Supplemental Material

A NAP-Family Histone Chaperone Functions in Abiotic Stress Response and Adaptation

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One Sentence Summary

A functional H3/H4 histone chaperone mediates abiotic stress adaptation via transcriptional regulation of diverse stress-related genes in rice.

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Predicted NLS in OsNAPL6 sequence

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MTAPADKGKKAKTDADGGAAEENEQIDGALVLSIEKLQEIQDELEKVNEE  50
ASDKVLEVEQKYSEIRRPVYLRRSDIIQTIPDFWLTAFLSHPLLSELLTE 100
EDQKMFKYLESVDVDDSKDVKSGYSITLTFSENPYFEDKELTKTYAFADD 150
GTTTINATCIKWKEGMEIANGNAKKKGSKRPLVEESFFTWFTDTEHKSLA 200
DGVQDEVAEIIKEDLWPNPLKYFNNEAEELGEDDDEEGSDADEGEEDEEE 250
EN                                                   252
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Predicted bipartite NLS

Pos.	Sequence	Score
6	DKGKKAKTDADGGAAEENEQIDGALVLSIEK	2.1
6	DKGKKAKTDADGGAAEENEQIDGALVLSIE	2.8

Figure S1: Sequence analysis of OsNAPL6 predicts a bipartite NLS near its N-terminus. The protein sequence of OsNAPL6 was searched for sequence elements which can potentially function as NLS using cNLS mapper. A putative bipartite NLS was found near the N-terminus of OsNAPL6 whereas no monopartite NLS was predicted. Putative NLS has been underlined and has also been shown (along with its position and score) in the bottom panel.

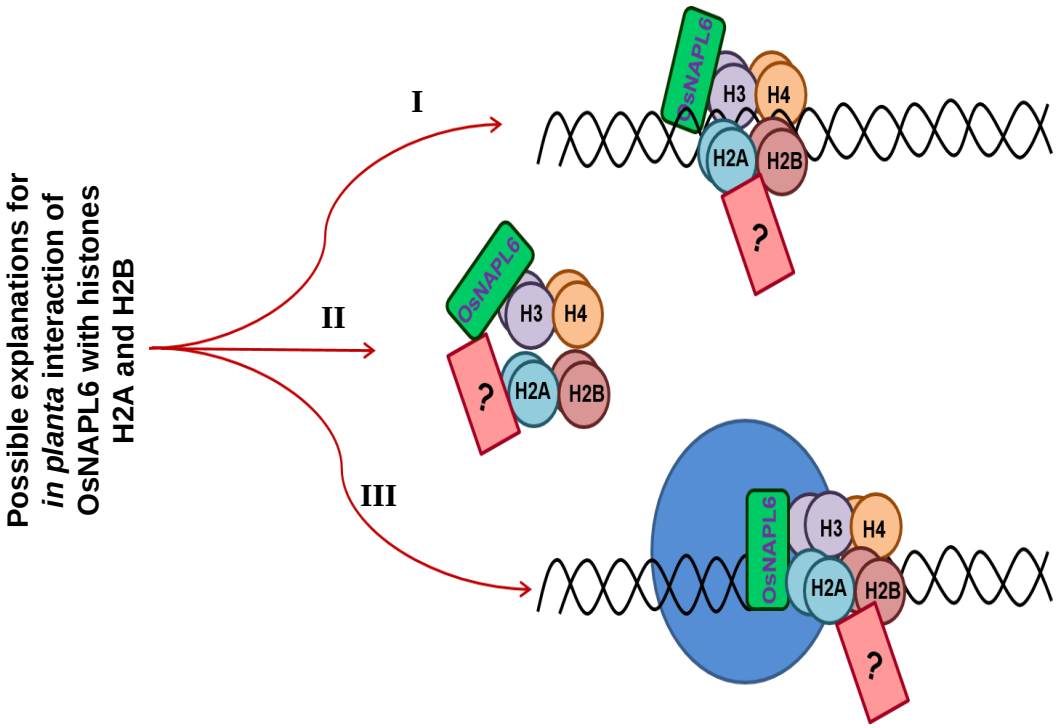

Figure S2: Model showing various possibilities explaining the apparent *in planta* interaction of OsNAPL6 with histones H2A and H2B. There are three possibilities which might explain the apparent interaction of OsNAPL6 with histones H2A and H2B as observed in Bimolecular Fluorescence Complementation (BiFC) assay. The first (marked as 'I') is that the histone chaperone OsNAPL6 interacts with DNA bound to all the four histones and hence the observed YFP fluorescence complementation is a result of the 'apparent' interaction which is mediated by DNA. The second (marked as 'II') is that the apparent interaction is mediated by an H2A-H2B histone chaperone (represented by a rectangle with question mark) which interacts with OsNAPL6. The third possibility (marked as 'III') is that OsNAPL6, all the four core histones, DNA and some yet unknown components together form a macromolecular complex leading to the apparent interaction of H2A-H2B with OsNAPL6.

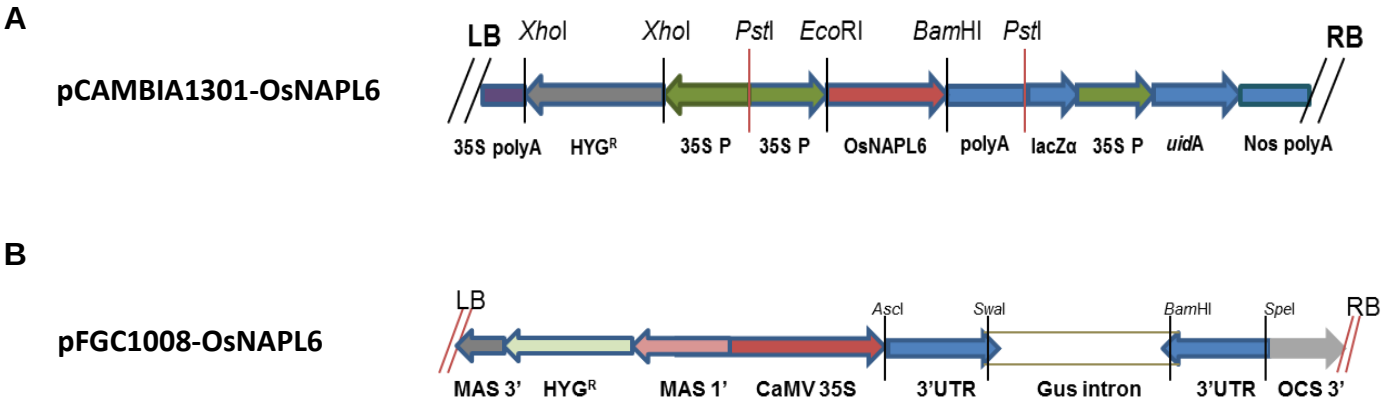

Figure S3: Schematic representation of OsNAPL6-overexpression and -knockdown vector constructs used for generation of transgenic rice plants overexpressing and underexpressing OsNAPL6. (A) For generation of the OsNAPL6-overexpression construct, the full-length coding sequence of *OsNAPL6* (from IR64 genotype) was cloned downstream to a 35S CaMV constitutive promoter in pCAMBIA1301 vector. **(B)** The shRNA (short hairpin RNA)-based OsNAPL6-knockdown construct was generated using a unique region from the 3' UTR of *OsNAPL6* which was cloned both in the sense and antisense direction in the RNAi vector pFGC1008. The pCAMBIA1301-OsNAPL6 overexpression and pFGC1008-OsNAPL6-RNAi (knockdown) constructs were then used for Agrobacterium-mediated transformation of rice.

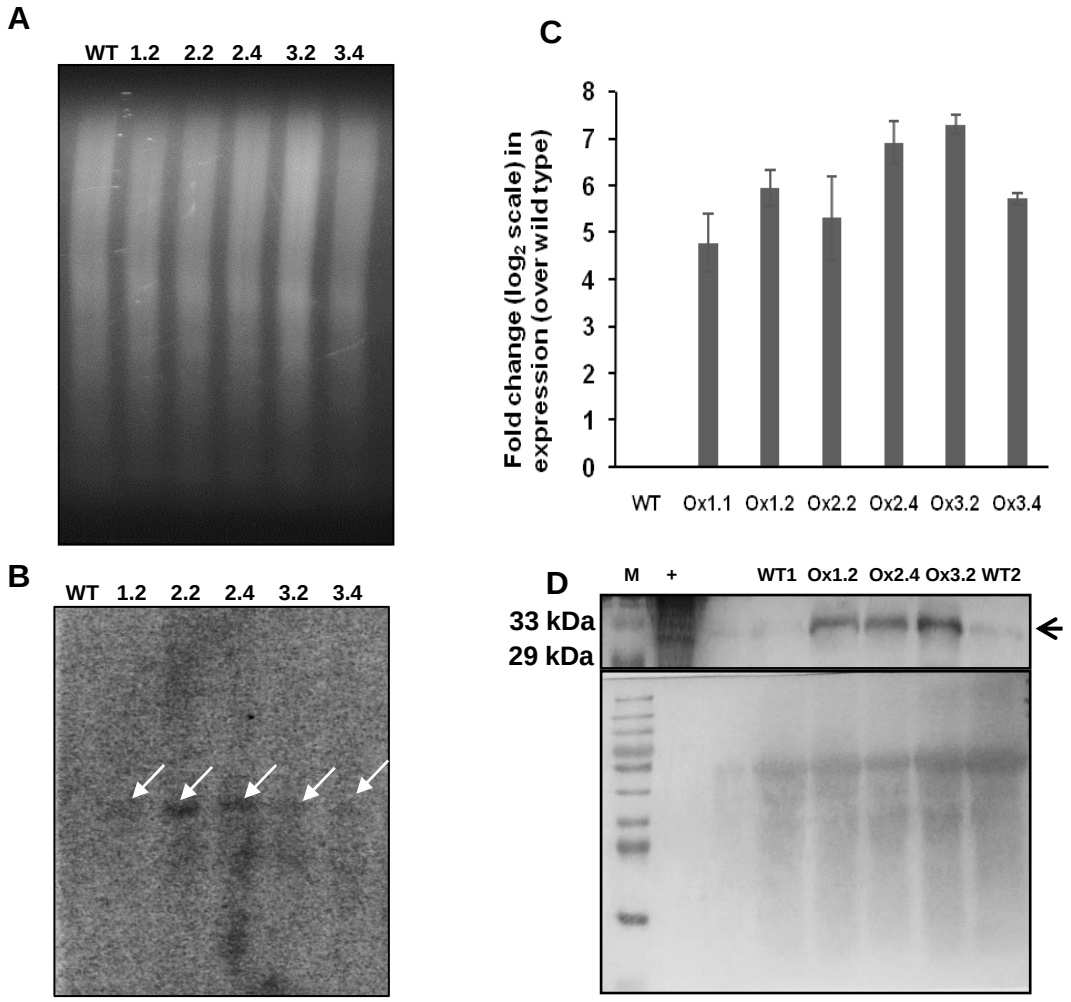

Figure S4: Molecular confirmation of OsNAPL6-overexpression transgenic plants via Southern hybridization, qRT-PCR and immunoblotting. (**A-B**) PCR-positive putative plants OsNAPL6-overexpression transgenic plants were further confirmed for their transgenic status by Southern blotting. For Southern blot analysis, 20 µg of genomic DNA from leaf tissue of putative transgenic plants (Ox) was digested with *Xho*I and run on a 0.8% agarose gel (**A**) followed by capillary transfer onto Nylon membrane. The corresponding blot (**B**) was probed with DIG-labeled *hpt*II (gene encoding hygromycin phosphotransferase) probe, the position of positive signals are shown by arrows. Numerals indicate transgenic line number; WT: wild type. **(C)** Overexpression of OsNAPL6 in the transgenic rice plants (Ox) was confirmed by qRT-PCR. For this, RNA was isolated from putative OsNAPL6-overexpression (Ox) as well as WT plants followed by first strand cDNA synthesis and real-time PCR. For normalization, OsNAPL6-UTR-specific primer set was used which would detect the endogenous gene only, while for quantitation OsNAPL6-CDS-specific primer set was used which would detect both the endogenous gene as well as the transgene. Numbers on the X- axis represent different transgenic lines. Data shown are mean fold change (over wild-type) ± SD (standard deviation); n=3. **(D)** For checking overexpression at the protein level, immunoblotting using anti-OsNAPL6 antibody was carried out for which, extract from rice leaves was subjected to SDS-PAGE followed by transfer onto a nitrocellulose membrane. The blot was first stained reversibly with Ponceau S (lower panel) followed by probing for OsNAPL6 (upper panel). Arrow marks the position of the band of interest. Numerals indicate transgenic line number; WT1 and WT2: represent extract from different wild type plants. '+' stands for positive control (lane loaded with recombinant 6x-His-OsNAPL6 protein).

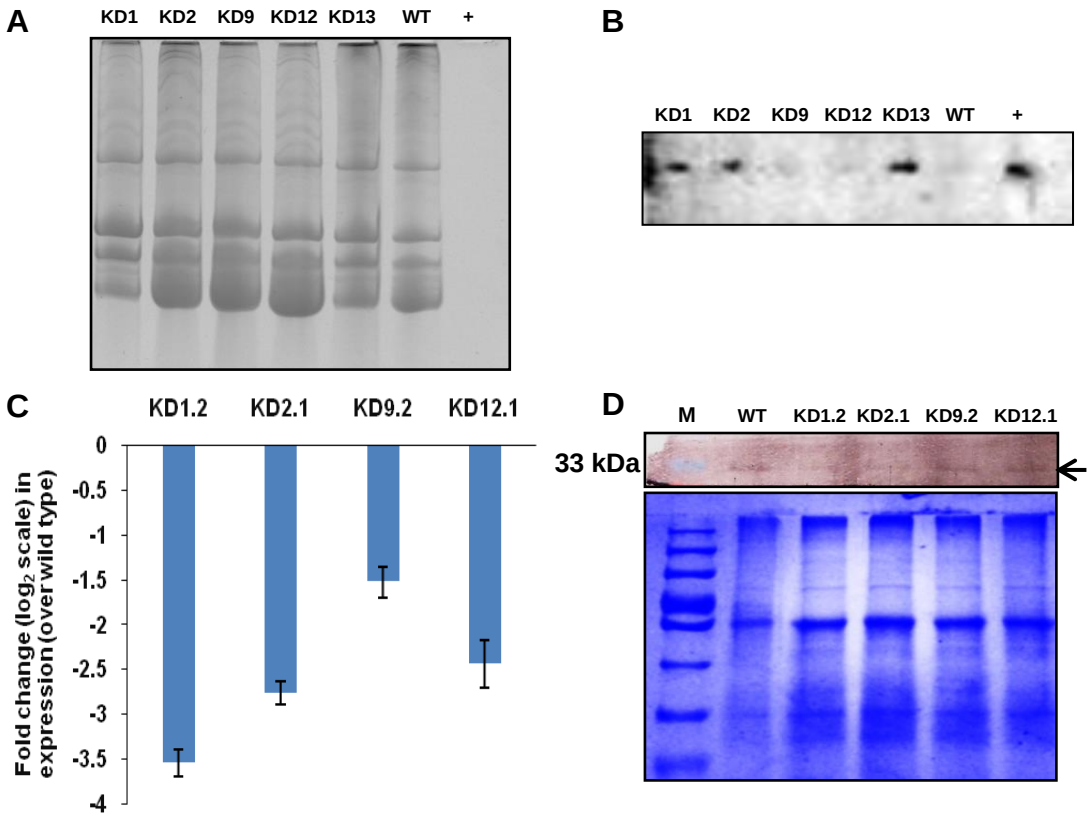

Figure S5: Confirmation of siRNA-mediated knockdown of OsNAPL6 in OsNAPL6-knockdown plants. The production of siRNAs against OsNAPL6 was checked by Northern blot analysis. For this, total RNA was isolated from WT and PCR-positive OsNAPL6-knockdown (KD) putative transgenic plants (T_1 generation) followed by enrichment of low molecular weight RNAs, resolution of the enriched fraction on a 15% denaturing PAGE **(A)** and electroblotting onto nylon membrane. The blot thus prepared was hybridized with α^{32}P-dATP-labelled OsNAPL6-UTR specific probe (79 bp) followed by detection on Phosphorimager **(B)**. '+' stands for positive control (lane loaded with 5 pg each of both the primers used for amplifying OsNAPL6-UTR-specific probe). **(C)** siRNA-mediated knockdown of OsNAPL6-expression in the KD plants (T_2 generation) was confirmed by qRT-PCR. For this, RNA was isolated from OsNAPL6-knockdown as well as WT plants followed by first strand cDNA synthesis and real-time quantitative PCR. For normalization, OsNAPL6-UTR-specific primer set was used which would detect the endogenous gene only, while for quantitation OsNAPL6-CDS-specific primer set was used which would detect both the endogenous as well as transgene. Numerals in labels on the X- axis represent different transgenic lines. Data shown are mean fold change (over wild-type) ± SD (standard deviation); n=3**. (D)** For checking reduction in expression of OsNAPL6 at the protein level, immunoblotting using anti-OsNAPL6 antibodies was carried out for which rice extract from rice leaves was subjected to SDS-PAGE followed either by Western blotting (upper panel) or Coomassie staining (lower panel). Numerals indicate transgenic line number; WT: wild type.

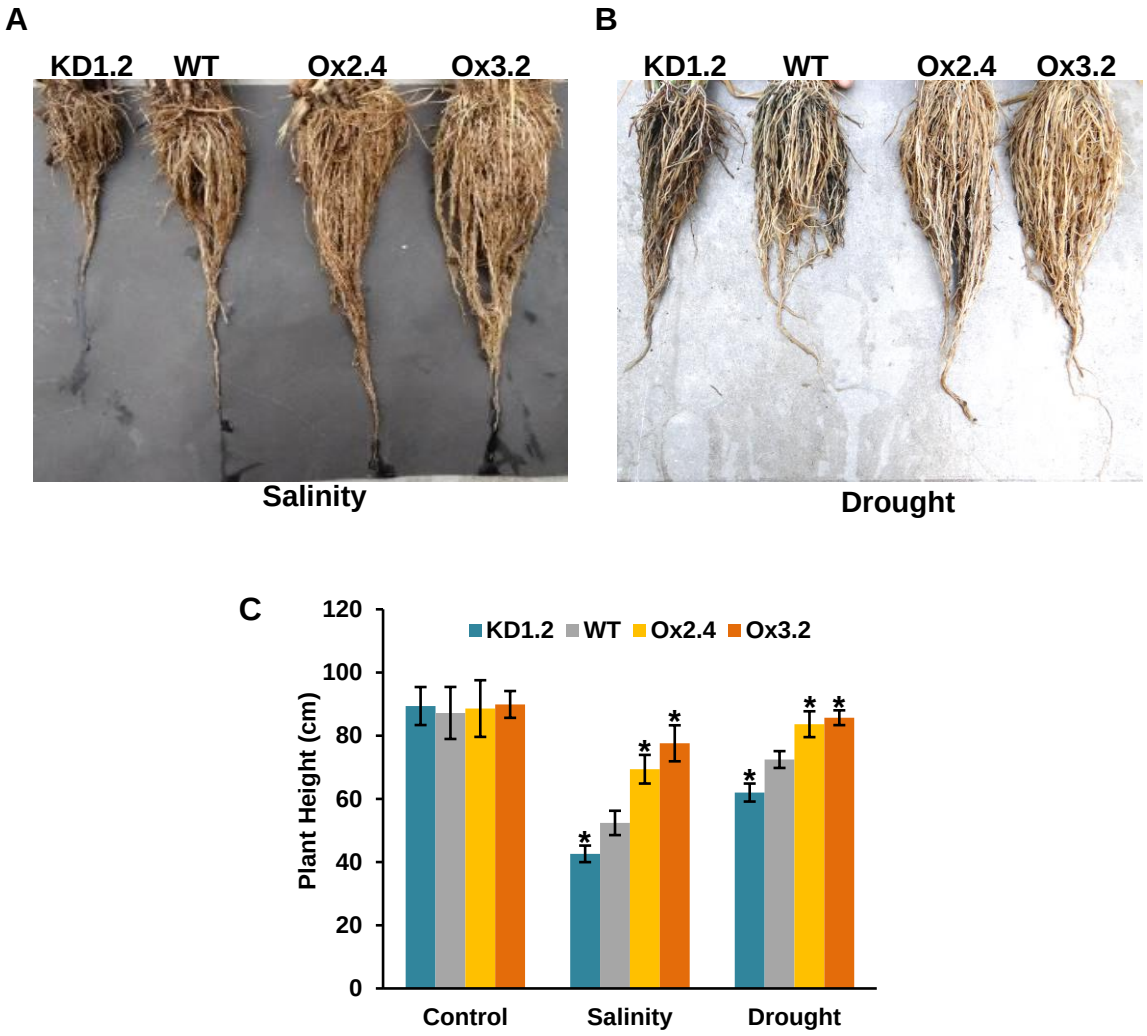

Figure S6: Root growth and plant height of wild type, OsNAPL6-overexpression and OsNAPL6-knockdown rice plants under salinity and drought stress conditions. Approximately two months old wild type, OsNAPL6-overexpression (lines Ox2.4 and Ox3.2) and OsNAPL6-knockdown (KD1.2) rice plants were subjected to salinity {regular fortnightly irrigation with a solution containing a mixture of salts leading to soil electrical conductivity (EC) of 10 dS/m} and drought stress. For drought stress, after 70 d of growth of plants under control conditions, water was withdrawn for 12 d following which the plants were recovered by rewatering. At maturity, the growth of the plants grown under salinity and drought (post-recovery) stress conditions was assessed via root growth **(A** and **B)** and plant height **(C)**. Plants irrigated with water served as controls. Data shown **(C)** are mean ± standard deviation; n=3. Statistically significant differences ($P < 0.05$), as compared to WT under the same condition, as tested by one-way ANOVA followed by post-hoc comparisons using Tukey-Kramer test are marked by '*'.

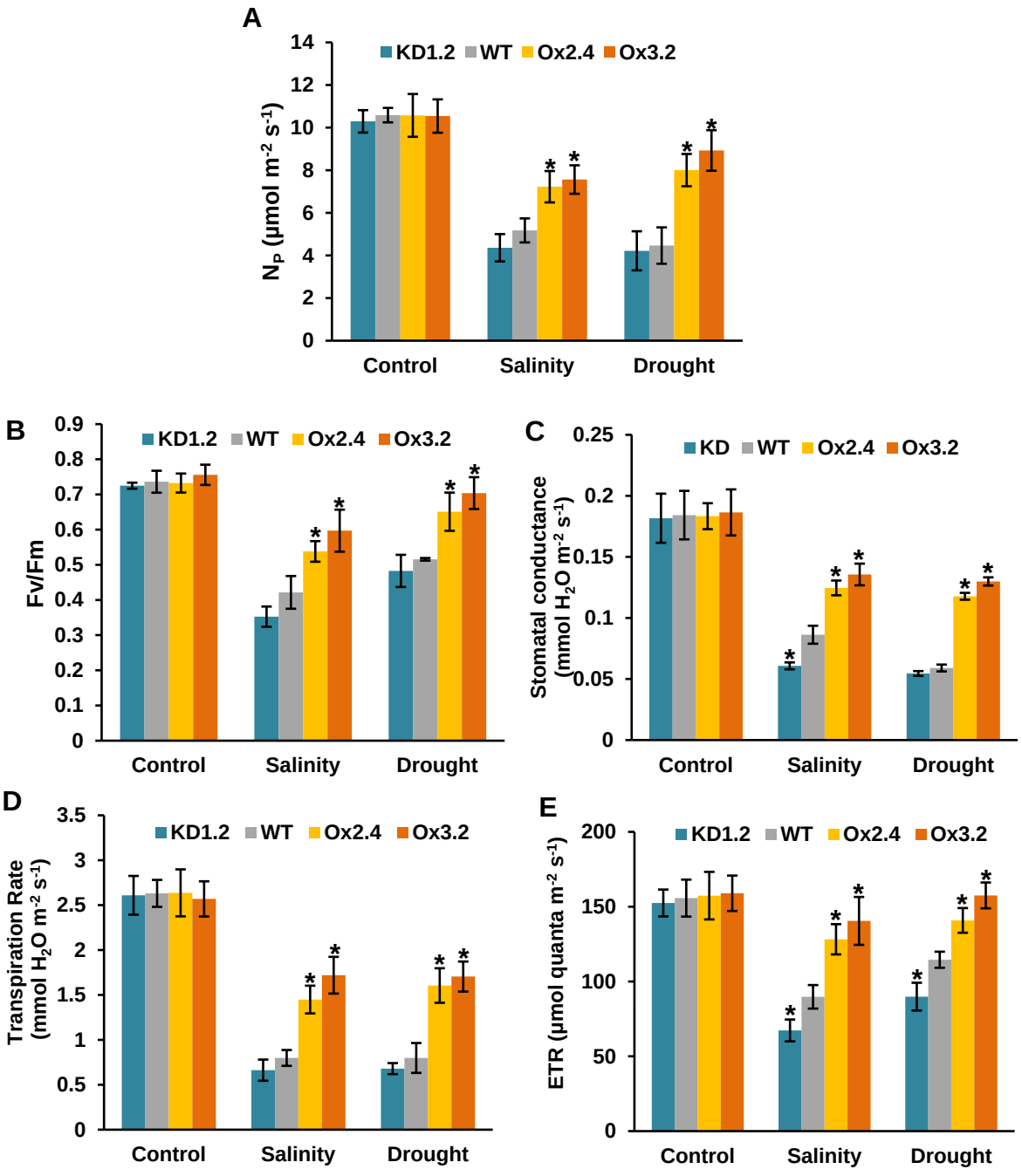

Figure S7: Various photosynthetic parameters of OsNAPL6-overexpression and OsNAPL6-knockdown rice plants under salinity stress and 3 days post-recovery from drought stress conditions. Approximately two months old wild type, OsNAPL6-overexpression (lines Ox2.4 and Ox3.2) and OsNAPL6-knockdown (KD1.2) rice plants were subjected to salinity stress {irrigation with a solution containing a mixture of salt leading to soil electrical conductivity (EC) of 10 dS/m} and drought stress. For drought stress, water was withdrawn for 12 d following which the plants were recovered by rewatering and grown to maturity. After 15 d of salinity stress and 3 days post-recovery from drought, various physiological parameters, viz. **(A)** net photosynthetic rate (N_P); **(B)** Fv/Fm; **(C)** stomatal conductance; **(D)** transpiration rate; **(E)** and electron transport rate (ETR); were measured and plotted as bar graphs. Plants irrigated with water served as controls. Data shown are mean ± standard deviation; n=3. Statistically significant differences ($P < 0.05$, as compared to WT under the same condition) as tested by one-way ANOVA followed by post-hoc comparisons using Tukey-Kramer test are marked by '*'.

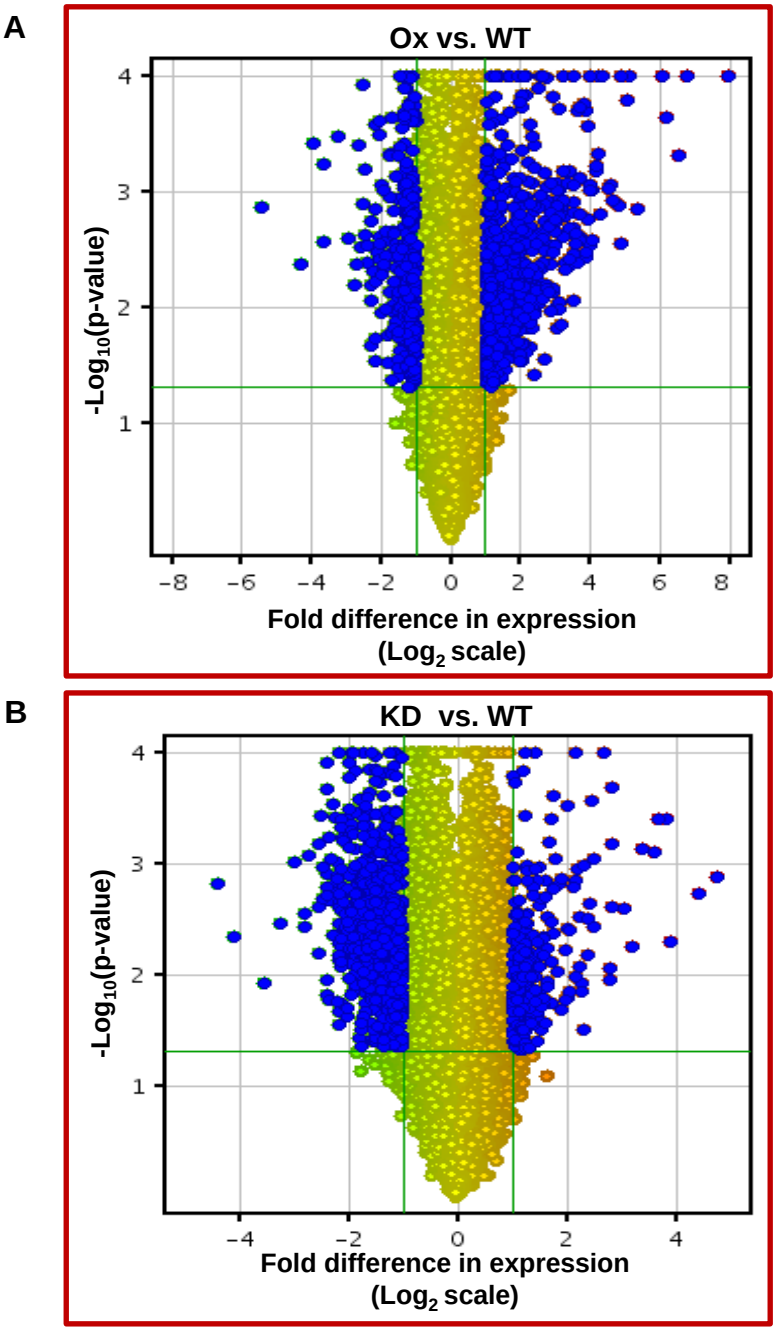

Figure S8: Volcano plot showing differentially expressed genes in OsNAPL6-overexpression and OsNAPL6-knockdown plants as compared to the wild type plants. X-axis represents fold difference in expression (in Log_2 scale) in Ox as compared to WT **(A)**, or KD as compared to WT **(B).** Y-axis represents the negative log_{10}-transformed P-values (with threshold set at $P < 0.05$ represented by the horizontal green line).

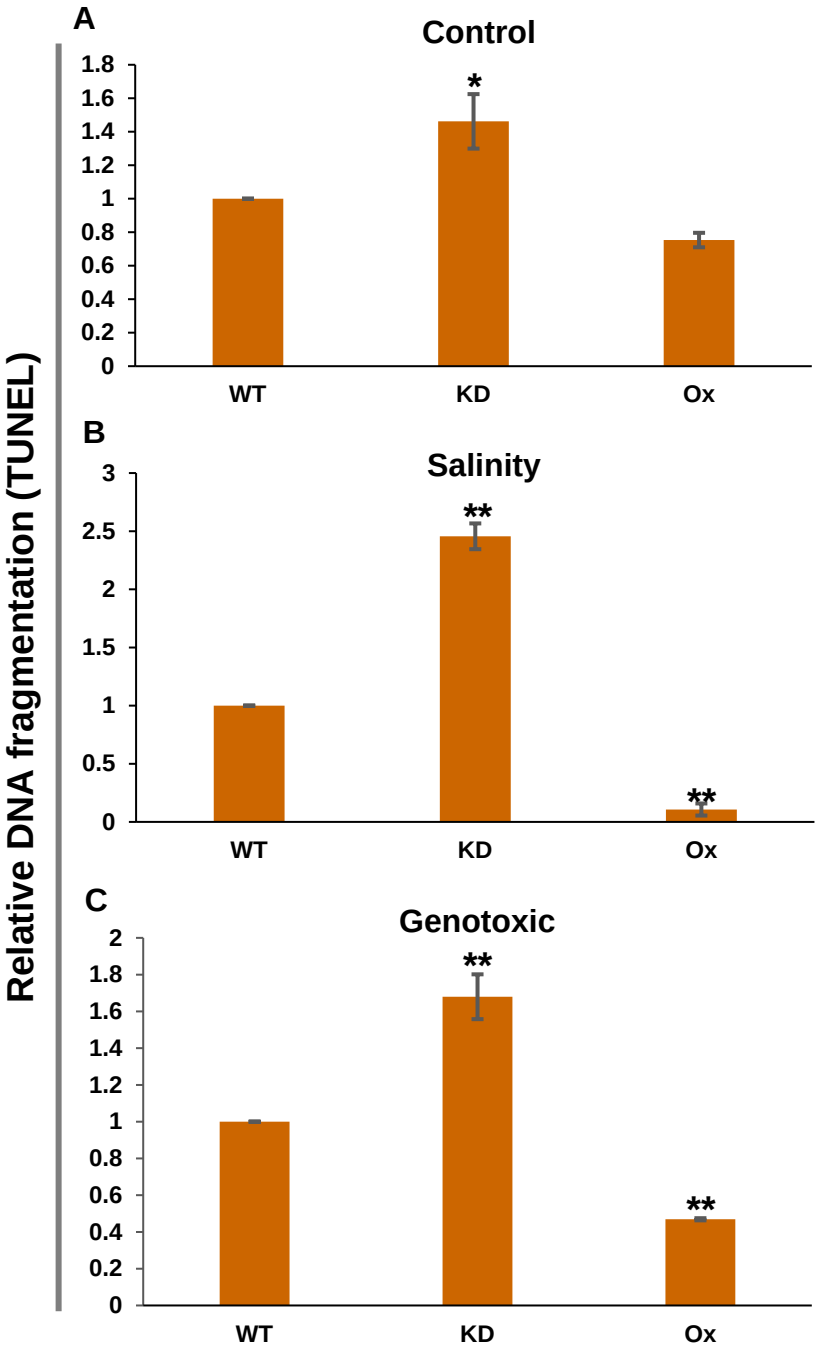

Figure S9: Extent of DNA fragmentation in root tips of OsNAPL6-overexpression and OsNAPL6-knockdown plants relative to the wild type plants as observed in TUNEL assay. Bar graphs show the extent of DNA fragmentation in the meristematic regions of roots of OsNAPL6-overexpression (Ox) and OsNAPL6-knockdown (KD) plants under control **(A),** salinity **(B)** and genotoxic **(C)** stress conditions with respect to that of the wild type (WT) plants under the respective conditions as observed in the TUNEL assay. The extent of DNA fragmentation was determined through measurement of fluorescence intensity (fluorescein) in the corresponding micrographs. Note that the incorporation of fluorescein-12-dUTP in the TUNEL reaction is directly proportional to the number of free DNA termini and hence is indicative of the extent of DNA fragmentation. Data shown is mean ± SE (standard error). * and ** represent statistically significant differences (as compared to WT under the same condition) at $P < 0.05$ and $P < 0.01$, respectively as tested by one-way ANOVA followed by post-hoc comparisons using Tukey-Kramer test.

Supplemental Table S1: Details of a few genes which were found to be differentially expressed in the high throughput comparative transcriptome analysis of OsNAPL6-overexpression, OsNAPL6-knockdown and wild type plants and were selected for further expression analysis by qRT-PCR. Amongst the genes which were found to be differentially-expressed in OsNAPL6-overexpression and OsNAPL6-knockdown plants as compared to the wild type, the following genes were selected on the basis of their known molecular, cellular and physiological function for further expression analysis by qRT-PCR**.** RGAP: Rice genome annotation project. See Figure 6, Supplemental Figure S8 and text in the 'Results' section.

Locus id	Nomenclature	Predicted product (RGAP7)	Known molecular function	Known function in stress	Reference
Genes functioning in DNA recombination and repair					
LOC_Os12g04980	*OsRad51*	DNA repair protein Rad51, putative, expressed	Involved in DNA repair	-	Wang et al. 2014
LOC_Os01g67510	*RecA-like*	recA protein, putative, expressed	Involved in homologous recombination	-	-
Genes with role in stress defense and response					
LOC_Os01g21250	*OsLEA9*	late embryogenesis abundant protein, putative, expressed	Macromolecular protection	Water stress (LEA family)	Wang et al. 2007
LOC_Os01g49720	*OsGST-U6*	glutathione S-transferase, putative, expressed	Redox balance	Oxidative stress	Edwards and Dixon, 2005
LOC_Os11g36960	*OsDnaJ/Hsp40*	DnaJ domain containing protein, expressed	Heat shock protein (Hsp40)	Heat stress	Sarkar et al. 2013
LOC_Os06g44220	*OsRCI2-9*	OsRCI2-9 - Putative low temperature and salt responsive protein, expressed	Stress-responsive transmembrane protein	Cold and salinity stress responsive	Medina et al. 2007
Genes encoding proteins involved in transcription with potential role in abiotic stress response					
LOC_Os09g35010	*OsDREB1C*	dehydration-responsive element (DRE)-binding protein, putative,	Transcription factor binding to DREs	Osmotic stress (due to cold and	Dubouzt et al. 2003

		expressed		drought)	
LOC_Os01g53040	*OsWRKY14*	WRKY14, expressed	Transcription factor	Abiotic stresses	Ramamoorthy et al. 2008
LOC_Os08g02070	*OsMADS26*	OsMADS26 - MADS-box family gene with MIKCc type-box, expressed	Transcription factor	Senescence, aging and cell death	Lee et al. 2008
LOC_Os09g13940	*OsAP2-63*	AP2 domain containing protein, expressed	APETALA 2 (AP2)-domain containing transcription factor	Osmotic stress response	Rashid et al. 2012
LOC_Os01g04110	*OsMed22*	mediator of RNA polymerase II transcription subunit 22, putative, expressed	Mediator of transcription	-	-
LOC_Os04g44440	*OsTCP10*	TCP family transcription factor, putative, expressed	Plant specific transcription factor	-	Yao et al. 2007
LOC_Os10g25850	*OsNF-YA8*	nuclear transcription factor Y subunit, putative, expressed	Nuclear transcription factor	Responsive to multiple abiotic stresses	Xu et al. 2014; Petroni et al. 2012
LOC_Os02g13310	*OsHDCP/BEL 1-like*	homeobox domain containing protein, expressed	TALE homeodomain transcription factor	-	-

Supplemental Methods

Cloning of OsNAPL6, its NLS-deleted mutant and histones

Standard procedure was followed for cloning various sequences. Sequences of different primers used for PCR amplification (using cDNA prepared from IR64 rice leaf tissue) for cloning are given below (Fw: forward; Re: reverse):

Name	Sequence (5'-3')	Purpose
OsNAPL6_Xba_Loc_Fw	GCTCTAGAATGACGGCGCCGGCGGACA	*In planta* localization of OsNAPL6
OsNAPL6_BamHI_Loc_Re	CGCGGATCCGCGTTCTCCTCCTCCTCATC	
OsNAPL6ΔN_Xba_Loc_Fw	CGGGATCCATGATCGAGAAGCTCCAGGAGATCC	Localization of the NLS-deleted mutant of OsNAPL6
OsNAPL6_BamHI_Loc_Re	CGCGGATCCGCGTTCTCCTCCTCCTCATC	
OsNAPL6_EcoRI_Fw	CGGAATTCATGACGGCGCCGGCGGACA	For cloning in pRT101
OsNAPL6_BamHI_Re	CGCGGATCCCTATCAGTTCTCCTCCTCCTCATC	
OsNAPL6_BamHI_Fw	CGCGGATCCATGACGGCGCCGGCGGACA	For cloning in pET28a and pGEX-4T-1
OsNAPL6_EcoRI_Re	CGGAATTCCTATCAGTTCTCCTCCTCCTCATC	
OsNAPL6_RNAi_AscI_Fw	GGCGCGCCTGTGGTCGCGCTGGCATTGT	For cloning OsNAPL6-UTR part (217 bp) in RNAi vector pFGC1008
OsNAPL6_RNAi_Swa_Re	ATTTAAATACCACCGTGTGGTTTGACGGT	
OsNAPL6_RNAi_SpeI_Fw	GACTAGTCTGTGGTCGCGCTGGCATTGT	
OsNAPL6_RNAi_BamHI_Re	CGGGATCCACCACCGTGTGGTTTGACGGT	
OsNAPL6_ HindIII_Fw	CCAAGCTTATGACGGCGCCGGCGGACA	Cloning of OsNAPL6 in split-YFP BiFC vector pSATN-cEYFP-N1
OsNAPL6_ EcoRI_Re	CGGAATTCTCAGTTCTCCTCCTCCTCATC	
H2A_HindIII_Fw	CCAAGCTTATGGCTGGTAGGGGCAAGGCGATC	Cloning of histone H2A in split-YFP BiFC vector pSATN-nEYFP-N1
H2A_EcoRI_Re	CGGAATTCTACTCGTCGTCGGCGGCGGC	
H2B_HindIII_Fw	CCAAGCTTATGGCGCCCAAGGCCGAGAAG	Cloning of histone H2B in split-YFP BiFC vector pSATN-
H2B_EcoRI_Re	CGGAATTCAGAGCTGGTGAACTTGGTG	

		nEYFP-N1
H3.1_HindIII_Fw	CCAAGCTTATGGCCCGCACCAAGCAGACG	Cloning of histone H3.1 in split-YFP BiFC vector pSATN-nEYFP-N1
H3.1_EcoRI_Re	CGGAATTCGGCCCTCTCGCCACGGATG	
H4_HindIII_Fw	CCAAGCTTATGTCTGGGCGCGGCAAGGGA	Cloning of histone H4 in split-YFP BiFC vector pSATN-nEYFP-N1
H4_EcoRI_Re	CGGAATTCGCCGCCGAAGCCGTAGAGG	

Subcellular localization by creating C-terminus GFP fusion and transient expression in onion peel epidermal cells

Subcellular localization of OsNAPL6 and its NLS-deleted mutant (OsNAPL6ΔNLS) was studied by gold particle bombardment-mediated transient expression in onion peel epidermal cells. For this, the coding sequences of *OsNAPL6* and *OsNAPL6ΔNLS* were cloned downstream to 35SCaMV promoter in plant localization vector pMBPII as done previously (Singh et al. 2012). Transcription and translation of the coding region in the vector thus generated (pMBPII with the gene for the protein whose localization is to be studied) would lead to the synthesis of plant protein-GFP fusion protein (GFP fused at the C-terminus). The resultant plasmid (pMBPII-OsNAPL6 or pMBPII-OsNAPL6ΔNLS) and the vector plasmid (pMBPII) were bombarded individually onto onion peel epidermal cells using biolistic method (Singh et al., 2012) with the help of macrocarrier and microcarriers. After 16 h of incubation at 28°C, slides were prepared and the peels were mounted using ProLong® Gold Antifade Mountant with DAPI (Molecular Probes, Life Technologies, USA). The localization was examined under a fluorescence microscope using GFP and DAPI filters (Axio Observer, Carl Zeiss, Germany).

GST-pull down assay for analyzing the histone specificity of OsNAPL6

OsNAPL6 coding sequence was cloned downstream to a GST gene in the bacterial expression vector pGEX-4T-1 (GE Healthcare, USA) at *Bam*HI and *Eco*RI restriction sites. The construct was transformed in *E. coli* BL-21 (DE3) strain and the expression was induced using 0.5 mM IPTG followed by cell lysis. Recombinant GST-tagged OsNAPL6 (GST-OsNAPL6) was

purified via glutathione sepharose affinity chromatography following standard procedure. The lysate was incubated with pre-equilibrated glutathione sepharose 4B beads for 30 min followed by washing with 10 column volumes of wash buffer (20 mM sodium phosphate pH 7.2, 300 mM NaCl, 5% Glycerol, protease inhibitor cocktail, Sigma Aldrich, USA). The protein was eluted using Elution buffer (20 mM sodium phosphate pH 7.2, 300 mM NaCl, 5% Glycerol, 20 mM reduced glutathione, protease inhibitor cocktail). Purity of the recombinant protein was determined by resolving it on a 12% SDS PAGE gel followed by staining with Coomassie Brilliant Blue. The protein was snap frozen in liquid nitrogen and stored at -80ºC until use.

For pull down assay, 2 µg of purified GST-OsNAPL6 was immobilized on 40 µl of glutathione-sepharose 4B beads (GE Healthcare, USA) pre-equilibrated in binding buffer (20 mM Tris-Cl pH 7.5, 0.15 M NaCl, 2 mM $MgCl_2$). GST-OsNAPL6 fusion protein immobilized on glutathione-sepharose beads was incubated, in a high stringency TNMT binding-cum-wash buffer (20 mM Tris-Cl pH 7.5, 0.5 M NaCl, 2 mM $MgCl_2$, 1% Triton X-100), with 1 µg each of H2A and H2B, or H3 and H4, or with all the four core histones (H2A, H2B, H3 and H4). Purified GST protein and heat-denatured OsNAPL6 immobilized on glutathione-sepharose beads were incubated with all the four core histones (H2A, H2B, H3 and H4) and these pull down mixtures served as negative controls. To remove non-specific binding, glutathione-sepharose beads bound to either GST, GST-NAPL6, or heat-denatured GST-NAPL6, were washed extensively with the binding-cum-wash buffer. Beads were recovered by centrifugation at 12000 X g for 10 min. Binding of GST or GST-OsNAPL6 with glutathione beads was reversed by addition of equal volumes of 2x Laemmli buffer followed by heating for 5 min at 95°C. The eluted proteins were resolved on a 15% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue.

***In planta* interaction using Bimolecular Fluorescence Complementation**

Bimolecular Fluorescence Complementation (BiFC) is based on the association of fragments of a fluorescent protein such as YFP (the fragments in isolation are non-fluorescent) when they associate (or come in very close proximity) on account of the interaction of two interacting proteins which have the non-fluorescent fragments (such as split-YFP tags) tagged to them (Citovsky, 2006; Kerppola, 2006). For BiFC, full length coding sequences of the putative

interacting proteins were cloned in complementary split-EYFP vectors pSATN-nEYFP-N1 and pSATN-cEYFP-N1 (Citovsky, 2006) at *Hind*III and *Eco*RI sites and the resultant plasmids were co-transformed into onion peel epidermal cells using biolistic bombardment method as described in the procedure for subcellular localization. After 16 h of incubation at 28°C, slides were prepared and the peels were mounted using ProLong® Gold Antifade Mountant with DAPI (Molecular Probes, Life Technologies, USA). Interaction was analyzed by observing EYFP (Enhanced yellow fluorescent protein) fluorescence under a fluorescence microscope (Axio Observer, Carl Zeiss, Germany).

Generation of transgenic plants overexpressing (Ox) and underexpressing (KD) OsNAPL6

For generating the overexpression construct for plant transformation, *OsNAPL6* coding sequence (759 bp) was PCR amplified using rice cDNA as the template and using primers with *Eco*RI and *Bam*HI restriction sites at the 5' end of the forward and reverse primers, respectively (see Table in the Cloning section above). The amplified fragment was then cloned in pRT101. Using pRT101-OsNAPL6 plasmid, the plant expression cassette comprising CaMV35S promoter-OsNAPL6-polyA was isolated by restriction digestion with *Pst*I. This cassette was then moved to plant transformation vector pCAMBIA1301 in which it was cloned at *Pst*I site. The pCAMBIA1301-OsNAPL6 plant transformation construct (**Figure S3a**) was used to over-express OsNAPL6 in *Oryza sativa* cv. IR64 using the transgenic approach.

To knockdown the expression of OsNAPL6, we chose an shRNA (short hairpin RNA)-based strategy to generate RNAi plants, for which a specific region from the 3'-UTR of OsNAPL6 was cloned both in the sense and antisense direction in the plant RNAi vector pFGC1008 (**Figure S3b**) using primers listed in the cloning section above.

For rice transformation, a methodology developed previously (Sahoo et al., 2011) was followed. The method utilized mature seed-derived scutellar calli which were Agro-infected followed by three rounds of selection in Hygromycin-containing medium. Regeneration of the transformed calli thus selected led to the development of putative transgenic plantlets which were further screened for their transgenic status.

Molecular confirmation of putative Ox and KD plants for their transgenic status

Putative transgenic plants were screened by PCR using REDExtract-N-Amp™ Plant PCR Kit (Sigma Aldrich, USA) following manufacturer's protocol. PCR-positive Ox plants were further confirmed by Southern blotting (using standard procedure) and expression analysis of the transgene using qRT-PCR and immunoblotting. In the case of KD plants, the production of siRNAs against OsNAPL6 was confirmed by Northern blot analysis. For this, total RNA was isolated from 14 d old seedlings of WT and putative knockdown (KD) lines. Enrichment of low molecular weight RNAs was carried out by differential precipitation of high molecular weight RNA using 10% PEG 8000 in the presence of 500 mM NaCl. Low molecular weight RNA-enriched fraction, thus obtained, was resolved on a 15% denaturing PAGE followed by electroblotting onto nylon membrane. The blot thus prepared was hybridized with α^{32}P-dATP-labelled OsNAPL6-UTR specific probe. For preparation of the probe, a fragment from the 3'UTR region of OsNAPL6 was PCR amplified using the primers: forward (5'-GCCAGTCTGATGGGCTGTTATT-3') and reverse (5'-GCACGGTTTCGTAAACAATGATC-3'). Pre-hybridization, hybridization and washing steps were carried out at 37°C followed by detection on Phosphorimager (GE Healthcare, USA) using standard procedure. Plants showing the production of siRNAs against OsNAPL6 were then confirmed for knockdown of OsNAPL6 expression using qRT-PCR and immunoblotting.

Leaf strip senescence assay and chlorophyll and carotenoid estimation

Healthy leaves of rice plants (both wild type and transgenic – overexpression and knockdown) of the same age and approximately same growth were selected for the leaf strip senescence assay. Leaf strips of two centimeter length were cut from each plant and floated on either aqueous solution containing different stress agents (200 mM NaCl, 5% PEG, 500 mM Mannitol, 5 mM H_2O_2) or sterile distilled water (for control). These leaf strips were used for measuring chlorophyll and carotenoid content. Chlorophyll and carotenoid content from the leaf strips was estimated spectrophotometrically after extraction in 80% acetone following the procedure described elsewhere (Arnon, 1949). Experiment was repeated thrice with three replicates each time (n=3).

High-throughput comparative transcriptome profiling

Microarray-based high-throughput comparative transcriptome profiling was carried out using leaf tissue from 12 d old Ox, KD and WT seedlings of the T_2 generation (grown under control conditions) as the starting material following a procedure as described previously (Kaur et al., 2015). Briefly, total RNA was isolated from 100 mg of leaf tissue corresponding to each of the samples using Trizol reagent (Life Technologies, USA) following the manufacturer's protocol. Following standard quality control, cDNA was synthesized. Labelling, hybridization onto Affymetrix 57K rice GeneChip (Affymetrix Inc., USA), washing, scanning and further processing was carried out following the steps described in Affymetrix GeneChip Expression Analysis Technical Manual (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf).

Normalization and probe summarization was carried out using Robust Multiarray Average algorithm. Normalized expression, thus obtained, was $\log_2$-transformed and the $\log_2$-tranformed expression values were used for further analysis. Differentially expressed genes in either OsNAPL6-overexpression (Ox) or -knockdown (KD) plants relative to the wild type (WT) plants were identified using unpaired t-test followed by adjusting for multiple-testing using the Benjamini-Hochberg method to correct for the false discovery rate. Genes showing a minimum fold difference in expression (with respect to the WT) of two folds ($\log_2$ fold difference in expression ≥ 1 for upregulation and $\log_2$ fold difference in expression ≤ -1 for downregulation; $P < 0.05$) were considered to be showing significant differential expression in the respective plant type (Ox or KD).

qRT-PCR

qRT-PCR was carried out as described previously (Tripathi et al. 2015) using primers listed below.

Primer Name	**Sequence (5'-3')**
OsNAPL6_qPCR_UTR_Fw	GCCAGTCTGATGGGCTGTTATT
OsNAPL6_qPCR_UTR_Re	GCACGGTTTCGTAAACAATGATC
OsNAPL6_qPCR_CDS_Fw	GCGCCCTCGTCCTCTCCATC
OsNAPL6_qPCR_CDS_Re	CCACATCGACAGACTCCAGGTACT

OsGST_qPCR_Fw	CTTTCTGCTTCGGGGATCTGT
OsGST_qPCR_Re	CACTGAACAGCGTGTCAACAT
OsWRKY14_qPCR_Fw	GCACACTGTTGATTGGAGAAAGG
OsWRKY14_qPCR_Re	TGGAGTTCCAATCCTTCTGTGT
OsRad51_qPCR_Fw	TGCAACCTCATTTATCCAGACGA
OsRad51_qPCR_Re	TCTGGGGAAAGTTACCATCATGT
OsMADS26_qPCR_Fw	TCCCGCAGGAAGTGTCTTTT
OsMADS26_qPCR_Re	TGTAATAAACGTTCCAGGAAACAA
OsTCP10_qPCR_Fw	TGCTTGATTTTAGGCCGGAGAA
OsTCP10_qPCR_Re	ACAGATGACAGAACCATGGCAA
OsHDCP/BEL1_qPCR_Fw	TCATTGGGAGATCGATGCGAA
OsHDCP/BEL1_qPCR_Re	GCCCCTAAGACGTACGCAAT
OsRecA_qPCR_Fw	GGATTCCTCGTCGGTTCTGG
OsRecA_qPCR_Re	CCCAAAACAATCGCACAGGA
OsDREB1C_qPCR_Fw	TGTGCCAGATTTCTCCCTCC
OsDREB1C_qPCR_Re	AAATCAGAGAAGAACTTGACCAAAA
OsLEA9_qPCR_Fw	GAGGAGAAGACGGCGTGG
OsLEA9_qPCR_Re	GCAGCCATCCTCTTGGAGTT
OsAP2-63_qPCR_Fw	ATTGTGGTGACAGACCGTCC
OsAP2-63_qPCR_Re	ACTATTCTTAGCTGTTCATTGCCT
OsNF-YA8_qPCR_Fw	TAGCAGCAAAGGTCAATCGC
OsNF-YA8_qPCR_Fw	AGCACACCGGCCAAATAGTTA
OsRCI2-9_qPCR_Fw	CTTCTGCAGCTCGGAGTTCG
OsRCI2-9_qPCR_Re	CGTCTTGAGGATGACGACCC
OsDNAJ_qPCR_Fw	GCCAACGTCAAAGCAGACTC
OsDNAJ_qPCR_Re	TTGGGCAAAACCGCAAAACA
OsMed22_qPCR_Fw	GTTGGGAACTTGGGATGCCT
OsMed22_qPCR_Re	GCAAACAGCTTCGTTTGGGT

Chromatin Immunoprecipitation (ChIP)

ChIP assay was performed according to Abcam ChIP manual with some modifications (Abcam USA; http://docs.abcam.com/pdf/chromatin/A-beginners-guide-to-ChIP.pdf). Briefly, 1 g of tissue from 12 d old rice seedlings of wild type as well as transgenic lines (grown under control and osmotic stress conditions) were washed thoroughly with double distilled water (ddH_2O) and fixed in 1% formaldehyde for 20 min by vacuum infiltration. The cross-linking reaction was stopped by the addition of 2 M glycine to attain a final concentration of 0.11 M, after which the tissue was washed twice with double-distilled water. The plant material was then crushed with liquid nitrogen to a fine powder using mortar and pestle and resuspended in 30 ml of pre-chilled extraction buffer 1 (400 mM Sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, and protease inhibitor cocktail, added just before use). Rest of the steps were performed strictly according to the protocol provided in the aforesaid manual. Antigen-purified anti-OsNAPL6 antibody was used for immunoprecipitation.

DNA was purified using QIAquick PCR purification kit following the manufacturer's protocol (Qiagen, GmBH). The DNA was eluted in 60 µl of ddH_2O and was analyzed by SYBR green based real-time qPCR with the indicated primer sets (see Table below). The data was normalized with the input DNA and the results were expressed as fold DNA enrichment over IgG control. The experiments were repeated thrice with three replicates each time (n=3).

Primer Name	**Sequence (5'-3')**
OsRad51_P_ChIP_Fw	CCGTTGCGTGTGTGAACAG
OsRad51_P_ChIP_Re	AATGCTCTTCCTGCCCTTGG
OsDREB1C_P_ChIP_Fw	GGTTGGAGACTTGGAGTTGGA
OsDREB1C_P_ChIP_Re	ACTCGTGCAGTCTCACCTTG
OsLEA9_P_ChIP_Fw	GGATGATCAACTTTCTTTCTCTCTC
OsLEA9_P_ChIP_Re	CCTTGCCTCTTGGACGTCTT

TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling) assay

Roots of 12 d old wild type as well as transgenic seedlings (OsNAPL6-overexpression and -knockdown) either control or treated with 200 mM NaCl, or 40 mg/l Aphidicolin (a DNA

damaging agent) for 48 h were fixed overnight at 4°C with 4% v/v paraformaldehyde (prepared in 1x PBS, pH 7.4). Next day, the fixed roots were washed twice with 100% ethanol at room temperature and incubated in 70% ethanol for 24 h at 4°C. These samples were then incubated in 1x PBS (at room temperature) and the solution was changed thrice (after every 20 min). Following this, the root samples were incubated in 100 mM sodium citrate buffer (pH 6.0) for 15 min followed by application of microwave (350 W) for 40 seconds. The samples were cooled rapidly by adding double-distilled water. Thereafter, the samples were incubated in permeabilization solution (0.1% Triton X-100 in 100 mM sodium citrate buffer, pH 6.0) at 37°C for 30 min. Proteins were digested using proteinase K (20 µg/ml final concentration, in 10 mM Tris-Cl, pH 7.5 in a total volume of 100 µl) at 37°C for 30 min. Then the root samples were washed with 1x PBS at room temperature.

For *in situ* TUNEL reaction, roots were cut approximately 1 cm length from the root tip and the TUNEL reactions were performed in microcentrifuge tubes (2 ml) using the DeadEnd™ Fluorometric TUNEL System (Promega, USA) following the manufacturer's instructions with some modifications. The reaction mix consisted of 100 µl equilibration buffer, 10 µl of fluorescein-12-dUTP -containing nucleotide mix and 1 µl of recombinant TdT (Terminal deoxynucleotidyl transferase) (Promega, USA). The reaction was carried out at 37°C for 1 h following which it was stopped by adding 1 ml of 2x Saline-sodium citrate buffer (2x SSC: 300 mM NaCl in 30 mM sodium citrate, pH 7.0). The root tips were then mounted onto slides with Prolong Gold Antifade reagent with DAPI (Life Technologies, USA) and the slides were visualized under a confocal microscope (Nikon A1R, Nikon, Japan) using a 20x objective.

Supplemental References

Arnon, D.I. (1949). Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in Beta Vulgaris. Plant Physiol. *24*, 1-15.

Citovsky, V., Lee, L.Y., Vyas, S., Glick, E., Chen, M.H., Vainstein, A., Gafni, Y., Gelvin, S.B., and Tzfira, T. (2006). Subcellular localization of interacting proteins by bimolecular fluorescence complementation in planta. J Mol. Biol. *362*, 1120-1131.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt-and cold-responsive gene expression. Plant J. *33*, 751-763.

Edwards, R., and Dixon, D.P. (2005). Plant glutathione transferases. Methods Enzymol. *401*, 169-186.

Kaur, C., Kushwaha, H.R., Mustafiz, A., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2015). Analysis of global gene expression profile of rice in response to methylglyoxal indicates its possible role as a stress signal molecule. Front. Plant Sci. *6*, 682.

Kerppola, T.K. (2006). Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat. Protoc. *1*, 1278-1286.

Lee, S., Woo, Y.M., Ryu, S.I., Shin, Y.D., Kim, W.T., Park, K.Y., Lee, I.J., and An, G. (2008). Further characterization of a rice AGL12 group MADS-box gene, OsMADS26. Plant Physiol. *147*, 156-168.

Medina, J., Ballesteros, M.L., and Salinas, J. (2007). Phylogenetic and functional analysis of Arabidopsis RCI2 genes. J Exp. Bot. *58*, 4333-4346.

Petroni, K., Kumimoto, R.W., Gnesutta, N., Calvenzani, V., Fornari, M., Tonelli, C., Holt, B.F., 3rd, and Mantovani, R. (2012). The promiscuous life of plant NUCLEAR FACTOR Y transcription factors. Plant Cell *24*, 4777-4792.

Ramamoorthy, R., Jiang, S.Y., Kumar, N., Venkatesh, P.N., and Ramachandran, S. (2008). A comprehensive transcriptional profiling of the WRKY gene family in rice under various abiotic and phytohormone treatments. Plant Cell Physiol. *49*, 865-879.

Rashid, M., Guangyuan, H., Guangxiao, Y., Hussain, J., and Xu, Y. (2012). AP2/ERF Transcription Factor in Rice: Genome-Wide Canvas and Syntenic Relationships between Monocots and Eudicots. Evol. Bioinform. Online *8*, 321-355.

Sahoo, K.K., Tripathi, A.K., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2011). An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. Plant Methods *7*, 49.

Sarkar, N.K., Kim, Y.K., and Grover, A. (2014). Coexpression network analysis associated with call of rice seedlings for encountering heat stress. Plant Mol. Biol. *84*, 125-143.

Singh, A.K., Kumar, R., Pareek, A., Sopory, S.K., and Singla-Pareek, S.L. (2012). Overexpression of rice CBS domain containing protein improves salinity, oxidative, and heavy metal tolerance in transgenic tobacco. Mol. Biotechnol. *52*, 205-216.

Tripathi, A.K., Singh, K., Pareek, A., and Singla-Pareek, S.L. (2015). Histone chaperones in Arabidopsis and rice: genome-wide identification, phylogeny, architecture and transcriptional regulation. BMC Plant Biol. *15*, 42.

Wang, X.-S., Zhu, H.-B., Jin, G.-L., Liu, H.-L., Wu, W.-R., and Zhu, J. (2007). Genome-scale identification and analysis of LEA genes in rice (Oryza sativa L.). Plant Sci. *172*, 414-420.

Wang, Y., Xiao, R., Wang, H., Cheng, Z., Li, W., Zhu, G., and Ma, H. (2014). The Arabidopsis RAD51 paralogs RAD51B, RAD51D and XRCC2 play partially redundant roles in somatic DNA repair and gene regulation. New Phytol. *201*, 292-304.

Xu, L., Lin, Z., Tao, Q., Liang, M., Zhao, G., Yin, X., and Fu, R. (2014). Multiple NUCLEAR FACTOR Y transcription factors respond to abiotic stress in Brassica napus L. PloS One *9*, e111354.

Yao, X., Ma, H., Wang, J., and Zhang, D. (2007). Genome-Wide Comparative Analysis and Expression Pattern of TCP Gene Families in Arabidopsis thaliana and Oryza sativa. J. Integr. Plant Biol. *49*, 885-897.