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Regulation of plant innate immunity through nonsense-mediated mRNA decay

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The great tragedy of science –
the slaying of a beautiful hypothesis by an ugly fact.
Thomas Henry Huxley

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1 Abstract

Nonsense-mediated mRNA decay (NMD) is a conserved eukaryotic RNA surveillance machinery that recognizes and degrades aberrant RNA transcripts, thereby ensuring the homeostasis of eukaryotic transcriptomes. Recently, beyond its role in RNA quality control, NMD has emerged as a potential gene regulatory mechanism that might be involved in the regulation of physiologically relevant transcripts. However, the biological processes controlled by NMD have remained elusive, mainly due to the broad impact of NMD deficiency on transcriptome homeostasis and the associated secondary effects.

In my PhD studies, I investigated the biological significance of NMD using the model plant *Arabidopsis thaliana*, and found that NMD is involved in the regulation of plant innate immunity. In a joint effort, Nina Riehs-Kearnan, a former colleague, and I dissected the severe phenotypic aberrations that are associated with the depletion of NMD factors in plants. We could demonstrate that stunting of NMD-impaired plants was caused by inappropriate activation of immune responses. Furthermore, I was able to delineate the genetic pathway that elicits autoimmunity in NMD mutants and provided evidence that the autoactivation of immune responses was conditioned by TIR-NB-LRR (Toll/Interleukin1 Receptor – Nucleotide Binding Site – Leucine-Rich Repeat) plant immune receptors. By using transcriptional profiling and RNA turnover studies, I showed that NMD directly processes numerous TIR-NB-LRR receptor transcripts, and thereby regulates TIR-NB-LRR expression through RNA decay. In a collaborative effort, colleagues and I demonstrated that deregulation of a single NMD-controlled TIR-NB-LRR transcript upon NMD impairment is sufficient to recapitulate all autoimmune symptoms observed in NMD mutants. Importantly, my data revealed that NMD is suppressed upon bacterial infections, which consequently leads to the overexpression of multiple TIR-NB-LRR transcripts. Finally, I demonstrated that NMD suppression upon bacterial infection is regulated through an autoregulatory loop involving the NMD core factor SMG7, and positively contributes to basal resistance in plants.

Taken together, my studies underscore the importance and complexity of RNA-based mechanisms in plant disease resistance, and add NMD as an important regulatory layer that shapes plant innate immunity.

2 Zusammenfassung

Nonsense-mediated mRNA decay (NMD) ist ein konservierter, eukaryotischer RNA-Kontrollmechanismus, der fehlerhafte messenger-RNA-Transkripte erkennt und abbaut, wodurch die Homöostase des Transkriptoms gewährleistet wird. Jüngste Forschungsergebnisse deuten allerdings auch auf eine Rolle für NMD jenseits der RNA-Qualitätssicherung hin und implizieren eine Funktion von NMD in der Regulierung physiologisch relevanter Transkripte. Die Untersuchung biologischer Prozesse, die von NMD reguliert werden gestaltet sich jedoch schwierig, da NMD-Defekte zu massiver Deregulierung des Transkriptoms führen und weitreichende sekundäre Effekte nach sich ziehen.

In Rahmen meiner Doktoratsarbeit habe ich die biologische Signifikanz von NMD in der Modellpflanze *Arabidopsis thaliana* untersucht und konnte eine Rolle für NMD in der Regulierung des pflanzlichen Immunsystems zeigen. Gemeinsam mit Nina Riehs-Kearnan, einer ehemaligen Kollegin, habe ich die schwerwiegenden phänotypischen Störungen untersucht, die mit dem Verlust von NMD-Faktoren einhergehen. Unsere Ergebnisse deuten darauf hin, dass die Wachstumsdefekte von NMD-Mutanten auf eine unkontrollierte Aktivierung des Immunsystems zurück zu führen sind. Des Weiteren habe ich durch genetische Analysen festgestellt, dass die Autoimmunität von NMD-Mutanten durch die deregulierte Aktivität von TIR-NB-LRR-Immunrezeptoren ausgelöst wird. Durch Transkriptomanalyse und RNA-Zerfallsstudien konnte ich beweisen, dass TIR-NB-LRR-Rezeptortranskripte direkt von NMD reguliert und abgebaut werden. Gemeinsam mit anderen KollegInnen konnte ich außerdem zeigen, dass die Deregulierung eines einzelnen Immunrezeptors ausreicht, um in NMD-Mutanten Autoimmunität auszulösen. Weiters zeigten meine Studien, dass eine Infektion von Pflanzen mit pathogenen Bakterien die Aktivität von NMD supprimiert, was die vermehrte Expression von NMD-regulierten TIR-NB-LRR-Rezeptoren zur Folge hat. Diese verminderte NMD-Aktivität wird durch eine autoregulatorische Schleife beeinflusst, die das für NMD wichtige SMG7 Protein einschließt, und hat einen positiven Effekt auf die Effizienz der pflanzlichen Immunantwort.

Die Ergebnisse meiner Doktoratsarbeit heben die Komplexität und Relevanz von RNA-basierten Kontrollmechanismen in der Ausprägung von Krankheitsresistenzen hervor und zeigen, dass die RNA-Qualitätskontrolle durch NMD ein wichtiges regulatorisches Element des pflanzlichen Immunsystems darstellt.

3 Introduction

3.1 Nonsense-mediated mRNA decay (NMD)

The central dogma of molecular biology represents a framework that describes the expression of genetic information as a flow of information from DNA molecules, that store genetic information, to messenger RNAs (mRNAs), that eventually serve as construction templates for the biosynthesis of proteins (Crick, 1970). Despite a battery of surveillance mechanisms that ensure the fidelity of gene expression all along the way to the final protein product (Moore, 2005), the transfer of information from DNA to RNA is inherently error-prone, mainly due to mutations of the genetic information, undetected or unrepaired inaccuracies in transcription, or aberrant post-transcriptional processing of RNA molecules (Doma and Parker, 2007; Houseley and Tollervey, 2009; Mühlemann and Jensen, 2012). As a result, a given cell can accumulate considerable amounts of aberrant RNAs, which when becoming translated might have adverse effects on physiology. Thus, it is essential for eukaryotic organisms to recognize and eliminate abnormal mRNA transcripts. All eukaryotic organisms have developed sophisticated RNA surveillance machineries that can recognize several types of aberrant RNAs (Mühlemann and Jensen, 2012; Schoenberg and Maquat, 2012).

The following chapters will give a brief glimpse on one of the better studied RNA surveillance machineries known as nonsense-mediated mRNA decay (NMD), which by its most original definition serves as a machinery to recognize and degrade mRNA species that encode for a premature termination codon (PTC) in their coding sequence (CDS) (Nicholson and Mühlemann, 2010).

3.1.1 Role of nonsense-mediated mRNA decay in RNA surveillance

Originally, NMD was defined as an RNA surveillance process that recognizes the occurrence of PTCs in capped and polyadenlyated mRNAs in a translation-dependent manner, and that degrades these aberrant transcripts through downstream acting RNA degradation pathways (reviewed in Isken and Maquat, 2008; Kervestin and Jacobson, 2012; Nicholson and Mühlemann, 2010; Schweingruber et al., 2013). Recently, NMD has also emerged as a potential regulator of physiologically relevant transcripts (Karam et al., 2013; Nicholson and Mühlemann, 2010; Rehwinkel et al., 2006). In support of this notion, transcriptome-wide analyses in different eukaryotic organisms have revealed that NMD also targets normal mRNA transcripts that lack

canonical PTCs in their CDS (Drechsel et al., 2013; Kurihara et al., 2009; Mendell et al., 2004; Ramani et al., 2009; Rehwinkel et al., 2005; Weischenfeldt et al., 2012). Numerous microarray studies and high-throughput RNA-sequencing (RNA-seq) studies have indicated that NMD might target up to 20% of transcripts in eukaryotic transcriptomes (Drechsel et al., 2013; Guan et al., 2006; Kurihara et al., 2009; Lelivelt and Culbertson, 1999; Mendell et al., 2004; Ramani et al., 2009; Weischenfeldt et al., 2012; Wittmann et al., 2006). In addition to PTCs, which might be the result of alternative splicing (AS), also the presence of upstream open reading frames (uORFs), unusually long 3' untranslated regions (3'UTRs) or intron-containing 3'UTRs might target a given transcript for NMD. Furthermore, transcriptome-wide studies have also demonstrated that RNA quality control by NMD is not limited to protein-coding genes, but that NMD also affects the expression of numerous non-coding RNAs (ncRNAs) (Drechsel et al., 2013; Kurihara et al., 2009; Tani et al., 2012). Subsequently, the definition of NMD targets has been modified, and it appears that NMD generally recognizes transcripts in which a potential stop codon is located in an unfavorable environment for efficient translation termination (Schweingruber et al., 2013).

3.1.2 Molecular mechanisms of NMD

Most of our current understanding of the molecular mechanisms underlying NMD comes from studies in yeast and mammalian cell systems. While many partially contradicting mechanistic models have been proposed, recently, a unified working model of NMD has emerged (Mühlemann, 2008), which I will briefly describe in the following paragraphs (see also Figure 3.1).

Degradation of NMD-sensitive transcripts requires the recognition of nonsense codons in a translation-dependent context. Upon stalling of an elongating ribosome at stop codons (UAA, UAG, UGA), the eukaryotic release factor I (eRF1) is recruited to the ribosomal acceptor site, to which usually charged tRNAs are recruited (Frolova et al., 1994; Jackson et al., 2012). eRF1 structurally mimics a tRNA with the capability to decode all three stop codons, and its activity is stimulated by the eukaryotic release factor (eRF3), leading to peptide release from the terminating ribosome, disassembly of ribosomal subunits, and their recycling (Frolova et al., 1994; Stansfield et al., 1995; Zhouravleva et al., 1995). Furthermore, translation termination was shown to require the interaction between eRF3 and the poly(A)-binding protein (PABP), which is associated with the poly(A)-stretch at the 3' end of eukaryotic mRNAs (Cosson et al., 2002; Hoshino et al., 1999). When a ribosome encounters a stop codon in an unfavorable environment in which eRF3 fails to interact with the PABP, it is thought that the translating ribosome stalls, which leads to NMD activation (Eberle et al., 2008; Ivanov et al., 2008; Singh et al., 2008). Notably, also the presence of long 3'UTRs between a termination codon and PABP could interfere with normal translation termination, which might explain why NMD can also be elicited by unusually long 3'UTRs (Amrani et al.,

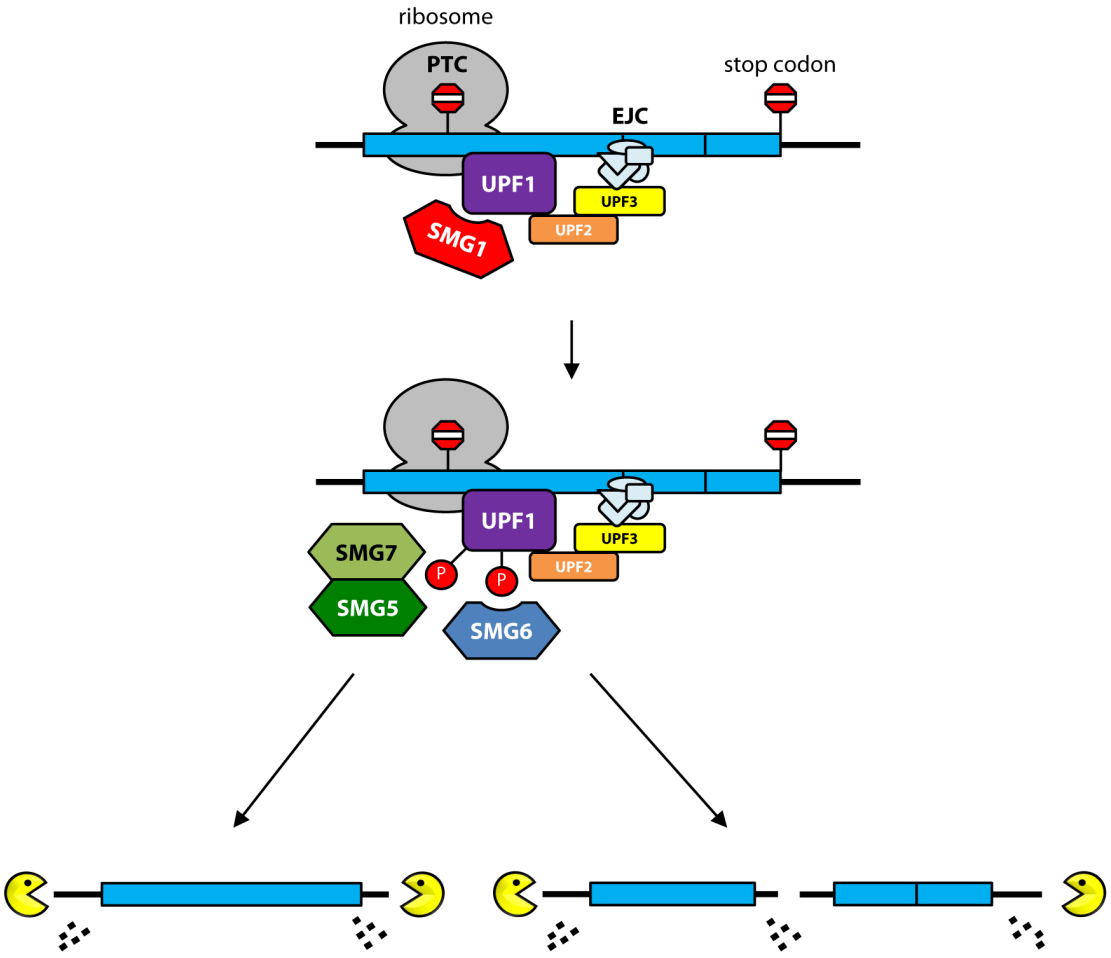

Figure 3.1: Simplified depiction of the early and late steps of NMD as proposed in the unified NMD model described by Mühlemann (2008).

2004; Behm-Ansmant et al., 2007; Eberle et al., 2008; Kerényi et al., 2008; Kertész et al., 2006; Nyikó et al., 2013; Silva et al., 2008).

Commonly, NMD requires the activity of three trans-acting NMD core proteins, which are known as the up-frameshift proteins UPF1, UPF2, and UPF3. Homologs of these proteins have been identified in all eukaryotes investigated so far. While these three NMD factors are dispensable for the survival of yeast (Cui et al., 1995; Leeds et al., 1991) and have only mild phenotypic impact in the model nematode *Caenorhabditis elegans* (*C. elegans*) (Hodgkin et al., 1989), NMD is essential for the survival of mice (McIlwain et al., 2010; Medghalchi et al., 2001; Weischenfeldt et al., 2012), zebrafish (Wittkopp et al., 2009), fruit flies (Avery et al., 2011; Metzstein and Krasnow, 2006), and plants (Arciga-Reyes et al., 2006; Hori and Watanabe, 2005; Riehs et al., 2008; Riehs-Kearnan et al., 2012; Yoine et al., 2006a,b).

At stalled ribosomes, the RNA helicase UPF1 is believed to compete with PABP for binding to eRF3 (Behm-Ansmant et al., 2007; Ivanov et al., 2008; Kashima et al., 2006; Singh et al., 2008). Upon binding of UPF1 to eRF3, UPF2 and UPF3 bind to UPF1 to form a ternary UPF1-UPF2-UPF3 complex, a process that stimulates the ATP-dependent RNA helicase activity of UPF1 (Chamieh et al., 2008; Kashima et al., 2006). Furthermore, metazoans and plants have an additional set of proteins, known as SMG proteins (suppressor with morphogenic effect on genitalia), that act downstream of the UPF proteins to mediate degradation of target RNAs, and also promote the recycling of UPF1 (reviewed in Kervestin and Jacobson, 2012; Schweingruber et al., 2013). In mammals, SMG1, a member of the phosphatidylinositol-3-kinase-related kinase (PIKK) family (Grimson et al., 2004; Yamashita et al., 2001), was shown to phosphorylate UPF1 at multiple sites (Isken et al., 2008; Ohnishi et al., 2003; Okada-Katsuhata et al., 2012; Page et al., 1999). The activity of SMG1 is modulated by SMG8 and SMG9 (Arias-Palomo et al., 2011; Fernández et al., 2011; Yamashita et al., 2001). Interestingly, while neither SMG1, SMG8, nor SMG9 homologs have been found in *Arabidopsis thaliana* (*A. thaliana*) (Grimson et al., 2004), a recent study identified SMG1 homologs in other plant species, suggesting that SMG1 is not an animal specific protein (Lloyd and Davies, 2013).

Upon UPF1 phosphorylation, SMG5 and SMG7, which form a heterodimer, are recruited to phosphorylated sites of UPF1 by virtue of their 14-3-3-like domain (Fukuhara et al., 2005; Jonas et al., 2013; Mérai et al., 2013; Okada-Katsuhata et al., 2012; Unterholzner and Izaurralde, 2004). Similarly, the SMG6 endonuclease binds phosphorylated UPF1 sites (Okada-Katsuhata et al., 2012), and mediates endonucleolytic cleavage of bound mRNAs in vicinity of the PTC (Eberle et al., 2009; Gatfield et al., 2003; Huntzinger et al., 2008). In contrast, the SMG5/7 heterodimer promotes decapping and deadenylation (Unterholzner and Izaurralde, 2004), and recruits both the XRN1 exoribonuclease and the exosome complex (Fukuhara et al., 2005), which catalyze the degradation of mRNAs in a 5'→ 3' and 3'→ 5' manner, respectively (Nicholson and Mühlemann, 2010). In plants, both SMG5 and SMG6

appear to be absent, but SMG7 was described to play a role in plant NMD (Kerényi et al., 2013; Riehs et al., 2008). Importantly, SMG5 and SMG7 have also been shown to promote recycling and dephosphorylation of UPF1 by recruiting protein phosphate 2A (PP2A) (Anders et al., 2003; Chiu et al., 2003; Ohnishi et al., 2003).

In plants and animals, the recognition of NMD substrates is greatly enhanced by the presence of the multi-subunit exon junction complex (EJC), which is deposited close to exon-exon boundaries after splicing of the intervening intron (Le Hir et al., 2000). The EJC comprises at least four proteins, Magoh, Y14, eIF4AIII and Barentsz (also known as MLN51 or CASC3) (Andersen et al., 2006; Bono et al., 2006), and serves as a binding platform for UPF2 and UPF3 (Le Hir et al., 2001). While EJCs that are positioned upstream of a stop codon are usually removed by the elongating ribosome, only EJCs located downstream of stop codons are expected to be present on an mRNA (Dostie and Dreyfuss, 2002; Gehring et al., 2009). EJCs deposited at exon-exon junctions that are located at least 50 nucleotides downstream of a PTC might place UPF2 and UPF3 in a favorable position for interaction with UPF1, thereby stimulating NMD (Le Hir et al., 2001). Recently, it was shown that UPF1 also independently associates with mRNAs in a 3'UTR length-dependent manner (Hogg and Goff, 2010). Consequently, the authors showed that transcripts containing long 3'UTRs are enriched for UPF1, which might lead to the formation of mRNP structures that are poised for NMD.

3.1.3 Stress-induced and cell-type specific regulation of NMD

Recently, evidence has emerged that the efficiency of NMD is dynamically controlled in a developmental and cell-type specific manner. Additionally, NMD intensity appears to be regulated in response to environmental and cellular stresses. This chapter aims at highlighting examples from the scarce literature in which NMD regulation was studied in a biologically relevant context. For a more complete overview, I refer the reader to several excellent reviews that extensively discuss the potential biological impact of NMD regulation (Huang and Wilkinson, 2012; Karam et al., 2013; Palacios, 2013).

Initially, stress-induced regulation of NMD was reported by Mendell and co-workers (2004) to occur upon amino acid starvation in mammalian cells. The authors suggested that amino acid starvation dampens global protein translation, which presumably also inhibits NMD due to its coupling to translation (Mendell et al., 2004). Interestingly, other cellular stresses such as hypoxia were demonstrated to have similar effects on translation and NMD (Gardner, 2008, 2010). Amino acid starvation and hypoxia have been demonstrated to activate a family of kinases that commonly phosphorylate the eukaryotic initiation factor 2α (eIF2α), such as GCN2 (specific for amino acid starvation), or PKR (activated by double stranded RNA) (Gardner, 2008; Wang et al., 2011; Zhang et al., 2002), which subsequently leads to translational arrest and NMD inhibition (Wengrod et al., 2013).

NMD also appears to be regulated by other RNA regulatory mechanisms. Recently, Bruno and colleagues (2011) identified that microRNA-128 (miR-128) regulates the levels of UPF1 in human and mouse cells by targeting the UPF1 transcript for post-transcriptional gene silencing (PTGS). The authors went on and demonstrated that the expression of miR-128 negatively influenced the efficiency of NMD. Notably, miR-128 is highly expressed during vertebrate brain development and differentiation of neuronal cells, which might lead to cell type- and tissue-specific suppression of NMD, and subsequently to stabilization of mRNA targets that are relevant for brain development (Bruno et al., 2011).

3.1.4 Autoregulation of NMD

The discovery of cell type- and stress-dependent regulation of NMD raised the question if mechanisms would exist that protect eukaryotic organisms from perturbations of NMD. Indeed, regulatory circuits have been identified that regulate the expression of NMD factors in mammalian cells and plants, and thus, alleviate the negative impact of environmental and genetic insults. Two recent studies have studied the existence of such autoregulatory feedback mechanisms in mammalian cells (Huang et al., 2011; Yepiskoposyan et al., 2011). Importantly, the authors found that depletion of the NMD core factors UPF1, UPF3B, SMG6, or SMG7 led to upregulation of other NMD factors on transcript and protein level. This feedback regulation was specific, since NMD-enhancing EJC components seemed to be unaffected by depletion of NMD factors. Subsequently, the authors also identified that the transcripts encoding UPF1, UPF2, SMG1, SMG5, SMG6, and SMG7 have abnormally long 3'UTRs, a common feature of NMD targets. Additionally, in transgenic assays, these 3'UTRs were shown to be sufficient to elicit NMD. Together, these results suggested that NMD factors negatively regulate their abundance through NMD to ensure robustness, and allow for buffering against undesired perturbations (Huang et al., 2011; Yepiskoposyan et al., 2011).

Feedback regulation of NMD does not seem to be limited to mammals, since autoregulatory loops that regulate NMD were also found to exist in plants. Recently, Nyikó and colleagues (2013) demonstrated that the abnormally long 3'UTR in the transcript of the *Arabidopsis* SMG7 homolog was sufficient to target the transcript for degradation by NMD, suggesting that *SMG7* levels are negatively regulated through an autoregulatory feedback loop. Interestingly, the existence of introns in the *SMG7* 3'UTR, a feature that seems to be conserved in all angiosperm, also induced NMD, indicating that both intron-based NMD and 3'UTR-based NMD control the levels of *SMG7*. Consistently, the *SMG7* transcript has been reported to be upregulated in NMD mutants impaired in UPF1 and UPF3 function (Nyikó et al., 2013; Rayson et al., 2012a). While EJC components seem to be unaffected by autoregulatory loops in mammalian NMD (Huang et al., 2011; Yepiskoposyan et al., 2011), the *Arabidopsis* homolog of Barentsz was also shown to be negatively regulated through NMD (Nyikó et al., 2013), suggesting an evolutionary diversification of the feedback mechanisms that govern NMD homeostasis.

3.1.5 Alternative Splicing coupled to NMD

Alternative splicing (AS) is an important gene regulatory mechanism that extends and diversifies eukaryotic transcriptomes and proteomes (Nilsen and Graveley, 2010). Alternative splicing events may include alternative 5' or 3' splice site selection, exon skipping/inclusion, excision of cryptic introns, and intron retention (IR) (Reddy, 2007; Syed et al., 2012). Previously, it has been demonstrated that alternative splicing is differentially regulated during development and upon stresses (Dubrovina et al., 2013; Mastrangelo et al., 2012; Staiger and Brown, 2013). Frequently, alternative splicing events lead to transcripts isoforms that encode for in-frame PTCs. The abundance of these PTC-containing transcripts can be regulated by NMD in a process that is known as "Regulated Unproductive Splicing and Translation" (RUST) or AS-coupled NMD (Lareau et al., 2007). Interestingly, AS-coupled NMD appears to be a common regulatory layer in determining the expression levels of numerous splicing factors and other RNA-binding proteins (Lareau et al., 2007; Palusa and Reddy, 2010; Stauffer et al., 2010; Wollerton et al., 2004), which suggests the existence of auto- and cross-regulatory loops that control the abundance and the splicing ratios of RNA-binding proteins. These processes on the one hand could ensure transcriptome homeostasis, but would also allow for transient perturbation by external and cellular cues, which might influence development and stress acclimatization (Staiger and Brown, 2013).

Transcriptome wide studies in *C. elegans* and mammals indicated that up to 30% of all genes produce alternative spliced variants that might be targeted by NMD (McIlwain et al., 2010; Ramani et al., 2009; Weischenfeldt et al., 2012). Similarly, Drechsel et al. (2013) have recently identified that in *Arabidopsis* at least 17% of all spliced protein-coding genes produce splicing variants are substrates for NMD, and similar numbers have been extrapolated by analyzing splicing events of a selected set of genes in *Arabidopsis* (Kalyna et al., 2012). The prevalence of AS-coupled NMD raises the question if these alternative splicing events are indeed unproductive, or rather represent stress and developmentally regulated events, that under certain conditions lead to the production of truncated proteins with novel functions (Kalsotra and Cooper, 2011; Keren et al., 2010; Staiger and Brown, 2013).

Analyses of alternative splicing events have indicated that exon skipping is by far the most prevalent event in humans, accounting for almost 40% of alternative splicing events (Keren et al., 2010; Pan et al., 2008). In contrast, intron retention appears to constitute the majority of splicing events in plants (Filichkin et al., 2010; Marquez et al., 2012). While transcripts that contain PTCs generated by retained introns are frequently processed by NMD (de Lima Morais and Harrison, 2010; Keren et al., 2010; Wong et al., 2013) in mammals, several studies have suggested that the majority of intron retention events escape NMD in *Arabidopsis* (Kalyna et al., 2012; Kim et al., 2009a; Marquez et al., 2012). The biological role of transcripts with retained introns that evade NMD in plants is currently unclear and warrants further investigation.

3.1.6 NMD in human diseases

The biological significance of NMD is underscored by the fact that up to 30% of all known mutations causing human diseases encode for PTCs (Holbrook et al., 2004). Therefore, NMD plays an important role in shaping disease severity and associated clinical symptoms. A prominent example for a beneficial impact of NMD on human diseases is a genetic disorder that affects the normal production of hemoglobin, known as β-thalassemia, which is a hereditary form of anemia (Holbrook et al., 2004). Patients with certain β-thalassemia forms carry mutations that generate a PTC in the β-globin transcript. Degradation activities of NMD on the β-globin transcript (Baserga and Benz, 1988) amend the effects of PTC mutations in β–thalassemia patients (Hall and Thein, 1994), and as a result, patients carrying only one affected allele are clinically asymptomatic.

While in many cases NMD activity is beneficial in amending disease symptoms caused by PTC-inducing mutations, NMD can also worsen disease phenotypes. In Duchenne muscular dystrophy (DMD), NMD-mediated clearance of PTC-containing transcripts, that encode partially functional dystrophin peptides, has been shown to cause severe forms of DMD (Kerr et al., 2001; Zubrzycka-Gaarn et al., 1988). In these cases, suppression therapies, which pharmacologically inhibit the activity of NMD by small molecules, represent attractive therapeutic approaches (Holbrook et al., 2004; Peltz et al., 2013). In conclusion, the clinical relevance of NMD underscores the importance of understanding the underlying molecular mechanisms of NMD, the role of NMD in the regulation of biologically relevant transcripts, and the impact of NMD on physiology.

3.2 Plant innate immunity

3.2.1 The plant immune response

Due to their sessile lifestyle, plants are constantly challenged by a variety of abiotic and biotic stresses. Extensive interaction with the surrounding microbiota requires plants to develop and employ a plethora of strategies to face threats from potential pathogens. The co-evolution of plants and plant pathogens drives an arms race on a molecular level, which puts both pathogens and plants under selective pressure to adapt and develop new strategies for virulence and counter-measures, respectively (Dodds and Rathjen, 2010; Jones and Dangl, 2006).

Several lines of defense confer immunity against plant pathogens (Figure 3.2). Besides mechanical and physical barriers such as the cell wall and stomata (Hématy et al., 2009; Melotto et al., 2008), plants also rely on the recognition of pathogen-specific molecules to mount effective immune responses. These pathogen-specific molecules can be located on the pathogen surface or can be translocated into the host cell.

As a first layer of defense, plants employ plasma membrane-localized pattern recognition receptors (PRRs) that recognize and interact with evolutionary conserved pathogen-associated molecular patterns (PAMPs) (Monaghan and Zipfel, 2012). Immunity mediated by surface PRRs confers rapid but transient immunity against the majority of non-adapted pathogens, a phenomenon known as non-host disease resistance (Jones and Dangl, 2006; Senthil-Kumar and Mysore, 2013). Recognition of conserved microbial features such as bacterial flagellin or the prokaryotic elongation factor EF-Tu by PRRs provides broad resistance and induces PAMP-triggered immunity (PTI) (Gómez-Gómez and Boller, 2000, 2002; Kunze et al., 2004; Zipfel et al., 2006) (Figure 3.3). Bacterial flagellin is likely to be the best-studied elicitor of PTI responses. The leucine-rich repeat (LRR) receptor kinase FLS2 (FLAGELLIN SENSITIVE 2) is capable of recognizing a minimum peptide of 22 amino acids (flg22) derived from bacterial flagellin, and subsequently, rapidly elicits multiple downstream signaling cascades (Chinchilla et al., 2006; Gómez-Gómez and Boller, 2000, 2002; Monaghan and Zipfel, 2012; Zipfel et al., 2004). The downstream signaling cascades involve the activation of mitogen-activated protein (MAP) kinases, production of reactive oxygen species, fortification of the cell wall through callose deposition, and large-scale transcriptional and metabolic reprogramming (Navarro et al., 2004; Schwessinger and Zipfel, 2008). Importantly, PTI induces the biosynthesis of the plant defense hormone salicylic acid (SA), that is required to establish both local and systemic acquired resistance (SAR) (Fu and Dong, 2013).

Adapted pathogens, for example certain strains of the bacterial pathogen *Pseudomonas syringae* (*P. syringae*), are capable of antagonizing or partially suppressing PTI by deploying race-specific effector proteins, that are also known as avirulence (Avr) factors (Alfano and Collmer, 2004; Jones and Dangl, 2006; Lindeberg et al., 2005; Navarro et al., 2008; Staskawicz

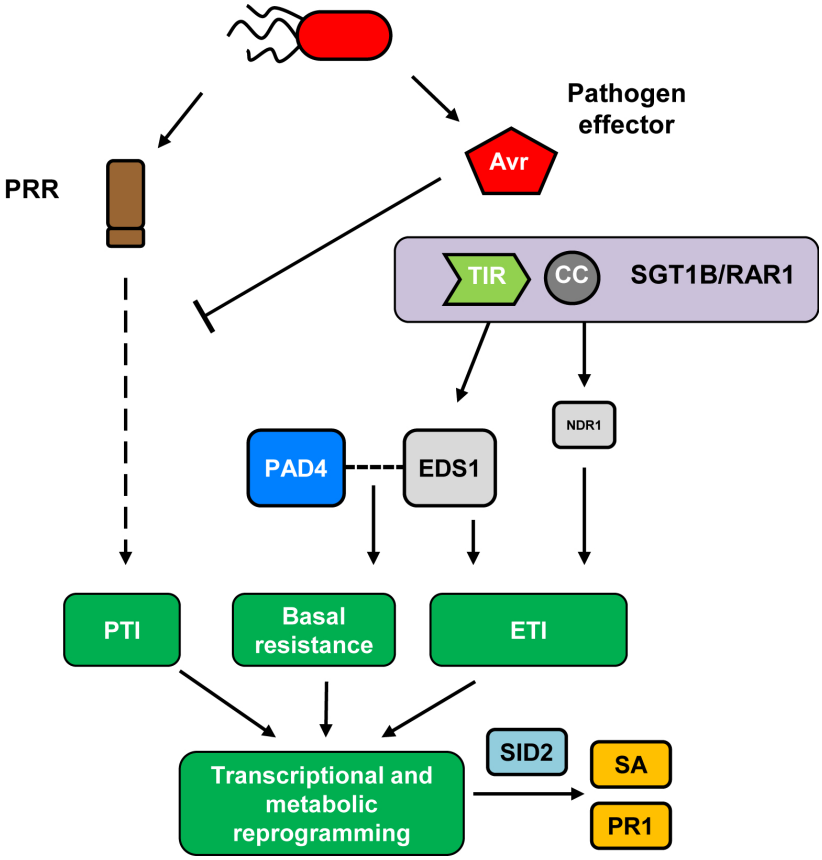

Figure 3.2: Schematic overview of disease resistance pathways. PTI involves the recognition of PAMPs via surface-PRRs. ETI relies on the recognition of pathogen effectors (Avr) by R-genes of the TIR- and CC-NB-LRR type, which have different signaling requirements. Basal resistance is a residual form of resistance that occurs upon infection with virulent pathogens and requires EDS1-PAD4 heterodimer formation. PTI, ETI and basal resistance lead to transcriptional and metabolic reprogramming, which involves the production of SA and PR1.

et al., 1984). Pathogen effectors interfere with signaling components required for PTI, which leads to host susceptibility (Guo et al., 2009; Mudgett, 2005). This phenomenon has been termed effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). In most cases bacterial effectors are delivered through a type III secretion system to host cells (Alfano and Collmer, 2004). A prominent example for a bacterial effector is the *Pseudomonas syringae* pv. *tomato* (*Pst*) effector AvrPto. AvrPto was previously shown to directly interact with the kinase domain of FLS2, the PRR that binds flagellin, thereby preventing the activation of FLS2 and its capability to mediate downstream PTI responses (Xiang et al., 2008). In addition, AvrPto was also shown to suppress the expression of microRNAs that have been implicated in PTI responses (Navarro et al., 2008).

Some plants, that have evolved the ability to recognize and respond to the action of pathogen effectors, can overcome ETS. This form of disease resistance is known as effector-triggered immunity (ETI) and represents a second branch of innate immunity (Chisholm et al., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006) (Figure 3.3). ETI relies on a diverse set of specific intracellular host immune receptors, known as resistance (R-)proteins, that are capable of directly recognizing pathogen effectors or monitor the status of host proteins that are targeted by pathogen effectors. Already in 1942, when no R-proteins nor avirulence factors were described yet, H.H. Flor proposed the resistance and recognition mediated by R-protein/avirulence factor interaction pairs in his gene-for-gene hypothesis (Flor, 1971).

ETI induces defense responses that represent amplified and prolonged versions of the weaker and transient resistance responses induced by PTI (Navarro et al., 2008; Tao et al., 2003; Tsuda and Katagiri, 2010). Commonly, ETI culminates in the induction of the hypersensitive response (HR). HR limits further colonization and spreading of pathogens at infected sites by rapid localized induction of host-programmed cell death (Greenberg and Yao, 2004). Recent studies suggest that many molecular responses that promote disease resistance are associated with both PTI and ETI, suggesting overlapping signaling machineries (Katagiri and Tsuda, 2010; Sato et al., 2010; Tsuda and Katagiri, 2010; Tsuda et al., 2009).

Effective ETI puts pathogenic microbes under selective pressure to evade R-protein-mediated resistance (Chisholm et al., 2006). The arms race between host and pathogens drives the evolution of alternative pathogen suppressors that suppress ETI (Chisholm et al., 2006; Jones and Dangl, 2006). Consequently, infected plants revert to basal resistance, that represents a minimal form of residual post-infection resistance. In their "zigzag" model, Jones and Dangl (2006) defined basal resistance as the combined effects of PTI and weak ETI, minus ETS (Figure 3.3). However, Zhang and co-workers (2010) found that PTI and ETI differentially contributed to basal resistance and suggested a predominant role of weak ETI in basal resistance against virulent bacterial pathogens. This co-evolutionary arms race forces plants to evolve new R-protein variants to recognize virulent pathogens, that in turn will develop new virulence strategies.

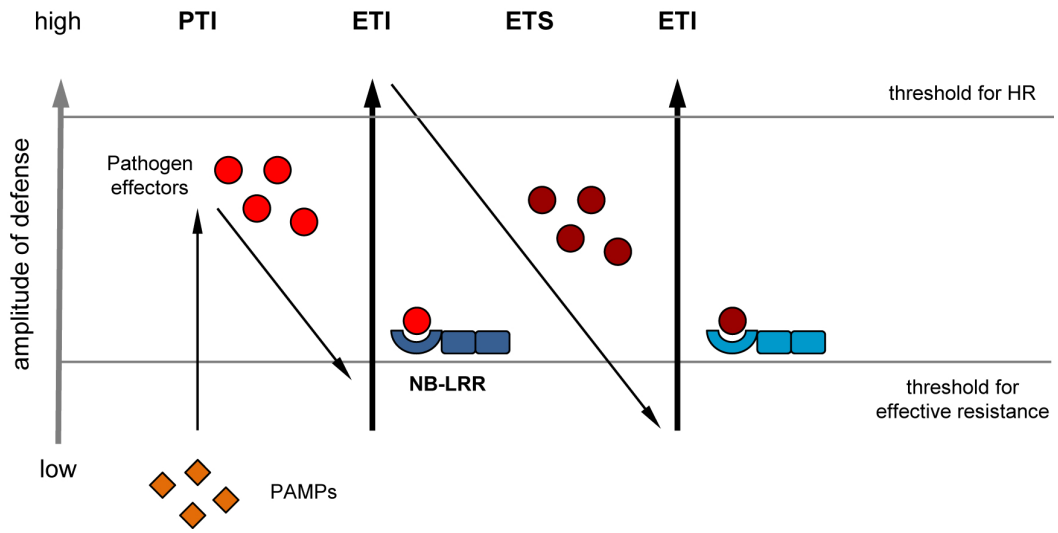

Figure 3.3: Depiction of the zigzag model as described by Jones and Dangl (2006). The amplitude of disease resistance or susceptibility is the sum of PTI-ETS+ETI.

3.2.2 Plant NB-LRR R-proteins

ETI relies on a diverse set of host R-proteins for the detection of pathogen effector activities in the host cell. The majority of R-proteins in *A. thaliana* belong to the NB-LRR class of R-proteins (NLRs) (Martin et al., 2003), which are named after their central nucleotide-binding domain, sometimes also referred to as NB-ARC (nucleotide-binding adaptor shared by Apaf-1, plant resistance proteins, and CED-4) domain, and their C-terminal leucine-rich repeat (LRR) domains (van der Biezen and Jones, 1998). NB-LRR proteins belong to a subclass of the STAND superfamily (signal transduction ATPases with numerous domains) (Danot et al., 2009), and resemble animal NOD (nucleotide-binding oligomerization domain)-like receptors that are also involved in innate immunity and cell death responses (Ausubel, 2005; Maekawa et al., 2011b).

In addition to NB-ARC domains and LRR domains, many plant NB-LRR proteins can also be classified by their additional N-terminal domains: TIR-NB-LRR (TNL) proteins contain an N-terminal TIR domain (Toll/interleukin-1 receptor, resistance protein) that is related to *Drosophila* Toll and human interleukin-1 receptor signaling domains (Bernoux et al., 2011), while CC-NB-LRR (CNL) proteins bear a coiled-coil (CC) domain (Maekawa et al., 2011a). However, some NB-LRR proteins contain uncharacterized or non-conserved N-terminal domains (Bonardi et al., 2012; Meyers et al., 2003).

Due to the multi-domain structure of NB-LRR proteins, NB-LRRs can simultaneously act as pathogen detectors, molecular switches, and mediate downstream signaling processes (Lukasik and Takken, 2009; Ma et al., 2012). Several conserved structural and sequence motifs are necessary for the correct function of plant NB-LRR proteins. The NB-ARC domain is

the most invariant domain in NB-LRR proteins (Takken and Goverse, 2012). Only recently the first crystal structures of plant NB-LRRs have been elucidated (Bernoux et al., 2011; Maekawa et al., 2011a; Wan et al., 2013), and most knowledge on structural features has been derived from model structures based on animal Apaf-1 and CED-4 (Riedl et al., 2005; van Ooijen et al., 2008; Yan et al., 2005).

Multiple proteins motifs located in the NB-ARC domain play crucial roles in ATP-binding and hydrolysis, which is thought to trigger conformational changes of NB-LRRs from a closed conformation (ADP bound) to an open conformation (ATP bound) (Danot et al., 2009; Takken et al., 2006). The Walker-A motif or p-loop (consensus sequence GxxxxGKS/T, where x stands for any amino acid) and Walker B motif (consensus sequence hhhhDD/E, where h indicates a hydrophobic residue) are required for ATP binding and hydrolysis (Takken et al., 2006). Mutations in these motifs have been demonstrated to impair NB-LRR function (Hanson and Whiteheart, 2005; Tameling et al., 2002). Similarly, the highly conserved MHD motif (consensus sequence hxhHD) is also embedded in the NB-ARC domain (Takken and Goverse, 2012). The MHD motif appears to have a critical role in controlling the activity of NB-LRR proteins, since mutations in this motif have been found to lead to autoactivation of several NB-LRRs, which often results in stunting or lethality of mutant plants (Bendahmane et al., 2002; Gao et al., 2011; Howles et al., 2005; Tameling et al., 2006; van Ooijen et al., 2008; Williams et al., 2011).

Based on domain-swapping experiments, intramolecular interactions between the NB-ARC domain and the LRR domain have been proposed to keep NB-LRR proteins in an auto-inhibited or resting state in the absence of pathogens (Heidrich et al., 2012; Lukasik and Takken, 2009; Ma et al., 2012; van Ooijen et al., 2008). The LRR domain is thought to confer pathogen recognition specificity (Farnham and Baulcombe, 2006; Ma et al., 2012). Upon detection of effectors or effector activity, a conformational change in the NB-ARC domain is induced, allowing for ADP to ATP exchange, a process that is believed to lead to the activation of downstream responses. NB-LRR-mediated pathogen sensing might rely on either direct physical interaction with effector proteins (Ellis et al., 2007), or on indirect recognition of effectors and their associated intracellular activities. Models for indirect effector recognition propose, that a host protein, that is a target of a pathogen effector, may mediate effector recognition. In this "guard hypothesis", the actions of the effector will induce a change in the host protein, and this change is sensed by the cognate NB-LRR protein, thereby acting as a guard of the host protein (the guardee) (Dangl and Jones, 2001). Alternatively, a duplication of the target gene might encode for a structural mimic of the actual effector target, thereby presenting a decoy, perturbation of which by the virulence effector might be monitored by a NB-LRR receptor (van der Hoorn and Kamoun, 2008).

The NB-LRR protein family is one of the most variable gene families in land plants and has undergone massive expansion in different plant species. The number of NB-LRR

proteins varies largely in flowering plants, ranging from approximately 150 NB-LRRs in the *A. thaliana* Columbia-0 (Col-0) accession (Meyers et al., 2003) to 460 NB-LRRs in rice (Li et al., 2010). Generally, NB-LRR genes are distributed unevenly in plant genomes and are often organized in linked cluster (Guo et al., 2011; Hulbert et al., 2001; Meyers et al., 2003). Rapid evolution of NB-LRRs is promoted by genomic re-arrangements, such as local and large-scale duplication, gene conversion, and ectopic recombination. Clustering of NB-LRR genes might facilitate these processes to evolve new recognition specificities (Michelmore and Meyers, 1998). Different selection mechanisms have been proposed to act on intragenic level to promote diversification processes against rapidly evolving pathogens (reviewed in Jacob et al., 2013). While NB-ARC domains generally appear to be under purifying selection to disfavor non-synonymous mutations, the variable LRR regions appear to be subject to positive diversifying selection (Chen et al., 2010; Mondragón-Palomino et al., 2002).

3.2.3 NB-LRR mediated defense-signaling networks

Genetic screens have identified different signaling requirements for TNL proteins and CNL proteins to mediate ETI (Figure 3.3). Disease resistance conditioned by CNL proteins generally requires *NDR1* (*NON-RACE DISEASE RESISTANCE 1*), which encodes a plasma membrane-localized protein with similarity to mammalian integrins that function in cell adhesion and signaling (Century et al., 1995, 1997; Coppinger et al., 2004; Knepper and Day, 2010; Knepper et al., 2011). NDR1 was shown to associate with RIN4, an *Arabidopsis* protein that is monitored by the CNL proteins RPM1 and RPS2 for perturbation by pathogen effectors (Day et al., 2006). Additionally, analysis of *ndr1* mutants revealed that NDR1 might also be involved in basal resistance, since loss of NDR1 allows for increased propagation of virulent pathogens (Zhang et al., 2010).

The lipase-like protein EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) has emerged as an important regulator in both ETI and basal resistance, and constitutes the main signaling hub for resistance elicited by TNL proteins (Parker et al., 1996; Wiermer et al., 2005). Recently, EDS1 was shown to interact with the TNL proteins RPS4, RPS6, and SNC1, suggesting that EDS1 and TNL proteins might be part of an immune signaling complex (Bhattacharjee et al., 2011; Heidrich et al., 2011). A model was proposed in which the main role of EDS1-associated TNL proteins is to guard EDS1 from manipulation by effector proteins. In this model, the guarding TNLs might sense perturbation of EDS1 complexes by pathogen effectors and subsequently elicit downstream defense responses. EDS1 complexes accumulate in the nucleus during ETI and basal immunity, which was shown to be required for transcriptional defense reprogramming (García et al., 2010; Heidrich et al., 2011).

EDS1 interacts with two more sequence related proteins, PAD4 (PHYTOALEXIN DEFICIENT 4) (Feys et al., 2001; Jirage et al., 1999), and SAG101 (SENESCENCE ASSOCIATED GENE 101) (Feys et al., 2005), and forms molecularly distinct EDS1/PAD4 and EDS1/SAG101

complexes (Rietz et al., 2011; Wagner et al., 2013). The combined activity of EDS1, PAD4, and SAG101 is essential for complete resistance conditioned by TNLs and basal resistance against virulent pathogens (Rietz et al., 2011). The activities of EDS1 and PAD4 stimulate the production of SA (Feys et al., 2001, 2005; Jirage et al., 1999; Rietz et al., 2011). Production of SA is required for SAR and involves the induction of plant systemic immunity in distal tissues emanating from the infection site (Boatwright and Pajerowska-Mukhtar, 2013; Vlot et al., 2009). *Arabidopsis* ICS1/SID2 (ISOCHORISMATE SYNTHASE 1/SALICYLIC ACID INDUCTION DEFICIENT 2) is required for SA biosynthesis and converts chorismate to isochorismate, a precursor of SA (Wildermuth et al., 2001). SA acts in a feed-forward and feed-back manner, which leads to increased expression of EDS1 and PAD4, thereby reinforcing and potentiating immune signaling (Falk et al., 1999; Jirage et al., 1999).

Balanced activities of the HSP90 (HEAT SHOCK PRTOTEIN 90) chaperone and the associated co-chaperones RAR1 (REQUIRED FOR MLA12 RESISTANCE 1) and SGT1B (SUPPRESSOR OF THE G2 ALLELE OF SKP1b) have been shown to regulate the assembly and stability of NB-LRR proteins (Seo et al., 2008; Shirasu, 2009). Mutations that compromise the function of these co-chaperones were shown to result in reduced accumulation of several NB-LRR proteins and loss of NB-LRR mediated resistance (Hubert et al., 2003; Takahashi et al., 2003). Interestingly, RAR1 and SGT1B appear to have overlapping, but also independent functions, since certain NB-LRR proteins require either RAR1, SGT1B or their combined activity for disease resistance signaling (Holt et al., 2005). In addition to their role in ETI, RAR1 and SGT1B appear to play a role in basal resistance against virulent pathogens (Zhou et al., 2008). The function of the HSP90/SGT1/RAR1 module seems to be evolutionary conserved, since mammalian NOD receptors also require the HSP90/SGT1/RAR1 module to mediate innate immunity (Maekawa et al., 2011b).

3.2.4 Alternative Splicing of NB-LRR transcripts

Alternative splicing appears to occur frequently in TNL type R-genes (Dinesh-Kumar and Baker, 2000; Gassmann et al., 1999; Kim et al., 2009c; Lawrence et al., 1995; Tan et al., 2007; Tang et al., 2013; Whitham et al., 1994). While alternatively spliced TNL transcripts have previously been shown to play a role in defense against pathogens in multiple plant species, to date their exact role in mediating disease resistance is less clear (Dinesh-Kumar and Baker, 2000; Tang et al., 2013; Zhang and Gassmann, 2003). Interestingly, alternative transcript variants of TNLs generally contain PTCs that potentially lead to the translation of truncated proteins, or could be substrates of NMD (reviewed in Gassmann, 2008; Jordan et al., 2002; Staiger and Brown, 2013; Staiger et al., 2013).

Initially, alternative splicing of the TIR-NB-LRR *N* gene from tobacco (*Nicotiana tabacum*) was reported (Dinesh-Kumar and Baker, 2000; Whitham et al., 1994). The *N* gene is required to confer complete resistance against tobacco mosaic virus (TMV) (Whitham et al., 1994).

Alternative splicing of the *N* pre-mRNA gives rise to two transcripts with different lengths, known as N_S and N_L. The short N_S transcript encodes for the full-length *N* protein with intact TIR, NB-ARC and LRR domains, while the longer N_L transcript lacks 13 of the 14 LRR repeats due to a PTC induced by inclusion of an alternative exon and subsequent frameshifting (Figure 3.4A). Alternative splicing of *N* is dynamically regulated during TMV infection. Interestingly, before TMV infection, N_S appears to be the dominant splice variant, while during infection the abundance of N_L increases dramatically relative to the N_S transcript (Dinesh-Kumar and Baker, 2000). Importantly, plants that expressed only N_S full length proteins were more susceptible than wild-type plants, suggesting that the expression of N_L-encoded truncated *N* protein, that lacks most of its LRRs, is required for complete resistance. Additionally, it was found that dynamic changes in the relative ratio of N_L and N_S are critical to confer resistance, since plants that expressed equal amounts of N_L and N_S only showed minimal resistance (Dinesh-Kumar and Baker, 2000).

Another well-studied example of alternative splicing of an NB-LRR transcript is the *A. thaliana* TNL *RPS4* (*RESISTANT TO P. SYRINGAE 4*). RPS4 mediates defense in response to *P. syringae* strains that express the bacterial effector protein AvrRps4 (Gassmann et al., 1999). In addition to the canonically spliced *RPS4* transcript, serveral alternatively spliced variants have been described (Zhang and Gassmann, 2003, 2007). These alternative transcript isoforms are generated by a combination of intron retention events and excision of a cryptic intron in the third exon, which in all cases generate PTCs and potentially lead to the production of truncated RPS4 proteins (Figure 3.4B). It has been shown that the presence of both full length and truncated RPS4 proteins is required for proper RPS4 function and induction of HR upon infection with avirulent *Pst* DC3000 expressing AvrRps4 (Zhang and Gassmann, 2003). Interestingly, splicing of the *RPS4* pre-mRNA was dynamically regulated during infection, leading to dynamic changes in transcript ratios (Zhang and Gassmann, 2007). The most abundant transcript isoform during infection retains intron 3, thereby encoding for a protein that only consists of the TIR and NB-ARC domains. Transient expression of truncated RPS4 proteins that corresponded to alternatively spliced *RPS4* isoforms was sufficient to induce HR in *Nicotiana benthamiana*, indicating an important role for alternative splicing of RPS4 in eliciting defense responses. However, alternative splicing of the *RPS4* mRNA is not limited to the RPS4-AvrRps4 interaction, but can also be induced by the unrelated effector proteins HopA1 and AvrRpt2 (Zhang and Gassmann, 2007).

AvrRps4-induced alternative splicing was also found to occur in unrelated RNA transcripts and suggested a more global effect on the splicing machinery than a specific effect on the *RPS4* mRNA (Zhang and Gassmann, 2007). In agreement, Xu and colleagues (2012) identified the *mos12-1* mutant that showed altered splicing patterns of *RPS4* and the TNL *SNC1* (Figure 3.4D). The *MODIFIER OF SNC1-12* (*MOS12*) encodes a cyclin L-like protein with similarity to serine-arginine splicing factors. Concomitantly with splicing defects observed in

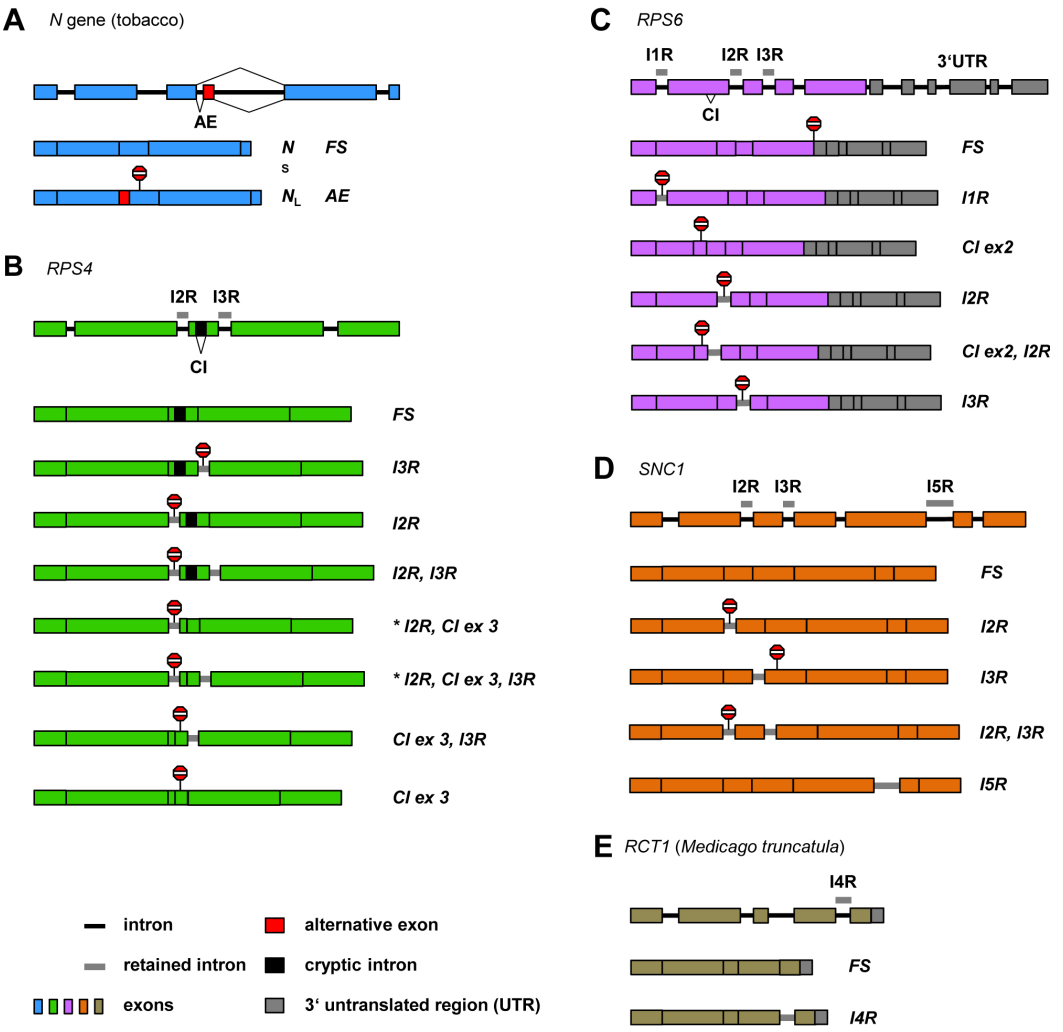

Figure 3.4: Alternative splicing of R-genes. (A) Scheme of the tobacco *N* mRNA. *N* exists in two mRNA isoforms designated N_S and N_L. N_L includes an alternative exon that introduces a PTC, leading to the translation of a truncated protein (Dinesh-Kumar and Baker, 2000). (B) Alternative splice variants of *A. thaliana RPS4*. Transcript isoforms marked with asterisks were predicted to exist but were not experimentally verified (Zhang and Gassmann, 2007). (C) Alternative splice variants of *A. thaliana RPS6* (Kim et al., 2009c). The alternative splicing event leading to retention of intron 3 (I3R) was identified by whole-transcriptome profiling (Marquez et al., 2012; Staiger and Brown, 2013). (D) Alternative splice variants of *A. thaliana SNC1* (Xu et al., 2012). Retention of intron 5 was identified by Marquez et al. (2012).(E) Alternative splice variants of *Medicago truncatula RCT1* (Tang et al., 2013). This figure was adapted from Staiger and Brown (2013).

mos12-1 mutants, also lower SNC1 protein levels were observed. Using co-immunoprecipitation experiments, the authors demonstrated that MOS12 interacts with the evolutionary conserved spliceosome-associated MOS4-complex (MAC), and components of the MAC have been implicated in basal resistance and NB-LRR mediated defense (Monaghan et al., 2009; Palma et al., 2007). MOS12 was also shown to be required for basal resistance and RPS4-mediated resistance (Xu et al., 2012), supporting an important role for alternative splicing of R-transcripts in fine-tuning disease resistance.

Alternative splicing was also described for the TNL *RESISTANT TO P. SYRINGAE 6* (*RPS6*) (Kim et al., 2009c). RPS6 confers resistance in response to the *P. syringae* effector HopA1 (formerly known as hopPsyA) (Gassmann, 2005; Kim et al., 2009c). Similar to *RPS4* and other TNL genes, *RPS6* generates multiple alternatively spliced isoforms (Figure 3.4C). Alternative splicing events include the retention of intron 1, excision of a cryptic intron that is embedded within exon 2, and optional retention of the downstream intron 2. Additional alternatively spliced forms of *RPS6* have been identified using RNA-seq (Marquez et al., 2012; Staiger and Brown, 2013). All splice variants found to date generate PTCs and encode truncated TIR-domain only or TIR-NB proteins. In addition, the *RPS6* mRNA features an unusually long 3'UTR and multiple 3'UTR-located introns, that might serve as recognition signals for the NMD machinery, thereby regulating *RPS6* mRNA abundance and protein accumulation (Kim et al., 2009c).

Recently, alternative splicing of a TNL type R-gene was also reported in *Medicago truncatula* (Tang et al., 2013). The TNL gene *RCT1* confers resistance to the hemi-biotrophic fungal pathogen *Colletotrichum trifolii* (Yang et al., 2008). An alternative splice event in *RCT1* leads to the retention of intron 4, resulting in a transcript that encodes for a truncated RCT1 protein (Figure 3.4E). Similar to the tobacco *N* gene and *A. thaliana RPS4*, combined expression of truncated and full-length *RCT1* transcripts is required to confer disease resistance (Tang et al., 2013).

NMD is likely to play a role in the turnover of alternative NB-LRR transcripts (Gassmann, 2008; Staiger and Brown, 2013; Staiger et al., 2013). Most alternatively spliced TNL transcripts contain PTCs that might target them for degradation by the NMD machinery. While steady state levels of PTC-containing TNL transcripts are generally low before infection, transcript abundance dramatically increases during the course of infection (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003, 2007), suggesting downregulation of NMD upon pathogen challenge. In agreement, Howard and co-workers (2013) reported that multiple PTC-containing transcripts were upregulated in an RNA-seq based study of *Pst* DC3000 infected leaf tissue, indicating that dampening of NMD might indeed occur during pathogen infection.

3.3 Aim of this work

RNA degradation machineries such as NMD play important roles in RNA surveillance, thereby ensuring transcriptome homeostasis (Mühlemann and Jensen, 2012; Pérez-Ortín et al., 2013). An increasing number of reports indicate that NMD might also act as regulator of endogenous gene expression, but so far only few biological processes have been implicated to be regulated by NMD (Bruno et al., 2011; Gardner, 2008; Mendell et al., 2004).

The NMD components of the SMG family (SMG5, SMG6 and SMG7) play pivotal roles in the turnover of aberrant mRNA transcripts (reviewed in Schweingruber et al., 2013). While core NMD factors have been extensively characterized in animals and yeast (Conti and Izaurralde, 2005), comparably little is known about NMD factors and the mechanism of NMD substrate recognition in plants. In addition, dissecting the biological processes regulated by NMD is a challenging task because of the complex phenotypes that arise from NMD impairment.

Previously, Nina Riehs-Kearnan, a former colleague, identified independent roles for the *Arabidopsis* SMG7 protein in the regulation of NMD and meiotic cell cycle progression (Riehs et al., 2008). Disruption of SMG7 led to severe phenotypic aberrations that were similar to those of *Arabidopsis* mutants with constitutive pathogen response activation (Riehs et al., 2008; Riehs-Kearnan et al., 2012). Parallel to our study on the cause of vegetative defects in *smg7* mutants (Riehs-Kearnan et al., 2012), similar studies using other NMD mutants were published during the course of my PhD studies (Jeong et al., 2011; Rayson et al., 2012a; Shi et al., 2012).

The aim of my PhD studies was to characterize a putative role for NMD in a biologically relevant process – the regulation of plant innate immunity. To this end, I genetically characterized disease resistance pathways that lead to phenotypic abberations and autoimmunity observed in *A. thaliana smg7* mutants. Furthermore, I investigated if constitutive defense signaling in SMG7-impaired plants, and mutants depleted of other NMD components, was correlating with impaired NMD. Moreover, using genetic approaches and whole-transcriptome profiling, I aimed to identify RNA substrates, that were regulated by NMD, and that were relevant for the regulation or execution of innate immune responses. Finally, I explored the physiological relevance of NMD during pathogen infection, and the contribution of NMD activity to disease resistance. The work presented here unravels the cause of phenotypic defects of *Arabidopsis* NMD mutants and identifies a regulatory role of NMD in plant innate immunity.

The majority of my work from my doctoral studies is presented in one peer-reviewed publication where I contributed as the second author, and another manuscript that is in preparation, in which I am the main contributing author. The findings from both studies are summarized and discussed in chapter 5.

4 Publications

4.1 Nonsense-mediated RNA decay deficiency triggers autoimmune-like response in *Arabidopsis thaliana*

Riehs-Kearnan Nina, Gloggnitzer Jiradet, Dekrout Bettina, Jonak Claudia, Riha Karel. Nucleic Acids Research. 2012, Jul;40(12):5615-24. doi: 10.1093/nar/gks195. Epub 2012 Feb 29.

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doi:10.1093/nar/gks195

Aberrant growth and lethality of *Arabidopsis* deficient in nonsense-mediated RNA decay factors is caused by autoimmune-like response

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ABSTRACT

Nonsense-mediated RNA decay (NMD) is an evolutionarily conserved RNA quality control mechanism that eliminates transcripts containing nonsense mutations. NMD has also been shown to affect the expression of numerous genes, and inactivation of this pathway is lethal in higher eukaryotes. However, despite relatively detailed knowledge of the molecular basis of NMD, our understanding of its physiological functions is still limited and the underlying causes of lethality are unknown. In this study, we examined the importance of NMD in plants by analyzing an allelic series of *Arabidopsis thaliana* mutants impaired in the core NMD components SMG7 and UPF1. We found that impaired NMD elicits a pathogen defense response which appears to be proportional to the extent of NMD deficiency. We also demonstrate that developmental aberrations and lethality of the strong *smg7* and *upf1* alleles are caused by constitutive pathogen response upregulation. Disruption of pathogen signaling suppresses the lethality of the *upf1-3* null allele and growth defects associated with SMG7 dysfunction. Interestingly, infertility and abortive meiosis observed in *smg7* mutants is not coupled with impaired NMD suggesting a broader function of SMG7 in cellular metabolism. Taken together, our results uncover a major physiological consequence of NMD deficiency in *Arabidopsis* and revealed multifaceted roles of SMG7 in plant growth and development.

INTRODUCTION

Nonsense-mediated RNA decay (NMD) is an important surveillance mechanism that detects and targets aberrant RNA transcripts for degradation (1,2). NMD substrates are typically transcripts containing a premature translation termination codon (PTC) that can arise by mutation and gene rearrangements, transcription errors or alternative splicing.

Research from various model organisms has provided reasonably detailed molecular insights into how aberrant RNAs are recognized and processed by the NMD machinery. One of the most defining features of NMD substrates is a PTC that gives rise to a long 3′ UTR, which is sensed, in a translation-dependent manner, by the evolutionarily conserved RNA helicase UPF1 (3). The current NMD model predicts that the decision of whether RNA will be degraded or not is determined by competition between cytoplasmic poly(A)-binding protein 1 and UPF1 for binding to translation release factors eRF1 and eRF3 at the terminating ribosome (1). This interaction may be modulated by numerous structural features within mRNA. For example, the presence of splicing boundaries downstream of a stop codon acts as a strong enhancer of NMD. This is due to the activity of two other conserved NMD components, UPF2 and UPF3, which associate with the exon junction complex that is deposited at the exon–exon boundaries and are thought to enhance interaction between UPF1 and the release factors (1,4). The UPF1-eRF binding stimulates UPF1 phosphorylation by SMG1 kinase and promotes RNA degradation. In many eukaryotic organisms, this process relies on the conserved SMG5–7 phosphoserine binding proteins that interact with phosphorylated UPF1 and promote its dephosphorylation by the

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protein phosphatase PP2A (5,6). In metazoans, these proteins are suggested to determine two distinct pathways of RNA degradation. One mechanism relies on SMG5 and SMG7, which are thought to direct aberrant RNAs destined for exonucleolytic degradation to cytoplasmic P-bodies (7,8). The alternative pathway initiates mRNA degradation by endonucleolytic cleavage in the vicinity of the PTC via the PilT N-terminus (PIN) nuclease domain of SMG6 (9,10).

Although NMD is primarily described as a quality control mechanism, studies in a number of organisms have shown that it affects the stability of many physiological mRNAs as well as non-coding transcripts and pseudogenes, indicating a broader role in gene regulation (11–14). Nevertheless, the biological function of NMD is still not fully understood, partially because NMD null mutations are lethal in higher eukaryotes. While NMD is dispensable for viability in yeast and worms (15–18), inactivation of the core NMD components such as UPF1, UPF2 or members of the SMG5–7 protein family confer embryonic lethality in flies, zebra fish and mice (19–23). However, the primary cause of the lethality in these organisms is unknown. Transcriptome analysis in aborted embryos of SMG1 mouse knock-outs revealed massive mis-expression of a number of genes, suggesting that defective embryogenesis in NMD mutants is underpinned by deregulation of multiple cellular pathways (24). Alternatively, NMD may be critical for a specific process, deregulation of which has fatal consequences. It is also possible that the essential functions of the above-mentioned proteins are linked to their roles in processes unrelated to NMD (2).

Nonsense-mediated RNA decay is also conserved in plants and several of its components have been functionally characterized in *Arabidopsis* (25–29). These studies demonstrated that NMD is essential for plant viability as null mutations in SMG7 and UPF1 cause embryo and seedling lethality, respectively. In our previous work, we took advantage of viable hypomorphic *smg7* alleles, which allowed us to study the role of SMG7 in plants. We showed that these mutants are infertile and that the infertility is caused by a specific defect in meiotic cell cycle progression that prevents formation of functional gametes (29,30). The *smg7* mutants also exhibited a range of vegetative phenotypes suggesting a pleiotropic role of SMG7 in plant development. Interestingly, these phenotypes were modulated by environmental conditions as the growth defects were largely suppressed by cultivation at higher temperature (27°C) or elevated humidity (80–100%). In this study, we demonstrate that the vegetative growth defects in hypomorphic *smg7* mutants are caused by deregulation of a specific pathway involved in pathogen response. Furthermore, we show that lethality of the UPF1 deficient plants is also caused by massive upregulation of pathogen response. Nevertheless, meiotic defects observed in *smg7* mutants do not appear to be a consequence of aberrant NMD and indicate a broader role of SMG7 in cellular metabolism.

MATERIALS AND METHODS

Plant growth conditions and treatments

All mutant strains used, in this study, were obtained from the *Arabidopsis* Stock Center (Supplementary Table S1). *Arabidopsis thaliana* ecotype Col-0 was used as a control line. Seeds were either directly sown on soil or were germinated on 0.5 × MS medium supplemented with 1% sucrose and 0.6% plant agar (Duchefa, Netherlands). In most experiments, plants were grown at 21°C and 60–70% humidity under long day conditions (16 h light/8 h dark). To examine phenotypic difference among *upf1* and *upf3* alleles, plants were grown at 60–70% humidity and 19°C during the photoperiod (16 h) and at 16°C in the dark (8 h). Plants used for infiltration with *P. syringae* were grown at 21°C and 60–70% humidity under short day conditions (8 h light/16 h dark). Infection of *Arabidopsis* plants with *P. syringae* was performed according to (31). In brief, an inoculum of *P. syringae* pv. maculicula ES4326 containing the LuxCDABE operon (32) was prepared by growing the bacterial culture at 28°C in King's B medium, followed by sedimentation and two washing steps. The bacterial pellet was diluted in water supplemented with 0.004% Silvet L-77 (Lehle Seeds) to an OD600 of 0.005 or 0.002. Six-week-old plants grown in short day conditions at 21°C were pre-conditioned for 3 days in a grow chamber under a 19/16°C temperature regime and then vacuum infiltrated with either the bacterial inoculum or with distilled water. Imaging of luciferase activity was performed using the VisiLuxx imaging system (Visitron System). For determination of the bacterial titer, discs of leaf tissue were excised from infiltrated leaves 48 h post-infection, washed and ground. The extract was then plated in a serial dilution on plates containing King's medium B and bacteria were allowed to grow at 28°C for 2 days, followed by quantification of colony forming units.

RNA analyses

Total RNA was isolated from plant tissues using TriReagent (Sigma). For Northern blot analysis, 10 µg of total RNA was fractionated by electrophoresis on a 1.2% formaldehyde agarose gel, blotted onto a nylon membrane (Hybond-NX, Amersham) and probed with ^{32}P-labeled probes specific for *PR1* (At2g14610), *PR5* (At1g75040) and *PDF1.2* (At1g55010). The autoradiogram was recorded on a Kodak Phospor Screen (Biorad) and scanned with the Molecular Imager FX (Biorad) imaging system. Equal loading of RNA samples was verified with ethidium bromide staining of the RNA gel. For analysis of alternatively spliced At2g45670 transcripts by RT-PCR, cDNA was reverse transcribed from 2 µg of total RNA using oligo-dT primers followed by PCR with primers listed in Supplementary Table S2. PCR products were separated on an 8% polyacrylamide gel, DNA was stained with SybrGreen I, and fluorescence signal was scanned using a Molecular Imager FX (Biorad). Cordycepin and cycloheximide treatments were carried out as previously described (33,34). RNA for qPCR analysis was pretreated with TURBO DNA-free DNAse

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(Ambion) and reverse transcribed with qScript Flex cDNA synthesis kit (Quanta Biosciences) and oligo dT in a 20 µl reaction. Two microlitres of the cDNA diluted in a 1:5 ratio were then used as a template in qPCR using the SensiMix SYBR & Fluorescein kit (Bioline) and the iQ5 cycler (BioRad) with PR1 and At2g45670 specific primers (Supplementary Table S2). The At4g26410 gene, expression of which does not alter after biotic stress, was used for normalization (35). All presented data are derived from three biological replicas, each of which represents an average of three technical replicas.

Histology and cytology

Necrotic lesions on leaves were detected by trypan blue staining according to (36). The stained leaves were examined under a phase-contrast stereo microscope (Leica). Meiosis in pollen mother cells and pollen viability were analyzed as described (29,37).

Salicylic acid quantification

Leaves of three 5-week-old plants were pooled and ground in liquid nitrogen to a fine powder. Free and total levels of salicylic acid were measured via high-performance anion-exchange chromatography as previously described (38).

RESULTS

Vegetative defects in *smg7* mutants can be genetically uncoupled from infertility

SMG7 is a member of the SMG5–7 protein family, which is characterized by an N-terminal TPR (tetratrico peptide repeat)-domain and a conserved central domain. SMG7 lacks the C-terminal PIN domain that is present in SMG5 and SMG6. SMG7 proteins are present in most metazoans and in plants, which appear to lack the SMG5 and SMG6 homologues (29). In our previous study, we analyzed three *Arabidopsis smg7* mutant lines carrying T-DNA insertions in the conserved N-terminal and central domains. While disruption of the N-terminal TPR-domain in *smg7-5* mutants is embryonic lethal, mutants with more distal insertions (*smg7-1* and *smg7-3*, Figure 1A) were viable. The *smg7-1* and *smg7-3* plants were infertile and exhibited pleiotropic growth phenotypes that included dwarf stature, narrow serrated leaves and suppressed apical dominance (29). To gain further insight into the role of SMG7 in plant growth and development, we analyzed three additional *smg7* T-DNA insertion alleles (Figure 1). The *smg7-2* plants, which harbour a disruption in the vicinity of the insertion site in the *smg7-3* allele disrupting the gene at the end of the central domain, show a similar set of phenotypes to *smg7-1* and *smg7-3* mutants and are completely sterile. Interestingly, the *smg7-6* and *smg7-4* alleles, which have insertions in the non-conserved C-terminal domain of SMG7, do not cause vegetative growth defects and are indistinguishable from wild-type. However, while *smg7-4* plants are fully fertile, the *smg7-6* mutants have significantly reduced fertility and only some late developing flowers give rise to seeds (Figure 1B).

To more fully understand the cause of impaired fertility in *smg7-6* mutants, we undertook a closer inspection of anthers and meiosis in pollen mother cells (PMC). Anthers of infertile *smg7-1* mutants are devoid of any pollen, because meiotic progression arrests in anaphase II. This arrest results in aberrant meiocytes that contain separated condensed chromatids that do not undergo cytokinesis [Figure 2; (29)]. Although analysis of meiosis in *smg7-6* PMCs revealed figures typical for meiocytes arrested in anaphase II (Figure 2I), we also detected normal telophase II stages with four haploid nuclei (Figure 2H). In addition, we often observed polyads containing a variable number of nuclei of different size (Figure 2J). These polyads underwent cytokinesis producing microspores of unequal size (Figure 2N). We conclude from these data that meiosis in *smg7-6* PMCs is delayed in anaphase II, but in contrast to the situation *smg7-1* mutants, it eventually proceeds to cytokinesis, even producing a small number of viable pollen (Figure 2C). Although *smg7-4* plants are fully fertile, we noticed that all four meiotic microspores tend to remain attached to each other, and this attachment is also preserved in mature pollen (Figure 2D, K, L) indicating that SMG7 function in the *smg7-4* germ line is still partially compromised. These data suggest that while the non-conserved SMG7 C-terminal domain is dispensable for normal vegetative growth, it is essential for proper germ-line development.

Vegetative defects in *smg7* mutants are caused by constitutive pathogen signaling

We next decided to decipher the cause of the severe vegetative phenotypes. We noticed that growth performance of *smg7-1* mutants is strongly influenced by environmental conditions. Plants grown at high humidity were much stronger and bigger than plants grown at low humidity (Figure 1C). We also observed similar modulation of the phenotype by temperature as *smg7-1* mutants grown at 27°C were almost indistinguishable from wild-type plants (data not shown). Under suboptimal conditions (either 19°C/16°C, 60–70% humidity or 21°C, 40–50% humidity), *smg7-1* plants often succumbed to massive necrosis within the first 3 weeks after germination [Supplementary Figure S4; (29)]. Necrotic lesions were apparent in all mutants with vegetative growth defects (*smg7-1,-2,-3*), but not in *smg7-6* plants (Figure 3C). Leaf necrosis and improved survival at high humidity or temperature are hallmarks of lesion mimic mutants (39–41). Such mutants have constitutively up-regulated pathogen signaling, characterized by high levels of salicylic acid (SA) and pathogen related (PR) transcripts, which leads to an enhanced hypersensitive response. Indeed, all strong *smg7* alleles (*smg7-1,-2,-3*) show elevated expression of *PR1* and *PR5* and up to ~10-fold increase in concentration of SA (Figure 3). In contrast, levels of PR transcripts and SA in the mild *smg7* mutants (*smg7-6,-4*) were comparable to wild-type plants.

A strong hypersensitive response is usually caused by effector triggered immunity, which is initiated by

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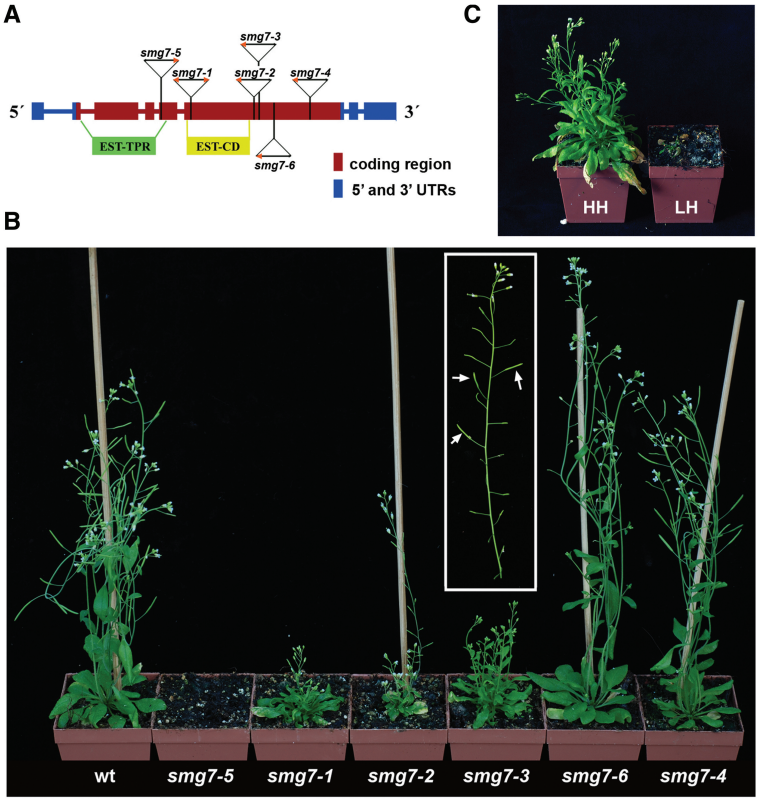

Figure 1. An allelic series of Arabidopsis *smg7* mutants. (**A**) Diagram of the *SMG7* gene that includes positions of T-DNA insertions with indicated orientation of T-DNA left borders (orange arrowhead). Boxes represent exons, regions coding for the conserved TPR (EST-TPR) and central (EST-CD) domains are also shown. Structure of T-DNA insertions in *smg7-4* and smg7-6 alleles is shown in Supplementary Figure 1. (**B**) Six-week-old *smg7* mutant plants grown at 60–70% humidity. In the inset is an older inflorescence from *smg7-6* mutant carrying partially fertile siliques that are indicated by arrows. (**C**) The *smg7-1* mutant plants grown at 60–70% (HH, high) and 40–50% (LH; low) humidity at 21°C.

activation of R-gene(s) in response to pathogen avirulence factors, and is further transduced, depending on the type of R-gene, through either the NDR1 protein or the PAD4/EDS1 complex (42–44). To determine whether inactivation of these pathways attenuates the constitutive pathogen signaling in SMG7 deficient plants, we generated *smg7-1 ndr1* and *smg7-1 pad4* double mutants. Whereas growth performance and PR1 transcription were not affected by the *ndr1* mutation (Supplementary Figure S2), inactivation of *PAD4* in *smg7-1* mutants led to full suppression of all vegetative defects. Furthermore, levels of SA and PR transcripts were restored to normal (Figure 3). Nevertheless, *smg7-1 pad4* still exhibited increased expression of the defensin *PDF1.2*, which is regulated by the jasmonic acid signaling pathway. Jasmonic acid mediates a subset of pathogen responses, but this pathway is usually antagonized by SA (45). Accordingly, expression of *PDF1.2* was suppressed in *smg7* mutants with high levels of SA (Figure 3B). The active jasmonic acid pathway in *smg7-1 pad4* mutants argues that *PAD4* inactivation only aborts the SA mediated response, but there is still ongoing defense signaling.

Enhanced SA signaling confers increased resistance to pathogens (46). To analyze the susceptibility of SMG7 deficient plants with up-regulated SA signaling to a bacterial pathogen, we compared growth of a virulent strain of *Pseudomonas syringae* pv. maculicola ES4326 containing the *luxCDAEB* operon (32) in leaves of wild-type and *smg7-1* mutants. By measuring luminescence and titer of *P. syringae*, we observed that bacterial growth was inhibited in *smg7-1* mutants by more than two orders of magnitude (Figure 4). In conclusion, upregulation of molecular markers, as well as increased resistance to *P. syringae*, demonstrate that SMG7 deficiency in *Arabidopsis* leads to auto-activation of the immune response.

Activation of pathogen response is caused by NMD deficiency

Although the *pad4* mutation rescues the vegetative growth phenotypes of *smg7* mutants, *smg7-1 pad4* mutants are

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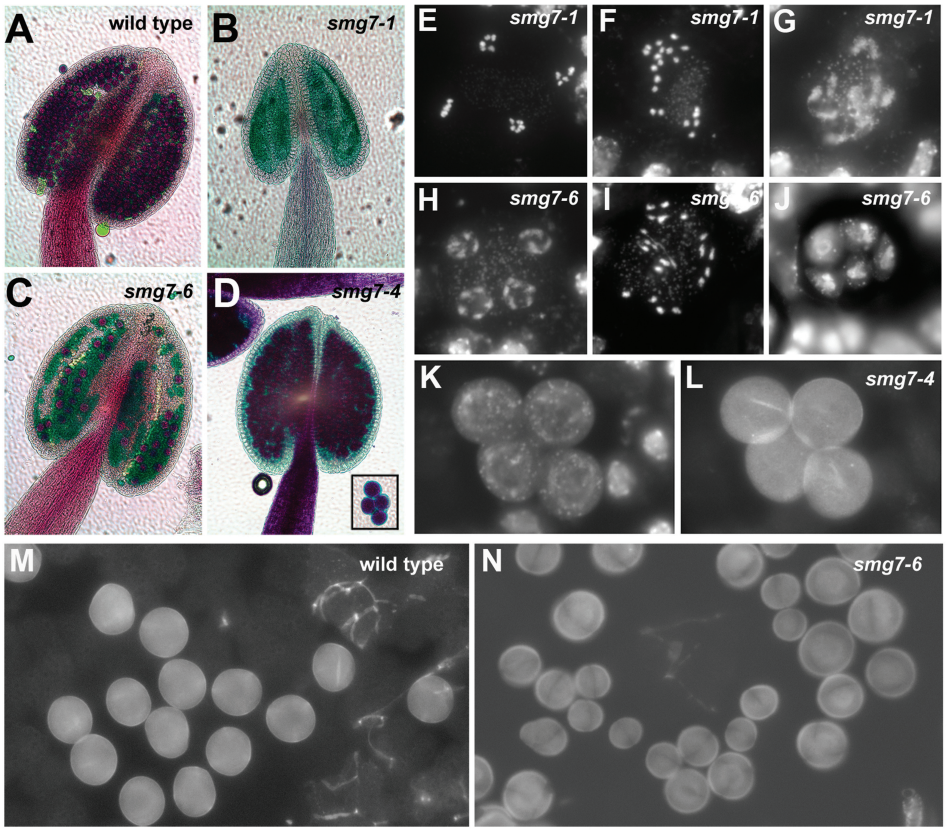

Figure 2. Development of male germ-line in *smg7-6* and *smg7-4* mutants. (**A–D**) Anthers of different *smg7* mutants after Alexander staining. Viable pollen stain red, no pollen is present in *smg7-1* anthers. Inset in (D) shows four joint pollen grains in *smg7-4* mutants. (**E–J**) End of second meiotic division in *smg7-1* and *smg7-6* mutants. Meiotic chromosomes are stained with DAPI: (E) regular anaphase II, (F, I) irregular anaphase II, (G) slowly decondensing chromosomes after anaphase II, (H) regular telophase II, (J) a polyad consisting of five separated cells with irregular nuclei. (**K**, **L**) Microspores typical for *smg7-4* mutants remain attached after meiotic division. Nuclei are stained with DAPI (K), cell walls detected due to their auto-fluorescence using FITC filters (L). (**M**, **N**) Fields of microspores in wild-type and *smg7-6* mutants.

still infertile. Furthermore, the mild *smg7-6* and *smg7-4* truncated alleles do not show pathogen response activation, but are still partially impaired in reproductive development. These observations indicate that SMG7 may participate in these processes through different molecular mechanisms. Because SMG7 is primarily known for its function in NMD, we assayed whether there was a correlation between NMD deficiency and any of the observed phenotypes. We used an alternatively spliced transcript of the At2g45670 gene harbouring a PTC as an endogenous NMD reporter (Figure 5A). The PTC-containing spliced variant is rapidly degraded after inhibition of transcription by cordycepin, and its amount is elevated after cycloheximide treatment that blocks translation (Supplementary Figure S3). This confirms that the alternatively spliced At2g45670 variant is a genuine NMD substrate (34). Quantitative PCR and RT-PCR analyses showed that the PTC-containing transcript is strongly increased relative to the transcript without PTC in *smg7-1, -2* and *-3* mutants confirming impaired NMD (Figure 5B and C). As expected, the *pad4* mutation did not rescue NMD deficiency in *smg7-1* plants. However, the ratios of +PTC and −PTC transcript levels in *smg7-4* and *smg7-6* mutants are comparable with wild-type suggesting that these plants are NMD proficient (Figure 5).

The detection of increased +PTC transcripts only in the *smg7* mutants with vegetative growth defects indicated that auto-activation of pathogen response is caused by aberrant NMD. In this case, deregulation of pathogen response should also occur in plants deficient in other NMD genes. In support of this prediction, a recent study showed slightly elevated levels of SA and PR1 transcripts in *Arabidopsis upf1-5* and *upf3-1* mutants that were not exposed to a pathogen (47). These mutants were also reported to exhibit abnormal growth phenotypes and altered response to *P. syringae* infection (47,48). We have also detected ~2- to 3-fold increase in SA accumulation in the *upf1-5* and *upf3-1* mutants along with mild growth defects, such as narrower and slightly smaller

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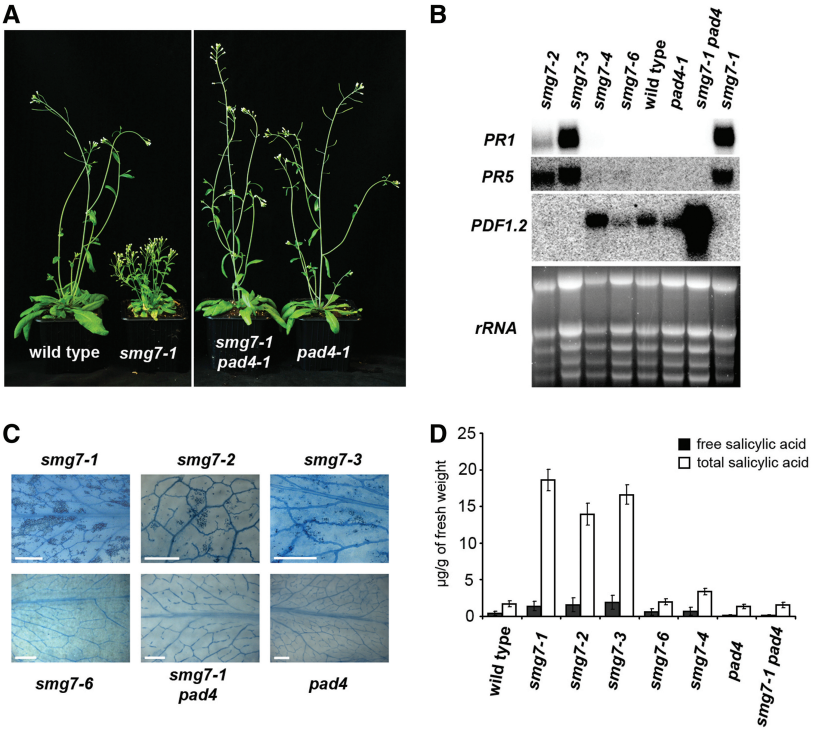

Figure 3. Aberrant growth of *smg7* mutants is associated with elevated pathogen response. (**A**) Six-week-old plants carrying different mutant combinations grown at 60–70% humidity. (**B**) Northern blot analysis of expression of pathogen related transcripts (*PR1* and *PR5*) and the defensin *PDF1.2* in leaves of *smg7* mutants. Ethidium bromide stained gel with *rRNA* species is shown as a RNA-loading control. (**C**) Necrotic lesions in leaves visualized by staining with trypan blue. Scale bar = 2 mm. (**D**) Concentrations of SA in leaves of *smg7* mutants. Each data point represents three biological replicates.

rosette leaves, when the plants were cultivated at 16°C. However, *smg7-1* plants grown at the same conditions exhibited much stronger growth retardation and more than 20-times higher concentration of SA in leaves than *upf1-5* and *upf3-1* mutants (Supplementary Figure S4).

The *upf1-5* allele is a hypomorphic mutation with a T-DNA insertion in the 3′UTR that leads to a reduction of *UPF1* expression (26). In contrast, the *upf1-3* allele harbours a T-DNA disruption in the conserved RNA helicase domain and is lethal (26,27). Seedlings homozygous for the *upf1-3* allele succumb to massive necrosis before development of the first true leaves and qRT-PCR analysis revealed that the *upf1-3* seedlings have highly elevated expression of *PR1* (Figure 6A and C). To determine whether the excessive hypersensitive response causes seedling lethality, we attenuated the pathogen response pathway by inactivating PAD4. Surprisingly, *upf1-3 pad4* mutant seedlings not only remained green, with most developing true leaves, many even survived to produce flowering adult plants (Figure 6A,B). The *upf1-3 pad4* plants grew more slowly than wild-type plants and also flowered much later, producing a large number of leaves before forming inflorescence bolts (Figure 6B). These data demonstrate that deregulation of pathogen response is the primary cause of lethality in UPF1 deficient plants. Analysis of At2g45670 expression revealed that while the relative quantity of the +PTC transcript is increased six times in *smg7-1 pad4* mutants (Figure 5C), the +PTC transcript is more than 40 times elevated in *upf1-3 pad-4* plants (Figure 6D). This data indicates that the extent of pathogen response upregulation is proportional to the decreased efficiency of NMD.

Interestingly, despite the more severe vegetative phenotypes, the *upf1-3 pad4* mutants are, in contrast to *smg7-1 pad4* plants, semisterile and produced viable pollen (Figure 6E). This observation further supports the notion that aberrant NMD does not underlie the meiotic defects observed in *smg7* mutants.

DISCUSSION

Nonsense-mediated RNA decay is an mRNA quality control mechanism that is highly conserved from yeast to humans. While the consequences of NMD dysfunction have a rather uniform molecular manifestation across all tested organisms, namely the increased stability of aberrant RNA transcripts, phenotypic outcomes differ

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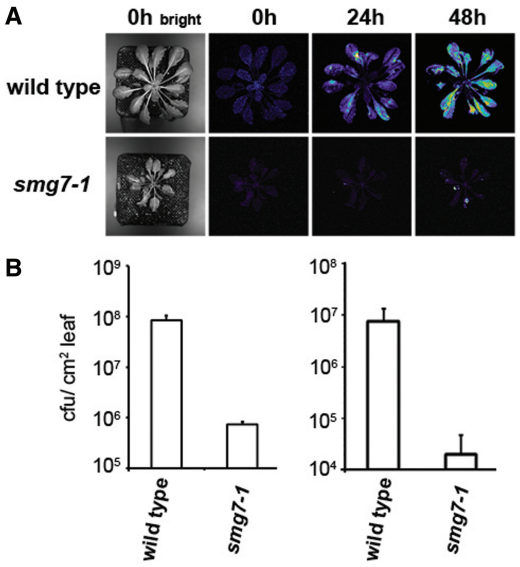

Figure 4. Resistance of *smg7-1* mutants to *P. syringae*. (**A**) Approximately six-week-old plants grown under short day conditions were infiltrated with *P. syringae* pv. maculicola ES4326 harbouring the LuxCDABE operon. Growth of bacteria in leaves was monitored at 24 and 48 h after inoculation by measuring the amount of light produced by bacteria within a 5 min interval. Pseudo-colouring of the images from blue through white and yellow to red reflects increasing light intensity. The left panel shows pictures of the same plants taken under bright light. (**B**) Concentration of bacteria in leaves determined by plating method in two independent experiments. Left chart shows concentration of bacteria 48 h after infection with higher concentration of bacteria (OD_{600} = 0.005). The right chart shows concentration of bacteria 72 h after infection with a lower concentration of bacteria (OD_{600} = 0.002). Standard deviations are derived from three independent biological replicates.

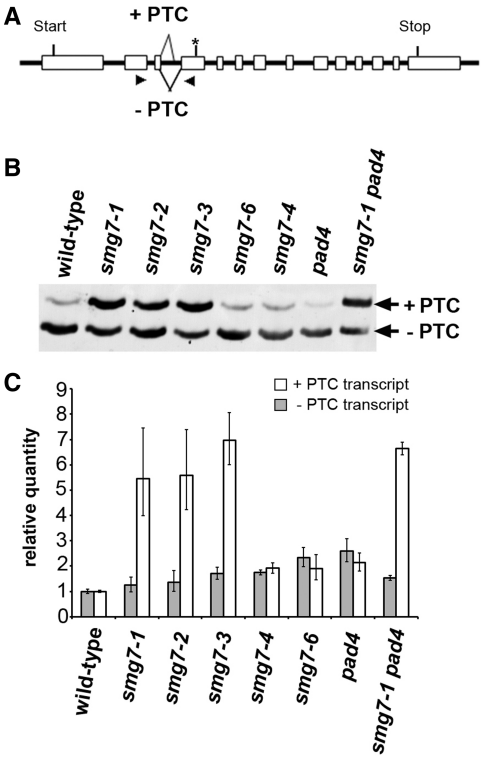

Figure 5. NMD efficiency in *smg7* mutants. (**A**) Alternative splicing of the At2g45670 results in a PTC in the 4th exon (indicated by an asterisk). Primers used to amplify the cDNA region spanning the alternatively spliced sites are indicated by arrowheads. (**B**, **C**) Effect of *smg7* alleles on the abundance of alternatively spliced At2g45670 transcripts analyzed by (B) RT-PCR and polyacrylamide gel electrophoresis (C) or qPCR. Error bars represent standard deviations from three independent RNA samples.

significantly between species. In budding and fission yeast, as well as in *Caenorhabditis elegans*, NMD is dispensable for viability, although NMD deficiency in worms causes mild morphological defects in reproductive organs (15–17). In contrast, inactivation of *UPF1* and *UPF2* genes in *Drosophila*, zebra fish and mice is embryonic lethal, although the underlying causes of lethality are not well understood (19–23). Based on genome-wide expression analysis in mammalian cell lines impaired in NMD, an estimated 6–9% of alternatively spliced genes may be subject to regulation by NMD (12,24,49). Similarly, up to 13% of *Arabidopsis* intron containing genes were predicted to be affected by NMD (50). Deregulation of such a large number of genes may interfere with essential cellular and developmental processes, thus causing lethality. However, some of the core components of the NMD machinery are implicated in diverse molecular pathways, such as DNA replication, telomere maintenance and cell cycle progression (2,51). Therefore, there is the possibility that the essential function of these genes is associated with a role outside of NMD.

In this study, we were able to pinpoint a specific pathway whose deregulation is responsible for the lethal consequences of NMD dysfunction in *Arabidopsis*. We show that aberrant NMD leads to constitutive autoactivation of the immune response. Importantly, disruption of the PAD4/EDS1 pathogen signaling pathway suppressed the vegetative defects and lethality of the strong *smg7* and *upf1* alleles, arguing that activation of pathogen signaling pathways is a major physiological response to declined NMD in *Arabidopsis*. Activation of an immune response upon NMD attenuation may not be limited to plants. A recent study showed that siRNA knock-down of SMG1 or UPF2 in tumour cells led to their immune mediated rejection in mice (52). The key question that remains is how NMD deficiency triggers an immune response. A strong hypersensitive response along with signaling through the PAD4/EDS1 complex indicates that impaired NMD leads to activation of effector triggered immunity in *Arabidopsis*. This defense mechanism relies on a set of R-proteins that act as intracellular sensors to recognize effector proteins, which are injected by an invading pathogen into plant cells to inhibit other pathogen signaling pathways. An R-protein can recognize an effector either through direct interaction, or can

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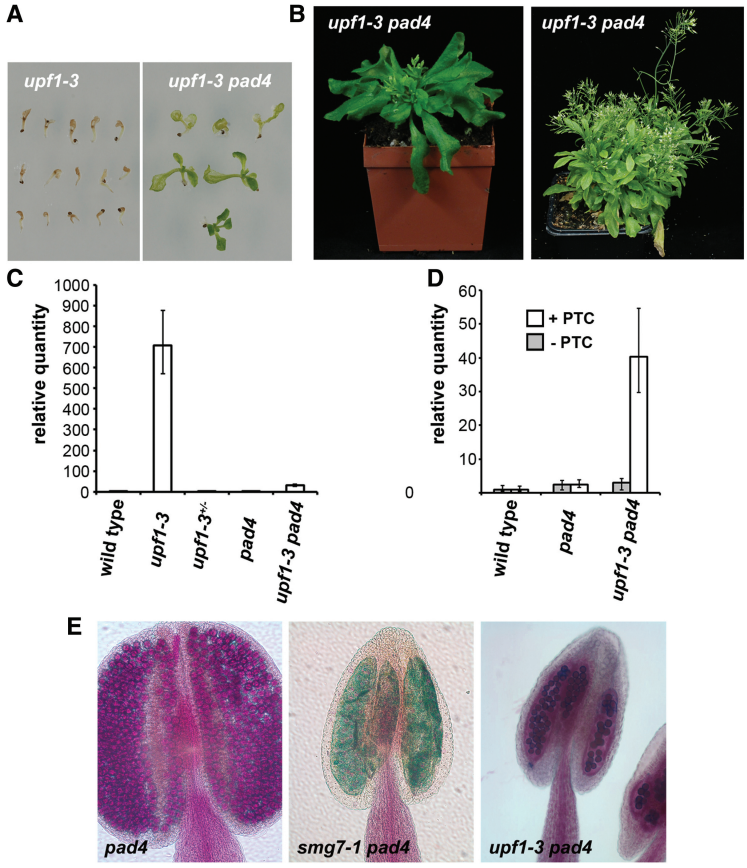

Figure 6. Pathogen response signaling causes lethality of *upf1-3* mutants. (**A**) Seedlings of *upf1-3* and *upf1-3 pad4* mutants grown for 10 days on agar plates. (**B**) Six- and 12-week-old *upf1-3 pad4* plants. (**C**) Levels of the PR1 transcript measured by qPCR in seedlings of *upf1-3* plants and corresponding controls. (**D**) Levels of alternatively spliced At2g45670 transcripts in *upf1-3 pad4* mutants. Error bars in (C) and (D) indicate standard deviations from three independent RNA samples. (**E**) Anthers of *pad4*, *smg7-1 pad4* and *upf1-3 pad4* mutants analyzed by Alexander staining. While *smg7-1 pad4* anthers are empty, a reduced number of viable pollen can be detected in *upf1-3 pad4* mutants.

sense its presence indirectly by monitoring host proteins that are targeted by the effectors (43,44,53). Two scenarios can be envisioned for triggering an autoimmune response by impaired NMD. One possibility is that activity of R-genes is unleashed by truncated or novel proteins that are expected to be produced under relaxed mRNA surveillance from transcripts carrying a PTC or a codon frame shift. A similar mechanism was proposed to be responsible for immune rejection of NMD deficient tumours in mice (52) and NMD-resistant transcripts with frame shift mutations were shown to yield novel protein epitopes in human tumour cells (54).

Another possibility is that NMD regulates expression of genes directly involved in pathogen perception or signaling. Indeed, expression of a number of pathogen response genes is altered in *Arabidopsis upf1-3* and *upf1-5* mutants. These include several WRKY transcription factors, *PAD4*, *EDS1* and *SNC1*, an R-gene whose mis-regulation leads to a lesion mimic phenotype (13,47,55). However, it is currently unclear whether these genes are direct targets of NMD, or whether their deregulation is a secondary consequence of elevated pathogen signaling. A promising group of putative NMD targets with a regulatory function in pathogen response are R-genes. There are about 150 NBS-LRR R-genes in the *Arabidopsis* genome. Their global expression analysis showed that at least 12 NBS-LRR R-genes produce alternatively spliced variants and 15 genes contain introns in 5′ or 3′ UTRs (56). Thus, a significant subset of R-gene transcripts carries features typical for NMD substrates.

Our data show that pathogen response upregulation has more detrimental consequences in *upf1-3* mutants than in *smg7-1* plants. Accordingly, the *upf1-3* allele has a greater effect on the +PTC transcript than *smg7-1,-2* and *-3* alleles. Thus, the extent of pathogen response deregulation appears to be proportional to the extent of NMD deficiency. It is likely that the *upf1-3* are NMD-null due to a T-DNA disruption in the highly conserved RNA

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helicase domain (27), while the *smg7-1, -2* and *-3* mutants still retain some NMD activity due to expression of the conserved TPR-domain. Alternatively, the role of UPF1 in NMD may be more important than the function of SMG7. Remarkably, the *smg7-4* and *-6* alleles are fully proficient in degrading the At2g45670 +PTC transcript indicating that, in contrast to mammals (7), the non-conserved C-terminus of SMG7 is dispensable for NMD in *Arabidopsis*.

Further phenotypic analysis of the *SMG7* allelic series demonstrates that the function of this protein goes beyond traditional NMD. Hypomorphic *smg7* alleles give rise to seemingly pleiotropic defects, which we were able to dissect into two specific pathways, namely pathogen response and meiotic progression (this study) (29,30). However, while the autoimmune response is caused by NMD deficiency, abnormalities in reproductive development also occur in *smg7* alleles, which are NMD proficient. Furthermore, NMD null *upf1-3 pad4* mutants do not exhibit the meiotic defects typical of *smg7* plants. These data argue that SMG7 function in meiosis is not mediated through NMD. A broader role for SMG7 in cellular metabolism is also inferred from the observation that, in contrast to the *upf1-3* mutation, we were not able to rescue embryonic lethality of the *smg7-5* allele by inactivating PAD4 (data not shown). An additional function, distinct from the other NMD factors, was also suggested for SMG7 in zebra fish (21).

In conclusion, our detailed functional and phenotypic analysis of SMG7 in *Arabidopsis* has revealed a multifaceted role for this protein in cellular metabolism. Identification of specific pathways that are affected by SMG7 dysfunction in *Arabidopsis* (e.g. pathogen signaling and meiosis) will facilitate more detailed understanding of molecular mechanisms that underlie the function of this evolutionary conserved gene and provide important research directions for elucidating the biological function of NMD in an organism with complex RNA regulatory networks.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–4, Supplementary Tables 1–2 and Supplementary References [57–59].

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Conflict of interest statement. None declared.

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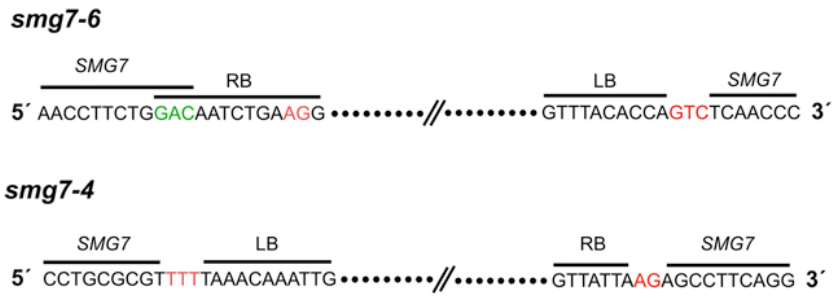
Supplementary Data

Supplementary Table S1. *Arabidopsis* mutant lines used in this study

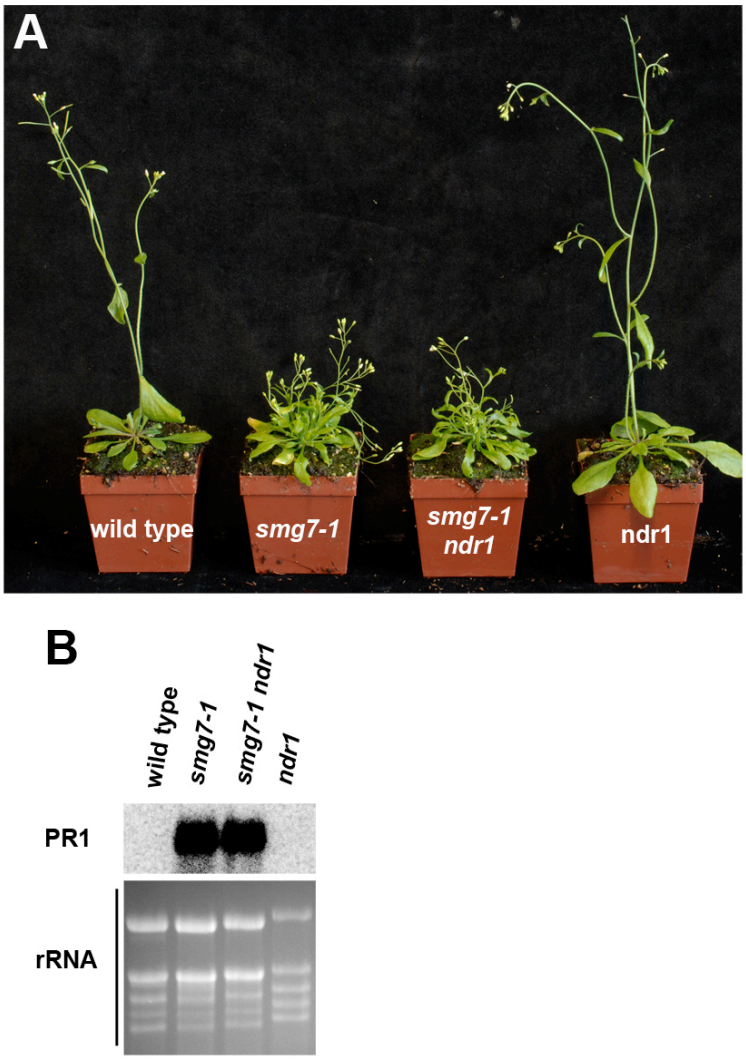
Mutation	T-DNA	References
smg7-1	SALK_073354	(1)
smg7-2	SALK_025699	this study
smg7-3	SALK_112476	(1)
smg7-4	SAIL_63F08	this study
smg7-5	SALK_144162	(1)
smg7-6	SALK_052532	this study
upf1-1/lba1	n.a.	(2)
upf1-2	SALK_004606	(3)
upf1-3	SALK_081178	(3) (4)
upf1-4	SALK_022721	(3) (4)
upf1-5	SALK_112922	(4)
upf3-1	SALK_025175	(4) (5)
upf3-2	SALK_097931	(4)
pad4-1	n.a.	(6)
snc1-11	SALK_047058	(7)
ndr1-1/PR1::GUS	n.a.	(8) (9)

Supplementary Figure S1



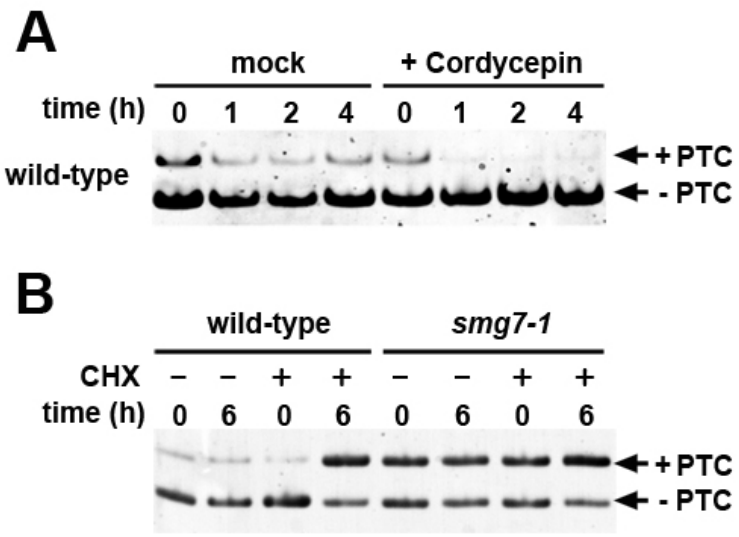
Supplementary Figure S1. Structure of T-DNA integration sites in *smg7-6* and *smg7-4* alleles. Sequences derived from the SMG7 gene and from T-DNA borders (LB, RB) are highlighted. Green letters indicate microhomology between SMG7 and T-DNA at the fusion junction; sequences formed *de novo* at the sites of insertions are in red.

Supplementary Figure S2



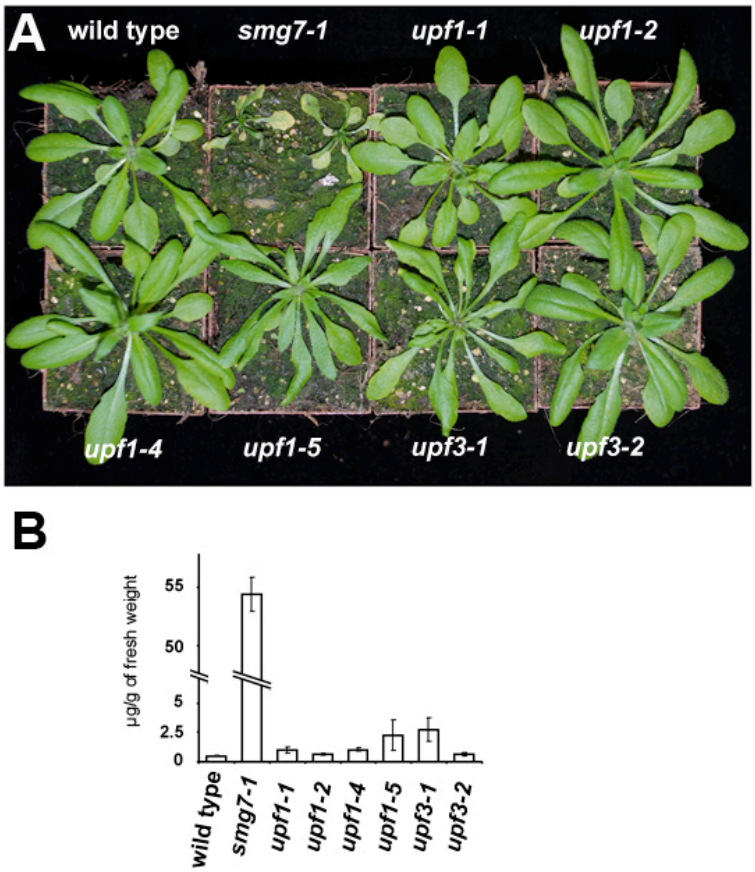
Supplementary Figure S2. Inactivation of NDR1 does not rescue growth defects of *smg7-1* mutants. **(A)** Six week old plants carrying different mutant combinations grown at 60% humidity. **(B)** Northern blot analysis of expression of *PR1* in leaves of *smg7-1 ndr1* mutants and control plants. Ethidium bromide stained gel with *rRNA* species is shown as a RNA loading control.

Supplementary Figure S3



Supplementary Figure 3. Alternatively spliced At2g45670 +PTC transcript is NMD target. **(A)** Stability of the +PTC transcript after inhibition of transcription with cordycepin in leaves of wild type plants. The +PTC transcript disappears within 1hr after cordycepin treatment **(B)** Effect of cycloheximide (CHX) on relative abundance of the +PTC transcript in wild type and *smg7-1* mutants. CHX treatment increases level of +PTC transcript in wild-type leaves.

Supplementary Figure S4



Supplementary Figure S4. *upf1-5* and *upf3-1* mutants exhibit subtler growth defects and a milder increase in SA than *smg7-1* plants. **(A)** Four week old NMD mutant plants cultivated at 16°C. **(B)** Levels of free SA in leaves of NMD mutants.

4.2 Regulation of plant innate immunity through nonsense-mediated RNA decay

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Manuscript in preparation

Regulation of plant innate immunity through nonsense-mediated mRNA decay

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Summary

Nonsense-mediated mRNA decay (NMD) is a conserved RNA surveillance mechanism that degrades aberrant mRNA transcripts, but also influences the expression of physiologically relevant transcripts. Loss of NMD in higher organisms, including plants, leads to severe phenotypes, which impeded the investigation of biological processes regulated by NMD. In *Arabidopsis*, disruption of NMD has adverse effects on development and growth, which we previously linked to the autoactivation of immune responses. Here, we provide evidence that NMD influences disease resistance pathways by directly regulating the expression of numerous resistance (R-) genes. Deregulation of a single R-gene upon NMD impairment is sufficient to recapitulate the autoimmunity symptoms observed in NMD mutants. Importantly, NMD is inhibited upon pathogen infection, leading to the derepression of NMD-regulated R-proteins, which positively contributes to disease resistance. Our data reveal a dynamic role of NMD in regulating the expression of R-genes, thereby extending the repertoire of RNA-based regulatory mechanisms that shape plant immunity.

Introduction

Nonsense-mediated mRNA decay (NMD) is a eukaryotic translation-coupled RNA surveillance mechanism that was originally discovered as a process that detects and degrades aberrant mRNA transcripts. A key feature of NMD targets are premature termination codons (PTC) that can arise as a consequence of mutations, transcription errors or as a result of alternative splicing events (Schweingruber et al., 2013). However, genome-wide analyses showed that many protein-coding mRNAs are also targeted by NMD due to the presence of additional signatures that can be recognized by NMD, including upstream open reading frames (uORFs), long 3' untranslated regions (UTRs), and introns located in 3'UTRs. Up to 20% of endogenous transcripts may be subjected to regulation by NMD in eukaryotic organisms (Drechsel et al., 2013; Kalyna et al., 2012; Kurihara et al., 2009; Mendell et al., 2004; Weischenfeldt et al., 2012). Thus, beyond its role in RNA surveillance, NMD may influence numerous biological processes by having adopted a more active role in regulating endogenous gene expression (Karam et al., 2013). In support of this notion, NMD was shown to be regulated in response to environmental and developmental cues. Several studies in mammalian cells described an inhibition of NMD under a variety of cellular stresses, including amino acid deprivation and hypoxia(Gardner, 2008; Mendell et al., 2004). Developmentally regulated inhibition of NMD by a microRNA targeting the core NMD factor UPF1 has recently been suggested to be involved in vertebrate brain development (Bruno et al., 2011) and was proposed to promote the expression of neural-specific transcripts. Furthermore, NMD is under the control of complex autoregulatory circuits that control NMD efficiency in a cell-type specific manner (Huang et al., 2010; Yepiskoposyan et al., 2011). These feedback mechanisms seem to be conserved from plants to animals, substantiating the importance of precise tuning of NMD activity for ensuring the homeostasis of eukaryotic transcriptomes (Kerényi et al., 2008; Nyikó et al., 2013).

While NMD has been implicated in the regulation of physiologically relevant transcripts, the biological significance of this regulation is currently unclear. This is largely because of a broad impact of NMD on the transcriptome and the severe pleiotropic phenotypes associated with NMD impairment, which obscure the identification of specific biological processes regulated by NMD. NMD-null mutations are embryonic lethal in *Drosophila*, zebrafish and mouse, but it is currently unclear whether this is caused by the production of truncated proteins from aberrant RNAs or by direct effects on physiological transcripts required for proper embryogenesis (Hwang and Maquat, 2011). NMD is also essential in plants as disruption of the *Arabidopsis* NMD core components UPF1 and SMG7 cause seedling lethality and retarded development, respectively (Arciga-Reyes et al., 2006; Riehs et al., 2008; Yoine et al., 2006). Notably, NMD impairment in *Arabidopsis* was linked to constitutive activation of plant pathogen responses (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012). In agreement, disruption of pathogen resistance signaling rescued lethality of UPF1-null plants and retarded growth of *smg7* mutants (Riehs-Kearnan et al., 2012). This argued that aberrant pathogen response signaling is a major physiological consequence of impaired NMD in *Arabidopsis* and indicated a role of NMD in regulating plant innate immunity.

The plant innate immune system employs multiple layers of defense to inhibit pathogen infection and to confer systemic resistance. A first defense layer relies on pattern recognition receptors located at the plasma membrane to detect various conserved pathogen-associated molecular patterns (PAMPs) (Monaghan and Zipfel, 2012). Detection of PAMPs leads to PAMP-triggered immunity (PTI), which involves transcriptional and metabolic reprogramming, and is sufficient to confer immunity against the majority of non-adapted pathogens (Jones and Dangl, 2006). Effector-triggered immunity (ETI) represents a second layer of defense and is mediated by intracellular immune receptors known as resistance (R-) proteins, which specifically detect the presence or action of pathogen-secreted effector proteins (Dodds and Rathjen, 2010). ETI induces defense responses that represent amplified and prolonged versions of the weaker and transient resistance responses induced by PTI (Navarro et al., 2004; Tsuda and Katagiri, 2010). Frequently, ETI culminates in the induction of the hypersensitive response (HR) that limits further colonization and spread of pathogens at infected sites by localized induction of programmed cell death (Greenberg and Yao, 2004). The majority of R-proteins belong to the nucleotide-binding site leucine-rich repeat (NB-LRR) class, and share homology to mammalian NOD-like immune receptors (Maekawa et al., 2011). Most plant NB-LRR proteins carry an N-terminal Toll/Interleukin-1 receptor domain (TIR-NB-LRRs) or a coiled-coil domain (CC-NB-LRR), and have

genetically distinct signaling requirements (Heidrich et al., 2011). The expression of NB-LRRs is tightly regulated on both transcriptional and post-transcriptional level (Staiger et al., 2013) and misexpression of NB-LRRs has previously been shown to be associated with severe fitness costs (Alcázar and Parker, 2011).

In this study, we deciphered the regulatory pathway that triggers inappropriate immune responses under NMD deficiency in *Arabidopsis*. In contrast to the generally held view that complex NMD phenotypes are caused by deregulation of multiple physiological processes, we show that developmental defects associated with SMG7 dysfunction are due to inappropriate activation of pathogen response pathways conditioned by TIR-NB-LRR (TNL) R-proteins. We demonstrate that NMD regulates a significant portion of TNL transcripts and that derepression of the TNL *RPS6* under NMD deficiency is sufficient to elicit autoactivation of defense responses. We further present evidence that pathogen infection leads to downregulation of NMD efficiency, which results in stabilization and accumulation of NMD-targeted mRNAs, including numerous TNL transcripts. Finally, we demonstrate that overexpression of SMG7 and disruption of NMD autoregulation sensitizes plants to pathogen infection. Our data provide compelling evidence that modulation of NMD efficiency represents a physiologically relevant gene regulatory mechanism that shapes plant innate immunity.

Results

Identification of genetic requirements of *smg7* associated autoimmunity

Previously, we reported that plants carrying *smg7* mutations exhibited autoimmunity, which is characterized by stunted growth, spontaneous formation of necrotic lesions and elevated levels of the plant defense hormone salicylic acid (SA). The aberrant growth phenotypes associated with impairment of *SMG7* could be suppressed by inactivation of the pathogen response regulator PAD4 (PHYTOALEXIN DEFICIENT 4), which is required for basal resistance and TNL-mediated disease resistance. In contrast, mutations in NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1), a mediator of CC-NB-LRR signaling, did not rescue *smg7-1* mutants (hereinafter referred to as *smg7*) (Riehs-Kearnan et al., 2012). To elucidate the signaling pathway triggering NMD-associated autoimmunity, we examined genetic interactions with additional factors involved in disease resistance signaling. Similar to PAD4, ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) is a central regulator of plant defense required for basal resistance and effector triggered immunity mediated by TNL proteins (Wagner et al., 2013; Wiermer et al., 2005). Inactivation of EDS1 fully suppressed the stunted phenotype and the expression of the SA-responsive marker gene PR1 in *smg7* plants (Figure 1A, B). Dependence of *smg7*-associated autoimmunity on PAD4/EDS1 and its independence on NDR1 indicated involvement of a TNL-conditioned signaling pathway. To further test this prediction, we analyzed plants deficient for the HSP90 co-chaperones RAR1 and SGT1B, which were implicated in stabilization and activity of many NB-LRRs proteins (Kadota et al., 2010). *Rar1* and *sgt1b* mutations largely suppressed stunted growth of *smg7* mutants, but mild alteration in leaf morphology remained (Figure 1A and Figure S1A). This coincided with strongly reduced, but still elevated expression of PR1 in *smg7 rar1* and *smg7 sgt1b* double mutant plants (Figure 1B). In addition, autoimmune symptoms and PR1 expression fully diminished in *smg7* mutants grown at elevated temperature (Figure 1D, E and Figure S1B). Modulation of immune responses by temperature has also been linked to TNL proteins, some of which were proposed to act as temperature-sensors determining resistance activation thresholds in given natural environments (Alcázar and Parker, 2011). To exclude the formal possibility that the suppression of autoimmune symptoms did occur through restoration of NMD proficiency, we tested the accumulation of endogenous PTC-containing transcripts that are targeted by NMD (Figure 1C, F). As expected, the *smg7* mutation led to the upregulation of aberrant mRNAs under all tested environmental and genetic conditions.

Pathogen defense signaling leads to the production of SA that potentiates defense mechanisms and promotes hypersensitive response (HR) and systemic acquired resistance (SAR) (Greenberg and Yao, 2004). Notably, inhibition of SA biosynthesis by inactivation of ISOCHORISMATE SYNTHASE 1 (ICS1/SID2) did not suppress stunted growth of *smg7* mutants, while it reduced PR1 expression and formation of necrotic lesions (Figures 1A, B and Figure

S1C). These genetic data argue that production of SA is not the primary cause of NMD-associated autoimmunity but rather reinforces defense responses, which are likely triggered by a TNL-conditioned disease resistance pathway.

TIR-NB-LRRs are regulated by NMD

The observation that NMD deficiency was accompanied by constitutive immune response activation raised the questions if NMD directly controls the expression of defense-related transcripts. To seek for putative NMD targets related to pathogen response, we performed RNA-seq analysis in SMG7-deficent plants. Published whole-genome expression data in NMD-impaired mutants indicated that NMD regulates a significant portion of the *Arabidopsis* transcriptome (Drechsel et al., 2013; Kurihara et al., 2009; Marquez et al., 2012). However, constitutive pathogen response and growth alterations in these mutants hinder direct identification of *bona fide* NMD targets, as a substantial part of genome deregulation may result from secondary physiological effects (Rayson et al., 2012). To curtail this problem, we took advantage of *smg7 pad4* mutants that are phenotypically normal, and in which pathogen defense signaling is abrogated (Riehs-Kearnan et al., 2012). We found that out of 17,269 mRNAs expressed in leaf tissue (reads per kilobase per million, RPKM>1), 2,653 mRNAs were significantly upregulated in *smg7 pad4* mutants relative to *pad4* control plants (Figure 2A and Figure S2A, Table S1; false discovery rate corrected p-value <0.05). GO-term classification of the upregulated transcripts did not reveal enrichment for the category "response to abiotic or biotic stimulus" (Fisher's exact test, p-value: 0.1193; Figure S2B) confirming abrogated pathogen signaling and showing that NMD regulated transcripts are proportionally distributed across all GO-term categories. Furthermore, we detected elevated expression of nearly 60% of transcripts that were previously reported to be increased in weak NMD mutants exhibiting no or only mild autoimmune phenotypes (Kurihara et al., 2009) (Figure S2C). This indicated that a large portion of upregulated transcripts in *smg7 pad4* mutants were subject to direct NMD regulation.

Our genetic analysis indicated involvement of TNLs in NMD-associated autoimmunity. Therefore, we next analyzed the RNA-seq data for the expression for TNLs. The *A. thaliana* Columbia-0 (Col-0) genome harbors 110 TNL genes (Meyers et al., 2003) and 72 of these were expressed in the RNA-seq dataset (Table S2). Importantly, 39 of the expressed TNL genes were upregulated in *smg7 pad4* mutants and none were downregulated, representing a statistically significant enrichment of TNL transcripts in the absence of immune responses (Fisher's exact test, p-value= 5.329E-16) (Figure 2B). Based on transcript features annotated in the *A. thaliana* genome (version TAIR 10) and identified by Tan et al. 2007 (Tan et al., 2007) we found that almost half of the upregulated TNLs carried putative NMD features (long 3'UTRs, 3'UTR introns, PTCs, uORFs) (Figure 2B and Figure S2D). Our results indicated that at least some of the upregulated TNL transcripts are directly processed by the NMD machinery.

Next, we validated the RNA-seq data by determining relative steady state levels of selected TNL transcripts by quantitative real-time PCR (qPCR) in *smg7 pad4* mutants (Figure 2C). Among the upregulated TNLs we found *RPS6* (*RESISTANT TO P. SYRINGAE6*) that has been previously described to undergo alternative splicing (Kim et al., 2009). Using splice-variant specific qPCR we determined that a subset of RPS6-derived PTC-containing isoforms was upregulated in *smg7 pad4* mutants indicating regulation by NMD (Figure S2E,F). In addition, we found that levels of the selected TNL transcripts were increased in wild type plants treated with the translation inhibitor cycloheximide that also blocks NMD (Figure 2D). To determine whether these TNL transcripts are indeed subjected to NMD, we assessed their stability after induction of a transcriptional block. While the RS2Z33-PTC and AT2G45670-PTC control transcripts were stable in both *pad4* and *smg7 pad4* plants, we observed increase in the half-lives of the corresponding PTC-containing transcripts in *smg7 pad4* plants (Figure 2E). With the exception of AT1G72940, we found similar stabilization of all tested TNL transcripts in *smg7 pad4* plants upon transcription inhibition (Figure 2E). This demonstrated that numerous TNL transcripts are direct targets of NMD.

Autoimmunity in *smg7* mutants is triggered by the TNL *RPS6*

The *smg7-1* allele used throughout this study was identified and characterized in the Col-0 genetic background (Riehs et al., 2008). We noticed that stunting and autoimmunity was diminished in a portion of *smg7* plants in the F2 population derived from a cross between *smg7* Col-0 and wild-type plants of the natural accession Landsberg *erecta*

(L*er*) (Figure S3A, S3B). This indicated existence of natural polymorphism(s) that suppressed constitutive pathogen signaling in *smg7* mutants. However, introgression of the *smg7* mutation in the L*er* background restored the autoimmune phenotype and stunted growth (Figure 3A). Thus, the genetic suppression of autoimmunity resulted from certain combinations of natural Col-0 and L*er* polymorphisms at multiple genetic loci. By analyzing several F2 populations derived from the backcrosses between *smg7* Col-0 and L*er*, we identified a genetic constellation where the suppressor phenotype was determined by a single genetic locus, which we called *REX* (*RIESENEXPERIMENT*) (Figure 3A and Figure S3A, S3C). Near isogenic lines (NILs) in this population harbor mostly L*er*-genome, but the *REX* suppressor was derived from the Col-0 genome. While SMG7-deficient $REX^{Ler/Ler}$ plants were dwarfed and exhibited increased level of SA and elevated PR1 expression, *smg7* $REX^{Col/Col}$ plants were phenotypically normal without symptoms of constitutive pathogen response signaling (Figure 3A-C). The REX^{Ler} allele was semidominant, which was consistent with a model in which autoimmunity associated with NMD deficiency is triggered by the REX^{Ler} allele in a dose-dependent manner. The *REX* locus did not affect NMD as the level of aberrant mRNAs was increased in the absence of SMG7 irrespective of the *REX* allele (Figure 3D), indicating that *REX* did not encode for a NMD modifier.

The *REX* locus was mapped to a 240 kb region on chromosome 5, encompassing 60 genes between At5g46250 and At5g46850. The mapped region contains a large cluster of genes related to pathogen signaling, including several TNLs (Figure 3E). Due to numerous structural rearrangements, this locus was deprived of meiotic recombination, which hampered further fine mapping. We reasoned that if the causative gene is regulated by NMD, its expression levels should be elevated in *smg7* mutants. Therefore, we performed RNA-seq on wild type and SMG7-deficient $REX^{Ler/Ler}$ plants, aligned the reads to the L*er* genome scaffold (Schneeberger et al., 2011) and compared the resulting expression profiles of the REX region (Figure 3F). Three out of six TNL genes located in the region showed significant increase in reads in *smg7* mutants: *RPS6*, At5g46490 and At5g46500. To identify the causative locus, we transformed *smg7*$^{+/-}$ $REX^{Col/Col}$ plants with genic constructs spanning the $RPS6^{Ler}$ and At5g46490-500^{Ler} loci. We then screened T2 *smg7* $REX^{Col/Col}$ progeny of the T1 transformants for autoimmunity symptoms. Only *smg7* $REX^{Col/Col}$ plants carrying the $RPS6^{Ler}$ construct showed stunted growth and elevated expression of PR1 (Figure 4A, 4B; 9 out of 11 independent transgenic lines). Ectopic expression of $RPS6^{Ler}$ in wild-type siblings neither resulted in adverse growth nor in elevated PR1 expression. This genetic complementation experiment demonstrated that in these plants autoimmunity was triggered by the $RPS6^{Ler}$ allele in an NMD-dependent manner. Consistent with this finding, *RPS6* transcript levels were only increased in *smg7* transformants, but not in *SMG7*-proficient siblings (Figure 4C).

RPS6 is a TNL gene that mediates resistance and HR upon elicitation with the *Pseudomonas syringae (P. syringae)* effector HopA1 (Gassmann, 2005). Annotation of the *RPS6* gene revealed a long 3'UTR containing multiple introns and we showed that stability of the *RPS6* transcript is regulated by NMD (Figure 2C-E). Interestingly, despite the fact that both $RPS6^{Col}$ and $RPS6^{Ler}$ variants are equally targeted by NMD (Figure S4A) only the $RPS6^{Ler}$ allele triggered autoimmunity. The $RPS6^{Ler}$ allele harbors one in-frame INDEL and six non-synonymous mutations in the coding region, but none of them affect conserved TNL motifs such as the p-loop, the Walker B motif or MHD motif (Figure 4D). Additionally, none of the polymorphisms appeared to affect a splice donor or acceptor site. Previously, a survey among 32 *Arabidopsis* accessions suggested natural variation in HR elicited in response to the *P. syringae* effector HopA1 (Gassmann, 2005). To investigate whether *RPS6* alleles mediate qualitatively different pathogen response, we inoculated wild-type $REX^{Col/Col}$ and $REX^{Ler/Ler}$ NILs with *P. syringae pv tomato* DC3000 (Pst DC3000) HopA1. While $REX^{Col/Col}$ plants were unaffected, the treatment of the $REX^{Ler/Ler}$ plants led to tissue collapse due to HR (Figure S4B). These data indicated that the absence of *smg7*-associated autoimmunity in $REX^{Col/Col}$ plants was caused by the reduced capability of the $RPS6^{Col}$ allele to elicit HR.

Pathogen infection reduces efficiency of NMD

We and others have previously shown that the inappropriate activation of immune responses in NMD deficient *Arabidopsis* mutants confers a substantial increase in resistance to virulent bacterial pathogens (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012). This poses an interesting question on whether NMD efficiency is modulated in response to pathogen infection and whether this modulation contributes to plant defense. To address

this question, we inoculated wild type plants with virulent *P. syringae* pv *tomato* (*Pst*) DC3000 and quantified endogenous NMD targets 72 hours after the inoculation. The efficiency of inoculation was monitored by PR1 expression, which was induced at both 48 and 72 hours time points (Figure 5A). Importantly, we found a significant increase in the levels of the PTC-containing transcripts derived from *RS2Z33* and elevated levels of the NMD components *SMG7* and *BARENTSZ* 72 hours after infection (Figure 5A). *SMG7* and *BARENTSZ* were shown to be subjected to autoregulation through NMD and thus, their increase is indicative of decreased NMD efficiency (Nyikó et al., 2013). Furthermore, we also found significant upregulation of several NMD-targeted TNLs including *RPS6*, as well as of AT1G72940, which does not appear to be regulated by NMD (Figure 5A). Therefore, we next decided to determine transcript stability to validate that the upregulation of NMD targets is indeed caused by impaired RNA decay rather than increased transcription. To this end, we induced a transcription block 72 hours after *Pst* DC3000 inoculation and analyzed RNA decay rates. While half-lives of all tested NMD-targeted transcripts were increased upon infection relative to mock treatment, stability of the PTC-free RS2Z33-PTC control and the TNL AT1G72940 mRNA did not substantially change. We conclude that infection with virulent *Pst* DC3000 leads to inhibition of NMD and stabilization of NMD-targeted mRNAs including TNL transcripts. Previous studies in mammalian cells indicated that stress-induced decline of NMD is mediated by phosphorylation of the eukaryotic translation initiation factor eIF2α by the GCN2 kinase and subsequent inhibition of translation (Gardner, 2008). However, this does not appear to be the mechanism causing NMD suppression upon pathogen infection in *Arabidopsis* (Figure S5).

Overexpression of SMG7 suppresses NMD downregulation and sensitizes plants to pathogen infection

We hypothesized that downregulation of NMD upon pathogen infection represents a physiologically relevant mechanism that contributes to plant defense. A key prediction of such model is that failure to suppress NMD during infection would lead to increased bacterial susceptibility. To test this prediction, we generated plants in which the *smg7* mutation was complemented by a construct that drives expression of the *SMG7* cDNA from an actin promoter. In addition, the construct lacked the endogenous *SMG7* 3'UTR with introns, rendering the transcript insensitive to NMD autoregulation. The construct fully complemented vegetative growth defects of *smg7* plants, but not infertility (Figure 6A and data not shown) and also suppressed autoimmunity as monitored by PR1 expression (Figure 6B). The complementation construct also led to a two-fold increase in *SMG7* mRNA compared to wild-type plants (Figure 6B). The complemented plants also had reduced levels of multiple NMD targets including the EJC component BARENTSZ1 to the wild-type levels demonstrating that NMD was fully restored (Figures 6B and S5). In fact, for some NMD targets we even detected significantly lower levels of PTC-containing transcripts in the *SMG7* overexpressor (OE) plants relative to wild-type plants, which may suggest a slight increase in NMD efficiency (Figure S6).

We next tested whether overexpression of SMG7 affected NMD regulation upon pathogen infection. While inoculation of wild-type plants with virulent *Pst* DC3000 led to a two-fold increase in *SMG7* mRNA, the *SMG7* transcript in OE plants remained constant at approximately two-fold level comparing to non-infected wild-type plants (Figure 6C). Strikingly, we observed that the SMG7 OE plants failed to upregulate the PTC-containing transcript of *RS2Z33*, as well as the NMD-targeted TNLs AT5G40920 and *RPS6* upon *Pst* DC3000 infection (Figure 6D). In contrast, these transcripts readily accumulated in infected wild-type plants (Figure 6D) and analysis of the pathogen response marker PR1 showed efficient infection in both settings (Figure 6E). These data indicated that SMG7 OE plants were unable to suppress NMD after *Pst* DC3000 infection. To determine whether this had any impact on disease resistance, we compared the magnitude of bacterial resistance in wild type and SMG7 OE plants to virulent *Pst* DC3000. We found that SMG7 OE plants had significantly higher bacterial titer three days after inoculation compared to wild type (Figure 6F). Susceptibility of the SMG7 OE plants to bacterial infection strongly supports the idea that suppression of NMD upon pathogen infection positively contributes to plant defense.

Discussion

The role of NMD as constitutively active RNA surveillance machinery is well accepted and recent research has shed new light on the mechanisms underlying substrate recognition and degradation (Schweingruber et al., 2013).

However, an increasing body of evidence suggests that the efficiency of NMD can vary considerably across cell-types, tissues and in response to cellular stresses (Huang and Wilkinson, 2012; Karam et al., 2013). While an increase in NMD efficiency was observed in developing B-lymphocytes, where it promotes the degradation of nonfunctional immunoglobulin transcripts (Delpy et al., 2004), the majority of studies described a decline in NMD efficiency during cell differentiation or upon stresses(Bruno et al., 2011; Gardner, 2008; Gong et al., 2009; Mendell et al., 2004). These observations led to the hypothesis that relaxed RNA surveillance may promote the expression of genes that directly contribute to stress adaptation or developmental responses(Karam et al., 2013). Nevertheless, there has only been circumstantial evidence supporting this idea, and several fundamental issues remain to be addressed. First, the biological role of NMD regulation was mainly investigated in *in vitro*, where NMD inhibition was shown to promote the differentiation of cultured myoblasts and neuronal stem cells (Bruno et al., 2011; Gong et al., 2009). However, the significance of this regulation remains to be established in the context of animal development. Secondly, the current strategy to determine the biological relevance of NMD targets is largely based on drawing correlations between the functional annotation of the candidate NMD transcript and its expression levels. Yet, such correlation does not necessarily indicate a causal link between the expression levels of the NMD target and the biological process that it is implicated in, and may only reflect secondary responses caused by perturbation of other cellular events. One of the first examples of NMD regulation was the finding that amino acid starvation led to NMD downregulation in human cell lines. As a result, the inhibition of NMD leads to the upregulation of genes involved in amino acid transport and biosynthesis, and to an increase of intracellular amino acid concentrations (Gardner, 2010; Mendell et al., 2004; Wengrod et al., 2013). However, increased amino acid concentrations were shown to be independent of the levels of NMD-regulated amino acid transporter transcripts, but were rather a consequence of the induction of autophagy in response to NMD inhibition (Wengrod et al., 2013). This finding underscores the importance of functional validation of NMD targets before their NMD-mediated regulation is asserted to have a biologically significant effect.

In this study we provide compelling evidence that NMD surveillance in plants is part of a stress response to pathogen attacks and unravel the mechanism which links NMD with plants defense (Figure 7). We genetically dissected the pathway that conditions NMD-triggered autoimmunity and found its dependence on the key regulators of basal resistance as well as NB-LRR co-chaperones. Transcriptional profiling of NMD-deficient plants with abrogated resistance signaling and RNA decay experiments revealed that TNLs are processed by NMD. In agreement, we found enrichment of NMD features in large portion of TNLs, but not in CNLs. This is consistent with our genetic data that NDR1 is dispensable for NMD-triggered autoimmunity. Studies with weak Arabidopsis NMD mutants indicated that even a slight decrease in NMD-efficiency triggers immune response sufficient to confer a pronounced resistance to bacterial infection (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012) (Gloggnitzer, unpublished data). By exploiting natural variation we found that NMD-mediated regulation of *RPS6* leads to a strong immune response, thereby establishing a causative link between NMD deficiency and disease resistance. The variation in genetic requirements for autoimmunity elicited by NMD deficiency in Col-0 and L*er* ecotypes indicate that the defense activation is conditioned by different complements of TNL variants present in the respective genomic background. Furthermore, we demonstrate that NMD is downregulated in response to pathogen attack. Notably, this downregulation leads to stabilization of TNLs underscoring the relevance of NMD regulation in an organismal context. Importantly, plants with constitutive NMD activity are more susceptible to bacterial infection, suggesting that regulation of NMD efficiency has a significant biological impact on potentiating plant basal defense.

NB-LRRs act as intracellular sensors that are activated upon perception of pathogen effector activities. Current models propose that NB-LRR proteins rest in an autoinhibitory conformation, which alters after pathogen perception and leads to protein activation and ETI (Maekawa et al., 2011). While the role of NB-LRRs in ETI is well documented, little is known about their contribution to basal resistance. Ample evidence exists that R-genes can also contribute to basal resistance mechanisms against virulent pathogens in the absence of recognizable ETI. Overexpression of the TNLs RPS4 and SNC1 was previously shown to be sufficient to elicit immune responses without requiring cognate pathogen effectors (Stokes et al., 2002; Wirthmueller et al., 2007). The CNL proteins of

the ADR1 family have also been shown to promote basal resistance against virulent pathogens (Roberts et al., 2013). Increased levels of NMD-controlled TNLs upon NMD suppression in plants infected with virulent pathogens may therefore sufficiently contribute to basal resistance in the absence of ETI. In addition, NMD is coupled to alternative splicing, a process that was previously proposed to regulate the expression of several TNLs (Gassmann, 2008). Previous studies indicated that *RPS6* is alternatively spliced upon pathogen infection, leading to isoforms that contain PTCs and potentially encode truncated TIR-only or TIR-NB domain proteins (Kim et al., 2009). Indeed, we found that these transcripts are subjected to NMD regulation supporting the idea that truncated TNLs are being produced due to relaxed NMD upon infection. While there is increasing evidence that alternative splicing of R-genes is an important aspect of plant immunity, the molecular functions of truncated R-proteins is less clear. Truncated R-proteins have been shown to efficiently induce HR when transiently expressed in plants (Bernoux et al., 2011; Michael Weaver et al., 2006; Swiderski et al., 2009). Notably, alternatively spliced isoforms of the N protein and RPS4 that encode truncated R-proteins have been demonstrated to be required for establishing full resistance (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003). A temporal decline in NMD efficiency may therefore permit the stabilization and translation of alternatively spliced transcripts, which in turn could promote disease resistance. It is currently unclear whether NMD downregulation upon infection with virulent bacteria is part of host-programmed immunity, or if PAMP-perception or effector action is required for NMD inhibition. However, Howard et al. (2013) (Howard et al., 2013) suggested that infection with avirulent *Pst* DC3000 AvrRps4 strains would lead to even stronger NMD inhibition when compared to plants challenged with virulent DC3000. Thus, ETI may amplify and reinforce NMD downregulation upon pathogen perception.

NB-LRR genes represent one of the largest and most variable plant gene family that undergoes rapid evolutionary diversification fueled by the arms race against pathogens attempting to evade the plant immune system (Jones and Dangl, 2006). Genetic changes associated with evolutionary diversification of NB-LRRs may often produce gene variants with potentially adverse effects on plant fitness (Jacob et al., 2013). Regulation of TNLs by NMD raises the intriguing possibility that in addition to its function in basal defense, NMD may act as an evolutionary capacitor. As such, NMD may mitigate the potentially harmful side effects of rapidly evolving R-genes in plant genomes, thus allowing the retention of genetic variation in R-genes, which may facilitate the evolution of new recognition specificities.

NB-LRR activation invokes extensive transcriptional and metabolic reprogramming and frequently culminates in localized cell death at the infection site. Considering the severe fitness costs associated with inappropriate activation of NB-LRRs, the expression of NB-LRRs seems to require regulation on multiple levels, including transcriptional gene silencing through RNA-directed DNA methylation {Yu, 2013 #4045;Yi, 2007 #3571} and post-transcriptional gene silencing (PTGS) by siRNAs and miRNAs (Boccara et al., 2014; Li et al., 2012; Shivaprasad et al., 2012; Zhai et al., 2011). It has been shown that PTGS can be inhibited either by viral and bacterial suppressors or by PAMP perception, which subsequently leads to de-repression of PTGS-controlled NB-LRRs. This mechanism has been proposed to contribute to both basal immunity as well as to race-specific resistance(Boccara et al., 2014; Shivaprasad et al., 2012). While PTGS seems to primarily regulate CNLs, in our work we determined that in *Arabidopsis* NMD predominantly targets TNLs. This preference may be due to the fact that most CNLs do not undergo splicing, and appear to be deprived of NMD-stimulating features. Thus, these two pathways seem to target different repertoires of NB-LRRs and may act complementary in plant defense. Interestingly, in analogy to PTGS, NMD has recently been identified as an important mechanism of plant antiviral defense and some viruses seem to have acquired the ability to suppress NMD (Olivier Voinnet, personal communication). Taken together, these findings underscore the importance and complexity of RNA-based mechanisms in plant defense and emphasize a role of NMD in regulating physiologically relevant processes

Experimental Procedures

Plant materials and growth conditions

Wild type *Arabidopsis thaliana* accessions used in this study were Columbia-0 (Col-0) and Landsberg *erecta* (L*er*). The *eds1-2* (Bartsch et al., 2006), *pad4-1* (Jirage et al., 1999), *sgt1b (eta3)* (Gray et al., 2003), *rar1-21* (Tornero et al., 2002), *sid2-1* (Nawrath and Métraux, 1999), and *smg7-1* mutant lines (Riehs et al., 2008), which all were in Col-0 background, were published previously. *Gcn2-2* seeds were a gift from Jean-Marc Deragon (Université de Perpignan Via Domitia) and Genetrap line GT8359 was obtained from Cold Spring Harbor Laboratory. Generally, plants were grown on soil with 16 hours photoperiod at 21°C and 60% relative humidity, except for temperature shift experiments where the temperature was set to 28°C. Plants used for pathogen treatments were grown at 21°C with 10 hours photoperiod. Stable transgenic *SMG7* cDNA overexpressors and *RPS6*L*er* transformants were generated by cloning of the corresponding cDNA or genomic region, respectively, followed by *Agrobacterium*-mediated transformation using the floral-dip method.

Gene expression analysis

Total RNA was isolated from plant tissue using peqGold RNA pure (peqLab). Genomic DNA was removed from RNA samples using Ambion Turbo DNA-free (Life Technologies) and 1 ug of RNA was reverse transcribed into cDNA using the Maxima H Minus First Strand cDNA synthesis kit. One fiftieth of synthesized cDNA was then amplified in qPCR reactions using Sso Advanced SYBR Green Supermix (Bio-Rad) and transcript-specific primers on a Bio-Rad iQ5 optical cycler. Relative quantification of mRNA levels was carried using the relative ΔΔCt method (Livak and Schmittgen, 2001). Transcript levels were normalized to the expression of At2g28390 (Czechowski et al., 2005) for steady state levels, and to the expression of *eIF4A1* (At3g13920) in experiments involving a transcriptional block. All data presented are derived from at least three biological replicates, each of which represents and average of three technical replicates. All primer sequences are listed in Table S3.

RNA-seq analysis

For whole transcriptome profiling of *pad4* and *smg7 pad4* mutants, three single-end (SE) second-strand specific libraries per genotype generated using the Scriptseq Complete Plant kit (Epicentre) were sequenced on the Illumina HiSeq2000 platform. Similarly, one library of *smg7* lines in L*er* background and wild type L*er* plants, each pooled from leaf material of three plants was prepared and sequenced as described above.

Pathogen assays

Five-week old soil-grown plants were spray-inoculated with with $1x10^8$ cfu/mL *P. syringae* pv *tomato* DC3000 (*Pst* DC3000) with 0.04% (v/v) Silwet L-77 (Lehle Seeds) or 10 mM $MgCl_2$ solution as mock control as previously described (Rietz et al., 2011). Gene expression levels in infected and mock-inoculated leaf tissue were assayed at 48 hours and 72 hours after treatment. Bacterial titers were measured 72 hours after inoculation by serial plating (Vlot et al., 2008). For determining differential hypersensitive response outputs, leaves of 4-week old plants were syringe infiltrated with $2x10^7$ cfu/mL *Pst* DC3000 HopA1 (Gassmann, 2005). Disease symptoms were recorded 24 hours after inoculation.

Salicylic acid quantification

Leaves of three 5-week old plants were pooled and ground in liquid nitrogen. Free and total levels of salicylic acid were measured by high-performance anion-exchange chromatography as previously described (Rozhon et al., 2005).

Translational and transcriptional inhibition

Translational inhibition was carried out in 14-day old seedlings for five hours using cycloheximide as previously described (Kalyna et al., 2012). Half-lives of mRNAs were determined by transcriptional inhibition as described by Johnson et al. (2000) (Johnson et al., 2000) with the following modifications. Detached rosette leaves of 4-5 week old plants were incubated in buffer (15 mM sucrose, 1 mM KCl, 1 mM Pipes, 1 mM sodium citrate, 0.08% Silwet L-77, pH 6.5) (Seeley et al., 1992) for 30 min before the addition of cordycepin (150 ug/mL, Sigma). Vacuum was applied for 5 minutes. Two leaves were then harvested after 1, 2 and 4 hours and the leaves were immediately frozen in liquid

nitrogen for subsequent RNA analysis. The mRNA half-lives were then calculated from qPCR data by non-linear least square regression with the Solver add-in of Excel (Microsoft), assuming exponential decay of mRNAs (Narsai et al., 2007; Rufener and Mühlemann, 2013).

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Figures

Figure 1

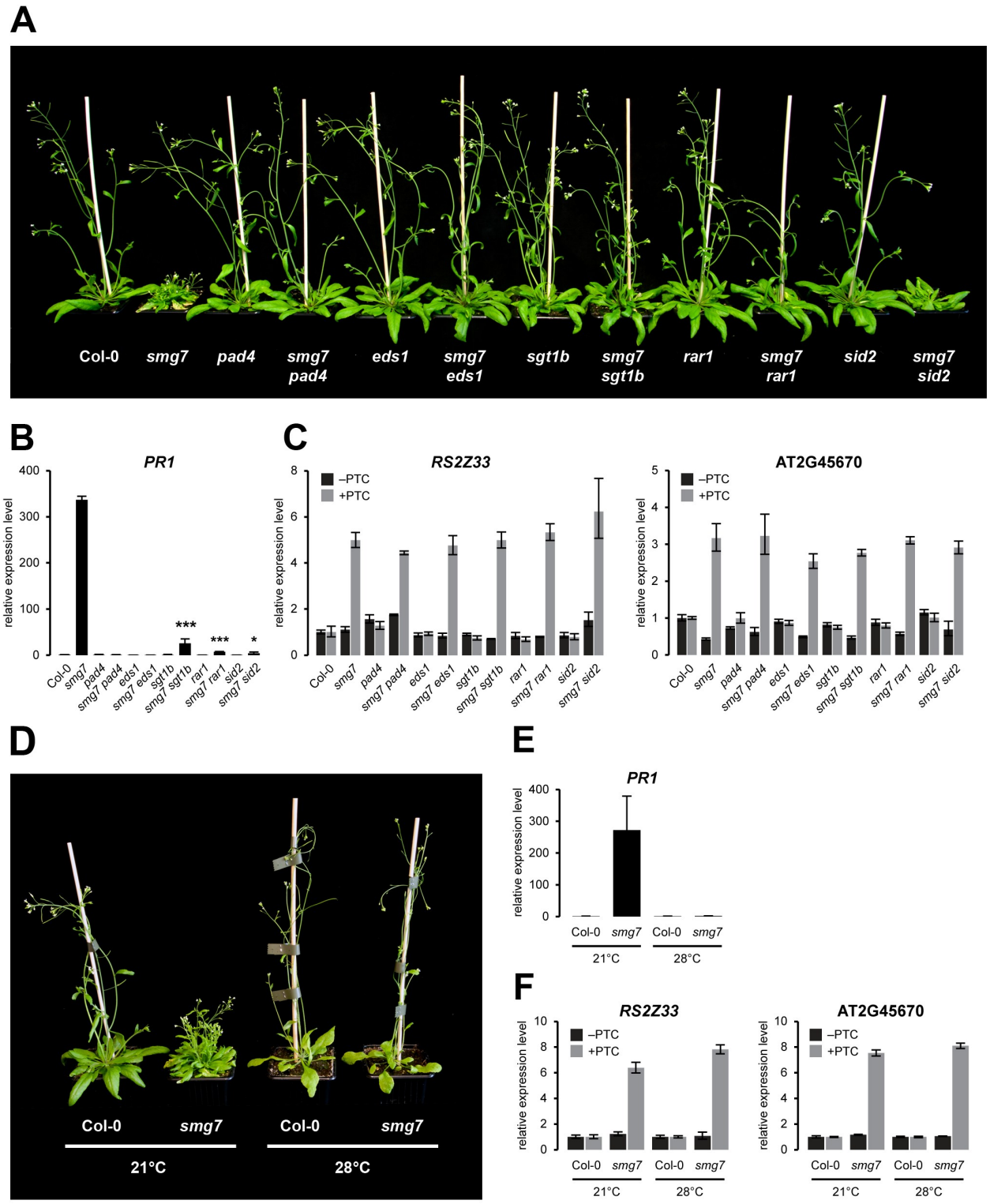
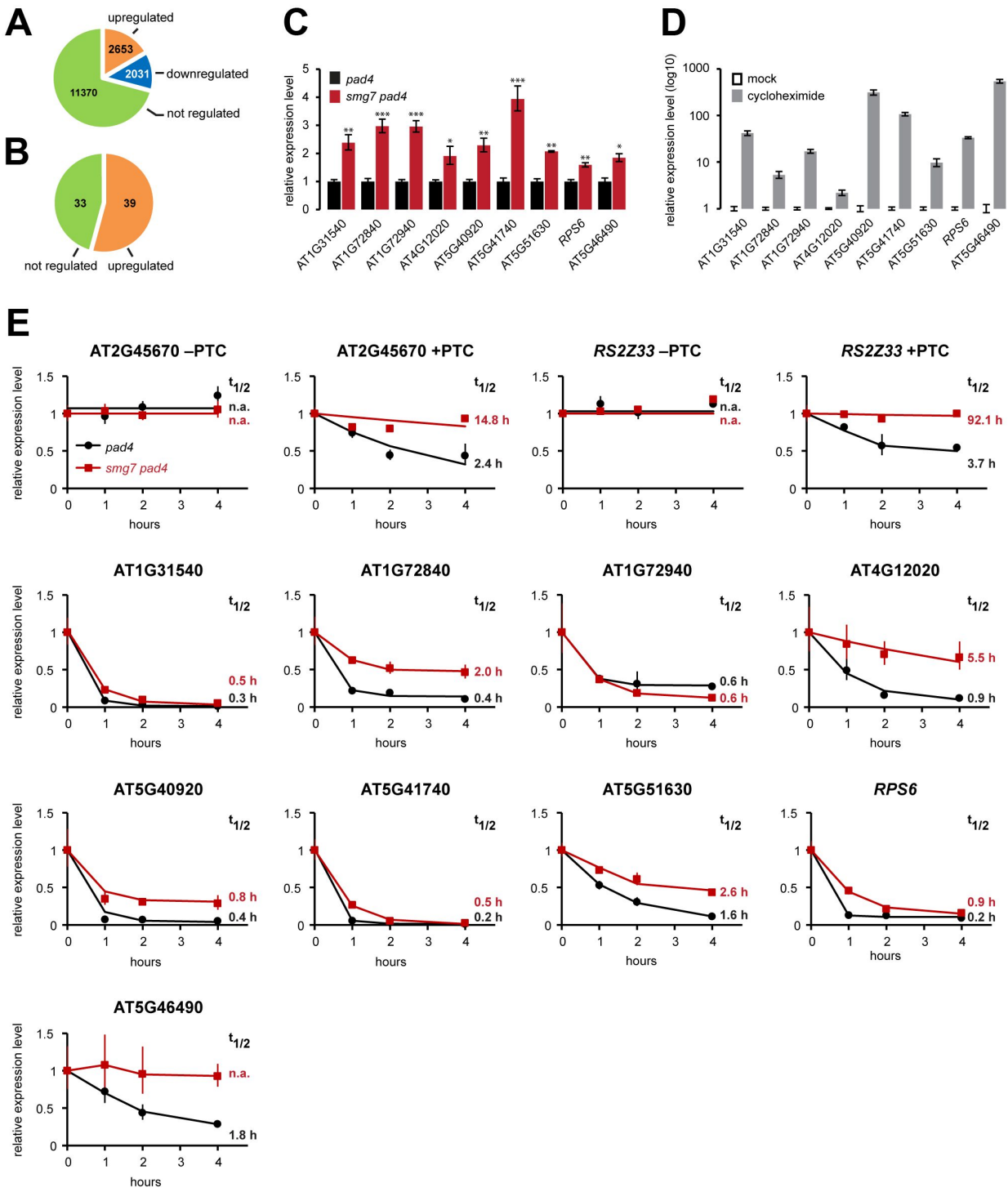


Figure 2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2653
2031
downregulated
11370
not regulated
B
33
39
not regulated
upregulated
C
relative expression level
pad4
smg7 pad4
AT1G31540
AT1G72840
AT1G72940
AT4G12020
AT5G40920
AT5G41740
AT5G51630
RPS6
AT5G46490
D
relative expression level (log10)
mock
cycloheximide
E
AT2G45670 –PTC
AT2G45670 +PTC
RS2Z33 –PTC
RS2Z33 +PTC
AT1G31540
AT1G72840
AT1G72940
AT4G12020
AT5G40920
AT5G41740
AT5G51630
RPS6
AT5G46490
t1/2
n.a.
14.8 h
2.4 h
92.1 h
3.7 h
0.5 h
0.3 h
2.0 h
0.4 h
0.6 h
5.5 h
0.9 h
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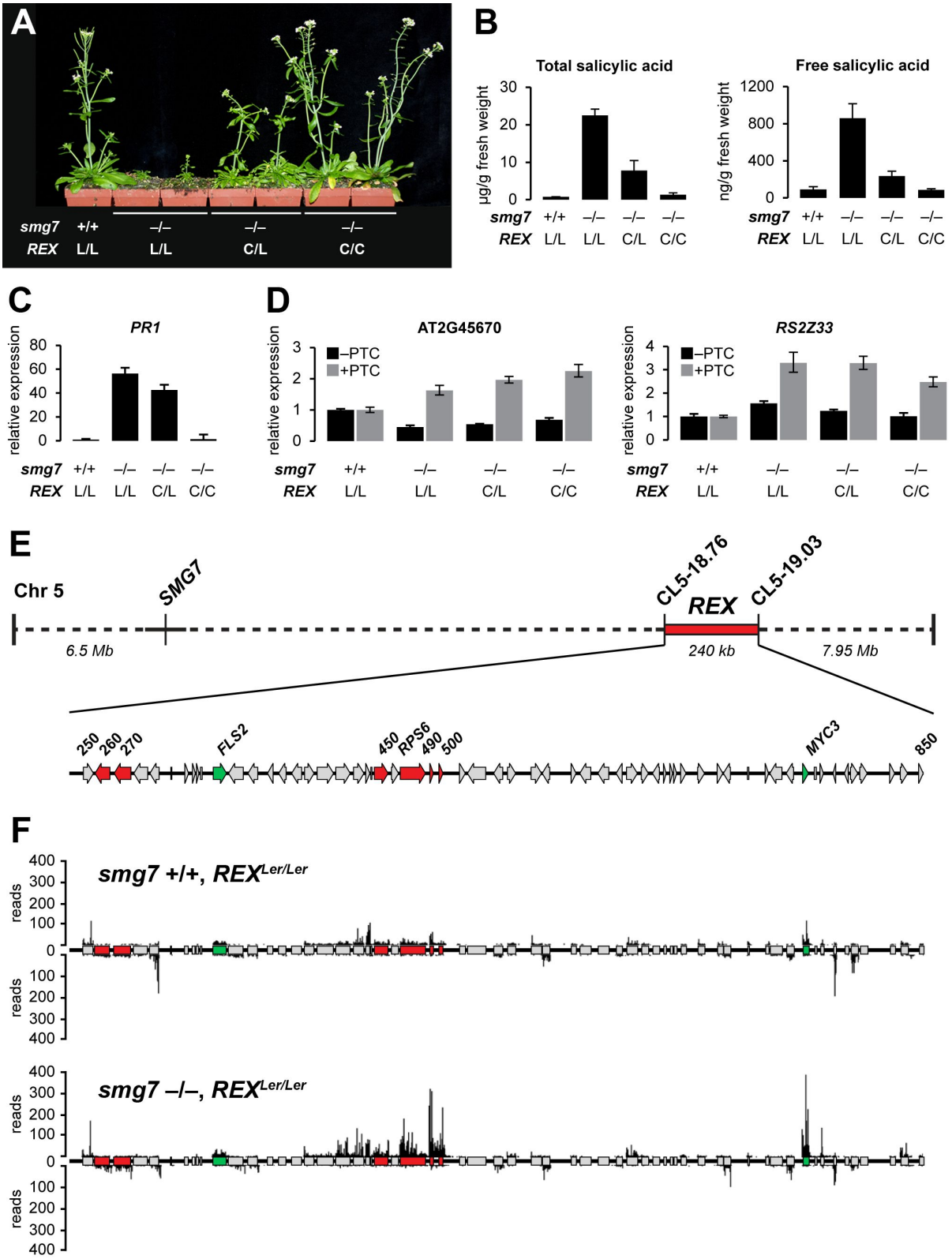
0.9 h
0.2 h
1.8 h
hours

Figure 3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10
0
Free salicylic acid
ng/g fresh weight
1200
800
400
0
smg7 +/+ –/– –/– –/–
REX L/L L/L C/L C/C
C
PR1
relative expression
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60
40
20
0
D
AT2G45670
RS2Z33
relative expression
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2
1
0
4
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+PTC
smg7 +/+ –/– –/– –/–
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E
Chr 5
SMG7
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6.5 Mb
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FLS2
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RPS6
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MYC3
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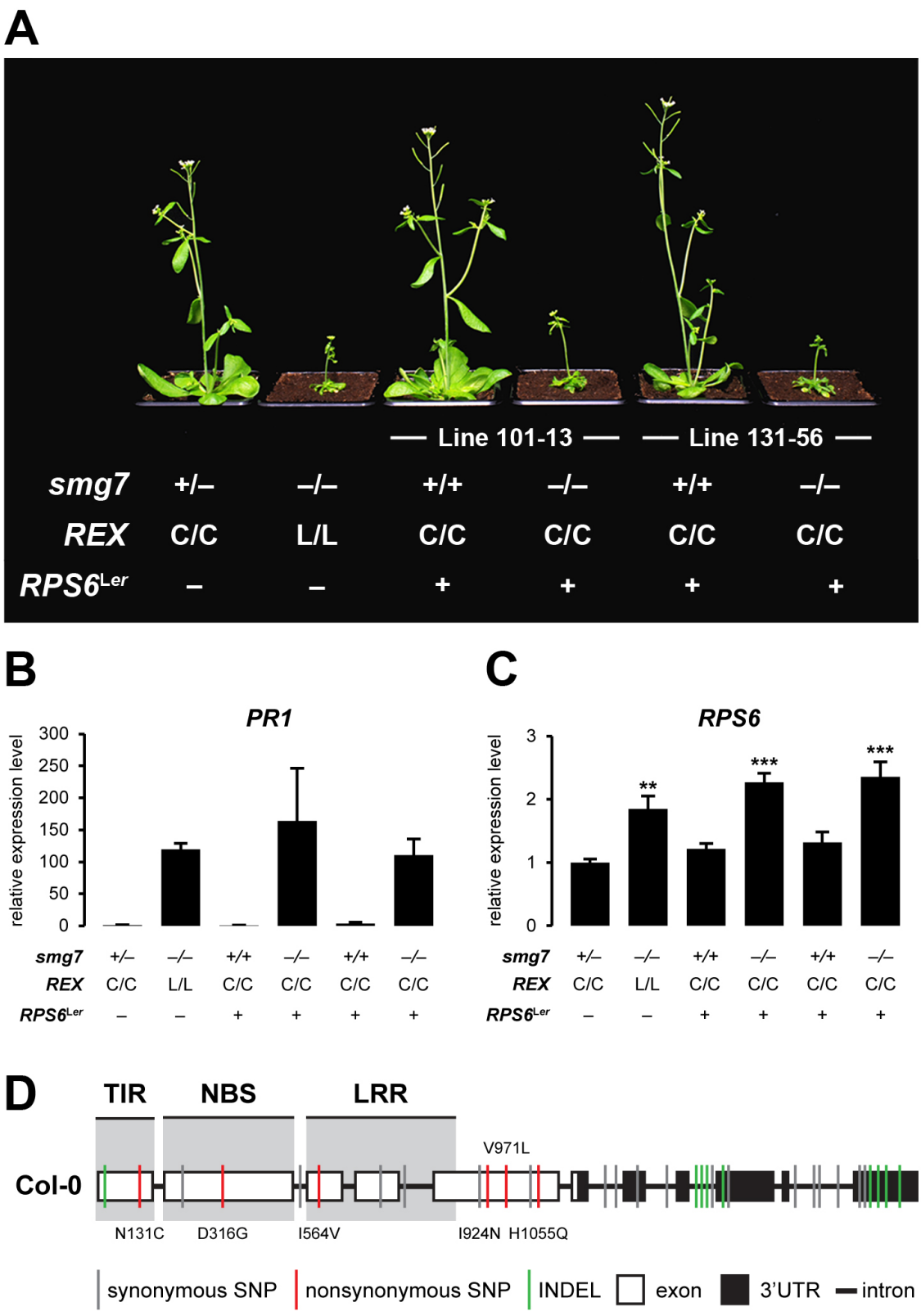
Figure 4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B
PR1
relative expression level
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250
200
150
100
50
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REX C/C L/L C/C C/C C/C C/C
RPS6Ler – – + + + +
C
RPS6
relative expression level
3
2
1
0
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smg7 +/– –/– +/+ –/– +/+ –/–
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RPS6Ler – – + + + +
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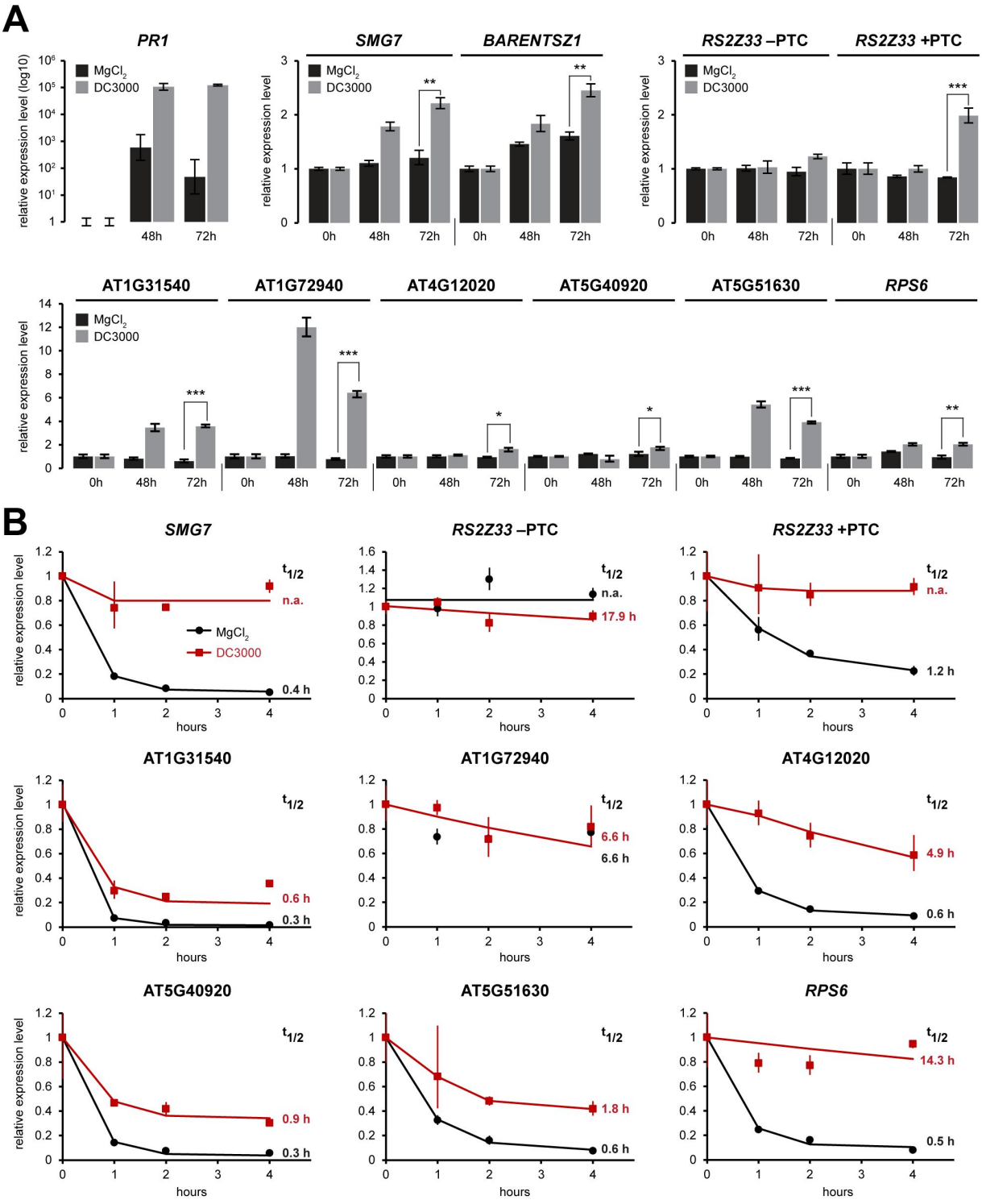
Figure 5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relative expression level
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48h
72h
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AT5G51630
RPS6
B
SMG7
RS2Z33 –PTC
RS2Z33 +PTC
t1/2
n.a.
0.4 h
17.9 h
1.2 h
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4.9 h
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hours

Figure 6

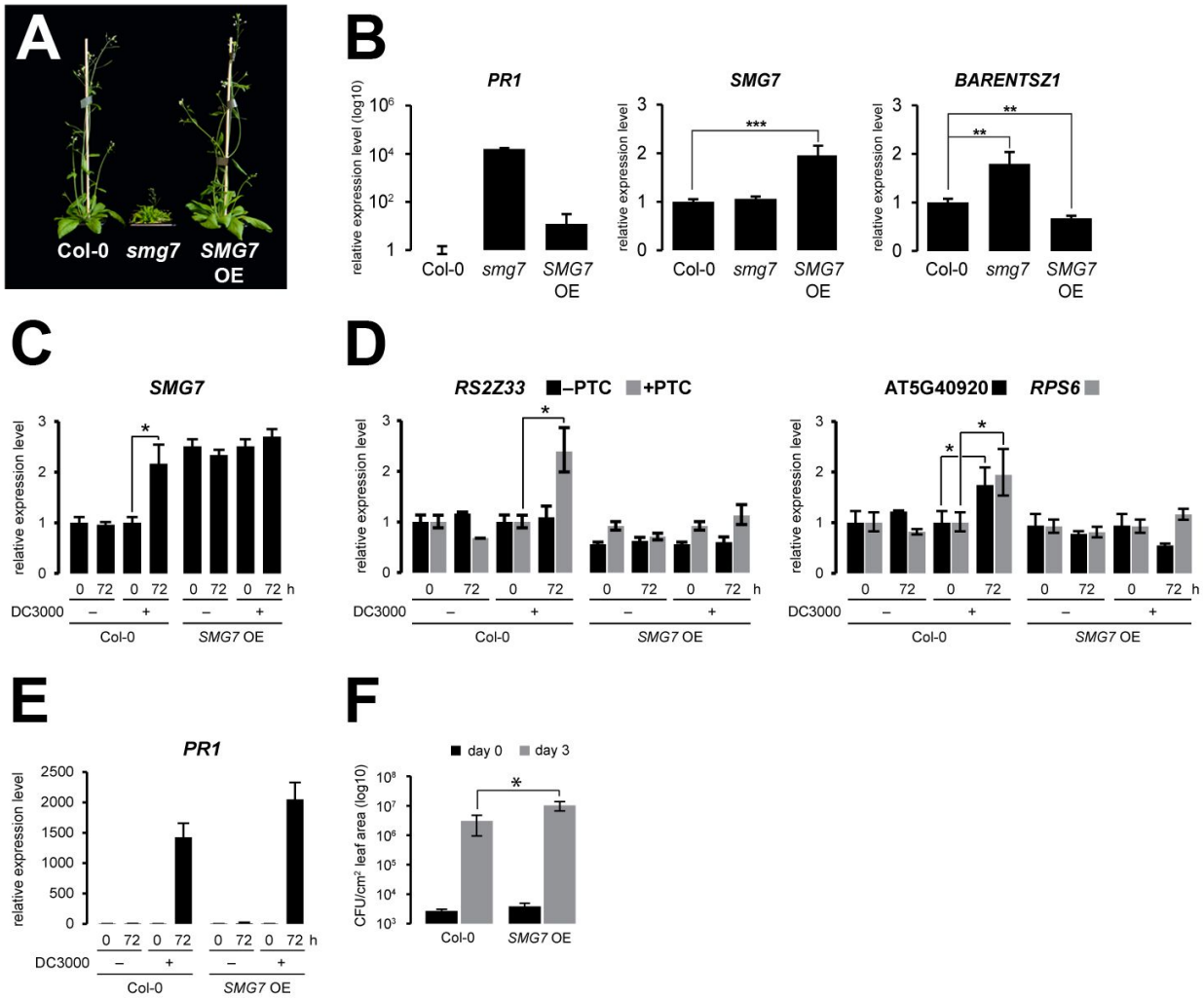


Figure 7

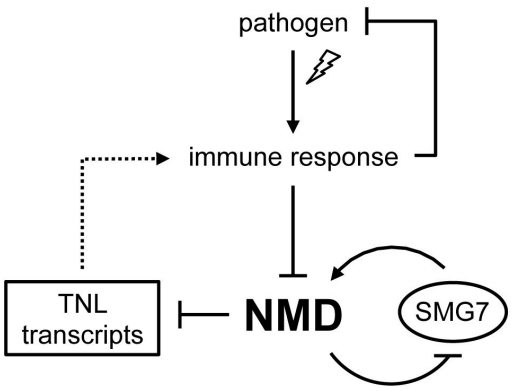


Figure Legends

Figure 1. Constitutive immune responses in *smg7* are suppressed by mutations in components required for NB-LRR signaling and by elevated ambient temperature.
(A) Morphology of five-week old plants of indicated genotypes. Stunted growth of *smg7* mutants is abolished in *smg7 pad4*, *smg7 eds1*, *smg7 sgt1b* and *smg7 rar1* plants.
(B) PR1 expression measured by qPCR in the indicated genotypes. Asterisks (*) indicate significant differences (*P<0.05, ***P<0.001) to the Col-0 wild type determined by Student's t-test (one-tailed).
(C) Expression levels of PTC-containing (+PTC) and PTC-free (-PTC) splice variants of the endogenous NMD reporters *RS2Z33* and At2g45670 measured by qPCR in the indicated genotypes.
(D) Morphology of five-week old Col-0 wild type plants and *smg7* mutants grown at 21°C and 28°C. Growth at 28°C suppresses stunting of *smg7*.
(E) *PR1* expression levels of plants shown in (D) measured by qPCR.
(F) Expression levels of PTC-containing (+PTC) and PTC-free (-PTC) splice variants of endogenous NMD reporters *RS2Z33* and At2g45670 measured by qPCR in plants shown in (D).
Expression levels shown in (B), (C), (E), and (F) are averages of four biological replicates. Values are normalized to the expression of At2g28390 (*SAND* gene). Error bars denote standard error of mean (SEM).

Figure 2. TIR-NB-LRR transcripts are direct targets of NMD.
(A) Classification of expressed transcripts that were significantly upregulated (adjusted *P*-value q<0.05), downregulated (q<0.05) or not significantly regulated (q≥0.05) in *smg7 pad4* plants relative to *pad4* control plants. The number of transcripts in the respective category is indicated.
(B) Classification of expressed TIR-NB-LRR transcripts that were significantly upregulated (q<0.05) or not significantly regulated (q≥0.05) in *smg7 pad4* plants relative to *pad4* control plants. The number of transcripts in the respective category is indicated.
(C) Steady state expression levels of selected TIR-NB-LRR transcripts measured by qPCR in *pad4* and *smg7 pad4* plants. The average values (±SEM) of three biological replicates are shown. Values are normalized to the expression of At2g28390. Asterisks (*) indicate significant differences (*P<0.05, **P<0.01, ***P<0.001) to *pad4* control samples (two-tailed Student's t-test).
(D) Expression levels of selected TIR-NB-LRR transcripts measured by qPCR after five hours of translational inhibition with cycloheximide or mock treatment (DMSO) in wild-type plants. The average values (±SEM) of three biological replicates are shown. Values are normalized to the expression of At2g28390.
(E) qPCR analysis of half-lives of selected TIR-NB-LRR transcripts and endogenous NMD reporters At2g45670 and *RS2Z33*. Transcription was inhibited in *pad4* and *smg7 pad4* plants using cordycepin and samples were harvested at 0, 1, 2 and 4 hours after treatment. Transcript half-lives (t1/2) were calculated by non-linear least square regression. The average values (±SEM) of three biological replicates are shown. Values are normalized to the expression of eIF4A1 (At3g13920).

Figure 3. The *REX* locus is sufficient to elicit autoimmunity in *smg7* mutants.
(A) Shown are five-week old *smg7* plants in L*er* genomic background carrying either Col-0 or L*er* alleles of *REX* on chromosome V. The respective allelic variant of *REX* is indicated with "L" (L*er*) or "C" (Col-0).
(B) HPLC-based quantification of total and free salicylic acid of plants shown in (A). Values and standard deviations are derived from three biological replicates.
(C) *PR1* expression measured by qPCR in plants shown in (A). Expression levels shown are averages of four biological replicates (±SEM). Values are normalized to the expression of At2g28390.
(D) Expression levels of PTC-containing (+PTC) and PTC-free (-PTC) splice variants of endogenous NMD reporters *RS2Z33* and At2g45670 measured by qPCR in plants shown in (A). Expression levels are averages of four biological replicates (±SEM) and are normalized to the expression of At2g28390.

(E) Genetic map of chromosome 5. The *REX* locus encompasses a 240 kb region (indicated by the red bar) and is located between markers CL5-18.76 and CL5-19.0. *REX* contains 60 genes in the L*er* accession, which are depicted as arrows (red arrows: TIR-NB-LRR genes, green arrows: other pathogen responsive genes). The three digits next to the genes are identifiers for the last corresponding AGI numbers (At5g46XXX).
(F) RNA-seq density profiles of normalized reads mapping to *REX* in wild type plants (*smg7*+/+) and mutant plants (*smg7*-/-) carrying the $REX^{Ler/Ler}$ allele.

Figure 4. *REX* encodes for the TIR-NB-LRR protein *RPS6*
(A) Shown are *smg7* mutant plants in L*er* background that carry different alleles of *REX*. Line-101-13 and line 131-56 are independent T2 lines derived from *smg7*+/- $REX^{Col/Col}$ plants (left) that were transformed with a genomic construct of $RPS6^{Ler}$. The $RPS6^{Ler}$ transgene elicits autoimmunity in transformed NMD deficient plants.
(B) *PR1* expression measured by qPCR in plants shown in (A). Expression levels shown are averages of four biological replicates (±SEM) and are normalized to the expression of At2g28390.
(C) Total levels of *RPS6* transcripts (derived from Col-0 and L*er*) measured by qPCR in plants shown in (A). Expression levels shown are averages of four biological replicates (±SEM) and are normalized to the expression of At2g28390. Asterisks (*) indicate significant differences (***P*<0.01, ****P*<0.001) to *smg7 +/-* $REX^{Col/Col}$ control samples (two-tailed, Student's t-test).
(D) Schematic representation of *RPS6*. Depicted polymorphisms were identified in the L*er* allele by Sanger sequencing.

Figure 5. NMD efficiency is downregulated upon pathogen infection
(A) Expression levels of *PR1*, the NMD components *SMG7* and *BARENTSZ1*, the endogenous NMD reporter *RS2Z33* and selected TNLs were measured by qPCR in wild type plants before and after 48 hours and 72 hours of treatment with either virulent *Pst* DC3000 or mock (MgCl2). Values are averages of three biological replicates (±SEM) and are normalized to At2g28390 expression. Asterisks (*) indicate significant differences (**P*<0.05, ***P*<0.01, ****P*<0.001) compared with the mock control at the respective time point (two-tailed Student's t-test). The experiment was repeated three times with similar results.
(B) qPCR analysis of half-lives of selected TIR-NB-LRR transcripts and endogenous NMD reporters AT2G45670 and *RS2Z33* after pathogen infection. Transcription was inhibited 72 hours after mock treatment or treatment with *Pst* DC3000 of wild type plants. Samples were harvested at 0, 1, 2 and 4 hours after treatment. Transcript half-lives (t1/2) were calculated by non-linear least square regression. The average values (±SEM) of three biological replicates are shown. Values are normalized to the expression of eIF4A1 (At3g13920). The experiment was repeated four times with similar results.

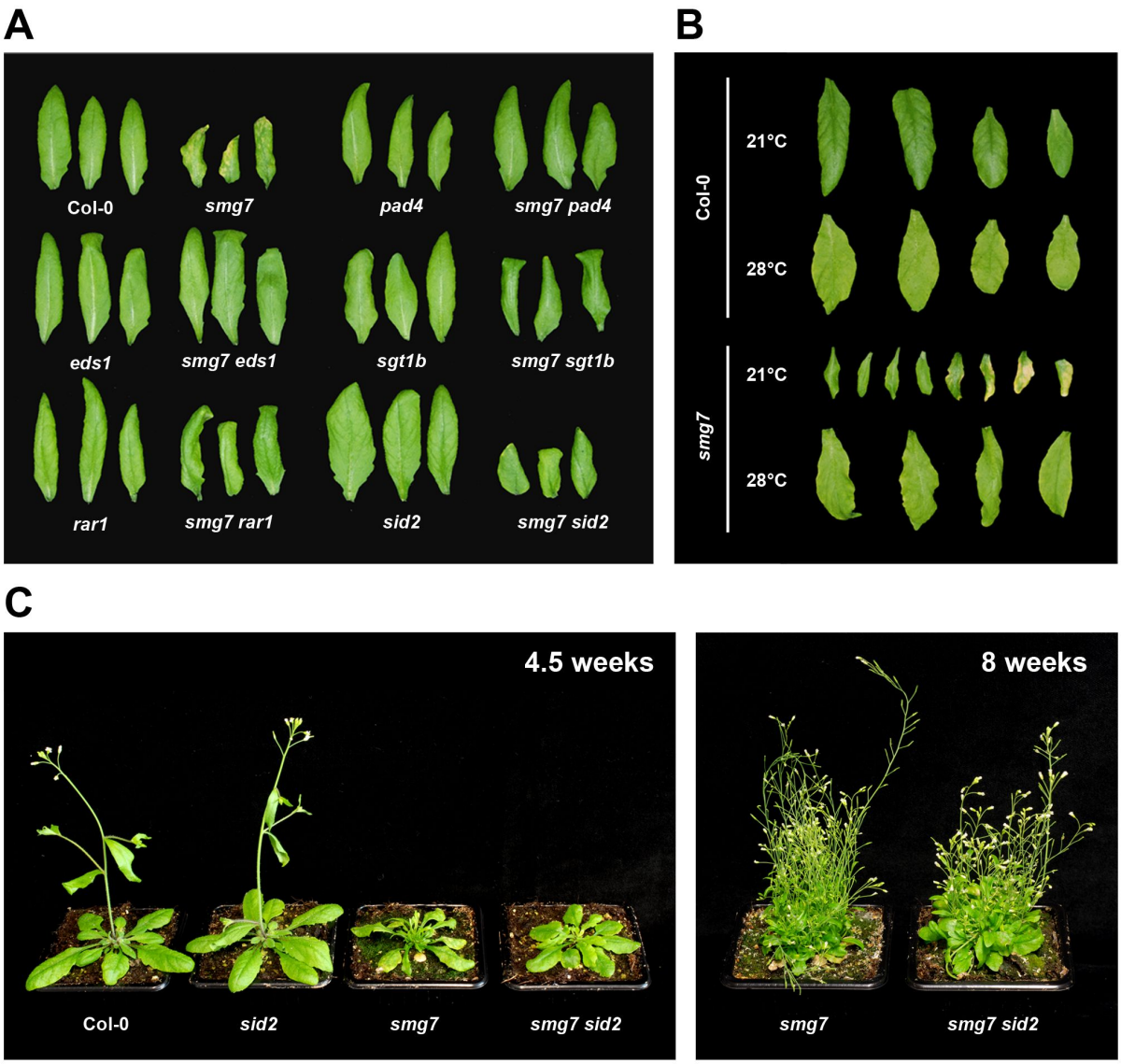
Figure 6. Overexpression of SMG7 has adverse effects on basal resistance
(A) Morphology of Col-0 wild type, *smg7* mutant and *SMG7* overexpressor *(*OE*)* plants. The actin promoter driven SMG7 cDNA transgene suppresses autoimmunity but not sterility observed in *smg7* mutants.
(B) Expression levels of *PR1* and the NMD components *SMG7* and *BARENTSZ1* measured by qPCR in plants shown in (A). Values for *SMG7* in *SMG7* OE plants represent total mRNA levels derived from the mutated endogenous *SMG7* copy and the functional *SMG7* cDNA transgene. Expression levels shown are averages of four biological replicates (±SEM) and are normalized to At2g28390 expression levels. Asterisks (*) indicate significant differences (***P*<0.01, ****P*<0.001) compared to Col-0 wild type plants (two-tailed Student's t-test).
(C-D) Expression levels of *SMG7*, the endogenous NMD reporter *RS2Z33* and the TNL*s RPS6* and At5g40920 measured by qPCR in wild type and *SMG7* OE plants before and after 72 hours of treatment with either virulent Pst DC3000 or mock (MgCl2). Plus signs (+) indicate inoculation with *Pst* DC3000, while minus signs (-) indicates mock treatment. Values are averages of three biological replicates (±SEM) and are normalized to the expression of At2g28390 (SAND family protein). Expression values are relative to steady state levels measured at time point 0

hours in Col-0 wild type plants. Asterisks (*) indicate significant differences (P<0.05) compared with the mock control at the respective time point (two-tailed Student's t-test).
(E) *PR1* expression measured by qPCR in the experiment outlined in (C). Values are averages of three biological replicates (±SEM) and are normalized to the expression of At2g28390 (SAND family protein).
(F) Bacterial growth measurement in Col-0 wild type plants and *SMG7* OE plants at day 0 and 3 days later after spray inoculation with *Pst* DC3000. Values (±SEM) represent the average of five biological replicates (each consisting of 4 leaves per plant). The asterisk (*) indicates a significant difference (P<0.05) determined by Student's t-test (two-tailed).

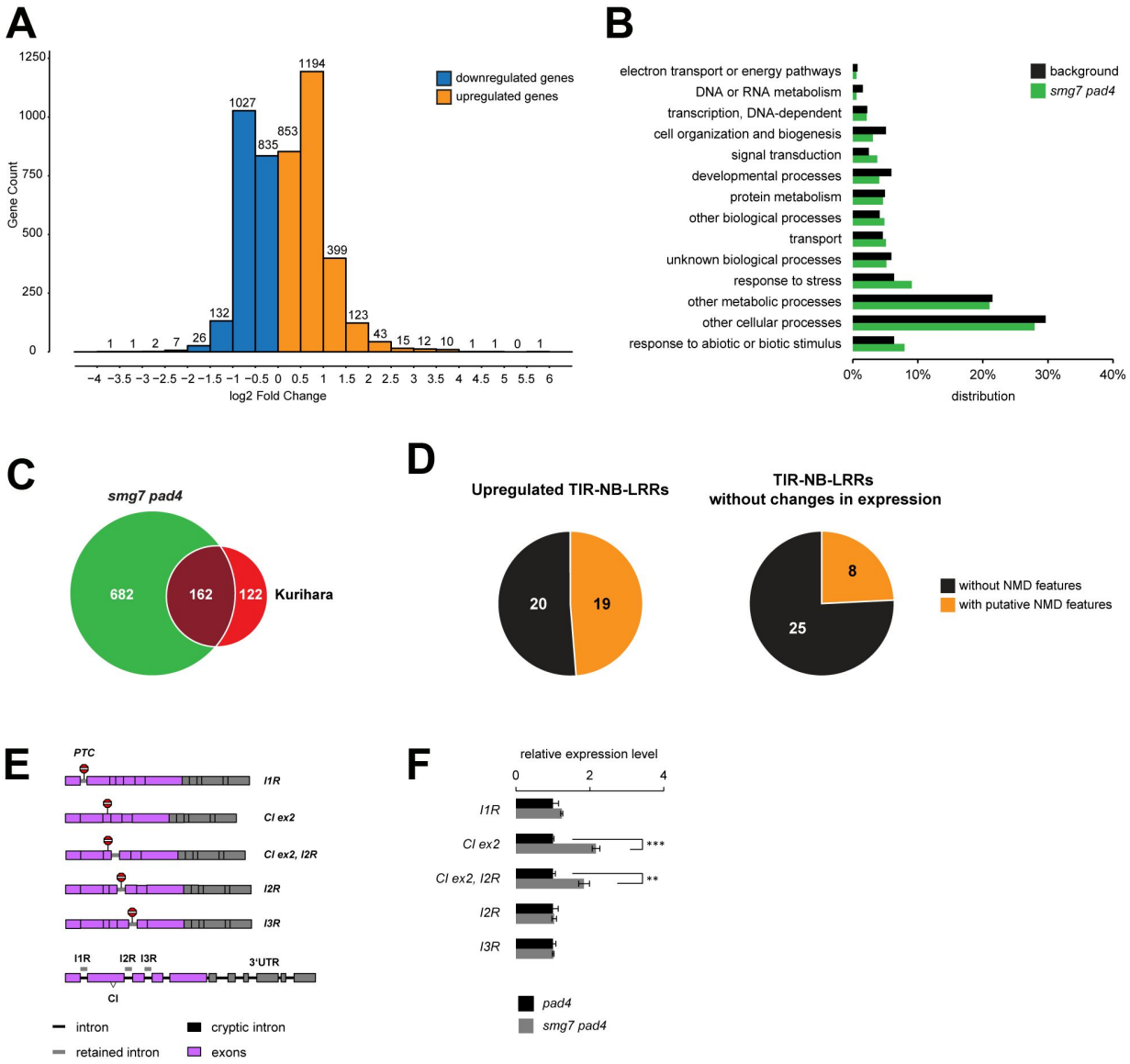
Figure 7. Model depicting the role of NMD in plant immunity
Downregulation of NMD in response to pathogen challenge leads to stabilization of TNL transcripts, which might contribute to disease resistance.

Supplemental Figures

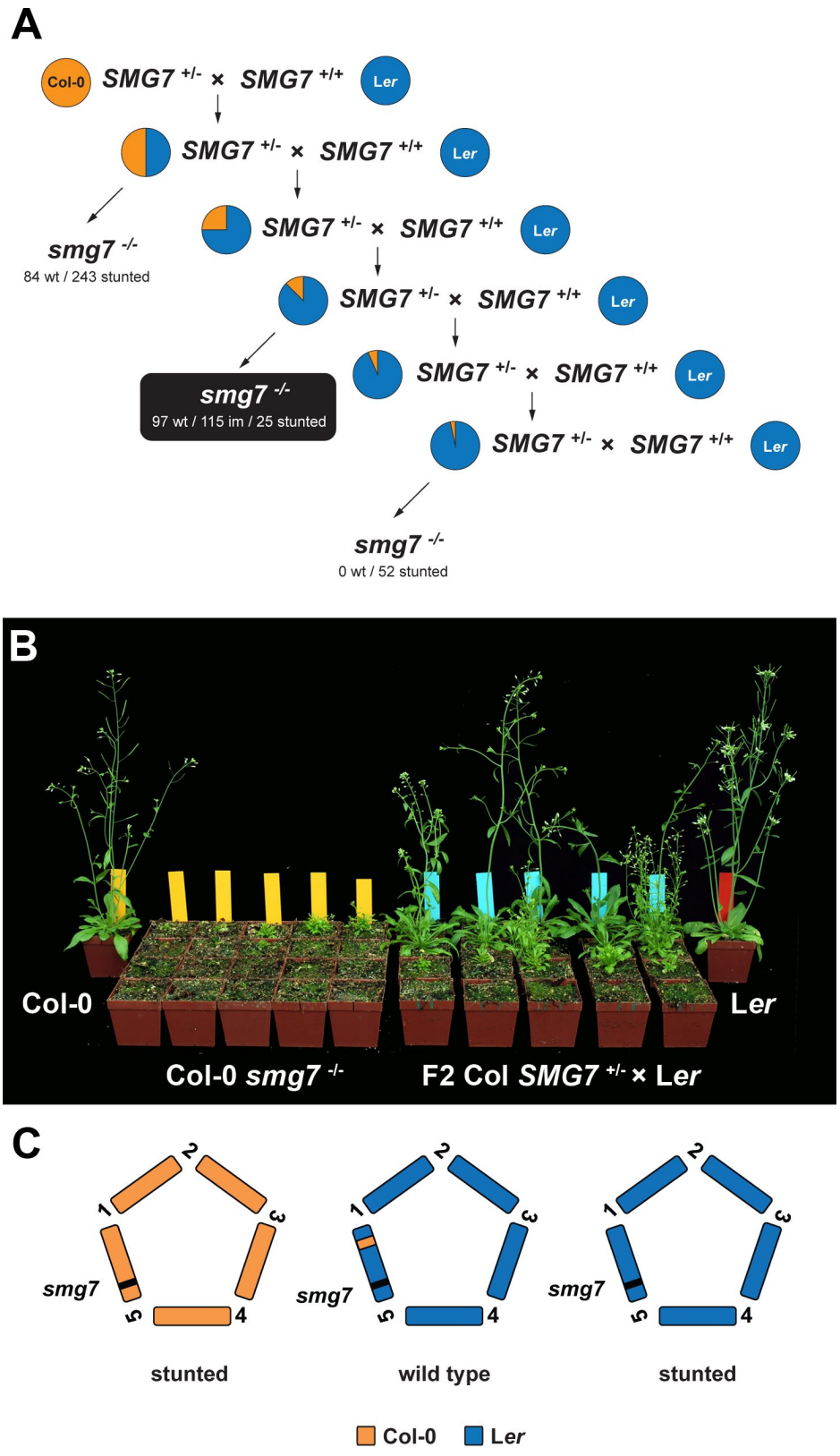
Supplemental Figure S1



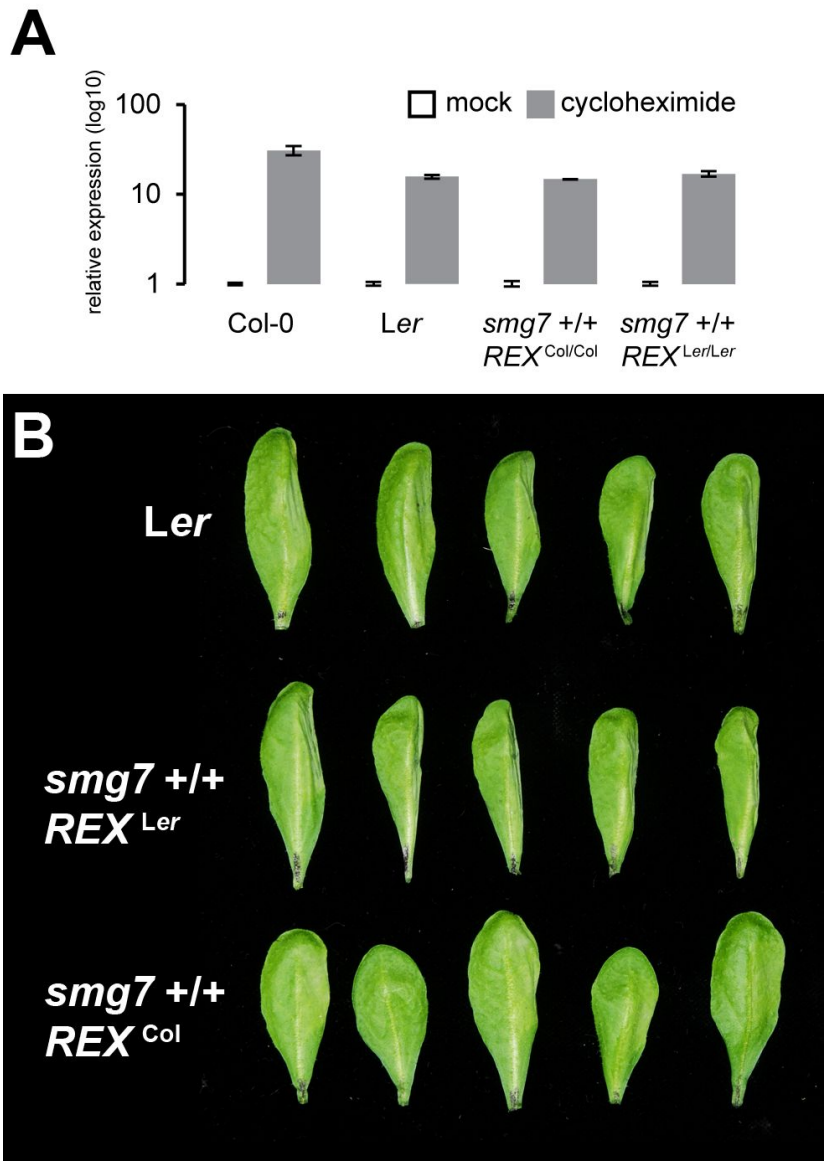
Supplemental Figure S2



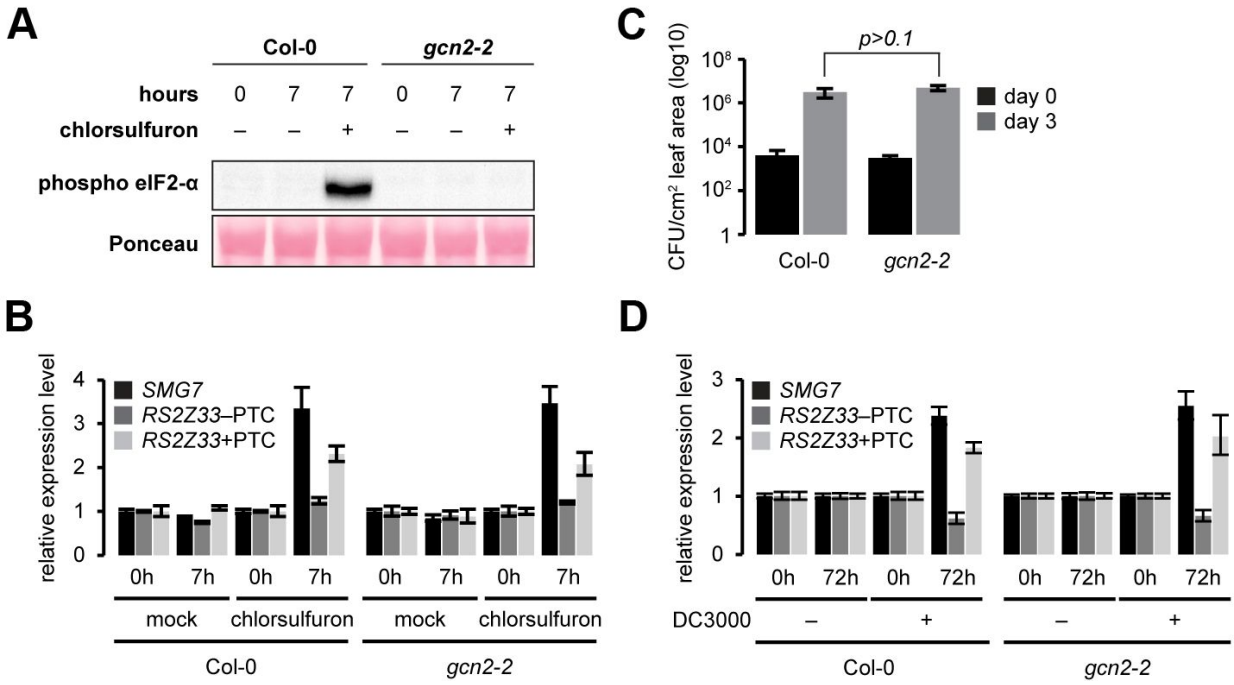
Supplemental Figure S3



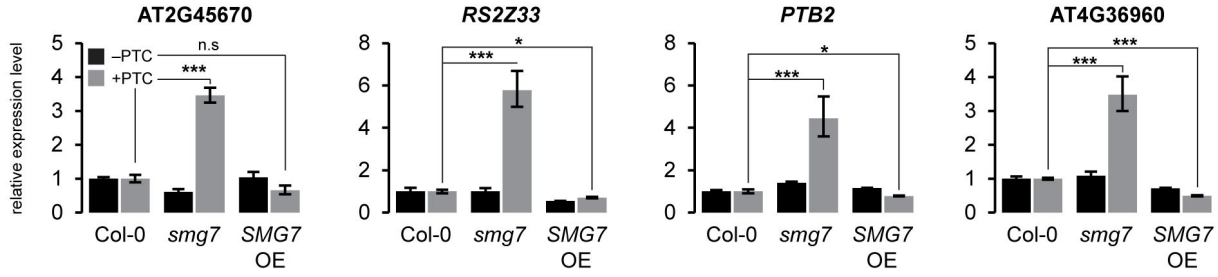
Supplemental Figure S4



Supplemental Figure S5



Supplemental Figure S6



Supplemental Figure Legends

Figure S1, Related to Figure 1. Genetic and environmental requirements for immune response activation in *smg7* mutants

(A) Detached leaves of five-week old plants of the indicated genotypes.

(B) Detached leaves of five-week old Col-0 and *smg7* plants grown at 21°C and 28°C.

(C) Shown are 4.5-week old Col-0, *smg7*, *sid2* and *smg7 sid2* plants. The *sid2* mutation failed to suppress stunting of *smg7* single mutants. The *smg7* and *smg7 sid2* plants shown in the left panel were propagated for a total of 8 weeks and are shown in the right panel.

Figure S2, Related to Figure 2. RNA-seq analysis of *smg7 pad4* mutants

(A) Histogram showing the fold-change (log2) distribution of significantly up- and downregulated transcripts in *smg7 pad4* mutant relative to *pad4* control plants (adjusted *P*-value *q*<0.05). Numbers on the bars indicate numbers of transcript in the respective fold-change range indicated on the x-axis.

(B) Distribution of functional gene ontology (GO) terms of genes that are significantly upregulated in *smg7 pad4* mutants (green bars) (adjusted *P*-value *q*<0.05), compared to GO terms present in the background set (black bars).

(C) Proportional Venn diagram illustrating the overlap of 284 genes that were found to be upregulated (fold change >1.8) in the NMD mutants *upf1-1* and *upf3-1* by Kurihara et al. (2009) {Kurihara, 2009 #1596} and 844 genes that were significantly upregulated (fold change 1.8, q<0.05) in *smg7 pad4* RNA-seq data sets. The two data sets have 162 genes in common, while 122 genes are uniquely present in the Kurihara data set and 682 genes are only present in the *smg7 pad4* set.

(D) Pie-charts showing the distribution of putative NMD eliciting features among expressed TIR-NB-LRR genes that were either found to be upregulated in *smg7 pad4* mutants (left) or that were not subjected to differential regulation (right).

(E) Scheme of previously reported *RPS6* splice variants (Kim et al., 2009). I1R=intron 1 retention, I2R = intron 2 retention, I3R=intron 3 retention, CI ex2= excision of a cryptic intron located in exon 2.

(F) qPCR-based quantification of RPS6 splice variants. Asterisk (*) indicate statistically significant differences determined by Student's –test (two-tailed, **P<0.01, ***P<0.001). Values are averages of three biological replicates and are normalized to the expression of AT2G28390. Error bars denote standard error of mean (SEM)

Figure S3, Related to Figure 3. Map-based cloning of *REX*

(A) A crossing scheme delineating the crosses performed for map-based cloning of *REX/RPS6.* Progeny of the first, third and fifth cross were allowed to self and *smg7*-/- mutants were phenotypically analyzed. Numbers below indicate the number of *smg7*-/- plants that were stunted, had wild-type appearance or showed and intermediate (im) phenotype. The population highlighted by a black box was used to fine-map *REX*.

(B) Plants shown are mutant *smg7* F2 progeny from a cross of *smg7*+/- plants (Col-0 background) to L*er* wild type plants. In this F2 population suppression of stunting was observed in 84 plants, while 243 remained stunted (see also Figure S3B).

(C) Illustration of genotypes that were obtained in map-based cloning of the *REX* locus. The *smg7* mutation was generated in the Col-0 accession (left panel) and is located on chromosome V. *SMG7*-deficient plants (Col-0 background) are stunted and suffer from autoimmunity. Introgression of the *smg7* mutation into L*er* genomic background by multiple backcrosses yields plants that are also stunted (right panel). However, *smg7* mutants in Ler background that retain Col-0 genome on the right arm of chromosome V (*REX* locus) have wild type appearance and no disease symptoms (middle panel).

Figure S4, Related to Figure 4. Col-0 and *Ler* alleles of *RPS6* are NMD targets and trigger different HR outputs.

(A) Expression levels of *RPS6* upon translational inhibition with cycloheximide in 14-day old seedlings of the indicated genotypes. Expression levels shown are averages of four biological replicates (±SEM). Values are normalized to the expression of AT2G28390.

(B) Syringe infiltration into the right side of leaves of the indicated genotypes with $2x10^7$ cfu/mL *Pst* DC3000 HopA1. Disease symptoms were recorded 24 hours after infiltration. Ler wild type plants and *SMG7* wild type plants (L*er* background) with the REX^{Ler} allele display tissue collapse in the infiltrated region, while this is not the case in *SMG7* wild type plants (L*er* background) with the REX^{Col} allele.

Figure S5, Related to Figure 5. The eIF2α-kinase GCN2 is dispensable for NMD downregulation in response to amino acid starvation and pathogen infection

(A) Western Blot analysis of 12-day old Col-0 wild type seedlings and *gcn2-2* mutant seedlings. To induce amino acid starvation, the seedlings were treated for 7 hours with 0.6 uM chlorsulfuron, an inhibitor of amino acid biosynthesis as described by Lageix

et al. (2008) (Lageix et al., 2008). Phosphorylation of eIF2α is readily detected in Col-0 seedlings after chlorsulfuron treatment, while no phosphorylation is detected in *gcn2-2* seedlings, confirming loss of function of *GCN2* in *gcn2-2* mutants. Similar results were obtained with another *gcn2* mutant line in Ler background (Genetrap line GT8359, Cold Spring Harbor Laboratory). Ponceau staining of Rubisco large subunit (rbcL) is shown as a loading control.
(B) Expression levels of *SMG7*, PTC-containing (+PTC) and PTC-free (-PTC) splice variants of *RS2Z33* measured by qPCR in Col-0 and *gcn2-2* seedlings upon amino acid starvation induced by chlorsulfuron. Expression levels shown are averages of four biological replicates (±SEM). Values are normalized to the expression of At2g28390. The NMD targets *SMG7* and *RS2Z33*+PTC are specifically upregulated in response to chlorsulfuron treatment in both Col-0 and *gcn2-2* seedlings, indicating that NMD downregulation occurs independently of the presence of a functional *GCN2* allele. Similar results were obtained with another *gcn2* mutant line in Ler background (Gentrap Line GT8359, Cold Spring Harbor Labs).
(C) Bacterial growth assay in Col-0 wild type plants and *gcn2-2* mutant plants at day 0 and 3 days later after spray inoculation with *Pst* DC3000 (1x108 cfu/ml). Values (±SEM) represent the average of five biological replicates (each consisting of 4 leaves per plant). Bacterial growth is not significantly different between infected Col-0 and *gcn2-2* mutants ($P>0.1$, two-tailed Student's t-test). These data suggest that the GCN2 kinase is dispensable for basal resistance against virulent *Pst* DC3000.
(D) Expression levels of *SMG7*, PTC-containing (+PTC) and PTC-free (-PTC) splice variants of *RS2Z33* measured by qPCR in Col-0 and *gcn2-2* plants at day 0 and 3 days later after spray inoculation with *Pst* DC3000 ($1x10^8$ cfu/ml). Values (±SEM) represent the average of three biological replicates. Accumulation of *SMG7* and *RS2Z33*+PTC was observed in *Pst* DC3000 treated Col-0 and *gcn2-2*, indicating that NMD downregulation upon *Pst* DC3000 treatment occurs independently of GCN2.

Figure S6, Related to Figure 6. Expression of NMD reporters in *SMG7* overexpressor plants.
Expression levels of PTC-containing (+PTC) and PTC-free (-PTC) splice variants of endogenous NMD reporters At2g45670, *RS2Z33*, *PTB2*, and At4g36960 measured by qPCR in Col-0, *smg7* mutants and *SMG7* OE plants. Expression levels shown are averages of four biological replicates (±SEM). Values are normalized to the expression of At2g28390. Asterisks (*) indicate significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) determined by Student's t-test (two-tailed).

Supplemental Tables

Table S1, Related to Figure 2. Differential gene expression analysis of pad4 and smg7 pad4 RNA-libraries
(this table will be included as a separate Excel file)

Table S2, Related to Figure 2. List of expressed TNL genes and their putative NMD features
(this table will be included as a separate Excel file)

Table S4, Related to Experimental Procedures. PCR markers used for map-based cloning
(this table will be included as a separate Excel file)

Table S3, Related to Experimental Procedures. Primers used in this study

PCR-genotyping of mutants

Name	Sequence (5'→3')	Remarks
Est1b-1	GACCTTGGTAGCTGGTCC TGAG	PCR genotyping of *smg7-1* and SMG7 cDNA wt: Est1b-1 + Est1b-2 mut: Est1b-1 + LBc-1
Est1b-2	GGACAACAGGCCAACCATTCAAC	
LBc-1	TGGACCGCTTGCTGCAACTCT	
pad4-1-F	GCGATGCATCAGAAGAG	PCR genotyping of *pad4-1* CAPS marker (BsmF1)
pad4-1-R	TTAGCCCAAAAGCAAGTATC	
105/E2	ACACAAGGGTGATGCGAGACA	PCR genotyping of *eds1-2,* multiplex PCR Wt: 1500 bp + 750 bp Mut: 1500 bp + 600 np
EDS4	GGCTTGTATTCATCTTCTATCC	
EDS6	GTGGAAACCAAATTTGACATTAG	
sgt1b-F dc_eta3_AluI-F	AGGATGAGAAGCTTGATGGAGATGCACC	PCR genotyping of *sgt1b (eta3),* CAPS marker (AluI)
sgt1b-F dc_eta3_AluI-R	CGTCCCATTCGACTCTGCCTGTCAAAGC	
rar1-21-F PM100	AACTTTTGCCACCGGTTATG	PCR genotyping of *rar1-21* CAPS marker (Cac8I)
rar1-21-R PM101	GGCCAGAACTGGTTTCTCAG	
SID2-F2	GCTCTGCAGCTTCAATGC	PCR genotyping of *sid2-1* CAPS marker (TruI1)
SID2-R2	CGAAGAAATGAAGAGCTTGG	

Primers for quantitative real-time PCR (qPCR)

Name	Sequence (5'→3')	Remarks
PR1-qF2	GCTCTTGTAGGTGCTCTTGTTCTTCC	qPCR for AT2G14610 (*PR1*)
PR1-qR2	AGTCTGCAGTTGCCTCTTAGTTGTTC	
NMD3-PTC-qF2	TACGGAAGAGTGCGAGATGTGG	qPCR for AT2G37340 (*RS2Z33*)–PTC splice variant
NMD3-PTC-qR2	GGGATCACCAAATTCAACGAAAGC	
NMD3+PTC-qF2	CGCCTTGGCGATTCTGTTTGTAG	qPCR for AT2G37340 (*RS2Z33*)+PTC splice variant
NMD3+PTC-qR2	GGGATCACCAAATTCAACGAAAGC	
NMD2-PTC-qF2	TGCTGTGCATGAAATAAAGAGAAAA	qPCR for AT2G45670–PTC splice variant
NMD2-PTC-qR2	TCGTGGTTCCTTCGGGGAAT	
NMD2+PTC-qF2	TGCTGTGCATGAAATAAAGGGATGT	qPCR for AT2G45670+PTC splice variant
NMD2+PTC-qR2	AGTCGTGGTTCCTTCGGGGA	
AT1G31540-qF1	GAAGCGCATGCGGCTATATCAC	qPCR for AT1G31540
AT1G31540-qR1	CCGTTTAGTTTGGACGTAGACACC	
AT1G72840-qF1	GGCAAGCATTCCAGAAATTGCG	qPCR for AT1G72840
AT1G72840-qR1	CTTGGCAGTCGACTTGAGATGC	
AT1G72940-qF1	GACGAGCATTGACCAGTTTGGC	qPCR for AT1G72940
AT1G72940-qR1	GCTTCGCTTCATCTTCACATTTGG	
AT4G12020-qF1	GGGTAACAACAGGTGATGCCTTG	qPCR for AT4G12020
AT4G12020-qR1	TGACGCCACTAGTGTCAACTCC	
AT5G41740-qF1	TGAAGCAGAAGGTTCCAAGTTCTC	qPCR for AT5G41740
AT5G41740-qR1	TCTTCTTCACTGACCAAGCTTCCG	
AT5G51630-qF1	TGGGATGTCTCTCCCAGAGTTC	qPCR for AT5G51630
AT5G51630-qR1	AGCTTCACCTTCACCTTTAACTGG	
AT5G40920-qF2	GTTGGCAAATGAAACAGGTACTGG	qPCR for AT5G40920
AT5G40920-qR2	GCATCCCTTCAAAGACTCGTTTCC	
RPS6-3UTR-qF2	ACCAAAGCATGGATCTCCCGGTC	qPCR for AT5G46470 (*RPS6*), amplifies Col-0 and L*er* alleles
RPS6-3'UTR-qR2	CGCACAGAGCTCTTCTCCACTGA	
RPS6-B-qF2	TGGTTGTGAAATGGTCTGTTTCCTT	qPCR for RPS6-I1R
RPS6-B-qR2	TCCCGACCATCCTCACTTCCTC	
RPS6-C-qF1	ATGTATTTTCAATGAGATCCCTTATC	qPCR for RPS6-CI ex2
RPS6-C-qR1	CCTTCTTAGTACCAGTGTTATGTTCAA	
RPS6-D-qF2	TGTATTTTCAATGAGATCCCTTATCTGT	qPCR for RPS6-CI ex2, I2R

RPS6-D-qR2	ACAATTCTGCACATATGAAATGACATGC	
RPS6-I2R-qF2	TCGGGCTAAAAAACCTTGTTGATAG	qPCR for RPS6-I2R
RPS6-I2R-qR2	ACCAATTCGTTACAATTCTGCACAT	
RPS6-I3R-qF1	TCTACCAGGATTAATATCGACATTGGAA	qPCR for RPS6-I3R
RPS6-I3R-qR1	CCCTGCAAGTGACTGAATTATATGCAA	
AT5G46490 -qF1	TCTACCGAATGGAATCGCGGA	qPCR fpr AT5G46490
AT5G46490-qR1	TCAGCGGTGTGATCTTCAATGCC	
SMG7-qF1	GCAGAGGGGGATTCCAGGAGCA	qPCR for AT5G19400 (*SMG7*) and *SMG7* cDNA
SMG7-qR1	TTGCAAGCTGATGATGTGGGTTTCC	
BARENTSZ1 AT1G80000.1+2-qF1	TGGTTCTTACCAAGCAGACAAACC	qPCR for AT1G80000 (*BARENTSZ1*)
BARENTSZ1 AT1G80000.1+2-qR1	TGGAACTGTTCTCTTGGCTGGAC	
PTB2-PTC-qF1	TGTCTTGCATCTGGTATTCTCAGC	qPCR for AT5G53180 –PTC splice variant
PTB2-PTC-R1	TGCTTGGTATCCGGCTGTCTTC	
PTB2+PTC-qF1	TCGAAGGCGATGATGCTCGTATG	qPCR for AT5G53180 +PTC splice variant
PTB2+PTC-qF1	TTGCCTTGACATGGCCCAGATG	
AT4G36960-PTC-qF1	AGAGATTTCAAAAGGAGGAAGAGG	qPCR for AT4G36960–PTC splice variant (Drechsel et al., 2013)
AT4G36960-PTC-qR1	GAAACCCTAACACACTCAAAAAG	
AT4G36960+PTC-qF1	GAAAGAGATGTGCTTGTGGTTTG	qPCR for AT4G36960+PTC splice variant (Drechsel et al., 2013)
AT4G36960+PTC-qR1	GAAACCCTAACACACTCAAAAAG	

Extended Experimental Procedures

Chlorsulfuron treatment

Seedlings were germinated on 0.5x Murashige and Skoog agar (supplemented with Gamborg vitamins) and were grown for 12 days with 16h photoperiod. Twenty-four hours before treatment, seedlings were carefully transferred into 6-well plates supplemented with 4 mL liquid 0.5 x MS medium. On the next day, seedlings were treated with 0.6 µM chlorsulfuron (Sigma) for 7 hours to induce amino acid starvation (Lageix et al., 2008). The seedlings were then harvested and flash frozen in liquid nitrogen for subsequent RNA and protein analysis.

Protein analysis

For total protein extraction, approximately 0.1g 12-day old seedlings were ground in liquid nitrogen. An equal volume of ice-cold Lacus buffer supplemented with protease inhibitor cocktail (complete Mini, Roche) and phosphatase inhibitor (phosSTOP, Roche) was added, followed by brief extraction on a vortex mixer. The sample debris was then removed by two centrifugation steps (30 min at 12000 rpm, 10 min at 12000 rpm, 4°C). The remaining supernatant was mixed with 5xLaemmli buffer and was separated on a 12% SDS-PAGE gel. Phosphorylation of eIF2-alpha was detected using a monoclonal phospho-specific primary antibody (clone ab23157, Abcam) at 1:500 dilution. The secondary antibody (mouse anti-rabbit HRP conjugate, Pierce #31460) was used at 1:20000. Signals were detected using a chemiluminescent substrate (ECL, Pierce) and were recorded on the Gel Doc XR System (Bio-Rad).

Bioinformatic analysis of RNA-seq data

Number of RNA-seq reads generated ranged from 52 to 67 million reads. Reads were mapped to the *Arabidopsis thaliana* reference genome TAIR10 (*http://www.arabidopsis.org*) using *Tophat v2.0.8b* (Trapnell et al., 2009) by allowing a maximum of three mismatches. In order to filter genes that are not expressed, RPKM values per gene per library were computed by using gene length from the representative gene model. Genes with RPKM >= 1 in at least three out of six libraries were alone considered expressed and retained for differential gene expression analysis. Counts per gene from the retained genes were then provided to the R/bioconductor package DESeq v1.10.1 (Anders and Huber, 2010) to detect differentially expressed genes between *pad4 and smg7 pad4*. Cutoffs of *q <= 0.05* were

taken as the criterion for significant differential gene expression. GO term analysis was carried out using the GO annotation search tool on TAIR. For RNA-seq analysis of *smg7* lines in L*er* strand-specific single end libraries were generated and sequenced on the Illumina HiSeq2000 platform. Reads were then mapped to the L*er* genome scaffold (Schneeberger et al., 2011) and were quantified using CLC Genomics Workbench 5.5.1 and custom-made Perl scripts. Read densities were visualized using Integrated Genome Browser (Nicol et al., 2009).

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5 Discussion

The role of NMD as a housekeeping RNA surveillance machinery that degrades aberrant RNA transcripts is well accepted, and the molecular mechanisms of substrate recognition and degradation have been extensively studied (reviewed in Kervestin and Jacobson, 2012; Schweingruber et al., 2013). However, the biological impact of NMD has remained elusive, mostly because of the pleiotropic phenotypes associated with NMD impairment. Recently, the idea, that NMD might also have an active role in the regulation of physiologically important transcripts, has emerged, but only few studies so far provided evidence that would support such a role for NMD (Huang and Wilkinson, 2012; Karam et al., 2013). In my studies, I investigated the biological significance of plant NMD and its putative role in regulating plant innate immunity. My data reveal a role of NMD in regulating the transcript levels of numerous TNLs, which represent a family of important plant immune receptors. Severe fitness costs are associated with inappropriate activation of TNLs in NMD-deficient background, underscoring the importance of NMD in controlling this class of transcripts. Using a combination of genetic and molecular approaches, we demonstrate that a single deregulated TNL transcript is sufficient to elicit autoimmunity in NMD-impaired plants. Finally, I show that NMD is suppressed upon pathogen infection, which positively contributes to plant disease resistance.

5.1 NMD mutants suffer from constitutive activation of defense responses

Several lines of evidence suggested that mutants, that were impaired in the core NMD components UPF1, UPF3, or SMG7, suffered from auto-activation of pathogen defense responses (Riehs-Kearnan et al., 2012). First, phenotypic aberrations of the hypomorphic *smg7-1*, *upf1-5*, and *upf3-1* mutants were reminiscent of previously described mutants with constitutively active immune responses (Lorrain et al., 2003; Moeder and Yoshioka, 2008). Secondly, we found that molecular markers of defense responses against biotrophic pathogens, such as SA and the SA-responsive transcript PR1, were upregulated in NMD mutants. Interestingly, increased SA and PR1 levels quantitatively correlated with the severity of phenotypic aberrations and the degree of NMD deficiency observed in the NMD mutants that we examined.

To quantify the degree of NMD deficiency in plant NMD mutants previous studies mostly relied on semi-quantitative RT-PCR detection of endogenous NMD targets, or on direct

sequencing of PCR products (Arciga-Reyes et al., 2006; Hori and Watanabe, 2005; Riehs et al., 2008; Yoine et al., 2006a,b). To improve the detection of quantitative differences in NMD efficiency, I developed a set of qPCR-based NMD assays, robustness of which I validated under multiple growth conditions and treatments. Using these qPCR assays, I was able to determine even small differences in NMD efficiency among several hypomorphic *smg7*, *upf1*, and *upf3* alleles, and found that NMD deficiency was most pronounced in *smg7-1* mutants while *upf1-5* and *upf3-1* alleles caused considerably less accumulation of aberrant RNAs (Riehs-Kearnan et al. 2012 Gloggnitzer & Riha, unpublished). Therefore, I suggest that qPCR-based quantification of NMD substrates is a suitable method to measure NMD efficiency, and that it should be consistently adopted in the future.

Generally, elevated levels of SA and PR1 correlate with increased resistance against virulent pathogens (Boatwright and Pajerowska-Mukhtar, 2013; Vlot et al., 2009). Thus, I hypothesized that increased SA and PR1 levels in *upf1-5*, *upf3-1* and *smg7-1* mutants should lead to increased bacterial against virulent *P. syringae* pv. *maculicoa* ES4326. In agreement with this idea, I found that all tested NMD mutants showed higher bacterial resistance than wild-type plants, again correlating with the severity of NMD impairment (Riehs-Kearnan et al. 2012; Gloggnitzer & Riha, unpublished).

While our first publication was under peer-review (Riehs-Kearnan et al., 2012), three studies were published, that also examined the underlying causes for the phenotypic aberrations of mild *upf1* and *upf3* mutants but did not study *smg7* mutants (Jeong et al., 2011; Rayson et al., 2012a; Shi et al., 2012). In agreement with our results, Jeong and colleagues (2011) demonstrated increased bacterial resistance in *upf1-5* and *upf3-1* mutants and detected elevated SA accumulation. The authors also examined the contribution of SA to autoimmunity in NMD-impaired plants and found that elevated SA levels correlated with increased *SID2/ICS1* transcript levels. Additionally, the authors also detected increased transcript levels of the immune regulators *NPR1*, *EDS1*, *PAD4* and multiple *WRKY* genes. The authors argued that upregulation of these transcripts led to the accumulation of SA in NMD-impaired mutants. While it is clear that these immune regulators are involved in SA-mediated defense response, it has also been reported that SA stimulates the expression of *NPR1*, *EDS1* and *PAD4* in a feed-forward manner (Falk et al., 1999; Jirage et al., 1999). Thus, SA might only reinforce disease symptoms in NMD mutants, and my genetic studies suggest that SA biosynthesis in NMD mutants plays only a minor role in conditioning autoimmunity (see section 5.2). Shi and co-workers (2012) also examined growth defects of mutants impaired in *UPF1* and *UPF3*, and additionally generated *UPF2* RNAi lines, an NMD component that had previously not been studied *A. thaliana*. Through analysis of endogenous NMD reporters, Shi et al. (2012) confirmed a role for UPF2 in NMD. In agreement, *upf1-5*, *up3-1* and *UPF2* RNAi lines displayed abnormal growth phenotypes, but the authors failed to detect differences in bacterial resistance in NMD mutants upon *Pst* DC3000 infection. Interestingly,

the authors also identified a photoperiod-dependent differential accumulation of jasmonic acid upon wounding. Based on microarray analysis of NMD mutants, Rayson et al. (2012a) also proposed a role for NMD components in regulating plant pathogen responses. Similar to our work, the three parallel independent studies published by Shi et al. (2012), Jeong et al. (2011), and Rayson et al. (2012a) suggested that NMD might play a role in regulating pathogen responses in *A. thaliana*.

5.2 Autoimmunity in NMD mutants is caused by TIR-NB-LRR-conditioned disease resistance signaling

For plants, mounting of immune responses is accompanied by high fitness costs, and requires fine-tuning to maintain a balance between defense against attacking pathogens and sustained growth. Consequently, integration of environmental cues such as temperature plays a critical role for plants in modulating their disease resistance pathways (reviewed in Alcázar and Parker, 2011). It was previously described in *A. thaliana* that stunting caused by inappropriate activation of defense responses is highly conditioned by temperature settings and relative air humidity. This was observed for incompatible hybrids of different natural variants of *A. thaliana* (Alcázar et al., 2009; Bomblies et al., 2007), but also for auto-activated mutants of TNL proteins, such as the *snc1* mutant and the *RPP4* mutant *chs2* (Huang et al., 2010; Yang and Hua, 2004; Zhang et al., 2003). To test if temperature settings also influenced stunting of *smg7* mutants, I analyzed growth performance and immune responses at different temperatures. I could show that elevated ambient temperature suppressed stunting and other autoimmunity symptoms in *smg7* mutants. However, higher growth temperature did not suppress NMD deficiency in SMG7-deficient plants. Previous studies indicated that the suppression of autoimmunity at elevated temperature might be associated with reduced stability or altered assembly of NB-LRR proteins (reviewed in Alcázar and Parker, 2011). Therefore, I suggested that SMG7 was involved in the regulation of an NB-LRR-conditioned disease resistance pathway.

To test if NMD impairment led to aberrant NB-LRR signaling, Nina Riehs-Kearnan and I investigated the genetic requirements for *smg7*-associated autoimmunity. Our genetic analyses revealed that disease resistance activation in SMG7-depleted plants required PAD4 (Riehs-Kearnan et al., 2012) and EDS1, which both are central regulators of basal resistance and ETI mediated by NB-LRRs. In addition, I demonstrated that mutations in RAR1 and SGT1B, which HSP90 co-chaperones that are required for stability of NB-LRR proteins, were sufficient to largely suppress autoimmunity symptoms in *smg7* mutant plants. In contrast, a mutation in *NDR1*, a signaling requirement for CNLs, failed to suppress *smg7*-associated autoimmunity. These findings led me to the conclusion that inappropriate activation of TNL-mediated disease signaling, but not disease resistance pathways requiring CNL proteins, might be causative

for stunting and autoimmunity observed in SMG7-impaired plants. Similarly, disruption of *PAD4* rescued seedling lethality of the *upf1-3* mutation. qPCR quantification of NMD targets and RNA-seq analysis revealed massive transcriptome deregulation, indicating that *upf1-3 pad4* double mutants might represent the strongest NMD mutant in *Arabidopsis*, and the first viable UPF1 null mutant in higher eukaryotes (Riehs-Kearnan et al., 2012).

A recent report genetically examined the role of SA in conditioning autoimmunity in the hypomorphic NMD mutant *upf1-5* (Rayson et al., 2012b). The authors suggested that abolishment of SA biosynthesis in *upf1-5* plants by introduction of the *sid2-1* mutation led to wild-type appearance of *upf1-5 sid2-1* double mutants. Therefore, the authors concluded that common phenotypes observed in NMD mutants were SA-dependent (Rayson et al., 2012b). In contrast to this report, I found that *smg7-1 sid2-1* double mutants did not revert to wild-type appearance, and only showed mild improvements in leaf morphology. In addition, crosses between the seedling lethal *UPF1* null allele *upf1-3* and *sid2-1* did not give rise to any viable double mutant progeny in the segregating F2 population (χ^2=4.26 at p=0.05). Therefore, I suggest that SA has only a minor role in contributing to phenotypic aberrations observed in hypomorphic NMD mutants and to lethality of the *upf1-3* null mutant. However, my findings do not exclude a role of SA-dependent feed-forward amplification of constitutive defense signaling in NMD mutants.

5.3 TIR-NB-LRR transcripts are *bona fide* NMD targets

Previously, transcriptional profiling analyses of NMD mutants indicated that NMD controls a significant portion of the *Arabidopsis* transcriptome (Drechsel et al., 2013; Kurihara et al., 2009). In addition to degrading RNAs bearing classical NMD features such as PTCs, NMD is also involved in the regulation of mRNA like non-coding RNAs and physiologically important mRNAs (Drechsel et al., 2013; Kalyna et al., 2012; Kurihara et al., 2009; Marquez et al., 2012). Additionally, microarray analysis identified an enrichment of transcripts that are associated with SA-dependent pathogen responses (Rayson et al., 2012a). Considering that the expression of numerous pathogen responsive genes is reinforced by positive amplification loops involving SA (Fu and Dong, 2013), it was unclear if upregulated defense-associated transcripts in NMD mutants were genuine NMD substrates or represented secondary effects caused by pathogen response signaling. The phenotypically normal *smg7 pad4* double mutant provided an unique opportunity to study the impact of a strong NMD mutation on the steady state transcriptome in the absence of ongoing defense.

In collaboration with Arunkumar Srinivasan (Max Planck Institute for Plant Breeding Research, Cologne), I analyzed differential gene expression in the *smg7 pad4* double mutant by RNA-seq. Our analyses revealed that NMD impairment led to significant upregulation of 15% of all expressed genes. Based on gene ontology (GO) term classification of upregulated genes,

we propose, that NMD has a widespread role in regulating numerous biological processes. In contrast, we did not find enrichment for GO terms associated with abiotic or biotic stresses and NMD regulated transcripts were proportionally distributed across all GO-term categories. Our results are in good agreement with studies published by Kurihara et al. (2009) and Drechsel et al. (2013). Drechsel and co-workers performed transcriptome analysis in the *upf1-1 upf3-1* double mutant and found that approximately 17% of all protein-coding genes in *A. thaliana* were targeted by NMD (Drechsel et al., 2013). Furthermore, similarly to the results of our GO term analysis, the authors suggested that NMD targets serve in diverse biological processes (Drechsel et al., 2013), which is in contrast to a previously published microarray study on *upf1-5*, *upf3-1*, and *smg7-1* mutants, which indicated over-representation of pathogen responsive genes (Rayson et al., 2012a). The different results might be explained by the fact that the study by Drechsel and co-workers only considered upregulated transcripts with putative NMD inducing features to be directly regulated by NMD (Drechsel et al., 2013). In contrast, Rayson and colleagues (2012a) defined the overlap of upregulated transcripts in *upf1-5*, *upf3-1* and *smg7-1* mutants as "common NMD target". These "common NMD target" are likely to include many transcripts that that do not present *bona fide* NMD targets, but rather have been induced by feed-forward signaling of autoactive disease resistance responses.

In addition to a widespread role of NMD in controlling endogenous gene expression, we found that TNL transcripts, including the TNL *RPS6*, were overrepresented among NMD regulated genes. These TNL transcripts were enriched for putative NMD features. Expression analysis of selected TNLs and determination of their transcript decay rates revealed that numerous TNL transcripts are directly turned over by NMD. My data implicate a role for NMD-mediated turnover in controlling TNL transcript levels, which might prevent the misexpression of TNL and the accompanying fitness costs.

In my studies, the determination of transcript decay rates turned out to be an invaluable tool for the validation of NMD targets. At least one TNL transcript had increased steady state levels in *smg7 pad4* mutants and upon treatment with the translational inhibitor cycloheximide, but did not show altered decay rates. Therefore, this TNL did not represent a *bona fide* NMD target. For the validation of candidate NMD targets, previous studies mostly relied on evaluation of steady-state transcript levels and transcript accumulation upon treatment with cycloheximide (Drechsel et al., 2013; Hori and Watanabe, 2005; Jeong et al., 2011; Kalyna et al., 2012; Kurihara et al., 2009), which might erroneously identify some transcripts as direct NMD targets. Therefore, I suggest that steady-state level determination and cycloheximide treatments might only be partially suitable for reliable candidate NMD target validation, and that future studies would strongly benefit from complementing mRNA half-life measurements.

Alternative splicing coupled to NMD, was previously suggested to be involved in the regulation of the TNL *RPS6* (Kim et al., 2009c). All previously characterized alternative *RPS6*

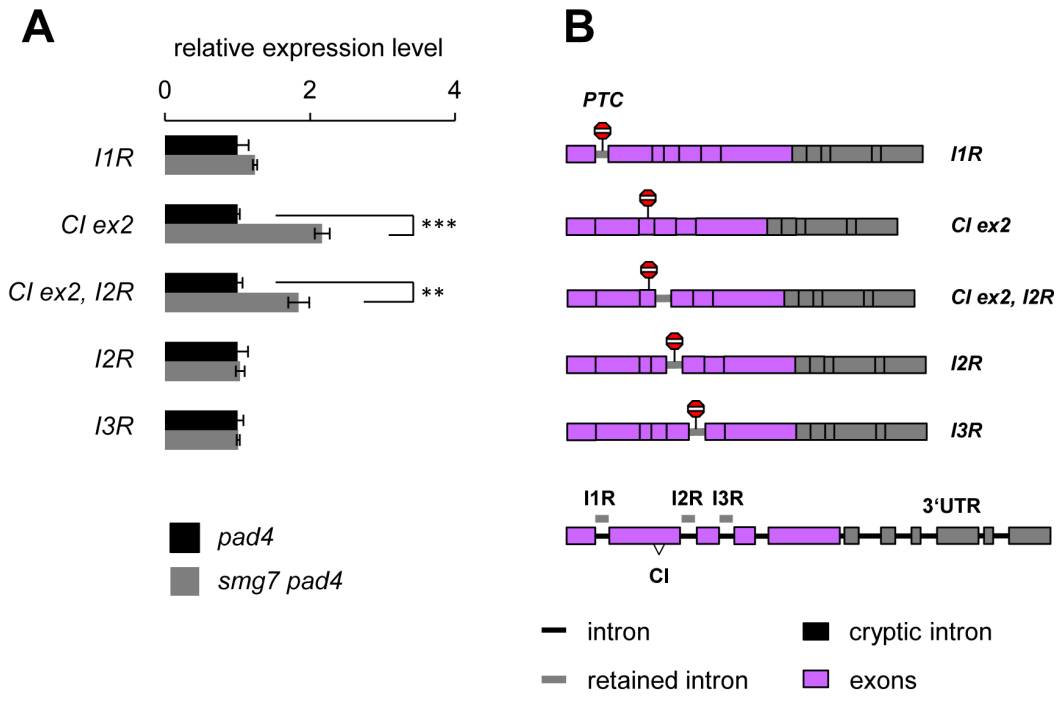

Figure 5.1: Alternative splice variants of *RPS6* are regulated by NMD. (A) qPCR-based quantification of *RPS6* splice variants in *pad4* plants (control) and *smg7 pad4* mutants. Asterisks (∗) indicate statistically significant differences determined by Student's t-test (two-tailed, $**p<0.01$, $***p<0.001$). Values are averages of three biological replicates and are normalized to the expression of At2g28390 (SAND gene). Error bars denote standard error of mean (SEM). (B) Depiction of *RPS6* splice variants quantified in (A). I1R=intron 1 retention, I2R=intron 2 retention, I3R=intron 3 retention, CI ex2=excission of a cryptic intron located in exon 2.

splice variants lead to the generation of a PTC that could potentially be recognized by NMD and/or could be translated into truncated proteins (see Figure 3.4C and Figure 5.1B). However, recent studies suggested that PTCs located within retained introns generally do not elicit NMD (Kalyna et al., 2012; Marquez et al., 2012). Our RNA-seq analysis confirmed that the *RPS6* transcript was upregulated in the NMD-impaired *smg7 pad4* double mutant. Interestingly, using splice variant specific qPCR, I found that only those *RPS6* alternative splicing events leading to the excision of a cryptic intron were accumulating in the NMD-deficient *smg7 pad4* double mutant (Figure 5.1A). In contrast, transcripts with alternative splicing events leading to the retention of intron 1, 2, or 3 were not affected by NMD impairment. Thus, the overall upregulation of *RPS6* in NMD-deficient plants is due to the derepression of two alternative splice variants that lack a cryptic intron. Additionally, these results support the idea that PTC-containing transcripts that were generated by intron retention events might not be targeted for NMD.

While there is increasing evidence that alternative splicing of R-genes is an important

aspect of plant disease resistance, the molecular functions of the potentially resulting truncated proteins is less clear. Truncated R-proteins that only encode for TIR or TIR-NB domains have been shown to efficiently induce HR in transient expression experiments, suggesting that they are sufficient to elicit defense response, and might represent the actual active signal of the full-length protein (reviewed in Gassmann, 2008). Truncated R-proteins might directly trigger downstream signals to activate defense responses or serve as adaptor proteins. Alternatively, truncated proteins could interact with the full-length R-protein to alleviate self-inhibition (Zhang and Gassmann, 2003). Considering the detrimental impact of defense responses on fitness, it is conceivable that the production of truncated isoforms must be tightly regulated and fine-tuned. In this respect, NMD might represent a post-transcriptional regulatory layer that controls the abundance of truncated NB-LRR proteins to balance growth and disease resistance responses.

5.4 The TIR-NB-LRR *RPS6* is sufficient to elicit *smg7*-dependent autoimmunity

Genetic crosses of the *smg7-1* mutation, which is in the Col-0 background, with the Landsberg *erecta* (L*er*) ecotype resulted in suppression of autoimmunity in a portion of *smg7* mutants in the F2 population derived from this cross. This indicated the existence of natural polymorphisms that suppressed autoactive disease resistance signaling in *smg7* mutants. In an collaborative effort using a combination of map-based cloning, RNA-sequencing, and complementation experiments, Nina Riehs-Kearnan, Svetlana Akimcheva, and I identified a genetic situation in which the naturally occurring L*er* allele of the TNL *RPS6* ($RPS6^{\mathrm{L}er}$) was sufficient to elicit autoimmunity in *smg7* plants that mostly contained L*er* genome. In contrast, the presence of the Col-0 allele of *RPS6* ($RPS6^{\text{Col-0}}$) did not lead to constitutive pathogen response in the same genetic background. Furthermore, both $RPS6^{\mathrm{L}er}$ and $RPS6^{\text{Col-0}}$ transcripts appeared to be equally processed by NMD, suggesting that autoimmunity in NMD-deficient background was not due to differential RNA decay of the *RPS6* transcript, but was rather conditioned by allelic variation. A previous study surveyed thirty-two *Arabidopsis* accessions for disease resistance activation upon treatment with the *P. syringae* effector HopA1 (Gassmann, 2005). The author found natural variation in HR in response to the HopA1 effector, which was suggested to be conditioned by allelic variation in *RPS6*. Importantly, Walter Gassmann found that Col-0 plants challenged with the HopA1 effector did not display any HR, while L*er* plants induced potent HR (Gassmann, 2005). Thus, I suggest that both $RPS6^{\mathrm{L}er}$ and $RPS6^{\text{Col-0}}$ are genuine NMD targets, and that NMD deficiency leads to ectopic expression of both variants. However, only overexpression of the $RPS6^{\mathrm{L}er}$ allele may elicit runaway autoimmunity and stunting in NMD-deficient background, while this is not the case for $RPS6^{\text{Col-0}}$ allele.

Our results from map-based cloning of *smg7* (Col) × L*er* recombinant populations hint to two more genetic loci that can individually suppress *smg7*-associated autoimmunity. Similar to *RPS6*, the additional suppressors also map to regions that are enriched for R-genes. Therefore, we suggest that autoimmunity in *smg7* mutants can be conditioned by natural variation at multiple genetic loci that presumably represent NB-LRR clusters. Certain allelic combinations at these loci might lead to autoimmunity in *smg7* mutants, while other allelic combinations might not be associated with adverse effects on plant fitness. Allelic variations in components of the plant immune system were previously described to condition variation in immune responses, and to drive incompatibility between hybrids of naturally occurring *A. thaliana* ecotypes (Alcázar et al., 2010, 2009; Alcázar and Parker, 2011; Gassmann, 2005; Kim et al., 2009b). Therefore, autoimmunity elicited by NMD deficiency might be conditioned by the complement of TNL variants present in the respective genomic background. Considering the variation in the R-gene repertoire among natural accessions of *A. thaliana*, I speculate that in certain naturally occurring genetic backgrounds, NMD deficiency might not lead to autoimmunity due to the lack of an NMD-regulated NB-LRR, deregulation of which would lead to inappropriate disease resistance activation.

5.5 NMD is suppresed upon pathogen infection

Inappropriate activation of immune responses appears to be a common phenotype in NMD mutants and is accompanied by considerable fitness costs (Jeong et al., 2011; Rayson et al., 2012a; Riehs-Kearnan et al., 2012; Shi et al., 2012). We and others have found that NMD impairment also resulted in a substantial increase in resistance to virulent bacterial pathogens (Jeong et al., 2011; Rayson et al., 2012a; Riehs-Kearnan et al., 2012). This led me to speculate that in wild-type plants, modulation of NMD efficiency might be part of a physiological response to pathogen infections. Regulated dampening of NMD during infection could contribute to plant defense by increasing the levels of defense-related transcripts such as TNLs. Thus, I expected that upon inoculation with virulent *Pst* DC3000, endogenous NMD target transcripts would accumulate. Indeed, I found that inoculation of wild-type plants with *Pst* DC3000 led to upregulation of PTC-containing splice variants of endogenous NMD reporters and to the upregulation of mRNAs encoding the NMD core factor SMG7 and the EJC component BARENTSZ1. Additionally, multiple NMD-targeted TNLs were upregulated upon infection. Determination of decay rates excluded the possibility that transcriptional activation was the cause for increased transcript levels of NMD targets, *SMG7*, and *BARENTSZ*. These results indicated that NMD efficiency was indeed downregulated in response to pathogen infection. Therefore, I suggested that suppression of NMD might be part of a physiological response to pathogen infection. Independent parallel studies from Jeong et al. (2011) and Howard et al. (2013) also identified increased levels of putative NMD targets upon infection

with virulent *P. syringae* strains. However, these studies did not examine changes in turn-over of putative NMD transcripts upon pathogen infection, which is essential for distinguishing between increased transcriptional activation or NMD suppression.

Previously, NMD was shown to be autoregulated in animals and plants (Huang et al., 2011; Nyikó et al., 2013; Rayson et al., 2012a; Yepiskoposyan et al., 2011). The *A. thaliana SMG7* and *BARENTSZ1* mRNAs both harbor NMD-eliciting features, and their transcript levels are negatively regulated through NMD. Accordingly, Nyikó et al. (2013) found elevated levels of the *SMG7* and *BARENTSZ1* mRNAs in mutants depleted for other NMD factors, indicating that a negative feedback loop regulates the levels of *SMG7* and *BARENTSZ1* to ensure NMD homeostasis. Negative feedback loops might be an important aspect in regulating the activity of NMD during pathogen infection. Considering the detrimental effects of constitutive NMD impairment, I speculate that NMD downregulation upon pathogen infection should only occur transiently, allowing the increase of NMD-regulated defense-related transcripts (e.g. TNLs) for a brief period of time to benefit from increased immune responses. Upon the decline of the infection negative regulatory feedback loops could then ensure return to normal NMD activity and restore transcriptome homeostasis.

While my *in vivo* studies showed that pathogen inoculation led to downregulation of NMD which causes increases in the steady-state transcript levels of selected NMD targets, among them TNLs, the extent of NMD regulation upon pathogen stress is currently unclear. To identify the transcriptome portion that is affected by NMD downregulation upon pathogen challenge, it would be highly interesting to examine transcriptome-wide RNA decay rates with and without pathogen treatment. A combination of RNA-seq and published algorithms for transcriptome-wide computation of turn-over rates (Thilmony et al., 2006) could identify transcripts with a significant difference in half-life between untreated and pathogen-challenged plants which would indicate regulation by NMD.

NMD downregulation upon pathogen infection increases the levels of NMD targets, but it remains an open question if increased NMD target transcript levels also lead to increased protein levels. Future investigations should focus on the expression of N-terminally tagged versions of NMD targets followed by pathogen infection. This would then allow the detection of quantitative differences in protein levels of NMD targets in unchallenged and pathogen-challenged conditions. Both transient expression in tobacco leaves and the use of stable transgenic *A. thaliana* lines would be suitable to address this question. Alternatively, quantitative proteomics approaches such as SILAC (Lewandowska et al., 2013) applied on uninfected and infected sample material could detect quantitative differences in the abundance of candidate NMD target proteins that have previously been identified in our RNA-seq analysis of *smg7 pad4* mutants.

5.6 Downregulation of NMD contributes to basal resistance

My observation that NMD efficiency was suppressed upon infection with virulent *Pst* DC3000 prompted me to investigate if suppression of NMD contributed to disease resistance.

Using plants that were deficient for endogenous SMG7, but expressed an actin promoter driven *SMG7* cDNA construct that lacked the natural 3'UTR, I was able to demonstrate that *SMG7* overexpressor (OE) plants expressed *SMG7* at higher levels than wild-type plants. Previously, overexpression of UPF1 in mammalian cells was shown to be sufficient to hyper-activate NMD and to increase the degradation of NMD targets (Wengrod et al., 2013). Similarly, I found that *SMG7* cDNA OE plants had significantly reduced levels of multiple endogenous NMD substrates.

I hypothesized that the lack of the long 3'UTR in *SMG7* cDNA OE plants should uncouple the transgenic *SMG7* mRNA from NMD autoregulation, allowing the overexpression of *SMG7*, which should lead to constitutive NMD activity. Thus, I expected that in *SMG7* OE plants, NMD would be constitutively active upon pathogen infection, while NMD would be suppressed in infected wild-type plants. In agreement with this hypothesis, *Pst* DC3000 infection of *SMG7* OE plants failed to downregulate NMD, resulting in unaltered levels of NMD-targeted TNL transcripts and NMD reporters. This was accompanied by significantly increased bacterial susceptibility of *SMG7* OE plants. Therefore, I suggest that NMD downregulation contributes to basal resistance against virulent pathogens, presumably by increasing the steady state level of multiple TNLs.

Interestingly, NMD downregulation does not appear to require recognition of *P. syringae* effectors by host R-proteins, since the virulent *Pst* DC3000 strain was sufficient to suppress NMD. However, Howard and co-workers (2013) found that avirulent *Pst* DC3000 AvrRps4 strains would lead to even stronger NMD inhibition when compared to plants infected with virulent *Pst* DC3000, suggesting that ETI might reinforce the inhibition of NMD. Based on preliminary data, I suggest that even the recognition of PAMPs such as flg22 is sufficient to transiently inhibit NMD (Figure 5.2). This should be further tested by inoculation of wild-type plants with the type III secretion apparatus mutant *Pst* DC3000 *hrcC*, which is unable to deliver effector proteins and is therefore only recognized by the host through PAMP perception.

While the role of R-genes in ETI is well documented, little is known about the contribution of R-genes to basal resistance. Ample evidence exists that NB-LRR proteins can also contribute to basal resistance against virulent pathogens in the absence of recognizable ETI. The CNL proteins of the ADR1 family have been shown to confer enhanced basal defense against virulent *Hyaloperonospora arabidopsidis* Emco 5 and *Pst* DC3000 strains (Bonardi et al., 2011; Roberts et al., 2013). Additionally, ectopic expression of the TNL proteins RPS4 and SNC1 was previously shown to elicit autoimmunity in the absence of cognate effector proteins (Stokes et al., 2002; Wirthmueller et al., 2007; Yi and Richards, 2009). Furthermore, Zhang

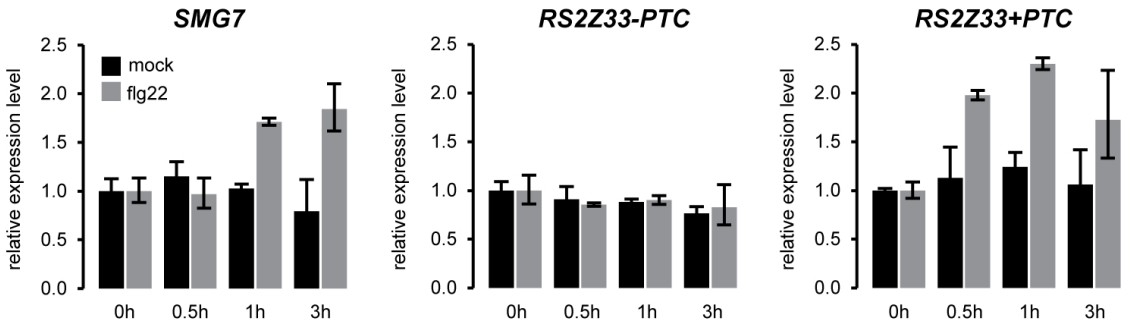

Figure 5.2: NMD targets are upregulated upon flg22 treatment. qPCR-based quantification of the NMD targets *SMG7* and the endogenous NMD reporter *RS2Z33*. *RS2Z33*+PTC is targeted for NMD, while the PTC-free splice variant (-PTC) is NMD-insensitive. Twelve day old seedlings were treated with 1μM flg22 for the indicated time. Values are averages of three biological replicates and are normalized to the expression of At2g28390 (SAND gene). Error bars denote standard error of mean (SEM). The experiment was repeated once with similar results.

et al. (2010) suggested that R-gene mediated resistance might contribute to basal resistance, since mutations in RAR1 and NDR1 compromised basal resistance against virulent pathogens. Therefore, I suggest a model in which NMD suppresses the expression of multiple TNL in the absence of pathogens to prevent the adverse effects that are associated with inappropriate TNL activation (Figure 5.3). In contrast, transient NMD suppression upon infection with virulent pathogens increases TNL levels, which might sufficiently contribute to basal resistance. NMD downregulation could be coupled with alternative splicing of R-genes, which would allow the production of truncated TNL proteins, which have been implicated to have an important role in disease resistance (Gassmann, 2008).

Considering the severe fitness costs associated with inappropriate activation of NB-LRRs, NB-LRR expression seems to be tightly regulated on multiple levels, including transcriptional activation, silencing through RNA-directed DNA methylation (Yu et al., 2013), post-transcriptional silencing by small RNAs (Boccara et al., 2014; Shivaprasad et al., 2012; Zhai et al., 2011), and alternative splicing (Gassmann, 2008; Staiger and Brown, 2013). Previous studied demonstrated that PTGS can be inhibited by both viral and bacterial suppressors and by PAMP perception, which consequently leads to the derepression of PTGS-regulated NB-LRRs (Boccara et al., 2014; Shivaprasad et al., 2012). Inhibition of PTGS was suggested to contribute to basal immunity as well as to race-specific resistance (reviewed in Pumplin and Voinnet, 2013). Interestingly, while PTGS appears to primarily regulate the expression of CNLs, my studies suggest that in *Arabidopsis* NMD preferentially targets TNLs. This preference might be due the fact that most CNLs do not undergo splicing, and that they are deprived of NMD-eliciting features. Thus, NMD and PTGS seem to target different

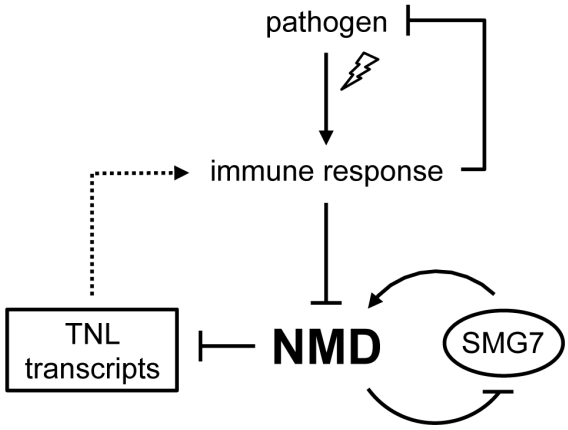

Figure 5.3: Model of regulation of plant innate immunity through NMD. In unchallenged conditions, NMD constitutively degrades numerous TNL transcripts. Suppression of NMD upon pathogen challenge leads to stabilization of TNL transcripts, which might contribute to disease resistance. NMD is regulated by an autoregulatory loop involving the NMD factor SMG7.

repertoires of NB-LRRs and might act complementary in plant defense. Altogether, these findings underscore the importance and complexity of RNA-based mechanisms in plant defense, and add NMD as an important regulatory layer that orchestrates the expression of numerous NB-LRRs, and thereby contributes to plant innate immunity.

5.7 The eIF2-alpha kinase GCN2 is dispensable for regulation of NMD

My finding that NMD efficiency declined during pathogen infection raised questions about the cues and mechanistic events that lead to NMD suppression upon infection. One of the first examples of modulation of NMD activity in response to stresses was the finding that amino acid starvation in mammalian cells led to translation-dependent inhibition of NMD (Mendell et al., 2004). Similarly, other stresses such as hypoxic environments were shown to dampen NMD (Gardner, 2008, 2010; Wengrod et al., 2013). These stresses were found to activate kinases, such as GCN2, that phosphorylate the eukaryotic initiation factor 2-alpha (eIF2α), which led to subsequent dampening of translation and NMD inhibition.

A. thaliana encodes for only one eIF2α kinase, which is homologous to yeast and mammalian GCN2 (Lageix et al., 2008). Previously, Lageix and co-workers (2008) reported that exogenous application of the plant defense hormones SA, methyl jasmonate, or ethylene, and the induction of amino acid starvation led to GCN2-mediated phosphorylation of eIF2α, followed by global translation inhibition. Based on this finding, I hypothesized that suppression of NMD upon pathogen infection might be regulated by GCN2. To test this hypothesis, I first examined

if the *Arabidopsis* homolog of GCN2 was required for the regulation of NMD upon amino acid depletion. To this end, I treated seedlings of two different mutant lines harboring *gcn2* mutations with chlorsulfuron, a drug that inhibits the biosynthesis of branched-chain amino acids(Zhang et al., 2008). Previously, chlorsulfuron treatment was described to activate GCN2, resulting in GCN2-dependent phosphorylation of eIF2α (Lageix et al., 2008; Zhang et al., 2008). In agreement, I was able observe GCN2-dependent phosphorylation of eIF2α upon chlorsulfuron treatment in both *gcn2* mutant lines.

Since amino acid starvation was previously shown to inhibit NMD in mammalian cells in a GCN2-dependent manner (Gardner, 2008, 2010; Mendell et al., 2004; Wang et al., 2011; Wengrod et al., 2013; Zhang et al., 2002), I investigated if GCN2 played a similar role in *A. thaliana*. Contrary to my expectations, expression analysis in wild-type seedlings and *gcn2* seedlings treated with chlorsulfuron revealed, that NMD targets were upregulated upon amino acid starvation independently of GCN2. Therefore, I concluded that like in mammals and yeast, chlorsulfuron-mediated amino acid starvation led to inhibition of NMD *A. thaliana*. However, in contrast to its mammalian and yeast counterparts, *Arabidopsis* GCN2 seems to be dispensable for NMD suppression upon amino acid depletion.

Interestingly, (Pajerowska-Mukhtar et al., 2012) demonstrated that *P. syringae* infection of adult plants induced eIF2α-phosphorylation, concomitant with the depletion of aromatic amino acids and reduced protein translation. This implicated a role of eIF2α-phosphorylation in regulating pathogen resistance. Upon pathogen infection, activation of GCN2 could be triggered by multiple cues such as altered levels of salicylic acid, jasmonic acid, ethylene or amino acid starvation, leading to eIF2α-phosphorylation. Subsequent translation inhibition might explain the observation that NMD is inhibited upon *Pst* DC3000 infection. However, I found that infection of wild-type plants and *gcn2-2* mutants with virulent *Pst* DC3000 led to the accumulation of NMD targets independently of GCN2. Plants depleted of GCN2 also did not show significantly altered resistance to *Pst* DC3000. Therefore, I suggest that the eIF2α kinase GCN2 is dispensable for basal resistance.

Taken together, I concluded that, in contrast mammals and yeast, GCN2 activity and subsequent eIF2α-phosphorylation are dispensable for the downregulation of NMD upon amino acid starvation or pathogen infection in *Arabidopsis*. My data indicate, that NMD is dynamically regulated in response to amino acid starvation and *Pst* DC3000, but that NMD suppression requires other cues than GCN2 activation and subsequent eIF2α-phosphorylation.

It is reasonable to speculate that in response to amino acid starvation or pathogen infection, plant NMD might either be regulated through (de-)phosphorylation of the core NMD factor UPF1, or through global translation inhibition mediated by other means than eIF2α-phosphorylation. While multiple phosphorylation sites have been identified in the *A. thaliana* UPF1 protein, the importance of these phosphorylation sites *in vivo* is only poorly understood (Kerényi et al., 2013; Mérai et al., 2013). Furthermore, *A. thaliana* lacks the

corresponding PIKK kinase SMG1, which is required for UPF1 phosphorylation in metazoans and other land plants (Lloyd and Davies, 2013). Examination of UPF1 phosphorylation sites upon pathogen challenge would provide insight into dynamic changes in UPF1 phosphorylation that might modulate the efficiency of NMD.

Translational control in response to environmental cues has been demonstrated to play an important role in plant development and in the adaptation to stresses (Deprost et al., 2007; Robaglia et al., 2004; Roy and von Arnim, 2013). In addition to GCN2, other signaling pathways have been implicated in translational control in metazoans and plants. The TOR-S6 kinase-signaling module is conserved in metazoans and plants and has been demonstrated to be a central regulator of cell growth by sensing energy balance and nutrient status (Deprost et al., 2007; Waller et al., 2012). Integration of stress signals leads to activation of TOR, followed by downstream phosphorylation of the S6 kinase, which in turn phosphorylates ribosomal components. While TOR signaling might be involved in the regulation of NMD in plants, the fact that TOR null mutants are embryonic lethal hampers further investigations (Deprost et al., 2007). However, *A. thaliana TOR* RNAi lines have been previously examined, but no change in eIF2α-phosphorylation was observed upon amino acid starvation (Lageix et al., 2008). In the future it would be interesting to examine the role of translational regulators such as TOR and the S6 kinase in modulating the efficiency of NMD in response to cellular and environmental cues.

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7 Abbreviations

ADP	adenosine diphosphate
AS	alternative splicing
ATP	adenosine triphosphate
Avr	avirulence factor
CC-NB-LRR	coiled-coil – nucleotide binding site – leucine-rich repeat
CC	coiled-coil
CDS	coding sequence
CNL	coiled-coil – nucleotide binding site – leucine-rich repeat
Col-0	Columbia-0
DNA	desoxyribonucleic acid
EJC	exon-junction complex
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
GO	gene ontology
HR	hypersensitive response
IR	intron retention
Ler	Landsberg *erecta*
LRR	leucine-rich repeat
miR	microRNA
mRNA	messenger ribonucleic acid
NB-ARC	nucleotide-binding adaptor shared by Apaf-1, plant resistance proteins, and CED-4
NB-LRR	nucleotide-binding adaptor shared by Apaf-1, plant resistance proteins, and CED-4 – leucine-rich repeat

ncRNA	non-coding RNA
NLR	NB-LRR
NMD	nonsense-mediated mRNA decay
NOD	nucleotide-binding oligmerization domain
OE	overexpressor
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain-reaction
PRR	pattern recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*
PTC	premature termination codon
PTGS	post-transcriptional gene silencing
PTI	PAMP-triggered immunity
qPCR	quantitative real-time polymerase chain reaction
R-protein/gene	resistance-protein/gene
RNA-seq	RNA-sequencing
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
TIR-NB-LRR	Toll/interleukin1 receptor – nucleotide binding site – leucine-rich repeat
TIR	Toll/interleukin1 receptor
TNL	Toll/interleukin1 receptor – nucleotide binding site – leucine-rich repeat
uORF	upstream open reading frame
UTR	untranslated region

8 Curriculum vitae

Personal Details

Date and place of birth	April 25th, 1985, Bangkok (Thailand)
Nationality	Austrian
Marital status	married
Language skills	German (mother tongue), English (fluent) Austrian Sign Language (advanced)

Education

University of Vienna & *Gregor Mendel Institute* Vienna, Austria October 09–April 14	*PhD student, International PhD Programme* *in Molecular Life Sciences* Thesis: RNA regulation of plant innate immunity Supervisor: Dr. Karel Riha
University of Vienna Vienna, Austria October 04–November 08	*Diploma studies in Molecular Biology* Graduation with distinction Thesis: Meiosis in *C. elegans* Supervisor: Prof. Dr. Verena Jantsch
Weizmann Institute of Science Rehovot, Israel June 06–September 06	*Karyn Kupcinet International Science School* Basic research on lipid metabolism in human cells Supervisor: Prof. Dr. Sima Lev
Weizmann Institute of Science Rehovot, Israel July 05	*Dr. Bessie F. Lawrence Summer Science Institute* Basic research on cellulose degradation Supervisor: Prof. Dr. Ed A. Bayer
Federal Higher Technical *Institute for Chemistry Industry* Vienna, Austria September 00–June 04	*A-levels and Vocational Education Training Diploma* *in Biochemistry, Biotechnology and Genetic Engineering* Graduation with distinction Graduation project: Development of biosensors Supervisor: Prof. Dr. Fritz Pittner, University of Vienna

Professional Experience

Vienna Open Lab Vienna, Austria October 06–June 10	*Tutor in Science Communication* Supervising and tutoring in a hands-on-laboratory
University of Vienna Vienna, Austria October 07–July 08	*Laboratory course instructor* for students of molecular biology
Phamarcon GmbH Vienna, Austria July 04	*Internship in pharcmacological synthesis* Synthesis, purification and quality testing of pharmaceutical compounds
Beiersdorf GmbH Vienna, Austria July 01 & August 02	*Internship in Quality Assurance and Marketing* QC of cosmetics, introduction into marketing activites

Oral and poster presentations

Keystone Symposium on Plant Innate Immunity Big Sky, MT, USA, 2013	*Poster* Deficiency in nonsense-mediated RNA decay triggers autoimmunity in *Arabidopsis thaliana*
Max Planck Institute for Plant Breeding Research Cologne, Germany, Feb 2013	*Invited oral presentation* Deficiency in nonsense-mediated RNA decay triggers autoimmunity in *Arabidopsis thaliana*
International Conference on *Arabidopsis* Research Vienna, Austria , 2012	*Oral presentation* (Plant RNA Workshop) Nonsense-mediated RNA decay deficiency triggers an autoimmune-like response in *Arabidopsis thaliana*
EMBO Meeting Vienna, Austria , 2011	*Poster* Nonsense-mediated RNA decay deficiency triggers an autoimmune-like response in *Arabidopsis thaliana*
Vienna Plant Network Meeting Vienna, Austria , 2011	*Oral presentation* Deficiency in nonsense-mediated RNA decay triggers immune responses in *Arabidopsis thaliana*
C. elegans Development and Evolution Meeting 2008 Madison, WI, USA	*Poster* Matefin/SUN-1 is required for homologous chromosome pairing in *C. elegans*

Awards

Austrian Federal Minister of Science and Research Austria, 2009	*Honorary Award of the Federal Minister of Science and Research*, annually presented to the fifty best graduates from all Austrian universities
University of Vienna 2006, 2009, 2010	*Merit scholarships*
Weizmann Institute of Science 2006	*Karyn Kupcinet International Science School* Scholarship
Weizmann Institute of Science 2005	*Dr. Bessie F. Lawrence Summer Science Institute* Scholarship

Publications

Regulation of plant innate immunity through nonsense-mediated RNA decay.
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MicroRNAs cross the line: the battle for mRNA stability enters the coding sequence. Nielsen AF, Gloggnitzer J, Martinez J. Molecular Cell. 2009 (mini-review)

Ars2 and the Cap-Binding Complex Team up for Silencing.
Nielsen AF, Gloggnitzer J, Martinez J. Cell. 2009 (mini-review)

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