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The RNA binding protein OsCP31A modulates the expression of NDH complex genes involved in abiotic stress tolerance mechanism

환경 저항성과 관계된 NDH complex 유전자의 발현을 조절하는
RNA 결합 단백질인 OsCP31A에 대한 연구

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ABSTRACT

The RNA binding protein OsCP31A modulates the expression of NDH complex genes involved in abiotic stress tolerance mechanism

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Drought is a serious threat to crops causing over $ 10 billion worth of damage annually around the world. To overcome this problem, biotechnological approaches have been made to change the root architecture or control the guard cell closure of plants. Another solution for drought tolerance modulation is via the homeostasis of chloroplast, which can be achieved by using chloroplast genes. The regulation of chloroplast genes including RNA processing is controlled by the nuclear encoded RNA binding protein, chloroplast ribonucleoprotein (cpRNP). Thus, we tried to isolate a cpRNP related to abiotic stresses in rice. Based on the phylogenetic tree between 10 *Arabidopsis* and 8 rice cpRNPs, we isolated Os09g0565200 designated, OsCP31A. We constructed overexpressors and knock down plants of *CP31A* for functional analyses. *OsCP31A* was mainly expressed in green tissues throughout development and was localized in the stroma of chloroplasts. Like cpRNPs in other crops, OsCP31A also had broad binding affinity towards chloroplast RNAs. Using

overexpression (*OsCP31A*^{OX}), non-transgenic (NT) and knock-down (*OsCP31A*^{RNAi}) plants, we treated drought stresses and discovered drought tolerance in *OsCP31A*^{OX}. To understand the OsCP31A-mediated drought tolerance mechanism, we examined expression levels of chloroplast genes in *OsCP31*^{OX} and *OsCP31*^{RNAi}. From this experiment, we finally identified NDH complex RNAs that were relatively stable in *OsCP31A*^{OX} and less stable in *OsCP31A* ^{RNAi}. The NDH complex, generally known as a protein complex forms a supercomplex with PSI and transports electrons under abiotic stress conditions. The NDH complex-controlling electron transport system is called a cyclic electron transport (CET). We assumed that active NDH complex-controlling CET in *OsCP31A*^{OX} may enhance drought tolerance under drought stress. To determine the active NDH-dependent CET in *OsCP31A*^{OX}, we observed the chlorophyll fluorescence in *OsCP31A*^{OX} and *OsCP31A*^{RNAi}. When actinic light was turned off, CET was more responsive in *OsCP31A*^{OX} compared to that of NT. However, the responsiveness was reduced in *OsCP31A*^{RNAi}. Finally, we analyzed ATP content produced by the NDH-dependent CET. Interestingly, ATP content of *OsCP31A*^{RNAi} was comparably low under normal growth conditions when compared to that of *OsCP31A*^{OX} and NT plants. Under drought conditions, the ATP content was highest in *OsCP31A*^{OX} followed by NT and *OsCP31A*^{RNAi}. Taken together, we propose overexpression of *OsCP31A* is sufficient for improving drought tolerance by regulating the expression of NDH complex genes.

Keywords: rice, chloroplast ribonucleoproteins, transgenic rice plants, drought tolerance, NADH dehydrogenase-like complex, cyclic electron transport

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List of Abbreviation

AL	Actinic light
ATP	Adenosine triphosphate
Bar	*Biallaphos-resitance gene*
CaMV 35S	*35S Cauliflower mosaic virus 35S promoter*
CDS	Coding region sequence
CET	Cyclic electron transport
CFP	*Cyan fluorescence protein*
cpRNP	Chloroplast ribonucleoprotein
Fm	Maximum fluorescence
Fv	Variable fluorescence
Fo	Minimal fluoresence
GFP	*Green fluorescence protein*
LET	Linear electron transport
NDH	NADH dehydrogenase-like
NPQ	Non-photochemical quenching
NT	Non-transgenic
OsCc1	*Oryza Sativa cytochrome c 1 promoter*
PAM	Pulse-amplitude modulation

PCR	Polymerase chain reaction
PPR	Pentatricopeptide-repeats
PSI	Photosystem I
PSII	Photosystem II
RbcS	*Rubisco small subunit*
RBP	RNA binding protein
RIP	RNA immunoprecipitation
Rrm	RNA-recognition motif
qE	Energy-dependent quenching
qRT-PCR	Quantitative Real-Time PCR
ΔpH	Proton Gradient

Introduction

Cyanobacteria are considered as the ancestors of chloroplasts which engulfed the eukaryotic cell around 1.5 billion years ago (Schirrmeister et al., 2011). In land plants, about 11-18% of the genes are originated from cyanobacteria and the majority of these genes are located in the nucleus (Nowack et al., 2010; Rujan and Martin, 2001). Around 100 genes remain in plastids and are mostly associated with photosynthesis (Clegg et al., 1994; Hiratsuka et al., 1989; Shinozaki et al., 1986). Expression of these plastid genes are regulated by a large number of nucleus-encoded proteins (Kroeger et al., 2009; Schmitz-Linneweber and Barkan, 2007; Stern et al., 2010). During the post-transcriptional event, these nucleus-encoded RNA-binding proteins (RBPs) are known to control stabilization, splicing, polyadenylation, localization and translation of mRNAs. This process is generally known as RNA processing. Common motifs of chloroplast RBPs are *pentatricopeptide-repeats* (PPR) and *RNA-recognition motifs* (RRM) which have specific RNA binding affinities (Jacobs and Kück, 2011). In addition, *chloroplast ribonuclease proteins* (cpRNP) are also involved in chloroplast RNA processing (Li and Sugiura, 1991; Li et al., 1991; Ye and Sugiura, 1992). The cpRNPs are characterized by an N-terminus signal peptide and two RNA recognition motifs separated by a short spacer at the C-terminus (Lorković and Barta, 2002; Ohta et al., 1995). Five cpRNPs have been identified in tobacco (Li and Sugiura, 1990; Ye et al., 1991) and ten in *Arabidopsis* (Lorković and Barta, 2002; Ruwe et al., 2011). However, none have been identified in rice. In *Arabidopsis*, plants deficient of *CP31A*

and *CP31B* genes induce mis-RNA editing and destabilize RNAs in chloroplast (Tillich et al., 2009). CP31A and CP31B proteins are also involved in chloroplast homeostasis under cold stress conditions (Kupsch et al., 2012). However, the molecular mechanism for the cpRNP-mediated abiotic stress tolerance is not completely understood.

Through photosynthesis, plants convert light energy into NADPH and ATP via linear electron transfer (LET). Then, NADPH and ATP are used for nitrogen and sulfur assimilation, protein synthesis and photorespiration. Though LET produces sufficient amounts of NADPH, the amount of ATPs produced are insufficient (Seelert et al., 2000; Wang et al., 2015; Yamori and Shikanai, 2016). So plants need alternative pathways to keep the ideal NADHP/ATP ratio. This is resolved by Photosystem I (PSI) cyclic electron transport (CET) which provides the extra ATPs required. CET also protects the photosystems from over-reduction of chloroplasts caused by environmental and/or physiological issues (Miyake, 2010; Shikanai, 2007). In these conditions, the CET induces qE (energy-dependent quenching) of NPQ (non-photochemical quenching) which dissipates excessive light energy as heat (Müller et al., 2001; Niyogi, 1999). The CET pathway consists of two pathways: the protein gradient regulation 5 (PGR5)/PGR-like 1 (PGRL1) ferredoxin-dependent pathway and the NADH dehydrogenase-like (NDH) complex-dependent pathway (Burrows et al., 1998; DalCorso et al., 2008; Horváth et al., 2000; Kofer et al., 1998; Munekage et al., 2002; Shikanai et al., 1998). The former is known to be the major pathway, whereas the latter is known to be minor under normal growth conditions. This is

because the NDH-dependent pathway contributes to only 1% of total electron transportation (Peng et al., 2012; Peng et al., 2011). The NDH complex consists of 11 plastid-encoded subunits and 18 nucleus-encoded subunits (Ifuku et al., 2011; Matsubayashi et al., 1987; Shikanai, 2016). Mutants of these subunits show little to no growth defect under normal growth conditions unlike *PGR5* and *PGRL1* mutants that have growth defects (Burrows et al., 1998; Munekage et al., 2004; Shikanai et al., 1998; Yamori et al., 2011). Chlorosis phenotype is observed in *PGR5* and *PGRL1* mutants under light, temperature and drought stress conditions (Burrows et al., 1998; Horváth et al., 2000; Li et al., 2004; Wang et al., 2006; Zhang and Sharkey, 2009). In rice, a *crr6* mutant which lacks *ndhK* also shows growth defects under low-temperature, low-light and fluctuating-light stress (Yamori et al., 2016; Yamori et al., 2011; Yamori et al., 2015). These studies support the argument that the NDH-dependent CET is essential for adapting to abiotic stress. PPR and cpRNPs have been known to be involved in NDH complex RNA editing (Hashimoto et al., 2003; Hirose and Sugiura, 2001; Kotera et al., 2005; Okuda et al., 2009; Okuda et al., 2007; Okuda et al., 2006; Yamazaki et al., 2004). Thus, the modulation of PPR and cpRNPs is considered a key challenge to improve various abiotic stresses for plants. Since overexpression of *PGR5* enhances CET and tolerance to high light and drought stresses (Long et al., 2008; Okegawa et al., 2005) in Arabidopsis, we hypothesized that controlling the expression of NDH complex genes would result in abiotic stress tolerance as well.

In this study, we investigated the function of the rice cpRNP OsCP31A using

overexpression and knock-down plants. The OsCP31A proteins had a broad binding affinity towards chloroplast RNAs and the expression of NDH complex genes were regulated by *OsCP31A* expression. Over two generations, we verified drought tolerance phenotype of *OsCP31A* overexpression plants (*OsCP31A*^{OX}). The NDH-dependent CET was more responsive in overexpression plants under stress conditions. Since NDH-dependent CET produces ATP, we analyzed the ATP content in *OsCP31A*^{OX}, NT and *OsCP31A*^{RNAi} under normal and drought conditions. Under normal conditions, ATP content of *OsCP31A*^{RNAi} was markedly low compared to *OsCP31A*^{OX} and NT. Under drought conditions, the ATP content was highest in *OsCP31A*^{OX}. These results suggest that overexpression of the *OsCP31A* is sufficient to improve drought tolerance through modulation of NDH-dependent CET.

Materials and methods

1. Vector construction and transformation

Overexpression and RNAi constructs were used in this study. For *OsCP31A* (Os09g0565200) overexpression plants, 969 base pair coding region sequence (CDS) was isolated from rice cDNA (*Oryza sativa* cv. Dongjin) and cloned into the pSB11 vector by the Gateway™ cloning system (Invitrogen, USA). Rice *OsCc1* was used as a constitutive promoter (Jang et al., 2002) and the potato derived 3'*pinII* as a terminator (*OsCP31A*^{OX}). *OsCP31A* CDS without the stop codon was isolated from rice cDNA and fused to the *green fluorescent protein* (*GFP*) for *GFP* tagged constructs (*OsCP31A-GFP*^{OX}). Rice *OsCc1* was used as a constitutive promoter and the potato derived 3'*pinII* as a terminator. Bar gene was used as an herbicide resistant selection marker, controlled by *CaMV 35S* promoter and 3'nos terminator. For the knock down construct (*OsCP31A*^{RNAi}), CDS was isolated from rice cDNA (*Oryza sativa cv* Dongjin) and cloned into the pGOS2-RNAi vector (Lee et al., 2016) containing the *bar* selection marker by the Gateway™ cloning system. Primers used for vector construction are listed in Table 1. All transgenic plants were produced by *Agrobacterium tumefaciens* (LBA4404)-mediated transformation and then tissue culture as previously described (Hiei et al., 1994). Homozygous lines of T_5 *OsCP31A*^{OX}, *OsCP31A-GFP*^{OX} and T_2 *OsCP31A*^{RNAi} were selected based on the gene expression level in this study.

2. Phylogenetic analysis and sequence alignment

Sequence alignment of *OsCP31A* and *AtCP31A* was performed using ClustalW (http://www.genome.jp/tools/clustalW). The phylogenetic tree was constructed by CLC workbench software version 7.0 (http://www.qiagenbioinformatics.com/products/clc-genomics-workbench.) via the neighbor-joining method using full sequence of 8 rice cpRNPs: *OsCP33* (Os07g0158300), *OsCP29A* (Os03g0376600), *OsCp31A* (Os09g0565200), *OsCP31B* (Os08g0557100), *OsCP28A* (Os02g0815200), *OsCP29C* (Os09g0279500) and *OsCP28B* (Os08g0117100) and 10 *Arabidopsis* cpRNPs: *AtCP33A*, *AtCP29A*, *AtCP32B*, *AtCP31A*, *AtCP31B*, *AtCP28A*, *AtCP29C*, *AtCP33B*, *AtCP33C*, *AtCP28B*. Bootstrap values shown as percentages were performed with 100 repetitions.

3. Confocal microscopy

Localization of OsCP31A was performed using *OsCP31A-GFP*^{OX} plants. The chloroplast transit peptide tagged with GFP (*TP-GFP*^{RbcS}) was used as control. RbcS was used as a promoter and the potato derived 3'*pinII* as a terminator. 10-day old rice plants grown in MS medium under chamber conditions (16 hr light at 28°C and light intensity between 170 ~ 180 μmol m^{-2} s^{-1} / 8 hr dark cycles at 25°C) was used in this study. Leaf sheath was selected for localization. Samples were mounted under water and then covered with a cover glass. Observations were made with the Leica SP8 STED laser scanning confocal microscope (Leica, Germany) at 40 X and 60 X of

magnification. GFP and chlorophyll were excited at 488 nm. Emitted light was detected between 512 and 580 nm and between 700 and 790 nm, respectively.

Alternatively, transient expression system using protoplasts were performed for subcellular localization of the OsCP31A. CDS of *OsCP31A* without the stop codon was transferred by In-Fusion® HD cloning system (Clontech, USA) into a pHBT vector containing GFP reporter protein controlled by the 35s promoter. *Bam*H1 and *Pst*I restriction sites were used for vector construction (New England Biolabs, USA). Protoplasts were isolated from 10-day old rice plants (O. sativa cv. Dongjin) by the method previously described (Jung et al., 2015). *OsCP31A-GFP*^{35OX} was co-transfected with *cyan fluorescence protein* (*CFP*) fused organelle markers (Nelson et al., 2007). Protoplasts were incubated in dark conditions for 12 hours at 28°C and then were harvested for confocal microscopy. CFP was excited at 458 nm and emitted light was detected between 460 and 510 nm.

4. Chlorophyll content measurement

Middle portion of the leaves were directly measured with the SPAD-502 PLUS chlorophyll meter (Konica Minolta, Japan) according to methods previously described (Yuan et al., 2016). The third leaf from the top was used and a total of 30 plants for each lines were analyzed in all measurements.

5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the leaves of *OsCP31A*^{OX}, non-transgenic Dongjin (NT) and *OsCP31A*^{RNAi} plants by Hybrid-R kit (GeneALL, Korea). Each sample was treated with 70µl of DNase reaction buffer (DRB) containing 2 µl of DNase I (GeneALL, Korea) for 10 minutes to avoid DNA contamination. To synthesize cDNA, 1 µl of RNA was used with 1 µl of oligo dT primer and 1 µl of RevertAid^{TM} reverse transcriptase (Thermo Fischer Scientific, USA). Reverse transcription was performed at 42°C for 90 minutes and terminated by incubating the reaction mixture for 5 minutes at 70°C. qRT-PCR was carried out with Mx3000p real-time PCR machine and Mx3000p software (Agilent Technologies, CA) with 20 µl reaction mixture containing 1 µl of cDNA template, 2 µl of primer, 0.04 µl of ROX reference dye (Invitrogen, USA), 1 µl of 20X Evagreen (SolGent, Korea), 10 µl of 2X premix, and dH_2O. Reactions were carried out by 1 cycle at 95°C for 10 minutes and 55 cycles at 95°C for 30 seconds, 58°C for 30 seconds and at 72°C for 30 seconds. *OsUbi1* (Os06g0681400) was used as an internal control in all experiments. Primers used for qRT-PCR are listed in Table 1.

6. RNA immunoprecipitation (RIP)

RIP was carried out by methods previously described (Keene et al., 2006; Peritz et al., 2006). All procedures were carried out at 4 ºC conditions. 14 day-old rice leaves were homogenized with liquid nitrogen and incubated with polysome lysis buffer for

20 minutes by shaking. The buffer consists of 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40, 1 mM DTT, RNase Out RNase inhibitor, 100 units ml^{-1} (Invitrogen, USA), 2 mM vanadyl ribonucleoside complexes solution (Sigma-Aldrich, USA) and Protease inhibitor cocktail tablets (Roche, Switzerland). The soluble supernatant was separated from the crud extract by centrifuging 16,000 x g for 20 minutes at 4 ºC. The proteins were quantified by the Bradford method and 1 mg aliquot of lysate was used for the next step. The OsCP31A protein fused with 6x myc epitope was confirmed by immuno-blotting analysis. Lysate was precleared before the immunoprecipitation step with 50% slurry of protein A-agarose beads equilibrated in lysis buffer containing 1 mg ml^{-1} BSA by rotating at 4 ºC for 2 hours. After incubating the lysate adding myc antibodies, pull down of protein-RNA complexes were carried out using protein A-agarose beads. The beads were washed four times with polysome lysis buffer to remove RNase and proteinase inhibitors. Then, they were washed four times with polysome lysis buffer containing 1 M of urea. RNAs were eluted from the beads with the polysome lysis buffer containing 0.1% SDS and 30 μg of proteinase K for the final step. The RNAs including total input were purified and enriched by using Trizol reagent (Invitrogen, USA) method. 20 μg of glycogen were added during the ethanol precipitation to get the RNA pellet.

7. Analysis of RNA editing

Total RNAs of 2-week-old *OsCP31A*^{OX}, NT and *OsCP31A*^{RNAi} plants were isolated by the Hybrid-R kit (GeneALL, Korea) according to the manufacturer's instructions. Each sample was treated with 70µl DRB containing 2 µl DNase I (GeneALL, Korea) for 10 minutes to remove excess DNA. Reverse transcription was carried out by using 0.5µ of oligo dT primers and 0.5µ of random primers. Sequences that include rice chloroplast RNA editing sites (*atpA*, *ndhB, ndhD, rpl2, rpoB, rps8, rps14*) (Corneille et al., 2000) were cloned by PCR using primers listed in Table 1. cDNAs containing the editing sites were directly sequenced to analyze changes in editing.

8. Evaluation of drought tolerance

NT, Homozygous single copy lines of *OsCP31A*^{OX} (9-1, 9-2, 2) and *OsCP31A*^{RNAi} (4-1, 4-2, 6) were selected to evaluate drought tolerance. For every line, 30 plants were grown in MS medium plates under dark condition (28°C) for 3days. The plates were then placed in chamber conditions for 2 days and finally transferred to soil. Plants were grown under greenhouse for 4 weeks thereafter. Drought stress was applied for 3 days by removing water from plants. Soil moisture was measured by SM150 Soil Moisture Sensor (AT Delta-T devices, UK) during this period to ensure equal treatment of drought stresses. After the drought treatment, plants were re-watered for 5 days to observe recovery. Phenotypic analysis of transgenic and NT plants under drought conditions were visualized by using NEX-5N camera (Sony, Japan) at 4 different time points.

9. Chlorophyll *a* fluorescence under drought stresses

NT, *OsCP31A*^{OX} (9-1, 9-2, 12) and *OsCP31A*^{RNAi} (4-1, 4-2, 6) plants were used for chlorophyll *a* fluorescence (Fv/Fm) measurement. 9 plants for each line were planted in the MS medium plates and grown under dark condition (28°C). Plants were then placed in chamber conditions for 2 days and finally transferred to soil containing pots. and transferred to separate pots containing soil in greenhouse. 6-week-old plants were treated with drought stresses by removing the water from the pots. Plants were adapted in dark for 2 hours before analysis sufficiently open the reaction centers. Fv/Fm values were measured using the middle portion of the leaf. 30 measurements were averaged per each line using the Handy PEA fluorimeter (Hanstech Instruments, UK).

10. Analysis of NADH dehydrogenase-like (NDH)-dependent cyclic electron transport (CET)

NT, *OsCP31A*^{OX} (12), and *OsCP31A*^{RNAi} (6) plants were selected for analysis. NDH-dependent CET was determined by monitoring chlorophyll *a* fluorescence through mini-PAM (Waltz, Germany) with methods previously described (Shikanai et al., 1998). Plants were adapted in growth chambers (24°C dark/ 35°C dark) for at least 30 minutes prior to measurement. Leaves were exposed to actinic light (AL: 200 l mol photons m^2 sec^{1}) for 5 minutes after the measuring light was turned on (*F*o level: minimum yield of Chlorophyll fluorescence) to drive electron transport between

photosystem II (PSII) and photosystem I (PSI). Maximum fluorescence (*F*m) and steady-state fluorescence (*F*s) were determined under these conditions. The transient increase in chlorophyll fluorescence was monitored after actinic light was turned off.

11. Determination of ATP content

Measurement of ATP content was performed by procedures provided in the ENLITEN® ATP Assay Kit (Promega, USA). Accurately weighed leave samples (0.05g) were put into 2ml tubes containing 1ml of Tris-HCl (pH 7.8). Tubes were then heated in a water bath at 100°C for 10 minutes and cooled to room temperature. To analyze the ATP content, 10 μl of the cooled samples were added to wells containing 100 μL of rL/L Reagent each. The ATP standard curve was obtained by ATP standard samples provided in the kit. Luminescence was measured by Infinite M200 PRO (TECAN, Switzerland). ATP content was determined via the ATP standard curve according to the concentration of solution and sample weight.

Results

1. Structure of *OsCP31A* and phylogenetic analysis

We isolated a chloroplast ribonucleoprotein (cpRNP) related to abiotic stresses in rice. We identified 8 cpRNP-like genes in rice genome by comparing the full-length sequence of cpRNP genes in *Arabidopsis thaliana*. Using the amino acid sequences of 8 rice and 11 Arabidopsis cpRNP genes, we generated a phylogenetic tree to understand inter-species relationship between cpRNP genes (Fig 1B). Based on the phylogenetic relationship between cpRNPs, they were named *OsCP28A* (Os02g0815200), *OsCP28B* (Os08g0117100) *OsCP29A* (Os03g0376600), *OsCP29B* (Os07g0631900), *OsCP29C* (Os09g0279500), *OsCp31A* (Os09g0565200), *OsCP31B* (Os08g0557100) and *OsCP33* (Os07g0158300). AtCP31 was identified as a key regulator for abiotic stress tolerance mechanism (Kupsch et al., 2012). However, the mechanism for AtCP31-mediated-abiotic tolerance is not well understood. To understand the molecular mechanism, we finally selected *OsCP31A* for further study in rice. The nuclear encoded *OsCP31A* gene consisted of 4 exons and 3 introns with a 969 base-pair coding sequence. The amino acid sequence analysis showed that OsCP31A consisted of a signal peptide located in the N terminal regions and two RNA recognition motifs (RRM) in the C terminal regions, separated by a short spacer (Fig 1A).

2. OsCP31A is localized in the chloroplast of green tissues

Since studies in Arabidopsis and tobacco show that cpRNPs are localized in the chloroplast (Li et al., 1991; Nakamura et al., 1999; Ohta et al., 1995) and present in green tissues, we examined the rice cpRNP, OsCP31A. Firstly, we determined the temporal expression pattern of *OsCP31A* through qRT-PCR, using rice cDNAs from 6 different developmental stages. *OsCP31A* was highly expressed 7 days after germination (DAG) to 60 DAG plants (Fig 3A). The expression levels were reduced in leaves of the flowering stage. On the other hand, *OsCP31A* expression was low in roots of all stages, indicating the genes are mostly related to the green tissues of rice. Next, we observed the localization of OsCP31A by monitoring GFP signals using leaves of GFP tagged plants (*OsCP31A-GFP*^{OX}, *TP1-GFP*^{RbCs}) through the confocal microscope. *TP1-GFP*^{RbCs} GFP signals were merged with the auto florescence of chloroplasts and *OsCP31A-GFP*^{OX} GFP signals were also merged with chloroplast but in an aggregated manner (Fig 3B). Alternatively, we carried out subcellular localization by the transient expression assay using protoplasts isolated from *OsCP31A-GFP*^{OX} plants. Organelle markers containing CFP were co-transfected in the protoplasts. GFP signals were merged with the CFP signals of the plastid maker (Fig 3C), verifying that OsCP31A is localized in chloroplasts.

3. OsCP31A is a chloroplast RNA binding protein (RBP)

We examined the chloroplast RNA binding affinity of OsCP31A through RNA

immunoprecipitation (RIP) to investigate similarities between other cpRNPs. OsCP31 proteins isolated from *OsCP31A-GFP*^{OX} leaves were preliminarily verified by western blot (Fig 4A). The RNA-protein complex was precipitated by using α-GFP antibodies in transgenic leaves and the non-specific NT control precipitated by same antibodies in NT leaves as a negative control. Next, we analyzed 23 plastid encoded genes of four major classes: ATP synthase (*atp*), photosystem I (*psa*), photosystem II (*psb*) and NADH dehydrogenase like (*ndh*). RNAs were isolated from the RNA-protein complex after the immunoprecipitation step and cDNA was synthesized for qRT-PCR analysis. Of the 23 mRNAs, 18 were enriched by more than 5 fold and 5 (*psaB*, *atpB*, *ndhC*, *ndhE*, and *ndhF*) exceeded 38 fold. These results show that OsCP31A have a broad target range to chloroplast RNAs, similar to that of Arabidopsis CP31A (Tillich et al., 2009).

4. OsCP31A has minor roles in chloroplast RNA editing

The tobacco CP31 and Arabidopsis CP31A are known to bind to chloroplast RNAs and control their editing (Hirose and Sugiura, 2001; Tillich et al., 2009). To investigate the possibility of this function to be conserved in rice OsCP31A, we observed the chloroplast RNA editing sites of rice plants. The 7 chloroplast transcripts containing 15 editing sites (Table 2) were sequenced. Only 1 transcript (*rpoB*) with 2 editing sites (codon 182, 187) showed abnormal editing in *OsCP31A*^{RNAi} plants (Fig 5). The rpoB transcript induced missense translation from Leucine (TTA) to

Serine(TCA). The other 6 transcripts had no defect, and were normally edited in all plants, indicating that *OsCP31A* has minor roles in chloroplast RNA editing.

5. Expression of the NDH complex genes are increased in *OsCP31A* overexpression plants

To understand the function of OsCP31A, we examined the expression levels through quantitative real time PCR (qRT-PCR). Of the 23 chloroplast genes screened, we discovered most of the NDH complex RNAs being affected in all transgenic plants. Expression of *ndhE* was the highest (about 2 fold) followed by that of *ndhF*, *ndhD* *ndhG*, *ndhB* and *ndhH* in $OsCP31A^{OX}$ plants while the expressions of same genes were decreased in $OsCP31A^{RNAi}$ plants (Fig 4B). Collectively, antagonistic regulation of NDH complex genes is regulated by the expression level of *OsCP31A,* suggesting that it is possible to increase NDH complex gene stability by OsCP31A RBPs. Interestingly, the NDH complex is a component of the cyclic electron transport (CET) which is known to relieve plants from abiotic stresses.

6. Overexpression of *OsCP31A* enhances drought tolerance

Next, we evaluated drought tolerance of transgenic plants by selecting elite lines of $OsCP31A^{OX}$ and $OsCP31A^{RNAi}$ plants based on their gene expression (Fig 6B).

Drought stress was mimicked for 3 days by removing water from the plants and placing them on dry trays. Soil was uniformly dried during this period (Fig 6C). Indicators of drought stress, mainly leaf rolling and tilting, were observed in plants at day 2 (Fig 6A). At day 3, *OsCP31A*^{OX} plants were the least damaged and mostly recovered after re-watering. The survival rates were 80% in 9-1, 83.33% in 9-2 and 76.67% in 12, indicating that the overexpression of OsCP31A enhances drought tolerance. Contrary to the overexpressors, NT and *OsCP31A*^{RNAi} plants had drier leaves which were mostly chlorotic. After re-watering, only 12.22% of recovered in NT. The survival rate of #4-1, #4-2 and #6 plants (*OsCP31A*^{RNAi}) was 6.67%, 6.67% and 13.33% respectively. In order to confirm drought tolerance, Chlorophyll fluorescence test was carried out by monitoring Fv/Fm values in the leaves of transgenic and NT plants. *OsCP31A*^{OX} and *OsCP31A*^{RNAi} were experimented individually. The pots containing NT and transgenic plants were equally dried by removing the water from soil (Fig 6D). The average values were 0.8 in all plants shortly after they were taken out of the water, indicating optimal growth condition (Maxwell and Johnson, 2000). Drought stress symptoms began at day 3 in *OsCP31A*^{OX} and NT plants. At day 4, Fv/Fm values were around 0.6 in *OsCP31A*^{OX} lines while the values decreased to 0.02 in NT plants. After re-watering the plants for 7 days, only *OsCP31A*^{OX} plants recovered. In *OsCP31A*^{RNAi} and NT plants, drought effect began at the sixth day. The Fv/Fm readings began dropping at day 7 and reached 0.2 at day 8 in *OsCP31A*^{RNAi} and NT plants. Non recovered after re-watering. This was identical to the drought visual analysis, confirming that overexpressing *OsCP31A* does improve drought tolerance.

7. Overexpressing *OsCP31A* regulates expression of NDH complex genes under drought stress

The expression of NDH complex genes in transgenic and NT plants were compared under normal and drought conditions. 2-week-old *OsCP31A*^{OX}, *OsCP31A*^{RNAi} and NT plants were air dried and sampled at 0 and 6 hours of treatment for qRT-PCR analysis. Of the various NDH genes, 10 plastid encoded RNAs were chosen since OsCP31A is a chloroplast RNA binding protein. Expression levels of *ndhC*, *ndhG*, *ndhH*, *ndhJ*, and ndhK were high in *OsCP31A*^{OX} plants and significantly higher in *ndhB* and *ndhF* (Fig 7). However, the expression of *ndhA*, *ndhD* and *ndhE* decreased in all plants. Collectively, overexpressing *OsCP31A* led to stabilization of NDH transcripts under drought conditions.

8. Lack of *OsCP31A* results in chlorosis under light stress

The NDH complex forms with photosystem I (PSI) and is known to activate CET under abiotic stresses (Endo et al., 1999; Horváth et al., 2000; Li et al., 2004; Munné-Bosch et al., 2005; Wang et al., 2006). This complex consists of various subunits encoded by multiple genes (Ifuku et al., 2011; Matsubayashi et al., 1987; Shikanai, 2016). We assumed that the regulation of the NDH complex genes by *OsCP31A* expression enhances NDH-dependent CET. To validate this, we decided to analyze the well-studied NDH mutant phenotypes under light stress (Endo et al., 1999; Horváth et al., 2000; Munekage et al., 2004). *OsCP31A*^{OX}, NT and *OsCP31A*^{RNAi}

plants were grown under light stress conditions (240 ~ 250μmol m^{-2} s^{-1}) to observe chlorosis and necrosis in leaves. After 2 weeks of treatment, the corresponding phenotypes were scattered across the leaves of *OsCP31A*^{RNAi} plants. 83.33% of line 8 plants displayed chlorosis and necrosis followed by 70% in line 6 and 63.33% in line 4 plants (Fig 8A). No visual symptoms were observed in *OsCP31A*^{OX} and NT plants. The phenotype was confirmed by direct measurement of the chlorophyll content in leaves with SPAD. As shown in Fig 8B, the values matched with the symptoms; chlorophyll content was similar between *OsCP31A*^{OX} and NT plants while the content was noticeably low in *OsCP31A*^{RNAi} plants. These results suggested the correlation between *OsCP31A* expression and NDH-dependent CET activity under stress conditions.

9. The NDH-dependent CET is more responsive in *OsCP31A* overexpression plants

The responsiveness of NDH-dependent CET was directly measured by mini-PAM. We monitored the increase in chlorophyll fluorescence after the offset of actinic light, which is caused by the NDH complex catalyzing the reduction of the plastoquinone pool (Shikanai et al., 1998). Before measurement, plants were transferred to dark chambers with different temperatures (24°C as normal and 35°C as heat stress). Plants were examined in 35°C chambers because heat stress can be treated rapidly and evenly. It is also easier to distinguish responsiveness of CET among plants under this

condition (Bukhov et al., 1999; Zhang and Sharkey, 2009). Under normal condition, similar increase in chlorophyll fluorescence was observed in all plants upon post illumination (Fig 9). After heat treatment, the responsiveness of NDH-dependent CET under dark condition was enhanced in *OsCP31A*^{OX} compared to that of NT. Chlorophyll fluorescence was significantly lower in *OsCP31A*^{RNAi} plants under heat stress. These observations showed that the responsiveness of NDH-dependent CET is higher in OsCP31A overexpression plants under heat stress.

10. Pronounced expression of NDH complex genes are observed in *OsCP31A* overexpression plants in the absence of light

Maximum CET is known to be achieved in the absence of light (Joliot et al., 2004; Joliot and Joliot, 2006). To understand the correlation between NDH complex gene expression and light, we observed its diurnal expression in *OsCP31A*^{OX}, NT and *OsCP31A*^{RNAi} plants. Leaves of transgenic and NT plants were sampled every 3 hours for this assay. Under light conditions, most of the expression of NDH complex genes were in a steady state (Fig 10). In dark conditions however, the expression of NDH complex genes began to increase in *OsCP31A*^{OX} and NT plants. Marginal changes were observed in *OsCP31A*^{RNAi} plants. Expression of NDH complex genes were distinguishably high in *OsCP31A*^{OX} plants at midnight. Among the 10 genes analyzed in this study, *ndhD*, *ndhE*, *ndhF*, *ndhG*, and *ndhH* were noticeably high in

overexpressors. In summary, the expression of NDH complex genes were comparably induced in *OsCP31A* ^{OX} plants upon post illumination.

11. ATP content is higher in overexpression plants under drought conditions

Finally, we examined the ATP content in plants produced by NDH-dependent CET activity. It is reported that CET enhanced plants, which are tolerant to abiotic stress, contain higher ATP content (He et al., 2015). The plants were sampled in normal and drought conditions. In normal condition, the ATP content of *OsCP31A*^{RNAi} plants were 29% lower than that of NT plants. The content of *OsCP31A*^{OX} were 3% higher than that of NT plants. After 1 hour of drought treatment, ATP content decreased by 3% in *OsCP31A*^{OX} while by 22% in NT and 11% in *OsCP31A*^{RNAi}. At 3 hours of treatment, the content decreased in all plants but ATP levels remained comparably high in *OsCP31A* ^{OX}. These findings indicate that overexpression plants produce more ATP content under drought conditions by improved NDH-dependent CET.

Table 1. Primer information

Gene	Specificity	Purpose	Primer Sequence	
			Forward	Reverse
OsCP31	gene	Cloning	5'- TATACAGAGGAGACTCGATTGA -3'	5'- TACATACACAGCTGATGTTGAC -3'
gfp	gene	Cloning	5'- CAGCACGACTTCTTCAAGTCC -3'	5'- CTTCAGCTCGATGCGGTTCAC -3'
OsCP31	coding sequences	Localization	5'- TTGCTCCGTGGATCCTCGATGGCCTCCTCCATCGCCAT -3'	5'- AAAGCGGCCGCAAATAAAGCCTCGGCGCGGTGGTCGC -3'
OsUbi1	gene	qRT-PCR	5'- ATGGAGCTGCTGCTGTTCTA -3'	5'- TTCTTCCATGCTGCTCTACC -3'
OsCP31	coding sequences	qRT-PCR	5'- CAGCTGTTCAGCGAGCATGG -3'	5'- CGCTCCTCTGCAACATTCAC -3'
OsCP31	3'UTR	qRT-PCR	5'- TGGGATAGCAGGCTTTGTGG -3'	5'- AGGAGCAGTAAACAGCAGGC -3'
psaA	gene	qRT-PCR	5'- AGTAGCAGTAGGCGGCAAAG -3'	5'- ACGGGAACTGCGAGCAAATA -3'
psaB	gene	qRT-PCR	5'- TGGACATCTTGTTTGGGCGA -3'	5'- GAGCCACGGGCTTATCTCTC -3'
psaC	gene	qRT-PCR	5'- AAGATAGAGCCATGCTGCGG -3'	5'- ACCGAAGATTGTGTGGGTTGT -3'
psbB	gene	qRT-PCR	5'- ACACATTTGGCATGGGGCTA -3'	5'- ACTGGCTGTCTCCCTGTAGT -3'
psbC	gene	qRT-PCR	5'- AATGTGGGATCTGCCCAAGG -3'	5'- CACGAAGGTCCCAAAAACGC -3'
psbD	gene	qRT-PCR	5'- GGCAACCGTGGAAAACACTC -3'	5'- AAAGCGATTAGCGGTGACCA -3'
psbE	gene	qRT-PCR	5'- ACGGAATTCCTTGTCGGCTT -3'	5'- CCCTATTCATTGCGGGTTGG -3'
psbF	gene	qRT-PCR	5'- CGTTGGATGAACTGCATTGCT -3'	5'- TTTTACAGTGCGATGGCTGG -3'
psbG	gene	qRT-PCR	5'- CCGCTTGCCTAGGACTTGAT -3'	5'- AGACTCTCTAGTTTATGGCCCCT -3'
psbH	gene	qRT-PCR	5'- CCAAGACAAACTCGCGTAGG -3'	5'- CGCGAATAAAGCCATTGCGA -3'
psbK	gene	qRT-PCR	5'- TTTCTTCGCCAAATTGCCCG -3'	5'- CAGCTTGCCAAACAAAGGCT -3'
atpA	gene	qRT-PCR	5'- TGAATCTCCTGCTCCGGGTA -3'	5'- GCTGTTTTGCCGGTTTGTCT -3'
atpB	gene	qRT-PCR	5'- CCTTATCGGCGTGGAGGAAA -3'	5'- CCCCTACTCCGCCAAATACG -3'
atpF	gene	qRT-PCR	5'- CCGACAACGGGTTTTCCAAC -3'	5'- ATTCCATGGCCCCGAGAATG -3'
atpE	gene	qRT-PCR	5'- TGTTAATGGGGGCGTGGTTT -3'	5'- TGACTCCTAAGCGAATTATTTGGG -3'
ndhA	gene	qRT-PCR	5'- CGAGCTGCCGCTCAATCTAT -3'	5'- AGGCTGACGCCAAAGATTCC -3'
ndhB	gene	qRT-PCR	5'- TACGAAGGATCCCCCACTCC -3'	5'- TCCAGAAGAAGATGCCATTCGT -3'
ndhC	gene	qRT-PCR	5'- GCCCAAGGGTAGAGAAAGACC -3'	5'- TTAGCTCCGGTTCGTGAAGG -3'
ndhD	gene	qRT-PCR	5'- GCTTCCCCATGGGTATCTGG -3'	5'- ACGGTTCCAACGAACCAAGA -3'
ndhE	gene	qRT-PCR	5'- AGCCGCAAGGGCTATAACAA -3'	5'- TGATCACAAGCCGAAACATGG -3'
ndhF	gene	qRT-PCR	5'- CGCAGTTTTTCGACAAGGGT -3'	5'- GACGAAATTCGACCTCCCCC -3'
ndhG	gene	qRT-PCR	5'- GTTGTGCCACAGCTACAAAGT -3'	5'- GGGGGTCTAGGGGTGGTATT -3'

Gene	Specificity	Purpose	Primer Sequence			
			Forward		Reverse	
ndhH	gene	qRT-PCR	5'- ATAAGGCAGATCGGCAGCAA	-3'	5'- TTATGGCAGATCTCGGTGCG	-3'
ndhJ	gene	qRT-PCR	5'- GTAAGGGCCAGCCTATCCAA	-3'	5'- TCCGATTTTCAAGAACGCGAAT	-3'
ndhK	gene	qRT-PCR	5'- TGTGGCTGGTAGTAAAACATCG	-3'	5'- GGTTGCCCACCTAAACCAGA	-3'
Dip1	gene	qRT-PCR	5'- GAGCTTGTCACCGGCATGGA	-3'	5'- AGCTGGAGCTGGAGCTGGAT	-3'
RbcS1	gene	qRT-PCR	5'- GGCAGGTACTGGACCATGTG	-3'	5'- TTGTCGAAGCCGATGATACG	-3'
atpA	gene	RNA editing	5'- CGCCTTTTAGAAAGAGCCGC	-3'	5'- ATTCTCGTAATCGTCGGCCC	-3'
ndhB1	gene	RNA editing	5'- CGGCCCTATTGTTCCGATGG	-3'	5'- CTAAAAGAGGGTATCCTGAGCAAT	-3'
ndhB2	gene	RNA editing	5'- GGACGAAACCAAGAAATAACCC	-3'	5'- CCCAAGTCCTACAGTGATGG	-3'
ndhB3	gene	RNA editing	5'- GGACGAAACCAAGAAATAACCC	-3'	5'- AGTAATAGCAAGGAGATTCCCCA	-3'
ndhD	gene	RNA editing	5'- GGCGCATTCTATCACAAGCC	-3'	5'- CATGGGGAAGCGCATTACAG	-3'
rpl2	gene	RNA editing	5'- ACGGCGAAACATTTATACAAAACAC	-3'	5'- TCAAAGGTAGGGCATTTCCCA	-3'
rpoB	gene	RNA editing	5'- GCTAAGTCCTGGTATTTACTACC	-3'	5'- GTCCCCACCTACACAAGCAA	-3'
rps8	gene	RNA editing	5'- TCCGCCCAAAACCTTAGGAA	-3'	5'- ATGGGCAAGGACACTATTGC	-3'
rps14	gene	RNA editing	5'- CAACTGGATCTTGTTGCACCC	-3'	5'- GAGAGAGGAAGCGGCAGAAAT	-3'

Table 2. Chloroplast RNA editing analysis of *OsCP31A* transgenic plants

The identified 7 rice chloroplast genes containing 16 editing sites were randomly chosen, cloned and analyzed by Sanger sequencing. + indicates edited deficiency and – indicates editing efficiency.

Editing Sites		Editing Defects		
Gene	Editing Sites	OX	NT	KD
atpA	1	-	-	-
ndhB	8	-	-	-
ndhD	1	-	-	-
rpl2	1	-	-	-
rpoB	3	-	-	++
rps8	1	-	-	-
rps14	1	-	-	-

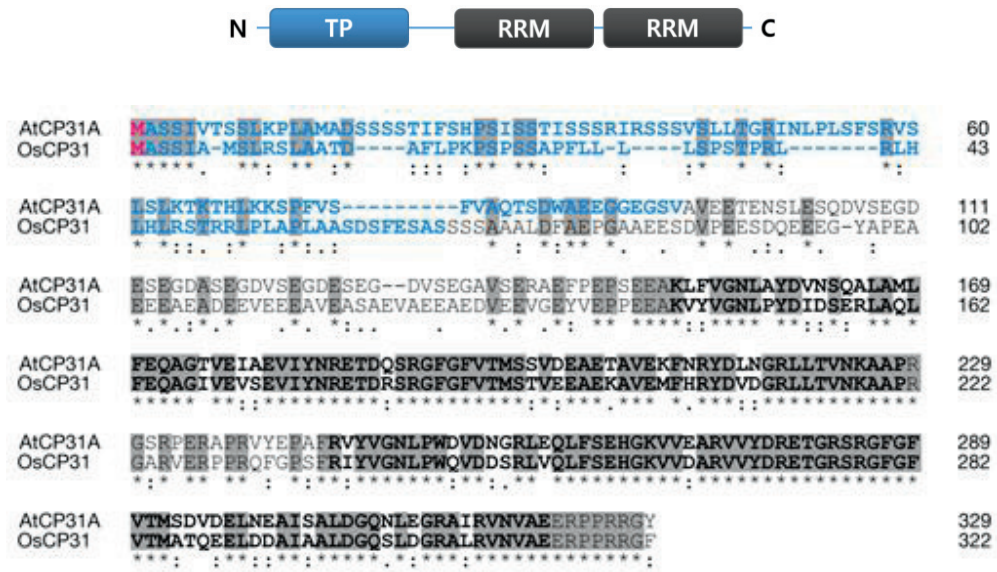
- indicates normal editing, + indicates editing defect

Fig 1. Characterization of *OsCP31A*

(A) Sequence alignment of *OsCP31A* and *AtCP31A* were performed using ClustalW (http://www.genome.jp/tools/clustalW). Conserved amino acid residues are marked with grey box. Blue letters represent the transit peptide (TP) and black letters represent the RNA recognition motif (RRM).

(B) Phylogenetic tree was constructed with CLC workbench software (http://www.qiagenbioinformatics.com) using full-length amino acid sequences of rice and *A. thaliana* cpRNPs by the neighbor-joining method.

(A)


N
TP
RRM
RRM
C
AtCP31A
OsCP31

(B)

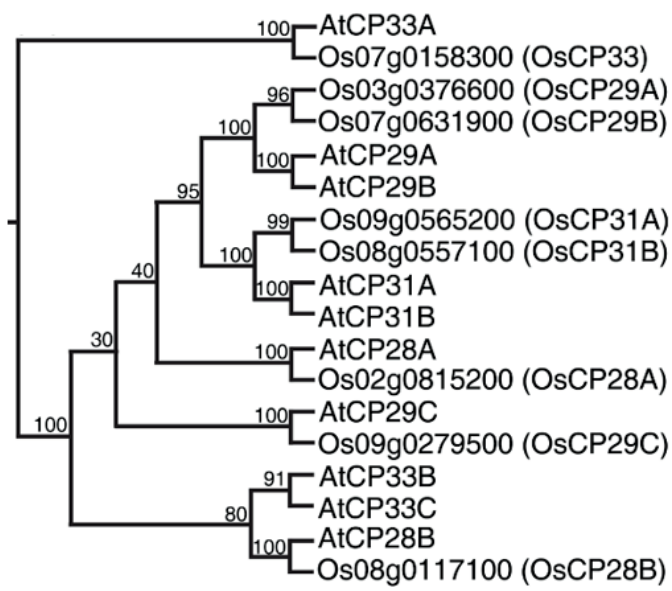
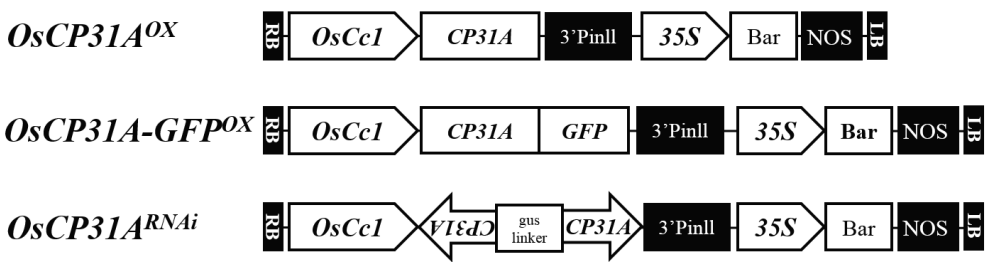

AtCP33A
Os07g0158300 (OsCP33)
Os03g0376600 (OsCP29A)
Os07g0631900 (OsCP29B)
AtCP29A
AtCP29B
Os09g0565200 (OsCP31A)
Os08g0557100 (OsCP31B)
AtCP31A
AtCP31B
AtCP28A
Os02g0815200 (OsCP28A)
AtCP29C
Os09g0279500 (OsCP29C)
AtCP33B
AtCP33C
AtCP28B
Os08g0117100 (OsCP28B)

Fig 2. Vectors used in this study

(A) Vector maps of *OsCP31A* transgenic plants used in the study. *OsCP31A*^{OX} represents the overexpression construct, *OsCP31A-GFP*^{OX} represents the GFP tagged construct and *OsCP31A*^{RNAi} represents the knock-down construct. (B) Vector map of *OsCP31A-GFP*^{35OX} for subcellular localization

(A)


OsCP31A^OX
RB
OsCc1
CP31A
3'PinII
35S
Bar
NOS
LB
OsCP31A-GFP^OX
RB
OsCc1
CP31A
GFP
3'PinII
35S
Bar
NOS
LB
OsCP31A^RNAi
RB
OsCc1
CP31A
gus linker
CP31A
3'PinII
35S
Bar
NOS
LB

(B)

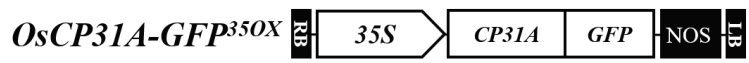

OsCP31A-GFP^35OX
RB
35S
CP31A
GFP
NOS
LB

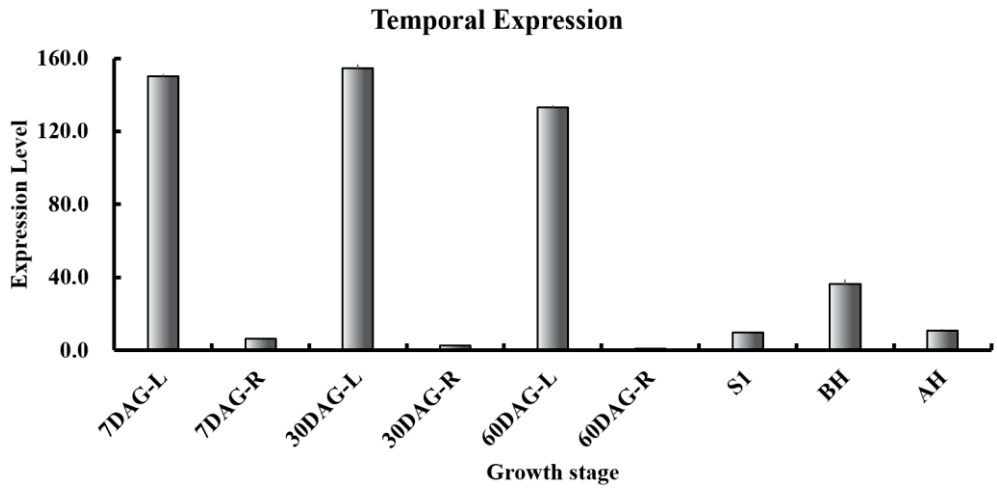
Fig 3. Temporal expression pattern of *OsCP31A*

(A) Expression of *OsCP31A* was analyzed at different time points of plant development in NT (*Oryza sativa* cv. Ilmi) including leaves (L), roots (R) and the flowering stage: less than 1 cm in panicle size (S1), before heading (BH) and after heading (AH). Data are shown as the mean ± standard deviation (SD) of two biological and two technical replicates.

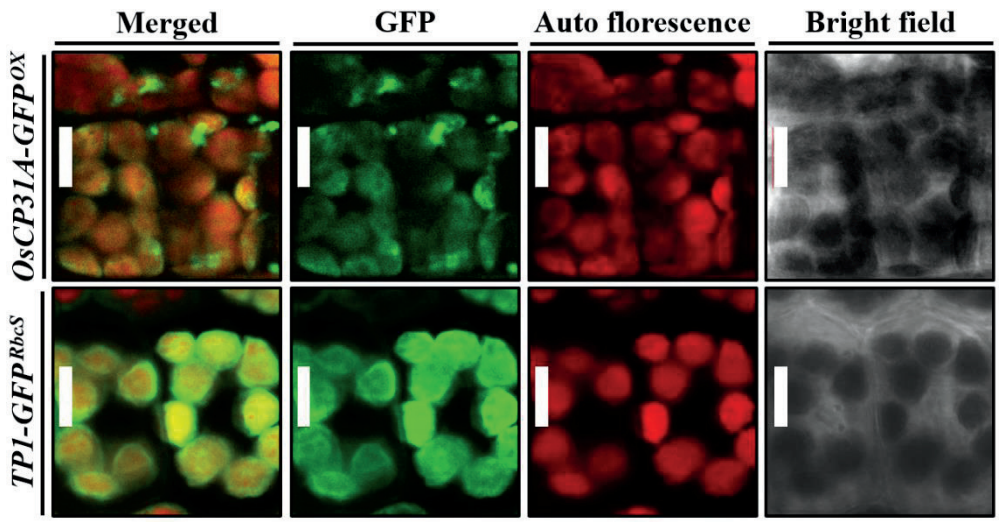
(B) Localization was confirmed by the observation of GFP fluorescence in 1-week-old *OsCP31A-GFP*^{OX} and *TP-GFP*^{RbcS} plants via Leica SP8 STED laser scanning confocal microscope (Leica, Germany). White scale bar represents 10μm.

(C) Rice protoplast transient expression system was used to identify foci. *OsCP31A-GFP*^{OX} vector and Plastid markers containing CFP were co-transfected. White scale bar represents 10 μm.

(A)


Temporal Expression
160.0
120.0
80.0
40.0
0.0
Expression Level
7DAG-L
7DAG-R
30DAG-L
30DAG-R
60DAG-L
60DAG-R
S1
BH
AH
Growth stage

(B)


Merged
GFP
Auto florescence
Bright field
OsCP31A-GFP OX
TP1-GFP RbcS

(C)

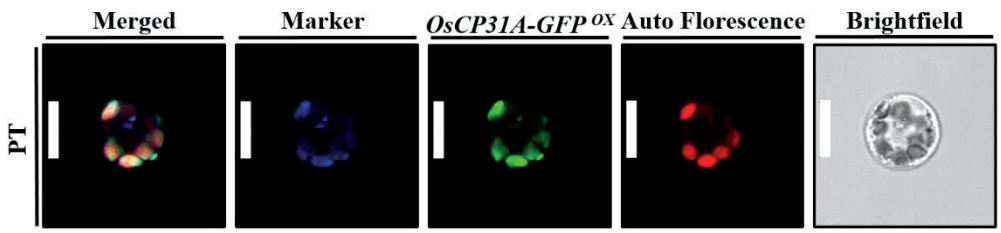
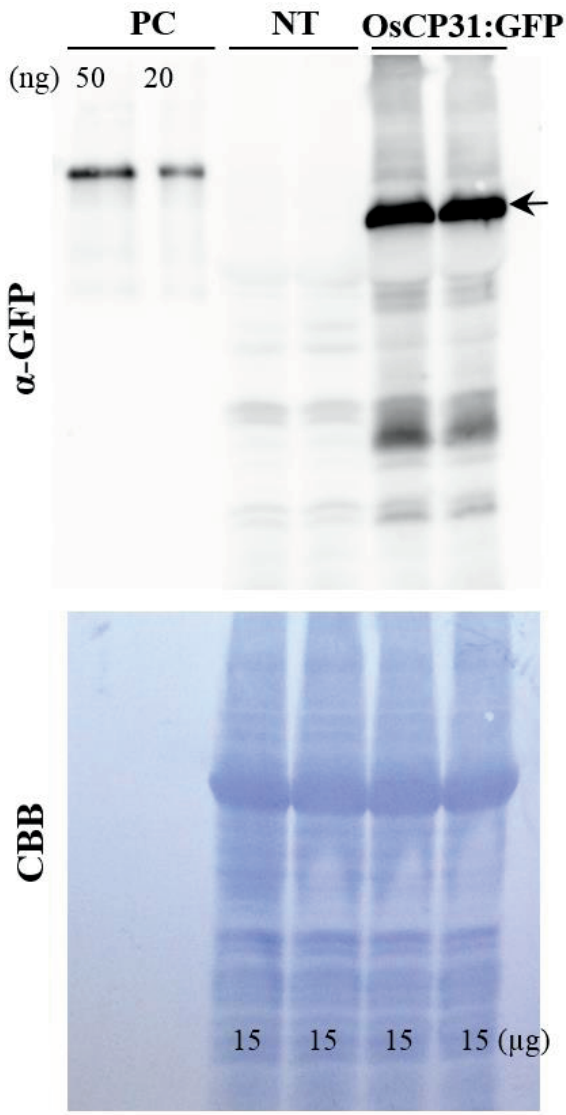

Merged
Marker
OsCP31A-GFP OX
Auto Florescence
Brightfield
PT

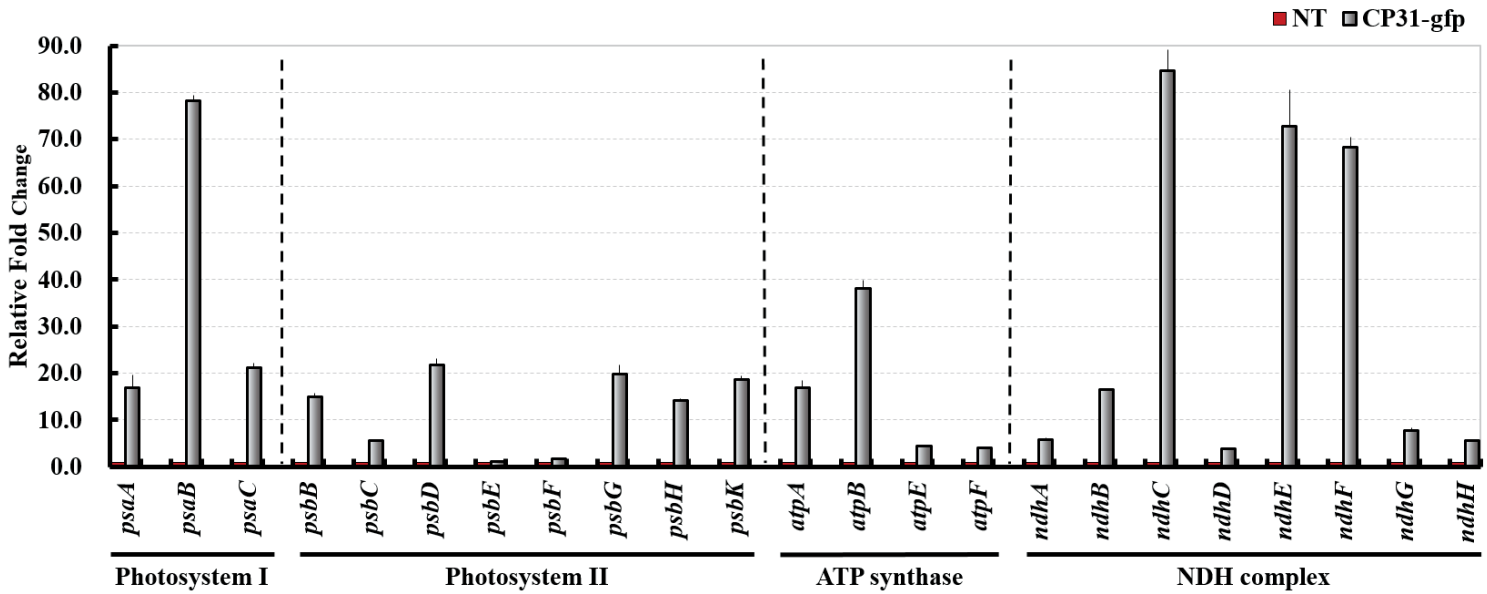
Fig 4. Chloroplast RNA targets of OsCP31A

(A) RNA-immunoprecipitation (RIP) assay was carried out by using 2-week-old *OsCP31A-GFP* ^{OX} to determine target chloroplast RNAs. OsCP31A proteins were confirmed by immunoblotting analysis. NT was normalized as 1 and compared with 23 genes related to photosynthesis.

(b) qRT-PCR was performed with the same 23 genes using cDNAs of *OsCP31A*^{OX} (#12), NT, and *OsCP31A*^{RNAi} (#6) 2-week-old plants to confirm stability. Values show the mean ± SD of two biological and two technical replicates.

(A)




NT
CP31-gfp
Relative Fold Change
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
psaA
psaB
psaC
psbB
psbC
psbD
psbE
psbF
psbG
psbH
psbK
atpA
atpB
atpE
atpF
ndhA
ndhB
ndhC
ndhD
ndhE
ndhF
ndhG
ndhH
Photosystem I
Photosystem II
ATP synthase
NDH complex

(B)

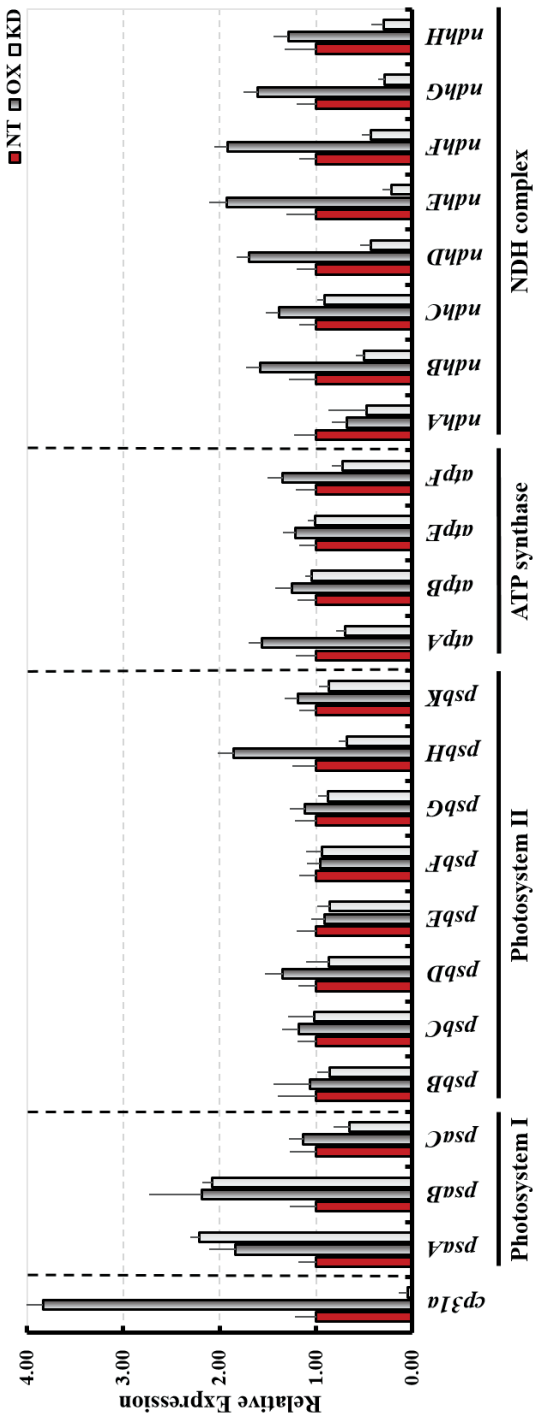

NT OX KD
Relative Expression
0.00
1.00
2.00
3.00
4.00
cp31a
psaA
psaB
psaC
psbB
psbC
psbD
psbE
psbF
psbG
psbH
psbK
atpA
atpB
atpE
atpF
ndhA
ndhB
ndhC
ndhD
ndhE
ndhF
ndhG
ndhH
Photosystem I
Photosystem II
ATP synthase
NDH complex

Fig 5. RNA editing analysis of transgenic and NT plants

Electropherogram of known RNA editing sites of rice in *OsCP31A*^{OX} (#12), NT, and *OsCP31A*^{RNAi} (#6). cDNA was synthesized of 2-week-old plant RNAs. Editing sites were cloned and analyzed by Sanger sequencing. *OsCP31A*^{RNAi} did not edit codon 182 and 187 in *rpoB* normally under normal growth condition. Purple Boxes indicate the altered sequence.

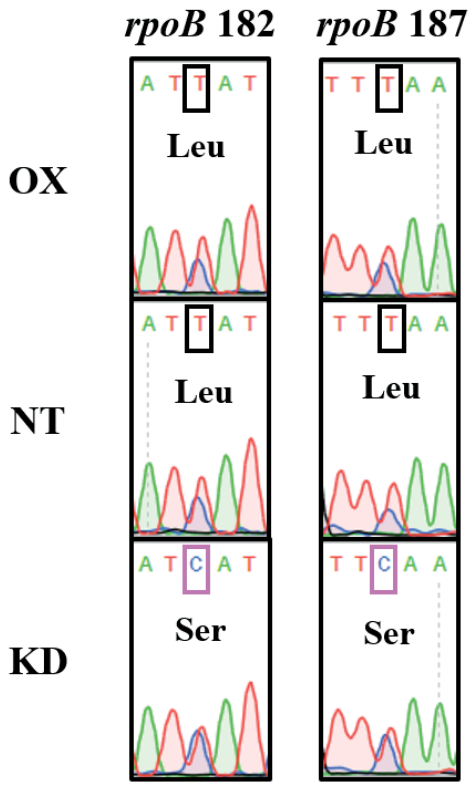

rpoB 182
rpoB 187
OX
NT
KD
A T T A T
T T T A A
Leu
A T C A T
T T C A A
Ser

Figure 6. Phenotypic analysis of transgenic and NT plants under drought stress

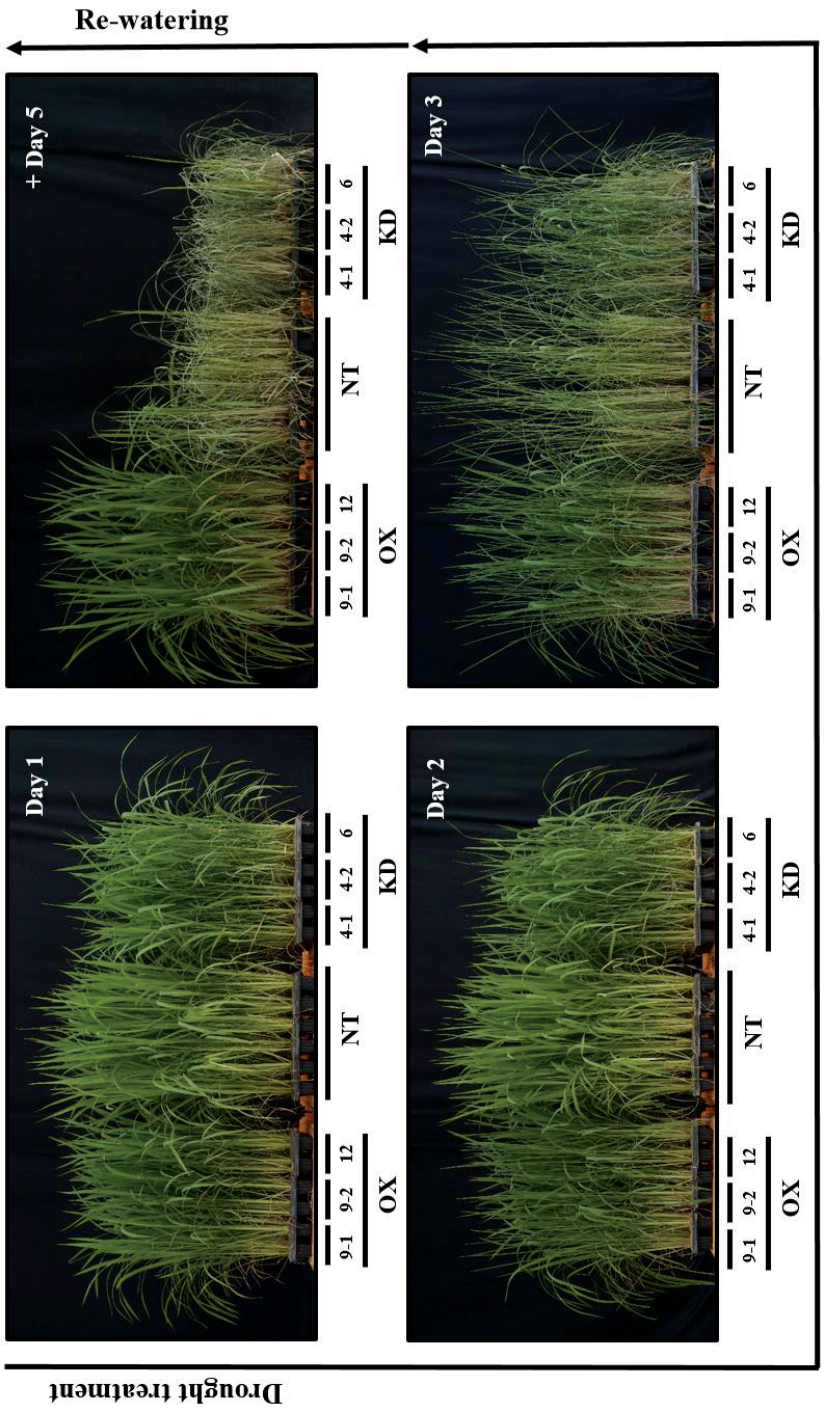
(A) Phenotypes of 5-week-old *OsCP31A*^{OX} (9-1, 9-2, 12), NT, and *OsCP31A*^{RNAi} (4-1, 4-2, 6) plants and under drought stress. Plants were dehydrated for 3 days followed by re-watering for 5days. Numbers indicate individual lines. A total of 30 plants were planted for each line.

(B) Expression level of *OsCP31A* of chosen lines for study. Plants were grown for 2 weeks and sampled. NT was set as 1 for normalization.

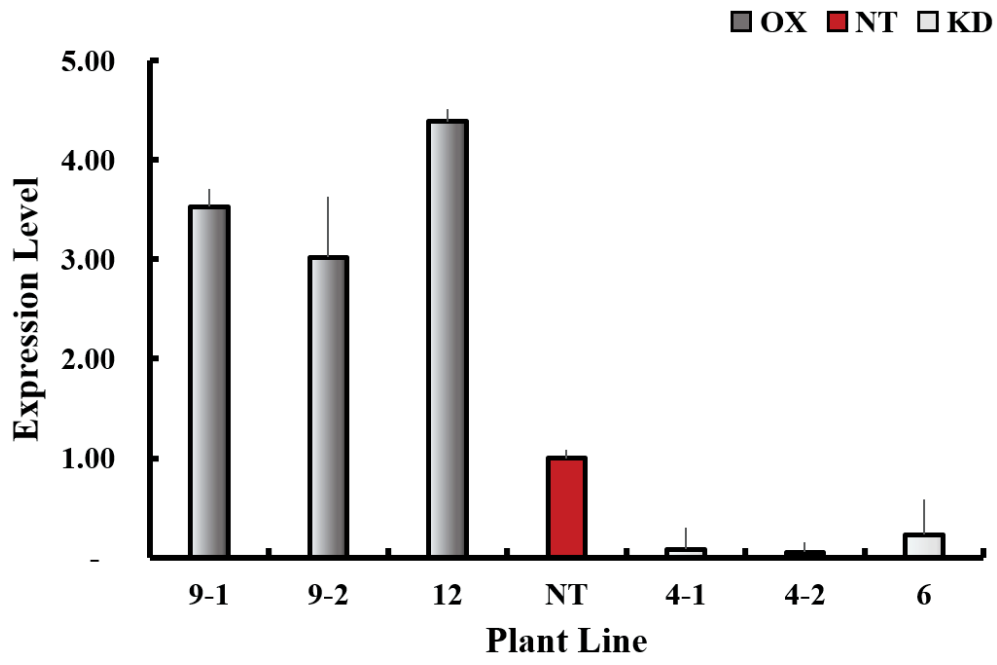
(C) Soil moisture of transgenic and NT plants. 10 measurements were averaged for each line.

(D). Chlorophyll fluorescence (Fv/Fm) was determined by measuring from the upper surface of the leaves with Handy PEA fluorimeter (Hanstech Instruments, UK). Plants were adapted in the dark for at least 2 hours prior to measurement. Each data point represents the mean ± SD (n=30).

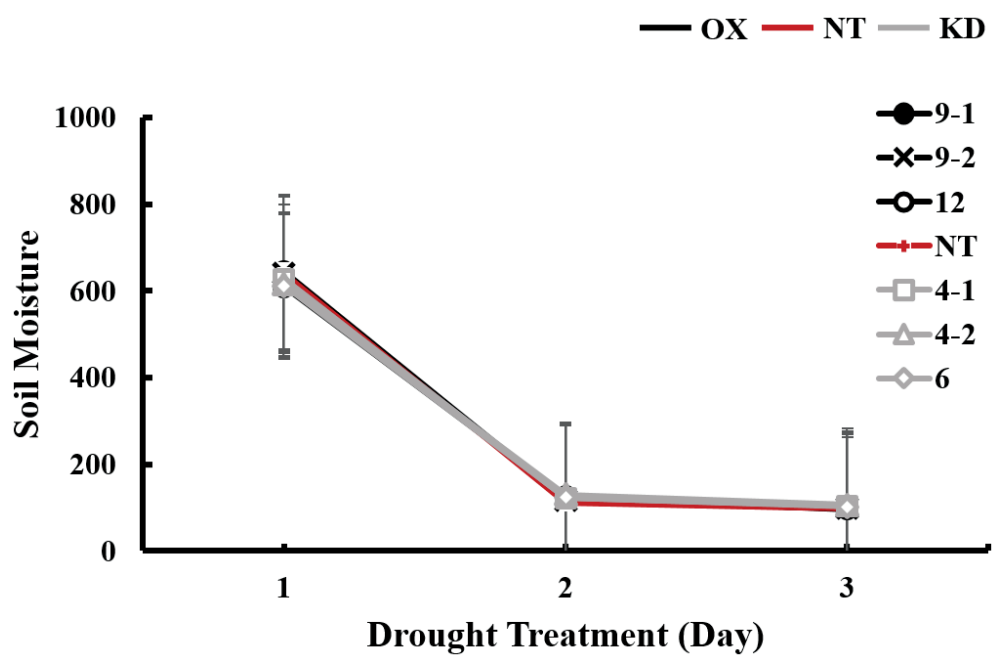
(A)


Re-watering
+ Day 5
Day 3
Day 1
Day 2
9-1 9-2 12
OX
NT
4-1 4-2 6
KD
Drought treatment

(B)


OX
NT
KD
5.00
4.00
3.00
2.00
1.00
-
Expression Level
9-1
9-2
12
NT
4-1
4-2
6
Plant Line

(C)


OX
NT
KD
1000
800
600
400
200
0
Soil Moisture
9-1
9-2
12
NT
4-1
4-2
6
1
2
3
Drought Treatment (Day)

(D)

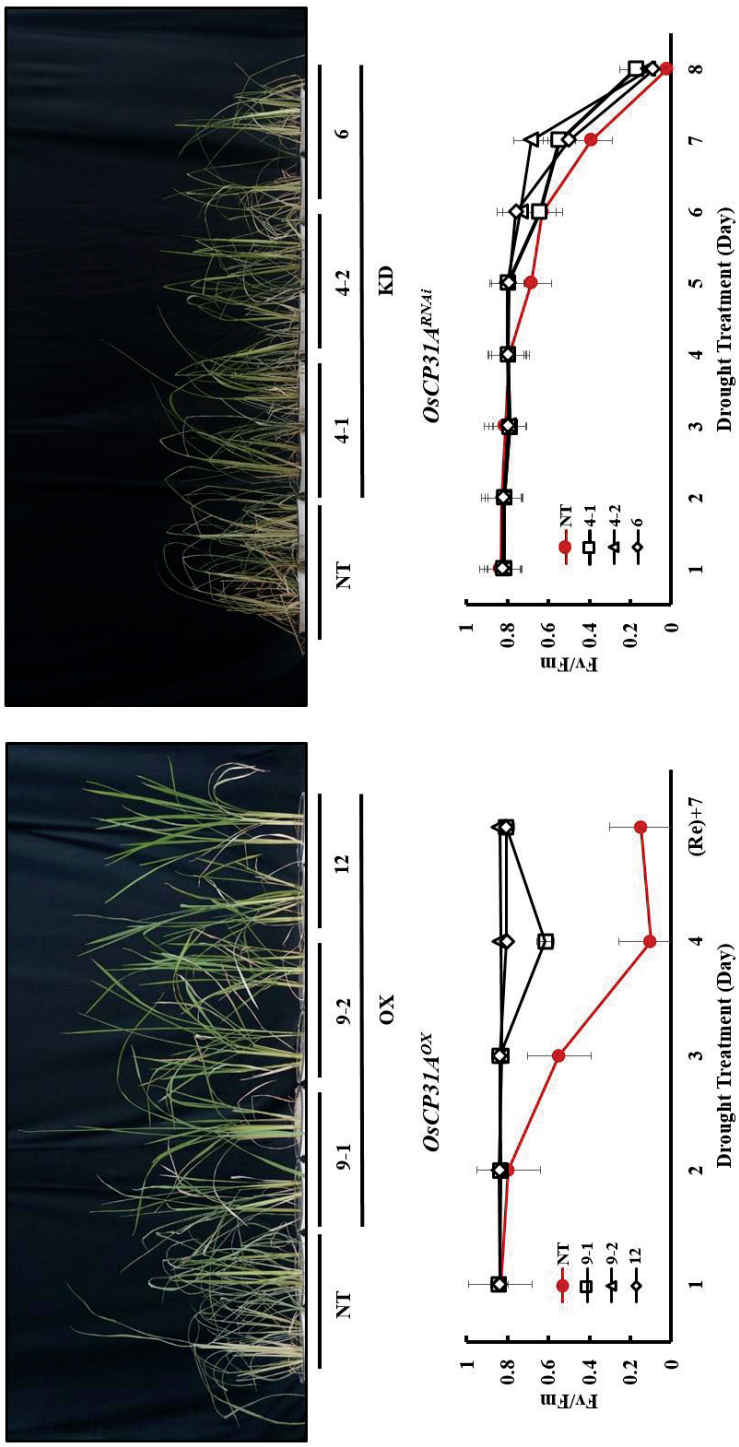

NT
9-1
9-2
12
OX
OsCP31A^OX
NT
9-1
9-2
12
Fv/Fm
Drought Treatment (Day)
(Re)+7
NT
4-1
4-2
6
KD
OsCP31A^RNAi
NT
4-1
4-2
6
Fv/Fm
Drought Treatment (Day)

Figure 7. RNA stability under drought condition

2-week-old *OsCP31A*^{OX} (OX), NT, and *OsCP31A*^{RNAi} (KD) plants were transferred from soil to water and adapted for 2 days prior to drought treatment. RNA was extracted from leaves of non-treated and 6 hour air-dried samples. Expression levels of 10 plastid encoded NDH genes were analyzed by qRT-PCR. *Ubi1* was used as internal control and each non-treated NDH genes were normalized as 1. Each data point represents the mean ± SD of three biological and three technical replicates.

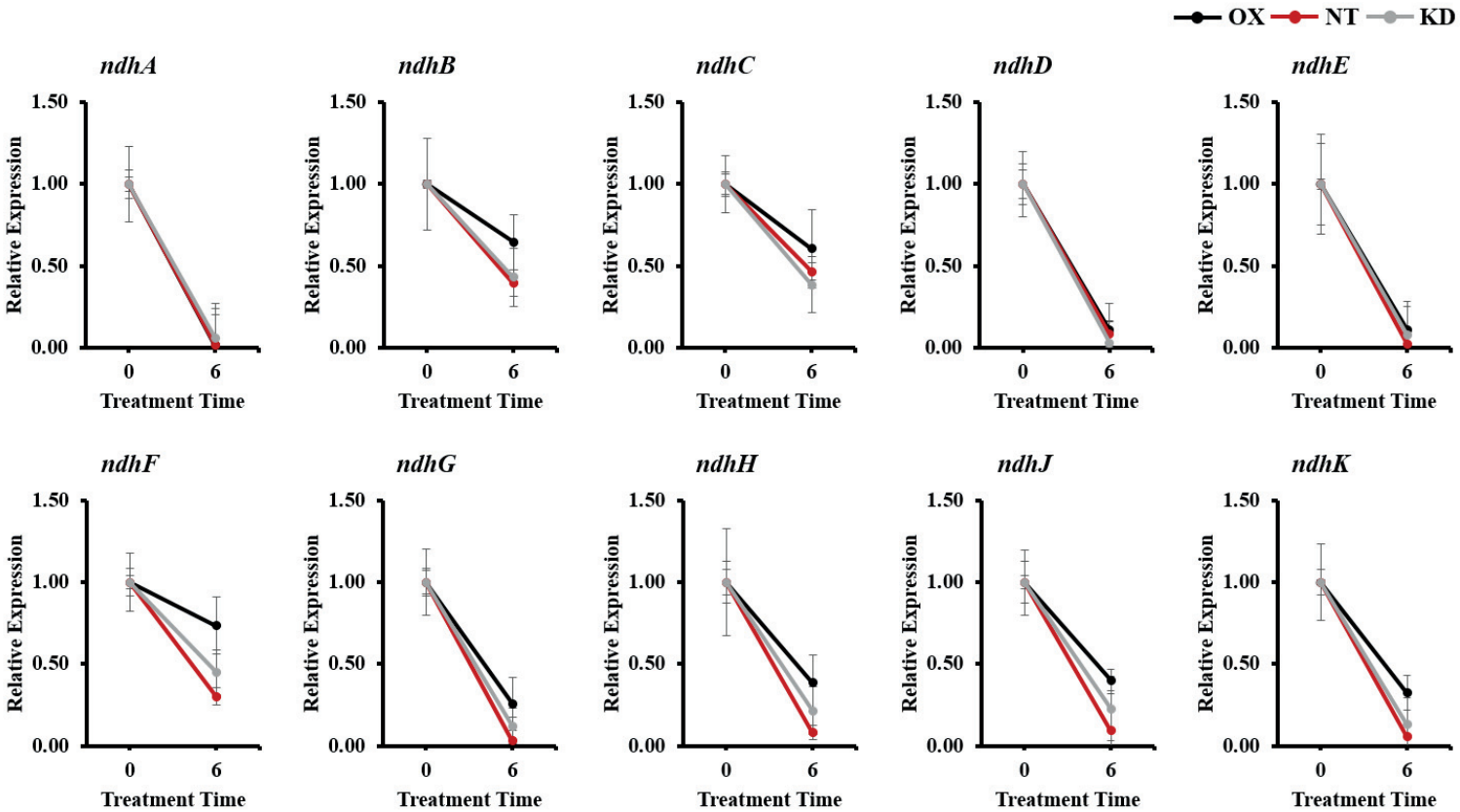
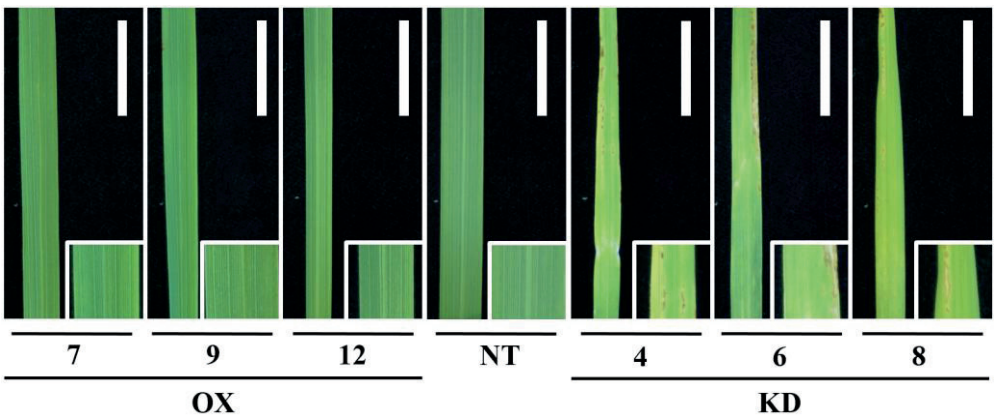

OX
NT
KD
ndhA
ndhB
ndhC
ndhD
ndhE
ndhF
ndhG
ndhH
ndhJ
ndhK
Relative Expression
1.50
1.00
0.50
0.00
0
6
Treatment Time

Figure 8. Phenotypic analysis of transgenic and NT plants under high light stress

(A) *OsCP31A*^{OX}, NT, and *OsCP31A*^{RNAi} plants were grown for 2weeks in chamber conditions and light intensities were 170 ~ 180 μmol m^{-2} s^{-1} prior to stress treatment. Then plants were transferred to light intensities of 240 ~ 240 m^{-2} s^{-1} to observe phenotypes. All light measurements were made with LI-250A Light Meter (LI-COR, USA). Photos were taken 2 weeks after treatment. White scale bar indicates 3 cm. The analysis was carried out by three biological repeats and three technical repeats. (B) The upper portion of the leaves were used for the analysis because chlorotic symptoms were severe in these areas. Third leaf from the top was used in all measurements. Values are the means + SD (n=30).

(A)


7
9
12
NT
4
6
8
OX
KD

(B)

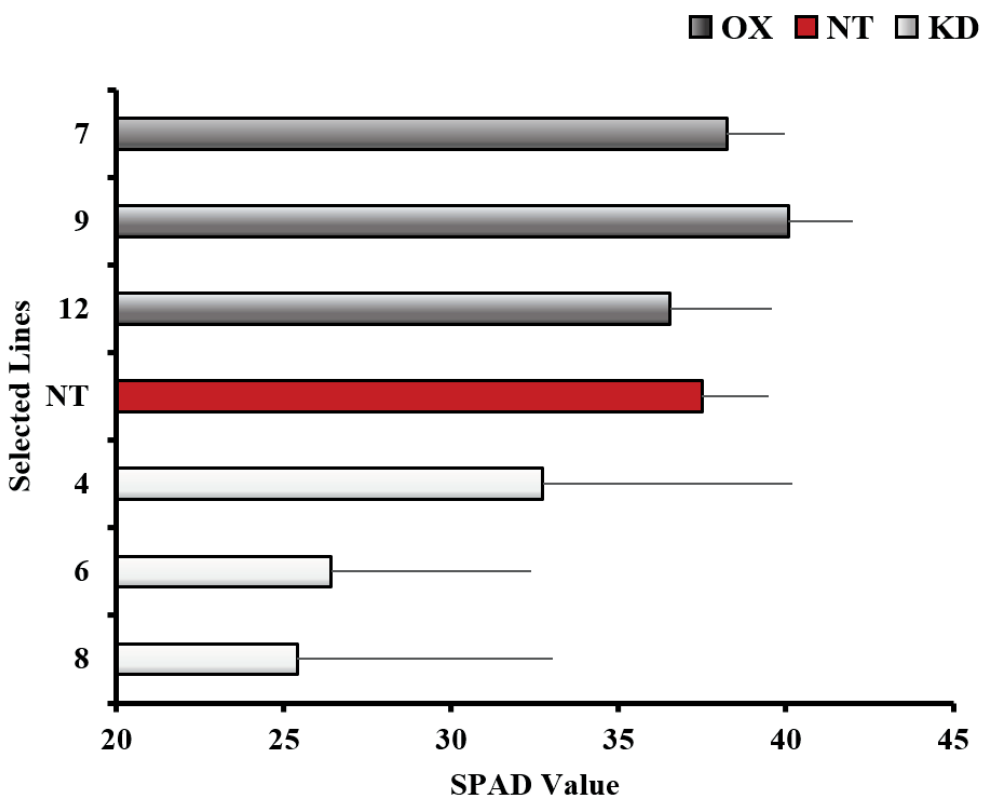
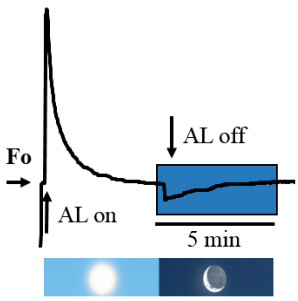

OX
NT
KD
Selected Lines
7
9
12
NT
4
6
8
20
25
30
35
40
45
SPAD Value

Figure 9. Monitoring NDH-dependent CET through Chlorophyll *a* fluorescence analysis

Chlorophyll *a* fluorescence of $OsCP31A^{OX}$, NT, and $OsCP31A^{RNAi}$ plants. 5-week-old plants grown in chamber conditions prior to analysis. Middle portions of leaves were measured with mini-PAM (Waltz, Germany). Fluorescence curve which represents the NDH-dependent CET was examined for 5 minutes after actinic light (AL) was turned off (blue box).


Fo
AL off
AL on
5 min

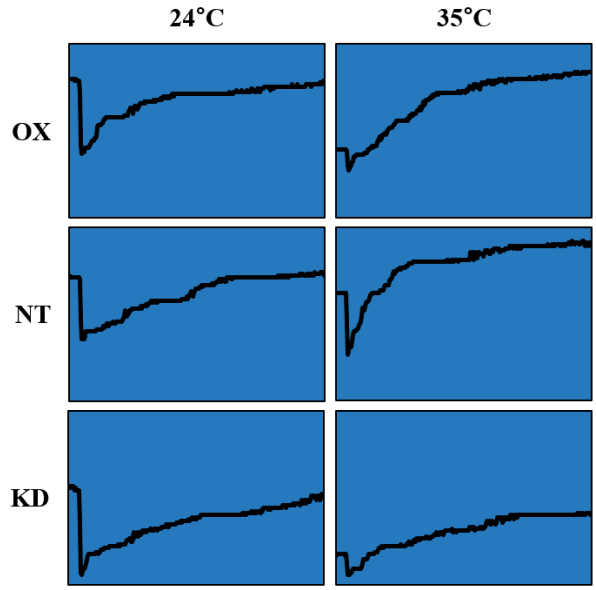

24°C
35°C
OX
NT
KD

Figure 10. Diurnal rhythm of NDH genes

Diurnal rhythm of *OsCP31A* and 10 NDH genes. 14DAG *OsCP31A* ^{OX}, NT and *OsCP31A*^{RNAi} plants grown in chamber conditions were sampled every 3 hours. The day condition in the chamber was set from 09:00 to 21:00 and the night condition was set from 21:00 to 09:00. RNA was extracted to synthesize cDNA and qRT-PCR was performed to determine relative expression. Genes were normalized to expression of NT at 09:00. Each data point represents the mean ± SD of three technical replicates.

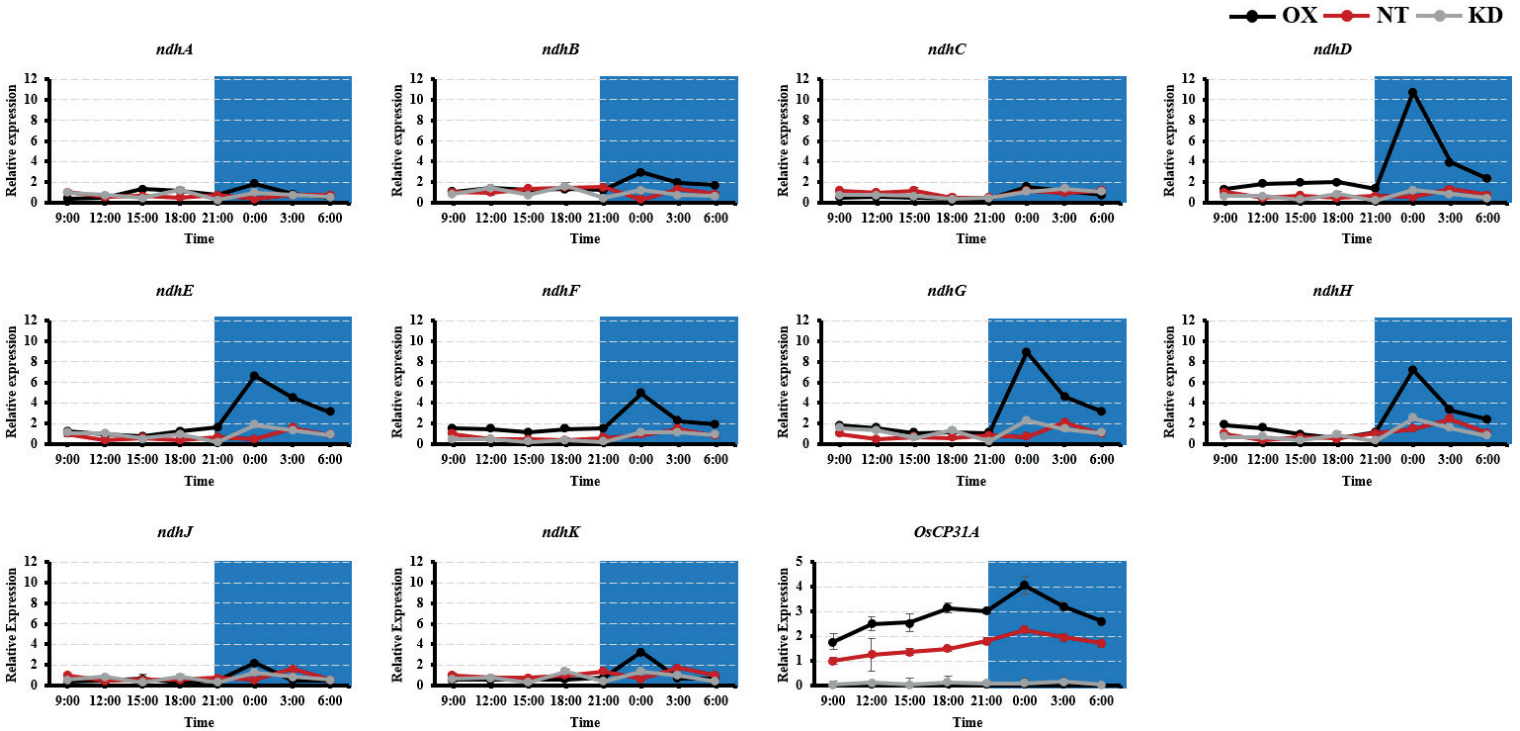

OX
NT
KD
ndhA
ndhB
ndhC
ndhD
ndhE
ndhF
ndhG
ndhH
ndhJ
ndhK
OsCP31A
Relative expression
Relative Expression
Time
9:00 12:00 15:00 18:00 21:00 0:00 3:00 6:00

Figure 11. ATP content under drought stress

ATP content of *OsCP31A*^{OX}, NT, and *OsCP31A*^{RNAi} plants under drought stress. 4-week-old plants were grown in soil and transferred to water for 2 days prior to drought treatment. Air-dried plants were sampled at 1 and 3 hour of treatment to compare ATP content. The middle portion of the second leaves from the top were accurately weighed. The samples were cooled to room temperature after moving them out of boiling water. Infinite M200 PRO was warmed up for 10 minutes prior to measurement and set to 10000 ms for integration time and 1000 ms for settle time. The wells were adapted in the device for at least 1 minute to remove background luminescence. Data are shown as the mean + SD of two biological and two technical replicates.

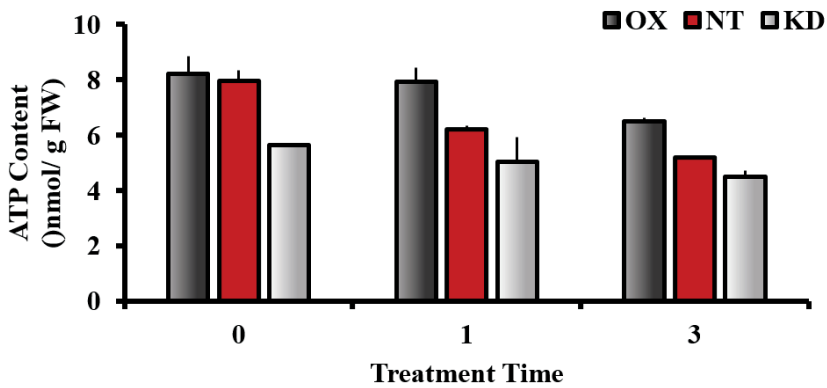
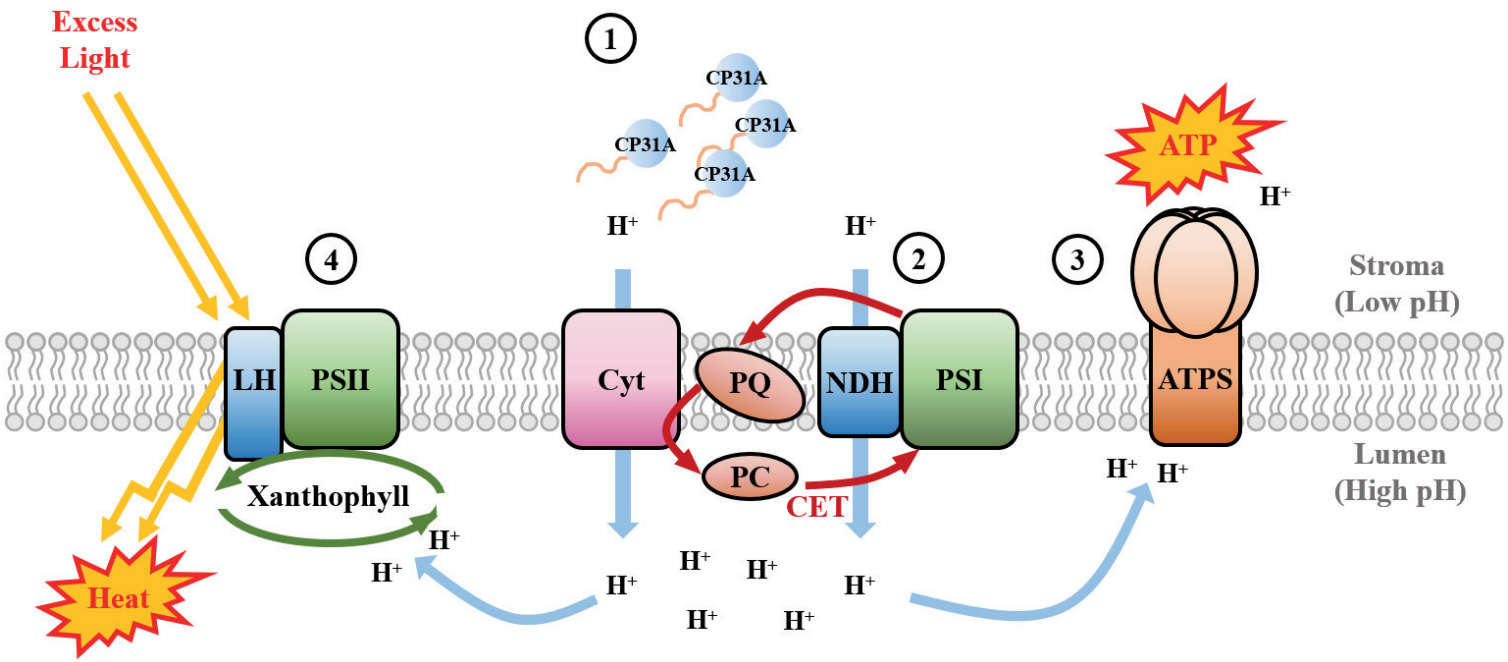

OX
NT
KD
10
8
6
4
2
0
ATP Content
()nmol/ g FW)
0
1
3
Treatment Time

Figure 12. Schematic model of NDH-dependent CET in *OsCP31A* overexpressors and physiological roles under drought condition

In *OsCP31A*^{OX} plants, NDH complex RNAs were stabilized (1) under drought condition, leading to steady state expression of NDH complex genes. This enhanced the NDH-dependent CET (2) in overexpressors. Studies show that when NDH dependent CET is active, protons from the stroma are transferred into the thylakoid lumen causing acidification. Some protons are used for ATP synthesis, (3) keeping the ideal NADPH/ ATP ratio and providing ATPs to other chloroplast metabolisms. Other protons are used in the xanthophyll cycle (4) which is involved in NPQ. The excess light energy is dissipated through heat in the light harvesting complex of PSII, preventing potential photo-oxidative damage. The red arrows show cyclic electron transport, the blue arrows show movement of protons, the green arrows show the xanthophyll cycle, and the yellow arrows show the movement of excess light energy. Abbreviations: ATPS, ATP synthase; CET, cyclic electron transport CP31A, OsCP31A; Cyt, cytochrome b_6f complex; H^+, proton; LH, light harvesting complex; NDH, NADH dehydrogenase-like complex; PSI, photosystem I; PSII, photosystem II; PC, plastocyanin; PQ, plastoquinone.


Excess
Light
1
CP31A
CP31A
CP31A
CP31A
ATP
H+
H+
H+
4
2
3
Stroma
(Low pH)
LH
PSII
Cyt
PQ
NDH
PSI
ATPS
PC
CET
Xanthophyll
Lumen
(High pH)
H+
H+
Heat
H+
H+
H+
H+
H+
H+
H+
H+

Discussion

In this study, we identified 8 chloroplast ribonucleoprotein(cpRNP)-like genes in rice through homology and phylogenetic analysis (Fig 1). Among the 8 rice cpRNPs, we finally isolated *OsCP31A*, which is homologous to the abiotic stress related Arabidopsis CP31A, and examined its function further. *OsCP31A* was localized in the stroma of chloroplasts (Fig 3). Similar to the Arabidopsis CP31A (Tillich et al., 2009), OsCP31A had a broad binding affinity towards chloroplast RNAs (Fig 4A). However, the expression of *OsCP31A* in rice did not show much association with chloroplast RNA editing (Fig 5). Through the qRT-PCR analysis, we confirmed that the expression of *OsCP31A* regulates the expression of NADH dehydrogenase-like (NDH) complex genes (Fig 4B). The expression of NDH complex genes were increased in overexpressors ($OsCP31A^{OX}$) and decreased in knock-down ($OsCP31A^{RNAi}$) plants. We speculated this was a result of RNA stability, a feature of cpRNPs. These proteins are known to bind directly to target chloroplast RNAs, possibly protecting them from ribonuclease activities (Nakamura et al., 2001). It is believed that chloroplast gene expression is influenced more by RNA stability than transcriptional regulation (Del Campo, 2009; Jiao et al., 2004). In Arabidopsis, the chloroplast gene expression is reduced when cpRNPs are absent (Tillich et al., 2009). Taken together, we suggest that OsCP31A is a chloroplast RNA binding protein which controls the expression of NDH complex genes through RNA stabilization.

Studies are reveling that the NDH complex is abiotic stress related. When plants are exposed to stress, reactive oxygen species (ROS) is formed which causes damage to plants. To prevent this, excessive light energy is dissipated as heat in light harvesting complex through non-photochemical quenching (NPQ) (Fig 12) (Müller et al., 2001; Niyogi, 1999). NPQ is achieved via the xanthophyll cycle, which is activated by protonation of the thylakoid lumen (Ruban et al., 2012). It is suggested that the NDH complex, which sides with photosystem I (PSI) supply this proton gradient (ΔpH) through cyclic electron transport (CET) (Shikanai, 2007; Wang et al., 2006). This NDH-dependent CET is essential for rice plants under low temperature (Yamori et al., 2011), low light (Yamori et al., 2015) and fluctuating light (Yamori et al., 2016) conditions. Interestingly, the expression of *CP31A* in Arabidopsis is required for chloroplast homeostasis under low temperature condition (Kupsch et al., 2012). It has been suggested that CP31A stabilizes certain RNAs under cold stress. In rice, we identified that *OsCP31A* modulates the expression of NDH complex genes. This might enhance NDH-dependent CET activity required in abiotic stress conditions. Two studies have shown that overexpression of the protein gradient regulation 5 (PGR5) gene in the PGR5/PGR-like 1 pathway increases CET (Long et al., 2008; Okegawa et al., 2005). This results in drought and light stress tolerance. Similar to this alternative pathway, we hypothesized that the increased expression of the NDH complex genes in *OsCP31* overexpressors enhances NDH-dependent CET. We found drought tolerance was enhanced in $OsCP31A^{OX}$ plants (Fig 6). Under drought condition, the overall expression of NDH complex genes were stabilized in $OsCP31A^{OX}$ plants (Fig 7) except *ndhA*, *ndhD*, and *ndhE* genes. Since the expression

of *ndhD* and *ndhE* was strongly altered in transgenic plants, we suspected that stabilization was not detectable due to the shorter half-lives. Unlike the two, we presumed that the *ndhA* is not the target of OsCP31A because minor changes in expression were detected under normal conditions. Under high light conditions, chlorotic phenotypes were observed in *OsCP31A*^{RNAi} plants (Fig 8). These are well-known characteristics of light-mediated oxidative damage, caused by deficient NDH-dependent CET (Endo et al., 1999; Horváth et al., 2000; Munekage et al., 2004). However, we could not distinguish *OsCP31A*^{OX} from NT plants under a continuous high light treatment because chlorotic phenotypes emerged in both plants eventually. The responsiveness of NDH-dependent CET was enhanced in overexpressors; this was confirmed by the measurement of the chlorophyll fluorescence (Fig. 9). In contrast, *OsCP31A*^{RNAi} plants had significantly lower readings upon post-illumination, indicating inefficiency in NDH-dependent CET. We observed expression of NDH complex genes were notably increased in *OsCP31A*^{OX} plants upon post illumination (Fig 10) when CET is known to reach the maximum rate (Joliot et al., 2004; Joliot and Joliot, 2006). The final experiment showed that more ATPs were present in *OsCP31A* ^{OX} plants than in NT and *OsCP31A*^{RNAi} plants under drought condition (Fig 11). This supports the argument that NDH-dependent CET is enhanced in *OsCP31A*^{OX} plants, providing extra ATPs for optimizing homeostasis under water-limiting conditions.

Taken together, we propose that the overexpression of *OsCP31A* improves drought tolerance by regulating the expression of NDH complex genes, enhancing the NDH-

dependent CET and the production of ATPs. Through further studies, we can confirm the NDH complex RNA stability with transcriptional inhibitor treatment. Electron transport rate of PSI, p700 redox and CO_2 assimilation could also be analyzed to clarify NDH-dependent CET activity for future applications. Since the increased CET is known to enhance plant tolerance to other abiotic stresses, it would be worthwhile to examine multi tolerance of overexpressors as well.

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ABSTRACT IN KOREAN

환경 저항성과 관계된 NDH complex 유전자를 조절하는 RNA 결합 단백질인 OsCP31A에 대한 연구

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한해에 가뭄에 의해 작물이 입는 패해가 세계적으로 100억 달러에 달하는 것으로 알려져 있다. 이를 극복하기 위해 생물공학적인 접근들이 이루어지고 있으며 대표적인 방법으로 식물의 뿌리 구조와 기공의 개폐 조절이 있다. 다른 방법은 엽록체 유전자를 통해 엽록체 항상성을 유지하는 것이다. 엽록체 유전자들은 RNA processing을 통해 발현을 조절하는데 RNA 결합단백질이 그 역할을 담당하며 chloroplast ribonucleoprotein (cpRNP)이 여기에 포함된다. 본 연구에서는 계통발생 분류로 환경저항성과 관련 있는 cpRNP-like인 Os09g0565200을 선별하여 OsCP31A로 명명하고, 과발현체와 저발현체 벼를 통해 그 기능을 관찰하였다. 관찰 결과 *OsCP31A* 유전자는 성장 단계 동안 엽록체의 스트로마(stroma)에 발현했

으며, OsCP31A 단백질은 다른 cpRNP와 유사하게 전반적인 엽록체 RNA 결합을 보였다. 여러 가뭄조건하에서 *OsCP31A* 과발현체가 저항성을 띄었으며, qRT-PCR을 통해 NDH complex 유전자가 비교적 안정화 되어있는 것이 관찰되었다. 이 NDH complex는 광계I에 위치하고 환경적인 스트레스에서 전자 전달을 한다고 알려져 있는데, 이를 NDH에 의한 순환전자전달이라 지칭한다. 본 연구에서는 NDH 순환전자전달에 의해 가뭄저항성이 향상된다고 가정하고 이를 증명하기 위해 형질전환 벼의 엽록소 발광을 측정하였다. 측정 결과 Actinic light가 차단된 상태에서 순환전자전달의 효율성이 과발현체에서 증대되었고 저발현체에서는 감소되었다. 마지막으로 NDH 순환전자전달의 생산물인 ATP content를 형질전환 벼와 재배종 벼에서 측정하였다. 일반 조건하에서 저발현체의 ATP content가 낮았으며 과발현체와 재배종은 유사했다. 그러나 가뭄 조건하에서는 과발현체의 ATP content가 가장 높았으며 다음으로 재배종, 저발현체의 순서였다. 이상의 관찰 결과를 종합하여 본 논문에서는 *OsCP31A*의 과발현을 통해 NDH complex 유전자들을 조절하여 가뭄저항성을 향상시킬 수 있다는 결론을 제시한다.

주요어: 벼, chloroplast ribonucleoproteins, 형질전환, 가뭄 저항성, NDH complex, 순환전자전달

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