Supplementary Information

SPAAC-NAD-seq, a sensitive and accurate method to profile NAD^+-capped transcripts

This PDF file includes:

Other supplementary materials for this manuscript include the following:

Datasets S1 to S9

Supplementary Methods

CuAAC-NAD reactions

CuAAC-NAD reactions were conducted in two steps. For the first step, ADPRC transglycosylation, an NAD^+ standard (2.5 mM) was incubated at 37℃ for 30 min with 2 μl 4-Pentyn-1-ol (Sigma-Aldrich), ADPRC (1.4 μM, Sigma-Aldrich), HEPES (50 mM) and $MgCl_2$ (5 mM) in a total volume of 30 μL. After the reaction, 2 μl of the mixture was diluted 200-fold in water for untargeted analysis by LC-MS. For the second step, 5 μl of the remaining mixture further underwent the CuAAC reaction in a 20 μl volume at 25℃ for 30 min with the addition of biotin-PEG3-azide (0.5 mM, Sigma-Aldrich), freshly prepared $CuSO_4$ (5 mM), sodium ascorbate (10 mM), HEPES (50 mM) and $MgCl_2$ (5 mM).

Untargeted analysis by LC-MS

Untargeted analysis by LC-MS was performed using a quadrupole time-of-flight mass spectrometer (Synapt G2-Si) coupled to an I-class UPLC system (Waters). Separations were performed on an HSS T3 Column (1.8 μm, 2.1 mm× 100 mm) (Waters) at a flow rate of 0.25 ml/min. The mobile phases were (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The injection volume was 5 μl. The gradient was as follows: 0 min, 1% B; 6 min, 47.5% B; 10 min, 75% B. Then, the column was washed and equilibrated for 5 min at 100% B before the next injection. The mass

spectrometer was operated in negative ionization mode (50 to 1200 m/z). All gases were nitrogen except for the collision gas, which was argon. Electrospray ionization was conducted with a capillary voltage of 2 kV. Desolvation gas was set to 1100 l/h and cone gas to 150 l/h. Source and desolvation temperatures were 150°C and 600°C, respectively. Data were acquired and analyzed using the Masslynx V4.1 software (Waters).

Targeted analysis by triple quadrupole LC-MS

Targeted analysis was performed on a TQ-XS triple quadrupole mass spectrometer (Waters) coupled to an I-class UPLC system (Waters). Separations were carried out on a ZIC-pHILIC column (2.1 x 150 mm, 5 µM) (EMD Millipore) at the flow rate of 0.2 ml/min. The mobile phases were (A) water with 15 mM ammonium bicarbonate adjusted to pH 9.6 with ammonium hydroxide and (B) acetonitrile. The injection volume was 2 µl. The gradient was as follows: 0 min, 90% B; 1.5 min, 90% B; 16 min, 20% B; 18 min, 1% B; 22 min, 1% B; 23 min, 90% B; 33 min, 90% B. The MS was operated in selected reaction monitoring (SRM) mode with electrospray ionization. All gases were nitrogen except for the collision gas, which was argon. ESI was conducted at a capillary voltage of 1 kV in positive ion mode and 2 kV in negative ion mode. Desolvation gas, cone gas, and collision gas were set to 1100 l/h, 150 l/h, and 0.15 ml/min, respectively. Source and desolvation temperatures were 150°C and 600°C, respectively. Authentic standards were used to define the parameters of retention time and to determine the daughter ions derived from the precursor ions for each target metabolite. The details of parameters are as follows (compound, mode, retention time, precursor ion (m/z), daughter ion (m/z) with corresponding collision energy): NAD^+, positive, 12.04 min, 664, 428 (26 V), 136 (39 V); m^7GpppA, positive, 13.14 min, 787, 166 (21), 136 (42); 7-methylguanine, positive, 7.65 min, 165, 149 (10 V), 124 (16 V). Data were acquired and analyzed using the Masslynx V4.1 software (Waters). Relative abundance was determined by peak area for each target compound.

Preparation of in vitro transcribed RNAs

The template for in vitro transcription was generated by annealing complementary oligonucleotides (20 µM) (primer sequences in *SI Appendix*, Table S1) in 0.2 M Tris-HCl (pH 8), 1 M NaCl and 0.1 M EDTA (pH 8) at 95°C for 2 min, followed by cooling down to room temperature for 45 min, such that the resulting template contained the T7

class II promoter (ϕ2.5) and an adenosine at the transcription start site. In vitro transcription was performed in a 100 µl reaction containing 1 µg of the above DNA template, 100 units T7 RNA polymerase (New England Biolabs, catalog# M0251L), 1× T7 polymerase buffer, 100 units RiboLock RNase Inhibitor (Thermo fisher), 1 mM CTP, GTP and UTP and 1 mM of cap substrate at 37℃ for 12 h. Cap substrates (ATP, ADP, NAD^+, m^7GpppA, FAD or NADH) used depended on the desired RNA product. After the reaction, the mixtures were incubated with 1 unit/µg DNase I (Roche) and 1× DNase I buffer (Roche) at 37℃ for 1 h to remove the DNA template, then RNA was extracted using acid phenol/chloroform and finally precipitated with 0.3 M sodium acetate (pH 5.5) and ethanol. Unincorporated nucleotides were removed with a Micro Bio-Spin P-30 Gel Column (Bio-Rad).

Total RNA extraction and poly(A) RNA isolation

Total RNAs were extracted from 12-day-old Col-0 seedlings with the TRIzol reagent (Molecular Research Center) and treated with 1 unit/µg DNase I (Roche) at 37℃ for 1 h. After DNase I treatment, total RNAs were acid phenol/chloroform extracted and ethanol precipitated. Poly(A) RNAs were isolated from total RNAs using Oligo d(T)25 Magnetic Beads (New England Biolabs) following the manufacturer's instructions. RNA concentration was determined by a Nanodrop spectrophotometer.

CuAAC-NAD reactions with RNAs

RNAs were incubated with 10 µl 4-Pentyn-1-ol (Sigma-Aldrich), ADPRC (0.85 µM, Sigma-Aldrich), HEPES (50 mM), $MgCl_2$ (5 mM) and 100 units RiboLock RNase Inhibitor (Thermo fisher) in a 100 µl volume at 37℃ for 30 min. 100 µl diethyl pyrocarbonate (DEPC) H_2O was then added and acid phenol/chloroform extraction was performed to stop the reaction. RNAs were precipitated by ethanol, redissolved in buffer containing HEPES (50 mM) and $MgCl_2$ (5 mM) and then treated with biotin-PEG3-azide (0.5 mM, Sigma-Aldrich), freshly prepared $CuSO_4$ (1 mM), sodium ascorbate (2 mM) and Tris (3-hydroxypropyltriazolylmethyl)amine (THPTA)(0.5 mM) at 25℃ for 30 min. 100 µl H_2O was then added, and acid phenol/chloroform extraction and ethanol precipitation were performed.

Dot blot and gel blot assays

After the CuAAC- or SPAAC-NAD reaction, biotin-labeled nucleic acids were

UV-crosslinked on both the dot blot and gel blot membranes, probed by stabilized Streptavidin-Horseradish Peroxidase (HRP), and subsequently detected with the Chemiluminescent Nucleic Acid Detection Module Kit (Thermo Fisher). The membranes were imaged by ChemiDoc XRS+ camera (BioRad). To visualize the input RNA signal on membranes, RNA gel blot analysis was performed with a probe corresponding to the in vitro transcription template and labeled with [α-^{32}P]-dATP. Signals were detected and quantified using the Typhoon phosphorimaging instrument.

Immuno-depletion of m^{7}G-RNAs

5 μg mRNAs or a mixture of in vitro transcribed NAD- and m^{7}G-RNAs (1 μg: 100 μg; 1:225 molar ratio) were denatured at 65℃ for 5 min and then incubated with 40 μl anti-m^{7}G antibody (RN017M, MBL) and 500 units RiboLock RNase Inhibitor (Thermo fisher) in 1× RIP reaction buffer (10 mM Tris-HCL pH 7.4, 150 mM NaCl) in a 800 μl volume at 4℃ for 8 h under rotation. 500 μl Dynabeads G (Thermo fisher) were washed twice with 1× RIP reaction buffer and blocked with 1 mg BSA (New England Biolabs) at 4℃ for 2 h under rotation. After blocking, Dynabeads G were washed three times, resuspended in 200 μl 1× RIP reaction buffer and incubated with the above 800 μl reaction for 8 h at 4℃ under rotation. Finally, supernatants were kept and RNAs were extracted by acid phenol/chloroform. RNAs were precipitated by ethanol and redissolved in 10 μl DEPC H_2O. RNAs were incubated without the anti-m^{7}G antibody as the mock-depletion control.

Examination of m^{7}G-RNA depletion efficiency

To examine the efficiency of m^{7}G-RNA depletion, anti-m^{7}G dot blot assays and nuclease P1 digestion followed by LC-MS detection were performed.

For anti-m^{7}G dot blot assays, 1 μl of treated RNAs was denatured and spotted on a nitrocellulose membrane (Bio-Rad) followed by UV crosslinking. The membrane was first stained by 0.03% methylene blue to visualize the RNAs and then blocked with 5% non-fat milk in 1× PBS buffer (pH 7.4) containing 0.1% Tween 20 at room temperature for 1 h and incubated overnight at 4℃ in 1% non-fat milk with 0.1% anti-m^{7}G antibody. After three washes with 1× PBS buffer (pH 7.4) containing 0.1% Tween 20, the membrane was subsequently incubated in 1% non-fat milk with 0.02% anti-mouse IgG at room temperature for 1 h. After three more washes and development with the ECL solution kit (GE Healthcare), the membrane was imaged immediately by ChemiDoc

XRS+ camera (BioRad).

For nuclease P1 digestion followed by LC-MS detection, 20 μg treated RNAs was incubated with 5 units nuclease P1 (Sigma-Aldrich) in sodium acetate (50 mM, pH 5.3) and $ZnSO_4$ buffer (2 mM) in a 20 μl reaction volume at 37°C for 2 h. Reaction mixtures were subsequently analyzed by targeted LC-MS. The targeted LC-MS conditions are described in detail in *SI Appendix*, *Supplementary Methods*. The relative amount of m^7GpppA or NAD^+ between m^7G-depletion and mock-depletion samples was calculated by the peak intensity to evaluate the efficiency of m^7G-RNA removal.

Validation of NAD-RNAs by real-time RT-PCR

NAD-RNAs after CuAAC- or SPAAC-NAD reactions were isolated using magnetic MyOne Streptavidin C1 beads and subsequently reverse transcribed into cDNA using oligo(dT) primers following manufacturer's instruction from the SuperScript III Reverse Transcriptase Kit (Invitrogen). Real-time PCR was performed using AzuraView GreenFast qPCR Mix (FroggaBio) with the specific primers listed in *SI Appendix*, Table S1. Transcript levels were determined by the comparative Ct method. Data were normalized to the ADPRC- negative controls set as 1. Two biological replicates and three technical replicates were performed.

Supplementary Figures and Tables

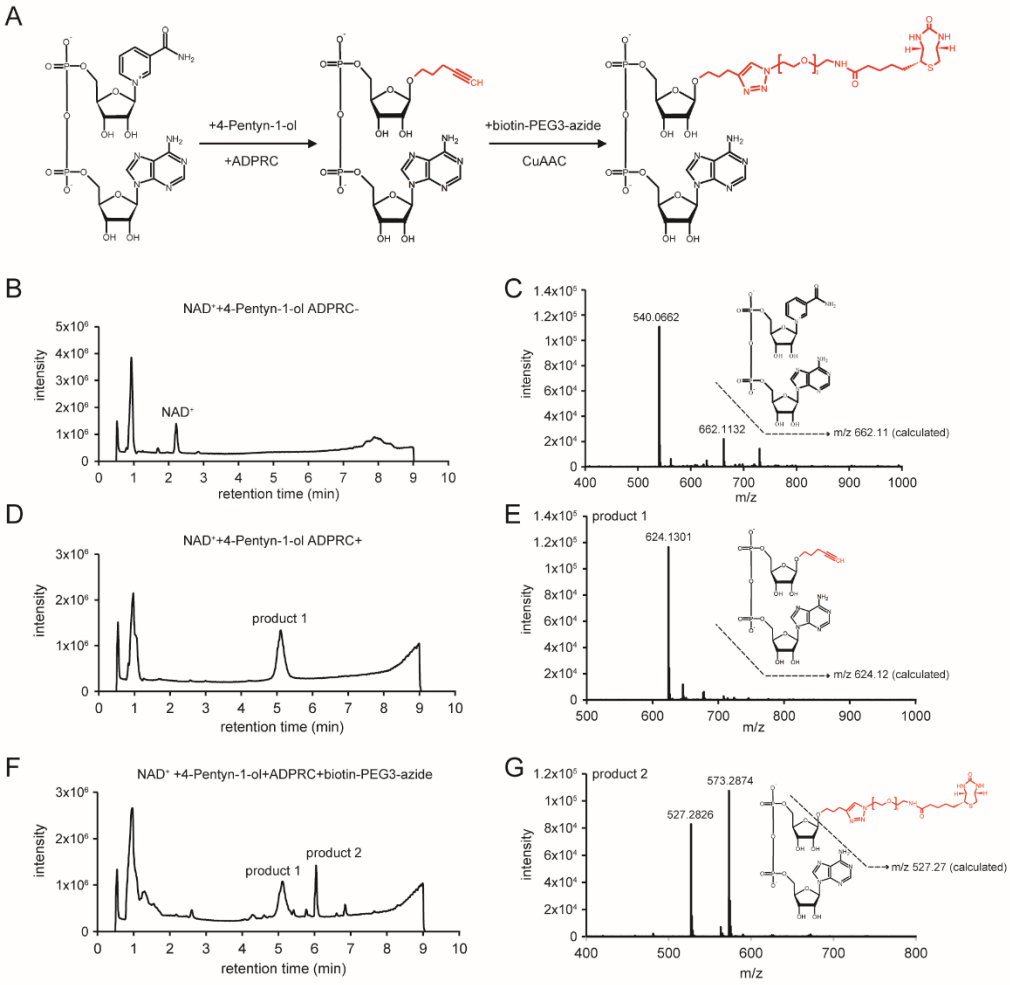

Fig. S1. Products of the CuAAC-NAD reaction

(*A*) Schematic diagram of the CuAAC-NAD reaction. ADPRC catalyzes the reaction replacing the nicotinamide moiety of NAD^+ with 4-Pentyn-1-ol, followed by CuAAC-based biotinylation with biotin-PEG3-azide. (*B*) HPLC chromatogram of the control reaction in which NAD^+ was incubated with 4-Pentyn-1-ol in the absence of ADPRC. The NAD^+ peak is marked. (*C*) Mass spectrum of the NAD^+ peak in (*B*). A compound of mass 662.113 matches NAD^+ in mass. (*D*) HPLC chromatogram of the ADPRC-catalyzed reaction of NAD^+ with 4-Pentyn-1-ol. A product is found as compared to (*C*). (*E*) Mass spectrum of product 1 in (*D*). A compound with mass 624.130 matches the expected product as diagramed. (*F*) HPLC chromatogram of the ADPRC-catalyzed reaction of NAD^+ with 4-Pentyn-1-ol followed by the CuAAC reaction of product 1 with biotin-PEG3-azide. Product 2 is formed as compared to (*D*). (*G*) Mass spectrum of product 2. The compound of mass 527.27 matches the moiety in red in the molecular diagram of product 2.

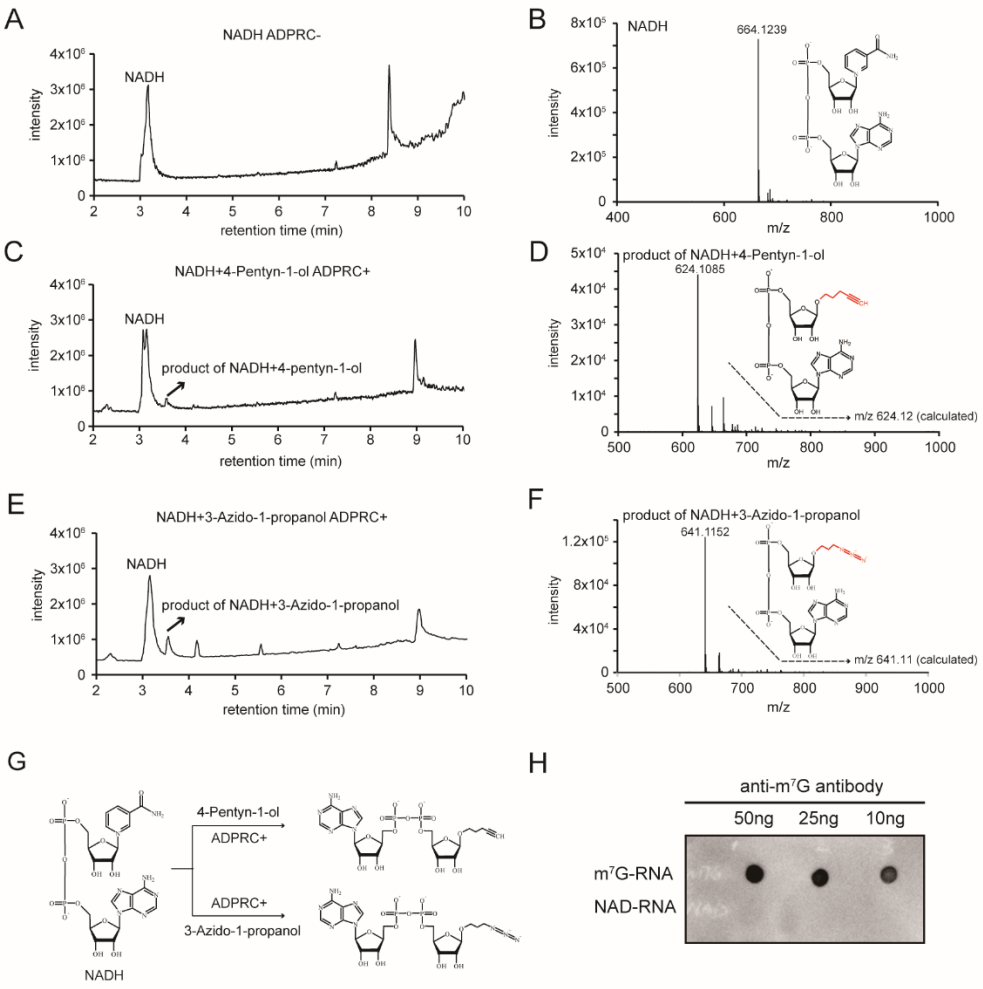

Fig. S2. The specificity of CuAAC- and SPAAC-NAD reactions and specificity of the anti-m^{7}G antibody

(*A*) HPLC chromatogram of NADH in the absence of ADPRC. (*B*) Mass spectrum of the NADH peak. The compound of mass 664.1239 matches NADH in mass. (*C*) HPLC chromatogram of the ADPRC-catalyzed reaction of NADH with 4-Pentyn-1-ol. The peak representing the reaction product is indicated. (*D*) The mass spectrum of the product in (*C*). The compound of mass 624.1085 matches the expected product as diagramed. (*E*) HPLC chromatogram of the ADPRC-catalyzed reaction of NADH with 3-Azido-1-propanol. The peak representing the reaction product is indicated. (*F*) The mass spectrum of the product in (*E*). The compound of mass 641.1152 matches the expected product as diagramed. (*G*) Schematic diagram showing the products of ADPRC-catalyzed transglycosylation between NADH and 4-Pentyn-1-ol or 3-Azido-1-propanol. (*H*) Specificity of the anti-m^{7}G antibody towards m^{7}G- and NAD-RNAs. Different amounts of in vitro transcribed m^{7}G- and NAD-RNAs were spotted on a nylon N$^+$ membrane and probed with the anti-m^{7}G antibody.

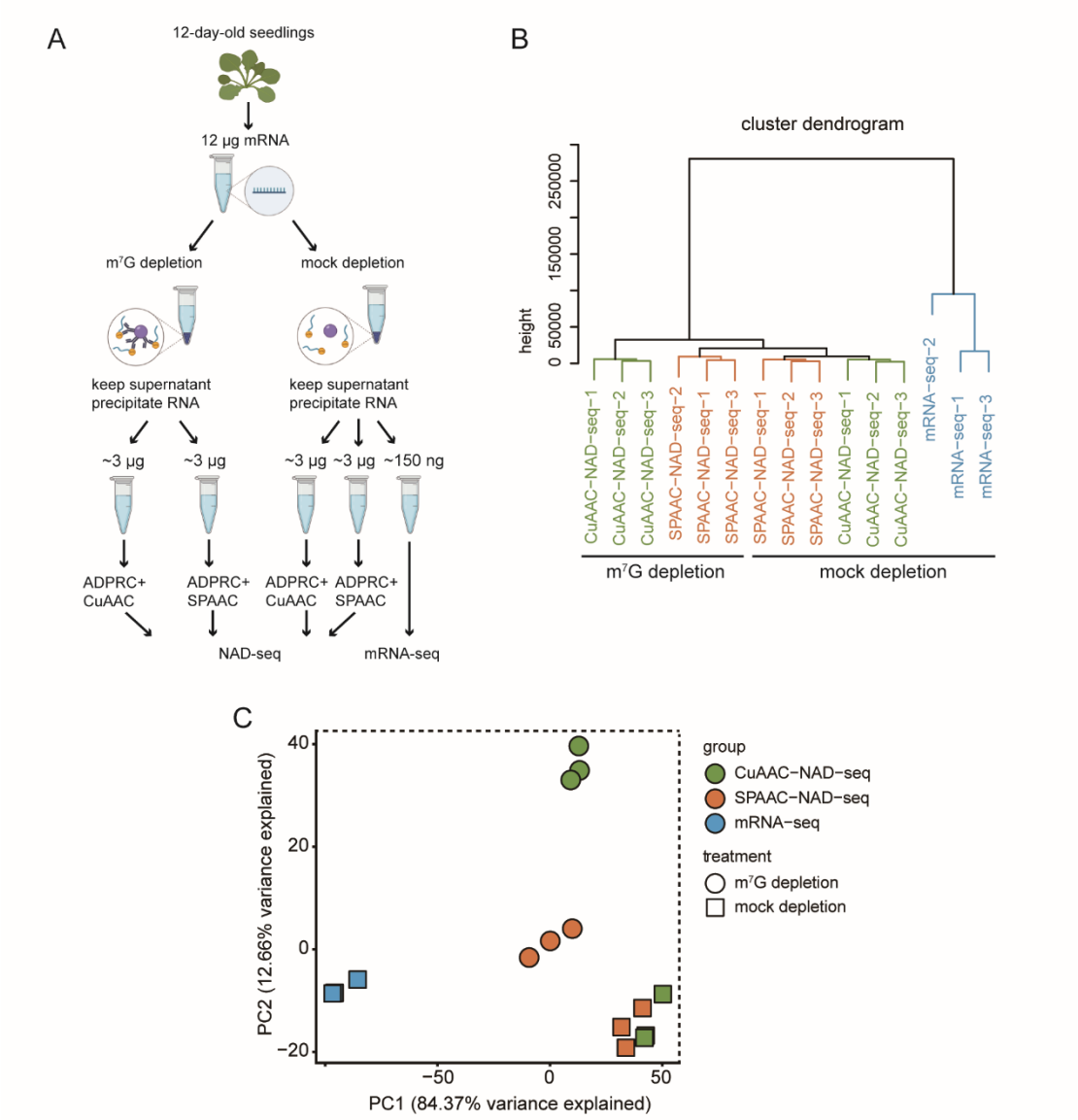

Fig. S3. NAD-seq workflow and quality control

(*A*) Schematic workflow of sample treatments. Poly(A) RNAs were isolated from 12-day-old seedlings and treated with (m^{7}G-depletion sample) or without (mock-depletion sample) the anti-m^{7}G antibody. mRNA-seq and NAD-seq were performed as described in Methods. Cartoons of the objects were created with BioRender.com. (*B*) Hierarchical clustering of all sequenced samples. The dendrogram was created using all expressed genes in each sample as a means to assay reproducibility. The three samples in the same color represent three biological replicates. (*C*) Principal Component Analysis of gene expression across all sequenced samples. PC1 and PC2 define the x and y axes, respectively.

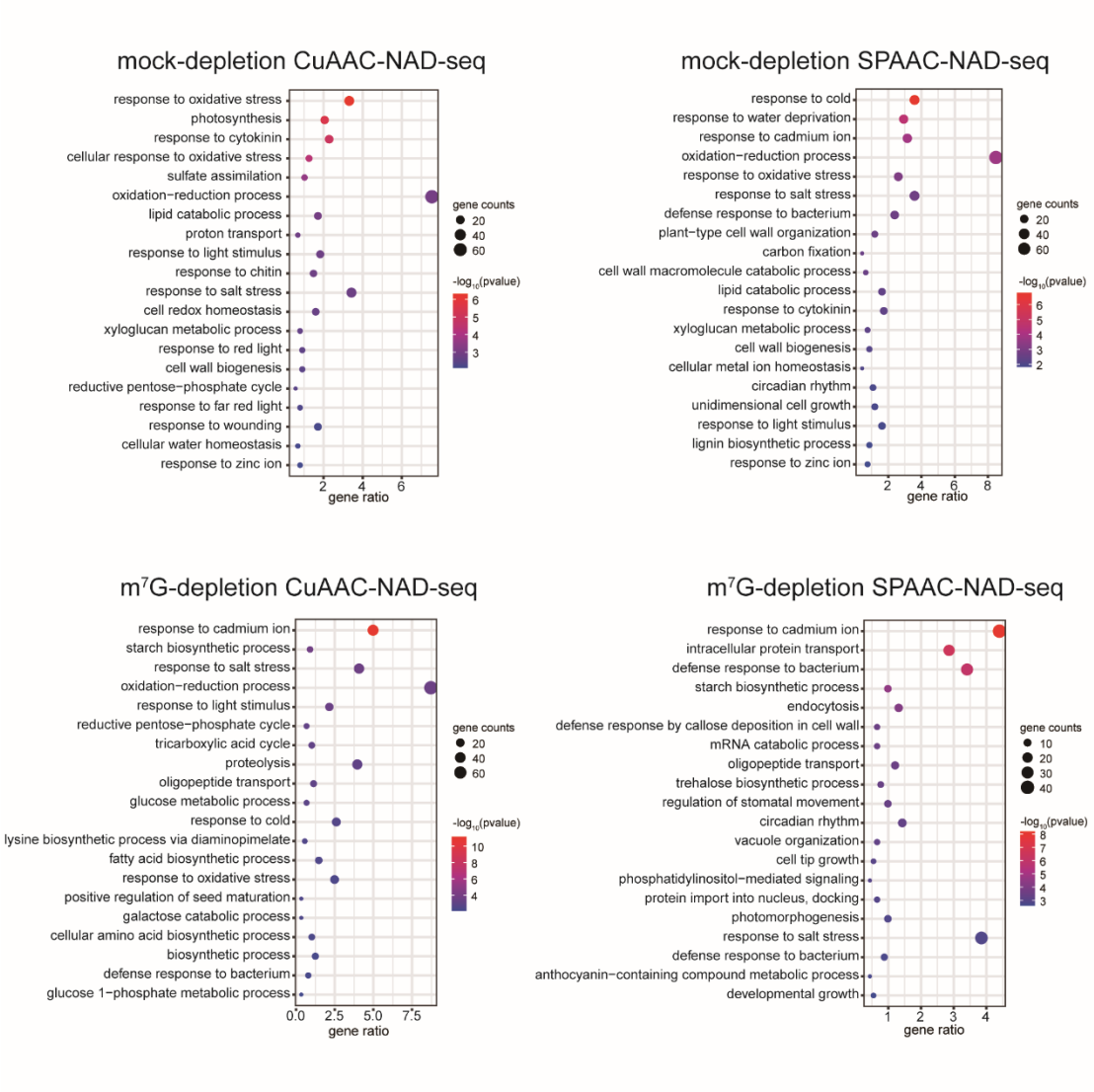

Fig. S4. GO enrichment analysis of NAD-RNAs

1000 NAD-RNAs with the highest NAD-seq/mRNA-seq FPKM ratio and FPKM mRNA-seq ≥ 5 were included in the analyses. GO terms were ordered by -$\log_{10}$ (p-value). The top 20 GO terms are shown.

Table S1. Oligonucleotides used in this study

Name	Sequence
31-nt template for in vitro transcription	CAGTAATACGACTCACTATTAGGCCTCTCGCTCT GCTGGGTGTGCGCTTGC
70-nt template for in vitro transcription	CAGTAATACGACTCACTATTAGGCCTCTCGCTCT GCTGGGTGTGCGCTTGCTTGGCTTGCTT GTGGTCTGCGGTTCGTTCCCGCTTTGGT
AT5G38420 qRT-PCR	Forward: AGTCATCCGCTTCTTTCCCG Reverse: ACGGTACACAAATCCGTGCT
ATMG01130 qRT-PCR	Forward: ACGAAGCCTCCTCCTCAGAT Reverse: CTATTCCTCGGTAAGCGGGC

The following files are provided separately:

Dataset S1. NAD-RNAs identified by CuAAC-NAD-seq in mock-depletion samples

Dataset S2. NAD-RNAs identified by SPAAC-NAD-seq in mock-depletion samples

Dataset S3. NAD-RNAs identified by CuAAC-NAD-seq in m^7G-depletion samples

Dataset S4. NAD-RNAs identified by SPAAC-NAD-seq in m^7G-depletion samples

Dataset S5. mRNA-seq in mock-depletion samples

Dataset S6. GO enrichment analysis of NAD-RNAs in CuAAC-NAD-seq of mock-depletion samples

Dataset S7. GO enrichment analysis of NAD-RNAs in SPAAC-NAD-seq of mock-depletion samples

Dataset S8. GO enrichment analysis of NAD-RNAs in CuAAC-NAD-seq of m^7G-depletion samples

Dataset S9. GO enrichment analysis of NAD-RNAs in SPAAC-NAD-seq of m^7G-depletion samples