Supporting information

1. **Supplementary Figures and Supplementary Methods** (this file)
2. **Four datasets submitted as separate files:**

Dataset S1. A list of NAD-RNAs identified in the total RNA tagging experiment
Dataset S2. A list of NAD-RNAs identified in the poly(A) RNA tagging experiment with enrichment of tagged RNAs
Dataset S3. A list of 2,000 highly abundant NAD-RNAs
Dataset S4. A list of NAD-RNAs identified in the poly(A) RNA tagging experiment without enrichment of tagged RNAs

Supplementary Figures

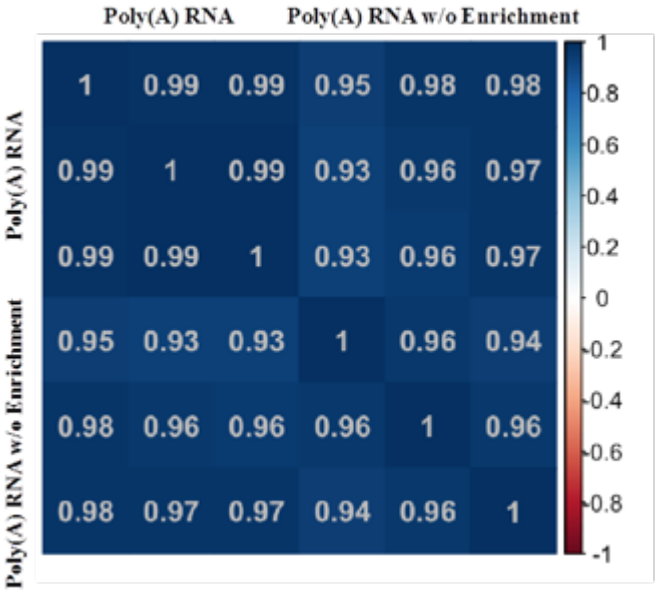

Fig S1. Correlation of the abundances of NAD-RNAs determined by the poly(A) RNA tagging experiments with or without enrichment of tagged RNAs
Pairwise Pearson correlations are shown for each replicate in the experiments. The darker the color, the higher the correlation between the paired samples.

Gene Name	Fold Enrichment	P-value	FDR
response to cytokinin	2.00E-47	5.91	2.15E-44
ribosome biogenesis	1.17E-45	6.6	6.31E-43
translation	7.16E-38	2.42	2.57E-35
photosynthesis	7.38E-34	5.75	1.98E-31
response to cadmium ion	3.06E-30	3.55	6.58E-28
cytoplasmic translation	5.65E-26	8.32	1.01E-23
response to cold	1.23E-23	3.38	1.89E-21
photosynthesis, light harvesting in photosystem I	3.22E-18	10.93	4.32E-16
photorespiration	1.17E-15	6.49	1.46E-13
response to oxidative stress	9.65E-15	2.81	1.04E-12
response to salt stress	3.31E-14	2.31	3.23E-12
cellular response to oxidative stress	2.25E-13	5.26	2.02E-11
cell redox homeostasis	3.50E-12	3.33	2.89E-10
nucleosome assembly	3.81E-12	5.12	2.93E-10
reductive pentose-phosphate cycle	1.71E-11	9.02	1.23E-09
oxidation-reduction process	1.77E-11	1.64	1.19E-09
sulfate assimilation	2.20E-11	5.42	1.39E-09
defense response to bacterium	9.87E-11	2.53	5.89E-09

Fig S2. Gene Ontology (GO) enrichment analysis for top 2,000 NAD-RNA-producing genes

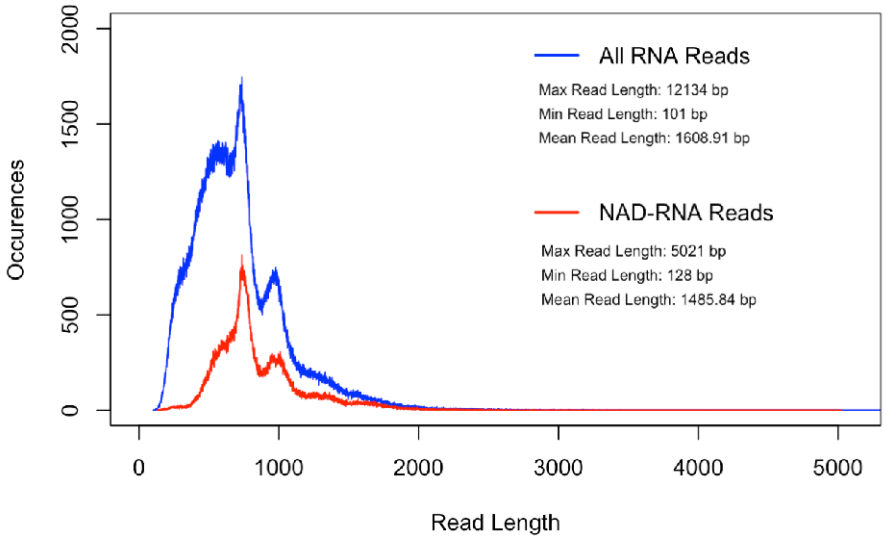

Fig S3. Read length distribution of NAD-RNAs and all RNAs detected in the poly(A) RNA tagging experiment

Blue line: all mapped RNA reads; red line: all mapped NAD-RNA reads. For a clearer view of the distribution, we only show the distribution of reads under 5,000 bp.

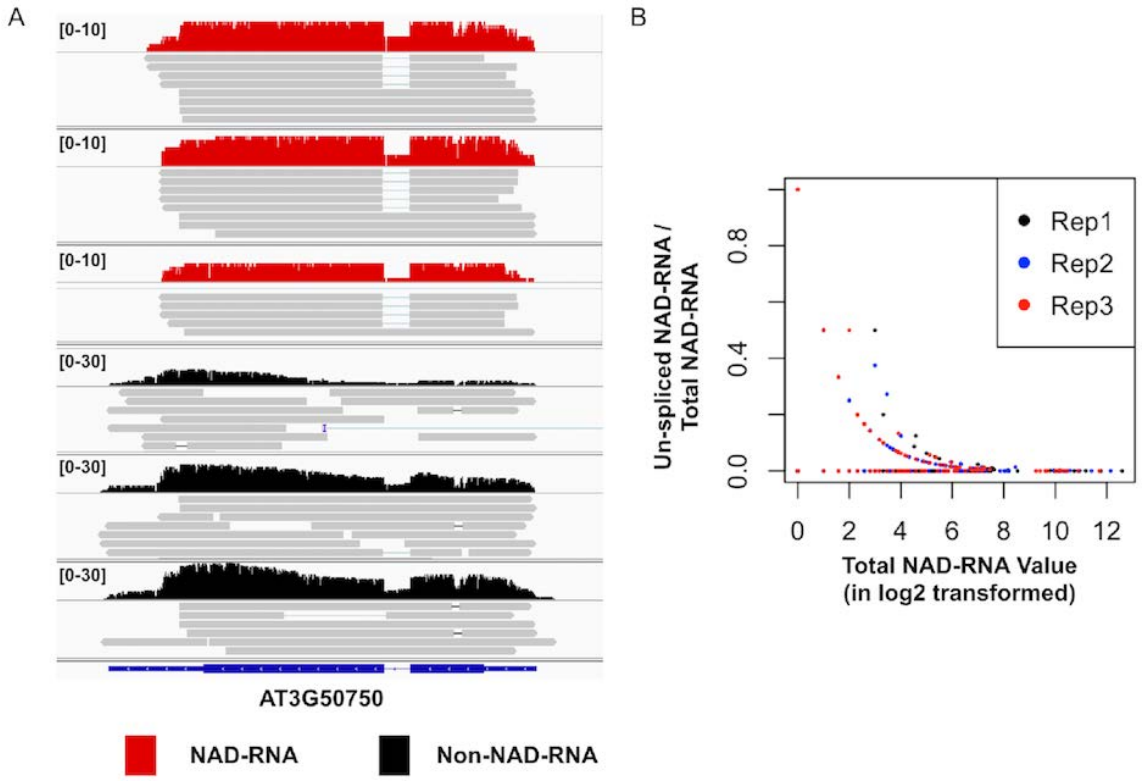

Fig S4. Some NAD-RNAs were unspliced
(A) An example of pre-mRNA transcripts detected in the NAD-RNA reads (red) and the non-NAD-RNA reads (black) produced from *AT3G50750* in three replicates. Overall, approximately one third of the transcripts were unspliced. The transcript model and protein model are shown at the bottom. (B) The ratios of unspliced NAD-RNAs reads to total NAD-RNA reads from corresponding genes detected in the poly(A) RNA tagging experiment.

Supplementary Methods

Detection of the NAD^+ moiety in NAD-RNAs by mass spectrometry. Total RNAs were digested by nuclease P1 in a 125 µL reaction containing 50 mM NaOAc (pH 5.4), 2 mM ZnSO4, 125 µg RNA, and 2.5 U P1 nuclease (Sigma) at 37°C for 2 hours. The control reaction used heat-inactivated P1 nuclease (100°C for 20 min). The retention time of the standard NAD^+ (Sigma) was determined by resolving NAD^+, 5'AMP, 5'CMP, 5'UMP, and 5'GMP, respectively, on a Waters HPLC system equipped with a 259 nm wavelength UV fluorometer and a fraction collector using a YMC C18 column (250 mm, 4.6 mm). Fractions in the nuclease P1-digested sample corresponding to the retention time of NAD^+ were collected and dried using a vacuum concentrator. The fraction was re-dissolved and applied to an Ultimate 3000 UHPLC (Thermo Fisher Scientific) LC-MS system coupled with a Q-Exactive ESI-Q-Orbitrap mass spectrometer (Thermo Fisher Scientific) in positive mode. The Thermo Xcalibur program was used to extract the area under the detected ion abundance peaks and to compare them with the product ions of the

standard compounds. NAD^+ product ions include 136.0614, 232.0821, 348.0694, 428.0355, and 524.0563.

HPLC setting for fractionation of P1-digested products. Mobile phase A was 0.37% acetic acid, and mobile phase B was 20% methanol. The flow rate was a constant 0.8 mL/min, and the mobile phase composition was set as follows: 100% A for 2 min; linear increase over 40 min to 100%, which was held for 5 min before returning linearly to 100% A.

Chromatographic separation. An Aquity UPLC BEH C18 column (1.7 μm particle size, 2.1 mm i.d., 100 mm length, Waters) was used. For each sample, 10 μL of the redissolved solution was injected. Mobile phase A was 0.1% aqueous NH_4OAc, and mobile phase B was 100% methanol. The flow rate was a constant 0.3 mL/min and the mobile phase composition was set as follows: 2% B for 1 min; a linear increase over 3 min to 15% B; a second linear increase over 2 min to 100% B, which was held for 1 min before returning linearly to 2% B over 0.5 min.

Mass spectrometry analysis. Electrospray ionization (ESI) was used with a spray voltage of 3.5 kV. The auxiliary gas flow rate was 10 arbitrary units and the sheath gas flow rate was 35 psi. The capillary temperature was 350°C. The mass resolution was set at 70,000. The scan range was m/z 75–800. The S-lens RF level was 55. The collision voltage was set as 30.0 V for MS/MS analysis.

***In vitro* transcription for NAD-RNA synthesis.** The T7 RNA polymerase used in in vitro transcription was 6x histidine-tagged protein expressed in *E. coli* and purified by us using Ni-NTA metal chelate chromatography. Transcription was performed in a 400 μL reaction containing 40 mM Tris-HCl (pH 8.0), 1 mM spermidine, 22 mM $MgCl_2$, 0.01% Triton-X-100, 5 mM DTT, 5% DMSO, 4 mM NAD^+, 1 mM GTP, 1 mM CTP, 1 mM UTP, 1 μM dsDNA oligo, 400 U Murine RNase inhibitor (NEB), and 10 μg T7 RNA polymerase at 37°C for 4 hours.

ADPRC catalyzed reaction. This reaction was conducted following the method used for NAD captureSeq (1). Briefly, the basic reaction contained RNA in 100 μL reaction containing 50 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 10 μL 4-pentyn-1-ol (Sigma), 100 U murine RNase inhibitor (NEB), and 0.85 μM ADP-ribosyl cyclase (Sigma). The reaction was carried out at 37°C for 30 min. Three different input RNAs were used in the different experiments; 450 ng NAD-38 nt RNA, 100 μg total RNA, or 40 μg poly(A) RNA mixed with 50 μg yeast tRNA (Invitrogen) as carrier molecules. The reaction without ADP-ribosyl cyclase was used as control**.** The ADPRC reaction was stopped by adding solutions supplied with the RNA clean kit (Zymo Research) and the RNAs were then purified with columns supplied with the kit following the manufacturer's instructions.

Removal of free tagRNAs after tagging with RNA samples. Unreacted azide-RNAs were separated from poly(A) RNAs using 1.5 mg Oligo (dT)25 magnetic beads (NEB) according to the manufacturer's instructions. The beads were first equilibrated with 400 μL equilibration buffer. The beads were then precipitated with a magnetic separation rack, and the supernatant was removed. The volume of purified RNA was adjusted to 150 μL

before an equal volume of 2x binding buffer was added to the beads. The mixture was incubated at room temperature with gentle vortexing for 10 min to allow poly(A)-containing RNA to hybridize with the oligo (dT)25. The beads were precipitated with a magnetic separation rack, and the supernatant was retained. The beads were washed twice with 400 μL wash buffer and once with 400 μL low salt buffer. Captured poly(A) RNAs were eluted by incubating the beads with 200 μL elution buffer at 50 °C for 2 min, with gentle vortexing. After elution, the beads were reused to capture poly(A) RNAs that remained in the supernatant. The second round of isolation was conducted as described above. The eluted poly(A) RNAs were pooled and purified using RNA clean kit (Zymo Research). Detailed recipes for the buffers are as follows: equilibration buffer (20 mM Tris-HCl, 500 mM LiCl, 1 mM EDTA, pH 7.5), 2x binding buffer (100 mM Tris-HCl, 1 M LiCl, 2 mM EDTA, pH 7.5), wash buffer (50 mM Tris-HCl, 500 mM LiCl, 1 mM EDTA, pH 7.5), low salt buffer (50 mM Tris-HCl, 150 mM LiCl, 1 mM EDTA, pH 7.5) and elution buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Enrichment of tagRNA-linked NAD-RNA. The DNA-biotin molecule for capturing tagRNAs by hybridization (see below for its sequence) was immobilized on streptavidin magnetic beads (NEB; 500 pmol DNA/600 μg beads) following the manufacturer's instructions. The beads were equilibrated three times with 300μL equilibration buffer. tagRNA-linked RNAs were captured by hybridization with the probe DNA in 150 μL 2x binding buffer. The mixture was first incubated at 65°C for 2 min. Then the temperature was gradually decreased to 30°C, with gentle vortexing. The beads were washed twice with 300 μL of wash buffer and once with 300 μL of low salt buffer. The hybridized RNAs were eluted by incubating the beads in 150 μL elution buffer at 80°C for 2 min, with gentle vortexing. The eluted RNAs were purified and concentrated using RNA clean kit (Zymo Research). RNA concentration was determined using the Qubit Fluorometer.

DNA and RNA oligos used in the study

DNA oligo 5'-GATCACTAATACGACTCACTATTACTGTGTCGTCGTCGTCTGCTGTCTCTCTCTCCGCGGGC-3' (Shaded letters denote T7 class II promoter (ϕ2.5) and its complementary sequence 5'-GCCCGCGAGAGAGAGACAGCAGACGACGACGACACAGTAATAGTGAGTCGTATTAGTGATC-3' were used as template for *in vitro* transcription. DNA oligo with a biotin modification at the 3' terminus (5'-TTCAGGTTCAGGTTCAGGTTCAGG-biotin-3') was used for enrichment of tagRNA-linked RNAs. All the DNA oligos were ordered from Beijing Genomic Institute (Shenzhen, China). Azide-modified RNA oligos were ordered from Integrated DNA Technologies. 25 nt tag (5'-GCCAUUGCCAUUGCCAUUGCCAUUG/3AzideN/-3') was used for tagging 38 nt NAD-RNA and tagRNA (5'-CCUGAACCUGAACCUGAACCUGAACCUGAACCUGAACCUG/3AzideN/-3') was used for tagging cellular RNA samples for NAD tagSeq analysis throughout this study.

Differentiation of NAD-RNA reads and non-NAD-RNA reads

Reads with or without the tagRNA sequence were differentiated using a Python script. It was estimated that around 10 nucleotides at the 5' end of the 40 nt tagRNA sequence

were probably uncalled due to the inability of the Nanopore sequencing technology to detect sequences at an end. Several bases near the junction of the tagRNA and the NAD cap also might not have been called. The reads whose 5' regions exactly matched at least 12 consecutive bases of the tagRNA sequence were considered to be NAD-RNAs. Native RNAs with a sequence similar to the tagRNA sequence were filtered.

References

1. Winz ML, *et al.* (2017) Capture and sequencing of NAD-capped RNA sequences with NAD captureSeq. *Nat Protoc* 12(1):122-149.