RBS1, an RNA Binding Protein, Interacts with SPIN1 and Is Involved in Flowering Time Control in Rice

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Abstract

The rice U-box/ARM E3 ubiquitin ligase SPL11 negatively regulates programmed cell death (PCD) and disease resistance, and controls flowering time through interacting with the novel RNA/DNA binding KH domain protein SPIN1. Overexpression of Spin1 causes late flowering in transgenic rice under short-day (SD) and long-day (LD) conditions. In this study, we characterized the function of the RNA-binding and SPIN1-interacting 1 (RBS1) protein in flowering time regulation. Rbs1 was identified in a yeast-two-hybrid screen using the full-length Spin1 cDNA as a bait and encodes an RNA binding protein with three RNA recognition motifs. The protein binds RNA in vitro and interacts with SPIN1 in the nucleus. Rbs1 overexpression causes delayed flowering under SD and LD conditions in rice. Expression analyses of flowering marker genes show that Rbs1 overexpression represses the expression of Hd3a under SD and LD conditions. Rbs1 is upregulated in both Spin1 overexpression plants and in the spin1 mutant. Interestingly, Spin1 expression is increased but Spin1 expression is repressed in the Rbs1 overexpression plants. Western blot analysis revealed that the SPIN1 protein level is increased in the Rbs1 overexpression plants and that the RBS1 protein level is also up-regulated in the Spin1 overexpression plants. These results suggest that RBS1 is a new negative regulator of flowering time that itself is positively regulated by SPIN1 but negatively regulated by SPL11 in rice.

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Introduction

Flowering time is controlled by endogenous and environmental signals. In Arabidopsis, a long day (LD) plant, extensive genetic analyses have identified four flowering pathways: photoperiod, autonomous, vernalization, and gibberellin induced pathways, as well as a series of signaling molecules [1,2,3,4,5]. Among them, the FLOWERING LOCUS T (FT) protein is the florigen signal, which moves from stem vascular tissue to the shoot apical meristem (SAM) to promote flowering [6]. CONSTANS (CO) is a key regulator of the photoperiod pathway. CO is a positive regulator of FT and is induced under LD conditions [7,8].

Rice, a short day (SD) plant, has a similar molecular mechanism to that of Arabidopsis. Rice heading date 3a (Hd3a) and heading date 1 (Hd1) are the orthologs of FT and CO, respectively [9,10]. Hd1 promotes flowering under SD conditions but inhibits Hd3a under LD conditions. The early heading date 1 (Ehd1) protein positively regulates the expression of Hd3a, influencing the flowering time in rice independent of Hd1. This pathway only exists in rice as there is no Ehd1 ortholog in Arabidopsis [11]. Previous research has shown that Hd3a and its homologue RICE FLOWERING LOCUS T1 (RFT1), act as rice florigens that activate the expression of OsMADS15 [12,13]. Recently, it has been shown that the rice florigen protein Hd3a interacts with 14-3-3 proteins in the apical cells of shoots, yielding a complex that translocates to the nucleus and binds to the OsFD1 transcription factor to regulate flowering time [14].

RNA binding proteins are involved in the synthesis, processing, transportation, translation, and degradation of various types of RNA molecules in the cell [15]. In Arabidopsis, there are 196 genes encoding RNA recognition motif (RRM)- containing proteins [16]. The RNA binding protein FGA has two RRMs and promotes flowering under LD condition [17]. Another RNA binding protein gene, FLK, regulates the autonomous flowering pathway via FLC in Arabidopsis [18]. More recently, another RRM-containing protein, LIF2, was identified in Arabidopsis and shown to be involved in flowering time control and cell fate [19]. In rice, there are at least 221 RNA binding proteins [20]. Among them, 31 contain the RRM RNA binding motif. The function of the RRM RNA binding proteins in rice is unknown.

We previously demonstrated that Spotted Leaf 11 (SPL11), a functional U-box/ARM E3 ligase, is involved in the regulation of programmed cell death (PCD), defense and flowering time in rice [21,22,23]. In a yeast-two hybrid screen using SPL11 as the bait, we identified the SPL11 Interact Protein 1, SPIN1, which is a KH domain RNA binding protein. SPL11 monoubiquitinates SPIN1 in vitro and interacts with SPIN1 in the nucleus [22]. Overexpression of Spin1 in transgenic rice causes late flowering under SD and LD conditions [22]. In order to identify proteins that interact...
with SPIN1, we performed a yeast two-hybrid screen using Spin1 full-length cDNA as the bait. We identified a novel RRM-containing protein that we have named RNA-binding and SPIN1-interacting 1 (RBS1). RBS1 interacts with SPIN1 in vitro and in vivo and binds RNA in vitro. Overexpression of Rbs1 in transgenic rice leads to late flowering under both SD and LD conditions. These results revealed that RBS1 plays a negative role in rice flowering and is a new component in the SPL11-mediated signaling pathway.

Materials and Methods

Measurement of the Flowering Time Under SD and LD Conditions

Rice (Oryza sativa) seeds of mutant, transgenic and wild-type plants were sterilized and germinated in half-strength Murashige and Skoog medium for 7 d, and then transferred to a pot with sterilized soil in a growth chamber with the same conditions as described by Vega-Sánchez et al. (2008). Rbs1-ox plants were generated in the cv. Nipponbare background, while the knockout mutant was obtained from the Postech collection in Korea (cv. Dongjin). For flowering time measurements, plants were grown either in 10/14 h light/dark for SD or 14/10 h light/dark for LD. Rice flowering time was measured in days from germination until emergence of the first panicle. For diurnal expression analyses, young leaves were harvested from wild-type Nipponbare, Rbs1 overexpression lines, Dongjing and rbs1 plants of 50 day-old (SD) or 60 day-old (LD) plants at 4 h intervals for a total of 24 h.

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 Spin1 cDNA was used as the bait by cloning it into the pDBleu vector (SalI and NotI sites). A rice cDNA library available into the pPC86 vector was used as the prey (22). 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 the BglII and SnaI sites into the pGDR vector to make pGRbs1-Red. The GFP-Spin1 translational fusion was made in the pGDG vector [pGSpin1-GFP] as described previously [22]. The pGRbs1-Red and pGSpin1-GFP constructs were transformed into Agrobacterium tumefaciens strain GV3101 and used to agroinfiltrate 4 week-old N. benthamiana plants as described previously [24]. Two days after infiltration, infiltrated leaves were observed under a fluorescent microscope to detect 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 transfor-
Reverse Transcription (RT)-PCR Analyses

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer’s protocol. 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. Real-time quantitative RT-PCR (qPCR) was performed in a final volume of 50 μL, including 25 μL iQ SYBR Green Supermix (Biorad), 2 μL of the diluted first-strand cDNA as templates and 0.2 μM of each primer. The reactions were carried out with the Biorad IQ5 system in the following program: 94°C for 4 min, 40 cycles of 95°C for 5 s, and 58°C for 20 s, 72°C for 30 s. Every experiment was repeated at least three times. The primers of Hd1, Hd3a, Ubq, Rbs1, Spin1 and Spl11 are listed in Table S1. The level of ubiquitin (UBQ) expression was used to normalize the expression ratio of each gene.

RNA Binding Assay

The Rbs1 coding sequence was cloned by PCR into the HindIII and Xhol sites of pET28a to obtain pET-Rbs1. As a negative control, unrelated protein Avr-Pita was cloned into BamHI and SalI site of the same vector and designated as pET-AvrPita. The 6XHIS-RBS1 or 6XHIS-Avr-Pita protein was induced in E. coli and purified using HIS-Select® Nickel Affinity Gel (Sigma) according to manufacturer’s instruction. Either purified 6XHIS-RBS1 or 6XHIS-Avr-Pita was incubated with beads containing polyuridylic, and polyguanylic ribohomopolymers as well as calf thymus single- and double stranded DNA purchased from Sigma.

Incubation was done in 500 μl of buffer KH2 (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 KH2 buffer 5 times and proteins retained in the beads were identified by Immunoblotting using anti-HIS antibody (Thermo Scientific).

Constructs

In general, constructs were generated by PCR amplification of the target gene using primers containing appropriate restriction enzyme sites and Spin1 or Rbs1 cDNA template, followed by ligation into a desired vector. For the BiFC assay, the Rbs1 and Spin1 constructs in the pA7-NYFP and pA7-CYFP vectors were constructed following the procedure by Chen et al. (2006). The Rbs1 overexpression construct was made into the Gateway (Invitrogen)-compatible vectors Ubix.nc1300.ntap.gck, following the Gateway cloning protocols. Two artificial miRNA constructs were made that targeted the 21 bp of Rbs1’s 3’UTR (gatggatttgtatataacttatga, 50 bp after the stop code), which was obtained from the WMD3 website (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) [26]. The first construct has one mismatch and the second construct has two mismatches against the target site.

BiFC Detection of the Interact between SPIN1 and RBS1

Fluorescence microscopy analyses were done by transfection of rice protoplasts with various constructs as described [25].

In vivo Co-IP assays. For in vivo Co-IP assay, Agrobacterium strain GV3101 carrying expression vectors of RFP:RBS1 and
were agroinfiltrated *N. benthamiana* leaves to express the proteins in vivo. Two days after agroinfiltration, *N. benthamiana* leaf tissues were harvested, and total proteins were extracted with a native buffer including 50mM K+ -HEPES (pH 7.4), 110mM KOAc, 2mM MgCl2, 0.1% Tween-20, 0.2% Triton, and plant protease inhibitor. A 15-μl volume of anti-HA agarose suspension (Sigma) was added to the protein samples loaded in the column, and the mixtures were kept at 4°C with head-to-tail shaking overnight. The samples were washed five times using 1X IP buffer following the manufacturer’s instruction (Sigma). After 50 μl of 1X SDS sample loading buffer was added to each column, samples were heated to 95°C for 5 min. Twenty microliters of each sample was loaded to the protein gel for immunoblot analysis using anti-HA (Sigma) and anti-RFP (BGI, China) antibodies.

### Western Blot

The protein extraction and protein gel blot assay were done as described [27]. Anti-HA tag antibody (Roche), Anti-RBS1 antibody (Beijing Protein Innovation, China), Anti-SPIN1 antibody (Beijing Protein Innovation, China) and Anti-peroxidase (PAP) antibody (Sigma-Aldrich) were used to detect these proteins. Chemiluminescence was detected by the ChemiDoc XRS system (Bio-Rad).

### Results

**RBS1 Interacts with SPIN1 in Yeast and in Rice Protoplasts and Encodes a Heterogeneous Nuclear Ribonucleoprotein R-type Protein**

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 the bait and a rice cDNA library as the 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 and appearance of blue colonies in an X-GAL assay (Figure S1). Sequencing analysis revealed that both 69-2 and 72 clones contained the same cDNA insert.

BLAST searches showed that the insert corresponded to the full-length cDNA of the rice gene LOC_Os11g14430. The deduced amino acid sequence of Os11g14430 encodes 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 RRMs (Figure 1A). The candidate gene was then named *Rbs1*, for RNA-binding and SPIN1-interacting 1. The RRM is one of the most common domains found in heterogeneous nuclear ribonucleoproteins (hnRNP) [16]. 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 [28]. 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 1B). So far, only the function of the *Arabidopsis* closest relative of RBS1 has been determined; it was shown that this protein, known as LIF2, interacts with the polycomb repressive complex protein LHP1 and is involved in flowering time and flower organ development [18].

**RBS1 Co-localizes and Interacts with SPIN1 in the Nucleus**

We have previously shown that SPIN1 is localized in the nucleus [22]. To determine the subcellular localization of RBS1, we expressed the fusion protein 6xHis-RBS1 in *Nicotiana benthamiana*. Red fluorescence was observed in the nuclear region in the infiltrated cells (Figure 2A), indicating the nuclear localization of RBS1. We then co-expressed 6xHis-RBS1 and GFP-SPIN1 in *N. benthamiana* and in rice protoplasts, separately. As shown in Figure 2B and 2C, SPIN1 and RBS1 co-localized in the nucleus of both *N. benthamiana* and rice cells.

To confirm the Y2H result, we used the bimolecular fluorescence complementation (BiFC) technique to test the
interaction between SPIN1 and RBS1 in rice protoplasts [25,28]. No fluorescence signal was detected when NYFP-SPIN1 or NYFP + CYFP-RBS1 (Figure 3A) were expressed in rice protoplasts, respectively. However, fluorescence was fully reconstituted in the nucleus only when full-length SPIN1 and RBS1 proteins were co-expressed in rice protoplasts as NYFP and CYFP fusions, respectively (Figure 3A), indicating that these proteins interact in vivo and that the interaction occurs in the nucleus. To further confirm the interaction in vivo, we performed a co-IP experiment by expressing SPIN-HA and RBS1-RFP fusion proteins in N. benthamiana by agroinfiltration. As can be seen in figure 3B, RBS1-RFP co-immunoprecipitated with SPIN-HA.

Figure 5. Flowering time of the Rbs1 overexpression plants and the expression pattern of flowering marker genes under SD and LD conditions. (A) Schematic diagram of the Rbs1 overexpression construct. The Rbs1 cDNA sequence fused with the HA tag is placed after the maize ubiquitin promoter (B) Flowering time (days) of the Rbs1 overexpression plants and wild type Nipponbare plants (NPB) was measured under SD and LD conditions (C) Real-time PCR analysis of the expression of Hd3a and Hd1 in the Rbs1 overexpression plants and wild type Nipponbare plants under SD and LD conditions.

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Figure 6. Gene expression and protein accumulation patterns of Rbs1, Spin1 and Spl11 in the Rbs1 and Spin1 overexpression plants. A. Q-PCR analysis of the expression pattern of Spin1 and Spl11 in the Rbs1 overexpression plants under LD and SD conditions. B. Expression of Rbs1 in the Spin1 overexpression and RNAi plants. C. Western blot detection of RBS1 and SPIN1 proteins in the Spin1 overexpression and RNAi plants. RBS1 was detected using a native antibody. The SPIN1-TAP fusion protein was detected in the Spin1-OX plants using the PAP antibody. D. Western blot detection of RBS1 and SPIN1 proteins in the Rbs1 overexpression lines; RBS1 and SPIN1 native antibodies were used in the immunoblot detection. doi:10.1371/journal.pone.0087258.g006
RBS1 has Nucleic Acids Binding Activity in vitro

Our bioinformatic analyses suggested that RBS1 is a putative RNA binding protein. To confirm the in silico data, we performed an in vitro RNA binding assay. 6xHis-RBS1 protein was purified from E. coli and incubated with ribonucleopolymer beads containing poly U and poly C RNA molecules, as well as calf thymus single and double-stranded DNA. Detection of proteins bound to nucleic acids was performed by western blot analysis using an anti-His antibody after SDS-PAGE and blotting. The assay showed that 6xHis-RBS1 bound to both RNA and DNA molecules in vitro (Figure 4). The unrelated avr-Pita protein was used as negative control in the same RNA and DNA binding experiments (Figure 4).

Rbs1 Overexpression Causes Late Flowering Under LD and SD Conditions

To analyze the function of Rbs1, we obtained the rbs1 T-DNA mutant, PFG_4A-01883.R, from the rice T-DNA insertion sequence database [29]. In the mutant, the T-DNA inserted in the fourth intron of the Rbs1 locus. The genotype was confirmed by PCR using Rbs1 and T-DNA specific primers (Figure S2A and B). Real-time PCR analysis revealed that the transcription of RBS1 was completely abolished (Figure S2C). However, no significant difference in flowering time in LD and SD was found between the rbs1 knockout mutant and the wild type Dongjin (Figure S2D). The Rbs1 silencing transgenic lines produced by the artificial microRNA technique showed similar result (data not shown).

We also constructed an Rbs1 overexpression vector in which the Rbs1 full-length cDNA was fused with an HA tag under the control of the maize ubiquitin (Ubi) promoter (Figure 5A). Through Agrobacterium-mediated transformation, we obtained more than twenty Rbs1 overexpression transgenic lines. Among them, two T3 homozygous lines with high expression of the transgene, RBS1-OX-3 and -11, were used for the flowering time and gene expression analyses. To confirm the overexpression of Rbs1 in the transgenic lines, we detected the RBS1 protein in the wild type and overexpression transgenic plants using both anti-RBS1 and anti-HA antibodies. Western blot analysis showed that the RBS1 protein level was high in both transgenic lines (Figure S3). On the contrary, no RBS1 band was detected in the wild type control plants.

In a previous study we showed that the Spin1 overexpression plants flower late under SD and LD conditions and that the spl11 mutant plants flower late under LD conditions. To evaluate whether Rbs1 functions in flowering time, we grew the Rbs1 overexpression and wild type Nipponbare plants under SD and LD conditions. Under both conditions, the Rbs1 overexpression plants flowered one week later than the wild type plants (Figure 5B).

To gain insights into the Rbs1-mediated flowering mechanism, we detected the diurnal expression of the flowering marker genes Hd3a and Hd1 in the Rbs1 overexpression and the wild type plants using real-time PCR. Under SD and LD conditions, the expression of Hd3a was repressed in the Rbs1 overexpression plants at most of the time points (Figure 5C), which is consistent with their late flowering phenotype and the previous report that Hd3a’s transcript is highly correlated with the flowering induction in the Spin1 overexpression lines [22] and other genotypes [30]. No significant change in Hd1 expression was observed between wild type NPB and Rbs1-Ox plants (Figure 5C). These results suggested that RBS1 may regulate the expression of Hd3a in SD to cause late flowering in rice.

RBS1 Negatively Regulates Spl11 Expression but Positively Regulates Spin1 Expression

Since SPIN1 interacts with SPL11 [22], we tested whether RBS1 also interacts with SPL11. No interaction between RBS1 and SPL11 was detected in yeast (data not shown). To determine the relationship among RBS1 and SPIN1 and SPL11 at the transcriptional level, we investigated the expression pattern of Spin1 and Spl11 in the Rbs1 overexpression plants. Real-time PCR analysis showed that the expression of Spin1 in the Rbs1 overexpression plants was higher than the wild type at 6 h after dawn but lower at 10 h in LD (Figure 6A, left upper panel). At midnight, the expression of Spl11 was repressed in the Rbs1 overexpression plants (Figure 6A, left lower panel). However, the expression of Spin1 and Spl11 in the Rbs1 overexpression plants in SD was not significantly different although both genes had higher expression at 14 h compared with that in wild type plants (Figure 6A, right upper and lower panels). We also detected the expression of Rbs1 in the Spin1 overexpression and RNAi plants and spl11 mutant plants. The expression of Rbs1 in the Spin1 overexpression plants was higher than in the wild type plants, but did not vary significantly in Spin1 RNAi plants (Figure 6B, Figure S4A). Rbs1 expression was upregulated in the spl11 mutant in the cultivar IR68 background at the old leaf stage when lesion mimics were present [31], correlating with the accumulation of many lesion mimics (Figure S4B). The upregulation of Rbs1 in the spl11 plants was also confirmed in the IR64 background (Figure S4C).

Furthermore, we determined the protein levels of RBS1 and SPIN1 in the Rbs1 and Spin1 overexpression plants using anti-RBS1 and anti-SPIN1 antibodies. Western blot analysis showed that enhanced accumulation of the RBS1 protein was detected in the Spin1 overexpression plants (Figure 6C) and that the accumulation of the SPIN1 protein was higher in the Rbs1 overexpression plants (Figure 6D). We also detected that the RBS1 protein level was increased in the spl11 mutant (Figure S4D), which is consistent with the expression data mentioned above. These results suggest that there is a positive relationship between RBS1 and SPIN1 but a negative relationship between SPL11 and RBS1.

Discussion

We identified RBS1 as a novel RNA binding protein of the hnRNP-R type in rice and showed that it interacts with SPIN1 in both yeast and rice protoplasts. To assess the functional relationship between Rbs1 and Spin1 in flowering time regulation in rice, we identified an Rbs1 T-DNA insertion mutant and generated both overexpression and artificial microRNA transgenic plants of the Rbs1 gene. Similar to the T-DNA mutant and the Spin1 RNAi plants [22], the Rbs1 artificial microRNA plants did not show any difference in flowering time compared with the wild type plants in both LD and SD conditions (Data not shown). However, like the Spin1 overexpression plants [22], the Rbs1 overexpression plants show later flowering under SD and LD conditions. Expression analysis showed that Rbs1 overexpression leads to the suppression of both Hd3a, the rice florigen [12]. Our results clearly demonstrate that RBS1 is a flowering suppressor that interacts with SPIN1, and this interaction could lead to the regulation of expression of flowering genes in rice.

Genome analysis showed that there are 196 RRM-containing proteins in Arabidopsis and 221 proteins in rice [16,20]. In rice, LOC_Os10g06130 also encodes an RRM-containing protein, which has 88% protein sequence similarity with RBS1. The expression of this gene is not affected in the Rbs1 overexpression and artificial microRNA plants (data not shown). This gene may
have redundant function with Rhs1 in rice flowering, which might explain our observation that the rbs1 knockout mutant and artificial microRNA plants did not show any significant flowering time change under SD and LD conditions.

In the Rhs1 overexpression plants, the expression of Spin1 is upregulated, which correlates with enhanced SPIN1 protein accumulation. We have previously shown that the Spin1 overexpression plants flower later than the wild type Nipponbare plants under SD and LD conditions [22]. In SD conditions, the expression of Hd1 is reduced in the Spin1 overexpression plants, indicating that SPIN1 influences flowering time via the Hd1 pathway in flowering-promoting conditions. However, in LD conditions, the Hd1 expression, is the same in Nipponbare and Spin1 overexpression plants [22], suggesting that SPIN1 may target an unknown factor to regulate Hd3a under LD conditions. By contrast, in this study we found that the expression of Hd1 is similar between the Rhs1 overexpression and wild type Nipponbare plants under both LD and SD conditions (Figure 5C). Interestingly, in the Rhs1 overexpression plants, the mRNA level of Hd3a is suppressed under both SD and LD conditions. These data suggest that RBS1 may function to regulate flowering in rice via the suppression of Hd3a-mediated mechanisms.

Our previous study revealed the negative relationship between SPIN1 and SPL11 [22]. In this study, we also found that the expression of Spl11 is repressed in the Rhs1 overexpression plants. In contrast, both the expression of Rhs1 and accumulation of the RBS1 protein are increased significantly in the spl11 mutant, indicating a negative relationship between RBS1 and SPL11. However, the direct relationship between these two proteins remains unclear. Yeast-two hybrid assays using SPL11 as the bait and RBS1 as the prey or vice versa did not detect any interaction between them (Vega-Sanchez and Wang, unpublished results). It is plausible that RBS1 is one of the components in the SPL11 protein complex and the interaction between RBS1 and SPL11 requires the presence of another protein that interacts with both proteins, for example SPIN1.

Because RBS1 is a putative RNA binding protein and has RNA binding activity in vitro, it might be involved in RNA metabolism to regulate flowering time. 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 [32,33]. Whether Rhs1 is involved in mRNA turnover in rice needs to be determined in future studies. It is interesting to note that the putative ortholog of RBS1 in Arabidopsis, LIF2, is also involved in flowering time control [19]. However, at this point, it is unclear whether LIF2 and RBS1 are functional homologs. LIF2 seems to be part of protein complex that regulates gene expression via epigenetic mechanisms [19]. Whether RBS1 acts similarly to regulate flowering and other developmental pathways needs to be explored. Complementation studies of the Arabidopsis lif2 loss-of-function mutants with the rice Rhs1 gene should provide clues as to whether these pathways are conserved or not between the two species.

Supporting Information

Figure S1 Identification of Rbs1 by yeast two-hybrid screen in a rice cDNA library using the full length Spin1 as the bait. Numbers 72 and 69-2 are independent yeast clones containing the pDBleu-Spin1 and pPC36-Rhs1 constructs. Positive clones were identified by an X-GAL assay. (TIF)

Figure S2 Molecular analysis and flowering time of the rbs1 mutant. A. Structure of the T-DNA insertion. T-DNA was inserted into the fourth intron of Rhs1. Arrows indicate the primers used for analyzing the insertion site. LB and RB represent the left and right borders of T-DNA; B. PCR genotyping of the rbs1 mutant. DJ denotes Dongjin, 1 is a segregated wild type, 2 and 3 are homozygous plants and 4 is a heterozygous plant; C. Expression of Rbs1 for the plants in 3S3; D. Flowering time of the rbs1 mutant under LD and SD conditions. (TIF)

Figure S3 Western blot analysis of the protein level of RBS1 in the Rhs1 overexpression and wild type plants. (TIF)

Figure S4 Expression analysis of Rhs1 in the Spin1 overexpression plants, spl11 mutant plants and wild type Nipponbare (NPB) plants. A. RT-PCR analysis of Rhs1 and Spin1 in the Spin1 overexpression and RNAi transgenic plants; B. RT-PCR of Rhs1 in the leaves of the wild type IR68 and spl11 mutant plants at different developmental stages. For spl11, young (Y) without lesions, fully expanded (FE) with few lesions and old (O) with many lesions were taken for RNA extraction; C. RT-PCR of Rhs1 in leaves from 55 day-old plants of the wild type IR64 and spl11 mutant. D. Western blot analysis of Rbs1 in the spl11 mutant and Spl11 overexpression lines. (TIF)

Table S1

(DOCX)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: GLW ZG. Performed the experiments: YC MVS. Contributed reagents/materials/analysis tools: YC MVS. Wrote the paper: YC MVS GLW.

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