The U-Box E3 Ligase SPL11/PUB13 Is a Convergence Point of Defense and Flowering Signaling in Plants[1]

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The ubiquitin-proteasome system (UPS) is involved in the selective degradation of proteins in the cells of eukaryotic organisms and consists of three main enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). In a typical ubiquitination reaction, E1, E2, and E3 enzymes participate in the transfer of the ubiquitin monomers to the ε-amino group of a Lys residue in the target protein. In the first step of the reaction, E1 catalyzes the activation of ubiquitin via ATP, creating a high-energy thiol intermediate. After activation, ubiquitin is transferred to the E2 and then to the E3 ligase for the final step in the process, which involves the creation of an isopeptide bond between the C-terminal Gly of ubiquitin and a Lys residue in the substrate protein (for review, see Vierstra, 2009; Ye and Rape, 2009). The UPS in plants has a hierarchical structure for the three enzymes: there are few E1s, about 50 E2s, but over 1,000 E3s. For example, the rice (*Oryza sativa*) genome encodes six E1 genes, 49 E2 and E2-like genes, and over 1,300 E3 genes (Du et al., 2009). The abundance of the E3 proteins in the UPS system allows plants to target substrates for many biological processes, because each E3 ubiquitin ligase acts for the ubiquitination of only one or a few target proteins. Based on the subunit component and action modes, E3 ligases can be divided into two major types: the single-subunit type (Homology to E6 carboxyl terminus [HECT] and RING/U-box) and the multisubunit type (Skp1 [for S-phase kinase-associated protein1]-CUL1 [for Cullin1]-F-box [SCF], Anaphase promoting complex [APC], CUL3-BTB [for Bric-a-Brac, tramtrack broad complex], CUL4-DDB [for damage-specific DNA binding protein] complex, and others; Vierstra, 2009; Fig. 1).

Depending on the number (single or multiple) of ubiquitins attached to the substrates and on the manner in which multiple ubiquitins are attached to the substrates, the ubiquitin-mediated protein modifications can be classified as monoubiquitination, multiubiquitination, and polyubiquitination (for review, see Hochstrasser, 2006; Mukhopadhyay and Riezman, 2007; Vierstra, 2009). Monoubiquitination and multiubiquitination usually alter substrate protein localizations, affect protein-protein interactions, and modulate protein activities. The polyubiquitination can differ in structure and function depending on how the seven Lys residues in ubiquitin (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63) are connected to each other and to the substrates. For example, Lys-48-mediated and Lys-11-mediated polyubiquitination target substrates for degradation by the 26S proteasome, while Lys-63-mediated polyubiquitination usually mediates DNA repair, membrane trafficking, and chromatin remodeling (for review, see Ye and Rape, 2009; Liu and Chen, 2011). The function of polyubiquitination mediated by the other four Lys residues is not clear.

The UPS functions in nearly all aspects of plant life, including the cell cycle, embryogenesis, photomorphogenesis/flowering, hormone signaling, and abiotic and biotic stress responses (for review, see Stone and Callis, 2007; Vierstra, 2009; Santner and Estelle, 2010). In this paper, we focus on the function of the rice U-box protein SPOTTED LEAF11 (SPL11) and its Arabidopsis (*Arabidopsis thaliana*) ortholog protein PLANT U-BOX13 (PUB13) in programmed cell death (PCD), defense, and flowering (Table I).

THE FUNCTION OF PCD IN PLANT DEFENSE

During the course of coevolution, plants have developed two layers of innate immune systems that rely on the pattern-recognition receptors (PRRs) and resistance (R) proteins to defend against pathogen attack (for review, see Jones and Dangl, 2006; Dodds and Rathjen, 2010). The first line of defense depends on the

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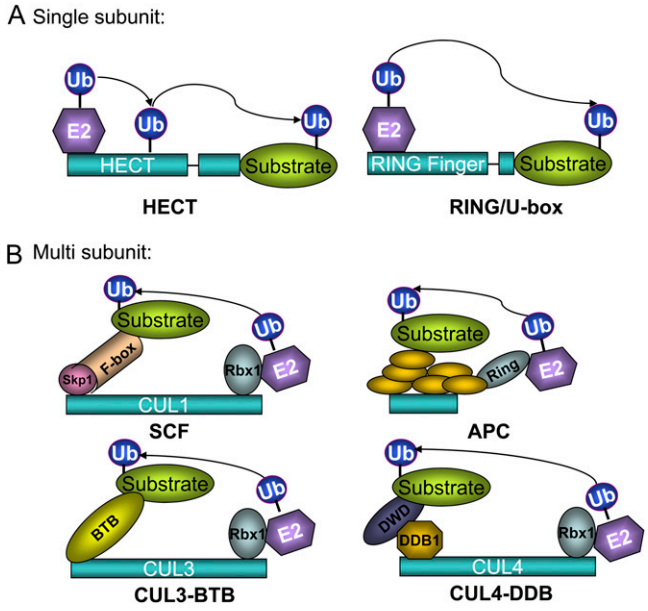

Figure 1. Two types of E3 ligases, their interaction components, and their modes of action.

activation of PRRs by the perception of pathogen-associated molecular patterns (PAMPs). The PAMP-triggered immunity (PTI) causes the accumulation of reactive oxygen species (ROS) and the deposition of phenolic compounds. To suppress PTI, pathogens have evolved effector proteins that can be secreted into the cytoplasm of host cells. In response, plants employ R proteins, such as nucleotide-binding site leucine-rich repeat (LRR) proteins (or NLRs), to monitor the entry of these effector proteins directly or indirectly, resulting in the second layer of defense, which is called effector-triggered immunity (ETI). ETI is more robust and effective than PTI and is often associated with a hypersensitive response (HR), which is characterized by the rapid death of cells in the local region surrounding an infection. Several recent studies, however, have challenged the distinction between PTI and ETI and have provided evidence for a continuum between the two types of immunities (Lee et al., 2009; Thomma et al., 2011).

Lesion-mimic (LM) mutants displaying spontaneous HR-like cell death under normal growth conditions represent a unique kind of PCD in plants. Many LM mutants have been identified and characterized in maize (*Zea mays*), Arabidopsis, rice, barley (*Hordeum vulgare*), and tobacco (*Nicotiana tabacum*). One of the common characteristics of numerous LM mutants is that they exhibit enhanced resistance to biotrophic or hemibiotrophic pathogens. Cloning and molecular characterization have revealed that the mutated genes encode various proteins involved in defense-related signaling pathways, including ion channels, ROS generation, sphingolipid metabolism, porphyrin/phenolics/chorophyll biosynthesis and metabolism, ubiquitination, and other signaling transduction (for review, see Love et al., 2008; Moeder and Yoshioka, 2008).

An intimate relationship between LM mutation and ETI was indicated by the genetic analysis of the Arabidopsis LM mutant *lesion simulating disease1* (*lsd1*). This analysis revealed that the phenotypes of the mutant were nullified by mutation in *ENHANCED DISEASE SUSCEPTIBILITY1* and *PHYTOALEXIN DEFICIENT4* (*PAD4*), two important genes required for the Toll-IL-1 receptor (TIR)-type NLR *R* gene-mediated resistance and also for basal resistance (Rustérucci et al., 2001; Wiermer et al., 2005). Both genes are also essential for cell death regulation in Arabidopsis under intense light and for the ROS- and salicylic acid (SA)-dependent defense signal amplification loop (Glazebrook, 2005; Mühlenbock et al., 2008). Recently, a suppressor screening of the *lsd1* mutant revealed that PHOENIX21 is a positive regulator of *lsd1* runaway cell death and is a member of the ACTIVATED DISEASE RESISTANCE1 family of the coiled coil (CC)-type NLR proteins (Bonardi et al., 2011). Thus, these results suggested that the HR and cell death in some LM mutants share common signaling pathways.

THE FUNCTION OF UPS IN PCD AND DEFENSE RESPONSES IN PLANTS

The role of UPS in regulating apoptosis in animals has been well established (for review, see Broemer and Meier, 2009). Recently, the role of UPS in plant PCD and defense responses has become clearer based on many studies with dicot plants. One of the first studies reported that Arabidopsis SGT1, a homolog of yeast SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1), interacts with SUPPRESSOR OF KINETOCHORE PROTEIN1 (SKP1) and CULLIN1 (CUL1), subunits of the Skp1-Cullin-F-box (SCF) E3 ligase, and is required for defense signaling mediated by multiple NLR-type *R* genes (Kitagawa et al., 1999; Austin et al., 2002; Azevedo et al., 2002; Tör et al., 2002; Moon et al., 2004). In tobacco, a set of E3 ligase genes induced by the fungal avirulence protein Avr9 are required for the HR of Cf9-mediated resistance (González-Lamothe et al., 2006; Yang et al., 2006; van den Burg et al., 2008). In Arabidopsis, two RING finger E3 ligases, RPM1-INTERACTING PROTEIN2 (RIN2) and RIN3, have also been implicated in the HR of both CC-type NLR R proteins RPM1- and RPS2-mediated resistance (Kawasaki et al., 2005), whereas a PUB E3 ligase, PUB17, is required for CC-type NLR R protein RPM1- and TIR-type NLR R protein RPS4-mediated resistance (Yang et al., 2006), indicating the critical role of UPS in ETI. Similarly, a homologous triplet of PUBs (PUB22, PUB23, and PUB24) and PUB12/13 in Arabidopsis have been demonstrated to negatively regulate PTI (Trujillo et al., 2008; Li et al., 2012a), suggesting the involvement of the UPS in plant PTI signaling.

The role of UPS in the regulation of PCD and defense of the monocot rice is also becoming clearer. Our

Table I. *Summary of the rice and Arabidopsis genes discussed in this paper*

Gene	Full Name	Species	Protein Encoding	Biological Function
SPL11	Spotted leaf11	Rice	U-box domain and ARM repeat domain protein	Negative regulator of PCD and defense, and positive regulator of flowering in LD conditions
SPIN1	SPL11-interacting protein1	Rice	K homology domain-containing RNA-binding protein	Suppressor of flowering in both SD and LD conditions
SPIN6	SPL11-interacting protein6	Rice	Small GTPase-activating protein	Component involved in SPL11-mediated PCD and defense
RBS1	RNA-binding and SPIN1-interacting1	Rice	RNA-binding protein	Component involved in SPL11/SPIN1-mediated flowering
OsRAC1	Rice homolog of mammalian Rac-GTPase RAC1	Rice	Small GTPase protein	Integrator of PTI and ETI
OsCERK1	Chitin elicitor receptor kinase1	Rice	LysM RLK	Positive regulator of defense by perceiving chitin elicitors
Pit	*Pyricularia grisea* resistance locus t	Rice	CC-nucleotide-binding site-LRR protein	Positive regulator by recognizing its cognate *M. oryzae* Avr effector AvrPit-triggered immune signaling
Hd1	Heading date1	Rice	Zinc finger protein and ortholog of Arabidopsis CO	Positive regulator of flowering in SD conditions but a negative regulator in LD conditions
Hd3a	Heading date3a	Rice	Similar to phosphatidylethanolamine-binding protein and ortholog of Arabidopsis FT	Positive regulator of flowering in both SD and LD conditions
MAPKs	Mitogen-activated protein kinases	Rice and Arabidopsis	Ser/Thr protein kinases	Components for signal transduction by phosphorylation
NOXs	NADPH oxidases	Rice and Arabidopsis	Membrane-bound enzyme complex	Regulator of ROS generation
PUB13	Plant U-box13	Arabidopsis	U-box and ARM repeat domain protein and ortholog of rice SPL11	Negative regulator of plant PCD, defense, and flowering in LD conditions
PAD4	Phytoalexin deficient4	Arabidopsis	Triacylglycerol lipase-like protein	Positive regulator of SA-dependent defense signaling
SID2	SA induction deficient2	Arabidopsis	Isochorismate synthase	Positive regulator of SA-dependent defense signaling and type I SA gene required for SA synthesis
WIN3	HOPW1-1-interacting3	Arabidopsis	Member of the firefly luciferase family	Positive regulator of SA-dependent defense signaling and type II SA gene regulating SA level
FLS2	Flagellin-sensing2	Arabidopsis	LRR-RLK	Positive regulator of plant defense by perceiving PAMPs (e.g. flagellin)
BAK1	Brassinosteroid-insensitive1-associated receptor kinase1	Arabidopsis	LRR-RLK	Component involved in hormone BRI1-mediated growth and development and plant PTI immune signaling
FLC	Flowering locus C	Arabidopsis	MADS box transcription factor	Suppressor of the flowering signal from the vernalization and autonomous pathways
FT	Flowering locus T	Arabidopsis	Similar to phosphatidylethanolamine-binding protein	Positive regulator of flowering in both SD and LD conditions
SOC1	Suppressor of overexpression of constans1	Arabidopsis	MADS box transcription factor	Integrator of multiple flowering signals from photoperiod, temperature, hormone, and age-related signals
HFR1	Long hypocotyl far-red light1	Arabidopsis	Atypical basic helix-loop-helix protein	Component involved in plant photomorphogenesis

laboratory's identification and characterization of SPL11 in rice as a U-box protein with E3 ligase activity provided, to our knowledge, the first direct evidence that ubiquitination controls resistance and PCD in rice (Zeng et al., 2004). In addition, a RING finger E3 ligase, BLAST AND BTH-INDUCED1, was found to positively regulate resistance against *Magnaporthe oryzae* by modifying the rice cell wall (Li et al., 2011). Another interesting protein is the RING finger E3 ligase XA21-BINDING PROTEIN3, which is required for the

accumulation of the rice bacterial blight R protein XA21 and XA21-mediated defense signaling against *Xanthomonas oryzae* pv *oryzae* (Wang et al., 2006). Surprisingly, XA21 was identified to be a PRR that binds a PAMP-like type I-secreted sulfated peptide, AxYS22, derived from the Ax21 protein (Lee et al., 2009). Further characterization of these PCD- and defense response-related UPS components will shed light on the molecular mechanisms underlying HR cell death through the UPS in rice.

THE FUNCTION OF UPS IN FLOWERING TIME REGULATION

Flowering is a well-defined developmental process that is controlled by environmental cues and intrinsic biological rhythms (for review, see Amasino, 2010). Extensive investigations in Arabidopsis have identified four major pathways that perceive and process different signals. The autonomous and GA pathways perceive and transduce internal signals to promote flowering. The external stimuli of variations in daylength and temperature mediate signal transduction in the photoperiodic and vernalization pathways, respectively. Ultimately, signaling in all pathways converges at the floral pathway integrators, a group of genes that are turned on or off in a manner that is consistent with the decision to flower. Among these integrators, the best characterized are FLOWERING LOCUS T (FT; Kardailsky et al., 1999) and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1; Borner et al., 2000; Lee et al., 2000; Moon et al., 2003). Both FT and SOC1 are activated by the photoperiodic protein CONSTANS (CO) and are repressed by FLOWERING LOCUS C (FLC), a negative regulator of the autonomous and vernalization pathways (for review, see Lee and Lee, 2010).

In recent years, the UPS has been found to contribute to the regulation of flowering time mainly by regulating the accumulation and stability of CO, GIGANTEA (GI), and FLC. The evening stability and morning instability of CO are affected by the blue light receptor CRYPTOCHROME2 and the light receptor PHYTOCHROME B, respectively (Valverde et al., 2004; Liu et al., 2008). Furthermore, the stability of the CO protein is controlled by the 26S proteasome, because the degradation of CO in the morning and in the dark is inhibited by proteasome inhibitors (Valverde et al., 2004). Intriguingly, the photomorphogenesis-related RING finger protein CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) was identified as the E3 ligase responsible for the degradation of CO in the dark (Liu et al., 2008), while another RING finger E3 ligase, HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1, was recently determined to interact synergistically with COP1 and to negatively regulate CO abundance, especially during the day (Lazaro et al., 2012). The stability of GI, a circadian-associated protein and promoter of CO, is also mediated by COP1, which acts with the clock-associated protein EARLY FLOWERING3 (ELF3) to regulate circadian function and photoperiodic flowering by degrading GI (Yu et al., 2008). GI stability is also mediated by COP1, which acts with the clock-associated protein ELF3 to regulate circadian function and photoperiodic flowering by degrading GI (Yu et al., 2008). In addition, two E2 ubiquitin-conjugating enzymes (UBC), AtUBC1 and AtUBC2, contribute to flowering time regulation by working with the E3 ligases HISTONE MONOUBIQUITINATION1 (HUB1) and HUB2 to monoubiquitinate the histone protein H2B; monoubiquitination of H2B is required for the transcriptional activation of FLC expression in Arabidopsis (Cao et al., 2008; Xu et al., 2009). Taken together, these studies have revealed a pivotal role for the UPS in flowering time regulation.

CONVERGENCE OF DEFENSE AND FLOWERING IN ARABIDOPSIS

As discussed above, defense and flowering are two distinct signaling pathways in plants. Interestingly, emerging evidence suggests that the two pathways are connected through the SA pathway. SA is an essential hormone for plant disease resistance (for review, see Vlot et al., 2009) and plant growth and development (Martínez et al., 2004; Wada et al., 2010; Rivas-San Vicente and Plasencia, 2011). *SIZ1*, which encodes a SUMO E3 ligase in Arabidopsis, suppresses SA- and PAD4-mediated signaling to regulate innate immunity (Lee et al., 2007a, 2007b). The *siz1* mutant flowers early under short-day (SD) conditions with elevated SA levels. Further study indicated that SIZ1 regulates flowering by operating a SA-dependent floral promotion pathway and repressing FLD activity through sumoylation to promote FLC expression (Jin and Hasegawa, 2008; Jin et al., 2008). Recently, HOPW1-1-INTERACTING3 (WIN3) was also found to regulate both plant innate immunity and flowering time in Arabidopsis (Wang et al., 2011). Among its multiple functions, WIN3 confers broad-spectrum disease resistance to biotrophic and necrotrophic pathogens, modulates cell death in the SA signaling mutant *accelerated cell death6-1*, and contributes to flg22-induced PTI (Wang et al., 2011). Additionally, WIN3 negatively modulates flowering under long-day (LD) conditions via the regulation of FLC and FT (Wang et al., 2011). These data indicated that WIN3 might be one of the connecting points for plant defense and flowering signaling pathways.

THE FUNCTIONS OF SPL11 AND PUB13 IN PCD AND DEFENSE

The rice LM mutant *spl11*, which was identified from an ethyl methanesulfonate-mutagenized population of IR68, is genetically controlled by a recessive gene (Singh et al., 1995; Yin et al., 2000). Disease resistance evaluations indicated that *spl11* confers enhanced non-race-specific resistance to both *X. oryzae* pv *oryzae* and

M. oryzae. When the LMs develop, the resistance is correlated with a constitutive activation of defense-related genes, including pathogenesis-related (*PR*) genes (*PR1*, *PBZ1*, *chintinaseIII*), oxalate oxidase genes involved in the production of ROS (*HvOxOa*, *HvOxOLP*), and genes encoding peroxidases (*POX8.1*, *POX22.3*; Yin et al., 2000). Subsequent comparison of the global transcripts between the wild-type and *spl11* plants in three types of leaf tissues with different lesion-development phenotypes revealed that the *spl11* mutation causes significant changes in the rice transcriptome (Zeng et al., 2006). Over 300 genes are highly induced in the fully expanded leaves of *spl11*, and nearly half are classified as oxidative stress/cell death- or defense-related genes. These results suggested a strong correlation between the non-race-specific resistance and the constitutive activation of genome-wide defense signaling in the *spl11* mutant. In addition, Kojo et al. (2006) showed that the ROS accumulation induced by elicitors is significantly suppressed by the NADPH oxidase (NOX) inhibitor imidazole in *spl11*, suggesting that SPL11 is important for the regulation of NOX-mediated ROS generation.

The *Spl11* gene was mapped on chromosome 12 (Zeng et al., 2002) and subsequently cloned by a map-based strategy (Zeng et al., 2004). Protein sequence analysis revealed that *Spl11* encodes a U-box and armadillo (ARM) repeat domain E3 ligase protein, which is a member of the PUB-ARM family (Zeng et al., 2004, 2008). The protein is localized in both the nucleus and cytoplasm. An E3 ligase activity assay indicated that the intact U-box domain is essential for SPL11 E3 ligase activity (Zeng et al., 2004).

To search for other components in the rice SPL11-mediated signaling pathway, we used the ARM domain in SPL11 as the bait in a yeast two-hybrid screen and identified eight SPL11-interacting proteins (SPINs; Vega-Sánchez et al., 2008). Among them, SPIN6 is a putative small GTPase-activating protein (GAP) and interacts with SPL11 in vivo (J.L. Liu and G.-L. Wang, unpublished data). GAP hydrolyzes the active GTP-bound small G protein to inactive the GDP-bound state, which leads to the activation of the cycle of small GTPase-mediated GTP-GDP exchange and downstream signaling (Tcherkezian and Lamarche-Vane, 2007). In rice, a small GTPase protein named OsRAC1 has been implicated in NOX-mediated ROS generation signaling (Kawano et al., 2010). Interestingly, the *spl11* mutant is defective in the regulation of the activity of NADPH oxidases (Kojo et al., 2006). We also found that *OsRac1* expression is induced in the *Spin6* RNA interference and knockout mutant plants and that *Spin6* expression is down-regulated in the *spl11* mutant (J.L. Liu and G.-L. Wang, unpublished data). These results suggested that SPL11/SPIN6 may regulate NOX-mediated ROS accumulation through OsRAC1. It will be interesting to see whether there is a direct interaction between the SPL11/SPIN6 complex and the OsRAC1-associated defensome and/or between the GAP protein SPIN6 and the small GTPase OsRAC1 in regulating ROS generation and immunity signaling. In addition, six suppressors of *spl11*-mediated lesion development have been identified. These mutants show reduced or no lesion development in the *spl11* background (G. Shirsekar and G.-L. Wang, unpublished data). Three have been molecularly mapped and phenotypically characterized. The reduction or elimination of lesions in *spl11* is closely related to the disease resistance level and defense gene expression. The SA level is significantly lower in the *spl11* suppressors than in the *spl11* plants, indicating that SA might play a role in the regulation of cell death and defense in rice. Cloning of these genes will provide new insight into the SPL11-mediated defense signaling pathway.

Among the 61 putative *PUB* genes in Arabidopsis (Zeng et al., 2008), *PUB13* is the closest ortholog of the rice U-box ligase gene *Spl11*. PUB13 has 73% identity in amino acids with SPL11 and also contains a U-box/ARM structure with E3 ligase activity. We recently characterized the *PUB13* gene and found that disruption of the gene by T-DNA insertion results in spontaneous cell death and accumulation of hydrogen peroxide (H_2O_2) and SA. Consistent with the phenotypes of *spl11*, the *pub13* mutant plants showed elevated resistance to biotrophic or hemibiotrophic pathogens but increased susceptibility to necrotrophic pathogens (Li et al., 2012a). We also found that cell death is suppressed in the *pub13sid2-2* double mutant compared with the clear cell-death phenotype in *pub13* under LD and high-humidity conditions. When another SA-deficient mutant, *pad4*, is crossed with *pub13*, the cell death in the *pub13pad4* double mutant is also suppressed under both conditions. These results demonstrated that the increase in cell death and H_2O_2 accumulation in *pub13* depends on the SA signal. In addition, the LM cell-death phenotype of *pub13* is partially rescued to the wild-type level after the overexpression of *Spl11* under LD and high-humidity conditions, indicating that *PUB13* and *Spl11* are functionally conserved in regulating cell death and defense.

Remarkably, Lu et al. (2011) recently reported that the PRR FLAGELLIN-SENSING2 (FLS2), a LRR receptor-like kinase (RLK), is a target of the E3 ligases PUB12 and PUB13 via the UPS. When induced by the bacterial PAMP elicitor flagellin, FLS2 is polyubiquitinated and degraded by PUB12/13. Moreover, the FLS2-PUB12/13 association is required for a PUB12/13 phosphorylation targeted by the LRR-RLK BRASSINOSTEROID-INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE1 (BAK1), an important component in the plant hormone brassinosteroid receptor BRI1-mediated signaling. These results revealed a significant role for E3 ubiquitin ligase PUB12/13-mediated protein degradation in plant PTI signaling.

Environmental factors such as light, temperature, and humidity affect the lesion development of LM mutants (Roden and Ingle, 2009; Mosher et al., 2010; Alcázar and Parker, 2011). In rice *spl11* mutant plants, LM formation is greater under SD conditions than under LD conditions (Fig. 2A). Conversely, cell-death

phenotypes are enhanced in *pub13* under LD conditions (Fig. 2, C and D), suggesting that lesion development in both *spl11* and *pub13* depends on light period or the circadian clock. Additionally, *pub13* exhibits increases in cell death, H_2O_2 accumulation, and resistance to biotrophic and hemibiotrophic pathogens after pretreatment with high humidity (Li et al., 2012a). Similarly, LM formation is accelerated when the *spl11* mutant is kept in a growth chamber with high humidity and under strong sunlight in a greenhouse in summer. These results suggested that both SPL11 and PUB13 are involved in responses to multiple environmental cues.

SPL11 REGULATES FLOWERING TIME VIA SPIN1

In addition to its function in cell death and defense regulation, SPL11 is also involved in regulating flowering time (Vega-Sánchez et al., 2008). The *spl11* mutant shows a delayed-flowering phenotype only under LD conditions. In contrast, the *spl11* suppressors partially recover the delayed flowering under the same conditions, suggesting that SPL11 is likely involved in flowering time regulation. Direct evidence for SPL11 involvement in flowering came from the analysis of the *Spin1* gene, which encodes a nuclear, RNA/DNA-binding protein that is a member of the STAR (for signal transduction and activation of RNA) family. SPL11 interacts with SPIN1 in yeast and in planta and monoubiquitinates but does not degrade SPIN1 (Vega-Sánchez et al., 2008). SPL11 negatively regulates *Spin1* expression during the light phase under both SD and LD conditions (Vega-Sánchez et al., 2008). Overexpression of *Spin1* causes late flowering under both SD and LD conditions in the growth chamber and field (Fig. 2B). SPIN1 represses flowering time via transcriptional perturbation of *Heading date3a* (*Hd3a*), which is daylength independent (Vega-Sánchez et al., 2008). These results demonstrated that SPL11 controls flowering time through the monoubiquitination of the flowering suppressor SPIN1 and that ubiquitination and RNA metabolism are linked (via the interaction between SPL11 and SPIN1) in the regulation of flowering time.

To identify the target(s) of SPIN1 in the regulation of flowering time, we performed a yeast two-hybrid screen using SPIN1 as the bait and found another RNA-binding protein, named RBS1 (for RNA-binding and SPIN1-interacting1; Y. Cai and G.-L. Wang, unpublished data). Preliminary analysis of the overexpression and RNA interference lines of *Rbs1* revealed that it also positively regulates flowering time (Y. Cai and G.-L. Wang, unpublished data). Based on this result and those mentioned above, we speculate that SPL11 might enter into the nucleus to form a protein complex with the nuclear proteins SPIN1 and RBS1 and that the modification of these two proteins by SPL11 can alter the flowering signaling pathway in rice.

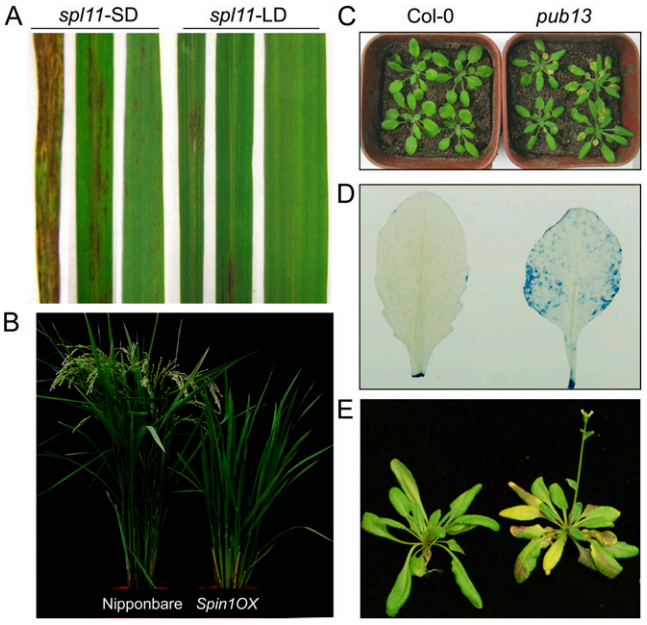

Figure 2. Phenotypes of *spl11*, the *Spin1* overexpression line, and *pub13*. A, Cell-death phenotypes of *spl11* under SD (10 h of light and 14 h of dark) and LD (14 h of light and 10 h of dark) conditions in growth chambers. B, Flowering phenotype of *japonica* rice 'Nipponbare' Nipponbare and *Spin1* overexpression (OX) plants under natural LD conditions (13 h of light and 11 h of dark). C and D, Cell death in *pub13* under LD conditions (16 h of light and 8 h of dark) in a growth room. Col-0 (ecotype Columbia) is the wild-type control. Trypan blue staining was used to detect the cell death of Col-0 and *pub13* in D. E, Flowering phenotype of *pub13* under LD conditions in a growth room. Both Col-0 and *pub13* were 32 d old.

PUB13 NEGATIVELY REGULATES FLOWERING TIME IN A PHOTOPERIOD- AND SA-DEPENDENT MANNER

We recently found that PUB13 is also involved in the regulation of flowering time in Arabidopsis (Li et al., 2012a). Unlike the rice mutant *spl11*, which has a delayed-flowering phenotype under LD conditions (Vega-Sánchez et al., 2008), the Arabidopsis mutant *pub13* displays an early-flowering phenotype under LD or middle-day conditions (Li et al., 2012a). The opposite functions of PUB13 and SPL11 in flowering time control are probably caused by differences in the photoperiodism of Arabidopsis (a LD plant) and rice (a SD plant) or by the divergence in molecular mechanisms controlling flowering in these two types of photoperiodic plants. For instance, GI is a promoter of flowering time in Arabidopsis, while its ortholog in rice, OsGI, is a suppressor of flowering time under both SD and LD conditions (Fowler et al., 1999; Hayama et al., 2003). In addition, CO is a positive regulator of flowering time under SD or LD conditions in Arabidopsis, but its ortholog in rice, Hd1, functions as a negative regulator of flowering time under LD

conditions but as a positive regulator under SD conditions (Yano et al., 2000). Intriguingly, overexpression of *Spl11* in *pub13* can complement the early-flowering phenotype of *pub13* (Li et al., 2012a, 2012b), suggesting that the function of these two E3 ligases, PUB13 and SPL11, in flowering time control is conserved and that the E3 ligase activity of the two homologous proteins may be similar in dicot and monocot plants.

Plant hormones determine many biological processes, such as growth, development, and responses to environmental stresses (for review, see Depuydt and Hardtke, 2011). The hormone SA is not only pivotal in plant responses to biotic stress but is also involved in flowering (for review, see Martínez et al., 2004). SA probably controls flowering time through the photoperiod and vernalization flowering pathways that are independent of FLC, CO, and FCA. The SA level is elevated in *pub13*, but once the increased level of SA in *pub13* is reduced by knockout of the two important components (*SID2* and *PAD4*) in the SA pathway, the early-flowering phenotype in *pub13* is restored to the wild-type level (Li et al., 2012a). These results suggested that PUB13 regulates flowering time mainly through an SA-dependent pathway. The function of SA in flowering control in *pub13* is consistent with previous reports that SA accelerates the transition from the vegetative to the reproductive phase by suppressing the floral repressor genes and activating the positive regulator of flowering (Martínez et al., 2004).

PUB13 INTERACTS WITH HFR1, A POSITIVE REGULATOR OF PHOTOMORPHOGENESIS

It is clear that SPL11 regulates flowering time through SPIN1 in rice (Vega-Sánchez et al., 2008). To identify a SPIN1-like ortholog in Arabidopsis, we performed a yeast two-hybrid screen using the PUB13 mutant protein PUB13^{V273R}, an E3 activity-compromised mutant, as the bait because we did not find any SPIN1-like interactor when the full-length PUB13 was used (Li et al., 2012b). One of the interacting proteins identified in the screen is LONG HYPOCOTYL IN FAR-RED LIGHT1 (HFR1), which encodes a basic helix-loop-helix-type transcription factor (Duek et al., 2004). The interaction between PUB13 and HFR1 was confirmed by a glutathione *S*-transferase pull-down assay (Li et al., 2012b). HFR1 promotes photomorphogenesis and is ubiquitinated and degraded by the E3 ligase COP1 in darkness, a protein that is involved in photomorphogenesis, plant growth, flower shape, and flowering time (Jang et al., 2008). How PUB13 regulates HFR1 to control flowering time and whether the regulation is associated with the COP1 protein complex are being investigated.

WORKING MODELS FOR SPL11- AND PUB13-MEDIATED REGULATION OF DEFENSE AND FLOWERING AND FUTURE RESEARCH DIRECTIONS

In the last 10 years, we have obtained considerable information on the function of SPL11 and PUB13 in the regulation of PCD, defense, and flowering in model monocot and dicot plants. Based on our results and those from other studies, we have proposed working models to illustrate the function of SPL11 and PUB13 and their interacting proteins in the regulation of defense (Fig. 3) and flowering (Fig. 4) in both species. Although the biological functions of SPL11 and PUB13 are similar, the components participating in SPL11- and PUB13-mediated signaling have become diverse during the course of speciation (Table I). In rice, SPL11, a negative regulator of cell death, physically associates with the putative GAP protein SPIN6 to suppress NOX-mediated ROS generation and *PR* gene activation and thereby to inhibit the autoactivation of defense responses (Fig. 3A). GAP

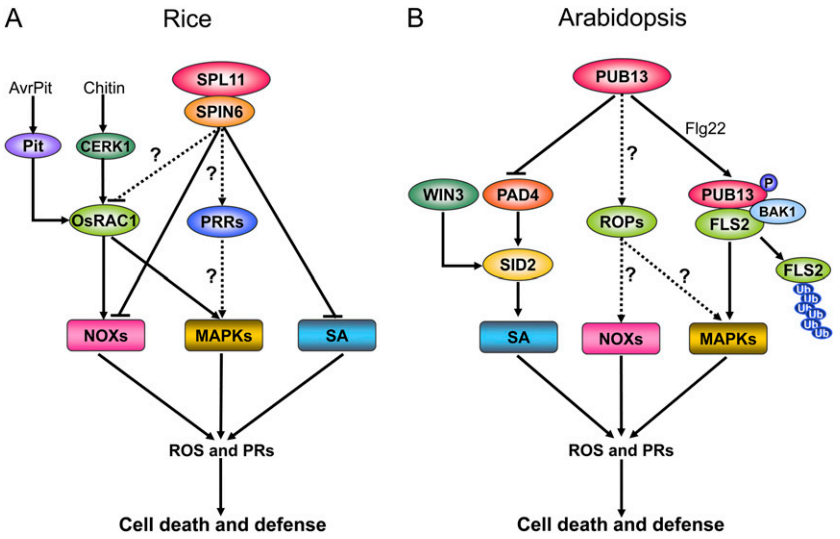

Figure 3. Proposed working models of cell death and defense signaling mediated by rice SPL11 (A) and Arabidopsis PUB13 (B). See details in the text.

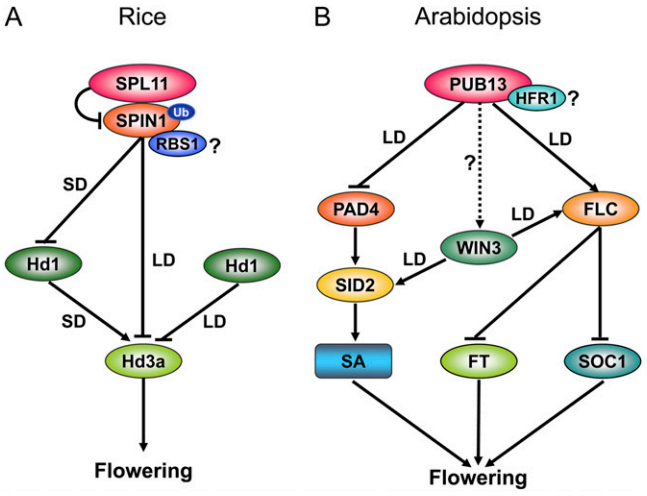

Figure 4. Proposed working models of flowering time regulation meditated by rice SPL11 (A) and Arabidopsis PUB13 (B). See details in the text.

regulates the activity of GTP-bound small GTPase proteins. The small G protein OsRAC1 is a signal integrator of OsCERK1-mediated PTI and Pit-mediated ETI signaling pathways (for review, see Kawano et al., 2010). Thus, we speculate that SPIN6 might biochemically inactive GTP-bound OsRAC1 to suppress the OsRAC1-mediated signaling and to prevent unnecessary cell death and defense activation when no pathogen is present. The SPL11 downstream defense signaling might be transduced to mitogen-activated protein kinase (MAPK) cascades and the NOX complex by OsRAC1. The SPL11 also negatively regulates SA accumulation, because mutation of *Spl11* elevates the SA level in the *spl11* mutant (Fig. 3A). In Arabidopsis, PUB13 negatively regulates the PAD4/SID2-dependent SA defense pathway and FLS2-mediated PTI signaling (Fig. 3B). SID2 is also regulated by WIN3, a type II regulator of the SA pathway (Wang et al., 2011). PUB13 interacts with BAK1 and is phosphorylated by BAK1. The PAMP elicitor flg22 stimulates PUB13 to associate with FLS2, an association that is required for the BAK1-mediated PUB13 phosphorylation. Polyubiquitination of FLS2 by PUB13 causes FLS2 degradation. After FLS2 is activated by flg22, the signal is transduced to the MAPK cascades to regulate ROS burst and *PR* gene expression and thereby to trigger cell death and defense responses. Because of the conserved function of SPL11 and PUB13 in defense, some FLS2-like PRRs might be targeted by SPL11 in rice or some OsRAC1-like proteins (called Rho-related GTPases from plants) might be targeted by PUB13 in Arabidopsis. These interesting questions remain to be answered in future work.

For flowering time regulation in rice, SPL11 monoubiquitinates SPIN1 and negatively regulates its activity (Fig. 4A). Under SD conditions, the suppression of SPIN1 by SPL11 promotes the H3a-dependent pathway by activating Hd1. Under LD conditions, SPIN1 and Hd1 may act additively to repress Hd3a, causing late flowering but high levels of SPL11 throughout the day in order to alleviate flowering repression. SPIN1 can also interact with RBS1 to regulate flowering time, but how the interaction affects flowering remains unknown. In Arabidopsis, however, PUB13 positively regulates the flowering suppressor FLC to delay flowering under LD conditions; FLC is a negative regulator of the flowering activator FT and SOC1 (Fig. 4B). PUB13 also physically associates with HFR1, a positive regulator of plant photomorphogenesis, but its role in PUB13-mediated flowering remains unclear. Furthermore, PUB13-mediated LD flowering also requires the PAD4/SID2-mediated SA pathway. Additionally, WIN3 positively regulates SID2-mediated SA signaling and the FLC-mediated flowering pathway under LD conditions (Wang et al., 2011). The relationship between PUB13 and WIN3 needs to be investigated.

Although much progress has been made in the dissection of the SPL11 and PUB13 pathways, several critical questions remain. How do the E3 ligases SPL11 and PUB13 regulate their substrates to modulate both defense and flowering? Is there an intimate connection between the SPL11/SPIN6 complex and the OsRAC1-mediated PTI and ETI defensome? Which genes are targeted by the SPL11/SPIN1-mediated RNA-processing complex in the regulation of flowering time? What is the relationship between SPL11/PUB13 and other hormone signaling pathways in plants? How does PUB13 regulate flowering through HRF1 and other COP1-associated proteins? Is PUB13 physically associated with resistance proteins, as has recently been demonstrated for FLS2 (Qi et al., 2011)? Answers to these questions will provide a better understanding of the dual functions of SPL11 and PUB13 in rice and Arabidopsis.

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