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 $0.5 \times$ 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

(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
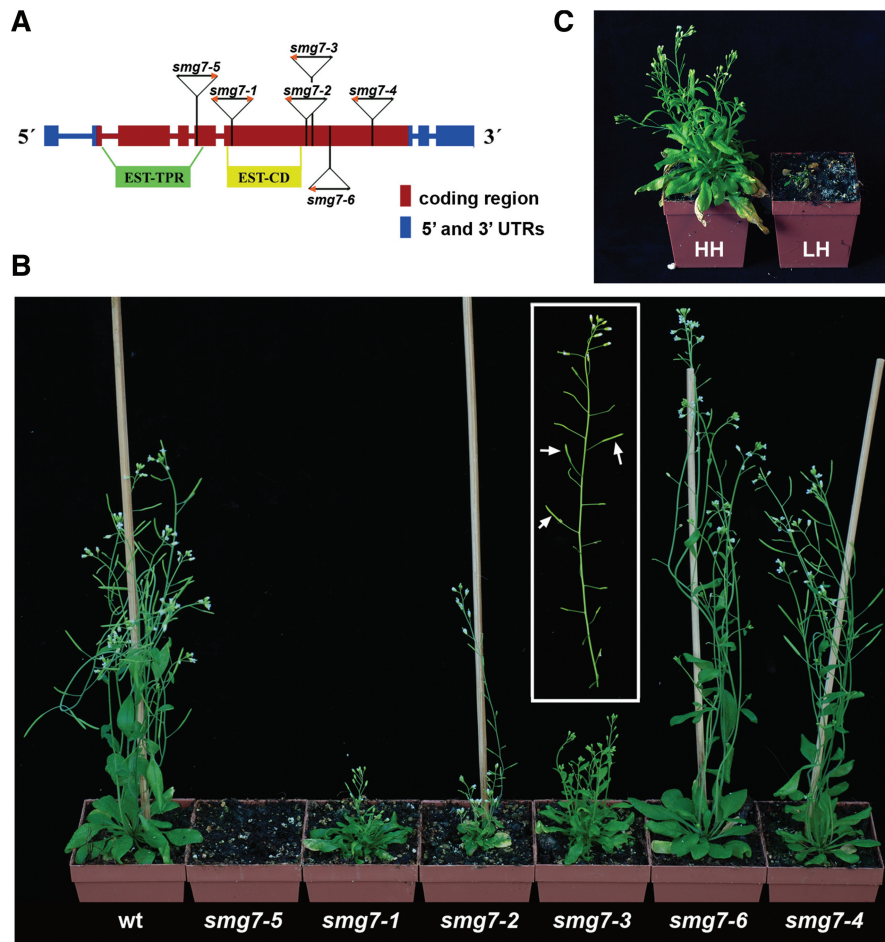
Figure 1. An allelic series of Arabidopsis *smg7* mutants. (**A**) Diagram of the *SMG7* gene that includes positions of T-DNA insertions with indicated orientation of T-DNA left borders (orange arrowhead). Boxes represent exons, regions coding for the conserved TPR (EST-TPR) and central (EST-CD) domains 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
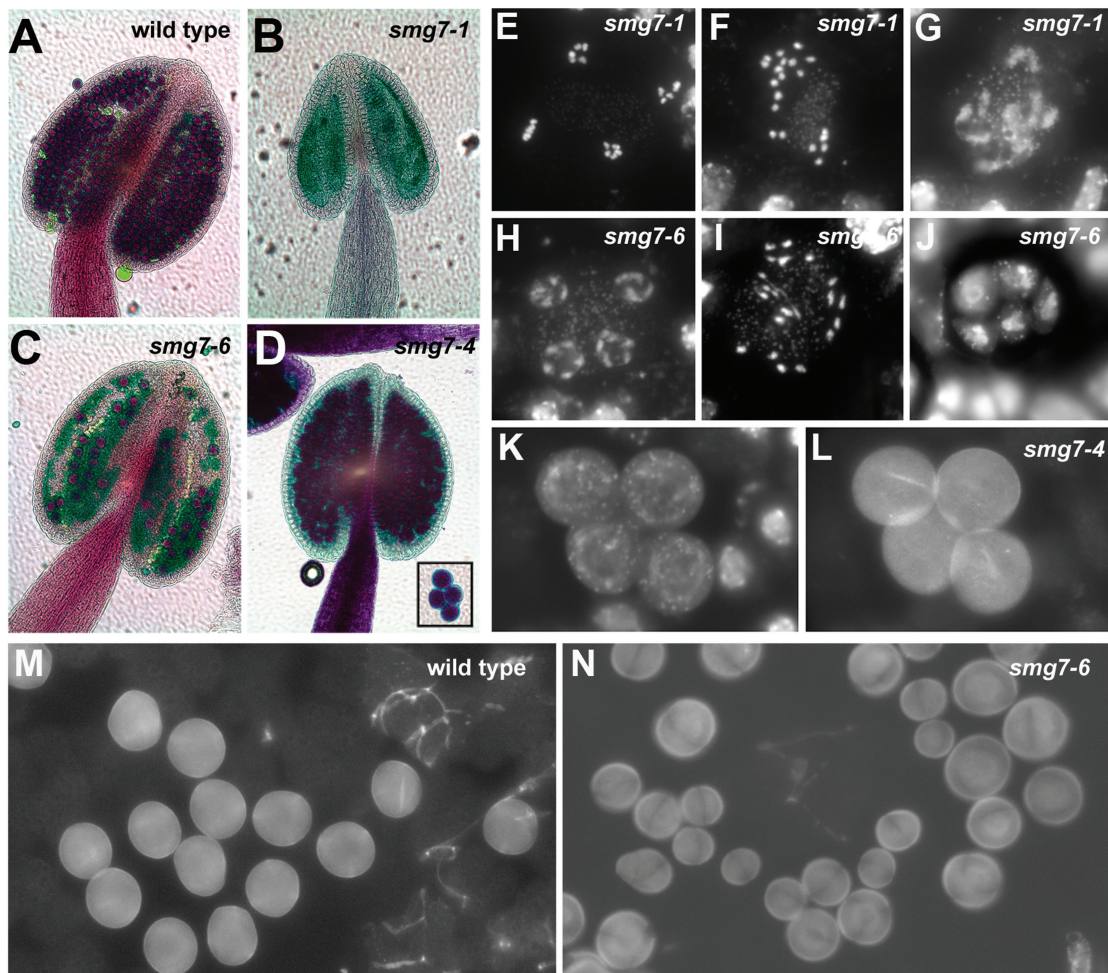
Figure 2. Development of male germ-line in *smg7-6* and *smg7-4* mutants. (A–D) Anthers of different *smg7* mutants after Alexander staining. Viable pollen stain red, no pollen is present in *smg7-1* anthers. Inset in (D) shows four joint pollen grains in *smg7-4* mutants. (E–J) End of second meiotic 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
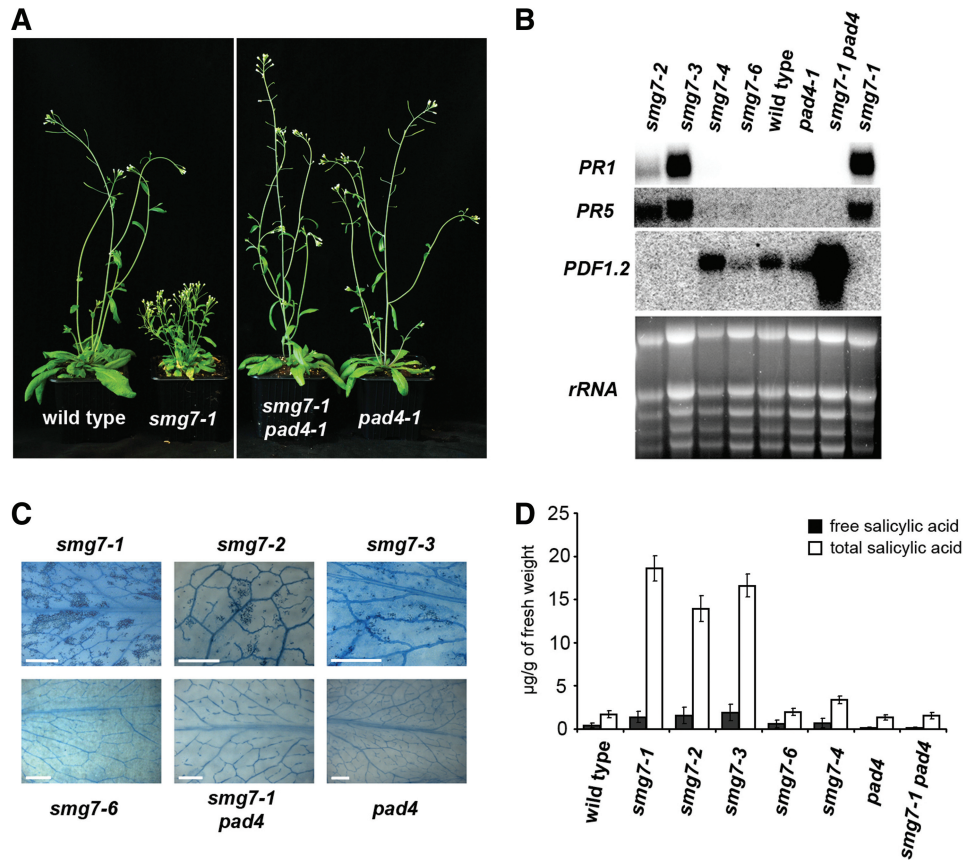
Figure 3. Aberrant growth of *smg7* mutants is associated with elevated pathogen response. (**A**) Six-week-old plants carrying different mutant combinations grown at 60–70% humidity. (**B**) Northern blot analysis of expression of pathogen related transcripts (*PR1* and *PR5*) and the defensin *PDF1.2* 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
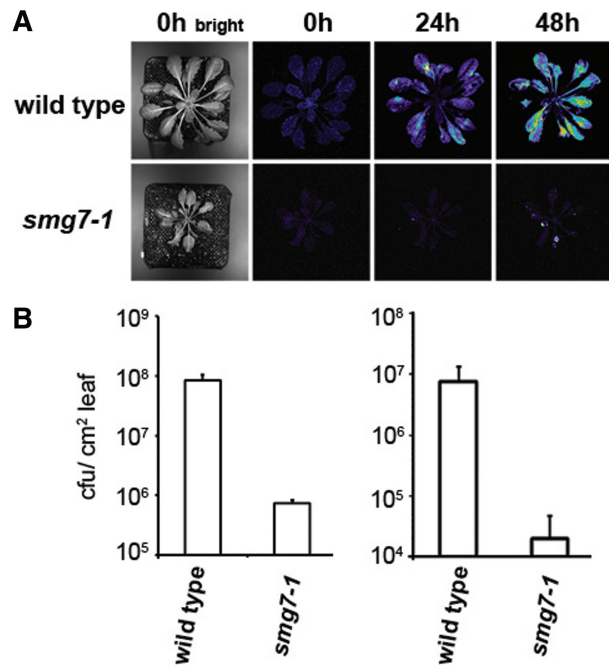
Figure 4. Resistance of *smg7-1* mutants to *P. syringae*. (**A**) Approximately six-week-old plants grown under short day conditions were infiltrated with *P. syringae* pv. maculicola ES4326 harbouring the LuxCDABE operon. Growth of bacteria in leaves was monitored at 24 and 48 h after inoculation by 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.

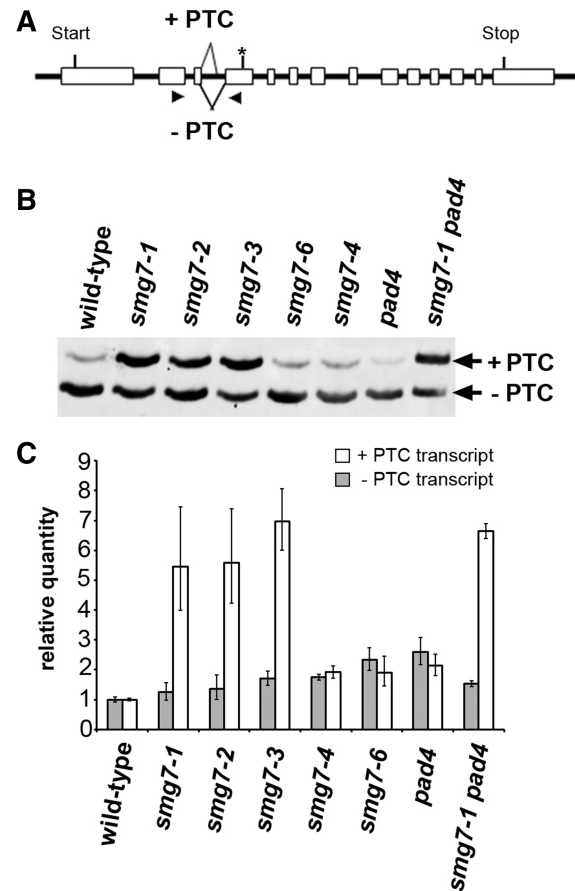In this study, we were able to pinpoint a specific pathway whose deregulation is responsible for the lethal consequences of NMD dysfunction in *Arabidopsis*. We show that aberrant NMD leads to constitutive auto-activation of the immune response. Importantly, disruption of the PAD4/EDS1 pathogen signaling 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 intra-cellular 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
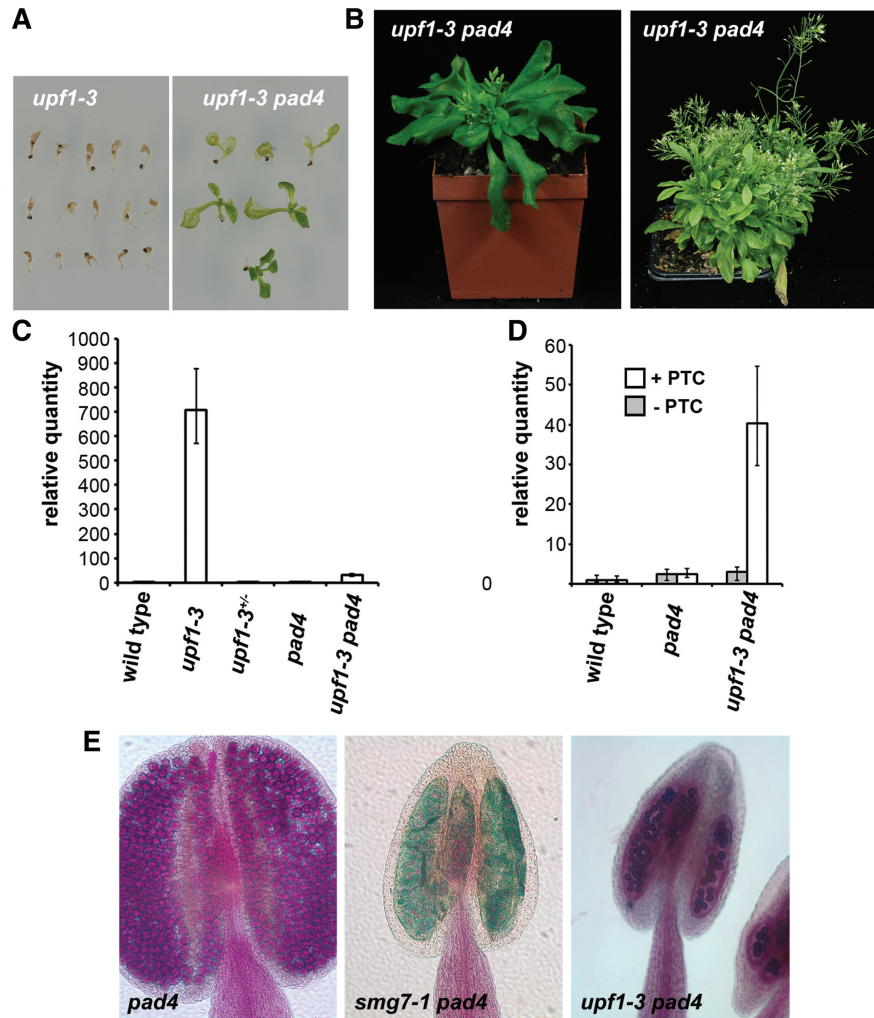
Figure 6. Pathogen response signaling causes lethality of *upf1-3* mutants. (**A**) Seedlings of *upf1-3* and *upf1-3 pad4* mutants grown for 10 days on agar plates. (**B**) Six- and 12-week-old *upf1-3 pad4* plants. (**C**) Levels of the PR1 transcript measured by qPCR in seedlings of *upf1-3* plants 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

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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