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Widespread Exon Junction Complex Footprints in the RNA Degradome Mark mRNA Degradation Before Steady-state Translation

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One-sentence summary: Exon junction complex footprints account for a sizable proportion of major RNA degradation intermediates and can serve as markers for mRNA degradation before steady-state translation.

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ABSTRACT

Exon junction complexes (EJCs) are deposited on mRNAs during splicing and displaced by ribosomes during the pioneer round of translation. Nonsense-mediated mRNA decay (NMD) degrades EJC-bound mRNA, but the lack of suitable methodology has prevented identification of other degradation pathways. Here, we show that the RNA degradomes of Arabidopsis thaliana, rice (Oryza sativa), worm (Caenorhabditis elegans) and human (Homo sapiens) cells exhibit an enrichment of 5′ monophosphate (5′ P) ends of degradation intermediates that map to the canonical EJC region. Inhibition of 5′-3′ exoribonuclease activity and overexpression of an EJC disassembly factor in Arabidopsis reduced the accumulation of these 5′ P ends, supporting the notion that they are in vivo EJC footprints. Hundreds of Arabidopsis NMD targets possess evident EJC footprints, validating their degradation during the pioneer round of translation. In addition to premature termination codons, plant microRNAs can also direct the degradation of EJC-bound mRNAs. However, the production of EJC footprints from NMD but not microRNA targets requires the NMD
factor SUPPRESSOR WITH MORPHOLOGICAL EFFECT ON GENITALIA PROTEIN 7 (SMG7). Together, our results demonstrating in vivo EJC footprinting in Arabidopsis unravel the composition of the RNA degradome, and provide a new avenue for studying NMD and other mechanisms targeting EJC-bound mRNAs for degradation before steady-state translation.

INTRODUCTION

Many eukaryotic pre-mRNAs undergo splicing, in which intervening sequences (introns) are excised and the exon junction complex (EJC) is deposited in a region 20-24 nt upstream of the exon-exon junction (Le Hir et al., 2000). During the pioneer round of translation, EJCs residing upstream of the termination codon are disassembled and removed through the ribosome-associated protein Partner of Y14 and Mago (PYM) (Gehring et al., 2009). For most eukaryotic mRNAs, the termination codon is present in the last exon. Hence, after the pioneer round of translation, these mRNAs no longer carry any EJCs and subsequently enter steady-state cycles of translation, which contribute to the bulk production of proteins. However, if a termination codon situated more than 50-55 nt upstream of an exon-exon junction is encountered by a ribosome during the pioneer round of translation, it is generally recognized as a premature termination codon (PTC) and promotes nonsense-mediated mRNA decay (NMD) (Nagy and Maquat, 1998).

Although many studies have demonstrated that the EJCs downstream of a termination codon are crucial for NMD, NMD can also occur in an EJC-independent manner. In the absence of EJCs, a long 3’ untranslated region (UTR) sequence is able to promote NMD of mRNAs harboring a natural termination codon (Behm-Ansmant et al., 2007). Budding yeast (Saccharomyces cerevisiae) lacks the genes encoding core EJC components but possesses the NMD pathway (Conti and Izaurralde, 2005; Bannerman et al., 2018). Additionally, in the worm Caenorhabditis elegans and the fly Drosophila melanogaster, PTC definition is independent of exon-exon boundaries and EJCs are dispensable for NMD.
While silencing of the EJC core components Mago and Y14 or overexpression of PYM inhibits NMD triggered by 3′ UTR-located introns in plants (Kerenyi et al., 2008; Nyiko et al., 2013), the number of studies of plant EJCs is limited and little is known about the binding sites of plant EJCs.

Translation termination at a PTC promotes the assembly of the UP FRAMESHIFT1 (UPF1) surveillance complex that activates NMD (Hug et al., 2016). In animal cells, PTC-containing mRNAs are destabilized through endonucleolytic cleavage near the PTC catalysed by SMG6/EST1A (a member of the gene family named after C. elegans Suppressor with Morphological effect on Genitalia or yeast Ever Shorter Telomeres 1) (Gatfield and Izaurralde, 2004; Huntzinger et al., 2008; Eberle et al., 2009) or through deadenylation or decapping (Chen and Shyu, 2003; Lejeune et al., 2003; Couttet and Grange, 2004). Although the NMD factors UPF1, UPF2 and SMG7 are conserved in animals and plants, plants appear to lack SMG6 orthologs and presumably remove PTC-containing mRNAs through exonucleolytic pathways (Kerenyi et al., 2008). Previously, identification or validation of NMD targets often relied on the detection of changes in gene expression in NMD mutants or NMD factor-depleted cells because the specific degradation products of NMD targets were poorly defined (He et al., 2003; Kalyna et al., 2012; Drechsel et al., 2013; Colombo et al., 2017; Lloyd et al., 2018). Nevertheless, relying on changes in gene expression could be problematic because impairment of the NMD pathway can alter the expression of many genes that are indirectly regulated by this pathway.

High-throughput approaches for profiling 5′ monophosphate ends of RNA degradation intermediates (hereafter referred to as 5′P ends) such as Degradome-Seq (Addo-Quaye et al., 2008), Parallel Analysis of RNA Ends (PARE) (German et al., 2008), Genome-wide Mapping of Uncapped and Cleaved Transcripts (GMUCT) (Gregory et al., 2008), and 5′ P sequencing (Pelechano et al., 2015), provide a more reliable way to detect
the direct targets of RNA degradation pathways. These approaches have been applied for transcriptome-wide identification of endonucleolytic cleavage events directed by microRNA (miRNA) in diverse plant species and catalyzed by SMG6 in human (*Homo sapiens*) cells as well as the substrates of exoribonuclease 4 (XRN4), which catalyzes 5'-3' RNA decay in Arabidopsis (*Arabidopsis thaliana*). For plant miRNA targets, 5'P ends predominantly correspond to the middle of miRNA complementary sites (Addo-Quaye et al., 2008; German et al., 2008; Li et al., 2010; Pantaleo et al., 2010; Shamimuzzaman and Vodkin, 2012; Li et al., 2013a). Arabidopsis miRNA-directed cleavage products over-accumulate in the *Arabidopsis xrn4* mutant (German et al., 2008). Global profiling of human SMG6-dependent 5’ P ends by PARE revealed a sequence motif enriched at the SMG6 cleavage site (Schmidt et al., 2015). Notably, depletion of SMG6 increased the number of 5’ P ends located at the mRNA cap site for many SMG6 substrates, suggesting competition between endonucleolytic cleavage and decapping in the human NMD pathway. Because SMG6 appears to be absent in plants, plant NMD targets are presumably degraded through XRNs and the exosome after decapping and deadenylation. Intriguingly, Arabidopsis *xrn4* not only over-accumulates intermediates with 5’P ends located at the cap sites of NMD targets but also intermediates with 5’P ends located in the coding region (CDS) or 3’ UTR of some targets (Nagarajan et al., 2019), suggesting that endonucleolytic cleavage might also account for the turnover of plant NMD targets.

Endonucleolytic cleavage is not the only mechanism that can result in predominant accumulation of 5’ P ends at specific sites. Blocking of 5’-3’ RNA decay by RNA binding proteins likely also yields 5’ P ends clearly delineated by the 5’ edge of the RNA binding protein (Hou et al., 2014). Consistent with this speculation, previous analyses of yeast and plant 5’ P end data indicated that a portion of 5’ P ends are ribosome-protected mRNA fragments generated through co-translational RNA decay (Pelechano et al., 2015; Hou et al.,
The in vivo ribosome footprints in the RNA degradome thus provide an alternative to Ribo-Seq as way to infer ribosome dynamics. Stress-induced pauses in translation at specific codons and gene regions associated with collided ribosomes have been uncovered through analyses of RNA degradome data. As many eukaryotic mRNAs are post-transcriptionally processed by splicing, EJCs might also make a noteworthy contribution to the pool of RNA degradation intermediates if they are able to hinder XRN-mediated RNA decay and leave footprints during in vivo mRNA degradation as ribosomes do. If this is the case, EJC footprints in the RNA degradome can serve as signatures of mRNA degradation before steady-state translation, as EJCs residing upstream of the termination codon are presumably removed after the pioneer round of translation. Theoretically, only mRNA degradation occurring before or during the pioneer round of translation, such as NMD, can lead to the accumulation of EJC footprints in CDSs.

Here, we provide multiple lines of evidence supporting the notion that the RNA degradome contains EJC footprints. We observed EJC footprints in some miRNA targets in addition to NMD targets, demonstrating that RNA degradome data can be applied to the study of mRNA degradation before steady-state translation.

RESULTS

5′ P Ends are Enriched in the Canonical EJC Region

If EJCs are able to block 5′-3′ decay of RNA and leave footprints in the RNA degradome as ribosomes do, it is expected that enrichment of 5′ P ends in the canonical EJC region will be observed. To test this possibility, we analyzed the previously reported 5′P end data of five evolutionarily distant species. Consistent with this prediction, metagene analyses of both Arabidopsis PARE data (German et al., 2008) and rice (Oryza sativa) Degradome-Seq data...
(Li et al., 2010) revealed that the relative occurrence of 5′P ends was higher in a region 25-30 nt upstream of the 3′ end of the exon (Figure 1A). As these two datasets only contain the 5′P ends of polyadenylated degradation fragments, we also examined Arabidopsis PARE data for nonpolyadenylated degradation fragments generated by Nagarajan et al. (2019). Interestingly, 5′P end enrichment was also detected in the same region of nonpolyadenylated degradation fragments but to a lesser degree compared with that in polyadenylated degradation fragments (Supplemental Figure 1A). Analysis of the Arabidopsis 5′P ends obtained using the GMUCT method (Willmann et al., 2014) gave an enrichment pattern in this region similar to that observed for the PARE data (German et al., 2008) (Supplemental Figure 1B). Similarly, a 5′P peak spanning the same region was also detected when analyzing the GMUCT data for human HEK293 cells (Willmann et al., 2014) (Figure 1A). Analysis of Degradome-Seq data for worm (Park et al., 2013), in which EJCs are not required for triggering NMD (Longman et al., 2007), also revealed an enrichment of 5′P ends in a similar region with a 1-nt offset (Figure 1A). However, analysis of the PARE data for budding yeast (Harigaya and Parker, 2012), which lacks EJCs and has only a few intron-containing genes (Bannerman et al., 2018), revealed no enrichment of 5′P ends in this region (Figure 1A). The common patterns observed in the 5′P end datasets of four evolutionarily distant species indicates that this phenomenon is truly conserved across species possessing EJCs. Given that the canonical deposition site of human EJCs is centered at a position 24 nt upstream of the exon-exon junction (Sauliere et al., 2012; Singh et al., 2012), and that deposition of an EJC results in the protection of an 8-nt fragment from complete RNase digestion (Le Hir et al., 2000) (Figure 1A, top), the 5′P peaks spanning the -25 to -30 region correspond to the 5′ edges of EJCs deposited in the canonical region. Therefore, these 5′P peaks are very likely to be EJC-protected 5′ termini of RNA degradation intermediates naturally produced in cells.
In addition to the analysis of 5′ P end distribution in a 50-nt region upstream of the exon-exon junction, we also investigated the most abundant 5′P peaks (maximum 5′ P peak) occurring in the canonical EJC region (positions -26 to -30) within transcripts of intron-containing genes. Analysis of previously published 5′ P end data revealed that 13.1% (2411 genes), 9.4% (1650 genes), 8.2% (710 genes) and 8.1% (1142 genes) of the maximum 5′ P peaks within transcripts of Arabidopsis, rice, human cells and worm, respectively, were located in the canonical EJC region (Figure 1B). These values were significantly higher than expected (P < 10^{-100}, \chi^2 test) as the total length of the canonical EJC regions represents less than 2% of the total length of spliced transcripts. By contrast, the percentages of maximum 5′ P peaks falling in the nearby 5-nt regions in Arabidopsis, rice and human cells were mostly lower than 2%, which is close to the expected value for a 5-nt region calculated by dividing the total length of each 5-nt region to that of spliced transcripts (Figure 1B). Hence, EJC footprints appear to account for a noticeable portion of major mRNA degradation intermediates.

**Exoribonucleases are Involved in in vivo EJC Footprinting**

To investigate whether EJC footprints in the RNA degradome are generated through 5′-3′ decay, as was previously reported for ribosome footprints (Pelechano et al., 2015; Yu et al., 2016), we profiled 5′ P ends in Arabidopsis wild type (WT) and two mutants defective in 5′-3′ RNA decay, *xrn4-6* and *fiery1-6 (fry1-6)*, and compared the abundances of 5′P ends mapping to canonical EJC regions. Although 5′ P ends remained enriched in the canonical EJC region in the two mutants (Figure 2A), *xrn4-6* had significantly fewer 5′ P ends in the canonical EJC region than WT in a global analysis of Arabidopsis exons (P < 0.001, Kruskal-Wallis test followed by a pairwise Wilcoxon test with FDR correction) (Figure 2B). The decrease in the number of 5′ P ends in the canonical EJC region was more evident in
fry1-6 (P < 0.001, Kruskal-Wallis test followed by a pairwise Wilcoxon test with FDR correction), wherein the activities of XRN2, XRN3 and XRN4 are likely all suppressed (Gy et al., 2007). Notably, the 5'P ends enriched around positions -20 to -15 are likely also fragments protected by RNA-binding proteins because the number of 5'P ends mapping to this region also appeared to be reduced in xrn4-6 and fry1-6. By contrast, the abundance of 5' P ends around the transcription start site (TSS) of genes harboring exons was largely increased in xrn4-6 and slightly but significantly elevated in fry1-6 compared with that in WT (P < 0.001, Kruskal-Wallis test followed by a pairwise Wilcoxon test with FDR correction) (Figure 2C). This result strongly suggests that EJCs can sterically hinder XRN-mediated 5' -3' decay, resulting in the EJC footprints observed in the RNA degradome. Moreover, fry1-6 exhibited a more pronounced reduction of EJC footprints than xrn4-6, implying that in addition to the cytoplasmic-localized XRN4 (Kastenmayer and Green, 2000), nuclear XRNs (XRN2 and XRN3) or other proteins with exoribonuclease activity may also contribute to in vivo EJC footprinting.

Prominent EJC Footprints are Prevalent in NMD Targets

Given that ribosomal arrest by a PTC can trigger decapping, followed by 5’ -3’ RNA decay, the EJCs deposited on the exons downstream of a PTC were predicted to hinder XRN-mediated degradation and lead to the accumulation of degradation intermediates with 5’ P ends in the EJC region. Indeed, three Arabidopsis genes, TFIIIA, LPEAT2, and RS2Z33, which are known to produce PTC-containing mRNAs due to alternative splicing (AS) (Yoine et al., 2006; Gloggnitzer et al., 2014), displayed prominent 5’ P peaks in the EJC regions of the exons 3’ to the PTC (Figure 3 and Supplemental Figure 2). Notably, in the WT, the maximum 5’ P peaks in these three genes were all located in the second exon 3’ end downstream of the PTC, although the maximum 5’ P peak in LPEAT2 was not in the
canonical EJC region but at a position 37 nt upstream of the exon-exon junction. Moreover, the abundances of these maximum peaks including the two in the canonical EJC region were reduced in both xrn4-6 and fry1-6 compared with that in the WT, but to a greater extent in fry1-6. However, a comparable or higher number of 5’P ends corresponding to the putative TSS and regions outside the canonical EJC site were observed in xrn4-6 and fry1-6 than in the WT, which excluded the possibility that a change in gene expression accounted for the reduction of EJC footprints in the mutants.

A previous analysis of AS events suppressed in a double mutant of the NMD factors UPF1 and UPF3 of Arabidopsis revealed a large number of genes producing splicing variants with classical NMD features (Drechsel et al., 2013). We thus used this gene list to test the association between the NMD-suppressed AS events and the occurrence of maximum 5’P peaks in the canonical EJC region. Using a threshold applied in the previous report (up-regulated in the double mutant of lba1 upf3-1 with FDR < 0.1; lba1 is also known as upf1-1) (Drechsel et al., 2013), 1082 genes with NMD-suppressed AS events were identified. Among them, 37.2% (402 genes) and 32.8% (355 genes) possessed a maximum 5’P peak in the EJC region in replicates 1 and 2 of the Arabidopsis 10-day-old WT seedling PARE data we generated, respectively (Figure 4A and B). By contrast, the total incidence of maximum 5’P peaks in the EJC region for all intron-containing genes in the Arabidopsis genome was 15.3% (3379 genes) and 13.8% (3064 genes) in replicates 1 and 2, respectively, which is significantly lower than the incidence of genes with NMD-suppressed AS events (P < 10^{-100}, binomial test). This result implies that EJC footprints are the major and common degradation products of putative NMD targets. The predominance of EJC footprints in many putative NMD targets also validated their degradation during the pioneer round of translation. Because it has been shown that many intron-retention events are not sensitive to NMD (Kalyna et al., 2012; Drechsel et al., 2013), we also compared the
incidence of maximum 5′ P peaks in the EJC region for four types of AS events suppressed by NMD. Interestingly, a significantly higher incidence of maximum 5′ P peaks in the EJC region was observed for alternative 5′ or 3′ splice site events than for intron-retention events (P < 10^-5, binomial test), although these events were all suppressed by NMD (Figure 4B). However, for 2306 intron-containing genes harboring a maximum 5′ P peak in the EJC region in both replicates of the PARE data we generated, only 293 genes (12.7%) had NMD-suppressed AS events (Figure 4A). A large number of maximum 5′ P peaks in the EJC region did not overlap with NMD-suppressed AS events reported previously (Drechsel et al., 2013), implying that either the list was not complete or there are additional pathways involved in the degradation of EJC-bound RNA.

AS events in CYP38, PPD6 and STR14 were suppressed by NMD (Drechsel et al., 2013), but were not annotated in the TAIR database. The maximum 5′ P peaks in these three genes were located in the EJC region, and these peaks had a 5′ P count ≥ 1000 tags per 40 million (TP40M) (Figure 4C and Supplemental Figure 3). Although the 5′ P end profiles of these three genes highly resembled those of validated plant miRNA targets reported previously (Addo-Quaye et al., 2008; German et al., 2008), the maximum 5′ P peaks were dampened in xrn4-6 and fry1-6. This indicates that these extremely abundant 5′ P peaks do not correspond to 5′ P ends derived from endonucleolytic cleavage but instead the protected fragments of EJCs. Notably, these peaks were located in the first or third exon 3′ end downstream of the predicted PTCs caused by AS. This again supports the notion that the dominant EJC footprints in the exon 3′ ends downstream of predicted PTCs could be evidence of NMD triggered by those PTCs.

PTC-triggered RNA Decay Leads to the Production of EJC Footprints
To verify that a PTC conditions the production of EJC footprints, we took advantage of existing nonsense mutants of *PHYB* and *PHO2* (Reed et al., 1993; Delhaize and Randall, 1995), in which a PTC was introduced by ethyl methanesulfonate mutagenesis. The PTCs in the nonsense mutants should direct mRNA decay through the NMD pathway and are predicted to trigger the production of EJC footprints downstream of the PTC. We compared degradation intermediates of *PHYB* and *PHO2* mRNAs in WT and the nonsense mutants *phyB-9* and *pho2* using a modified RNA-ligase mediated 5′ rapid amplification of cDNA ends (RLM 5′ RACE) protocol. In both nonsense mutants, the 5′ ends of the most abundant amplification products of the mRNA degradation fragments were mainly mapped to positions 27-28 nt upstream of the exon-exon junction downstream of the PTCs (Figure 5A and B). However, these degradation intermediates were not prominent in the WT although the miR159-guided 3′ cleavage product of *MYB65*, the positive control for modified RLM 5′ RACE, was detected in both the WT and the nonsense mutants. The modified RLM 5′ RACE results for *phyB-9* and *pho2* mimic the PARE data of the three known NMD targets with PTCs introduced by AS (Figure 3), and together these results support the conclusion that PTCs elicit accumulation of EJC footprints in downstream exons. Moreover, PTCs caused by AS or point mutations tended to result in the generation of 5′P ends mainly in the first or second EJC region downstream of PTCs (Figures 3, 4C, 5A and 5B), suggesting that EJC footprints may serve as an immediate readout of PTC-mediated NMD irrespective of the cause of the PTC.

We further validated the contribution of XRN4 to EJC footprint production from nonsense mRNAs by generating the *phyB-9 xrn4-6* and *pho2 xrn4-6* double mutants. As expected, a lower number of putative EJC footprints was observed in the double mutants than in the nonsense mutants (Figure 5C). The result again confirms the role of XRN4 in the turnover of PTC-containing mRNAs and the generation of EJC footprints.
Analysis of EJC Footprints Reveals NMD Targets

Using the common features of EJC footprints in the NMD targets shown in Figures 3, 4 and 5, we globally validated potential targets of NMD triggered by AS or introns in the 3’ UTR that were annotated in the Arabidopsis and rice genome databases. For protein-coding transcripts with AS events, if the maximum 5’P peak was located in the first or second canonical EJC region downstream of an AS site, they were identified as putative targets of NMD triggered by AS, which frequently results in a PTC. In addition, transcripts possessing an intron-containing 3’ UTR were also identified as putative NMD targets if the maximum 5’ P peak was located in the first or second canonical EJC region of the exon 3’ end at least 50 nt downstream of the annotated termination codon. Analysis of the PARE data for Arabidopsis WT seedlings and flowers that we generated and the previously published Degradome-Seq data for rice WT seedlings (Li et al., 2010) revealed 254 Arabidopsis transcripts and 168 rice transcripts as putative NMD targets (Figure 6A and Supplemental Datasets 1 and 2). Remarkably, 19.3% and 44% of the putative NMD targets identified from the analysis of Arabidopsis and rice 5’P end data, respectively, have an intron-containing 3’ UTR (Figure 6A). Compared with the large number of NMD-sensitive AS events reported by Drechsel et al. (2013), the number of Arabidopsis NMD targets we identified is small because many AS events reported by Drechsel et al. (2013) including AS of CYP38, PPD6 and STR14 were not annotated in TAIR 10. However, 224 of the targets we identified in Arabidopsis did not show evidence of NMD-suppressed AS in the previous report (up-regulated in the double mutant of lba1 upf3-1 with FDR < 0.1) (Drechsel et al., 2013) (Supplemental Dataset 1). Some of the novel NMD targets such as Arabidopsis ARR8 and KCBP and rice transcripts encoding an SPA4-like protein (LOC_Os01g52640) and a transposon protein (LOC_Os04g03884) had a high number of EJC footprints in the 3’ UTR (Figure 6B and C). For the Arabidopsis NMD
targets we obtained, the analysis of gene ontology (GO) terms revealed the highest enrichment for transcripts involved in “regulation of alternative mRNA splicing, via spliceosome” (22.27-fold enrichment, $P < 0.001$, Fisher's exact test with Bonferroni correction) (Supplemental Table 1). This enriched GO term is consistent with the previous finding that in Arabidopsis, NMD is an important mechanism regulating the splicing of genes encoding serine/arginine proteins, which play key roles in splicing (Palusa and Reddy, 2010). Other significantly enriched GO terms unrelated to splicing included “organic cyclic compound metabolic process,” “nitrogen compound metabolic process,” and “chloroplast” ($P < 0.05$, Fisher's exact test with Bonferroni correction). For the rice NMD targets we identified, however, no enriched GO terms were recovered. The difference in GO enrichment analysis results between the two species might be due to differences in the 5′P end datasets we analyzed, the frequency of introns in the 3′ UTR, or the annotations of splicing variants in two genomes. It may also suggest that the role of the NMD pathway in gene regulation could be distinct among plant species.

**Dysfunction of SMG7 Reduces the Number of EJC Footprints in Putative NMD Targets**

To verify that EJC footprints in NMD targets are degradation products generated by the NMD pathway, we profiled 5′P ends in Arabidopsis WT and mutants of key NMD factors, and compared the abundances of EJC footprints between them. In Arabidopsis, the crucial roles of *UPF1* and *SMG7* in the NMD pathway have been demonstrated (Arciga-Reyes et al., 2006; Riehs-Kearnan et al., 2012). Given that null mutants of *UPF1* are seedling lethal (Arciga-Reyes et al., 2006), *smg7-1*, a severe but viable mutant of *SMG7* with a T-DNA insertion in the CDS, was chosen for the comparison (Riehs et al., 2008). However, homozygous *smg7-1* plants are sterile and morphologically different from WT plants when grown at 22°C due to an autoimmune response (Riehs et al., 2008; Riehs-Kearnan et al.,
To reduce the possibility that differences might be caused by the autoimmune response, we selected \textit{smg7-1} homozygous plants by genotyping and grew them at 28°C, under which the autoimmune response is repressed and WT and \textit{smg7-1} plants more closely resemble each other (Gloggnitzer et al., 2014). We then collected the inflorescences of WT and \textit{smg7-1} plants and isolated RNA for PARE library construction. Similar to the WT, \textit{smg7-1} displayed an enrichment of 5' P ends in the canonical EJC region (Figure 7A). However, in \textit{smg7-1} the abundance of 5' P ends was significantly lower in the canonical EJC region but higher at the TSS compared with WT (P < 0.01, Wilcoxon rank-sum test) (Figure 7B and C). Furthermore, the median of the log$_2$ fold change in maximum EJC footprint abundance between \textit{smg7-1} and WT was significantly lower for transcripts experiencing NMD-suppressed AS events than for other transcripts (P < 10$^{-4}$, Kolmogorov-Smirnov two-sided test) (Figure 7D), supporting the notion that SMG7 plays a crucial role in the production of EJC footprints from NMD targets. Consistent with the analysis of PARE data (Figures 3 and 4C), the analysis of modified RLM 5' RACE data also confirmed involvement of SMG7 in the production of EJC footprints for three NMD targets in both flowers and 14-day-old seedlings (Supplemental Figure 4). The reduction in the number of EJC footprints in \textit{smg7-1} seedlings supports the notion that the effect was due to the NMD function of SMG7 instead of its unique function in meiosis (Riehs et al., 2008). Like the 5' P peaks in the EJC regions of six NMD targets shown in Figures 3 and 4C, the maximum 5' P peak in \textit{LPEAT2}, which was positioned outside the canonical EJC region, was also dampened in \textit{smg7-1} (Figure 3 and Supplemental Figure 2), indicating that it might also be a degradation product of the SMG7-dependent NMD pathway. Moreover, for three previously reported NMD-sensitive transcripts that had \textit{xrn4}-enhanced 5'P peaks in the CDS or 3' UTR (Nagarajan et al., 2019), \textit{xrn4}-enhanced 5'P peaks in \textit{eRF1-1} (\textit{Eukaryotic Release Factor 1-1}, NMD factor and target) were detected in the flowers of WT but were absent in
those of smg7-1 (Supplemental Figure 5). This result further confirms the notion that the
xrn4-enhanced 5′P peaks in eRF1-1 are also intermediates of degradation initiated by the
NMD pathway (Nagarajan et al., 2019).

Some miRNA Targets Possess EJC Footprints

Surprisingly, 5′ P peaks corresponding to putative EJC footprints were also observed
downstream of miRNA-guided cleavage sites in the targets of Arabidopsis miR159 (MYB33),
mR160 (ARF10), and miR396 (GRF1) (Figure 8A and Supplemental Figure 6). Likewise,
rice miR159, miR160, and miR396 targets also harbored EJC footprints in the exon ends
downstream of the miRNA target sites (Figure 8B). The accumulation of putative EJC
footprints in the three Arabidopsis miRNA targets was reduced in xrn4-6 and nearly
abolished in fry1-6; however, the amplitude of miRNA-guided cleavage peaks was increased
in both xrn4-6 and fry1-6 compared with that in the WT. We also confirmed the decreased
number of EJC footprints and the increased number of miRNA-guided cleavage 3′
remnants for these three miRNA targets in xrn4-6 and fry1-6 by performing modified RLM 5′
RACE assays (Supplemental Figure 7). We also assayed XRN2 and XRN3 function in the
production of EJC footprints from these three miRNA targets. The levels of 3′ remnants of
miRNA-guided cleavage and EJC footprints were comparable between the WT, xrn2-1 and
xrn3-3 (Supplemental Figure 7). We thus further examined the level of EJC footprints in the
XRN triple mutant xrn2-1 xrn3-3 xrn4-6. Like xrn4-6, the triple mutant also accumulated a
higher number of miRNA-guided cleavage 3′ remnants but fewer EJC footprints compared
with the WT. However, the accumulation of these two types of degradation fragments in the
triple mutant was neither different from that in xrn4-6 nor comparable to that in fry1-6. This
result does not completely rule out a role for XRN3 in EJC footprinting because xrn3-3 is a
knock-down mutant with a T-DNA insertion in the promoter region (Gy et al., 2007). These
results confirm that XRN4 degrades the 3′ cleavage remnants of plant miRNA targets (Souret et al., 2004), and suggest that in addition to XRN4, other enzymes with 5′-3′ exonuclease activity also contribute to the turnover of miRNA cleavage remnants and EJC footprinting.

Notably, not all of the 5′ P peaks evident at miRNA target sites were associated with prominent EJC footprints in downstream exon ends. For instance, while a high number of 5′P ends corresponding to the miR398 target site of Arabidopsis CSD1 and CSD2 were observed, no or poor 5′ P peaks appeared in the canonical EJC regions 3′ to the miR398 target site (Figure 9A and Supplemental Figure 8). More intriguingly, the miR398 target site is close to the canonical EJC region in CSD1 and overlaps with the canonical EJC region in CSD2. Hence, EJCs might mask the miR398 target site on the CSD1 and CSD2 mRNAs that have not been translated, and prevent miRNA regulation before the pioneer round of translation. Consistent with this result, rice miR398-regulated CSD1 and two miR444-regulated MADS genes, which possess a miRNA target site in or near the canonical EJC region, also lacked evident EJC footprints (Figure 9B). In addition to the miR398 and miR444 targets, a miR408 target encoding plantacyanin (ARPN) in Arabidopsis also possessed a prominent 5′ P peak at the miRNA-guided cleavage site but poor peaks in the EJC region. However, unlike the miR398 and miR444 targets, the miRNA target site on ARPN is located at a position about 200 nt upstream of an exon-exon junction that is unlikely to be masked by EJCs. Taken together, these results suggest that there might be multiple factors controlling the interaction between plant miRNAs and EJC-bound targets.

**Arabidopsis miRNAs Direct EJC-bound mRNA Degradation**

Unlike NMD targets, Arabidopsis miRNA targets did not exhibit changes in the number of EJC footprints in smg7-1 (Figure 8A and Supplemental Figure 6). Indeed, a global analysis
of maximum EJC footprint abundance between smg7-1 and WT revealed a median log_2 fold change value close to 0 for miRNA targets. The log_2 fold change of miRNA targets is significantly higher than that of other transcripts (P < 0.01, Kolmogorov-Smirnov two-sided test) (Figure 7D). This trend is opposite to that observed for NMD-suppressed AS events and suggests that most EJC footprints in miRNA targets are likely produced through an NMD-independent pathway.

Given that EJC footprints were evident downstream of miRNA target sites but rare in the upstream regions, we hypothesized that plant miRNAs are able to target EJC-bound mRNAs for degradation and trigger the production of EJC footprints. To test this hypothesis, we first assayed the role of AGO1, which mediates the function of most plant miRNAs, in the production of EJC footprints from the three miRNA targets shown in Figure 8A in the null ago1-3 mutant (Arribas-Hernandez et al., 2016). In ago1-3 there were fewer miRNA-guided cleavage remnants and EJC footprints corresponding to the miR160 target ARF10 and the miR396 target GRF1 (Figure 10A). Interestingly, for the miR159 target MYB33, which exhibited no change in mRNA level in ago1-3 (Arribas-Hernandez et al., 2016), the levels of miRNA-guided cleavage remnants and EJC footprints in ago1-3 were comparable to those in WT. Also, the mutation of AGO1 did not affect EJC footprint accumulation in the two NMD targets CYP38 and LPEAT2. Hence, these results confirm the link between the function of AGO1 and the production of EJC footprints in some miRNA targets.

To further validate the ability of plant miRNAs to direct EJC footprint accumulation, we created an artificial miRNA target by fusing the 5’ UTR of Arabidopsis NITROGEN LIMITATION ADAPTATION (NLA), which contains a target site of miR827, to an intron-containing GUS gene and introduced this construct (I-GUS827) into Arabidopsis (Figure 10B). As miR827 is barely detectable when phosphate levels are sufficient but is highly induced upon phosphate starvation (Hsieh et al., 2009) (Figure 10C), we thus were
able to test the ability of miR827 to induce EJC footprint production by comparing RNA degradation intermediates produced from the I-GUS827 construct under phosphate sufficient and starvation conditions using modified RLM 5′ RACE. As expected, distinct amplification products of the RNA degradation intermediates with 5′ P ends predominantly mapping to positions 26-27 nt upstream of the exon-exon junction were detected in the plants grown under phosphate starvation conditions but not in those grown with sufficient phosphate (Figure 10D and E). In contrast to the I-GUS827 construct, the control construct containing an intron-free GUS gene fused with the NLA 5′ UTR (GUS827) did not result in the accumulation of RNA degradation fragments truncated at the same site under phosphate starvation conditions (Figure 10D). Nevertheless, the number of RNA degradation fragments with the 5′ end determined by miR827-guided cleavage was comparable between plants expressing the two constructs. This result validates the ability of plant miRNAs to target the mRNAs associated with EJCs for degradation and initiate the accumulation of transcripts with 5′ P ends in the EJC region.

Unexpectedly, AS was observed in the I-GUS827 construct we created. In the AS form, the dominant sequence matching cloned 5′ P ends was shifted further upstream along with the alternative 5′ splice site compared with that in the normal splice form (Figure 10E). However, the distance between the predominant 5′ P peak and the exon-exon junction remained 27 nt in the AS form. As the deposition of EJC on mRNA is dependent on splicing, the shift of the 5′ P peak in the AS form reinforces our hypothesis that the 5′ P ends matching a region 26-30 nt upstream of the exon-exon junction mostly represent in vivo EJC footprints.

**Overexpression of an EJC Disassembly Factor Eliminates EJC Footprints**
To provide direct evidence that the 5′ P ends in the EJC region are EJC-protected fragments, we cloned an EJC disassembly factor, PYM, from Arabidopsis and transiently expressed a PYM overexpression construct in Arabidopsis protoplasts to disassemble EJCs (Figure 11). We predicted that disassembly of EJCs by PYM overexpression would promote the degradation of existing EJC-protected degradation intermediates and inhibit the production of new EJC footprints. For NMD targets, the production of intermediates with 5′ P ends matching the canonical EJC regions of CYP38 and PPD6 was abolished when PYM was overexpressed (Figure 11). Degradation fragments corresponding to the maximum 5′ P peak of LPEAT2, which was positioned outside the canonical EJC region and SMG7-dependent (Figure 3), were also absent in PYM-overexpressing protoplasts. This result indicates that this maximum 5′P peak is an EJC footprint and supports the notion that EJCs are also deposited on non-canonical sites in Arabidopsis. In addition to investigating the SMG7-dependent 5′ P peaks in NMD targets (Figures 3 and 4C), we also examined the effect of PYM overexpression on the SMG7-independent 5′ P peaks in the EJC region of miRNA targets shown in Figure 8A. Compared with the number of degradation intermediates in control protoplasts, the number of 3′ cleavage remnants directed by miRNAs in the three miRNA targets (MYB33, ARF10 and GRF1) was increased, whereas degradation intermediates with 5′ P ends corresponding to the EJC region were barely detectable in the PYM-overexpressing protoplasts (Figure 11). Taken together, these results strongly indicate that the 5′ P ends mapping to the canonical EJC regions of these transcripts are in vivo EJC footprints irrespective of their dependency on SMG7.

DISCUSSION

The RNA degradome is composed of assorted RNA degradation intermediates derived from diverse RNA degradation pathways. While endonucleolytic cleavage results in the
production of cleavage remnants with well-defined 5′ P ends, growing evidence supports
the idea that the hindrance of XRN-mediated 5′-3′ decay by RNA-binding proteins such
as Pumilio/fem-3 mRNA binding factors and ribosomes can also precisely delineate the 5′
P ends of degradation products (Hou et al., 2014; Pelechano et al., 2015; Hou et al., 2016;
Yu et al., 2016). Here, we demonstrate that like ribosomes, EJCs also have the capacity to
stop 5′-3′ decay and leave precise footprints in the RNA degradome (Figures 12). Because
EJCs are deposited on RNA during splicing and are displaced by ribosomes during the
pioneer round of translation (Figure 12A and B), EJC footprints can serve as markers for the
degradation of mRNA before steady-state translation. If a spliced mRNA is degraded after
the pioneer round of translation, 5′-3′ mRNA decay should not yield EJC footprints
upstream of the termination codon while co-translational mRNA decay during steady-state
translation may yield ribosome footprints (Figure 12B) (Pelechano et al., 2015; Hou et al.,
2016; Yu et al., 2016). By contrast, NMD targets are mainly degraded during the pioneer
round of translation and may produce EJC footprints downstream of PTCs (Figure 12C).
Additionally, some plant miRNA targets seem to be attacked by miRNAs before translation,
which may result in EJC footprints located 3′ to the miRNA target sites (Figure 12D). As
some EJC footprints appear not to be linked to NMD or miRNA pathways (Figures 4A and
7D), other post-transcriptional gene regulation mechanisms may also account for
EJC-bound mRNA degradation before or during the pioneer round of translation. In
summary, our study demonstrates that the RNA degradome contains EJC footprints, which
account for a sizable portion of major RNA degradation intermediates. The analysis of EJC
footprints in the RNA degradome confirms the degradation of plant NMD targets during the
pioneer round of translation and reveals EJC-bound mRNAs as targets of some plant
miRNAs.
Location and Abundance of EJC Footprints in the Plant RNA Degradome

Although CLIP-seq (cross-linked immunoprecipitation followed by next generation sequencing) has not been adopted to profile EJC binding regions on a genome-wide scale in Arabidopsis and rice, the identification of a conserved hot spot for 5′ P ends located in a region 25-30 nt upstream of the exon-exon junction (Figure 1) implies that the deposition of EJCs on plant and animal RNAs presumably follows similar rules. Two transcriptome-wide studies of human EJC binding regions using CLIP-seq revealed that only about half of the EJC binding sites mapped to the canonical position (Sauliere et al., 2012; Singh et al., 2012). The binding of EJCs to non-canonical sites likely also occurs in plants and accounts for the 5′ P peaks positioned outside the canonical EJC regions. For instance, the maximum 5′ P peak in Arabidopsis LPEAT2 appeared to be the footprint of an EJC deposited at a non-canonical site as it was located immediately downstream of the PTC, and the peak was dampened in smg7-1, and largely reduced when EJC disassembly factor PYM was overexpressed (Figures 3 and 11). If half of EJCs are deposited at non-canonical sites in plants as in humans, the contribution of EJC footprints to maximum 5′ P peaks located within transcripts is likely to have been underestimated as we only considered the maximum 5′ P peaks positioned in the canonical EJC region. Given that EJC footprints in the RNA degradome can be prominent, abundant, and prevalent as we demonstrated in this study, investigations of inference by endonucleolytic cleavage directed by miRNAs or other mechanisms using RNA degradome data should be undertaken cautiously. Dependency on XRN activity could serve as a key to differentiate between protected fragments derived from RNA binding proteins and endonucleolytic cleavage products.

The number of 5′ P ends mapping to EJC regions varied dramatically within PTC-containing mRNAs and miRNA targets (Figures 3 and 8). Intriguingly, the maximum EJC-protected 5′ P peaks tended to reside in the first or second exon end downstream of a
PTC or an miRNA-directed cleavage site. This result implies that in both cases 5′-3′ decay is more frequently blocked by the first EJC met by the XRN. However, in some cases, the exons further downstream also had 5′ P ends mapping predominantly to the EJC region, although to a lesser degree, implying that sometimes XRN-mediated decay might be able to overcome the hindrance of EJCs. Notably, in PTC-containing transcripts such as Arabidopsis LPEAT2 and RS2Z33, some exons possessed poor 5′ P peaks in the canonical EJC region while both the 5′ and 3′ neighboring exons had prominent EJC peaks (Figure 3). Since no other evident 5′ P peaks were present in these exons, which lacked canonical EJC footprints, it appears that EJCs are less frequently deposited on these exons than their neighboring exons. This possibility is supported by the previous finding that the loading of human EJCs varies among exons even within the same transcript (Sauliere et al., 2012; Singh et al., 2012). Differential EJC loading probably also accounts for poor accumulation of 5′ P ends in some EJC regions downstream of a PTC or miRNA target site within the same transcripts in plants.

**Application of EJC Footprints to the Study of NMD**

Previously, the identification or the validation of endogenous NMD substrates required NMD mutants or treatment with NMD inhibitors such as cycloheximide, which is not feasible or problematic in some species or tissues. Moreover, the up-regulation of some assayed RNAs is an indirect consequence of NMD pathway impairment. Our study shows that EJC footprints can be a direct readout of NMD; these footprints will be useful in the validation of potential NMD substrates and inference of NMD activity. The presence of a predominant 5′ P peak in the EJC region located at the first or second exon end downstream of the predicted PTC is strong evidence of NMD-mediated degradation. Using 5′ P end data for WT species alone, we have validated hundreds of NMD targets in Arabidopsis and rice,
where NMD is potentially triggered by AS or intron-containing 3’ UTRs (Supplemental Datasets 1 and 2). As RNA degradome data have been generated in many plant species for miRNA target identification, the analysis of publicly available RNA degradome datasets may advance our understanding of gene regulation mediated by the NMD pathway in different species. Nevertheless, this application might be limited to NMD targets with at least one intron after the PTC; NMD events triggered by long intron-free 3’ UTRs are unlikely to be confirmed by this approach. Furthermore, RNA degradome data may allow assessment of the impact of stress on the transcriptome through the NMD pathway. In plants, abiotic stress conditions such as high temperature, drought and salt stress have been shown to largely alter the accumulation of splicing variants, which mostly have features associated with NMD (Chang et al., 2014; Feng et al., 2015; Thatcher et al., 2016; Jiang et al., 2017). However, whether NMD activity is regulated by these stresses has not been addressed. Measuring EJC footprints from well-characterized NMD targets provides a straightforward approach to investigate the regulation of NMD activity by stress.

Targeting of EJC-bound mRNAs by Plant miRNAs

Here, we showed that the cleavage events guided by some, but not all, Arabidopsis and rice miRNAs were notably associated with the production of EJC footprints (Figure 8 and Supplemental Figure 6). If plant miRNAs only direct cleavage of the mRNAs that have finished the pioneer round of translation, the mRNA decay initiated by plant miRNAs would not lead to the accumulation of EJC footprints in the CDS. This result therefore suggests that at least a fraction of some plant miRNAs such as miR159, miR160 and miR396 target EJC-bound mRNAs that have not completed the pioneer round of translation (Figure 12D). Although prominent EJC peaks are only evident downstream of the miRNA-guided cleavage site in some targets (Figure 8), EJCs upstream of the miRNA target site likely remain
associated with the mRNA during miRNA-target interaction (Figure 12D). After cleavage
initiated by miRNAs, if these upstream EJCs can stop 3′-5′ RNA decay by the exosome, they
may also leave footprints in the RNA degradome. However, 3′-5′ RNA decay of
nonpolyadenylated 5′ cleavage remnants will generate degradation intermediates with a 3′
end delineated by the 3′ edge of the EJC. Hence, even if the EJC footprints upstream of the
miRNA-guided cleavage sites are present in the RNA degradome, they cannot be captured
by PARE or similar methods, which profile the 5′ ends but not 3′ ends of degradation
fragments with a polyadenylated tail.

While several previous studies have demonstrated that AGO1 is associated with
polysomes (Lanet et al., 2009; Li et al., 2013b; Li et al., 2016), the miRNA-mediated
regulation of EJC-bound mRNAs appears not to require prior translation. There are two
scenarios for the targeting of EJC-bound mRNAs by plant miRNAs. Since the loading of
mature plant miRNAs to AGO1 can occur in the nucleus (Bologna et al., 2018), it is possible
that some miRNA-induced silencing complexes (miRNA-RISCs) can function before being
exported from the nucleus, which is rich in EJC-bound mRNAs. Alternatively, cytoplasmic
miRNA-RISCs might be able to recognize their targets, which remain associated with EJCs,
and direct cleavage before translation. Considering these two scenarios, the miRNA targets
with a lower efficiency of nuclear export or translation during the pioneer round may have a
higher chance of being cleaved before translation and accumulating EJC footprints. The
analysis of the 5′P ends of miRNA targets in nuclear and cytoplasmic fractions will help to
answer this question. By contrast, for miRNAs such as miR398 and miR444, which pair with
target sites close to the EJC region and direct cleavage without triggering EJC footprint
production (Figure 9), prior translation of targeted mRNAs is likely to be essential for their
regulation. Other miRNA targets that possess target sites outside the EJC region but lack
prominent EJC footprints, such as Arabidopsis ARPN (Figure 9), may have few EJCs
deposited on the transcripts. Alternatively, their cleavage remnants might be mainly
degraded through the 3′-5′ RNA decay pathway, which is not able to yield EJC-protected 5′
RNA ends.

Interplay between the miRNA and NMD Pathways

In human cells, it was demonstrated that miRNAs are able to be loaded on EJC-bound
mRNAs, thereby repressing the translation and decay of some NMD targets (Choe et al.,
2010). As our study also demonstrated that some plant miRNAs can target EJC-bound
mRNAs, this raises the possibility that some plant transcripts are targeted by both RNA
degradation pathways. Interestingly, a previous analysis of miRNA and NMD targets in
Arabidopsis showed that there was little overlap between the two groups and that these
groups were enriched in distinct biological processes (Zhang et al., 2013). In spite of these
findings, interplay between these two RNA degradation pathways might occur in other plant
species. In Solanum and Fabaceae species, 22-nt miRNAs repress the expression of a large
number of nucleotide binding site leucine-rich repeat (NBS-LRR) resistance genes (Zhai et
al., 2011; Shivaprasad et al., 2012). Interestingly, normal transcripts of some NBS-LRR
genes, which harbor long or intron-containing 3′ UTRs, are NMD targets in Arabidopsis
(Gloggnitzer et al., 2014). If NMD also regulates NBS-LRR genes in Solanum or Fabaceae
species, the miRNA and NMD pathways might be interchangeable or cooperate to inhibit the
plant immune system.

As total RNA is generally used for genome-wide profiling of 5′ P ends, the existence of
EJC footprints in the RNA degradome allows us to infer that mRNA degradation occurs
before steady-state translation in the cytoplasm and nucleus by known and unknown
mechanisms. Our work leads to new possibilities for obtaining a greater understanding of
mRNA degradation before steady-state translation, which is currently under appreciated and has many unknowns.

**METHODS**

**Plant Materials and Growth Conditions**

All WT, mutant and transgenic *Arabidopsis thaliana* plants used in this study are in the Columbia-0 background. Arabidopsis mutants *phyB-9* (CS6217), *pho2* (CS8508), *xrn4-6* (SALK_014209), *xrn2-1* (SALK_041148), *xrn3-3* (SAIL_1172C07), *xrn2-1 xrn3-3 xrn4-6*, *fry1-6* (SALK_020882), *smg7-1* (SALK_073354) and *ago1-3* were described previously (Reed et al., 1993; Delhaize and Randall, 1995; Bohmert et al., 1998; Gy et al., 2007; Riehs et al., 2008; Hirsch et al., 2011). The double mutants *phyB-9 xrn4-6* and *pho2 xrn4-6* were identified in an F2 population by genotyping.

All plants except for *smg7-1* and its WT controls were grown at 22°C with a 16 h light (110-140 μmol m⁻² s⁻¹, photosynthetically active radiation)/8 h dark photoperiod before harvesting for RNA extraction. For *smg7-1* and its WT control, the seedlings were first grown at 22°C, but after genotyping *smg7-1* homozygous individuals and WT control plants were transferred to 28°C in order to inhibit autoimmune responses in *smg7-1*. For PARE library construction and modified RLM 5′ RACE assays, RNA was extracted from the inflorescences of soil-grown plants or from seedlings grown on 0.8% Bacto-agar plates containing half-strength Murashige and Skoog medium (pH 5.7) and 1% sucrose. For phosphate starvation treatment of WT plants and Arabidopsis transgenic lines carrying artificial miRNA targets, seedlings were grown on Pi-sufficient agar plates for 7 days and then transferred to Pi-deficient or Pi-sufficient agar plates for an additional 7 days before harvesting for RNA extraction. The Pi-sufficient and Pi-deficient agar plates contained 1%
Bacto-agar, half-strength modified Hoagland nutrient solution and 1% sucrose supplemented with 250 μM and 10 μM KH$_2$PO$_4$, respectively.

**PARE Library Construction and Sequencing**

For each genotype, about 80 μg of total RNA isolated from two separate biological replicates using PureLink Plant RNA Reagent (Thermo Fisher) was used for PARE library construction following the protocol described previously (Zhai et al., 2014). PARE libraries were sequenced on the Illumina HiSeq 2500 platform.

**Pre-processing and Mapping of PARE and GMUCT Reads**

Previously published PARE data for Arabidopsis and budding yeast (Saccharomyces cerevisiae), Degradome-Seq data for rice (Oryza sativa) and worm (Caenorhabditis elegans), and GMUCT data for Arabidopsis and human (Homo sapiens) HEK293 cells were downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/). Accession numbers and details for the publicly available 5'P end data analyzed are given in Supplemental Dataset 3. For PARE and Degradome-Seq data, trimmed reads of 20-21 nt with a quality score ≥ 30 were mapped to the corresponding genome and cDNA sequences downloaded from The Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/; TAIR 10), the MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/; RGAP 7), the Saccharomyces Genome Database (https://www.yeastgenome.org/; S288C), and the WormBase database (https://www.wormbase.org/; WS269) using BOWTIE 1.2.1.1 with 0 mismatches (Langmead et al., 2009). PARE and Degradome-Seq reads with > 10 genomic hits or that mapped to the chloroplast genome, mitochondria genome, ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs) or small nucleolar RNAs (snoRNAs) were
discarded. For the human GMUCT data, trimmed reads of 30-50 nt and with a quality score ≥ 30 were mapped to the human genome and cDNA sequences downloaded from the Ensembl database (http://www.ensembl.org; GRCh38p12) using BOWTIE 1.2.1.1 with 2 mismatches. Human GMUCT reads with > 10 genomic hits or that mapped to the mitochondria genomes, rRNAs, tRNAs, snRNAs or snoRNAs were discarded. The processing and mapping of the Arabidopsis GMUCT data was similar to that of the human GMUCT data but the Arabidopsis sequences were used for mapping and filtering. The abundance of PARE, Degradome-Seq or GMUCT sequences was first normalized to the total remaining reads to TP40M and then assigned to the genomic or gene position corresponding to the first nucleotide of the sequence. As many genes have multiple isoforms, only exons with a size ≥ 50 nt in the longest isoform of a gene were included in the following exon analyses.

Metagene Analysis of 5′P Ends

For metagene analysis of 5′P ends mapping to the 50-nt region upstream of the exon-exon junction, the normalized occurrence of 5′P ends (P_i) starting at each position was calculated using the formula

\[ P_i = \frac{\sum_{j=1}^{n} C_{i,j}}{\sum_{i=0}^{49} C_{i,j}} \]

where \( C_{i,j} \) is the 5′P end count starting at position \( i \) of exon \( j \), and \( n \) is the number of analyzed exons with a size ≥ 50 nt and a 5′P end raw count ≥ 1. Then, the relative frequency of normalized 5′P end occurrences in the 50-nt region was plotted.
For comparison of 5′P ends mapping to the canonical EJC region in the WT and mutants, exons possessing a total 5′P end count ≤ 10 TP40M in the canonical EJC region (26-30 nt upstream of the exon-exon junction) in all of the samples were excluded. The abundance of 5′P ends at the TSS of genes harboring the exons included in the comparison of 5′P ends in the EJC region was calculated as the total 5′P end count in the 21-nt region symmetrically flanking the TSS based on the TAIR 10 annotation. The TSS regions with a total 5′P end count ≤ 5 TP40M in all of the samples were excluded from the comparison.

Identification and GO analysis of NMD Targets with 5′P End Data

The WT Arabidopsis PARE data generated in this study and the Degradome-seq data for WT rice produced by Li et al. (2010) were used in the identification of putative NMD targets. These targets were identified through the analysis of EJC footprints using a custom R script (https://github.com/LabHMChenABRC/EJC-DegradomeAnalysis). We first selected protein-coding genes with at least one intron-containing gene model and a maximal 5′P peak with a count of ≥ 10 TP40M in representative gene models. If the position of the maximum 5′P peak matched one of the following two criteria, the transcript was identified as a putative NMD target: (1) The maximum 5′P peak was located in the first or second canonical EJC region downstream of an alternative splice site based on the existence of a non-representative gene model with an altered translation start or termination site compared with that of the representative gene model. NMD of this type of target was classified as being triggered by AS. (2) The maximum 5′P peak was located in the first or second canonical EJC region of the exon 3′ end at least 50 nt downstream of the annotated stop codon. NMD of this type of target was classified as being triggered by an intron-containing 3′ UTR. The
analysis of enriched GO terms for putative NMD targets was performed using PANTHER version 14 (Mi et al., 2019).

**Artificial miRNA Target Construction and Arabidopsis Transformation**

The Cauliflower Mosaic Virus (CaMV) 35S promoter, a 330 bp fragment of the Arabidopsis *NLA* 5′ UTR, and the intron-free β-glucuronidase gene (*GUS*) from the pBI121 vector or the intron-containing *GUS* gene from the pBISN1 vector were fused and then cloned into the pCAMBIA1390 vector for Arabidopsis transformation. Homozygous T3 transgenic lines were selected and used for phosphate treatments. The PCR primers used for cloning artificial miRNA targets are listed in Supplemental Table 2.

**Modified RLM 5′ RACE assay and Quantification of Steady-state mRNA Levels**

The GeneRacer kit (Thermo Fisher) was used to detect the 5′ P ends of specific genes. Total RNA (2-3 μg) isolated using the PureLink Plant RNA Reagent (Thermo Fisher) or mirVana™ miRNA Isolation Kit (Thermo Fisher) was used as a template in modified RLM 5′ RACE assays that were performed according to the manual of the GeneRacer kit, except that the calf intestinal phosphatase and tobacco acid pyrophosphatase (TAP) treatments were skipped. After RNA was ligated to the 5′ RNA adapter, an oligo-dT primer was used to synthesize cDNA, which served as the template for PCR analysis with a GeneRacer 5′ primer and a gene-specific primer. For the modified RLM 5′ RACE of *PHO2*, nested PCR was performed with a GeneRacer 5′ nested primer and a gene-specific nested primer. PCR products were gel purified and cloned into the pJET1.2 vector or pCR4-TOPOTA vector (Thermo Fisher) for sequencing. Control HeLa total RNA included in the GeneRacer kit was treated with TAP for a spike-in control. The same cDNA was used for the
quantification of steady-state mRNA levels of *PYM* and *ACT8* with pairs of gene specific primers. The PCR primers are listed in Supplemental Table 2.

**RNA Gel Blot Analysis of Small RNAs**

Total RNA (5 µg) isolated using the PureLink Plant RNA Reagent (Thermo Fisher) was used for small RNA gel blot analysis following the procedures described previously (Lee et al., 2015). The probes used for the detection of miR827 and U6 are listed in Supplemental Table 2.

**PYM Overexpression in Protoplasts**

The Arabidopsis *PYM* coding region amplified from cDNA was cloned into pJD301 to replace the firefly luciferase sequence using the NEBuilder HiFi DNA Assembly Master Mix according to the manufacturer’s recommendations (NEB). The PCR primers used for *PYM* cloning are listed in Supplemental Table 2. Protoplast isolation and transfection were performed as described previously with minor modifications (Hou et al., 2016). Arabidopsis mesophyll protoplasts were isolated from 17-day-old rosette leaves. Together with 5 µg of transfection control plasmids, 30 µg of pJD301 or pJD-PYM was transfected into 4 x 10^4 protoplasts using PEG solution. The transfected protoplasts were incubated at 22°C in the dark for 19-20 h. Then, 2 x 10^5 transfected protoplasts were pooled and lysed for RNA extraction with the mirVana™ miRNA Isolation Kit (Thermo Fisher).

**ACCESSION NUMBERS**

The PARE data generated in this study are available in the GEO database under the accession number GSE118215. The previously published PARE data, Degradome-Seq data
and GMUCT data used in this study are available in the GEO database under the accession numbers shown in Supplemental Dataset 3. Sequences of individual genes included in 5’ P end analysis using PARE data or modified RLM 5’ RACE assays can be found in TAIR or the MSU Rice Genome Annotation Project database under the locus numbers indicated. The Arabidopsis mutants analyzed and genes cloned can be found in the TAIR database under the following accession numbers: AT5G42540 for xrn2-1, AT1G75660 for xrn3-3, AT1G54490 for xrn4-6, AT5G63980 for fry1-6, AT2G18790 for phyB-9, AT2G33770 for pho2, AT5G19400 for smg7-1, AT1G02860 for NLA, AT1G48410 for ago1-3 and AT1G11400 for PYM.

SUPPLEMENTARY DATA

Supplemental Figure 1. 5’P End Analyses of Polyadenylated and Deadenylated Degradation Fragments, Using PARE and GMUCT Data, all Show an Enrichment in the Canonical EJC Region.

Supplemental Figure 2. The EJC Regions Downstream of PTCs Harbor SMG7-dependent 5’ P Peaks in Arabidopsis.

Supplemental Figure 3. Genes Producing NMD-suppressed AS Variants Possess a Maximum 5’ P Peak in the EJC Region Downstream of the PTC.

Supplemental Figure 4. Dysfunction of SMG7 Reduces the Number of EJC Footprints in Putative NMD Targets.

Supplemental Figure 5. The xrn4-enhanced 5’P Peaks in eRF1-1 are Dampened in smg7-1.

Supplemental Figure 6. Some Arabidopsis miRNA Targets Possess Dominant EJC Footprints Downstream of miRNA-guided Cleavage Sites.

Supplemental Figure 7. XRN4 Contributes to the Turnover of 3’ Cleavage Remnants and Production of EJC footprints in miRNA Targets.
Supplemental Figure 8. Some Arabidopsis miRNA Targets Possess Poor EJC Footprints Downstream of miRNA-guided Cleavage Sites.

Supplemental Table 1. GO Terms Enriched in Putative Arabidopsis NMD Targets Identified from the Analysis of EJC Footprints in the RNA Degradome.

Supplemental Table 2. Sequences of Primers for Modified RLM 5’ RACE, Quantification of Steady-state mRNA, Cloning, and RNA Gel Blot Analysis.

Supplemental Dataset 1. Putative Arabidopsis NMD Targets Identified from the Analysis of EJC Footprints in the RNA Degradome.

Supplemental Dataset 2. Putative Rice NMD Targets Identified from the Analysis of EJC Footprints in the RNA Degradome.

Supplemental Dataset 3. Summary of 5’P End Datasets Used in this Study.

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AUTHOR CONTRIBUTIONS

H.-M.C. designed the research. B.-H.H., C.-Y.H., S.-M.T., P. K., and H.-M.C. performed the computational analyses. W.-C.L. carried out the experiments. B.-H.H., W.-C.L. and H.-M.C. wrote the paper. All authors read and approved the final manuscript.

FIGURE LEGENDS

Figure 1. The Canonical EJC Region is a Conserved Hot Spot for 5’ P Ends.

(A) Distribution of the relative frequency of 5’ P end occurrences in a 50-nt region upstream of the exon-exon junction. PARE data for Arabidopsis WT inflorescences (German et al., 2008) and yeast (Harigaya and Parker 2012), Degradome-Seq data for rice WT seedlings (Li et al., 2010) and adult stage worms (Park et al., 2013), and GMUCT data for human embryonic kidney 293 T cells (Willmann et al., 2014), were used in the metagene analyses. The illustration at the top shows the canonical position of an EJC and the size of a fragment protected by an EJC. The numbers of exons ≥ 50 nt in size and with a 5’ P end count ≥ 1 that were included in the analysis are shown in parentheses.

(B) Distribution of the maximum 5’ P peaks within the transcripts of intron-containing genes in a 50-nt region upstream of the exon-exon junction. The numbers of intron-containing genes that had the maximum 5’ P peak with a count ≥ 3 and were included in the analyses are shown in parentheses.

Figure 2. XRNs are Involved in the Production of 5’ P Ends Corresponding to the Canonical EJC Region in Arabidopsis.

(A) Distribution of 5’ P ends in a 50-nt region upstream of the exon-exon junction in WT, xrn4-6, and fry1-6 seedlings. The distributions for two independent biological replicates (R1 and R2) are shown. The numbers of exons ≥ 50 nt in size with a 5’ P end count ≥ 1 that were included in the analysis are shown in parentheses.

(B) and (C) Comparison of 5’ P end abundance in the canonical EJC region (EJC) (B) and at the transcription start site (TSS) (C) of the genes harboring the selected exons in Arabidopsis WT, xrn4-6, and fry1-6 seedlings. The numbers of EJC regions (count > 10 TP40M) and TSSs (count > 5 TP40M) included in the analysis are shown in parentheses. Different letters above box plots indicate significant differences between groups (P < 0.001) determined by the Kruskal-Wallis test followed by the pairwise Wilcoxon test with FDR correction. Box boundaries and whiskers indicate quartiles and the range of 5’ P end abundance, respectively.
Figure 3. The EJC Regions Downstream of PTCs Harbor SMG7-dependent 5’ P Peaks in Arabidopsis.

Positional distribution of 5’ P ends in three Arabidopsis genes producing PTC-containing mRNAs due to AS. The 5’P ends were identified in the PARE data generated by this study. Within the 5’P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak in 10-day-old (10d) WT seedlings; blue arrow, peak corresponding to the putative TSS. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; red asterisk, PTC. Only the 5’ P ends residing in exons are displayed in the plots. Data for replicate 2 are shown in Supplemental Figure 2.

Figure 4. Genes Producing NMD-suppressed AS Variants More Frequently Possess a Maximum 5’P peak in the EJC Region.

(A) Venn diagram showing the overlap between genes producing NMD-suppressed AS variants (NMD+ AS) and genes possessing a maximum 5’P peak (M5’P) in the EJC region. The NMD-suppressed AS variants were extracted from the lba1 upf3-1 data reported by Drechsel et al. (2013). Two replicates (R1 and R2) of PARE data for 10-day-old (10d) seedlings generated by this study were used for the identification of genes possessing a maximum 5’P peak with a count ≥ 3 located in the EJC region.

(B) Relative frequency of genes possessing a maximum 5’P peak in the EJC region. Frequencies are shown for genes containing introns (intron+ gene), and those producing transcripts derived from the following AS events: NMD+ AS, intron-retention, exon-skipping, alternative 5’ and 3’ splice site (alt. 5’ss and alt. 3’ss). The number of genes in each category possessing a maximum 5’P peak in the EJC region is shown above the bar. Asterisks indicate a significant difference between categories based on the binomial test (*, P < 10^-5; **, P < 10^-15; ***, P < 10^-100).

(C) Positional distribution of 5’P ends (from PARE data generated by this study) in three Arabidopsis genes producing NMD-suppressed AS variants. Within the 5’P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak in 10-day-old WT seedlings. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; red asterisk, PTC; orange box, region altered by NMD-suppressed AS events according to Drechsel et al. (2013). Only the 5’P ends residing in exons are displayed in the plots. Data for replicate 2 are shown in Supplemental Figure 3.

Figure 5. PTCs Trigger Formation of EJC Footprints in Downstream Exons.

(A) and (B) Modified RLM 5’ RACE assays of RNA degradation intermediates generated from WT and nonsense mutant mRNAs of PHYB (A) and PHO2 (B). Arrowheads indicate
the RACE products excised and cloned for Sanger sequencing (left panel). The positional distribution of the 5′ P ends of the cloned RACE products from nonsense mutants is shown in the right panel. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; asterisk, PTC; half arrow, gene-specific primer used for modified RLM 5′ RACE. The 3′ remnant of MYB65 derived from miR159-guided cleavage was used as a positive control for modified RLM 5′ RACE.

(C) Comparison of EJC footprints from nonsense mutant mRNAs between phyB-9 and phyB-9 xrn4-6, and between pho2 and pho2 xrn4-6 using modified RLM 5′ RACE assays. Arrowheads indicate the RACE products sequenced in (A) and (B).

Figure 6. Analysis of EJC Footprints reveals Targets of NMD Triggered by AS and Intron-containing 3′ UTRs.

(A) Fraction of putative NMD targets where NMD is triggered by AS or an intron-containing 3′ UTR (intron+ 3′ UTR). The numbers of total putative NMD targets recovered from the analysis are in parentheses.

(B) and (C) Positional distribution of 5′ P ends for targets of NMD triggered by intron-containing 3′ UTRs in Arabidopsis (B) and rice (C). 5′P ends in Arabidopsis were identified using the Arabidopsis PARE data (replicate 1) generated by this study, and those in rice were identified using the Degradome-seq data for rice WT seedlings reported by Li et al. (2010). Within the 5′P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron. Only the 5′ P ends residing in exons are displayed in the plots.

Figure 7. EJC Footprints in NMD Targets but not miRNA Targets are Less Abundant in smg7-1.

(A) Distribution of 5′ P end counts in a 50-nt region upstream of the exon-exon junction. Counts are shown for two replicates (R1 and R2) each from WT and smg7-1 flowers. The numbers of analyzed exons that are ≥ 50 nt in size and with a 5′ P end count ≥ 1 TP40M are shown in parentheses.

(B) and (C) Comparison of 5′ P end counts in the canonical EJC region (EJC) (B) and at the TSS (C) of the genes harboring the selected exons between WT and smg7-1. Asterisks indicate significant difference between groups (P < 0.01, Wilcoxon rank-sum test). Box boundaries and whiskers indicate quartiles and the range of 5′ P end abundance, respectively. The numbers of analyzed EJC regions (count > 10 TP40M) or TSSs (count > 5 TP40M) are shown in parentheses.

(D) MA and density plots depicting log2 fold change (smg7-1/WT) and log2 average 5′P end counts for the maximum EJC peaks in genes. Each data point represents a maximum EJC
peak with an average 5′P end count $\geq 10$. EJC peaks for genes producing
NMD-suppressed AS variants (NMD$^+$ AS), miRNA targets and other genes (other) are
shown. Arabidopsis genes producing NMD-suppressed AS variants and genes that are
validated miRNA targets were extracted from datasets reported by Drechsel et al. (2013)
and Zheng et al. (2012), respectively. The number of analyzed genes in each category is
shown in parentheses. The density plots to the right cover all data points in each category,
and straight lines indicate the median of each distribution. Asterisks indicate significant
differences in the comparisons of the NMD$^+$ AS group and the miRNA group to the group of
other genes (Kolmogorov-Smirnov two-sided test). The color and location of asterisks
indicate the associated category and the direction of the shift in the distribution.

Figure 8. Some Arabidopsis and Rice miRNA Targets Possess Dominant EJC Footprints
Downstream of miRNA-guided Cleavage Sites.
(A) and (B) Positional distribution of 5′ P ends of selected Arabidopsis (A) and rice (B)
miRNA targets with prominent EJC footprints based on the Arabidopsis PARE data
generated in this study and the rice Degradome-Seq data generated by Li et al. (2010).
Within the 5′ P end plot: red peak, peak in the canonical EJC region; red arrowhead, peak
corresponding to the miRNA-guided cleavage site. Within the gene model: light grey box,
UTR; dark grey box, CDS; thin line, intron. Only 5′ P ends residing in exons are displayed in
the plots. Data for replicate 2 of Arabidopsis are shown in Supplemental Figure 6.

Figure 9. Some Arabidopsis and Rice miRNA Targets Possess Poor EJC Footprints
Downstream of miRNA-guided Cleavage Sites.
(A) and (B) Positional distribution of 5′ P ends of selected Arabidopsis (A) and rice (B)
miRNA targets with poor EJC footprints based on the Arabidopsis PARE data generated in
this study and the rice Degradome-seq data generated by Li et al. (2010). Within the 5′ P
end plot: red peak, peak in the canonical EJC region; red arrowhead, peak corresponding to
the miRNA-guided cleavage site. Within the gene model: light grey box, UTR; dark grey box,
CDS; thin line, intron. Only 5′ P ends residing in exons are displayed in the plots. Data for
replicate 2 of Arabidopsis are shown in Supplemental Figure 8.

Figure 10. AGO1 Function, Splicing and miRNA Expression Determine EJC Footprint
Production in miRNA Targets.
(A) RLM 5′ RACE assays of degradation intermediates generated from three miRNA targets
and two NMD targets in WT and ago1-3. Open and solid arrowheads indicate miRNA-guided
cleavage sites and EJC binding sites, respectively, in gene models and the corresponding
RACE products separated by gel electrophoresis. An arrow indicates the maximum 5′ peak
in the LPEAT2 gene model and in the corresponding RACE product in an electrophoretic gel. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; asterisk, PTC; half arrow, gene-specific primer. The steady-state mRNA level of Arabidopsis ACT8 was used to control the input amount of total RNA and reverse transcription among samples. An equal amount of TAP-treated total RNA from HeLa cells was spiked into each sample, and the TSS of human β-actin was used to control for differences in ligation efficiency among samples.

(B) Constructs expressing miR827 artificial targets: GUS with an intron (I-GUS827) and without an intron (GUS827). 35S, CaMV 35S promoter; box, exon; thin line, intron; half arrow, gene-specific primer used for modified RLM 5′ RACE.

(C) RNA gel blot analysis of miR827 in Arabidopsis transgenic lines harboring miR827 artificial targets. Transgenic seedlings grown under phosphate sufficient (+P) or deficient conditions (−P) were used for the assays. U6 was used as a loading control. Numbers to the right of the blot show sizes in nucleotides.

(D) Modified RLM 5′ RACE assays of RNA degradation intermediates generated from miR827 artificial targets in Arabidopsis transgenic lines. An open arrowhead indicates the expected RACE product for the 3′ cleavage remnant of the artificial targets directed by miR827. A solid arrowhead indicates the RACE product excised and cloned for Sanger sequencing. The miR159-guided 3′ cleavage remnant of MYB65 was used as a positive control for the modified RLM 5′ RACE assay.

(E) Positional distribution of the 5′ P ends of cloned RACE products from I-GUS827 transgenic plants grown under −P conditions.

Figure 11. Overexpression of PYM Abolishes EJC Footprints in NMD and miRNA Targets. Arabidopsis PYM was cloned and transiently overexpressed in Arabidopsis protoplasts. The steady-state mRNA levels of PYM and a housekeeping gene, ACT8, in control (−) and PYM-transfected (+) protoplasts were measured using the cDNA from the modified RLM 5′ RACE assays. The amounts of RNA degradation intermediates from three NMD targets and three miRNA targets in control and PYM-overexpressing protoplasts were compared using modified RLM 5′ RACE assays. An equal amount of TAP-treated total RNA from HeLa cells was spiked into each sample, and the TSS of human β-actin was used to infer ligation efficiency. Open and solid arrowheads indicate the miRNA-guided cleavage sites and EJC binding sites, respectively, in gene models and the corresponding RACE products separated by gel electrophoresis. An arrow indicates the maximum 5′ peak in the LPEAT2 gene model and the corresponding RACE product in an electrophoretic gel. The identity of all marked RACE products was confirmed by Sanger sequencing. Within the gene model: light
grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; asterisk, PTC; half arrow, gene-specific primer.

Figure 12. Model for EJC-bound mRNA Degradation Before and During Steady-state Translation.

(A) During splicing, EJCs are deposited on mRNAs at a position so that the distance between the 5′ edge of the EJC and the exon-exon junction is about 26-30 nt. 

(B) After the pioneer round of translation, all EJCs upstream of the termination codon are removed from the mRNAs. The XRN-mediated 5′ to 3′ co-translational mRNA decay occurring during steady-state translation yields ribosome footprints but not EJC footprints in the CDS.

(C) During the pioneer round of translation of PTC-containing mRNAs, ribosomes displace the EJCs upstream of the PTC while triggering NMD when stalling at the PTC. The XRN-mediated 5′ to 3′ decay of PTC-containing mRNAs produces footprints of the EJCs positioned downstream of the PTC.

(D) Before the pioneer round of translation, endonucleolytic cleavage directed by miRNAs results in EJC-bound cleavage remnants, which are further trimmed by XRNs and become EJC footprints.

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Figure 1. The Canonical EJC Region is a Conserved Hot Spot for 5′P Ends.

(A) Distribution of the relative frequency of 5′P end occurrences in a 50-nt region upstream of the exon-exon junction. PARE data for Arabidopsis WT inflorescences (German et al., 2008) and yeast (Harigaya and Parker 2012), Degradome-Seq data for rice WT seedlings (Li et al., 2010) and adult stage worms (Park et al., 2013), and GMUCT data for human embryonic kidney 293 T cells (Willmann et al., 2014), were used in the metagene analyses. The illustration at the top shows the canonical position of an EJC and the size of a fragment protected by an EJC. The numbers of exons ≥ 50 nt in size and with a 5′P end count ≥ 1 that were included in the analysis are shown in parentheses.

(B) Distribution of the maximum 5′P peaks within the transcripts of intron-containing genes in a 50-nt region upstream of the exon-exon junction. The numbers of intron-containing genes that had the maximum 5′P peak with a count ≥ 3 and were included in the analyses are shown in parentheses.
Figure 2. XRNs are Involved in the Production of 5′P Ends Corresponding to the Canonical EJC Region in Arabidopsis. 

(A) Distribution of 5′P ends in a 50-nt region upstream of the exon-exon junction in WT, xrn4-6, and fry1-6 seedlings. The distributions for two independent biological replicates (R1 and R2) are shown. The numbers of exons ≥ 50 nt in size with a 5′P end count ≥ 1 that were included in the analysis are shown in parentheses. 

(B) and (C) Comparison of 5′P end abundance in the canonical EJC region (EJC) (B) and at the transcription start site (TSS) (C) of the genes harboring the selected exons in Arabidopsis WT, xrn4-6, and fry1-6 seedlings. The numbers of EJC regions (count > 10 TP40M) and TSSs (count > 5 TP40M) included in the analysis are shown in parentheses. Different letters above box plots indicate significant differences between groups (P < 0.001) determined by the Kruskal-Wallis test followed by the pairwise Wilcoxon test with FDR correction. Box boundaries and whiskers indicate quartiles and the range of 5′P end abundance, respectively.
Figure 3. The EJC Regions Downstream of PTCs Harbor SMG7-dependent 5′P Peaks in Arabidopsis. Positional distribution of 5′P ends in three Arabidopsis genes producing PTC-containing mRNAs due to AS. The 5′P ends were identified in the PARE data generated by this study. Within the 5′P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak in 10-day-old (10d) WT seedlings; blue arrow, peak corresponding to the putative TSS. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; red asterisk, PTC. Only the 5′P ends residing in exons are displayed in the plots. Data for replicate 2 are shown in Supplemental Figure 2.
Figure 4. Genes Producing NMD-suppressed AS Variants More Frequently Possess a Maximum 5′P peak in the EJC Region.

(A) Venn diagram showing the overlap between genes producing NMD-suppressed AS variants (NMD+ AS) and genes possessing a maximum 5′P peak (M5′P) in the EJC region. The NMD-suppressed AS variants were extracted from the lba1 upf3-1 data reported by Drechsel et al. (2013). Two replicates (R1 and R2) of PARE data for 10-day-old (10d) seedlings generated by this study were used for the identification of genes possessing a maximum 5′P peak with a count ≥ 3 located in the EJC region.

(B) Relative frequency of genes possessing a maximum 5′P peak in the EJC region. Frequencies are shown for genes containing introns (intron+ gene), and those producing transcripts derived from the following AS events: NMD+ AS, intron-retention, exon-skipping, alternative 5′ and 3′ splice site (alt. 5′ss and alt. 3′ss). The number of genes in each category possessing a maximum 5′P peak in the EJC region is shown above the bar. Asterisks indicate a significant difference between categories based on the binomial test (*, P < 10^-5; **, P < 10^-15; ***, P < 10^-100).

(C) Positional distribution of 5′P ends (from PARE data generated by this study) in three Arabidopsis genes producing NMD-suppressed AS variants. Within the 5′P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak in 10-day-old WT seedlings. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; red asterisk, PTC; orange box, region altered by NMD-suppressed AS events according to Drechsel et al. (2013). Only the 5′P ends residing in exons are displayed in the plots. Data for replicate 2 are shown in Supplemental Figure 3.
Figure 5. PTCs Trigger Formation of EJC Footprints in Downstream Exons. (A) and (B) Modified RLM 5’ RACE assays of RNA degradation intermediates generated from WT and nonsense mutant mRNAs of PHYB (A) and PHO2 (B). Arrowheads indicate the RACE products excised and cloned for Sanger sequencing (left panel). The positional distribution of the 5’P ends of the cloned RACE products from nonsense mutants is shown in the right panel. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; asterisk, PTC; half arrow, gene-specific primer used for modified RLM 5’ RACE. The 3’ remnant of MYB65 derived from miR159-guided cleavage was used as a positive control for modified RLM 5’ RACE. (C) Comparison of EJC footprints from nonsense mutant mRNAs between phyB-9 and phyB-9 xrn4-6, and between pho2 and pho2 xrn4-6 using modified RLM 5’ RACE assays. Arrowheads indicate the RACE products sequenced in (A) and (B).
Figure 6. Analysis of EJC Footprints reveals Targets of NMD Triggered by AS and Intron-containing 3′ UTRs.

(A) Fraction of putative NMD targets where NMD is triggered by AS or an intron-containing 3′ UTR (intron + 3′ UTR). The numbers of total putative NMD targets recovered from the analysis are in parentheses.

(B) and (C) Positional distribution of 5′P ends for targets of NMD triggered by intron-containing 3′ UTRs in Arabidopsis (B) and rice (C). 5′P ends in Arabidopsis were identified using the Arabidopsis PARE data (replicate 1) generated by this study, and those in rice were identified using the Degradome-seq data for rice WT seedlings reported by Li et al. (2010). Within the 5′P end plot: red peak, peak in the canonical EJC region; black arrow, the maximum peak. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron. Only the 5′P ends residing in exons are displayed in the plots.
Figure 7. EJC Footprints in NMD Targets but not miRNA Targets are Less Abundant in smg7-1.

(A) Distribution of 5′P end counts in a 50-nt region upstream of the exon-exon junction. Counts are shown for two replicates (R1 and R2) each from WT and smg7-1 flowers. The numbers of analyzed exons that are ≥ 50 nt in size and with a 5′P end count ≥ 1 TP40M are shown in parentheses.

(B) and (C) Comparison of 5′P end counts in the canonical EJC region (EJC) (B) and at the TSS (C) of the genes harboring the selected exons between WT and smg7-1 flowers. Asterisks indicate significant difference between groups (P < 0.01, Wilcoxon rank-sum test). Box boundaries and whiskers indicate quartiles and the range of 5′P end abundance, respectively. The numbers of analyzed EJC regions (count > 10 TP40M) or TSSs (count > 5 TP40M) are shown in parentheses.

(D) MA and density plots depicting log\(_2\) fold change (smg7-1/WT) and log\(_2\) average 5′P end counts for the maximum EJC peaks in genes. Each data point represents a maximum EJC peak with an average 5′P end count ≥ 10. EJC peaks for genes producing NMD-suppressed AS variants (NMD+ AS), miRNA targets and other genes (other) are shown. Arabidopsis genes producing NMD-suppressed AS variants and genes that are validated miRNA targets were extracted from datasets reported by Drechsel et al. (2013) and Zheng et al. (2012), respectively. The number of analyzed genes in each category is shown in parentheses. The density plots to the right cover all data points in each category, and straight lines indicate the median of each distribution. Asterisks indicate significant differences in the comparisons of the NMD+ AS group and the miRNA group to the group of other genes (Kolmogorov-Smirnov two-sided test). The color and location of asterisks indicate the associated category and the direction of the shift in the distribution.
**Figure 8.** Some Arabidopsis and Rice miRNA Targets Possess Dominant EJC Footprints Downstream of miRNA-guided Cleavage Sites.  
(A) and (B) Positional distribution of 5′P ends of selected Arabidopsis (A) and rice (B) miRNA targets with prominent EJC footprints based on the Arabidopsis PARE data generated in this study and the rice Degradome-Seq data generated by Li et al. (2010). Within the 5′P end plot: red peak, peak in the canonical EJC region; red arrowhead, peak corresponding to the miRNA-guided cleavage site. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron. Only 5′P ends residing in exons are displayed in the plots. Data for replicate 2 of Arabidopsis are shown in Supplemental Figure 6.
Figure 9. Some Arabidopsis and Rice miRNA Targets Possess Poor EJC Footprints Downstream of miRNA-guided Cleavage Sites.

(A) and (B) Positional distribution of 5′P ends of selected Arabidopsis (A) and rice (B) miRNA targets with poor EJC footprints based on the Arabidopsis PARE data generated in this study and the rice Degradome-seq data generated by Li et al. (2010). Within the 5′P end plot: red peak, peak in the canonical EJC region; red arrowhead, peak corresponding to the miRNA-guided cleavage site. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron. Only 5′P ends residing in exons are displayed in the plots. Data for replicate 2 of Arabidopsis are shown in Supplemental Figure 8.
Figure 10. AGO1 Function, Splicing and miRNA Expression Determine EJC Footprint Production in miRNA Targets.

(A) RLM 5′ RACE assays of degradation intermediates generated from three miRNA targets and two NMD targets in WT and ago1-3. Open and solid arrowheads indicate miRNA-guided cleavage sites and EJC binding sites, respectively, in gene models and the corresponding RACE products separated by gel electrophoresis. An arrow indicates the maximum 5′ peak in the LPEAT2 gene model and in the corresponding RACE product in an electrophoretic gel. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; asterisk, PTC; half arrow, gene-specific primer. The steady-state mRNA level of Arabidopsis ACT8 was used to control the input amount of total RNA and reverse transcription among samples. An equal amount of TAP-treated total RNA from HeLa cells was spiked into each sample, and the TSS of human β-actin was used to control for differences in ligation efficiency among samples.

(B) Constructs expressing miR827 artificial targets: GUS with an intron (I-GUS827) and without an intron (GUS827). 35S, CaMV 35S promoter; box, exon; thin line, intron; half arrow, gene-specific primer used for modified RLM 5′ RACE.

(C) RNA gel blot analysis of miR827 in Arabidopsis transgenic lines harboring miR827 artificial targets. Transgenic seedlings grown under phosphate sufficient (+P) or deficient conditions (−P) were used for the assays. U6 was used as a loading control. Numbers to the right of the blot show sizes in nucleotides.

(D) Modified RLM 5′ RACE assays of RNA degradation intermediates generated from miR827 artificial targets in Arabidopsis transgenic lines. An open arrowhead indicates the expected RACE product for the 3′ cleavage remnant of the artificial targets directed by miR827. A solid arrowhead indicates the RACE product excised and cloned for Sanger sequencing. The miR159-guided 3′ cleavage remnant of MYB65 was used as a positive control for the modified RLM 5′ RACE assay.

(E) Positional distribution of the 5′P ends of cloned RACE products from I-GUS827 transgenic plants grown under −P conditions.
Overexpression of PYM Abolishes EJC Footprints in NMD and miRNA Targets.

Arabidopsis PYM was cloned and transiently overexpressed in Arabidopsis protoplasts. The steady-state mRNA levels of PYM and a housekeeping gene, ACT8, in control (−) and PYM-transfected (+) protoplasts were measured using the cDNA from the modified RLM 5′ RACE assays. The amounts of RNA degradation intermediates from three NMD targets and three miRNA targets in control and PYM-overexpressing protoplasts were compared using modified RLM 5′ RACE assays. An equal amount of TAP-treated total RNA from HeLa cells was spiked into each sample, and the TSS of human β-actin was used to infer ligation efficiency. Open and solid arrowheads indicate the miRNA-guided cleavage sites and EJC binding sites, respectively, in gene models and the corresponding RACE products separated by gel electrophoresis. An arrow indicates the maximum 5′ peak in the LPEAT2 gene model and the corresponding RACE product in an electrophoretic gel. The identity of all marked RACE products was confirmed by Sanger sequencing. Within the gene model: light grey box, UTR; dark grey box, CDS; thin line, intron; orange box, region altered by AS; asterisk, PTC; half arrow, gene-specific primer.
Figure 12. Model for EJC-bound mRNA Degradation Before and During Steady-state Translation.

(A) During splicing, EJCs are deposited on mRNAs at a position so that the distance between the 5′ edge of the EJC and the exon-exon junction is about 26-30 nt.

(B) After the pioneer round of translation, all EJCs upstream of the termination codon are removed from the mRNAs. The XRN-mediated 5′ to 3′ co-translational mRNA decay occurring during steady-state translation yields ribosome footprints but not EJC footprints in the CDS.

(C) During the pioneer round of translation of PTC-containing mRNAs, ribosomes displace the EJCs upstream of the PTC while triggering NMD when stalling at the PTC. The XRN-mediated 5′ to 3′ decay of PTC-containing mRNAs produces footprints of the EJCs positioned downstream of the PTC.

(D) Before the pioneer round of translation, endonucleolytic cleavage directed by miRNAs results in EJC-bound cleavage remnants, which are further trimmed by XRNs and become EJC footprints.
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Widespread Exon Junction Complex Footprints in the RNA Degradome Mark mRNA Degradation Before Steady-state Translation

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