CHARACTERIZATION AND CLONING OF DEFENCE-RELATED SUPPRESSORS OF

mos4-1 snc1 IN *Arabidopsis thaliana*

by

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ABSTRACT

In response to pathogen infection, plants typically use RESISTANCE (R) proteins to recognize and induce a strong defence response. SNC1 belongs to a class of R-proteins, and the gain of function *snc1* mutant has constitutively active immune responses. *MOS4* has been identified through a *snc1* suppressor screen. MOS4 interacts in a complex called the mos4-associated complex (MAC) which is homologous to splicing-related complexes in yeast and humans. However, no splicing defects have been observed in any MAC single mutants. Two MAC proteins, AtCDC5 (a transcription factor) and MAC3A/3B (an E3 ubiquitin ligase) could be responsible for defence signalling downstream of the MAC. Since *mos4-1* has the same defence phenotype as both *atcdc5* and *mac3a/3b*, mutations to any of these genes probably has the same effect on perturbing the MAC. We performed a *mos4-1 snc1* suppressor screen to identify signalling components downstream of the MAC.

The suppressor screen identified 31 dwarf mutants that all had either high *PR2* defence gene expression or resistance to a virulent pathogen, *H.a.* Noco2, suggesting that the mutations affect defence signalling. Three mutants, 60B-1, 83-2 and 39-1, were characterized in greater detail and each of their respective mutations were mapped. 60B-1 carries a mutation to a known negative regulator of defence signalling, *BON1*. 83-2 carries a further gain of function mutation to *snc1*, however, unlike *snc1* which causes snc1 protein accumulation, snc1 protein accumulation in 83-2 does not appear to be affected, suggesting that the protein is converted into a more active form. The molecular lesion in 39-1 was mapped near the southern telomere of chromosome 1, its exact location awaits discovery.

We have shown that a *mos4 snc1* suppressor screen can successfully identify both recessive negative regulators of defence and dominant positive regulators of defence.

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LIST OF ABBREVIATIONS

aa – Amino acid

AtCDC5 – *Arabidopsis thaliana* cell division cycle 5

BAC – Bacterial artificial chromosome

BG2 – Col-0 with *pPR2::GUS* in genetic background

BLAST – Basic local alignment search tool

BON1 – Bonzai 1

cDNA – Complementary DNA

cfu – Colony forming units

Col-0 – *Arabidopsis*, Columbia ecotype

CPN1 – Copine 1

CTAB – Hexadecyl trimethyl-ammonium bromide

dNTP – Deoxynucleotide triphosphates

EMS – Ethyl methanesulfonate

ETI – Effector-triggered immunity

FTA – Fast technology for analysis (of nucleic acids)

GUS – β-glucuronidase

H.a. Noco2 – *Hyaloperonospora arabidopsidis* Noco2

Het – Heterozygous

HR – Hypersensitive response

IN/DEL – Insertion/Deletion

Kb – Kilo-base

L*er* – *Arabidopsis*, Landsberg *erecta* ecotype

LRR – Leucine rich repeat domain

MAC – MOS4-associated complex

MAC3A/3B – Mos 4 associated complex 3 homologs "A" and "B"

MOS – Modifier of *snc1*

MS – Murashiga and Skooge

NB – Neucleotide binding domain

NPR1 – Non-expresser of *PR* genes, 1

NTC – Nineteen complex

PAMP – Pathogen associated molecular pattern

PTI – PAMP-triggered immunity

P.s.m. – Pseudomonas syringae pathovar maculicola

PCR – Polymerase chain reaction

pPR2::GUS – PR2 promoter fused to a *GUS* reporter

PR gene – *Pathogenesis-related* gene

PRR – Pathogen recognition receptor

PRL1 – Pleiotropic regulatory locus, 1

R-gene – *RESISTANCE* gene

rpm – revolutions per minute

R-protein – RESISTANCE protein

RT-PCR – Two-step reverse transcription PCR

SA – Salicylic acid

SNC1 – Suppressor of *npr1-1* constitutive 1

SNP – Single nucleotide polymorphism

SSLP – Simple sequence length polymorphism

Taq – *Thermus aquaticus*

T-DNA – Transfer-DNA

TE – Tris-EDTA buffer

TIR – Toll-interleukin receptor domain

UTR – Untranslated region

UV – Ultra-violet

V – Volt

Ws – Wassilewskija ecotype

WT – Wild-type

AKNOWLEDGMENTS

Chapter 1: **INTRODUCTION AND LITERATURE REVIEW**

Plants are able to defend themselves against pathogens in a variety of manners. Recent conceptual models suggest that the plant immune system generally has three layers: non-host resistance, pathogen associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). In non-host resistance, pathogens are not able to infect a plant, usually due to pre-formed physical barriers such as the waxy cuticle and rigid cell wall, or due to the secretion of plant-derived antimicrobial secondary metabolites (Nurnberger *et al*., 2005). In contrast, host resistance, where plants detect pathogens and subsequently initiate defence signalling, is viewed as having two layers: PTI and ETI (Jones and Dangl, 2006). In PTI, plant pathogen recognition receptors (PRR) are able to detect conserved features of pathogens (*e.g.* bacterial flagella), termed PAMPs, to mount a relatively weak defence response. To mount a stronger defence response, ETI, plants use RESISTANCE (R) proteins which can detect secreted pathogen-derived compounds, termed effectors, either directly or indirectly. ETI is characterized by two major events: a localized hypersentive response (HR) which kills plant cells at the site of infection to prevent further pathogen colonization, and systemic acquired resistance (SAR) which increases the plant's ability to resist future infections (Durrant and Dong, 2004). Although the non-host, PTI, ETI model has been a useful framework for studying plant immunity, several examples that do not conform to this model have recently shown that plant defence probably exists along a continuum rather than in layers (Thomma et al., 2011). These findings will undoubtedly lead to an updated model of plant defence in the coming years.

Between R-protein activation and the resulting defence responses, many complex intracellular signalling events take place (Figure 1-1). Salicylic acid (SA) is the primary

hormone involved in plant resistance to biotrophic pathogens, and SA induction is required for SAR (*i.e.* a defence response where a localized infection stimulates a heightened ability for the entire plant to resist future infection, which usually lasts for the remaining life of the plant). Expression of *pathogenesis-related* (*PR*) genes is correlated with SAR induction. Studies of SA biosynthesis mutants *enhanced disease susceptibility 5* (*eds5*) and *SA induction deficient 2* (*sid2*) have shown that in SA deficient plants, defence signalling is not completely abolished, suggesting that both SA-dependent and SA-independent defence pathways exist (Zhang *et al.*, 2003) (Figure 1-1).
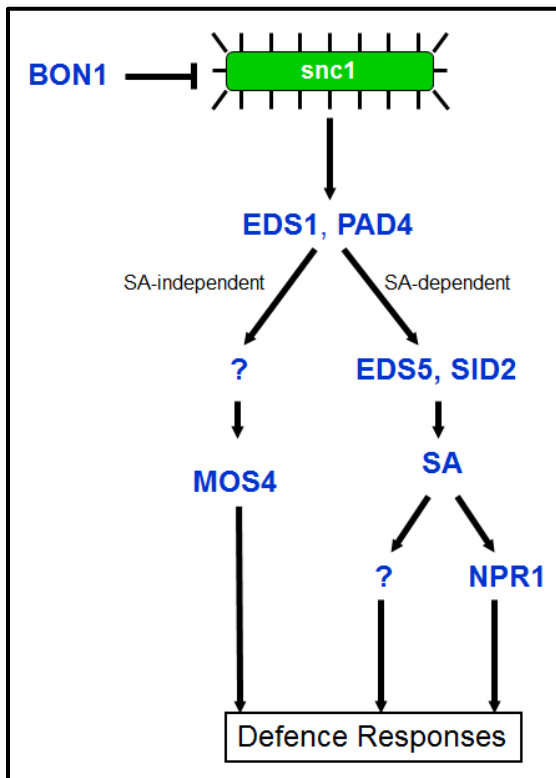
Figure 1-1. Signalling pathways used by the snc1 R-protein.

Non-expressor of pathogenesis-related genes 1 (*npr1*) was identified in a mutant screen for mutants with compromised SAR and *PR* gene expression (Cao *et al.*, 1994). However, in the *npr1* mutant, SAR is not completely compromised; *npr1* plants pre-treated

with SA were more resistant to the virulent *Pseudomonas syringae* pv. *maculicola* (P.s.m.) ES4326 than non-SA pre-treated Col-0 controls. This result suggests that there are *npr1*-dependant and *npr1*-independent pathways downstream of SA accumulation (Figure 1-1).

The *suppressor of npr1 constitutive 1* (*snc1*) mutant was identified in a mutant screen designed to search for mutants with enhanced defence signalling (Li *et al*., 2001). Using the *npr1* mutant with a *pPR2::GUS* defence marker gene reporter construct in its genetic background, mutagenized plants were screened for the up-regulation of the β-glucuronidase (*GUS*) reporter. The *snc1* lesion was cloned and found to be a gain-of-function mutation to a TIR-NB-LRR (Toll-interleukin receptor, nucleotide binding and leucine rich repeat domain) type *R*-gene, which results in the constitutive defence signalling in the absence of pathogen effectors (Zhang *et al*., 2003). Like all characterized TIR-NB-LRR R-proteins, *snc1* is fully suppressed by mutations to *enhanced disease susceptibility 1*(*eds1*) and *phytoalexin deficient 4* (*pad4*) (Figure 1-1).

Since *snc1* functions at or near the beginning of defence signalling, it became apparent that it could be used to identify downstream defence-related genes. To identify genes downstream of *snc1*, multiple *snc1* suppressor screens were carried out; a reversion from the *snc1* phenotype to a wild-type (WT)-like phenotype in double mutants was indicative of *snc1*suppression. These screens identified fifteen *modifier of snc1* (*mos*) genes. The nine fully characterized MOS proteins function in RNA processing, nucleocytoplasmic transport, protein modification and epigenetic control of gene expression, which have revealed the complexity of signalling downstream of R-protein activation (Palma *et al*., 2005; Zhang *et al*., 2005; Zhang *et al*., 2005; Goritschnig *et al*., 2007; Palma *et al*., 2007;

Goritschnig *et al*., 2008; Cheng *et al*., 2009; Germain, *et al*., 2010; Li *et al*., 2010; Wiermer *et al.,* 2010).

The *mos4-1* mutation is recessive and completely suppresses all the phenotypes of *snc1* (Palma *et al*., 2007). In the *mos4-1 snc1* mutant, *snc1*-mediated dwarfism, constitutive *PR* gene expression, heightened SA and enhanced resistance to both *Hyaloperonospora arabidopsidis* (*H.a.*) Noco2 and *P.s.m.* ES4326 are all reverted to WT. MOS4 was placed in the SA-independent pathway because avirulent pathogen induced SA accumulation was not compromised in the *mos4-1* single mutant (Palma *et al*., 2007) (Figure 1-1). Furthermore, *npr1* and *mos4* had additive effects on susceptibility to infection, suggesting that they function in two separate pathways.

The MOS4 protein is a small (253 aa) evolutionarily conserved coiled-coil protein that is predicted to be involved in protein-protein interactions. Yeast two hybrid and co-immunoprecipitation assays found that MOS4 associates with at least twenty other proteins *in planta*, forming the MOS4-associated complex (MAC) (Palma *et al*., 2007; Monaghan *et al*., 2009). Furthermore, many homologs of MAC proteins are found in homologous complexes of yeast (nineteen complex) and human (Prp19/CDC5L complex), suggesting that the complex is highly conserved. In yeast, the NTC plays an essential role in spliceosome assembly (Wahl *et al*., 2009). Therefore, we hypothesized that *mos4-1* causes impaired splicing (Palma *et al*., 2007). However, splicing defects have not been observed in *mos4-1* or other MAC component single mutants. Interestingly, combined MAC double mutants are lethal, suggesting that the MAC performs an essential function. These results suggest that the MAC performs a function in defence (and possibly splicing) which is disabled in *mos4-1*.

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Recently the molecular structure of the human Prp19/CDC5L complex was characterized (Grote *et al*., 2010). The core components of the Prp19/CDC5L complex (*Arabidopsis* MAC) are PRP19 (MAC3), SPF27 (MOS4), PRL1 (PRL1) and CDC5L (AtCDC5). Grote *et al*., (2010) found that SPF27 forms a hub to which four units of PRP19, and one unit each CDC5L and PRL1, bind. From these data we can infer that MOS4 could be an important protein holding parts of the MAC together, and with the mutant mos4, the MAC protein interactions could be altered, thereby reducing the function of the complex.

The hard question remains: how does the MAC function in defence? Two of the core components of the MAC, AtCDC5 and MAC3 offer some possibilities. AtCDC5 is an atypical R2R3-Myb transcription factor and MAC3A and MAC3B (MAC3A/3B) are two redundant U-box E3 ubiquitin ligases (Monaghan *et al*., 2009). Given the aforementioned data, it is possible that MOS4 tethers AtCDC5 and MACA/3B to the MAC. AtCDC5 could promote the transcription of defence related transcripts, whereas MAC3A/3B could ubiquitinate negative regulators of defence signalling, leading to their degradation through the proteosome pathway. In the *mos4-1* mutant, AtCDC5 and MAC3A/3B binding to the MAC could be impaired leading to a reduction or complete loss of their functions. In support of this hypothesis, we found that a*tcdc5* mutants partially suppress *snc1* (Palma *et al.,* 2007) and *mac3a/3b* double mutants have exactly the same phenotypes as *mos4-1* (Monaghan *et al*., 2009).

To search for the targets of the MAC, a suppressor screen in the *mos4 -1 snc1* background was conducted. If our hypothesis that the MAC targets negative regulators of defence for degradation (through MAC3A/3B) is correct, then this screen should allow us to find those targets. A loss of function to one of these negative regulators could lead to up-

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regulated defence signalling. Suppressors found in the screen could also function in MAC-independent defence signalling. Although not anticipated, rare gain-of-function dominant mutants carrying mutations in positive regulators of defence could also be found. Targets of AtCDC5 could also be identified.

Chapter 2: **MATERIALS AND METHODS**

2.1   **PLANT CARE, CROSSING AND CO-SEGREGATION ANALYSIS**

Seeds were sterilized by soaking them in a solution of 15% bleach and 0.1% Tween-20 for up to 5 min followed by two rinses with sterile water. Seeds were cold treated for at least 2 days prior to seeding. Plants grown on MS media were kept at 22°C and exposed to a16 h light, 8 h dark regimen. Plants were grown on plates for up to two weeks before experimentation or transplantation to soil. Seeds for soil grown plants were sown on the soil surface and the flat was covered with a transparent dome for 2-4 days to allow germination. Soil-grown plants were exposed to 16 h of light at 23°C and 8 h of dark at 20°C per day.

Plants were crossed by removing the developing sepals, petals and stamens from the female parent just before flowering occurred. Stamens from the male parent were obtained from open flowers and used to pollinate the carpels of the female parent. In all cases, reciprocal crosses were attempted.

2.2   **MUTAGENESIS AND THE PRIMARY SCREEN**

Approximately 10000 *mos4-1 snc1* seeds were treated with 20 mM ethyl methansulfonate (EMS) for 18 h (M_1). M_1 seeds were planted and seeds from ~2500 M_1 plants were harvested in pools of ~100 (25 plants per pool). M_2 seeds were planted and ~50000 M_2 plants were screened for dwarf or a *snc1*-like morphology. Seeds from individual mutant plants were collected and the M_3 seeds were grown to confirm heritability of the phenotype. All mutants with a heritable phenotype were assayed in the secondary screen.

2.3   **THE SECONDARY SCREEN**

To determine whether the mutants collected from the primary screen were in fact defence-related dwarves, a secondary screen was conducted. Mutations to many genes that are unrelated to immunity are known to cause a dwarf phenotype (*e.g.* genes involved in bassinosteroid biosynthesis). To exclude dwarf mutants that were not involved in defence signalling, defence marker gene expression, *mos4-1* genotyping and a pathogen resistance assays were performed.

2.3.1   **GUS HISTOCHEMICAL STAINING**

The parental *mos4-1 snc1* line used for mutagenesis has the *Arabidopsis PR2* promoter fused to a *GUS* reporter gene in the genetic background. *PR2* is a well-established defence marker gene. If *PR2* expression was induced in the mutants, the GUS enzyme would be produced. Later, when X-gal was infiltrated into the cells, wherever the GUS enzyme was produced, X-gal would be cleaved to yield a blue colour. Having this construct in the genetic background allowed us to determine whether or not defence signalling was activated in the mutants.

Six seeds for each line were grown on MS media in a 22°C growth chamber for two weeks. Col-0, *mos4-1*, *snc1* and *mos4-1 snc1* plants were included as controls. Seedlings were removed from the plates and transferred to a 24 well plate (3.3 mL per well) containing approximately 1.5 mL of GUS staining solution (1.0M $NaPO_4$, 0.5M EDTA, 10% Triton X-500, $K_3Fe(CN)_6$, 0.1M X-Gluc). The plate was put inside a vacuum chamber and vacuum infiltration was performed until all the leaves of the seedlings were translucent. The plate was closed, covered with aluminum foil and incubated at 37°C overnight. GUS staining solution was removed and the seedlings were de-stained twice with 70% ethanol for 6 h to

remove background pigment from the seedlings. The level of staining was rated on a four point scale. If no staining was observed a rating of "-" was given; if some weak staining was observed, a score of "+" was given; "++" meant strong and "+++" meant very strong staining. The seedlings were photographed and the 70% ethanol was replaced with 50% glycerol for long term storage.

2.3.2   *mos4-1*

To ensure that none of the mutants were *snc1* single mutant contaminants, *MOS4* genotyping was performed. If the mutants were homozygous for the mutant form of *mos4-1* we could be confident that they were not *snc1* contaminants. The *mos4-1* mutation is a 2.2 Kb insertion. Primers mos4genoF (5'-ctggcttttggaacttaaccac- 3') and mos4genoR (5'-gatctgtgtctcaagcatggc- 3') flanking the insertion were used for genotyping. During the PCR, a 1 min extension time was used. If the genotype of the plant was homozygous or heterozygous for *MOS4* then a band would be visible on an agarose gel. If the plant was homozygous for *mos4-1*, no band would be produced because the 1 min extension time would be too short to amplify the ~2.5 Kb fragment. FTA card based PCR was used for this experiment (see section 2.4.1.3 – PCR AND GEL ELECTROPHORESIS). At least two PCR reactions per line were performed and a Col-0 control was included in every assay to ensure the PCR did not fail.

2.3.3   **PLANT PATHOGEN INFECTIONS**

To directly assess the ability of the mutants to resist pathogen infection, resistance to the obligate biotrophic oomycete pathogen *H.a.* Noco2, was assayed. Approximately sixteen plants for each line were grown on soil for 2 weeks. Col-0, *mos4-1*, *snc1* and *mos4-1 snc1* plants were included as controls. *H.a.* Noco2 spores were collected from infected Col-0 or

eds1-2 leaves. Leaves were placed in 50 mL tube containing ddH$_2$O. The tube was vortexed to dislodge and suspend the spores. Infection was performed by spraying a flat of plants with 10 mL of the spore solution (5×10^4 spores per mL). Flats were covered with a clear plastic dome; the gap between the flat and the dome was sealed with masking tape and the flats were incubated in a growth chamber (18°C) for one week. Diseases severity was scored by observing the number of leaves and number of spores per leaf for each line.

For the lines 60B-1, 83-2 and 39-1, more precise infection data was gathered. The infections were done in the manner described above; however, the disease severity rating was done in a more quantitative manner. For each line, aerial tissues from every plant were weighed and placed into a test tube. Twenty µl of water for each mg of tissue was added to each tube. Tubes were vortexed for 30 sec to suspend the spores. The concentration of spores for each line was measured using a hemocytometer. To take into account any errors incurred by variations in spore distribution while spraying the flats, three technical replications were performed.

2.4 MAP-BASED CLONING

To identify the molecular lesion in the mutants, map-based cloning was performed. To do this we took advantage of the genetic polymorphisms between the Col-0 ecotype and L*er* ecotype. Linkage between the mutant phenotype and genetic markers allowed us to map each mutation to a small region of the genome. Sequencing within the final region was performed, and once the mutation was found, a complementation test was performed to confirm that the correct gene was mapped.

2.4.1 CRUDE MAPPING

To crude map the mutations, very dwarf plants from an F2 cross with L*er* were genotyped with markers throughout the genome that can distinguish Col-0 from L*er* DNA. Since the mutagenesis was performed in Col-0 plants, a bias towards the Col-0 genotype was used as a guide to direct us towards the mutation. Crude mapping is complete when the gene is flanked (*i.e.* when a marker on each side of the mutation has been identified).

2.4.1.1 CROSSES

Each homozygous mutant line was crossed with L*er*. The F1 was observed to determine if each mutation was dominant or recessive. The F1 was allowed to self and the F2 population was used for crude mapping. Since all three mutant alleles were segregating, only the very dwarf plants (plant of similar size or smaller than the triple mutant) were selected for crude mapping. The very dwarf plants must have at least one copy of the third mutation. Sixteen to 24 very dwarf plants were used for crude mapping.

2.4.1.2 DNA EXTRACTION

Genomic DNA was extracted from the plants using a CTAB DNA extraction protocol. Whole plants or leaves, depending on the size of the mutant, were collected in a 1.5 mL tube containing 250 µl of 65°C CTAB extraction buffer. Plant tissues were ground in the extraction buffer using a micropestle and incubated at 65°C for 5 min. To remove proteins and other impurities, 200 µl of chloroform (24:1 chloroform:isoamyl alcohol) was added to each tube. The tubes were vortexed twice and spun down at $16\,110 \times g$ for 5 min. The supernatant containing the DNA was removed and dispensed into a new tube. To precipitate the DNA, 140 µl of isopropanol was added to each tube. The tubes were mixed by inversion several times, incubated at room temperature for 5 min and centrifuged for 5

min at 16 110 × g to pellet the DNA. The remaining liquid was discarded and the tubes were allowed to dry for 5 min by leaving them open and inverted on a piece of paper towel. The DNA pellet was washed with 200 μl of -20°C 80% ethanol. The tubes were centrifuged for 5 min at 16 110 × g and the liquid was discarded. To dry the pellet, the samples were centrifuged in a speed-vac for 8 min. The DNA was re-suspended in 50 μl of TE buffer (pH 8.0) and heat treated for 15 min at 70°C to help dissolve the DNA and degrade DNAses. The samples were stored at 4°C.

2.4.1.3 **PCR AND GEL ELECTROPHORESIS**

To search for a bias towards the Col-0 genotype throughout the genome of each plant, insertion/deletion (IN/DEL) (also known as simple sequence length polymorphism) markers with size differences in Col-0 relative to L*er* were used (Figure 2-1). For each marker the same PCR conditions were used. The PCR reaction contained the following components: 14.5 μl ddH$_2$O, 2 μl 10 × PCR buffer, 1.6 μl of 10mM dNTPs, 0.3 μl of each primer (20 μM each), 0.3 μl of Taq polymerase and 1 μl of CTAB extracted DNA. PCR reactions were always prepared on wet ice. The thermal cycling program consisted of a 2 min incubation at 95°C, then 45 cycles of 95°C for 20 sec, 55°C for 30 sec and 72°C for 90 sec. A final incubation at 72°C for 5 min was performed after the 45 cycles. After PCR, 10× gel loading buffer (30% glycerol, 0.1M EDTA, 1% SDS, 0.25% bromophenol blue) was added to each sample and 15-20 μl of sample was loaded into a 1, 1.5, 2 or 3% agarose gel (depending on the size difference between the Col-0 and L*er* bands; the larger the size difference, the lower percentage of agarose in the gel) pre-stained with ethidium bromide. The agarose gels were run at approximately 150 V until band separation was evident (usually ~40 min) and analyzed using a UV transilluminator gel documentation unit.
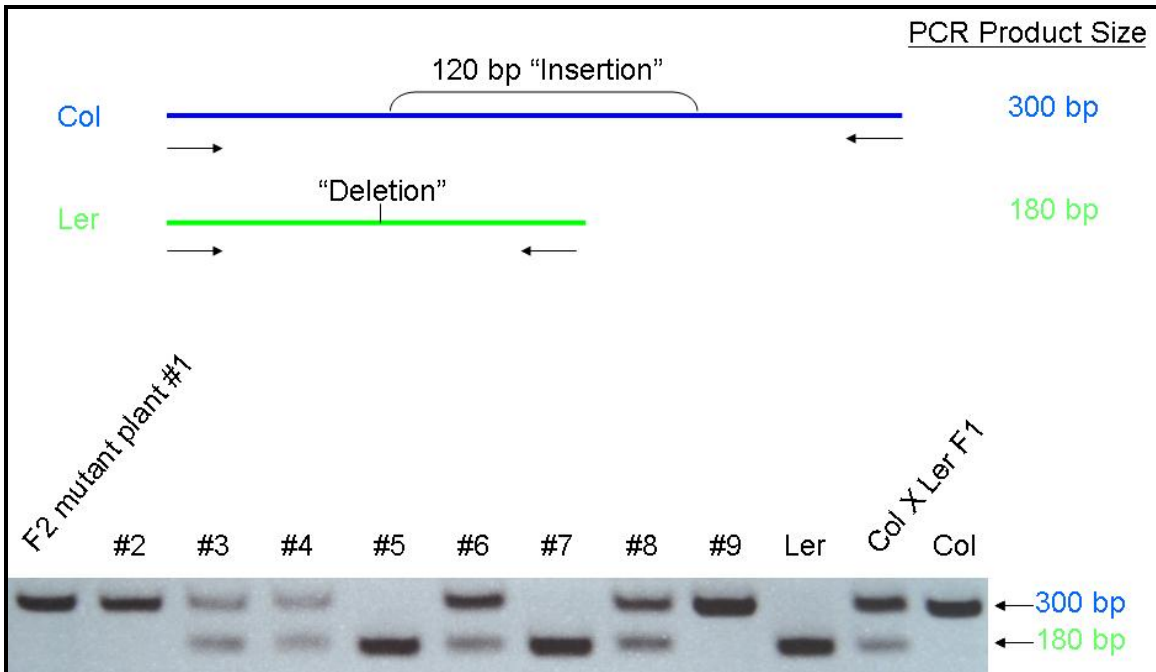
Figure 2-1. Insertion/deletion markers.
Markers were designed by creating primers that amplify a region containing a known Col-0/L*er* sequence length polymorphism (top: 120bp difference). The forward and reverse primers are capable of amplifying both Col-0 and L*er* DNA. Using these markers, plants could be PCR genotyped at the marker loci. When run on an agarose gel (bottom), homozygous (Col-0 or L*er*) plants have a single band, whereas heterozygous plants have two bands: the Col-0 band and the L*er* band.

Table 2-1. Insertion/deletion markers used for mapping.
A. Mapping markers. B. Fine mapping markers. Genome positions are based on TAIR 10. Primer sequences are listed 5' to 3'. "Top Band" indicates which ecotype produces a fragment of greater molecular weight (*e.g.* for marker F9L1, the L*er* fragment will be heavier than the Col-0 fragment). The BAC position of the markers were used as their names, a sub-name (in parentheses) was made when more than 1 marker per BAC was designed. The recommended percentage of agarose gel to resolve both the Col-0 and L*er* band is also listed (electrophoresis conditions: 150V for approximately 40 min).

A

Chromosome	Position (Mb)	BAC	F Primer	R Primer	Top Band	% Agarose Gel
1	5.2	F9L1	atgctttcgagccttcgatg	aagcgaagacagcttactgg	Ler	2
	13.7	F28J9	cacttccccagcaaccgaga	ccgtcagagacgtgaaggca	Col-0	1
	25.6	T2E12	cgactagccagtccgataca	cgttttgggagccacgtttc	Col-0	1
2	4.1	T4D8	gacccgaaaggaatgcgg	caacatggtatgtttgcgg	Col-0	2
	9.3	F2G1	cgtcgtcggaagtttcagag	gaataagaagaacacatgcgtc	Col-0	1
	15.6	T2N18	gtcgtctagtgtacttgtagc	ttggtcactagtaagatcttg	Col-0	2
3	4.9	M1E1	ctaagttcttccaccatctg	caaggagcatctagccagag	Col-0	1
	11.5	T13J10	attcggacaagatcggtgc	tgattcttctgagcatagag	Col-0	1
	20.7	F18O21	attaggtggacaagactctac	ttgggtgagatcggtcttga	Ler	2
4	5.0	T13D4	cataccaagcctacgtcaac	aaactccctggatcaggcag	Col-0	1
	11.0	F9F13	cttggttagtgaccaagcctta	gatgaatcgtggatacattcagc	Col-0	1
	17.0	F8D20	tctccctgtgttaacttgcc	cggtaggtaacgagatccag	Col-0	1
5	5.9	MPI7	gtgaattccaattagaccgca	tccttgataccgaccggtga	Col-0	1
	14.7	MJG14	ccaacacctccacctggaag	tgatgtcgattccgaagctg	Col-0	1
	24.7	K11J9	gcaacggtgtaaccagcaag	ctcctcgtgtttggctgacc	Col-0	1

B

Chromosome	Position (Mb)	BAC	F Primer	R Primer	Top Band	% Agarose Gel
1	26.83	F23N20	actaggcaagcttgttttcc	tgtaagacctttgatggtcg	Col-0	2
	27.36	F28P22	caagattcacaatgctgagg	tccaccgtagcatcttcacg	Col-0	2
	28.42	F10A5	aagctcgctttgcagttc	ctccaatttaccagctacat	Col-0	2
	30.21	F5I6 (PG#3)	ttcatccgaatggcacaaaca	actcccaactttccctccac	Col-0	3
	30.25	F5I6 (PG#2)	gtttgactttgcacgtctag	ccagggatcaatcattacgc	Col-0	3
	30.27	F5I6 (PG)	gaggccacatgtgaagtaaaa	taactgctatccattcgcaag	Col-0	2
5	24.25	F15L12	ctatgaagcggaaactgtg	tcaatacgccaggtttcg	Col-0	3
	24.50	MSL3	cgattgcaaatctaacaaaaga	ggagatgaggtgccaaaatg	Col-0	3
	24.61	MAF19	agcccacgttgataactttt	ccagtaaaacattatttcccaca	Ler	2
	24.78	K11J9 (#2)	tcataaaatggacgtaccaaaa	attacaaatctatcatgtcacttaacg	Col-0	2
	24.82	MAC9	cactgctctcagtcttaaggt	gcaacaatcaaaatcacaac	Col-0	3
	24.99	MMI9	aaaaccaatccattgaaact	tgcataacattgtcactcct	Col-0	3
	25.06	K19B1 (#1)	gaaaacagagaaacattttgg	tagtcaatgttgggaatttg	Col-0	3
	25.29	MJH22	tttgcaggagtgatggagac	gatctttctttattgagtatgtgacga	Col-0	3

2.4.1.4 FLANKING THE MUTATION

The first step to flanking the mutation was to identify biases towards the Col-0 genotype (Table 2-2A). The following methods will assume the mutation is recessive. Regions of bias towards the Col-0 genotype were identified by calculating the map distance between a given marker and the potential mutation (Table 2-2B). To calculate the map distance, the number of recombination events between the marker and the mutation were divided by the total number of chromosomes (number of plants multiplied by two) analyzed.

To help determine the precise location of the mutation relative to the markers, arrows were drawn on the spreadsheet. These arrows point away from regions where the mutation could not possibly be and towards the region containing the mutation (Table 2-2C). If phenotyping was done correctly, all the arrows point to a region that has the Col-0 genotype for all plants tested. Once this region was found, we searched for flanking markers that were roughly equidistant from the mutation with at least two recombinant arrows pointing towards the mutation (Table 2-2D). This was done to ensure the mutation was confidently flanked. If the mutation is not confidently flanked it can lead to wasted effort by fine mapping the wrong region. Once the mutation was confidently flanked, fine mapping was performed.

Table 2-2. The process of crude mapping.

A. Twelve mutant plants were selected and genotyped using all the crude mapping markers (Table 2-1) and the data was entered in a spreadsheet (c = Col-0 genotype, h = heterozygous genotype, l = L*er* genotype). The results for chromosome 5 are shown. Since mutagenesis was done in the Col-0 ecotype, at the site of the mutation all mutant plants have the Col-0 genotype. B. The map distance between each marker and the mutation was calculated ((number of cross overs between the marker and the mutation ÷ total number of chromosomes) × 100). C. Arrows that point towards the mutation were added; these arrows point away from heterozygous or L*er* genotypes towards Col-0 genotypes. The data shown in C suggests that the mutation is at a position >14.7 Mb. To flank the mutation, a marker at a position >25 Mb must be assayed. D. Data gathered using KIL20 allows the mutation to be flank the mutation between 14.7 and 26.5 Mb, an 11.8 Mb region. Data from K19E20 further narrows down the flanking region to a 6.5Mb region.

A

	Chromosome 5		
BAC	MPI6	MJG14	K11J9
Position	6 Mb	14.7 Mb	25 Mb
Plant 1	l	l	c
Plant 2	h	c	c
Plant 3	h	h	c
Plant 4	h	h	c
Plant 5	l	h	c
Plant 6	h	h	c
Plant 7	c	c	c
Plant 8	l	l	c
Plant 9	h	h	c
Plant 10	c	c	c
Plant 11	l	h	c
Plant 12	l	c	c

B

	Chromosome 5		
BAC	MPI6	MJG14	K11J9
Position	6 Mb	14.7 Mb	25 Mb
Plant 1	l	l	c
Plant 2	h	c	c
Plant 3	h	h	c
Plant 4	h	h	c
Plant 5	l	h	c
Plant 6	h	h	c
Plant 7	c	c	c
Plant 8	l	l	c
Plant 9	h	h	c
Plant 10	c	c	c
Plant 11	l	h	c
Plant 12	l	c	c
cM calculation	15/24	10/24	0/24
cM	63	42	0

C

	Chromosome 5		
BAC	MPI6	MJG14	K11J9
Position	6 Mb	14.7 Mb	25 Mb
Plant 1	l	l	c
Plant 2	h →	c	c
Plant 3	h	h →	c
Plant 4	h	h →	c
Plant 5	l	h →	c
Plant 6	h	h →	c
Plant 7	c	c	c
Plant 8	l	l →	c
Plant 9	h	h →	c
Plant 10	c	c	c
Plant 11	l	h →	c
Plant 12	l →	c	c
cM calculation	15/24	10/24	0/24
cM	63	42	0

D

	Chromosome 5				
BAC	MPI6	MJG14	K19E20	K11J9	KIL20
Position	6 Mb	14.7 Mb	20 Mb	25 Mb	26.5 Mb
Plant 1	l	l →	c	c	c
Plant 2	h →	c	c	c	c
Plant 3	h	h	h →	c	c
Plant 4	h	h →	c	c	c
Plant 5	l	h →	c	c ←	h
Plant 6	h	h →	c	c	c
Plant 7	c	c	c	c	c
Plant 8	l	l	h →	c	c
Plant 9	h	h →	c	c	c
Plant 10	c	c	c	c	c
Plant 11	l	h →	c	c	c
Plant 12	l →	c	c	c ←	h
cM calculation	15/24	10/24	2/24	0/24	2/24
cM	63	42	8	0	8

2.4.2   **FINE MAPPING**

Fine mapping involves using the flanking markers from crude mapping to screen many plants for recombinants. Using these recombinants and markers progressively closer to the mutation allowed us to narrow down the mutation to a small region of the genome (approximately 100 Kb).

2.4.2.1   **IDENTIFICATION OF SUITABLE LINES**

To simplify fine mapping, careful attention was given to selecting a suitable fine mapping line. The F2 population from the L*er* cross was screened to identify lines for fine mapping. These F2 plants were genotyped using markers flanking *SNC1* and *MOS4*. Plants that were homozygous for both *SNC1* and *MOS4* flanking markers, and heterozygous for both markers flanking the third mutation, were selected. Since random alleles segregating from L*er* can interfere with phenotyping, it was important to observe the segregation of a few lines with the desired genotypes. Roughly 20 F3 seeds from each potential fine mapping line were planted and the segregation pattern, if any, was observed. Only lines with a perfect 3 to 1 segregation pattern and an easily distinguishable phenotype were used for fine mapping.

To confirm that the phenotypic segregation was a result of genotypic segregation in the flanked region, co-segregation analysis was performed; all ~20 plants were genotyped using the markers flanking the unknown mutation. All WT plants were expected to have a L*er* or Het genotype and all mutant plants were expected to have the Col-0 genotype.

2.4.2.2   **FTA CARD-BASED PCR**

To speed the pace of PCR-based genotyping, FTA card-based PCR was used for fine mapping. FTA card-based PCR essentially bypasses the need for a DNA extraction. Instead, leaves were removed and placed on FTA paper. A piece of parafilm was used to cover the

leaf, and a glass 15 mL round bottom tube was used to press the leaf tissues into the FTA

paper. A green leaf-shaped mark remained on the paper. The paper was allowed to dry for at

least 30 min. Using a 1.2 mm Harris Micro-punch, a punch of the green coloured paper was

removed and transferred to a 0.2 mL PCR tube. 50 µl FTA wash solution ($50 \times$ stock is 10

mM Tris pH 7.5, 2 mM EDTA, 0.1% Tween 20) was added to the punch and incubated at

room temperature for 5 min. The wash solution was removed and the punch was washed

with 180 µl of TE^{-1}, twice. To obtain robust and consistent PCR results, it was very

important to spin down and remove any residual TE^{-1} from the tube. PCR master mix was

added directly to the tube. PCR and gel electrophoresis were performed as described in

section 2.4.1.3 (PCR and Gel Electrophoresis); however, since no liquid DNA sample is

added, 15.5 µl of H_2O per reaction was used instead of 14.5 µl.

2.4.2.3   CHROMOSOME WALKING

To confirm no errors were incurred from phenotyping or PCR during fine mapping,

each recombinant plant was transplanted out, a new FTA card sample was taken and the

sample was genotyped again using the flanking markers. Only recombinants that were

confirmed using this method were used for fine mapping.

Recombinants were used to narrow down the mutation using chromosome walking

(Table 2-3). In general, this process involves screening all the recombinant plants using a

new genotyping marker that is closer to the mutation. At this point some plants will no

longer be recombinants; these plants will not be useful for further mapping. The remaining

recombinant plants are tested for yet another new genotyping marker that is closer to the

mutation. This process is repeated until only 2 recombinants remain, or the region is

narrowed down to ~100 Kb. While designing the new genotyping markers, the location of

the marker was important. Generally, as a useful guideline, the markers should be 50%

closer to mutation (*e.g.* if the flanked region is between 18 and 20 Kb then the new

genotyping markers for fine mapping should be designed at 18.5 Kb and 19.5 Kb).

Table 2-3. The process of fine mapping.

Once a suitable fine mapping line is found, several hundred seeds of that line (usually F3 seeds) are planted and all plants are genotyped using the flanking markers that were identified through crude mapping. A. Over 400 F3 seeds are planted and genotyped for the crude mapping flanking markers (MMN10 and K19B1). Six recombinant plants are identified (*i.e.* the genotypes for the two flanking are different (*e.g.* #38 is a recombinant because it is Het for MMN10 and Col-0 for K19B1)). Arrows are drawn for Col-0-Het type recombinants but not Het-L*er* type recombinants. B. To narrow down the mutation further the recombinants were genotyped using another marker (MUF9). Relative to the two flanking markers (MUF9 and K19B1), plant #38 is no longer a recombinant, so it will not be of any use for further fine mapping. C., D., E., and F. New makers are designed and used to screen the remaining recombinants until the region containing the mutation is narrowed down to approximately 100 Kb, or no more recombinants remain. G. To confirm the genotyping and phenotyping data is correct, the F4 of each recombinant line is planted. F4 segregation pattern (if any) is noted and 3 big and 3 small plants are genotyped using markers flanking the recombination event that occurred in each line. H. Updated fine mapping data based on the F4 results. Plant #38 was not a real recombinant (its genotype for MMN10 was Col-0, a PCR error led to it being called Het). The F4 data also allow us to draw arrows for the Het-L*er* type recombinants (#140 and 300). Based on the data from this figure it can be concluded that the mutation is in the 250 Kb region between MAE1 and MAC9. To narrow down the mutation to a region of 100Kb, more recombinants are needed.

A

	BAC	MMN10	MUF9	MAE1	MAC9	K22G18	K19B1
	Location (Mb)	24.1	24.35	24.475	24.725	24.85	25.1
38	mutant	h →					c
76	mutant	c				←	h
140	WT	h					l
205	WT	h				←	c
300	WT	l					h
432	mutant	h →					c

B

38	mutant	h → c					c
76	mutant	c	c			←	h
140	WT	h	h				l
205	WT	h	h			←	c
300	WT	l	l				h
432	mutant	h	h →				c

C

38	mutant	h → c					c
76	mutant	c	c			c ←	h
140	WT	h	h			l	l
205	WT	h	h		←	c	c
300	WT	l	l			h	h
432	mutant	h	h →			c	c

D

38	mutant	h → c					c
76	mutant	c	c	c		c ←	h
140	WT	h	h	l		l	l
205	WT	h	h	h	←	c	c
300	WT	l	l	l		h	h
432	mutant	h	h	h →		c	c

E

38	mutant	h → c					c
76	mutant	c	c	c		c ←	h
140	WT	h	h	l		l	l
205	WT	h	h	h ← c		c	c
300	WT	l	l	l	h	h	h
432	mutant	h	h	h → c		c	c

F

38	mutant	h → c					c
76	mutant	c	c	c		c ←	h
140	WT	h	h	l		l	l
205	WT	h	h	h ← c		c	c
300	WT	l	l	l	h	h	h
432	mutant	h	h	h → c		c	c

G

	F3 phenotype	F4 Segregation	Left Marker	Right Marker
38	mutant	all mutant	MMN10-all Col-0	MUF9-all Col-0
76	mutant	all mutant	K22G18-all Col-0	K19B1-2 Col-0,4 Het,2 Ler
140	WT	all WT	MUF9-2 Col-0,4 Het,2 Ler	MAE1-all Ler
205	WT	3 WT: 1 mutant	MAE1- 3 Mut.- all Col-0, 3WT- 2 Het, 1 Ler	MAC9-all Col-0
300	WT	3 WT: 1 mutant	MAE1-all Ler	MAC9-3 Mut.- all Col-0, 3WT- 2 Het, 1 Ler
432	mutant	all mutant	MAE1-2 Col-0,4 Het,2 Ler	MAC9-all Col-0

H

38	mutant	c ✗ c					c
76	mutant	c	c	c		c ←	h
140	WT	h	h ···> l	l		l	l
205	WT	h	h	h ← c		c	c
300	WT	l	l	l ···> h	h	h	h
432	mutant	h	h	h → c		c	c

To confirm the location of the recombination event, relative to the fine mapping markers in each recombinant F3 plant, ~20 F4 seeds were planted for co-segregation analysis. Phenotypic segregation patterns (if any) were observed, and FTA card-based PCR genotyping using the fine mapping markers flanking the recombination event was performed on 6 plants. If segregation was observed, 3 mutant and 3 WT plants were selected for PCR genotyping. If no segregation was observed, 3 smaller plants and 3 larger plants were selected for PCR testing.

2.4.3 DNA SEQUENCING

When a promising candidate gene, usually a gene known to be involved in immunity, was identified during fine mapping, the gene was sequenced using Sanger sequencing. Primers amplifying all the exons of the gene were designed and DNA from the mutant plants was used as a template to amplify the region. The fragments were gel purified using the QIAquick gel extraction kit (Cat no. 28706); the manufacturer's instructions were followed, including all optional steps. The purified DNA was quantified using spectrophotometer and used as a template for a PCR sequencing reaction (BigDye terminator v3.0 cycle sequencing kit, Cat no. 4337455). Each 10 µl reaction consisted of 20 ng of DNA template, 5 pmol of primer, 3 µl of BigDye dilution mix. The thermal cycler program consisted of a 1 min incubation at 96°C, then 25 cycles of 96°C for 10 sec, 50°C for 4 sec and 72°C for 4 min. The PCR reaction was purified using a sephadex-based column and the samples were run on a sequencing gel.

When no obvious candidate genes were present in the fine mapped region, Solexa sequencing was used to sequence the entire genome of the mutant. Solexa sequencing is a

sequence-by-synthesis type of massively parallel sequencing method. It makes use of reversible terminators that only allow extension to occur one base at a time. When a fluorescently labelled reversible terminator is incorporated, fluorescence is detected by a camera, the 3'-protection group (prevents elongation by more than one nucleotide at a time) is reverted to a 3'-hydroxyl group (allows a new reversible terminator to be added) and a new reversible terminator can be incorporated. By sequencing numerous small fragments in parallel, and by aligning these fragments with the fine mapped region of the *Arabidopisis* genome, the causal mutation could be found.

2.4.4   COMPLEMENTATION TEST

To determine whether 60B-1 and *BON1* where homozygous for mutations to the same gene, a complementation test was performed. Two *BON1* T-DNA insertion mutant lines, SALK_ 070435 (intron) and SALK_122769 (5'-UTR), were planted and their phenotypes were observed. Plants with the *bon1* phenotype were crossed with 60B-1. If 60B-1 and *BON1* were homozygous for mutation to the same gene, the F1 was expected to have the *bon1* phenotype.

2.5   RT-PCR

Two-step RT-PCR was used to measure the abundance of transcripts. The Totally RNA kit (Ambion) was used to extract RNA from 4-week-old soil-grown plants that were either mock infected or infected with *P.s.m*. AvrRpm1. Reverse transcription was carried out using the Superscript II reverse transcriptase kit (Invitrogen). The resulting cDNA was used as template for PCR (as described in section in 2.4.1.3). Thirty PCR cycles were performed instead of 45.

2.6   **PROTEIN EXTRACTION AND WESTERN BLOT**

Leaf tissue (~0.1 g) from 2-week-old soil or MS plate grown plants were collected into 2 mL tubes containing two 5 mm glass beads and frozen in liquid nitrogen. Samples were ground into a fine powder using a TissueLyser (Qiagen Retsch). Tubes were placed on wet ice, suspended in extraction buffer (100mM Tris-HCl pH 8, 0.1% SDS, 2% β-mercaptoethonol) and spun down for 5 min at $16\,110 \times g$ in a 4°C microcentrifuge. The supernatant was transferred to a 1.5 mL tube, boiled for 5 min at 100°C then $4 \times$ SDS loading buffer was added. Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane by electro-blotting. The membrane was probed with an anti-SNC1 primary antibody then an anti-rabbit secondary antibody conjugated to horse radish peroxidase. The membrane was treated with a horse radish peroxidase substrate, SuperSignal West Chemiluminescent substrate (Thermo), and exposed to photographic film to visualize protein expression.

Chapter 3: **RESULTS AND DISCUSSION**

3.1   **CHARACTERIZATION OF SUPPRESSORS OF *mos4-1 snc1***

From the *mos4-1 snc1* suppressor screen, 108 M_2 mutant lines were identified. In the

M_3, 56 of these lines were confirmed to carry a heritable mutation. These 56 lines were

analyzed using the secondary screen. After the secondary screen, 31 mutants with auto-

activated defence phenotypes were identified. All 31 lines were smaller than the parental

line; some were extremely dwarfed (Figure 3-1). Most of the lines also had *snc1*-like

morphology: they had twisted, dark leaves. Using GUS staining, we found that every line

except 5-1, 59-1 and 97-1 had plants with a visible blue colouration, indicating expression of

the *PR2* defence marker gene (Figure 3-2). Most lines were also resistant to *H.a.* Noco 2

infection (Figure 3-2). Since all 31 lines had a *snc1*-like phenotype, elevated *PR2* expression

or enhanced resistance to *H.a.* Noco 2, our laboratory crude mapped all of them. Three

mutants, 60B-1, 39-1 and 83-2, will be discussed in this thesis. These three mutants were

selected because they all had obvious phenotypes, which made them amenable to mapping,

they all had different phenotypes, which suggests that none of the mutants were allelic, and

they all had high *PR2* expression and enhanced resistance to infection, suggesting that the
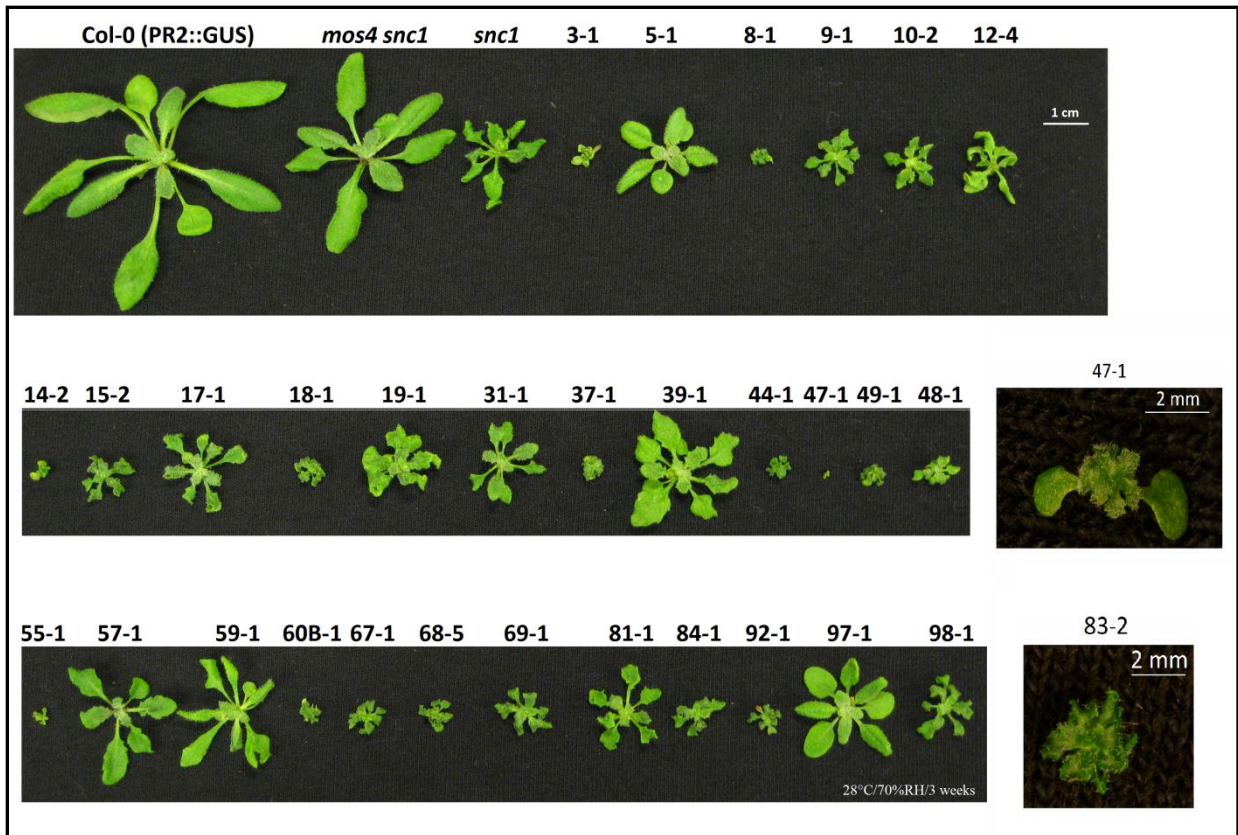
mutations effect defence signalling.

Figure 3-1. Morphology of *mos4-1 snc1-1* suppressor mutants.
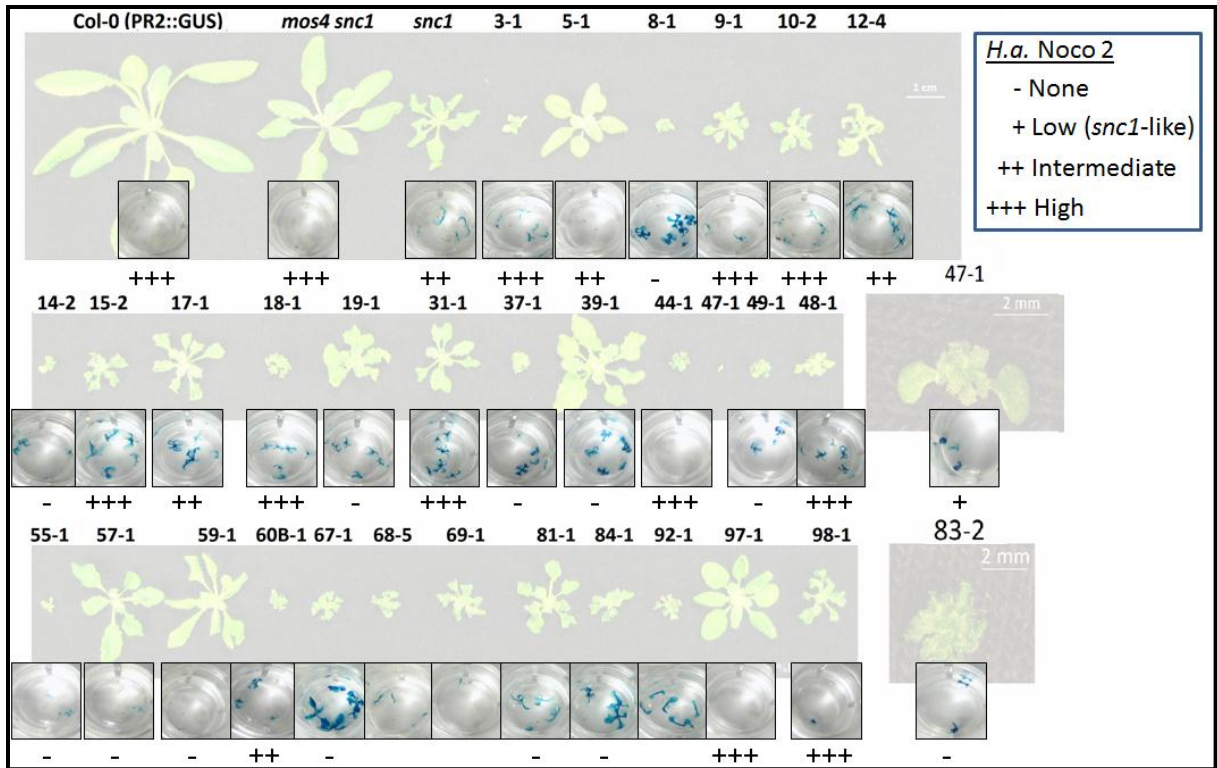Plants were grown on soil at 28°C and 70% relative humidity for 3 weeks.

Figure 3-2. Suppressor of *mos4-1 snc1* secondary screen.
The intensity of blue GUS staining is a reporter for *PR2* defence gene expression. The amount of *H.a.* Noco 2 spores from conidiophores on each mutant is reported. For 47-1 there are two pictures, the GUS staining and *H.a.* Noco2 data for 47-1 is only shown under the close up photo. 68-5, 69-1 and 92-1 were not assayed for resistance to *H.a.* Noco2 because not enough seeds could be obtained to perform the assay.

3.2   60B-1

60B-1 is a recessive, early flowering dwarf with dark, twisted leaves (Figure 3-3).

GUS staining was visible in the aerial tissues but absent in the roots. Staining was most

prominent in the veins, but most of the leaf, trichome, stem and cotelydon tissues had some

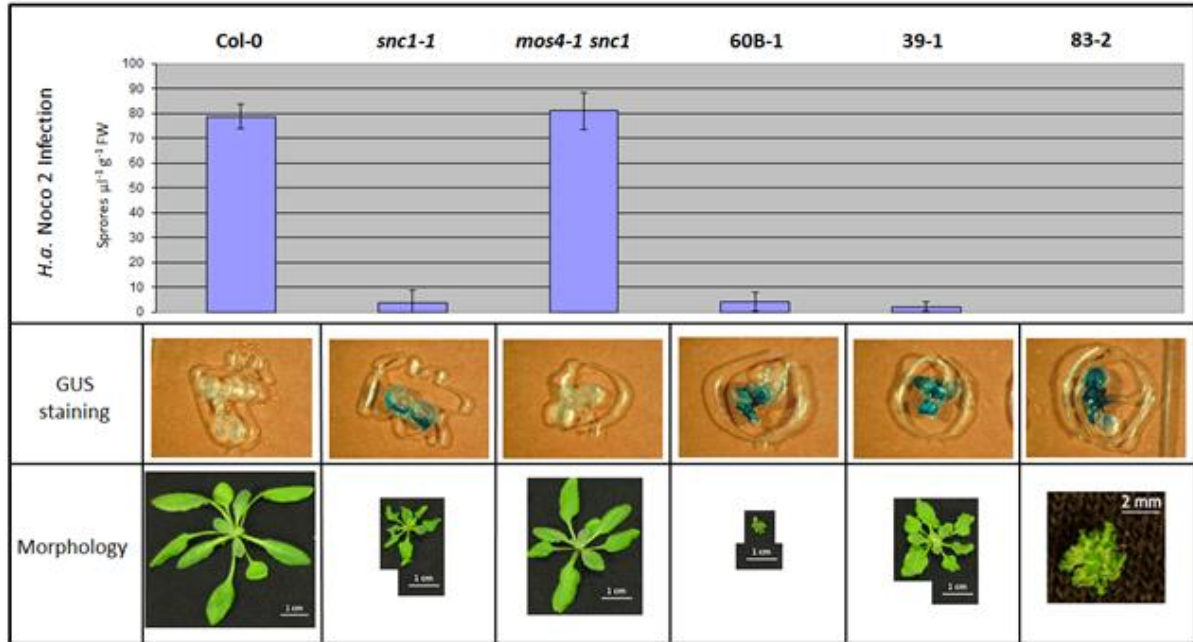blue colouration. The 60B-1 mutant is as resistant to *H.a.* Noco2 infection as *snc1*.

Figure 3-3. Characterization of 60B-1, 83-2 and 39-1.
For the H.a. Noco2 infections three technical replicates were performed. No spores were ever found in samples from 83-2. All GUS staining images were taken with identical camera settings and lighting using a dissecting microscope. All morphology images have 1 cm size bars, except for 83-2 which has a 2 mm size bar. Plants were grown on soil at 28°C and 70% relative humidity for 3 weeks.

3.2.1   GENETIC MAPPING

Crude mapping found that marker K11J9 (chromosome 5 at 24.7 Mb) was closely linked to the mutation; 1 recombinant out of 24 F2 plants was found. Because K11J9 is very close to the mutation, we decided not to use it to flank the mutation because this could have easily led to an error in flanking the mutation. Instead, we sought out two different flanking markers that were further from the mutation. 60B-1 was confidently flanked using K19E20 (chromosome 5 at 20Mb) and KIL20 (chromosome 5 at 26.5 Mb).

A F2 line homozygous for *snc1* and *MOS4* and heterozygous at both K19E20 and KIL20 had perfect 3 (*snc1*-like) to 1 (60B-1-like) segregation in the F3 population. Fine mapping was performed using 701 plants from this line. 60B-1 was fine mapped between

MAC9 and MMI9 (Figure 3-4 A). At this point it was evident that the mutation was mapping to a region of the genome containing a known negative regulator of defence, *BON1*.

BON1 is a plasma membrane localized calcium responsive-phospholipid binding protein. The *bon1* mutant is a dwarf, temperature-sensitive mutant with constitutive defence responses. Studies found that *bon1* has a natural suppressor in the Ws ecotype that is not present in Col-0 (Hua *et al*., 2001). Through genetic studies it was determined that *bon1* only has a phenotype when *SNC1* is present (SNC1 is present in Col-0 but not in Ws) (Yang *et al.*, 2004). Since both *bon1* and *snc1* mutations lead to constitutive defence, and *SNC1* is required for the phenotypes of *bon1*, it was concluded that BON1 is a negative regulator of SNC1, upstream of SNC1 (Figure 1-1).

Recent studies have begun to reveal the function of BON1 in defence signalling. Lee *et al*., (2009) found that *bon1* plants had dose-dependent *PR* gene accumulation in response to calcium ionophore A23187 infiltration. These preliminary results suggest that BON1 may be involved in preventing calcium-induced defence signalling.

3.2.2   60B-1 CARRIES A MUTATED ALLELE OF *BON1*

Using Sanger sequencing, *BON1* was sequenced in 60B-1. A single G to A mutation leading to an early stop codon was found (Figure 3-4 B). G to A mutations are consistent with EMS treatment. To further confirm that the mutation to *BON1* was responsible for the phenotypes of 60B-1, a complementation test was performed. When planted, SALK_ 070435 (T-DNA in the 3rd of 15 introns) segregated 3 WT to 1 *bon1*-like. The *bon1*-like plants were crossed with 60B-1. In the F1 we found that 60B-1 did not complement SALK_070435; the F1plants were *bon1*-like (Figure 3-4 C). This result confirmed that the 60B-1 and *bon1* are homozygous for mutations to the same gene. The phenotype of the

plants was more severe than the *bon1* single mutants. This is likely because the *snc1* mutation is heterozygous in the 60B-1 × *bon1* F1 plants and contributes to the mutant phenotype. However, since the 60B-1 × Col-0 F1 is WT, we can be confident that observed result in the 60B-1 × *bon1* F1 plants is not a false positive caused by the co-dominant defence signalling phenotype of *snc1*.
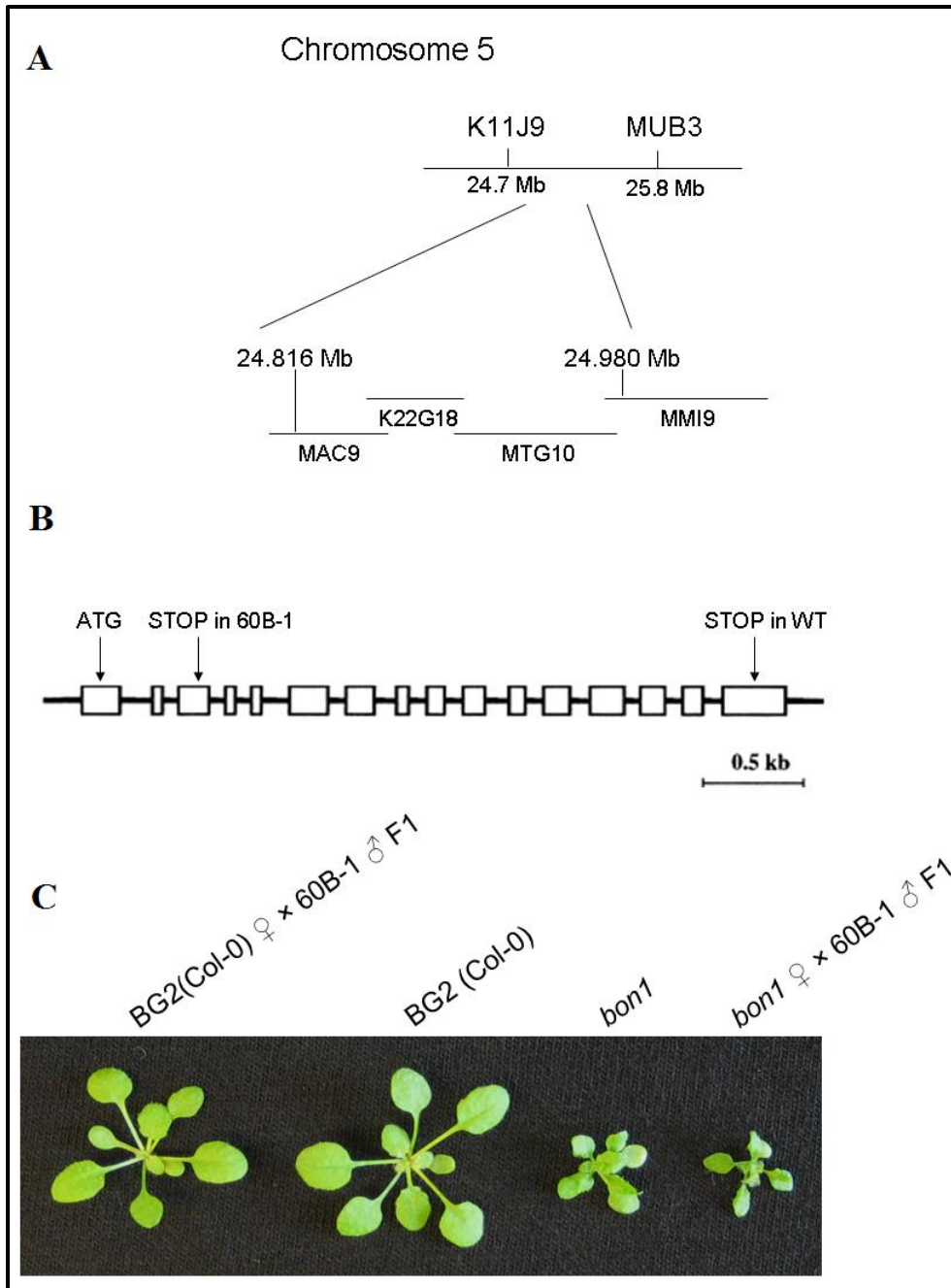
Figure 3-4. Map-based cloning of 60B-1.
A. Diagrammatic representation of the mapping of 60B-1. The final 164 Kb region containing the 60B-1 mutation is shown. Genome positions are based on TAIR10. B. Diagram of *BON1* gene structure modified from Hua *et al.*, (2001). The 60B-1 point mutation leads to an early stop codon in the third exon. C. *bon1* × 60B-1 complementation test. Three-week-old soil grown plants. The *bon1* is the SALK_070435 T-DNA mutant.

3.2.3 BON1 AND THE MAC

Based on the finding that the 60B-1 phenotype is caused by a mutation to *BON1*, it is tempting to propose that BON1 is a negative regulator targeted by MAC3A/3B. However, a direct protein-protein interaction is highly unlikely because BON1 is localized to the plasma membrane (Hua *et al*., 2001; Li *et al*., 2010), whereas the MAC is localized exclusively to the nucleus (Palma *et al*., 2007; Monaghan *et al.*, 2009). Another possibility is that ATCDC5, a transcription factor known to bind to CTCAGCG sequences (Hirayama et al., 1996), targets the *BON1* promoter. *BON1* is known to be induced during defence responses and Lee *et al*. (2009) found that a 280 bp upstream sequence of the *BON1* promoter was required for pathogen induced *BON1* expression. Perhaps ATCDC5 is responsible for pathogen-induced *BON1* expression. As an initial test of this hypothesis, an alignment between the ATCDC5 target sequence and *BON1* (including 1500 bp upstream of the ATG start-site) was performed. No potential ATCDC5 binding sites were identified. Therefore, it is unlikely that ATCDC5 directly influences *BON1* expression. If any interaction between the MAC and BON1 exists, it could be an indirect interaction. For example, when defence signalling is activated, MAC3A/3B could cause the degradation of transcription repressors on the *BON1* promoter. If this were the case then we would expect *BON1* expression to be low in uninfected and infected *mac3a/3b* mutants. This is one of many possible examples. However, if a case similar to this exists, *BON1* expression in *mac* mutants should change compared to WT. To test this hypothesis, RT-PCR was used to analyze *BON1* expression in various *mac* mutants under infected and uninfected conditions (Figure 3-5). Consistent with previous reports, *BON1* expression increased upon pathogen infection (Jambunathan *et al.*,

2003). In uninfected and infected plants *BON1* expression was not drastically affected in any

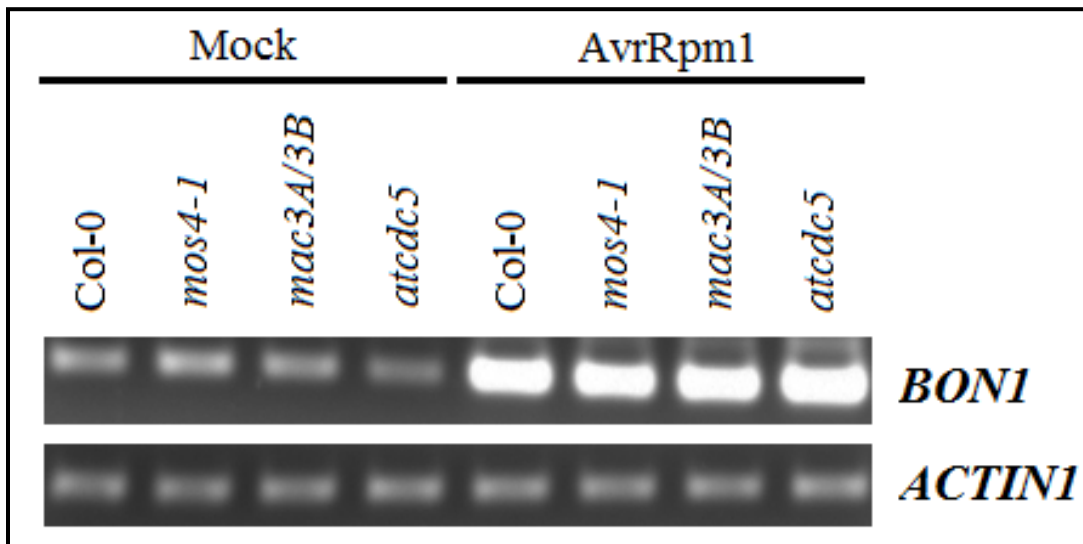of the *mac* mutants tested, suggesting that the MAC does not regulate *BON1* expression.

Figure 3-5. *BON1* gene expression in infected and uninfected *mac* mutants.
Plants were grown on soil for 4 weeks prior to infiltration. Leaves were infiltrated on their abaxial side with a needleless 1 mL syringe containing either a *P.s.m.* AvrRpm1 (10^6 cfu/mL) or a 10 mM $MgCl_2$ solution (mock treatment). Plants were allowed to dry for a few minutes, and then were covered with a clear plastic dome. RNA was extracted 24 hours post-infection. *BON1* expression was measured by RT-PCR. *ACTIN1* is a stably expressed gene.

3.2.4   SUMMARY

Using map-based cloning, the 60B-1 mutant was mapped near 25 Mb on chromosome

5. The mapped region contained a well characterized negative regulator of defence, *BON1*.

When *BON1* was sequenced in 60B-1, a single G to A mutation leading to an early stop

codon was found. The 60B-1 mutant failed to complement a *bon1* T-DNA mutant line,

indicating that 60B-1 and *bon1* are homozygous for mutations in the same gene. To

determine if *BON1* expression is regulated by the MAC, *BON1* expression was measured in

uninfected and infected *mac* mutants. *BON1* expression was unaltered in the *mac* mutants,

suggesting that *BON1* expression is not regulated by the MAC.

3.3 **83-2**

83-2 is a semi-dominant extreme dwarf with twisted dark green leaves and reduced

fertility (Figure 3-3). Heterozygous mutants gave rise to homozygous 83-2 progeny that

usually set ~5-10 seeds at 28°C. Homozygous 83-2 mutants do not given rise to fertile

progeny, regardless of growth conditions. Heterozygous 83-2 plants set many seeds at both

22°C and 28°C. The GUS staining pattern in 83-2 was identical to 60B-1 (it was most

prominent in the veins but also present in leaf, trichome, stem and cotelydon tissues) but the

intensity of staining was much greater in 83-2. *H.a.* Noco2 spores were never found on the

mutant, indicating that pathogen infection was extremely low or absent in 83-2 plants.

3.3.1 **GENETIC MAPPING**

During crude mapping, linkage was found only on chromosome 4 near *SNC1*. Other

regions of potential linkage were investigated and excluded as possible mutation sites using

co-segregation analysis.   After crude mapping, we suspected the mutation was a further

gain-of-function mutation to *snc1*.

In addition to the crude mapping result, several lines of evidence suggested that 83-2

was a further gain-of-function mutation to *snc1*. Like *snc1*, 83-2 is co-dominant in its

defence signalling phenotype as observed by GUS staining. 60B-1 carries a mutation to a

negative regulator of *SNC1*.   60B-1 and 83-2 had identical GUS staining patterns, suggesting

that common molecular pathways are activated in both mutants. One of the features that

makes the *snc1* mutant unique among auto-activated *R*-gene mutants is that it does not

exhibit constitutive HR lesioning. Like *snc1*, 83-2 does not exhibit constitutive HR

lesioning. Finally, our laboratory has transformed a *snc1* null mutant with *snc1* and the

tranformants phenocopy 83-2 (X. Li, personal communication, June 29, 2011). To determine

if 83-2 carries a mutation to *snc1* or any other *R*-gene, all *R*-genes were sequenced in 83-2.

3.3.2   **83-2 CARRIES A MUTATION TO *SNC1***

In 83-2, a single G to A mutation was found in *snc1*, at the beginning of the LRR

(Figure 3-6 A). The 83-2 mutation leads to the substitution of glycine 705 to glutamic acid.

The properties of these amino acids are quite different; glycine is small and bears no charge,

whereas glutamic acid is negatively charged, hydrophilic and more than twice the mass of

glycine. Therefore, this amino acid change could profoundly alter protein structure and

function. Conservation of the substituted residue across other closely related *Arabidopsis*

TIR-NB-LRR R proteins was assessed (Figure 3-6 B).

Unfortunately, we could not perform transgene complementation to show that the 83-

2 phenotype is caused by the early LRR mutation to *snc1*. *SNC1* expression is known to be

suppressed by its location in the genome, likely due to its DNA methylation status or

surrounding histone structure (Li et al., 2007). Since 83-2 is already a severe dwarf mutant,

it is very likely that any complementing lines would be lethal. If surviving lines were found,

they would be expected to be severe dwarfs. However, this result would also be expected if a

snc1 null mutant were transformed with the *snc1* allele; *snc1* transformed into a *snc1* null

mutant phenocopies 83-2 (X. Li, personal communication, June 29, 2011). Therefore, it

would be very difficult, if not impossible to use transgene complementation to show that the

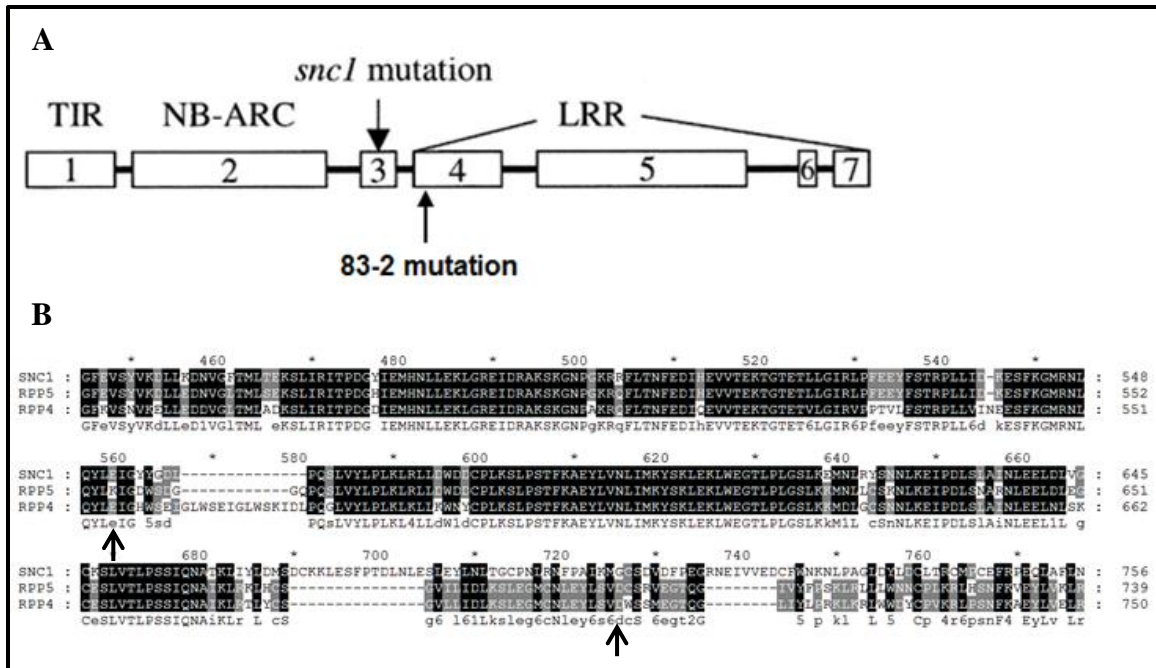83-2 mutation in *snc1* contributes to its mutant phenotype.

Figure 3-6. Identification and Characterization of the 83-2 Mutation.
A. *SNC1* gene structure with mutation sites shown. Boxes represent exons, while lines represent introns. The approximate locations of protein domains are shown. This figure is modified from Zhang *et al.,* (2003) B. *Arabidopsis* TIR-NB-LRR R-protein alignment using ClustalX2. Arrows indicate the locations of the amino acid substitution caused by the 83-2 mutation (G705) and the original *snc1* mutation (E552).

The *snc1* mutation increases snc1 protein accumulation (Figure 3-7) (X. Li, personal communication, June 30, 2011). In addition, *SNC1* gene expression is not significantly altered in *snc1* (Zhang *et al*., 2003). Therefore, the accumulation of snc1 protein is likely caused by an increase in snc1 protein stability. To determine if the 83-2 mutation further increases snc1 protein accumulation, a western blot using an SNC1 antibody was performed. Three replicates using both soil and plate grown seedlings showed that snc1 protein levels were similar 83-2 and *snc1* mutants. One of these replicates is shown in Figure 3-7. These data suggest that the 83-2 mutation probably does not affect snc1 protein stability.
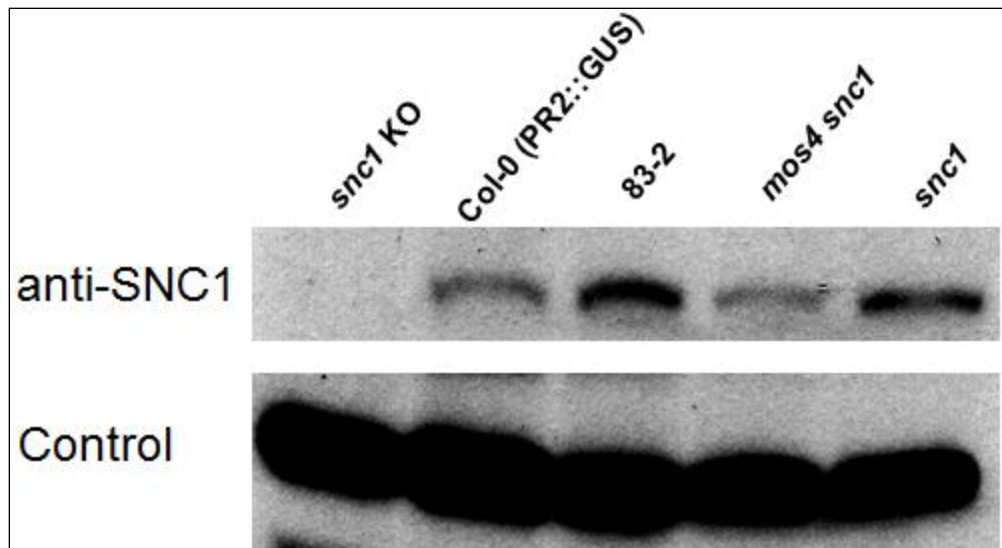
Figure 3-7. SNC1 protein accumulation in 83-2.
Western blot using a SNC1 antibody as probe. Plants were grown on MS media for 2 weeks prior to protein extraction. The loading control is a non-specific band from the same blot. A null allele of *SNC1, snc1* KO is shown.

Based on the western blot results, 83-2 probably has the effect of increasing SNC1 signalling activity without affecting its stability. To directly test this hypothesis, more research on the biochemical activity of SNC1 will have to be conducted first. Specifically, if we can identify a downstream target of SNC1, that target can be monitored in the 83-2 mutant to determine if defence signalling is augmented in the mutant.

In most well characterized R-proteins that have direct effector-R protein interactions, the effector interacts with the LRR of the R-protein (Padmanabhan *et al*., 2009). It is tempting to speculate that since the 83-2 mutation affects an amino acid that is not conservation in the LRR, it could mimic the biochemical changes that occur when the effector that activates SNC1 is present, thus leading to a constitutively active R-protein. However the location of the effector binding site on the LRR is unpredictable, and in some cases, such as the tobacco R-protein, N, the effector binds to the TIR domain, not the LRR.

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Another possibility is that the inactive form of SNC1 inhibits itself through intramolecular interactions. This type of self-inhibition, which thus far has always been shown to involve the LRR, has been found in several R-proteins (Padmanabhan *et al.*, 2009). These studies have also found that when the effector is present, the R-protein self-association no longer occurs, suggesting that the active R-protein does not self-associate. Perhaps the 83-2 mutation prevents SNC1 self-association, leading to a constitutively active protein (Figure 3-8).
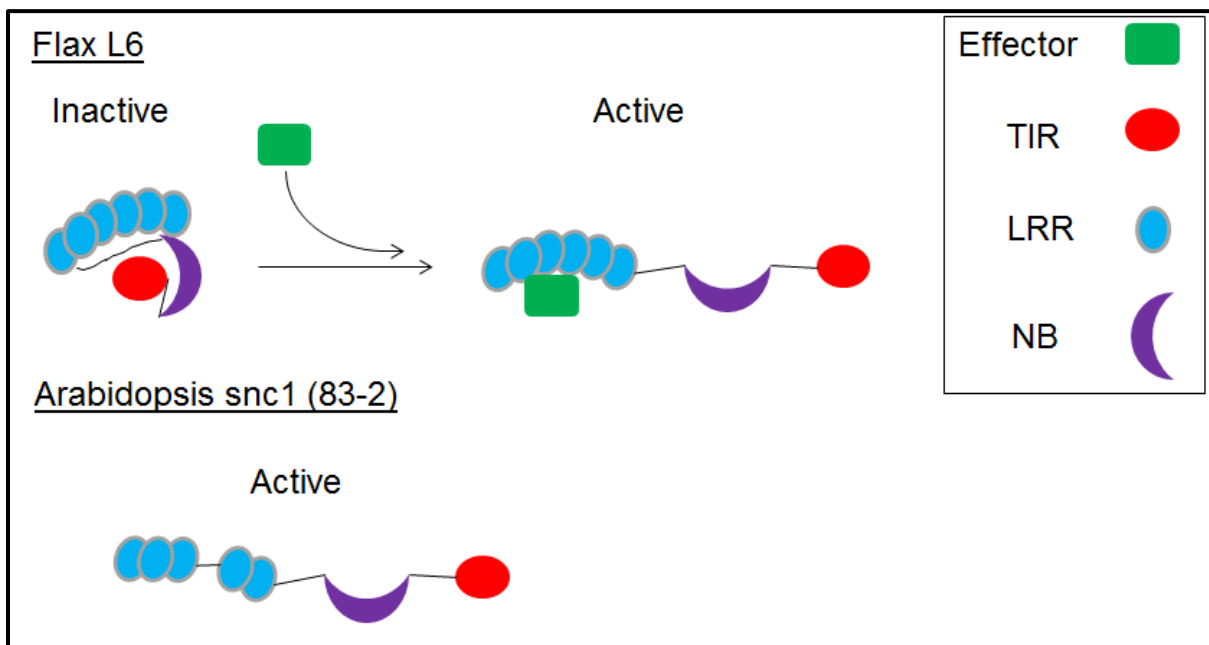

Figure 3-8. Model for snc1 auto-activation in the 83-2 mutant.
Most R-proteins are regulated in a similar manner (Bernoux *et al.,* 2011). The TIR-NB-LRR R-protein, Flax L6, is shown as an example. Inactive L6 is self-inhibited through intramolecular interactions. Upon recognition of its effector, AvrL567, self-inhibition is repressed and this allows active defence signalling to occur. The 83-2 mutation could change the structure of the snc1 LRR and prevent self-inhibition, leading to a constitutively active R-protein.

Using co-immunoprecipitation, our lab has found that SNC1 self-associates, likely in a di- or multi-meric complex, but it is not clear if this interaction occurs when SNC1 is active or inactive (X. Li, personal communication, June 29, 2011). The 83-2 mutation could enhance or prevent SNC1 di- or multi-mer formation, leading to constitutive defence

signalling. By studying the biochemical events that occur when SNC1 is activated and inactivated (specifically the role of the LRR in SNC1 function) the effect of the 83-2 mutation on SNC1 may eventually be revealed.

3.3.3 SUMMARY

The 83-2 mutant is an extreme dwarf, autoimmune mutant. It was crude mapped near *SNC1* on chromosome 4. Sequencing of all the *Arabidopsis R* genes revealed a single G to A mutation in the LRR of *snc1*. Western blot showed that snc1 accumulation is not enhanced in 83-2 relative to *snc1*. We concluded that 83-2 is a further gain-of-function mutation to *snc1* that likely enhances snc1 signalling ability, probably by changing protein structure into the active form.

3.4 39-1

The 39-1 mutant is a recessive, late flowering, pale green dwarf with twisted leaves that tend to fold perpendicular to the mid rib. GUS staining was only observed in aerial tissues including the leaves, trichomes and stem (Figure 3-3). GUS staining was relatively even throughout the leaves (*i.e.* it was not restricted to the veins); however, staining in the cotyledons was very low compared to the leaves. In terms of disease resistance, the 39-1 mutant was as resistant as *snc1* to *H.a.* Noco2 infection (Figure 3-3).

3.4.1 MAPPING

The 39-1 mutation was mapped to chromosome 1 between 28.55 Mb and the southern telomere (30.43 Mb). Since the mutation was close to the telomere, it could not be flanked during crude mapping. The mutation was subsequently fine mapped in two rounds. The mutation was flanked during the first round of fine mapping, and mapped between 30.211 Mb and 30.245 Mb using a population of ~400 F3 plants. There are 7 genes within this

region. Five of these genes, AT1G80400, AT80420, AT1G80440, AT1G80450 and AT1G80840, were sequenced and no mutation was found. The last two genes were not sequenced because when the genome of 39-1 was sequenced, no confident mutations (mutations found consistently in >2 sequence reads that would alter the predicted amino acids sequence of a gene) were found between 29.87 Mb and the telomere. Since the flanking markers in the first round of fine mapping were close together (< 0.5 Mb apart), we suspected that the mutation might not have been properly flanked. To test this hypothesis, a second round of fine mapping using mutant progeny from three independent lines was performed. Using these 214 mutant progeny, and by mapping with flanking markers that were ~5 Mb apart (25.7 Mb and 30.41 Mb), the mutation was once again mapped between 29.87 Mb and the telomere.

Although we did not find any confident mutations while analyzing the genome sequence, single read mutations (mutations found in regions with 1 fold sequence coverage of the genome) that would result in an amino acid substitution were found in 2 genes between 29.87 Mb and the telomere: an RNAse THREE-like protein (AT1G80650) and an F-box and LRR containing protein (AT1G80960). The putative mutations within these genes were sequences in 39-1. Neither of the mutations were confirmed, indicating that the mutation identified in the genome sequence were false positives.

Further analysis is necessary to identify the molecular lesion in 39-1. The mutation could be in the promoter of a gene. If this is the case, the mutation can be found by analyzing intergenic sequences in the fine mapped region. In addition, there were gaps in the genome sequence. These gaps can be filled in using Sanger sequencing. However, at this point there is too much data to analyze it efficiently; the flanking region is too large. To

improve the likelihood of success, further fine mapping will have to be performed first.

Once the region is narrowed down to ~100 Kb, the intergenic regions can be analyzed and

any gaps can be sequenced.

3.4.2 SUMMARY

The 39-1 mutation was mapped between 29.87 Mb and the southern telomere on

chromosome 1. Whole genome sequencing was unable to confidently identify the molecular

lesion. Fine mapping the mutation to a ~100 Kb region followed by further analysis of the

sequencing data and sequencing gaps in the genome should allow us to identify the molecular

lesion.

Chapter 4: **FUTURE DIRECTIONS AND CONCLUSION**

4.1 **60B-1**

The 60B-1 mutant could be used to better characterize SNC1 signalling pathways. The MOS genes are known to suppress *snc1,* but the number of pathways and the detailed relationship between different *MOS* genes is largely unknown. 60B-1 could be crossed with *mos snc1* double mutants and the F2 could be screened using PCR genotyping markers for *mos4-1*, mos and *bon1*. To facilitate faster genotyping, the 60B-1 mutation (a SNP) could be replaced, through crossing, with the SALK_070435 T-DNA allele. This strategy would allow us to determine if other *MOS* genes function in the same pathway as *MOS4* or different pathways.

A rise in cytosolic calcium levels is a well-established response to pathogen infection (Lee *et al*., 2009). Calcium analog infiltration was found to induce *PR* gene expression in *bon1* but not in Col-0. If the calcium-induced *PR* gene expression is dependent on SNC1, this would suggest that SNC1 might be responsible for calcium-induced defence signalling. This could be tested by repeating the calcium infiltration experiment with a *bon1 snc1* (both null mutant) double mutant. If SNC1 is responsible for calcium-induced defence signalling then Col-0-like *PR* gene expression would be expected.

Although the MAC does not appear to influence *BON1* expression, we found that *mos4-1* probably suppresses *bon1*. In the F2 of a cross between 60B-1 and Col-0, 17 out of 111 plants (15.3%) had a *bon1*-like phenotype. If *bon1* is suppressed by *mos4-1*, then 14.1% (9/64) of these plants would be expected to have been *bon-1*-like. If *bon1* is not suppressed by *mos4-1*, then 18.8% (12/64) of these plants would be expected to have been *bon-1*-like.

This suggests that *bon1*, like *snc1*, is suppressed by *mos4-1*. This is not surprising given that *bon1* is a negative regulator of *snc1*.

The contrast between the morphology of *mos4-1 snc1* and 60B-1plants was an unexpected finding. Since BON1 negatively regulates SNC1, it could reasonably be expected that when the *bon1* mutation is integrated into *mos4-1 snc1* plants, a *bon1*-like or *snc1*-like phenotype would be expected. However, the 60B-1 phenotype is much more severe than *bon1* or *snc1* single mutants. The SA-independent pathway is known to be involved in the positive amplification of defence signalling (Zhang *et al*., 2003). Perhaps, the SA-independent pathway is only required for amplification of low level signals (*e.g.* those present in *snc1* or *bon1* single mutants), and above a certain threshold of signalling, the SA-independent pathway is no longer required for positive amplification (Figure 1-1). In the *mos4-1 snc1* mutant, *PR1* expression is not fully suppressed (Palma et al., 2007), suggesting that a low level of signalling still occurs through the SA- and NPR1-dependent pathways. In addition, SA has long been known to positively regulate itself. When plants are treated with SA, endogenous SA levels increase. This could explain why 60B-1 has a severe phenotype, whereas *mos4-1 snc1* is WT-like. In *mos4-1 snc1* the level of signalling by *snc1* may be insufficient to induce constitutive defence without positive amplification from the SA-independent pathway. However, in 60B-1, *bon1* enhances snc1 signalling to an extent that SA can positively regulate itself and amplification from the SA-independent pathways is no longer required, leading to a severe auto-immune phenotype.

4.2 **83-2**

The data from 83-2 supports the hypothesis that the SA-independent pathway is only required for the positive amplification of low-level defence signalling. The 83-2 mutation

appears to enhance snc1 signalling such that SA could be increased enough to positively regulate itself.

Recently, our laboratory and others around the world have attempted to purify an R protein complex. These experiments have proven to be extremely challenging and largely unsuccessful. Because the 83-2 mutant could have a more active form of the SNC1 complex, it could be used to improve our chances of isolating the SNC1 complex. Transforming a *snc1* null mutant with tagged 83-2 in an effort to purify the SNC1 complex may be worth attempting. However, since the *SNC1* locus suppresses *SNC1* expression, even heterozygous transformants would probably be lethal.

4.3   **39-1**

The future experiments performed on 39-1 will depend on the identity of the mutated gene. Further mapping, bioinformatics analysis of the genome sequence and sequencing of candidate sequences should be performed to identify the molecular lesion.

4.4   **CONCLUSION**

Three defence-related suppressors of *mos4-1 snc1* were characterized and map-based cloning was used to search for their respective mutations. 60B-1 carries a mutation in *BON1*, a well-studied negative regulator of defence. *BON1* expression was not altered in any *mac* mutants, suggesting that the MAC does not regulate *BON1* expression. 83-2 was found to carry a second mutation to *SNC1*. Unlike the *snc1* mutation, 83-2 does not cause a further increase in snc1 protein accumulation. The 83-2 mutation probably changes snc1 protein structure into a more active form. The mutation in 39-1 was mapped between 29.87 Mb and the southern telomere of chromosome 1, but the causal mutation was not found. This research has shown that a *mos4 snc1* suppressor screen can successfully identify both recessive

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negative regulators of defence and dominant positive regulators of defence. Identification of

the remaining 29 mutations will likely reveal a significant number of new components in

plant defence signalling.

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