OsSPL14 promotes panicle branching and higher grain productivity in rice

Kotaro Miura, Mayuko Ikeda, Atsushi Matsubara, Xian-Jun Song, Midori Ito, Kenji Asano, Makoto Matsuoka, Hidemi Kitano & Motoyuki Ashikari

Rice is a staple food for nearly half of the world’s population, with more than 10,000 rice varieties providing almost one-quarter of the global per capita dietary energy supply. Grain number, grain size, panicle size and branch number in a panicle are directly associated with rice productivity. Several genes have been shown to influence panicle size and branch number in a panicle. Grain number, grain size, panicle size and branch number in a panicle are directly associated with rice productivity.

To investigate genes that regulate rice grain productivity, we selected two rice lines, Nipponbare and ST-12 (from the Stocked rice collection of Togo field and Nagoya University-12), which differ in number of grains per panicle. The typical japonica rice variety, Nipponbare, has approximately 152 grains in the main panicle; in contrast, ST-12 has approximately 475 grains in the main panicle (Fig. 1a–c). Nipponbare and ST-12 have 10.5 and 28.9 primary branches in the main panicle, respectively, and thus the difference in grain number between the two lines is primarily due to the difference in the number of their primary branches (Fig. 1b,d).

To identify the gene responsible for the increased number of primary panicle branches in ST-12, we produced an F2 population derived from a cross between Nipponbare and ST-12 plants and observed the number of primary branches in 192 of these plants (Supplementary Fig. 1e). Quantitative trait locus (QTL) analysis of F2 plants with 118 molecular markers detected two major QTLs with a log10 odds (LOD) score greater than 3.0 on chromosomes 1 (LOD score = 4.952, additive effect = 3.022) and 8 (LOD score = 13.229, additive effect = 4.275) (Supplementary Table 1). Because the QTL on chromosome 1 included the Gnia locus, we sequenced and detected the same mutation as the dominant Habataki allele. We concluded that the detected QTL on chromosome 1 in this study might be affected by Gnia.

To further understand the molecular mechanisms of the production of primary panicle branches, we focused on the positional cloning of the QTL detected on chromosome 8, named here WFP (WEALTHY FARMER’S PANICLE), which acts in a semidominant manner (Supplementary Fig. 2). Analysis of 3,000 F2 plants narrowed the candidate region to between RM223 and RM264 (Fig. 1e). Further high-resolution mapping using F1 and F2 recombinant lines narrowed the candidate region down to a 2.6-kb area (Fig. 1e and Supplementary Figs. 3 and 4). The Rice Annotation Project Database (RAP-DB) has predicted that the gene at the Os08g0509800 locus encodes a hypothetical protein in this region (RAP-DB; see URLs and Fig. 1e). We compared the sequence of the 2.6-kb candidate region between the Nipponbare and ST-12 lines but found no difference in nucleotide sequence. We performed expression analysis of the gene at Os08g0509800 but did not detect expression in either Nipponbare or ST-12 plants (data not shown). We could find no evidence of transcripts from the gene at Os08g0509800 in available databases, suggesting Os08g0509800 may contain a pseudogene.

Analysis of neighboring genes revealed that the candidate 2.6-kb region was upstream of Os08g0509600 (also predicted as LOC_Os08g39890 in the Rice Genome Annotation Project; see URLs and Fig. 1e), which encodes the plant-specific transcription factor OsSPL14 (ref. 10). Phylogenetic analysis indicated that the OsSPL gene at Os08g0509600 is categorized as OsSPL14 and is conserved in sorghum, wheat, maize and Arabidopsis thaliana (Supplementary Fig. 5ab). Sequence analysis revealed that there was no difference in the coding region of OsSPL14 between Nipponbare and ST-12 plants.

Quantitative RT-PCR analysis of OsSPL14 detected a difference in expression between Nipponbare and ST-12 in the shoot apices and
young panicles, with higher expression in ST-12 than in Nipponbare (Fig. 2a). Because primary branches begin differentiation in the early stages of panicle development, we investigated OsSPL14 expression level at several developmental stages of the panicle. The expression of OsSPL14 at the 1–2-mm and 2–5-mm stages was about ninefold higher in ST-12 than in Nipponbare (Fig. 2b). We also assessed the expression pattern by in situ hybridization in young panicles (Fig. 2c–j). By this means we detected OsSPL14 expression around the branch meristems in Nipponbare and ST-12 (Fig. 2c–e,g–i), and again, we detected higher OsSPL14 expression in young panicles of ST-12 (Fig. 2c–e,g–i).

To investigate whether OsSPL14 is the gene underlying the WFP QTL, we cloned 11-kb genomic fragments of OsSPL14, including the 2.6-kb candidate region, five SNPs around the candidate region, and the coding region, from both Nipponbare and ST-12 (pNip::OsSPL14 and pST-12::OsSPL14; Supplementary Fig. 6a,b); we then transformed each genomic fragment into Nipponbare. Both transgenic plant lines showed a higher primary branch number than Nipponbare plants transformed with a vector control (Fig. 2k,l). These results confirmed that OsSPL14 functions in the regulation of primary branch number, and thus we concluded that the WFP QTL encodes OsSPL14.

Heritable differences in gene expression not due to DNA sequence changes are defined as epigenetic alleles11. Epigenetic alleles have been reported in Arabidopsis12–17 and rice18. We considered whether heritable epigenetic marks in the endogenous OsSPL14 promoter may be related to different expression levels of OsSPL14. To test this, we performed bisulfite sequencing to compare DNA methylation levels of the 2.6-kb candidate region in Nipponbare and ST-12; overall, there was no significant difference in total DNA methylation in the
### Figure 3 Effect of microRNA excision on OsSPL14 expression.

(a–d) Expression of GUS-fused OsSPL14 without or with a mutation in the microRNA-targeted site driven by the Nipponbare promoter. (a–d) The expression of unmutated OsSPL14-GUS and mutated mOsSPL14-GUS in leaves (a,b) and in the shoot base (c,d). (e,f) Gross morphology of unmutated (pNip::OsSPL14) and mutated (pNip::mOsSPL14) transgenic plants with OsSPL14 driven under the Nipponbare promoter at the vegetative stage. (g) Comparison of tiller number among transgenic plants of the OsSPL14 Nipponbare allele (pNip::OsSPL14), mutated OsSPL14 (pNip::mOsSPL14) and OsSPL14 Aikawa 1 allele (pNip::OsSPL14<sub>Aikawa1</sub>). (h) Nucleotide change in Aikawa 1 from C to A at the OsmiR156-targeted site in OsSPL14. (i–k) Gross morphologies of Nipponbare, ST-12 and Aikawa 1 plants. (l) Comparison of tiller number per plant among Nipponbare, ST-12 and Aikawa 1. (m) Relative expression of OsSPL14 in shoots of Nipponbare, ST-12 and Aikawa 1. (n) RNA hybridization of OsmiR156. U6 RNA was used as a control probe and ethidium bromide was used as the loading control. Arrowhead indicates 20-nucleotide (nt) signal of OsmiR156. (o) Number of primary branches per panicle for Nipponbare, ST-12 and Aikawa 1. (p) Relative expression of OsSPL14 in the 2–5-mm stage of young panicles of Nipponbare, ST-12 and Aikawa 1. Scale bars: a–d, 1 cm; e,f,i–k, 20 cm. Values are means and bars are s.d. (n = 40 plants in g,l,o; n = 3 times in m,p).

region (Supplementary Fig. 7). However, methylation differences were observed at several cytosines near 1,070 base pairs, with higher levels of methylation in Nipponbare (68–79%) compared to ST-12 (0–24%) (Supplementary Fig. 8). These results warrant further investigation to determine if OsSPL14 is regulated by a heritable epigenetic mechanism in ST-12.

We also evaluated whether the candidate 2.6-kb region possesses enhancer or repressor activity by comparing β-glucuronidase (GUS) expression between GUS constructs with and without the 2.6-kb candidate interval. We did not detect significant differences in GUS expression (Supplementary Fig. 9), suggesting that the 2.6-kb region does not have enhancer or repressor activity in callus tissue. Given the lack of regulatory activity in this fragment, it is unclear how DNA methylation changes at a specific site may be related to heritable expression changes.

OsSPL14 contains a microRNA (miRNA)-targeted sequence in the third exon (Fig. 1e). To test the effect of the miRNA, known as OsmiR156, on OsSPL14 mRNA, we produced OsSPL14-GUS fusion constructs (pNip::mOsSPL14-GUS and pNip::OsSPL14-GUS) driven by the Nipponbare promoter (Supplementary Fig. 10a,b) with and without synonymous mutations in the OsmiR156-targeted site, respectively (Supplementary Fig. 10c). We transformed these constructs into Nipponbare (Fig. 3a–d) and transgenic plants possessing a mutated OsmiR156-targeted site and observed that they showed high levels of GUS staining in leaves and in the basal part of the shoot (Fig. 3b,d). In contrast, the transgenic plants lacking a mutation in the OsmiR156-targeted site had no observable GUS signal (Fig. 3a,c). These results suggest that OsSPL14 mRNA is targeted and cleaved by OsmiR156.

We then examined the effect of OsmiR156 on plant architecture. We produced Nipponbare plants carrying an OsSPL14 transgene with either the unmutated (pNip::OsSPL14) or the mutated OsmiR156-targeted site (pNip::mOsSPL14) (Supplementary Figs. 6a and 10d). The pNip::OsSPL14 plants had 6.4 tillers each and pNip::mOsSPL14
plants had 1.8 tillers each (Fig. 3e–g). These transgenic plants suggest that OsSPL14 is involved in the regulation of shoot branching in the vegetative stage.

We hypothesized that mutation of the OsmiR156-targeted site in OsSPL14 may lead to repression of shoot branching, so we screened low-tiller lines and varieties for mutations in the OsmiR156-targeted site of OsSPL14. We found that the japonica cultivar Aikawa 1 plant line has a single nucleotide change from C to A at the OsmiR156-targeted site in OsSPL14 (Fig. 3f) and has an average tiller number (4.1 tillers) much lower than that of Nipponbare (15.8 tillers) and ST-12 (10.9 tillers) (Fig. 3i–l). In addition, in Aikawa 1, OsSPL14 expression was higher in the shoot than in Nipponbare and ST-12 (Fig. 3m).

To confirm the function of OsSPL14Aikawa1 allele, we prepared Nipponbare plants carrying OsSPL14Aikawa1. Transgenic plants with the Aikawa1 OsSPL14 allele driven under the Nipponbare promoter (pNip::OsSPL14Aikawa1) had an average of 2.2 tillers (Fig. 3g). These results confirmed that the low-tiller number seen in Aikawa 1 plants was caused in a dominant manner by the single nucleotide mutation in the OsmiR156-targeted site in OsSPL14. We concluded that OsSPL14 functions to repress shoot branching in the vegetative stage. In Nipponbare, the OsSPL14 transcript would be cleaved by OsmiR156, but the transcript derived from OsSPL14Aikawa1 would not be cleaved because of the mutation in the OsmiR156-targeted site in Aikawa 1.

In fact, the expression level of OsSPL14 was higher in Aikawa 1 than in Nipponbare (Fig. 3m); this could be due to non-cleavage of the OsSPL14Aikawa1 transcript. The cleavage of the OsSPL14 transcript by OsmiR156 in the vegetative stage was supported by the results of the WFP-GUS transgenic assay performed in this study. GUS staining was not observed in OsSPL14-GUS transgenic plants (Fig. 3a,c) but was observed in mOsSPL14-GUS transgenic plants (Fig. 3b,d), suggesting that the OsSPL14 transcript is targeted by OsmiR156. Furthermore, OsmiR156 is highly expressed in vegetative leaves, roots and the shoot apex but is repressed in the early stages of panicle development (Fig. 3m); overexpression of OsmiR156 leads to a significantly increased numbers of tillers10. These results are consistent with OsSPL14 expression being regulated by OsmiR156 and with OsSPL14 functioning to repress shoot branching in the vegetative stage.

The primary branch number of the panicle in Aikawa 1 was higher than in Nipponbare (Fig. 3o). In the first part of this study, we concluded that higher OsSPL14 expression at the young panicle stage increased primary branches in the panicle. Because WFP-Aikawa1 has a mutation in the OsmiRNA156 target site, it is not cleaved by OsmiRNA156, which may cause accumulation of OsSPL14 transcripts in the panicle. In fact, a higher level of OsSPL14 transcripts was detected in young panicles of Aikawa 1 compared with Nipponbare (Fig. 3p). This higher level of OsSPL14Aikawa1 transcripts in young panicles of Aikawa 1 may lead to more primary branches in panicles than occurred with Nipponbare. It was previously reported that overexpression of OsmiR156 leads to increased shoot branching in the vegetative stage and to a reduced number of spikelets and grains per panicle16. Our results, that higher OsSPL14 expression in the vegetative stage represses shoot branching that and higher OsSPL14 expression in young panicles promotes branching, correspond with the previously reported expression and function of OsmiR15610.

Because OsSPL14WFP was found to lead to increased primary branch number in panicles, which could in turn lead to an increase in grain productivity, we evaluated the effect of the QTL WFP (on chromosome 8) and the QTL on chromosome 1 from the ST-12 line on rice grain yield. Four types of plants with different allele combinations of the two QTLs were selected in the BC4 F2 population using molecular markers (Fig. 4a). Rice plants with both chromosomes 1 and 8 from Nipponbare had an average of 11.6 primary branches, 159.7 grains in the main panicle and 2,232 grains in each plant; however, plants containing chromosome 1 from Nipponbare and the OsSPL14WFP allele on chromosome 8 had an average of 21.4 primary branches, 241.6 grains in a panicle and 3,142 grains in each plant (Fig. 4b–d). Thus, the OsSPL14WFP allele on chromosome 8 was associated with an increase of approximately 40% in primary branch and grain number. Furthermore, plants possessing chromosome 1 of ST-12 and the OsSPL14WFP allele on chromosome 8 produced an average of 23.8 primary branches, 272.2 grains in a panicle and 3,396 grains in a plant, which is an increase of 12.2 primary branches and 1,164 grains (52%) as compared to plants with both Nipponbare alleles. These results indicate that OsSPL14WFP has a strong effect on grain production and that QTL pyramiding, based on combinations of alleles with molecular markers, may be an efficient method for breeding higher-yielding rice19.

In this study, we identified WFP as a QTL that affects the number of primary branches in rice panicles. The SQUAMOSA PROMOTER BINDING PROTEIN was first found as a transcription factor, binding to the promoter of the snapdragon MADS-box gene, SQUA
(SQUAMOSA); now, 16 putative SPL (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE) genes in Arabidopsis and 19 genes in rice have been predicted from the genomic sequences of these plants\textsuperscript{10}. Several studies have recently reported SPL function in Arabidopsis and rice\textsuperscript{20–25}. In this study, we demonstrated the function of OsSPL14 in the regulation of branching in panicles and vegetative shoots in rice. However, the mechanism(s) by which OsSPL14 regulates branching are still unknown. From the results of the test of its subcellular localization and transcriptional activity, it is possible that OsSPL14 may be a transcription factor (Supplementary Note and Supplementary Fig. 11). Further investigation, such as screening for target genes that are regulated by OsSPL14, may provide clues for understanding the molecular mechanism.

It was also determined that OsSPL14 expression is regulated by post-transcriptional gene silencing, as we demonstrated that the expression of OsSPL14 is controlled by OsmiR156. Taken together, our results suggest that OsSPL14 may not only be useful for increasing grain yields in rice but may also be applicable to other cereal crops.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

We thank K. Inamura for providing the detailed protocol for in situ hybridization, S. Mizuno for maintenance of the paddy field and E. Kouketsu and K. Sakata for helping to produce transgenic plants. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan (Integrated Research Project for Plants, Insects and Animals using Genome Technology, QTL-1001).

AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/naturegenetics/

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ONLINE METHODS
Plant materials and growth conditions. All nontransgenic plants were grown under natural field conditions in a research field at Nagoya University, Togo, Aichi, Japan. Seeds were immersed in water for 2 d and then sown in a nursery bed. One-month-old seedlings were transplanted to a paddy field with a spacing of 20 x 35 cm. Aikawa 1 was identified by screening of low-tiller lines from the rice collections of the Togo field in Nagoya University.

QTL analysis. QTL analysis was performed with 192 F2 plants as previously reported26. The linkage map was calculated using Mapmaker27. LOD value, phenotypic variation and additive effect were calculated using Mapmaker/QTL3. Primers used in positional cloning are listed in Supplementary Table 2.

Transgenic analysis. All transgenic plants were grown in a closed greenhouse. pNip::OsSPL14, pST-12::OsSPL14 and pNip::OsSPL14Aikawa1 constructs were transformed into the binary vector pYLTAC728 (provided by RIKEN Biocentre). The binary vector was transformed into Agrobacterium tumefaciens strain EHA10529 by electroporation, and rice plants were transformed with this strain as previously reported2. Control plants were generated by introducing the empty vector. Transgenic plants possessing two copies of the transgene were selected and used in this study.

RNA isolation and quantitative RT-PCR (qRT-PCR) analysis. Total RNAs were isolated from various rice tissues using the TRIzol reagent (Invitrogen). cDNAs were synthesized using an Omniscript reverse transcription kit (Qiagen GmbH). qRT-PCR was performed using a SYBR Green RT-PCR kit (Qiagen) with a LightCycler (Roche Diagnostics GmbH). Os Ubiquitin (OsUbq1) primers were used as the internal control. Primers used in qRT-PCR are listed in Supplementary Table 2.

In situ hybridization. Plant materials were fixed in 4% (wt/vol) paraformaldehyde and 0.25% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer at pH 7.4 overnight at 4 °C, dehydrated through a graded ethanol series followed by a r-butanol series, and finally embedded in Paraplast Plus (Sherwood Medical). Microtome sections (7 μm) were mounted on glass slides treated with silane. Digoxigenin-labeled RNA probes were transcribed with T7 RNA polymerase. The probes were amplified using the primer set of the OsSPL14 in situ probe and cloned into the pCR4 blunt TOPO vector (Invitrogen; Supplementary Table 2). Hybridization and immunologic detection of the hybridized probes were performed according to the method described in ref. 30.

Phylogenetic tree. SPL family proteins were screened by BLAST searching of the protein databases of GRAMEINE and TAIR (GRAMEINE, http://www.grameine.org/; TAIR, http://www.arabidopsis.org/). The phylogenetic tree of the SPL family based on the protein sequences of SBP domains was aligned as previously reported2.

RNA blotting of microRNA. Total RNAs were loaded into a 15% polyacrylamide gel containing 7 M urea, transferred to Hybond N+ membranes and hybridized with 32P-labeled oligonucleotide probes for 18 h at room temperature (23–25 °C). PerfectHyb Plus Hybridization Buffer 1x (Sigma H7033) was used for hybridization. The membranes were washed with wash solution (6x SSC buffer, 0.2% SDS solution) for 5 min at room temperature three times and at 42 °C for 10 min once. The probe sequences used in this assay were U6 RNA: 5'-TGATCGTTCAATTATATCGGATGT-3'; miR156: 5'-GTGTCACCTCTCTCTCTGTC-3'.

Bisulfite sequencing. Genomic DNA was bisulfite treated using the EpiTect Bisulfite kit (Qiagen 59104). The candidate 2.6-kb region was amplified using bisulfite primers and cloned into the PCR-4 vector. Each fragment was sequenced in at least 24 clones. The sequences of bisulfite primers used in this assay are listed in Supplementary Table 2.

Promoter GUS assay. The promoter and coding sequence surrounding the microRNA-targeted site of WFP were amplified by PCR using the genomic DNA as a template and cloned into pCR4 TOPO. These partial WFP sequences were introduced in front of the GUS reporter gene of pBI-Hm (ref. 2) to produce a fusion with the GUS reporter gene. These constructs were transformed into Nipponbare. GUS activity was assessed as described previously2.

Subcellular localization analysis. The clones of WFP cDNA were amplified using XbaI site–linked primers and then ligated into the XbaI site of a vector containing the CaMV35S-GFP-NOS cassette31. Fused 35S-OsSPL14-GFP fragments were cloned into the pUC119 vector. Gold particles were coated with the 35S-OsSPL14-GFP constructs and used to bombard onion epidermis with a PDS-1000/He biolistic system (Bio-Rad). The onion epidermis was incubated in the dark at 25 °C. After 18 h, the cell layers were soaked in 2 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride ·hydrate (DAPI, Dojindo) solution for visualization of the nucleus and analysis of the nuclear localization of 35S-OsSPL14-GFP derivatives.

Transactivation activity assay. The GAL4-based Matchmaker Two-Hybrid System 3 (Clontech) was used for the transactivation activity assay. For construction, the full-length coding sequence was amplified using PCR and then cloned. The cloned fragment was then inserted into pGBKT7 and fused with the GAL4-binding domain. The GAL4-based Matchmaker Two-Hybrid System 3 (Clontech) was used for the transactivation activity assay. For construction, the full-length coding sequence was amplified using PCR and then cloned. The cloned fragment was then inserted into pGBKT7 and fused with the GAL4-binding domain. The GAL4-binding construct was then transformed into the yeast strain AH109. Each yeast liquid culture was serially diluted to 10−3, serially plated on the yeast synthetic complete medium lacking leucine, tryptophan and histidine, and incubated in the dark at 25 °C. After 18 h, the cell layers were soaked in 2 μg/ml 4',6-diamidino-2-phenylindole dihydrochloride ·hydrate (DAPI, Dojindo) solution for visualization of the nucleus and analysis of the nuclear localization of 35S-OsSPL14-GFP derivatives.

**Supplementary information**

**Supplementary Note**

**ST-12 WFP allele is semi-dominant**

To investigate the heredity of the *WFP* gene, we selected plants that were homozygous for chromosome 1 of Nipponbare and heterozygous for Nipponbare and ST-12 *WFP* alleles (Nip / ST-12) from the BC$_2$F$_2$ plants. Because the primary branch number of *WFP*$_{Nip / ST-12}$ was intermediate between those of the homozygote plants, *WFP*$_{Nip / Nip}$ and *WFP*$_{ST-12 / ST-12}$ (Supplementary Fig. 6), the ST-12 *WFP* allele is semi-dominant.

**Function of OsSPL14 as a transcription factor**

The *WFP* gene encodes the putative transcription factor, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL), and this protein has a conserved SQUAMOSA PROMOTER BINDING PROTEIN (SBP) domain that includes a nuclear localization signal. To confirm the protein’s function as a transcription factor, we observed its subcellular localization and performed transactivation analysis of OsSPL14. Subcellular
localization analysis using green fluorescent protein (GFP) fusion OsSPL14 proteins detected the GFP signal localized to nuclei (Supplementary Fig. 11a). Additionally, analysis using the yeast one-hybrid system showed that the OsSPL14 protein had transcription-activating abilities (Supplementary Fig. 11b). These results suggest that OsSPL14 has the potential to act as a transcription factor.
Supplementary Figure 1. Phenotypic distribution of F$_2$ plants derived from the cross between Nipponbare and ST-12.

Number of primary branches per main panicle in 192 plants. The means are indicated by blue and red arrowheads for Nipponbare and ST-12, respectively.
Supplementary Figure 2. Semi-dominant effect of \textit{WFP}.

Comparison of primary branch numbers per panicle among homozygotes of Nipponbare allele of \textit{WFP} (Nip / Nip), heterozygotes of Nipponbare and the ST-12 allele of \textit{WFP} (Nip / ST-12), and homozygotes of the ST-12 allele of \textit{WFP} (ST-12 / ST-12). These three genotypes were selected from BC$_2$F$_2$ plants. Values are means, with bars showing SD ($n = 40$ plants).
Supplementary Figure 3. Graphical genotype and phenotype of recombinant plants in F$_2$ generation.

The graphical genotypes of 14 recombinant plants in F$_2$ generation. Blue and yellow bars indicate Nipponbare and ST-12 chromosomes respectively. The primary branch number of each recombinant plants are shown in right side.
Supplementary Figure 4. Graphical genotype and phenotype of recombinant lines in $F_3$ and $F_4$ generation.

The graphical genotypes of 14 recombinant lines in $F_3$ and $F_4$. Blue and yellow bars indicate Nipponbare and ST-12 chromosomes respectively. The primary branch number of each recombinant plants are shown in right side.
Supplementary Figure 5. Phylogenetic tree and homology analysis of SPL proteins.

(a) Phylogenetic tree of SPL proteins among rice, sorghum, wheat, maize, and Arabidopsis. OsSPL14/WFP is highlighted by the red rectangle. (b) Homology analysis of SPL domains of the WFP clade. The SBP-domain includes the Zn-1, Zn-2, and NLS domains.
Supplementary Figure 6. The constructs used in the transgenic analysis.

(a) The construct of OsSPL14 driven under the Nipponbare promoter. (b) The construct of OsSPL14 driven under the ST-12 promoter. Five polymorphisms were detected in the upstream region of OsSPL14. The numbers under the construct indicate the position of the polymorphism.
**Supplementary Figure 7. Bisulfite sequence of the candidate 2.6-kb region.**

DNA methylation levels (%) are indicated by blue (Nipponbare) and red (ST-12) bars.

The numbers under the graph indicate the position in the candidate 2.6-kb region.
Supplementary Figure 8. Magnified figure of bisulfite sequence of 1061-1080 bases in candidate 2.6-kb region.

DNA methylation levels (%) are indicated by blue (Nipponbare) and red (ST-12) bars. The numbers under the graph indicate the position in the candidate 2.6-kb region. The nucleotide sequences are shown under the positions.
Supplementary Figure 9. Analysis of promoter activity of candidate 2.6-kb region.

The GUS gene was fused with OsSPL14 just downstream of the mutated microRNA-targeted site, driven by the Nipponbare promoter. (b) The GUS gene was fused with OsSPL14 just downstream of the mutated microRNA-targeted site, driven by the Nipponbare promoter lacking the 2.6-kb candidate region. (c) GUS activity of pNip::mOsSPL14-GUS in callus. (d) GUS activity of pNip-2.6kb::mOsSPL14-GUS in callus.
Supplementary Figure 10. The constructs used in the transgenic analysis for microRNA

(a) The GUS gene was fused with OsSPL14 just downstream of the microRNA targeted site driven under the Nipponbare promoter. (b) The GUS gene was fused with OsSPL14 just downstream of the mutated microRNA-targeted site, driven by the Nipponbare promoter. (c) The mutated OsmiR156 targeted site of OsSPL14. The mutated targeted site contains five synonymous mutations. The mutation sites are highlighted in red characters. (d) The mutated OsSPL14 driven by the Nipponbare promoter.
Supplementary Figure 11. Subcellular localization and transactivation analysis of OsSPL14 protein.

(a) Subcellular localization of OsSPL14 was analyzed using the GFP fusion OsSPL14 protein driven by the 35S promoter. Nuclei were stained with DAPI. (b) The full-length cDNA of OsSPL14 and SK1-Full, as a positive control, were fused to the GAL4-DNA binding domain. The pGBK7-vector is the negative control.
## Supplementary Table 1. Summary of QTL analysis for primary branch number

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## Supplementary Table 2. Primers used in this study

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