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.