The De-Etiolated 1 Homolog of Arabidopsis Modulates the ABA Signaling Pathway and ABA Biosynthesis in Rice

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DEETIOLATED1 (DET1) plays a critical role in developmental and environmental responses in many plants. To date, the functions of OsDET1 in rice (Oryza sativa) have been largely unknown. OsDET1 is an ortholog of Arabidopsis (Arabidopsis thaliana) DET1. Here, we found that OsDET1 is essential for maintaining normal rice development. The repression of OsDET1 had detrimental effects on plant development, and led to contradictory phenotypes related to abscisic acid (ABA) in OsDET1 interference (RNAi) plants. We found that OsDET1 is involved in modulating ABA signaling in rice. OsDET1 RNAi plants exhibited an ABA hypersensitivity phenotype. Using yeast two-hybrid (Y2H) and bimolecular fluorescence complementation assays, we determined that OsDET1 interacts physically with DAMAGED-SPECIFIC DNA-BINDING PROTEIN1 (OsDDB1) and CONSTITUTIVE PHOTOMORPHOGENIC10 (COP10); DET1- and DDB1-ASSOCIATED1 binds to the ABA receptors OsPYL5 and OsDDB1. We found that the degradation of OsPYL5 was delayed in OsDET1 RNAi plants. These findings suggest that OsDET1 deficiency disturbs the COP10-DET1-DDB1 complex, which is responsible for ABA receptor (OsPYL) degradation, eventually leading to ABA sensitivity in rice. Additionally, OsDET1 also modulated ABA biosynthesis, as ABA biosynthesis was inhibited in OsDET1 RNAi plants and promoted in OsDET1-overexpressing transgenic plants. In conclusion, our data suggest that OsDET1 plays an important role in maintaining normal development in rice and mediates the cross talk between ABA biosynthesis and ABA signaling pathways in rice.

Leaf senescence is the final stage of leaf development. The most obvious phenotypic change during senescence is leaf yellowing caused by chlorophyll degradation (Bleecker and Patterson, 1997; Lim et al., 2007). Leaf structure, cellular metabolism, and gene expression also undergo dramatic changes during this process. Valuable nutrient components in the cell are remobilized to young tissues, while senescent leaves die slowly (Hörtensteiner and Feller, 2002; Buchanan-Wollaston et al., 2005; Lim et al., 2007). Leaf senescence is controlled by a complex regulatory network that is governed by developmental age. Leaf senescence also can be affected by other endogenous factors (such as phytohormones, the levels of some metabolites, and the state of photosystem complexes) and external factors (biotic and abiotic stresses). Among these, darkness is one of the most effective known external stimuli that cause leaf senescence, and it is frequently used to simulate synchronous senescence (Kim et al., 2006; Kong et al., 2006; Liang et al., 2014). Abscisic acid (ABA) plays a central role in regulating leaf senescence governed by developmental age (Breeze et al., 2011; Lee et al., 2011). The ABA content increases during leaf senescence in many plants, such as oat (Avena sativa; Gepstein and Thimmann, 1980), rice (Oryza sativa; Philosoph-Hadas et al., 1993), and Arabidopsis (Arabidopsis thaliana; Zhao et al., 2010; Breeze et al., 2011). Simultaneously, ABA biosynthesis and signaling genes are up-regulated during age-dependent leaf senescence (Tan et al., 2003; Buchanan-Wollaston et al., 2005). Moreover, numerous biotic and abiotic stresses usually induce ABA biosynthesis and increase ABA levels, activate ABA signaling pathways, and lead to leaf senescence. Exogenously applied ABA increases the expression of chlorophyll degradation-related genes.

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ABA signaling is mediated by the pyrabactin resistance/pyrabactin resistance-like/regulatory components of the ABA receptor (PYR/PYL/RCAR; Cutler et al., 2010; Hubbard et al., 2010). In the presence of ABA, PYR/RCARs bind with ABA and clade A phosphatases type 2C (PP2Cs; these include ABA INSENSITIVE1, HYPERSENSITIVE TO ABA1 [HAB1], and HAB2) to form a PP2C-ABA-PYL ternary complex (Park et al., 2009; Cutler et al., 2010; Hubbard et al., 2010), inhibiting the phosphatase activity of PP2Cs, allowing SUCROSE NONFERMENTING1-related subfamily 2 kinase (SnRK2) activation and phosphorylation of the ABA-responsive element binding factor family transcription factors, ultimately regulating the transcriptional response to ABA (Cutler et al., 2010; Hubbard et al., 2010). Simultaneously, the PYL ABA receptors are recognized and ubiquitinated by DET1-AND DDB1-ASSOCIATED1 (DDA1) and the COP10-DET1-DDB1 (CDD) complex to alleviate the adverse effects of continuous ABA responses. CDD components cooperate to regulate PYL stability. DETIOLATED1 (DET1), part of the CDD complex, also modulates ABA responses (Irigoyen et al., 2014).

DET1 is a well-known repressor of photomorphogenesis (Pepper et al., 1994). This nucleus-localized protein interacts with DAMAGED-SPECIFIC DNA-BINDING PROTEIN1 (DDB1) to regulate plant photomorphogenesis (Schroeder et al., 2002). DET1 and DDB1 interact with CONSTITUTIVE PHOTOMORPHGENIC10 (COP10) to form the CDD complex in living plant cells (Yanagawa et al., 2004). The CDD complex cooperates with CULLIN4 (CUL4) in response to light and mediates plant development (Chen et al., 2006; 2010). DET1 also was shown recently to interact directly with phytochrome-interacting factors and to modulate their stability, thereby further repressing photomorphogenesis (Dong et al., 2014). A mutation in DET1 damages the normal functioning of the CDD complex in Arabidopsis. Furthermore, adding a C-terminal GFP tag interferes with the formation of the CDD complex by DET1, DDB1, and COP10 and influences the normal functioning of DET1 in Arabidopsis (Schroeder et al., 2002; Irigoyen et al., 2014). In addition to regulating photomorphogenesis, DET1 also plays a key role in many biological processes in plants. DET1 functions in some stress responses, and it joins with CUL4, DDB1, and DDB2 to form a complex to maintain genome integrity in Arabidopsis during UV light stress (Castells et al., 2011). DET1 also influences plant responses to ABA by modulating the functioning of CDD in Arabidopsis. The CDD complex interacts with DDA1 to form the COP10-DET1-DDB1-DDA1 (CDD) complex, which provides substrate specificity for CUL4-RING E3 ubiquitin ligase (CRL4) ubiquitination of the PYR/PYL/RCAR family of ABA receptors. Reduced CDD function causes ABA hypersensitivity in Arabidopsis. As part of the CDD substrate adaptor module, DET1 indirectly affects the recognition and ubiquitination of PYLs by DDA1. The Arabidopsis det1-1 mutant exhibits an increased response to the ABA-mediated inhibition of germination and seedling establishment compared with wild-type plants (Irigoyen et al., 2014). Interestingly, the detached leaves of the Arabidopsis det1-1 mutant exhibit significantly delayed dark-induced leaf senescence compared with the wild type (Chory et al., 1994).

Although DET1 plays an important role in plant development, the roles of OsDET1 in rice remain unclear, except for its role in chlorophyll biosynthesis (Huang et al., 2013). Here, we report that OsDET1 influences the sensitivity of rice plants to ABA. As OsDET1 is an ortholog of Arabidopsis DET1, we found that OsDET1 deficiency caused pleiotropic phenotypes related to ABA hypersensitivity in OsDET1 RNA interference transgenic plants (OsDET1 RNAi plants). We also observed similar ABA hypersensitivity phenotypes in OsDET1-GFP plants. yeast two-hybrid (Y2H) and bi-molecular fluorescence complementation (BiFC) assays showed that OsDET1 interacts physically with OsDD1 and OsCOP10 in vivo. OsDD1 also binds to the ABA receptors OsPYL5 and OsDD1. Further experiments demonstrated that the degradation of OsPYL5 was delayed in OsDET1 RNAi and OsDET1-GFP plants. These results suggest that deficiency or structural changes to OsDET1 may disturb the functioning of CDD and cause ABA hypersensitivity in rice. Surprisingly, OsDET1 also influenced ABA biosynthesis and inactivation. The level of endogenous ABA was reduced in OsDET1 RNAi plants, while ABA biosynthesis was promoted in OsDET1-overexpressing transgenic plants (OE-OsDET1 plants), suggesting that OsDET1 also modulates ABA biosynthesis in rice. Overall, our results suggest that OsDET1 mediates the cross talk between ABA biosynthesis and ABA signaling pathways in rice.

RESULTS

Stress-Induced and Spatial Expression Profiles of OsDET1 in Rice

DET1 plays an important role in regulating plant development. To investigate the role of OsDET1 in rice development, we constructed the vector P_\text{DET1-GFP} and introduced it into cv Nipponbare rice. Consistent with our previous results (Huang et al., 2013), GUS activity was detected in almost all tissues and organs, such as young callus, roots, stems, nodes, internodes, leaf sheaths, leaves, and panicles (Fig. 1A). GUS activity was stronger in leaves, leaf sheaths, and internodes than in roots and panicles. Surprisingly, GUS staining was observed distinctly in the stigma and ovary, suggesting that OsDET1 affects the fertility of rice spikelets.
OsDET1 Factor Accelerates Dark-Induced Leaf Senescence

Detached leaves of the det1-1 mutant exhibit delayed dark-induced leaf senescence compared with the wild type in Arabidopsis (Chory et al., 1994). Therefore, we investigated the role of OsDET1 in leaf senescence in rice by generating transgenic OsDET1 RNAi plants. The expression of OsDET1 was reduced markedly in these plants (Fig. 2A). Therefore, we utilized the penultimate leaves of these lines at the tillering stage for further dark-induced leaf senescence experiments. OsDET1 RNAi plants exhibited significantly accelerated leaf yellowing and reduced chlorophyll contents compared with the wild type during dark-induced leaf senescence (Fig. 2, B and C). We also examined the ratio of variable fluorescence to maximum fluorescence ($F_v/F_m$) and the membrane ion leakage values in these plants. $F_v/F_m$ values reflect PSII activity and membrane ion leakage reflects cell death, and they are frequently used as parameters of leaf senescence (Morita et al., 2009). As shown in Figure 2D, the $F_v/F_m$ values decreased rapidly with treatment time in OsDET1 RNAi plants, indicating that chloroplast damage was accelerated in the leaves of these plants. Similarly, the membrane ion leakage values increased sharply in transgenic plant leaves (Fig. 2E), indicating that the process of programmed cell death was accelerated in OsDET1 RNAi plant leaves. Furthermore, the expression of major key CDGs (including PAO, SGR, NYC1, NYC3, RCCR1, PPH, and NOL) and SAGs (including OsNAP, OsI57, and OsI85) was markedly higher in leaves of OsDET1 RNAi plants than in wild-type leaves during 4 d of dark induction (Fig. 2, F–H; Supplemental Fig. S1, A and B; Morita et al., 2009; Rong et al., 2013; Yamatani et al., 2013; Liang et al., 2014).

Given that OsDET1 deficiency accelerated the expression of CDGs and chlorophyll degradation during dark-induced leaf senescence, we performed western-blot analysis to investigate the stability of photosynthetic proteins in OsDET1 RNAi plants and the wild type during dark-induced leaf senescence. After 4 d of dark treatment, the PSII proteins (Lhcb1, Lhcb4, Lhcb6, and D1) and PSI proteins (Lhca1 and PSa) were quickly degraded in OsDET1 RNAi plants compared with the wild type, whereas Lhcb2 and RbcL were retained in OsDET1 RNAi plants and wild-type plants (Supplemental Fig. S2). These results suggest that OsDET1 deficiency impairs the stability of photosynthetic proteins during dark-induced leaf senescence in rice.

We observed the chloroplast ultrastructure in the plants using transmission electron microscopy. Interestingly, the
grana stacks were thicker in OsDET1 RNAi plants than in wild-type plants at the tillering stage (Supplemental Fig. S3, A, B, F, G, K, L, P, and Q), suggesting that OsDET1 also modulates chloroplast development in rice. During dark incubation, the grana stacks and stroma membranes were rapidly degraded in OsDET1 RNAi plants compared with wild-type plants. After 4 d of dark treatment, the grana stack arrays and intergranal lamellae were only slightly disordered in the wild type. By contrast, the volume of the thylakoid membrane was reduced significantly in OsDET1 RNAi plants, and the grana and intergranal lamellae were fused and disordered (Supplemental Fig. S3, C, D, H, I, M, N, R, and S). After 6 d of dark treatment, the chloroplast components were almost decomposed in OsDET1 RNAi plants (Supplemental Fig. S3, C, D, H, I, M, N, R, and S). After 6 d of dark treatment, the chloroplast components were almost decomposed in OsDET1 RNAi plants (Supplemental Fig. S3, C, D, H, I, M, N, R, and S). After 6 d of dark treatment, the chloroplast components were almost decomposed in OsDET1 RNAi plants (Supplemental Fig. S3, C, D, H, I, M, N, R, and S). After 6 d of dark treatment, the chloroplast components were almost decomposed in OsDET1 RNAi plants (Supplemental Fig. S3, C, D, H, I, M, N, R, and S).

Overall, unlike the phenotype of the Arabidopsis det1-1 mutant, our results demonstrate that OsDET1 deficiency accelerates leaf senescence during dark treatment in rice. Furthermore, OsNAP, an important link between ABA signaling and leaf senescence, is induced specifically by ABA, which directly activates the expression of CDGs and other SAGs (Liang et al., 2014). Thus, it appears that ABA signaling is intensified in OsDET1 RNAi plants during dark-induced leaf senescence. However, while the increased dark-induced leaf senescence phenotype is usually closely linked with premature leaf senescence in rice (Morita et al., 2009; Rong et al., 2013; Yamatani et al., 2013; Liang et al., 2014), in this study, OsDET1 deficiency accelerated dark-induced leaf senescence, but no visible difference in leaf senescence was observed in OsDET1 RNAi plants compared with wild-type plants at the vegetative and late filling stages (Fig. 2; Supplemental Fig. S4, A and B).

Inhibited OsDET1 Expression Accelerates ABA-Induced leaf Senescence

ABA plays a central role in modulating leaf senescence (Lim et al., 2007). To further investigate the intrinsic
functions of OsDET1 in leaf senescence, we explored leaf senescence phenotypes in response to ABA treatment. Detached leaves from the tillering stage were treated with ABA in the light. As expected, the detached leaves of OsDET1 RNAi plants exhibited obviously accelerated senescence. The chlorophyll contents decreased rapidly and membrane ion leakage increased quickly in OsDET1 RNAi plants (Fig. 3, A–C). Simultaneously, the expression of CDGs and SAGs was up-regulated significantly in OsDET1 RNAi plants compared with the wild type (Fig. 3D). Taken together, these results demonstrate that OsDET1 deficiency accelerates ABA-induced leaf senescence, implying that ABA signaling is enhanced in OsDET1 RNAi plants.

**OsDET1 Influences ABA Biosynthesis in Rice**

To help explain the discrepancy between the leaf senescence phenotype during dark and ABA stress and the normal growth of OsDET1 RNAi plants, we investigated the ABA contents in OsDET1 RNAi plants and wild-type plants at the vegetative and late filling stages; T2 OE-OsDET1 plants served as a positive control (Supplemental Fig. S5A). Surprisingly, compared with the wild type, the ABA contents were reduced in OsDET1 RNAi plants and increased in OE-OsDET1 plants (Fig. 4A; Supplemental Fig. S6A). We also analyzed the expression of key ABA biosynthesis genes (OsNCED1, OsNCED3, OsNCED4 [for 9-cis-epoxycarotenoid dioxygenase], and OsZEP [for zeaxanthin epoxidase]) and ABA inactivation genes (OsABA8ox1, OsABA8ox2, and OsABA8ox3) in these plants. As expected, the transcript levels of ABA biosynthesis genes declined in OsDET1 RNAi plants compared with the wild type (Fig. 4, E and H). Conversely, OsNCED1, OsNCED3, and OsNCED4 were up-regulated in OE-OsDET1 plants (Fig. 4, E–G). In addition, the ABA inactivation genes OsABA8ox1, OsABA8ox2, and OsABA8ox3 were markedly up-regulated in OsDET1 RNAi plants but down-regulated in OE-OsDET1 plants (Fig. 4, B–D). Taken together, these results suggest that OsDET1 acts as a positive regulator of ABA biosynthesis by modulating the expression of ABA biosynthesis and inactivation genes. Therefore, although OsDET1 deficiency enhances ABA signaling and the ABA contents were reduced in OsDET1 RNAi plants, this new balance maintained the leaf in normal functioning at the vegetative and filling stages in OsDET1 RNAi plants.

Darkness forcefully stimulates ABA biosynthesis (Gepstein and Thimann, 1980) and causes the rapid senescence of leaves in OsDET1 RNAi plants. Senescence also is a powerful factor for promoting ABA biosynthesis. The contents of ABA are increased tremendously...
with the leaves in senescence in many plants (Zhao et al., 2010; Breeze et al., 2011). By contrast, the repression of ABA biosynthesis by OsDET1 deficiency was gentle in OsDET1 RNAi plants (Fig. 4; Supplemental Fig. S6A). Therefore, in dark treatment, the balance was shifted by darkness. Consistent with the premature leaf senescence, the ABA contents were much higher in OsDET1 RNAi plants than in the wild type after a 4-d dark treatment (Supplemental Fig. S7A). Moreover, qRT-PCR demonstrated that ABA biosynthesis was promoted and ABA inactivation was repressed in OsDET1 RNAi plants compared with the wild type (Supplemental Fig. S7, B–H). These results suggest that the factors of darkness and senescence are more powerful than OsDET1 in regulating ABA biosynthesis during dark-induced leaf senescence and clearly indicate that the enhanced ABA signaling is the main reason for the rapid leaf senescence of OsDET1 RNAi plants in dark incubation.

Since ABA promotes leaf senescence in plants, activating endogenous ABA biosynthesis causes ABA hypersensitivity in many plants (Suzuki et al., 2002; Oliver et al., 2007; Zhu et al., 2009). To further assess the function of OsDET1 in ABA biosynthesis, we examined the leaf senescence phenotype of OE-OsDET1 plants during dark incubation. As expected, OE-OsDET1 plants also exhibited an accelerated leaf senescence phenotype (Supplemental Fig. S5). These results suggest that the positive regulation of ABA biosynthesis by OsDET1 also causes ABA hypersensitivity in OE-OsDET1 plants during dark treatment.

OsDET1 Influences Seed Germination and Seedling Growth

To further explore the role of OsDET1 in linking ABA signaling and ABA biosynthesis, we performed germination and postgermination assays to investigate the ABA-related phenotypes of the transgenic lines. Compared with the wild type, OsDET1 RNAi plants showed delayed germination on one-half-strength Murashige and Skoog (MS) medium (Fig. 5, A and B). Moreover, ABA treatment significantly delayed the germination of OsDET1 RNAi seeds (Fig. 5, C and D). After germination, wild-type and transgenic seedlings of similar size were transferred to one-half-strength MS medium containing 2 μM ABA. In the absence of ABA, shoot growth was inhibited markedly. Conversely, root growth was promoted in the OsDET1 RNAi lines compared with the wild type (Fig. 5, E, G, and H). Application of ABA strongly inhibited root and shoot growth in the OsDET1 RNAi lines compared with the wild type (Fig. 5, F–H). Overall, these finding indicate that down-regulating OsDET1 confers ABA hypersensitivity in OsDET1 RNAi plants during seed germination and seedling growth.

Some phenotypes of the OE-OsDET1 plants were similar to those of the OsDET1 RNAi lines. For example, on one-half-strength MS medium, seed germination was obviously delayed, shoot growth was inhibited, and root growth was promoted (Fig. 5). These results imply that higher levels of endogenous ABA also cause ABA hypersensitivity in OE-OsDET1 plants during seed germination and seedling growth. However, unlike the OsDET1 RNAi lines, the inhibition of root and shoot growth was slight in the OE-OsDET1 lines compared with the wild type during ABA treatment (Fig. 5, F–H).

OsDDA1 Interacts with OsPYL5 and the CDD Complex in Vivo

To further unravel the mechanism by which OsDET1 deficiency causes ABA hypersensitivity in rice, we performed yeast two-hybrid (Y2H) assays to identify
and H, Root and shoot growth rates of the wild type (WT) and carried out with similar results. Representative graphs are shown (RNAi and OE- growth and promoted root growth. F, Overexpression or suppression of seeds in each experiment). *, identi ortolog of Arabidopsis DDB1 (Kimura et al., 2004), was (Supplemental Fig. S8). OsDDB1 (Os05g0592400), an proteins that interact physically with OsDET1. The OsDET1 coding sequence was inserted into PGBKT7 as bait, and a cv Nipponbare complementary DNA (cDNA) library previously generated in our laboratory was used for yeast two-hybrid (Y2H) screening. Nine candidate interacting proteins were identi for yeast two-hybrid (Y2H) screening. Nine candidate library previously generated in our laboratory was used as bait, and a cv Nipponbare complementary DNA (cDNA) coding sequence was inserted into PGBKT7 as OsDET1 Influences OsPYL5 Degradation in Rice

Indeed, a series of studies within the last decade demonstrated that DET1 interacts with COP10 and DDB1 to form the CDD complex in Arabidopsis and tomato (Solanum lycopersicum; Kimura et al., 2004; Lau and Deng, 2012). The CDD complex interacts with DDA1 to form the CDDD complex, which mediates the ubiquitination of the PYR/PYL/RCAR family of ABA receptors in Arabidopsis (Irigoyen et al., 2014). Since our results suggest that OsDET1 deficiency increases ABA sensitivity in rice, we hypothesized that a similar mechanism might occur in rice. Therefore, we cloned the orthologous genes of DDA1, COP10, and PYL8-related from cv Nipponbare. The full-length coding sequences of OsDDB1, OsCOP10, and OsDDA1 and truncated versions of the ABA receptor gene OsPYL5 (encoding amino acids 90–209) were fused separately to pGADT7 or PGBK7T7, and a yeast two-hybrid (Y2H) assay was performed to determine the interaction of OsDET1 with OsDDB1, OsCOP10, OsDDA1, and OsPYL5 (Ishibashi et al., 2003; Kim et al., 2012, 2014; Irigoyen et al., 2014). We found that OsDET1 interacts physically with OsDDB1 and OsCOP10 (Fig. 6A) and that OsDDA1 binds to OsPYL5 and OsDDB1 in yeast (Y2H) cells (Fig. 6B).

To confirm the results of the yeast two-hybrid (Y2H) assay, we performed BiFC assays using agroinfiltrated onion (Allium cepa) epidermal cells. The full-length coding sequences of OsDET1, Os-DDB1, OsCOP10, OsDDA1, and OsPYL5 were fused separately with the N- or C-terminal portions of yellow fluorescent protein (YFP). OsDET1 interacted with OsDDB1 and OsCOP10 in the BiFC assays (Fig. 6C), further indicating that the CDD complex may be present in rice. In addition, OsDDA1 interacted with OsPYL5 and OsDDB1 (Fig. 6D), suggesting that OsDDA1 functions in combining the CDDD complex with OsPYL5 in rice. Taken together, these results support the notion that OsDET1 interacts with OsCOP10 and OsDDB1 and modulates OsPYL degradation in rice.

OsDET1 Influences OsPYL5 Degradation in Rice

To determine whether OsDET1 takes part in the regulation of OsPYL degradation in rice, we monitored the expression of OsPYL5 by qRT-PCR. The expression of OsPYL5 was reduced slightly in OsDET1 RNAi plants after 12 h of ABA treatment compared with the wild type (Fig. 7A). Conversely, western blotting showed that OsPYL5 levels were markedly higher in OsDET1 RNAi plants than in wild-type plants (Fig. 7C), implying that the degradation of OsPYL5 was delayed in OsDET1 RNAi plants. In addition, we observed no significant differences of OsPYL5 level in OE-OsDET1 plants and the wild type (Fig. 7F), suggesting that the overexpression of OsDET1 was unable to promote the degradation of OsPYL5 in OE-OsDET1 plants. The expression of ABA-inducible marker genes, including OsRAB16A, OsLEA, and OsLIP9, is promoted by OsPYL5 in plants under ABA treatment (Kim et al.,

Figure 5. OsDET1 influences seed germination and seedling growth. A, Overexpression or suppression of OsDET1 inhibited seed germination in transgenic plants. B, Germination time courses on one-half-strength MS medium. C, Overexpression or suppression of OsDET1 inhibited seed germination in transgenic plants during ABA treatment. D, Germination time courses on one-half-strength MS medium containing 2 μM ABA. E, Overexpression or suppression of OsDET1 inhibited shoot growth and promoted root growth. F, Overexpression or suppression of OsDET1 inhibited the growth of shoot and root during ABA treatment. G and H, Root and shoot growth rates of the wild type (WT) and OsDET1 RNAi and OE-OsDET1 plants. Three independent experiments were carried out with similar results. Representative graphs are shown (n = 30 seeds in each experiment). *, P ≤ 0.05 and **, P ≤ 0.01 (Student’s t test).
Therefore, we investigated the transcription of these genes in the transgenic lines. Consistent with the higher level of OsPYL5, the expression of OsRAB16A, OsLEA, and OsLIP9 was significantly higher in OsDET1 RNAi plants than in wild-type plants (Fig. 7, B–D). Taken together, these results suggest that the degradation of OsPYL5 is impaired in OsDET1 RNAi plants, leading to ABA hypersensitivity.

**Pleiotropic Function of OsDET1 in Rice**

OsDET1 deficiency caused pleiotropic phenotypes in addition to accelerated dark- and ABA-induced leaf senescence. Excessive RNA interference treatment led to plant death in T0 transgenic plants at the vegetative growth stage or a failure of T1 transgenic seeds to germinate (Supplemental Fig. S9, A–C). In addition, OsDET1 deficiency caused an overall reduction in biomass. The number of tillers, panicles, and spikelets as well as fertility were reduced significantly in OsDET1 RNAi plants (Fig. 8A; Supplemental Fig. S9, D and E; Supplemental Table S1). Meanwhile, the plant height and leaf length were reduced in OsDET1 RNAi plants compared with the wild type (Supplemental Fig. S9, F and G). Conversely, OE-OsDET1 plants were vigorous and fertile (Supplemental Figs. S10A and S11). The leaf length was increased in transgenic plants compared with wild-type plants, which caused drooping leaves in OE-OsDET1 plants (Supplemental Fig. S10, A and B).

We observed some phenotypes related to ABA hypersensitivity in OsDET1 RNAi plants. The transgenic
plants had fewer filled spikelets than wild-type plants (Fig. 8A) as well as slightly smaller pollen grains and a higher proportion of irregularly shaped pollen grains (Fig. 8, B and C). Similarly, more stomatal pores were completely closed in OsDET1 RNAi plants compared with the wild type, as revealed by scanning electron microscopy (SEM: Fig. 9, A and B). These results further support the notion that OsDET1 deficiency increases ABA sensitivity in rice. Interestingly, less wax crystallization was observed on the OsDET1 RNAi leaf surface compared with the wild type (Fig. 9A), and more rapid water loss occurred in OsDET1 RNAi plants as well (Fig. 9C). Wax crystallization functions as the outermost barrier against nonstomatal water loss (Zhou et al., 2015). Therefore, the accelerated water loss may have been due to the deficient wax crystallization in OsDET1 RNAi plants. ABA promotes cuticular wax biosynthesis in many plants. Thus, these phenotypes are consistent with the reduced ABA levels in OsDET1 RNAi plants, but they appear to contradict the ABA sensitivity of these plants.

In this study, ABA biosynthesis was accelerated in OE-OsDET1 plants. We noticed that more wax crystallization existed on the OE-OsDET1 leaf surface than on wild-type leaves (Supplemental Fig. S10C), which was consistent with the increase of endogenous ABA in OE-OsDET1 plants. But the gentle increase of ABA was unable to change the morphology of pollen in OE-OsDET1 plants. The ratio of normal pollen in OE-OsDET1 plants showed only a slight decrease compared with that in the wild type (Supplemental Fig. S11, B and C). In addition, the spikelet fertility of OE-OsDET1 plants was normal (Supplemental Fig. S11A). Taken together, these pleiotropic phenotypes of OE-OsDET1 and OsDET1 RNAi plants suggest the diverse role of OsDET1 in rice development.

Overexpressing OsDET1-GFP Enhances ABA Sensitivity in Rice

Adding a C-terminal GFP tag to DET1 disrupts the functioning of the CDD complex in Arabidopsis (Schroeder et al., 2002). We hypothesized that a C-terminal GFP tag also would disrupt the functioning of OsDET1 in rice. Therefore, we introduced the pCOMBIA1301-OsDET1-GFP vector into cv Nipponbare rice. OsDET1-GFP transcript levels were significantly higher than endogenous OsDET1 levels in OsDET1-GFP transgenic plants (Supplemental Fig. S12A). Different from OE-OsDET1 plants, the phenotype of OsDET1-GFP plants was similar to that of OsDET1 RNAi plants. The transgenic plants showed growth retardation, dwarf, and some ABA sensitivity phenotypes (Supplemental Fig. S12B). The morphology of OsDET1-GFP pollen grains also was changed, and the spikelets were almost completely infertile in OsDET1-GFP plants (Supplemental Fig. S11). Moreover, OsDET1-GFP plants exhibited an accelerated leaf senescence phenotype during dark or ABA treatment (Supplemental Figs. S13 and
These results demonstrate that overexpressing OsDET1-GFP enhances ABA sensitivity in rice. Meanwhile, unlike OE-OsDET1 plants, the ABA content was reduced in OsDET1-GFP leaves in plants at the tillering stage (Supplemental Fig. S6B), and OsPYL5 degradation was delayed in OsDET1-GFP plants compared with the wild type after ABA treatment (Fig. 7E). These results suggest that the ABA hypersensitivity phenotypes of OsDET1-GFP plants are not caused by posttranscriptional gene silencing or overexpression of OsDET1. Instead, the misshapen OsDET1-GFP protein might disturb the normal functioning of OsDET1. Overall, these findings suggest that structural changes in OsDET1 may impair the normal functioning of the CDD complex and further influence ABA receptor stability in rice.

Transcriptome Analysis of OsDET1 RNAi Plants

To obtain clues about the pleiotropic roles of OsDET1 in rice, we performed mRNA sequencing (RNA-SEQ) to examine the genome-wide effects of the OsDET1 gene in OsDET1 RNAi plants (Supplemental Table S2). A total of 4,752 differentially expressed genes (DEGs)
were identified after comparing the transcriptome profiles of DRI-16 versus wild-type plants under normal growth conditions based on the criteria of DESeq (Anders et al., 2015). There were more up-regulated genes (2,580) than down-regulated genes (2,172; Fig. 10A; Supplemental Table S3). The DEGs were classified into different functional categories based on Gene Ontology (GO) annotation analysis. A wide range of categories were affected in OsDET1 RNAi plants, including biological process, molecular function, and cellular component GO terms (Supplemental Table S4). A number of GO terms, including chlorophyll catabolic process and chlorophyllase activity, were assigned to the DEGs (Supplemental Table S4). In addition, OsDET1 deficiency strongly affected the expression of photosynthesis- and thylakoid-related genes (Supplemental Table S5). DET1 modulates photomorphogenesis by regulating large numbers of light-mediated genes in Arabidopsis, as determined by microarray-based expression profiling analyses (Ma et al., 2003; Dong et al., 2014). Together, these findings suggest that OsDET1 modulates chlorophyll metabolic processes and chloroplast development and that the role of DET1 in photomorphogenesis is conserved in rice.

Furthermore, 15 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched in OsDET1 RNAi plants based on corrected *P* < 0.05 values (Fig. 10B; Supplemental Table S6). This result is consistent with the multiple phenotypes of OsDET1 RNAi plants and further supports the pleiotropic functions of OsDET1 in rice. In addition, plant hormone signal transduction pathways were markedly affected by OsDET1 deficiency. In particular, 16 differentially expressed ABA signal transduction genes were detected, most of which were significantly up-regulated in OsDET1 RNAi plants; eight genes encoding PP2C family proteins were markedly
up-regulated in these plants (Table I). ABA increases the expression levels of all OsPP2C genes from subfamily A (Xue et al., 2008). In this study, three basic Leu zipper transcription factor (bZIP) family genes were markedly up-regulated (Table I); bZIPs play important roles in the ABA signaling pathway and were induced by ABA in previous studies (Nijhawan et al., 2008; Amir Hossain et al., 2010). Moreover, OsPYL1 was down-regulated in OsDET1 RNAi plants, whereas OsPYL10 was up-regulated (Table I). These results are consistent with previously reported OsPYL expression patterns after ABA treatment (Tian et al., 2015). Overall, these results indicate that the OsDET1 RNAi plants suffered from ABA stress, further supporting the notion that OsDET1 deficiency leads to ABA hypersensitivity.

**DISCUSSION**

DET1 encodes an evolutionarily conserved protein. DET1, a central repressor of plant photomorphogenesis, indirectly regulates the expression of a vast number of light-regulated genes by modulating the stability of some key transcription factors involved in light signaling. The mutation of DET1 causes pleiotropic phenotypes in Arabidopsis and tomato (Chory et al., 1994; Pepper et al., 1994; Mustilli et al., 1999; Huang et al., 2014). In Arabidopsis, DET1 represses light-induced seed germination. The det1 mutants display light-grown phenotypes in the dark. The mutation of DET1 also leads to chlorophyll accumulation in Arabidopsis and tomato leaves during the vegetative stage. In addition, DET1 play a negative role in controlling flowering time in Arabidopsis (Kang et al., 2015). Therefore, DET1 plays a prominent role in modulating plant development (Lau and Deng, 2012). Despite the rapid progress in dissecting the roles of DET1 in many plants several decades ago, little is known about the role of OsDET1 in rice. This study demonstrates that changes in OsDET1 expression cause pleiotropic phenotypes in transgenic rice. Specifically, OsDET1 deficiency had strong detrimental effects on basic plant development, indicating that OsDET1 is essential for maintaining normal development in rice and is required throughout the plant life cycle.

Notably, the pollen grain morphology was altered in OsDET1 RNAi plants (Fig. 8, B and C). Similar phenotypes were observed in rice treated with exogenous ABA or with increased endogenous ABA content (i.e. transgenic plants overexpressing OsAP2-39; Oliver et al., 2007; Yaish et al., 2010). In addition, more stomatal pores were closed in OsDET1 RNAi plants than in wild-type plants (Fig. 9, A and B). ABA, which regulates stomatal movement, is biosynthesized mainly in vascular parenchyma cells and is transported to guard cells by ABA transporters (such as AtBCG25 and AtABCG40; Dörrffling and Tietz, 1985; Cheng et al., 2002; Kotwai et al., 2004; Endo et al., 2008; Pandey et al., 2009; Kanno et al., 2010). Subsequently, ABA is recognized by the PYR/PYL/PCAR ABA receptor and...
OsDET1 Affects ABA Signaling in Rice

Table 1. Examples of the DEGs in DRI-16 transgenic lines that are predicted to be involved in the ABA signaling pathway

<table>
<thead>
<tr>
<th>Gene Identifier</th>
<th>Log2 Fold Change</th>
<th>Associated Gene Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS03G0268600</td>
<td>1.6772</td>
<td>Protein phosphatase 2C</td>
<td></td>
</tr>
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<td>OS09G0325700</td>
<td>1.3708</td>
<td>Protein phosphatase 2C</td>
<td></td>
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<td>2.1824</td>
<td>Protein phosphatase 2C</td>
<td></td>
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Ser/Thr-protein kinase SRK2

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<th>Description</th>
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<tr>
<td>OS10G0564500</td>
<td>−1.2467</td>
<td>SAPK3</td>
<td></td>
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<tr>
<td>OS03G0390200</td>
<td>0.61378</td>
<td>SAPK1</td>
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<tr>
<td>OS04G0691100</td>
<td>−0.62471</td>
<td>SAPK5</td>
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ABA receptor PYR/PYL family

<table>
<thead>
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<th>Log2 Fold Change</th>
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<tr>
<td>OS02G0255300</td>
<td>0.5072</td>
<td>OSJNBA0052K15.19</td>
<td>Polyketide cyclase/dehydrase</td>
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<tr>
<td>OS01G0827800</td>
<td>Infinitesimal</td>
<td>B1088C09.11</td>
<td>Polyketide cyclase/dehydrase</td>
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<tr>
<td>OS03G0297600</td>
<td>−2.3167</td>
<td>OS03G0297600</td>
<td>Polyketide cyclase/dehydrase</td>
</tr>
</tbody>
</table>

ABA-responsive element-binding factor

<table>
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<th>Log2 Fold Change</th>
<th>Associated Gene Name</th>
<th>Description</th>
</tr>
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<tr>
<td>OS01G0867300</td>
<td>−1.2092</td>
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<td>B1342C04.26</td>
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</table>

further associates with PP2Cs to form the PP2C-ABA-PYL ternary complex, which activates the ABA response. The accumulation of ABA in guard cells leads to stomatal closure (Cutler et al., 2010; Kim et al., 2010; Gonzalez-Guzman et al., 2012). Therefore, these phenotypes imply that OsDET1 RNAi plants suffered from ABA stress. However, the ABA content was reduced in OsDET1 RNAi leaves (Fig. 4A; Supplemental Fig. S6). These seemingly contradictory results indicate that OsDET1 deficiency most likely increases ABA sensitivity in OsDET1 RNAi plants. ABA mediates plant germination and early seedling growth (Kim et al., 2012, 2014). In this study, down-regulation of OsDET1 produced ABA-hypersensitive phenotypes during seed germination and early seedling growth in OsDET1 RNAi plants (Fig. 5), which strongly supports the role of OsDET1 in ABA sensitivity. This notion was further confirmed by our investigation of dark- and ABA-induced leaf senescence (Figs. 2 and 3; Supplemental Figs. S1–S3), where leaf senescence was accelerated under dark and ABA treatment in OsDET1 RNAi plants, indicating that OsDET1 deficiency increases the sensitivity of ABA signaling in rice.

ABA signaling is mediated by the PYR/PYL/RCAR family of ABA receptors (Gonzalez-Guzman et al., 2012; Wang et al., 2013; Rodriguez et al., 2014). DET1 negatively regulates ABA signaling by modulating the ubiquitination of the PYL ABA receptor in Arabidopsis. DET1 interacts with COP10 and DDB1 to form the CDD complex, which further associates with DDA1 to form the CDDD complex. This complex provides substrate specificity for CRL4 by the recognition of DDA1 for the ubiquitination targets. Although the CDD complex does not interact with PYLs in vivo, it influences the recognition of DDA1 by PYLs (Irigoyen et al., 2014). In Arabidopsis, damage to the CDD complex obstructs the ubiquitination of PYLs, leading to their accumulation. The det1-1 mutants exhibit increased responses to ABA-mediated inhibition of germination and seedling establishment compared with the wild type (Irigoyen et al., 2014). Given that OsDET1 deficiency also leads to ABA hypersensitivity in OsDET1 RNAi plants and that the orthologs of DDA1, COP10, and PYL8 were discovered in rice, we speculate that a similar ABA signal regulatory mechanism also exists in rice (Fig. 11). The results of protein interaction analysis and the abundance of PYR/PYL/RCAR ABA receptors strongly support this model. First, we found that OsDET1 interacts with OsDDB1 and OsCOP10 in vivo (Fig. 6). Next, we demonstrated that OsDDA1 interacts with OsPYL5 and OsDDB1 (Fig. 6). Moreover, the degradation of OsPYL5 was impaired in OsDET1 RNAi plants. In addition, OsPYL5 was shown previously to promote the expression of ABA-inducible marker genes (including OsRAB16A, OsLEA, and OsLIP9) during ABA treatment and to play an important role in mediating ABA signaling during seed germination and early seedling growth (Kim et al., 2012, 2014). In this study, these ABA-inducible marker genes were up-regulated significantly in OsDET1 RNAi plants during ABA treatment compared with wild-type plants (Fig. 7). These results are consistent with the finding that OsPYL5 accumulated in OsDET1 RNAi plants, which further supports our hypothesis. The correct structure of DET1 is necessary to maintain the function of the CDD complex: a C-terminal GFP tag on DET1 hinders the formation of the CDD complex by DET1, COP10, and DDB1 in Arabidopsis (Schroeder et al., 2002). We found that overexpressing OsDET1-GFP also increased plant sensitivity to ABA signaling (Supplemental Figs. S11–S14) and delayed the degradation of OsPYL5 in
OsDET1-GFP plants (Fig. 7E). These findings suggest that structural changes in OsDET1 impair the normal functioning of CDD and may affect ABA receptor stability in rice, further supporting the role of OsDET1 in modulating ABA signaling.

Interestingly, OsDET1 also influences ABA biosynthesis in rice. Endogenous ABA levels are controlled by the balance of ABA biosynthesis and inactivation. ZEP and NCED are rate-limiting enzymes in ABA biosynthesis. ABA 8′-hydroxylase is the major enzyme for ABA inactivation (Nambara and Marion-Poll, 2005). Our results show that the ABA contents decreased in OsDET1 RNAi and OsDET1-GFP plants and increased in OE-OsDET1 plants compared with wild-type plants (Fig. 4A; Supplemental Fig. S6). Further analysis suggested that OsDET1 regulates ABA biosynthesis by fine-tuning the expression of ABA biosynthesis and inactivation genes (Fig. 4). ABA mediates plant germination and early seedling growth (Mulkey et al., 1983; Cornish and Zeevaart, 1985). Stimulating ABA biosynthesis inhibits seed germination as well as root and shoot growth in rice (Ikeda et al., 2002; Zhu et al., 2009; Cai et al., 2015). We found that seed germination was delayed in OE-OsDET1 plants and that root and shoot growth was inhibited during ABA treatment (Fig. 5). Similarly, mutation of the SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE1 gene (SAUL1) increases ABA biosynthesis and causes early leaf senescence under low light conditions in the saul1 mutants (Raab et al., 2009). OE-OsDET1 plants also showed premature leaf senescence during dark treatment (Supplemental Fig. S5). These phenotypes are in accordance with the higher ABA levels in OE-OsDET1 plants, and they also support our conclusion that OsDET1 plays a positive role in ABA biosynthesis. In addition, ABA promotes cuticular wax biosynthesis in many plants (Fricke et al., 2006; Seo et al., 2011; Lee and Suh, 2015; Zhou et al., 2015). Interestingly, this study found that there was less wax crystallization on the leaf surface of OsDET1 RNAi plants than in wild-type plants (Fig. 9A). On the contrary, more wax crystallization existed on the OE-OsDET1 plant leaf surface than in the wild type (Supplemental Fig. S10C). These results are consistent with the change of ABA contents

Figure 11. Model of how OsDET1 deficiency leads to contradictory phenotypes related to ABA in OsDET1 RNAi plants. OsDDB1 interacts with OsCOP10, OsDET1, and OsDDA1 to form the CDDD complex. The reduction of CDDD complex function hinders the ubiquitination of OsPYLs and causes the accumulation of OsPYLs. The accumulation of PYLs and ABA promotes the stability of the PP2C-ABA-PYL ternary complex (Irigoyen et al., 2014). However, OsDET1 deficiency also leads to a decline in the content of ABA in normal conditions. Thus, due to these opposing factors, OsDET1 deficiency only partly causes the ABA hypersensitivity phenotype, such as closed stomatal pores and changes of pollen grain morphology, although it is unable to induce leaf senescence in normal development. The modulation of cuticular wax biosynthesis by the ABA signaling pathway is distinct from the governing stomatal regulation, and the accumulation of cuticular waxes is reduced in OsDET1 RNAi plants. During dark treatment, ABA is induced by continuous dark, which further enhances the ABA response and finally leads to increased leaf senescence. The leaf senescence in turn promotes the synthesis of ABA. Finally, OsDET1 RNAi plants exhibit a significantly accelerated leaf senescence phenotype.
OsDET1 modulates ABA biosynthesis.

Therefore, we propose a model explaining the contradictory ABA-related phenotypes of OsDET1 RNAi plants (Fig. 11). The PP2C-ABA-PYL ABA signaling pathway regulates leaf senescence. OsDET1 deficiency damages the functioning of the CDD complex and causes the accumulation of PYLs. Meanwhile, OsDET1 deficiency also reduces ABA contents in OsDET1 RNAi plants. PYLs and ABA positively regulate the stability of the PP2C-ABA-PYL ternary complex, leading to ABA responses (Irigoyen et al., 2014; Zhao et al., 2016). Therefore, the reduced ABA content alleviates the damage caused by ABA hypersensitivity in OsDET1 RNAi plants, thereby preventing early leaf senescence in these plants. Nonetheless, OsDET1 RNAi plants also partially exhibit ABA hypersensitivity phenotypes, including stomatal closure and changes in pollen grain morphology. Furthermore, ABA promotes cuticular wax biosynthesis, but not via the PP2C-ABA-PYL ABA signaling pathway (Seo et al., 2011; Gonzalez-Guzman et al., 2012). Thus, wax crystallization and accumulation are weakened in OsDET1 RNAi plants. ABA signaling is induced by dark treatment, which increases dark-induced leaf senescence, in turn promoting ABA biosynthesis. Indeed, the OsDET1 RNAi plants exhibited rapidly accelerated leaf senescence.

In conclusion, the disruption of OsDET1 expression leads to a range of altered phenotypes. OsDET1 deficiency or structural changes impair the degradation of OsPYL5, stimulating ABA signaling, thereby causing ABA hypersensitivity in rice. OsDET1 also plays a positive role in ABA biosynthesis. Due to these two effects, OsDET1 RNAi plants exhibited some seemingly contradictory phenotypes related to ABA. In addition, overexpression of OsDET1 also caused ABA hypersensitivity in OE-OsDET1 plants. Overall, our work demonstrates that OsDET1 not only is involved in regulating the ABA signaling pathway but also regulates the ABA biosynthesis pathway in rice, implying that this protein has diverse effects on the ABA signaling pathway.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Rice (Oryza sativa japonica ‘Nipponbare’) plants were used in this study. Recombinant plasmids were transformed into rice callus by Agrobacterium tumefaciens (EHA105) medium transformation as described previously (Huang et al., 2013). The plants were grown in a greenhouse under a 12-h-light/12-h-dark cycle (300 mmol photons m⁻² s⁻¹) at 30°C and 60% relative humidity.

For OsDET1 induction analysis, cv Nipponbare seeds were germinated and cultured in one-half-strength MS liquid medium in a greenhouse for 2 weeks at 30°C under a 12-h-light/12-h-dark cycle. Different types of phytohormones were added to the nutrition solution, including gibberellin (50 μM), auxin (50 μM), 2-benzylaminopurine (50 μM), salicylic acid (50 μM), and ABA (50 μM). In addition, the plants were treated with NaCl (200 mM) or polyethylene glycol 6000 (20%) for 8 h for various lengths of time. For UV light treatment, the seedlings were treated with UV light for 40 min. Five plants were collected per treatment for RNA isolation. All experiments were repeated three times independently.

Physiological Measurements

The penultimate leaves at the tillering stage were used for dark- and ABA-induced leaf senescence tests. Photosynthetic pigments were extracted with 80% (v/v) acetone from dark-induced senescent and fresh leaves. Chlorophyll contents were determined as described (Lichtenthaler, 1987). Fv/Fm was measured with a PAM 2000 fluorometer (Heinz Walz) as described previously (Sakuraba et al., 2014). To measure membrane ion leakage, freshly excised leaves were washed several times with Milli-Q water and incubated in Milli-Q water in the dark at 30°C. Conductivity was measured with a DDSJ-318 conductivity meter (INESA). To measure water-loss rates, the penultimate leaves were detached from rice plants and weighed over time at room temperature at the tillering stage. To determine leaf stomatal closure status, the penultimate leaves were detached from rice plants of tillering stage and monitored by SEM (S-3400; Hitachi; Sang et al., 2012). To determine pollen fertility, pollen iodine staining was performed as described previously (Yaish et al., 2010). To explore leaf cell death and chlorophyll degradation, leaves were examined by transmission electron microscopy (H-7650; Hitachi) using a voltage of 80 kV as described previously (Morita et al., 2009). Leaf ABA contents were measured using a PhytoDectek competitive ELISA kit (Agdia). ABA was extracted as described previously and measured following the manufacturer’s instructions (Fukumoto et al., 2013). Three independent biological repeats were performed.

Histochemical Staining for GUS Activity

The vector pCAMBIA-1381Z was used to construct the fusion plasmid P₀DET1::GUS, containing the 2-kb OsDET1 promoter region and the GUS reporter gene. The construct was stably transformed into cv Nipponbare rice. GUS staining and observation were carried out as described previously (Liang et al., 2014).

Dark- and ABA-Induced Leaf Senescence

To analyze dark- and ABA-induced senescence, the penultimate leaves were excised from the plants at the tillering stage. The detached leaves were incubated in water at 30°C in the dark to analyze dark-induced leaf senescence. Detached leaves were incubated in 50 μM ABA at 30°C in the light to analyze ABA-induced leaf senescence.

Germination and Postgermination Assays

After surface sterilization, the seeds were planted on one-half-strength MS medium supplemented with 0 and 2 μM ABA. Seeds that formed green shoots were scored as germinated. Seed germination was record every day until day 10. For postgermination assays, seedlings were grown on one-half-strength MS medium for 4 to 5 d. Similarly sized transgenic plants were transferred to one-half-strength MS medium supplemented with 0 and 2 μM ABA. Seedling growth was measured at 7 d after transfer. The plants were grown in a greenhouse under a 12-h-light/12-h-dark cycle (300 mmol photons m⁻² s⁻¹) at 20°C and 60% relative humidity.

Expression Analysis

Total RNA was extracted using RNAiso Plus (Takara; http://www.takara.com.cn), and the RNA was reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) according to the manufacturer’s instructions. Transcript levels of each gene were measured by qRT-PCR usingCFX1000 (Bio-Rad) with the SYBR Premix Ex Taq II Kit (Takara). Primer sets used for qRT-PCR are listed in Supplemental Table S7. The relative expression levels of each gene were normalized to the expression of OsACTIN1. Values are means ± SD of three biological repeats.

Yeast Two-Hybrid and BiFC Assays

yeast two-hybrid (Y2H) analysis was performed using the Matchmaker Gold Yeast Two-Hybrid System as described in the Yeastman Yeast Transformation System 2 User Manual (Clontech). First, the coding sequence of OsDET1 was amplified and inserted into PGBK7T7 as bait using the primers listed in Supplemental Table S7. Then, the PGBK-OsDET1 plasmid was transformed into Y2H Gold yeast cells. After autoactivation and toxicity experiments were performed, the cv Nipponbare cDNA library (generated previously in our laboratory) was screened with PGBK-OsDET1 as bait in the Y2H Gold strain (Clontech) on SDO/X-a-gal/aurobasidin A and synthetic dextrose/-Trp media.
The plasmids were purified from positive clones and transformed into *Escherichia coli* DH5α. Plasmids with an open reading frame fused in frame to *PGADT7* were identified by nucleotide sequencing, followed by BLAST analysis against the National Center for Biotechnology Information database. To confirm the positive interactions, bait and prey plasmids were cotransformed into Y2H Gold yeast cells, followed by incubation on synthetic dropout (SD) medium lacking tryptophan (Trp), leucine (Leu), histidine (His) and adenine (-Ade) and containing the X-a-gal, Azurobasinidin A and 3-amino-1, 2, 4-triazole (SD/-Trp/-Leu/-His/-Ade/X/Aba/3-AT).

To confirm protein interactions, full-length OsDDAO1, OsDDB1, and OsCOP10 cDNA were cloned into pGBKT7. The full-length OsDET1 and truncated versions of OsPYL5 (amino acids 84–207) were fused to pGADT7, and the plasmids were cotransformed into Y2H Gold yeast cells, followed by incubation on the SD/-Trp/-Leu/-His/-Ade/X/Aba/3-AT medium.

The vectors (pFGC-N-YFP and pFGC-C-YFP) used in the BIFC assay were a kind gift from Chen Zhixiang. For BIFC analysis, the full-length coding sequences of OsDET1 and OsDDAO1 without terminators were subcloned adjacent to pFGC-N-YFP to form the pFGC-OsDET1-YFP and pFGC-OsDDAO1-YFP constructs, respectively. The full-length coding sequences of OsCOP10, OsPYL5, and OsDDB1, without terminators, were subcloned adjacent to pFGC-N-YFP to form the pFGC-OsCOP10-YFP, pFGC-OsPYL5-YFP, and pFGC-OsDDB1-YFP constructs, respectively. The primers used are listed in Supplemental Table S7. Pairs of plasmids were introduced into onion (*Allium cepa*) epidermal cells by *Agrobacterium*mediated transformation as described previously. The onion cells were then incubated at 28°C for 48 h. YFP fluorescence was examined and photographed by confocal microscopy (A1 R ; Nikon) at 48 to 72 h after infiltration.

**Antibody Production**

Two specific polypeptides of OsPYL5 were synthesized (BMRRLLSHAPGE and AVQSPITPSLEQ) and injected separately into rabbits. Rabbit preimmune serum was used to check for anti-OsPYL5 specificity.

**Immunoblot Analysis**

Total leaf protein was prepared essentially as described (Sangon Biotech; http://www.sangon.com/). SDS-PAGE and subsequent immunoblot analysis were performed as described previously (Morita et al., 2009). Protein content was detected by the Bradford method (Bio-Rad). The following antibodies were used: polyclonal antibodies against LHCa1, LHCb1, LHCb2, LHCb4, LHCb6, Rubisco det (www.sangon.com/). SDS-PAGE and subsequent immunoblot analysis were performed as described previously (Morita et al., 2009). Protein content was detected by the Bradford method (Bio-Rad). The following antibodies were used: polyclonal antibodies against LHCa1, LHCb1, LHCb2, LHCb4, LHCb6, Rubisco det (www.sangon.com/).

**RNA-SEQ Analysis**

The penultimate leaves were dissected from 8-week-old wild-type and DRI-16 plants. Samples from five siblings grown in the same bucket were pooled as one biological repeat; three biological repeats were performed per genotype. The RNA-SEQ experiments were performed by Novogene. Total RNA was extracted with TRIzol reagent (Invitrogen). Library construction was performed according to Illumina instructions and sequenced on a HiSeq 2000 sequencer. All paired-end reads were mapped to the rice cv Nipponbare genome using TopHat2 (Langmead et al., 2009; Trapnell et al., 2009; Kim and Salzberg, 2011). Expression levels were calculated using the reads per kb per million reads using TopHat2 (Langmead et al., 2009; Trapnell et al., 2009; Kim and Salzberg, 2011). Expression levels were calculated using the reads per kb per million reads using TopHat2 (Langmead et al., 2009; Trapnell et al., 2009; Kim and Salzberg, 2011).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** OsDET1 deficiency accelerates the expression of CDGs and SAGs during dark-induced leaf senescence.

**Supplemental Figure S2.** Photosynthetic protein degradation during dark-induced leaf senescence.

**Supplemental Figure S3.** Ultrastructural changes of chloroplasts in *OsDET1* RNAi lines and the wild type during dark incubation.

**Supplemental Figure S4.** Characterization of *OsDET1* RNAi plants at the late filling stage.

**Supplemental Figure S5.** Overexpression of *OsDET1* accelerates dark-induced leaf senescence.

**Supplemental Figure S6.** *OsDET1* deficiency and overexpressing *OsDET1-GFP* decrease the ABA content in leaves.

**Supplemental Figure S7.** ABA biosynthesis of *OsDET1* RNAi lines during dark treatment.

**Supplemental Figure S8.** *OsDET1* interacts with both candidate proteins in vivo.

**Supplemental Figure S9.** *OsDET1* deficiency leads to pleiotropic phenotypes in *OsDET1* RNAi lines.

**Supplemental Figure S10.** Overexpressing *OsDET1* causes pleiotropic phenotypes in OE-*OsDET1* plants.

**Supplemental Figure S11.** Different functions of overexpressing *OsDET1-GFP* or *OsDET1* in pollination and fertilization processes in the flower.

**Supplemental Figure S12.** Phenotypes of *OsDET1-GFP* plants.

**Supplemental Figure S13.** Overexpressing *OsDET1-GFP* accelerates ABA-induced leaf senescence.

**Supplemental Figure S14.** Overexpressing *OsDET1-GFP* accelerates dark-induced leaf senescence in rice.

**Supplemental Table S1.** Agronomic traits of wild-type and *DRI-16* plants.

**Supplemental Table S2.** Summary of the mRNA sequencing data mapping.

**Supplemental Table S3.** Transcriptome analysis identified 4,752 DEGs in *DRI-16* transgenic plants.

**Supplemental Table S4.** GO biological process assignments and their enrichments.

**Supplemental Table S5.** Examples of the DEGs in *OsDET1* RNAi plants that are predicted to be involved in photosynthesis and thylakoid.

**Supplemental Table S6.** KEGG pathway enrichment results for DEGs.

**Supplemental Table S7.** Primers used in this study.

**ACKNOWLEDGMENTS**

We thank Bing Yang (Iowa State University) for advice on the RNAi vector, Zhiqiang Chen (Purdue University) for providing the vectors pFGC-N-YFP and pFGC-C-YFP, and Guanghua He (Southwest University, China) for SEM analyses. Received January 13, 2016; accepted April 27, 2016; published May 2, 2016.

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