Coordinated regulation of vegetative and reproductive branching in rice

Lei Wang (王磊), Shengyuan Sun, Jiye Jin, Debao Fu, Xuefei Yang, Xiaoyu Weng, Caiguo Xu, Xianghua Li, Jinghua Xiao, and Qifa Zhang (张启发)[1]

National Key Laboratory of Crop Genetic Improvement and National Center of Plant Gene Research, Huazhong Agricultural University, Wuhan 430070, China

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Grasses produce tiller and panicle branching at vegetative and reproductive stages; the branching patterns largely define the diversity of grasses and constitute a major determinant for grain yield of many cereals. Here we show that a spatiotemporally coordinated gene network consisting of the *MicroRNA 156* (*miR156*/)*miR529*/*SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) and *miR172*/*APETALA2* (*AP2*) pathways regulates tiller and panicle branching in rice. SPL genes negatively control tillering, but positively regulate inflorescence meristem and spikelet transition. Underproduction or overproduction of *SPL*s reduces panicle branching, but by distinct mechanisms: *miR156* and *miR529* fine-tune the *SPL* levels for optimal panicle size. *miR172* regulates spikelet transition by targeting AP2-like genes, which does not affect tillering, and the AP2-like proteins play the roles by interacting with TOPLESS-related proteins (TPRs). *SPL*s modulate panicle branching by directly regulating the *miR172*/*AP2* and *PANICLE PHYTOMER2* (*PAP2*)/*Rice TFL1/CEN homolog 1* (*RCN1*) pathways and also by integrating other regulators, most of which are not involved in tillering regulation. These findings may also have significant implications for understanding branching regulation of other grasses and for application in rice genetic improvement.

Oryza sativa | lateral branch | panicle | spikelet | microRNA

The architecture of grasses is largely determined by the branching patterns. Tillers and inflorescence branches are produced at vegetative and reproductive stages, respectively, and their patterns greatly contribute to the diversity of grasses and constitute a major determinant of grain yield of major cereals.

Rice branching has attracted much attention because of its importance in food production. Axillary buds produce tillers during the vegetative stage. However, only the early ones formed from the unelongated internodes outgrow as tillers, whereas later ones formed from the upper internodes remain dormant. After reproductive transition, the shoot apical meristem is converted to inflorescence meristem to produce panicle. Rice panicle morphology is largely determined by the timing of identity transition among the different types of meristems (SI *Appendix*, Fig. S1). Therefore, fine-tuning of meristem phase change at reproductive stage defines the size and architecture of the rice panicle (1).

Many genes have been identified as regulators of rice branching. Generally, genes involved in axillary bud initiation control both vegetative and reproductive branching, whereas genes under axillary bud outgrowth have specific roles only at certain stages (2, 3). *LAX PANICLE 1* (*LAX1*) and *MONOCULM1* control axillary bud initiation; mutation in either of them results in reduction of both tiller and panicle branches (4, 5). Other genes such as *Grain number*, *plant height, and heading date7* exclusively control panicle branching (6). As a third class, many genes, including *Ideal Plant Architecture 1* (*IPA1*)/*Wealthy Farmer's Panicle* (*WFP*) and genes related to strigolactone, play opposite roles in tiller and panicle branches (7–9). Therefore, there are both commonalities and distinctions in the mechanisms regulating vegetative and reproductive branching. An interesting and fundamental question is how the tillers and panicle branches are coordinately regulated. Elucidating the shifting gene regulatory networks underlying branch outgrowth following the developmental stages should provide understanding of the coordinated regulation and offer guidance for plant breeding practice.

MicroRNA 156 (*miR156*) targets the plant-specific transcription factor *SQUAMOSA PROMOTER BINDING PROTEIN LIKE* (*SPL*) gene families. In *Arabidopsis*, *miR156*/*SPL* plays vital roles in both vegetative and reproductive phase changes (10, 11), whereas *miR172* shows an opposite role in phase change by targeting *APETALA2* (*AP2*)-like transcription factors (10). The sequential actions of *miR156* and *miR172* in regulating vegetative phase change has been reported in many plant species (12). Compared with *Arabidopsis*, grass inflorescence development and phase changes are more complicated, involving different types of meristems. Whether these transitions are also related to the *miR156*/*miR172* pathway is still unknown. Both *miR156* and *miR172* play as regulators of inflorescence and tiller development in rice and maize (13–16). Unlike *Arabidopsis*, SPL genes are also regulated by *miR529* in grasses (17). However, further studies are required to understand the regulatory network and coordination of these three miRNAs in lateral branching.

In this study, we elucidated the roles of *miR156*, *miR172*, *miR529* and their target genes in regulating rice tiller and panicle branching. Our findings suggest that the miRNAs and transcription factors in coordination regulate the vegetative and reproductive branching by shifting gene regulation networks.

Results

Effects of *miR156* and *miR529a* on Tiller and Panicle Branching. Two groups of genes exhibited complementary expression profiles from early to late stages of panicle development (18). Among them, *SPL7*, *SPL14*, and *SPL17* showed decreased expression from early to late stages. They are targets of *miR156* and *miR529*, which together with *miR172* were reported to control developmental timing in plants (12). Thus, we analyzed these three miRNAs and their target genes in branching.

Compared with wild-type (WT) plants, the *miR156* overexpressors (designated as *miR156OE*) had shorter plastochron length (*SI Appendix*, Fig. S2 *A* and *B*). Accompanied with higher

Significance

The patterns of lateral branching, including tillers and inflorescence branches, determine grain yields of many cereals. In this study, we characterized a regulatory network composed of microRNAs and transcription factor that coordinately regulate vegetative (tiller) and reproductive (panicle) branching in rice. The findings hold tremendous promise for application in rice genetic improvement and may also have general implications for understanding branching regulation of grasses.

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[1]To whom correspondence should be addressed. Email: qifazh@mail.hzau.edu.cn.

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leaf initiation rate, the tiller bud was produced as early as 7 d after germination in *miR156OE* plants, whereas it was 15 d in WT (*SI Appendix*, Fig. S3*A*). A tiller bud was produced from the axil of each leaf except the flag leaf, and the ones from the elongated internode were usually dormant (*SI Appendix*, Fig. S3 *B* and *D*), whereas ectopic tiller bud from the axil of flag leaf and higher-order tillers were produced in *miR156OE* plants, resulting in many more tillers (Fig. 1*A* and *SI Appendix*, Fig. S3 *C*–*G*). Thus, *miR156* regulates both initiation and outgrowth of vegetative branching. The panicles of *miR156OE* were very small (Fig. 1*B*), with the number of spikelets only 6.95% of the WT on average (*SI Appendix*, Table S1). This phenotype was consistent with previous results (15). Smaller inflorescence meristem and fewer primary branch primordia were produced in *miR156OE* plants than WT, as revealed by scanning electron microscopy (Fig. 1 *G* and *H*). The meristem marker gene *OSH1* (3) was expressed in much fewer primordia in *miR156OE* plants than in WT, as detected by using in situ hybridization (Fig. 1 *I*–*K*). Therefore, *miR156* negatively regulates inflorescence meristem activity and the initiation of reproductive branching. Manually removing the newborn tillers every other day from seedling to maturation stages led to bigger panicles in WT, but not *miR156OE*, plants (Fig. 1 *C* and *D*), implying that the smaller panicles in *miR156OE* plants were not the trade-off of the higher number of tillers. Thus, *miR156* regulates tiller and panicle branching through distinct pathways.

The target mimicry approach (19) was used to interfere the activity of *miR156*, which reduced the level of *miR156* significantly in the transgenic plants (*MIM156*) (*SI Appendix*, Fig. S4*A*), accompanied by elevated levels of the target SPL genes (*SI Appendix*, Fig. S4*B*). The rates of leaf and tiller production in *MIM156* plants were slower than WT (*SI Appendix*, Figs. S2 *A* and *B* and S3 *A* and *F*), resulting in fewer tillers with bigger panicles (Fig. 1 *A* and *B* and *SI Appendix*, Table S1), exactly opposite from the phenotype of *miR156OE*. Moreover, the defects of *miR156OE* could largely be corrected by *MIM156* (Fig. 1 *A* and *B* and *SI Appendix*, Fig. S4 *C*–*H*), suggesting that overexpression and target mimicry of *miR156* could counteract each other in planta.

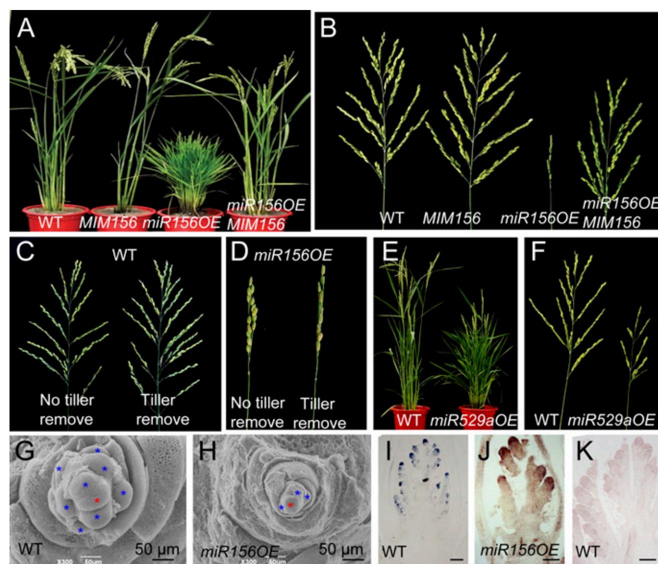

Fig. 1. Tillering and panicle branching regulated by *miR156* and *miR529*. (*A* and *B*) The plants (*A*) and panicles (*B*) at adult stage of WT, *MIM156*, *miR156OE*, and the hybrid between *miR156OE* and *MIM156* plants. (*C* and *D*) Main panicles of WT (*C*) and *miR156OE* (*D*) plants with or without tiller removal every other day. Values are means ± SEM ($n = 15$). (*E* and *F*) Plants (*E*) and panicles (*F*) of WT and *miR529aOE* plants at adult stage. (*G* and *H*) Scanning electron microscopic images of the panicles at the primary branch initiation stage in WT (*G*) and *miR156OE* (*H*) plants. The red and blue asterisks indicate inflorescence and primary branch meristems, respectively. (Scale bars: 50 μm.) (*I*–*K*) In situ hybridization of *OSH1* in WT (*I* and *K*) and *miR156OE* (*J*) plants by using antisense (*I* and *J*) and sense (*K*) probes. [Scale bars: 200 μm (*I*) and 100 μm (*J* and *K*).]

miR529 sharing 14-nt homology with *miR156* also targets SPL genes, mostly at later panicle stage (*SI Appendix*, Fig. S5*A*) (17). The reporter gene *firefly luciferase* (LUC) fused with the *miR529* binding site of *SPL17* was significantly repressed by *miR529a*, but not *miR529b* or *AtmiR172b*, in a transient expression assay demonstrating that *miR529a* regulated *SPL* in planta (*SI Appendix*, Fig. S5 *B* and *C*). Accordingly, *miR529a*, but not *miR529b*, overexpressor (*miR529aOE*) produced similar phenotypes to *miR156OE* in Zhonghua 11 (ZH11), although to a lesser extent (Fig. 1 *E* and *F*). Transcript levels of *SPL14* and *SPL17* were also reduced in *miR529aOE* (*SI Appendix*, Fig. S5 *D* and *E*), suggesting that *SPL*s were regulated by *miR529a* as well.

Effects of *SPL7*, *SPL14*, and *SPL17* on Tillering and Panicle Branching. Among the *SPL* gene family (*SI Appendix*, Fig. S6), *SPL7*, *SPL14*, and *SPL17* showed the highest expression in panicles, as revealed in microarray data (18), and the patterns could be confirmed by quantitative RT-PCR (qRT-PCR) in 16 other tissues (*SI Appendix*, Fig. S7). A T-DNA insertion mutant 4A-00131 for *SPL7* in Dongjin genetic background (*spl7-1*) and RNAi lines of *SPL14* and *SPL17* in ZH11 (*SPLxRi*) were obtained (*SI Appendix*, Fig. S8 *A*–*G*). Not much change in branching and leaf emergence rate was observed in *spl7-1* (*SI Appendix*, Figs. S2*C* and S8 *H* and *I* and Table S1), whereas the lateral organ initiation rates and overall architectures of *SPL14Ri* and *SPL17Ri* plants were reminiscent of *miR156OE* plants (*SI Appendix*, Figs. S2*B*, S3*A*, and S8 *J*–*O*). Panicle branching and spikelets were heavily reduced in both *SPL14Ri* and *SPL17Ri* plants (*SI Appendix*, Table S1), indicating that *SPL*s positively regulated the activities of inflorescence and branch meristems. Each of the RNAi transformants showed gene-specific repression as expected, whereas *SPL7* showed slight, but insignificantly, increased expression in both RNAi lines (*SI Appendix*, Fig. S8 *E*–*G*). Double RNAi lines of *SPL14* and *SPL17* enhanced phenotypic effects compared with individual RNAi plants, but still were weaker than the *miR156OE* plants (*SI Appendix*, Fig. S8 *P* and *Q*), implying that SPL genes play a redundant function in rice development.

To further dissect the roles of SPL genes, we overexpressed *SPL7*, *SPL14*, and *SPL17* in ZH11 (*SPLxOE*). The overexpressors of all three genes greatly reduced tiller numbers (Fig. 2 *A*–*C* and *SI Appendix*, Fig. S9 *A*–*F*), of which *SPL7* had the strongest effect, such that many of the positive transgenic plants were of monoculm and died before maturation. Panicle branching and spikelets also decreased significantly in the overexpressors (*SI Appendix*, Table S1), such that secondary branches, rather than the lateral spikelets produced on the primary branches, were reduced (Fig. 2*D*), implying that the early arising lateral meristems on the primary branches were precociously converted to spikelets. Correspondingly, the expression level of *Frizzy Panicle* (*FZP*), the marker of spikelet meristem in grasses (20, 21), was elevated in *SPL14OE* and *MIM156* lines (Fig. 2*E*). *FZP* was transiently expressed in the spikelet meristem in WT (Figs. 2*F* and 3*O*) (20). However, in situ hybridization showed that *FZP* was ectopically expressed in the branch meristem of *SPL14OE* plants (Fig. 2*G*), suggesting that SPL genes promoted the transition from branch to spikelet meristem. The resistant *SPL7* (*rSPL7*) produced by changing the recognition site of *miR156* and *miR529*, but not the protein sequence driven by its native promoter (designated *rSPL7HA*), also showed similar phenotypes to the *SPL7OE* plants (*SI Appendix*, Fig. S9 *G*, *H*, and *L*–*O*). GFP fused to the C-terminal SPL14 (termed as *SPL14GFP*) showed a similar phenotype to *SPL14OE* plants (*SI Appendix*, Fig. S9 *I*–*K* and *P*–*S*). Together, SPL genes promoted the conversion of branch to spikelet meristem and had a general function in reducing branching.

Effects of *miR172* and its Target Genes on the Transition of Spikelet Meristem Identity. In rice, five *AP2*-like transcription factors (*SNB*, *OsIDS1*, *SHAT1*, *OsTOE1*, and *OsGL15*) are targeted by

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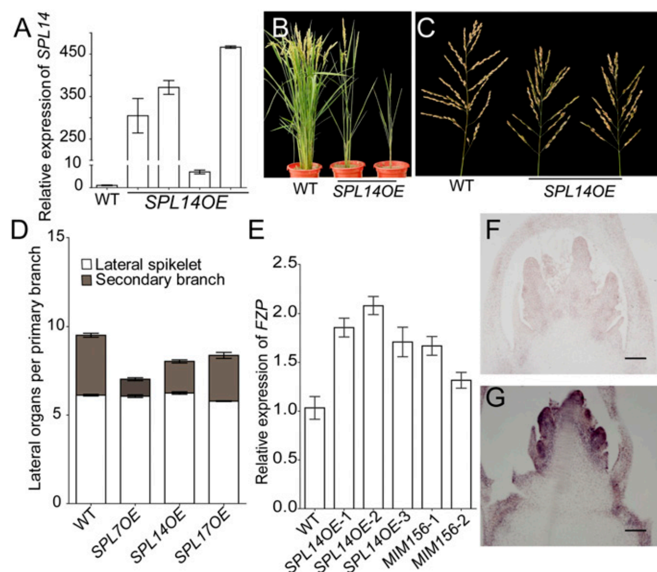

Fig. 2. The transition from branch to spikelet meristem promoted by SPL genes. (*A*) Relative expression level of *SPL14* in flag leaf of its overexpressing plants. Values are means ± SEM (n = 3). (*B* and *C*) The plants (*B*) and panicles (*C*) of *SPL14OE* compared with WT. (*D*) Numbers of secondary branches and lateral spikelets produced by primary branches in *SPL* overexpressors. Values are means ± SEM (n = 15). (*E*) Relative expression level of *FZP* in the young panicle (<1 mm) of *SPL14OE* and *MIM156* plants compared with WT. Values are means ± SEM (n = 3). (*F* and *G*) In situ hybridization of *FZP* in the panicles at the primary branch initiation stage in WT (*F*) and *SPL14OE* (*G*) plants. (Scale bars: 100 μm.)

miR172 (*SI Appendix*, Fig. S10*A*) (16). *miR172* overexpressor (*miR172OE*) did not show marked difference in plant height and tillering (Fig. 3*A* and *SI Appendix*, Fig. S10*B*), but the panicle branching was greatly reduced (Fig. 3*B* and *SI Appendix*, Table S2). Like the overexpressors of SPL genes, secondary branches, rather than lateral spikelets on primary branches, decreased significantly in *miR172OE* plants (Fig. 3*C* and *SI Appendix*, Fig. S10 *F* and *G*).

The target mimicry approach was also used to interfere the *miR172* in ZH11 (*MIM172*) (*SI Appendix*, Fig. S10 *C–E*). The *MIM172* plants were markedly shorter than the WT, whereas the numbers of tillers were comparable (Fig. 3*D*). Strikingly, the panicle architecture changed drastically in *MIM172* (Fig. 3 *E* and *F* and *SI Appendix*, Table S2). Larger numbers of secondary branches, rather than lateral spikelets on primary branches, were produced in *MIM172* (*SI Appendix*, Fig. S10 *F* and *G*), and even tertiary branches occasionally occurred, suggesting that the transition from branch to spikelet meristem was delayed. Consistently, the scanning electron microscopic image revealed that the flower primordia were differentiated in the panicles ∼1-mm in length in WT, but not in *MIM172*, plants (Fig. 3 *M* and *N*). In addition, the lack of *FZP* expression in *MIM172* revealed by in situ hybridization also showed that the spikelet transition was delayed (Fig. 3 *O* and *P*). Together, *miR172*, like SPL genes, also positively promoted the transition from branch to spikelet meristem. Given that *miR172* inhibited the transition from spikelet to floret meristem (16), it apparently had dual roles in establishing and maintaining the spikelet meristem identity.

To investigate the roles of *miR172* targeted genes, we analyzed the effects of *SNB* and *OsTOE1* as the representative of AP2-like genes. Consistent with previous results (16), RNAi lines of *SNB* and *OsTOE1* in ZH11 produced smaller panicles but normal tiller branching (*SI Appendix*, Fig. S10 *H–K* and Table S2). Constructs of *OsTOE1* and *miR172*-resistant *SNB* (*rSNB* by changing the *miR172* binding site, but not protein sequence, and fused with GFP) both driven by the 35S promoter were also introduced into ZH11 (designated as *OsTOE1OE* and *rSNBOE*) (*SI Appendix*, Fig. S10*A*). Overexpressors of both genes showed similar, but weaker, phenotypic changes compared with *MIM172* (Fig. 3 *G–L*)—i.e., dense panicle with significant increase in numbers of branches and spikelets (*SI Appendix*, Table S2) and the secondary branches, but not the lateral spikelets, increased on the primary branches (*SI Appendix*, Fig. S10 *F* and *G*). Our results, together with the fact that *FZP* was ectopically expressed in the double mutant of *snb osids1* (16), suggested that *OsTOE1* and *SNB* dampened the transition from branch to spikelet meristem. Together, *miR172* and its target genes had crucial roles in regulating reproductive, but not vegetative, branching in rice.

SNB and OsIDS1 fused to the GAL4 DNA binding domain attenuated the activity of the reporter 4×*UAS:LUC* in the transient expression assay, suggesting that SNB and OsIDS1 might act as transcriptional repressors (*SI Appendix*, Fig. S11 *A* and *B*). Accordingly, two putative ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motifs, which usually interacted with the transcriptional corepressor TOPLESS-related proteins (TPRs), were found in the proteins of SNB, OsIDS1, and OsTOE1 at the N and C termini (termed as EAR^N and EAR^C respectively) (*SI Appendix*, Fig. S11*C*). One of the three rice TPRs, *TPR2*—also termed *ABERRANT SPIKELET AND PANICLE1* (*ASP1*) or *Lissencephalytype-1-like1* (*OsLIS-L1*)—was involved in regulating panicle development (22, 23). Yeast two-hybrid assay showed that SNB, OsIDS1, and OsTOE1 interacted with TPR1 and ASP1 in yeast, and a series of protein mutation and truncation assays showed that the EAR^C motif of SNB, OsIDS1, and OsTOE1, and the LisH (lissencephaly type I-like homolog) domain of ASP1 were responsible for the interactions (Fig. 4 *A–D* and *SI Appendix*, Fig. S12). Both firefly LUC complementation imaging assay (LCI) (24) and bimolecular fluorescence complementation (BiFC) further substantiated the physical interactions of SNB, OsTOE1, and OsIDS1 with ASP1 in planta (Fig. 4 *E* and *F*).

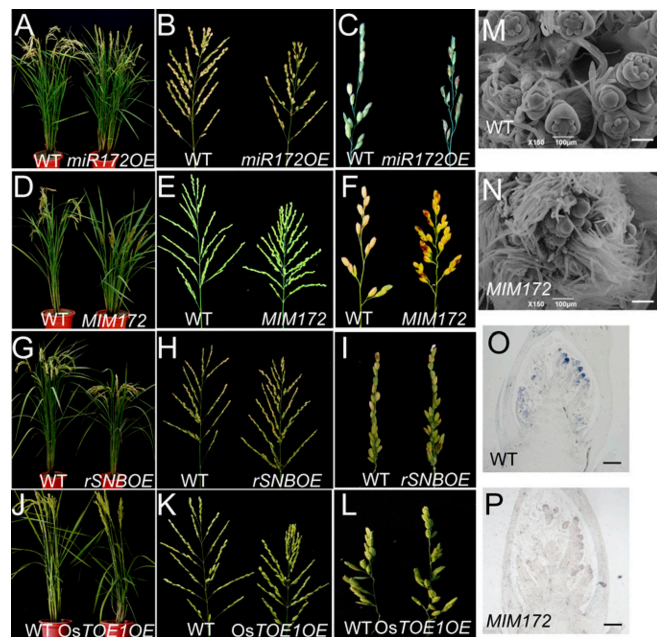

Fig. 3. Regulation of panicle branching, but not tillering, by the *miR172/AP2* pathway. (*A–L*) The plants (*A*, *D*, *G*, and *J*), panicles (*B*, *E*, *H*, and *K*), and primary branches (*C*, *F*, *I*, and *L*) of *miR172OE* (*A–C*), *MIM172* (*D–F*), *rSNBOE* (*G–I*), and *OsTOE1* (*J–L*) transformants. (*M* and *N*) Scanning electron microscopic images of developing panicles ∼1 mm in length of WT (*M*) and *MIM172* (*N*) plants. (Scale bars: 100 μm.) (*O* and *P*) In situ hybridization of *FZP* in panicles ∼1 mm in length of WT (*O*) and *MIM172* (*P*) plants. (Scale bars: 200 μm.)

Fig. 4. Interactions of SNB, OsIDS1, and OsTOE1 with TPRs. (*A*) Schematic representation of the EAR motif in SNB, OsIDS1, and OsTOE1 proteins. Leu (L) to Met (M) or Ile (I) substitution mutations in the EAR motif were used to investigate its role in the interaction with TPRs. EAR^N and EAR^C indicate the N and C termini of the EAR motif, respectively. (*B*) Schematic representation of the protein domains of ASP1. ASP1N and ASP1ΔCTLH are the truncated versions of ASP1 used for investigating the roles of each domain in the protein– protein interaction. (*C*) Yeast two-hybrid assay showing the EAR^C of SNB to be essential for the interaction with ASP1 in the yeast strain *AH109*. AD, GAL4 activation domain; BD, GAL4 DNA binding domain. (*D*) Yeast two-hybrid assay showing the CTLH domain of APS1 to be essential for the interaction with SNB, OsIDS1, and OsTOE1 in yeast. (*E*) LCI assay showing interactions of ASP1 with SNB, OsIDS1, and OsTOE1 in *Arabidopsis* protoplast. Data are normalized to the internal control *35S:GUS* cotransformed in the assay. Values are means ± SEM (n = 3). (*F*) BiFC assay confirming the interaction between ASP1 and SNB in tobacco leaf cells. BF, bright field. (*G* and *H*) Panicles of *asp1* (*G*) and *ASP1Ri* (*H*) plants compared with WT. (*I* and *J*) Plants (*I*) and panicles (*J*) of WT, *MIM172*, and the *ASP1Ri* in the *MIM172* genetic background.

The panicles of the *ASP1* RNAi lines in ZH11 were reminiscent of its null-allele mutant *asp1* (*oslis-l1-1*), in which the panicle branching and spikelet were heavily perturbed (Fig. 4 *G* and *H*). The same panicle defects were also observed in the *ASP1Ri* plants in *MIM172* background (Fig. 4 *I* and *J*), suggesting that *ASP1* was essential for the function of *miR172* and its target genes in regulating rice panicle development.

Interactions Between *SPL* Genes and *miR172* in Regulating Panicle Branching. The results that both SPL genes and *miR172* promoted spikelet transition led us to investigate their possible interactions in regulating panicle branching. *miR156* and *miR172* had spatially–temporally complementary expression patterns in a range of tissues (*SI Appendix*, Fig. S13 *A* and *B*), implying that they might play complementary roles in many developmental processes beyond the vegetative phase change (12). The collective levels of the precursors of *miR156*, *miR172*, and *miR529* were similar to their mature miRNAs (*SI Appendix*, Fig. S13 *C–G*), suggesting that the complementary expression patterns of mature miRNAs were modulated at the transcriptional level.

miR172 and its precursors were elevated in *MIM156* and *SPL14OE* plants (Fig. 5 *A–C*), suggesting that *SPLs* regulated *miR172* expression. Several *GTAC* motifs, the binding site of SPL proteins, were found in the promoters of *pri-miR172b* and *-miR172d* (*SI Appendix*, Fig. S13*H*). Chromatin immunoprecipitation (ChIP)-PCR showed that SPL14 bound to the promoter fragments of both *pri-miR172b* and *-miR172d*, but not the control *actin* gene, in vivo (Fig. 5*D*). Moreover, yeast one-hybrid (Y1H) assay showed that SPL14 bound the promoter fragments of *pri-miR172b* and *-miR172d*, thus activating the expression of reporter gene *LacZ* in yeast (Fig. 5*E*). Also, electrophoresis mobility shift assay (EMSA) showed that the recombinant protein GST-SPL14N but not GST alone physically bound the promoter fragment of *pri-miR172d* in vitro (Fig. 5*F*). Thus, *SPL14* might regulate *miR172* expression directly.

To investigate the possibly genetic interaction between *miR156* and *miR172* in rice panicle development, we knocked down both miRNAs simultaneously (referred as *MIM156–172*). The transgenic plants showed similar tiller phenotypes to *MIM156* at vegetative stage, whereas their panicle morphology was similar to *MIM172* plants (*SI Appendix*, Fig. S13 *I–J*), suggesting that *miR156* and *miR172* regulated rice vegetative and reproductive branches in coordination. The panicle branching defects and precocious transition of spikelet in *SPL7OE* and *SPL14OE* plants could

Fig. 5. Regulation of spikelet transition by *SPL14* via *miR172*. (*A–C*) Relative expression levels of mature *miR172* (*A*), *pri-miR172b* (*B*), and *pri-miR172d* (*C*) in the young panicles (<1 mm) of *SPL14OE* and *MIM156* plants compared with WT. Values are means ± SEM (n = 3). (*D*) ChIP assays of *pri-miR172b* and *-miR172d* in the young panicles (<10 mm) collected from *Ubi:GFP* and *SPL14GFP* plants. Samples were precipitated with anti-GFP antibody and IgG protein. The values were first normalized to the input values, then divided by the *Ubi:GFP* value to get the enrichment fold. Values are means ± SEM (n = 3). The amplification fragments are shown in *SI Appendix*, Fig. S13*H*. (*E*) Y1H assay of SPL14 with the promoters of *pri-miR172b* and *-miR172d* in yeast. (*F*) EMSA of GST and GST-SPL14N recombinant proteins incubated with biotin-labeled probes of *pri-miR172d*. (*G–J*) Panicles (*G* and *I*) and primary branches (*H* and *J*) of *WT*, *SPL7OE*, *SPL14OE*, *MIM172*, and the corresponding hybrids.

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largely be corrected to normal by *MIM172* (Fig. 5 *G–J* and *SI Appendix*, Fig. S13 *K* and *L*). Similar results were also obtained by crossing *rSNBOE* with *SPL7OE* and *SPL14OE* plants (*SI Appendix*, Fig. S13 *M–P*). Together, the results suggested that the spikelet transition promoted by SPL genes was largely through *miR172*.

Other Elements in the *SPL* Pathway in Branching Regulation. *PANICLE PHYTOMER2* (*PAP2*) encoding MADS34 was another positive regulator of spikelet meristem identity in rice by suppressing *Rice TFL1/CEN homolog* (*RCNs*) (25–27), and this genetic module was conserved in *Arabidopsis* in determining its inflorescence architecture (27). In *Arabidopsis*, SPL genes controlled flowering time by regulating MADS genes (11, 28); thus, we speculated that SPL genes might also regulate spikelet transition by *PAP2/MADS34-RCN* besides the *miR172-AP2* pathway in rice. Indeed, *PAP2* was down-regulated in both *miR156OE* and *SPL14Ri* plants and up-regulated in *SPL14OE* and *MIM156* plants (Fig. 6 *A* and *B*), whereas *RCN1* was up-regulated in the panicles of *miR156OE* plants (*SI Appendix*, Fig. S15*B*). Several *GTAC* motifs were found in the promoters of *PAP2* (*SI Appendix*, Fig. S14*A*), ChIP assay showed that SPL14 directly bound to the *PAP2* promoter, but not the control, in vivo (Fig. 6*C*). Although Y1H assay could not reveal the interaction of SPL14 with the promoter fragments of *PAP2* due to their high autoactivation activity in yeast, physical interactions in vitro were indicated by EMSA (Fig. 6*D*). These results demonstrated that *PAP2* was also directly regulated by *SPL14* in rice.

Because of unavailability of overexpressor or mutant of *PAP2* in ZH11 genetic background, we used *RCN1* to test the genetic interaction with *miR156-SPL*. Overexpressing *RCN1* in ZH11 greatly delayed spikelet meristem transition, thus producing much more secondary branches and spikelets (*SI Appendix*, Fig. S14 *B* and *C*). Strikingly, tillering was also reduced in *RCN1OE* plants (*SI Appendix*, Fig. S14 *D–K*), suggesting that *RCN1* regulated both tiller and panicle branching in rice. The high number of tillers of *SPL14Ri* and *SPL17Ri* plants could be corrected to normal by *RCN1OE* or vice versa (*SI Appendix*, Fig. S14 *D–K*). The defects of secondary, but not primary, branches of *SPL14Ri*, *SPL17Ri*, and *SPL14OE* plants could be rescued by *RCN1OE* (Fig. 6*E* and *SI Appendix*, Fig. S14 *D–K*), suggesting that SPL genes regulated spikelet transition, but not inflorescence meristem activity by *RCN1*.

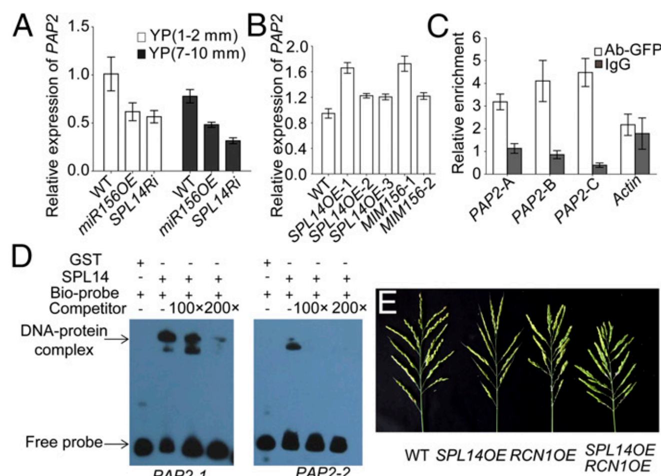

Fig. 6. *SPL14* also regulated spikelet transition by *PAP2/MADS34-RCN* pathway. (*A* and *B*) Relative expression levels of *PAP2* in the panicles (<1 mm) of *miR156OE*, *SPL14Ri* (*A*), *SPL14OE*, and *MIM156* (*B*) plants. Values are means ± SEM (n = 3). (*C*) ChIP assay of *PAP2* in the young panicles (<10 mm) collected from *Ubi:GFP* and *SPL14GFP* plants. The details are as in Fig. 5*D*. Values are means ± SEM (n = 3). (*D*) EMSA of GST and GST-SPL14N recombinant proteins incubated with biotin-labeled probes of *PAP2*. (*E*) The panicles of WT, *SPL14OE*, *RCN1OE*, and the corresponding hybrid.

To further investigate the downstream genes regulated by the *miR156-SPL* pathway in rice panicle development, we compared the transcriptomes of very young panicles (<1 mm) between *miR156OE* and WT plants and found that expression of a large number of genes, including the ones related to transcriptional regulation, were altered in *miR156OE* plants ($P < 0.05$, cutoff >1.5-fold; *SI Appendix*, Fig. S15*A* and Dataset S1). Besides *PAP2/MADS34* and the SPL genes, which were down-regulated in *miR156OE* plants as expected, many other known genes related to rice panicle development were also down-regulated, including *LAX1*, *LONELY GUY* (*LOG*), and *Rice Leafy Homolog* (*RFL*), all of which are important regulators of rice panicle development (4, 29, 30). Some differentially expressed panicle regulators were further confirmed by qRT-PCR (*SI Appendix*, Fig. S15*B*).

Besides spikelet transition, SPL genes also regulated rice panicle primary branching. *LAX1* and *RFL*, as the important genes for rice panicle branch development (4, 30), were highly coexpressed with *SPL7*, *SPL14*, and *SPL17* (18) and down-regulated in the panicles of the *miR156OE* plant. Thus, we also investigated the functions of these two genes in the *miR156-SPL* pathway. The larger panicles of *MIM156* could be recovered by RNAi of *LAX1* and *RFL* (*SI Appendix*, Fig. S15 *C* and *D*). EMSA and Y1H assay revealed that SPL14 bound to the *LAX1* promoter (*SI Appendix*, Fig. S15 *E–G*), implying that *LAX1* might also be directly regulated by *SPL14*. Moreover, *Leafy*, as the ortholog of *RFL*, was directly regulated by SPL genes in *Arabidopsis* (28). These results suggested that *miR156-SPL* might also regulate panicle branching by *LAX1* and *RFL*.

Discussion

Our study revealed the gene networks regulated by *miR156*, *miR172*, *miR529*, and their target genes that coordinately control rice vegetative and reproductive branching, which we attempted to summarize as a model in *SI Appendix*, Fig. S16. At early vegetative stage, *miR156* promotes tillering by inhibiting *SPL*s. Our results revealed that *RCN1* and SPL genes might be involved in the same genetic pathway in regulating tillering. It should be noted in this connection that *TEOSINTE BRANCHED 1* (*TB1*), another negative regulator of tillering, is also directly regulated by *SPL14* (31). Therefore, the *miR156/SPL* pathway regulates tillering at least involving *RCN1* and *TB1* as the downstream elements, although details of the actions and their relationships with other known genes are still lacking for constructing the pathway.

After reproductive transition, SPL genes reach the highest levels at the early panicle stage. It is remarkable that panicle branches were reduced in both RNAi and overexpression lines of SPL genes, indicating that the expression of SPL genes must be fine-tuned to optimal levels for reproductive branching: Either above or below the optimal levels would reduce branching. Therefore, the *miR156* and *miR529* at reproductive stage would maintain the *SPL* expression to the more optimal levels, thus promoting panicle branching. At the downstream, the *miR172/AP2* pathway is used in regulating panicle, but not tiller, branching. Therefore, the activities of the *miR156/miR529/SPL* and *miR172/AP2* pathways harmoniously coordinate vegetative and reproductive branching by shifting gene networks in different developmental stages.

Our results showed that *miR156* negatively regulated inflorescence meristem activity; therefore, *SPL*s had positive roles in maintaining the activity of inflorescence meristem. *LOG* encoding an enzyme for activating the cytokinin is directly regulated by *SPL14* (29, 31), suggesting that SPL genes may play these roles by regulating cytokinin signaling in rice. It was reported that more branches and spikelets were produced in rice genotype containing the allele *IPA1/WFP* of *SPL14* (7, 8). In light of our results, *SPL14* has negative roles on panicle branches by promoting the transition of spikelet meristem, thus negatively regulating secondary branches. Therefore, the total number of spikelets represents a balance

among the multiple roles of *SPL14*. The SPL genes could increase spikelets only when their level is optimal. Plants with moderately high activity of *SPL14*, such as the ones containing the *IPA1*/*WFP* allele or *MIM156*, produce more spikelets, because the higher activity of inflorescence meristem results in more primary branches, which compensates the negative effect of precocious transition of spikelet meristem identity. Conversely, very high activity of SPL genes reduces total spikelets by precocious transition of spikelet meristem. Therefore, *SPL*s have multiple roles in regulating panicle branches, depending on the expression levels. Fine-tuning of the expression of SPL genes to the most favorable levels may provide a strategy for increasing rice productivity in breeding application.

Both molecular and genetic evidence demonstrated that *SPL14* positively regulates the transition of spikelet from branch meristem via both *miR172*/*AP2* and *PAP2*/*RCN1* pathways. Natural variations in *miR172* and its targets AP2-like genes have crucial roles in the domestication and evolutionary processes of grasses (14, 32, 33). However, the relationship of natural variation of these genes with agronomic traits had not been demonstrated in rice. We showed that more spikelets were produced in *MIM172* and *rSNBOE* plants, which may be used for rice breeding by genome manipulating technologies. *SNB* and *OsIDS1* have intrinsic transcriptional repressor activity, and they interacted with the transcriptional corepressor TPRs, which usually recruit the histone deacetylase to modify the epigenetic states of their downstream genes (34); thus, AP2-like genes might repress the expression of their target genes. *ASP1* might serve as another link between the *miR156*/*SPL* and *miR172*/*AP2* pathways, because it was also directly targeted by *SPL14* (31).

Some of the *SPL* and *AP2* genes had been studied in maize, barley, and wheat (14, 32, 33, 35), and our results uncovered that functions of these genes are mostly conserved in rice. Moreover, *miR156*-regulated vegetative phase change via *miR172* is well known to be conserved in many plant species, including maize and rice (12), and the gene regulatory networks revealed in this study may shed light for understanding the ancient and conserved mechanisms. However, there are also distinctions. For example, the branch meristems were converted to spikelet meristems in the mutant *asp1* in rice (22), whereas exactly the reverse was the case in the mutant of its maize ortholog *RAMOSA ENHANCER LOCUS 2*, in which spikelet meristems were converted to branch meristems (36). Thus, functional divergence of the pathways had taken place during evolution. Furthermore, there are questions remaining to be addressed in future studies, regarding whether the panicle branching regulation by physically interaction between AP2 and TPRs is conserved and how *miR156*/*SPL*s and *PAP2*/*RCN1* pathways are related in other grasses.

Materials and Methods

The rice variety Zhonghua 11 (ZH11) was used for transformation in most of this study. The T-DNA insertion mutant *spl7-1* was obtained from the Postech rice mutant library (37). Details of experimental methods are given in *SI Appendix, SI Materials and Methods*.

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