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Spotted leaf11, a Negative Regulator of Plant Cell Death and Defense, Encodes a U-Box/Armadillo Repeat Protein Endowed with E3 Ubiquitin Ligase Activity [W]

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The rice (*Oryza sativa*) *spotted leaf11* (*spl11*) mutant was identified from an ethyl methanesulfonate–mutagenized *indica* cultivar IR68 population and was previously shown to display a spontaneous cell death phenotype and enhanced resistance to rice fungal and bacterial pathogens. Here, we have isolated *Spl11* via a map-based cloning strategy. The isolation of the *Spl11* gene was facilitated by the identification of three additional *spl11* alleles from an IR64 mutant collection. The predicted SPL11 protein contains both a U-box domain and an armadillo (ARM) repeat domain, which were demonstrated in yeast and mammalian systems to be involved in ubiquitination and protein–protein interactions, respectively. Amino acid sequence comparison indicated that the similarity between SPL11 and other plant U-box-ARM proteins is mostly restricted to the U-box and ARM repeat regions. A single base substitution was detected in *spl11*, which results in a premature stop codon in the SPL11 protein. Expression analysis indicated that *Spl11* is induced in both incompatible and compatible rice–blast interactions. In vitro ubiquitination assay indicated that the SPL11 protein possesses E3 ubiquitin ligase activity that is dependent on an intact U-box domain, suggesting a role of the ubiquitination system in the control of plant cell death and defense.

INTRODUCTION

In multicellular organisms, cell death can occur as either a physiological cell death or a nonphysiological cell death (Vaux and Korsmeyer, 1999). Physiological cell death, also coined as programmed cell death (PCD), refers to a process programmed by the organism to kill its own cells in purpose. The most predominant form of PCD in animals is apoptosis, which is morphologically characterized by membrane blebbing, cell volume loss, nuclear condensation, and DNA fragmentation (Kerr et al., 1972). In plants, PCD occurs during both normal development and in response to pathogen infection. Prominent examples of developmentally PCD include the degeneration of cereal aleurone cells, the development of treacheary elements in xylogenesis, leaf senescence, and cell death in plant reproduction (Kuriyama and Fukuda, 2002). In plant–microbe interactions, PCD occurs during both plant hypersensitive response (HR) to avirulent pathogen infection and plant disease susceptibility under virulent pathogen attack (Greenberg, 1997).

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HR cell death is characterized by the rapid localized cell death that occurs at the site of infection caused by avirulent pathogens. This response appears to be triggered through the recognition of an avirulent factor by a corresponding resistance (R) protein in the plant. A large number of mutants characterized by misregulated cell death phenotypes mimicking the HR have been identified in maize (*Zea mays*) (Walbot et al., 1983), *Arabidopsis thaliana* (Lorrain et al., 2003), barley (*Hordeum vulgare*) (Wolter et al., 1993), and rice (*Oryza sativa*) (Yin et al., 2000). The constitutive activation of cell death and defense pathways in some of the mutants suggests that these mutations might define genes involved in the regulation of HR in wild-type plants. These mutants are collectively called lesion mimics based on their spontaneous lesion formation in the absence of pathogen infection. More than a dozen genes controlling lesion mimics have been isolated to date. The proteins encoded by these genes fall into various functional groups, including membrane associated protein (Büschges et al., 1997), ion channel (Balagué et al., 2003), zinc-finger protein (Dietrich et al., 1997), heat stress transcription factor (Yamanouchi et al., 2002), and components involved in the biosynthesis/metabolic pathways of fatty acid/lipids (Kachroo et al., 2001), porphyrin (Hu et al., 1998), and phenolics (Gray et al., 1997). Studies of these lesion mimic mutants have begun to shed light on the control of PCD and its connections to disease resistance in plants. For example, analyses of Arabidopsis double mutants between the lesion stimulating disease mutant *lsd1* (Dietrich et al., 1997) and mutants for two positive regulators

for *R* gene function, enhanced disease susceptibility1 (EDS1) and phytoalexin deficient4 (PAD4), have indicated that both EDS1 and PAD4 are required for runaway cell death in the *lsd1* mutant (Rusterucci et al., 2001). It was suggested that *EDS1* and *PAD4*, two signaling genes that mediate some but not all R responses in Arabidopsis, regulate a reactive oxygen intermediates/salicylic acid–dependent defense signal amplification loop that is modulated by LSD1 (Rusterucci et al., 2001).

The ubiquitin/proteasome pathway is the major selective protein degradation system in eukaryotes. It is initiated by the formation of a thiol-ester linkage between the ubiquitin molecule and the Cys residue at the active site of the ubiquitin-activating enzyme (E1) in an ATP-dependent manner. The activated ubiquitin is then transferred to the active site of the ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin-ligase (E3) binds E2 and catalyzes the formation of an isopeptide linkage between the activated ubiquitin and the substrate protein. In the last decade, ubiquitination has emerged as one of the key regulatory mechanisms of apoptosis in mammalian systems (Lee and Peter, 2003). In plants, ubiquitination-mediated protein degradation has been shown to play a significant role in multiple cellular processes, such as photomorphogenesis and regulation of hormone signaling (Sullivan et al., 2003). Recent data suggest that ubiquitination may also play an important role in plant defense against pathogens. The identification of two F-box proteins and several RING-type E3 ubiquitin ligases in the regulation of plant defense as well as the finding of a possible SGT1-mediated link between ubiquitination and R gene–mediated resistance have suggested a possible role for ubiquitination in plant disease resistance signaling (Devoto et al., 2003). Nevertheless, direct evidence for the involvement of the ubiquitination/proteolysis pathway in signaling and regulating plant PCD and disease resistance has not been established.

Many lesion mimic mutants have been identified in rice, and some of these mutants display altered early defense signaling or disease resistance (Takahashi et al., 1999; Yin et al., 2000). Disruption of a heat stress transcription factor was found recently to be responsible for the phenotype of the stress-inducible rice lesion mimic mutant *spl7* (Yamanouchi et al., 2002). The rice lesion mimic mutation *spotted leaf11* (*spl11*) was identified from an ethyl methanesulfonate–mutagenized *indica* cultivar IR68 population and was shown to be inherited in a recessive monogenic fashion (Singh et al., 1995). Phenotypic characterization showed that *spl11* confers enhanced, nonrace-specific resistance to both *Magnaporthe grisea* and *Xanthomonas oryzae* pv *oryzae*, the pathogens that cause rice blast and bacterial blight diseases, respectively (Yin et al., 2000). In addition, correlation between the lesion development on leaves and the activation of several defense-related genes and enhanced resistance of the *spl11* mutant to pathogens was also observed. To understand the molecular basis by which *Spl11* suppresses cell death and the relationship between the spontaneous cell death and the activation of defenses in *spl11*, we have isolated the *Spl11* gene by a map-based cloning strategy. The isolation of the *Spl11* gene was facilitated by the identification of three additional *spl11* alleles from an IR64 mutant collection (Leung et al., 2001). The *Spl11* gene encodes a novel protein with both a U-box domain and six armadillo (ARM) repeats. A point mutation was identified in *spl11* that resulted in a premature stop codon in the SPL11 protein. We also showed that SPL11 possesses an E3 ubiquitin ligase activity in vitro, and the intact SPL11 U-box domain is essential for this activity, suggesting an involvement of ubiquitination in the control of plant PCD and pathogen defense.

RESULTS

Genetic and Physical Mapping of the *Spl11* Locus

Spl11 was previously physically delimited to a 160-kb DNA segment by markers R1709 and CG16C-7 in the BAC clone BAC78 (Figure 1A) (Zeng et al., 2002). BAC78 was partially sequenced using a shotgun sequencing approach. Assembly of the sequences produced two continuous sequence contigs that cover 95.7% of BAC78 (Figure 1B). To narrow down the *Spl11* gene to a smaller region, two new mapping populations were generated by crossing the *spl11* mutant with two *japonica* cultivars, TP309 and Nipponbare. The *spl11* mutation was first backcrossed into TP309. Homozygous progenies derived from the cross showing the same lesion mimic phenotype as *spl11* (designated as TP309$^{spl11/spl11}$) were then used as the pollen donor to cross with Nipponbare. Plants from the F2 and F3 generation of the cross were used for subsequent mapping analysis. Seven cleaved amplified polymorphism sequence markers (Weining and Langridge, 1991) were developed from the sequences available in the 160-kb DNA region. Recombination analysis of 297 F2 lesion mimic plants and 1846 F3 individuals indicated that the *Spl11* locus was localized within a 27-kb DNA region bracketed by CG47 and CG111 (Figure 1B).

The gene prediction programs GENSCAN (Burge and Karlin, 1997) and Fgenesh (Solovyev et al., 1995) were then used to identify possible genes in the 27-kb DNA region. Three DNA intervals with high probability of containing a coding region were detected. We designated the putative genes encoded by these DNA intervals G1, G2, and G3 (Figure 1C). BAC78 was subcloned into a modified transformation-competent bacterial artificial chromosome (TAC) vector using *Not*I as the cloning restriction enzyme (Qu et al., 2003). The subclone that covers the 27-kb region, named TAC20, was then used as a probe to fingerprint *spl11* and IR68 genomic DNA by restiction fragment length polymorphism (RFLP) analysis (Figure 1C). Among the 19 restriction enzymes analyzed, TAC20 showed polymorphism between *spl11* and IR68 only with restriction enzyme *Bsl*I (Figure 1D, 1). Based on these results, we postulated that a mutation is most likely located within the *Spl11* gene, which resulted in the absence of a *Bsl*I restriction site normally present in wild-type IR68 genomic DNA (Figure 1C). To test this hypothesis, DNA spanning the putative mutated *Bsl*I cutting site was then amplified from the TAC20 plasmid DNA and used as a probe to hybridize with the blot previously used in the fingerprinting experiment. The second hybridization gave exactly the same polymorphism pattern between *spl11* and IR68 (Figure 1D, 2), indicating that a mutation did occur in the vicinity of the *Bsl*I restriction site.

Sequence alignment between the mutated *Bsl*I region and the 27-kb DNA stretch indicated that the putative deleted *Bsl*I restriction site (designated as Δ*Bsl*I) in *spl11* is located in the

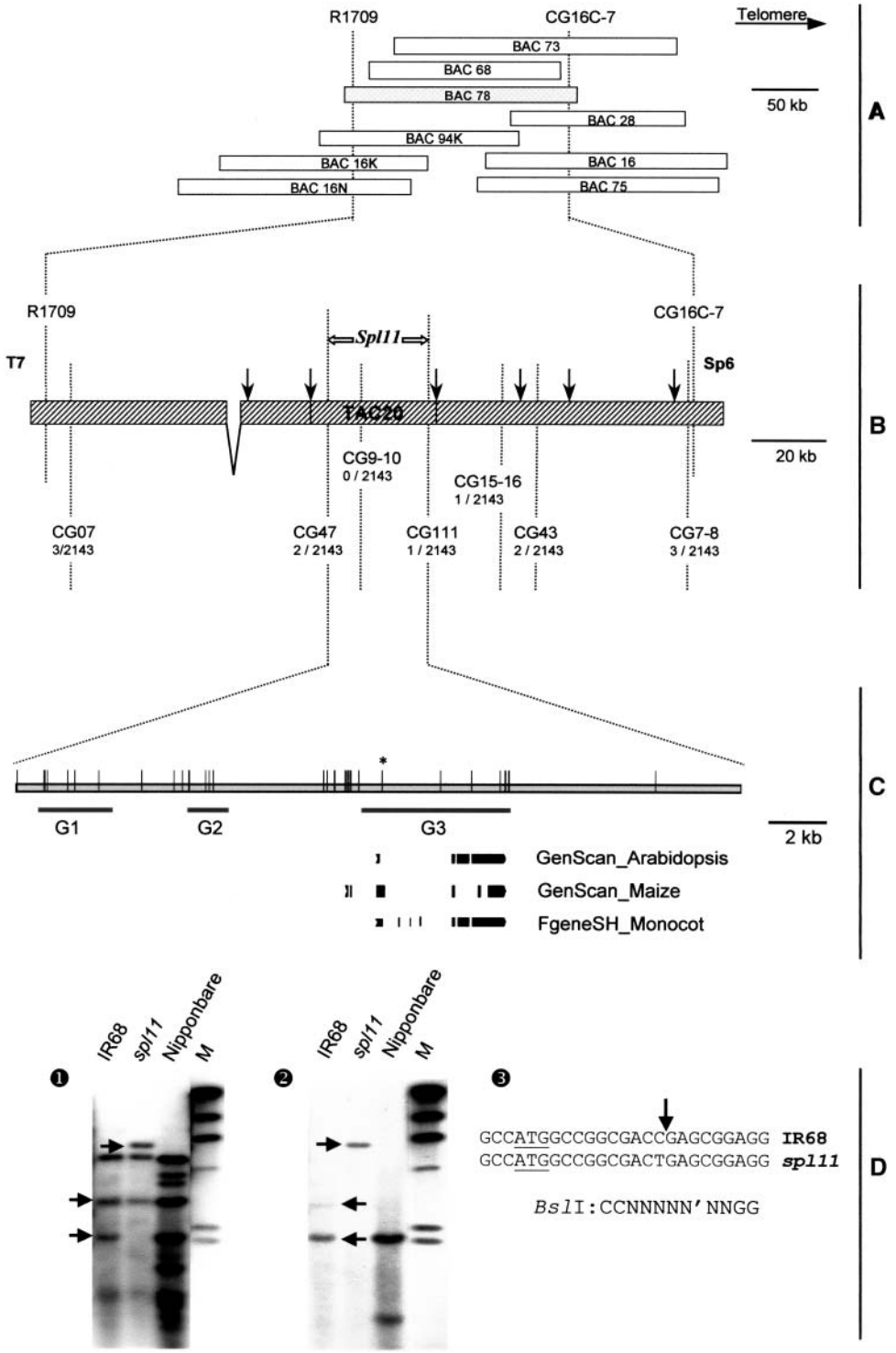

Figure 1. Physical Delineation of the *spl11* Mutation.

(A) Schematic representation of the BAC contig spanning the *Spl11* locus. The overlaps between BAC inserts are displayed to scale as open bars. The dotted vertical lines mark the positions of DNA markers. The BAC insert containing the *Spl11* locus is highlighted in light gray. Orientation of rice chromosome 12 is indicated in the top right corner.

first predicted exon of G3 shortly downstream of the start codon (Figure 1C). A search for ESTs in the 27-kb DNA sequence using the BLAST2 algorithm and available databases identified rice ESTs matching the 3′ end of G3 only, which suggests that G3 was most probably the candidate *Spl11* gene.

Identification of *spl11* Alleles in an IR64 Mutant Collection

To facilitate the cloning of the *Spl11* gene, we searched for lesion mimic mutants with a similar phenotype to that of the *spl11* mutant from an IR64 mutant collection (Figure 2A). Three mutants were identified from either diepoxybutane-treated (DB2487) or radiation (γ-ray)-treated (GR5612 and GR5717) IR64 populations (Leung et al., 2001). Allelism tests indicated that the mutations that occurred in all three IR64 lesion mimic mutants are recessive and allelic to *spl11* (Table 1). Moreover, alterations at the *Spl11* locus were detected in two of the three mutants by RFLP analysis (Figure 2B). PCR analysis was also performed on these mutants using several *Spl11*-specific primer pairs. A combination of the PCR and DNA gel blot analysis data suggested a 2.5-kb genomic DNA deletion at the 5′ end of the *Spl11* locus in mutant GR5612 and a 1.4-kb deletion near the *Spl11* start codon in mutant GR5717 (data not shown). However, no visible change at the *Spl11* locus was detected in mutant DB2487 when 20 enzymes were used in the RFLP analysis. This could reflect the fact that these enzymes could not detect the small deletion or point mutation in DB2487. Therefore, we evaluated ~2000 F2 plants from the two reciprocal crosses between *spl11* and DB2487. All the F2 plants were lesion mimics, suggesting that the mutation in DB2487 was allelic to the *spl11* mutation. The segregation ratio in the F2 generation of the cross between IR64 and DB2487 fitted 3 to 1 (85 wild type to 23 lesion mimic, $\chi^2 = 0.79$, $P = 0.37$), further indicating that the mutation in mutant DB2487 was controlled by a single recessive locus.

To test whether the mutation at the *Spl11* locus affected the transcript accumulation of the gene in these mutants, RT-PCR analysis using *Spl11*-specific primers was performed. As shown in Figure 2C, the expression of the candidate *Spl11* gene in mutant GR5612 was completely disrupted, corroborated by DNA gel blot analysis data showing deletion of a 2.5-kb fragment (Figure 2B). There was only a trace expression of *Spl11* in mutant GR5717. The expression of *Spl11* was reduced in mutant DB2487 compared with that of wild-type IR64. The perfect association between genomic changes at the *Spl11* locus and the lesion mimic phenotype of the three IR64 *spl11* alleles, along with changes observed in candidate gene expression of the mutants, provided strong evidence that the candidate gene within the G3 fragment encodes *Spl11*.

Functional Complementation of *spl11*

To get final confirmation that the candidate gene encoded within the G3 DNA fragment was *Spl11*, we made a pCAMBIA1301-derived binary plasmid, pGW78, that contains the full-length *Spl11* genomic DNA and a 2.55-kb fragment of the upstream sequence to complement the *spl11* mutation. To improve the efficiency of transformation, the *spl11* mutation was first introgressed into *japonica* cultivar TP309, the most commonly used cultivar in rice transformation. The seeds produced by the lesion mimic plant TP309$^{spl11/spl11}$ were then used for the complementation test. Plasmid pGW78 was transferred into the *spl11* mutant via the *Agrobacterium tumefaciens*–mediated transformation system (Qu et al., 2003). In total, 44 independently transformed transgenic lines were generated, among which 40 lines were successfully complemented. DNA gel blot analysis of the transgenic plants revealed that all the plants carry the mutation originally introgressed from the *spl11* plant and zero to two copies of the transgene (data not shown). None of the transgenic plants containing the completely integrated transgene showed any development of lesions within 2 months after the regeneration (Figure 3). These results confirmed that the gene encoded within the G3 DNA interval was responsible for the phenotype of the *spl11* mutant.

The *Spl11* Gene Encodes a U-Box/ARM Protein with Homology to *Drosophila melanogaster* ARM

The successful complementation of the *spl11* mutation prompted us to obtain the cDNA in the G3 DNA interval. RNA gel blot analysis using G3 genomic DNA as a probe revealed an

Figure 1. (continued).

(B) Fine physical mapping of *Spl11* in BAC78. The two cross-hatched gray bars denote the sequenced regions in BAC78. The vertical dotted lines denote the positions of the respective cleaved amplified polymorphism sequence markers. The number of recombinants/number of segregants tested is indicated for each marker. Arrows above the bars mark the *Not*I cutting sites of the BAC78 insert. T7 and Sp6 indicate the orientation of the insert cloned into the BAC vector pBeloBAC11. The position of the subclone TAC20 insert that contains the *Spl11* gene is displayed.

(C) Prediction of potential coding sequences in the 27-kb region of TAC20 where the *Spl11* gene was physically delimited. The gray bar depicts the sequenced area. The three solid gray lines designated as G1, G2, and G3 indicate the regions with high coding probability. The vertical lines mark the *Bsl*I cutting sites. The asterisk denotes the putative mutation site in *spl11*. Exons predicted in G3 by the programs GENSCAN and Fgenesh using different matrixes are displayed in dark gray.

(D) RFLP fingerprinting of IR68, *spl11*, and Nipponbare genomic DNA at the *Spl11* locus and detection of a point mutation detected in the *spl11* gene. Nineteen restriction enzymes were analyzed but only the results of *Bsl*I are shown. (1) Genomic DNA was digested with *Bsl*I and then separated on a 1.0% agarose gel. A TAC20 insert digested with *Hin*dIII was used as the probe for hybridization. (2) The same blot hybridized with a TAC20 insert in (1) was used. DNA spanning the putative mutated *Bsl*I site was amplified from TAC20 and used as the probe. Gray arrows denote the polymorphic bands. M, λ/*Hin*dIII DNA marker (New England Biolabs, Beverly, MA). (3) DNA sequence in the vicinity of the *spl11* mutation. The C-to-T point mutation in *spl11* is denoted by an arrow. This point mutation causes a premature stop codon as marked by the underline. The asterisk marks the start codon for the SPL11 protein.

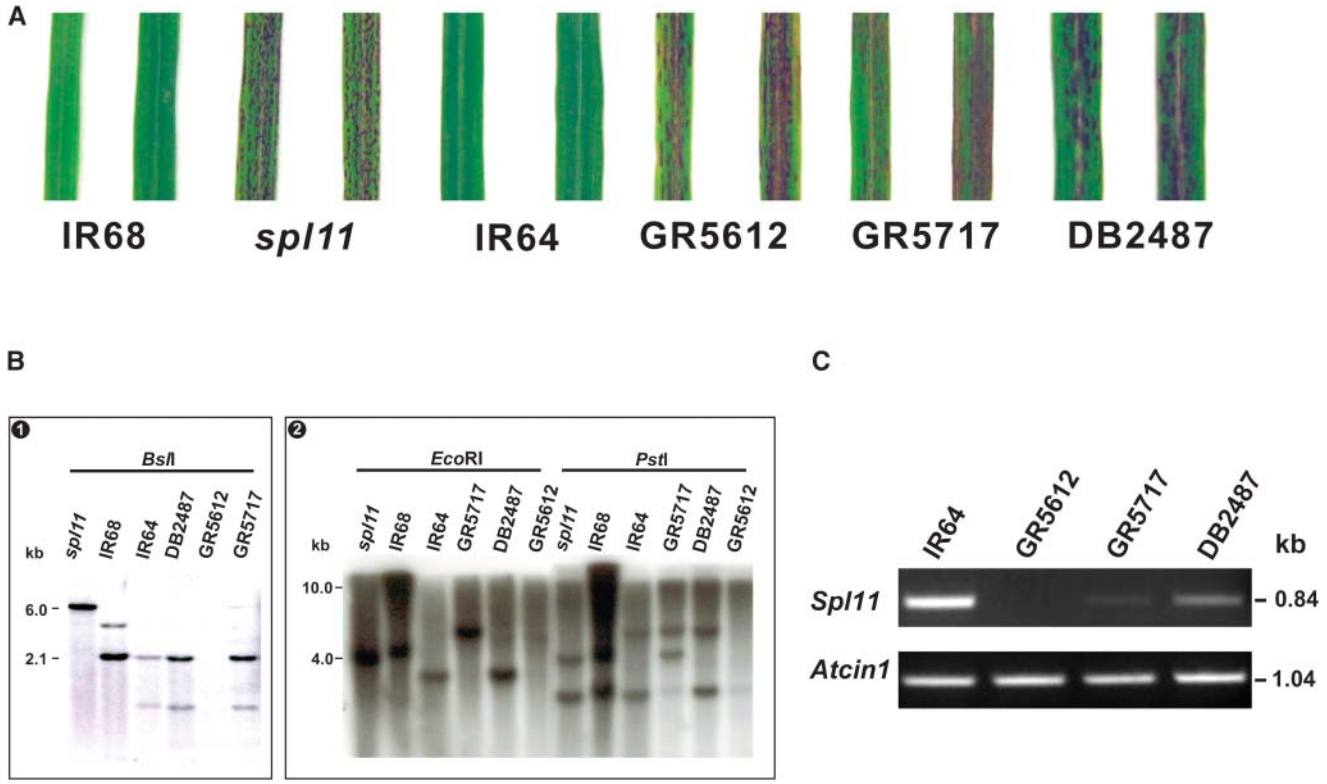

Figure 2. Analysis of IR64 Lesion Mimic Mutants Allelic to *spl11*.

(A) Lesion phenotype of *spl11* and IR64 background lesion mimic mutants. Picture was taken of leaves from 2-month-old plants.

(B) DNA gel blot analysis of the *Spl11* locus in wild-type plants and different mutant lines. (1) Genomic DNA was restricted by *Bsl*I and then separated on a 1.0% agarose gel. A 2.5-kb genomic DNA fragment at the 5′ end of the *Spl11* gene was used as the probe. (2) Genomic DNA was digested by *Eco*RI and *Pst*I, respectively. The same probe in (1) was used for the hybridization. Changes detected at the *Spl11* locus in the IR64 mutants are described in the text.

(C) Transcript analysis of *Spl11* in IR64 lesion mimic mutants by RT-PCR. *Spl11*-specific primers were used to amplify a 0.84-kb *Spl11* cDNA fragment from total RNA. The rice *Actin1*-specific primers were used in the RT-PCR to quantify the cDNA template. The experiment was repeated three times.

~2.6-kb mRNA in the leaf, stem, and root of IR68 (Figure 4A). *Spl11* shows highest expression in the leaf, and lowest in the root. A 2106-bp cDNA fragment that covers the central region and 3′ end of the G3 gene was identified from a Nipponbare leaf cDNA library. The 2106-bp cDNA sequence completely matches the corresponding predicted exons in G3. A modified rapid amplification of cDNA ends (RACE) amplification method using primers derived from the predicted *Spl11* gene sequence was then used to obtain the 5′ *Spl11* cDNA. A full-length cDNA sequence of 2518 bp for the G3 DNA interval was generated when the RACE result and the cDNA clone sequence were combined (Figure 4B). An open reading frame of 2085 bp starting at position 81 was detected in this full-length cDNA. The deduced protein of the complete open reading frame had 694

Table 1. Allelism Tests between *spl11* and Three IR64 Lesion Mimic Mutants[a]

Cultivar/Mutant Line	IR64	GR5717	GR5612	DB2487	*spl11*
IR64	–[b]	11[c] WT/12[d]	13 WT/14	12 WT/13	–
GR5717		–	71 LM/71	65 LM/65	33 LM/33
GR5612			–	35 LM/35	56 LM/56
DB2487				–	58 LM/58

[a] When crossed to IR64, the lesion mimic mutants were used as the female parent. WT, wild type; LM, lesion mimic.

[b] Corresponding cross was not made.

[c] The number of F1 plants with corresponding phenotype.

[d] Total number of F1 plants of the corresponding cross.

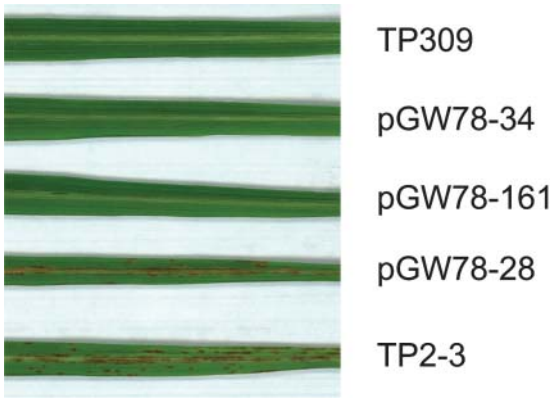

Figure 3. Functional Complementation Test of the *Spl11* Candidate Gene.

The leaves of 2-month-old plants are shown. Wild-type TP309 and mutant TP2-3 (TP309$^{spl11/spl11}$) are used as nontransgenic controls. Lines pGW78-34 and pGW78-161 are *Spl11* transgenic plants: *spl11* mutant TP2-3 (TP309$^{spl11/spl11}$) with the 8.06-kb *Xba*I-*Pac*I fragment of the wild-type gene. Line pGW78-28 indicated as an example of failure in transformation.

amino acids and a molecular mass of 75.3 kD, with a predicted isoelectric point of pH 5.2 (http://us.expasy.org/tools/#primary).

A database search with the deduced SPL11 amino acid sequence showed that the 75 amino acids at positions 272 to 346 of SPL11 share high similarity to the consensus U-box domain sequence that was first identified in the yeast protein UFD2 (Koegl et al., 1999) (Figure 4C). The U-box domain contains ~70 amino acids and is conserved among fungi, plants, and animals (Aravind and Koonin, 2000). It has been shown that the U-box domain is indispensable for E3 ubiquitin ligase activity of several U-box proteins (Jiang et al., 2001). The presence of the U-box domain in the SPL11 protein therefore suggests a probable E3 ubiquitin ligase activity for SPL11.

The database search also showed that the central and C-terminal regions of the SPL11 protein share similarity with the ARM repeats of β-catenin, the vertebrate homolog of Drosophila segment polarity protein ARM (Riggleman et al., 1989). The ARM repeats are tandemly repeated copies of the ARM motif, each containing 38 to 45 amino acid residues (Peifer et al., 1994). Structural characteristics of the ARM motif suggest its involvement in protein–protein interactions, which has been demonstrated in several cases (Huber et al., 1997). In total, six ARM repeat motifs were detected in SPL11 (Figure 4C). Alignment of the ARM repeats in SPL11 with β-catenin repeats 1 to 6 is shown in Figure 4D. Despite the significant variability in sequence among individual motifs, the chemical nature of the residues within each motif is generally conserved. Homologous modeling between the corresponding SPL11 and β-catenin ARM repeat region indicated that their structure matches well (data not shown) (Guex and Peitsch, 1997). This suggests that the ARM repeats of SPL11, like that of β-catenin, might be in physical contact with its interactor(s).

Only two plant proteins bearing both U-box and ARM repeat domains similar to those of SPL11 have been functionally characterized so far. One of them, ARC1, was isolated in a yeast two-hybrid screen for *S* receptor kinase–interacting proteins and was shown to possess an E3 ubiquitin ligase activity that positively regulates self-incompatibility of Brassica (Stone et al., 2003). The other one is PHOR1, which is a photoperiod-responsive protein involved in gibberellin signaling (Amador et al., 2001). In the Arabidopsis genome, more than 40 U-box-ARM proteins were identified using sophisticated data-mining approaches (Mudgil et al., 2004). In addition to Arabidopsis and rice U-box/ARM repeat proteins homologous to SPL11, BLAST2 algorithm search of the National Center for Biotechnology Information database identified several expressed U-box-ARM proteins from other plant species as well. Two of them, ACRE276 and NtPUB4, were isolated from tobacco (*Nicotiana tabacum*) and were speculated to be involved in Cf9/*Avr9* elicited defense and tobacco development signaling, respectively (Durrant et al., 2000; Kim et al., 2003). The parsley (*Petroselinum crispum*) CMPG1 responded immediately after pathogen infection and the mangrove (*Bruguiera gymnorrhiza*) bg55 was induced in high salinity stress (Kirsch et al., 2001; Banzai et al., 2002). SPL11 is related to these proteins in amino acid sequence and overall structure. The sequence similarity between SPL11 and these proteins is mostly restricted to the U-box and ARM repeats. The sequence identity between SPL11 and these proteins in the U-box domain ranges from 75 to 47%, with those amino acid residues key to the U-box function highly conserved (Ohi et al., 2003) (Figure 5A). The overall distribution and position of the ARM repeats in these proteins are shown in Figure 5B. The number of ARM repeats in these proteins is different, varying from 3 to 7. Sequence comparison between the ARM repeats of these proteins and SPL11's ARM repeats indicated a sequence identity ranging from 19 to 71% in the ARM domain (Figure 5B; see Supplemental Figure 1 online). Phylogenetic analysis between Arabidopsis U-box/ARM repeat proteins (Azevedo et al., 2001) and SPL11 indicated that SPL11 is evolutionally most close to AtPUB13 (Figure 5C; see Supplemental Figure 2 online). Compared with other rice U-box/ARM repeat proteins, of which full-length cDNAs are available in the public database, SPL11 is most highly related to the protein deduced from the cDNA AK121978, with overall 57% sequence identity (data not shown). No significant SPL11 homolog was identified in human, animals, and yeast, suggesting SPL11 might be unique to plants.

Molecular Properties of the *Spl11* Gene and *Spl11* Analogs in the Rice Genome

To determine the exact mutation site of the *Spl11* gene in the *spl11* mutant, the genomic DNA fragments that span the Δ*Bsl*I restriction site were identified from both *spl11* and IR68. DNA sequencing revealed a unique nucleotide substitution of T for C in the *spl11* gene, a substitution that eliminates the *Bsl*I restriction site originally present in the wild-type IR68 genome (Figure 1D, 3). This point mutation occurs in the first exon of the *Spl11* gene, resulting in a premature stop codon in the *spl11* transcript.

DNA gel blot analysis of nine rice *japonica* or *indica* cultivars indicated that the rice genome contains a single copy of the *Spl11* gene (data not shown). Nevertheless, a whole genome scale sequence analysis revealed 83 annotated U-box proteins

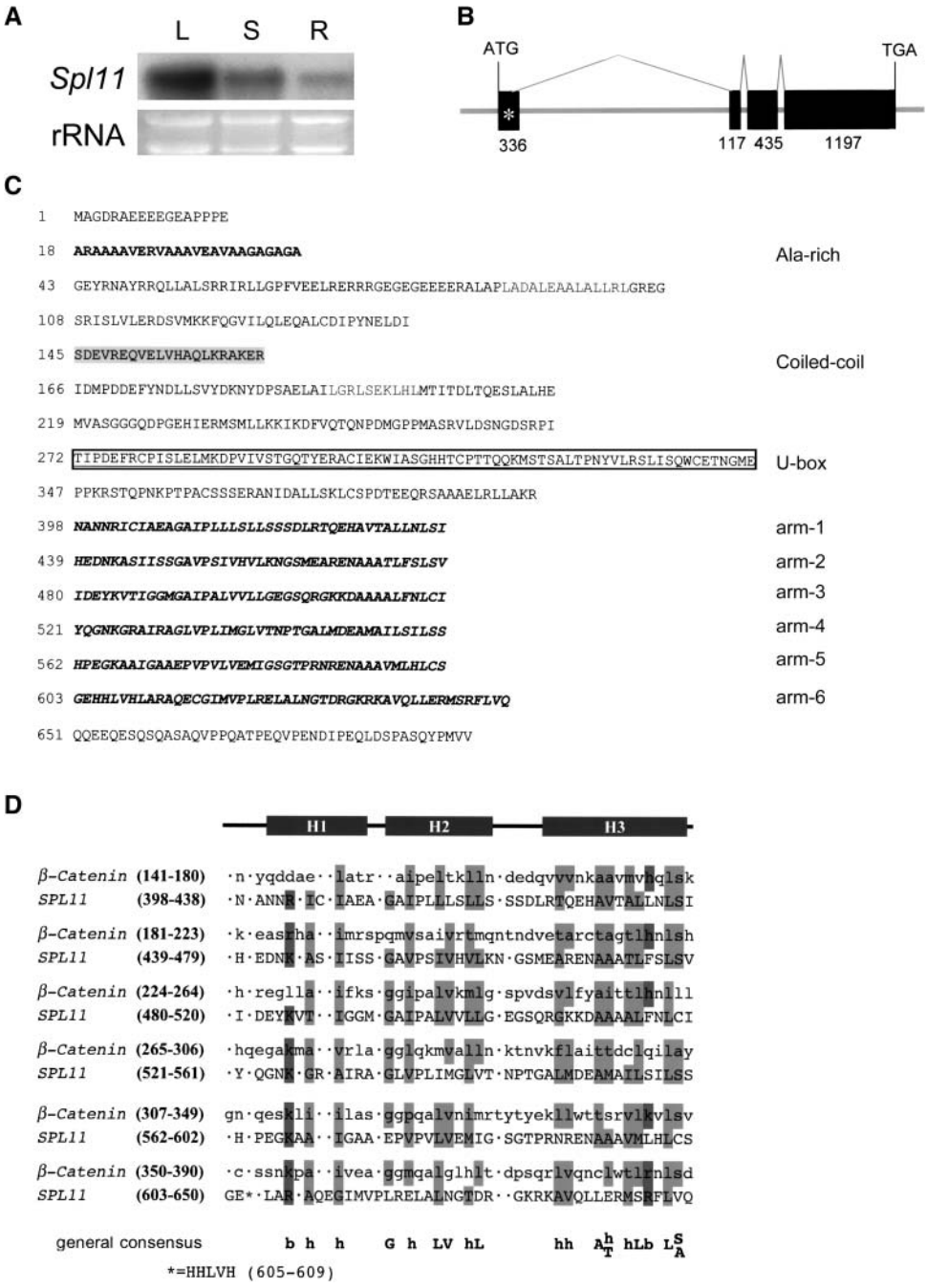

Figure 4. Transcript Abundance of *Spl11* in Different Tissues, Gene Structure, and Deduced Amino Acid Sequence of *Spl11*.

(A) RNA gel blot analysis of the *Spl11* transcript accumulation in different tissues. Total RNA from 3-week-old leaves (L), stems (S), and roots (R) of IR68 were probed with a 0.84-kb cDNA fragment at the 3′ portion of *Spl11*. Ethidium bromide staining of rRNA was used as a loading control.
(B) *Spl11* gene structure. Exons are denoted as black boxes. The number below each exon indicates the length of the exon in base pairs.
(C) Deduced amino acid sequence of *Spl11*. The N-terminal Ala-rich region is in bold. Amino acids of the coiled-coil domain (145 to 165) are highlighted. The U-box domain (272 to 346) is boxed. Amino acids of the ARM repeat motifs are displayed in italics and boldface.
(D) Sequence alignments of the ARM repeat of β-catenin and SPL11. Numbers of the ranges of amino acids composing each repeat are shown on the left. The repeats are structurally similar, with each repeat containing three helices, H1, H2, and H3, as indicated. The chemically conserved hydrophobic and polar residues are highlighted in dark and light gray, respectively.

in rice, among which 32 showed a U-box-ARM overall structure (L.-R. Zeng, unpublished data). A search for SPL11-like proteins in the rice full-length cDNA database KOME (http://cdna01.dna.affrc.go.jp/cDNA/) identified 11 U-box-ARM proteins in addition to a partial cDNA of *Spl11* (see Supplemental Table 1 online). The existence of a large number of U-box-ARM proteins in the rice genome suggests their probable involvement in a wide range of cellular processes.

SPL11 Possesses E3 Ubiquitin Ligase Activity in Vitro, and the U-Box Domain Is Essential for the E3 Ligase Activity

Because one of the important features of U-box containing proteins is to function as E3 ubiquitin ligases (Hatakeyama et al., 2001), we wanted to determine whether SPL11 also possesses E3 ligase activity. SPL11 (residues 112 to 694) was expressed in *Escherichia coli* as a fusion with maltose binding protein (MBP) and was purified by affinity chromatography. Mouse E3 ubiquitin ligase CBL (GI:38605691) was included in the experiment as a positive control.

In the presence of wheat (*Triticum aestivum*) E1 and an Arabidopsis E2 AtUBC9, ubiquitination activity was observed for the purified MBP-SPL11 and MBP-CBL proteins (Figure 6A, lanes 5 and 6 from the left, respectively), whereas in the absence of any of the E1, E2, or E3 (lanes 1 to 4 from the left), no signal was detected (Figure 6A). These results indicated that SPL11 possesses E3 ligase activity.

It has been shown that the U-box is essential for the E3 ligase activity of U-box proteins (Hatakeyama et al., 2001). To test if an intact U-box domain is required for SPL11 E3 ligase activity as well, two versions of SPL11 bearing mutation in the U-box domain were tested for E3 activity. The first version carried a single mutation that results in a Val^{290} to Arg^{290} single amino acid residue change (Figure 5A, asterisk). This Val is highly conserved among different U-box proteins and a Val to Ile point mutation in the yeast protein Prp19p U-box domain leads to pre-mRNA splicing deficiency in vivo (Ohi and Gould, 2002). The second version carried a three–amino acid residue deletion ($\Delta C^{314}P^{315}T^{316}$) in the U-box domain (Figure 5A, arrows). The C^{314} and P^{315} also are highly conserved and were reported to be essential for correct folding of the U-box domain to form an appropriate interface interacting with E2 ubiquitin conjugase (Ohi et al., 2003). In vitro ubiquitination analysis indicated that the E3 ligase activity was completely abolished in both versions of SPL11 (Figure 6B), indicating that an intact U-box domain is required for SPL11's E3 ligase activity.

Expression Pattern of *Spl11* in Blast-Infected Rice Plants

It is assumed that *Spl11* is involved in rice defense signaling based on the enhanced resistance of the *spl11* mutant to rice pathogen attack (Yin et al., 2000). To investigate the role of *Spl11* in defense against rice pathogens, the expression of *Spl11* in rice blast-infected resistant [carrying the *Pi2*(*t*) *R* gene] and susceptible [without the *Pi2*(*t*) gene] plants was monitored by RNA gel blot and RT-PCR analyses (Liu et al., 2002). RNA was isolated from both resistant and susceptible plants 0, 12, 24, and 72 h after inoculation with isolate PO6-6 avirulent to *Pi2*(*t*). Both RNA gel blot hybridization and RT-PCR results indicated that *Spl11* was induced at 12 and 24 h after blast inoculation in resistant and susceptible interactions (Figure 7). No difference was detected in the pattern and level of *Spl11* expression between the resistant and susceptible plants. Inoculation with PO6-6 on IR68 plants (resistant reaction) confirmed the induction of *Spl11* by rice blast at early infection stages (data not shown). These results suggest that *Spl11* is not *R* gene dependent and might be involved in the basal defense signaling against rice blast.

DISCUSSION

To expand our understanding of cell death in plant disease resistance, we previously characterized nine rice lesion mimic mutants for their resistance to fungal and bacterial pathogens (Yin et al., 2000). The clear cell death phenotype and the intimate association of cell death with defense activation in the *spl11* mutant suggested that the activation of a cell death pathway that is coordinated with the induction of defense responses leads to the *spl11* phenotype. In plant–pathogen systems, coordination of PCD and defense responses occurs in both incompatible and compatible interactions. The activation of cell death and defense responses in these two types of disease reactions is dissimilar in terms of the time, location, and magnitude of their occurrence as demonstrated in Arabidopsis (Tao et al., 2003; Wu et al., 2003). In *spl11*, the two processes are difficult to distinguish because of the constitutive activation of both. The induction of *Spl11* in both incompatible and compatible rice–blast interactions suggests that it might be involved in basal resistance instead of *R* gene–mediated race-specific defense. The specific role of *Spl11* in fighting against rice blast invasion remains to be elucidated. The enhanced resistance of *spl11* to rice blast and bacterial blight diseases supports the argument that the cell death pathway activated in *spl11* might be interconnected with the defense pathways to the two rice pathogens.

A link between U-box–mediated ubiquitination and cell death has not been clearly established in plants. Our finding that *Spl11* encodes a U-box protein endowed with E3 ubquitin ligase activity and the U-box domain is essential for its E3 activity is significant for our understanding of PCD and disease resistance in plants. The ubiquitination related to the spontaneous cell death phenotype of *spl11* is analogous to the wide involvement of ubiquitination in the regulation of apoptosis in mammals (Lee and Peter, 2003). Mechanistically, regulation of apoptosis by ubiquitination always occurs via the ubiquitination of key pro- and anti-apoptotic regulators. In animals, a large number of components forming a complicated signaling network involved in the regulation and execution of apoptosis have been identified in the last decade. The HECT domain protein family and RING-finger domain–containing protein family, including the Skp1-Cdc53/Cullin1-F-box (SCF) multisubunit E3 complexes, have been implicated in targeting these apoptotic regulators for degradation (Wilson et al., 2002; Wing et al., 2002; Miyazaki et al., 2003). Although it is unclear at present how SPL11-mediated ubiquitination is regulated and how it functionally contributes to PCD and defense activation in the *spl11* mutant, the indication of E3 activity for SPL11 suggested an involvement of a new family of E3 ubiquitin ligases in plant PCD and defense.

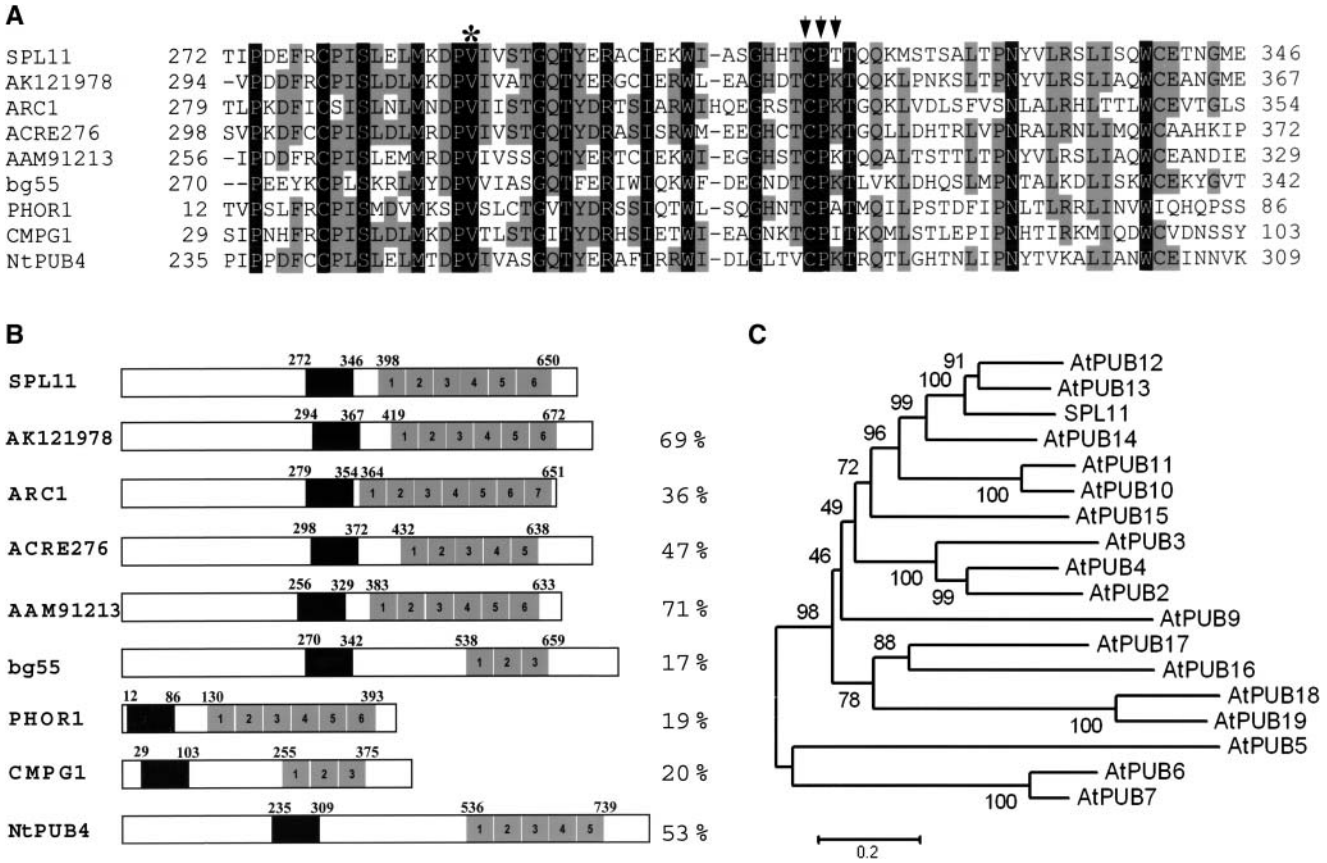

Figure 5. Amino Acid Sequence Alignments between SPL11 and U-Box-ARM Proteins from Other Plant Species.

(A) Sequence alignments in the highly conserved U-box domain of SPL11 and those of other U-box-ARM proteins. The numbers on the left or right indicate the amino acid residues. Gaps, which were introduced to maximize alignment, are indicated by dashes. The residues conserved among the compared sequences are boxed in black or light gray based on the degree of conservation. AK121978 from *O. sativa* (GI:37991601), ARC1 from *B. napus* (GI:2558938), ACRE276 from *N. tabacum* (GI:30013679), AAM91213 from *A. thaliana* (GI:22136270), bg55 from *B. gymnorrhiza* (GI:14149112), PHOR1 from *Solanum tuberosum* (GI:13539578), CMPG1 from *P. crispum* (GI:14582202), and NtPUB4 from *N. tabacum* (GI:28974687) are shown. Only the one most highly related to SPL11 from rice and Arabidopsis is included in the alignment because of the large number of U-box-ARM proteins present in the rice and Arabidopsis genomes. The asterisk and arrows marked the amino acids that were mutated in the SPL11 E3 ligase activity assay.
(B) Schematic representation of SPL11 and other U-box-ARM proteins of plants. The black box indicates the U-box domain, and the individual ARM repeat of the ARM domain is indicated by a numbered, shaded box. The percentage of sequence identity of the ARM repeats from plant U-box-ARM proteins to their most homologous ARM repeats in SPL11 is indicated. Detail sequence alignment in the ARM domain of SPL11 with those of other U-box-ARM proteins is indicated in Supplemental Figure 1 online.
(C) Phylogenetic relationship between SPL11 and Arabidopsis U-box/ARM repeat proteins. The phylogenies were generated with neighbor joining with 400 bootstrap replicates and were rooted at midpoint. The bootstrap values are shown as percentages. AtPUB8 (locus At4g21350) was not included in the tree because no EST, SAGE tag, or cDNA was identified for the corresponding predicted gene.

Non-U-box protein–mediated ubiquitination recently has been shown to be associated with plant disease resistance in several cases. For example, several RING-finger–type E3 ubiquitin ligases were induced after elicitor or pathogen treatments (Durrant et al., 2000; Takai et al., 2002). Recently, the plant SGT1 protein, which interacts with a convergence component of multiple *R* gene–mediated signaling pathways, RAR1, was found to interact with the SCF ubiquitin ligase complex as well as the COP9 signalosome (Azevedo et al., 2002). Despite all these findings, key questions remain to be addressed, such as (1) determining which substrates are targeted by the ubiquitination in plant defenses, and (2) determining when and where (i.e., at what level) ubiquitination operates in regulating the defense reactions. It is possible that one or more substrates targeted by SPL11 are functionally related in PCD and defense signaling pathways. In animals, the inhibitors of apoptosis proteins (IAPs) contain at least one baculoviral IAP repeat (BIR) domain at the N terminus and often a RING domain at the C terminus. The combination of BIR-mediated binding, and hence inactivation of proteins and RING-mediated proteolysis of proteins, has been shown to be central to the role of IAPs in regulating apoptosis (Lee and Peter, 2003). In this regard, it would be interesting to

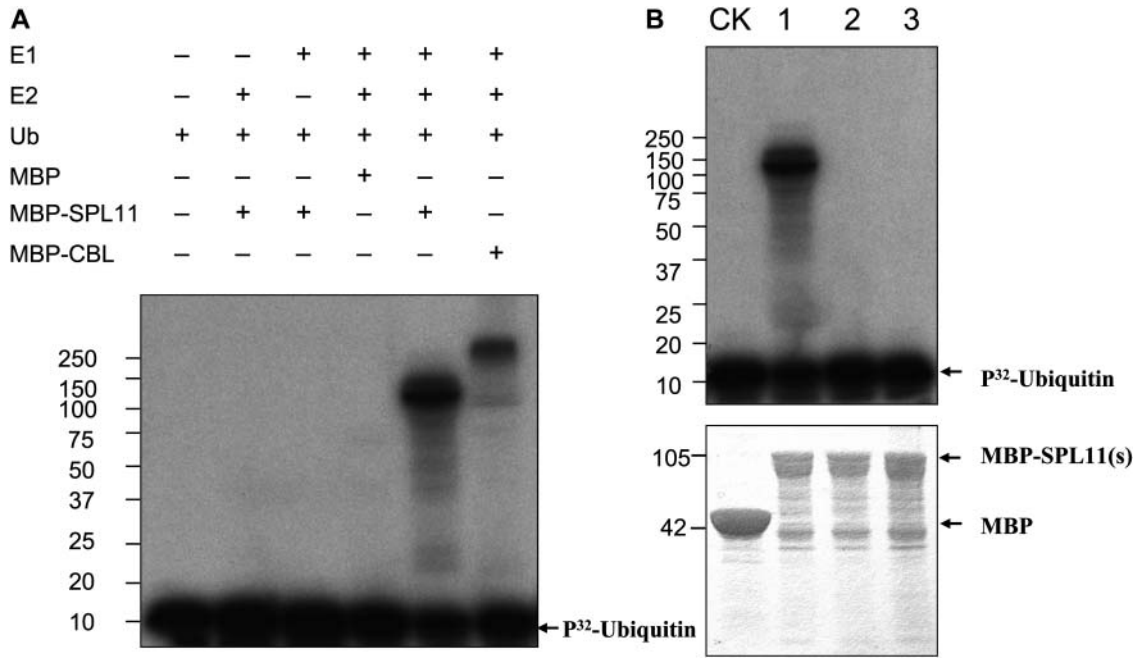

Figure 6. E3 Ubiquitin Ligase Activity of SPL11.

(A) MBP-SPL11 and MBP-CBL fusion proteins were assayed for E3 activity in the presence of E1 (from wheat, GI:136632), E2 (AtUBC9, GI:20136191), and ^{32}P-labeled ubiquitins. The numbers on the left denote the molecular mass of marker proteins in kilodaltons. Mouse E3 ubiquitin ligase CBL (GI:38605691) was used as a positive control. ^{32}P-ubiquitin is indicated by an arrow. MBP itself was used as a negative control.

(B) E3 ligase activity of SPL11 and its mutants. CK, MBP; lane 1, wild-type SPL11; lane 2, SPL11 ($V^{290}R$); lane 3, SPL11$\Delta C^{314}P^{315}T^{316}$. ^{32}P-ubiquitin is indicated by arrow. The bottom panel shows two times of the corresponding amount of MBP and MBP fusion proteins used in the E3 activity assay.

determine whether the ARM repeat domain in SPL11 functions with the U-box domain in a way similar to that between the BIR domain and the RING domain in animal IAPs. The identification and characterization of the substrates in SPL11-mediated ubiquitination will be essential to answer such questions and to obtain an in-depth understanding of the role of the SPL11-mediated ubiquitination in PCD and defense.

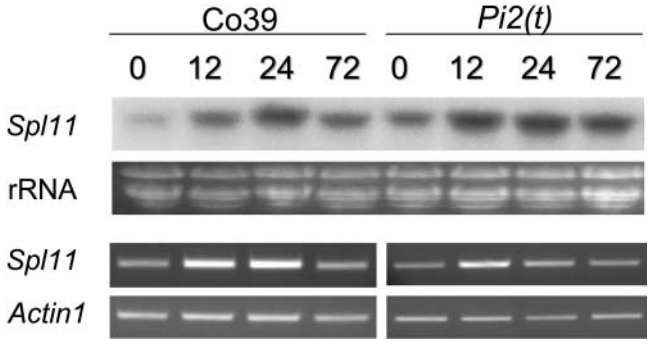

Figure 7. Expression Patterns of *Spl11* in Rice–Blast Interaction.

Total RNA was isolated from infected leaves at the indicated hours after rice blast inoculation. Approximately 10 μg of total RNA was loaded in each lane in RNA gel blotting. ^{32}P-labeled *Spl11* cDNA fragment (0.84 kb) was used as the probe in the RNA hybridization. A pair of *Spl11*-specific primers was used in the RT-PCR analysis. The rRNA gel shows the loading quantification of the RNAs in the RNA gel blot analysis. The amplification of the rice *Actin1* gene was used as a control for equal amount of total RNAs in the RT-PCR analysis. The numbers denote the hours after rice blast inoculation.

The *Spl11* mutant phenotype could be caused by the disruption of negative regulation of PCD and defense activation or simply be a reflection of perturbed cellular homeostasis. Although it is difficult to distinguish these two possibilities with certainty, two lines of evidence support the first explanation. First, most of the genes differentially expressed in *spl11* in our microarray hybridization experiment of *spl11* are related to cell death and defense (L.-R. Zeng, T. Zhu, and G.-L. Wang, unpublished data). By contrast, few genes involved in other well-defined cellular processes could be identified, suggesting that the *spl11* mutant, like the Arabidopsis mutant *acd11*, is not excessively pleiotropic (Brodersen et al., 2002). Second, we have identified several putative *spl11* suppressors in the screening for mutants with alleviated *spl11* phenotype from a diepoxybutane-treated *spl11* population (H. Leung and G.-L. Wang, unpublished data). Multiple genes might suppress the lesion formation because there was a wide range of lesion phenotypes in terms of lesion numbers among these mutants. The identification of *spl11* suppressors suggests that the *spl11* phenotype is genetically programmed.

Only a few U-box proteins can be identified in the genomes of human, *Caenorhabditis elegans*, and Drosophila (Azevedo et al., 2001). By contrast, dozens of U-box proteins were identified in Arabidopsis and the rice genome (Mudgil et al., 2004; L.-R. Zeng and G.-L. Wang, unpublished data). Such proteins likely exist widely in other plants as well. The existence of a large number of

such proteins in plants suggests that they may play diverse roles in multiple processes. So far, SPL11 represents the first case of a U-box/ARM repeat structure protein related to both PCD and defense. Our results indicated that ubiquitination is involved in PCD and defense in plants. This is consistent with the emerging plant disease defense model suggesting that the signaling components/regulators in the plant PCD and defense pathways need to be inactivated and degraded in a temporally and spatially ordered manner similar to what was observed in animals. This strongly suggests that the ubiquitination pathway could play a significant role in regulating plant PCD and defense as well. Therefore, the cloning and characterization of the *Spl11* gene hereby opens a way to dissect the mechanism by which the ubiquitination system contributes to the control of PCD and disease resistance in plants.

METHODS

Plant Growth

The F2 population derived from the cross between TP309$^{spl11/spl11}$ and Nipponbare used for the mapping analysis was grown in the greenhouse in the winter season at Columbus, OH, at 28 to 18°C day and night temperatures. The F3 population of the same cross was grown in the greenhouse in the summer at Columbus, OH, at 32 to 26°C day and night temperatures. Genomic DNA was prepared from young leaves of plants around 45 d old.

Identification of *spl11* Allelic IR64 Mutants

A large deletion mutant bank was established at the International Rice Research Institute from chemical- and irradiation-treated IR64 populations as described (Leung et al., 2001). Among the morphological mutants collected in the bank, more than 30 were lesion mimic mutants (C. Wu and H. Leung, unpublished data). Mutants with phenotypes similar to that of *spl11* were then crossed with IR64 and *spl11* or were crossed with each other for genetic analysis. Those mutant lines allelic to *spl11* were then subjected to molecular analysis.

DNA Gel Blot Analysis

Genomic DNA from young leaves was extracted and purified according to the method described (Dellaporta et al., 1984) with extraction buffer modification. The extraction buffer included 100 mM Tris-HCl buffer, pH 8.0, 25 mM EDTA, pH 8.0, 2% (w/v) sorbitol, 0.25% (w/v) hexadecyl trimethyl ammonium bromide, 0.25% (w/v) polyvinyl polyprolidone, 1% *N*-lauroyl sarcosine, and 1.4 M sodium chloride. Approximately 2 μg of rice (*Oryza sativa*) genomic DNA was digested with an appropriate enzyme and fractionated on a 1.0% agarose gel by electrophoresis. For DNA gel blotting analysis, the gel was first soaked in 0.25 M HCl for 10 to 20 min, rinsed with distilled water, and then soaked in 0.4 M NaOH for 10 min. The fractionated DNA was then transferred to a Hybond N^+ nylon filter under alkaline conditions (0.4 M NaOH). The prehybridization, hybridization, and washing of the filter were conducted using standard procedures (Sambrook and Russell, 2001).

RNA Gel Blot Analysis

Total RNA was isolated with the RNAwiz RNA isolation reagent (Ambion, Austin, TX) from 3- to 4-week-old rice leaves according to the protocol provided by the manufacturer. Approximately 10 μg of total RNA from each sample was mixed with an equal volume of northernMax gel loading solution (Ambion), heated at 50°C for 30 min, then cooled on ice to denature the RNA. The denatured samples were then separated on a 1.4% agarose gel in 1× BPTE electrophoresis buffer (10 mM Pipes, 30 mM Bis-Tris, and 1 mM EDTA) and blotted to a Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ) with 20× SSC solution (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate). The prehybridization and hybridization were performed using standard procedures (Sambrook and Russell, 2001). After hybridization, the blot was washed twice in 1× SSC and 0.5% SDS solution at 65°C for 5 min, followed by washing in 0.5× SSC and 0.5% SDS solution at 65°C for 10 min.

RT-PCR Amplification

To detect changes in *Spl11* expression in IR64 mutants, 1 μg of total RNA per sample was used to synthesize the first-strand cDNA using the AMV reverse transcriptase system (Promega, Madison, WI). The synthesis was conducted according to the protocol provided by the manufacturer. The 20-μL first-strand cDNA product was diluted to 120 μL final volume with 1× TE buffer. For PCR amplification of the *Spl11* cDNA fragment, 1.5 μL of the diluted first strand cDNA was used in a 25-μL reaction volume with the *Taq* enzyme from New England Biolabs. *Spl11*-specific primers Uc-3 (5′-GATGCTTGCCTTATTGTCCTCA-3′) and Uc-4 (5′-ACGGATTGATA-TGCCTGACGAT-3′) were used for the amplification. The reaction mixture was cycled through the following temperature profiles: 94°C for 210 s for one cycle, followed by 94°C for 40 s, 62°C for 40 s, and 72°C for 60 s for 22 cycles, and a final incubation at 72°C for 5 min. For amplification of the rice *Actin1* gene, primer pair Actin-F (5′-CGTCTGCGATAATGGAACTGG-3′) and Actin-R (5′-CTGCTGGAATGTGCTGAGAGAT-3′) were used.

For amplification of the 5′ end of the *Spl11* cDNA, ~1.6 μg mRNA was first purified from total RNA using the Oligotex mRNA mini kit (Qiagen, Valencia, CA). Approximately 12.5% DMSO (final concentration) was added to the first-strand cDNA synthesis reaction to break the secondary structures of the RNA. The oligo(dT) primer was replaced by the *Spl11*-specific primer Uc-3 (5′-GATGCTTGCCTTATTGTCCTCA-3′) in the synthesis reaction. The reaction was performed at 38.5°C for 1 h using the AMV reverse transcriptase system and was then denatured at 95°C for 5 min. The reverse transcription was followed by PCR that was performed with primers RACE1 (5′-CGTCAGGCATATCAATCCGTTCTTT-3′) and URACE3 (5′-CCCCACTATTTACCATTCTGCCACT-3′) using approximately one-tenth of the reverse transcription products. Two percent DMSO and 0.25 M betaine (trimethylglycine) were added to the reaction mixture to overcome the difficulty in the amplification of the high GC content region. The reaction mixture was cycled through the following temperature profiles using the ThermalACE Taq enzyme (Invitrogen, Carlsbad, CA): 98°C for 180 s for one cycle, followed by 98°C for 30 s, 54°C for 40 s, and 72°C for 45 s for 32 cycles, and a final incubation at 72°C for 10 min.

E3 Ubiquitin Ligase Activity Assay

DNA fragments of *Spl11* containing sequence for both the U-box domain and the ARM domain (1749 bp) and mouse E3 ubiquitin ligase gene CBL (GI:38605691) were cloned into the pMAL-c2 vector (New England Biolabs) and expressed in *Escherichia coli*. The fusion proteins were prepared according to the manufacturer's instructions. For the E3 ubiquitin ligase activity assay of the fusion proteins, wheat (*Triticum aestivum*) E1 (GI:136632) and *Arabidopsis thaliana* E2 AtUBC9 (GI:20136191) were used for the assay. Both wheat E1 and AtUBC9 were cloned in frame into vector pET28a (Novagen, Madison, WI, now part of EMD Biosciences, San Diego) and expressed in *E. coli* strain BL21. Protein from the E1- or E2-expressing *E. coli* was used in the E3 ubiquitin ligase assay in which ~50 ng of E1, 50 ng of E2, and 1 μg of E3 were added. The two SPL11 mutants that contain mutation in the U-box

domain are prepared using the Quickchange site-directed mutatgenesis kit (Stratagene, La Jolla, CA) according to the protocol provided by the manufacturer. The sequence of the primer pair used for the preparation of the Val^{290} to Arg^{290} mutant is as follows: M1F (5′-CTTGAGCTGATGAAGGATCCTAGAATAGTGTCTACAGGGCAGACA-3′) and M1R (5′-TGTCTGCCCTGTAGACACTATTCTAGGATCCTTCATCAGCTCAAG-3′). The primers for the preparation of the mutant containing a small deletion ($\Delta C^{314}P^{315}T^{316}$) in the U-box are as follows: M2F (5′-ATAGCATCAGGCCATCATACCACGCAACAGAAGATG-3′) and M2R (5′-CATCTTCTGTTGCGTGGTATGATGGCCTGATGCTAT-3′). The in vitro E3 ligase assays were performed as described (Xie et al., 2002).

Complementation

The BAC78 that contains the *Spl11* gene was first subcloned into the modified transformation-competent BAC vector pTAC8 (Qu et al., 2003) using *Not*I as the restriction enzyme. The insert of different subclones was determined by pulse-field gel eletrophoresis and PCR amplification using primer pairs specific to each *Not*I-digested fragment of BAC78. The subclone containing the *Spl11* gene, TAC20, was then digested with *Pac*I and separated on a 0.8% agarose gel. The 9.4-kb fragment from the *Pac*I-digested TAC20 that contains the *Spl11* gene was recovered from the gel and then digested with *Xba*I to remove the 1.4-kb *Pac*I-*Xba*I fragment. A 365-bp *nos* terminator DNA was amplified from vector pBI221 (Clontech, Palo Alto, CA) with primers that contain adapter sequences harboring the *Pac*I and *Hin*dIII restriction sites, respectively. The 8.0-kb *Xba*I-*Pac*I genomic DNA fragment containing the entire *Spl11* gene and sufficient *cis* element (a 2.6-kb DNA fragment upstream of the start codon) was then ligated together with the *nos* terminator DNA fragment into the binary vector pCAMBIA1301 (CAMBIA, Canberra, Australia). This final binary construct (pGW78) was used for the complementation of the *spl11* mutation. The pGW78 was mobilized into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by electroporation and was used to transform *spl11* plants ($TP309^{spl11/spl11}$) (Qu et al., 2003). The phenotype of the T1 transformants was scored under standard plant growth conditions as described above.

Protein Sequence Alignments and Phylogenetic Analysis

All the protein sequence alignments were conducted using the program ClustalX (Thompson et al., 1997). The aligned sequence data was then inputted into the MEGA2 program (Kumar et al., 2001) to construct the phylogenetic tree.

Sequence data for the mRNA and genomic DNA of the *Spl11* gene have been deposited with the EMBL/GenBank data libraries under accession numbers AY652589 and AY652590, respectively.

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