THESIS FOR DEGREE OF MASTER OF SCIENCE

Regulatory Role of *ONAC016* in the promotion of Leaf Senescence in Rice (*Oryza sativa L.*)

벼 잎 노화에 관여하는 전사인자 *ONAC016*의 조절 기작 규명

BY

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SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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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, *ONAC016.* We found that *ONAC016* expression level increased during senescence. *onac016* T-DNA KO mutants showed stay green phenotype during dark-induced and natural senescence condition, while *onac016-D* T-DNA overexpression mutants showed early senescence phenotype, suggesting *ONAC016* acts as senescence promoting NAC TF. Furthermore, we found that *ONAC016* expression is induced by ABA, and *onac016* mutants showed stay-green phenotype during ABA induced senescence. By microarray and qRT-PCR analysis, several Senescence-Associated Genes (*NOL, OsNAP)*, Chlorophyll Degradation Genes (*OsSGR, OsNYC1)*, and ABA signaling genes (*ABF1)* were down-regulated in *onac016* under Dark-Induced

Senescence condition. These results suggest that *ONAC016* plays an important role in promotion of leaf senescence through ABA-signaling pathway.

Keywords: rice, leaf senescence, NAC, Transcription factor, *ONAC016,* Knock out, overexpression, stay-green

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ABBREVIATION

NAC	NAM, ATAF1, 2 and CUC2
TF	Transcription Factor
senNAC	Senescence-associated NAC
ABA	Abscisic Acid
SGR	Senescence-Associated Gene
CDG	Chlorophyll Degradation Gene
WT	Wild Type
DIS	Dark-Induced Senescence
DT	Days after Treatment
DDI	Days of Dark Incubation
DAH	Days After Heading
DAT	Day after Transplanting

INTRODUCTION

Leaf senescence, the final stage of leaf development in plants, involves extensive degradation of macromolecules and dramatic changes in gene expression [1]. The initiation and progression of leaf senescence is regulated by endogenous factors including phytohormones, levels of some metabolites, and the state of photosynthesis [2-4], and by external factors, such as drought, high salinity, light, nutrient starvation and pathogen attack [5-8].

One of the best approaches for understanding leaf senescence mechanisms is the isolation and analysis of stay-green mutants, which show delayed leaf senescence. To date, a large number of stay-green mutants have been identified in model and crop plant species, and characterization of these mutants, as well as examination of transcriptional changes in senescence in the wild type (WT) have identified many senescence-associated genes [9-10]. Stay-green plants can be classified into two types: functional and non-functional (or cosmetic) stay-green plants. Functional stay-green plants retain both leaf greenness and photosynthetic competence significantly longer than the WT, while nonfunctional stay-green plants retain leaf greenness without delaying the progression of the other aspects of senescence [11]. Among these two types, some functional stay-green plants showed increases in crop yield, because they retain their photosynthetic capacity during the reproductive stage [12-14]. Thus, the identification and analysis of stay-green

plants may enable improvements in crop yield, particularly in cereal crops.

Large numbers of transcription factors (TFs) are involved in the regulation of leaf senescence in the model plant Arabidopsis and in crop species. Work in Arabidopsis has identified >40 senescence-associated TFs (senTFs) that increase in mRNA levels during senescence; these senTFs include the plant-specific NAM/ ATAF1/ ATAF2/ CUC2 (NAC) TFs [15].

NAC TFs is one of the largest plant-TF family and regulates various plant process including senescence in many species, such as soybean [16], wheat [17,18], rice [19, 20], and Arabidopsis [21, 22]. 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 [23, 24]. 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 VNDINTERACTING2 (ANAC083/VNI2) have been identified as negative regulator of leaf senescence; Overexpressing plants of JUB1 and VNI2 showed delayed senescence phenotype [25, 26]. 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 [25]. As described above, several NAC TFs regulate leaf senescence positively or negatively.

Considering that Arabidopsis has >40 senNACs, other crop plant species, such as rice and barley, also should have >10 senNAC TFs, but the study of senNAC TFs in other plant species remains considerably limited. The Oryza sativa genome contains 151 NAC genes (OsNACs; Nuruzzaman et al. 2010), but the functions of most OsNAC TFs are still unknown. Recent work identified and characterized a rice senNAC TF named OsNAP (Os03g0327800). The OsNAP-OX rice plants showed an accelerated leaf senescence phenotype, while OsNAP-RNAi (RNA interference) plants showed delayed leaf senescence [27, 28] , indicating that OsNAP functions as a senescence-promoting senTF, like Arabidopsis NAP [29]. Furthermore, OsNAP directly or indirectly controls the genes associated with ABA synthesis [30-32], indicating that OsNAP is a key regulator of ABA synthesis and signaling. Actually, the function of OsNAP is considerably similar to that of Arabidopsis NAP [33].

Although only one senTF (OsNAP) has been identified in rice, a few OsNAC TFs were considered to have potential functions as senNAC TFs. For example, previous work reported that the expression levels of OsNAC5 and OsNAC6 were upregulated during leaf senescence [34].

Here we identify and characterize the rice senNAC TF ONAC016. The expression of ONAC016 significantly increased during leaf senescence.

onac016 mutants showed stay-green during DIS and natural senescence condition. In microarray analysis, the expression of several SAGs, including *NOL, OsNAP, OsNYC1* and *OsSGR* was down-regulated in *onac016* mutants during DIS. Expression levels of *ONAC016* were up-regulated by ABA, indicating that ONAC016 modulated ABA-mediated leaf senescence pathway. Indeed, *onac016* leaves showed significant delayed senescence phenotype during ABA-mediated leaf senescence.

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MATERIALS AND METHODS

Plant materials and growth conditions

The T-DNA insertion knockdown mutant of *ONAC016* (PFG_3A-09456.L; *onac016*) and T-DNA insertion overexpression mutant of *ONAC016* (PFG_1B-15060.L; *onac016-D*) were obtained from Kyung-Hee University, Korea [35, 36 (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, 30°C). For DIS experiment, detached leaf discs from 4-weeks-old plants were incubated in complete darkness.

Chlorophyll Quantification

For total chlorophyll concentrations, pigments were extracted from leaf tissues with 80 % ice-cold acetone. Chlorophyll concentrations were determined by spectrophotometry as described previously [36].

Measurement of the *Fv/Fm* ratio

The *Fv/Fm* ratio was measured using the OS-30p+ instrument (Opti-Sciences). The middle part of each flag leaf of plants in natural paddy field were used and more than three experimental replicates per plant were conducted.

Measurement of Ion Leakage

Ion leakage was measured as described previously [37 (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 20min. The ion leakage is expressed as the percentage of initial conductivity divided by total conductivity.

Dark Incubation and ABA Treatment on Detached Leaves.

For dark incubation and ABA treatment, middle part of second leaves of 4-month-old plants in growth chamber. Detached leaves were floated on 3 mM MES (pH 5.8) buffer with the abaxial side up and incubated in complete darkness at 28 ℃. In ABA treatment experiment, detached leaves were floated on 3 mM MES buffer containing 5 μM ABA and incubated in continuous light condition at 30 ℃.

SDS-PAGE and immunoblot analysis

Protein extracts were prepared from leaf tissues. To extract total proteins, leaf tissues from 4 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, Lhcb1, PsaA, and PsaD) 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 [38] 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).

Microarray analysis

Total RNA was extracted from the leaves of 4-week-old soil-grown wild type plants and osnac016 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 42,000 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. 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).

Table1. Information of primers used in this study.

Marker	Forward primer (5'→3')	Reverse primer(5'→3')
A. Primers for verification of transgenic plants		
PFG 3A-09456.L	CTAGAGTCGAGAATTCAGTACA	ACCAACAGAAGCACTCCAGG
PFG 1B-15010.L	CTAGAGTCGAGAATTCAGTACA	ACGAACAGAAGCACTCCAGG
B. Primers used for gene cloning		
ONAC016	ATGGCGCAATGCGGCGGCGGCGACGTTTCACGACA	TTACATTTCCATTTTGAGATTGTCTAGC
C. Primers used for qRT-PCR		
ONAC016 qRT	CTGGAGATGGTCGTCAGTAACCAG	ACGGTGCATCAAATCCACTGAAC
NOL qRT	GTCGACATCCAGGCCAAGAC	TCTGCCTTGAGAGCGTCAGA
NAP qRT	GAAAAATAGTTCTCGCATAGGTAGTG	GGTCATTTTACAAGCAGCACAAC
SGR qRT	AGCTGCCTGGCACTAGGCTCTACAGATCAC	ATGTTGTCGGAGATGAGCTCGTCGGTGAGC
PAO qRT	CGTCGCCCACCTACAACCTC	TCGCCCAAATCGCAGCAT
Osl2 qRT	GAGTTGATCTTGGGAATGCTGC	CACTAGGTATCGTTCCGCTTATGTT
NYC1 qRT	TTCTCTCCAAGCTTCAGGCCCT	TGCACGTCTCCTGCAAAACCCT
UBQ5	TCCAAGCTCCAGTCCCTCCT	CGGTGCAGGCTCTTGATGTA

RESULTS

ONAC016 is senescence associated NAC TF

We examined the expression pattern of *ONAC016* in rice under various condition by RT-qPCR. First we checked the expression level of *ONAC016* during dark-induced senescence (DIS) condition. We incubated detached leaves of WT plants in complete darkness from 0 to 4 days. Its expression increased dramatically until 4 days of dark incubation (4 DDI; Fig. 1A). In naturally senescing rice leaf blades, we checked *ONAC016* expression levels in different regions of wild type. *ONAC016* showed high expression in the yellowing sector (Tip) compared with the green sectors (Middle and Bottom; Fig 1B). In the flag leaf tissues, mRNA levels of *ONAC016* increased gradually before heading, but it increased dramatically with the beginning of grain filling (Fig 1C). Taken these results together, *ONAC016* expression was increased during both DIS and natural senescence, suggesting that *ONAC016* might have a regulatory role in the promotion of leaf senescence.

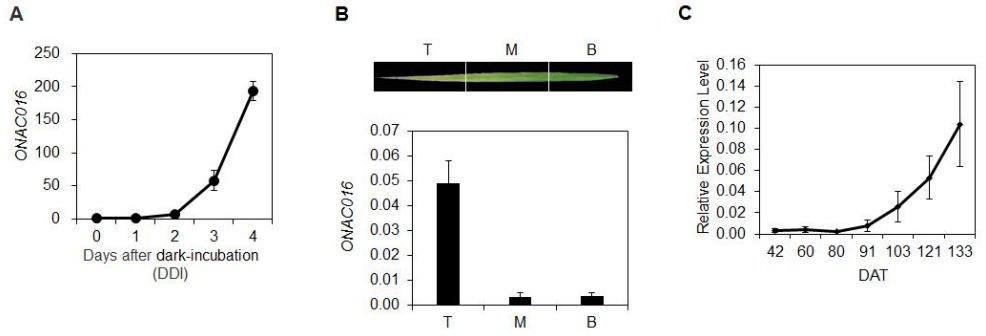

Figure 1. The expression pattern of *ONAC016* during senescence.

The expression level of ONAC016 during dark induced senescence (A), in natural senescing leaf (B), and during natural paddy field (C) were determined by normalizing the expression level of UBQ5. Mean and SD were obtained from more than three biological replicates.

ONAC016 T-DNA insertion knockout mutant shows delayed senescence phenotype during DIS

To identify the function of *ONAC016* in the leaf senescence, we used two different T-DNA insertion mutants, PFG_1B-15010 and PFG_3A-09456, selected as homo lines. *ONAC016* consists of two exons and one intron. Each of the T-DNA fragments was integrated in the 2nd exon and promoter region (Fig. 2A). By semi quantitative RT-PCR and RT qPCR, we confirmed that *ONAC016* transcripts did not accumulate in knockout lines, indicating that this T-DNA insertion line is *onac016* knockout (KO) mutant allele (hereafter termed *onac016).* While T-DNA insertion-mediated enhancer-tagged mutants of *ONAC016* (hereafter termed *onac016-D)* accumulated *ONAC016* transcripts much higher than parental japonica cultivar, Dongjin (hereafter termed wild type, WT; Fig. 2B and 2C).

At first, we checked leaf phenotype of *onac016* during DIS. At vegetative stage, leaf color of *onac016* is same as WT (Fig. 3A, 0 DDI). At 3 DDI and 4 DDI, however, *onac016* exhibited strong stay-green phenotype while WT became completely yellow (Fig. 3A). Consistent with visible phenotype, photosystem proteins, including Photosystem Ⅰ complex subunit (Lhca1 and PsaA) and Photosystem Ⅱ complex subunits (Lhcb1 and PsbD), highly remained in *onac016* (Fig. 3B). In addition, *onac016* showed relatively high Fv/Fm ratio, which indicates the efficiency of photosynthesis (efficiency of PS

Ⅱ) than WT. Chlorophyll content of *onac016* retained higher than that of WT (Fig. 3D). We also found that ion leakage rate, an indicator of membrane disintegration, was considerably lower in *onac016* compared with WT during DIS (Fig. 3E).

Subsequently, we compared chloroplast structure of the WT and *onac016* before and after 4 days of dark incubation (4 DDI). Before dark treatment, WT and *onac016* leaves have similar chloroplast structure (Fig. 4, upper panels). After 4 days of dark treatment, the grana stacking of *onac016* leaves retained, while that of WT leaves was degraded (Fig. 4, lower panels).

Taken these results together, mutation of *ONAC016* 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.

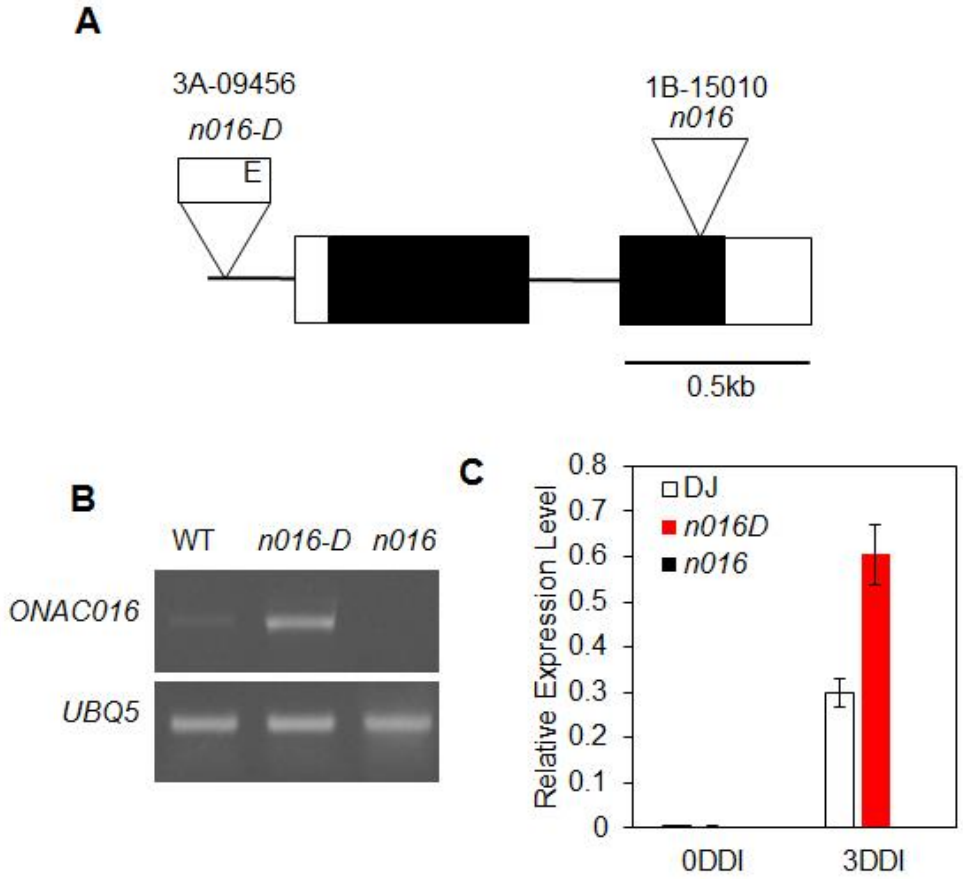

Figure 2. Characterization of *onac016* T-DNA insertion KO mutant and *onac016-D* dominant mutant

Gene structure of ONAC016 and T-DNA insertion point (inverted triangle) in promoter region (PFG_3A-09456) and 2nd exon (PFG_1B-15010). Exons are shown as black boxes and introns are shown as connecting lines (A). The dominant and null mutant were confirmed by semi RT-PCR (B) and RT-qPCR (C).

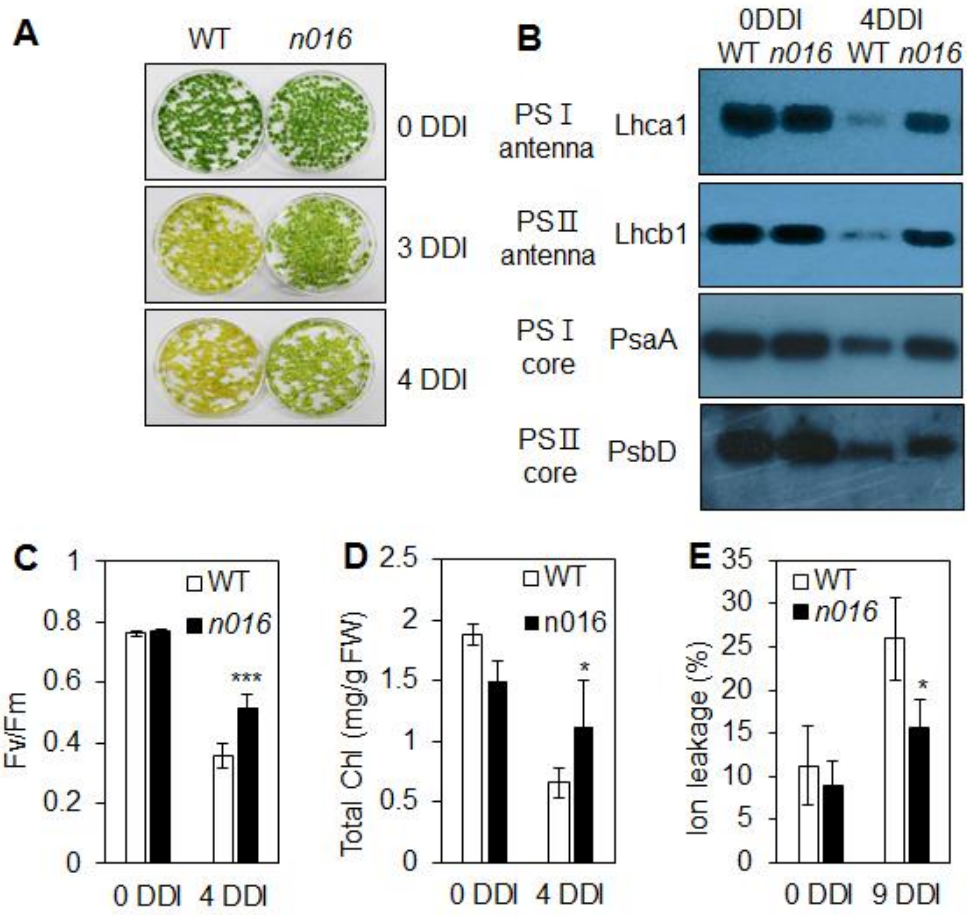

Figure 3. Characterization of stay-green phenotype of *onac016* under DIS condition

The phenotype of detached leaves of WT and onac016 during dark-induced senescence (A). Immunoblot analysis of photosystem proteins in detached leaves of 4-weeks-old WT and onac016 plants at 0, 4 DDI (B). Changes in the level of *Fv/Fm* ratio (C), total chlorophyll contents (D), and ion leakage rate (E).

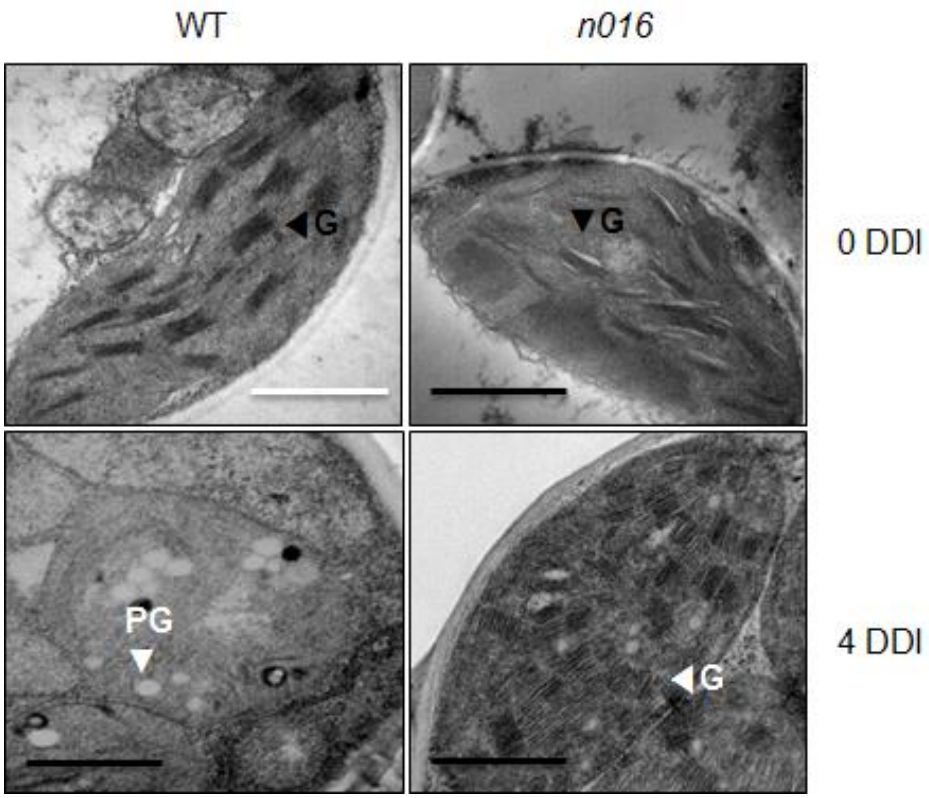

Figure 4. Transmission electron microscopy of WT and onac016 leaves under DIS conditions.

Morphological changes in the chloroplasts and structure of grana thylakoids of WT and onac016 leaves before and after 4 DDI. G, Grana; PG, plastoglobule. Bars = 200nm.

onac016 is Insensitive to Exogenous Treatment of ABA

Leaf senescence proceeds with age, but the process is intricate and comprehensive. It is modulated by diverse external and internal factors including abiotic stress, biotic stress, and phytohormones [39 (4[]. Especially, phytohormones such as ABA, MeJA, and SA promote leaf senescence. To determined *ONAC016* expression is modulated by phytohormones, second leaves from 4-weeks-old WT plants were exogenously treated by several phytohormones, such as Abcisic acid (ABA), Methyl Jasmonate (MeJA), 1-aminocyclo-propane-1-carboxylic acid (ACC, one of the ethylene intermediates), Gibberellic Acid (GA), Indole-3-acetic acid (IAA, one of the auxin class), and Kinetin. After 12 h of treatments, *ONAC016* expression was significantly increased up to 5-fold in ABA-treated WT (Fig. 5A). When treated with ABA, the detached leaves of *onac016* maintained green color, while that of WT was absolutely changed to yellow (Fig 5B). The chlorophyll content of *onac016* leaves treated with ABA was also highly retained than WT.

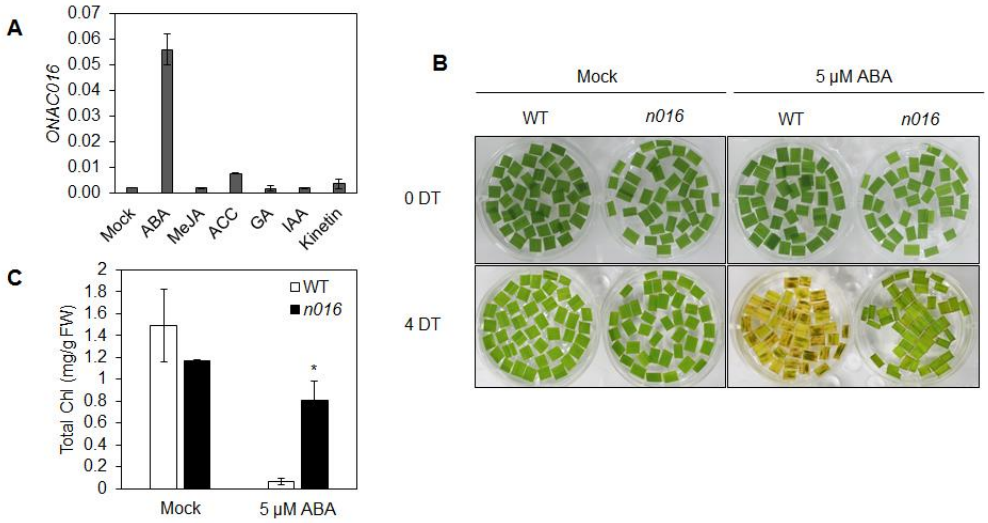

Figure 5. Hyposensitivity phenotype of onac016 to exogenous treatment of ABA.

Expression of ONAC016 under exogenous phytohormone treatments (A). Phenotype (B) and total chlorophyll levels (C) of WT and onac016 plants before (0 DT) and after 4 days of ABA treatment (4 DT).

ONAC016 regulates expression of several SAGs, CDGs and ABA signaling gene

To examine downstream target genes and the regulatory network of *ONAC016*-mediated leaf senescence, we conducted a genome-wide microarray analysis to identify differentially expressed genes (DEGs) between the WT and *onac016.* The detached leaves of 4-weeks-old plants were incubated in the dark for 3 days (3 DDI), and their leaves were sampled for microarray analysis (Fig. 6A). From analysis of the microarray data, we identified 711 and 452 genes that were significantly upregulated (*onac016*/WT; >2-fold) and 417 and 240 genes that were down-regulated (*onac016*/WT; <2-fold) at 0 and 3 DDI, respectively (Fig. 6B and C).

In the microarray data set, we first examined whether known SAGs are differentially expressed in *onac016* compared with the WT (*onac016*/WT). We found that SAGs, *NOL* and *OsNAP,* and CDGs, *OsSGR, OsNYC1* were down-regulated in *onac016* leaves at 3 DDI (Fig. 6D). We next examined whether phytohormone-associated genes are differentially expressed in *onac016* compared with the WT. We found that ABA signaling gene, ABF1 were down-regulated in onac016 leaves at 3 DDI (Fig. 6D).

To confirm the result of microarray analysis, we checked the expression of several SAGs and CDGs during DIS condition by qRT-PCR analysis. In addition to SAGs described above (NOL, OsNAP, OsSGR, OsNYC1), we checked expression of OsPAO and Osl2. At 0 DDI, expression levels of NOL,

OsNAP, OsSGR, OsPAO, Osl2, and OsNYC1 in onac016 was almost the same as those in WT, while these gene expression was significantly down-regulated in onac016 at 3DDI (Fig. 7). These results suggest that ONAC016 is involved in the leaf senescence by directly or indirectly regulating typical SAGs.

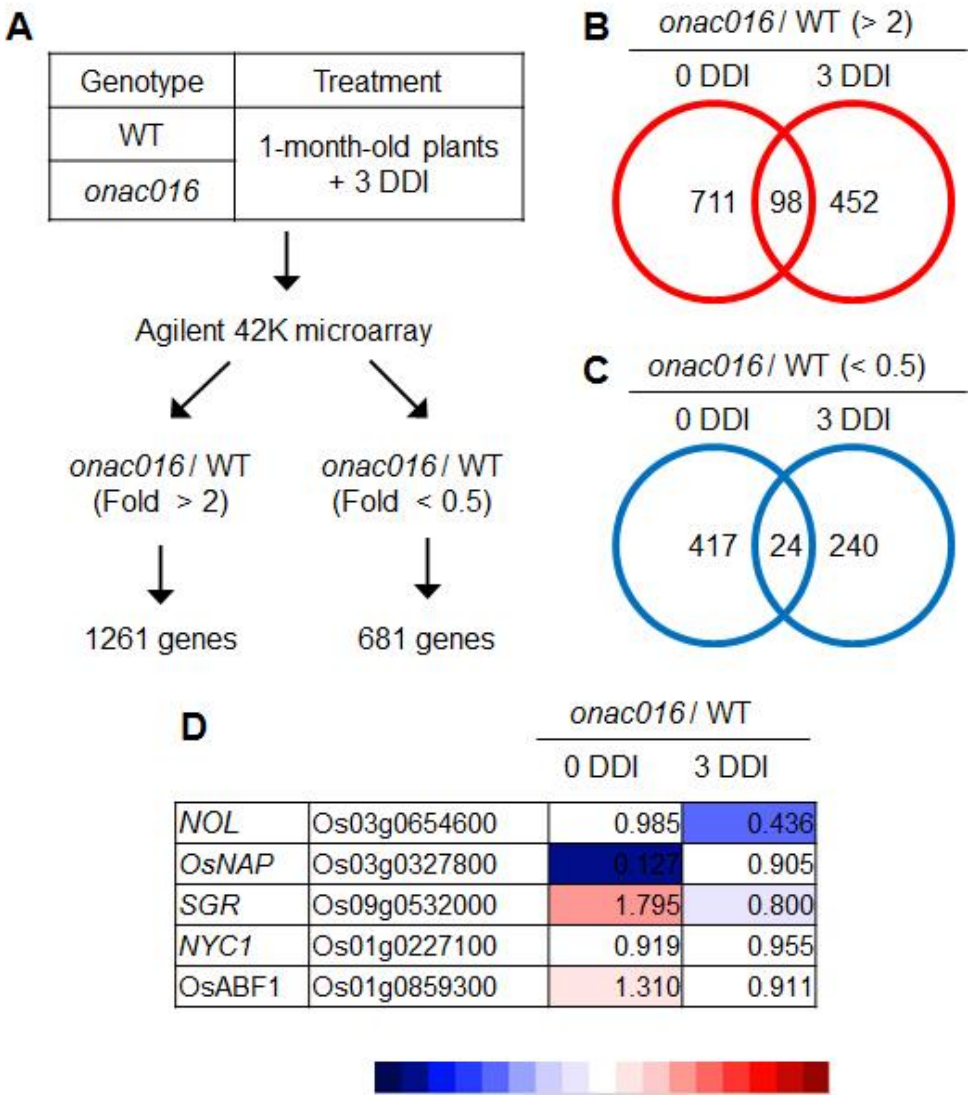

Figure 6. Summary of microarray analysis between WT and onac016 at 0 DDI and 3 DDI

Hierarchical linkage map of the gene that are differently expressed in WT and onac016 (A). Ven diagram representing the number of genes up-regulated (B) and down-regulated (C) at 0 DDI and 3 DDI in WT and onac016. The ratios of expression levels (onac016/WT) for known SAGs at 0 and 3 DDI are illustrated (D).

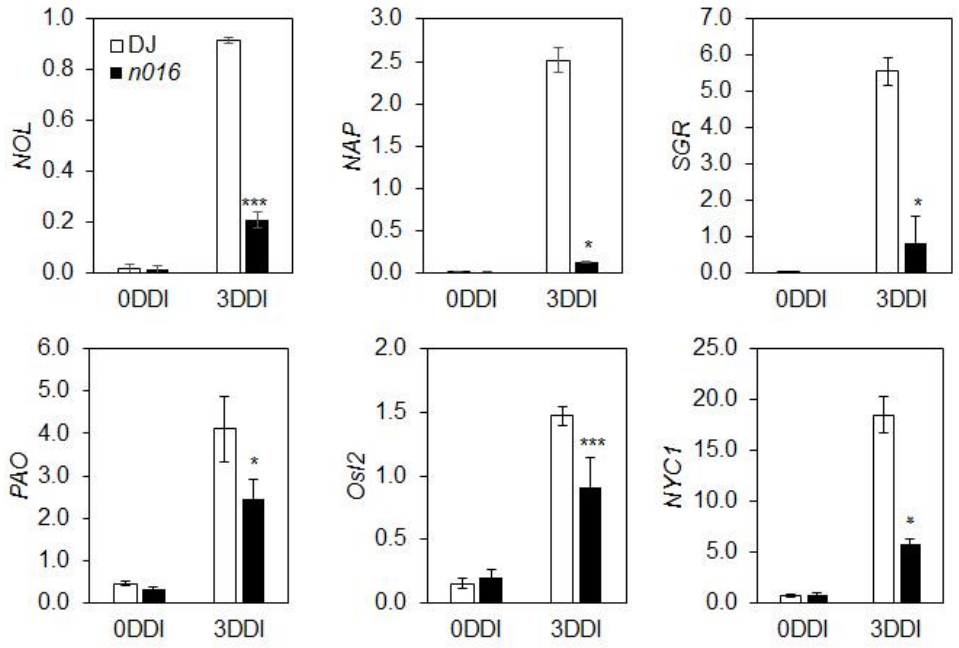

Figure 7. Altered expression of SAGs and CDGs in onac016 under DIS

Total RNA was extracted from second leaves of 4-week-old WT and onac016 plants before and after dark incubation. Relative expression levels of NOL, OsNAP, OsSGR, OsPAO, Osl2, OsNYC1 were determined by RT-qPCR and normalized to the transcript levels of UBQ5.

The *onac016* mutants exhibit a non-functional stay-green phenotype during natural senescence

We examined the senescence phenotype of onac016 during natural senescence. To this end, plants were grown in the paddy field under natural long-day (LD) conditions (~14 h light per day at 37 N latitude, Suwon, South Korea). Throughout the vegetative stage, the growth rate and size of onac016 and WT plants were almost the same (Fig. 9A, left panel) and they flowered at almost the same time (Fig. 9B). During grain filling, however, onac016 exhibited a delayed-senescence phenotype (Fig. 9A, right panel). Consistent with the delayed-senescence phenotype, onac016 plants retained total Chl levels during senescence, unlike the WT, which showed the expected decrease in Chl (Fig. 9D). To examine the efficiency of photosynthesis, we measured the Fv/ Fm ratio (efficiency of PSII) after heading. However, the Fv/Fm ratio in both the WT and onac016 drastically decreased after 40 days after heading (40 DAH). These results indicate that onac016 is a non-functional stay-green plant.

Previous work reported that functional stay-green crops can potentially increase yield by prolonging photosynthetic activity during grain filling [39]. Thus, we examined several agronomic traits in onac016 plants at 180 days after sowing (DAS). We found that the panicles were shorter in onac016 than that in the WT (Fig. 10B). Moreover, the number of panicles per plant was smaller than WT (Fig. 10C). Spikelet fertility (Fig. 10G) and 500-grain weight

(Fig. 10F) were lower in onac016 plants than in the WT. Overall, grain yield per plant was greater in WT than in onac016 (Fig. 10H), indicating that onac016 is the non-functional stay green plant.

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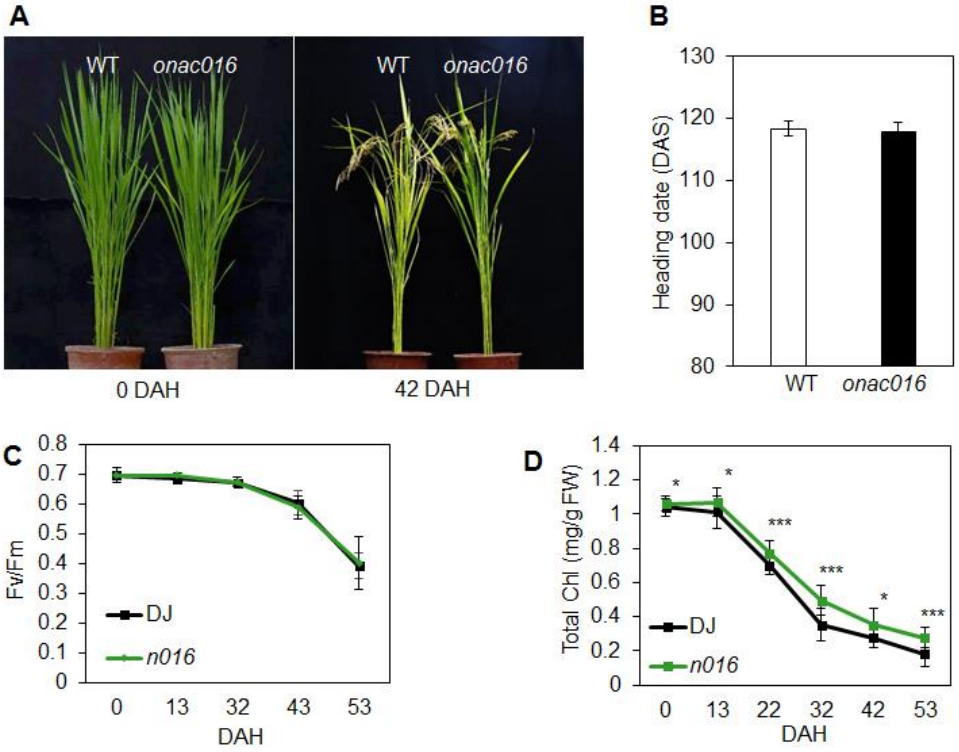

Figure 8. Characterization of stay-green phenotype of onac016 in natural paddy field condition

Phenotype of WT and onac016 plants at 0 DAH and 42 DAH (A). The heading date of WT and onac016 (B). Changes in the level of Fv/Fm ratio (C) and total chlorophyll contents (D)

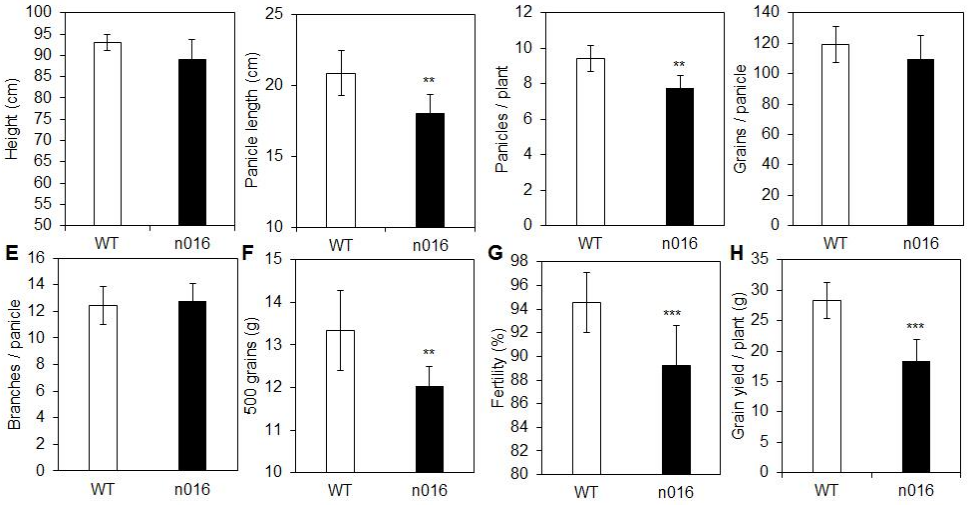

Figure 9. Agronomic traits of onac016

(A-H) Eight agronomic traits were examined at harvest: plant height (A), panicle length (B), number of panicles per plant (C), number of grains per panicle (D), number of branches per panicle (E), 500-grain weight (F), spikelet fertility (G), and yield per plant (H) in WT and onac016 mutants.

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 [38]. 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, ONAC016. onac016 mutant showed stay-green phenotype during both dark induced senescence (Fig. 3) and natural senescence (Fig. 8), strongly indicating that ONAC016 is senescence promoting TFs. We also analyzed downstream cascade of ONAC016 dependent leaf senescence pathway by microarray and RT-qPCR analysis. We found several typical senescence associated genes, such as NOL, OsNAP, OsSGR, OsNYC1 were significantly down-regulated in onac016 during DIS (Fig. 6 and 7).

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 [39], 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. 5). Indeed, onac016 mutant showed stay-green phenotype during ABA induced senescence (Fig. 5), suggesting that ONAC016 is involved in the promotion of leaf senescence through ABA, similar to OsNAP [17].

In this study, we also found that three Chl degradation associated genes, OsSGR, and OsNYC1 were significantly down-regulated in onac016 mutant during DIS (Fig. 6 and 7). 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 and OsNYC1 for activation of these genes (Liang et al. 2014). It is also possible that the impairment of ABA signaling pathway in onac016 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 [40].

Also, we cannot exclude the possibility that ONAC016 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.

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초 록

이 연구는 벼의 생장 발달 과정에서 야생형보다 잎의 노화가 지연되는 *onac016* T-DNA 삽입 돌연변이체를 이용하여 잎의 노화에 관여해 stay-green 형질을 조절하는 *ONAC016* 유전자의 기능을 밝힌 연구이다. 야생형으로 사용된 동진벼의 노화가 진행될수록 잎에서 *ONAC016* 의 발현이 증가되었고, Knock-out 돌연변이체는 야생형에 비해 노화가 지연되고 과발현 돌연변이체는 야생형에 비해 노화가 촉진되는 것을 보아 *ONAC016* 유전자가 잎의 노화에 관여한다는 것을 알 수 있었다. 또한 다양한 식물호르몬을 처리한 후 *ONAC016* 의 발현을 확인한 결과, ABA 를 처리한 잎에서 발현이 증가하였고, ABA 에 의한 노화과정에서 *onac016* 돌연변이체가 야생형에 비해 노화가 억제되었다. 전사 인자인 *ONAC016* 의 보다 정확한 역할을 규명하기 위해 암 처리 후 microarray 를 실시한 결과 야생형에 비해 *onac016* 돌연변이체에서 잎 노화에 관여하는 유전자 (SAG, Senescence-Associated Gene) 인 *OsNAP, OsNOL* 과 엽록소 분해에 관여하는 유전자 (CDG, Chlorophyll Degradation Gene) 인 *OsSGR, OsNYC1* 그리고 ABA 신호전달에 관여하는 유전자인 *OsABF1* 의 발현이 down-regulation 되는 것을 확인하였다. 또한 qRT-PCR 을 통해 microarray 에서는 발현의 차이가 없는 다른 노화관련 유전자들의 발현을 확인하여 하위 유전자들을 예측하였다. 이러한 결과들은 *ONAC016* 이 잎의 노화에 있어 노화관련 유전자들을 직접적 혹은 간접적으로 조절할 뿐만 아니라, 식물호르몬 신호전달에도 관여하여 노화를 촉진한다는 것을 보여준다. 암 처리 상태뿐만 아니라 자연 장일 조건 하에서도 *onac016* 돌연변이체가 야생형에 비해 잎의 노화가 지연되는 표현형을 보였다. 엽록소는 분해되지 않고 유지되는 반면 *Fv/Fm ratio* 가 야생형과 차이가 없음을 통해 non-functional stay-green 임을 확인하였다. 기능성 녹색 유지 형질은 작물의 생산량 증대와 관련이 있다는 이전 연구가 있는데, *onac016* 돌연변이체는 자연 장일 조건 하에서 야생형에 비해 키, panicle 당 낱알 수와 가지 수는 차이가 없지만, panicle 길이, 식물체 당

panicle 수, 500 립중, 생식율이 감소하고 그 결과 생산량이 감소하는 것을 보았다. 이는 *ONAC016* 전사인자는 상위 유전자이며, 노화 관련된 유전자들을 조절할 뿐만 아니라 농업적 형질과 관련된 유전자들을 조절하기 때문이라 추측하였다. 실제로 microarray 분석을 통해 panicle 발달, 꽃 기관 발달 관련 유전자들인 *EP2, API5, MOC1, SP1* 의 발현이 *onac016* 돌연변이체에서 변화가 있음을 확인하였다.