Genes Genet. Syst. (2007) **82**, p. 361–366

Rice tillering dwarf mutant *dwarf3* has increased leaf longevity during darkness-induced senescence or hydrogen peroxide-induced cell death

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(Received 4 June 2007, accepted 17 July 2007)

Senescence or cell death in plant leaves is known to be inducible by darkness or H_2O_2. When the *Arabidopsis* gene *MAX2/ORE9* is disrupted, leaf senescence or cell death in response to the above stimuli is delayed. Because the rice (*Oryza sativa* L.) gene *DWARF3* (*D3*) is orthologous to *MAX2/ORE9*, we wished to know whether disruption of *D3* also results in increased longevity in leaves. We found that darkness-induced senescence or H_2O_2-induced cell death in the third leaf [as measured by chlorophyll degradation, membrane ion leakage and expression of senescence-associated genes (SAGs)] in a *d3* rice mutant was delayed by 1–3 d compared to that in its reference line Shiokari. Moreover, the mRNA levels of *D3*, *HTD1* and *D10*, which are orthologs of *Arabidopsis MAX2/ORE9*, *MAX3* and *MAX4*, respectively, increased during cell death. These results suggest that D3 protein in rice, like MAX2/ORE9 in *Arabidopsis*, is involved in leaf senescence or cell death.

Key words: *d3*, MAX2/ORE9, *Oryza sativa*, oxidative stress, senescence

Leaf senescence is an important step in the plant life cycle and occurs not only by normal aging but also by external factors (*e.g.*, insufficient light/darkness, temperature, drought, nutrient deficiency and pathogen attack) or internal factors (*e.g.*, plant hormones and growth regulators) (for review, see Lim et al., 2003; Yoshida, 2003). Among the internal factors, ethylene, abscisic acid (ABA), and jasmonic acid (JA) act as promoters or modulators of leaf or flower senescence and leaf abscission, whereas cytokinins and polyamines work as antagonists of leaf senescence (Gan, 2003).

In addition to these plant hormones and growth regulators, an unidentified inhibitor of shoot branching (for reviews, see McSteen and Leyser, 2005; Beveridge, 2006) may be involved in leaf senescence (Snowden et al., 2005). Genetic and molecular analyses of the roles of the shoot branching inhibitor in axillary bud outgrowth have been undertaken using mutants of *Arabidopsis* [*more axillary growth* (*max*) mutants *max1*, *max2*, *max3* and *max4* (McSteen and Leyser, 2005)], pea [*ramosus* (*rms*) mutants *rms1*, *rms2*, *rms3*, *rms4* and *rms5* (Beveridge, 2000; Johnson et al., 2006)], petunia [*decreased apical dominance1* (*dad1*) (Snowden et al., 2005)] and rice [*dwarf3* (*d3*; Ishikawa et al., 2005), *high-tillering dwarf1* (*htd1*; Zou et al., 2005; Zou et al., 2006) and *d10* (Arite et al., 2007)], all of which show increased branching or tillering phenotypes. The pathway involved in these gene products is referred as the MAX pathway (McSteen and Leyser, 2005; Stirnberg et al., 2007) or the shoot-multiplication signal (SMS) pathway (Beveridge, 2006). In addition to having an increased branching phenotype, the petunia *dad1* mutant has a delayed leaf senescence phenotype (Snowden et al., 2005). The *DAD1* gene encodes a hypothetical carotenoid cleavage dioxygenase (CCD) (Snowden et al., 2005), which is an ortholog of *Arabidopsis MAX4/AtCCD8* gene and which is probably responsible for the biosynthesis of the shoot branching inhibitor (Sorefan et al., 2003). On the other hand, the *Arabidopsis max2* mutant was originally isolated by screening for mutants that show a delay of senescence [Woo et al., 2001; in this paper it is referred to as the *oresara9* (*ore9*)

Edited by Toru Terachi
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mutant]. The product of the disrupted gene in this mutant, MAX2/ORE9, was identified as a member of the F-box leucine-rich repeat (LRR) family of proteins, and probably plays a role in ubiquitin-proteasome-mediated protein degradation (Woo et al., 2001; Stirnberg et al., 2002; Stirnberg et al., 2007). The *Arabidopsis max2/ore9* mutant also shows resistance to oxidative stress induced by reactive oxygen species (ROS)-generating agents such as H_2O_2 (Woo et al., 2004). On the other hand, it seems that MAX2/ORE9-mediated protein degradation regulates the ENHANCED DISEASE RESISTANCE 1 (EDR1)-involved senescence pathway in *Arabidopsis* (Tang et al., 2005). The mutation in *EDR1* enhances ethylene-induced senescence, whereas the double mutant in both *EDR1* and *MAX2/ORE9* suppresses the ethylene-induced senescence, suggesting that MAX2/ORE9 negatively affects the function of EDR1 in ethylene-induced senescence (Tang et al., 2005).

Ishikawa et al. (2005) previously characterized five tillering dwarf mutants (*d3*, *d10*, *d14*, *d17* and *d27*) in rice, which reduced plant stature and increased tiller number. Among the disrupted genes in these mutants, the *D3* gene and the *D10* gene were identified by map-based cloning and were shown to encode proteins orthologous to *Arabidopsis* MAX2/ORE9 (Ishikawa et al., 2005) and MAX4 (Arite et al., 2007), respectively. This suggests that the mechanism controlling outgrowth of axillary bud is at least partly shared by eudicots and monocots. In this study, we wished to know whether leaf senescence or cell death in the rice *d3* mutant is delayed as they are in the *Arabidopsis max2/ore9* mutant.

In the rice *d3* mutant, the *D3* gene is disrupted by insertion of a putative transposon in exon 1, thereby causing an alteration of the amino acid sequence of D3 protein and generating a stop codon in the open reading frame (Ishikawa et al., 2005). The *d3* mutant used in this study was introduced to the cultivar Shiokari by 5 rounds of back-crossing and the resultant inbred line was designated I*d3* (Ishikawa et al., 2005). To examine whether the D3 protein is involved in leaf senescence or cell death, I*d3* and its reference line Shiokari were germinated and grown for 7 d under light conditions (white light; 80 μmol m^{-2} sec^{-1}), and then the seedlings were transferred to darkness to induce leaf senescence. First, we measured chlorophyll contents and membrane ion leakage, which are often used as senescence-associated physiological markers (Woo et al., 2001; Woo et al., 2004), of the third leaf of I*d3* and Shiokari seedlings. During darkness-induced leaf senescence, degradation of chlorophyll was observed in both I*d3* and Shiokari, but the chlorophyll breakdown was later in I*d3* than in Shiokari (Fig. 1A, B). As shown in Fig. 1C, the increase of membrane ion leakage in the third leaf was also later in I*d3* than in Shiokari, indicating that darkness-induced senescence in I*d3* was delayed. Moreover, to confirm the delay of darkness-induced senescence in I*d3*, we examined the expression of three Senescence Associated Genes (SAGs; *Osl20*, *Osl85* and *Osl295*) that are known to be induced during leaf senescence (Lee et al., 2001). mRNA levels in the third leaf were monitored with quantitative reverse transcription-PCR (qRT-PCR) using a LightCycler (Roche Diagnostics, Mannheim, Germany) and QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA). The mRNA levels of the three SAGs started to increase dramatically in Shiokari at 1 d after transfer to dark conditions, peaked at 3 d, and then decreased (Fig. 1D). However, in I*d3*, induction of the SAGs was delayed for 1-3 d compared with the induction observed in the Shiokari (Fig. 1D).

Senescence is known to involve oxidative reactions induced by reactive oxygen species such as superoxide anion, H_2O_2 and hydroxyl radical, which result in lipid peroxidation and membrane leakiness (Buchanan-Wollaston, 1997; Lam, 2004). In the *Arabidopsis* delayed leaf senescence mutant *max2/ore9*, leaf senescence was delayed even more by oxidative stress (Woo et al., 2004). Thus, we examined whether I*d3* also is more tolerant of oxidative stress. To check the dose-dependence of H_2O_2 on oxidative damage to rice leaves, the detached third leaf of Shiokari seedlings was floated on H_2O_2 solutions with different concentrations (0, 10, 50, 100 and 500 mM) under light conditions (white light; 80 μmol m^{-2} sec^{-1}), and chlorophyll content in each leaf was measured once every 24 h after the treatment. Chlorophyll degradation was dose-dependently accelerated by H_2O_2 (data not shown), and we used 100 mM H_2O_2 solution for further analyses. Chlorophyll degradation and the increase of membrane ion leakage induced by H_2O_2 occurred about 1 d later in I*d3* than in Shiokari (Fig. 2A, B). In contrast, the rates of chlorophyll degradation and membrane ion leakage in detached leaves treated with water (negative control) were comparable between I*d3* and Shiokari (Fig. 2A, B). H_2O_2 treatment dramatically increased the mRNA levels of *Osl20*, *Osl85* and *Osl295* in both I*d3* and Shiokari, but the expressions began 1–2 d later in I*d3* than in Shiokari (Fig. 2C). Taken together, these results indicate that the mutation in the *D3* gene in rice results in increased leaf longevity during darkness-induced senescence or H_2O_2-induced cell death, and suggest that the roles of this F-box LRR protein in leaf longevity are at least partly shared by eudicots (*e.g.*, *Arabidopsis*) and monocots (*e.g.*, rice). However, pea *RMS4*, which is orthologous to *Arabidopsis MAX2/ORE9* and rice *D3*, does not appear to have a role in senescence (Johnson et al., 2006), suggesting that the MAX/SMS pathway does not affect leaf longevity in all plant species.

Next, we examined whether the gene expressions of *D3* (Ishikawa et al., 2005), *HTD1* (Zou et al., 2005; Zou et al., 2006) and *D10* (Arite et al., 2007), which are orthologous to *Arabidopsis MAX2/ORE9*, *MAX3* and *MAX4*, respec-

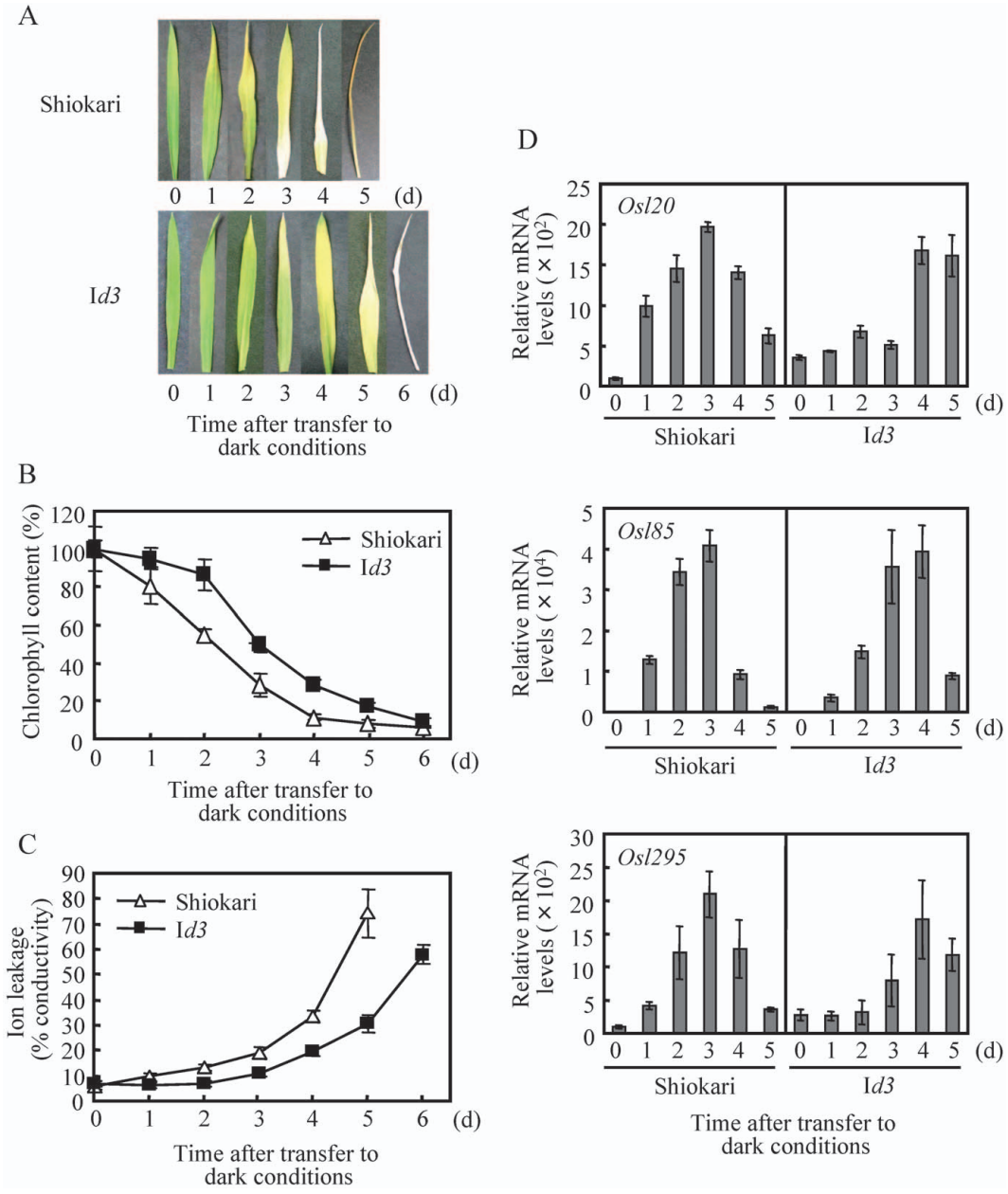

Fig. 1. Chlorophyll degradation and membrane ion leakage in leaves of Shiokari and I*d3* during darkness-induced leaf senescence. **A.** Representative third leaves of 7-d-old light-grown Shiokari and I*d3* seedlings transferred to darkness for the indicated times. **B.** Changes of chlorophyll contents in the third leaves of Shiokari and I*d3* during darkness-induced leaf senescence. Chlorophyll was extracted from the third leaf with 80% acetone. Chlorophyll content was determined spectrophotometrically at 663.2 nm and 646.8 nm according to Lichtenthaler (1987). **C.** Membrane ion leakage in the third leaves of Shiokari and I*d3* during darkness-induced leaf senescence. Membrane ion leakage was determined by measuring electrolytes leaked from leaves. A 2-cm-length leaf segment cut from the midst of the third leaf was immersed in 2 ml distilled water at 28°C for 1 hr, and then the initial conductivity of the media was measured using an electrical conductivity meter (B-173, Horiba, Kyoto, Japan). Total conductivity was determined after boiling for 10 min. The conductivity was expressed as the percentage of the initial conductivity versus the total conductivity. Results are expressed as the percentage of conductivity (initial conductivity/total conductivity × 100). **D.** mRNA levels of Senescence-Associated Genes (SAGs), *Osl20*, *Osl85* and *Osl295*, in Shiokari and I*d3* during darkness-induced leaf senescence. For qRT-PCR, 0.1 μg of total RNA extracted from the third leaves of 7-d-old light-grown seedlings that had been transferred to darkness for the indicated times was used as a template. All mRNA levels were normalized to the 17S rRNA level as a control and were presented as a percent of the value observed at 0 d in Shiokari. qRT-PCR was performed three times for each RNA template. Data show mean ± SD of three separate PCR analyses. The RT-PCR experiments were done twice using different RNA samples for the template. Similar results were obtained for each experiment.

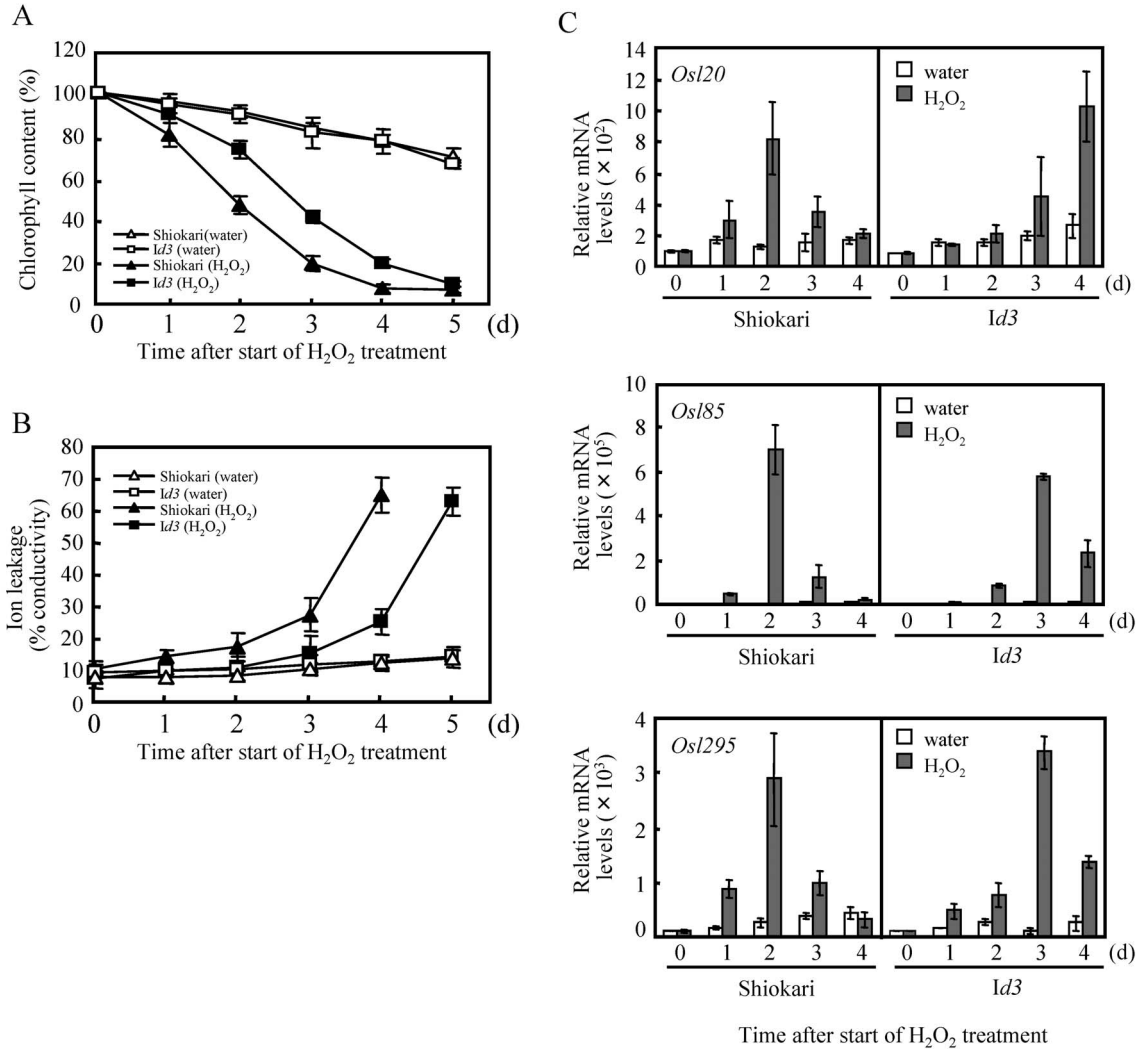

Fig. 2. Chlorophyll degradation and membrane ion leakage in Shiokari and I*d3* during H_2O_2-induced leaf cell death. **A, B.** Changes of chlorophyll contents (**A**) and membrane ion leakage (**B**) in the third leaves of Shiokari and I*d3*, which were floated on 100 mM H_2O_2 solution and water (negative control) under light conditions for the indicated times. **C.** mRNA levels of Senescence-Associated Genes (SAGs), *Osl20*, *Osl85* and *Osl295*, in Shiokari and I*d3* during H_2O_2-induced leaf cell death. The third leaves of Shiokari and I*d3* were floated on 100 mM H_2O_2 solution and water (negative control) for the indicated times. Transcript levels were measured as described in Fig. 1D.

tively, are changed during darkness-induced senescence or H_2O_2-induced cell death. In Shiokari, the *D3* transcripts increased by darkness or H_2O_2 and then decreased, whereas the *D3* mRNA levels were maintained at relatively low levels in I*d3* (Fig. 3A, B). The *HTD1* mRNA began to increase 1–2 d after the transfer to darkness and peaked at 3 d (Fig. 3A) or began to increase 1 d after H_2O_2 treatment and peaked at 2 d (Fig. 3B). The *HTD1* gene expression pattern was comparable between Shiokari and I*d3* (Fig. 3A, B). *D10* mRNA increased from 1–2 d after the transfer to darkness and peaked at 4 d in Shiokari or peaked at 3 d in I*d3* (Fig. 3A). On the other hand, *D10* mRNA started to increase at 1 d after H_2O_2 treatment in both in Shiokari and I*d3* (Fig. 3B). *D10* mRNA levels were slightly higher in I*d3* than in Shiokari (Fig. 3A, B). These results suggest that expression of the *D3*, *HTD1* and *D10* genes is induced during senescence or cell death in leaves, but the expressions of *HTD1* and *D10*, unlike the expression of SAGs, is not delayed by the deficiency of *D3*. It would be of interest to examine whether leaf senescence or cell death was also delayed in the rice *htd1* and *d10* mutants like they are in the *d3* mutant. In fact, in the petunia *dad1* mutant, in which a gene orthologous to *Arabidopsis MAX4* and rice *D10* is deficient, leaf senescence is delayed (Snowden et al., 2005).

As shown in Fig. 3, the *D10* mRNA levels were slightly higher in I*d3* than in Shiokari. These results are consistent with the data reported by Arite et al. (2007), in which *D10* gene expression was upregulated in the rice *d3*, *d14*,

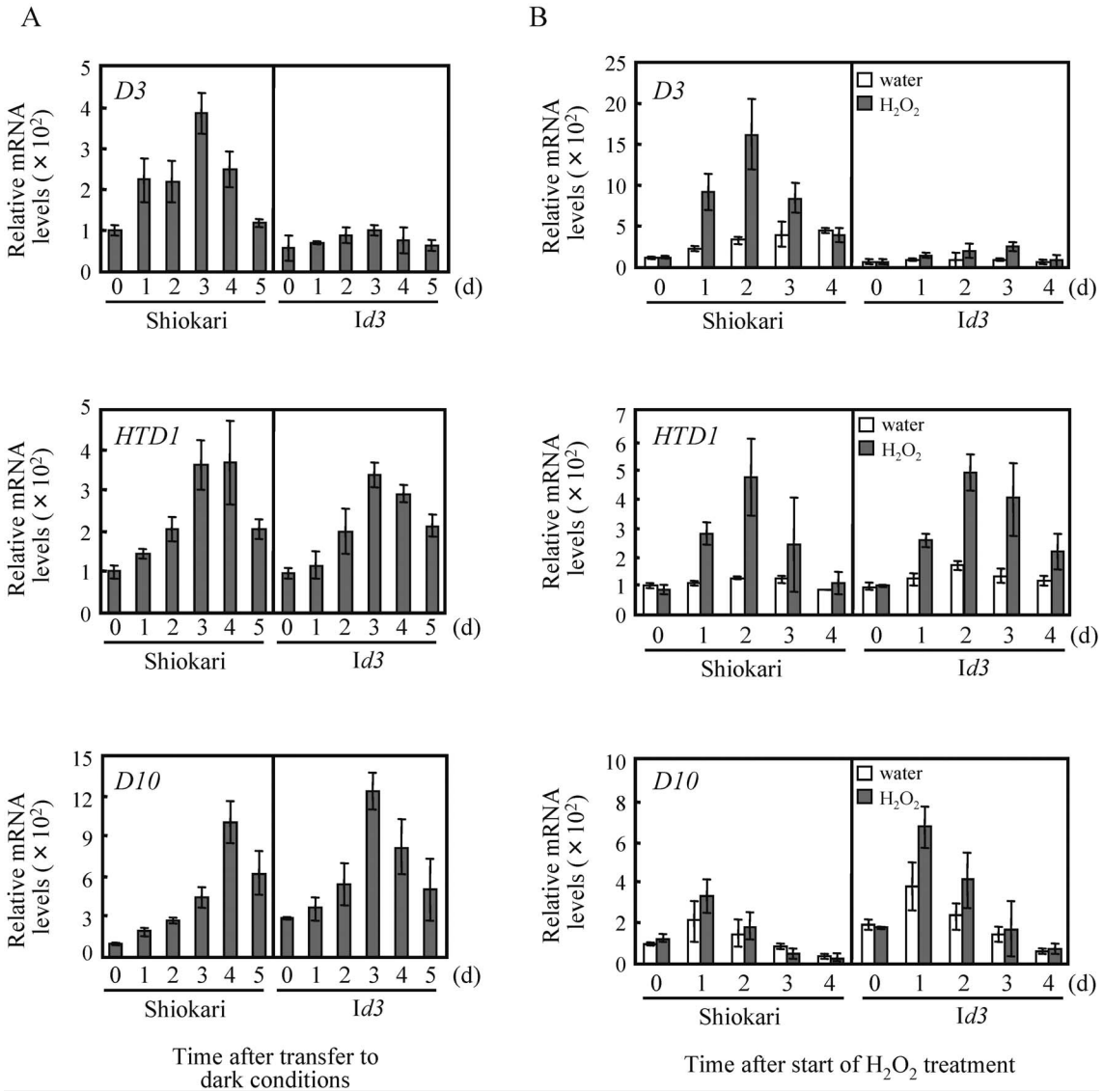

Fig. 3. mRNA levels of the MAX/SMS pathway genes, *D3*, *HTD1* and *D10*, in Shiokari and I*d3* during darkness-induced leaf senescence (**A**) and H_2O_2-induced leaf cell death (**B**). Used samples were the same as those used in Figs. 1D and 2C.

d17 and *d27* mutants. Similar results were obtained from studies of pea *RMS1* (a gene orthologous to *Arabidopsis MAX4* and rice *D10*; Foo et al., 2005), petunia *DAD1* (Snowden et al., 2005) and *Arabidopsis MAX4* (Bainbridge et al., 2005). For example, in the pea *rms4* mutant, in which a gene orthologous to *Arabidopsis MAX2* and rice *D3* is deficient, *RMS1* gene expression is upregulated (Foo et al., 2005). *MAX4* expression is slightly enhanced in hypocotyls of the *Arabidopsis max2* mutant (Bainbridge et al., 2005). These data suggest that the expressions of rice *D10*, pea *RMS1*, petunia *DAD1* and *Arabidopsis MAX4* are controlled by feedback regulation from the MAX/SMS pathway.

As mentioned above, plants appear to have natural shoot branching inhibitors. These inhibitors are probably carotenoid derivatives (Schwartz et al., 2004; McSteen and Leyser, 2005; Beveridge, 2006). The target protein of *Arabidopsis* MAX2/ORE9 or rice D3 is not known. Thus, future characterization of the shoot branching inhibitor and D3-target protein appears to be necessary to understand the D3-dependent control of leaf senescence or cell death and to understand the relationship between tillering and senescence (cell death) in rice.

We thank Drs. M. Kusaba, M. Iino, K. Haga, E. Nambara, S. Yamaguchi, Y. Jikumaru, S. Arimura, K. Yoshinaga and Mr. T. Arite for their stimulating discussions. This work was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from Ministry of Agriculture, Forestry and Fisheries of Japan.

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