THE E3 UBIQUITIN LIGASE SPL11 REGULATES BOTH PROGRAMMED CELL
DEATH AND FLOWERING TIME IN RICE

DISSERTATION

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By

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ABSTRACT

The rice E3 ubiquitin ligase SPL11 was previously characterized as a negative regulator of both programmed cell death (PCD) and broad-spectrum disease resistance, suggesting its putative role in the ubiquitination/protein degradation machinery, or other ubiquitin-mediated pathway. A novel role for SPL11 in flowering time control was identified via its interaction with SPIN1 (SPL11-interacting protein 1), a nuclear, RNA/DNA binding protein and STAR (signal transduction and activation of RNA) family member. In addition to ubiquitinating SPIN1 in vitro, SPL11 downregulated the expression of Spin1 during the light phase to promote flowering. Overexpression of Spin1 inhibited flowering in both short and long days and affected Spl11 expression as well. These results link ubiquitination and RNA metabolism in flowering time control. Isolation of spl11-mediated cell death suppressor mutants (sds) provided additional evidence that PCD is under genetic control in the spl11 mutant. The sds mutants showed various levels of cell death suppression, had wild-type levels of resistance to bacterial blight, and repressed the elevated defense gene expression of spl11. The delayed flowering phenotype of spl11 in long days was partially repressed in sds plants independently of cell death suppression levels, suggesting that the PCD and flowering pathways partially overlap. Another RNA binding protein was identified in a yeast two-
hybrid screen designed to isolate SPIN1-interacting partners. Rbs1 (RNA binding and SPIN1-interacting 1) was shown to promote cell death when overexpressed in both *Nicotiana benthamiana* and rice. Rbs1 was upregulated in lesion mimic-expressing leaves of *spl11*, suggesting that it may contribute to the cell death phenotype. RBS1 may constitute a link between the flowering and PCD pathways controlled by SPL11. The data presented here provides the first evidence of a link between cell death control and the switch to reproductive development in plants.
Dedicated to my family
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PUBLICATIONS

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CHAPTER 1

INTRODUCTION

The role of ubiquitination in programmed cell death (PCD) and flowering time control in plants

Post-translational modifications are key factors in the regulation of protein activity and stability. In eukaryotes, protein alteration by the addition of ubiquitin, a small 76 amino acid polypeptide, rivals other essential processes such as transcription and protein kinase cascades in the regulation of cellular homeostasis (Vierstra, 2003; Moon et al., 2004). This fact has been recently acknowledged by the awarding of the 2004 Nobel Prize in Chemistry to the discoverers of ubiquitination. In plants, the ubiquitin-26S proteasome has been implicated in a variety of processes including cell cycle, circadian rhythm control, hormone signaling, senescence and disease resistance (Devoto et al., 2003; Itoh et al., 2003; Moon et al., 2004; Zeng et al., 2006).

Ubiquitination and the 26S proteasome

The ubiquitin-26S proteasome pathway is involved in the selective degradation of proteins in the cells of eukaryotic organisms (Glickman and Ciechanover, 2002). Target proteins are tagged by one or more ubiquitin molecules and ultimately directed to the 26S proteasome for proteolysis (Hatakeyama and Nakayama, 2003a). In a typical
ubiquitination reaction, ubiquitin-activating (E1), -conjugating (E2) and –ligase (E3) enzymes participate in the process of transferring the ubiquitin monomers to the ε-amino group of a lysine residue in the target protein (Hatakeyama and Nakayama, 2003a). In the first step in the reaction, E1 catalyzes the activation of ubiquitin via ATP creating a high-energy thiol intermediate. Following activation, ubiquitin is transferred to the E2, which then transfers it to the E3 ligase for the final step in the process involving the creation of an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine residue in the substrate protein (Hershko, 1983; Hatakeyama and Nakayama, 2003a).

The addition of a chain of ubiquitin molecules to a target protein via lysine-48 linkages in the ubiquitin monomers, known as polyubiquitination, is generally recognized by the 26S proteasome as a signal for degradation (Thrower et al., 2000).

The 26S proteasome is a multi-protein component that has proteolytic activities (Voges et al., 1999). It is formed by the 20S, barrel-shaped, core particle and two subunits of the 19S complex at each end of the 20S particle (Voges et al., 1999; Hatakeyama and Nakayama, 2003a). The 19S portion represents the regulatory subunit involved in the recognition, deubiquitination and unfolding of target proteins, whereas the 20S core particle is the catalytic unit with proteolytic function (Groll et al., 1997; Voges et al., 1999; Hatakeyama and Nakayama, 2003a). In plants, similar proteasome architecture has been demonstrated (Yang et al., 2004), which indicates conservation of the protein degradation machinery across kingdoms.

Although a major function of ubiquitination is the degradation and removal of proteins via the 26S proteasome pathway, there is increasing evidence for a non-proteasomal role as well. As mentioned above, polyubiquitination via lysine-48 linkages
of ubiquitin is generally associated with proteasomal degradation (Thrower et al., 2000). However, monoubiquitination has been shown to be required for entry of cargo protein into the secretory pathway (Hicke, 2001) and polyubiquitination via lysine-63 linkages is associated with cellular functions as diverse as DNA damage repair and ribosomal protein synthesis (Pickart and Fushman, 2004). Other functions of ubiquitination have been elucidated in transcription, endocytosis and replication (Mukhopadhyay and Riezman, 2007) arguing for diverse regulatory functions of ubiquitin pathways beyond protein degradation.

**E3 ligases and the importance of ubiquitination in plants**

A preliminary inventory of ubiquitin-26S proteasome components in the *Arabidopsis thaliana* genome revealed that approximately 5% of the proteome is devoted to this pathway representing almost nine times and more than double that of the yeast and *Drosophila* components, respectively (Vierstra, 2003). Such a number of genes imply that ubiquitination is a major player in the life cycle of plants.

Of the more than 1300 loci in the *A. thaliana* genome that are involved in the ubiquitin-26S proteasome pathway, roughly 1200 encode for E3 ligases (Vierstra, 2003). E3 ubiquitin ligases confer substrate specificity to the pathway and can be divided into five classes based on their mode of action and domain characteristics: HECT (for Homology to E6-AP C-terminus), SCF (for SKP1-CDC53-F-box), RING/U-Box, APC (for Anaphase-Promoting Complex) and CUL3-BTB (for Broad-complex, Tramtrack, Bric-a-Brac) types (Hatakeyama and Nakayama, 2003b; Vierstra, 2003; Moon et al.,
2004). The HECT, RING finger and U-Box classes represent single subunit E3 ligases, while SCF, APC and CUL3-BTB form multi-subunit E3 complexes (Moon et al., 2004).

The HECT proteins are unique among the E3 ligases because they can form a covalent bond with ubiquitin prior to transfer to the target (Hatakeyama and Nakayama, 2003b). In plants, HECT are known as UPL, for Ubiquitin Protein Ligase (Moon et al., 2004). The RING finger proteins are characterized by a domain capable of binding zinc as well as for interacting with the E2 enzyme (Jackson et al., 2000). Interestingly, all multisubunit E3 ligases contain a RING finger protein as part of their complex. The SCF complexes are composed of four proteins that together form a functional E3 ligase: SKP1 (or ASK in plants), CDC3 (also known as Cullin), F-Box, and RBX1. The RBX1 subunit is a RING finger protein, SKP1 binds to the F-Box, which selects the target protein, and Cullin acts as a scaffold to bring together RBX1 to the SKP1-F-box unit (Deshaies, 1999). CUL3-BTB also contains RBX1 and a Cullin subunit but form complexes with a protein containing a BTB domain (Figueroa et al., 2005). The APC are E3 ligases composed of 11 subunits of which two, APC2 and APC11, are homologous to Cullin and a RING finger protein, respectively (Tang et al., 2001). More recently, a new family of E3 enzymes containing a U-box domain, which is a RING finger-like motif lacking the characteristic metal chelating residues, has been identified (Azevedo et al., 2001; Hatakeyama and Nakayama, 2003b). Unlike the HECT E3s, RING and U-box proteins do not form an intermediate ubiquitin-E3 complex during transfer of the activated ubiquitin to the target (Jackson et al., 2000).

As the vast majority of the ubiquitination machinery in plants includes E3s, it is not surprising that most of the advances in the field have come from the functional
characterization of E3 ligases and their targets in the context of various developmental processes. Of specific interest for this dissertation is the role of a unique rice U-box E3 enzyme, SPL11, which was previously shown to negatively regulate both programmed cell death (PCD) and broad-spectrum disease resistance (Yin et al., 2000; Zeng et al., 2004). *Spl11* is one of a handful of plant U-box genes characterized to date and the first one to directly show a link between the ubiquitination process and PCD control. The study of ubiquitination pathways in the regulation of PCD and flowering time (see chapter 2 for the latter) is in its infancy. The remainder of this chapter will deal with a review of recent advances made in the last five years aiming at elucidating how ubiquitination participates in regulating these two key processes in plants.

**An overview of PCD in plants**

PCD entails the participation of the cellular machinery in destroying itself and requires active metabolism (Jones, 2001). In multicellular organisms, PCD is an essential mechanism to ensure proper development and appropriate responses to biotic and abiotic challenges. In plants, PCD occurs at various stages of development such as tracheary element formation (Fukuda, 2000), aleurone and root cap cell disposal, or senescence (Pennell and Lamb, 1997). In response to pathogen attack, a form of PCD termed the hypersensitive response (HR) is activated in resistant plants (Heath, 2000; Greenberg and Yao, 2004). Environmental stimuli such as hypoxia (Pennell and Lamb, 1997) or exposure to ozone (Hoeberichts and Woltering, 2003), for example, also trigger PCD in plants. How and when a plant commits its cell for suicide is an intensive area of research
that, despite recent breakthroughs, is not yet well characterized as it is in animals (van Doorn and Woltering, 2005; Della Mea et al., 2007).

A recent review on mechanisms of PCD recognizes that autophagy and non-lysosomal cell death are the most frequent types of PCD observed in plants (van Doorn and Woltering, 2005). Autophagy is the engulfing of cytoplasmic contents by autophagosomes, which are vesicles that are then taken to the vacuole or to lysosomes for degradation (van Doorn and Woltering, 2005; Patel et al., 2006). Non-lysosomal PCD is broadly defined as a cell death mechanism that does not involve lysosomal degradation but rather activates cell death by either unknown mechanisms or by membrane destabilization (van Doorn and Woltering, 2005). Most developmental forms of plant PCD show morphological features that resemble different forms of autophagy, while most of the ultrastructural hallmarks of HR cell death seem to be related to non-lysosomal PCD (van Doorn and Woltering, 2005). However, silencing of the autophagy-related gene Beclin-1 in plants infected with a pathogen that triggers the HR caused uncontrollable spread of cell death to non-infected tissue and compromised the induction of autophagosomes in HR sites (Liu et al., 2005). These results suggest that autophagy is required to prevent the spread of HR cell death in plants and that autophagic cell death is also observed during the HR (Liu et al., 2005; Patel et al., 2006). Due to the different roles of PCD in plants, cell death mechanisms vary according to the context. This is clearly apparent by the observation of distinct ultrastructural cellular features and PCD markers in dying cells. For example, during the formation of the xylem in plants the vacuole collapses, organelles are degraded and protoplasts are removed but the secondary cell walls are thickened (Fukuda, 2000), whereas during aerenchyma formation cell walls
are completely disintegrated (van Doorn and Woltering, 2005). On the other hand, it has been suggested that two universal features in plant PCD are the involvement of Ca^{2+} fluxes and the collapse of the vacuole (Jones, 2001). An increase in cytosolic Ca^{2+} is an early event in HR cell death (Heath, 2000) as well as in xylogenesis and senescence (Hoeberichts and Woltering, 2003), while vacuole collapse assures that hydrolase enzymes that are stored in it are released in a timely manner to process the cellular corpse (Jones, 2001). Thus, both shared and unique mechanisms exist in the execution and manifestation of cell death pathways in plants.

Plant hormones have been shown to regulate, either positively or negatively, different PCD pathways (Kuriyama and Fukuda, 2002). For example, ethylene mediates senescence in flowers triggered after pollination (Rogers, 2006), the death of barley aleurone cells is promoted and antagonized by giberellic acid (GA) and abscisic acid (ABA), respectively (Hoeberichts and Woltering, 2003), and brassinosteroids (BA) induce both PCD and secondary cell wall formation in differentiating tracheary elements (Kuriyama and Fukuda, 2002). In the resistance response upon pathogen recognition, salicylic acid (SA) has been extensively characterized as an important signaling molecule in both HR cell death and in the establishment of systemic acquired resistance (SAR) (Shirasu and Schulze-Lefert, 2000; Hoeberichts and Woltering, 2003). The involvement of hormones in PCD control in plants argues that different cell death signaling pathways are activated. Elucidating the convergent and divergent points between these pathways is an ongoing challenge in plant science. In addition to hormones, reactive oxygen species (ROS), ion fluxes and nitric oxide have also been implicated as possible signals in plant PCD, especially during the HR (Shirasu and Schulze-Lefert, 2000; Hoeberichts and
Woltering, 2003; Greenberg and Yao, 2004). Interestingly, studies of the mammalian NADPH-oxidase subunit homologs in *Arabidopsis* (*Atrboh*) have convincingly shown that ROS actually prevent, rather than promote, the spread of cell death during the HR (Torres and Dangl, 2005; Torres et al., 2005). This finding contradicts earlier dogma that stated that ROS during infection are executioners of PCD in plants (Heath, 2000). It is possible, however, that ROS can be involved in both promotion and prevention of cell death in plants and that additional oxidases in plants, other than the *Atrboh* family members, are responsible for PCD execution (Torres et al., 2005).

The role of proteases in plant PCD has also been documented (Hoeberichts and Woltering, 2003). For example, in soybean cell cultures, cysteine proteases are activated after oxidative stress treatment that induced PCD (Solomon et al., 1999). In animals, a hallmark of apoptosis, a type of PCD, is the induction of cysteine proteases named caspases (Hoeberichts and Woltering, 2003; Della Mea et al., 2007). As no true apoptosis occurs in plants, direct homologs of caspases have not been found in any plant species (van Doorn and Woltering, 2005). However, peptide inhibitors of caspases were shown to inhibit the HR in plants in response to a bacterial pathogen (del Pozo and Lam, 1998). More recently, a vacuolar protease, VPE, was shown to provide caspase-1 activity in tobacco infected with tobacco mosaic virus (TMV) (Hatsugai et al., 2004). Silencing of *VPE* not only abolished HR cell death in response to TMV infection but prevented vacuolar collapse, directly implicating a vacuolar protease in plant PCD (Hatsugai et al., 2004). These results suggest that HR cell death functions through caspase-like activities.

Many mutants with spontaneous lesion formation or constitutive cell death have been isolated in plants (Walbot et al., 1983; Yin et al., 2000; Lorrain et al., 2003). Lesion
mimics will be discussed in more detail in chapter 3 but in general terms, the study of these lesion mimics has been instrumental in understanding the molecular basis of PCD in plants as many of the genes responsible for the mutant phenotypes can represent regulators of cell death pathways. For example, the *Arabidopsis LSD1* gene, encoding for a zinc finger protein (Dietrich et al., 1997), is a negative regulator of a pro-death pathway mediated by SA (Torres et al., 2005). Another lesion mimic gene, *ACD5*, encodes for CERK, a kinase that is responsible for the phosphorylation of C2 ceramides to attenuate cell death, implicating ceramides as modulators of PCD in plants (Liang et al., 2003).

**Ubiquitination and PCD in plants**

The ubiquitin-26S proteasome pathway has a well-established role in regulating apoptosis, a type of PCD in animals (Jesenberger and Jentsch, 2002; Yang and Yu, 2003). Both positive and negative regulatory functions of ubiquitination have been unraveled in mediating apoptosis. For example, proteasomal degradation of the pro-apoptotic protein Bax is associated with survival of human cancer cells (Li and Dou, 2000), whereas the autoubiquitination and subsequent degradation of some inhibitor of apoptosis proteins (IAPs) that contain a RING finger domain is required for the activation of PCD in *Drosophila melanogaster* (Jesenberger and Jentsch, 2002; Vaux and Silke, 2005). Interestingly, caspases have been recently shown to cleave several subunits of the 19S regulatory particle, which suggests that inactivation of the proteasome is a mechanism to amplify apoptosis (Sun et al., 2004). Similarly, an increasing body of evidence implicates the proteasome and several E3 ligases in the control of PCD in plants.
Roles of the 26S proteasome in plant PCD

In *Nicotiana benthamiana* silencing of two different subunits of the 26S proteasome induces cell death (Kim et al., 2003). Several markers of PCD were observed including nuclear condensation, DNA fragmentation and cytochrome c release from mitochondria (Kim et al., 2003). ROS and some defense-related genes were also induced, but not the typical HR cell death markers such as PR1 (Kim et al., 2003). In a follow-up experiment, microarrays were used to compare the cell death pathways induced during the HR and by proteasome inhibition (Kim et al., 2006). Only a small subset of genes were upregulated in both treatments, notably genes encoding some proteases and anti-oxidant enzymes, which indicated that the two PCD pathways appeared to be differentially regulated with only minor overlap (Kim et al., 2006). Further support for the notion that the proteasome has anti-PCD activities has been recently shown by overexpressing a ubiquitin mutant that is unable to form polyubiquitin chains in plants (Schlogelhofer et al., 2006). PCD was activated in plants expressing this ubiquitin variant, but the cell death was not associated with increased ROS and was SA-independent (Schlogelhofer et al., 2006). In addition to the anti-cell death functions, the proteasome has also been associated as a positive regulator of developmental PCD. One of the first examples linking ubiquitination to PCD in plants was the finding that proteasome inhibitors such as MG132 prevented the differentiation of tracheary elements in *Zinnia* mesophyll cells (Woffenden et al., 1998), suggesting a positive role of the proteasome in xylogenesis. In *Arabidopsis*, the F-box E3 ligase ORE9 is involved in the progression of the senescence program mediated by several hormones as the *ore9* mutant.
shows enhanced leaf longevity and delayed senescence when treated with ethylene, ABA and jasmonic acid (Woo et al., 2001). Thus, it seems apparent that the ubiquitin-26S proteasome has both positive and negative regulatory roles in plant PCD depending on the cellular and physiological context.

*E3 ligases and HR/PCD in plants*

One of the first studies linking ubiquitination to the plant defense response and the HR came from the identification of the *Arabidopsis* homolog of yeast *Sgt1*. In yeast, SGT1 is a key protein involved in cell cycle regulation and it interacts with the SKP1 component of SCF E3 ligases (Kitagawa et al., 1999). Plant SGT1 also interacts with SCF components (Azevedo et al., 2002) and is required for Resistance (R) gene-mediated disease resistance signaling (Austin et al., 2002; Azevedo et al., 2002; Tor et al., 2002; Muskett and Parker, 2003). Indeed, the RPP5 R protein-mediated HR in response to avirulent *Peronospora parasitica* is significantly delayed and reduced in *sgt1b* mutants and is observed as a trailing cell death (Austin et al., 2002; Muskett and Parker, 2003). However, direct evidence on how plant SGT1 functions to regulate HR and other defenses through ubiquitination is still lacking.

Identification and characterization of SPL11 as a U-box protein with E3 ligase activity provided the first direct evidence that ubiquitination controls resistance and PCD in plants (Zeng et al., 2004). The *spl11* mutation causes a premature stop codon in the first exon of the *Spl11* gene (Zeng et al., 2004) and is characterized by spontaneous cell death in leaves and enhanced disease resistance to a bacterial and a fungal pathogen in rice (Yin et al., 2000). Thus, SPL11 is a negative regulator of PCD in plants, which is in
agreement with the negative role of the proteasome in cell death control (see above). More recently, a set of E3 ligases identified as rapidly induced genes in Avr9-treated Cf-9 tobacco cell cultures have been identified as positive regulators of the HR (Gonzalez-Lamothe et al., 2006; Yang et al., 2006; van den Burg et al., 2008). Avr9 is an effector from the leaf mold pathogen Cladosporium fulvum that is recognized by the R protein Cf-9 in tomato or transgenic tobacco plants (Durrant et al., 2000). In Cf-9 N. benthamiana plants, silencing and overexpression of tobacco CMPG1, encoding a U-box protein, reduced and enhanced the HR triggered by Avr9 infiltration, respectively (Gonzalez-Lamothe et al., 2006). Silencing of another U-box-containing E3 ligase, ACRE276, also compromised the HR in this system (Yang et al., 2006). Importantly, a mutant Arabidopsis homolog protein lacking ubiquitination activity was unable to restore the HR in tobacco plants silenced for ACRE276, demonstrating that the E3 ligase activity is necessary for HR induction (Yang et al., 2006). Similarly, silencing of the F-box protein ACIF1 compromised the HR triggered by different elicitors and by activation of different R genes in tobacco plants (van den Burg et al., 2008). Another pair of E3 ligases, the Arabidopsis RING finger proteins RIN2 and RIN3 have been implicated in signaling through two different R proteins since a double rin2 rin3 mutant shows reduced RPM1 and RPS2-dependent HR (Kawasaki et al., 2005). Further characterization of these PCD-related E3 ligases will shed light on the molecular mechanisms underlying HR cell death in plants. Most notably, the identification of their substrates is of major importance to understanding their roles in HR/PCD regulation, as no ubiquitination target for any plant E3 ligase involved in PCD has been isolated to date.
Overview of flowering time regulation in plants

Flowering at the right time is critical for successful sexual reproduction and the ensuing development of seeds and fruits in plants. The control of flowering is achieved by the perception of both environmental cues and endogenous factors followed by the integration of such information in signal transduction cascades that lead to the switch from vegetative to reproductive development. In *Arabidopsis*, four different flowering pathways have been characterized (Simpson and Dean, 2002; Boss et al., 2004; Putterill et al., 2004). The autonomous and giberellin pathways perceive and transduce internal signals to promote flowering (King and Evans, 2003; Simpson, 2004). External stimuli, such as seasonal variations in daylength and temperature, mediate signal transduction in the photoperiodic and vernalization pathways, respectively (Henderson and Dean, 2004; Searle and Coupland, 2004). Ultimately, signaling in all pathways converge at the floral pathway integrators, a group of genes that are turned on or off in a manner that is concomitant with the decision to flower (Simpson and Dean, 2002; Boss et al., 2004; Putterill et al., 2004). Among these flowering pathway integrators, the best characterized are FT (Kardailsky et al., 1999) and SOC1 (Borner et al., 2000; Lee et al., 2000). FT and SOC1 are targeted for both positive and negative regulation. For example, the photoperiodic protein CO (Putterill et al., 1995) activates FT to promote flowering (Samach et al., 2000; Suarez-Lopez et al., 2001), whereas the negative regulator of the autonomous and vernalization pathways, FLC (Michaels and Amasino, 1999), represses FT function to inhibit floral initiation (Boss et al., 2004; Henderson and Dean, 2004). Regulation of FT is a key process since it has been recently shown that this protein is the
'florigen' signal that moves from leaves to the shoot apex where it activates floral meristem identity genes that promote the transition to flowering (Corbesier et al., 2007). In this manner, plants are able to integrate various environmental and endogenous cues in the control of the flowering process.

In Arabidopsis, the photoperiodic pathway promotes flowering under long day (LD) conditions and delays it on short days (SD). In contrast, rice flowers earlier in SD but late in LD. Interestingly, despite the opposite response to photoperiod, several homologs of the Arabidopsis flowering genes exist in rice, which suggest an ancient origin for these loci, predating the split between dicots and monocots. Rice Hd1, Hd3a and Hd6 are orthologs of Arabidopsis CO, FT and the α subunit of kinase CK2, respectively (Takahashi et al., 1998; Yano et al., 2000; Kojima et al., 2002). However, the flowering promoter gene Ehd1 in rice seems to be specific for monocots as no Arabidopsis homolog has been identified (Doi et al., 2004). Similarly, no evidence for the floral repressor FLC has been found in the rice genome (Izawa et al., 2003; Tadege et al., 2003), which is consistent with the observation that an autonomous pathway has not been characterized in rice. Thus, both conserved and unique flowering time components exist between rice and Arabidopsis. In addition, one key difference in the regulation of photoperiodic floral induction between the two species is that in rice, Hd1 can be both a activator and a repressor of flowering, while Arabidopsis CO only promotes flowering (Onouchi et al., 2000; Izawa et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004). Indeed, in SD, Hd1 activates Hd3a but represses it in LD, which is a key factor in the adaptation of rice to SD conditions (Hayama et al., 2003).
Ubiquitination and flowering time control

As described above, daylength is a major regulator of flowering time. In *Arabidopsis*, one of the well-established modes of photoperiodic control of flowering is the regulation of *CO* mRNA and protein levels in what has been termed the external coincidence model. In this model, the circadian clock controls *CO* levels and high *CO* expression occurs in late afternoon; the coincidence of high *CO* levels in the light in LD triggers flowering by activation of *FT* (Thomas, 2006). In recent years, the role of ubiquitination in the regulation of *CO* accumulation has been elucidated by elegant molecular genetic studies.

*CO* abundance studies demonstrated that the protein is unstable in the morning and in the dark in LD (Valverde et al., 2004). Treatment of LD-grown seedlings with proteasome inhibitors stabilized *CO* both in the morning and in the dark, suggesting that *CO* is targeted for degradation via the 26S proteasome (Valverde et al., 2004). Recently, the E3 ligase responsible for the degradation of *CO* in the dark was identified as the photomorphogenesis-related RING finger protein COP1 (Liu et al., 2008). Interestingly, the light receptor Phytochrome B (PhyB) was shown to be responsible for *CO* protein instability in the morning (Valverde et al., 2004), while the blue light receptor Cryptochrome 2 (Cry2) contributed to the stabilization of *CO* in the evening and in the dark (Valverde et al., 2004; Liu et al., 2008). Thus, a role for Cry2 as antagonist of PhyB and COP1 has been suggested (Valverde et al., 2004; Liu et al., 2008). Cry2 is itself targeted for blue light-mediated phosphorylation and subsequent degradation via the 26S proteasome and functions in the nucleus as a blue light receptor to promote flowering.
in LD (Yu et al., 2007). However, the significance of Cry2 proteosomal degradation is not clear. CO regulation via Cry2, PhyB and COP1 activities assures that its protein levels are high late in the day when plants grown under LD are exposed to the light. How CO levels are maintained high at late afternoon has been elucidated by the interaction of the F-Box protein FKF1 with the transcriptional repressor CDF1. FKF1 is a positive regulator of CO (Imaizumi et al., 2003). CDF1 binds to the CO promoter and represses its transcription (Imaizumi et al., 2005). Late in the afternoon, FKF1 targets CDF1 for proteosomal degradation, which in turn liberates the repression on the CO promoter allowing CO protein levels to rise and promote flowering (Imaizumi et al., 2005). Taken together, these and other studies have shown that circadian rhythms, light receptor proteins and ubiquitination tightly regulate CO mRNA and protein levels to assure the accumulation of stabilized CO at a critical phase, allowing the flowering signal to proceed by activation of FT. Whether similar mechanisms exist in regulation of Hd1 levels in rice needs to be determined.

As described in this chapter, ubiquitination is emerging as a key regulatory process in both PCD and flowering time in plants. Although recent progress has been made towards the understanding of how ubiquitination controls flowering time, the same cannot be said about the mechanisms involving ubiquitination in plant PCD. The functional characterization of the U-box E3 ligase SPL11, especially the identification of its substrates, should greatly advance our knowledge of ubiquitination and cell death control. The objective of the research presented here was to elucidate the function of Spl11 in PCD and other processes. Two different but complementary approaches were
undertaken: one involving reverse genetics to isolate SPL11-interacting proteins by yeast two-hybrid, and another using forward genetics to identify spel11-mediated cell death suppressor mutants. The yeast two-hybrid analysis was carried out before and described elsewhere, thus, only the functional analysis of one of the candidates identified in the screen is presented in detail. The mutational screen for spel11 suppressors is introduced for the first time here. As will be demonstrated by the work described in this dissertation, Spl11 has dual functions in PCD and flowering time control.

References


CHAPTER 2

SPIN1, a K homology domain protein negatively regulated and ubiquitinated by the E3 ubiquitin ligase SPL11, is involved in flowering time control in rice

Introduction

Flowering in plants is triggered by both endogenous and environmental cues. Molecular genetic studies in *Arabidopsis thaliana* have identified at least four major pathways that regulate flowering, each perceiving and processing different signals (Simpson and Dean, 2002; Boss et al., 2004; Putterill et al., 2004). The photoperiodic and vernalization pathways mediate responses to changes in day-length and temperature associated with seasonal variation, respectively, whereas the autonomous and gibberellin pathways transduce internal developmental and physiological signals to promote flowering (Simpson and Dean, 2002; Putterill et al., 2004). These signaling pathways form a multi-level regulatory complex where input signals ultimately converge at a small set of key regulatory genes known as the floral pathway integrators (Simpson and Dean, 2002; Boss et al., 2004; Putterill et al., 2004). For example, the flowering activators *FT* (*FLOWERING LOCUS T*) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CO1*) are not only positively regulated via the photoperiodic pathway by *CO* (*CONSTANS*) (Samach et al., 2000; Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002), but also negatively by the floral repressor *FLC* (*FLOWERING LOCUS C*) through the
In *Arabidopsis*, flowering is promoted under long day (LD) conditions. In contrast, rice flowers earlier under short days (SD). Despite this obvious difference in response to photoperiod, molecular genetic studies in rice have revealed the existence of both conserved and unique components in the day length response pathway between the two species (Izawa et al., 2003; Hayama and Coupland, 2004; Izawa, 2007). Rice genes *Heading date* (*Hd*)1, *Hd3a* and *Hd6* are orthologs of *Arabidopsis* *CO*, *FT* and the α subunit of kinase *CK2*, respectively (Takahashi et al., 1998; Yano et al., 2000; Kojima et al., 2002). Moreover, the homolog of *GIGANTEA* (*GI*), a gene regulating both circadian clock inputs and the control of flowering (Fowler et al., 1999), and the homolog of the floral pathway integrator *SOC1* have also been isolated in rice (Hayama et al., 2002; Hayama et al., 2003; Tadege et al., 2003). In the current model for flowering control in rice, phytochromes (light receptor proteins) are essential in determining the photoperiod sensitivity (Izawa et al., 2000; Izawa et al., 2002). Under non-inductive, LD conditions, the coincidence of phytochrome signaling with *Hd1* expression in the light makes *Hd1* a repressor of the floral activator *Hd3a* (Izawa et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004). This is in contrast to *Arabidopsis* where overexpression of *CO* promotes flowering independently of day length (Onouchi et al., 2000). On the other hand, high level expression of *Hd1* occurs in the dark under SD which in turn activates *Hd3a* to promote flowering (Izawa et al., 2002; Kojima et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004). Hence, through fine-tuning of the
activation/accumulation of Hd3a via Hd1 in response to photoperiod, flowering is
delayed under LD and induced under SD in rice.

Ubiquitination is one of the major types of post-translational modifications in the
cell (Nalivaeva and Turner, 2001). It is achieved by a cascade of enzymatic reactions
that involve coordinated activities of three enzymes: ubiquitin-activating (E1), ubiquitin-
conjugating (E2), and ubiquitin-ligase (E3) (Ciechanover, 1998). Ubiquitination
regulates various aspects of plant life including disease resistance (Zeng et al., 2006),
hormone signaling (Itoh et al., 2003), and many developmental processes (Moon et al.,
2004) such as flowering time control. In Arabidopsis, CO is degraded via the 26S
proteasome in darkness (Valverde et al., 2004) by the E3 ligase COP1 (Liu et al., 2008),
while its transcriptional repressor CYCLING DOF FACTOR 1 (CDF1) is degraded by
the F-box protein FKF1 (Imaizumi et al., 2005). Thus, by means of directly targeting CO
or its negative regulators through ubiquitination, tight regulation of CO levels is achieved
in response to day length variations. Whether ubiquitination functions to control rice
flowering time remains to be elucidated.

In addition to ubiquitination, RNA processing also plays an important role in the
regulation of plant flowering time. The autonomous pathway genes FCA and FPA encode
proteins containing RNA recognition motifs (Macknight et al., 1997; Schomburg et al.,
2001). The FLK gene also encodes a protein with putative RNA processing function
(Lim et al., 2004; Mockler et al., 2004). The FLK protein contains three K homology
(KH) domains, a widely-found motif involved in RNA-binding activity (Adinolfi et al.,
1999). FCA, FPA and FLK were shown to negatively regulate FLC expression
(Macknight et al., 1997; Schomburg et al., 2001; Lim et al., 2004; Mockler et al., 2004).
Recent evidence suggests that FPA and FCA mediate siRNA-directed DNA methylation of *FLC* chromatin (Baurle et al., 2007; Liu et al., 2007).

A recent bioinformatic analysis revealed that E3 ligase proteins containing domains associated with RNA binding could be found in animals, plants and fungi (Lucas et al., 2006) suggesting that the link between ubiquitination and RNA metabolism is an ancient, evolutionarily-conserved mechanism. The rice gene *Spl11*, encoding a U-box domain/ARM repeats type E3 ubiquitin ligase, was previously shown to negatively regulate programmed cell death (PCD) and disease resistance (Yin et al., 2000; Zeng et al., 2004). Here, we present evidence that *Spl11* also controls flowering time via its interaction with a novel RNA/DNA binding KH domain protein called SPIN1 (*Spl11* interacting protein 1). The interaction between SPL11 and SPIN1 occurs in the nucleus suggesting that such interaction may be related to RNA-processing. SPL11 both ubiquitinates SPIN1 *in vitro* and negatively regulates *Spin1* mRNA levels. Overexpression of *Spin1* significantly delays rice flowering under both SD and LD conditions, while mutation of *Spl11* only delays flowering in LD. Our data suggest that the SPL11-SPIN1 interaction regulates a post-transcriptional mechanism involved in flowering time control in rice.

**Results**

*Flowering time phenotypes of the spl11 mutation*

The *spl11* mutation is characterized by enhanced, non race-specific resistance to both *Magnaporthe oryzae* and *Xanthomonas oryzae pv. oryzae* (*Xoo*), the pathogens that cause rice blast and bacterial blight diseases, respectively (Yin et al., 2000). A more detailed
analysis revealed that the \emph{spl11} mutant showed alterations in flowering time, in addition to previously reported phenotypes. Under SD, no significant difference in flowering time between the wild type IR64 and the \emph{spl11} mutant GR5717 was observed (Figure 1A). However, under non-inductive LD conditions, flowering was significantly delayed in the \emph{spl11} mutant compared to wild type (Figure 1A, B). Flowering in rice is promoted under SD by activating the \emph{CO} homolog \emph{Hd1}, which in turn activates \emph{Hd3a}, the rice homolog of \emph{FT} (Hayama et al., 2003). Under LD, \emph{Hd1} negatively regulates flowering by repressing \emph{Hd3a} (Hayama et al., 2003). To test whether the late-flowering phenotype of the \emph{spl11} mutant in LD is due to changes in flowering time gene expression, a quantitative RT-PCR/Southern blot (Yanovsky and Kay, 2002) analysis was conducted in wild type and \emph{spl11} plants over a 24 hour period. Both IR64 and \emph{spl11} showed similar diurnal regulation of mRNA levels for \emph{Hd1} and \emph{OsSOC1} (Figure 1C). However, \emph{Hd3a} transcript levels were significantly reduced in the mutant compared to wild type in both light and dark-growth periods (Figure 1C & D). Moreover, \emph{Spl11} showed a diurnal expression pattern under SD conditions that is typical of many flowering regulators with mRNA levels peaking in the dark and decreasing in mid-afternoon (Figure 6C, right panel). Interestingly, the diurnal regulation of \emph{Spl11} mRNA level was abolished in LD (Figure 6D, right panel). Taken together, these results confirm a role for the \emph{Spl11} gene in the regulation of flowering time.
Identification and subcellular localization of SPIN1

To elucidate the molecular basis by which Spl11 controls flowering time and/or programmed cell death, yeast two-hybrid screens were performed using the ARM domain region of the SPL11 protein as the bait (reported in Zeng et al., 2005).

One of the putative interactors, named SPIN1, accounted for nearly one third of the positive clones identified in the screens. The Spin1 full-length cDNA was obtained from the library used for the yeast two-hybrid analysis and consisted of an 846 bp open reading frame encoding a 281 amino acid protein with a theoretical pI of 9.48. Searches in various protein databases using the deduced amino acid sequence revealed that Spin1 encodes a novel protein containing a single K homology (KH) domain (Figure 2). Sequence similarity searches using the BLAST algorithm on the NCBI website showed that SPIN1 belongs to a sub-family of KH domain-containing proteins known as the STAR (signal transduction and activation of RNA) domain (Figure 2A) (Vernet and Artzt, 1997). The STAR domain is a tripartite motif with a single KH domain flanked by two sub-domains termed QUA1 and QUA2 (Figure 2A) (Vernet and Artzt, 1997). RNA-binding proteins in the STAR family include proteins involved in the regulation of key developmental processes in animals such as Gld-1 in Caenorhabditis elegans, How in Drosophila melanogaster, or Sam-68 in mouse and human (Vernet and Artzt, 1997; Ryder et al., 2004). The sequence homology between SPIN1 and the animal STAR family members is limited to the STAR region (Figure 3). The SPIN1 N-terminal region shows no homology to any known domain present in the databases. The STAR domain in SPIN1 is most similar to mammalian Splicing factor 1 (Sf-1) (Berglund et al., 1997)
that lacks the QUA1 sub-domain (Figure 2A) with 59% similarity and 42% identity at the amino acid level.

Several SPIN1-like sequences were identified in rice and other plant species, including *Arabidopsis*, tobacco and *Medicago truncatula* when SPIN1 was used as a query in database mining (Figure 2B). The existence of six other *Spin1*-like genes in the rice genome suggested that *Spin1* belongs to a small gene family. Phylogenetic analysis indicated that SPIN1 has highest homology to one of its paralogs (Os NP_001059216) and the homology between SPIN1 and its dicot orthologs is higher than that of the rest of its rice homologs (Figure 2B). Sequence alignment revealed that high level of amino acid similarity spanning the whole protein sequence exists among the SPIN1-like homologs, suggesting that these proteins are evolutionarily conserved.

A protein’s localization in the cell is usually closely related to its biological function. GFP-SPIN1 and GFP-SPL11 fusions were expressed in rice protoplasts and their sub-cellular localizations were determined. While GFP-SPIN1 was localized strictly in the nuclear region (Figure 4A), GFP-SPL11 accumulated all over the cell (Figure 4B). This data suggests that SPIN1 may function at the level of nuclear-related RNA metabolism such as transcription or splicing, while SPL11 may be involved in a broader range of cellular functions. Interestingly, the fluorescence signal for GFP-SPIN1 did not cover the entire nucleus (Figure 4A) suggesting a distinct subnuclear localization pattern for SPIN1.
**SPIN1 interacts with SPL11 in vitro and in rice protoplasts**

To validate the yeast two-hybrid data, both *in vitro* and *in vivo* experiments were performed to test the SPIN1-SPL11 interaction. Recombinant Glutathione S-Transferase (GST) fusion proteins containing the SPL11 ARM domain or full length SPL11 were purified from *E. coli* and incubated with *in vitro* translated and biotinylated SPIN1 protein. Results from the GST pull-down assay showed that SPIN1 was present in reactions containing either GST-ARM or GST-SPL11, but not GST, confirming the *in vitro* interaction between SPIN1 and SPL11 (Figure 5A). To determine which region of the SPIN1 protein is minimally required for the interaction with SPL11, an interaction domain mapping assay using the yeast two-hybrid approach was performed. Different domains of SPIN1 were co-expressed in yeast with the ARM region of SPL11 (Figure 5C). Only the N-terminus region of SPIN1 interacted with the ARM domain of SPL11 as seen by a positive X-GAL assay (Figure 5C). As the SPIN1 N-terminal region shows no homology to any known domain in the existing protein databases, these results suggested that the N-terminal region of SPIN1 encodes a novel protein-protein interaction domain.

Testing of the interaction in living cells was performed by bi-molecular fluorescence complementation (BiFC) in rice protoplasts (Bracha-Drori et al., 2004; Chen et al., 2006). YFP fluorescence was reconstituted when full-length SPIN1 and SPL11, ARM and SPIN1, or SPIN1-N and SPL11 proteins were co-expressed in protoplasts (Figure 5B) indicating that these proteins interact *in vivo*. Control experiments co-expressing the SPIN1-C terminus with SPL11, or the unrelated protein β-Glucuronidase (GUS) with either SPIN1 or SPL11, showed no fluorescence (Figure 4B) confirming that the SPIN1-SPL11 interaction is specific. Moreover, the BiFC technique allowed us to clearly
identify the sub-cellular localization of the SPL11-SPIN1 interaction in the nucleus (Figure 5B). As an attempt to more clearly define this interaction, the spinach nucleolar protein PRH75 (Lorkovic et al., 1997) was used as a marker to pinpoint the sub-nuclear localization of the SPIN1-SPL11 interaction relative to the nucleolus. As shown in Figure 4C, the SPIN1-SPL11 interaction occurs in the vicinity of, and partially overlapping with, the nucleolus.

Spin1 is ubiquitously expressed in rice, shows diurnal expression patterns and its expression is negatively regulated by Spl11 in the light.

RT-PCR analyses revealed that Spin1 is expressed in most tissues from stems to old leaves (Figure 6A) and flowers (Figure 6B). Spin1 transcript level was highest in stems and mature leaves and almost undetectable in young roots, although it was present in mature roots (Figure 6A). Interestingly, both Spin1 and Spl11 seem to be upregulated at flowering since their expression levels were higher in mature leaves at booting stage compared to leaves prior to booting (Figure 6B). The booting stage represents the swelling of the flag leaf sheath caused by the growth of the inflorescence. This increase in expression at booting was followed by an increased expression in immature panicles for Spin1, but not for Spl11 (Figure 6B).

Similar to Spl11, Spin1 expression showed a diurnal regulation under SD over a 24 h period (Figure 6C). However, high Spl11 expression tended to correlate with decreased Spin1 levels and highest Spin1 expression occurred when Spl11 levels were the lowest, especially during the light period (Figure 6C, left and right panels). Surprisingly, in the spl11 mutant background in SD, Spin1 maintained its diurnal regulation but at a
phase opposite to the one observed in the wild type IR64 (WT) (Figure 6C, middle panel). These results suggest that Spl11 negatively regulates Spin1 transcript levels. The Spin1 gene expression phase change between IR64 and spl11 during light was also observed in LD (Figure 6D, left and middle panels). While Spl11 mRNA accumulation was aphasic and remained constant throughout the day, its expression peaked at dawn under LD conditions (Figure 6D, right panel). Importantly, the elevated Spl11 transcript levels in LD correlated with a decrease in Spin1 mRNA accumulation that coincided with the extended light period during morning and early evening (Figure 6D, time points 0, 4 and 12, left and right panels). This decrease of Spin1 expression in LD in IR64 plants at morning and early evening was reversed in the spl11 mutant (Figure 6D, middle panel), which demonstrated that it is under the control of Spl11.

Taken together, the expression analyses of Spin1 revealed the ubiquitous expression of this gene in rice tissues and developmental stages, as well as its negative regulation in both SD and LD by Spl11 especially during light periods. The aphasic Spl11 transcript accumulation in LD suggests that a photoperiod-sensitive clock component controls Spl11 expression levels.

**SPL11 targets SPIN1 for mono-ubiquitination**

SPL11 was shown to possess E3 ubiquitin ligase activity (Zeng et al., 2004). The interaction between SPL11 and SPIN1 both in vitro and in vivo suggests that SPL11 might target SPIN1 for ubiquitination. To test this possibility, an in vitro ubiquitination assay was performed using recombinant GST-SPIN1-HA, GST-SPL11, E1, E2 and ubiquitin (Ub) (Figure 7). Ubiquitination was observed as higher molecular weight bands
compared to free GST-SPIN1-HA when GST-SPL11 was present in the reaction (Figure 7, lane 4). An unrelated E3 ligase, OsSINAT5, homologous to Arabidopsis SINAT5 (Xie et al., 2002) failed to ubiquitinate SPIN1 (Figure 7, lane 8) indicating that SPL11 specifically targets SPIN1 for ubiquitination. Importantly, the size of the ubiquitinated protein suggested mono or di-ubiquitination of SPIN1 by SPL11.

**SPIN1 has both RNA and DNA binding activities** in vitro

Our bioinformatic analyses indicated that SPIN1 is a member of the STAR family of RNA binding proteins (Figure 2). To confirm its biochemical function, recombinant GST-SPIN1-HA protein was incubated in vitro with ribohomopolymer-bound beads representing poly(A), poly(U), poly(G) and poly(C), as well as single and double stranded calf thymus DNA. Western blot analysis following incubation and washing of beads revealed that GST-SPIN1-HA bound to all RNA and DNA polymers tested (Figure 8). As a control, GST did not show any RNA or DNA binding activity. These results confirmed that SPIN1 has RNA/DNA binding activity in vitro.

**Overexpression of Spin1 causes late flowering under both SD and LD**

To functionally characterize Spin1, RNAi and overexpression transgenic lines were generated in the rice japonica cultivar Nipponbare (NPB). Twenty six RNAi lines successfully silencing Spin1 and 13 lines overexpressing the gene with an N-terminal TAP (tandem affinity purification) tag (Spin1-OX) were generated. As an example of successful silencing and overexpression of the transgenes, RT-PCR analysis indicating
reduction or increase of Spin1 expression in some of the transgenic lines is shown in Figure 9.

Neither Spin1-RNAi nor Spin1-OX lines showed signs of lesion mimic or cell death-related phenotypes. Moreover, no enhanced resistance or susceptibility were observed when both Spin1-silenced and overexpressed lines were inoculated with virulent strains of M. oryzae and Xoo, compared to non-transformed NPB (Figure 10). These results suggested that SPIN1 might not be involved in the cell death and disease resistance signaling that was reported to be associated with Spl11. Nevertheless, examination of Spin1-OX lines revealed that they showed delayed flowering time compared to the RNAi and NPB plants (Figure 11). Under both SD and LD conditions, no significant difference in the appearance of the first panicle was observed between Spin1-RNAi lines and NPB or segregant transgenic lines not carrying the transgene (Figure 11B). However, flowering time was significantly delayed in Spin1-OX lines under the same conditions (Figure 11B). The delayed flowering phenotype correlated with the overexpression of the TAP-SPIN1 protein in T4 transgenic lines (Figure 11A) and was due to the overexpression of the Spin1 gene since transgenic lines overexpressing the TAP tag alone in the Kitaake background had no differences in flowering time compared to non-transformed plants (Figure 12).

The expression of Hd1, Hd3a and OsSOC1 was monitored over a 24 hour period in both Spin1-OX and NPB plants under SD and LD conditions. Higher expression of Hd1 at mid-morning, early and late night correlated with significant reduction of Hd3a expression in Spin1-OX in SD when compared to NPB (Figs. 11C, D & F) suggesting that Spin1 represses Hd3a expression via Hd1. In contrast, the expression levels of
OsSOC1 did not vary significantly between Spin1-OX and NPB under SD (Figure 11C). Under LD, Hd1 transcript levels either did not vary significantly between NPB and Spin1-OX or variations did not correlate with the delayed flowering phenotype (Figs. 11C & E). In contrast, significant differences in Hd3a expression between NPB and Spin1-OX at mid-morning and especially at midnight, correlated with the delayed flowering in Spin1-OX plants (Figs. 11C & G). The enhanced downregulation of Hd3a transcript levels at midnight correlated with significant reduction of OsSOC1 expression at this time point in Spin1-OX lines compared to NPB, but not at the other time points (Figure 11C). These results indicated that Spin1 represses flowering independently of day-length via a mechanism that primarily targets the flowering promoter Hd3a, especially in SD.

**Discussion**

We have shown that SPL11 interacts with and ubiquitinates SPIN1, a nuclear RNA/DNA binding protein of the STAR family. In addition, we have shown that Spl11 negatively regulates Spin1 expression during light periods in both SD and LD. The spl11 mutant has a delayed flowering phenotype in LD, while overexpression of Spin1 causes delayed flowering in both SD and LD. Our data is in agreement with SPL11 acting as a positive regulator of flowering via negative regulation of the SPIN1 flowering repressor. Our results reveal that transcriptional regulation of a gene encoding an RNA/DNA binding protein, possibly via mono-ubiquitination, is the mechanism of flowering time control by this E3 ligase in rice.
Putative paralogs and orthologs of SPIN1 were identified in rice and other plant species, respectively. The high level of conservation at the amino acid level between monocot and dicot SPIN1-like sequences suggests that their functions might be conserved across distantly related species. In total, seven paralogs including SPIN1 were found in rice. It is likely that these genes have redundant functions in rice which might explain our observation that silencing of Spin1 did not significantly affect flowering time. Functional redundancy was confirmed for the SPA family and the AGL15-AGL18 floral repressors in Arabidopsis, where double or triple, but not single, loss-of-function mutations cause early flowering (Laubinger et al., 2006; Adamczyk et al., 2007). We have checked the expression of the closest rice paralog of Spin1, Os07g0227400 (encoding Os_NP001059216, Figure 2B), in our transgenic plants and found a slight to non-significant decrease in its transcript levels depending on the RNAi line tested (Figure 13). Os07g0227400’s coding sequence shows 79% identity with Spin1 at the nucleotide level and 90% similarity in the amino acid sequence (Figure 2B). Therefore, the lack of phenotype in our RNAi lines is most likely due to genetic redundancy. Silencing Spin1 and one or a few paralogous members simultaneously will be central to testing such functional redundancy.

Spl11 was initially characterized as a gene that negatively regulates PCD and broad-spectrum disease resistance (Zeng et al., 2004). Our results in this study add a role for SPL11 in the control of flowering time. In plants, examples have been reported of an E3 ligase that plays different roles depending on the biological processes. For example, the F-box protein ORE9 regulates both senescence and shoot branching in Arabidopsis (Woo et al., 2001; Stirnberg et al., 2007). The Spin1-RNAi transgenic plants did not
show disease resistance or cell death-related phenotypes suggesting that SPIN1 might not be involved in these pathways. Due to possible functional redundancy in the Spin1 gene family (see above) we cannot rule out a role of SPIN1 in PCD and pathogen resistance. However, Spin1-OX lines were not affected in defense or cell death either. Moreover, Spin1 is upregulated in leaves at booting stage and in panicles, which is in agreement with its involvement in flowering control. We have identified other interactors of SPL11 in the yeast two-hybrid screen. Whether other SPL11-interacting partners play a role in PCD and defense is under investigation. Preliminary studies on SPIN6 have implicated it in cell death control (Wang et al, *unpublished results*).

Our results showed that SPIN1 is ubiquitinated *in vitro* by SPL11 (Figure 7). Poly-ubiquitination through Lys48 ubiquitin residues usually serves as a signal for degradation by the 26S proteasome (Thrower et al., 2000). However, poly-ubiquitination through lysine residues other than the Lys48 as well as mono-ubiquitination have been implicated in non-proteolytic cellular processes (Terrell et al., 1998; Schnell and Hicke, 2003; Sigismund et al., 2004; Mukhopadhyay and Riezman, 2007). Our initial attempts to test whether SPL11 regulates SPIN1 protein stability using a cycloheximide-chase experiment indicated that SPIN1 is not degraded when SPL11 is overexpressed in rice protoplast. However, the experiment was inconclusive due to our failure to detect the ubiquitinated form of SPIN1 in protoplasts. Nevertheless, it is likely that SPIN1 is not degraded by SPL11 *in vivo* based on the results indicating that SPIN1 is mono-ubiquitinated by SPL11 and by a time-course analysis of the TAP-SPIN1 protein accumulation under SD and LD over a 24h period (Figure 14): no significant variations in protein levels were observed throughout the day which would not be expected if
SPIN1 was a substrate for proteolysis. Although the TAP-SPIN1 protein does not represent the endogenous levels of SPIN1 accumulation, in the case of the flowering repressor CDF1 in *Arabidopsis*, which is targeted for degradation by FKF1-mediated ubiquitination, the levels of CDF1 protein in plants overexpressing an HA-CDF1 construct were significantly reduced in the dark in a proteasome-dependent manner (Imaizumi et al., 2005). Several examples of U-box type E3 ligases targeting their substrates for non-proteolytical ubiquitination have been reported. In animals, the U-box co-chaperone CHIP ubiquitinates, but does not degrade, Hsc70 (Jiang et al., 2001). Similarly, the *Arabidopsis* homologue of CHIP, AtCHIP, mono-ubiquitinates the A subunit of phosphatase 2A (PP2A); since overexpression of At CHIP increased the activity of PP2A, mono-ubiquitination of PP2A does not promote its degradation (Luo et al., 2006).

Overexpression of Spin1 under both SD and LD delays flowering (Figure 11B). However, the spl11 mutant shows no significant difference in flowering time under SD, but flowers late in LD (Figure 1). These results suggest that Spin1 acts as a flowering repressor and Spl11 acts as a positive regulator of flowering time in rice. Consistent with this notion, the expression levels of the flowering promoter gene Hd3a are significantly reduced in plants overexpressing Spin1, especially in SD, and in the spl11 mutant in LD. In *Arabidopsis*, the Hd3a ortholog FT activates some of the floral meristem identity genes involved in flowering transition at the shoot apical meristem (Simpson and Dean, 2002). Recently, both FT and Hd3a proteins have been shown to move from the leaf to the shoot apex where floral organ identity genes are activated (Corbesier et al., 2007; Tamaki et al., 2007). Hence, it is not surprising that many floral repressor genes function...
by targeting, either directly or indirectly, FT/Hd3a accumulation. The MADS domain proteins AGL15 and AGL18 directly downregulate FT expression (Adamczyk et al., 2007). Many genes indirectly target FT by negatively regulating CO at transcript or protein levels. For example, CDF1 binds to the CO promoter to repress its expression (Imaizumi et al., 2005) while SPA1 interacts with and negatively controls CO protein levels (Laubinger et al., 2006). COP1 has been recently shown to negatively regulate flowering by ubiquitinating CO and promoting its degradation in the dark (Liu et al., 2008). It is unclear whether similar negative regulation is conserved in rice. Knowledge in the regulation of Hd1 is limited compared to its Arabidopsis ortholog CO. However, it has been shown that under LD conditions Hd1 acts as a repressor of Hd3a expression (Izawa et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004) which is in contrast to the function of CO in Arabidopsis.

Based on our results, it appears that Spin1 negatively regulates flowering via Hd1-dependent and independent mechanisms in SD and LD, respectively. In flowering-promoting SD conditions, downregulation of Hd1 at mid-morning and early and late evening correlates with decreased Hd3a levels in the Spin1-OX line compared to wild type NPB plants (Figure 11C, D & F). In contrast, under flowering-restrictive LD conditions, Hd1 mRNA levels do not vary significantly between NPB and Spin1-OX. Hd3a levels are very low in both Spin1-OX and NPB in LD, except at time point 16 h (around midnight) when Hd3a transcript accumulation is significantly higher in NPB (Figure 11C & G). It is unlikely, however, that the decrease in Hd3a mRNA level at midnight is solely responsible for the delayed flowering phenotype of Spin1-OX plants in LD. Similar to what we observed in Spin1-OX plants, Hd3a expression was strongly
repressed in LD conditions in rice plants overexpressing Os GI (Hayama et al., 2003).
Moreover, the Os MADS51 mutant shows no significant difference in flowering time compared to wild type in LD although Hd3a expression is greatly reduced in the mutant (Kim et al., 2007). Therefore, additional factors regulated by SPIN1 in LD might also contribute to the delay in flowering compared to WT.

Spl11 and Spin1 have opposite expression patterns in immature panicles but are both upregulated in leaves at booting stage (Figure 6B) and their mRNA abundances are diurnally regulated in SD (Figure 6C). These results support the notion that both of these genes are related to the plant flowering process. We know that Spl11 negatively regulates the accumulation of Spin1 mRNA in the light and that in the absence of Spl11, Spin1 transcript accumulates with an opposite phase compared to WT (Figure 6C&D). The only times this change in phase correlates with changes in flowering time is when Spin1 is upregulated in the morning and during extended light hours corresponding to early evening in LD in the spl11 mutant (Figure 6D, middle panel). Thus, high expression of Spin1 in the morning and early evening in the light period is required to repress flowering. This explains why there is no difference in flowering time between spl11 and WT in SD since high Spin1 expression does not coincide with the light at these time points (Figure 6C, left and middle panels). However, high Spin1 expression during LD in WT does not coincide with the light either (Figure 6D, left panel), and yet flowering is delayed compared to SD. Delayed flowering in WT plants under LD can thus be attributed to other flowering repressing mechanisms, most likely via Hd1, a strong floral repressor in LD (Izawa et al., 2002; Hayama et al., 2003; Hayama and Coupland, 2004). This is in agreement with the observation that in the spl11 mutant,
flowering is further delayed in LD compared to WT, which can be explained by the additive contributions of Hd1 and SPIN1. In Spin1-OX lines high accumulation of Spin1 throughout the day, regardless of light and dark phases, causes delayed flowering in both SD and LD (Figure 11B). This is reminiscent of Hd1 regulation in SD versus LD in which high Hd1 accumulation in the dark promotes flowering in SD, while the coincidence of high Hd1 levels occurring in the light inhibits flowering in LD (Hayama and Coupland, 2004). However, a major difference is that the phase of Hd1 accumulation is not altered by changes in day length as is the case for Spin1.

Interestingly, overexpression of Spin1 affects Spl11 diurnal regulation. As shown in Figure 15, Spl11 shows diurnal regulation in LD but not in SD in Spin1-OX, which is the opposite of what we observed in WT IR64 plants (see Figure 6C&D, right panels). These results suggest that Spin1 regulates Spl11 expression. In LD, loss of Spl11 diurnal regulation may be a mechanism to keep SPL11 levels high enough to inhibit Spin1 expression in the light that could lead to a delay in flowering time. Thus, the restoration of Spl11 diurnal expression pattern in LD when Spin1 is overexpressed would serve to decrease SPL11 levels during the light phase to inhibit flowering. However, the role, if any, of the loss of Spl11 diurnal pattern in SD when Spin1 is overexpressed is less clear. The fact that Spl11 loses diurnal regulation in LD in WT while Spin1 retains its diurnal regulation regardless of photoperiod argues that clock-related components act differentially in the control of oscillation patterns for the two genes. Supporting the notion of circadian control of flowering regulators, it has been widely documented that loss-of-function mutants in clock feedback loop components causes changes in CO protein and mRNA levels that result in early or late flowering depending on the mutant
(Simpson and Dean, 2002; Putterill et al., 2004; Izawa, 2007). Taken together, we propose the following model of flowering time control (Figure 16): under SD both Spin1 and Spl11 are diurnally regulated with opposing phases; negative regulation of Hd1 and Hd3a by SPIN1 is inhibited by SPL11 either on Spin1 mRNA, protein levels or both, and flowering is promoted. In the absence of Spl11 the phase of Spin1 expression changes, but, since high expression levels do not coincide with the light during morning and early evening flowering is not affected. By contrast, in Spin1-OX lines high accumulation of SPIN1 throughout the day is enough to repress flowering and to cause loss of diurnal Spl11 expression by an unknown mechanism. Under LD, SPIN1 and Hd1 may act additively to repress Hd3a and possibly other components. Loss of Spl11 diurnal regulation might be required for the negative control of Spin1 transcript and/or protein levels by keeping high levels of SPL11 throughout the day and alleviate flowering repression; in the absence of Spl11, high levels of Spin1 coincide with the light in the morning and early evening which triggers increased inhibition of flowering time.

Similarly, overexpression of Spin1 maintains high SPIN1 levels throughout the day, which both represses Hd3a and other factors and restores the diurnal regulation of Spl11, which ultimately decreases SPL11 overall levels during the light and delays flowering.

Our results indicate that SPL11 negatively regulates Spin1 expression and that SPIN1 affects the Spl11 expression pattern as well. The mechanism(s) underlying the transcriptional regulation of Spin1 by SPL11 and vice versa are unknown at this point. As a nuclear RNA binding protein, SPIN1 might bind directly to the Spl11 pre-mRNA or to the pre-mRNA of putative Spl11 circadian clock regulators. In addition, SPIN1 most likely represses flowering by binding to other targets in the cell. We speculate that it is at
this level that the SPL11-mediated ubiquitination of SPIN1 affects flowering time by negatively-regulating these interactions. Negative regulation by non-proteasomal-mediated ubiquitination has been recently shown in the case of endocytic proteins such as Sts2 where mono-ubiquitination causes intramolecular interactions that impair Sts2 binding to other proteins abolishing its regulatory function in receptor trafficking (Hoeller et al., 2006). Similarly, mono-ubiquitination of SPIN1 might affect its RNA-binding activity or its interaction with other proteins, which are questions to be addressed in future studies.

**Materials and methods**

**Plant material**

Seeds of wild type, mutant and transgenic rice were first sterilized by treatment in 75% ethanol for 1 minute followed by immersion in 2% sodium hypochlorite for 15-20 minutes. After thoroughly washing with sterile distilled water, seeds were germinated in ½ MS medium for 7-10 days and then transferred to soil. Growth chamber conditions were 10h light, 26º C, 80% relative humidity (RH), followed by 14h dark, 20º C, 60% RH, unless otherwise specified.

**Flowering time measurements**

Plants were grown as described above with the following modifications: for SD, plants were grown either in 12h light/12h dark or 10h light/14h dark; LD were 16h light/8h dark for 120 days, then 14h light/10h dark for the rest of the experiment. Heading date or flowering time was measured in days from sowing until emergence of the first panicle.
Plasmids

In general, constructs were generated by PCR amplification of the target gene using primers containing appropriate restriction enzyme sites and Spin1 or Spl11 cDNA template followed by ligation into a desired vector. For a list of primers and constructs see Table 1. For the BiFC method Spl11, Spin1 and GUS constructs in pA7-NYFP and pA7-CYFP vectors are described elsewhere (Chen et al., 2006). Overexpression and RNAi constructs were made into the Gateway (Invitrogen)-compatible vectors Ubix.nc1300.ntap.gck (Chern et al., 2005) and pANDA (Miki and Shimamoto, 2004), respectively, following Gateway cloning protocols.

Yeast two-hybrid screen [Previously described in detail in (Zeng et al., 2005)]

The ProQuest system (Invitrogen) was used to screen for SPL11-interacting proteins following the manufacturer’s protocol. Briefly, a construct that contains the SPL11 ARM repeat domain (amino acid number 379-653) was cloned by PCR into the NcoI and NheI sites of Proquest’s pDBleu vector; a rice cDNA library was built into the expression vector pPC86 of the ProQuest system using the restriction enzymes NotI and SalI, using mRNA isolated from three-week-old seedlings of rice line 75-1-127. Total RNA was isolated using Trizol reagent (Invitrogen) and mRNA was then purified using Absolutely mRNA Purification Kit (Stratagene), according to the protocol provided by the manufacturer.
GST pull-down

GST pull-down was performed as described (Liu et al., 2002) with the following modifications: a Spin1 construct in pET28-a (Novagen) driven by a T7 promoter was used for in vitro transcription/translation using the TNT rabbit reticulocyte lysate translation system (Promega) in the presence of biotinylated lysine to label the protein (Transend non-radioactive translation detection system, Promega). Detection of bound SPIN1 was performed by Streptavidin-HRP chemiluminescent detection using the Transend non-radioactive detection system (Promega).

Bi-molecular fluorescence complementation and fluorescence microscopy analyses

Fluorescence microscopy analyses were done by transfection of rice protoplasts with various constructs as described (Chen et al., 2006).

Gene expression analyses

RNA was extracted with Trizol reagent (Invitrogen) and treated with DNAse-I before being used for reverse transcription using the Promega reverse transcription system. Two to three microliters of first strand cDNA were used for the PCR reactions. Details on the collection of tissue for RNA samples of rice developmental stages are as described (Nobuta et al., 2007). The exponential range of amplification was determined for each gene in a pilot experiment using incremental PCR cycles (15-30 cycles); 24 cycles were optimal for Spl11 and Ubiquitin, 25 for Spin1, Hd1 and OS_SOC1, and 26 for Hd3a. Spin1 RT-PCR cycling conditions were denaturing at 94º C for 1 min, annealing at 55º C for 1 min, extension at 72º C for 1 min; 25 cycles. Ubiquitin was used as internal control.
with denaturing at 94º C for 30s, annealing at 50º C for 30s, extension at 72º C, for 24 cycles. Spl11 cycling conditions were as described (Chen et al., 2006). For flowering marker genes, primers were the same as described (Hayama et al., 2003) (Tadege et al., 2003). For quantification of gene expression, band intensity from Sybr Gold-stained agarose gels was determined using the ImageJ software. Gene expression values at each time point were divided by their corresponding ubiquitin value to correct for loading, and the highest number was given the arbitrary quantity of 100. The rest of values were normalized relative to 100 to correct for variations in band intensity between replicate gels. For flowering marker gene expression, RT-PCR followed by Southern blotting was done as described (Yanovsky and Kay, 2002). Briefly, RT-PCR was done as described above and 8µl of the reaction was loaded on an agarose gel. DNA was blotted onto a nylon membrane and hybridized using gene-specific probes labeled with ³²P-dCTP. Blot images were scanned with a phosphorimager (Molecular Dynamics) and band intensity was quantified using the ImageQuant TL image analysis software (GE Healthcare). Gene expression analysis using the data from the phosphorimager was done following the same protocol described above. RNA was extracted from 55 day-old plants every four hours starting at time 0 (7 AM) and ending at time 20 (3 AM, the next day). The values of 3 independent experiments were averaged and plotted for all quantitative RT-PCR assays performed.

In vitro ubiquitination assays
The ubiquitination assay was done as described (Zeng et al., 2004) with the following modifications: in a 30µl final volume 100ng of E1, 100ng of E2, 200ng of GST-SPIN1-
HA, 600ng of GST-SPL11 or GST-OsSINAT5, 12µg of ubiquitin (Boston biochem), and 3µl of 10X ubiquitination buffer (500mM Tris-HCl pH7.5, 50mM ATP, 50mM MgCl₂, 20mM DTT, 30mM creatine phosphate, 0.05mg/ml creatine phosphokinase) were mixed and incubated at 30º C for 1.5 hours. Samples were denatured, loaded on a 10% SDS-polyacrylamide gel and ubiquitination was detected by Western blot analysis with anti-HA (Roche) antibodies. AtUBC9 was used in the reaction using GST-SPL11 and AtUBC7 with GST-OsSINAT5.

**RNA/DNA binding assay**

Beads containing polyadenylic, polycytidylic, polyuridylic and polyguanylic ribohomopolymers, as well as calf thymus single and double stranded DNA were purchased from Sigma. Approximately 30ng of recombinant GST-SPIN1-HA was incubated with the beads in 500µl of KHN buffer (150mM KCl, 20mM HEPES pH7.9, 0.01% Nonidet P-40, complete protease inhibitors) under rotation for 10 min. Beads were washed in KHN buffer 3 times and proteins retained in the beads were identified by Western blot analysis using either anti-HA (Roche) or anti-GST (Invitrogen) antibodies.

**Protein extraction and Western blot analysis**

Protein extraction from transgenic Spin1-TAP plants was done by grinding leaves in liquid nitrogen and homogenizing the ground tissue in buffer 2 (8M urea, 4% CHAPS, 40mM Tris, 2mM TBP) from the ReadyPrep sequential extraction kit from Biorad (Hercules, CA, USA) following the kit’s protocol. For Western blots, membranes were blocked for 1h in 5% milk in 1X TBST, incubated 1h in 2% milk in 1X TBST containing
the appropriate primary antibody, and finally incubated for 1h in 2% milk in 1X TBST with the conjugated, secondary antibody. Proteins in blots were detected by chemiluminescence using the Pierce ECL western blotting substrate (Rockford, IL, USA) or the Amersham ECL plus western blotting detection reagents (GE healthcare, Buckinghamshire, UK), following the manufacturer’s instructions. TAP-tagged proteins were detected with a Peroxidase-anti-peroxidase (PAP) antibody (Sigma, St. Louis, MO, USA).

*Phylogenetic analysis*

The dendogram was constructed using the MEGA3.1 software (Kumar et al., 2004) using the Neighbor Joining algorithm. Boostrapping was performed with 1000 replicates. *Arabidopsis* FLK, a KH domain-containing protein unrelated to the SPIN1-like sequences, was used as an outgroup. Multiple sequence alignment was done using ClustalX and conserved residue shading performed using GeneDoc.

**References**


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**Table 1.** List of primers and constructs used in Chapter 2
Table 1 continued

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Figure 1. Flowering time phenotype of the spl11 mutation. A. Days to heading in short days (SD) and long days (LD) for IR64 (WT) and IR64-GR5717 (spl11). N= 5-10 plants; +/- standard deviation is shown. B. Delayed flowering phenotype of spl11 mutant (left) compared to WT (right) in LD. C. RT-PCR/Southern blot analysis of Hd1, Hd3a, SOC1 and Ubiquitin in IR64 and spl11 under LD in 55 day-old plants. D. Quantification of mRNA abundance relative to Ubiquitin (Ubq) of the Hd3a blot shown in C. White and black rectangles represent light and dark conditions over a 24-hour period, respectively. Error bars: +/- standard deviation of three independent experiments.
Figure 2. SPIN1 is a member of the STAR family of RNA binding proteins. A. Schematic representation of the STAR (Signal transduction and activation of RNA) domain. Gld-1, from C. elegans; Sf1 from human. Note that similarly to Sf1, SPIN1 lacks the QUA1 subdomain. B. Phylogenetic analysis of plant SPIN1 homologs from rice (Os), Arabidopsis thaliana (At), tobacco (Nt) and Medicago truncatula (Mt). The dendogram was constructed using the MEGA3.1 software with the Neighbor Joining algorithm; Arabidopsis FLK, a KH domain-containing protein unrelated to the SPIN1-like sequences, was used as an outgroup. Amino acid percentage similarity to SPIN1 is shown.
Figure 3. Alignment of the STAR domain of SPIN1 with mammalian protein members of the family. Gld-1, from *C. elegans*; Sf1 from human; How, from *Drosophila melanogaster*. Multiple sequence alignment was done using ClustalX and conserved residue shading performed using GeneDoc. Amino acid similarity between SPIN1 and GLD-1, HOW and SF-1 is 69%, 69% and 59%, respectively.
Figure 4. Subcellular localization of SPIN1 and SPL11 in rice protoplasts. A. SPIN1-GFP localizes to the nuclear region. 1: bright field; 2: green fluorescence; 3: bright and green merged. Arrow indicates the nucleus. B. SPL11-GFP is localized in the entire cell. 1: bright field; 2: green fluorescence. C. Subnuclear localization of the SPIN1-SPL11 interaction using BiFC. Green fluorescence: SPL11-NYFP + SPIN1-CYFP; red fluorescence: nucleolar protein PRH75-RFP. 1. Bright field; 2. green fluorescence; 3. red fluorescence; 4: green and red merged. Scale bars = 20 microns.
Figure 5. SPIN1 interacts with SPL11 both in vitro and in vivo. A. GST pull-down assay shows in vitro interaction of GST-SPL11 (full length) and GST-ARM (ARM domain only) with SPIN1. In vitro-translated and biotinylated SPIN1 was incubated with GST fusion proteins in glutathione beads. SPIN1 was pulled-down in beads containing GST-SPL11 and GST-ARM but not GST. B. Bimolecular fluorescence complementation (BiFC) detection of the SPIN1 and SPL11 interaction in rice protoplasts. Cells were observed on a fluorescence microscope under bright and UV lights to detect cells and green fluorescence, respectively. C. SPIN1 interaction domain mapping with SPL11-ARM in yeast. SPL11-ARM and different domains of SPIN1 were fused to the GAL4 DNA binding and activation domains, respectively. Blue colonies in X-GAL assay indicate interaction in yeast.
Figure 6. Expression analysis of Spin1 and Spl11. A. Quantitative RT-PCR of Spin1 expression in vegetative rice tissues. B. RT-PCR analysis of Spin1 and Spl11 expression patterns in mature leaf and flowering tissues. C. RT-PCR of Spin1 and Spl11 expression in leaves under SD over a 24 hour period. D. RT-PCR of Spin1 and Spl11 expression in leaves under LD over a 24 hour period. White and black rectangles represent light and dark conditions over a 24 hour period, respectively. Error bars: +/- standard deviation of three independent experiments in A, B, C and D.
Figure 7. Ubiquitination of SPIN1 by SPL11. SPL11 mono-ubiquitinates SPIN1 \textit{in vitro} (arrowhead indicates position of mono-ubiquitinated SPIN1; arrow indicates un-ubiquitinated SPIN1). The unrelated E3 ligase Os SINAT5 was used as negative control (At UBC7 was used as the E2 enzyme in the Os SINAT5 reaction while At UBC9 was used in the SPL11 reaction).
Figure 8. SPIN1 has both RNA and DNA binding activities in vitro. GST-SPIN1-HA binds to ribohomo-polymers and both single and double stranded calf thymus DNA in vitro. GST-SPIN1-HA was purified from E. coli and incubated with beads containing different nucleic acid polymers. Binding of SPIN1 to RNA or DNA was confirmed by a Western blot using Anti-HA antibodies. Lower panel: GST negative control; Anti-GST WB.
Figure 9. RT-PCR analysis of *Spin1* expression in T2 transgenic RNAi and TAP (overexpression) lines. NPB: Nipponbare (recipient cultivar).
Figure 10. Rice blast and bacterial blight inoculation response of Spin1-RNAi and Spin1-OX lines. A. Lesion length of Spin1 transgenic plants inoculated with X. oryzae pv. oryzae race J22. TAP lines are lines overexpressing Spin1. B. Lesion area of Spin1 transgenic plants inoculated with M. oryzae isolate Che86. Error bars: +/- standard deviation. A minimum of 10 leaves per genotype were inoculated.
Figure 11. Overexpression of Spin1 causes delayed flowering under both SD and LD conditions. A. Western blot detection of the accumulation of TAP-SPIN1 protein in a T4 line overexpressing Spin1-TAP. Line 32-3-5 is segregating for flowering time in SD. PAP: peroxidase anti-peroxidase antibody. B. Days to heading under SD and LD in T4 RNAi and OX Spin1 (Spin1-TAP) lines, and on the untransformed Nipponbare recipient cultivar (NPB). (+) and (−) indicate siblings from the same T3 family that do or do not contain the RNAi or OX construct. Asterisk: did not flower after 230 days. C. RT-PCR/Southern blot analysis of Hd1, Hd3a, SOC1 and Ubq in Spin1-TAP line 11-15 and NPB under SD and LD, over a 24 hour period in 55 day-old plants. D-G. Quantification of gene expression relative to Ubq of the Hd1 and Hd3a blots shown in C. White and black rectangles represent light and dark conditions over a 24 hour period. Error bars: +/- standard deviation from three independent experiments.
Figure 12. Days to heading in NTAP transgenic lines transformed with the empty TAP tag vector used for generation of Spin1-TAP lines. Recipient cultivar for transformation is Kitaake.
Figure 13. Expression analysis of Spin1 and its closest paralog Os07g0227400 by RT-PCR in T5 Spin1-RNAi and Spin1-TAP lines. +/- indicates sibling containing or not the transgene, respectively.
**Figure 14.** Accumulation of TAP-SPIN1 protein in SD and LD over a 24 h period. Ponceau S staining of the blot after transfer is shown as loading control. Western blot performed with PAP antibody.
Figure 15. RT-PCR analysis of Spl11 expression in Spin1-TAP (OX) line #11-15 in SD and LD over a 24h period. White and black boxes indicate light and dark periods. Error bars are +/- standard deviation from an average of three experiments.
Figure 16. Proposed model for the involvement of SPIN1 and SPL11 in flowering time control under SD and LD. In SD, SPL11 negatively regulates Spin1 expression to allow early flowering. Overexpression of Spin1 affects Spl11 expression by unknown mechanisms and causes repression of Hd3a via Hd1, which ultimately leads to delayed flowering. In LD, Hd1 represses Hd3a causing late flowering; however, since Spl11 accumulates at high levels throughout the day, SPIN1 is repressed, which attenuates the late flowering effect. Overexpression of Spin1 affects Spl11 expression by unknown mechanisms and causes an increase in late flowering by targeting unknown factors and Hd3a. Black lines: regulation in WT; grey lines: regulation in Spin1-Ox lines. Arrows: positive regulation; blunt ends: negative regulation. Question marks: unknown additional components.
CHAPTER 3

Identification and characterization of \textit{spl11}-mediated cell death suppressor mutants (\textit{sds}) in rice

\textbf{Introduction}

Programmed cell death (PCD) is an active physiological process triggered in multicellular organisms to kill its own cells on purpose. It has been widely established that PCD is essential for many aspects in the life cycle of plants including cellular differentiation and various developmental programs (Pennell and Lamb, 1997; Jones, 2001; Hoeberichts and Woltering, 2003), and is also one of the hallmarks of the hypersensitive response (HR), a rapid and localized cell death reaction associated with resistance to pathogens (Heath, 2000; Greenberg and Yao, 2004). Although many of the cytological and biochemical features related to the death of a cell vary depending on the context, it seems clear that Ca$^{2+}$ influx and vacuole collapse are shared mechanisms in all types of PCD observed in plants (Jones, 2001). In addition, a burst of reactive oxygen intermediates (ROI), ion fluxes and the activation of cysteine proteases are also manifestations of HR cell death (Heath, 2000).

The identification and characterization of mutants defective in the initiation and propagation of cell death has not only been pivotal in discovering the molecular mechanisms of PCD in plants, but also support the notion that the cell death program is
under genetic control. These mutants, collectively called lesion-mimics due to the spontaneous generation of cell death in plant tissue, have been studied in *Arabidopsis* (Lorrain et al., 2003), rice (Yin et al., 2000), barley (Wolter et al., 1993) and maize (Walbot et al., 1983). In general, many characteristics of most lesion mimic mutations resemble the systemic acquired resistance (SAR) response: elevated levels of salicylic acid (SA) (mostly in *Arabidopsis* mutants), constitutive defenses with high expression of pathogenesis-related genes and enhanced resistance to otherwise virulent pathogens (Lorrain et al., 2003). However, although not all lesion mimic mutants can be unequivocally associated with disease resistance or HR cell death regulation (see below), they most likely control essential cellular functions that, when compromised, trigger PCD (Pennell and Lamb, 1997). In this regard, the study of lesion mimics has provided many insights into the general mechanisms of cell death control in plants.

Many lesion mimic mutants represent loss-of-function alleles that indicate that PCD is under a tightly regulated negative control and that the genes underlying these mutations have functions in cell death suppression in wild type plants. The *Mlo* gene in barley is a negative regulator of cell death and disease resistance to powdery mildew disease and encodes a protein containing six membrane-spanning helices (Buschges et al., 1997). One of the most well-characterized cell death suppressors is the zinc finger protein LSD1 of *Arabidopsis* (Dietrich et al., 1997). The *lsd1* mutant can not contain the spread of cell death initiated by the HR or by defense responses mediated by SA (Rusterucci et al., 2001) (Aviv et al., 2002). LSD1 has been recently shown to sequester in the cytoplasm a positive regulator of the HR and disease resistance, the transcription factor bZIP10, to prevent the activation of cell death and basal defenses (Kaminaka et al., 2002).
Other negative regulators of the HR, although not originally identified as lesion mimics, are BECLIN-1 and Adi3. Silencing BECLIN-1 in tobacco mosaic virus (TMV)-inoculated plants causes HR cell death to spread to sites both adjacent and distal to the infection site, suggesting that BECLIN-1 is required to contain PCD to the site of inoculation (Liu et al., 2005). Similarly, silencing Adi3, an AGC protein kinase from tomato, causes localized cell death (Devarenne et al., 2006). In unchallenged plants, Adi3 is phosphorylated and activated by another kinase, Pdk1, to suppress cell death (Devarenne et al., 2006). Since Adi3 interacts with, and is phosphorylated by the Avr-Pto-activated resistance protein Pto, it has been suggested that this interaction renders Adi3 inactive in order to release the negative control of cell death (Devarenne et al., 2006). As expected, some lesion mimic genes also negatively control PCD that is not related to the HR and disease resistance, such as cell death triggered by accumulation of phototoxic compounds produced by chlorophyll catabolism. The maize pheophorbide a oxigenase LLS1 and its Arabidopsis ortholog ACD1 are responsible for the degradation of pheophorbide a, which causes light-dependent PCD in the lls1 and acd1 mutants (Gray et al., 1997; Gray et al., 2002; Pruzinska et al., 2003). Similarly, accumulation of porphyrin compounds, due to mutation of the red chlorophyll catabolite reductase enzyme in the acd2 mutant in Arabidopsis, causes a lesion mimic phenotype (Mach et al., 2001). In rice, mutation of a heat shock transcription factor, causing PCD associated with environmental stress, is responsible for the spl7 phenotype (Yamanouchi et al., 2002). Thus, many lesion mimic mutations can result from de-regulation of cellular homeostasis while others are related to disease resistance signaling or HR cell death. However, discrimination between the two types is not always straightforward.
The rice lesion mimic mutant *spl11* was identified in an ethyl methanesulfonate-mutagenized population of cultivar IR68 (Yin et al., 2000). The *spl11* mutation is characterized by spontaneous, developmentally regulated cell death on leaves, which correlates with both enhanced disease resistance and defense-related gene expression (Yin et al., 2000). *Spl11* encodes a protein with U-box and ARM repeat domains (Zeng et al., 2004) that are characteristic features of a group of evolutionarily-conserved proteins involved in the ubiquitin-26S proteasome-mediated protein degradation/modification system in eukaryotes (Hatakeyama and Nakayama, 2003; Vierstra, 2003; Mudgil et al., 2004). In the ubiquitin-proteasome pathway, target proteins are tagged with multi-ubiquitin chains and ultimately directed to the 26S proteasome for degradation (Hatakeyama and Nakayama, 2003). Ubiquitin-activating (E1), -conjugating (E2) and –ligase (E3) enzymes participate in the process of transferring the ubiquitin monomers to the ε-amino group of a lysine residue in the target protein (Hatakeyama and Nakayama, 2003). SPL11 possesses E3 ubiquitin-ligase activity *in vitro* (Zeng et al., 2004). Recent studies have demonstrated that two U-Box proteins, CMPG1 and AtPUB17, and the F-Box E3 ligase ACIF1 are required for cell death and R-gene mediated disease resistance, which provides conclusive evidence linking ubiquitination to defense and PCD in plants (Gonzalez-Lamothe et al., 2006; Yang et al., 2006; van den Burg et al., 2008). Interestingly, evidence is also accumulating that the 26S proteasome negatively regulates PCD (Kim et al., 2003; Kim et al., 2006; Schlogelhofer et al., 2006). These results support the emerging view that the ubiquitination process is a key regulator of PCD and disease resistance in plants. At this point, however, the actual mechanisms linking the plant’s defense response to ubiquitination, the substrate proteins, as well as
the level of involvement of the ubiquitination process in PCD are unknown. In order to understand how Spl11 controls PCD, we have used a forward genetics approach targeted at the identification of suppressor mutants of the cell death phenotype of spl11. Here, we present data characterizing four spl11-mediated cell death suppressors (sds) and discuss the implications of such suppressors in the context of PCD regulation via ubiquitination.

Results

Identification of spl11 suppressor mutants

Seeds of the spl11 mutant GR5717-1-6-3 in the background of cultivar IR64 were treated with EMS in order to isolate mutants that suppress lesion formation. This specific IR64 mutant was selected for mutagenesis because it contains a small deletion at the Spl11 locus that was easily detected by Restriction Fragment Length Polymorphism (RFLP) analysis (Zeng et al., 2004). More than 7500 M1 lines were screened for lesion suppression phenotype at the International Rice Research Institute (IRRI), yielding 91 putative suppressor mutants. Two categories of suppressor mutants were identified: type A and type B putative suppressors, showing total and partial lesion suppression, respectively (Figure 17). All putative suppressors were selfed and advanced to the M3 generation. Genotyping by RFLP of all putative suppressor mutants revealed that all except one of type A suppressors were either heterozygotes for the spl11 mutation or wild type contaminants (data not shown) whereas all type B suppressors and type A suppressor 0963 were homozygotes for the spl11 mutation (Figure 18). Phenotyping and genotyping confirmed one type A suppressor line and six type B suppressor lines with delayed and/or reduced lesion formation phenotypes, compared to IR64 GR5717-1-6-3
Lesion suppression phenotypes

As mentioned above, one complete and several partial suppressor mutants were identified in the screen. The extent of cell death suppression in leaves of 3 month-old plants is shown in Figure 19A. Although the 0963 suppressor is considered “complete”, minute lesions could be seen sporadically, depending on the growing conditions, and lesions sometimes formed very late in development, usually after flowering time and only in leaves (data not shown and Figure 19B). In the partial suppressor mutants, we observed that the appearance of lesions was delayed in comparison to spl11 plants, starting from 20 days to four weeks after spl11, with 3787 and 2143 being the earliest, and 1902 and 6455 the latest. Once the lesions appeared, the degree of suppression varied quantitatively depending on the suppressor mutant (Figure 19A & B). The 1902 mutant was the strongest partial suppressor showing very few lesions on the leaves and almost never lesions on the stems, followed by 6455, which showed a few lesions on stems and leaves (Figure 19A & B). Suppressor 1326 was intermediate (Figure 19A) but a few plants showed no difference compared to spl11, indicating that it was still segregating in the M3 generation. Suppressor 2143 was unusual as it showed some lesions on the leaves but had considerably more lesion formation on the stem (Figure 19A & B), which was only surpassed by 3787 (data not shown). The mutants were named spl11-mediated cell death suppressors (sds). Based on the level of lesion mimic suppression, the mutants were
designated as follows: \textit{sds}1 (0963), \textit{sds}2 (1902), \textit{sds}3 (6455), \textit{sds}4 (1326), \textit{sds}5 (2143) and \textit{sds}6 (3787). Due to the weak suppression phenotype of \textit{sds}6 and to the inability of obtaining homozygous seed of \textit{sds}4, these two mutants were no longer considered in subsequent analyses. In summary, suppression was expressed as both delayed lesion appearance and reduced number of lesions in the \textit{spl11} partial suppressor mutants. Interestingly, timing of lesion appearance was negatively correlated with degree of lesion reduction.

\textit{Inheritance of spl11 suppressors reveals dominant mutations}

Homozygous suppressor lines for \textit{sds}1, \textit{sds}2, \textit{sds}3 and \textit{sds}5 were crossed to the japonica \textit{spl11} mutant line in the TP309 background, designated as TP309-spl11. The original intention of the crosses was to generate mapping populations (see Materials and Methods), and since TP309-spl11 contains the \textit{spl11} mutation, these crosses were informative to study the inheritance of the suppressor mutations. The F1 progeny for all the crosses except the TP309-spl11x\textit{sds}5 had a lesion-suppressed phenotype, suggesting these mutations were dominant. The identity of these F1s as true hybrids was confirmed using random amplified polymorphic (RAPD) markers (data not shown). The RAPD analysis also confirmed that the putative F1s from the TP309-spl11x\textit{sds}5 cross were actually selfed TP309-spl11 (data not shown), which was consistent with the lack of suppressed lesion mimics. F2 progenies segregated with a 3:1 suppressed:lesion mimic ratio (Table 3), confirming that the mutations in \textit{sds}2 and \textit{sds}3 were due to a dominant, single gene. In the case of the TP309-spl11x\textit{sds}1 cross, the F2 data was inconsistent with a 3:1 segregation (P value <0.05; Table 3); it fit a 13:3 suppressed to lesion mimic
ratio (Table 3), suggesting that one dominant and one recessive gene were segregating. Importantly, since the GR5717 spl11 allele is due to a deletion in the Spl11 locus caused by the gamma ray mutagenesis (Zeng et al., 2004), there is no possibility that these suppressor mutations are revertants or second site mutants within the spl11 allele. This was also confirmed by the RFLP analysis on the M3 suppressors (Figure 18).

*Enhanced resistance to bacterial blight is abolished in spl11 suppressor mutants*

It had been previously shown that spl11 has enhanced disease resistance against virulent *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the pathogen that causes bacterial blight (Yin et al., 2000). To test whether the suppressor mutants were affected in their resistance response to the pathogen, *Xoo* race 6 was inoculated onto 2 month-old plants and lesion length was measured 14 days after. As shown in Figure 20A-B, all suppressor mutants lost their resistance to *Xoo*, showing full susceptibility similar to the IR64, WT control, compared to *spl11*.

*Defense-related gene expression correlates with loss of resistance to Xoo*

The loss of enhanced resistance to bacterial blight in the *spl11* suppressor mutants prompted us to check the expression of several defense-related genes, which were shown to be upregulated in *spl11* at the lesion forming stages (Yin et al., 2000). RT-PCR analysis of *PR1a*, *Chitinase III* and *PBZ1* were all downregulated in the suppressor mutants compared to *spl11* (Figure 21). For *PR1a* in particular, the level of expression correlated very nicely with the degree of lesion mimic phenotype (Figure 21). *PR1a* was highly expressed in *spl11*, was intermediate in *sds5*, low in *sds2* and *sds3*, and not
detectable in \textit{sds1} and IR64 (Figure 21). This suggests that \textit{PRIa} transcript accumulation is a good marker for the onset of cell death in these mutants. \textit{PBZ1} expression was lower in the suppressor mutants compared to \textit{spl11} but it was higher than in IR64, even in the \textit{sds1} mutant that showed no lesions (Figure 21). Chitinase III expression levels were the same in the suppressor mutants as in WT IR64 (Figure 21). Taken together, these results suggest that the elevated defenses typical of the \textit{spl11} mutant are downregulated in the suppressor mutants. This explains the lack of enhanced disease resistance to \textit{Xoo}. Interestingly, the expression level of these marker genes, with the exception of \textit{PRIa}, did not necessarily correlate with the degree of cell death suppression, suggesting cell death-dependent and independent gene expression regulation in these mutants.

\textit{Flowering phenotypes of \textit{spl11} suppressor mutants}

We have shown that \textit{spl11} mutant plants show a delayed flowering phenotype in long days (LD) in Chapter 2. To test the effect of the suppressor mutations on flowering, plants were grown under controlled conditions in 10h light/14 h dark and 14h light/10h dark cycles to simulate short and long days, respectively. In short days (SD), no significant differences in flowering time between IR64, \textit{spl11} or the suppressor mutants were observed (Figure 22A). However, in long days (LD), flowering was significantly delayed in \textit{spl11} compared to IR64, with suppressor mutants showing intermediate late flowering (Figure 22B). Interestingly, the degree of lesion suppression was independent of the flowering response in LD since the complete suppressor 0963 (\textit{sds1}) was significantly later flowering than the partial suppressors, most notably 2143 (\textit{sds5}) (Figure 22B). These results suggest that cell death control and flowering time are
separate pathways controlled by SPL11, although some overlap may exist as spl11 still flowers later than any of the suppressor mutants.

*RRT-PCR analysis of SPL11-interacting (Spin) genes in suppressor mutants*

Several *SPL11-interacting* genes were identified in the yeast two-hybrid analysis reported in Chapter 2 and elsewhere. *Spin1* encodes an RNA binding protein of the signal transduction and activation of RNA (STAR) family (Vernet and Artzt, 1997). *Spin3* encodes a protein with limited homology to *Arabidopsis* MOM (maintenance of methylation), which is involved in chromatin methylation (Amedeo et al., 2000). Interestingly, *Spin4* has sequence homology to *Arabidopsis* PCFS4, a pre-mRNA cleavage complex-related protein recently shown to regulate flowering time via the autonomous pathway (Xing et al., 2008). The SPIN6 protein contains a Rho GTPase-activating domain, typical of proteins involved in G protein signal transduction (Wu et al., 2000). To test the possibility that any of the spl11 suppressor mutants may be affected in the expression of putative SPL11-interactors, a preliminary RT-PCR analysis was performed. The expression of *Spin1*, *Spin3* and *Spin4* did not seem to vary significantly between IR64, spl11 and the suppressors (Figure 23). *Spin6* was significantly upregulated in the sds1 suppressor compared to IR64, spl11 and the rest of suppressor mutants (Figure 23). The significance of these results is unclear at present, especially due to recent data indicating that overexpression on *Spin6* in rice causes enhanced resistance to both bacterial blight and rice blast (Liu and Wang, unpublished results).
Screening for polymorphic SSR markers between IR64 and TP309-spl11

In order to obtain markers useful for mapping the suppressor mutations into the rice linkage map, a set of SSR markers was screened for polymorphism between IR64 and TP309-spl11. Out of 300 markers tested, 112 were shown to be reproducibly polymorphic between the two genotypes and were distributed across all 12 rice chromosomes (Table 4). An example of a typical polyacrylamide gel after PCR of SSR markers is shown in Figure 24. These polymorphic markers are being used to locate the suppressor mutations in the rice linkage map (data not shown).

Discussion

The Spl11 gene was previously shown to negatively regulate PCD and broad-spectrum disease resistance to pathogens in rice (Yin et al., 2000). In the present study, we have undertaken a forward genetics approach to identify mutations in rice that can suppress the cell death phenotype in spl11. The spl11 suppressor mutants had varying degrees of lesion mimic suppression, had wild-type levels of resistance to Xoo and did not upregulate the expression of defense-related genes. Taken together, these results show that all previously characterized phenotypes of spl11 were suppressed in these mutants. These results further confirm that cell death and disease resistance in spl11 are under genetic control.

Prior to this study, only two other lesion mimic cell death suppressor screens have been reported: one characterizing suppressors of lsd5 in Arabidopsis (Morel and Dangl, 1999), and the other mapping of a large QTL in maize, slm1, that suppresses the lesion mimic les23 (Penning et al., 2004). In both studies, the lesion suppression phenotype is
characterized by delayed onset of cell death coupled to significant decrease in severity of lesion mimics (Morel and Dangl, 1999; Penning et al., 2004), which is in agreement with the spl11 suppressors phenotype. Both strong and weak lsd 5 suppressors, named phx, were isolated (Morel and Dangl, 1999), similarly to our observation that both total and partial lesion mimic suppression is possible in spl11. The fact that the les23 suppressor was identified as a QTL (Penning et al., 2004) further reinforces the notion of partial cell death suppression in these types of mutants. Moreover, the slm1 QTL as well as some of the phx mutants are dominant (Morel and Dangl, 1999; Penning et al., 2004). Our data indicated that sds2, sds3 and one of the two genes segregating in sds1 were also shown to be inherited as dominant mutations.

In the Arabidopsis phx suppressors, PR1 expression, which is constitutive in lsd5 plants, is only detectable in weak suppressor lines (Morel and Dangl, 1999). This is consistent with our expression data for PRIa, which was positively correlated with cell death severity. We had previously shown that both enhanced defense-related gene expression and resistance in spl11 correlates with the appearance of lesion mimics in leaves (Yin et al., 2000). Although the loss of enhanced resistance to Xoo in the spl11 suppressor mutants correlated with overall decrease in expression of defense-related genes, the patterns of expression of Chitinase III and PBZ1 did not necessarily reflect changes in lesion mimic suppression levels. These results are consistent with the observations that cell death and defense-related gene expression can be uncoupled in studies with double mutants in lesion mimics. For example, in the lesion mimic mutants lsd2, lsd4 and ssi4, enhanced PR gene expression, but not spontaneous cell death, is abolished in the NahG background (Hunt et al., 1997; Shirano et al., 2002), indicating
that depletion of SA due to overexpression of *NahG* uncouples defense-related gene expression from cell death. In addition, the *snc1* mutant causes enhanced disease resistance and elevated expression of *PR* genes in the absence of lesion mimics in *Arabidopsis* (Zhang et al., 2003). More recently, silencing of the gene encoding VPE protease in *Nicotiana benthamiana* plants infected with tobacco mosaic virus (TMV) abolished HR cell death but not the expression of defense-related genes (Hatsugai et al., 2004). These studies indicate that, although cell death and *PR* gene expression seem to be associated in lesion mimic mutants and during a resistance response to a pathogen, they can be genetically and mechanistically isolated from each other. Interestingly, disease resistance seems to be associated with enhanced *PR* gene expression rather than cell death, since SA depletion in *lsd2*, *lsd4* and *ssi4* causes suppression of enhanced resistance to oomycete and bacterial pathogens (Hunt et al., 1997; Shirano et al., 2002). For viral pathogens, on the other hand, HR cell death is required since loss of cell death in VPE-silenced plants compromises the resistance to TMV (Hatsugai et al., 2004).

Taken together, the expression levels of *Chitinase III* and *PBZ1* in our *spl11* suppressor lines are in agreement with the observation that cell death and elevated defenses can be separated, even though we still observed loss of enhanced resistance in the absence of lesion mimics.

*Spl11* encodes an E3 ubiquitin ligase protein with U-Box and ARM repeat domains (Zeng et al., 2004). These characteristics implicate ubiquitination in negative control of PCD and defense. In support of this notion, accumulating evidence suggest that the 26S proteasome and polyubiquitination in plants have anti-PCD activities. Silencing of proteasome subunits causes activation of PCD in *N. benthamiana* (Kim et
al., 2003), while overexpression of a ubiquitin variant unable to form polyubiquitin chains induces cell death in *Arabidopsis* (Schlogelhofer et al., 2006). Interestingly, several E3 ligases recently characterized in plants have also implicated ubiquitination as a positive regulation mechanism in HR cell death and disease resistance (Gonzalez-Lamothe et al., 2006; Yang et al., 2006; van den Burg et al., 2008). It is unclear how SPL11 or any other plant E3 ligases regulate PCD as none of their ubiquitination substrates related to cell death control have been identified to date. However, a bacterial effector with E3 ligase activity negatively regulates the HR by promoting the ubiquitination and degradation of the tomato Fen kinase, inducing susceptibility (Rosebrock et al., 2007). It has been recently suggested that SPL11 might function by regulating NADPH oxidase activity, as *spl11* mutant suspension culture cells accumulate increased levels of H₂O₂ in response to fungal elicitor (Kojo et al., 2006). NADPH oxidase is responsible for the generation of reactive oxygen species (ROS) typically produced upon pathogen recognition and during the HR (Torres and Dangl, 2005). Recent evidence indicate that ROS are actually required to prevent, rather than promote, the spread of cell death in *lsd1* mutant plants (Torres et al., 2005). Thus, the significance of the suggested negative regulation of NADPH oxidase by SPL11 needs to be further investigated. The future cloning and functional characterization of the *spl11* suppressor mutants identified in this study will prove instrumental in elucidating the role of SPL11 in negative control of PCD. Most of the suppressors we have isolated represent dominant mutations. Many recessive mutations, such as *eds1* and *npr1*, have been shown to suppress lesion mimics in *Arabidopsis* via double mutant analyses (Rusterucci et al., 2001; Brodersen et al., 2002; Shirano et al., 2002). However, no dominant mutations,
except for overexpression of the bacterial *NahG* transgene in *ssi, acd6, acd11* or *lsd1* mutants, for example (Rate et al., 1999; Shah et al., 1999; Aviv et al., 2002; Brodersen et al., 2002) have been isolated to date that suppress lesion mimic cell death. Dominant mutations may sometimes represent gain-of-function mutants, resulting in constitutive activation or overexpression of the wild type allele. In the context of suppression of *spl11*-mediated cell death, these dominant mutations might reveal additional negative regulators of cell death in plants. We found that *Spin6* is upregulated in the *sds1* suppressor. Whether this gene is responsible for the lesion mimic suppression phenotype needs to be investigated.

In Chapter 2, we identified a novel function for SPL11 in positive control of flowering time. The finding that the *spl11* suppressors all had intermediate delayed flowering compared to IR64 and *spl11* and yet the strongest suppressor was still significantly more delayed than the partial suppressors, argues for partial overlap between PCD regulation and flowering time control by SPL11. Further characterization of *spl11* suppressors will clarify the relationship between cell death and flowering in plants.

**Materials and methods**

*Mutagenesis by EMS and screening for lesion suppression*

Seeds of the *spl11* mutant G 5717-1-6-3 were used for mutagenesis using ethyl methanesulfonate (EMS) as described (Yin et al., 2000). Seed of the M1 generation was planted in a greenhouse at the International Rice Research Institute (IRRI) in the Philippines and plants were visually scouted for the reduction or absence of lesions in leaves. Putative suppressor lines were tagged and allowed to self-pollinate in order to
obtain next generation mutants for further analysis. A total of 92 putative suppressors were selected for RFLP analysis.

*Restriction Fragment Length Polymorphism (RFLP) analysis*

DNA extraction and DNA gel blot hybridization were performed as described (Zeng et al., 2004). Prior to transfer to the nylon membrane, 10 µg of genomic DNA was digested with EcoR1. A 2.5 Kb genomic fragment from the 5’ region of the Spl11 gene (Zeng et al., 2004) was used as probe in the hybridization reaction.

*Bacterial blight inoculation*

Rice plants were grown in a growth chamber with 12h light, 26°C, 80% relative humidity (RH), followed by 12 h dark, 20°C and 60% RH. Xoo race 6 was plated onto potato sucrose agar medium and grown for 3 days in the dark at 25°C. Bacteria were scraped from the plates and resuspended in sterile distilled water. The bacterial inoculum was diluted to a concentration of 0.5 OD600 and inoculated in 2 month-old rice plants by dipping scissors in the bacterial suspension and clipping the tip of the rice leaf. Plants were kept in the growth chamber for 14 days before lesion measurement.

*RT-PCR analyses*

Total RNA was extracted from leaf tissue of 2 month-old rice plants grown in a growth chamber using the Trizol reagent following the manufacturer’s recommended protocol (Invitrogen). After extraction, RNA was treated with DNase-I (Invitrogen) and 1.5 ug was used for reverse transcription using the Promega RT kit. PCR amplifications
conditions for the ubiquitin control were as follows: 5 min denaturation at 94°C; 24 cycles of 30s at 94°C, 30s at 50°C, and 30s at 72°C; a final extension of 10 min at 72°C. For the rest of genes tested, conditions were the same as ubiquitin except in annealing temperature and number of cycles performed: 58°C annealing, 25 cycles for Chitinase III and PBZ1; 63°C annealing, 25 cycles for PR1a; 50°C annealing, 24 cycles for Spin6; 55°C annealing, 26 cycles for Spin1; 55°C annealing, 25 cycles for Spin4; and 50°C annealing, 25 cycles for Spin3. PCR products were run on 1% agarose gels and visualized on a UV transilluminator to capture the images.

Flowering time experiments
Plants were grown in growth chambers as described above for the Xoo inoculation experiments with the following modifications: for SD, a 10h light/14h dark photoperiod was used; for LD, 14h light/10h dark conditions were used. Heading date was recorded as days from planting until the emergence of the first panicle.

Generation of mapping populations
Once homozygous lines of the spl11 suppressors were confirmed after the M3-M4 generation after selfing, four suppressors were selected to generate mapping populations: 0963, 1902, 2143 and 6455. The japonica ssp. TP309-spl11 line, generated previously (Zeng et al., 2004), was selected as a suitable parent for the cross due to being genetically distant from indica ssp. IR64 and for containing the spl11 mutation. As both the suppressor parent and TP309-spl11 contain the spl11 mutation, the progenies will only segregate for the suppressor loci. The crosses were advanced to the F2 generation, and
phenotypic ratios were determined by visual examination of lesion presence/absence. Due to TP309-spl11 having the spl11 mutation, the ratios could also be used as data to determine the inheritance of the suppressor mutations, similarly to a backcross.

_Screening of rice SSR markers and identification of polymorphic markers between TP309-spl11 and IR64_

In order to identify polymorphic markers between the two parents used in the generation of mapping populations (see above), a set of 300 rice SSR markers was screened for polymorphism between IR64 and TP309-spl11 plants. Ten nanograms of genomic DNA for each parent were used in PCR with the following conditions: denaturation at 94°C for 4 min; 35 cycles of 94°C for 30s, 50-61°C for 30s (depending on the annealing temperature for the pair of primers), 72°C for 30s; and final extension of 72°C for 4 min. PCR products were resolved in 6.5% non-denaturing polyacrylamide gels run at 60V for approximately 4 h. Gels were stained with 0.1% SYBR gold (Invitrogen) dye solution for 30 min and observed on a UV transilluminator. Bands were scored by visual examination of gels, and only polymorphic markers with reproducible results were selected. Out of 300 markers, a set of 112 polymorphic SSR markers was identified, spanning the 12 rice chromosomes (Table 4).
References


Table 2. Segregation after selfing in the M3 generation for \textit{spl11} suppressor mutants.
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**Table 3.** Inheritance of suppressor mutations in crosses with TP309-spl11 (F\(_2\) data).
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*Table 4.* Polymorphic SSR markers between TP309-spl11 and spl11-GR5717
Table 4 continued

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Figure 17. Screening for *spl11* lesion mimic suppressors. 1. *spl11* old leaf; 2. *spl11* fully expanded leaf; 3. putative suppressor fully expanded leaf.
**Figure 18.** Restriction fragment length polymorphism (RFLP) analysis of confirmed type B spl11 suppressor mutants. A 2.5 Kb genomic fragment from TAC20 in the Spl11 genomic region was used as the probe. Genomic DNA was digested with EcoRI.
Figure 19. Lesion suppression phenotypes of *splII*-suppressors.  

**A.** Fully expanded leaf stage, 3 month-old plants.  **B.** Sheath and stem of 3 month-old plants.
Figure 20. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) race 6 inoculation.  

**A.** Lesion length 14 days after inoculation. Ten leaves per genotype were inoculated on 2 month-old plants. Error bars: +/- standard deviation.  

**B.** Pictures showing representative inoculated leaves from A. 1. 6455 (*sds3*); 2. 2143 (*sds5*); 3. 1902 (*sds2*); 4. 0963 (*sds1*); 5. IR64; 6. *spl11*. Scale bar: 4 cm.
**Figure 21.** RT-PCR analysis of defense-related genes in WT, *spl11* and *spl11* suppressors.
Figure 22. Flowering phenotype of *spl11* suppressors. **A.** Days to flower in short days (10h light/14 dark). **B.** Days to flower in long days (14h light/10h dark). Error bars: +/- standard dev.
Figure 23. RT-PCR analysis of Spin genes in spl11 suppressors.
Figure 24. Polyacrylamide gel of SSR marker screen to test for polymorphism between IR64 and TP309-spl11. 1. IR64; 2. TP309-spl11.
CHAPTER 4

Overexpression of an RNA binding protein causes cell death in rice and *Nicotiana benthamiana*

**Introduction**

Programmed cell death (PCD) entails the self-destruction of the cellular machinery and is essential for plant development as well as for accurate responses to abiotic and biotic challenges. For example, removal of the aleurone layer in cereals, senescence, response to ozone exposure, and defense against pathogen infection all require PCD (Jones, 2001; Kuriyama and Fukuda, 2002; Hoeberichts and Woltering, 2003). Many internal and external stimuli can trigger PCD, and the way plant cells are committed to die depends on the type of tissue involved and the inducing agent. For instance, in the vascular tissue, cell contents are eliminated but secondary walls are reinforced to generate tracheary elements (Fukuda, 2000), which is different from the hypersensitive response (HR) cell death triggered in response to pathogens that is intimately linked to disease resistance (Greenberg and Yao, 2004). The study of the unique and shared signaling events between different PCD pathways in plants is under intense investigation. However, despite recent advances in the field, a clear understanding of how PCD is executed and knowledge of the extent of overlap between the numerous cell death programs are both currently lacking in plants.
Due to its importance in various developmental processes and for general homeostasis, it is not surprising that PCD in plants is under tight genetic control. Both negative and positive regulators of cell death have been discovered by molecular genetic studies. Among the negative regulators, the plant Bax inhibitor-1 (BI-1) gene has been implicated in suppressing numerous cell death programs including those triggered by the toxin fumonisin B1 (Watanabe and Lam, 2006), the Magnaporthe oryzae cell wall extract elicitor (Matsumura et al., 2003), and by the lesion mimic mutant mlo (Huckelhoven et al., 2003). This makes BI-1 the best candidate to date for a general plant PCD inhibitor (Watanabe and Lam, 2006), suggesting it may act to suppress cell death at a convergent point common to different pro-death pathways. Among the positive regulators of PCD in plants, many have been identified in disease resistance signaling. For example, EDS1 is a lipase-like protein not only required for the HR activated by R protein receptors during recognition of avirulent pathogens (Falk et al., 1999), but also for cell death in some mutants known as “lesion mimics” (Rusterucci et al., 2001; Brodersen et al., 2002; Shirano et al., 2002). The transcription factor AtMYB30 is required for the initiation of HR cell death in Arabidopsis (Vailleau et al., 2002), whereas HR and disease susceptibility PCDs are both positively-regulated by the MAPKKKα protein in N. benthamiana plants (del Pozo et al., 2004). Recently, the overexpression of an acyltransferase in rice was shown to be responsible for the cell death phenotype of the Spl18 mutant (Mori et al., 2007). Similarly, overexpression or constitutive activation of R genes due to gain-of-function mutations causes cell death (Rathjen et al., 1999; Shirano et al., 2002), confirming that R receptors regulate pro-death pathways that are kept under negative control.
RNA binding proteins are involved in the synthesis, processing, transport, translation, and degradation of various types of RNA molecules in the cell (Fedoroff, 2002). In animals, the RNA binding protein TIA-1, a pre-mRNA splicing factor that regulates the alternative splicing of the apoptosis-promoting protein Fas, has been implicated in PCD (Forch et al., 2000; Izquierdo et al., 2005). To date, the only RNA binding protein shown to have a role in PCD control in plants is the eukaryotic initiation factor 5A (eIF-5A) (Thompson et al., 2004; Feng et al., 2007). eIF-5A isoform 2 positively controls senescence and cell death triggered by fumonisin B1 in addition to various cell growth and division pathways involved in development of vegetative and reproductive organs in Arabidopsis (Feng et al., 2007). We present here evidence for the role of another RNA binding protein in cell death control. RNA binding and SPIN1-interacting 1 (Rbs1) was identified in a yeast two-hybrid screen using the full-length Spin1 cDNA as bait. Overexpression of Rbs1 in both N. benthamiana and rice protoplasts caused cell death. Rbs1 was modestly upregulated in the spl11 lesion mimic mutant compared to WT, suggesting that it might be a positive regulator of PCD in this mutant.

Results

RBS1 interacts with SPIN1 in yeast and in rice protoplasts and encodes a heterogeneous nuclear ribonucleoprotein R-type protein.

In order to identify proteins that interact with the RNA binding protein SPIN1, a yeast two-hybrid screen was performed using the full-length Spin1 cDNA as bait and a rice cDNA library as prey. Approximately half a million yeast colonies were screened and
140 putative positive clones were selected for validation after the first round of screening. Only clones 69-2 and 72 were able to reproduce the interaction based on yeast growth on different selection media (data not shown) and appearance of blue colonies in an X-GAL assay (Figure 25A). Plasmid extraction and sequencing revealed that both the 69-2 and 72 clones contained the same cDNA insert.

Sequence analysis revealed that the insert corresponded to the full-length cDNA of the rice gene *Os11g14430*. The deduced amino acid sequence of *Os11g14430* encoded a protein of 465 residues with a predicted molecular weight of 51.2 KDa and a theoretical pI of 5.15. Searches in various protein databases, including GenBank and Pfam, using the deduced amino acid sequence revealed that the candidate SPIN1 interactor was a putative RNA binding protein containing three RNA Recognition Motifs (RRMs) (Figure 26A). The candidate gene was then named *Rbs1*, for RNA-binding and SPIN1-interacting 1. The RRM is one the most common domains found in heterogeneous nuclear ribonucleoproteins (hnRNP) (Lorkovic and Barta, 2002). Indeed, the RRM motifs in RBS1 were most similar to metazoan Apobec-1 complementation factor 1 (ACF1), a type of hnRNP-R protein involved in apolipoprotein B mRNA editing (Mehta et al., 2000). The homology of RBS1 to ACF1 was limited to the RRM region with 36% identity and 53% similarity. Several plant RBS1-like proteins were identified in *Arabidopsis*, grape (*Vitis vinifera*) and the moss *Physcomitrella patens* subsp. *patens* (Figure 26B).

To determine if SPIN1 and RBS1 interact *in vivo*, a protoplast two-hybrid (P2H) assay was performed. The full-length *Spin1* and *Rbs1* coding sequences were cloned as translational fusions to the yeast GAL4 DNA binding (DB) and activation (AD) domains,
and protein-protein interaction was determined by activation of a GUS (β-glucuronidase) reporter gene driven by a promoter containing GAL4 binding sites. As is shown in Figure 25B, when both SPIN1-DB + RBS1-AD and SPIN1-AD + RBS1-DB proteins were expressed in rice protoplasts, GUS activity significantly increased above background levels indicating that SPIN1 and RBS1 interact in rice cells. The interaction between mammalian Jun and Fos proteins was included as a positive control in the P2H assay (Figure 25B).

**RBS1 co-localizes with SPIN1 in the nucleus**

We have previously shown that SPIN1 is localized in the nucleus (Chapter 2). To determine the subcellular localization of RBS1, the fusion protein DsRed-RBS1 was expressed in both *N. benthamiana* and rice protoplasts. Red fluorescence was observed in the nuclear region in both cases (Figure 27A-B), confirming the nuclear localization of RBS1. DsRed-RBS1 and GFP-SPIN1 were co-expressed in rice protoplasts and in *N. benthamiana*. As shown in Figure 27C and D, SPIN1 and RBS1 co-localize in plant cells.

**Rbs1 expression is induced in spl11 mutant plants**

Since SPIN1 interacts with the E3 ligase SPL11, we tested whether RBS1 and SPL11 also interact in yeast. No interaction between full-length RBS1 and the ARM domain of SPL11 was detected in yeast cells (data not shown). However, since RBS1 and SPIN1 do interact, the expression pattern of *Rbs1* was analyzed by RT-PCR in both WT (IR68) and *spl11* mutant plants. *Rbs1* expression was upregulated in the *spl11* mutant at the old leaf
stage, correlating with the accumulation of many lesion mimics (Figure 28A). The upregulation of \( Rbs1 \) in \( spl11 \) plants was also confirmed in the IR64 background (Figure 28B).

**RBS1 has RNA binding activity in vitro**

Our bioinformatic analyses suggested that RBS1 is an RNA binding protein. To confirm the *in silico* data, we performed an *in vitro* RNA binding assay. RBS1 protein was translated and labeled with biotin using the rabbit reticulocyte lysate system and incubated with ribohomopolymer beads containing poly A, poly U, poly C and poly G RNA molecules. Detection of protein bound to RNA was performed with streptavidin-HRP substrate after SDS-PAGE and blotting. RBS1 bound to poly A, poly U and poly G but not poly C RNA (Figure 29), confirming it has RNA binding activity *in vitro*.

**Overexpression of \( Rbs1 \) in rice protoplasts and in \( N. \) benthamiana causes cell death**

We overexpressed \( Rbs1 \) by agroinfiltration in \( N. \) benthamiana plants. Unexpectedly, after 4-5 days, a patch of dead cells appeared in the infiltrated area with 35S:\( Rbs1 \) but not with the empty vector control (Figure 30A). The extent and intensity of the cell death induced by \( Rbs1 \) was not as strong as with the \( Pantoee stewartii \) effector \( WisE \) used as positive control (Figure 30A). Out of 35 infiltrated leaves, 32 showed the same cell death reaction with overexpression of \( Rbs1 \) compared to zero out of 35 for the empty vector. As expected, overexpression of \( Spin1 \) did not cause any cell death in \( N. \) benthamiana (Figure 30B), which confirms the results observed in transgenic rice plants reported in Chapter 2. In rice protoplasts, GUS activity was significantly reduced when \( Rbs1 \) was
overexpressed compared to empty vector control (Figure 30C). Treatment of GUS-
expressing protoplasts with the rice blast fungal elicitor, which is known to induce PCD
in rice, had a similar effect of significantly reducing GUS activity (Figure 30C). These
results indicate that overexpression of Rbs1 also caused cell death in rice protoplasts.

Unfortunately, these results could not be confirmed by the generation of stable
transgenic lines silencing or overexpressing Rbs1. Transgenic lines carrying an RNAi
construct targeting a 3’ region of the Rbs1 cDNA failed to show any decrease in the
expression of endogenous Rbs1 (Figure 31A-B), even in lines showing high expression of
the transgene (Gus linker, Figure 31A). These results were confirmed in rice protoplasts
transiently expressing RNAi constructs targeting 5’ or 3’ regions of the Rbs1 cDNA:
neither endogenous nor overexpressed Rbs1 levels could be silenced in rice protoplasts
(Figure 31B-C). In addition, when we overexpressed Rbs1 fused to an N-terminal TAP
(tandem affinity purification) tag in transgenic plants, no evidence of cell death was
observed (data not shown). We later found that the presence of the N-terminal TAP tag
fused to RBS1 interfered with the cell death inducing activity, as TAP-Rbs1
overexpression in N. benthamiana plants failed to trigger PCD compared to Rbs1 without
the tag (Figure 31D).

Discussion

We have identified RBS1 as a novel RNA binding protein of the hnRNP-R type in rice
and shown that it interacts with the flowering repressor SPIN1 in both yeast and rice
protoplasts. RBS1 has RNA binding activity in vitro, which, together with the finding
that it contains three RRM domains, suggests that it might be involved in RNA
metabolism. In mammals, it has been recently shown that hnRNP-R binds to the 3’UTR of Serotonin N-acetyltransferase and to the AU rich element of c-Fos mRNA to promote their degradation (Kim et al., 2005; Huang et al., 2008). Whether RBS1 is involved in mRNA turnover needs to be determined in future studies. Overexpression of Rbs1 causes cell death in N. benthamiana and in rice, which suggests that the signaling components involved in RBS1-mediated PCD are conserved between monocots and dicots. In agreement with the latter, several RBS1-like proteins were identified in grape, Arabidopsis and the moss P. patens subsp. patens by sequence homology. Our results suggest that RBS1 is a positive regulator of cell death in plants.

Both SPIN1 and RBS1 interact in vivo and co-localize in nuclei of N. benthamiana and rice, which supports the notion that these two proteins are functionally related. However, overexpression of Spin1 in rice plants (Chapter 2) and in N. benthamiana (this study) did not cause any cell death phenotype. Since SPIN1 was shown to be a flowering repressor (Chapter 2), it is possible that its interaction with RBS1 is part of the flowering pathway and not PCD, which would indicate that RBS1 may have cell death-dependent and independent functions in plants. Unfortunately, it was not possible to test the function of Rbs1 in flowering control due to problems generating RNAi and overexpression lines. Moreover, although RBS1 and SPL11 (an E3 ligase that negatively controls PCD) do not interact in yeast, Rbs1 expression is modestly upregulated in leaves of the spl11 mutant showing lesions. These results suggest that upregulation of Rbs1 can be associated with PCD in the spl11 mutant. We have shown in Chapter 2 that SPL11 and SPIN1 interact in the nucleus of rice protoplasts and that, in addition to its role as a negative regulator of PCD, SPL11 also controls flowering time.
Thus, it is tempting to speculate that RBS1 serves as a link between the cell death and flowering pathways controlled by SPL11. The generation of functional transgenic lines silencing and overexpressing Rbs1 is needed to confirm this hypothesis.

It was unexpected to find that we could not silence Rbs1 using RNAi in rice. As shown in Figure 31, neither transient nor stable transformation of rice with double-stranded RNA molecules targeting Rbs1 could suppress its expression. The RNAi constructs were expressed at high levels in either stably-transformed rice plants or transiently-transfected protoplasts as seen by accumulation of the Gus linker transcript, so failure of silencing can not be attributed to defects in the delivery or integration of the transgene. The target sequences selected were in both 5’ and 3’ regions of the Rbs1 cDNA, which argues against sequence bias in the failure to trigger RNAi in both transgenic plants and rice protoplasts. Using virus-induced gene silencing (VIGS), a GFP transgene could be silenced more efficiently than the endogenous phytoene desaturase (PDS) gene in N. benthamiana or Arabidopsis (Vaistij et al., 2002). The difference in silencing efficiency was related to the ability of the GFP transgene, but not PDS, in supporting secondary siRNA amplification via the RNA-dependent RNA polymerase RDR6 (Vaistij et al., 2002; Herr et al., 2006). It is possible that the endogenous PDS transcript has primary or secondary structures that prevent binding by RDR6 therefore causing inefficient silencing (Vaistij et al., 2002). Similarly, sequence-specific features that prevent the proper interaction of the RNAi machinery with the Rbs1 mRNA could be the explanation for the lack of silencing. Thus, functional analysis of this gene using T-DNA insertion mutants will be necessary. In addition, the TAP tag in the overexpression lines interfered with the function or expression of RBS1 as can be
seen in Figure 31D. Generation of transgenic lines overexpressing \textit{Rbs1} without a tag or with a small epitope tag such as HA will allow confirmation of the cell death phenotype in whole plants.

The only other RNA binding protein with a role in PCD regulation in plants is eIF-5A. Mutation in eIF-5A isoform 2 causes severe developmental and growth defects in addition to delayed senescence and resistance to the PCD-inducing toxin fumonisin B1 in \textit{Arabidopsis} (Feng et al., 2007). Three isoforms of eIF-5A exist in \textit{Arabidopsis} and it has been suggested that they are involved in the transport of target mRNAs across the nuclear membrane to facilitate their translation in the cytoplasm (Thompson et al., 2004). RBS1 may be involved in the processing of cell death-related transcripts. The identification of RBS1 mRNA targets will be instrumental in elucidating its function in PCD control.

\textbf{Materials and methods}

\textit{Yeast two-hybrid screen}

The ProQuest yeast two-hybrid system (Invitrogen) was used to screen for SPIN1-interacting proteins following the manufacturer’s protocol. A full-length \textit{Spin1} cDNA identified in chapter 2 was used as the bait by cloning it into the pDBleu vector (\textit{SalI} and \textit{NotI} sites). A rice cDNA library already available into the pPC86 vector was used as the prey (see Chapter 2). Putative interacting candidates were identified by sequencing of the inserts in pPC86 at the Plant-Microbe Genomics Facility (PMGF) at the Ohio State University.
Subcellular localization experiments

The DsRed-Rbs1 translational fusion was obtained by cloning an Rbs1 coding sequence PCR fragment containing BglII and Sall sites into the pGDR vector (Goodin et al., 2002) to make pGRbs1-Red. The GFP-Spin1 translational fusion was made in the pGDG vector as described in chapter 2 (pGSpin1-GFP). The pGRbs1-Red and pGSpin1-GFP constructs were transformed into Agrobacterium tumefasciens strain GV3101 and used to agroinfiltrate 4 week-old N. benthamiana plants as described in (Goodin et al., 2002). Two after infiltration, infiltrated leaves were observed under a fluorescent microscope to observed the localization of DsRed-RBS1 and/or GFP-SPIN1 fusion proteins. For subcellular localization in rice cells, the same constructs used for agroinfiltration were used to transform rice seedling protoplasts. The rice protoplast isolation and PEG-mediated transformation is described in (Chen et al., 2006). Fluorescence microscope settings and filters were as described in (Chen et al., 2006).

Reverse transcription-PCR analyses

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol. Tissue of IR68 and spl11-IR68 at three different developmental stages (young, fully expanded and old leaves) was collected from 2 month-old, greenhouse-grown plants. Tissue was also collected from 55 day-old, growth chamber-grown plants of IR64 and spl11-GR5717 showing no and many lesions, respectively. To check for silencing of Rbs1 in Rbs1-RNAi lines, leaf tissue from 1 month-old transgenic plants in the T2 generation was collected. For the protoplast transient RNAi assay, frozen protoplasts
previously transfected with the desired construct were used for RNA extraction using the Qiagen Plant RNA easy kit. RNA was treated with DNAse1 (Invitrogen) and 1.5 µg was used for first strand cDNA synthesis with the reverse transcription system from Promega, following the kit’s instructions. *Rbs1* RT-PCR cycling conditions were denaturing at 94°C for 4 min, annealing at 50°C for 30s, extension at 72°C for 30s, for either 23 or 25 cycles with primers *Rbs1*-F (5’-TTATAACCACGCTTGTGCAGAC-3’) and *Rbs1*-R (5’-CTCTTATGCCCATCCTTAGCAG-3’). *Actin* and *Ubiquitin* genes were used as internal controls in RT-PCR. The Gus-linker sequence corresponds to the pANDA and pANDA-mini vectors used for RNAi in rice and is described in (Miki and Shimamoto, 2004). Gus linker RT-PCR conditions were denaturing at 94°C for 4 min, annealing at 50°C for 30s, extension at 72°C for 1min, for 25 cycles, using primers GusPF (5’-CATGAAGATGCGGACTTACG-3’) and GusPR (5’-ATCCACGCGCTATTCCG-3’).

**RNA binding assay**

The *Rbs1* coding sequence was cloned by PCR into the *HindIII* and *XhoI* sites of pET28a to obtain pET-Rbs1. The pET-Rbs1 construct was used for *in vitro* coupled transcription/translation of the RBS1 protein using the TNT rabbit reticulocyte lysate system (Promega) following the manufacturer’s protocol. The Transcend non-radioactive labeling system (Promega) was added to the *in vitro* translation reaction in the rabbit reticulocyte lysate to label the protein with biotin tags fused to lysine residues. *In vitro* translated RBS1 was incubated with beads containing polyadenylic, polycytidyllic, polyuridylic and polyguanylic ribohomopolymers purchased from Sigma. Incubation was done in 500 µl of buffer KHN (150 mM KCl, 20 mM HEPES pH7.9, 0.01% NP-40,
complete protease inhibitors) for 10 min under rotation. Beads were then washed in KHN buffer 3 times and proteins retained in the beads were identified after SDS-PAGE and blotting using streptavidin conjugated to horseradish peroxidase substrate.

Protoplast isolation, transfection and GUS assay

The protocol for rice seedling protoplast isolation and transfection is published elsewhere (Chen et al., 2006). For the GUS activity cell death assay, the pUbi-Rbs1 construct was made as follows: pGRbs1-Red was digested with SalI and blunt-ended; the pUbi-GUS construct described in (Chen et al., 2006) was digested with SacI and blunt ended as well; cut and blunted pGRbs1-Red was digested with BglII to release the Rbs1 sequence, whereas cut and blunted pUbi-GUS was digested with BamHI to remove the GUS gene; finally, the BglII-blunt Rbs1 fragment was ligated to the BamHI-blunt pUbi backbone. pUbi-GUS alone or in combination with pUbi-Rbs1 were transfected into rice protoplasts. Four hours post-transfection, rice blast fungal elicitor was added to the positive control at a concentration of 1mg/ml. GUS activity was measured as described in (Chen et al., 2006).

Agroinfiltration experiments

The Rbs1 and Spin1 coding sequences were PCR-amplified and cloned into the BglII/SalI and XhoI/BamHI sites, respectively, of the pGD vector (Goodin et al., 2002). The TAP-Rbs1 sequence was transferred from the pRbs1-TAP construct made into the Ubix.nc1300.ntap.gck Gateway system vector (see below) to the pGD vector (Goodin et al., 2002). The pRbs1-TAP plasmid was digested with Ascl and blunted, followed by
digestion with XhoI and ligation into XhoI/SmaI-digested pGD vector to obtain pGTAP-Rbs1. The pGWtsE plasmid is described elsewhere (Ham et al., 2006). Constructs were transformed into A. tumefasciens strain GV3101 and used to infiltrate N. benthamiana plants as described in (Goodin et al., 2002).

RNAi and overexpression constructs for rice transformation

The Gateway (Invitrogen)-compatible vectors pANDA and pANDA-mini for RNAi in stable transgenic and transient expression in rice (Miki and Shimamoto, 2004), respectively, were used to generate Rbs1-RNAi constructs. As shown in Figure 31B, a 3’ fragment of Rbs1 was selected for cloning into pANDA, whereas both 5’ and 3’ fragments were cloned into pANDA-mini, following Invitrogen’s Gateway technology. To obtain pRbs1-TAP, the Gateway-compatible rice overexpression vector Ubix.nc1300.ntap.gck, which contains an N-terminal TAP tag fusion (Chern et al., 2005), was used to clone the Rbs1 coding sequence using Invitrogen’s Gateway system protocol. Rice transformation with either pANDA-Rbs1 or pRbs1-TAP was done following a standard protocol described in (Zeng et al., 2004). RNAi constructs made in pANDA-mini were used for transient expression in protoplasts as described above.

Protoplast two-hybrid assay

To make the constructs for the assay, the pA7-NYFP plasmid reported in (Chen et al., 2006) was digested with SpeI and SacI to remove the NYFP fragment followed by self-ligation to create plasmid pA7-Nos. The GAL4-AD and GAL4-DB sequences in the pDBleu and pPC86 plasmids from the ProQuest yeast two-hybrid system (Invitrogen),
respectively, were amplified by PCR using primers GAD-F (5’-ATAGTCGACATGCCCAAGAAGAAG-3’) and GAD-R (5’-TTAGTCGACCACCTCTTTTTTTT-3’) for the AD domain, and GBD-F (5’-ATAGTCGACATGAAGCTACTGTCT-3’) and GBD-R (5’-TTAGTCGACCCTCGACGATACAG-3’) for the BD domain. The GBD and GAD sequences were then ligated into pA7-Nos to make pA7-GAD and pA7-GBD. Protoplast transfection and GUS activity assay was performed as described in (Chen et al., 2006).

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Figure 25. RBS1 interacts with SPIN1 in yeast and in rice protoplasts. A. Identification of Rbs1 by yeast two-hybrid screen of a rice cDNA library using full length Spin1 as the bait. 72 and 69-2 are independent yeast clones containing the pDBleu-Spin1 and pPC86-Rbs1 constructs. Positive clones were identified by an X-GAL assay. B. Protoplast two-hybrid (P2H) assay confirming the in vivo interaction between SPIN1 and RBS1. GUS activity was measured after transfection of rice protoplasts with GAL4 activation (AD) and DNA binding (BD) fusions to SPIN1 and/or RBS1. The interaction between AD-JUN and BD-FOS is shown as a positive control for the P2H assay. Error bars: +/- standard deviation. The assay was repeated twice with similar results.
Figure 26. RBS1 is an RNA binding protein with three RNA Recognition Motifs (RRM). A. Schematic representation of the RBS1 protein. B. Multiple sequence alignment of RBS1 and its putative plant orthologs. At4g00830, from Arabidopsis thaliana; CAO24311 from grape (Vitis vinifera); XP_0017722 From the moss Physcomitrella patens subsp. patens. The alignment was performed with ClustalX and shading of amino acid residues with Genedoc.
Figure 28. Expression analysis of Rbs1 in WT and spl11 mutant plants.  

A. RT-PCR of Rbs1 in leaves of WT (IR68) and mutant spl11 plants at different stages of development and lesion expression. For spl11, young, fully expanded and old leaves have no, few and many lesion mimics, respectively.  

B. RT-PCR of Rbs1 in 55 day-old leaves of short day-grown IR64 (WT) and spl11 mutant plants.
Figure 29. RNA binding assay for RBS1. *In vitro* translated RBS1 protein was incubated with ribohomo-polymers to test for *in vitro* RNA binding activity. RBS1 binds preferentially to Poly G RNA.
Figure 30. Overexpression of Rbs1 causes cell death. A. Agroinfiltration of 35S:Rbs1 in N. benthamiana. WtsE is a bacterial effector that induces a strong cell death and was used as positive control; pGD: empty vector control. B. Agroinfiltration of 35S:Spin1 in N. benthamiana. Notice the lack of cell death. C. GUS activity in rice protoplasts transfected with pUbi-Rbs1 (a construct overexpressing Rbs1), empty vector (control) or treated with the M. grisea cell wall elicitor. The M. g elicitor was used as a positive control for cell death in rice.
Figure 31. Failure to silence \textit{Rbs1} via RNAi and interference of the TAP tag \textit{in planta}. 
\textbf{A.} RT-PCR of \textit{Rbs1} in T1 transgenic lines transformed with a 3′-\textit{Rbs1}-RNAi construct. NPB is the untransformed recipient cultivar. The GUS linker amplification product indicates that the double-stranded RNA is expressed in transgenic plants. 
\textbf{B.} Schematic representation of the \textit{Rbs1} cDNA and coding sequence (cds) and the location of the RNAi sequences targeted for silencing. 
\textbf{C.} RT-PCR analysis of \textit{Rbs1} in rice protoplasts transfected with \textit{Rbs1}-RNAi and 35S:\textit{Rbs1} constructs. 
\textbf{D.} Agroinfiltration of \textit{N. benthamiana} with 35S:\textit{Rbs1} and 35S:TAP-\textit{Rbs1} to check for cell death induction. Notice absence of cell death when leaf is infiltrated with a construct containing the TAP tag.
CHAPTER 5

*Spl11* links programmed cell death to flowering time control in plants

The regulation of programmed cell death (PCD) in plants is an active area of research. In rice, the *spotted leaf 11 (spl11)* lesion mimic mutation is characterized by spontaneous cell death in leaves, elevated defense-related gene expression, and enhanced resistance to virulent strains of *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae*, the pathogens that cause rice blast and bacterial blight, respectively (Yin et al., 2000). The identification of *Spl11* as a gene encoding an E3 ubiquitin ligase provided the first direct evidence in plants of a role of ubiquitination in PCD and disease resistance (Zeng et al., 2004). Ubiquitination is emerging as a key mechanism of PCD control in plants with both positive and negative regulatory functions (see Chapter 1). However, only a handful of plant E3 ligases have been identified to date having a role in the cell death program (Woo et al., 2001; Zeng et al., 2004; Gonzalez-Lamothe et al., 2006; Yang et al., 2006; van den Burg et al., 2008). Moreover, the actual mechanism(s) by which ubiquitination controls PCD as well as the ubiquitination substrates of any plant PCD-related E3 ligase are unknown. Thus, the study of *Spl11* function provides an unprecedented opportunity to unravel the mechanisms of PCD control via ubiquitination. Work done previously suggested that *Spl11* is a negative regulator of PCD in rice due to the loss-of-function phenotype of the *spl11* mutant (Yin et al., 2000; Zeng et al., 2004).
With that premise, one of the goals of the research presented here was to understand how the rice E3 ligase SPL11 exerts its function in PCD and disease resistance.

In Chapter 2, we showed that SPL11 has a novel function in flowering time control via its interaction with SPIN1, an RNA/DNA binding protein of the signal transduction and activation of RNA family. In addition to proving that SPIN1 is a ubiquitination substrate of SPL11, we were able to show that SPL11 promotes flowering by maintaining low expression levels of SPIN1 during the light phase. SPIN1 inhibits flowering in both short and long days and affects Spl11 expression as well. Reciprocal regulation of SPIN1 and SPL11 is a novel mechanism of flowering time control in rice. Among the SPL11-interacting genes isolated in a previously reported yeast two-hybrid screen, SPIN4 was identified as a putative pre-mRNA splicing factor and SPIN6 as a protein containing a Rho GTPase-activating (GAP) domain (Zeng, 2005). Interestingly, a recent search in GenBank using the amino acid sequence of SPIN4 revealed that it has sequence homology to Arabidopsis Pcf11-p similar protein 4 (PCFS4), a protein involved in flowering time control (Xing et al., 2008). Indeed, the pcfs4 mutant has a delayed flowering phenotype and the PCFS4 protein forms a complex with FY, a known polyadenylation factor involved in the autonomous flowering pathway (Simpson et al., 2003). If the function of PCFS4 is conserved between dicots and monocots, it is possible that SPIN4 has also a role in flowering time control in rice. Preliminary data on the Arabidopsis Spl11 ortholog (At Spl11) using a previously identified T-DNA insertion line (Zeng, 2005) have shown that the mutant has both environmentally induced cell death and early flowering in long days (G. L. Wang and I. P. Ahn, unpublished results).

Moreover, initial characterization of Spin6 RNAi and overexpression lines have revealed
that SPIN6 may have important functions regulating disease resistance and flowering time. Similar to SPL11, SPIN6 localized to both the cytosol and the nucleus (Vega-Sanchez and Wang, unpublished results). Spin6-RNAi lines showed increased susceptibility whereas Spin6-OX plants displayed enhanced resistance to bacterial blight and rice blast inoculation, respectively (Liu and Wang, unpublished results).

Additionally, silencing and overexpression of Spin6 caused early and delayed flowering, respectively (Liu and Wang, unpublished results), suggesting that SPIN6 may negatively regulate flowering time, similar to SPIN1. Taken together, these results strongly support our finding that Spl11 has dual function in PCD control and flowering time and suggest that its function is conserved across distantly related plant species.

In Chapter 3, we have successfully isolated mutants that suppress the lesion mimic, the elevated defense gene expression and the enhanced disease resistance phenotypes of spl11. These spl11 suppressor mutants, or sds, provide additional evidence that the PCD function of Spl11 is genetically-controlled and constitute valuable materials for the identification of additional cell death regulators in plants. All except one of the sds mutants partially suppressed the lesion mimic phenotype, which suggests that more than one cell death signaling branch is controlled by Spl11. Alternatively, if the sds mutants represent gain-of-function mutations, then it is possible that this causes only partial inhibition of cell death. Interestingly, the sds mutants showed an intermediate delayed flowering phenotype in long days resembling more spl11 than the WT IR64. This delayed flowering phenotype was independent of the degree of lesion suppression since sds1, the strongest cell death suppressor, was the one with the latest
flowering. These results suggest that the PCD and flowering pathways controlled by Spl11 partially overlap.

We were unable to identify any cell death or disease resistance phenotype in our transgenic plants silencing and overexpressing Spin1 (Chapter 2). Spin1 is part of a seven-member gene family in rice and it is possible that the lack of phenotype in the RNAi lines was due to functional redundancy. The lack of PCD or resistance phenotype when Spin1 was overexpressed in rice (Chapter 2) or in N. benthamiana (Chapter 4) suggest that this gene is not involved in PCD. However, we cannot completely rule out a role of Spin1 in cell death control until we address the functional redundancy issue. We identified RBS1, an hnRNP-R protein, as an interacting partner of SPIN1 that causes cell death when overexpressed in plants (Chapter 4). We were not able to test the involvement of RBS1 in flowering time due to problems generating transgenic lines successfully silencing and overexpressing Rbs1 (Chapter 4). Rbs1 is moderately upregulated in old leaves that show lesions in the spl11 mutant, suggesting it may contribute to the cell death phenotype. Thus, it is tempting to speculate that RBS1 acts at a junction linking the SPL11-mediated PCD and flowering time control pathways. Taken together, the data presented in this dissertation can be incorporated into a model of flowering time and PCD regulation by the E3 ligase SPL11 (Figure 32).

How does SPL11 control flowering and PCD? The involvement of RNA binding proteins such as SPIN1, RBS1 (shown in this work), and possibly SPIN4 (Zeng, 2005) strongly suggests that SPL11 may form a complex associated with RNA metabolism. SPIN1 and SPIN4 have sequence homology to pre-mRNA splicing factors (Berglund et al., 1997; Xing et al., 2008) whereas RBS1 may either have RNA editing or RNA
turnover functions based on its sequence similarity to hnRNP-R proteins (Mehta et al., 2000; Kim et al., 2005; Huang et al., 2008). A recent bioinformatics analysis revealed that several E3 ligase proteins containing domains associated with RNA binding could be found in animals, plants and fungi (Lucas et al., 2006) suggesting that the link between ubiquitination and RNA metabolism is an ancient, evolutionarily conserved mechanism. In yeast, a link between pre-mRNA splicing and ubiquitination has been proposed following studies on the pre-mRNA splicing factor Prp19p. Prp19p, along with its interactor proteins form the nineteen complex (Ntc), which is believed to represent a subcomplex of the spliceosome (Ohi and Gould, 2002). Prp19p is a U-box protein with WD40 repeats, and it was recently shown that it functions as a ubiquitin ligase in vivo (Ohi et al., 2003). It has also been demonstrated that mutations in the U-box domain of Prp19p abolish both its pre-mRNA splicing ability and its E3 ligase activity (Ohi and Gould, 2002; Ohi et al., 2003). Thus, it is likely that SPL11 functions in a complex regulating splicing or other post-transcriptional mechanism. The identification and functional analysis of mRNA targets of SPIN1 and RBS1 is necessary to test this hypothesis. The identification of SPIN6 as a putative Rop GAP suggests that G protein signaling is also involved in the regulation of PCD and flowering by SPL11. A heterotrimeric G protein and OsRac1 have been shown to regulate hydrogen peroxide-induced cell death and disease resistance in rice (Kawasaki et al., 1999; Suharsono et al., 2002). OsRac1 and Heterotrimeric G protein alpha seem to activate OsMAPK6 in response to a sphingolipid elicitor (Lieberherr et al., 2005). How PCD and especially flowering are controlled via G protein signaling needs to be determined in future studies. As a Rop GAP protein, SPIN6 may inactivate a Rop GTPase involved in either positive
control of flowering or cell death. This would explain why Spin6-RNAi plants show a lesion mimic phenotype at late maturity (Liu and Wang, unpublished results). Figure 33 shows a model of SPL11 signaling incorporating all its putative components.

Cell death control and flowering are seemingly two very distinct mechanisms. The data presented in this dissertation strongly support the idea that SPL11 links cell death regulation to the switch to reproductive development in rice. It is not clear, at this point, why these two processes are linked in the context of Spl11-mediated signaling. It is widely known that plants can accelerate flowering in response to environmental stress. For example, *Arabidopsis* plants treated with UV-C light show precocious flowering (Martinez et al., 2004). Interestingly, the same study also proved that salicylic acid (SA) is required for flowering in both stressed and non-stressed plants, as SA-deficient plants are late flowering (Martinez et al., 2004). SA has been extensively characterized as a signaling molecule required for systemic resistance to pathogens, the hypersensitive response (HR) and in some cases for cell death progression in some lesion mimic mutants (Weymann et al., 1995; Rate et al., 1999; Shah et al., 1999; Shirasu and Schulze-Lefert, 2000; Aviv et al., 2002; Brodersen et al., 2002; Hoeberichts and Woltering, 2003). Thus, SA seems to link plant stress responses, such as UV-C and defense, to reproductive development, a mechanism that assures the survival of a plant population under unfavorable environmental conditions (Martinez et al., 2004). In rice, Spl11 acts as a negative regulator of cell death and spl11 mutants have delayed flowering compared to WT in long days (Chapter 2). If we assume that the lesion mimic phenotype and elevated defenses of spl11 are a source of stress, then we should expect the mutant to flower earlier, not later. Another explanation could be drawn from the evolutionary theory of
senescence as it applies to plants. In brief, this theory states that natural selection will favor mutations or traits that allow reproductive success rather than extending the lifespan of an individual (Kirkwood and Austad, 2000). In plants, a key characteristic of the senescence program is the recycling of cellular components and nutrients for growth of other tissues and organs. In that respect, it has been suggested that cell death in senescence is the detrimental consequence of natural selection’s recruitment of multiple biochemical pathways involved in nutrient salvage to assure reproductive success (Bleecker, 1998). This has been demonstrated for the cpr5 lesion mimic mutant of Arabidopsis (Jing et al., 2007). The cpr5 mutant is pleiotropic and different alleles have been identified by independent groups in mutant screens for ethylene-induced senescence (Jing et al., 2007), enhanced resistance to pathogens (Bowling et al., 1997), abnormal trichome development (Kirik et al., 2001) and dark-induced senescence (Yoshida et al., 2002). CPR5 is then required for normal growth and development, including the repression of premature cell death in young plants (Jing et al., 2007). As the plant ages, however, the level of CPR5 expression increases and it has been shown that overexpression of the gene causes accelerated senescence at late developmental stages, after flowering has occurred (Jing et al., 2007). Thus, this explains how a gene can have multiple roles in plant development and in negative control of PCD. Indeed, many senescence-associated genes have also been found to possess important functions in development. For example, the ORE9 F-Box protein controls the senescence program mediated by several plant hormones in addition to its role in lateral shoot branching (Woo et al., 2001; Stirnberg et al., 2002). Spl11 has not been shown to be associated with senescence, however it is expressed constitutively (Zeng et al., 2004) which suggests it
may be involved in many cellular processes. In addition, cell death in the *spl11* mutant is only triggered in fully expanded and mature leaves but not in young leaves (Yin et al., 2000), suggesting that the cell death program is developmentally regulated. Interestingly, a microarray analysis comparing the WT and *spl11* mutant expression profiles revealed that several photosynthesis associated transcripts were downregulated in the mutant (Zeng, 2005). It is widely known that chlorophyll degradation is a hallmark of senescence. Taken together, the characteristics of the *spl11* mutation could be associated with a premature senescence program similar to CPR5, and SPL11 may suppress a PCD program to avoid early senescence and promote normal development. SPL11 also acts as a repressor of the late flowering phenotype conferred by overexpression of *Spin1* in long days (Chapter 2). Since long days are flowering restrictive conditions in rice, SPL11 could attenuate the late flowering effect of long day conditions to assure reproductive development. Knocking out *Spl11* would then cause premature senescence in both short and long days coupled to very delayed flowering in long days only. It would be interesting to see whether SPL11 has a positive role in senescence after flowering, similar to CPR5 (Jing et al., 2007).

Altogether, the data presented in this dissertation suggests that the E3 ligase SPL11 positively controls flowering time by a process that involves ubiquitination and RNA metabolism, and negatively regulates cell death by yet-to be discovered mechanisms. The control of PCD and flowering time seems to involve partially overlapping pathways, as suggested by the *sds* mutants. Further characterization of SPL11, its interacting partners as well as the cloning of the suppressor mutants will
provide the framework to understand how ubiquitination and RNA metabolism regulate
PCD and flowering time in plants.

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Figure 32. Model of SPL11 dual role in PCD and flowering time control. Arrows indicate signaling in the pathway. Question mark: unknown but suggested role.
Figure 33. Model of SPL11 signaling in the cell. 1: SPL11 and SPIN6 may shuttle between the nucleus and the cytoplasm. 2: Cytosolic SPIN6 may inactivates a putative Rop protein by facilitating GTPase activity; this promotes enhanced disease resistance and suppresses both PCD and flowering; alternatively, SPIN6 negatively controls PCD directly (3); SPL11 may suppress disease resistance by targeting SPIN6 (4). 5: SPL11 represses PCD directly or by recruiting the SDS proteins. SDS1 may also contribute to flowering regulation. 6: in the nucleus, SPL11 forms a complex with SPIN1 and possibly SPIN4 to regulate flowering time; both SPIN1 and SPIN6 repress flowering and SPL11 promotes flowering by negative regulation of SPIN1 and SPIN6. RBS1 may also contribute to flowering time control. 7: RBS1 positively controls PCD possibly by processing cell death-promoting transcripts; SPIN1 may not be involved in this RBS1 function. Arrows: activation/positive control; blunt arrows: repression/negative control. Dotted lines: putative function/role. Question mark: unknown component/mechanism. Rop: plant Rho protein.
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