THESIS FOR DEGREE OF MASTER OF SCIENCE

Regulatory mechanism of the rice *RADIALIS-LIKE3* (*OsRL3*) in leaf senescence and salt stress response

벼의 ***RADIALIS-LIKE3*** 전사인자의 노화 지연 및 염 민감성 연구

BY

DAYEA PARK

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MAJOR IN CROP SCIENCE AND BIOTECHNOLOGY

DEPARTMENT OF PLANT SCIENCE

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UNDER THE DIRECTION OF DR. NAM-CHON PAEK
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DAYEA PARK

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The rice *RADIALIS-LIKE3* (*OsRL3*) connects leaf senescence and salt stress response through ABA signaling pathway

DA YEA PARK

ABSTRACT

Abscisic acid (ABA) is associated with multiple developmental processes in higher plants, especially to respond to abiotic stresses. ABA synthesis is triggered by various environmental factors and ABA sensing promotes leaf senescence or stress tolerance. Here, we demonstrate a rice MYB transcription factor RADIALIS-LIKE3 (*OsRL3*) which promotes dark-induced senescence (DIS) and salt stress response. The gene expression is slightly induced under DIS and some osmotic stress conditions, highly expressed under salt stress. The T-DNA insertion *osrl3* knockout mutant delayed leaf senescence in the dark with a significant retention of chlorophylls and photosynthetic capacity. Furthermore, in high salinity conditions, *osrl3* mutation increased stress response with reduced expression of proline biosynthetic genes OsP5CS1 and OsP5CS2 in the leaves. In an exogenous ABA treatment, *OsRL3* expression increased in the WT leaves and *osrl3* mutant exhibit a stay-green phenotype. Under both dark and salt stress treatments, ABA signaling-associated genes were down-regulated in *osrl3*. Yeast one-hybrid assay revealed that *OsRL3* directly targets the promoters of OsNAP and OsRAB16D that accelerate leaf senescence and abiotic stress response. Taking together, we conclude that *OsRL3* functions as a

transcriptional activator for the genes involved in ABA signaling which promotes leaf senescence and abiotic stress response in rice.

Keywords: rice, ABA, MYB transcription factor, Dark-induced senescence, *OsRL3*, Salt stress response.

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ABBREVIATION

OsRL3	*rice RADILIA-LIKE3*
TF	Transcription factor
WT	Wild-type
DIC	Differential interference contrast
DDI	Day(s) after dark treatment
DT	Day(s) after treatment
HT	Hour(s) after treatment
DAF	Day(s) after flowering
SAG	Senescence-associated gene
Chl	Chlorophyll

INTRODUCTION

Leaf senescence, the final step of leaf development, is genetically programmed deteriorative process that ultimately leads to the death of an annual plant. This process can be triggered by external factors including drought, high salinity stress, ultraviolet light, and pathogen attacks [1–3],or by endogenous factors such as phytohormones, some metabolites and the states of photosystem complex [4,5]. In senescence, chlorophyll (Chl) is converted to colorless breakdown products in a chlorophyll catabolic pathway. This pathway consists of several Chl catabolic enzymes (CCE) [6,7]. The genetic regulatory mechanism induce senescence via signal transductions and transcription factors (TFs) to modulate the expression of CCE genes and senescence-associated genes (SAGs) dynamically[8]. The promoters of several SAGs consist of diverse sequence elements, suggesting that the regulation of SAG expression is multifactorial. It is postulated that there may be several genetic regulations, including TFs. [9].

TFs represent a regulatory component of the genome and are the most important targets for engineering aging and stress tolerance. Rice TFs were classified into 84 families [10]. MYB transcription factor, one of the largest TF family, is a key factor in regulatory networks controlling plant development, metabolism and responses to biotic and abiotic stresses in rice [11,12]. MYB protein has two distinct regions, an N-terminal involved MYB DNA binding domain and a various C-terminal modulator region which is responsible for the regulatory activity of the protein. MYB domain consists of up to four imperfect amino acid sequence repeats (R) of about 52 amino acids, each forming a-helices [13]. Each function of MYB Transcription factor depends on the number of adjacent R repeats [14]. The R2R3-MYB genes, the largest MYB subfamily, have been extensively studied in diverse biological functions, such as phenylpropanoid [15,16], cell shape and differentiation [17,18], biotic and abiotic stress [19–21], and hormone responses [22]. MYB –related genes,

evolutionarily older R1/R2- type, including *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*), encode key components of the central circadian oscillator [23]. Function of genes that are involved in the other two MYB subfamilies were not well known.

In plant, Abscisic acid (ABA) has substantial function in stress response and regulates various plant developments, including seed germination and dormancy, organ abscission, and leaf senescence [24–26]. In particular, ABA is has well been known for participating in osmotic stress response and leaf senescence [22,27]. There are three core components of ABA signaling; pyrabactin resistance (PYR)/pyrabactin resistance-like(PYL)/regulatory component of ABA receptors (RCAR), protein phosphatase 2C (PP2C) and SNF1 (Sucrosenon-fermenting)- related protein kinase 2 (SnRK) [28]. Under normal conditions, PP2C negatively regulates SnRK2; however, once abiotic stresses or developmental cues up-regulate endogenous ABA, PYR/PYL/RCAR binds ABA and interacts with PP2C to inhibit protein phosphatase activity [29]. Then, the core complex directly regulates ABA – responsive gene expression by phosphorylation of transcription factors [30]. In this signal transduction, transcription factors, especially MYB TF, are vital for the regulation of ABA related gene networks, activating some stress-inducible genes [31]. For example, MYB TFs in Oryza sativa namely *OsMYB48-1*, *OsMYB2*, and *OsMYB91* involved in ABA-dependent stress response to osmotic stresses [21,32,33]. However, there is no report that MYB TFs are related to leaf senescence in rice, even though in Arabidopsis thaliana.

In this study, we show that Os02g47744, a novel MYB transcription factor, acts as a positive regulator in leaf senescence and salinity stress response. It was identified as a *rice RADIALIS-like3* (*OsRL3*) in NCBI database similar with Arabidopsis thaliana *RADIALIS-like3*. *OsRL3* is involved in MYB-related gene, which has only one activation domain, SANT domain. The *osrl3* knock out mutants stayed green during dark induced senescence (DIS). Under high

salinity stress, *osrl3* has a high sensitivity. In diverse phytohormones, *OsRL3* expression is significantly upregulated by exogenous ABA. In ABA-induced senescence, *osrl3* shows delayed senescence. During both DIS and salt stress, ABA signaling genes were downregulated in *osrl3*. By promoter binding assay to identify the target genes, our work indicates that *OsRL3*, as a upstream TF of *OsNAP* and *OsRAB16D*, positively regulates SAGs and ABA signaling genes to promotes leaf senescence and salt stress resistance mechanism.

MATERIALS AND METHODS

Plant Growth Conditions and Stress treatments

The parental japonica cultivar ‘Dongjin’ and *OsRL3* plants were grown in a natural long day conditions. The *OsRL3* seeds were obtained from the Crop Biotech Institute at Kyung Hee University, Korea (Jeon et al., 2000, 2000). Wild-type and *osrl3* mutants grown in soil were used to determine the salt stress tolerance. For treatment with salt stress, 3-week old plants grown in pots were watered with 200 mM NaCl for 5 days and recovered for another 8 days.

Subcellular localization

To determine its subcellular localization, *OsRL3* cording sequence was amplified by PCR, then subcloned into binary vector pEG103 to produce YFP tagging protein. The recombinant vector and empty vector were bombarded into onion (*Allium cepa*) epidermal cells with using a DNA particle delivery system (Biolistic PDS-1000/He,Bio-Rad). 16-24 hours after bombarding onion cells into the incubation at 22_C, the green fluorescence signal was detected by a confocal laser scanning microscope II (LSM710, Carl Zeiss).

Quantification of total chlorophyll and Malondialdehyde (MDA)

To measure the photosynthesis pigments levels, Chlorophyll was extracted with 80% acetone from dark and hormone treated leaf discs. Chlorophyll contents were valued using a UV/VIS spectrophotometer as described previously (Porra et al., 1989)

The malondialdehyde (MDA) content was measured following the

methods described by Duan et al. Briefly, rice leaves were homogenized in 5 ml of 10% trichloroacetic acid containing 0.25% thiobarbituric acid. The mixture was incubated in water at 95 _C for 30 min and the reaction stopped in ice. The mixture was centrifuged at 13,000 rpm for 20 min and the absorbance of the supernatant was measured at 450, 532, and 600 nm.

Ion leakage

Ion leakage is a representative measurement of a membrane leakage. With a small modification of the previous measurement of membrane leakage protocol (Fan et al., 1997), it was conducted by measurement of ions leaked from leaf tissues under each treatment. Six pieces of the detached leaf from each treatment were immersed in 8ml of the 400 mM Mannitol for 3 hours, and then the solution was measured by conductivity meter (CON6 NETER, LaMOTTE). The solutions including leaf discs were treated 90_C for 40 minutes at water bath. After the heat treatment, total conductivity was measured again. The rate of the ion leakage was valued to the percentage of initial conductivity by total conductivity.

SDS–PAGE and immunoblot analysis

Protein extracts were prepared from leaf tissues. To extract total proteins, leaf tissues were ground in liquid nitrogen homogenized with sample buffer (50mM Tris, pH 6.8, 2mM EDTA, 10% glycerol, 2% SDS and 6% 2-mercaptoethanol). Homogenates were centrifuged at 13000rpm for 3 min and supernatants were denatured at 100℃ for 5 min. Each sample was subjected to 12% (w/v) polyacrylamide SDS–

PAGE and resolved proteins were transfered to a Hybond-P membrane (GE Healthcare). Antibodies against the photosystem proteins Lhcb1, Lhcb2, Lhcb4, Lhca1, Lhca2, PsaA, and D1 (Agrisera) were used for immunoblot analysis. Each protein level was examined using the ECL system with WESTSAVE (AbFRONTIER) according to the manufacturer's protocol.

Abiotic stress and phytohormone treatments

To detect the transcription level of *OsRL3* under various abiotic stress and phytohormone treatment, seeds of wild type (Dongjin) were sterilized in 75% ethanol for 10 min and in 20% NaClO for 20 min, and were washed with sterile water thoroughly for three times. The sterilized seeds were germinated at 30_C continuous light condition for 10 days in MS solid medium. 10-day-old plants were treated with stress including dehydration stress (the plants were exposed in the air without water supply), high salinity stress using 200 mM NaCl solution, and oxidative stress using 3 mM H2O2 solution and 100 mM mannitol.

To identify the expression changes of *OsRL3* in various phytohormone treatments, 100 uM abcisic acid (ABA), 100 μM Methyl Jasmonic acid (MeJA), 100 μM Salicylic acid and 100 μM ACC were treated at 10-day-old WT plant. The WT plants were immersed in MS liquid media (pH 5.7) with each hormone. It was treated for 0 to 12 hours in 30_C chamber. As a leaf disk to figure out the hormone treated phenotypes, 2-week old plants were detached and floated on 3 mM MES buffer (pH 5.7). Hormone treatment was performed at 30_C under continuous light (90 μM m-2 s-1).

RNA Isolation and qRT-PCR Analysis

Total RNA was isolated from the rice leaves using RNA kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. The RNA was reverse-transcribed into first-strand cDNA with M-MLV reverse transcriptase (TaKaRa, Dalian, China), according to the manufacturer's instructions. A five folds dilution of the resultant first strand cDNA was used as template for PCR. For quantitative real-time PCR (qPCR), a 20uL mixture was prepared including 2ul cDNAs from RNA reverse transcription. 10 ul 2X GoTaq master mix (Promega), 6 ul distilled water, and gene-specific forward and reverse primers (Table S1). qPCR was performed using a Light Cycler 480 (Roche Diagnostics). The rice Ubiqutin 5 was used as the endogenous control for data normalization, and each analysis was repeated at three times. All the primers used in experiment were listed in Table S1.

Yeast one-hybrid assays

The *OsRL3*, acting as a prey, cording sequence was inserted in the pGAD vector (Clontech). As a bait, DNA fragments involved in the promoter region of ABA signaling genes (*OsRAB16D*, Os*RK1*, and *OsLEA3*) and senescence associated genes (*SAG12*, *OsNAP*, *RCCR*) were inserted in pLacZi vector (Clontech). Each of the promoter size was 1000 to 1200 bp. Primers in used are listed in Supplemental Table S1. YM4271 was used as a yeast strain for baits and preys and the enzyme assay, b-galactosidase activity assay, was conducted with the liquid assay using chlorophenol red–b-D-galactopyranoside (CPRG; Roche Applied Science) according to the Yeast Protocol Handbook (Clontech).

Table1. Primers used in this study.

A. Primers for verification of *OsRL3* T-DNA insertion		
Product	Forward primer (5'→3')	Reverse primer (5'→3')
PFG_ 3D-02666	CTGGAGGCGACTTGTTTCTC	CTTTCGGCTTTCGAGATGTC
PFG_1B-05327	GAGAAAACCCCAGGGAGAAG	ATCACCGCATCATCTCCTTC
PGA_2715_RB	CGTCCGCAATGTGTTATTAAG	
PGA_2715_LB	CTAGAGTCGAGAATTCAGTACA	
B. Gene-specific primers used for qRT-PCR		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
OsRL3	AAGTTGTGGTACCTCCTCCTCCTGT	AAGGACGTGGATCGCATTGA
OsRK1	AGTACACCAAGCAGGTGAAGCA	GCAACAGCAAAGCTTGAACTCA
OsLEA3	TTTCTGACGGGTGTGGGTGATG	AACACAGACGAGAAACTCTGACG
OsRAB16D	GCGCACTTACATACAGTGCTACGT	CGGGTAAACAATAAAGTCGTGATG
OsRAB16C	TTCCCGGCCAGCACTAAAT	AAACTGCACGTACATCACGACAT
OsRAB21	CACACCACAGCAAGAGCTAAGTG	TGGTGCTCCATCCTGCTTAAG
OsSGR1	AGGGGTGGTACAACAAGCTG	GCTCCTTGCGGAAGATGTAG
OsNAP	AACCATTTCATCGCGAACAAC	CAGTGACGATCCCTGCAAGG
OsNOL1	TCTTATGTCTGGTGCCACAAC	GGATGTAGGTTGGTTTCATGG
OsRCCR	CCAGTCCTCACTACACTGCAA	AGAAGGTCCAGAAGCACCAC
OsNYC1	GAATCCGTAATTGGGCTGAA	CTGGAAGAGGTCCACCTGAG
OsPAO	GTGTTGCCTTCCACTGTCCT	ACTGAACATCCGCAGGAATC
OsP5CS1	CCCGTCCCGGAGCTTCGTGAG	CCTAAGTCGCTGTCGCCCCAC
OsP5CS2	GCTGCCGTCGGTCAGAGTG	CTCGTATGGTTGCCTCCTGGT
OsHKT1;1	TTCACCACTCTTGCGGCTATG	TGTTTGTAGCCAGTCTCCCAG
OsHKT1;5	CCACCTTTTCCTTTTCCATGC	GGTCTTCATCGGCAGAGCTTT
OsAPX5	CCGGTGGCCCCAAGAT	GGTGGGCATTCTTCACCATC
OsCATb	GCTGGTGAGAGATACCGGTCA	TCAACCCACCGCTGGAGA
OsSNAC1	GCCAAGAAGGGATCTCTCAGGTTG	TCTTCTTGTTGTACAGCCGACAC
OsNAC6	AGAAGAACAGCCTCAGGTTGGATG	AGCCCGCCTTCTTGTTGTAAATC
OsABI5	ATGGCATCGGAGATGAGCAAGAAC	GCTTCTTTGTCAGTAGAACCGTCTTC
OsNCED2	TCCGTTGCCCAAGATCAAG	CGTCCAACCGTGCAATCAC
OsNCED4	GATTGCACGGCACCTTCATT	CTCTGTAATTTGATTTTTCACTGGCTAAT
OsNCED5	GGATGGGCTGAACTTCTTCCAG	CAGCACATTCGTGATGAACCCT

OsUBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
C. Gene-specific primers used for yeast one hybrid assay		
Gene	Forward primer (5'→3')	Reverse primer (5'→3')
OsRL3	GAATTCATGAGTTCATCCTGGACAACA	GAGCTTCACTGAAAGTTGTGGTACCT
OsRK1	GAATTCTCTGCTACTTGCTTTGCTTG	CTCGAGCATCGCAACAACACTTGT
OsRAB16D	AAGCTTCTGCGTTGTGGTTGCACAC	CTCGAGCATTCTCGCTATACCTAGC
OsNAP	AAGCTTAAATTTGCTCAATGTCTCC	CTCGAGATCATTTCTCTTGCTGTTTC
OsSAG12	CCCGGGAGTCTAGACCAGGTTCTGT	GGTACCACACCGAAGTTGCCCGA
OsRCCR	CTCGAGAGGAAGAGATAGGGAATAA	GTCGACAGTGAGAAGGATTCCGTGAA

RESULTS

***OsRL3* responds to Dark induced senescence (DIS) and Salinity stress.**

OsRL3 is a MYB-related TF from NCBI database as predicted, and a nuclear localization signal was detected in *OsRL3*-YFP transformed onion epidermal cell while free YFP showed ubiquitous distribution of signal in the whole cells (Figure 1).

To conduct the *OsRL3* gene functional study, we identified the basal *OsRL3* expression profiling. Tissue specificity may be associated with specific biological function. We characterized the expression patterns of *OsRL3* with qRT-PCR in various organs at seedling stage. As shown in Figure 2A, *OsRL3* was mainly expressed in leaf.

As one of the natural leaf phenotype, we determined the role of *OsRL3* in a leaf senescence, verifying the expressional patterns in dark induced senescence (DIS). Under DIS, *OsRL3* expression level was low in the green leaves, 0 to 3 days after dark incubation (DDI); however, when leaves turn yellow, 4 to 5 DDI, the gene expression was increased (Figure 2B).

Based on the previous work of genome-wide analysis under abiotic stress using microarray, the result indicates that *Os02g47744* (*OsRL3*) should be a osmotic stress responsive gene (Katiyar et al., 2012). We figured out the gene expression levels under various osmotic stress treatments. We treated dehydration stress by air dry, 200 mM NaCl, 100 mM Mannitol, and 3 mM H2O2 to the 10-day old WT plants. In each stress conditions, gene expression is up-regulated under dehydration and salinity stress treatments. Especially, in salinity stress condition, expression of *OsRL3* increased almost five folds compared to the expression in the mock condition (Figure 2C). This result suggests that *OsRL3* is closely related to leaf senescence and salt stress mechanism.

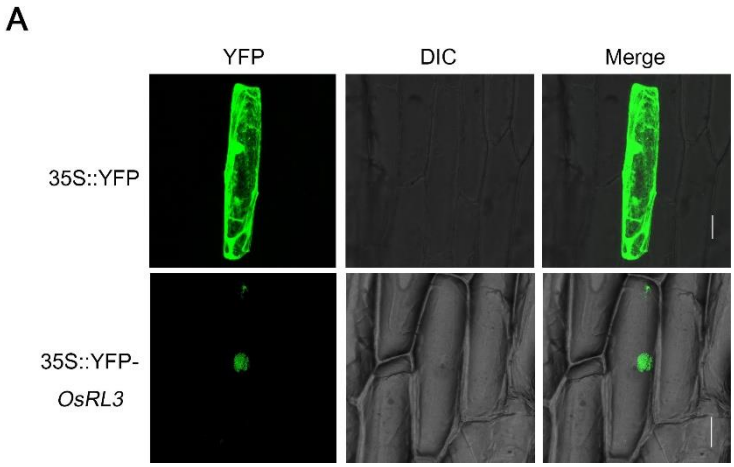

Figure 1. Subcellular localization

The *OsRL3*-YFP fusion protein was transiently expressed in onion epidermal cells and visualized by fluorescence microscopy. The upper panels exhibits single YFP as a control in a transient assay, while bottom panels show the localization of the localization of *OsRL3*-YFP in onion cells. Scale bar = 50μm

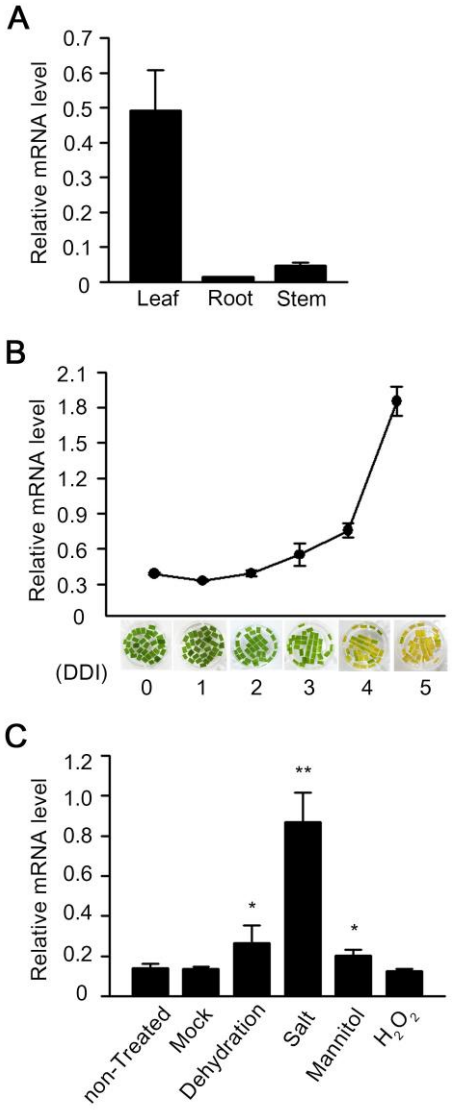

Figure 2. Expression patterns of OsRL3.

(A) *OsRL3* is mainly expressed in leaves. (B) *OsRL3* mRNA level increases under dark-induced senescence conditions. Leaf disks detached from 3-week-old WT plants were incubated in the dark at 28℃, and then sampled at the each day. DDI, day(s) of dark incubation. (C) The *OsRL3* expression

levels examined by qRT-PCR. 10-day old WT treated dehydration, salt, mannitol, H2O2 under continuous light condition at 28℃. Air-dry method was used for dehydration. 200 mM NaCl, 300 mM mannitol, or 3 mM H2O2 was treated. Each stress treated at 10-day old WT plants. Mock; -, Dehydration; D, Salt; S, Mannitol; M, H2O2; H. The relative transcript levels of *OsRL3* determined by RT–qPCR and normalized to transcript levels of OsUBQ5. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01, ***P<0.001)

OsRL3 exhibits stay green during DIS.

To identify the function of *OsRL3* in senescence and stress, we used two different T-DNA mutants, PFG_3D-02666.R and PFG_1B-05327.L, selected as homo lines. *OsRL3* has two exons and one intron. Each of the T-DNA mutant is inserted at the 42bp and 1033bp of the intron (Figure 3A). We checked the *OsRL3* transcriptional levels in mutants by semi qRT-PCR. As the *OsRL3* gene expression was not shown in the mutants, it turns out to be knock out mutants (Figure 3B). In natural field, no difference in development and senescence process were observed between WT and *osrl3* mutants (Figure 4).

These two different mutants exhibit a delayed senescence during dark incubation. The dark incubation treated on leaf disks from 2-month old WT, *osrl3* mutant plants. The leaf disks were floated on MES (pH 5.7) buffer for 3 days. At the 3 DDI, mutants showed stay green phenotypes, whereas leaf discs of the WT turned yellow (Figure 4C). Stay green phenotype accompanies maintenance of chlorophyll contents. To detect the changes of the chlorophyll contents in leaf tissues, we examined the total chlorophyll contents and the photosystem proteins levels. Total chlorophyll contents lasted longer in the *osrl3* mutants during dark incubation (Figure 4D). Through immunoblot analysis, *osrl3* mutants showed high levels of photosystem proteins, such as PSII antenna proteins (Lhcb1 and Lhcb2) and core protein (D1), PSI antenna proteins (Lhca1 and Lhca2) and core protein (PsaA), and one of enzyme of Calvin cycle (rbcL) during the dark condition (Figure 4E). By stabilizing the chlorophyll content and photosynthesis protein complexes during DIS, *osrl3* mutants showed stay green phenotype.

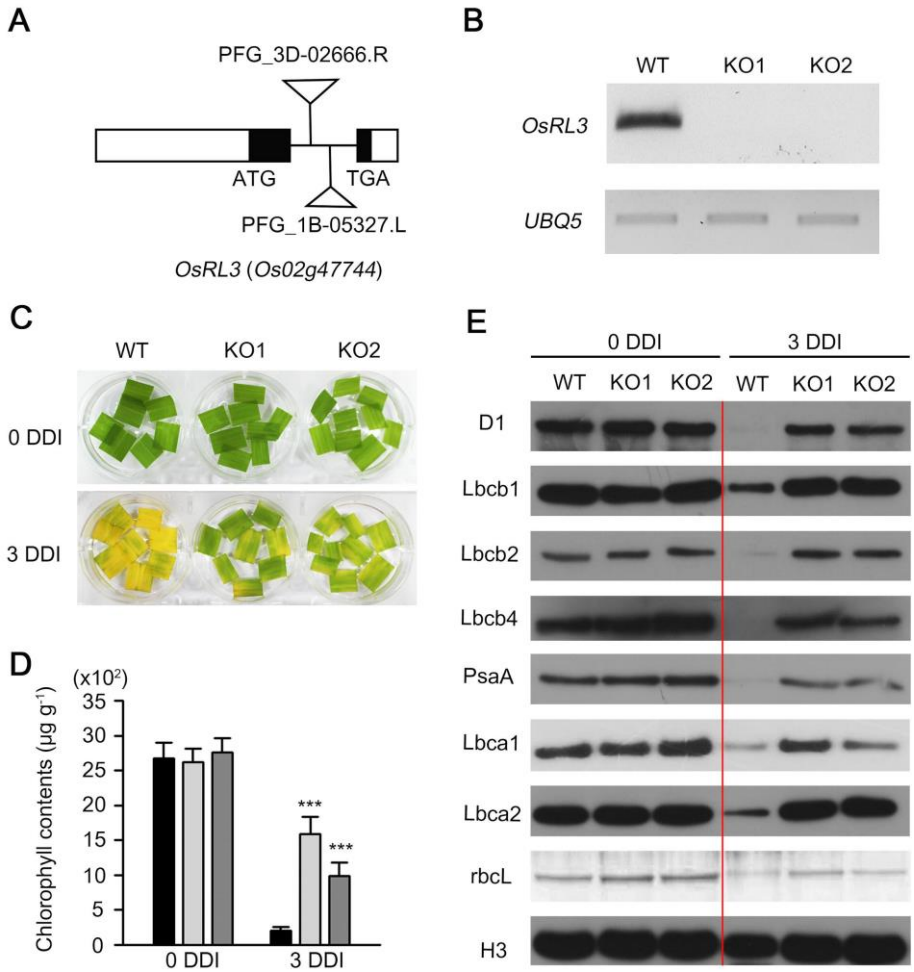

Figure 3. *OsRL3* shows stay green during dark induced senescence.

(A) T-DNA insertion sites in the *OsRL3* gene structure. (B) *OsRL3* expressions in T-DNA mutants was verified by semi-qRT PCR. (C) WT and two individual *OsRL3* knock-out plants grown for 2 month under natural long day condition were transferred to darkness as a leaf disk shapes at 28℃ for 3 DDI. The changes of detached leaf color in the leaf discs of the WT and *osrl3* during dark induced senescence. The incubation was going on 3mM MES (pH 5.8) buffer with the abaxial side up at 28℃ in darkness, then sampled at the specified 0 DDI and 4 DDI for each experiment. (D) Changes

in total Chl contents of detached leaves WT and *osrl3* mutants after 0 and 3 DDI. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological sample (Student's t-test, ***P<0.001) (E) Antibodies against PSII antenna (Lhcb1, Lhcb2 and Lhcb4), PSI antenna (Lhca1 and Lhca2), PSII core (D1) and PSI core (PsaA) proteins were used for detection, and the Rubisco large subunit (RbcL) was detected by Coomassie brilliant blue staining.

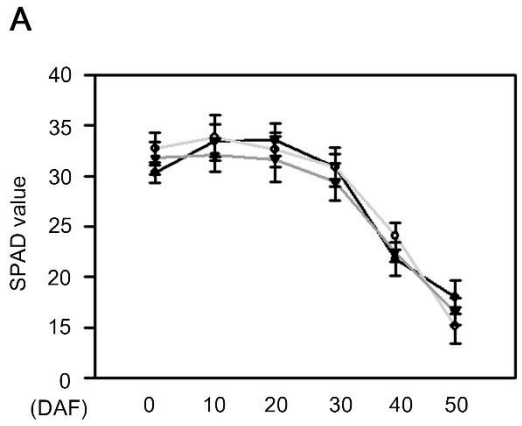

Figure 4. OsRL3 T-DNA line phenotype in natural field.

(A) Changes of total Chl level was measured through SPAD value (D) in the WT and *OsRL3* during grain filling stage (0–50 DAF). The mean and SD were obtained from more than five biological replicates. Black, gray and dark gray lines represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. These experiments were repeated twice with similar results. WT, wild type; DAF, day (s) after flowering.

OsRL3 regulates expression of several SAGs.

Leaf senescence is accompanied with decreasing expression of photosynthesis genes and increasing expression of senescence-associated genes (SAGs). In a leaf senescence process, several TFs regulate downstream genes such as SAGs. To examine the regulatory mechanism of *OsRL3* in senescence, we used qRT-PCR to identify expression transition of senescence-associated genes (SAGs) in dark. We found that chlorophyll catabolic enzyme (CCE) genes *OsNOL*, OsNYC1, *OsPAO* and *OsRCCR* are strongly down-regulated in *osrl3* mutants at 3DDI. We also examined whether there is expressional changes of senNACs in *osrl3* mutants. The senNAC transcription factors potentially includes *OsNAP*, *OsORE1*, *SNAC1*, *OsNAC5* and *OsNAC6*. Among these genes, expression of *OsNAP* was substantially decreased in the *osrl3* mutants at 3DDI. Then, we observed changes in other major SAGs such as *OsSGR1* and *OsNAP*. Similar to other SAGs that were mentioned earlier, these two genes expression was dramatically down-regulated in mutants (Figure 5). This result indicates that *OsRL3* regulates SAGs which leads the *osrl3* to stay green during DIS.

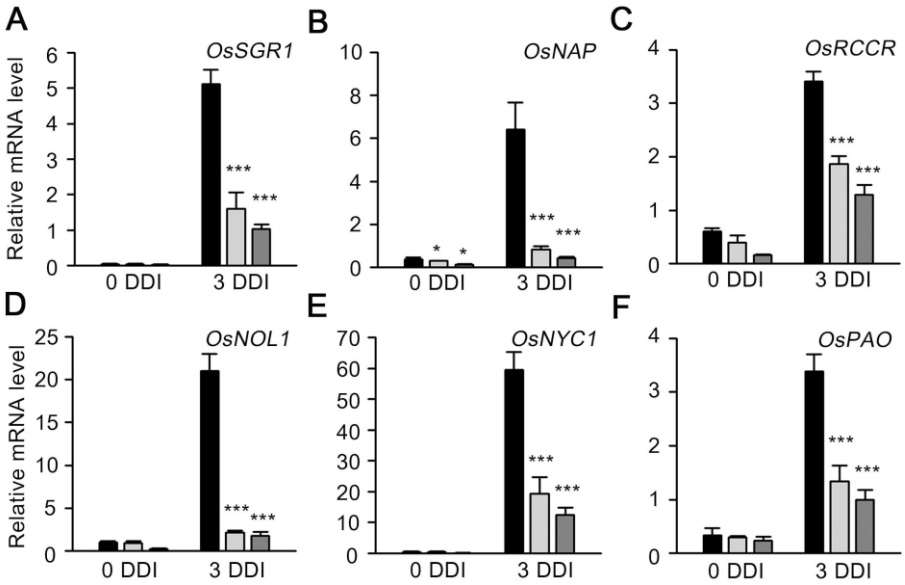

Figure 5. Expression patterns of SAGs.

Expressional Plants grown in 2- month at long day condition transferred to darkness at 28℃ as leaf disk forms, and incubated in complete darkness.

Total RNA from dark treated leaf disks was used for RT-qPCR. (A, B) Relative expression levels of SAGs (*OsSGR1*, *OsNAP*). (C-F) Transcription levels of CCE genes (Os*RCCR*, Os*NOL, OsNYC1, OsPAO*). The each gene's relative mRNA levels were determined by RT–qPCR and normalized to transcript levels of *OsUBQ5*. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

***OsRL3* responds to Salt stress.**

As the *OsRL3* transcript expression is induced by multiple abiotic stresses especially in salt stress treatments, we exposed 200 mM NaCl to 2-week WT and *osrl3* mutants, planted in the same pot for each mutant line. Under salt stress treatment, *osrl3* mutants have sensitive phenotypes with over 40% lower survival rate than WT (Figure 6A and 6B). We measured the stress level of each plant by ion leakage and MDA contents. Following a sensitive phenotype, ion leakage of *osrl3* mutants is significantly lower than wild type after the salt stress treatment (Figure 6C). Under abiotic stressed conditions, ROS is accumulated in plant tissues leading oxidative damage, indicated by the membrane lipid peroxidation. Malondialdehyde (MDA) is a lipid peroxidation product and regarded as a biomaker for membrane lipid peroxidation. A shown in Figure 6D, salt treatments enhanced MDA production in the both WT and mutants; however, mutants contained much more melandehydrate (MDA) than WT. It suggests that *osrl3* mutants are sensitive to salt stress in the vegetative phase.

In plant, there are three common adaptive mechanism for plant responses to stress condition, ion homeostasis, water homeostasis and damage repairing system (Verslues et al., 2006). *OsRL3* regulates proline biosynthesis genes, OsP5CS1 and OsP5SC2 involved in water homeostasis regulation. However, the expression levels of ion homeostasis-related genes (OsHKT1;1 and OsHKT1;5) and damage repairing system-related genes (CATb and APX5) showed non-significant differences between the *osrl3* and WT under salt stress condition. In abiotic stress condition, some NAC TFs regulate stress responsive genes. SNAC1 and OsNAC6 were downregulated in *osrl3* mutants under salt stress condition (Figure 7). This result indicates that *OsRL3* responds to salt stress via regulation of water homeostasis and NAC TFs.

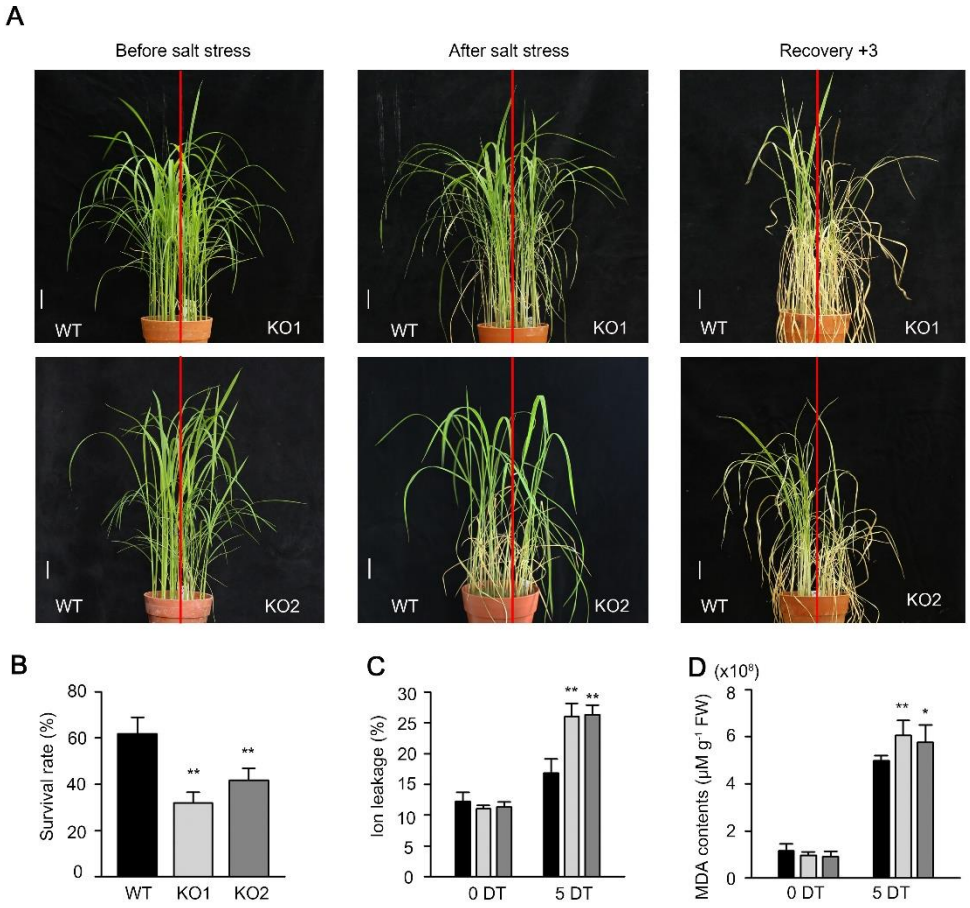

Figure 6. The osrl3 mutants increases salt stress response.

(A) Mimic physiological dehydration stress tolerance assay of the *osrl3* mutants and WT plants under 200 mM NaCl treatment. The plant grew in natural long day for 3-weeks (21 days) and transfered to 200 mM NaCl solution for 5 day. Then we recovered with pure water for 3 days. (B) Survival rates of transgenic and WT plants testing in (A). Values are means 6 SE (n = 3). (C-D) Ion leakage (C) and Malondialdehyde (MDA) (D) of stress treated plants. ion leakage and MDA contents were measured in the second leaves of salt stress treatment conditions. (B-D) The mean and SD were obtained from three biological replicates. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

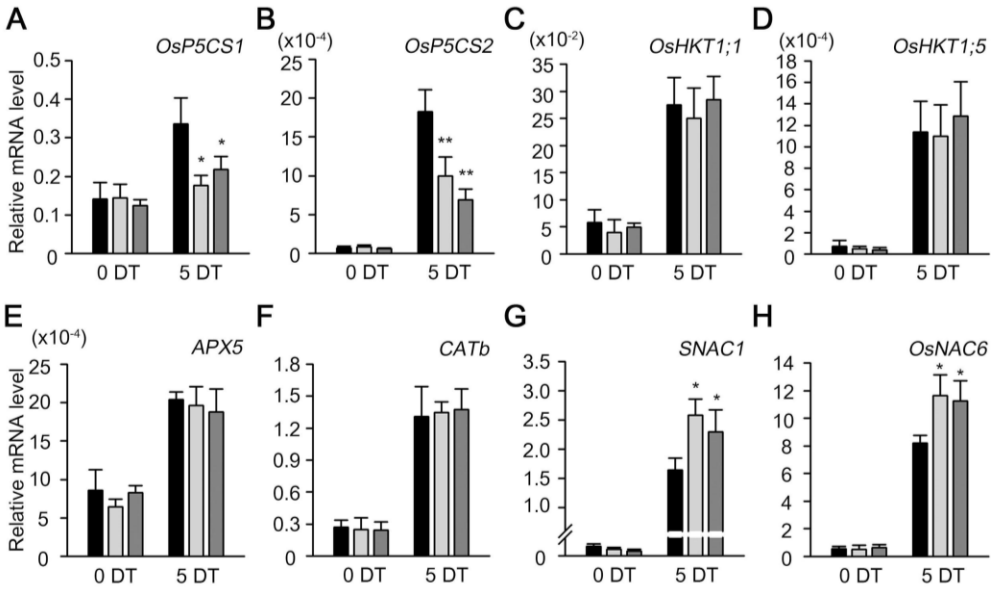

Figure 7. Transcript transition of genes involved in salt stress response mechanism.

Expression of salt stress responsive genes, water homeostasis genes (A-B), ion homeostasis genes (C-D), damage repairing system associated genes (E-F), and salt stress responsive TF (G-H) were determined in *OsRL3* knock out mutants and WT. The qRT-PCR analysis was performed with total RNA from *OsRL3* plants and WT under 0 DT and 5 DT after salt stress treatments. DT, day (s) after treatment. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

The *osrl3* mutants are insensitive to ABA by regulation of ABA signaling genes.

Certain hormones are able to hasten or repress senescence (Jibran et al., 2013). We verified transcriptional changes of *OsRL3* under various hormone treatments such as 1-aminocyclo-propane-1-carboxylic acid (ACC), absicisic acid (ABA), and methyl-jasmonic acid (MeJA). 10-day old WT plants were used and each of the samples was obtained 1, 3, 6, and 12 hours after the treatments, respectively. *OsRL3* expression level is stable at hormone treatments except ABA. High value was maintained for 1 to 12 hours after ABA treatments (Figure 8A). To examine the ABA treated phenotype, hormone-induced senescence was conducted with leaf discs from 3-week old WT and *osrl3*. Leaf discs were floated on the each hormone-treated MES buffer (pH 5.7) at the continuous light, 50 mM ACC, 3 μM ABA, 100 μM MeJA, 100 μM GA, and 100 μM IAA . We found that leaf discs of *osrl3* mutants show stay green only in ABA treatment (Figure 8B and Figure 9). Additionally, higher chlorophyll contents of the mutants proved that *osrl3* mutants showed stay green during ABA induced senescence (Figure 8C).

ABA induces visible yellowing and genes, which directly response to those stresses and hormones. Next, we examined expressional changes of ABA signaling genes from ABA, dark, and stress treated leaf by qRT-PCR. ABA signaling genes were generally divided into two groups, which are ABA early responsive genes and ABA late responsive genes. As an ABA early responsive gene, *OsRK1* and *OsPP2C* is involved. Plus, *OsRAB16C*, *OsRAB16D*, and *OsRAB21* act as ABA late responsive genes. Expression levels of the four ABA signaling genes, *OsRK1* and *OsRAB16D*, were significantly decreased under exogenous ABA treatment in *osrl3* mutants (Figure 8D-E). OsRK1 is significantly down-regulated after DIS in *osrl3* mutants (Figure 30A). Furthermore, an ABA late responsive genes, OsRAB16D dramatically decreased by dark incubation (Figure 10B). In a salt stress treatment, transcription levels of *OsRK1* and *OsRAB16D* in *osrl3*

mutants down-regulated in 5 DT (Figure 10C-D). In addition, the expression of other ABA late responsive genes, *OsLEA3*, *OsRAB21*, and *OsRAB16C*, was also decreased in each treatment (Figure 11). However, transcript levels of ABA biosynthesis genes did not change in DIS and salt stressed condition (Figure 12). This result strongly suggested that *OsRL3* regulates expression of the ABA signaling genes.

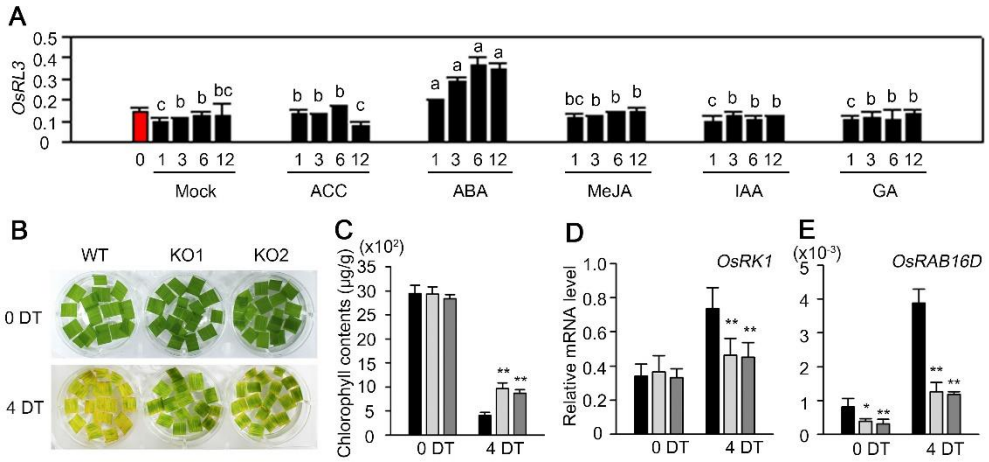

Figure 8. OsRL3 response to ABA treatment.

(A) Various hormone, such as 1-aminocyclo-propane-1-carboxylic acid (ACC), abscisic acid (ABA), and methyl jasmonic acid (MeJA), were treated at 10-day-old WT plants. Every treated hormone concentration was treated 100 μM. The means and SDs were obtained from three biological replicates. and sampled at 0, 3, 6 and 12 HT, for RT–qPCR analysis using leaf tissue. The relative expression levels of *OsRL3* were normalized to transcript levels of OsUBQ5. (B) Delayed senescence was induced by ABA in the mutants. Leaf disks of the 4-week plants were treated by 1 mM ABA under continuous light condition. MES buffer (pH 5.7) were used as a control. The detached leaves were floated on MES buffer as a control and 1 mM ABA. Total chlorophyll (C) examined at each points. DT, Day after treatment (D-E) Transcriptional levels of ABA signaling genes, OsRK1 and OsRAB16D under ABA treatment were determined by qRT-PCR. The relative mRNA levels were normalized to transcript levels of OsUBQ5. (C-E) Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

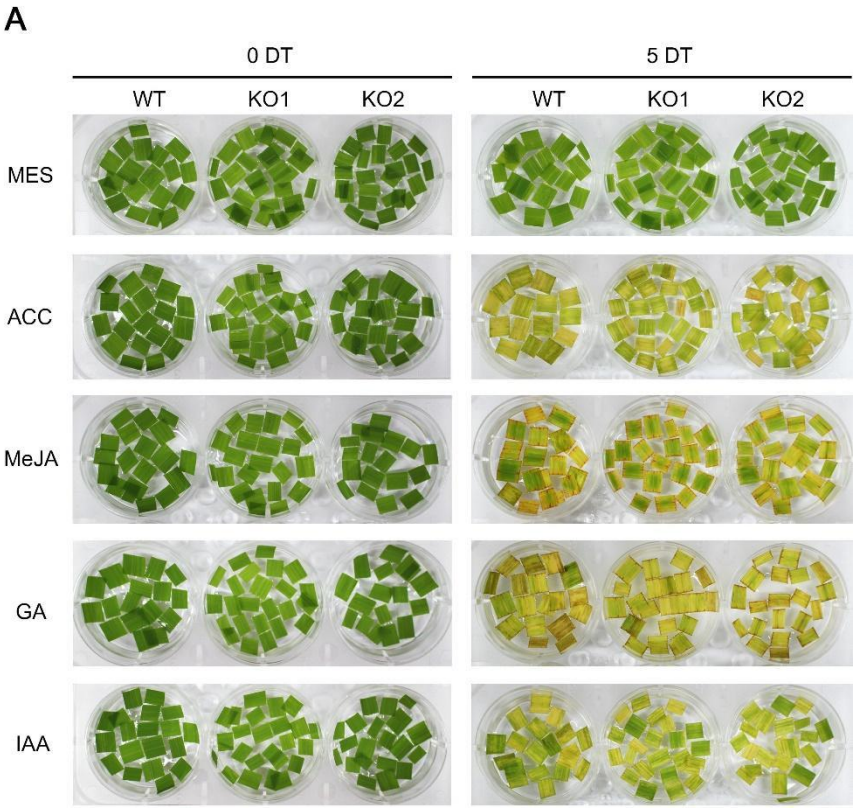

Figure 9. Phenotype of the *osrl3 mutants* under hormone induced senescence.

(A) Hormone induced senescence. Leaf disks of the 4-week plants were treated by 50 mM ACC, 100 μM MeJA, 100 μM GA, and 100 μM IAA under continuous light. 3 mM MES buffer (pH 5.7) were used as a control.

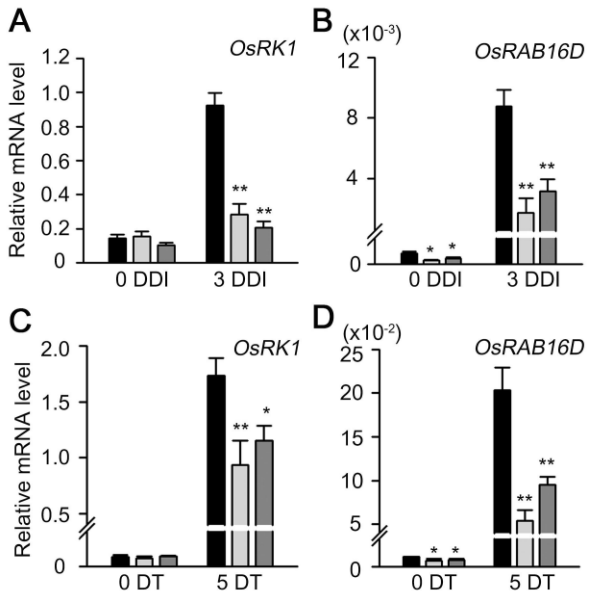

Figure 10. Transcriptional changes of ABA signaling genes.

Transcription levels of ABA signaling genes under senescence and stress condition. (A, B) Relative expression levels of OsRK1 (A) and OsRAB16D (B) in DIS. (C-D) Under high salinity stress condition, the expression levels of ABA signaling genes, OsRK1 (C) and OsRAB16D (D). The each gene's relative mRNA levels were determined by RT–qPCR and normalized to transcript levels of OsUBQ5. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

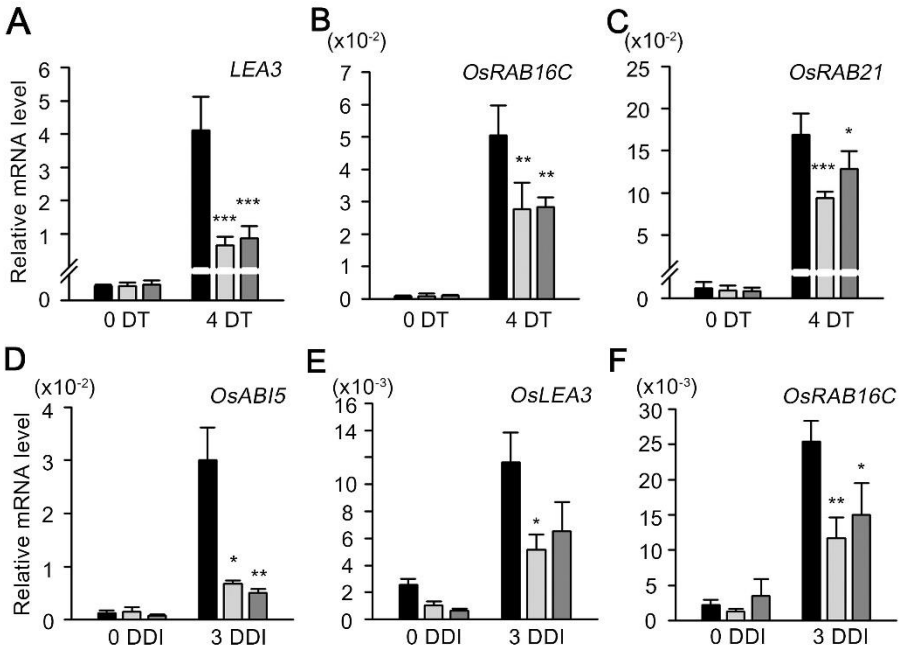

Figure 11. Transcript levels of others ABA signaling and biosynthesis genes under ABA- and dark-induced senescence.

(A-C)Transcription levels of ABA signaling genes, *OsLEA3*, *OsRAB16C*, and *OsRAB21*, in *osrl3* mutants and WT under ABA treatment. The qRT-PCR analysis was performed with total RNA from *osrl3* plants and WT at 0 DT and 4 DT. (D-F) Transcription levels of ABA signaling genes, *OsABI5*, *OsLEA3*, and *OsRAB16C*, in mutants and WT during DIS. The qRT-PCR analysis was performed with total RNA from OsRL3 plants and WT under 0 DDI and 5 DDI. DDI, day (s) after Dark Incubation. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

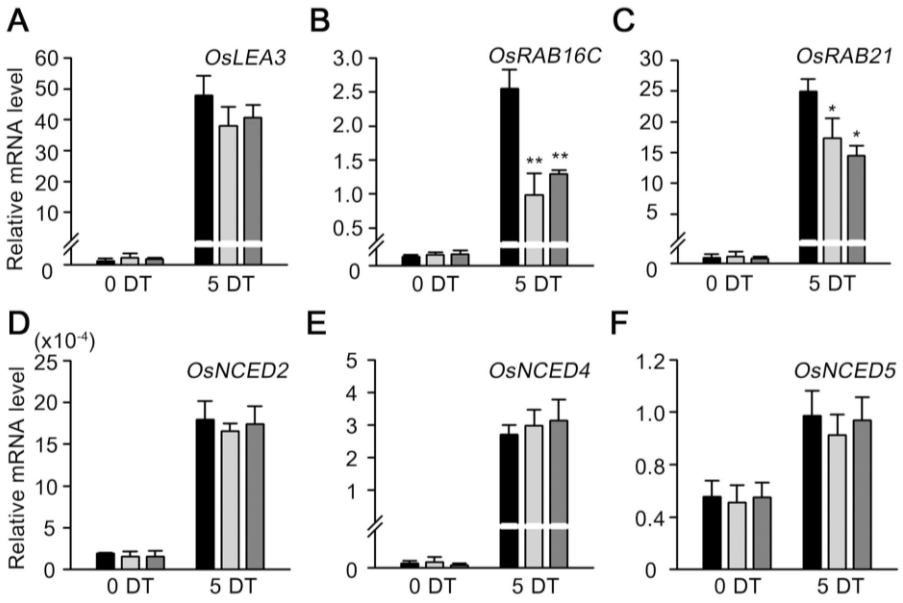

Figure 12. Transcriptional changes of ABA signaling genes and biosynthesis genes under high salinity condition.

(A-C) Expression levels of the ABA signaling genes, OsLEA3, OsRAB16C, and OsRAB21, in stressed condition. (D-F) mRNA levels of ABA biosynthesis genes, *OsNCED2*, *OsNCED4*, and *OsNCED5* in salt stress treatment by qRT PCR. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, *P<0.05, **P<0.01)

***OsRL3* directly binds to *OsNAP* and *OsRAB16D*.**

Overall, it could be considered that *OsRL3* controls DIS and salt stress response via regulation of ABA signaling genes. Therefore, we examined whether *OsRL3* directly regulates ABA signaling genes and SAGs by promoter binding assay through yeast one hybrid assay. A few genes, which are significantly downregulated by senescence and salt stress, were selected as candidate target genes of *OsRL3*. Among ABA signaling genes, transcriptional levels of *OsRK1* and *OsRAB16D*, *RAB21* and *LEA3* made a substantial differences compared with WT. In *SAGs*, *SAG12* and *OsNAP* have significant changes of expression levels. We found that the *OsRL3* protein directly binds to the promoter of *OsNAP* and *OsRAB16D*, but not to the other seven promoter regions (Figure 13A). It signified that *OsRL3* regulates ABA signaling genes by direct binding to *OsNAP* and *OsRAB16D* promoter.

To determine the epistasis within *OsNAP* and *OsRL3*, we examined transcript levels of each genes in WT, *osrl3* knock out and OsNAP overexpression (*osnap-D*) plant. The qRT-PCR analysis was performed with total RNA from 10-day old plants. Transcript levels of OsNAP in *osrl3* mutants are lower than in WT; however, the expression of *OsRL3* in *osnap-D* is similar with WT (Figure 13B-C). Expression of OsRAB16D, a downstream gene of OsNAP, is down-regulated in *osrl3* mutants and up-regulated in *osnap-D* under normal condition (Figure 14). It suggests that *OsRL3* directly regulates OsNAP and OsRAB16D in ABA signaling pathway.

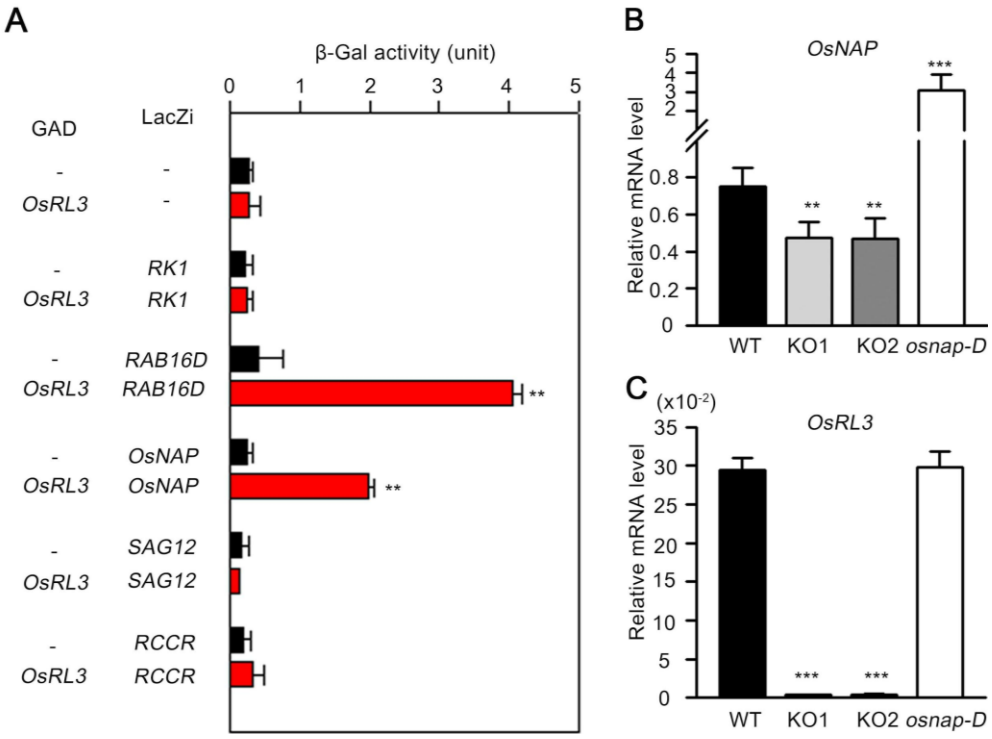

Figure 13. *OsRL3* directly regulate *OsNAP* and *OsRAB16D*.

(A)The binding activity of the *OsRL3* to the promoter of the ABA signaling genes (*OsRK1* and *OsRAB16D*) and senescence associated genes (*OsNAP, SAG12,* and *RCCR*) was examined using yeast one-hybrid assays. Each promoter was amplified with the specific primers listed in Table 2. As a negative control we used empty bait and prey plasmids (-). Means and SD were obtained from more than five independent colonies. These experiments were repeated more than three individual cells and twice with similar results. (B-C) Transcription levels of *OsNAP*, and *OsRL3* in WT, *osrl3* knock out mutants and *OsNAP* overexpression plants. Black, gray, dark gray, and white bars represent WT, *osrl3*-KO1, *osrl3*-KO2, and *osnap-D* respectively. The plants were grown in MS medium for 10 days. The mean and SD were obtained from more than three biological samples. (Student's t-test, **P<0.01, ***P<0.001)

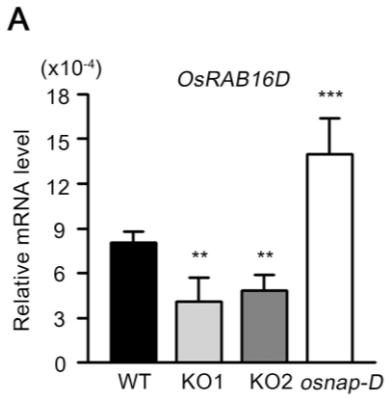

Figure 14. Expression levels of OsRAB16D in normal condition.

(A) Transcription levels of *OsRAB16D* in WT, *osrl3* knock out mutants and *OsNAP* overexpression plants. The plants were grown in MS medium for 10 days. These experiments were repeated more than three individual cells and twice with similar results. Black, gray and dark gray bars represent WT, *osrl3*-KO1 and *osrl3*-KO2, respectively. The mean and SD were obtained from more than three biological samples. (Student's t-test, **P<0.01, ***P<0.005)

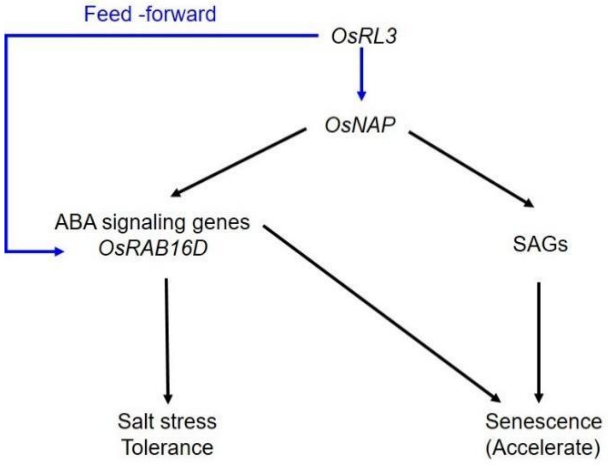

Figure 15. Proposed model for *OsRL3* function in rice leaf senescence and salt stress response mechanism.

DISCUSSION

***OsRL3* participates in the leaf senescence and stress response.**

In the rice genome, 183 MYB-encoding genes plays diverse roles in developmental processes and defense response (Yanhui et al., 2006). There is an emerging evidence to support that many MYB proteins are associated with response to abiotic stress. So far, only two genes, *MYBS3*, known as a cold stress resistance gene, and *OsMYB48-1*, known as a positive regulator of drought and salt stress, are the identified in rice MYB-related genes (Su et al., 2010; Xiong et al., 2014). Several studies of the MYB-related genes, which are involved in abiotic stress response have been identified in rice; however, the function of MYB-related genes have not been reported as a regulator of the senescence.

In this study, we investigated the function of *OsRL3* as a positive regulator of leaf senescence and abiotic stress responsive. The *osrl3* delayed senescence in dark and highly response to salt stress. In DIS, stay green appears along with expressional changes of SAGs including *SGR1*, *OsNAP*, and several CCEs in the *osrl3* mutants (Figure 5). In abiotic stress condition, plants generally resisted low water potential and related stresses by modifying water uptake and water loss, accumulating solutes and modifying the properties of cell walls. Mechanisms of ion homeostasis and water/osmotic homeostasis are intended to restore the cellular ions or water contents. In addition, plant uses the protective proteins and mechanisms to tolerate reduced water content by preventing or repairing cell damage (Ruiz-Lozano et al., 2012; Verslues et al., 2006). Under salt stress, the *OsRL3* related with water homeostasis regulating proline biosynthesis genes, *OsP5CS1* and *OsP5CS2*; however, it did not regulate other genes involved in ion homeostasis (*OHKT1;1* and *OsHKT1;5*), or damaged repairing system (*APX1* and *CATb*). In addition, *OsRL3* negatively regulated some NAC TF, a

response to abiotic stress (Figure 9). The results confirmed that *OsRL3* responded to senescence and abiotic stress along with gene expressional regulations.

***OsRL3* response to ABA among regulation of signaling genes.**

ABA plays a key role in senescence inhibitor as well as in response to various stress (Tuteja, 2007). In a recent report, some TFs are involved in these two mechanisms through ABA biosynthesis and signaling pathway. For instance, overexpression of *OsNAC2* dramatically accelerated leaf senescence while its knockdown plants showed a delay in leaf senescence via direct regulation of ABA biosynthesis genes (*OsNCED4*, *OsZEP1* and *ABA8ox1*) (Mao et al., 2017). Some studies reported that certain TFs regulate stress signal transduction mediating response by ABA-dependent and independent mechanism. *OsNAC2* regulates both abiotic stress responses and ABA-mediated responses, and acts at the junction between the ABA and abiotic stress pathways (Shen et al., 2017). MYB TFs, otherwise NAC TF, has not been studied on the functions about senescence and ABA signaling regulation in rice. Our results indicate that *OsRL3* have unique features as compared to other MYB TFs. *OsRL3* affects senescence and abiotic stress through the regulation of ABA signaling genes. It is highly expressed by exogenous ABA treatment compared to other phytohormone treatment, suggesting that it possibly play an important role in ABA signaling transduction pathway. In DIS, *OsRL3* regulates ABA signaling genes, such as *OsRK1*, *OsRAB16D*, *LEA3, OsRAB16C*, and *OsRAB21* (Figure 11). Under salinity stress, transcription levels of ABA signaling genes were changed in *osrl3* mutants (Figure 10) whereas expressions of ABA biosynthesis genes were unconverted (Figure12). It strongly suggests that *OsRL3* regulates ABA signaling genes in both leaf senescence and salt stress response mechanism. It is the first report explaining that *OsRL3,* a novel MYB related type TF, regulates ABA signaling pathway mediating

senescence and stress response.

The *OsRL3* regulatory networks leaf senescence and salt stress signaling pathways

Further analysis revealed that *OsRL3* can directly regulates SAGs and ABA signaling genes (Figure 13A). Recently, *OsNAP*, a transcriptional activator of senescence and abiotic stress response mechanism, is found to be a key gene in ABA biosynthesis and signaling pathway. During the senescence, *OsNAP* directly or indirectly regulates SAGs and CCEs, and represses ABA biosynthesis (Liang et al., 2014). On the other aspect, in abiotic stress condition, especially drought and salt stress, *OsNAP* upregulates stress response genes, including *OsPP2C* and stress-related TFs (Chen et al., 2014). Importantly, *osnap-D* showed early senescence phenotype in DIS and salt stress tolerance phenotype in high salinity condition, opposite phenotype of *osrl3* (Figure 4B and 4A). It was probably that *OsRL3* might not activate the expression of downstream genes without targeting of *OsNAP*, which indicated that *OsNAP* was the targets of *OsRL3* directly (Figure 13A). Furthermore, expression levels of *OsNAP* down-regulated in *osrl3* mutants, whereas transcript levels of *OsRL3* is stable in *osnap-D*, which indicates that *OsRL3* is epistasis to *OsNAP* (Figure 13B and 7C). In conclusion, *OsRL3* accelerates senescence and osmotic stress response via induction of ABA signaling pathway in rice.

Based on the above results, we proposed a following model for the role of *OsRL3* in leaf senescence and salt stress response pathway (Figure 15). *OsRL3* controls leaf senescence and abiotic stress response via regulation of *OsNAP*. In senescence process, *OsRL3*, regulating *OsNAP*, accelerates senescence via increasing the transcription of chlorophyll degradation genes and other SAGs. During stress condition, *OsRL3* up-regulates stress responsive genes which are downstream of ABA signaling pathway, via *OsNAP* regulation. In addition, *OsRL3* directly binds to *OsRAB16D*, one of

ABA late signaling gene (Figure 14). *OsRAB16D* is known as a representative stress-response TF, directly regulated by some TFs and ABA contents (Nakashima et al., 2014; Yamaguchi-Shinozaki et al., 1990). Following the initiation of ABA signal transduction with PYR/PYL/RCAR complex and Os*PP2C*, *OsRAB16D* is expressed by some TFs. It is possible that *OsRL3* regulates not only *OsNAP*, also downstream gene *OsRAB16D* as a feed-forward regulation. Thus, it strongly suggests that *OsRL3*, a novel MYB transcription factor, acts as a key regulator of leaf senescence process and abiotic stress response mechanism linked with ABA signaling pathway.

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초 록

ABA 는 식물의 생장 과정 중 특히 abiotic stress 에 대한 반응성에 밀접히 연관되어 있다. ABA 는 여러 환경적인 요소에 의해 합성되며 신호전달과정을 통해 잎의 노화 및 stress 에 대한 저항성 mechanism 이 작동하게 된다. 본 연구는 벼의 MYB 전사인자 중 하나인 *RADIALIS-LIKE3* (*OsRL3*)의 유전자 기능으로서 노화 및 염 민감성에 대한 연구를 수행하였다. 유전자의 발현은 암 처리를 통한 노화에서 증가하였고, 여러 abiotic stress 를 처리 한 경우 염 처리 시 발현율이 가장 크게 증가하였다. 유전자의 기능을 밝히고자 T-DNA 돌연변이체를 이용하였고, 암 처리시 돌연변이체에서 낮은 엽록체 분해율과 및 광합성 효율과 함께 stay green 표현형을 확인할 수 있었다. 또한 높은 농도의 염 처리를 했을 때, 돌연변이체에서는 염에 대한 높은 민감성을 보였고, 이는 항상성 유지에 관여하는 여러 osmolyte 중 하나인 proline 을 합성하는 유전자의 발현이 감소를 통해 나타났다. *OsRL3* 는 노화 및 stress 를 조절하는 여러 phytohormone 중, ABA 처리시 유전자의 발현이 증가하며, ABA 를 통한 노화 유도 실험을 통해 *osrl3* 돌연변이체가 암 처리를 통한 노화 실험과 동일하게 노화 지연 현상이 나타남을 알 수 있다. 또한 암 처리 노화와 염 처리시, 돌연변이체에서 ABA 신호전달 유전자의 발현이 감소하였다. 직접적인 *OsRL3* 의 유전자 조절을 확인하기 위해 Yeast one hybrid 실험을 통한 promoter binding assay 에서는 *OsRL3* 가 *OsNAP* 과 *OsRAB16D* 의 직접적인 binding 을 통해 노화 및 stress 반응 mechanism 을 조절한다는 것을 알 수 있다. 따라서 본 연구를

통해 *OsRL3* 가 전사촉진인자로서 ABA 신호전달 유전자의 발현을 촉진하여 노화과정과 염에 대한 반응성 mechanism 을 조절함을 규명한다.