THESIS FOR DEGREE OF MASTER OF SCIENCE

Functional analysis of *Oryza sativa NAC054* transcription factor during leaf senescence

벼의 잎 노화에 관여하는 전사인자 *OsNAC054*의 조절 기작 규명

BY

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MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Leaf senescence, the final stage of leaf development, is regulated by complex network of senescence associated genes (SAGs). Several NAC transcription factors have been found to regulates leaf senescence in Arabidopsis, but only a few senescence associated NAC TFs have been identified in rice. In this study, we identified new senescence-associated NAC TF in rice, *OsNAC054*. We found *OsNAC054* expression increased during senescence. *osnac054-1* T-DNA KO mutants stayed green during dark-induced and natural senescence condition, suggesting *OsNAC054* acts as senescence promoting NAC TF. By microarray and qRT-PCR analysis, several SAGs (*OsORE1.1, OsNAP, OsEIL1*) and Chl degradation associated genes (*OsSGR, OsNYC1, OsPAO*) were down-regulated in *osnac054-1* under DIS condition. Furthermore, we found that *OsNAC054* expression is induced by ABA, and *osnac054-1* mutants showed stay-green phenotype during ABA induced senescence, strongly indicating that *OsNAC054* regulates leaf senescence through ABA-signaling pathway. Also, *OsNAC054* is Membrane bound TFs (MTFs) that have a transmembrane (TM) domain at its C-terminus. By transient expression assay using epidermal onion cell, we identified TM region of OsNAC054. We found that TM deleted OsNAC054 localized only in

the nucleus, while full length OsNAC054 localized both inside and outside of nucleus. Thus, it is possible that TF activity of *OsNAC054* is controlled by proteolytic cleavage of TM. Our data suggest that *OsNAC054* is involved in the promotion of leaf senescence through ABA-signaling pathway.

Keywords: rice, leaf senescence, NAC, Transcription factor, Transmembrane, *OsNAC054*, Alternative splicing, Stay-green

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ABBREVIATION

NAC	NAM, ATAF1, 2 and CUC2
TF	Transcription Factor
senNAC	Senescnece induced NAC gene
JA	Jasmonic Acid
ABA	Abscisic Acid
MTF	Membrane-bound Transcription Factor
NTL	NAC with Transmembrane motif 1-Like
SAG	Senescence-Associated Gene
RDV	Rice Dwarf Virus
RIM1	Rice dwarf virus Multiplication 1
MeJA	Methyl jasmonate
DIS	Dark Induced Senescence
DDI	Days of Dark Incubation
TM	Transmembrane
DAH	Days After Heading
DEG	Differently Expressed Gene
DT	Days after Treatment

INTRODUCTION

Leaf senescence is final stage of leaf development, which actively destabilizes intercellular organellas, including decomposition of macromolecules, to translocate nutrients into developing tissues and strage organs. Although leaf senescence occurs autonomously in an age-dependent manner, it is also regulated by various external factors, like drought, cold, and high salinity stresses [1-3], and internal factors, like phytohormone level, and the state of photosynthesis [4]. And then, these factors affect expression level of senescence-associated transcription factors [5], leading to the downstream leaf senescence cascade [4, 5].

NAM, ATAF1, 2 and CUC2 (NAC) TFs is one of the largest plant-TF family and regulates various plant process including senescence in many species, such as soybean [6], wheat [7, 8], rice [9, 10], and Arabidopsis [11, 12]. In Arabidopsis, some senescence-induced NACs (senNACs) have been identified. For example, *ORESARA1 (ORE1/ANAC092*) and *AtNAP* (*ANAC029*) play a positive regulator of leaf senescence [13, 14]. Mutation of *ORE1* and *AtNAP* delays leaf senescence, while overexpression of *ORE1* and *AtNAP* promotes leaf senescence, indicating that *ORE1* and *AtNAP* playing important role in leaf senescence.

On the other hand, *JUNGBRUNNEN1* (*JUB1/ANAC042*) and *VND-INTERACTING2* (*ANAC083*/*VNI2*) have been identified as negative regulator of leaf senescence; Overexpressing plants of *JUB1* and *VNI2*

showed delayed senescence phenotype [15, 16]. In addition, both *JUB1* and *VNI2* overexpression plants showed salt tolerance phenotype. *VNI2* expression was induced by ABA-treatment, but reduced in the ABA-deficient *aba3-1* mutant at high salinity condition. Thus, probably *VNI2* acts as a molecular link that integrates ABA-dependent salt stress signals into leaf senescence by regulating salt-stress responsive COR and RD genes [15]. As described above, several NAC TFs regulate leaf senescence positively or negatively.

In rice, however, only a few senNACs have been characterized. One of the leaf senescence positive regulator is *OsNAP*. *OsNAP* overexpressing plants showed early leaf senescence, while *osnap* mutant exhibits delayed leaf senescence [10, 17]. *OsNAP* is involved in JA-related leaf senescence pathway through regulation of JA-biosynthesis genes expression [10]. The other group reported that *OsNAP* expression is up-regulated in salt and drought stress condition and *OsNAP* overexpression plants showed salt and drought tolerance phenotype [18]. In addition, *OsNAP* expression induced by ABA and reduced in ABA biosynthesis mutants [17]. Thus, *OsNAP* functions as an important link among JA signaling, ABA signaling, and leaf senescence.

Those transcription factor activities are regulated by multiple steps, such as transcriptional level regulation, post-transcription modification, translation and post-translational modification [19].

Recently, membrane bound transcription factors (MTFs) has been identified that regulated by proteolytic cleavage. MTFs are initially made as dormant membrane-bound form. When MTFs recognize any stimuli, however, MTFs released from the membranes through proteolytic cleavage by intracellular signal and integrate into the nucleus [20]. To date, 12 MTF NACs identified in Arabidopsis [1], and the function of several MTF NACs have been already studied [1, 21, 22]. These NAC MTFs are involved in abiotic stress response and leaf senescence.

NTL9 expression is increased in osmotic stress condition. Proteolytic cleavage of *NTL9* also increased in same condition. In addition, SAGs(Senescence-Associated Genes) expression up-regulated in pale-green overexpressing activated form of *NTL9* plants. Leaf senescence is delayed in *ntl9* mutants. These results suggesting that *NTL9* regulates osmotic stress response during leaf senescence by proteolytic cleavage of NTL9 protein. *NTL6* plays a role in the cold induction pathogen resistance and ABA-related drought-resistance response [22, 23]. Proteolytic cleavage of NTL6 protein triggered by cold, ABA and phosphorylation of active form NTL6 by SnRK2.8 is needs for imported into nucleus in drought-resistance response [23]. On the other hand, the studies of NAC MTF of rice are still considerably limited.

The physiological function of *RICE DWARF VIRUS MULTIPLICATION 1* (*RIM1*), one of the rice NAC MTFs, were previously studied. *rim1* mutants

showed resistance to *Rice dwarf virus* (RDV), and RDV capsid proteins content was reduced in *rim1* mutants [24], indicating that RIM1 involved in multiplication of RDV in rice. In addition, using Microarray analysis, Yoshii et al. (2010) found that several JA-biosynthesis genes expression is reduced in *rim1* mutants. And these JA-inducible gene expressions was repressed in *RIM1* overexpressing plants. Also, RIM1 protein is degraded by 26S proteasome complex when JA treated condition. These results suggest that *RIM1* is a transcriptional repressor of JA pathway [25]. In this regards, it is possible that RIM1 also has an important role in the leaf senescence because MeJA is known as one of the senescence promoting phytohormones [26]. However they didn't mention about the role of transmembrane and *RIM1* functions in leaf senescence.

Here we show identified NAC MTF, RIM1/OsNAC054 (hereafter *OsNAC054*) acts as a positive regulator in leaf senescence*. osnac054-1* mutants showed stay-green during DIS and natural senescence condition. In microarray analysis, the expression of several SAGs, including *OsABI5, OsORE1, and OsNAP* was down-regulated in *osnac054-1* mutants during DIS. Expression levels of *OsNAC054* and SAGs were up-regulated by ABA, indicating that OsNAC054 modulated ABA-mediated leaf senescence pathway. Indeed, *osnac054-1* leaves showed significant delayed senescence phenotype during ABA-mediated leaf senescence. Furthermore,

we identified TM domain in C-terminal region of OsNAC054, and probably it has an important role in the regulation of OsNAC054 activity.

MATERIALS AND METHODS

Plant materials and growth conditions

osnac054-1 T-DNA insertion mutant was obtained from Kyung-Hee University, Korea [27, 28]. *Japonica rice* cv 'Dongjin' was used as WT. WT and mutant plants were grown in the paddy field (natural long day in Suwon, South Korea, 37N) or growth chamber (12-h light/12-h dark, 500 μmol m^{-2} s^{-1}, 30°C). For DIS experiment, detached leaf discs from 4-weeks-old plants were incubated in complete darkness.

Chlorophyll (Chl) Quantification

Total Chl was extracted from the second or third leaves using 80% ice-cold acetone at 4°C. Chl was quantified spectrophotometrically as previously described [29].

Measurement of Ion Leakage

Ion leakage was measured as described previously [30] with minor modifications. Membrane leakage was determined by measurement of electrolytes (or ions) leaking from rice leaf disc (1 cm^2). Three leaf discs from each treatment were immersed in 10 mL of 0.4 M mannitol at room temperature with gentle shaking for 3 h, and conductivity of the solution measured with a conductivity meter (CON6 METER, LaMOTTE Co.) Total

conductivity was determined after sample incubation at 85℃ for 20 min. The ion leakage is expressed as the percentage of initial conductivity divided by total conductivity.

SDS-PAGD and immunoblot analysis

Protein extracts were prepared from leaf tissues. To extract total proteins, leaf tissues from 2-month-old rice plants grown in the growth chamber (14 h light/ 10 h dark) were ground in liquid nitrogen and 10 mg aliquots were homogenized with 100 μL of sample buffer (50 mM Tris, pH 6.8, 2 mM EDTA, 10% glycerol, 2% SDS, and 6% 2-mercaptoethanol). Homogenates were centrifuged at 10,000 × *g* for 3 min, and supernatants were denatured at 80°C for 5 min. 4 μL of each sample was subjected to 12% (w/v) polyacrylamide SDS-PAGE and resolved proteins were electroblotted onto a Hybond-P membrane (GE Healthcare). Antibodies against photosystem proteins (Lhca1, Lhca2, Lhcb2, Lhcb4, PsaA, and CP43) were used. And their secondary antibody activities were visualized using the WEST SAVE chemiluminescence detection kit (AbFRONTIER).

Transmission electron microscopy (TEM) analysis

TEM analysis was performed as described previously [31] with minor modifications. Segments of leaf tissues were fixed with a modified Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde and 50mM

sodium cacodylate buffer, pH 7.2) and washed three times with 50 mM sodium cacodylate buffer (pH 7.2), at 4℃ for 10 min. The samples were fixed with 1% osmium tetroxide in 50 mM sodium cacodylate buffer (pH 7.2) at 4℃ for 2 h, and briefly washed twice with distilled water at 25℃. Samples were stained with *en bloc* in 0.5% uranyl acetate at 4℃ for a minimum of 30 min, dehydrated in a gradient series of ethanol and propylene oxide, and finally embedded in Spurr's resin. After polymerization at 70℃ for 24 h, ultrathin sections were prepared with a diamond knife on an ultramicrotome (MT-X) and mounted on Formvarcoated copper grids. The sections on the grids were stained with 2% uranyl acetate for 5 min and with Reynolds' lead citrate for 2 min at 25℃ and then examined using a JEM-1010 EX electron microscope (JEOL).

Measurement of *Fv/Fm* ratio

Maximal photochemical efficiency of photosystem II (*Fv/Fm*), and minimum (F0) and maximum fluorescence (Fm) of photosystem II were measured using a PAM 2000 fluorometer (Heinz Walz, Germany). Plants were dark-adapted for 10 min and the *Fv/Fm* ratios of the second or third leaves were measured.

Microarray analysis

Total RNA was extracted from the leaves of 4-week-old soil-grown wild type plants and os*nac054-1* mutants using the TRIzol Reagent, according to the manufacturer's protocol (Invitrogen). Total RNA quality was checked using a 2100 Bioanalyzer (Agilent Technologies). Microarray analysis was performed using the Rice Gene Expression Microarray, design identifier 015241 (Agilent), containing 43,803 rice gene. Total RNA (150 ng) was used to prepare Cy3-labeled probes, using the low-RNA-input linear amplification kit PLUS (Agilent). Labeled RNA probes were fragmented using the Gene Expression Hybridization buffer kit (Agilent). All microarray experiments, including data analysis, were performed according to the manufacturer's manual (http://www.chem.agilent.com/Scripts/PCol.asp?lPage=494). The arrays were air-dried and scanned using a high-resolution array scanner (Agilent) with the appropriate settings for two-color gene expression arrays. GeneSpring GX (Agilent) was used to calculate the intensity ratio and fold changes.

RT-PCR and qPCR Analysis

For RT-PCR analysis, total RNA was isolated from leaves using the TRIzol Reagent according to the manufacturer's protocol (Invitrogen). First-strand cDNAs were prepared with 2 μg of total RNA in a 25 μl reaction volume using M-MLV reverse transcriptase and oligo$(dT)_{15}$ primer (Promega), and

diluted with water to 100 µl. The qPCR mixture contained 2 µl of cDNA template, 10 µl of 2X SYBR Green PCR Master Mix (Qiagen) and 0.25 mM of the forward and reverse primers for each gene. Primer sequences for each gene are listed in (Table 1). qPCR was performed using the Light Cycler 480 (Roche Diagnostics). Transcript levels of each gene were normalized to ubiquitin 5 (*UBQ 5*).

Table 1. Information of primers used in this study

Primer name	Forward (5' - 3')	Reverse (5' - 3')
A. Primers for verification of *OsNAC054* T-DNA insertion		
PFG_3A-07241.L	ATGGCGCCCGTGAGTTTGCCT	TTAGTGATCGCACTCAGCT
B. Primers used for gene cloning		
OsNAC054	ATGGCGCCCGTGAGTTTGCCT	TTAGTGATCGCACTCAGCT
OsNAC054⊿TM1	ATGGCGCCCGTGAGTTTGCCT	TTAGACGTCAACCTTCTCGTACAG
OsNAC054⊿TM2	ATGGCGCCCGTGAGTTTGCCT	TTATGTGGTTTCTTTGCTGCC
OsNAC054⊿TM3	ATGGCGCCCGTGAGTTTGCCT	TTAGCTGGAAATGCCCTTCCC
C. Primers used for qRT-PCR		
OsNAC054	CCCCGTTACGACCAAAGTTA	CAGTAGGGCAGCCATTGATT
OsORE1.1	CTGGTGCAAXTCCTGGAGAG	AGATCTCCGGGTTCACGTC
OsNAP	AACCATTTCATCGCGAACAAC	CAGTGACGATCCCTGCAAGG
OsSGR	GGAGTGGAAGAAGGTGCA	CCTTGCGGAAGATGTAGTAG
OsPAO	GTGTTGCCTTCCACTGTCCT	ACTGAACATCCGCAGGAATC
OsNYC1	GAATCCGTAATTGGGCTGAA	CTGGAAGAGGTCCACCTGAG
OsEIL1	ATCTTCCCGGCAACCTACAA	CATGATCGTGGCATTGTCGT
OsABI5	ATGGCATCGGAGATGAGCAAGAAC	GCTTCTTTGTCAGTAGAACCGTCTTC
OsABF2	GCTGAGGTGGCAAAACTGAA	ATGGACCAGTCAGTGTTCGT
UBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

Phytohormone and Abiotic stress treatments

For hormone and abiotic stress treatments, the second leaves of 4-weeks-old WT plants were detached and floated on 3 mM MES (pH5.8) buffer solution in the presence and absence of 100 μM ABA or 100 μM MeJA, 500 μM ACC, 200 mM NaCl. Hormone and salt stress treatments were performed at 23°C under continuous light for 2 days. For cold stress treatment, leaves were incubated at 4°C for 2 days under continuous light condition.

Subcellular localization of OsNAC054 proteins

PCR amplified *OsNAC054* cDNA was ligated into pCR8/GW/TOPO plasmid (Invitrogen). *OsNAC054* cDNA was inserted into vector pMDC43 with two CaMV 35S promoter and GFP coding sequence by LR recombination reaction (Invitrogen). For subcellular localization analysis, GFP-OsNAC054 vector bombarded into onion epidermal cells using a DNA particle delivery system (Biolistic PDS-1000/He, BioRad). 16-24h after bombardment, epidermal cells were analyzed using a Confocal Laser Scanning Microscope II (LSM710, Carl Zeiss).

RESULTS

OsNAC054 is senescence associated NAC TF

In Arabidopsis, several senescence associated NAC TFs (senNAC TFs), including *ORE1*, *NAP*, *JUB1* and *NAC016* have been reported [1, 13, 14, 16]. On the other hand, in rice the studies of senNACs are still limited. To find rice senNACs, we constructed phylogenic tree using all of Arabidopsis and rice NAC genes (Fig. 1). In this phylogenic tree, we found *OsNAC054* is phylogenetically close to Arabidopsis senNACs like *JUB1* and *ORE1*. Because it was previously reported that senescence associated NAC genes expression significantly increased during senescence [1, 13, 16], we checked *OsNAC054* expression levels in different regions of the senescenced flag leaf of WT at 50 days after heading (DAH) in the paddy field.

OsNAC054 expression was comparatively higher in the yellowish top area of flag leaf than in the yellow green bottom area (Fig. 2A). We also checked expression of *OsNAC054* during dark-induced senescence (DIS) condition. We incubated detached leaves of WT plants in complete darkness from 0 to 4 days. Before dark incubation (0 DDI), *OsNAC054* expression was considerably lower, and increased gradually until 4 DDI as detached leaves turned yellow (Fig. 2B). Taken these results together, *OsNAC054*

expression was increased during both natural senescence and DIS, indicating that *OsNAC054* is related with leaf senescence.

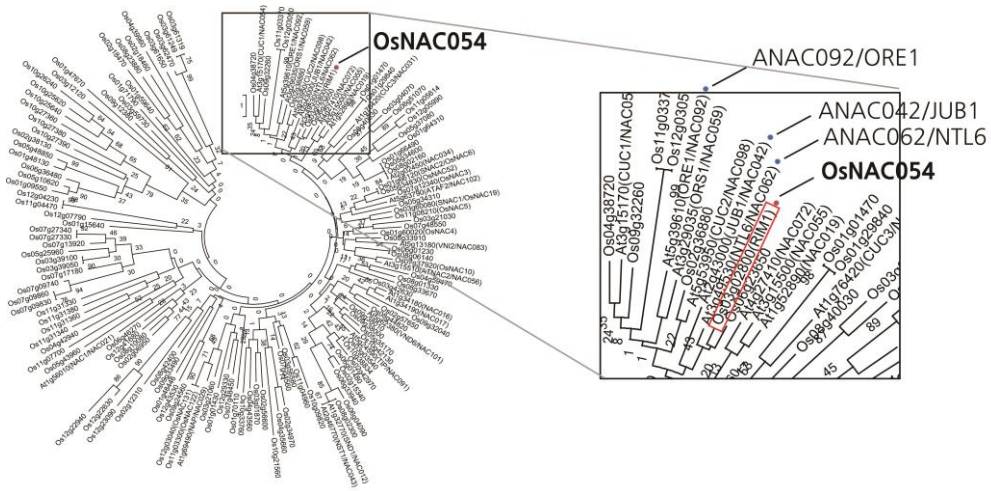

Fig. 1 Phylogenic tree of all of Arabidopsis and rice NAC genes.

The sequences of NAC domain in Arabidopsis and rice NAC TFs were used for constructing phylogenic tree. Phylogenic tree was constructed using MEGA5 software.

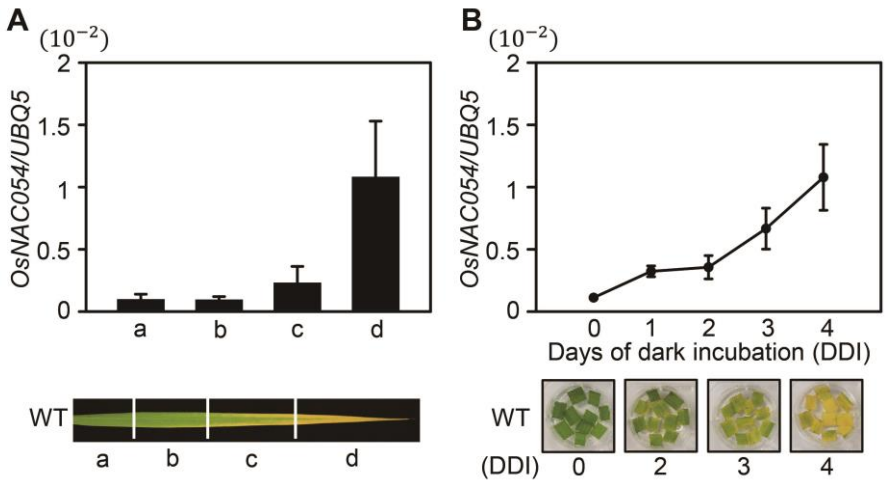

Fig. 2 The expression pattern of *OsNAC054* during senescence.

The expression level of *OsNAC054* in senescent leaves (A) and during dark induced senescence (B) were determined by normalizing the expression level of *UBQ5*. Mean and SD were obtained from more than three biological replicates.

OsNAC054 T-DNA insertion knockout mutant shows delayed senescence phenotype

To examine the function of OsNAC054 in the leaf senescence, we obtained one possible T-DNA insertion knockout lines. (PFG_3A-07241.L) has a single T-DNA fragments inserted in the 6th exon *OsNAC054* (Fig. 3A). By semi quantitative RT-PCR, we confirmed that *OsNAC054* transcripts did not accumulate in this T-DNA line leaves (Fig. 3B), indicating that this T-DNA insertion line is *osnac054* knockout (KO) mutant allele (hereafter, os*nac054-1*). At first, we checked leaf phenotype of *osnac054-1* during DIS. At vegetative stage, leaf color of *osnac054-1* is almost the same as WT (Fig. 3C, 0 DDI). At 4 DDI, however, *osnac054-1* exhibited strong stay-green phenotype while WT became completely yellow (Fig. 3C).

Consistent with visible phenotype, total Chl and most photosystem proteins, including Photosystem I complex subunits (Lhca1, Lhca2, and PsaA) Photosystem II complex subunits (Lhcb2, Lhcb4 and CP43) highly retained in os*nac054-1* (Fig. 4A & 4B). We also found that ion leakage rate, an indicator of membrane disintegration, was considerably lower in os*nac054-1* compared with WT during DIS (Fig. 4C).

Subsequently, we compared chloroplast structure of the WT and *osnac054-1* before and after 4 days of dark incubation (4 DDI). It was previously reported that in many stay-green plants chloroplast structure and

grana thylakoid structure retained during DIS (Park et al. 2007; Kim et al. 2013). Before dark treatment, WT and *osnac054-1* leaves have similar chloroplast structure (Fig. 5A, B, upper panels). At 4 DDI, however, chloroplasts in *osnac054-1* leaves retained thick grana thylakoid structure, while it was hardly detected in WT leaves (Fig. 5A, B, lower panels).

Taken these results together, mutation of *OsNAC054* delays leaf senescence by maintaining the balance of photosystem complexes and the persistence of cell membrane integrity much longer than in WT under DIS conditions.

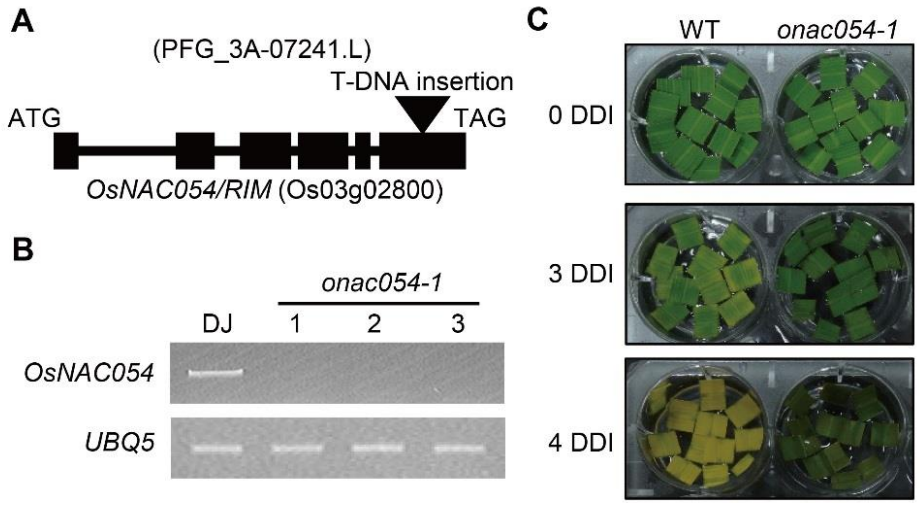

Fig. 3 Characterization of *osnac054-1* T-DNA insertion KO mutant

(A) Gene structure of *OsNAC054* and T-DNA insertion point (inverted closed triangle) in the sixth exon of *OsNAC054* (PEG_3A-07241.L). Exons are shown as black boxes and introns are shown as connecting lines. (B) The absence of *OsNAC054* transcripts in *osnac054-1* was confirmed by RT-PCR. *UBQ5* was used as a loading control. (C) The phenotype WT and *osnac054-1* leaf discs during dark-induced senescence (DIS) condition. Detached leaves from 1-month-old WT and *osnac054-1* plants were used.

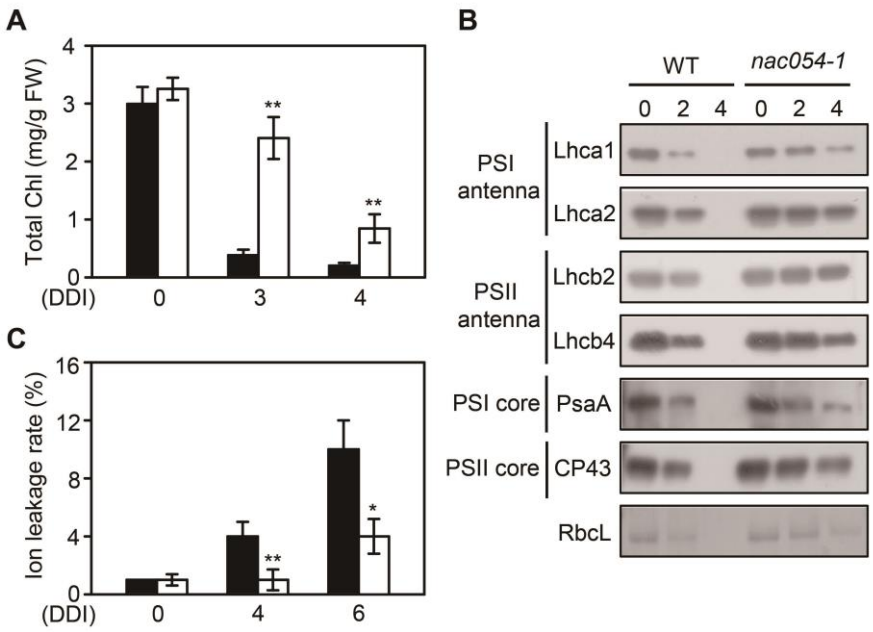

Fig. 4 Characterization of stay-green phenotype of *osnac054-1*

(A, B) Changes in total Chl levels (A) and ion leakage rate (B) in WT and *osnac054-1* during DIS condition. For these analysis, detached leaves from 1-month-old WT and *osnac054-1* plants were used. Mean and SD values were obtained from more than three biological replicates. (C) Immunoblot analysis of photosystem proteins in detached leaves of 2-month-old WT and *osnac054-1* plants at 0, 2, 4 DDI. Antibodies against PSI complex subunits (Lhca1, Lhca2, and PsaA) PSII complex subunits (Lhcb2, Lhcb4 and CP43) were used for detection. RbcL (Rubisco large subunit) protein was visualized by Coomassie Brilliant Blue staining.

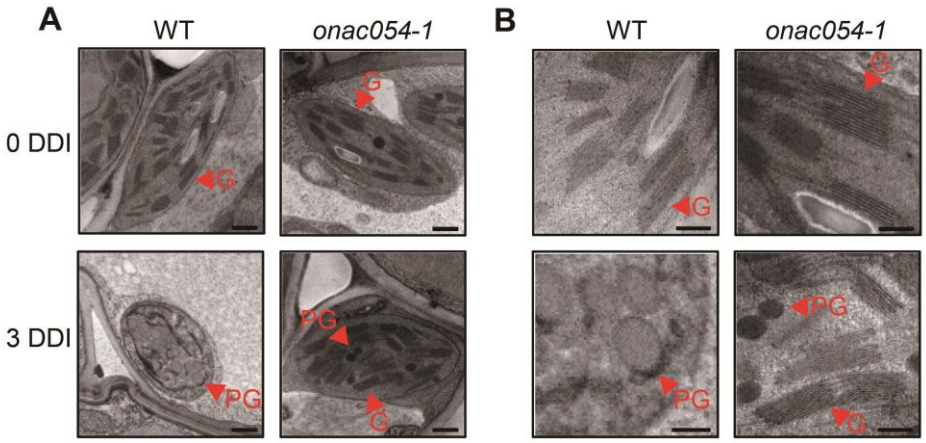

Fig. 5 Transmission electron microscopy of WT and *osnac054-1* leaves under DIS conditions.

(A) Morphological changes in the chloroplasts of detached leaves of WT and *osnac054-1* leaves before and after 3 DDI. Bars = 0.5 μm. DDI, days of dark incubation. (B) The structure of grana thylakoids in chloroplasts of WT and *osnac054-1* leaves before and after 3 DDI. G, granum; PG, plastoglobule. Bars = 200 nm.

The *osnac054-1* mutants exhibit a functional stay-green phenotype during natural senescence

Stay-green mutants are divided into two groups, functional and nonfunctional mutants. The differences between two groups are retention of green color and photosynthetic activity. Nonfunctional stay-green mutants showed green leaf color without photosynthetic activity during senescence, while functional stay-green mutants retain both green color of leaves and photosynthetic activity [32, 33]. Thus delaying senescence of functional stay-green mutants is positively affect plant yield [18, 34]. To determine whether *osnac054-1* stay-green phenotype is functional or nonfunctional mutant during natural senescence condition, we checked phenotype of WT and *osnac054-1* in the paddy field under natural long day conditions. At 60 days after heading, during grain filling period, *osnac054-1* showed delayed leaf senescence (Fig. 6A, B). Subsequently, we examined total Chl levels in second leaves of *osnac054-1* and WT at 40 days after heading (DAH). Total Chl levels in *osanc054-1* mutant were significantly higher than those in WT (Fig. 6C). We also checked photosynthetic capacity in WT and *osnac054-1* by measuring *Fv/Fm* ratio after heading. The *Fv/Fm* ratio in WT and *osnac054-1* were almost same before heading. During grain filling stage, *Fv/Fm* ratio in WT was decreased while it was not much altered in *osnac054-1* (Fig. 6D). These results strongly suggest that *osnac054-1* is the functional stay-green mutant.

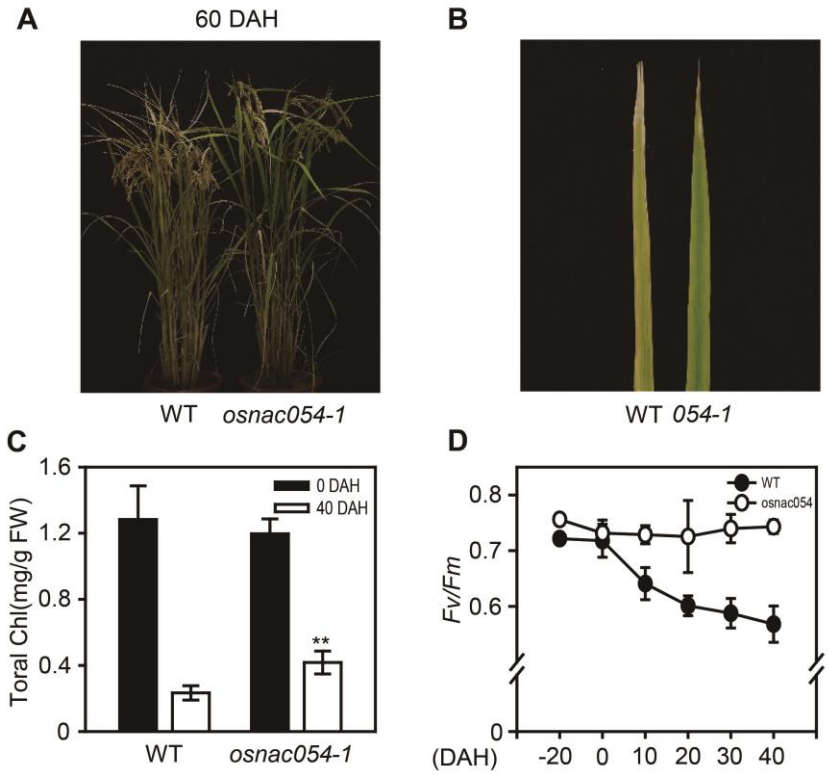

Fig. 6 Phenotype of *osnac054-1* during natural senescence

(A) Phenotype of WT and *osnac054-1* at 60 days after heading (DAH). (B) Phenotype of flag leaves in WT and *osnac054-1* at 60 DAH. (C) Total Chl levels of WT and *osnac054-1* at 0 (black bars) and 40 DAH (white bars). (D) Temporal changes of *Fv/Fm* ratio in WT and *osnac054-1*. The second leaves were used. Filled and opened circles indicate WT and *osnac054-1*, respectively. Mean and SD values were obtained from three biological replicates.

Analysis of regulatory network of *OsNAC054* dependent leaf senescence pathway

To identify downstream target genes and pathways regulated by *OsNAC054* during leaf senescence, we checked differentially expressed genes (DEGs) between WT and *osnac054-1* plants at 0DDI and 2DDI using the Agilent Rice Gene Expression Microarray. Linkage map analysis revealed that compared with 0DDI a large portion of genes were differentially expressed at 2DDI (Fig. 7A), indicating that *OsNAC054* has a important role in the induction of leaf senescence. In these DEGs, 757 and 2605 genes were up-regulated at 0DDI and 2DDI respectively, and 283 genes were shared by two datasets (*osnac054-1*/WT; >2-fold, Fig. 7B). On the other hand, 336 and 1511 genes were down-regulated at 0DDI and 2DDI respectively, and 61 genes were shared by two datasets (*osnac054-1*/WT; < 2-fold, Fig. 7C). In this microarray analysis, we found several SAGs, including one of the rice homologs of Arabidopsis senescence associated NAC TFs *ORESARA1* (*OsORE1.1*; [13, 35]) and *OsNAP* [14], and Chlorophyll catabolism associated genes *OsSGR* [36] and *PHEOPHORBIDE A OXYGENAGE* (*OsPAO*; [37]), *OsNYC1* [38] were also differentially expressed in *osnac054-1.*

To confirm the result of microarray analysis, we checked several SAG genes expression during DIS condition by qRT-PCR analysis. In addition to SAGs described above (Os*ORE1.1*, *OsNAP*, *OsSGR*, *OsPAO, OsNYC1*),

we checked expression levels of *OsEIL1*, a rice homolog of *Arabidopsis ETHYLENE INSENSITIVE3* (*EIN3*) [39]. Arabidopsis *EIN3* is one of the major SAGs and it directly promotes the expression of Arabidopsis *ORE1* [40]. At 0 DDI, expression levels of *OsORE1, OsNAP, OsSGR, OsPAO, OsNYC1* and *OsEIL1* in *osnac054-1* was almost the same as those in WT, while these gene expression was significantly down-regulated in *osnac054-1* at 3 DDI (Fig. 8A-8F). These results suggest that *OsNAC054* is involved in the leaf senescence by directly or indirectly regulating typical SAGs.

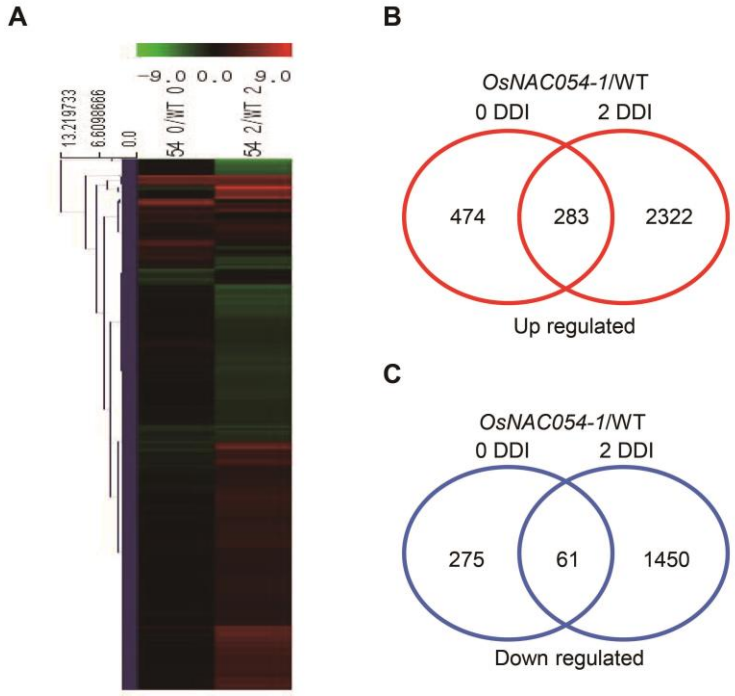

Fig. 7 Summary of microarray analysis between WT and *osnac054-1* at 0 and 2 DDI.

(A) Hierarchical linkage map of the genes that are differently expressed in WT and *osnac054-1*. (B, C) Venn diagram representing the number of genes up-regulated (B) and down-regulated (C) at 0DDI and 2DDI in WT and *osnac054-1*.

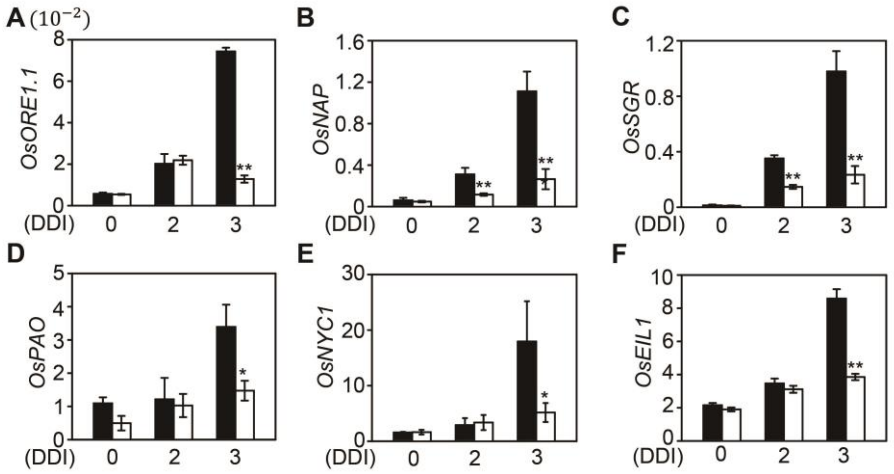

Fig. 8 Expression patterns of SAGs in the *OsNAC054* dependent leaf senescence pathway

(A-I) Total RNA was extracted from second leaves of 2-month-old WT and osnac054-1 plants before and after dark incubation. Relative expression levels of *OsORE1.1* (A), *OsNAP* (B), *OsSGR* (C), *OsPAO* (D), *OsNYC1* (E), *OsEIL1*(F) were determined by RT-qPCR and normalized to the transcript levels of *UBQ5*. Means and SDs were obtained from more than three biological replicates. These experiments were replicated at least twice with similar results. Student's t-test was used to calculate statistical significance (*P<0.05; **P<0.01).

OsNAC054 modulates ABA-mediated leaf senescence pathway

Leaf senescence is often modulated by senescence associated phytohormones and abiotic stresses [4, 41]. To determined *OsNAC054* expression is modulated by phytohormones and abiotic stress treatments, Second leaves from one-month-old WT plants were treated by several phytohormones, such as Abcisic acid (ABA), 1-aminocyclo-propane-1-carboxylic acid (ACC, one of the ethylene intermediates), and Methyl Jasmonate (MeJA), and abiotic stresses, such as at salt and cold, and *OsNAC054* expression levels were examined by qRT-PCR analysis. After 24h of treatments, *OsNAC054* expression was significantly increased up to 3-fold in ABA-treated WT, while it was hardly changed under other treatment conditions (Fig. 9), indicating that OsNAC054 has an important role in the ABA signaling responsive pathway. Thus, we subsequently checked expression of SAGs *(OsNAP, OsORE1, OsSGR,* and *OsNYC1*) that down-regulated in *osnac054-1* during DIS (Fig. 8). These SAGs expression also increased until 48h after ABA treatment (Fig 10A-10D).

In microarray analysis, we found ABA signaling related genes, such as rice homolog of *Arabidopsis ABA INSENSITIVE5* (*OsABI5;* [42]) and *Oryza sativa ABA-RESPONSIVE ELEMENT BINDING FACTOR 2* (*OsABF2*; [43]) were differently expressed in *osnac054-1*. We confirmed the expression of these genes during DIS condition by qRT-PCR analysis. At 3 DDI, these genes expression is down-regulated in *osnac054-1* (Fig. 11A & 11B). We

also confirmed that *osnac054-1* showed stay-green phenotype with highly retaining Chl during ABA induced senescence (Fig. 12). Collectively, these results suggest that OsNAC054 controls leaf senescence pathway by promoting ABA signaling responsive pathway.

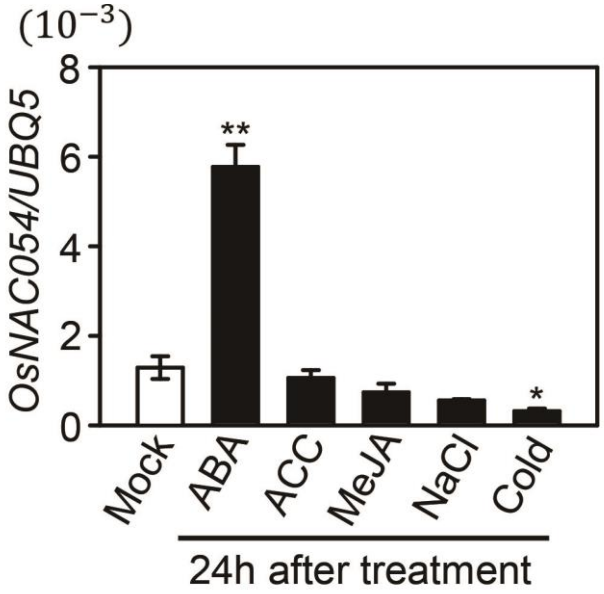

Fig. 9 Expression of *OsNAC054* under phytohormones and abiotic stress conditions.

Second leaves of one-month-old WT plants were floated in 3 mM MES buffer (pH5.8) containing 100 μM ABA, 500 μM ACC, 100 μM MeJA, 200 mM NaCl, and incubated for 24h under continuous light. The cold treatment was performed at 4℃. The expression level of *OsNAC054* were determined by normalizing the expression level of *UBQ5*. Mean and SD were obtained from more than three biological replicates. Student's t-test was used to calculate statistical significance (*P<0.05; **P<0.01). ABA, Abcisic acid; ACC, 1-aminocyclo-propane-1-carboxylic acid; MeJA, Methyl Jasmonate.

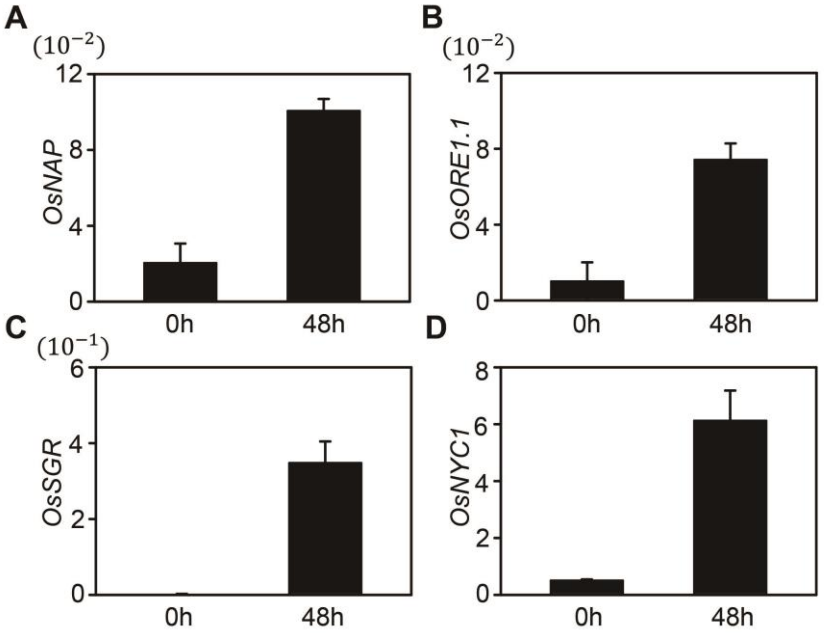

Fig. 10 The expression level of SAGs under ABA treatment condition.

Second leaves from one-month-old WT plants were treated by 100 μM ABA for 48h under continuous light. The expression level of *OsNAP* (A), *OsORE1.1* (B), *OsSGR* (C), *OsNYC1* (D) were determined by normalizing the expression level of *UBQ5*. Mean and SD were obtained from more than three biological replicates. Student's t-test was used to calculate statistical significance (*P<0.05; **P<0.01).

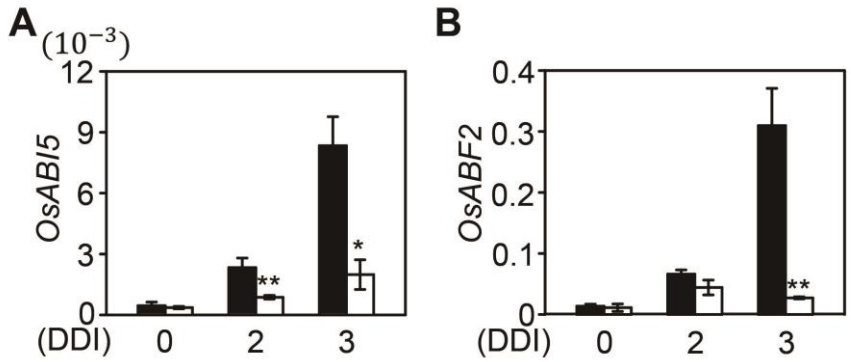

Fig. 11 The expression pattern of ABA-related genes in *osnac054-1* under DIS conditions.

Total RNA was extracted from second leaves of 2-month-old WT and *osnac054-1* plants before and after dark incubation. Relative expression levels of *OsABI5* (A), Os*ABF2* (B) were determined by RT-qPCR and normalized to the transcript levels of *UBQ5*. Means and SDs were obtained from more than three biological replicates. These experiments were replicated at least twice with similar results. Student's t-test was used to calculate statistical significance (*P<0.05; **P<0.01).

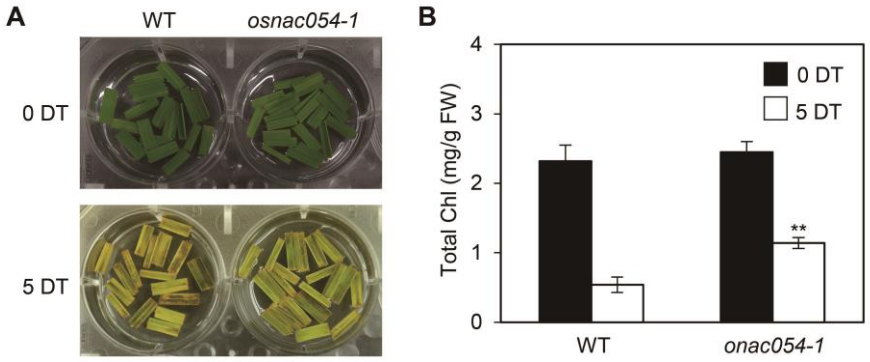

Fig. 12 Phenotype characterization of *osnac054-1* plants under ABA stress condition.

Phenotype (A) and total Chl levels (B) of WT and *osnac054-1* plants before (0 DT) and after 5 days of ABA treatment (5 DT). Detached leaves of one-month-old plants were used. Mean and SD were obtained from more than three samples. Student's t-test was used to calculate statistical significance (*P<0.05; **P<0.01).

The localization of OsNAC054 is partly regulated by alternative splicing

Recent studies have shown that several NAC TFs are associated with membranes and their activity is regulated by proteolytic cleavage of transmembrane(TM) domain in C-terminal region [44]. Membrane-bound transcription factors (MTFs) have larger size than non-membrane-associated NAC TFs because of TM domain in C-terminal region [45].

We found that protein size of OsNAC054 is much larger than the other rice senescence associated NAC TFs (Fig. 13). we analyzed whether *OsNAC054* have TM domain in C-terminal region using DAS (Dense Alignment Surface method) transmembrane prediction site (http://www.sbc.su.se/~miklos/DAS/) showing that *OsNAC054* contains at least 3 TM prediction site at its C-terminus (Fig. 14A). Thus it is possible one of the 3 prediction site is TM of OsNAC054 and it control the sublocalization on the OsNAC054. To this end, GFP was fused to the N-terminus of the OsNAC054-FL(OsNAC054a, 1-650aa), OsNAC054△TM1(1-480aa), OsNAC054△TM2(1-567aa), OsNAC054△TM3(1-626aa) sequences (Fig. 14B). The localization of these fused proteins was observed in onion epidermal cells by confocal microscopy (CLSM). We found the full length of OsNAC054, OsNAC054△TM2 and OsNAC054△TM3 were targeted to both inside and outside of nucleus while

OsNAC054△TM1 localized only in the nucleus (Fig.14C), indicating TM1 is TM domain of OsNAC054 that controls the localization and TF activity.

We also found *OsNAC054* undergo alternative splicing producing two splicing variants. *OsNAC054* alternative splicing is mediated by 7 bp the retention of intron 6 resulting premature stop codon is introduced into *OsNAC054* gene, leading to the truncated form lacking the TM domain (OsNAC054*β*, Fig. 15A). Thus, we subsequently checked the subcellular localization of OsNAC054*β* in onion epidermal cells. We found that OsNAC054*β*-GFP was completely targeted to the nucleus (Fig. 15B), as well as OsNAC054△TM1-GFP (Figure 14C). Taken these results together, *OsNAC054* is MTFs which have TM domain in C-terminal region, and activation of OsNAC054 is controlled by not only proteolytic cleavage but also alternative splicing.

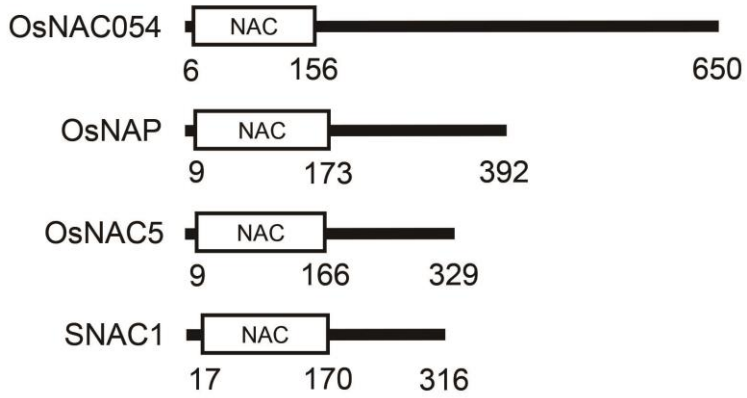

Fig. 13 Comparison of OsNAC054 protein with other rice senNACs.

Protein stricture of OsNAC054 and other senescence associated NAC TFs, OsNAP, OsNAC5, and SNAC1 are compared. White boxes indicate NAC domain, which was predicted by PROSITE (http://prosite.expasy.org/).

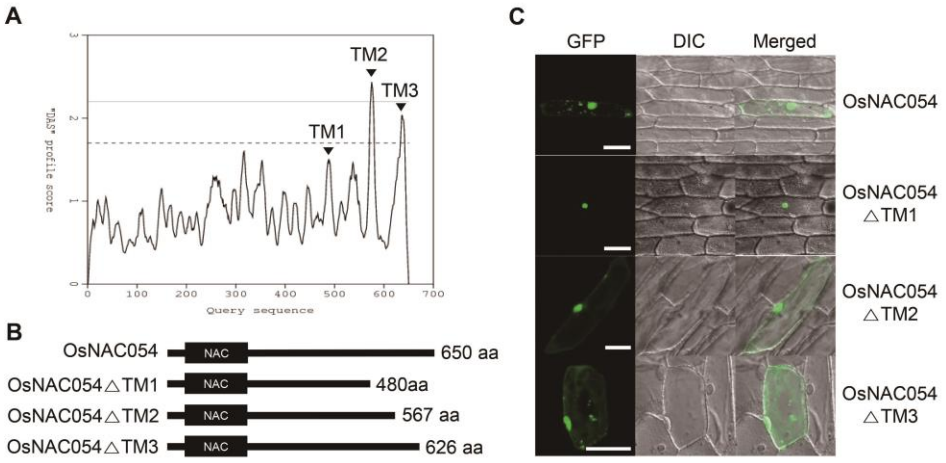

Fig. 14 OsNAC054 TF activity is controlled by C-terminal transmembrane. (A) Black arrowhead indicated TM prediction site (TM1, TM2, TM3) predicted by DAS. Structure (B) and subcellular localization (C) of OsNAC054 protein and TM prediction site deleted OsNAC054 proteins (OsNAC054△TM1 (1-480 aa), OsNAC054△TM2(1-567 aa), OsNAC054△TM3(1-626 aa). GFP fused proteins were transiently expressed in onion epidermal cells. GFP fluorescence was collected at 525 nm. Bars = 100 μm

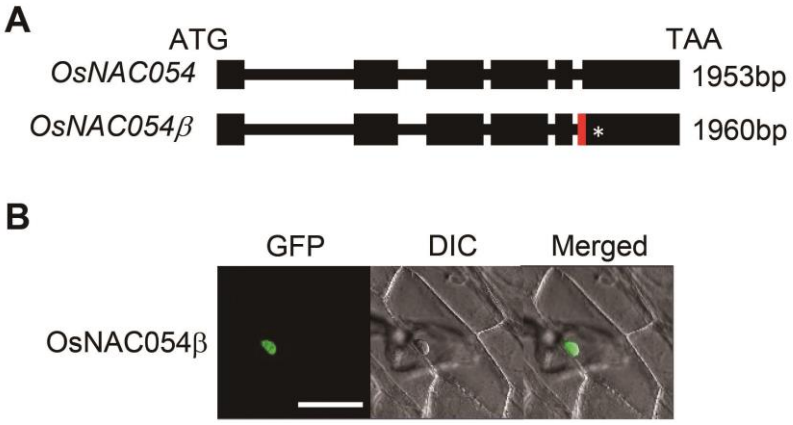

Fig. 15 OsNAC054 TF activity is controlled by alternative splicing.

(A) Gene structure of *OsNAC054* and *OsNAC054β*. Red box indicates the 7bp retention of sixth intron and white star shows premature stop codon of *OsNAC054β* (Splicing variant). (B) Subcellular localization of OsNAC054β proteins. GFP-OsNAC054β protein was transiently expressed in onion epidermal cells. GFP fluorescence was collected at 525 nm. Bars = 100 μm.

DISCUSSION

The initiation and progress of leaf senescence is controlled by a great number of TFs. Among TFs, NAC TFs are considered to have important role in regulating leaf senescence because the expression levels of many of Arabidopsis NAC TFs significantly increased during senescence [46]. Although several senescence associated NAC TFs have been identified [1, 13, 14, 16]. However, in rice only a few senescence associated NAC TFs have been reported [9, 17].

Here we identified new rice senescence associated NAC TF, *OsNAC054*. *osnac054-1* mutant showed stay-green phenotype during both dark induced senescence (Fig. 3) and natural senescence (Fig. 6), strongly indicating that *OsNAC054* is senescence promoting TFs. We also analyzed downstream cascade of *OsNAC054* dependent leaf senescence pathway by microarray and RT-qPCR analysis. We found several typical senescence associated genes, such as *OsORE1.1*, *OsNAP*, and *OsEIL1/OsEIN3* were significantly down-regulated in *osnac054-1* during DIS (Fig. 8). It was previously reported that in Arabidopsis *EIN3* binds to the promoters of *ORE1* and *NAP*, and directly promotes their expression [40, 47]. Thus, it is possible that *OsEIL1/OsEIN3* also directly activates *OsORE1* and *OsNAP* during senescence, and *OsNAC054* is involved in the activation of this *OsEIL1-OsORE1*/*OsNAP* cascade (Fig. 16).

In both Arabidopsis and rice, *NAP* is also known as an ABA signaling related gene. In Arabidopsis, *NAP* directly activates one of ABA responsive genes *SAG113*, *a protein phosphatase 2C* (*PP2C*; [48]). KO mutant of *sag113* showed delayed senescence phenotype as well as *nap* mutant [48], strongly indicating *SAG113* is one of the important downstream genes in leaf senescence pathway. Similar to Arabidopsis *NAP*, KO mutant of *OsNAP* showed delayed senescence phenotype [17]. In addition, *OsNAP* expression is significantly induced by ABA, while its expression is repressed in two ABA biosynthesis mutants, *aba1* and *aba2* [17]. Taken together with these results, both Arabidopsis *NAP* and *OsNAP* have important role in the leaf senescence through ABA signaling. In this study, we found that *OsNAC054* is also strongly induced by ABA treatment (Fig. 9). Furthermore, two ABA bZIP TFs, *OsABI5* and *OsABF2* were significantly down-regulated in *osnac054-1* mutant during DIS (Fig. 11). Indeed, *osnac054-1* mutant showed stay-green phenotype during ABA induced senescence (Fig. 12), suggesting that *OsNAC054* is involved in the promotion of leaf senescence through ABA, similar to *OsNAP* [17]. In Arabidopsis, *ABI5* is known as one of the senescence associated genes. *abi5* KO mutant showed stay-green phenotype, and *ABI5* directly activates the expression of *ORE1*, *SGR1*, and *NYC1* [47]. Thus, it is possible that *OsABI5* is important downstream genes in *OsNAC054* dependent leaf senescence pathway, as well as *OsNAP*.

In this study, we also found that three Chl degradation associated genes, *OsSGR*, *OsPAO*, and *OsNYC1* were significantly down-regulated in *osnac054-1* mutant during DIS (Fig. 8). One possibility is that the down-regulation of *OsNAP* in *osnac054-1* led to the repression of Chl degradation related genes, because *OsNAP* binds to the promoters of *OsSGR*, *OsNYC1*, and *OsPAO* for activation of these genes (Liang et al. 2014). It is also possible that the impairment of ABA signaling pathway in *osnac054-1* mutant led to the down-regulation of Chl degradation related genes. It is well known that Arabidopsis *SGR1* and *NYC1* transcriptional levels are tightly regulated by ABA signaling genes. Both *SGR1* and *NYC1* are directly activated by *ABI5* and *EEL* (*ABI5* functional homolog) during senescence [47]. Furthermore, during seed degreening *SGR1* and *NYC1* are also regulated by other ABA bZIP TFs *ABI3* and *ABF4*, respectively [49, 50]. Also, we can't exclude the possibility that *OsNAC054* directly activates these Chl degradation related genes. For demonstrating these hypotheses, in vitro and in vivo promoter binding assays, such as yeast one-hybrid assay and chromatin immunoprecipitation (ChIP) assay are necessary.

One unique characteristic of *OsNAC054* is TM region at its C-terminus (Fig. 14). Thus, *OsNAC054* is much longer than other senNACs in rice (Fig. 13). By transient expression assay using onion epidermal cells, we identified TM region of *OsNAC054*. Full-length OsNAC054-GFP was dispersed in the onion cells, while GFP fluorescence was target to only nucleus when TM

region was cleaved (Fig. 14C), indicating TM region of *OsNAC054* controls its TF activity in nucleus. In Arabidopsis, one of the Arabidopsis senNACs *NAC016* contains the TM region [1], although the relationship between TF activity of *NAC016* and its TM is still unknown. The study of *NTL4*, an Arabidopsis TM containing NAC TF, well showed the importance of TM region. Arabidopsis transgenic plants that overexpressing full-length NTL4 did not show phenotype, while transgenic plants that overexpressing NTL4△TM showed different phenotypes, like growth retardation and curled leaves even under normal growth condition [51], indicating the importance of TM region for conditional control of TF activity. Similar to the case of *NTL4*, TF activity of *OsNAC054* is conditionally controlled by its TM region. The preparation of transgenic plants of *35S:OsNAC054* and *35S:OsNAC054△TM* is needed for revealing the exact role of TM region of *OsNAC054*.

Another intriguing matter is the functional homolog of *OsNAC054* in Arabidopsis. In the phylogenic tree of Arabidopsis and rice NAC genes, *OsNAC054* is considerably close to other Arabidopsis senNACs such as *ORE1* and *JUB1*, and other abiotic stress associated NAC TFs, such as *NTL6*, *NAC019*, *NAC055*, and *NAC072* (Fig. 1). Arabidopsis *ore1* KO mutant showed stay-green phenotype (Kim et al. 2009), similar to *osnac054-1* mutant (Fig. 3 & Fig. 6). Unlike *OsNAC054*, however, *ORE1* did

not contain TM region (Kim et al. 2013). Furthermore, *ore1* KO mutant showed tolerance phenotype to high salinity stress [52], while *osnac054-1* mutant did not show tolerance phenotype under salt stress (data not shown). Considering these other characteristics, the function of *OsNAC054* is not so similar with *ORE1*. Although *JUB1* is one of the senescence associated NAC TFs, it is senescence regulating NAC TFs. Thus, *JUB1-OX* palnts showed stay-green phenotype while *jub1* mutant showed early senescence phenotype [16], which is completely opposite to *osnac054-1* mutant. *NTL6* contains TM at its C-terminus as well as *OsNAC054*, but its senescence phenotype was not observed [23]. *NAC019*, *NAC055*, and *NAC072* are also well known as ABA signaling related genes, and their overexpressing plants showed tolerance phenotype to drought stress and osmotic stress [53, 54]. However, it is still not clear whether they also have important role in the leaf senescence pathway. Further physiological analyses of KO mutants and OX plants of *OsNAC054* and Arabidopsis NAC genes will be needed for revealing this relationship.

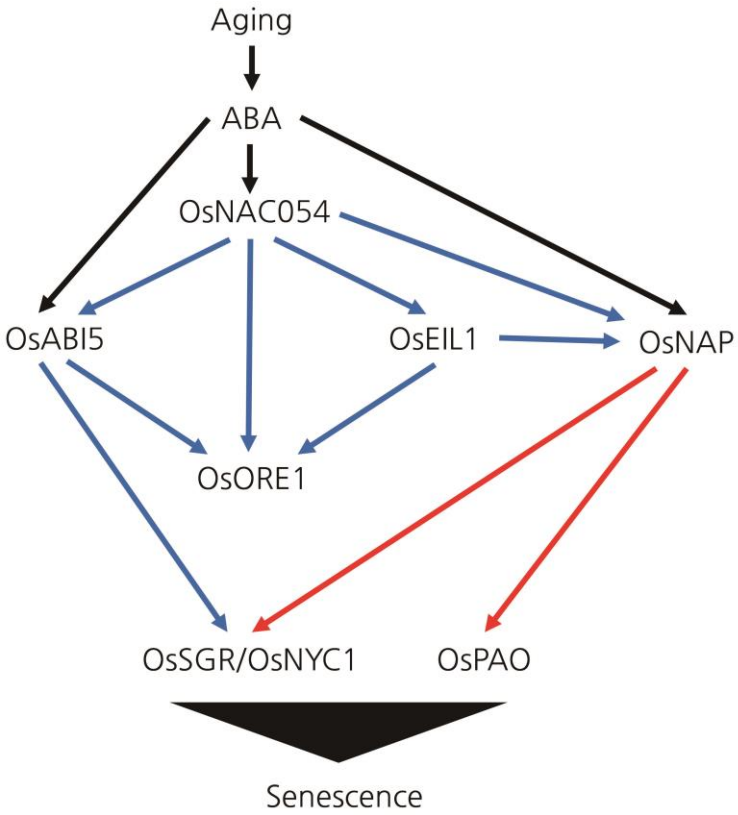

Fig. 16 Tentative model of *OsNAC054* dependent leaf senescence pathway. *OsNAC054* is involved in leaf senescence through the up-regulation of *OsABI5*, *OsORE1*, and *OsNAP* expression by crosstalk with ABA-related pathway. Red arrows indicate transcriptional regulation that previously reported. Blue arrows indicate transcriptional regulation that may be regulated by *OsNAC054.*

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초 록

벼의 생장발달의 마지막 단계인 잎의 노화가 지연되는, 즉 잎의 녹색과 광합성능이 비교군보다 높게 유지되는 T-DNA 삽입 돌연변이체인 *osnac054-1* 을 이용하여 잎의 노화에 관여하여 stay-green 형질을 조절하는 *OsNAC054* 유전자의 특성을 분석하였다. 비교군으로 사용된 동진벼의 노화가 진행될수록 *OsNAC054* 의 발현이 촉진되었고 돌연변이체가 비교군보다 잎의 노화가 지연되는 것으로 보아 *OsNAC054* 유전자가 잎의 노화에 관여한다는 것을 알 수 있었다. 전자조절인자인 *OsNAC054* 의 보다 정확한 역할을 규명하기 위해 암처리 전후의 식물체를 이용해 Microarray 를 실시한 결과 암 처리 후 *osnac054-1* 돌연변이체에서 엽록소 합성에 관여하는 *OsSGR, OsNYC1, OsPAO* 와 잎 노화에 관여하는 유전자인 *OsNAP, ORESARA1* (*ORE1*), *OsEIL1* 을 포함하고 있었다. 뿐만 아니라 *osnac054-1* 돌연변이체는 식물호르몬인 ABA 로 인한 노화 과정에서도 노화가 억제되었고, *OsNAC054* 의 발현이 ABA 를 처리했을 때 증가하는 것을 확인하였다. 이러한 결과들은 *OsNAC054* 가 엽록소 합성과 잎의 노화에 관여하는 유전자들의 발현을 ABA-신호 전달 회로와 상호작용하여 간접 혹은 직접적으로 조절함으로써 잎이 노화를 촉진한다는 것을 보여준다.