RESEARCH ARTICLE

Pathogen-associated Molecular Pattern-triggered Immunity Involves Proteolytic Degradation of Core Nonsense-mediated mRNA Decay Factors During the Early Defense Response

Ho Won Junga, Gagan Kumar Panigrahib,c,d, Ga-Young Junga, Yu Jeong Leea, Ki Hun Shinb,c, Annapurna Sahoo,h,c, Eun Su Choa, Eunji, Leea, Kyung Man Kimb,c,e, Seung Hwan Yangg, Jong-Seong Jeonf, Sung Chul Leea, and Sang Hyon Kimb,c,h

aDepartment of Applied Bioscience, Dong-A University, Busan 49315, Korea
bDepartment of Biosciences and Bioinformatics, Myongji University, Yongin 17058, Korea
cRNA Genomics Center, Myongji University, Yongin 17058, Korea
dSchool of Applied Sciences, Centurion University of Technology and Management, Odisha 752050, India
eDepartment of Biotechnology, Chonnam National University, Yeosu 59626, Korea
fGraduate School of Biotechnology and Crop Biotech Institute, Kyung Hee University, Yongin 17104, Korea
gSchool of Biological Sciences, Chung-Ang University, Seoul 06974, Korea
hCorresponding Author: kim6189@mju.ac.kr

Short title: Induction of UPF protein ubiquitination

One-sentence summary: Nonsense-mediated mRNA decay is a major regulatory process by which pattern recognition receptors fine-tune resistance gene transcript levels to reduce fitness costs and achieve effective immunity.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the instructions for authors (www.plantcell.org) is Sang Hyon Kim (kim6189@mju.ac.kr)

ABSTRACT

Nonsense-mediated mRNA decay (NMD), an mRNA quality control process, is thought to function in plant immunity. A subset of fully spliced (FS) transcripts of Arabidopsis thaliana resistance (R) genes are upregulated during bacterial infection. Here, we report that 81.2% and 65.1% of FS natural TIR-NBS-LRR (TNL) and CC-NBS-LRR (CNL) transcripts, respectively, retain characteristics of NMD regulation, as their transcript levels could be controlled posttranscriptionally. Both bacterial infection and the perception of bacteria by pattern recognition receptors (PRRs) initiated the destruction of core NMD factors UP-FRAMESHIFT1 (UPF1), UPF2, and UPF3 in Arabidopsis within 30 minutes of inoculation via the independent ubiquitination of UPF1 and UPF3 and their degradation via the 26S proteasome pathway. The induction of UPF1 and UPF3 ubiquitination was delayed in mitogen-activated protein kinase3 (mpk3) and mpk6, but not in salicylic acid-signaling mutants, during the early immune response. Finally, previously uncharacterized TNL-type R transcripts accumulated in upf mutants and
conferring disease resistance to infection with a virulent *Pseudomonas* strain in plants. Our findings demonstrate that NMD is one of the main regulatory processes through which PRRs fine-tune *R* transcript levels to reduce fitness costs and achieve effective immunity.

INTRODUCTION

Plant immunity, a counterattack mechanism against microbial infection, is exquisitely controlled by two different immune receptors known as extracellular immune receptors (pattern recognition receptors, PRRs) and intracellular immune receptors [resistance (R) proteins, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) or nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins], which recognize microbe-associated molecular patterns (MAMPs) and pathogen-derived effectors, respectively. In general, pattern-triggered immunity (PTI), which is controlled by PRRs, confers moderate disease resistance to a broad spectrum of pathogens, and effector-triggered immunity (ETI), which is controlled by R proteins, is responsible for resistance to a specific pathogen carrying a cognate avirulence gene (Chisholm et al., 2006; Jones and Dangl, 2006). In addition to their predominance, *R* genes are also indispensable for establishing basal immunity to virulent pathogen infection and maintaining the balance between growth and defense (Karasov et al., 2017; Li et al., 2001; Maekawa et al., 2011; Palma et al., 2010; Shirano et al., 2002). The maintenance of the switched-off state and posttranslational modification of R proteins by other cellular components are crucial for maintaining the proper homeostasis of R activity (DeYoung and Innes et al., 2006; Li et al., 2015; Karasov et al., 2017). Transcriptional controls, such as histone modification and DNA methylation, are fundamental for fine-tuning *R* transcript levels (Halter and Navarro, 2015; Lai and Eulgem, 2018). Alternative splicing (AS) can secure the accumulation of diverse transcript isoforms for full immunity (Dinesh-Kumar and Baker, 2000; Xu et al., 2012; Yang et al., 2014; Zhang and Gassmann, 2007). A few fully spliced (FS) and AS versions of *R* transcripts are targets of miRNAs and nonsense-mediated mRNA decay (NMD) (Gloggnitzer et al., 2014; Shivaprasad et al., 2012; Zhai et al., 2011; Zhang et al., 2016), indicating that *R* gene expression is also under posttranscriptional control in plants.

NMD is a translation-coupled mRNA surveillance process in eukaryotes that prevents mRNAs containing premature termination codons (PTCs) from being translated. PTC-containing transcripts can arise from mutations or AS, and NMD is usually triggered by the presence of a downstream splice junction or long 3’ untranslated region (UTR) (Peccarelli and Kebaara, 2014;
Rebbapragada and Lykke-Andersen, 2009). FS natural transcripts can also be subject to NMD by virtue of containing NMD-triggering characteristics such as one or more introns in the 3’UTR, long 3’UTRs, or upstream open reading frames (uORFs) encoding no less than 35 amino acids (Kalyna et al., 2012; Kerényi et al., 2008; Kertesz et al., 2006; Le Hir et al., 2016; Nyikó et al., 2009).

The canonical NMD process occurs when a translating ribosome encounters a PTC and fails to properly terminate translation. This leads to the phosphorylation and activation of the core NMD factor UPF1, a process promoted by the interaction of UPF1 with UPF2 and UPF3, which associate with a downstream exon-junction complex (EJC) (Banihashemi et al., 2006; Kim et al., 2001; Schweingruber et al., 2013). In a transient wild tobacco (Nicotiana benthamiana) assay, NMD factor Suppressor for Morphological defects of Genitalia 7 (SMG7) bound to the phosphorylated C-terminal region of UPF1 for subsequent recruitment of the decapping and deadenylation complexes prior to the decay of the target mRNA (Merai et al., 2013). A recent study provided evidence that the dephosphorylation of UPF1 involves an SMG7 function (Kesarwani et al., 2019). NMD is involved in flowering, sucrose metabolism, and stress responses in plants (Filichkin et al., 2015; Garcia et al., 2014; Gloggnitzer et al., 2014; Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012; Shi et al., 2012; Sureshkumar et al., 2016; Yoine et al., 2006).

The most noteworthy function of NMD is its participation in the regulation of plant immunity. A null mutation in UPF1 or UPF3 confers disease resistance via increased levels of salicylic acid (SA) and elevated expression of defense genes, but this mutation also causes seedling lethality and late flowering in Arabidopsis thaliana (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012; Shi et al., 2012). A subset of FS natural TIR-NBS-LRR (TNL)-type R gene transcripts carrying NMD-eliciting features accumulated with known AS-coupled NMD (AS-NMD) transcripts in both smg7-1, an NMD-compromised Arabidopsis mutant showing autoimmunity, and a wild-type (WT) plant infected with Pseudomonas syringae pv. tomato DC3000 (PstDC3000) (Gloggnitzer et al., 2014). In addition, the autoimmune phenotype of smg7-1 requiring RESISTANT TO P. SYRINGAE 6 (RPS6) was suppressed by the simultaneous mutation of PHYTOALEXIN DEFICIENT 4 (PAD4) or ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1), which are important for TNL-dependent signaling (Gloggnitzer et al., 2014). These findings suggest that autoimmunity in NMD-compromised mutants results from
the activation of TNL-type R-dependent immunity and that bacterial infection might
immediately suppress the NMD machinery in Arabidopsis. However, the pathway and timing of
the decrease in NMD efficiency during Pseudomonas infection are still unclear.

Here, we analyzed the architecture of R genes expressed in NMD-compromised
Arabidopsis mutants and found that most R genes produce transcripts that are targets of NMD.
The recognition of bacterial infection by PRRs leads to the accumulation of a group of TNL- and
CC-NBS-LRR (CNL)-type transcripts by triggering the decay of UPF proteins through the
ubiquitin-26S proteasome system (UPS). These findings demonstrate that the expression of a
subset of R genes is controlled by NMD in the face of pathogen infection.

RESULTS

Simultaneous mutation of UPF1 and UPF3 causes severe autoimmune responses

Arabidopsis mutants with a defect in any of the genes encoding NMD core components
(e.g., UPF1, UPF3, and SMG7) accumulate NMD-sensitive transcripts and exhibit autoimmunity
and developmental arrest (Drechsel et al., 2013; Gloggnitzer et al., 2014; Jeong et al., 2011).
When we performed reciprocal crosses of upf1-5 and upf3-1, the resulting double mutants upf1-5
upf3-1 and upf3-1 upf1-5 exhibited distinctly dwarfed, curly leaves and seedling lethal
phenotypes when grown under typical growth conditions (22°C, 16-hour day/8-hour night) but
not when grown at a high temperature (28°C, 16-hour day/8-hour night) (Figure 1A and 1B).
When the plants were grown at 22°C under a 12-hour day/12-hour night photoperiod, the
growth-arrested phenotypes were partially rescued in the double mutants (Supplemental Figure
1A). Thus, we used Arabidopsis plants grown under this neutral-day condition for
pathophysiological studies.

To determine whether the simultaneous mutation of UPF1 and UPF3 would also
reinforce resistance responses against bacterial infection, we analyzed the responses of WT,
upf1-5, upf3-1, upf1-5 upf3-1, and upf3-1 upf1-5 plants to infection with PstDC3000 and
Pseudomonas cannabina pv. alisalensis ES4326 (PcaES4326; formerly P. syringae pv.
maculicola ES4326; Bull et al., 2010). Although the number of bacteria in upf1-5 upf3-1 and
upf3-1 upf1-5 leaves was comparable to that in upf1-5 and upf3-1, these double mutants, but not
any single mutant, exhibited severe necrotic symptoms three days after Pseudomonas infection.
(Figure 1C). Considering the atypical symptom development observed in \textit{upf1-5 upf3-1} and \textit{upf3-1 upf1-5} after virulent \textit{Pseudomonas} infection, we tested whether basal defense responses are boosted in these double mutants after infection. The expression of \textit{FLG22-INDUCED RECEPTOR-LIKE KINASE 1 (FRK1)}, \textit{NDRI-HINI-LIKE 10 (NHL10)} (MAMP-responsive genes) and \textit{PATHOGENESIS-RELATED PROTEIN 1 (PRI)} was not significantly altered in the double mutants after \textit{PstDC3000} infection compared to the single mutant or WT plants (Supplemental Figure 1B and 1C). Before infection, however, the expression levels of these genes were higher in the \textit{upf3-1 upf1-5} mutant than in the other plants. These results suggest that immune-related genes are positively regulated in the double mutant. In addition, accelerated SA accumulation was observed in the double mutant both before and after \textit{PstDC3000} infection (Supplemental Figure 1C).

Next, we examined the level of electrolytic leakage in \textit{Arabidopsis} leaves after \textit{PstDC3000} infection. Electrolyte leakage was more severe in the leaf disks of \textit{upf3-1 upf1-5} than in WT, \textit{upf1-5}, and \textit{upf3-1} grown at 22°C during infection, while the level in every single mutant was not significantly different from that of WT plants (Supplemental Figure 1D, left panel). However, an increase in electrolyte leakage was not observed in the double mutant grown at 28°C (Supplemental Figure 1D, right panel). The transcripts of 39 TNL-type \textit{R} genes were more stable in \textit{smg7 pad4} than in \textit{pad4}, 20 of which showed NMD-sensitive features (Gloggnitzer et al., 2014). To determine whether the accumulation of TNLs is a representative physiological characteristic in these \textit{upf} mutants, we measured the mRNA levels of selected TNLs in plants under both NMD-compromised conditions [\textit{upf1-5, upf3-1, upf1-5 upf3-1, upf3-1 upf1-5}, and cycloheximide (CHX)-treated WT] and WT plants grown at two different temperatures. We analyzed well-characterized FS \textit{R} gene transcripts showing NMD sensitivity (Gloggnitzer et al., 2014). We treated WT and mutant plants with actinomycin D (ActD) for 4 hours to inhibit transcriptional elongation (Sobell, 1985), as described in a previous analysis of \textit{upf3-1}, in which the 4-hour treatment was found to be optimal without damaging plant tissues (Hori & Watanabe, 2005). This blockage of \textit{de novo} transcription enables the NMD sensitivity of individual transcripts to be evaluated at the posttranscriptional level (Kuroasaki et al., 2014). The known NMD-sensitive TNL transcripts (Gloggnitzer et al., 2014) accumulated under potent NMD-compromised conditions (in the double mutants and CHX-treated WT plants) regardless of temperature (Supplemental Figure 2). These results suggest that double mutants possessing an
excessive amount of TNL transcripts remain under the control of temperature-dependent EDS1-mediated immune responses, as described for smg7-1 (Alcazar and Parker, 2011; Carstens et al., 2014; Gloggnitzer et al., 2014; Heidrich et al., 2013; Stuttmann et al., 2016; Wang et al., 2009). Notably, the differences in transcript levels among the WT and upf mutants were reduced when de novo transcription continued to occur compared to ActD-treated plants (DMSO in Supplemental Figure 2), indicating that continuous transcription is necessary for maintaining a steady-state level of R gene transcripts in Arabidopsis (Drechsel et al., 2013). Altogether, we propose that the accumulation of a variety of NMD-sensitive R transcripts leads to enhanced basal immunity in these mutants.

A significant number of R gene transcripts retain NMD-sensitive characteristics

To comprehensively examine the extent to which the expressed R transcripts differ both quantitatively and qualitatively across WT and single- and double-mutant plants, we analyzed the FS natural transcripts and the AS variants of TNLs and CNLs based on our RNA-seq data of ActD-treated plants (Supplemental Data Set 1) and determined whether each transcript carried NMD-eliciting features (Supplemental Data Set 2; Supplemental Files 1 to 7). Among the 85 TNL and 43 CNL genes expressed, 19 and 8 of the TNL (22.3%) and CNL genes (18.6%) analyzed, respectively, contained at least one intron in the 3’UTR. A long 3’UTR was the most common NMD feature of the FS R transcripts (63.5%, 54 TNLs out of 85; 53.4%, 23 out of 43 CNLs), ranging from 350 to 3,180 nt in length. Thirty-two TNLs (37.6%) and 16 CNLs (37.2%) carried uORFs that either overlapped with the main ORFs (mORFs) or encoded peptides composed of no fewer than 35 amino acids (Table 1). In summary, the FS natural transcripts of 69 TNLs (81.2%) and 28 CNLs (65.1%) retained at least one of the three NMD-sensitive features (Figure 1D; Supplemental Data Set 2; Supplemental Files 6 and 7). The FS natural transcripts of 16 TNL and 15 CNL genes carried no NMD signals, but a range of their splicing variants (12 TNLs and 5 CNLs) were targets of AS-NMD (Table 1; Supplemental Data Set 2). Overall, 81 TNLs (95.3%) and 33 CNLs (76.7%) are potential NMD substrates. Previously, in Arabidopsis studies, 1–2% of the natural transcripts and 13–17.4% of intron-containing genes were shown to be upregulated in NMD-deficient plants (Drechsel et al., 2013; Kalyna et al., 2012; Kurihara et al., 2009; Yoine et al., 2006), demonstrating that plant R genes are highly enriched in NMD regulation.
Next, we tested the stability of these NMD-susceptible FS R transcripts in *upf3-1 upf1-5* mutants by measuring the half-lives of R transcripts from leaf samples taken from WT and mutant plants at 0, 1, 2, and 4 hours after blocking *de novo* transcription. We used *At2g29210* and *LYSOPHOSPHATIDYLETHANOLAMINE ACYLTRANSFERASE 2* (*LPEAT2*) AS variants (PTC+) as NMD controls (Gloggnitzer et al. 2014). The half-lives of these NMD markers and R transcripts carrying the NMD-sensitive features were elevated in *upf3-1 upf1-5* compared to normal-looking transcripts showing similar levels of stability between WT and *upf3-1 upf1-5* (Figure 1E to 1G; Supplemental Figure 3), indicating that NMD is one of the main processes controlling the expression of TNL and CNL genes. Other transcripts bearing NMD-sensitive features, however, showed no differences in RNA stability between WT and *upf3-1 upf1-5*, e.g., *RPP5* (Supplemental Figure 3). This result suggests that not all R genes bearing NMD-sensitive features are genuine NMD targets, as widely observed in eukaryotic genes (Kurosaki et al., 2014; Peccarelli and Kebaara, 2014; Rebbapragada and Lykke-Andersen, 2009).

To determine whether FS natural TNL and CNL transcripts are post-transcriptionally regulated, we measured the transcription rates of the randomly selected genes by performing a chromatin immunoprecipitation (ChIP) assay of WT and *upf3-1 upf1-5* plants using an α-RNA polymerase II (α-RNAPII) C-terminal domain (CTD) antibody and primer sets specific to regions between the transcription and translation initiation sites. The ChIP signals in the TNL genes were largely constant between WT and *upf3-1 upf1-5* (Supplemental Figure 4A), indicating that most TNL-type R genes with NMD-sensitive features are constitutively transcribed in *Arabidopsis* leaves and that the resulting transcripts are subject to immediate turnover by NMD in the WT. Unlike TNLs, half of the CNL genes tested here showed higher enrichment of the RNAPII elongation complex in *upf3-1 upf1-5* than in WT, e.g., *RPP7* and *RPM1* (Supplemental Figure 4B), suggesting that these genes are transcriptionally upregulated in the *upf3-1 upf1-5* mutant. Taken together, these findings support the notion that NMD is one of the regulatory pathways controlling the steady-state levels of both FS and AS R transcripts carrying NMD-sensitive features (Gloggnitzer et al., 2014).

Bacterial infection results in turnover of UPF proteins

Since bacterial infection upregulates a subset of NMD-sensitive transcripts (Gloggnitzer et al., 2014), we monitored the transcript levels of core NMD factors in *PstDC3000*-infected WT
leaves to detect any decrease in transcript levels upon bacterial infection. The FS natural transcripts encoding *Arabidopsis* UPF1, UPF3, and SMG7 are known NMD substrates (Degtiar et al., 2015; Gloggnitzer et al., 2014; Kesarwani et al., 2019; Nyikó et al., 2013). Our RNA-seq data also revealed that the *UPF1* transcript, a possible NMD target, carries a 508-nt-long 3′UTR (Supplemental Figure 5A). These transcripts were upregulated after *PstDC3000* infection, while the level of the FS *UPF2* transcript remained constant (Supplemental Figure 5A). The upper products, which accumulated during infection, were identified as AS variants of *UPF1* and *UPF2* produced by unspliced introns 26 and 14, respectively, and they eventually retained NMD-triggering features owing to the long 3′UTR containing two introns (single and double asterisks, Supplemental Figure 5A and 5B). The results indicate that the plant NMD system becomes impaired at an early stage of infection, despite the increasing levels of transcripts of NMD factors (Degtiar et al., 2015; Gloggnitzer et al., 2014; Kerényi et al., 2008; Nyikó et al., 2013; Shaul, 2015).

The inconsistency between the increased transcript levels of *UPF* genes and the reduction in NMD activity after infection (Gloggnitzer et al., 2014) suggests that bacterial infection might disrupt the plant NMD system by affecting the integrity of the machinery at the protein level. To address this hypothesis, we prepared monoclonal antibodies against *UPF1* (α-UPF1), *UPF2*, and *UPF3* and used them for immunoblot analysis (Supplemental Figure 6B and 6C). α-UPF1 detected a faint signal of *UPF1* in the knockdown mutant *upf1*-5 and in the putative knockdown mutant *upf1*-4. However, the protein was undetectable in the amino acid substitution mutant *upf1*-1 for unknown reasons (Supplemental Figure 6B). We examined the levels of *UPF1*, *UPF2*, and *UPF3* in WT leaves infected with *PstDC3000* using α-UPF1, α-UPF2, and α-UPF3. The levels of *UPF1* and *UPF3* were reduced to less than 40% (*UPF1*) to 50% (*UPF3*) within 0.5 hours post inoculation (hpi) and became undetectable from 10 to 30 hpi, whereas the level of *UPF2* was maintained for up to 6 hpi. All three NMD factors were undetectable by 20 hpi (Figure 2A). The turnover of *UPF1* occurred more quickly than that of *UPF3*, *i.e.*, *UPF3* was still detectable at 10 hpi when *UPF1* was absent (Figure 2A, upper panel).

Since *UPF1* decay began as early as 30 minutes post inoculation ( mpi) (Figure 2A, upper panel), we wondered how early the turnover of UPF proteins was initiated. We monitored the stability of UPF proteins at various time points beginning at 1 mpi with *PstDC3000*. Surprisingly, the reduction in *UPF1* levels was clearly observed at 5 mpi, and a sharp decrease occurred
between 15 and 20 mpi, after which only approximately 50% UPF1 remained. A drastic decrease in UPF3 levels was observed between 15 and 25 mpi, during which more than 90% of UPF2 remained (Figure 2A, lower panel). These findings suggest that the turnover of UPF proteins is an early response to *PstDC3000* infection.

To determine whether other protein components involved in NMD (Kerényi et al., 2008) are also unstable upon bacterial infection, we generated transgenic *Arabidopsis* plants overexpressing the SMG7 and EJC proteins. SMG7 and the other EJC components, Y14, MAGO, eIF4AII, and CASC3/Barentsz (BTZs), were stably maintained during infection compared to the overexpressed UPF proteins, although the overexpressed versions of UPF proteins (the first to third rows of the left panel in Figure 2B) were destabilized later than the native proteins (Figure 2B). Thus, we conclude that bacterial infection initiates the specific destruction of UPF proteins.

To determine whether the decay of UPF proteins is initiated by only virulent *Pseudomonas* infection, we inoculated WT plants with an attenuated strain, *PstDC3000 hrcC*, and two avirulent derivatives of *PstDC3000* carrying *AvrRpm1* or *AvrRps4* and subjected them to immunoblot analysis using an anti-UPF1 polyclonal antibody. Like the turnover pattern of UPF1 in *PstDC3000*-infected plants, infection with these different strains also triggered the decay of UPF1 proteins in *Arabidopsis* (Supplemental Figure 7A and 7B), indicating that the sensing of *P. syringae* is sufficient to initiate the decay of the core NMD factors. Unlike the immunoblotting results obtained using monoclonal α-UPF1 antibody, the UPF1 level at 6 hpi was somehow recovered, which was more pronounced after infection with the *PstDC3000 hrcC* strain. Furthermore, a putative UPF1 degradation product (single asterisk in Supplemental Figure 7) was also observed in parallel with UPF1 dynamics and was not detected in the blots using monoclonal α-UPF1 antibody. We speculate that UPF1 might undergo a conformational change during the decay process and that the monoclonal antibody is unable to cross-react with this transitional form of UPF1. The detection of a small product by the polyclonal antibody even before infection (single asterisk in Supplemental Figure 7) suggests that the UPF1 level may be controlled posttranslationally under normal conditions.

**UPF1 and UPF3 are destroyed via ubiquitination at an early stage of infection**

To examine whether UPF proteins are post-translationally degraded during an early stage of infection, MG132, an inhibitor of the proteasome, was coinfiltrated into WT *Arabidopsis* leaves
with PstDC3000. While the coapplication of the solvent DMSO alone with PstDC3000 did not affect the protein dynamics of UPF1, UPF2, or UPF3, MG132 coinfiltrated with PstDC3000 interrupted the infection-induced destruction of these proteins (Figure 3A). These results support our observation that the reduction and subsequent disappearance of UPF proteins resulting from Pseudomonas infection (Figure 2) is due to the decay of UPF proteins rather than the translational inhibition of mRNAs encoding these NMD factors.

To confirm that the decay of the UPF proteins occurs via the UPS during bacterial infection, we monitored the levels ubiquitinated UPF proteins in the α-UPF1, α-UPF2 and α-UPF3 immunoprecipitates by immunoblot analysis using an α-ubiquitin (α-UBQ) antibody. The ubiquitination of UPF1 and UPF3 was strongly induced at 30 mpi and 1 hpi, respectively (Figure 3B; Supplemental Figure 8A), which is consistent with the dynamics of these proteins (Figure 2A). These results suggest that the ubiquitination of UPF1 and UPF3 is a temporally controlled early response in plant immunity. UPF1 was already ubiquitinated prior to infection (Figure 3B), which likely explains the previous findings that the turnover of UPF1 occurs within 4 hours in ActD-treated upf1-5 plants (Supplemental Figure 6C) and that the putative degradation product was detected by polyclonal α-UPF1 antibody even before infection (single asterisks in Supplemental Figure 7). These results suggest that, in Arabidopsis, UPF1 levels are also regulated by ubiquitination under normal growth conditions. The induced ubiquitination signals of UPF1 and UPF3 were maintained for up to 6 and 10 hpi (Figure 3B), respectively, when the proteins were still present (Figure 2A). The ubiquitinated UPF1 and UPF3 signals, however, were no longer evident at 10 hpi and 20 hpi, respectively, and at 20 hpi, both were nearly or entirely undetectable (Figure 3B), indicating that the induction of UPF1/UPF3 ubiquitination is responsible for the decay of these proteins. To validate this result, we treated plants with MG132 and PstDC3000 infection and found that the induced level of UPF1 and UPF3 ubiquitination signals was maintained through 20 hpi (Figure 3C; Supplemental Figure 8B), supporting the suggestion from Figure 3B that the disappearance of the induced ubiquitination signals was due to the absence of both proteins at this stage. We did not detect multiple bands larger than UPF1 and UPF3 (estimated molecular masses of 136.9 and 54.0 kDa, respectively), suggesting that these proteins are degraded by the 26S proteasome via monoubiquitination or multi-monoubiquitination rather than polyubiquitination (Braten et al., 2016; Ciechanover and Stanhill, 2014; Shabek et al., 2012).
UPF2, which is also decayed during *PstDC3000* infection (Figures 2A and 3A), was not ubiquitinated following infection (Figure 3B and 3C). Since UPF2 and UPF3 are present in complexes from the nucleolus to the cytoplasm (Kerényi et al., 2008; Kim et al., 2009), we reasoned that UPF2 might enter the 26S proteasome complex with UPF3 during infection. Indeed, UPF2 coimmunoprecipitated with UPF3 under both uninfected and *PstDC3000*-infected conditions (Supplemental Figure 9). To test whether UPF1/UPF3 decay upon bacterial challenge is universal among plants, we subjected *N. benthamiana* plants to infection with the known bacterial pathogen *P. syringae* pv. *syringae* B728a (*PssB728a*). The UPF proteins of the *N. benthamiana* plants were also destroyed by 30 hpi (Figure 3D). The induction of UPF1 and UPF3 ubiquitination was detected at 2 and 6 hpi, respectively (Figure 3E), suggesting that plant immunity involves the suppression of NMD via the control of UPF protein levels in members of the *Solanaceae* family.

Since TNL genes are known to be deregulated upon *Pseudomonas* infection due to decreased NMD efficiency (Gloggnitzer et al., 2014), we investigated whether the kinetics of the NMD-sensitive TNL and CNL transcripts reflect the decay of UPF proteins upon *PstDC3000* infection. We monitored the stability of TNL and CNL transcripts from ActD-treated infected leaves taken at 0, 1, 2, and 4 hpi by qRT-PCR analysis. The transcripts of NMD markers as controls, namely, *At2g29210* and *LPEAT2* retaining PTC (*At2g29210 PTC+* and *LPEAT2 PTC+*), were more stably maintained in infected WT leaves than in noninfected leaves (Figure 4A). Similarly, the TNL and CNL transcripts bearing NMD-sensitive features, *e.g.*, *SIKIC3*, *RPS6*, *RPM1*, and *RPP7*, were more long-lived in infected leaves than in noninfected leaves (Figure 4B and 4C; Supplemental Figure 10). The half-lives of *SNC1* and *RPP5*, which are representative targets of small-interfering RNA (siRNA) (Yi and Richards, 2007), and non-NMD targets, namely, *SUMM2* and *RPS4*, were similar between infected and noninfected leaves (Figure 4B and 4C; Supplemental Figure 10). The gaps between the NMD target *R* transcripts in infected and noninfected plants were less distinct than those between *upf3-1 upf1-5* and WT (Figures 1F, 1G, 4B, and 4C; Supplemental Figures 3 and 10), suggesting that NMD may have still occurred by 4 hpi, when UPF1 is still present (Figures 2A and 3B).

In parallel, we analyzed the accumulation of *R* transcripts in infected leaf samples taken at the same time points (Figures 2 and 3) used for protein analysis. As a result, we were able to sort the *R* genes bearing NMD-sensitive features into three groups. The early-response *R* genes,
including RPM1 and At3g44400, were upregulated as early as 30 mpi of PstDC3000 (Figure 4; Supplemental Figure 11), suggesting that the transcripts of these genes are instantly stabilized when the UPF1 level is reduced to less than 40%. The second group, the so-called late-response genes, e.g., SOC3, At3g14470, At4g14610, and At5g38340, appeared to be upregulated later than 10 hpi, when UPF1 is completely decayed (Supplemental Figure 10). The nonresponsive genes, including RRS1 and At5g35450, were not regulated by Pseudomonas infection (Supplemental Figure 10).

The miRNA target CNL-type R genes At1g50180 and RSG2 (Bocca et al., 2014; Cai et al. 2018), carrying uORF/long 3’ UTR and long 3’ UTR, respectively, are though to also be targets of NMD, since their transcripts are stabilized upon Pseudomonas infection (Supplemental Figure 10B). By contrast, the half-lives of other known miRNA targets including RPS5, At1g12290, and At1g61180 were not altered or shortened by the mutation of UPF1/UPF3 or Pseudomonas infection (Supplemental Figure 11A), suggesting that the steady-state level of a subset of CNL transcripts bearing NMD-sensitive features may be under the control of miRNA-mediated gene silencing rather than NMD. Moreover, three TNL-type R transcripts, At3g44400, At4g14370, and At5g41740, which were upregulated as early as 30 mpi by Pseudomonas infection, were more stable in infected vs. noninfected plants. By contrast, the stability of these transcripts was reduced in upf3-1 upf1-5 vs. WT plants (Supplemental Figure 11B). These results suggest that susceptibility to NMD varies across FS R transcripts carrying NMD-sensitive features (Kurosaki et al., 2014; Peccarelli and Kebaara, 2014; Rebprapagada and Lykke-Andersen, 2009) and that the control of the expression of these genes might involve other processes, e.g., transcriptional regulation, under a variety of conditions. Based on these results, we propose that NMD is one of the main posttranscriptional control pathways involved in maintaining R transcript levels in Arabidopsis during the immunity response.

The induction of UPF3 ubiquitination partially depends on UPF1

The cysteine- and histidine-rich (CH) domain of UPF1, which serves as a Really Interesting New Gene (RING)-related E3 ligase, is responsible for the UPF3-dependent self-ubiquitination required for activation of NMD in yeast (Takahashi et al., 2008). To determine whether UPF3 is required for UPF1 ubiquitination and vice versa in Arabidopsis, we monitored the ubiquitination and turnover of UPF proteins in upf1-1, upf2-12, and upf3-1 plants at 1 hour
after *PstDC3000* infection. The ubiquitination level of UPF1 was similar across WT, *upf2-12*, and *upf3-1* (Figure 5A; 2nd row in Figure 5B), suggesting that the induction of UPF1 ubiquitination upon *PstDC3000* infection occurs independently of UPF2 or UPF3. By contrast, the level of ubiquitinated UPF3 decreased 1.24 ± 0.042-fold in *upf1-1* compared to WT (4th row in Figure 5B; 6th row in Figure 5C). The UPF3 level stabilized from 1 to 10 hpi in *upf1-1* compared to WT (3rd and 6th rows in Figure 5C), most likely due to decreased ubiquitination. These results are consistent with the observation that the decay of UPF1 occurs prior to that of UPF3 (Figures 2A, 3B, and 3C) and that UPF1 protein itself or ubiquitinated UPF1 modulates the ubiquitination of UPF3 upon bacterial infection. Bacterial infection induces the ubiquitination of UPF1/UPF3 for the decay of three UPF proteins, which differs from the UPF3-dependent self-ubiquitination of UPF1 required for NMD in yeast (Takahashi et al., 2008).

**Microbial perception by PRRs initiates the decay of UPF proteins**

NMD-sensitive transcript levels also increased in WT plants treated with 1 µM of the bacterial elicitor flg22, while the expression of these transcripts was suppressed in *flagellin sensitive 2* (*fls2*) mutants (Gloggnitzer et al., 2014), suggesting that MAMP recognition can also affect the integrity of UPF proteins. Compared to mock treatment, in which the levels of UPF proteins were unchanged, both UPF1 and UPF3 started to degrade at 1 and 2 hours posttreatment (hpt) with flg22 in WT plants, respectively, while UPF2 levels were significantly reduced at 6 hours (Figure 6A). flg22 treatment did not trigger the decay of UPF proteins in *fls2* or the co-receptor mutant *bri1-associated receptor kinase 1-5* (*bak1-5*), although UPF1 in *bak1-5* disappeared within 30 hours (Figure 6B). These findings suggest that the flg22-FLS2 association is pivotal for the effect of flg22 on UPF proteins. Consistent with UPF degradation via the UPS during *Pseudomonas* infection, flg22 treatment also caused the ubiquitination of UPF1 and UPF3 but not UPF2, and induced ubiquitination was observed at 1 and 2 hpt for UPF1 and UPF3, respectively (Figure 6C; Supplemental Figure 8C and 8D), when these proteins began to be destroyed (Figure 6A). Ubiquitination of UPF1 and UPF3 was uninducible in *fls2* or delayed to 20 hpt in *bak1-5* compared with 1 to 2 hpt in WT plants (Figure 6D). These results indicate that the perception of MAMPs is sufficient for initiation of the ubiquitination of UPF1 and UPF3 during infection. The decay of UPFs was still triggered in *fls2* and *bak1-5* after *PstDC3000* infection, although the proteins were more stable in these mutants than in the WT (Figure 6E).
The relatively high dose of flg22 described above did not induce as strong a response in UPF proteins as PstDC3000 infection (compare Figures 2A and 3B with Figures 6A and 6C, respectively). These findings suggest that microbial sensing by PRRs leads to the decay of UPF proteins, that other pathogen-derived molecules might simultaneously act during this early event and that unidentified bacterial effectors might also contribute to the decay process.

**MPK3 and MPK6 regulate the induction of UPF1 and UPF3 ubiquitination**

The perception of microbial infection rapidly triggers an oxidative burst and dual phosphorylation of MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and MPK6 in plants (Nicase et al., 2009). To examine whether the degradation of UPF proteins is associated with these early events, we observed the decay of these proteins in WT and mutant plants defective in early signaling. UPF1 turnover occurred by 20 hpi after PstDC3000 infection in mpk3 and mpk6 compared to 10 hpi in WT. UPF1 was still detectable until 20 hpt after flg22 treatment in mpk6 cells (Figure 7A). To address whether the induction of UPF1/UPF3 ubiquitination is affected by mutations in MPK3 and MPK6, we monitored the α-UPF1 and α-UPF3 immunoprecipitates from flg22-treated mpk3 and mpk6 leaves by immunoblotting using an α-UBQ antibody. The induction of UPF1 ubiquitination was initiated at 2 and 6 hpt in mpk3 and mpk6, respectively, compared with 1 hpt in WT (Figures 6C, 6D and 7B). The ubiquitination of UPF3 was unaffected in mpk3, but it was delayed to 6 hpt in mpk6 from 2 hpt in WT (Figures 6C, 6D and 7B). By contrast, the induction of UPF1/UPF3 ubiquitination was unaffected by mutations in the RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD) and RBOHF genes, although UPF2 was not degraded in the flg22-treated rbohD rbohF mutant compared to the responses in WT (Figure 7A and 7B; Supplemental Figure 12A). These findings indicate that the induction of UPF1/UPF3 ubiquitination requires the mitogen-activated protein kinase (MAPK) signaling cascade, the crucial early response in PTI, and that reactive oxygen species might stimulate the turnover of UPF2. Indeed, transgenic plants expressing constitutively active MPK6 (MPK6CA) showed a low level of UPF1 compared to the WT (Supplemental Figure 12B), supporting the idea that MAPK signaling participates in the decay of UPF proteins after sensing microbial infection.

We examined the effects of SA-related defense responses on the decay event. The levels of UPF proteins were not significantly altered in the SA signaling mutants *eds1-1, sa induction*
deficient 2-1 (sid2-1) and nonexpresser of pr genes 1 (npr1-1) compared to WT after PstDC3000 infection and flg22 treatment (Figure 7C). Exogenous treatment with BTH, a SA agonist, did not have any effect on the stability of UPF proteins in WT plants (Supplemental Figure 12C). The induction of UPF1/UPF3 ubiquitination upon flg22 treatment was also unaltered in these mutants (Supplemental Figure 12D). However, UPF2 persisted in sid2-1 and npr1-1 leaves for up to 20 hours. These results suggest that the complete decay of UPF2 might involve SA-related events during a late stage of bacterial infection, which would explain the stability of UPF2 during early stages of PstDC3000 infection, i.e., 0.5 to 6 hpi (Figures 2A and 7C). Based on these results, we suggest that the decay of UPF1 and UPF3 by the UPS involves as-yet-unidentified events downstream of the MAPK pathway triggered by bacterial recognition rather than subsequent SA-related events.

Unannotated fused TNL-type transcripts accumulate in WT plants after bacterial infection

Finally, we aimed to establish a model explaining why the suppression of NMD and the resulting upregulation of NMD-susceptible TNL transcripts lead to enhanced net immunity. The best way to illustrate this concept would be to produce a plant overexpressing the whole body of NMD-susceptible TNLs without suppressing NMD, which is impossible. As an alternative, we took previously uncharacterized fusion transcripts from RNA-seq data that were overrepresented in upf1-5 upf3-1 and upf3-1 upf1-5 (Supplemental Data Set 1 and 2) and comprised four pairs of adjacent genes annotated as independent genes: At1g57630-At1g57650, At2g17055-At2g17050, At5g38344-At5g38350, and At5g46490-At5g46500 (Supplemental Figures 13 and 14). The individual genes in pairs encoded N- and C-terminal fragments of TNLs, such that the fusion transcripts effectively encoded full-length TNLs (Supplemental File 6 and Supplemental Figure 13). All the fusion transcripts appeared to become upregulated in WT plants within 30 mpi with PstDC3000 except At2g17055-At2g17050, although the levels were not significantly different from those under noninfection condition. Later, the fusion transcripts were overrepresented within 30 hpi (Figure 8A). Except at the loci of At5g38344 and At5g38350, RNAPII enrichment at the other three loci was not significantly altered by the simultaneous mutation of UPF1 and UPF3: the ChIP signals were similar in both upf3-1 upf1-5 and WT plants (Figure 8B). Again, these results demonstrate that the accumulation of TNL-type transcripts is regulated by NMD and/or transcription.
Finally, to test whether these fusion transcripts were functional, we produced transgenic Arabidopsis plants expressing the At1g57630-At1g57650 and At5g38344-At5g38350 cDNAs under the control of the CaMV 35S promoter and terminator. Stable overexpression of these fusion transcripts, which do not retain NMD-eliciting features, conferred greater resistance to virulent Pseudomonas infection compared to WT plants (Figure 8C, left panel), showing that these transcripts are involved in basal resistance to bacterial infection. However, the bacterial numbers of PstDC3000 hrcC from these transgenic lines were comparable to those from WT plants (Figure 8C, right panel). In contrast to upf3-1 upf1-5, which produces a high level of the PRI transcript even before pathogen infection (Supplemental Figure 1C), the PRI mRNA levels were reduced in the transgenic plants in the absence of pathogen infection (Figure 8D). The conformational changes in translated NLRs crucial for NLR activation may not occur under infection by the disarmed strain and noninfection conditions (Sukarta et al., 2016). Overall, we suggest that the suppression of NMD via the degradation of UPFs and the resulting stabilization of TNL transcript levels from an early stage of bacterial infection contribute to the basal immunity of plants to virulent pathogen infection.

DISCUSSION

Microbial perception by PRRs and R proteins occurs at the forefront of the battle between plants and microbes. In this study, we demonstrated that MAMP recognition by PRRs controls the levels of R transcripts carrying NMD-sensitive features via the induction of UPF1/UPF3 ubiquitination, followed by their 26S-proteasome-dependent degradation at an early stage of infection. Following their export from the nucleus, virtually all mRNAs undergo mRNA surveillance processes, including NMD and miRNA-mediated gene silencing, by which only those certified as “normal” are deemed competent at translation (Eulalio et al., 2007; Lai and Eulgem, 2018). Because most FS R transcripts retain one of the NMD-triggering characteristics, they are thought to be either prevented from undergoing translation or immediately decayed by the NMD machinery, which may be essential for avoiding the unnecessary costs of immunity under normal growth conditions (Karasov et al., 2017; Maekawa et al., 2011). The observation that a majority of TNL and CNL genes carry NMD-sensitive features suggests that the NMD machinery and R genes coevolved in plants. The coexistence of both normal genes and those
with NMD-regulated features in a few single R gene clusters (Supplemental Figure 15) suggests that gene duplication events including unequal crossing-over (Meyers et al., 2003) may have occurred prior to genetic drift, followed by subsequent positive selection. Such gene clusters might have evolved in land plants to facilitate the switching of R transcript levels (Raxwal and Riha, 2016).

Most FS R transcripts of the genes bearing NMD-eliciting features that we tested were highly stable in upf3-1 upf1-5, except for those of a few genes, supporting previous observations (Gloggnitzer et al., 2014). However, the gaps in the stability of these FS R transcripts between noninfected and Pseudomonas-infected plants were smaller than those between WT and upf3-1 upf1-5 under our experimental conditions. In addition, the steady-state levels of a few R transcripts carrying typical NMD features might also be regulated by the putative miRNA-mediated control system during Pseudomonas infection. Based on these findings, we speculate that NMD in Pseudomonas-infected plants still occurs until the UPF proteins are entirely eliminated. It is also likely that other mRNA control processes, including miRNA-mediated gene silencing, are deregulated in Arabidopsis during infection, since the intergenic pri-mRNAs may contain uORFs or long 3′ UTRs due to their weak protein-encoding capability as noncoding mRNAs or primary transcripts of small nucleolar RNAs and are thus NMD targets (Drechsel et al., 2013; Kurihara et al., 2009). The predicted NMD-sensitivity of R transcripts did not fully explain their actual biological NMD susceptibility, as the R transcripts were upregulated, unregulated, or even downregulated at a variety of time points after Pseudomonas infection, although most were upregulated or highly stable in the upf3-1 upf1-5 mutant.

In addition to the FS R transcripts, their AS variants also predominantly accumulated in upf mutants (Supplemental Files 6 and 7), which suggests that the atypical cell death-like symptoms observed in upf1-5 upf3-1 and upf3-1 upf1-5 after virulent Pseudomonas infection resulted from the increased heterogeneity of the NMD-sensitive R transcript repertoire as well as RPS6 (Gloggnitzer et al., 2014). Therefore, we suggest that the diversified expression of R genes, not their canonical functions in ETI, confers basal immunity in NMD-compromised mutants. NMD-sensitive R transcripts also accumulate in WT leaves after virulent P. syringae infection by suppressing NMD. How do virulent P. syringae strains overcome the induced barriers resulting from the accumulated R proteins? One possible explanation is that WT Col plants do not produce any functional R proteins capable of recognizing effectors delivered from PstDC3000 and
PcaES4326 and are unable to trigger ETI in response to infection with these virulent pathogens, even when they accumulate high levels of NMD-sensitive R transcripts. If this is the case, these R transcripts may not function in the susceptible response in plants. During ETI, the accumulation of various sensor and helper NLRs is necessary for full immunity against avirulent pathogen infections (Jones et al., 2016; Wu et al., 2017). To achieve full immunity against a countless number of different pathogens, plants may have evolved diverse NLRs, known as the NLRome and pan-NLRome, via an arms race against a variety of pathogens (Meyers et al., 2003; Van de Weyer et al., 2019). Unlike miRNAs that directly regulate the levels of their target R transcripts, NMD covers both AS and FS R transcripts as long as they carry NMD-triggering characteristics. Thus, NMD-mediated posttranscriptional regulation can diversify the expressed R repertoire to maximize the chances to achieve full resistance to unexpected pathogen infection. Our data do not provide details on every upregulated FS R transcript and its AS variants during pathogen infection, although we described their RNA-seq coverage data extracted from upf mutants (Supplemental Files 6 and 7). It would be interesting to monitor the dynamic changes in AS variants in real time after infection, since most splicing factor genes have AS forms, and many are also NMD sensitive (Palusa and Reddy, 2010).

The UPS regulates diverse fundamental processes in plants, including hormonal signaling and immune responses (Dudler, 2013; Kelley and Estelle, 2012; Marino et al., 2012). The latter has been referred to as a double-edged sword, in which E3 ligases function as either positive or negative regulators of plant immunity (Marino et al., 2012). The best examples are the E3 ligases PLANT U-BOX 12 (PUB12)/PUB13 and PUB22, which are involved in the decay of flagellin-induced FLS2 and EXO70B2 (EXOCYST SUBUNIT EXO70 FAMILY PROTEIN B2), respectively, resulting in attenuated plant immunity (Lu et al., 2011; Stegmann et al., 2012). The homologous triplet PUB22/PUB23/PUB24 can dampen early immune responses stimulated by MAMPs (Trujillo et al., 2008). By contrast, the transcription of ATL9 (ARABIDOPSIS TOXICOS EN LEVADURA9), encoding a RING zinc-finger E3 ligase, is induced by a variety of external stimuli, including chitin, and the mutant shows enhanced susceptibility to Golovinomyces cichoracearum and reduced reactive oxygen species production after infection (Berrocal-Lobo et al., 2010; Ramonell et al., 2005). Thus, the early immune response is either suppressed or activated by ubiquitination (Marino et al., 2012). We report here that the decay of UPF1 and UPF3 via the UPS is linked to the suppression of NMD. These processes are governed the roles
of PRRs as sensors, reporting dire circumstances to the downstream components of PTI, leading to the suppression of NMD and involving the decay of even nonubiquitinated UPF2.

Unlike the well-characterized target proteins of the UPS, e.g., receptors and transcription factors, UPF proteins participate in diverse cellular processes in plants by controlling the transcriptome (Drechsel et al., 2013; Kurihara et al., 2009). Why must UPF proteins decay upon bacterial infection at the expense of such pleiotropic effects? The most likely answer is that a considerable variety of \( R \) proteins must accumulate during an early stage of infection to protect the host plants from attack from unpredictable pathogens before they successfully colonize the plants. When \( R \) proteins monitor their cognate avirulence proteins derived from the pathogen, plants may only have to pay a reasonable cost for subsequent immunity. If not, the accumulated \( R \) proteins can confer a defense response to plants. Thus, we suggest that the consecutive events between MAMP perception by PRRs and the accumulation of \( R \) transcripts are well programmed in the “black box”.

The decay of UPF proteins and the accumulation of \( R \) transcripts occur in infected leaves at an early phase after bacterial infection. Interestingly, the significant delay in the induction of ubiquitination and subsequent decay of UPF1 in \( mpk3 \) and \( mpk6 \) suggests that the MAPK cascade is associated with these events. Because the \( mpk3 \) \( mpk6 \) double mutant was lethal, we could not quantify the ubiquitination of UPF1 and UPF3 in these plants. However, the results sufficiently indicate that the MAPK cascade not only modulates transcription activators/coactivators that regulate target genes involved in plant immunity (Rasmussen et al., 2012), but it also links bacterial sensing and the maintenance of \( R \) transcripts by participating in the induction of the ubiquitination of proteins, including UPF1 and UPF3. Therefore, our findings point to an additional layer of plasticity between the PTI and ETI by NMD.

METHODS

Plants and pathogens

Wild-type Arabidopsis thaliana Columbia-0 (WT) and the NMD-compromised mutants \( upf1-1 \) (Yoine et al., 2006), \( upf1-4 \) (Salk_022721), \( upf1-5 \) (Salk_112922), \( upf2-12 \) (SAIL_512_G03), \( upf3-1 \) (Salk_025175), \( upf3-2 \) (Salk_097931), \( upf3-3 \) (Salk_061923), \( smg7-1 \), \( upf1-5 \) \( upf3-1 \), and \( upf3-1 \) \( upf1-5 \) were grown in environmentally controlled growth chambers [22±1°C, 16-hour day
(120 µmol photons m\(^{-2}\)s\(^{-1}\))/8-hour night for developmental studies or 12-hour day/12-hour night for pathophysiological experiments] (Arciga-Reyes et al., 2006; Hori and Watanabe, 2005; Merchante et al., 2015; Riehs et al., 2008). The upf1-5 upf3-1 and upf3-1 upf1-5 mutants were maintained at a high temperature (28°C, 16-hour day/8-hour night) until the flowering stage. The fsl2 (Salk_093905), bak1-5, rbohD rohhF, mpk3-1, mpk6-2, eds1-1, sid2-1, and npr1-1 mutants and MPK6CA transgenic plants described elsewhere (Boutrot et al., 2010; Cao et al., 1997; Hudik et al., 2014; Nawrath and Metraux, 1999; Parker et al., 1996; Schwessinger et al., 2011; Torres et al., 2002; Wang et al., 2007) were grown at 22±1°C with a 12-hour day/12-hour night photoperiod.

The ORFs of UPF1, UPF2, UPF3, SMG7, Y14, MAGO, elF4AIII, CASC3/Barentsz elF4AIII-binding protein (BTZ1), and BTZ2 were cloned into CTAPI, a plant transformation vector (Rohila et al., 2004), for stable expression in Arabidopsis. The oligonucleotide sequences used to amplify the cDNA fragments are listed in Supplemental Data Set 3. Transgenic plants were produced by the floral dip method (Clough and Bent, 1998). Seeds were screened on MS medium (Duchefa) containing 50 µg ml\(^{-1}\) DL-phosphinothricin (Duchefa). T\(_3\) homozygotes were isolated and used for analysis.

Freshly incubated bacterial strains PstDC3000, PcaES4326, PstDC3000/AvrRpm1, PstDC3000/AvrRps4 and PstDC3000 hrcC\(^{\prime}\) in King’s B medium supplemented with the appropriate antibiotics were diluted in 10 mM MgSO\(_4\) to different concentrations as follows: OD\(_{600}\)=0.05 to measure ion leakage, OD\(_{600}\)=0.01 to evaluate pathophysiological responses, and OD\(_{600}\)=0.0001 (PstDC3000 and PcaES4326) or OD\(_{600}\)=0.001 (PstDC3000 hrcC\(^{\prime}\)) to count the number of bacteria in leaf disks. The number of bacteria in infected leaves was determined 3 days after inoculation following a typical serial dilution method, and the infected leaves were photographed on the same day.

RNA-seq

Three-week-old Arabidopsis plants were vacuum infiltrated for 5 minutes in 0.5x MS medium containing 80 µM ActD and/or 20 µM CHX and incubated in the dark for 4 hours prior to RNA isolation (Ambion) (Hori and Watanabe, 2005). After confirming the RNA purity with a bioanalyzer using the Agilent RNA 6000 Pico Kit (Agilent), the total RNA was processed for mRNA sequencing library preparation using a TruSeq Stranded mRNA Sample Preparation Kit
according to the manufacturer’s instructions (Illumina). In brief, mRNAs were isolated from 400 ng total RNA with RNA purification beads using poly(A) capture followed by enzyme shearing. After first and second-strand cDNA synthesis, A-tailing and end repair were performed for the ligation of proprietary primers incorporating unique sequencing adaptors with an index for tracking Illumina reads from multiplexed samples run on a single sequencing lane. All samples were processed in two biological replicates per genotype, each from individual plants. For each library, an insert size of approximately 270 bp was confirmed with a bioanalyzer, and the library was quantified by RT-PCR using the CFX96 system (BioRad). Each library was sequenced for 75 bp paired-end reads with a NextSeq500 High Output 150 Cycle Kit on the NextSeq500 platform (Illumina).

The raw image data were transformed by base-calling into sequence data and stored in FASTQ format. The RNA-seq data were aligned by TopHat2 and Bowtie2 to detect the canonical and noncanonical intron motifs (Kim et al., 2013; Langmead and Salzberg, 2012). Unaligned reads using the above platforms were mapped by BLAT (Trapnell et al., 2010). AS events were extracted from the TAIR10 gene models (www.arabidopsis.org). To evaluate NMD-triggering features (see the next section “Evaluation of R transcripts for NMD-sensitive features”), the datasets were mapped in the Integrative Genomics Viewer (IGV) browser (Robinson et al., 2011; Thorwaldsdottir et al., 2013). To quantify the total transcript mass in fragments per kilobase of transcript per million mapped reads (FPKM), biological replicates of ActD-treated WT Col-0, ActD/CHX-treated WT Col-0, upf1-5, upf3-1, and upf3-1 upf1-5 were separately aligned with the A. thaliana TAIR10 gene model using the Cufflinks package (Saeed et al., 2003).

Evaluation of R transcripts for NMD-sensitive features

Although many normal-looking mRNAs can be regulated by NMD via low translation efficiency of the main ORF (mORF) or out-of-frame translation due to ribosome read-through (Celik et al., 2017), we ruled out these possibilities due to the absence of sufficient evidence in Arabidopsis genes. We examined three NMD-triggering features to judge whether the individual TNL or CNL transcripts are putatively NMD regulated:

Intron in the 3’ UTR: Introns occurring more than 55 nt downstream of the termination codon (TC) trigger NMD, by which the ribosome-bound UPF1 and EJC-bound UPF2-UPF3
complex are well spaced to activate UPF1 (Kertesz et al., 2006; Le Hir et al., 2016; Nyikó et al., 2013).

**Long 3'UTR:** Long 3'UTR (≥350 nt)-containing transcripts are also NMD targets in Arabidopsis (Kalyna et al., 2012; Kerényi et al., 2008; Kertesz et al., 2006), wherein UPF1, which otherwise falls off the normal mRNA template due to its own ATPase activity, binds in a size-dependent manner for the phosphorylation and activation of NMD (Kurosaki et al., 2014; Peccarelli and Kebaara, 2014).

**Upstream ORF (uORF):** Although uORF-driven NMD has also been considered an EJC type, exactly how it can trigger NMD is still unclear, since the translation of uORFs is often leaky. Approximately 20% of Arabidopsis genes contain uORFs (Kochetov et al., 2002), of which only approximately 2% are putative NMD targets that contain uORFs encoding ≥35-amino-acid peptides that are likely to be stably expressed (Nyikó et al., 2009, 2013). The MAGNESIUM/PROTON EXCHANGER (AtMHX) uORF, encoding a 13-amino-acid peptide, induces NMD in a downstream intron-dependent manner (Saul et al., 2009). Here, however, we considered CNL and TNL transcripts containing only uORFs encoding peptide(s) ≥35 amino acids as NMD targets based on general observations (Nyikó et al., 2009). The transcripts carrying uORFs that overlap with the authentic initiation codons were also considered to have putative NMD-sensitive features since this event showed strong correlations with sensitivity to NMD, even in the transcripts with uORFs encoding ≤35 amino acids (Kalyna et al., 2012).

**RNA analysis**

Leaves of 4-week-old Arabidopsis (~ 0.1 g) infiltrated with the PstDC3000 strain or 80 µM ActD/20 µM CHX were harvested at the indicated time points after treatment. RNA was extracted from the leaves following the manufacturer’s instructions (TRIzol reagent, Thermo Fisher Scientific). TURBO DNase™ (2 units) (Thermo Fisher Scientific) was used to remove contaminating genomic DNA. After phenol/chloroform/isoamyl alcohol extraction, the purified RNA was dissolved in DEPC-treated water. Five micrograms of total RNA was used to synthesize first-strand cDNA by SuperScript™-II RTase (Thermo Fisher Scientific), and the first-strand cDNA was diluted 10-fold for standard RT-PCR or qRT-PCR. qRT-PCR was performed with SYBR® Premix Ex Taq™ (TaKaRa Bio) as described previously (Jung et al., 2009). All experiments were performed in three biological replicates with two or three technical
repeats. The oligonucleotide sequences used in this study are presented in Supplemental Data Set 3.

**Quantification of cell death-like responses**

Electrolyte leakage measurements were performed to quantify the hypersensitive response in plants after infection (Sohn et al., 2012). After infiltrating *PstDC3000* into the leaves of WT and mutant plants, 10 leaf disks per biological replicate (n = 4) were collected from the infected leaves of different plants, rinsed with distilled water for 30 minutes, and submerged in 2 mM MgSO₄. Conductivity was measured from 1 to 36 hpi with a LAQUAtwin compact water quality meter (Horiba Sci.).

**flg22 treatment and SA measurement**

flg22 (1 µM, Peptron) was infiltrated into *Arabidopsis* leaves, and the treated plants were transferred to a growth chamber (22±1°C, 12-hour day/12-hour night). The leaves were collected at the indicated time points after treatment to examine the levels of UPF proteins. Free-SA levels were also quantified using HPLC coupled with a fluorescence detector (Agilent) as described previously (Jung et al., 2009; Seskar et al., 1998).

**Monoclonal and polyclonal antibodies against UPF proteins, IP, and immunoblot analysis**

Following analysis of the UPF1, UPF2, and UPF3 primary sequences using the SEAL™ package (http://www.ab-mart.com), 4 peptide fragments per protein were designed to yield monoclonal antibodies (Supplemental Figure 16). After vigorous tests for 3 to 4 antibodies raised from each peptide using ELISA and immunoblot analysis, one epitope each from the four peptides against each protein appeared to generate a specific antibody: UPF1 (41-GSPTAWPTPSDS-52), UPF2 (933-ENGEAHGEESDS-944) and UPF3 (192-NKPSPRPSKRNS-203). These antibodies were used for further immunoblot and immunoprecipitation (IP) analyses. The specificity and sensitivity of the selected monoclonal antibodies were verified by immunoblotting using different *upf1*, *upf2*, and *upf3* mutant alleles (Supplemental Figure 6).

GST-UPF1(1-232), covering the N-terminal and CH domains, was bacterially expressed and purified by glutathione-agarose chromatography (Green and Sambrook, 2012), followed by
extraction from SDS-polyacrylamide gels, and used to raise an anti-UPF1 rabbit polyclonal antibody (Youngin Biotech, Korea).

For IP, leaf samples were ground in extraction buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM NaCl (for Figure 5; Supplemental Figure 9) or 150 mM NaCl (for Figures 3, 6, and 7; Supplemental Figures 8 and 12), 1 mM PMSF, and 1x proteinase inhibitor and clarified at 15,000 x g for 10 minutes at 4°C. The homogenate was incubated with protein G-magnetic beads (Invitrogen) that were coated with the α-UPF1, α-UPF2, or α-UPF3 monoclonal antibody for an hour at 4°C. The immune complex on the magnetic beads was washed five times in buffer containing 20 mM Tris-Cl (pH 8.0), 1 mM EDTA, 100 mM (or 150 mM for high stringency) NaCl, and 0.5% Triton X-100, resuspended in SDS sample buffer, and loaded on SDS-polyacrylamide gels for subsequent immunoblot analysis.

Finely ground leaf powder was homogenized in an equal volume of protein extraction buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 0.1% SDS, 5 mM dithiothreitol, and 1x proteinase inhibitor and loaded onto SDS-polyacrylamide gels after clarification for immunoblot analysis as described previously (Green, 2012). The antibodies and manufacturers were as follows: α-UBQ (AS08 307, Agrisera), α-PR1 (AS10 687, Agrisera), and α-PAP (P1291, Sigma-Aldrich). The dilution of primary antibodies used in this study was 1:5,000. The signal was visualized with a chemiluminescent substrate (Intron).

### ChIP-qPCR

Nuclei from the leaves of 4-week-old WT and upf3-1 upf1-5 plants were extracted and purified as described previously (Jaskiewicz et al., 2011). The purified nuclei were resuspended in nucleus lysis buffer [50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 1% SDS, and complete proteinase inhibitor (Roche)], and the resulting chromatin was sheared by sonication to obtain fragment sizes ranging from 200 to 800 bp (Biorupter, Diagenode). The ChIP assay was carried out using an anti-CTD (phospho-S5) antibody (ab5131, Abcam) as described by the manufacturer (Pierce® Agarose ChIP Kit, Thermo Fisher Scientific). RNA polymerase II enrichment between the transcription start sites and translation initiation sites of representative TNL- and CNL-type R genes was determined by qRT-PCR and calculated by the percent input method (Lin et al., 2012). The primers used in this study are listed in Supplemental Data Set 3.
**Statistical analysis**

One-way analysis of variance (ANOVA) with a post hoc Tukey honest significant difference (HSD) test was used for multiple comparisons. To analyze significant differences between the control and treatment groups, a two-tailed Student’s t-test was employed in this study. The sample size (n) and type of error bar are indicated in the figure legends. All pathophysiological experiments were performed in at least triplicate. The results of all statistical analyses performed in this study are presented in Supplemental Data Set 4 and 5.

**Accession numbers**

The accession numbers for the genes examined and discussed in this article are as follows:

- ACTIN2 (At3g18780), CASC3/Barentsz eIF4AIII-binding protein (BTZ1) (At1g80000), BTZ2 (At1g15280), EDS1 (At3g48090), eIF4AIII (At3g19760), FRK1 (At2g19190), ICS1/SID2 (At1g74710), LPEAT2 (At2g45670), MAGO (At1g02140), MPK3 (At3g45640), MPK6 (At2g43790), NHL10 (At2g35980), NPR1 (At1g64280), PAD4 (At3g52430), PRI (At2g14610), RBOHD (At5g47910), RBOHF (At1g64060), RPM1 (At3g07040), RPP5 (At4g16950), RPP7 (At1g58602), RPS2 (At4g26090), RPS4 (At5g45250), RPS5 (At1g12220), RPS6 (At5g46740), RRS1 (At5g45260), RSG2 (At5g43730), SIKIC3 (At4g16960), SMG7 (At5g19400), SNC1 (At4g16890), SOC3 (At1g17600), SUMM2 (At1g12280), UPF1 (At5g47010), UPF2 (At2g39260), UPF3 (At1g33980), and Y14 (At1g51510).

RNA-seq data have been deposited in The National Agricultural Biotechnology Information Center (NABIC) (http://nabic.rda.go.kr/) under accession numbers NN-5098, NN-5102, NN-5103, NN-5104, and NN-5105.

**Supplemental Data**

**Supplemental Figure 1.** upf1-5 upf3-1 and upf3-1 upf1-5 exhibit severe necrotic responses and enhanced immune responses against infection with virulent *Pseudomonas* strains.

**Supplemental Figure 2.** Representative R transcripts that accumulated in the leaves of NMD-compromised mutants
Supplemental Figure 3. Stability of the mRNAs of TNLs and CNLs in WT and upf3-1 upf1-5 plants.

Supplemental Figure 4. RNAPII enrichment at FS R genes in WT and upf3-1 upf1-5 plants.

Supplemental Figure 5. Upregulation of the FS natural UPF1, UPF3, and SMG7 transcripts and AS versions of UPF1 and UPF2 in WT leaves during infection.

Supplemental Figure 6. Morphology of the WT and NMD-compromised mutants and the specificities of monoclonal antibodies used in this study.

Supplemental Figure 7. Stability of UPF1 in WT leaves infected with different P. syringae strains.

Supplemental Figure 8. Levels of UPF proteins in the immune complexes shown in Figures 3B, 3C, and 6C.

Supplemental Figure 9. UPF2 physically associates with UPF3 regardless of bacterial infection.

Supplemental Figure 10. Steady-state levels and stability of the mRNAs of TNLs and CNLs in WT leaves infected with a virulent PstDC3000 strain.

Supplemental Figure 11. Steady-state levels and stability of putative miRNA-target R transcripts.

Supplemental Figure 12. Induction of UPF1 and UPF3 ubiquitination occurs independently of reactive oxygen species burst or SA signaling.

Supplemental Figure 13. Structures of the fusion transcripts encoded by two separately annotated genes and their protein products.

Supplemental Figure 14. RNA-seq coverage plots of the At1g57630-57650 fusion transcript.

Supplemental Figure 15. Physical locations of Arabidopsis R genes.

Supplemental Figure 16. Amino acid sequences of UPF1, UPF2, and UPF3.

Supplemental Data Set 1. Read counts and FPKM values of the Arabidopsis NLR genes determined by RNA-seq in the WT and NMD-compromised mutants 4 hours after ActD treatment.
Supplemental Data Set 2. NMD-sensitive features in the TNL- and CNL-type genes expressed in Arabidopsis leaves.

Supplemental Data Set 3. Oligonucleotides used in this study.

Supplemental Data Set 4. Statistical analysis of the results of shown in the figures.

Supplemental Data Set 5. Statistical analysis of the results of shown in the supplemental figures.

Supplemental File 1. Nucleotide sequences of the genomic DNA region of each TNL-type gene in the Arabidopsis genome.

Supplemental File 2. Genomic DNA sequences of Arabidopsis CNL-type genes.

Supplemental File 3. Nucleotide sequences of the FS natural transcripts and the AS variants of each TNL-type gene obtained by RNA-seq analysis.

Supplemental File 4. Transcripts of the CNL-type genes identified by RNA-seq analysis.

Supplemental File 5. Legends of Supplemental Files 1 and 2.

Supplemental File 6. The 85 FS natural transcripts and their AS variants from the Arabidopsis TNL genes expressed in leaves of the WT and upf mutants.

Supplemental File 7. The 43 FS natural transcripts and their AS variants from the Arabidopsis CNL genes expressed in leaves of the WT and upf mutants.

Table 1. Detailed NMD-sensitive characteristics of 85 TNL and 43 CNL genes expressed in Arabidopsis leaves.

| NMD-sensitive features                        | Number of genes (percent) |      |
|----------------------------------------------|---------------------------|------|
|                                              | TNLs                      | CNLs |
| Intron in the 3′ UTR                         | 19 (22.3%)                | 8 (18.6%) |
| Long 3′ UTR                                  | 54 (63.5%)                | 23 (53.4%) |
| uORF                                         | 32 (37.6%)                | 16 (37.2%) |
| AS-NMD                                       | 43 (50.6%)                | 13 (30.2%) |
| Alternative promoters to NMD                 | 12 (27.9%)                | 7 (16.3%) |
| Alternative polyadenylation to NMD           | 15 (17.6%)                | 3 (7.0%) |
| Fusion transcripts between the flanking genes resulting in NMD | 9 (10.6%) | 7 (16.3%) |
ACKNOWLEDGMENTS

We thank J. W. S. Brown (Dundee U., UK), S. B. Choi (Myongji U.), K. H. Sohn (Postech), and D. Choi (Seoul National U.) for critical reading and discussions. We thank W. T. Kim (Yonsei U.) and O. Kim (Korea U.) for the rbohD rbohF seeds, J.-H. Ko (Kyung Hee U.) for the MPK6CA seeds, and C. Zipfel (The Sainsbury Laboratory) for the bak1-5 seeds. This work was carried out with the support of the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01365101)" Rural Development Administration, Republic of Korea (S. H. K) and Basic Science Research Program from National Research Foundation of Korea (2019R1I1A3A01063543 to H. W. J).

AUTHOR CONTRIBUTIONS

H.W.J. and S.H.K. conceived the study, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. H.W.J., G.K.P., G.Y.J., Y.J.L., K.H.S., A.S., E.S.C., E.L., K.M.K., S.H.Y., J. S. J. and S.C.L. performed the experiments.

REFERENCES

Alcazar, R., and Parker, J.E. (2011). The impact of temperature on balancing immune responsiveness and growth in *Arabidopsis*. Trends Plant Sci. 16: 666-675.

Arciga-Reyes, L., Wootton, L., Kieffer, M., and Davies, B. (2006). UPF1 is required for nonsense-mediated mRNA decay (NMD) and RNAi in *Arabidopsis*. Plant J. 47: 480-489.

Banihashemi, L., Wilson, G.M., Das, N., and Brewer, G. (2006). Upf1/Upf2 regulation of 3' untranslated region splice variants of AUF1 links nonsense-mediated and A+U-rich element-mediated mRNA decay. Mol. Cell. Biol. 26: 8743-8754.

Berrocal-Lobo, M., Stone, S., Yang, X., Antico, J., Callis, J., Ramonell, K.M., and Somerville, S. (2010). ATL9, a RING zinc finger protein with E3 ubiquitin ligase activity implicated in chitin- and NADPH oxidase-mediated defense responses. PLoS One 5: e14426.

Bocca, B., Sarazin, A., Thiebaud, O., Jay, F., Voinnet, O., Navarro, L., and Colot, V. (2014). The Arabidopsis miR472-RDR6 silencing pathway modulates PAMP- and effector-triggered immunity through the post-transcriptional control of disease resistance genes. PLoS Pathog. 11: e1004814.

Boutrot, F., Segonzac, C., Chang, K.N., Qiao, H., Ecker, J.R., Zipfel, C., and Rathjen, J.P. (2010). Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1. Proc. Natl. Acad. Sci. USA 107: 14502-14507.

Braten, O., Livneh, I., Ziv, T., Admon, A., Kehat, I., Caspi, L.H., Gonen, H., Bercovich, B., Godzik, A., Jahandideh, S., et al. (2016). Numerous proteins with unique characteristics are
degraded by the 26S proteasome following monoubiquitination. Proc. Natl. Acad. Sci. USA 113: E4639-4647.

Bull, C. T., Manceau, C., Lydon, J., Kong, H., Vinatzer, B. A., and Fischer-Le Saux, M. (2010) Pseudomonas cannabina pv. cannabina pv. nov., and Pseudomonas cannabina pv. alisalensis (Cintas Koike and Bull, 2000) comb. nov., are members of the emended species Pseudomonas cannabina (ex Sutic & Dowson 1959) Gardan, Shafik, Belouin, Brosch, Grimont & Grimont 1999. Syst. Appl. Microbiol. 33: 105–115.

Cai, Q., Liang, C., Wang, S., How, Y., Gao, L., Liu, L., He, W., Ma, W., Mo, B., and Chen, X. (2018). The disease resistance protein SNC1 represses the biogenesis of microRNAs and phased siRNAs. Nat. Comm. 9: 5080

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57-63.

Carstens, M., McCrindle, T.K., Adams, N., Diener, A., Guzha, D.T., Murray, S.L., Parker, J.E., Denby, K.J., and Ingle, R.A. (2014). Increased resistance to biotrophic pathogens in the Arabidopsis constitutive induced resistance 1 mutant is EDS1 and PAD4-dependent and modulated by environmental temperature. PLoS One 9: e109853.

Celik, A., Baker, R., He, F., and Jacobson, A. (2017). High-resolution profiling of NMD targets in yeast reveals translational fidelity as a basis for substrate selection. RNA 23: 735-748.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124: 803-814.

Ciechanover, A., and Stanhill, A. (2014). The complexity of recognition of ubiquitinated substrates by the 26S proteasome. Biochim. Biophys. Acta 1843: 86-96.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735-743.

Degtiar, E., Fridman, A., Gottlieb, D., Vexler, K., Berezin, I., Farhi, R., Golani, L., Shaul, O. (2015) The feedback control of UPF3 is crucial for RNA surveillance in plants. Nucleic Acids Res. 43: 4219–4235

DeYoung, B.J., Innes, R.W. (2006) Plant NBS-LRR proteins in pathogen sensing and host defense. Nat. Immunol. 7:1243-1249.

Dinesh-Kumar, S.P., and Baker, B.J. (2000). Alternatively spliced N resistance gene transcripts: their possible role in tobacco mosaic virus resistance. Proc. Natl. Acad. Sci. USA 97: 1908-1913.

Drechsel, G., Kahles, A., Kesarwani, A.K., Stauffer, E., Behr, J., Drewe, P., Ratsch, G., and Wachter, A. (2013). Nonsense-mediated decay of alternative precursor mRNA splicing variants is a major determinant of the Arabidopsis steady state transcriptome. Plant Cell 25: 3726-3742.

Dudler, R. (2013). Manipulation of host proteasomes as a virulence mechanism of plant pathogens. Ann. Rev. Phytopathol. 51: 521-542.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007). P bodies: at the crossroads of post-transcriptional pathways. Nat. Rev. Mol. Cell Biol. 8: 9-22.

Filichkin, S.A., Cumbie, J.S., Dharmawardhana, P., Jaiswal, P., Chang, J.H., Palusa, S.G., Reddy, A.S., Megraw, M., and Mockler, T.C. (2015). Environmental stresses modulate abundance and timing of alternatively spliced circadian transcripts in Arabidopsis. Mol. Plant 8: 207-227.
Garcia, D., Garcia, S., and Voinnet, O. (2014). Nonsense-mediated decay serves as a general viral restriction mechanism in plants. Cell Host Microbe 16: 391-402.

Gloggnitzer, J., Akimcheva, S., Srinivasan, A., Kusenda, B., Riehs, N., Stampfl, H., Bator, J., Dekrout, B., Jonak, C., Jimenez-Gomez, J.M., et al. (2014). Nonsense-mediated mRNA decay modulates immune receptor levels to regulate plant antibacterial defense. Cell Host Microbe 16: 376-390.

Green, M.R., Sambrook, J. (2012). Molecular Cloning: A laboratory Manual, Fourth Edition edn (Cold Spring Harbor Laboratory Press, NY).

Halter, T., and Navarro, L. (2015). Multilayer and interconnected post-transcriptional and co-transcriptional control of plant NLRs. Curr. Opin. Plant Biol. 26: 127-134.

Heidrich, K., Tsuda, K., Blanvillain-Baufume, S., Wirthmüller, L., Bator, J., and Parker, J.E. (2013). Arabidopsis TNL-WRONKY domain receptor RRS1 contributes to temperature-conditioned RPS4 auto-immunity. Front. Plant Sci. 4: 403.

Hori, K., and Watanabe, Y. (2005). UPF3 suppresses aberrant spliced mRNA in Arabidopsis. Plant J. 43: 530-540.

Hudík, E., Berriri, S., Hirt, H., and Colcombet, J. (2014) Identification of constitutively active AtMPK6 mutants using a functional screen in Saccharomyces cerevisiae. Methods Mol. Biol. 1171: 67-77.

Jaskiewicz, M., Peterhansel, C., and Conrath, U. (2011). Detection of histone modifications in plant leaves. Journal of visualized experiments. J. Vis. Exp. 55: 3096

Jeong, H.J., Kim, Y.J., Kim, S.H., Kim, Y.H., Lee, I.J., Kim, Y.K., and Shin, J.S. (2011). Nonsense-mediated mRNA decay factors, UPF1 and UPF3, contribute to plant defense. Plant Cell Physiol. 52: 2147-2156.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature 444: 323-329.

Jones, J.D., Vance, R.E., and Dangl, J.L. (2016). Intracellular innate immune surveillance devices in plants and animals. Science 354: aaf6395.

Jung, H.W., Tschaaplinski, T.J., Wang, L., Glazebrook, J., and Greenberg, J.T. (2009). Priming in systemic plant immunity. Science 324: 89-91.

Kalyna, M., Simpson, C.G., Syed, N.H., Lewandowska, D., Marquez, Y., Kusenda, B., Marshall, J., Fuller, J., Cardle, L., McNicol, J., et al. (2012). Alternative splicing and nonsense-mediated decay modulate expression of important regulatory genes in Arabidopsis. Nucl. Acids Res. 40: 2454-2469.

Karasov, T.L., Chae, E., Herman, J.J., and Bergelson, J. (2017). Mechanisms to Mitigate the Trade-Off between Growth and Defense. Plant Cell 29: 666-680.

Kelley, D.R., and Estelle, M. (2012). Ubiquitin-mediated control of plant hormone signaling. Plant Physiol. 160: 47-55.

Kerényi, Z., Merai, Z., Hiripi, L., Benkovics, A., Gyula, P., Lacomme, C., Barta, E., Nagy, F., and Silhavy, D. (2008). Inter-kingdom conservation of mechanism of nonsense-mediated mRNA decay. EMBO J. 27: 1585-1595.

Kertesz, S., Kerényi, Z., Merai, Z., Bartos, I., Palfy, T., Barta, E., and Silhavy, D. (2006). Both introns and long 3'-UTRs operate as cis-acting elements to trigger nonsense-mediated decay in plants. Nucl. Acids Res. 34: 6147-6157.

Kesarwani, A.K., Lee, H.C., Ricca, P.G., Sullivan, G., Faisss, N., Wagner, G., Wunderling, A., and Wachter, A. (2019). Multifactorial and species-specific feedback regulation of the RNA surveillance pathway nonsense-mediated decay in plants. Plant Cell Physiol. 60:1986-1999.
Kim, D., Pertea, G., Trapnell, C., Pimentel, H., Kelley, R., and Salzberg, S.L. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. 14: R36.

Kim, S.H., Koroleva, O.A., Lewandowska, D., Pendle, A.F., Clark, G.P., Simpson, C.G., Shaw, P.J., and Brown, J.W. (2009). Aberrant mRNA transcripts and the nonsense-mediated decay proteins UPF2 and UPF3 are enriched in the Arabidopsis nucleolus. Plant Cell 21: 2045-2057.

Kim, V.N., Kataoka, N., and Dreyfuss, G. (2001). Role of the nonsense-mediated decay factor hUpf3 in the splicing-dependent exon-exon junction complex. Science 293: 1832-1916.

Kochetov, A.V., Syrnik, O.A., Rogozin, I.B., Glazko, G.V., Komarova, M.L., and Shumnyi, V.K. (2002). [Context organization of mRNA 5’-untranslated regions of higher plants]. Molekuliarnaia biologiia 36: 649-656.

Kurihara, Y., Matsui, A., Hanada, K., Kawashima, M., Ishida, J., Morosawa, T., Tanaka, M., Kaminuma, E., Matsushima, A., et al. (2009). Genome-wide suppression of aberrant mRNA-like noncoding RNAs by NMD in Arabidopsis. Proc. Natl. Acad. Sci. USA 106: 2453-2458.

Kurosaki, T., Li, W., Hoque, M., Popp, M.W., Ermolenko, D.N., Tian, B., and Maquat, L.E. (2014). A post-translational regulatory switch on UPF1 controls targeted mRNA degradation. Genes Dev. 28: 1900-1916.

Lai, Y., and Eulgem, T. (2018). Transcript-level expression control of plant NLR genes. Mol. Plant Pathol. 19: 1267-1281.

Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat. Methods 9: 357-359.

Le Hir, H., Sauliere, J., and Wang, Z. (2016). The exon junction complex as a node of post-transcriptional networks. Nat. Rev. Mol. Cell Biol. 17: 41-54.

Li, X., Clarke, J.D., Zhang, Y., and Dong, X. (2001). Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. Mol. Plant Microbe Interact. 14: 1131-1139.

Li, X., Kapos, P., and Zhang, Y. (2015) NLRs in plants. Curr. Opin. Immunol. 32: 114-121

Lin, X., Tirichine, L., and Bowler, C. (2012). Protocol: Chromatin immunoprecipitation (ChIP) methodology to investigate histone modifications in two model diatom species. Plant Methods 8: 48.

Lu, D., Lin, W., Gao, X., Wu, S., Cheng, C., Avila, J., Heese, A., Devarenne, T.P., He, P., and Shan, L. (2011). Direct ubiquitination of pattern recognition receptor FLS2 attenuates plant innate immunity. Science 332: 1439-1442.

Maekawa, T., Kufer, T.A., and Schulze-Lefert, P. (2011). NLR functions in plant and animal immune systems: so far and yet so close. Nat. Immunol. 12: 817-826.

Marino, D., Peeters, N., and Rivas, S. (2012). Ubiquitination during plant immune signaling. Plant Physiol. 160: 15-27.

Merai, Z., Benkovics, A.H., Nyikó, T., Debreczeny, M., Hiripi, L., Kerényi, Z., Kondorosi, E., and Silhavy, D. (2013). The late steps of plant nonsense-mediated mRNA decay. Plant J. 73: 50-62.

Merchante, C., Brunmos, J., Yun, J., Hu, Q., Spencer, K.R., Enriquez, P., Binder, B.M., Heber, S., Stepanova, A.N., and Alonso, J.M. (2015). Gene-specific translation regulation mediated by the hormone-signaling molecule EIN2. Cell 163: 684-697.
Palusa, Riehs, N., Akimcheva, S., Puizina, J., Bulankova, P., Idol, R.A., Siroky, J., Schleiffer, Riehs, Rebbapragada, I., and Lykke-Rasmussen, M.W., Ro-
Ramonell, K., Berrocal-Peccarelli, M., and Kebaara, B.W.
Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J.
Nyikó, T., Kerényi, F., Szabadkai, L., Benkovics, A.H., Major, P., Sonkoly, B., Merai, Z.,
Nawrath, C., and Metraux, J.P.
Meyers, B.C., Kozik, A., Griego, A., Kuang, H., and Michelmore, R.W. (2003). Genome-
wide analysis of NBS-LRR-encoding genes in Arabidopsis. Plant Cell 15: 809-834.
Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of
Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen
inoculation. Plant Cell 11: 1393-1404.
Nicaise, V., Roux, M., and Zipfel, C. (2009). Recent advances in PAMP-triggered immunity
against bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol.
150: 1638-1647.
Nyikó, T., Sonkoly, B., Merai, Z., Benkovics, A.H., and Silhavy, D. (2009). Plant upstream
ORFs can trigger nonsense-mediated mRNA decay in a size-dependent manner. Plant Mol.
Biol. 71: 367-378.
Palma, K., Thorgrimsson, S., Malinovsky, F.G., Fiil, B.K., Nielsen, H.B., Brodersen, P.,
Hofius, D., Petersen, M., and Mundy, J. (2010). Autoimmunity in Arabidopsis acd11 is
mediated by epigenetic regulation of an immune receptor. PLoS Pathog. 6, e1001137.
Palusa, S.G., Reddy, A.S. (2010) Extensive coupling of alternative splicing of pre-mRNAs of
serine/arginine (SR) genes with nonsense-mediated decay. New Phytol. 135, 83-89.
Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J. (1996).
Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora
parasitica specified by several different RPP genes. Plant Cell 8, 2033-2046.
Peccarelli, M., and Kebaara, B.W. (2014). Regulation of natural mRNAs by the nonsense-
mediated mRNA decay pathway. Euk. Cell 13: 1126-1135.
Ramonell, K., Berrocal-Lobo, M., Koh, S., Wan, J., Edwards, H., Stacey, G., and
Somerville, S. (2005). Loss-of-function mutations in chitin responsive genes show increased
susceptibility to the powdery mildew pathogen Erysiphe cichoracearum. Plant Physiol. 138:
1027-1036.
Rasmussen, M.W., Roux, M., Petersen, M., and Mundy, J. (2012). MAP Kinase Cascades in
Arabidopsis Innate Immunity. Front. Plant Sci. 3: 169.
Raxwal, V.K. and Riha, K. (2016) Nonsense mediated RNA decay and evolutionary
capacitance. Biochim. Biophys. Acta 1859: 1538-1543.
Rayson, S., Arciga-Reyes, L., Wootton, L., De Torres Zabala, M., Truman, W., Graham,
N., Grant, M., and Davies, B. (2012). A role for nonsense-mediated mRNA decay in plants:
pathogen responses are induced in Arabidopsis thaliana NMD mutants. PLoS One 7: e31917.
Rebbapragada, I., and Lykke-Andersen, J. (2009). Execution of nonsense-mediated mRNA
decay: what defines a substrate? Current Opin. Cell Biol. 21: 394-402.
Riehs-Kearnan, N., Gloggnitzer, J., Dekrout, B., Jonak, C., and Riha, K. (2012). Aberrant
growth and lethality of Arabidopsis deficient in nonsense-mediated RNA decay factors is
caused by autoimmune-like response. Nucl. Acids Res. 40: 5615-5624.
Riehs, N., Akimcheva, S., Puizina, J., Bulankova, P., Idol, R.A., Siroky, J., Schleiffer, A.,
Schweizer, D., Shippen, D.E., and Riha, K. (2008). Arabidopsis SMG7 protein is required
for exit from meiosis. J. Cell Sci. 121: 2208-2216.
Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G.,
and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. 29: 24-26.
Rohila, J.S., Chen, M., Cerny, R., and Fromm, M.E. (2004). Improved tandem affinity purification tag and methods for isolation of protein heterocomplexes from plants. Plant J. 38: 172-181.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., et al. (2003). TM4: a free, open-source system for microarray data management and analysis. BioTechniques 34: 374-378.

Saul, H., Elharrar, E., Gaash, R., Eliaz, D., Valenci, M., Akua, T., Avramov, M., Frankel, N., Berezin, I., Gottlieb, D., et al. (2009). The upstream open reading frame of the Arabidopsis AtMHX gene has a strong impact on transcript accumulation through the nonsense-mediated mRNA decay pathway. Plant J. 60: 1031-1042.

Schweingruber, C., Rufener, S.C., Zund, D., Yamashita, A., and Muhlemann, O. (2013). Nonsense-mediated mRNA decay - mechanisms of substrate mRNA recognition and degradation in mammalian cells. Biochim. Biophys. Acta 1829: 612-623.

Schwessinger, B., Roux, M., Kadota, Y., Ntoukakis, V., Sklenar, J., Jones, A., and Zipfel, C. (2011). Phosphorylation-dependent differential regulation of plant growth, cell death, and innate immunity by the regulatory receptor-like kinase BAK1. PLoS Genet. 7: e1002046.

Seskar, M., Shulaev, V., and Raskin, I. (1998). Endogenous Methyl Salicylate in Pathogen-Infected Tobacco Plants. Plant Physiol. 116: 387-392.

Shabek, N., Herman-Bachinsky, Y., Buchbaum, S., Lewinson, O., Haj-Yahya, M., Hejjajoui, M., Lashuel, H.A., Sommer, T., Brik, A., and Ciechanover, A. (2012). The size of the proteasomal substrate determines whether its degradation will be mediated by mono- or polyubiquitylation. Mol. Cell 48: 87-97.

Shaoul, O. (2015). Unique Aspects of Plant Nonsense-Mediated mRNA Decay. Trends Plant Sci. 20: 767-779.

Shi, C., Baldwin, I.T., and Wu, J. (2012). Arabidopsis plants having defects in nonsense-mediated mRNA decay factors UPF1, UPF2, and UPF3 show photoperiod-dependent phenotypes in development and stress responses. J. Integr. Plant Biol. 54: 99-114.

Shirano, Y., Kachroo, P., Shah, J., and Klessig, D.F. (2002). A gain-of-function mutation in an Arabidopsis Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell 14: 3149-3162.

Shivaprasad, P.V., Chen, H.M., Patel, K., Bond, D.M., Santos, B.A., Baulcombe, D.C. (2012). A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. Plant Cell 24: 859-874.

Sobell H (1985) Actinomycin and DNA transcription. Proc. Natl. Acad. Sci. USA 82: 5328-5331

Sohn, K.H., Hughes, R.K., Piquerez, S.J., Jones, J.D., and Banfield, M.J. (2012). Distinct regions of the Pseudomonas syringae coiled-coil effector AvrRps4 are required for activation of immunity. Proc. Natl. Acad. Sci. USA 109: 16371-16376.

Stegmann, M., Anderson, R.G., Ichimura, K., Pecenkova, T., Reuter, P., Zarsky, V., McDowell, J.M., Shirasu, K., and Trujillo, M. (2012). The ubiquitin ligase PUB22 targets a subunit of the exocist complex required for PAMP-triggered responses in Arabidopsis. Plant Cell 24: 4703-4716.

Stuttmann, J., Peine, N., Garcia, A.V., Wagner, C., Choudhury, S.R., Wang, Y., James, G.V., Griebel, T., Alcazar, R., Tsuda, K., et al. (2016). Arabidopsis thaliana DM2h (R8) within the Landsberg RPP1-like Resistance Locus Underlies Three Different Cases of EDS1-Conditioned Autoimmunity. PLoS Genet. 12: e1005990.
Sukarta, O.C.A., Slootweg, E.J., and Goverse A. (2016). Structure-informed insights for NLR functioning in plant immunity. Semin. Cell Dev. Biol. 56:134-149.

Sureshkumar, S., Dent, C., Seleznev, A., Tasset, C., and Balasubramanian, S. (2016). Nonsense-mediated mRNA decay modulates FLM-dependent thermosensory flowering response in Arabidopsis. Nat. Plants 2: 16055.

Takahashi, S., Araki, Y., Ohya, Y., Sakuno, T., Hoshino, S., Kontani, K., Nishina, H., and Katada, T. (2008). Upf1 potentially serves as a RING-related E3 ubiquitin ligase via its association with Upf3 in yeast. RNA 14: 1950-1958.

Thorvaldsdottir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. 14: 178-192.

Torres, M.A., Dangl, J.L., and Jones, J.D. (2002). Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. USA 99: 517-522.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28: 511-515.

Trujillo, M., Ichimura, K., Casais, C., and Shirasu, K. (2008). Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in Arabidopsis. Curr. Biol. 18: 1396-1401.

Van de Weyer, A-L., Monteiro, F., Furzer, O.J., Nishimura, M.T., Cevik, V., Witek, K., Jones, J.D.G., Dangl, J.L., Weigel, D., and Bemm, F. (2019) A species-wide inventory of NLR genes and alleles in Arabidopsis thaliana. Cell 178: 1260-1272.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., and Zhang, S. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell 19: 63-73.

Wang, Y., Bao, Z., Zhu, Y., and Hua, J. (2009). Analysis of temperature modulation of plant defense against biotrophic microbes. Mol. Plant Microbe Interact. 22: 498-506.

Wu, C.H., Abd-El-Haliem, A., Bozkurt, T.O., Belhaj, K., Terauchi, R., Vossen, J.H., and Kamoun, S. (2017). NLR network mediates immunity to diverse plant pathogens. Proc. Natl. Acad. Sci. USA 114: 8113-8118.

Xu, F., Xu, S., Wiermer, M., Zhang, Y., and Li, X. (2012). The cyclin L homolog MOS12 and the MOS4-associated complex are required for the proper splicing of plant resistance genes. Plant J. 70: 916-928.

Yang, S., Tang, F. and Zhu, H. (2014) Alternative splicing in plant immunity. Int. J. Mol. Sci. 15: 10424–10445.

Yi, H., and Richards, E.J. (2007) A cluster of disease resistance genes in Arabidopsis is coordinately regulated by transcriptional activation and RNA silencing. Plant Cell 19: 2929-2939.

Yoine, M., Ohto, M.A., Onai, K., Mita, S., and Nakamura, K. (2006). The lb1 mutation of UPF1 RNA helicase involved in nonsense-mediated mRNA decay causes pleiotropic phenotypic changes and altered sugar signalling in Arabidopsis. Plant J. 47: 49-62.
D.J., Meyers, B.C. (2011). MicroRNAs as master regulators of the plant NB-LRR defense gene family via the production of phased, trans-acting siRNAs. Genes Dev. 25: 2540-2553.

Zhang, X.C., and Gassmann, W. (2007). Alternative splicing and mRNA levels of the disease resistance gene RPS4 are induced during defense responses. Plant Physiol. 145: 1577-1587.

Zhang, Y., Xia, R., Kuang, H., Meyers, B.C. (2016). The diversification of plant NBS-LRR defense genes directs the evolution of microRNAs that target them. Mol. Biol. Evol. 33: 2692-705.
Figure 1. The upf1 upf3 double mutants exhibit intensified autoimmunity. (A) and (B) Five-week-old Arabidopsis thaliana ecotype Columbia-0 (WT), upf1-5, upf3-1, upf1-5 upf3-1, and upf3-1 upf1-5 plants were grown at 22±1°C (A) or 28±1°C (B) with a 16-hour day/8-hour night photoperiod. Simultaneous mutation of UPF1 and UPF3 arrested Arabidopsis growth at 22°C, while the growth defect was moderately recovered by a temperature shift to 28°C. Bars = 5 cm.

(C) Bacterial counts and disease symptoms in the WT and NMD-compromised mutants on day 3 after PstDC3000 and PcaES4326 infection. Different letters indicate statistically significant differences. P<0.01; one-way analysis of variance (ANOVA). Error bars indicate SE (n = 8).

(D) Venn diagrams of TNL- and CNL-type R genes illustrating the number of genes carrying different NMD features. Numbers in brackets are the total number of expressed genes [*] and the total number of genes bearing each NMD event (**).

(E) to (G) Stability of NMD reporters (E), representative TNL transcripts (F) and representative CNL transcripts (G). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis was performed using total RNA extracted from ActD-treated leaves of the WT and upf3-1 upf1-5 (u3u1) mutants that had been collected at 0, 1, 2, and 4 hours posttreatment. The half-lives were calculated by nonlinear least-squares regression analysis [average ± SD, n = 3 (three biological replicates with three technical repeats)]. Stability analyses of other R transcripts are provided in Supplemental Figure 3. Sequences of the individual primers used in this study are presented in Supplemental Data Set 3.
Figure 2. UPF1, UPF2, and UPF3 decay during an early phase of *PstDC3000* infection.

(A) Dynamics of UPF1, UPF2, and UPF3 proteins up to 30 hours post inoculation (hpi; upper panel) and 50 minutes post inoculation (mpi; lower panel). Immunoblot analyses were performed for leaf samples taken from WT Col-0 plants that had been infected with *Pseudomonas* and collected at the indicated time points using an anti-UPF1 monoclonal antibody (α-UPF1), α-UPF2 or α-UPF3. The right panel shows UPF protein levels in infected leaves at the indicated time points (average ± SD, n = 4). Different letters above the bars indicate statistically significant differences (P<0.05, one-way ANOVA). Successful infection was verified by examining the levels of PR1. Both experiments were performed with four biological replicates.

(B) The NMD factor SMG7 and the EJC core protein components, but not UPF1, UPF2, or UPF3, remained stable during *PstDC3000* infection. The leaves of transgenic *Arabidopsis* plants (T3) stably expressing protein A-fused UPF1, UPF2, UPF3, SMG7, Y14, MAGO, elF4A-III, BTZ1, and BTZ2 were infected with *PstDC3000* and collected at the indicated time points after infection for immunoblot analysis. GFP-protein A was used as a representative stable protein. The recombinant proteins were detected using an α-PAP antibody. M indicates a prestained protein ladder.
Figure 3. *P. syringae* infection induces ubiquitination of UPF1 and UPF3 in *Arabidopsis* and *N. benthamiana* plants.

(A) Effects of the 26S proteasome inhibitor MG132 on the decay of UPF1, UPF2, and UPF3 during infection. DMSO alone or MG132 dissolved in DMSO was coinfiltrated into WT leaves with *Pst* DC3000. Immunoblot analyses were performed using α-UPF1, α-UPF2, or α-UPF3. Successful infection was verified by examining the levels of PR1.

(B) Ubiquitination of UPF1 and UPF3, but not UPF2, in WT leaves after *Pst* DC3000 infection. Immunocomplexes with the α-UPF1, α-UPF2, or α-UPF3 antibody were subjected to immunoblotting with an anti-ubiquitin antibody (α-UBQ).

(C) Immunoprecipitation assay with proteins extracted from MG132+*Pst* DC3000-treated WT plants. The levels of UPF proteins in IP complexes in (B) and (C) are shown in Supplemental Figure 8A and 8B, respectively.

(D) Degradation of UPF proteins in *N. benthamiana* leaves infected with *P. syringae* pv. *syringae* B728a (*Pss*B728a).

(E) Induction of UPF1 and UPF3 ubiquitination in *N. benthamiana* leaves during *Pss*B728a infection. Levels of ubiquitinated UPF1 and UPF3 were determined by IP with α-UPF1 or α-UPF3 and subsequent immunoblot analysis with α-UPF1, α-UPF3, and α-UBQ.
Figure 4. Steady-state levels and the stability of R transcripts in PstDC3000-infected WT plants.

(A) Stability of NMD markers Atg29210 PTC+ and LPEAT2 PTC+ in infected WT leaves.

(B) and (C) Transcript levels and half-lives of representative TNL transcripts, the non-NMD target RPS4, and SIKIC3 and RPS6 bearing NMD-sensitive features (B), and CNL transcripts, the non-NMD target SUMM2, and the NMD-targets RPM1 and RPP7 (C).

(Left panel) Steady-state levels of selected R transcripts were measured in infected WT leaves during PstDC3000 infection without ActD treatment. Bars indicate the average ± SD of three biological replicates with two technical repeats (P<0.01, one-way ANOVA, n = 3).

(Right panel) Either ActD alone or ActD together with PstDC3000 was infiltrated in WT leaves, and the treated leaves were collected at the indicated time points. Data points are the average ± SD of three biological replicates with three technical repeats (n = 3). Sequences of the gene-specific primers are presented in Supplemental Data Set 3.
Figure 5. Induction of UPF1 ubiquitination occurs independently of UPF2 or UPF3, while UPF1 positively affects the induction of UPF3 ubiquitination.

(A) Levels of UPF1, UPF2, and UPF3 in the leaves of the WT (UPF1 UPF3 and UPF2) and the mutant (upf1-1 UPF3, UPF1 upf3-1, and upf2-12) plants before and after PstDC3000 infection (1 hpi).

(B) Induction of UPF1 and UPF3 ubiquitination in WT and mutant plants. Protein complexes with the α-UPF1 or α-UPF3 antibody were subjected to immunoblot analyses with an α-ubiquitin (α-UBQ) antibody. Notably, depletion of UPF3 did not affect the induction of UPF1 ubiquitination (2nd row), while the induced ubiquitination signal of UPF3 was reduced in the upf1-1 allele (4th row). M indicates a prestained protein ladder. The experiment was performed with three biological replicates, which showed similar results.

(C) Depletion of UPF1 reduces the ubiquitination level of UPF3 during PstDC3000 infection. Total proteins were extracted from WT and upf1-1 that had been infected with PstD3000 and immunoprecipitated with α-UPF3 to examine whether UPF3 is differentially ubiquitinated between the WT and upf1-1 mutant at the indicated time points. The immunocomplexes were subjected to immunoblotting with α-UPF3 or α-UBQ.
Figure 6. The recognition of the representative MAMP flg22 is sufficient to trigger UPF protein decay in Arabidopsis.

(A) Dynamics of UPF1, UPF2, and UPF3 proteins in WT leaves treated with 10 mM MgSO₄ (WT-mock) or 1 µM flg22 (WT-flg22) up to 30 hpt.

(B) The extracellular immune receptor (FLS2) and the coreceptor (BAK1) are required to initiate degradation of the UPF proteins upon flg22 treatment.

(C) Perception of flg22 induces ubiquitination of UPF1 and UPF3 in WT leaves. Levels of the UPF proteins in the IP complexes are shown in Supplemental Figure 8C and 8D.

(D) No or delayed induction of UPF1/UPF3 ubiquitination is observed after flg22 treatment in the FLS2 and BAK1 mutants, respectively.

(E) Levels of UPF1, UPF2, and UPF3 proteins in WT, fls2, and bak1-5 leaves during PstDC3000 infection.
Figure 7. Initiation of UPF protein decay involves a MAPK pathway. 
(A) Levels of UPF1, UPF2, and UPF3 in the leaves of *mpk3*, *mpk6* (defective in the MAPK cascade), and *rbohD rbohF* (defective in the ROS burst) mutants after recognition of flg22. 
(B) MPK3 and MPK6, but not RBOHD or RBOHF, are genetically required for the induction of UPF1 and UPF3 ubiquitination. 
(C) *EDS1* (a key player in TNL-type R-triggered immunity and SA regulation), *ICS1/SID2* (SA biosynthesis), and *NPR1* (SA response) are dispensable for the decay of UPF1, UPF2 and UPF3. 
WT and mutant plants were either inoculated with *Pst*DC3000 (*Pst*) or treated with 1 µM flg22 and then used for immunoblotting. Col-0 is the parental line of *mpk3*, *mpk6*, *rbohD rbohF*, *sid2-1*, and *npr1-1*, while Ws (Wassilewskija) is the parental line of *eds1-1*. 
Figure 8. Stable expression of previously uncharacterized fusion transcripts enhances the basal defense response to P. syringae infection in Arabidopsis.

(A) Dynamics of fusion TNL-type R transcript accumulation in WT upon PstDC3000 infection. Data points are the average ± SD with three biological replicates with two technical repeats (n = 3). Statistically significant differences are shown using different letters above the bars (P<0.01, one-way ANOVA).

(B) RNAPII enrichment at the genomic loci containing the split genes in WT (white bars) and upf3-1 upf1-5 (gray bars). Four independent biological experiments were performed in technical triplicates (average ± SE, **P<0.01, two-tailed Student’s t-test, n = 4).

(C) Bacterial growth in WT (white bars) and in two independent transgenic Arabidopsis Col-0 plants expressing either At1g57630-At1g57650 or At5g38344-At5g38350 (light and dark gray bars, respectively) on day 3 after PstDC3000 and PstDC3000 hrcC- infection. Average ± SE values are plotted. Different letters above the bars indicate statistically significant differences (P<0.01, one-way ANOVA, n = 8). The numbers of PstDC3000 in WT plants were compared with those in independent transgenic lines expressing the same fusion transcript, as different transgenic plants had been grown separately.

(D) PR1 mRNA expression in noninfected WT and transgenic Arabidopsis plants used in (C). Different letters above the bars indicate statistically significant differences among the genotypes (average ± SD, P<0.05, one-way ANOVA, n = 3). Three biological experiments were performed with three technical repeats.
Pathogen-associated Molecular Pattern-triggered Immunity Involves Proteolytic Degradation of Core Nonsense-mediated mRNA Decay Factors During the Early Defense Response
Hyon Kim, Eun Su Choi, Eunji Lee, Kyung Man Kim, Seung Hwan Yang, Jong-Seong Jeon, Sung Chul Lee and Sang Ho Won Jung

Plant Cell; originally published online February 21, 2020;
DOI 10.1105/tpc.19.00631

This information is current as of May 6, 2020