Protein modification in plant innate immunity

by

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Abstract

Plant diseases cause major crop losses worldwide. Crop protection strategies enhancing the plants' own defence mechanisms could be a sustainable solution to ensure future food supply. This thesis describes my research effort to better understand the innate defence mechanisms in plants.

Specific resistance responses towards invading pathogens are mediated by Resistance (R) proteins. They recognize pathogen-derived molecules and activate signalling cascades, initiating physiological responses to limit pathogen spread in infected cells while minimizing harmful effects on the rest of the plant.

We use the unique gain-of-function *R* gene *snc1* as a tool to identify components of resistance signalling in *Arabidopsis thaliana.* In a screen for suppressors of *snc1*-mediated constitutive resistance, we identified a number of *modifier of snc1* (*mos*) mutants. My thesis focuses on the identification and characterization of *mos5* and *mos8.* Both mutations partially suppress *snc1*-associated morphological phenotypes and revert susceptibility to virulent pathogens to wild type levels.

mos5 contains a deletion in one of two ubiquitin activating enzyme genes in *Arabidopsis.* The mutation in *mos5* lies in a putative binding domain, potentially disrupting interaction with downstream ubiquitin acceptors. The *mos5* single mutant displays enhanced susceptibility to virulent bacteria, as well as to bacteria carrying the effector protease AvrRpt2, indicating a role of ubiquitination in both specific and basal resistance. A mutation in the *mos5* homolog *UBA2* does not affect resistance, however, a double mutant *mos5 uba2* is lethal, indicating that the two genes are partially redundant.

mos8 is allelic to *enhanced response to abscisic acid 1* (*era1*), which encodes the beta subunit of protein farnesyltransferase. Mutations in the gene are known to affect development and abscisic acid signalling. *mos8* displays enhanced susceptibility to virulent and avirulent pathogens and acts additively with *NPR1.* Defects in geranylgeranylation, a protein modification similar to farnesylation, do not affect resistance responses against virulent or avirulent pathogens.

Taken together, my data reveals the importance of post-translational modification of yet to be identified regulatory proteins in plant innate immunity. Further research will aim at unravelling the mechanisms by which *mos5* and *mos8* affect resistance signalling.

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List of Abbreviations

ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
ATP	adenosine triphosphate
Avr	avirulence
BAC	bacterial artificial chromosome
bp	basepair
C	cytosine
C	carboxy
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CC	coiled coil
cDNA	complementary DNA
cfu	colony forming units
DNA	deoxyribonucleic acid
dpi	days post inoculation
E1	ubiquitin activating enzyme
E2	ubiquitin conjugating enzyme
E3	ubiquitin ligase
ET	ethylene
g	gram
G	guanine
GTP	guanosin triphosphate
GUS	beta glucuronidase
h	hour
HPLC	high performance liquid chromatography
HR	hypersensitive response
JA	jasmonic acid
kb	kilobases
LRR	leucine rich repeat
m	milli
Mb	Megabases

MgCl2	magnesium chloride
µ	micro
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog
N	amino
NBS	nucleotide binding site
ºC	degree Celsius
OD_{600}	optical density at 600 nm
P	probability
P.p.	*Peronospora parasitica*
P.s.m.	*Pseudomonas syringae pv. maculicola*
P.s.t.	*Pseudomonas syringae pv. tomato*
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PFT	protein farnesyltransferase
PGGT	protein geranylgeranyltransferase
PR	pathogenesis related
PRR	pathogen recognition receptor
R	resistance
RING	really interesting new gene
RLK	receptor-like kinase
RNA	ribonucleic acid
ROI	reactive oxygen intermediates
RT-PCR	reverse transcriptase polymerase chain reaction
RUB	related to ubiquitin
SA	salicylic acid
SAIL	Syngenta Arabidopsis Insertion Library
SAR	systemic acquired resistance
SUMO	small ubiquitin-like modifier
TAIR	The Arabidopsis Information Resource

T-DNA	transfer DNA
TIR	Toll/Interleukin 1-receptor
TLR	Toll-like receptor
UTR	untranslated region

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Meinen Grossmüttern
Katharina (in Memoriam)
und Annemarie

Co-Authorship Statement

The work described in this thesis is the culmination of research from 2003 to 2006. Below is a list of papers that have been accepted for publication or are in preparation as a result of this work, and the contribution made by the candidate:

Goritschnig, S., Zhang, Y. and Li, X. The ubiquitin pathway is required for innate immunity in *Arabidopsis*. Plant J. in press.

The candidate performed all experiments described in the paper and wrote the manuscript. Y. Zhang was involved in the mutant screen from which the mutant was isolated and in the map-based cloning of the mutation. X. Li designed the suppressor screen and supervised the work and manuscript preparation.

Goritschnig, S., Zhang, Y. and Li, X. A novel role for *Arabidopsis* farnesyltransferase in plant innate immunity (in preparation).

The candidate performed all experiments described and wrote the manuscript. Y. Zhang was involved in the mutant screen from which the mutant was isolated and in the map-based cloning of the mutation. X. Li designed the suppressor screen and supervised the work and manuscript preparation.

1. Introduction

Plants provide the basic nutritional resource for most other living organisms on the planet. They are constantly challenged by microbial pathogens, such as bacteria, fungi and viruses and thus have evolved a multi-layered and complex network of defence responses, including physical barriers (e.g. bark and cuticle), pre-formed and *de novo* produced chemical deterrents (e.g. secondary metabolites and phytoalexins), and pathogen-specific induced responses. In the face of fitness costs of resistance, it is clear that induced defences have to be tightly regulated, and aspects of their genetic basis are currently emerging.

In animals, specialized cells of the circulatory blood system, such as lymphocytes or macrophages, execute immune responses. In plants, on the other hand, every cell is a potential target of the attacking pathogen, and thus has to autonomously possess the ability to signal and respond in the case of an infection.

In the following section, our current understanding of the different layers of plant defence and a number of important components and signalling cascades identified by molecular biology approaches are reviewed.

1.1 Non-host resistance in plant immunity

Despite the large number of potential phyto-pathogenic species, disease is a rare occurrence. The predominant form of plant resistance is the so-called "non-host" resistance, whereby an entire plant species is resistant to a certain pathogen species (Heath, 2000). Non-host resistance is conferred by a number of pre-formed and induced defences that prevent microbial entry and generally provide an unfavourable environment for microbial proliferation. Among the constitutive barriers are cuticular wax layers, antimicrobial secondary metabolites and peptides (Heath, 2000). Recent reports suggest a particularly important role of the plant cell wall in non-host interactions (Schulze-Lefert, 2004). This is not surprising, considering the physico-chemical properties of the cell wall, which shields and stabilizes the protoplast. Cell wall fortification, callose deposition and polarized vesicle transport

constitute important inducible responses in non-host resistance to fungal pathogens (Collins et al., 2003; Nishimura et al., 2003).

Once a pathogen manages to overcome this first obstacle, it is facing another level of defence that likely contributes to defence against non-host pathogens, induced by general pathogen-associated molecular patterns (PAMPs). PAMPs are molecular structures common to a wide range of microbes important for their specific lifestyles and include chitin, lipopolysaccharides, and bacterial flagellin. These conserved microbial features are recognized by pattern recognition receptors (PRRs), which share similarities between animal and plant kingdoms (Ausubel, 2005). In animals, Toll-like receptors (TLRs) involved in innate immunity are localized on the cell surface and contain extracellular leucine rich repeat domains (LRR), a membrane spanning domain and an intracellular TIR (for Toll/Interleukin 1-receptor) signalling domain (Underhill and Ozinsky, 2002). Signalling through the TIR domain proceeds using several adaptor molecules and induction of serine/threonine kinases, subsequent translocation of NFκB (nuclear factor kappa B) to the nucleus and induction of defence genes (Beutler et al., 2003).

In plants, recognition of PAMPs is accomplished by receptor-like kinases (RLK), which, like TLRs, contain extracellular LRRs, but instead of the intracellular TIR domain possess a serine/threonine kinase domain, which is involved in signal transduction (Dievart and Clark, 2004). Cellular responses triggered by PAMP recognition constitute a plant's basal defence, in which physiological changes, such as changes in ion fluxes, production of reactive oxygen intermediates (ROI) and other signalling molecules occur to limit pathogen spread. Recognition of PAMPs in non-host resistance results in activation of signalling cascades that show significant overlap with those associated with cultivar-specific resistance and are discussed below.

1.2 Gene-for-gene resistance

In an evolutionary arms race, pathogens have acquired genes encoding effector proteins that are essential for virulence and are believed to function in subverting basal defence responses (Nomura et al., 2005). Plants, in return, have evolved receptors that specifically recognize the virulence action of pathogen effectors, resulting in avirulence of the pathogen and resistance of the plant. This cultivar-specific recognition is best described by the gene-for-gene concept (Flor, 1971), whereby an incompatible interaction is observed when the plant possesses a *Resistance* (*R*) gene and the attacking pathogen the cognate *Avirulence* (*Avr*) gene. All other compatible interactions, where plants do not recognize pathogen effectors, either because they do not contain the *R* gene or the pathogen does not have the *Avr*-gene, lead to disease (Flor, 1971).

The major class of R proteins identified predominantly in *Arabidopsis thaliana* are cytosolic proteins containing conserved nucleotide-binding site (NBS) and leucine rich repeat (LRR) domains, presumably involved in ATP or GTP binding and hydrolysis and protein-protein interaction, respectively (McHale et al., 2006). The domain architecture of the NBS-LRR proteins is reminiscent of another class of mammalian innate immunity receptors, the intracellular NOD (nucleotide-binding oligomerization domain) proteins, again stressing similarities between plant and animal innate immune signalling (Inohara and Nunez, 2003). Based on their N-terminal domains, NBS-LRR proteins can be further subdivided into Toll-Interleukin1 receptor (TIR) or coiled-coil (CC) NBS-LRRs, and these subclasses activate distinct but overlapping branches of resistance signalling pathways (Aarts et al., 1998). The sequenced *Arabidopsis* genome contains approximately 150 NBS-LRR genes, a small number for the vast diversity of potentially invading pathogens (Meyers et al., 2003).

In vivo physical interaction between R protein and pathogen Avr elicitor has only been shown in a few instances (Jia et al., 2000; Deslandes et al., 2003). In the majority of cases, recognition of pathogen elicitors occurs indirectly. In the guard model described initially by van der Biezen and Jones, R proteins are hypothesized to monitor (or "guard") a small set of host proteins, which function as pathogen

effector targets and might play a role in basal resistance (Van der Biezen and Jones, 1998). Modification of guarded proteins by the pathogen effector results in activation of the R protein and a defence response. In the absence of the respective R protein, the effectors provide a selective advantage for the pathogen in colonization of the host, by suppressing host basal defences and/or promoting disease (Chang et al., 2004).

The activation of R proteins triggers rapid physiological changes in the plant cell, similar to the ones triggered by PAMP recognition. These include redox changes, production of signal molecules such as ROI or salicylic acid (SA), and induction of defence gene transcription. Those responses usually culminate in localized cell death at the site of infection, referred to as the hypersensitive response (HR), which limits pathogen spread. Race-specific resistance signalling is thought to be superimposed on the basal defence response, which acts more slowly and at a lower amplitude to restrict pathogen growth (Dangl and Jones, 2001; Tao et al., 2003; Eulgem et al., 2004). Our understanding of how recognition of pathogens by NBS-LRR R proteins is transduced into a defence response is the focus of intensive investigations. Several regulators of plant disease resistance have been identified, and their potential functions shed light on signalling events involved in R protein mediated defence.

1.3 Signalling components in plant immunity

A series of elegant genetic screens were applied to identify components of resistance signalling pathways, some of which were also shown to be involved in basal defence. According to the "guard" hypothesis, NBS-LRR R proteins act within macromolecular complexes (Shirasu and Schulze-Lefert, 2003). Their abundance and activity has to be tightly regulated to avoid inappropriate defence responses and the associated fitness costs.

R protein complex association and stability has been shown to depend on several chaperonins. Heat shock protein 90 (Hsp90) interacts with several NBS-LRR proteins and has been shown to be essential for their function (Hubert et al., 2003; Lu et al., 2003; Liu et al., 2004). Hsp90 might act as a chaperonin to stabilize the

protein and/or complex in anticipation of elicitation. Required for Mla12 Resistance 1 (RAR1) and Suppressor of G2 Allele of SKP1 1b (SGT1b) were independently identified as essential components in responses by several R proteins (Azevedo et al., 2002; Muskett et al., 2002). Together with Hsp90 they have subsequently been shown to associate with and to affect stability of some R protein complexes (Hubert et al., 2003; Bieri et al., 2004; Holt et al., 2005). Together they appear to function to maintain a certain R protein threshold level, enough for rapid initiation of defence upon pathogen recognition, but too little to cause deleterious effects due to inappropriate activation of defence responses (Bieri et al., 2004; Azevedo et al., 2006). The detrimental impact of inappropriate activation has been shown for the R protein RPS2 (Resistance to *Pseudomonas syringae* 2), which is activated upon disappearance of its negative regulator RIN4 (RPM1 interacting protein 4). In a *rin4* null mutant RPS2 is hyperactivated, resulting in the death of the plant (Mackey et al., 2003).

RIN4, a protein that physically interacts with the two R proteins RPM1 (Resistance to *Pseudomonas syringae* pv. *maculicola* 1) and RPS2, serves as an excellent example of the "guard hypothesis", as it is a direct target of several pathogen effectors that differentially modify RIN4 to suppress basal defence. Infection with *Pseudomonas syringae* carrying the effectors AvrB or AvrRPM1 triggers phosporylation of RIN4 resulting in activation of RPM1-mediated defence responses (Mackey et al., 2002). RIN4 is also a target for proteolytic cleavage by the cysteine protease AvrRpt2, and subsequent degradation of RIN4 results in activation of RPS2-mediated resistance (Mackey et al., 2003; Kim et al., 2005).

Other examples of R protein activation through proteolytic activity of bacterial effectors are RPS5 in *Arabidopsis* and Cf-2 in tomato. RPS5 is activated upon cleavage of the protein kinase PBS1 (avrPphB susceptible 1) by the *P. syringae* effector AvrPphB. However, direct interaction between RPS5 and PBS1 has not been documented (Shao et al., 2003). The resistance of tomato against the fungal pathogen *Cladosporium fulvum* carrying the effector Avr2 depends on the R protein Cf-2. Cf-2 is a transmembrane protein with extracellular LRRs involved in indirect recognition of the pathogen effector, which requires the secreted plant protease Rcr3 (Thomas et al., 1998; Rooney et al., 2005).

Apart from these direct and indirect R protein interactors primarily involved in R protein activation, a number of downstream components of resistance signalling pathways have been identified. The membrane-localized protein NDR1 (non-race-specific disease resistance 1) is required for resistance mediated by the CC-NBS-LRR R proteins RPM1, RPS2 and RPS5, and its overexpression results in enhanced disease resistance (Century et al., 1995; Aarts et al., 1998; Coppinger et al., 2004). TIR-NBS-LRR proteins require the action of EDS1 (Enhanced Disease Susceptibility 1), which was first identified as a suppressor of RPP5 (Resistance to *Peronospora parasitica* 5) mediated resistance towards pathogenic oomycetes (Parker et al., 1996). EDS1 forms temporally and spatially distinct associations with its homologs PAD4 (PhytoAlexin Deficient 4) and SAG101 (Senescence Associated Gene 101), and these distinct complexes are important in basal defence signalling, particularly in promoting the HR (Feys et al., 2001; Feys et al., 2005; Wiermer et al., 2005). The EDS1 protein associations might shuttle between the nucleus and cytoplasm, however, their biochemical role in resistance responses remain to be determined. EDS1 and PAD4 are required for induced production of the signalling molecule salicylic acid (SA) upon R protein activation (Feys et al., 2001). SA accumulation is required to potentiate defence responses and to propagate systemic acquired resistance (SAR; Vernooij et al., 1994; Shah, 2003). SAR is a durable state of heightened resistance in distal parts of the plant that is brought on by a primary infection (Ryals et al., 1996). Mutants with defects in pathogen-inducible SA-production, such as *sid2* (*salicylic acid induction deficient 2*; Nawrath and Metraux, 1999; Wildermuth et al., 2001) or *eds5* (Nawrath et al., 2002), do not accumulate SA and are defective in mounting appropriate local and systemic defence responses. In addition to its role in transducing defence responses to biotrophic pathogens, SA also acts antagonistically to responses mediated by the jasmonic acid/ethylene (JA/ET) signalling pathway controlling defences against necrotrophic pathogens and herbivores (Glazebrook et al., 2003).

NPR1 (non-expressor of pathogenesis related genes 1) is an important signalling component downstream of SA induction. Mutant *npr1* alleles were identified in several screens, looking for non-responsiveness to SA or lack of SA-induced resistance (Cao et al., 1994; Ryals et al., 1997; Shah et al., 1997). NPR1

oligomerizes in the cytosol and SA-induced redox changes cause hydrolysis of the connecting disulphide bonds, allowing NPR1 monomers to translocate to the nucleus where they bind to a subclass of TGA transcription factors to activate transcription of *PR* genes (Zhang et al., 1999; Mou et al., 2003).

A number of additional regulatory genes with a variety of potential roles in resistance have been identified, but much work is still necessary to further define signal transduction cascades and to identify correlations between gene expression patterns and disease resistance.

1.4 The *Arabidopsis* suppressor of npr1-1 constitutive 1 (snc1) auto-immune model

In an effort to dissect the involvement of NPR1 in plant defence signalling, a suppressor screen for mutations that restore SA responsiveness was conducted. Two classes of suppressors of *npr1*-mediated abrogation of SA-induced resistance were identified. One class is represented by *sni1 (suppressor of npr1-1 inducible 1),* a novel transcriptional repressor, which restored the SA responsiveness of the *npr1-1* mutant (Li et al., 1999; Mosher et al., 2006). The other class constitutively suppresses *npr1*-associated phenotypes, even without induction by SA. The first mutant cloned in the second class was *suppressor of npr1-1 constitutive 1 (snc1),* which encodes a TIR-NBS-LRR R protein homolog of RPP5, located in the *RPP4* cluster of *R* genes on chromosome 4 (Parker et al., 1997; Noel et al., 1999; Li et al., 2001a; van der Biezen et al., 2002; Zhang et al., 2003). A point mutation resulting in a Glu to Lys amino acid change in the linker region between the NBS and LRR domains renders the snc1 R protein constitutively active even without pathogen presence (Zhang et al., 2003). Interestingly, a similar mutation in the linker region of the human NBS-LRR protein NOD2 has been associated with the auto-inflammatory Crohn's disease (Eckmann and Karin, 2005; Inohara et al., 2005), implying conserved mechanisms in NBS-LRR protein activation (Belkhadir et al., 2004). The constant state of alertness in the *snc1* plants presumably results in mis-allocation of resources, thus affecting development of the *snc1* mutant plant. In addition to dwarf and curly leaf morphology, *snc1* exhibits constitutive expression of several

pathogen-responsive PR genes and increased levels of endogenous SA, which is required for the manifestation of the *snc1* phenotypes. Furthermore, and most importantly, *snc1* displays enhanced resistance to virulent biotrophic bacterial and oomycete pathogens (Li et al., 2001a; Zhang et al., 2003).

Epistasis analyses have identified signalling components important for *snc1*-mediated constitutive resistance. Like other R proteins of the TIR-NBS-LRR class, *snc1* signalling is fully dependent on EDS1 and PAD4 (Li et al., 2001a; Zhang et al., 2003). The increased endogenous levels of SA in the *snc1* mutant favoured a model in which *snc1*-mediated resistance is caused by high levels of this signal molecule. However, mutants impaired in SA production, such as *eds5*, are unable to fully suppress *snc1* associated phenotypes, indicating the existence of an SA-independent branch of signalling downstream of *snc1* (Figure 1.1; Zhang et al., 2003).

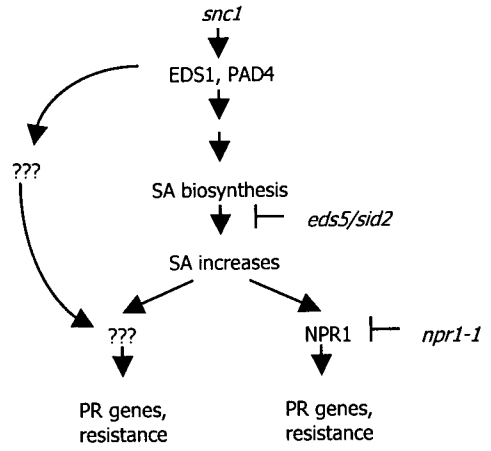

Figure 1.1. Model of downstream signalling pathways in *snc1*.

Known regulators are indicated and explained in the text. *snc1* signaling involves pathways both dependent and independent of SA and *NPR1*. Question marks indicate potential positions for regulators identified in the *snc1* suppressor screen.

The fact that some of the known components of plant defence signalling also affect *snc1*-mediated resistance and the finding that *snc1*-signalling likely involves a complicated network of signalling pathways, incited the use of *snc1* as a unique model for auto-immunity in plants. The identification of novel components required for *snc1* signalling would increase our understanding of both basal and R protein defence signalling pathways. One way to identify such components is using a suppressor screen approach.

1.5 Identification of R protein signalling components in a *snc1* suppressor screen

Most of the previously identified defence signalling components were discovered based on greatly enhanced disease resistance or susceptibility. Mutations in defence genes which only have a subtle effect on disease severity, or which are redundant, were missed by such attempts. Complete and partial suppression of *snc1*-associated phenotypes in epistasis analyses with *eds1* and *eds5*, respectively, suggested the use of the unique *snc1* innate immune model for the identification of novel players in plant immunity. Mutations in genes required for signal transduction of the constitutively active R protein snc1 can be identified based on suppression of the easily visible *snc1*-mediated morphological phenotype.

Fast-neutron mutagenesis, a method which mainly causes deletion mutations (Li et al., 2001b), of *snc1* and *snc1 npr1-1* seeds created a population of potential suppressors, which restore wild-type morphology and abolish constitutive *PR* gene expression. Because this approach uses an activated R protein in the background, it could be expected that suppressor mutants identified in this screen would include loss-of-function alleles of novel positive regulators with specific roles in defence responses. Mutagenesis of *snc1* and *snc1 npr1-1* seeds was used in parallel in an attempt to isolate suppressors acting on different branches of the *snc1* signalling pathways, dependent or independent of NPR1. In the initial screen, 15 complementation groups of *modifier of snc1 (mos)* mutants were isolated, including several alleles of *pad4*, demonstrating the efficiency of the applied selection scheme.

1.6 Thesis objectives

The primary aim of the work presented here was the identification of genes involved in R protein mediated and basal defence signalling, which is crucial for understanding the basic mechanisms of resistance. Using the *snc1 Arabidopsis* auto-immune model, it is possible to isolate mutations in genes that were previously unknown to be important in defence signalling.

During a screen for suppressors of *snc1*-mediated morphology and resistance in *snc1 npr1-1*, a number of mutants were identified. For the screen, *snc1 npr1* double mutant seeds were mutagenized by fast-neutron bombardment (60 Gy) by Andrea Kodym (Agriculture and Biotechnology Laboratory, International Atomic Energy Agency, Vienna, Austria). M_1 plants were allowed to self-pollinate and seeds of 10-20 plants were pooled. M_2 plants were screened for bigger size and wild-type morphology. Seeds of those putative suppressors were collected and examined for lack of expression of the *pBGL2-GUS* reporter gene. Semi-dominant mutations isolated in the screen comprised mainly deletions in the *SNC1* gene, reverting the effects of the gain-of-function mutation in *snc1* (Zhang et al., 2003). This screen also yielded a number of recessive mutants, which suppressed the *snc1 npr1-1* phenotypes either completely or partially, and were chosen for further studies.

This work describes the identification, positional cloning and in-depth characterization of two *mos* mutants, *mos5* and *mos8,* isolated in the *snc1 npr1-1* background. Both mutations suppress *snc1*-mediated phenotypes partially and affect R protein mediated and basal defences. Pleiotropic phenotypes of the mutants indicate the existence of tightly regulated and interconnected signalling networks integrating development and responses to biotic and abiotic stresses.

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2. The ubiquitin pathway is required for innate immunity in *Arabidopsis*[1]

2.1 Introduction

Plants are constantly challenged by a variety of biotic and abiotic stresses, and have evolved a range of sophisticated mechanisms to cope with them. Attacking pathogens often encounter an unfavorable environment or preformed defences in non-host plants. On host plants, the pathogens can either cause disease or are recognized and rapidly contained at the site of infection by a mechanism usually involving the programmed death of the cells surrounding the infection, referred to as hypersensitive response (HR) (Hammond-Kosack and Jones, 1996; Nimchuk et al., 2003). This specific recognition of pathogens is mediated by *Resistance* (*R*) gene products, which directly or indirectly interact with pathogen derived *avirulence* (*Avr*) gene products to induce a resistance response. R proteins are proposed to associate into multimolecular complexes and most likely sense the presence of the pathogen indirectly through the action of its elicitors in the host cell (Belkhadir et al., 2004).

Most *R* genes encode proteins with highly conserved structural domains, which are also frequently found in mammalian immune system modules (Dangl and Jones, 2001; Ausubel, 2005). For example, the majority of R proteins share a central nucleotide binding site (NBS) domain, and carboxy terminal leucine rich repeats (LRR), similar to mamalian NOD proteins involved in innate immunity (Inohara et al., 2005). The amino-terminal portions distinguish two classes of R proteins: those containing the Toll/Interleukin1-receptor-like (TIR) domain with high homology to the cytoplasmic signalling domain of mammalian and *Drosophila* Toll-like receptors, involved in recognizing microbe-specific antigens in innate immunity (Athman and Philpott, 2004), or proteins with a coiled coil (CC) motif potentially involved in protein-protein interactions (Burkhard et al., 2001; Martin et al., 2003; Belkhadir et al., 2004).

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Upon recognition of the cognate avirulence elicitor, TIR-NBS-LRR and CC-NBS-LRR R proteins activate a range of defence responses. Their requirements of downstream regulators are sometimes, but not always, overlapping. Generally, signalling downstream of TIR-NBS-LRR R proteins is dependent on the lipase-like protein EDS1, whereas CC-NBS-LRR R proteins depend on NDR1 (Aarts et al., 1998). Other signalling components, such as RAR1, SGT1b and Hsp90, are shared by TIR and CC class R protein pathways (Hubert et al., 2003; Muskett and Parker, 2003; Takahashi et al., 2003).

RAR1 and *SGT1b* were independently identified in screens for suppressors of various *R* gene mediated responses (Shirasu et al., 1999; Austin et al., 2002; Tor et al., 2002; Tornero et al., 2002). In *Arabidopsis*, both are required for resistance conferred by RPP5 (Resistance to *Peronospora Parasitica* 5) (Austin et al., 2002). Recent studies have revealed the direct interaction between several R Proteins and SGT1b (Bieri et al., 2004; Leister et al., 2005) as well as Hsp90 (Liu et al., 2004). No direct interaction has been reported between RAR1 and R proteins, but RAR1 interacts with both Hsp90 and SGT1b and might thus be indirectly involved in the assembly and stability of R protein complexes (Bieri et al., 2004; Azevedo et al., 2006). RAR1 and SGT1b have also been shown to act antagonistically as positive and negative regulators, respectively, on R protein accumulation prior to infection (Holt et al., 2005).

A gain-of-function mutation in a close homolog of *RPP5*, *suppressor of npr1-1 constitutive* 1 (*snc1*), results in constitutive activation of basal defence responses manifested as resistance to the virulent pathogens *Pseudomonas syringae* pv. *maculicola (P.s.m.)* ES4326 and *Peronospora parasitica (P.p.)* Noco2, elevated levels of the endogenous signalling molecule salicylic acid (SA) and dwarf morphology (Li et al., 2001; Zhang et al., 2003). As opposed to other constitutively resistant mutants, *snc1* does not exhibit spontanous lesions. However, like other TIR-NBS-LRR *R* genes, *snc1*-mediated resistance fully depends on functional EDS1 and PAD4, but is only partially dependent on SA accumulation (Li et al., 2001; Zhang et al., 2003).

To further understand the signalling downstream of TIR-NBS-LRR R-proteins, a suppressor screen was performed in the *snc1* and *snc1 npr1-1* backgrounds. This

screen identified a number of *modifier of snc1* (*mos*) mutants, including several alleles of *pad4*. The identities of MOS3 (a putative nucleoporin 96; (Zhang and Li, 2005) and MOS6 (an importin alpha 3 homolog; (Palma et al., 2005) reveal an essential role for nucleo-cytoplasmic trafficking in resistance signalling. MOS2, a nuclear protein with putative RNA-binding motifs, highlights the importance of RNA processing in plant innate immunity (Zhang et al., 2005).

Here, we report the identification and cloning of *mos5*, a modifier of constitutive disease resistance in *snc1*, which suppresses *snc1*-associated phenotypes and shows differential responses to avirulent bacteria. Cloning of *MOS5* revealed that it encodes an essential component of the ubiquitin pathway and implicates a requirement for ubiquitination in R protein signalling. We also show that the resistance regulators RAR1 and SGT1b are not required for *snc1*-mediated constitutive resistance.

2.2 Results

2.2.1 Isolation and genetic analysis of *mos5 snc1 npr1-1*

The screen for suppressors of *snc1*-mediated resistance has been previously described (Zhang and Li, 2005). *mos5* was identified in the *snc1 npr1-1* double mutant background based on its ability to revert *snc1* morphology to wild type and to abolish constitutive expression of the *pBGL2-GUS* reporter transgene (Figure 2.1 a,b). Lack of *snc1*-induced constitutive expression of pathogenesis related genes in *mos5 snc1 npr1-1* was further confirmed by semi-quantitative RT-PCR (Figure 2.1 c).

When *mos5 snc1 npr1-1* was backcrossed with *snc1 npr1-1*, F_1 progeny displayed the characteristic *snc1* morphology indicating that the mutation is recessive, and GUS-staining of the F_2 progeny segregated 75:21 (staining:non-staining), demonstrating that the phenotype of *mos5* is caused by a single recessive mutation (expected 3:1, $\chi^2 = 0.5$, $P = 0.48$).

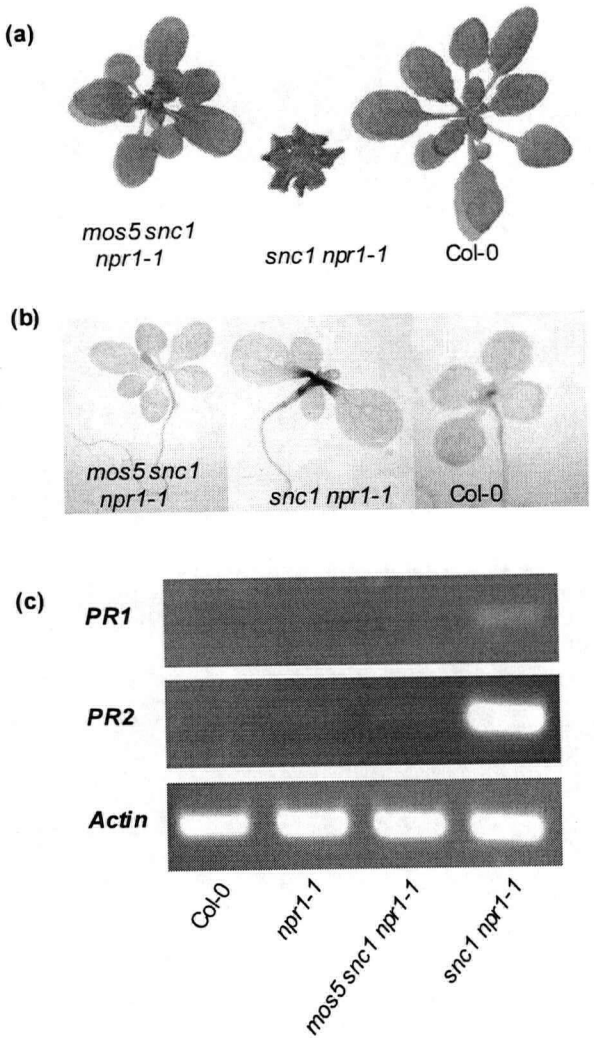

Figure 2.1: Phenotypic characterization of *mos5 snc1 npr1-1*

(a) Morphology of soil grown plants. The picture was taken 5 weeks after planting. (b) Expression of *pBGL2-GUS* reporter gene. 20-day-old seedlings grown on MS plates were stained for GUS activity. (c) Semi-quantitative RT-PCR of *pathogenesis-related* genes. RNA was extracted from 20-day-old seedlings grown on MS-plates and reverse transcribed to obtain total cDNA. The cDNA samples were normalized using the *actin* probe (Zhang *et al.*, 2003). *PR1*, *PR2* and *Actin* were PCR-amplified in 30 cycles using equal amounts of cDNA.

2.2.2 Characterization of defence related phenotypes of *mos5 snc1 npr1-1*

Salicylic acid (SA) is an important signalling molecule in plant defence responses and is associated with systemic resistance (Ryals et al., 1996; Durrant and Dong, 2004). *snc1 npr1-1* mutant plants have high endogenous levels of SA (Li et al., 2001). The *mos5 snc1 npr1-1* mutant exhibits an approximately 12-fold reduction in endogenous levels of both free and total SA as compared to *snc1 npr1-1* (Figure 2.2 a,b). However, the mutant still displays 10 and 5-fold higher levels of SA than either Col-0 or the *npr1-1* single mutant, respectively. This indicates that suppression of high SA levels in *snc1* by *mos5* is not complete.

The *snc1 npr1-1* double mutant exhibits enhanced resistance to the virulent pathogens *P.p.* Noco2 and *P.s.m.* ES4326 as compared to Col-0 wild type (Li et al., 2001). To investigate the role of *MOS5* in disease resistance, plants were inoculated with either pathogen. The *mos5* mutation suppresses *snc1*-mediated constitutive resistance to *P.p.* Noco2, resulting in wild type susceptibility (Figure 2.2 c). Furthermore, *mos5 snc1 npr1-1* supports wild type levels of growth of *P.s.m.* ES4326 (Figure 2.2 d), however, the mutation is not sufficient to restore *npr1*-like susceptibility against the bacterial pathogen, again indicating that suppression of *snc1* by *mos5* is incomplete.

Taken together, these results demonstrate that the mutation in *MOS5* strongly impairs defence signalling in *snc1 npr1-1*, affecting responses to bacterial and oomycete pathogens as well as accumulation of SA.

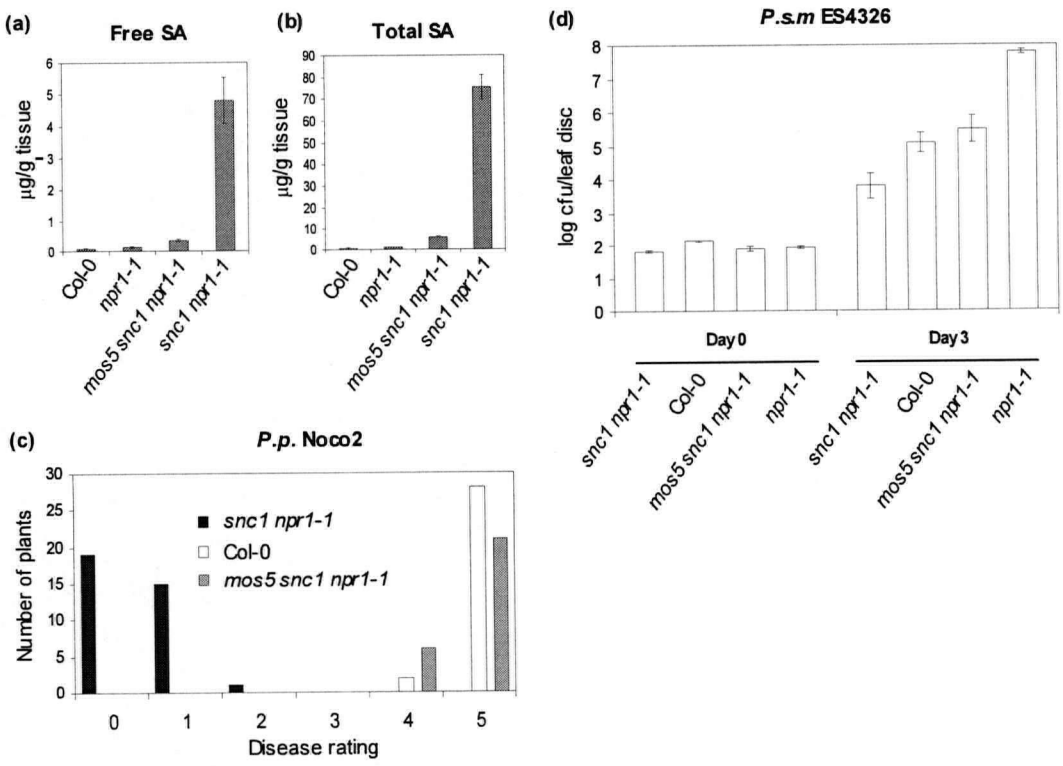

Figure 2.2: Suppression of constitutive resistance in *mos5 snc1 npr1-1*

(a,b) Levels of endogenous SA are reduced in *mos5 snc1 npr1-1*. Free (a) and total (b) SA was extracted from 5-week-old soil grown plants and analyzed with HPLC. (c,d) *mos5* suppresses resistance against virulent oomycete and bacterial pathogens. Bars represent the average of four (a,b) and six (c) biological replicates, error bars represent standard deviation. (c) two-week-old seedlings were infected with *Peronospora parasitica* Noco2 conidiospores (10^4/ml) and disease symptoms were assessed and rated 7 days post-inoculation (dpi) as follows: 0 – no conidiophores on entire plant; 1 – at least one leaf with 1-5 conidiophores; 2 – some infected leaves with 6-20 conidiophores, but most with 1-5; 3 – most infected leaves with 6-20 conidiophores; 4 – all infected leaves with >5 conidiophores, or most infected leaves with >20; 5 – all infected leaves with >20 conidiophores. (d) 4-week-old plants were infected with *Pseudomonas syringae* pv. *maculicola* ES4326 (OD_{600} = 0.0001) and bacterial growth was measured by quantifying colony forming units (cfu) at day 0 and day 3. Experiments were repeated at least twice with similar results.

2.2.3 Map-based cloning of *mos5*

In order to map the recessive modifier of *snc1* in the Col-0 ecotype, *mos5 snc1 npr1-1* was crossed with Ler-*snc1*, in which *snc1* had been introgressed into the Landsberg *erecta* ecotype by repeated backcrossing as described previously (Zhang and Li, 2005). Using 68 plants homozygous for *mos5* from the F_2 progeny of the mapping cross, the approximate position of *mos5* was determined to be between markers T8O18 (12.28 Mb) and T16B12 (13.25 Mb) on the lower arm of Chromosome 2 (Figure 2.3 a). The progeny of F_2 plants heterozygous for *mos5* and homozygous for the *pBGL2-GUS* transgene were used for fine mapping. Out of 747 F_3 plants, 46 recombinants between markers T8O18 and T16B12 were identified. The phenotypes of these recombinants were confirmed by following segregation of the F_4 progeny on soil as well as with GUS staining. The *mos5* mutation was ultimately mapped to a 95 kb region between T27E13-2 and T9D9, with 1 and 4 remaining recombinants, respectively (Figure 2.3 a). Open reading frames located in this region were sequenced from *mos5 snc1 npr1-1* and a 15 bp deletion was found in the coding region of *At2g30110*. Sequence analysis indicated that the deletion is located in the last exon of *At2g30110*, leading to an amino acid substitution (Arg to Ser) and the deletion of the following 5 amino acids (Figure 2.3 b,c).

At2g30110 encodes one of two ubiquitin activating (E1) enzymes in *Arabidopsis (AtUBA1)*. AtUBA1 is very similar to AtUBA2 (81 % amino acid sequence identity) and E1 enzymes in other organisms, indicating the evolutionary conservation of this essential enzyme (Hatfield et al., 1997). Ubiquitin activating enzymes from all kingdoms share regions of high homology that are involved in binding of the ubiquitin molecule, including the catalytic cysteine residue and ATP-binding regions for the nucleotides that provide the energy for ubiquitin activation.

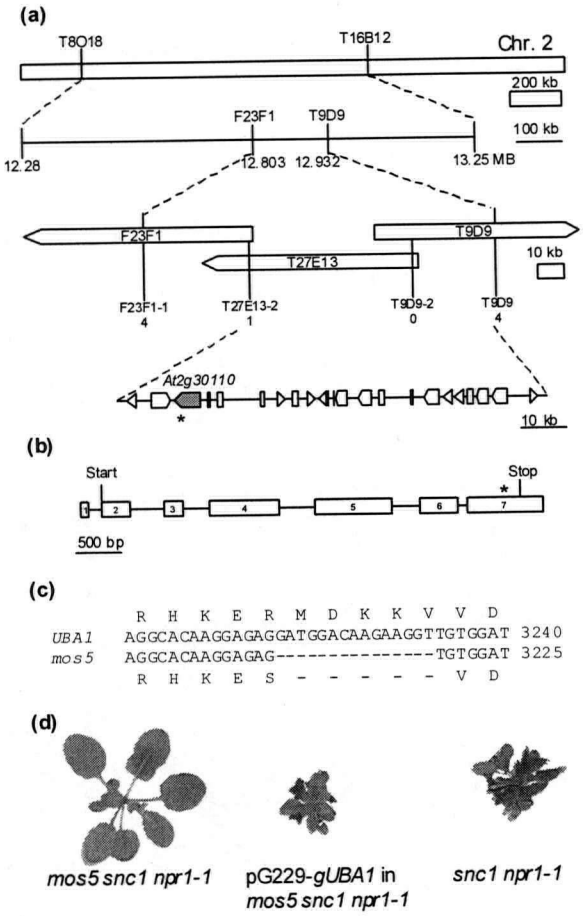

Figure 2.3: Map-based cloning of *mos5*

(a) Mapping of *mos5* to the bottom of chromosome 2. Markers used for crude and fine mapping are indicated with the number of recombinants below. Open reading frames between the final markers T27E13-2 and T9D9 were sequenced and a deletion was identified in *At2g30110* (indicated by *). (b) intron-exon structure of *At2g30110*. The mutation in *mos5* (*) lies close to the stop codon in exon 7. (c) DNA and amino acid sequence alignment of *mos5* and *UBA1*. The 15 bp deletion in *At2g30110* results in an amino acid substitution and deletion of 5 amino acids. (d) Complementation of *mos5* by *AtUBA1* genomic DNA. *mos5 snc1 npr1-1* plants carrying the 7kb transgene of the genomic sequence of *AtUBA1* show typical *snc1* morphology. Representative plants are shown, photographed at 5 weeks.

The mutation in *mos5* is located in the C-terminal domain of the E1, outside the highly conserved nucleotide and ubiquitin binding regions. Interestingly, the C-termini of ubiquitin E1s from different kingdoms are also very similar, suggesting a conserved function of this region in the structure or activity of the enzyme (Figure 2.4).

To confirm that the deletion in *mos5* is responsible for the suppression of the *snc1* phenotype, a 7 kb genomic clone including the complete *At2g30110* ORF as well as 2 kb of the 5' promoter region and the 3' UTR were cloned into pGreen229 (Hellens et al., 2000) and transformed into the *mos5 snc1 npr1-1* mutant. 31 out of 33 T_1 transformants displayed *snc1* morphology (Figure 2.3 d) indicating that *At2g30110* can complement the mutation in *mos5 snc1 npr1-1* and that *MOS5* is *AtUBA1*.

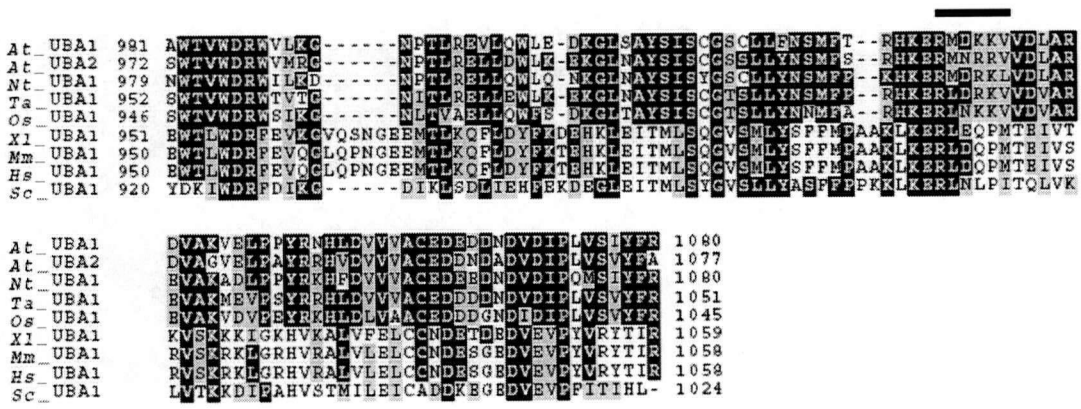

Figure 2.4: Alignment of the C-terminal domains of ubiquitin activating enzymes from different species

Protein sequences were aligned using ClustalX and shaded using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Conserved residues are shaded in black and similar residues are shaded in grey. The location of the deletion in *mos5* is indicated with a bar. AtUBA1: *Arabidopsis thaliana* UBA1 (accession AAC16961); AtUBA2: *A. thaliana* UBA2 (accession BAB08968); Ta_E1: *Triticum aestivum* (accession A38373); Os_E1: *Oryza sativa* (accession NP_910456); Nt_E1: *Nicotiana tabacum* (accession BAD00983); Hs_E1: *Homo sapiens* (accession CAA40296); Mm_E1: *Mus musculus* (accession AAF00149); Xl_E1: *Xenopus laevis* (accession BAB19357); Sc_Uba1p: *Saccharomyces cerevisiae* (accession NP_012712).

2.2.4 The *mos5* single mutant displays enhanced disease susceptibility

The *mos5* single mutant was generated by crossing *mos5 snc1 npr1-1* with Col-0 (carrying the *pBGL2-GUS* transgene) and identified in the F_2 progeny by genotyping using PCR. *mos5* single mutant plants are phenotypically indistinguishable from *mos5 snc1 npr1-1* and grow slightly smaller than Col-0 wild type plants (Figure 2.5 a).To test for effects of *mos5* on basal disease resistance, plants were infected with a low dose of the virulent bacterial pathogen *P.s.m.* ES4326 (OD_{600} = 0.0001). Compared to the Col-0 wild type, *mos5* supports five-fold more bacterial growth (Figure 2.5 b). These data indicate a minor involvement of *mos5* in basal resistance against virulent bacteria.

2.2.5 *mos5* exhibits differential susceptibility to avirulent pathogens

To investigate whether *MOS5* affects resistance mediated by R proteins other than snc1, the *mos5* single mutant was infected with bacteria expressing *Avr* genes. *mos5* plants support wild type growth of bacteria expressing *AvrB* or *AvrRps4*, determinants recognized by the CC-type R protein RPM1 and the TIR-type R protein RPS4, respectively (Figure 2.5 d,e). Only when infected with bacteria expressing *AvrRpt2*, the avirulence determinant recognized by the CC-type R protein RPS2, was a reproducible 10-fold increase in bacterial growth observed (Figure 2.5 c). These data suggest that activation or downstream signalling of certain R proteins depends on a functional ubiquitination machinery.

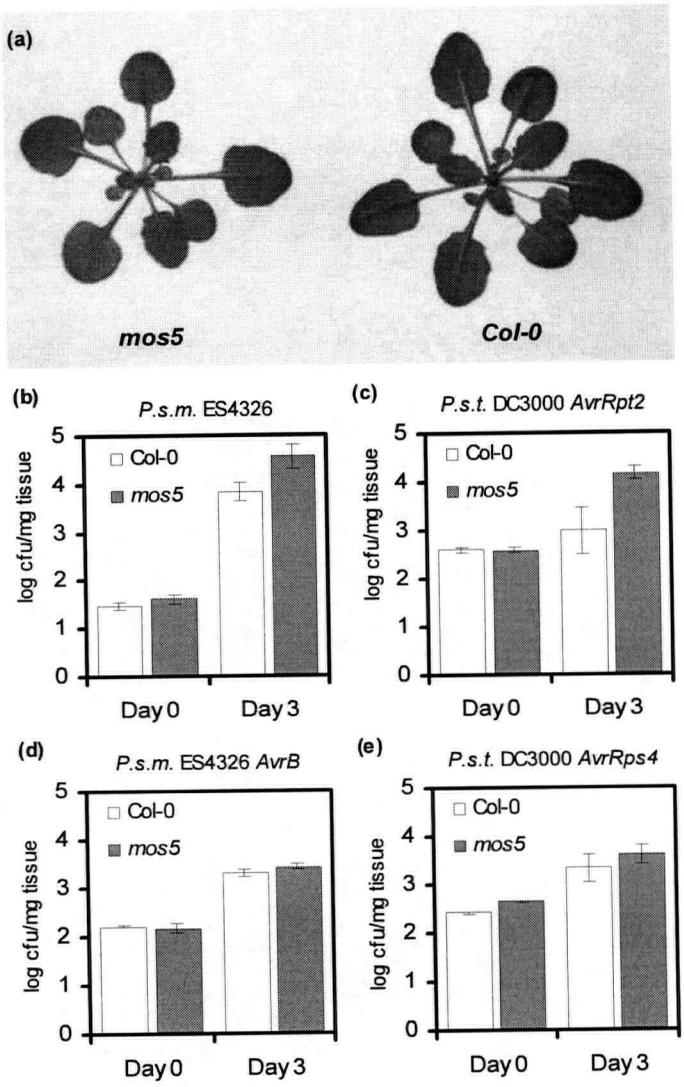

Figure 2.5: Requirement for MOS5 in basal and *R* protein mediated resistance

(a) Morphology of the mos5 single mutant compared to Col-0 wild type. Photograph was taken of five-week-old soil-grown plants. (b) Basal resistance is affected by *mos5*. 4-week-old plants were infected with *P.s.m.* ES4326 (OD_{600} = 0.0001) and bacterial growth measured as described. (c-e) Response of *mos5* to avirulent bacteria. Plants were infected with *P.s.t.* DC3000 *AvrRpt2* (c), *P.s.m.* ES4326 *AvrB* (d) or *P.s.t.* DC3000 *AvrRps4* (e) at OD_{600} = 0.001. Bars represent the average of 4 replicates, error bars represent standard deviation. Experiments were repeated at least twice with similar results.

2.2.6 UBA2 is not required for resistance

Arabidopsis contains two E1 paralogs. To investigate whether *AtUBA2* (At5g06460) is also involved in disease resistance we obtained a T-DNA insertion line from the ABRC (Salk_108047; Alonso et al., 2003), containing an insertion in the 5th exon of *UBA2* (Figure 2.6 a), henceforth referred to as *uba2*.

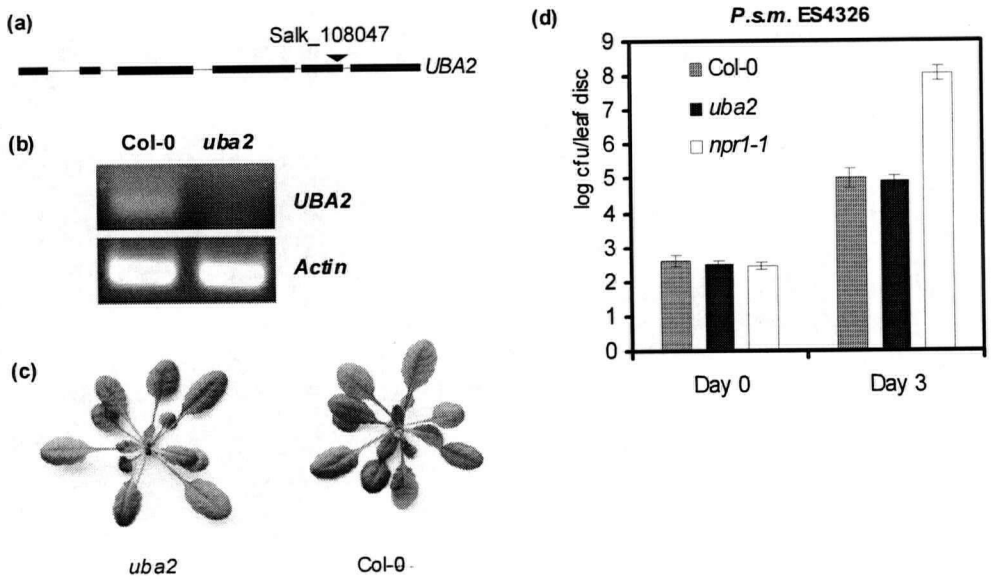

Figure 2.6: UBA2 is not essential for plant innate immunity

(a) Exon-intron structure of UBA2. The position of the T-DNA insertion in SALK_108047 is indicated by a rectangle. (b) Salk_108047 does not express *UBA2* mRNA. PCR was performed using cDNA specific primers. (c) *uba2* plants are morphologically indistinguishable from Col-0 wild type plants. The picture was taken of 5-week-old soil-grown plants. (d) Resistance to virulent pathogens is not affected in *uba2*. 4-week-old plants were infected with *P.s.m.* ES4326 (OD_{600} = 0.0001) and bacterial growth was measured as described. Bars represent the average of six biological replicates, error bars represent standard deviation. Experiments were repeated at least twice with similar results.

RT-PCR analysis showed that *uba2* does not accumulate *UBA2* mRNA (Figure 2.6 b), indicating that the mutation causes a loss of function. In contrast to *mos5*, the *uba2* mutant plants were phenotypically indistinguishable from Col-0 wildtype plants (Figure 2.6 c), suggesting that the loss of *UBA2* function has no major effect on development. In bacterial infection assays, *uba2* plants did not exhibit increased susceptibility to virulent *P.s.m.* ES4326 (Figure 2.6 d). We crossed *uba2* with *snc1* to obtain the double mutant and *uba2 snc1* plants displayed the typical *snc1* stunted morphology (not shown). This indicates that a loss of UBA2 function is unable to suppress *snc1*, in contrast to the *mos5* mutation in *UBA1*. These data suggest that UBA2 activity is not required in resistance responses.

2.2.7 A *mos5 uba2* double mutant is lethal

Since *uba2* does not have an obvious phenotype whereas *mos5* is defective in innate immunity and responses to the plant hormone auxin (S.G., unpublished data), we investigated whether *mos5* could be a peculiar allele of *UBA1* by two genetic approaches. We first attempted to find insertion alleles that knock out *UBA1* function from ABRC seed stocks. Unfortunately, all available alleles with putative T-DNAs in exons of *UBA1* did not actually carry an insertion in the gene (for a list of tested T-DNA insertion lines, see Table 2.1).

Table 2.1: T-DNA insertion alleles for *UBA1* and *UBA2* tested in this study.

gene	line ID	T-DNA position	insertion verified[1)]	expression[2)]
At2g30110 UBA1	Salk_020406	exon	no	-
	Salk_078543	5'-UTR	yes	no difference
	Salk_149288	5'-UTR	yes	no difference
	Salk_0066771	Promoter	yes	no difference
	SAIL_1237_E11	exon	no	-
At5g06460 UBA2	Salk_047313	5'-UTR	yes	no difference
	Salk_047314	5'-UTR	no	-
	Salk_023058	exon	no	-
	Salk_108047	exon	yes	no expression
	SAIL_840_C08	exon	no	-

[1)] Insertion of T-DNA in the gene was verified using gene-specific primers paired with T-DNA specific primers Lba1 or Sail-1F. Lines were T-DNA insertions could be detected are indicated.
[2)] Expression of *UBA1* or *UBA2* in the homozygous T-DNA insertion lines was determined using real-time RT-PCR using mRNA-specific primers (available upon request) and is indicated relative to levels observed in Col-0.

Furthermore, T-DNA insertions in the promoter and 5'-UTR regions of *UBA1* did not cause any discernible phenotype and transcription of the gene was unaffected as determined by RT-PCR (data not shown). We could thus not distinguish whether *mos5* is a complete or only a partial loss-of-function allele of *UBA1*. We then attempted to generate a *mos5 uba2* double mutant. Out of 183 randomly-chosen F2 plants, none were homozygous for both mutations, indicating that a combination of *mos5* and *uba2* is lethal. Furthermore, plants homozygous for one mutation and heterozygous for the other (thus only containing one fully functional copy of E1) are statistically under-represented with 15 plants of *MOS5/mos5 uba2/uba2* and 10 plants *mos5/mos5 UBA2/uba2*, vs. 23 expected for either combination ($\chi^2 = 9.96$, df = 1; $P<0.005$). All other classes are represented close to expected values. These data suggest that the two copies of *UBA* present in *Arabidopsis* are partially redundant and that the loss of both genes causes lethality. Thus *mos5* is most likely a complete loss-of-function allele of *UBA1*, unless a null mutation in *UBA1* alone is lethal.

2.2.8 Resistance in *snc1* is independent of *SGT1b* and *RAR1*

RAR1 and SGT1b were previously identified as necessary components in RPP5-mediated resistance responses and are also required in RPP4-mediated resistance (Austin et al., 2002; Muskett et al., 2002). SGT1b was shown to interact with components of ubiquitin E3 ligases of the Skp/cullin/F-box (SCF) type in yeast and *Arabidopsis* (Kitagawa et al., 1999; Gray et al., 2003), indicating an additional function in protein degradation. Since *MOS5* encodes an essential component of the plant's protein degradation machinery and given the fact that *snc1* encodes an RPP5-homolog that is located in the *RPP4* cluster (Zhang et al., 2003), snc1 resistance signalling might also be dependent on either or both proteins.

To investigate a potential role for SGT1b and RAR1 in snc1 signalling, *sgt1b-1* (in *Landsberg erecta* ecotype) and *rar1-21* (in Col-0) were crossed with *snc1 npr1-1*. As expected, the F_1 progeny of both crosses looked phenotypically like wild type. In the F_2 generation, 85 out of 353 plants of the *rar1-21* x *snc1 npr1-1* cross showed typical *snc1*-like morphology, indicating that *rar1* does not suppress the *snc1* growth phenotype (expected ratio 1:3, $\chi^2 = 0.16$, $P = 0.69$). The *snc1 rar1-21* double mutant was then isolated using genotype-specific markers. *SGT1b* is closely linked to *SNC1* on chromosome 4, resulting in a skewed ratio in F_2 progeny of the *snc1* x *sgt1b-1* cross. The *snc1 sgt1b-1* double mutant was isolated by genotyping and the presence of both mutations was subsequently confirmed by sequencing.

Both the *snc1 sgt1b-1* and the *snc1 rar1-21* double mutant plants display similar morphological phenotypes as *snc1*, i.e. small stature, dark green colour and curly leaves (Figure 2.7 a).

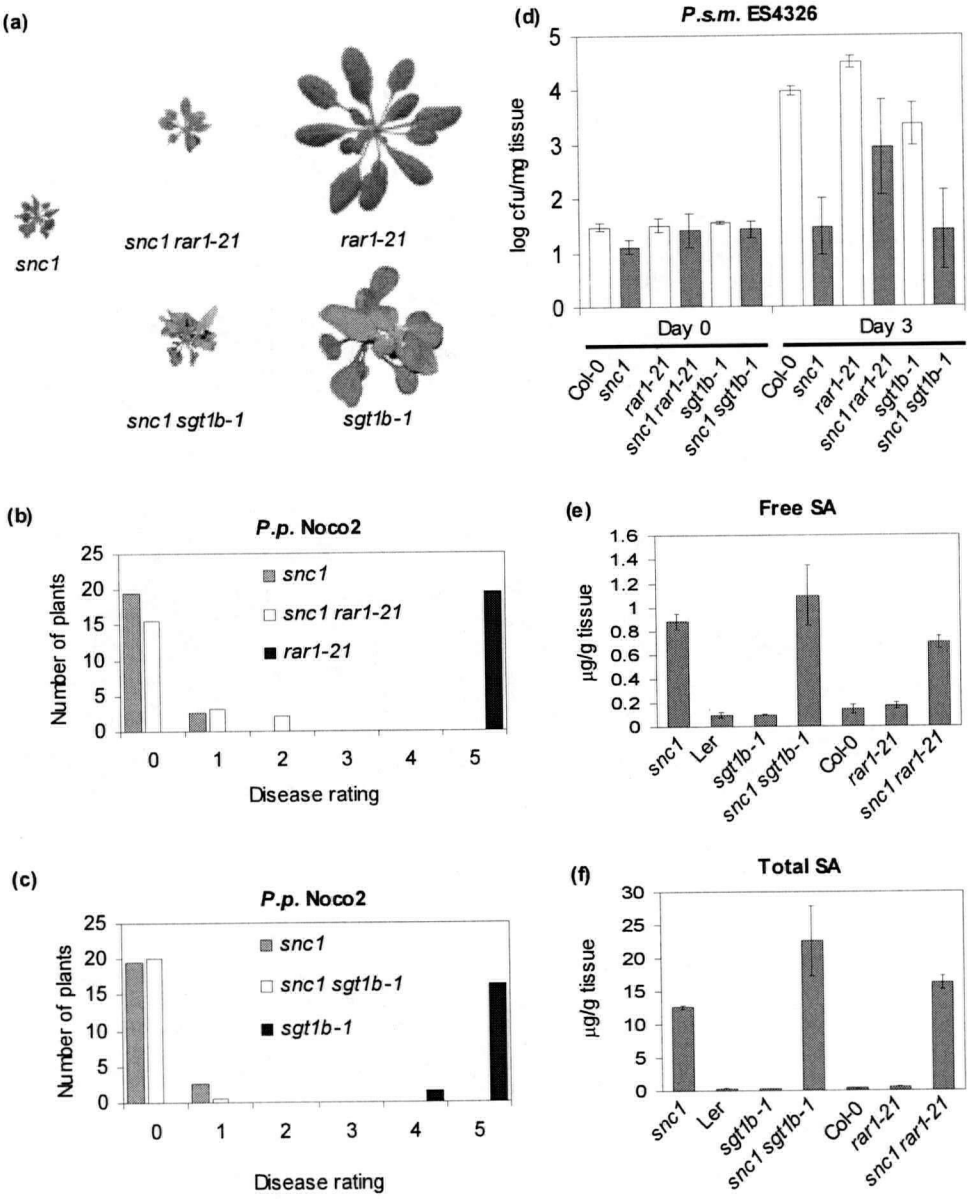

Figure 2.7: SGT1b and RAR1 are not required for snc1-mediated resistance

(a) Morphology of single and double mutants. Pictures show 4-week-old soil grown plants. (b-d) *sgt1b* and *rar1* do not suppress constitutive resistance towards virulent pathogens in *snc1*. (b,c) Plants were infected with *P.p.* Noco2 conidiospores and disease ratings assessed 7 dpi as described in Figure 2. (d) Plants were infected with *P.s.m.* ES4326 (OD_{600} = 0.0001) and bacterial growth measured as desribed. (e,f) *sgt1b* and *rar1* do not suppress elevated endogenous SA-levels in *snc1*. Free (e) and total (f) SA was extracted from 5-week old plants and analyzed by HPLC as described. Bars represent the average of six (d) and four (e,f) biological replicates, error bars represent standard deviation. All experiments were repeated at least twice with similar results.

To test whether the *rar1* and *sgt1b* mutations affect *snc1*-mediated constitutive resistance towards *P.p.* Noco2, a pathogen for which *rar1* and *sgt1b* are hyper-susceptible (Austin et al., 2002), two week-old seedlings were sprayed with a conidiospore suspension. As shown in Figure 2.7 (b+c), the *snc1 rar1-21* double mutant and *snc1* had a few infected leaves whereas *snc1 sgt1b-1* is completely resistant against *P.p.* Noco2. We also investigated the effect of both mutations on *snc1* mediated increased basal resistance towards virulent *P.s.m.* ES4326. As expected, both the *rar1-21* and the *sgt1b-1* single mutant showed much higher bacterial growth compared to *snc1* (Figure 2.7 d). In the *snc1 sgt1b-1* double mutant, *snc1*-like resistance was completely restored, whereas the *snc1 rar1-21* double mutant could partially restore resistance. These data indicate that constitutive resistance against *P. p.* Noco2 and *P.s.m.* ES4326 in *snc1* is not mediated by these two regulators.

To fully evaluate the involvement of RAR1 and SGT1b, the levels of endogenous SA in the single and double mutants were also measured. Both double mutants exhibited elevated levels of SA, similar to those found in the *snc1* mutant (Figure 2.7 e,f). Thus, our results show that neither *sgt1b* nor *rar1* suppress *snc1*-mediated phenotypes including stunted morphology, constitutive pathogen resistance and elevated endogenous SA levels.

2.3 Discussion

In a screen for suppressors of constitutive resistance responses in *snc1 npr1-1*, we identified *mos5*, a mutant that restores wild type morphology and susceptibility to virulent bacterial and oomycete pathogens. The mutation abolishes constitutive expression of *PR* genes and mutant plants accumulate reduced levels of endogenous SA, an important signalling molecule in R protein mediated resistance. Using a map-based approach, the *mos5* mutation was identified in *AtUBA1*, one of two ubiquitin activating E1 enzymes in *Arabidopsis*. Both E1s have previously been shown to bind ubiquitin and to transfer it to various ubiquitin conjugating E2 enzymes (Hatfield et al., 1997). *UBA1* is expressed in all parts of the plant,

predominantly in young cells and dividing tissue, as determined by promotor-GUS fusion experiments (Hatfield et al., 1997).

The identification of *mos5* reveals an essential role for ubiquitination in plant defence signalling. In animal systems, ubiquitination has been shown to have a conserved role in different immunity pathways. In the mammalian immune system, ubiquitination is associated with processing of the transcription factor NF-κB precursors into functional products and their activation through the degradation of the inhibitory protein IκB (Ben-Neriah, 2002). Apart from these proteolysis-associated ubiquitination events, a number of regulatory functions for ubiquitination in animal immune signalling have been identified. These include signal termination via ubiquitin-associated receptor endocytosis, inhibition of T-cell activation, modulation of ubiquitination through the activity of deubiquitinating enzymes and activation of IκB kinase (IKK) via ubiquitination of the upstream E3-ligase TRAF6 (Ben-Neriah, 2002). TRAF6 ubiquitination is mediated by a hetero-dimeric ubiquitin conjugating enzyme complex consisting of Ubc13 and UEV1a, and does not lead to its degradation by the proteasome (Deng et al., 2000). The *Drosophila* homologs of Ubc13 and UEV1a are similarly required for IKK activation and induction of an immune response (Zhou et al., 2005), revealing a strong evolutionary conservation in eukaryotic immune systems. In plants, the role of ubiquitinaton in R protein signalling has been elusive, although ubiquitin-dependent protein degradation has previously been implicated in plant disease resistance responses (Devoto et al., 2003). Tobacco plants expressing an ubiquitin variant unable to form polyubiquitin chains necessary for recognition by the 26S proteasome show altered responses to infection with tobacco mosaic virus (Becker et al., 1993). Jasmonate-dependent responses to wounding and necrotrophic pathogens have been shown to require the action of the SCF^{COI1} ubiquitin ligase complex (Xie et al., 1998; Xu et al., 2002). Two RING ubiquitin ligases, RIN2 and RIN3, which were initially identified as interactors of the R protein RPM1, affect the hypersensitive response mediated by RPM1 and RPS2 without having an effect on pathogen proliferation (Kawasaki et al., 2005). In tomato, a set of U-Box ubiquitin ligases that are induced upon elicitor treatment appear to regulate the hypersensitive response, adding further evidence for a role of

ubiquitination in specific defence signalling (Gonzalez-Lamothe et al., 2006; Yang et al., 2006).

Interestingly, the *mos5* mutation affects resistance responses of only one R protein tested. This might reflect divergent signalling pathways employed by different *R* gene products. Indeed, studies of RIN4, a protein involved in activation of both RPM1- and RPS2-mediated resistance showed that these R proteins are differentially regulated by RIN4. Induction of RPM1-mediated resistance involves phosporylation of RIN4 (Mackey et al., 2002), whereas the proteolytic processing of RIN4 is necessary for activation of RPS2 (Axtell et al., 2003; Chisholm et al., 2005), where MOS5 is also required (Figure 2.5 b).

Since *snc1* was originally identified as a gain-of-function *R* gene rather than from traditional gene-for-gene interactions, the cognate *Avr* gene product recognized by wild type SNC1 is not known. We are therefore not able to speculate on the requirement of MOS5 in wild-type SNC1-mediated resistance signalling.

Apart from its effect on resistance, *mos5* also showed slightly enhanced resistance towards several natural and synthetic auxins (data not shown). These findings are not surprising, since ubiquitination and targeted degradation of Aux/IAA proteins are involved in auxin signalling (Dharmasiri and Estelle, 2004). The auxin resistant mutant *axr1* contains a mutation in the RUB-activating enzyme, analogous to UBA1 (Leyser et al., 1993). We therefore conclude that the defects observed in *mos5* are likely to be due to alterations in ubiquitination of target proteins.

mos5 has a deletion of 15 bp very close to the C-terminus of *UBA1*, resulting in a substitution of arginine to serine and the deletion of 5 amino acids. The mutation lies in a region with high homology among ubiquitin E1s from different organisms (Figure 2.4), and some recent structural studies of E1s of ubiquitin-like proteins point to a possible function of the C-terminus of UBA1. Walden and co-workers reported the three-dimensional structure of the E1 for the human RUB1 ortholog Nedd8, a heterodimer composed of APPBP1 and UBA3 (Walden et al., 2003b; Walden et al., 2003a). Interestingly, the C-terminus of UBA3 adopts a ubiquitin-like fold, which plays a role as an adapter domain for the docking of the cognate conjugating enzyme UBC12 (Huang et al., 2005). The human SUMO1 E1 heterodimer Sae1/Sae2 adopts a very similar structure as APPBP1/UBA3 and also features a C-

terminal ubiquitin-fold domain (Lois and Lima, 2005). In both cases the ubiquitin fold is necessary for binding of the cognate E2s as determined by mutational analyses. Given the strong evolutionary conservation among E1s of different ubiquitin-like proteins over all kingdoms (Walden et al., 2003a), it seems plausible that UBA1 has a similar structure.

It might be speculated that the *mos5* mutation somehow disrupts the putative ubiquitin-fold domain in UBA1 resulting in reduced binding affinity of some, if not all, conjugating enzymes. This disruption or alteration of the ubiquitination cascade may result in increased stability of negative regulatory proteins, the degradation of which might be necessary during *snc1*-mediated resistance responses. Alternatively, activation of positive regulators by ubiquitination could be affected by the *mos5* mutation.

Our data support a model in which pathogen elicitors target a population of host proteins, some of which might be involved in basal defence. Modification of these proteins is perceived by the corresponding R proteins, which are thus activated to initiate defence signalling. The ubiquitination of those target proteins might be impaired in the *mos5* mutant, affecting activation of the corresponding R proteins. We cannot exclude the possibility, however, that R protein pathways which are unaffected by *mos5* involve signalling via pathways independent of ubiquitination or, alternatively, that UBA2 might act partially redundantly in these pathways. We have identified a T-DNA insertion mutant line for *UBA2* (SALK_108047), containing the T-DNA in an exon and likely resulting in a complete loss-of-function phenotype. The *uba2* mutant plants, however, do not exhibit any morphological phenotype different from Col-0 wild type and are unaffected in disease susceptibility (Figure 2.6), indicating that *UBA2* is not essential. In addition, no T-DNA insertion mutants for *UBA1* could be identified and a *mos5 uba2* double mutant is lethal. This could hint at a primary requirement for *UBA1*, and the mutation in *mos5* could reduce the activity of UBA1. Most species contain only one copy of the E1 enzyme, and the lack of phenotypic effect in the *uba2* insertion mutant indicates that there might be a preferential recruitment of UBA1 in the ubiquitination process. Similarly, Arabidopsis contains two homologs of SGT1 and, while mutations in *SGT1a* cause no phenotypical abnormalities, only *sgt1b* mutants display disease-related phenotypes.

Interestingly, a *sgt1a/b* double mutant is also lethal, reminiscent of the *mos5/uba2* double mutant (Azevedo et al., 2006).

Ultimately, however, the specificity of protein ubiquitination is likely dependent on the action of ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3), and not on E1. Furthermore, ubiquitination might be a strategy of the pathogen to eliminate host proteins involved in basal defence in order to facilitate infection. The *Pseudomonas syringae* protein AvrPtoB has recently been shown to possess ubiquitin ligase activity and to inhibit the hypersensitive response in susceptible tomato plants (Janjusevic et al., 2006). The pathogenicity of the human pathogen *Shigella flexneri* relies on OspG, a protein that specifically binds ubiquitinated E2s to inhibit an immune response in the host (Kim et al., 2005). Pathogens are thus able to exploit the host's ubiquitination machinery to suppress basal defences.

SGT1b and RAR1 are essential signalling components of a number of *R* gene products (Muskett and Parker, 2003). SGT1 was first identified as an interactor of SCF ubiquitin ligases in yeast (Kitagawa et al., 1999), and its plant homolog SGT1b has been shown to be essential for ubiquitin dependent responses to auxin and jasmonic acid (Gray et al., 2003). These data suggested an involvement of SGT1b in ubiquitination-dependent plant defence responses. Our data, however, indicate that SGT1b and RAR1 act independently from the ubiquitin-proteasome pathway in resistance signalling mediated by *snc1*, because, unlike *mos5, sgt1b* and *rar1* do not suppress the constitutive defence responses against virulent pathogens in *snc1*. Our data support other reports positioning RAR1 and SGT1 upstream of R protein activation, where they are potentially involved as co-chaperones in the assembly and stability of putative R protein recognition complexes (Bieri et al., 2004; Azevedo et al., 2006). RAR1 was shown to affect the steady state levels of the R proteins Mla1 and Mla6 in barley although no direct interaction was observed (Bieri et al., 2004). In the same study, SGT1 was identified as an interactor of the LRR C-terminal region of Mla1 in a yeast two-hybrid screen, which was not observed when the bait also contained the N-terminal NBS domain. This suggests a steric hindrance of the R protein structure and might reflect an intramolecular switch (Bieri et al., 2004). In another study, intramolecular association of the R protein Bs2 was shown to be dependent on SGT1b (Leister et al., 2005), and accumulation of the R proteins

Rx and N in tobacco is dependent on the presence of SGT1 (Azevedo et al., 2006). These reports indicate that SGT1b and RAR1 directly or indirectly interact with several NBS-LRR R proteins, controlling their abundance on the one hand and their intra- and intermolecular interactions on the other. Interestingly, SGT1b was also shown to act as RAR1 antagonist in negatively regulating R protein accumulation (Holt et al., 2005). Thus, SGT1b and RAR1 presumably act as co-chaperones in the formation of R protein recognition complexes and may be necessary for effector recognition but are likely dispensable once the R protein is activated. This would explain why the constitutively active *R* protein snc1 is unaffected by mutations in *RAR1* and *SGT1b*. However, we cannot exclude the possibility that these co-chaperones are important in stabilizing the wild type SNC1 complex.

Our results suggest that the ubiquitination pathway is essential for the activation of some, but not all R protein mediated resistance responses, as well as for basal defence. Ubiquitination appears to act both positively on promoting defence responses in the plant as well as negatively to suppress defences when employed by the attacking pathogen. The balance of these positive and negative aspects may determine the outcome of a plant-pathogen interaction.

2.4 Experimental procedures

2.4.1 Plant growth and mutant phenotypic characterization

All plants were grown at 22°C under 16 h-light/8 h-dark cycles. The screen for suppressors of *snc1 npr1-1* double mutants was described elsewhere (Zhang and Li, 2005). *pBGL2-GUS* reporter gene expression was tested on 20 day-old plants grown on MS plates as described previously (Zhang et al., 2003). Infection experiments with *Pseudomonas syringae* and *Peronospora parasitica* were performed as described in (Li et al., 2001). Endogenous salicylic acid (SA) was extracted from 4 week-old soil grown plants and determined by HPLC as described previously (Li et al., 1999). RNA was extracted from 20 day-old seedlings grown on MS plates using the Totally RNA kit (Ambion, Austin, TX) and reverse transcribed to cDNA using the RT-for-PCR kit (Clontech, Palo Alto, CA). Expression levels of the

pathogenesis related genes *PR1* and *PR2* were determined as described previously (Zhang et al., 2003). Expression of *uba2* was determined using the cDNA specific primers UBA2-RT-F (5'-tccagtttgaaaaggacgatg-3') and UBA2-RT-R (5'-tcaccactttaggtggaacc-3').

2.4.2 Map-based cloning of *mos5*

The markers used to map *mos5* corresponding to the respective BAC-clones were derived from insertion-deletion (InDel) and single sequence polymorphisms (SSLP) between the Col-0 and Ler *Arabidopsis* ecotypes, identified by mining the available genomic sequences of both ecotypes as well as a database provided by Monsanto on the TAIR homepage (Jander *et al.*, 2002; http://www.arabidopsis.org/Cereon/). Marker T8O18 was amplified using primers T8O18-NF (5'-tgtgatgtgaaccaagattg-3') and T8O18-R (5'-agcttcgagtggattctac-3') yielding PCR-fragments of 728 and 401 bp in Col-0 and Ler, respectively. Marker T16B12 was amplified using primers T16B12-F (5'-atactattaccgtactcatg-3') and T16B12-R (5'-acgcatgcattagacaacg-3') yielding PCR-fragments of 542 and 238 bp in Col-0 and Ler, respectively. Marker F23F1-1 was amplified using primers F23F1-1F (5'-ctctgtttccagcttgtatg-3') and F23F1-1R (5'-gtgacgtacactactttctc-3') yielding PCR-fragments of 231 and 206 bp in Col-0 and Ler, respectively. Marker T9D9 was amplified using primers T9D9-F (5'-tatgtttagtcaacgcctcc-3') and T9D9-R (5'-cattaccatactaacgtacg-3') yielding PCR-fragments of 267 and 222 bp in Col-0 and Ler, respectively. The marker T27E13-2 was amplified using primers T27E13-2F (5'-gattagtgtcacaagttcttg-3') and T27E13-R (5'-tctaaagtcagaaccaactag-3') yielding 291 bp PCR-fragments in both ecotypes, but only the Col-0 derived product was digested with *Hin*P1I.

2.4.3 Complementation of *mos5*

A genomic clone of *At2g30110* encompassing 7 kb was amplified by PCR using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) in a two-step reaction using primers with modified restriction enzyme cleavage sites (underlined). The N-terminal fragment UBA1-N was amplified using primers mos5-KpnI (5'-cttggtaccaggtttcaactgcatc-3') and mos5-intR (5'-ggctgtttcacttgagtgac-3') and the C-terminal fragment UBA1-C was amplified using primers mos5-intF (5'-catacaggcatcattgcgtc-3') and mos5-NotI (5'-ttttccttttgcggccgcgaaacaccacctgcaag-3'). Both fragments were first cloned into pBluescript (Alting-Mees et al., 1992) using the restriction enzymes *KpnI*/*SalI* and *SalI*/*NotI*, respectively and sequenced to ensure that no mutations were introduced. UBA1-C was then subcloned into pBS-UBA1-N to create pBS-UBA1g. UBA1g was subsequently cloned into the binary vector pGreen229 (Hellens et al., 2000) to create pG229-UBA1g. *mos5 snc1 npr1-1* plants were transformed with *Agrobacterium* containing pSoup and pG229-UBA1g using the floral dip method and T_1 plants containing the UBA1g transgene were selected by spraying with glufosinate.

2.4.4 Creating the *mos5* single and *mos5 uba2* double mutants

The *mos5* single mutant was obtained by crossing *mos5 snc1 npr1-1* with Col-0 carrying the *pBGL2-GUS* transgene. F_1 progeny of the cross displayed wild type morphology and were allowed to self-pollinate and set seed. *mos5* single mutants were identified among the F_2 progeny using genotype-specific markers. Presence of the *mos5* deletion was determined by PCR using the primers mos5del-F (5'-aactcttcgtgaggtgttgc-3') and mos5del-R (5'-actcgactttcgcaacatcc-3'), which amplify fragments of 181 and 166 bp in Col-0 and *mos5*, respectively.

The *uba2* homozygous T-DNA insertion mutants were identified in SALK_108047 using the gene-specific primers 1972900F (5'-ctcacctcactgagaactatg-3') and 1974050R (5'-tcaccactttaggtggaacc-3'). One *uba2* line was crossed with *mos5* to yield F_1 progeny with wild type phenotype. Segregating progeny in the F_2 were genotyped using the PCR markers described above. Presence of the T-DNA

was determined using the combination of T-DNA specific LBa1 (5'-tggttcacgtagtgggccatcg-3') and 1974050R.

2.4.5 Creating the *snc1 sgt1b-1* and *snc1 rar1-21* double mutants

In order to create the *snc1 sgt1b-1* double mutant, *snc1* (in the Col-0 background) was crossed with *sgt1b-1* (in the Ler background). F_2 plants that were homozygous Col-0 at the *SNC1* locus, but heterozygous Col-0/Ler at the *SGT1b* locus were selfed and their progeny screened for lines homozygous for *sgt1b-1*. The obtained double mutant line was confirmed by sequencing to be homozygous for both *snc1* and *sgt1b-1* and used for further analysis.

To create the *snc1 rar1* double mutant, *snc1 npr1-1* was crossed with *rar1-21* (both in the Col-0 background). Selfed F_2 progeny segregated 1 : 3 *snc1*-like : wild type and plants displaying the characteristic *snc1*-like phenotype were screened with genetic markers specific for the *npr1-1* and *rar1-21* mutations. Nine plants out of 32 *snc1*-like plants were homozygous for *rar1-21*. Among those, two plants were homozygous for the segregating *npr1-1* mutation, three plants were heterozygous and four plants were homozygous for wild type *NPR1*. The presence of the *rar1-21* mutation in those four plants was confirmed by sequencing the locus and one line was used for further characterization.

2.6 References

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3. A novel role for *Arabidopsis* farnesyltransferase in plant innate immunity[2]

3.1 Introduction

Plant immunity to microbial pathogens requires an intricate signalling network, components of which are subjects of current investigation. An integral part of pathogen-specific defence is mediated by so-called Resistance (R) proteins, which may recognize pathogenic effector molecules or, more likely, the results of their pathogenic activity, such as attempted suppression of the plant defence responses by pathogenic avirulence factors. According to their predicted structure, most R proteins belong to the NBS-LRR class, with carboxy-terminal leucine-rich repeats (LRRs) and a central nucleotide binding site (NBS) domain. The amino-terminal domains divide the NBS-LRR proteins into two subclasses, the TIR-NBS-LRR class with a Toll/Interleukin-1-receptor domain, and the CC-NBS-LRR class with a coiled coil-domain potentially involved in protein-protein interaction (Belkhadir et al., 2004). NBS-LRR proteins are similar to receptor modules in mammalian innate immunity, the Toll-like receptors (TLR) and NOD-proteins, which recognize general pathogen associated molecular patterns (PAMPs) as a first step in an innate immune response (Ausubel, 2005). Activation of R proteins initiates discrete and overlapping signalling events, usually culminating in programmed death of cells at the site of infection (Hypersensitive Response, HR) and containment of the invading pathogen (Nimchuk et al., 2003).

Several components involved in R protein signalling have been identified. For example, the Enhanced Disease Susceptibility1/Phytoalexin Deficient4/Senescence Associated Gene101 (EDS1/PAD4/SAG101) complex functions genetically downstream of TIR-NBS-LRR R proteins and is present in both the nucleus and cytoplasm in varying composition (Feys et al., 2005; Wiermer et al., 2005). Non-expressor of *Pathogenesis-Related* genes1 (NPR1)

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is activated by salicylic acid (SA) and, upon redox-changes in the cytoplasm as a result of SA induction, relocates to the nucleus and interacts with TGA-transcription factors to induce expression of defence-related genes (Zhang et al., 1999; Mou et al., 2003). Like NPR1, many proteins involved in plant defence signalling are regulated post-translationally, ensuring a rapid initial response that can be further amplified by transcriptional activation. Proper localization and interaction with other signalling components are imperative for successful defence responses, and these often depend on post-translational modifications. For example, membrane association has been demonstrated for the negative regulatory and avirulence target protein RPM1 interacting protein4 (RIN4), which is tethered to the plasma membrane most likely via palmytoylation at its C-terminus. RIN4 release from the membrane after proteolytic cleavage by the *P. syringae* type III effector AvrRpt2 results in its degradation by the proteasome and activation of the associated R protein Resistance to *P. syringae* 2 (RPS2) (Kim et al., 2005). Recent reports are proposing a role for the versatile and reversible protein modification by ubiquitination in plant defences. Ubiquitination has been implicated in plant-virus interactions (Becker et al., 1993) and a number of ubiquitin ligases have been shown to be important in plant resistance responses (Kim and Delaney, 2002; Kawasaki et al., 2005; Gonzalez-Lamothe et al., 2006; Yang et al., 2006). We have recently identified a unique loss-of-function mutant allele of the ubiquitin-activating enzyme UBA1, which affects some aspects of basal and R protein mediated defence responses (Goritschnig et al., Plant Journal, in press).

To search for additional components required for R protein signalling, we took advantage of the plant auto-immune model *suppressor of npr1 constitutive 1 (snc1)*, a unique gain-of-function allele of a TIR-NBS-LRR *R* gene homologous to *RPP4* (*Resistance to Peronospora parasitica* 4) and *RPP5*. Apart from constitutive resistance against virulent bacterial and oomycete pathogens, the *snc1* mutant also displays several constitutive defence-related phenotypes, including increased levels of endogenous SA and constitutive expression of several *PR* genes (Li et al., 2001; Zhang et al., 2003). *snc1*-mediated resistance completely depends on EDS1/PAD4 and involves several branches of the

downstream signalling network, dependent or independent of SA and NPR1, or both (Zhang et al., 2003). The unique properties of the *snc1* auto-immune model facilitate the identification of novel signalling components downstream of activated *R* proteins.

Here we present *mos8 (modifier of snc1 8)*, another suppressor of *snc1*-mediated defence responses. *mos8* is a novel allele of *ERA1* (*Enhanced Response to ABA1*), which encodes the protein farnesyltransferase beta subunit and has been shown to be important in development and hormonal responses (Cutler et al., 1996; Yalovsky et al., 2000; Ziegelhoffer et al., 2000). *mos8* affects both basal resistance against virulent pathogens as well as some R protein mediated resistance responses. We also show that defence responses have a specific requirement for farnesylation, which cannot be substituted by geranylgeranylation. This novel function of farnesylation in response to biotic stresses adds another layer of complexity to the signalling network integrating biotic and abiotic stress responses.

3.2 Results

3.2.1 *mos8* suppresses constitutive resistance in *snc1 npr1-1*

The suppressor screen of *snc1 npr1-1* was described elsewhere (Zhang and Li, 2005). *mos8* was isolated based on a partial suppression of the small size and constitutive expression of the *pBGL2-GUS* reporter gene in the *snc1 npr1-1* mutant background (Figure 3.1 a and data not shown). The mutant, however, displayed a very distinct morphological phenotype with flat, dark green leaves and delayed flowering. When crossed with *snc1 npr1-1*, the F_1 progeny exhibited the characteristic *snc1* phenotype, and in the F_2 progeny, 424 out of 556 plants had *snc1*-like morphology (expected ¾, χ^2: 0.47, P = 0.5), confirming that the phenotype is caused by a recessive mutation in a single gene.

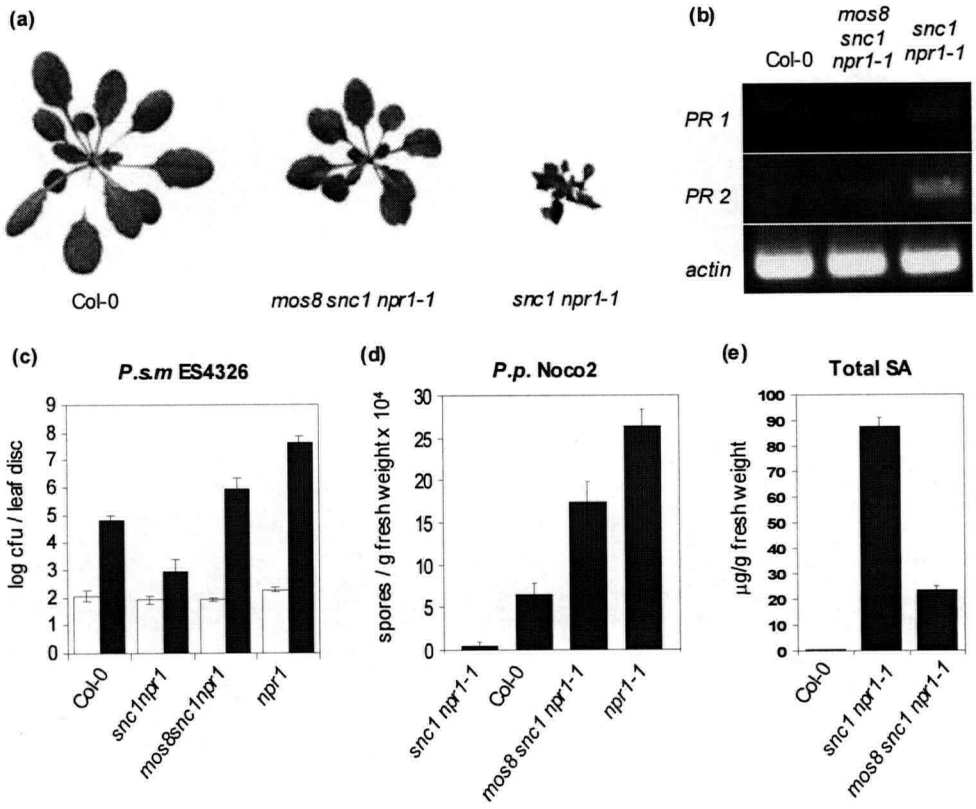


Figure 3.1: *mos8* suppresses *snc1*-mediated resistance phenotypes

(a) Morphology of five-week-old soil-grown plants of indicated phenotypes. (b) Semi-quantitative RT-PCR of pathogenesis related genes *PR1* and *PR2*. Fragments were amplified from standardized cDNA in 30 cycles. Actin is included as a normalization control. (c+d) *mos8* completely suppresses constitutive resistance to virulent bacteria and oomycetes. (c) 5-week-old soil-grown plants were infected with *P.s.m.* ES4326 (OD_{600}=0.0001) and colony forming units (cfu) quantified at 0 (white bars) and 3 (dark bars) days post inoculation (dpi). (d) 2-week-old seedlings were inoculated with *P.p.* Noco2 and conidia were quantified 7 dpi. (e) *mos8* reduces endogenous salicylic acid (SA) in *snc1 npr1*. Total SA was extracted and analyzed with HPLC. Bars represent the average of six (c) and four (d,e) biological replicates, error bars represent standard deviation. All experiments were repeated at least twice with similar results.

At the same time, constitutive *PR* gene expression and enhanced resistance is abolished in *mos8 snc1 npr1-1* (Figure 3.1). In infection experiments with virulent *Peronospora parasitica (P.p.)* Noco2, *mos8 snc1 npr1-1* restores more than wild type-like susceptibility (Figure 3.1 d). When infected with virulent bacteria *Pseudomonas syringae* pv. *maculicola (P.s.m.)* ES4326, *mos8 snc1 npr1-1* sustained high levels of bacterial growth, significantly greater than Col-0

wild type (Figure 3.1 c; P-value <0.0001, t-test). Furthermore, elevated levels of endogenous SA, which are observed in *snc1 npr1-1* mutant plants, are drastically reduced in *mos8 snc1 npr1-1* (Figure 3.1 e), indicating that *mos8* most likely functions upstream of or in parallel with SA synthesis.

3.2.2 *mos8* contains a mutation in a farnesyltransferase subunit

The mutation in *mos8* was identified using a map-based approach. *mos8 snc1 npr1-1* was crossed with *snc1* in Landsberg *erecta* background (Ler-*snc1;* Zhang and Li, 2005) to generate a mapping population in the F_2 progeny. The approximate map position of *mos8* was determined on the bottom arm of chromosome 5 (Figure 3.2 a).

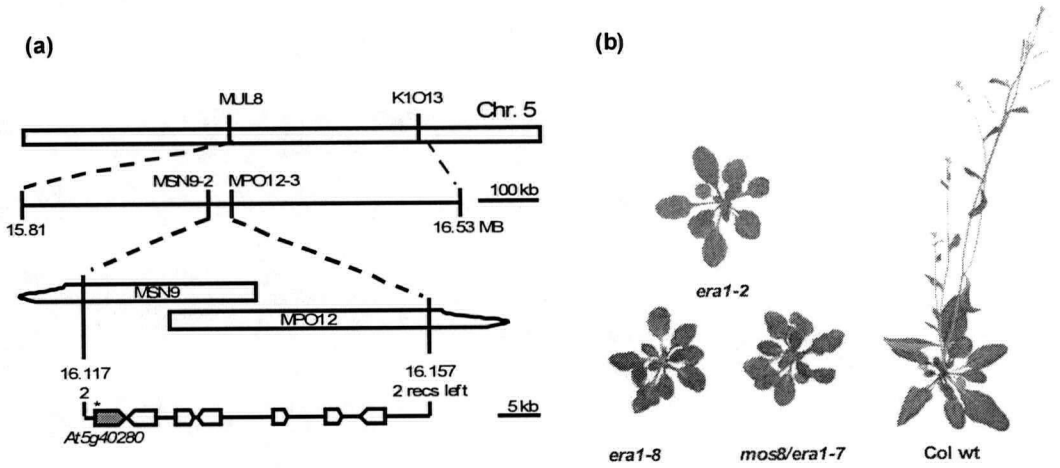


Figure 3.2: *mos8* is allelic to *era1*

(a) Positional cloning of *mos8*. BAC clones and recombinants are indicated. A mutation (*) was identified in *At5g40280/ERA1*. (b) Two additional alleles of *era1* display the same late flowering phenotype as the *mos8 (era1-7)* single mutant. Pictures were taken at 7 weeks after planting.

Using 918 F_2 plants, the region containing the *mos8* mutation was localized to a 723 kb region between markers MUL8 and K1O13, with 56 recombinants remaining. The phenotypes of these recombinants were confirmed in the next generation and they were used to narrow down the region containing *mos8*. The final position of *mos8* was flanked by the markers MSN9-2 and MPO12-4, with two remaining recombinants, respectively. This region encompassed 40 kb, containing 7 genes. Sequencing of the coding regions of these genes revealed a point mutation in *mos8*, substituting G to C in *At5g40280* (Figure 3.4 a).

At5g40280 encodes the beta subunit of protein farnesyltransferase. Plants with loss-of-function mutations in *At5g40280* have been described previously and display several characteristic developmental phenotypes, including delayed germination in *enhanced response to abscisic acid 1* (*era1*; Cutler et al., 1996) and increased floral meristem size and delayed flowering in *wiggum* mutants (Ziegelhoffer et al., 2000). Closer inspection of *mos8* mutant plants revealed an increase in the number of floral organs, as described for the *wiggum* mutants (Running et al., 1998; data not shown).

wiggum mutants obtained from Dr. E. Meyerowitz and *era1-2* mutants from Dr. P. McCourt, as well as a T-DNA insertion allele from the Arabidopsis stock centre (Salk_110517; Alonso et al., 2003) were crossed with *mos8 snc1 npr1-1* to test for complementation. All available mutant alleles of *At5g40280* were unable to complement the *mos8* mutation, confirming that *MOS8* is allelic to *ERA1/WIGGUM* (Table 3.1). Therefore, *mos8* was subsequently referred to as *era1-7,* and Salk_110517 was renamed *era1-8*. Figure 3.2 b shows the similarity in the morphological phenotypes of three *era1* alleles, which are late flowering and have darker rosette leaves than the Col-0 wild type. Moreover, *era1-2 snc1* and *era1-8 snc1* double mutants were obtained, and both alleles were able to suppress *snc1*-associated phenotypes and resistance, further confirming that *mos8* is an allele of *era1* (Table 3.2 and data not shown).

Table 3.1: Allelism test between several *era1*-alleles and *mos8 snc1 npr1-1.*

cross[1]	F_1 morphology[2]	F_2 phenotypic segregation [3]			
		total	wt-like	*snc1*-like	*era1*-like
era1-8 X *mos8 snc1 npr1-1*	*era1*-like	197	0	0	197
mos8 snc1 npr1-1 X *era1-5*	*era1*-like	150	0	0	150
mos8 snc1 npr1-1 X *era1-6*	*era1*-like	125	0	0	125
era1-2 X *era1-7*	*era1*-like	205	0	0	205

[1] The crosses are indicated with the female parent first, the male parent second. *era1-4,5,6* are in Ler ecotype, *era1-2,8* are in Col-0 ecotype.
[2] F_1 phenotypes were scored for seedlings sown on MS-plates and transferred to soil, based on flowering time and morphology.
[3] F_2 phenotypes were scored for seedlings stratified for seven days and sown directly on soil. wt, wild type.

Table 3.2: Multiple *era1* alleles suppress *snc1*.

cross[1]	F_1 morphology [2]	F_2 phenotypic segregation [3]						
		total	wt-like	*snc1*-like	*era1*-like	hypothesis[4]	χ^2	P
snc1 X *era1-4*	wt-like	142	80	28	34	9 : 3 : 4	0.1346	0.93
era1-5 X *snc1*	wt-like	104	61	20	23	9 : 3 : 4	0.4658	0.79
snc1 X *era1-6*	wt-like	168	92	35	41	9 : 3 : 4	0.4788	0.79
era1-8 X *snc1*	wt-like	327	175	66	86	9 : 3 : 4	1.0136	0.60
era1-2 X *snc1*	wt-like	206	118	41	47	9 : 3 : 4	0.5782	0.75

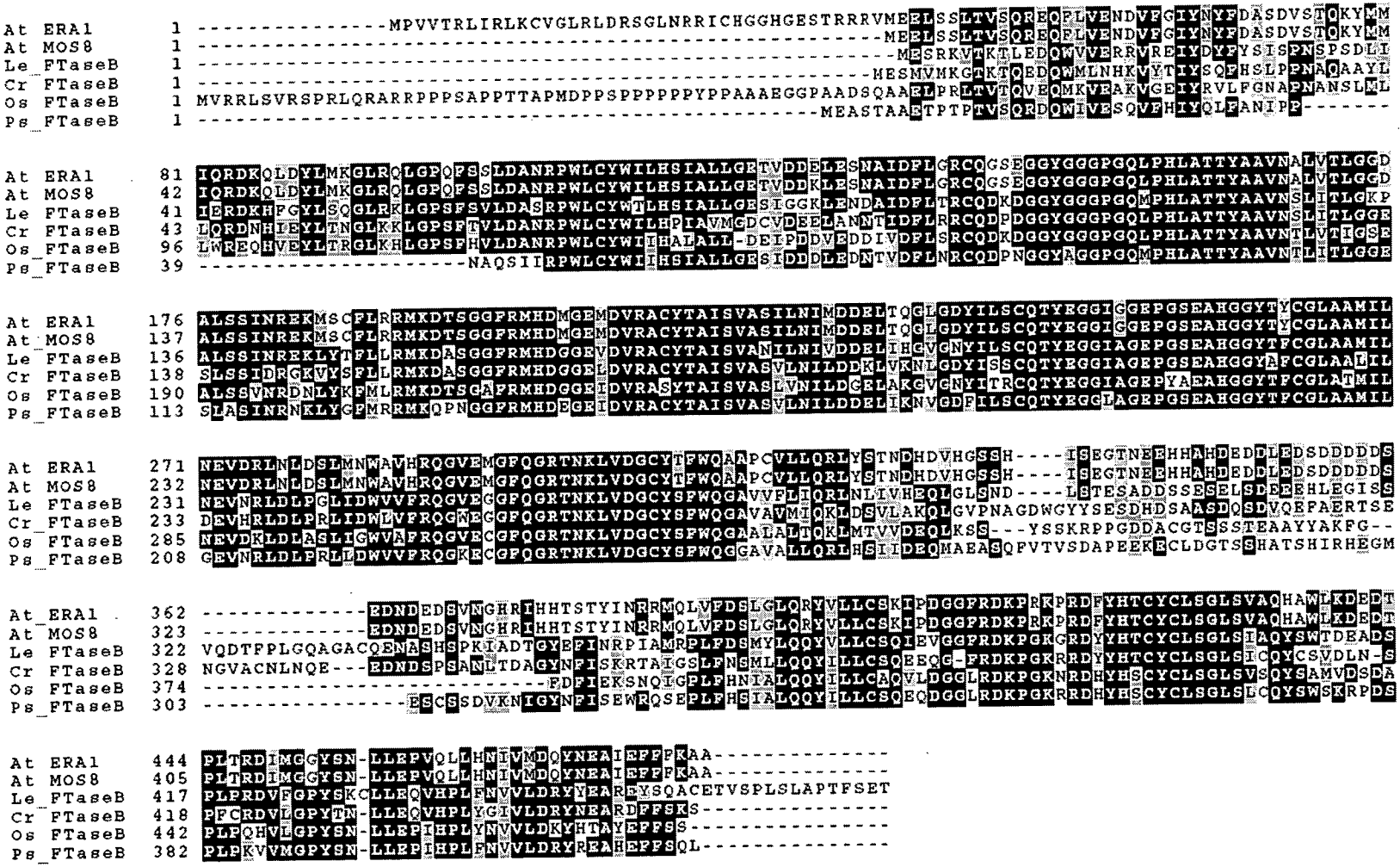
[1] The crosses are indicated with the female parent first, the male parent second. *era1-4,5,6* are in Ler ecotype, *era1-2,8* are in Col-0 ecotype.
[2] F_1 phenotypes were scored for seedlings sown on MS-plates and transferred to soil, based on flowering time and morphology. wt, wild type.
[3] F_2 phenotypes were scored for seedlings stratified for seven days and sown directly on soil.
[4] The hypothesis for suppression of *snc1* in the F_2 is 9 wild type : 3 *snc1*-like : 4 *era1*-like.

3.2.3 The *era1-7* mutation affects the start codon of *ERA1*

The phenotype of *era1-7* is comparable to the deletion mutant allele *era1-2*, suggesting that *era1-7* is likely a complete loss-of-function allele. The G/C point mutation in *era1-7* changes a methionine to an isoleucine. Based on the annotation of the published *ERA1* open reading frame (Ziegelhoffer et al., 2000), this mutated Met is placed 40 amino acids downstream of the start codon. Sequence comparison with ERA1 homologs in other plant species however, suggests that the annotated cDNA for *ERA1* is too long and that translation initiates at a later start codon (Figure 3.3). In accordance with the null-allele phenotype in *era1-7*, we hypothesized that the actual start codon of *ERA1* is absent in *era1-7*, resulting in an altered protein product as the translation initiates at the next available ATG (Figure 3.3 and 3.4 a). To test this hypothesis, we cloned the *ERA1* cDNA beginning with the predicted start codon into the binary vector pBI1.4 (Mindrinos et al., 1994) under control of the CaMV35S promoter and transformed the construct into *mos8 snc1 npr1-1* plants using *Agrobacterium*-mediated floral dip transformation. T_1 seeds were screened for plants that restored *snc1* phenotypes, and 20 independent primary transformant lines were obtained. Constitutive expression of the *ERA1* cDNA starting at the ATG start codon mutated in *mos8* fully complemented *mos8* (Figure 3.4 b), indicating that the ERA1 protein may indeed be smaller than previously suggested. Complementation was also observed when *era1-2* and *era1-7* single mutant plants were transformed with *35S::ERA1* (data not shown).

Figure 3.3: Alignment of Farnesyltransferases from different plant species.

Amino acid sequences were aligned using clustalW (http://www.ebi.ac.uk/clustalw/) and shaded using boxshade (http://www.ch.embnet.org/software/BOX_form.html). Conserved residues are shaded in black and similar residues are shaded in grey. At_ERA1 *Arabidopsis thaliana* (accession NP_198844); Le_FTaseB *Lycopersicon esculentum* (tomato, accession AB69757); Cr_FTase B: *Catharanthus roseus* (Madagascar periwinkle, accession AAQ02809); Os_FTaseB *Oryza sativa* japonica cultivar-group (rice, accession NP_001044183); Ps_FTaseB: *Pisum sativum* (pea, accession Q04903).


At ERA1
At MOS8
Le FTaseB
Cr FTaseB
Os FTaseB
Ps FTaseB

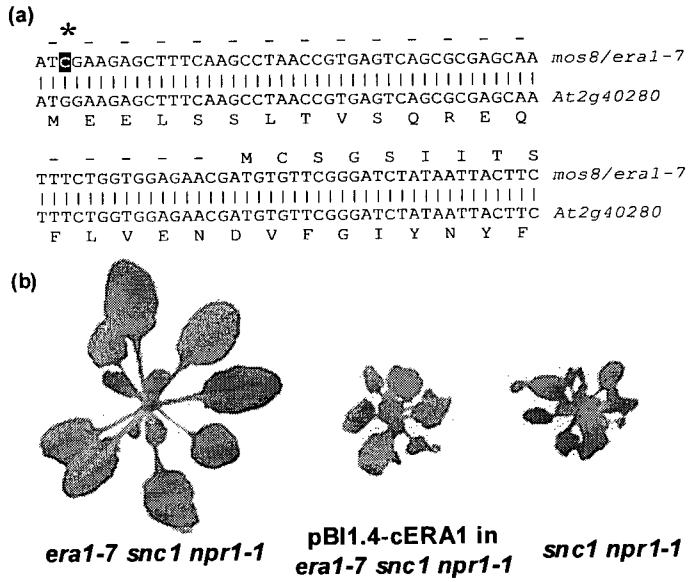


Figure 3.4: The mutation in *mos8/era1-7* affects the *ERA1* start codon

(a) The G to C mutation in *era1-7* (indicated by *) causes a loss of the endogenous ATG start codon. Translation of the wild type and the mutant gene are indicated below and above the alignment, respectively. (b) Complementation of *era1-7 snc1 npr1-1* with *ERA1* cDNA initiating at the start codon mutated in *era1-7* restores *snc1*-phenotype. Representative 5-week-old plants are shown.

3.2.4 *era1* confers enhanced disease susceptibility

Since *mos8 snc1 npr1-1* was shown to be more susceptible than Col-0 to virulent bacteria, we suspected that *era1* might also play a role in basal resistance. We generated the *mos8/era1-7* single mutant by back crossing with wild type and tested it together with the other available alleles in Col-0 background for infection assays. The *era1-7* single mutant showed about 20-fold more growth of virulent *P.s.m.* ES4326 compared with Col-0, similar to *era1-2* and *era1-8.* (Figure 3.5 a). Furthermore, the *era1* alleles in Col-0 background were more susceptible to the virulent oomycete *P.p.* Noco2 (Figure 3.5 b). These data suggest that *ERA1* plays a role in basal defence signalling in responses to different virulent pathogen species.

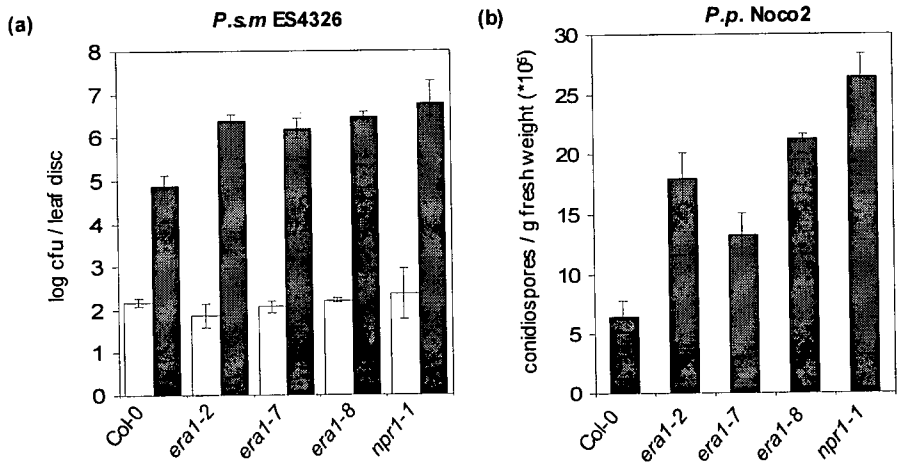


Figure 3.5: *era1* confers enhanced susceptibility to virulent *P. syringae* and *P. parasitica*.

(a) 5-week old plants were infected with *P.s.m.* ES4326 (OD_{600}=0.0001) and bacterial growth quantified at 0 (white bars) and 3 dpi (black bars). (b) Quantification of *P.p.* Noco2 conidiospores 7 dpi. Susceptibility in the *era1* single mutants is significantly enhanced compared to Col-0 wild type ($P<0.0001$, t-test). Bars represent the average of 6 biological replicates, error bars indicate standard deviation. Experiments were repeated twice with similar results.

3.2.5 *era1* affects resistance to avirulent pathogens

Several of the *mos* mutants identified in the *snc1* suppressor screen have previously been shown to exhibit reduced resistance towards avirulent pathogens (Palma et al., 2005; Zhang et al., 2005; Zhang and Li, 2005; Goritschnig et al., in press). To investigate whether a mutation in *ERA1* also affects other R protein signalling pathways, single mutant plants were infected with avirulent bacterial and oomycete pathogens. We did not observe significant differences compared with Col-0 in susceptibility towards *P. syringae* expressing the effectors AvrB or AvrRpt2, which trigger defence signalling of the R proteins RPM1 and RPS2, respectively (data not shown). However, in infection experiments with *P. syringae* pv. *tomato* (*P.s.t.*) DC3000 expressing AvrRpm1 or AvrRps4, effectors recognized by the R proteins RPM1 and RPS4, respectively, we observed significantly increased susceptibility in *era1-7* compared to Col-0 wild type (Figure 3.6 a,b).

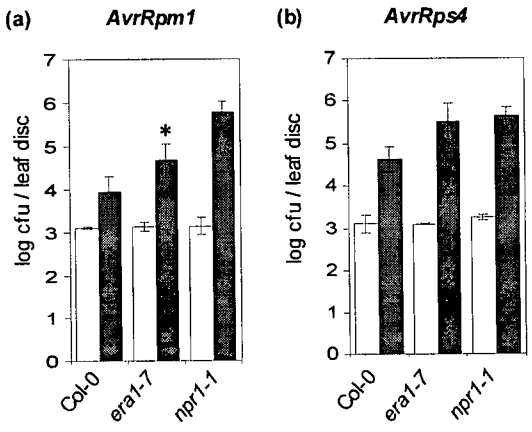


Figure 3.6: *era1* affects R protein signaling

5-week-old plants were infected with *P.s.t.* DC3000 expressing *AvrRpm1* (a) or *AvrRps4* (b) at OD_{600}=0.001 and bacterial growth quantified at 0 (white bars) and 3 dpi (black bars). Susceptibility towards *AvrRpm1* in the *era1* single mutant is significantly enhanced compared to Col-0 wild type (P=0.0071, t-test). Bars represent the average of six biological replicates, error bars indicate standard deviation. Experiments were repeated twice with similar results.

These data indicate that ERA1 might be preferentially required in the interaction between some pathogen effectors and their cognate R protein.

We also took advantage of the availability of *era1* alleles in different genetic backgrounds to investigate their responses to avirulent oomycetes. *P.p.* Noco2 is a virulent pathogen for the Col-0 ecotype but avirulent on Ler, which contains the *RPP5 R* gene. Infection assays with *P.p.* Noco2 on *era1* alleles in different ecotypes can thus provide insight into the involvement of *ERA1* in both compatible and incompatible interactions. *era1* mutants in the Col-0 genetic background showed significantly more growth of the oomycete pathogen, as determined by quantification of conidiospores (Figure 3.5 b). Inoculation of Ler plants induced a rapid hypersensitive response, which is apparent in trypan blue staining (Figure 3.7 c). This staining method is used to reveal hyphal structures and dead cells, whereas live cells are not stained. The *wiggum* alleles *era1-4* and *era1-6* in the Ler background were able to suppress RPP5-mediated resistance

towards *P.p.* Noco2, visualized by hyphal growth in infected tissues and the formation of conidiophores (Figure 3.7 a+c).

In infection experiments with *P.p.* Emwa1, which is recognized by RPP4 in Col-0, the *era1* mutant plants were more susceptible than Col-0 (Figure 3.7 b+c). *era1* alleles allowed sporulation of the pathogen to which Col-0 is resistant to, albeit to a lesser extent than the susceptible ecotype control *Wassilewskija* (Ws). Strong resistance towards *P.p.* Emwa1 in Ler conferred by RPP5 and RPP8 was also compromised in the *wiggum* alleles, as demonstrated by extensive trailing necrosis and occasional sporulation on the mutant plants (Figure 3.7 c and data not shown). Taken together, these findings indicate that the farnesyltransferase encoded by *ERA1* and therefore farnesylation might be important in a subset of R protein-mediated resistance responses, that confer resistance to bacterial and oomycete pathogens.

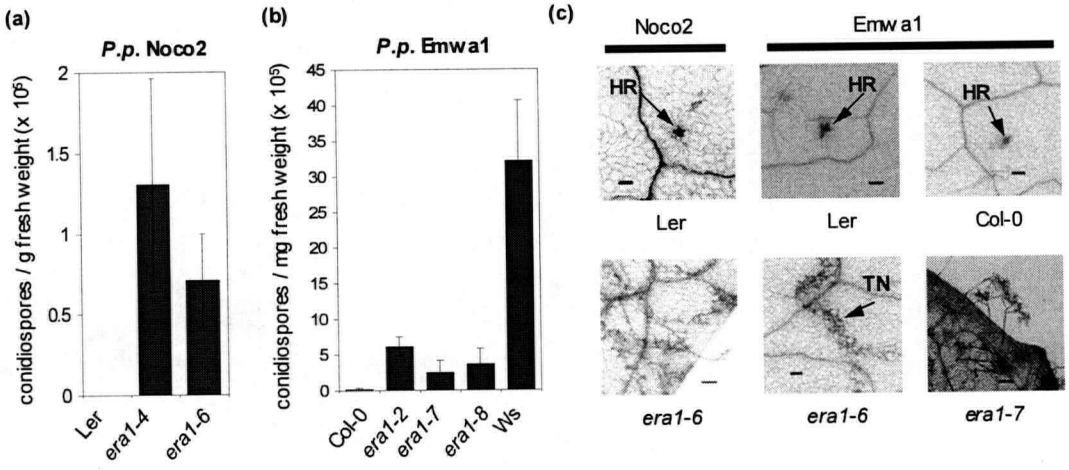


Figure 3.7: *era1* confers enhanced susceptibility to avirulent oomycete pathogens.

(a-b) Growth of *P. parasitica* isolates Noco2 and Emwa1 on *era1* alleles in different ecotypes. *P.p.* Noco2 is avirulent on Ler ecotype (a), and *P.p.* Emwa is avirulent on Col-0 (b). Ws is included as a susceptible control. Conidiospores were harvested and quantified 7 dpi. Bars represent the average of four biological replicates, error bars indicate standard deviation. Experiments were repeated twice with similar results. c) Visualization of oomycete growth by lactophenol trypan blue staining at 7 dpi. HR, hypersensitive response. TN, trailing necrosis. Bar in (c) represents 100 μm.

3.2.6 *ERA1* acts additively with *NPR1* in resistance signalling

Resistance signalling downstream of *snc1* has been shown to combine the contributions of at least three distinct signalling pathways, dependent and independent on either SA or NPR1, or both (Zhang et al., 2003).

In order to genetically dissect the contribution of *era1* in the *snc1* signalling pathway, we generated *era1-7 snc1* and *era1-7 npr1-1* double mutants and compared them with their respective controls in bacterial infection assays (Figure 3.8 a).

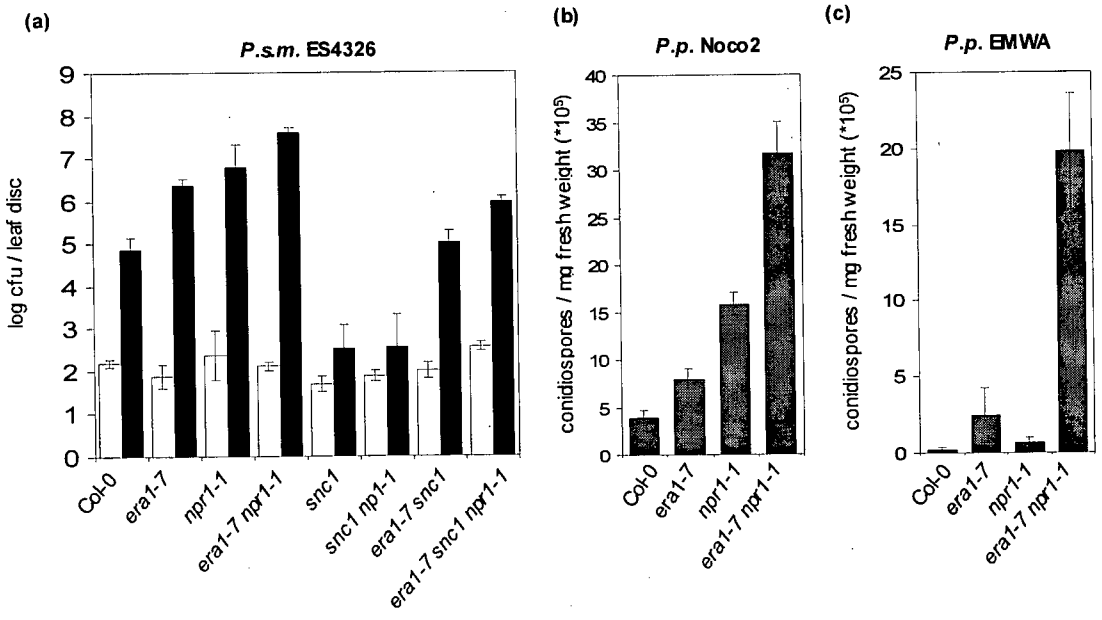


Figure 3.8: *era1* and *npr1* act additively to confer enhanced disease susceptibility.

(a) Enhanced susceptibility to virulent *P. s. m.* ES4326 in *era1* is potentiated by *npr1-1*. Bacterial growth was determined 0 (white bars) and 3 (dark bars) dpi. (b+c) *era1* and *npr1* have additive effects in susceptibility to virulent *P.p.* Noco2 (b) and avirulent *P.p.* Emwa1 (c). Conidiospores were harvested and quantified 7 dpi. Bars represent the average of six (a) and four (b,c) biological replicates, error bars indicate standard deviation. Experiments were repeated twice with similar results.

The *era1-7 snc1* double mutant plants exhibited susceptibility comparable to Col-0, indicating complete suppression of *snc1*-mediated resistance by *era1*. The *npr1-1* mutation confers greatly enhanced susceptibility towards virulent bacteria, an effect that is slightly increased in the presence of the *era1-7* mutation. The additive effects of the *npr1* and *era1* mutations are apparent in *Peronospora* infection assays, where the double mutant resulted in increased growth of virulent *P.p.* Noco2 (Figure 3.8 b). Interestingly, we also observed a similar additive effect of *era1* and *npr1-1* in infection assays with avirulent *P.p.* Emwa1 (Figure 3.8 c). We therefore concluded that *ERA1* most likely acts in an *npr1* independent pathway to mediate resistance signalling and that both genes act synergistically in basal and RPP4-mediated resistance responses.

3.2.7 Geranylgeranylation is not required in defence responses

Protein farnesyltransferase (PFT) is a modular enzyme in which the alpha subunit forms a scaffold for the barrel-shaped beta subunit that performs the addition reaction (Park et al., 1997). In an alternative prenylation pathway, protein geranylgeranyltransferase 1 (PGGT1) utilizes the same alpha subunit but a distinct beta subunit to transfer geranylgeranyl units to target proteins. The enhanced susceptibility of the *era1* mutant towards virulent and avirulent pathogens prompted us to investigate a potential role of geranylgeranylation in defence responses. We obtained a T-DNA insertion line with a defect in At2g39550, the gene encoding the geranygeranyltransferase beta subunit *GGB*, from the Arabidopsis stock center (Salk_040904). The Salk_040904 line, representing the *ggb-2* allele, carries the T-DNA insertion in the first exon. Although *ggb-2* represents a null allele with no detectable GGB transcript, the *ggb-2* mutant did not exhibit significant morphological differences compared with wildtype (Johnson et al., 2005). However, the mutant has been described to be involved in several aspects of hormone signalling (Johnson et al., 2005).

We challenged the *ggb-2* mutant with virulent *P.s.m.* ES4326 and avirulent *P. syringae* and, in contrast to the *era1* alleles, the mutant did not exhibit enhanced susceptibility (Figure 3.9 a and data not shown). The *ggb-2* mutant

showed a slight but not statistically significant increased susceptibility to *P.p.* Noco2 compared to wild type (P=0.3307, t-test), but the mutation did not result in the extensive oomycete growth observed in *era1* (Figure 3.9 b). Furthermore, RPP4-mediated resistance towards *P. p.* EMWA1 was only mildly affected in *ggb-2* as visualized by the development of trailing necrosis and delayed HR in the mutant (Figure 3.9 c). These data imply a very minor involvement of geranylgeranylation in some aspects of defence responses. To test whether the *ggb-2* mutation had an effect on *snc1*-mediated resistance, we generated a double *snc1 ggb-2* mutant. However, we did not observe suppression of *snc1*-associated phenotypes (data not shown). Taken together, our findings suggest that farnesylation, and not geranylgeranylation, plays an important role in both basal and R protein defence signalling.

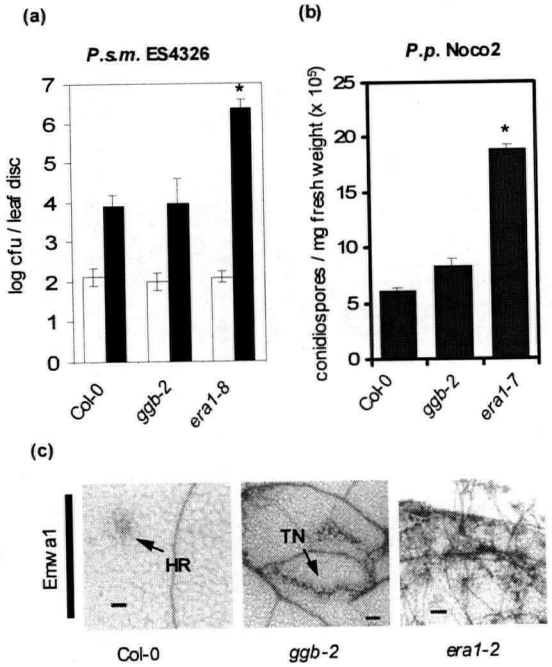


Figure 3.9: Involvement of geranylgeranylation in defence responses

Col-0 wild type, *ggb-2* and *era1* plants were infected with virulent *P.s.m.* ES4326 (a), virulent *P.p.* Noco2 (b) and avirulent *P.p.* EMWA (c). Bacterial growth in (a) was determined as colony forming units 0 (white bars) and 3 (dark bars) dpi. Oomycete growth was determined by quantifying conidiospores (b) and trypan-blue staining of infected plant tissue (c) 7 dpi. Experiments were repeated twice with similar results. Bars represent the average of six (a) and four (b,c) biological replicates, error bars indicate standard deviation. Asteriks indicate statistically significant differences (t-test). Bars in (c) represent 100 μm.

3.3 Discussion

Proteins are frequently altered post-translationally to modify their solubility, compartmentalisation or interaction with other proteins. The most common lipid modification, prenylation, involves the covalent attachment of farnesyl- or geranylgeranyl-diphosphate moieties to the C-terminus of a small group of target proteins, which contain a conserved CaaX motif (Galichet and Gruissem, 2003). Unlike in yeast and *Drosophila*, mutations in prenyltransferases are not lethal in *Arabidopsis*, suggesting that plants may have evolved alternative mechanisms to bypass the essential requirement for protein farnesylation (Trueblood et al., 1993; Therrien et al., 1995).

Protein prenyltransferases are modular enzymes and mutations in several subunits have been described in *Arabidopsis* (Galichet and Gruissem, 2003; Running et al., 2004; Johnson et al., 2005). *Enhanced response to ABA 1 (era1)*, a mutant in the farnesyltransferase beta subunit, was first identified based on delayed germination in the presence of abscisic acid (ABA) (Cutler et al., 1996) and was later found to display a variety of morphological phenotypes, including an increase in floral organ number and enlarged meristems (Bonetta et al., 2000; Yalovsky et al., 2000; Ziegelhoffer et al., 2000). Guard cell responses to ABA are enhanced in *era1*, resulting in enhanced drought resistance of the mutant plants (Pei et al., 1998). This phenomenon was used in the production of transgenic *Brassica napus,* in which silencing of endogenous *ERA1* by an antisense construct results in increased drought resistance (Wang et al., 2005).

Here we show that farnesylation is not only important in development and abiotic stress responses, but also in biotic interactions. The enhanced susceptibility of *mos8/era1-7* towards virulent bacterial and oomycete pathogens indicates the involvement of farnesylation in basal defence responses. In addition, our findings indicate that signalling mediated by several R proteins relies on functional ERA1, further demonstrating the existence of divergent signalling events downstream of different R protein classes.

Interestingly, a mutation in the alternative prenyltransferase β-subunit, *ggb-2* resulted in no detectable defence-related phenotype and it is unable to

suppress constitutive resistance mediated by *snc1*. Geranylgeranyltransferase-beta (GGB) was previously shown to partially compensate a lack of farnesyltransferase activity, probably due to the larger substrate binding-pocket in GGB, which might accommodate the farnesyl-diphosphate (Johnson et al., 2005). Overexpression of GGB complemented the *era1* phenotype and the double mutant *ggb-2 era1-4* was shown to exhibit a phenotype similar to *pluripetala (plp)*, a mutant in the α-subunit of prenyltransferase which exhibits an aggravated *era1*-like phenotype (Running et al., 2004; Johnson et al., 2005). However, in our cross between *ggb-2* and *era1-7*, both in the Col-0 ecotype background, we did not see the drastic increase of floral meristem size and the plants resembled *era1-7* (data not shown). It has been shown previously for the *wig* mutants that backcrosses with Col-0 increased the number of floral organs (Running et al., 1998). Hence, the *plp*-like phenotype observed in the *ggb-2 era1-4* double mutant may result from the hybrid Col-0 and Ler ecotype cross (*era1-4* is *wig1* isolated in the Ler ecotype).

The differential requirement for farnesylation and geranylgeranylation in defence responses suggests that one or more targets of farnesyltransferase are involved in defence signalling. Farnesyl- and geranylgeranyltransferase target proteins differ with respect to their C-terminal CaaX consensus sequence. The C represents the prenylation target cysteine, whereas 'a' indicates an aliphatic amino acid. While the X in farnesylated proteins stands for methionine, alanine, glutamine, serine or cysteine, the geranylgeranylated proteins usually have a leucine in this position (Rodriguez-Concepcion et al., 1999). Since sequencing of the *Arabidopsis* genome has been completed, more than 100 proteins with a PFT CaaX consensus sequence at the C-terminus have been identified, roughly twice the number identified in the human genome (Galichet and Gruissem, 2003; Roskoski, 2003). Among putative prenylated proteins with a CaaX consensus, cell cycle regulators, metal-binding proteins and signalling proteins represent the majority (Galichet and Gruissem, 2003).

It is tempting to speculate about a requirement for specific farnesylated proteins in defence responses, and some prenylated proteins have been associated with plant-pathogen interactions. *AIG1 (avrRpt2 induced gene 1*) was

identified as rapidly upregulated transcriptionally in response to infection with *P.s.m.* ES4326 AvrRpt2 (Reuber and Ausubel, 1996) and the amino acid sequence of the predicted protein terminates in the geranylgeranylation consensus sequence CSIL. The *Arabidopsis* genome contains a small family of AIG1-like proteins clustered on chromosome 1, and their function is predicted to involve GTP-binding. Other G-proteins, that contain the geranylgeranylation consensus sequence, are members of the plant ras (rat sarcome oncogene product) related C3 botulinum toxin substrate (RAC)/Rho of plants (ROP) family, which have been implicated in the susceptible interaction between barley and the powdery mildew *Blumeria gramins f.sp. hordei* (*Bgh*) (Schultheiss et al., 2003). The functional analogs of RAC/ROP proteins in animals, Rho, are involved in cytoskeleton reorganization (Takai et al., 2001). Interestingly, one of the RAC proteins in barley has been connected to cell polarization and actin rearrangements in the interaction with *Bgh* (Opalski et al., 2005). Both of these examples concern geranylgeranylation targets involved in defence responses, leaving an open question as to why mutations in farnesyltransferase, but not geranylgeranyltransferase so severely affected resistance responses in our study.

We attempted to investigate potential farnesylated signalling components by a reverse genetics approach using available T-DNA insertion lines from the Arabidopsis stock center and a collection from P. McCourt. After testing 25 homozygous knockout lines, we did not find any mutants that were affected in their response to avirulent *P.p.* Emwa1 to the same extent as *era1* (S.G. unpublished data). However, one has to be cautious with the interpretation of these results, given that not all potential targets were tested due to unavailability of T-DNA insertions in a number of interesting genes (such as *AIG1*) or potential redundancies among protein family members.

Another perspective for the role of ERA1 in defence signalling is a link to abiotic stresses via its involvement in ABA signalling. Recent studies using mutants impaired in ABA production and signalling indicate a negative correlation between ABA and susceptibility to biotrophic and necrotrophic pathogens (Mauch-Mani and Mauch, 2005). For example, reduced levels of ABA in *aba1-1*

mutant plants correlated with increased resistance towards virulent and avirulent *Peronospora* isolates, but the ABA insensitive mutant *abi1-1* displayed wild type susceptibility (Mohr and Cahill, 2003). Interestingly, *era1* suppresses ABA insensitivity of *abi1* and the two genes are hypothesized to function in parallel pathways in guard cell responses (Pei et al., 1998). The fact that *era1* and *aba1* mutations, but not *abi1*, affect defence responses might therefore indicate the existence of a complicated network integrating ABA and resistance signalling. Indeed, gene expression profiling experiments unveiled antagonistic interactions between ABA and wound-responsive signalling pathways employing jasmonic acid (JA) and ethylene (Anderson et al., 2004). The transcriptional activator AtMYC2 was shown to be involved in ABA- and JA-responsive gene activation during drought stress and pathogen attack (Abe et al., 2003; Lorenzo et al., 2004). In the tomato – *Botrytis cinerea* interaction, antagonistic interactions between ABA and SA signalling were observed (Audenaert et al., 2002). Finally, ABA signalling in guard cells has been shown to play an important role in the initial recognition of bacterial pathogens on the leaf surface and their potential for invasion through the stomata in an SA-dependent manner (Melotto et al., 2006).

Taken together, previous results and data presented in this study indicate complex cross-talk between development, biotic and abiotic stress signalling, shedding light on a more extensive involvement of ABA in defence responses than previously suggested. It will be interesting to further dissect the detailed function of farnesylation and ABA in disease resistance.

3.4 Experimental procedures

3.4.1 Plant growth and mutant characterization

Plants were grown at 22°C under long-day conditions (16h light/8h dark). Seeds were surface-sterilized using 5% hypochlorite solution and stratified for 7 days at 4°C before sowing. The screen for suppressors of the *snc1 npr1-1* double mutant was described previously (Zhang and Li, 2005). pBGL2-GUS expression was determined by histochemical staining of 20-day old seedlings grown on MS following standard protocols. Measurements of endogenous SA levels were performed as described (Li et al., 1999). RNA extraction and semi-quantitative RT-PCR were performed as described (Zhang et al., 2003).

3.4.2 Pathogen assays

For *Pseudomonas* infection assays, 5-week old soil-grown plants were infiltrated with a bacterial suspension in 10 mM $MgCl_2$ at an optical density of OD_{600} = 0.0001 for virulent *P. s.m.* ES4326 or OD_{600} = 0.001 for avirulent *P.s.m.* and *P.s.t.* using a blunt syringe. Bacterial growth was measured 3 days post-infection (dpi) by harvesting leaf discs and determining colony forming units (cfu). *Peronospora parasitica* isolates Noco2 and Emwa1 were inoculated on 2-week-old seedlings at a concentration of 5 x 10^5 conidiospores per ml. At 7 dpi, conidiospores were quantified by harvesting replicate samples of 15 plants, vortexing and counting using a hemocytometer. Plant necrosis and hyphal growth were visualized using lactophenol trypan blue staining following a protocol by Koch and Slusarenko (1990). All experiments were repeated at least twice with similar results.

3.4.3 Positional cloning of *mos8*

The markers used to map *mos8* were derived from the Ler/Col-0 polymorphism database provided by Monsanto on the TAIR homepage (Jander *et al.*, 2002; http://www.arabidopsis.org/Cereon/). Marker MUL8 was amplified using primers MUL8-F (5'-aaggttaatagacctgtcgg-3') and MUL8-R (5'-aagcacaagccatttgacca-3') yielding PCR-fragments of 285 and 174 bp in Col-0 and Ler, respectively. Marker K1O13 was amplified using primers K1O13-F (5'-tgatcacaacttcaccattg-3') and K1O13-R (5'-aatgtaaacaccaaagctgc-3') yielding PCR-fragments of 352 and 230 bp in Col-0 and Ler, respectively. Marker MSN9-2 was amplified using primers MSN9-2F (5'-gtggagaagtgggtttatgg-3') and MSN9-2R (5'-cgggaagatttgagagcagc-3') yielding PCR-fragments of 265 bp in Col-0 and Ler, which were digested with *Hinc*II only in Ler. The marker MPO12-3 was amplified using primers MPO12-3F (5'-agacgtttatagcttcggag-3') and MPO12-3R (5'-actggttggagatggaatcg-3') yielding 397 bp PCR-fragments in both ecotypes, which upon digestion with *Taq*1 generated four fragments in Col-0 and three in Ler.

3.4.4 Complementation of *mos8* with *ERA1* cDNA

ERA1 cDNA was amplified using Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) with primers ERA1short_5'_EcoR1: 5'-agaattcatggaagagctttcaagcc-3' and ERA1short_3'_Not1: 5'-ttttgcggccgctcatgctgctttaaagaagaac-3', containing novel restriction sites (underlined). The PCR fragment was subcloned into pBluescript (Alting-Mees et al., 1992) using *Eco*R1 and *Not*1, and sequenced. The fragment was subcloned into *pBIN1.4 (Mindrinos et al., 1994)* under the control of the CaMV-35S promoter and transformed into *mos8 snc1 npr1-1* using the *Agrobacterium* floral dip method (Clough and Bent, 1998). Transformants were identified based on kanamycin resistance.

3.4.5 Generating the *era1-7* single and double mutants

To generate the *era1-7* single and the *era1-7 snc1* and *era1-7 npr1-1* double mutant, *mos8 snc1 npr1-1* was crossed with Col-0 carrying the pBGL2::GUS reporter transgene. Mutant combinations were identified among the F_2 progeny of selfed F_1 plants based on morphological phenotypes and genotyping. Homozygosity of *snc1* in the *era1-7 snc1* double mutant was confirmed by backcrossing with *snc1*, all F_1 progeny displayed *snc1* morphology.

3.4.6 Identification of homozygous T-DNA mutant plants

The *era1-8* allele was identified in Salk_110517 using the gene-specific primers Salk_110517-A (5'-agaacacaaggggctgcctg-3') and Salk_110517-B (5'-tgcttccctcttccttgatg-3'). Homozygous *ggb-2* mutant plants were identified in Salk_040904 using gene-specific primers Salk_040904-A (5'-tagtaggaaaggcctggaag-3') and Salk_040904-B (5'- gatccaagtgtccttgaacg-3'). Presence of either T-DNA was verified using a combination of Lba1 and Salk_040904-A or Salk_110517-A

3.5 References

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4. Discussion

4.1 Use for the auto-immune *snc1* model in plant resistance studies

Agricultural crop production registers significant losses annually due to phytopathogenic attacks. In plant-pathogen interactions, R proteins have been identified as the principal sentries responsible for surveillance in the plant cell. Upon recognition of pathogenic elicitors, R protein activation triggers major transcriptional reprogramming leading to pathogen containment and resistance. On the one hand, much is known about the physiological outcome of R protein activation, such as production of reactive oxygen intermediates, induction of SA and phytoalexin production and expression of pathogenesis-related proteins with toxic effects to the attacking pathogen. On the other hand, only a few key signalling players responsible for those physiological changes have been characterized in depth so far, and much is yet to be learned about the detailed pathways involved in defence responses.

This thesis describes the discovery of two genes with important roles in plant resistance, which were identified based on suppression of a gain-of-function *R* gene allele conferring constitutive resistance, *snc1*. The *snc1* mutant is unique in that it constitutes the only reported gain-of-function *R* gene in which enhanced resistance is not associated with constitutive cell death, as observed in several lesion mimic mutants. For example, mutations in the NBS and LRR regions of the potato *R* genes *Rx* or of *ssi4* in *Arabidopsis* result not only in enhanced resistance, but also in constitutive HR and the formation of spontaneous lesions (Bendahmane et al., 2002; Shirano et al., 2002). The position of the *snc1* mutation in the linker region connecting the NBS and LRR domains suggests that constitutive activation of SNC1 results from a conformational rearrangement between the domains, thereby potentially losing the ability to associate with a negative regulator (Zhang et al., 2003). The absence of deleterious side effects, such as spontaneous lesion formation, make *snc1* an excellent model to study signalling downstream of activated R proteins. Suppressors of constitutive HR in lesion-mimic mutants such as *Rx* or *ssi4* would likely constitute components of

the cell-death pathway rather than genes involved in R protein signalling, and thus make it difficult to discern between the two.

4.2 Identification of novel defence signalling components using *snc1*

Using the *snc1* mutant as a background for a suppressor screen, a number of *mos* (*modifier of snc1*) mutants were identified that completely or partially suppress *snc1*-mediated morphological and resistance phenotypes. The identity of some of them unveiled new perspectives on defence signalling pathways not only in plants, but also in animal innate immunity, where their homologs might perform similar functions. MOS2, a nuclear protein with putative RNA binding-activity, is involved in basal and R protein mediated defences and might control transcript levels of regulatory genes during resistance responses (Zhang et al., 2005). The significance of nucleo-cytoplasmic trafficking was obvious from the fact that defence responses coincide with transcriptional reprogramming, including the *de novo* expression of pathogenesis-related genes. The important regulatory protein NPR1 was shown to shuttle between cytoplasm and nucleus, where it associates with transcription factors to actively initiate PR gene transcription (Zhang et al., 1999; Mou et al., 2003). Three *mos* mutants affecting nucleo-cytoplasmic transport were identified in the *snc1* suppressor screen, with lesions in the importin alpha homolog MOS6 and the nucleoporins MOS3 and MOS7 (Palma et al., 2005; Zhang and Li, 2005; Cheng, Y. et al., unpublished data). All of these affect basal and R protein resistance. MOS3 likely serves as a gateway for mRNA export while MOS6 and MOS7 are involved in protein import (Vasu et al., 2001; Palma et al., 2005; Cheng, Y. et al., unpublished data).

The *mos* mutants described in this work reveal a requirement for ubiquitination and farnesylation in defence responses, implying a global role for protein modification in defence signalling.

4.3 Post-translational protein modification in plant defence

An effective immune response requires intricate networks of regulatory signalling pathways. Appropriate spatial and temporal distribution of the contributing proteins is imperative for proper function. A variety of post-translational modifications assists in correct targeting of the protein to the right cell compartment, facilitates secretion or membrane association or helps in the assembly of multi-molecular protein complexes. Proteins are frequently modified by attachment of carbohydrate moieties, fatty acids or prenyl groups, all of which alter the surface properties of the protein and aid in their interaction with hydrophilic and hydrophobic environments or other proteins. Chemical modification of amino acids in a protein, such as methylation, can also alter the protein properties. Transient modifications include phosphorylation or ubiquitination, and these are typically employed to alter the activity and stability of proteins.

An association between protein modification and defence signalling has been reported for protein phosphorylation, where Mitogen Activated Protein (MAP) Kinase cascades play a pivotal role in resistance to viruses and non-host pathogens (Zhang and Klessig, 2001). There is, however, a limit to the complexity of how proteins can be modified by phosphorylation. Recent reports are proposing an emerging role for the versatile and reversible protein modification by ubiquitination in plant defenses. Ubiquitination has been implicated in plant-virus interactions (Becker et al., 1993) and a number of ubiquitin ligases have been shown to be important in plant resistance responses (Kim and Delaney, 2002; Kawasaki et al., 2005; Gonzalez-Lamothe et al., 2006; Yang et al., 2006).

The work presented here provides novel aspects for the involvement of protein modification in defence responses. MOS5 pinpoints the requirement of ubiquitination in plant innate immunity, and MOS8/ERA1 constitutes the first report of the involvement of farnesylation in defence.

4.4 Perspectives for future research

The fact that *mos5* as well as *mos8* contain mutations in enzymes involved in the first step of two important modification pathways adds another layer of complexity to defence signalling networks. It is tempting to speculate about regulatory roles of the proteins targeted for modification, but the identity of those so far remains elusive.

In an initial attempt, a number of available T-DNA insertion mutant lines in genes coding for potential farnesyltransferase target proteins were screened for alterations in disease phenotypes. No significant difference was observed compared to wildtype Col-0 for the lines tested. However, since the number of farnesylation targets is limited by their carboxy-terminal consensus sequence, a systematic reverse genetics approach could be used to identify the one(s) involved in defence signalling. Screening a complete selection of homozygous insertion mutant lines for defence-related phenotypes is labour intensive but doable. One difficulty using this approach could be the potential redundancy of target proteins, since some of them are present in large families in *Arabidopsis*. It might be necessary to generate multiple knockouts for those or, alternatively, RNA interference-based approaches could be used to knock down expression of closely related genes simultaneously. Alternatively, defence-related target proteins of farnesyltransferase could be identified in an *era1* suppressor screen, looking for abrogation of enhanced susceptibility towards virulent and avirulent pathogens.

A similar approach could be used to identify MOS5 target proteins, except here one would have to target the enzymes catalyzing the second step of ubiquitination, the conjugating enzymes, or E2s. The complexity of the ubiquitination network, with around 40 E2s and hundreds of E3s, which work together to specifically ubiquitinate proteins to alter their stability or functionality, poses a significant challenge in this respect (Bachmair et al., 2001). The *mos5* mutation lies within the carboxy-terminal region of UBA1, which is potentially involved in binding of E2s. Thus, protein-protein interaction assays could aid in identifying the E2s, which might discriminate between UBA1 and MOS5.

However, it cannot be ruled out that binding of all E2s is affected by the *mos5* mutation, and that it can be partially compensated for by the alternative E1, UBA2. This hypothesis is supported by the observation that a double mutant *mos5 uba2* is lethal.

A different approach to unravel the contribution of MOS5 in defence signalling would be to investigate the *mos5* mutant in the context of E3 ligases that have been documented to participate in defence responses. One could generate mutant combinations with *mos5* and E3 mutants, if available, or one could compare E3 functions between *mos5* and the wild type.

4.5 Conclusion

The identification of *mos5* and *mos8* in a screen for suppressors of a constitutively active R protein has added significantly to the complexity of signalling networks in plant disease resistance. Transient and stable modifications are known to be important in specifying a protein's localization and activity, and the data described in this thesis highlight the particular importance of ubiquitination and farnesylation in plant defence responses. However, identification of a defence-related role for the ubiquitin activating enzyme and farnesyltransferase represent only the "tip of the iceberg", and the much larger body of the downstream targets remains to be brought to light. Much research is still needed to fully understand the role and involvement of these protein modification pathways in resistance responses, and this thesis presents a first stepping stone.

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