REGULATION OF PLANT INNATE IMMUNITY:
THE ROLE OF PROTEIN IMPORT AND THE NOVEL
MOS4-ASSOCIATED COMPLEX

by

Kristoffer Palma

B.Sc. (First Class Honors), Simon Fraser University, 1999
M.Sc., Simon Fraser University, 2002

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Abstract

Plants have evolved sophisticated defence systems against pathogen infection. Initiation of induced defence signalling often involves specific recognition of invading pathogens by the products of specialized host Resistance (R) genes. Consequently, the pathogen is stopped at the site of infection. A unique dominant mutant in Arabidopsis thaliana, snc1, constitutively expresses pathogenesis-related (PR) genes and exhibits enhanced resistance to bacterial and oomycete pathogens. SNC1 encodes an R-gene – a single amino acid change renders this protein constitutively active without interaction with pathogens. snc1 displays a stunted phenotype that may be caused by both the accumulation of toxic compounds and energy squandered on unnecessary defence instead of normal growth. The distinctive morphological phenotype of snc1 is intimately associated with the other resistance phenotypes, and provides a robust genetic tool for dissecting the signalling events downstream of snc1.

To identify genes important for defence signalling, we carried out a suppressor screen to identify modifier of snc1 (mos) mutants that restore the wild type size and morphology in the snc1 background. Furthermore, in most cases, a loss of sneakiness in mos mutants correlated with a reduced or abolished constitutive PR gene expression, SA accumulation and pathogen resistance in snc1 plants. These loss of function mutants represent defects in positive regulators of the snc1 pathway. I cloned and characterized two mos mutants, and showed that they both have roles in Arabidopsis innate immunity as well.

mos6 partially suppresses snc1 and exhibits enhanced disease susceptibility (EDS) to an oomycete pathogen. MOS6, identified by map-based cloning, encodes an alpha-importin subunit, one of 8 found in Arabidopsis, and has a demonstrated role in nucleocytoplasmic partitioning (protein import). Two other genes cloned by others from this screen, MOS3 and MOS7, encode components of the nuclear pore complex, implicating nuclear trafficking as a key regulator in plant innate immunity.

mos4 exhibits EDS to virulent and avirulent bacterial and oomycete pathogens. There is evidence that MOS4-mediated resistance is independent of the signalling protein NPR1. MOS4 encodes a protein with homology to human Breast Cancer Amplified Sequence 2 and with
predicted protein-protein interaction domains. Subcellular localization of MOS4-GFP shows that MOS4 is localized to the nucleus. To illuminate the biochemical function of MOS4, a yeast-2-hybrid screen was conducted. One MOS4-interactor was a putative myb transcription factor, MOS4-Associated Complex Protein 1 (MAC1), also known at AtCDC5. MAC1 interacts directly with MOS4 in vitro and in planta. mac1 insertional mutants exhibit defects in immune responses to pathogens similar to that of mos4. In addition, mac1 also partially suppressed snc1 morphology and enhanced resistance.

Both MOS4 and MAC1 have homologs in humans and fission yeast that are members of a discrete protein complex that has been implicated in several different biological processes including RNA splicing, apoptosis and protein degradation. Using proteomics data from yeast and human, we found genes with homology to additional components of the orthologous complex in Arabidopsis, and isolated insertion mutants in these. Mutations in PRL1, which encodes a WD protein, display similar disease phenotypes to that of mos4 and mac1. AtCDC5 has DNA binding activity, suggesting that this complex may regulate defence responses through transcriptional control. Since the complex components along with their interactions are highly conserved from fission yeast to Arabidopsis and human, they may also have a yet-to-be identified function in mammalian innate immunity.
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Preface

The following manuscript-based thesis is divided into five chapters: Chapter One was written as an in-depth and current literature review on intracellular dynamics of plant pathogen response (submitted for publication in early 2008), however, for the purposes of this thesis I cut half of the text out (detailed descriptions on exocytosis, endocytosis, etc) since these sections do not relate to the actual thesis work presented in the following chapters. The remainder serves as the literature review and introduction to what I believe is one of the most interesting aspects of molecular plant pathogen interactions – intracellular dynamics. The significance of this theme becomes apparent when discussing MOS6 and future MOS4 work. This is followed by Chapter 2, a short introduction to the snc1 autoimmune model and thesis objectives. Chapter Three and Four, which constitute the primary data of the Ph.D. research, describe two novel positive regulators of plant innate immunity (MOS6 and MOS4, respectively). These chapters have been published as separate manuscripts and therefore have their own introduction, results, materials and methods, and discussion as per the publication style. The final chapter (Chapter 5) briefly relates the significance of this research to the field and highlights prospects for future work.

The University of British Columbia permits this style of thesis as an alternative to the “classical” style of thesis. For this reason, there is some disconnect between chapters (for example, the Chapter 1 review discusses MOS6 even though the MOS6 chapter follows later). Because there is a distinct discussion in each manuscript chapter, the final chapter is meant to be a short summary with future perspectives. Although my thesis work does not appear to deal with some of the themes discussed in the introductory chapter (endocytosis, exocytosis), I believe it is useful to present the data in the broader context of plant defence.
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Special thanks to all of my current and former colleagues, especially those in the Li lab: A big thank you goes to Yuelin Zhang, who taught me the strategy and tactics behind successful cloning. Sandra Goritschnig, my Austrian twin, and I started on the same day in January, 2003... we mapped our genes together and went through the ups and downs together at the same time, and I am thankful for her support and fantastical beer-drinking abilities, not to mention her parties at the Red Light District. Thanks to Yu Ti Cheng (always reliable Yu Ti!), who always knew where everything was in the lab, and helped with many of my experiments. Thanks to Jacqui Monaghan, who did some crucial experiments for the MOS4 story... and for letting me pass you the torch of the MAC project. Thanks to Marcel Wiermer, the calmest member of the lab; never stressed, always efficient. We`ve collaborated on two reviews together and the process was great. Thanks to Tabea Weihmann, the newest grad student, for taking on the prl1 suppressor screen... and for keeping us all in line. And last but not least, the numerous undergrads (summer students, work-study, etc.) who`ve had a hand in various experiments in both the MOS6 and MOS4 stories (acknowledged personally in Manuscript Acknowledgment sections of this thesis). Also, they washed many flats so I didn`t have to. Best luck to all of you!

Great thanks goes to members of Yuelin Zhang`s lab at NIBS in Beijing who had a huge part in pushing out the MOS4 story by addressing reviewer comments, especially Qingguo Zhao who did some very nice nuclear protein Co-IP work, and Wei Cheng and Dongling Bi. I have never met you three, but I thank you nonetheless!

My sincerest gratitude to the various colleagues in the field who gave us seeds, clones, advice, and shared unpublished data; these are addressed in the separate chapter acknowledgements. A special tip of the hat to Jane Parker (MPIZ, Cologne) who invited us to write a chapter for an upcoming book – this became my literature review for this thesis (Chapter 1).

Thank you to my thesis supervisory committee; Jim Kronstad, George Haughn, and Carl Douglas. My committee meetings went smoothly, and I thank you for your feedback and for reading the various manuscripts. I also thank you for the speedy timeframe in which I am sure you will digest this thesis so I can submit it on time!

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I am eternally grateful to my family and friends for supporting me during my lengthy graduate student career – especially my parents, my wife Betty (who gracefully put up with many all-nighters writing these papers), and my grandmother (nanny) who supported me in many ways, and to whom this thesis is dedicated.

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As in my M.Sc. thesis, I also thank Beer. Beer, you have always been there for me, and did not desert me during my Ph.D... in fact we have become much closer. Sweet elixir of life!
To Johanna Reimer

My Grandmother
Co-Authorship Statement

The work described in this thesis is the culmination of research from 01/2003 to 09/2007. Below is a list of manuscripts (published or in press) that comprise this thesis, and the contribution made by the candidate.


- The candidate wrote the manuscript and drew most of the original figures. M. Wiermer created Figure 1.2, and assisted in revisions. X. Li supervised the manuscript preparation. While the chapter was submitted for invited publication in an edited book chapter, the thesis chapter is presented in its unedited form.


- The candidate performed all experiments described in the paper and wrote the manuscript. Y. Zhang was involved in the mutant screen and assisted with multiple aspects of experimental design. X. Li designed the *snc1* suppressor screen and supervised the work and manuscript preparation.


- The candidate performed most of the experiments and designed or contributed to the remainder, and wrote the manuscript. Q. Zhao performed nuclear IP-Western experiments appearing in the paper and generated Figure 4.11. Y. Cheng collaborated on multiple experiments with the candidate such as SA level determination by HPLC and some pathogen assays. D. Bi generated some of the fluorescence micrograph images. W. Cheng generated polyclonal rabbit antibodies against AtCDC5 for use in IP-Westerns. J. Monaghan conducted the flowering time experiment and generated Figure 4.3 under the candidate’s supervision. Y. Zhang (NIBS, Beijing) and X. Li (MSL, UBC) supervised the work and X. Li supervised the manuscript preparation. X. Li designed the *snc1* suppressor screen.
1 Marshalling the troops: Intracellular dynamics in plant-pathogen defence

1 A version of this chapter has been submitted for publication in a volume called “Molecular Aspects of Plant Disease Resistance” in the 'Annual Plant Reviews' series (Blackwell Publishing) for publication as a book in early 2008. Edited by Jane Parker.
1.1 Introduction

Plants have evolved a multi-layered immune system to defend against potentially infectious pathogens. Unlike vertebrates, they lack specialized circulatory cells, a somatic adaptive immune system and immunological “memory”, except at the scale of evolutionary time (Jones and Dangl, 2006). Therefore, plants rely on a large repertoire of pre-existing immune receptors in probably almost every cell to trigger local and systemic innate immune responses upon infection (Chisholm et al., 2006). The innate immune system is considered to be multi-layered because it involves “pre-invasion immunity”, expressed principally at the plant surface and “post-invasion immunity”, where virulence factors of adapted pathogens are recognized in a microbial strain/plant cultivar-specific manner. In nature, most plants are resistant to most potential pathogens. Non-host resistance occurs when all isolates of a microbe are non-pathogenic on a particular host. Often, only plants of a single genus are hosts for a particular pathogen; this is the case for many powdery mildew, rust and bacterial pathogens (Thordal-Christensen, 2003). Resistance to a non-adapted pathogen can be the consequence of preformed barriers or a lack of signals from the plant to induce spore germination or express essential pathogenicity genes; however, it is increasingly evident that many instances of non-host resistance rely on active cellular defences. Recent work has shown that resistance to inappropriate fungal pathogens depends on aspects of two layers of defence; the first, pre-invasive, is expressed at the cell wall and apoplastic space to prevent penetration at the attempted pathogen entry sites, whereas the second post-invasive layer mediates resistance to fungi that have successfully penetrated, and often results in a localized suicide of cells at the site of infection (termed the hypersensitive response, HR). The dissection of these cooperative layers of non-host resistance has revealed that dynamic intracellular processes play an important role in both pre- and post-invasive defence.

Pre-invasion immunity may involve the perception of pathogen-associated molecular patterns (PAMPs), highly conserved molecules shared by many microbes. Examples of PAMPs include lipopolysaccharide, a major membrane component of gram-negative bacteria, and flg22, a 22-
amino acid peptide epitope conserved in the N terminus of flagellin, the subunit of bacterial surface structure flagellum (Nürnberger et al., 2004; Zipfel and Felix, 2005). Recognition of PAMPs by extracellular receptor-like kinases promptly triggers defence responses which require signalling through mitogen activated protein kinase (MAPK) cascades and transcriptional reprogramming mediated in part by plant WRKY transcription factors (Asai et al., 2002; Eulgem, 2006). Pathogen perception rapidly results in the mobilization of antimicrobial compounds, reactive oxygen species (ROS), actin filament rearrangement, and cell wall thickening (cell wall apposition; CWA) at the site of attempted invasion. Some of these pre-invasion responses remain operative even in susceptible plants to limit pathogen growth, contributing to a “basal resistance” of the host.

The second layer of defence acts post-invasively and is termed effector-triggered immunity. Successful pathogens have evolved effector molecules that target multiple host proteins to evade or suppress basal resistance to promote virulence. Pathogenic bacteria deliver effectors across the host plasma membrane (PM); little is known about how fungal and oomycete effectors are delivered to the cell, although some are released in the apoplast to counteract host antimicrobial compounds (Chisholm et al., 2006). Effector-mediated resistance is triggered by immune receptors encoded by Resistance (R) genes, which recognize these effectors either directly or by detection of the effector’s virulence activity on host cellular targets – the so-called “guard hypothesis” (Dangl and Jones, 2001). Most cloned R genes encode intracellular receptors with a C-terminal leucine-rich repeat (LRR) domain and central nucleotide binding site (NB). The NB-LRR class of R protein can be further divided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor (TIR) NB-LRR according to their N-terminal domain. The N terminus influences the requirement for downstream defence-response components (Aarts et al., 1998). The recognized cognate effector of any R protein is termed an avirulence (Avr) protein; this pairwise association has been characterized genetically as gene-for-gene resistance (Flor, 1971), although one R protein can guard multiple effector targets (Marathe and Dinesh-Kumar, 2003; see below). Once an R protein is triggered, resistance is activated, resulting in the initiation of defence signalling, HR, and inhibition of pathogen growth. Besides the HR, many of the responses to inappropriate pathogens (non-host resistance), virulent pathogens (basal resistance) and avirulent pathogens (R protein-mediated resistance) overlap. Indeed, effector-
triggered immunity has been described as a faster and stronger version of basal resistance (Tao et al., 2003; Jones and Dangl, 2006). The local defence response also primes uninfected systemic cells against a broad spectrum of pathogens in a process called systemic acquired resistance (SAR; (Durrant and Dong, 2004). SAR is dependent on the defence-signalling molecule salicylic acid (SA) and on the key regulator NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1). PAMP- and R protein-dependent responses both result in the induction of Pathogenesis-Related (PR) gene expression. PR genes encode a diversity of peptides, some of which have antimicrobial properties (van Loon et al., 2006).

Although many R genes and their corresponding pathogen effectors have been cloned, direct binding between them has rarely been demonstrated. The question of how R proteins are indirectly activated by their cognate Avr proteins has been a major endeavour in recent years. The majority of bacterial effectors known thus far possess enzyme activity and modify plant proteins (see Takken et al., 2006; Chisholm et al., 2006). Therefore, plants have evolved to monitor changes associated with pathogen virulence effector activity on host proteins, rather than developing receptors for every possible effector. For example, the cleavage of the host protein PBS1 of P. syringae by AvrPphB, a cysteine protease, is indirectly detected by the R protein RPS5 to activate resistance (Shao et al., 2003). An even better characterized example is the activation of resistance responses by perturbations of the Arabidopsis protein RPM1 interacting protein 4 (RIN4). RIN4 is monitored by at least two NB-LRR R proteins, RPM1 and RPS2. RPM1 recognizes two unrelated P. syringae effector proteins, AvrRpm1 and AvrB (Bisgrove et al., 1994). When either effector is delivered to the plant cell, RIN4 is hyperphosphorylated, leading to RPM1-mediated resistance (Mackey et al., 2003). Importantly, proteolytic cleavage and subsequent degradation of RIN4 by a third P. syringae effector, AvrRpt2, a cysteine protease, is recognized by the R protein RPS2 (Coaker et al., 2005). RPM1 and RPS2 have both been shown to associate with RIN4 in planta (Axtell and Staskawicz, 2003; Mackey et al., 2002). It is likely that RPM1 and RPS2 evolved to recognize the effects of these multiple Avrs due to their virulence activities on host proteins – it is unknown what they target (if anything) besides RIN4. This hypothesis is supported by experiments that show AvrRpm1 and AvrRpt2 inhibit PAMP-triggered defence responses, presumably through their modification of RIN4 and other host proteins (Kim et al., 2005).
Do intra- or inter-molecular interactions negatively regulate NB–LRR signalling? Several reports indicate that NB–LRR proteins are subject to constitutive negative regulation (reviewed in Rathjen and Moffett, 2003). It has been hypothesized that intramolecular interactions between R-protein domains may function as negative regulators of activation – mutations that affect the integrity of the TIR/CC, NB or LRR domains can result in gain-of-function mutations that are often deleterious. Moffett et al. (2002) demonstrated that physical interactions occur in vivo between the NB–LRR domain and the amino-terminal CC of the potato R protein Rx, which mediates resistance to Potato Virus X (PVX). In addition, interactions can occur between the LRR and the CC-NB. Importantly, these intramolecular interactions were disrupted in the presence of the relevant avirulence protein, in this case the PVX coat protein (CP; Moffett et al., 2002). Alternatively, intermolecular regulation and R protein complex association and stability (Hubert et al., 2003; Bieri et al., 2004; Holt et al., 2005) have been proposed to regulate activation of R proteins and thus downstream resistance responses. A third possibility, differential subcellular localization, is a newer idea supported by recent experimental evidence (see below).

The existence of constitutively active R protein variants, such as the mutant TIR-NB-LRR proteins snc1 and ssi4 (discovered in separate npr1 insensitivity to SA suppressor screens), that exhibit enhanced resistance to multiple pathogens, suggest their use as tools to elucidate downstream signalling events (Li et al., 2001; Shirano et al., 2002; Zhang et al., 2003). After expression of defence genes, the cell must deliver and concentrate antimicrobial products to the site of infection. Also, the transcriptional reprogramming of the cell must be tightly controlled to achieve rapid triggering of the defence response while avoiding spurious activation. These goals are achieved through regulated directional transport and partitioning of defence regulators between organelles and across membranes.

1.2 The front line – exocytosis, endocytosis and vesicle transport

Unlike many mammalian pathogens, potential microbial pathogens of plants remain outside the host cell – bacteria live in the apoplastic space whereas biotrophic fungi and oomycetes form special feeding structures (haustoria) that facilitate parasitism by intimate association
with the host PM. Therefore, the cell wall and apoplastic space represent the “front line” for active defence against microbial pathogens. The primary defence against invasion of fungal and oomycete pathogens is penetration resistance, the central features of which are a localized reinforcement of the cell wall (papilla formation) by callose deposition, localized accumulation of antimicrobial secondary metabolites (phytoalexins) and secretion of a suite of defence proteins (Hammerschmidt, 1999; Dixon, 2001; Maor and Shirasu, 2005; van Loon et al., 2006; Hardham et al., 2007). In addition, microscopic observations show that various membranous subcellular structures, for instance \( \text{H}_{2}\text{O}_{2} \)-containing vesicles and the so-called multivesicular bodies, accumulate at infection sites, possibly to release their cargos of antimicrobial compounds (Hückelhoven et al., 1999; An et al., 2006). These responses are dependent on secretion/exocytosis and vesicle transport.

A recent report by Bais et al. (2005) provides indirect evidence for the role of secretion in non-host resistance in Arabidopsis-bacteria interactions. The authors show that tissue-specific resistance to a wide range of bacterial pathogens is dependent on the exudation of root-derived antimicrobial metabolites, and that \textit{Pseudomonas syringae pv. tomato} is capable of blocking their synthesis and/or secretion to infect the roots and cause disease in a type III secretion-dependent manner (Bais et al., 2005). Another important finding on the activation of the secretory system in response to pathogens came from an extensive survey of genes up-regulated by NPR1 in an inducible system (Wang et al., 2005). Along with the expected \textit{PR} genes (\textit{PR}-1, \textit{PR}-5), the expression profile included various genes encoding protein folding and secretory machinery components such as \textit{BiP2}, \textit{DAD1} and \textit{SEC61a}. Indeed, following treatment with an SA analog, null mutants in these genes resulted in compromised secretion of \textit{PR}-1 and enhanced susceptibility to \textit{P. syringae pv. maculicola} compared to wild type. The NPR1-responsive regulation of secretion-related genes is through \textit{TL1}, a novel cis element distinct from and independent of the TGA-binding \textit{os}-1 element (Wang et al., 2005). This data suggests that preparation of the cell for enhanced secretion is controlled by different, unknown NPR1-dependent transcription factors (TFs). Similarly, in animals, B-cells boost the capacity of their secretion machinery before antibody secretion (van Anken et al., 2003).
Exocytosis is dependent on vesicle transport, docking, and membrane fusion at the PM. In all eukaryotes, the fusion of membrane-coated vesicles with lipid bilayer target membranes is mediated by a specialized class of proteins, the soluble N-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs). While plants and animals share mechanisms for vesicle formation and fusion, plant cells exhibit a more complex endomembrane system than animal cells, with distinct storage and lytic compartments and different vesicle trafficking routes (Jürgens, 2004). Consequently, about 60 genes in Arabidopsis encode SNAREs, compared to 24 in yeast and at least 35 in humans (Sanderfoot, 2007). Functional classification divides SNAREs into either target-membrane or vesicle-associated SNAREs (syntaxins or VAMPs, respectively) (Lipka et al., 2007). A breakthrough study that focused attention on vesicle-mediated secretion and plant immunity came from the genetic dissection of non-host resistance using the grass powdery mildew fungus Blumeria graminis f. sp. hordei (Bgh), a barley pathogen that does not normally colonize Arabidopsis. The majority of penetration attempts by this pathogen result in failure at the cell wall and absence of intracellular fungal structures. In pen (penetration) mutants, Bgh is able to evade this pre-invasive immunity, resulting in enhanced penetration (Collins et al., 2003). Further growth of haustoria and hyphae are subsequently halted after penetration, suggesting that pen mutants do not affect post-invasive immunity. The activation of post-invasive defences after successful Bgh penetration resulted in two phenotypes detectable by fluorescence microscopy; autofluorescence resulting from single-cell death and aniline blue epifluorescence staining of callose deposition (Collins et al., 2003). Because post-invasive responses are not highly activated in the absence of penetration in a wild type background, these phenotypes were utilized in two separate screens for pen mutants. At least four PEN genes were discovered in these screens. PEN1 (also called SYNTAXIN OF PLANTS 121 [AtSYP121]) encodes a PM-resident syntaxin, indicating that the secretory machinery is essential for pre-invasive defence (Collins et al., 2003). PEN2 and PEN3 (also called PLEIOTROPIC DRUG RESISTANCE 8 [PDR8]) encode a glycosyl hydrolase and an ABC transporter, respectively (Lipka et al., 2005; Stein et al., 2006).

While well-studied in animals, surface receptor endocytosis is a new research field in plant cell biology and especially in the study of plant immune responses (Robatzek, 2007). Recent data suggests that this innate immune response is shared between kingdoms. For example,
endocytosis of *Xanthomonas campestris* LPS occurs in non-host tobacco, although a cognate receptor is not yet known (Gross et al., 2005). Also, the fungal PAMP ethylene-inducing xylanase (EIX) induces PR expression and HR in specific cultivars of tomato and tobacco. The tomato transmembrane receptor *LeEix2*, which binds to EIX and mediates downstream responses, contains the conserved endocytosis signal YxxΦ in its cytoplasmic tail; mutation of this putative signal abolishes EIX-dependent HR (Ron and Avni, 2004). Similarly, the receptor mediating recognition of the bacterial elongation factor Tu (EF-Tu), and the tomato R proteins *Ve*, *Cf4* and *Cf9* all possess the endocytosis signature at their C-terminus, suggesting a conserved role of receptor endocytosis in PAMP- and R protein-mediated responses (Kawchuk et al., 2001; Zipfel et al., 2006; Robatzek, 2007).

The first direct evidence of endocytosis of a plant immune receptor comes from a landmark study by Robatzek et al. (2006), in which they show that the PM-resident receptor-like kinase FLS2 is internalized in response to stimulation by its ligand, flg22. Within minutes after elicitation, FLS2-GFP fluorescence disappeared from the PM and accumulated in intracellular vesicles – these vesicles are likely trafficked for degradation, since prolonged treatment resulted in a complete loss of GFP fluorescence (Robatzek et al., 2006). Upon withdrawal of flg22, PM-residing FLS2-GFP was replenished in a cyclohexamide-sensitive manner, indicating that FLS2 protein is synthesized *de novo* and not recycled. Furthermore, FLS2 contains a ubiquitination motif that, when mutated, compromises receptor endocytosis and downstream signalling but not early responses such as flg22-triggered ROS production (Robatzek et al., 2006). Therefore, the authors suggest that FLS2-mediated flg22 signalling is initiated at the PM and continues from intracellular compartments where it FLS2 is subjected to degradation (Robatzek, 2007).

The role of exocytosis/secretion and endocytosis has come to the forefront of the field – recent genetic, biochemical and cell biology work has begun to elucidate the molecular components of this phenomenon. These are summarized in Figure 1.1, and the reader is directed to several excellent reviews on the subject (Schulze-Lefert, 2004; Lipka and Panstruga, 2005; Robatzek, 2007; Hückelhoven, 2007). While these processes highlight the cell periphery as the site of pathogen perception and initiation of defence responses, recent studies revealed multiple
aspects of nucleocytoplasmic trafficking that link pathogen perception and cytoplasmic signal transduction to defence gene expression.

**Figure 1.1: Conceptual summary of microbe-triggered dynamic cellular responses in plants**

On the left side flg22-mediated internalization of the pattern recognition receptor FLS2 and its subsequent accumulation via endosomes in multivesicular bodies (MVBs) is shown. On the right side, cellular responses to attempted fungal ingress are shown; these involve actin filament mediated focal transport of vesicles to and SNARE protein-dependent fusion of vesicles with the plasma membrane (PM), a process that is thought to be negatively regulated by MLO. Vesicle trafficking pathways are embedded into the cellular membrane trafficking system linking the PM with the endoplasmic reticulum (ER), Golgi and vacuoles. Dashed arrows indicate vesicle movement and protein re-localizations. ROS, reactive oxygen species. ADF, actin depolymerising factor. P, phosphorylation. Ub, ubiquitination. Based on unpublished data and information in reviews by Schulze-Lefert (2004), Lipka and Panstruga (2005), Robatzek (2007) and Hückelhoven (2007).
Figure 1.1: Conceptual summary of microbe-triggered dynamic cellular responses in plants
1.3 Nucleocytoplasmic trafficking in plant immunity

Nucleocytoplasmic trafficking is a fundamental process in all eukaryotes that mediates the bidirectional import and export of all proteins and RNA through the nuclear pore complex (NPC). The NPC is a large macromolecular structure that spans the double membrane of the nuclear envelope, and is composed of nucleoporins (Nups) that localize to protein subcomplexes within the greater pore complex. While some Nups are structural and required for NPC assembly, recent studies show that certain Nups are mobile and not permanently associated with the NPC (Tran and Wente, 2006). Nups lining the interior of the nuclear pore exhibit phenylalanine-glycine (FG) repeats which are thought to provide transient, low-affinity binding sites for transport receptors (Frey et al., 2006). These receptors, collectively termed karyopherins (Kaps), recognize exposed nuclear localization signals (NLSs) and nuclear export signals (NESs) to mediate the bidirectional nucleocytoplasmic shuttling of these cargo proteins. While Nups are well characterized in humans and yeast, relatively little is known in plants. For an overview on nuclear transport in plant cells and the composition of the plant NPC, the reader is referred to comprehensive reviews by Merkle (2001; 2003) and Meier (2007).

The relationship between plant pathogen defence and nucleocytoplasmic trafficking has been under special focus as of late. The nucleus is obviously the site for all transcription and therefore transcription factors (TFs) involved in regulating pathogen-responsive genes must be imported at some point. It is the rapid and dynamic regulation of these TFs in response to pathogen attack, in many cases by interacting signal transducers or phosphorylation cascades that signal from the cytoplasm to the nucleus, which determines the efficacy of the host response. Nucleocytoplasmic partitioning is a well-studied feature of innate immunity in animals, a process that largely depends upon the induced translocation of NF-κB from the cytoplasm to the nucleus. In uninduced cells, NF-κB proteins are predominantly cytoplasmic and interact with members of the inhibitory IκB family (Ghosh et al., 1998). Depending on the type of IκB, these proteins either mask NF-κB’s classic NLS, sequestering it in the cytoplasm, or preclude high steady-state levels of these complexes in the nucleus by virtue of a nuclear export signal (NES) in IκB (Huang et al., 2000; Malek et al., 2001). Upon induction, IκB is
degraded, and activated NF-κB accumulates in the nucleus, stimulating the expression of immune-response genes. MAPK signalling cascades terminating in the nucleus are another essential feature shared by animal and plants, fulfilling important regulatory functions that modulate defence against pathogens (Tena et al., 2001; Nürnberger et al., 2004). Activated MAPKs correlate with the upregulation of many PR genes in several experimental systems, suggesting that they play an important role in gene transcription (Pedley and Martin, 2005). MAPKs have been shown to translocate to the nucleus in rice (Cheong et al., 2003) and in cultured parsley cells in response to Pep13, a peptide elicitor derived from the oomycete pathogen Phytophthora (Lee et al., 2004).

Several recent studies point to nucleocytoplasmic trafficking as a key regulatory step of effector-mediated and basal resistance. Components of the nuclear transport machinery have been found in genetic screens for regulators of R protein-mediated resistance, and as interactors of R proteins. Key regulators of plant defence are reported to be regulated across the nuclear envelope in a spatio-temporal manner reminiscent of NF-κB. Most recently, intracellular R proteins themselves have been shown to have functions in the nucleus. Two studies of paramount interest showed that NB-LRR R proteins from barley (MLA, CC-type) and tobacco (N, TIR-type) have functions in the nucleus (Burch-Smith et al., 2007; Shen et al., 2007). This localization is surprising, since both lack a discernable nuclear localization signal (NLS) – the prevailing hypothesis until now was that R proteins, being intracellular immune receptors, were cytoplasmic or associated with the PM to directly or indirectly recognize pathogen effectors (Belkhadir et al., 2004; Jones and Dangl, 2006). However, these results fit in light of the accumulating evidence that some pathogen effector proteins are localized to the nucleus, and that defects in nuclear trafficking machinery affect R protein-mediated resistance (see below).

Another topical study revealed that RPS4, an Arabidopsis TIR-NB-LRR R protein, has nuclear activity, although in this case a putative bipartite NLS was predicted in the C-terminal extension domain of RPS4 (L. Wirthmueller, Y. Zhang, J.D.G. Jones and J.E. Parker, personal communication). In all three cases, a nuclear export signal (NES) sequence fused to the NB-LRR R proteins indicated that their nuclear localization is absolutely required for effector-mediated resistance. Furthermore, recent work with the autoimmune mutant snc1 has underlined the importance of nucleocytoplasmic trafficking in R protein activity (see further chapters).
1.4 R protein activity in the nucleus

Shen et al. (2006) analyzed allelic barley CC-NB-LRRs MLA1, MLA6 and MLA10 that confer resistance to powdery mildew *Bgh* expressing the specific effectors AVR$_{A1}$, AVR$_{A6}$ and AVR$_{A10}$, respectively. *In vivo* imaging of yellow fluorescent protein (YFP)-tagged MLA10 transiently expressed in leaf epidermal cells showed that MLA10-YFP localized to the cytoplasm and nucleus, and likely shuttles between the two compartments. An NES fusion to MLA10-YFP prevents its nuclear accumulation and suppresses defence responses. In stable transgenic barley lines, epitope-tagged MLA1 was also detected in both the nuclear and cytoplasmic fractions, suggesting that the MLA10-YFP localization in the single-cell system reflects a physiological condition. In addition, a time-course revealed an increase in the MLA1 nuclear pool in response to inoculation of *Bgh* carrying its cognate effector AVR$_{A1}$, but not *Bgh* lacking AVR$_{A1}$, suggesting that recognition of the effector triggers dynamic nuclear changes in the behaviour of the R protein (Shen et al., 2007). The invariant CC domain shared by all three MLAs interacts with two closely related barley WRKY domain-containing TFs, *HvWRKY1* and *HvWRKY2*, which repress defence responses to *Bgh*. In agreement with this, silencing of *HvWRKY1/2* significantly enhanced resistance to virulent *Bgh*, whereas overexpression of *HvWRKY1/2* resulted in enhanced susceptibility to virulent *Bgh* (Shen et al., 2007). Furthermore, this overexpression fully compromises MLA1-, MLA10- and MLA12-mediated resistance against avirulent *Bgh* isolates expressing the cognate AVR$_{A}$ effectors. The Arabidopsis functional homologs of *HvWRKY1/2*, *AtWRKY18* and *AtWRKY40*, also exhibit redundant repressor activities in the virulent powdery mildew *G. orontii*-Arabidopsis interaction (Shen et al., 2007), and were formerly implicated in repressing basal defence to virulent *Pseudomonas* (Xu et al., 2006). In barley, transiently expressed MLA10-YFP and cyan fluorescent protein (CFP)-*HvWRKY2* fusion proteins co-localize in epidermal cell nuclei; protein association was monitored by Förster resonance energy transfer (FRET) between the YFP and CFP fluorescent tags in the presence and absence of Avr$_{A10}$. Importantly, using quantitative fluorescence lifetime imaging (FLIM) to detect FRET, the association of MLA10 and *HvWRKY2* was dependent on the presence of the cognate effector (Ridout et al., 2006; Shen et al., 2007). The direct nuclear association between MLAs and WRKY transcriptional repressors, induced by the fungal
effector, imply a short signalling pathway conducive to rapid defence signal transduction. This data is summarized in Figure 1.2A.

Another notable report demonstrating R protein activity in the nucleus comes from Burch-Smith et al. (2007). The TIR type NB-LRR protein N, along with its cognate effector, the p50 helicase domain of the tobacco mosaic virus (TMV) replicase, are found in both the cytoplasm and nucleus. Recognition of p50 by N triggers an HR and confers resistance to TMV (Whitham et al., 1994; Erickson et al., 1999). As with MLA10, fusing an NES to N abolished p50-dependent HR, indicating that N’s nuclear localization is indispensable for Avr-triggered defence responses. In contrast to this, using the same strategy it was shown that the nuclear localization of p50 is not required for HR. These data suggest that N recognizes p50 in the cytoplasm, and that the nuclear pool of N has another function (Burch-Smith et al., 2007). Using bimolecular fluorescence complementation (BiFC) imaging in living cells and biochemical assays, this study showed that the TIR domain of N associates with p50, likely in the context of other host factors. This was unexpected, since the LRR was previously proposed to be involved in ligand recognition. Based on this and other studies, a complex, multi-step model of N receptor activation by the TMV replicase is emerging, perhaps resulting in activated N shuttling from the cytoplasm to the nucleus subsequent to Avr-dependent activation, Figure 1.2B (Burch-Smith et al., 2007; Sheen and He, 2007). Intriguingly, plant-specific transcription factors have been previously shown to interact with the LRR of N – in the context of this study, this finding hints at
Figure 1.2: A model for nuclear R protein signalling

The barley MLA10 (CC-NB-LRR) receptor (A) and tobacco N (TIR-NB-LRR) receptor (B) are present in both the cytoplasm and the nucleus. Nuclear localization of either R protein is indispensable for downstream signalling and defence responses. According to the previous model outlined in Wiermer et al. (2007), in an un-induced cell, some NB-LRR R proteins may continuously cycle between the cytoplasm and the nucleus, and equilibrium is maintained. Upon pathogen effector perception, the nuclear pool of activated R protein increases and after reaching a threshold concentration de-represses defence gene expression. (A) The N-terminal CC domain of barley MLA10 interacts with WRKY1/2. It is unknown whether AvrA10 is also present in the nucleus or where perception occurs. (B) The viral effector p50 is present in both subcellular fractions, and is recognized by the N-terminal TIR domain of tobacco N. Host factors may assist this association in vivo. It is unknown if activated N also binds to WRKY negative transcriptional regulators. See text for details on these two NB-LRR R proteins. TFs, transcription factors.
a previously undescribed role for R proteins regulating gene transcription (Liu et al., 2004).

A third piece of direct evidence highlighting the nucleus as a key location for the function of intracellular immune receptors is the demonstration that a sub-pool of the Arabidopsis R-protein RPS4, which recognizes the bacterial Type III effector AvrRps4, localizes to the nucleus (L. Wirthmueller, Y. Zhang, J.D.G. Jones and J.E. Parker, personal communication). Through cellular fractionation and imaging of YFP-tagged fusion proteins, RPS4 was found to distribute constitutively between the cytoplasm and nuclei. As with MLA and N, a nuclear exclusion strategy was employed to determine whether the nuclear localization of RPS4 was required to trigger immune responses. Transient overexpression of RPS4 in tobacco induces an AvrRps4-independent HR (Zhang et al., 2004); fusion of an NES to YFP-RPS4 abolished cell death in almost all transfected leaves. Moreover, YFP-RPS4-NES resulted in a partial redistribution of YFP-RPS4 from the nucleus to the non-nuclear compartment in N. benthamiana leaves. As mentioned, RPS4 has a predicted NLS in its C-terminus. Mutation of this NLS resulted in both nuclear exclusion and failure to complement an rps4 mutant when challenged with P. syringae expressing AvrRps4. These data strongly suggest that nuclear localization of RPS4 is essential for both AvrRps4-dependent and -independent immune responses. An important observation is that AvrRps4 was not observed in the nucleus, indicating that a spatial separation between RPS4 receptor activation and defence triggering exists (L. Wirthmueller, Y. Zhang, J.D.G. Jones and J.E. Parker, personal communication).

Before the identification of MLA10, N and RPS4 as nuclear-targeted R proteins, the only other concrete example of an R-protein with activity in the plant nucleus was the atypical RRS1-R of Arabidopsis. RRS1-R is unusual in that it contains, along with TIR-NB-LRR domains similar to other TIR-class R proteins, a C-terminal extension with a putative NLS and a 60–amino acid DNA binding motif characteristic of the WRKY family of TFs (Deslandes et al., 2002). The presence of WRKY and NLS domains in RRS1-R suggests that this R protein mediates Avr perception and subsequent transcriptional activation of the defence-related transcripts, representing an extremely condensed signalling pathway. It was recently discovered that an in-frame 3 bp insertion mutation in the WRKY domain of RRS1-R, which impairs its DNA-binding activity, results in constitutive activation of defence responses, suggesting that resistance signalling may
be negatively regulated by the WRKY domain in the R protein (Noutoshi et al., 2005). One interpretation of this data is that other, typical NB-LRR R proteins might directly activate gene expression by interacting with WRKYs, and that RRS1-R represents an evolutionary “Rosetta stone” of domain fusion events of functionally related proteins that physically interact (Martin et al., 2003). However, a direct interaction between an R protein and WRKY TFs has only been reported recently in the case of MLA, as described above (Shen et al., 2007). In this context, it is interesting that the LRR domain of N was found to interact with plant-specific TFs (Liu et al., 2004).

RRS1-R confers resistance to the bacterial pathogen Ralstonia solanacearum expressing PopP2, a member of the YopJ/AvrRxv family of effector proteins that is targeted to the plant cell nucleus. It has been suggested that PopP2 may bind an importin α via its NLS, and that this protein may be a potential virulence target of the avirulence effector that is guarded by RRS1-R (Lahaye, 2004). Nevertheless, RRS1-R and PopP2 interact directly in a yeast-2-hybrid system; this interaction is essential for inducing accumulation of fluorescent protein-tagged RRS1-R in the nucleus (Deslandes et al., 2003). It remains unknown whether these two proteins first interact in the cytoplasm, subsequently shuttling RRS1-R into the nucleus, or if PopP2 interacts with a pre-existing nuclear pool of RRS1-R and somehow suppresses its export, accumulating the R protein to levels detectable by fluorescence microscopy. Evidence suggests that other pathogen effector proteins are active in the nucleus, based on conserved NLSs and/or DNA-binding motifs (Zhu et al., 1998; Yang et al., 2000; Szurek et al., 2001; Kemen et al., 2005; Kanneganti et al., 2007). For example, AvrBs3 and AvrBs4, two nearly identical bacterial type III effectors from Xanthomonas campestris pv. vesicatoria (Xcv) that are specifically detected by the cognate pepper Bs3 and tomato Bs4 R proteins, respectively, are targeted to the nucleus (Schornack et al., 2006). These Avr proteins exhibit potential C-terminal acidic transcription activation domains (AD) and monopartite NLSs that could possibly hijack the host nuclear transport machinery to gain access to the nucleus; in the case of AvrBs3, the disruption of either the AD or the NLSs affect both Xcv virulence and Bs3 recognition (Szurek et al., 2001). The guard hypothesis suggests that Avr-induced modifications of a host target protein trigger activation of matching plant R proteins (Dangl and Jones, 2001). According to this model, the R protein Bs3 is predicted to be nuclear. In contrast, the Bs4-dependent avirulence activity of
AvrBs4 is not compromised by deletion of its NLS nor AD domains, suggesting that Bs4 recognizes and is activated by AvrBs4 during its transit through the cytoplasm (Bonas et al., 1993; Schornack et al., 2006). While this result points to a separation of virulence and avirulence activity, contrary to predictions based on the guard model, the effect of the C-terminal truncation on virulence of AvrBs4 and direct localization of Bs4 remain to be tested.

The discovery that NB-LRR R receptors have activity in the nucleus led Shen et al. (2007) and Burch-Smith et al. (2007) to propose a conceptual model, which we extended in a recent review (Wiermer et al., 2007). According to this model, in an uninduced cell, some NB-LRR R proteins continuously cycle between the cytoplasm and nucleus and equilibrium is maintained. Upon pathogen effector recognition, the nuclear pool of R proteins increases. After the nuclear R protein concentration reaches a certain threshold, defence gene expression is induced by derepression of WRKY TFs. This accumulation of the nuclear pool of R proteins could be accomplished by an increase in the rate of nuclear import or a decrease in the rate of nuclear export. Some R proteins are targeted to the nucleus by an NLS and some are not, therefore, effector-mediated activation could expose an obscured NLS via conformational changes or recruit additional interacting proteins to affect nuclear shuttling or retention. This model predicts that defects in the NPC or transport machinery might alter resistance by upsetting the ratio of cytoplasmic- to nuclear-localized R proteins. Furthermore, elevated defence gene expression associated with overexpression of R genes and constitutively active R protein variants (i.e. snc1, ssi4) may be explained by altered nucleocyttoplasmic distribution (Shirano et al., 2002; Zhang et al., 2003a). The connection between R protein nuclear activity and effector perception is unknown and awaits further research. Also, it is very likely that the cytoplasmic R protein pool is involved in other, non-transcriptional downstream signalling events, such as those that lead to the HR.

These nucleocytoplasmic R protein regulation models highlight the significance of the identities of several positive regulators of an autoimmune mutant, suppressor of npr1-1, constitutive 1 (snc1). snc1 carries a gain-of-function mutation in a TIR-NB-LRR class R gene resulting in spurious activation of downstream defence responses in the absence of pathogens, such as constitutive PR gene expression, accumulation of SA, and enhanced resistance to virulent P.
syringae bacteria and *Hyaloperonospora parasitica* oomycete pathogens without spontaneous cell death (Li et al., 2001; Zhang et al., 2003a). The pathogen effector recognized by the wild type protein is unknown. Several genes cloned by our group in a *snc1* suppressor screen, *MOS3*, *MOS7* and *MOS6* (for *MODIFIER OF snc1*), encode components of the plant nucleocytoplasmic trafficking machinery, revealing the importance of this process in deregulated R protein signalling. Subsequent analysis showed that the products of *MOS* genes are required for both basal resistance to virulent pathogens and for resistance mediated by other *R* genes in addition to *snc1*.

*MOS3* is the Arabidopsis homolog of vertebrate nucleoporin 96 (Nup96) and *Saccharomyces cerevisiae* C-Nup145p, which are components of the conserved Nup107-160 nuclear pore subcomplex (termed the Nup84 complex in yeast) (Zhang and Li, 2005b). This complex has a crucial role in nuclear pore assembly, since complete depletion of the Nup107–160 complex leads to nuclei entirely lacking pores (Harel et al., 2003; Walther et al., 2003). Additionally, this subcomplex, which localizes to both sides of the nuclear pore, has been implicated in both mRNA export and mitotic spindle assembly (Belgareh et al., 2001; Vasu et al., 2001; Orjalo et al., 2006). Faria et al. (2006) recently established a selective role for Nup96 in innate and adaptive immunity from studies of Nup96-depleted mice; upon immunization, mice with low levels of Nup96 show impaired antigen presentation and T cell proliferation. This and previous experiments showed that Nup96 is regulated by interferons and actively participates in the interferon-dependent induction of major histocompatibility complex (MHC) I and MHC II (Enninga et al., 2002; Faria et al., 2006). While the total levels of MHC I and II mRNA in Nup96-depleted and control mice are similar, the ratios of nuclear to cytoplasmic levels of these mRNAs are partially but significantly increased in Nup96-depleted macrophages. This partial nuclear retention likely contributes to lower expression of these proteins. Taken together, these data suggest an ancient role for Nup96 in immunity, possibly due to its role in nuclear export of specific classes of mRNAs, although it remains to be determined whether the defects in disease resistance observed in *mos3* are specific or a pleiotropic phenotype based on nuclear mRNA retention. *MOS3* was also found independently in a genetic screen for suppressors of auxin-resistant 1 (*sar* mutants) and designated SAR3 (Parry et al., 2006). Another gene, *SAR1*, encoding the Arabidopsis homolog of Nup160, was also found in this screen. *sar1* single-mutant
plants exhibit defects in auxin response and in tolerance to cold stress, mos3 single-mutant plants are early-flowering, and sar1 mos3 double-mutants present variable phenotypes such as severe developmental defects, seedling lethality, and infertility, along with nuclear accumulation of mRNA (Parry et al., 2006). In corroboration with data from animal systems, the sar1 mos3 double-mutants could represent a non-functional orthologous Arabidopsis Nup107-160 subcomplex and the severe phenotypes may be due to impaired NPC assembly. Conversely, this indicates that AtNup96 itself might have specific functions and is not purely a structural protein. Intriguingly, homologs of two additional members of the Nup107-160 complex, Nup85 and Nup133, are essential for microbial symbiosis in the legume Lotus japonicus (Kanamori et al., 2006; Saito et al., 2007).

MOS7 encodes another Nup that is homologous to vertebrate Nup88 (Y. Cheng and X. Li, unpublished data). In addition to snc1-mediated resistance, MOS7 is essential for resistance to avirulent pathogens mediated by other R proteins, basal resistance, and SAR. Its homolog in Drosophila, members only (mbo), is found on the cytoplasmic side of the NPC and is selectively required for activation of innate immune responses to bacterial infection (Uv et al., 2000). In Drosophila, Toll signalling upon bacterial infection leads to degradation of the IκB homolog, Cactus, and nuclear accumulation of the Rel/NFκB TFs Dorsal and Dif. In mutant larvae lacking DmNup88, Dorsal and Dif fail to accumulate in the nucleus (Uv et al., 2000). While overexpression of Nup88 interferes with nuclear export of a GFP-NES reporter construct, the cytoplasmic accumulation of GFP-NES is accelerated in mbo mutants – this and other data suggests that Nup88 is likely involved in modulating protein retention in the nucleus (Roth et al., 2003; Xylourgidis et al., 2006). A possible mechanism for Nup88’s role in innate immunity is by selectively trapping endogenous TFs and thereby modulating the expression of Rel/NFκB target genes upon activation of the defence response. Although mbo mutant flies fail to accumulate these Rel proteins in the nucleus, they are not affected in the nuclear translocation of several other, unrelated proteins (Xylourgidis et al., 2006). It will be interesting to test if MOS7 is involved in protein retention of any known plant defence regulators. As expected for components of the NPC, GFP-tagged MOS3 and MOS7 localize exclusively to the nuclear rim (Zhang et al., 2005; Y. Cheng and X. Li, unpublished data).
MOS6 encodes importin-α3, one of at least eight putative α importins encoded by the Arabidopsis genome. A defect in this importin subunit partially suppresses the constitutive defence responses in snc1 and displays enhanced susceptibility to a virulent H. parasitica oomycete pathogen (Palma et al., 2005). Importins are a conserved family of Kaps, mobile targeting receptors that mediate the bidirectional trafficking of macromolecules across the nuclear envelope. The best-understood function of the α-subunit is to serve as an adaptor that links NLS-containing proteins to importin β, which mediates translocation through the NPC by its ability to interact with Nups (Goldfarb et al., 2004). The deduced MOS6 protein has the typical architecture of all importin-α proteins (except for AtImp-α8); an N-terminal autoinhibitory importin-β binding domain, armadillo repeats containing NLS binding pockets, and a C-terminal acidic patch that is implicated in the interaction with a specific export receptor for recycling back to the cytoplasm (Goldfarb et al., 2004). The importin-α gene family has undergone considerable expansion during the course of eukaryotic evolution. Whereas the yeast genome encodes a single importin-α, multicellular eukaryotes all contain multiple proteins with deduced importin-α function. The existence of multiple importin-α isoforms in animals and plants has raised questions about the specificity and efficiency in transport of different NLS-containing proteins; because of high sequence similarity, it is probable that some Arabidopsis importins are functionally redundant. For example, MOS6 (importin α3) is almost identical to importin α6, and these two proteins are closely related phylogenetically (Palma et al., 2005). Because all alleles of mos6 only partially suppress snc1 phenotypes, it will be interesting to see if defects in both AtImpα6 and MOS6 more completely suppress snc1.

The significance of encoding multiple importin α homologs can be inferred from studies in humans, in which at least seven different importin-α proteins have been described (Pemberton and Paschal, 2005). The downregulation by RNAi of two ubiquitously expressed human importins, α3 and α5, specifically inhibit cellular proliferation and the import of the nuclear protein Ran guanine nucleotide exchange factor (RCC1) – both defects can be rescued by microinjection of recombinant importin-α3 (Quensel et al., 2004). Therefore, it can be concluded that nuclear import of RCC1 is dependent on importin-α3 only, in humans. In another example, importin-α3 and importin-α4 were identified as the main importin-α isoforms mediating TNF-α-stimulated NF-κB translocation into the nucleus in humans (Fagerlund et al.,
So far, limited examples of importin specificity have been described in plants. In rice, nuclear import of constitutive photomorphogenic 1 (COP1), a repressor of photomorphogenesis regulated by nucleo-cytoplasmic partitioning, is mediated preferentially by the rice importin-α1b (Jiang et al., 2001). A further recent example comes from a convincing report by Kanneganti et al. (2007) showing that *N. benthamiana* importin-α isoforms have distinct preferences for different NLS-carrying proteins. VIGS of two importins, *NbImpα1* and *NbImpα2*, inhibit the nuclear targeting of fluorescent protein-tagged *P. infestans* candidate effectors Nuk6 and Nuk7. In contrast, the nuclear import of *P. infestans* Nuk12 and YFP-SV40NLS (a reporter shown previously to require importin-α for nuclear import) is unaffected in these silenced plants (Kanneganti et al., 2007). This study demonstrates *in vivo* that importin-α isoforms have distinct preferences for different NLS-carrying proteins. Although it is possible that other unknown *N. benthamiana* importin-α genes were targeted by VIGS, it is likely that not all of these were silenced, as nuclear import of the YFP–SV40NLS fusion was not inhibited in the silenced plants. The most plausible explanation for this result is that the nuclear import of the YFP–SV40NLS fusion depends on other *N. benthamiana* importin-α isoforms than *NbImpα1* and *NbImpα2* and close homologs (Kanneganti et al., 2007). This study shows that VIGS is a viable strategy to further dissect the roles of different importin-α proteins in specific trafficking of nuclear localized defence proteins and plant pathogen effectors. Another strategy for use in *Arabidopsis*, although tedious, is analysis of double-, triple- and higher-order mutant lines with T-DNA insertions in *Arabidopsis* importin-α-encoding genes, to test nucleocytoplasmic trafficking of candidate immune proteins.

A recent report by Tameling and Baulcombe (2007) implicates nucleocytoplasmic trafficking in R-protein mediated immunity to a plant virus. Resistance to *Potato virus X* (PVX) is mediated by the potato CC-NB-LRR protein Rx via recognition of the viral coat protein (CP) (Bendahmane et al., 1995). In a targeted proteomics approach, proteins in the Rx signalling complex were identified by affinity purification and mass spectrometry after transient coexpression of tagged Rx fragments (CC or LRR domains) in *N. benthamiana* leaves. Interestingly, in many independent experiments, the purified Rx-CC fragment copurified with a Ran GTPase activating protein (RanGAP) protein, whereas the purified Rx-LRR fragment did not (Tameling and Baulcombe, 2007). Further characterization revealed that the Rx-CC-interacting protein was
RanGAP2, one of two RanGAPs in *N. benthamiana* that are homologous to the two RanGAP proteins found in Arabidopsis. Silencing of RanGAP2 by VIGS suppresses Rx-mediated resistance to PVX but does not affect resistance mediated by other NB-LRR proteins such as N or Pto, suggesting that RanGAP2 is required specifically for Rx function. In an independent study, RanGAP2 interacted with not only Rx but also the CC-domains of the close homologs Gpa2, which confers resistance to the potato cyst nematode, and Rx2 (Sacco et al., 2007). The small GTPase Ran is required for directionality of nucleocytoplasmic trafficking through the NPC; the association of Ran-GTP with Kaps inside the nucleus causes the release of import cargos and binding of export cargos, whereas Ran-GTP hydrolysis in the cytoplasm triggers the dissociation of Ran-GDP-Kap-export cargo complexes. Back in the nucleus, Ran–GDP is converted to Ran-GTP by RCC1 (Merkle, 2001). RanGAP stimulates the GTPase activity of Ran-GTP on the cytoplasmic side of the nuclear envelope. Thus, a resulting Ran-GDP versus Ran-GTP gradient over the NE is established by the spatial sequestering of the Ran accessory proteins, which is involved in maintaining the directionality of nucleocytoplasmic transport (Meier, 2007). As for the role of RanGAP2 in Rx-mediated resistance, the authors offer three alternative hypotheses: 1) the PVX CP binds to or somehow indirectly perturbs RanGAP2 function and Rx, as predicted by the prevailing Guard Hypothesis, senses the activity of the effector and is activated, 2) Rx itself is targeted to the nucleus, and interaction with RanGAP acts as a carrier protein at the NE, or 3) subsequent to activation by CP, Rx interacts with RanGAP to influence the trafficking of resistance-related proteins (Tameling and Baulcombe, 2007). The first hypothesis would indicate that the CC domain of Rx is involved in effector recognition, against the popular point of view that the LRR is more important for this function. The recent data revealing that the TIR domain of N, and not the LRR, associates with the TMV effector p50 negates one of the strongest arguments for this hypothesis (Burch-Smith et al., 2007). Also, a similar mechanism has been proposed for the HRT-dependent resistance to turnip crinkle virus; the CP effector binds to and blocks the nuclear accumulation of a putative TF (Ren et al., 2005). Evidence for the latter two hypotheses is circumstantial, but a growing body of work continues to shed light on the role of nucleocytoplasmic trafficking in resistance signalling. One supporting piece of data is that Rx has been detected in the nucleus (J. Bakker, personal communication). If RanGAP2 itself functions as the carrier for nuclear Rx, which has no detectable NLS, perhaps it can be shown experimentally that RanGAP2 is also required for nuclear accumulation of
MLA10, N, and other R proteins lacking NLSs. Alternatively, since RanGAP2 is involved in hydrolysis of Ran-GTP and cytoplasmic dissociation of export cargo, inhibition of RanGAP2 by Rx could alter the Ran-GDP/Ran-GTP gradient and thus suppress nuclear export of R proteins or defence regulators, increasing the relative concentration of their nuclear pool. Further work is required on the mechanism of RanGAPs on nucleocytoplasmic localization of defence proteins. Components of the nucleocytoplasmic trafficking machinery implicated in plant–microbe interactions are summarized in Figure 1.3.

1.5 Regulation of defence proteins by nucleocytoplasmic partitioning

Besides R proteins, shuttling of downstream regulators across the nuclear envelope is a key regulatory step in defence. In Arabidopsis, TIR-NB-LRR-triggered and basal defence responses are dependent on the lipase-like ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and its sequence related interacting partners PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE ASSOCIATED GENE 101 (SAG101), which together constitute a central regulatory node in diverse immune responses (Aarts et al., 1998; Feys et al., 2001; Feys et al., 2005). While neither the biochemical activities of EDS1, PAD4 and SAG101, nor the signal they transduce are known, there is evidence that they process ROS-derived signals as part of a signal potentiation loop (Rustérucci et al., 2001; Wiermer et al., 2005). Several molecularly distinct EDS1 complexes have been identified in uninduced Arabidopsis cells; these have differential nucleocytoplasmic distribution, providing a handle to explore the role of trafficking on EDS1-dependent defence responses. Without pathogen infection, EDS1 and PAD4 localize to both the cytoplasm and the nucleus whereas SAG101 is exclusively nuclear. EDS1 forms homodimers in the cytoplasm but not the nucleus, where it was shown to interact with SAG101 by FRET (Feys et al., 2005). PAD4 is found in both compartments, where the entire cellular pool likely associates with a small fraction of EDS1, at least in unchallenged cells. The defence regulatory functions of PAD4 and SAG101 are functionally redundant in both basal and TIR-NB-LRR-mediated resistance and HR; indeed the pathology of pad4 sag101 double-mutants in both types of resistance was more extreme than that of of eds1 mutants (Feys et al., 2005). However, SAG101 is only partially able to compensate for loss of PAD4, implying a unique capability of PAD4, potentially as a
Figure 1.3: Components of the nucleocytoplasmic trafficking machinery implicated in plant–microbe interactions

A schematic cross-section of a plant NPC and overview of karyopherin-mediated transport is shown, as described in the text. α, importin α; β, importin β. Nup, nucleoporin. RanGAP2, Ran GTPase activating protein 2.
consequence of the nuclear restriction of SAG101. In another study discussed earlier, Lipka et al. (2005) found that combined PAD4 and SAG101 contributions to post-invasion nonhost resistance greatly exceed those of the single components EDS1, PAD4, or SAG101 alone, furthering the conclusion that PAD4 and SAG101 are partially redundant. Furthermore, a synergistic effect was seen between the glycosyl hydrolase PEN2 and PAD4-SAG101. While pen2 mutants exhibited enhanced penetration of nonhost E. pisi in Arabidopsis but little microcolony formation, penetration rates on pad4 sag101 double-mutant lines were indistinguishable from that of wild type yet microcolony formation (resulting from rare successful penetration) was greatly enhanced. In the pen2 pad4 sag101 triple-mutant lines, the timing and extent of E. pisi colonization became indistinguishable from wild type interactions between Arabidopsis and virulent pathogen species, strongly suggesting that PEN2 and the EDS1-PAD4-SAG101 signaling complex have separate functions in preinvasion and postinvasion resistance to non-host powdery mildews (Lipka et al., 2005).

While the molecular role of EDS1 remains elusive, one study concluded that the strict requirement of TIR-type NB-LRR receptors for EDS1 is likely not at the level of receptor assembly or distribution in the cell, since RPS4 accumulation and nucleocytoplasmic partitioning were not altered in an eds1 mutant (L. Wirthmueller and J.E. Parker, personal communication). To examine the significance of the nucleocytoplasmic distribution of EDS1 complexes, it will be important to localize these complexes in pathogen-induced cells or in autoimmune mutants such as snc1 or ssi4, versus non-challenged cells. Furthermore, as important defence regulatory proteins that presumably traffic through the NPC, the subcellular distribution of EDS1 and PAD4 in mutants with defective nuclear import/export machinery (e.g. mos3, etc) should be examined. Real-time in vivo imaging technologies can be employed to elucidate stimulus-dependent subcellular localization and interactions of defence regulators in living cells (Koh and Somerville, 2006).

The zinc-finger protein LESION SIMULATING DISEASE RESISTANCE1 (LSD1) negatively regulates an EDS1- and PAD4-dependent cell death pathway to protect plant cells from reactive oxygen-induced stress. Arabidopsis lsd1 mutants exhibit normal HR after infection by various incompatible pathogens, but runaway cell death is initiated subsequently at the margins of
these sites (Dietrich et al., 1994). In addition, *lsd1* mutants have enhanced resistance to several virulent pathogens in a pre-lesion state. Evidence suggests that LSD1 responds to a ROS signal emanating from an infection site, and that it is a negative regulator of cell death by acting as a cellular redox sensor (Jabs et al., 1996; Dietrich et al., 1997). Results from Rustérucci et al. (2001) indicate a genetic link between the disease resistance-promoting functions of *EDS1* and *PAD4* and the negative regulation of plant cell death exerted by *LSD1*, possibly due to an *EDS1-PAD4*-dependent processing of ROS-derived signals. The molecular function of LSD1 has been further characterized in a notable study by Kaminaka and colleagues (2006); in both yeast and in protoplasts, LSD1 antagonizes the nuclear activity of the Arabidopsis basic region leucine zipper 10 (bZIP10) transcription factor by sequestering a considerable amount of bZIP10 in the cytoplasm. This observation is not seen when *AtbZIP10* is overexpressed, because LSD1 retention activity is titrated. *AtbZIP10* is a positive regulator of ROS-induced cell death and basal defence, and likely shuttles between the cytoplasm and the nucleus via NLS-dependent nuclear import and NES-dependent nuclear export. Antagonistic functions of LSD1 and *AtbZIP10* modulate disease resistance response to an avirulent oomycete (*H. parasitica*) isolate recognized by the R protein RPP2 (Despres et al., 2000), presumably via their antagonistic control of host cell death. A simplified model states that the cytoplasmic *AtbZIP10–LSD1* complex dissociates upon perception of a ROS-derived signal perception and subsequently unmasks the NLS, permitting the nuclear shuttling of *AtbZIP10* and bZIP10-related target gene expression (Kaminaka et al., 2006). So far, the HR- and basal defence-related genes targeted by *AtbZIP10* remain unknown, but the quantitative output in gene expression depends on the relative intracellular amounts of LSD1 and *AtbZIP10*, the retention activity of LSD1 and the *AtbZIP10* export rate. Moreover, it is likely that LSD1 regulates other proteins in addition to *AtbZIP10*, as the *lsd1-2 atbzip10* double-mutant exhibits the *lsd1* phenotype (Kaminaka et al., 2006).

Redox-regulated translocation from the cytoplasm to the nucleus is an essential feature of another key regulator of the plant pathogen defence, NPR1. The *NPR1* gene encodes a protein containing a bipartite NLS and two protein-protein interaction domains (Cao et al., 1997). Defects in *NPR1* compromise immune responses downstream of SA, including SAR and basal resistance (Cao et al., 1997). In the absence of pathogen infection, NPR1 forms an oligomeric
complex in the cytoplasm through intermolecular disulfide bonds (Mou et al., 2003). In response to pathogen attack, an oxidative burst and subsequent biphasic change in the cellular redox environment leads to the reduction of disulfide bonds. NPR1 monomers translocate into the nucleus, possibly due to exposure of normally obscured NLSs, where they regulate downstream PR gene expression through interaction with the TGA subfamily of bZIP transcription factors. Mou et al. (2003) showed that two critical cysteine residues were essential for NPR1 monomerization. Indeed, a GFP-tagged constitutive monomeric version of the protein with either critical cysteine changed to alanine exhibited an enhanced nuclear accumulation as assayed by fluorescence microscopy and nuclear fractionation experiments. Additional evidence for redox modulation of the interaction between NPR1 and certain TGA TFs inside the nucleus to activate PR-1 expression has recently been reported (Rochon et al., 2006). As discussed at the beginning of this chapter, NPR1 also directly controls the expression of the protein secretory pathway genes through interaction with an unknown TF that binds to the TL1 element. This element was shown to have a specific protein-binding activity in an electrophoretic mobility shift assay. Intriguingly, when whole-cell extracts were used for this assay, the TL1 binding activity was not affected by SA treatment, whereas in nuclear extracts, this specific binding was enhanced in SA-treated samples, suggesting that the TL1-binding protein also translocates to the nucleus (Wang et al., 2005).

Recent advances in understanding the bridge between cytoplasmic signal transduction and transcription in the nucleus highlight another possible control-point based on nucleocytoplasmic partitioning. Pathogen-stimulated MAPK cascades end in transcription of defence-related genes. A MAPK in Arabidopsis, MAP KINASE 4 (AtMPK4), was identified as a negative regulator of SAR (Petersen et al., 2000). mpk4 mutants exhibit a constitutive SAR phenotype that includes elevated levels of SA, increased resistance to virulent pathogens, and constitutive expression of PR genes; the de-regulation of the SA pathway is dependent on EDS1 and PAD4 (Brodersen et al., 2006). Andreasson and colleagues found that GFP-tagged MPK4 and its substrate MAP KINASE 4 SUBSTRATE 1 (MKS1) both localize to the nucleus in planta, and that MPK4 interacts with WRKY transcription factors implicated in defence gene expression (Andreasson et al., 2005).
Localization in the cytoplasm ensures that transcriptional regulators have rapid access to cytoplasmic and PM-resident receptors for activation. The examples of bZIP10–LSD1 and NPR1 demonstrate that spatial restriction of transcription factors or their coactivators by the nuclear envelope is a powerful regulatory mechanism utilized by plants to link cytoplasmic signal transduction to rapid defence gene activation in a stimulus-dependent manner. The subcellular localization of MAPK substrates, whether they are TFs or cofactors such as MKS1, could also be regulated in a similar manner, although this requires further study. It remains to be determined how nuclear translocation of signal transducers or changes in their nucleocytoplasmic dynamics are mediated upon pathogen perception, and which transport receptors and components of the NPC are involved. Nucleocytoplasmic distribution of some known defence regulatory proteins in the Arabidopsis cell is reviewed in Figure 1.4.

Figure 1.4: Nucleocytoplasmic distribution of known defence regulatory proteins in the Arabidopsis cell

The sequestration of the HR-inducing bZIP10 transcription factor in the cytoplasm by LSD1 is released upon pathogen-induced ROS signal perception. Basal defence responses and signalling mediated by TIR type NB-LRR R proteins are dependent on EDS1, PAD4 and SAG101. EDS1 interacts with SAG101 only in the nucleus and forms homodimers exclusively in the cytoplasm of un-induced cells; EDS1 is possibly continuously cycling between the two compartments. EDS1 also interacts with PAD4, and this complex is found in both the nucleus and cytoplasm. It is unknown whether the nucleocytoplasmic distribution of EDS1 or EDS1–PAD4 changes as a result of pathogen recognition. The defence regulator NPR1 is sequestered as an oligimer in the cytoplasm of un-induced cells. Following pathogen perception, an ROS burst and subsequent shift in redox balance results in an SA-dependent NPR1 monomerization and exposure of its NLS as described in the text. The NPR1 monomer is translocated to the nucleus, where it regulates defence gene expression through its interaction with TGA bZIP transcription factors. ROS, reactive oxygen species. SA, salicylic acid.
Figure 1.4: Nucleocytoplasmic distribution of known defence regulatory proteins in the Arabidopsis cell
1.6 Conclusions

Intracellular trafficking of vesicles to and from the PM and macromolecules across the nuclear pore have emerged as key dynamic processes in all layers of pathogen defence. Mobilization of antimicrobial proteins and compounds to the site of attempted pathogen attack involves the concerted involvement of cytoskeletal rearrangement, vesicle transport and fusion, and PM microdomain formation; while a few of the genes involved in these events have been isolated, much work remains to elucidate the molecular mechanisms of regulation.

Genetic analysis in Arabidopsis has shown that defects in the nuclear trafficking machinery suppress multiple layers of the defence response. Very recent work in barley, tobacco and Arabidopsis has identified intracellular NB-LRR R protein receptors in the nucleus; this nuclear pool is essential for defence signalling. Other defence regulators, such as NPR1, LSD1, MKS1, EDS1 and SAG101, exhibit nuclear trafficking and, as in NPR1 and LSD1, may be regulated by nucleocytoplasmic partitioning. What are the roles of nuclear and cytoplasmic R protein pools and how is their complex composition and distribution regulated? Moreover, do autoimmune mutants, such as snc1, exhibit R protein mis-localization? Is the localization of R proteins or other known defence regulators (e.g. EDS1) affected by defects in the nuclear trafficking machinery? Again, detailed biochemical analysis coupled with live-cell fluorescent imaging after pathogen elicitation is key to determining changes in nuclear versus cytoplasmic concentrations of R proteins and other defence regulators. Novel regulators of R protein function, discovered in suppressor and interaction screens, may play a role in nucleocytoplasmic localization of R protein-containing complexes. Proteomics of defence regulator complexes isolated from nuclei of infected versus non-infected cells might illuminate this phenomenon. Do pathogen effectors specifically interfere with nuclear transport to promote virulence? Previously, several Avr proteins were implicated in direct or indirect interaction with components of the nuclear transport machinery, for example AvrBS3 with α-importin. This has been interpreted as evidence for hijacking of the host nuclear transport machinery for nuclear import. Instead, it is possible that the nuclear import machinery is targeted for disruption to promote pathogen virulence. Very recent data showing that the Rx interaction with RanGAP2 is required for...
recognition of PVX CP may indicate that RanGAP2 may be a target of the effector. In light of the importance of dynamic nucleocytoplasmic regulation in pathogen defence, we expect some newly characterized pathogen effectors may target this process.

In conclusion, exocytosis, endocytosis and nucleocytoplasmic trafficking in pathogen defence are emerging research fields in plant science – future studies that address these and other fundamental questions will add important insights into eukaryotic innate immunity.
1.7 References


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2 The snc1 autoimmune model

As discussed previously, NON-EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1) is an important signalling component in plant innate immunity. NPR1 is essential for SAR and responsiveness to SA, and has been shown to be regulated by nucleocytoplasmic partitioning. In an effort to further dissect the involvement of NPR1 in plant defence signalling, a suppressor screen for mutations that restored SA responsiveness in npr1-1 was conducted. Two classes of suppressor mutants were identified. One class, represented by suppressor of npr1-1, inducible 1 (sni1), a novel transcriptional repressor, suppressed npr1-mediated abrogation of SA-induced resistance (Li et al., 1999). The other class, represented by the gain-of-function mutant suppressor of npr1-1, constitutive 1 (snc1), constitutively suppressed npr1-associated phenotypes without induction by SA by activating NPR1-independent signalling pathways (Li et al., 2001). The snc1 single-mutant also constitutively expresses PR genes, exhibits enhanced resistance to the virulent bacterial and oomycete pathogens, and accumulates SA. SNC1 encodes an R protein of the TIR-NB-LRR class – a point mutation in snc1 a Glu to Lys amino acid change in the linker region between the NB and LRR domains renders this protein constitutively active without interaction with pathogens (Zhang et al., 2003). The snc1 mutation may affect the interaction between SNC1 and its negative regulator. Interestingly, mutations in the corresponding linker region of NOD2, a mammalian NB-LRR–containing protein involved in host defense against pathogens, also results in constitutive activation and has been associated with the auto-inflammatory Crohn’s disease (Tanabe et al., 2004; Eckmann and Karin, 2005; Inohara et al., 2005). One of the potential negative regulators of SNC1 is BONZAI1 (BON1). Loss-of-function mutations in BON1 constitutively activate SNC1-dependent PR gene expression and pathogen resistance (Yang and Hua, 2004). The genetic interaction between BON1 and SNC1 resembles the interactions between RIN4 and the R proteins RPS2 or RPM1, as rin4 constitutively activates defense response in an RPM1- and RPS2-dependent fashion (Belkhadir et al., 2004a). Whereas RIN4 associates with RPM1 and RPS2 in a multiprotein complex (reviewed in Belkhadir et al., 2004b), it remains to be determined whether BON1 and SNC1 interact with each other in vivo.
The downstream signalling induced by the activation of *snc1* requires multiple components (Li et al., 2001; Zhang et al., 2003). Like other R proteins of the TIR-NB-LRR class, *snc1* signalling is fully dependent on the previously mentioned EDS1 and PAD4. Responses downstream of *snc1* diverge into SA-dependent and SA-independent pathways (Zhang et al., 2003). The *eds5* mutant, which is deficient in SA accumulation, exhibits *PR-2* gene expression in response to avirulent pathogens (Nawrath and Metraux, 1999). EDS5 is an essential component of SA-dependent disease resistance signalling, and is probably involved in SA synthesis (Nawrath et al., 2002). Constitutive *PR-2* gene expression was not affected in *snc1 eds5-3* mutant plants, which had intermediate size and curly leaves but were susceptible to virulent pathogens (Zhang et al., 2003). Some responses downstream of *snc1*, such as constitutive *PR-1* expression, are dependent on NPR1 whereas others (*PR-2*) are not, suggesting that the *snc1* signal transduction pathway also includes NPR1-dependent and NPR1-independent branches (Figure 2.1). Previously NDR1, RAR1, SGT1b, and HSP90 have also been shown to be important regulators of *R*-gene function (reviewed in Muskett and Parker, 2003). Whereas EDS1 and PAD4 are essential for the resistance specified by the TIR-NB-LRR proteins, *NDR1* is only important for the resistance conferred by several coiled-coil–NB-LRR proteins (Century et al., 1995; Aarts et al., 1998). Unlike EDS1, PAD4 and NDR1, RAR1 and SGT1 are required for resistance mediated by R-proteins in both groups (Shirasu et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Liu et al., 2002; Muskett et al., 2002). HSP90 associated with both RAR1 and SGT1 and has been shown to be essential for resistance mediated by multiple R-proteins (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004). Although *ndr1-1* does not suppress *snc1*-mediated resistance (Li et al., 2001), it is not yet known whether RAR1, SGT1b, or HSP90 are required for *snc1*-mediated resistance signalling.

*snc1* displays stunted growth and curly leaves; the dilapidated morphology of *snc1* is likely caused by both the accumulation of SA, which is toxic at high concentrations (Yalpani, 1992), and by the metabolic shift associated with misregulated activation of defence responses. The activation of R-proteins generally results in the death of plant cells that are in contact with the pathogen, a phenomenon called the hypersensitive response (HR) (Hammond-Kosack et al., 1994; Greenberg and Yao, 2004). Spontaneous HR is common to previously described mutants that display constitutive defence responses, including *ssi* (Shah et al., 1999; Shirano et al.,
2002), *acd* (Greenberg et al., 1994; Lu et al., 2003), and some *cpr* (Bowling et al., 1997; Yoshioka et al., 2001) mutants. However, no macroscopic or microscopic cell death has been observed in *snc1* (Li et al., 2001), suggesting that defence response pathways are activated independent of HR. Many previously identified defence signalling components were identified in genetic screens based on either suppression of R protein-mediated HR or exceedingly enhanced susceptibility to pathogens. It is known that some resistance responses and cell death (HR) are separable events activated by R proteins; however, mutations in genes required for HR-independent resistance would be masked if cell death was still initiated. Moreover, the contribution of a complicated network of signalling pathways to defence means that mutations in defence regulators specific to one contributing pathway with subtle effects on disease severity, or those with partial functional redundancy, could be missed in such screens. Because *snc1* does not manifest constitutive HR, we hypothesized that either only a subset of defence responses are triggered by this misregulated R protein, or most likely the amplitude of activation is such that it does not cross the threshold for HR.

The *snc1* suppressor screen is the basis of this thesis work. *snc1* and *snc1 npr1-1* seeds were mutagenized by fast-neutron bombardment (60 Gy) by Andrea Kodym (Agriculture and Biotechnology Laboratory, International Atomic Energy Agency, Vienna Austria). Mutations in genes required for signal transduction downstream of the constitutively active *snc1* can be identified based on a restoration of wild type morphology in M2 plants by suppression of the easily scored *snc1* morphological phenotypes. A secondary screen for suppression of constitutive *PR2* gene expression, based on lack of GUS staining in seedlings stably transformed with a *pPR2-GUS* reporter construct, was utilized to confirm a defence signalling related defect in progeny of M2 plants with wild type size and morphology. The suppressors identified in this genetic screen, termed *modifier of snc1* (*mos*) mutants, were hypothesized to be positive regulators with specific roles in defence responses. In the initial screen, 15 complementation groups of *mos* mutants were isolated, including several alleles of *pad4*, validating the efficacy of the screen. Besides MOS6 and MOS4, to be discussed in the following chapters, the *mos* screen has resulted in the cloning and characterization of several other genes that encode proteins with signalling functions downstream of *snc1* and in plant innate immunity in general. These include MOS2, a putative RNA-binding protein (Zhang et al., 2005); MOS3 (Zhang et al., 2003).
and MOS7 (Y. Cheng and X. Li, unpublished data), components of the nuclear pore complex; and MOS5 (Gortischning et al., 2007) and MOS8 (S. Goritchnig and X. Li, unpublished data), protein modification enzymes involved in ubiquitination and farnesylation of unknown targets. Other mos complementation groups have been isolated from the screen and await cloning.

### 2.1 Thesis Objectives

The primary aim of this thesis research was to identify novel genes encoding positive regulators of R protein mediated and basal defence signalling, using a suppressor screen of the snc1 autoimmune mutant. To this end, this work describes the identification, positional cloning and in-depth characterization of two mos mutants; mos6 and mos4. Both mutations suppress snc1 responses and affect basal defences as well as resistance mediated by R proteins. The “big picture” goal of this research is to further our understanding of innate immunity in plants and, since some aspects of innate immunity may be conserved across kingdoms, multicellular eukaryotes in general. Furthermore, this research increases knowledge about how plants fight off microbial pathogens, which may in the future aid in wide-spectrum crop protection strategies based on manipulation of the plant’s endogenous defence mechanisms.
Figure 2.1: Model for pathways activated in snc1 plants
2.2 References


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3 An importin-α homolog, MOS6, plays an important role in plant innate immunity\(^1\)

\(^1\) A version of this chapter has been published. Kristoffer Palma, Yuelin Zhang and Xin Li. (2005) *Current Biology* **15**:1129-1135.
3.1 Summary

Plant disease resistance is the consequence of an innate defense mechanism mediated by Resistance \((R)\) genes (Nimchuk et al., 2003). The conserved structure of one class of R-protein is reminiscent of Toll-like receptors (TLRs) and Nucleotide-binding oligomerisation domain (NOD) proteins – immune response perception modules in animal cells (Belkhadir et al., 2004; Nurnberger et al., 2004; Philpott and Girardin, 2004). The Arabidopsis \(snc1\) (suppressor of \(npr1-1\), constitutive, 1) mutant contains a mutation in a TIR-NBS-LRR-type of R-gene that renders resistance responses constitutively active without interaction with pathogens (Zhang et al., 2003a). Few components of the downstream signaling network activated by \(snc1\) are known. To search for regulators of R-gene-mediated resistance, we screened for genetic suppressors of \(snc1\). Three alleles of the mutant \(mos6\) (\(modifier\) of \(snc1\), \(6\)) partially suppressed constitutive resistance responses and immunity to virulent pathogens in \(snc1\). Furthermore, the \(mos6-1\) single mutant exhibited enhanced disease susceptibility to a virulent oomycete pathogen. \(MOS6\), identified by positional cloning, encodes importin-\(\alpha3\), one of eight \(\alpha\) importins in Arabidopsis (Merkle, 2001). \(\alpha\)-Importins mediate the import of specific proteins across the nuclear envelope. We previously reported that \(MOS3\), a protein homologous to human nucleoporin 96, is required for constitutive resistance in \(snc1\) (Zhang and Li, 2005b). Our data highlights an essential role for nucleo-cytoplasmic trafficking, especially protein import, in plant innate immunity.

3.2 Results and Discussion

3.2.1 Identification of three \(mos6\) alleles from a suppressor screen of \(snc1\) and \(snc1\) \(npr1-1\)

The \(modifier\) of \(snc1\), \(6\) (\(mos6\)) mutant, generated by fast neutron mutagenesis in the \(snc1\) background, was identified in the suppressor screen described earlier (Zhang and Li, 2005b). While \(snc1\) plants exhibit constitutive Pathogenesis-related (\(PR\)) gene expression and have a
distinctive morphology (stunted growth and dark, curly leaves) (Li et al., 2001; Zhang et al., 2003a), the mos6-1 snc1 mutant plants are intermediate in size between snc1 and wild type, and have leaves that are lighter green and less curly than in snc1 (Figure 3.1A). Two other mutants isolated in the snc1 npr1-1 background were mapped to the same chromosomal arm. These were subsequently found to be allelic to mos6-1 snc1 by complementation test (see below), and named mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1. mos6-2 and mos6-3 partially suppress the severe dwarf phenotype of snc1 npr1-1. The constitutive pBGL2-GUS reporter gene expression in snc1 and snc1 npr1-1 is suppressed by all three alleles of mos6 (Figure 3.1B). Furthermore, RT-PCR analysis showed that constitutive endogenous PR-2 (BGL2) and PR-1 expression were largely suppressed in mos6-1 snc1 and mos6-2 snc1 npr1-1 (Figure 3.1C).

When mos6-1 snc1 was backcrossed with snc1 (both carrying the reporter gene pBGL2-GUS), the F1 progeny have a snc1 morphology, indicating that mos6-1 is a recessive mutation. Out of 42 F2 progeny, 31 had GUS staining, suggesting that the suppression of constitutive pBGL2-GUS expression by mos6-1 is caused by a single recessive mutation (expected ratio is 3:1, $\chi^2 = 0.032; P>0.1$). To test for allelism, mos6-1 snc1 was crossed with mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1. In both cases no complementation was observed. All of the F1 progeny had parental morphology (did not exhibit a snc1 phenotype), and there was no constitutive pBGL2-GUS expression in F2 seedlings (data not shown).

### 3.2.2 mos6 suppresses elevated SA levels in snc1 and snc1 npr1-1

Defense responses with varying degrees of efficacy in limiting pathogen growth have been shown to have differential dependency on SA, jasmonic acid and ethylene signaling (Glazebrook et al., 2003). The snc1 mutant accumulates high levels of SA (Li et al., 2001). Previously, we found that SA levels are only partially responsible for snc1 phenotypes, since the eds5 mutant, which is deficient in SA accumulation (Nawrath et al., 2002), does not affect constitutive PR-2 gene expression in snc1 (Zhang et al., 2003a). Moreover, snc1 eds5-3 mutant plants had intermediate size and curly leaves but were susceptible to virulent pathogens.

Free and total SA in mos6-1 snc1 plants was extracted and measured, to determine whether
Figure 3.1. Suppression of snc1-associated morphology and constitutive PR gene expression by mos6 alleles

(A) Morphology of wild type (WT) Columbia, snc1, snc1 npr1-1, mos6-1 snc1, mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1. All plants were 4 weeks old, and grown parallel on soil. (B) Suppression of snc1-induced pBGL2-GUS reporter gene expression in representative 3-week-old seedlings grown on MS medium. Seedlings were stained for GUS activity and cleared as in Bowling et al. (1994). (C) PR-1 and PR-2 (BGL2) gene expression in WT, snc1, snc1 npr1-1, mos6-1 snc1 and mos6-2 snc1 npr1-1. RNA was extracted from 3-week-old plants grown on MS medium and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real-time PCR using an Actin 1 probe. PR-1, PR-2 and Actin 1 were amplified by 30 cycles of PCR using equal amounts of total cDNA. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.
Figure 3.1: Suppression of snc1-associated morphology and constitutive PR gene expression by mos6 alleles
mos6-1 suppresses SA accumulation in snc1. As shown in Figure 3.2A and B, free and total SA in mos6-1 snc1 is about 4.5-fold and 7-fold lower than that in snc1, respectively. Both mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 had a ~10-fold reduction in free SA levels and a 6- to 7-fold reduction in total SA levels compared to snc1 npr1-1 (Figure 3.2A and B). snc1 npr1-1 accumulates significantly more SA than snc1 alone, and thus mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 accumulate more SA than mos6-1 snc1. This effect is probably due to the contribution of npr1-1, since NPR1 is involved in feedback regulation of SA levels (Cao et al., 1997). Because mos6 suppresses SA accumulation and constitutive PR-2 expression in snc1, we tentatively place MOS6 downstream of snc1 and upstream of SA accumulation in the snc1-signaling pathway (Zhang et al., 2003a).

3.2.3 mos6 partially suppresses constitutive pathogen resistance in snc1 and snc1 npr1-1

snc1 and snc1 npr1-1 are resistant to the virulent bacterial pathogen Pseudomonas syringae (P.s.m.) ES4326 and oomycete pathogen Hyaloperonospora parasitica (H.p.) Noco2 (Zhang et al., 2003a). To determine whether the mos6 mutations affected this resistance, the mos6-1 snc1 double mutant and mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 triple mutant plants were infected with both pathogens and compared to snc1 and snc1 npr1-1, respectively. While snc1 had no disease symptoms and supported two orders of magnitude less bacterial growth than wild type at 3 days post-infection, mos6-1 snc1 had intermediate disease symptoms and about 8-fold higher titer of bacteria than snc1 (Figure 3.2C). Similarly, in mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1, P.s.m. ES4326 grew about 100- and 40-fold more than in snc1 npr1-1, respectively (Figure 3.2C). All three mos6 alleles also partially suppressed snc1-mediated resistance to the H.p. Noco2 (Figures 3.2D, E). These data together suggests that snc1-mediated resistance signaling is dependent on MOS6.
**Figure 3.2.** Suppression of snc1-associated salicylic acid accumulation and resistance to virulent pathogens by mos6 alleles

(A) Free and (B) Total SA in leaves of 4-week-old soil-grown WT, snc1, mos6-1 snc1, npr1-1, snc1 npr1-1, mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 plants. SA was extracted and analyzed by HPLC as previously described (Li et al., 1999). The values presented are averages of four replicates ± SD. (C) Growth of *P. s.m. ES4326*. The leaves of 4-week-old soil-grown plants were infiltrated with a suspension of the bacteria at OD$_{600}$ = 0.001. Leaf discs within the inoculated areas were taken immediately (Day 0) and three days after infection. The log-transformed values presented are averages of four replicates ± SD. Cfu, colony-forming units. (D) and (E) Growth of *H. parasitica* Noco2. Two-week-old seedlings were sprayed with a conidiospore suspension of 5x10$^4$ spores per ml of water. The infection was rated as follows 7 days after infection by counting the number of conidiophores per infected leaf: 0, no conidiophores on the plants; 1, no more than five conidiophores per infected leaf; 2, 6-20 conidiophores on a few of the infected leaves; 3, 6-20 conidiophores on most of the infected leaves; 4, 5 or more conidiophores on all infected leaves; 5, 20 or more conidiophores on all infected leaves. Data is presented for 20 plants of each genotype. All experiments were repeated at least twice with similar results.
Figure 3.2: Suppression of snc1-associated salicylic acid accumulation and resistance to virulent pathogens by mos6 alleles
3.2.4 The mos6-1 single mutant exhibits enhanced disease susceptibility (EDS) to a virulent oomycete pathogen

The mos6-1 single mutant was isolated by crossing mos6-1 snc1 with Col containing pBGL2-GUS. This allele had no noticeable morphological phenotype when compared to wild type. mos6-1 single-mutant plants were tested against avirulent Pseudomonas strains expressing AvrB and AvrRpt2, and the avirulent Hyaloperonospora parasitica race Emwa. No significant increase in susceptibility to these avirulent pathogens was observed in mos6-1 when compared to wild type (data not shown). To test if basal defense was compromised in the mos6-1 single mutant, plants were infected with virulent P.s.m. ES4326 and H.p. Noco2. While there was no significant difference in growth of the bacterial pathogen in mos6-1 as compared to wild type (data not shown), mos6-1 supported two-fold higher growth of H.p. Noco2 (Figure 3.3).

Mutations in PAD4 and EDS1, which completely block snc1 signaling (Li et al., 2001; Zhang et al., 2003a), exhibit EDS to both bacterial and oomycete virulent pathogens (Glazebrook et al., 1996; Parker et al., 1996). Differential susceptibility to pathogen class has been described for other mutants with EDS such as eds14, eds15, eds17 (Dewdney et al., 2000) and esa1 (Tierens et al., 2002). Because the mos6 alleles only partially block snc1 phenotypes, and the single mutant has EDS against H.p. Noco2 but not P.s.m. ES4326, MOS6 may be a component of snc1 signal transduction pathway that is more important for limiting growth of a specific subset of pathogens, such as oomycete pathogens.

3.2.5 Map-based cloning of mos6

To map mos6, mos6-1 snc1 in Columbia (Col-0) ecotype (containing pBGL2-GUS) plants were crossed with Ler-snc1, where the snc1 mutation was introgressed into Ler (Zhang and Li, 2005b), to produce a segregating population. mos6-1 was subsequently mapped to a region of about 120 kb (Figure 3.4). Overlapping 2 kb regions of genomic DNA in this interval were then amplified by PCR to look for potential deletions. A 5.3 kb chromosomal rearrangement was detected in mos6-1 snc1 that affects several genes, including the first predicted exon of
Figure 3.3: Enhanced susceptibility of mos6-1 to virulent H.p. Noco2

Growth of H. parasitica Noco2 on the mos6-1 single-mutant. Two-week-old seedlings were sprayed with a conidiospore suspension of 5x10^3 spores per ml of water. The infection was assayed by collecting 5 plants per mL of water for each replicate and counting the total number of conidiospores with a hemacytometer. The values presented are averages of six replicates ± SD. The experiment was repeated twice with similar results.
At4g02150 (accession # NM_116447). PCR fragments of this region were subsequently amplified from the genomic DNA of mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 and sequenced. For mos6-2 snc1 npr1-1, a single bp deletion was discovered in the first exon of At4g02150, introducing a frameshift mutation at bp 45 from the ATG (Figure 3.5A). A 10-bp deletion in the last exon of the same locus was found in mos6-3 (Figure 3.5A).

To further confirm that MOS6 is At4g02150, a full-length cDNA clone of At4g02150 was transformed into mos6-1 snc1 under the control of the CaMV 35S promoter. 10 out of 10 transgenic plants exhibited complementation of mos6-1 by displaying snc1 morphology (Figure 3.6), constitutive pBGL2-GUS reporter gene expression (data not shown), and restored resistance to H.p. Noco2 (Figure 3.5B). The complementation experiment was repeated for mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 with similar results in morphology (Figure 3.6) and resistance to H.p. Noco2 (data not shown).

### 3.2.6 Subcellular localization of MOS6

The subcellular localization of the MOS6 protein was examined by fusing GFP in-frame to the C-terminus of MOS6 under control of the CaMV 35S promoter. The GFP fluorescence signal is concentrated in the nucleus, and co-localizes with stained DNA (Figure 3.7A). This is similar to the nuclear localization pattern described before for importin-α proteins in tobacco protoplasts (Smith et al., 1997). In contrast, GFP alone driven by the 35S promoter did not have a localization pattern similar to that of MOS6-GFP (Figure 3.7B). To ensure that this data reflects the natural localization of MOS6, 35S-MOS6-GFP was transformed into mos6-1 snc1 plants and the transgenic progeny examined. Out of 12 transgenic plants selected, all exhibited snc1 morphology and constitutive pBGL2-GUS expression (data not shown), indicating that 35S-MOS6-GFP complements the mos6-1 mutation in the snc1 background and the MOS6-GFP fusion protein is correctly localized.
Figure 3.4: Map-based cloning of MOS6

(A) Crude 5-megabase (Mb) map of the mos6-1 locus on chromosome 4, co-segregating with marker T10P11-2. Left is telomeric, right is centromeric. Positions of the markers used for mapping are indicated. (B) Four recombinants pointed the mos6-1 mutation to the centromeric side of marker T7B11 and one recombinant pointed the mutation to the telomeric side of marker T2H3. (C) Fine map of the BACs and mapping markers surrounding the mos6-1 locus. *, physical location of the mos6-1 locus. (D) Sequenced genomic region and associated genes. *, region of the mos6-1 defect and affected genes.
Figure 3.5. Sequence analysis of MOS6

(A) Exon/intron and predicted protein structure of MOS6. The exons are indicated with boxes. Locations of the mos6-2 and mos6-3 deletions are indicated, relative to the mos6-1 defect affecting the first exon. Different predicted protein domains and their relative location are indicated by shape. IBB, importin-β binding domain; ARM, armadillo repeat. Base pair (bp) and amino acid (aa) lengths are indicated by size bars. (B) MOS6 restores constitutive disease resistance in mos6-1 snc1 plants. Procedures and P. p. Noco2 disease ratings were as described in Figure 3.2. Two of the independent complementing lines are shown as representatives. (C) Amino acid sequence comparison of MOS6 (AtImpα3, accession NP_192124) and other Arabidopsis importin-α homologs. Sequences of AtImpα1/AtKapα1 (accession NP_850524), AtImpα2 (accession NP_567485), AtImpα4 (accession NP_849623), AtImpα5 (accession NP_199742), AtImpα6 (accession NP_973743), AtImpα7 (accession NP_187223), and AtImpα8 (accession NP_200013) were compared to MOS6 using ClustalW. Amino acids identical among at least 50% of the proteins are shaded black and amino acids with similar properties are shaded gray.
Figure 3.5: Sequence analysis of MOS6
Figure 3.6: Complementation of mos6-1 phenotypes by full-length At4g02150 cDNA

Expression of the full-length MOS6 cDNA driven by the 35S promoter complements the mos6-1, mos6-2 and mos6-3 mutants and restores the snc1 morphology in mos6-1 snc1, mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1. Plants shown are 4 weeks old, soil-grown, representative of at least 2 independent transgenic lines for each genotype.
Figure 3.7. Localization of MOS6 in live cells

GFP localization (green) and DNA staining (DAPI, blue) determined by confocal fluorescence microscopy in transgenic plants expressing (A) 35S-MOS6-GFP and (B) 35S-GFP. Images were taken from the root tip regions of representative plants. Similar patterns of localization were seen in several independent transgenic lines, for each construct. (C) Close-up view of GFP fluorescence from several representative cells expressing 35S-MOS6-GFP.
Figure 3.7: Localization of MOS6 in live cells
**MOS6 encodes a putative nuclear import receptor subunit**

Sequence analysis of *At4g02150* shows that MOS6 encodes a paralog of Arabidopsis Karyopherin α (AtKapα), (Smith et al., 1997). Karyopherins are a conserved family of mobile targeting receptors that mediate the bidirectional trafficking of macromolecules across the nuclear envelope, and include classes such as α-importins, β-importins, and exportins (Goldfarb et al., 2004). The best understood function of importin-α is to serve as an adaptor that links nuclear localization signal (NLS)-containing proteins to importin-β, forming a trimeric complex (Goldfarb et al., 2004). AtKapα, for example, binds to three different nuclear import signals *in vitro*, namely a classical NLS, a basic bipartite NLS, and a Mat-like signal (Smith et al., 1997), and also to importin-β, which mediates translocation through the nuclear pore complex (NPC) owing to its ability to interact with nucleoporins (Goldfarb et al., 2004). In mammalian cells, after entering the nucleus, RanGTP binds to importin-β and displaces the cargo-importin-α heterodimer. This process is similar in plant cells, and nucleo-cytoplasmic partitioning of proteins has been implicated in the regulation of various environmental and developmental signals (Merkle, 2003).

The importin-α gene family has undergone considerable expansion during the course of eukaryotic evolution. Whereas the yeast genome encodes a single importin-α, *Drosophila*, *C. elegans*, mammalian and higher plant genomes all contain multiple proteins with deduced importin-α function. The Arabidopsis genome encodes 8 importin-α homologs (Figure 3.5C), of which the expression of AtImp-α1 through AtImp-α4 (Schledz, 1998) were confirmed experimentally and AtImp-α5 through AtImp-α8 (Merkle, 2001) were deduced from the genomic sequences and therefore still lack experimental verification. MOS6 was previously classified as importin-α3 (AtImpα3) (Schledz, 1998). The deduced MOS6 protein has the typical architecture of all importin α proteins (except for AtImp-α8): an amino-terminal importin β-binding domain (IBB) that is rich in arginine residues, armadillo (ARM) repeats that make up the core of the protein, and contain the NLS-binding pockets, and a carboxy-terminal acidic patch that is implicated in recycling back to the cytoplasm (Herold et al., 1998) (Figure 3.5A). Phylogenetic analysis of plant importin-α proteins (8 Arabidopsis, 5 rice, 2 pepper, 1 tomato) and several animal α importins (3 each from *Drosophila* and *C. elegans*) shows that, except for
one rice importin, OsImpα-2, all plant homologs fell into a distinct clade (Figure 3.8). Since homologs from the same species do not cluster together within the plant clade, it is likely that α-importins diverged in a common ancestor of all higher plants, presumably to perform cell and tissue specific roles in development and differentiation. It is possible that some Arabidopsis importins are functionally redundant. MOS6 (importin-α3) is very similar to importin-α6 (accession NP_973743), and these two proteins are closely related phylogenetically (Figure 3.8). Because all alleles of mos6 only partially suppress snc1 phenotypes, it will be interesting to see if defects in both AtImpα6 and MOS6 more completely suppress snc1.

The question of why there are so many importin-α proteins has not so far been addressed in plants. In animals, tissue-specific expression has been shown for different α-importin homologs, however many of these interact with the same substrate (Goldfarb et al., 2004). The significance of encoding multiple importin-α homologs can be inferred from studies in humans, in which seven different importin-α proteins have been described (Kohler et al., 1999). Recently, in an effort to examine specificity of these proteins, RNAi was used to specifically down-regulate the expression of ubiquitously expressed human α importins (Quensel et al., 2004). The down-regulation of two human importins, α3 and α5, specifically inhibited cellular proliferation and the import of the nuclear protein Ran guanine nucleotide exchange factor (RCC1) – both defects were rescued by injection of recombinant importin-α3. Therefore, it was concluded that, in humans, nuclear import of RCC1 was dependent on importin-α3 only (Quensel et al., 2004). More recently, importin α3 and importin α4 have been identified as the main importin α isoforms mediating TNF-α stimulated NF-κB p50/p65 heterodimer translocation into the nucleus in humans (Fagerlund et al., 2005b). Since all importin-α proteins bind NLS sequences, this finding further suggests that there is additional import substrate specificity. So far, the only example of this type of specificity in plants has been described in rice. Nuclear import of constitutive photomorphogenic 1 (COP1), a repressor of photomorphogenesis regulated by nucleo-cytoplasmic partitioning, is mediated preferentially by the rice importin-α1b (Jiang et al., 2001).
Figure 3.8. Phylogenetic analysis of MOS6

Phylogenetic relationship of MOS6 and other importin-α proteins in plants, Drosophila melanogaster, Caenorhabditis elegans, and yeast. Amino acid sequences of MOS6, Arabidopsis importin-α homologs, Oryza sativa (rice) importin-α1a (OsImpα1a; accession NP_912763), OsImpα1b (accession NP_910164), OsImpα2 (accession NP_909338), Os importin-α-like A (OsImpα-like A; accession NP_908847), Os importin-α-like B (OsImpα-like B; accession XP_479607), Capsicum annuum (pepper) importin-α1 (CaImpα1; accession AAk38726), CaImpα2 (accession AAK38726), Lycopersicon esculentum (tomato) importin-α1 (LeImpα1; accession AAC23722), C. elegans (Ce) importin-α1 (CeIMA-1; accession NP_505854), CeIMA-2 (accession NP_491824), CeIMA-3 (accession NP_501227), D. melanogaster (Dm) importin-α1 (DmIMA-1; accession NP_524167), DmIMA-2 (pendulin; accession NP_477041), DmIMA-3 (accession NP_788614) and yeast Srp1p (accession NP_014210) were compared phylogenetically. The consensus neighbor-joining tree was constructed with BIONJ phylogenetic analysis software (Gascuel, 1997) using the Jones-Taylor-Thornton distance matrix (Jones et al., 1992). Bootstrap re-sampling was performed using PHYLIP (Felsenstein, 1989). Confidence levels for the tree branches are shown as numbers of alignments out of 500 that support each branch.
Figure 3.8: Phylogenetic analysis of MOS6
3.2.7 Nucleo-cytoplasmic trafficking has an essential role in innate immunity

The most obvious potential role for MOS6 is in protein import, where MOS6 may specifically or preferentially import unknown cargo proteins involved in disease response signaling into the nucleus. In Arabidopsis, bZIP transcription factors TGA2, TGA5 and TGA6 are essential for SA-induced PR gene expression and pathogen resistance (Zhang et al., 2003b). Binding of these transcription factors to the promoter of PR-1 is regulated by NPR1 (Johnson et al., 2003), a critical component of SA-dependent defense responses (Dong, 2004). Interestingly, NPR1 is sequestered in the cytoplasm of uninduced cells and imported to the nucleus after induction by SA (Kinkema et al., 2000; Mou et al., 2003). It remains to be determined whether MOS6 is involved in the translocation of NPR1 from cytoplasm to nucleus.

In future studies, it will be important to analyze the dynamics of the MOS6 protein in response to pathogen infection. In addition, the possibility of functional redundancy of importin-α proteins needs to be addressed. Nevertheless, the identification of mos6 as a suppressor of snc1 resistance, coupled with our previous report on MOS3, which is a nucleoporin 96 homolog that may be involved in RNA export (Zhang and Li, 2005b), highlights the significance of nucleo-cytoplasmic trafficking in plant immunity.

3.3 Experimental Procedures

3.3.1 Mutant screen and characterization of mos6

The plant growth conditions and mos suppressor screen were described previously (Zhang and Li, 2005b). The pBGL2-GUS reporter gene was described earlier (Bowling et al., 1994).

RNA used for gene expression analysis was extracted from 3-week-old seedlings grown on MS medium using the Totally RNA kit from Ambion (Austin, TX). Reverse transcription (RT) was carried out using the RT-for-PCR kit from Clontech (Palo Alto, CA). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit from Qiagen (Valencia, CA). The primers used for amplification of Actin1, PR-1, and PR-2 were described previously (Zhang et al., 2003a).
Infection of Arabidopsis with *P.s.m.* ES4326 was carried out on 4-week-old soil-grown plants and infection of *H.p.* Noco2 was performed on 2-week-old seedlings as described (Li et al., 1999). Salicylic acid was extracted and measured using a previously described procedure (Li et al., 1999), proportionally scaled down using 100 mg tissue per sample.

### 3.3.2 Map-Based Cloning of *mos6*

Mapping markers were designed based on ecotype-specific insertions, deletions, and single nucleotide polymorphisms (SNPs) found in the Monsanto Arabidopsis polymorphism and Landsberg sequence collections (Jander et al., 2002). Marker primers used include T10P11-2, 5’-acacagggcgttaattaatggc-3’ and 5’-tgcttaggttatgatagttgg-3’ (256 bp in Col, 223 bp in Ler); F2N1, 5’-gatgtcggaccaccagtgt-3’ and 5’-gaaccttctgagctgtttagg-3’ (274 bp in Col, 246 bp in Ler); T7M24, 5’-ttactcgcccatagctttagg-3’ and 5’-ttcccatcagcttctctc-3’ (219 bp in Col, 186 bp in Ler); T10M13-1, 5’-cgaagctcatcaaccatgac-3’ and 5’-gcacggcacaacctgtataac-3’ (174 bp in Col, 196 bp in Ler); T7B11-4, 5’-ccctaacatcaccaaatcacc-3’ and 5’-ccctctgtacatcacagcatc-3’ (369 bp in Col and Ler, digestion with PsiI in Col only); and T2H3, 5’-gccctacgcctttttg-3’ and 5’-aacgtgtatgcacggtagac-3’ (1518bp in Col, 534 bp in Ler). The PCR fragments containing the mutations responsible for *mos6*-2 and *mos6*-3 were amplified and sequenced using the primers T10M13-77851F (5’-tatctgatctgcatttccagc-3’) and T10M13-79988R (5’-ctccataagctacgcgtctt-3’), and T10M13-75270F (5’-tagctacacaccaggtccag-3’) and T10M13-77926R (5’-ccagcaagcgtctctgtagc-3’), respectively.

*F*₁ plants from the mapping cross were selfed, and 44 plants from the *F*₂ progeny lacking *snc1* morphology, therefore homozygous at the *mos6*-1 locus, were selected for linkage analysis. Linkage was found at the top of chromosome 4. A further 36 *F*₂ plants with *snc1* morphology, thus heterozygous or wild type at MOS6, were selected for crude mapping. There was perfect co-segregation when the marker T10P11-2, located on the bacterial artificial chromosome (BAC) T10P11, was used on all *F*₂ plants. At the telomeric marker F2N1, 4 recombinants were identified, whereas at the centromeric marker T7M24, only 1 different recombinant was found. This data indicated that the *mos6*-1 locus was flanked by F2N1 and T7M24, and closely linked to T10P11, a total physical distance of about 1.3 Mb (Figure 3.4A).
To fine-map mos6-1, 858 plants were randomly selected from the F3 progeny of F2 plants genotyped to be heterozygous for both flanking markers, F2N1 and T7M24, and homozygous for the pBGL2-GUS reporter gene. 97 recombinants were found between F2N1 and T7M24 and the seeds collected. The phenotypes of the recombinant plants were determined by both segregation of GUS staining of seedlings on MS and snc1 morphological phenotype of soil-grown plants in the F4 progeny. The phenotypes of the recombinant plants co-segregated perfectly with the genotype of the plants at the marker T10M13-1, suggesting that the mos6-1 locus was very closely linked to this marker. Further polymorphism markers were used to narrow the flanking interval; with the last remaining recombination events located between markers T7B11-4 (2 recombinations) and T2H3 (1 recombination), a region of about 120kb (Figure 3.4B, C). Since fast neutron is known to cause deletions/rearrangements (Li and Zhang, 2002), and three alleles of mos6 were available, overlapping ~2kb regions of genomic DNA in this interval were amplified by PCR to look for potential deletions. Several fragments were absent in mos6-1 snc1 but not in the Col-0 control; this defect was subsequently narrowed down to a ~5kb region between bp 79300 and 84618 of BAC T10M13 that would not amplify in the mutant. Further analysis determined that the mutation was a chromosomal rearrangement affecting several genes (Figure 3.4D). The mutations responsible for the other two alleles of mos6 were discovered by sequence analysis of the genomic region affected by the rearrangement found in mos6-1.

To isolate the mos6-1 single mutant, mos6-1 snc1 was crossed with wild type plants containing pBGL2-GUS, and F2 plants were genotyped by PCR. Primers used to detect mos6-1 homozygotes, based on a lack of product when compared to wild type, were T10M13-80080F (5’-cattaataacgaacctgccac-3’) and T10M13-81067R (5’-tgctagaaccaaattgcagc-3’); the primers used to detect the snc1 point mutation were described previously (Zhang et al., 2003a). Lines homozygous for mos6-1 without the snc1 mutation were designated as mos6-1 single mutants and used for further analysis.

The full-length cDNA for At4g02150 (U12574) was obtained from the Arabidopsis Biological Resource Center (Yamada et al., 2003). The cDNA was cloned into a modified pBl1.4 vector (Mindrinos et al., 1994), and transformed into mos6-1 snc1, mos6-2 snc1 npr1-1 and mos6-3 snc1 npr1-1 plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were
selected for antibiotic resistance on MS plates containing 75 µg/ml kanamycin, and analyzed for complementation of the mutant.

### 3.3.3 Subcellular Localization of MOS6

Full-length *At4g02150* cDNA lacking a stop codon was cloned into the pBS-GFP5 vector with GFP in-frame at the C-terminus (Haseloff et al., 1997). The resulting *MOS6-GFP* fusion construct was sequenced to confirm an in-frame fusion without PCR errors, and subsequently excised and cloned into pBI1.4 to obtain pBI-*MOS6-GFP*. This construct was used to transform wild type and *mos6-1 snc1* plants. Transgenic plants were selected on MS medium containing 75 µg/ml kanamycin. Roots of transgenic seedlings were stained with 1 µL/mL 4',6-diamidino-2-phenylindole (DAPI) and examined for green and blue fluorescence with a Radiance 2000 multi-photon confocal microscope (Bio-Rad Laboratories, Hercules, CA), as described previously (Haseloff et al., 1997). A construct expressing GFP alone (*35S-GFP*) was used as a control as described previously (Zhang and Li, 2005b).

### 3.4 Manuscript Acknowledgements

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3.5 References


rice, by which constitutive photomorphogenic 1 (COP1) nuclear localization signal (NLS)-protein is preferentially nuclear imported. J Biol Chem 276, 9322-9329.


Regulation of plant innate immunity by three proteins in a complex conserved across the plant and animal kingdoms

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4.1 Introduction

Innate immunity is the front-line defense system of multicellular eukaryotes, and is activated by a limited number of pattern-recognition receptors (PRRs). Most PRRs in animals contain leucine-rich repeat (LRR) motifs including Toll-like receptors (TLRs) and proteins with a nucleotide oligomerization domain (NOD) (Akira et al., 2006). There is considerable evidence that plant and animal innate immune systems are conserved as a consequence of convergent evolution (Ausubel, 2005). Initiation of induced plant defense signaling involves the recognition of specific pathogen effectors by the products of specialized host Resistance (R) genes (Belkhadir et al., 2004). TIR-NB-LRR-type R-proteins combine features of two different classes of PRRs in animal innate immunity; TLRs and NOD proteins (Philpott and Girardin, 2004). In the unique Arabidopsis snc1 mutant, a gain-of-function mutation in a TIR-NB-LRR-type R-gene results in constitutive defense responses against a wide spectrum of pathogens – the specific pathogen effector detected by wild type SNC1 in nature is unknown. The snc1 mutation is located in the linker region between the NB and LRR (Zhang et al., 2003a). Intriguingly, mutations in the corresponding linker region of some mammalian NODs also result in constitutive self-activation. Dominant mutations in NODs can lead to autoimmune disorders such as Crohn’s disease and Blau syndrome (Eckmann and Karin, 2005). To identify additional components required for plant innate immunity, a suppressor screen was performed to search for mutations that revert the autoimmune phenotypes of snc1 to wild type. One mutant, modifier of snc1, 4 (mos4), completely abolished enhanced resistance to the virulent pathogens in snc1. MOS4 encodes a nuclear protein with homology to human Breast Cancer Amplified Sequence 2 (BCAS2). MOS4 directly interacts in planta with AtCDC5, an atypical R2R3 Myb transcription factor with significant homology to human CDC5L, which is a key component of the spliceosome-associated PRP Nineteen Complex (NTC). BCAS2 also belongs to NTC. AtCDC5 was previously shown to have sequence-specific DNA binding activity (Hirayama and Shinozaki, 1996), thus it most likely functions as a transcriptional regulator. A third member of the NTC that interacts directly with CDC5L is PLRG1 (Ajuh et al., 2001). In Arabidopsis, one of the PLRG1 homologs, PRL1, interacted with AtCDC5 in planta. MOS4, AtCDC5, and PRL1 are all required for
pathogen resistance in Arabidopsis. Thus, the evolutionarily conserved NTC plays an essential regulatory role in plant innate immunity, and may have a similar function in animals.

4.2 Results

4.2.1 mos4-1 suppresses the autoimmunity-related phenotypes of snc1

In plants, innate immunity against microbial pathogens is governed by R-proteins that are responsible for recognizing pathogen effectors and initiating downstream defense responses (Belkhadir et al., 2004). The most common R-proteins are TIR- and CC-class NB-LRR proteins (Chisholm et al., 2006). Although many R-genes have been cloned, the signal transduction events downstream of R-proteins remain scarcely detailed. In order to identify more signaling components downstream of R-protein activation, we carried out a suppressor screen using snc1 (Zhang et al., 2005b; Zhang and Li, 2005a). The mos4-1 snc1 double mutant plants were the same size as wild type, and had leaves that were less curly than in snc1 (Figure 4.1A). Constitutive pBGL2-GUS reporter gene expression in snc1 was completely abolished by mos4-1 (Figure 4.2A). Furthermore, RT-PCR analysis showed that endogenous PR-2 (BGL2) was no longer constitutively expressed in mos4-1 snc1 plants, and that the expression of PR-1 was partially suppressed (Figure 4.2B). Genetic analysis indicated that mos4-1 is a single recessive mutation. In addition, defects in MOS4 affected flowering time and male fertility (Figure 4.3). It was determined that fertility defects were due to male sterility because crosses using mos4-2 or mos4-3 as female were always successful whereas crosses using pollen from mos4-2 or mos4-3 were never successful.

The snc1 mutant accumulates high levels of the defense-signaling molecule salicylic acid (SA). Previously, we found that blocking SA synthesis only partially suppressed snc1 phenotypes (Zhang et al., 2003a). In mos4-1 snc1, levels of free SA were about 8-fold lower than in snc1 and levels of total SA were about 30-fold lower than in snc1 (Figure 4.1B); these SA levels were comparable to wild type controls, suggesting that MOS4 functions upstream of SA synthesis.
Two of the most amenable plant-pathogen model systems for the genetic dissection of host responses are those between Arabidopsis and 1) *Pseudomonas syringae* pv *maculicola* (*P.s.m.*) and pv tomato (*P.s.t.*), two strains of a hemi-biotrophic bacterial species that cause disease symptoms ranging from leaf spots to stem cankers, on a broad range of plant hosts and; 2) *Hyaloperonospora parasitica* (*H.p.*), an obligate biotrophic oomycete. Oomycetes grow fungus-like hyphae but have cellulosic cell walls and are phylogenetically unrelated to fungi. *snc1* is resistant to *P.s.m.* ES4326 and *H.p.* Noco2, both of which are normally virulent in Arabidopsis Col-0 plants. While *snc1* had no disease symptoms and supported about 50 times less bacterial growth than wild type at 3 days post-infection (DPI), *mos4-1 snc1* harbored an approximately wild type titer of bacterial growth (Figure 4.1C) and exhibited some disease symptoms (data not shown). Furthermore, *mos4-1* completely suppressed resistance to *H.p.* Noco2 in *snc1*, as shown in Figure 4.1D.

### 4.2.2 MOS4 encodes the Arabidopsis homolog of human BCAS2

*MOS4* (*At3g18165*) was cloned using a map-based approach (Figure 4.4). The mutation in *mos4-1 snc1* was found to be an insertion in *At3g18165*, and *35S-At3g18165* was able to complement the *mos4*-related phenotypes (Figure 4.4E, F). In addition, other T-DNA alleles of *At3g18165* were able to suppress *snc1* (Figure 4.4G), confirming that *MOS4* is indeed *At3g18165*. Sequence analysis revealed that MOS4 is similar to human BCAS2, with 29% identity and 48% similarity at the protein level. An amino acid alignment between Arabidopsis MOS4 and related proteins from other eukaryotes shows highly conserved regions (Figure 4.5). Since the identity of MOS4 did not reveal the detailed biochemical mechanism of its regulation of innate immunity, we employed three approaches to further elucidate MOS4’s detailed modes of action: 1) genetic analysis to identify the pathway in which MOS4 acts; 2) subcellular localization of MOS4; and 3) identification of MOS4-interacting proteins.
4.2.3 MOS4 is essential for signaling in the NPR1-independent pathway

To understand how MOS4 contributes to plant innate immunity, we used a genetic approach to examine the pathway in which it functions. Signaling downstream of snc1 diverges into multiple pathways dependent on different signaling intermediates (Zhang et al., 2003a). NPR1, an ankyrin-repeat protein with redox-regulated nuclear translocation, controls basal resistance downstream of SA and regulates PR-1 expression through interaction with the TGA sub-family of bZIP transcription factors (Dong, 2004). Since npr1-1 does not block PR-2 expression or resistance to virulent P.s.m. ES4326 in the snc1 npr1-1 double-mutant, snc1 activates NPR1-dependent and independent responses (Zhang et al., 2003a). Because mos4-1 completely suppressed the constitutive PR-2 expression of snc1 (Figure 4.2B), we surmised that MOS4 might be required for the NPR1-independent pathway. To test the epistatic relationship between MOS4 and NPR1, mos4-1 npr1-1 double and mos4-1 npr1-1 snc1 triple mutants were created. When challenged with virulent and avirulent pathogens, mos4-1 completely suppressed the enhanced resistance and morphology of snc1 npr1 in the mos4-1 npr1-1 snc1 triple-mutant, and supported at least a 10-fold higher titer of bacteria than mos4-1 snc1, suggesting that a defect in MOS4 blocks NPR1-independent pathways downstream of snc1 (Figure 4.6A). Furthermore, in the absence of the snc1 mutation, mos4-1 npr1 plants were more susceptible to a normally subclinical concentration of P.s.m. ES4326 than npr1-1 or mos4-1 alone, supporting 3- to 7-fold more bacteria 3 DPI (Figure 4.6B). The additive nature of the enhanced disease susceptibility of mos4-1 and npr1-1 shown by these data suggests that two separate pathways contributing to basal defense are compromised in the double mutant. In addition, mos4-1 npr1-1 was more susceptible to avirulent H.p. Emwa1 than either the npr1-1 or mos4-1 single mutants (Figure 4.6C), indicating that NPR1-independent signaling downstream of R-protein activation – in this case, RPP4 – is mediated by MOS4.

To date, a fairly exclusive group of NPR1-independent defense signaling components have been described – these include MOS2, a putative RNA-binding protein isolated earlier from the snc1 suppressor screen (Zhang et al., 2005b), and FMO1 (FLAVIN-DEPENDENT MONOXYGENASE 1), a positive regulator of EDS1 signaling that is independent of SA accumulation (Bartsch et al.,
Although the genetic relationship of FMO with NPR1 remains to be determined, it is likely to be NPR1-independent.

Because the mos4-1 single-mutant is able to accumulate SA in response to avirulent pathogen infiltration (Figure 4.7), MOS4 does not seem to contribute to SA accumulation caused by pathogen infection. The reason why mos4-1 snc1 has low SA is probably due to the SA-independent pathway having a positive amplification role in SA synthesis (Zhang et al., 2005b). A schematic model of MOS4 signaling in plant innate immunity is shown in Figure 4.8.

### 4.2.4 MOS4 is a nuclear protein

To determine the subcellular localization of MOS4, GFP was cloned in-frame at the C-terminus of MOS4 and transformed into mos4-1 plants. GFP fluorescence was detected exclusively in the nucleus of various cell-types in several independent MOS4-GFP transgenic lines, including guard cells (Figure 4.9A), epidermal cells, and root cells (data not shown), suggesting that MOS4 is a nuclear protein. When MOS4-GFP was transformed into mos4-1 snc1 plants and the transgenic progeny examined, out of 9 transgenic plants selected, all exhibited snc1 phenotypes, indicating that the expression of MOS4-GFP driven by its native promoter complements the mos4-1 mutation in the snc1 background (Figure 4.9B). In addition, MOS4-GFP complemented the enhanced disease susceptibility (EDS) phenotype of the mos4-1 single-mutant (Figure 4.9C). Although mos4-1 with MOS4-GFP is wild type-like, whereas mos4-1 snc1 with MOS4-GFP is snc1-like, we did not observe any difference in GFP localization or intensity of GFP fluorescence between the two genetic backgrounds (data not shown).

### 4.2.5 MOS4 interacts in yeast with AtCDC5, an atypical R2R3 Myb transcription factor with homology to human CDC5L

Analysis of the MOS4 amino acid sequence predicted that the C terminal half had the potential to form an amphipathic α-helix, a motif known to mediate protein-protein interactions through parallel two-stranded coiled-coil structure. We thus employed a GAL4-based yeast two-hybrid screen to search for proteins that are capable of interacting with MOS4. One of the interacting proteins found in the screen was AtCDC5 (Figure 4.10A), an 844 amino-acid polypeptide with
similarity to R2R3-type MYB transcription factors that exhibits in vitro sequence-specific DNA-binding activity (Hirayama and Shinozaki, 1996). To confirm in planta interaction of MOS4 and AtCDC5, HA or GFP was cloned in frame at the C-terminus of MOS4 driven by its own promoter, and transformed into mos4-1. The fusion clone is able to fully complement mos4-related phenotypes (data not shown), indicating that the fusion proteins function the same as the wild type. Immunoprecipitation using GFP-column followed by western blot analysis with AtCDC5 antibody showed that AtCDC5 co-immunoprecipitated with MOS4-GFP (Figure 4.11A). Co-IP with HA-column yielded similar results (data not shown).

The human homologs of MOS4 and AtCDC5, BCAS2 (also known as hSPF27) and hCDC5L, respectively, have both been isolated as components of the multi-protein spliceosome complex by proteomic analysis (Neubauer et al., 1998; Zhou et al., 2002). Using different purification strategies and mass spectrometry, hCDC5L co-purified with at least 5 non-snRNA proteins in a discrete complex, one of which was BCAS2, the MOS4 homolog (Ajuh et al., 2000). CDC5L orthologs in S. pombe and S. cerevisiae, Cdc5p and Cef1p respectively, also co-purified with a core complex nearly identical in composition to that in human cells (Ohi and Gould, 2002). The complex containing Cef1p was termed the PRP Nineteen Complex, or NTC, based on the identity of another complex member, Prp19p (Cwf8p in S. pombe) (Tsai et al., 1999). The hNTC has also been termed the NMP200 Complex and PSO4 Complex (Ohi and Gould, 2002). In vitro immunodepletion of the orthologous human NTC from HeLa nuclear extracts inhibits formation of pre-mRNA splicing products (Ajuh et al., 2000). Taken together, the conserved interaction between MOS4 and AtCDC5 homologs in yeast, Arabidopsis, and human suggests an evolutionarily conserved function of MOS4 through its interaction with AtCDC5.

4.2.6 AtCDC5 interacts with PRL1

A third component of the human NTC is PLRG1, a WD40 repeat protein shown to bind directly to CDC5L (Ajuh et al., 2001). An Arabidopsis homolog of PLRG1, the previously described Pleiotropic Regulatory Locus 1 (PRL1, At4g15900), encodes a conserved nuclear WD-protein that functions as a pleiotropic regulator of glucose and hormone responses (Nemeth et al., 1998). Using yeast-two-hybrid assay, we showed that AtCDC5 interacted directly with PRL1
(Figure 4.10B). In addition, PRL1-GFP was localized to the nucleus (Figure 4.12). To test for \textit{in planta} interaction, GFP or HA tag was cloned in frame at the C-terminus of PRL1, and transformed into \textit{prl1}-1. The fusion clone fully complemented all the \textit{prl1}-phenotypes (data not shown). Using IP-Western procedure similar to what was done with MOS4, it is concluded that AtCDC5 co-immunoprecipitated with PRL1-GFP (Figure 4.11B). Co-IP with HA-column yielded similar results (data not shown).

4.2.7 A mutation in \textit{AtCDC5} suppresses \textit{snc1}

To determine if AtCDC5 is also involved in \textit{snc1}-dependent signaling, a T-DNA insertion line (SAIL\_207\_F03) in the 5'-UTR of \textit{AtCDC5} (At1g09770) was obtained from the Arabidopsis Biological Resource Center (ABRC). The morphological phenotypes of \textit{Atcdc5-1} were very similar to \textit{mos4-1}; like \textit{mos4-1}, \textit{Atcdc5-1} flowered late, but was slightly smaller than \textit{mos4-1}, and exhibited complete male sterility (Figure 4.3). All of these phenotypes were complemented by a genomic clone of wild type \textit{AtCDC5} (data not shown). The mutation in \textit{AtCDC5} partially suppressed \textit{snc1} morphology, as the \textit{Atcdc5-1 snc1} double mutant plants grew bigger and had less curly leaves than in \textit{snc1} (Figure 4.13A). \textit{Atcdc5-1 snc1} plants also partially suppressed \textit{PR} gene expression (Figure 4.13B) and supported higher bacterial growth than \textit{snc1} (Figure 4.13C). Thus, the mutation in \textit{AtCDC5} suppressed enhanced disease resistance responses in \textit{snc1} similar to mutations in \textit{MOS4}. Subsequently, a second T-DNA insertion line (GABI\_278B09) in an exon of \textit{AtCDC5} (\textit{Atcdc5-2}) was obtained and determined to be allelic to \textit{Atcdc5-1}. \textit{Atcdc5-2} also suppressed \textit{snc1} (data not shown).

4.2.8 \textit{MOS4}, \textit{AtCDC5}, and \textit{PRL1} are all required for basal defense

Three T-DNA insertions in \textit{PRL1} were obtained from the ABRC. The morphological phenotypes of all three \textit{prl1} alleles were identical to each other and similar to those of \textit{mos4-1} and \textit{Atcdc5-1}, except with no flowering time defect (Figure 4.3). In addition, a genomic clone of wild type PRL1 was able to fully complement \textit{prl1} phenotypes in 20 out of 22 transgenic T1 plants (data not shown). Because \textit{PRL1} and \textit{SNC1} are very closely linked on chromosome 4, we were unsuccessful in our effort to identify the \textit{prl1 snc1} double mutant (data not shown).
Since homologs of MOS4, AtCDC5, and PRL1 all belong to the NTC in fission yeast and human, an immediate question is whether or not these three genes affect innate immunity in a similar manner. We tested the mos4, Atcdc5, and prl1 single mutants for enhanced disease susceptibility to a subclinical concentration of the virulent bacterial pathogen P.s.m. ES4326. At 3 DPI, disease symptoms were evident in infected leaves of all three mutants and absent in the wild type Col-0 control (data not shown). Furthermore, mos4 alleles and Atcdc5-1 consistently supported about 25-50 fold more bacterial growth than wild type plants, while prl1 alleles supported an even higher titer of bacteria (at least 10-fold further) in the infected leaves, indicating that MOS4, AtCDC5, and PRL1 are all required for basal resistance to P.s.m. ES4326 (Figure 4.14A). Similarly, mos4, Atcdc5 and prl mutants exhibited enhanced susceptibility to other virulent pathogens such as H.p. Noco2 (data not shown) and Pseudomonas syringae pv tomato (P.s.t.) DC3000 (Figure 4.15A).

Since alleles of prl1 are consistently more susceptible than alleles of mos4 or Atcdc5 (Figure 4.14A), we crossed mos4-1 with Atcdc5-1 or prl1-1 to screen for respective double mutants to see if each putative complex member contributes quantitatively to plant immunity. We were unable to identify a mos4-1 Atcdc5-1 or mos4-1 prl1-1 double mutant plant in the F2 generation. After selecting several F2 plants homozygous for one mutation and heterozygous for the other by PCR, for both mutant combinations, we were unable to find double-homozygous plants in several F3 populations of over 100 plants. This strongly suggests that mos4-1 Atcdc5-1 and mos4-1 prl1-1 double homozygous plants are probably lethal.

To assay whether these mutants affected PR gene expression during pathogen infection, mos4-1, Atcdc5-1, prl1-1 and npr1-1 plants were inoculated with a clinical dose of virulent P.s.m. ES4326 and the RNA was extracted after 24 hours. Real-time RT-PCR revealed that PR-1 gene expression was absent in npr1-1 and much lower in the other mutants tested, compared to wild type (Figure 4.15B).

Is enhanced susceptibility to P.s.m ES4326 in these mutants caused by a deficiency in SA accumulation? To answer this question, we inoculated quarter-leaves of Col-0, mos4-1, Atcdc5-1 and prl1-1 with a high dose of avirulent P.s.m. ES426 carrying AvrB and collected the remaining leaf tissue 24 hours later, at which time macroscopic cell death was observed. Total
SA was extracted and measured in pathogen- versus mock-inoculated leaves. As seen in Figure 4.13, SA was able to accumulate in all three mutants as in wild type in response to an avirulent pathogen, indicating that MOS4 and its interacting partners do not contribute to SA accumulation after infection.

To test if defects in MOS4, AtCDC5 or PRL1 affected non-host resistance, we inoculated plants with a high dose of P.s.t. DC3000 lacking HrpA, which encodes a pilus subunit required for the secretion of effectors into the host cytoplasm, and assayed for bacterial growth (Figure 4.16A). At 4-DPI, there was insignificant growth of P.s.t. DC3000 hrpA- in wild type and all three mutant plants, suggesting that no defect in non-host resistance exists as in mutants such as nho1 (Lu et al., 2001). We also tested the response of these mutants to a model pathogen-associated molecular pattern (PAMP), flg22, the peptide epitope of the bacterial flagellin protein that is recognized by the receptor-like kinase FLS2 (Felix et al., 1999; Gomez-Gomez et al., 1999). The defense responses mediated by FLS2 are associated with a measurable inhibition of seedling growth upon prolonged treatment with flg22 (Gomez-Gomez et al., 1999). Ten days after application of 20µM flg22, seedling growth inhibition was similar in wild type Col-0 and in mos4-1, Atcdc5-1 and prl1-1 seedlings compared to growth in media alone (Figure 4.16B, C). In addition, injection of flg22 peptide followed by inoculation with P.s.t. DC3000 24 hours later resulted in enhanced resistance, and consequently lower bacterial growth at 2-DPI, versus mock treatment in wild type and in all three mutants (Figure 4.16D). The Arabidopsis ecotype Wassilewskija-0 (WS-0), which has a natural defect in FLS2, and the fls2 mutant in Col-0, were not responsive to flg22 in either experiment, as expected. This data suggests that response to flg22 is not compromised in mos4-1, Atcdc5-1 or prl1-1, although we cannot exclude the possibility that these mutants are compromised in an FLS2-independent response to other PAMPs.

4.2.9 MOS4, AtCDC5, and PRL1 are all essential for R-protein-mediated resistance

To test if the mos4, Atcdc5, and prl1 mutations affect resistance mediated by R-proteins other than SNC1, we tested several Avr-R combinations that result in incompatible interactions in Arabidopsis. At least two distinct disease resistance pathways have been described for
Arabidopsis, governed by R-protein structural type rather than pathogen class (Aarts et al., 1998). The R-genes *RPM1* (CC-NB-LRR type) and *RPP4* (TIR-NB-LRR type) confer resistance to bacterial and oomycete pathogens expressing specific cognate avirulence gene products (Grant et al., 1995; van der Biezen et al., 2002). *mos4-1, Atcdc5-1, and prl1-1* were more susceptible than wild type to both avirulent *P.s.m. ES4326 carrying AvrB* and avirulent *H.p. Emwa1*, based on higher growth of pathogens in both mutants (Figure 4.14B, C). Unlike EDS1 and NDR1, which are specifically required for signaling of TIR-NB-LRR and CC-NB-LRR-type R-proteins, respectively, MOS4, AtCDC5 and PRL1 are required for resistance mediated by both types of R-proteins. Other proteins required for both TIR and CC-NB-LRR R-proteins include SGT1b, RAR1 and HSP90, which seem to function in stabilization of the R-protein complex rather than in downstream signaling (Austin et al., 2002; Azevedo et al., 2002; Hubert et al., 2003).

Because mutants in *MOS4, AtCDC5* and *PRL1* exhibit very similar phenotypes and defects in basal and specific defense responses, MOS4 and PRL1 directly interact with AtCDC5, and homologs of all three proteins belong to the NTC in humans and yeast, we speculate that these three proteins must function together in a multi-protein complex orthologous to the human NTC to regulate innate immunity in Arabidopsis. We term this multi-protein assembly the MOS4-Associated Complex (MAC).

### 4.3 Discussion

#### 4.3.1 The Arabidopsis MAC and RNA splicing

It is reasonable to propose that the Arabidopsis MAC is orthologous to the NTC, which has been indicated to be essential for splicing (Ajuh et al., 2001), based on studies in human and yeast. Since the majority of plant genes are interrupted by non-coding introns, a defect in general splicing of mRNA would likely result in catastrophic phenotypes or death. For example, several general splicing mutants are embryo lethal, based on data in the SeedGenes database (Tzafrir et al., 2003). Since mutations in *MOS4, PRL1, or AtCDC5* alone only have minor effects on the plants' normal growth and development, it is unlikely that the protein products of these genes are essential components of splicing. However, since *mos4-1 Atcdc5-1* and *mos4-1 prl1-1*
double homozygous mutants seem to be lethal, MAC as a whole could be required for an essential process, such as spliceosome assembly, as suggested from studies in yeast and humans (Tarn et al., 1993; Grillari et al., 2005)

In some instances, the splicing machinery can process the same pre-mRNA differently by selectively joining different exons or retaining specific introns. This alternative splicing potentially leads to the generation of structurally and/or functionally distinct proteins. Several TIR-NB-LRR-class R-genes, along with TLRs in animals, are alternatively spliced, although the functional relevance is unknown (Jordan et al., 2002). For example, the stoichiometry of two tobacco N gene-derived splice variants undergoes a 50-fold change in their relative molar ratios upon pathogen challenge (Dinesh-Kumar and Baker, 2000). The Arabidopsis R-gene RPS4 produces alternative transcripts with truncated open reading frames. The dominant alternative RPS4 transcripts are generated by retention of intron 3 or introns 2 and 3, which contain in-frame stop codons and lie downstream of the NB-encoding exon – the combined presence of regular and alternative RPS4 transcripts is necessary for RPS4 function in resistance (Zhang and Gassmann, 2003).

Although the functional relevance of alternative splicing in innate immunity is unknown, the association of the NTC with the spliceosome led us to hypothesize that MOS4, AtCDC5, and PRL1 may be involved in alternative splicing. We tested several alternatively spliced genes, including RPS4, AtSRp30, AtSRp34, U1snRNP, ANP1, and POT1, for relative levels of transcript variants of each in the mos4-1, Atcdc5-1 and prl1-1 mutants by real-time RT-PCR. In all cases, there were no significant differences in transcript variant levels between wild type and the mos4-1, Atcdc5, and prl1 mutants (Figure 4.17). Our data suggests that MOS4, AtCDC5 and PRL1, while possibly associated with a spliceosome complex, are individually not involved in general or alternative RNA splicing. We cannot discount the possibility that the intact MAC complex is required for spliceosome assembly as the NTC is in yeast, or that the individual members regulate the splicing of an unknown RNA species.
4.3.2 What is the function of the MAC and NTC in plants and animals

In human cells, in addition to being associated physically with the spliceosome, the MOS4 ortholog BCAS2 and hCDC5L have been reported to have roles in cancer malignancy, apoptosis, estrogen receptor-mediated transcription and DNA repair (Lee et al., 2002; Maass et al., 2002; Mahajan and Mitchell, 2003; Qi et al., 2005; Zhang et al., 2005a). The detailed biochemical and biological function of BCAS2, hCDC5L, and other members of the human NTC remains elusive in part because of the challenge of genetic analysis at a whole-organism level. Our analysis of the mos4, Atcdc5 and prl1 mutants highlights the advantage of using Arabidopsis genetics to dissect biological processes unambiguously and provides an exceptional opportunity to define the function of the NTC. The NTC is an evolutionarily conserved protein complex that interacts in an as-yet undetermined way with the spliceosome. Future yeast-2-hybrid or proteomic efforts using MOS4 epitope-tagged lines will enable us to identify more Arabidopsis components of the complex and analyze their functions using reverse genetic approaches. It is important to note that, although we show that MOS4, AtCDC5 and PRL1 are required for innate immunity, there is as of yet no direct evidence that their interactions are required for innate immunity.

Defects of MOS4, PRL1, and AtCDC5 are confined to innate immunity, flowering time, and fertility, indicating that targets of MAC/AtNTC regulation are limited. Members of the human NTC include PSO4, an E3 ubiquitin ligase, hCDC5L, a transcription factor, and several proteins with protein-interaction domains (e.g. BCAS2 and PLRG1), hinting to a complex regulatory module (Loscher et al., 2005; Zhang et al., 2005a). Since AtCDC5 has sequence-specific DNA binding capability (Hirayama and Shinozaki, 1996), the simplest and most likely explanation is that the MAC fulfills its role through regulating transcription of downstream target genes. The spliceosome may assist the NTC in fulfilling this regulatory function. Alternatively, the NTC may merely co-localize with the spliceosome based on its association with the nuclear structure. A third possibility could be that the MAC contributes to regulatory processes mediated by microRNA (miRNA) and small interfering RNA (siRNA), both of which have been shown recently to be involved in the regulation of development and innate immunity (Katiyar-Agarwal et al., 2006; Navarro et al., 2006). It is interesting that endogenous small RNAs are also involved in the regulation of developmental processes, such as flowering time via FLC (Swiezewski et al., 2007).
Future detailed genetic and biochemical analysis using Arabidopsis *mac* mutants will help reveal these underlying mechanisms. It will also be very interesting to test whether the NTC functions in the regulation of animal innate immunity.

### 4.4 Materials and Methods

#### 4.4.1 Mutant screen and characterization of *mos4*

All plants were grown under a 16h light/8h dark regime. The *mos* (*modifiers of snc1*) suppressor screen and GUS staining protocol have been described previously (Zhang and Li, 2005a).

RNA used for gene expression analysis was extracted from 3-week-old seedlings grown on Murashige and Skoog (MS) medium using the Totally RNA kit (Ambion). Reverse transcription (RT) was carried out using the RT-for-PCR kit (Clontech). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (Qiagen). The primers used for amplification of *Actin1*, *PR-1*, and *PR-2* were described previously (Zhang et al., 2003a).

SA was extracted and measured from 4-week-old soil-grown leaves using a previously described procedure (Li et al., 1999), proportionally scaled down using approximately 100 mg tissue per sample. 4-week-old soil-grown Arabidopsis plants were infected with *P.s.m.* ES4326. Leaf discs of 0.32 cm$^2$ were taken with a standard paper hole-punch. Infection of plants with *H.p.* Noco2 and *H.p.* Emwa1 was performed on 2-week-old seedlings. These procedures were as described (Li et al., 1999).

#### 4.4.2 *Hyaloperonospora parasitica* Disease Rating

Two-week-old seedlings were sprayed with a virulent *H.p.* Noco2 conidiospore suspension with a concentration of $5 \times 10^4$ spores per ml of water. Seven days after infection, the numbers of conidiospores per infected leaf were counted and disease rated as follows: 0, no conidiophores on any leaf; 1, no more than 5 conidiophores per infected leaf; 2, 6-20 conidiophores on a few
of the infected leaves; 3, 6-20 conidiophores on most of the infected leaves; 4, 5 or more conidiophores on all infected leaves; 5, 20 or more conidiophores on all infected leaves. For avirulent \( H.p. \) Emwa1, two-week-old plants were infected with a suspension of \( 1 \times 10^5 \) spores per ml of water. After 7 days, 5 leaves per treatment were collected, weighed, and vortexed in 1mL water. Conidiophores were counted on a hemocytometer.

### 4.4.3 Map-Based Cloning of \( mos4 \)

To map \( mos4-1, mos4-1 \ snc1 \) in the Columbia (Col-0) ecotype (containing \( pBGL2-GUS \)) was crossed with Landsberg erecta (Ler) ecotype plants in which the \( snc1 \) mutation was previously introgressed (Zhang and Li, 2005a). \( F_1 \) plants were selfed and 48 plants from the \( F_2 \) progeny lacking \( snc1 \) morphology, and therefore homozygous at the \( mos4-1 \) locus, were selected for linkage analysis. Linkage was found at the top arm of chromosome 3. A further 32 \( F_2 \) plants with \( snc1 \) morphology, and thus heterozygous or wild type at \( MOS4 \), were selected for crude mapping. At the telomeric marker MIE1, three recombinants were identified, whereas at the centromeric marker MYM9, one different recombinant was found. This indicated that the \( mos4-1 \) locus was flanked by MIE1 and MYM9, a total physical distance of about 3.5 Mb (Figure 4.4A).

To fine-map \( mos4-1 \), 925 plants were randomly selected from the \( F_3 \) progeny of \( F_2 \) plants genotyped to be heterozygous for both flanking markers, MIE1 and MYM9, and homozygous for the \( pBGL2-GUS \) reporter gene. A total of 36 recombinants were obtained and their phenotypes were determined by segregation analysis in the \( F_4 \) progeny by GUS staining of plants grown on MS and \( snc1 \) morphological phenotype on soil. Additional polymorphic markers were used to narrow the flanking interval between the remaining 5 recombinants at MIE1 and 31 at MYM9 (Figure 4.4A). Mapping markers were designed based on ecotype-distinct insertions, deletions, and single nucleotide polymorphisms (SNPs) found in the Monsanto Arabidopsis polymorphism and Landsberg sequence collections (Jander et al., 2002). Marker primers used include MIE1, 5’-CTAAGTTCTCCACCATCGT-3’ and 5’-CAAGGACATCTAGGCCAGAG-3’ (449 bp product in Col-0, 135 bp shorter in Ler); MYM9, 5’-GGCTTAGGTGTAACGTAAG-3’ and 5’-GTAACCTTCATCCCGG-3’ (288 bp product, 17 bp
longer in Ler); MEB5, 5'-GATGGTCAAGATGAGTTCG-3’ and 5’- TTGAAGAGTGCACGTTATCC-3’ (528 bp product, 49 bp longer in Ler); MYF24, 5’-AAACCTACCGACCAACTACC-3’ and 5’- TATACCACTGCGTGCAAC-3’ (576 bp product, 25 bp shorter in Ler); MIE15-2, 5’-TCCGTCAAACCTATCC-3’ and 5’-TCTTCATCGGTAGTGATGGTG-3’ (454 bp fragment digested by MseI in Col only); MRC8-7, 5’-CTCTACACTGCGTGTGCAAC-3’ and 5’- AAGTTGGAATGCTCTCTGTCG-3’ (817 bp fragment digested by HincII in Ler only). The fragment with the apparent insertion was amplified with the primers MRC8-51725F (5’- TTGGGTAGTAGAGGAGATGG-3’) and MRC8-53900R (5’- GATCTGTGTCTCAAGCATGG-3’); the sequencing primer that revealed the defect in this fragment was MRC8-53013F (5’- CACCTGCCAATAAGAGGAATG-3’). The insertion in mos4-1 was confirmed by PCR, using MRB17-43260R (5’-GTAGTGTGCACAGTTCTTC-3’) and MRB17-42641F (5’- CCGTTGATTGCTACTGTGAC-3’) paired with MRC8 flanking primers.

The last recombination events were located between markers MIE15-2 and MRC8-7, a 69.2 kb region (Figure 4.4B, C). Next, overlapping ~2kb regions of genomic DNA in this interval were amplified by PCR to look for mutations potentially associated with mos4-1. One fragment, between bp 51725 and 53900 of P1 clone MRC8, was significantly larger in mos4-1 snc1 than in the Col-0 control. Sequencing of this PCR product revealed a 2.2 kb insertion in the 5th exon of At3g18165 (accession number NM_112699; Figure 4.4D). The insertion was determined to be genomic DNA from chromosome 5, corresponding to bp 42454-44648 of the P1 clone MRB17, a region predicted to be non-coding. To confirm the existence of the 2.2kb insertion in mos4-1 snc1, primers were designed in an outward orientation from the putative insertion (based on MRB17 sequence), and paired with MRC8 flanking primers. PCR products of the predicted size were amplified in mos4-1 snc1 and not in the wild type control (data not shown).

Two strategies were used to confirm that MOS4 is At3g18165. First, full length cDNA clone U11032 was obtained from the ABRC (Alonso et al., 2003; Yamada et al., 2003). The cDNA was cloned into a modified pBl1.4 vector with cauliflower mosaic virus (CaMV) 35S promoter (Mindrinos et al., 1994), and transformed into mos4-1 snc1 plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected for antibiotic resistance on MS plates containing 75 µg/ml kanamycin, and analyzed for complementation of the mutant. Plants homozygous for the T-DNA insertion in mos4-2 and mos4-3 were identified by PCR using the
primers MRC8-52199F (5’-GGTCTCTCCATCTTTCTCAG-3’) and MRC8-52913R (5’-CATTCCCTTTATTGGCAGTG-3’). Among 18 transgenic plants obtained, 14 displayed snc1 morphology (Figure 4.4E). Expression of the pBGL2-GUS reporter gene (data not shown), and resistance to H.p. Noco2 were restored in the homozygous progeny of transgenic plants with snc1 morphology (Figure 4.4F), demonstrating complementation of mos4-1 by At3g18165.

Secondly, two additional mutant alleles of At3g18165 were obtained from the ABRC (Alonso et al., 2003), mos4-2 (Salk_019535) and mos4-3 (Salk_141393), each containing T-DNA insertions in the second intron of At3g18165 (Figure 4.4D). mos4-2 and mos4-3 were indistinguishable from mos4-1, with the exception that they were completely male sterile and had to be propagated as heterozygotes. To test whether these insertion mutants were allelic to mos4-1, mos4-2 and mos4-3 were crossed with mos4-1 snc1. Out of a total of 36 randomly chosen F2 progeny from each of these crosses, none had snc1 morphology. Additionally, mos4-2 snc1 and mos4-3 snc1 were identified in the corresponding F2 progeny, and in both cases, the double mutants were indistinguishable from mos4-1 snc1 (Figure 4.4G). Therefore, both T-DNA insertions in At3g18165 suppressed snc1 phenotypes, and were allelic to mos4-1.

Amino acid alignments were generated using ClustalW (http://www.ebi.ac.uk/clustalw/) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The coiled-coil domain in MOS4 was predicted with COILS 2.2 (http://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991).

### 4.4.4 Isolation of the mos4-1 single mutant

To identify the mos4-1 single mutant, mos4-1 snc1 was crossed with wild type Col-0 plants containing pBGL2-GUS, and F2 plants were genotyped by PCR. Primers used to detect the ~2.2 kb insertion in mos4-1 were MRB17-42641F and MRC8-53900R (438 bp in mos4-1, a PCR fragment that is not amplified in wild type. SNC1 was amplified using the primers 5’-GTGGAGTTCCATCTGAACATC-3’ and 5’-CCCATTGATTGCTGGAAG-3’. To differentiate between WT and snc1, the fragment was digested with XbaI – the mutation in snc1 alters the restriction site and therefore only wild type fragments are cleaved. Lines homozygous for
mos4-1 without the snc1 mutation were designated as mos4-1 single mutants and were used for further analysis.

4.4.5 Obtaining the prl1 and additional Atcdc5 mutants

Three mutant alleles of At4g15900 (PRL1) were obtained from the ABRC (Alonso et al., 2003); prl1-1 (Salk_008464) and prl1-2 (Salk_039427) contain T-DNA insertions in exons while prl1-3 (Salk_096289), contains a T-DNA insertion in an intron of PRL1. prl1-2 and prl1-3 were indistinguishable from prl1-1. The second T-DNA insertion line (GABI_278B09) in the third exon of AtCDC5 (Atcdc5-2) was obtained from the Max Planck Institute for Plant Breeding Research, Cologne, Germany (Rosso et al., 2003). Sequences of PCR primers used to confirm insertions will be supplied upon request.

4.4.6 MOS4-GFP Cloning

A genomic fragment spanning the full-length MOS4 gene (gMOS4), without the stop codon but including a 1.5 kb sequence upstream of the translation initiation codon, was amplified from Col-0 DNA using Phusion Taq (Finnzyme) and cloned into pENTR using the Gateway® pENTR/D-TOPO Cloning Kit (Invitrogen). The primers used to amplify the genomic fragment (with CACC incorporated at the 5’ end of the forward primer to facilitate directional TOPO cloning) were 5’-CACCACACTGCTAGAGGTCTTGG-3’, and 5’-TTGCATTTGAAGTGGCTCGAC-3’. Similar techniques were used to clone PRL1-GFP.

4.4.7 Subcellular localization of MOS4

The Gateway® LR Clonase enzyme (Invitrogen) was used to catalyze the recombination between pENTR-gMOS4 and a destination vector which is a modified binary vector with TOPO destination recombination sites with GFP fused in-frame to the C-terminus. The MOS4-GFP expression clone was sequenced to confirm in-frame fusion and a lack of PCR errors. Transgenic plants were selected on MS medium containing 75 μg/ml kanamycin. Guard cells of transgenic seedlings were examined for GFP fluorescence as described previously (Zhang et al., 2005b).
4.4.8 Yeast-2-Hybrid Cloning

The full-length cDNA of MOS4 (cMOS4 – stock number U11032) in pUNI-51, obtained from the ABRC and verified by sequencing, was used as template for amplification by PCR using Pfx polymerase (Invitrogen). Restriction enzyme recognition sites were incorporated at the 5' and 3' ends of the primers to facilitate directional cloning of the cMOS4 fragment into the bait (GAL4-DB) plasmid pBI-880 (Kohalmi et al., 1998). cMOS4 was ligated as a $Xho$I-$Xba$I fragment into pBI-880 cut with $Sal$I and $Xba$I to create GAL4(DB)-cMOS4.

4.4.9 Yeast Two-Hybrid Assay

We used the GAL4-based Y2H system described previously (Kohalmi et al., 1998). Briefly, a GAL4(DB)-cMOS4 construct was introduced into Y2H strain YPB2 and used to screen an Arabidopsis cDNA expression library (kindly provided by William Crosby) encoding Arabidopsis proteins as C-terminal fusions to the GAL4 transcription activation domain (TA). Putative MOS4-interacting proteins were selected on the basis of histidine prototrophy and further screened on the basis of $lacZ$ expression.

4.4.10 Confirmation of MOS4-interacting proteins found by Y2H screen

Yeast plasmid DNA was extracted from colonies found in the screen and amplified by PCR with prey plasmid- (pBI-881) specific primers. Half of the reaction was digested with $Nla$III to identify unique clones, and the remainder was used for sequencing. GAL4-interacting and self-activating proteins were eliminated by reintroducing prey clones into YPB2 containing pGAL4(DB)-MOS4 or pBI-880 alone (GAL4(DB) without a C-terminal fusion).

4.4.11 Preparation of Nuclear Extracts

Aerial Arabidopsis tissues (30g) of 20-day old transgenic plants expressing MOS4-GFP or PRL1-GFP were ground in liquid nitrogen and homogenized in 1.5 volume of lysis buffer (20 mM Tris-HCl, pH 7.4, 25% glycerol, 20 mM KCl, 2 mM EDTA, 2.5 mM MgCl$_2$, 250 mM sucrose) at 4ºC.
Lysates were filtered through 100 μm, 70μm and 30μm filters in sequence, and subsequently centrifuged at 1000g for 10 min to pellet the nuclei at 4°C. The nuclear pellet was washed three times in nuclei re-suspension buffer (20 mM Tris-HCl, 25% glycerol, 2.5 mM MgCl₂, with 0.5% Triton X-100). The nuclear fraction (about 5×10⁸ nuclei) was re-suspended in 1.0 ml of high-salt buffer (50 mM Tris-HCl, pH 7.4, 400 mM KCl, 0.5 Triton X-100, 1.0 mM DTT and protease inhibitors), and centrifuged at 35,000g for 10 min to lyse nuclei. The high salt buffer of the nuclear extraction was adjusted to low salt buffer (50 mM Tris-HCl, pH 7.4, 100 mM KCl, 0.5 Triton X-100, 1.0 mM DTT and protease inhibitors) before immunoprecipitation experiments.

4.4.12 In planta protein interaction assay

Nuclear extracts of transgenic mos4-1 complemented with gMOS4-GFP or gMOS4-HA and prl1-1 complemented with gPRL1-GFP or gPRL1-HA were mixed with 50 µL Anti-GFP or Anti-HA MicroBeads (Miltenyi Biotec) to magnetically label the epitope-tagged target protein. After an overnight incubation on ice, the MicroBead-bound target protein was magnetically precipitated on columns according the manufacturer’s instructions (μMACs, Miltenyi Biotec) for subsequent immunoblotting analysis. Immunoprecipitated proteins eluted from the column were detected respectively with anti-GFP or anti-HA antibody, and the co-immunoprecipitated endogenous AtCDC5 protein was detected by a polyclonal antibody raised against the N-terminus 144 amino acid fragment of AtCDC5.
Figure 4.1: Suppression of snc1-associated morphology, SA accumulation and pathogen resistance by mos4-1

(A) Morphology of wild type Columbia (Col-0), snc1 and mos4-1 snc1. Image is of representative plants. (B) Free and Total SA in leaves of Col-0, snc1 and mos4-1 snc1 plants. (C) Growth of Pseudomonas syringae pv maculicola (P.s.m.) ES4326 at 0- and 3-DPI with OD600 = 0.001. The log-transformed values presented are averages of four replicates ± SD. (D) Growth of Hyaloperonospora parasitica (H.p.) Noco2. Data is presented for 20 plants of each genotype. The experiments were repeated at least twice with similar results.
Figure 4.2: Suppression of snc1-induced constitutive PR gene expression by mos4-1

(A) pBGL2-GUS reporter gene expression in representative 3-week-old seedlings grown on MS medium. Seedlings were stained for GUS activity and cleared as previously described (Zhang and Li 2005). (B) PR-2 (BGL2) and PR-1 gene expression in Col-0, snc1 and mos4-1 snc1. RNA was extracted from 3-week-old plants grown on MS medium and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real-time PCR using an Actin 1 probe. PR-1, PR-2 and Actin 1 were amplified by 30 cycles of PCR using equal amounts of total cDNA. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The results reported are representative of several experiments.
Figure 4.3: Fertility and flowering time phenotypes of mos4-1, prl1-1 and Atcdc5-1

(A) Representative mature silique morphology in Col-0, mos4-1, prl1-1 and Atcdc5-1. (B) Fertility was assessed in mos4-1, prl1-1 and Atcdc5-1 plants by counting the number of seeds per silique compared to Col-0. Five siliques were chosen at random from the middle section of the primary bolt and the total number of seeds was determined. The average number of seeds per silique from five individual plants is shown for each genotype, ± SD. Statistical significance was determined by student’s t-test (** P<0.0001; * P<0.01). (C) Flowering time was assessed in mos4-1, prl1-1 and Atcdc5-1 plants by counting the number of rosette leaves when the primary bolt was 6-10 cm, as described (Weigel and Glazebrook 2002). Plants were germinated on soil and then transplanted to individual (14.52 cm³) pots and grown in a long-day photoperiod (14 h light/10 h dark) at 20-22°C. Five to ten plants of each genotype were scored. This experiment was repeated four times and under a short-day photoperiod (8 h light/ 16 h dark) with similar results (data not shown). Statistical significance was determined by student’s t-test.
Figure 4.3: Fertility and flowering time phenotypes of *mos4-1*, *prl1-1* and *Atcdc5-1*
Figure 4.4: Map-based cloning of MOS4

(A) Crude 10-Megabase (Mb) and 3.5-Mb maps of the mos4-1 locus on chromosome 4. Left is telomeric, right is centromeric. *, markers bordering fine map in (B). Relative positions of the markers used for mapping are indicated, as are the number of recombinants remaining at each marker position. (B) Fine ~120-kilobase (kb) map of the mos4-1 locus showing final recombinants flanking a region of 69.2 kb. *, physical location of the mos4-1 locus. (C) Detail of 69.2 kb genomic region between final recombinants, with associated genes and P1 clones. *, gene with the defect as determined by sequencing. (D) Gene structure of At3g18165. The exons are indicated by shaded boxes, introns and UTRs by dashed lines. Locations of the mos4-1 defect and mos4-2 (Salk_019535) and mos4-3 (Salk_141393) T-DNA insertions are indicated. Base pair (bp) length is indicated by a size bar. (E) Expression of the full-length MOS4 cDNA driven by the 35S promoter complements the mos4-1 mutant and restores snc1 morphology in mos4-1 snc1. Plants shown are 4 weeks old, soil-grown, and mos4-1 snc1 containing 35S:MOS4 is a representative of 6 independent transgenic lines with similar phenotypes. (F) MOS4 restores constitutive disease resistance in mos4-1 snc1 plants. Procedures and H. p. Noco2 disease ratings were as described in Chapter 3. Two of the independent complemented lines are shown as representatives. (G) The mos4-3 T-DNA insertion line suppresses the snc1 morphological phenotype. Plants are 4 weeks old, soil-grown, and the genotypes were confirmed by PCR.

Figure 4.5: Amino acid alignment of MOS4 and MOS4 homologs from other eukaryotes

Amino acid sequences from Arabidopsis MOS4 (accession number NP_566599), Oryza sativa (rice; Os) P0453A06.28 (accession number BAD81257), human BCAS2 (accession number NP_005863), Danio rerio (zebra fish; Dr) ZGC101730 (accession number AAH85427), D. melanogaster (fruit fly; Dm) CG4980 (accession number NP_651596), C. elegans (nematode; Ce) T12A2.7 (accession number NP_498360), and S. pombe (fission yeast, Sp) Cwf7 (accession number AAF67744) are compared. Identical amino acids are shaded black, and amino acids with similar properties are shaded grey.
Figure 4.4: Map-based cloning of MOS4
Figure 4.5: Amino acid alignment of MOS4 and MOS4 homologs from other eukaryotes
Figure 4.6: mos4-1 affects the NPR1-independent signaling pathway

(A) Growth of P.s.m. ES4326 in Col-0, snc1 npr1-1, mos4-1 snc1, mos4-1 snc1 npr1-1, and npr1-1 after infiltration with OD$_{600}$ = 0.0001. (B) Growth of P.s.m. ES4326 in Col-0, mos4-1, npr1-1, and mos4-1 npr1-1 after infiltration with OD$_{600}$ = 0.00005. The log-transformed values presented are averages of four replicates ± SD. (C) Growth of avirulent H.p. Emwa1 on Col-0, mos4-1, npr1-1 and mos4-1 npr1-1 plants. Results are averages of six replicates ± SD. The log-transformed values presented are averages of four replicates ± SD. The experiments were repeated at least twice with similar results. a, b, c, d; Statistically significant ($P<0.0001$) difference as determined by unpaired student’s t-test.
Figure 4.7: MOS4, AtCDC5 and PRL1 are not upstream of pathogen-dependent SA synthesis

Total SA in leaves of Col-0, mos4-1, Atcdc5-1, and prl1-1 plants 24 hours after inoculation (quarter-leaves) with avirulent P.s.m. ES4326 expressing AvrB (OD$_{600}$ = 0.2) or MgCl$_2$ buffer alone (mock treatment). snc1 is included as a control, and was not inoculated. Results are averages of four replicates, ± SD. The experiment was repeated with similar results.
Figure 4.8: Simplified model of MOS4 signaling in plant innate immunity
Figure 4.9: Subcellular localization of MOS4

(A) Guard cells of transgenic mos4-1 plants expressing the genomic MOS4:GFP fusion protein were examined by confocal microscopy – a representative plant is shown. Top panel, DAPI staining of the nucleus; Center panel, MOS4-GFP fluorescence; Bottom panel, merged fluorescence channels and brightfield image. (B) Morphology of mos4-1 snc1 complemented with gMOS4-GFP. (C) Growth of P.s.m. ES4326 in Col-0, mos4-1, and two independent complementing lines of gMOS4-GFP transformed into mos4-1 mutants at 0- and 3-DPI, showing complementation of EDS in mos4-1 gMOS4-GFP. A subclinical concentration of P.s.m. (OD$_{600}$ = 0.0001) was infiltrated. The log-transformed values presented are averages of four replicates ± SD. The experiment was repeated twice with similar results.
Figure 4.10: MOS4 and AtCDC5 interact directly with PRL1 in yeast

(A) Yeast-2-hybrid assay of pBI880-MOS4 (MOS4-BAIT) and either pBI-881 alone or pBI881-AtCDC5 (AtCDC5-PREY) in YPB2. (B) Yeast-2-hybrid assay of pBI880-PRL1 (PRL1-BAIT) and either pBI-881 alone or pBI881-AtCDC5 (AtCDC5-PREY) in YPB2. -Leu-Trp, growth on media without leucine or tryptophan. Growth of yeast indicates presence of pBI-800 and pBI-881. -Leu-Trp-His, growth on media without leucine, tryptophan or histidine. Growth of yeast indicates interaction of fusion proteins. β-Gal, X-Gal filter-lift assay. Accumulation of blue precipitate indicates β-galactosidase activity due to protein interaction. EV = empty vector.
Figure 4.11: MOS4, AtCDC5 and PRL1 interact in planta

(A) AtCDC5 co-immunoprecipitates with MOS4-GFP in nuclear extracts from mos4-1 complemented by genomic MOS4 fused to GFP (gMOS4-GFP) transgenic plants. (B) AtCDC5 co-IPs with PRL1-GFP in nuclear extracts from prl1-1 complemented by gPRL1-GFP transgenic plants. The aerial plant tissue was harvested for nuclear fractionation, and nuclear extracts were subjected to immunoprecipitation (IP) as indicated. Equal amounts by number of nuclei in nuclear extracts (input) and flow-through supernatant after IP (unbound) were loaded for immunoblotting. The elution fraction for immunoblotting with anti-GFP or anti–HA antibody was ~20 times more concentrated than nuclear extract, that of anti-AtCDC5 antibody was ~ 40 times. Protein from MOS4–HA (A) and PRL1-HA (B) transgenic plants were used as negative controls in anti-GFP IPs.
Figure 4.12: Subcellular localization of PRL1

Fluorescence microscopy of guard cells of transgenic complemented prl1-1 plants expressing genomic PRL1-GFP driven by the endogenous PRL1 promoter. A representative plant is shown. Top panel, PRL1-GFP fluorescence. Middle panel, DAPI staining of the nucleus. Bottom panel, merged fluorescence channels and bright-field image.
Figure 4.13: Suppression of snc1 by Atcdc5-1

(A) Phenotypes of Col-0, snc1 and Atcdc5-1 snc1 plants showing suppression of snc1 morphology by Atcdc5-1. Image is of representative plants. (B) PR-2 (BGL2) and PR-1 gene expression in Col-0, snc1 and Atcdc5-1 snc1. RNA was extracted from 3-week-old plants grown on MS medium and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real-time PCR using an Actin 1 probe. PR-1, PR-2 and Actin 1 were amplified by 27 cycles of PCR using equal amounts of total cDNA. PCR products were analyzed by agarose gel electrophoresis and EtBR staining. The results reported are representative of several experiments. (C) Atcdc5-1 suppresses enhanced resistance of snc1 to virulent P.s.m. ES4326, after infiltration at OD$_{600}$ = 0.001. The experiment was repeated twice with similar results.
Figure 4.14: Characterization of mos4, Atcdc5 and prl1 in innate immunity

(A) Growth of P.s.m. ES4326 in Col-0, mos4-1, mos4-3, Atcdc5-1, prl1-1, prl1-2 and npr1-1 single-mutants after infiltration at OD$_{600}$ = 0.0001. (B) Growth of avirulent P.s.m. ES4326 expressing AvrB. The log-transformed values presented are averages of four replicates ± SD. (C) Growth of avirulent H.p. Emwa1 on Col-0, mos4-1, Atcdc5-1 and prl1-1 plants. Results are averages of six replicates ± SD. The experiments were repeated three times with similar results.
Figure 4.15: Further characterization of *mos4-1, Atcdc5-1* and *prl1-1* in innate immunity

(A) Growth of virulent *P.s.t.* DC3000 in Col-0, *mos4-1, Atcdc5-1, prl1-1*, and *npr1-1* single-mutants after infiltration at OD$_{600}$ = 0.0002. (B) *PR-1* expression after pathogen infection of single-mutants. Five-week-old plants (whole leaves) were inoculated with virulent *P.s.m.* ES4326 (OD$_{600}$. = 0.001) or buffer (mock); RNA was extracted from leaves immediately (0h) and 24h later and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real-time PCR using an *Actin 1* probe. *PR-1, PR-2* and *Actin 1* were amplified by 22 (*PR-1*) or 27 (*Actin*) cycles of PCR using equal amounts of total cDNA. The PCR products were analyzed by agarose gel electrophoresis and EtBr staining. The results reported are representative of several experiments. Because no *PR-1* expression is evident in any genotypes at 0h, this data is not shown.
Figure 4.16: Response of mos4-1, Atcdc5-1 and prl1-1 to hrpA- bacteria and the model PAMP flg22

(A) Growth of P.s.t. DC3000 hrpA- strain in Col-0, mos4-1, Atcdc5-1, and prl1-1 4-DPI at OD$_{600}$ = 0.002. (B, C) Seedlings grown for 7 days on MS agar plates were transferred to liquid MS medium alone or liquid MS medium supplied with 20µM flg22 peptide as previously described (Gomez-Gomez et al. 1999). The effect of treatment with the different peptides on seedling growth of Col-0, WS-0, mos4-1, Atcdc5-1 and prl1-1 was analyzed after 10 or 15 days by weighing (fresh weight) or photography. Bars show average and standard deviation of $n = 20$ seedlings per treatment, ± SD. The experiment was repeated twice with similar results. The WS-0 ecotype has a natural mutation in FLS2. (D) Col-0, fls2, mos4-1, Atcdc5-1 and prl1-1 plants were pretreated for 24 h by leaf infiltration with 20µM flg22 or water. Subsequently, leaves were infected with P.s.t. DC3000 (OD$_{600}$ = 0.00005), and bacterial growth was assessed at 2-DPI, as described before (Zipfel et al. 2004).
Figure 4.16: Response of *mos4-1, Atcdc5-1* and *prl1-1* to *hrpA*- bacteria and *flg22*
RNA was extracted from leaves and reverse transcribed to obtain total cDNA. The cDNA samples were normalized by real-time PCR using an Actin probe. PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Schematic diagrams of alternative splicing are shown at right. (A) Alternative splicing of RPS4 results in 5 transcript variants (TV) of different sizes, as determined by PCR using nested primers as previously described (Zhang and Gassmann 2003). In this case, TV1 retained both introns in the schematic diagram, whereas TV2 was composed of two transcript species of similar size lacking either the first intron (TV2A) or second intron (TV2B). TV3 corresponds to the regular transcript with both introns removed. An additional cryptic intron was spliced out in TV4. Primers were designed across intron/exon boundaries to specifically amplify TV3 and TV2B from diluted PCR products of the distal nested primers. As determined by real time PCR, and shown in the far right panel, there was no significant difference in relative abundance of either alternatively spliced RPS4 transcripts between Col-0, mos4-1, Atcdc5-1 and prl1-1. (B) Alternative splicing of U1-70K featuring intron retention (Savaldi-Goldstein et al. 2003). Primers were designed across intron/exon boundaries to specifically amplify the retained-intron transcript of U1-70K and relative abundance in Col-0 and MAC mutants was assayed by real time PCR; as determined by real time PCR, and shown in the far right panel, there was no significant difference in relative abundance of retained-intron U1-70K transcript (U1-R). (C) AtSRp34/SR1 showing 5’ and 3’ alternative splicing (Savaldi-Goldstein et al. 2003). (D) AtSRp30 showing 5’ and 3’ alternative splicing (Savaldi-Goldstein et al. 2003). (E) Actin control, 30 cycles. Alternative splicing of ANP1 and POT1 (Tani and Murata 2005) in the MAC mutants resulted in similar patterns, (data not shown).
Figure 4.17: Levels of transcript variants in several alternatively spliced genes in Col-0, mos4-1, Atcdc5-1 and prl1-1
4.5 Manuscript Acknowledgements

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4.6 References


5 Discussion and Future Perspectives

The *snc1* suppressor screen has yielded many novel and unexpected new details of resistance signalling, and many of the genes we discovered have homologs in animal innate immunity that may serve similar functions. Thus, the *MOS* genes represent initiation points for further research and new perspectives on innate immunity. At least three *MOS* genes encode components of the nuclear trafficking machinery; MOS3 and MOS7 are nucleoporins, whereas MOS6 is a mobile nuclear import receptor subunit (Zhang and Li, 2005; Palma et al., 2007; Y. Cheng and X. Li, unpublished data). The fact that so many suppressors of *snc1* encode proteins with a putative role in nucleocytoplasmic trafficking highlights the essentiality of this process in signalling downstream of *snc1*. Because defects in all of these also affect other aspects of disease resistance independent of *snc1*, nucleocytoplasmic regulation is clearly an important aspect of defence. MOS2 encodes a nuclear protein with putative RNA-binding activity that might control expression levels of regulatory genes during resistance responses (Zhang et al., 2005). Although there is no experimental evidence for interaction of MOS2 and components of the MOS4 Associated Complex (MAC), the assertion that homologs of MOS4-associated proteins in other kingdoms are related to RNA-binding proteins and the spliceosome machinery suggests a possible link. MOS5 and MOS8 are involved in post-translational protein modification – the targets of these remain to be elucidated (Goritschnig et al., 2007; S. Goritschnig and X. Li, unpublished data). Other *mos* mutants are currently being characterized, and should soon shed light into how these and other functions are related to resistance signalling.

It will be interesting to determine whether components of the MAC are regulated by nucleocytoplasmic partitioning. In an intriguing report, a yeast-2-hybrid screen for PRL1 (MAC2) interactors pulled out an importin-α, which just happens to be the importin subsequently identified as MOS6 (Nemeth et al., 1998). While *mos6* alleles do not share the pleiotropic phenotypes of *prl1* alleles, we suspect that MOS6 may have some functional redundancy with other α importins, as these proteins do in humans. To test this hypothesis, double and higher-order mutants can be created between *mos6* and SALK insertion mutants in the other seven α importins. One of these, identified as importin-α6, is the closest MOS6 homolog
phylogenetically (Figure 3.8). Unfortunately, we only recently acquired an exonic T-DNA insertion mutant (from GABI-KAT) in the gene encoding importin-α6. If the MAC is specifically transported by MOS6 and its closest homolog, we predict the double-mutant would resemble the MAC mutants and also exhibit more severe disease susceptibility symptoms. Another future experiment would be to examine subcellular distribution of MOS4, PRL1 and AtCDC5 in the mos6 mutant background. In addition, great efforts are underway in the Li Lab to elucidate the subcellular localization of snc1 – because several suppressors encode defected components of the nuclear trafficking machinery, and due to recent reports of other Arabidopsis NB-LRR R proteins with functions in the nucleus (see Chapter 1), we predict that snc1 or a portion of snc1 may be nuclear. What are the effects of mos6, mos3, and mos7 on the subcellular distribution of snc1? This is an important question that is being addressed. As discussed in Chapter 1, other proteins involved in defence signalling exhibit nucleocytoplasmic distribution and regulation as well.

Much additional work is required to establish a molecular function for the MAC. While I present data that suggest it is not involved in general or alternative RNA splicing as suggested by orthologs in yeast, we cannot rule out the possibility that the splicing of specific genes is accomplished by this complex (Tsai et al., 1999). Another possibility is that the MAC contributes to regulatory processes mediated by microRNA (miRNA) and small interfering RNA (siRNA), both of which have been shown recently to be involved in the regulation of development and innate immunity (Katiyar-Agarwal et al., 2006; Navarro et al., 2006; Swiezewski et al., 2007). It should be straightforward to check for changes in known miRNA and siRNA levels in mac mutants.

*MOS4*, the originally cloned suppressor of snc1, appears to encode an “adaptor” protein with structural function, since we have no evidence for any molecular role excepting protein-protein interaction. While we have demonstrated that MOS4 interacts with AtCDC5, the significance of this interaction remains unclear (Palma et al., 2007). Does MOS4 stabilize the MAC components, or act as a bridge to another MAC protein? Could MOS4 be involved in anchoring of the subcomplex to the spliceosome or to a subnuclear locale? While no other subsequently identified MAC protein was found in the MOS4 yeast-2-hybrid screen, interaction data from
other model systems could be used to determine whether MOS4 interacts with anything other than AtCDC5.

AtCDC5 (MAC1), identified by virtue of its interaction with MOS4, also suppresses snc1 and basal resistance independent of snc1 (Palma et al., 2007). While it was shown that AtCDC5 localizes to the nucleus, further characterization of this protein is required. Because this protein is predicted to be a transcription factor and has demonstrated DNA binding activity, it is possible that the MAC is a direct or co-regulator of transcription and that disruption of this activity accounts for the shared phenotypes in mutated MAC components (Hirayama and Shinozaki, 1996). Potential targets for gene regulation by AtCDC5 are currently being explored by chromatin immunoprecipitation experiments.

PRL1 (MAC2) was the first MAC protein identified by a reverse genetics approach using yeast and human proteomic data. Arabidopsis PRL1 encodes a WD-protein that has been implicated in pleiotropic regulation of metabolic, hormonal and stress responses through its interaction with and inhibition of Snf1-related protein kinases involved in proteasomal binding of a ubiquitin ligase (Bhalerao et al., 1999; Farras et al., 2001). Because PRL1 is closely linked to snc1, we were initially unable to isolate a prl1 snc1 double-mutant to determine whether this protein is required for snc1 responses. While the isolation of this double-mutant proceeds, we have strong evidence that defects in PRL1 affect resistance to virulent and avirulent pathogens in an NPR1-independent manner (Palma et al., 2007; K. Palma and X. Li, unpublished data).

Other members of the MAC have been isolated using a reverse genetics approach based on proteomic data from Arabidopsis (Q. Zhao, and X. Li, unpublished data) and other kingdoms. For instance, two novel proteins with unknown function, MOS4-Associated Complex Protein 3 (MAC3) and MAC4, interact specifically with AtCDC5. Defects in these genes suppress snc1, and the mutants exhibit enhanced susceptibility to virulent pathogens (J. Monaghan, K. Palma, Y. Zhang and X. Li, unpublished data). Further characterization of MAC3 and MAC4, and the isolation of more MAC proteins, will be crucial in unravelling the function of this complex and how it affects disease resistance signalling.

One question that remains unresolved is whether the constituents of the MAC change in response to a pathogen trigger or in autoimmune mutants. It is likely that, together with MOS4,
AtCDC5, PRL1, MAC3 and MAC4, additional proteins are elements of this discrete complex. Besides nucleocytoplasmic localization, these individual proteins may interact differently depending on defence state. Does phosphorylation, ubiquitination or other post-translational protein modifications differ in uninduced versus induced cells? By employing the stably expressing epitope-tagged lines and antibodies generated during the course of this thesis research, future work could focus on proteomic analysis of the MAC and MAC components in wild type versus snc1 versus pathogen-induced plants.

**Conclusion**

The identification and cloning of mos6 and mos4 in the snc1 suppressor screen has added significantly to our knowledge of signalling networks in plant disease resistance. In particular, the MAC represents a new signalling node that is essential for the little-known NPR1-independent branch of defence. Much further work is required to fully elucidate the functional role of MOS4, AtCDC5, PRL1 and other MAC components – this thesis introduces the MAC and acts as a “stepping stone” to future work on this important complex. The MOS6 story is timely in that it precedes the unexpected identification of NB-LRR R proteins in the nucleus. The topic of nucleocytoplasmic regulation of defence regulators, including R proteins, is currently one of the forefronts of molecular plant-microbe interaction research. This research adds to understanding the fundamental biology of plant defence, and thus enriches the repertoire of tools available for eventual crop protection.
References


