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Nonsense-Mediated mRNA Decay Modulates Immune Receptor Levels to Regulate Plant Antibacterial Defense

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SUMMARY

Nonsense-mediated mRNA decay (NMD) is a conserved eukaryotic RNA surveillance mechanism that degrades aberrant mRNAs. NMD impairment in *Arabidopsis* is linked to constitutive immune response activation and enhanced antibacterial resistance, but the underlying mechanisms are unknown. Here we show that NMD contributes to innate immunity in *Arabidopsis* by controlling the turnover of numerous TIR domain-containing, nucleotide-binding, leucine-rich repeat (TNL) immune receptor-encoding mRNAs. Autoimmunity resulting from NMD impairment depends on TNL signaling pathway components and can be triggered through deregulation of a single TNL gene, *RPS6*. Bacterial infection of plants causes host-programmed inhibition of NMD, leading to stabilization of NMD-regulated TNL transcripts. Conversely, constitutive NMD activity prevents TNL stabilization and impairs plant defense, demonstrating that host-regulated NMD contributes to disease resistance. Thus, NMD shapes plant innate immunity by controlling the threshold for activation of TNL resistance pathways.

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a eukaryotic translation-coupled RNA surveillance mechanism that degrades aberrant mRNA transcripts. Key features of NMD targets are premature termination codons (PTCs) arising as a consequence of mutation, transcription errors, or alternative splicing events (Schweingruber et al., 2013). In mammals, the core NMD machinery relies on the conserved proteins UPF1, UPF2, and UPF3, which participate in NMD target recognition (Isken and Maquat, 2008). Improper translation termination due to PTCs is thought to recruit UPF1 to stalled ribosomes, which leads to UPF1 phosphorylation (Kashima et al., 2006). Phosphorylated UPF1 facilitates the recruitment of the proteins SMG5, SMG7, and SMG6, which then promote mRNA degradation (Eberle et al., 2009; Okada-Katsuhata et al., 2012; Unterholzner and Izaurralde, 2004). Although UPF1, UPF2, and UPF3 homologs are present in all eukaryotes, homologs of SMG5 and SMG6 appear to be absent from plant genomes (Riehs et al., 2008). Plant and animal NMD target recognition is enhanced by the presence of the exon junction complex (EJC) downstream of PTCs, consisting of the core EJC factors Magoh, Y14, eIFAIII, and Barentsz (Ballut et al., 2005; Nyikó et al., 2013).

Genome-wide analyses showed that up to 20% of endogenous transcripts, including many functional protein-coding as well as noncoding RNAs, are also targeted by NMD due to the presence of NMD-eliciting signatures, including upstream open reading frames (uORFs), long 3′ untranslated regions (UTRs), and introns in 3′ UTRs (Drechsel et al., 2013; Kurihara et al., 2009; Mendell et al., 2004; Weischenfeldt et al., 2008). Thus, beyond its role in RNA surveillance, NMD might also regulate a wide spectrum of biological processes. This is supported by studies in mammalian cells that describe modulation of NMD by various cellular stresses and developmental cues (Bruno et al., 2011; Gardner, 2008; Mendell et al., 2004). Conserved autoregulatory circuits regulate NMD efficiency in mammals and plants, underscoring the importance of precise tuning of NMD to ensure transcriptome homeostasis (Huang et al., 2011; Nyikó et al., 2013; Yepiskoposyan et al., 2011). NMD null mutations are embryonic lethal in *Drosophila*, zebrafish, and mouse, and this has impeded identification of NMD-regulated processes (Hwang and Maquat, 2011). NMD is also essential in plants since disruption of *Arabidopsis* NMD components UPF1 and SMG7 caused seedling death and retarded development, respectively (Arciga-Reyes et al., 2006; Riehs et al., 2008; Yoine et al., 2006). NMD impairment in *Arabidopsis* was linked to constitutive

activation of plant immune responses, characterized by elevated expression of defense genes, production of the plant defense hormone salicylic acid (SA), and enhanced resistance to bacterial infection (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012). Importantly, disruption of disease resistance signaling rescued the adverse effects of *upf1* and *smg7* mutations (Riehs-Kearnan et al., 2012). Thus, aberrant pathogen response signaling is a major physiological consequence of impaired NMD in *Arabidopsis*, suggesting a role for NMD in regulating plant immunity.

The plant innate immune system employs successive defense layers to inhibit pathogen infection. One defense layer relies on plasma membrane-localized receptors that detect conserved pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs triggers transcriptional reprogramming, known as PAMP-triggered immunity (PTI), that is sufficient to confer resistance against nonadapted pathogens (Jones and Dangl, 2006). Infectious (virulent) pathogenic strains deliver a battery of effectors to host cells to interfere with defense programs. However, these pathogens encounter a postinfection resistance barrier (hereafter referred to as postinfection basal resistance or immunity), controlled by intracellular complexes of the immune regulators EDS1 and PAD4, which reinstate resistance to slow infection (Rietz et al., 2011), in part via the SA defense signaling pathway (Fu and Dong, 2013). Basal resistance to virulent pathogens is further reinforced by intracellular nucleotide-binding, leucine-rich repeat (NLR) receptors, which detect the presence or actions of specific pathogen effector proteins to confer effector-triggered immunity (ETI) (Dodds and Rathjen, 2010). NLRs represent one of the largest and most variable plant gene families that have undergone rapid evolutionary diversification, likely driven by pathogens (Guo et al., 2011). ETI engages components of the basal resistance machinery to amplify defense programs, often culminating in programmed cell death at local infection sites, which is also known as the hypersensitive response (HR) (Maekawa et al., 2011). Plant NLRs are broadly divided into two subclasses carrying an N-terminal Toll/interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain (referred to, respectively, as TNLs and CNLs), with genetically distinct signaling requirements (Heidrich et al., 2012). The expression of NLRs is tightly regulated at transcriptional and postranscriptional levels (Staiger et al., 2013), and NLR misexpression often triggers autoimmunity associated with severe stunting and fitness costs (Alcázar and Parker, 2011).

Here we elucidate the mechanism underlying autoimmunity caused by NMD deficiency in *Arabidopsis*. We show that autoimmune phenotypes associated with SMG7 dysfunction are due to TNL receptor transcript misregulation and that host-modulated NMD dynamically regulates the abundance of TNL receptors, thus influencing immune response thresholds. We conclude that NMD represents a physiological gene regulatory mechanism contributing to plant innate immunity.

RESULTS

Genetic Requirements of *smg7* Autoimmunity

Arabidopsis accession Columbia (Col) carrying the loss-of-function *smg7-1* mutation (hereafter referred to as *smg7*) exhibits autoimmunity, characterized by stunting, spontaneous formation of necrotic lesions, and elevated SA levels. The *smg7* aberrant phenotypes were suppressed by mutations in the basal and TNL immunity regulator *PAD4* (Figures 1A, 1B, and S1A). By contrast, defects in a CNL receptor signaling component, NDR1, did not rescue the *smg7* mutant (Riehs-Kearnan et al., 2012). We explored further the genetic basis for NMD autoimmunity by crossing *smg7* with a loss-of-function mutant of the key basal and TNL immunity regulator EDS1. Mutation of *EDS1* fully suppressed *smg7* stunting and expression of the SA-responsive marker gene *PR1* (Figures 1A, 1B, and S1A). Combinations of *smg7* with mutations in the HSP90 cochaperones *RAR1* and *SGT1b*, which assist the activation of many NLR receptor complexes (Kadota et al., 2010), also suppressed *smg7* stunting and strongly reduced *PR1* expression, but retained some altered *smg7* leaf morphology (Figures 1A, 1B, and S1A). Notably, inhibition of SA biosynthesis by mutation of *ICS1*/*SID2* did not suppress *smg7* stunting, but reduced *PR1* expression and chlorosis (Figures 1A, 1B, and S1C). The above *smg7* genetic requirements point to involvement of TNL receptor signaling in *smg7* autoimmunity, since TNLs immediately engage EDS1/PAD4 basal immunity signaling components (Heidrich et al., 2011; Rietz et al., 2011; Wagner et al., 2013). This is further supported by the suppression of autoimmunity in *smg7* plants grown at 28°C compared to 21°C (Figures 1D, 1E, and S1B), because TNL resistance is temperature sensitive (Alcázar and Parker, 2011). By contrast, early PTI defense signaling outputs, such as generation of reactive oxygen species (ROS) and induction of PTI marker genes *FRK1* and *WRKY29* upon treatment with the flagellin-derived PAMP elicitor flg22, were unaltered in *smg7* and *smg7 pad4* plants, despite the fact that *smg7* plants showed elevated *FRK1* steady-state levels (Figures S1D and S1E). We also tested growth in plants of virulent *Pseudomonas syringae pv tomato* (*Pst*) DC3000 and the type III secretion-defective *Pst* DC3000 *hrcC*$^{-}$ strain, which fails to inject effector proteins into host cells to suppress PTI (Dodds and Rathjen, 2010). While *smg7* mutants were highly resistant to virulent *Pst* DC3000 and *Pst* DC3000 *hrcC*$^{-}$ compared to wild-type, enhanced resistance was abolished in *smg7 pad4* plants (Figure S1F). These data suggested that increased resistance of *smg7* mutants to virulent *Pst* DC3000 and *Pst* DC3000 *hrcC*$^{-}$ depends on *PAD4*, consistent with its role in basal and TNL resistance (Rietz et al., 2011). The above findings further substantiate a role for NMD in TNL receptor regulation, but not in early PTI responses to pathogens.

Next, we examined whether alleviation of *smg7* autoimmunity in certain resistance signaling mutant backgrounds could be explained by restored NMD efficiency. For this, we measured the accumulation of endogenous PTC-containing (PTC+) splice variants of *RS2Z33* and AT2G45670 that are targeted by NMD (Kalyna et al., 2012). Aberrant PTC+ mRNA levels were increased in all *smg7* immunity mutant backgrounds (Figure 1C), as well as in *smg7* plants grown at 28°C or 21°C (Figure 1F). Therefore, suppression of TNL resistance pathways does not alter the NMD defect in *smg7* but rather the consequences of altered NMD efficiency.

Stability of TNL Receptor Transcripts Is Directly Regulated by NMD

Since reduced NMD leads to autoimmunity, we asked whether NMD controls the expression of immunity-related transcripts.

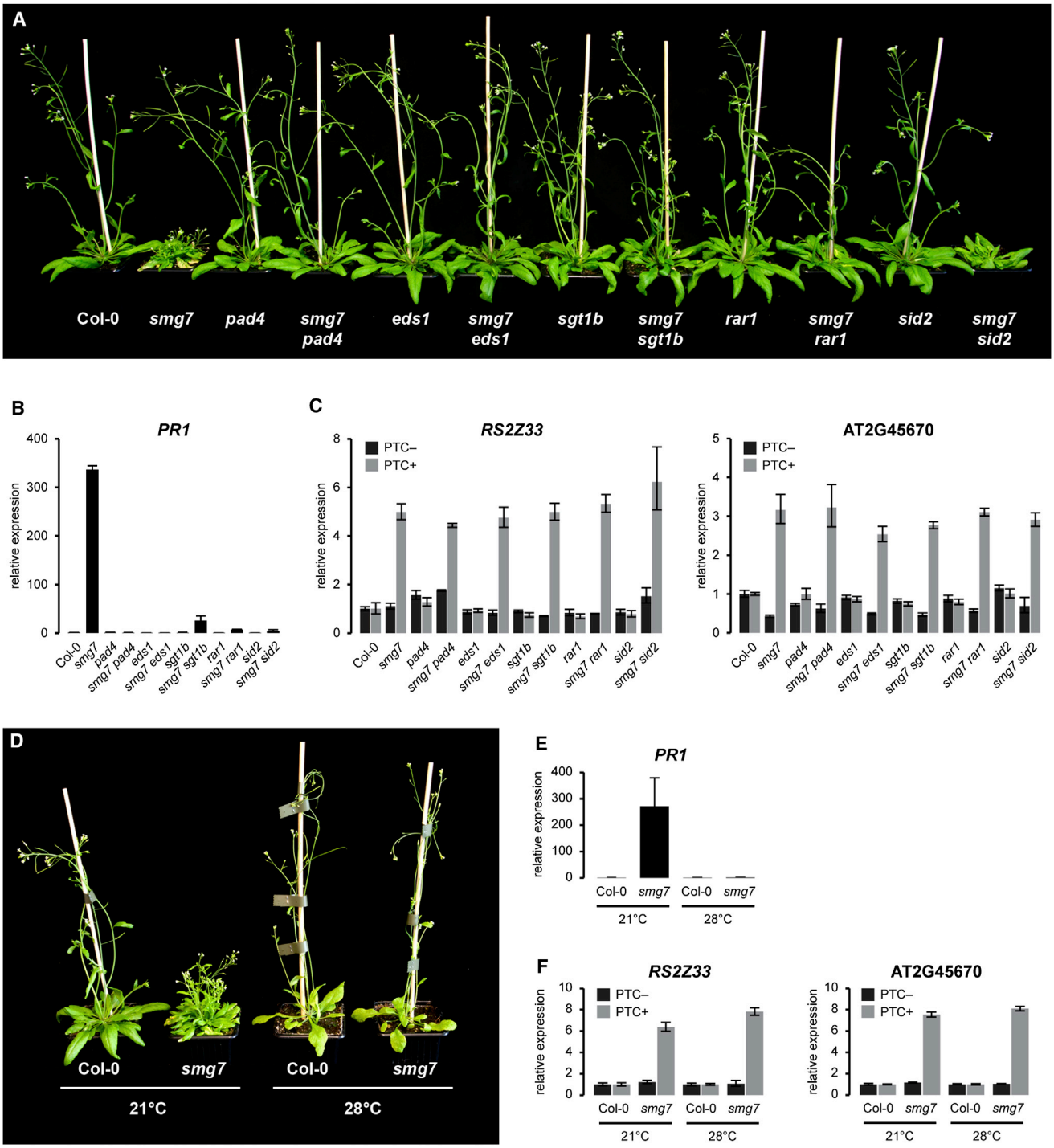


Figure 1. *Smg7* Autoimmunity Is Conditioned by TNL Signaling Components

(A) Morphology of 5-week-old plants of the indicated genotypes.

(B) *PR1* expression measured by qRT-PCR in the indicated genotypes.

(C) Expression of PTC−/+ splice variants of NMD reporters *RS2Z33* and AT2G45670 in the indicated genotypes.

(D) Morphology of 5-week-old Col and *smg7* plants grown at 21°C and 28°C.

(E) *PR1* expression in plants shown in (D).

(F) Expression of NMD reporters *RS2Z33* and AT2G45670 in plants shown in (D).

Expression levels in (B), (C), (E), and (F) are mean ± standard error of mean (SEM) of four biological replicates. See also Figure S1.

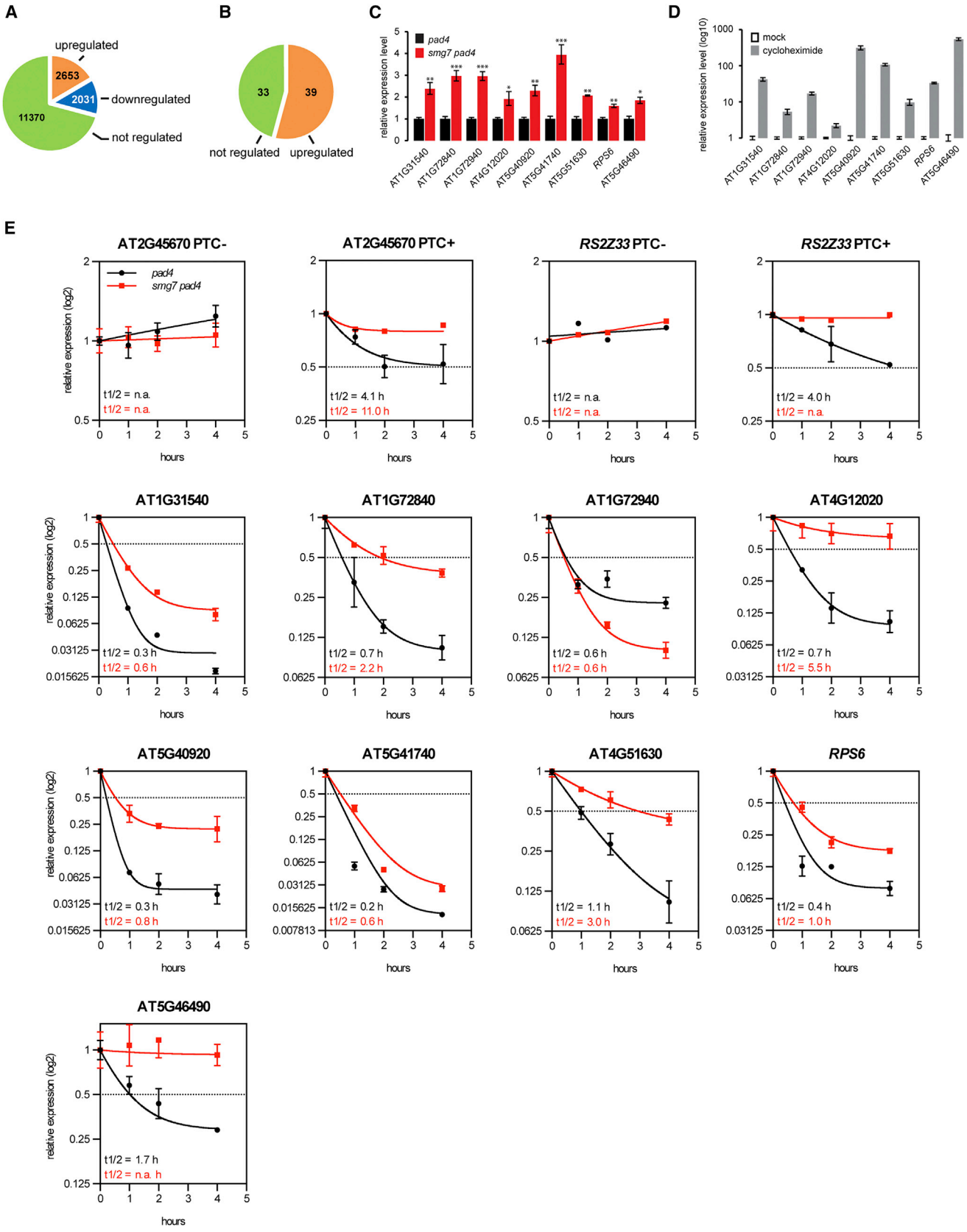
Published whole-genome expression data in NMD-impaired *Arabidopsis* mutants showed that altered NMD affects a significant portion of the transcriptome (Drechsel et al., 2013; Kurihara et al., 2009), but developmental abnormalities of these mutants impede identification of direct NMD targets. To avoid this problem, we performed RNA-seq analysis of *smg7 pad4* mutants that develop normally due to the block in immunity signaling (Figure 1A). Of 17,269 mRNAs expressed in leaf tissues, 2,653 mRNAs (~15%) were significantly upregulated in *smg7 pad4* compared to *pad4* control plants (false discovery rate corrected p value $q < 0.05$; Figures 2A and S2A and Table S1). Gene Ontology (GO)-term classification of upregulated transcripts showed no enrichment for the category "response to abiotic or biotic stimulus" (Fisher's exact test, p = 0.1193; Figure S2B), consistent with abrogated defense signaling in *pad4* and showing that NMD-regulated transcripts are broadly distributed across all GO-term categories. In addition, comparison of *smg7 pad4* upregulated transcripts (1.8-fold threshold) revealed substantial overlap with expression data of two weak NMD mutants, *upf1-1* and *upf3-1*, which exhibit extremely mild autoimmune phenotypes (Kurihara et al., 2009) (Figure S2C). We then examined the RNA-seq data for differences in the expression of NLRs. *Arabidopsis* accession Col contains 110 annotated TNLs and 59 CNLs (Meyers et al., 2003), of which 72 TNLs and 42 CNLs were expressed in our RNA-seq data set (Table S2). Thirty-nine expressed TNLs and 11 CNLs were upregulated in *smg7 pad4* mutants, representing a statistically significant enrichment of TNL transcripts in the absence of autoimmunity (Fisher's exact test, $p = 5.329^{-16}$), while CNL transcripts were not significantly enriched (p = 0.155) (Figures 2B and S2D). Based on transcript annotations (TAIR 10 and by Tan et al. [2007]), we established that ~50% of the upregulated TNLs carry putative NMD features (Figure S2E and Table S2). By contrast, canonical NMD features were absent in upregulated CNLs, suggesting that they are not NMD targets (Table S2). We validated the RNA-seq trends by measuring steady-state levels of selected TNL and CNL transcripts by qRT-PCR in *smg7 pad4* and *pad4* leaves (Figures 2C and S2F). Among the *smg7 pad4* upregulated TNLs, we identified *RPS6* that was reported to undergo alternative splicing (Kim et al., 2009). We found that a subset of *RPS6* PTC+ splice variants was upregulated in *smg7 pad4*, indicating regulation by NMD (Figure S2G). Transcript levels of other selected TNLs were also increased in wild-type plants treated with the NMD and translation inhibitor cycloheximide (Figure 2D). To determine whether TNL and CNL transcripts are regulated by NMD, we assessed their mRNA degradation kinetics after imposing a transcriptional block (Johnson et al., 2000). Similar to the PTC+ splice variants of NMD reporters AT2G45670 and *RS2Z33*, we found increased half-lives for all tested TNL transcripts in *smg7 pad4* plants, with the exception of AT1G72940, which is likely to be transcriptionally regulated (Figure 2E). In agreement with previous studies in mammalian cells, most NMD-targeted TNLs and NMD control transcripts showed biphasic degradation kinetics (Trcek et al., 2013). By contrast, *smg7 pad4* upregulated CNLs did not display changes in half-lives, suggesting that they are not processed by NMD (Figure S2H). Taken together, our results show that NMD specifically targets a large subset of TNL receptor transcripts.

A Natural Variant of TNL *RPS6* Confers Autoimmunity in NMD-Impaired Plants

Given the extensive natural variation in NLR repertoires across *Arabidopsis* accessions (Guo et al., 2011), we examined the impact of a different genetic background, that of accession Landsberg *erecta* (L*er*), on *smg7* autoimmunity. When we crossed Col *smg7* with L*er*, we found that stunting was suppressed in a portion of recombinant F2 *smg7* Col/L*er* progeny, suggesting the existence of natural polymorphism(s) affecting constitutive resistance (Figures S3A and S3B). Since introgression of the *smg7* mutation into a L*er* background by four further crosses to L*er* completely restored the stunting phenotype (Figures S3A and S3C), we concluded that the suppression of *smg7* autoimmunity in the F2 population arises out of a combination of Col and L*er* polymorphisms at multiple genetic loci. Genetic analysis of the L*er*-derived *smg7* suppressors pointed to the existence of at least two independent suppressor loci that displayed recombination suppression, impeding further mapping. However, we found that the suppressor phenotype of *smg7* plants in L*er* (hereafter referred to as L*er-smg7* plants) was determined by a single Col locus, denoted *REX* (*RIESENEXPERIMENT*) (Figures 3A, S3A, and S3C).

While L*er-smg7* plants harboring $REX^{Ler/Ler}$ exhibited hallmarks of autoimmunity, L*er-smg7* plants carrying the $REX^{Col/Col}$ suppressor locus did not exhibit constitutive resistance signaling (Figures 3A–3C). The REX^{Ler} allele was semidominant, consistent with a model in which L*er-smg7* autoimmunity is conferred by the REX^{Ler} locus in a dose-dependent manner (Figures 3A–3C). Since NMD reporter levels increased in L*er-smg7* mutants irrespective of the *REX* allele present, we concluded that *REX* does not encode an NMD modifier (Figure 3D). We mapped the *REX* locus to a 240 kb region on chromosome 5, encompassing 60 genes between AT5G46520 and AT5G46850 in the L*er* reference sequence, including disease resistance signaling genes and several TNLs (Figure 3E). RNA-seq analysis of L*er-SMG7* $REX^{Ler/Ler}$ and L*er-smg7* $REX^{Ler/Ler}$ plants revealed that of six TNL genes in the REX^{Ler} region, three (*RPS6*, AT5G46490, and AT5G46500) were overexpressed in L*er-smg7* $REX^{Ler/Ler}$ plants compared to L*er-SMG7* $REX^{Ler/Ler}$ (Figure 3F). To identify the causative TNL, we transformed wild-type-appearing L*er-SMG7*$^{+/-}$ $REX^{Col/Col}$ plants with genomic constructs of upregulated candidate TNLs and scored transformed L*er-smg7* $REX^{Col/Col}$ T2 progeny for autoimmunity. L*er-smg7* $REX^{Col/Col}$ plants transformed with $RPS6^{Ler}$ displayed autoimmunity, but segregating *SMG7* siblings did not (Figures 4A and 4B; 9 out of 11 independent transgenic lines). Also, $RPS6^{Ler}$ mRNA levels increased in L*er-smg7* transformants but not in transformed *SMG7* siblings (Figure 4C). These results suggest that the TNL $RPS6^{Ler}$ locus underlies L*er-smg7* autoimmunity in an NMD deficiency-dependent manner.

Both $RPS6^{Ler}$ and $RPS6^{Col}$ transcripts are NMD targets since their levels increased similarly upon NMD inhibition by cycloheximide (Figure S4A). Therefore, the $RPS6^{Ler}$-conditioned autoimmunity in L*er-smg7* plants is not explained by its specific targeting for NMD. The $RPS6^{Ler}$ allele harbors one in-frame INDEL and six nonsynonymous mutations in the coding region, but none were within conserved TNL motifs such as the P-loop, Walker B, or MHD motifs that would account for a functional difference between $RPS6^{Col}$ and $RPS6^{Ler}$ (Figure 4D). Additionally, no



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polymorphism was predicted to affect splice donor or acceptor sites. Previously, a survey of 32 *Arabidopsis* accessions revealed *RPS6*-conditioned natural variation in immune responses to *Pst* effector HopA1. Some *RPS6* alleles, including $RPS6^{Ler}$, induced a pronounced HR upon HopA1 effector recognition, while this was not the case for $RPS6^{Col}$ (Gassmann, 2005). In agreement, we found that L*er-SMG7* $REX^{Ler/Ler}$ plants inoculated with *Pst* DC3000-HopA1 displayed a strong HR, whereas L*er-SMG7* $REX^{Col/Col}$ plants produced no macroscopically discernable HR (Figure S4B). These data suggest that autoimmunity in L*er-smg7* $REX^{Ler/Ler}$ plants is caused by derepression of $RPS6^{Ler}$, which induces a strong HR. Collectively, the data show that naturally occurring variation in TNLs can influence immunity and cell death thresholds in NMD-deficient plants, and that in certain genetic backgrounds deregulation of a single NMD-regulated TNL is sufficient to trigger autoimmunity.

Virulent Bacterial Pathogen Infection Reduces NMD Efficiency

Inappropriate activation of immune responses in *Arabidopsis* NMD mutants leads to increased resistance against infectious bacterial pathogens (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012). Therefore, we asked whether NMD efficiency is itself modulated in response to pathogen infection, as a possible target for pathogen interference and/or means for the plant to promote resistance. To test this, we spray-inoculated Col wild-type plants with virulent *Pst* DC3000 and measured the effect on endogenous NMD targets. Coinciding with increased *PR1* levels at 48–72 hr postinoculation (hpi) (Figure 5A), we observed elevated transcript levels of NMD factors *SMG7* and *BARENTSZ1* at 48 and 72 hpi and an increase in *RS2Z33* PTC+ at 72 hpi (Figure 5A). Since *SMG7* and *BARENTSZ1* levels are autoregulated by NMD, their increased accumulation reflects decreased NMD efficiency (Nyikó et al., 2013). Expression of several NMD-targeted TNLs, including *RPS6*, was also increased at 72 hpi (Figure 5A). We then examined transcript stability in order to discern whether upregulation of the NMD targets was a result of impaired NMD. Analysis of mRNA decay rates upon imposing a transcriptional block 72 hr after *Pst* DC3000 infection revealed that half-lives of NMD reporters and NMD-targeted TNLs increased after *Pst* DC3000 inoculation compared to mock treatment (Figure 5B). Therefore, we concluded that NMD target upregulation was a result of impaired NMD. In line with our previous analysis of TNL stability in *smg7 pad4* plants (Figure 2E), the decay rate of TNL AT1G72940 did not change after *Pst* DC3000 treatment (Figure 5B), arguing that its increased expression was due to increased transcription. We concluded that virulent bacterial infection leads to reduced NMD and thus stabilization of NMD targets, including TNL transcripts.

To test whether NMD inhibition is a result of pathogen effector activities targeting NMD or is part of an intrinsic host defense program, we inoculated wild-type plants with virulent *Pst* DC3000 or *Pst* DC3000 $hrcC^{-}$ strain that triggers strong PTI. We used leaf syringe infiltration for both strains because the *Pst* DC3000 $hrcC^{-}$ strain grows poorly on wild-type plants. Transcripts of *SMG7*, *RS2Z33* PTC+, and the TNL *RPS6* were upregulated at 24–48 hpi in response to *Pst* DC3000 and *Pst* DC3000 $hrcC^{-}$ (Figure 6A). Similar expression trends were observed after treatment of wild-type seedlings with the PAMP-elicitor flg22 (Figure 6B). Because PAMP perception alone reduced NMD, we concluded that NMD suppression is an integral part of the host-programmed immune response.

Previous studies in mammalian cells showed that stress-induced phosphorylation of the eukaryotic translation initiation factor eIF2α by GCN2 kinase, for example, upon amino acid starvation, leads to inhibition of translation and thus suppression of NMD (Mendell et al., 2004). We therefore tested if GCN2-dependent phosphorylation of eIF2α inhibits NMD in *Arabidopsis*. Amino acid starvation led to NMD suppression in both Col and *gcn2-2* mutant seedlings, suggesting that GCN2 is not required for NMD inhibition (Figures S5A and S5B). Similarly, the *gcn2-2* mutation did not abolish NMD inhibition when we spray-inoculated plants with virulent *Pst* DC3000 bacteria (Figure S5C). Furthermore, *gcn2-2* mutants did not have altered basal resistance to virulent *Pst* DC3000 (Figure S5D), arguing that GCN2-dependent phosphorylation of eIF2α is dispensable for stress-induced NMD inhibition and does not contribute to immune responses.

Disruption of *SMG7* Autoregulation Sensitizes Plants to Infection

We reasoned that dampening of NMD upon bacterial pathogen infection might represent a physiological mechanism for bolstering defenses. If this were the case, failure to suppress NMD during infection would lead to increased disease susceptibility. To test this, we generated *smg7* mutants expressing *SMG7* cDNA from an actin promoter. The *SMG7* construct lacked an endogenous *SMG7* 3′ UTR with introns, rendering its transcript insensitive to NMD autoregulation (*SMG7-ΔUTR*). The *SMG7-ΔUTR* transgene fully complemented *smg7* autoimmunity in two independent lines (Figures 7A, 7B, S6A, and S6B). Both lines expressed ~2-fold higher *SMG7* mRNA levels (Figures 7B and S6C) than wild-type and had lower levels of multiple NMD targets, demonstrating increased NMD efficiency (Figures 7B, S6D, and S6E).

Figure 2. NMD Targets TNL Transcripts

(A) Classification of significantly upregulated (adjusted p value $q < 0.05$), downregulated ($q < 0.05$), or not significantly changed transcripts ($q \geq 0.05$) in *smg7 pad4* relative to *pad4* control plants. The number of transcripts in each category is indicated.

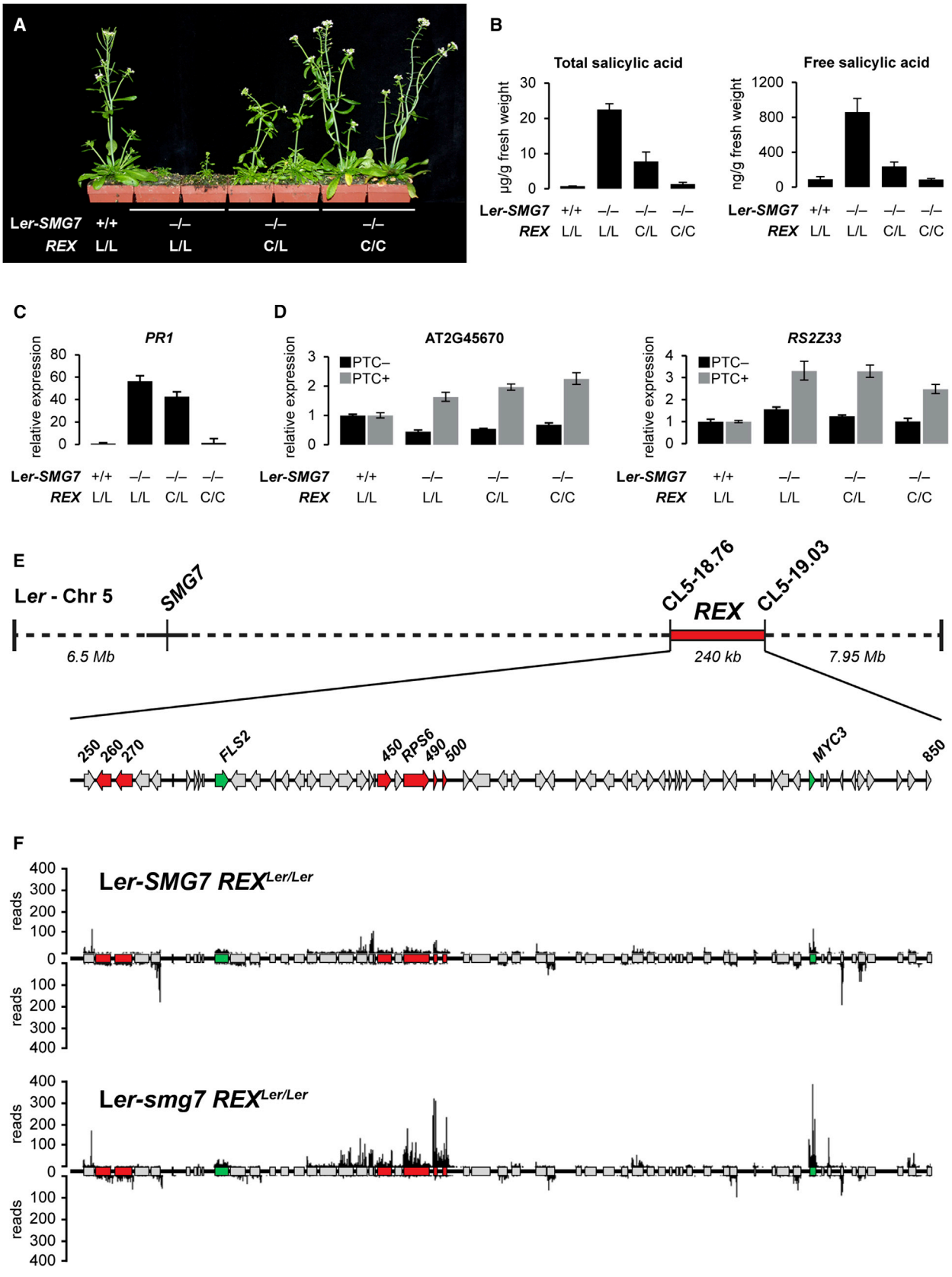
(B) Classification of significantly upregulated ($q < 0.05$) or expressed but not significantly changed TNL transcripts ($q \geq 0.05$) in *smg7 pad4* relative to *pad4* plants. The number of transcripts in each category is indicated.

(C) Steady-state levels of selected TNL transcripts in *pad4* and *smg7 pad4* measured by qRT-PCR (mean ± SEM, three biological replicates). Significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) compared to *pad4* control samples are indicated (two-tailed Student's t test).

(D) Expression of selected TNL transcripts 5 hr after translational inhibition with cycloheximide in wild-type plants (mean ± SEM, three biological replicates).

(E) qRT-PCR analysis of half-lives of TNL transcripts and NMD reporters AT2G45670 and *RS2Z33* in *pad4* and *smg7 pad4*. Half-lives ($t_{1/2}$) were calculated by nonlinear least-square regression (data points are mean ± SEM, three biological replicates). The experiment was repeated twice with similar results.

See also Figure S2 and Tables S1 and S2.



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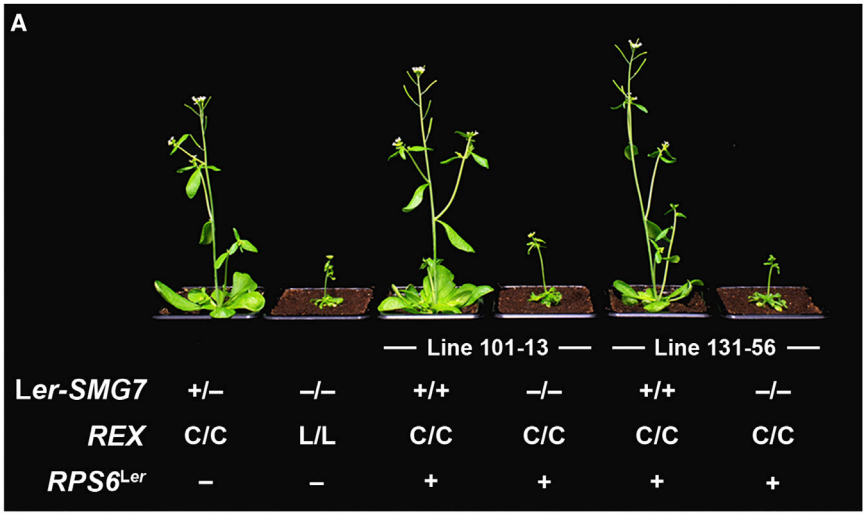


Figure 4. The TNL *RPS6* Underlies L*er-smg7* Autoimmunity

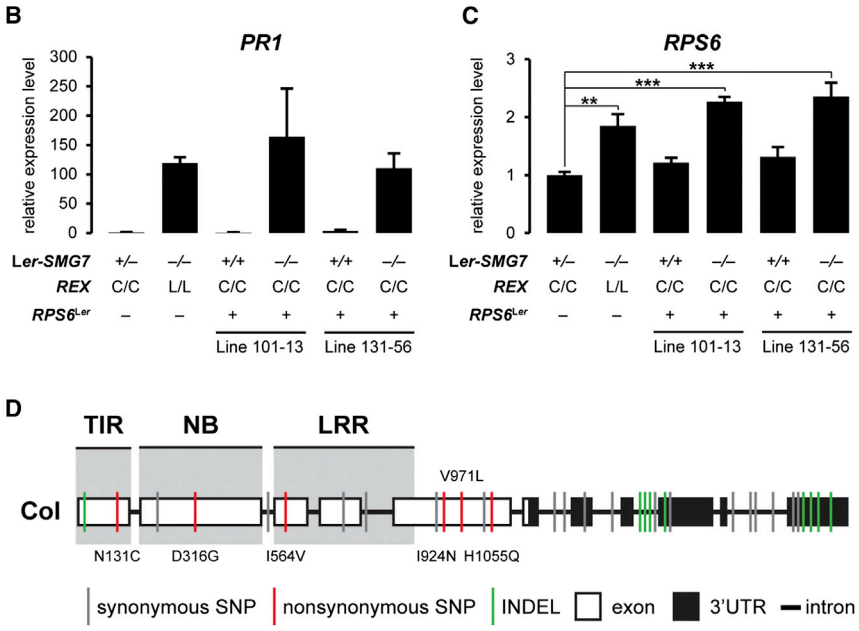
(A) Independent T2 progeny lines 101-13 and 131-56 derived from L*er-SMG7*$^{+/-}$ plants harboring *REX*$^{Col/Col}$ (left) transformed with a genomic construct of *RPS6*^{Ler}. L*er-smg7 REX*$^{Ler/Ler}$ plants (second to the left) are shown as control for the stunting phenotype. The allelic variant of *REX* is indicated with "L" (L*er*) or "C" (Col). L*er-SMG7*$^{+/-}$ control plants are shown on the left.

(B) *PR1* expression in plants shown in (A).

(C) Total levels of *RPS6* transcripts (derived from Col and L*er* alleles) in plants shown in (A). Asterisks indicate significant differences (**p < 0.01, ***p < 0.001) to L*er-SMG7*$^{+/-}$ *REX*$^{Col/Col}$ (two-tailed Student's t test).

(D) Domain organization and exon/intron structure in *RPS6*^{Col}. The depicted polymorphisms were identified in *RPS6*^{Ler} by Sanger sequencing.

Values shown in (B) and (C) are mean ± SEM of four biological replicates. See also Figure S4.



We next tested whether expression of *SMG7-ΔUTR* influenced NMD regulation after bacterial infection. Whereas spray inoculation of wild-type plants with virulent *Pst* DC3000 led to a 2-fold increase in *SMG7* mRNA, *SMG7* transcript levels in *SMG7-ΔUTR* plants remained constant (Figures 7C and S6F). Unlike wild-type, *SMG7-ΔUTR* plants did not upregulate *RS2Z33* PTC+ and NMD-targeted TNL mRNAs AT5G40920 and *RPS6* upon *Pst* DC3000 infection (Figures 7D and S6G). However, *PR1* induction was unaffected in *SMG7-ΔUTR* plants, suggesting that SA-dependent defense responses are not altered by constitutive NMD activation (Figures 7E and S6H). Similarly, the induction of PTI marker genes *WRKY29* and *FRK1* and generation of ROS upon flg22 treatment remained unaffected in *SMG7-ΔUTR* plants (Figures S6I and S6J). Our data show that removal of *SMG7* autoregulatory elements increases NMD efficiency and prevents NMD inhibition upon *Pst* DC3000 infection without affecting early PTI or SA-dependent outputs.

We then determined whether the failure to suppress NMD in *SMG7-ΔUTR* plants impacts disease resistance by comparing *Pst* DC3000 and *Pst* DC3000 *hrcC*$^{-}$ growth in wild-type and *SMG7-ΔUTR* plants. Similar to *eds1-2* mutants, *SMG7-ΔUTR* plants supported significantly higher *Pst* DC3000 bacterial titers than wild-type at 3 days postinoculation, while low *Pst* DC3000 *hrcC*$^{-}$ growth remained unaffected in *SMG7-ΔUTR plants*

Figure 3. The *REX*^{Ler} Locus Underlies Autoimmunity in L*er-smg7* Mutants

(A) Five-week-old *smg7* plants in L*er* genomic background (L*er-smg7*) carrying either Col (C) or L*er* (L) alleles of *REX*. L*er-SMG7* control plants are shown on the left.

(B) Quantification of total and free SA of plants shown in (A) (mean ± standard deviation, three biological replicates).

(C) *PR1* expression in plants shown in (A).

(D) NMD reporter expression in plants shown in (A).

(E) Genetic map of L*er* chromosome 5. The *REX* locus is indicated by a red bar, located between markers CL5-18.76 and CL5-19.0 (Table S4). Genes are shown as arrows (TNL genes in red, other pathogen responsive genes in green). The three digits next to the genes are identifiers for the last corresponding AGI numbers (AT5G46XXX).

(F) Normalized RNA-seq read profiles mapping to *REX*^{Ler} in L*er-SMG7* and L*er-smg7* plants.

Values shown in (C) and (D) are mean ± SEM of four biological replicates. See also Figure S3.

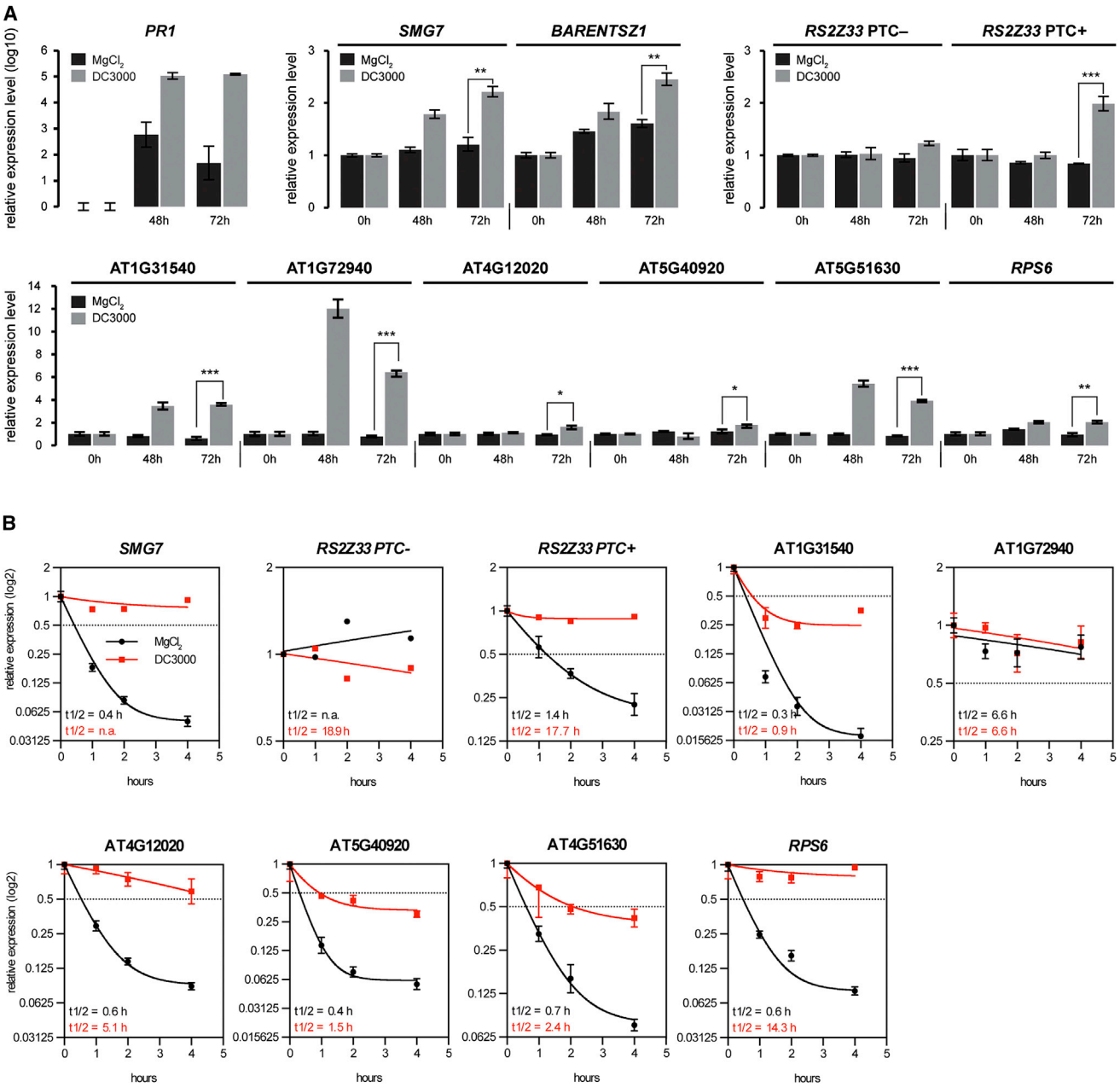


Figure 5. NMD Efficiency Declines upon Pathogen Infection

(A) Expression of *PR1*, NMD factors *SMG7* and *BARENTSZ1*, the NMD reporter *RS2Z33*, and selected TNLs in wild-type plants after treatment with *Pst* DC3000 or mock ($MgCl_2$) (mean ± SEM of three biological replicates, normalized to 0 hpi). Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) to mock treatment at the respective time point (two-tailed Student's t test).

(B) qRT-PCR analysis of half-lives of selected TNL transcripts and NMD reporters AT2G45670 and *RS2Z33* after treatment with *Pst* DC3000 or mock. Data points are mean ± SEM of three biological replicates.

Experiments shown in (A) and (B) were repeated three times with similar results. See also Figure S5.

(Figure 7F). The increased susceptibility of *SMG7-ΔUTR* plants to virulent *Pst* DC3000, together with the normal PTI responses observed in these lines, supports the idea that host-directed suppression of NMD contributes to postinfection basal resistance mechanisms downstream of early PTI responses.

DISCUSSION

An increasing body of evidence suggests that, beyond its role in mRNA surveillance, NMD is also important for regulating physiological gene expression. For example, NMD efficiency was reported to vary between cell types and tissues and in response

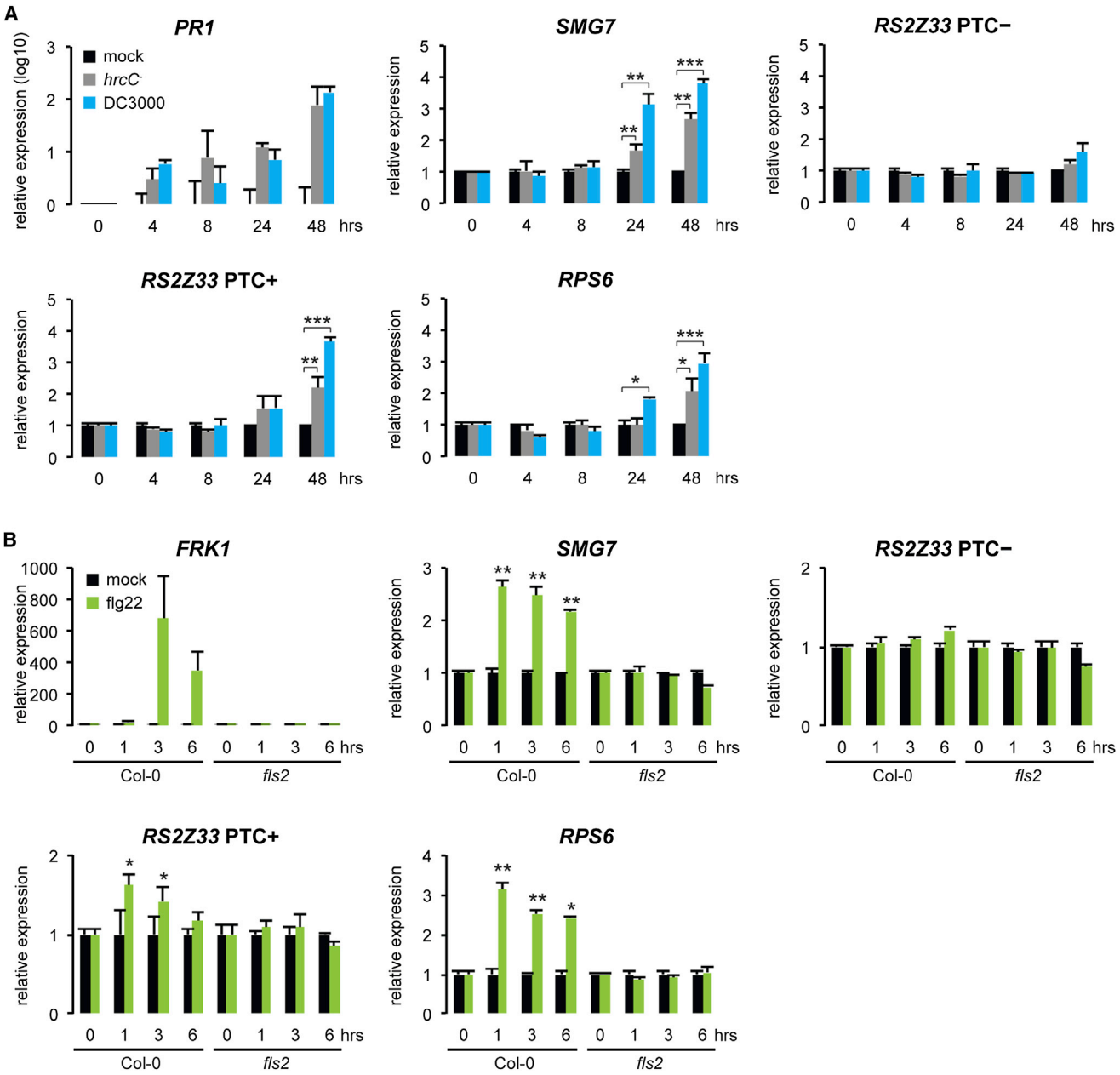


Figure 6. PAMP Perception Dampens NMD

(A) Expression of *SMG7*, NMD reporter *RS2Z33*, and TNL AT5G40920 upon syringe infiltration with *Pst* DC3000 and *Pst* DC3000 *hrcC*$^{-}$ (mean ± SEM of four biological replicates). *PR1* expression serves as a marker for SA-dependent defense responses.

(B) Expression of *SMG7*, NMD reporter *RS2Z33*, and TNL AT5G40920 upon flg22 treatment (1 μM) of wild-type and *fls2* seedlings, which serve as a negative control for flg22-induced responses (mean ± SEM of three biological replicates). *FRK1* expression serves as a marker for PTI induction.

Expression values in (A) and (B) were normalized to mock treatment at the respective time point. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared to mock (two-tailed Student's t test). Both experiments were repeated with similar results.

to cellular stress (Karam et al., 2013). Most studies describe a decline in NMD efficiency during cell differentiation or exposure to stresses (Bruno et al., 2011; Gardner, 2008; Mendell et al., 2004), suggesting that relaxed RNA surveillance might promote the expression of genes involved in development or stress adaptation (Karam et al., 2013). However, the significance of this regulation is unknown. Also, approaches to identify biologically relevant NMD targets have largely drawn correlations between functional annotations of candidate NMD transcripts and expression, leaving causal relationships unclear (Karam et al., 2013). Here we identify a causal link between NMD impairment and pathogen resistance in *Arabidopsis* and show that NMD surveillance is an integral part of the plant immune response. We establish that NMD regulates the turnover of multiple TNL

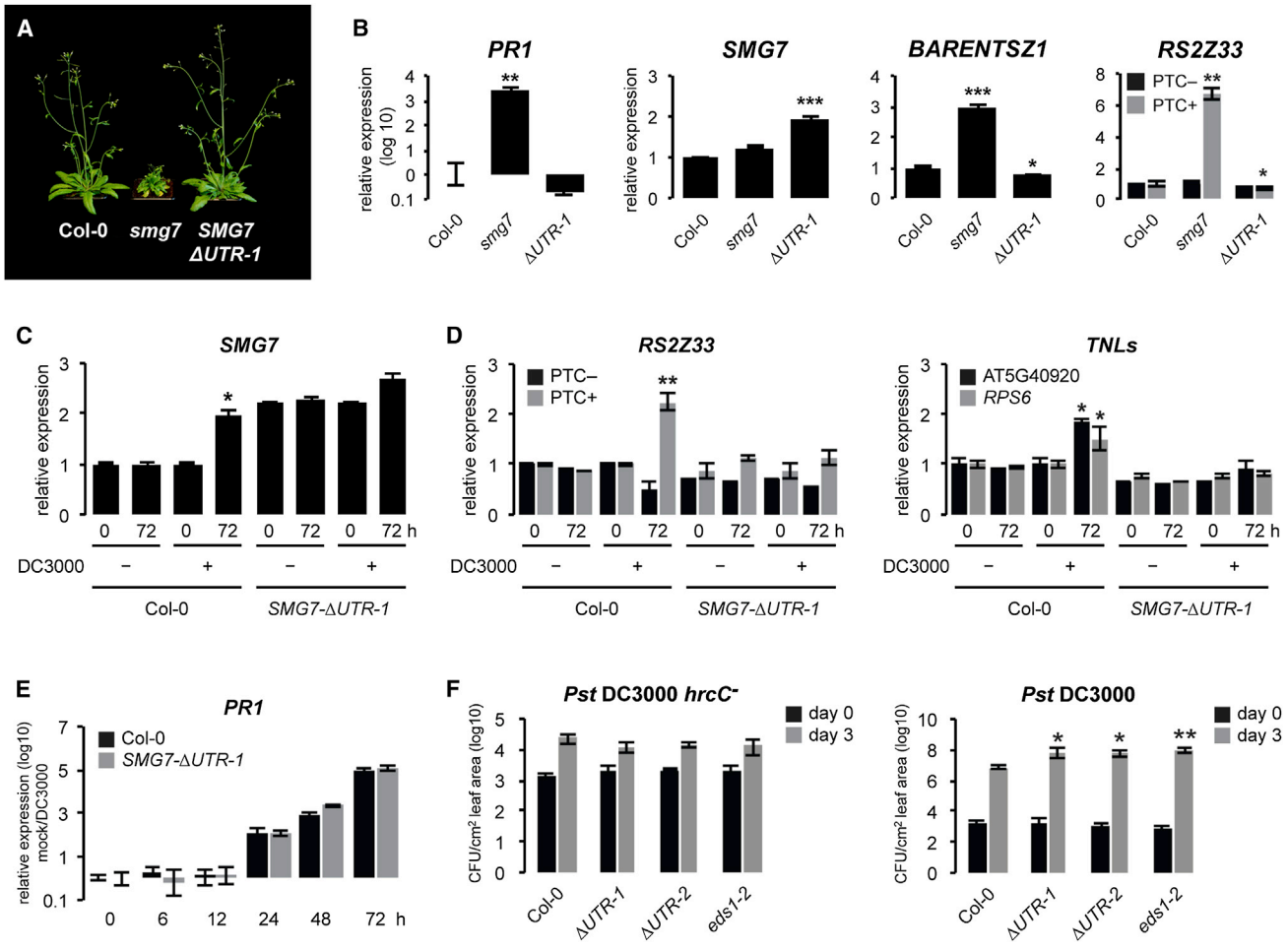


Figure 7. Impairment of NMD Autoregulation Reduces Basal Resistance

(A) Five-week-old Col, *smg7*, and a transgenic *smg7* mutant line complemented with *SMG7* cDNA lacking the endogenous 3′ UTR (*SMG7-ΔUTR-1*) are shown.

(B) *PR1*, *SMG7*, and *BARENTSZ1* expression in plants shown in (A) (mean ± SEM, four biological replicates). Values for *SMG7* in *SMG7-ΔUTR* plants are total levels of endogenous *SMG7* and *SMG7-ΔUTR* transgene.

(C and D) Expression of *SMG7*, *RS2Z33*, and TNLs *RPS6* and AT5G40920 in Col and *SMG7-ΔUTR-1* plants after spray inoculation with *Pst* DC3000 (+) or mock inoculation with $MgCl_2$ (–). Values are averages of three biological replicates (±SEM) and are relative to Col levels at 0 hpi. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$; two-tailed Student's t test) compared with the mock at the respective time point. The experiment was repeated with similar results.

(E) *PR1* expression in Col and *SMG7-ΔUTR-1* plants upon spray inoculation with virulent *Pst* DC3000. Expression values (mean ± SEM; averages of three biological replicates) are normalized to mock treatment.

(F) Bacterial titers of *Pst* DC3000 and *Pst* DC3000 *hrcC*$^-$ in Col-0, two *SMG7-ΔUTR* lines, and *eds1-2* mutants (mean ± SEM of five biological replicates; cfu = colony forming units). The experiment was repeated with similar results.

Asterisks in (B) and (F) indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; two-tailed Student's t test) compared to Col-0. See also Figures S6 and S7.

immune receptor transcripts. By so doing, NMD operates post-transcriptionally to control the threshold for activation of plant TNL and basal resistance pathways.

While *smg7* autoimmunity relies genetically on key regulators of TNL and basal resistance, disruption of SA biosynthesis in *smg7 sid2* mutants only mildly mitigated autoimmune symptoms (Figures 1A, 1B, S1A, and S1C). This fits with SA serving more as a defense signal amplifier in systemic tissues and often being dispensable for NLR local immunity (Fu and Dong, 2013; Tsuda et al., 2013). Although *smg7* mutants displayed enhanced resistance to virulent *Pst* DC3000 and poorly virulent *Pst* DC3000 *hrcC*$^-$ (Figure S1F), they had unaltered early PTI responses (Figures S1D and S1E), suggesting that NMD-regulated TNLs contribute to EDS1/PAD4-regulated postinfection basal resistance pathways at a point downstream of early PTI mechanisms.

By exploring *Arabidopsis* natural variation, we found that NMD deficiency-conditioned autoimmunity can be elicited by a *Ler* allelic form of the TNL *RPS6*, reinforcing the link between NMD deficiency and TNL receptor actions. While *RPS6* is causal for autoimmunity in the *Ler* accession (Figures 4A–4C), at least two other yet unidentified genetic loci underlie autoimmunity in Col plants. Similarly, suppression of autoimmunity was observed in a portion of *smg7* F2 plants derived from a cross between Col and accession Ws (data not shown). Thus, autoimmunity caused

by NMD deficiency might be conditioned by different TNLs in different *Arabidopsis* accessions. This is supported by our transcriptome data showing that NMD affects a broad set of TNL transcripts. We therefore propose that one physiological role of NMD is to prevent inappropriate TNL activation and its adverse effects on growth and fitness.

In contrast to TNLs (Figures 2A–2E), CNLs do not appear to be regulated by NMD (Figures S2D and S2F), which is consistent with the lack of NMD-eliciting features in CNL transcripts (Table S2). Several TNLs have been reported to undergo alternative splicing, a process that is frequently coupled to NMD (Gassmann, 2008). TNL *RPS6* in accession RLD is alternatively spliced upon pathogen infection, producing transcripts that contain PTCs and potentially encode truncated TIR-only or TIR-NB domain proteins (Kim et al., 2009). We found that a subset of *RPS6* splice isoforms generated by the excision of a cryptic intron were upregulated by NMD impairment, whereas transcript variants containing PTCs resulting from an intron retention (IR) event were unaffected (Figure S2G). This fits with the observation that PTCs generated by IR events generally do not elicit NMD in plants due to nuclear retention of these transcripts (Göhring et al., 2014).

Studies of weak *Arabidopsis* NMD mutants showed that even a slight attenuation of NMD increases immunity against bacterial infection (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012). We find that NMD efficiency declines in response to pathogen infection, leading to stabilization of TNL mRNAs and elevated mRNA levels of the NMD components *SMG7* and *BARENTSZ1* (Figures 5A and 5B). Disruption of NMD autoregulation by expressing the *SMG7-ΔUTR* construct led to constitutive NMD activity (Figures 7B, S6D, and S6E), suggesting that SMG7 might be a rate-limiting factor in plant NMD. Notably, *SMG7-ΔUTR* plants failed to stabilize NMD-regulated TNL transcripts upon bacterial infection and were more susceptible to virulent bacteria (Figures 7D, 7F, and S6G). The level of disease susceptibility was comparable to that of *eds1-2* mutants, suggesting that infection-modulated NMD significantly potentiates antimicrobial defenses. *PR1* induction upon infection was unaltered in *SMG7-ΔUTR* plants compared to wild-type (Figures 7E and S6H), suggesting that NMD promotes an EDS1/PAD4-regulated SA-independent resistance mechanism (Bartsch et al., 2006).

Some virulent pathogen effectors target proteins involved in RNA metabolism as a host cell reprogramming infection strategy (Pumplin and Voinnet, 2013). Our data argue that NMD suppression is part of an intrinsic host defense program to bolster resistance against pathogen infection (Figures 6A, 6B, and S7D). While the precise molecular events underlying NMD suppression during infection have yet to be clarified, one mechanism might involve downregulation of NMD factors, as indicated by a study reporting downregulation of *UPF1* and *UPF3* during *Pst* DC3000 infection (Jeong et al., 2011). However, we were unable to confirm these findings (Figures S7A–S7C). Alternatively, NMD suppression might result from infection-induced translation inhibition. Infection of plants with *Pst* DC3000 inhibits host translation (Pajerowska-Mukhtar et al., 2012), and translation inhibition plays an important role in *Drosophila* and *Caenorhabditis elegans* innate immunity (Chakrabarti et al., 2012; McEwan et al., 2012). In mammals, a major pathway for stress-induced NMD suppression requires GCN2-mediated translational inhibition (Karam et al., 2013). However, we found that reduced NMD upon amino acid starvation or pathogen infection is independent of GCN2 in *Arabidopsis* (Figures S5A–S5D). Other translational regulators such as TOR, which has overlapping functions with GCN2 in yeast (Staschke et al., 2010), might be involved in infection-triggered NMD inhibition.

Plant NLRs are intracellular sensors of pathogen effector activities. However, there is evidence that NLRs also contribute to basal resistance mechanisms against virulent pathogens in the absence of recognizable ETI. For example, overexpression of the TNLs RPS4 and SNC1 elicits immune responses without effector triggering (Stokes et al., 2002; Wirthmueller et al., 2007). Thus, suppression of NMD leading to increases in NMD-controlled TNLs would be expected to boost basal resistance.

NLR expression requires exquisite control at multiple levels to prevent inappropriate activation of immune responses (Staiger et al., 2013). Transcriptional gene silencing through RNA-directed DNA methylation (RdDM), posttranscriptional gene silencing (PTGS) by different small RNA species, and alternative splicing all impose a constraint. Epigenetic control of NLR expression was demonstrated for the TNL *RMG1*, which is suppressed by RdDM (Yu et al., 2013). At a posttranscriptional level, microRNAs regulate the expression of numerous NLRs in *Medicago*, tobacco, tomato, and *Arabidopsis* (Boccara et al., 2014; Li et al., 2012; Shivaprasad et al., 2012; Zhai et al., 2011). Interference with PTGS by viral and bacterial suppressors can lead to derepression of PTGS-controlled NLRs and thus re-establishment of basal and NLR-conditioned immunity (Boccara et al., 2014; Shivaprasad et al., 2012). While PTGS appears to regulate both TNLs and CNLs, our analysis shows that NMD targets mainly TNLs (Figures 2B–2E, S2E, and S2G). Therefore, PTGS and NMD pathways might affect different NLR gene sets to modulate defense thresholds. Taken together, our findings emphasize the role of NMD in physiological processes and underscore the importance of RNA-based regulatory mechanisms in plant immunity.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions

Wild-type *Arabidopsis thaliana* accessions used were Columbia-0 (Col) and Landsberg *erecta* (Ler). Mutant and transgenic lines are described in the Supplemental Experimental Procedures. Plants were grown on soil with 16 hr light/8 hr dark photoperiod (21°C, 60% relative humidity), except for temperature shift experiments (28°C, 60% relative humidity) and experiments involving pathogen assays.

Pathogen Assays

Five-week-old soil-grown plants (8/16 hr photoperiod, 21°C) were spray inoculated with 1×10^8 cfu/ml *P. syringae pv tomato* DC3000 (*Pst* DC3000), or *Pst* DC3000 *hrcC*$^-$ with 0.04% (v/v) Silwet L-77 (Lehle Seeds), or 10 mM $MgCl_2$ as mock control, and bacterial titer was determined 72 hpi by serial dilution as previously described (Rietz et al., 2011). Samples for gene expression analysis were harvested from the same plant material. For comparison of NMD inhibition upon *Pst* DC3000 and *Pst* DC3000 *hrcC*$^-$ inoculation, leaves of 4- to 5-week-old Col plants (8/16 hr photoperiod, 21°C) were syringe infiltrated with 5×10^6 cfu/ml bacteria. For testing of HR outputs, leaves of 4- to 5-week-old plants were syringe infiltrated with 1×10^7 cfu/ml *Pst* DC3000 HopA1, and disease symptoms were recorded 24 hr after inoculation. Flg22 treatments of 12-day-old seedlings with 1 μM flg22 were performed as described by Tsuda et al. (2013). Alternatively, 1 μM flg22 was syringe infiltrated into

leaves of 4- to 5-week-old plants grown at 21°C with 8/16 hr photoperiod as described by Boccara et al. (2014). Flg22 peptide (Genscript) was a gift from Lionel Navarro.

Gene Expression Analysis

Total RNA was isolated from plant tissue using peqGOLD RNAPure (peqLab). Genomic DNA was removed from RNA samples using Ambion TURBO DNA-free (Life Technologies), and 1 μg of DNA-free RNA was reverse transcribed using the Maxima H Minus First Strand cDNA synthesis kit (Fermentas). One-fiftieth of cDNA was then amplified in qRT-PCR reactions using SsoAdvanced SYBR Green Supermix (Bio-Rad) and transcript-specific primers on a Bio-Rad iQ5 optical cycler. Expression was normalized to AT2G28390 or AT4G26410 (Czechowski et al., 2005) for steady-state levels and to *eIF4A1* (AT3G13920) expression in transcriptional block experiments. All qRT-PCR data presented are from at least three biological replicates, each of which represents the average of three technical replicates. All primer sequences are listed in Table S3.

RNA-Seq Analysis

Single-end, strand-specific, and ribosomal RNA-depleted RNA-seq libraries were generated and sequenced using the ScriptSeq Complete Plant kit (Epicentre) on the Illumina HiSeq2000 platform. Experimental details are described in the Supplemental Experimental Procedures.

Transcriptional and Translational Inhibition

Transcriptional inhibition was performed in detached leaves of 4- to 5-week-old plants as previously described (Johnson et al., 2000) using a modified incubation buffer containing 1 mM PIPES (pH 6.25), 1 mM sodium citrate, 1 mM KCl, 15 mM sucrose, and 0.08% Silwet L-77. Leaves were soaked in incubation buffer for 30 min before the addition of cordycepin (150 μg/ml, Sigma). Vacuum was applied for 5 min and two leaves were then harvested after 1, 2, and 4 hr for subsequent RNA analysis. Transcript half-lives were calculated from qRT-PCR data by nonlinear least-square regression using GraphPad Prism 6.0. Translation inhibition was carried out for 5 hr using cycloheximide as previously described (Kalyna et al., 2012).

ACCESSION NUMBERS

The GEO accession number for the Illumina RNA-seq data set reported in this paper is GSE55884.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.08.010.

AUTHOR CONTRIBUTIONS

J.G., J.E.P., and K.R. designed the experiments and wrote the manuscript. J.G., S.A., N.R., H.S., J.B., and B.D. performed experiments. A.S., B.K., and J.M.J.-G. performed computational analyses. J.G., C.J., H.S., N.R., J.E.P., and K.R. analyzed the data.

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