RNS2, a conserved member of the RNase T2 family, is necessary for ribosomal RNA decay in plants

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RNase T2 enzymes are conserved in most eukaryotic genomes, and expression patterns and phylogenetic analyses suggest that they may carry out an important housekeeping role. However, the nature of this role has been elusive. Here we show that RNS2, an intracellular RNase T2 from Arabidopsis thaliana, is essential for normal ribosomal RNA recycling. This enzyme is the main endoribonuclease activity in plant cells and localizes to the endoplasmic reticulum (ER), ER-derived structures, and vacuoles. Mutants lacking RNS2 activity accumulate RNA intracellularly, and rRNA in these mutants has a longer half-life. Normal RNA turnover seems essential to maintain cell homeostasis because rns2 mutants display constitutive autophagy. We propose that RNS2 is part of a process that degrades rRNA to recycle its components. This process appears to be conserved in all eukaryotes.

ribophagy

Ribonucleases (RNases) belonging to the RNase T2 family are acidic endonucleases without base specificity that are either extracellular or targeted to the secretory pathway (1). This family is conserved in the genome of almost all eukaryotic organisms so far analyzed, suggesting that it performs an important function that has been maintained throughout evolution (2, 3). Phylogenetic analyses have identified three subclasses present in plant genomes (3, 4). Class I proteins are highly diversified and show evidence of gene sorting, and their expression is commonly tissue-specific and/or regulated by biotic and abiotic stress (3). Class III includes mostly members of the S-RNases, enzymes involved in the process of gametophytic self-incompatibility in three plant families (5). Finally, class II includes proteins that are highly conserved in all plant genomes and are normally expressed at high levels in most plant tissues (3). On the basis of their conservation and gene expression characteristics, we proposed that class II RNases may have a housekeeping role in plant cells (3). Similar gene expression and phylogenetic studies in animals led us to the hypothesis that metazoan RNase T2 enzymes also have a housekeeping role and may be equivalent to class II enzymes from plants (2).

RNS2, one of five RNase T2 genes in the Arabidopsis genome, encodes the only class II protein in this model organism (3, 4). This RNase is present in all tissues at high levels (6, 7) and is localized in an intracellular compartment. RNS2 expression is increased even further during senescence and during inorganic phosphate (Pi) starvation (6, 8). Thus, it was hypothesized that RNS2 is part of a phosphate scavenging system that rescues plants that are under nutritional stress (9). However, because RNS2 and other class II RNase T2 proteins accumulate to high levels even under optimal growth conditions, this rescue function is unlikely to be the main role of these enzymes. Moreover, in vertebrates, in which RNase T2 enzymes are absolutely conserved (2), the mechanisms that control the response to phosphate starvation seem to be specific to the intestine and kidney (10), whereas RNase T2 genes are expressed constitutively in all tissues (2, 3, 11). Therefore, the biological function that has led to the conservation of these enzymes in all eukaryotic organisms is still unknown.

Here we show that RNS2 is localized to the endoplasmic reticulum (ER) or ER-derived compartments and to the vacuole in Arabidopsis cells. Although a large fraction of the protein is present in vacuoles, the enzyme has a neutral pH optimum, suggesting that it may also function in the ER or another neutral pH compartment. We found that plants lacking RNS2 activity accumulate RNA intracellularly, most likely in the vacuole. Ribosomal RNA is degraded at a slower rate in mutant than in wild-type (WT) plants; thus, RNS2 is necessary for normal RNA decay. In turn, deficient rRNA decay results in constitutive autophagy in mutant plants. Our results indicate that rRNA turnover is carried out by RNS2 in vacuoles or ER-derived compartments and that this process is necessary for normal cell homeostasis. A similar finding for an RNase T2 enzyme from zebrafish (12) suggests that this mechanism for rRNA recycling is conserved in all eukaryotes.

Results

RNS2 Localizes to ER and Vacuoles. Previous work had shown that RNS2 is an intracellular protein, and the presence of a C-terminal extension suggested either a vacuolar or ER localization (7). To determine more definitively the localization of RNS2, we fused a cyan fluorescent protein (CFP) to the RNS2 polypeptide. RNS2 has an N-terminal secretion signal that targets the protein to the secretory pathway, in addition to the putative C-terminal extension, which could be an ER retention or vacuolar targeting signal. To avoid disrupting any potential localization signals, the CFP peptide was fused in frame after the N-terminal secretion signal (Fig. S14). This construct was expressed in transgenic plants under the control of a strong constitutive promoter. The fusion protein is expressed well in plants and maintains RNS2 activity (Fig. S1B). Expression of CFP-RNS2 is also accompanied by an increase in RNS2 activity, suggesting that the CFP tag is removed from some of the proteins. Several new RNAse bands appeared in the profile of plants expressing CFP-RNS2. These bands could be the result of different levels of glycosylation, a posttranslational modification common in RNase T2 enzymes. To test this hypothesis, plant extracts were treated with N-glycosydase F and then analyzed in the in gel assay (Fig. S2). We found that RNS2 is glycosylated and that the multiple bands in the CFP-RNS2 size range correspond to different glycosylation states of the protein.

Confocal microscopy showed that the CFP-RNS2 fusion protein localizes to ER structures in leaf protoplasts, as determined...
by its colocalization with the ER marker YFP-HDEL, which contains a C-terminal HDEL tetrapeptide that promotes retrieval to the ER (13) (Fig. 1 and Fig. S3). Diffuse CFP-RNS2 signal is also observed in the protoplast vacuole. Confocal imaging of roots also revealed CFP-RNS2 fluorescence in ER bodies and vacuoles (Fig. S4). To confirm the microscopy results, we separated ER body-rich fractions (P1 and P8) and an ER network-rich fraction (P100) by subcellular fractionation (14) and analyzed the fractions using RNase activity in gel assays (Fig. 2A). We confirmed that RNS2 is present both in a fraction corresponding to small ER bodies (P8) and in the ER network (P100). However, a large fraction of RNS2 activity was found in the S100 that contains soluble cytoplasmic and vacuolar proteins. To confirm vacuolar localization, vacuoles were purified from leaf protoplasts (Fig. 2E) and then assayed for RNS2 activity (Fig. 2C). RNS2 activity was enriched in the vacuolar fraction, although not as much as the vacuolar marker acid phosphatase (Fig. 2F). Our results also confirm previous results by Carter et al. (15), who identified RNS2 as a vacuolar protein in a survey of vacuolar proteins through a proteomics approach.

**RNS2 Is Not an Acidic RNase.** The presence of RNS2 in ER, ER-derived bodies, and vacuoles could mean that the enzyme functions in more than one cellular location. Alternatively, RNS2 could function only in the vacuole, and the high level of CFPRNS2 signal in the ER network could be the result of overexpression of the fusion protein. Also, ER bodies are considered an alternate pathway for protein transport to the vacuole (16). The environment in the vacuole is different from that found in the ER. Although the vacuolar pH is acidic, the ER interior is maintained at a neutral pH (17, 18). Thus, determination of the pH preference of RNS2 activity may indicate whether its biological function is carried out in the ER or in the vacuole.

RNS2 was expressed in yeast cells using a system that facilitates collection of the protein and limits its potential toxic effect (19). Two class I RNase T2 genes from Arabidopsis, RNS1 and RNS3, were also expressed for comparison (Fig. 3A). The activities of the three RNases were tested over a range of pHs (Fig. 3B). We found that RNS1 and RNS3 have an acidic pH preference, as is common for most RNase T2 enzymes (1). In contrast, RNS2 has a pH preference near 7.5 and the activity at acidic pH is only a fraction of that at neutral pH. This finding suggests that RNS2 is more suited to carry out a biological function in the ER or ER-derived compartments than in the vacuole, although the high level of activity found in the vacuole suggests that RNS2 may function in both compartments. To confirm these results using native RNS2, plant extracts were analyzed using the RNase activity in gel assay at different pHs. Again, we found that RNS2 activity is higher at neutral pH (Fig. 3C), although the pH range of native RNS2 seems broader than that observed using yeast-expressed RNS2. On the other hand, RNS1 activity is detected only at acidic pH.

**Mutants Lacking RNS2 Activity Have Longer-Lived rRNA, and RNA Accumulates in Vacuoles.** A proposed function for RNS2 and other class II RNase T2 enzymes in plants is the scavenging of phosphate from bulk RNA during starvation. We hypothesized that the constitutive presence of this enzyme at high levels could indicate that RNS2 is involved in RNA recycling as part of normal cell homeostasis. Because the largest fraction of RNA in a cell is rRNA, we suspected that rRNA is a substrate for RNS2 activity.

We obtained an Arabidopsis line carrying a T-DNA insertion in the fifth intron of the RNS2 gene, truncating the encoded RNS2 protein before the second conserved active site motif (Fig. 4A and Fig. S5). Protein extracts from homozygous T-DNA insertion individuals were analyzed using RNase activity in gel assays (Fig. 4B). These mutants lack the main RNase activity found in Arabidopsis extracts. This RNase activity is present at high levels in all tissues of WT plants, and it is induced by Pi starvation and senescence, as described for the RNS2 transcript (7, 20). Thus, we identified this band as RNS2 and confirmed
In yeast, mutation, both
fi
0.05) be-
AS-RNS2
gene and the T-DNA
mutant was identi-
mutants show constitutive
15.2 h). The same decay analysis was
±
mutant plants. A strong RNase ac-
line does not fully phenocopy the
expression. As previously reported,
mutants (61.8
±
mutant roots showed a clear increase in
RNS2
mutant plants decays at a signi-
4.4 h for WT and
plants. Autophagy is not commonly
plants did not show any obvious morphological
this mutant
rns2-2

Isolation of
AS-RNS2
construct (Fig. S7
B)

Fig. 3. Characterization of the pH preference of RNS2. (A) Recombinant
Arabidopsis RNases RNS1 (lane 1), RNS2 (lane 2), and RNS3 (lane 3) were
obtained from a yeast expression system and analyzed by an RNase activity in
gel assay. Each lane contains 1 μg of protein. (B) RNase activity of yeast-
expressed RNS1 (●), RNS2 (▲ and ■), and RNS3 (▲) was assayed in vitro at
different pHs as indicated. The activity of each enzyme relative to the maxi-
imum activity (at optimal pH) is reported. RNS2 activity (solid lines) near the pH
optimum was assayed using two different buffers, Tris (●) or phosphate (■),
to discard any buffer artifact. (C) pH preference of native RNS2. Leaf protein
extracts (10 μg) from WT and rns2-2 mutant plants were analyzed by an RNase
activity in gel assay, with incubations at different pHs as indicated (below the
panels). The position of RNS2 is indicated. At pH 6, it is possible to detect
activity of RNS1 as an RNase band below the RNS2 activity.
that the T-DNA insertion produced a null mutation. We named
this mutant
ms2-2.

The
ms2-2
plants did not show any obvious morphological
phenotype, nor did they have any reproductive deficiency. We
used an RNA-specific dye, SYTO-RNASElect, to test for changes in RNA accumulation in these plants. Comparison of
WT and
ms2-2
mutant roots showed a clear increase in fluo-
rescence in mutant cells (Fig. 5
A–D). Quantification of the
fluorescence signal from WT and mutant roots showed a roughly
10-fold increase in staining in mutant roots (Fig. 5E). This
differential RNA accumulation seems to occur mainly in vacuoles,
although in some cells the fluorescence is more evident around
the contour of the vacuole, probably in the cytoplasm. A similar
comparison was carried out using leaf protoplasts. About 10% of
mutant protoplasts showed a large fluorescent body in the vac-
ucle (Fig. 5
F–G); this body was never found in WT protoplasts.

We hypothesized that the substrate for RNS2 is rRNA. Lack
of RNS2 would result in a longer half-life of rRNA. Because
rRNA is long-lived, it is not possible to analyze its decay with the
use of transcriptional inhibitors. Thus, we designed an in vivo
labeling experiment to follow rRNA decay in mutant and WT
plants. For this, 1-wk-old plants grown in liquid medium were
incubated with [3H]-uridine for 30 min and then transferred to
cold medium. Samples were taken 24, 48, and 96 h later, and the
ratio of radioactive/total 28S rRNA was calculated. We found
that 28S rRNA in the
ms2-2
mutant plants decays at a signifi-
cantly slower rate than in WT plants (Fig. 6A). We calculated the
28S rRNA half-life following standard methods described for
mRNA (21) and found a significant difference (P < 0.05) be-
between the 28S rRNA half-life in WT plants (38.0 ± 4.2 h) and in
ms2-2
mutants (61.8 ± 15.2 h). The same decay analysis was

Lack of RNS2 Activity Causes Constitutive Autophagy. In yeast,
ribosomes are selectively targeted for degradation by an
autophagy-like process (termed ribophagy) in response to nu-
tritional stress (22). RNS2 could be involved in a similar process
in plant cells. Thus, we analyzed the autophagy process in
WT,
ms2-2,
and
AS-RNS2
plants. Autophagy is not commonly
detected in WT cells of plants growing under normal nutrient
conditions. However, when plants are subjected to nutritional
stress, such as sucrose starvation, autophagosomes can be easily
detected (Fig. 7 and Fig. S7). In contrast,
ms2-2
and
AS-RNS2
plants showed constitutive autophagy even under normal growth
conditions, but the level of autophagy in these lines was not
different from WT under extended sucrose starvation (Fig. 7 and
Fig. S7). The number of autophagosomes per cell under normal
growth conditions was almost 10-fold higher in
ms2-2
than in
WT
plants.

Discussion
We found that RNS2 is essential for normal degradation of
ribosomes, as mutants lacking RNS2 activity have rRNA with
an extended half-life, which accumulates intracellularly, probably
in the vacuole. We found that
ms2
mutants show constitutive
autophagy, indicating that recycling of ribosomes is essential for
maintaining cellular homeostasis. We propose that RNS2 is part
of a housekeeping mechanism that recycles ribosomes through-
out the life of a cell. This mechanism could be similar to
ribophagy, the recycling of ribosomes observed under nutrient
deficiency in yeast (22).

The mechanisms of ribosome degradation in eukaryotes are
only now being investigated. Several reports have examined the
fate of aberrant ribosomes with
cis-acting mutations, in which
rRNA sequences are mutated, or with
trans-acting mutations, in
which mutant proteins are absent from or fail to interact with the
rRNA (23). These studies focus on the surveillance mechanisms
involved in dysfunctional rRNA decay (NRD) and indicate that
small and large ribosomal subunits are processed by independent
decay pathways. Small ribosomal subunit NRD in yeast shares

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some components with the no-go mRNA decay pathway that targets mRNA in stalled ribosomes for degradation (24). 18S NRD involves the action of the exosome, and substrates for this process accumulate in P-bodies (24), conserved cytoplasmic granules that contain untranslabeled mRNA, a set of translational repressors, the mRNA decapping machinery, and the 5′-3′ exonuclease XRN1 (25). 25S NRD seems to use a different mechanism in which ubiquitylation is important (24, 26). Large subunit NRD substrates accumulate in the cytoplasm in a perinuclear compartment. Additionally, a quality control mechanism surveys the proper assembly of ribosomes in the nucleolus, and misfolded ribosomes are targeted for degradation by the TRAMP-exosome pathway (27, 28). All of these mechanisms target aberrant, short-lived RNA, such as NRD.

Some clues regarding the degradation of ribosomes have come from studies of cells undergoing nutritional stress. When cells are starved, portions of the cytosol, including entire organelles, are recycled to obtain essential material for cell survival. The best-characterized process involved in this recycling is macroautophagy. Macroautophagy is nonselective and entails the formation of double-membrane vesicles in the cytoplasm called autophagosomes (29). In yeast, a unique type of selective autophagy occurs in parallel with nonselective autophagy. Yeast cells under nitrogen starvation specifically target ribosomes for degradation by ribophagy, which requires the action of Ubp3, a ubiquitin protease, and its cofactor Bre5 (22). Ribophagy also depends on some components of the basal, nonselective autophagy machinery. We hypothesize that RNS2 is an essential component of ribophagy, or a similar mechanism, and that this mechanism not only is active during nutritional stress, but also has a housekeeping role. This housekeeping role, missing in the absence of RNS2, is the degradation of effete ribosomes, probably to recycle essential nutrients such as nucleotides. Thus, we propose that the absence of RNS2 activity produces a nutrient imbalance in the cell that triggers constitutive production of autophagosomes in an attempt to restore the housekeeping function of the ribophagy-like process.

Our results suggest that RNS2 participates in a ribophagy-like mechanism rather than one of the mechanisms proposed for degradation of aberrant, short-lived RNA, such as NRD. Mutants lacking RNS2 activity accumulate rRNA mostly in the vacuole. This observation is consistent with a ribophagy-like

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**Fig. 5.** Differential staining of mutant and WT cells using the RNA-specific dye SYTO RNASelect. Seven-day-old seedlings were transferred to liquid MS medium, stained with 5 μM SYTO RNASelect green, and observed using confocal microscopy. Green fluorescence (Left panels in A and B) and bright-field images (Right panels in A and B) were collected for WT (A) and rns2-2 (B) roots. A magnified image of the Inset boxed regions is shown in C and D. In all cases, the root tip lies on the right side of the image. (Scale bars, 75 μm.) Metamorph software was used to measure the mean fluorescence of six lateral lines across the elongation zone in the WT and rns2-2 roots stained with SYTO RNASelect (E). Leaf protoplasts were also stained with 5 μM SYTO RNASelect green and observed using confocal microscopy. Fluorescence images were collected for WT (F) and rns2-2 (G) protoplasts. Chlorophyll fluorescence (Center) are shown. Merged images (Right) show chlorophyll in red and SYTO RNASelect in green. (Scale bar, 10 μm.)

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**Fig. 6.** Decay of rRNA in wild-type plants and in lines with altered levels of RNS2 expression. One-week-old seedlings grown in liquid medium were incubated with [3H]-uridine for 30 min and then transferred to cold medium. At the indicated time points samples were extracted and total RNA was isolated. To determine the fraction of [3H]-RNA versus total RNA for the 28S rRNA, the samples were analyzed by agarose gel electrophoresis and transferred to nitrocellulose. Total 28S RNA was determined by EB staining, then bands were excised and the tritium signal was quantified with a scintillation counter. (A) Comparison of the decay of 28S rRNA in WT and the rns2-2 mutant. (B) 28S rRNA decay in WT and in a RNS2 antisense line (AS-RNS2). Asterisks indicate statistically significant differences with respect to WT (P < 0.05).

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**Fig. 7.** Loss of RNS2 activity leads to constitutive autophagy. One-week-old WT, rns2-2, and AS-RNS2 seedlings were transferred to fresh solid MS medium with or without sucrose in the dark for 4 d, followed by staining with MDC. The number of autophagosomes per cell was quantified. Asterisks indicate statistically significant differences (P < 0.05) with respect to WT.
process because ribophagosomes fuse with the vacuole; in contrast, NRD is associated with P-bodies or is cytoplasmic, depending on the rRNA subunit. A ribophagy-like role is also consistent with the observed localization of the RNS2 enzyme in the ER or ER-derived structures and the vacuole. However, the existence of ribophagy in plants has not been shown, and this hypothesis needs to be tested further.

A nutritional role for RNase T2 enzymes has been proposed previously. Expression of class II RNAs, including RNS2, is normally high in most tissues (3, 8, 30, 31); however, these class II RNAs are induced by senescence and phosphate starvation in plants (6, 30). It was proposed that these enzymes, along with several class I RNase T2 enzymes that are also induced by starvation, are part of a phosphate-scavenging system that rescues plants under nutritional stress (9). It is possible that a ribophagy-like mechanism also participates in this scavenging system.

Another argument in support of a housekeeping role for RNS2 and other class II RNases is the high degree of conservation and expression patterns of these enzymes among all higher eukaryotes (2, 3). This conservation suggests that the proposed rRNA decay mechanism in plants may also be conserved in other eukaryotes. This seems to be the case. Most vertebrates have only one RNase T2 gene in their genomes, but zebrafish and other bony fishes have two (2). One of the two copies (RNase T2a) is a fish-specific clade, whereas the other (RNase T2b) belongs to the same clade that is conserved in all vertebrates, and in zebrafish is expressed constitutively in all tissues (2). Haud et al. (12) show that zebrafish mutants lacking RNase T2b accumulate rRNA in the lysosome. They also propose that RNase T2a (or RNASET2, as it is called in humans) is part of the ribophagy machinery. Lack of RNASET2 results in engorged lysosomes that are particularly apparent within neurons of the brain, leading to white matter lesions. These lesions resemble neuropathologies that are associated with mutations in the RNASET2 gene in human infants (32).

Recently, another function was proposed for RNS2 T2 enzymes, in particular Rny1, the only RNase T2 enzyme present in Saccharomyces cerevisiae. Yeast cells under oxidative stress accumulate cleaved tRNAs (33). Rny1 is responsible for this cleavage after release of the enzyme from the vacuole into the cytoplasm (34). Rny1 induces cell death in yeast in a process that is independent of its catalytic activity (34). Cleavage of tRNA is a conserved response to oxidative stress: accumulation of cleaved tRNAs was also observed in human and Arabidopsis (33).

These results suggest that RNS2 could function in this process; thus, it is possible that the conservation of class II RNases in all eukaryotic organisms is related to this stress response. However, in humans the tRNA cleavage function is carried out by angiotensin-converting enzyme 2 (ACE2), which is also expressed in the brain, suggesting that this function may be important in the central nervous system (35). In plants, Rny1 has been shown to be involved in the degradation of damaged tRNAs (36). However, it is not yet clear how this function is related to the ribophagy machinery in plants. It is possible that Rny1 and other RNase T2 enzymes participate in a conserved mechanism that recycles rRNA and could have ribophagy-like functions in other eukaryotes.

Materials and Methods

Plant Material. Arabidopsis thaliana ecotype Columbia-0 was used for all of the experiments. The rns2-2 mutant (SALK_096588) was obtained from the Arabidopsis Biological Resource Center. The antisense RNS2 line (79c6) was described before (7). Soil-grown plants were kept in chambers with 16 h light at 60% humidity and 21 °C. For seedling experiments, seeds were surface-sterilized and germinated on Arabidopsis growth medium as described (6).

Standard cloning techniques were used to build the CFP-RNS2 construct. The RNS2 cDNA (6) was obtained from Pamela Green (University of Delaware, Newark, DE). The CaMV 35S promoter region was amplified from pCambia 2301 as a PCR fragment (37). The cauliflower mosaic virus 35S promoter was obtained from the pGDS vector (38). After assembly, the 35S-CFP-RNS2 construct was inserted into the binary vector pCAMBIA 2301. The plasmid spyTPFP-HDEL (13) was provided by Christa Hawes (Oxford Brookes University, Oxford). Plants were transformed according to Bariola et al. (7). Mesophyll protoplasts were prepared from leaves using a modified version of the method described by Yoo et al. (39). See SI Materials and Methods for details.

RNA and Protein Preparation and Subcellular Fractionation. RNA was prepared as previously described (40). Protein extracts were prepared as previously described (41), but with the addition of 2-mercaptoethanol. ER-enriched fractions were obtained following the method of Matsuhashima et al. (14). Vacuoles were purified from protoplasts according to Robert et al. (42). Quality of purified vacuoles was determined by Neutral Red staining and visualization with a bright-field microscope (43). Vacuole enrichment was measured by assaying the vacuolar marker enzyme acid phosphatase as described (43).

Ribo nuclease Activity Assays. RNase activity in gel assays were performed as described by Yen and Green (44), using high-molecular-weight purified torula yeast RNA (Sigma). In vitro RNAase activity was assayed as described by McClure et al. (45), using the same substrate. For pH optima assays, acetate, phosphate, or Tris buffers at the indicated pH were used in place of the standard reaction buffers.

Analysis of RNA Decay. Approximately 200 Arabidopsis seeds were surface-sterilized, cold treated, and grown in 125-mL sterile flasks with 20 mL of Murashige and Skoog (MS) media (Phytotechnology) and 1% sucrose on an orbital shaker at 150 rpm at 22 °C under 16 h light. After 7 d, 0.5 mCi of [5-33P]uridine (1 mCi/mL, 15–30 Ci/mmol; Moravek Biochemicals) were added to the medium. The flasks were subjected to vacuum for 1 min and shaken at 80 rpm for 30 min. The seedlings were then washed with sterile MS medium and resuspended in 20 mL of germination medium. Samples were taken 24, 48, and 96 h later. RNA was prepared using the Qiagen RNeasy Plant Mini Kit. Five micrograms of total RNA was separated by electrophoresis in the presence of 6% polyacrylamide and 8 M urea in the gel and blotted onto nylon membranes as previously described (46). Total 28S rRNA amount was determined by scanning the membrane on a Typhoon scanner using the fluorescence setting and adjusting the photomultiplier tube (PMT) setting to 550 to eliminate signal saturation. ImageQuant 5.2 software was used for quantification. Triticum labeling was measured by cutting out the fragment of nylon membrane corresponding to each ribosomal band. Background controls for each lane were used by cutting out a similar-sized membrane piece above the ribosomal bands. Membranes were analyzed on a liquid scintillation counter. The fraction (tritium signal/EtBr signal) of radioactive RNA remaining at each time was calculated. Half-life was calculated as described (21).

Autophagy Induction and Monodansylcadaverine Staining. Autophagy was induced by sucrose starvation and monitored by monodansylcadaverine (MDC) staining as described (47). Seedling samples were harvested 4 d after transfer. The cells in the elongation zone were observed near the central focal plane of the root. The number of motile MDC-labeled autophagosomes in all cells visible in the focal plane was determined. This number was divided by the total number of cells visible in the focal plane and designated the number of autophagosomes per cell for that region. The data from all regions in a sample were averaged together to obtain a mean number of autophagosomes per cell. At least 150 cells were observed per sample.

Specific RNA Staining Using SYTO RNASelect. Wild-type and rns2-2 seedlings were germinated and grown for 7 d on solid medium. Whole seedlings were transferred to liquid MS medium and stained with 5 μM SYTO RNASelect.
green (in nitrogen) for 3 h at room temperature. After staining, roots were washed three times with fresh MS and observed using confocal microscopy. Leaf protoxylem protoplasts were transferred to MS medium and stained with 5 μM SYTO RNASelect green for 12 h. After staining, cells were washed three times with fresh MS and observed using confocal microscopy (microscopy details are in SI Materials and Methods).

**Statistical Analysis and Experiment Repetitions.** All experiments were repeated at least three times. For gels, a representative sample is shown. Numerical data were analyzed with t-test (for samples with an equal number of repetitions) or z-test (for samples with an unequal number of repetitions), and means were considered different when P < 0.05. Error bars in figures indicate SE.

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