Evidence that XRN4, an *Arabidopsis* homolog of exoribonuclease XRN1, preferentially impacts transcripts with certain sequences or in particular functional categories

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ABSTRACT

One of the major players controlling RNA decay is the cytoplasmic 5'-to-3' exoribonuclease, which is conserved among eukaryotic organisms. In *Arabidopsis*, the 5'-to-3' exoribonuclease XRN4 is involved in disease resistance, the response to ethylene, RNAi, and miRNA-mediated RNA decay. Curiously, XRN4 appears to display selectivity among its substrates because certain 3' cleavage products formed by miRNA-mediated decay, such as from *ARF10* mRNA, accumulate in the *xrn4* mutant, whereas others, such as from *AGO1*, do not. To examine the nature of this selectivity, transcripts that differentially accumulate in *xrn4* were identified by combining PARE and Affymetrix arrays. Certain functional categories, such as stamen-associated proteins and hydrolases, were over-represented among transcripts decreased in *xrn4*, whereas transcripts encoding nuclear-encoded chloroplast-targeted proteins and nucleic acid-binding proteins were over-represented in transcripts increased in *xrn4*. To ascertain if RNA sequence influences the apparent XRN4 selectivity, a series of chimeric constructs was generated in which the miRNA-complementary sites and different portions of the surrounding sequences from *AGO1* and *ARF10* were interchanged. Analysis of the resulting transgenic plants revealed that the presence of a 150 nucleotide sequence downstream from the *ARF10* miRNA-complementary site conferred strong accumulation of the 3' cleavage products in *xrn4*. In addition, sequence analysis of differentially accumulating transcripts led to the identification of 27 hexamer motifs that were over-represented in transcripts or miRNA-cleavage products accumulating in *xrn4*. Taken together, the data indicate that specific mRNA sequences, like those in *ARF10*, and mRNAs from select functional categories are attractive targets for XRN4-mediated decay.

Keywords: ribonuclease; RNase; XRN1; XRN4; RNA decay; RNA degradation

INTRODUCTION

Survival of all living organisms is dependent on the ability to alter gene expression in response to both environmental and developmental stimuli. This exquisite control of gene expression occurs at many levels, including mRNA decay. The majority of RNA is degraded through three major pathways involving the exosome, decapping followed by 5'-to-3' exoribonuclease decay or endonucleolytic cleavage (Chiba and

Green 2009). The exosome is a multiprotein complex that degrades RNA in the 3'-to-5' direction. Recently, it was shown that the exosome along with deadenylases and RNA binding proteins co-localize to cytoplasmic granules called cytoplasmic exosome granules (Graham et al. 2006; Lin et al. 2007). The decapping/5'-to-3' pathway is also associated with cytoplasmic granules, called processing bodies (P-bodies reviewed in Balagopal and Parker 2009), which are distinct from the exosome granules. In the decapping/5'-to-3' pathway, deadenylation or uridylation often precedes decapping by DCP1/DCP2 (Badis et al. 2004; Muhlrud and Parker 2005; Rissland and Norbury 2009). Removal of the cap structure produces RNAs with 5' monophosphates, which are the preferred substrates of the 5'-to-3' exoribonucleases such as XRN1 in yeast or its functional homolog XRN4 in *Arabidopsis* (Stevens 1979; Muhlrud et al. 1994; Kastenmayer and Green

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2000). The endonucleolytic pathway involves initiation of decay by endonucleolytic cleavage followed by 3'-to-5' and 5'-to-3' exonucleolytic decay of the 5' and 3' cleavage products, respectively (Chiba and Green 2009).

Most other decay pathways funnel transcripts into the three major pathways. For example, ARE-mediated decay uses both the decapping/5'-to-3' and the exosome pathways, whereas nonsense-mediated decay (NMD), whose components can target transcripts to P-bodies, uses the decapping/5'-to-3' decay route (He and Jacobson 2001; Lejeune et al. 2003; Sheth and Parker 2006). In addition, it was recently found that NMD can also act through endonucleolytic cleavage (Gatfield and Izaurralde 2004; Huntzinger et al. 2008; Eberle et al. 2009). Small RNA-mediated decay by small interfering RNAs (siRNAs) and microRNAs (miRNAs) also can work through endonucleolytic cleavage in plants (Vaucheret 2006). siRNAs are produced from double-stranded RNA precursors, whereas miRNAs are generated from RNA hairpins. However, both siRNAs and miRNAs associate with protein complexes containing Argonaute proteins, which cleave target mRNAs sharing complementarity to the small RNAs (Meister et al. 2004; Vaucheret 2006).

Loss of XRN4 in *Arabidopsis* affects both siRNA and miRNA-mediated decay. In the absence of XRN4, increased gene silencing has been observed (Gazzani et al. 2004). This can be attributed to the accumulation of uncapped RNAs in *xrn4*. These can be substrates of the RNA-dependent RNA polymerase RDR6, leading to the formation of double-stranded RNA precursors used for siRNA biogenesis (Gazzani et al. 2004). For miRNA-mediated decay, XRN4 contributes to the decay of a subset of 3' cleavage products generated after miRNA-mediated cleavage (Souret et al. 2004). Indeed, only select 3' cleavage products accumulate in *xrn4* (Souret et al. 2004), even though in principle all should be functionally equivalent substrates for XRN4.

Due to its involvement in a wide range of mRNA decay pathways, the selective XRN4-mediated decay observed for miRNA-mediated cleavage products may extend to other RNAs as well. In this study, we examine the extent and potential causes of this selectivity by combining Parallel Analysis of RNA Ends (PARE) with microarray analysis to profile differentially expressed transcripts in *xrn4*. Detailed analysis of these transcripts revealed that the selectivity extends beyond miRNA targets, because transcripts increasing in *xrn4* are over-represented in select functional groups. Also, the expression of chimeric genes in transgenic plants demonstrated that a specific region of the *ARF10* RNA has a strong impact on accumulation in *xrn4*. Correspondingly, a number of hexamer motifs were found to be over-represented in transcripts that increase in *xrn4*, including the aforementioned region of *ARF10*. These findings indicate that XRN4 selectively impacts RNAs in certain pathways or that contain particular motif sequences.

RESULTS

Identification of XRN4 regulated transcripts using microarrays and PARE

As previously shown (Souret et al. 2004; Gregory et al. 2008), XRN4 is responsible for degrading and/or causing differential accumulation of a number of transcripts, including select 3' cleavage products of miRNA-mediated cleavage and uncapped endogenous transcripts. To get a more comprehensive view of XRN4 function, we compared the transcript levels in wild-type (WT, Col-0) and *xrn4* inflorescence using complementary technologies: two types of Affymetrix GeneChip arrays and PARE (German et al. 2008). The first type of array was the ATH1 array, which has probes representing approximately 24,000 *Arabidopsis* genes. Total RNA from WT and *xrn4* mixed-stage inflorescences was labeled and hybridized to ATH1 arrays. Two biological replicates were completed for each sample for a total of four chips. When a twofold mean difference was the cutoff for significance with a minimum difference of 1.5-fold in both replicates, 34 transcripts decreased in *xrn4*, whereas 33 increased (Tables 1, 2).

On ATH1 arrays, each gene is represented by 11 probes, predominantly at the 3' end. Accordingly, it detects a mixture of full-length capped transcripts and poly(A)-containing decay products from 5'-to-3' exonucleolytic decay. Due to this, the accumulation of decay products in *xrn4* may be overshadowed by non-accumulating full-length transcripts. To get a better picture of the decay products, data from WT and *xrn4* PARE libraries from German et al. (2008) were examined. The PARE method is a deep-sequencing approach that captures the first 20 nt from 5' monophosphorylated and polyadenylated transcripts (German et al. 2009). Such RNAs include intermediates of RNA decay, for example, decapped RNAs and 3' cleavage products of endonucleolytic decay. Aligning the resulting sequences to the annotated cDNAs presents a picture of the intermediates of RNA decay. The raw data were normalized to transcripts per 10 million (TP10M), and the abundances of the sequences found in both libraries

TABLE 1. mRNA transcripts with decreased accumulation in the *xrn4* mutant as defined by ATH1 Affymetrix arrays, the two PARE analyses, and Affymetrix tiling arrays

	ATH1	5PARE	APARE	Tiling
ATH1	29 ^a			
5PARE	2	147 ^a		
APARE	4	16	545 ^a	
Tiling	1	2	3	25 ^a
Total ^b	34	164	565	30

A twofold cutoff was used for the ATH1 arrays, whereas a fivefold cutoff was used for analysis of the PARE libraries. Dark gray cells represent the overlap between two analyses.

^aNumber of transcripts decreased in only one analysis.

^bNumber of nonredundant transcripts decreased in each analysis.

TABLE 2. mRNA transcripts with increased accumulation in the *xrn4* mutant

	ATH1	5PARE	APARE	Tiling
ATH1	22 ^a			
5PARE	11	2,863 ^a		
APARE	0	91	366 ^a	
Tiling	2	12	0	40 ^a
Total ^b	33	2,975	457	52

The same cutoffs were used as for Table 1. Dark gray cells represent the overlap between two analyses.

^aNumber of transcripts uniquely increased in each analysis.

^bNumber of nonredundant transcripts increased in each analysis.

were compared. On average, the distinct cDNA-matched PARE sequences identified in both WT and *xrn4* libraries were 1.43 times more abundant in the *xrn4* library. Although the increase in *xrn4* could be a global increase in decay products due to loss of XRN4 function, it could also be an artifact of normalization; thus, to be conservative, all WT abundances were multiplied by 1.43 to remove any potential artifacts before comparing WT and *xrn4* PARE data.

The PARE data were analyzed in two ways to maximize detection of differential accumulation in *xrn4*. These analyses, referred to as APARE and 5PARE, were designed to detect altered overall decay of the transcripts and accumulation of decapped transcripts, respectively. For the APARE analysis, the abundance of all the PARE sequences matching each annotated cDNA was summed. cDNAs from the chloroplast and mitochondrial genomes were removed to focus on nuclear-encoded transcripts in this and all subsequent analyses. After filtering out transcripts expressed at very low levels (<10 TP10M), 565 transcripts decreased and 457 increased in *xrn4* at least fivefold in the APARE analysis. When developing the 5PARE analysis, one consideration taken into account was that ~30% of the protein-coding annotated cDNAs lack reported 5' UTR sequences in The *Arabidopsis* Information Resource (TAIR) database. To potentially increase detection of full-length decapped transcripts, the abundances from PARE sequences matching the region from -50 to +50 relative to the start of the cDNA were summed to create the 5PARE data. Using the same filtering and fivefold cutoff as for the APARE analysis, 164 transcripts decreased and 2975 transcripts increased in *xrn4* compared to WT in the 5PARE comparison.

The overlap between the two PARE analyses and the ATH1 array is shown in Tables 1 and 2. Approximately 18% of the transcripts decreased in *xrn4* on the ATH1 array were also down in APARE or 5PARE, whereas 33% of those increased in *xrn4* on the ATH1 array were increased in APARE or 5PARE. A second set of Affymetrix data from tiling arrays of the *xrn4* mutant *ein5* (Gregory et al. 2008) and WT also was compared to the PARE data. The tiling arrays consist of 25-mer probes with 10 nt between probes for the non-repetitive portions of the *Arabidopsis* genome. The overlap

between the tiling array and the APARE and 5PARE was comparable to that of the ATH1 array, with 17% and 21% of transcripts decreasing or increasing in *xrn4*, respectively, overlapping. Those transcripts decreased or increased in *xrn4* in two or more of the four analyses will hereafter be called the down or up sets, and represent 22 and 112 genes, respectively.

To confirm the validity of the up set and down set, candidates were selected from the down and up sets, which cover a wide range of fold differences, for validation by Northern blot (Fig. 1; Supplemental Table 1). Five of the six candidates from the up set and all four of the candidates from the down set differentially accumulated in two or more biological replicates. In addition, transcripts that were differentially regulated in one analysis were also surveyed (Fig. 1; Supplemental Table 1). Two of the five candidates that were increased in *xrn4* and three of the eight that were decreased in *xrn4* in one library validated in three or more biological replicates. Although some transcripts that differentially accumulated in one analysis also validate, the lower validation rates suggest that too much noise would be introduced if we had reduced the stringency of our analysis. Such high stringency misses cleaved miRNA targets that are up in Northern blots as well (e.g., ARF10 and the two targets shown in Fig. 1B, AT1G30210 and AT1G10120). Most importantly though, the up and down sets have a low false-positive rate and therefore represent robust sets of transcripts for further study.

Transcripts with differential accumulation in *xrn4* cluster into several functional categories

The gene ontology (GO) annotations for the annotated cDNAs were obtained from TAIR. The number of genes with each

FIGURE 1. Transcripts decreased (A) or increased (B) in *xrn4*. Transcripts were quantified in three to five biological replicates by Northern blot. Only transcripts for which all replicates decreased or increased by 10% or more are shown. Values for individual replicates can be found in Supplemental Table S3. Fold represents the abundance relative to WT.

annotation was compared using Z-tests between the down or up sets and all transcripts to determine if XRN4 showed a preference for any category of transcript. Keyword categories that were over-represented in the down and up sets with a Z-score >3.09 (P -value <0.001) are found in Tables 3 and 4, respectively. The down set was enriched in transcripts encoding GO categories for hydrolases, other molecular functions, response to abiotic or biotic stimulus, and other binding (Table 3). Transcripts producing nuclear-encoded chloroplast-targeted (n-chlor) proteins were greatly enriched in the up set (Table 4). More than 33% of the up set genes for which there were GO annotations are predicted to fall into this category (Supplemental Fig. S1). Many other GO categories showed a high degree of overlap with the chloroplast-targeted set (Supplemental Table S2, gray entries). To rule out that these other categories were over-represented solely due to the elevation of n-chlor proteins, the Z-score after their removal was also calculated, and transcripts encoding proteins containing nucleic acid, DNA-, and RNA-binding activities still were enriched in the up set (Table 4).

Although previous studies have surveyed the accumulation of RNA in *xrn4* mutants (Souret et al. 2004; Gregory et al. 2008), none have validated transcripts decreasing in *xrn4*. Although it is likely that these transcripts differentially accumulate due to a secondary effect of XRN4 mutation, they provide insight into the pathways influenced by XRN4-mediated decay. Figure 1A shows the average fold differences for six genes whose transcripts decreased in *xrn4* in four biological replicates that were assessed by Northern blot analysis (Supplemental Table S3 contains data from individual replicates). Interestingly, all were associated with stamens (Honys and Twell 2004; Wellmer et al. 2004). In fact, $>45\%$ of the transcripts with decreased accumulation in *xrn4* (10 out of the 22) have been shown to be predominately expressed in stamens (Honys and Twell 2004; Wellmer et al. 2004). In contrast, none of the 112 transcripts in the up set were predominately expressed in stamens, indicating that the large proportion of stamen-specific transcripts in the down set is not solely due to there being a large number of stamen-specific transcripts in inflorescence tissue. This observation suggests that XRN4 may be involved in stamen development; however, this role may be partially redundant since the gross morphology and fertility of the

TABLE 3. GO categories over-represented in the down set

Keyword category	Functional category	Z-score
GO Molecular Function	Hydrolase activity	4.06
GO Molecular Function	Other molecular functions	4.71
GO Biological Process	Response to abiotic or biotic stimulus	3.24
GO Molecular Function	Other binding	3.12

TABLE 4. GO categories over-represented in the up set compared to all transcripts

Keyword category	Functional category	Z-score
GO Cellular Component	Chloroplast ^a	7.27
GO Molecular Function	Nucleic acid binding	5.45
GO Molecular Function	DNA or RNA binding	3.99

^aNuclear genes encoding chloroplast-targeted proteins.

stamens appeared normal in the *xrn4* mutants (data not shown).

Figure 1B shows the average fold differences for seven transcripts with increased expression levels in *xrn4* in three or more biological replicates (Supplemental Table S3). Three transcripts (AT1G20070, AT2G252850, and AT5G51720) corresponded to n-chlor proteins based on their annotations in TAIR. This coincides with the GO annotation results, where transcripts encoding n-chlor proteins were over-represented in the up set. Taken together, these data suggest that mRNA decay by XRN4 may be one way the nucleus controls chloroplast function.

Targets of miRNA-mediated decay do not accumulate in *xrn2xrn3* or exosome mutants

The enrichment of transcripts with certain GO annotations in the *xrn4* mutant suggests that XRN4 displays selectivity in the transcripts it affects. This mirrors previous data showing that select 3' cleavage products of miRNA-mediated cleavages accumulate in *xrn4* (Souret et al. 2004; Gregory et al. 2008), whereas others do not, even though they should all have 5' monophosphates, the preferred substrate of XRN4. This differential accumulation was not due to altered miRNA levels in the mutant (Souret et al. 2004; Gregory et al. 2008). In order to gain insight into why some 3' cleavage products of miRNA targets accumulate in *xrn4* and others do not, AGO1 and ARF10 were chosen for detailed analysis. The 3' cleavage product of ARF10 strongly accumulates in *xrn4*, whereas the 3' cleavage product of AGO1 does not (Souret et al. 2004). One hypothesis for why the AGO1 cleavage product does not accumulate in *xrn4* is that it is degraded by another ribonuclease, such as the nuclear 5'-to-3' exoribonucleases, XRN2 or XRN3, or the exosome. To investigate this hypothesis, we checked its accumulation in the *xrn2xrn3* mutant (Fig. 2). Neither the full-length nor 3' cleavage product of AGO1 accumulated in *xrn2xrn3*, suggesting that AGO1 is not cleaved and degraded by the corresponding enzymes in the nucleus. It is also unlikely that the exosome is the predominant enzyme that degrades the AGO1 cleavage products or any 3' cleavage products of miRNA-mediated cleavage, because none of the validated miRNA targets showed altered RNA accumulation in the tiling arrays for exosome (*rrp4*, *rrp41*, or *csf4*) RNAi lines (Chekanova et al. 2007). Therefore, 3' cleavage products of miRNA-mediated

FIGURE 2. 3' cleavage products from miRNA-mediated cleavage were not degraded by XRN2 or XRN3. RNA blots were probed with radiolabeled *ARF10* or *AGO1* PCR products. (Arrows) The cleavage products. Hybridization with *eIF-4A* was used as a loading control.

cleavages were unlikely to be degraded mainly by XRN2, XRN3, or the exosome.

The accumulation of 3' cleavage products of *AGO1* and *ARF10* in *xrn4* is sequence-dependent

Given the lack of evidence for involvement of XRN2, XRN3, and the exosome, it seemed that RNA sequence differences might provide the best alternative reason for why some 3' cleavage products accumulate differentially in *xrn4* and some do not. Accordingly, the role of putative sequence determinants affecting the accumulation of the *AGO1* and *ARF10* 3' cleavage products was investigated. The most likely sequence determinants directing accumulation of the 3' cleavage products in *xrn4* are near the miRNA complementary sites (miCS), because after cleavage by the RISC complex, the distal halves of these sites would be the first sequence potentially interacting with XRN4. For this reason, the sequences surrounding the miCS in *AGO1* and *ARF10* were examined.

The role of RNA sequence in XRN4-mediated decay was tested initially by removing ~300 nucleotides (nt) centered over the miCS site from *ARF10* and substituting it for the miCS from *AGO1*, and vice versa. This was done using 35S-driven constructs to create ARF::AGO and AGO::ARF, respectively (Fig. 3A). The chimeric transcripts were tagged with short HA sequence motifs inserted into the 3' fragments so as to differentiate them from the endogenous transcripts. After plant transformation with these constructs, RNA was isolated from inflorescences of T2 plants and evaluated by Northern blot (Fig. 3B). Each of the transgenic lines exhibited slightly different levels of transgene expression, likely due to position effects. Since the formation of the 3' cleavage products should be dependent on the level of full-length transcript, the intensity of the cleavage product was calculated relative to that of the full-length RNA. The WT cleavage product/full-length ratio was set to one to allow for easy comparison between constructs, and the accumulation of the cleavage products in WT and *xrn4* was determined (Fig. 3C). While the relative levels of 3' cleavage products generated from the ARF::AGO transcripts were only slightly higher in

the *xrn4* mutant, the relative abundance of the AGO::ARF cleavage products accumulating in the *xrn4* mutant was more than twofold greater than in the WT. Together, these data indicate that the 300 nt surrounding the miCS from *AGO1* or *ARF10* controls the level of accumulation of the 3' cleavage products in *xrn4*.

To ascertain whether the 300 nt that surrounded the *AGO1* miCS contained sequence elements that block XRN4-mediated RNA accumulation, additional *ARF10* variants containing the 150 nt upstream of the miCS site plus the miCS (ARF::AGO5'), the miCS alone (ARF::AGOMi), or the miCS plus the 150 nt downstream from AGO (ARF::AGO3') were created (Fig. 4A). If *AGO1* contains sequence elements that inhibit XRN4 activity, the 3' cleavage products should accumulate in the *xrn4* background when they are removed. The 3' cleavage products from ARF::AGOMir and ARF::AGO3' accumulate to a slightly greater extent in *xrn4* than ARF::AGO5' and ARF::AGO (Fig. 4B). Although the increase in ARF::AGOMir and ARF::AGO3' compared to ARF::AGO5' is hardly dramatic, it cannot be ruled out that *AGO1* may contain weak sequence elements upstream of the miCS that inhibit accumulation in the *xrn4* mutant.

A similar set of deletion constructs was created for the AGO::ARF series (Fig. 4A) to assess whether *ARF10* contained sequence elements that could promote XRN4-mediated

FIGURE 3. (A) Chimeric constructs were generated where the 300-nt regions surrounding the miRNA-complementary sites for *ARF10* and *AGO1* were interchanged. (Vertical gray arrows) The miCS. (B) RNA blots were probed with a 5'-end-labeled oligo complementary to the HA sequence and radiolabeled *eIF-4A* PCR products. (V) The vector control; (1–3) independent transgenic lines; (horizontal arrows) cleavage products. (C) The average ratio of cleavage product to full length was calculated using ImageQuant software. The average WT ratio was set to 1, and the *xrn4* value was adjusted accordingly for easy comparison between constructs.

FIGURE 4. (A) Chimeric constructs were generated where the 300-nt regions surrounding the miCS for *ARF10* and *AGO1* from Figure 3 were subdivided as shown. (Vertical gray arrows) The miCS. (B) RNA was extracted from pooled T1 plants. RNA blots were generated and probed as in Figure 3. The average ratio of cleavage product to full length was calculated. The WT ratio was set to 1, and the *xrn4* value was adjusted accordingly.

RNA accumulation. While the 3' cleavage products of plants expressing *AGO::ARF5'* and *AGO::ARFmi* did not accumulate in *xrn4* compared to WT, a twofold increase in 3' cleavage product accumulation of *AGO::ARF3'* was observed compared to the levels in WT plants (Fig. 4B). This increase was much more dramatic than that observed for any of the *ARF::AGO* constructs in Figure 4, and it can completely explain the twofold increase in accumulation of the *AGO::ARF* transcript (Figs. 3C, 4B). This demonstrates that sequences within the 150 nt downstream from the *ARF10* miCS are sufficient to confer the effect of *XRN4* on RNA levels.

Hexamers over-represented in potential *XRN4* substrates cluster at the 5' end of the RNAs

Since the 150-nt sequence stretch downstream from the miCS in *ARF10* facilitates *XRN4*-mediated accumulation, we hypothesized that a select set of smaller sequence elements or motifs might be preferentially found among transcripts accumulating in *xrn4*. Such motifs would therefore be over-represented in the up set. To search for motifs with this characteristic, the distribution of hexamers among transcripts was examined, similar to the analysis in Narsai et al. (2007), for (1) the annotated cDNAs, (2) the data set used for the 5PARE analysis, and (3) the first 150 nt downstream from the miRNA-mediated cleavage sites. First, the number of transcripts containing each hexamer was calculated. This was compared to either the up set or the mirup set, which consisted of the eight miRNAs targets whose 3' cleavage products accumulate in *xrn4* on Northern blots (Fig. 1B; Souret et al. 2004). For controls, the up set or mirup set was

shuffled as described in the Materials and Methods to create five shuffle sets for each analysis. Any hexamer that was found proportionally higher (Z -score >3.09 , P -value <0.001) in the up set or the mirup set compared to all transcripts or the validated miRNA targets, respectively, and to all five of their respective shuffle sets, was considered a sequence potentially influencing *XRN4*-mediated RNA accumulation.

Although no hexamer motifs were found to be over-represented in the up set when full-length cDNAs were examined, 20 hexamer motifs were found proportionally higher in the 5' ends of the up set (Table 5) when the 5PARE data set was examined. In addition, eight hexamers were found to be over-represented in the mirup set. Only one hexamer, CTCCGT, was found in both the 5PARE and the miRNA targets hexamer analyses. To determine if these hexamers cluster to particular regions of the transcripts, the starting positions of the over-represented hexamer motifs

TABLE 5. Hexamers over-represented in transcripts that increased in *xrn4*

Analysis	Hexamer	All transcripts ^a	Up set ^a	Z-score ^b
3' CL	AGCCAG	5.97	37.50	3.24
3' CL	CACCAT	8.21	50.00	3.74
3' CL	CAGTGG	5.22	37.50	3.47
3' CL	CCAATA	3.73	37.50	4.02
3' CL	CTCCGT	5.22	37.50	3.47
3' CL	GAAATC	5.97	37.50	3.24
3' CL	GAGCCA	5.22	37.50	3.47
3' CL	TCAATC	11.19	50.00	3.13
5' cDNA	AGCTGG	0.95	7.14	6.69
5' cDNA	CCGTCC	1.54	5.36	3.26
5' cDNA	CGTCAA	1.75	7.14	4.33
5' cDNA	CGTGTG	0.98	5.36	4.65
5' cDNA	CTCAGT	2.36	7.14	3.32
5' cDNA	CTCCGT	1.87	6.25	3.40
5' cDNA	CTTATC	3.20	11.61	5.02
5' cDNA	GAACAA	5.01	11.61	3.19
5' cDNA	GAGACT	2.28	8.04	4.06
5' cDNA	GCTCAG	1.57	8.04	5.45
5' cDNA	GGGCCT	0.58	4.46	5.35
5' cDNA	GGTTAG	1.44	10.71	8.14
5' cDNA	GTGGCT	1.39	5.36	3.56
5' cDNA	GTGTGG	1.14	5.36	4.16
5' cDNA	TAGAGC	1.68	8.04	5.20
5' cDNA	TCTCCG	3.52	8.93	3.10
5' cDNA	TGGAGC	1.47	5.36	3.39
5' cDNA	TGGTTA	2.84	11.61	5.55
5' cDNA	TTAGAG	2.83	12.50	6.13
5' cDNA	TTGACT	2.79	8.93	3.92

Either the first 150 nt of the 3' cleavage products from miRNA targets (3' CL), or the 5' ends of annotated cDNAs ± 50 nt (5' cDNA) were examined. The percent of transcripts, in which each hexamer was found in the entire sequence set ("all transcripts") and in the up set is provided. Hexamers having Z -scores >3.09 (P -value <0.001) when comparing the up set values to all transcripts are shown.

^aThe percent of transcripts containing each hexamer.

^bThe Z -score of the up set compared to the all transcripts set.

were sorted into 50-nt bins and graphed according to the percent of all potential hexamers in that bin (Supplemental Fig. S2A,B) for the entire transcript (up set hexamers) or the miRNA cleavage products (mirup hexamers). In both cases, the hexamers were found significantly more often (Z -score >3.09 , P -value <0.001) in the first 50 nt after the start of the cDNA (up set hexamers) (Supplemental Fig. S2A), or miRNA cleavage product (mirup hexamers) (Supplemental Fig. S2B). Finding that the over-represented hexamers from both the miRNA targets and 5' ends of cDNA cluster in the first 50 nt, and that the 150 nt after the miCS controls accumulation in *xrn4*, indicate that short sequences near the 5' end of the uncapped RNAs influence accumulation in *xrn4*. In fact, when the distribution of the over-represented mirup hexamers was examined for *ARF10*, six hexamer motifs were over-represented in the 150 nt after the miCS, including four of them present within the first 50 nt (Supplemental Fig. S3). All of these data point to sequence elements, particularly near the 5' ends of uncapped RNAs controlling susceptibility to XRN4.

DISCUSSION

In eukaryotes, XRN cytoplasmic 5'-to-3' exoribonucleases are involved in many processes, such as decay of decapped transcripts (Hsu and Stevens 1993), nonsense-mediated decay (NMD) (He and Jacobson 2001), suppression of silencing (Gazzani et al. 2004), and miRNA-mediated decay (Souret et al. 2004). Here, we identified more than 20 transcripts with decreased accumulation in *xrn4* and more than 100 with increased abundance using PARE analysis and Affymetrix arrays. Both the down and up sets clustered into functional groups. While the down set was enriched for transcripts preferentially expressed in stamens, the up set was enriched for transcripts encoding n-chlor proteins and nucleic acid-binding proteins. For the up set, our data indicate that this specialization may be, in part, due to sequence elements because the 150 nt after the miCS clearly caused accumulation in *xrn4* (Fig. 4B). In addition, 27 hexamers were found to be over-represented in the 5' ends of RNAs that accumulated in XRN4. This further supports the finding that specific sequence motifs dictate the impact of XRN4 on RNA abundance and most likely RNA decay.

Stamen association of transcripts that decrease in *xrn4* mutants

Although transcripts that decrease in *xrn4* have been reported in a transcriptome study (Gregory et al. 2008), they have not been validated or analyzed experimentally. Figure 1A shows six of the transcripts whose accumulations were reduced in *xrn4* in multiple independent experiments. Theoretically, the presence of the XRN4 protein could directly stabilize these transcripts in WT plants. However, it seems more likely that their decrease is a secondary effect of

the *xrn4* mutation. One of the phenotypes of the *xrn4* mutants is higher levels of RNA silencing (Gazzani et al. 2004). Indeed, *xrn4* mutants in *Arabidopsis* are less susceptible to infection by cauliflower mosaic virus and turnip mosaic virus due to gene silencing of viral genes (Gy et al. 2007; Vogel et al. 2010). One possibility is that the down set is decreased in *xrn4* due to the production of secondary siRNA generated from their uncapped transcripts and subsequent siRNA-mediated decay. Three of the 22 transcripts in the down set matched to at least fivefold more small RNAs in an *xrn4* mutant (see * in last columns of Supplemental Table S1) and maybe subject to such regulation. However, based on available data (Gregory et al. 2008), this does not seem to be a general feature of the down set transcripts. Therefore, it seems more likely that the majority are regulated by another secondary effect of the gene products differentially accumulating in *xrn4*.

Interestingly, $>45\%$ of the down set and all of the transcripts in Figure 1A are preferentially expressed in stamens (Honys and Twell 2004; Wellmer et al. 2004). This may be due to altered expression of stamen-specific transcription factors or repressors. To determine if XRN4 is associated with a particular stage of stamen development, our data were compared to the microarray data of *apetala3* (*ap3*), *sporocyteless/nozzle* (*spl/nzz*), and *male sterility1* (*ms1*) (Alves-Ferreira et al. 2007), which block stamen development at stamen stages 1, 3, and 7, respectively. Only three of the 10 stamen-specific transcripts in the down set overlapped with the *ap3* set, and none of the three are predicted to encode transcription factors or repressors. In contrast, eight and nine transcripts from the stamen-specific down set transcripts overlapped with the *spl/nzz* and *ms1* data, respectively. The transcripts encoding *SPL/NZZ* and *MS1* were not differentially expressed in *xrn4*. Nevertheless, our data suggest that XRN4 likely influences stamen development sometime during or after stage 3 of stamen development.

Transcripts that accumulate in *xrn4* mutants are enriched for those encoding nucleic acid-binding proteins and chloroplast proteins and demonstrate a sequence dependence and/or hexamer association

Transcripts encoding two types of proteins are prominent in the up set: nucleic acid-binding proteins and n-chlor proteins. Nucleic acid-binding proteins include proteins influencing transcription by binding to the DNA, and proteins controlling RNA stability or translation by binding to the RNA. One of the phenotypes of *xrn4* mutants is insensitivity to exogenously applied ethylene (Olmedo et al. 2006; Potuschak et al. 2006). Although several components of the ethylene response pathway are differentially regulated in *xrn4* (Souret et al. 2004; Olmedo et al. 2006; Potuschak et al. 2006), the RNA decay rates of these transcripts are not altered. It is possible that one or more of the nucleic acid-binding proteins enriched in the up set influence the

transcription of these ethylene-responsive factors or somehow influence the stability or translation of other members of the ethylene response pathway.

The chloroplast proteins encoded by the up set transcripts are diverse and include proteins involved in transcription, translation, and light harvesting. Thus, they do not appear to be functionally distinguished from other n-chlor proteins. Several n-chlor transcripts have been found to localize to the surface of the chloroplasts in wheat and *Arabidopsis* (Gibson et al. 1996; Marrison et al. 1996). While the nature of this targeting is unknown, it has been speculated that correctly targeted RNAs may be protected from degradation, whereas those not clustered around the chloroplasts would be subject to decay (Okita and Choi 2002). If so, RNA from the n-chlor transcripts in the up set may not be efficiently transported to the chloroplasts, leaving them vulnerable to XRN4 decay.

The over-representation of certain functional groups and the fact that most transcripts did not accumulate in *xrn4* suggest that its major role is not bulk decay, but rather preferential degradation of select substrates, as suggested previously (Souret et al. 2004). Our experimental studies indicated that this specificity may be associated with RNA sequence elements based on results from the chimeric construct analysis, where the regions surrounding the *ARF10* and *AGO1* miCS were interchanged. This could not be caused by changing the miCS, and thus the miRNA that initiates cleavage, because transcripts containing *ARF5'*, *ARFmi*, and *ARF3'* all contain the same miR160 recognition sequence, yet only cleavage products from transcripts containing *ARF3'* accumulate in *xrn4*. These experiments indicated that the 150-nt sequence region present downstream from the *ARF10* miRNA cleavage site (*ARF3'*) (Fig. 4B) in *AGO1* was responsible for the accumulation of the cleavage products in *xrn4*.

To gain further insight as to which specific sequences may be involved in differential RNA accumulation in *xrn4*, the association of hexamers with transcripts in the miRNA cleavage products that accumulate in *xrn4* and the up set were examined. This led to the identification of eight hexamer motifs over-represented in miRNA cleavage products that accumulate in *xrn4* and 20 hexamers over-represented in the 5' end of transcripts from the up set. These hexamer motifs were compared with sequences previously identified as over-represented in uncapped enriched or depleted transcripts (Jiao et al. 2008) or in unstable transcripts (Narsai et al. 2007). Although there was no overlap between those sequences over-represented in uncapped enriched or depleted transcripts, seven had been previously identified as hexamers found in the 5' UTR of RNAs with half-lives of <60 min (Narsai et al. 2007). Overall, the up set did not significantly (Z -score >3.09, P -value <0.001) correlate with shorter half-lives (Supplemental Fig. S4), even when the half-lives of only the subset of the up set containing one or more of the overlapping hexamers in the 5PARE region were evaluated. This is consistent with prior data that showed that highly unstable transcripts (half-lives <60 min) were not substrates

of XRN4 (Souret et al. 2004), and the fact that there were only two transcripts in the up set previously classified as GUTs (genes with unstable transcripts) (Gutierrez et al. 2002).

The clustering of the hexamers in the first 50 nt of the annotated cDNAs and the 3' cleavage products from miRNA-mediated decay argues for the importance of the sequence near the 5' end of uncapped RNA in the 5'-to-3' decay pathway. This is consistent with several studies that have shown that the 5' UTR contains sequence elements that stabilize (Ruiz-Echevarria and Peltz 2000; Hua et al. 2001), destabilize RNA (Iliev et al. 2002; Li et al. 2009), or direct RNA localization (Saunders and Cohen 1999). For the human *Rrp41* gene, a destabilization element has been shown to reside within the first 33 nt of the 5' UTR (Li et al. 2009). This 33 nt folds into a stem-loop that directly binds DCP2. The first 50 nt of the majority of the up set and miRNA cleavage products containing over-represented hexamers did not fold into obvious stem-loop structures (data not shown). Even so, this does not exclude the possibility that these hexamers directly interact with the decapping complex or XRN4, or perhaps provide binding sites for RNA-binding proteins that do. Another possibility is that the hexamers may direct RNAs to P-bodies where XRN4 localizes (Weber et al. 2008). For the *Gurken* mRNA in *Drosophila*, the first 35 nt of the 5' UTR can drive RNA localization to the posterior pole of the oocyte during early development (Saunders and Cohen 1999). RNA localization signals have been identified in plants (Washida et al. 2009a,b), but thus far, none reside in the 5' UTR.

In summary, the data presented here highlight the association of XRN4 with transcripts encoding select functional groups and specific sequence motifs. This suggests that these RNAs may be expressly targeted to the 5'-to-3' decay pathway for degradation by XRN4 in *Arabidopsis*, potentially controlling gene expression in response to developmental or environmental cues. RNA sequence plays a major role in this targeting because the 150-nt stretch following the miCS in *ARF10* strongly influences accumulation of the 3' cleavage products in *xrn4*. Also, 27 hexamers were over-represented in the transcripts accumulating in *xrn4*. Their concentration within the first 50 nt of transcripts places them in an ideal position to interact directly or indirectly with XRN4. Lastly, six of the 27 hexamers are present in the *ARF10* 3' cleavage product, four of which are present in the first 50-nt sequence stretch downstream from the miCS. Taken together, these results demonstrate that RNA sequence is responsible for XRN4 selectivity. The animal cytoplasmic homolog of XRN4, XRN1, was also shown to degrade cleaved small RNA targets (Bagga et al. 2005; Orban and Izaurralde 2005; Behm-Ansmant et al. 2006), similar to XRN4 (Souret et al. 2004). Although little is known about XRN1 sequence specificity, the work presented here should provide a foundation for comparative analysis and for further elucidating the biological and mechanistic implications of XRN-mediated decay.

MATERIALS AND METHODS

Plant growth and tissue collection

The lines used in this study were Col-0 (WT), *xrn4* (*xrn4-5*) (Souret et al. 2004), and *xrn2xrn3* (*xrn2-1xrn3-3*) (Gy et al. 2007). All plants were grown under long-day conditions (16 h light, 8 h dark) at 21°C. For chimeric constructs, seeds were germinated on Murashige and Skoog (MS) medium containing 1% sucrose, 1× Gamborg's vitamins, and 40 µg/ mL Hygromycin and grown for 2 wk before transfer to soil. After flowering, mixed-stage inflorescences were collected by trimming the inflorescences just above the first mature flower. Plants that displayed the *AGO1* co-suppression phenotype (Mallory and Vaucheret 2009) were discarded. For *AGO::ARF* and *ARF::AGO* lines, inflorescences were pooled from multiple siblings from a self-crossed T1 parent for three individual transformants per construct. For the remaining lines, two T1 pools were collected for each construct by harvesting five inflorescences from each of 10 or more T1 plants.

ATH1 array analysis

Total RNA was extracted using the RNeasy plant kit (QIAGEN) according to the manufacturer's instructions. Fifteen micrograms of total RNA from WT or *xrn4* inflorescences was labeled and hybridized to the ATH1 (25K) GeneChip according to the manufacturer's instructions. Two biological replicates were examined for each line for a total of four chips. All analysis was performed using the Affymetrix GCOS package. A comparative data analysis was performed to calculate the fold values displayed in Supplemental Table S1 by using *xrn4* results as the experimental chip and the WT chip as baseline. Transcripts were only considered differentially accumulating if all signal *P*-values were <0.05. In addition, to be considered as decreased in *xrn4*, they required fold *P*-values >0.995, maximum folds ≤0.667, and mean folds ≤0.5. To be considered as increased, transcripts were required to have fold *P*-values <0.005, a minimum elevation of ≥1.5-fold and a mean fold of ≥2. Array data have been deposited at NCBI (accession number GSE23027).

PARE analysis

The Col-0 and *xrn4* PARE libraries analyzed were generated previously (German et al. 2008). All sequences matching the TAIR8 genome (<http://www.arabidopsis.org>) more than five times or to short sequence repeats were discarded. The remaining analyses were performed on the nuclear-encoded TAIR8 cDNAs (<http://www.arabidopsis.org>). The APARE analysis consisted of matching the PARE sequences to the cDNAs and summing the abundances of the PARE sequences on the positive strand. For the 5PARE analysis, the genomic sequences 50 nt upstream of the cDNAs were concatenated with the first 50 nt of each annotated cDNA, and the PARE sequences were matched and summed as per the APARE analysis. The results of the APARE and 5PARE analyses are reported in Supplemental Table S1. To remove potential bias due to low abundance transcripts, a minimum of 10 TP10M in one library was required to be considered for differential accumulation. After that, a fivefold or greater difference was set as the minimum cutoff for differential accumulation.

GO analysis

The Gene Ontology (GO) annotations from TAIR were obtained for the annotated cDNAs. The number of genes with each annotation in the down and up sets was compared, using *Z*-tests, to the number with the corresponding annotation in all transcripts. Annotations that had a *Z*-score >3.09 (*P*-value <0.001) were considered significant. For the up set, *Z*-tests were run again after removal of genes with the Chloroplast GO annotation.

Hexamer analysis

A Practical Extraction and Report Language (Perl) script was written to collect the number of genes in which each hexamer was present from a given sequence set. The gene sets were the first 150 nt downstream from the miCS from 134 validated miRNA-mediated cleavage products (132 transcripts total), the 5PARE sequences described above, and the annotated nuclear-encoded TAIR8 cDNAs. The number of genes containing each hexamer was collected from each data set. To control for sequence composition, the respective mirup or up set sequences from each data set were shuffled and added to a shuffle set. This was repeated 10 times to create shuffle sets that had 10 times the number of sequences as the respective mirup or up set. Five such shuffle sets were constructed for each sequence set. In order for a hexamer to be considered significant, the proportion of the hexamer in the up set or mirup set had to have a *Z*-score >3.09 (*P*-value <0.001) relative to the proportion of the hexamer in the corresponding all transcripts set and each of the five shuffle sets. *Z*-scores were calculated as previously described (Narsai et al. 2007).

To determine if the significant hexamers were found in certain regions of the transcripts at a higher rate, the positions of the hexamers were binned into 50-nt intervals. Since all the transcripts were not of equal lengths, the total number of hexamers for each bin was also calculated. For each bin, *Z*-scores were calculated comparing the number of over-represented hexamers in the up set or mirup set to over-represented hexamers in their respective "all transcripts" set; *Z*-scores >3.09 (*P*-value <0.001) were considered significant.

RNA isolation and blot analysis

Inflorescence tissue was ground under liquid nitrogen and extracted with TRI reagent (Molecular Research Center Cincinnati OH) according to the manufacturer's instructions with the exception that a second chloroform extraction was added prior to the isopropanol precipitation step. Poly(A) RNA was purified from total RNA using the NucleoTrap mRNA kit (Macherey-Nagel), and 1.5 µg of poly(A)-selected RNA was fractionated on a 1.2% denaturing formaldehyde gel and transferred to Hybond N+ membrane (GE Healthcare Life Sciences). Northern blots were hybridized using oligo primers end-labeled with optikinase (Promega) according to the manufacturer's instructions, or PCR products generated using the primers supplied in Supplemental Table S5 and labeled using the random priming method (Feinberg and Vogelstein 1983). For *ARF10* and *AGO1* probes, PCR products corresponding to the 150 nt downstream from miSC from *ARF10'* and *AGO1* were used. Membranes were prehybridized for 1 h and hybridized overnight with either Ultrahyb Oligo (Ambion) at 40°C or Church and Gilbert's buffer (Church and

Gilbert 1984) at 65°C. All membranes were washed twice in 2× SSC, 0.1% SDS for 20 min. The Typhoon system and ImageQuant software (Amersham Biosciences) were used to analyze signal intensities, and *eIF-4A* (Taylor et al. 1993) was used as a control for equal loading.

Plasmid construction and plant transformation

The *AGO1* and *ARF10* chimeric constructs presented in Figures 3 and 4 were created using standard techniques as described in the Supplemental Methods and the primer sequences described in Supplemental Table S5.

For plant transformation, plasmids were introduced into *Agrobacterium tumefaciens* strain GC3101 (C58C1 Rif^r) pMP90 via electroporation (Koncz and Schell 1986). *Arabidopsis* plants were transformed with *Agrobacterium* by growing a 50-mL culture to a density of OD₅₉₅ = 1.5. Cells were centrifuged and the cell pellet was resuspended in 50 mL of 5% sucrose, 0.05% Silwet L-77. The *Agrobacterium* suspension was dripped onto all inflorescences and meristematic regions. Plants were covered in plastic wrap for 24 h to maintain high humidity before a second treatment with *Agrobacterium* and re-covered. After 24 h, the plants were uncovered and grown normally.

All materials are available upon request.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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