An investigation into the role of SUMO proteases OVERLY TOLERANT to SALT1 and -2 in salicylic acid mediated defense signalling in *Arabidopsis thaliana*

Toward understanding the role of SUMOylation in SA signalling

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

The work presented (including data generated and data analysis) was carried out by the author except where explicitly stated otherwise.

-Mark Bailey
Summary

Enzymatic, covalent attachment of the Small Ubiquitin-like Modifier (SUMO) protein to a substrate protein, SUMOylation, is a stress inducible post-translational modification conserved throughout eukaryotes. SUMO conjugation to proteins alters protein interactions, regulating signalling pathways in the cell, and modulating response. SUMO proteases process SUMO into its mature form as a prerequisite to conjugation, in addition to providing reversibility to the SUMOylation pathway by cleaving SUMO from substrate proteins.

Salicylic acid (SA) is a key hormone in propagating defense activation and signalling against biotrophic pathogens in plants. An investigation into the role of SUMO proteases OVERLY TOLERANT to SALT1 and -2 (OTS1 and -2) in SA regulation was performed using Arabidopsis thaliana mutants and transgenic over expressing lines. OTS1 and -2 were required for the restriction of SA biosynthesis and signalling in unchallenged plants. Further, SA treatment promoted OTS1 degradation and accumulation of SUMO conjugates, suggesting a positive relationship between SUMO conjugation and SA synthesis.

Mutants of the SUMO E3 ligase SAP and MIZ1 (SIZ1) possess reduced levels of SUMO conjugates whilst displaying elevated SA content and activated defenses. This apparent contradiction was investigated using single siz1 and triple ots1 ots2 siz1 mutants, which were found to possess comparable SA related phenotypes to the ots1 ots2 double mutant. Finally it was concluded that there is more to the regulation between SA biosynthesis and SUMOylation than the presence or absence of SUMOylated proteins, and further, that promotion of SUMO conjugates by SA may facilitate modulation of other signalling pathways.
# Nomenclature

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<tr>
<td>ABA</td>
<td>Abscisic acid</td>
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<tr>
<td>Avr</td>
<td>Avirulence gene</td>
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<tr>
<td>BAK1</td>
<td>BRASSINOSTEROID INSENSITIVE 1 ASSOCIATED KINASE 1</td>
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<td>CPR1</td>
<td>CONSTITUTIVE EXPRESSER OF PR GENES 1</td>
</tr>
<tr>
<td>CRL</td>
<td>CULLIN RING E3 Ubiquitin Ligase</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage Associated Molecular Pattern</td>
</tr>
<tr>
<td>EDS1</td>
<td>ENHANCED DISEASE SUSCEPTIBILITY 1</td>
</tr>
<tr>
<td>EFR</td>
<td>EF-Tu RECEPTOR</td>
</tr>
<tr>
<td>EIL1</td>
<td>ETHYLENE INSENSITIVE 3-LIKE 1</td>
</tr>
<tr>
<td>EIN3</td>
<td>ETHYLENE INSENSITIVE 3</td>
</tr>
<tr>
<td>eLRR</td>
<td>extracellular Leucine Rich Repeat</td>
</tr>
<tr>
<td>ELS1</td>
<td>ESD4-LIKE 1</td>
</tr>
<tr>
<td>ESD4</td>
<td>EARLY in SHORT DAYS 4</td>
</tr>
<tr>
<td>ETI</td>
<td>Effector Triggered Immunity</td>
</tr>
</tbody>
</table>
FLC  FLOWERING LOCUS C
FLS2  FLAGELLIN-SENSING2
GA  Gibberellic acid
HPY2  HIGH PLOIDY 2
HR PCD  Hypersensitive Response associated Programmed Cell Death
HSP90  HEAT SHOCK PROTEIN 90
ICE1  INDUCER OF CBF EXPRESSION1
ICS1/2  ISOCHORISMATE SYNTHASE1/2
JA  Jasmonic acid
JAZ  JASMONATE-ZIM-DOMAIN PROTEIN
KAPP  KINASE-ASSOCIATED PROTEIN PHOSPHATASE
LC-MS  Liquid chromatography-mass spectrometry
LYK5  LYSM-CONTAINING RECEPTOR-LIKE KINASE 5
MKK  Mitogen-Activated Protein Kinase Kinase
MMS21  METHYL METHANESULFONATE-SENSITIVITY protein 21
MOS  MODIFIER OF SNC1
MUSE  MUTANT SNC1-ENHANCING
NB LRR  Nucleotide Binding site Leucine Rich Repeat
NCBI  National Center for Biotechnology Information
NDR1  NON RACE-SPECIFIC DISEASE RESISTANCE 1
NIM1  NON-INDUCIBLE IMMUNITY1
NINJA  NOVEL INTERACTOR OF JAZ
NPR1  NONEXPRESSER OF PR GENES 1
OTS1 and -2  OVERLY TOLERANT to SALT1 and -2
PAD4  PHYTOALEXIN DEFICIENT 4
PAL1-4  PHENYLALANINE AMMONIA LYASE1-4
PAMP  Pathogen Associated Molecular Pattern
PBL  PBS1-LIKE
PBS1  
AVRPPHB SUSCEPTIBLE 1

PIAL1 and -2  
PROTEIN INHIBITOR of ACTIVATED STAT-LIKE1 and -2

PRR  
Pattern Recognition Receptor

Pst.  
Pseudomonas syringae pv. tomato DC3000

Pst. AvrRPM1  
Pseudomonas syringae pv. tomato DC3000 AvrRPM1

PTI  
PAMP Triggered Immunity

PTM  
Post translation modification

PUB  
PLANT U-BOX

R  
Resistance gene

RAR1  
REQUIRED FOR MLA12 RESISTANCE 1

RBOHD  
RESPIRATORY BURST OXIDASE HOMOLOGUE D

RIN4  
RPM1 INTERACTING protein 4

RLCK  
Receptor-Like Cytosolic Kinase

RLK  
Receptor-Like Kinase

RLP  
Receptor-Like Protein

ROS  
Reactive oxygen species

SA  
Salicylic acid

SAE1  
SUMO-Activating Enzyme 1

SAE2  
SUMO-Activating Enzyme 2

SAG  
SA 2-O-β-D-glucoside

SAG101  
SENESCENCE-ASSOCIATED GENE 101

SAR  
Systemic acquired resistance

SARD1  
SAR DEFICENT 1

SCE1  
SUMO-Conjugating Enzyme 1

SCF  
SKP1–CULLIN–F-box

SGT1  
SUPRESSOR OF THE G2 ALLELE OF SKP1

SIM  
SUMO-interacting motif

SIZ1  
SAP and MIZ1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNI1</td>
<td>SUPPRESSOR of NPR1-1 INDUCIBLE</td>
</tr>
<tr>
<td>SON1</td>
<td>SUPPRESSOR OF NIM1-1</td>
</tr>
<tr>
<td>SRFR1</td>
<td>SUPPRESSOR OF RPS4-RLD1</td>
</tr>
<tr>
<td>SSN2</td>
<td>SUPPRESSOR of SNI1-2</td>
</tr>
<tr>
<td>STUBL</td>
<td>SUMO TARGETED UBIQUITIN LIGASE</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small Ubiquitin-like Modifier</td>
</tr>
<tr>
<td>TAIR</td>
<td>The Arabidopsis Information Resource</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll Interleukin 1 Receptor</td>
</tr>
<tr>
<td>ULP1</td>
<td>UBIQUITIN-LIKE PROTEIN-SPECIFIC PROTEASE1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
1. Introduction

1.1. Preamble

Post-translational modifications (PTMs) provide a rapid method to moderate cellular signalling in response to an organism’s changing circumstances, using the current population of proteins within cells. This is achieved via attachment of chemical groups or small proteins, at specific amino acids (Prabakaran et al., 2012). Enzymatic covalent attachment of the Small Ubiquitin-like Modifier (SUMO) protein to target proteins, SUMOylation, is one such post-translational modification. It is conserved throughout eukaryotes. SUMO conjugation to a protein can alter its interactions or functioning, modulating signalling pathways within the cell. SUMO proteases process SUMO into its mature form as a prerequisite to conjugation, as well as provide reversibility to the SUMOylation pathway by cleaving SUMO from target proteins (Miura et al., 2007a; Park and Yun, 2013).

Hormones are by definition, small signalling molecules and work to disperse signals within and between cells and tissues. Salicylic acid (SA) is a key plant hormone in propagating defense activation and response, against a wide range of plant pathogens (Vlot et al., 2009). A growing body of evidence is indicating a close relationship between SUMOylation, SA and immunity in plants. Here I introduce these concepts in detail, with focus upon the model plant Arabidopsis thaliana, although orthologues of key components and mechanisms are published upon in crop species (Chaikam and Karlson, 2010; Park et al., 2011c; Novatchkova et al., 2012).
1.2. Plant immunity

1.2.1. Summary

During the life of a plant it may encounter any of a diverse range of pathogens, varying in infection strategy and in the set of disease promoting molecules they release. Despite this, most plants are resistant to most infectious organisms and host defenses are rarely breached by the array of pathogens present in the environment (Dickinson, 2003).

Plants possess constitutive passive defenses consisting of natural barriers reinforced with cutin, suberin, pectin, lignin and cellulose, in addition to preformed antimicrobial compounds and inhibitors such as phytoanticipins (Malinovsky et al., 2014; Serrano et al., 2014). The vast majority of pathogens are unable to overcome these basal defenses but those that do gain entry through natural openings (stomata and hydathodes), wounds, or by actively penetrating the plant cell wall, such is seen in fungus appressorium development or aphid stylets (Glazebrook, 2005). Once within the cellular spaces of the plant, the pathogen must then contend with plant innate inducible defenses. Each plant cell is inherently capable of pathogen detection and subsequent signalling to activate downstream defense responses, acting to limit the spread of the pathogen through the host (Spoel and Dong, 2012; Dodds and Rathjen, 2010).

Pathogens are broadly grouped by infection lifestyle. Biotrophs gain nutrition from living tissue, whilst necrotrophs take their nutrients from dead or dying tissue (Glazebrook, 2005). Here I am principally concerned with interactions with biotrophic or hemi-biotrophic pathogens although the underlying principles of recognition, signalling, and response are essentially the same. The \textit{Pseudomonas syringae pv. tomato - Arabidopsis thaliana} pathogen-host interaction is a widely used model for the study of plant immunity and has provided the basis of much of our current understanding (Katagiri et al., 2002).
1.2.2. PAMP Triggered Immunity

Initial perception of a potentially infectious agent of disease, a pathogen, is through the recognition of widely conserved Pathogen Associated Molecular Patterns (PAMPs) such as bacterial flagellin, bacterial elongation factor (EF-Tu) and the fungal cell wall component chitin (Boller and Felix, 2009). Detection is achieved through transmembrane Pattern Recognition Receptors (PRRs) such as FLAGELLIN-SENSING2 (FLS2), EF-Tu RECEPTOR (EFR) and chitin receptors CHITIN ELICITOR RECEPTOR KINASE1 (CERK1) and LYSM-CONTAINING RECEPTOR-LIKE KINASE 5 (LYK5) respectively (Dunning et al., 2007; Zipfel et al., 2006; Kombrink et al., 2011; Cao et al., 2014).

PAMP Recognition Receptors

Pattern recognition receptors are surface localised proteins including Receptor-Like Kinases (RLK) and Receptor-Like Proteins (RLP). Both RLKs and RLPs contain ligand binding extracellular domains (ectodomains) which may contain leucine rich repeats, lysine motifs, lectin motifs or epidermal growth factor (EGF)-like domains (Macho and Zipfel, 2014). PRRs span the membrane with a single pass transmembrane domain while only RLKs possess an intracellular kinase domain to propagate signalling. Presumably RLPs associate with other membrane-associated kinases. CERK1 possesses a lysine motif triplicate containing ectodomain, responsible for binding to chitin oligomers (Miya et al., 2007; Petutschnig et al., 2010). Upon binding chains of chitin LYK5 phosphorylates CERK1 stimulating its homo-dimerisation forming an activated kinase within the cytoplasm (Liu et al., 2012b; Cao et al., 2014). Similarly FLS2 dimerises upon binding to flagellin peptide via its leucine-rich repeats, except in this case FLS2 forms heterodimers with another transmembrane kinase BRASSINOSTEROID INSENSITIVE1 ASSOCIATED KINASE1 (BAK1), required to amplify the phosphorylation mediated signal initiated by FLS2 flagellin binding. EFR, like FLS2,
contains extracellular LRRs and EF-Tu perception and signalling is also dependent on BAK1 (Macho and Zipfel, 2014; Chinchilla et al., 2007; Zipfel et al., 2006).

**DAMP self recognition**

A number of plant pathogens produce hydrolytic enzymes to gain access to, or nutrition from, plant hosts (Abramovitch et al., 2006; Knogge, 1996). Although plant derived, hydrolysed products can serve as recognition ligands to unique PRRs allowing the plant to detect the activity of a pathogen rather than the pathogen itself (Boller and Felix, 2009). They have been termed Damage Associated Molecular Patterns (DAMPs) although still fit under the concept of a molecular pattern which is associated with pathogen attack (i.e. a PAMP). DAMPs include host structural components such as oligogalacturonides and cutin monomers, in addition to peptides such as PROPEP family (cleaved to PEPs) from Arabidopsis and SYSTEMIN from Solanaceae, recognised upon cleavage of precursor proteins (D’Ovidio et al., 2004; Kauss et al., 1999; Huffaker et al., 2006; Bartels et al., 2013; Pearce et al., 1991). To date only a handful of DAMP recognising PRRs have been identified. PEP RECEPTOR1 and -2 (PEPR1 and -2) binding to PEP, is structurally akin to FLS2. SR160, the SYSTEMIN receptor, is an LRR-RLK related to the brassinosteroid hormone receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) (Huffaker and Ryan, 2007; Yamaguchi et al., 2006; Krol et al., 2010; Scheer and Ryan, 2002).

**Signalling**

Downstream signalling after PRR ligand binding is highly regulated, involving a complex network of molecular interactions (Thomma et al., 2001). RLKs associate with Receptor-Like Cytosolic Kinases (RLCK) to direct protein phosphorylation and activate response gene transcription. By far the most studied is FLS2 mediated signalling. Two RLCKs, BOTRYTIS-INDUCED KINASE1 (BIK1),
which constitutively associates with BAK1 and FLS2, and BR-signalling KINASE1 (BSK1), which associates with BAK1, are phosphorylated by BAK1 upon flg22 binding to the FLS2-BAK1 co-receptor (Shi et al., 2013; Lu et al., 2010). This results in RLCK mediated phosphorylation of BAK1 and FLS2 and RLCK disassociation from the receptor (Zhang et al., 2010a). The role of BSK1 has yet to be defined. Recently BIK1 has been shown to phosphorylate and thus activate the reactive oxygen species (ROS) producing NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD)(Kadota et al., 2014; Li et al., 2014) (see section, Response). Additional RLCKs PBS1-LIKE (PBL)-1, -2 and -5 are related to BIK1 and may function partially redundantly in the regulation of flg22 mediated ROS production (Liu et al., 2013; Zhang et al., 2010a). RLCKs interact with a number of RLKs and may provide crosstalk between signalling pathways. BIK1 for example, interacts with CERK1 and EFR suggesting there may be some convergence in defense signalling pathways (Zhang et al., 2010a). BSK1 and BIK1 in addition to the transmembrane kinase BAK1 have all been shown to interact with non-PRR RLKs such as the brassinosteroid receptor BRI1 (Tang et al., 2008; Sun et al., 2013). This may provide antagonism between defense activation and growth regulation involving downstream components such as the transcriptional regulator BRASSINAZOLE-RESISTANT 1 (BZR1) (Lozano-Duran et al., 2013). Being resource limited, plants must moderate responses to pathogen challenge to an appropriate level in order to prevent pathogen colonisation whilst not expending unnecessary energy (Alcazar et al., 2011). Many negative regulators of immune signalling prevent inappropriate response. Phosphatases, such as KINASE-ASSOCIATED PROTEIN PHOSPHATASE (KAPP) and ubiquitin E3 ligases, such as PLANT U-BOX12 and -13 (PUB12 and -13), target FLS2. Presumably KAPP deactivates the phosphorylated FLS2 kinase domain, whilst PUB12 and -13 ubiquitinate the ligand bound receptor causing its subsequent invagination (Lu et al., 2011; Gómez-Gómez et al., 2001). Mechanisms such as these are believed to prevent activated receptors from perpetuating
1.2 Plant immunity

defense signalling beyond the point at which the pathogen threat has been suppressed.

Response

Single or multiple PRRs bound to ligands result in rapid responses such as ion fluxes, Mitogen-Activated Protein Kinase (MAPK) activation cascades and reactive oxygen species generation (ROS). Shortly followed by transcriptional response, stomatal closure and SA accumulation. Some time later, followed by callose deposition mediating cellular reinforcement. These events and others yet to be described result in PAMP Triggered Immunity (PTI), thought to be sufficient to prevent infection by non-adapted pathogens (Jones and Dangl, 2006; Gimenez-Ibanez and Rathjen, 2010; Melotto et al., 2006; Vlot et al., 2009; Tsuda et al., 2008; Mishina and Zeier, 2007).

Suppression

In order to combat this cellular surveillance system, pathogens have adapted and evolved to either, evade host defense perception, or to actively suppress it using ‘effectors’ (or virulence factors). Effectors are molecules injected into the host through the secretion systems of pathogens and include enzymes, hormones and toxins capable of interfering with plant basal defense signalling or downstream responses (Abramovitch et al., 2006). Just a few examples of the many strategies used by pathogens to overcome PTI will be discussed below.

Amino acid sequence polymorphisms and post-translational modifications have been observed within PAMPs between pathogen species and subspecies, resulting in loss of PAMP recognition by the host (Sun et al., 2006). Effectors such as AvrPto, AvrRpt2 and AvrRpm1 are secreted via the type three secretion system of Pseudomonas syringae pv. tomato (Pst.), and compromise defenses such as host callose deposition, resulting in susceptibility (Hauck et al., 2003; Kim
1.2 Plant immunity

et al., 2005a). As discussed, PTI depends largely on a phosphorylation cascade to transmit the signal, these have been targeted by a number of pathogen effectors in order to suppress host defense gene expression and response (He et al., 2006; Orth et al., 1999). AvrPto and AvrPtoB from Pst. do this upstream at the plasma membrane, targeting the PRRs FLS2 and CERK1 respectively (Gimenez-Ibanez et al., 2009; Xiang et al., 2008, 2011). AvrPto blocks FLS2 phosphorylation hence activation, whilst AvrPtoB has E3 ligase activity, ubiquitinating CERK1 and marking it for degradation. AvrAC from Xanthomonas blocks phosphorylation of Arabidopsis BIK1 and RIPK RLCK activation sites, through uridine conjugation (Feng et al., 2013). Another Pst. effector, HopF2, targets Mitogen-Activated Protein Kinase Kinase (MKK) and BAK1, blocking the downstream phosphorylation cascade (Zhou et al., 2014; Wu et al., 2011).

Pst. toxin coronatine (COR) is another well studied suppressor of basal host responses (Bender et al., 1987; Uppalapati et al., 2005). COR mimics the biologically active conjugates of the plant hormone jasmonic acid (JA–isoleucine). COR binds to the JA receptor CORONATINE-INSENSITIVE PROTEIN1 (COI1) leading to the degradation of the JA signal suppressing JASMONATE ZIM-DOMAIN (JAZ) proteins (Zhao et al., 2003; Brooks et al., 2005; Thines et al., 2007; Thilmony et al., 2006). While activation of JA signalling is associated with the activation of responses to wounding and necrotrophic pathogens, it is antagonistic to biotrophic pathogen associated salicylic acid mediated immune signalling (see below, Salicylic acid) (Glazebrook, 2005). Thus, through the secretion of COR virulent Pst. is able to suppress SA mediated defenses and inhibit callose deposition (Geng et al., 2012).

1.2.3. Effector Triggered Immunity

Pathogens which have successfully evolved mechanisms of overcoming host basal PTI, colonise a susceptible plant and establish disease (Lapin and Van den Ackerveken, 2013). To combat these pathogen evolutionary innovations, plants have
evolved a second layer of inducible defense provided by resistance gene (R) products. R proteins recognise specific pathogen effector molecules, or their effects on the host (the genes encoding effectors recognised by the host are known as avirulence genes (Avr)). Originally identified as ‘gene-for-gene’ resistance, host R protein recognition of pathogen effectors leads to rapidly induced Effector Triggered Immunity (ETI) (Jones and Dangl, 2006; Flor, 1971).

**Resistance Proteins**

Resistance proteins can be classified into two common groups based on domain structure. **Nucleotide Binding Site Leucine Rich Repeat (NBS LRRs)** which have N-terminal domains containing either a Coiled Coil (CC) or Toll Interleukin 1 Receptor homology (TIR) such as RPS2, RPM1, RPS5 (CC NB LRRs) and RPS4 (TIR NB LRR) conferring resistance to *Pst* strains containing recognised effectors (Bent et al., 1994; Grant et al., 1995; Simonich and Innes, 1995; Hinsch and Staskawicz, 1996; Muthamilarasan and Prasad, 2013). NB domains are associated with nucleotide binding, commonly ATP or GTP, whilst LRR domains typically act as surfaces for protein interactions. TIR homology can encode protein localisation signals and, like CCs, provide another surface for protein interactions (McHale et al., 2006). The second most common R protein structures are extracellular Leucine Rich Repeats (eLRRs) and include members of RLPs, RLPKs and PolyGalacturonase-Inhibiting Proteins (PGIPs). The tomato R protein Cf9 is an eLRR that provides resistance to *Cladosporium fulvum* expressing the Avr9 effector protein (Shanmugam, 2005; Hammond-Kosack et al., 1998). Structural analyses of NBS LRR proteins led to the proposal of a mechanism for switching between the inactive, and active ligand-bound state (Lukasik and Takken, 2009; Takken and Tameling, 2009; Bernoux et al., 2011; McHale et al., 2006). In an inactive state the NB domain interacts either intramolecularly with the LRR domain or with another host protein, acting to limit NB site nucleotide exchange. Upon LRR-ligand binding, this inhibition is alleviated leading to nu-
cleotide hydrolysis hence phosphorylation and receptor activation. R protein
stability and abundance also appear be to a key regulatory mechanism in R pro-
tein activation and deactivation, with overexpression of some R proteins leading
to auto-activation (Oldroyd and Staskawicz, 1998; Stokes et al., 2002; Mysore
et al., 2003).

Resistance protein ligands

To date numerous resistance (R) genes and corresponding avirulence (Avr) genes
have been found in a diverse set of host and pathogen species. Despite the appar-
ent direct correlation in ETI between host R proteins recognising the presence of
particular pathogen Avirulence factors (effectors) proteins, interaction between
the two has proven surprisingly rare. This led to the proposal of the ‘Guard Hypo-
thesis’ (Van der Biezen and Jones, 1998), whereby a resistance gene product
indirectly recognises the presence of a pathogen by monitoring another presumably
host component which is altered by the activity of the pathogens effector
molecule. Strong support for this model came from the RPM1 INTERACTING
protein 4 (RIN4), which interacts with both RPM1 and RPS2 R proteins in unin-
fected cells (Mackey et al., 2002, 2003). RIN4 is targeted by the bacterial effectors
AvrB and AvrRPM1 which enhance its phosphorylation. RIN4 phosphorylation
results in the activation of RPM1 and subsequent ETI (Liu et al., 2011). Sim-
ilarly the AvrRpt2 effector cleaves RIN4 with cysteine protease activity, leading
to RPS2 activation and resistance (Axtell and Staskawicz, 2003; Mackey et al.,
2003). Significantly RIN4 contains two separable domains, both of which can sup-
press PTI. In the absence of RPM1 and RPS2 RIN4 appears to be targeted by
these Pst. effectors to suppress PTI, indicating RPM1 and RPS2 monitor RIN4-
consistent with the guard hypothesis (Kim et al., 2005a; Afzal et al., 2011). Sim-
ilarly AvrPphB from Pst., which encodes a cysteine protease, is recognised by
RPS5 indirectly through monitoring the integrity of the host RLCK AVRPPHB
SUSCEPTIBLE 1 (PBS1) (Zhang et al., 2010a; Ade et al., 2007).
1.2 Plant immunity

**Downstream signalling**

Although our understanding of R proteins- structure, activation and to some extent the downstream responses they initiate- is improving, the components connecting activation to response have been illusive in genetic screens (Shirasu and Schulze-Lefert, 2003; McHale et al., 2006). Nonetheless a few downstream components have been identified for subsets of R proteins, although these do not appear to be common to all.

REQUIRED FOR MLA12 RESISTANCE 1 (RAR1) zinc binding protein is required for R gene mediated resistance to- fungal powdery mildew in barley, and bacterial and oomycete pathogens in Arabidopsis (Shirasu, 2009; Tornero, 2002; Muskett et al., 2002). RAR1 interactor SUPPRESSOR OF THE G2 ALLELE OF SKP1 (SGT1), interacts with S PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1) and CULLIN homologues of the SCF (SKP1–CULLIN–F-box) complex CULLIN RING E3 Ubiquitin Ligase (CRL) subunits in yeast. Mutation or silencing of SGT1 results in the breakdown of defense responses orchestrated by multiple R genes, including suppression of ROS generation and Hypersensitive Response associated Programmed Cell Death (HR PCD) (Austin et al., 2002; Azevedo et al., 2002). HEAT SHOCK PROTEIN 90 (HSP90) interacts with RAR1 and SGT1. Inhibitor studies indicated it’s homologues are required for RPS2 and RPM1 mediated resistance in Arabidopsis as well as R mediated resistance in other plant species (Takahashi et al., 2003; Hubert et al., 2003; Shirasu, 2009). Rar1 and sgt1b mutants, or silencing of HSP90 and SGT1 indicate these proteins regulate R protein accumulation during effector perception and ETI activation (RAR1, (Bieri et al., 2004; Holt et al., 2005) SGT1, (Holt et al., 2005; Azevedo et al., 2006; Mestre and Baulcombe, 2006; Boter et al., 2007) HSP90, (Lu et al., 2003; Boter et al., 2007)). Direct association of SGT1 and HSP90 with R proteins may provide a mechanism of regulating R protein stability through SCF associated ubiquitination (Hubert et al., 2003; Liu et al., 2004b; Bieri et al., 2004; Leister et al., 2005). Equally, SGT1-HSP90 may chaperone R proteins,
regulating subcellular relocalisation to the nucleus (Noël et al., 2007; Shirasu, 2009). RAR1 is not required for all R mediated pathways, rather acts to enhance HSP90 and SGT1 interaction (Leister et al., 2005; Zhang et al., 2010b).

ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and NON RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) are essential to largely discrete subsets of R protein mediated immune signalling (Aarts et al., 1998). EDS1 and its interactors PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101 (SAG101) possess lipase like homology and an uncharacterised EP domain (Wiermer et al., 2005). All three proteins are involved in TIR NB LRR, but not CC NB LRR R protein triggered immunity, with partial redundancy between SAG101 and PAD4 (Feys et al., 2001; Zhu et al., 2011). EDS1 interacts with R proteins RPS4 and RPS6 and their corresponding avirulent pathogen effectors, AvrRps4 and AvrHopA1 (Heidrich et al., 2011; Bhattacharjee et al., 2011). This may indicate EDS1 is guarded by RPS4 and RPS6. EDS1, PAD4 and SAG101 are involved in redox signalling and pathogen induced SA accumulation. While only EDS1 has an essential role in HR PCD, with \textit{eds1} being fully defective in pathogen induced SA accumulation (Feys et al., 2001; Aarts et al., 1998; Zhu et al., 2011).

NON RACE-SPECIFIC DISEASE RESISTANCE 1 (NDR1) is essential for the activation of defenses by multiple CC NB LRR R proteins (Century et al., 1995). NDR1 interaction with the R protein regulator, RIN4, occurs upon its cleavage by the avirulent \textit{Pst.} effector AvrRpt2, and is essential to RPS2 mediated ETI activation (Day et al., 2006, 2007). In the case of one R protein, RPS5, NDR1 is required for ETI signalling but works independently of RIN4. Activities of NDR1 beyond RIN4 are yet to be defined, although bioinformatic structural analysis of NDR1 indicated similarity to mammalian integrins, suggesting a role in mediating plasma membrane wall adhesions and maintenance of cell integrity (Knepper et al., 2011).

Like PTI, ETI requires careful regulation and it is likely much of the negative
1.2 Plant immunity

regulation is shared, if not at the component level, certainly at the mechanistic level. SUPPRESSOR OF RPS4-RLD1 (SRFR1), is a negative regulator of ETI, with \textit{srfr1} mutants displaying enhanced resistance to pathogens. SRFR1 interacts with EDS1 and SGT1 in the negative regulation of R protein accumulation and auto-activation (Kim et al., 2010; Li et al., 2010a; Bhattacharjee et al., 2011). Similarly the CONSTITUTIVE EXPRESSER OF PR GENES 1 (CPR1) F-box, negatively regulates R protein receptor SNC1 and RPS2 accumulation, presumably through CRL mediated ubiquitination (Cheng et al., 2011; Gou et al., 2012). A plant E4 ubiquitin conjugation factor, which in yeast is involved in ubiquitin chain assembly, also regulates SNC1 protein levels cooperatively with CPR1 (Huang et al., 2014; Koegl et al., 1999).

\textbf{Response}

ETI results in ion fluxes, ROS generation and transcriptional reprogramming, often culminating in a localized HR PCD. HR PCD is believed to isolate the pathogen and starve it from nutrition, but may be the final result of some other unknown lethal response (Abramovitch et al., 2006).

\textbf{Evolutionary Competition}

Pathogen effector recognition versus evasion in the host, is a key determinant of resistance versus susceptibility thus has led to evolutionary pressure at the genomic level of pathogen and host organisms. This evolutionary competition has promoted a great diversity in host R genes and pathogen effector genes over generations (Holub, 2001; Flor, 1971).

R protein mediated pathogen detection results in defense activation- the pathogen is avirulent on the host. This places strong selection pressure for change on the pathogen and it’s effector genes, in order to facilitate evasion or suppression of host responses. If this adaptation occurs the host becomes susceptible-
the pathogen is infectious. This places strong selective pressure for change on
the host and it’s R genes, in order to detect the pathogen once more, host-
pathogen co-evolution is cyclical (Fig. 1.1) (Jones and Dangl, 2006). This is clas-
sically demonstrated by effector allele diversity of ATR13 found in the oomycete
Hyaloperonospora arabidopsisidis which is matched by R gene RPP13 diversity in
Arabidopsis (Allen et al., 2004).

Although amplitude of response is clearly different between ETI and PTI, it
should be noted that these pathways may not be distinct (Abramovitch et al.,
2006; Sun et al., 2006). With ETI and PTI sharing significant molecular overlap,
from the domain structures of the pathogen perceiving receptors, to the mecha-
isms of signalling and downstream response. This is exemplified in comparative
gene expression studies, and highlights likely integration between PTI and ETI
pathways (Navarro et al., 2004; Tao et al., 2003; Thilmony et al., 2006).

![Figure 1.1.: Host-Pathogen evolutionary interface in plant immunity. Immune activation by PRR recognition of pathogen encoded PAMPs, suppressed by pathogen effectors. Host R proteins act to detect the defense suppressing activities of pathogen effectors and trigger ETI. Plant host components in green: PRR= pattern recognition receptor, R= resistance protein. Pathogen components in orange: E= effector. Evolutionary time axis (grey) represents the evolutionary pressure on the pathogen and host to overcome immunity or susceptibility respectively; driving forward the diversification of effectors and resistance proteins.](image-url)
1.2 Plant immunity

1.2.4. Systemic Acquired Resistance

Although PTI and ETI are on a cellular level they trigger signalling throughout the plant providing defense activation and Systemic Acquired Resistance (SAR) throughout the organism. SAR is broad spectrum resistance to pathogens, lasting for up to months in some cases (Kachroo and Robin, 2013). Avirulent and at high levels, virulent, pathogens have been shown to induce SAR. Induced locally, the mobile signal inducing SAR in distant tissues has attracted much research attention (Durrant and Dong, 2004). When applied locally, a number of chemicals are capable of inducing resistance in distal tissues. Perhaps the strongest candidates for the mobile SAR signalers are methyl SA, azelaic acid (AZA), dehydroabietal (DA) and glycerol-3-phosphate (G3P) (Park et al., 2007; Jung et al., 2009; Chaturvedi et al., 2012; Chanda et al., 2011). Two lipid transferases DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) and AZELAIC ACID INDUCED 1 (AZI1) have proven to be cooperative and essential in SAR induction (Yu et al., 2013). Mutants of these transferases are unresponsive to AZA, DA or GP mediated SAR induction. Given the range of pathogens and a logical requirement for differential acquired resistance dependent on the infection strategy of the pathogen, it is likely that multiple mobile signals may be transported upon pathogen challenge.

The mechanisms and components involved in perception of the SAR inducing mobile signal(s) are largely unknown (Kachroo and Robin, 2013). Mutation of enzymes required for cutin or fatty acid biosynthesis lead to defective SAR signal perception but the reasoning behind this has not been deciphered (Xia et al., 2009, 2010, 2012).

SAR induction requires both the phytohormone salicylic acid and the transcriptional regulator NONEXPRESSER OF PR GENES 1 (NPR1), which work together to activate SA mediated defenses (discussed in detail below, see, *Salicylic Acid*).
1.3. SUMOylation

1.3.1. Summary

The Small Ubiquitin-like Modifier, SUMO, takes its name based on similarity to the well studied post-translational modifier ubiquitin and is conserved throughout all kingdoms of eukaryotes (Müller et al., 2001). Despite low amino acid sequence similarity to *Homo sapiens* ubiquitin, hsSUMO1 shares a high degree of secondary and tertiary protein structure with Ubiquitin (Bayer et al., 1998). Akin to ubiquitination, covalent attachment of SUMO results from the sequential activity of three enzymes an E1, E2 and E3, yet unlike ubiquitination, the principal role of SUMOylation is not to target proteins for degradation, but rather moderate protein interactions and activities. In plants, SUMOylation has been implicated in many life processes with a principal role in stress responses (Park and Yun, 2013). Exposure to abiotic stresses such as heat shock and high salt concentrations, leads to accumulation of SUMO conjugates (Castro et al., 2012).

1.3.2. Machinery

In Arabidopsis there are eight SUMO genes (SUMO1-8) (Novatchkova et al., 2004, 2012). The paralogues SUMO1 and -2 are highly expressed and capable of forming poly-SUMO chains. Amongst the other six SUMO isoforms evidence of conjugation for only SUMO3 and -5 has been shown, SUMO3 forming monomers only. Basal expression of the other isoforms (SUMO4, -6, -7 and -8) is very low if present at all (Budhiraja et al., 2009; Colby et al., 2006; Chosed et al., 2006).

Synthesized as an inactive precursor, SUMO proteins are processed to there mature form by cysteine proteases (see section, *SUMO proteases*), which cleave the C-terminal tail from SUMO exposing a diGlycine motif, the site at which SUMO is attached to permissible lysine residues in substrate proteins. The small and large, E1 SUMO-Activating Enzyme subunits, SAE1(a or b) and SAE2, act as
a heterodimer. Utilising ATP, the SAE1-SAE2 dimer adenylates the C-terminal glycine residue of SUMO, leading to thio-ester bond formation between SUMO and the active cysteine residue of SAE2. Transesterification results in SUMO transfer to the cysteine active site of the E2 SUMO-Conjugating Enzyme, SCE1. SCE1 finally catalyses SUMO isopeptide bond formation to target proteins, in conjunction with MIZ-type zinc finger containing E3 SUMO ligases, such as HIGH PLOIDY 2 (HPY2 otherwise know as METHYL METHANESULFONATE- SENSITIVITY protein 21 (MMS21)), or SAP and MIZ1 (SIZ1), in Arabidopsis (Summarised, Fig. 1.2). SUMO conjugation in plants most frequently occurs on proteins containing a somewhat conserved motif, $\psi$-K-V-D/E ($\psi$- hydrophobic residue). Localisations of the conjugation enzymes, SUMO isoforms and of SUMOylated substrates, indicate the vast majority of SUMOylation occurs in the nucleus, although SUMOylated proteins and proteases have been observed in other cellular compartments (Miura et al., 2007a; Saracco et al., 2007; Ishida et al., 2012; Novatchkova et al., 2012; Miura et al., 2005; Huang et al., 2009; Kurepa et al., 2003; Lois et al., 2003; Miller et al., 2010). Unlike Ubiquitination, the E3 ligase may not be necessary. E1, E2 and mature SUMO expressed in Escherichia coli are sufficient to catalyse SUMO conjugation and the E2 conjugating enzyme has been found to bind to SUMOylated targets directly (Castaño-Miquel et al., 2011). Given that siz1 mutants have significantly diminished SUMO conjugation, it is believed that the E3 acts to enhance and amplify SUMO conjugation (Okada et al., 2009; Lee et al., 2006; Gareau and Lima, 2010).

Two additional SUMO ligases were identified by the presence of a MIZ1 zinc finger, PROTEIN INHIBITOR of ACTIVATED STAT-LIKE1 and -2 (PIAL1 and -2). PIAL1 and -2 have been shown to possess E4 SUMO chain extending activity in vitro (Novatchkova et al., 2012; Tomanov et al., 2014).
1.3 SUMOylation

**Figure 1.2.: Overview of (de)SUMOylation cycling.** SUMO isoforms are synthesised as inactive precursors which require processing by SUMO proteases. The sequential activities of SUMO, activating (E1), conjugating (E2) and ligase (E3) enzymes, covalently attaches SUMO to protein substrates. SUMO protease activity can also counter this pathway by cleaving SUMO from bound substrates.

### 1.3.3. SUMO proteases

In addition to their SUMO processing activities, SUMO proteases also possess deconjugative activity, capable of cleaving SUMO from conjugated proteins or chains- providing reversibility and buffering to the pathway (Mukhopadhyay and Dasso, 2007; Hickey et al., 2012) (Fig. 1.2).

The first SUMO protease to be described was the *Saccharomyces cerevisiae* UBIQUITIN-LIKE PROTEIN-SPECIFIC PROTEASE1 (ULP1), a member the Cysteine-48 group of proteases (Li and Hochstrasser, 1999; Mukhopadhyay and Dasso, 2007). Four SUMO proteases related to ULP1 have been characterised in Arabidopsis, OVERLY TOLERANT to SALT1 and -2 (OTS1 and -2/ Ulp1d and c), EARLY IN SHORT DAYS4 (ESD4) and ESD4-LIKE1 (ELS1/ ulpa) (Murtas et al., 2003; Hermkes et al., 2010; Conti et al., 2008) (Fig. 1.3). Based on sequence homology to ULP1, further cysteine SUMO proteases are present in the
Arabidopsis genome but remain to be characterised (Kurepa et al., 2003; Colby et al., 2006; Novatchkova et al., 2012). Work in other organisms has identified SUMO proteases outside the ULP1-like cysteine-48 proteases. A yeast metallopeptase WEAK SUPPRESSOR of SMT3 PROTEIN1 (WSS1), and, a human Permutated Papain fold Peptidases of Ds-RNA viruses and Eukaryotes (PPPDE) class protease DESUMOYLATING ISOPEPTIDASE1 (DeS1), have been shown to cleave conjugated SUMO (Mullen et al., 2010; Shin et al., 2012). Given the presence of Arabidopsis orthologues, this indicates a wider diversity in SUMO protease structure than previously thought (Novatchkova et al., 2012).

Figure 1.3.: Alignment of the C-terminal domains of SUMO proteases. Sequences from Arabidopsis thaliana, Saccharomyces cerevisiae and Homo sapiens were aligned using EMBL-EBI Clustal Omega. Conserved catalytic residues are highlighted on a black background, other conserved residues by* and conservative substitutions by: (Sievers et al., 2011; McWilliam et al., 2013).

Unlike the SUMO activating and conjugating enzymes (E1 and E2), SUMO pro-
teases possess isoform specificity, in both their SUMO processing and SUMO deconjugation activities (Colby et al., 2006; Chosed et al., 2006). In vitro cleavage assays with recombinant SUMOs show specificity between the characterised SUMO proteases in Arabidopsis (Chosed et al., 2006). OTS1, OTS2, ESD4 and ELS1 were all able to process SUMO1 and -2. All four proteases deconjugated SUMO1 and SUMO2 from a substrate protein with the exception of OTS1, which deconjugated SUMO1 but not SUMO2. Only ELS1 showed some capacity to process but not deconjugate SUMO3 while non of the proteases appeared to act upon SUMO5 (summarised in Table 1.1). Interestingly the activity of ESD4 and ELS1 required an intact N-terminus while for OTS1 and OTS2 just the C-terminal catalytic domain was sufficient. Protein structure studies in yeast and mammals further indicate that SUMO processing is isoform specific between proteases, yet it has been reported that SUMO deconjugation was less selective (Mossessova and Lima, 2000; Reverter and Lima, 2006; Shen et al., 2006a). Despite this, capacity for substrate specificity has been observed (Lima and Reverter, 2008; Shen et al., 2006b). Localisation of SUMOylation enzymes and substrates offers another level of regulation over SUMO substrate targeting. Differential localisations between ESD4, ELS1, OTS1 and OTS2 have been reported (summarised in Table 1.1) (Murtas et al., 2003; Conti et al., 2008; Hermkes et al., 2010). *Saccharomyces cerevisiae* ULP1 is sequestered during alcohol stress conditions leading to the accumulation of SUMO conjugates (Sydorskyy et al., 2010). Similarly the ULP1-like human SENTRIN (SUMO)-SPECIFIC PROTEASEs (SENPs) are shuttled between the nucleus and cytoplasm in the regulation of their deSUMOylation activities (Kim et al., 2005b; Goeres et al., 2011). These findings illustrate the dynamic localisation of SUMO proteases warrants further investigation in plants.

### 1.3.4. Role of SUMO

In plants SUMO conjugation has been shown to be inducible by abiotic stresses including heat, cold, high salt, drought, oxidative and ethanol (Kurepa et al.,
Table 1.1.: SUMO protease activities characterised in Arabidopsis thaliana. In vitro SUMO isoform processing and deconjugating specificities, and in vivo cellular localisations observed, for the four SUMO proteases characterised in Arabidopsis EARLY IN SHORT DAYS4, ESD4-LIKE1 and OVERLY TOLERANT to SALT1 and -2.
1.3 SUMOylation

2003; Conti et al., 2008; Miura and Ohta, 2010; Catala et al., 2007). Through the phenotypic characterisation of SUMO machinery mutants and the cataloguing of SUMOylated substrates by mass spectrometry SUMOylation has been implicated in a wide variety of processes beyond abiotic stress response, such as growth & development and metabolism (Miller et al., 2010; Miller and Vierstra, 2011; Miura et al., 2009; van den Burg et al., 2010; Miura et al., 2011a; Xu and Yang, 2013). In some cases the regulatory role of SUMO within plant signalling pathways is becoming defined.

**Cold tolerance**

Chilling Arabidopsis induces SUMO conjugate accumulation (Miura et al., 2007b). Characterisation of the *siz1* E3 ligase mutant led to the discovery that SIZ1 mediates SUMOylation of INDUCER OF CBF EXPRESSION1 (ICE1) in the activation of cold responsive gene expression. Mutation of the SUMOylated lysine of ICE1 led to chilling sensitivity and reduced cold responsive gene expression. Further it was shown that this is due to salicylic acid accumulation (Miura et al., 2007b; Miura and Ohta, 2010).

**Heat stress**

Exposing Arabidopsis to heat shock, leads to SUMO conjugate accumulation (Kurepa et al., 2003). Both Heat shock proteins (HSPs)- the molecular chaperones and protein protectants during heat stress, and heat shock factors (HSFs)- the heat responsive transcription regulators, have been identified as SUMO substrates (Miller and Vierstra, 2011; Elrouby and Coupland, 2010). HSFA2 activity, required for maintenance of HSP gene expression inducibility after repeated heat shock treatments, is repressed by SUMO1 conjugation (Cohen-Peer et al., 2010; Charng et al., 2007). In vitro the Arabidopsis E1 and E2 enzyme activities increase with temperature, up to an optimum of 42°C (Castaño-Miquel et al., 2011,
1.3 SUMOylation

Given the apparent significance of SUMOylation in heat stress signalling, the molecular implications require further investigation.

**Nutrition**

SUMOylation provides regulation in nutrient metabolism and response to nutrient availability pathways in plants.

1 **Phosphate** The $siz1$ mutant is sensitive to phosphate deficiency (Miura et al., 2005). The phosphate starvation responsive transcription factor PHOSPHATE STARVATION RESPONSE1 (PHR1) is SUMOylated, and two starvation responsive genes that PHR1 regulates are down-regulated in the $siz1$ mutant. This suggests SUMOylation plays a positive role in the phosphate starvation response. Mutants of the transcription factor LOW PHOSPHATE ROOT2 (LPR2) are unresponsive to phosphate starvation. LPR2 is also SUMOylated although crosses with $siz1$ suggest its attenuation of the phosphate starvation response is independent of SIZ1 (Wang et al., 2010c; Miller et al., 2010).

2 **Nitrogen** NITRATE REDUCTASE1 and -2 (NIA1 and -2) help catalyse the breakdown of nitrogen to ammonium for protein synthesis. SIZ1 mediated SUMOylation of NIA1 and -2 positively regulates their activities, with $siz1$ mutants displaying nitrogen deficiency (Park et al., 2011a). Supplementing $siz1$ mutants with ammonium recovers their dwarf phenotype of $siz1$.

3 **Copper** SUMOylation is induced by excess of copper ions, presumably by ROS production (Chen et al., 2011). The $siz1$ mutant displays perturbed copper distribution and metal transporter gene expression. Metal transporters are normally down-regulated under excess copper, yet are up-regulated in the $siz1$ mutant when subjected to this stress. The molecular role of SUMOylation requires further investigation.
1.3 SUMOylation

Flowering time

Mutants of the SUMO proteases, *ots1 and ots2, esd4* or, of the SUMO E3 ligase *siz1*, all result in early flowering (Jin et al., 2007; Murtas et al., 2003; Conti et al., 2008). The *siz1* mutant displays reduced expression of the floral repressor *FLOWERING LOCUS C* (*FLC*). SIZ1 facilitates SUMOylation of FLOWERING LOCUS D, in the promotion FLC gene expression hence flowering repression. Mutation of the lysines to which SUMO is conjugated, leads to reduced FLC expression and reduced acetylation of histones at FLC4 chromatin (Jin et al., 2007). Interestingly, SUMOylation and acetylation have been proposed to work antagonistically upon histones in gene repression and activation (See section, *SUMO PTMs*) (Nathan et al., 2003). FLC is also a substrate for SUMO conjugation and results in suppression of flowering (Son et al., 2014). Surprisingly FLC interaction with the SIZ1 negatively regulates its SUMOylation, rather SIZ1 interaction appears to stabilise FLC (Son et al., 2014). Taken together, findings demonstrate the requirement of SUMOylation and SIZ1 in floral repression.

Abscisic acid (ABA) signalling

Increased levels of SUMOylation caused by overexpression of SUMO1 attenuates growth inhibition under ABA treatment (Lois et al., 2003). *siz1* mutants are hyper-responsive to ABA, in terms of root growth inhibition, and induction of ABA responsive gene expression. Mutants of the transcription factors *aba-insensitive5* (*abi5*) are unresponsive to ABA, while *myb30* mutants are hypersensitive to ABA (Finkelstein and Lynch, 2000; Zheng et al., 2012). Both ABI5 and MYB30 were found to be SUMOylated via SIZ1 E3 ligase activity (Miura et al., 2009; Zheng et al., 2012). Crossing *siz1* mutants with *abi5* and *myb30* mutants had different effects. *siz1 abi5* double mutants suppressed the ABA hyper-responsiveness of the *siz1* mutants, whereas *siz1 myb30* double mutants where additive in there responsiveness to ABA. While both ABI5 and MYB30
are SUMOylated their opposing roles may be down to a fine balance in SUMOylation levels mediated by SIZ1 (Zheng et al., 2012). It is also possible, like in FLC regulation, that SIZ1 is regulating one of these components through direct interaction rather than having a singular role in the promotion of SUMO conjugation to them both (Son et al., 2014).

**Brassinosteroid (BR) signalling**

CESTA is a transcription factor involved in the activation of BR synthesis and responsive genes (Poppenberger et al., 2011). CESTA BR-induced relocalisation is regulated by SUMO conjugation (Khan et al., 2014). Mutation of SUMOylated residues prevented CESTA relocalisation into nuclear bodies in response to BR treatment, after pretreatment with a BR synthesis inhibitor. Interestingly CESTA SUMOylation was shown to be antagonised by phosphorylation of two residues within close proximity to the SUMOylated residue (Khan et al., 2014). These studies highlight the control that SUMOylation can provide to signalling pathways in plants. This, considered with the number of stresses that induce SUMO conjugation, provides a mechanism of remodeling cellular signalling in stress conditions.

**1.3.5. Mechanisms of influence**

Once covalently bound SUMO can alter a conjugated protein’s stability and functionality. SUMO may facilitate new protein-protein interactions through SUMO-interacting motifs (SIMs) or interface with other posttranslational modifications such as ubiquitination, and acetylation (Kerscher, 2007; Müller et al., 2001).

**SUMO-interacting motifs (SIMs)**

SIMs are made up of a hydrophobic core followed by a stretch of indeterminate amino acids, usually accompanied by a cluster of acidic amino acids (Hecker et al.,
These structural features facilitate hydrophobic binding into a groove of SUMO, creating a non-covalent interaction, which, in the case of hsSUMO1 and hsSUMO2- SIM interactions, appears to possess a far greater affinity than that seen with ubiquitin to ubiquitin binding proteins (Hecker et al., 2006; Kerscher, 2007). Given the presence of single or multiple SIM(s) on proteins, SUMO conjugation introduces a new surface for interactions to substrates. Significantly, the residues identified to be critical for SIM interactions in human SUMOs, are conserved in AtSUMO1 and AtSUMO2 but not AtSUMO3 and AtSUMO5 (Castaño-Miquel et al., 2011). Despite this, a recent yeast two hybrid screen identified two SIM containing proteins from an Arabidopsis cDNA library which exclusively interacted with SUMO3 (Elrouby et al., 2013). The authors identified a further twelve proteins which interacted with SUMO1 and SUMO2 but not SUMO3, indicating discrete differences in SIM structure facilitate SUMO isoform selectivity. The SUMO binding proteins included the SUMOylation enzymes, orthologues of SUMO TARGETED UBIQUITIN LIGASEs (STUBLs) and enzymes involved in methylation. The limited number of interactors discovered may be due to the experimental approach. GAL4 and DNA binding domain fusions may impede SUMO-SIM and reporter interactions, or, given the reported use of full-length mature SUMOs, covalent conjugation to substrates may occur by endogenous yeast, or library encoded SUMOylation enzymes (Elrouby et al., 2013; Murtas et al., 2003). Another approach used recombinant SUMO1 bound to a column to identify SUMO interacting proteins from total plant protein extracts, by mass spectrometry (Park et al., 2013). Fifteen candidate proteins were then confirmed by yeast two hybrid and transiently in planta, using split luciferase assays. Considering over five hundred SUMO substrates have been identified in Arabidopsis (Elrouby and Coupland, 2010; López-Torrejón et al., 2013; Miller et al., 2013, 2010; Miller and Vierstra, 2011; Budhiraja et al., 2009; Park et al., 2011b), these numbers of SIM containing proteins are surprisingly low. As both studies identified a discrete set of proteins, this likely indicates the technical difficulties of
identifying low affinity, non-covalent, SUMO interactions.

Recently the function of a plant SIM was illustrated in gibberellic acid (GA) hormone signalling. The growth suppressing DELLA proteins GAI and RGA were shown to be SUMOylated (Conti et al., 2014). DELLA SUMOylation was proposed to facilitate the sequestration of the GID1 receptor through a SIM, pulling it away from its role as a cofactor in the SLY1 E3 ligase. Subsequent accumulation of DELLA proteins results in SUMOylation-induced growth inhibition (Conti et al., 2014).

**SUMO and other PTMs**

In other eukaryotes, SUMOylation has been shown to interface with the majority of known PTMs, including: ubiquitination, acetylation, methylation and phosphorylation (Perry et al., 2008; Bossis and Melchior, 2006; Nathan et al., 2003; Gareau and Lima, 2010). SUMO does so either directly, competing or cooperating at residues where other PTMs occur, or, through moderating protein-protein interactions of the enzymes involved in PTM pathways. Although many of these activities have yet to be observed in plants, SUMOylation and other PTMs are highly conserved across Kingdoms, hence it is highly likely these do occur in plants also. I have therefore included a brief overview of the activities shown in plants and other eukaryotes to date.

**Phosphorylation**

In mammalian cell lines phosphorylation at sites adjacent to SUMOylation motifs have been shown to increase SUMO conjugation, through enhanced support for the E2 SUMO activating enzyme (Gareau and Lima, 2010; Hietakangas et al., 2003; Mohideen et al., 2009). Phosphorylation dependent SUMOylation has been shown to switch transcription factors from activators to repressors (Yamashita et al., 2004). Conversely in some instances phosphorylation can inhibit SUMO conjugation (Gareau and Lima, 2010). Such as in the case of the Brassi-
nosteroid responsive CESTA transcription factor discussed above (Khan et al., 2014) (see, *Brassinosteroid (BR) signalling*). A large number of proteins which are SUMOylated in plants have been previously shown to be phosphorylated, including, ABI5, ICE1, FLC and DELLA (Liu and Stone, 2013; Miura et al., 2009, 2011b; Robertson et al., 2008; Fu et al., 2004). As discussed, ABA signalling is regulated by SUMOylation, and interestingly, ABA is also implicated in the inhibition of phosphatases (Park et al., 2009) (see above, *Abscisic acid (ABA) signalling*). Phosphate groups have been shown to provide a negative charge at sites juxtaposing hydrophobic cores in *hsPIAS1*, creating a phospho-conditional SIM (Stehmeier and Muller, 2009). Phosphorylation of SUMO itself has been shown in multiple organisms and is likely to occur in plants, although its significance is not yet understood (Matic et al., 2008; Skilton et al., 2009; Watts, 2013).

**Ubiquitination**

SUMOylation has been shown to block ubiquitination and degradation of proteins by competing for lysine residues targeted by ubiquitin (Kim et al., 2008b; Desterro et al., 1998). Conversely, ubiquitin E3 ligases from yeast and animals have been found to contain SIMs targeting them to polySUMOylated substrates, which they then ubiquitinate, marking them for proteasomal degradation (Sriramachandran and Dohmen, 2014; Perry et al., 2008). Six SUMO TARGETED UBIQUITIN LIGASEs (STUBLs) have been identified in Arabidopsis, their cellular targets in plants pose an interesting line of research (Elrouby et al., 2013). Alternatively Ubiquitin has been found to conjugate directly to SUMO to form hybrid chains, moderating substrate activities (Tatham et al., 2011, 2013; Praefcke et al., 2011).

**Acetylation**

As with ubiquitin, acetylation has been shown to compete for the same lysines as SUMO. The observation that this occurs at histones has led to the proposal of a SUMO-Acetyl switch, whereby acetylation activates gene transcription, and SUMOylation represses it (Nathan et al., 2003, 2006; Shalizi et al., 2006). Al-
though given the observation in human cells, that chromatin SUMOylation co-incides with gene expression of key enzymes in protein synthesis, this does not appear to be true in all cases (Rosonina et al., 2010; Liu et al., 2012a). Acetylation of hsSUMO itself has recently been shown to block SUMO-SIM interactions (Ullmann et al., 2012). Deacetylases in animals and plants are substrates of SUMO. SUMOylation of FLD reduced acetylation of histone 4 and this may be due to recruitment of histone deacetylases through SUMO-SIM mediated interactions (Wilkinson and Henley, 2010; Jin et al., 2007).

Methylation

Methylation may also occur upon the same lysines as SUMOylation (yet to be shown), ubiquitination and acetylation, but appears to be a more stable modification (Watts, 2013). Like Ubiquitin, SUMO, and acetyl modifications, methylation acts upon histones in the stable activation or repression of gene expression (Nathan et al., 2003; Zhang and Reinberg, 2001).

1.4. Salicylic acid signalling

1.4.1. Summary

The plant cell is constantly perceiving changes in it’s surroundings, from changes in temperature or light, to interactions with other organisms. Processing this information is essential in mounting appropriate and timely responses. Hormone signalling provides a mechanism whereby plants are able to interpret and integrate a vast range of stimuli and activate specific responses, facilitating coordinated regulation in all aspects of plant life. Salicylic acid (SA) is a plant hormone with a central role in mounting effective defenses during pathogen challenge, locally and systemically, in addition to being implicated in the regulation of growth and development (Vlot et al., 2009).
1.4 Salicylic acid signalling

1.4.2. Biosynthesis

Plants produce SA from chorismic acid through two biosynthetic pathways. One catalyzed by the PHENYLALANINE AMMONIA LYASEs (AtPAL1-4), and the other, catalyzed by the ISOCHORISMATE SYNTHASEs (AtICS1 and 2) (Dempsey et al., 2011). In Arabidopsis the PAL catalysed pathway converts phenylalanine into cinnamic acid via the activity of four, partially redundant, PALs (PAL1-4). Oxidative or hydroxylative steps then convert cinnamic acid into either benozic acid or ortho-coumaric acid intermediates, and then finally produce SA (Huang et al., 2010). The ICS catalysed pathway is present in prokaryotes, in Arabidopsis it depends on the activity of ICS1/2 for isochorismic acid production (Dempsey et al., 2011). In bacteria subsequent isochorismate pyruvate lyase activity is required to produce SA but these enzymes, at least on the sequence level, are not conserved in the Arabidopsis genome. The path from isochorismic acid to SA production still remains to be fully defined (Dempsey et al., 2011; Chen et al., 2009b).

1.4.3. Induction

SA has been shown to be a key signal in systemic acquired resistance (Durrant and Dong, 2004). SA accumulation is induced by pathogen challenge (Métraux et al., 1990; Malamy et al., 1990; Yalpani et al., 1991a). This is exemplified in Arabidopsis ics1 mutants (also known as salicylic acid induction-deficient2 mutant, sid2), which are defective in pathogen induced SA production and defense signalling (Nawrath and Métraux, 1999; Wildermuth et al., 2001). ICS1 is required for 90-95% of SA produced during avirulent Pseudomonas syringae challenge. Wildermuth et al. (2001) showed that ICS1 expression is induced by pathogen challenge and that its expression coincides with SA accumulation. To date three proteins have been shown to bind to the promoter of ICS1 and to enhance its expression: CAMODULIN-BINDING PROTEIN60-LIKE G (CBP60G), SAR DE-
1.4 Salicylic acid signalling

FICIENT1 (SARD1) and WRKY28 (van Verk et al., 2011; Zhang et al., 2010d). A regulator of ethylene signalling, ETHYLENE INSENSITIVE3 (EIN3), has been shown to bind ICS1 promoter, and along with ETHYLENE INSENSITIVE3-LIKE1 (EIL1), is involved in the restriction of ICS1 gene expression (Chen et al., 2009a). These findings provide insight into the mechanism behind ICS1-mediated SA production, although need to be brought into context during pathogen infection.

Although ICS1 is responsible for the majority of *Pst.* induced SA production, induction of PAL gene expression has been reported in response to bacterial extracts and at the site of biotrophic pathogen entry into host tissues (Edwards et al., 1985; Davis and Ausubel, 1989; Mauch-Mani and Slusarenko, 1996). Screening for mutants with heightened sensitivity to a putative SA precursor downstream of PAL activity- benzoic acid- led to the isolation of *benzoic acid hypersensitive1-Dominant* (*bah1-D*) (Yaeno and Iba, 2008). BAH1-D encodes a RING E3 ligase previously implicated in nitrogen limitation adaptation (Peng et al., 2007). *Bah1-D* mutants accumulated SA under benzoic acid treatment and *Pseudomonas* inoculation. *Bah1-D* mutants crossed with the *ics1* mutant (*sid2*) reduced SA accumulation, although the double mutant still accumulated relatively more SA than the single *bah1-D* mutant in response to pathogen challenge. This suggests potential crosstalk between the ICS and PAL mediated pathways (Yaeno and Iba, 2008).

### 1.4.4. Immune signalling

Three groups independently identified mutations in the same gene which compromised disease resistance and SAR (*Nonexpresser of PR genes1* (*npr1*)/ *Non-inducible immunity1* (*nim1*)/ *Salicylic acid–insensitive* (*sai*)) (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). This gene has become widely known as *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) and encodes the central signalling molecule in the SA pathway (Dong, 2004). Works
stemming from the genetic and proteomic interactions of NPR1 have led to a framework of the molecular mechanism behind SA-mediated defense signalling.

Discovery of SA binding capacity in NPR1, and of its paralogues NPR3 and NPR4, has led to the belief that, collectively, they are the canonical SA receptors (Attaran and He, 2012; Zhang et al., 2006b; Liu et al., 2004a). NPR1, through interaction with members of the TGA family of bZIP transcription factors, co-activates SA mediated defense gene transcription (Dong, 2004; Cao et al., 1997, 1994; Kesarwani et al., 2007). NPR3 and NPR4 participate as subunits in Cullin RING ubiquitin E3 Ligase-mediated ubiquitination. Differential affinity of NPR3 and NPR4 for SA and opposing mediation of SA binding upon their interaction with NPR1 have provided a molecular mechanism for sensing SA levels in the cell, in order to activate defense responses appropriately (Kawano and Bouteau, 2013; Fu et al., 2012).

Upon recognition of a pathogen, biosynthesis of SA is induced (Yalpani et al., 1991b). As SA levels rise, the disassociation of oligomerised NPR1 proteins sequestered in the cytoplasm is promoted by a thioredoxin catalysed redox change, resulting in NPR1 transloca-
1.4 Salicylic acid signalling

tion into the nucleus (Tada et al., 2008). Upon SA binding NPR1 intramolecular auto-inhibition is released, exposing its transactivation domain (Wu et al., 2012). NPR4 with its high affinity for SA, preferentially binds SA ahead of NPR3. NPR4-SA binding inhibits NPR4 interaction with NPR1, thus blocking ubiquitination of NPR1 (Fu et al., 2012). Once stabilised, NPR1 is able to interact with members of the TGA family of bZIP transcription factors. The TGA proteins are required for promoter binding and subsequent co-activation of \textit{PATHOGENESIS-RELATED1} (\textit{PR1}) and multiple other defense related genes (Johnson et al., 2003). Within cells in close proximity to the recognised pathogen, SA levels continue to increase up to, and beyond, the capacity of NPR4 binding. Unbound SA now becomes available to NPR3 despite its lower affinity. Conversely to NPR4-SA binding, this stabilises NPR3 interaction with NPR1 and through its adapter function, brings together CUL3 mediated ubiquitination of NPR1, marking it for proteasomal degradation (Fu et al., 2012). Being an activator of defense NPR1 promotes cell survival and its degradation is thought to allow progression to programmed cell death, isolating the invading pathogen from living tissue (Spoel et al., 2009; Zhang et al., 2010c). Further away from the site of infection, cellular SA levels remain lower and once the distance from the site of infection is sufficient, SA levels in the cell are lower than the capacity of NPR4 proteins to bind it. NPR4-SA binding prevents the formation of SA bound NPR3 and negates NPR1 interaction and ubiquitination. Stabilised, NPR1 mediates the activation of defenses thus protecting the tissues surrounding the area of infection (Kaltdorf and Naseem, 2013; Attaran and He, 2012; Moreau et al., 2012; Mukhtar et al., 2009).

1.4.5. Other components

\textit{Eds1} and \textit{pad4} mutants are defective in pathogen induced SA accumulation, implicating EDS1 and PAD4 in the regulation of SA biosynthesis, as well as R protein mediated signalling (Falk et al., 1999; Jirage et al., 1999) (discussed
previously see Section, 1.2.3, Downstream signalling). Two mutants with constitutively activated defense phenotypes, suppressor of SA insensitive2 (ssi2/fatty acid biosynthesis2 (fab2)) and the double syntaxin of plants -121, -122 (syp121 and -122) mutant, require further mutation of EDS1 and ICS1 before a significant reduction in defense activation is observed (Zhang et al., 2008; Venugopal et al., 2009). These results suggest EDS1 is partially redundant with ICS1 activity in activation of responses such as R gene expression and ETI. Enhanced disease susceptibility5 (eds5) is also defective in SA accumulation in response to Pst. ESD5 encodes a member of the multi-drug and toxin extrusion (MATE) family, which appears to play a role in exporting SA out from the chloroplast (Nawrath and Métraux, 1999; Nawrath et al., 2002; Serrano et al., 2013).

A number of genetic screens were performed looking for mutants capable of recovering the susceptibility caused by the npr1 mutation (sai/npr1/nim1). Suppressor of npr1-1 constitutive mutant (snc1) encodes a single amino acid substitution in-between the NB and LRR domains of an R protein, leading to autoimmunity and elevated SA levels (Zhang et al., 2003a). Mutations in PAD4 fully recovered the snc1 phenotype, while mutation of EDS5 led to partial recovery. These results indicate SNC1 signalling is fully dependent on PAD4 and partially on SA. A further screen for suppressors of the enhanced resistance of the snc1 npr1-1 double has identified thirteen MODIFIER OF SNC1 (MOS) components implicated in this immune pathway (Johnson et al., 2012). All of the MOS mutations reduced the levels of SA in the snc1 npr1-1 double mutant and were comparatively more susceptible to virulent pathogens (Li et al., 2010b; Zhang et al., 2005; Zhang and Li, 2005; Palma et al., 2007; Goritschnig et al., 2007; Palma et al., 2005; Cheng et al., 2009; Goritschnig et al., 2008; Xia et al., 2013; Zhu et al., 2010; Germain et al., 2010; Xu et al., 2012, 2011). The MOS proteins included multiple components involved in RNA processing, protein trafficking and posttranslational modification (specifically- methylation, ubiquitination and farnesylation). An snc1 enhancer screen has just begun to identify negative regulators of this
immune pathway, 'mutant, snc1-enhancing' (muse). MUSE proteins identified include a mitochondrial membrane protein implicated in protein import into the mitochondria, and an E4 Ubiquitin conjugation factor (Huang et al., 2013, 2014) (see section, 1.2.3. Downstream signalling).

Arabidopsis suppressor of nim1-1 (son1) was identified from a mutant screen for silencers of NPR1 mutant non-inducible immunity1 (nim1). SON1 is an F-box protein although it’s targets have not been investigated (Kim and Delaney, 2002).

Suppressor of npr1-1 inducible (sni1) mutants also recover the defense deficient phenotype of npr1 mutants but only upon induction by a SA functional analogue (Li et al., 1999). SNI1 regulates the binding of proteins to the promoter of PR1 (Pape et al., 2010; Wang et al., 2010b; Kim et al., 2012). Suppressor screens of sni1 revealed mutations in RAD51D, BREAST CANCER 2 (BRCA2) and SUPPRESSOR of SNI1-2 (SSN2) could suppress the immune responsiveness of the npr1 sni1 double mutant (Durrant et al., 2007; Wang et al., 2010b). RAD51 interacts with BRCA2 and SSN2 and orthologues of these proteins are involved in homologous recombination and double strand DNA break repair. SSN2, BRCA2 and RAD51 are all recruited to the promoter of PR1 during SA stimulated immune activation (Wang et al., 2010b; Song et al., 2011). SNI1 and SSN2 interact, although there interaction is reduced in the presence of SA. SNI1 and SSN2 have opposing binding activities at the PR1 promoter in the presence or absence of SA (Song et al., 2011). This led to the hypothesis that SNI1 binding to the chromatin holds it in a non-permissible conformation repressing transcription. Upon SA induction, NPR1 is thought to facilitate SSN2 displacement of SNI1 allowing positive regulators such as NPR1-TGA co-factors, to promote gene expression of PR1(Song et al., 2011).

In addition to heightened levels of homologous recombination, the sni1 mutants were recently shown to possess a constitutive DNA damage phenotype (Yan et al., 2013). This led to the surprising discovery that exogenous application of SA results in visible damage to the DNA of plant nuclei. SNI1 was shown to interact
with Structural Maintenance of Chromosome proteins (SMC5 and SMC6B) in planta; components of a highly conserved family of complexes essential in the maintenance of chromosome architecture and stability (Hirano, 2006). Collectively these findings have demonstrated the central role of chromatin remodelling in the regulation of SA induced immunity.

1.4.6. Beyond immunity

Given that there is significant cross regulation between SA with other hormones such as GA and ABA, it is no surprise that SA plays a significant role in regulating plant growth and development (Wang and Irving, 2011; Grant and Jones, 2009). Numerous SA biosynthesis and signalling mutants have altered growth phenotypes (Rivas-San Vicente and Plasencia, 2011). Similar to it’s role in immunity, the concentration of SA appears to define the growth related responses it induces. For example, high concentrations of SA inhibited seed germination whilst low concentrations of SA promoted germination under abiotic stress (Rajjou et al., 2006; Alonso-Ramirez et al., 2009). SA acts as a regulatory signal from seed germination through to flowering and senescence, with roles in metabolism, photosynthesis and respiration (Rivas-San Vicente and Plasencia, 2011). Salicylic acid also plays a role in abiotic stress responses, including response to heat or chilling, osmotic stress, drought and high light (Horváth et al., 2007; Scott et al., 1999). Despite these wide implications the molecular mechanisms behind SA’s role in growth and development largely remain to be investigated.

1.5. SUMO in immunity and salicylic acid signalling

1.5.1. Summary

While SUMOylation has been implicated in many aspects of plant life, the role it plays in signalling pathways is only just being established. Mutation or overex-
pression of endogenous components of the SUMO pathway have indicated a role in salicylic acid biosynthesis and plant immunity (Lee et al., 2006; Kim, 2009; van den Burg et al., 2010; Villajuana-Bonequi et al., 2014). Here I discuss this connection and our progress in understanding the relationship at the molecular level.

1.5.2. Perturbations in the Arabidopsis SUMO system result in SA accumulation

*Siz1*, SUMO E3 ligase mutants produce higher levels of salicylic acid and constitutively activate pathogen defense responses, such as elevated PR1 expression, callose deposition and cell death (Lee et al., 2006; Kim, 2009). *Siz1* autoimmunity was alleviated by the introduction of the bacterially derived salicylate hydroxylase (NahG) gene, encoding an enzyme which hydrolyses SA. Crosses with *npr1* and *ndr1* mutants did not recover the autoimmune phenotype but *pad4* did (Lee et al., 2006). Although like *siz1* mutants, *hpy2/mms21* SUMO E3 ligase mutants are dwarf in stature, they do not appear to possess the misregulated accumulation of SA; expression of *nahG* or mutation of ICS1 did not recover the *hyp2* mutant phenotype at the morphological level (Ishida et al., 2012).

EARLY IN SHORT DAYS4 SUMO protease mutants (*esd4*), like *siz1*, are dwarf and flower early under short day lighting conditions (Murtas et al., 2003). Despite original reports that mutation of SA biosynthesis gene ICS1 (*sid2*) did not recover this phenotype, a recent mutant screen in the *esd4* background demonstrated that mutation of ICS1 led to partial recovery of the phenotype (Hermkes et al., 2010; Villajuana-Bonequi et al., 2014). This suggests elevated SA levels may be contributing to the phenotype of *esd4*, similarly to *siz1*.

Double *SUMO1 SUMO2* mutants are lethal, whilst single *SUMO1* or *SUMO2* mutants are phenotypically comparable to wild-type plants, indicating their redundancy (Saracco et al., 2007). Silencing *SUMO2* in *sumo1* mutants (*sum1-
1.5 SUMO in immunity and salicylic acid signalling

amiR-SUM2) led to the accumulation of SA derivative, SA-O-β-glucoside (SAG) and activated SA-dependent defense responses, resulting in resistance to Pst. (van den Burg et al., 2010). Overexpression of wild-type SUMO1, SUMO2, or conjugation deficient mutants (lacking the double glycine attachment site) also led to an increase in SA levels and activated defenses compared to wild-type plants. SUMO3 mutants are developmentally comparable to wild-type plants (van den Burg et al., 2010). Overexpression of wild-type and non-conjugatable mutated SUMO3, also led to the accumulation of SA but not SAG, with activated defenses. SUMO isoforms show differential responsiveness to SA and the bacterial PAMP flagellum peptide, flg22 (van den Burg et al., 2010). Flg22 and SA strongly induced SUMO3 gene expression, with SUMO1 showing some degree of SA responsiveness, whilst SUMO2 expression did not appear responsive (van den Burg et al., 2010).

Despite the mounting evidence indicating a significant connection between the regulation of the SUMO system and SA, the molecular underpinnings are unclear.

1.5.3. Phytopathogens target the SUMO system

A large number of plant pathogens (including pathovars of: Xanthomonas campes-tris, Ralstonia solanacearum, Pseudomonas syringae, Erwinia pyrifoliae and Rhizobium spp.) secrete effector proteins with ULP1 SUMO protease homology, into host cells (Roden et al., 2004; Bartetzko et al., 2009; Hotson et al., 2003; Kim et al., 2013a; Deslandes et al., 2003; Orth et al., 1999, 2000; Hotson and Mudgett, 2004). A number of these have been found to interact with host components although only one, XopD, has been shown to deSUMOylate its host target (Summarised in Table 1.2). In fact, some YopJ related effectors possess acetyltransferase activities and it is unclear as to whether they may possess additional SUMO protease activity or not (Üstün et al., 2013). XopJ and XopD effectors reduce pathogen induced SA accumulation further implying a link between SUMOylation and salicylic acid in plants (Üstün et al., 2013; Kim et al., 2008a).
<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Effector</th>
<th>SUMO protease activity</th>
<th>Host Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xanthomonas campestris</em> (euvesicatoria)</td>
<td>AvrBsT</td>
<td>protease catalytic core required host recognition- acetyltransferase</td>
<td>pepper SGT1, acetylates microtubule associated AtACIP1</td>
<td>(Orth et al., 2000; Hwang et al., 2012; Cheong et al., 2014; Kim et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>AvrXV4</td>
<td>protease catalytic core required host recognition, reduces SUMO conjugates in planta</td>
<td>tomato AvRxv Interactor 1 AR1 (14-3-3-type protein)</td>
<td>(Roden et al., 2004; Whalen et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>XopJ</td>
<td>protease catalytic core required suppress host defense-</td>
<td>proteasomal subunit RPT6</td>
<td>(Bartetzko et al., 2009; Üstün et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>XopD</td>
<td>DeSUMOylates SIERF4 and reduces SUMO conjugates in planta</td>
<td>Tomato SIERF4 transcription factor, Arabidopsis MYB30 transcription factor</td>
<td>(Hotson et al., 2003; Kim et al., 2008a, 2013a; Canonne et al., 2011)</td>
</tr>
<tr>
<td><em>Ralstonia solanacearum</em></td>
<td>PopP2</td>
<td>?</td>
<td>acetylates Arabidopsis RRS1-R Resistance protein</td>
<td>(Deslandes et al., 2003; Tasset et al., 2010)</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>YopJ</td>
<td>reduces SUMO conjugates in planta</td>
<td>MAPK kinase kinases</td>
<td>(Orth et al., 1999, 2000)</td>
</tr>
</tbody>
</table>

**Table 1.2:** Phytopathogen effectors with putative SUMO protease activity. Evidence of ULP1-like SUMO protease activity of plant pathogen effectors described in the literature (see column, references).
1.5 SUMO in immunity and salicylic acid signalling

An increasing number of pathogenic viruses have been found to target SUMO cascade enzymes in human cells (reviewed by (Boggio and Chiocca, 2006; Wimmer et al., 2011; Wilson, 2012)). This includes viral targeting of the SUMO system and STUBLs, to host proteins to circumvent host defense responses, in addition to SUMOylation of viral proteins themselves (Müller and Dejean, 1999; Endter et al., 2001; Rosas-Acosta et al., 2005; Palacios et al., 2005; Izumiya et al., 2005; Lamsoul et al., 2005; Marcos-Villar and Campagna, 2011). Although these viral activities have yet to be described in plant-virus interactions they further demonstrate the importance of SUMOylation in host cell signalling and defense.

1.5.4. SUMOylated substrates linked to immunity

AgriGO (Du et al., 2010) gene ontology analysis of the datasets produced by mass spectromic or Y2H approaches indicate a number of the SUMOylated substrates identified have been previously implicated in plant immunity (Elrouby and Coupland, 2010; López-Torrejón et al., 2013; Miller et al., 2013, 2010; Miller and Vierstra, 2011; Budhiraja et al., 2009; Park et al., 2011b)(Fig. 1.5). These include MODIFIER OF SNC1, -2, (MOS2), a MOS4 interactor, a number of defense implicated WRKY transcription factors, HISTONE DEACETYLASE19 (mutants of which possess elevated SA content (Choi et al., 2012)), an RPM1 resistance protein interactor, jasmonic acid responsive transcription factor MYC2, ETHYLENE RESPONSE FACTORs, an ethylene biosynthesis enzyme, and an as yet uncharacterised TIR NB LRR resistance protein. The role of these modifications remains to be established in the context of plant immunity.

The link between SUMOylation, salicylic acid and immunity is apparent, but this is just a small facet in the life processes of plants that SUMO appears to exert it’s regulation (Fig. 1.5).
1.6 Future prospects

Figure 1.5.: Gene Ontology enrichment analysis of biological processes of SUMOylated substrates in *Arabidopsis thaliana*. Based upon proteins identified by mass spectrometry or yeast two hybrid (Elrouby and Coupland, 2010; López-Torrejón et al., 2013; Miller et al., 2013, 2010; Miller and Vierstra, 2011; Budhiraja et al., 2009; Park et al., 2011b) compared to the entire *Arabidopsis thaliana* genome using AgriGO (Du et al., 2010). Implicated biological process annotations: green bars (input list)- genes whose products have been identified as being SUMOylated by mass spectrometry; blue bars (background/ reference)- whole Arabidopsis genome.

1.6. Future prospects

As illustrated above, significant progress has been made in the fields of plant immunity, salicylic acid signalling and more recently SUMOylation. I will now briefly summarise some of the gaps in these areas and future opportunities to further our understanding.

Despite all avirulent interactions converging on HR PCD, the identity of downstream executioners remain elusive. SA accumulation has been shown to precede cell death, along with apparent NPR1 degradation but a clear mechanism is lacking (Durrant and Dong, 2004; Zhang et al., 2010c). SA induced DNA damage may prove to be a central component (Yan et al., 2013). How salicylic acid syn-
thesis is stimulated by pathogen detection via PRR and R activation is unclear. Mutant screens have provided insight into the components of SA mediated signalling, it is surprising that SA biosynthesis mutants, such as \textit{ics1}, have not been utilised in such approaches.

The increased sensitivity and affordability of mass spectrometry, has facilitated the identification of SUMOylated substrates (López-Torrejón et al., 2013; Miller et al., 2013, 2010; Miller and Vierstra, 2011; Budhiraja et al., 2009; Park et al., 2011b). This now needs to be coupled with the identification of SUMO binding proteins containing SIMs. The success in identification of SUMO binding proteins has been limited, perhaps in part due to the use of SUMO monomers (Elrouby et al., 2013; Park et al., 2013). The use of in vitro synthesised poly-SUMO chains could prove to be a more sensitive approach. Coupling the identification of SUMOylated proteins and their SIM mediated interactors holds the potential to put plant SUMOylation into biological context. Furthermore an open mind must be maintained in order to not prematurely exclude the possibility that SUMO modification may have novel mechanistic implications in the regulation of eukaryotes.
1.7 Study objectives

Previously, overexpression of the SUMO protease OVERLY TOLERANT to SALT1 (OTS1) has been shown to result in salt stress tolerance (Conti et al., 2008). Double mutants of *ots1* and its closest homolog *ots2*, were hypersensitive to salt. Given the associations made between SUMOylation and SA mediated pathogen defenses it was hypothesised that OTS1/2 regulate SA mediated defense signalling through their SUMO deconjugative activities.

Main objectives of this work:

1. Investigate the role of OTS1 and -2 in plant immunity.
2. Investigate the regulation of OTS1/2 and SUMOylation in SA-mediated defense responses.
2. Materials

2.1. Enzymes

Name (Supplier- Order No.)

Polymerases

MyTaq™ Red Mix (Bioline- BIO-25044)
Q5® Hot Start High-Fidelity DNA Polymerase (New England BioLabs- MD493L)
Sigma-Aldrich SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma- S4438)

Gateway

Life Technologies pENTR D-TOPO (Fisher- 10780335)
Life Technologies Gateway cassette LR clonase II (Fisher- 11791020)

Reverse Transcription

Invitrogen SuperScript® II Reverse Transcriptase (Fisher- 18064014)
Invitrogen RNaseOUT™ Recombinant Ribonuclease Inhibitor (Fisher- 10777-019)

Restriction Enzymes

MluI (New England BioLabs- R0198L)
2.2 Chemicals and solutions

2.2. Chemicals and solutions

Name (Supplier- Order No.)

2,6-Dichloropyridine-4-carboxylic acid, INA (Sigma- 456543)

50X TAE (Fisher- 10490264)

Acetic Acid glacial (Fisher- 10394970)

Acrylamide (Sigma- A3574)

Agarose (Melford- MB1200)

Agar, plant (Sigma- A1296 )

Agar, microbiological (Melford- M1002)

Ammonium persulfate APS (Fisher- 10396503)

Brilliant blue R250 (Fisher- 10573165)

Bromophenol Blue (Fisher- 10679733)

Calcium chloride, dihydrate (Fisher- 10158280)

Chloral Hydrate (Sigma- C8383)

Di-Potassium hydrogen orthophosphate (Fisher- 10101490)

Ethidium bromide (Fisher- 10132863)

Ethylenediaminetetraacetic acid EDTA (Sigma- E5134)

Glucose, D- (Melford- G1400)

Glufosinate-ammonium (Sigma- 45520)

Glycerol (Fisher- 10021083)

Glycine (Fisher- 10773644)

Hydrochloric acid, 35-38% (Fisher- 10000180)

Lactic acid (Fisher- 10141430)

LB (Fisher- 12871650)
2.2 Chemicals and solutions

Liquid herbicide basta (marketed as Harvest) was kindly supplied by Ian Cockram, Technical enquiry Manager at Bayer CropScience Limited

Magnesium Chloride, hexahydrate (Melford- M0533)

Magnesium Sulfate, heptahydrate (Fisher- 10553355)

Methanol (Fisher- 10785484)

MG132 (Enzo Life Sciences- BML-PI102)

Murashige & Skoog medium, Basal salt mixture (Duchefa- M0221.0050)

N-ethylmaleimide (Sigma- E3876)

Peptone (Melford- P1328)

Phenol, Saturated pH 4.3 (Fisher- 10513135)

PIPES (Melford- B2004)

Potassium Chloride (Melford- P0515)

Salicylic acid, SA (Sigma- 247588)

Sigma agar (Sigma- A4550)

Silwett-L77® surfactant Sodium Chloride (VWR- 27810.364)

Sodium Chloride (VWR- 27810.364)

Sodium dodecyl sulfate, SDS (Melford- B2008)

Sodium hypochlorite (Fisher- 10296650)

Sucrose (Melford- 57-50-1)

TEMED (Fisher- 10549960)

Tris (VWR 28811.364)

Trypan blue (Fisher- 10174110)

Tryptone (Melford- T1332)

Tween-20 (Sigma- P2287)

β-mercaptoethanol (Sigma- M3148)
2.3 Antibiotics

### 2.3. Antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock solution</th>
<th>Working concentration</th>
<th>Supplier- Order No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin monosulphate</td>
<td>water 50mg/ml</td>
<td>50μg/ml</td>
<td>Melford- K0126</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>100% methanol 25mg/ml</td>
<td>50μg/ml</td>
<td>Fisher- 10562975</td>
</tr>
<tr>
<td>Gentamicin sulphate</td>
<td>70% ethanol 25mg/ml</td>
<td>25μg/ml</td>
<td>Melford- G0124</td>
</tr>
</tbody>
</table>

2.4. Kits

Name (Supplier- Order No.)

Sigma Plant Spectrum total RNA extraction kit (Sigma- STRN250)

Qiagen QIAquick Gel Extraction Kit (Qiagen- 28704)

Qiagen QIAprep Spin Miniprep kit (Qiagen- 27106)

2.5. Ladders

Name (Supplier- Order No.)

**DNA**

HyperLadder™ 1kb (Bioline- BIO-33026)

Ready Ladder™ 50 bp (VWR- N746)

**Protein**

Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder (Fisher 26620)
2.6. Vectors

**TOPO cloning**

Life Technologies pENTR D-TOPO (Fisher-K240020)

**Plant transformation vectors**

pEarley Gate201 (Earley et al., 2006)

2.7. Bacterial strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Resistance*</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agrobacterium tumefaciens</em></td>
<td>GV3101:pMP90</td>
<td>Rifampicin and Gentamicin</td>
<td>Arabidopsis transformation (see, 2.2.3)</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>DH5α</td>
<td>None used (nalidixic acid)</td>
<td>Laboratory strain, plasmid maintenance and propagation (see, 2.2.1)</td>
</tr>
<tr>
<td><em>Pseudomonas syringae pv. tomato</em></td>
<td>DC3000 (Pst.),</td>
<td>Rifampicin only</td>
<td>Arabidopsis infection (see, 2.2.2.4)</td>
</tr>
<tr>
<td>AvrRPM1 (Pst, AvrRPM1),</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas syringae pv. tomato</em></td>
<td>DC3000 AvrRPM1</td>
<td>Rifampicin and Kanamycin</td>
<td>Arabidopsis infection (see, 2.2.2.4)</td>
</tr>
</tbody>
</table>

*see 2.3 *virulence on Arabidopsis thaliana ecotype Col-0

**Table 2.1.: Details of bacterial strains used.**

Long term storage: All bacterial strains were grown in liquid culture before transfer into 2 ml eppendorfs with glycerol at a final concentration of 20%, these were snap frozen in liquid nitrogen before long term storage at minus 80°C.

Short term storage: *A. tumefaciens* and *E. coli* were grown on LB agar with appropriate antibiotics (see, 2.3) and grown at, 28°C for 48 hours or 37°C for 16 hours respectively. *Pseudomonas* strains were streaked from glycerols onto King’s B agar with appropriate antibiotics (see, 2.3), incubated at 28°C for up to 48 hours before use.
2.8 Antibodies

2.8. Antibodies

**Primary**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Working concentration (TBST)</th>
<th>Supplier-Order No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-HA</td>
<td>Rat</td>
<td>1:10 000</td>
<td>Roche-3F10 11867423001</td>
</tr>
<tr>
<td>anti-SUMO1/2</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>Abcam-Ab5316</td>
</tr>
<tr>
<td>anti-Ubquitin11</td>
<td>Rabbit</td>
<td>1:5000</td>
<td>Agrisera-AS08 307A</td>
</tr>
</tbody>
</table>

See appendix for specificity (anti-SUMO: Fig. 5.9, anti-HA: Fig. 4.10(a)).

**Secondary**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Working concentration (TBST)</th>
<th>Supplier-Order No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-RAT-Hrp</td>
<td>1:20 000</td>
<td>Sigma-A5795</td>
</tr>
<tr>
<td>anti-Rabbit-Hrp</td>
<td>1:20 000</td>
<td>Sigma-A0545</td>
</tr>
</tbody>
</table>

2.9. Arabidopsis

**Mutants**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Gene ID</th>
<th>SALK code</th>
<th>Source</th>
<th>Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Overly tolerant to salt1</em> (<em>ots1</em>)</td>
<td>At1g60220</td>
<td>022798</td>
<td>Dr Lucio Conti</td>
<td>Fig. 4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>(Conti et al., 2008)</em></td>
<td></td>
</tr>
<tr>
<td><em>Overly tolerant to salt2</em> (<em>ots2</em>)</td>
<td>At1g10570</td>
<td>001579</td>
<td>Dr Lucio Conti</td>
<td>Fig. 4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>(Conti et al., 2008)</em></td>
<td></td>
</tr>
<tr>
<td><em>Isochorismate synthase1</em> (<em>ics1</em>)</td>
<td>At1g74710</td>
<td>088254</td>
<td>Dr Harold van den Burg</td>
<td>Fig. 4.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>(University of Amsterdam)</em></td>
<td></td>
</tr>
<tr>
<td><em>Sap and Miz1</em> (<em>siz1</em>)</td>
<td>At5g60410</td>
<td>034008</td>
<td>Dr Lucio Conti</td>
<td>Fig. 6.1</td>
</tr>
</tbody>
</table>

*ots1 ots2* double mutant lines were isolated by Dr Lucio Conti, as described previously *(Conti et al., 2008)* (Fig. 4.1). *Ots1 ots2 siz1* triple mutant was isolated by Dr Lucio Conti (Fig. 6.1).

2.10. Suppliers

Abcam PLC. http://www.abcam.com/
2.10 Suppliers


Duchefa Biochemie B.V. http://www.duchefa-biochemie.com/


Fisher Scientific UK Ltd. http://www.fisher.co.uk/


Melford Laboratories Ltd. http://melford.co.uk/


QIAGEN Ltd. http://www.qiagen.com/

Roche Products Ltd. http://www.roche.co.uk/

SARSTEDT Ltd. http://www.sarstedt.com/

Sigma-Aldrich Co. https://www.sigmaaldrich.com/united-kingdom.html

STARLAB (UK) Ltd. http://www.starlab.co.uk/


VWR International. https://uk.vwr.com/app/Home
3. Methods

3.1. Plant growth and treatments

3.1.1. Growth

*Arabidopsis thaliana* was sown in moist Levington F2 plus sand compost. Seed was stratified for two days at 4°C before transferring to Panasonic MLR Plant Growth Chambers. Chambers were set to either: long day conditions- day-night cycle of 16 hours light at 22°C and 8 hours dark at 20°C, or short day conditions- day-night cycle of 10 hours light at 22°C and 14 hours dark at 20°C with a constant relative humidity of 70%.

3.1.2. Seed sterilisation

~12 mg of seed in 1.5 ml eppendorfs were placed in an upright rack, within an airtight box situated in a fume hood. Within the box, 3 ml of concentrated Hydrochloric acid was added to a beaker containing 100 ml of hypochlorite and the box sealed immediately. This was left overnight (~14 hr). The box was opened, the beaker removed and the box resealed. Seeds transferred to sterile laminar flow, aired for 1 hour before transfer to medium.
3.1 Plant growth and treatments

3.1.3. Plant medium

2.15 g Murashige & Skoog (MS) medium (half strength), 5 g Sucrose, 10 g Sigma agar (excluded for liquid media), made up to 1 litre with purified water and autoclaved for 15 minutes at 121°C.

Media were allowed to cool below 50°C before adding supplements (see 3.1.6 & 3.1.8).

3.1.4. Infections

King’s B Media 10 g Peptone, 0.75 g di-potassium hydrogen orthophosphate, 6 g agar (excluded for liquid media), 400 ml distilled water, 5 ml glycerol and mixed. The mixture was made up to 500 ml with water and autoclaved for 15 minutes at 121°C. Media were allowed to cool below 50°C before adding 3.05 ml 1 M magnesium sulfate followed by appropriate antibiotics (see, Table 2.1).

Procedure

Pst. strains were grown on King’s B agar with appropriate antibiotics (see, Table 2.1) and incubated for two days at 28°C. Liquid King’s B with appropriate antibiotics was inoculated from freshly grown Pst. plates, and grown at 28°C overnight with shaking at 200 rpm. Cells were centrifuged at 4500 rpm at room temperature and resuspended in sterile water. This was repeated once and the final suspension diluted to an optical density of OD_{600} equal to 0.002 (1X10^6 cfu/ml). Five leaves from 12 four week old short day grown plants were pressure infiltrated with the suspension and returned to the growth chamber. Leaf discs were cut from three different plants and macerated in 200 µl of sterile water. This suspension was moved to a microtitre plate and serially diluted 1 in 5 down the plate (40 µl suspension plus 160 µl water). 15 µl of each dilution was spotted onto King’s B agar plates with appropriate antibiotics and allowed
3.1 Plant growth and treatments

to dry. Plates were incubated for 36 hours before counting colonies. This was repeated three times per genotype, per day assayed (Katagiri et al., 2002).

3.1.5. Trypan blue staining

**Trypan blue stock**  25% saturated phenol (pH 4.3), 25% glycerol, 25% lactic acid, 25% water, plus 0.025% weight/volume of trypan blue.

**Chloral-hydrate**  Chloral-hydrate was mixed with an equal weight/volume of purified water in the fume hood until dissolved.

**Procedure**

Visualisation of dead cells using trypan blue staining was performed on four week old Arabidopsis plants pressure infiltrated with *Pst.* (as described in, 3.1.4) with a bacterial suspension with an OD\(_{600}\) of 0.2 (1\(\times\)10\(^8\)).

All steps of the staining procedure were undertaken within a fume hood. Leaves were detached at given time-points (untreated, 6, 12, 24 and 36 hours post infiltration). A working stock of trypan blue was made by mixing the trypan blue stock solution 1:1 with 96% ethanol. 600 ml of water was brought to the boil in a 1 L beaker on a hot plate. Labelled 15 ml centrifuge tubes were filled with 6 ml of working trypan blue solution, capped loosely, and placed in a water bath to bring to the boil (~5 minutes). Tubes were removed with tweezers and allowed to cool ~20 seconds in an upright rack. Detached leaves were dropped into the solution with tweezers. The tubes were recapped and placed back into a boiling water bath with tweezers and incubated for 10 minutes, after which they were removed and left staining overnight at room temperature. The next morning the stain was poured off into a labelled waste Duran using tweezers to prevent the leaves falling out. 12 ml of chloral-hydrate destain was added and the tubes inverted to mix. The tubes were left for 4 hours before inverting once again. After a further
4 hours the chloral-hydrate was poured into a waste Duran and 12 ml of fresh chloral hydrate solution was added. The tubes were inverted and left overnight. The following morning the choral hydrate was poured into the waste. Leaves were imaged in water or 50% glycerol. Triplicate leaves were photographed with a Nikon D30 with a macro lens on a light box. Single leaf images were taken using Olympus SZH10 Research Stereo dissecting light microscope and QImaging QICAM camera. 10 X magnification images of leaves were taken using a Zeiss Axioskop light microscope and QImaging RETIGA 2000R camera.

3.1.6. Hormone treatments

**SA spray**

400mM salicylic acid stock was made in 100% ethanol. The stock was diluted in sterile water to a final concentration of 2mM and supplemented with Silwett-L77® surfactant to a final concentration of 0.005%. A control spray solution was made with equivalent volume of ethanol and silwett. Equal volumes were sprayed evenly across trays of 4 week old short day grown plants. Plants were sealed with propagator lids and returned to Panasonic growth chambers. The aerial tissues of three plants were removed at each time-point and four leaves per plant frozen in liquid nitrogen for RNA extraction, or whole plants for protein extraction (see, 3.2.6.1, and 3.3.1 respectively).

**SA liquid treatment**

12 mg of seed were sterilised in 2 ml eppendorf tube (see, 3.1.2). 1.5 ml of sterile water was added, mixed by inversion and moved to 4°C for stratification. The suspended seed was transferred into 50 ml of MS liquid in a 250 ml glass conical (see, 3.1.3). These were incubated with gentle shaking (<100 rpm) in long day light conditions for 10 days before treatment. Preincubation with MG132 was performed at a final concentration of 20 μM (stock 10 mM dissolved in DMSO) or
3.1 Plant growth and treatments

solvent (control) for 1 hour. A final concentration of 0.5 mM SA or an equivalent volume of ethanol was pipetted and mixed into the media. These were incubated for 0.5 hour before seedlings were collected and snap frozen in liquid nitrogen for protein extraction (see, 3.3.1).

INA plates

40 mM INA (2,6-dichloropyridine-4-carboxylic acid) stock was made in 100% ethanol. MS agar was supplemented with INA to final concentrations of 20 or 40 µg/ml, or an equal volume of ethanol for control plates (see, 3.1.3). Sterilised seed were spread over plates and sealed with micropore tape (see, 3.1.2). Plates were stratified for 48 hours at 4°C and moved into a Panasonic growth chamber set to long day conditions (see, 3.1.1).

3.1.7. SA measurement

Measurements were kindly performed by Dr Hannah Florance and Dr Venura Perera at Professor Murray Grant’s Laboratory (Exeter University).

10 mg freeze dried leaf powder was extracted in 0.8 ml 80% methanol containing a 100 µM internal standard. After centrifugation (10 min at 16,100 x g, 4°C), the samples were filtered through a 0.2 µm (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK).

Hormone quantitative analysis was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system. 20 µl of sample extract were loaded onto a Zorbax Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B; 7 min post time. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l min-1, nebuliser pressure
3.1 Plant growth and treatments

35 psig, capillary voltage ±4 kV. The fragmentor voltage and collision energies were optimised for each compound.

3.1.8. Arabidopsis transformation

Buffers adjusted to the correct pH using thermo scientific orion 3 star ROSS combination pH electrode using HCl or NaOH where appropriate.

**Agrobacteria competent cells**

**TE buffer** 10mM Tris pH 8 and 1 mM EDTA pH 8 in water.

**Procedure**

*Agrobacterium tumefaciens* (GV3101 pMP90) was streaked out from a glycerol stock onto fresh LB agar with rifampicin and gentamicin (see, 2.3), and incubated at 28°C for 48 hours. A single colony was used aseptically to inoculate 10 ml of liquid LB plus appropriate antibiotics, and grown for 24 hours at 28°C with shaking at 220 rpm. 200 ml of liquid LB with appropriate antibiotics was inoculated with 1 ml of the culture and grown at 28°C for a further 18 hours with shaking at 220 rpm. The culture was transferred to chilled tubes and centrifuged at 4000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended in 10ml of refrigerated TE buffer. The resuspension was centrifuged again at 4000 rpm for 15 minutes at 4°C and the supernatant discarded. The cell pellet was resuspended in 20ml of refrigerated liquid LB. 200 µl aliquots were made in chilled 1.5 ml eppendorfs, which were snap frozen in liquid nitrogen. These were stored in a minus 80°C freezer.
3.1 Plant growth and treatments

**Agrobacterium tumefaciens transformation**

Cells were thawed on ice, 1 µg of construct added and mixed briefly by flicking the tube. Tubes were incubated on ice for 5 minutes, moved to liquid nitrogen and incubated for 5 minutes, before being suspended in a water bath at 37°C for a further 5 minutes. 1 ml of liquid LB medium was added to the cells and incubated at 28°C with shaking at 200 rpm for 2 hours. Cells were spun down at 4500 rpm for 1 minute and 1 ml of the supernatant was discarded. Cells were resuspended in the remaining 200 µl by gentle pipetting before spreading onto LB agar plates with appropriate antibiotic selection. Plates were incubated for 48 hours before screening colonies by PCR for uptake of the vector (see, 3.2.4.2).

**Plant transformation**

*A. tumefaciens* (GV3101 pMP90) transformed with the OTS1 and OTS2 pEarly Gate 201 constructs were used to transform Arabidopsis plants by the floral dip method (Zhang et al., 2006a). In brief, agrobacterium was initially grown on agar plates with rifampicin, gentamicin and kanamycin (see, 2.3). 10 ml of liquid LB with equivalent selection was inoculated from freshly grown plates and grown overnight at 28°C with shaking at 200 rpm. Agrobacterium was subcultured into 200 ml of liquid LB with appropriate selection and grown as before for 24 hours. The cells were centrifuged at 4500 rpm for 10 minutes and the supernatant disposed. The cells were resuspended in 400 ml 5% (w/v) of sucrose in sterile water. Silwett-L77® was added to a concentration of 0.02% before dipping the unopened inflorescence of bolted Arabidopsis (4-5 week old grown under long day conditions) for ~5 seconds with agitation. Dipped plants were laid down in trays and placed into an autoclave bag for 24 hours before standing upright and allowing to set seed.
Plant selection

Primary transformants were selected for by spreading seed on soil soaked in 0.1% solution of herbicide (basta, marketed as Harvest). After nine days green, resistant seedlings were pricked out into fresh soil (no selection) and grown in long day conditions. Once siliques begin to form plants were bagged up individually and allowed to set seed.

Secondary transgenic seed was sterilised (see, 3.1.2) and spread aseptically on MS supplemented with a final concentration of 20 μg/ml glufosinate-ammonium and sealed with micropore tape. Plates were stratified for 48 hours before moving into Panasonic growth chambers set to long day growth conditions. After eleven days, plates were screened for resistant seedlings at a ratio of 3:1 (resistant:susceptible) in order to select transgenics containing a single transformation insert. Selected lines were pricked out to fresh soil (no selection) and grown in long day conditions. Once siliques begin to form plants were bagged up individually and allowed to set seed.

Tertiary transgenic seed was sterilised (see, 3.1.2) and spread aseptically on MS supplemented with a final concentration of 20 μg/ml Glufosinate-ammonium, and sealed with micropore tape. Plates were stratified for 48 hours before moving into Panasonic growth chambers set to long day growth conditions. After eleven days plates were screened for complete resistance indicating homozygous transgenics. Selected lines were pricked out to fresh soil (no selection) and grown in long day conditions. Once siliques begin to form plants were bagged up individually and allowed to set seed.

3.2. Nucleic acid

Buffers adjusted to the correct pH using thermo scientific orion 3 star ROSS combination pH electrode using HCl, NaOH or KOH where appropriate.
3.2 Nucleic acid

3.2.1. Oligonucleotides

The Arabidopsis Information Resource (TAIR) was used to retrieve Arabidopsis thaliana gene coding DNA sequences, or in the case of plasmid sequences, from relevant literature (Lamesch et al., 2012; Earley et al., 2006). Primers were designed to gene targets using Serial Cloner software (see, 3.4 Software Packages), or the National Center for Biotechnology Information (NCBI) primer BLAST web application (Geer et al., 2010) (see, A.1. Primers).

3.2.2. Agarose gel electrophoresis

0.8-2.5% weight/ volume of agarose was added to 1XTAE buffer and heated in a microwave until dissolved. This was allowed to cool before adding ethidium bromide to a final concentration of 0.0001%. The agarose solution was poured into a gel mould and a 20 well comb inserted. Gels were allowed to set for ~20 minutes before placing into a electrophoresis tank filled with 1 X TAE. Prior to loading DNA samples into wells, 5 X sample buffer was added (supplied with Bioline HyperLadder™ 1 kb), (omitted if MyTaq™ Red Mix was used- dye included within mix). Samples were loaded alongside a DNA ladder (0.8-1.5% agarose-Bioline HyperLadder™ 1 kb or 1.5-2.5 % agarose- Ready Ladder™ 50 bp). Gels were run at 70-110 V dependent on percentage of agarose. Gels were imaged using a BioRad Gel Doc 2000.

3.2.3. Gateway recombination cloning

Entry clones of OTS1 and OTS2 were cloned into pD-TOPO previously by Dr Lucio Conti (Conti et al., 2008). Destination vector pEG201 encodes the same antibiotic resistance as the entry vector pENTR™/D-TOPO® (Kanamycin), thus entry clones were first digested before performing ligation reactions (Earley et al., 2006).
3.2 Nucleic acid

**Restriction digest**

0.5 μg plasmid, 2 μl buffer and 0.5 μl MluI enzyme, was made up to 20 μl with water. Reactions were mixed gently and incubated at 37°C for 1 hour, then 80°C for 20 minutes.

10 μl of digestions was mixed with 5 X sample buffer and run on a 0.8% agarose gel. Bands containing the gene insert with recombination sites, were extracted from the gel using Qiagen QIAquick Gel Extraction kit as per manufacturer’s instructions.

**Ligation Reaction**

6 μl of gel extracted digested entry clone, 75 ng destination vector (pEG201), 1 μl TE buffer, 0.5 μl LR clonase II were mixed in a 1.5 ml eppendorf by gentle flicking and centrifuged briefly at low speed. Reactions were incubated at room temperature for 1 hour 30 minutes before proceeding to *E. coli* transformation.

**3.2.4. Competent *E. coli***

**SOB medium** 20 g tryptone, 5 g yeast extract, 0.59 g sodium chloride, 0.187 g potassium chloride, 2.03 g magnesium chloride hexahydrate, 2.47 g magnesium sulfate heptahydrate, made up to 1 litre with water and autoclaved for 15 minutes at 121°C.

**TB buffer** 10 mM PIPES pH 6.7, 55 mM magnesium chloride, 15mM calcium chloride, 250mM potassium chloride, sterilised by passing through a 0.2 μm syringe filter.

**Procedure**

DH5α was streaked out on LB agar plate with no selection and incubated at 37°C overnight (~16 hours). 10 ml of liquid LB medium (no selection) was inoculated
3.2 Nucleic acid

with a single colony from the plate and grown overnight at 37°C with shaking at 220 rpm. The whole culture was poured into 250ml of SOB medium in a 1 litre conical flask. This was grown at 18°C with shaking (220 rpm) until an OD$_{600}$ of 0.6 (~24 hours) was reached. The conical was submerged in an ice bucket for 10 minutes before centrifuging at 4000 rpm at 4°C for 10 minutes. The supernatant was disposed and the pelleted cells were resuspended in 80 ml of refrigerated TB buffer and returned to an ice bucket for 10 minutes. The cells were again centrifuged (4°C at 4000 rpm) for 10 minutes and the supernatant disposed. The pellet was resuspended in 20 ml of refrigerated TB buffer. 1.5 ml of DMSO was added to the resuspended cells and gently mixed. 100 μl aliquots were made in chilled 1.5 ml eppendorfs, which were snap frozen in liquid nitrogen. These were stored in a minus 80°C freezer.

3.2.4.1. Transformation

SOC medium  As SOB medium (see, 3.2.4) with the addition of 20 ml of 1 M glucose, sterilised by passing through a 0.2 μm syringe filter.

Procedure

One tube per transformation of chemically competent cells was thawed on ice. The entire ligation reaction was added to the cells and gently mixed. The cells were incubated for 10 minutes on ice before heat shocking for 30 seconds in a water bath at 42°C. 1 ml of SOC medium was aseptically added to the tubes and incubated at 37°C for one hour with shaking at 220 rpm. 50 μl of the cells were aseptically spread on LB agar with kanamycin (50 μg/ml). The remaining cells were centrifuged at 4500 rpm for one minute. Supernatants were removed leaving approximately 100 μl for resuspension of the bacterial pellet. Resuspended cells were spread on another LB agar plate with kanamycin (50 μg/ml). Plates were incubated at 37°C overnight (~16 hrs).
3.2.4.2. Colony PCR

Six colonies per transformation were screened for successful recombinants. Each colony was resuspended in 20 μl of autoclaved water for checking by PCR using a forward primer designed to the HA tag of pEG201 and a reverse primer from the end of OTS1/2 (see, A.1.):

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resuspended cells</td>
<td>2</td>
</tr>
<tr>
<td>Forward HA primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse gene primer</td>
<td>0.5</td>
</tr>
<tr>
<td>MyTaq™ Red Mix</td>
<td>6</td>
</tr>
<tr>
<td>H₂O</td>
<td>2</td>
</tr>
</tbody>
</table>

28 cycles

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>95</th>
<th>95</th>
<th>58</th>
<th>72</th>
<th>72</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min “sec”)</td>
<td>1’</td>
<td>30”</td>
<td>30”</td>
<td>1’/kb</td>
<td>5’</td>
<td>∞</td>
</tr>
</tbody>
</table>

Whole PCR reactions were run on an agarose gel to check that PCR products were the correct size, corresponding to the gene insert size plus the HA gene fusion. Remaining cell suspensions from two correct colonies were used to inoculate 10 ml of liquid LB with kanamycin (50 μg/ml) and incubated at 37°C with shaking (220 rpm) overnight (~16 hrs).

3.2.4.3. Plasmid Prep

After making glycerols from overnight cultures (see, 2.7), the remaining volume of cultures were centrifuged at 5000 rpm for 10 minutes. Bacterial pellets were lysed and plasmid purified using Qiagen QIAprep Spin Miniprep kit as per manufacturer’s guidelines. Purified plasmids were confirmed by PCR using a forward
3.2 Nucleic acid 

primer designed to the HA tag of pEG201 and a reverse primer from the end of OTS1/2 (see, A.1.):

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted plasmid (1:30)</td>
<td>1</td>
</tr>
<tr>
<td>Forward HA primer</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse gene primer</td>
<td>0.5</td>
</tr>
<tr>
<td>MyTaq™ Red Mix</td>
<td>6</td>
</tr>
<tr>
<td>H2O</td>
<td>2</td>
</tr>
</tbody>
</table>

25 cycles

Temperature (°C) 95 95 58 72 72 10
Time ('min “sec) 1’ 30” 30” 1’/kb 5’ ∞

Whole reactions were run on a 0.8% agarose gel alongside a 1:15 dilution of plasmids mixed with 5 X loading buffer (supplied with Bioline ladder). PCR product amplicon and vector band sizes were used to confirm correct recombination of gene insert into destination vector.

3.2.5. Arabidopsis genotyping

3.2.5.1. Arabidopsis genomic DNA extraction

**Extraction Buffer** 200 mM tris pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS.

**Procedure**

A single leaf disc was cut using the wide end of p10 pipette tip. This was ground with a mini pestle briefly in a 1.5 ml eppendorf tube. 150 µl of extraction buffer was added and the mixture ground again until homogenous. Samples were centrifuged at 13500 rpm for 5 minutes. 100 µl of the supernatant was transferred
to a new tube. 100 µl of isopropanol was added and mixed by inversion. This mixture was allowed to stand for 5 minutes. Samples were again centrifuged at 13500 rpm for 10 minutes and the supernatant was discarded. The pellet was mixed gently with 500 µl of 70% ethanol. Tubes were centrifuged at 13500 rpm for 5 minutes and the supernatant was discarded. Tubes were centrifuged at 13500 rpm for 1 minute and the remaining supernatant was removed using a pipette. Tubes were left open upside down on tissue for ~10 minutes until pellets dried. Pellets were then dissolved in 50 µl of 10 mM Tris pH 8.5. Extractions were checked by PCR.

All Arabidopsis mutants used in this study were produced by the SALK institute using transfer DNA insertion mutagenesis using the pROK2 plant transformation vector (see, 2.9.) (Baulcombe et al., 1986). This means that all lines will contain the same T-DNA sequence and thus mutant alleles can be detected using a primer designed to the left border of this insert- LBb1.3 (see Primers, A.1), with a gene specific primer.

3.2.5.2. Arabidopsis direct leaf PCR for screening crosses

Primers were designed which straddle either side of T-DNA insertion sites from OTS1, OTS2 and ICS1 mutant lines (see, A.1, 2.1.9). Primers were confirmed on wild-type genomic DNA with a gradient across the heat cycler for the annealing step. The PCR mix was setup as follows:

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis gDNA (see, 2.2.1.10 )</td>
<td>1</td>
</tr>
<tr>
<td>F primer</td>
<td>1</td>
</tr>
<tr>
<td>R primer</td>
<td>1</td>
</tr>
<tr>
<td>MyTaq™ Red Mix</td>
<td>6</td>
</tr>
<tr>
<td>H₂O</td>
<td>3</td>
</tr>
</tbody>
</table>
3.2 Nucleic acid

Once the optimal annealing temperature was determined, the specificity of the primers was confirmed using genomic DNA from homozygous T-DNA insertion lines alongside wild-type positive control template.

**Leaf disc excision**

Using the narrow end of a p200 pipette tip two pieces were cut from an individual leaf. The tip containing the leaf pieces was placed in an open labelled PCR tube in a rack. Positive (wild-type) and negative controls (tip only) were included throughout. The PCR rack was placed into a heating oven at ~50°C (+/-10°C) for a minimum of 2 hours (up to overnight). Dried leaf discs were ejected from the tip into the PCR tube pushing a gloved finger on the back of the tip or carefully flicking the top of tip. PCR mix was added directly to the tubes:

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1</td>
</tr>
<tr>
<td>MyTaq™ Red Mix</td>
<td>6</td>
</tr>
<tr>
<td>H₂O</td>
<td>4</td>
</tr>
</tbody>
</table>

The mixture was centrifuged at 1000 rpm for 1 minute and PCR tubes placed into a the heat cycler with the following cycling conditions:

<table>
<thead>
<tr>
<th>35 cycles</th>
<th>Temperature (°C)</th>
<th>Time (‘min “sec’)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95</td>
<td>1’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>95 *</td>
<td>30”</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>30”</td>
<td></td>
</tr>
<tr>
<td></td>
<td>* gradient 50-65°C</td>
<td>1’/kb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5’</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>∞</td>
<td></td>
</tr>
</tbody>
</table>
3.2 Nucleic acid

*optimal annealing temperature determined by genomic DNA test (see above)*

Whole reactions were analysed by gel electrophoresis. Candidate homozygous T-DNA alleles were confirmed using a genomic DNA extraction (see, 3.2.5.1.).

3.2.6. Quantitative PCR

3.2.6.1. RNA Extraction

Procedure

Frozen leaf tissue was ground to a fine powder with a pre-chilled pestle and mortar. Sigma-Aldrich Spectrum™ Plant Total RNA Kit was used to extract RNA as per manufacturers recommendations. RNA was quantified by measuring absorbance at wavelengths of 260 and 280 nm using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

3.2.6.2. cDNA synthesis

**DEPC water**  In a fume hood 1 ml of DEPC was added to 1 litre of sterile water in a Duran. The cap was screwed on loosely and left overnight. The following morning the treated solution was autoclaved at 121°C for 20 minutes.

**DNase Treatment**

1.5 μg of RNA was DNase treated with 1 μl Promega DNase1 with supplied buffer in a 10 μl reaction made up with DEPC water. The mix was incubated at 37°C for 30 minutes. 1 μl RQ1 stop solution was added and the reaction incubated at 65°C for 10 min, as per manufacturers guidelines.
3.3 Protein

Procedure

1 μl oligo dT (500 μg/ml) and 1 μl dNTP mix (10 mM each) was added to the DNase treated RNA and heated at 65°C for 5 minutes, chilled briefly on ice and span down. 4 μl 5 X First Strand buffer, 2 μl 0.1 M DTT, 1 μl RNaseOUT was added to the reaction tube and mixed gently. The reaction was heated at 42°C for 2 minutes before adding 1 μl SuperScript® II Reverse Transcriptase and heated at 42°C for 50 minutes. Finally the reaction was terminated at 70°C for 15 minutes. The resultant cDNA was made up to 100 μl with 79 μl H₂O of purified water. cDNA was tested by PCR using ACTIN7 primers spanning an exon junction (see, A.1).

3.2.6.3. Real-time

Quantitative PCR primers were designed to gene targets using the NCBI primer BLAST (Geer et al., 2010) and primer annealing was tested using gradient PCR. Relative expression was compared between genotypes and treatments using target primers and primers to the housekeeping gene ACTIN7 (At5g09810) for normalization. Housekeeping gene suitability for normalisation and stability across SA treatments is demonstrated in the Appendix (see, Fig. A.2 and Fig. A.1). Sigma-Aldrich SYBR® Green JumpStart™ Taq ReadyMix™ was used in conjunction with Qiagen Rotor- Gene® Q and analysis was undertaken with provided software using the comparative quantification method (Warton et al., 2004).

3.3. Protein

Buffers adjusted to the correct pH using thermo scientific orion 3 star ROSS combination pH electrode using HCl or NaOH where appropriate.
3.3 Protein

3.3.1. Plant total protein extraction

**Extraction buffer**  50 mM tris pH 8.5; 4% SDS; 2% β-mercaptoethanol, 10 mM EDTA, 50 mM NEM in water.

**Procedure**

Frozen leaf tissue was ground to a fine powder with a chilled pestle and mortar. Extraction buffer was added 1:1 (weight/ volume). The mixture was centrifuged at 13500rpm at room temperature for 10 minutes. Samples were diluted 1:5 and protein concentration was determined using an EMD Millipore Corporation Direct Detect™ Infra-red Spectrometer and samples equalized with the addition of extraction buffer before adding 4X protein sample buffer (40% glycerol, 240 mM tris-HCl pH 6.8, 8% SDS, 0.04% bromophenol blue, 50 mM EDTA, 5% β-mercaptoethanol).

3.3.2. SDS PAGE and Western Blotting

**10XRunning Buffer**  1% SDS, 0.25 M tris, 1.92 M glycine in water.

1X solution made up with water.

**10XTransfer Buffer**  0.25 M tris, 1.92 M glycine in water.

1X solution made up with water and methanol to a final concentration of 20%.

**10XTBS**  0.2 M tris, 1.37 M sodium chloride pH 7.6 in water.

1X solution made with water.

To make TBST, Tween-20 was added to a final concentration of 0.1%.

**Chemical Luminescence Reagent**  Stock A- 250 mM luminol in DMSO.

Stock B- 90 mM p-coumaric acid in DMSO.
Solution 1. 1ml stock A, 0.44 ml stock B, 10 ml 1 M tris pH 8.5 made up to 100 ml with water.

Solution 2. 64 μl 30% H$_2$O$_2$, 10 ml 1 M Tris pH 8.5 make up to 100 ml with water.

Procedure

The BIORAD Mini PROTEAN system was used for gel casting, running and transfer. 10% polyacrylamide gels (resolving gel: 0.38 M tris-HCl pH 8.8, 10% (w/v) acrylamide 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.07% TEMED; stacking gel: 132 mM tris-HCl pH 6.8, 4% (w/v) acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.15% (v/v) TEMED) were used to separate protein samples by gel electrophoresis (at 90 V). Separated proteins were transferred to PVDF membranes overnight at 4°C at 30 V.

Membranes were blocked and probed with TBST (see, above) on a STUART SCIENTIFIC STR9 3D rocking platform set to 35 rpm. Blotted membranes were blocked with 5% semi-skimmed milk in TBST for one hour at room temperature. Membranes were washed twice by hand with gentle agitation changing TBST each time. Membranes were probed with primary antibodies diluted in TBST (anti-HA 1:10000, anti-SUMO1/2 and anti-Ubiquitin at 1:5000) for three hours. Membranes washed twice more by hand, followed by three 5 minute fresh TBST washes on the platform mixer. Membranes were probed with appropriate secondary antibodies diluted in TBST (1:20 000) for one hour. Membranes were washed twice by hand, followed again by three 5 minute TBST washes on the platform mixer. TBST was poured off and a mix of 1:1 chemical luminescence reagent solution 1 and 2 was added to the membranes and incubated for one minute. Excess reagent was allowed to run off and membranes were sealed between two sheets of acetate film in a HI-SPEED-X intensifying screen binder. X-ray film (FUJIFILM SUPER RX) was exposed to the blot in a dark room for between 1 second and ten minutes, dependent on protein sample and antibody.
Films were developed with an Xograph Compact X4 Automated Processor.

### 3.3.3. Coomassie blue staining

**Stain** 0.05% weight/volume R250 Brilliant blue, 50% acetic acid, 20% methanol, made up with distilled water.

**Destain** 50% methanol, 10% acetic acid made up with distilled water.

**Procedure**

PVDF membranes were submerged with coomassie stain and shaken for 20 minutes. Stain was poured off and membranes were washed with distilled water. Membranes were submerged in destain and shaken for 15 minutes. Destain was poured off and membranes rinsed in water. Membranes were left to air dry for ~5 minutes before scanning with an EPSON Perfection V5000 PHOTO flatbed scanner.

### 3.4. Software packages

**Plasmid sequence analysis and oligo design**

Serial Cloner Version 2.6.1 © 2004-2013 Franck Perez [SerialBasics].

**Image capture**

OPEN LAB 5 Version 5.5.0 © 1994-2002 Improvision Ltd.

Used with Olympus SZH10 Dissecting Microscope

Q Capture Pro 7 © 2010 QImaging.

Used with Zeiss Axioskop Microscope
3.4 Software packages

Quantity One ® 1-D Analysis Software Version 4.6.5 © 2006 Bio-Rad Laboratories.

Used with Bio-Rad Gel Doc 2000

Data analysis

GraphPad Prism 6 for Mac OS x Version 6.0d © 1994-2013 GraphPad Software, Inc.

Used for graph generation and statistical analysis (unpaired Student’s t-test, one-way and multiway ANOVA with Tukey test post hoc where appropriate)

Qiagen Rotorgene Q Version 1.7 © 1990-1999 Info-ZIP Pty. Ltd.

Figure preparation

ImageJ Version 1.47

Inkscape Version 0.48.1 © 1989, 1991 Free Software Foundation, Inc.


Microsoft ® PowerPoint ® for Mac 2011 Version 14.4.4 © 2010 Microsoft Corporation. All rights reserved.

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4. Characterisation of immune response status in the OTS SUMO protease mutants

Chosed et al. (2006) showed in vitro catalytic activity of four yeast ULP1-like SUMO proteases from plants (see section, 1.3.3. SUMO proteases). An activation-tagging insertional mutant approach, led to the discovery of a biological role for two of these proteases in plants (ULP1c and -d) (Conti et al., 2008). An insertion into the promoter region of ULP1d resulting in it’s overexpression, led to enhanced salt tolerance. ULP1d was henceforth know as OVERLY TOLERANT TO SALT1 (OTS1). A close homologue OVERLY TOLERANT TO SALT2 (OTS2/ ULP1c) was found to act redundantly with OTS1 in the regulation of salt stress responses, with ots1 ots2 double mutants showing increased sensitivity to salt. Further, Conti et al. (2008) also found salt stress promoted the turnover of OTS1.

Mutants of the SUMO E3 ligase SIZ1 display reduced levels of SUMO conjugates and enhanced resistance to the virulent bacterial pathogen Pseudomonas syringae, relative to wild-type (WT) Arabidopsis plants (Miura et al., 2005; Lee et al., 2006). These findings led to the hypothesis that SUMOylation of transcription factors may suppress the expression of defense-related genes (van den Burg and Takken, 2010). Conti et al. (2008) have shown that the ots1 ots2 double SUMO protease mutant possesses higher levels of SUMO conjugates than
4.1 Enhanced resistance to virulent Pseudomonas in the ots double mutant

that of WT plants. In order to test the hypothesis of SUMO mediated negative regulation of immune signalling, here I characterised the status of immune signalling and defense in the single ots1, ots2 and double ots1 ots2 SUMO protease mutants.

4.1. Enhanced resistance to virulent Pseudomonas in the ots double mutant

The status of defense responses was assessed in the ots SUMO protease mutants previously isolated by Dr Lucio Conti (see, Materials 2.8.). Mutants were confirmed by PCR from genomic DNA extracts. Full length gene cloning primers were used to amplify wild-type alleles and insertional mutant alleles were amplified using a left border T-DNA insert primer with gene specific forward primers (Fig. 4.1) (see, Methods 3.2.5 and Apendix Primers A.1). Additionally, reverse transcription PCR using primers 3’ of T-DNA insertion sites was performed to confirm loss of gene expression in mutants (Fig. 4.2) (see, Appendix A.1 and, Methods 3.2.6.1 & 3.2.6.2). The Arabidopsis thaliana - Pseudomonas syringae interaction is a widely used host-pathogen model for studying plant immunity, and is becoming well characterised at a physiological and molecular level (Katagiri et al., 2002; Navarro et al., 2004; Jones et al., 2006; Muthamilarasan and Prasad, 2013).

Surprisingly, growth of the virulent strain Pseudomonas syringae pv. tomato DC3000 (Pst.) appeared to be more than ten times lower in the ots1 ots2 double mutant compared to WT (Fig.4.3a). Single T-DNA insertion mutants ots1 or ots2 did not differ significantly when compared to WT plants (when analysed using ANOVA). This indicates OTS1 and OTS2 act redundantly in defense suppression, as has been shown previously in salt stress response (Conti et al., 2008). The infections were repeated with just WT and ots1 ots2 double mutant plants with greater replication. This led to statistically significant lower Pst.
4.1 Enhanced resistance to virulent Pseudomonas in the *ots* double mutant

Figure 4.1.: Genotyping of the *ots* mutants. PCR products from genomic DNA extracts from wild-type (WT), *ots1*, *ots2*, and *ots1 ots2* lines were analysed by agarose gel electrophoresis. (a) The full-length *OTS1* and *OTS2* gene PCR product (3579bp and 3464bp, respectively), (b) the T-DNA insert PCR product, amplified using *OTS1* and *OTS2* forward primers with a reverse left border T-DNA primer (~3763bp and ~2952bp, respectively), and (c) *ACTIN1* PCR product from all DNA extracts as an extraction control.
4.1 Enhanced resistance to virulent Pseudomonas in the *ots* double mutant

growth in the *ots* double mutant compared to WT at three days post infiltration (ANOVA-Tukey test) (Fig. 4.3b).

Virulent *Pst.*, causes host cell death as disease progresses (Katagiri et al., 2002). Trypan blue staining to visualise the cell death resulting from *Pst.* infection showed that cell death progression in the *ots1 ots2* double mutants began earlier, with staining six hours post infiltration, versus twelve hours observed for WT plants (Fig. 4.4a) (see, Methods 3.1.5). Interestingly, control infiltration (water) also resulted in some cell death six hours after infiltration in the *ots1 ots2* double mutant, unlike WT or single mutants. (Fig. 4.4b). This did not increase at later time-points and suggests that there may be a greater sensitivity to cell death elicitation in the *ots1 ots2* double mutant, with the damage caused by infiltration enough to trigger cell death. Over the 36 hours assayed comparable staining was observed in the single *ots* mutants (*ots1* and *ots2*) to that of WT plants treated with *Pst.*, with signs of cell death presenting at 12 hours and increasing at the 24 and 36 hour time-points. These observations are consistent with previous indications that OTS1 and OTS2 act redundantly in immune pathways (Fig. 4.3a).

Taken together these results suggest OTS1 and OTS2 work redundantly in restricting cell death, but by doing so they compromise defense against virulent *Pst.* in WT Arabidopsis.
4.1 Enhanced resistance to virulent Pseudomonas in the ots double mutant

Figure 4.2.: Loss of OTS gene expression in the ots mutants. PCR products from cDNA prepared from RNA extracts from wild-type (WT), ots1, ots2, and ots1 ots2 lines were analysed by agarose gel electrophoresis. Reverse transcription PCR was performed using gene specific primers 3’ of genomic T-DNA insertion sites.

Figure 4.3.: The ots1 ots2 double mutant displays enhanced resistance to virulent Pseudomonas syringae. Colony forming unit counts of Pseudomonas syringae pv tomato DC3000 from the leaves of 4 week old Arabidopsis plants: (a) Wild-type (WT), single ots1 and ots2 mutants, and the double ots1 ots2 mutants 2, 3 and 4 days post infiltration, (b) WT and ots1 ots2 double mutants on the day of infiltration (day 0) and 3 days later. Error bars represent Standard Error of the Mean. ** p value 0.001-0.01 (one-way ANOVA with Tukey test post hoc).
4.1 Enhanced resistance to virulent Pseudomonas in the *ots* double mutant

Figure 4.4.: The *ots1 ots2* double mutant is more sensitive to cell death elicitation. Trypan blue dead cell staining of the leaves of wild-type (WT), single *ots1* and *ots2* mutants, and the double *ots1 ots2* mutant, infiltrated with (a) high titre *Pst.* or (b) water (control) over 36 hours.
4.2 SA signalling is up-regulated in the ots double mutant

In order to understand more about the defense phenotype of the ots mutants, quantitative PCR (qPCR) was used to measure basal gene expression of PATHOGENESIS-RELATED (PR) defense genes- PR1, PR2, and PR5 (Sels et al., 2008). These were found to be significantly up-regulated (relative to housekeeping gene- ACTIN7. See, A.1.) in the ots1 ots2 double mutant compared to WT (Fig. 4.5).

Plant immune signalling and response to biotrophic pathogens, such as that to the initial phase of colonisation by Pst. are antagonistic to necrotrophic pathogen associated responses (Spoel et al., 2007). Given the resistance observed to Pst. in the double ots mutant, necrotrophic defense mediated by jasmonic acid (JA) may be suppressed. Expression of the necrotrophic pathogen-associated defense marker PLANT DEFENSIN1.2 (PDF1.2) was not found to differ significantly in untreated ots mutants compared with WT plants, although high variability in WT PDF1.2 expression may be masking differences (Fig. 4.5, bottom right panel). Resistance to Pst. and expression of the defense marker PR1 are associated with SA signalling, this raised the possibility that the SA pathway may be activated in the ots1 ots2 mutants.

In order to identify potential perturbations in the SA mediated defense pathway, basal expression of key components of the pathway was determined using qPCR. Expression of the bZIP TGA transcription factors, implicated in PR1 promoter binding and associated gene expression, was measured (see, Introduction 1.4.4). TGA1 and TGA5 were significantly up-regulated in the ots1 ots2 double mutant relative to WT (one-way ANOVA-Tukey test) (Fig. 4.6). TGA2, TGA5 and TGA6 are partially redundant and essential in the generation of SAR (Zhang et al., 2003b). TGA1 and 4 are also redundant, with tga1 tga4 double mutants displaying susceptibility to pathogens and conversely increased PR1 expression.
4.2 SA signalling is up-regulated in the *ots* double mutant

Figure 4.5.: Immune defense related gene expression is activated in the *ots1 ots2* double mutants. Quantitative PCR analysis of gene expression from 4 week old wild-type (WT), single *ots1* and *ots2* mutants, and the double *ots1 ots2* mutant of, *PATHOGENESIS-RELATED1, -2, -5* (*PR1, PR2, PR5* respectively) and *PLANT DEFENSIN1.2* (*PDF1.2*) (normalised to *ACTIN7*). Error bars represent standard error of the mean. P values for differences between WT and mutants: ** 0.001-0.01, *** 0.0001-0.001, and **** <0.0001, respectively (one-way ANOVA with Tukey test post hoc).
4.2 SA signalling is up-regulated in the *ots* double mutant

(Kesarwani et al., 2007; Lindermayr et al., 2010; Shearer et al., 2012; Gatz, 2013). These results indicate that while TGA1 and TGA4 play a role in the suppression of basal defense, they also appear to play a positive role in defense upon pathogen challenge, which may explain up-regulation in the autoimmune *ots1 ots2* double mutant. Increased expression was also found for the SA receptors, significantly so for *NPR3* (Fig. 4.6). NPR3 has been proposed to promote cell death at high SA concentrations, and may be facilitating the enhanced *Pst.* associated cell death, and with elevated TGA expression, may be leading to the resistance seen in the *ots1 ots2* double mutant (Fig. 4.4a)(Fu et al., 2012).
4.2 SA signalling is up-regulated in the *ots* double mutant

Figure 4.6.: Salicylic acid related defense gene expression is up-regulated in the *ots1* *ots2* double mutant. Quantitative PCR analysis of gene expression from 4 week old wild-type (WT), single *ots1* and *ots2* mutants, and the double *ots1* *ots2* mutant, of salicylic acid signalling pathway components, the TGA transcription factors TGA1, -2, -3, -4, -5, -6 and NON EXPRESSOR OF PRI1, NPR1, and its paralogues NPR1-LIKE PROTEIN3 and -4 (normalised to ACTIN7). Error bars represent standard error of the mean. P values for differences between WT and mutants: * 0.01-0.05, ** 0.001-0.01, and **** < 0.0001, respectively (one-way ANOVA with Tukey test post hoc).
4.3 Spontaneous lesions of dead cells are formed in the ots double mutant

4.3. Spontaneous lesions of dead cells are formed in the ots double mutant

Given the activated defense and immune signalling observed in the ots double mutant (Fig. 4.3, Fig. 4.4, Fig. 4.5 and Fig. 4.6), light microscopy was used to examine trypan blue staining of the untreated leaves of the ots double mutants and WT plants. This revealed that two week old ots1 ots2 double mutants displayed spontaneous lesions of cell death absent in WT plants (Fig. 4.7).

Figure 4.7.: The ots1 ots2 double mutant displays spontaneous lesions. Trypan blue dead cell staining with comparable leaves from two week old WT and ots1 ots2 double mutant plants.

4.4. SA biosynthesis is elevated in the ots double mutant

In order to ascertain if increased SA biosynthesis is the cause of the increased resistance and cell death observed in the ots mutant, relative gene expression of the
4.4 SA biosynthesis is elevated in the *ots* double mutant

Key enzymes responsible for catalysing SA biosynthesis was determined (Dempsey et al., 2011). In the *ots1 ots2* double mutants expression of *PHENYLALANINE LYASE1-4* (*PAL1-4*) was comparable to wild type, whereas *ISOCHORISMATE SYNTHASE1* and -2 expression (*ICS1* and -2) differed significantly (Fig. 4.8). *ICS1* was up-regulated over six fold, whilst *ICS2* appeared to be down-regulated six fold, relative to WT gene expression levels.

The opposing regulation of *ICS1* and *ICS2* gene expression prompted us to determine SA content in the *ots* double mutants. Collaborators Dr Hannah Florance and Dr Venura Perera (Prof. Murray Grant’s lab, Exeter University) used liquid chromatography-mass spectrometry (LC-MS) to measure the abundance’s of SA and glycosylated SA (SA 2-O-β-D-glucoside, SAG) in WT and the *ots* mutants (see, Methods 3.1.7). SA and SAG levels were significantly higher in the *ots1 ots2* double mutant than WT and single mutants (Fig. 4.9). Taken with Fig. 4.8, this suggests up-regulation of *ICS1* leads to increased SA levels in the *ots* double mutant. This is consistent with previous findings, which showed that *ICS1* is responsible for the majority of pathogen induced SA synthesis (Wildermuth et al., 2001). This is further substantiated by the recent finding that the mutant phenotype of EARLY FLOWERING IN SHORT DAYS4 (ESD4) SUMO protease, can be partially recovered by mutation of *ics1* (Villajuana-Bonequi et al., 2014). Jasmonic acid (JA) levels were also measured in the *ots* mutants, and these appear reduced in the *ots1 ots2* double mutant compared to WT, similar to *PDF1.2* gene expression. Although neither of these differences are statistically significant (one-way ANOVA). Repression of the JA pathway during necrotrophic pathogen challenge is not ruled out and warrants future investigation.
4.4 SA biosynthesis is elevated in the *ots* double mutant

Figure 4.8.: Salicylic acid synthesis catalysing enzyme gene expression is altered in the *ots1 ots2* double mutant. Quantitative PCR analysis of gene expression from 4 week old wild-type (WT), single *ots1* and *ots2* mutants, and the double *ots1 ots2* mutant, of salicylic acid biosynthesis genes *ISOCHORISMATE SYNTHASE1* and -2 (*ICS1* and -2) and *PHENYLALANINE AMMONIA LYASE1, -2, -3, -4* (*PAL1-4*) (normalised to *ACTIN7*). Error bars represent standard error of the mean. P values for differences between WT and mutants: * 0.01-0.05, and *** 0.0001-0.001, respectively (one-way ANOVA with Tukey test post hoc).
4.4 SA biosynthesis is elevated in the *ots* double mutant

**Figure 4.9.:** Salicylic acid content is higher in the *ots1 ots2* double mutant.

Liquid chromatography mass spectrometry quantification of salicylic acid (SA), glycosylated SA (SA 2-O-β-D-glucoside, SAG) and jasmonic acid abundance’s in wild-type (WT), single *ots1* and *ots2* mutants, and the double *ots1 ots2* mutant. Internal standards were unavailable for SAG, hence values as relative abundances. Error bars represent standard error of the mean. P values for differences between WT and mutants: *** 0.0001-0.001 and **** < 0.0001, respectively (one-way ANOVA with Tukey test post hoc).
Further confirmation that the absence of OTS1 and OTS2 were causing the heightened defenses present in the *ots* double mutant was sought out. Dr Cunjin Zhang (Dr Sadanandom’s lab, Durham University) transformed WT plants with a pEarley Gate 201 construct expressing OTS1 with an N-terminal fusion to a human influenza hemagglutinin (HA) epitope tag, behind a constitutive 35S viral promoter (OTS1-HOx1 and 2) (see, Methods 3.2.3 and 3.1.8) (Earley et al., 2006). OTS1 overexpression was confirmed by western blotting with anti HA antibodies and by *OTS1* qPCR (see, Methods 3.3). Specific bands corresponding to the approximate molecular weight for the HA-OTS1 fusion were detected (~67.2 + 9.5 = 76.7kDa) whilst no band was observed from WT extracts, demonstrating antibody specificity (Fig. 4.10a). qPCR demonstrates *OTS1* was significantly overexpressed in the OTS1-HOx1 and -HOx2 lines, with expression in HOx2 by far the greatest (Fig. 4.10c). Homozygosity of fourth generation transgenic lines was confirmed by germinating seed on glufosinate-ammonium MS agar plates, a chemical herbicide to which resistance is encoded within the BAR gene in the pEarly Gate vector (Fig. 4.10b) (see, Methods 3.3.) (Earley et al., 2006; Thompson et al., 1987).

Given the increased resistance seen in the *ots1 ots2* double mutants, *Pst.* infections were undertaken to see if overexpression of OTS1 would increase susceptibility. After three days post inoculation, although there appeared to be slightly higher levels of *Pst.* in the overexpressing lines, the difference was not significant from WT (one-way ANOVA) (Fig. 4.11a). Comparative expression of *ICS1* and *PR1* was measured in the OTS1 overexpressor lines compared to the *ots* double mutant and WT. Although previous results indicate OTS1 plays a role in restriction of the SA pathway, OTS1 overexpression did not lead to *PR1* suppression in untreated plants when compared to WT (Fig. 4.11b). *ICS1* expression did show
4.5 Confirming the cause of the *ots* double mutant phenotype

Figure 4.10.: Characterisation of the OTS1 overexpressing lines, OTS1-HOx1 and OTS1-HOx2. (a) Western blot probed with anti-HA monoclonal antibodies shows protein expression in each line. (Coomassie blue staining of blots is shown as a loading control). (b) Seedlings (T4 generation) showing resistance to glufosinate-ammonium (+). (c) Quantitative PCR gene expression analysis of *OTS1* in WT, *ots1 ots2* double mutant, OTS1-HOx1 and OTS1-HOx2 lines (normalised to *ACTIN7*). Error bars represent standard error of the mean. P values for differences between WT and overexpressing lines: * 0.01-0.05, and **** < 0.0001, respectively (one-way ANOVA with Tukey test post hoc).
some indication of reduction in the OTS1 overexpressing lines relative to WT, although again this was not statistically significant (one-way ANOVA-Tukey test). The low expression of ICS1 and PR1 in untreated WT plants may be masking differences.

Figure 4.11.: The effect of OTS1 overexpression on susceptibility to virulent Pseudomonas syringae and SA signalling. (a) Colony forming unit counts of Pseudomonas syringae pv tomato DC3000 in the leaves of 4 week old wild-type (WT), ots1 ots2 double mutant and OTS1 overexpressing lines (OTS1-HOx1 and OTS-HOx2), on the day of infiltration (day 0) and 3 days later. (b) Quantitative PCR analysis of gene expression from 4 week old WT, ots1 ots2 double mutants and OTS1-HOx1 and OTS-HOx2, of PATHOGENESIS-RELATED1 (PR1) and ISOCORISMATE SYNTHASE1 (ICS1) (normalised to ACTIN7). Error bars represent standard error of the mean. Difference between WT and ots double mutant, **** p < 0.0001 (one-way ANOVA with Tukey test post hoc).
4.5 Confirming the cause of the *ots* double mutant phenotype

To confirm that ICS1 was indeed responsible for the elevated SA content and activated defenses, the *ots1 ots2* double mutant was crossed with a single *ics1* T-DNA insertional mutant (Fig. 4.12). As *OTS1*, *OTS2* and *ICS1* all lie on chromosome 1 of *Arabidopsis thaliana*, the success of obtaining a triple mutant depended on the occurrence of homologous recombination in the region between *ICS1* and *OTS1* (less than 5.9Mbp) (see, Fig. 4.13). In order to streamline screening and improve the chances of finding a triple mutant, a protocol for PCR direct from Arabidopsis leaves was developed and optimised (see, Methods 3.2.5.2 and Fig. 4.14). Progeny from the cross was allowed to self before screening the F2 generation using primers that straddle either side of T-DNA insertion sites in *OTS1*, *OTS2* and *ICS1* mutant lines by leaf PCR (see Appendix, A.1). A range of lines with one or more homozygous mutant alleles were then chosen for genomic DNA extracts to confirm mutant allele zygosity and check for the presence of heterologous T-DNA alleles by full-length and T-DNA left border PCR. Only progeny with all three T-DNA alleles were carried through to the next generation (confirmed as either homozygous or heterozygous). This screening procedure was repeated at the F3 and F4 generations (success summarised in Fig. 4.15). Out of the 351 total plants screened across generations, none were homozygous for the three mutant alleles.
4.5 Confirming the cause of the *ots* double mutant phenotype

**Figure 4.12.** Genotyping of the *ics1* mutant. PCR products from genomic DNA extracts from WT and *ics1* single mutant, analysed by agarose gel electrophoresis. **(a)** The full-length *ICS1* PCR product (3572bp), and the T-DNA insert PCR product, amplified using a *ICS1* forward primer and reverse left border T-DNA primer (~545bp) **(b)** *ACTIN1* PCR product from all extracts as a positive extraction control.

**Figure 4.13.** Positions of *OTS1 OTS2* and *ICS1* loci on *Arabidopsis thaliana* ecotype Columbia Chromosome 1. Schematic based upon TAIR Sequence viewer (Lamesch et al., 2012).
4.5 Confirming the cause of the ots double mutant phenotype

Figure 4.14.: Validation and demonstration of leaf PCR Method used for triple ots1 ots2 ics1 genotype screening. PCR products analysed by agarose gel electrophoresis. (a) Confirmation of the T-DNA straddling primers using genomic DNA extracts from WT and respective, ots1, ots2 and ics1 single mutants. (b) Representative example of PCR directly from the leaves of progeny from ots1 ots2 crossed with ics1, screening for homozygous ots1, ots2 and ics1 mutant alleles. 18 plants shown, 3 lines have been labelled for reference.
Confirming the cause of the *ots* double mutant phenotype

Figure 4.15.: Mutant allele screening of progeny from the *ots1 ots2* double mutant cross with the *ics1* mutant. (a) Frequency of progeny with homozygous mutant allele genotypes (other alleles either homozygous or heterozygous), as detected by leaf PCR with T-DNA insert spanning primers. Yellow highlighted cells indicate the genotypes taken to the following generation (other alleles confirmed heterozygous). (b) Graphical representation of (a).
4.6 Discussion

In hindsight this was perhaps not the most efficient strategy. Instead, F1 cross progeny should be allowed to self as before, but the F2 generation should have been screened for all three T-DNA insertions but not for any homozygous T-DNA alleles. This would allow for the event whereby a single cross over had occurred leading to the inclusion of all three T-DNA insertions on the one chromosome. Assuming this occurred, it would lead to ~1 in 4 chance of producing a homozygous plant at the next generation (F3), which could then be screened for using the T-DNA straddling primers used within this study. This sequence of events was prevented by my approach. Nonetheless, there would have been no way of knowing if mutant alleles were within the same chromosome at F2, so screening large numbers of plants is unavoidable. An alternative approach would have been the production of an ICS1 knockdown line by RNA interference, which may have facilitated partial confirmation that elevated SA levels in the ots double mutant are down to ICS1 expression. As highlighted, a recent screen for suppressors of the esd4 SUMO protease mutant phenotype discovered that mutation of ICS1 reduced the esd4 mutant’s dwarf stature, early flowering, SA accumulation and SUMO conjugate accumulation (Villajuana-Bonequi et al., 2014). This taken with evidence here of elevated ICS1 expression in the ots1 ots2 double knock out, is highly suggestive that ICS1 is responsible for catalysing the SA accumulation (leading to increased resistance) in the ots double mutant also, despite failure to isolate a triple ots1 ots2 ics1 mutant.

4.6. Discussion

A Common Phenotype

Loss of OTS1 and OTS2 SUMO proteases has been shown to result in elevated SA content, heightened defense signalling and increased resistance against a virulent pathogen (Fig. 4.3, Fig. 4.4, Fig. 4.5, Fig. 4.6, Fig. 4.7, Fig. 4.8 and Fig. 4.9). The proposed cause of this defense activation is elevated ICS1 expression (Fig. 4.16).
A large number of Arabidopsis mutants have been shown to abnormally accumulate SA (Rivas-San Vicente and Plasencia, 2011). These include many lesion mimic mutants, which, like the ots double mutant, produce characteristic clusters of dead cells (Lorrain, 2003). The majority of these mutants also possess constitutively activated defense related gene expression and resistance to pathogens. In some cases this has been associated with ICS1 activity, hence may provide further clues into the role of SUMO proteases OTS1 and -2 in SA and immune regulation (Durrant and Dong, 2004). Using The Arabidopsis Information Resource (TAIR) (Lamesch et al., 2012), publications associated with ICS1 revealed sixteen mutants shown to accumulate SA attributed to ICS1 activity (Table 4.1). This association was made through elevated ICS1 expression in the mutants, and/or reversion of mutant phenotypes by the introduction of a further mutation in ICS1. These mutants mainly comprise of loss of enzyme or transcription factor function, with common implications within metabolism, growth, protein trafficking and hormone signalling. This underlines the diversity of processes associated with salicylic acid regulation and the variety of processes that OTS1 and OTS2 may be impacting upon. Significantly, mutants of characterised negative regulators of immune signalling, pub13, srfr1 and cpr1 were all shown to accumulate SA dependent upon ICS1 (Lu et al., 2011; Li et al., 2012; Gou et al., 2012; Li et al., 2010a; Kim et al., 2010; Gou et al., 2009) (see, Introduction 1.2). It seems likely that mutants of other negative regulators of PTI and ETI, such as KAPP and the two MUSE proteins identified to date, may also exhibit similarly elevated SA through ICS1 activity but this has not been tested (Gómez-Gómez et al., 2001; Huang et al., 2013, 2014). Table 4.1 is not exhaustive and other mutants displayed elevated SA levels but ICS1 implication was untested (Tang...
et al., 2005; Yaeno and Iba, 2008; Zhang et al., 2003a; Bowling et al., 1997). Similarly, mutation of other components leading to constitutive defenses and associated gene expression has been reported without subsequent measurement of SA (Wang et al., 2010a; Ichimura et al., 2006). Further SA accumulating mutants may have been published upon but may have not been annotated sufficiently for TAIR to associate them with ICS1. $Srfr1$ and $cpr1$ are examples of this, absent in the ICS1 associated publications on TAIR but have been included in Table 4.1.
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Table 4.1: SA accumulating mutants mediated by ICS1. Based on a TAIR search of (CS1) related publications (Lamesch et al., 2012).
4.7 Conclusions

Results show that the double *ots1 ots2* SUMO protease mutant possesses increased *Pst.* resistance and forms spontaneous lesions of dead cells. Presumably this is the result of greater SA content due to elevated *ICS1* expression in the *ots1 ots2* double mutant. Recently, mutants of the SUMO protease EARLY IN SHORT DAYS4 (ESD4) have also been shown to contain elevated SA content, and a suppressor screen indicated this was down to ICS1 activity (Villajuana-Bonequi et al., 2014). Both SUMO protease mutants (*ots1 ots2* double and *esd4* single) have been reported to possess higher levels of SUMO conjugates than WT plants (Conti et al., 2008; Villajuana-Bonequi et al., 2014). These findings suggest that the hypothesis made elsewhere that SUMO conjugation to transcription factors suppresses the expression of defense-related genes may be incorrect (Lee et al., 2006; van den Burg and Takken, 2010). Rather, it appears that there may be more to the cross regulation between SA and SUMOylation than simply the presence or absence of SUMOylated proteins.
5. The response of OTS1, OTS2 and SUMOylation to salicylic acid

Results presented in Chapter 4 indicate the SUMO proteases OTS1 and -2 are required to restrict SA biosynthesis and defense signalling in unchallenged plants. Elsewhere, other perturbations of the SUMOylation pathway have been shown to lead to SA accumulation and auto-immunity (Lee et al., 2007; van den Burg et al., 2010; Villajuana-Bonequi et al., 2014). Despite the apparent connection, the inductive relationship between SA and SUMOylation is poorly characterised. Building upon Fig. 4.16, a hypothesis that SA would down-regulate OTS1/2 gene expression, reducing OTS protein levels, and that this would impact on SUMO1/2 conjugation, was investigated. This was extended upon to other aspects of SUMOylation in order to gain further insight into the impact of SA on the SUMO PTM system.

5.1. Neither OTS1 nor OTS2 gene expression is SA responsive

Activation of defense related SA synthesis and signalling in the ots1 ots2 double mutants suggests OTS1 and OTS2 inhibit the SA-mediated defense pathway.
5.1 Neither OTS1 nor OTS2 gene expression is SA responsive

Thus, OTS1 and OTS2 should be down-regulated during defense activation in wild type (WT) plants. To test this hypothesis WT plants were sprayed with SA and samples taken over a 36 hour time-course for RNA extraction and cDNA synthesis (see, Methods 3.1.6 and 3.2.6). Quantitative PCR (qPCR) indicated OTS1 and OTS2 expression responded approximately equally to the control and SA spray (Fig. 5.1a and b) (see, Appendix Fig. A.1 for normalisation gene stability over treatments). Significant changes in OTS1 or OTS2 gene expression in response to SA were not detected compared to control (solvent water sprays) at the most differential time-points analysed by Student’s t-test (12hr OTS1 and 24hr OTS2). The SA responsive gene PR1 was clearly up-regulated under SA indicating the efficacy of treatments (Fig. 5.1c). This suggests OTS1 and OTS2 gene expression may be responsive to a common perturbation caused by both sprays—such as abrasion, the solvent (ethanol), or changes in humidity.

To further substantiate that OTS1 and OTS2 gene expression was not responsive to SA, the functional analogue 2,6-Dichloropyridine-4-carboxylic acid (INA) was utilised in plate treatments (Ward et al., 1991; Uknes et al., 1992; Delaney et al., 1994) (see, Methods 3.1.6). Seed were germinated on MS medium containing INA or solvent, hence removing the variability associated with performing an aerosol treatment. Comparative qPCR similarly indicated OTS1 and OTS2 expression did not significantly differ between INA treatment and control (unpaired Student’s t-tests). Both PR1 and ICS1 expression were up-regulated significantly under INA exposure, confirming treatment efficacy (Fig. 5.2). INA induced up-regulation of ICS1 further highlights the feedback loop between SA and ICS1 expression reported previously (Hunter et al., 2013).
5.1 Neither OTS1 nor OTS2 gene expression is SA responsive

**Figure 5.1:** OTS1 and OTS2 gene expression is unresponsive to SA treatment. Quantitative PCR gene expression analysis of (a) OVERLY TOLERANT to SALT1 (OTS1), (b) OTS2, and (c) PATHOGENESIS-RELATED1 (PR1) (normalised to ACTIN7), in 4 week old WT plants sprayed with salicylic acid or solvent control over a time-course of 48 hours. Error bars represent standard error of the mean.
5.1 Neither OTS1 nor OTS2 gene expression is SA responsive

Figure 5.2.: OTS1 and OTS2 gene expression is unresponsive to INA treatment. Quantitative PCR gene expression analysis of OVERLY TOLERANT to SALT1 (OTS1), OTS2, PATHOGENESIS-RELATED1 (PR1) and ISOCHORIS-MATE SYNTHASE1 (ICS1) (normalised to ACTIN7), in 10 day old WT seedlings grown in the presence of INA or solvent (Control). Error bars represent standard error of the mean. ** p value 0.001-0.01 (unpaired Student’s t-test).
5.1 Neither OTS1 nor OTS2 gene expression is SA responsive

Results from the previous chapter indicate that OTS1 and OTS2 negatively regulate SA biosynthesis and signalling. \( PR1 \) expression was measured in the \( ots \) mutants after SA treatment in order to determine if there was further capacity for SA induction of defense signalling. SA spray treatment of the \( ots1\ ots2 \) double mutant was undertaken alongside WT plants (these were analysed for \( OTS1 \) and \( OTS2 \) expression above (Fig. 5.1)). \( PR1 \) gene expression in \( ots1\ ots2 \) double mutant was similar between SA and control treated plants, although at the twelve hour time-point there did appear to be some increase in \( PR1 \) expression up to similar levels as WT under SA treatment (Fig. 5.3a). These observed differences are not statistically significant and appear to be masked by the high basal expression and variability between samples (Student’s t-test).

INA plate treatments were again used to provide further clarification of the responsiveness of \( PR1 \) expression in the \( ots \) double mutants compared to WT. Additionally, the \( OTS1 \) overexpressing lines (OTS1-HOx1 and -HOx2), were also tested to see if overexpression of \( OTS1 \) is able to suppress SA responsive gene expression under hormone induction (Ox lines characterised previously, Fig. 4.10). Both the \( ots \) double mutant and WT appeared to respond to INA displaying elevated \( PR1 \) expression under 20\( \mu \)m INA (Fig.5.3b). Interestingly the double \( ots \) mutant showed further capacity for induction of \( PR1 \) under 40\( \mu \)m INA (significant when analysed by multi-way ANOVA with Tukey test post hoc). \( ICS1 \) expression was also examined, with the \( ots1\ ots2 \) mutant showing significantly higher induction of \( ICS1 \) expression than WT. These results suggest \( ICS1 \) gene expression is deregulated in the \( ots \) mutant. INA induction of \( PR1 \) and \( ICS1 \) was not significantly suppressed in the \( OTS1 \) overexpressing lines (ANOVA).

Previously, basal expression of \( ICS1 \) in four week old plants was not found to be suppressed in the two \( OTS1 \) overexpressing lines (Fig.4.11b). In ten day old seedlings \( PR1 \) is significantly repressed in OTS1-HOx1, the lower \( OTS1 \) overexpressor of the two (Fig.5.3b control). This may indicate a disproportionately significant role of OTS1 in restricting initiation of the SA biosynthesis pathway.
5.1 Neither OTS1 nor OTS2 gene expression is SA responsive

in early development, which manifests in the ots1 ots2 double mutant gradually.

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**Figure 5.3.:** The ots1 ots2 double mutant is responsive to SA treatment. Quantitative PCR gene expression analysis of (a) PATHOGENESIS-RELATED1 (PR1) in 4 week old WT and ots1 ots2 double mutant plants treated with salicylic acid or control (solvent) sprays over 48 hours, (b) PR1 and ISOCHORISMATE SYNTHASE1 (ICS1) in 10 day old WT, ots1 ots2 double mutant and transgenic OTS1 overexpressing (OTS1-HOx1 and -HOx2) plants grown in the presence of INA (20µg/ml or 40µg/ml) or solvent (control). Error bars represent standard error of the mean. P values for differences between WT and mutants: ** 0.001-0.01, and **** < 0.0001, respectively (multi-way ANOVA with Tukey test post hoc).
5.2 SA promotes degradation of the SUMO protease OTS1

Previously the differences between *ICS1* and *PR1* gene expression in the *ots* mutants compared to WT had been undertaken in mature plants (four week old) (Fig. 4.5 and Fig. 4.8). It is noticeable that in ten day old seedlings expression of these genes is lower and differences between WT and the *ots* mutants much smaller. Examination of *ICS1* expression in samples taken from plants of different ages, indicates expression increases as plants mature and that this appears to be less restricted and more incremental in the *ots1 ots2* double mutant over 28 days (Fig. 5.4). These preliminary observations further indicate the role that OTS1 and OTS2 play in restricting SA biosynthesis over development.

![Figure 5.4: *ICS1* gene expression increases with maturation. Quantitative PCR gene expression analysis of *ICS1* (normalised to *ACTIN 7*) in 10 day, 18 day and 28 day old WT and *ots1 ots2* double mutant plants. Error bars represent standard error of the mean.](image)

5.2. SA promotes degradation of the SUMO protease OTS1

Mutation of *OTS1* and *OTS2* leads to higher SA levels (shown previously Fig. 4.9), whilst OTS1 and OTS2 gene expression did not respond to SA or the functional analogue INA (Fig. 5.1 and Fig. 5.2). These results suggest that SA-mediated regulation of the OTS proteases occurs post-translationally. The effects of SA upon OTS stability was investigated using the OTS1 overexpressing line OTS1-HOx2.
5.2 SA promotes degradation of the SUMO protease OTS1

The human influenza hemagglutinin (HA) epitope tag fusion to the N-terminus of OTS1 was used to monitor OTS1 protein stability by western blotting with HA antibodies (see, Materials 2.8 and Methods 3.3). Specificity of the antibody and expression of the full length HA-OTS1 fusion was confirmed previously (Fig. 4.10). Plants sprayed with SA show a depletion of OTS1 after 1 hour with no HA-OTS1 visible at 3 hours after treatment, whilst HA-OTS1 levels appear stable in control sprayed plants (Fig. 5.5a). The 26S proteasome is a conserved enzyme complex, which provides precise and selective degradation of proteins in the cell (Schrader et al., 2009; Vierstra, 2009). A complementary approach to examine SA induced OTS1 degradation was undertaken using the 26S proteasomal inhibitor MG132 (see, Methods 3.1.6). Seedlings treated with SA for thirty minutes showed greater depletion of OTS1 relative to control seedlings, with MG132 preincubation partially stabilising OTS1 under SA treatment (Fig. 5.5b). Clearly OTS1 SUMO protease abundance is SA-dependent providing further evidence of the apparent cross-link between SUMOylation and SA signalling. *Pst.* challenge causes SA accumulation and therefore, should also induce the degradation of OTS1 (Mishina and Zeier, 2007). This was investigated with the avirulent AvrRPM1 expressing *Pst.* strain, which induces rapid SA accumulation through host ETI. Western blots of protein extracts from OTS1-HOx2 plants infiltrated with *Pst.* AvrRPM1, indicate OTS1 degradation was maintained throughout the ten hours tested (Fig. 5.5c). OTS1 degradation was also observed one hour after control and virulent *Pst.* treatments, indicating OTS1 appears to be degraded by the wounding caused during infiltration.
Figure 5.5.: OTS1 degradation is promoted by salicylic acid and ETI. Western blots probed with anti-HA monoclonal antibodies showing OTS1 stability using the transgenic line OTS1-HOx2: (a) 4 weeks old, sprayed with SA or solvent (control) over a 6 hour time-course; (b) 10 days old, grown in liquid half MS treated with SA (+SA) or solvent (-SA) following pre-incubation with 26S proteasome inhibitor MG132 (+) or solvent (-); (c) 4 weeks old, infiltrated with water (control), *Pseudomonas syringae pv tomato* DC3000 and *Pst*. AvrRPM1, over a 10 hour time-course. Asterisks (*) in-line with HA-OTS1 bands. (Coomassie blue staining of blots is shown as a loading control)
5.3. SA promotes SUMO conjugate accumulation without SUMO1/2 induction

It has been reported that SUMO1 and -2 gene expression is not induced by SA, rather expression being constitutively high in untreated tissues (van den Burg et al., 2010; Budhiraja et al., 2009). SUMO proteases play dual roles in processing immature SUMO and deconjugating mature SUMO from its substrates. Given that SA promotes OTS1 degradation, the hypothesis that SA would impact upon net SUMO1/2 conjugation was tested.

SUMO conjugate levels in equalised total protein extracts was determined by western blotting with SUMO1/2 antibodies (see, Materials 2.8, Methods 3.3 and below, for antibody specificity Fig. 5.9). Exogenous SA treatments of wild-type (WT) plants led to the accumulation of SUMO1/2 conjugates compared to control, within one hour of SA application and their levels remained elevated for up to six hours (Fig. 5.6a). SUMO1/2 conjugate accumulation coincides with OTS1 degradation seen previously (Fig. 5.5). Treatments were repeated with sampling at 0.5 and 1 hour post SA treatment, indicating SUMO conjugate accumulation occurs between thirty minutes and one hour under SA (Fig. 5.6b). SA treatment of the ots1 ots2 double mutant also showed SUMOylated proteins accumulated within an hour of SA treatment (Fig. 5.6c). Increased levels of high molecular weight (~250kDa) conjugates appeared to accumulate in SA treated ots1 ots2 mutant plants compared to WT, 1 hour after treatment, indicative of polySUMOylated conjugates. As SA promotes OTS1 degradation, lower levels of SUMO conjugates in SA treated WT plants relative to the ots double mutant suggests either- SA differently regulates OTS2 (i.e. SA does not promote OTS2 degradation or it’s degradation is less sensitive to SA), or- that de novo OTS1/2 protein synthesis is dampening SUMO conjugate accumulation in WT plants. SUMO1/2 monomers (~11kDa) also appear reduced in the ots double mutants (Fig. 5.6c). It is likely that this is due to reduced recycling from deSUMOylation
of conjugates because, although SA appears to induce more SUMO conjugation, there is no evidence for additional production of SUMO or processed SUMO in the double mutant lines. Under experimental conditions the timing of OTS1 degradation and SUMO1/2 conjugate accumulation preceded SA responsive gene expression, indicating defense-related gene expression may be controlled by SUMO conjugation (Fig. 5.5, Fig. 5.6 and Fig. 5.1c).
5.3 SA promotes SUMO conjugate accumulation without SUMO1/2 induction

Figure 5.6.: SUMO conjugate accumulation is promoted by salicylic acid. Western blots probed with anti-SUMO1/2 polyclonal antibodies showing SUMO1/2 and their conjugates in 4 week old plants sprayed with salicylic acid or solvent (control): (a) wild-type (WT) over 6 hours, (b) WT at 0.5 and 1 hour, and (c) WT compared to ots1 ots2 double mutants over 6 hours. (Coomassie blue staining of blots is shown as a loading control).
5.3 SA promotes SUMO conjugate accumulation without SUMO1/2 induction

*SUMO1* and *SUMO2* have been previously reported as unresponsive to SA in spray treatments (van den Burg et al., 2010). As differences may be masked by the difficulty in producing a homogenous spray treatment, this result was verified using the same set of cDNA from previous INA plate treatments. Comparative qPCR indicates that *SUMO1* and *SUMO2* expression did not differ significantly between INA and control treatments (Student’s t-tests), while the SA responsive SUMO isoform, SUMO3 did, confirming previous reports (Fig. 5.7) (van den Burg et al., 2010). These findings lead to the conclusion that SUMO protease stability regulates the accumulation of SUMO1/2 conjugates in response to SA, not induction of *SUMO1/2* expression.

![SUMO1 and SUMO2 gene expression](image)

**Figure 5.7.:** *SUMO1* and *SUMO2* gene expression is unresponsive to INA treatment. Quantitative PCR gene expression analysis of *SUMO1*, *SUMO2* and *SUMO3* (normalised to *ACTIN7*) in 10 day old WT seedlings grown in the presence of INA or solvent (control). Error bars represent standard error of the mean. ** p value 0.001-0.01 (unpaired Student’s t-test).

To further demonstrate that INA plates are representative of salicylic acid treatments, total protein extracts were prepared from seedlings grown on MS medium containing INA or solvent control. Western blotting with SUMO1/2 antibodies indicated elevated levels of SUMO1/2 conjugates were present in extracts from
5.3 SA promotes SUMO conjugate accumulation without SUMO1/2 induction

seedlings grown on INA compared to control (Fig. 5.8), suggesting this chemical is analogous to SA.

![Western blots probed with anti-SUMO1/2 polyclonal antibodies showing SUMO1/2 conjugates in 10 day old WT seedlings grown in the presence of INA or solvent (control). (Coomassie blue staining of blots is shown as a loading control).](image)

**Figure 5.8.: SUMO conjugate accumulation is promoted by INA.** Western blots probed with anti-SUMO1/2 polyclonal antibodies showing SUMO1/2 conjugates in 10 day old WT seedlings grown in the presence of INA or solvent (control). (Coomassie blue staining of blots is shown as a loading control).

The validity of these findings is dependent on the specificity of the Abcam SUMO1/2 polyclonal antibody. The protein samples used in Fig. 5.8 were probed with a ubiquitin antibody (Agrisera) showing comparably little difference between INA and control treatments (Fig. 5.9). Upon discussion with a technical representative at Abcam I was referred to papers characterising the antibodies (Kurepa et al., 2003; Saracco et al., 2007). In these papers the isoform specificity of the SUMO1/2 antibody is demonstrated, indicating they do not cross-react with recombinant or endogenous SUMO3 in *Arabidopsis thaliana.*
5.4. Discussion

Assuming expression of the SUMO machinery, continual occurrence of SUMO conjugation and deconjugation to substrates can occur. Post-translational regulation of the (de-)SUMOylation enzymes is a key mechanism of shifting the balance between conjugation and deconjugation (Watts, 2013). Here degradation of OTS1 and accumulation of SUMO conjugates in response to SA has been shown (Fig. 5.5 and Fig. 5.6). Previously Conti et al. (2008) demonstrated that OTS1 was also degraded under salt stress, leading to SUMO conjugate accumulation. In addition to degradation, inactivation- by heat or hydrogen peroxide, and sequestration of SUMO proteases have been shown to promote SUMO conjugate accumulation in yeast and mammals (Sydorskyy et al., 2010; Pinto et al., 2012; Xu et al., 2008; Yan et al., 2010; Kim et al., 2005b; Goeres et al., 2011).

In humans, the balance between SUMOylation and deconjugation is regulated by SENP SUMO protease levels, and appears to be critical in the progression of some forms of cancer (Bawa-Khalfe and Yeh, 2010; Xirodimas and Lane, 2008). These findings in other organisms support the hypothesis that down-regulation
of SUMO proteases is a key mechanism in promoting accumulation of SUMO conjugates, as shown here in response to SA in plants.

In the previous chapter study of the ots mutants indicated that OTS1 and OTS2 negatively regulate ICS1 gene expression and SA biosynthesis. Here, SA promoted degradation of OTS1 and SUMO conjugate accumulation has been shown to precede SA stimulated defense gene expression. These results extend our model (Fig. 4.16) and support the hypothesis that OTS1 and -2 play a vital role as part of a SUMO-mediated positive SA feedback loop, acting to amplify immune responses (Fig. 5.10).

Beyond the model, further levels of negative regulation would be required to avoid inappropriate escalation of SA mediated defenses once pathogen threats have subsided. These could be through additional regulation of SUMO protease stability or independent pathways. Additionally, this model does not incorporate initiation of the SA defense pathway and further efforts are required to decipher the mechanisms and regulation behind activation of SA biosynthesis during pathogen challenge. One possibility is through ICS1 independent SA biosynthesis by other pathogen responsive pathways, such as the PAL catalysed route (Edwards et al., 1985; Davis and Ausubel, 1989; Mauch-Mani and Slusarenko, 1996) (see, Fig. 5.12).
A logical progression from findings here would be to determine what is being SUMOylated after SA treatments using established SUMO centric mass spectrometric approaches (Park et al., 2011b; Budhiraja et al., 2009; Elrouby and Coupland, 2010; López-Torrejón et al., 2013; Miller et al., 2013, 2010). Despite the large number of proteins already identified as SUMOylated substrates, there appears to be a degree of substrate selectiveness dependent on the type of stress plants are exposed to (Fig. 5.11) (Miller et al., 2013), indicating the value in this line of investigation.

**SUMOylation of regulators of ICS1 expression**

If the model holds true, SUMOylation may be exerting control over *ICS1* gene expression in a number of ways. Repressors of *ICS1* expression such as ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1) may be SUMOylated and sequestered from their role (Chen et al., 2009a). Alternatively activators of *ICS1* expression such as CAMODULIN-BINDING PROTEIN 60-LIKE G (CBP60G), SAR DEFICIENT 1 (SARD1) and WRKY28 may be SUMOylated, facilitating interactions with coactivators, or their stabilisation (van Verk et al., 2011; Zhang et al., 2010d). There is also the possibility that *ICS1* itself could be a direct target for SUMOylation. Although the majority of SUMOylation occurs in the nucleus, proteins localised in the chloroplast (where *ICS1* resides) have been detected as SUMOylated targets, whether this occurs...
Figure 5.12.: Model of ICS1 mediated defense amplification. Pathogen-responsive PAL-catalysed SA production leads to the degradation of OTS SUMO proteases. SUMO conjugates accumulate, leading to activation of ICS1 gene expression. ICS1 catalyses the production of large amounts of SA, escalating defense activation.

within the chloroplast has yet to be established (Elrouby and Coupland, 2010; López-Torrejón et al., 2013).

5.5. Conclusions

Results presented here indicate that although OTS1 and OTS2 gene expression is unresponsive to SA, SA promotes degradation of OTS1, and quite possibly other SUMO proteases. Further, this results in the accumulation of SUMO1/2 conjugates. Building upon our examination of the ots double mutant (Chapter 4), we hypothesise that the induction of ICS1 is caused by the downstream effects of this SUMO conjugate accumulation. Increased levels of ICS1 will in turn lead to further elevation of SA production, propagating and amplifying biotic stress signalling. Overall, the data indicate that OTS1 and OTS2 are key components of SA mediated defense signalling.
5.5 Conclusions

These findings conflict with conclusions made by van den Burg and Takken (2010) who suggest SUMO conjugation has a suppressive role in SA signalling.
6. A comparison between OTS SUMO protease and SIZ1 E3 ligase mutants

Results presented in Chapters 4 and 5 have led to the hypothesised model of SA induced SUMOylation increasing ICS1 expression, Fig. 5.10. For the model to hold true, you would expect mutation of the SIZ1 SUMO E3 ligase—displaying greatly reduced levels of SUMO conjugation—to be restricted in ICS1 gene expression. Given reported SA accumulation in the siz1 mutants, this does not appear to be the case (Lee et al., 2006).

Here a comparison between siz1 single, ots1 ots2 double, and ots1 ots2 siz1 triple mutants was performed, in order to test the model and address conflicting evidence presented here, and in the literature, regarding the regulation between SUMO conjugate levels and SA biosynthesis (Chapters 4 and 5, (Lee et al., 2007; van den Burg et al., 2010; van den Burg and Takken, 2010; Villajuana-Bonequi et al., 2014)).
6.1 The *ots* double mutant shares phenotypic similarities to the *siz1* SUMO E3 ligase mutant

**6.1. The *ots* double mutant shares phenotypic similarities to the *siz1* SUMO E3 ligase mutant**

Dr Lucio Conti previously performed a cross between the *ots1 ots2* double SUMO protease mutant and the *siz1* single SUMO E3 ligase mutant, and isolated a homozygous triple mutant. I confirmed the triple mutant genotype alongside the *siz1* single and *ots* double mutants using genomic PCR. Forward cloning primers were used with reverse cloning or left border T-DNA primers, to amplify WT and mutant alleles (Fig. 6.1) (see, Methods 3.2.5). Further, reverse transcription PCR using primers 3’ of T-DNA insertion sites was performed to confirm loss of gene expression in mutants (Fig. 6.2) (see, Methods 3.2.6.1 & 3.2.6.2).
6.1 The *ots* double mutant shares phenotypic similarities to the *siz1* SUMO E3 ligase mutant

![Figure 6.1: Genotyping of the triple *ots1 ots2 siz1* mutant.](image)

(a) The full-length *OTS1* gene PCR product (3579bp) and T-DNA insert confirmation PCR product (~3763bp)
(b) Full-length *OTS2* (3464bp) and T-DNA insert confirmation PCR product (~2952bp)
(c) Full-length *SIZ1* (5567bp) and T-DNA insert confirmation PCR product (~4514bp)
(d) *ACTIN1* PCR from all DNA extracts as an extraction control.
6.1 The *ots* double mutant shares phenotypic similarities to the *siz1* SUMO E3 ligase mutant

**Figure 6.2.: Loss of OTS and SIZ1 gene expression in the triple *ots1 ots2 siz1* mutant.** PCR products from cDNA prepared from RNA extracts from wild-type (WT), *ots1*, *ots2*, and *ots1 ots2* lines were analysed by agarose gel electrophoresis. Reverse transcription PCR was performed using gene specific quantitative PCR primers 3’ of genomic T-DNA insertion sites.

The confirmed SUMOylation pathway mutants were grown under short and long day light conditions, to see if any physiological characteristics were shared between them (See, Methods 3.1.1., Fig. 6.3, Fig. 6.4). Under short day light, dwarf phenotypes of all of the mutants were apparent from four weeks of the sowing date, when compared to WT (Fig. 6.3). The *siz1* single and the triple mutants display a more pronounced reduction in growth size than the *ots* double mutant, with additional wrinkling of the leaves. Short day light conditions suppress bolting and flowering (Mouradov et al., 2002). WT and the *ots* double mutants did not bolt within the nine week observation period. However, most of the triple mutant plants and some of the *siz1* plants bolted within six weeks. This suggests flowering repression is alleviated in the *siz1* mutant, as reported elsewhere (Jin et al., 2007). Further, mutation of *ots1* and *ots2* in addition to *siz1* appears to be additive to this reduction in floral suppression. If this is due to further restriction of the SUMO conjugation pathway by loss of the SUMO processing activities of OTS1 and OTS2 on top of loss of the SUMO E3 ligase, then these observations further support findings that SUMOylation has a suppressive role in flowering promotion (Jin et al., 2007; Son et al., 2014) (see, Introduction 1.3.4.).

Under long day conditions the dwarf phenotypes of all the mutants is less obvious in the first four weeks (Fig. 6.4). Rosette growth appears to arrest in all of the mutants from four weeks, whilst WT rosettes continue to expand. Again early flowering is observed in the *siz1* and triple mutants compared to WT. Although
6.1 The \textit{ots} double mutant shares phenotypic similarities to the \textit{siz1} SUMO E3 ligase mutant under short day light WT plants and the \textit{ots} mutant did not flower within nine weeks, the \textit{ots} double mutants flower earlier than WT in long days, as reported previously (Conti et al., 2008).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{growth.png}
\caption{Growth of the \textit{ots1 ots2} and \textit{siz1} mutants in short day conditions. WT, and double \textit{ots1 ots2}, single \textit{siz1} and triple \textit{ots1 ots2 siz1} mutants, grown in short day conditions. Photographs taken over nine weeks.}
\end{figure}
6.1 The *ots* double mutant shares phenotypic similarities to the *siz1* SUMO E3 ligase mutant

Figure 6.4.: Growth of the *ots1 ots2* and *siz1* mutants in long day conditions. WT, and double *ots1 ots2*, single *siz1* and triple *ots1 ots2 siz1* mutants, grown in long day conditions. Photographs taken over six weeks.
6.2 SA signalling is similarly up-regulated in *ots* SUMO protease and *siz1* SUMO E3 ligase mutants

6.2. **SA signalling is similarly up-regulated in *ots***
**SUMO protease and *siz1* SUMO E3 ligase mutants**

It has been previously shown that, like the *ots1 ots2* double mutants, *siz1* mutants accumulate SA coupled with displaying constitutively active pathogen defense responses ([Lee et al., 2006; Kim, 2009](#)). As discussed previously SA accumulation can result in the formation of lesions of dead cells ([Lorrain, 2003](#)). This has been reported in the *siz1* mutants and similarly observed here, in the *ots1 ots2* double mutant ([Kim, 2009](#)) (Fig. 4.7). In order to confirm the *siz1* cell death phenotype and ascertain the effects of simultaneous mutations in *ots1*, *ots2* and *siz1*, trypan blue dead cell staining was undertaken of untreated plants. All of the mutants displayed spontaneous lesions of cell death at the two week developmental stage assayed (Fig. 6.5). These observations indicate loss of any of these SUMOylation pathway enzymes involved in SUMO conjugation and deconjugation results in similarly activated defenses.

To establish if the SA mediated defense pathway is up-regulated in the triple *ots1 ots2 siz1* mutant, as has been shown for the other mutants, quantitative PCR was utilised to compare gene expression of the SA defense pathway marker *PR1* in untreated plants. *Siz1*, *ots1 ots2* and *ots1 ots2 siz1* mutants all possessed similarly elevated *PR1* gene expression (Fig. 6.6). Although elevated SA signalling and content has been established in the *siz1* mutant it has not been shown if this is related to *ICS1*. *ICS1* gene expression was also elevated in all of the mutants (Fig. 6.6). These results further underline the similarities between the activated defense phenotypes of the *siz1* and *ots1 ots2* mutants. Unlike in flowering suppression, the triple mutant does not appear to be additive to the up-regulation of SA signalling or the promotion of cell death compared to the *ots1 ot2* double and *siz1* single mutants.
Figure 6.5.: The *ots1 ots2* SUMO protease and the *siz1* SUMO E3 ligase mutants display spontaneous lesions. Trypan blue dead cell staining with comparable leaves from two week old WT, and double *ots1 ots2*, single *siz1* and triple *ots1 ots2 siz1* mutant plants.
6.3 The *ots* double, *siz1* single and *siz1 ots1 ots2* triple mutants possess salicylic acid responsiveness

Figure 6.6.: Salicylic acid-related gene expression is elevated in the double *ots1 ots2*, single *siz1* and triple *ots1 ots2 siz1* mutants. Quantitative PCR analysis of basal gene expression from 2 week old wild-type (WT), *ots1 ots2*, *siz1* and *ots1 ots2 siz1* lines, of salicylic acid biosynthesis gene *ISOCHORISMATE SYNTHASE1 (ICS1)* and SA signalling gene *PATHOGENESIS RELATED1 (PR1)* (normalised to *ACTIN7*). Error bars represent standard error of the mean. Difference between WT and *ots1 ots2* *p* value of 0.01-0.05 (ANOVA with Tukey test post hoc).

### 6.3. The *ots* double, *siz1* single and *siz1 ots1 ots2* triple mutants possess salicylic acid responsiveness

The *siz1* mutants possess greatly reduced levels of drought and heat-shock induced SUMOylation (Miura et al., 2005; Catala et al., 2007). Results above describing SA mediated signalling and activated defense responses in the *siz1* and triple *siz1 ots1 ots2* appear to contradict the proposed model of *ICS1* gene expression induction via SA induced SUMOylation (Fig. 5.10). As the model is based upon SA induction, INA responsiveness of the *siz1* and triple mutants was tested (see, Methods 3.1.6). Seed of all three genotypes were germinated on hormone (INA) or solvent plates and allowed to grow for ten days before harvesting tissue for RNA extraction and cDNA synthesis (see, Methods 3.2.6). *PR1* and *ICS1* gene expression was responsive to INA in all of the mutants (Fig. 6.7). As observed previously in the *ots* double mutant, INA induced *ICS1* expression to significantly greater levels in all of the mutants compared to WT (multi-way
6.4 Discussion

ANOVA-Tukey test). There was no significant difference in INA induced \textit{ICS1} gene expression between the mutants whereas the triple mutant possessed significantly higher \textit{PR1} expression than the other mutants. The increase in \textit{PR1} induction in the triple mutant indicates an additive effect of mutation of \textit{siz1} and \textit{ots1 ots2} in \textit{PR1} gene regulation and appears to suggest further disruption of the SUMOylation pathway releases repression of \textit{PR1} induction.

![Figure 6.7](image)

\textbf{Figure 6.7.} \textit{PR1} and \textit{ICS1} gene expression is responsive to INA treatment in the \textit{ots1 ots2} double, \textit{siz1} single and \textit{ots1 ots2 siz1} triple mutants. Quantitative PCR gene expression analysis of \textit{PR1} and \textit{ICS1} (relative to \textit{ACTIN7}) in 10 day old, wild-type (WT), double \textit{ots1 ots2}, single \textit{siz1}, and triple \textit{ots1 ots2 siz1} mutant seedlings, grown in the presence of INA (+) or solvent (-). Error bars represent standard error of the mean. Difference p values: * 0.01-0.05, *** 0.0001-0.001, and **** <0.0001, respectively (multi-way ANOVA with Tukey test post hoc).

\section*{6.4. Discussion}

Here the similarities between the physiological and immune-related phenotypes of the \textit{ots1 ots2} SUMO protease double mutant, the \textit{siz1} SUMO E3 ligase single mutant, and the \textit{siz1 ots1 ots2} triple mutant have been shown. Results confirm that the regulation of SUMOylation is critical in restricting inappropriate activation of SA mediated immune responses in unchallenged plants. Results from Chapters 4
and 5 indicated that SUMO1/2 conjugation might act to enhance or amplify SA signalling (see, Chapters 4 and 5). The apparent contradiction here, that $siz1$ and $ots1$ $ots2$ mutant phenotypes share up-regulated SA biosynthesis whilst possessing opposing levels of SUMO conjugation suggest this model is insufficient given the complexity of the relationship. Other aspects of the functioning of these three proteins ($OTS1$ $OTS2$ and $SIZ1$), within SUMO processing, (de-)conjugation, and beyond, are likely to be important in the regulation of SA biosynthesis.

Reports that $SIZ1$ related mammalian SUMO E3 ligases exert control over transcriptional regulators through SUMOylation-dependent and -independent mechanisms (Sharrocks, 2006), suggest that there is likely to be more to these mutant phenotypes than simply the presence or absence of SUMOylated substrates. The dual roles that $SIZ1$ plays in flowering time and ABA signalling may be an indicator of this in plants (Son et al., 2014; Zheng et al., 2012). The protein-protein interactions of $SIZ1$ mediated by a putative SUMO Interacting Motif (SIM) appear to be important in SA biosynthesis regulation (Cheong et al., 2009). $Siz1$ mutants complemented with the $SIZ1$ protein with a mutated SIM under its endogenous promoter, possessed wild-type comparable heat stress induced SUMO E3 activity, yet still accumulated significantly more SA than $siz1$ mutants complemented with wild-type $SIZ1$ (Cheong et al., 2009; Garcia-Dominguez et al., 2008). It must be noted, however, that expression of the SIM mutated $SIZ1$ protein did not lead to accumulation SA up to the same levels as the untransformed $siz1$ mutant.

Western blots indicating net accumulation or reduction of SUMOylation are insufficient if it is the SUMOylation state of just a few key regulators of SA biosynthesis causing accumulation of SA in these mutants. Although SUMOylation is reduced in the $siz1$ mutant, $SIZ1$ has been shown to negatively regulate the SUMOylation of one substrate, therefore SUMOylation as a positive mechanism in SA biosynthesis should not be completely ruled out (Son et al., 2014). The Arabidopsis genome encodes other SUMO proteases and SUMO E3 ligases, these...
may be adding to the complexity of differentiating between these mutant phenotypes through redundancy or antagonism with OTS1, OTS2, SIZ1 or SUMO substrate modulation (see, Introduction 1.3.2 & 1.3.3). Given observed differences in SUMOylated substrates between stresses, different SUMO E3 ligases and SUMO proteases may play differentially significant roles dependent on the type of stress (Miller et al., 2013) (Fig. 5.11); once again highlighting the need to identify SUMOylated substrates under SA.

The dual role of SUMO proteases in SUMO deconjugation and SUMO processing adds difficulty in interpretation of SUMO protease mutant phenotypes. Presumably, within shared SUMO paralogue specific SUMO proteases, there is significant redundancy in SUMO processing. Structural studies of SUMO proteases in yeast and mammals indicate they are largely promiscuous in SUMO-substrate deconjugation (Mukhopadhyay and Dasso, 2007), whilst some SUMO proteases possess specificity between substrates bound by SUMO. Crystal structures of mammalian SENP7 indicate additional surfaces beyond SUMO binding, involved in substrate interactions (Lima and Reverter, 2008). Further, SENP1 has been shown to possess differential deconjugating activity toward two hsSUMO1 bound substrates (Shen et al., 2006b).

An alternative hypothesis has been suggested, that the balance in SUMOylation levels may be important in regulating SA biosynthesis (van den Burg et al., 2010). van den Burg et al. (2010) proposed that SUMO1 and SUMO2 prevent SA accumulation in non-infected plants with both knockdown and overexpression leading to SA accumulation. Data presented within this thesis may support this idea, although the events of SUMO protease degradation and SUMO conjugate accumulation promoted by SA still highlight a positive relationship between SUMO1/2 conjugate accumulation in SA signalling.
7. Final Discussion

The results presented in Chapters 4, 5 and 6 underline the significance of SUMOylation in the regulation of SA production and signalling. The apparent disagreement within the role that (de)SUMOylation plays in the regulation of SA production (Chapters 4 and 6) and the observations of OTS1 degradation and SUMO conjugate accumulation under SA (Chapter 5), do not align well. Thus, these topics will be discussed separately in sections below (‘SUMO and SA biosynthesis’ and ‘SA promoted SUMO conjugate accumulation’).

SUMO and SA biosynthesis

Results in Chapter 4 indicated that ots1 ots2 double mutants produce more SA due to the elevated expression of the biosynthetic ICS1 gene. This led to a model describing an amplification loop in ICS1 expression facilitated by SA-mediated OTS degradation and SUMO conjugate accumulation (Fig. 5.10). The model was challenged with siz1 lines because SIZ1 mutants accumulate fewer SUMO conjugates, despite also showing elevation of ICS1 and SA synthesis. No reduction in the induction of defense-related gene expression was observed, indicating the model to be inaccurate. It is clear we do not yet have the full picture of how SUMOylation and salicylic acid interface. Nonetheless the SUMOylation status of known (see, Chapter 5 SUMOylation of regulators of ICS1 expression) and unknown promoter-associated regulators of ICS1 gene expression could be further investigated using reverse chromatin immunoprecipitation pro-
teomics (pulling down proteins bound to the chromatin), a technique developed in other organisms but yet to be used in plants (Mittler et al., 2008; DEjardin and Kingston, 2009; Rusk, 2009). Defining the relationships between SUMO isoforms and SUMOylation enzymes, and how non-covalent SUMO-led interactions impact on this pathway, require further investigation before we can fully decipher the regulatory connection between the two. The availability of materials such as SUMO3 antibodies and lethality of sumo1/2 and siz1/hpy2 double mutants have hampered progress interpreting SA-related SUMO phenotypes, such as that observed in the ots1 ots2 double mutant here (Ishida et al., 2012; Saracco et al., 2007). However, suggestions can be derived from the literature. For example, overexpression of WT and mutated SUMO1 and -2 lacking the attachment residues for conjugation led to heightened SA synthesis and defense-related phenotypes, indicating not just SUMO conjugation but also accumulation of free SUMO impacts upon SA biosynthesis (van den Burg et al., 2010).

The significance of the deconjugative activities of OTS1 and -2 has largely been the focus of this study. SUMO proteases also play a critical role in the maturation of free SUMO as a prerequisite to SUMO conjugation. Anti-SUMO1/2 blotting in chapter 5 (Fig. 5.6c) indicated that the ots double mutant possesses significantly less free SUMO than that of WT plants. Further, the elevated levels of SUMO conjugates in the mutant may suggest depletion of available mature SUMO in the absence of the OTS SUMO proteases. Without mature SUMO new conjugates cannot be catalysed allowing parallels to be drawn between the ots1 ots2 mutant and the conjugation deficient siz1 mutants. This is also equivalent to the SUMO mutant over expression (van den Burg et al., 2010), with all cases where free SUMO cannot be conjugated leading to SA accumulation. Conversely, SA promoted SUMO1/2 conjugation in the ots double mutant (Fig. 5.6c), therefore the effects of loss of OTS SUMO protease function and SA upon unprocessed and mature SUMO1/2 levels requires closer examination before further conclusions can be made. Miller and Vierstra (2011) and Miller et al. (2013) show that
during heat stress the amount of SUMO conjugates goes up as the pool of free SUMO is depleted yet within four hours the amount free SUMO is restored to basal levels, demonstrating precise regulation of SUMO availability. The amount of free mono SUMO may be significant in limiting SUMO monomer interference with SIM interactions or access to SUMO pathway enzymes. This raises the possibility that the abundance of free SUMO modulates the whole SUMOylation pathway through interference—too much free SUMO blocks SUMO conjugation enzymes and SIM interactions, whilst too little frees enzymes up to conjugate SUMO until the pool is depleted. Hence, rather than accumulation of SUMO conjugates a hypothesis can be proposed that the regulation of SA biosynthesis is dependent on the size of this pool of SUMO monomers. This hypothesis may explain the SA related phenotypes of the ots1 ots2 mutant, with a restricted pool of SUMO monomers, and the siz1 mutant, which, unable to conjugate, presumably possesses an overaccumulation of SUMO monomers. This could be investigated through expression of mature SUMO1 behind an inducible or endogenous promoter in the background of the ots1 ots2 double mutant, which may lead to temporary reduction of SA biosynthesis. Further, the use of high percentage acrylamide gels and inhibitors of protein synthesis such as cycloheximide and puromycin could help to dissect the dynamic regulation of SUMO production versus conjugation in SA signalling (Častorálová et al., 2012).

**SA promoted SUMO conjugate accumulation**

Despite the contradictions of the siz1 and ots mutant lines, Chapter 5 clearly demonstrates that OTS1 is degraded under SA and that SUMO conjugates accumulate. The potential significance of this observation will be discussed here.
**R protein regulation**

In most cases mutation of negative regulators of immune responses appears to result in constitutive activation of immune receptors or R proteins (Gou et al., 2012; Lu et al., 2011; Li et al., 2012; Bhattacharjee et al., 2011; Li et al., 2010a; Kim et al., 2010) (see, Chapter 4 Table 4.1). This auto-immune phenotype is shared by the ots1 ots2 double mutant, posing a role for OTS1 and OTS2 in suppression of PRR or R protein activation (see, Introduction 1.2.2 & 1.2.3). The stability of R proteins is central to the activation of immune signalling in response to pathogen challenge, highlighted by observations that R protein over-expression leads to their constitutive activation (Stokes et al., 2002; Oldroyd and Staskawicz, 1998). SUMOylated substrates discovered to date include a putative TIR NB LRR R protein, and it is tempting to hypothesise that SUMOylation of R proteins acts to stabilise them (Park et al., 2011b). Further, while host SUMO proteases appear to be degraded during the activation of immune responses (Fig. 5.5), pathogens secrete SUMO protease-like effectors into host cells to subvert defense activation (Table 1.2), including PopP2 from *Ralstonia solanacearum* shown to bind to the R protein RRS1 (Deslandes et al., 2003; Tasset et al., 2010). Taken together a hypothetical mechanism can be drawn whereby OTS1/2 act to deSUMOylate PTI or ETI immune receptors in defense signalling suppression. This hypothesis is not negated by findings that the siz1 mutants are responsive to SA treatment as this regulation could lie upstream of SA mediated responses and may not be linked to the SA accumulating phenotype of the siz1 mutant.

OTS1 and OTS2 are localised in the nucleus when transiently overexpressed in *Nicotiana benthamiana* (Conti et al., 2008). If this truly reflects their localisation in unchallenged cells, regulation of immune receptors would have to occur during or immediately after protein synthesis, before receptors are exported to the membrane or cytoplasm. As transient expression is facilitated by *Agrobacterium tumefaciens* infection of *N. Benthamiana* OTS1 and -2 localisation requires closer
Hormone signalling

Rather than amplifying defense signalling, SA mediated promotion of SUMO conjugates may facilitate crosstalk with other signalling pathways. SUMOylation has been implicated in multiple hormone pathways in plants, including abscisic acid (ABA), auxin, gibberellic acid (GA), brassinosteroid (BR) and jasmonic acid (JA) (Lois et al., 2003; Miura et al., 2009; Conti et al., 2014; Zheng et al., 2012; Khan et al., 2014; Elrouby and Coupland, 2010; Miller et al., 2013). These reports pose the possibility that SUMOylation brought about by SA biosynthesis may provide a mechanism to facilitate hormone crosstalk.

The growth suppressing DELLA proteins GAI and RGA were recently shown to be SUMOylated, leading to their stabilisation and subsequent SUMOylation-induced growth inhibition (Conti et al., 2014). Here the promotion of SUMO conjugation by the defense signalling hormone SA has been demonstrated. Taken together, these findings may facilitate a SUMO orchestrated mechanism for pathogen induced switching of resources away from growth into defense - a frequently highlighted paradigm in plant host defense signalling (Alcazar et al., 2011).

Proteins in JA, ABA, and BR pathways have been found to be SUMOylated (Elrouby and Coupland, 2010; Miller et al., 2013; Miura et al., 2009; Zheng et al., 2012; Khan et al., 2014). All three are antagonistic to SA in immunity, although ABA and BR appear to play positive roles in abiotic stress tolerance (Szepesi et al., 2009; Seo and Park, 2010; Divi et al., 2010). Two JA regulatory proteins have been identified in SUMO substrate screens, MYC2 and TOPLESS (Elrouby and Coupland, 2010; Miller and Vierstra, 2011). TOPLESS forms a repressor complex with the JASMONATE-ZIM-DOMAIN PROTEIN (JAZ) group and NOVEL INTERACTOR OF JAZ (NINJA), binding to the MYC2 transcription factor and blocking JA responsive transcription (Chini et al., 2007; Pauwels
et al., 2010). Through SUMO-SIM interactions this may be resulting in repression of the JA pathway in the ots1 ots2 double mutant, due to SA promoted SUMOylation. TOPLESS acts in multiple other hormone pathways including auxin and ABA, indicating the potential global influence of SUMOylation upon hormone regulation though SIM mediated interactions (Causier et al., 2012).

An emerging mechanism in hormone pathways is stimulation of ubiquitin E3 ligases leading to the degradation of signalling repressors, such as the JAZ and DELLA proteins (Dharmasiri et al., 2013). The significance of hormone crosstalk, and SA promoted OTS1 degradation, indicate the regulation of SUMO protease stability by hormone stimulated ubiquitin E3 ligases such as NPR1, -3 and -4 is worthy of investigation.

**SUMO conjugation antagonism**

Ginkgolic acid, inhibits E1-SUMO intermediate formation in mammalian cells (Fukuda et al., 2009). If effective and specific in plants, it could provide a useful investigative tool, facilitating investigation into the significance of SUMOylation in defense signalling. Significantly, ginkgolic acid consists of salicylic acid and a long-carbon chain conjugate. Taken with findings here in plants, this proposes possible antagonism between SA and other SA conjugates in plants (Pastor et al., 2012).

**Future prospects**

Gene mutants and gene overexpression provide useful insights into biological roles of single genes at the whole organism level, a powerful tool in the molecular biologist’s repertoire. Nonetheless these perpetual biological changes can display complex phenotypic traits and caution needs to be exercised during interpretation. The complexity of mutant phenotypes is apparent here, assessing the
Final Discussion

significance of highly regulated inductive processes like SUMOylation and salicylic acid signalling. Integrative global approaches such as whole transcriptome sequencing and proteome studies, are crucial in deciphering underlying molecular mechanisms responsible for these traits, and facilitate far less investigative bias than has ever been possible previously. As proteomic technologies improve it has become apparent that PTMs do not operate in isolation but rather facilitate concerted integration of signals upon proteins in signalling pathways- the necessity to study PTMs as a whole is coming to head (Ulrich, 2012; Mertins et al., 2013). For example, salicylic acid is utilised experimentally in mammalian cell culture to induce kinase activity (Hawley et al., 2012), posing the question- is SA induced SUMO conjugate accumulation in plants facilitated by phosphorylation at phospho-dependent SUMOylation sites?- integrative proteomic approaches may provide the answer.

Knowledge transfer

The model plant Arabidopsis thaliana facilitates rapid discovery and elucidation of signalling and response pathways in plants. Once clearly defined pathways have been experimentally determined, care needs to be taken translating knowledge gained into crop species for improvement. Existing genome and transcriptome resources represent an opportunity to incorporate evolutional awareness when applying findings across genera. Traditional approaches of constitutive increase or reduction in expression have yielded limited success. The natural intrinsic complexity and elegant regulation behind the expression of individual genes is indicative of the necessity to update and innovate our approach. Given the conserved nature of SUMOylation sites, technical advances in genome editing techniques such as CRISPR provide far more precise and less intrusive ways of engineering crops than traditional crop transformation approaches (Shan et al., 2013). Given the implications of SUMOylation in abiotic, and demonstrated here, biotic stress
Final Discussion

pathways, continued research will provide opportunities to help maintain crop yields under intermittent and persistent stresses present in the environment.
# A. Appendix 1.

## A.1. Primers

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A.1 Primers

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<tbody>
<tr>
<td>MX171 PR5 RTR</td>
<td>GCTCCGGTACAAGTGAGGGTGCTCGTT</td>
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**Quantitative PCR SUMO pathway**

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<tr>
<td>MX331 OTS1 RTF</td>
<td>TCTCACCCTGCTCTAAACAGAGT</td>
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<tr>
<td>MX332 OTS1 RTR</td>
<td>ACCTATGAGTCTGTAAGCCA</td>
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<tr>
<td>MX333 OTS2 RTF</td>
<td>CAGTGCTCAAGTTGGTTCAGCTCT</td>
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<td>MX334 OTS2 RTR</td>
<td>AGCAATGTTGGGCTTTTTATTCGGA</td>
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<td>MX174 SUMO1 RTF</td>
<td>TGCTTACTGTGACCCGGAAT</td>
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<tr>
<td>MX175 SUMO1 RTR</td>
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<td>MX339 SUMO2 RTR</td>
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<td>MX336 SUMO3 RTF</td>
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<td>MX337 SUMO3 RTR</td>
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<td>MX176 SIZ1 RTF</td>
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<td>MX177 SIZ1 RTR</td>
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**Genotyping**

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<td>MX222_LBb1.3</td>
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<td>MX229_Siz1FLF</td>
<td>ATGGATTTGGAAGCTAATTGTAAGG</td>
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<td>MX330_Siz1 FLR</td>
<td>TAAAACCTCCGGTGCTCCTG</td>
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<td>MX178 ICS1 FLF</td>
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<td>MX179 ICS2 FLF</td>
<td>CACCATGGCGGTGCTCAG</td>
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<td>MX16 OTS1 FLF</td>
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<td>MX18 OTS1 FLR</td>
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<td>MX21 OTS2 FLF</td>
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<td>MX23 OTS2 FLR</td>
<td>TTAATCGTTGTGGTACCCTTGC</td>
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<tr>
<td>MX223 ICS1 TDNAStradR</td>
<td>AGCAGCAGAATCGGATTTGTC</td>
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<td>MX224 OTS1 TDNASradF</td>
<td>GGTCTGAAAATGTACGACATTTAC</td>
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<tr>
<td>MX225 OTS1 TDNAStadR</td>
<td>TCTGTCTGGTACGACAC</td>
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### A.1 Primers

<table>
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<th>Primer</th>
<th>Sequence</th>
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<td>MX226 OTS2</td>
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<tr>
<td>MX227 OTS2</td>
<td>CAAGTGAATTATAGTCAATCCCG</td>
</tr>
<tr>
<td>TDNASTradR</td>
<td>CAAGTGAATTATAGTCAATCCCG</td>
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A.2 Quantitative PCR

A.2. Quantitative PCR

Housekeeping selection

Figure A.1.: *ACTIN7* housekeeping gene stability under SA treatment. Amplification curves using *ACTIN7* primers over three biological reps of SA spray treatments, showing no indication of clustering between treatment and control demonstrating suitability of housekeeping gene for normalisation. Red lines=untreated, green lines=SA spray, blue lines=control (solvent) spray.
A.2 Quantitative PCR

Figure A.2.: Comparison of two different housekeeping genes for normalising \textit{ICS1} expression in the \textit{ots} mutants. Quantitative PCR analysis of gene expression from 4 week old wild-type (WT), single \textit{ots1} and \textit{ots2} mutants, and the double \textit{ots1 ots2} mutant, of salicylic acid biosynthesis genes \textit{ISOCHORISMATE SYNTHASE1} normalised to \textit{\textit{\beta}-TUBELIN} (left panel) or \textit{ACTIN7} (right panel). Error bars represent standard error of the mean. \textit{P} values for differences between WT and ots double mutants: * \textit{0.01-0.05}, and *** \textit{0.0001-0.001}, respectively (one-way ANOVA with Tukey test post hoc).
A.2 Quantitative PCR
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