A Novel Nuclear-Localized CCCH-Type Zinc Finger Protein, OsDOS, Is Involved in Delaying Leaf Senescence in Rice[1][W]

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Leaf senescence is a developmentally programmed degeneration process, which is fine tuned by a complex regulatory network for plant fitness. However, molecular regulation of leaf senescence is poorly understood, especially in rice (*Oryza sativa*), an important staple crop for more than half of the world population. Here, we report a novel nuclear-localized CCCH-type zinc finger protein, *Oryza sativa* delay of the onset of senescence (OsDOS), involved in delaying leaf senescence in rice. The expression of *OsDOS* was down-regulated during natural leaf senescence, panicle development, and pollination, although its transcripts were accumulated in various organs. RNAi knockdown of *OsDOS* caused an accelerated age-dependent leaf senescence, whereas its overexpression produced a marked delay of leaf senescence, suggesting that it acts as a negative regulator for leaf senescence. A genome-wide expression analysis further confirmed its negative regulation for leaf senescence and revealed that, in particular, the jasmonate (JA) pathway was found to be hyperactive in the *OsDOS* RNAi transgenic lines but impaired in the *OsDOS* overexpressing transgenic lines, indicating that this pathway is likely involved in the OsDOS-mediated delaying of leaf senescence. Furthermore, methyl JA treatments of both seeds and detached leaves from the RNAi and the overexpressing transgenic lines showed hyper- and hyporesponses, respectively, consistent with the negative regulation of the JA pathway by *OsDOS*. Together, these results indicate that OsDOS is a novel nuclear protein that delays leaf senescence likely, at least in part, by integrating developmental cues to the JA pathway.

Leaf senescence is a developmentally programmed degeneration process that constitutes the final step of leaf development and is controlled by multiple developmental and environmental signals (Lim et al., 2003). During senescence, leaf cells undergo dramatic changes in cellular metabolism and a sequential degeneration of cellular structures (Nam, 1997). Previous efforts to elucidate the underlying molecular mechanism of leaf senescence began with the identification of *senescence-associated genes* (*SAGs*; defined as genes with expression up-regulated during leaf senescence), and most of them are found to encode proteases, nucleases, lipases, hydrolases, enzymes involving nutrient recycling, stress-responsive proteins, and transcriptional regulators (Nam, 1997; Quirino et al., 2000; Buchanan-Wollaston et al., 2003; Gepstein et al., 2003; Lin and Wu, 2004). Analyses of expression profiles of those *SAGs* indicate that a complex regulatory network functions in leaf senescence processes (Park et al., 1998; Weaver et al., 1998; Quirino et al., 1999; He et al., 2001; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005), implying that some key regulators may take active roles in regulating senescence. In fact, several senescence-associated regulatory genes have recently been identified. They are predicted to encode transcription factors such as *WRKY6* (WRKY DNA-binding protein 6; Robatzek and Somssich, 2001) and *WRKY53* (Hinderhofer and Zentgraf, 2001; Miao et al., 2004) in Arabidopsis (*Arabidopsis thaliana*), and receptor-like kinases such as the Arabidopsis *SIRK* (senescence-induced receptor-like kinase; Robatzek and Somssich, 2002) and the bean (*Phaseolus vulgaris*) *SARK* (senescence-associated receptor-like kinase; Hajouj et al., 2000). However, only a very limited number of the regulators were functionally confirmed through genetic approaches to date (Woo et al., 2001; Jing et al., 2002; Miao et al., 2004).

Although leaf senescence occurs in an age-dependent manner, the initiation and progression of senescence can be influenced by a variety of plant hormones (Hensel et al., 1993; Gan and Amasino, 1997; Lim et al., 2003). Cytokinins have been found to play a role in delaying leaf senescence (Gan and Amasino, 1995; McCabe et al., 2001; Kim et al., 2006), whereas ethylene, jasmonates (JAs), brassinosteroids, and salicylic acids are known to promote the process of senescence

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(Grbic and Bleecker, 1995; Oh et al., 1997; Morris et al., 2000; He and Gan, 2001; He et al., 2002; Jing et al., 2002; Yin et al., 2002). For example, JA could rapidly induce the expression of several *SAGs* (Park et al., 1998; Schenk et al., 2000). The Arabidopsis *SAG101* encodes an acyl hydrolase, which is involved in degradation of lipid and might be responsible for release of α-linoleic acid that could be used for the synthesis of JA. Antisense suppression of *SAG101* retarded the progression of leaf senescence, whereas its overexpression had a promotive role (He and Gan, 2002). Treatment of JA usually causes premature senescence in both attached and detached leaves in wild-type Arabidopsis but failed to induce precocious senescence in JA-insensitive mutant *coi1* plants (He et al., 2002). Recent comparative transcriptome analysis revealed that the JA pathway was clearly active in age-dependent leaf senescence as well as dark-induced and starvation-induced ones (Buchanan-Wollaston et al., 2005). However, little is known about the regulation, especially the developmental regulation, of the JA pathway in leaf senescence.

So far, the molecular control of leaf senescence in rice (*Oryza sativa*) remains largely unknown, though the rice is an important model plant and feeds more than half of the world population (Sasaki and Burr, 2000). Current understanding on leaf senescence in rice is limited to physiological and cytological studies, characterization of several senescence up-regulated genes (Inada et al., 1998, 1999; Lee et al., 2001, 2004; Hung and Kao, 2004; Tang et al., 2005), rudimentary quantitative trait loci-based genetic analysis (Jiang et al., 2004; Abdelkhalik et al., 2005), and the isolation and characterization of a premature mutant (Li et al., 2005) and stay-green mutants (Cha et al., 2002). Importantly, previous studies revealed that 60% to 90% of total carbon in the panicles at harvest is derived from photosynthesis after heading (Mae, 1997), and that leaf senescence during reproductive and ripening stages is directly related to biomass production and grain yield of rice crop (Ray et al., 1983). Therefore, deciphering the molecular control of leaf senescence and delaying senescence during the late growth period will be important for genetic improvement of rice grain yield. Here, we report the isolation and functional characterization of a novel nuclear-localized C-x8-C-x5-C-x3-H (CCCH)-type zinc finger protein designated *Oryza sativa* delay of the onset of senescence (OsDOS) in rice. We provide evidence to show that OsDOS acts as a negative regulator for leaf senescence in rice, at least in part mediated by the JA pathway.

RESULTS

Identification and Structural Features of OsDOS Protein

Previously, we constructed a 10 K cDNA microarray (http://plantbiol.genetics.ac.cn) that was used to monitor gene expression profiles, and subsequently found that 253 cDNAs exhibited differential expression ($e \pm 2$-fold changes) during pollination (Lan et al., 2004). Among them, a pollination-repressed cDNA y712d12 (accession nos. CR293108 and CR293228) encoding a putative zinc finger protein was selected for further functional studies. Homology search in GenBank (http://www.ncbi.nlm.nih.gov) revealed that it is an intronless gene encoding a predicted protein of 386 amino acids in length with a calculated molecular mass of 42.5 kD. Database search also revealed that it contained two tandem CCCH-type zinc-binding motifs, separated by 18 amino acids. The CCCH-type proteins belong to an unusual family of zinc finger proteins containing tandem zinc-binding motifs characterized by three Cyss followed by one His (Varnum et al., 1991; Blackshear, 2002). They have been shown to be associated with RNA metabolism in various organisms (Brown, 2005; Hall, 2005). Best known among them is the TTP, which is an RNA-binding protein that binds to AU-rich elements in the 3′ untranslated regions of tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor mRNAs for degradation of the messages, and thus regulates the level of protein expression (Carballo et al., 1998, 2000). In Arabidopsis, HUA1 functions in floral reproductive organ identity by binding *AGAMOUS* pre-mRNA and facilitating its processing (Li et al., 2001; Cheng et al., 2003).

The two zinc finger motifs of OsDOS were compared with other members of this class (Fig. 1A). The spacing between the highly conserved Cys and His residues is variable, as is the length of the linker between the two zinc finger domains. In Arabidopsis, only one CCCH motif is found in the ZFWD subfamily (Terol et al., 2000), whereas HUA1 has six tandem CCCH motifs (Li et al., 2001). So far, to our knowledge, none of the predicted CCCH-type zinc finger proteins has been studied in rice.

Subcellular Localization of OsDOS

To examine the subcellular localization of OsDOS, a *green fluorescent protein* (*GFP*) reporter gene was fused in frame to the last codon of the OsDOS coding region to produce an OsDOS-GFP fusion protein in transgenic rice plants. As shown in Figure 1B, the OsDOS-GFP green fluorescent signal was detected predominantly in the nuclei of the transgenic rice plants. In total, we have produced five transgenic plants and similar results were observed in all of them (data not shown). In onion (*Allium cepa*) epidermal cell transient expression assays, the OsDOS-GFP also was found to be in the nucleus, whereas GFP alone was present throughout the cell (data not shown). These results indicate that OsDOS is a nuclear-localized protein and presumably functions as a transcript regulator as predicted for other CCCH-type proteins. However, no known nuclear localization signal (NLS) sequences were detected in OsDOS, suggesting that there may be an unidentified

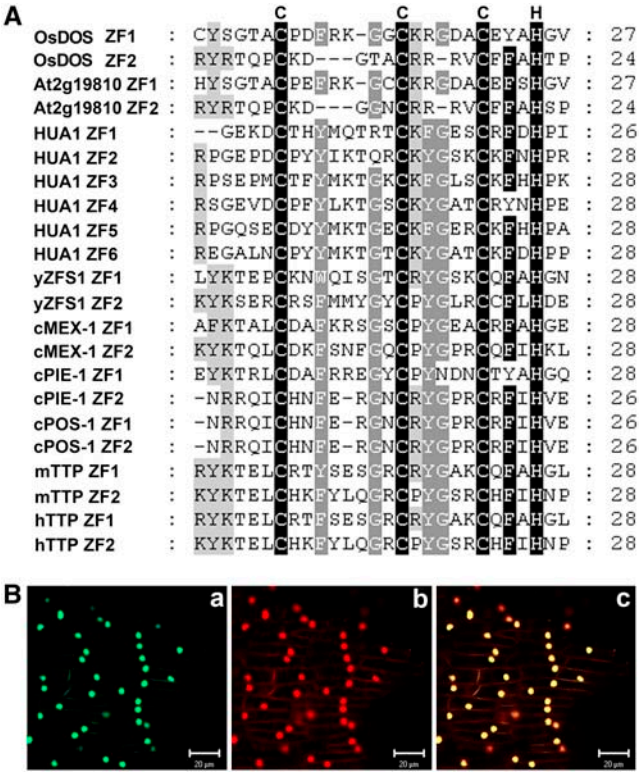

Figure 1. Structural feature and nuclear localization of the OsDOS protein. A, A Clustal W alignment of two zinc finger motifs (ZF1 and ZF2) of OsDOS with zinc fingers from other CCCH-type zinc finger proteins. Identical and similar residues are shaded with dark and light gray, respectively. Zinc fingers (numbers given behind the protein names) are from rice OsDOS (Os01g09620), Arabidopsis At2g19810 and HUA1 (NP_187874), yeast (*Saccharomyces cerevisiae*) ZFS1(P47979), *C. elegans* MEX-1 (U81043), PIE-1 (AAB17868), and POS-1 (T37246), mouse TTP (S04743), and human TTP (P26651). B, Nuclear localization of OsDOS. The figure shows the OsDOS-GFP green fluorescence in the nuclei of root cells in the transgenic rice plant (a), the same cells stained with propidium iodide (b), and their merged image (c). Bars = 20 μm.

NLS in OsDOS or it may be imported into the nucleus through an NLS-independent mechanism.

Expression Patterns of *OsDOS*

To examine the expression profile of *OsDOS*, real-time PCR analysis was performed. The *OsDOS* transcripts were detected throughout the plant, in panicles and leaves with a higher expression at the early stages (Fig. 2). Interestingly, the temporal expression profiles of the *OsDOS* transcripts indicated a down-regulation of the *OsDOS* gene expression during natural leaf senescence (Fig. 2, A and B). Down-regulation of the *OsDOS* expression also occurred during the panicle development process. In addition, the expression level of *OsDOS* was reduced after pollination (Fig. 2C), consistent with our previous results (Lan et al., 2004).

To further examine tissue-specific expression patterns of *OsDOS*, we conducted RNA in situ hybridization analysis. Strong *OsDOS* expression was detected in the leaf primordium, incipient leaf, and shoot meristem, but weak expression was observed in the elder leaf tissue (Fig. 2D).

Taken together, these results suggest that *OsDOS* is developmentally regulated and probably functions during leaf and panicle development as well as pollination.

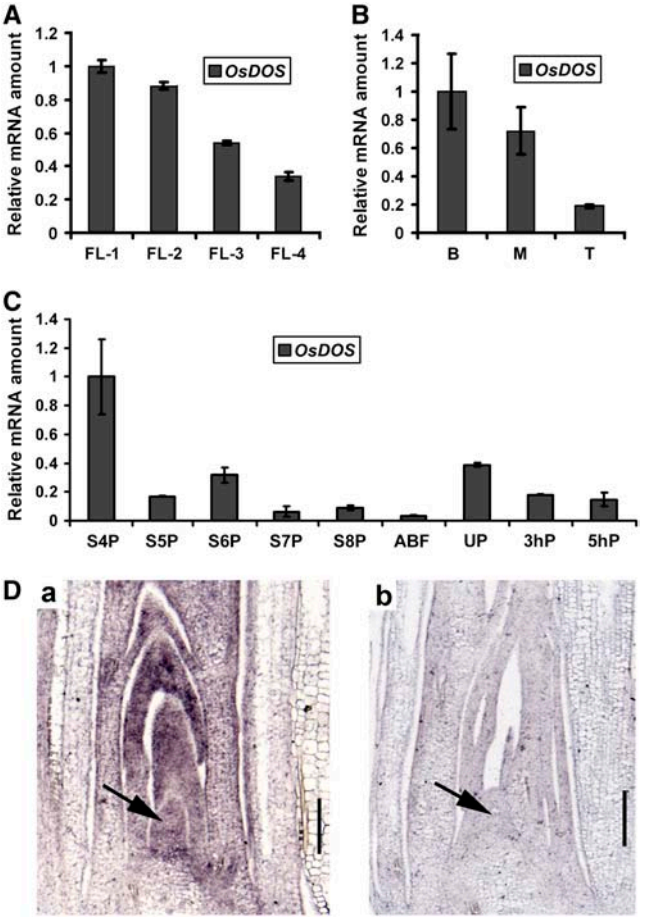

Figure 2. Expression patterns of *OsDOS*. A, *OsDOS* expression in leaves at different growth stages. FL-1, 2, 3, and 4 were flag leaf at booting, heading, grain-filling, and seed maturation stage, respectively. B, *OsDOS* expression in a different part of a senescent leaf (flag leaf at grain maturation stage). B, Base; M, middle; T, tip. C, *OsDOS* expression during panicle development process and pollination. Panicles were from stages 4 (immature panicle at stamen and pistil primordium differentiating stage, S4P), 5 (immature panicle at pollen mother cell formation stage, S5P), 6 (immature panicle at pollen mother cell meiotic division stage, S6P), 7 (immature panicle at pollen-filling stage, S7P), and 8 (mature panicle at heading stage, S8P). Anthers before flowering (ABF) and unpollinated pistils (UP) were dissected from mature florets at heading stage. 3hP and 5hP represent the pollinated pistils 3 and 5 h after pollination, respectively. Primers specific for *OsDOS* were used in real-time PCR analysis (A, B, and C); the rice *18 s rRNA* was used as an internal control. D, In situ localization of *OsDOS* transcripts in the shoot apex of three-leaf stage rice seedling. Longitudinal sections of the shoot apex were hybridized with digoxigenin-labeled antisense *OsDOS* RNA probes (a). A section was hybridized with the sense *OsDOS* RNA probe as a negative control (b). Arrows indicate the region of the shoot apical meristem. Bars = 100 μm.

RNAi Knockdown of *OsDOS* Expression Caused an Accelerated Age-Dependent Leaf Senescence

To examine the function of *OsDOS* in vivo, we utilized RNAi interference to silence the expression of *OsDOS*. An RNAi construct driven by a maize (*Zea mays*) ubiquitin promoter (Fig. 3A) was introduced into rice, and four independently transformed lines were generated (see Supplemental Fig. 1A). Further real-time PCR analysis showed that the transcription of *OsDOS* was drastically reduced in all the four transgenic lines (line i-1, 0.39 ± 0.10; line i-2, 0.28 ± 0.05; line i-3, 0.31 ± 0.08; line i-4, 0.14 ± 0.04; and wild type, 1.00 ± 0.11; Fig. 3B). In the sequenced rice genome, *Os05g10670*, another CCCH-type zinc finger gene, is found to be most similar to *OsDOS* with a nucleotide identity of 70%. We examined the expression of *Os05g10670* in the *OsDOS* RNAi lines, showing that *Os05g10670* expression did not exhibit a significant difference between the RNAi lines and the wild-type control. The result indicated that the *OsDOS* RNAi construct did not affect the expression of endogenous *Os05g10670* (see Supplemental Fig. 1, B and C), indicating a gene-specific effect of the RNAi construct used. To further confirm the inheritance of the transgene, we detected its expression in both T_1 lines and the wild-type control. Real-time PCR analysis showed that the *OsDOS* transcripts were sharply reduced in the T_1 lines compared with the wild-type control (line i-2, 0.12 ± 0.03; line i-3, 0.30 ± 0.05; line i-4, 0.23 ± 0.05; and wild type, 1.00 ± 0.16), indicating that the reduction of *OsDOS* in the transgenic lines was stably inherited into their T_1 progeny (Fig. 3C).

To examine possible phenotypes of the transgenic lines, T_1 progeny of the *OsDOS* RNAi lines and the wild-type plants were grown in the field under identical conditions. Forty T_1 progeny from each of the four independent T_0 lines were cultivated for the phenotypic analysis. The RNAi T_1 lines appeared normal during the vegetative growth. However, beginning at the time of late booting stage, slight but consistent leaf yellowing was clearly observed in the RNAi lines and then an accelerated leaf senescence occurred compared with that in the wild-type plants. At the grain-filling stage, striking leaf yellowing appeared in the whole plant, except the flag leaf showing a slightly less yellowing, and the senescence symptom was correlated with the reduction of the expression level of *OsDOS*. Compared with the *OsDOS* RNAi lines, the wild-type plants stayed much more green (Fig. 4A). Further genetic analysis showed that the T_1 progeny of both lines i-3 and i-4, which were generated from the T_0 lines containing a single copy insertion, conformed to a segregation ratio of 3:1 (early senescence phenotype to wild-type phenotype), 31:9 ($\chi^2 = 0.0333$, $P = 0.866$), and 33:6 ($\chi^2 = 1.444$, $P = 0.229$), respectively. These results suggest that *OsDOS* plays a negative role in leaf senescence.

Since the loss of chlorophyll associated with leaf yellowing is one of the typical symptoms of leaf senescence (Oh et al., 1997), the time-course changes in the chlorophyll content of the upper five leaves were compared between the RNAi T_1 lines and wild-type plants after heading. To precisely evaluate the leaf senescence, the chlorophyll measurements were performed at five identical time points beginning at heading date, and the comparisons were conducted between age-matched RNAi T_1 and the wild-type leaves. Compared with the wild-type plants, the RNAi T_1 leaves displayed a slight chlorophyll loss at 1 week after heading (WAH). With the progress of grain filling, chlorophyll loss in the RNAi T_1 leaves sharply accelerated. Remarkably, compared with the wild-type control, the earlier senescence (chlorophyll loss) sequentially occurred in the top five leaves; the younger the leaf, the

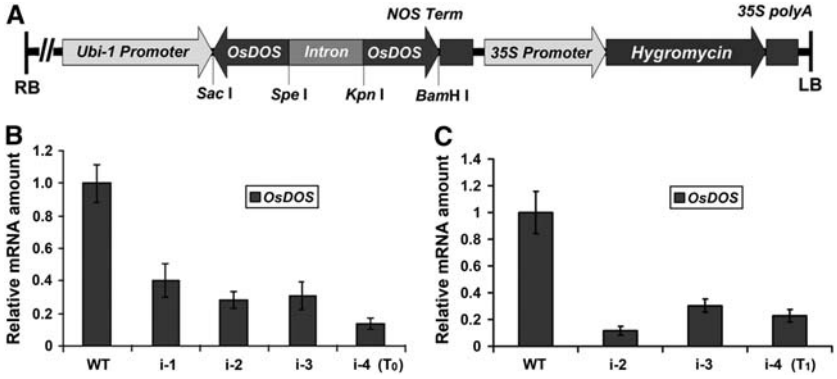

Figure 3. Molecular analyses of the *OsDOS* RNAi transgenic lines. A, A schematic representation of the RNAi construct of *OsDOS* used for rice transformation. The hairpin structure consisting of an antisense *OsDOS* fragment, a rice intron, and the sense *OsDOS* fragment was inserted between the maize ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303. B, Reduced *OsDOS* expression in the RNAi T_0 lines. Flag leaf total RNA samples of the four independent RNAi T_0 plants and the wild-type (WT) control at heading stage were used for real-time PCR analysis. C, Reduced *OsDOS* expression in the RNAi T_1 lines. Fully expanded flag leaf total RNA samples of the three RNAi T_1 lines and the wild-type control at the booting stage were used for real-time PCR. The samples are biological replicates of those in Affymetrix GeneChip analysis (see below).

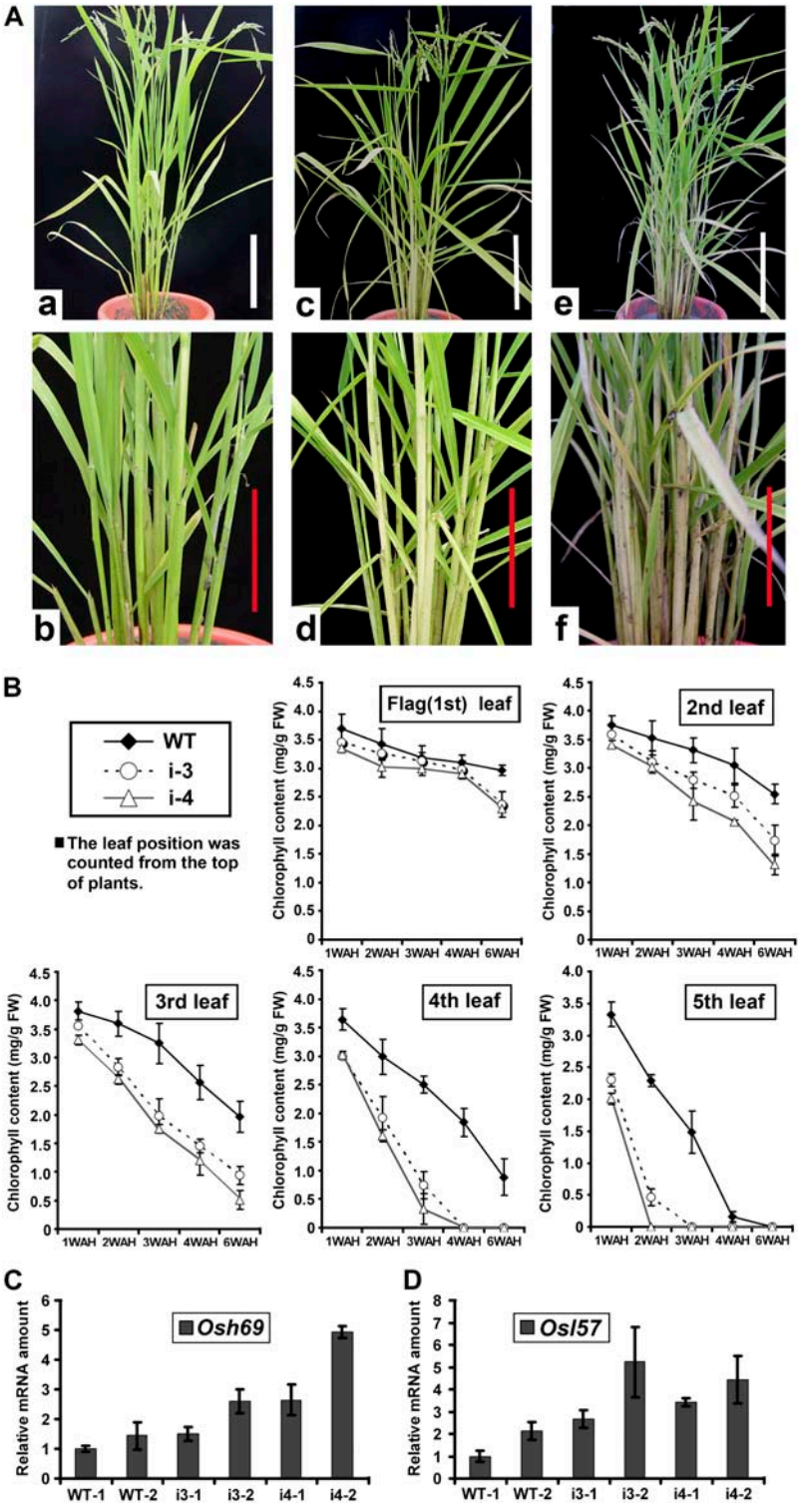

Figure 4. The *OsDOS* RNAi T_1 plants showed accelerated age-dependent leaf senescence. A, Gross morphology of two *OsDOS* RNAi T_1 lines and the wild-type plants at grain-filling stage. a and b, The wild-type plant. c and d, The RNAi line-3 (i-3). e and f, The RNAi line-4 (i-4). b, d, and f are the basal part close ups of a, c, and e, respectively. The plants grew in the field under identical conditions. White bars = 25 cm; red bars = 15 cm. B, The time-course changes in the chlorophyll content of the upper five leaves from the main culm were compared between the RNAi T_1 lines (i-3 and i-4) and the wild-type plants after heading. Results are shown as mean ± SD of at least three replicates. FW, Fresh weight. C and D, Expression of two SAGs, *Osh69* (C) and *Osl57* (D), in the RNAi lines and the wild-type plants at 6 WAH stage. The first leaf (1) and second leaf (2) were harvested for the real-time PCR analysis. The leaf position was counted from the top of plants. Accession numbers: AF251068 (*Osh69*) and AF251076 (*Osl57*).

less the differences between the RNAi lines and the wild type (Fig. 4B). In addition, the senescence physiology was well correlated with the expression level of *OsDOS*. The results clearly indicated that the early senescence occurred in the *OsDOS* RNAi lines in an age-dependent manner. Moreover, two selected SAGs, *Osh69* and *Osl57* (Lee et al., 2001, 2004), were expressed at higher levels in the RNAi leaves compared with the age-matched wild-type leaves, indicating that specific molecular events were in correlation with the overall physiology (Fig. 4, C and D). Together, these results indicated that knockdown of *OsDOS*

caused an accelerated age-dependent leaf senescence and suggested that *OsDOS* plays a negative regulatory role in developmental senescence.

Premature senescence would lead to poor grain quality and the yield loss of crop plants (Jiang et al., 2004). Compared with the wild-type control, the *OsDOS* RNAi lines appeared shorter in statue and had slightly earlier flowering; in addition, they showed shorter panicle and slightly reduced seed setting (see Supplemental Table I).

Overexpression of the *OsDOS* Caused a Marked Delay of Leaf Senescence

To further explore the function of *OsDOS*, we introduced its coding sequence (CDS) into rice under the control of the maize ubiquitin promoter (see Supplemental Fig. 2A). In total, 21 independently transformed lines were generated (see Supplemental Fig. 2B) and could be arranged into three phenotypic groups: normal lines (overexpressing [OX] lines 1, 4, 10, and 17) that were indistinguishable from the untransformed wild-type plants; mild lines (OX lines 5–9, 11–13, 15, and 18–21) that displayed a delay in growth and partly exserted panicles; and strong lines (OX lines 2, 3, 14, and 16) that showed a severe delay in growth and enclosed panicles.

To examine the expression of the *OsDOS*, the inflorescences from eight transgenic lines (OX lines 1–3 and 5–9) that represented the three phenotypic series and the wild-type plants were used for real-time PCR analysis. The result showed that the transcription of the *OsDOS* in seven T_0 lines (OX lines 2, 3, and 5–9) was sharply increased. Among them, two lines (OX lines 2 and 3) showed a higher expression level than that of the rest. However, the expression of the *OsDOS* in line 1 (OX1) was indistinguishable from the untransformed wild-type plant (wild type, 1.00 $\pm$ 0.18; line 1, 1.13 $\pm$ 0.19; line 2, 40.1 $\pm$ 7.1; line 3, 46.0 $\pm$ 4.5; line 5, 31.3 $\pm$ 5.6; line 6, 31.4 $\pm$ 8.6; line 7, 31.2 $\pm$ 9.8; line 8, 33.3 $\pm$ 0.9; and line 9, 33.6 $\pm$ 3.5; Fig. 5A). The results indicated that the different phenotypes were correlated with the increased expression levels of the transgene.

Interestingly, the *OsDOS* OX lines displayed an extended leaf longevity (Fig. 5, B–D). Given a delay in growth of the OX lines, to precisely evaluate the leaf senescence, the time-course changes in the chlorophyll content of the age-matched upper four leaves were compared between the RNAi T_1 lines and wild-type plants. The result showed that the *OsDOS* OX lines exhibited a marked delay of leaf senescence (Fig. 5E). In addition, induction of *Osh69* and *Osl57* were delayed in the OX lines (Fig. 5, F and G). Darkness is one of the most potent external stimuli for inducing leaf senescence. To gain further insight into the role of *OsDOS* on leaf senescence, dark treatment on detached leaves was performed. The result showed that, compared with the wild-type control, the dark-induced leaf senescence was accelerated in the RNAi lines (data not shown), but delayed in the OX lines (Fig. 6, A and B). Moreover, induction of *Osl57* was also delayed in these leaves (Fig. 6C). Taken together, these results indicated that the overexpression of *OsDOS* directly affected various senescence-associated symptoms, suggesting that *OsDOS* normally functions to negatively regulate leaf senescence in rice.

In addition, the *OsDOS* OX lines showed a delay in growth, shorter stature, abnormally developed panicle, deferred heading, and severe sterility. We also found that the florets of the *OsDOS* OX lines failed to open or scarcely opened after heading. Further phenotypic analysis showed that defects in anther dehiscence and pollen viability may be responsible for the severe sterility of the OX lines (see Supplemental Fig. 3). Together, these results indicated that the ectopic overexpression of *OsDOS* has a pleiotropic impact on rice development.

Global Gene Expression Changes Regulated by *OsDOS*

Based on the potential function in mRNA processing and developmental down-regulation of the *OsDOS*, we predicted that the genes directly regulated by *OsDOS* would be up-regulated in the *OsDOS* RNAi lines and down-regulated in the *OsDOS* OX lines, respectively. To dissect possible pathways in which OsDOS is involved, we used Affymetrix GeneChip analysis to assess global gene expression changes mediated by *OsDOS*. The result revealed that 214 genes with putative annotations showed reproducible 2.5-fold changes of up-regulation in the two RNAi T_1 lines. Among them, 68 genes were down-regulated genes with at least 2-fold changes and 101 genes did not show significant changes in the OX lines (see Supplemental Table II). Of the 214 up-regulated genes in the RNAi lines, the majority encoded proteins involved in degradation of macromolecules, transport, as well as oxidative damage and defense responses such as lipases, acyl hydrolases, and many types of proteases, including Cys, Ser, and aspartyl proteases, which are involved in the degradation of the respective macromolecules during leaf senescence (Thompson, et al., 2000; He and Gan, 2002; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005). To further confirm whether these genes are truly involved in leaf senescence, we monitored global gene changes during natural leaf senescence (expression profile comparison between nascent leaves and senescent leaves in the wild-type plants). The result showed that 81 of the 214 genes were up-regulated in senescent leaves with at least 2-fold changes. Together, these results indicate that *OsDOS* is involved in the leaf senescence program in rice (see Supplemental Table II).

Among the 68 genes that showed differential expressions in opposite directions as postulated, most notably, a number of genes involved in the JA pathway, including JA-biosynthetic genes encoding putative lipases, lipoxygenases, 12-oxophytodienoate reductases, and JA-responsive genes encoding proteinase

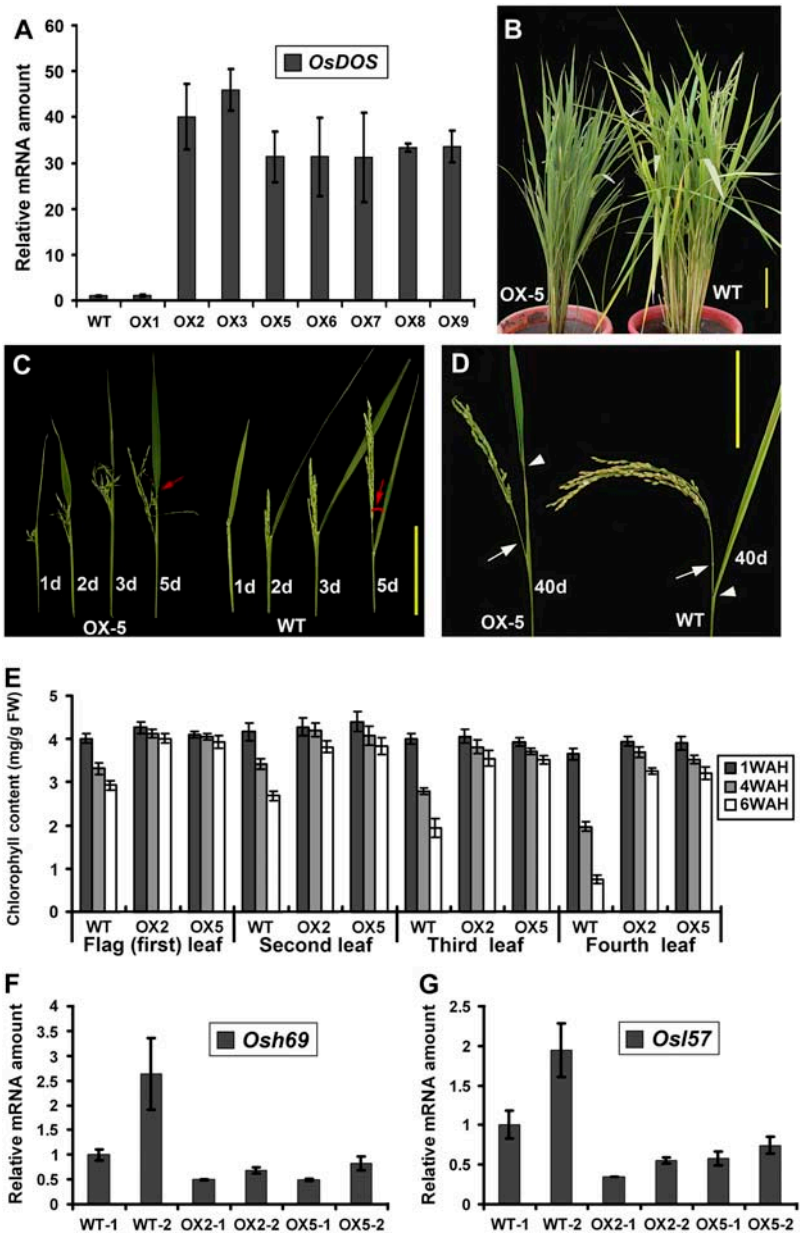

Figure 5. Marked delay of leaf senescence displayed by the *OsDOS* OX transgenic plants. A, Overexpression of the *OsDOS* mRNA transcription in the OX T_0 lines. Panicle total RNA samples of the OX lines and the wild-type control at heading stage were used for real-time PCR analysis. B, Gross morphology of the OX line and the wild-type plant grown in the field under identical conditions. The OX line exhibited dwarfism and marked delay of leaf senescence. Bar = 10 cm. C and D, Comparison of leaf senescence symptom and panicle morphology between the OX line and the wild-type plant at 1, 2, 3, and 5 (C) and 40 d (D) after heading, respectively. The OX line showed a strikingly delay of senescence in flag leaf, partly exerted panicle, as well as sterility. The red arrows indicate the flag leaf angle. The white arrows indicate the position of the panicle base and the white arrowheads the position of the lamina joint of the flag leaf. Bars = 10 cm. E, The time-course changes in the chlorophyll content of the upper four leaves were compared between the OX lines and the wild-type plants after heading. FW, Fresh weight. Results are shown as mean ± SD of at least three replicates. F and G, Expression of *Osh69* (F) and *Osl57* (G) in the OX lines and the wild-type plants at 6 WAH stage. The first leaf (1) and second leaf (2) were harvested for the real-time PCR analysis.

inhibitors, thionins, and thaumatin-like proteins, were up-regulated in the *OsDOS* RNAi lines. By contrast, most of them were down-regulated or did not show a significantly differential expression in the *OsDOS* OX lines (see Supplemental Table III). Further promoter analysis showed that the majority have one to approximately eight cis-acting regulatory elements involved in methyl JA (MeJA) responsiveness (see Supplemental Table III). To confirm the results of the GeneChip analysis, three known genes involved in JA pathway, *OsLOX8* (Peng et al., 1994), *OsAOC1* (Agrawal et al., 2003a), and *OsOPR1* (Agrawal et al., 2003b), were used for real-time PCR analyses (Fig. 7, A–C). The results showed that the expression profiles obtained by the GeneChip analysis were similar to those obtained by real-time PCR analysis in the three selected genes. In addition, real-time PCR analysis showed that the transcription of *OsDOS* was not significantly altered during MeJA treatment (Fig. 7D), indicating that *OsDOS* itself is not a JA-responsive gene. Taken together, these results indicate that the JA pathway was hyperactive in the *OsDOS* RNAi lines but impaired in the *OsDOS* OX lines, suggesting that *OsDOS* likely regulates the JA pathway.

Negative Regulation of the JA pathway by *OsDOS*

To gain further insight into the cross talk between *OsDOS* and JA pathway, MeJA treatment assay was performed on the transgenic plants with the altered

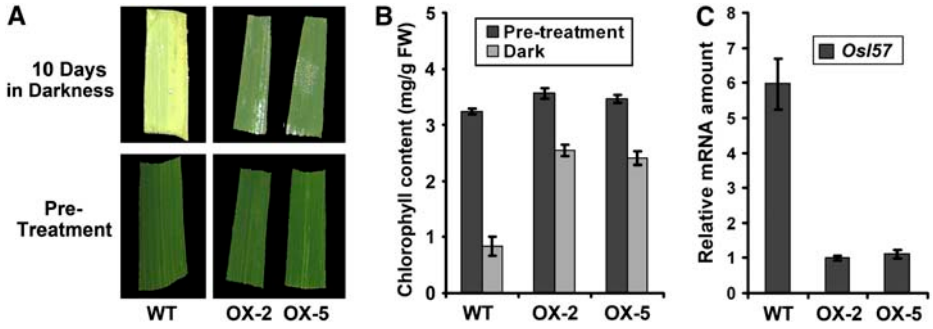

Figure 6. Delay of dark-induced leaf senescence displayed by the *OsDOS* OX lines. A, The *OsDOS* OX leaves showed a delay of dark-induced leaf senescence. Detached flag leaves from the OX lines and wild-type plants at heading stage were incubated with water for 10 d in darkness. B, Chlorophyll contents of the leaves corresponding to A. Results are shown as mean ± SD of at least three replicates. C, Expression of *Osl57* in the detached leaves incubated with water for 10 d in darkness of A.

OsDOS expression levels. The age-matched leaves from the *OsDOS* RNAi, *OsDOS* OX, and wild-type plants were detached and mock treated or treated with 100 μM MeJA for 4 d in darkness. Under the treatment for 4 d, the *OsDOS* RNAi leaves showed a severe senescence symptom, but the JA-induced senescence was delayed in the *OsDOS* OX leaves (Fig. 8, A and B). Further expression analysis showed that expression of *Osl57* was well correlated with the differential senescence symptoms (Fig. 8C). In addition, seed germination assay also showed that severe inhibition of root elongation occurred, even at a low MeJA concentration, in the *OsDOS* RNAi lines (Fig. 8, D and E). Together, these results showed that MeJA treatments of both seeds and detached leaves from the RNAi and the OX transgenic lines showed hyper- and hyporesponses, respectively, suggesting that JA pathway is negatively regulated by *OsDOS*.

DISCUSSION

OsDOS Encodes a Nuclear Protein Required for Delaying the Leaf Senescence

Leaf senescence is an integral part of plant development, and multiple interconnected pathways appear to form a regulatory network to control leaf senescence (Gan and Amasino, 1997). So far, only a limited number of the regulators have been identified through molecular and genetic approaches, such as AtWRKY53, a WRKY protein, and ORE9, an F-box protein (Hinderhofer and Zentgraf, 2001; Woo et al., 2001; Miao et al., 2004). Most of them act positively during leaf senescence. In this study, we have found that *OsDOS* acts as a novel negative regulator during leaf senescence in rice. This role is consistent with its down-regulated expression during natural leaf senescence (Fig. 2, A and B). *OsDOS* encodes a CCCH-type zinc finger protein without known signal peptide. Apparently, unlike the cytoplasm localization of Pie-1, Mex-1, and Pos-1, three *Caenorhabditis elegans* proteins with similar zinc fingers (Guedes and Priess, 1997; Tabara et al., 1999; Tenenhaus et al., 2001), OsDOS was found to be a nuclear-localized protein (Fig. 1B) similar to HUA1, a nuclear-localized CCCH-type zinc finger protein in Arabidopsis (Li et al., 2001). The nuclear localization and the presence of the CCCH-type zinc finger motif of the OsDOS protein imply that OsDOS may play a regulatory role, dissimilar to AtWRKY53 (Miao et al., 2004) and ORE9 (Woo et al., 2001), during leaf senescence. To our knowledge, OsDOS is the first CCCH-type

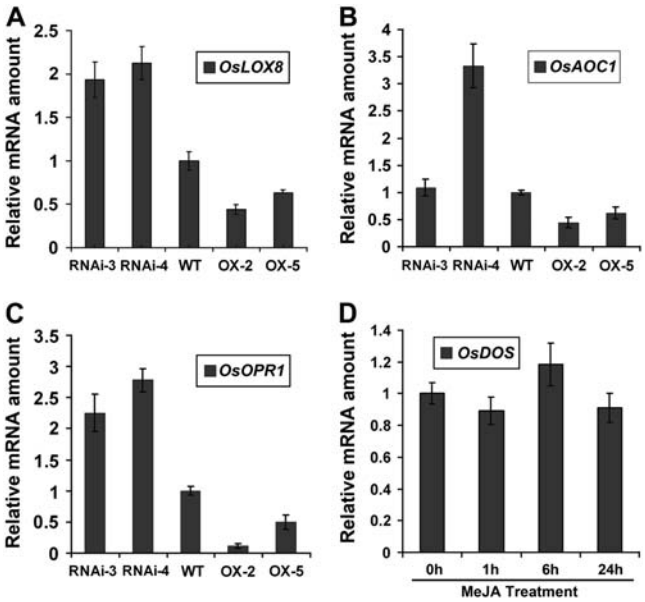

Figure 7. Real-time PCR analyses for the expression profiles of three JA-biosynthetic genes, *OsLOX8* (A), *OsAOC1* (B), and *OsOPR1* (C) in the *OsDOS* RNAi, OX, and wild-type plants and the expression of *OsDOS* in response to MeJA (D). Flag leaves at heading stage from the two RNAi lines (i-3 and i-4), two OX lines (OX-2 and OX-5), and the wild-type plants were used in A, B, and C. Three-leaf stage wild-type seedlings were treated with 100 μM MeJA for 0 and 1 h, 6 and 24 h, respectively; total leaf RNA of the four time points were used in D. The The Institute for Genomic Research Rice Gene Index (OGI) of *OsDOS* and three selected genes: *OsDOS*, Os01g09620; *OsLOX8*, Os08g39840; *OsAOC1*, Os03g32310; and *OsOPR1*, Os06g11290.

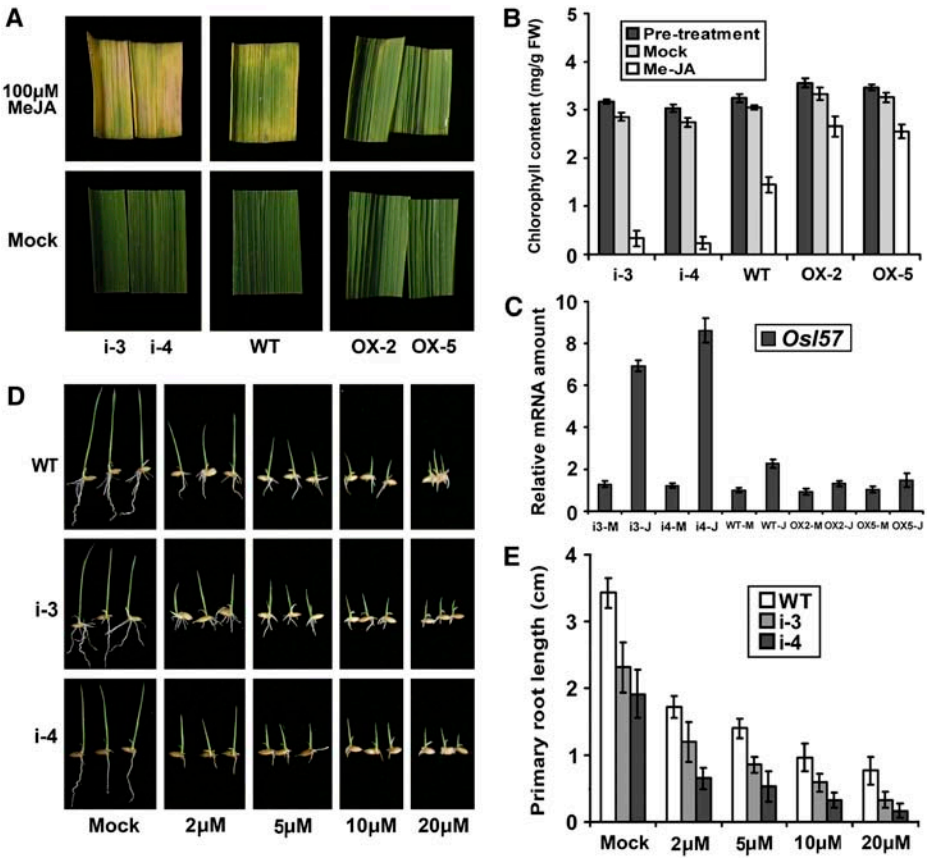

Figure 8. Differential MeJA responsiveness of the *OsDOS* RNAi, *OX* lines, and wild-type plants. A, Detached flag leaves from the *OsDOS* RNAi, OX lines, and wild-type plants at heading stage were mock treated (water) or treated with 100 μM MeJA for 4 d in darkness. B, Variable chlorophyll contents of the leaves of the pretreatment and different treatments corresponding to A. Results are shown as mean ± SD of at least three replicates. C, Expression of *Osl57* in the leaves under different treatments corresponding to A. M, Mock treatment; J, JA treatment. D, Identically sprouted seeds from both the *OsDOS* RNAi lines and wild-type plants grown for 5 d on the half-strength Murashige and Skoog plates without (mock) or with MeJA of the indicated concentrations. E, Primary root lengths of the 5-d-old seedlings in the different growth conditions corresponding to D. Measurements were performed using at least 30 germinated seeds. Results are shown as mean ± SD of at least three replicates.

zinc finger protein that is identified and functionally characterized in rice, and this is the first report showing that a CCCH-type zinc finger protein plays a role during leaf senescence in plants. In addition, OsDOS also may be involved in regulating the panicle development and pollination/fertilization process because the *OsDOS* RNAi lines showed a reduced seed setting, and the *OsDOS* OX lines displayed abnormal panicle development and sterility. However, its molecular mechanisms in regulating theses processes remain to be established.

The JA Pathway Is Involved in the OsDOS-Mediated Senescence Process

We have shown that RNAi knockdown of *OsDOS* caused accelerated age-dependent leaf senescence (Fig. 4). However, leaf senescence is influenced not only by age-dependent internal factors but also by a range of other internal and environmental factors, such as phytohormones, darkness, drought, pathogen attack, and oxidative stress (Hensel et al., 1993; Gan and Amasino, 1997; Quirino et al., 2000). Many studies have revealed that the JA signaling is involved in the regulation of leaf senescence and JAs have a role in promoting leaf senescence (Park et al., 1998; Schenk et al., 2000; He et al., 2002; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). In this study, first, we have found that the JA pathway was hyperactive in the *OsDOS* RNAi lines but impaired in the *OsDOS* OX lines (Fig. 7, A–C; Supplemental Table III), strongly suggesting that the JA signaling is implicated in the OsDOS-mediated negative regulation of leaf senescence. Second, further methyl MeJA treatments of both seeds and detached leaves from the RNAi and the OX transgenic lines showed hyper- and hyporesponses (Fig. 8), respectively, consistent with the negative regulation of the JA pathway by *OsDOS*.

However, it remains to be determined how *OsDOS* and the JA signaling pathway cross talk. It is known that the JA pathway can be triggered and regulated by multiple stresses and developmental cues (Turner et al., 2002; Devoto and Turner, 2003; van der Graaff et al., 2006). WIPK, a mitogen-activated protein kinase, was proposed as a positive regulator upstream of the JA pathway. When wild-type tobacco (*Nicotiana tabacum*) plants were mechanically wounded, activation of WIPK was required for triggering the JA-mediated wounding signal transduction cascade (Seo et al., 1995, 1999). JAs also affect a variety of plant developmental processes including leaf senescence (Creelman and Mullet, 1997). However, developmental regulation of JA remains largely unknown. Given the fact that a regulatory role for the JA pathway by *OsDOS* and its developmentally down-regulated expression, it may represent a factor mediating the integration of developmental

cues to the JA pathway. It is possible that developmental cues or age-related factors could influence the age-dependent senescence by initiating the senescence program and further activating downstream signaling cascade, including the JA pathway. Similarly, onset of leaf death1 (OLD1) might function as a repressor for integrating ethylene action into leaf senescence, because the *old1-1* mutant displayed a phenotype with an earlier onset of senescence, which was further accelerated by ethylene exposure in an age-dependent manner (Jing et al., 2002).

It is unclear how *OsDOS* acts during leaf senescence. The presence of the CCCH-type zinc fingers in OsDOS provides a clue to its potential molecular function. Many proteins with the similar zinc fingers have been shown to be RNA-binding proteins and to be associated with RNA metabolism in various organisms, such as the murine TTP (Carballo et al., 1998, 2000) and the Arabidopsis HUA1 (Li et al., 2001; Cheng et al., 2003). Thus, based on the nuclear localization of the OsDOS protein, we propose that OsDOS possibly plays a role at posttranscriptional level in delaying leaf senescence in rice. Potential targets of OsDOS might be key positive elements that are required to promote the leaf senescence program. During senescence, the plant cells should perceive and process senescence signals such as age and other internal and external factors. Receptor-like kinases can potentially function as a key component in the perception of senescence signals and in subsequent phosphorylation cascades involved in leaf senescence programs (Lim and Nam, 2005). Interestingly, our transcriptional profiling analysis revealed several transcripts, encoding putative Leu-rich repeat receptor-like kinases and putative protein kinases, upregulated in the *OsDOS* RNAi lines but suppressed in the *OsDOS* OX lines (see Supplemental Table II). However, additional studies are required to confirm whether these transcripts are potential targets of OsDOS and encode predicted positive elements promoting leaf senescence.

In conclusion, our results strongly suggest that *OsDOS* acts as a novel negative regulator for leaf senescence by integrating developmental cues to senescence signaling cascades including the JA pathway. Elucidation of possible OsDOS targets will help to understand how these two processes are orchestrated. Given a significant correlation between leaf senescence during reproductive and ripening stages and grain yield potential in rice (Ray et al., 1983), it is also worthwhile to test whether enhanced expression of *OsDOS* in an age-dependent manner has a positive impact on grain yield by delaying the onset of leaf senescence.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rice (*Oryza sativa* subsp. *japonica* var. Nipponbare) was used for various experiments in this study, including expression analysis, transformation, and MeJA treatment. Transgenic plants (RNAi T_0, T_1, T_2 lines, and overexpression T_0 lines) and the untransformed wild-type control were grown in the field under natural conditions within two growing seasons, respectively. The first growing season was from May to October, in the experimental farm of the Institute of Genetics and Developmental biology, Beijing, China. The second growing season was from December to next April, in an experimental farm in Hainan, China.

RNA in Situ Hybridization

RNA in situ hybridization was performed as previously described (Lai et al., 2002). A fragment of approximately 400 bp that was amplified from 3′ untranslated region of *OsDOS* with primers 5′-GAGGAGGAGAAGGTG-GCGGTAAG-3′ and 5′-GCAACAAACGATGAAC AGAGGATAAAC-3′ was cloned into pGEM-TEasy vector. Plasmid DNA was linearized by use of *Sal*I and *Nco*I, and then transcribed in vitro with T7 and SP6 RNA polymerase, respectively. The digoxigenin-labeled antisense and sense riboprobes were synthesized using a DIG northern starter kit (catalog no. 2039672, Roche) according to the manufacturer's instructions. These riboprobes were hydrolyzed into small fragments of about 150 nucleotides in length.

Shoot apexes of rice seedlings at the three-leaf stage were fixed with formalin-acetic acid-alcohol fixative solution at 4°C overnight followed by dehydration steps, and then embedded in paraffin (Paraplast Plus, Sigma). The tissues were sliced into 8 μm sections with a microtome (Leica RM2145) affixed to microscope slides. Images were observed under bright field through a microscope (Leica DMR) and photographed using a Micro Color CCD camera (Apogee Instruments).

Plasmid Constructs

OsDOS RNA Interference

A fragment of approximately 450 bp was amplified from *OsDOS* with two primers, 5′-GG GGTACC ACTAGT CGTCCTCGCCGCTGGC-3′ (*Kpn*I and *Spe*I sites underlined) and 5′-CG GGATCC GAGCTC TCCGGACTCCA CCCTCTGG-3′ (*Bam*HI and *Sac*I sites underlined), containing two restriction enzymes at their 5′ ends, respectively. The plasmid was constructed as previously described (Wang et al., 2004). The hairpin structure consisting of an antisense *OsDOS* fragment, a rice intron, and an *OsDOS* sense fragment was inserted between the maize (*Zea mays*) ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303 (see Fig. 3A). The construct was completely sequenced to ensure that it did not contain PCR or cloning errors.

OsDOS Overexpression

The *OsDOS* CDS was inserted between the maize ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303. The *OsDOS* CDS was PCR amplified by Pyrobest DNA polymerase (TaKaRa) using the wild-type genomic DNA as template. The PCR primers used were as follows: 5′-AAGGATCCATGATGATGATGGGG-3′ (*Bam*HI site underlined) and 5′-GGACTAGTTCAGTTGATGAGGTC-3′ (*Spe*I site underlined). The resulting PCR product was digested with *Bam*HI and *Spe*I, and was inserted between the maize ubiqutin1 promoter and the nopaline synthase terminator of the vector pTCK303. The construct was completely sequenced to ensure that it did not contain PCR or cloning errors.

OsDOS-GFP Fusion

The whole CDS of *OsDOS* was amplified using two primers (5′-CGGGATCCATGATGATGATGGGGGAAGG-3′, *Bam*HI site underlined, and 5′-CAAAGCTTGCAGTTGATGAGGTCGGACA CC-3′, *Hind*III site underlined). The resulting PCR product was subcloned into a rebuilt vector pCAMBIA 1301 to generate *p1301-OsDOS-GFP* containing an *OsDOS-GFP* fusion construct under the control of the maize ubiquitin1 promoter. The construct was sequenced to verify the in-frame fusion and no nucleotide mutations.

Plant Transformation and Generation of Transgenic Plants

Plant transformation was performed as previously described (Ge et al., 2004). Briefly, rice embryonic calli were induced on scutella from germinated

seeds and transformed with strain EHA105 of *Agrobacterium tumefaciens* containing the desired binary vector. Transgenic plants were selected in half-strength Murashige and Skoog medium containing 50 mg L^{-1} hygromycin (Roche, 843555). Hygromycin-resistant plants from calli, defined as transgenic plants of T_0 generation, were transplanted into the field. T_0 plants were further used for Southern-blot analysis. For phenotypes, analysis of the *OsDOS* OX lines, T_0 generation, and the untransformed control were transplanted into the field and grown under identical conditions. For phenotypic analysis of the *OsDOS* RNAi lines, seeds of both the *OsDOS* RNAi T_0 lines and the wild-type control were germinated and then transplanted into the field under identical conditions. The *OsDOS* RNAi T_1 lines were identified and confirmed from T_0 progeny by β-glucuronidase staining and real-time PCR analysis. *OsDOS* RNAi T_2 lines were selected in the same way. For analysis of localization of OsDOS-GFP, hygromycin-resistant *Ubi*::*OsDOS-GFP* T_0 lines were confirmed by β-glucuronidase staining and were then used for observation.

Real-Time PCR

Total RNA preparation and real-time PCR were performed as previously described (Lan et al., 2004). In brief, reverse transcription was performed using TaqMan Reverse Transcription Regents kit (Applied Biosystems). The cDNA samples were diluted to 5 and 1.25 ng/μL. Triplicate quantitative assays were performed on 1 μL of each cDNA dilution using the SYBR GreenMaster mix (Applied Biosystems, PN 4309155) with an ABI 7900 sequence detection system according to the manufacturer's protocol (Applied Biosystems). The gene-specific primers were designed by using PRIMER-EXPRESS software (Applied Biosystems). The relative quantification method (Delta-Delta CT) was revised used to evaluate quantitative variation between replicates examined. The amplification of 18S rRNA was used as an internal control to normalize all data. Gene-specific primers for *OsDOS* were 5′-ATG ATGATGATGGGGGAAGG-3′ and 5′-CTCACGGGGAGGTGAGACC-3′, for *Os05g10670* 5′-AACCCAACAAGGCGACAGC-3′ and 5′-CCTGCTACCTTC TGCTTCCAC-3′, for *OsLOX8* 5′-CGATCGACATCAGGGATCTCA-3′ and 5′-CCACATTGTGCGCGTAGCT-3′, for *OsAOC1* 5′-GAGGCTTCTTGGTAG-TA GGTGGA-3′ and 5′-CGTAGTGGCGGTCGTTGTAGT-3′, for *OsOPR1* 5′-ACAAGCACCCAGGGTTTCTCTA-3′ and 5′-TCTTCCTTCGTCCTTCG AT-TATG-3′, for *Osh69* 5′-GGCCTTGTGAACCCTGACA-3′ and 5′-CTCCATC-GATCCCAG CAGAT-3′, for *Osl57* 5′-GCTGCTGCTGAGTCTCATAGGA-3′ and 5′-TGTAGG CACCGGAACAATCTC-3′, and for *18S rRNA* 5′-CGGCTAC-CACATCCAAGGAA-3′ and 5′-TGTCACTACCTCCCCGTGTCA-3′.

Southern-Blot Analysis

Genomic DNA isolation and Southern-blot analysis were performed as described previously (Qiao et al., 2004). DNA (5 μg) was digested, separated on 0.8% agarose gel, and transferred onto Hybond Nt (Amersham) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. *HPT* probe was labeled with ^{32}P by random priming using the Prime-a-Gene labeling system (Promega). The *HPT* primers were 5′-GCAAGGAATCGGTCAATACAC-3′ and 5′-TCCACTATC-GGC GAGTACTTC-3′.

Subcellular Localization

The *p1301-OsDOS-GFP* construct was transformed into wild-type rice plants. The root tip of the transgenic rice plant was sectioned longitudinally, stained for 30 min with 2 μg mL^{-1} propidium iodide (in 30 mM 2-[*N*-morpholino]-ethanesulphonic acid and 100 mM mannitol, pH 5.9), and visualized with a laser-scanning confocal microscope (LSM 510, Zeiss).

Chlorophyll Content Measurement

Chlorophyll was extracted from 50 mg leaf samples in 10.0 mL 80% acetone for 16 h in the darkness and was determined spectrophotometrically at 652 nM using the method of Arnon (1949).

Observations of Pollen Viability and Pollen Germination

Mature anthers (before flowering) of *OsDOS* OX lines and the wild-type plants were harvested and the pollen grains stained with a 1% iodine-potassium iodide solution for the observation of starch accumulation. Stained pollen grains were observed under an optical microscope.

For the pollen-tube germination test, pistils of *OsDOS* OX lines and the wild-type plants were collected 5 h after flowering, stained with aniline blue, and observed under a fluorescent microscope, as described by Lan et al. (2004).

Affymetrix GeneChip Analysis

Total RNA was extracted using TRizol reagent (Invitrogen, P/N 15596-018) and purified by using Qiagen RNeasy columns (QIAGEN, catalog no. 74104). For Affymetrix GeneChip analysis, 8 μg of total RNA was used for making biotin-labeled cRNA target. The RNA samples were from fully expanded flag leaves of two independent *OsDOS* RNAi T_1 lines (T_1 lines i-3 and i-4) and the wild-type control at booting stage, the panicles of the *OsDOS* OX-5 line and the wild-type control at heading stage, the nascent flag leaves at booting stage (FL-1), and the senescent flag leaves at grain-filling stage (FL-3). All the processes for cDNA and cRNA synthesis, cRNA fragmentation, hybridization, washing and staining, and scanning, were conducted according to the GeneChip standard protocol (Eukaryotic Target Preparation, Affymetrix). Poly-A RNA Control kit and the One-Cycle cDNA Synthesis kit were used in this experiment as described in the Web site: http://www.affymetrix.com/products/arrays/specific/rice.affx. The information about GeneChip Rice Genome Array (MAS 5.0) could be accessed from Affymetrix Web site: http://www.affymetrix.com/products/arrays/specific/rice.affx. GCOS software (Affymetrix GeneChip Operating Software) was used for data collection and normalization. The overall intensity of all probe sets of each array was scaled to 500 to guaranty that hybridization intensity of all arrays was equivalent; each probe set is assigned with "P," "A," and "M," and p-value from algorithm in GCOS.

Promoter Prediction

Potential promoter sequences (2 kb upstream elements) were used to search for the Plant Cis-Acting Regulatory Elements Database (Rombauts et al., 1999; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

JA Treatments

The flag leaves at heading stage were detached for JA treatments. Detached leaves were incubated in water (mock) and a solution containing 100 μM MeJA in darkness for 4 d, and then were photographed.

RNAi T_1 and the wild-type seeds used in the germination assays were sterilized and pregerminated by soaking for 24 h and incubating in sterile water for another 24 h at 32°C to promote synchronous sprouting. Identically sprouted seeds (plumule approximately 2 mm) were transplanted on the half-strength Murashige and Skoog plates without (mock) or with MeJA of a concentration gradient (2 μM, 5 μM, 10 μM, and 20 μM) for 5 d. Then the seedlings were photographed and the length of the primary roots measured.

Sequence data from this article can be found in the GenBank under accession numbers CR293108 and CR293228.

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