Natural Variation in the Flag Leaf Morphology of Rice Due to a Mutation of the *NARROW LEAF 1* Gene in *Oryza sativa* L.

Fumio Taguchi-Shiobara,*[,1,2] Tatsuya Ota,[†] Kaworu Ebana,*[,3] Taiichiro Ookawa,[‡] Masanori Yamasaki,[§] Takanari Tanabata,*[,4] Utako Yamanouchi,* Jianzhong Wu,* Nozomi Ono, Yasunori Nonoue,*[,5] Kazufumi Nagata,* Shuichi Fukuoka,* Hideyuki Hirabayashi,[††] Toshio Yamamoto,* and Masahiro Yano*[,6]**

*Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan, [†]Department of Evolutionary Studies of Biosystems, SOKENDAI (The Graduate University for Advanced Studies), Hayama, Kanagawa 240-0193, Japan, [‡]Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan, [§]Food Resources Education and Research Center, Graduate School of Agricultural Science, Kobe University, Kasai, Hyogo 675-2103, Japan, **Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan, and [††]Rice Research Division, NARO Institute of Crop Science, Tsukuba, Ibaraki 305-8518, Japan

ABSTRACT We investigated the natural variations in the flag leaf morphology of rice. We conducted a principal component analysis based on nine flag leaf morphology traits using 103 accessions from the National Institute of Agrobiological Sciences Core Collection. The first component explained 39% of total variance, and the variable with highest loading was the width of the flag leaf (WFL). A genome-wide association analysis of 102 diverse Japanese accessions revealed that marker RM6992 on chromosome 4 was highly associated with WFL. In analyses of progenies derived from a cross between Takanari and Akenohoshi, the most significant quantitative trait locus (QTL) for WFL was in a 10.3-kb region containing the *NARROW LEAF 1* (*NAL1*) gene, located 0.4 Mb downstream of RM6992. Analyses of chromosomal segment substitution lines indicated that a mutation (G1509A single-nucleotide mutation, causing an R233H amino acid substitution in NAL1) was present at the QTL. This explained 13 and 20% of total variability in WFL and the distance between small vascular bundles, respectively. The mutation apparently occurred during rice domestication and spread into *japonica*, *tropical japonica*, and *indica* subgroups. Notably, one accession, Phulba, had a *NAL1* allele encoding only the N-terminal, or one-fourth, of the wild-type peptide. Given that the Phulba allele and the histidine-type allele showed essentially the same phenotype, the histidine-type allele was regarded as malfunctional. The phenotypes of transgenic plants varied depending on the ratio of histidine-type alleles to arginine-type alleles, raising the possibility that H^{233}-type products function differently from and compete with R^{233}-type products.

KEYWORDS rice; flag leaf width; natural variation; *Oryza sativa* L.; *NARROW LEAF 1*

doi: 10.1534/genetics.115.181040
Manuscript received April 1, 2015; accepted for publication August 3, 2015; published Early Online August 13, 2015.
Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181040/-/DC1.
[1]Present address: Soybean Applied Genomics Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan. E-mail: fstagu@affrc.go.jp
[2]Corresponding author: Rice Applied Genomics Research Unit, Agrogenomics Research Center, National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki 305-8602, Japan. E-mail: fstagu@affrc.go.jp.
[3]Present address: Genetic Resources Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan.
[4]Present address: Center for Sustainable Resource Science, RIKEN, Tsukuba, Ibaraki 305-0074, Japan.
[5]Present address: Iwate Biotechnology Research Center, Kitakami, Iwate 024-0003, Japan.
[6]Present address: NARO Institute of Crop Science, Tsukuba, Ibaraki 305-8518, Japan.

THE leaf of grasses typically consists of a relatively narrow blade and sheath enclosing the stem, and venation is parallel in the blade and the sheath (Esau 1977). Because large leaves intercept more light, the leaf area of the blade strongly affects final yield in cereal crops (Watson 1952). To produce plants that intercept light efficiently, leaf angle has been a target in breeding programs because erect leaves can capture more sunlight (Sinclair and Sheehy 1999). It was demonstrated that a brassinosteroid-deficient mutant with erect leaves showed increased grain yield under dense planting conditions (Sakamoto *et al.* 2005). It is also essential to understand the mechanism of development and the natural variations in morphology of the flag leaf since photosynthesis in the top three leaf blades of the plant, especially flag leaf,

Table 1 Eigenvectors for nine flag leaf morphology traits in principal component analysis of 103 rice accessions from NIAS Core Collection

Principal component		1st	2nd	3rd
Eigenvalue		3.52	2.47	1.21
Contribution ratio		39.2	27.5	13.5
Cumulative contribution		39.2	66.6	80.1
Trait	Abbreviation			
Length of flag leaf	LFL	−0.13	0.08	0.78
Width of flag leaf	WFL	0.50	−0.05	0.21
No. of large vascular bundles	NLVB	0.39	−0.27	0.03
No. of small vascular bundles	NSVB	0.48	−0.24	−0.07
No. of small vascular bundles between large vascular bundles	NSVB bet LVB	0.43	−0.16	−0.10
Distance between small vascular bundles	DSVB	0.28	0.26	0.50
Thickness of flag leaf				
At point of large vascular bundle[a]	TFL_LVB	0.15	0.42	−0.25
At point of small vascular bundle[b]	TFL_SVB	0.13	0.57	−0.03
At point of motor cell[b]	TFL_MC	0.22	0.51	−0.17

[a] Second large vascular bundle from midrib.
[b] Near second large vascular bundle from midrib.

makes the largest contribution to the grain yield of rice (Tanaka 1958; Yoshida 1972).

The developmental processes of the flag leaf are the same as those of other leaves. In rice, the longitudinal strands in the leaf comprise the midrib, large vascular bundles, and small vascular bundles (Hoshikawa 1989). According to Inosaka (1962) and Itoh *et al.* (2005), the midrib and large vascular bundles initiate at the base of the leaf primordium and develop acropetally in the leaf and basipetally in the culm (stage P2 in leaf development). It takes about one plastochron to initiate a small vascular bundle after the initiation of the midrib. When the leaf sheath and blade start to differentiate (the beginning of stage P3), small vascular bundles become visible between the large vascular bundles at the base of leaf primordia. Small vascular bundles form acropetally in the leaf blade and basipetally in the stem. Later (stage P3), a small vascular bundle develops between the midrib and a large vascular bundle near the leaf tip and extends basipetally through the leaf blade. Then, more small vascular bundles form between large vascular bundles sequentially from the midrib toward the leaf margin. After the rapid elongation of the leaf blade (stage P4) and the leaf sheath (stage P5), the leaf becomes mature and growth is complete (stage P6).

Natural variations in flag leaf size have been reported for 491 rice accessions from Japan and 666 accessions from other countries (Matsuo 1952). Flag leaves were wider in accessions from Java, western China, and Latin America and narrower in those from north China, central China, and Russia. Flag leaves were longer in accessions from Java and India, but shorter in those from Taiwan, central China, and south China. In genome-wide association studies (GWAS) of 413 diverse accessions, significant loci accounted for ∼24% of variance in the width of the flag leaf (WFL), and three loci on chromosomes 1, 4, and 7 contributed 5.7, 5.0, and 6.1% of phenotypic variance, respectively (Zhao *et al.* 2011).

The *NARROW LEAF 1* (*NAL1*) gene was located at ∼31.2 Mb on chromosome 4 (hereafter, all genomic positions are based on Os-Nipponbare-Reference-IRGSP-1.0), close to one of the single-nucleotide polymorphisms contributing to the variation in WFL reported by Zhao *et al.* (2011). *NAL1* was originally isolated as a gene affecting vascular patterns in a study on a classic dwarf mutant with a narrow leaf (Qi *et al.* 2008) and was later shown to affect WFL, total spikelet number per panicle, photosynthetic rate, and chlorophyll content (Chen *et al.* 2012; Fujita *et al.* 2013; Takai *et al.* 2013; Zhang *et al.* 2014). *NAL1* encodes a plant-specific protein, and the *nal1* mutant with an in-frame deletion of 10 amino acids in exon 4 showed reduced basipetal polar auxin transport and fewer longitudinal veins, compared with wild type (Qi *et al.* 2008). A recent analysis of mutant rice with a *NAL1* null allele implied that *NAL1* is also involved in control of the cell cycle and cell division from the initial stage of leaf development onward (Jiang *et al.* 2015).

The *NAL1* gene exhibits natural variations that are associated with plant morphology in rice. The *NAL1* allele in Koshihikari, a *temperate japonica* cultivar, has three amino acid differences (R233H, A475V, and V484I) compared with *NAL1* in the *indica* cultivar Takanari. A study on near-isogenic lines revealed that the Koshihikari *NAL1* allele decreased the thickness of the flag leaf and increased the ratio of leaf area to dry mass, *i.e.*, specific leaf area (Takai *et al.* 2013). The *NAL1* allele in Daringan, a *tropical japonica* landrace, has the same predicted amino acid sequence as that of *NAL1* in Koshihikari. When the Daringan allele was introduced into an *indica* cultivar with the same NAL1 protein as Takanari (in an IR64 background), the progeny showed increased flag leaf width, more vascular bundles, greater root biomass, more spikelets, and increased grain yield per square meter (Fujita *et al.* 2013), suggesting that *NAL1* affects various yield-related traits.

In this study, we extensively examined world-wide collections of rice accessions using a variety of analyses, including principal component analysis (PCA) of morphological characters, GWAS, quantitative trait loci (QTL) analysis, and positional cloning, to explore the cause of the natural variations

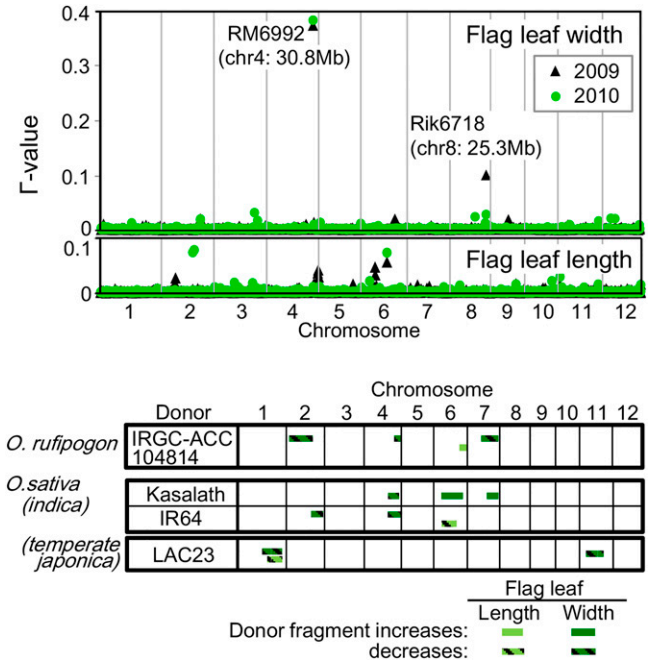

Figure 1 Regions associated with flag leaf width and/or length in rice. (A) Genome-wide association analysis of flag leaf width (top) and length (bottom) using 102 rice accessions and 1596 DNA markers in 2009 and 2010. (B) Regions associated with flag leaf width and/or length were detected by evaluating four sets of CSSLs in which the whole genome was covered by at least one donor fragment. Each CSSL was compared with the background accession Koshihikari. When the CSSL is significantly different from the background, regions covered by donor segment are shown in green or light green boxes.

in the flag leaf morphology of rice. We found that an amino acid-altering mutation in *NAL1*, which occurred during rice domestication, significantly contributes to the natural variations in rice flag leaf morphology.

Materials and Methods

Plant materials

The rice accessions used in this study are summarized in Supporting Information, Table S1, and details are provided in File S1.

A total of 103 accessions from the National Institute of Agrobiological Sciences (NIAS) Core Collection represented natural variations in *Oryza sativa* L.; 56 accessions were from the World Rice Core Collection (WRC) (Kojima *et al.* 2005), and 47 accessions were from the Core Collection of Japanese Landraces (Ebana *et al.* 2008). Seeds of all of these accessions were planted in 2013 to collect data for the PCA (Table S1 and Table S2).

A total of 102 accessions (Table S1 and Table S3) were selected to represent the breeding history of rice in Japan in the GWAS. These accessions were selected because their population structure was simpler than that of the accessions in the NIAS Core Collection (Yamasaki and Ideta 2013). Seeds of these accessions were planted in 2009 and 2010.

Seeds of world-wide collections of rice accessions, including those described above, hybrid populations derived from a Takanari × Akenohoshi cross, and most of the chromosomal segment substitution lines (CSSLs) were sown in April. The seedlings were transplanted in May in 2009–2013 into an experimental field at NIAS, Tsukuba, Japan. These plants were used to evaluate flag leaf morphology traits such as the length and width of the flag leaf, number of vascular bundles, distance between vascular bundles, and thickness of the flag leaf. Seeds of the CSSLs that were used to evaluate the Phulba allele of *NAL1* were sown in May 2013, and seedlings were transplanted in June.

Seeds of recombinant fixed lines containing either Akenohoshi or Takanari fragments were sown on April 20 and May 20, and seedlings were transplanted on May 20 and June 18 in 2010 and 2011, respectively, in an experimental field at the Tokyo University of Agriculture and Technology, Fuchu, Japan. These plants were used to evaluate yield and yield-related traits such as flag leaf size, dry weight of leaf and culm, and number of spikelets per plant.

Evaluation of flag leaf morphology

Plants with a heading date between July 20 and September 15 were used to evaluate flag leaf morphology. Flag leaves were sampled in the field, and their width and/or length were measured. In 2012, the widest part of the flag leaf was measured as WFL in the field using a ruler. To perform the measurement tasks efficiently, we developed an application program to record measured data using an iPod touch (Apple Inc. Cupertino, CA). Additional details about evaluating flag leaf morphology are provided in File S1.

Principal component analysis

A flag leaf was sampled from the main or equivalent culm of each plant, and nine traits were scored to identify the primary factors affecting flag leaf morphology by PCA (Table 1). Scores from five plants were averaged to represent each accession. In addition to length, width, and thickness of the flag leaf, the number of small or large vascular bundles and the distance between vascular bundles were recorded. The thickness of the flag leaf was represented by the thickness at three points around the second vascular bundle next to the midrib, *i.e.*, the point of the large vascular bundle, the point of the small vascular bundle, and the point of the motor cell. All nine traits were used for the correlation analysis and PCA, which was conducted using JMP version 10.0 (SAS Institute, Cary, NC).

Genome-wide association analysis

We conducted the GWAS of flag leaf morphology using 102 rice accessions (Table S3) and 1596 DNA markers (Nagasaki *et al.* 2010; Yamasaki and Ideta 2013) tagged by Tagger (De Bakker *et al.* 2005). The GWAS model included the effects of multiple QTL and population structure and was based on the Bayesian method (Iwata *et al.* 2007; Yamasaki and Ideta 2013). A marker was regarded as significant if its mean

Table 2 Four amino acid-altering differences in 10.3-kb region harboring QTL for flag leaf width: amino acid-altering nucleotide differences present in exon regions in 10.3-kb region

	NARROW LEAF 1 (*NAL1*)			*Os04t0615200*
	3rd exon	5th exon		2nd exon
Nucleotide site	1509	2727	2753	No. of CTG repeats starting from 115th nucleotide
Amino acid residue	233	475	484	No. of glutamine residues starting from 39th amino acid
Parents used in F_2 analysis				
Takanari	G (R: arginine)	C(A: alanine)	G(V: valine)	3
Akenohoshi	A (H: histidine)	T(V: valine)	A (I: isoleucine)	4

gamma value was higher than the specific threshold of gamma = 0.1.

Quantitative trait loci analysis

Simple sequence repeat (SSR) markers spread throughout the genome were used for the QTL analysis. The SSR markers were selected from those described in McCouch *et al.* (2002) and the International Rice Genome Sequencing Project (2005). Polymorphisms were detected as described by Ebana *et al.* (2011). Genomic DNA was extracted from leaves of each plant by the cetyl trimethylammonium bromide method. A total of 96 and 149 markers were used to analyze 93 F_2 (Akenohoshi/Takanari) plants and 95 backcrossed inbred lines (BILs) (Jarjan/Koshihikari//Koshihikari), respectively. Linkage maps were constructed with MAPMAKER/EXP 3.0 software (Lander *et al.* 1987) using the Kosambi map function.

The length and width of the flag leaf were the averages of the three largest flag leaves per plant in the F_2 population (Akenohoshi/Takanari) or averages of the largest flag leaf of five plants per line in the BIL population (Jarjan/Koshihikari//Koshihikari).

The QTL analyses were performed by composite interval mapping as implemented by QTL Cartographer 2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). Genome-wide threshold values ($\alpha = 0.05$) were used to detect QTL based on the results of 1000 permutations (Churchill and Doerge 1994).

One-way analysis of variance

The 103 accessions from the NIAS Core Collection were divided into two groups according to the 1509th nucleotide (233rd amino acid) of their *NAL1* gene; 62 accessions had a guanine (arginine) residue, and 41 accessions had an adenine (histidine) residue. To test the equality of the two groups, one-way analysis of variance for length and width of the flag leaf, number of vascular bundles, distance between small vascular bundles, and thickness of the flag leaf was performed using Microsoft Excel 2007. The variance between two groups was divided by the total variance to obtain the coefficient of determination (R^2).

Production and evaluation of transgenic plants

Details of the production of transgenic plants are provided in File S1.

The copy number of transgenes in T_0 and T_1 plants was determined based on the amount of *NAL1* genes relative to ubiquitin 2 (*RUBQ2*, AF184280) genes, as determined by quantitative real-time PCR. The primers used are shown in Table S4. The T_1 plants were classified into groups depending on the copy number of transgenes, and each group was compared to the vector control group using the F-test and *t*-test in Microsoft Excel 2007. To identify the T_1 plant groups that differed significantly from the vector control group, we used Tukey's "Honest Significant Difference" method in the "multcomp" package of R v. 3.2.0 software (R foundation, Vienna).

Molecular phylogenetic analysis

The transcribed region of *NAL1* was amplified from the 56 accessions from the WRC (Kojima *et al.* 2005) by PCR with the primers shown in Table S4. The PCR products were sequenced, and phylogenetic analyses were conducted with MEGA6 (Tamura *et al.* 2013). The complete deletion option was selected to use only sites shared among aligned sequences, and the T92 + G model was selected as the best-fit model with the lowest Bayesian information criterion score. The maximum-likelihood method was used to infer the phylogenetic tree and ancestral nucleotide sequences.

Homology searches in databases of other species

Homology searches were conducted using TBLASTN with the predicted NAL1 protein sequence of an accession from the NIAS Core Collection, Bei Khe (WRC 03), as the query. The searches were conducted against the genome databases of plants shown in Table S9B, and the sequence with the lowest *E*-value was selected for each species.

Real-time PCR analysis

Details on determining the transcript levels of *NAL1* at various developmental stages and the transgene copy number in transgenic plants are provided in File S1. The primers used for real-time PCR are shown in Table S4. TaqMan real-time PCR was performed as described previously (Kojima *et al.* 2002). All TaqMan probes (Operon) were 3′-labeled with Black Hole Quencher-1a dye. The *RUBQ2* probe was 5′-labeled with VIC, and *HPT* and *NAL1* probes were 5′-labeled with the reporter dye FAM.

Data availability

Accessions of NIAS Rice Core Collection (Table S2 and Table S8) are available at https://www.gene.affrc.go.jp/databases-core_collections_wr_en.php for WRC, and

Table 3 Four amino acid-altering differences in 10.3-kb region harboring QTL for flag leaf width: flag leaf width of CSSLs in the Koshihikari background

		NARROW LEAF 1 (*NAL1*)			*Os04t0615200*				
		3rd exon	5th exon		2nd exon				
	Nucleotide site	1509	2727	2753	No. of CTG repeats starting from 115th nucleotide				
	Amino acid residue	233	475	484	No. of glutamine residues starting from 39th amino acid				
						Flag leaf width (mm; n = 5)			
	Accession	Nucleotide (amino acid)				Year	CSSL	Control (Koshihikari)	*P*
Background	Koshihikari	A (H)	T(V)	A(I)	4				
Donor of CSSL	Hayamasari	A (H)	T(V)	A(I)	4	2012	11.2 ± 0.45	11.6 ± 0.55	NS
	Tupa 121-3	A (H)	T(V)	A(I)	3	2012	12.0 ± 0.00	12.4 ± 0.55	NS
	Khao Nam Jen	G (R)	T(V)	A(I)	3	2012	9.0 ± 0.00	12.4 ± 0.89	6.2*E*-05
	Naba	G (R)	T(V)	A(I)	3	2013	9.4 ± 0.55	12.4 ± 0.55	2.5*E*-05
	Shuusoushu	G (R)	T(V)	A(I)	3	2013	9.6 ± 0.55	13.2 ± 1.10	1.7*E*-04
	Basilanon	G (R)	C(A)	A(I)	3	2013	9.8 ± 0.45	11.8 ± 0.84	1.5*E*-03
	Kasalath	G (R)	C(A)	G(V)	3	2013	9.0 ± 0.71	11.4 ± 0.55	9.4*E*-05
	IR64	G (R)	C(A)	G(V)	3	2012	10.2 ± 0.45	12.6 ± 0.55	3.2*E*-05
	Bei Khe	G (R)	C(A)	G(V)	3	2012	8.8 ± 0.45	12.2 ± 0.45	1.1*E*-06
	Deng Pao Zhai	G (R)	C(A)	G(V)	3	2013	10.8 ± 0.45	12.2 ± 0.45	1.1*E*-03
	Bleiyo	G (R)	C(A)	G(V)	3	2013	8.6 ± 0.55	13.2 ± 0.45	4.9*E*-07

https://www.gene.affrc.go.jp/databases-core_collections_jr_en.php for JRC.

Accessions having NIAS Genebank accession number (JP No) (Table S3) is accessible by 'Plant Search' in NIAS Genebank Databases (https://www.gene.affrc.go.jp/databases_en.php). As for wild rice (Table S9), accessions of Wild Core Collection Rank 1 provided by National Institute of Genetics (NIG) are available at http://www.shigen.nig.ac.jp/rice/oryzabase/strain/wildCore/about.

Results

First component of PCA explained 39% of total variance and WFL had highest loading

To identify the morphological characters of the flag leaf that primarily contribute to the natural variations in rice, we evaluated the following nine traits: length of the flag leaf (LFL), WFL, number of large vascular bundles (NLVB), number of small vascular bundles (NSVB), number of small vascular bundles between large vascular bundles (NSVB bet LVB), distance between small vascular bundles (DSVB), and thickness of flag leaf at the point of the large vascular bundle (TFL_LVB) at the point of the small vascular bundle (TFL_SVB) and at the point of the motor cell (TFL_MC) (Table S2, right). There were positive correlations (1) among the four traits WFL, NLVB, NSVB, and NSVB bet LVB; (2) between DSVB and WFL, TFL_SVB, or TFL_MC (Table S5); and (3) among the three thickness of flag leaf traits. The highest estimated correlation coefficient was 0.87.

The above nine traits were used in the PCA. This analysis covered 103 accessions from the NIAS Core Collection that represented the natural variations in *O. sativa* (Table 1). In the PCA, the first three components accounted for >80% of the total variance. PC1 and PC2 accounted for 39.2 and 27.5% of total variance, respectively, and the following variables showed substantial loadings: WFL, NLVB, NSVB, and NSVB bet LVB for PC1; TFL_LVB, TFL_SVB, and TFL_MC for PC2; and LFL and DSVB for PC3. WFL and its correlated traits were, therefore, the most significant characters to distinguish the natural variations in the flag leaf morphology of rice.

Scatter plots of PC1/PC2 or PC2/PC3 exhibited overlapping but distinct distributions of the *indica*, *temperate japonica*, and *tropical japonica* subgroups (Figure S1; see also Table S6) and reflected the morphological features of the three cultivars. Flag leaves tended to be thin and short with a moderate width in *indica* accessions; narrow, thick, and long in *temperate japonica* accessions; and wide, thick, and short in *tropical japonica* accessions.

QTL on chromosomes 4 and 8 contributed to natural variations in WFL

The GWAS for WFL (Figure 1A) identified a marker RM6992 at 30.8 Mb on chromosome 4 with a gamma value >0.1 over 2 years. Another marker, Rik6718, at 25.3 Mb on chromosome 8, also had a gamma value >0.1 in 2009, while no marker with gamma values >0.1 were detected for LFL.

We conducted whole-genome analyses of four CSSLs: two with *indica* accessions of *O. sativa* (Kasalath and IR64) as the donor, one with a *temperate japonica* accession (LAC23) as the donor, and one with *Oryza rufipogon* (IRGC-ACC104814) as the donor, in the Koshihikari background. These analyses,

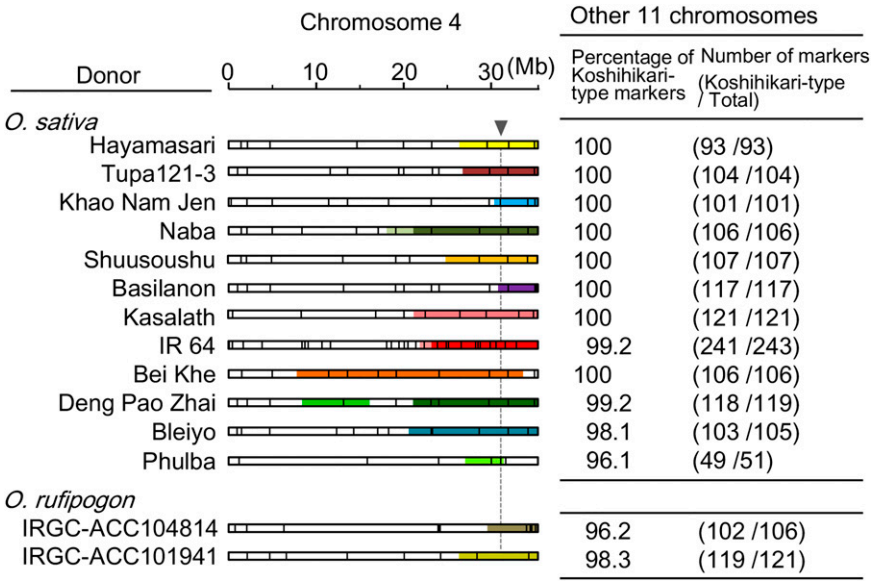

Figure 2 Chromosome 4 of 14 CSSLs in which the 10.3-kb region harboring the QTL for flag leaf width was replaced by a fragment from the donor. Vertical lines on chromosomes indicate positions of SSR or INDEL markers. White and colored regions indicate Koshihikari background and donor fragments, respectively. Triangle indicates 10.3-kb region. In three CSSLs with Naba, IR 64, or Deng Pao Zhai as the donor, heterozygous regions are shown in light color. Background of CSSLs in the remaining 11 chromosomes was largely from Koshihikari, as shown at right. Of 12 CSSLs with *O. sativa* as donor, 11 CSSLs (except for 1 with a Phulba fragment) were used to determine cause of variation in flag leaf width harbored in the 10.3-kb region.

except for one using the LAC23 as the donor, identified a QTL for WFL on the long arm of chromosome 4 but no QTL on chromosome 8 (Figure 1B).

The variation in WFL was further studied by a QTL analysis followed by a mapping study using recombinant fixed lines. The analyses of the F_2 population derived from a Takanari × Akenohoshi cross revealed two QTL, one of which was located in the region near 32.2 Mb on chromosome 4 (Table S7 and Figure S2A). Three QTL, including one located in the region near 18.8 Mb on chromosome 8, were identified in the analysis of BILs derived from a BC_1F_1 (Jarjan/Koshihikari//Koshihikari) plant (Table S7). In both analyses, QTL for WFL were not linked to markers for heading date (HD), whereas QTL for LFL and for HD on chromosome 6 were linked to the same marker.

QTL for WFL on chromosome 4 was located within a 10.3-kb region

We analyzed the effect of the QTL on chromosome 4 on WFL using a pair of lines with the same fixed Takanari/Akenohoshi background except at two regions: one at 31.2–35.0 Mb on chromosome 4 and the other at 2.3–4.5 Mb on chromosome 7, which has no QTL (Figure S2, A and B). Compared with the line with the Takanari-derived allele, the line with the Akenohoshi-derived allele at the 31.2- to 35.0-Mb region on chromosome 4 showed greater WFL, spikelet number, hulled grain weight per plant, and panicle weight per growing area (Figure S2B). This result indicated that the 31.2- to 35.0-Mb region on chromosome 4 likely contained a QTL for WFL and/or yield.

We further examined an additional 77 recombinant fixed lines with the same background as that of F_4 line_10-7-58-16-1 or F_4 line_10-7-58-16-5 (Figure S2B). These analyses further restricted the QTL to a 10.3-kb region within the 31.2- to 35.00-Mb region on chromosome 4. This region contained only three ORFs: Os04t0615000 (*NARROW LEAF 1*), Os04t0615100, and Os04t0615200 (Figure S2C). Among the three genes, *NAL1* had three nucleotide differences that would alter the encoded amino acid (one in exon 3 and the

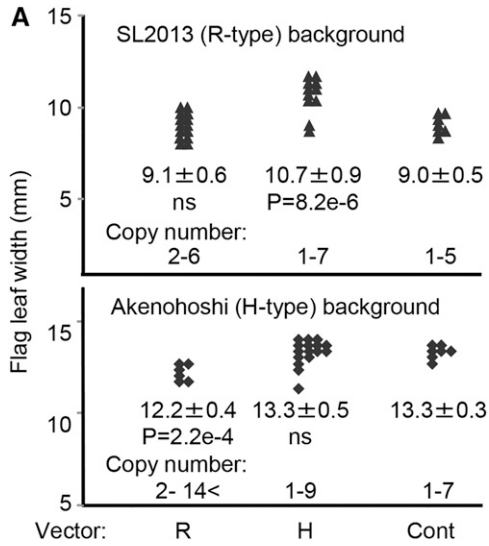

Figure 3 Flag leaf morphology of plants transformed by the *NAL1* gene with the endogenous promoter. Vectors or background with arginine or histidine at the 233rd amino acid residue of *NAL1* are R-type and H-type, respectively. SL2013 and Takanari are R-type; Koshihikari and Akenohoshi are H-type. Vector with Takanari allele is R vector; R vector in which the 233rd arginine is replaced by histidine is H vector. (A) T_0 transgenic plants harboring R vector, H vector, and empty vector as control in SL2013 or Akenohoshi background. (B) T_1 transgenic plants harboring R vector, H vector, and vector control in SL2013 background. Copy number of introduced vectors, total copy number of *NAL1*, and proportion of H-type are shown below. Each experimental group was compared to vector control (*t*-test), and *P*-values are shown above plots. Different letters below plots indicate significant differences ($P < 0.05$, Tukey's HSD test). (C) T_1 transgenic plants harboring H vector in Akenohoshi background. (D) T_1 transgenic plants harboring H vector or R vector in SL2013 background.

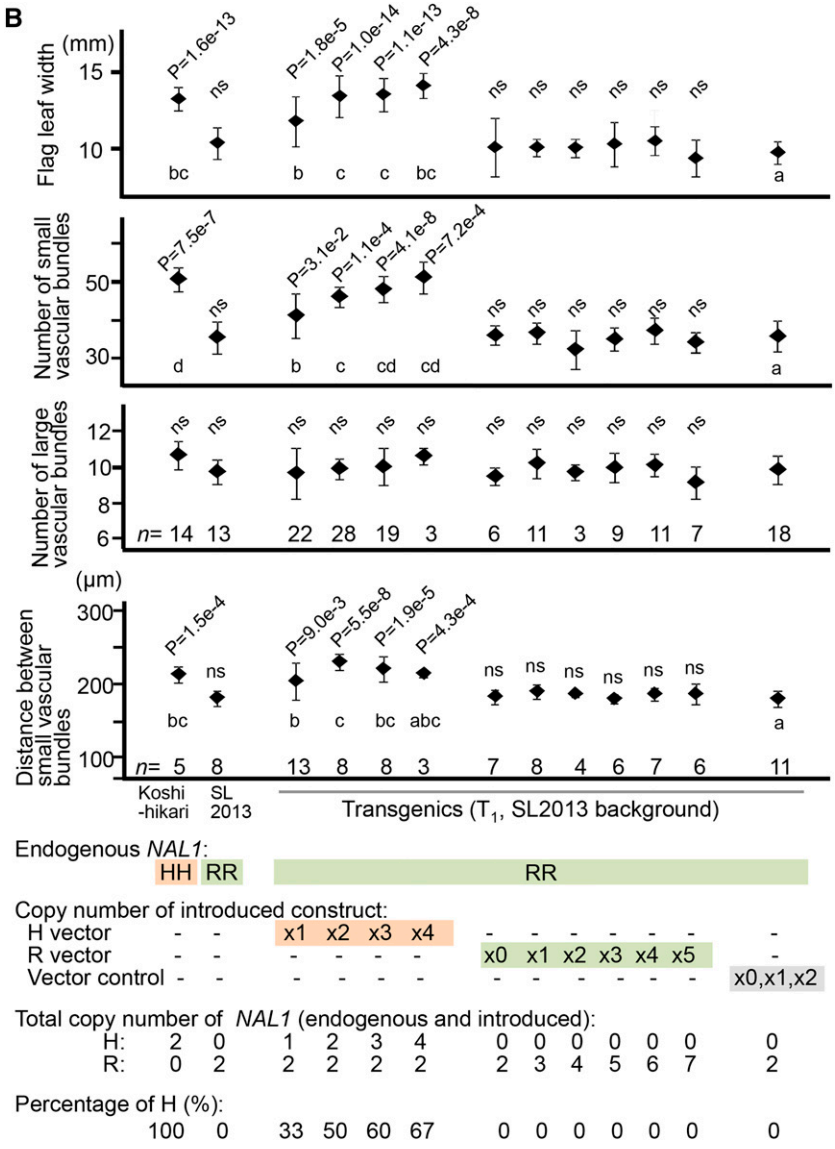

Figure 3 Continued.

other two in exon 5). Another difference between Takanari- and Akenohoshi-derived alleles was the length of a poly-glutamine region, that is, a difference in the number of CTG repeats, in exon 2 of Os04t0615200 (Table 2 and Figure S2C).

Given that *NAL1* was the best candidate for the QTL for WFL among the three ORFs, we examined its promoter region, since a previous report suggested that some *NAL1* alleles were expressed at different levels (Zhang *et al.* 2014). A pair of recombinant fixed lines, F_5 line_10-7-52-84-3-1 and -2 (Figure S2D), which had either the Takanari or Akenohoshi fragment in the upstream promoter region but shared the Akenohoshi coding region, exhibited the same WFL and yield. The promoter region accounted for none or little of the variations in WFL and/or yield (Figure S2D), suggesting that mutations in the coding region, not the promoter region, caused the variation in WFL in this case.

R233H amino acid substitution in NAL1 caused variation in WFL and its phenotypic effect depended on the ratio of copy number of R^{233} type to H^{233} type

Eleven CSSLs with different donor fragments at the 10.3-kb region in the Koshihikari background (Figure 2) were used to identify the causative mutation of the QTL for WFL. Of the 11 CSSLs shown in Table 3, only 2 had the same WFL as Koshihikari (Table 3, column *P*). The donors of these CSSLs were Hayamasari or Tupa121-3. Among the four differences mentioned above, only the R233H amino acid difference in NAL1 coincided with the observed differences in WFL (Table 3).

To gain insight into the R233H amino acid mutation, we compared eight traits between Koshihikari and CSSLs whose donor was one of four *O. sativa* accessions (Hayamasari, Tupa 121-3, Khao Nam Jen, Bei Khe) or one of two *O. rufipogon* accessions (IRGC-ACC104814, IRGC-ACC101941) (Figure

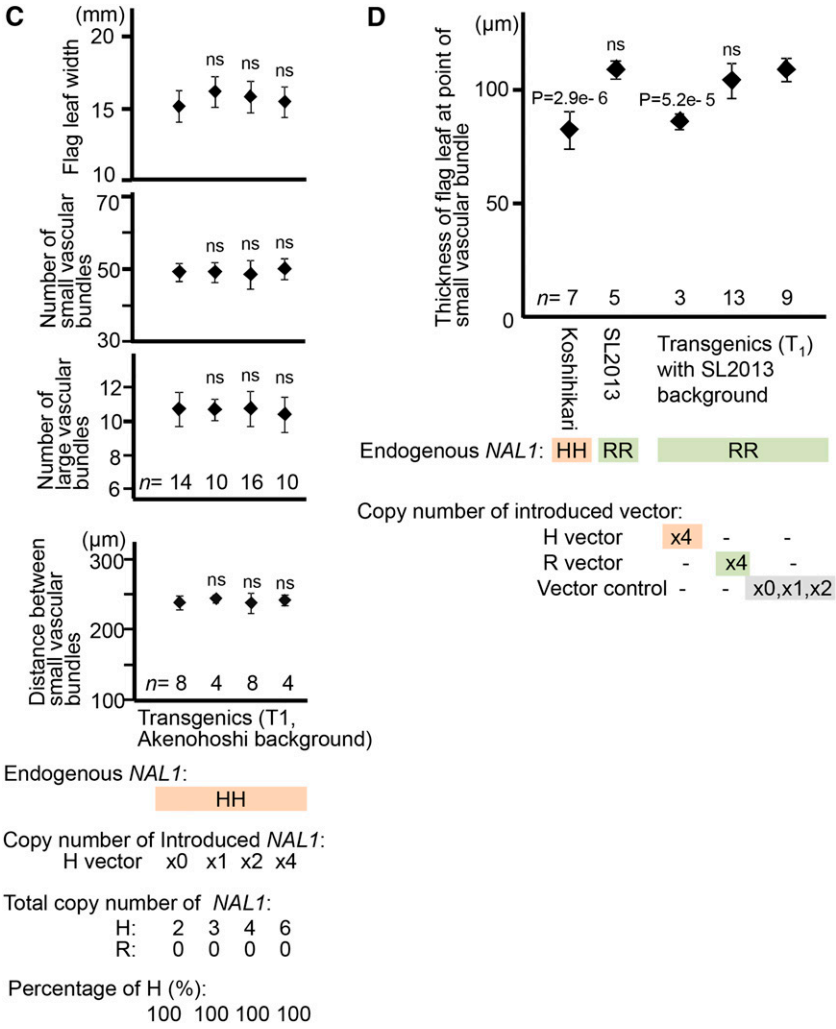

Figure 3 Continued.

S3 and Figure 2). The seven traits in addition to WFL were LFL, NLVB, NSVB, TFL_SVB, DSVB, number of cells between motor and epidermal cells (NCME), and number of cells between large vascular bundle and epidermal cells (NCLVBE). We also compared these traits between a pair of recombinant fixed lines that had a Takanari fragment (F_4_10-7-58-16-5) or an Akenohoshi fragment (F_4_10-7-58-16-1) at the 31.2- to 35.00-Mb region (Figure S2B). These analyses confirmed that the 233rd amino acid substitution in NAL1 affected NSVL, NCME, and NCLVBE, as well as WFL (Figure S3).

To further explore the effect of the R233H amino acid mutation on WFL, we transformed two lines (SL2013 with R-type NAL1 and Akenohoshi with H-type NAL1) with either Takanari NAL1 or the Takanari NAL1_R233H vector (see File S1). We observed the phenotypes of the T_0 and T_1 generations (Figure 3) and found that the WFL increased when the H construct was introduced into the R-type background, whereas the WFL decreased when the R construct was introduced into the H-type background. There was no apparent effect when the R construct was introduced into the R-type background or when the H construct was introduced into the H-type background. In the R-type background (Figure 3B), WFL was correlated with NSVB and DSVB but not with NLVB. Interestingly, the WFL, NSVB, and DSVB of transgenic plants increased as the copy number of H-type alleles increased from zero to two, but their values did not exceed those of Koshihikari (H-type) even if the copy number of H-type alleles was more than two. In the H-type background (Figure 3C), the copy number of the H-type allele had little or no effect on traits related to WFL. Therefore, the proportion rather than the actual copy number of introduced vectors (H-type alleles) was important for WFL. Interestingly, the wider flag leaves with increased NSVB and DSVB were thinner at the point of the small vascular bundles (Figure 3D).

R233H amino acid mutation occurred during O. sativa domestication and accounted for 13% of total variation in WFL among rice accessions from the global collection

We sequenced the transcribed region of the *NAL1* gene from 69 accessions from the NIAS Global Core Collection, the four parents of the CSSLs, and the two parents of the hybrid population used in the QTL analysis (Table S8A). The obtained

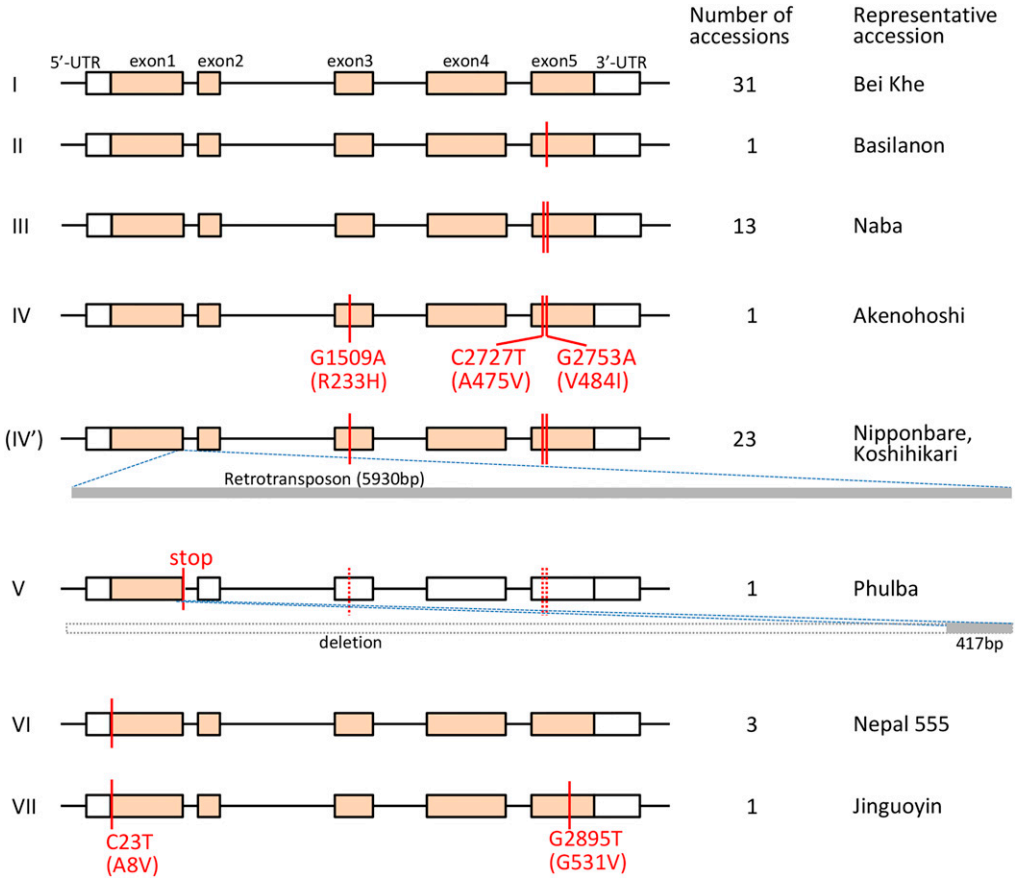

Figure 4 Seven types of *NAL1* genes observed in nature. Type IV′ with retrotransposon insertion presumably encodes the same amino acid sequence as that encoded by type IV. In total, 75 accessions (69 accessions from NIAS Global Core Collection, 4 donors of CSSLs not included in Core Collection, and 2 parents used to produce hybrid population for mapping QTL for flag leaf width) were classified into seven types. Boxes indicate transcribed regions; regions translated into polypeptide are shown in orange. Gray bar shows inserted retrotransposon.

sequences were classified into seven types according to their predicted amino acid sequences (Figure 4). Notably, 23 accessions including Nipponbare or Koshihikari had a retrotransposon insertion in exon 1 with duplicated regions at the 3′ end of exon 1 and the 5′ end of exon 2 (type IV′), although the nucleotide sequences in the coding region were intact and essentially the same as that in Akenohoshi (type IV). The type IV′ allele was also present in LAC23 and Jarjan, which explained why no QTL on chromosome 4 was detected in the analysis of a CSSL with LAC23 as donor and from BILs derived from BC_1F_1 (Jarjan/Koshihikari//Koshihikari). One accession, Phulba, had an allele in which the deletion of a large part of the inserted retrotransposon resulted in a stop codon near the end of exon 1 (type V).

Using the nucleotide sequences of only the shared region, and excluding the retrotransposon region, the maximum-likelihood tree was estimated by MEGA6 by inferring ancestral amino acid sequences under the best-fit evolutionary model of T92 + gamma (Figure 5). As for the 233rd amino acid, accessions with R^{233} or H^{233} were distributed in all three subgroups: *indica*, *temperate japonica*, and *tropical japonica*. However, most of the accessions with R^{233} were in the *indica* subgroup.

Exon 3 of *NAL1* was sequenced for 23 accessions from 10 wild rice species, and all had G at the 1509th nucleotide and encoded the amino acid R at the 233rd residue (Table S9A). Interestingly, the survey of *NAL1* homologs in the genome databases of plants of various taxa (Table S9B) revealed that species in the Streptophyta, including land plants (Embryophyta) had *NAL1* homologs with R at the corresponding amino acid position, while species in the Rhodophyta or Chlorophyta had no detectable *NAL1* homolog. This finding suggested that the original amino acid residue at this position in the rice *NAL1* was R, which later mutated into H.

We conducted a one-way analysis of variance of 103 accessions from the NIAS Core Collection to examine the functional significance of the G1509A nucleotide substitution (R233H amino acid substitution). The results showed that the substitution accounted for 13, 20, and 6% of the total variation in WFL, DSVB, and NSVB, respectively (Table 4).

H^{233}-type NAL1 gene is likely to be a malfunctional allele

One accession in the NIAS Core Collection, Phulba, had an allele encoding a truncated NAL1 protein of only 148 amino acids, in contrast to the full NAL1 with 582 amino acids (type V in Figure 4). The phenotypes of Koshihikari with the H-type allele (type IV′ in Figure 4) and a CSSL containing a Phulba-derived fragment of *NAL1* in the Koshihikari background were compared (Figure 2). There were no significant differences in any of the traits (Figure 6), indicating that the phenotypes of the Phulba allele and H-type allele were essentially the same and that both were likely to be malfunctional.

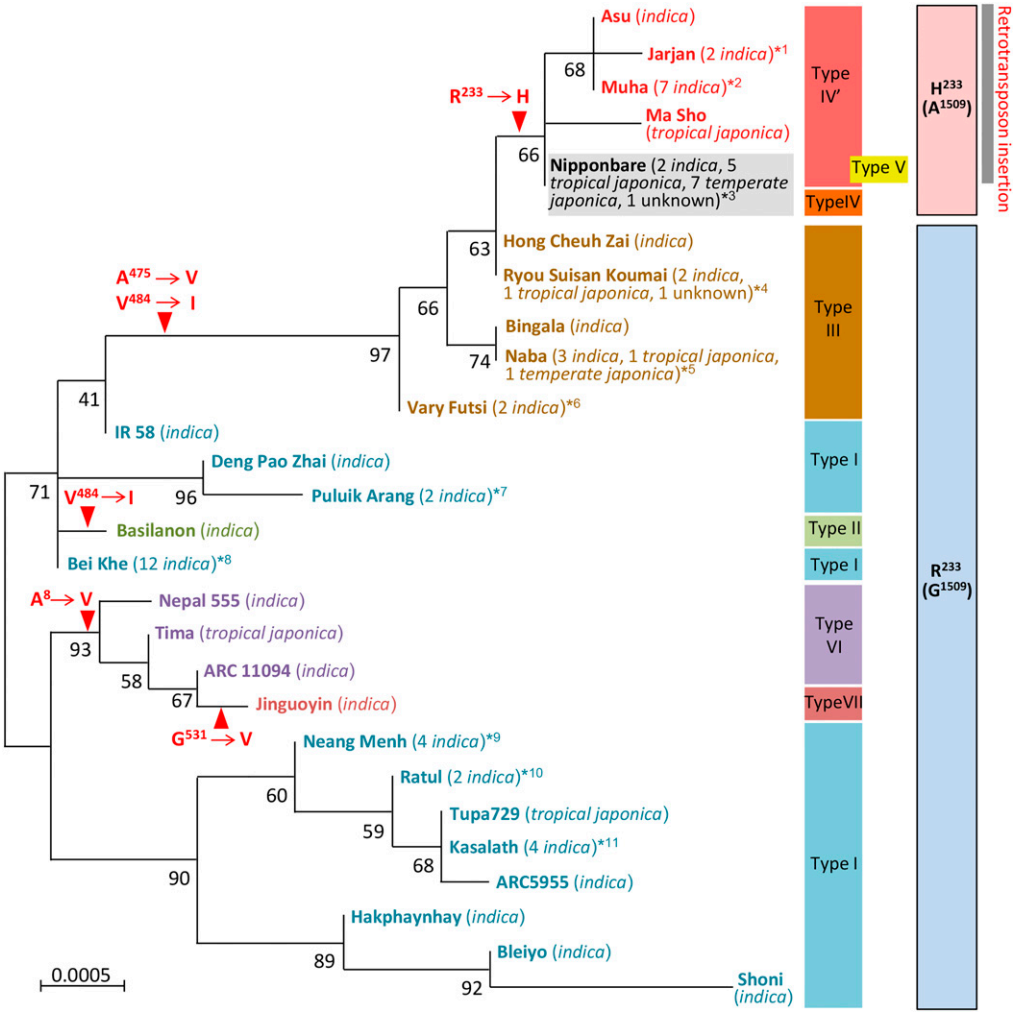

Figure 5 Maximum-likelihood tree of rice *NAL1* genes from 75 accessions. Types of NAL1 protein (see Figure 4) and numbers of accessions for each subgroup (*indica*, *tropical japonica*, and *temperate japonica*) are shown. Note that the nucleotide sequences used in the phylogenetic analysis are same for the 15 accessions included by "Nipponbare*3", and that Nipponbare*3 includes type IV, type IV′ and type V at the encoded protein level due to indels of retrotransposon. (Right) The 233th amino acid of NAL1, 1509th nucleotide of the NAL1 gene, and the retrotransposon insertion are shown. *1Jarjan and Kalo Dhan. *2Muha, Jhona 2, Napal 8, Anjana Dhan, Tupa 121-3, Surjamukhi, and ARC7291. *3Includes one type IV (Akenohoshi, *temperate japonica*,), 13 type IV′ (two *indica*, *i.e.*, Shwe Nang Gyi and Kaluheenati, five *tropical japonica*, *i.e.*, Khao Nok, Jaguary, Padi Perak, Rexmont, and Urasan 1, five *template japonica*, *i.e.*, Nipponbare, Dianyu 1, Koshihikari, Hayamasari, and LAC23, and one unknown, Calotoc) and one type V (Phulba, *temperate japonica*). *4Ryou Suisan Koumai, Tadukan, Pinulupot 1, and Khau Mac Kho. *5Naba, Shuusoushu, Keiboba, Khau Tan Chiem, and Khao Nam Jen. *6Vary Futsi and Deejiaohualuo. *7Puluik Arang and Neang Menh. *8Bei Khe, Davao 1, Co 13, Qingyu (Seiyu), Lebed, Milyang 23, Neang Phtong, Pokkari, Chin Galay, Vandaran, IR64, and Takanari. *9Neang Menh, Radin Goi Sesat, Kemashin, and Rambhog. *10Ratul and Local Basmati. *11Kasalath, Jena 035, ARC 7047, and Badari Dhan.

NAL1 expression peaked at stage P3 when small vascular bundles form

Immature flag leaves were sampled to survey the transcript levels of *NAL1* throughout flag leaf development. Leaves at stage P2 and at the beginning of stage P3 were too small to excise intact, and so the whole shoot apex was used instead. In the period from stage P2 to P6, *NAL1* transcript levels peaked at stage P3. At this stage, there was no difference in *NAL1* transcript levels among IR64 (R-type; type I in Figure 4), Koshihikari (H-type; type IV′), and Phulba (truncated protein; type V) (Figure 7).

Discussion

H^{233} in NAL1 was likely selected during rice domestication

Seven different NAL1 types have been detected among the rice accessions examined so far. The progenitor of cultivated rice had R^{233} in its NAL1 protein, given that all 23 accessions from 10 wild rice species including *O. rufipogon*, from which *O. sativa* originated, had R^{233} (Table S9A). The presence of the R-type allele of *NAL1* in land plants also suggests that the R-type allele is the ancestral form and that the H-type allele is derived from it (Figure 5). The R233H amino acid mutation, the insertion of a retrotransposon, and the subsequent deletion of the retrotransposon distinguished type IV from type III, type IV′ from type IV, and type V from type IV′, respectively (Figure 4). Our analyses raise the possibility that the three major subgroups of cultivated rice differentiated after the insertion of the retrotransposon into the H-type allele as both R^{233} (type I, II, III, VI, and VII) and H^{233} (type IV and IV′) were present among the *indica*, *temperate japonica*, and *tropical japonica* subgroups (Figure 4 and Table S8A).

According to Huang *et al.* (2012), ancient *japonica* was first domesticated from a specific population of *O. rufipogon* in southern China. Subsequently, *indica* originated from crosses between ancient *japonica* and local *O. rufipogon* as the initial cultivar of *temperate japonica* spread into Southeast and South Asia. Considering this pattern of distribution, we

Table 4 One-way analysis of variance based on the 1509th nucleotide (233rd amino acid) in 3rd exon of *NAL1* using 103 accessions from NIAS Core Collection

Trait	1509th nucleotide (233rd amino acid) in 3rd exon		One-way analysis of variance	
	G (arginine), n = 62 Average	A (histidine), n = 41 Average	P	R^2
Length of flag leaf (mm)	330 ± 5165	349 ± 3984	NS	—
Width of flag leaf (mm)	13.2 ± 5.0	15.3 ± 9.4	1.39*E*-04	0.13
No. of large vascular bundles	12.4 ± 1.8	12.9 ± 3.1	NS	—
No. of small vascular bundles	44.9 ± 78.1	49.8 ± 109.8	1.32*E*-02	0.06
No. of small vascular bundles between two large vascular bundles	4.63 ± 0.24	4.90 ± 0.35	1.52*E*-02	0.06
Distance between small vascular bundles (μm)	196 ± 368	215 ± 250	2.13*E*-06	0.20
Thickness of flag leaf (μm)				
At point of large vascular bundle[a]	231 ± 860	232 ± 508	NS	—
At point of small vascular bundle[b]	115 ± 191	115 ± 142	NS	—
At point of motor cell[b]	98.2 ± 126.5	98.5 ± 92.6	NS	—

[a] Second large vascular bundle from midrib.
[b] Near second large vascular bundle from midrib.

propose an evolutionary scenario in which the R233H amino acid substitution and the following retrotransposon insertion occurred at the differentiation of ancient *japonica* and that the *temperate japonica*, *indica*, and *tropical japonica* accessions with H-type alleles originated from crosses between *O. rufipogon* and ancient *japonica* harboring the mutation.

Although further population genetics studies are required to confirm this scenario, the current data suggest that the H-type allele has been selected for during rice domestication. The evidence for this hypothesis can be summarized as follows: the angiosperms examined so far have retained R-type NAL1 homologs for a long time during evolution, implying a selective advantage of R-type alleles over others in the natural environment. However, both H-type and R-type alleles have persisted among the three rice subgroups during the history of rice cultivation. The H-type allele directly or indirectly leads to increased WFL, increased number of spikelets per panicle, and often increased yields, although its effects vary among accessions (see below). Therefore, the H-type allele seems to have provided some desirable characters, *e.g.*, increased grain yields, with little, if any, adverse effects under artificially controlled environments. We propose that *NAL1* has been one of the important genes in the domestication of cultivated rice.

Amino acid change in NAL1 is important for increased WFL

Previous reports have suggested that there are differences among rice accessions in terms of the quantity and quality of *NAL1* gene expression. It was reported that the H-type allele in HB277 but not the R-type allele in D50 was subject to extensive alternative splicing, while both were expressed at similar levels (Chen *et al.* 2012). In Koshihikari (type VI′), only 20% of *NAL1* transcripts contained no retrotransposon, and a NAL1 protein the same size as that encoded by the R-type allele was synthesized (Takai *et al.* 2013). Furthermore, it was shown that *NAL1* expression was significantly higher in Nipponbare (type IV′) than in 93-11 (type I), although the expression level was shown to vary among developmental stages (Zhang *et al.* 2014). Overexpression of the Nipponbare allele in Nipponbare increased FWL and LWL through increasing the distance between vascular bundles (Zhang *et al.* 2014), indicating that the *NAL1* transcript level is an important factor in leaf morphology.

Nonetheless, most of the current evidence indicates that the different effects of NAL1 types on flag leaf phenotypes are mainly due to the amino acid at the 233rd position, rather than to changes in transcript levels. First, there was no phenotypic difference between a pair of recombinant fixed lines with the same H^{233}-type NAL1 driven by either the Takanari or Akenohoshi promoter (Figure S2D). Second, there was no difference in gene expression among the IR64 allele (type I), the Koshihikari allele (type IV′), and the Phulba allele (type V) at stage P3 (Figure 4 and Figure 7B). Third, transformation by two vectors driven by the same Takanari promoter with different amino acids at the 233rd residue resulted in marked differences in phenotype (Figure 3).

Products of the malfunctional H^{233}-type allele still regulate WFL and compete with R^{233}-type products

Although the H-type allele is thought to be malfunctional, it must retain some function because the phenotype of plants homozygous for H-type alleles differed from that of plants homozygous for the null allele. The leaves of plants with the null allele were 50% narrower and shorter than those of the H-type plants (Nipponbare, type IV′) (Jiang *et al.* 2015). The H-type allele was neither recessive nor dominant, as transgenic plants with both the R-type allele and the H-type allele exhibited intermediate phenotypes (Figure 3B). The effect on phenotype did not depend on the dosage of R-type or H-type genes, but on the ratio of the copy numbers of R-type to H-type (Figure 3). Therefore, the H^{233}-type NAL1 products likely compete with R^{233}-type products to regulate WFL.

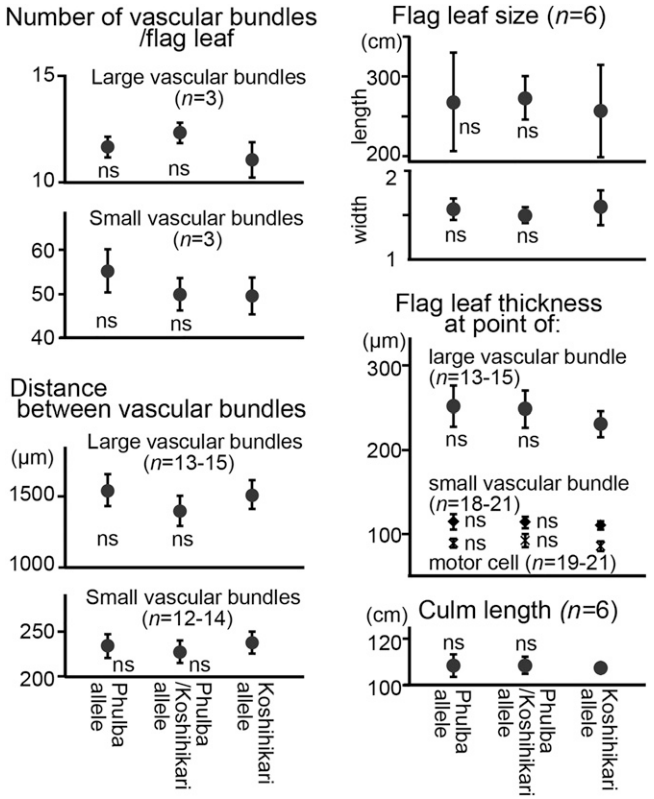

Figure 6 Comparison of flag leaf morphology and culm length among three genotypes: Phulba allele (type V), Koshihikari allele (type IV′), and Phulba/Koshihikari allele. No significant differences were observed among the three types.

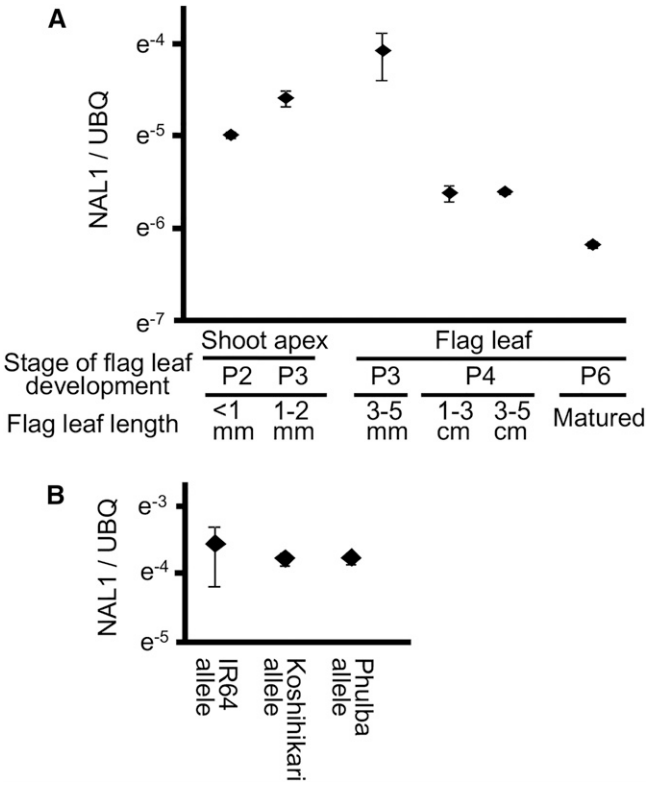

Figure 7 Gene expression of *NAL1* relative to that of ubiquitin (*UBQ*). (A) Changes in gene expression throughout early developmental stages of flag leaf in Koshihikari (type IV′) (mean ± SD, $n = 3$). Note that shoot apex includes flag leaf primordia. Stages of leaf: P2, hood-shaped primordium; P3, formation of blade-sheath boundary; P4, rapid elongation of leaf blade, P6, growth is completed. (B) Comparison of IR64 allele (type I), Koshihikari allele (type IV′), and Phulba allele (type V) in Koshihikari background at stage P3 (mean ± SD, $n = 3$).

Notably, the deletion of two amino acids in exon 1 resulted in a phenotype similar to that of the null mutant, with a 50% reduction in the width and length of the leaf (Jiang *et al.* 2015). Compared to a deletion in exon 1, a 10-amino-acid deletion in exon 4 had a milder effect: it reduced the blade width by 37% and the blade length by 13% (Qi *et al.* 2008). The Phulba allele, which produces a truncated protein consisting of only exon 1 (type V), resulted in a phenotype similar to that of the H-type allele (type IV′), which appeared to have no obvious defects (Figure 6). Therefore, the protein domain encoded by exon 1 seems to be essential for the regulation of WFL and LFL, as well as the competition of R^{233}-type and H^{233}-type products.

NAL1 regulates flag leaf width and exhibits various pleiotropic effects

NAL1 derived from Nipponbare (type IV′) was shown to promote cell division in the anticlinal direction and suppress it in the periclinal direction with no or little effect on cell size in the leaf (Jiang *et al.* 2015). This is consistent with our observations, where WFL, NSVB, DSVB were larger and TFL_SVB was smaller in the line transformed with the H-type allele (Figure 3). Takai *et al.* (2013) reported that the R-type allele derived from Takanari (type I) increased leaf thickness and the H-type allele derived from Koshihikari (type IV′) decreased leaf thickness. These results can be explained if the R-type allele is regarded as an allele with a weaker effect than the H-type allele on cell division in the anticlinal and periclinal directions. This would also explain the increase in WFL when the Daringan allele (H-type allele) was introduced into IR64 (R-type allele) (Fujita *et al.* 2013) and when the Nipponbare allele (H-type allele) was introduced into 93-11 (R-type allele) (Zhang *et al.* 2014). However, it could not explain the results of Chen *et al.* (2012), where the R-type allele increased WFL in mapping of QTL for WFL using hybrid populations originated from a cross between D50 (R-type allele) and HB277 (H-type allele). This discrepancy might be because of the presence of a large number of alternatively spliced forms of the H-type allele and a low level of the NAL1 protein (Chen *et al.* 2012). However, further analyses are required to identify the exact reason for this discrepancy.

Gene expression of *NAL1* was observed as early as the formation of leaf primordia (Jiang *et al.* 2015). *NAL1* was highly expressed in the vascular bundle, especially in the phloem (Qi *et al.* 2008), and its expression peaked at the P3 stage when the small vascular bundle formed. Therefore, its main effect appears to be on the formation of vascular

bundles, especially small vascular bundles, which subsequently affect leaf width and morphology. Nonetheless, the R233H amino acid mutation of NAL1 exhibits a pleiotropic effect. In addition to the increase in WFL and the number of vascular bundles, *Nal1* increased the total number of spikelets per panicle, root dry weight, and the rate of filled grains (Fujita *et al.* 2013). Improvements to the panicle, such as increased panicle length, more spikelets per panicle, and more secondary branches per panicle, were also observed after introducing the H-type allele (Zhang *et al.* 2014). Since *NAL1* is expressed in the culm, coleoptile, crown root, lateral root, and panicles (Qi *et al.* 2008; Fujita *et al.* 2013; Jiang *et al.* 2015), its regulation of the cell cycle and cell division in these tissues likely explains the pleiotropic effects of the *Nal1* mutation.

With respect to the grain yields, the effects of R-type and H-type alleles seem to vary. Higher photosynthetic rate per area was attained when the Koshihikari allele (H-type) was replaced by the Takanari allele (R-type) due to the increased number of mesophyll cells between vascular bundles and the larger total mesophyll area between the vascular bundles (Takai *et al.* 2013). Meanwhile, when IR64 (R-type allele) was replaced by the Daringan allele (H-type allele), grain yield per area increased despite the decreased number of panicles per plant and lower-1000-grain weight (Fujita *et al.* 2013). When the 93-11 allele (R-type) was replaced by the Nipponbare allele (H-type), the yield and chlorophyll content in the leaf increased, and panicle morphology was affected (Zhang *et al.* 2014). Grain yield is also affected by other factors including the primary structure and expression level of *NAL1*, the background genome, and the growth conditions/environmental factors. Therefore, the outcomes of different types of NAL1 alleles in various rice accessions are not easily predicted.

NAL1 may be involved in auxin transport and response

It has been speculated that *NAL1* is involved in polar auxin transport, since a *nal1* mutant lacking 10 amino acids from exon 4 produced fewer vascular bundles and contained lower levels of OsPIN1 protein, compared with wild type (Qi *et al.* 2008). The fact that the expression of the *AUXIN RESPONSE FACTOR* gene family (*ARF1*, *ARF2*, and *ARF3*) and *PIN1* was reduced in the *NAL1* null mutant (Jiang *et al.* 2015) supports the involvement of NAL1 in the auxin response. In this regard, it is notable that a *NAL1* homolog was found in *Klebsormidium flaccidum*, a terrestrial alga with a primitive body plan in the phylum Streptophyta (Table S9B). This species produces multicellular and nonbranching filaments without differentiated or specialized cells, but its genome contains most of the genes required for auxin biosynthesis, auxin receptors, auxin sensing, and auxin transport. Also, auxin (indole-3-acetic acid) was detected in tissues of this terrestrial alga by mass spectrometry (Hori *et al.* 2014). Future research, including studies on the phylum Streptophyta, will shed light on the function of NAL1 and its involvement in the auxin response.

Acknowledgments

We thank Akemi Tagiri for thin sectioning and Haruko Onodera for production of transgenic plants. The wild rice accessions used in this study were obtained from the National Institute of Genetics, which is supported by the National Bioresource Project, Ministry of Education, Culture, Sports, Science, and Technology, Japan. This work was supported by the National Institute of Agrobiological Sciences technical support system and by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics for Agricultural Innovation NVR-0001 and QTL-1002 and Genomics-based Technology for Agricultural Improvement IVG-2003).

Literature Cited

Chen, M., J. Luo, G. Shao, X. Wei, S. Tang *et al.*, 2012 Fine mapping of a major QTL for flag leaf width in rice, *qFLW4*, which might be caused by alternative splicing of *NAL1*. Plant Cell Rep. 31: 863–872.

Churchill, G. A., and R. W. Doerge, 1994 Empirical threshold values for quantitative trait mapping. Genetics 138: 963–971.

de Bakker, P. I. W., R. Yelensky, I. Pe'er, S. B. Gabriel, M. J. Daly *et al.*, 2005 Efficiency and power in genetic association studies. Nat. Genet. 37: 1217–1223.

Ebana, K., Y. Kojima, S. Fukuoka, T. Nagamine, and M. Kawase, 2008 Development of mini core collection of Japanese rice landrace. Breed. Sci. 58: 281–291.

Ebana, K., T. Shibaya, J. Wu, K. Matsubara, H. Kanamori *et al.*, 2011 Uncovering of major genetic factors generating naturally occurring variation in heading date among Asian rice cultivars. Theor. Appl. Genet. 122(6): 1199–1210.

Esau, K., 1977 *Anatomy of Seed Plants*. Ed. 2. Wiley & Sons, New York.

Fujita, D., K. R. Trijatmiko, A. G. Tagle, M. V. Sapasap, Y. Koide *et al.*, 2013 *NAL1* allele from a rice landrace greatly increases yield in modern indica cultivars. Proc. Natl. Acad. Sci. USA 110: 20431–20436.

Hori, K., F. Maruyama, T. Fujisawa, T. Togashi, N. Yamamoto *et al.*, 2014 *Klebsormidium flaccidum* genome reveals primary factors for plant terrestrial adaptation. Nat. Commun. 5: 3978.

Hoshikawa, K., 1989 *The Growing Rice Plant: An Anatomical Monograph*. Nobunkyo, Tokyo.

Huang, X., N. Kurata, X. Wei, Z.-X. Wang, A. Wang *et al.*, 2012 A map of rice genome variation reveals the origin of cultivated rice. Nature 490: 497–501.

Inosaka, M., 1962 Studies on the development of vascular system in rice plant and the growth of each organ viewed from the vascular connection between them. Bull. Fac. Agric. Univ. Miyazaki 7: 15–116.

International Rice Genome Sequencing Project, 2005 The map-based sequence of the rice genome. Nature 436: 793–800.

Itoh, J.-I., K.-I. Nonomura, K. Ikeda, S. Yamaki, Y. Inukai *et al.*, 2005 Rice plant development: from zygote to spikelet. Plant Cell Physiol. 46: 23–47.

Iwata, H., Y. Uga, Y. Yoshioka, K. Ebana, and T. Hayashi, 2007 Bayesian association mapping of multiple quantitative trait loci and its application to the analyses of genetic variation among *Oryza sativa* L. germplasms. Theor. Appl. Genet. 114: 1437–1449.

Jiang, D., J. Fang, L. Lou, J. Zhao, S. Yuan *et al.*, 2015 Characterization of a null allelic mutant of the rice *NAL1* gene reveals its role in regulating cell division. PLoS One 10(2): e0119169.

Kojima, S., Y. Takahashi, Y. Kobayashi, L. Monna, T. Sasaki *et al.*, 2002 *Hd3a*, a rice ortholog of the Arabidopsis *ft* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. Plant Cell Physiol. 43: 1096–1105.

Kojima, Y., K. Ebana, S. Fukuoka, T. Nagamine, and M. Kawase, 2005 Development of an RFLP-based rice diversity research set of germplasm. Breed. Sci. 55: 431–440.

Langdale, J. A., B. Lane, M. Freeling, and T. Nelson, 1989 Cell lineage analysis of maize bundle sheath and mesophyll cells. Bull. Fac. Agric. Univ. Miyazaki 133: 128–139.

Matsuo, T., 1952 Genecological studies on cultivated rice. Bulletin of Nat. Inst. Agr. Sci. D 3: 1–111.

McCouch, S. R., L. Teytelman, Y. Xu, K. B. Lobos, K. Clare *et al.*, 2002 Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res. 9: 199–207.

Nagasaki, H., K. Ebana, T. Shibaya, J. Yonemaru, and M. Yano, 2010 Core-single nucleotide polymorphisms: a tool for genetic analysis of the Japanese rice population. Breed. Sci. 60: 648–655.

Nagata, K., T. Ando, Y. Nonoue, T. Mizubayashi, N. Kitazawa *et al.*, 2015 Advanced backcross QTL analysis reveals complicated genetic control of rice grain shape in a japonica x indica cross. Breed. Sci. 65: 308–318.

Qi, J., Q. Qian, Q. Bu, S. Li, Q. Chen *et al.*, 2008 Mutation of the rice Narrow leaf1 gene, which encodes a novel protein, affects vein patterning and polar auxin transport. Plant Physiol. 147: 1947–1959.

Sakamoto, T., Y. Morinaka, T. Ohnishi, H. Sunohara, S. Fujioka *et al.*, 2005 Erect leaves caused by brassinosteroid deficiency increase biomass production and grain yield in rice. Nat. Biotechnol. 24: 105–109.

Sinclair, T. R., and J. E. Sheehy, 1999 Erect leaves and photosynthesis in rice. Science 283: 1455.

Takai, T., S. Adachi, F. Taguchi-Shiobara, Y. Sanoh-Arai, N. Iwasawa *et al.*, 2013 A natural variant of *NAL1*, selected in high-yield rice breeding programs, pleiotropically increases photosynthesis rate. Sci. Rep. 3: 2149.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar, 2013 MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol. 30: 2725–2729.

Tanaka, A., 1958 Studies on the characteristics of the physiological function of leaf at definite position on stem of rice plant. (Part 11): comparison of photosynthetic activity of leaves at various position on main stem and translocation of photosynthetic products within plant. Jpn. J. Soil Sci. Plant Nutr. 29: 327–333.

Watson, D. J., 1952 The physiological basis of variation in yield. Adv. Agron. 4: 101–145.

Yamasaki, M., and O. Ideta, 2013 Population structure in Japanese rice population. Breed. Sci. 63: 49–57.

Yoshida, S., 1972 Physiological aspects of grain yield. Annu. Rev. Plant Physiol. 23: 437–464.

Zhang, G.-H., S.-H. Li, L. Wang, W.-J. Ye, D.-L. Zeng *et al.*, 2014 *LSCHL4* from *japonica* cultivar, which is allelic to *NAL1*, increase yield of *indica* super rice 93–11. Mol. Plant 7: 1350–1364.

Zhao, K., C.-W. Tung, G. Eizenga, M. Wright, M. L. Ali *et al.*, 2011 Genome-wide association mapping reveals a rich genetic architecture of complex traits in *Oryza sativa*. Nat. Commun. 2: 467.

Communicating editor: A. H. Paterson

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181040/-/DC1

Natural Variation in the Flag Leaf Morphology of Rice Due to a Mutation of the *NARROW LEAF 1* Gene in *Oryza sativa* L.

Fumio Taguchi-Shiobara, Tatsuya Ota, Kaworu Ebana, Taiichiro Ookawa, Masanori Yamasaki, Takanari Tanabata, Utako Yamanouchi, Jianzhong Wu, Nozomi Ono, Yasunori Nonoue, Kazufumi Nagata, Shuichi Fukuoka, Hideyuki Hirabayashi, Toshio Yamamoto, and Masahiro Yano

DOI: 10.1534/genetics.115.181040

File S1

Supporting Materials and Methods

Plant materials

We evaluated flag leaf length and width using four sets of CSSLs in which the donor fragments covered the whole genome. Two sets of CSSLs, one with IR 64 as the donor (Nagata *et al*. 2015; http://www.rgrc.dna.affrc.go.jp/ineKIRKCSSL42.html) and one with LAC23 as the donor (Abe *et al*. 2013), were planted and evaluated in 2011. Another set of CSSLs, which had Kasalath as the donor (Ebitani *et al*. 2005; http://www.rgrc.dna.affrc.go.jp/ineKKCSSL39.html), was planted and evaluated in 2013. The set of CSSLs for which the donor was *O. rufipogon* IRGC-ACC104814 (Hirabayashi *et al*. 2010) was planted and evaluated in 2012.

The two cultivars Takanari and Akenohoshi, which are commonly grown for animal feed, were used to produce the hybrid population for mapping QTLs for flag leaf width and length. We selected these cultivars because we expected that their large plant body would allow for easier evaluation of flag leaves. Takanari was crossed with Akenohoshi to obtain F_1 seeds, and an F_1 plant was selfed to obtain F_2 seeds. A total of 93 F_2 plants were planted in 2013 to detect QTLs for flag leaf width and length.

Two lines, TUAT1-5-6 and TUAT1-5-32, which originated from a Takanari × Akenohoshi cross, were used to evaluate the effect of the QTL and to narrow down the region of the QTL. The panicle weight and number of spikelets were greater in both of these lines than in their parents. TUAT1-5-6 and TUAT1-5-32

were crossed to obtain F_1 seeds (F_1_10-7), and an F_1 plant was selfed five times to obtain F_6 seeds. Through the generations, marker-assisted selection was performed to obtain recombinant fixed lines. Out of 187 F_2 plants, one plant, F_2_10-7-58, was selected because it had a heterologous region in the 29–35 Mb region on chromosome 4, and had the Takanari allele or the Akenohoshi allele in 86 out of 90 SSR markers dispersed throughout the whole genome except for this region. F_2_10-7-58 was selfed to obtain F_3 seeds, and one F_3 plant, F_3_10-7-58-16, was selected because it retained the heterologous region at 29–35 Mb on chromosome 4 and had Takanari or Akenohoshi alleles at all 90 SSR markers. F_3_10-7-58-16 was selfed to obtain F_4 seeds. To verify the effect of the QTL on chromosome 4 on flag leaf width, we selected a pair of F_4 plants that had a Takanari fragment (F_4_10-7-58-16-5) or an Akenohoshi fragment (F_4_10-7-58-16-1) in the 31.2–35.0 Mb region, and verified their genotypes using 768 SNPs (Yonemaru *et al*. 2014). This pair of F_4 plants was selfed to obtain F_5 seeds, and the F_5 plants were evaluated in 2010. To map the QTL, F_4 plants with recombination in the 30–34 Mb region were selected from 823 plants and selfed to obtain F_5 seeds. In total, 77 F_5 plants with fixed genotypes were selected and selfed. F_6 plants were evaluated as recombinant fixed lines in 2010 and 2011.

To identify whether the promoter region of *NAL1* was responsible for the variation in flag leaf width, we selected an F_4 (TUAT1-5-6/TUAT1-5-32) plant, F_4_10-7-52-84-3. This plant was selected because it had a heterologous fragment covering about 300 kb upstream of the start codon of *NAL1*, and because 94 out of 96 markers in the whole genome were fixed to either Takanari or Akenohoshi. F_4_10-7-52-84-3

was selfed to obtain F_5 seeds, and a pair of F_5 plants that had a Takanari fragment (F_5_10-7-52-84-3-1) or an Akenohoshi fragment (F_5_10-7-52-84-3-2) in the promoter region was selected. This pair of F_5 plants was selfed to obtain F_6 seeds, and the F_6 plants were evaluated in 2011.

A series of CSSLs with 13 donor fragments around 30.8 Mb on chromosome 4 in the Koshihikari background were used to identify candidate variations in the 10.3-kb region that may be responsible for flag leaf width. Previous reports have described the CSSLs with the donors Kasalath (Ebitani *et al*. 2005; http://www.rgrc.dna.affrc.go.jp/ineKKCSSL39.html), IR64 (Nagata *et al*. 2015; http://www.rgrc.dna.affrc.go.jp/ineKIRKCSSL42.html), and *O. rufipogon* IRGC-ACC104814 (Hirabayashi *et al*. 2010).

Nine other CSSLs, except for one CSSL whose donor was Phulba, were developed as follows: an F_1 plant was crossed with Koshihikari to obtain BC_1F_1, and then backcrossed with Koshihikari three additional times to obtain BC_4F_1 seeds. Throughout backcrossing, SSR markers were used to select BC_nF_1 plants with a donor fragment on the long arm of chromosome 4 in the Koshihikari background. BC_4F_1 plants were selfed three or four times and BC_4F_4 or BC_4F_5 plants were planted as CSSLs in 2012 or 2013.

To develop a CSSL with Phulba as the donor, most of the plants were grown in a growth chamber to shorten the generation time. Also, the embryo rescue technique was used to obtain immature embryos at 10–14 days after pollination to skip the period necessary for seed maturation and dormancy breaking. An F_1 plant was crossed with Koshihikari to obtain BC_1F_1, and then backcrossed with Koshihikari to obtain

BC_2F_1 seeds. BC_2F_1 plants were selfed three times to obtain BC_2F_4 seeds. Throughout backcrossing and selfing, SSR and INDEL markers were used to select plants with a Phulba fragment on the long arm of chromosome 4 in the Koshihikari background. BC_2F_4 plants were planted as CSSLs in 2013.

To detect the QTL for flag leaf width on chromosome 8, 95 backcrossed inbred lines (BILs) originating from the cross between Jarjan (WRC 28) and Koshihikari (Abe *et al*. 2011; Taguchi-Shiobara *et al*. 2011; http://www.rgrc.dna.affrc.go.jp/ineJKKBIL95.html) were planted in 2010. To develop CSSLs with a Jarjan fragment in the Koshihikari background, Jarjan was crossed with Koshihikari to obtain F_1 seeds, and an F_1 plant was backcrossed with Koshihikari to obtain BC_1F_1 seeds. A BC_1F_1 plant was backcrossed twice with Koshihikari to obtain BC_3F_1 seeds (Taguchi-Shiobara *et al*. 2013), and BC_3F_1 plants were selfed six times to obtain BC_3F_7 seeds. Throughout backcrossing and selfing, SSR markers and SNPs were used to select BC_nF_n plants with a Jarjan fragment containing the QTL for flag leaf width on chromosome 8 in the Koshihikari background. Selected BC_3F_6 plants were selfed and the BC_3F_7 plants were evaluated in 2013.

Evaluation of flag leaf morphology

In the PCA and measurement of transgenic plants, sections were hand-cut and the thickness of the flag leaf was measured under a Nikon ECLIPSE E200MV microscope equipped with a DS-L2 controller. A 5-mm portion was cut from the widest part of the flag leaf, fixed in FAA, and then cleared in chloral hydrate solution (1.6 g/1 ml water) at 80°C for 20 min. The chloral hydrate solution was replaced with

50% (v/v) glycerol, and then 80% (v/v) glycerol. The cleared samples were examined under a Leica MZ16F stereomicroscope and a Nikon Optiphot microscope to count the number of vascular bundles. The distance between vascular bundles was measured using ImageJ software version 1.46r (Rasband 1997–2014).

We also evaluated leaf thickness and vascular bundle traits for the CSSL series in which donor fragments covered *NAL1* on chromosome 4 in the Koshihikari background and a pair of recombinant fixed lines consisting of Takanari and Akenohoshi genomes. For these plants, a fixed flag leaf sample was embedded in a paraffin block, and paraffin sections stained with toluidine blue were examined to measure the thickness of the flag leaf, the distance between vascular bundles, and the number of vascular bundles.

Production and evaluation of transgenic plants

Two vectors, “Takanari NAL1” and “Takanari NAL1_R233H”, were constructed. To produce the “Takanari NAL1” vector, the BAC clone “Takanari_03G22” was double-digested with *Sal*I/*Acc*65I, and a 7.6-kb fragment containing the Takanari *NAL1* gene with the 3.2-kb region upstream of start codon was obtained. This 7.6-kb fragment was inserted into the restriction site (*Sal*I/*Kpn*I) of the pPZP2H-lac binary vector, a derivative of pPZP200 containing the CaMV 35S promoter-HPT-*nos* terminator and a multi-cloning site (Fuse *et al*. 2001).

To swap the 233rd arginine in exon 3 of NAL1 for histidine, we used two internal *Bsr*I sites; one in intron 2 and one in intron 4 of *NAL1*. The pPZP2H-lac vector containing the 7.6-kb Takanari fragment was linearized by *Bsr*I digestion, and a 1.8-kb fragment covering the region from intron 2 to intron 4 was amplified by PCR from the BAC clone "Akenohoshi_14E16". The linearized vector and the 1.8-kb fragment were fused using an In-Fusion HD Cloning Kit (Clontech, Palo Alto, CA, USA) to produce the "Takanari NAL1_R233H" vector.

The two vectors "Takanari NAL1" and "Takanari NAL1_233H" were introduced into the *Agrobacterium tumefaciens* strains EHA101 and EHA105, respectively, and then into Akenohoshi or SL2013, respectively. SL2013 is a CSSL with an IR 64 fragment in the long arm of chromosome 4 in the Koshihikari background (Nagata *et al*. 2015; http://www.rgrc.dna.affrc.go.jp/ineKIRKCSSL42.html). T_0 transgenic plants were produced as described previously (Toki 1997).

Real-time PCR analysis

To determine the transcript levels of *NAL1*, we used *RUBQ2* and *NAL1* probes. Three samples of total RNA were extracted from immature flag leaves or shoot apices about 30-days before flowering with an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Tissues were excised using a scalpel, frozen immediately in liquid nitrogen, and ground using a mortar and pestle. Immature flag leaves at the P2 stage (<1 mm) and those at the P3 stage (1–2 mm) were too small to excise, so the whole shoot apex,

including the immature flag leaf, was used instead. Immature flag leaves at the P3 stage (3–5 mm in length), those at the early P4 stage (1–3 cm), those at the late P4 stage (3–5 cm), and those at the P6 stage (green mature leaf) were collected as the other samples. RNA samples from three independent harvests were separately converted into first-strand cDNA by SuperScript III (Invitrogen, Carlsbad, CA, USA). For each cDNA sample, three replicate real-time PCRs were performed. Relative amounts were calculated as the ratio of the copy number of *NAL1* to that of *RUBQ2*.

To determine the transgene copy numbers, *RUBQ2* and *HPT* probes were used. Three genomic DNA samples were extracted from leaves of a transgenic seedling in extraction buffer without CTAB (Hori *et al*. 2015), and three replicate real-time PCRs were performed for each plant. Relative amounts were calculated as the ratio of the copy number of *HPT* to that of *RUBQ2*.

Literature Cited

Abe, T., Y. Nonoue, N. Ono, M. Omoteno, M. Kuramata *et al*., 2013 Detection of QTLs to reduce cadmium content in rice grains using LAC23/Koshihikari chromosome segment substitution lines. Breed Sci. 63: 284–291.

Abe, T., F. Taguchi-Shiobara, Y. Kojima, T. Ebitani, M. Kuramata *et al*., 2011 Detection of a QTL for accumulating Cd in rice that enables efficient Cd phytoextraction from soil. Breed. Sci. 61: 43–51.

Ebitani, T., Y. Takeuchi, Y. Nonoue, T. Yamamoto, K. Takeuchi *et al*., 2005, Construction and Evaluation of Chromosome Segment Substitution Lines Carrying Overlapping Chromosome Segments of indica Rice Cultivar 'Kasalath' in a Genetic Background of japonica Elite Cultivar 'Koshihikari'. Breed. Sci. 55:65-73.

Fuse, T., T. Sasaki, and M. Yano, 2001 Ti-plasmid vectors useful for functional analysis of rice genes. Plant Biotechnol. 18: 219-222.

Hirabayashi, H., H. Sato, Y. Nonoue, Y. Kuno-Takemoto, Y. Takeuchi *et al*., 2010 Development of introgression lines derived from *Oryza rufipogon* and *O. glumaepatula* in the genetic background of japonica cultivated rice (*O. sativa* L.) and evaluation of resistance to rice blast. Breed. Sci. 60: 604–612.

Nagata, K., T. Ando, Y. Nonoue, T. Mizubayashi, N. Kitazawa *et al*., 2015 Advanced backcross QTL analysis reveals complicated genetic control of rice grain shape in a *japonica* x *indica* cross. Breed. Sci. 65: 308-318.

Rasband, W. S., 1997-2014 ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/.

Taguchi-Shiobara, F., Y. Kojima, T. Ebitani, M. Yano, K. Ebana, 2011 Variation in domesticated rice inflorescence architecture revealed by principal component analysis and quantitative trait locus analysis. Breed. Sci. 61: 52–60.

Taguchi-Shiobara, F., H. Ozaki, H. Sato, H. Maeda, Y. Kojima *et al*., 2013 Mapping and validation of QTLs for rice sheath blight resistance Breed. Sci. 63:301-308.

Toki, S., 1997 Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol. Biol. Rept. 15: 16-21.

Yonemaru, J., K. Ebana, M. Yano, 2014 HapRice, a SNP haplotype database and a web tool for rice. Plant Cell Physiol. 55(1): e9(1–12) doi: 10.1093/pcp/pct188.

Tables S1-S9

Available for download as Excel files at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181040/-/DC1

Table S1 Populations and traits evaluated in this study.

Table S2 Details of varieties used in principal component analysis and their flag leaf morphology. Varieties were among 103 accessions from NIAS core collection.

Table S3 Details of 102 japonica accessions used in genome-wide association analysis.

Table S4 Primers for positional cloning and molecular analysis.

Table S5 Nine flag leaf morphology traits and correlation coefficients for 103 rice accessions from NIAS Core Collection used in principal component analysis.

Table S6 Comparison of principal components among *indica*, *temperate japonica*, and *tropical japonica*.

Table S7 Putative QTL for flag leaf size and heading date detected in QTL analysis using two hybrid populations.

Table S8 Natural variation in rice *NAL1* genomic sequence.

Table S9 Amino acid substitution in exon 3 of *NAL1* homolog in wild rice and other plant species.

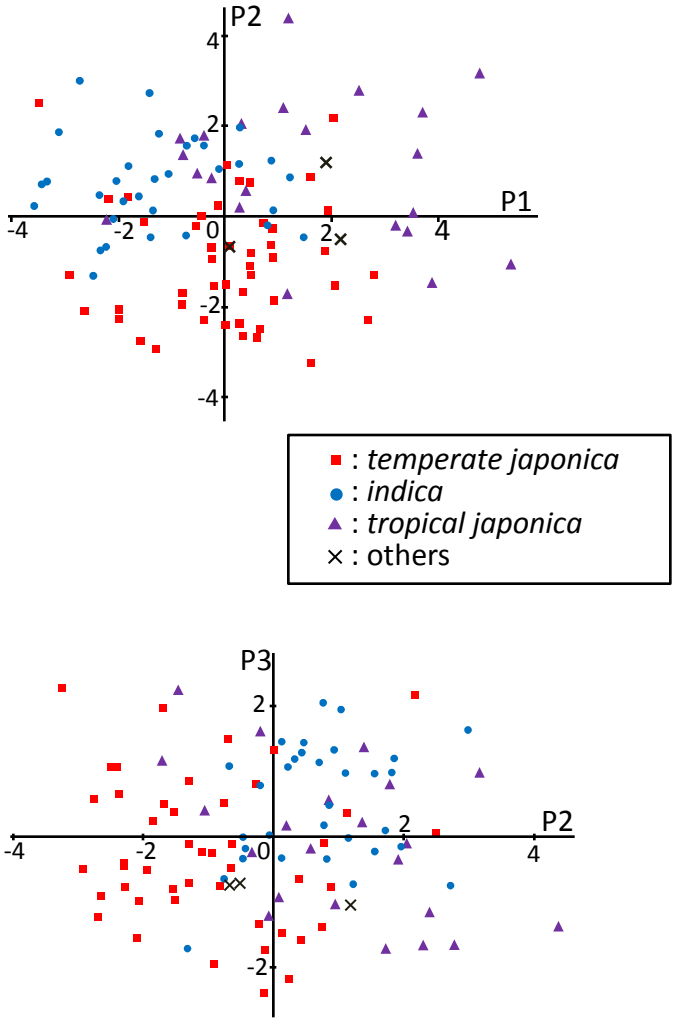

FIGURE S1 Scatter plots for first and second (top), and second and third (bottom) principal components of principal component analysis of morphological variation in the flag leaf. Points represent 103 rice accessions from NIAS core collection including 46 *indica*, 32 *temperate japonica*, 22 *tropical japonica*, and three other accessions.

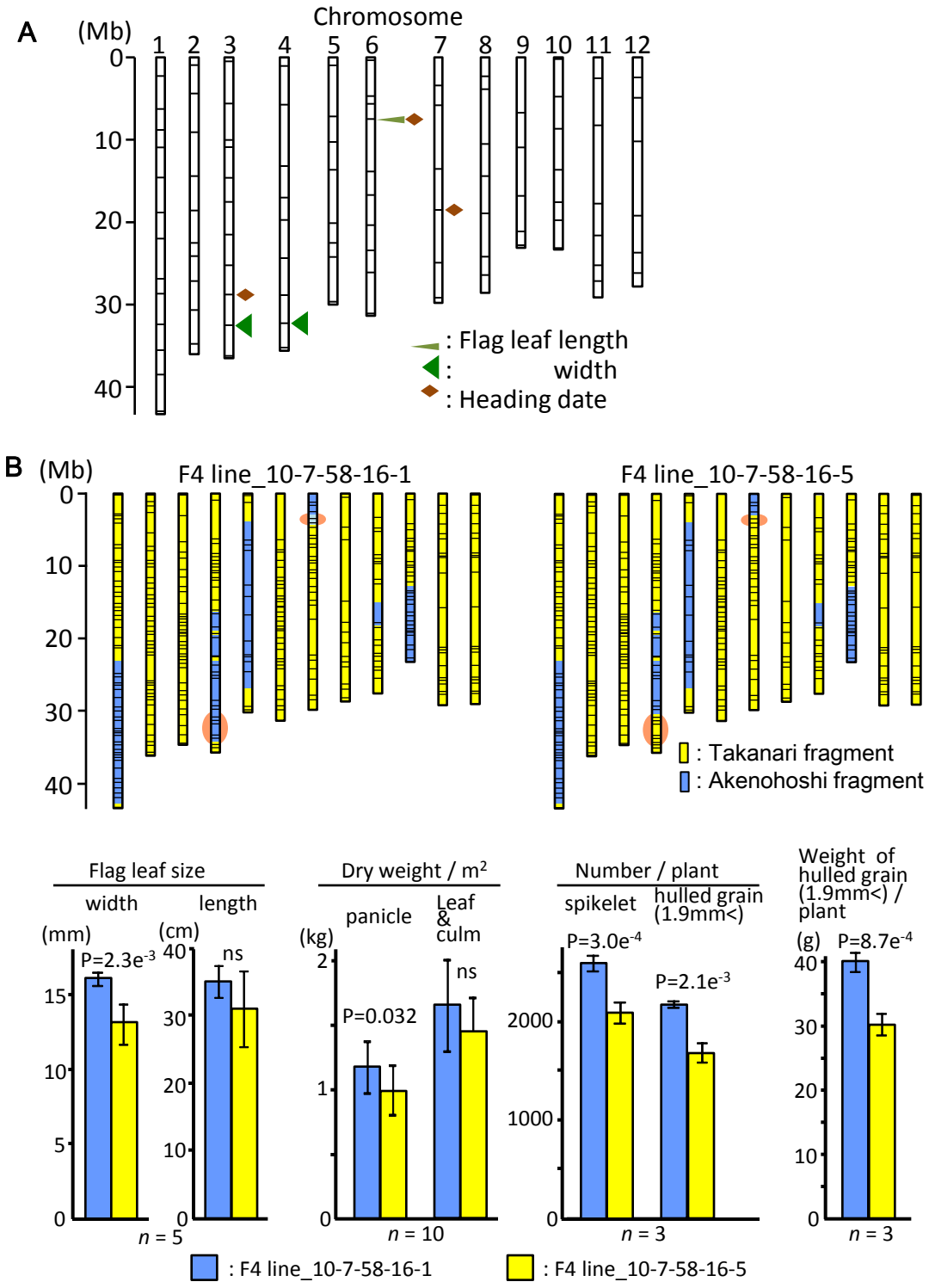

FIGURE S2 Quantitative trait locus (QTL) for flag leaf width was detected on chromosome 4 among hybrid populations derived from a Takanari × Akenohoshi cross. (A) QTL detected for flag leaf size and heading date among F_2 population of a Takanari × Akenohoshi cross. Triangles and squares indicate nearest markers to QTLs. (B) Genotype of recombinant fixed lines containing either Akenohoshi or Takanari fragment around 32 Mb on chromosome 4 in almost the same background (F_4 line_10-7-58-16-1 and -5). Plants with Akenohoshi fragment (31.2-35.0 Mb) on chromosome 4 have wider flag leaf and greater yield.

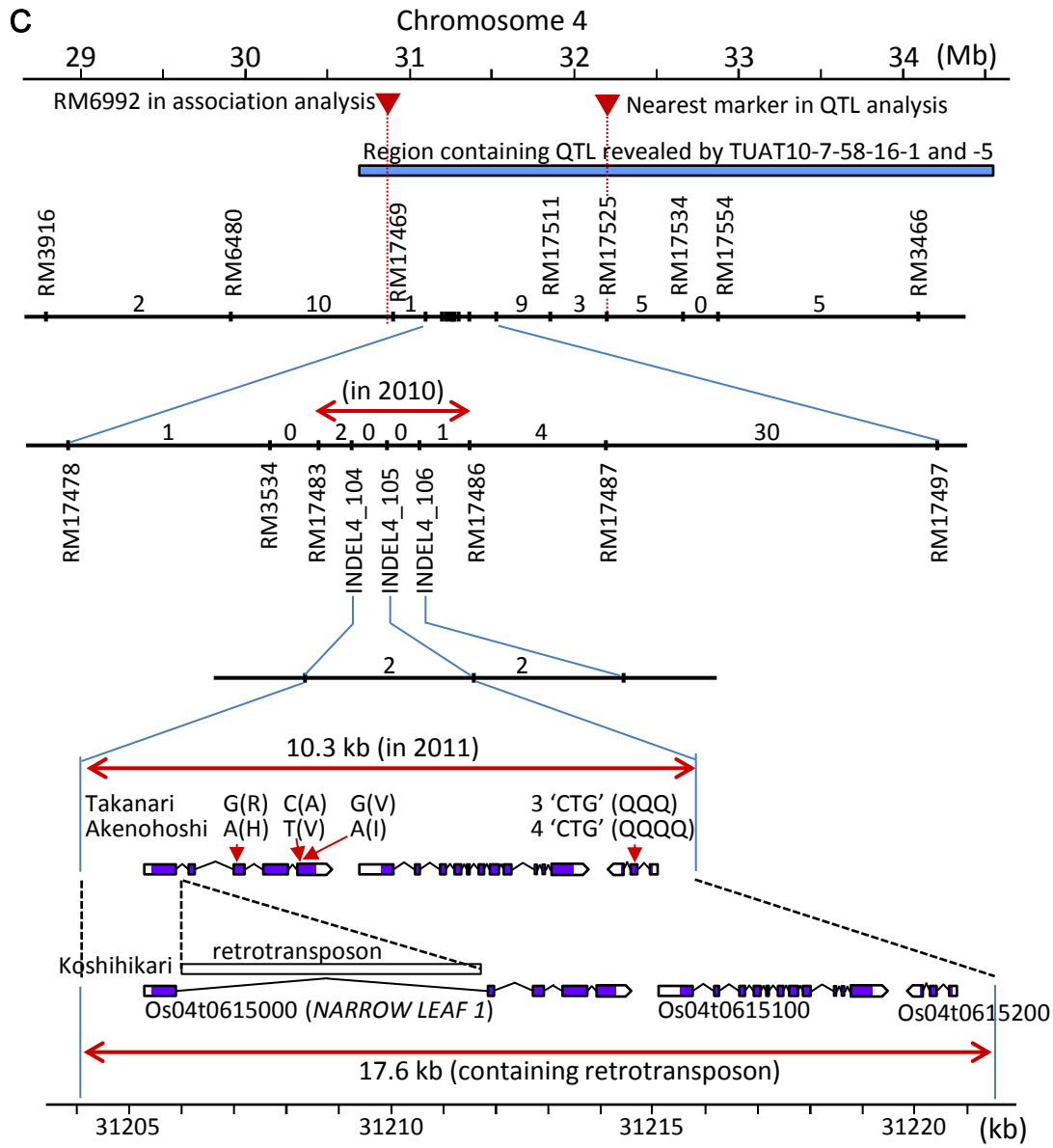

FIGURE S2 (continued)
(C) QTL for flag leaf width on chromosome 4 found among 77 recombinant fixed lines whose background was the same as that of F_4 line_10-7-58-16-1 or F4 line_10-7-58-16-5. Numbers on lines indicate recombination frequency between neighboring markers observed among 77 lines. QTL was located within a 10.3-kb region containing three ORFs, where three nonsynonymous nucleotide substitutions and one deletion of a three-nucleotide repeat were present. Nucleotide positions at the bottom are based on Nipponbare genome (IRGSP-1.0).

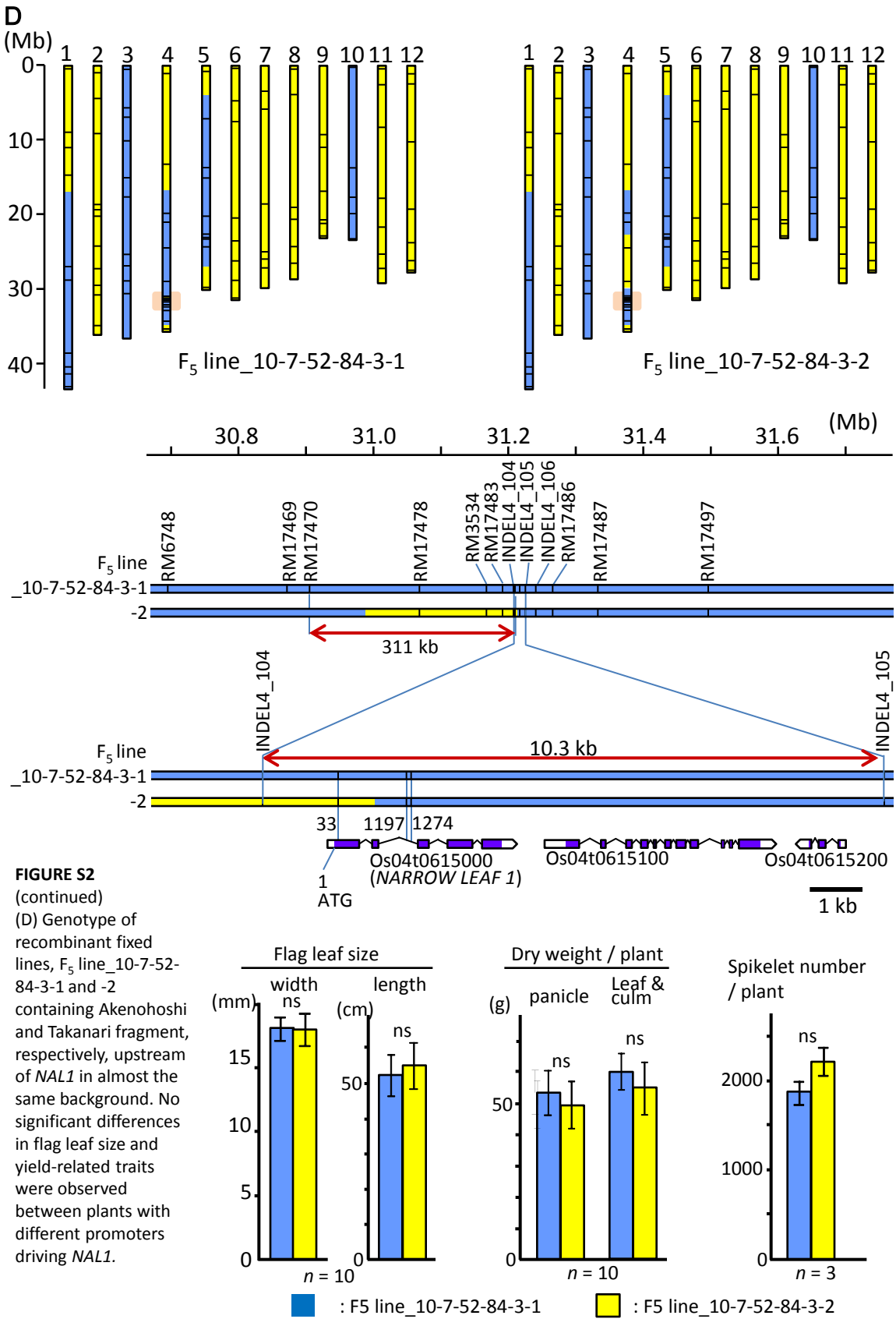

FIGURE S2
(continued)
(D) Genotype of recombinant fixed lines, F_5 line_10-7-52-84-3-1 and -2 containing Akenohoshi and Takanari fragment, respectively, upstream of *NAL1* in almost the same background. No significant differences in flag leaf size and yield-related traits were observed between plants with different promoters driving *NAL1*.

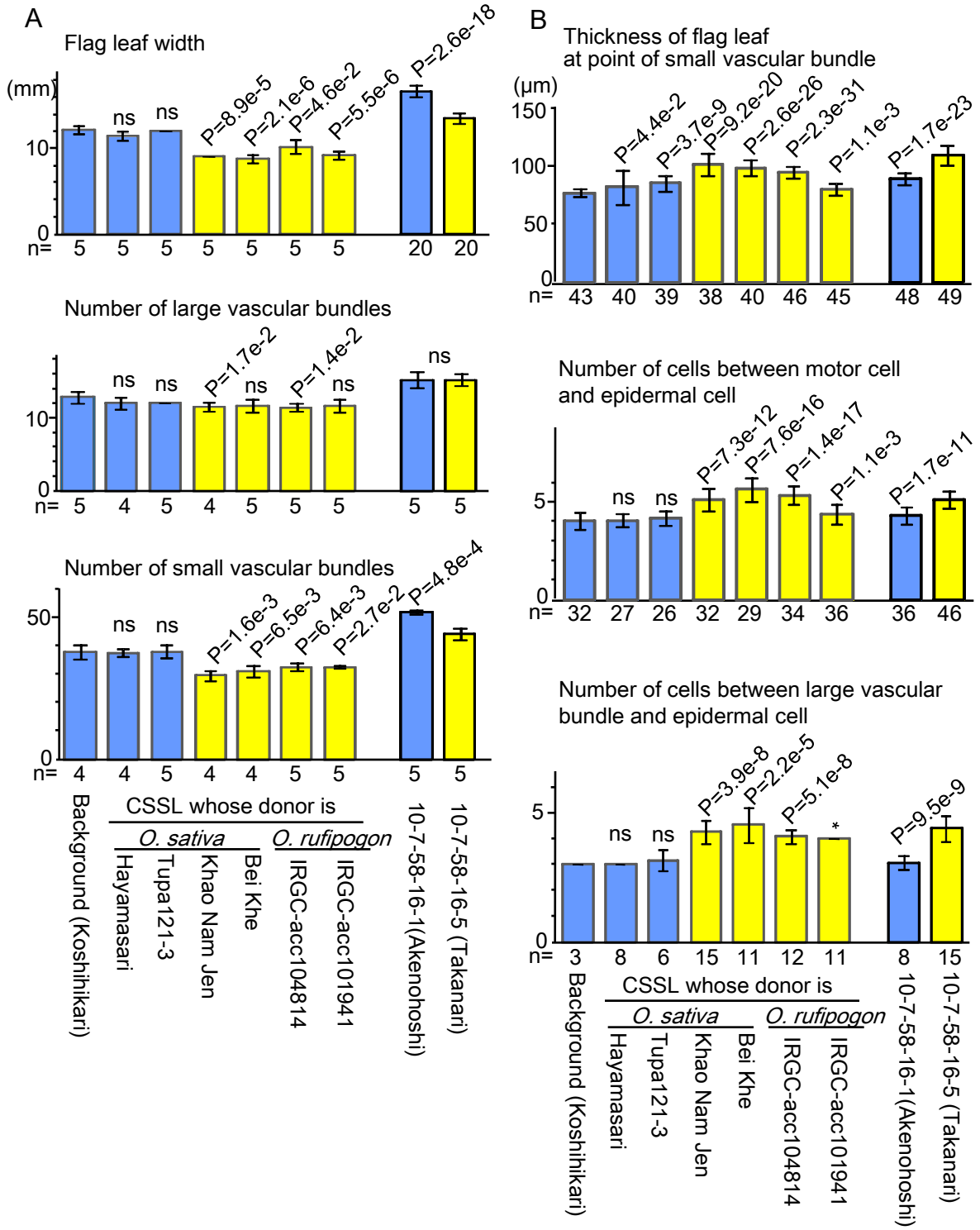

FIGURE S3 Flag leaf morphology of six CSSLs and two recombinant fixed lines. (1) Six CSSLs with Koshihikari background, each with a different fragment at QTL for flag leaf width derived from respective donor accession. (2) Pair of recombinant fixed lines containing either Akenohoshi- or Takanari-derived region around 32 Mb on chromosome 4 with almost the same background (F_4 line_10-7-58-16-1 and -5). Yellow and blue indicate that 233rd amino acid of NAL1 is R and H, respectively. (A) Width of flag leaf and number of vascular bundles. (B) Thickness of flag leaf.

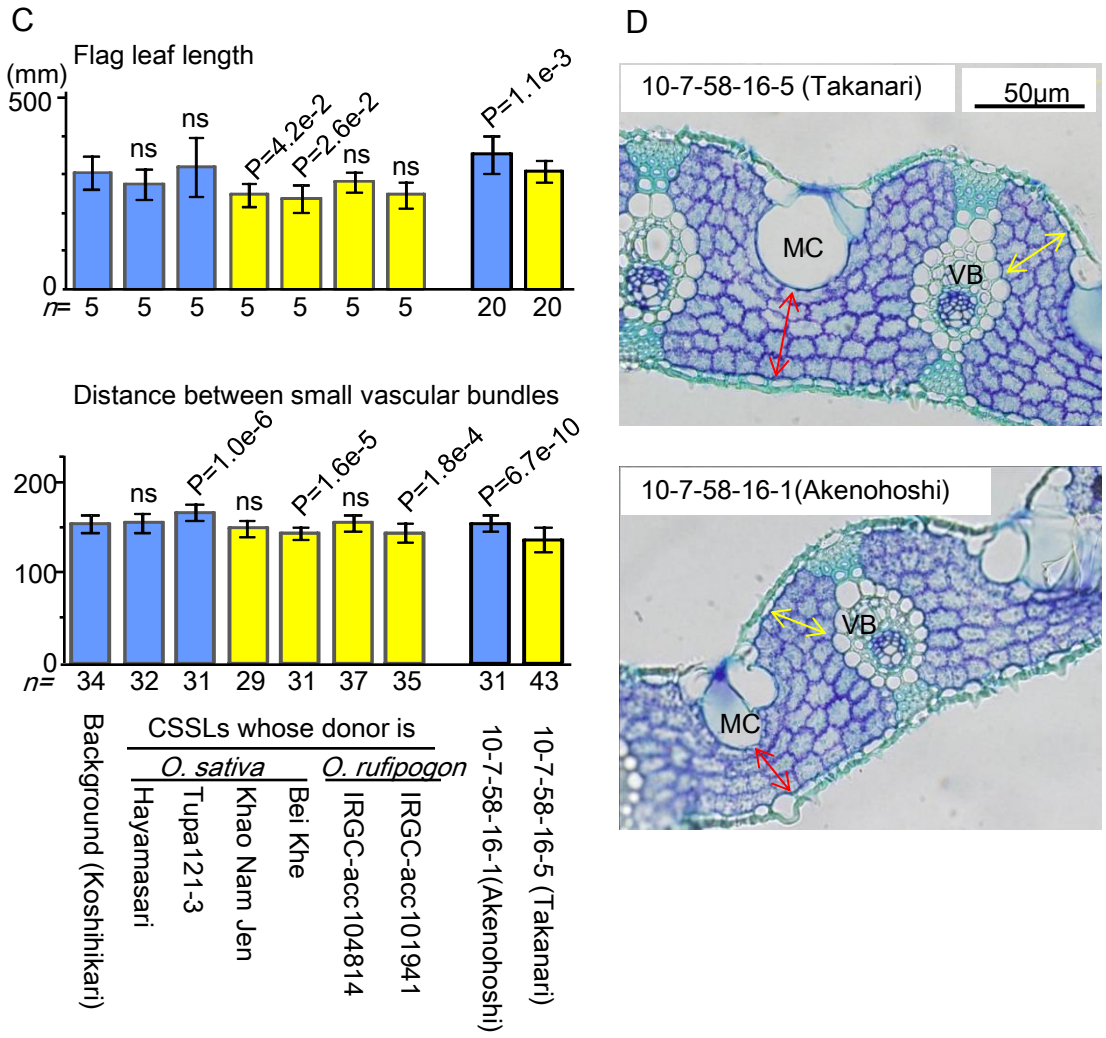

FIGURE S3 (continued)
(C) Length of flag leaf and distance between vascular bundles. Vertical bars show SD. P values were obtained from comparison between CSSL and Koshihikari or between pair of recombinant fixed lines; asterisk (*) indicates that P value was not obtained due to SD=0. (D) Transverse section of F_4 line_10-7-58-16-1 (Akenohoshi fragment) and -5 (Takanari fragment). VB, vascular bundle. MC, motor cell. Number of cells between motor cell and epidermal cell (red arrow) and number of cells between large vascular bundle and epidermal cell (yellow arrow) were counted.