THESIS FOR DEGREE OF MASTER OF SCIENCE

Regulatory Roles of *DNA-binding One Zinc Finger 24* (*OsDof24*) in Leaf Senescence in Rice (*Oryza sativa*)

벼 잎 노화에 관여하는 *OsDof24*의 조절 기작 규명

BY

YEJIN SHIM

FEBRUARY, 2018

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

Regulatory Roles of *DNA-binding One Zinc Finger 24* (*OsDof24*) in Leaf Senescence in Rice (*Oryza sativa*)

UNDER THE DIRECTION OF DR. NAM-CHON PAEK
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

BY
YEJIN SHIM

MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY
DEPARTMENT OF PLANT SCIENCE

FEBRUARY, 2018

APPROVED AS A QUALIFIED DISSERTATION OF YEJIN SHIM
FOR THE DEGREE OF MASTER
BY THE COMMITTEE MEMBERS

FEBRUARY, 2018

CHAIRMAN

Hee-Jong Koh, Ph.D.

VICE-CHAIRMAN

Nam-Chon Paek, Ph.D.

MEMBER

Hak Soo Seo, Ph.D.

Regulatory Roles of *DNA-binding One Zinc Finger 24* (*OsDof24*) in Leaf Senescence in Rice (*Oryza sativa*)

YEJIN SHIM

ABSTRACT

Leaf senescence is a final stage of leaf development, which largely affects agronomic traits in cereal production. Although many genetic factors have been reported, it is still necessary to determine the key regulators that delay leaf senescence during grain filling in staple food crops including rice. Here we show that *OsDOF24*, one of DOF (DNA-binding One zinc Finger) transcription factor family, acts as a repressor of leaf senescence in rice. The T-DNA insertion-mediated enhancer-tagged overexpression of *OsDOF24* (*osdof24-D*) in rice exhibits a stay-green phenotype during both age-dependent natural and dark-incubated senescence. The stay-green phenotype was further confirmed with the detached leaves of transgenic rice overexpressing *OsDOF24* by *35S* CaMV promoter. To elucidate the molecular mechanism of leaf senescence in *osdof24-D* mutants, we performed RT-qPCR analysis, revealing that senescence-associated genes (SAGs), *Osl85* and *Osl57*, and chlorophyll degradation genes (CDGs), *OsNYC1*, *OsNYC3*, and *OsSGR*, are downregulated in *osdof24-D* mutants during dark incubation.

In addition, the detached leaves of *osdof24-D* mutants were less sensitive to MeJA (methyl-jasmonate)-induced senescence. Consistent with this stay-green phenotype, expression levels of MeJA-responsive genes, *OsJAmyb* and *OsCOI1a*, decreased in *osdof24-D* mutants. Taken together, our results demonstrate that *OsDOF24* suppresses the induction of leaf senescence during vegetative growth by downregulating MeJA-responsive signaling.

Keywords: rice, *DNA-binding One Zinc Finger 24* (*OsDof24)*, transcription factor, leaf senescence, overexpression, stay-green

Student number: 2016-21355

CONTENTS

LIST OF TABLES AND FIGURES

ABBREVIATION

ABA	Abscisic acid
CDG	Chlorophyll degradation gene
Chl	Chlorophyll
Dof	DNA binding one zinc finger
DIS	Dark Induced Senescence
DDI	Days after Dark Incubation
JA	Jasmonate
MeJA	Methyl jasmonate
PS	Photosystem
SA	Salicylic acid
SAG	Senescence-associated gene
WT	Wild type

INTRODUCTION

As the final stage of leaf development, leaf senescence is a pivotal factor to increase crop yield, which is largely controlled by each species-specific genetic program. While onset of leaf senescence is generally initiated by developmental age, the progress of leaf senescence is largely affected by various environmental cues including phytohormone, extreme temperature, water deficit, pathogen infection, nutrient deficiency, and deep shading [1]. A number of senescence-associated genes (SAGs) have been identified and their expression is up-regulated during natural and dark-induced leaf senescence [2, 3]. Especially, transcription factors that is responsive to senescence play key roles in regulating the senescence processes. For example, expression of Arabidopsis *AtNAP* and its rice ortholog, *OsNAP* correlates with leaf senescence. Null mutation of *AtNAP* and *OsNAP* shows a delayed leaf senescence phenotype in Arabidopsis and rice, respectively [5, 6]. Moreover, prolonged grain-filling period due to deficiency of *OsNAP* contributes to improved grain yield in rice [6]. Overexpression of *AtORE1* and its paralog *AtORS1* causes precocious leaf senescence [7,8]. *AtJUB1, AtVNI2* and *OsNAC106* act as negative regulator for leaf senescence of which overexpression exhibits delayed senescence phenotype [9-11]. *AtWRKY22*, whose expression was markedly induced by H2O2, positively regulates leaf senescence in arabidopsis [12]. Plants with overexpression in *OsWRKY42*

that binds to promoter of *OsMT1d*,that encodes non-enzymatic ROS scavenger, show early senescence phenotype [13].

As the internal factor of senescence regulator, jasmonate (JA) and its derivatives play important roles in regulating leaf senescence. Exogenous application of JA to attached and detached leaves promotes leaf senescence in Arabidopsis, suggesting that JA serves as a powerful senescence inducer [14]. Therefore, null mutation of *OsCOI1b*, that encodes a JA receptor and functions in JA signaling gene, exhibits stay-green phenotype during dark-induced and natural senescence conditions [15]. Overexpression of *OsNAP* elevates JA contents, that is coincide with increase of JA biosynthetic genes including *LOX* and *AOC*, resulting in early senescence [16]. Plant-specific DOF transcription factors contain the DNA-binding domain usually located close to the N-terminal region of the protein [17, 18]. DOF domains that is conserved region of 52 amino acid recognize an AAAG motif or the reverse complement CTTT [18]. DOF transcription factors have important functions in many physiological processes such as stress response [19], flowering time [20, 21], hypocotyl elongation [22], seed germination [23, 24] and photosynthesis [25, 26]. Although rice genome has 31 DOF TFs [27], regulatory mechanisms in a few genes have been elucidated in rice. For example, heterologous expression of *OsDof25* in arabidopsis have revealed that *OsDof25* affects carbon and nitrogen metabolism with simultaneous expression of relevant genes encoding ammonium transporters (*AtAMT1.1* and *AtAMT2.1*) and nitrate transporter (*AtNRT2.1*) [28]. However, the

mechanism of leaf senescence in DOF TFs has never been identified.

In this study, we found that in rice, transcript levels of *OsDOF24* decreased in both natural and dark-incubated senescence conditions. Overexpression of *OsDOF24* generated by enhancer tagging (*osdof24-D*) and by *35S:OsDOF24* transformation (OsDOF24-OE) delayed leaf senescence compared to wild type. *OsDOF24* negatively regulated the expression of CDGs and SAGs that were closely associated with leaf senescence. Furthermore, detached leaves were almost insensitive to exogenous application of MeJA, and the expression of JA responsive genes such as *OsCOI1a* and *OsJAmyb* were downregulated by *OsDOF24* during dark-incubated leaf senescence. Therefore, these findings suggest that *OsDOF24* plays important roles in regulating leaf senescence though MeJA signaling pathway.

MATERIALS AND METHODS

Plant materials and growth conditions

The T-DNA insertion osdof24-D (PFG_3A-00724.R) mutants were obtained from Crop Biotech Institute at Kyung Hee University, Korea [29]. The WT japonica cultivar “Dongjin”, and osdof24-D mutants were grown in paddy field under natural long days (>14 h light/dark) in Suwon, South Korea (37N latitude).

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. [40]

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 paddy field were used and more than three experimental replicates per plant were conducted.

Dark Incubation and MeJA Treatment on Detached Leaves

For dark incubation and MeJA treatment, middle part of second leaves of 4-month-old plants. For dark incubation and MeJA treatment, middle

part of second leaves of 4-month-old plants grown in paddy field were used. Detached leaves were floated on 3 mM MES (pH 5.8) buffer with the abaxial side up and incubated in complete darkness at 30 ℃. In MeJA treatment experiment, detached leaves were floated on 3 mM MES buffer containing 100 μM MeJA and incubated in continuous light condition at 30 ℃. For both experiments, detached leaves floating on 3 mM MES under continuous light were used as control.

Transmission Electron Microscopy

To perform transmission electron microscopy, a previously described method [41] was used, with some modifications. The middle part of the second leaves of fully expanded plants were used for this experiment. Small leaf pieces were fixed with modified Karnovsky fixative (2 % paraformaldehyde, 2 % glutaraldehyde, and 50 mM sodium cacodylate buffer, pH 7.2). After fixation, samples were washed with 0.05 M sodium cacodylate buffer, pH 7.2 at 4 ℃, three times for 10 min each. The samples were post-fixed with 1 % osmium tetroxide in 50 mM sodium cacodylate buffer, pH 7.2, at 4 ℃ for 2 h and washed twice with distilled water at room temperature. Samples were stained en bloc in 0.5 % uranyl acetate at 4 ℃ overnight and dehydrated in an ethanol gradient solution with propylene oxide, then infiltrate

with Spurr's resin. Samples were polymerized at 70 ℃ for 24 h and sectioned with an Ultramicrotome (MT-X). The sections were mounted on copper grids and stained with 2 % uranyl acetate for 7 min and with Reynolds' lead citrate for 7 min. Micrographs were made using a LIBRA 120 transmission electron microscope.

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR) Analysis

Total RNA was extracted from rice leaf tissues with the MG Total RNA Extraction Kit (Macrogen, Korea). First-strand cDNA was synthesized with 2 μg of total RNA in a 25 μl volume using M-MLV reverse transcriptase and oligo(dT)15 primer (Promega), and diluted with 75 μl water. The 20 μl of qPCR mixture was prepared including 2 μl of the first-strand cDNA mixture, 10 ul of 2X GoTaq PCR Mix (Roche), and 1 μl of 10pM qRT primer pairs. qPCR was conducted on the LightCycler 2.0 instrument (Roche Diagnostics). The qPCR conditions were 95 ℃ for 2 min, followed by 50 cycles at 95 ℃ for 5 s, 59 ℃ for 15 s, and 72 ℃ for 10 s. OsUBQ5 (Os01g0328400) was used as an internal control. Primers used for qRT-PCR analysis are listed in

Supplemental Table 1.

Plasmid Construction and Transformation

A full-length cDNA of OsDOF24 was ligated into the pMDC32 gateway binary vector containing the 35S promoter and then 35S:OsDOF24 were introduced into calli generated from the mature embryos of Dongjin seeds by Agrobacterium (strain EHA105)-mediated transformation method [42]. Agrobacterium-infected calli were transferred to solid media containing cytokinin and auxin and plantlets were regenerated from the calli grown under continuous light condition. Transgenic plants overexpressing OsDOF24 were confirmed by qRT-PCR using the specific primers listed in Supplemental Table 1.

Statistical Analysis

Data in figures are expressed as the mean ± standard errors (S.E.) of more than three independent biological replicates. The data were statistically analyzed by Student’s t-test.

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-00724.L	TGGCTGCTCTGGTCCCTC	ATCCAGTGAAATCCAGGCAG
B. Primers used for gene cloning		
OsDof24	ATGCAGGAGCAGCAGCCGGAGAC CGG	TCATGGGAGGTTGAGGAACACGGC GGTC
C. Primers used for qRT-PCR		
OsDof24	GTTCACGGACCTCCTGCAGCC	CGGCCACTCGAAGTGCAGGTC
OsCoi1a	GAT GCC CTC CCT GAG ATA CA	AGT CAG ACC TCC TTC CAG CA
OsJAmyb	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT
Osl85	GAGCAACGGCGTGGAGA	GCGGCGGTAGAGGAGATG
Osl57	ACCCTAAAGTAAATGAAGTC	CCTGCTCTTGTCTTGTTA
OsNAP	CAAGAAGCCGAACGGTTC	GTTAGAGTGGAGCAGCAT
OsNYC1	CATGCAACACCAACAAAAGG	GACCATTCCAGGAGAAGCAG
OsNYC3	TGTCGTTGCCATGTGAAGAT	TTGGTCACGCCACAAATCTA
OsSGR	AGGGGTGGTACAACAAGCTG	GCTCCTTGCGGAAGATGTAG
OsUBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

RESULTS

Expression of OsDOF24 Decreases during Leaf Senescence

First we examined the expression pattern of OsDOF24 in rice under various growth conditions by RT-qPCR. The OsDOF24 transcripts gradually decreased after the transition from vegetative stage to reproductive stage (Figure 1A). In the flag leaf tissues, mRNA levels of OsDOF24 were higher in the green sector (bottom) rather than in the yellow sector (tip) during natural senescence (Figure 1B). Similarly, drastic decrease of OsDOF24 expression was observed in the detached leaves during dark-induced senescence (Figure 1C). It suggests that OsDOF24 is down-regulated by the onset of leaf senescence in rice. OsDOF24 transcripts were found in all the vegetative organs in rice, although its level was the highest in root tissues (Figure 1D). Taking these results together, we alternately speculated that OsDOF24 might have a regulatory role in repressing leaf yellowing during vegetative growth.

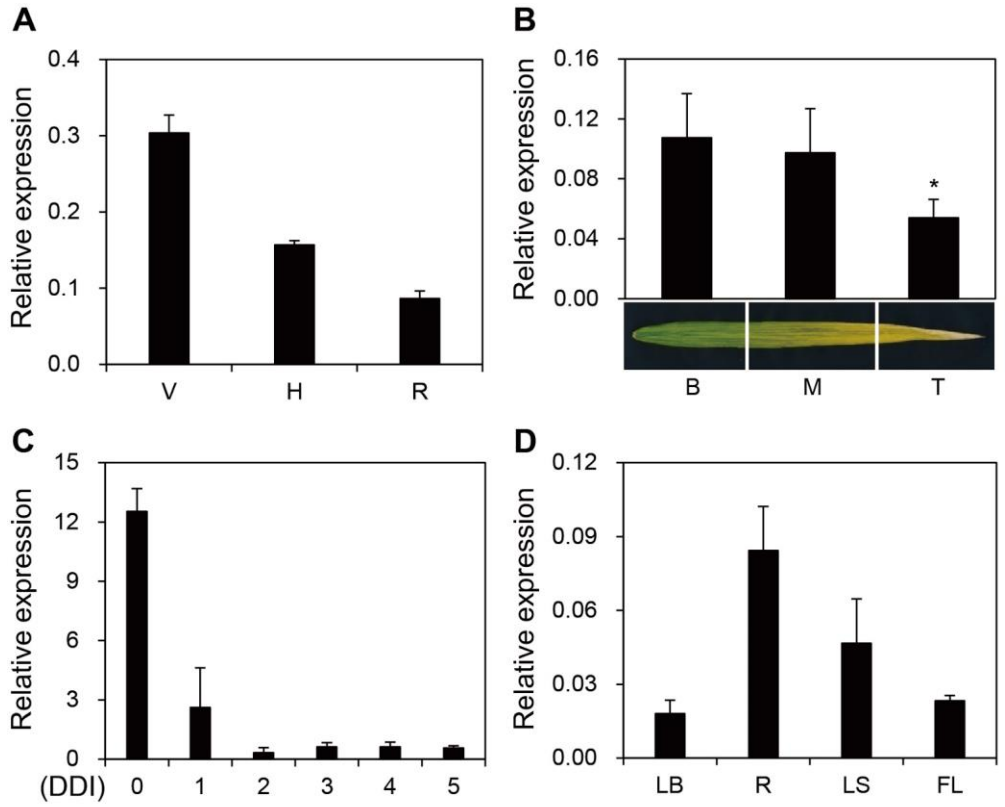

Figure 1. Expressional profile of OsDOF24

(A) The relative expression levels of OsDOF24 at different developmental stages. V, vegetative stage at 89 days after sowing (DAS); H, heading stage at 118 DAS; R, reproductive stage at 123 DAS. (B) OsDOF24 expression in three different sectors of naturally senescing flag leaves. B, bottom; M, middle; T, tip. (C) OsDOF24 transcript levels of dark incubated senescing detached rice leaves. (D) Expression of OsDOF24 in various organs including leaf blade (LB), root (R), leaf sheath (LS) and flag leaf (FL). Mean and SD value were obtained from more than three biological repeats. Asterisks indicate a significant difference between B and T of the Figure 1B (Student′s t-test, *P < 0.05).

osdof24-D Mutants Exhibits A Functional Stay-Green Phenotype during Natural Senescence.

To verify the role of OsDOF24 in leaf senescence, we examined several senescence parameters in the T-DNA insertion-mediated enhancer-tagged mutant of OsDOF24 (hereafter termed *osdof24-D*) [29], in which a T-DNA fragment with 4 repeats of CaMV 35S constitutive promoter, 35S(4x), was integrated in the promoter region (Figure 2A), by comparing with its parental japonica cultivar, Dongjin (hereafter termed wild type, WT), grown in natural long days (>14 h light/day in Suwon, Korea, 37 oN latitude). RT-qPCR analysis revealed that *osdof24-D* accumulated OsDOF24 transcripts much higher than WT (Figure 2B). Overexpression of OsDOF24 did not alter heading date (Figure 3). At 40 days after heading, however, *osdof24-D* showed distinctly delayed leaf yellowing compared with WT (Figure 2C). Consistent with its persistance of leaf green color, chlorophyll content of *osdof24-D* retained higher than that of WT (Figure 2E). In addition, *osdof24-D* showed relatively high Fv/Fm ratio, which indicates the efficiency of photosynthesis (efficiency of PS II) (Figure 2D). The stay-green phenotype can be roughly divided into two types [30,31]. The functional stay-green types maintain leaf greenness and retained photosynthetic activity due to well-preserved chloroplast structures during seed filling, resulting in increasing grain yield. While the nonfunctional stay-green types show green color without sustaining photosynthetic competence. Therefore, our observation

that *osdof24-D* retained not only its green color but also its photosynthetic capacity, strongly supports that *osdof24-D* is a functional stay-green plant.

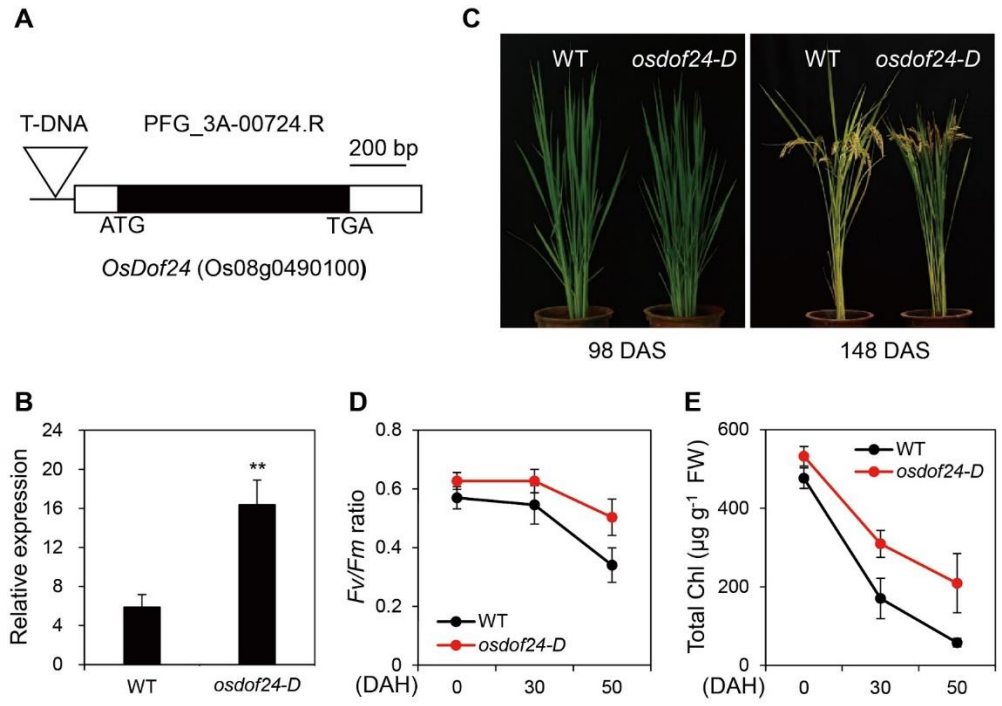

Figure 2. Functional stay-green phenotype of *osdof24-D* under natural long day (NLD) conditions

(A) Schematic diagram of *osdof24-D* mutant. The black box and white boxes stand for exon and untranslated regions, respectively. The triangle represent for the T-DNA insertion position in the promoter region of OsDOF24. (B) The overexpression of OsDof24 in *osdof24-D* was confirmed by RT-qPCR. The total RNA was extracted from the second leaves of 3-month-old plants grown under NLD. OsUBQ5 was used as an internal control. (C) Phenotype of WT and *osdof24-D* plants at 98 DAS and 148 DAS. Changes in the level of *Fv/Fm* ratio (D) and total chlorophyll contents (E) of the flag leaves after heading. Mean and SD value were obtained from more than ten biological repeats. Asterisks indicate a significant difference between WT and *osdof24-D* (Student's t-test, **P < 0.01). DAS, days after sowing.

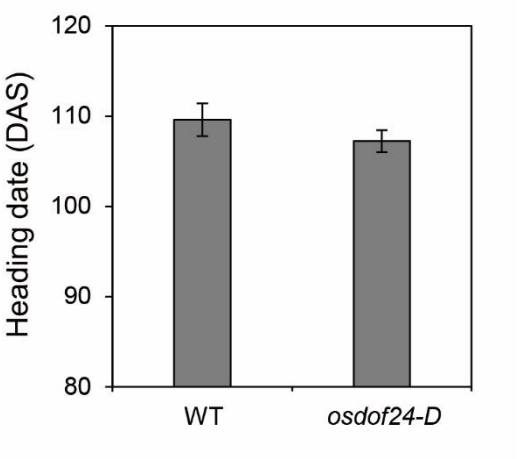

Figure 3. Heading dates of WT and *osdof24-D* mutants grown under natural long day condition

Mean and SD were obtained from more than fifteen biological repeats. DAS, days after sowing.

osdof24-D Mutants Retain Chloroplast Structures Under DIS Conditions

Dark treatment is an effective method that induces leaf senescence artificially [32,33]. Detached leaves of *osdof24-D* exhibited delayed senescence during dark incubation, compared with wild-type (Figure 4A). This observation was consistent with higher chlorophyll contents in *osdof24-D* relative to wild-type (Figure 4B). We also compared chloroplast structure of the leaf blades between WT and *osdof24-D* before and after 4 days of dark incubation (DDI). Transmission electron microscopy analysis revealed that at 0 DDI, the chloroplast ultrastructures of *osdof24-D* leaves were very similar to that of WT leaves (Figure 5A, B). At 4 days of dark incubation, however, chloroplasts retained grana thylakoid structure much longer in the *osdof24-D* leaves (Figure 5D), but little detectable structure remained in the WT leaves (Figure 5C). These results can be further evidence suggesting that *osdof24-D* is a functional stay-green mutant. To confirm the function of OsDOF24 in leaf senescence, we generated several independent lines overexpressing *OsDOF24* under the CaMV 35S promoter (OsDOF24-OE). A delayed senescence phenotype was also observed in the detached leaves of OsDOF24-OE lines during dark incubation (Figure 6), which is the same as the *osdof24-D* leaves.

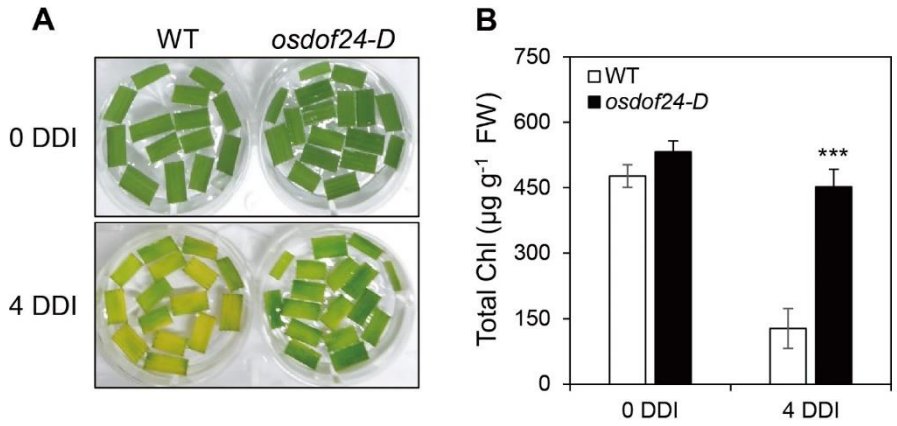

Figure 4. Delayed leaf senescence phenotype of *osdof24-D* mutants during dark incubation.

(A) Phenotype of WT and *osdof24-D* leaf discs 0 DDI and 4 DDI. The second leaf sectors (1-cm long) of 3-month-old plants grown under natural long day condition were floated on 3 mM MES buffer (pH 5.8) with the abaxial side up and incubated in complete darkness at 30℃. (B) The changes of total chlorophyll level of WT and *osdof24-D* in (A). Mean and SD values were obtained from the leaf samples of three independent plates. Asterisks indicate a significant difference between WT and *osdof24-D* (Student's t-test, ***P < 0.001). DDI, day(s) after dark-incubation.

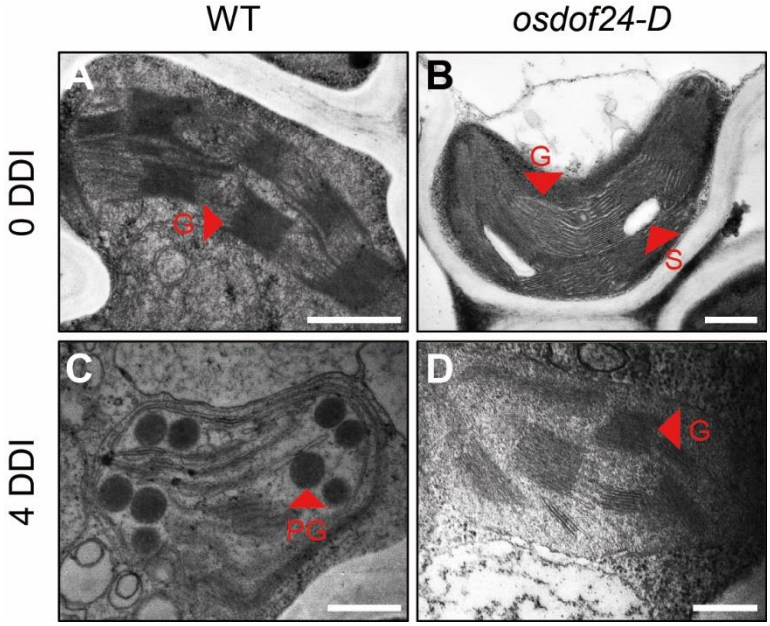

Figure 5. Transmission electron microscopy of chloroplast structures of WT and *osdof24-D* leaves during dark induced senescence.

The second leaves of 3-month-old plants grown under natural long day condition were detached and floated on 3 mM MES buffer (pH 5.8) with the abaxial side up. Then, they were incubated in complete darkness at 30℃ for 4 days. Chloroplast in the mesophyll cells of developing leaves in WT (A) and osdof24-*D* mutant (B) at 0 DDI and that of senescing leaves in WT (C) and *osdof24-D* mutant (D) at 4 DDI. G, grana thylakoid; PG, plastoglobule; S, starch; DDI, day(s) after dark-incubation. Scale bars = 5 μm.

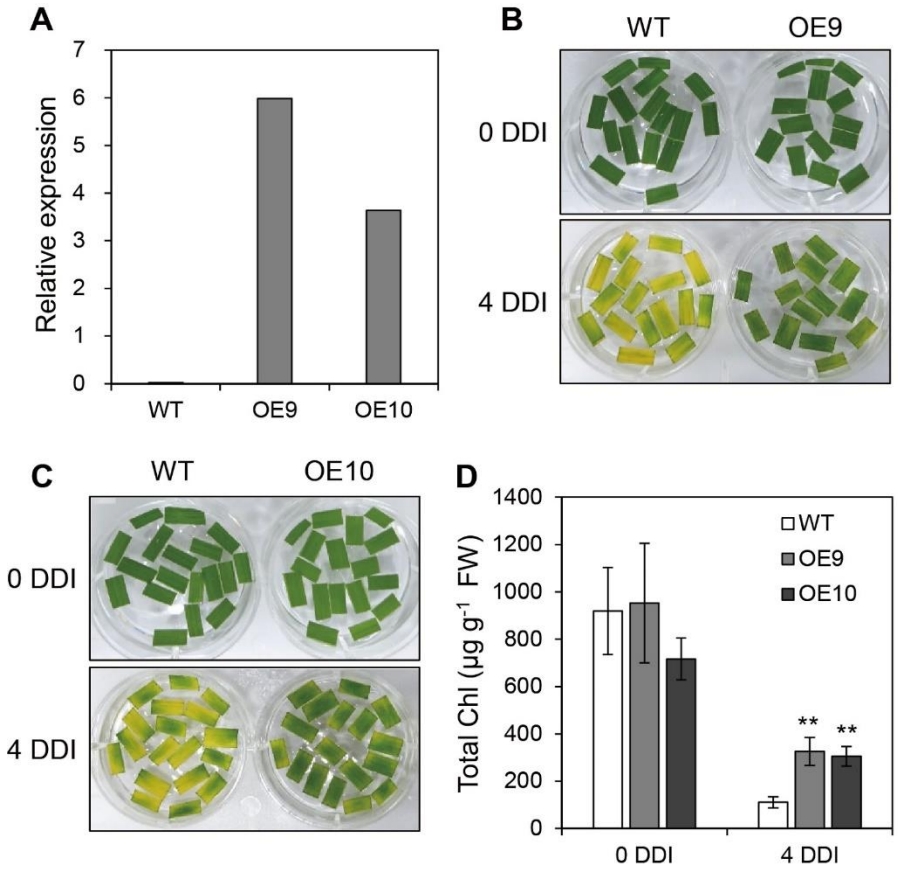

Figure 6. Detached leaves of transgenic plants (35s:OsDOF24) exhibited stay-green phenotype under dark-induced senescence conditions.

(A) Accumulation of OsDOF24 transcripts was highly in two OsDOF24 overexpressed-transgenic plants (OE9 and OE10). (B-D), Stay-green phenotype (B, C) and chlorophyll contents (D) of the wild type and OsDOF24-OE. Detached leaves from 4-weeks old rice plants were incubated with 3mM MES buffer for 3 days at 28℃ in darkness. Mean and SD value were obtained from more than three biological repeats. Asterisks indicate a significant difference between WT and OsDOF24-OE (Student′s t-test, **P < 0.01). DDI, day(s) after dark-incubation.

OsDOF24 Downregulates Expression of SAGs and CDGs during Dark Incubation

Many genes involved in the senescence regulatory networks have been identified and termed as senescence-associated genes (SAGs) [4]. Likewise, genes that have function in chlorophyll degradation were clustered as chlorophyll degradation genes (CDGs) [34, 35]. To elucidate the regulatory function of OsDOF24 as a transcription factor, we examined whether SAGs were was differently expressed in osdof24-D mutants by RT-qPCR analysis. The results showed that the expression of SAGs, including Osl85 and Osl57 didn′t increased that much in osdof24-D mutants while that of WT increased sharply 4 days after dark incubation (Figure 7A, B). In addition, the mRNA level of OsNAP, the transcription factor well known as a strong positive regulator of leaf senescence, was also significantly low in osdof24-D at both 0 and 4 DDI (Figure 7C). Similarly, CDGs such as OsSGR, OsNYC1 and OsNYC3 were also found to be lowered in osdof24-D mutants during dark incubation (Figure 7D, E, F). These result suggested that OsDOF24 delayed leaf senescence in the way of downregulating several SAGs and CDGs under senescence-promoting conditions. Interestingly, previous report indicates that OsNAP binds

directly to the promoters of OsSGR, OsNYC1, OsNYC3 and Osl57 [6]. Our results suggested that OsDOF24 regulates the expression of both OsNAP and its target genes that are placed in regulatory loop of OsNAP, such as OsSGR, OsNYC1, OsNYC3 and Osl57. These findings provided possibility that OsDOF24 may be concerned in feed-forward regulation of CDGs and SAGs through OsNAP (Figure 8). However, further binding assays like yeast one-hybrid and ChIP are required to figure out whether OsDOF24 regulates these genes directly or indirectly as a transcription factor.

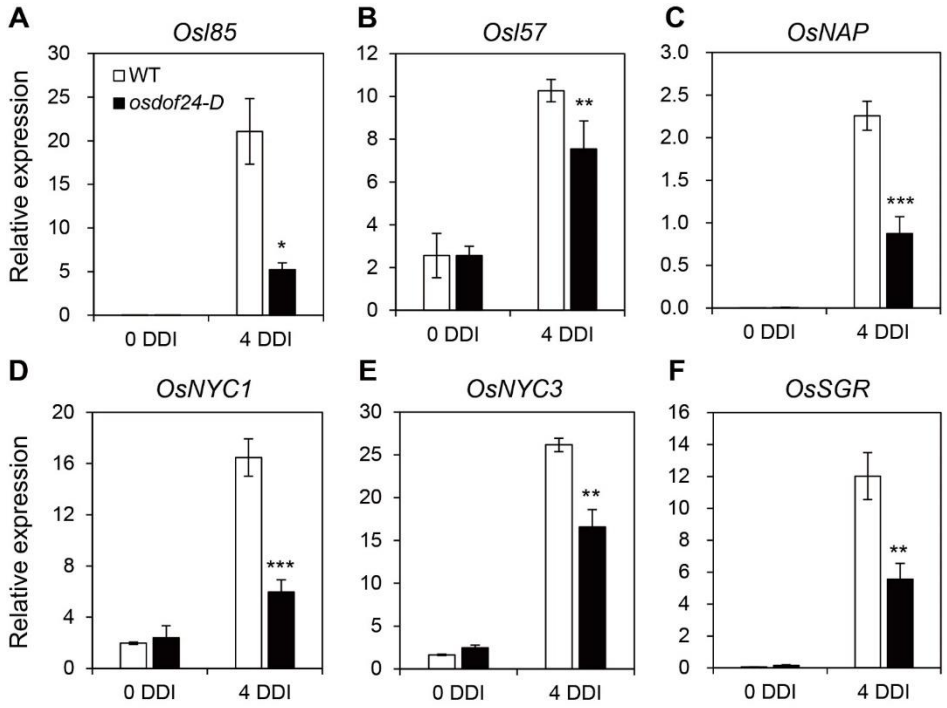

Figure 7. Altered gene expression in the osdof24-D leaves during dark incubation.

The relative expression levels of senescence-associated genes (A-C) and chlorophyll degradation genes (D-E) were measured by RT-qPCR analysis. OsUBQ5 was used as an internal control. Total RNA was extracted from detached leaves of the WT and osdof24-D shown in Figure 3A. Mean and SD value were obtained from more than three biological repeats. Asterisks indicate a significant difference between WT and osdof24-D (Student′s t-test, *P < 0.05, **P < 0.01, ***P < 0.001). DDI, day(s) after dark-incubation.

osdof24-D Is Less Sensitive to Exogenous Treatment of MeJA

Many phytohormones act as internal signal to initiate and progress leaf senescence [1]. Especially, phytohormones such as ABA, MeJA, and SA promote leaf senescence. To examine whether OsDOF24 is associated with the signal transduction of these hormones in leaf senescence, we made exogenous treatments of ABA, MeJA, and SA to the detached leaves of osdof24-D and WT. No significant difference in sensitivity was observed during ABA and SA treatments (data not shown). When treated with MeJA, however, the detached leaves of osdof24-D maintained green color (Figure 8A), in accordance with their chlorophyll contents (Figure 8B). The progress of leaf senescence is affected by endogenous JA levels and JA signal transduction by altering expression of JA biosynthesis and signaling genes, respectively [16]. Therefore, our observation that osdof24-D showed hyposensitive phenotype under MeJA treatment can be speculated that JA signal transduction is involved in OsDOF24-mediated senescence process. Indeed, RT-qPCR analysis showed that transcription levels of JA responsive genes such as OsCOI1a and OsJAmyb were significantly downregulated in osdof24-D detached leaves at 4 DDI (Figure 8C, D). Furthermore, three jasmonic acid-responsive elements (one JARE, TCCTGA, two G-box, CACGTG/T)

existing in OsJAmyb promoter support the possibility that OsJAmyb is closely associated with JA signal transduction [36]. OsCOI1a is a homolog of OsCOI1b and considered to promote JA signaling-mediated senescence additively with OsCOI1b [15]. Considering that JA signaling pathway plays an important role in leaf senescence regulatory system [37], these results implied that OsDOF24 is closely associated with regulation of leaf senescence through JA signaling.

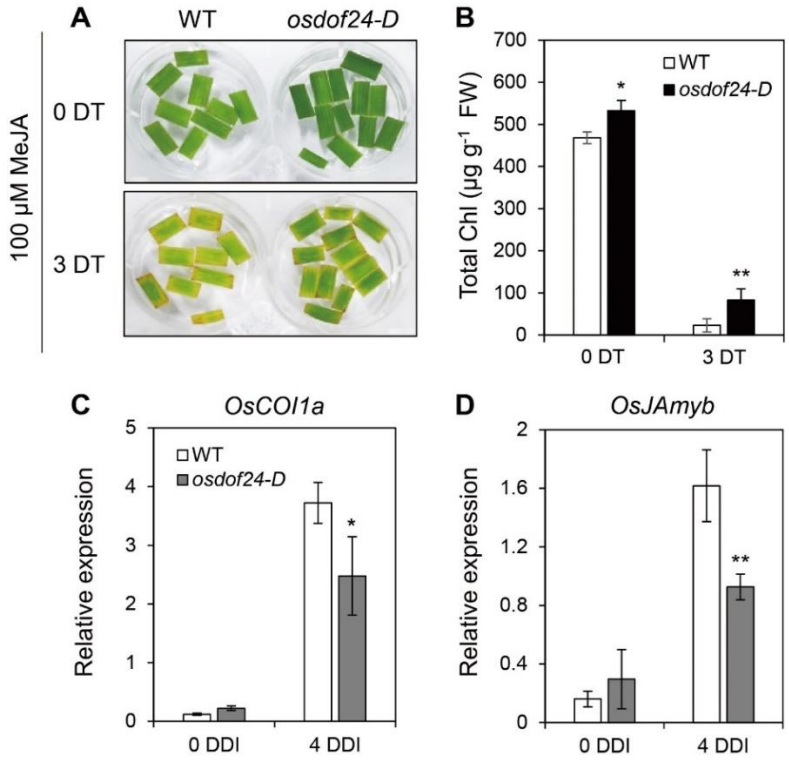

Figure 8. Hyposensitivity of osdof24-D to exogenous treatment of MeJA. The changes of detached leaf color (A) and its chlorophyll level (B). The second leaves (1-cm long) of 3-month-old plants grown under natural long day condition were floated on 3 mM MES buffer supplemented with 100 μM MeJA with the abaxial side up and incubated under continuous light condition at 30 ℃. Altered expression of genes associated in MeJA response in dark-incubated WT and osdof24-D leaves (C-D). The total RNA used in Figure 3A were used for this analysis. The relative expression levels of OsCOI1a (C) and OsJAmyb (D) were measured by RT-qPCR analysis. OsUBQ5 was used as an internal control. Mean and SD value were obtained from more than three biological repeats. Asterisks indicate a significant difference between WT and osdof24-D (Student′s t-test, *P < 0.05, **P < 0.01). DT, day(s) after treatment; DDI, day(s) after dark-incubation.

OsDOF24 Negatively Affects Plant Height, Panicle Length, and Grain Yield

As previously reported, functional stay-green phenotype can increase grain yield in cereal crops by prolonging photosynthetic capacity during grain filling [38]. To identify whether *OsDOF24* affected yield components, we evaluated several agronomic traits in *osdof24-D* mutants and WT grown under natural long day conditions, including plant height, main panicle length, number of panicles per plant, 500-grain weight, grains per panicle, and spikelet fertility. The *osdof24-D* mutants and WT had similar number of panicles per plant and 500-grain weight (Figure 9D, F). However, number of grains and spikelet fertility of *osdof24-D* were significantly lower than those of WT (Figure 9E, G), consequently decreasing total grain yield (Figure 9H). Additionally, plant height and panicle length of *osdof24-D* mutants were observed shorter than that of WT (Figure 9B, C).

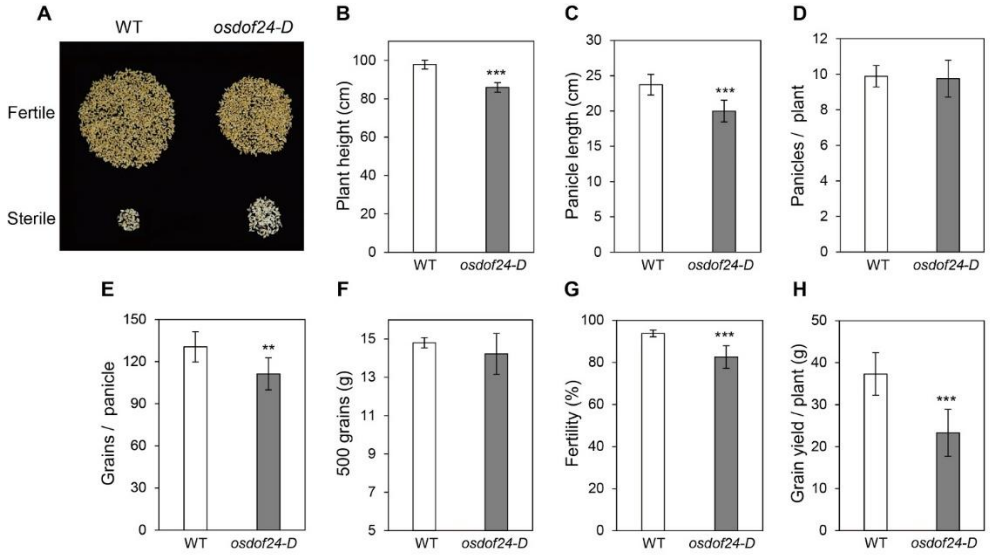

Figure 9. Agronomical traits of osdof24-D.

(A) Photograph of fertile and sterile spikelets of WT and osdof24-D. (B-H) Seven agronomic traits were examined at harvest: plant height (B), panicle length (C), number of panicles per plant (D), number of grains per panicle (E), 500-grain weight (F), spikelet fertility (G), and yield per plant (H) in WT and osdof24-D mutants. Mean and SD were obtained from more than ten biological repeats. Asterisks indicates significant difference between WT and osdof24-D (Student's t-test p-value, **P<0.01, ***P<0.001).

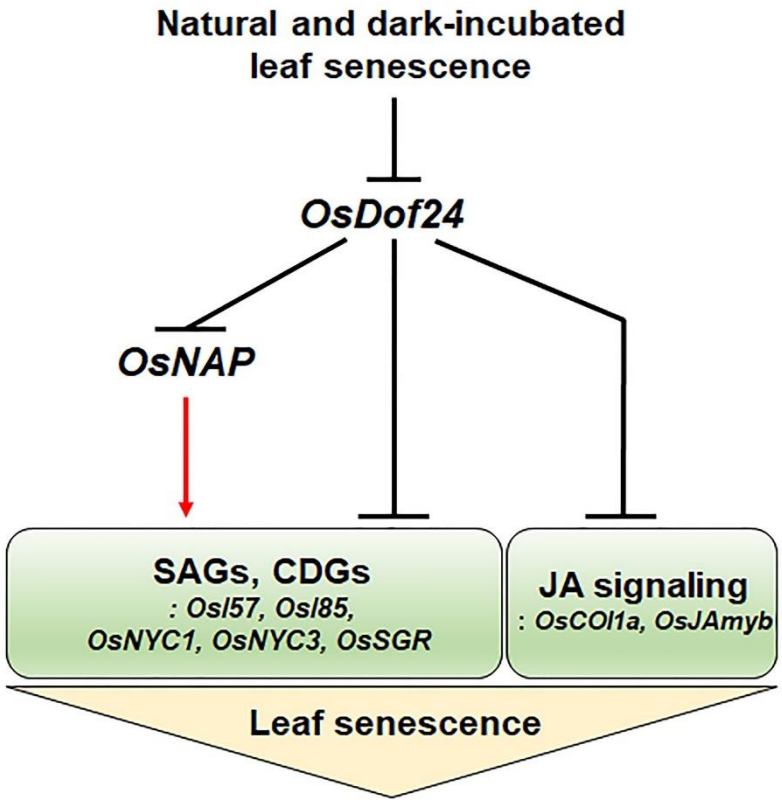

Figure 10. Tentative model of OsDOF24 roles in leaf senescence.

Red arrow represents the previously known regulatory mechanism of *OsNAP* [6]. SAGs, senescence-associated genes; CDGs, chlorophyll degradation genes.

DISCUSSION

DOF, plant specific transcription factor family, has been reported to have roles in seed germination, carbon nitrogen assimilation, photoperiodic flowering time, and abiotic stress response [18]. However, any of DOF transcription factors was revealed to be involved in leaf senescence regulatory pathway. Here we report *OsDof24* acts as a negative regulator of leaf senescence.

osdof24-D, *OsDOF24*-overexpressed T-DNA insertional mutant, retained not only its green color but also photosynthetic capacity during natural senescence process, which implied *osdof24-D* is a functional stay green type [31].

As previously reported, functional stay-green phenotype can increase grain yield in cereal crops by prolonging photosynthetic capacity during grain filling [38]. Based on its functional stay-green phenotype, we expected increased grain yield in *osdof24-D*. However, relatively lowered total grain yield was found in *osdof24-D* compared with WT due to poor spikelet fertility. Endogenous JA contents and its signal transduction can affect seed fertility by regulating spikelet development [15, 39]. Deficiency of *OsCOI1b* gene that functions in JA signal transduction exhibits typical stay green phenotype; prolonged photosynthetic activity and remained chloroplast structure during grain filling. However, this stay green traits doesn’t promise increasing grain

yield. Instead, consistent with observation of *osdof24-D*, null mutation of *OsCOI1b* reduces seed fertility that cause to low total grain yield compared with wild type [15]. Furthermore, previous report has been identified the functions of *EG1 (extra glumes 1*) encoding a lipase for JA biosynthesis and *EG2/OsJAZ1* that is high sequence similarity with the Arabidopsis *JAZ* proteins. The *eg1-3* and *eg2-1D* mutants, that occurred amino acid substitution in codon region of *EG1* and *JAZ* domain of *EG2/OsJAZ1* protein, exhibit abnormal spikelet morphology [39]. Therefore, JA signal transduction that is associated with *OsDOF24*-mediated senescence probably have a negative effect on spikelet development during seed filling.

REFERENCES

1. Lim, *et al:* **Leaf Senescence**. *Annual Review of Plant Biology*. (2007): 115–136.
2. Liu, *et al:* **Identification of early senescence-associated genes in rice flag leaves**. *Plant Molecular Biology*. 67 (2008): 37–55.
3. Buchanan-Wollaston, *et al:* **The molecular biology of leaf senescence.** *Journal of Experimental Botany*. (1997), 48, 181–199.
4. Lee, *et al:* **Leaf senescence in rice plants: cloning and characterization of senescence up-regulated genes.** *Journal of Experimental Botany*. (2001), 52, 1117–1121.
5. Guo, Y *et al:* **AtNAP, a NAC family transcription factor, has an important role in leaf senescence**. *The Plant Journal*. (2006), 46, 601–612.
6. Liang, C *et al:* **OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice**. *Proceedings of the National Academy of Sciences*. (2014), 111, 10013–10018.
7. Uauy, C *et al:* **A NAC Gene Regulating Senescence Improves Grain Protein, Zinc, and Iron Content in Wheat**. *Science*. (2006), 314, 1298–1301.
8. Balazadeh, S *et al:* **ORS1, an H2O2-Responsive NAC Transcription Factor, Controls Senescence in Arabidopsis thaliana**. *Molecular Plant*. (2011), 4, 346–360.
9. Wu, A *et al:* **JUNGBRUNNEN1, a Reactive Oxygen Species-Responsive NAC Transcription Factor, Regulates Longevity in Arabidopsis**. *Plant cell*. (2012), 24, 482–506.
10. Yang, S.-D *et al:* **The Arabidopsis NAC Transcription Factor VNI2 Integrates Abscisic Acid Signals into Leaf Senescence via the COR / RD Genes**. *Plant Cell*. (2011), 23, 2155–2168.
11. Sakuraba, Y. *et al:* **Rice ONAC106 Inhibits Leaf Senescence and Increases Salt Tolerance and Tiller Angle**. *Plant and Cell Physiology*. (2015), 56, 2325–2339.

12. Zhou, X. *et al:* **WRKY22 transcription factor mediates dark-induced leaf senescence in Arabidopsis**. *Molecules and Cells*. (2011), 31, 303–313.
13. Han, M *et al:* **OsWRKY42 Represses OsMT1d and Induces Reactive Oxygen Species and Leaf Senescence in Rice**. *Molecules and Cells*. (2014), 37, 532–539.
14. He, Y.; *et al:* **Evidence Supporting a Role of Jasmonic Acid in Arabidopsis Leaf Senescence***. PLANT PHYSIOLOGY*. (2002), 128, 876–884.
15. Lee, S.-H.; *et al:* **Mutation of Oryza sativa CORONATINE INSENSITIVE 1b (OsCOI1b) delays leaf senescence: OsCOI1b in leaf senescence.** *Journal of Integrative Plant Biology*. (2015), 57, 562–576.
16. Zhou, Y.; *et al:* **Identification and functional characterization of a rice NAC gene involved in the regulation of leaf senescence**. *BMC Plant Biology.* (2013), 13, 132.
17. Riechmann, J. L *et al:* **A genomic perspective on plant transcription factors.** *Current Opinion in Plant Biology*. (2000), 3, 423–434.
18. Yanagisawa, S. **The Dof family of plant transcription factors**. *Trends in Plant Science*. (2002), 7, 555–560.
19. Zhang, B. **Interactions between Distinct Types of DNA Binding Proteins Enhance Binding to ocs Element Promoter Sequences**. *Plant cell*. (1995), 7, 2241–2252.
20. Li, D.; *et al:* **Functional characterization of rice OsDof12.** *Planta*. (2009), 229, 1159–1169.
21. Corrales, A.-R *et al:* **Characterization of tomato Cycling Dof Factors reveals conserved and new functions in the control of flowering time and abiotic stress responses.** *Journal of Experimental Botany*. (2014), 65, 995–1012.
22. Gualberti, G. **Mutations in the Dof Zinc Finger Genes DAG2 and DAG1 Influence with Opposite Effects the Germination of Arabidopsis Seeds.** *Plant cell*. (2002), 14, 1253–1263.
23. Papi, M.; *et al:* **Identification and disruption of an Arabidopsis zinc finger gene controlling seed germination.** *Genes* Dev. (2000), 14, 28–33.

24. Washio, K. **Functional Dissections between GAMYB and Dof Transcription Factors Suggest a Role for Protein-Protein Associations in the Gibberellin-Mediated Expression of the RAmy1A Gene in the Rice Aleurone.** *PLANT PHYSIOLOGY*. (2003), 133, 850–863.
25. Yanagisawa, S. **Involvement of Maize Dof Zinc Finger Proteins in Tissue-Specific and Light-Regulated Gene Expression.** *Plant cell*. (1998), 10, 75–90.
26. Yanagisawa, S. **Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize**. *The Plant Journal*. (2000), 21, 281–288.
27. Noguero, M. *et al:* **The role of the DNA-binding One Zinc Finger (DOF) transcription factor family in plants**. *Plant Science*. (2013), 209, 32–45.
28. Santos, L. A *et al:* **OsDof25 expression alters carbon and nitrogen metabolism in Arabidopsis under high N-supply**. *Plant Biotechnology Reports. (*2012), 6, 327–337.
29. Jeong, D.-H. **T-DNA Insertional Mutagenesis for Activation Tagging in Rice.** *PLANT PHYSIOLOGY*. (2002), 130, 1636–1644.
30. Howard, T *et al:* **Crops that stay green**. *Annals of Applied Biology*. (1993), 123, 193–219.
31. Thomas, H *et al:* **Five ways to stay green**. *Journal of Experimental Botany.* (2000), 51, 329–337.
32. Kim, H. J.; *et al:* **Cytokinin-mediated control of leaf longevity by AHK3 through phosphorylation of ARR2 in Arabidopsis**. *Proceedings of the National Academy of Sciences.(*2006), 103, 814–819.
33. Kong, Z.; *et al:* **A Novel Nuclear-Localized CCCH-Type Zinc Finger Protein, OsDOS, Is Involved in Delaying Leaf Senescence in Rice**. *PLANT PHYSIOLOGY*. (2006), 141, 1376–1388.
34. Oda-Yamamizo, *et al:* **The NAC transcription factor ANAC046 is a positive regulator of chlorophyll degradation and senescence in Arabidopsis leaves.** *Scientific Reports*. (2016), 6.
35. Morita, R *et al:* **Defect in non-yellow coloring 3, an α/β hydrolase-fold family protein, causes a stay-green phenotype during leaf senescence in rice.** *The Plant Journal*. (2009), 59, 940–952.

36. Cao, W.-L *et al:* **OsJAMyb, a R2R3-type MYB transcription factor, enhanced blast resistance in transgenic rice.** *Physiological and Molecular Plant Pathology. (*2015), 92, 154–160.

37. Hu, Y.; *et al:* **Jasmonate regulates leaf senescence and tolerance to cold stress: crosstalk with other phytohormones**. *Journal of Experimental Botany. (*2017), 68, 1361–1369.

38. Robson, P *et al:* **New ways to stay green***. IGER Innov*. 2001, 5, 12-15.

39. Cai, Q *et al:* **Jasmonic acid regulates spikelet development in rice**. *Nature Communications*. (2014), 5.

40. Porra, R. J *et al:* **Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy.** *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. (1989), 975, 384–394.

41. Inada, N *et al:* **Three-dimensional analysis of the senescence program in rice (Oryza sativa L.) coleoptiles**. *Planta*. (1998), 206, 585–597.

42. Jeon, J.-S *et al:* **T-DNA insertional mutagenesis for functional genomics in rice***. The Plant Journal*. (2000), 22, 561–570.

초 록

본 연구에서는 다양한 유전자의 promoter sequence 에 binding 함으로서 그 발현을 조절하는 전사인자들 중 하나인 *OsDof24* 의 기능을 T-DNA 삽입 돌연변이체를 이용하여 밝혀내었다. *OsDof24* 유전자는 자연 장일 조건 하에서 영양 생장기에서 생식 생장기로 이행될 때 그 발현이 감소하는 경향을 보였으며, 노화가 진행 중인 잎에서도 완전히 노화가 완료된 노란 부분에서, 노화가 아직 진행 중인 연두색 부분보다 적은 양의 mRNA 가 관찰되었다. 잘라낸 잎을 암처리를 하여 인위적으로 노화 유도한 상황에서도 역시 *OsDof24* 유전자의 발현이 급격히 감소하는 것을 확인할 수 있었다. 이러한 발현 패턴을 통해 *OsDof24* 는 노화에 대한 억제 조절자 역할을 할 것이라고 예상하였다. 실제로 *OsDof24* 유전자가 과다발현 된 osdof24-*D* 돌연변이체는 자연 장일 조건 하에서 잎의 노화가 지연되는 표현형을 보였으며, 잎의 녹색뿐만 아니라 광합성 효율과 엽록체 구조 또한 유지된다는 것을 *Fv/Fm ratio* 와 전자투과현미경을 이용한 단면 사진을 통해 확인하였다. 전사인자인 *OsDof24* 가 어떤 하위 유전자들의 발현을 조절 함으로서 이러한 표현형을 나타내는지 확인하기 위해 암처리로 노화를 유도한 잎 조각으로부터 RNA 를 추출해 cDNA 를 합성한 후 qRT-PCR 을 수행하였다. 그 결과 노화 시 발현이 증가한다고 알려진 SAG(Senescence-Associated Gene)에 속하는 *Osl57* 과 *Osl85* 의 발현이 야생형에 비해 억제된 것을 알 수 있었다. 또한 엽록소 분해 과정에 참여한다고 알려진 CDG(Chlorophyll Degradation Gene)에 속하는 *OsNYC1, OsNYC3*, 그리고 *OsSGR* 의 발현 역시 야생형보다 적은 것을 확인하였다. 벼에서 잎의 노화를 가속화시킨다고 보고된 *OsNAP* 이라는 전사인자의 발현 역시 감소하였다. 노화 과정에서 더욱 상위에서 발달 단계에 대한 신호를 인지하고 다양한 유전자들의 발현을 변화시키는 역할을 하는 것은 식물 호르몬인데, 이러한 호르몬에 의한 조절 과정에도 참여하는지 확인하기 위하여 노화 과정에 역할을 한다고 알려져 있는 호르몬인 ABA 와 MeJA, 그리고 SA 를 외부적으로 처리해보았다. 그 결과

ABA 와 SA 에 대해서는 야생형과 별다른 차이를 보이지 않았지만 MeJA 가 유도하는 노화 상황에 대해 *osdof24-D* 돌연변이체가 그 반응성이 더 낮은 것을 확인할 수 있었다. 암처리한 야생형과 돌연변이체의 cDNA 로부터 MeJA 반응성에 관여한다고 알려진 유전자인 OsCoi1a 와 OsJAmyb 의 발현이 낮은 것을 확인하였고, 이를 통해 노화 과정에서도 노화를 촉진하는 역할을 하는 호르몬인 MeJA 의 signaling 을 약화 시킴으로서 노화지연 표현형을 나타낼 것이라고 예상해볼 수 있었다. 마지막으로 기능성 녹색유지 형질은 작물의 수량 증대와 관련이 있다는 이전의 연구결과와 부합할 지 확인해보기 위해 본 돌연변이체에서 농업적 형질을 측정하였다. 그 결과 panicle 수와 500 립중은 야생형과 큰 차이를 보이지 않았으나, 총 grain 수와 임실률이 유의미하게 감소함으로서 결과적으로 수량이 감소하였다는 것을 알 수 있었다. 이는 높은 광합성률이 오랜 시간 지속됨에도 불구하고 spikelet 의 형성에 도움을 주는 기능을 하는 JA 의 signaling 이 약화된 원인으로 추측할 수 있었다.