Two putative RNA-binding proteins function with unequal genetic redundancy in the MOS4-associated complex

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ABSTRACT

The MOS4-associated complex (MAC) is a highly conserved nuclear protein complex associated with the spliceosome. We recently purified the MAC from Arabidopsis thaliana nuclei, identified its potential components by mass spectrometry, and showed that at least five core proteins in the MAC are required for defense responses in plants. Here we report the characterization of a putative RNA-binding protein identified in the MAC named MAC5A and its close homolog MAC5B. We confirmed that MAC5A is a component of the MAC through co-immunoprecipitation with the previously described MAC protein AtCDC5. In addition, like all other characterized MAC proteins, MAC5A-GFP localizes to the nucleus. Double mutant analysis revealed that MAC5A and MAC5B are unequally redundant and that a double mac5a mac5b mutant results in lethality. Probably due to this partial redundancy, mac5a and mac5b single mutants do not exhibit enhanced susceptibility to virulent or avirulent pathogen infection. However, like other MAC mutations, mac5a-1 partially suppresses the autoimmune phenotypes of snc1, a gain-of-function mutant that expresses a deregulated TIR-NB-LRR type Resistance (R) protein. Our results suggest that MAC5A is a component of the MAC that contributes to snc1- mediated autoimmunity.
INTRODUCTION

Higher plants have evolved a sophisticated innate immune system against microbial pathogen infection. Pattern recognition receptors (PRRs) and Resistance (R) proteins represent two major classes of receptors in plants that recognize the presence of pathogens and trigger cell-autonomous defense responses (Jones and Dangl, 2006). Many PRRs are transmembrane receptor-like kinases (RLKs) that contain extracellular leucine-rich repeats (LRRs) and are involved in the perception of non-self epitopes common to whole groups of microbes called microbe-associated molecular patterns (MAMPs) (Zipfel, 2009). Most cloned R genes encode intracellular proteins containing a central nucleotide binding (NB) site and C-terminal LRRs and are involved in the recognition of effector proteins secreted by pathogens during infection. The activation of NB-LRRs is often associated with the onset of a cell-death program known as the hypersensitive response (HR), in which the plant cell kills itself to restrict pathogen growth. Most mutants with deregulated NB-LRR proteins exhibit seedling lethality, suggesting that NB-LRRs are under tight negative regulation to avoid unnecessary cell death (Lukasik and Takken, 2009). SUPPRESSOR OF NPR1-1, CONSTITUTIVE, 1 (SNC1) encodes an NB-LRR with an N-terminal Toll/Interleukin1 receptor (TIR) domain (Li et al., 2001; Zhang et al., 2003). The unique gain-of-function mutant snc1 exhibits constitutive defense responses in the absence of cell death, representing an autoimmune model amendable to genetic analysis. A forward genetic screen for suppressors of snc1 revealed that snc1-mediated defense includes components involved in nucleo-cytoplasmic partitioning (Palma et al., 2005; Zhang and Li, 2005; Cheng et al., 2009), protein modification (Goritschnig et al., 2007; Goritschnig et al., 2008), and RNA processing (Zhang et al., 2005; Palma et al., 2007). Importantly, like most other TIR-NB-LRRs, snc1 signaling requires the function of PHYTOALEXIN DEFICIENT4 (PAD4) and ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) (Li et al., 2001; Zhang et al., 2003).

MODIFIER OF SNC1, 4 (MOS4) is the founding member of the MOS4-associated complex (MAC) (Palma et al., 2007), a highly conserved spliceosome-associated complex homologous to the Prp19-complex (NTC) in yeast (Tarn et al., 1994) and the CDC5L complex in human (Ajuh et al., 2000). The MAC is composed of over 20 proteins in Arabidopsis, many of
which have predicted roles in RNA processing and/or splicing (Monaghan et al., 2009). Accordingly, several MAC proteins are encoded by essential genes including AtSKIP, EMB2765, CLO/VAJ/GFA/MEE5, EMB1507, ESP3/EMB2733 and SUS2/EMB177 (Tzafrir et al., 2004; Pagnussat et al., 2005; Herr et al., 2006; Moll et al., 2008; Lim et al., 2009; Liu et al., 2009; Yagi et al., 2009) (Table S1). We previously demonstrated that the MAC core proteins MOS4, AtCDC5, PRL1, and MAC3A/3B associate in planta and are all required for plant immunity, as loss-of-function mutations in the genes encoding these proteins result in plants exhibiting enhanced susceptibility to infection by virulent pathogens (Palma et al., 2007; Monaghan et al., 2009). In addition, these loci are broadly required for R protein-mediated defense pathways and snc1 autoimmune signaling.

Here we characterize two unequally redundant putative RNA binding proteins, MAC5A and MAC5B, with sequence similarity to the human RNA-binding motif protein RBM22. MAC5A was previously isolated as a component of the MAC (Monaghan et al., 2009), but has otherwise not yet been studied in plants. We show that MAC5A localizes to the nucleus and interacts with AtCDC5 in planta, confirming its association with the MAC. In addition, we show that MAC5A and its close homolog MAC5B are partially redundant in a dosage-dependent manner, and that a double mac5a mac5b mutant is lethal. Although single mac5a and mac5b mutants do not exhibit obvious enhanced susceptibility to pathogen infection, we found that mac5a-1 suppresses snc1-associated phenotypes. Overall, our results indicate that MAC5A is component of the MAC that functions in the snc1 signaling pathway.

RESULTS

Isolation of mac5a, mac5b, and mac5c T-DNA insertion mutants

We used the MAC5A protein sequence as a query in the basic local sequence alignment tool (BLAST) and identified two additional proteins with significant sequence similarity to MAC5A (At1g07360) encoded in the Arabidopsis genome. We named these proteins MAC5B (At2g29580) and MAC5C (At5g07060). MAC5A and MAC5B are proteins of approximately
480 amino acids in length that are 82% identical and contain a CCCH-type zinc-finger domain and an RNA recognition motif (RRM) (Figure 1A and Figure S1). Conversely, MAC5C is a truncated protein of 363 amino acids that contains only a zinc-finger domain and no RRM (Figure 1A and Figure S1). The phylogenetic relationship between these proteins indicates that MAC5A and MAC5B are more closely related to each other than to MAC5C (Addepalli and Hunt, 2008; Wang et al., 2008). In addition, according to publically available microarray data (Winter et al., 2007), MAC5A and MAC5B are expressed in similar tissue types, although MAC5A is expressed at a much higher level (Figure S2). Conversely, MAC5C is expressed at very low levels in dry seeds, senescent leaves and floral organs, but not at all in any other tissues (Winter et al., 2007) (Figure S2). MAC5A, MAC5B, and MAC5C share homology with the human protein RBM22/hECM2/fSAP47 (42-50% identity at the amino-acid level), and share very weak homology to the yeast protein Ecm2p/Slt11p (13-16% identity) (Figure S1). These proteins have been repeatedly isolated as components of the NTC/MAC in several eukaryotes (Ohi et al., 2002; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008; Herold et al., 2009; Monaghan et al., 2009).

To study the biological functions of MAC5A, MAC5B and MAC5C, we obtained T-DNA insertion alleles from Arabidopsis seed centers. Salk_132881 (mac5a-1) carries an insertion in the second exon of MAC5A, whereas Salk_142085 (mac5a-2) and Salk_072670 (mac5a-3) carry insertions in the first intron of MAC5A (Figure 1A). Semi-quantitative RT-PCR indicated that all alleles were genuine mac5a knock-out mutants as no MAC5A transcript could be detected compared to Col-0 (Figure S3B). The leaves of these mac5a mutants appeared slightly serrated, twisty, and often exhibited elongated petioles (Figure 1B and Figure S3A). In addition, mac5a mutants displayed shortened roots compared to Col-0 plants (Figure S3C), were early-flowering (Figure S3A) and had reduced seed yield (data not shown). Only plants homozygous for the mac5a mutations displayed the same morphological phenotypes, indicating that the alleles are recessive and that the phenotypes co-segregate with the mutations. Importantly, F1 progeny from crosses between these mutants did not exhibit phenotypic complementation (Figure S3A), confirming that they are allelic.

GK419F11 carries an insertion in the first exon of MAC5B (Figure 1A). This particular T-DNA insertion line is annotated as also being in the promoter region of PCNA2, a locus that
neighbours *MAC5B* in the opposite orientation on chromosome II (Figure S4A). Because of this, we examined the expression level of both genes in GK419F11 compared to Col-0 using semi-quantitative RT-PCR. We found that expression of *MAC5B* was strongly reduced in this line, whereas expression of *PCNA2* was not affected (Figure S4B). This indicated that GK419F11 is a null allele of *MAC5B* so we named it *mac5b-1*. Plants homozygous for the insertion did not exhibit any aberrant phenotypes and appeared morphologically similar to wild type Col-0 plants (Figure 1B). Although our *in silico* analysis indicated that *MAC5C* is barely expressed in *Arabidopsis*, we sought to test the biological function of this locus using a knock-out mutant as well. For this, we obtained WiscDsLox262F09, which carries an insertion in the fourth intron of *MAC5C*, very close to an intron-exon junction (Figure 1A). Like *mac5b-1* mutant plants, these *mac5c-1* plants also appeared indistinguishable from Col-0 (Figure 1B).

**MAC5A and MAC5B are unequally redundant**

Because of the sequence similarity between MAC5A, MAC5B, and MAC5C, we were interested to test for possible genetic redundancy between these loci. To do this, we crossed homozygous mutants together to create *mac5a-1 mac5b-1, mac5a-1 mac5c-1*, and *mac5b-1 mac5c-1* double mutants. The *mac5a-1 mac5c-1* double mutants appeared morphologically similar to *mac5a-1* single mutants (Figure S5A), and the *mac5b-1 mac5c-1* double mutants appeared morphologically similar to Col-0 wild-type plants (Figure S5B). These data suggest that there are no redundant functions shared between *MAC5C* and *MAC5A* or *MAC5B*. In contrast, *MAC5A* and *MAC5B* seem to perform some redundant functions in plants, as a double *mac5a-1 mac5b-1* mutant is lethal. Out of 127 randomly chosen progeny from a parent that was homozygous for *mac5b-1* but heterozygous for *mac5a-1*, 52 were wild-type for *MAC5A*, 75 were heterozygous for *mac5a-1*, and none were homozygous for *mac5a-1*, indicating a 1:2:0 ratio rather than a normal 1:2:1 ratio. A chi-squared test using these values indicates that a combination of *mac5a-1* and *mac5b-1* is lethal (χ²=36.86 with 2 degrees of freedom; *p*<0.0001). From another segregating population in which the parent was heterozygous for both *mac5a-1* and *mac5b-1*, we recovered very few plants (typically 1 or 2 out of ~100 plants) that were homozygous for *mac5a-1* and heterozygous for *mac5b-1*. These plants, in which there was only
one functional copy of MAC5B and no functional copies of MAC5A, displayed severe
developmental defects including dwarfism, delayed growth, abnormal floral organs and sterility
(Figure 2A). Terminal outgrowths known as enations were also observed extending from the leaf
margins of these plants (Figure 2B). To further investigate the redundant roles of MAC5A and
MAC5B, we tested if over-expression of MAC5B can compensate for the loss of MAC5A in
mac5a-1 mutants. For this, we stably expressed MAC5B under the control of the constitutive
CaMV P35S promoter in homozygous mac5a-1 plants by Agrobacterium-mediated
transformation. Analysis of transgenic plants revealed that expression of P35S-MAC5B was
indeed able to complement mac5a-1 (Figure 2A). Over-expression of MAC5B in the transgenics
was confirmed by semi-quantitative RT-PCR (Figure 2C). These results further supported
redundant functions between MAC5A and MAC5B, and suggested that the major difference
between these loci is linked to their steady-state expression levels. Thus, because a double
mac5a-1 mac5b-1 mutant is lethal, and over-expression of MAC5B can compensate for the loss
of MAC5A, we conclude that MAC5A and MAC5B are unequally redundant as defined by (Briggs
et al., 2006), and that MAC5A is the dominant contributor of the pair.

**mac5a-1 suppresses snc1 autoimmunity**

MOS4, AtCDC5, and MAC3A/3B are required for snc1-mediated immunity, as deduced from
double mutant analysis between snc1 and mos4, Atcdc5, or mac3a mac3b mutants (Palma et al.,
2007; Monaghan et al., 2009). To test if MAC5A, MAC5B, or MAC5C are likewise required for
snc1 signaling, we created snc1 mac5a-1, snc1 mac5b-1, and snc1 mac5c-1 double mutants and
conducted suppression analysis of snc1 related phenotypes. The loss of MAC5A (Figure 3), but
not MAC5B or MAC5C (Figure S6), suppressed snc1-associated dwarfism, as snc1 mac5a-1
plants were more than twice the size of snc1 (Figure 3A). Heightened resistance to
Hyaloperonospora arabidopsidis (H.a.) isolate Noco2 and Pseudomonas syringae pathovar
tomato (P.s.t.) strain DC3000 was also suppressed in snc1 mac5a-1, as indicated by
quantification of pathogen growth in these genotypes compared to Col-0 wild-type. As shown in
Figure 3B, whereas H.a. Noco2 was unable to colonize snc1 plants, snc1 mac5a-1 mutants were
as susceptible as Col-0 to this pathogen. Infection with P.s.t. DC3000 indicated that snc1
mac5a-1 plants sustain 100-fold higher bacterial growth over three days compared to snc1 (Figure 3C). Furthermore, quantitative RT-PCR showed that the constitutive expression of PR-1 and PR-2 in snc1 is partially suppressed by mac5a-1 (Figure 3D). Importantly, accumulation of the SNC1 transcript did not differ between the snc1 and snc1 mac5a-1 mutants (Figure 3D), indicating that altered SNC1 expression is not the likely cause for the observed suppression.

We previously demonstrated that MAC5A and MAC5B are unequally redundant and that MAC5A is the dominant contributor. Thus, it is likely that the reason mac5b-1 was unable to suppress snc1 autoimmunity was simply due to normal expression of MAC5A in the double mutants. To investigate the role of MAC5B in snc1 autoimmunity, we stably expressed P35S-MAC5B in snc1 mac5a-1 plants and tested for restoration of the snc1 phenotype. As a control, we also stably expressed PMAC5A-MAC5A in snc1 mac5a-1. Out of fifteen P35S-MAC5B transgenics, nine restored snc1 morphology (Figure S7). Similarly, out of twelve PMAC5A-MAC5A transgenics, nine restored snc1 morphology (Figure S7). These results indicate that MAC5B can function in the snc1 pathway and further demonstrates unequal genetic redundancy between MAC5A and MAC5B in Arabidopsis.

The loss of MAC5A or MAC5B does not result in enhanced disease susceptibility

Mutations in MOS4, AtCDC5, PRL1, and MAC3A/3B lead to enhanced susceptibility to pathogen infection (Palma et al., 2007; Monaghan et al., 2009). To test if any of the three MAC5 homologs are necessary for the full expression of basal defense responses in Arabidopsis, mac5a, mac5b, and mac5c mutants were challenged with P.s.t. DC3000 and bacterial growth was assayed after three days. We observed similar bacterial growth in Col-0 and the mutants (Figure 4A and Figure S8), whereas the well-known enhanced susceptibility mutant npr1-1 (Cao et al., 1994), harboured an over 10-fold higher titre of bacteria in all experiments. In addition, when we infected plants with H.a. Noco2, we consistently observed slightly higher oomycete colonization of the mac5a mutants compared to Col-0, however this difference could not be repeatedly supported by statistical analysis (Figure 4B).
To test if R protein mediated defenses other than *snc1* autoimmunity require *MAC5A* or *MAC5B*, we also infected plants with pathogens that express avirulence effectors specifically recognized by R proteins in Col-0. *P.s.t. avrRps4* and *P.s.t. avrPphB* (recognized by RPS4 and RPS5, respectively) grew to similar levels in Col-0 and the *mac5a* and *mac5b* mutants (Figure 4C-D). In addition, the *H.a.* isolates Emwa1 and Cala2, expressing avirulence effectors recognized by RPP4 and RPP2, respectively, grew to similar levels in Col-0 and *mac5a-1* (not shown), and both genotypes were able to mount an HR at infection sites as indicated by lactophenol trypan blue staining on leaf tissue seven days after inoculation (Figure S9).

Together, these data reveal that although *MAC5A* is required for *snc1*-mediated defense, *MAC5A* and *MAC5B* may not be required for other R protein mediated pathways or basal defense in plants. However, the involvement of *MAC5A* or *MAC5B* in these pathways may be masked by the partial redundancy between the two loci.

**MAC5A localizes to the nucleus and associates with AtCDC5 in planta**

To confirm that MAC5A is a MAC component, we tested whether MAC5A localizes to the nucleus and if it is capable of associating with the MAC protein AtCDC5 in a co-immunoprecipitation assay. For this, we stably transformed either *PMAC5A-MAC5A-GFP* or *PMAC5A-MAC5A-HA* into *mac5a-1* plants by *Agrobacterium*-mediated transformation. The majority of transgenic lines (24/25 independent GFP lines; 21/22 independent HA lines) complemented *mac5a-1* phenotypes such as morphology (Figure 5A) and root length (Figure 5B). Because these fusion proteins complemented *mac5a-1* phenotypes, they should function similarly to endogenous *MAC5A*. We confirmed that the fusion proteins were expressed *in vivo* by extracting total protein from representative transgenic lines followed by Western blot analysis using antibodies against GFP or HA (not shown).

All *MAC5A-GFP* complementing lines showed clear nuclear localization when viewed under a fluorescence microscope. Root cells from one representative line are shown in Figure 5C, but nuclear localization was also observed in other tissues including leaves (not shown). This localization was not unexpected, as the MAC5A homolog in zebrafish, RBM22, also localizes to the nucleus (Montaville et al., 2006), as do the MAC core proteins MOS4, AtCDC5,
PRL1, and MAC3A/3B. To test if MAC5A associates with AtCDC5, we isolated total nuclear protein and immunoprecipitated MAC5A-HA using anti-HA microbeads. Western blot analysis using an anti-AtCDC5 antibody revealed that AtCDC5 was present only in the eluted fraction from transgenic plants and not from control Col-0 plants (Figure 6). Association between MAC5A and AtCDC5 has been shown in other eukaryotes as well (McDonald et al., 1999; Ohi et al., 2002; Deckert et al., 2006; Gavin et al., 2006; Bessonov et al., 2008). These data indicate that MAC5A localizes to the nucleus and associates with AtCDC5 in planta. Thus, we conclude that MAC5A is indeed a member of the MAC.

Analysis of constitutive splicing in higher-order MAC mutants

Because of the close association between the MAC and the spliceosome, we previously tested if general splicing was impaired in the MAC mutants. For this, we monitored the presence of several known alternative transcripts in MAC mutants compared to Col-0 (Palma et al., 2007). We did not observe obvious differences in the presence of U1-70K, AtSRp34/SR1, or AtSRp30 alternative transcript variants (Palma et al., 2007), leading us to conclude that the MAC is not required for general splicing in Arabidopsis. However, although we have been unable to show aberrations in general/constitutive splicing, the MAC as a whole appears to be essential for plant viability, as all higher-order MAC mutants so far described (mos4-1 Atcdc5-1, mos4-1 prl1-1, mos4-1 mac3a mac3b, and mac3a mac3b prl1-1) result in lethality (Palma et al., 2007; Monaghan et al., 2009). Probably because of the partial redundancy between MAC5A and MAC5B, we were able to successfully isolate double mutants when we crossed mos4-1, Atcdc5-1, prl1-2, or mac3a mac3b with mac5a-1. The morphological phenotypes of the double mutants looked like a combination of phenotypes between the respective loci (Figure 7A). The most striking phenotype was observed with mac5a-1 prl1-2 double mutant plants that exhibited severe developmental defects and often displayed leaf enations similar to the mac5a-1/mac5a-1 MAC5B/mac5b-1 mutant. Interestingly, when we infected plants with P.s.t. DC3000 and monitored growth three days later, we did not observe a further increase in bacterial titre in any of the combination mutants (Figure S10 and data not shown), indicating that the additional loss of MAC5A does not further impair basal defenses. When we tested the fidelity of the splicing
machinery in these higher-order MAC mutants, we again did not observe any changes in the presence of U1-70K, AtSRp34/SR1, or AtSRp30 alternative transcript variants (Figure 7B). We also did not observe any changes in these transcripts in the mac5a-1; MAC5B/mac5b-1 mutant (not shown). These data indicate that the spliceosome is functional in MAC single and higher-order mutants and further demonstrates that individually MOS4, AtCDC5, PRL1, MAC3A/3B, and MAC5A/5B do not contribute key functions to general splicing in plants, even though the MAC is closely associated with proteins that form the spliceosome. It is important to keep in mind, however, that our analysis is not stringent enough to observe even several-fold differences in the abundance of splice variants, so we are not able to address splicing efficiency in the MAC mutants at this time. We also cannot rule out the possible involvement of the MAC in specific splicing events such as those that may be important during the plant immune response.

**DISCUSSION**

The MAC is a spliceosome-associated protein complex homologous to the well-studied NTC in yeast and the CDC5L complex in human (Palma et al., 2007; Monaghan et al., 2009). It contains proteins with diverse functions, including the scaffolding protein MOS4, the Myb transcription factor AtCDC5, the WD-40 protein PRL1, the E3 ubiquitin ligases MAC3A and MAC3B, as well as several nucleic-acid binding proteins and snRNP subunits that are predicted to be integral components of the spliceosome (Monaghan et al., 2009). Here we present evidence suggesting that the putative RNA-binding protein MAC5A is unequally redundant with its close homolog MAC5B and functions in snc1-mediated autoimmunity as a component of the MAC.

MAC5A and MAC5B are predicted to be RNA-binding proteins by virtue of containing both an RRM and a CCCH type zinc-finger motif. Although the intrinsic ability of MAC5A/5B to bind RNA species has yet to be shown experimentally, proteins containing CCCH zinc-fingers or RRMs have been demonstrated to bind RNA *in vitro* (Jessen et al., 1991; Burd and Dreyfuss, 1994; Wang et al., 2008). The most abundant RNA binding domain in eukaryotes is the RRM (Lorkovic and Barta, 2002), often found in proteins also containing CCCH motifs, such as in
MAC5A/5B. There are 196 RRM-containing proteins encoded in the *Arabidopsis* genome, including several predicted poly(A)-binding proteins (PABPs), arginine-rich (SR) proteins, and small nuclear ribonucleoproteins (snRNPs) (Lorkovic and Barta, 2002). Importantly, it has been demonstrated that the RRM is necessary and sufficient for RNA binding (Jessen et al., 1991; Burd and Dreyfuss, 1994). In addition to the RRM, there are several other RNA-binding domains such as the CCCH zinc-finger motif. This family is also fairly large in *Arabidopsis*, with 68 proteins encoded in the genome (Wang et al., 2008). Whereas most zinc-fingers are involved in DNA binding, several zinc-fingers containing CCCH motifs have been shown to function post-transcriptionally and have RNA binding capability (Wang et al., 2008). For example, the *Arabidopsis* protein ENHANCER OF AG-4,1 (HUA1) contains 6 tandem CCCH motifs and binds RNA *in vitro* (Li et al., 2001). Likewise, the *Arabidopsis* cleavage and polyadenylation specificity factor AtCPSF30 contains 3 tandem CCCH motifs and has also been shown to bind RNA (Delaney et al., 2006; Wang et al., 2008). Interestingly, a recent study demonstrated that five CCCH zinc finger proteins in *Arabidopsis*, representing proteins from diverse subfamilies, are capable of degrading RNA *in vitro*, uncovering the intriguing possibility that ribonuclease activity may be a common property of CCCH proteins in *Arabidopsis* (Addepalli and Hunt, 2008).

It is not hard to imagine roles for RNA-binding proteins and/or ribonucleases in plant defense and *snc1* autoimmunity. A large number of genes are differentially regulated following the perception of pathogens, and this massive change in gene expression must be correlated with subsequent RNA processing and/or degradation events. A relevant example is the glycine-rich RRM-containing protein AtGRP7 which was recently shown to be targeted by HopU1, a *Pseudomonas* type-III effector with mono-ADP-ribosyltransferase activity, to suppress the plant immune response and increase virulence (Fu et al., 2007). Loss-of-function *Atgrp7* mutants were shown to display enhanced susceptibility to infection by *Pseudomonas* in the same study. In addition, MOS2, a protein containing RNA-binding G-patch and KOW motifs, is likewise required for innate immunity in *Arabidopsis* (Zhang et al., 2005), although the precise function of this protein is unclear. Our finding that MAC5A is partly required for *snc1*-mediated autoimmunity points to a possible role for this RNA-binding protein in plant defense. However, we did not observe enhanced susceptibility in *mac5a* or *mac5b* single mutants when we infected
plants with a variety of pathogens, possibly complicated by the partial redundancy between MAC5A and MAC5B. The lethality of the mac5a-1 mac5b-1 double mutant prevented us from testing whether plants lacking both MAC5A and MAC5B exhibit enhanced disease susceptibility like the other MAC mutants characterized to date.

Importantly, whereas the MAC core components MOS4, AtCDC5, PRL1, and MAC3A/3B are not encoded by essential genes in Arabidopsis, both MAC5A and MAC5B are required for plant viability. Because of the physical association between the MAC and components of the spliceosome, it is very likely that the essential functions employed by MAC5A and MAC5B have something to do with RNA processing. That said, we have not yet been able to directly link the function of the MAC to splicing. We can only speculate that this association has to do with transcriptional co-regulation or alternative splicing of currently unknown gene targets. The presence of transcript variants for three known alternatively spliced genes, U1-70K, AtSRp34/SRI, and AtSRp30, was not affected in mac5a-1, mac5a-1; MAC5B/mac5b-1, or any other MAC mutants – broadly suggesting that the spliceosome is functional despite these lesions. And yet, the loss of any two MAC components in combination results in synthetic lethality, indicating that the MAC as a whole is required for an essential function, such as splicing or spliceosome assembly, as in yeast (Chan et al., 2003). In this respect, the identification of MAC substrates and downstream signaling components is of particular interest. Uncovering what genes are regulated by AtCDC5, what proteins are ubiquitinated by MAC3A/3B, and what RNA species are bound by MAC5A/5B will truly allow us to elucidate the molecular function of the MAC. A major hurdle, however, will be deciphering which downstream components are specific to the plant defense response. It is clear that MAC proteins are employed by several signaling networks, as indicated by the pleiotropic phenotypes observed for MAC mutants described to date. All MAC mutants, to one extent or another, show defects in flowering time, leaf development, fertility, root length, hormone signaling, sugar signaling, and innate immunity (Nemeth et al., 1998; Palma et al., 2007; Monaghan et al., 2009) (J.M. and X. L., unpublished data). Based on these observations, it is possible that the MAC acts as a key regulatory node utilized by many signaling pathways in plants. A joint effort to study the molecular function of this complex across kingdoms could shed light on the role of the MAC/NTC in signal
transduction relay and may lead to a better understanding of how plants utilize this complex to achieve immunity.

MATERIALS AND METHODS

Plant growth and pathology assays

Plants were grown on soil in a 16h light / 8h dark regime or on Murashige and Skoog (MS) medium supplemented with 0.5% sucrose and 0.3% phytogel or 0.8% agar, depending on the experiment. Bacterial and oomycete infections were performed as described in (Li et al., 2001). Briefly, bacterial pathogens were inoculated on the abaxial leaf surfaces of four-week old plants using a needless syringe. Leaf discs (with an area of 0.38cm²) were collected on the day of infection (Day 0) and three days later (Day 3) from different plants. *H. a.* isolates were spray-inoculated onto adaxial leaf surfaces of two-week old seedlings and stained using lactophenol trypan blue seven days later using a protocol described in (Koch and Slusarenko, 1990).

Mutant isolation and genetic crosses

T-DNA mutants were obtained from the ABRC or NASC and genotyped by PCR using primers flanking the insertions. *mac5a-1* was isolated and genotyped using the primers 5’-CACTCCTTAGGGAGGTATC-3’ and 5’-GGTGTTTAGGTGCGACCTGG-3’. The other two alleles, *mac5a-2* and *mac5a-3*, were identified first by phenotypic comparison to *mac5a-1* and then confirmed by crossing to test for allelism. For this, homozygous *mac5a-1* females were crossed with males homozygous for either the *mac5a-2* or *mac5a-3* alleles and the F₁ was analyzed for complementation. Heterozygosity at the *mac5a-1* locus in the F₁ plants was confirmed by genotyping. *mac5b-1* was isolated and genotyped using the primers 5’-CAGCTTCAACACTAAGAAAC-3’ and 5’-TAGAGTGTGGATCGAAACGG-3’. *mac5c-1* was isolated and genotyped using the primers 5’-GGTCAGTGTAAAAGAGGTGCC-3’ and 5’-GCTTGAAACCACATCCTCTTTC-3’. Isolation and genotyping of *snc1* (Li et al., 2001; Zhang et al., 2003), *mos4-1*, *Atcdc5-1*, *prl1-2* (Palma et al., 2007), and *mac3a mac3b* (Monaghan et al., 2009) have been previously described, as has isolation of *npr1-1* (Cao et al., 1994), *eds1-2* (Col)
(Bartsch et al., 2006), and ndr1 (Century et al., 1995). The sncl mac5a-1, sncl mac5b-1 and sncl mac5c-1 double mutants were obtained by crossing a homozygous sncl single mutant plant with homozygous mac5a-1, mac5b-1 or mac5c-1 plant. The F1 was allowed to self and the doubles were isolated in the F2 using a combination of phenotyping and PCR-based genotyping. A similar procedure was used to create the mac5a-1 mac5b-1, mac5a-1 mac5c-1, mac5b-1 mac5c-1, mac5a-1 mos4-1, mac5a-1 Atecdc5-1, mac5a-1 mac3a mac3b and mac5a-1 prl1-2 double and triple mutants. All genotypes were confirmed by genotyping with mutation-specific primers.

**Molecular cloning and expression analysis**

A genomic fragment spanning the full-length MAC5A gene including its native promoter was amplified from Col-0 DNA using the primers 5’-

CGGGGTACCCGGTTCAATGTCACCGGCAG-3’ (Kpn1) and 5’-

AAAACTGCAGCTGAGACGAACCAGTAGCTGT-3’ (Pst1) and cloned into pGreen0229 in-frame with a C-terminal HA or GFP tag (Hellens et al., 2000), and confirmed by sequencing. The open-reading frame of MAC5B was amplified from Col-0 cDNA using the primers 5’-

CACCATGGGCGCATAGAATCTGAG-3’ and 5’-TTGAGACGAACCAGTAGTAAC-3’. This Gateway-adapted PCR fragment was cloned into pENTR using the Gateway pENTR/D-Topo kit (Invitrogen). Entry vectors were confirmed by sequencing using the M13F and M13R primers. Recombination into a destination binary vector containing a constitutive 35S promoter was carried out with Gateway LR Clonase (Invitrogen). Transgenic seedlings were selected on soil with the herbicide Basta and confirmed by PCR. For mRNA expression analysis, RNA was extracted from 20 day old seedlings grown on MS medium using the Totally RNA Kit (Ambion). Reverse transcription was performed using Superscript II reverse transcriptase (Invitrogen). The primers used to monitor MAC5A expression were either 5’- TGCAAGATATGTACACGACC-3’ and 5’-AGTGCCCATCTCACCAGCTT-3’ (fragment size; 525bp), or 5’-

ACGCGTCCGACGATGGCTGCTCAGAAATACTGAG-3’ and 5’-

ATAGTTTAGCGGCCGCGCTCCTGAATAGCGGAAA-3’ (full length transcript; 1528 bp). The primers used for MAC5B expression were 5’-GCAATCTGCTCTCCAAGGT-3’ and 5’-
GCCGGGTACAGATCTTACAC-3’. The primers used to monitor the expression of PCNA2 were 5’-GATGGTAGCGACACTGTTAC-3’ and 5’- CCGATATCACCTGCTGTTGA-3’. The primers used to amplify PR-1, PR-2 and Actin 1 have been described previously (Zhang et al., 2003). Primers used to detect alternative transcript variants for U1-70K, AtSRp34/SRI and AtSRp30 are described in (Savaldi-Goldstein et al., 2003). TUBULIN was amplified using the primers 5’-ACGTATCGATGTCTATTTCAACG-3’ and 5’-ATATCGTAGAGAGCCTATTTGAAG-3’.

Nuclear protein extraction and immunoprecipitation

Approximately 15g of leaf tissue from complementing mac5a-1 plants expressing PMAC5A-MAC5A-HA was used to isolate the nuclear protein fraction with a procedure described in (Monaghan et al., 2009). Immunoprecipitation was carried out using anti-HA microbeads (Miltenyi Biotec Inc.) as described in (Monaghan et al., 2009). The eluted fraction was loaded on a 12% SDS-PAGE gel followed by Western Blot analysis using anti-HA (Roche) or anti-AtCDC5 (Palma et al., 2007) antibodies.

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FIGURE LEGENDS

Figure 1. Isolation of mac5a, mac5b, and mac5c loss-of-function mutants.

(A) Gene structures of MAC5A (At1g07360), MAC5B (At2g29580), and MAC5C (At5g07060) showing the positions of T-DNA insertions. Lines indicate introns and boxes indicate exons. The location of translation start (ATG) and stop (TGA) codons are indicated. The predicted protein domain structures are shown to the right of the gene structures. The phylogenetic relationship between the encoded proteins is indicated to the left, as predicted by (Addepalli and Hunt, 2008; Wang et al., 2008). (B) Morphology of homozygous mac5a-1, mac5b-1, and mac5c-1 mutants compared to Col-0. Soil-grown plants were photographed 3 weeks after germination. Size bar indicates 1 cm.

Figure 2. MAC5A and MAC5B are unequally redundant.

(A) Morphology of soil-grown plants, photographed 4 weeks after planting. Size bar represents 1 cm. (B) Close-up of a plant with the genotype mac5a-1; MAC5B/mac5b-1 taken approximately 6 weeks after germination. Arrows indicate leaf enations. Size bar represents 1 cm. (C) Expression analysis of MAC5A and MAC5B compared to ACTIN in the respective genotypes, as indicated by equal cycles of semi-quantitative RT-PCR. Primers used to amplify the MAC5A and MAC5B transcripts are described in the Methods section; the MAC5A fragment shown above is 525 bp and the MAC5B fragment is 232 bp. This experiment was repeated using cDNA isolated from four independent experiments with similar results.

Figure 3. The loss of MAC5A suppresses snc1 autoimmune phenotypes.

(A) Morphology of Col-0 compared to snc1, mac5a-1, and the double snc1 mac5a-1 mutant. Soil-grown plants were photographed 4 weeks after planting. Size bar represents 1 cm. (B) Growth of H.a. isolate Noco2 seven days after infection with 4 x 10^4 spores/ml water. Values represent an average of two replicates of 20 seedlings each ± SD. Statistically significant groups were calculated using multiple unpaired Student’s t-tests comparing the means of all samples; a indicates no significant difference compared to Col-0, b indicates a statistically significant difference from group a (p<0.008). This experiment was repeated three times with similar results. (C) Growth of P.s.t. DC3000 at 0 and 3 days after infection. Values represent an average
of four replicates ± SD. Statistically significant groups were calculated using multiple unpaired Student’s $t$-tests comparing the means of all samples; a indicates no significant difference compared to Col-0, b indicates a statistically significant difference from group a ($p<0.0008$). This experiment was repeated six times with similar results. (D) Steady-state expression analysis of PR-1, PR-2, and SNC1 relative to Col-0 and normalized against ACTIN1 using quantitative RT-PCR. Values represent the average of three experimental replicates ± SD. This experiment was repeated using cDNA isolated from five independent trials with similar results.

**Figure 4.** mac5a and mac5b mutants do not exhibit enhanced disease susceptibility.

(A) Growth of the bacterial pathogen *P.s.t.* DC3000 at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. Statistically significant groups were calculated using multiple unpaired Student’s $t$-tests comparing the means of all samples; a indicates no significant difference compared to Col-0, b indicates a statistically significant difference from group a ($p<0.0005$). This experiment was repeated six times with similar results. (B) Growth of *H.a.* isolate Noco2 seven days after infection with 2.5 x 10⁴ spores/ml in water. Values represent an average of four replicates of 20 seedlings each ± SD. Statistically significant groups were calculated using multiple unpaired Student’s $t$-tests comparing the means of all samples; a indicates no significant difference compared to Col-0, b indicates a statistically significant difference compared to group a ($p<0.005$). This experiment was repeated three times with similar results. (C-D) Growth of the bacterial pathogens *P.s.t.* avrRps4, and *P.s.t.* avrPphB, at 0 and 3 days post-inoculation. Values represent an average of four replicates ± SD. Statistically significant groups were calculated using multiple unpaired Student’s $t$-tests comparing the means of all samples; a indicates no significant difference compared to Col-0, b indicates a statistically significant difference from group a ($p<0.0001$). This experiment was repeated six times with similar results.

**Figure 5.** Sub-cellular localization of MAC5A-GFP.

(A) Full-length genomic MAC5A-GFP driven by its native promoter complements mac5a-1 morphology. Soil-grown plants were photographed 4 weeks after germination. Size bar represents 1 cm. (B) Root length assay demonstrating complementation of mac5a-1 by the MAC5A-GFP transgene. Plants were grown on MS media supplemented with 0.5% sucrose and
0.3% phytagel for 8 days. Values represent the mean of 10 replicates ± SD. Statistically significant groups were calculated using multiple unpaired Student’s $t$-tests comparing the means of all samples against Col-0; $a$ indicates no significant difference compared to Col-0, $b$ indicates a statistically significant difference from $a$ ($p<0.0001$). This experiment was repeated three times with similar results. (C) $MAC5A$-$GFP$ localizes to the nucleus. Confocal microscopy was used to examine the localization of $MAC5A$-$GFP$ in the transgenic line shown in A. Root cells from a representative line are shown. 4,6-diamidino-2-phenylindole (DAPI) was used as a control for nuclear localization.

**Figure 6. MAC5A-HA and AtCDC5 co-immunoprecipitate in planta.**

Total nuclear extracts were isolated from a complementing $mac5a-1$ transgenic line expressing $PMAC5A$-$MAC5A$-$HA$ (+) and Col-0 (-). MAC5A-HA was immunoprecipitated using anti-HA microbeads. MAC5A-HA and AtCDC5 were detected in the eluted fractions (20x concentrated) by Western blot analysis using antibodies against HA or AtCDC5.

**Figure 7. Analysis of constitutive splicing in higher-order MAC mutants.**

(A) Morphology of soil-grown plants, photographed 4 weeks after planting. Size bar represents 1 cm. (B) The presence of alternative transcript variants for the genes $UI-70K$, $AtSRp34/SRI$, and $AtSRp30$ was monitored in Col-0 compared with the MAC mutants $mos4-1$, $Atcdc5-1$, $prl1-2$, $mac3a$ $mac3b$, $mac5a-1$ and the higher-order mutants. An equal amount of RNA from all genotypes was used to make cDNA for subsequent analysis by semi-quantitative RT-PCR. $TUBULIN$ is included as a control. This experiment was repeated four times from independent trials. The schematic to the right represents the nature of the alternative transcript variants as adapted from (Palma et al., 2007).
