

OsSPL14 promotes panicle branching and higher grain productivity in rice

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Identification of alleles that improve crop production and lead to higher-yielding varieties are needed for food security. Here we show that the quantitative trait locus WFP (WEALTHY FARMER'S PANICLE) encodes OsSPL14 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14, also known as IPA1). Higher expression of OsSPL14 in the reproductive stage promotes panicle branching and higher grain yield in rice. OsSPL14 controls shoot branching in the vegetative stage and is affected by microRNA excision. We also demonstrate the feasibility of using the OsSPL14^{WFP} allele to increase rice crop yield. Introduction of the high-yielding OsSPL14^{WFP} allele into the standard rice variety Nipponbare resulted in increased rice production.

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 supply¹. 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 these rice traits: *GN1A* and *APO1* regulate grain number^{2,3}; *GS3*, *GW2* and *qSW5* regulate grain size^{4–6}; *DEP1* controls panicle size⁷; and *FZP* and *LAX1* (ref. 8,9) control branch number per panicle. However, identification of other genes that regulate these traits would help us further understand the molecular mechanisms that regulate rice productivity and would also facilitate breeding of new varieties that increase rice crop yield.

To investigate genes that regulate rice grain productivity, we selected two rice lines, Nipponbare and ST-12 (from the Stocked rice collections 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. 1). 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 *GN1A* locus, we sequenced and detected the same mutation as the dominant Habataki allele². We concluded that the detected QTL on chromosome 1 in this study might be affected by *GN1A*.

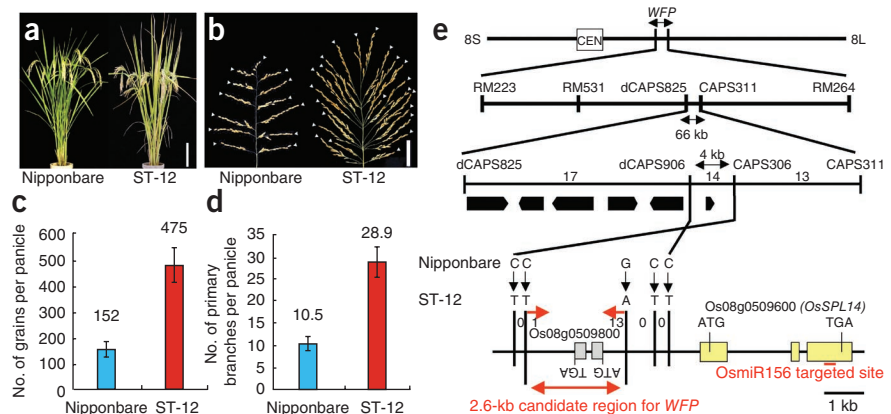
To further understand the molecular mechanisms of the production of primary panicle branches, we focused on the positional cloning of the QTL detected on chromosome 8, named here *WFP* (WEALTHY FARMER'S PANICLE), which acts in a semidominant manner (Supplementary Fig. 2). Analysis of 3,000 F₂ plants narrowed the candidate region to between RM223 and RM264 (Fig. 1e). Further high-resolution mapping using F₃ and F₄ 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. 5a,b)¹⁰. 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

How to know it is on chromosome 8???

Figure 1 Characterization and cloning of the *WFP* QTL. (a) The gross morphologies of Nipponbare and ST-12. Scale bar, 20 cm. (b) The panicle morphologies of Nipponbare and ST-12. Arrowheads indicate primary branches. Scale bar, 5 cm. (c) Comparison of grain number per main panicle between Nipponbare and ST-12. (d) Comparison of primary branch number per main panicle between Nipponbare and ST-12. (e) The *WFP* locus was detected between RM223 and RM264 on chromosome 8. Positional cloning narrowed the *WFP* locus to a 66-kb region between dCAPS825 and CAPS311, and six genes are predicted to be located in this region by RAP-DB. Further analysis used 14 recombinant plants to narrow the candidate region to 2.6 kb. Numbers on the map indicate the number of recombinants. Predicted open reading frames and sequence differences between Nipponbare and ST-12 around the *WFP* candidate region are shown. Values are means, with bars showing s.d. ($n = 40$ plants in c,d).



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 *Arabidopsis*^{12–17} 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

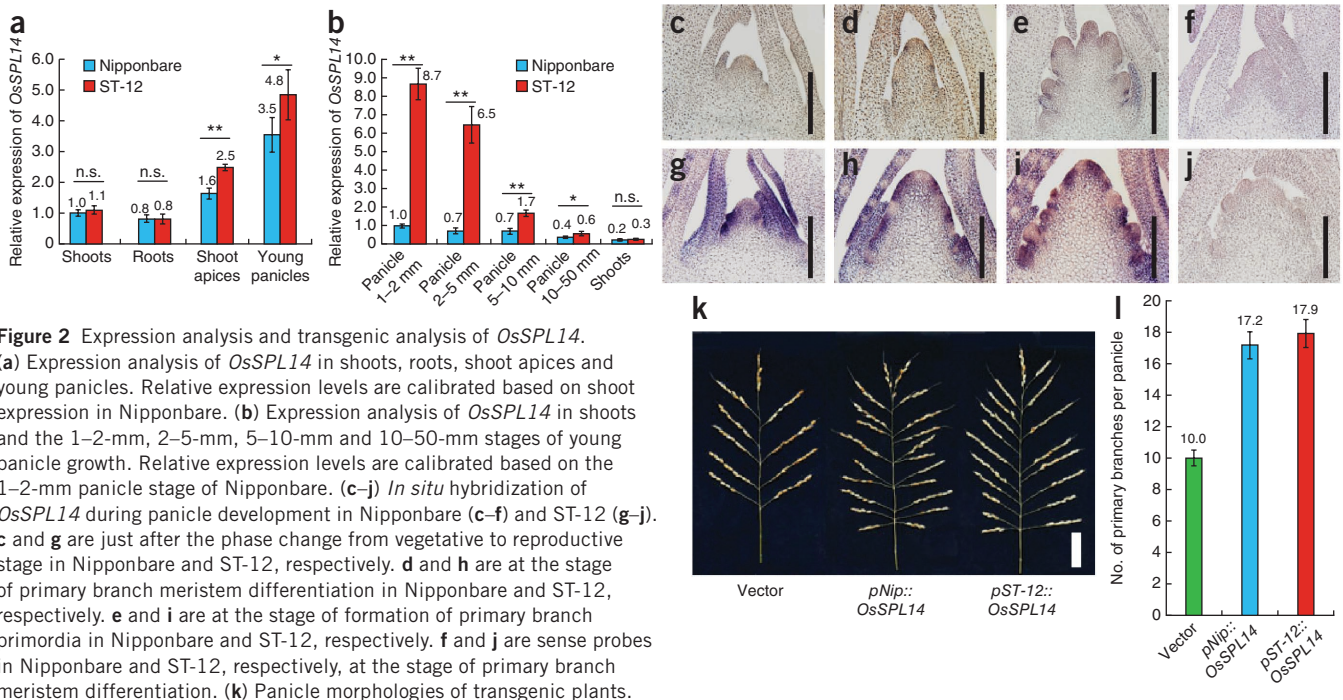
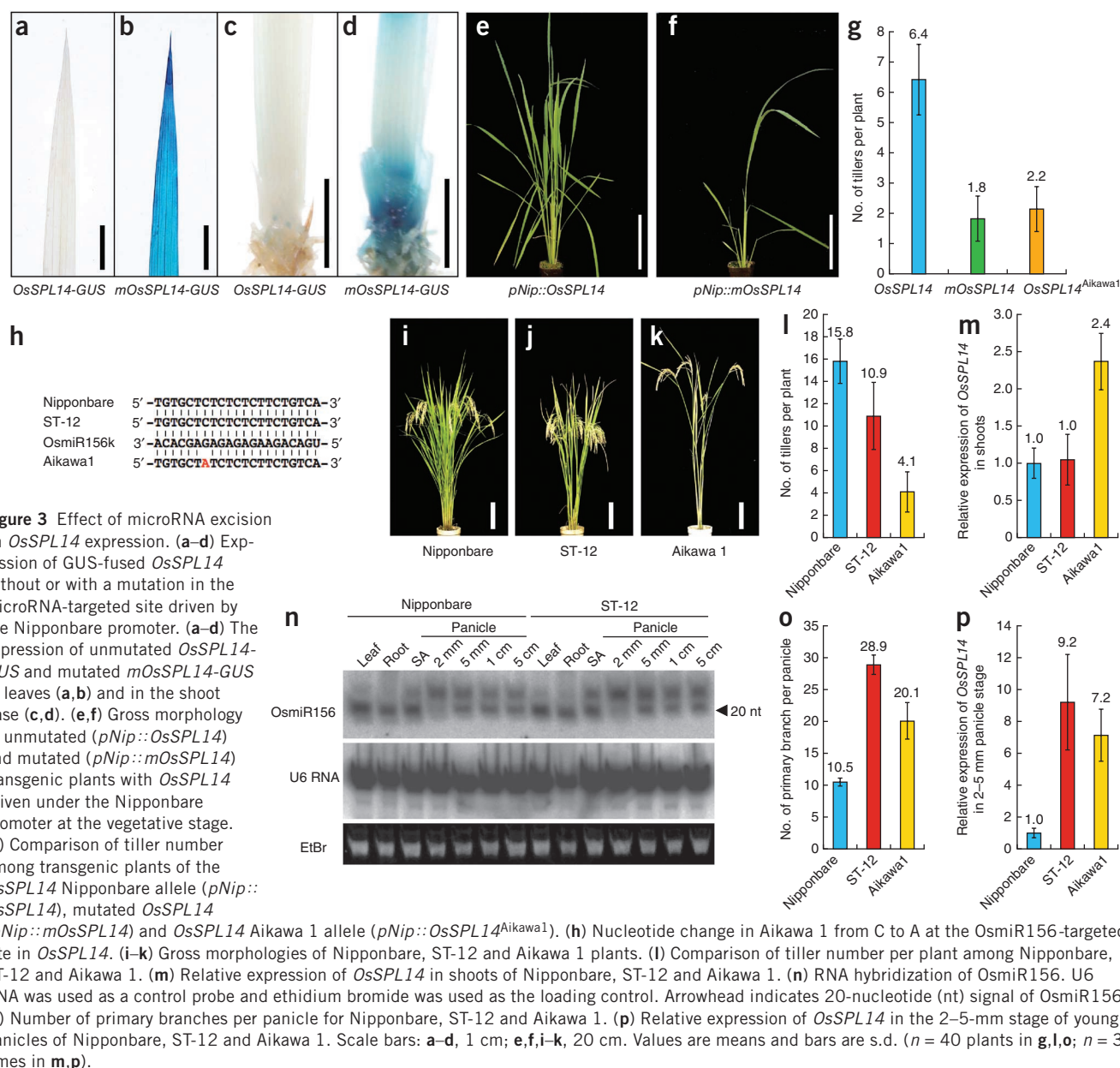


Figure 2 Expression analysis and transgenic analysis of *OsSPL14*. (a) Expression analysis of *OsSPL14* in shoots, roots, shoot apices and young panicles. Relative expression levels are calibrated based on shoot expression in Nipponbare. (b) Expression analysis of *OsSPL14* in shoot and the 1–2-mm, 2–5-mm, 5–10-mm and 10–50-mm stages of young panicle growth. Relative expression levels are calibrated based on the 1–2-mm panicle stage of Nipponbare. (c–j) *In situ* hybridization of *OsSPL14* during panicle development in Nipponbare (c–f) and ST-12 (g–j). c and g are just after the phase change from vegetative to reproductive stage in Nipponbare and ST-12, respectively. d and h are at the stage of primary branch meristem differentiation in Nipponbare and ST-12, respectively. e and i are at the stage of formation of primary branch primordia in Nipponbare and ST-12, respectively. f and j are sense probes in Nipponbare and ST-12, respectively, at the stage of primary branch meristem differentiation. (k) Panicle morphologies of transgenic plants. *OsSPL14* driven by the Nipponbare and ST-12 promoter, indicated as *pNip::OsSPL14* and *pST-12::OsSPL14*, respectively. Vector, the TAC7 vector control. Scale bar, 5 cm. (l) Comparison of primary branch number per main panicle of transgenic plants. Values are means, with bars showing s.d. ($n = 3$ times in a,b; $n = 40$ plants in l). Scale bars in c–j indicate 200 μm. **Significant at 1% level; *significant at 5% level; n.s., not significant.



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*

To observe whether the fused gene expressed or NOT.

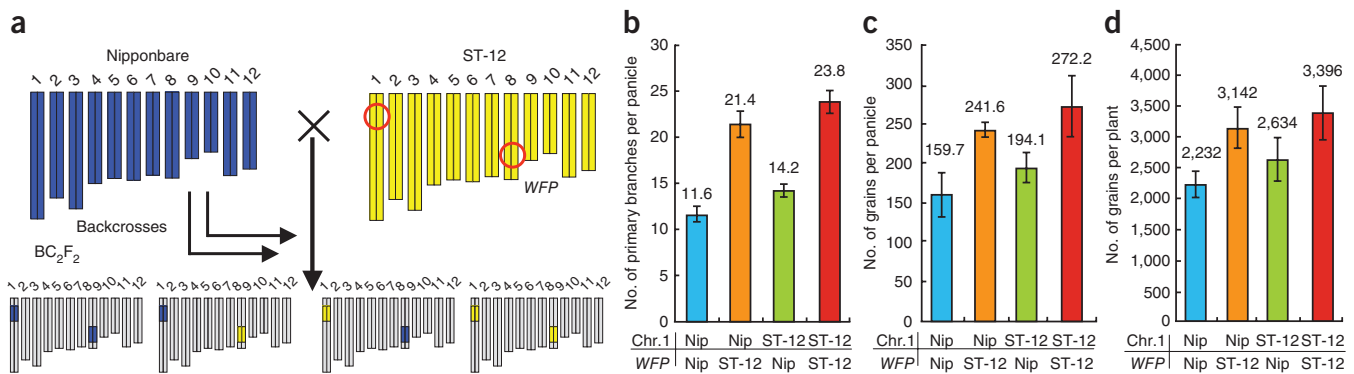


Figure 4 Effect of *OsSPL14*^{WFP} on grain yield. (a) Graphical genotypes of BC₂F₂ plants derived from a cross between Nipponbare and ST-12. Blue and yellow bars indicate chromosomes of Nipponbare and ST-12, respectively. Gray bars indicate chromosomes of undetermined genotypes. Red circles on ST-12 indicate the positions of QTLs controlling the number of primary branches. (b) Comparison of primary branch number per main panicle among the four BC₂F₂ genotypes. (c) Comparison of grain number per main panicle among the four BC₂F₂ genotypes. (d) Comparison of grain number per plant among the four BC₂F₂ genotypes. The Nipponbare and ST-12 genotypes in b–d are indicated as Nip and ST-12 on the x axes, respectively. Values in b–d are means, with bars showing s.d. (*n* = 40 plants).

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. 3h) 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 *OsSPL14*^{Aikawa1} allele, we prepared Nipponbare plants carrying *OsSPL14*^{Aikawa1}. Transgenic plants with the Aikawa1 *OsSPL14* allele driven under the Nipponbare promoter (*pNip::OsSPL14*^{Aikawa1}) 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 *OsSPL14*^{Aikawa1} 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 *OsSPL14*^{Aikawa1} 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. 3n); 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 *OsmiR156* target site, it is not cleaved by *OsmiR156*, 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 *OsSPL14*^{Aikawa1} 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 panicle¹⁰. 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 *OsmiR156*¹⁰.

Because *OsSPL14*^{WFP} 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 BC₂F₂ 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 *OsSPL14*^{WFP} 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 *OsSPL14*^{WFP} 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 *OsSPL14*^{WFP} 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 *OsSPL14*^{WFP} 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 rice^{2,19}.

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 plants¹⁰. Several studies have recently reported *SPL* function in *Arabidopsis* and rice^{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.

URLs. RAP-DB, <http://rapdb.dna.affrc.go.jp/>; Rice Genome Annotation Project, <http://rice.plantbiology.msu.edu/>.

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.

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AUTHOR CONTRIBUTIONS

H.K., M.M. and M.A. designed the research. K.M., M.I., A.M., X.-J.S., M.I. and K.A. conducted the research. K.M., X.-J.S., H.K. and M.A. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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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 × 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::OsSPL14^{Aikawa1}* constructs were transformed with the binary vector pYLTA7²⁸ (provided by RIKEN Bioresource Center). 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 (OsUbiq) 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 *t*-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 GRAMENE and TAIR (GRAMENE, <http://www.gramene.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 1× (Sigma H7033) was used for hybridization. The membranes were washed with wash solution (6× 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'-TGTATCGTTCCAATTTTATCGGATGT-3'; miR156: 5'-GTGCTCACTCTCTTCTGTGCA-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/l 4',6-diamidino-2-phenylindole dihydrochloride *n*-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 construct was then transformed into the yeast strain AH109. Each yeast liquid culture was serially diluted to A₆₀₀ = 0.6, and 2 μl of each dilution were inoculated onto tryptophan- and histidine-negative synthetic dropout medium. SK1-full was used as a positive control³².

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