seen with proteins expressed under the control of this strong promoter, a dramatic increase in OXI1-YFP levels together with the observation that cellulase caused degradation of OXI1-YFP. Application of MG132 to OXI1::OXI1-YFP transgenic seedlings resulted in very weak expression of OXI1-YFP and as before no OXI1-YFP protein was detected in the H_2O or cellulase treated samples (Figure 3.18 B). The absence of OXI1-YFP in the cellulase sample pre-treated with MG132 might be due to the difference in protein loading (Figure 3.18 B), however it is more likely that OXI1-YFP would not be detected in this sample since the level of OXI1-YFP without MG132 is beyond detection in this transgenic line and furthermore cellulase would cause degradation of OXI1-YFP. Interestingly, the expression of GST1 was not significantly increased in either the 35S::OXI1-YFP or OXI1::OXI1-YFP transgenic line after 1 hour with MG132 treatment (Figure 3.18 C and D). Therefore GST1 has a much lower degradation rate than OXI1-YFP and it also backs up the earlier observation based on the CHX experiments that GST1 is a stable protein.

In general the above data suggest that OXI1 has a short half-life and is subject to degradation by the proteasome. Transcription of OXI1-YFP is high in the 35S::OXI1-YFP transgenic line (Figure 3.8) therefore OXI1-YFP is most likely post transcriptionally regulated either due to a low rate of translation or high rate of degradation to maintain low protein levels. Furthermore, the fast degradation of OXI1-YFP in response to cellulase treatment is most likely due to the regulation of OXI1 signalling during this response.

3.10 A 2-D SDS PAGE approach to identify targets of OXI1 protein kinase

It was established that OXI1 was required for the full activation of MPK3 and MPK6 in response to both H_2O_2 and cellulase treatment (Rentel *et al.*, 2004). However, it is not known whether the interaction of OXI1 with MPK3 and MPK6 is direct or indirect. In an attempt to find targets of OXI1 2-D SDS PAGE analysis was performed. In this experiment the *oxi1* complemented line which represents wild type Arabidopsis, since the root hair phenotype and susceptibility to *H. parasitica* of the *oxi1* mutant was rescued in this line (Rentel *et al.*, 2004), and the *oxi1* mutant were used. Ten day old seedlings were treated with H_2O or 0.1% cellulase for 30 min. This time point was chosen since OXI1 protein kinase was shown to be active within 15 min following 0.1% cellulase treatment (Rentel *et al.*, 2004) therefore to allow for signalling events, such as phosphorylation cascades, to occur samples were harvested 15 min later. Furthermore, even though OXI1-YFP was demonstrated to be targeted for degradation by the proteasome in response to cellulase treatment, it was not fully degraded within 30 min hence an OXI1-mediated signalling cascade was still likely to occur within this time frame (Figure 3.17). In order to perform statistical analysis on the results, the experiment was performed three times and 2-D SDS PAGE of all 3 biological replicates was carried out on the same day to minimise gel to gel variation.

The Pro-Q® Diamond Phosphoprotein Gel stain was employed to selectively stain for phosphorylated proteins (Figure 3.19 A) and Colloidal Coomassie was used to stain for total protein (Figure 3.19 B). To identify differentially phosphorylated proteins between H_2O and cellulase treated samples for each genotype, the volume of each protein spot was normalised against the total spot density of that gel, and the phosphorylation intensity for each protein spot was expressed relative to the total amount of protein for that specific spot as determined by coomassie staining. This analysis accounts for the fact that increased phosphorylation intensity may be due to protein abundance. A total of six phosphorylated proteins were identified to be differentially phosphorylated between H_2O and cellulase treated samples (Figure 3.20 A). However, only three of these proteins displayed differential phosphorylation intensities between the *oxi1* mutant and the *oxi1* complemented line (Figure 3.20 A, spots 3-5). Interestingly, one of these proteins was identified as being the ATPase α-subunit (atpA) present in the choloroplast genome (Table 3.5). The activity of ATPases has been reported to have an important

role in chloroplast proteolysis (Adam and Clarke, 2002). The phosphorylation intensity of atpA was increased in response to cellulase treatment in the *oxi1* complemented line but was reduced in the *oxi1* mutant (Figure 3.20 A, spot 5). This indicates that the activity of OXI1, either directly or indirectly, is required for phosphorylation of atpA. Cellulase treatment also triggered phosphorylation changes of proteins independent of OXI1 since differences in phosphorylation intensities of the remaining 3 proteins occurred to a similar extent in both genotypes (Figure 3.20 A, spots 1,2 and 6). MS analysis revealed a chloroplast RNA-binding protein displayed reduced phosphorylation in response to cellulase treatment (Table 3.5 and Figure 3.20 A, spot 6). This presents an interesting finding since chloroplastic RNA binding proteins have been shown to play a role in mRNA stability (Nakamura *et al.*, 2001). The ability of RNA-binding proteins to regulate mRNA stability may be dependent on the phosphorylation status of the RNA-binding protein. Therefore cellulase treatment may be causing down regulation of chloroplastic proteins by (i) increasing proteolysis, which may in part require OXI1 to phosphorylate atpA and (ii) decreasing mRNA stability through inhibition of the activity of RNA binding proteins thereby limiting protein synthesis. Protein identities of the other four phosphorylated proteins could not be determined either due to insufficient protein for MS analysis or the peptide fragments obtained did not match any known proteins in the database.

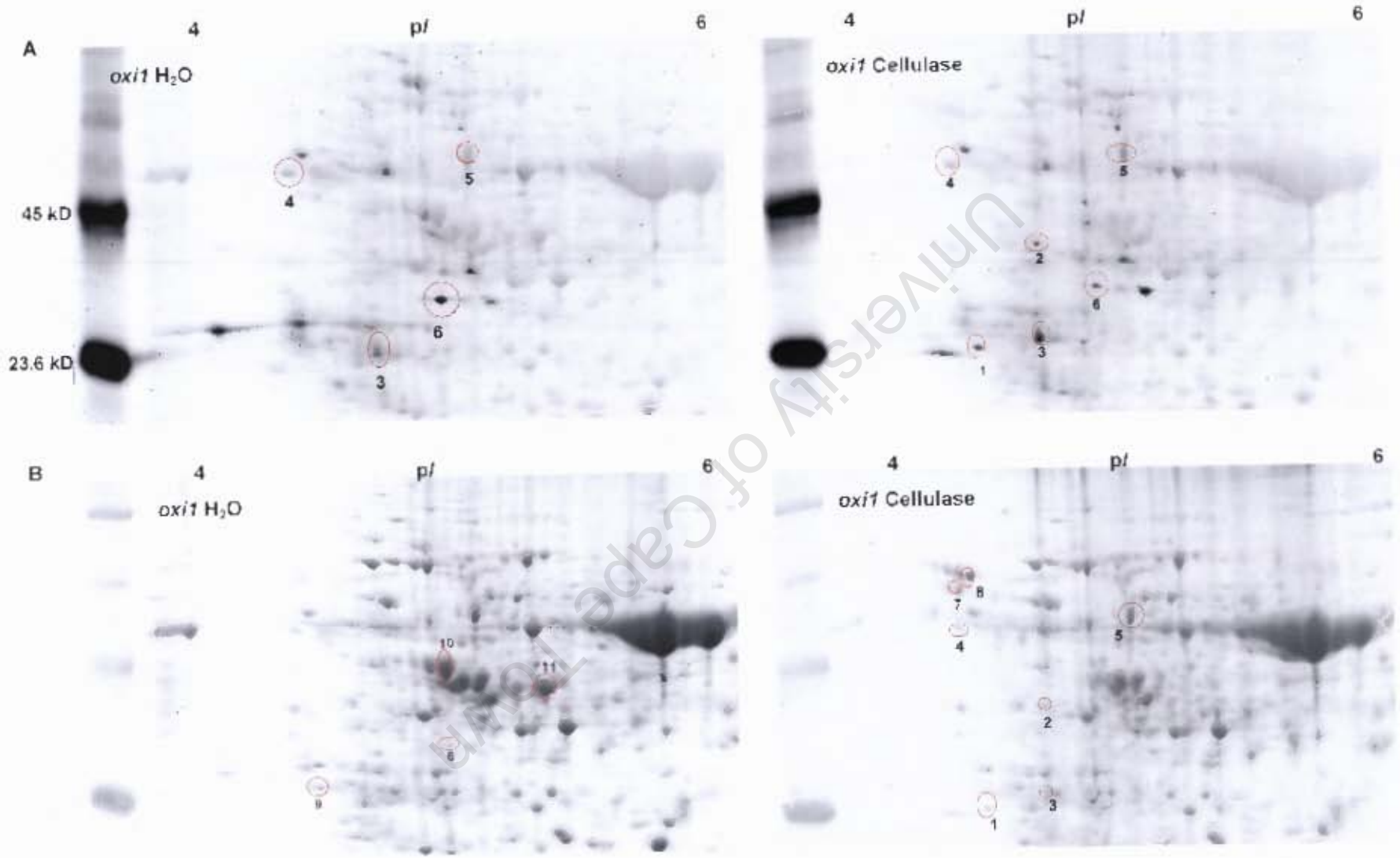
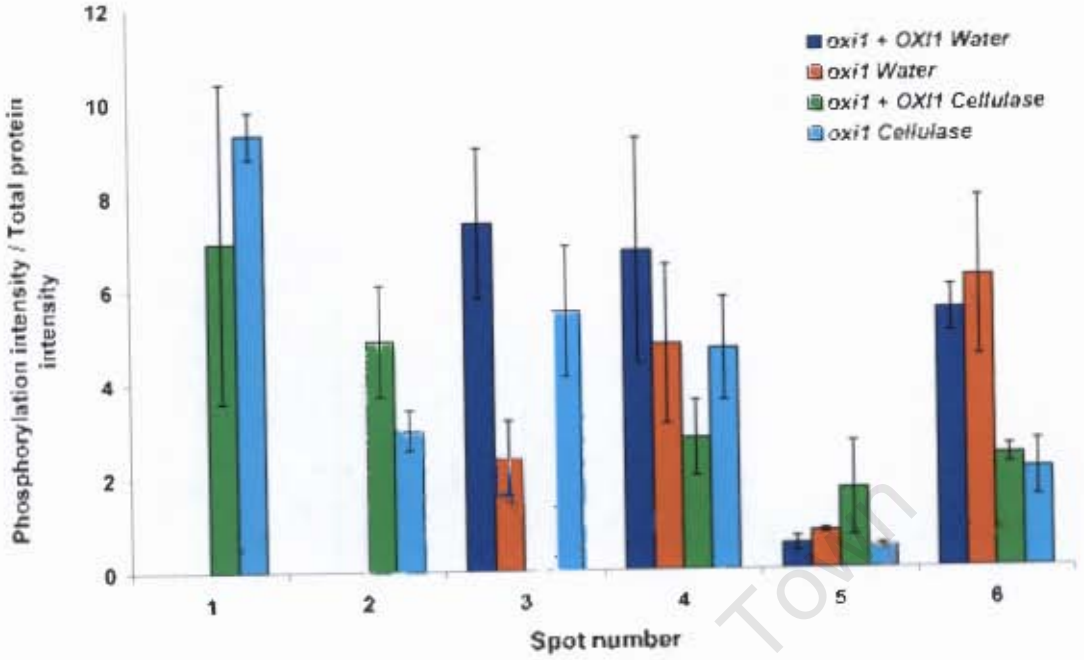


Figure 3.19 2-D SDS PAGE analysis of OXI1 following cellulase treatment

Ten day old seedlings of the *oxi1* knockout and *oxi1* complemented line were treated with either H_2O or 0.1% cellulase for 30 min. Each treatment was performed on 40 seedlings per genotype, in triplicate. 2-D SDS PAGE analysis was performed in an attempt to identify phosphorylated and total proteins that were differentially regulated in the *oxi1* complement line versus the *oxi1* knockout. Both the *oxi1* knockout and *oxi1* complemented line yielded practically identical profiles therefore the *oxi1* knockout gels are shown as representative samples. (A) Proteins were stained with Pro-Q® Diamond Phosphoprotein gel stain to identify phosphorylated proteins. Those phosphorylated proteins which are differentially expressed between the H_2O and cellulase treated samples are circled in red and numbered. The one dimensional lane on each gel represents the Peppermint stick phosphoprotein molecular weight standards (45 and 23.6 kD) and the double lane in (B) is due to overloading of protein on the 1-D IPG strip. (B) The Phosphoprotein stained gels were subsequently stained with Colloidal coomassie to determine the amount of total protein present on each gel as well as to identify whether there were any proteins whose expression level was differentially regulated by OXI1. Those proteins that are differentially expressed in the *oxi1* H_2O sample versus the cellulase treated samples are numbered and circled in red. The p*I* of each gel was in the approximate range of 4 to 6 as indicated above each gel.

A



B

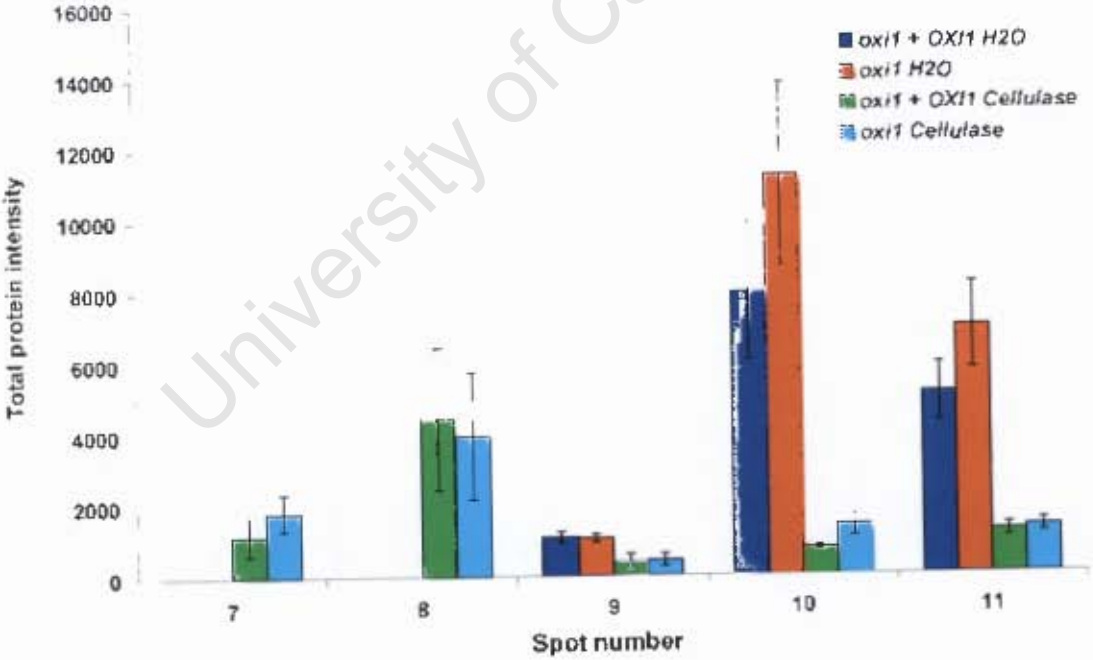


Figure 3.20 Comparison of relative signal intensities of phosphorylated and total proteins between the *oxi1* complemented line and the *oxi1* knockout

The Phosphoprotein and Colloidal Coomassie stained 2-D SDS PAGE gels were analysed using PDQuest software. (A) Each phosphoprotein stained gel was normalised against the total spot density of the gel and differentially phosphorylated proteins between the four samples (i.e. *oxi1* + *OXI1* complemented line and oxi1 knockout treated with H_2O or 0.1% cellulase for 30 min) were expressed relative to the abundance of those specific proteins. This form of analysis takes into account that the higher phosphorylated signal for a specific protein spot may be due to the fact that there may be more protein present for that spot. The bars represent the phosphorylation intensity of a specific protein relative to the abundance of that protein; an average of 3 samples and the standard error is shown. Protein 5 is increased and decreased in phosphorylation intensity in the *oxi1* complemented line and *oxi1* knockout respectively and may be a potential target of OXI1. (B) Colloidal Coomassie stained gels were normalised against total spot density of the gels. The bars represent total intensity of non-phosphorylated proteins; an average of 3 samples and the standard error is shown. Non-phosphorylated proteins 7 and 8 were induced by cellulase treatment whereas proteins 9-11 were down regulated in response to cellulase treatment in both genotypes. See Figure 3.19 for location of protein spots represented here.

Cellulase treatment also rendered changes in abundance of possibly non-phosphorylated proteins, which were all independent of OXI1 (Figure 3.20 B). Two proteins were induced whereas three proteins were decreased in response to cellulase treatment in both genotypes. MS analysis identified the elongation factor 1B α-subunit and rubisco activase as the proteins down regulated by cellulase treatment (Table 3.5). The former evidence is line with cellulase treatment resulting in down regulation of proteins since elongation factors are responsible for the elongation process during protein synthesis (Héricourt and Jupin, 1999). Rubisco activase exits in two isoforms arising from alternative splicing and both isoforms are capable of regulating the activity of rubisco (Zhang and Portis, 1999). Cellulase treatment results in reduction of both isoforms of rubisco activase which suggest that rubisco is possibly inactivated hence decreasing photosynthesis in response to cellulase. Alternatively, it is possible that the down regulation of chloroplastic proteins identified in this study is due to the indirect effect of cellulase treatment causing a change in the redox state of the plant cell since the chloroplast is sensitive to redox changes. The identity of the proteins up regulated in response to cellulase treatment could not be identified. Although, this 2-D SDS PAGE analysis together with the Pro-Q® Diamond phosphoprotein stain has provided some insight into the proteins and possible cellular processes regulated by cellulase treatment, it has not proved a very useful technique in the identification of phosphorylated targets of OXI1 protein kinase.

Table 3.5 Identification of proteins that were differentially regulated in response to cellulase by Mass Spectrometry

Spot number	Gene ID	Protein ID/Description	Molecular Weight
5	844790*	ATPase α-subunit chloroplast genome	55.2 kD
6	At4g24770	RNA-binding Protein 3 chloroplast	31 kD
9	At5g19510	Elongation factor 1B α-subunit	24 kD
10	At2g39730	Rubisco activase	46 kD
11	At2g39730	Rubisco activase	43 kD

* Chloroplast gene

3.11 Discussion

3.11.1 *OXI1* expression does not correlate with an essential function

Microarray analysis indicated that *OXI1* was expressed in response to a variety of stresses associated with an increase in AOS, for example, salt, cold, heat and the bacterial pathogen *P. syringae* (Figure 3.9 and Table 3.1). These findings were supported by previous work (Rentel, 2002) and northern analysis performed in this study (Figure 3.10). Protein kinases are common elements in signal transduction pathways (Cheng *et al.*, 2002; Bögre *et al.*, 2003; Pitzschke and Hirt, 2006) therefore the increased expression of *OXI1* in response to several stimuli suggest that OXI1 may a have a role in the plant response to these stresses.

OXI1 expression was one of the highest genes induced during salt stress but interestingly the *oxi1* mutant was no more impaired than wild type in salt tolerance. Similarly, examination of the effect of heat stress on the *oxi1* mutant yielded no difference in heat tolerance levels in comparison to wild type. The lack of phenotype of the *oxi1* mutant in response to stresses that causes induction of the *OXI1* gene has two important implications. Firstly, it shows the importance of empirically determining the significance of gene expression data through mutational, genetic and/or biochemical data to gain an understanding of gene function. Secondly, it raises the question as to why would *OXI1* be upregulated in response to stress without playing an essential role. There are two possibilities, *OXI1* expression in response to these AOS generating stimuli is an indirect effect and therefore has no biological relevance or *OXI1* expression is of biological relevance but it is not essential or functionally redundant in that process. Although, proving the latter hypothesis would be difficult it is conceptually possible. For example, signalling pathways conferring basal and acquired thermotolerance to heat stress involves a diverse set of components including heat shock proteins, calcium, abscisic acid, ET and SA (Larkindale and Knight, 2002; Baniwal *et al.*, 2004; Larkindale *et al.*, 2005). *OXI1* gene expression also correlated with the expression of *HSF4*, calcium binding proteins such as calmodulin and calcium dependent protein kinases, and the *ethylene-responsive element-binding protein 1* (Tables 3.2-3.4). Therefore it is also possible that OXI1 may function in a heat induced signal transduction pathway but owing to the complexity of processes involved in establishing basal and acquired

thermotolerance, other compensatory mechanisms are activated in the *oxi1* mutant hence it acts as wild type in response to heat stress.

A role for OXI1 in auxin signalling was first postulated with the demonstration that auxin induced the activity of OXI1 in Arabidopsis cell culture with maximal activity occurring around 10 hrs (Anthony *et al.*, 2004). However, auxin failed to induce *OXI1* gene expression in Arabidopsis seedlings (Rentel, 2002) while microarray data illustrated that the auxin transport inhibitor TIBA induced *OXI1* expression 17 fold (Table 3.1). The lack of induction of *OXI1* by auxin in whole seedlings was most likely due to the time at which gene expression was investigated i.e. 1 hour, alternatively auxin might have caused an increase in OXI1 activity but not an increase in expression. The induction of *OXI1* by TIBA may be an indirect effect of the chemical since other inhibitors of auxin transport within the same microarray experiment did not induce *OXI1* expression. Therefore it is likely that OXI1 could play a role in auxin signalling particularly since microarray analysis show that several auxin responsive genes have reduced expression in the *oxi1* mutant in comparison to wild type in response to H_2O_2 (Rentel, 2002). Additionally, in this report it was demonstrated that gene expression of an auxin responsive GH3 family protein correlated with *OXI1* expression (Table 3.4). Given the requirement for OXI1 in normal root hair development under conditions of stress (Rentel, 2002; Anthony *et al.*, 2004) and the role of auxin and H_2O_2 in root development and gravitropism (Joo *et al.*, 2001) OXI1 may indeed be involved in these auxin responsive pathways but further investigation is required. However, considering the complexity of auxin signalling and cross talk between pathways such a role for OXI1 may not be discernable.

Interestingly *OXI1* gene expression was induced in response to virulent, avirulent and non-host strains of the bacterial pathogen *P. syringae* (Figure 3.9) and together with the involvement of OXI1 in basal resistance to *H. parasitica* (Rentel *et al.*, 2004), points to a role for OXI1 in disease resistance to a range of pathogens. Additionally expression of a number of characterised genes involved in defence related processes such as *WRKY6*, *CCR2*, *SYP122* and two putative *GST* genes correlated with that of *OXI1* (Table 3.2). Therefore OXI1 protein kinase may indeed be vital for the establishment of plant defence responses to pathogen attack and a more detailed analysis is described in Chapter 4.

Despite transcriptional increases in *OXI1* gene expression in response a variety of conditions a functional link for OXI1 in some of these processes could not be established. It has been difficult to map OXI1 to signal transduction networks because it did not strongly correlate with any defined set of genes known to be involved in a particular pathway, even when all or different subsets of microarray experiments were used to perform correlation studies. Added to that is the difficulty in determining cut off values for correlation coefficients which represent significant correlation. In one report correlation values of 0.9 were considered statistically significant because most other ribosomal proteins displayed such values when a ribosomal gene was used as the driver (Jen *et al.*, 2006). Nonetheless, any gene which shows strong correlation with another would still need to be proved experimentally. Of course there is the possibility that genes which actually do regulate biological processes in concert with OXI1 may not be identified during correlation studies if their expression is constitutive or not co-expressed with *OXI1*. An alternative strategy to find a biological role for *OXI1* gene expression would be to identify proteins that either interact with or are phosphorylated by OXI1 perhaps through yeast-2-hybrid or immunoprecipitation pull down assays.

3.11.2 A cytosolic localisation for OXI1

Overexpression of an OXI1-YFP protein fusion in tobacco and Arabidopsis revealed a cytosolic localisation for OXI1 protein kinase through confocal microscopy and was confirmed by subcellular fractionation studies (Figures 3.13 to 3.15). Cellulase treatment does not cause translocation of OXI1-YFP protein suggestive that not only is OXI1 located in the cytosol but the site of OXI1 action is also cytosolic. This conclusion has further implications in that the presumable dependence on OXI1 for the phosphorylation of the chloroplastic ATPase α-subunit, as identified by 2-D SDS PAGE gel analysis and MS (Figure 3.20 A and Table 3.5), in response to cellulase treatment is most likely indirect requiring either a single or multiple intermediary protein kinases. Furthermore, OXI1-YFP expression was not restricted to a particular cell type or part of the plant since expression occurred in all cell types in both root and shoot tissue. Taken together with its cytosolic localisation and the accumulation of *OXI1* mRNA transcript in response to different AOS generating stimuli strengthens a possible role for OXI1 as a signal transducer. The challenge lies in determining the significance of OXI1 mediated signal

transduction cascades particularly due to lack of phenotype in the *oxi1* mutant in response to various stresses inducing *OXI1* gene expression.

A previous report has suggested a developmentally regulated subcellular translocation for OXI1 during root hair growth (Anthony *et al.*, 2004). Constitutive expression of an N-terminal GFP-OXI1 in Arabidopsis demonstrated that localisation of GFP-OXI1 occurred on the internal surface of immature root hairs and as root hairs developed, GFP-OXI1 accumulated first at the tips and later around the apex of root hairs (Anthony *et al.*, 2004). GFP-OXI1 was also detected in nuclei at the base of mature root hairs (Anthony *et al.*, 2004). However, this study could not discern such a change in localisation patterns and generally weak expression of OXI1-YFP, in root hairs, was only detected along the internal surface of the root hair (Figure 3.14 G). Perhaps the difference in expression pattern of OXI1 is due to a difference either in the importance of OXI1 signalling between the two ecotypes, OXI1-YFP and GFP-OXI1 in Ws-2 and Col-0 background respectively, or laboratory growth conditions. Interestingly, even though expression of OXI1-YFP was usually weak, the signal intensity of OXI1-YFP at the root tip was in most occasions generally stronger in comparison to other parts of the root (Figure 3.14 H). Similarly GFP-OXI1 accumulated in the meristematic zones of the primary root tip (Anthony *et al.*, 2004). In conjunction with the observation that *OXI1* mRNA expression in response to H_2O_2 is calcium dependent and both H_2O_2 and $[Ca^{2+}]_c$ are required for root hair elongation as well as root gravitropism (Joo *et al.*, 2001; Rentel, 2002; Dolan and Davies, 2004), the aforementioned observations suggest OXI1 signalling may mediate tip growth of both roots and root hairs and requires further investigation.

It has also been shown that the phosphorylation and activation of OXI1 is dependent upon its interaction with the 3'-phosphoinositide-dependent protein kinase1 (PDK1) (Anthony *et al.*, 2004). PDK1 is a central regulator of lipid-derived signals in animal systems and a similar role is emerging in plants (Belham *et al.*, 1999; Peterson and Schreiber, 1999; Bögre *et al.*, 2003). PDK1 is recruited to the membrane through binding of its pleckstrin homology domain to membrane bound phosphoinositide lipids (Peterson and Schreiber, 1999). Therefore it is plausible that OXI1 may be translocated to the membrane, despite the lack of OXI1 translocation discerned in this study, during lipid-derived signalling and activation by PDK1 possibly through the putative myristoylation

sites within OXI1 given that PDK1 is the only Arabidopsis AGC kinase with an identifiable lipid binding domain (Bögre *et al.*, 2003).

3.11.3 Below the radar: regulation of OXI1 protein levels

The induction of *OXI1* in the presence of CHX suggests that *OXI* gene expression is potentially suppressed by a negative regulator (Figure 3.9 C). This potential negative regulator has a short half-life because CHX treatment was only applied for 3 hrs and *OXI1* gene expression increased more than 100 fold within that time frame. The increased expression of *oxi1* mutant transcript compared to wild type *OXI1* mRNA in response to stimulus (Rentel *et al.* (2004) and Figure 3.4) further suggest negative regulation of the *OXI1* gene and given that OXI1 has a short half-life points to a role for OXI1 protein in negatively regulating its own promoter. This study demonstrated that this negative regulation of the *OXI1* promoter was unlikely because complementation of the *oxi1* mutant with wild type *OXI1* did not consistently reduce the expression of *oxi1* mutant transcript and the complemented *OXI1* gene was also highly expressed. Furthermore, the *OXI1* complemented gene contains more than 2 kb sequence upstream of the start codon of *OXI1* thus it is likely to harbour most binding sites for potential transcriptional repressors (Rentel, 2002). Therefore the increased expression of *OXI1* complemented gene could be due to the position effect, in transcriptionally active chromosomal 'hot spots', or the actual copy number of the inserted transgene (Dean *et al.*, 1988; van Leeuwen *et al.*, 2001). The increased expression of the *oxi1* mutant transcript could result from increased mRNA stability, which could either be due to lack of recognition of *oxi1* mutant transcript by the basal mRNA degrading machinery or sequence specific controls which is absent in the mutant transcript (Gutiérrez *et al.*, 1999; Rentel, 2002).

Nonetheless, a definitive experiment to test for OXI1 negative regulation on its own promoter would be to cross the *OXI1* promoter-*GUS* transgenic line with the *oxi1* knockout as well as the 35S::OXI1 or 35S::OXI1-YFP lines and determine *OXI1* promoter function through GUS activity. If stronger GUS expression is observed in the *oxi1* mutant background in comparison to the OXI1::GUS transgenic line, under conditions known to induce large amounts of *OXI1* transcript, this would indicate negative regulation of OXI1 on its own promoter. Additional evidence for negative

regulation would be if GUS expression is fainter in the 35S::OXI1 transgenic line in comparison to the OXI1::GUS line. Negative regulation of WRKY6 on its own promoter was successfully demonstrated using the aforementioned approach (Robatzek and Somssich, 2002). WRKY6 positively regulates the expression of defence related genes and it was suggested that negative regulation of WRKY6 on its own promoter was established once a certain threshold of WRKY6 was reached (Robatzek and Somssich, 2002). This negative regulation of *WRKY6* gene expression, either directly or indirectly, may function to confine the plant responses to specific cell layers surrounding the pathogen therefore limiting the cost to the plant for mounting a defence response (Robatzek and Somssich, 2002). OXI1 may be able to mediate numerous signal transduction networks in response to a variety of stimuli even though biological significance may be questionable. Therefore it could be envisaged that OXI1 similarly to WRKY6 negatively regulates its own promoter as a control mechanism to 'switch off' and limit the cost of OXI1 activated signal transduction cascades. Recently, a transgenic line carrying the *OXI1* promoter fused to the firefly *luciferase* reporter gene has been generated (Dr Robert Ingle, Department of Molecular and Cell Biology, University of Cape Town) and similar experiments with the *oxi1* knockout and 35S::OXI1 could be performed with this *OXI1* promoter-reporter fusion.

An interesting observation is that the large increase in *OXI1* transcript in response to cellulase or overexpression of the *OXI1* gene is not mirrored by increased OXI1 protein levels. It appears that the rate of degradation of OXI1 is greater than the rate of OXI protein synthesis. This study demonstrated that OXI1-YFP has a short half life and was targeted for degradation by the proteasome (Figures 3.18). The significance of the rapid turnover of OXI1 is currently unknown. One possibility, as alluded to earlier, is that given the induction of *OXI1* by a host of AOS generating stimuli and the apparent lack of biological significance for OXI1 in cellular processes mediating stress tolerance, the high turnover rate may be to control the increased transcription (in response to AOS) and limit non essential activation of OXI1 signal transduction pathways. In response to stimuli, which do require a *bona fide* activation of OXI1 signal transduction pathways, either the simultaneous activation of other molecular components together with low levels of OXI1 or perhaps prolonged activation of OXI1 may be sufficient to transduce a signalling cascade. Once OXI1 protein is activated in response to a stimulus, it mediates a phosphorylation cascade and degradation of activated OXI1 may be required to dampen

the signal. Consequently, even though transcript levels increase, protein accumulation remains low due to the combination of the short half-life of OXI1 and the added degradation following activation of a signalling cascade. Although the rate of OXI1 protein synthesis is unknown, it may be that OXI1 has a slow rate of synthesis and this would also contribute to low levels of OXI1. The model above is consistent with what was observed in response to cellulase treatment. It was shown that *OXI1* gene expression is upregulated in response to cellulase and OXI1 protein is activated within 15 min of treatment (Rentel *et al.*, 2004). This study reported rapid degradation of OXI1 following cellulase treatment which was caused at least in part by the proteasome. It was also noted that OXI1-YFP protein levels in samples treated with cellulase and the proteasome inhibitor MG132 were still lower than seedlings treated with MG132 alone. This data suggest that other mechanisms for OXI1 degradation are activated, such as proteases, during cellulase treatment and/or there is a lack of new protein synthesis in response to cellulase. Experiments with CHX were employed to address the question of rate of OXI1 protein synthesis following cellulase treatment but the results obtained were unclear.

3.11.4 Summary

The lack of phenotype of the *oxi1* mutant in response to salt and heat stress, the low level of OXI1-YFP protein even in transgenic plants overexpressing OXI1-YFP as well as the draw back in identifying targets of OXI1 has made it difficult to characterise the function of OXI1 or the processes it is involved in. It is possible that the laboratory conditions employed in this study or the stresses chosen may not have been suitable to produce a phenotype in the *oxi1* mutant. The problem is to find or mimic such conditions where a phenotype can be discerned.

Nonetheless the transcriptional induction of OXI1 in response to the variety of biotic and abiotic stimuli suggests that there may be two modes of OXI1 action (Figure 3.21). In the first instance different processes may activate OXI1 which in turn activates a signal transduction cascade specific to that stress. Although the abiotic stresses tested in this study have not shown a pivotal role for OXI1 function, other stresses which induce *OXI1* mRNA may well prove that the induction or activity of OXI1 is crucial to the

establishment of a response to that particular stress. Alternatively, all stimuli resulting in the accumulation of AOS may converge to activate OXI1 which then induces a single pathway. The response that this OXI1 pathway mediates may not be of sufficient magnitude to cope with every stress that induces it. Therefore other compensatory pathways may come into play independent of OXI1 and consequently the *oxi1* mutant would not show a phenotype in response to stresses that would induce either *OXI1* transcript or protein. To reconcile the involvement of OXI1 in particular plant responses it is imperative that the cellular targets of OXI1 be identified, if the same components are found to interact with OXI1 or be phosphorylated by OXI1 in response to different stresses it would suggest that OXI1 activates the same signal transduction pathway in response to different stimuli. The processes which can be employed to identify these interacting components or targets of OXI1 will be discussed in Chapter 5.

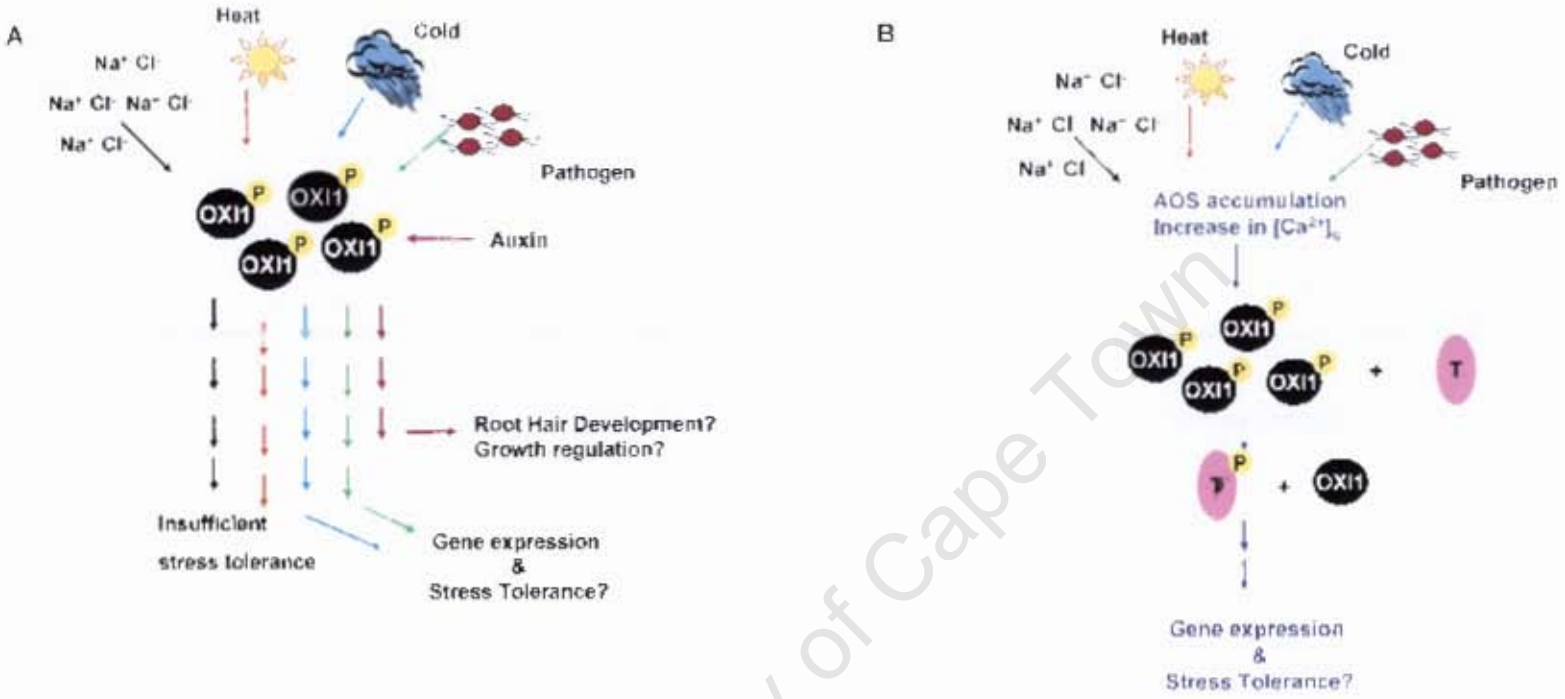


Figure 3.21 OXI1 mediated signal transduction cascades

(A) Biotic and abiotic stresses as well as the plant hormone auxin activate OXI1 and initiates distinct signal transduction cascades for each particular stress. These different OXI1 mediated signal transduction cascades facilitate the desired end response to assist the plant to cope with the particular stimuli being imposed upon it. In the case of heat and salt (Na^+ Cl^-) stress the initiated signal transduction cascade and the end responses are insufficient to promote salt and heat tolerance. (B) Different stresses all induce the accumulation of AOS which possibly together with changes in $[Ca^{2+}]_c$ mediates the activation of OXI1. OXI1 activates T its target protein which then initiates a single signal transduction cascade that may be sufficient to some but not all forms of stress.

CHAPTER 4

The role of Oxidative Signal-Inducible1 Protein Kinase in Plant Defence and Disease Resistance

CHAPTER 4: The role of Oxidative Signal-Inducible1 protein kinase in plant defence and disease resistance

4.1 Rationale

The importance of the signalling capacity of AOS and protein kinase activity in disease resistance pathways has already been highlighted in Sections 1.2.4.1 and 1.3.2. To briefly reiterate, the biphasic oxidative burst induced during gene-for-gene resistance is essential for the establishment of plant defence responses such as the HR and defence gene expression (Levine *et al.*, 1994; Alvarez *et al.*, 1998). Compatible interactions with virulent pathogens produce a weak monophasic oxidative burst that does not trigger the HR and expression of defence related genes are reduced and delayed hence disease ensues (Levine *et al.*, 1994; Lamb and Dixon, 1997). Direct functions of AOS such as AOS toxicity (Peng and Kuc, 1992) and oxidative cross-linking of plant cell walls (Bradley *et al.*, 1992) also aid in the limitation of pathogen spread in response to biotrophic pathogen infection. In contrast to the beneficial role for AOS in disease resistance responses to biotrophic pathogens, necrotrophic pathogens, which obtain their nutrients from dead tissue, exploit the oxidative burst and ensuing HR to aid fungal infection. It has been shown that the accumulation of O_2^- and H_2O_2 is directly proportional to the growth of the fungal pathogen *B. cinerea* and severity of disease (Govrin and Levine, 2000).

Protein kinase activity is required at various stages of disease resistance responses ranging from perception of the pathogen to downstream activation of defence gene expression. For example, the RLK FLS2 binds to bacterial flagellin and either directly or indirectly induces a complete MAPK cascade that culminates in the induction of *WRKY22*, *WRKY29* and *GST* expression (Gomez-Gomez *et al.*, 2001; Asai *et al.*, 2002; Chinchilla *et al.*, 2006). Protein kinases also provide a node of functional overlap between gene-for-gene resistance and basal defence mechanisms. Silencing of *MPK6* renders transgenic plants with increased susceptibility to both virulent and avirulent strains of *P. syringae* (Menke *et al.*, 2004). Similarly a single protein kinase can play a role in resistance to different types of pathogens. For example, constitutive activation of MEKK1 kinase domain or constitutively active MKK4 and MKK5 conferred Arabidopsis leaves with enhanced resistance to *P. syringae* and *B. cinerea* indicative that defence

responses activated by this MAPK cascade is effective against both bacterial and fungal pathogens (Asai *et al.*, 2002). More recently it was demonstrated that transgenic potato plants harbouring a constitutively active form of a MAPKK (StMEK1) driven by a pathogen-inducible promoter displayed increased resistance to virulent *Phytophthora infestans* through AOS accumulation and an HR-like phenotype and were also more resistant against infection with the necrotroph *Alternaria solani* (Yamamizo *et al.*, 2006). This data also places a putative MAPK signalling pathway upstream of H_2O_2. It has been demonstrated that H_2O_2 induces the activity of the MAPKKK ANP1 which in turn activates MPK3 and MPK6 and induces the expression of the defence genes *GST6* and *HSP18.2* (Kovtun *et al.*, 2000) hence MAPK signalling also occurs downstream of H_2O_2. Therefore identification of proteins that can sense the change in H_2O_2 accumulation during pathogen attack and consequently direct the activation of MAPK cascades will prove invaluable in our understanding of disease resistance responses and likely candidates include protein kinases.

Protein kinase activity may also modulate the activity of antagonist signalling responses in disease resistance pathways. The *mpk4* mutant displays enhanced tolerance to virulent *P. syringae* via SA-dependent pathways whereas it is compromised in resistance to *A. brassicicola* and lacks responsiveness to JA and ET in defence gene induction (Petersen *et al.*, 2000; Brodersen *et al.*, 2006). This is not surprising since resistance to biotrophic pathogens is mediated through SA-signalling pathways while JA/ET-dependent pathways regulate resistance to necrotrophs (Glazebrook, 2005). Furthermore, numerous reports suggest that JA and SA have antagonistic effects on each other (Thomma *et al.*, 1998; Petersen *et al.*, 2000; Thomma *et al.*, 2001). Therefore MPK4 kinase activity mostly likely mediates the balance between SA and JA responses during pathogen attack and it may achieve this function by regulating the activity of EDS1 and PAD4 which suppresses both activators of SA-dependent and repressors of JA/ET-dependent defence responses (Brodersen *et al.*, 2006).

The induction of *OXI1* gene expression by AOS, its requirement for full activation of MPK3 and MPK6 in response to wounding (through cellulase treatment) and direct application of H_2O_2 suggest a role for OXI1 in plant defence signalling (Rentel *et al.*, 2004). The necessity for OXI1 in response to virulent *H. parasitica* infection demonstrated a role for OXI1 in basal defence against this pathogen (Rentel *et al.*,

2004). Additionally, microarray analysis indicated that virulent, avirulent and nonhost strains of *P. syringae* induced the expression of *OXI1* (Figure 3.9 B). Therefore the aim of this work was to examine whether OXI1 is involved in defence against other pathogens and whether it has a role in gene-for-gene mediated signalling as well as activation of basal defence systems. To determine whether OXI1 is a component of defined defence signalling pathways such as SA- and/or JA-mediated defence pathways, the effect of the *oxi1* mutation on gene expression of characterised defence associated genes in response to pathogen challenge was investigated. Furthermore, given the requirement of AOS for the establishment of SAR (Alvarez *et al.*, 1998) it was also investigated whether OXI1 is required for SAR.

4.2 Induction of *ECS1* is unlinked to the *oxi1* mutation

The *ECS1* gene encodes a plant cell wall-associated protein and is induced by virulent strains of the phytopathogenic bacterium *Xanthomonas campestris* pv. *campestris,* virulent and avirulent strains of *H. parasitica* and SA (Aufsatz and Grimm, 1994; Aufsatz *et al.*, 1998; Rentel, 2002). This gene expression profile suggests a role for *ECS1* in disease resistance and hence *ECS1* expression was investigated in the *oxi1* mutant in response to *H. parasitica* challenge and SA (Rentel, 2002). The accumulation of *ECS1* transcript was significantly reduced in the *oxi1* mutant compared to wild type in response to both treatments which suggests a role for OXI1 in the regulation of *ECS1* gene expression (Rentel, 2002).

To determine whether the reduction in *ECS1* expression is really due to the disruption of the *OXI1* gene, separate T_3 lines isolated from a single T_2 parent heterozygous for the *oxi1* mutation, were analysed for *ECS1* expression. The T_3 progeny were treated with cellulase to induce *OXI1* expression and distinguish between the *OXI1* mutant or wild type transcripts. Those T_3 lines that expressed both *OXI1* transcripts were considered to be heterozygous for the *oxi1* mutation whereas those that exhibited either the mutant or wild type *OXI1* transcript only were homozygous *oxi1* mutant or wild type lines respectively. Additionally the genotype of the segregating T_3 seedlings was confirmed through kanamycin screening since the T-DNA insertion rendering the *OXI1* gene non-functional is linked to the antibiotic kanamycin resistance marker. Seedlings that

exhibit 100% sensitivity or resistance to kanamycin are homozygous wild type or *oxi1* mutant lines respectively. The aforementioned approach was utilised to test for linkage between the *oxi1* mutation and reduced *ECS1* expression since the *oxi1* + *OXI1* complemented line had not been isolated at the time of this experiment.

Approximately 40 seedlings from each T_3 line were treated either with cellulase or SA to cause induction of the *OXI1* and *ECS1* gene respectively. Two T_3 lines expressed the wild type *OXI1* gene but had reduced *ECS1* expression (Figure 4.1 A, lines 10 and 12). Conversely, lines F and I expressed the *oxi1* mutant transcript and induced the *ECS1* gene to levels comparable to wild type (Figure 4.1 B). However, lines F and I may have been heterozygous for the *oxi1* mutation since expression of the wild type *OXI1* transcript was not detectable in this experiment (Figure 4.1 B). The genotype for the segregating progeny was confirmed through a kanamycin screen whereby 100 % of seedlings of lines 10 and 12 were completely susceptible to the antibiotic indicating that these lines do not harbour the T-DNA insertion and are therefore wild type seedlings (data not shown). The above data demonstrates that the observed reduction in *ECS1* gene expression was not caused by the *oxi1* mutation but was possibly due to additional insertions of parts of the T-DNA in the *oxi1* mutant since the *oxi1* mutant had not been backcrossed into wild type to obtain a 'clean' mutation.

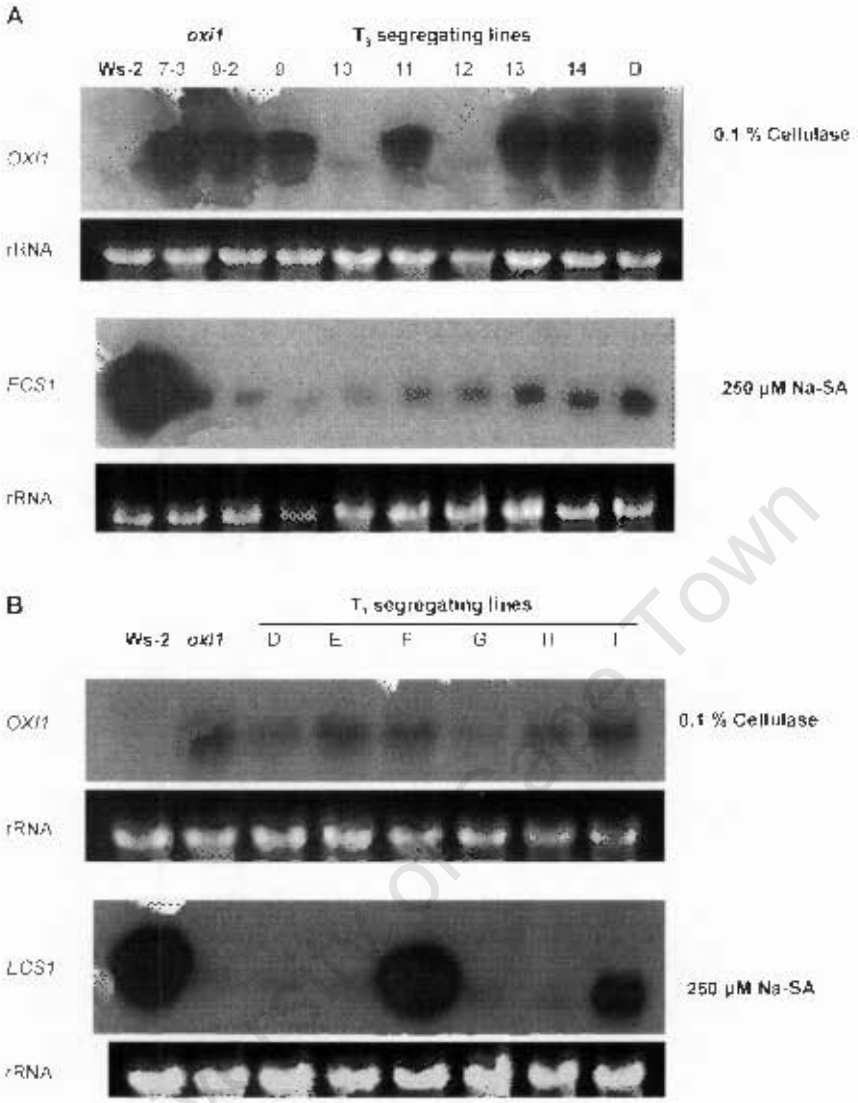


Figure 4.1 Reduced *ECS1* gene expression is not linked to the *oxi1* mutation.

Northern analysis of the *OXI1* gene induced by 0.1% cellulase treatment for 1 hour demonstrates the genotype of T_3 lines (lines 9-14 and D to I) isolated from a single parent heterozygous for the *oxi1* mutation (A and B). *ECS1* expression was induced by treatment of approximately 40 seedlings of each T_1 line as well as genotypes Ws-2 and *oxi1* mutant. with 250 µM Sodium salicylate (Na-SA) for 6 hours (A and B). T_3 lines 10 and 12 expressed the wild type *OXI1* transcript and displayed reduced *ECS1* expression (A). Mutant *oxi1* transcript was detected in lines F and I which had wild type *ECS1* levels (B). The ethidium bromide stained rRNA was used as a loading control.

4.3 *OXI1* gene expression after *P. syringae* infection correlates with the oxidative burst

Analysis of global gene expression data revealed that expression of *OXI1* was induced by both virulent and avirulent strains of *P. syringae* (Figure 3.9 B). Since *OXI1* is induced in response to AOS and AOS is known to accumulate during early stages of pathogen infection, it was examined whether *OXI1* expression correlated spatially and/or temporally with AOS production after infection with an avirulent strain of *P. syringae* (*Pst*DC3000 AvrB). The OXI1::GUS transgenic line harbouring the OXI1 promoter fused to the GUS reporter gene was employed for this investigation since it allowed for *in vivo* visualisation of both the oxidative burst and reporter gene expression. Both the accumulation of H_2O_2 and induction of *OXI1* is confined to the region of the leaf infiltrated with *Pst*DC3000 *avrB* visualised through DAB staining (Thordal-Christensen *et al.*, 1997) and GUS activity respectively (Figure 4.2). H_2O_2 accumulation precedes *OXI1* gene expression since DAB staining could be visualised 3 hrs post infection whereas GUS activity was first detected at 8 hrs post infection (Figure 4.2). The delay in GUS activity suggests that either the oxidative burst during incompatible interactions occurs prior to *OXI1* expression or the GUS protein takes time to be synthesised following activation of the *OXI1* promoter. It should also be noted that translation of *GUS* mRNA could be regulated differently to *OXI1* mRNA. In OXI1::GUS transgenic plants infected with virulent *Pst*DC3000 the generation of H_2O_2 and *OXI1* gene expression were again limited to the infected region of the leaf but were only detectable 24 hrs post treatment (Figure 4.2) consistent with the late establishment of plant defence responses during compatible interactions. Taken together the data demonstrates spatial correlation between the oxidative burst and *OXI1* expression and suggests that H_2O_2 or the oxidative burst may cause induction of *OXI1* transcript during pathogen infection.

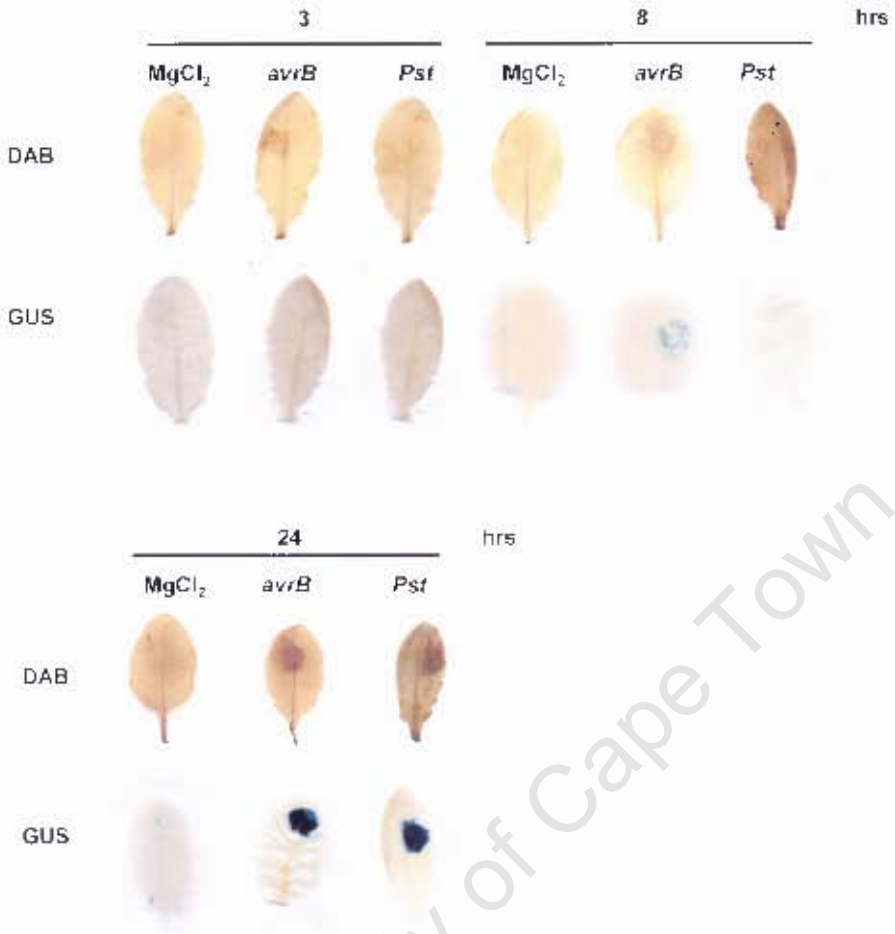


Figure 4.2 *OXI1* expression correlates with the oxidative burst during *P. syringae* infection

A small area of leaves from 4 week old OXI1::GUS transgenic plants was pressure inoculated with either 10 mM $MgCl_2$ alone, avirulent *Pst*DC3000 *avrB* or virulent *Pst*DC3000 (*Pst*) suspension in 10 mM $MgCl_2$ at 5×10^8 cfu/ml. At 3, 8 and 24 hrs post infection leaves were excised and stained for the presence of an oxidative burst, detected with DAB staining, and *OXI1* expression visualised through GUS activity. Three leaves were analysed per treatment per time point; one representative leaf of each sample is shown. Both the oxidative burst and *OXI1* expression occurred within the infected area. This experiment was repeated twice with similar results.

4.4 NADPH-produced AOS causes induction of *OXI1* during an incompatible *P. syringae* infection

To determine whether H_2O_2 generated during the oxidative burst is indeed responsible for the induction of *OXI1* during an incompatible pathogen infection, *OXI1* gene expression was examined in the respiratory burst oxidase mutant *atrbohD*. The Arabidopsis *AtrbohD* gene, homologous to human $gp91^{phox}$ encoding the catalytic subunit of NADPH oxidase, is responsible for the majority of AOS production during plant defence responses (Torres *et al.*, 1998; Torres *et al.*, 2002). The generation of AOS is greatly reduced in the *atrbohD*-null mutant infected with either avirulent *Pst*DC3000 *avrRpm1* or virulent *H. parasitica* Emco5 (Torres *et al.*, 2002). In this study the *atrbohD* mutant lines also contained the calcium sensitive photoprotein aequorin (Knight *et al.*, 1991) therefore Col plants expressing the *aequorin* gene were used as the control or wild type genotype. To ensure that the site of insertion of the *aequorin* gene does not have an affect on the *atrbohD* mutation three independently transformed lines were analysed. DAB staining confirmed that the *atrbohD* mutant lacked the oxidative burst in response to *Pst*DC3000 *avrB* infection (Figure 4.3 A). *OXI1* expression in this mutant background was reduced compared to wild type (Figure 4.3 B). This implies that during incompatible interactions AOS generated through NADPH oxidase is partly responsible for the induction of *OXI1*.

A role for AOS generated through NADPH oxidase in pathogen induced expression of *OXI1* is further strengthened by experiments using OXI1::GUS transgenic plants. Leaves from OXI1::GUS plants co-infiltrated with *Pst*DC3000 *avrB* and 10 µM diphenylene iodonium (DPI), a chemical inhibitor of NADPH oxidase, exhibited reduced GUS activity in comparison to leaves infiltrated with *Pst*DC3000 *avrB* alone (Figure 4.4).

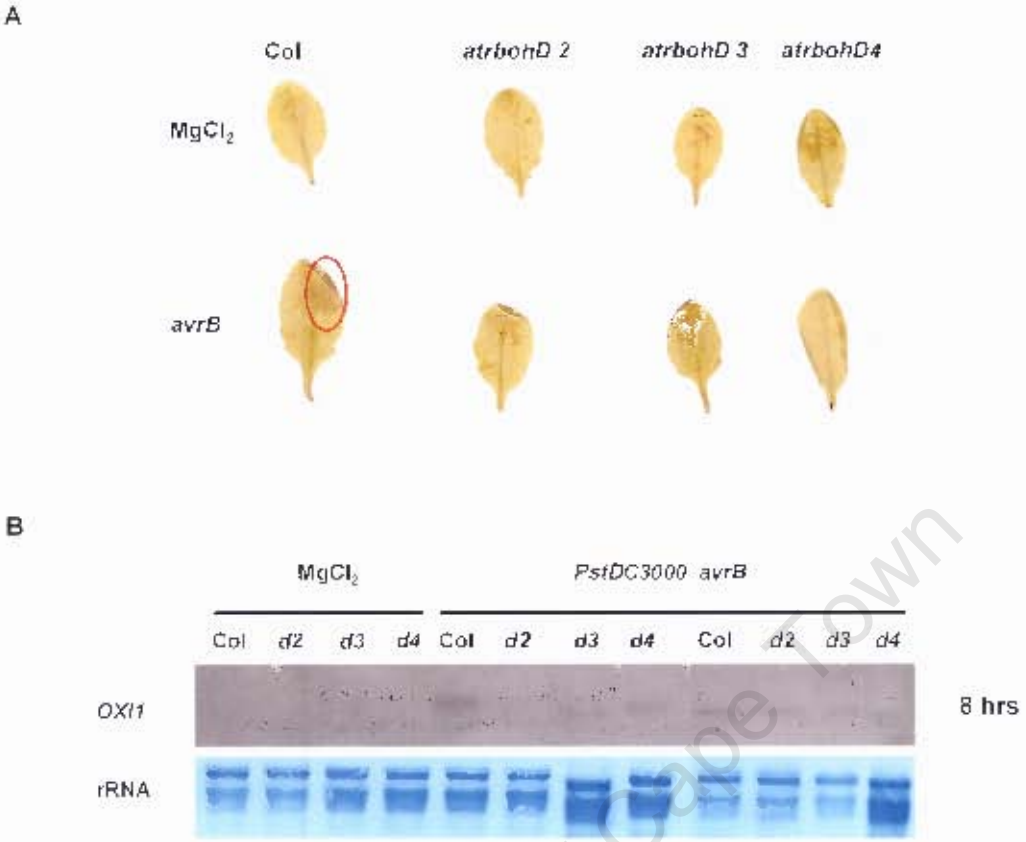


Figure 4.3 The respiratory burst oxidase mutant *atrbohD* lacks the oxidative burst in response to *Pst*DC3000 *avrB* infection and displays reduced *OXI1* mRNA levels

The *atrbohD* mutant and Col-0 both expressing the calcium sensitive photoprotein aequorin, were pressure inoculated with either 5 X 10^6 cfu/ml of avirulent *Pst*DC3000 *avrB* in 10 mM $MgCl_2$ or 10 mM $MgCl_2$ alone in a small region of the leaf. Mutant *atrbohD 2 (d2), 3 (d3) and 4 (d4)* represents 3 lines of *atrbohD* that were independently transformed with the aequorin gene. (A) DAB staining demonstrates lack of oxidative burst or H_2O_2 production in *atrbohD* mutants at 6 hrs post infection. A representative of 3 leaves per treatment for each genotype is shown and the red circle indicates the infected area exhibiting an oxidative burst. (B) Northern analysis demonstrates reduction of *OXI1* mRNA levels in the *atrbohD* mutant in response to pathogen. The entire leaf was pressure inoculated with either 10 mM $MgCl_2$ alone or *PstDC3000 avrB* in 10 mM $MgCl_2$ at 5 X 10^7 cfu/ml. A minimum of 4 leaves per sample were harvested at 8 hrs post treatment. Northern blots were probed with a full length *OXI1* DNA fragment. The methylene blue stained RNA was used as a loading and transfer control.

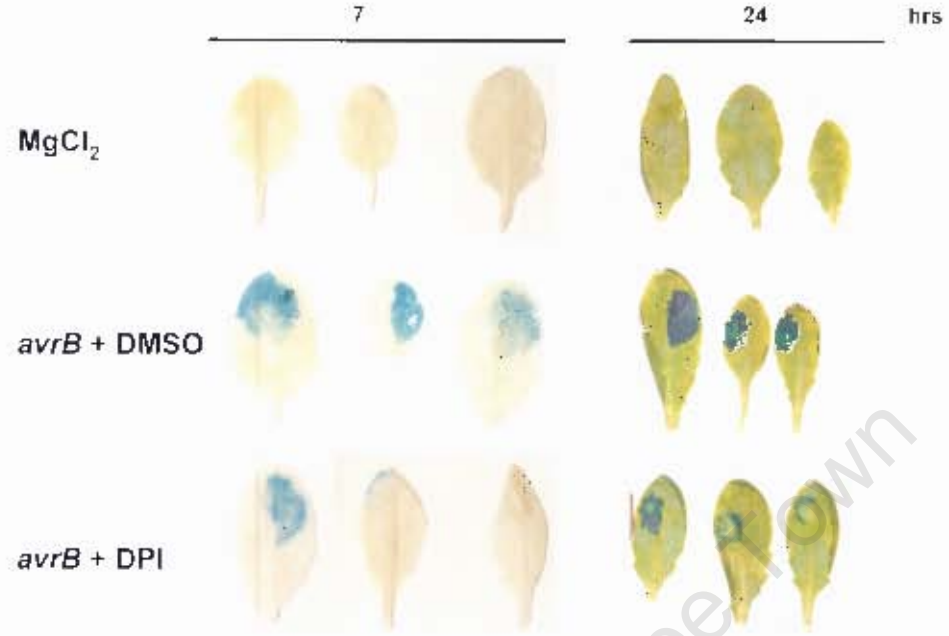


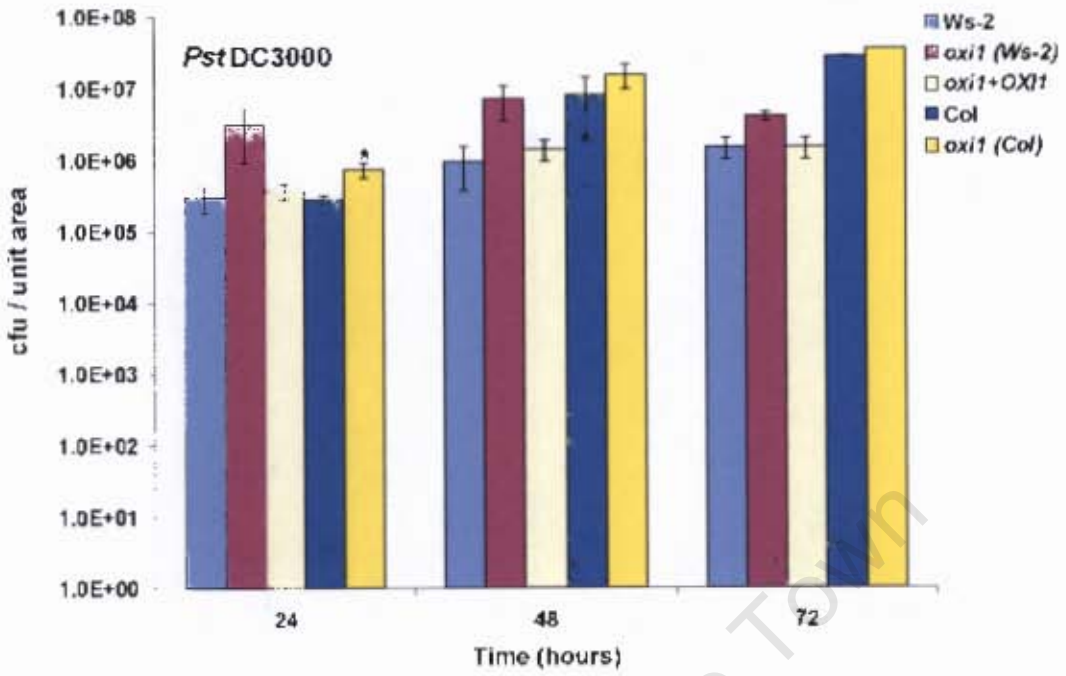
Figure 4.4 Full *OXI1* expression in response to avirulent *P. syringae* infection requires NADPH oxidase activity

A small region of leaves from 4 week old OXI1::GUS transgenic plants was pressure inoculated with either 10 mM $MgCl_2$ alone or avirulent *Pst*DC3000 *avrB* suspension in 10 mM $MgCl_2$ containing either 0.1% DMSO (*avrB* + DMSO) or 10 µM DPI (*avrB* + DPI) at 5 X 10^7 cfu/ml. DPI is a chemical inhibitor of NADPH oxidase and co-infiltration with *Pst*DC3000 *avrB* and DMSO is used as a control for DPI treatment. Two independent experiments are shown where in the first experiment GUS activity was first visualised at 7 hrs post treatment whereas in the second experiment GUS activity was only detected 24 hrs post treatment. This data illustrates that there are different infection rates of *Pst*DC3000 *avrB* between different experiments which is either due the integrity of the plant or *P. syringae* at the time of infection even though growth conditions of Arabidopsis and bacteria are similar between different experiments. Three leaves per treatment per time point for each experiment are shown.

4.5 The *oxi1* mutant displays increased susceptibility to virulent and avirulent *P. syringae* challenge

Given the expression profile of *OXI1* during *P. syringae* infection, it was hypothesised that *OXI1* was involved in the signalling cascade mediating defence against *P. syringae*. *Oxi1* null mutants in both Ws-2 and Col backgrounds, their corresponding wild type Arabidopsis as well as the *oxi1* (Ws-2) complemented line (*oxi1* + *OXI1*) were challenged with virulent *Pst*DC3000 or the avirulent *Pst*DC3000 *avrB* isolate and the extent of bacterial growth assessed. Both *oxi1* knockouts exhibited increased susceptibility to both virulent and avirulent *P. syringae* in comparison to wild type (Figure 4.5). Although significant differences in bacterial titres between the *oxi1* mutant and wild type varied between time points of different experiments, the increased susceptibility phenotype of the *oxi1* mutant in response to virulent and avirulent *P. syringae* infection is a consistent trend and therefore more than one data set for each experiment is shown. Importantly the heightened susceptibility phenotype of the *oxi1* mutant was rescued in the complemented line in response to both the virulent and avirulent strain, which demonstrated that the increased susceptibility phenotype was caused by the *oxi1* mutation (Figure 4.5 A and C). Differences in bacterial titres between *oxi1* mutants and their respective wild types occurred as early as 24 hrs post infection in response to virulent *P. syringae* (Figure 4.5 A) whereas it was observed from 48 to 72 hrs post infection with the avirulent strain (Figure 4.5 C and D). This suggests that rather than preventing the initial stages of infection, OXI1 may play a role in slowing subsequent bacterial growth and since bacterial titres reach higher levels faster during virulent infection, differences in bacterial growth between the *oxi1* mutant and wild type are observed earlier. It was noted that the difference in bacterial counts between the *oxi1* mutant and wild type Col for both virulent and avirulent *P. syringae* was not as large as in the Ws-2 background (Figure 4.5 A and D), which could likely be accounted for by natural variation in the importance of OXI1 signalling between various ecotypes. Alternatively, in response to virulent *P. syringae*, the weaker phenotype of the *oxi1* (Col) mutant in response to *P. syringae* infection could be due to the higher bacterial titres obtained in Col in comparison to Ws-2 which possibly (Figure 4.5 A) masks the *oxi1* phenotype.

A



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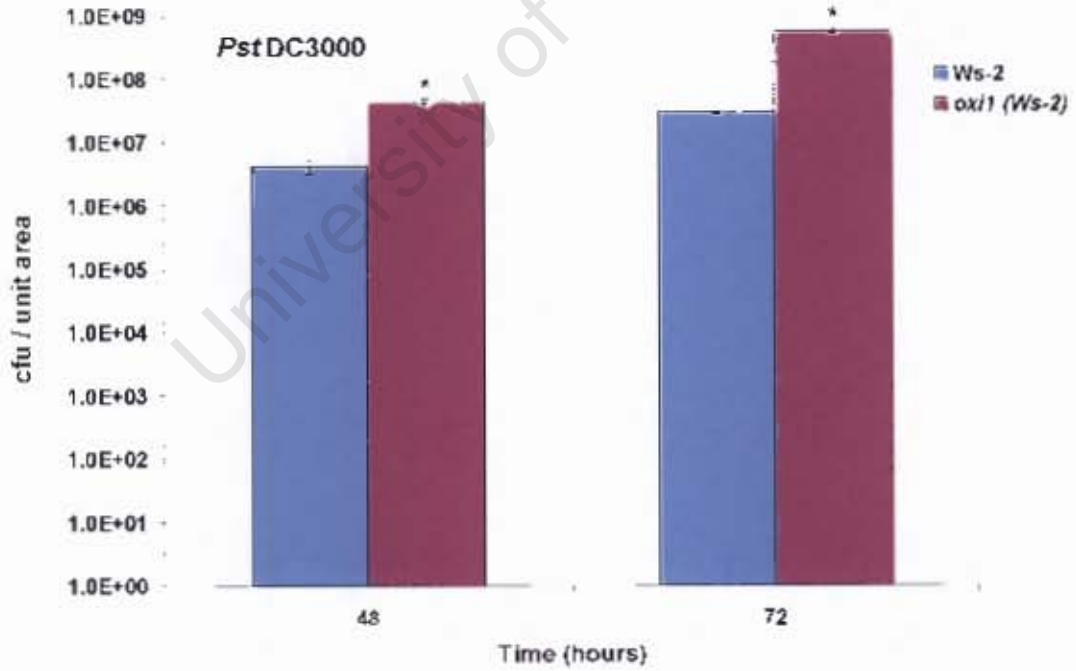

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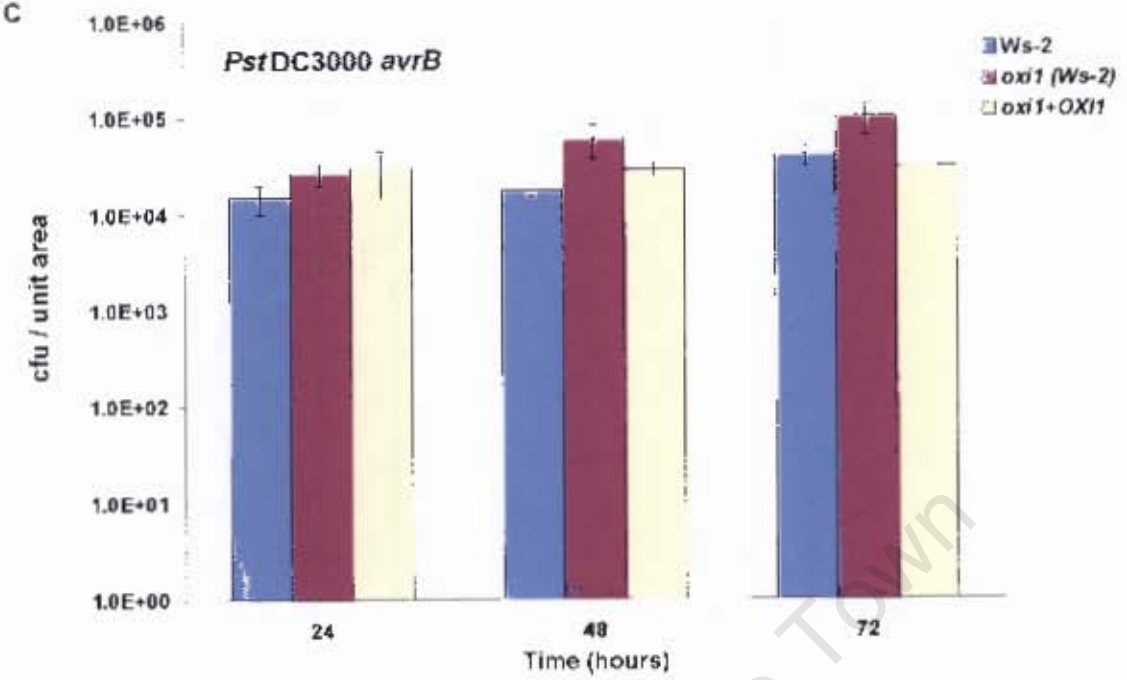

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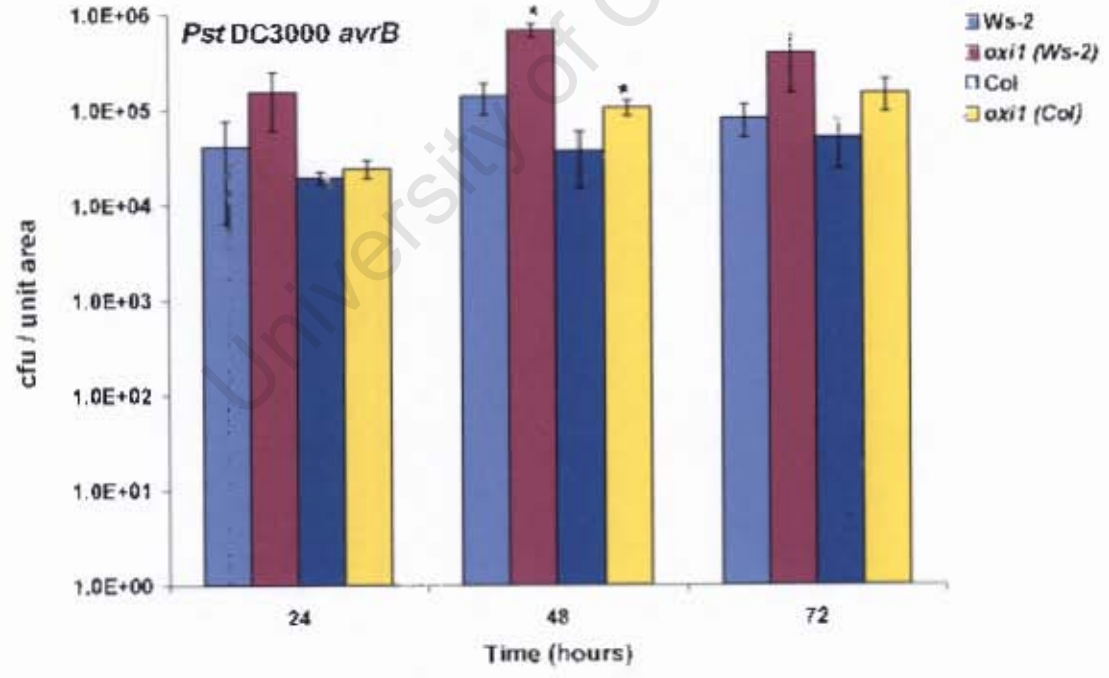

Figure 4.5 *oxi1* mutants exhibit increased susceptibility to both virulent and avirulent strains of *P. syringae*

The entire abaxial surface of 3 week old leaves of wild type Ws-2 and Col, *oxi1* knockouts in both backgrounds (*oxi1 (Ws-2)* and *oxi1 (Col)*) and the *oxi1* + *OXI1* complemented line were pressure inoculated with either virulent *PstDC3000* (A and B) or avirulent *PstDC3000 avrB* (C and D) at 5 X 10^5 cfu/ml. For each genotype leaf discs of 0.5 cm^2 were harvested in triplicate per sample and 3 samples were collected for each time point (n=9). The bars represent the log of bacterial growth expressed as cfu/unit area at 24, 48 and 72 hrs post infection and the standard error with a 95% confidence interval is shown. * indicates a significant increase in pathogen growth compared to wild type (student's t-test, $P<0.05$). These experiments were performed three times with similar results and graphs presented here represent two of those experiments for each *P. syringae* strain.

OXI1 appears to have a role in limiting the extent of pathogen colonisation during both compatible and incompatible interactions. To determine whether this role extended to all or only specific incompatible interactions both *oxi1* mutants were infected with *Pst*DC3000 expressing the avirulence factor avrRpt2. *Pst*DC3000 *avrRpt2* is recognised by Arabidopsis plants harbouring the resistance gene *RPS2* which is found in both Ws-2 and Col ecotypes (Kunkel *et al.*, 1993). The *oxi1 (Ws-2)* mutant was more susceptible to *Pst*DC3000 *avrRpt2* infection than Ws-2 (Figure 4.6). However, the *oxi1 (Col)* mutant displayed no appreciable difference in bacterial titre in comparison to wild type (Figure 4.6). A 10-fold difference in bacterial titre was also observed between Ws-2 and Col which suggests that the gene-for-gene mediated resistance in response to *Pst*DC3000 *avrRpt2* was weaker in the Col ecotype under the experimental conditions employed in this study. This further demonstrates natural variation among different ecotypes in disease resistance pathways. The above data suggests that *OXI1* is involved in diverse *R* gene-mediated resistance pathways and the importance of its function relative to other signalling mechanisms might differ between ecotypes.

In general the *oxi1* mutant is more susceptible to *P. syringae* infection in comparison to wild type. It was investigated whether overexpression of the *OXI1* gene would lead to decreased susceptibility to *P. syringae*. Surprisingly, both the 35S::OXI1 and 35S::OXI1-YFP transgenic lines displayed enhanced susceptibility to both virulent and avirulent strains of *P. syringae* (Figure 4.7). The increased susceptibility phenotype of the transgenic lines overexpressing *OXI1* was not due to the site of insertion of the transgene or as an indirect consequence of the YFP tag. Somewhat contradictory, increasing levels of OXI1 protein has the same effect as the *oxi1* knockout in response to pathogen attack i.e. modulating levels of OXI1 either higher or lower leads to increased susceptibility.

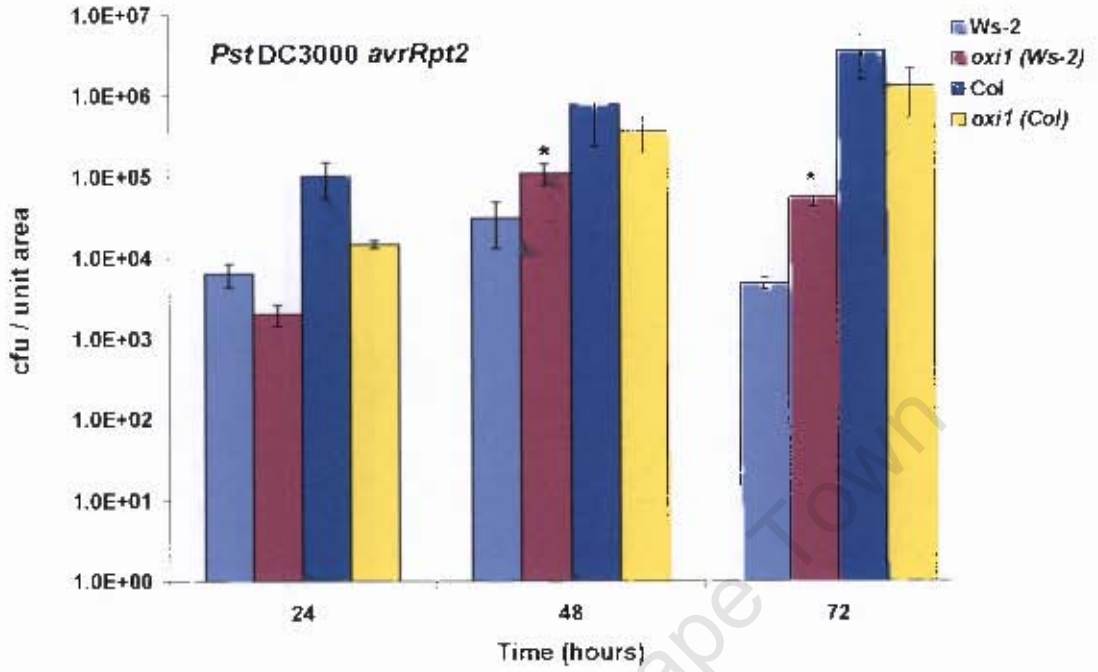

Figure 4.6 *oxi1* mutants display differential resistance to the avirulent *Pst*DC3000 *avrRpt2* strain of *P. syringae*

Leaves of 3 week old wild type (Ws-2 and Col) and *oxi1* mutant (*oxi1 (Ws-2)* and *oxi1 (Col)*) plants were infected with *Pst*DC3000 *avrRpt2* at 5 X 10^5 cfu/ml. For each genotype leaf discs of 0.5 cm^2 were harvested in triplicate per sample and 3 samples were collected for each time point (n=9). The bars represent the log of bacterial growth expressed as cfu/unit area at 24, 48 and 72 hrs post infection and the standard error with a 95% confidence interval is shown. * indicates a significant increase in pathogen growth compared to wild type (student's t-test, $P<0.05$). This experiment was performed twice with similar results.

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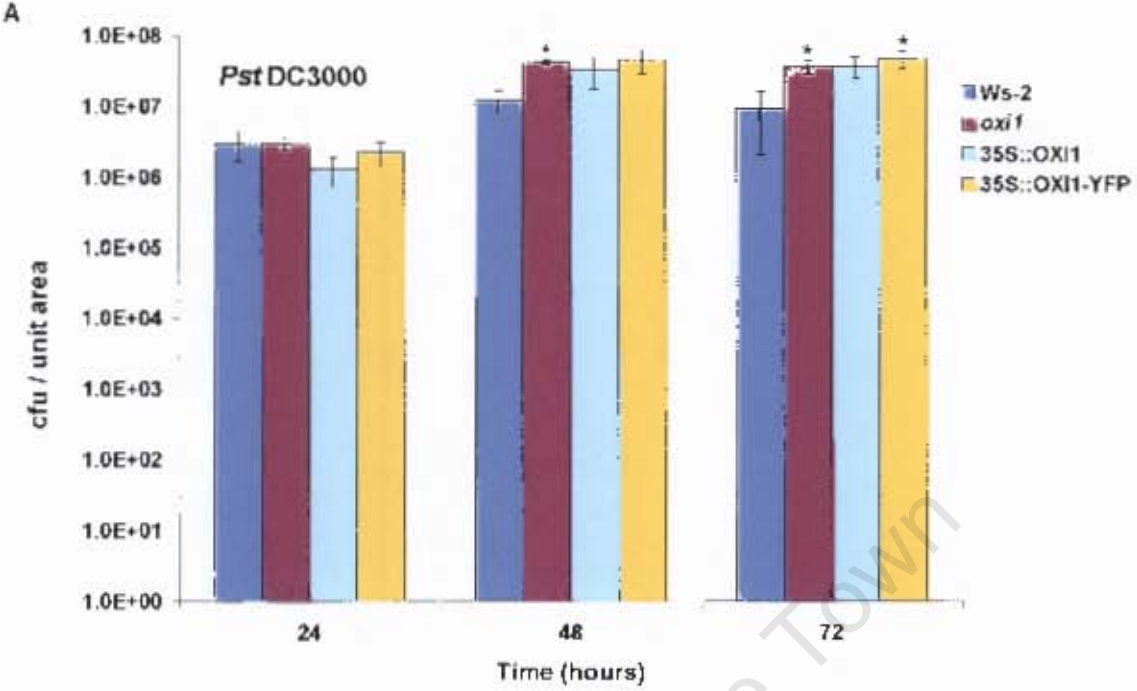

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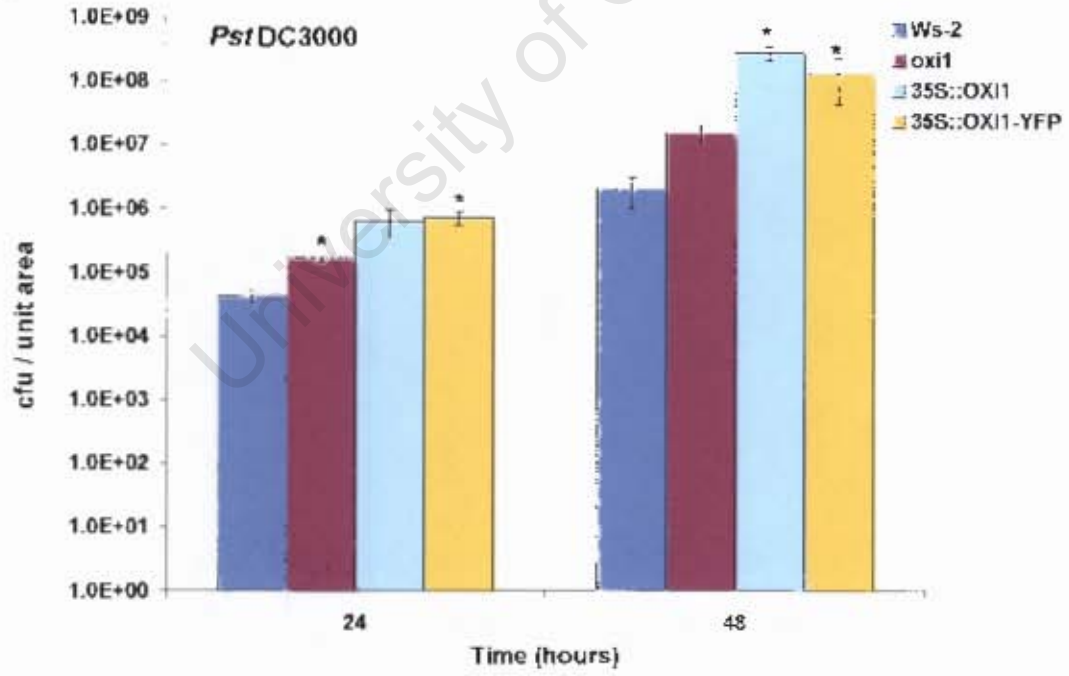

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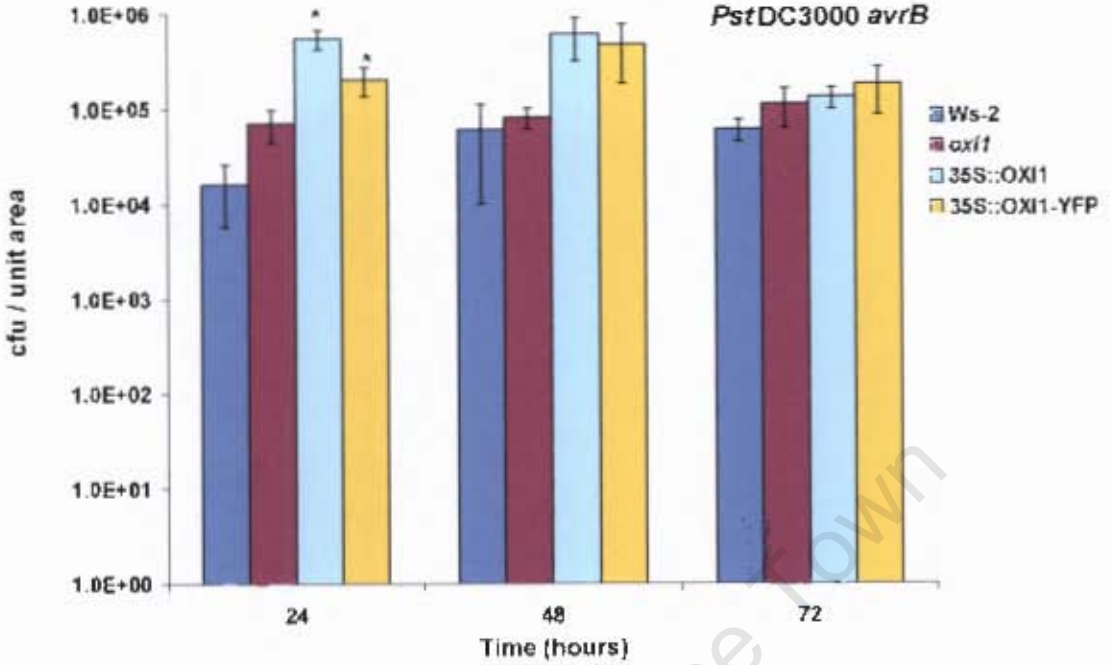

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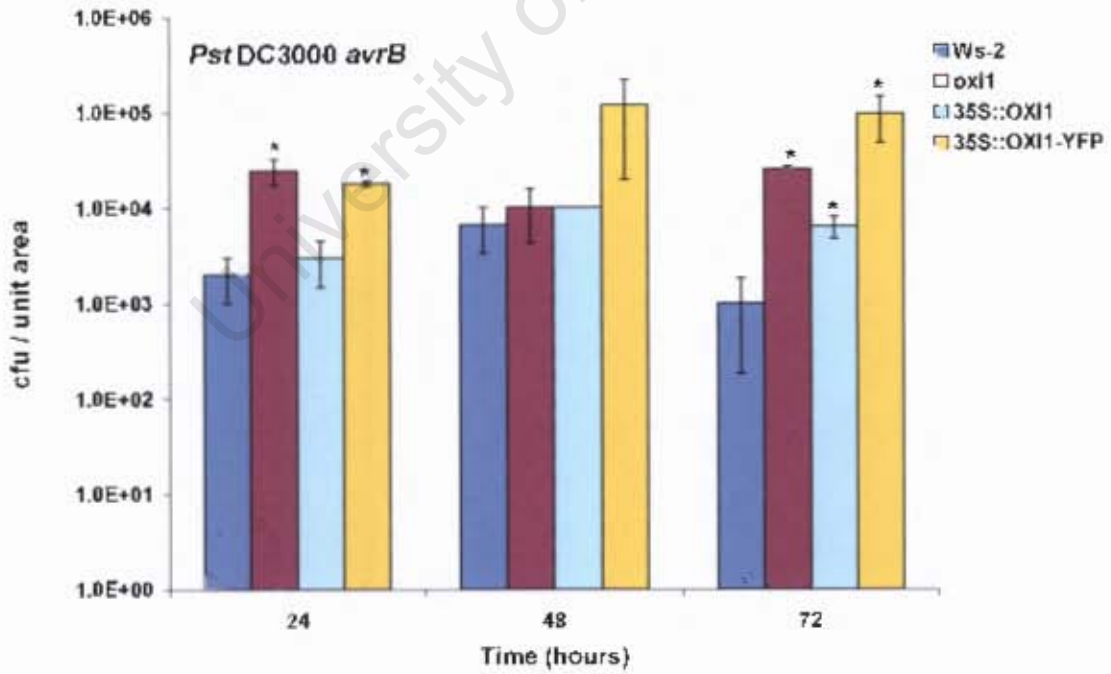


Figure 4.7 Transgenic lines overexpressing *OXI1* show increased susceptibility to both virulent and avirulent *P. syringae*

Three week old leaves of Ws-2, 35S::OXI and 35S::OXI1-YFP were pressure inoculated with either 5 X 10^5 cfu/ml virulent *Pst*DC3000 (A and B) or avirulent *Pst*DC3000 *avrB* (C and D). Leaf discs were harvested in triplicate at 24, 48 and 72 hrs post infection to determine the extent of bacterial growth. The bars represent the log of the bacterial growth expressed as cfu/unit area and the standard error with a 95% confidence interval is indicated. * indicates a significant increase in pathogen growth compared to wild type (student's t-test, $P<0.05$). Two independent experiments for each *P. syringae* strain are shown.

4.6 Overexpression of OXI1 causes increased susceptibility to virulent *H. parasitica*

The *oxi1* null mutant is more susceptible than wild type to virulent but not avirulent *H. parasitica* and importantly this increased susceptibility is rescued in the *oxi1* mutant complemented with the wild type *OXI1* gene (*oxi1* + *OXI1*) (Rentel *et al.*, 2004). This suggests a role for OXI1 in basal resistance to *H. parasitica* infection. OXI1 may either have a redundant function or it is not involved during gene-for-gene mediated resistance to *H. parasitica*. Therefore although basal resistance may be impaired, gene-for-gene signalling overrides basal resistance and resistance ensues in the *oxi1* mutant in response to avirulent *H. parasitica* infection. If OXI1 does play a pivotal role in basal defence against *H. parasitica* then overexpressing the OXI1 gene may lead to increased resistance. The 35S::OXI1 and 35S::OXI1-YFP transgenic lines were challenged with the virulent *H. parasitica* strain, Emco5. The degree of fungal sporulation was enhanced in both overexpressing transgenic lines in comparison to wild type after infection with Emco5 (Figure 4.8), as observed with *P. syringae* infection (Figure 4.7). Therefore both the lack of OXI1 protein and overexpression of OXI1 protein led to increased susceptibility to biotrophic pathogens.

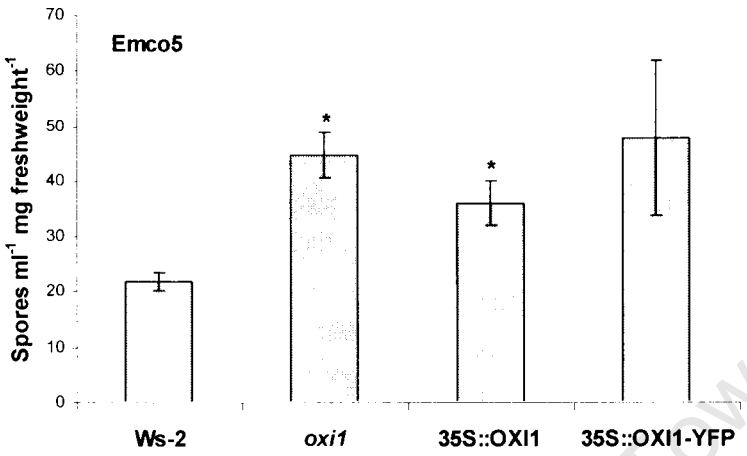


Figure 4.8 Overexpression of *OXI1* increases susceptibility to virulent *H. parasitica*

Seven day old seedlings of genotypes Ws-2, *oxi1*, 35S::OXI1 and 35S::OXI1-YFP were sprayed with spores of the virulent *H. parasitica* strain Emco5 at a suspension of 5-6 X 10^4 spores per ml. The bars represent the average amount of sporulation of 4 samples for each genotype, 7 days post infection. * indicates a significant increase in pathogen growth compared to wild type (student's t-test, $P<0.05$). This experiment was repeated twice with similar results.

4.7 OXI1-YFP protein levels do not change following *P. syringae* infection

It was shown that cellulase treatment, which breaks down components of the cell wall and therefore mimics a wound response and/or elicitor treatment, caused degradation of OXI1 protein kinase (Figure 3.17). The 35S::OXI1-YFP transgenic line was used to investigate what effect *P. syringae* infection has on OXI1 protein levels i.e. does *P. syringae* infection similarly target OXI1-YFP for degradation or does it induce OXI1-YFP. Therefore the levels of OXI1-YFP protein in the 35S::OXI1-YFP transgenic line in response to virulent and avirulent *P. syringae* infection was determined. OXI1-YFP protein levels did not change upon challenge with virulent or avirulent *P. syringae* since a basal level of OXI1-YFP protein was present in all samples throughout the duration of the experiment (Figure 4.9). Therefore it is likely that activation and not protein expression of OXI1 occurs after pathogen infection, which plays a role in disease resistance pathways. Perhaps constitutive expression of OXI1 may be affecting other signalling pathways that have an indirect effect on disease resistance hence the overexpressing transgenic lines behave as *oxi1* knockouts.

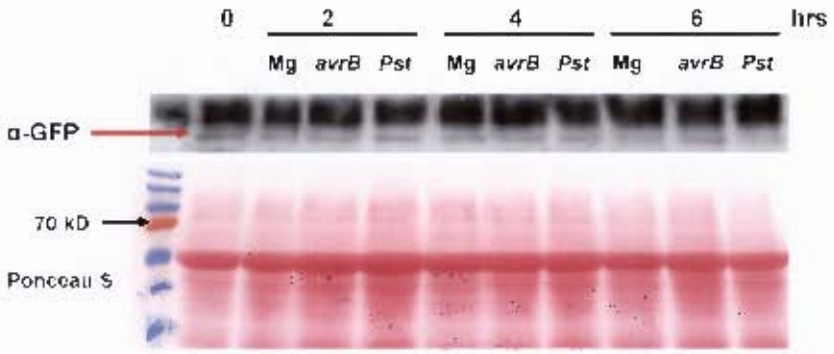


Figure 4.9 OXI1-YFP protein is not targeted for degradation following *P. syringae* challenge

Three week old leaves of 35S::OXI1-YFP transgenic line were pressure inoculated with either 10 mM $MgCl_2$ alone (Mg) avirulent (*avrB*) or virulent (*Pst*) *Pst*DC3000 both in a 10 mM $MgCl_2$ suspension at 5 X 10^6 cfu/ml. Leaves from 3 separate plants for each sample were harvested before treatment (0 hrs) or at 2, 4 and 6 hrs post treatment. Immunoblot analysis of OXI1-YFP protein with use of the α-GFP antibody at a 1:125 dilution revealed constitutive basal expression of OXI1-YFP (red arrow) in all samples. The top band of the immunoblot represents non-specific binding of the antibody since this band is present in the marker as well. The Ponceau S stained immunoblot was used as a loading control and the black arrow points to the 70 kD orange band of protein marker which is in the region OXI1-YFP protein resides since it is 77.6 kD. This experiment was performed twice with similar results.

4.8 Defence gene expression is not compromised in the *oxi1* mutant

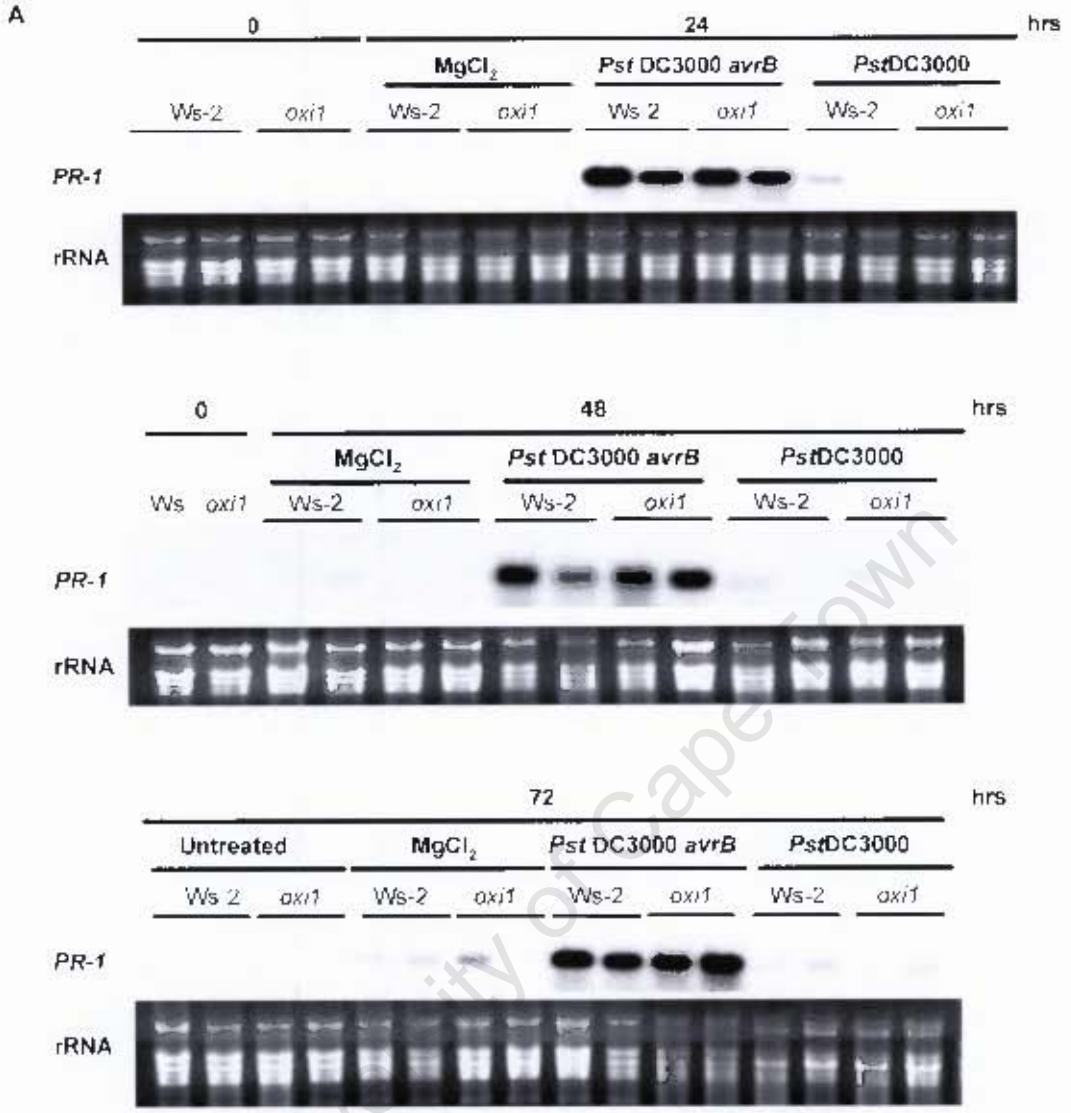
The expression of defence related genes is a crucial factor in establishing an effective defence response to limit colonisation of the invading pathogen and prevent disease (Glazebrook *et al.*, 2003). Plants deploy multiple signalling pathways that may work independently, in concert or antagonistically with each other to initiate expression of the appropriate end response genes in an attempt to defend against a particular pathogen (Pieterse and Loon, 2004). The expression profile of various defence related genes mediated by different signalling pathways was investigated in the *oxi1* mutant to determine the basis of *oxi1* susceptibility to *P. syringae* challenge.

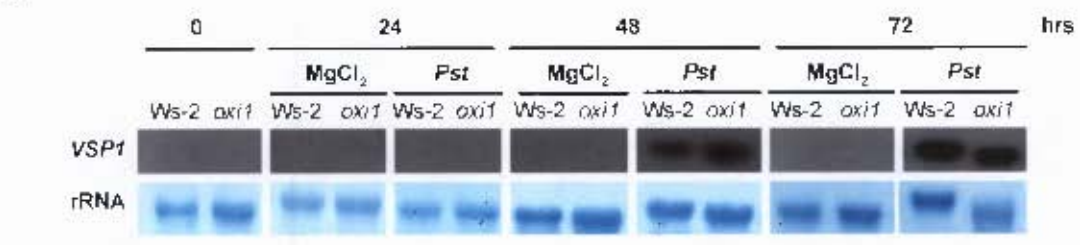
As expected, *PR-1* gene expression was induced in response to avirulent *P. syringae* within 24 hrs post infection. *PR-1* induction was delayed, only detected 72 hrs post infection, and much lower in response to virulent *P. syringae* infection, in which interaction the plant is unable to mount an effective defence response. *PR-1* expression was uncompromised in *oxi1* in response to either virulent or avirulent *P. syringae* infection (Figure 4.10 A). Induction of the defence related gene *Vegetative Storage Protein 1* (*VSP1*) by virulent *Pst*DC3000 requires MPK6 activity (Menke *et al.*, 2004). OXI1 is required for the full activation of MPK3 and MPK6 in response to AOS and cellulase treatment (Rentel *et al.*, 2004), which suggests that OXI1 may act upstream of MPK6 to regulate *VSP1* expression. However, induction of *VSP1* is unaffected in the *oxi1* mutant in response to virulent *Pst*DC3000 infection (Figure 4.10 B).

The WRKY superfamily of plant-specific transcription factors are involved in regulating a wide variety of physiological processes (Eulgem *et al.*, 2000). One such member *WRKY53* is induced by both wounding and pathogen and an Arabidopsis *wrky53*-null mutant shows increased susceptibility to virulent *Pst*DC3000 challenge (Dr Shane Murray pers. comm.). Given that OXI1 has a role in both wounding and pathogen defence, the expression of *WRKY53* was consequently investigated in the *oxi1* mutant to determine whether OXI1 was required for the induction of *WRKY53*. It was previously shown that *WRKY53* was induced by virulent *P. syringae* at 48 hrs post infection (Dr Shane Murray pers. comm.) therefore this time point was chosen to investigate the expression profile of *WRKY53* in the *oxi1* mutant. It was found that *WRKY53* transcript

levels increased in the $MgCl_2$ control in both Ws-2 and the *oxi1* knockout hence *WRKY53* expression was indeed responsive to wounding (Figure 4.10 C). However, *WRKY53* was not induced in either Ws-2 or the *oxi1* mutant in response to virulent *P. syringae* infection under the experimental conditions used in this study since its expression was not higher in either genotype treated with virulent *P. syringae* in comparison to $MgCl_2$ treatment, taking RNA loading into account (Figure 4.10 C). Additionally, there was no significant difference in expression level of *WRKY53*, taking RNA loading into account, between Ws-2 or the *oxi1* mutant in either the $MgCl_2$ control or *P. syringae* infection (Figure 4.10 C). These results suggest that *WRKY53* expression is independent of OXI1 and that these two components probably operate in separate signalling pathways.

The data presented here demonstrates that the increased susceptibility phenotype of *oxi1* mutant in response to *P. syringae* challenge is not a consequence of aberrant expression of the defence related genes tested in this study. OXI1 protein may be linked to other as yet unidentified components of the plant defence signalling network. Alternatively, rather than OXI1 mediating defence gene expression, OXI1 may be exerting its effects on pathogen susceptibility in some other way.





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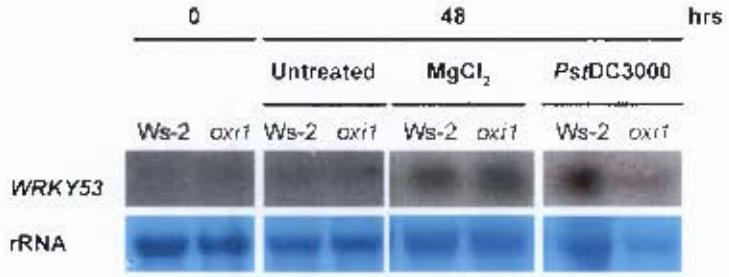


Figure 4.10 Expression of defence associated genes in the *oxi1* mutant

The entire abaxial surface of 3 week old leaves of wild type (Ws-2) or the *oxi1* mutant was pressure inoculated with either 10 mM $MgCl_2$ alone ($MgCl_2$) or 5 X 10^5 cfu/ml avirulent *PstDC3000 avrB* (*avrB*) or virulent *PstDC3000* both in a 10 mM $MgCl_2$ suspension. Leaves were harvested either before the start of the experiment (0 hrs) or at 24, 48 and 72 hrs post infection and expression of *PR-1* (A), *VSP1* (B) and *WRKY53* (C) were assessed. The untreated samples in A and C represent leaves from plants that were grown along side the treated plants but were not infected and harvested at the times indicated. The ethidium bromide stained RNA (A) or methylene blue stained RNA (B-C) was used as a loading control. These experiments were performed twice with similar results.

4.9 SA induction of *OXI1* does not require changes in $[Ca^{2+}]_c$

OXI1 was identified during a differential display searching for SA-regulated protein kinases (Figure 4.11). Both the free acid and sodium salt (Na-SA) forms of SA have been used interchangeably to cause induction of PR-1 protein and therefore both forms are capable of mediating SA-dependent signalling pathways. The non-functional analogue of SA, 4-hydroxybenzoic acid (4-HBA), does not induce PR-1 expression and has been used as a negative control. This was verified by testing PR-1 expression after SA treatment (Figure 4.12 A). To confirm SA regulation of *OXI1* gene expression northern analysis was performed. *OXI1* expression was induced at 6 hrs following treatment with Na-SA and was not induced in response to treatment with 4-HBA (Figure 4.12 B). Since SA accumulation is required for the establishment of defence responses following pathogen attack and SA induces *OXI1* mRNA, the increased susceptibility phenotype of the *oxi1* mutant in response to biotrophic pathogens might be because the *oxi1* mutation disrupts signalling downstream of SA in a SA-dependent PR1-independent pathway.

It has been suggested that Ca^{2+} functions as a second messenger of SA since extracellular Ca^{2+} was required for SA-induced chitinase activity in both tobacco leaves and carrot suspension culture (Raz and Fluhr, 1992; Schneider-Müllar *et al.*, 1994). A study in tobacco cell culture revealed that exogenously applied SA induced a rapid and transient increase in O_2^- levels which was followed by a transient increase in $[Ca^{2+}]_c$ (Kawano *et al.*, 1998). Furthermore, during an incompatible *avrRpm1/RPM1* interaction with avirulent *P. syringae* the Arabidopsis RPM1 resistance gene product activated an increase in $[Ca^{2+}]_c$ that was required for AOS accumulation and the HR (Grant *et al.*, 2000b). Transgenic tobacco defective in catalase activity generated excess H_2O_2 in response to high light which stimulated SA accumulation, expression of PR proteins and increased tolerance to virulent *P. syringae* infection (Chamnongpol *et al.*, 1998). This suggests that $[Ca^{2+}]_c$ and H_2O_2 function both up- and down-stream of SA in a potential positive feedback loop (Van Camp *et al.*, 1998). It was demonstrated that induction of *OXI1* expression in response to cellulase was inhibited by the extracellular Ca^{2+} blocker

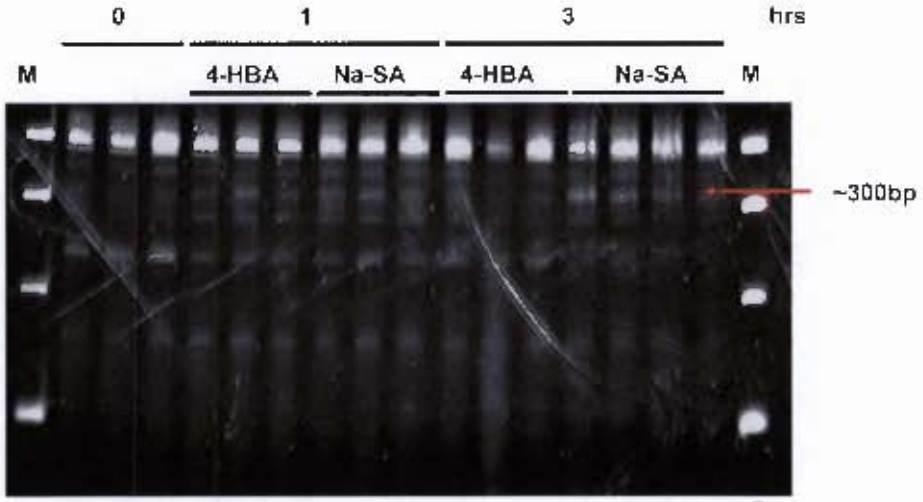


Figure 4.11 *OXI1* kinase identified through differential display of kinase cDNAs induced by 1 mM Na-SA

The entire abaxial surface of 3 week old leaves of Col plants was pressure inoculated with either 1 mM Na-SA or 1 mM 4-HBA. Five leaves from different plants per sample were harvested before the experiment (0) or at 1 and 3 hrs post treatment. Each treatment was performed in triplicate. cDNA synthesis was carried out on RNA from each sample and followed by PCR amplification with degenerate kinase specific primers. The bands represent PCR products of each sample and the 100 bp DNA ladder (M) was electrophoresed along side samples to determine size of bands. An approximate 300 bp (indicated by the red red arrow) band was induced in the Na-SA at 3 hrs and this band was excised and cloned. Sequencing data revealed that the band encoded the *OXI1* gene.

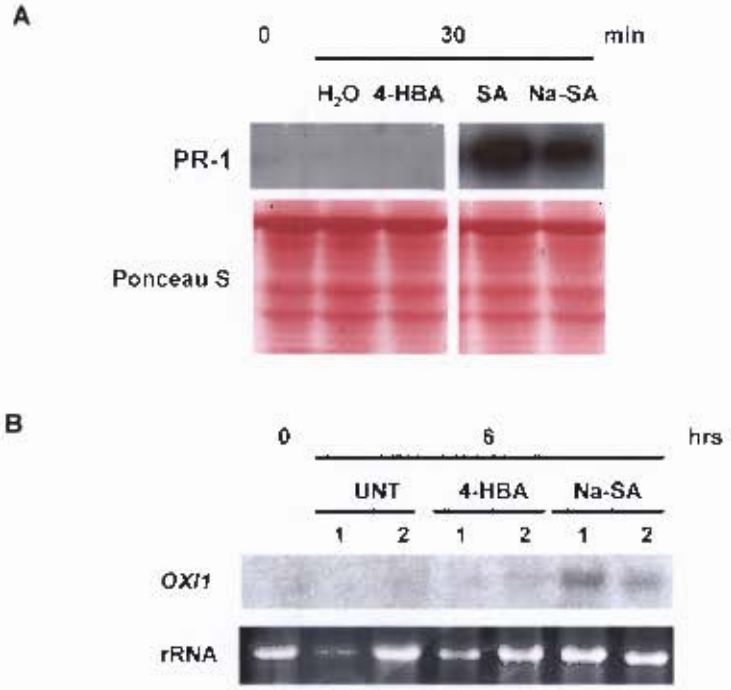


Figure 4.12 SA induces PR-1 protein and *OXI1* gene expression

Two week old Col-0 seedlings were incubated in H_2O, 1 mM 4-HBA, 1 mM SA or 1 mM Sodium salicylate (Na-SA) for 30 min and were subsequently placed in water. Seedlings were harvested before the experiment (0) or 48 hrs following treatment to allow for protein production (A). Immunoblot analysis was performed with PR-1 antiserum at a 1:125 dilution. The Ponceau S stained membrane was used as a loading control. (B) The abaxial surface of leaves of 4 week old Col-0 plants were infiltrated with 1 mM Na-SA, 1 mM 4-HBA or left untreated (UNT). Leaves were harvested before any treatment (0) and at 6 hrs after treatment and steady state *OXI1* mRNA determined. Duplicate samples (1 and 2) represent leaves from two independent plants. The ethidium bromide stained rRNA was used as a loading control. These experiments were performed twice with similar results.

lanthanum indicative of a requirement for Ca^{2+} in *OXI1* gene expression (Rentel, 2002). Therefore it was investigated using transgenic Arabidopsis seedlings expressing the calcium sensitive photoprotein aequorin (Knight *et al.*, 1991) whether exogenously applied SA induced an increase in $[Ca^{2+}]_c$ and, if so, whether this SA-induced $[Ca^{2+}]_c$ was essential for SA-mediated *OXI1* expression.

SA induced a transient increase in $[Ca^{2+}]_c$ that reached maximal levels around 2 min and decreased within 6 min whereas 4-HBA was unable to induce similar increases in $[Ca^{2+}]_c$ (Figure 4.13 A). Consequently, this transient induction of $[Ca^{2+}]_c$ by SA was thought to be specific to SA since it was also distinct from changes in $[Ca^{2+}]_c$ produced in response to other environmental stimuli, for example cold induced a single $[Ca^{2+}]_c$ transient lasting approximately 30 seconds (Knight *et al.*, 1996) whereas fungal elicitors induced a much longer biphasic increase in $[Ca^{2+}]_c$ (Blume *et al.*, 2000) . However, treatment of transgenic Arabidopsis seedlings with Na-SA failed to induce an increase in $[Ca^{2+}]_c$ (Figure 4.13 A). The initial $[Ca^{2+}]_c$ spike occurring within 10 sec in response to all three treatments is due to the touch response (Knight *et al.*, 1991) which results from the addition of the chemicals (Figure 4.13 A). It was considered that perhaps the SA induced $[Ca^{2+}]_c$ increase was due to the acidity of SA since 1 mM SA has a pH of 3.5 while 1 mM 4-HBA has a pH of 4.5 and 1 mM Na-SA failed to induce an increase in $[Ca^{2+}]_c$. Alternatively, Na-SA may cause a weak increase in $[Ca^{2+}]_c$ that is undetectable in the whole plant aequorin system particularly since not all cells are exposed to treatment nor are the cells exposed to treatment at the same time which adds to the difficulty in detecting a weak response. Therefore attention was focussed on Arabidopsis cell cultures expressing the aequorin gene as SA should be equally accessible to all cells simultaneously. It was found that only Na-SA concentrations 25-fold above that which was routinely used to study the effect of SA on disease resistance, resulted in an increase in $[Ca^{2+}]_c$ that mimicked the SA induced $[Ca^{2+}]_c$ transient (Figure 4.13 B).

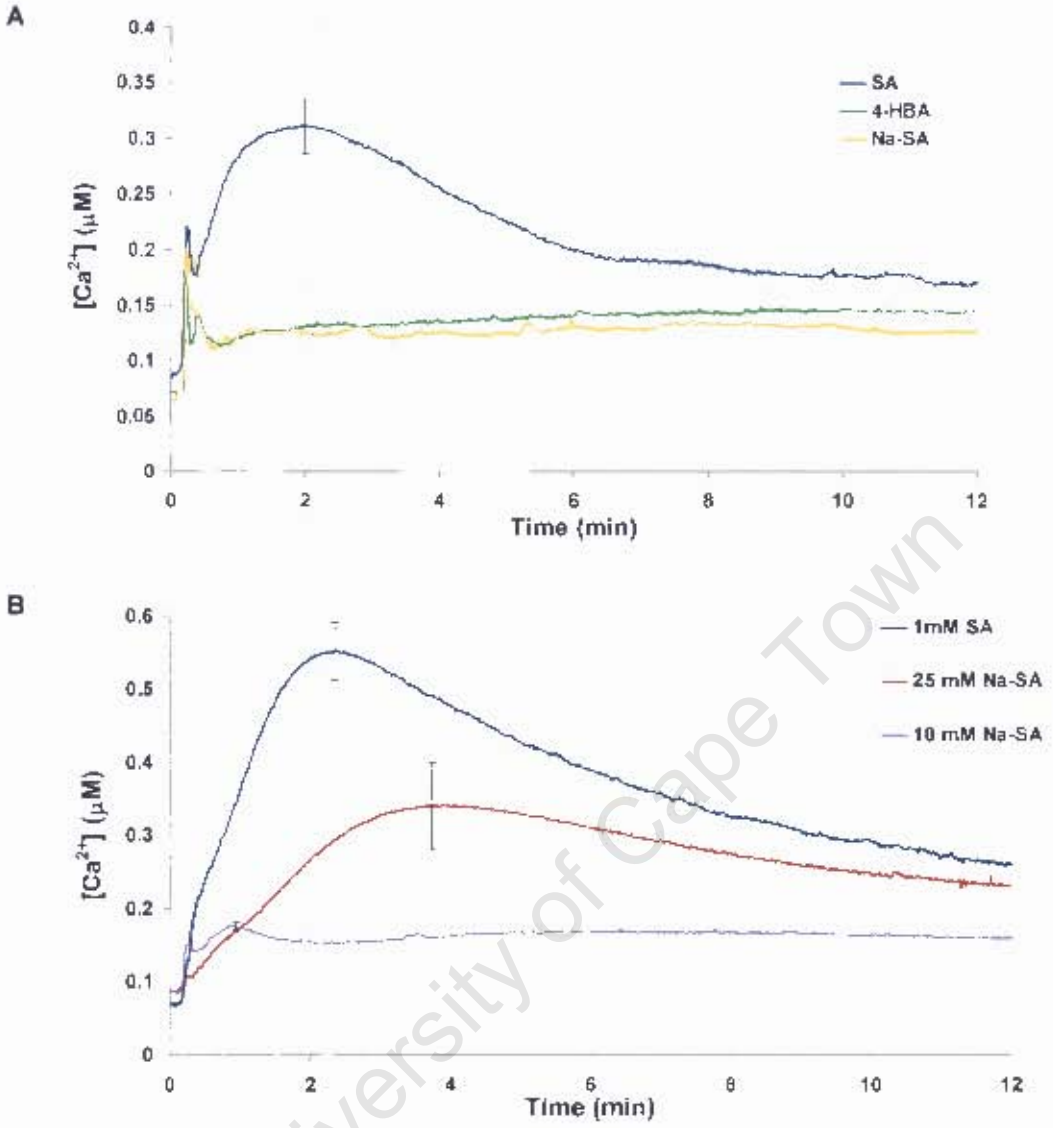


Figure 4.13 A SA-induced $[Ca^{2+}]_c$ increase

(A) Seven day old transgenic Arabidopsis seedlings (Col-0) expressing the calcium sensitive protein aequorin were treated with 1 mM SA, its non functional analogue 4-HBA or 1 mM Na-SA with only SA inducing a significant increase in $[Ca^{2+}]_c$. (B) Arabidopsis cell cultures expressing the aequorin gene were treated with 1 mM SA or a 10 and 25 mM Na-SA to determine whether Na-SA induces $[Ca^{2+}]_c$ in a dose dependent manner. Bioluminescence measurements from several plants or cell culture samples (n=8) for each treatment were recorded over time and converted to $[Ca^{2+}]_c$ (Knight et al., 1996). Traces represent the average increase in $[Ca^{2+}]_c$ over time and the standard error of the mean at peak maxima is shown. These experiments were performed twice with similar results.

To assess whether lower, biologically relevant, concentrations of Na-SA induced increases in $[Ca^{2+}]_c$ that were still below detection even in the cell culture system, the amount of aequorin that remained after treatment was used as a measure to determine if Ca^{2+} had been increased in the cytosol during treatment. Aequorin interacts with 3 Ca^{2+} ions in an irreversible reaction to produce apoaequorin, coelenteramide and emit blue light. Therefore if $[Ca^{2+}]_c$ is increased during a given response it will reduce the pool of aequorin present in the cell and consequently the bigger the $[Ca^{2+}]_c$ response the lower the amount of aequorin remaining. Arabidopsis cell cultures were treated for either 30 min or 6 hrs with Na-SA or for control treatments incubated in water or left in solution with the chromophore coelentrazine for 6 hrs and the relative amount of aequorin that remained was determined by the amount of light emitted following discharge of aequorin with excess calcium (Figure 4.14 A). It would be expected that if small changes in $[Ca^{2+}]_c$ had occurred in response to Na-SA then the amount of remaining aequorin would be significantly lower in the treated samples in comparison to the controls. However, no significant difference in total luminescence was observed between the control and Na-SA treated cell cultures (Figure 4.14 A). Buffering the acid form of SA to pH 7 in a sodium phosphate buffer (SA-PO_4) did not cause increases in $[Ca^{2+}]_c$ since the total luminescence produced after SA-PO_4 treatment was similar to that produced in the water and Na-SA treated cell cultures while SA treatment induced large changes in $[Ca^{2+}]_c$ since no aequorin remained (Figure 4.14 B). Therefore the SA-induced $[Ca^{2+}]_c$ increase is most likely an acid effect and it could be postulated that *in planta* SA does not induce changes in $[Ca^{2+}]_c$ to mediate its defence related processes. Furthermore the induction of *OXI1* gene expression by Na-SA is calcium-independent. However, it is possible that the SA-induced $[Ca^{2+}]_c$ transient may have an effect on other SA mediated pathways unrelated to defence.

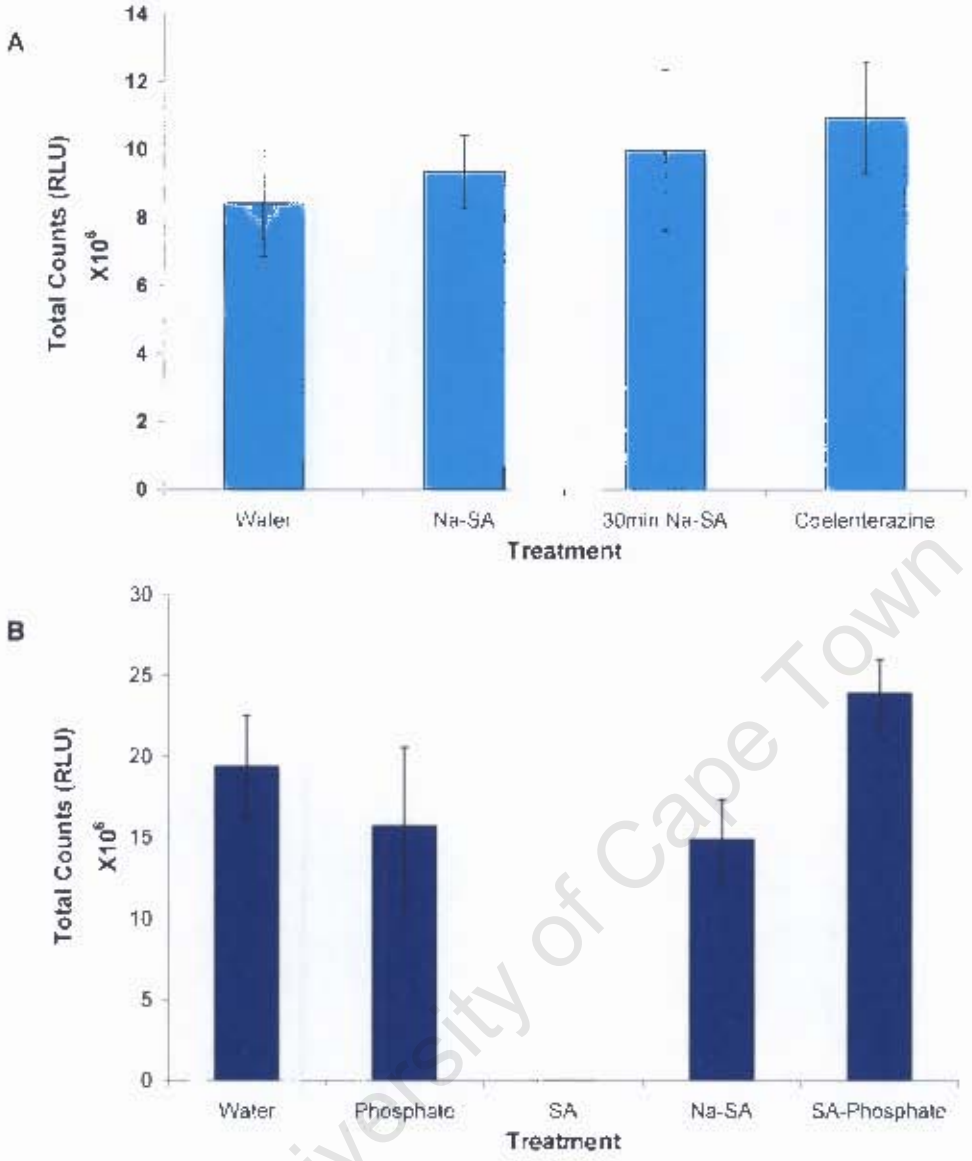


Figure 4.14 Na-SA does not induce small changes in $[Ca^{2+}]_c$ in Arabidopsis cell culture

(A) Arabidopsis cell cultures expressing the calcium sensitive photoprotein aequorin were treated with Na-SA for 30 min (30 min Na-SA) or 6 hrs (Na-SA), water for 6 hrs or left in coelenterazine for a further 6 hrs following overnight incubation to show that difference in the amount of reconstituted aequorin within the cell cultures were not affecting the experimental results. (B) Arabidopsis cell cultures were treated with water, sodium phosphate buffer pH 7 (Phosphate), SA, Na-SA or the acid form of SA buffered in sodium phosphate buffer pH 7 (SA-Phosphate) for a duration of 6 hrs. For both A and B, the remaining aequorin was discharged with excess $CaCl_2$ in 20 % ethanol. The bars represent the bioluminescence counts recorded as relative light units (RLU) of an average of 5 samples per treatment and the standard error is shown

4.10 The *oxi1* mutant can establish SA-mediated SAR

A role for OXI1 in SA-mediated SAR was examined since the accumulation of SA has proven vital for the establishment of SAR and SA induced *OXI1* gene expression (Figure 4.12 B and (Dempsey *et al.*, 1999)). Leaves were first inoculated with 1 mM SA to induce SAR or mock inoculated with 0.5% ethanol. The induction of PR-1 protein is a characteristic molecular marker used for the establishment of SAR (Cao *et al.*, 1994). Western analysis revealed that after 48 hrs SA treatment, PR-1 protein was induced in the *oxi1* mutant and 35S::OXI1 transgenic line to similar levels as that observed in the *oxi1* complemented line (Figure 4.15 A). In some instances PR-1 was detected in different genotypes in the ethanol treated samples between different experiments, however PR-1 expression was always very weak in these samples and was consistently induced to a much greater level in SA treated samples for all genotypes. The *npr1* mutant displays increased susceptibility to *P. syringae* infection, is unable to induce the expression of PR-1 and fails to establish SAR (Cao *et al.*, 1994). As expected no PR-1 protein was detected in the *npr1* mutant in response to SA treatment (Figure 4.15 A). Therefore this suggests that all genotypes, apart from *npr1*, had established SAR and modulation of *OXI1* expression levels had no effect on SA-induced gene expression.

Following challenge with virulent *P. syringae*, the SA pre-treated leaves exhibited significantly lower bacterial growth in comparison to the bacterial growth yielded from the mock (ethanol) inoculated leaves for all genotypes tested (Figure 4.15 B). However, as expected the *npr1* mutant did not show reduced bacterial growth in leaves pre-treated with SA. Both the *oxi1* mutant and 35S::OXI1 transgenic line were more susceptible than the *oxi1* complemented line with and without SA pre-treatment but did show reduced susceptibility after SA pre-treatment compared to ethanol pre-treatment (Figure 4.15 B). Consequently these results suggest that both the *oxi1* mutant and the transgenic line overexpressing *OXI1* are capable of mounting an effective SAR against subsequent pathogen attack hence OXI1 is not essential for the establishment of SA-mediated SAR.

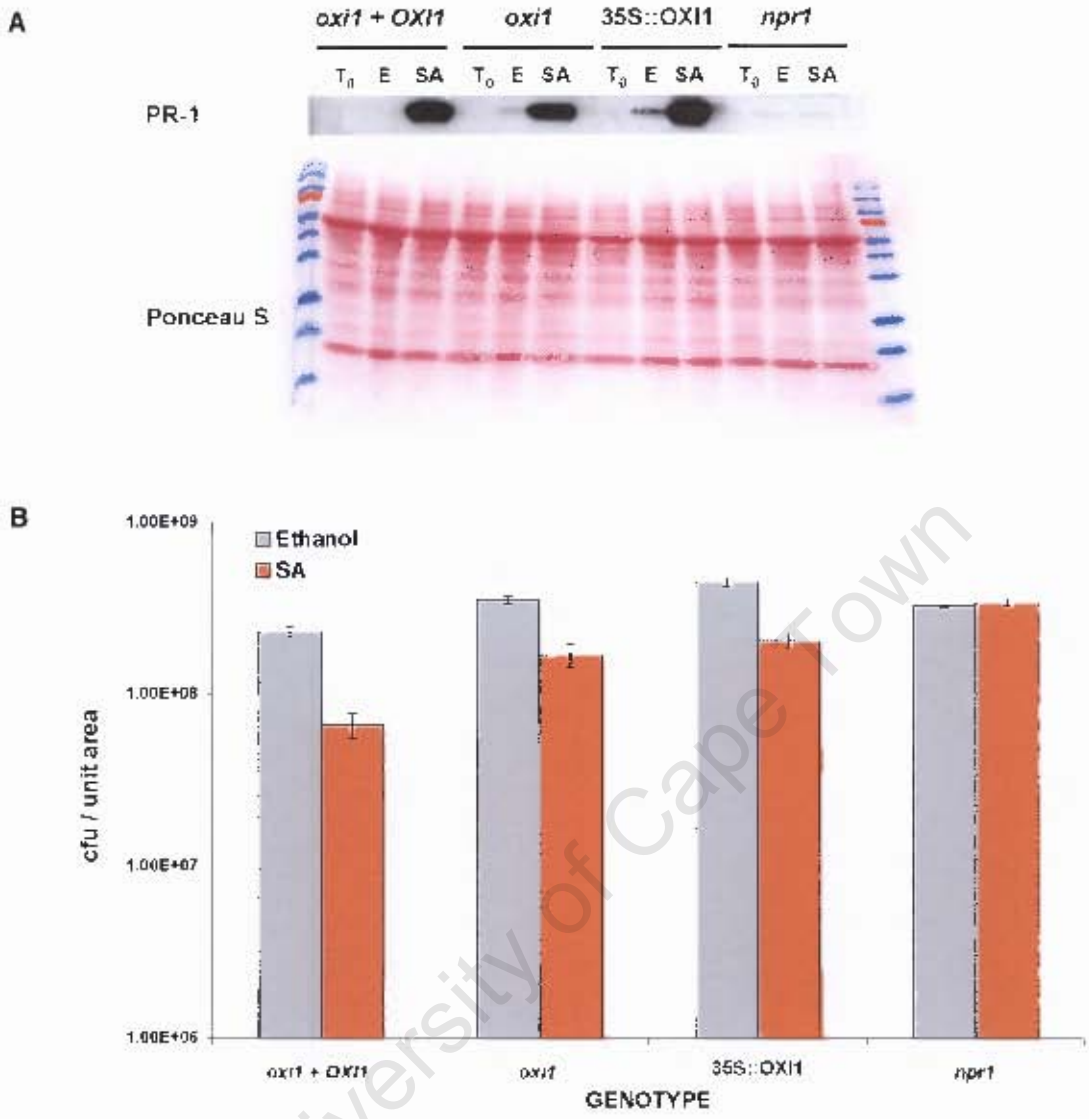


Figure 4.15 Systemic acquired resistance is not compromised in the *oxi1* mutant

Systemic acquired resistance was induced in individual plants (genotypes: *oxi1* + *OXI1* complement, *oxi1* mutant, 35S::OXI1 and *npr1*) by infiltrating the same leaves with 1 mM SA, 48 hrs prior to infection with virulent *PstDC3000*. Control plants were infiltrated with a solution of 0.5 % Ethanol prior to infection. (A) Leaves were harvested before treatment (T_0) or 48 hrs post SA (SA) or ethanol (E) treatment. Western analysis was performed with PR-1 antiserum at a 1:125 dilution and the Ponceau S stained immunoblot was used as a loading control. (B) Leaves treated with either SA or ethanol, were pressure inoculated 2 days post treatment with 5×10^5 cfu/ml virulent *PstDC3000*. The bars represent the log of bacterial growth at 48 hrs post infection and the standard error with a 95 % confidence level is shown.

4.11 *P. syringae* root pathogenicity is not more severe in the *oxi1* mutant

Although *P. syringae* is not a classical soil borne pathogen, it was demonstrated to colonise Arabidopsis roots and cause extensive necrosis (Bais *et al.*, 2004). The root exudates of wild type Arabidopsis mediate resistance to several non pathogenic *P. syringae* strains as well as to virulent *P. syringae* with a dysfunctional type III secretion system (Bais *et al.*, 2005). The basal level of *OXI1* expression is particularly high in the roots and OXI1 is required for normal root development under conditions of mild stress (Rentel *et al.*, 2004). Therefore it was investigated whether the *oxi1* mutant displayed increased susceptibility to root pathogenicity inflicted by virulent *Pst*DC3000 challenge. The severity of disease was not increased in the *oxi1* mutant compared to wild type during a root infection with *Pst*DC3000 (Figure 4.16). Furthermore, the onset of disease and eventual death occurred at similar times in all the Arabidopsis genotypes tested including the *npr1* mutant (Figure 4.16). Although this is a crude assay, the data suggests that the high basal expression of *OXI1* within the root zone does not affect the entry and/or growth of *P. syringae* colonisation of Arabidopsis roots.

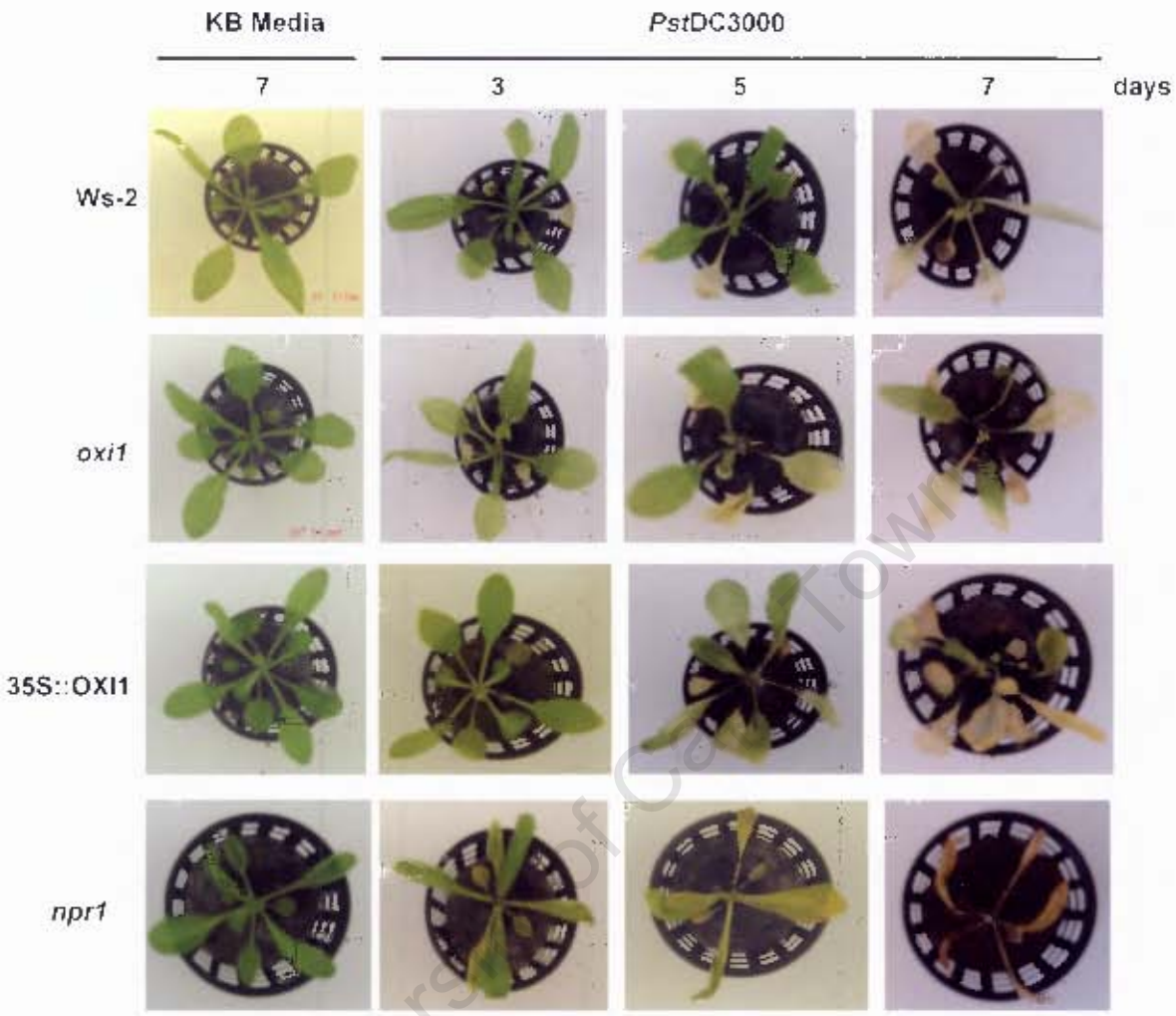


Figure 4.16 The *oxi1* mutant does not display increased susceptibility to *Pst*DC3000 in the root pathogenicity assay

Three week old plants of genotype Ws-2, *oxi1* mutant, 35S::OXI1and *npr1* were infected with 10 ml of either KB media supplemented with 50 µg/ml rifampicin or a virulent *Pst*DC3000 culture at OD_{600} = 0.2. The onset of disease symptoms was examined visually for seven consecutive days post treatment and no appreciable difference in the extent of disease was detectable between the different genotypes. Ten plants per line were treated with KB only or *Pst*DC3000 and all ten plants within a genotype exhibited similar susceptibility phenotypes on any given day. A representative plant for each genotype is shown at 3, 5 and 7 days post *Pst*DC3000 infection and 7 days post treatment for KB control. This experiment was performed twice with similar results.

4.12 *Botrytis cinerea* induces *OXI1* gene expression

Necrotrophic pathogens exploit the plant defence responses of AOS accumulation and the HR, processes that cause plant cell death to contain biotrophic pathogens, to facilitate infection (Govrin and Levine, 2000; Glazebrook, 2005). It has been suggested that Arabidopsis employs different defence mechanisms against various *B. cinerea* isolates since distinct quantitative trait loci governing susceptibility to *B. cinerea*, were identified for two isolates (Denby *et al.*, 2004). Given the induction of *OXI1* by biotrophic pathogens (*H. parasitica* and *P. syringae*) it was investigated whether infection with a necrotrophic pathogen also induces *OXI1* expression. It was also determined if different isolates of *B. cinerea* differentially regulated *OXI1* expression. Three isolates of *B. cinerea* were used to infect excised leaves of OXI1::GUS transgenic plants. Lesions were visible from 48 hrs post infection for all three isolates and the accumulation of H_2O_2, visualised through DAB staining, occurred in and around the infection site (Figure 4.17). *B. cinerea* is known to cause induction of AOS which is initially localised at the site of infection (6 hrs post infection) and with the progress of *B. cinerea* growth, AOS production spreads several cell layers away from the fungal hyphae facilitating spread of the developing lesion (Govrin and Levine, 2000). Histochemical GUS staining revealed that *OXI1* was induced by all three isolates of *B. cinerea* at 48 hrs post infection and that *OXI1* expression occurred around the lesion (Figure 4.17). Since lesion formation, H_2O_2 accumulation and OXI1::GUS expression were all first detected at the same time i.e. 48 hrs under these experimental conditions it is difficult to say whether it is the generation of H_2O_2 by *B. cinerea* that causes *OXI1* gene expression. Nonetheless, the induction of *OXI1* in response to *B. cinerea* may, like in response to biotrophic pathogens, play a role in limiting pathogen spread. The control leaves drop inoculated with 50 % (v/v) grape juice display no lesion formation, H_2O_2 accumulation or GUS activity for the entire duration of the experiment (Figure 4.17 A).

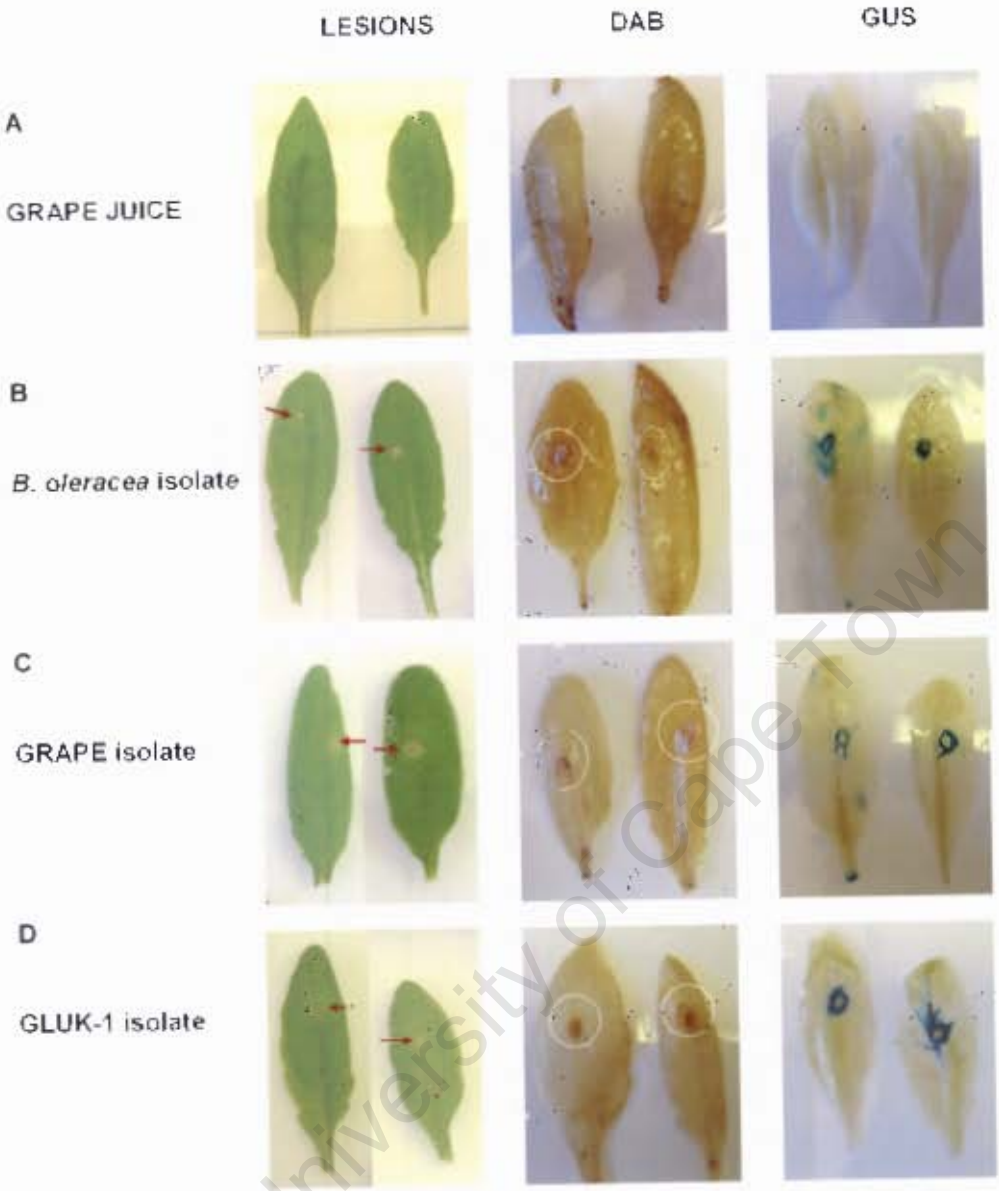


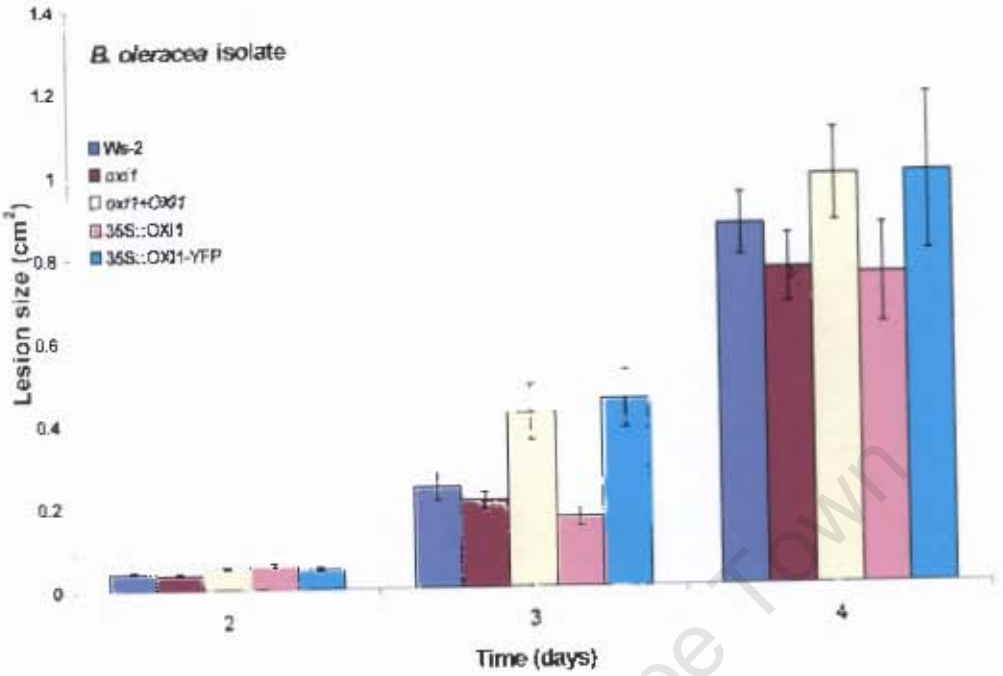
Figure 4.17 *B. cinerea* induces an oxidative burst and *OXI1* expression

Excised leaves of 4 week old OXI1::GUS transgenic plants were drop inoculated with either 50 % (v/v) Grape Juice (**A**) or one of the three *B. cinerea* isolates at a spore concentration of 5 X 10^4 spores/ml. *B. oleracea* (**B**), grape (**C**) or GLUK-1 (**D**). Leaves were assayed for lesion formation, an oxidative burst and *OXI1* expression 48 hrs post infection. The arrows (→) indicate where the lesion had developed. The presence of an oxidative burst (white circles) was determined by staining the leaves with 1 mg/ml DAB solution, which forms a reddish-brown precipitate with H_2O_2. *OXI1* expression was visualised through *in vivo* GUS staining. This experiment was performed twice with similar results.

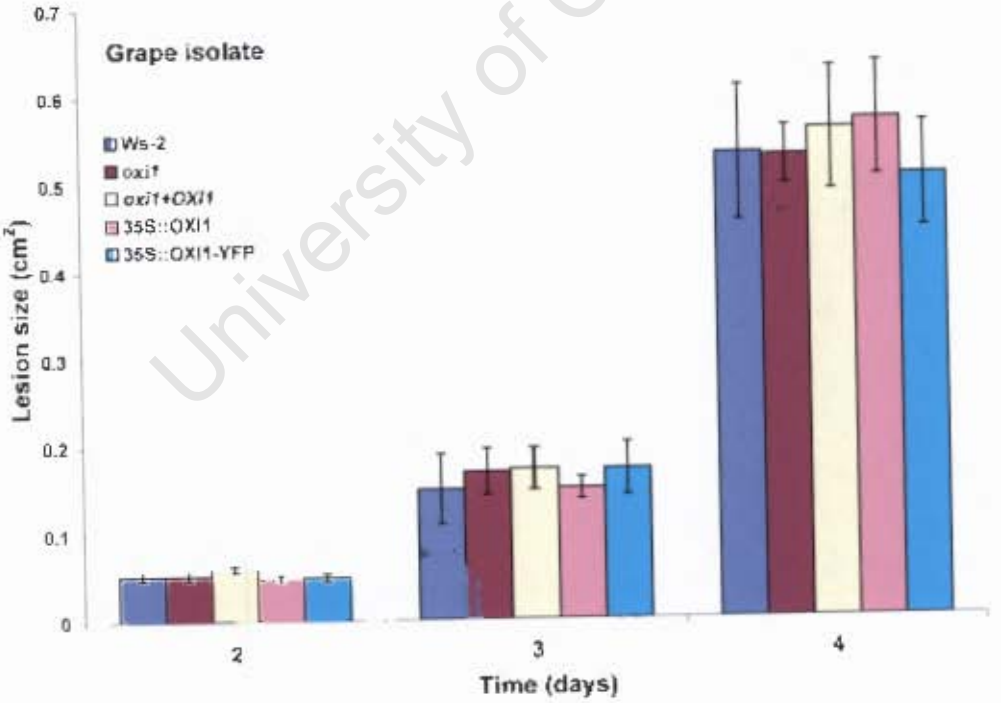
4.13 The *oxi1* mutant is not more susceptible to *B. cinerea* infection

The induction of *OXI1* by all three *B. cinerea* isolates suggests that OXI1 could play a role in resistance to this pathogen. Employing a detached leaf assay (Denby *et al.*, 2004), the degree of susceptibility in the different transgenic lines of OXI1 to *B. cinerea* infection was assessed. Following inoculation with *Botrytis*, the area of the developing lesion is measured over time and the size of the developing lesion has previously been shown to directly correlate with plant susceptibility, fungal growth and disease severity i.e. the more susceptible the plant, the greater the lesion size and pathogen biomass hence the greater the extent of disease (Govrin and Levine, 2000; Ferrari *et al.*, 2003; Denby *et al.*, 2004; Murray *et al.*, 2005). The *oxi1* mutant exhibits wild type lesion size in response to all three *B. cinerea* isolates (Figure 4.18 A-C) at all time points tested. Consequently, the *oxi1* mutant is not more susceptible than wild type (Ws-2) to *B. cinerea* infection. At three days post infection, the *oxi1* + *OXI1* complemented and the 35S::OXI1-YFP lines displayed increased lesion size whereas the 35S::OXI1 transgenic line showed reduced lesion size in comparison to wild type and the *oxi1* mutant in response to infection with the *B. oleracea* isolate (Figure 4.18 A). However, the lesion size between all genotypes was not significantly different at four days post infection (Figure 4.18 A). The difference observed in lesion size at day three may possibly be the result of variability between the two biological systems within each experiment. In an independent repeat experiment both overexpressing *OXI1* lines had marginally larger lesions (difference of 0.1 to 0.15 cm^2) than wild type in response to infection with the *B. oleracea* isolate at three days post infection whereas four days post infection there was no significant difference in lesion size between all genotypes (data not shown). Furthermore, the lesion size in both experiments was not limited by the size of the leaf. There was no significant difference observed in the lesion size between all genotypes in response to the grape and GLUK-1 isolates (Figure 4.18 B and C). Taken together the data suggests that although *OXI1* is induced by *B. cinerea* it is not required for the establishment of defence responses to limit fungal growth.

A



B



C

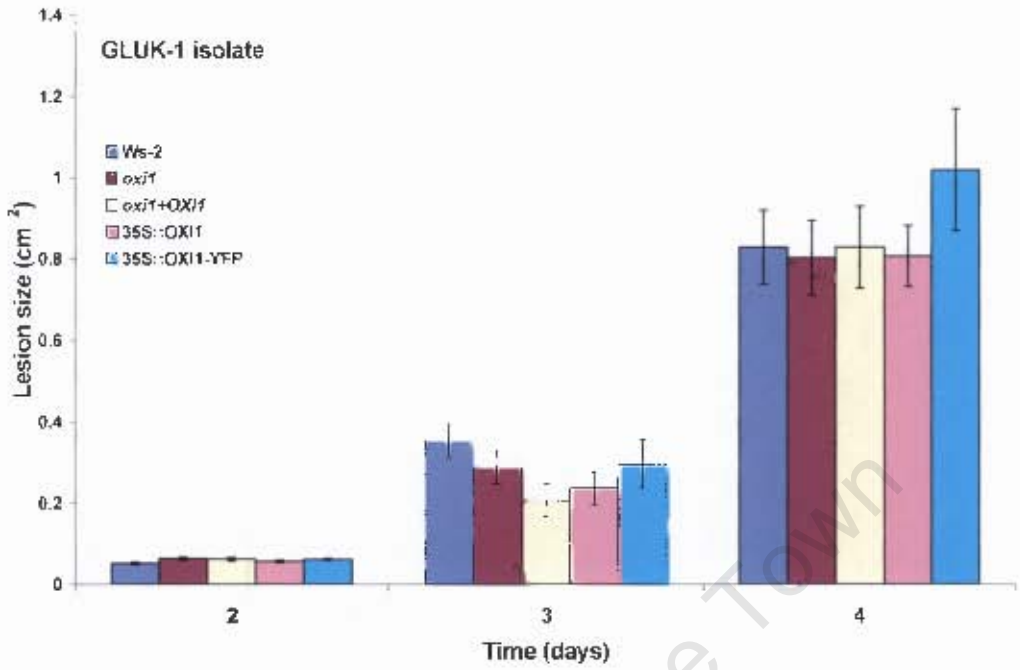


Figure 4.18 Susceptibility of *OXI1* genotypes to *B. cinerea* infection

Leaves of 3 week old *Arabidopsis* plants; wild type (Ws-2), *oxi1*, complemented line (*oxi1* + *OXI1*) and the *OXI1* overexpressing transgenic lines (35S::OXI1 and 35S::OXI1-YFP), were excised and drop inoculated with 4µl of 5 X 10^4 spores/ml of *B. cinerea* isolates: *B. oleracea* (A), grape (B) and GLUK-1 (C). At least 10 leaves from different plants per *Arabidopsis* line were used for each isolate. Photographs of developing lesions (including the size of the ruler) were taken 2, 3 and 4 days post infection and the lesion area was measured with ImageJ 1.34n software. The bars represent the mean lesion size and the standard error is shown. The experiment was performed twice with similar results.

4.14 *PDF1.2* expression uncompromised in the *oxi1* mutant

JA and ET signalling pathways have been implicated in resistance to necrotrophic pathogens and the *Plant defensin 1.2* (*PDF1.2*) gene is a marker for these JA/ET-dependent pathways (Murray *et al.*, 2002; Mengiste *et al.*, 2003; Veronese *et al.*, 2004). *PDF1.2* expression is induced by *B. cinerea* and thought to mediate resistance to this pathogen (Penninckx *et al.*, 1998). However, conflicting reports have uncoupled *PDF1.2* expression from resistance to *B. cinerea* since normal induction of *PDF1.2* was observed in mutants that exhibited enhanced susceptibility to *B. cinerea* (Ferrari *et al.*, 2003; Veronese *et al.*, 2004). Evidence for the involvement of SA and its signalling components to combat susceptibility to necrotrophic pathogens is also emerging (Ferrari *et al.*, 2003). It was investigated whether OXI1 affected *PDF1.2* expression, even though the *oxi1* mutant was not more susceptible than wild type to *B. cinerea* challenge, since both genes were induced by *B. cinerea* infection (Figure 4.13 and Penninckx *et al.*, (1998)) and to also determine whether OXI1 could be involved in JA/ET-dependent defence pathways. The expression of *PDF1.2* in response to infection with the grape isolate of *B. cinerea* was not compromised in the *oxi1* mutant compared to wild type (Figure 4.19). This data suggests that OXI1 is not likely to be a component of JA/ET-signalling pathway mediating induction of *PDF1.2*.

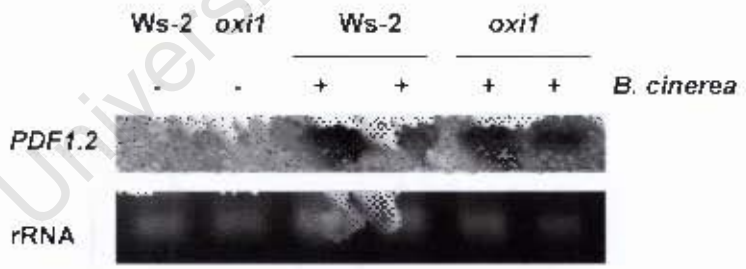


Figure 4.19 *Plant Defensin 1.2* gene expression is not compromised in the *oxi1* mutant in response to *B. cinerea* infection

Detached leaves of 3 week old wild type (Ws-2) and *oxi1* plants were drop inoculated with the grape isolate of *B. cinerea* at 5 X 10^4 spores/ml (+) or 50 % grape juice (-). Visible lesions began to develop around 3 days post infection and the induction of *PDF1.2* expression in response to *B. cinerea* challenge was determined at 4 days post infection. The ethidium bromide stained RNA was used as a control for equal RNA loading.

4.15 Discussion

4.15.1 Demonstration of a role for OXI1 in defence against *P. syringae*

This work established a role for OXI1 in defence against *P. syringae.* Not only do steady state mRNA levels of *OXI1* increase after infection with both virulent and avirulent *P. syringae* but the *oxi1* null mutant exhibits increased susceptibility in response to *P. syringae* infection which is rescued in the *oxi1* complemented line (Figures 4.3 and 4.6). The induction of *OXI1* in response to *P. syringae* infection correlates with the oxidative burst suggesting that the accumulation of H_2O_2 is responsible for *OXI1* gene expression (Figure 4.3). As previously described the *atrbohD* mutant, defective in NADPH oxidase (Torres *et al.*, 2002) lacks the oxidative burst in response to challenge with avirulent *Pst*DC3000 *avrB* (Figure 4.4 A). *OXI1* gene expression was decreased in the *atrbohD* mutant when infected with *Pst*DC3000 *avrB* which suggests that AOS produced through NADPH oxidase during incompatible interactions is at least partly responsible for *OXI1* induction (Figure 4.4 B). Additionally, OXI1::GUS transgenic plants co-infiltrated with *Pst*DC3000 *avrB* and DPI, a chemical inhibitor of NADPH oxidase, exhibited a reduction in GUS activity in comparison to avirulent treatment alone (Figure 4.5). This further implicates a role for NADPH oxidase in *OXI1* gene expression. Alternative mechanisms for AOS production during pathogen attack have been demonstrated. Expression analysis and enzymatic activity assays illustrates that oxalate oxidases produce AOS during the incompatible interaction between barley and *Erysiphe cichoracearum* (Zhou *et al.*, 1998a). In tobacco cell suspension culture SA treatment induces the generation of O_2^- and H_2O_2 through peroxidase-catalysed reactions which in turn causes an increase in $[Ca^{2+}]_c$ (Kawano and Muto, 2000). Both SA and changes in the $[Ca^{2+}]_c$ concentration are necessary for the establishment of plant defences during incompatible interactions in Arabidopsis (Dempsey *et al.*, 1999; Grant *et al.*, 2000b) thus peroxidase activity could be important in mediating such defence processes. Neither the *OXI1* transcript in the *atrbohD* mutant nor GUS activity from co-infiltration of *Pst*DC3000 *avrB* and DPI is completely abolished. Therefore a role for either other AOS generating mechanisms such as cell wall bound peroxidases or active oxygen species other than H_2O_2, in the expression of the *OXI1* gene during incompatible interactions is likely.

4.15.2 OXI1 is involved in resistance against biotrophic pathogens

OXI1 positively regulates resistance responses to biotrophic pathogens since the *oxi1* null mutant has compromised resistance in response to virulent *H. parasitica* and both virulent and avirulent *P. syringae* challenge. Therefore OXI1 has a role in basal defence systems effective against *H. parasitica* and *P. syringae* infection as well as a role during the incompatible interaction with *P. syringae*. It is possible that OXI1 may have the same or similar functions in both resistance responses. Perhaps OXI1 triggers phosphorylation events that are common to both basal and R gene-mediated resistance and this signalling cascade may in turn activate defence responses to restrict or slow the process of pathogen growth. This is not without precedent since molecular components affecting both basal defence and *R* gene-mediated resistance have been identified. For example, a mutation in the *EDS1* gene which encodes a protein with similarity to triacyl glycerol lipases results in *eds1* plants being more susceptible to both virulent and avirulent isolates of *H. parasitica* (Parker *et al.*, 1996). Similarly, the Arabidopsis *eds5*, *npr1*, *salicyclic acid-induction deficient2* (*sid2*) and *pad4* mutants display increased susceptibility to virulent and avirulent strains of *H. parasitica* and *P. syringae* (Cao *et al.*, 1994; Zhou *et al.*, 1998b; Nawrath and Métraux, 1999). Interestingly, silencing of *MPK6* compromised resistance to both virulent and avirulent strains of *P. syringae*, as did OXI1, but these plants showed enhanced susceptibility to avirulent and not virulent *H. parasitica* (Menke *et al.*, 2004). Since OXI1 is required for full activation of MPK6 during cellulase and H_2O_2 treatment (Rentel *et al.*, 2004), OXI1 may play a role in basal defence and R gene-mediated resistance through MPK6 at least in response to *P. syringae*. The difference in response of the *mpk6* and *oxi1* mutants to *H. parasitca* infection suggests that MPK6 and OXI1 are either not involved in the same signal transduction cascades activated by this pathogen or the lack of one kinase may lead to the activation of another to compensate for its function. The latter argument is supported by the observation that a given MAPKK can activate different MAPKs in response to a single stimulus. For example, during elicitor treatment of tobacco suspension-cultured cells NtMEK2 was shown to activate both SIPK and WIPK (Yang *et al.*, 2001) and similarly treatment of Arabidopsis protoplasts with flg22 triggered a MAPK cascade module whereby MKK4 and MKK5 activated both MPK3 and MPK6 (Asai *et al.*, 2002).

In a previous study, the *oxi1* mutant was not compromised in resistance to the avirulent isolate of *H. parasitica* Emoy2 (Rentel, 2002). This suggests that OXI1 does not play a role in R-mediated resistance responses to *H. parasitica*. However, Emoy2 contains three avirulence factors, AvrRPP1A and AvrRPP1B which are recognised by the R gene products RPP1A and B in Ws-2 ecotype and AvrRPP4 recognised by RPP4 in the Col ecotype (Holub *et al.*, 1994; van der Biezen *et al.*, 2002). It was demonstrated here that the importance of OXI1 signalling in response to different avirulence gene products varied amongst the different Arabidopsis ecotypes. In response to *Pst*DC3000 *avrRpt2* challenge the *oxi1* null mutant displayed increased susceptibility in the Ws-2 background but showed wild type resistance in the Col-0 background (Figure 4.6), indicating that OXI1 signalling is more important relative to other defence signalling pathways in the former ecotype during this incompatible interaction. The requirement for OXI1 signalling during the incompatible interaction with *Pst*DC3000 *avrB*, however, is similar in the different ecotypes since both *oxi1* knockouts displayed increased susceptibility to infection with this strain (Figure 4.5 D). Therefore investigating the effect of resistance in the *oxi1* knockout in the Col ecotype following challenge with the avirulent *H. parasitica* Emoy2 strain may demonstrate whether OXI1 signalling is required for the AvrRPP4-RPP4 interaction or if OXI1 is indeed not involved in resistance against Emoy2.

Given the importance of OXI1 signalling in basal defence systems against *H. parasitica* and *P. syringae* infection, it is likely that OXI1 is also involved in nonhost resistance. Similar basal defence systems or signalling pathways are activated in response to infection with a virulent pathogen or a nonhost microorganism, except that this basal defence system is effective at preventing colonisation of the nonhost microoganism but unable to prevent disease by the virulent pathogen (Ingle *et al.*, 2006). Additionally, the isolation of mutants defective in both nonhost and R gene-mediated resistance provides overlap between these two pathways. The *nonhost1* (*nho1*) mutant is defective in resistance to the nonhost pathogen *P. syringae* pv *phaseolicola* and displays enhanced susceptibility to *P. syringae* expressing the avirulent effector proteins RPS2, RPS4, RPS5 and RPM1 (Lu *et al.*, 2001). Similarly, *eds1* exhibits increased susceptibility to several avirulent pathogens and is compromised in nonhost resistance against isolates of *H. parasitica* and *Alternaria candida* (Parker *et al.*, 1996; Aarts *et al.*, 1998). Therefore since the *oxi1* mutant is more susceptible to avirulent *P. syringae* carrying the effector proteins RPS2 and AvrB it would be interesting to determine whether *oxi1* has reduced

resistance to nonhost species of *Pseudomonas* as well as other nonhost pathogens of Arabidopsis.

In contrast to biotrophic pathogens, necrotrophs induces the generation of AOS to aid the spread of infection. The induction of *OXI1* expression in response to infection with all three isolates of *B. cinerea*, possibly through AOS accumulation, suggests OXI1 may play a role in defence related processes against *Botrytis* (Figure 4.16). However, unlike the response to biotrophic pathogens, the *oxi1* null mutant was not more susceptible than wild type to *B. cinerea* challenge (Figure 4.17). The reason for this may be two fold. Firstly, a role for OXI1 in defence against *B. cinerea* may be masked either by the severity of disease caused by *B. cinerea* infection or functional redundancy of OXI1 by another protein kinase. For instance assuming OXI1 has a role in defence against *B. cinerea*, the induction of OXI1 in response to *B. cinerea* may activate signal transduction cascades that are insufficient, in isolation, to induce plant defence mechanisms of great magnitude. Therefore even though this putative cascade may be impaired in the *oxi1* mutant it does not phenotypically affect the rate of lesion development. It could also be postulated that other protein kinases might be able to compensate for the lack of functional OXI1 protein. Secondly, the most likely scenario would be that OXI1 does not mediate signal transduction cascades in response to *B. cinerea* infection to facilitate resistance to this necrotrophic pathogen and OXI1 expression is an indirect consequence of AOS produced by the pathogen during infection. Wild type expression levels of *PDF1.2* in the *oxi1* mutant following infection with the grape isolate of *B. cinerea* supports this conclusion (Figure 4.18). Although, *PDF1.2* expression has been uncoupled from resistance to *B. cinerea* since mutants that showed enhanced susceptibility to *B. cinerea* exhibited normal induction of *PDF1.2* (Ferrari *et al.*, 2003; Veronese *et al.*, 2004). Therefore the role of *PDF1.2* in resistance to *B. cinerea* is questionable but its expression is independent of OXI1 removing a role for OXI1 in JA/ET signal transduction pathways, which facilitates resistance to necrotrophic pathogens, operating through PDF1.2. Recently, a membrane-anchored serine/threonine protein kinase has been shown to be required for resistance to *B. cinerea*. *BOTRYTIS-INDUCED KINASE1* (*BIK1*) was transcriptionally up regulated in response to *B. cinerea* infection and like *OXI1* was shown to be required for normal root hair development (Rentel *et al.*, 2004; Veronese *et al.*, 2006).The *bik1* mutant was more susceptible to *B. cinerea* infection but displayed enhanced resistance to virulent

*Pst*DC3000 in comparison to wild type whereas the response to avirulent *P. syringae* was uncompromised (Veronese *et al.*, 2006). This is in contrast to the effects observed in the *oxi1* mutant, which was not compromised in susceptibility to *B. cinerea* but displayed enhanced susceptibility to both virulent and avirulent isolates of *P. syringae.* Additionally, kinase assays revealed that BIK1 was not required for AtMPK3 and AtMPK6 activity, two downstream targets of OXI1 (Rentel *et al.*, 2004; Veronese *et al.*, 2006). Hence it was not surprising that BIK1 did not interact with OXI1 in yeast-two-hybrid assays nor was it required for activity of OXI1 (Veronese *et al.*, 2006). The aforementioned data place BIK1 and OXI1 in distinct disease resistance pathways and OXI1 does not have a pivotal role in resistance to *Botrytis*. Nonetheless this highlights the importance of protein kinases in mediating the signal transduction network of plant defence responses.

4.15.3 Defence gene expression is uncompromised in the *oxi1* mutant

Apart from signalling events, inducible basal defence systems and R gene-mediated resistance also employ similar defence end response strategies to prevent disease, which include the accumulation of AOS and NO, callose deposition and expression of defence related genes (Dangl and Jones, 2001; Thomma *et al.*, 2001; Gomez-Gomez, 2004; Zeidler *et al.*, 2004). It was investigated whether the increased susceptibility phenotype of the *oxi1* mutant was due to aberrant changes in expression of defined defence related genes between the *oxi1* mutant and wild type. Transcript levels of the SA-inducible *PR-1* gene were uncompromised in the *oxi1* mutant suggesting OXI1 is not required for *PR-1* expression. The senescence-related transcription factor WRKY53 was demonstrated to be induced by H_2O_2 (Miao *et al.*, 2004) and the Arabidopsis *wrky53* knockout displayed an enhanced susceptibility phenotype (Dr Shane Murray pers. comm.), like *oxi1*, in response to virulent *P. syringae* infection. Therefore it is probable that WRKY53 may have a role in basal defence to *P. syringae*. However, under the experimental conditions employed in this study *WRKY53* expression was induced in response to wounding rather than pathogen infection since its expression was higher in the control samples for both *oxi1* and wild type. Furthermore, there was no appreciable difference in the transcript levels of *WRKY53* between the *oxi1* knockout and wild type. Therefore OXI1 acts either independently or downstream of WRKY53 in basal defence

and OXI1 is not required for *WRKY53* expression in response to wounding. Investigation of *OXI1* expression in the *wrky53* knockout would illustrate whether WRKY53 regulates OXI1 expression and if these two basal defence pathways are linked. Somewhat surprising, the expression of *VSP1* was uncompromised in the *oxi1* mutant challenged with virulent *P. syringae* despite the fact that OXI1 was shown to regulate MPK6 activity in response to H_2O_2 and both the *oxi1* and *mpk6* mutants were more susceptible to virulent and avirulent *P. syringae* infection. This data suggest that either MPK6 induction of *VSP1* gene expression is independent of OXI1 or perhaps another kinase may be functionally redundant for OXI1 to mediate MPK6 activation of *VSP1* expression.

Despite the *oxi1* mutant displaying increased susceptibility to *P. syringae* challenge in comparison to wild type, the increased susceptibility is not attributable to alterations in tested defence gene expression. This finding suggests that OXI1 signalling is either not required or functionally redundant for the expression of these defence related genes but it does not rule out the possibility that OXI1 may be required for the expression of other defence associated genes. It may also be that the time course employed in this study was not suitable for gene expression studies. For example, if OXI1 signalling increases the timing in which a defence response is established then perhaps a difference in defence gene expression between the *oxi1* mutant and wild type may be observed at earlier time points. Alternatively, OXI1 signalling may be involved in other defence responses such as the synthesis of phytoalexins, such as camalexin, or other secondary metabolites which act as toxins to the invading pathogen and thereby restricting growth.

4.15.4 OXI1 and SA-mediated signal transduction

The induction of *OXI1* gene expression in response to SA and *Pst*DC3000 *avrB* infection points to a role for the kinase in SA-mediated defence responses (Figure 4.9). SA signalling is important in conferring resistance responses to the biotrophic pathogens *H. parasitica* and *P.syringae* and has many facets (Dempsey *et al.*, 1999). It has been observed that transgenic Ws-0 plants expressing *NahG*, a bacterial salicylate hydroxylase gene that converts SA to its catechol, displayed increased susceptibility to infection with virulent Emco5 suggesting that SA mediates basal defence in response to this isolate (McDowell *et al.*, 2005). As a result OXI1 protein kinase may be involved in

SA-mediated basal resistance to Emco5 since *oxi1* is more susceptible than wild type to this isolate. However, OXI1 is not essential for the expression of SA-inducible *PR-1* gene nor for the establishment of SA mediated SAR since the *oxi1* null mutant displays wild type responses during these two processes (Figures 4.10 and 4.14). This implies that either OXI1 does play a role in SA-mediated defence responses but it is functionally redundant or OXI1 does not play a role in SA signal transduction pathways in relation to disease resistance. Due to the complexity of plant disease resistance and the emergence of multiple components facilitating cross talk and redundancy between the numerous signalling pathways it is tempting not to dismiss the former argument (Nürnberger and Scheel, 2001; Glazebrook *et al.*, 2003; Glazebrook, 2005). Consequently, investigation of *OXI1* expression and/or activity in response to pathogen challenge in mutants that either show constitutive SA signalling as well as enhanced disease resistance, such as *cpr5* or *cpr6* (Bowling *et al.*, 1997; Clarke *et al.*, 1998; Jirage *et al.*, 2001), or mutants that are defective in SA responses (*npr1*) (Cao *et al.*, 1994) may help to reconcile the involvement of OXI1 in SA mediated defence signalling.

4.15.5 Modulation of OXI1 protein levels compromises resistance

If OXI1 protein kinase plays a positive regulatory role in plant disease resistance to *H. parasitica* and *P. syringae* infection it could be envisaged that overexpressing the kinase would either lead to increased resistance or have no added effect on resistance compared to wild type. This follows from observations that overexpression of a protein kinase involved in a particular stress response often leads to constitutive activation of signalling pathways and enhanced tolerance to that stress. For example, transgenic Arabidopsis overexpressing the *NDP kinase 2* gene displays elevated activation of downstream targets MPK3 and MPK6 in comparison to wild type in the absence of stimulus and also has enhanced tolerance to several environmental stimuli that generates the accumulation of AOS (Moon *et al.*, 2003). Similarly, MEKK1 is a component of the MAPK cascade module that activates defence responses to bacterial flagellin and transient overexpression of the kinase domain of MEKK1 confers Arabidopsis leaves with increased resistance to bacterial and fungal pathogens (Asai *et al.*, 2002). This study reports however, that both the 35S::OXI1 and 35S::OXI1-YFP transgenic lines have a similar phenotype as the *oxi1* mutant with increased

susceptibility to virulent Emco5 infection (Figure 4.8) and virulent and avirulent isolates of the bacterial pathogen *P. syringae* (Figure 4.7). Furthermore, the observed phenotype was reproducible in both independently transformed OXI1 overexpressing lines indicating that the increased susceptibility was not due to the position of integration of the transgene. This finding presents an apparent paradox in that overexpressing *OXI1* appears to suppress basal defence mechanisms mediating resistance to *H. parasitica* and *P. syringae* as well as gene-for-gene mediated resistance to *P. syringae*, two pathways it is postulated to positively regulate. It was hypothesised with the observation that cellulase treatment triggered the degradation of OXI1-YFP protein (Figure 3. 17), that overexpression of *OXI1* possibly constitutively activated similar pathogen-induced degradation pathways that targeted OXI1 for degradation. However, it was observed that OXI1-YFP protein levels were neither reduced nor increased upon infection with either virulent or avirulent *P. syringae* but remained at a constitutive basal level (Figure 4.9).

One possibility is that perhaps OXI1 is not constitutively active in the overexpressing transgenic lines and inactive OXI1 has the same affinity for binding to upstream activators or downstream cellular targets as active OXI1. This is not without precedence since it was shown that the C-terminal hydrophobic domain of OXI1 was required for interaction with PDK1, and although phosphorylation of OXI1 at its activation site was essential for the activity of OXI1, it was not required for binding of OXI1 to PDK1 (Anthony *et al.*, 2004). Thus following pathogen attack OXI1 may be activated either by H_2O_2 itself or an upstream H_2O_2 sensing protein but this activation of OXI1 could be rate limiting due to the intracellular redox state or amount of 'activator' protein. It has been recently reviewed that that intracellular redox state is crucial in determining which putative H_2O_2 sensing proteins are modified, possibly through their thiol groups, at a particular time to initiate the appropriate signal transduction cascade (Hancock *et al.*, 2006). Consequently, inactive OXI1 may out compete active OXI1 for binding to downstream cellular targets and prevent OXI1 signalling hence the overexpressing transgenic OXI1 lines behave as *oxi1* null mutants in response to pathogen infection. Alternatively, overexpressing the *OXI1* gene may be affecting other signalling pathways unrelated to defence responses and as an indirect consequence cause increased susceptibility to virulent *H. parasitica* and both virulent and avirulent *P. syringae* infection. To understand the signalling mechanisms taking place in the overexpressing *OXI1* transgenic lines it will be imperative to determine the activity of OXI1 in these lines in

response to pathogen challenge as well as to isolate an OXI1 antibody to compare OXI1 protein levels in wild type plants and the 35S::OXI1 transgenic line.

4.15.6 Summary

To summarise *OXI1* is a positive regulator of disease resistance in response to biotrophic pathogens but dispensable in resistance to the necrotrophic pathogen *B. cinerea*. However, the mechanism of action remains elusive. *OXI1* could possibly act through SA-mediated defence pathways. Key experiments to better understand the nature of OXI1 protein kinase in disease resistance would be to investigate *OXI1* expression in other disease resistance impaired Arabidopsis mutants and/or the construction of double mutants with *oxi1* to determine the effect on the disease resistance profile of *oxi1*. Arabidopsis mutants exhibiting enhanced or deficient SA signalling are proposed for such a study as well as the *wrky6* knockout (Robatzek and Somssich, 2002). AtWRKY6 was among the top 10 genes identified that could possibly be co-regulated with OXI1 (Table 3.2) and plays a role in senescence and defence related processes (Robatzek and Somssich, 2001). Furthermore identification of molecular targets of, or proteins that interact with, OXI1 will provide valuable information in understanding the molecular mechanisms of OXI1-induced resistance pathways. It can be envisaged that plausible targets of OXI1 may include kinases involved in MAPK cascades, WRKY transcription factors, putative peroxidases and SA-regulated defence genes since these are common elements displaying differential expression in response to pathogen challenge evoking both basal and *R* gene-mediated resistance responses against biotrophs and necrotrophs (Glazebrook *et al.*, 2003; Eulgem *et al.*, 2004; Thilmony *et al.*, 2006; Truman *et al.*, 2006).

CHAPTER 5

General Discussion

CHAPTER 5: General Discussion

5.1 Protein phosphorylation mediates signal transduction

Activation of an appropriate end response following perception of a given stimulus requires the careful interplay between different signalling components. Protein kinases encompass a very important part of these signalling components since phosphorylation of proteins can affect activation, localisation and/or turnover of target proteins as well as change protein-protein interactions (Huber and Hardin, 2004; de la Fuente van Bentem *et al.*, 2006b). There are over 1000 genes encoding different types of protein kinases in the Arabidopsis genome (Initiative, 2000) and kinase activity regulates diverse cellular processes ranging from embryogenesis to stress and growth responses. For example, the NDR family of AGC kinases regulate cell cycle and morphogenesis (Tamaskovic, 2003), CDPK activity has been implicated in stomatal opening and closure through phosphorylation of a vacuolar anion channel and a voltage-gated K^+ influx channel respectively (Pei *et al.*, 1996; Li *et al.*, 1998; Cheng *et al.*, 2002) and finally members of the large family of RLK respond to both biotic and abiotic stress signals (Morris and Walker, 2003). Phosphorylation cascades, particularly the mitogen activated protein kinases, are conserved amongst eukaryotes, aid signal amplification and result in the activation of downstream targets such as transcription factors to elicit a defined end response. A full MAPK cascade in response to the bacterial elicitor flg22 illustrated a signal transduction cascade in defence related processes (Asai *et al.*, 2002) however components of this cascade are also activated in response to other stimuli e.g. the involvement of AtMPK3 and AtMPK6 in response to salt and cold stress (Tiege *et al.*, 2004). This indicates a single kinase can be involved in very different cellular processes and that there is cross talk between different signalling pathways. Currently knowledge is lacking concerning how specificity is achieved by a protein kinase to elicit the appropriate end response but specificity may reside in the upstream activators or which domains of the given kinase are phosphorylated (Jonak *et al.*, 2002). This study attempted to decipher the functionality of a member of the AGC protein kinase family OXI1 and the cellular processes it regulates.

5.2 Significance of transcriptional upregulation?

For the most part transcriptional activation of any gene in response to internal or external cues denotes involvement of that gene in a given process. Transcriptional upregulation of signalling components has been proposed to act as a positive feedback mechanism to reinforce transduction of the signalling pathway through increasing the availability of its components (Yamamoto *et al.*, 1998; Hirt, 1999). Studies employing loss-of-function mutants have shown that the absence of an important signalling component in a particular stress activated pathway leads to decreased expression of downstream targets and compromised tolerance to that stress. Additionally, overexpression of signalling components results in constitutive activation of downstream components and enhanced stress tolerance and such is the case for *AtNDPK2* in response to oxidative stress and *MKK2* in response to salt and cold stress (Moon *et al.*, 2003; Tiege *et al.*, 2004). However, such a role corresponding to the induction of *OXI1* transcript in response to a variety of AOS mediated stimuli could not be discerned.

Firstly, there was a general lack of phenotype in the *oxi1* mutant in response to salt and heat stress which removes biological significance for OXI1 in these processes and/or alternatively indicates functional redundancy for the OXI1 protein kinase. This is not surprising given the complexity and cross talk that is continually emerging in different stress response pathways (Thomma *et al.*, 2001; Jonak *et al.*, 2002; Kreps *et al.*, 2002). Transcriptional increases are likely not to be of biological significance if the induction does not translate into protein production. Post-transcriptional as well as post-translational modification mechanisms, such as mRNA degradation or protein phosphorylation and/or thiol modification, serve to control the fate of gene expression. This could be the situation for OXI1 where, in this study, it was not possible to detect OXI1 protein in wild type seedlings in response to stresses that induced its gene expression. Perhaps plants do not expend energy increasing OXI1 protein if it is not essential in certain processes. However, it is possible that stimuli which do cause an increase in OXI1 protein may still not be detected owing to the short half-life of OXI1 and the suggestion that low levels of OXI1 may be sufficient to transduce a signal since protein levels were not strongly enhanced in transgenic Arabidopsis overexpressing OXI1-YFP.

In order to reconcile a *bona fide* involvement of OXI1 in response to a given stress or stimulus a measurable phenotype needs to be discerned in the *oxi1* mutant. This could encompass altered gene expression, reduced activity of key regulatory phosphoproteins or synthesis of secondary metabolites. However it is appreciated that this type of approach is equivalent to searching for a needle in a haystack if no obvious visible phenotype can be identified in the *oxi1* mutant or transgenic *OXI1* overexpressing lines in comparison to wild type. Additionally other methods need to be employed to increase the sensitivity of detection of abundance and/or activity of OXI1 protein to gain a better understanding of OXI1 function and regulation. These methods may include production of a new OXI1 antibody or the use of a tertiary antibody to further amplify the signal produced by the bound primary and secondary antibodies. Perhaps OXI1 plays a more important role in response to other stresses which induce its expression such as UV irradiation or genotoxic stress. However, the findings presented in this study illustrate that results based solely on transcriptional changes may not be meaningful unless supported by mutational and/or biochemical data.

5.3 OXI1 is involved in disease resistance

OXI1 gene expression is induced in response to pathogen challenge and is preceded by and is spatially correlated with the accumulation of AOS (Figure 4.2). The generation of AOS through NADPH oxidases is partly responsible for the induction of *OXI1* gene expression therefore other mechanisms which produce AOS during pathogen challenge could also evoke *OXI1* expression. The *oxi1* mutant is more susceptible than wild type in response to infection with virulent *H. parasitica* and both virulent and avirulent isolates of *P. syringae* (Figures 4.5 and 4.8). Taken together this data suggests a positive role for OXI1 in disease resistance responses to biotrophic pathogens. The precise mechanism of action of OXI1 in defence still remains unknown since expression of key disease resistance genes and features of SA signalling (e.g. SAR) were unaltered in the *oxi1* mutant in response to pathogen infection.

Activation of various components of MAPK cascades has been demonstrated in response to H_2O_2, PAMPs and during gene-for-gene resistance in a range of plant species and proven essential for the initiation of defence responses such as the HR and

defence gene expression (Romeis, 2001; Zhang and Klessig, 2001; Nakagami *et al.*, 2005). For example, MEK2 activation of WIPK and SIPK (the orthologues of MPK3 and MPK6 respectively) during *N* gene-mediated resistance in tobacco preceded a HR-like cell death and silencing of any of these genes compromised resistance against TMV (Yang *et al.*, 2001; Jin *et al.*, 2003). Similarly, in Arabidopsis the bacterial elicitor flg22 and H_2O_2 induced separate MAPK cascades that differ at the MAPKKK level involving MEKK1 and ANP1 respectively. However, both pathways activate MPK3 and MPK6 and are required for resistance to pathogen challenge and expression of WRKY transcription factors in response to flg22 and ANP1 induced *GST6* and *HSP18.2* expression (Kovtun *et al.*, 2000; Asai *et al.*, 2002). It is likely that OXI1 may initiate a MAPK cascade in response to pathogen challenge given that OXI1 is required for full activation of MPK3 and MPK6 in response to H_2O_2 *in vivo* (Rentel *et al.*, 2004). This putative OXI1-inducible MAPK cascade may be required to slow the process of bacterial growth by accelerating plant defence responses. For example, silencing of *WIPK* in tobacco delayed HR-like cell death suggesting that WIPK activation may be required to accelerate the cell death process (Zhang and Liu, 2001). Therefore such a scenario might reconcile why the *oxi1* knockout is not compromised in gene expression, particularly in the case of MPK6-dependent *VSP1* expression, or SAR if the OXI1 pathway acts in parallel with other MAPK cascades to elicit plant defence responses. This is not without precedent since activation of multiple MAPK cascades has been shown in response to a single pathogen, for example the AvrPto-Pto incompatible interaction in tomato and tobacco discussed in Section 1.3.2.

It was also demonstrated that MAPKs may be involved in signal amplification loops since the MEK2 pathway was shown to act upstream of respiratory burst oxidase genes in tobacco during fungal infection (Yoshioka *et al.*, 2003). Constitutive activation of MKK4 and MKK5 (the upstream activators of MPK3 and MPK6 in Arabidopsis) resulted in H_2O_2 production and cell death (Ren *et al.*, 2002). Furthermore, SA causes the induction of OXI1 and both SA and H_2O_2 are implicated in signal amplification loops during pathogen challenge (Van Camp *et al.*, 1998) hence OXI1 may also form part of such mechanisms. Although the requirement for OXI1 to function in MAPK cascades is likely, for all these arguments to hold it would need to be shown that OXI1 is not only active but activates downstream MAPK components in response to pathogen challenge and SA. It would also be interesting to determine whether virulent and avirulent isolates (or different

biotrophs) induce the same MAPK, if such an OXI1-inducible MAPK pathway in response to pathogen challenge does exist.

Recently, it was reported that repression of auxin signalling mediated through a plant miRNA restricted growth of *P. syringae* in Arabidopsis (Navarro et al., 2006). This suggests that auxin negatively regulates plant defence responses. Given that OXI1 most likely positively regulates plant defence responses as well as the fact that *OXI1* expression positively correlates with the expression of a number of auxin responsive genes, this presents an apparent paradox. However, any interaction between OXI1 and auxin signalling may be different under different biological conditions. For example, during root growth auxin signalling and OXI1 may interact co-operatively to regulate root development (Section 5.6) whereas during pathogenesis these two components might display an antagonistic relationship to establish a plant defence response. This may reconcile why *OXI1* is induced by auxin transport inhibitors as well as being activated by auxin. However, the possible involvement and role of OXI1 in various auxin-mediated signalling pathways requires further investigation

Different types of protein kinases have been implicated in various stages of the plant defence response from pathogen perception to activation of ion channels and transcription factors (Romeis, 2001). Therefore it is possible that OXI1 may phosphorylate key regulatory proteins or transcription factors in defence signal transduction pathways independent of MAPK cascades. Again this would require a demonstration that OXI1 interacts with these cellular players and is capable of phosphorylating them.

5.4 OXI1 protein is differentially regulated in response to different stresses

OXI1-YFP has a short half life and even though transcript levels increased and OXI1 is required for full activation of MPK3 and MPK6 in response to cellulase treatment, OXI1-YFP protein is subject to degradation following cellulase treatment (Figure 3.17). Degradation of proteins by the Ub/26S proteasome pathway is another important aspect of signal transduction in many plant biological processes rather than just mediating termination of a protein's life (Vierstra, 2003). For example, auxin action requires the

degradation of short-lived AUX/IAA proteins which block the AFR family of transcription factors that upregulate auxin-responsive genes and it was recently shown that auxin directly activates the SCF^{TIR} complex (Gray, 2001; Zenzer *et al.*, 2001; Kepinski and Leyser, 2005). CONSTITUTIVE PHOTOMORPHOGENIC (COP) proteins, components of Ub/26S pathway, facilitate the rapid degradation of the HY5 family of bZIP transcription factors in the dark thereby preventing photomorphogenesis proceeding in the dark (Osterlund, 2000; Suzuki *et al.*, 2002). It is proposed that the degradation of OXI1, mediated in part by the proteasome, may switch off or dampen the OXI1-induced signalling cascade.

In contrast, OXI1-YFP levels remained unchanged in response to both virulent and avirulent pathogen infection (Figure 4.9), albeit at a low level due to the rapid turnover of this protein. These findings illustrate that OXI1 is differentially regulated in response to different stresses and consequently OXI1 is likely to mediate different cascades in response to different stimuli. Additionally, given that OXI1 protein levels are low even in the transgenic lines overexpressing *OXI1* indicate that OXI1 is subject to tight regulation. Protein kinases are regulated by a number of factors such as phosphorylation, binding of inhibitory proteins and association of scaffold proteins to target individual kinases into specific complexes (Zhang and Klessig, 2001; Cheng *et al.*, 2002; Johnson and Ingram, 2005). Therefore the activation of specific OXI1 signal transduction cascades may be a consequence of the duration and concentration of AOS accumulation, or which scaffold proteins recruit OXI1 into a given pathway pending on the stimulus or it could be due to post translational protein modification of OXI1. Identifying components or mechanisms which regulate specificity will be invaluable to the understanding of OXI1 and it is appreciated that this may prove a difficult task given the short half-life and low abundance of OXI1 protein.

5.5 A cytosolic localisation for OXI1

Overexpression of an OXI1-YFP construct revealed a cytosolic localisation for OXI1 (Figures 3.14 and 3.15). Additionally OXI1 expression occurred in all parts of the plant with slightly stronger expression occurring in the root tips. An independent report also utilising a constitutively expressed GFP-OXI1 fusion protein yielded similar results,

although these authors did report nuclear localisation for GFP-OXI1 in mature root hairs (Anthony *et al.*, 2004). However, due to the low level of detection of OXI1-YFP under the experimental conditions used in this study, it has been difficult to determine translocation of OXI1 during root hair growth and requires further investigation. Again more sensitive techniques and perhaps a more rigorous time course would be needed to address this issue.

It is appreciated that overexpression of the OXI1 kinase may affect the localisation pattern however expression of this construct under control of its native promoter failed to produce detectable levels of the kinase and also OXI1 protein levels are not enhanced to any great extent in the overexpressors. One experiment to reconcile the effect of overexpression on the localisation or expression pattern of OXI1 would be to transform the OXI1-YFP construct into the *oxi1* mutant background and determine whether it complements the *oxi1* phenotype e.g. in terms of the root hair phenotype. If this construct does complement the mutant phenotype then it follows that overexpression of OXI1 is not altering the localisation of OXI1.

5.6 OXI1 and root hairs

OXI1 is required for normal root hair development under conditions of mild stress since the *oxi1* mutant displays shorter root hairs than wild type seedlings (Anthony *et al.*, 2004; Rentel *et al.*, 2004). The root hair phenotype of the *oxi1* mutant was not explored in this study since the phenotype of the mutant was unclear and proved to be very variable under the growth conditions employed in this study. It has however emerged through microarray analysis that the molecular mechanisms governing the involvement of OXI1 in root hair development may occur through auxin signalling, particularly since several auxin responsive genes are reduced in the *oxi1* mutant background in response to H_2O_2 (Rentel, 2002) and the expression of an auxin responsive GH3 family protein correlated with *OXI1* expression (Table 3.4). Evidence for auxin signalling in root development include auxin mediation of AOS accumulation to direct root gravitropism, the AUX/IAA protein SHY2/IAA3 promotes root hair initiation and growth and lastly the auxin response mutant *axr1* also displays short root hairs (Joo *et al.*, 2001; Knox *et al.*, 2003). Therefore given the induction of OXI1 activity by auxin (Anthony *et al.*, 2004) it would be interesting

to investigate whether OXI1 activity or expression is regulated by these molecular components of auxin signalling.

Phospholipase Dζ1 (AtPLDζ1) positively regulates root hair outgrowth and placement and its activity is repressed in non-hair cells by GLABRA2, an inhibitor of root hair formation (Ohashi *et al.*, 2003). Considering that chemical inhibition of AtPLDζ1 slightly reduced OXI1 activity (Anthony *et al.*, 2004), it could be that OXI1 functions downstream of AtPLDζ1, in part, during root hair development under conditions of stress but this would require further investigation.

RHD2 encodes the *AtrbohC* NADPH oxidase gene and the *rhd2* mutant is unable to control H_2O_2 production required for root hair elongation and consequently displays short root hairs (Forman *et al.*, 2003). It was further illustrated that a RhoGDP dissociation inhibitor (AtrhoGDI1 or SCN1) spatially regulated growth in root hair cells by controlling the activation of the RHD2/AtrbohC NADPH oxidase since the phenotype of *scn1* mutants (too much delocalised AOS and spatially deregulated root hair growth) is suppressed in the *rhd2* mutant background (Carol *et al.*, 2005). Given the requirement for NADPH oxidases in the expression of *OXI1* mRNA during disease resistance it was hypothesised that the constitutive expression of OXI1::GUS in root hairs (Rentel *et al.*, 2004) was dependant on RHD2/AtbohC NADPH oxidase activity. However, both Northern and Reverse Transcription PCR analysis revealed that OXI1 expression was not compromised in the *rhd2* or *scn1* mutant in either root or shoot tissue (data not shown). Hence OXI1 is unlikely to control root hair development through pathways mediated by these proteins. Further investigation is required to unravel the role of OXI1 in root signal transduction networks.

5.7 Future work

In order to better grasp an understanding of OXI1 protein kinase it is clear that a wider range of techniques need to be employed to study its function and the molecular components that either interact with or are targeted by OXI1 need to be identified. The approaches that can be employed to address this will be discussed in turn.

5.7.1 Generation of an OXI1 antibody

The difficulty in studying OXI1 protein levels was due to the low level of detection *in planta*. One of the first tasks should be to generate a new OXI1 high-titre antibody using either the entire protein (even though this has draw backs, particularly if the protein is low in abundance and affinity purification of whole proteins can be problematic) or the generation of a synthetic peptide, encompassing a surface orientated hydrophilic region of OXI1 protein and different to the C-terminal amino acids originally used (Rentel *et al.*, 2004). A few things to consider when selecting a peptide sequence include the peptide length (around 10-20 residues), stability (avoidance of amino acids that make peptides unstable e.g. Cys, Asp-Gly, Asn-Gly etc.) capping (to eliminate potential charge on the peptide not present in the native protein) and avoidance of sequence motifs to eliminate cross-reactivity. Most commercial companies that produce antibodies have various programmes to identify the best sequences of the protein of interest for antigen design.

5.7.2 Microarray analysis

This study has shown that transcriptional changes for a specific gene may not relate to biological significance for that gene in a given process therefore results based on solely on transcriptional information should be viewed with caution. However, microarray analysis of the *oxi1* knockout in response to pathogen infection in comparison to wild type may provide insightful clues into the components and potentially pathways regulated by OXI1. To provide meaningful data the design of the microarray experiment is of utmost importance. Here one could have 3 experimental sets (performed in triplicate) comprising of infecting the *oxi1* mutant and wild type Arabidopsis plants with virulent and avirulent *P. syringae* as well as virulent *H. parasitica* and using $MgCl_2$ or H_2O as the control infections respectively. The recommended avirulent strain would be *Pst*DC3000 *avrB* since this isolate exhibited a similar phenotype in the *oxi1* mutant in both genetic backgrounds (Figure 4.5). Comparison across all 3 experimental sets and the controls should reveal whether components regulated by OXI1 are shared amongst virulent and avirulent interactions or between different biotrophic pathogens. Additionally, a thorough time course would also need to be employed. This is particularly true if OXI1 does cause defence responses to be set up more quickly. It is proposed that transcriptional changes be monitored in the first few hours before and after the second phase of the oxidative

burst, for example at 0, 1 and 3 hrs, followed by 6, 12 and 24 hrs to encompass later responses. It would also be interesting to compare transcriptional changes between both *oxi1* mutants in the Col and Ws-2 background together with their respective wild types in response to *Pst*DC3000 *avrRpt2* infection since during this incompatible interaction the importance of OXI1 signalling appears to differ between the two ecotypes (Figure 4.6). Taken together, this form of large scale analysis would hopefully provide insightful clues as to what molecular components are involved downstream of OXI1 signalling during disease resistance and are shared between the different types of resistance responses. Additionally, any components that are identified on the basis of transcriptional changes would need to be backed up by either biochemical data (such as direct interaction with OXI1 or requirement for kinase activity) or mutational analysis.

5.7.3 Phosphoproteomics

Advances made in the field of phosphoproteomics have facilitated the large scale analysis of the phosphoproteome which includes not only the identification of kinase substrates but also which residues are phosphorylated under different conditions in yeast, animal and plant systems (de la Fuente van Bentem *et al.*, 2006a; Peck, 2006). Some phosphoproteins are low in abundance and development of purification techniques of phosphoproteins or phosphopeptides from complex mixtures to aid their enrichment include affinity purification with phosphospecific antibodies, cationic or anionic exchange chromatography and/or immobilised metal affinity chromatography (IMAC), which makes use of trivalent metal cations such as Fe^{3+} to bind phosphate groups. The isolated phosphopeptides can then be processed by tandem mass spectrometry to obtain identification profiles of these phosphopeptides.

A recent technique called iTRAQ (Applied Biosystems) has facilitated quantitative proteome comparison of up to four different protein samples in a single experiment. To explain simply, following protein purification from different samples e.g. isolation of phosphopeptides from a stress and control treatment, the phosphopeptides from each sample are labelled at the N-terminus with a different isobaric (same mass) reagent. There are four isobaric reagents which upon fractionation in MS/MS yield four unique reporter ions. The control and stress sample mixtures are then combined and the mixture analysed by LC/MS/MS for protein identification and quantitation. Therefore this

technique can be employed to identify downstream kinase substrates of OXI1 by comparing the phosphoproteome of the *oxi1* knockout in response to pathogen challenge (or any stress) to that of wild type seedlings with the appropriate controls for each genotype. Recently this technique was successfully utilised to identify five phosphoproteins potentially involved in basal defence responses against *Pst*DC3000 challenge in Arabidopsis (Jones *et al.*, 2006). These authors compared changes in the phosphoproteome between a control sample and three different bacterial challenges (Jones *et al.*, 2006). It should be noted that this type of analysis is extremely costly and requires a large amount of handling time. Therefore experimental design should include choosing an appropriate time point where OXI1 activity is presumed to be most active and also which method of phosphoprotein isolation would be best.

5.7.4 Immunoprecipitation

The success of this technique to identify proteins that interact with OXI1 requires the successful production of a high affinity binding antibody for OXI1 (see 5.7.1) as well as large scale protein purification due to the low abundance of OXI1 in plant extracts. Immunoprecipitation of OXI1 from plant extracts under different conditions of stress can be compared by electrophoresis under both denaturing and non-denaturing conditions. Depending on the experimental conditions employed different protein profiles may be obtained in response to specific stresses i.e. if different proteins interact with OXI1 under different conditions. This could well be the case if OXI1 does direct different signal transduction cascades and scaffold proteins ensures that it is directed to the appropriate pathway. Additional proteins obtained during immunoprecipitation of OXI1 can be identified by MS and their interaction with OXI1 would need to be validated by co-immunoprecipitation or yeast-two-hybrid studies. An advantage of this technique is that one could potentially identify proteins that are required for OXI1 activation and not just downstream targets of OXI1.

5.7.5 Yeast-two-hybrid

Although, similar to the above strategy this technique does not rely on a specific stimulus to induce OXI1 and interact with particular components nor does it rely on the amount of OXI1 *in planta*. OXI1 can be used as bait to screen an Arabidopsis cDNA

yeast-two-hybrid library made from either Arabidopsis root tissue and/or Arabidopsis leaves infected with *P. syringae* and *H. parasitica* to assess putative partners interacting with OXI1 during root development and pathogen defence respectively. It is possible that both upstream and downstream OXI1 interacting proteins can be identified using this approach. Identification of these putative OXI1 interacting proteins may also place OXI1 into signalling networks not formerly associated with OXI1. Once again biological significance of this interaction would need to be proved *in planta* either through mutational analysis or biochemical in gel kinase assays due to the high false positive rate of yeast-two-hybrid assays.

5.7.6 Mutational analysis

The aforementioned approaches will provide information as to which proteins interact with or are regulated by OXI1. Due to the large available resources of Arabidopsis T-DNA knockout populations it should be relatively easy to isolate knockouts of genes thought to interact with OXI1. Alternatively, construction of constitutive or inducible RNAi lines for the gene of interest can also be utilised. These null mutants can be crossed with the *oxi1* knockout and the double mutant can be analysed to determine if it is more sensitive to or compromised in the establishment of end responses of a particular pathway which these genes together with OXI1 are thought to regulate. In the case of negative regulation the double mutant may show an enhanced phenotype in response to a particular stress or stimulus.

Another approach to identify upstream activators of OXI1 is currently underway. A 1.61 kb genomic DNA fragment upstream of the *OXI1* start codon was fused to the firefly *luciferase* gene and transformed into Arabidopsis. Transgenic lines that contained one insert and displayed high luciferase activity in response to cellulase treatment have been mutagenised by EMS (Dr R Ingle, pers. comm.). Mutant plants which show either constitutively high luciferase or abolishment of luciferase activity in response to a particular stress will presumably encode negative and positive regulators of *OXI1* gene expression respectively.

5.8 Conclusion

Further investigation into the regulation and role of OXI1 protein kinase has revealed that it is a cytosolic protein differentially regulated in response to different stresses. Its role in basal defence mechanisms in response to *H. parasitica* has been extended to be effective against virulent *P. syringae* and a positive regulatory role for OXI1 in gene-for-gene resistance against avirulent *P. syringae* has also emerged. It is interesting that OXI1 is transcriptionally up regulated in response to a wide variety of stimuli but it is so tightly controlled at the translational level. Therefore this study sets the stage to further investigate OXI1 protein with its unique characteristics and identification of its downstream targets will enable OXI1 to be placed into specific signal transduction networks.

REFERENCES

REFERENCES

Aarts, N., Metz, M., Holub, E., Staskawicz, B., Daniels, M., and Parker, J. (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two *R* gene-mediated signaling pathways in Arabidopsis. *PNAS* **95,** 10306-10311.

Adam, Z., and Clarke, A.K. (2002). Cutting edge of chloroplast proteolysis. *Trends in Plant Science* **7,** 451-456.

Ahfors, R., Macioszek, V., Rudd, J., Brosche, M., Schlichting, R., Scheel, D., and Kangasjarvi, J. (2004). Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thalina* druing ozone exposure. *Plant Journal* **40,** 512-522.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shin, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-Wide Insertional Mutagenesis of Arabidopsis thaliana. *Science* **301** , 653-657.

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A., and Lamb, C. (1998). Reactive Oxygen Intermediates Mediate a Systemic Signal Network in the Establishment of Plant Immunity. *Cell* **92,** 773-784.

Anthony, R.G., Henriques, R., Helfer, A., Mészáros, T., Rios, G., Testerink, C., Munnik, T., Deák, M., Loncz, C., and Bögre, L. (2004). A protein kinase target of a PDK1 signalling pathway is involved in root hair growth in *Arabidopsis*. *The EMBO Journal* **23,** 572-581.

Asada, K. (1999). The water-water cycle in chloroplasts: scavenging of active oxygen and dissipation of excess photons. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 601-639.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausebel, F.M., and Sheen, J. (2002). MAP Kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* **415,** 977-983.

Assaad, F.F., Qiu, J.-L., Youngs, H., Ehrhardt, D., Zimmerli, L., Kalde, M., Wanner, G., Peck, S.C., Edwards, H., Ramonell, K., Somerville, C.R., and Thordal-Christensen, H. (2004). The PEN1 Syntaxin Defines a Novel Cellular Compartment upon Fungal Attack and Is Required for the Timely Assembly of Papillae. *Molecular Biology of the Cell* **15,** 5118-5129.

Aufsatz, W., and Grimm, C. (1994). A new, pathogen-inducible gene of *Arabidopsis* is expressed in an ecotype-specific manner. *Plant Molecular Biology* **25,** 229-239.

Aufsatz, W., Amry, D., and Grimm, C. (1998). The *ECS1* gene of *Arabidopsis* encodes a plant cell wall-associated protein and is potentially linked ot a locus influencing resistance to *Xanthomonas campestris*. *Plant Molecular Biology* **38,** 965-976.

Bais, H.P., Fall, R., and Vivanco, J.M. (2004). Biocontrol of *Bacillus subtillis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiology* **134,** 307-319.

Bais, H.P., Prithiviraj, B., Jha, A.K., Ausubel, F.M., and Vivanco, J.M. (2005). Mediation of pathogen resistance by exudation of antimicrobials from roots. *Nature* **434,** 217-221.

Baniwal, S.K., Bharti, K., Chan, K.Y., Fauth, M., Ganguli, A., Kotak, S., Mishra, S.V.K., Nover, L., Port, M., Scharf, K.-D., Tripp, J., Weber, C., Zielinski, D., and Koskull-Döring, P.V. (2004). Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. *J. Bioscience* **29,** 471-487.

Belham, C., Wu, S., and Avruch, J. (1999). Intracellular signalling: PDK1- a kinase at the hub of things. *Current Biology* **9,** 93-96.

Benjamins, R., Ampudia, C.S.G., Hooykaas, P.J.J., and Offringa, R. (2003). PINOID-Mediated Signaling Involves Calcium-Binding Proteins. *Plant Physiology* **132,** 1623-1630.

Benjamins, R., Quint, A., Weijers, D., Hooykaas, P.J.J., and Offringa, R. (2001). The PINOID protein kinase regulates organ development in *Arabidopsis* by enhancing polar auxin transport. *Development* **128,** 4057-4067.

Binder, B.M., Harper, J.F., and Sussman, M.R. (1994). Characterization of an *Arabidopsis* calmodulin-like domain protein kinase purified from *Escherichia coli* using an affinity sandwich technique. *Biochemistry* **33,** 2033-2041.

Blume, B., Nürnberger, T., Nass, N., and Dierk, S. (2000). Receptor-Mediated Increase in Cytoplasmic Free Calcium Required for Activation of Pathogen Defence in Parsley. *Plant Cell* **12,** 1425-1440.

Bögre, L., Ökrész, L., Henriques, R., and Anthony, R.G. (2003). Growth signalling pathways in *Arabidopsis* and the AGC protein kinases. *Trends in Plant Science* **8,** 424-431.

Bolwell, G.P., and Wojtaszek, P. (1997). Mechanisms for the generation of reactive oxygen species in plant defence - broad perspective. *Physiological and Molecular Plant Pathology* **51,** 347-366.

Bonhert, H.J., and Sheveleva, E. (1998). Plant stress adaptations, making metabolism move. *Current Opinion in Plant Biology* **39,** 295-311.

Borsani, O., Valpuesta, V., and Botella, M.A. (2001). Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. *Plant Physiology* **126,** 1024-1030.

Boursiac, Y., Chen, S., Luu, D.-T., Sorieul, M., Dries, N.v.d., and Maurel, C. (2005). Early Effects of Salinity on Water Transport in Arabidopsis Roots. Molecular and Cellular Features of Aquaporin Expression. *Plant Physiology* **139,** 790-805.

Bowling, S.A., Guo, A., Cao, H., Gordan, A.S., Klessig, D.F., and Dong, X. (1997). The *cpr5* mutant of Arabidopsis expresses both *NPR1*-dependent and *NPR1*-independent resistance. *The Plant Cell* **9,** 1573-1584.

Bradford, M.M. (1976). A rapid and sensitive method for quantification of microgram quantities of protein utlizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248-254.

Bradley, D.J., Kjellbom, P., and Lamb, C.J. (1992). Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant-cell wall protein - a novel, rapid defense response. *Cell* **70,** 21-30.

Brady, J.D., and Fry, S.C. (1997). Formation of di-isodityrosine and loss of isodityrosine in the cell walls of tomato cell-suspension cultures treated with fungal elicitors or H_2O_2. *Plant Physiology* **115,** 87-92.

Breyne, P., Rombaut, D., Gysel, A.V., Montagu, M.V., and Gerats, T. (1999). AFLP analysis of genetic diversity within and between Arabidopsis thaliana ecotypes. *Mol Gen Genet* **261,** 627-634.

Brodersen, P., Petersen, M., Nielsen, H.B., Zhu, S., Newman, M.-A., Shokat, K.M., Rietz, S., Parker, J., and Mundy, J. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *The Plant Journal* **47,** 532-546.

Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonreponsive to inducers of systemic acquired resistance. *Plant Cell* **6,** 1583-1592.

Carol, R.J., Takeda, S., Linstead, P., Durrant, M.C., Kakesova, H., Derbyshire, P., Drea, S., Zarsky, V., and Dolan, L. (2005). A RhoGDP dissociation inhibitor spatially regulates growth in root hair cells. *Nature* **438,** 1013-1016.

Chamnongpol, S., Willekens, H., Moeder, W., Langebartels, C., Sandermann, H.J., Montagu, M.V., Inze, D., and Camp, W.V. (1998). Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic plants. *PNAS* **95,** 5818-5823.

Chang, L., and Karin, M. (2001). Mammalian MAP kinase signalling cascades. *Nature* **410,** 37-40.

Chaudhuri, S., Seal, A., and Gupta, M.D. (1999). Autophosphorylation-dependent activation of a calcium-dependent protein kinase from groundnut. *Plant Physiology* **120,** 859-866.

Cheng, S.-H., Willmann, M.R., Chen, H.-C., and Sheen, J. (2002). Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family. *Plant Physiology* **129,** 469-485.

Chevalier, F., Martin, O., Rofidal, V., Devanuchelle, A.D., Barteau, S., Sommerer, N., and Rossignol, M. (2004). Proteomic investigation of natural variation between Arabidopsis ecotypes. *Proteomics* **4,** 1372-1381.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. (2006). The Arabidopsis Receptor Kinase FLS2 Binds flg22 and Determines the Specificity of Flagellin Perception. *Plant Cell* **18,** 465-476.

Chinnusamy, V., Schumaker, K., and Zhu, J.-K. (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Journal of Experimental Botany* **55,** 225-236.

Chinnusamy, V., Ohta, M., Kanrar, S., Lee, B.-H., Hong, X., Agarwal, M., and Zhu, J.-K. (2003). ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis. Genes and development* **17,** 1043-1054.

Chivasa, S., Ndimba, B.K., Simon, W.J., Robertson, D., Yu, X.L., Knox, J.P., Bolwell, P., and Slabas, A.R. (2002). Proteomic analysis of the *Arabidopsis thaliana* cell wall. *Electrophoresis* **23,** 1754-1765.

Chomczynski, P., and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162,** 156-159.

Christie, J.M., Reymond, P., Powell, G.K., Bernasconi, P., Raibekas, A.A., Liscum, E., and Briggs, W.R. (1998). *Arabidopsis* NPH1: a flavoprotein with the properties of a photoreceptor for phototropism. *Science* **282,** 1698-1701.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci.* **81,** 1991-1995.

Clarke, J.D., Volko, S.M., Ledford, H., Ausubel, F.M., and Dong, X. (1998). Uncoupling *PR* gene expression from *NPR1* and bacterial resistance: characterization of the dominant Arabidopsis *cpr6-1* mutant. *The Plant Cell* **10,** 557-569.

Conklin, P.L., and Last, R.L. (1995). Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. *Plant Physiology* **109,** 499-511.

Corpas, F.J., Barroso, J.B., and del Río, L.A. (2001). Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. *Trends in Plant Science* **6,** 145-150.

Craigon, D.J., James, N., Okyera, J., Higgins, J., Jotham, J., and May, S. (2004). NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. *Nucleic Acid Research* **32,** D575-577.

Czarnecka-Verner, E., Yuan, C.-X., Scharf, K.-D., Englich, G., and Gurley, W.B. (2000). Plants contain a novel multi-member class of heat shock factors without transcriptional activator potential. *Plant Molecular Biology* **43** 459-471.

Dalton, D.A., Russel, S.A., Hanus, F.J., Pascoe, G.A., and Evans, H.J. (1986). Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *PNAS* **83,** 3811-3815.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. *Nature* **411,** 826-833.

de la Fuente van Bentem, S., Roitinger, E., Anrather, D., Csaszar, E., and Hirt, H. (2006a). Phosphoproteomics as a tool to unravel plant regulatory mechanisms. *Physiologia Plantarum* **126,** 110-119.

de la Fuente van Bentem, S., Anrather, D., Roitinger, E., Djamei, A., Hufnagl, T., Barta, A., Csaszar, E., Dohnal, I., Lecourieux, D., and Hirt, H. (2006b). Phosphoproteomics reveals extensive *in vivo* phosphorylation of Arabidopsis proteins involved in RNA metabolism. *Nucleic Acid Research* **34,** 3267-3278.

Deak, M., Casamayor, A., Currie, R.A., Downes, P., and Alessi, D.R. (1999). Characterisation of a plant 3-phosophoinositide-dependent protein kinase-1 homologue which contains a pleckstrin homology domain. *FEBS Letters* **451,** 220-226.

Dean, C., Jones, J., Favreau, M., Dunsmuir, P., and Bedbrook, J. (1988). Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobacco plants. *Nucleic Acid Research* **16,** 9267-9283.

Deblaere, R., Bytebier, B., Greve, H.D., Deboeck, F., Schell, J., Montagu, M.V., and Leemans, J. (1985). Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. *Nucl. Acids Res.* **13,** 4777-4788.

del Pozo, O., Pedley, K.F., and Martin, G.B. (2004). MAPKKKa is a positive regulator of cell death associated with both plant immunity and disease. *EMBO Journal* **23,** 3072-3082.

del Río, L.A. (1992). Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Rad. Biol. Med.* **13,** 557-580.

del Río, L.A., Sandalio, L.M., Corpas, F.J., López-Huertas, E., Palma, J.M., and Pastori, G.M. (1998). Activated oxygen-mediated metabolic functions of leaf peroxisomes. *Physiologia Plantarum* **104,** 673-680.

Delledonne, M., Xia, Y., Dixon, R.A., and Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature* **394,** 585-588.

Demidchik, V., Shabala, S.N., Coutts, K.B., Tester, M.A., and Davies, J.M. (2003). Free oxygen radicals regulate plasma membrane Ca^{2+} and K^{+}-permeable channels in plant root cells. *J Cell Sci* **116,** 81-88.

Dempsey, D.M.A., Shah, J., and Klessig, D.F. (1999). Salicylic Acid and Disease Resistance in Plants. *Critical Reviews in Plant Sciences* **18,** 547-575.

Denby, K.J., Kumar, P., and Kliebenstein, D.J. (2004). Identification of *Botrytis cinerea* susceptibility loci in *Arabidopsis thaliana*. *The Plant Journal* **38,** 473-486.

Denby, K.J., Jason, L.J.M., Murray, S.L., and Last, R.L. (2005). *ups1*, an *Arabidopsis thanliana* camalexin accumulation mutant defective in multiple defence signalling pathways. *The Plant Journal* **41,** 673-684.

Deprés, C., Chubak, C., Rochon, A., Clark, R., Bethune, T., Desveaux, D., and Fobert, P.R. (2003). The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confers redox regulation of DNA binding activity to basic domain/leucine zipper transcription factor TGA1. *Plant Cell* **15,** 2181-2191.

Desikan, R., A-H-Mackerness, S., Hancock, J.T., and Neill, S.J. (2001a). Regulation of the *Arabidopsis* transcriptome by oxidative stress. *Plant Physiology* **127,** 159-172.

Desikan, R., Hancock, J.T., Ichimura, K., Shinozaki, K., and Neill, S.J. (2001b). Harpin induces activation of the *Arabidopsis* mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiology* **126,** 1579-1587.

Desikan, R., Hancock, J.T., Bright, J., Harrison, J., Weir, I., Hooley, R., and Neill, S.J. (2005). A role for ETR1 in hydrogen peroxide signalling in stomatal guard cell closure. *Plant Physiology* **137,** 831-834.

DeWald, D.B., Torabinejad, J., Jones, C.A., Shope, J.C., Cangelosi, A.R., Thompson, J.E., Prestwich, G.D., and Hama, H. (2001). Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-triphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. *Plant Physiology* **126,** 759-769.

Dixon, D.P., Cummins, L., Cole, D.J., and Edwards, R. (1998). Glutathione-mediated detoxification system in plants. *Current Opinion in Plant Biology* **1,** 258-266.

Dolan, L., and Davies, J. (2004). Cell expansion in roots. *Current Opinion in Plant Biology* **7,** 33-39.

Donaldson, L., Ludidi, N., Knight, M.R., Gehring, C., and Denby, K. (2004). Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels. *FEBS Letters* **569,** 317-320.

Drögre, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews* **82,** 47-95.

Droillard, M., Boudsocq, M., Barbier-Brygoo, H., and Lauriere, C. (2002). Different protein kinase families are activated by osmotic stress in *Arabidopsis thaliana* cell suspensions. Involvement of the MAP kinases AtMPK3 and AtMPK6. *FEBS Letters* **527,** 43-50.

Dupre, S., Urban-Grimal, D., and Haguenauer-Tsapis, R. (2004). Ubiquitin and endocytic internalization in yeast and animal cells. *Biocheimica et Biophysica Acta* **1695,** 89-111.

Durner, J., Wendehenne, D., and Klessig, D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP-ribose. *PNAS* **95,** 10328-10333.

Durrant, I., and Fowler, S. (1994). Nonradioactive oligonucleotide probe labelling. *Methods in Molecular Biology* **31,** 103-175.

Durrant, J.R., Giorgi, L.B., Barker, J., Klug, D.R., and Porter, G. (1990). Characterisation of triplet states in isolated photosystem II reaction centres: oxygen quenching as a mechanism for photodamage. *Biochimica et Biophysica Acta* **1017,** 167-175.

Edwards, K., Johnstone, C., and Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acid Research* **19,** 1349.

Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., and Martin, G.B. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *The Plant Journal* **36,** 905-917.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, E. (2000). The WRKY superfamily of plant transcription factors. *Trends in Plant Science* **5,** 199-206.

Eulgem, T., Weigman, V.J., Chang, H.-S., McDowell, J.M., Holub, E.B., Glazebrook, J., Zhu, T., and Dangl, J.L. (2004). Gene Expression Signatures from Three Genetically Seperable Resistance Gene Signaling Pathways for Downy Mildew Resistance. *Plant Physiology* **135,** 1129-1144.

Falk, A., Feys, B.F., Frost, L.N., Jones, J.D.G., Daniels, M.J., and Parker, J.E. (1999). *EDS1*, an essential component of R-gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. *PNAS* **96,** 3292-3297.

Farmer, P.K., and Choi, J.H. (1999). Calcium and phospholipid activation of a recombinant calcium-dependent protien kinase (DcCPK1). *Biocheimica et Biophysica Acta* **1434,** 6-17.

Feilner, T., Hultschig, C., Lee, J., Meyer, S., Immink, R.G., Koenig, A., Possling, A., Seitz, H., Beveridge, A., and Scheel, D. (2005). Hight throughput identification of potential Arabidopsis mitogen-activated protein kinases AtMPK4 and AtMPK6. *Plant Physiology* **126,** 1579-1568.

Ferrari, S., Plotnikova, J.M., Lorenzo, G.D., and Ausubel, F.M. (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2,* but not *SID2, EDS5* or *PAD4*. *The Plant Journal* **35,** 193-205.

Fobert, P.R., and Després, C. (2005). Redox control of systemic acquired resistance. *Current Opinion in Plant Biology* **8,** 378-382.

Forman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., Davies, J.M., and Dolan, L. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* **422,** 442-446.

Fourcroy, P., Vansuyt, G., Kushnir, S., Inzé, D., and Briat, J.-F. (2004). Iron-Regulated Expression of a Cytosolic Ascorbate Peroxidase Encoded by the *APX1* Gene in Arabidopsis seedlings. *Plant Physiology* **134,** 605-613.

Friml, J., Yang, X., Michniewicz, M., Weijers, D., Quint, A., Tietz, O., Benjamins, R., Ouwerkerk, P.B.F., Ljung, K., Sandberg, G., Hooykaas, P.J.J., Palme, K., and Offringa, R. (2004). A PINOID-Dependent Binary Switch in Apical-Basal PIN Polar Targeting Directs Auxin Efflux. *Science* **306,** 862-865.

Frugoli, J.A., McPeek, M.A., Thomas, T.L., and McClung, C.R. (1998). Intron loss and gain during evolution of the catalase gene family in angiosperms. *Genetics* **149,** 355-365.

Fry, S.C., Willis, S.C., and Patterson, A.E.J. (2000). Intraprotoplasmic and wall-localized fromation of arabinoxylan-cound diferulates and larger ferulate coupling products in maize cell-suspension cultures. *Planta* **211,** 679-692.

Frye, C.A., Tang, D., and Innes, R.W. (2001). Negative regulation of defence responses in plants by a conserved MAPKK kinase. *PNAS* **98,** 373-378.

Ganguly, S., and Singh, M. (1999). Purification and characterization of a protein phosphatase from winged bean. *Phytochemistry* **52,** 239-246.

Gelhaye, E., Rouhier, N., Navrot, N., and Jacquot, J.P. (2005). The plant thioredoxin system. *Cell Mol Life Sci* **62,** 24-35.

Glazebrook, J. (2005). Constrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. *Annu. Rev. Phytophathol.* **43,** 9.1-9.23.

Glazebrook, J., Chen, W., Estes, B., Chang, H.-S., Nawrath, C., Metraux, J.-P., Zhu, T., and Katagiri, F. (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. *The Plant Journal* **34,** 217-228.

Godiard, L., Sauviac, L., Torii, K.U., Grenon, O., Mangin, B., Grimsley, N.H., and Marco, Y. (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. *Plant Journal* **36,** 353-365.

Gomez-Gomez, L. (2004). Plant perception systems for pathogen recognition and defence. *Molecular Immunology* **41,** 1055-1062.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: a LRR receptor-like kinase involved in recognition of the flagellin elicitor in Arabidopsis. *Mol. Cell* **5,** 1003-1020.

Gomez-Gomez, L., and Boller, T. (2002). Flagellin perception: a paradigm for innate immunity. *Trends in Plant Science* **7,** 251-256.

Gomez-Gomez, L., Bauer, Z., and Boller, T. (2001). Both the extracellular leucine-rich-repeat domain and the kinase activity of FLS2 are required for flagellin binding and signalling in *Arabidopsis*. *The Plant Cell* **13,** 1155-1163.

González-Lamothe, R., Tsitsigiannis, D.I., Ludwig, A.A., Panicot, M., Shirasu, K., and Jones, J.D.G. (2006). The U-Box Protein CMPG1 Is Required for Efficient Activation of Defense Mechanisms Triggered by Multiple Resistance Genes in Tobacco and Tomato *The Plant Cell* **18,** 1067-1083.

Gopalan, S., Wei, W., and He, S.Y. (1996). *hrp* gene-dependent induction of hin1: A plant gene activated rapidly by both harpins and the *avrPto* gene-mediated signal. *Plant Journal* **10,** 591-600.

Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology* **10,** 751-757.

Grant, J.J., and Loake, G.J. (2000). Role of Reactive Oxygen Intermediates and Cognate Redox Signaling in Disease Resistance. *Plant Physiology* **124,** 21-29.

Grant, J.J., Byung-Wook, and Loake, G.J. (2000a). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *The Plant Journal* **24,** 569-582.

Grant, M.R., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J.W. (2000b). The *RPM1* plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *The Plant Journal* **23,** 441-450.

Gray, W.M. (2001). Auxin regulates SCF^{TIR}-dependent degradation of AUX/IAA proteins. *Nature* **414,** 271-276.

Gupta, R., and Luan, S. (2003). Redox control of protein tyrosine phosphatases and mitogen activated protein kinases in plants. *Plant Physiology* **132,** 1149-1152.

Gutiérrez, R.A., Macintosh, G.C., and Green, P.J. (1999). Current perspectives on mRNA stability in plants: multiple levels and mechanisms of control. *Trends in Plant Science* **4,** 429-438.

Haffani, Y.Z., Gaude, T., Cock, J.M., and Goring, D.R. (2004). Antisense suppression of thioredoxin h mRNA in Brassica napus cv. Westar pistils causes a low level constitutive pollen rejection response. *Plant Molecular Biology* **55,** 619-630.

Halfter, U., Ishitani, M., and Zhu, J.-K. (2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. *PNAS* **97,** 3735-3740.

Halliwell, B., and Gutteridge, J.M.C. (1989). Free radicals in biology and medicine. (Oxford: Claredon Press).

Hancock, J., Desikan, R., Harrison, J., Bright, J., Hooley, R., and Neill, S. (2006). Doing the unexpected: proteins involved in hydrogen peroxide perception. *Journal of Experimental Botany* **57,** 1711-1718.

Hancock, J.T., Henson, D., Nyirenda, M., Desikan, R., Harrison, J., Lewis, L., Hughes, J., and Neill, S.J. (2005). Proteomic identification of glyceraldehyde 3-phosphate dehydrogenase as an inhibitory target of hydrogen peroxide in *Arabidopsis*. *Plant Physiology and Biochemistry* **43,** 828-835.

Haugh, G.W., and Sommerville, C. (1986). Sulfonylurea-resistant mutants of *Arabidopsis thalina*. *Molecular and General Genetics* **204,** 430-434.

Héricourt, F., and Jupin, I. (1999). Molecular cloning and characterization of the *Arabidopsis thaliana* a-subunit of elongation factor 1B *FEBS Letters* **464,** 148-152.

Hirt, H. (1999). Transcriptional upregulation of signaling pathways: more complex than anticipated? *Trends in Plant Science* **4,** 7-8.

Holdaway-Clarke, T.L., Feijo, J.A., Hackett, G.R., Kunkel, J.G., and Hepler, P.K. (1997). Pollen Tube Growth and the Intracellular Cytosolic Calcium Gradient Oscillate in Phase while Extracellular Calcium Influx is Delayed. *The Plant Cell* **9,** 1999-2010.

Holub, E.B., Beynon, L.J., and Crute, I.R. (1994). Phenotypic and genotypic characterisation of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. *Mol. Plant-Microbe Interact.* **7,** 223-239.

Hong, S.-W., and Vierling, E. (2000). Mutants of *Arabidopsis thaliana* defective in the acquisition of tolerance to high temperature stress. *Proc. Natl. Acad. Sci.* **97,** 4392-4397.

Hu, X.Y., Neill, S.J., Cai, W.M., and Tang, Z.C. (2004). Induction of defence gene expression by oligogalacturonic acid requires increases in both cytosolic calcium and hydrogen peroxide in *Arabidopsis thaliana*. *Cell Research* **14,** 234-240.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J., and Kieber, J.J. (2003). Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signalling in *Arabidopsis*. *The Plant Journal* **33,** 221-233.

Huber, S.C., and Hardin, S.C. (2004). Numerous posttranscriptional modifications provide opportunities for the intricate regulation of metabolic enzymes at multiple levels. *Current Opinion in Plant Biology* **7,** 318-322.

Huckelhoven, R., Dechert, C., and Kogel, K.-H. (2001). Non-host resistance of barley is associated with a hydrogen peroxide burst at sites of attempted penetration by wheat powdery mildew fungus. *Molecular Plant Pathology* **2,** 199-205.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K. (2000). Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. *Plant Journal* **24,** 655-665.

Ichimura, K., Mizoguchi, T., Irie, K., Morris, P., Giraudat, J., Matsumoto, K., and Shinozaki, K. (1998). Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in *Arabidopsis*. *Biochemical and Biophysical Research Communication* **253,** 532-543.

Iida, A., Kazuoka, T., Torikai, S., Kikuchi, H., and Oeda, K. (2000). A zinc finger protein RHL41 mediates the light acclimatization response in *Arabidopsis*. *The Plant Journal* **24,** 191-203.

Ingle, R.A., Carstens, M., and Denby, K.J. (2006). PAMP recognition and the plant-pathogen arms race. *BioEssays* **28,** 880-889.

Initiative, T.A.G. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408,** 796-810.

Ishitani, M., Xiong, L., Stevenson, B., and Zhu, J.-K. (1997). Genetic analysis of osmotic an cold stress signal transduction in *Arabidopsis*: Interactions and convergence of acscisic acid-dependent and abscisic acid-independent pathways. *Plant Cell* **9,** 1935-1949.

Ishitani, M., Liu, J., Halfter, U., Kim, C.S., Wei, M., and Zhu, J.K. (2000). SOS3 function in plant stress tolerance requires myristolyation and calcium-binding. *Plant Cell* **12,** 1667-1677.

Ivanov, B., and Khorobrykh, S. (2003). Participation of Photosynthetic Electron Transport in Production and Scavenging of Reactive Oxygen Species. *Antioxidants and Redox Signalling* **5,** 43-53.

Jabs, T., Dietrich, R.A., and Dangl, J.L. (1996). Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. *Science* **273,** 1853-1856.

Jabs, T., Tschope, M., Colling, C., Hahlbrock, K., and Scheel, D. (1997). Elicitor-stimulated ion fluxes and O_2^- from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. *PNAS* **94,** 4800-4805.

Jarillo, J.A., Gabrys, H., Capel, J., Alonso, J.M., Ecker, J.R., and Cashmore, A.R. (2001). Phototropin-related NPL1 controls chloroplast relocation induced by blue light. *Nature* **410,** 952-954.

Jen, C.-H., Manfield, I.W., Michalopoulos, I., Pinney, J.W., Willats, W.G.T., Gilmartin, P.M., and Westhead, D.R. (2006). The Arabidopsis co-expression tool (ACT): a WWW-based tool and database for microarray-based gene expression analysis. *Plant Journal* **46,** 336-348.

Jin, H., Liu, Y., Yang, K.Y., Kim, C.Y., Baker, B., and Zhang, S. (2003). Function of a mitogen-activated protein kinase pathway in N gene-mediated resistance in tobacco. *Plant Journal* **33,** 719-731.

Jirage, D., Zhou, N., Cooper, B., Clarke, J.B., Dong, X., and Glazebrook, J. (2001). Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires PAD4. *The Plant Journal* **26,** 395-407.

Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.F., Parker, J.E., Ausubel, F.M., and Glazebrook, J. (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. *PNAS* **96,** 13583-12588.

Johnson, K.L., and Ingram, G.C. (2005). Sending the right signals: regulating receptor kinase activity. *Current Opinion in Plant Biology* **8,** 648-656.

Jonak, C., Okresz, L., Bogre, L., and Hirt, H. (2002). Complexity, cross talk and intergration of plant MAP kinase signalling. *Current Opinion in Plant Biology* **5,** 415-424.

Jones, A.M., Bennet, M.H., Mansfield, J.W., and Grant, M. (2006). Analysis of the defence phosphoproteome of *Arabidopsis thaliana* using differential mass tagging. *Proteomics* **6,** 4155-4165.

Joo, J.H., Bae, Y.S., and Lee, J.S. (2001). Role of Auxin-Induced Reactive Oxygen Species in Root Gravitropism. *Plant Physiology* **126,** 1055-1060.

Katagiri, F., Thilmony, R., and He, S.Y. (2002). The Arabidopsis Thaliana-Pseudomonas Syringae Interaction. In The Arabidopsis Book, A.S.o.P. Biologists, ed, pp. 1-39.

Kawano, T., and Muto, S. (2000). Mechanism of peroxidase actions for salicylic acid-induced generation of active oxygen species and an increase in cytosolic calcium in tobacco cell suspension. *Journal of Experimental Botany* **51,** 685-693.

Kawano, T., Sahashi, N., Takahashi, K., Uozumi, N., and Muto, S. (1998). Salicylic acid induces extracellular superoxide generation followed by an increase in cytosolic calcium ion in tobacco suspension culture: the earliest events in salicylic acid signal transduction. *Plant Cell Physiology* **39,** 721-730.

Kepinski, S., and Leyser, O. (2005). The *Arabidopsis* F-box protein TIR is an auxin receptor. *Nature* **435,** 446-451.

Kepler, L.D., Baker, C.J., and Atkinson, M.M. (1989). Activated oxygen production during a bacteria induced hypersensitive reaction in tobacco suspension cells. *Phytopathology* **79,** 555-562.

Keppler, L.D., Baker, C.J., and Atkinson, M.M. (1989). Activated oxygen production during a bacteria induced hypersensitive reaction in tobacco suspension cells. *Phytopathology* **79,** 974-978.

Kiegle, E., Moore, C.A., Haseloff, J., Tester, M.A., and Knight, M.R. (2000). Cell-type specific calcium responses to drought, salt and cold in the *Arabidopsis* root. *Plant Journal* **23,** 267-278.

Kim, J.H., Lee, S., Park, J.B., Lee, S.D., Kim, J.H., Ha, S.H., Hasumi, K., Endo, A., Suh, P.-G., and Ryu, S.H. (2003). Hydrogen peroxide induces association between glyceraldehyde 3-phosphate dehydrogenase and phospholipase D2 to facilitate phospholipase D2 activation in PC12 cells. *Journal of Neurochemistry* **85,** 1228-1236.

King, E.O., Ward, M.K., and Raney, D.E. (1954). Two simple media for the demonstration of phycocynin and fluorescein. *J. Lab. Clin. Med.* **44,** 301-307.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence Arabidopsis /*Botrytis* interactions: variation in host production and pathogen sensitivity. *The Plant Journal* **44,** 25-36.

Knight, H. (2000). Calcium Signaling during Abiotic Stress in Plants. *International Review of Cytology* **195,** 269-234.

Knight, H., Trewavas, A.J., and Knight, M.R. (1996). Cold Calcium Signaling in Arabidopsis Involves Two Cellular Pools and a Change in Calcium Signature after Acclimation. *Plant Cell* **8,** 489-503.

Knight, H., Trewavas, A.J., and Knight, M.R. (1997). Calcium signalling in Arabidopsis thaliana in response to drought and salinity. *The Plant Journal* **12,** 1067-1078.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352,** 524-526.

Knox, K., Grierson, C.S., and Leyser, O. (2003). AXR3 and SHY2 interact to regulate root hair development. *Development* **130,** 5769-5777.

Koorneef, M., Léon-Kloosterziel, K.M., Schwartz, S.H., and Zeevaart, J.A.D. (1998). The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in *Arabidopsis*. *Plant Physiology Biochemistry* **36,** 83-89.

Kovtun, Y., Chiu, W.L., Zeng, W., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *PNAS* **97,** 2940-2945.

Kreps, J.A., Wu, Y., Chang, H.-S., Zhu, T., Wang, X., and Harper, J.F. (2002). Transcriptome Changes for Arabidopsis in Response to Salt, Osmotic, and Cold Stress. *Plant Physiology* **130,** 2129-2141.

Kumar, D., and Klessig, D.F. (2000). Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene and jasmonic acid. *Mol. Plant-Microbe Interact.* **13,** 347-351.

Kunkel, B.N., Bent, A.F., Dahlbeck, D., Innes, R.W., and Staskawicz, B.J. (1993). *RPS2*, an *Arabidopsis* disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. *The Plant Cell* **5,** 865-875.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I. (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signalling in Arabidopsis. *EMBO Journal* **22,** 2623-2633.

Laloi, C., Apel, K., and Danon, A. (2004). Reactive oxygen signalling: the latest news. *Current Opinion in Plant Biology* **7,** 323-328.

Laloi, C., Przybyla, D., and Apel, K. (2006). A genetic approach towards elucidating the biological activity of different reactive oxygen species in *Arabiopsis thaliana*. *Journal of Experimental Botany* **57,** 1719-1724.

Lamb, C., and Dixon, R.A. (1997). The Oxidative Burst in Plant Disease Resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 251-275.

Larkindale, J., and Knight, M.R. (2002). Protection against Heat Stress-Induced Oxidative Damage in Arabidopsis Involves Calcium, Abscisic Acid, Ethylene and Salicylic Acid. *Plant Physiology* **128,** 682-695.

Larkindale, J., Hall, J.D., Knight, M.R., and Vierling, E. (2005). Heat Stress Phenotypes of Arabidopsis Mutants Implicate Multiple Signaling Pathways in the Acquisition of Thermotolerance. *Plant Physiology* **138,** 882-897.

Lauvergeat, V., Lacomme, C., Lacombe, E., Lasserre, E., Roby, D., and Grima-Pettenati, J. (2001). Two cinnamoyl-CoA reductase (CCR) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* **57,** 1187-1195.

Levine, A., Tenhaken, R., Dixon, R., and Lamb, C. (1994). H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* **79,** 583-593.

Leyser, O. (1998). Auxin signalling: Protein stability as a versatile control target. *Current Biology* **8,** R305-R307.

Li, J., Lee, Y.-R.J., and Assmann, S.M. (1998). Guard cells possess a calcium-dependent protein kinase that phosphorylates the KAT1 potassium channel. *Plant Physiology* **116,** 785-795.

Lindermayr, C., Saalbach, G., and Durner, J. (2005). Proteomic identification of *S*-nitrosylated proteins in *Arabidopsis*. *Plant Physiology* **137,** 921-930.

Liu, J., Ishitani, M., Halfter, U., Kim, C.-S., and Zhu, J.-K. (2000). The *Arabidopsis thaliana* SOS2 gene encodes a protein kinase that is required for salt tolerance. *PNAS* **97,** 3730-3734.

Liu, Y., Jin, H., Yang, K.Y., Kim, C.Y., Baker, B., and Zhang, S. (2003). Interaction of between two mitogen-activated protein kinases during tobacco defense signaling. *Plant Journal* **34,** 149-160.

Lopez-Huertas, E., Charlton, W.L., Johnson, B., Graham, I.A., and Baker, A. (2000). Stress induces peroxisome biogenesis genes. *EMBO Journal* **19,** 6770-6777.

López-Huertas, E., Charlton, W.L., Johnson, B., Graham, I.A., and Baker, A. (1999). Characterisation of membrane polypeptides from pea leaf peroxisomes involved in superoxide radical generation. *Biochem. J.* **337,** 531-536.

Lu, M., Tang, X., and Zhou, J.-M. (2001). *Arabidopsis NHO1* is required for general resistance against *Psuedomonas* bacteria. *The Plant Cell* **13,** 437-447.

Mackey, D., III, B.F.H., Wiig, A., and Dangl, J.L. (2002). RIN4 Interacts with *Pseudomonas syringae* Type III Effector Molecules and Is Required for RPM1-Mediated Resistance in *Arabidopsis*. *Cell* **108,** 743-754.

Martin, G.B., Brommonschenkel, D., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earfe, E.D., and Tanksley, S.D. (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science* **265,** 212-219.

Maxwell, D.P., Wang, Y., and McIntosh, L. (1999). The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. *PNAS* **96,** 8271-8276.

McAinsh, M.R., and Hetherington, A.M. (1998). Encoding the specificity in Calcium Signaling systems. *Trends in Plant Science* **3,** 32-36.

McClung, C.R. (1997). Regulation of catalases in *Arabidopsis*. *Free Rad. Biol. Med.* **23,** 489-496.

McDowell, J.M., Williams, S.G., Funderburg, N.T., Eulgem, T., and Dangl, J.L. (2005). Genetic Analysis of Developmentally Regulated Resistance to Downy Mildew (*Hyaloperonospora parasitica*) in *Arabidopsis thaliana*. *Molecular Plant-Microbe interactions* **18,** 1226-1234.

McLusky, S.R., Bennett, M.H., Beale, M.H., Lewis, M.J., Gaskin, P., and Mansfield, J.W. (1999). Cell wall alterations and localized accumulation of feruloyl-3'-methoxytyramine in onion epidermis at sites of attempted penetration by Botrytis allii are associated with actin polymerisation, peroxidase activity and suppression of flavonoid biosynthesis. *Plant Journal* **17,** 523-534.

Mehler, A.H. (1951). Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. *Archives of Biochemistry* **33,** 65-77.

Meinhard, M., and Grill, E. (2001). Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from *Arabidopsis*. *FEBS Letters* **508,** 443-444.

Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R. (2003). The *BOTRYTIS SUSCEPTIBLE1* Gene Encodes an R2R3MYB Transcription Factor Protein That is Required for Biotic and Abiotic Stress Responses in Arabidopsis. *The Plant Cell* **15,** 2551-2565.

Menke, F.L.H., Pelt, J.A.v., Pieterse, C.M.J., and Klessig, D.F. (2004). Silencing of the Mitogen-Activated Protein Kinase MPK6 Compromises Disease Resistance in Arabidopsis. *The Plant Cell* **16,** 897-907.

Meskiene, I., Bögre, L., Glaser, W., Balog, J., Brandstötter, M., Zwerger, K., Ammerer, G., and Hirt, H. (1998). MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. *PNAS* **95,** 1938-1943.

Meskiene, I., Baudouin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P.L., Jelinek, H., and Hirt, H. (2003). Stress-induced Protein Phosphatase 2C Is a Negative Regulator of a Mitogen-activated Protein Kinase. *The Journal of Biological Chemistry* **278,** 18945-18952.

Miao, Y., Laun, T., Zimmermann, P., and Zentgraf, U. (2004). Targets of the WRKY53 transcription factor and its role during leaf senescence in *Arabidopsis*. *Plant Molecular Biology* **55,** 853-867.

Miles, G.P., Samuel, M.A., Zhang, Y., and Ellis, B.E. (2005). RNA interference-based (RNAi) suppression of AtMPK6, an Arabidopsis mitogen-activated protein kinase, results in hypersensitivity to ozone and misregulation of AtMPK3. *Environ. Pollut.* **138,** 230-237.

Millar, A.H., and Day, D.A. (1997). Alternative solutions to radical problems. *Trends in Plant Science* **2,** 289-290.

Millar, A.H., and Leaver, C.J. (2000). The cytotoxic lipid peroxidation product, 4-hydroxy-2-nonenal, specifically inhibits decarboxylating dehydrogenases in the matrix of plant mitochondria. *FEBS Letters* **481,** 117-121.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7,** 405-410.

Mizoguchi, T., Irie, K., Hirayama, T., Hayashida, N., Yamaguchi-Shinozaki, K., Matsumoto, K., and Shinozaki, K. (1996). A gene encoding a mitogen-activated protein kinase kinase kinase is induced simultaneously with genes for a mitogen activated protein kinase and an S6 ribosomal protein kinase by touch, cold and water stress in *Arabidopsis thaliana*. *PNAS* **93,** 765-769.

Moller, I.M. (2001). Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 561-591.

Moller, I.M. (2006). Reactive Oxygen Species (ROS) and Plant Respiration. In Plant Physiology Online, L. Taiz and E. Zeiger, eds.

Moon, H., Lee, B., Choi, G., Shin, D., Prasad, D.T., Lee, O., Kwak, S.-S., Kim, D.H., Nam, J., Bahk, J., Hond, J.C., Lee, S.Y., Cho, M.J., Lim, C.O., and Yun, D.-J. (2003). NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. *PNAS* **100,** 358-363.

Morita, S., Kaminaka, H., Masumura, T., and Tanaka, K. (1999). Induction of rice cytosolic ascorbate peroxidase mRNA by oxidative stress, the involvement of hydrogen peroxide in oxidative stress signalling. *Plant Cell and Physiology* **40,** 417-422.

Morris, E.R., and Walker, J.C. (2003). Receptor-like kinases: the keys to response. *Current Opinion in Plant Biology* **6,** 339-342.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* **113,** 935-944.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* **15,** 473-497.

Murata, Y., Pei, Z.M., Mori, I.C., and Schroeder, J. (2001). Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. *Plant Cell* **13,** 2513-2523.

Murray, S.L. (2001). Identification and Characterisation of *Arabidopsis* Systemic Acquired Resistance Mutants isolated by Luciferase Imaging. In Institute of Cell and Molecular Biology (Edinburgh: University of Edinburgh), pp. 148.

Murray, S.L., Denby, K.J., Berger, D.K., and Loake, G.J. (2002). Disease resistance signalling in *Arabidopsis:* applications in the study of plant pathology in South Africa. *South African Journal of Science* **98,** 161-165.

Murray, S.L., Adams, N., Kliebenstein, D.J., Loake, G., and Denby, K.J. (2005). A constitutive *PR-1::luciferase* expression screen identifies Arabidopsis mutants with differential disease resistance to both biotrophic and necrotrophic pathogens. *Molecular Plant Pathology* **6,** 31-41.

Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F., and Giraudat, J. (2002). *Arabidopsis* OST1 protein kinase mediates the regulation of stomatal apperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14,** 3089-3099.

Mysore, K.S., and Ryu, C.-M. (2004). Nonhost resistance: how much do we know? *Trends in Plant Science* **9,** 97-104.

Nakagami, H., Pitzschke, A., and Hirt, H. (2005). Emerging MAP kinase pathways in plant stress signalling. *Trends in Plant Science* **10,** 339-346.

Nakamura, T., Ohta, M., Sugiura, M., and Sugita, M. (2001). Chloroplast Ribonucleoproteins Function as a Stabilizing Factor of Ribosome-free mRNAs in the Stroma. *The Journal of Biological Chemistry* **276,** 147-152.

Navari-Izzo, F., Pinzino, C., Quartacci, M.F., and Sgherri, C.L. (1999). Superoxide and hydroxyl radical generation, and superoxide dismutase in PSII membrane fragments from wheat. *Free Rad. Res.* **31,** 3-9.

Navarro, L., Zipfel, C., Roland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J.D.G. (2004). The Transcriptional Innate Immune Response to flg22. Interplay and Overlap with Avr Gene-Dependent Defense Responses and Bacterial Pathogenesis. *Plant Physiology* **135,** 1113-1128.

Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Vionnet, O., and Jones, J.D.G. (2006). A Plant miRNA Contributes to Anitbacterial Resistance by Repressing Auxin Signaling. *Science* **312,** 436-439.

Nawrath, C., and Métraux, J.-P. (1999). Salicylic Acid Induction-Deficient Mutants of Arabidopsis Express *PR-2* and *PR*-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. *Plant Cell* **11,** 1393-1404.

Neill, S.J., Desikan, R., and Hancock, J.T. (2003). Nitric oxide signalling in plants. *New Phytologist* **159,** 11-35.

Noctor, G., and Foyer, C. (1998). Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49,** 249-279.

Nuccio, M.L., Rhodes, D., McNeil, S.D., and Hanson, A.D. (1999). Metabolic engineering of plants for osmotic stress resistance. *Current Opinion in Plant Biology* **2,** 128-134.

Nürnberger, T., and Scheel, D. (2001). Signal transmission in the plant immune response. *Trends in Plant Science* **6,** 372-379.

Ohashi, Y., Oka, A., Rodrigues-Pousada, R., Possenti, M., Ruberti, I., Morelli, G., and Aoyama, U. (2003). Modulation of phospholipid signalling by GLABRA2 in root-hai pattern formation. *Science* **300,** 1427-1430.

Orlandi, E.W., Hutcheson, S.W., and Baker, C.J. (1992). Early physiological responses associated with race-specific recognition in soybean leaf tissue and cell suspensions treated with *Pseudomonas syringae* pv. *glycinea*. *Physiol. Mol. Plant Pathol.* **40,** 173-180.

Osterlund, M.T. (2000). Targeted destabilisation of HY5 during light-regulated development of *Arabidopsis*. *Nature* **405,** 462-466.

Oyama, T., Shimura, Y., and Okada, K. (2002). The *IRE* gene encodes a protein kinase homologue and modulates root hair growth in *Arabidopsis*. *Plant Journal* **30,** 289-299.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996). Characterisation of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. *Plant Cell* **8,** 2033-2046.

Pavet, V., Olmos, E., Kiddle, G., Mowla, S., Kumar, S., Antoniw, J., Alvarez, M.E., and Foyer, C.H. (2005). Ascorbic acid deficiency activates cell death and disease resistance responses in Arabidopsis. *Plant Physiology* **139,** 1291-1303.

Peck, S.C. (2006). Phosphoproteomics in *Arabidopsis*: moving from empirical to predictive science. *Journal of Experimental Botany* **57,** 1523-1527.

Pedley, K.F., and Martin, G.B. (2004). Identification of MAPKs and their possible MAPK kinase activators involved in the Pto-mediated defence response of tomato. *Journal of Biological Chemistry* **279,** 49229-49235.

Pei, Z.M., Ward, J.M., Harper, J.F., and Schroeder, J.I. (1996). A novel chloride channel in *Vicia faba* guard cell vacuoles activated by the serine/threonine kinase, CDPK. *EMBO Journal* **15,** 6564-6574.

Pei, Z.M., Murata, Y., Benning, G., Thomine, S., Klusener, B., Allen, G.J., Grill, E., and Schroeder, J.I. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406,** 731-734.

Peng, M., and Kuc, J. (1992). Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf discs. *Phytopathology* **82,** 696-699.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. *The Plant Cell* **10,** 2103-2113.

Persson, S., Wei, H., Milne, J., Page, G.P., and Sommerville, C.R. (2005). Identification of genes required for cellulose synthesis by regression analysis of public microarray data sets. *PNAS* **102,** 8633-8638.

Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattson, O., Jensen, A.B., and Mundy, J. (2000). Arabidopsis MAP Kinase 4 Negatively Regulates Systemic Acquired Resistance. *Cell* **103,** 1111-1120.

Peterson, R.T., and Schreiber, S.L. (1999). Kinase phosphorylation: Keeping it all in the family. *Current Biology* **9,** R512-R524.

Pieterse, C.M.J., and Loon, L.C.V. (2004). NPR1: the spider in the web of induced resistance signaling pathways. *Current Opinion in Plant Biology* **7,** 456-464.

Pitzschke, A., and Hirt, H. (2006). Mitogen-Activated Protein Kinases and Reactive Oxygen Species Signaling in Plants. *Plant Physiology* **141,** 351-356.

Planchais, S., Samland, A.K., and Murray, J.A.H. (2004). Differential stability of *Arabidopsis* D-type cyclins: CYCD3; is a highly unstable protein degraded by a proteasome-dependent mechanism. *Plant Journal* **38,** 616-625.

Polverari, A., Molesini, B., Pezzotti, M., Buonaurio, R., and Marte, M. (2003). Nitric oxide-mediated transcriptional changes in *Arabidopsis thaliana*. *Mol Plant Microbe Interact* **16,** 1094-1105.

Purvis, A.C. (1997). Role of alternative oxidase in limiting superoxide production by plant mitochondria. *Physiologia Plantarum* **100,** 165-170.

Queitsch, C., Hong, S.-W., Vierling, E., and Lindquist, S. (2000). Heat Shock Protein 101 Plays a Crucial Role in Thermotolerance in Arabidopsis. *The Plant Cell* **12,** 479-492.

Qui, Q.S., Guo, Y., Quintero, F.J., Pardo, J.M., Schumaker, K.S., and Zhu, J.K. (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. *PNAS* **99,** 8436-8441.

Rao, M.V., Paliyath, G., Ormrod, D.P., Murr, D.P., and Watkins, C.B. (1997). Influence of Salicylic Acid on H_2O_2 Production, Oxidative Stress and H_2O_2-Metabolizing Enzymes. *Plant Physiology* **115,** 137-149.

Rathjen, J.P., Chang, J.H., Staskawicz, B.J., and Michelmore, R.W. (1999). Constitutively active Pto induces a Prf-dependent hypersensitive response in the absence of avrPto. *The EMBO Journal* **18,** 3232-3240.

Raz, V., and Fluhr, R. (1992). Calcium requirement for ethylene-dependent responses. *Plant Cell* **4,** 1123-1130.

Ren, D., Yang, H., and Zhang, S. (2002). Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. *Journal of Biological Chemistry* **277,** 559-565.

Rentel, M. (2002). Signal transduction in response to active oxygen species in *Arabidopsis thaliana*. In Department of Plant Sciences (Oxford: University of Oxford).

Rentel, M.C., and Knight, M.R. (2004). Oxidative Stress-Induced Calcium Signalling in Arabidopsis. *Plant Physiology* **135,** 1471-1479.

Rentel, M.C., Lecourieux, D., Ouaked, F., Usher, S.L., Petersen, L., Okamoto, H., Knight, H., Peck, S.C., Grierson, C.S., Hirt, H., and Knight, M.R. (2004). OXI1 kinase is necessary for oxidative burst-mediated signalling in Arabidopsis. *Nature* **427,** 858-861.

Rivas, S., Rougon-Cardoso, A., Smoker, M., Schauser, L., Yoshioka, H., and Jones, J.D. (2004). CITRX thioredoxin interacts with the tomato Cf-9 resistance protein and negatively regulates defence. *EMBO Journal* **23,** 2156-2165.

Rizhsky, L., Liang, H., and Mittler, R. (2003). The Water-Water Cycle is Essential for Chloroplast Protection in the Absence of Stress. *The Journal of Biological Chemistry* **278,** 38921-38925.

Rizhsky, L., Hallak-Herr, E., Van Breusegem, F., Rachmilevitch, S., Barr, J.E., Rodermal, S., Inzé, D., and Mittler, R. (2002). Double antisense plants lacking ascorbate peroxidase and catalase are less sensitve to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. *The Plant Journal* **32,** 329-342.

Robatzek, S., and Somssich, I.E. (2001). A new member of the Arabidopsis WRKY transcription factor family, AtWRKY6, is associated with both senescence- and defence-related processes. *Plant Journal* **28,** 123-133.

Robatzek, S., and Somssich, I.E. (2002). Targets of *At*WRKY6 regulation during plant senescence and pathogen defence. *Genes and development* **16,** 1139-1149.

Robatzek, S., Chinchilla, D., and Boller, T. (2006). Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Plant Cell* **13,** 1155-1163.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romanschuk, M., and He, S.Y. (1997). Hrp pilus: An hrp-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *PNAS* **94,** 3459-3464.

Romeis, T. (2001). Protein kinases in the plant defence response. *Current Opinion in Plant Biology* **4,** 407-414.

Romeis, T., Piedras, P., and Jones, J.D.G. (2000). Resistance gene-dependent activation of a calcium-dependent protein kinase in plant defence responses. *Plant Cell* **12,** 803-815.

Romeis, T., A.Ludwig, A., Martin, R., and D.G.Jones, J. (2001). Calcium-dependent protein kinases play an essential role in a plant defence response. *The EMBO Journal* **20,** 5556-5567.

Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H., and Jones, J.D.G. (1999). Rapid Avr9- and Cf-9-Dependent Activation of MAP Kinases in Tobacco Cell Cultures and Leaves: Convergence of Resistance Gene, Elicitor, Wound and Salicylate Responses. *The Plant Cell* **11,** 273-287.

Romero, H.M., Berlett, B.S., Jenson, P.J., Pell, E.V., and Tien, M. (2004). Investigations into the role of plastidial peptide methionine sulfoxide reductase in response to oxidative stress in *Arabidopsis*. *Plant Physiology* **136,** 3784-3994.

Rouhier, N., Gelhaye, E., and Jacquot, J.P. (2004). Plant glutaredoxins: still mysterious reducing systems. *Cell Mol Life Sci* **61** 1266-1277.

Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B.-H., Matsumoto, T.K., Koiwa, H., Zhu, J.-K., Bressan, R.A., and Hasegawa, P.M. (2001). AtHKT1 is a salt-tolerance determinant that controls Na^+ entry into plant roots. *PNAS* **98,** 14150-14155.

Sadanandom, A., Poghosyan, Z., Fairbairn, D.J., and Murphy, D.J. (2000). Differential regulation of plastidial and cytosolic isoforms of peptides methionine sulfoxide reductase in *Arabidopsis*. *Plant Physiology* **123,** 1299-1309.

Saha, P., and Singh, M. (1995). Characterization of a winged bean (*Psophocarpus tetragonolobus*) protein-kinase with calmodulin-like domain regulaton by autophosphorylation. *Biochem J* **305,** 205-210.

Saijo, Y., Hata, S., Kyozuka, J., Simamoto, K., and Izui, K. (2000). Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *The Plant Journal* **23,** 319-327.

Samuel, M.A., Miles, G.P., and Ellis, B.E. (2000). Ozone treatment radiply activates MAP kinase signalling in plants. *Plant Journal* **22,** 367-376.

Sanchez, J.P., and Chua, N.H. (2001). Arabidopsis PLC1 is required for secondary responses to abscisic acid signals. *Plant Cell* **13,** 1143-1154.

Sandalio, L.M., Fernandez, V.M., Ruperez, F.L., and del Río, L.A. (1988). Superoxide free radicals are produced in glyoxysomes. *Plant Physiology* **87,** 1-4.

Sanders, D., Brownlee, C., and Harper, J. (1999). Communicating with Calcium. *Plant Cell* **11,** 691-706.

Scheel, D. (1998). Resistance response physiology and signal transduction. *Current Opinion in Plant Biology* **1,** 305-310.

Schneider-Müllar, S., Kurosaki, F., and Nishi, A. (1994). Role of salicylic acid and intracellular Ca^{2+} in the induction of chitinase activity in carrot suspension culture. *Physiological and Molecular Plant Pathology* **45,** 101-109.

Sharma, P.C., Ito, A., Shimizu, T., Terauchi, R., Kamoun, S., and Saitoh, H. (2003). Virus-induced silencing of *WIPK* and *SIPK* genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotinana Benthamiana*. *Mol Gen Genomics* **269,** 583-591.

Sheen, J. (1996). Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science* **274,** 1900-1902.

Sheng, J., D'Ovidio, R., and Mehdy, M.C. (1991). Negative and positive regulation of a novel proline-rich protein mRNA by fungal elicitor and wounding. *Plant Journal* **3,** 345-354.

Shin, R., and Schachtman, D.P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *PNAS* **101,** 8827-8832.

Shiu, S.H., and Bleecker, A.B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis. Plant Physiology* **132,** 530-543.

Singh, K.K. (2000). The *Saccharomyces cerevisiae* SLN1P-SSK1P two-component system mediates response to oxidative stress and in an oxidant-specific fashion. *Free Radicals in Biology and Medicine* **29,** 1043-1050.

Somssich, I.E., and Hahlbrok, K. (1998). Pathogen defence in plants - a paradigm of biological complexity. *Trends in Plant Science* **3,** 86-90.

Stahl, W., and Sies, H. (2003). Antioxidant activity of carotenoids. *Molecular Aspects of Medicine* **24,** 345-351.

Suzuki, G., Yanagawa, Y., Kwok, S.F., Matsui, M., and Deng, X.W. (2002). *Arabidopsis* COP10 is a ubiquitin-conjugating enzyme variant that acts together with COP1 and the COP9 signalosome in repressing photomorphogenesis. *Genes and development* **16,** 554-559.

Sweetlove, L.J., Heazlewood, J.L., Herald, V., Holtzapffel, R., Day, D.A., Leaver, C.J., and Millar, A.H. (2002). The impact of oxidative stress on *Arabidopsis* mitochondria. *The Plant Journal* **32,** 891-904.

Tamaskovic, R. (2003). NDR family of AGC kinases - essential regulators of the cell cycle and morphogenesis. *FEBS Letters* **546,** 73-80.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and the Pto kinase. *Science* **274,** 2060-2063.

Thilmony, R., Underwood, W., and He, S.Y. (2006). Genome-wide transcriptional analysis of the *Arabidopsis thaliana* interaction with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the human pathogen *Escherichia coli* O157:H7. *Plant Journal* **46,** 34-53.

Thomma, B.P.H.J., Penninckxx, I.A.M.A., Broekaert, W.F., and Cammue, B. (2001). The complexity of disease signaling in *Arabidopsis. Current Opinion in Immunology* **13,** 63-68.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., and Vogelsang, R. (1998). Separate jasmonate-dependent and salicylate-dependent defense response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *PNAS* **95,** 15107-15111.

Thordal-Christensen, H. (2003). Fresh insights into processes of nonhost resistance. *Current Opinion in Plant Biology* **6,** 351-357.

Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D.B. (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powedery mildew interaction. *The Plant Journal* **11,** 1187-1194.

Tiege, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., Dangl, J.L., and Hirt, H. (2004). The MKK2 Pathway Mediates Cold and Salt Stress Signaling in *Arabidopsis*. *Molecular Cell* **15,** 141-152.

Torres, M.A., Dangl, J.L., and Jones, J.D.G. (2002). *Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for the accumulation of reactive oxygen intermediates in the plant defence response. *PNAS* **99,** 517-522.

Torres, M.A., Onouchi, H., Hamada, S., Machida, C., Hammond-Kosack, K.E., and Jones, J.D.G. (1998). Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*gp91^{phox}*). *Plant Journal* **14,** 365-370.

Trewavas, A.J., and Malhó, R. (1997). Signal Perception and Transduction: The origin of the Phenotype. *The Plant Cell* **9,** 1181-1195.

Truman, W., Zabala, M.T.d., and Grant, M. (2006). Type III effectors orchestrates a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *Plant Journal* **46,** 14-33.

Turrens, J.F. (1997). Superoxide production by the mitochondrial respiratory chain. *Biosci. Rep.* **17,** 3-8.

Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J. (1992). Acquired Resistance in Arabidopsis. *The Plant Cell* **4,** 645-656.

Ulm, R., Revenkova, E., Sansebastiano, G.-P.d., Bechtold, N., and Paszkowski, J. (2001). Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in *Arabidopsis*. *Genes and development* **15,** 699-709.

Ulm, R., Ichimura, K., Mizoguchi, T., Peck, S.C., Zhu, T., Wang, X., Shinozaki, K., and Paszkowski, J. (2002). Distinct regulation of salinity and genotoxic stress responses by *Arabidopsis* MAP kinase phosphatase 1. *EMBO Journal* **21,** 6483-6493.

Urban, P., Mignotte, C., Kazmaier, M., Delorme, F., and Pompon, D. (1997). Cloning, yeast expression and characterisation of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. *Journal of Biological Chemistry* **272,** 19176-19186.

Van Breusegem, F., Vranová, E., Dat, J.F., and Inzé, D. (2001). The role of active oxygen species in plant signal transduction. *Plant Science* **161,** 405-414.

Van Camp, W., Inzé, D., and Van Montagu, M. (1997). The regulation and function of tobacco superoxide dismutases. *Free Rad. Biol. Med.* **23,** 515-520.

Van Camp, W., Van Montagu, M., and Inze, D. (1998). H_2O_2 and NO: redox signals in plant signal transduction. *Trends in Plant Science* **3,** 330-334.

van der Biezen, E.A., Freddie, C.T., Kahn, K., Parker, J.E., and Jones, J.D.G. (2002). *Arabidopsis RPP4* is a member of *RPP5* multigene family of TIR-NB-LRR genes and confers downy mildew resistance through multiple signalling components. *Plant Journal* **29,** 439-451.

van Leeuwen, W., Ruttink, T., Borst-Vrenssen, A.W.M., van der Plas, L.H.W., and van der Krol, A.R. (2001). Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. *Journal of Experimental Botany* **52,** 949-959.

van Mieghem, F.J.E., Nitschke, W., Mathis, P., and Rutherford, A.W. (1989). The influence of the quinone-iron electron acceptor complex on the reaction centre photochemistry of photosystem II. *Biochimica et Biophysica Acta* **977,** 207-214.

Vandenabeele, S., Der Kelen, K.V., and Dat, J. (2003). A comprehensive analysis of hydrogen peroxide-induced gene expression in tobacco. *PNAS* **100,** 16113-16118.

Vanderauwera, S., Zimmermann, P., Rombauts, S., Vandenbeele, S., Langebartels, C., Gruissem, W., Inzé, D., and Van Breusegem, F. (2005). Genome-wide analysis of hydrogen peroxide-regulated gene expression in *Arabidopsis* reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. *Plant Physiology* **139,** 806-821.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.C. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70,** 535-602.

Veronese, P., Chen, X., Bluhm, B., Salmeron, J., Dietrich, R., and Mengiste, T. (2004). The *BOS* loci of *Arabidopsis* are required for resistance to *Botrytis cinerea* infection. *The Plant Journal* **40,** 558-574.

Veronese, P., Nakagami, H., Bluhm, B., AbuQmar, S., Chen, X., Salmeron, J., Dietrich, R.A., Hirt, H., and Mengiste, T. (2006). The Membrane-Anchored BOTRYTIS-INDUCED KINASE1 Plays Distinct Roles in Arabidopsis Resistance to Necrotrophic and Biotrophic Pathogens. *The Plant Cell* **18,** 257-273.

Vierstra, R.D. (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends in Plant Science* **8,** 135-142.

Volotovski, I.D., Dubovskaya, L.V., and Molcham, O. (2003). Photoreceptor Phytochrome regulates the cyclic guanosine 3',5'-monophosphate synthesis in *Avena Sativa* L. Cells. *Bulg. J. Plant Physiology* **29,** 3-12.

Vranová, E., Inzé, D., and Van Breusegem, F. (2002). Signal Transduction during oxidative stress. *Journal of Experimental Botany* **53,** 1227-1236.

Wendehenne, D., Durner, J., and Klessig, D.F. (2004). Nitric oxide: a new player in plant signalling and defence responses. *Current Opinion in Plant Biology* **7,** 449-455.

Whitmarsh, A.J., and Davis, R.J. (1998). Structural organization of MAP-kinase signaling modules by scaffold proteins in yeast and mammals. *Trends in Biochemical Science* **23,** 481-485.

Widmann, C., Gibson, S., Jarpe, M.B., and Johnson, G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **79,** 143-180.

Willekens, H., Villarroel, R., Montague, M.V., Inzé, D., and Camp, W.V. (1994). Molecular identification of catalases from *Nicotiana plumbaginifolia*. *FEBS Letters* **352,** 79-83.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Montague, M.V., Inzé, D., and Camp, W.V. (1997). Catalase is a sink for H_2O_2 and is indispensible for stress defence in C-3 plants. *EMBO Journal* **16,** 4806-4816.

Winger, A.M., Millar, A.H., and Day, A.D. (2005). Sensitivity of plant mitochondrial terminal oxidases to the lipid peroxidation product 4-hydroxy-2-nonenal (HNE). *Biochem. J.* **387,** 865-870.

Wu, G., Shortt, B.J., Lawrence, E.B., Leon, J., Fitzsimmons, K.C., Levine, E.B., Raskin, I., and Shah, D.M. (1997). Activation of host defence mechanisms by elevated production of H_2O_2 in transgenic plants. *Plant Physiology* **115,** 427-435.

Wu, G.S., Short, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C., and Shah, D.M. (1995). Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. *Plant Cell* **7,** 1357-1368.

Xing, T., Higgins, V.J., and Blumwald, E. (2001). Ectopic expression of an Arabidopsis calmodulin-like domain protein-kinase enhanced NADPH oxidase activity and oxidative burst in tomato protoplasts. *Molecular Plant-Microbe Interactions* **14,** 1261-1264.

Xiong, L., Schumaker, K.S., and Zhu, J.-K. (2002). Cell Signaling during Cold, Drought and Salt stress. *The Plant Cell* **Supplement,** S165-S183.

Yamamizo, C., Kuchimura, K., Kobayashi, A., Katou, S., Kawakita, K., Jones, J.D.G., Doke, N., and Yoshioka, H. (2006). Rewiring Mitogen-Activated Protein Kinase Cascade by Positive Feedback Confers Potato Blight Resistance. *Plant Physiology* **140,** 681-692.

Yamamoto, Y.Y., Mitsui, M., and Deng, X.-W. (1998). Positive feedback in plant signalling pathways. *Trends in Plant Science* **3,** 374-375.

Yang, K.Y., Liu, Y., and Zhang, S. (2001). Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *PNAS* **98,** 741-746.

Yoshioka, H., Numata, N., Nakajima, K., Katou, S., Kawakita, K., Rowland, O., Jones, J.D., and Doke, N. (2003). *Nicotiana benthamiana* gp91phox homologs NbrbohA and NbrbohB participate in H_2O_2 accumulation and resistance to *Phytophthora infestans. Plant Cell* **15,** 706-718.

Zeidler, D., Zahringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P., and Durner, J. (2004). Innate immunity in Arabidopsis thaliana: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *PNAS* **101,** 15811-15816.

Zenzer, N., Ellsmore, A., Leasure, C., and Callis, J. (2001). Auxin modulates the degradation rate of Aux/IAA proteins. *PNAS* **98,** 11795-11800.

Zhang, N., and Portis, A.R. (1999). Mechanism of light regulation of Rubisco: A specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f. *PNAS* **96,** 9438-9443.

Zhang, S., and Klessig, D.F. (1998). Resistance gene N-mediated de novo synthesis and activation of a tobacco mitogen-activated protein kinase by tobacco mosaic virus infection. *PNAS* **95,** 7433-7438.

Zhang, S., and Liu, Y. (2001). Activation of salicylic acid-induced protein kinase, a mitogen-activated protein kinase, induces multiple defence responses in tobacco. *Plant Cell* **13,** 1877-1889.

Zhang, S., and Klessig, D.F. (2001). MAPK cascades in plant defence signaling. *Trends in Plant Science* **6,** 520-527.

Zhang, Z., Colinge, B., and Thordal-Cristensen, H. (1995). Germin-like oxalate oxidase, a H_2O_2 producing enzyme, accumulates in barley attacked by powdery mildew fungus. *Plant Journal* **8,** 139-145.

Zhou, F., Zhang, Z., Gregersen, P.L., Mikkelsen, J.D., Neergaard, l.d., Collinge, D.B., and Thordal-Christensen, H. (1998a). Molecular Characterization of the Oxalate Oxidase Involved in the Response of Barley to the Powdery Mildew Fungus. *Plant Physiology* **117,** 33-41.

Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J. (1998b). *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* **10,** 1021-1030.

Zhu, J.-K. (2002). Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology* **53,** 247-273.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.G., Felix, G., and Boller, T. (2004). Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* **428,** 764-767.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D.G., Boller, T., and Felix, G. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* **125,** 749-760.