The *Arabidopsis* NMD Factor UPF3 Is Feedback-Regulated at Multiple Levels and Plays a Role in Plant Response to Salt Stress

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Nonsense-mediated mRNA decay (NMD) is a eukaryotic RNA surveillance mechanism that degrades aberrant transcripts and controls the levels of many normal mRNAs. It was shown that balanced expression of the NMD factor UPF3 is essential for the maintenance of proper NMD homeostasis in *Arabidopsis*. UPF3 expression is controlled by a negative feedback loop that exposes UPF3 transcript to NMD. It was shown that the long 3′ untranslated region (3′ UTR) of UPF3 exposes its transcript to NMD. Long 3′ UTRs that subject their transcripts to NMD were identified in several eukaryotic NMD factors. Interestingly, we show here that a construct that contains all the regulatory regions of the UPF3 gene except this long 3′ UTR is also feedback-regulated by NMD. This indicates that UPF3 expression is feedback-regulated at multiple levels. UPF3 is constitutively expressed in different plant tissues, and its expression is equal in leaves of plants of different ages. This finding is in agreement with the possibility that UPF3 is ubiquitously operative in the *Arabidopsis* NMD pathway. Expression mediated by the regulatory regions of UPF3 is significantly induced by salt stress. We found that both a deficiency and a strong excess of UPF3 expression are detrimental to plant resistance to salt stress. This indicates that UPF3 plays a role in plant response to salt stress, and that balanced expression of the UPF3 gene is essential for coping with this stress.

**Keywords:** IME, intron-mediated enhancement, NMD regulation, NaCl, negative feedback loop, nonsense-mediated mRNA decay, RNA stability, salt stress

**INTRODUCTION**

Nonsense-mediated mRNA decay (NMD) is an RNA surveillance mechanism that functions in all eukaryotes (reviewed by Celik et al., 2015; Fatscher et al., 2015; He and Jacobson, 2015; Shaul, 2015; Smith and Baker, 2015). Early studies on yeast, nematodes, and mammalian cells showed that transcripts harboring premature termination codons (PTCs) are rapidly degraded (Kinniburgh et al., 1982; Leeds et al., 1991; Pulak and Anderson, 1993). The elimination of PTC-containing transcripts by the NMD mechanism prevents the accumulation of truncated, potentially deleterious, proteins. Moreover, NMD controls the levels of many normal transcripts with features that lead to the recognition of their termination codons (TCs) as premature. The NMD mechanism in mammalian cells is briefly illustrated here. Upon reaching a TC, the ribosome binds the eukaryotic release factors eRF1 and eRF3. Normal translation termination and ribosome recycling,
which prohibit NMD, depend on the interaction between eRF3 and the poly(A)-binding protein (PABP; Fatscher et al., 2014; Joncourt et al., 2014). When the ribosome terminates translation far upstream to the poly(A) due to a PTC or a long 3′ untranslated region (3′ UTR), an eRF3-PABP interaction is prevented. Consequently, eRF3 interacts with the NMD factor UPF1 to form the SURF complex, which also includes eRF1 and the protein kinase SMG1 (Kashima et al., 2006). The interaction between the SURF complex and the NMD factors UPF2 and UPF3 leads to the activation of SMG1, which phosphorylates UPF1 and leads to NMD activation (Kashima et al., 2006; Arias-Palomo et al., 2011; Melero et al., 2014).

Introns located \( \geq 50–55 \) nucleotides (nt) downstream of TCs facilitate NMD (Zhang et al., 1998; Singh et al., 2008). The NMD factor UPF3 interacts with the exon-junction complex (EJC) [a complex of proteins deposited on the mRNA 20–24 nt upstream of exon–exon junctions (EEJ)] before export of the mRNA to the cytoplasm (Lykke-Andersen et al., 2000; Kim et al., 2001). If a TC is located \( \geq 50–55 \) nt upstream of an EEJ, the terminating ribosome does not remove the EJC. The EJC-bound UPF3 then interacts with UPF2, which bridges between UPF3 and UPF1 (Singh et al., 2007), thereby leading to NMD activation. UPF1 can also bind the mRNA in an EJC-independent, 3′ UTR-length-dependent manner (Hogg and Goff, 2010; Shigeka et al., 2012; Kurosaki and Maquat, 2013; Zund et al., 2013; Fiorini et al., 2015). Thus, although introns \( \geq 50–55 \) nt downstream of TCs increase NMD efficiency, they are not essential for this process, and NMD can be activated by long 3′ UTRs alone. NMD can also be activated by upstream open reading frames (uORFs), whose TCs can be recognized as premature due to their long distance from the poly(A) tail.

The basic features of mammalian NMD are conserved in plants, although there are certain regulatory and mechanistic differences between plant and mammalian NMD (reviewed by Shaul, 2015). Plant NMD degrades PTC-containing mRNAs (Hori and Watanabe, 2005; Arciga-Reyes et al., 2006; Yoine et al., 2006b), alternatively spliced transcript isoforms (Kalyna et al., 2012; Drechsel et al., 2013), as well as transcripts derived from pseudogenes, transposable elements, potential natural antisense RNAs, and aberrant mRNA-like non-coding RNAs (Kurihara et al., 2009). Plant NMD also controls the levels of many normal mRNAs that have long (\( \geq 300–350 \) nt) 3′ UTRs (Kertesz et al., 2006; Schwartz et al., 2006; Hori and Watanabe, 2007; Kalyna et al., 2012), introns \( \geq 50–55 \) nt downstream of TCs (Kertesz et al., 2006; Hori and Watanabe, 2007; Nyiko et al., 2013), or uORFs (although not all uORFs activate NMD; Nyiko et al., 2009; Saul et al., 2009; Kalyna et al., 2012; Rayson et al., 2012).

Since the NMD pathway controls the fate of many normal and aberrant plant transcripts, understanding how this pathway is regulated can shed more light on the control of gene expression in plants and plant physiology. However, compared to the current knowledge available about the NMD mechanism, much less is known about how this pathway is regulated. We showed that the maintenance of balanced expression of the Arabidopsis UPF3 gene is very important for the overall regulation of plant NMD (Degtiar et al., 2015). It is, therefore, important to obtain a full understanding of the regulation of UPF3 expression. We also showed that UPF3 is controlled by a negative feedback loop that increases its expression when NMD is inhibited, and restricts its expression when NMD functions properly (Saul et al., 2009). The Arabidopsis UPF3 transcript is sensitive to NMD owing to its long 3′ UTR (Degtiar et al., 2015). The transcripts of several other eukaryotic NMD factors were also shown to be sensitive to NMD owing to long 3′ UTRs or 3′ UTR introns (Mendell et al., 2004; Rehwinkel et al., 2005; Kerenyi et al., 2008; Huang et al., 2011; Yepiskoposyan et al., 2011; Rayson et al., 2012; Nyiko et al., 2013; reviewed by Huang and Wilkinson, 2012; Karam et al., 2013; Shaul, 2015). However, very little is known about other mechanisms that control the expression of eukaryotic NMD factors. In this work, we show that a construct containing the regulatory regions of UPF3 but lacking its long 3′ UTR, is also feedback-regulated by NMD. This indicates that UPF3 expression is feedback-regulated at multiple levels.

It was also interesting to determine whether UPF3 is differentially expressed in certain plant tissues or growth stages. This can shed light on the question of whether UPF3 is ubiquitously utilized in the NMD of wild type (WT) plants or, alternatively, if there are certain tissues or growth stages in which a UPF3-independent branch of the NMD pathway is normally operative. The latter situation is possible, since Arabidopsis plants with a null mutation in the UPF3 gene are viable. Because a complete loss of NMD function in Arabidopsis is lethal (Arciga-Reyes et al., 2006; Yoine et al., 2006a), NMD should still be functional in plants with a loss of UPF3 function [although with a much lower efficiency (Hori and Watanabe, 2005)]. In mammalian cells, there are branches of the NMD pathway that do not require the involvement of all known NMD factors (Gehring et al., 2005).

A link between NMD and plant-stress response was established. Biotic stress inhibits NMD in plants, thereby initiating a signaling cascade that elevates plant defense (Jeong et al., 2011; Rayson et al., 2012; Riehs-Kearnan et al., 2012; Shi et al., 2012; Gloggnitzer et al., 2014; reviewed by Shaul, 2015). It was also reported that the expression of UPF1 and UPF3 is downregulated by biotic stress (Jeong et al., 2011).

Compared to the response to biotic stress, much less is known about the correlation between NMD and other types of plant stress responses. It was shown that NMD is inhibited by salt stress (Drechsel et al., 2013), but it is not known whether salt stress affects the expression of NMD factors. To increase our understanding of the correlation between NMD and plant stress responses, we examined the impact of salt stress on UPF3 expression. We also examined the impact of deficient or excess UPF3 expression on the response to salt stress.

We show here that UPF3 is expressed in all plant organs and at all growth stages, in agreement with the possibility that UPF3 is ubiquitously operative in the Arabidopsis NMD pathway. Our finding that a construct including UPF3 regulatory regions but lacking its long 3′ UTR is also controlled by NMD, indicates that UPF3 expression is feedback-regulated by NMD at multiple levels. We also show that UPF3 is significantly induced by salt stress. We found that both a deficiency and a strong excess in
UPF3 expression are detrimental to plant resistance to salt stress. This indicates that UPF3 plays a role in plant response to salt stress, and that the balanced expression of the UPF3 gene is essential for coping with this stress.

MATERIALS AND METHODS

Mutant Lines, Plant Transformation, and Expression Analysis
The WT Arabidopsis thaliana (L.) plants were of the Col-0 accession, and the mutant plants were homozygous and in the Col-0 background. The selection of homozygous progenies of the Arabidopsis T-DNA insertion mutants (Alonso et al., 2003) SALK_112922 (upf1-5; Arciga-Reyes et al., 2006) and SALK_025175 (upf3-1; Hori and Watanabe, 2005) was described (Saul et al., 2009). The plants were transformed using the floral dip technique and selected on 20 µg/mL hygromycin B. The plants used for expression analysis were germinated on MS (Duchefa Biochemie BV) plates, and grown in climate-controlled growth rooms or greenhouses in a photoperiod of 16 h light and 8 h dark. Growth in hydroponics was carried out as described (Berezin et al., 2013).

Generation of Constructs
Construct U3::GUS is similar to construct U3::R described in Degtiar et al. (2015), except that the coding sequence of UPF3 was replaced with that of β-glucuronidase (GUS). The U3::GUS construct included the UPF3 promoter, the UPF3 5′ UTR (which includes an uORF), and the UPF3 terminator (which includes the last intron of this gene). The cloning of these sequence elements, which were similar to those utilized in the U3::R construct, was described (Degtiar et al., 2015). The first intron of UPF3, which was present in the coding sequence of UPF3 in the U3::R construct, was inserted into the GUS coding sequence as described in Supplementary Figure 1. Construct U3::GUS-In was identical to construct U3::GUS except that it did not include the first intron of UPF3 (but did include all the extra sequences added to the GUS coding sequence to allow the splicing of this intron—see Supplementary Figure 1B). Construct U3::GUS-NOS was identical to U3::GUS except that the native terminator of UPF3 was replaced with the short nopaline synthase (NOS) terminator. The generation of the U3::NR construct was previously described (Degtiar et al., 2015). All these constructs were cloned into a binary vector that included the coding sequence of Escherichia coli hygromycin phosphotransferase, which confers hygromycin B resistance, immobilized into Agrobacterium tumefaciens strain EHA105, and used for the stable transformation of Arabidopsis (Col-0) plants.

RNA Extraction and Northern Blot Analysis
Total RNA was extracted using the TRI-Reagent (Sigma). RNA samples were denatured with glyoxal (Sigma) and fractionated on 1% agarose gels. Gel preparation and fractionation were carried out with 10 mM NaPi buffer, pH 7.0. The gels were blotted onto a Zeta-Probe GT membrane (Bio-Rad) with 25 mM NaPi buffer, pH 7.0. RNA was fixed by UV. The membranes were stained by 0.02% methylene blue in 0.3 M sodium acetate (pH 5.5) to visualize the ribosomal RNA, and then rinsed in H2O. Hybridization was carried out using the DIG-labeling system (Roche Diagnostics GmbH) according to the manufacturer’s instructions. The primers used for probe preparation were: UPF3, 5′-AAGGCACCAGAAGATG-3′ and 5′-GGATCCACATTGGCTTCTCAT-3′; EF1a (AT1G0...
sequences were: \( \text{UPF3} \) gene showed very similar transcript levels among all samples. Using the primers indicated in Czechowski et al. (2005), to Arabidopsis \( \text{YLS8} \) of four biological replicates, with three technical replicates of \( \pm \) efficiencies of all primer pairs were 100 of a representative cDNA sample measured in triplicate. The efficiencies were determined using a fivefold dilution series 'no template controls' contained water instead of cDNA. Primer carried out using the Bio-Rad CFX Manager software v3.1. The (Tiangen). Primer efficiency calculations and data analyses were a Bio-Rad CFX96 Real-Time PCR system (Bio-Rad Laboratories) Kit (Thermo Scientific). Quantitative RT-PCR was performed on transcription using the RevertAid First Strand cDNA Synthesis Kit (Tiangen). Gene (AT5G08290) was used as an internal standard (Czechowski et al., 2005), using the primers indicated in Czechowski et al. (2005), to normalize the variations of cDNA concentrations. This control gene showed very similar transcript levels among all samples. The thermal cycling program was 95°C for 15 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Primer sequences were: \( \text{UPF3}, 5'-\text{AAGAAGAGGTGGTGATTGG}-3' \) and \( 5'-\text{GAATCCACATTGTGTTCTCA3'-3} \); \( \text{YLS8}, 5'-\text{TTACTGT TTCGTTGTTCATATT3'-3} \) and \( 5'-\text{CCTGAATCAGTT CGAAGCAGT3'-3} \). Quantitative GUS Analysis Quantitative measurement of GUS activity was carried out using the fluorometric assay (Breyne et al., 1993). Plant material was ground in liquid nitrogen and extracted in a buffer containing 100 mM \( \text{NaPO}_4 \), pH 7, 10 mM \( \text{Na}_2\text{EDTA} \), pH 8, 10 mM \( \beta \)-mercaptoethanol, and 0.1% (v/v) Triton X-100. Following centrifugation (5 min, 14,000 \( \times \) g, 4°C), the supernatant was collected and the concentration of proteins was determined using the Bradford reagent (Sigma). Samples including equal amounts of protein were suspended in 250 \( \mu \)l extraction buffer including 1.2 mM (final concentration) of the fluorescent GUS substrate 4-methylumbelliferyl-\( \beta \)-D-glucuronide (MUG; Duchefa Biochemie BV). GUS activity was assayed on a 96-well fluorescent plate reader (Fluoroscan II, Lab Systems) with the excitation wavelength set at 350 nm and the emission wavelength at 460 nm. GUS activity (milli units-mg protein\(^{-1}\)) was calculated from the slope of the line generated from measurements taken at 3-min intervals during 1 h, with respect to the slope of commercial, pure GUS enzyme (Roche Diagnostics GmbH). 7920), \( 5'-\text{CACGTCGATTCTGGAAGTC}-3' \) and \( 5'-\text{TGATAAC ACCGACTGCAACAG3'-3} \).

### Histochemical GUS Analysis

For histochemical analysis of GUS activity, plants were incubated for 1–2 days at 37°C in a medium that included 50 mM \( \text{NaPO}_4 \), pH 7.2, 0.5 mM \( \text{K}_3\text{Fe(CN)}_6 \), 20% (v/v) methanol, and 1 mM X-Gluc A (Duchefa Biochemie BV). This solution was vacuum-infiltrated into the plants. Stained plants were cleared in 70% (v/v) ethanol. Photographs were taken with an Olympus AH3-RFCA microscope or with an Olympus SZX-12 binocular.

### RESULTS

#### The First Intron of UPF3 Is Essential for its Expression

Proper balancing of \( \text{UPF3} \) expression is crucial for NMD homeostasis in *Arabidopsis* (Degtiar et al., 2015). We thus wanted to obtain a full understanding of the regulation of \( \text{UPF3} \) expression. For this, we created the U3:GUS construct, in which the reporter GUS was fused to all the regulatory elements of the \( \text{UPF3} \) gene (**Figure 1A**). These elements included the native promoter, 5' UTR, terminator, and the first and last introns of this gene. The last (12th) intron of \( \text{UPF3} \) is located in its 3' UTR. However, we found that this intron, which is located less than 50 nt downstream of the TC, did not affect \( \text{UPF3} \) expression (Degtiar et al., 2015). The first intron of \( \text{UPF3} \) was also included in the U3:GUS construct because some introns, particularly first introns, can play important roles in gene expression through the intron-mediated enhancement (IME) mechanism [reviewed by Gallegos and Rose (2015)]. However, many introns are incapable of affecting expression. The first intron of \( \text{UPF3} \) is considerably longer than other introns of this gene (Supplementary Figure 1A). To determine if this intron plays a role in \( \text{UPF3} \) expression, we created the U3:GUS-In (-In stands for minus intron) construct, which did not include this intron (**Figure 1A**).

In the U3:GUS construct, the first intron was inserted into the GUS coding sequence flanked by several nucleotides of the original coding sequence of \( \text{UPF3} \), to ensure the proper splicing of this intron (Supplementary Figure 1B). The reading frame of these few nucleotides was altered (Supplementary Figure 1B), to prevent the translation of the first amino acids of \( \text{UPF3} \) and a consequent putative dominant negative effect. In the U3::GUS-In construct, GUS was fused to essentially the same sequences, including the few flanking nucleotides, but excluding the intron. The two constructs were stably transformed into *Arabidopsis*, and GUS transcript level was determined (**Figure 1B**). GUS transcript in plants expressing the construct that lacked the intron was below the detection limit. The *EF1a* mRNA was used as a control to demonstrate that this did not result from mRNA degradation in the U3::GUS-In plants. This indicated that the first intron of \( \text{UPF3} \) is important for expression of this gene. To ensure reliable data, expression analysis was carried out in plants expressing the U3::GUS construct, which included this intron.
**UPF3** Is Ubiquitously and Stably Expressed in Different Plant Organs and Growth Stages

Young seedlings of transgenic plants expressing the U3::GUS construct showed relatively uniform GUS staining in true leaves and in roots (Figures 2A,H). GUS staining in the cotyledons was lower than in true leaves. Staining was observed in all parts of 25-day-old plants, including roots and leaves (Figure 2B). Expression was also observed in all parts of plants at the reproductive stage, including roots, rosette and cauline leaves, inflorescence stems, flowers, and siliques (Figures 2C–G). Thus, unless UPF3 has other, yet-undefined functions, this expression pattern is in agreement with the possibility that the NMD process is functional in all plant parts and at all developmental stages, and that UPF3 is ubiquitously utilized in the NMD pathway of WT Arabidopsis plants.

Although GUS staining was observed in plants at different ages, quantitative GUS analysis was necessary to determine if there were differences in the level of expression at different growth stages. It was found that the expression in rosette leaves of plants at different growth stages was equal (Figure 3A). Expression was also equal in younger and older leaves of the same plant (Figure 3B). The results obtained using the reporter gene were supported by analysis of **UPF3** mRNA levels (Figure 3C). **UPF3** transcript levels were similar in younger and older leaves of 25-day-old plants, and were also similar in the leaves of older plants. Altogether, these data indicated that **UPF3** expression in leaves is stable throughout plant growth.
The Induction of UPF3 under NMD Inhibition Depends Not Only on Its Long 3’ UTR but Also on Other Regulatory Elements of This Gene

We previously showed that the long 3’ UTR of UPF3 subjects the transcript of this gene to NMD (Degtiar et al., 2015). It was interesting to learn whether, besides the long 3’ UTR, other regulatory elements of the UPF3 gene can also confer NMD sensitivity. To address this question, we compared the expression in WT and NMD mutant plants of the U3::GUS construct, which contained all the regulatory regions of UPF3, including the long (545 nt) 3’ UTR, with that of the U3::GUS-NOS construct, which was identical except having the short (∼180 nt long; Bevan et al., 1983) NOS 3’ UTR (Figure 4A). The NMD mutant plants utilized were upf1-5 and upf3-1. The upf1-5 mutants have severely reduced, but not completely eliminated expression of UPF1 (a null mutation in UPF1 is lethal in Arabidopsis; Arciga-Reyes et al., 2006; Yoine et al., 2006a). The upf3-1 mutants do not have intact UPF3 mRNA (Hori and Watanabe, 2005).

As expected, the expression of the U3::GUS construct was higher in stably transformed NMD mutants as compared with transformed WT (Col-0) plants (Figure 4B). This can be explained by the presence in the U3::GUS construct of the long 3’ UTR of UPF3, which renders it NMD-sensitive (Degtiar et al., 2015). The higher expression of the U3::GUS construct in upf3-1 compared with upf1-5 mutants is in accord with the indication that, in terms of NMD impairment, the upf3-1 allele is stronger than the upf1-5 allele (Kalyna et al., 2012). Interestingly, increased expression in transformed NMD mutants compared with transformed WT plants was also observed for the U3::GUS-NOS construct, which did not include the long 3’ UTR of UPF3 (Figure 4C). This indicated that besides the long 3’ UTR, other regulatory elements of the UPF3 gene can also confer NMD sensitivity. We previously showed that the uORF of UPF3 does not expose it to NMD (Degtiar et al., 2015). It is, thus, possible that the UPF3 promoter includes NMD responsive elements. The extent of induction by NMD inhibition was higher for plants expressing the U3::GUS as compared with the U3::GUS-NOS construct (Figures 4B,C). This indicates that the impacts of the long 3’ UTR and the other regulatory elements that confer NMD responsiveness to the UPF3 gene are cooperative.

The Regulatory Regions of UPF3 Are Induced by Salt Stress

It was shown that NMD plays a role in plant response to biotic stress, and the expression of UPF1 and UPF3 is affected by biotic stress (Jeong et al., 2011; Rayson et al., 2012; Richards-Kearnan et al., 2012; Shi et al., 2012; Gloggnitzer et al., 2014; reviewed by Shaul, 2015). Here, we investigated whether salt stress affects the expression of UPF3 regulatory regions. For this, plants expressing the U3::GUS and U3::GUS-NOS constructs were grown on plates including 100 mM NaCl. We compared the impact of NaCl, which imposes both salt and osmotic stress, with that of sorbitol, which imposes osmotic stress only. The sorbitol concentrations used were 200 mM (providing equal osmolarity to 100 mM NaCl) and 250 mM (providing increased osmolarity).

As exhibited by plants expressing both constructs, salt treatment induced the expression mediated by the regulatory regions of UPF3 (Figures 5A,B). Increased expression, although to a lower extent, was also observed upon treatment with sorbitol (Figures 5A,B). We attempted to estimate the extent of growth inhibition mediated by these stress treatments (NaCl and sorbitol) by measuring the plants’ fresh weight.
These plants, in which the coding sequence (named U3::NR plants) were obtained in a previous study, expression of UPF3 plants, in a larger extent than did 100 mM NaCl (in the presence of 250 mM sorbitol, which inhibited growth to a lesser extent than did leaves of plants grown on the control medium did not show reduced fresh weight compared with plants grown in the control medium (Figure 6B). A similar observation was made for upf1 mutants and the U3::NR-L plants, which had a twofold increase in UPF3 expression (Figures 6A,B). Interestingly, both upf3 mutants and plants with about a sixfold increase in UPF3 expression exhibited a significant reduction in their fresh weight under salt stress compared with plants grown in the control medium (Figure 6B). These findings are reflected by the phenotype of these plants (Figure 6C). U3::NR-H plants and upf3 mutants grown on the control medium did not show reduced fresh weight compared with WT plants (Figure 6B). To conclude, plants with either deficient or highly excessive UPF3 expression exhibit impaired salt tolerance. This indicates that balanced expression of UPF3 is essential for plant resistance to salt stress (see Discussion for a detailed explanation of this conclusion).

**DISCUSSION**

Very little is known about NMD regulation in plants and the mechanisms controlling the expression of plant NMD factors. The proper regulation of UPF3 expression is crucial for NMD homeostasis in Arabidopsis (Degtiar et al., 2015). It is, therefore, interesting to obtain a full understanding of the mechanisms that regulate UPF3 expression. To address this topic, we utilized a reporter construct that included all the regulatory regions of the UPF3 gene. We first showed that the first intron of UPF3 is essential for the expression of this gene. Some introns, particularly first introns, are capable of enhancing gene expression in a process termed IME (reviewed by Gallegos and Rose, 2015). While in some cases no expression is seen in the absence of introns, many introns do not affect gene expression. Whereas no expression could be observed in the absence of the first intron of UPF3, the last intron located in the 3′ UTR of UPF3 was unable to restore the enhancing function of the first intron (Figure 1). Due to its essential function, the first intron of UPF3 was included in all reporter gene constructs utilized in this study. This increased the reliability of the data obtained.

UPF3 showed rather uniform expression in different plant tissues. Although some proteins involved in the NMD process are also engaged in other functions (Isken and Maquat, 2008), NMD-unrelated functions have not yet been assigned to UPF3. Thus, unless such functions are identified, the expression pattern
observed here is in agreement with the possibility that UPF3 is ubiquitously utilized in the NMD of WT plants. Expression was similar in both the young and mature leaves of the same plant, and was also stable in plants at different growth stages. This suggests that the NMD process is functional at all growth stages.

In both plant and mammalian cells, the feedback loops controlling the expression of NMD factors have been attributed only to long 3′ UTRs, 3′ UTR introns, and/or uORFs (see Introduction). We previously showed that the long 3′ UTR of UPF3 exposes this gene to NMD (Degtiar et al., 2015). Interestingly, we show here that a construct that contains all the regulatory regions of the UPF3 gene except this long 3′ UTR is also feedback-regulated by NMD. Since the uORF of UPF3 does not expose its transcript to NMD (Degtiar et al., 2015), it is possible that this additional layer of NMD sensitivity results from elements in the UPF3 promoter. Our findings revealed that UPF3 is feedback-regulated at multiple levels. However, the effects of the long 3′ UTR and the other element(s) conferring
FIGURE 6 | Plants with deficient or highly excessive UPF3 expression exhibit impaired salt tolerance. In order to compare salt tolerance, WT (Col-0) plants, upf1-5 and upf3-1 mutants, as well as plants overexpressing UPF3 under the control of its own regulatory sequences (U3::NR-L and U3::NR-H), were germinated in the absence or presence of NaCl. (A) qRT-PCR analysis showing the extent of UPF3 overexpression in each of the two homozygous transformed plants (U3::NR-L and U3::NR-H) used in this experiment. Samples from four biological replicates (four different plates including ∼50 plants each), each analyzed using three technical replicates, were used for determination of UPF3 transcript content in each plant type, and data were normalized to UPF3 expression in WT plants. Error bars represent the SE. An asterisk indicates a significant difference (p < 0.05) between the WT and the transformed plant, as determined by Student’s t-test. (B) Each column represents the mean fresh weight of 10 biological replicates grown on the control medium (gray columns) or on plates containing 100 mM NaCl (black columns). Each replicate was the average fresh weight of a single plant determined according to the weight and number of 2-week-old plants grown in a single plate (thus, each column shows the average weight of about 500 plants grown in 10 plates). It was verified that all seeds germinated at the same time. All data were normalized to the control treatment of WT plants. Error bars represent the SE. An asterisk indicates a highly significant difference (p < 0.001) between the control and the NaCl treatment of the same plant genotype, as determined by Student’s t-test. (C) Representative plates at the time of harvesting.

NMD responsiveness were cooperative, and regulation of UPF3 by its long 3′ UTR was essential for the maintenance of NMD homeostasis in Arabidopsis (Degtiar et al., 2015).

Interestingly, the expression of the U3::GUS and U3::GUS-NOS constructs was induced by salt (Figures 5A,B). Salt excess imposes two major adverse effects on plants: osmotic stress and ion (Na⁺ and Cl⁻) toxicity (Greenway and Munns, 1980). The induction of U3::GUS and U3::GUS-NOS expression was stronger in the presence of NaCl than upon treatment with sorbitol at equivalent or even higher osmolarity. This indicates that the induction of expression under salt stress results from both the osmotic and the ion toxicity components of the stress.

Our finding that upf3 mutants have increased sensitivity to salt stress as compared to the WT plants points to a role of UPF3 in plant response to salt stress. The increased salt sensitivity of upf3 mutants could be a secondary impact related to the role of UPF3 in NMD management. According to this suggestion, NMD impairment alters the expression of genes that are essential for plant response to salt stress. The finding that upf1 mutants are less sensitive to salt stress than upf3 mutants (Figure 6) may be explained by the observation that the upf3-1 allele is stronger than the upf1-5 allele in terms of NMD impairment (Kalyna et al., 2012).

It was shown that salt stress inhibits NMD (Drechsel et al., 2013). It is possible that the moderate increase in the expression of UPF3 during salt stress is helpful for coping with this inhibition. It was, therefore, interesting to determine the impact of increased expression of UPF3 on plant response to salt stress. However, plants with a twofold increase in UPF3 expression (the U3::NR-L plants) did not show increased salt tolerance as compared with the WT plants (Figure 6). It is reasonable that increased expression of UPF3 is just one of several mechanisms utilized by plants to cope with salt stress, but alone, it is not sufficient for eliminating this stress. Interestingly, plants with a strong increase in UPF3 expression (the U3::NR-H plants) showed, similar to the upf3 mutants, reduced salt tolerance compared with the WT plants (Figure 6). This finding can be explained by our previous observation that not only a deficiency but also an excess of UPF3 can impair NMD (Degtiar et al., 2015).
Altogether, our data show that both deficiency and a strong excess in UPF3 expression are detrimental to the plant resistance to salt stress. This indicates that UPF3 plays a role in the plant response to salt stress, and that a balanced expression of the UPF3 gene (avoiding both a deficiency and a strong excess) is essential for coping with this stress. Thus, the feedback regulation of UPF3 expression, which prevents the imbalanced expression of this gene, is essential for plant salt response.

AUTHOR CONTRIBUTIONS

KV, MAC, IB, LG, ML, and HS performed the experiments and participated in data analysis. OS designed the experiments, participated in data analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01376

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