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Supplemental information

OsCPK12 phosphorylates OsCATA and OsCATC to regulate H_2O_2 homeostasis and improve oxidative stress tolerance in rice

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Running title: OsCPK12 improves oxidative tolerance in rice

OsCPK12 phosphorylates OsCATA and OsCATC to regulate H_2O_2 homeostasis and improve oxidative tolerance in rice

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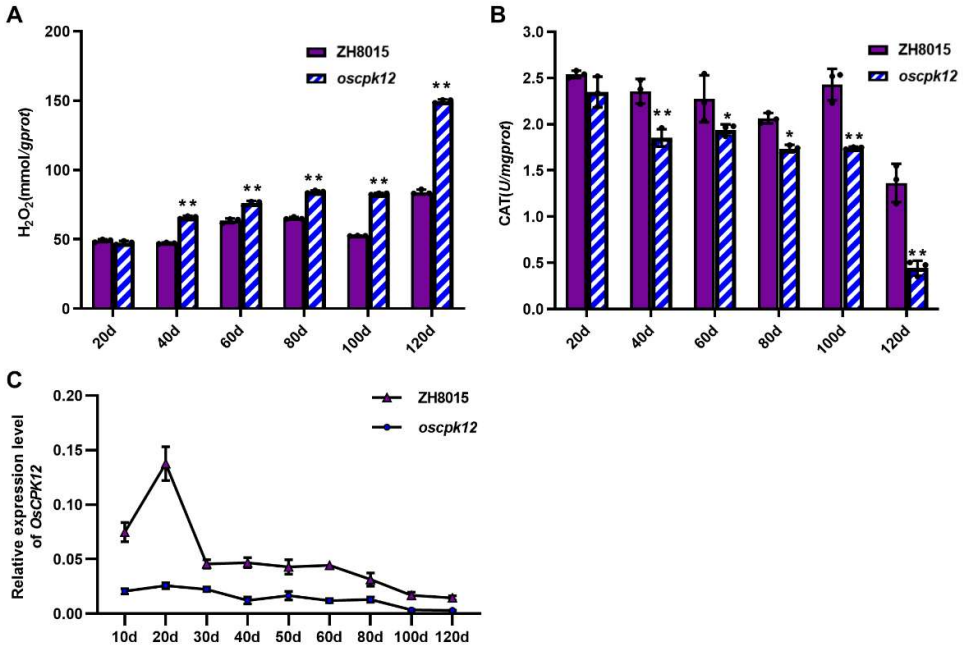
Short summary: OsCPK12 functions in signal transduction pathways, the low-nitrogen stress response, and salt stress processes. However, how OsCPK12 regulates the antioxidant defense system remains largely unknown. Our findings demonstrate that OsCPK12 phosphorylates OsCATA and OsCATC at Ser11 to increase their activity for maintaining H_2O_2 homeostasis, and overexpression of *OsCPK12* improved tolerance to oxidative stress in rice.

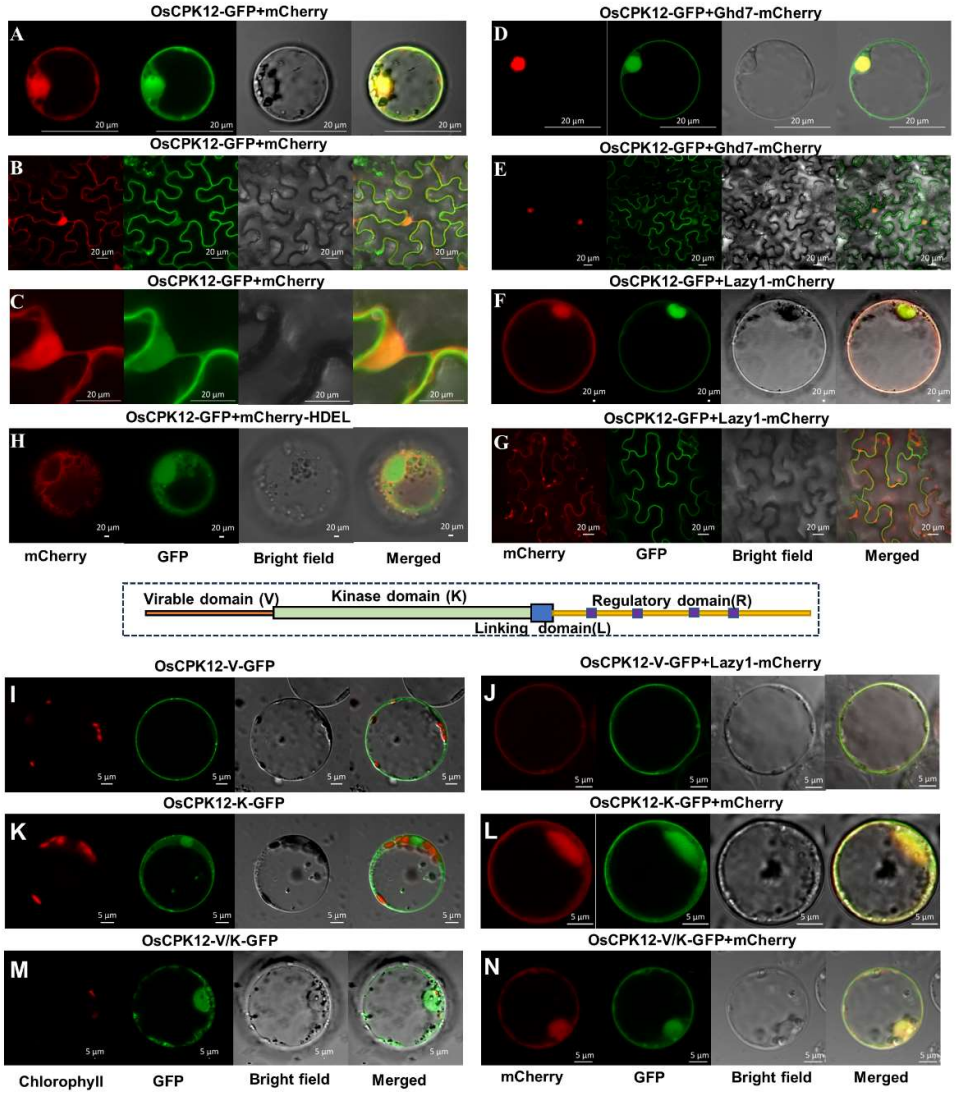
SUPPLEMENTAL INFORMATION

Supplemental Figures

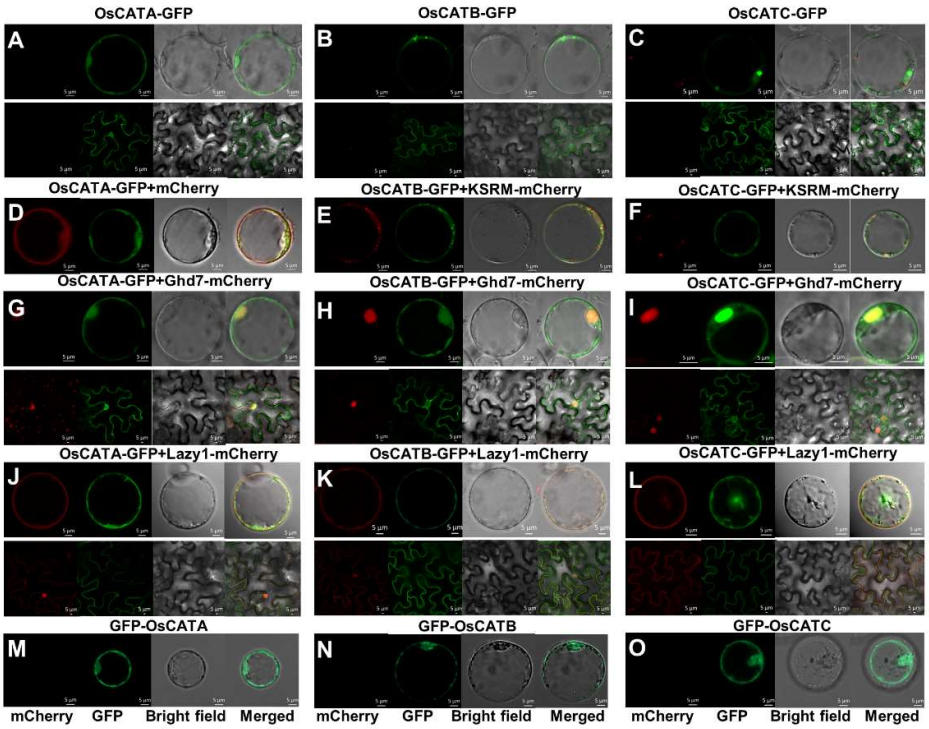


Supplemental Figure S1. H_2O_2 content and CAT activity in ZH8015 and *oscpk12*, and expression pattern of *OsCPK12*.

(A) The content of H_2O_2 in leaves of ZH8015 and *oscpk12* plants at different stages. Data are presented as mean±SD (n = 3). The content of H_2O_2 in leaves of ZH8015 and *oscpk12* plants at different stages were analyzed using Student's *t*-test, **P*<0.05, ***P*<0.01. **(B)** The enzymatic activities of catalase (CAT) in leaves of ZH8015 and *oscpk12* plants at different stages. Data are presented as mean±SD (n = 3). The enzymatic activities of catalase (CAT) in leaves of ZH8015 and *oscpk12* plants at different stages were analyzed using Student's *t*-test, **P*<0.05, ***P*<0.01. **(C)** Expression pattern of *OsCPK12* in the leaves of ZH8015 and *oscpk12*.The 3^{td} leaves of ZH8015 and *oscpk12* at different time points were used for qRT-PCR. Data are presented as mean±SD (n=3).

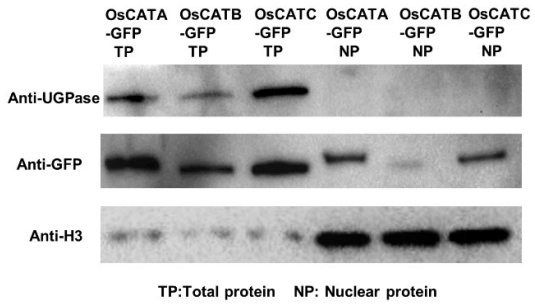


Supplemental Figure S2. Subcellular localization of OsCPK12 and truncated OsCPK12. **(A-C)** Subcellular localization of OsCPK12 by colocalization with mCherry in rice protoplast and in leaves of *N. benthamiana*. Scale bars =20 μm. **(D-E)** Subcellular localization of OsCPK12 by colocalization Ghd7-mCherry in rice protoplast. Scale bars =20 μm. **(F-G)** Subcellular localization of OsCPK12 by colocalization with Lazy1-mCherry in rice protoplast. Scale bars =20 μm. **(H)** Subcellular localization of OsCPK12 by colocalization mCherry-HDEL in rice protoplast. Scale bars =20 μm. **(I)** Subcellular localization of OsCPK12-V-GFP in rice protoplast. Scale bars =5 μm. **(J)** Subcellular localization of OsCPK12-V-GFP by colocalization with Lazy1-mCherry in rice protoplast. Scale bars =5 μm. **(K)** Subcellular localization of OsCPK12-K-GFP in rice protoplast. Scale bars =5 μm. **(L)** Subcellular localization of of OsCPK12-K-GFP by colocalization with mCherry in rice protoplast. Scale bars =5 μm. **(M)** Subcellular localization of OsCPK12V/K-GFP in rice protoplast. Scale bars =5 μm. **(N)** Subcellular localization of OsCPK12V/K-GFP by colocalization with mCherry in rice protoplast. Scale bars =5 μm.



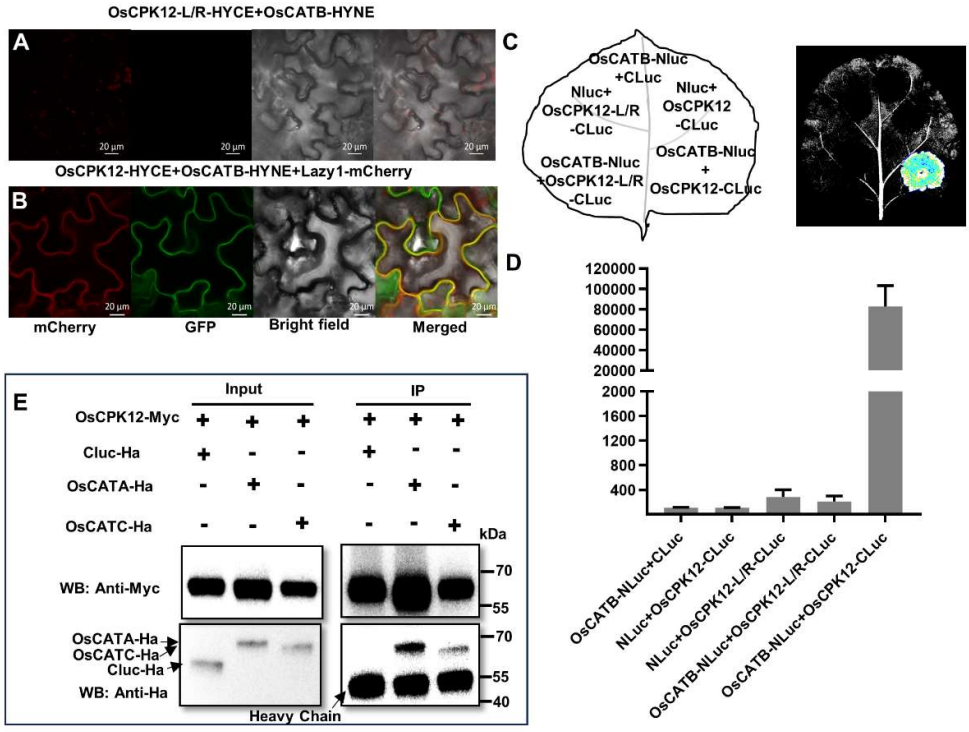
Supplemental Figure S3. Subcellular localization of OsCATA, OsCATB, and OsCATC. Bar = 5 μm.

(A-C) Subcellular localization of OsCATA-GFP, OsCATB-GFP, and OsCATC-GFP. **(D)** Subcellular colocalization of OsCATA-GFP with mCherry. **(E-F)** Subcellular colocalization of OsCATB-GFP, and OsCATC-GFP with KSRM-mCherry. **(G-I)** Subcellular colocalization of OsCATA-GFP, OsCATB-GFP, and OsCATC-GFP with Ghd7-mCherry. **(J-L)** Subcellular colocalization of OsCATA-GFP, OsCATB-GFP, and OsCATC-GFP with Lazy1-mCherry. **(M-O)** Subcellular localization of GFP-OsCATA, GFP-OsCATB, and GFP-OsCATC.



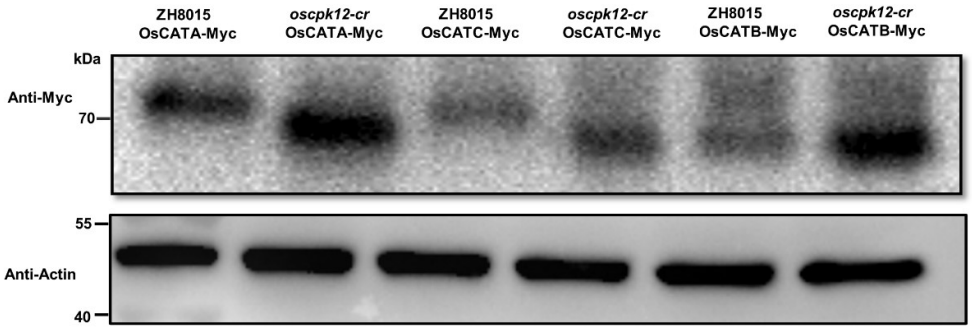
Supplemental Figure S4. Immunoblot analysis of OsCATA-GFP, OsCATB-GFP, and OsCATC-GFP.

The protoplast protein in ZH8015 of transiently transformed OsCATA-GFP, OsCATB-GFP, and OsCATC-GFP were extracted. Antiserum against GFP-tag (anti-GFP), antiserum against UDP-glucose pyrophosphorylase (cytoplasm marker) (anti-UGPase) and antiserum against Histone H3 (Nuclear marker) (anti-H3) were used in blotting. TP, total protein; NP, nuclear protein.

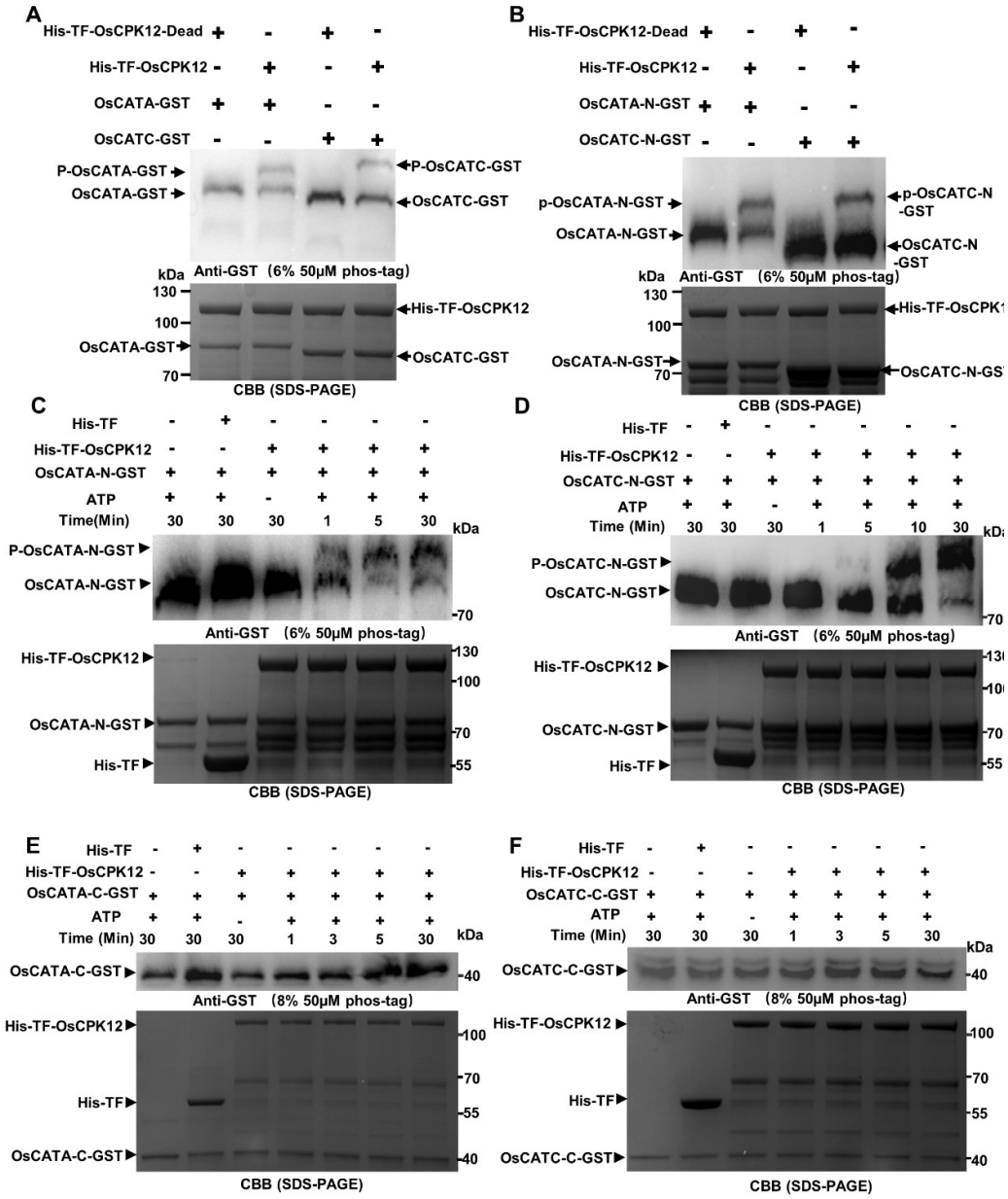


Supplemental Figure S5. OsCPK12 interacts with OsCATs.

(A-B) BIFC assay of OsCPK12 interacted with OsCATB. The indicated constructs were transiently expressed in *N. benthamiana* by an agroinfiltration method, and four days after infiltration (DAI), fluorescence was observed using a laser scanning confocal microscope (ZEISS 750). OsCPK12-L/R-HYCE was used as a negative control. Lazy1-mCherry was used as a cell membrane marker. Scale bars=20 μm. **(C-D)** OsCPK12 interacts with OsCATB as indicated by luciferase complementation imaging (LCI) assay. OsCPK12-CLuc and NLuc-OsCATB were transiently expressed in *Nicotiana benthamiana* by coinfiltration. NLuc and CLuc were the negative controls. Luminescences were monitored with a low-light, cooled, CCD imaging apparatus at 2 days after infiltration (dai). Data are presented as mean±SD (n = 3). **(E)** Co-immunoprecipitation assay for OsCPK12 interacting with OsCATA and OsCATC. The total protein extracts from rice protoplast co-transfected OsCPK12-Myc with OsCATA-Ha, OsCATC-Ha or Cluc-Ha were immunoprecipitated with anti-Myc Sepharose beads. Proteins from crude lysates (left, input) and immunoprecipitated proteins (right) were detected with anti-Ha and anti-Myc antibody.



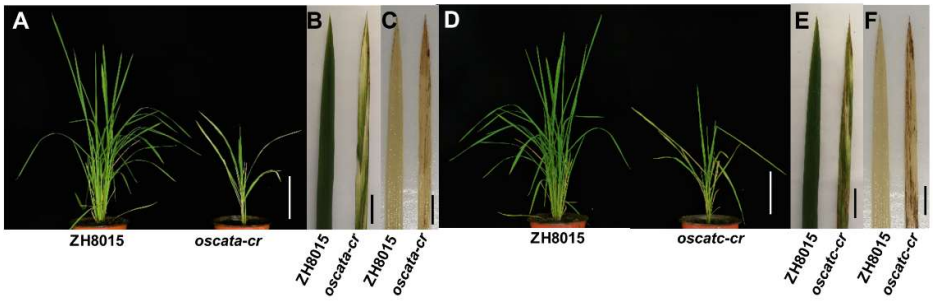
Supplemental Figure S6. OsCPK12 phosphorylates OsCATA and OsCATC *in vivo*.
OsCATA, OsCATB and OsCATC from rice protoplast proteins in 10-d-old ZH8015 and Cr-*OsCPK12* transgenic plants were separated by Phos-Tag SDS-PAGE and detected by immunoblot analysis using an anti-Myc antibody, anti-Actin were used in blotting.



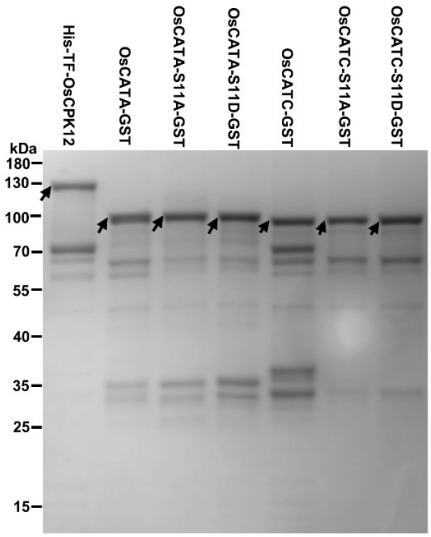
Supplemental Figure S7. OsCPK12 phosphorylates OsCATA-N and OsCATC-N, but does not phosphorylates OsCATA-C and OsCATC-C *in vitro*.

(A) OsCPK12 phosphorylates OsCATA and OsCATC *in vitro*. The input proteins OsCATA-GST, OsCATC-GST, and His-TF-OsCPK12 or His-TF-OsCPK12-Dead (denatured His-TF-OsCPK12) were detected by Coomassie Brilliant Blue (CBB) staining (lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag SDS-PAGE. **(B)** OsCPK12 phosphorylates OsCATA-N and OsCATC-N *in vitro*. The input proteins OsCATA-N-GST, OsCATC-N-GST, and His-TF-OsCPK12 or His-TF-OsCPK12-Dead (denatured His-TF-OsCPK12) were detected by CBB staining (lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag SDS-PAGE. **(C)** OsCPK12 phosphorylates OsCATA-N *in vitro*. The input proteins GST-OsCATA-N and His-TF-OsCPK12 were detected by CBB staining(lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag

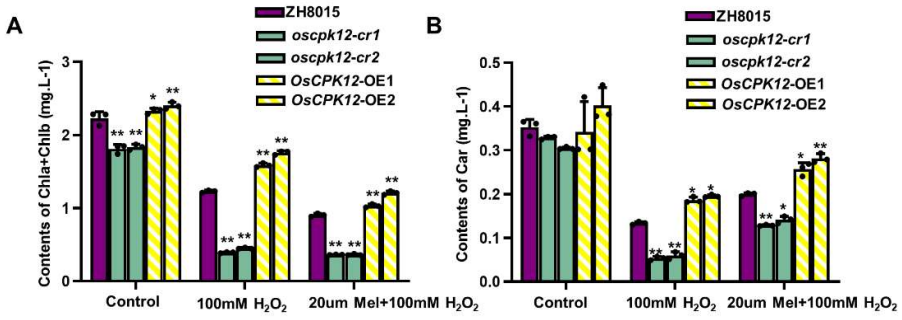
SDS-PAGE. OsCATA-N indicate 1-403 N-terminal amino acids of OsCATA. **(D)** OsCPK12 phosphorylates OsCATC-N *in vitro*. The input proteins GST-OsCATC-N and His-TF-OsCPK12 were detected by CBB staining (lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag SDS-PAGE. OsCATC-N indicate 1-403 N-terminal amino acids of OsCATC. **(E)** OsCPK12 cannot phosphorylate OsCATA-C *in vitro*. The input proteins GST-OsCATA-C and His-TF-OsCPK12 were detected by CBB staining (lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag SDS-PAGE. OsCATA-C indicate 404-493 C-terminal amino acids of OsCATA. **(F)** OsCPK12 cannot phosphorylate OsCATC-C *in vitro*. The input proteins GST-OsCATC-C and His-TF-OsCPK12 were detected by CBB staining (lower). Phosphorylation activity was detected by immunoblot analysis using Phos-Tag SDS-PAGE. OsCATC-C indicate 404-493 C-terminal amino acids of OsCATC.



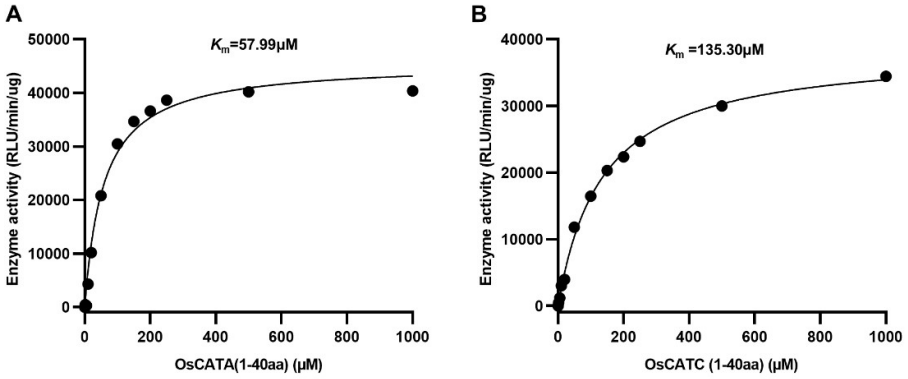
Supplemental Figure S8. The phenotypes of *oscata-cr* and *oscatc-cr* lines.
(A) The phenotypes of *oscata-cr* plants, Bar=20 cm. **(B)** The leaf phenotypes of in (A), Bar=2 cm. **(C)** DAB staining of leaves in (B), Bar= 2cm. **(D)** The phenotypes of *oscatc-cr* plants, Bar=20 cm. **(E)** The leaf phenotypes of in (D), Bar= 2cm. **(F)** DAB staining of leaves in **(E)**, Bar=2 cm.



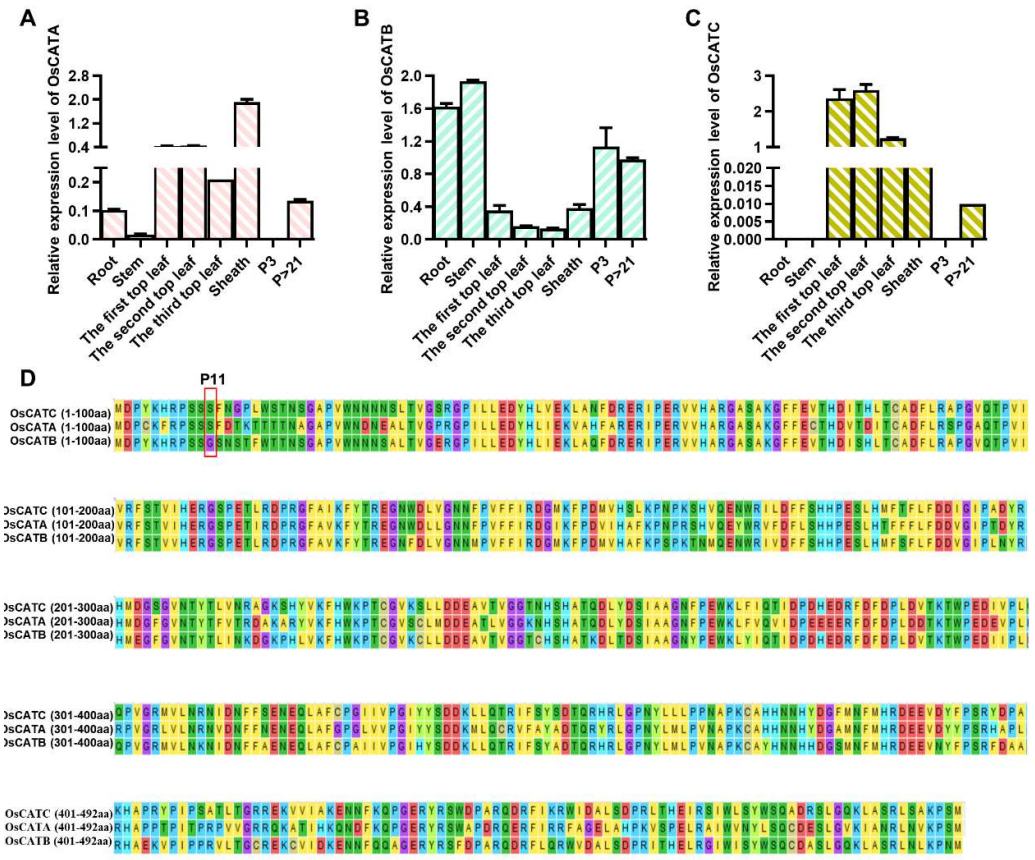
Supplemental Figure S9. SDS-PAGE analysis of purificated His-TF-OsCPK12, OsCATA and OsCATA^{S11A}, OsCATA^{S11D}, OsCATC, OsCATC^{S11A}, OsCATC^{S11D}.



Supplemental Figure S10. OsCPK12 positively affects oxidative tolerance in rice.
10-d-old seedlings were transplanted into medium supplemented with 100 mM H_2O_2 or 100 mM H_2O_2 and 20 μM melatonin for 6 d.
(A) Car content in leaves of rice plants subjected to H_2O_2 stress and H_2O_2 + melatonin stressed. Data are presented as mean±SD (n = 3). Data were analyzed using Student's t-test, **P*<0.05, ***P*<0.01. **(B)** H_2O_2 content in leaves of rice plants subjected to H_2O_2 stressed and H_2O_2 + melatonin stressed. Data were presented as mean±SD (n = 3). Data were analyzed using Student's t-test, **P*<0.05, ***P*<0.01.

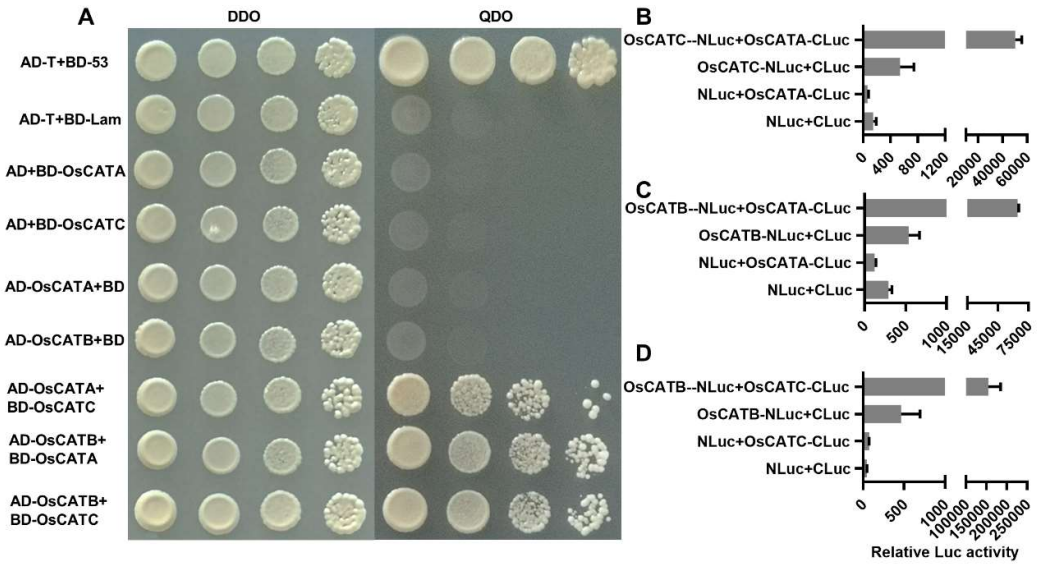


Supplemental Figure S11. Determination of K_m values of OsCPK12 towards substrate OsCATA(1-40aa) **(A)** and OsCATC(1-40aa) **(B)**
RLU values represent the decrease of sample against control, which are positively proportional to the kinase activity. Data are presented as mean±SD (n = 3).



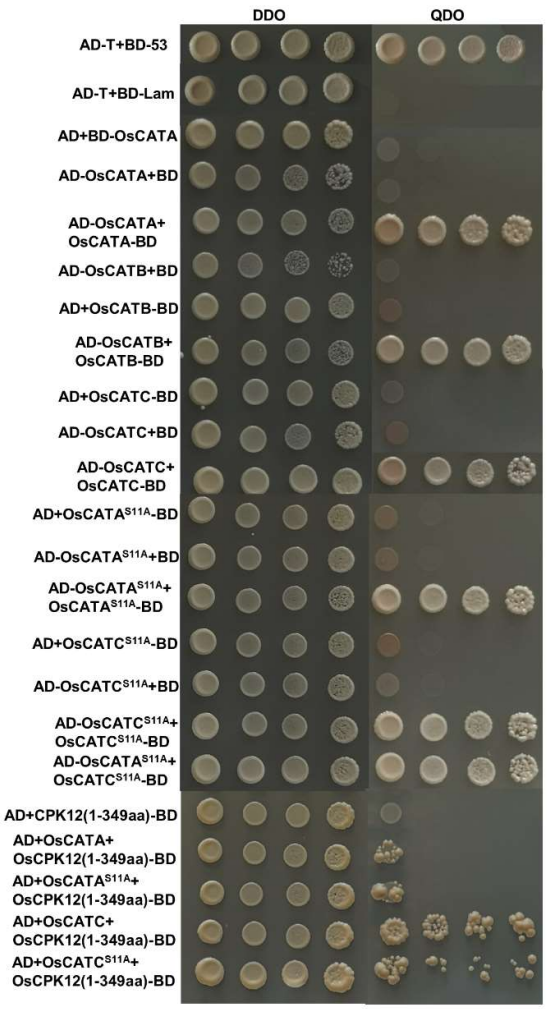
Supplemental Figure S12. Gene expression analysis of *OsCATA*, *OsCATB*, and *OsCATC*, and alignment of the amino acid sequence of the rice OsCATs family members.

(A) Gene expression analysis of *OsCATA*. Data are presented as mean±SD (n=3). **(B)** Gene expression analysis of *OsCATB*. Data are presented as mean±SD (n=3). **(C)** Gene expression analysis of *OsCATC*. Data are presented as mean±SD (n=3). P3, young panicles of 2-3cm; P>21, young panicles of more than 21cm. **(D)** The alignment of the amino acid sequence between the rice OsCATs family members. P11 indicates that OsCPK12 mediates the phosphorylation of OsCATA and OsCATC at Ser11.



Supplemental Figure S13. OsCATA, OsCATB, and OsCATC can interact with each other in yeast.

(A) Yeast two-hybrid assay of OsCATA, OsCATB and OsCATC interact with each other. **(B)** OsCATA interacts with OsCATC as indicated by luciferase complementation imaging (LCI) assay. Error bars represent the SD; n= 3. **(C)** OsCATA interacts with OsCATB as indicated by LCI assay. Error bars represent the SD; n= 3. **(D)** OsCATB interacts with OsCATC as indicated by LCI assay. Error bars represent the SD; n= 3.



Supplemental Figure S14. Yeast two-hybrid assay between non-phosphorylated OsCATA^{S11A} and OsCATC^{S11A} with OsCATs and OsCPK12.

Supplemental Tables

Supplemental Table S1. Phosphorylated residues of OsCATA and OsCATC by OsCPK12 *in vitro*, as revealed by LC-MS/MS.

Sample	phosphorylation sites	Peptide score	Modified peptide sequence	Sequence coverage (%)
GST-OsCATA	T 351	31.77	VFAYADtQR	83.74
GST-OsCATA	T 105	20.27	FStVIHER	83.74
GST-OsCATA	S 11	47.59	FRPSSsFDTK	83.74
GST-OsCATA	S 10	47.59	FRPSsSFDTK	83.74
GST-OsCATA	S 437	33.49	YRsWAPDR	83.74
GST-OsCATA	S 104	20.27	FsTVIHER	83.74
GST-OsCATA	S 164	37.62	sHVQEYWR	83.74
GST-OsCATC	T 414	40.11	YPIPSATLtGR	80.28
GST-OsCATC	T 412	45.25	YPIPSAtLTGR	80.28
GST-OsCATC	T 351	26.79	IFSYSDtQR	80.28
GST-OsCATC	T 19	61.73	HRPSSSFNGPLWStNSGAPVWNNNNSLTVGSR	80.28
GST-OsCATC	S 11	79.28	HRPSSsFNGPLWSTNSGAPVWNNNNSLTVGSR	80.28
GST-OsCATC	S 10	79.28	HRPSsSFNGPLWSTNSGAPVWNNNNSLTVGSR	80.28
GST-OsCATC	S 9	79.28	HRPsSSFNGPLWSTNSGAPVWNNNNSLTVGSR	80.28
GST-OsCATC	S 347	54.93	IFsYSDTQR	80.28
GST-OsCATC	S 21	61.73	HRPSSSFNGPLWSTNsGAPVWNNNNSLTVGSR	80.28
GST-OsCATC	S 18	61.73	HRPSSSFNGPLWsTNSGAPVWNNNNSLTVGSR	80.28