Surveillance of 3′ Noncoding Transcripts Requires FIERY1 and XRN3 in Arabidopsis

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ABSTRACT Eukaryotes possess several RNA surveillance mechanisms that prevent undesirable aberrant RNAs from accumulating. Arabidopsis XRN2, XRN3, and XRN4 are three orthologs of the yeast 5′-to-3′ exoribonuclease, Rat1/Xrn2, that function in multiple RNA decay pathways. XRN activity is maintained by FIERY1 (FRY1), which converts the XRN inhibitor, adenosine 3′, 5′-bisphosphate (PAP), into 5′AMP. To identify the roles of XRNs and FRY1 in suppression of non-coding RNAs, strand-specific genome-wide tiling arrays and deep strand-specific RNA-Seq analyses were carried out in fry1 and xrn single and double mutants. In fry1-6, about 2000 new transcripts were identified that extended the 3′ end of specific mRNAs; many of these were also observed in genotypes that possess the xrn3-3 mutation, a partial loss-of-function allele. Mutations in XRN2 and XRN4 in combination with xrn3-3 revealed only a minor effect on 3′ extensions, indicating that these genes may be partially redundant with XRN3. We also observed the accumulation of 3′ remnants of many DCL1-processed microRNA (miRNA) precursors in fry1-6 and xrn3-3. These findings suggest that XRN3, in combination with FRY1, is required to prevent the accumulation of 3′ extensions that arise from thousands of mRNA and miRNA precursor transcripts.

During developmental transitions and environmental fluctuations, unnecessary RNAs are destined for degradation, and new functional RNAs are transcribed. Several RNA turnover pathways are involved in controlling gene expression profiles, which is important to maintain normal cells and tissues (Houseley and Tollervey 2009). Generally, mRNA decay starts by deadenylation of the 3′ poly(A) tail and then by decapping of the 5′ cap structure, followed by degradation in the 5′-to-3′ and/or 3′-to-5′ directions by exonucleases (Chiba and Green 2009).

In yeast, cytoplasmic Xrn1 and nuclear Rat1 are particularly prominent 5′-to-3′ exonucleases. Xrn1 catalyzes 5′-to-3′ mRNA degradation in multiple decay pathways, whereas Rat1 functions in the processing of ribosomal RNAs (rRNA) and small nucleolar RNAs (Garneau et al. 2007; Houseley and Tollervey 2009). The Arabidopsis thaliana genome contains three XRN genes named XRN2, XRN3, and XRN4, which are structurally similar to Rat1 in yeast (Kastenmayer and Green 2000). XRN2 and XRN3 are localized in the nucleus, whereas XRN4 is localized in the cytoplasm. XRN4 not only acts as an mRNA-degrading enzyme similar to the yeast Xrn1 enzyme but also acts to degrade the 3′ products that result from microRNA (miRNA)-mediated cleavage of target mRNAs (Souret et al. 2004; Gy et al. 2007; Gregory et al. 2008; Rymarquis et al. 2011). XRN4, also referred to as ETHYLENE INSENSITIVE 5 (EIN5), is required for proper ethylene signaling. It functions by directly or indirectly promoting the degradation of miRNAs of two F-box proteins that mediate protein degradation of ETHYLENE INSENSITIVE3 (EIN3), a transcription factor that elicits the ethylene response (Roman et al. 1995; Olmedo et al. 2006; Gregory et al. 2008). XRN2 is required for primary cleavage of pre-ribosomal RNAs and redundantly acts with XRN3 in pre-ribosomal RNA processing (Zakrzewska-Placzek et al. 2010). In addition to the respective functions of each family member, all XRN proteins redundantly act as endogenous RNA silencing suppressors, probably through eliminating the free 5′ ends of single-stranded RNA templates that can be recognized by RNA-dependent

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RNA polymerases (Gazzani et al. 2004; Gy et al. 2007). Although XRN3 has limited roles in cleavage of pre-ribosomal RNAs, its primary role in RNA processing has yet to be determined.

**FIERY1 (FRY1),** which is an ortholog of *HAL2* from yeast, was first identified as a negative regulator of gene expression during stress responses (Xiong et al. 2001). This gene family encodes a 3′(2′),5′-bisphosphate nucleotidase that catalyzes 3′-phosphoadenosine 5′-phosphate (PAP), a product of sulfur assimilation, into 5′AMP and Pi (Dichtl et al. 1997; Gil-Mascarell et al. 1999; Gy et al. 2007). FRY1 was identified as an endogenous RNA silencing suppressor similar to the XRN gene family, because PAP is a strong inhibitor of XRN enzymatic activity (Gy et al. 2007). Therefore, repression of FRY1 activity leads to dysfunction of all XRN proteins through PAP overaccumulation. This effect also causes accumulation of looped RNA molecules derived from miR164b and miR168a precursors in *xrn*1-6 as well as slight accumulation in *xrn*2*xrn*3 double mutants (Gy et al. 2007). Moreover, *fry*1 mutants show severe developmental defects, such as altered root architecture, reduced growth, late flowering, and an ethylene-insensitive phenotype likely due to inhibition of XR/N/EIN5 activity (Gy et al. 2007; Kim et al. 2009; Olmedo et al. 2006; Chen and Xiong 2010). *fry*1 mutants also exhibit drought resistance, which can be mimicked by the *xrn*2*xrn*3*xrn*4 triple mutant (Hirsch et al. 2011).

Several recent reports revealed that numerous long non-coding RNAs, including intergenic and antisense transcripts, are abundant in the transcriptomes of many organisms, including *Arabidopsis thaliana* (Yamada et al. 2003; Luca and Dean 2011). Some of these transcripts possess important developmental functions through gene regulation by way of chromatin modifications. For example, a non-coding RNA arises from the antisense strand of *FLOWERING LOCUS C* (FLC), a major repressor of the floral transition (Liu et al. 2010). This antisense transcript uses two proximal and distal polyadenylation sites that are controlled by two RNA binding proteins, FCA and FPA, which in turn promote polyadenylation specifically at the proximal site (Liu et al. 2007; Hornyk et al. 2010). The antisense transcript that is adénylated at the proximal site triggers histone 3 lysine 4 demethylation and transcriptional deactivation of FLC, thereby inducing the transition to floral development.

However, many non-coding RNAs can be recognized by RNA surveillance mechanisms and eliminated from cells (Chekanova et al. 2007; Kurihara et al. 2009). One such RNA surveillance mechanism is nonsense-mediated decay (NMD), which fundamentally eliminates aberrant mRNAs with premature termination codons or relatively long 3′ UTRs (Maquat 2004). *UP-FRAMESHIFT (UPF)* genes (*UPF1*, *UPF2*, and *UPF3*) are essential for normal NMD and are evolutionarily conserved in eukaryotic organisms (Behm-Ansment et al. 2007). Previous reports using genome-wide tiling arrays showed that many of the mRNA-like non-coding RNAs, including antisense transcripts, overaccumulate in *Arabidopsis upf1* and *upf3* knockdown mutants. This is likely due to the long 3′ UTRs that many of these mRNA-like non-coding RNAs possess downstream of short ORFs, which do not encode proteins and can act as a trigger for NMD (Kurihara et al. 2009). These results also reveal that NMD eliminates non-coding RNAs as well as aberrant mRNAs.

The exosome, a 3′-to-5′ exoribonuclease complex, also plays a principal role in eliminating non-coding RNAs. Previous genome-wide tiling array analysis using inducible RNAi mutants of *RRP4* and *RRP41* genes that encode for components of the exosome core, detected not only accumulation of hundreds of mRNAs, 5′ remnants of miRNA precursors, and several classes of structural RNAs (such as small nuclear RNAs, small nucleolar RNAs, and transfer RNAs) but also accumulation of large classes of uncharacterized non-coding RNAs (Chekanova et al. 2007). Many of these RNAs are transcribed from repetitive elements and siRNA-generating loci of which genomic DNA is often highly methylated, indicating a close relationship between exosome-mediated RNA decay and DNA methylation via siRNAs. The other non-coding RNAs that accumulate in *rrp4* and *rrp4*1 RNAi mutants include aberrant transcripts that originate from the 5′ ends (first exon) of protein-coding genes. Thus, the exosome acts as a quality control of several kinds of non-coding RNAs. Studies of the exosome and NMD have uncovered hidden layers of eukaryotic transcriptomes that comprise many classes of non-coding RNAs as well as canonical transcripts.

As described above, elimination of non-coding RNAs is often an essential mechanism for maintenance of gene expression. Here, we investigated the roles of the FRY1- and XRN-mediated non-coding RNA regulation using both whole-genome tiling arrays and next-generation RNA-sequencing (RNA-Seq) methods. In *fry*1, we detected the accumulation of several thousand non-coding transcripts that mapped to the 3′ ends of genes. We also identified accumulation of many of these same transcripts in *xrn*3, but we did not detect significantly more accumulation in *xrn*2*xrn*3 and *xrn*3*xrn*4 double mutants than in *xrn*3, indicating that the main activity of XRN3 is to eliminate these non-coding transcripts. Additionally, we detected accumulation of the 3′ remnants of miRNA precursors in *fry*1 and *xrn*3. Therefore, we suggest that FRY1 and XRN3 are required to prevent spurious accumulation of non-coding transcripts from the 3′ end of genes.

**MATERIALS AND METHODS**

**Plant materials**

All the wild-type (WT) and mutant *Arabidopsis* plants used in this study are from the Columbia (Col-0) background. *fry*1-6 (Salk_020882), *xrn*2-1 (Salk_041148), *xrn*3-3 (Sail_117207), *xrn*4-6 (Salk_014209), *xrn*2-1*xrn*3-3, *xrn*2-1*xrn*4-6, and *xrn*3-3*xrn*4-6 were described previously (Gy et al. 2007). *xrn*2-4 (Salk_073255) was recovered from the Salk T-DNA insertion collection [http://signal.salk.edu/cgi-bin/tdnaexpress and Alonso et al. (2003)].

**Tiling array analysis**

The GeneChip *Arabidopsis* tiling array set (1.0F Array and 1.0R Array; Affymetrix, Santa Clara, CA) was used (Zhang et al. 2006). Plant growth conditions, RNA extraction, probe synthesis, and array hybridization were carried out as described previously (Matsui et al. 2008; Kurihara et al. 2009). The ARTADE-based method (P-initial < 10−8) was used to predict novel (unannotated) transcripts from the expression data (ARTADE v1.2.1.1) (Toyoda and Shinozaki 2005). The *Arabidopsis* genome annotation used in tiling array analysis was based on the TAIR8 [ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR8_genome_release/]. The transcripts predominantly upregulated in the mutants were identified by the Mann-Whitney *U*-test (FDR *α* = 0.05).

**RNA-Seq analysis**

Wild-type, *fry*1-6, *xrn*3-3, *xrn*2-1*xrn*4-6, and *xrn*3-3*xrn*4-6 were grown in Linsmaier and Skoog (LS; Caisson Laboratories, North Logan, UT) media containing 1% sucrose and 0.85% agar at 23°C under conditions of 16 hr of light and 8 hr of darkness for 2 weeks. Wild-type (WT3w) and *xrn*2-1*xrn*3-3 were grown in soil (Metro Mix 250; Grace-Sierra, Boca Raton, FL) at 23°C under conditions of 16 hr of light and 8 hr of darkness for 3 weeks. Total RNA was extracted from the plants using Trizol reagent (Invitrogen, Carlsbad, CA). Poly(A)+ fraction was separated from 80 μg of total RNA using Oligotex mRNA Mini Kit
(Qiagen, Valencia, CA) and fragmented using Fragmentation Reagents (Applied Biosystems/Ambion, Austin, TX) at 70°C for 15 min. Fifty nanograms of the fragmented RNA was used to generate strand-specific RNA-Seq library according to the Directional mRNA-Seq Library Preparation Protocol (Illumina). RNA-Seq libraries were sequenced for 42 cycles using the Illumina Genome Analyzer Ix (WT and fyu-1-6) or for 50 cycles using the HiSeq 2000 (xrn3-3, xrn3-3-xrn4-6, 3-week-old WT and xrn2-xrn3-3) according to manufacturer’s instruction. Image analysis and base calling were performed with the standard Illumina pipeline. Read sequences were aligned with the TopHat software to the TAIR9 reference genome (Trapnell et al. 2009). Reads that aligned to multiple positions were discarded. Reads per kilobase of transcript per million (RPKM) values were calculated using the Refiner Genome module developed by Genedata Expressionist (Genedata Inc., Lexington, MA). The Arabidopsis genome annotation used was based on the TAIR9 release (ftp://ftp.arabidopsis.org/home/tair/Genes/TAIR9_genome_release/). The transcripts predominantly upregulated in the mutants were identified by using a Student t-test (P-value < 0.1).

**MethylC-Seq analysis**

Wild-type and fyu-1-6 were grown in LS (Caisson Laboratories) media containing 1% sucrose and 0.85% agar at 23°C under conditions of 16 hr of light and 8 hr of darkness for 2 weeks. One microgram of gDNA was isolated from this tissue using the Qiagen Plant DNeasy Kit (Qiagen). DNA was sheared to ~100 bp using the Covaris S2 System using the following parameters: cycle number = 6, duty cycle = 20%, intensity = 5, cycles/burst = 200, and time = 60 sec. Sonicated DNA was purified with a mini-elucon column (Qiagen). Sequencing libraries were constructed for the NEBNext DNA Sample Prep Reagent Set 1 (New England Biolabs, Ipswich, MA) following the manufacturer’s instructions, except for the following modifications. Methylated adapters were used instead of the standard genomic DNA adapters from Illumina. Ligation was purified with AMPure XP beads (Beckman, Brea, CA). A total of 450 ng of DNA was bisulfite treated using the MethylCode Kit (Invitrogen) according to the manufacturer’s instructions and then PCR amplified using Phusion Cx Turbo (Agilent, Santa Clara, CA) instead of using the Phusion Taq included in the NEBNext kit using the following PCR conditions (2 min at 95°C, 4 cycles of 15 sec at 98°C, 30 sec at 60°C, 4 min at 72°C, and 10 min at 72°C).

**Analysis of differentially methylated regions**

DMRs were identified using the methylPipe package in R (Lister et al. 2011; Pelizzola, unpublished results). Each specific methylation context (CG, CHG, and CHH) was scanned genome-wide requiring at least 10 mC differences within a 100 bp window. The methylation level of the sites within a window was then compared across all samples using a using a Kruskal-Wallis test. Next, these potential DMRs were consolidated by joining neighboring DMRs that occur in the least methylated and most methylated sample was required.

**Northern blot analysis**

Fifteen micrograms of total RNA were loaded into 1% denaturing agarose gel containing 18% formaldehyde and MOPS buffer (0.2 M MOPS, 80 mM Na-acetate, and 10 mM EDTA, pH 7.0), subjected to electrophoresis at 100 V in MOPS buffer, and transferred onto Hybond N+ membrane (GE Healthcare, Piscataway, NJ) by capillary blotting method in 20 × SSC buffer (3 M sodium chloride, 300 mM trisodium citrate, pH 7.0), followed by UV-crosslinking with 120,000 μJ/cm². Prehybridization and hybridization with each specific probe were performed with the PerfectHyb hybridization buffer (Sigma-Aldrich, St. Louis, MO) at 65°C for 1 hr and overnight, respectively. The membrane was washed in the first wash buffer (2 × SSC, 0.1% SDS) for 10 min twice and then in the second wash buffer (0.5 × SSC, 0.1% SDS) for 20 min three times. The membrane was exposed to Imaging Plate BAS-MX2040 for 2 hr and the signals were detected using Bio-Image analyzer BAS-2500 (Fujifilm, Tokyo, Japan).

DNA probes were constructed by random priming reactions with a 32P-dCTP using the Megaprime DNA-labeling system (GE Healthcare) according to manufacturer’s instructions. DNA fragments used for these reactions were PCR amplified from genomic DNA using the following primer sets: mRNA_F and mRNA_R for mRNA detection, 3’ext_F and 3’ext_R for 3’ extension detection, and TUB8_F and TUB8_R for TUB8 mRNA detection (supporting information, Table S1).

**Self-igation-mediated RT-PCR**

Total RNA was extracted using Trizol reagent (Invitrogen) from 2-week-old seedlings grown in LS media as described above. Poly(A)+ RNA was separated from total RNA using Poly(A) Purist kit (Applied Biosystems/Ambion) according to the manufacturer’s instructions. The RNA (500 ng) was treated with or without calf intestinal phosphatase (CIP, 10 U; New England Biolabs, Cambridge, MA) in a 20 μL volume and then tobacco acid pyrophosphatase (TAP, 2 U; Epicentre Biotechnologies, Madison, WI) in a 30 μL volume. The modified or unmodified RNA molecules were self-ligated using T4 RNA ligase (20 U; Promega) in a 50 μL volume using Phire Hot Start DNA polymerase (New England Biolabs) and the following primer sets: 3’ext_F and 3’ext_R for 3’ extension detection, and TUB8_F and TUB8_R for TUB8 mRNA detection (supporting information, Table S1).

**Quantitative RT-PCR analysis**

Total RNA was extracted from 20-day-old seedlings grown in LS media containing 1% sucrose and 0.85% agar at 23°C under conditions of 16 hr of light and 8 hr of darkness. Ten micrograms of total RNA were digested in 50 μL of TURBO DNase (Applied Biosystems/Ambion) for 30 min and recovered by acid phenol:chloroform extractions and ethanol precipitation. Two micrograms of the DNase-treated RNA was used for the reverse-transcription reaction in 20 μL using the SuperScript II reverse transcriptase (Invitrogen) and Oligo dT primer in a 20 μL volume. As a PCR template, 0.25 μL of RT product was used. The PCR was carried out in a 20 μL volume using Phire Hot Start DNA polymerase (New England Biolabs) and the following primer set (circle_F and circle_R; Table S1). The amplification program was 35 cycles of 5 sec at 95°C, 15 sec at 55°C, and 20 sec at 72°C. The resulting PCR product was loaded into a 1.5% agarose gel, subjected to electrophoresis in TBE (50 mM Tris-HCl, 48.5 mM boric acid, 2 mM EDTA, pH 8.0) buffer at 100 V and visualized under UV light.
Accession numbers
Raw data from tiling arrays and RNA-Seq were deposited in Gene Expression Omnibus under the accession numbers GSE32977 and GSE34654, respectively. Raw data from MethylC-Seq were deposited in the Sequence Read Archive (SRA) of NCBI under accession number SRA049100.

RESULTS
Identification of novel transcripts extending from the 3′ ends of mRNAs
Strand-specific whole-genome tiling array analysis of fry1-6 and xrn single mutants (xrn2-4, xrn3-3, xrn4-6) was performed to identify the roles of XRNs and FRY1 in transcript accumulation. The xrn2-4 allele (Salk_073255) has a T-DNA insertion in the 12th intron, resulting in extremely reduced mRNA expression (see Figure S1A). These data were normalized together with previous tiling array data generated for upf mutants (Kurihara et al. 2009) to reduce misidentification of unannotated transcripts, such as non-coding RNAs. The ARTADE program, which predicts novel transcripts de novo (Toyoda and Shinozaki 2005) uncovered 171 novel transcripts located less than 300 nucleotides (nt) downstream of the 3′ ends of upstream mRNAs in fry1-6 [Fold (fry1-6/WT) > 2, P-initial < 10^{-8}, FDR α = 0.05] (Figure 1A and Table S2). Henceforth, we will refer to these novel transcripts as 3′ extensions. Interestingly, 61% (104) of the estimated lengths of the 3′ extensions were less than 800 nt (Figure 1B). The results from the tiling array analyses can be observed on OmicBrowse at http://omicspace.riken.jp/gps/group/psca7.

We suspected that many low abundance 3′ transcript extensions may not be detected using tiling array analysis. To overcome this limitation, strand-specific RNA-Seq was carried out using an Illumina Genome Analyzer IIx to investigate these RNA populations at single base resolution. Unlike the tiling array technique, the RNA-Seq method does not suffer from background noise (cross-hybridization of RNAs with multiple probes) because the vast majority of reads can be uniquely mapped in the Arabidopsis reference genome (Lister et al. 2008). First, three biological replicates of RNA-Seq were carried out using mRNA prepared using wild-type and fry1-6 seedlings. We identified 33.2 and 35.0 million sequenced reads that uniquely aligned to the TAIR9 reference genome sequence in wild-type and fry1-6, respectively. For quantification of transcript levels, RPKM values were calculated for the regions extending 500 nt downstream from the 3′ ends of all annotated genes. (Figure 1C). This range was selected because the lengths of most of 3′ extensions observed from previous tiling array analysis were less than 800 nt in length (Figure 1B). RPKM values of these 3′ regions tended to be higher in fry1-6 than in wild-
mRNAs (Kim et al. 2004; West et al. 2008). To examine whether the 5' extensions were detected at the 3' ends of the RNA. Only capped RNA species can be amplified without CIP and TAP treatment. Specific amplification products from 3' extensions were detected only in fry1-6, and not in wild-type, when RNAs were not pretreated with these enzymes (Figure 2B). The smeary bands observed in lane 4 most likely indicate that the position of 5' and/or 3' ends of 3' extensions is not uniform, which is probably due to degradation and/or variable poly(A) tail and nonstop PCR amplification. On the other hand, when RNAs were pretreated with TAP and CIP, specific amplification products from ACT2 mRNA were detected in both WT and fry1-6, demonstrating that the enzyme treatments were effective. These results indicate that the 3' extensions detected in fry1-6 mutants are very likely equivalent to Pol II 3' read-through products. **mRNA 3' extensions often occur in abundantly expressed transcripts** When the set of 2230 mRNAs with 3' extensions were analyzed by gene ontology (GO) annotations (categories cellular component, molecular component, and biological component), there were few significant differences in represented GO terms between all Arabidopsis genes expressed in fry1-6 and the 2230 mRNAs. The only exception was that in all three categories, the percentages of genes of unknown function were significantly lower for the 2230 mRNAs with 3' extensions than for all genes expressed in fry1-6 (Figure S2A). Therefore, these data suggest that FRY1 does not target specific classes of genes but, instead, is required for general surveillance that prevents these 3' extensions.

Next, we compared the accumulation of these 2230 mRNAs and their 3' extensions between wild-type and fry1-6. Although accumulation of 3' extensions was apparently higher in fry1-6 than in wild-type, accumulation of the 5' mRNAs was comparable between both genotypes (Figure 3A). However, the majority of the mRNAs with 3' extensions have relatively higher RPKM values compared with all expressed genes (Figure 3B and Figure S2B), indicating that actively transcribed genes tend to possess 3' extensions.

**XRN3 represses 3' extension transcripts** FRY1 is required for catalyzing conversion of the XRN inhibitor PAP into 5' AMP, thereby promoting XRN activities (Gy et al. 2007; Kim et al. 2009). In addition to the fry1-6 mutant, slightly increased accumulation of some 3' extensions in xrn3-3 were observed from the tiling array analysis (Figure S3A). However, it was difficult to detect 3' extensions in xrn3-3, possibly because xrn3-3 is a knockdown allele with a T-DNA insertion in the promoter region that results in reduced expression of XRN3 mRNA rather than in a complete knockout (Figure S1A; Gy et al. 2007). Therefore, the effect on accumulation of 3' extensions might be limited due to the allele used and low detection sensitivity of the tiling array in this experiment. As knockout mutant of XRN3 gene is lethal, we could not use it here (Gy et al. 2007).

To increase detection sensitivity, two biological replicates of directional RNA-Seq (on the Illumina HiSeq 2000 platform) were performed using xrn3-3 to examine whether XRN3 activity degrades the 3' read-through products described above. Additionally, two biological replicates of RNA-Seq using xrn2-1xrn3-3, xrn2-1xrn4-6, and xrn3-3xrn4-6 double mutants were carried out to investigate possible redundancies among the three XRN enzymes. The total numbers of sequenced reads mapped to the TAIR9 reference genome sequence for each genotype were 65.7, 58.7, 60.8, 35.7, and 58.5 million in xrn3-3, xrn3xrn4, xrn2xrn4, 3-week-old wild-type, and xrn2xrn3, respectively. Analyses of the results revealed that RPKM values of the 3' regions composed of the 500 nt regions downstream of genes were typically greater in xrn3-3, xrn2xrn3, and xrn3xrn4 (but not xrn2xrn4) when compared with wild-type (Figure 4A). When compared with fry1-6 (Figure 1D), RPKM values were lower, which is consistent with a

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**Table 1 Summary of 3' extensions in RNA-Seq**

| Genotype   | 3' Extension | Raw | Platform | Replicate | Total Number of Reads (in millions) |
|------------|--------------|-----|----------|-----------|------------------------------------|
| fry1-6     | 2,230        | 6,901 | GALI     | 3         | 35.0                               |
| xrn3-3     | 528          | 16,570 | HiSeq    | 2         | 65.7                               |
| xrn2xrn3   | 473          | 16,471 | HiSeq    | 2         | 58.5                               |
| xrn2xrn4   | 64           | 16,834 | HiSeq    | 2         | 60.0                               |
| xrn3xrn4   | 392          | 14,028 | HiSeq    | 2         | 58.7                               |

a The numbers of 3' extension candidates were estimated by filtering RNA-Seq data by four parameters (Fold > 5, RPKM > 1, P-value < 0.1, non-pri-miRNA).

b This category indicates the numbers of new transcripts that have RPKM values on 500 nt downstream from the 3' ends of genes.
previous report that xrn3-3 is a weak allele that results in fewer morphological defects than fry1-6 (Gy et al. 2007).

When these RNA-Seq data were stringently filtered using four parameters (Fold change > 5, RPKM average > 1, P-value < 0.1, non-pri-miRNA), we identified 528, 473, and 392 3' extension candidates in xrn3, xrn2xrn3, and xrn3xrn4, respectively (Table 1 and Table S3). These candidates significantly overlapped with the 2230 3' extensions observed in fry1-6 (Figure 4B). The abundance of the 2230 mRNAs with 3' extensions identified in fry1-6 showed little change in xrn3, xrn2xrn3, and xrn3xrn4 compared with wild-type. However, the abundance of the concomitant 2230 3' extensions was notably increased in xrn3, xrn2xrn3, and xrn3xrn4, but not in xrn2xrn4 (Figure S3B). The candidates in xrn3-3 do not seem to widely overlap with those in xrn3 double mutants (Figure 4B). Of the 528 candidates in xrn3-3, 20.3% and 19.1% overlap with those in xrn2xrn3 and xrn3xrn4, respectively. However, this result is due, in part, to the limitation of threshold values used for candidate identification. For example, when alternative (less stringent) threshold values are used to identify 3' extension candidates (Fold change > 2, RPKM average > 1, non-pri-miRNA), 44.8% and 51.5% of newly calculated candidates in xrn3-3 overlap with those in xrn2xrn3 and xrn3xrn4, respectively. These results reveal that significant redundancy in suppressing 3' extensions was not detected between XRN3 and XRN2/XRN4, indicating the XRN3 enzyme possesses unique specificity in 3' end processing/turnover.

Quantitative RT-PCR (qRT-PCR) analysis was used to examine the abundance of selected 5' mRNAs and 3' extensions in all genotypes. Two primer sets were used to amplify the 5' mRNAs (mRNA_F and mRNA_R) and 3' extensions (3'ext_F and 3'ext_R), respectively (Figure 4C and Table S3). These experiments revealed the accumulation of 3' extensions was not correlated with accumulation of the 5' mRNAs, consistent with the results of the RNA-Seq analyses (Figure 4B and Figure S3C). In this assay, we observed either compensation for reduced XRN3 activity in xrn3-3 by XRN2/XRN4 or a dependency on the combination of mutations in the double mutants. For example, in the case of At3g45160, accumulation of the 3' extension in xrn3xrn4 was higher than in xrn3-3. By contrast, in the case of At1g19670, accumulation of the 3' extension observed in xrn3xrn4...
was lower than in xrn3-3. These results suggest that XRN3 is the main component eliminating 3' extensions and that other XRNs redundantly act to compensate, in some cases, for reduced XRN3 activity in the xrn3-3 mutant. In this context, it is apparent that XRN3 possesses at least one of the features that define yeast Rat1-type enzymes: involvement in termination of transcription. However, the other Arabidopsis Rat1 orthologs, XRN2 and XRN4, exhibit little or none of this function. Surprisingly, XRN2 does not seem to act redundantly with XRN3 in targeting of 3' extensions for degradation, even though both XRN2 and XRN3 localize to the nucleus (Kastenmayer and Green 2000).

Transcript 3' extensions do not lead to DNA methylation of the genomic region of origin

A total of 953 3' extension candidates potentially overlapped with flanking opposite-strand transcripts from neighboring genes while a total of 24 pairs of 3' extension candidates were confirmed to overlap with each other (Figure 5A). We defined these 3' extensions by postulating that their lengths are ~500 nt. This range was used because the predicted lengths of most of 3' extensions observed in tiling array analysis were less than 800 nt (Figure 1B). It is hypothesized that overlap between neighboring Xrn-sensitive antisense transcripts provides a source of double-stranded RNAs which may lead to pools of siRNA species that in turn could mediate de novo DNA methylation of these 3' extension-generating loci (Zhang and Zhu 2011). To investigate whether these 3' extensions correlated with de novo DNA methylation, we performed genome-wide MethylC-Seq analysis in fry1-6 and wild-type (Lister et al. 2008; Schmitz and Zhang 2011).

A total of 52.8 and 53.0 million reads were aligned to the reference genome, resulting in ~42x coverage (~21x per strand coverage) of both wild-type and fry1-6, respectively. In total, we identified 35 differentially methylated regions (DMR) in fry1-6 compared with wild-type (P-value > 0.01, 8-fold difference in methylation levels as determined by combining levels from all three methylation contexts, CG, CHG, and CHH). However, none of the DMRs correlated with the identified 3' extensions from our previous analysis (Figure 5A and Figure 4).
Table S4), indicating that 3' extensions do not lead to de novo DNA methylation of the 3' extension originating loci.

A recent report using transcriptome tiling array analysis (Sonmez et al. 2011) revealed that expression of some 3' downstream regions were increased in an fcafpa double mutant, resulting in 3' transcript extensions similar to those reported here. Moreover, the expression of one transposon (At1TE93275) was reported to be increased, and DNA methylation at this locus was reduced in fcafpa. Similarly, we found that expression of antisense transcripts originating from this transposon was increased, and DNA methylation was drastically reduced in fry1-6 (Figure 5B). However, xrn2 and xrn4 mutations more strongly affected the expression of antisense transcripts originating from this locus compared with xrn3 mutations, and the combination of the xrn3 mutation with xrn2 or xrn4 mutations suppressed the expression of this transcript by way of an unknown mechanism (Figure 5C and Figure S5). Therefore, the mechanism regulating expression of this transposon seems to be independent of the XRN3-controlled 3' extension.

**FRY1 and XRN3 repress 3' remnants of miRNA precursors**

MicroRNAs (miRNAs) are regulatory small RNAs produced through DICER-LIKE1 (DCL1)-mediated cleavage of the stem-loop structure of primary miRNA precursors (pri-miRNA) (Kurihara and Watanabe 2004; Song et al. 2010). They execute endonucleolytic cleavage of target mRNAs with target sites complementary to the miRNAs (Chapman and Carrington 2007; Voinnet 2009). Previous reports have demonstrated that XRN4 and FRY1 cooperatively repress the accumulation of 3' extensions similar to those reported here. Moreover, the expression of one transposon (At1TE93275) was reported to be increased, and DNA methylation at this locus was reduced in fcafpa. Similarly, we found that expression of antisense transcripts originating from this transposon was increased, and DNA methylation was drastically reduced in fry1-6 (Figure 5B). However, xrn2 and xrn4 mutations more strongly affected the expression of antisense transcripts originating from this locus compared with xrn3 mutations, and the combination of the xrn3 mutation with xrn2 or xrn4 mutations suppressed the expression of this transcript by way of an unknown mechanism (Figure 5C and Figure S5). Therefore, the mechanism regulating expression of this transposon seems to be independent of the XRN3-controlled 3' extension.

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to repress the accumulation of the 3’ remnants of pri-miRNAs, preventing examination of whether other exoribonucleases act redundantly to repress the 3’ remnants. Taken together, the results indicate that XRN3 degrades unnecessary 3’ remnants of DCL1-mediated cleavage of pri-miRNAs.

Lithium induces accumulation of 3’ extension transcripts

A previous report showed that lithium ion could inhibit activity of HAL2, a yeast FRY1 ortholog, and then deplete activity of exoribonucleases, such as Rat1 and Xrn1, which induced accumulation of many of non-coding RNAs in yeast (Dichtl et al. 1997; Van Dijk et al. 2011). It was also reported that lithium and sodium ions could inhibit the recombinant FRY1 protein from catalyzing PAP, although the effect of sodium was weaker than that of lithium (Xiong et al. 2004). The depletion of FRY1 activity by lithium in wild-type plants may induce accumulation of 3’ extension transcripts through suppression of XRN exoribonuclease activities including XRN3 (Figure 7A). To test this hypothesis, accumulation of some 3’ extensions and pri-miRNA_3’ under some abiotic stresses in wild-type seedlings was examined using quantitative RT-PCR. Accumulation of 3’ transcripts was strongly induced by LiCl stress in wild-type (Figure S7). This result supports the idea that depletion of FRY1 activity leads to accumulation of the 3’ transcripts by following suppression of XRN activities.

DISCUSSION

Using two genome-wide technologies, tiling arrays and RNA-Seq, we report the identification of transcript extensions from the 3’ ends of thousands of genes as well as 3’ remnants of pri-miRNAs that accumulate in fry1-6 mutants. We also observed accumulation of these non-coding RNAs in xrn3-3 mutants. These studies may suggest that
XRN3 and FRY1 act cooperatively to eliminate two kinds of non-coding RNAs in plants: 3' extensions from transcribed mRNAs and 3' remnants of pri-miRNAs (Figure S8). This finding is supported by the fact that accumulation of the 3' transcripts was induced in wild-type plant under high-salinity stresses that are inhibitors of FRY1 activity (Figure 7). Most notably, this is the first report of endogenous XRN3-specific activity in plants and genome-wide base resolution identification of thousands of 3' gene transcript extensions.

**Gene transcript extensions are equivalent to 3' read-through products**

In yeast, the exoribonuclease Rat1 degrades 3' read-through products that are transcribed by RNA polymerase II after endonucleolytic cleavage of the mRNA at the poly(A) site, and transcription terminates upon complete degradation by Rat1 (Kim et al. 2004; West et al. 2004). This Rat1-mediated reaction is closely coupled with transcription. It is very likely that the 3' extensions detected in fry1-6 mutants are equivalent to such 3' read-through products and that only XRN3 (not XRN2 or XRN4) exclusively possesses this Rat1 function which can terminate the transcription reaction in *Arabidopsis*, regardless of the fact that XRN2 and XRN4 are also Rat1 orthologs (Kastenmayer and Green 2000). This indicates that some yeast Rat1 functions are eliminated of pri-miRNAs, because we could not detect significant accumulation of pri-miR156c_3' expression in wild-type and fry1 mutants. When we examined whether mRNAs tailed with 3' extensions were transcribed could partially escape from degradation.

**Characteristics of the mRNAs with 3' extensions**

Many, but not all, mRNAs showed 3' extensions in the *xrn* or *fry1* mutants. When we examined whether mRNAs tagged with 3' extensions possess specific characteristics, we noticed a significant number of the mRNAs with 3' extensions had relatively higher RPKM values compared with all expressed genes. These data revealed that actively transcribed genes tend to possess 3' extensions.

Importantly, mRNAs with 3' extensions showed comparable expression in wild-type and fry1-6, regardless of increased accumulation of 3' extensions. This result indicates that 3' extensions themselves do not affect the expression levels of the 5' mRNAs.

It has been proposed that elevated accumulation of PAP inhibits XRN3 activity, as well as XRN2 and XRN4 activities, in *fry1* and that 3' extensions being transcribed could partially escape from degradation.
by reduced XRN3 activity in both fry1 and xrn3. However, transcription might terminate at unknown downstream positions, such as poly(A) addition sites. *Arabidopsis* uses not only the AAUAAA consensus sequence conserved in all eukaryotes but also several other AU-rich consensus sequences as poly(A) signals that often appear in intergenic regions (Loke et al. 2005). This mechanism of alternative transcription termination could define the length of 3′ extensions in *fry1* and *xrn3* mutant backgrounds. Further studies are needed to identify why some mRNAs may possess 3′ extensions whereas others do not in these mutants.

The role of XRN3- and FRY1-mediated non-coding RNA suppression

The RNA-binding proteins FCA and FPA are known as positive regulators of the floral transition by downregulating expression of the MADS box floral repressor FLC (Koornneef et al. 1991). A recent study using tiling analysis detected 3′ read-through signals at the 3′ ends of several genes in an *fcafpa* double mutant (Sonmez et al. 2011). However, these findings differ from our study in that the 3′ extensions we identified are generally independent molecules from 5′ mRNAs, whereas 3′ read-through products in *fcafpa* mutants are likely connected directly with mRNAs.

Sonmez et al. (2011) also found accumulation of transcripts of one transposon (At1TE93275) that was associated with reduced DNA methylation in *fcafpa*. Our study also revealed much greater accumulation of this antisense transcript originating from this transposon in *fry1-6*, *xrn2-4*, and *xrn4-6* compared with those in other backgrounds, as well as strongly reduced DNA methylation in *fry1-6*, indicating that regulation of this transposon is quite different from that of 3′ extensions, which is primarily mediated by XRN3 activity. In future studies, it will be interesting to reveal the relationship between FRY1- and XRN3-mediated transcription termination and the RNA-binding proteins FCA and FPA.

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