SUPPLEMENTARY MATERIAL

Supplementary Materials and Methods

Yeast strains, plasmids and media

Full-length *AtXRN2* cDNA (p1486, {Kastenmayer, 2000 #59}) and a fragment encoding first 546 amino acids of AtXRN2 had been inserted into a p424TEF vector {Mumberg, 1995 #195} between sites SpeI-EcoRI and introduced into the yeast *xrn1Δ* strain (yRP884) as described {Gietz, 1992 #197}. Strains were grown at 30°C in synthetic complete (SC, 0.67% yeast nitrogen base, 2% galactose, supplemented with required amount of amino acids and nucleotide bases) or in SC media without tryptophan.

Southern blot analysis

The number of T-DNA insertions in the genome was determined by Southern blot analysis using genomic DNA isolated from 2-week-old plants with Plant DNAzol Reagent (Invitrogen). Digested DNA (8 μg) was separated on a 0.8% agarose gels, depurinated in 0.25 M HCl, and blotted onto Hybond-N^+ membranes (GE Healthcare) by capillary transfer. DNA probes were labelled with α-^{32}P by random hexamer priming (GE Healthcare) using gel-purified PCR products as templates.

Exoribonuclease activity in yeast

Analysis of reporter poly(G)-containing *PGK1* and *MFA2* mRNAs expressed in wild-type, *xrn1Δ*, *xrn1Δ* + [p1486/AtXRN2] and *xrn1Δ* + [p424TEF/5'AtXRN2] was performed as described {Kastenmayer, 2000 #59}.

Microarray analysis and half-life measurements

Genome-wide expression and mRNA stability profiles in wild-type and *xrn2-3* plants were performed using Affymetrix ATH1 Gene Chips on total RNA from seedlings treated with cordycepin. Time-course experiments were carried out as described {Souret, 2004 #109}. 2-week-old seedlings were transferred to flasks containing a buffer (1mM Pipes pH 6.25, 1mM sodium citrate, 1mM KCl, 15mM sucrose) and incubated for 30 min. Cordycepin was added (150mg/l) and incubation was continued for 120 min. Total RNA samples corresponding to 0 and 120 minute time points after transcriptional inhibition were extracted using RNeasy Plant Mini Kit (Qiagen). Biotin-labeled target RNA was prepared from 15 μg of total RNA, fragmented, and hybridized according to manufacturer's instructions (Affymetrix). Samples were stained with streptavidin-phycoerythrin conjugate using a GeneChip Fluidics Station 400 and probe arrays were scanned with Agilent GeneArray Scanner. Obtained data were analyzed using Affymetrix GeneChip Operating Software.

Supplementary Table S1: Oligonucletides used in this study.

Oligo	Region	Sequence
p1	5'ETS-1	5'-CCTAGGCGGATCCATGCTTTCCAAC
p2	5'ETS-2	5'-CATCGATCACGGCAATTCCCCGC
p3	5'ITS1	5'-GGTCGTTCTGTTTTGGACAGGTATC
p4	3'ITS1 (27SA)	5'- CGTTTTAGACTTCAGTTCGCAG
p5	5'ITS2	5'-GCAAAGGATGGTGAGGGACGACG
p6	3'ETS	5'-CGTTAAGGAGCTGTTGCTTTGTTAGTGTAG
p7	5.8S	5'-GATTCTGCAATTCACACCAAGTATC
p8	25S-5'	5'-CTCCGCTTATTGATATGCTTAAAC
p9	25S-3'	5'-GATGACCAATTGTGCGAATCAACGG
p10	18S-5'	5'-CATATGACTACTGGCAGGATCAACC
p11	18S-3'	5'-GATCCTTCCGCAGGTTCACCTACG
p12	5S	5'-GCACGCTTAACTGCGGAGTTCTG
p13	7SL	5'-ACTGGGCAGCCCAGAAACATGC
p15	XRN2-5'F	5'-ATGGGAGTTCCGTCGTTCTACAG

p16	XRN2-5'R	5'-TGTTCTCCATTTCCTGACCTGAACC
p17	XRN2-3'F	5'-CATGCTCTCCCAGAGTGCTATAGG
p18	XRN2-3'R	5'-ATGAGATGCCTTCCCAAGCCTACC
p19	XRN3F	5'-AACGCAGCACATCGTCTTGTTTCC
p20	XRN3R	5'-CAATATCGATGTCTAGGATTCC
p21	eIF-4E-F	5'-TCATGAGAGCTTTGATGCCATGG
p22	eIF-4E-R	5'-GATGAGAACACGGGAGGAACCAG
p23	35S-53P	5'-GTTCCAACTACTCTACCGAAGTAC
p24	ETSa-280	5'-GTGTAAACCAAACTCAACAATTCC
p25	5'ETSas3	5'-GGAGAATCCATGTCAGCCCATG
p26	5'ETS-seq2	5'-CGAGTTTTGTTGATGTGTTTCCGAG
p27	18S-56	5'-GTCTGAATTCGTTCATACTTACAC
p28	RT-PCR ETS-seq	5'-CATGGGCTGACATGGATTCTCC
p29	RT-PCR 3-ETS	5'-GGAGTGATTTAGGGGAGGGTCG
p30	RT-PCR 25S-3'	5'-GATCCTTCGATGTCGGCTCTTCC
p31	cRT-PCR-ETS-P	5'-CCTAGGCGGATCCATGCTTTCCAAC
p32	cRT-PCR-5ETSa3	5'-GGAGAATCCATGTCAGCCCATG
p33	cRT-PCR-ETS-s2	5'-CGAGTTTTGTTGATGTGTTTCCGAG
p34	5'ETS	5'-CCGGACGGTCGGTCATTCCTCGTG
p35	ITS2	5'-CGTCGTCCCTCACCATCCTTTGC
p36	25S	5'-CGCCCGATTGGGGCTGCATTCC
p37	U14	5'-CATTAACTCTCAAGCCTGGCGAAAG
p38	U4	5'-TTGGAAATAGTTTTCAACCAGC
p39	U3pe b	5'-TCAAGGAAACAGAGGTACGAGC
p40	snoR10	5'-GTCATTCTCATACACAGTAAACTG
p41	snoR30	5'-GATTCTGCCAGCAATTCTCTTCAGC
p42	ITS1	5'-CCACGGATCCGGCGGGCAAGG
p43	ITS1	5'-ATGCCAGCCGTTCGTTTGCATG
p44	3'ITS2	5'-GGACTTTGGGTCATCTACAGCTTC
X3-1	X3Hind1s	5'-ACTGATAAGCTTGATCTGCTAAAGATGCATCGG
X3-2	X3Xba_a1	5'-TTGACTTCTAGAGAATCCTCATCTCAAGCTCCAG
X3-3	X3Kpn_s1	5'-TTGACTGGTACCGATCTGCTAAAGATGCATCGG
X3-4	X3Xho_a1	5'-TTGACTCTCGAGGAATCCTCATCTCAAGCTCCAG
X3-5	X3Xho22a	5'-TTGACTCTCGAGGCCTTGTGCACCAGGTGGAGCC
X3-6	X3Kpn22s	5'-TTGACTGGTACCCTCATCGTCTAGCTGAACTCGTC
X3-7	X3Hind2s	5'-ACTGATAAGCTTCTCATCGTCTAGCTGAACTCGTC
X3-8	X3Xba22a	5'-TTGACTTCTAGAGCCTTGTGCACCAGGTGGAGCC
pROK_LB2		5'-TTGCTGCAACTCTCTCAGGGCCAGG
SALK_258		5'-GTAAGTCATCTCGTATCCGAGG
X2endoAS		5'-TGAGGATGACCAGAAACTGACC
SALK_657		5'-AGGAAACTACACAGTAGAAACC
X3endoAS		5'-GGTAAAACGACGGTACACCCATTC
AtXrnl		5'-GCGGGTACCATGGGAGTTCCGTCGTTCTAC
AtXrnr		5'-GCGCGGCCGCTCAAGCTGTTTTGGGAGGAG
ETS_a		5'-CATCGATCACGGCAATTCCCCGC
ETS-seq2		5'-CGAGTTTTGTTGATGTGTTTCCGAG
ETS_S		5'-GTACTTCGGTAGAGTAGTTGGAAC
18S900		5'-CATAAATCCAAGAATTTCACCTCTG
15s	eIF-4E	5'-TCATGAGAGCTTTGATGCCATGG
15as	eIF-4E	5'-GATGAGAACACGGGAGGAACCAG
NPTs	NPTII	5'-CTCCGGCCGCTTGGGTGGAGAGGC
NPTas	NPTII	5'-CAAGAAGGCGATAGAAGGCGATGCGC
AP2-s1	AP2	5'-GCTGCAGCATCATCAGGATTCTC
AP2-as	AP2	5'-CAAGAAGGTCTCATGAGAGG
ARF8-s1	ARF8	5'-CTTAGATCAGGCTGGCAGCTTG

ARF8-as	ARF8	5'-CTAGAGATGGGTCGGGTTTTGC
2995ARFs	ARF10	5'-ATATCTAGAGCAGGAATACAGGGAGCCAG
2995ARFa	ARF10	5'-TCATCCAAGCTTAGCCTGATAATGATGATGC
SPL10-s1	SPL10	5'-CTACAGTGCTCTCTCTCTTCTG
SPL10-as	SPL10	5'-CAGATGAAATGACTAGGGAAAG
ACT1	yeast *ACT1*	5'-TCTTGTCTACCGACGATAGATGGGAAGACAGCA
PGK1pG	*PGK1*+poly(G)	5'-AATTGATCTATCGAGGAATTCC
MFA2pG	*ACT1*+poly(G)	5'-ATATTGATTAGATCAGGAATTCC

Supplementary Results

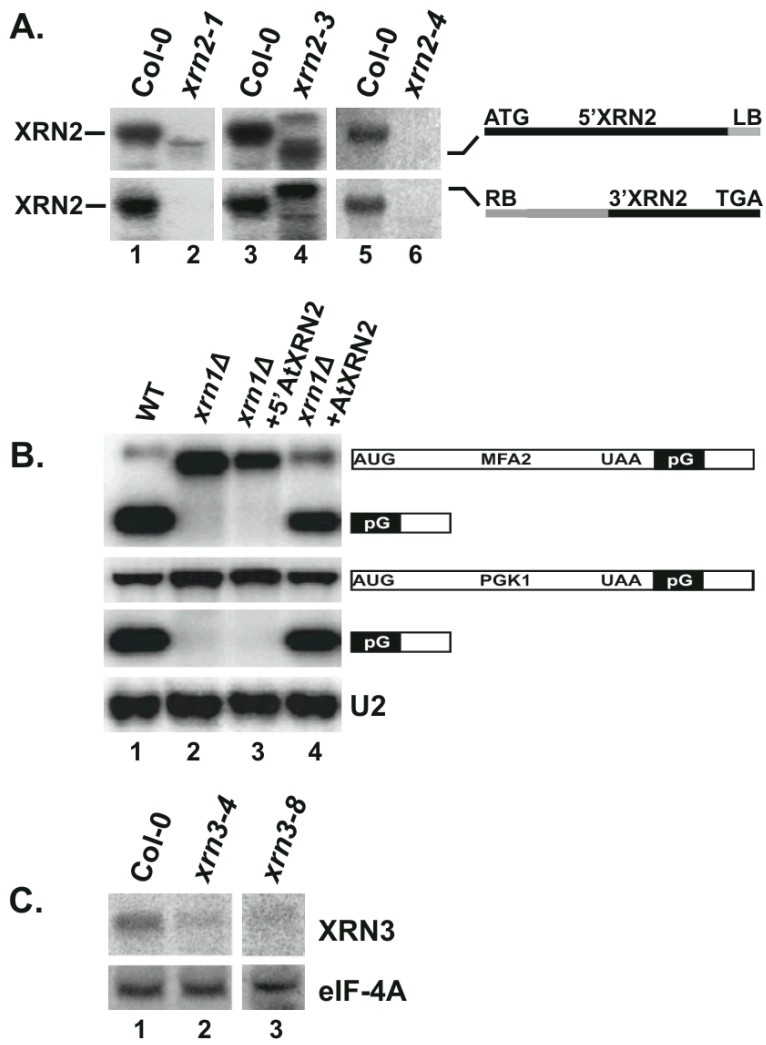
Isolation of mutant lines in* AtXRN2 *and* AtXRN3 *genes

Three xrn2 mutant lines were tested by PCR screening and Southern hybridization. Only one of them, *xrn2-3*, had a single T-DNA insert disrupting the 12th exon in *AtXRN2*; the other two, *xrn2-1* and *xrn2-4*, had further inserts at unknown locations in addition to inserts in the 12th exon and 11th intron, respectively (data not shown). Total RNA was isolated from *xrn2-1*, *xrn2-3* and *xrn2-4* seedlings to test for the expression of *AtXRN2* mRNA using probes directed against its different regions (Supplementary Figure S1A). The full-length mRNA was present only in wild-type plants (Col-0 ecotype), however, a considerable amount of shorter and longer RNAs containing *AtXRN2* sequences was detected in *xrn2-3* and some residual mRNA in *xrn2-1*, but none in *xrn2-4* mutants (Supplementary Figure S1A). The identity of the two transcripts in the *xrn2-3* line was confirmed by hybridization with a probe against *NPT2* from the T-DNA insert and by cloning and sequencing cDNAs corresponding to these RNAs. The truncated RNA contains the 5' end of the *AtXRN2* transcript that extends to the insert and is fused to the sequence originating from the T-DNA. The longer RNA initiates within the T-DNA and continues into the 3' end of *AtXRN2*. These RNAs could potentially encode proteins that retain some exonucleolytic activity. This is, however, unlikely for the 3'-*AtXRN2* transcript, since it contains hardly any of the sequences that encode conserved domains of the Xrn2/Rat1 family {Kastenmayer, 2000 #59}.

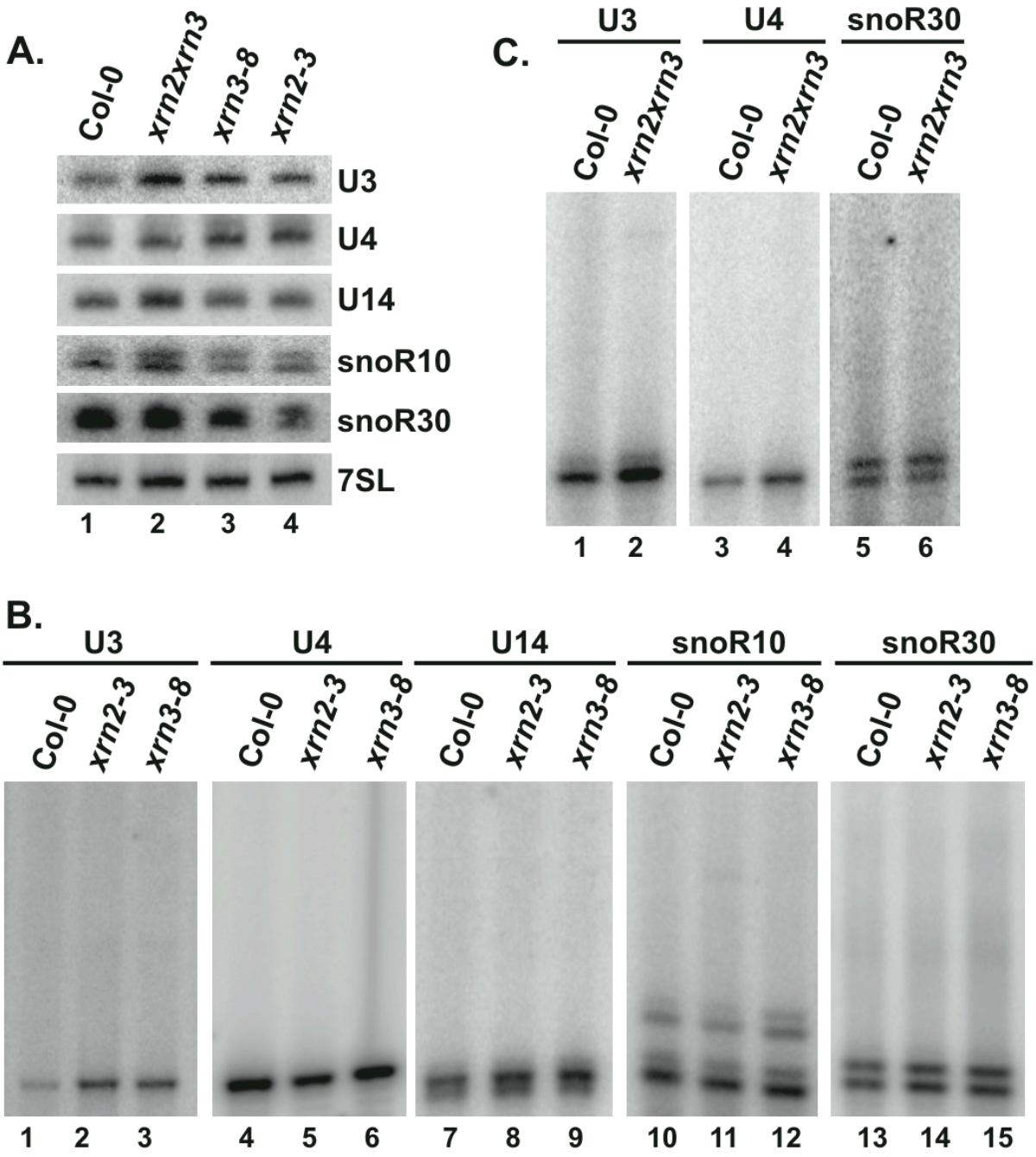
To assess the enzymatic activity conferred by the 5'-truncated AtXRN2, a complementation test was performed *in vivo* {Souret, 2004 #109}. This was based on the fact that active AtXRN proteins, when expressed in yeast cells, are able to replace endogenous Xrn1p and carry out its function in mRNA degradation. cDNA corresponding to the 5'-*AtXRN2* fragment present in *xrn2-3* was cloned into a high-copy number yeast vector and introduced into the *xrn1Δ* strain expressing reporter *PGK1* and *MFA2* transcripts with poly(G) tracts that block the progression of 5'→3' exonucleases, resulting in the appearance of specific products. In wild-type yeast and in cells expressing full-length AtXRN2, degradation intermediates derived from poly(G)-containing mRNAs were clearly detectable, indicating 5'→3' exonucleolytic activity (Supplementary Figure S1B, lanes 1 and 4). In contrast, in the absence of any XRN protein, as well as when only the 5'-truncated AtXRN2 was produced, there was no similar accumulation, showing that the 5' portion of AtXRN2 expressed in *xrn2-3* does not possess enzymatic activity (Supplementary Figure S1B, lanes 2 and 3).

During selection of *xrn3* RNAi transformants, following preliminary screening five *xrn3* lines RNAi were tested for the reduction of *AtXRN3* mRNA by northern blot and RT-PCR and two transgenic lines exhibited 52% and 43% of mRNA depletion, respectively (Supplementary Figure S1C and data not shown). Silencing effects in *xrn3* lines were stably inherited and actually improved in the next generations.

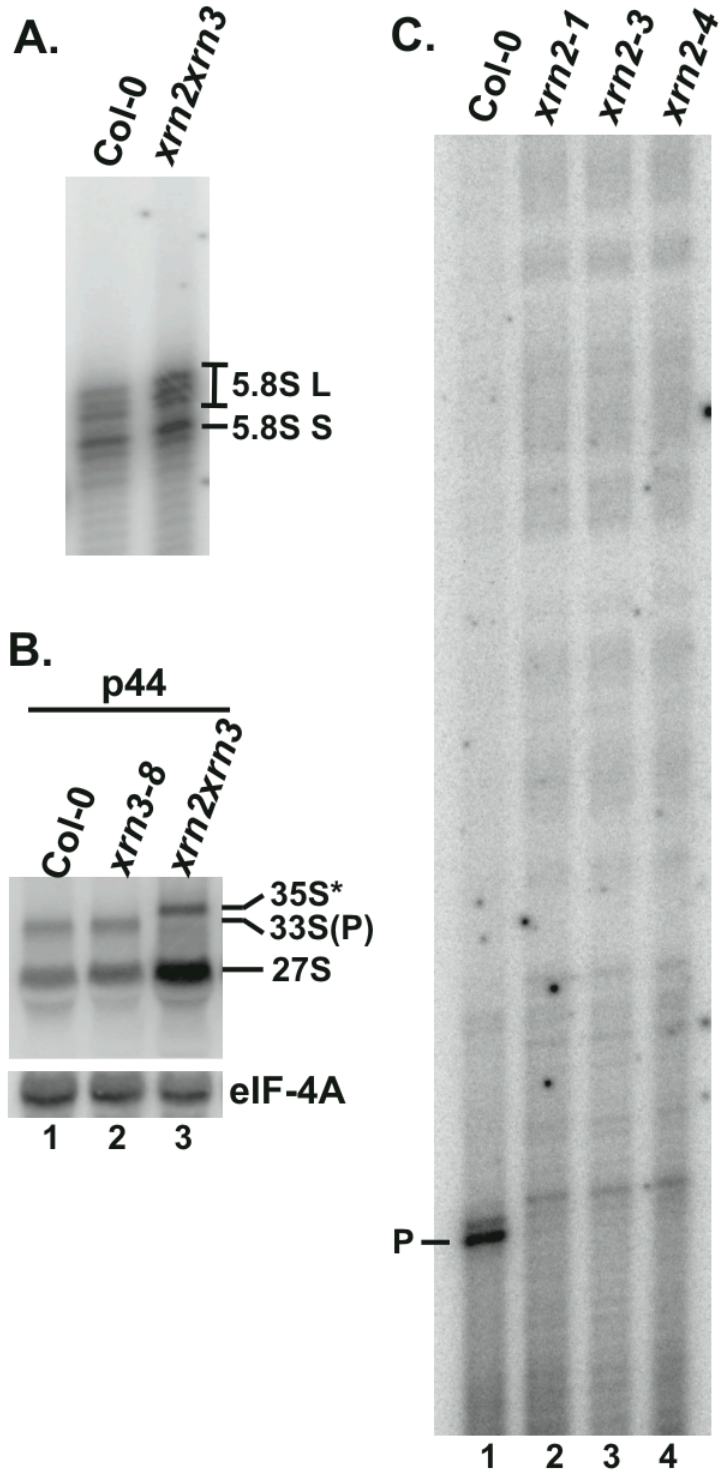
Supplementary Figures



Supplementary Figure S1. (A) Northern analysis of *AtXRN2* mRNA in wild-type and mutant plants. PCR products covering the 5' (0.7 kb, top panel) or 3' (1.5 kb, bottom panel) portion of *AtXRN2* were used as probes. Schematic representation on the right shows *AtXRN2* transcripts detected in the *xrn2-3* mutant: the shorter RNA contains residues 1–1640 of *AtXRN2* (546 amino acids) and enters the T-DNA left border; the longer transcript initiates within the T-DNA and covers residues 1663–3039 of *AtXRN2* (458 amino acids). (B) 5'→3' exoribonucleolytic activity in yeast strains expressing *MFA2* (top panel) and *PGK1* (bottom panel) reporters containing poly(G) tracts analyzed by northern blot. Strains used are wild-type, *xrn1Δ* and *xrn1Δ* expressing full-length or truncated AtXRN2, respectively. Structures of detected poly(G)-containing RNAs are shown on the right. (C) Northern analysis of *AtXRN3* mRNA in *xrn3* RNAi-silenced mutants using probes specific for *AtXRN3* and a loading control, *eIF-4A*. The level of *AtXRN3* silencing is 52% and 43% in *xrn3-4* and *xrn3-8*, respectively.



Supplementary Figure S2. AtXRN2 and AtXRN3 do not participate in snoRNA 5' processing. (A) Northern analysis of total RNA extracted from 14-day old seedlings of wild-type and mutants: *xrn2-3*, *xrn3-8* and *xrn2-1 xrn3-3*. Oligonucleotide probes specific for snoRNAs and for 7SL RNA (loading control) were used for hybridizations. (B-C) Primer extension detecting snoRNA 5' ends performed on total RNA from wild-type (Col-0), *xrn2-3* and *xrn3-8* (B) and from Col-0 and *xrn2-1 xrn3-3* (C) plants.

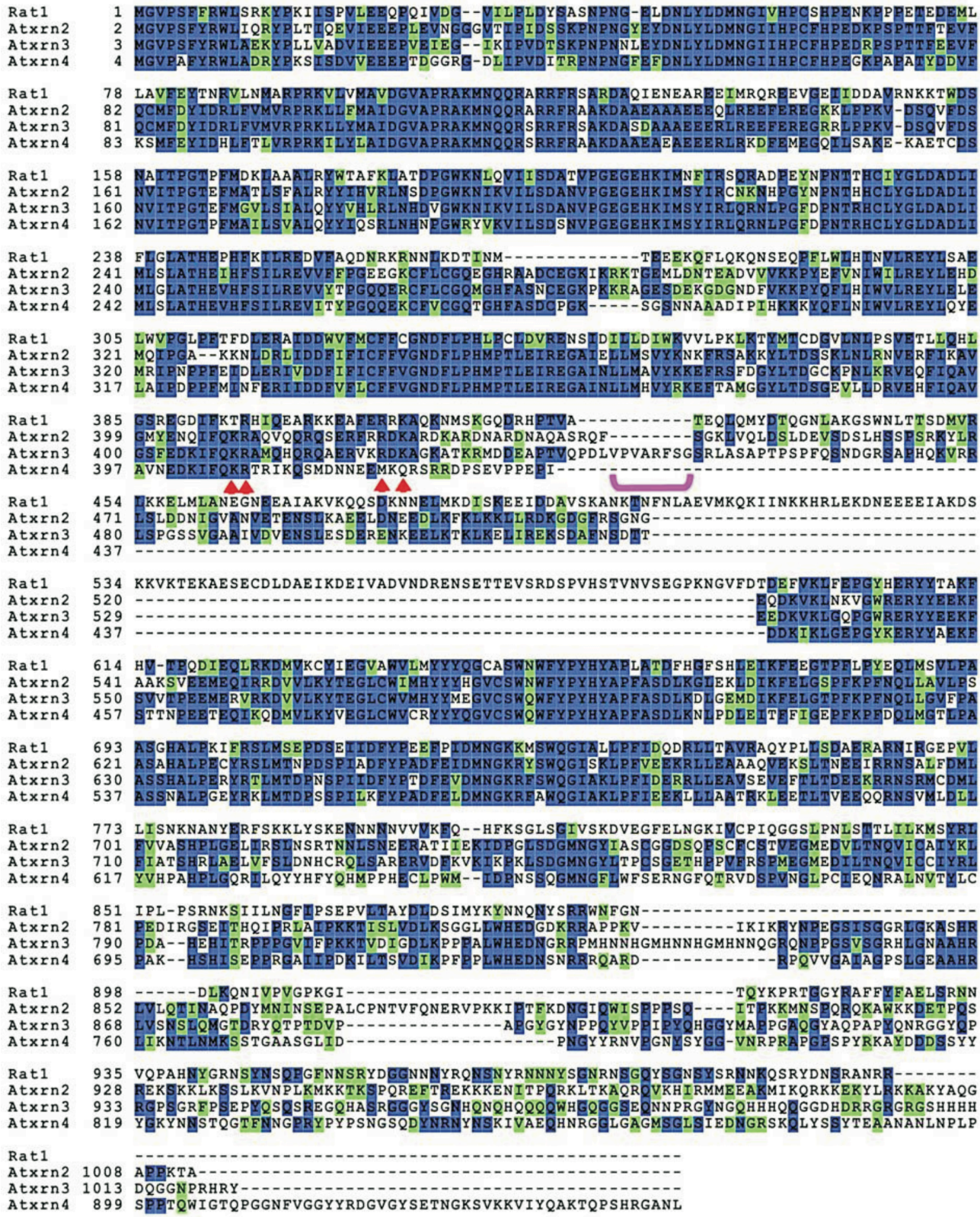


Supplementary Figure S3

(A) Primer extension for 5' ends of mature 5.8S rRNA species, $5.8S_S$ and $5.8S_L$, in wild-type (Col-0) and *xrn2-1 xrn3-3* lines using primer *p7*.

(B) Northern analysis for wild-type (Col-0), *xrn3-8* and *xrn2-1 xrn3-3* lines using probe *p44* located upstream of the mature 25S rRNA 5' end. *eIF-4A* mRNA was used as a loading control.

(C) Primer extension for cleavage at site P and upstream of site P in wild-type (Col-0) and *xrn2* plants using primer *p23*.



Supplementary Figure S4. Sequence alignment generated by CLUSTAL-W of Rat1, AtXRN2, AtXRN3 and AtXRN4 proteins. Identical and similar amino acids are marked blue and green, respectively; red arrows indicate nuclear localization signals (NLS) and a pink bracket shows the region of 8 amino acid deletion in AtXRN2 relative to AtXRN3.