**Master's Thesis of Science in Agriculture**

# Overexpression of *OsNAC14*, a NAC domain transcription factor, enhances tolerance to drought stress in rice.

## OsNAC14 전사인자 과발현 벼의 내건성 향상에 대한 연구

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# ABSTRACT

## Overexpression of *OsNAC14*, a NAC domain transcription factor, enhances tolerance to drought stress in rice.

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Environmental stresses such as drought, negatively affects crop production and food security, hence the development of stress tolerant crops has been greatly needed for modern agricultural areas. Transcription factors (TFs) are good candidates for genetic engineering to improve stress tolerance in crops because of their roles as master regulators of many stress-responsive genes. Here, we generated transgenic rice plants overexpressing *OsNAC14* to examine its function in drought tolerance. Overexpression of *OsNAC14* in rice improved drought tolerance not only during the vegetative stage but also during the reproductive stage. In addition, RNA sequencing analysis showed that overexpression of *OsNAC14* altered the expression of around 273 genes, directly or indirectly

OsNAC14-dependent manner. From these differentially regulated genes, we selected 16 genes based on gene ontology, including programmed cell death (GO:0012501) and DNA repair (GO:0006281) for further verification. These were confirmed through the qRT-PCR analysis with a transgenic rice and transient assay system using protoplast. Most noticeably, *OsRAD51A1*, a DNA repair related protein gene, and Piz-t, Pyricularia oryzae resistance Z, were significantly up-regulated in *OsNAC14* transgenic rice. Furthermore, OsNAC14 directly interacts with the *OsRAD51A1* promoter region as revealed through ChIP analysis *in vivo*. Finally, these results suggest that *OsRAD51A1* is directly regulated by the OsNAC14 transcription factor and overexpression of *OsNAC14* improves drought tolerance of rice.

**Keyword:** NAC transcription factors, OsNAC14, Transgenic rice, Drought tolerance, Target gene validation, protoplast, RNA sequencing, ChIP

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABA | Abscisic acid |
| *bar* | *Bialaphos-resistance gene* |
| BLAST | Basic Local Alignment Search Tool |
| CDS | Coding region sequence |
| ChIP | Chromatin Immunoprecipitation |
| *Dip* | *Dehydration stress inducible protein* |
| *GFP* | *Green fluorescent protein* |
| LB | Left border |
| *NAM* | *No apical meristem* |
| *nos* | *nopaline synthase gene* |
| NT | Non-transgenic |
| NC | Negative control |
| PCR | Polymerase chain reaction |
| *PGD1* | *Phosphogluconate dehydrogenase 1* |
| *Pin Ⅱ* | *Potato proteinase inhibitor Ⅱ* |
| qRT-PCR | Quantitative real-time PCR |
| RB | Right border |
| *RbcS* | *Rubisco small subunit* |

| | |
|---|---|
| SAM | Shoot apical meristem |
| TF | Transcription factor |
| *Wsi18* | *Water-stress inducible protein 18* |
| *35S* | *Cauliflower mosaic virus 35S promoter* |

# Introduction

Rice (*Oryza sativa* L.) is the most important grain crop in the world. More than half of the world's population relies on rice as it is primary staple food (Tang *et al*., 2009). Moreover, rice is a model crop for monocotyledonous plant since its whole genome sequence is already unveiled. Therefore, studies on transgenic rice with stress resistance genes will be a meaningful attempt to move towards a sustainable crop production system.

Recent climate change affects many aspects of agriculture, especially growing conditions for crops. Various abiotic stresses including high temperature, cold, drought and flooding, cause poor field conditions and reduce grain yield of crops. Among them, drought is one of the serious issues to crop production (Miura *et al*., 2010; Jeong *et al*., 2013). Even though severe water deficits are a rare event in viable agriculture, drought impact on crop production is extremely huge. Thus, better understanding of the effects of drought on crops is vital for improved management practices in agriculture (Chaves et al., 2003).

Plants have adapted to diverse stresses by inducing expression of numerous genes which lead to physiological and biochemical changes that can overcome water deficit stress and increase the chance of plant survival (Kazuko *et al*., 2006; Zheng *et al*., 2009). Drought resistance has three concepts, which has drought escape via a short life cycle of developmental plasticity, drought avoidance via enhanced water uptake and reduced water loss, and drought tolerance via

osmotic adjustment, antioxidant capacity and desiccation tolerance (Yue *et al*., 2006; Hasebe *et al*., 2014; Xu *et al*., 2014). Recently, many researches have been focused on specific factors which enhance drought tolerance and grain yield, yet the whole regulatory mechanism still require further elucidation. Drought tolerance is an intricate mechanism which is controlled by multiple genes and their expressions are influenced by various environmental stresses and controlled by multiple genes. Their expressions are influenced by various environmental stresses, making the development of drought tolerant transgenic crops a challenging task (Rachmat *et al*., 2014).

Transcription factors (TFs) play important roles in stress responses by regulating a large number of target genes. The interaction between transcription factor and the cis-acting elements at the target gene promoters regulate target gene expression and eventually has a direct effect on cellular activities (Yue *et al*., 2005). NAC transcription factor family is one of the largest families of plant-specific transcription factors and functions in plant development and environmental stress responses. The NAC proteins contain a highly conserved N-terminal DNA- binding domain, which is referred as the NAC domain (Ooka *et al*., 2003). The NAC domain was identified based on sequences from Petunia NAM and Arabidopsis ATAF and CUC proteins. Recently, a genome-wide analysis of the NAC transcription factor family in rice and Arabidopsis has identified 151 and 117, respectively (Nuruzzaman *et al*., 2010). The first reported NAC gene in petunia, NAM (No Apical Meristem), participates in shoot apical meristem (SAM)

development which was determined from the position of the SAM (Souer *et al*., 1996). In case of Arabidopsis, *ATAF1* mediate multiple functions on abiotic and biotic stress adaptation (Lu *et at*., 2007). In rice, the stress responsive NAC (SNAC) transcription factors have been already well known as key players in drought tolerance *OsNAC5, OsNAC9* and *OsNAC52* etc. (Gao *et al*., 2010; Redillas *et al*., 2012; Jeong *et al*., 2013).

In order to identify the relationship of TFs and target genes, we generated transgenic plants and characterized them through physiological and molecular approach. We used RNA sequencing analysis to determine differentially expressed genes following the overexpression of *OsNAC14* in rice. As a result, we found 273 putative target genes dependent on *OsNAC14* expression and found the direct target *OsRAD1A1* through ChIP analysis. The *OsRAD1A1* promoter region showed association and highly enriched by *OsNAC14*. Regulation of *OsRAD1A1* expression by OsNAC14 may have promoted DNA repair under drought stress providing the plants with tolerance to stress. Thus, these results suggest that *OsNAC14* has a regulatory role in drought stress tolerance via modulation of expression of *OsRAD1A1*. These changes in expression profiles resulted in improved drought tolerance at the vegetative and reproductive stages of growth.

# Materials and Methods

## 1. Vector construction and rice transformation

The full-length coding region of *OsNAC14* (*Os01g0675800*) was amplified from rice cDNA (*Oryza sativa* cv. Nipponbare). Primers used for cloning are listed in Table 3. For *OsNAC14* overexpression in rice, the full-length cDNA was recombined into the p700 vector carrying *PGD1* promoter (Park et al., 2012) and the Gateway system (Invitrogen). The final construct, *proPGD1:OsNAC14* was transformed into rice (*Oryza sativa* cv. Nakdong) through Agrobacterium-mediated transformation method (Hiei *et al*., 1994) to generate *OsNAC14* overexpressing transgenic rice. Single copy insertion lines, were identified by Taq-Man PCR.

## 2. Plant growth and drought stress treatment

Transgenic and NT (non-transgenic) (*Oryza sativa* cv. Nakdong) seeds were germinated on agar medium containing 1x Murashige and Skoog medium salt (including vitamin), 3% sucrose and 0.3% phytagel in dark condition at 28℃ for 3 days (d) and transferred to light condition at 28℃ for 1 d. Seedlings were transplanted into soil and grown in the greenhouse (16 h-light/8 h-dark cycle) at 30℃. Thirty plants from each transgenic and NT plants were grown in pots (5×10 tray; 3 plants per pot) and grown for 5 weeks. Five week-old plants were subject to drought stress by withholding water for 3 d, followed by 7 d of re-watering. The

number of plants that survived and continued to grow were then scored.

## 3. JIP test

Transient chlorophyll *a* fluorescence in the plants were measured using the Handy PEA fluorimeter (Hansatech Instruments). 2-month-old soil-grown plants were adapted in darkness for at least 1 h to ensure sufficient opening of the reaction centers. Twenty spots were chosen from three independent homozygous lines of *proPGD1:OsNAC14* and NT plants. All leaves of the plant were measured at their top, middle and base parts. Fluorescence transients were induced by a red light (650 nm) of 3500 μmol photons provided by the three light-emitting diodes, focused on a spot of 5 mm in diameter and recorded for 1 s with 12-bit resolution. Normalization of data was performed using the Biolyzer 4HP software program, according to the equations of the JIP test. The transients exhibited the typical polyphasic rise O-J-I-P having the same variable fluorescence ($F_M - F_O = F_V$), indicating that the photosynthetic systems of plants were functioning.

## 4. Evaluation of the agronomic traits of rice plants grown in the field

The *proPGD1:OsNACC14* transgenic and NT plants were grown under both the normal and drought semi-field conditions. Three independent homozygous lines of the pro*PGD1:OsNAC14* plants, together with NT plants, were transplanted to the paddy field (Kyungpook National University, Gunwi, Korea). A randomized design

was employed with three replicates using three plots each of 5 ㎡. Yield parameters were scored from 30 and 18 plants each per line grown under normal and drought conditions, respectively. Yield parameters contain that culm length, panicle length, number of grain, number of total grain, number of filled grain, filling rate, total grain weight, 1000 grain weight. When the plants were grown under normal and drought conditions had reached maturity and the grains had ripened, they were harvested and threshed by hand. The results from ANOVA ($p < 0.05$ level) with Fisher's least significant difference for multiple comparisons were compared between transgenic and NT plants. SPSS version 23.0 software was used to perform these statistical analyses.

## 5. RNA-sequencing analysis

Total RNA was extracted from rice leaves (2 weeks old, grown soil) using Trizol reagent (Invitrogen) and purified with RNeasy Mini Kit (Qiagen). The libraries were prepared using the TruSeq RNA sample Prep Kit (v2) (Macrogen). RNA sequencing was repeated twice with samples from *proPGD1:OsNAC14* transgenic and NT plants. Single-end sequences were obtained using IRGSP (v 1.0) and raw sequence reads were trimmed to remove adaptor sequences and their qualities were checked using the Trimmomatic 0.32 software. After mapping reads to a reference genome (Os-Nipponbare-Reference-IRGSP-1.0), differentially expressed genes were analyzed and validated by more than 2-fold change value and independent T-test ($p$-value < 0.05), then 554 transcripts were selected for further analysis.

## 6. Real-time PCR analysis

Total RNAs were extracted from *proPGD1:OsNAC14* transgenic (Line 11) and NT plants, which grown 2-weeks-old in soil, using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using 1 μl total RNA and oligo $dT_{18}$ primers by 200 U of the RevertAid M-MuLV Reverse Transcriptase (Thermo Scientific) at 42℃ for 60 min, and then the reaction was terminated by incubating at 70℃ for 5 min. Subsequent quantitative RT-PCR was performed with 2x Real-Time PCR smart mix (SRH72-M10h, SolGent) with EvaGreen (31000-B500, SolGent). The reaction was performed by initial denaturation at 95℃ for 15 min, followed by 40 cycles of 95℃ for 20 s, 60℃ for 20 s, and 72℃ for 30 s, in a 20 μl reaction mixture containing 1 μl of 20 X EvaGreen, 10 μM primers, and ROX reference dye. Thermocycling and fluorescence detection were performed using a Strategene Mx300p real-time PCR machine and Mx3000p software (Stratagene). Transcript levels were normalized to *Ubi1* (*Os06g0681400*) gene expression.

## 7. Protoplast isolation and transient gene expression

Polyethylene Glycol (PEG)-mediated protoplast transformation system was used to express OsNAC14 transiently and verify the correlation between OsNAC14 and its target genes (Ohnuma *et al*., 2008). Rice seedlings (*Oryza sativa* cv. Ilmi) were grown in the dark for 10 d and transferred to the light (16 light / 8 dark) for 1 d.

Leaf sheaths of 100 rice seedlings were cut into 0.5 mm pieces using a sharp blade on a glass. The pieces were transferred into 0.6 M mannitol solution and incubated at least 10 min at room temperature. After removal of mannitol solution, the pieces were soaked in enzyme solution [1.5% Cellulase R-10 (Yakult, Japan), 0.75% Macerozyme R-10 (Yakult, Japan), 0.5 M mannitol, 10 mM MES (pH 5.7), 0.1% BSA, 10 mM $CaCl_2$, and 5 mM β-mercaptoethanol] for cell wall degradation. Vacuum was applied to the enzyme solution for 15 min and the digestion was carried out in the dark at 28℃ for 4.5 h. The enzyme solution was filtered twice through 70μm and 40μm nylon meshs (Falcon, USA). The flow-through was centrifuged at 300g and the protoplast pellet was resuspended in W5 solution [154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES (pH 5.7)]. The protoplast concentration was measured under the microscope using a hemocytometer (Marienfeld) and adjusted to $7.0\times10^7$ protoplasts/ml. The protoplast suspension were centrifuged and resuspend in MMG solution [0.5 M mannitol, 15 mM $MgCl_2$, 4 mM MES (pH 5.7)]. Fifty microliters of protoplasts were mixed with 1 μg of plasmid DNA and 130 μl of PEG solution [0.2 M mannitol, 100 mM $CaCl_2$, 40% (wt/vol) PEG4000] in a 2 ml Eppendorf tube. The transfection mixture was incubated for 15 min in the dark, and then W5 solution was added and centrifuged at 300 g for 2 min. Lastly, the protoplast pellet was resuspended in incubation solution [0.5 M mannitol, 20 mM KCl, 4 mM MES (pH 5.7)] at room temperature overnight in the dark .

## 8. Subcellular localization of *OsNAC14*

The full length coding region of *OsNAC14* was amplified from the cDNA clone using the In-fusion system (Clonetech) and specific primers. The PCR product was ligated into the pHBT vector carrying GFP-myc. The final construct (*pro35S:OsNAC14-GFP*) and the control vectors (*pro35S:GFP* and *pro35S:mCherry*) were transfected into protoplasts (*Oryza sativa* cv. Ilmi). GFP and mCherry signals were observed under laser scanning confocal fluorescence microscope (Leica SP8 STED) after twelve hours protoplast transfection. GFP and chlorophyll were excited at 488 nm and then their fluorescence signals were detected between 512 and 580 nm and between 700 and 790 nm, respectively. mCherry was simultaneously excited at 400 nm and its fluorescence signal was detected between 430 and 450 nm.

## 9. Chromatin Immunoprecipitation (ChIP) assay

ChIP assay was performed according to Nelson et al (2006) with some modifications. Protoplasts from leaf sheath of rice (*Oryza sativa* cv. Ilmi), which were grown in the MS media during 10 d in the dark and transferred to the light for 1 d, were used for ChIP.

**Formaldehyde cross-linking**

The transfected protoplasts were cross-linked with 1% formaldehyde by vacuum infiltration for 15 min, 6 h after protoplast transfection. The cross-linking reaction

was stopped by the addition of 2 M glycine to a final concentration of 125 mM.

**Chromatin isolation**

The protoplasts pellet was resuspended in 10 ml of pre-chilled extraction buffer 1 [0.4 M Sucrose, 10mM Tris-HCl (pH 8.0), 5 mM β-mercaptoethanol, 0.1 mM PMSF, protease inhibitors]. After centrifugation for 20 min at 2880g at 4℃, the pellet was resuspended in extraction buffer 2 [0.25 M Sucrose, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, protease inhibitors]. After subsequent centrifugation for 10 min at 12000g at 4℃, the pellet was resuspended in extraction buffer 3 [1.7 M Sucrose, 10 mM Tris-HCl (pH 8.0), 0.15% Triton X-100, 2 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.1 mM PMSF, protease inhibitors].

**Chromatin shearing**

The chromatin pellet was resuspended in 300 µl Nuclei lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, PMSF, protease inhibitors]. To shear DNA to approximately 100-200 bp DNA fragments, the chromatin solution was sonicated in ice using Bioruptor (Diagenode) for 6 min with 30 s ON and 30 s OFF cycle.

**Immunoprecipitation**

The sonicated chromatin extract were centrifuged for 5 min at 12000g at 4℃ and the supernatant was diluted 10 times with ChIP dilution buffer [1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, PMSF, protease inhibitors]. To preclear the diluted chromatin solution, the solution was incubated

with protein A agarose beads (Temecula) for 1 h at 4℃ with gentle agitation. Then, after centrifugation for 1 min at 200g at 4℃, the supernatant was incubated with anti-myc polyclonal antibody (Sc 789x, Santa Cruz) at 4℃ overnignt. Further incubated with protein A agarose beads with gentle agitation. The beads were successively washed with low salt, high salt, LiCl and TE wash buffers.

**DNA isolation**

The pellet was resuspended in elution buffer [1% SDS, 0.1 M $NaHCO_3$] and vortex at 65℃ at 15 min. After cenentrifugation for 1 min at 200g at 4℃, the total DNA was elute reverse-crosslinking with 5 M $NaCl_2$ at 65℃ overnight, and add the RNase A solution 10mg/ml. The DNA was purified using DNA purification kit (Gene all).

**Data analysis.**

The purified DNA was analyzed by following Kuo *et al.* (1999) with some modification. The purified DNA was used in quantitative real-time PCR reactions using Mx3000P Real-Time PCR system (Agilent Technologies). The relative enrichment was normalized to the total input.

# Results

## 1. Structure and phylogenetic analysis of *OsNAC14*

OsNAC family is divided into two major groups. Group A and group B. These groups are further divided into 7 and 9 subgroups, respectively (Nuruzzaman *et al*., 2010). In group A, the subgroups are ONAC Ⅰ, ONAC Ⅱ, ONACⅢ, ONACⅣ, ONAC Ⅴ, ONACⅥ, and ONACⅦ. Likewise, SNAC (stress-associated NAC), NEO, NAM/CUC3, and OMNAC subgroups are assigned to group B. *OsNAC14* is belongs to the ONAC Ⅱ subgroup of Group A. Group A does not include any analyzed member of NAC and its members have less homology with the NACs in Arabidopsis. Especially, among the subgroups of the group A, the members of the second largest subgroup ONAC Ⅱ, show no pairs of segmentally duplicated genes and no introns were observed. For further analysis, the phylogenetic relationship of *OsNAC14* and other already known NAC proteins from rice was constructed based on the full-length sequences of proteins *in silico* (Fig. 1). This analysis shows that the SNAC family are separated with *OsNAC14* gene, and *OsNAC14* showed higher bootstrap values with the other ONAC Ⅱ subfamily. The high degree of homology is shared between *OsNAC14* and ONAC Ⅱ suggesting that they may have similar characteristic with ONAC Ⅱ subfamily members.

Previously, we performed expression profiling with the Rice 3'-Tiling microarray using total RNA from leaves of rice subjected to drought, high salinity,

abscisic acid (ABA), and low temperature (Oh *et al*., 2009). Results showed that *OsNAC14* responds to drought and high-salinity stress. Especially in drought stress, the fold change value was more than 10 times than NT (Fig. 2A). Moreover, RNA sequencing data analysis on drought-treatment samples (Chung *et al*., 2016) showed that *OsNAC14* have drought response and is up-regulated over a time course along with other already reported NAC transcription factors *OsNAC5*, *OsNAC6*, *OsNAC9* and *OsNAC10* in response to drought (Fig. 2B). Based on these data, we selected *OsNAC14* for further study. The genomic nucleotide sequence of *OsNAC14* (Os01g0675800) is a 1,319 bp and contain a single open reading frame (ORF) of 290 amino acids. *OsNAC14* has a motif of NAM superfamily domain in 5'-end region and also has the nuclear localization signal in *OsNAC14* protein sequence (Fig. 3).

## 2. Expression profiling and localization of *OsNAC14*

The expression pattern of *OsNAC14* under various abiotic stress treatments such as drought, high salinity, ABA, and low temperature was investigated using qRT-PCR at specific time course (2 h, 4 h, and 6 h). When treated with high-salinity, ABA, and low temperature, the expression level was slightly induced in both leaf and root tissue. On the other hand, under the drought treatments, the expression level was highest in both leaf and root tissue (Fig. 4A). The expression pattern of *OsNAC14* in various tissues and developmental stage in rice (*Oryza sativa* L. cv. Ill-mi) was also investigated (Fig. 4B). It was shown that the expression level is highest in flag

leaf and leaf at the meiosis stage.

The subcellular localization of *OsNAC14* was performed using rice protoplasts. The green fluorescent protein (GFP) was translationally fused to *OsNAC14* with 35S promoter, nos terminator for generating the constructs *pro35S:OsNAC14:GFP* (Fig. 5A). *OsNF-YA7:mCherry* was used for control to confirm the nucleus targeting protein and *P35S:GFP* vector used for *GFP* signal as a control (Lee *et al*., 2015). GFP fluorescence was observed in the nuclei of protoplasts through the merged image of both *OsNAC14:GFP* signal and *OsNF-YA7: mCherry* signal. It indicates that *OsNAC14* is a nuclear-localized protein (Fig. 5B)

## 3. Overexpression of *OsNAC14* in rice

For characterizing the function of *OsNAC14*, the overexpression vector was generated by fusing the cDNA of *OsNAC14* with the PGD1 promoter to enable whole-body overexpression (Fig. 6A). The expression vector was transformed using the Agrobacterium-mediated method (Hiei et al., 1994) and 30 transgenic plants were produced per construct. T4 generation seeds from these transgenic rice that grew normally with no stunting were collected, and 4 independent T5 homozygous lines were selected for this study. To determine the expression level of *OsNAC14* in the transgenic rice, qRT-PCR analysis were performed with of leaf and root tissues of 14-d-ole seedlings grown under normal conditions. Increased expression level of *OsNAC14* transcripts was detected in both leaves and roots

compared to those of NT plants (Fig. 6B).

## 4. The overexpression of *OsNAC14* in transgenic rice improved drought tolerance in the vegetative stage

To evaluate the tolerance of *proPGD1:OsNAC14* in drought conditions, 5-week-old transgenic and NT plants were grown in a greenhouse and subjected to drought stress by with-holding water. Over the time course of drought treatments, transgenic plants showed stress-induced damage, such as wilting and leaf-rolling with the concomitant loss of chlorophyll though lesser than the NT controls. The transgenic plants also recovered better than NT during re-watering 5 days. (Fig. 7A). The soil moisture contents were monitored during the drought treatment using a Soil Moisture Sensor SM150 (Delta-T Devices, UK). Even though no significant difference in soil moisture among the pot, the survival rates represent the difference between transgenic and NT. The transgenic plants ranged from 83% to 92%, whereas the NT and transgenic line 2 plants showed no signs of recovery (Fig. 7C). As a result, we did not select the line number 2 transgenic plant further experiments. To further verification of the stress tolerant phenotype, we measured the Fv/Fm values of transgenic lines with NT controls (Fig. 7D). Fv/Fm (Fv, the variable fluorescence; Fm, the maximum fluorescence) value is an indicator of the photochemical efficiency of photosystem Ⅱ (PSⅡ) in the dark- adapted state. Fv/Fm values were higher in *proPGD1:OsNAC14* plants than those of NT control plants during 11 d drought conditions, suggesting that *proPGD1:OsNAC14*

transgenic line 8 and 11 has dramatic tolerance under drought stress. Finally, our results indicate that overexpression of *OsNAC14* enhances tolerance of plants to drought stress in the vegetative stage.

## 5. Overexpression of *OsNAC14* improved grain yield under drought conditions

The yield components of *proPGD1:OsNAC14* plants and NT control plants were evaluated in year 2016 in a semi-paddy field under both normal and drought conditions. Three independent homozygous lines (T4) of *proPGD1:OsNAC14* plants and NT plants were planted in a paddy field and grown to maturity. The yield parameters were scored for 30 plants in normal condition and 18 plants in drought condition per transgenic line with three replicates. The filling rate and total grain weights of transgenic *proPGD1:OsNAC14* plants in normal condition, were similar or slightly increased than those of NT plants (Fig. 8, Table 2). To evaluate the growth response of *OsNAC14* transgenic rice under drought conditions, three independent T4 lines were tested at the panicle heading stage. In the drought-treated *proPGD1:OsNAC14* transgenic rice, the filling rate increased by 36.43% ($P < 0.05$) than the NT plants. Filling rate is one of the parameters used to evaluate the grain yield, thus our results suggest that overexpression of *OsNAC14* increases the grain yield of rice under the drought-stressed condition.

## 6. Transcriptome profiling of *OsNAC14* overexpressing

## transgenic plants using RNA sequencing

To identify the stress-inducible genes regulated by the *OsNAC14*, we performed the expression profiling using RNA-sequencing (Macrogen). Total RNA from the 14-d-old seedling of transgenic (T4 generation, line 11) and NT rice were extracted and submitted for analysis. A total of 273 genes showed significant expression relative to the NT (more than 2.0-fold change value and $p$-value < 0.05) and among them, 122 genes were up-regulated and 151 genes were down-regulated. Using 122 up-regulated genes, we compared these with expression pattern with previous drought treatment RNA-sequencing data (Chung *et al*., 2016). In addition, through GO (Gene Ontology) analysis, 16 genes were selected and considered to be candidates target genes of *OsNAC14* (Table 2).

We validated the RNA sequencing-based expression patterns of six genes which were selected on the basis of their observed expression pattern and molecular function via qRT-PCR. These genes encode for proteins involved in DNA repair (GO:0006281), programmed cell death (GO:00125010), and nucleotide binding (GO:0051170). qRT-PCR based expression analysis showed that six genes were evidently up-regulated in *proPGD:OsNAC14* plants than in NT and demonstrate an expression pattern similar to those observed in the RNA sequencing-based analysis (Fig 9, C, the white bar). To gain more insights into the drought responsive pattern of genes, we prepared a 3-day drought-treated *proPGD1:OsNAC14* transgenic and NT plants. Prior to the experiment, we measured the expression of *Dip1* (Dehydration Stress-inducible Pritein1,

*Os02g0669100*) and *RbcS* (Small subunit of Rubisco, *Os12g0274700*) genes as reference genes, whose expressions has been reported to be drought-inducible and drought-repressed respectively (Chung et al., 2016). *Dip1* expression increased in controls in 3rd day of drought but lower in *proPGD1:OsNAC14* than in the NT plants. On the other hand, *RbcS* expression decreased until the 3rd day of drought in controls, and showed slower decrease in the *proPGD1:OsNAC14* than in the NT plants. These findings suggest that *OsNAC14* overexpression caused the plants to be less sensitive to drought stress.

In the case of the target genes, we measured its expression patterns in the *proPGD1:OsNAC14* and NT plants under the drought conditions. Two of the putative target gene candidates, *Os04g0398000* and *Os11g0615700* showed that the *proPGD1:OsNAC14* transgenic plants have a lower or similar expression than NT plants, while another four of target gene candidates, *Os09g0357400*, *Os07g0158900*, *Os06g0286700* and *Os11g0615800* show that the *proPGD1:OsNAC14* transgenic plants have higher expression than NT plants. Interestingly, one of the candidate target gene, *Os11g06975800*, exhibited an increasing pattern as drought exposure progressed.

In order to select the direct target gene of *OsNAC14* more accurately and directly, we performed the qRT-PCR using rice protoplasts. The objective using the protoplast is for the direct target confirmation. The transient and prompt DNA transfection in the protoplast cell is considered to directly regulate expression of target gene rather than in already developed plants. To confirm the constitutive

expression protoplasts, NT and *proPGD1:OsNAC14* transgenic rice were prepared. Moreover, 35S:GFP and 35S:OsNAC14 vector was transfected in NT plants protoplast for transient expression. As a result, four of target gene candidates, *Os09g0357400*, *Os07g0158900*, *Os06g0286700* and *Os11g0615800* have higher expression levels in constitutive expression condition than transient expression condition. On the other hand, two of candidate target genes, *Os06g0286700* (*Piz-t*), and *Os11g0615800 (OsRAD51A1)* have higher expression levels in transient expression condition than the constitutive expression condition (Fig. 9B). Thus, *Piz-t* and *OsRAD51A1* expected to direct target gene of *OsNAC14*.

## 7. Direct target validation of *OsNAC14* using ChIP

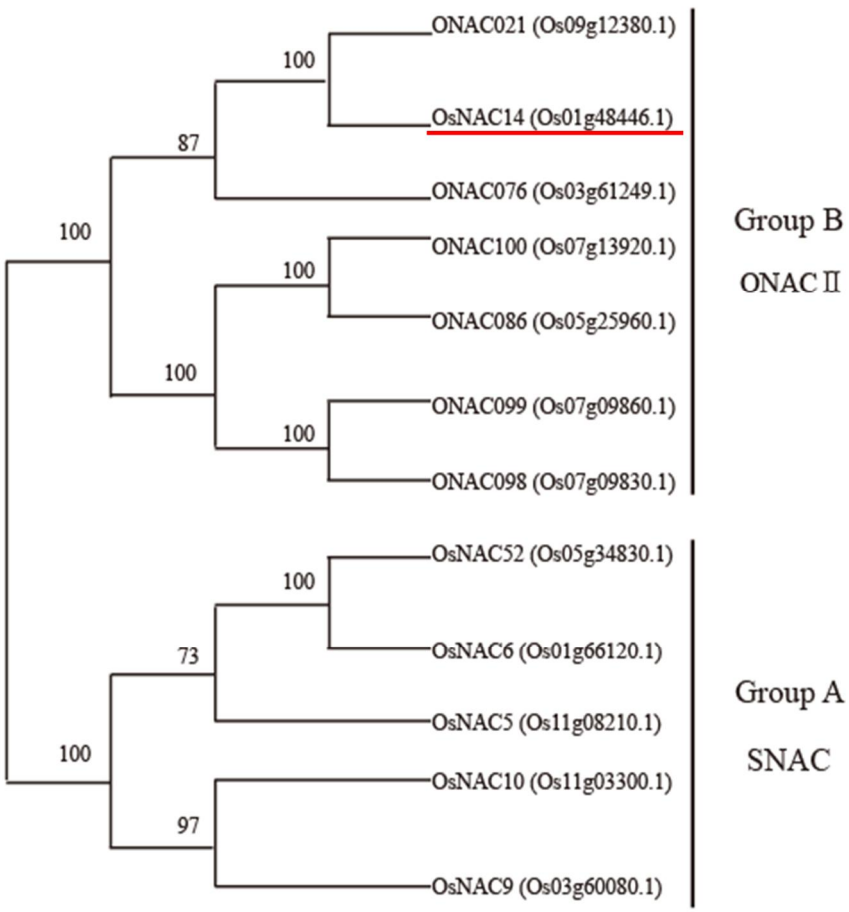
Chromatin Immunoprecipitation (ChIP) can resolve the TF-DNA interactions. In this experiment, ChIP was used to verify the direct target genes of *OsNAC14* in rice protoplasts. Both *35S:OsNAC14:myc* tagging vector and *35S:GFP:myc* vector for control were prepared. After cross-linking of formaldehyde, the chromatin is sheared through sonication into small fragments containing the cross linked protein and DNA. The total length of protein and DNA is around 100 to 200 bp, as seen in the 2 % agarose gel electrophoresis (Fig. 10B). Co-expression of some genes with OsNAC14 can be attributed to a variety of possible mechanisms involving the direct or indirect role of OsNAC14 in modulating the expression of such genes. In this study, we are more interested in delineating the direct targets of OsNAC14, i.e. those genes whose expression is regulated by OsNAC14. Therefore, we used ChIP-

based assays to figure out the recruitment of OsNAC14 to the promoter regions of two genes (Os11g0615800, and Os06g0286700) chosen on the basis of their physiological role and the results of our studies. Os11g0615800 (OsRad51A1) belongs to a class of eukaryotic recombination that play a role during recombination-associated with meiosis, and more importantly, during DNA repair (Tripathi et al., 2016). Piz-t (Os06g0286700) is involved in programmed cell death, which generally functions in ROS response.

Results of ChIP-qRT PCR assay showed that OsNAC14 are recruited to the promoter regions of OsRad51A1 (Fig. 10C), while no significant binding of OsNAC14 on Piz-t promoter region. Specifically, the P1 and P2 region of OsRad51A1 evidently showed highly enriched with OsNAC14. Furthermore, under drought stress conditions, the recruitment of OsNAC14 was more on OsRad51A1 as drought exposure time (Fig. 9A, the last graph), suggesting a potential link between elevated expression under drought stress and OsNAC14 function. These results show that OsNAC14 is recruited to the promoter regions of OsRad51A1, and the stimulation of their expression upon OsNAC14 overexpression may possibly be linked to the recruitment of OsNAC14 and drought response.

## Fig. 1 Phylogenetic analysis of *OsNAC14*

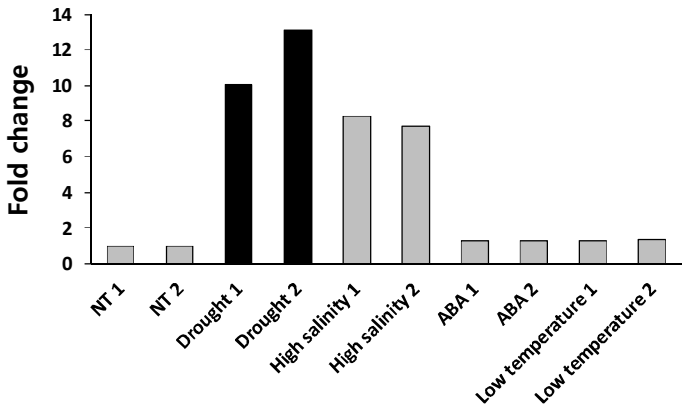
Multiple sequence alignment with12 full-length OsNAC proteins was performed using ClustalW (www.genome.jp/tools/clustalw), and the phylogenetic tree was constructed by the neighbor-joining method with 100 bootstrap repetitions.


ONAC021 (Os09g12380.1)
OsNAC14 (Os01g48446.1)
ONAC076 (Os03g61249.1)
ONAC100 (Os07g13920.1)
ONAC086 (Os05g25960.1)
ONAC099 (Os07g09860.1)
ONAC098 (Os07g09830.1)
OsNAC52 (Os05g34830.1)
OsNAC6 (Os01g66120.1)
OsNAC5 (Os11g08210.1)
OsNAC10 (Os11g03300.1)
OsNAC9 (Os03g60080.1)
100
87
100
100
100
100
73
100
97
Group B
ONAC Ⅱ
Group A
SNAC

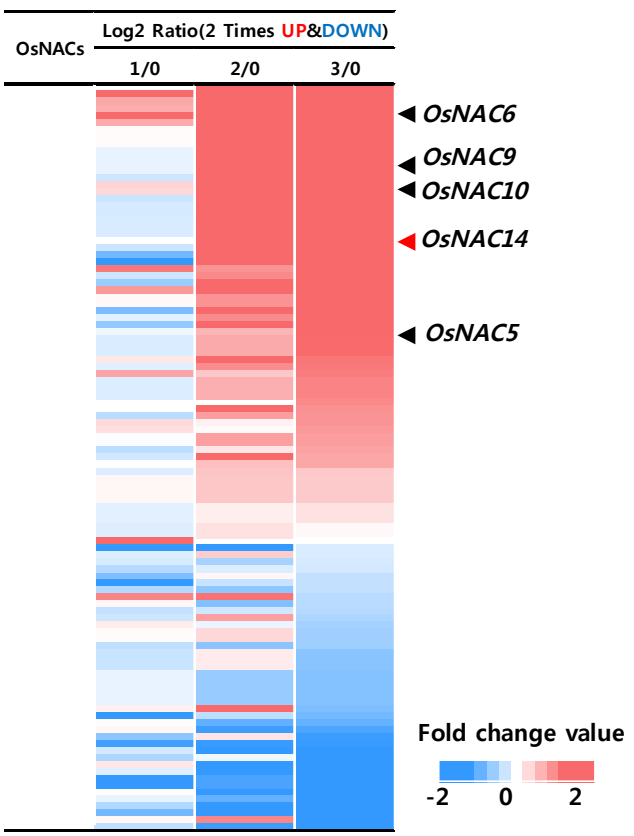
## Fig 2. Expression pattern of *OsNAC14* under the abiotic stress conditions through microarray and RNA-sequencing

(A) The relative expression pattern of *OsNAC14* was detected by microarray data (Oh *et al*., 2009). (B) Heatmap represents drought response of OsNAC family genes in rice. RNA sequencing data represent the drought-treated rice leaves (Chung *et al*., 2016). Among 151 genes, 66 genes are up-regulated and 41 genes are down-regulated in drought conditions. Red triangle represents the *OsNAC14* transcription factor and the black triangle represents *OsNAC6*, *OsNAC9*, *OsNAC10* and *OsNAC5*, respectively. The bar at the bottom of the heat map represents fold change values. 0, control., 1, drought for 1 d., 2, drought for 2 d., 3, drought for 3 d.

**(A)**

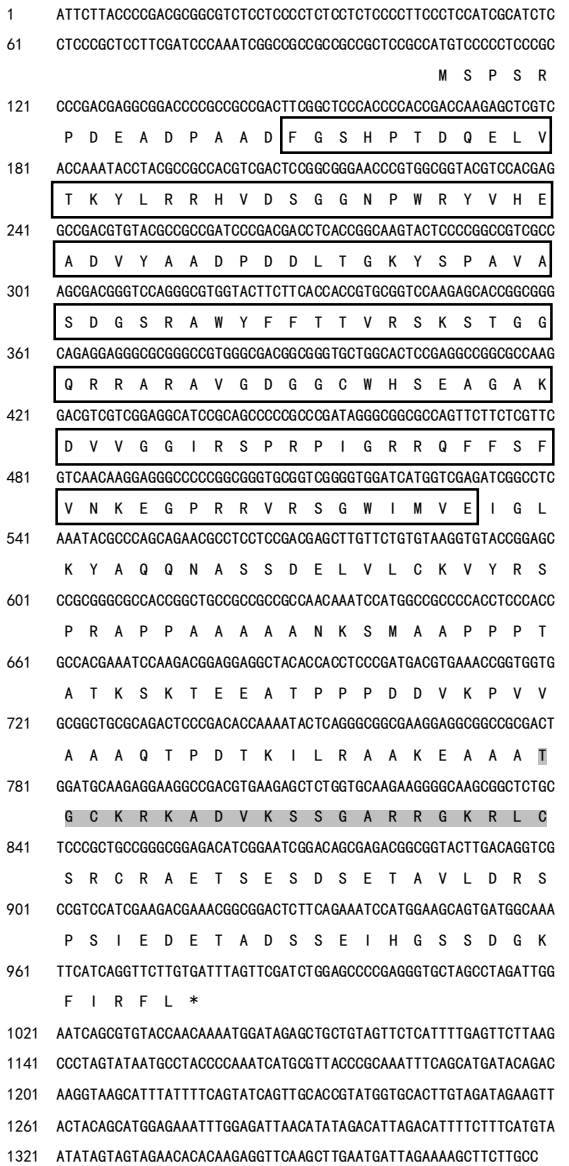

Fold change
14
12
10
8
6
4
2
0
NT 1
NT 2
Drought 1
Drought 2
High salinity 1
High salinity 2
ABA 1
ABA 2
Low temperature 1
Low temperature 2

**(B)**


OsNACs
Log2 Ratio(2 Times UP&DOWN)
1/0
2/0
3/0
OsNAC6
OsNAC9
OsNAC10
OsNAC14
OsNAC5
Fold change value
-2
0
2

## Fig.3 Full-length CDS and amino acid sequences of *OsNAC14*

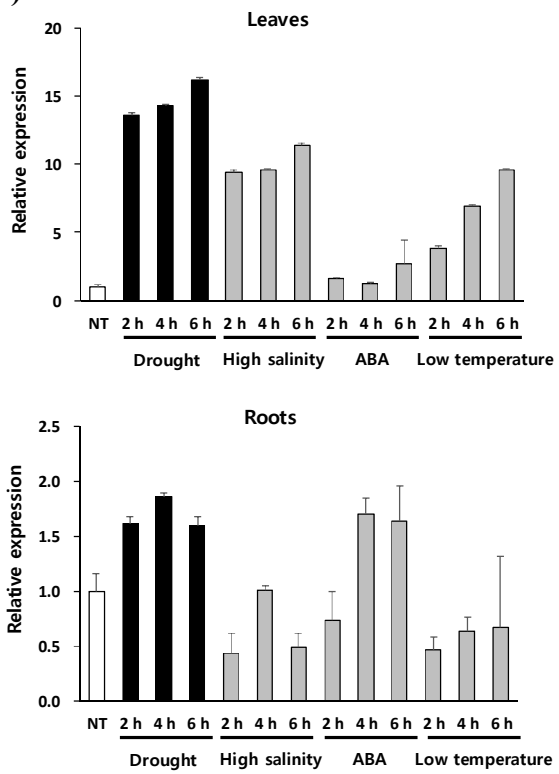
The length of the total genomic sequence is 1,318 bp, with 873 bp CDS and 290 aa coding protein. The NAM motif, also known as conserved motif sequence, is *boxed*. Nuclear localization signal (NLS) is *shaded*.


1 ATTCTTACCCCGACGCGGCGTCTCCTCCCCTCTCCTCTCCCCTTCCCTCCATCGCATCTC
61 CTCCCGCTCCTTCGATCCCAAATCGGCCGCCGCCGCCGCTCCGCCATGTCCCCCTCCCGC
M S P S R
121 CCCGACGAGGCGGACCCCGCCGCCGACTTCGGCTCCCACCCCACCGACCAAGAGCTCGTC
P D E A D P A A D F G S H P T D Q E L V
181 ACCAAATACCTACGCCGCCACGTCGACTCCGGCGGGAACCCGTGGCGGTACGTCCACGAG
T K Y L R R H V D S G G N P W R Y V H E
241 GCCGACGTGTACGCCGCCGATCCCGACGACCTCACCGGCAAGTACTCCCCGGCCGTCGCC
A D V Y A A D P D D L T G K Y S P A V A
301 AGCGACGGGTCCAGGGCGTGGTACTTCTTCACCACCGTGCGGTCCAAGAGCACCGGCGGG
S D G S R A W Y F F T T V R S K S T G G
361 CAGAGGAGGGCGCGGGCCGTGGGCGACGGCGGGTGCTGGCACTCCGAGGCCGGCGCCAAG
Q R R A R A V G D G G C W H S E A G A K
421 GACGTCGTCGGAGGCATCCGCAGCCCCCGCCCGATAGGGCGGCGCCAGTTCTTCTCGTTC
D V V G G I R S P R P I G R R Q F F S F
481 GTCAACAAGGAGGGCCCCCGGCGGGTGCGGTCGGGGTGGATCATGGTCGAGATCGGCCTC
V N K E G P R R V R S G W I M V E I G L
541 AAATACGCCCAGCAGAACGCCTCCTCCGACGAGCTTGTTCTGTGTAAGGTGTACCGGAGC
K Y A Q Q N A S S D E L V L C K V Y R S
601 CCGCGGGCGCCACCGGCTGCCGCCGCCGCCAACAAATCCATGGCCGCCCCACCTCCCACC
P R A P P A A A A A N K S M A A P P P T
661 GCCACGAAATCCAAGACGGAGGAGGCTACACCACCTCCCGATGACGTGAAACCGGTGGTG
A T K S K T E E A T P P P D D V K P V V
721 GCGGCTGCGCAGACTCCCGACACCAAAATACTCAGGGCGGCGAAGGAGGCGGCCGCGACT
A A A Q T P D T K I L R A A K E A A A T
781 GGATGCAAGAGGAAGGCCGACGTGAAGAGCTCTGGTGCAAGAAGGGGCAAGCGGCTCTGC
G C K R K A D V K S S G A R R G K R L C
841 TCCCGCTGCCGGGCGGAGACATCGGAATCGGACAGCGAGACGGCGGTACTTGACAGGTCG
S R C R A E T S E S D S E T A V L D R S
901 CCGTCCATCGAAGACGAAACGGCGGACTCTTCAGAAATCCATGGAAGCAGTGATGGCAAA
P S I E D E T A D S S E I H G S S D G K
961 TTCATCAGGTTCTTGTGATTTAGTTCGATCTGGAGCCCCGAGGGTGCTAGCCTAGATTGG
F I R F L *
1021 AATCAGCGTGTACCAACAAAATGGATAGAGCTGCTGTAGTTCTCATTTTGAGTTCTTAAG
1141 CCCTAGTATAATGCCTACCCCAAATCATGCGTTACCCGCAAATTTCAGCATGATACAGAC
1201 AAGGTAAGCATTTATTTTCAGTATCAGTTGCACCGTATGGTGCACTTGTAGATAGAAGTT
1261 ACTACAGCATGGAGAAATTTGGAGATTAACATATAGACATTAGACATTTTCTTTCATGTA
1321 ATATAGTAGTAGAACACACAAGAGGTTCAAGCTTGAATGATTAGAAAAGCTTCTTGCC

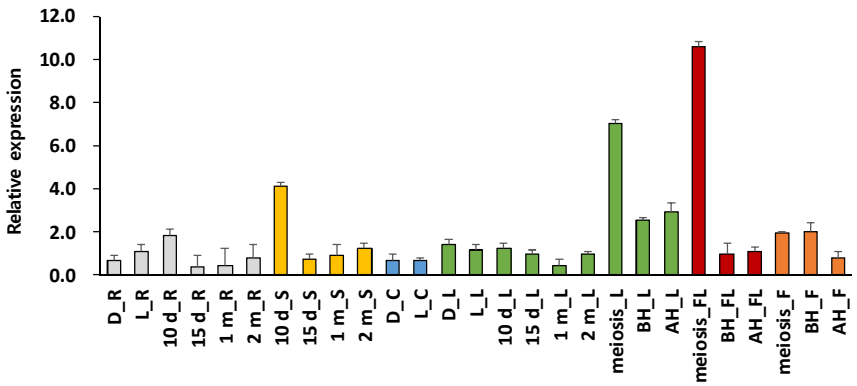
## Fig 4. The expression pattern of *OsNAC14* detected by qRT-PCR in diverse condition

(A) The relative expression patterns of *OsNAC14* in response to four different abiotic stresses. Drought, high salinity, ABA and low-temperature treatment of WT leaves and roots (*Oryza sativa*. L. Japonica cv. Nakdong) grown for 2 weeks. The 0, 2, 4, and 6 (x-axis) indicates the treatment time (hour) under corresponding abiotic stresses. (B) The organ-specific expression of *OsNAC14* was analyzed with diverse time at the developmental stage in the NT (*Oryza sativa*. L. Japonica cv. Ilmi). d, day; D, Dark; BH, Before heading; AH, After heading; C, Coleoptiles; R, Root; L, Leaves; FL, Flag leaves; F, Flower. The rice *Ubi* (AK121590) was used as an internal control to normalize the data. The error bar indicates the mean value and the ± standard deviation of three independent experiments

**(A)**


Leaves
Relative expression
20
15
10
5
0
NT
2 h
4 h
6 h
Drought
High salinity
ABA
Low temperature
Roots
Relative expression
2.5
2.0
1.5
1.0
0.5
0.0

**(B)**


Relative expression
12.0
10.0
8.0
6.0
4.0
2.0
0.0
D_R
L_R
10 d_R
15 d_R
1 m_R
2 m_R
10 d_S
15 d_S
1 m_S
2 m_S
D_C
L_C
D_L
L_L
10 d_L
15 d_L
1 m_L
2 m_L
meiosis_L
BH_L
AH_L
meiosis_FL
BH_FL
AH_FL
meiosis_F
BH_F
AH_F

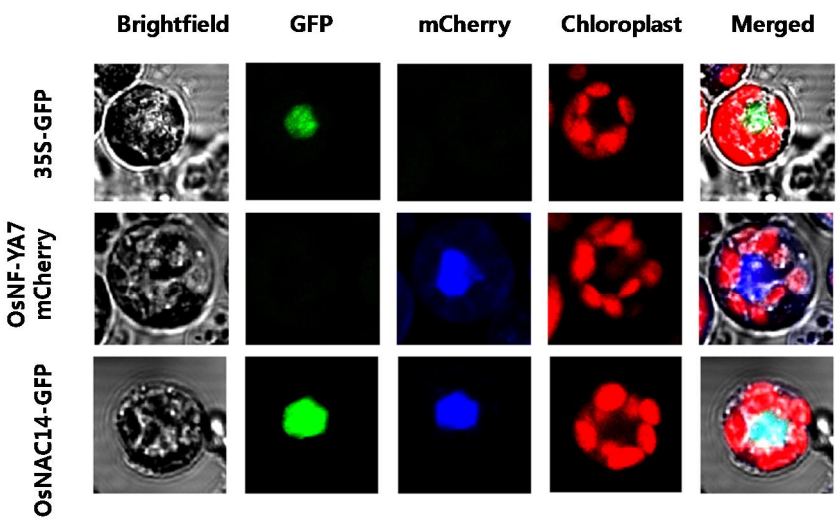
## Fig. 5 Subcellular localization of *OsNAC14*

(A) The schematic represents the expression vector for *sGFP* fusion. RB, right border., *35S*, CaMV 35S promoter., *nos*, terminator., (B) Individual and merged images of GFP, chlorophyll auto-fluorescence, mCherry, and visible protoplasts. The ONAC-GFP signals are the merged image of the green fluorescent signal from the sGFP protein and blue fluorescent signal from the mCherry protein (nucleus proteins), and the red fluorescent signal from chlorophyll in the chloroplast.

(A)


BamH I
Not I
Pst I

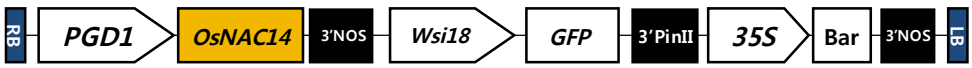
(B)


Brightfield
GFP
mCherry
Chloroplast
Merged
35S-GFP
OsNF-YA7 mCherry
OsNAC14-GFP

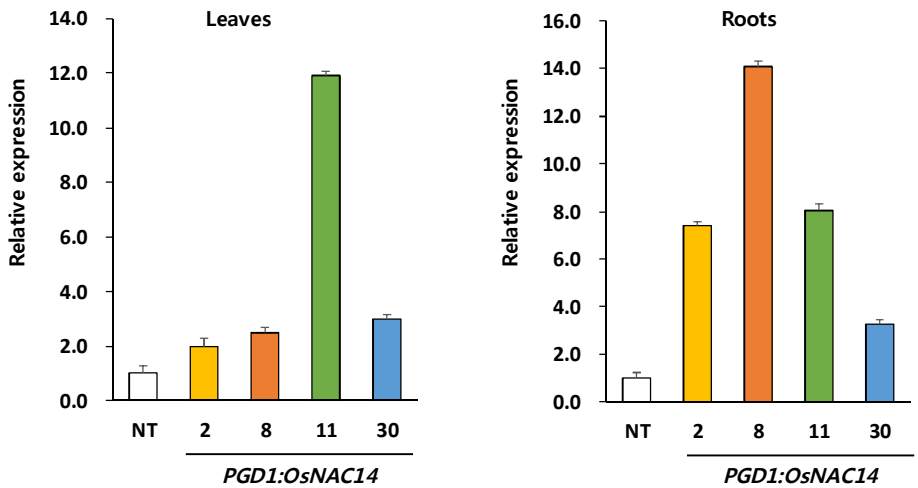
## Fig. 6 Vector construct of overexpressed transgenic plant of *OsNAC14* and analysis of expression levels in transgenic plants

(A) Vector constructs have a *PGD1* constitutive promoter for overexpression of *OsNAC14* in rice. A stress-inducible promoter Wsi18 drives *GFP* gene in stress condition, which is used as a selection marker. (B) Relative expression of *OsNAC14* in transgenic and non-transgenic (NT) plants. Total RNA from the roots and leaves of four homozygous T4 lines, 2, 8, 11, and 30 of *proPGD1:OsNAC14* plants and NT plants. Four independent T4 lines of *proPGD1:OsNAC14* and NT controls were grown for 2 weeks.

**(A)**

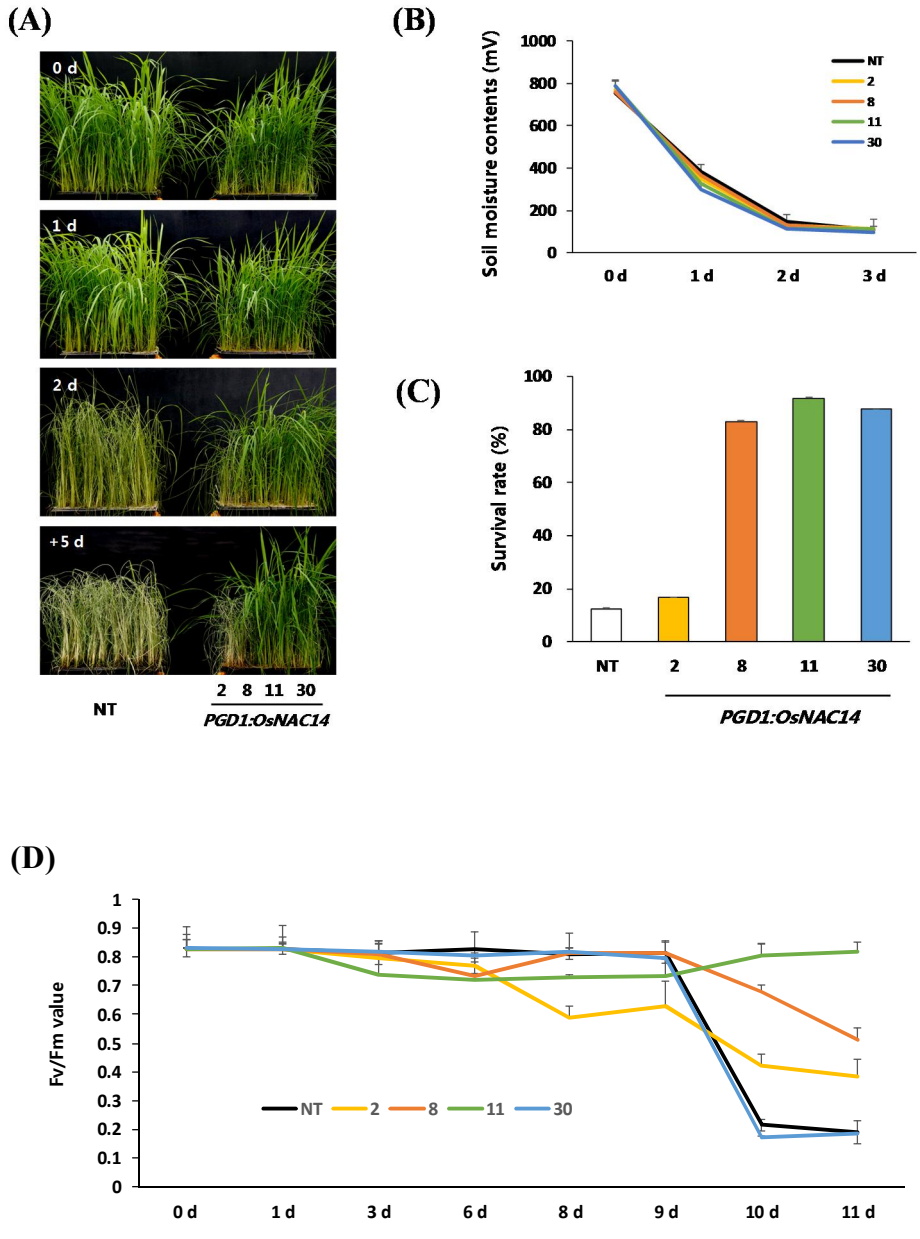

RB
PGD1
OsNAC14
3'NOS
Wsi18
GFP
3'PinII
35S
Bar
3'NOS
LB

**(B)**


Leaves
Relative expression
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
NT
2
8
11
30
PGD1:OsNAC14
Roots
Relative expression
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
NT
2
8
11
30
PGD1:OsNAC14

## Fig 7. Drought stress tolerance experiment of *proPGD1:OsNAC14* transgenic plants in the vegetative stage

(A) The phenotype of transgenic rice during drought stress. Four independent homozygous T4 lines of *proPGD1:OsNAC14* plants and non-transgenic (NT) controls were grown in soil for 5 weeks, subjected to 3 d of drought stress followed by 5 d of re-watering in the greenhouse. Each individual line of *proPGD1:OsNAC14* were planted 30 plants. (B) Measurement of soil moisture contents (mV). Each measurement was performed at different points of soil (n=30). (C) The survival rate of transgenic plants and NT after recovering through re-watering for 5 days (n=30). (D) Changes in the chlorophyll fluorescence (Fv/Fm) of plants under drought stress condition during the 12 days. The values were measured in the dark using a Pulse Amplitude Modulation (PAM) fluorometer. Each data point represents the mean value (n=20).

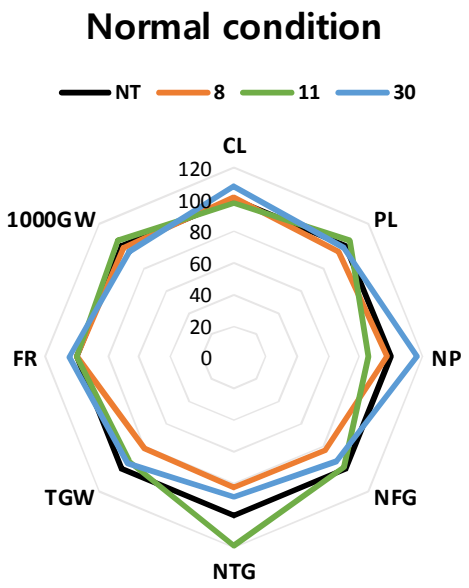

(A)
0 d
1 d
2 d
+5 d
NT
2 8 11 30
PGD1:OsNAC14
(B)
Soil moisture contents (mV)
1000
800
600
400
200
0
NT
2
8
11
30
0 d
1 d
2 d
3 d
(C)
Survival rate (%)
100
80
60
40
20
0
NT
2
8
11
30
PGD1:OsNAC14
(D)
Fv/Fm value
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NT
2
8
11
30
0 d
1 d
3 d
6 d
8 d
9 d
10 d
11 d

## Fig 8. Agronomic traits compare with *proPGD1:OsNAC14* and NT control

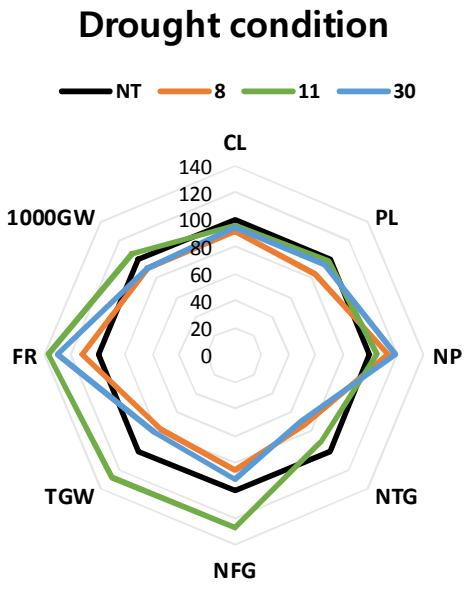
Spider plots of the agronomic traits with three independent homozygous T4 lines, which is *proPGD1:OsNAC14* plants and corresponding NT control under both normal and drought conditions. Each data represents the percentage of the mean value (n=30) with the NT plants in normal condition and mean values (n=18) with the NT plants in drought condition respectively, listed in Table 1. The mean measurements from the NT controls were assigned a 100% reference value. Normal condition (A) and drought condition (B) were harvested in year 2016.

CL, column length; PL, panicle length; NP, number of panicles per hill; NFG, number of filled grains; NTG, number of total grains; TGW, total grain weight; FR, filling rate; 1000GW, 1000 grain weight

**(A)**


Normal condition
NT
8
11
30
CL
PL
NP
NFG
NTG
TGW
FR
1000GW
120
100
80
60
40
20
0

**(B)**

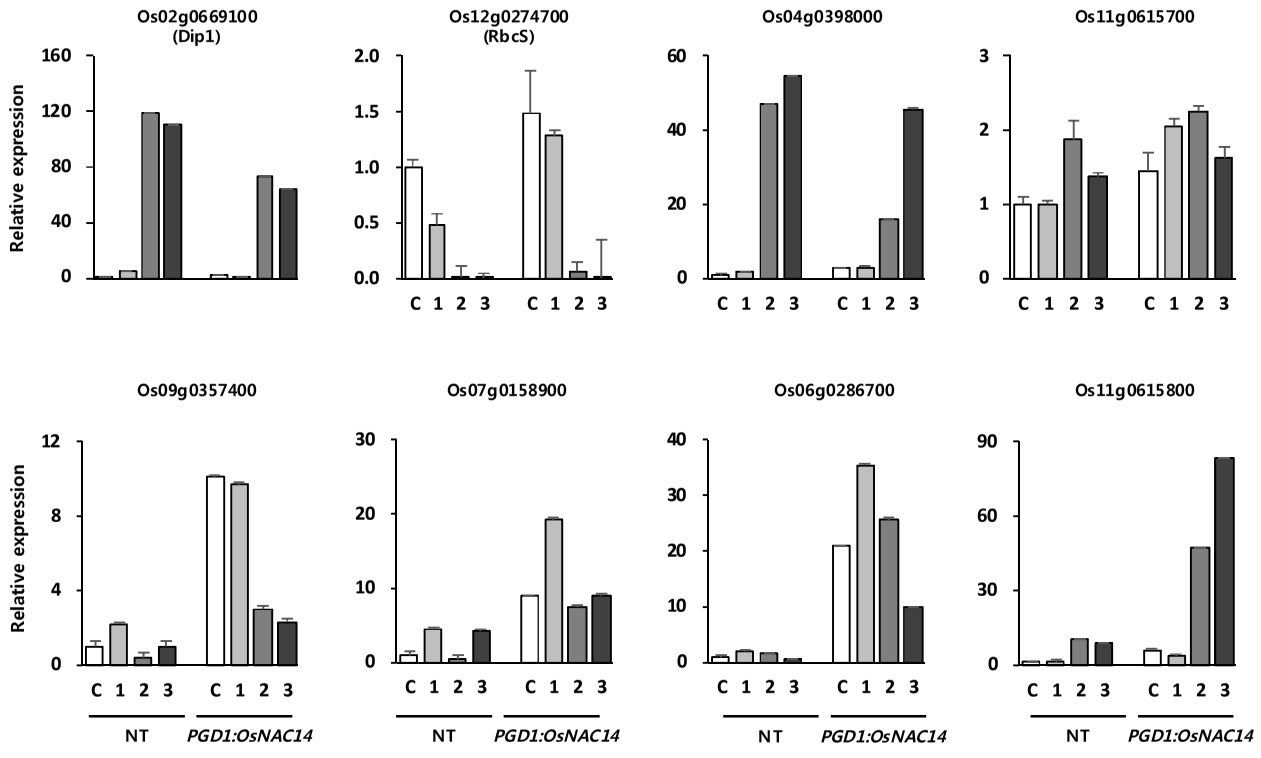

Drought condition
NT
8
11
30
CL
PL
NP
NTG
NFG
TGW
FR
1000GW
140
120
100
80
60
40
20
0

## Fig 9. Validation of *OsNAC14* target genes using qRT-PCR from RNA sequencing

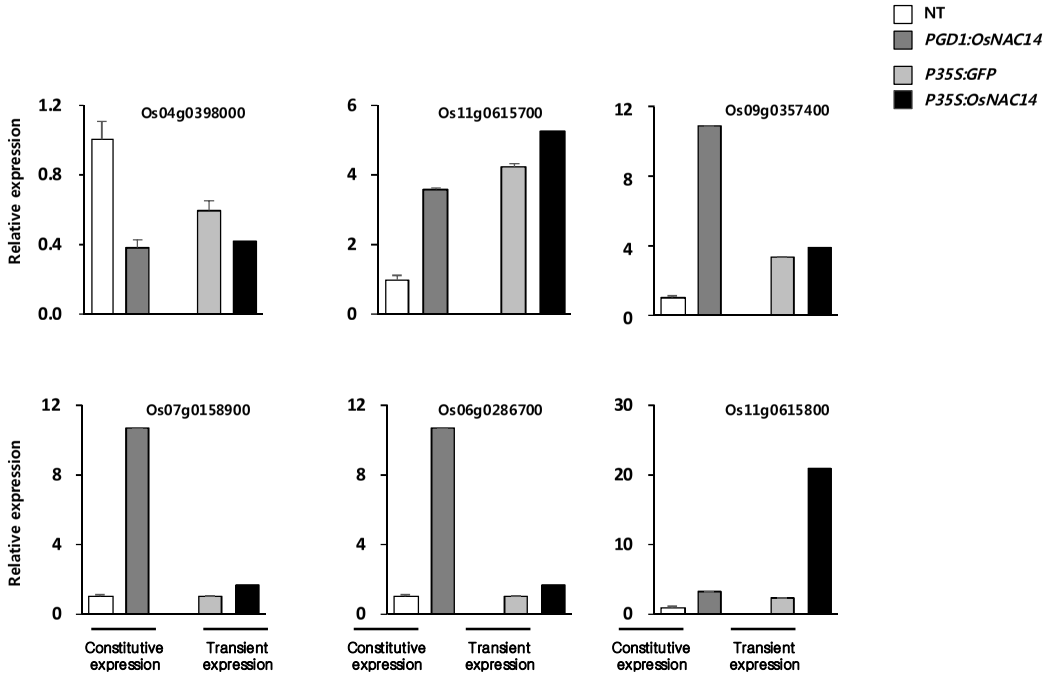
(A) The relative expression pattern of the target gene of *OsNAC14* in 1-month-old transgenic and NT leaves under drought condition. Total RNA was extracted from the leaf tissue that was subjected to drought stress for 0 to 3 days. Six target genes were validated by qRT-PCR. The transcript levels of *Dip1* (Dehydration stress-inducible protein1, *Os02g0669100*) and *RbcS1* (Small subunit of Rubisco, *Os12g0274700*) were used as stress marker genes. (B) The expression pattern of the candidate target gene of *OsNAC14* in rice protoplasts was validated by qRT-PCR. Front two bars indicate the constitutive expression level of protoplast without external DNA transfection. The other two bars indicate the transient expression level of protoplast with *pro35S:GFP* transfection into NT plants as control and *P35S:OsNAC14* transfection.

*Os04g0398000* (Pathogenesis-related transcriptional factor and ERF domain containing protein), *Os11g0615700* (20S Proteasome subunit alpha type 5), *Os09g0357400* (Disease resistance protein domain), *Os07g0158900* (Cyclin-like F-box domain containing protein), *Os06g0286700* (Similar to Piz-t), and *Os11g0615800* (DNA repair and recombination, RecA-like domain protein).

**(A)**

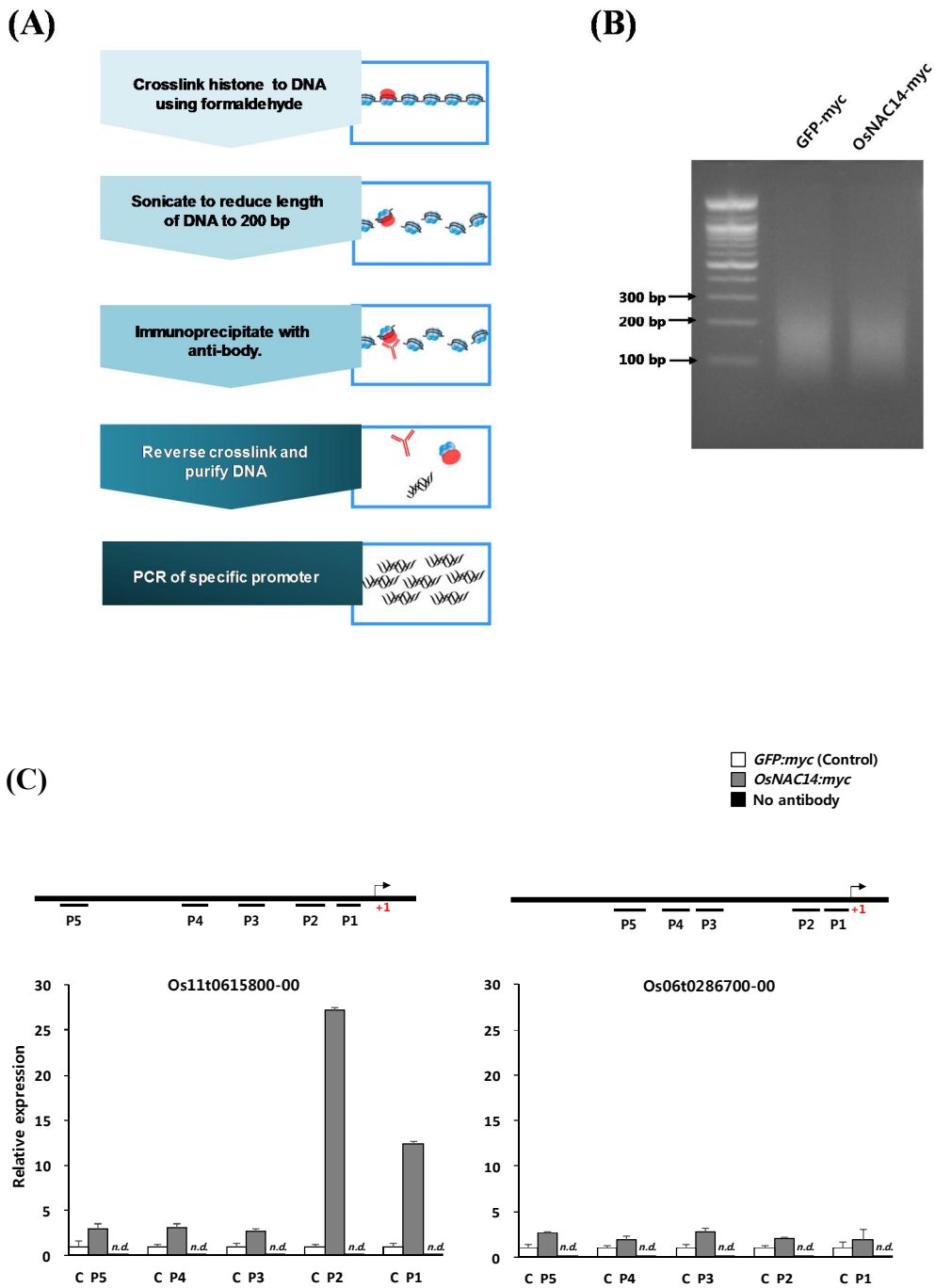

Os02g0669100
(Dip1)
Os12g0274700
(RbcS)
Os04g0398000
Os11g0615700
Os09g0357400
Os07g0158900
Os06g0286700
Os11g0615800
Relative expression
C 1 2 3 C 1 2 3
NT
PGD1:OsNAC14

**(B)**


NT
PGD1:OsNAC14
P35S:GFP
P35S:OsNAC14
Relative expression
Os04g0398000
Os11g0615700
Os09g0357400
Os07g0158900
Os06g0286700
Os11g0615800
Constitutive expression
Transient expression

## Fig. 10 Promoter analysis using Chromatin Immunoprecipitation (ChIP)

(A) The illustration of a method for ChIP analysis. (B) Agarose gel electrophoresis were conducted to confirm the shearing state of DNA after sonication using 2 % agarose gel. The size was around 100 to 200 bp of sheared genomic DNA. (C) ChIP qRT-PCR of target gene candidate (*Os11g0615800*) and another target gene candidate (Os06g0286700). Five different primer sets were designed in the promoter region, which is located in 2 kb up-stream region of the start codon (+1). The size of all products is less than 200 bp. The relative expression level of the final product from ChIP was determined by qRT-PCR (using the primers listed in Table 3).


(A)
Crosslink histone to DNA using formaldehyde
Sonicate to reduce length of DNA to 200 bp
Immunoprecipitate with anti-body.
Reverse crosslink and purify DNA
PCR of specific promoter
(B)
GFP-myc
OsNAC14-myc
300 bp
200 bp
100 bp
(C)
GFP:myc (Control)
OsNAC14:myc
No antibody
P5
P4
P3
P2
P1
+1
Os11t0615800-00
Os06t0286700-00
Relative expression
30
25
20
15
10
5
0
n.d.
C P5
C P4
C P3
C P2
C P1

## Table 1. Agronomic traits of *proPGD1:OsNAC14* plants grown in the field under both normal and drought conditions

Each parameter value represents the mean for *proPGD1:OsNAC14* plants and respective NT controls, drought condition (n=18) and drought condition (n=30). Percentage differences (%Δ) between the values for the *proPGD1:OsNAC14* plants and NT controls are listed. An asterisk (*) indicates a significant difference ($P<0.05$) and asterisk (**) indicates a significant difference ($P<0.01$).

| Drought | | Culm length (cm) | Panicle length (cm) | No. of Panicle (/ hill) | Filling rate (%) | No. of total grain (/ hill) |
|---|---|---|---|---|---|---|
| NT | | 79.00 | 17.63 | 19.13 | 33.62 | 1751.44 |
| ***PGD1-OsNAC14-8*** | | 66.17 | 15.00 | 21.63 | 37.74 | 1272.56 |
| | ***%Δ*** | -16.24 | -14.89 | 13.07 | 12.24 | -27.34 |
| | ***p*** | 0.01* | 0.00** | 0.14 | 0.47 | 0.00** |
| ***PGD1-OsNAC14-11*** | | 69.56 | 17.19 | 20.25 | 45.87 | 1601.69 |
| | ***%Δ*** | -11.95 | -2.48 | 5.88 | **36.43** | -8.55 |
| | ***p*** | 0.20 | 0.49 | 0.51 | 0.03* | 0.18 |
| ***PGD1-OsNAC14-30*** | | 68.11 | 16.50 | 22.69 | 43.81 | 1215.50 |
| | ***%Δ*** | -13.78 | -6.38 | **18.62** | 30.31 | -30.60 |
| | ***p*** | 0.09 | 0.08 | 0.04* | 0.08 | 0.00** |

| Normal | | Culm length (cm) | Panicle length (cm) | No. of Panicle (/ hill) | Filling rate (%) | No. of total grain (/ hill) |
|---|---|---|---|---|---|---|
| NT | | 71.41 | 20.47 | 17.10 | 87.62 | 1688.19 |
| ***PGD1-OsNAC14-8*** | | 72.19 | 19.37 | 16.54 | 87.12 | 1385.44 |
| | ***%Δ*** | 1.09 | -5.36 | -3.28 | -0.58 | -17.93 |
| | ***p*** | 0.54 | 0.00** | 0.65 | 0.79 | 0.15 |
| ***PGD1-OsNAC14-11*** | | 70.66 | 20.33 | 14.17 | 86.96 | 1650.44 |
| | ***%Δ*** | -1.05 | -0.65 | -17.14 | -0.75 | -2.24 |
| | ***p*** | 0.39 | 0.74 | 0.01** | 0.73 | 0.93 |
| ***PGD1-OsNAC14-30*** | | 76.89 | 20.00 | 18.13 | 90.55 | 1468.33 |
| | ***%Δ*** | 7.67 | -2.28 | 6.02 | 3.34 | -13.02 |
| | ***p*** | 0.00** | 0.25 | 0.38 | 0.13 | 0.31 |

**Table. 2 List of target gene of *OsNAC14***

| No. | Transcript_ID | Description | OsNAC14/ NT fold change | GO (gene ontology) | Log2 Ratio(2 Times UP&DOWN) | | |
|---|---|---|---|---|---|---|---|
| | | | | | 1/0 | 2/0 | 3/0 |
| 1 | Os06t0286700-00 | Similar to Piz-t. | 4.1 | Cell death | 0.2 | -1.7 | -4.7 |
| 2 | Os09t0357400-00 | Disease resistance protein domain containing protein. | 2.5 | Cell death | 0.3 | -1.9 | -4.3 |
| 3 | Os11t0590700-01 | NB-ARC domain containing protein. | 3.6 | Cell death | 0.3 | -3.3 | -5.0 |
| 4 | Os06t0287000-00 | Similar to NBS-LRR type R protein, Nbs4-Pi. | 2.4 | Cell death | 0.1 | -1.5 | -4.1 |
| 5 | Os11t0491600-00 | Hypothetical conserved gene. | 2.8 | Cell death | 0.0 | -1.8 | 1.6 |
| 6 | Os12t0281600-02 | Hypothetical conserved gene. | 2.1 | Cell death | 0.2 | -1.2 | -1.1 |
| 7 | Os12t0458100-01 | Transferase family protein. | 2.3 | Transferase | 0.1 | -1.3 | -3.9 |
| 8 | Os11t0691100-00 | Similar to Protein kinase. | 33.6 | Nucleotide-binding | | 1.8 | 1.0 |
| 9 | Os01t0705200-01 | Late embryogenesis abundant protein repeat containing protein. | 3.2 | _ | 1.4 | 7.8 | 8.1 |
| 10 | Os04t0398000-02 | Pathogenesis-related transcriptional factor and ERF domain containing protein. | 2.1 | _ | 1.0 | 4.9 | 4.6 |
| 11 | Os09t0332700-01 | Similar to PDR-type ABC transporter 2 (Fragment). | 2.9 | _ | 0.0 | 0.8 | 0.9 |
| 12 | Os11t0615800-00 | DNA repair and recombination, RecA-like domain containing protein. | 2.0 | DNA repair | -0.3 | 2.8 | 3.7 |
| 13 | Os11t0134300-01 | Similar to Serine/threonine kinase. | 8.2 | Nucleotide-binding | 0.3 | 2.8 | 1.2 |
| 14 | Os07t0158900-01 | Cyclin-like F-box domain containing protein. | 11.1 | _ | 0.1 | -0.6 | -2.2 |
| 15 | Os07t0249800-01 | Similar to IAA-amino acid hydrolase ILR1-like 8. | 2.1 | Peptidase activity | -0.6 | 2.7 | 2.5 |
| 16 | Os11t0615700-01 | Proteasome subunit alpha type 5 (EC 3.4.25.1) (20S proteasome alpha subunit E) (20S proteasome subunit alpha-5). | 16.0 | Peptidase activity | -0.2 | 0.2 | 0.1 |

**Table. 3 Primer information**

| Primer name | Forward | Reverse | Experiments |
|---|---|---|---|
| >NAC14 (Os01g0675800) | ATG TCC CCC TCC CGC C | TCA CAA GAA CCT GAT GAA TTT | Cloning |
| >NAC14 -qRTPCR | CAAGACGGAGGAGGCTACAC | GTCGGCCTTCCTCTTGCATC | RT-PCR |
| >NAC14-GFP | CGGGATCCATGTCCCCCTCCCGC | TATAGCGGCCGCCCAAGAACCTGATG | Cloning |
| >RbcS1 (Os02g0669100) | GGC AGG TAC TGG ACC ATG TG | TTG TCG AAG CCG ATG ATA CG | RT-PCR |
| >Dip1 (Os12g0274700) | GAG CTT GTC ACC GGC ATG GA | AGC TGG AGC TGG AGC TGG AT | RT-PCR |
| >Ubi1 (Os06g0681400) | ATG GAG CTG CTG CTG TTC TA | TTC TTC CAT GCT GCT CTA CC | RT-PCR |
| >1 (Os06g0286700) | CCA TGC TGA TAC TTG GGG CAT TGC | CAA GCC CAG CCA CTT TAC TTC CG | RT-PCR |
| >2 (Os09g0357400) | CGA AGC CTC ATC CAA CCA TCA GG | CCT CAC TTG TGA CAT CTT CAG GTT G | RT-PCR |
| >3 (Os11g0590700) | GGG CGA GAT AAC CGA TGA TGG C | GGT GTG GAA TAC GCA CGG AAT CC | RT-PCR |
| >4 (Os06g0287000) | GCA CTT CAT TGG GTG GTC TAG AC | GCT TGC TTA ACT GCC CCA GCT | RT-PCR |
| >5 (Os11g0491600) | GGT GAT GCC CAA CGG GTG TTT G | CTA ACA AAA TCC CAC TGT GCC TTC | RT-PCR |
| >6 (Os12g0281600) | GAC CAG GGA TCT CGT ATC AGC C | CCC TCG CTC ACT CGA GAA TCG | RT-PCR |
| >7 (Os12g0458100) | GAA TGC ACG TGG GAG ATG GAA G | GTA CGG TTT TTC ACG CCA CAA TGG | RT-PCR |
| >8 (Os11g0691100) | CGT GCT TCA TGG ACC TGA CCT G | CTT CCT TGG TTG CAA GGC CTA AC | RT-PCR |
| >9 (Os01g0705200) | CAG ACC GGG AGC TAC ATT GGA C | GTT ATC GCC TGA CAT CCC GAG C | RT-PCR |
| >10 (Os04g0398000) | CTA CAG AGG TGT TCG ACA GCG | GGG GAA GTT GAG CTT GGC CTT G | RT-PCR |
| >11 (Os09g0332700) | CCC AGG ACG AGC CCT TTT CAT G | GTG CGT TCA ACA GAC ACA ACT GG | RT-PCR |
| >12 (Os11g0615800) | CAG GTT CGG GTT GAA TGG TGC TG | CAT TTG CCT CGC TGA GAG CTC C | RT-PCR |
| >13 (Os11g0134300) | AGA TCT GCT CCC CTT GCC TAA C | CTT GTG AAA TTC GAG GGT GTC GCC | RT-PCR |
| >14 (Os07g0158900) | GCG AGC TAC GGA GTC TTC CAC | CCT TCC AAG GGT GAC AGC ATC G | RT-PCR |
| >15 (Os07g0249800) | CTG AAT CTG CAT CGC TGG GAG | GTT GGC CTC GCC GAG CAT C | RT-PCR |
| >16 (Os11g0615700) | CCA TTG AGG CCA TCA AGT TGG GG | CAC TCA TGG CAC AGC CTA TGT G | RT-PCR |
| >P1 (Os11g0615800) | GGG CAA TAG GCC TAT AGG GTG | GCC CAG TTT GTT TTT CTT GTA CGT C | ChIP |
| >P2 (Os11g0615800) | GCA GTG AGC AAT CAT AGG TGC TAT TG | GTT GGG GTC AAC TGC GAT CGG | ChIP |
| >P3 (Os11g0615800) | CGC CCA ACT CAT CCT GCA AAT C | CAG GTC GAG TAC GCC ATT GAG | ChIP |
| >P4 (Os11g0615800) | CAC GAT CTG ATT ACC TCG TGA GG | CCG CAA GAA GAA CAA CAC CGC | ChIP |
| >P5 (Os11g0615800) | GTG CCG CAG CCT CTA GGT TTT TG | GGA ATT GAA GGC GGC AAC GCG | ChIP |
| >P1 (Os06g0286700) | CGT ATG ACA TTG AAG ATT CCC TGA TG | GCC GGA GGA AAT AGG CTC GAC | ChIP |
| >P2 (Os06g0286700) | GAG TTG GGT TAT ACT TGA GAT ATC C | CAG GTC ACG TAT TTG CTC TGC CC | ChIP |
| >P3 (Os06g0286700) | GTG CAG CGC TAT CGA CAT GTG G | CAA GCC CTT GAT CAA CTA GGT G | ChIP |
| >P4 (Os06g0286700) | CAC TCT TGG ATG GTG GTG TCC | GAA CTA GCA CTG TGC ACC TAA CC | ChIP |
| >P5 (Os06g0286700) | CAT CTC TTT ACA TAG AGG CCG C | GGA GGT ACT AGG GTT TGT CAC C | ChIP |
| >Os11g0615800 | GCC AGT GCC AAG CTT TAA GGC CAC TCA TGG CAC AG | TGG CGT CTT CCA TGG GCG GAA TCC AGG AGG CCC TC | Dual Luciferase |

# Discussion

Drought is a serious environmental issue and causes food crisis worldwide. Scientists are focusing on the regulatory protein that regulates expression of stress-responsive genes such as transcription factors. There have been numerous reports suggesting that overexpression of NAC transcription factors can increase the tolerance of plants to water deficit conditions. For instance, *OsNAC52* showed drought tolerance in transgenic plants (Gao *et al*., 2010) and *OsNAC5* enhanced drought tolerance and increased grain yield in rice (Jeong *et al*., 2013). *OsNAC9* also enhanced drought resistance of rice and grain yield under field conditions (Redillas *et al*., 2012). In this study, we characterized the *OsNAC14* transcription factor that was up-regulated under drought stress conditions. To analyze the *OsNAC14* characteristics, qRT-PCR was performed with the rice plants exposed to various stress conditions, such as drought, high salinity, ABA and low temperature. Owing to the high-response of *OsNAC14* in drought stress condition, numerous downstream gene and network of the gene involved therein together determine the outcome of the stress response. So transgenic rice plants were developed to confirm the effect of *OsNAC14* improving stress resistance. The overexpression of *OsNAC14* under the constitutive (PGD1) promoter in transgenic rice was found to enhance plant tolerance to drought at both the vegetative stage and the reproductive stage. In the vegetative stage, the *proPGD1:OsNAC14* plants are less responsive to

drought than NT in visual test and more efficient and have stable photosynthetic systems than NT in JIP analysis under the drought conditions. In addition, *proPGD1:OsNAC14* plants had higher filling rates than NT plants under the drought conditions, which reflects *proPGD1:OsNAC14* has drought tolerance characteristic also in the reproductive stage. To validate the target gene of *OsNAC14*, our RNA sequencing experiment identified the 273 *OsNAC14*-dependent genes. Among them, six genes are selected based on gene ontology analysis and drought response. To confirm the candidate target gene, we conduct qRT-PCR using the material with drought-treated *proPGD1:OsNAC14* and NT plants, as well as in protoplasts. Two of the target gene candidates were expected to be a direct target, thus ChIP was conducted to confirm the genes as direct targets. A transcription factor, which can drive the expression of drought stress-associated genes, is significant in the context of the stress response. Results showed that OsNAC14 is recruited to the promoter region of OsRAD51A1. The promoter region of *OsRAD51A1* (*Os11g0615800*) was shown to interact with the *OsNAC14* transcription factor, seemingly a direct target. *OsRAD51A1* is known as homologous recombination protein, which regulates DNA repair in rice. *OsRAD51A1* encode for proteins involved in homologous recombination protein, which regulates DNA repair in rice. It is a multifunctional enzyme that plays a role in DNA repair by induction of the SOS response. As a result, the involvement of efficient DNA repair in stress adaptation mediated by OsNAC14, possibly via *OsRAD51A1*, provides a tolerance to drought stress. Moreover, the DNA repairs

are generated due to environmental factors such as exposure to drought stress. This process generates genetic variability, considered to be important for survival and evolution (Rajanikant *et al*., 2008). DNA repair is induced in response to DNA damage and have the important function of stress defense mechanism and adapt to various stresses in plants due to DNA repair involved delayed cell division and determined the DNA accessibility. Therefore, the up-regulation of *OsRAD51A1,* a downstream gene of *OsNAC14,* is expected to have an effect on DNA repair, which has functions in drought tolerance.

In conclusion, our results suggest that *OsNAC14* has important roles in regulating drought stress responses in rice. Importantly, we validated the direct target gene through the RNA sequencing and ChIP analysis. This study allowed us to assess the advantages of using a regulatory gene, *OsNAC14*, to improve stress tolerance in a commercially important crop.

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# ABSTRACT IN KOREAN

OsNAC14 전사인자 과발현 벼의
내건성 향상에 대한 연구

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가뭄 스트레스 같은 환경조건은 작물 생산에서부터 식량안보에 이르기까지 부정적인 영향을 끼치기 때문에 스트레스에 내성을 가지는 작물의 개발이 필요하다. 최근 전사인자 조절을 통해 여러 환경적인 스트레스에 내성을 가지는 식물체 개발이 전세계적으로 떠오르고 있다. 특히 전사인자는 여러 스트레스 관련 유전자들을 조절하는 역할을 갖고 있기 때문에 저항성 식물체를 만들기 위한 좋은 재료이다. 식물 특이적인 전사인자인 NAC 전사인자는 가뭄 같은 스트레스 조건에서 중요한 역할을 한다고 알려져 있다. 이 연구에서는 NAC 그룹 중 하나인 OsNAC14 전사인자를 벼에 과발현 시킨 형질전환체를 만들어 OsNAC14 의 기능에 대해 알아봤다. OsNAC14 를 과발현 시킨 형질전환체는 성장시기뿐만 아니라 생장시기에서까지 가뭄조건에서 저항성이 향상되는 결과를 나타냈다. 이어 RNA sequencing 실험을 통해 OsNAC14 가 273 개의 유전자 발현을 조절한다는 것을 확인했으며, 실제로 direct 하게 조절되는 유전자를 찾고자 이 후의 실험을 진행했다. Gene ontology 분석 후 16 개의 유전자를 골라 형질전환체와 protoplast 를 재료로 해 qRT-PCR 을 통해 타겟 유전자임을 증명하였다. 이 중 두 개의 유전자가 direct 하게 조절될 것이라

예상 한 뒤, DNA repair 와 관계 있다고 알려진 *OsRAD51A1* 와 programmed cell death 와 관계 있다고 알려진 *Piz-t* 유전자의 프로모터 분석을 수행했다. 그 결과, OsNAC14 전사인자가 *OsRAD51A1* 의 프로모터 부분에 binding 한다는 것을 ChIP 실험을 통해 증명했으며, 최종적으로 *OsRAD51A1* 의 프로모터 부분에 OsNAC14 전사인자가 관여 해 가뭄에 저항성을 가지게 될 것이라 예상된다.

**키워드** : NAC 전사인자, OsNAC14, 형질전환체, 가뭄저항성, 타겟 유전자 분석, 원형질체, RNA sequencing, ChIP