A Novel Protein RLS1 with NB–ARM Domains Is Involved in Chloroplast Degradation during Leaf Senescence in Rice

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ABSTRACT  Leaf senescence, a type of programmed cell death (PCD) characterized by chlorophyll degradation, is important to plant growth and crop productivity. It emerges that autophagy is involved in chloroplast degradation during leaf senescence. However, the molecular mechanism(s) involved in the process is not well understood. In this study, the genetic and physiological characteristics of the rice rls1 (rapid leaf senescence 1) mutant were identified. The rls1 mutant developed small, yellow-brown lesions resembling disease scattered over the whole surfaces of leaves that displayed earlier senescence than those of wild-type plants. The rapid loss of chlorophyll content during senescence was the main cause of accelerated leaf senescence in rls1. Microscopic observation indicated that PCD was misregulated, probably resulting in the accelerated degradation of chloroplasts in rls1 leaves. Map-based cloning of the RLS1 gene revealed that it encodes a previously uncharacterized NB (nucleotide-binding site)-containing protein with an ARM (armadillo) domain at the carboxyl terminus. Consistent with its involvement in leaf senescence, RLS1 was up-regulated during dark-induced leaf senescence and down-regulated by cytokinin. Intriguingly, constitutive expression of RLS1 also slightly accelerated leaf senescence with decreased chlorophyll content in transgenic rice plants. Our study identified a previously uncharacterized NB–ARM protein involved in PCD during plant growth and development, providing a unique tool for dissecting possible autophagy-mediated PCD during senescence in plants.

Key words: Rice; RLS1; NB–ARM domains; chloroplast degradation; senescence.

INTRODUCTION

Programmed cell death (PCD) is an important process used to remove unwanted cells in eukaryotes (Jacobson et al., 1997). In plants, PCD is necessary for a number of developmental processes and stress responses, including the formation of tracheary elements, the hypersensitive response (HR), and the development of unisexual floral organs (Lam, 2004; Williams and Dickman, 2008). Some key components, such as Apaf1 and Ced4, have been revealed to play critical roles in the regulation of PCD in animal cells (Zou et al., 1997; Mace and Riedl, 2010). Apaf1, Ced4, and other cell death proteins, including those involved in responses to microbial infection, share a conserved region termed nucleotide-binding site (NB) domain (Inohara and Nunez, 2001). The NB domain consists of three motifs: kinase 1a, kinase 2, and kinase 3a. This signaling domain is also found in most plant disease resistance (R) proteins encoded by resistance genes (R) (van der Biezen and Jones, 1998). R proteins are key components in mediating HR, a form of PCD that results from an incompatible interaction between plants and microbes (Morel and Dangl, 1997). This conserved domain has been referred to as NB-ARC (nucleotide-binding, Apaf-1, R proteins, and Ced-4) or NOD (nucleotide oligomerization domain) (Inohara and Nunez, 2001). The universal prevalence of such a domain implies an evolutionary conservation of cell death effectors between animals and plants (van der Biezen and Jones, 1998).

Two major types of PCD have been recognized in animals: apoptosis and autophagy (Clarke, 1990; Baehrecke, 2003).
Particularly, autophagy is a highly conserved process in eukaryotes during which cytoplasmic components are wrapped into double-membrane vesicles, which are then delivered into lysosomes/vacuoles for degradation and recycling of the resulting molecules (Ohsumi, 2001; Levine and Klionsky, 2004). The autophagy-related (ATG) genes have been identified in yeast. Studies of some yeast atg mutants have revealed that autophagy is involved in sporulation and survival during nutrient starvation (Kanki and Klionsky, 2010). The presence of the autophagy system in plants has been recently unveiled, which is required for several biological processes including nutrient recycling, maintaining cellular activities in plants (Swanson et al., 1998; Di Sansebastiano et al., 2001; Toyooka et al., 2001; Liu et al., 2005). Mutations in Arabidopsis ATG genes result in accelerated leaf senescence (Doelling et al., 2002; Hanaoka et al., 2002). Leaf senescence is a complex physiological event that constitutes the last stage of leaf development, integrating multiple developmental and environmental signals (Lim and Nam, 2005; Balazadeh et al., 2011). Cell death occurring in leaf senescence is a type of PCD, but it occurs more slowly than other acute forms of PCD, such as HR (Lim et al., 2003; Farage-Barhom et al., 2011). During leaf senescence, the process of cellular degeneration is initiated with chloroplast degradation (Orzáez and Granell, 1997; Stettler et al., 2009). It has been observed that a part of the chloroplast stroma could be mobilized to the vacuole in a specialized type of autophagic vesicles, mostly as Rubisco-containing bodies (RCBs), during leaf senescence (Chiba et al., 2003; Ishida et al., 2008). Although autophagy has been observed and its roles have been recognized in plants, the molecular mechanisms involved in the process are largely unknown (Reumann et al., 2010).

Nevertheless, the identification of mutants in which cell death is misregulated provides a powerful tool for the study of PCD pathways in plants. These mutants are called lesion mimics because they exhibit spontaneous cell death resembling disease lesions (Lorrain et al., 2003). Most of the lesion mimic genes encode proteins involved in defense responses or cellular signal components. For example, maize RPI encodes a NB-LRR resistance protein (Hu et al., 1996). Arabidopsis CPR5/HYS1 encodes a novel putative transmembrane protein that was proposed to play roles in defense responses to pathogen infection and the initiation of leaf senescence (Yoshida et al., 2002). In the model cereal crop, rice, several lesion mimic genes have also been identified. For example, SPL7 encodes a transcription factor functioning in response to heat stress (Yamanouchi et al., 2002). SPL11 encodes an E3 ligase that negatively regulates disease resistance to bacterial and fungal pathogens (Zeng et al., 2004). Recently, the mutations in a CC-NB-LRR-type R gene, NLS1, led to semi-dominant necrotic leaf sheath phenotypes with constitutive activation of defense responses against bacterial pathogens in rice (Tang et al., 2011).

We have collected and characterized several types of rice lesion resembling disease mutants (lr27-44) (Wang et al., 2004). Here, we report the map-based cloning and functional analysis of LRD43, renamed as RLS1 (RAPID LEAF SENESCENCE 1). The rls1 mutant displayed accelerated leaf senescence in both natural and dark-induced senescence, in comparison with the wild-type. We found that PCD involved in chloroplast degradation was misregulated in the leaf cells of rls1. Map-based cloning revealed that RLS1 encodes a novel NB containing protein with an ARM domain that presents only in sorghum, grape, and black cottonwood genomes. Our study reveals a role for RLS1, the unconventional NB-containing protein, in the autophagy-like process of chloroplast degradation during rice leaf senescence.

## RESULTS

### Characterization of the rapid leaf senescence Mutant rls1

The rls1 mutant (previously named lrd42) was isolated in genome-wide screening for lesion resembling disease mutants in rice (Wang et al., 2004). The mutant displayed small, yellow-brown lesions scattering over the whole surfaces of leaves that were developmentally programmed and only appeared after 6 weeks post germination under natural field conditions between 25 and 35°C (Figure 1A). Transmission electron microscopy (TEM) observation showed that mesophyll cells with fully developed chloroplasts were found in leaves of the wild-type, whereas completely destroyed chloroplasts and membrane-bound bodies distributed in the cytoplasm were found in the section of spotted area of the mutant leaf, which are characteristic of ongoing PCD (Figure 1B). The leaves of rls1 also displayed more accelerated senescence than those of wild-type plants (Figure 1C), resulting in accelerated plant death (Figure 1D). As a consequence of the expending senescence, the rls1 plants produced fewer grains (lower fertility) compared to the wild-type (Supplemental Figure 1). We did not find changes in disease resistance to rice blast and bacterial leaf blight in inoculation experiments as previously reported (Yang et al., 2008; Li et al., 2011; data not shown). These results suggested that the RLS1 gene might be involved in PCD that affects specific cellular processes.

### Physiological Alterations of Leaf Senescence in rls1

Leaf senescence is a chronic form of PCD, which is different from other acute PCD found during tissue wounding or pathogen infection, for example (Lim et al., 2003). To further confirm the role of RLS1 in leaf senescence, we examined senescence induced by dark treatment of intact plants—a method that is commonly used to artificially induce senescence (Lee et al., 2001; Chrost et al., 2004; Kusaba et al., 2007). After 2 d with dark treatment, some leaves of rls1 mutant displayed yellowing, whereas the leaves of wild-type plants were still green. At day 3, most of the older leaves were yellow and showed collapse of leaf structure in the mutant, whereas only a few leaves of wild-type plants showed yellowing (Figure 2A).

We next examined chlorophyll levels and found that mutant plants showed a more rapid loss of chlorophyll than wild-type...
plants, especially at days 2 and 3 after dark treatment. Chlorophyll levels decreased by 54% at day 2 and by 95% at day 4 in rls1 leaves, whereas the levels in wild-type leaves were reduced by only 18% at day 2 and by 70% at day 4 (Figure 2B). Maximal photochemical efficiency of photosystem II (PSII), a typical senescence-associated physiological marker, was measured as Fv/Fm (John et al., 1995; Fan et al., 1997). The value of Fv/Fm was 0.83 in both wild-type and rls1 plants before dark treatment. During dark treatment, Fv/Fm values fell to 0.43 in wild-type and to 0.34 in rls1 at day 4, indicating that the decrease in photosynthetic efficiency of PSII in rls1 is not significantly faster than that in wild-type (Figure 2C). Cell death and membrane damage in cut leaves were estimated by measuring ion leakage, which began to increase at day 2 and increased similarly in the wild-type and rls1 during dark incubation (Figure 2D), indicating that membrane damage was similar in wild-type and rls1 cells.

We further examined physiological changes during natural senescence. The flag and the second leaves were examined at 21 d after flowering, when senescence normally occurs in the rice leaves. Chlorophyll content was significantly lower in the rls1 leaves than that in the wild-type leaves (Supplemental Figure 2A; t-test, P < 0.05). The Fv/Fm values and the membrane

Figure 1. Phenotypes of the Wild-Type and rls1 Mutant.
(A) Leaves phenotypes of 30-, 45-, and 70-day-old wild-type and rls1 mutant plants. Cell death lesions began to appear on 45-day-old rls1 leaves.
(B) TEM analysis of cells of the wild-type leaf and rls1 spotted leaf, indicating the destroyed chloroplasts in the rls1 cell in the lesion spot. c, chloroplast; v, vacuole.
(C) Naturally senescent leaves of the wild-type and rls1 at 25 d after flowering. f, the flag leaf; 2, the second leaf; 3, the third leaf.
(D) Matured wild-type and rls1 plants 45 d after flowering. Note that most parts of the rls1 plant were dead. Bar = 15 cm.
ion leakage values of the leaves were also similar between \textit{rls1} and wild-type plants (Supplemental Figure 2B and 2C). To confirm that precocious senescence is actually induced in the \textit{rls1} mutant, we examined the expression of a senescence marker gene, \textit{Osh69} (Lee et al., 2001; Park et al., 2007). \textit{Osh69} transcript levels were much higher in the second leaves of the \textit{rls1} mutant than in the leaves of the wild-type (Supplemental Figure 2D), indicating accelerated senescence in the \textit{rls1} mutant. These observations suggest that the process of natural senescence in \textit{rls1} is most likely identical to that in dark-induced senescence.

**RLS1 Is Necessary for Proper Chloroplast Degradation**

To investigate the cause of the lower chlorophyll level in the \textit{rls1} mutant during senescence, we compared the ultrastructures of \textit{rls1} mutant and wild-type cells at different developmental stages using TEM. No obvious difference in the chloroplast structure was observed between wild-type and \textit{rls1} at the seedling stage (21 d old) (Supplemental Figure 3). At the tillering stage (45 d), when \textit{rls1} developed lesion mimics, a striking difference in the structure of chloroplasts was observed between wild-type and \textit{rls1} cells. In wild-type leaves, well-developed mesophyll cells were found with fully

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**Figure 2. Physiological Changes in Wild-Type and \textit{rls1} Leaves during Dark-Induced Senescence.**

(A) Wild-type and \textit{rls1} plants were grown for 3 weeks under 13/11 h light (30°C)/dark (28°C), and then transferred to the dark for 5 d to induce leaf senescence.

(B) Content of chlorophyll during dark incubation. Dotted lines, wild-type; solid lines, \textit{rls1}. Error bars indicate standard deviations (SD), \(n=6\).

(C) FvFm values. Dotted lines, wild-type; solid lines, \textit{rls1}. Error bars indicate SD, \(n=6\).

(D) Membrane ion leakage. Dotted lines, wild-type; solid lines, \textit{rls1}. Error bars indicate SD. \(n=6\).
developed chloroplasts (Figure 3A–3C), whereas damaged chloroplasts were found in the section surrounding yellowing spots on the rls1 leaf (Figure 3D). We further observed that plastoglobuli became obvious, and the array of grana stacks and intergrana became slightly disordered in the rls1 cells (Figure 3E and 3F). In addition, we occasionally observed double-membrane vesicles/spherical bodies that contained chloroplast materials in the rls1 cells (Figure 3G). These spherical bodies, with a diameter of 0.6–1.2 μm, seemed to be derived from the chloroplast envelope. We also observed the fusion of the spherical bodies with the vacuole in some rls1 cells (Figure 3H), which were further degraded in the vacuole (Figure 3I). It has been proposed that the spherical bodies mobilized to the vacuole are a specialized type of autophagic vesicles that are like Rubisco-containing bodies (RCBs) (Chiba et al., 2003; Ishida et al., 2008). The appearance of RCBs is most remarkable when the mutation in the rls1 mutant revealed a single C-to-T nucleotide substitution in the second exon of LOC_Os02g10900 (MSU Rice Genome Annotation Project: http://rice.plantbiology.msu.edu/), which results in Ser-to-Phe change at 994 residue (Figure 4C).

To confirm that RLS1 is LOC_Os02g10900, transformation of rls1 with the DNA fragment encompassing a wild-type copy of LOC_Os02g10900 under the control of its own promoter produced 12 T0-independent transformants, all of which resembled wild-type plants. None of the progeny plants (T1 and T2) containing the transgene showed any development of lesions or accelerated leaf senescence after the regeneration (Figure 4D and 4E). The seed set of transgenic plants was also similar to that of wild-type plants (Figure 4F). Therefore, RLS1 is indeed LOC_Os02g10900.

**Positional Cloning of the RLS1 Gene**

To identify the function of RLS1, we used a map-based cloning strategy to isolate the RLS1 gene. We crossed the rls1 mutant with *indica* cultivar Zhenshan 97 to generate a segregation population for gene mapping. The F1 plants from the cross were normal and segregation occurred in the F2 generation in the proportion of 3:1 (wild-type: mutant), indicating that rls1 was a recessive allele. The RLS1 locus was roughly mapped on chromosome 2. Subsequent fining mapping delimited the RLS1 locus to a 60-kb region between markers M17 and M24 (Figure 4A and 4B). DNA sequencing analysis of the entire region in the rls1 mutant revealed a single C-to-T nucleotide substitution in the second exon of LOC_Os02g10900 (MSU Rice Genome Annotation Project: http://rice.plantbiology.msu.edu/), which results in Ser-to-Phe change at 994 residue (Figure 4C).

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**Analysis of the RLS1 Protein and Homologs**

This predicted ORF of LOC_Os02g10900 was confirmed experimentally (Supplemental Figure 5A and 5B). The ORF of the RLS1 predicts a protein of 1040 amino acids. RLS1 is unrelated to any known proteins, but it contains the NB-ARC domain at the N terminus and the ARM motif at the C terminus (Supplemental Figure 5D). The NB-ARC domain is a conserved signaling motif shared by plant R proteins and regulators of apoptosis in animals whose activation results in cell death (van der Biezen and Jones, 1998; Takken and Tameling, 2009; Elmore et al., 2011). The ARM motif, containing 38–45 amino acid residues, has been implicated in mediating protein–protein interactions (Peifer et al., 1994; Huber et al., 1997; Tewari et al., 2010).

There is only one copy of the RLS1 gene in the rice genome. The RLS1 homolog genes with unknown function were only found in sorghum, grape, and black cottonwood by a BLAST search (Supplemental Figure 6). The putative NB domain of RLS1 contains the conserved motifs found in a large number of proteins binding ATP or GTP, and shows a significant similarity to that of homologous proteins from sorghum, grape, and black cottonwood. Moreover, it is also similar (23–30% similarity) to those of various NB-containing R proteins (Figure 5A). We then assessed the sequence diversity of the selected homologs, by generating a phylogenetic tree.
using the NB sequences (Figure 5B). The NB domains from 10 amino acids N-terminal to the first Gly in the Kinase 1a motif to 10 amino acids beyond the kinase 3a motif were used for analysis. Intriguingly, RLS1 was more closely related to the CC-NB-LRR proteins, Rx, I2, R3a (Moffett et al., 2002; Couch et al., 2006; Jia et al., 2010) than to TIR-NB-LRR proteins, N, RRS1-R, CHS3 (Deslandes et al., 2002; Konagaya et al., 2004; Yang et al., 2010).

**RLS1 Expression Is Affected by Dark-Induced Senescence and Cytokinin Treatment**

We first compared RLS1 transcript levels in rls1 mutant and wild-type plants using RT–PCR. No change in RLS1 transcript levels in the flag and second leaves was observed between rls1 and wild-type plants (Supplemental Figure 5C). Therefore, the point mutation did not affect RLS1 mRNA levels. The expression profile of RLS1 was investigated in various rice tissues at the adult stage with the quantitative real-time PCR approach. The results showed that RLS1 transcripts accumulated constitutively during leaf development, from young to senescent leaves (Figure 6A). In addition, RLS1 was constitutively but weakly expressed in the root of seedling, panicle, and spikelet. The highest level of the RLS1 transcript was detected in mature leaves, consistent with the phenotype of rls1.

Leaf senescence is a developmentally controlled, degenerative process affected by exogenous and endogenous factors such as cytokinin (Lim et al., 2007). Exogenous application or endogenous enhancement of cytokinin could delay senescence (Gan and Amasino, 1995). Leaf senescence is also regulated by the coordinated expression of specific genes (Buchanan-Wollaston, 1997; Lee et al., 2001). In order to know how RLS1 is affected during leaf senescence, we investigated the expression of RLS1 in the rice seedlings under dark-induced senescence and cytokinin treatment. The result showed that the expression of RLS1 was significantly induced in the seedlings transferred into darkness and reached the maximum level 12 h after treatment (Figure 6B). Accumulation of the RLS1 transcript was markedly reduced in the seedlings treated with 20 μM trans-zeatin (tZ) for 24 h (Figure 6C). These results suggested that RLS1 is involved in leaf senescence.

**Overexpression of RLS1 Displays a Weak Phenotype of Accelerated Leaf Senescence**

To further characterize the function of RLS1, we transformed wild-type plants with the RLS1 genomic DNA fused to the 35S promoter of Cauliflower mosaic virus (CaMV) in a 35S-C1301 vector (Zhu et al., 2006). T0 plants were isolated and levels of RLS1 expression were determined by RT–PCR for independent T0 plants (Figure 7A). None of the T0 plants, including those with highly increased levels of RLS1 transcripts, developed obvious morphological phenotype (data not shown). This was confirmed further by analyzing 30–40 T1 progeny plants of independent RLS1 overexpression lines H426 and H427.

Intriguingly, these transgenic T2 plants showed a weak accelerated leaf senescence phenotype (Figure 7B). To next compared the physiological change in the transgenic plants and wild-type during natural senescence. The flag leaf and the second leaf were examined 21 d after flowering. Chlorophyll content was lower in the leaves of RLS1-overexpressing plants than that of wild-type plants (t-test, P < 0.05) (Figure 7C). The Fv/Fm values and the membrane ion leakage values were similar between transgenic plants and wild-type plants (Figure 7D and 7E).
When senescence was induced by dark treatment of detached leaves, leaf senescence occurred more quickly (Weaver and Amasino, 2001). Under this condition, leaves of RLS1-overexpressing plants senesced more rapidly than did wild-type leaves. Individual leaves from wild-type plants became pale green after 4 d of dark treatment, whereas leaves from the RLS1-overexpressing plants showed severe yellowing. This was consistent with chlorophyll content of detached leaves during dark treatment (Supplemental Figure 7A and 7B). These results indicated that the RLS1 protein might be involved in chloroplast degradation during rice leaf senescence.

DISCUSSION

To elucidate the components involved in PCD signaling in the model cereal crop, we have been screening for lesion mimic mutations in rice. One recessive mutant, rls1, was identified based on its spontaneous cell death lesions and accelerated senescence. Map-based cloning of the RLS1 gene revealed that it encodes a novel protein with an N-terminal NB domain and a C-terminal ARM domain. RLS1 expression was induced during dark-induced leaf senescence and suppressed by cytokinin treatment. Intriguingly, overexpression of RLS1 in wild-type
plants showed no obvious lesions, but resulted in a weak accelerated leaf senescence phenotype, suggesting that RLS1 is positively involved in PCD during senescence.

NB domains critical for ATP or GTP binding have been found in many prokaryotic and eukaryotic proteins; the nucleotide-binding ability of the domain is essential for biological activity of proteins (Tameling et al., 2002). NB domains also share sequence similarities with the central regions of pro-apoptotic effector proteins such as Apaf-1 from human and CED4 from C. elegans. In plants, NB domains are conserved among R proteins. These proteins also share a similar structure with an N-terminal effector domain (CARD in Apaf-1 and TIR/CC in R proteins) to a C-terminal domain often involved in protein–protein interactions (WD domain in Apaf-1 and LRR domain in R proteins) (van der Biezen and Jones, 1998; Glowacki et al., 2010). Structural characteristics of the ARM domain also suggest its implication in protein–protein interaction (Huber et al., 1997). In rice, SPL11 represents a case of ARM-containing protein that is involved in PCD regulation (Zeng et al., 2004). So far, RLS1 represents the first case of a unique NB–ARM structure protein related to PCD. The Ser-to-Pro substitution caused by the rls1 mutation occurred in a non-conserved region just downstream of the ARM domain (Supplemental Figure 5D). However, it is unclear whether this mutation affects the ARM domain of RLS1.

It is generally recognized that NB-containing proteins in plants encoded by dominant or semi-dominant R genes initiate signal transduction cascades that lead to rapid localized host cell death at the site of pathogen attack (Shirano et al., 2002; Tameling et al., 2006). However, the mechanism(s) of NB-containing proteins inherited recessively is poorly understood. For example, the Arabidopsis RRS1-R gene encoding an NB-containing R protein with a C-terminal WRKY domain found in some plant transcription factors shows recessive inheritance. However, RRS1-R behaves in a dominant fashion when integrated as a transgene into RRS1-S (the susceptible allele of RRS1) genotypes (Deslandes et al., 2003). It was supposed that the RRS1-R and RRS1-S proteins compete for the components involved in defense signal transduction pathways, which lead to the HR (Lahaye, 2002; Deslandes et al., 2003). Arabidopsis CHS3 is another unconventional NB-containing protein with the NB domain at the N terminus and the LIM domain, a modular protein-binding interface to mediate protein–protein interaction, at the C-terminus (Yang et al., 2010). The chs3-1 mutant plants transformed with the wild-type copy CHS3 under its own promoter exhibited a wild-type phenotype. However, three T-DNA insertion lines of CHS3 in which CHS3 expression was abolished did not display any chs3-1 mutant phenotype. The F1 progeny of a cross between chs3-1 and any one of the T-DNA insertion lines showed a weaker vision of the chs3-1 phenotype. Thus, the chs3-1, shown to be recessive in the Col background, is a gain-of-function mutant. It was proposed that the wild-type and mutated CHS3 proteins formed a heterodimer and thus suppressed the activity of mutated CHS3 in the heterozygous plants (Yang et al., 2010). These two examples illustrate mutated NB-containing proteins encoded by recessive alleles that control PCD in plants. Here, rls1 was defined as a recessive NB-containing gene involved in cell death. The rls1 plants transformed with a wild-type copy under its own promoter exhibited a wild-type phenotype (Figure 4). Suppressing expression of the wild-type RLS1 using an RNA interference (RNAi) strategy did not display any obvious defects such as lesion mimic (data not shown). Thus, the rls1 mutant is the first case of a recessive NB-containing mutation in rice. The function of a mutant NB-containing RLS1 protein inherited recessively, probably like the way of the mutated CHS3 protein, should be studied further.

The rls1 mutant plants showed accelerated leaf senescence under both natural and dark-induced conditions (Figures 1C, 1D, and 2A). Therefore, the accelerated senescence of the rls1 mutant appears not to be directly induced by the formation of spontaneous lesions. Chlorophyll content began to decrease earlier in rls1 leaves than in wild-type leaves (Figure 2B). However, there was no significant difference in the efficiency of

Figure 6. Expression Analysis of *RLS1*. (A) Expression patterns of *RLS1* in the leaf of seedling (LS), root of seedling (RS), flag leaf during heading stage (LH), leaf sheath (LS), young panicle (PH) and spikelet (SH) of the wild-type. Values are means ± SD; n = 3. (B) *RLS1* expression in leaves of 3-week-old wild-type plants after transferred to darkness. RNA was isolated at 0 (control), 4, 8, 12, 24, 48, and 72 h after being transferred to darkness. Values are means ± SD; n = 3. (C) *RLS1* expression was suppressed by tZ (20 μM) in 3-week-old wild-type plants. RNA was isolated at 0, 12, 24, 48, and 72 h after tZ treatment. Values are means ± SD; n = 3.
photosystem II and ion leakage between rls1 and wild-type leaves (Figure 2C and 2D). All these features suggested that the rapid loss of chlorophyll was the main cause of accelerated leaf senescence in rls1.

We observed that PCD was misregulated in the leaves of rls1 plants (Figure 3), providing evidence for the involvement of RLS1 in the regulation of PCD for partial degradation of the chloroplast, which probably involves autophagy. Autophagy is a survival cellular mechanism that protects cells from damages under the unfavorable environmental conditions such as senescence, microbe infection, oxidative stress, and the aggregation of damaged proteins (Mizushima et al., 1998; Maiuri et al., 2007; Kwon and Park, 2008). Although appropriate autophagy suppresses cell death and thus promotes cell survival and stress adaptation, excessive autophagy causes cell death (Kwon and Park, 2008). That may contribute to the accelerated leaf senescence and cell death in rls1 plants.

Expression of RLS1 was found to be enhanced during dark-induced leaf senescence and suppressed by cytokinin treatment (Figure 6C and 6D). Interestingly, overexpression of RLS1 in wild-type plants showed no obvious lesions on the leaves, but resulted in a weak accelerated leaf senescence phenotype (Figure 7). Genetic studies have provided evidence that NB-containing R proteins in plant, such as RPS4, RPM1, can activate cell death during HR execution (Hofius et al., 2009). All these features indicated that RLS1 is involved in PCD for partial degradation of chloroplast during senescence. With this scenario, we also observed that rls1 seeds display accelerated germination (data not shown). Considering that seed germination induces PCD in aleurone layers, the mutation in the
RLS1 protein could also accelerate PCD in aleurone layers, resulting in earlier dormancy release. We then propose that the RLS1-mediated cellular process plays an important role in cell death and senescence in rice. The precise mechanism of the unique NB–ARM protein in PCD regulation should be further investigated by screening suppressor(s) or identifying interaction partner(s). Nevertheless, the characterization of the RLS1 gene will contribute to the elucidation of mechanisms involved in the PCD during leaf senescence and to strengthen our understanding of functions of NB-containing proteins in stress response.

METHODS

Plant Materials

The rls1 (previously named lrd43) mutant was isolated from our collection of γ-ray mutagenized population of japonica variety Zhonghua 11 (Wang et al., 2004). Plants were grown in the paddy field for observation of lesion mimic development and agronomic traits. Seedlings were incubated in Yoshida’s culture solution with a photoperiod of 13/11 h light (30°C)/dark (28°C). Three-week-old seedlings were transferred to complete darkness to induce senescence. For leaf dark treatment, detached leaves were incubated in distilled water at 28°C. For cytokinin treatment, 3-week-old seedlings were transferred to Yoshida’s culture solution containing 20 μM trans-zeatin (tZ) and grown for an additional 3 d. Leaves were excised and stored at –80°C for RNA preparation.

Map-Based Cloning and Complementation

About 1 300 F2 mutant plants derived from the cross of rls1 and Zhenshan 97 were selected for PCR-based mapping (Supplemental Table 1). The RLS1 was mapped to a 60-kb region on chromosome 2. The predicted rls1 locus was PCR-amplified and sequenced for mutation detection. The Nipponbare BAC OSJNBb12P08 containing the entire 7.9-kb genomic DNA of the candidate RLS1 gene, including the coding and the promoter regions. The 1300–RLS1 was then introduced into the rls1 mutant plants by Agrobacterium tumefaciens-mediated transformation for genetic complementation. More than 10 independent lines were generated that restored to the wild-type phenotypes.

Phylogenetic Analysis

The BLAST search program (www.ncbi.nlm.nih.gov/BLAST/) was used to look for protein sequences homologous to RLS1. The sequences of NB domains were aligned using ClustalX software and the neighbor-joining tree was generated with the Poisson correction method using MEGA version 4.0 software (Tamura et al., 2007). Bootstrap replication (1000 replications) was used for a statistical support for the nodes in the phylogenetic tree.

Overexpression of RLS1

A 4-kb fragment from the BAC OSJNBb12P08 digested with EcoRI was collected and sequenced, which contains the entire coding region of RLS1 and 78 bp upstream of the ATG start codon. The fragment was inserted into the overexpression vector 35S-C1301 (Zhu et al., 2006) and the resulting plasmid (35S-RLS1) was transformed into japonica cultivar Nipponbare to generate more than 20 T0-independent RLS1 overexpression plants. The T1 and T2 progenies of the transformants were analyzed.

RNA Preparation and Transcript Analysis

Total RNA was prepared from leaves of rls1 and wild-type plants and different tissues of wild-type plants using Trizol reagent according to the manufacturer’s instructions (Invitrogen). The RLS1 transcripts were detected by RT-PCR using the primers RLS1_F and RLS1_R (Supplemental Table 2) that amplify a 1055-bp fragment of the RLS1 cDNA, using the following PCR conditions: 94°C for 3 min, followed by 35 cycles at 94°C for 20 s, 60°C for 30 s, and 72°C for 40 s and an extension step at 72°C for 10 min. The rice ubiquitin (Ubi-1) cDNA served as an internal control (Zhu et al., 2006). The quantitative real-time PCR experiments were performed for RLS1 using a Roche LightCycler with LightCycler FastStart DNA Master SYBR Green I kit (Roche, www.roche.com/). Primer sequences can be found in Supplemental Table 2.

Chlorophyll Content, Photochemical Efficiency, and Membrane Ion Leakage

Leaf tissues were ground with liquid nitrogen and chlorophyll was extracted with 80% acetone. Chlorophyll content was measured according to the method described previously (Lichtenthaler, 1987). PSII maximum photochemical efficiency (Fv/Fm; Fv, variable fluorescence; Fm, maximum fluorescence) was measured using an Imaging-PAM fluorometer according to the manufacturer’s instructions (Heinz Walz GmbH, Germany). For measurement of membrane ion leakage, leaves were cut to 5 mm in length and placed into de-ionized water in a tube, followed by incubation in the dark at 28°C for 6 h. The membrane ion leakage (value IL1) was measured using an electrolyte leakage apparatus. Then, the samples were incubated at 121°C for 20 min to completely kill the tissues and release electrolytes. The final ion leakage (value IL2) was measured. The relative membrane ion leakage (IL) was obtained following the formula IL = IL1/IL2 × 100.

Transmission Electron Microscopy

For TEM assay, leaves of plants grown under the field conditions were fixed in a solution of 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 4 h at 4°C. The tissue was rinsed and post-fixed overnight at 4°C in 1% OsO4, then processed as previously described (Li et al., 2011), embedded and viewed via an electron microscopy (Hitachi, Tokyo, Japan).
Accession Numbers
Sequence data from this article can be found in GenBank databases under the following accession numbers: rice RLS1 (EAZ22148); Rx (CAB50786); I2 (ABB00532); R3a (AAW48299); RRS1-R (NP_199339); CHS3 (NP_197291); N (BAD12594); APAF1 (CAB55585); CED4 (AAAX2294).

SUPPLEMENTARY DATA
Supplementary Data are available at Molecular Plant Online.

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