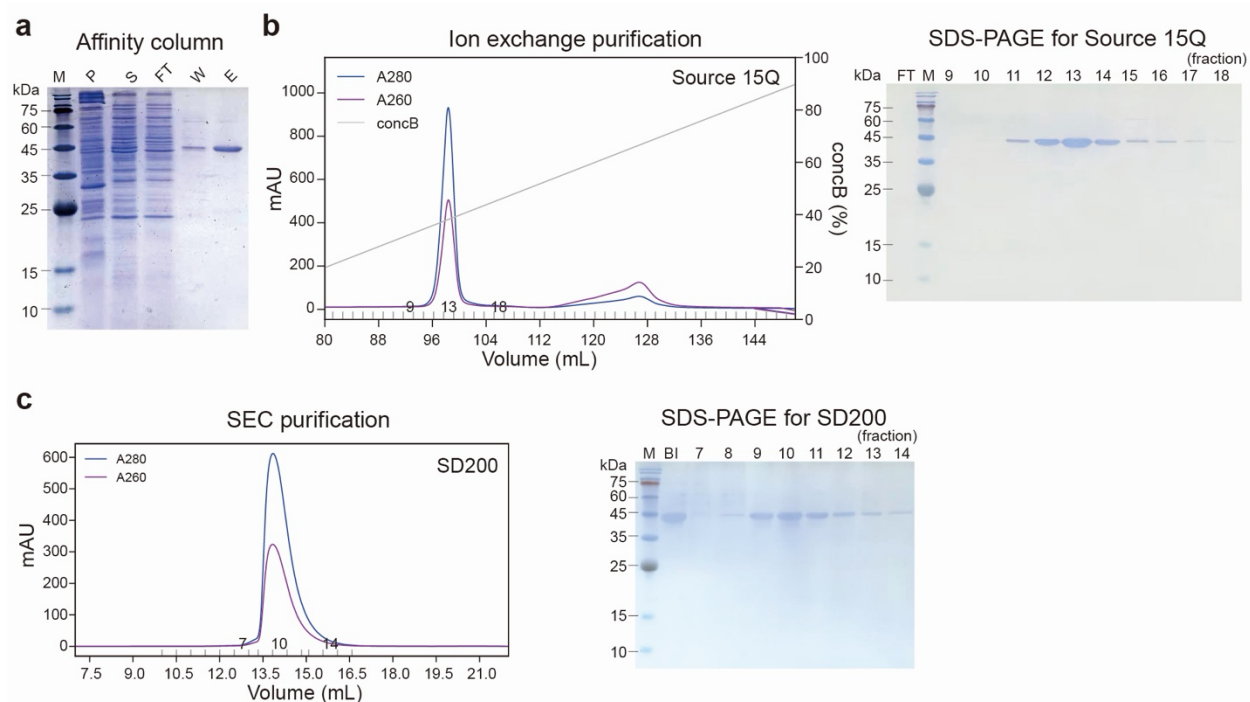
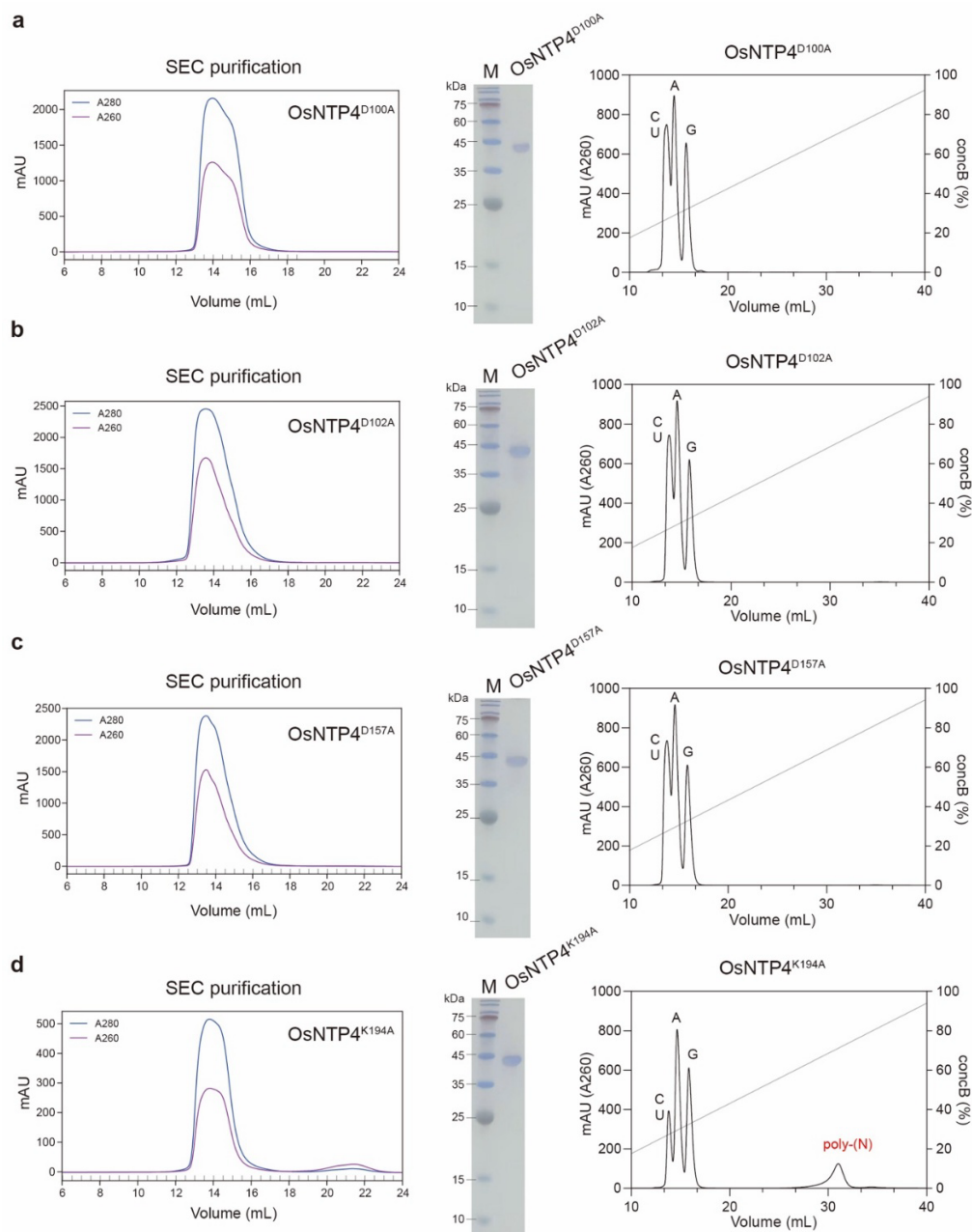
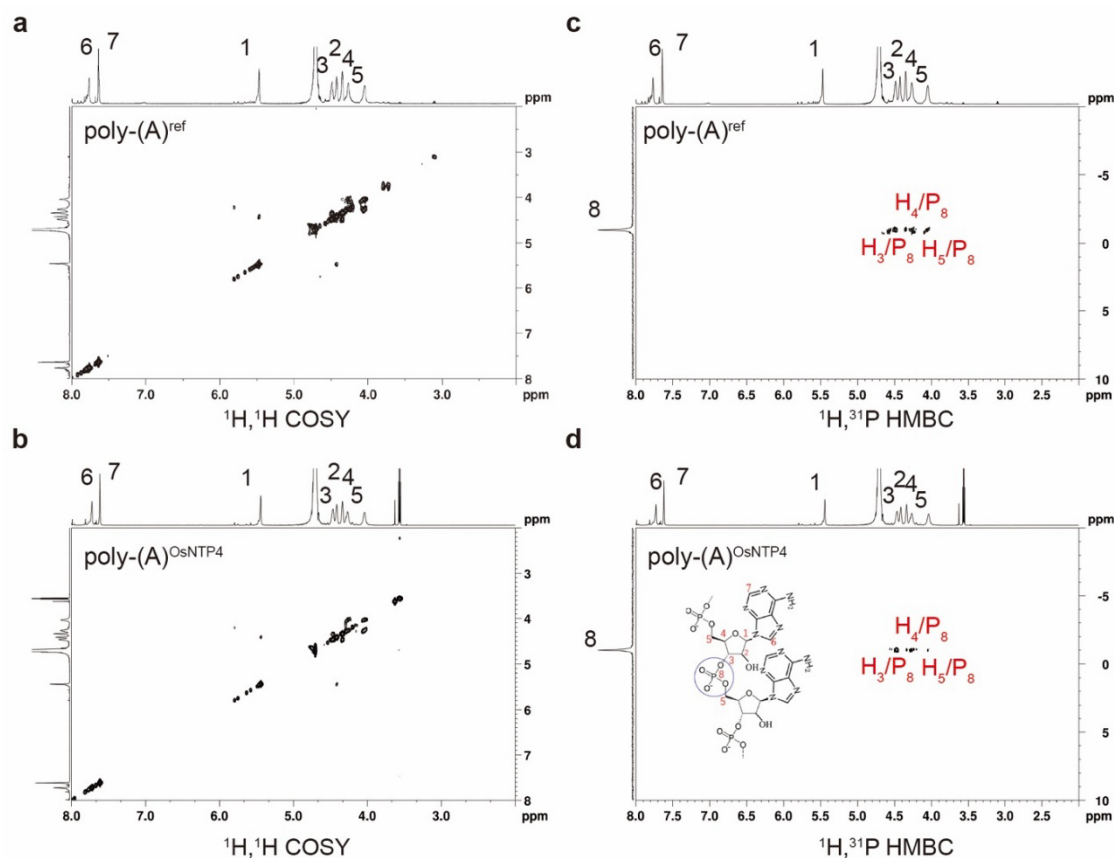




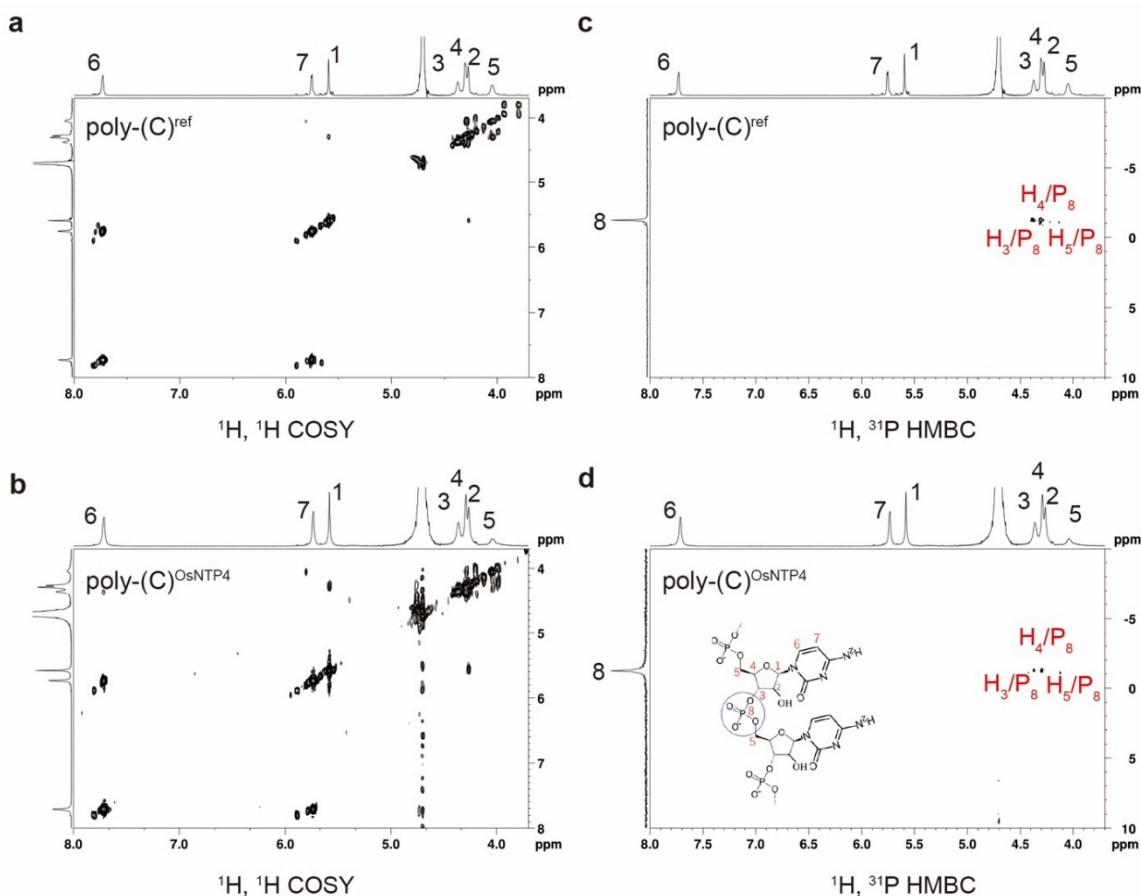
Supplementary Fig. S1 Biochemical purification of the recombinant full-length OsNTP4. a Ni-NTA affinity purification of OsNTP4. Shown here is an SDS-PAGE gel stained by Coomassie blue. M, molecular-mass marker; P, pellet after centrifugation; S, supernatant after centrifugation; FT, flow-through from the column; W, wash fraction; E, imidazole-eluted fraction. **b** Further purification of OsNTP4 by anion exchange chromatography using a Source 15Q column. Shown here are the chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel). Absorbances at 280 nm and 260 nm are shown in blue and purple lines, respectively. **c** Final purification of OsNTP4 by size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column. Shown here are the SEC chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel).



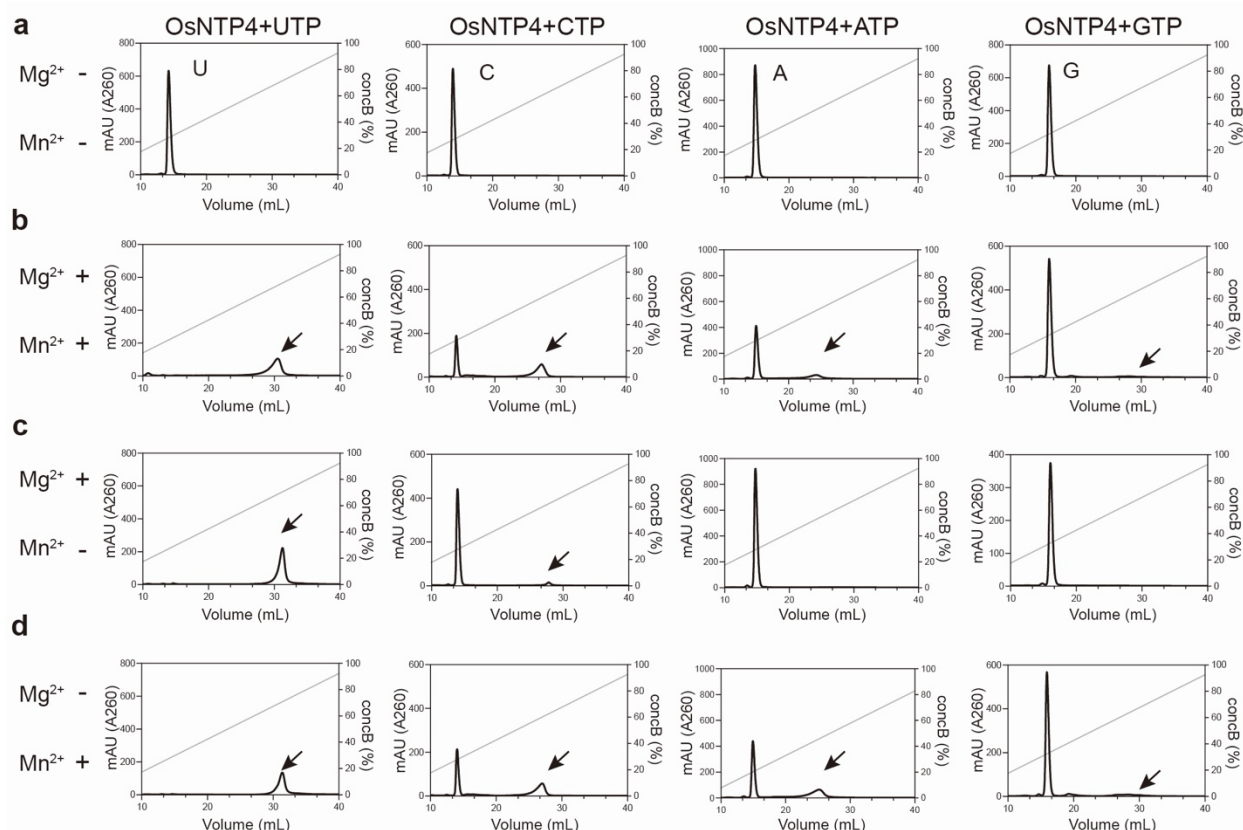
Supplementary Fig. S2 Conserved acidic residues in OsNTP4 are required for its *de novo* RNA synthesis activity. **a** The missense mutation D100A results in abrogation of RNA synthesis by OsNTP4. Shown here are the chromatogram of Superdex 200 Increase 10/300 GL purification for OsNTP4 variant D100A (left panel), the peak fraction visualized on an SDS-PAGE gel stained by Coomassie blue (middle panel), and the Capto Q anion-exchange chromatography of the reaction using OsNTP4 variant D100A (right panel). **b** The missense mutation D102A results in abrogation of RNA synthesis by OsNTP4. **c** The missense mutation D157A results in abrogation of RNA synthesis by OsNTP4. **d** The missense mutation K194A has no impact on RNA synthesis by OsNTP4.



Supplementary Fig. S3 The OsNTP4 reaction products, RNA polymers from ATP only, have a canonical 3'–5' phosphodiester linkage as revealed by NMR analysis. **a** The ^1H , ^1H COSY spectra of a reference poly-(A) sample. **b** The ^1H , ^1H COSY spectra of the reaction product of OsNTP4 in the presence of ATP only. **c** The ^1H , ^{31}P HMBC spectra of a reference poly-(A) sample. **d** The ^1H , ^{31}P HMBC spectra of the reaction product of OsNTP4 in the presence of ATP only. Red labels identify correlations between the bridging phosphate (P8) and H3' and H5' ribose protons, indicating a 3'–5' phosphodiester linkage.

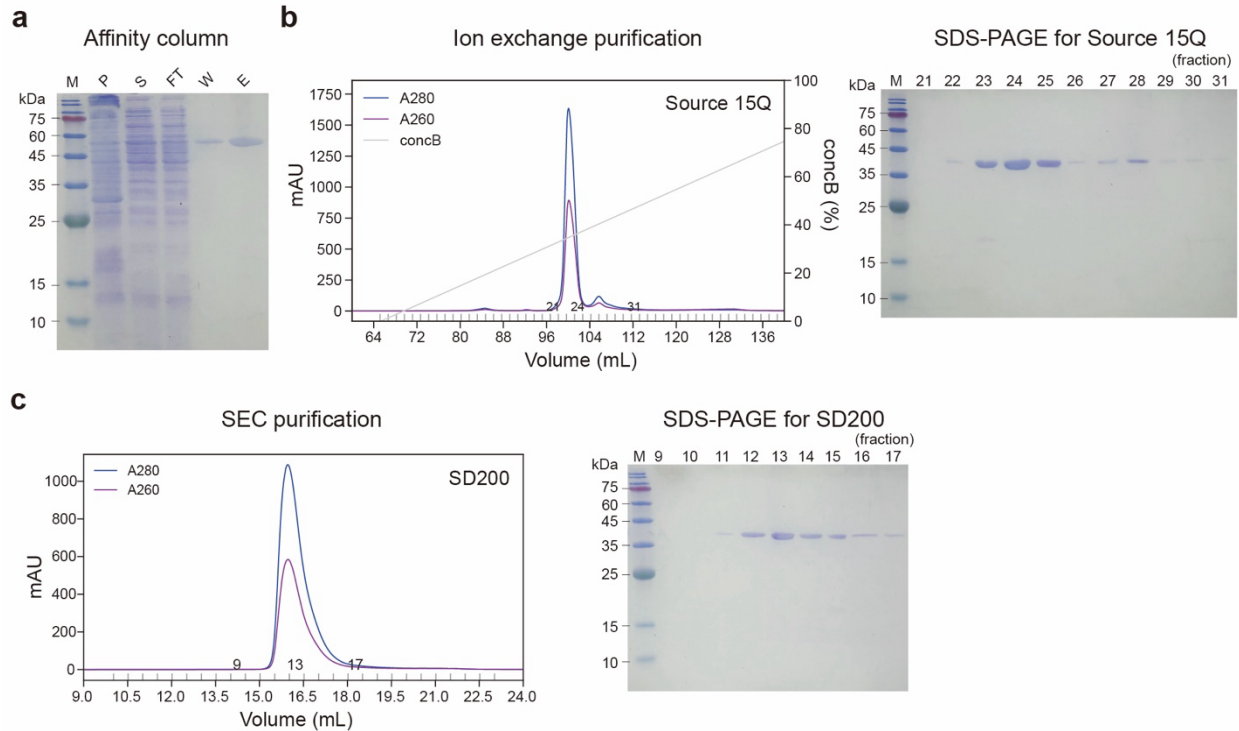


Supplementary Fig. S4 The OsNTP4 reaction products, RNA polymers from CTP only, have a canonical 3'-5' phosphodiester linkage as revealed by NMR analysis. **a** The ^1H , ^1H COSY spectra of a reference poly-(C) sample. **b** The ^1H , ^1H COSY spectra of the reaction product of OsNTP4 in the presence of CTP only. **c** The ^1H , ^{31}P HMBC spectra of a reference poly-(C) sample. **d** The ^1H , ^{31}P HMBC spectra of the reaction product of OsNTP4 in the presence of CTP only. Red labels identify correlations between the bridging phosphate (P₈) and H_{3'} and H_{5'} ribose protons, indicating a 3'-5' phosphodiester linkage.



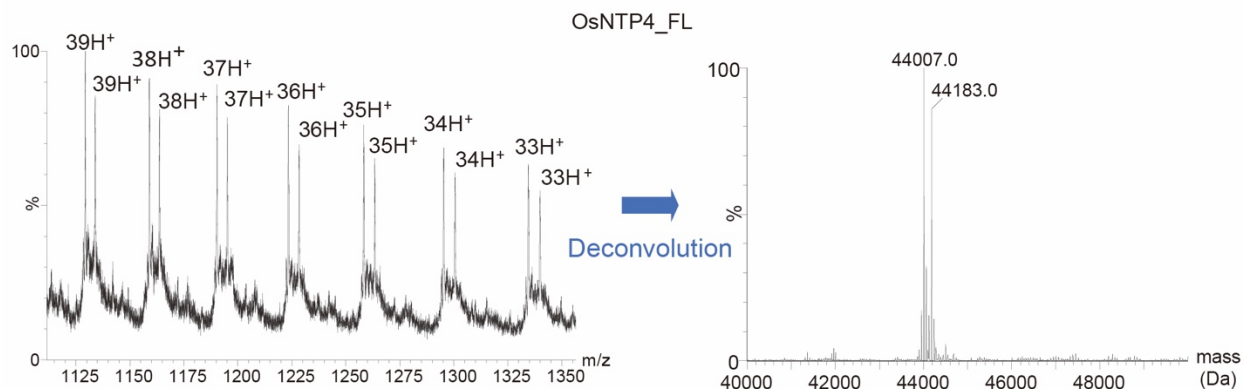
Supplementary Fig. S5 *De novo* RNA synthesis by OsNTP4 requires divalent metal ions. a

OsNTP4 has no activity in the absence of both Mg²⁺ and Mn²⁺ ions. Shown here are four chromatograms of Capto Q anion-exchange chromatography of the reaction products of OsNTP4 in the presence of UTP, CTP, ATP, or GTP (from left to right). **b** OsNTP4 exhibits *de novo* RNA synthesis activity in the presence of both Mg²⁺ and Mn²⁺ ions. **c** OsNTP4 exhibits *de novo* RNA synthesis activity in the presence of Mg²⁺ only. **d** OsNTP4 exhibits *de novo* RNA synthesis activity in the presence of Mn²⁺ only. Arrows indicate the reaction product peaks.

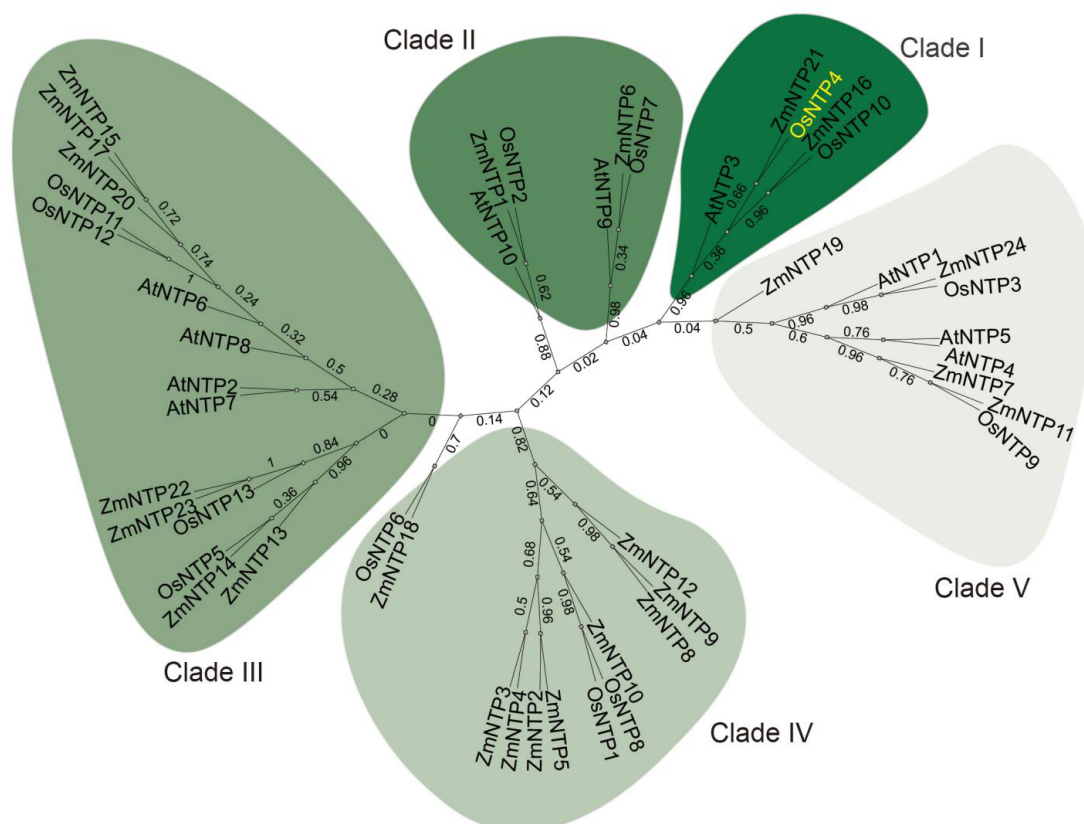


Supplementary Fig. S6 Biochemical purification of the OsNTP4 catalytic domain

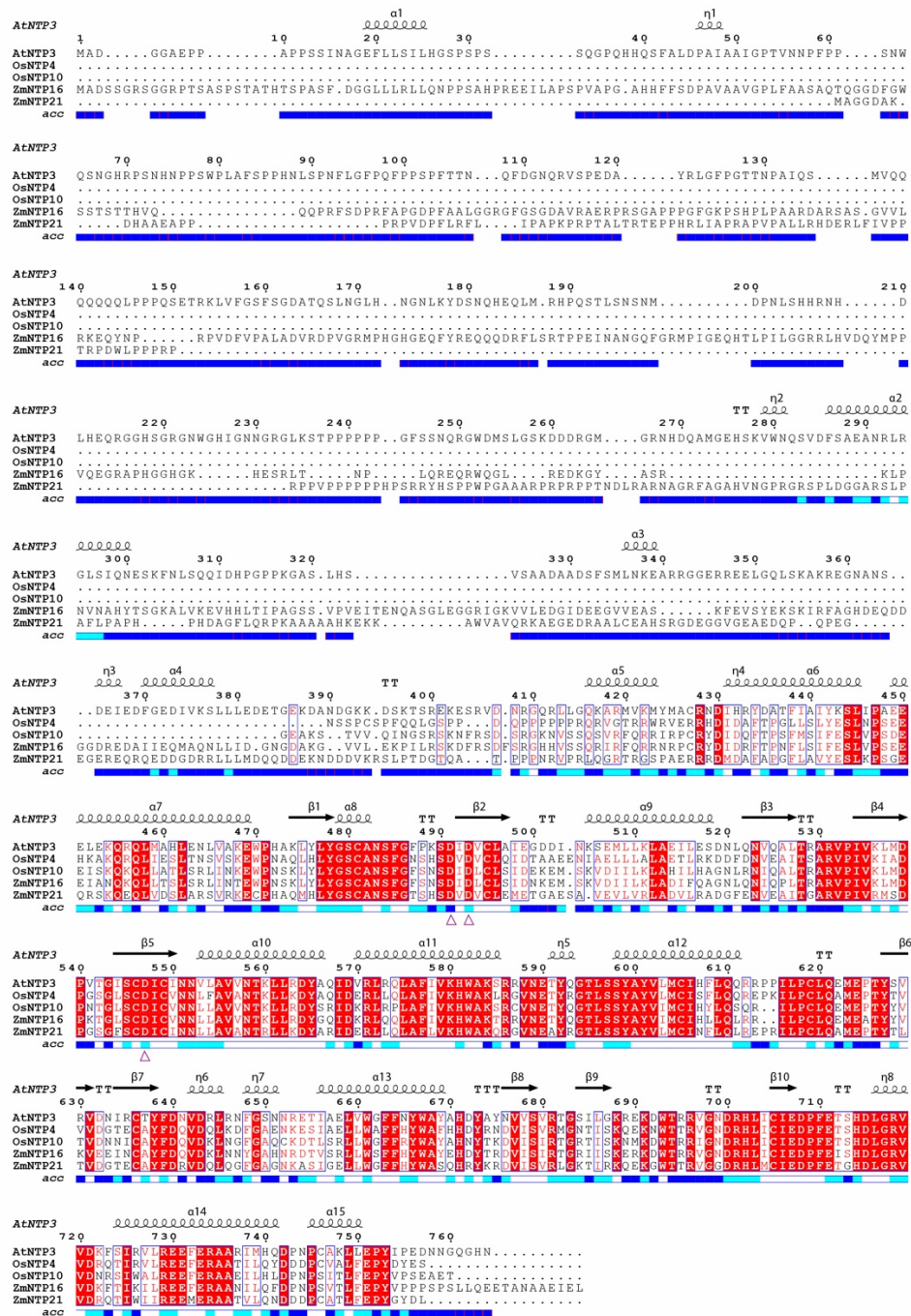
(OsNTP4_CD). **a** Ni-NTA affinity purification of OsNTP4_CD. Shown here is an SDS-PAGE gel stained by Coomassie blue. M, molecular-mass marker; P, pellet after centrifugation; S, supernatant after centrifugation; FT, flow-through from the column; W, wash fraction; E, imidazole-eluted fraction. **b** Further purification of OsNTP4_CD by anion exchange chromatography using a Source 15Q column. Shown here are the chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel). Absorbance at 280 nm and 260 nm is shown in blue and purple lines, respectively. **c** Final purification of OsNTP4_CD by size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column. Shown here are the SEC chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel).



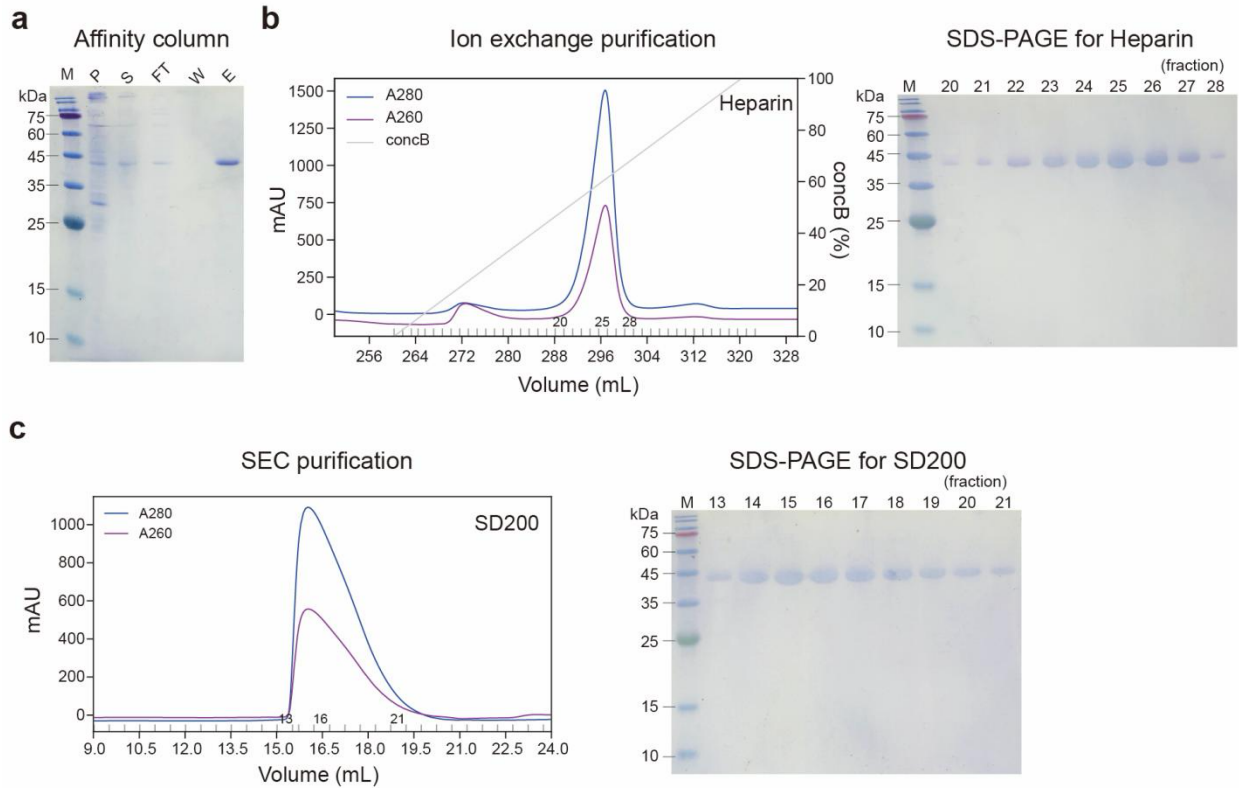
Supplementary Fig. S7 Mass spectrometry (MS) of the full-length OsNTP4. Charge-state series (left) and deconvoluted mass spectrum (right) of purified full-length OsNTP4. The theoretical average mass with an N-terminal N-formylmethionine (fMet) is 44,166.60 Da. The observed peaks at 44,007 Da and 44,183 Da correspond to the fMet-cleaved form (-159 Da) and the oxidized fMet-containing form (+16 Da), respectively. Mass errors for all species are within 1 Da.



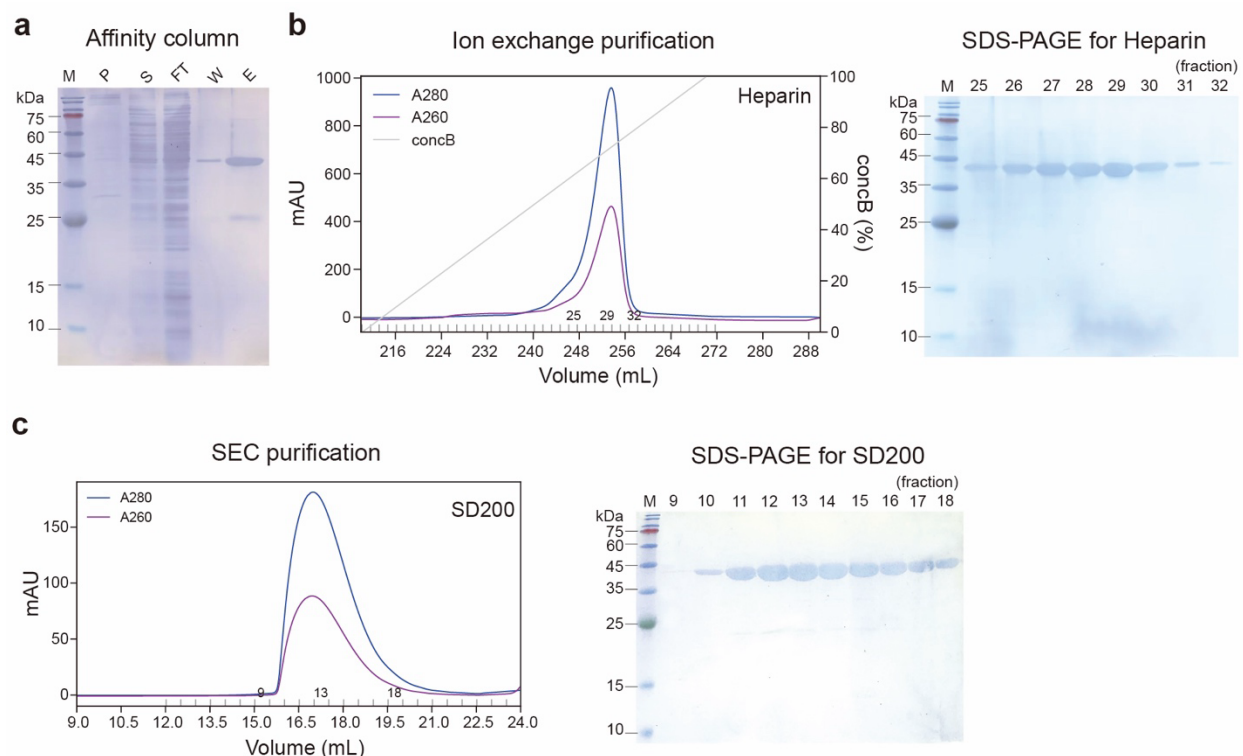
Supplementary Fig. S8 Phylogenetic analysis of the plant NTP-family proteins. Shown here is a phylogenetic tree of NTP-family proteins from three plant species: *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays*. Five major clades are revealed. OsNTP4 is highlighted in yellow. OsNTP10 and AtNTP3 (also known as AtURT1) are in Clade I. Bootstrap support values are labeled at each internal node.



Supplementary Fig. S9 Sequence alignment among Clade I plant NTP proteins. Sequence alignment of the Clade I NTP-family proteins. The sequences include AtNTP3, OsNTP4, OsNTP10, ZmNTP16, and ZmNTP21. The amino-terminal regions are divergent, whereas the carboxyl-terminal nucleotidyltransferase core is conserved. Conserved residues are highlighted, and secondary-structure elements are shown above the alignment.

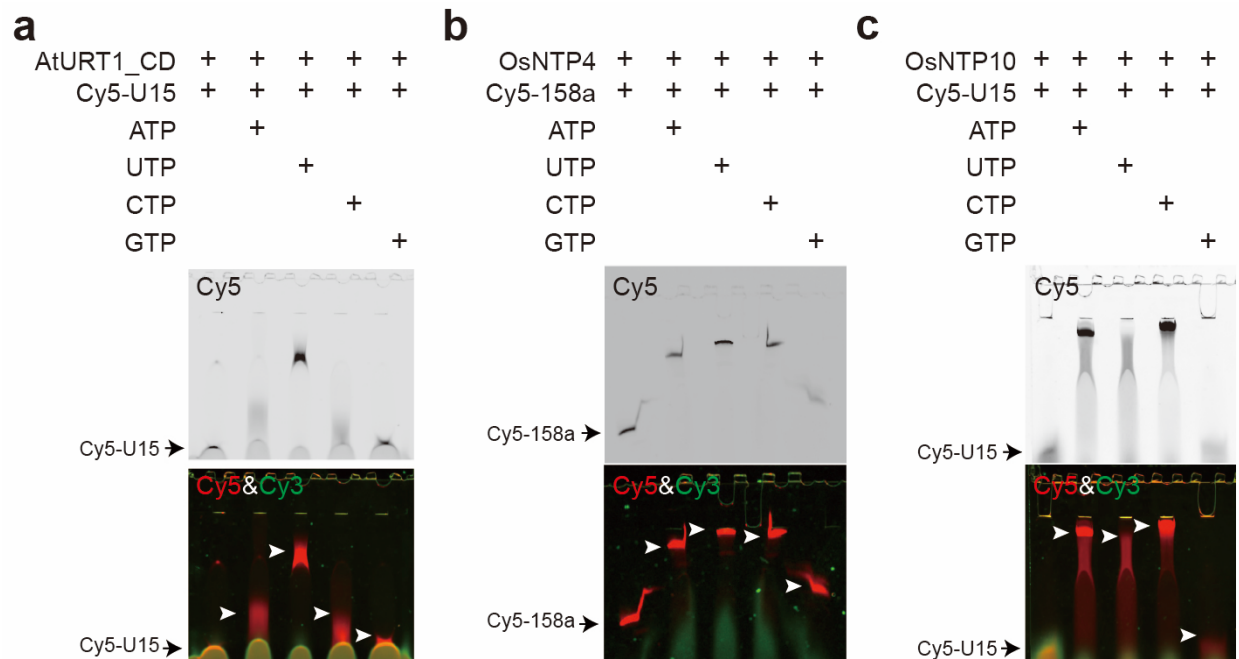


Supplementary Fig. S10 Biochemical purification of the full-length OsNTP10 protein. **a** Ni-NTA affinity purification of OsNTP10. Shown here is an SDS-PAGE gel stained by Coomassie blue. M, molecular-mass marker; P, pellet after centrifugation; S, supernatant after centrifugation; FT, flow-through from the column; W, wash fraction; E, imidazole-eluted fraction. **b** Further purification of OsNTP10 by anion exchange chromatography using a Heparin column. Shown here are the chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel). Absorbances at 280 nm and 260 nm are shown in blue and purple lines, respectively. **c** Final purification of OsNTP10 by size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column. Shown here are the SEC chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel).

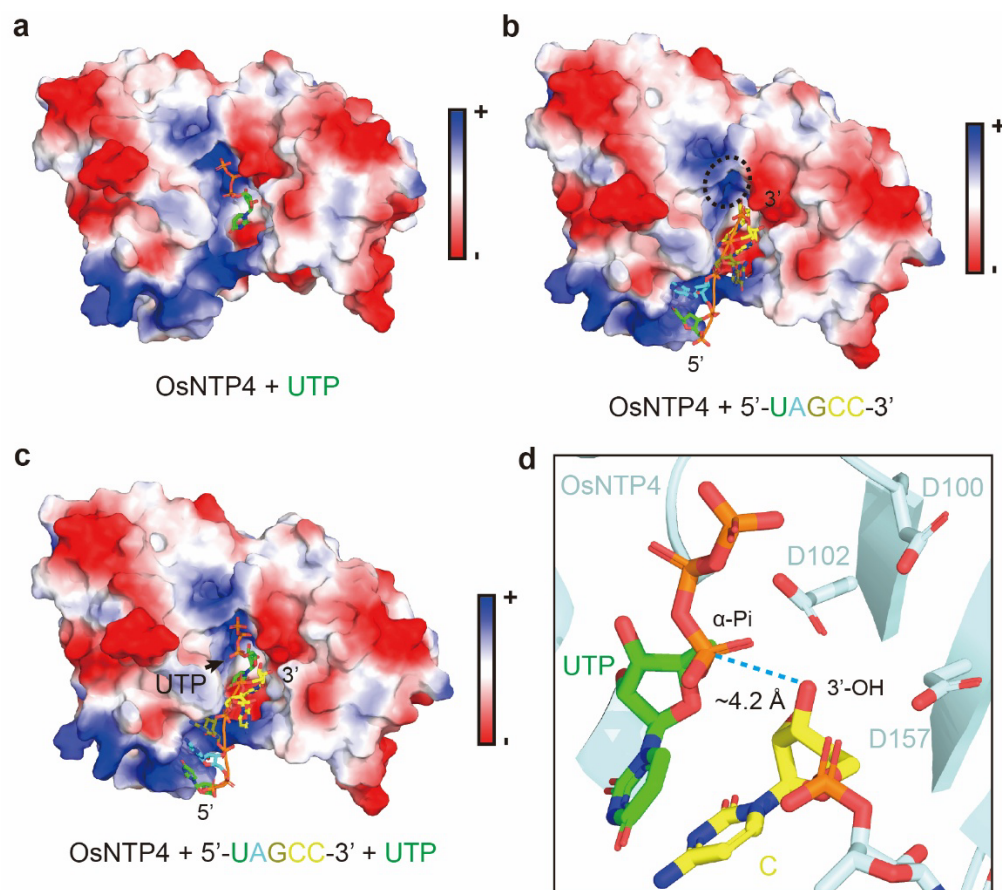


Supplementary Fig. S11 Biochemical purification of the AtURT1 catalytic domain

(AtURT1_CD). **a** Ni-NTA affinity purification of AtURT1_CD. Shown here is an SDS-PAGE gel stained by Coomassie blue. M, molecular-mass marker; P, pellet after centrifugation; S, supernatant after centrifugation; FT, flow-through from the column; W, wash fraction; E, imidazole-eluted fraction. **b** Further purification of AtURT1_CD by anion exchange chromatography using a Heparin column. Shown here are the chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel). Absorbances at 280 nm and 260 nm are shown in blue and purple lines, respectively. **c** Final purification of AtURT1_CD by size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 GL column. Shown here are the SEC chromatogram (left panel) and the peak fractions visualized on an SDS-PAGE gel stained by Coomassie blue (right panel).



Supplementary Fig. S12 NTP-dependent RNA 3'-end extension by plant NTP proteins. a Extension of Cy5-labeled U15 RNA by AtURT1_CD in the presence of individual NTPs. **b** Extension of Cy5-labeled miR158a RNA by OsNTP4. **c** Extension of Cy5-labeled U15 RNA by OsNTP10. Upper images show the Cy5 channel; lower images show merged Cy5 and Cy3 channels. White arrowheads indicate extension products. Reaction components are indicated above each gel.



Supplementary Fig. S13 Putative reaction mechanism of *de novo* RNA synthesis by OsNTP4. **a** Electrostatic surface representation of the AlphaFold-predicted model of OsNTP4 in the presence of UTP. The UTP molecule is placed near the conserved catalytic pocket. Electrostatic surface potential is shown (red, negative potential; blue, positive potential). **b** The AlphaFold-predicted model of OsNTP4 in the presence of an RNA penta-nucleotide. The short RNA penta-nucleotide, 5'-UAGCC-3', was placed into a positively charged cleft near the active site of OsNTP4. The potential binding site for the next NTP molecule is indicated by a dashed oval. **c** The AlphaFold-predicted model of OsNTP4 in the presence of an RNA penta-nucleotide and UTP. The UTP molecule was indeed placed into the positively charged pocket as indicated in panel b. **d** A close-up view on the active site of the predicted structure from panel c. UTP and the 3'-end of the RNA penta-nucleotide are placed close to the three acidic residues Asp100, Asp102, and Asp157. The modeled separation between the RNA 3'-OH and the UTP α -phosphate is approximately 4.2 Å.