Artificial selection for a green revolution gene during *japonica* rice domestication

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The semidwarf phenotype has been extensively selected during modern crop breeding as an agronomically important trait. Introduction of the semidwarf gene, *semi-dwarf1* (*sd1*), which encodes a gibberellin biosynthesis enzyme, made significant contributions to the "green revolution" in rice (*Oryza sativa* L.). Here we report that *SD1* was involved not only in modern breeding including the green revolution, but also in early steps of rice domestication. We identified two SNPs in *O. sativa* subspecies (ssp.) *japonica SD1* as functional nucleotide polymorphisms (FNPs) responsible for shorter culm length and low gibberellin biosynthetic activity. Genetic diversity analysis among *O. sativa* ssp. *japonica* and *indica*, along with their wild ancestor *O. rufipogon* Griff, revealed that these FNPs clearly differentiate the *japonica* landrace and *O. rufipogon*. We also found a dramatic reduction in nucleotide diversity around *SD1* only in the *japonica* landrace, not in the *indica* landrace or *O. rufipogon*. These findings indicate that *SD1* has been subjected to artificial selection in rice evolution and that the FNPs participated in *japonica* domestication, suggesting that ancient humans already used the green revolution gene.

Plant domestication involves the genetic modification of wild species to create a new plant to meet human needs (1). During this domestication, ancient humans subjected common agronomic traits to artificial selection, thereby increasing the seed or fruit size, synchronization of growth and flowering, loss of seed dispersal, changes in plant architecture, and other characteristics comprising the "domestication syndrome" (2). These traits have contributed to more efficient cultivation, higher yields, and more valuable products for human use. Consequently, crop species have undergone extensive selection for these agronomically important traits, and genes impacted by artificial selection can be essential genetic factors in the domestication process (3).

Asian rice, *Oryza sativa*, was domesticated from its wild ancestor, *O. rufipogon*, ~10,000 y ago (4–6). Recently, genes that control the domestication syndrome have been isolated in rice (7–9). In fact, several of these genes have been subjected to artificial selection during domestication and modern breeding (10–13). Although the genes controlling plant architecture are agriculturally important, little is known about their respective alterations during rice domestication (8, 9). In this study, we focused on *Semi-dwarf1* (*SD1*), a null allele of which is known as a "green revolution" gene that has been used extensively in rice modern breeding over the last 50 y (14–17). We found that *SD1* was also involved in the rice domestication process by controlling culm length (CL) in ancient *japonica* landraces. Ancient humans had selected mutations in the green revolution gene long before the green revolution of the 20th century.

Results

Quantitative Trait Locus Analysis for CL and Positional Cloning of *qCL1a*. To identify genes controlling CL in rice domestication, we conducted the quantitative trait locus (QTL) analysis for CL in a set of backcross inbred lines (BILs) derived from a cross between two *O. sativa* ssp., the *japonica* variety Nipponbare and the *indica* variety Kasalath (18). Because *japonica* and *indica* have distinctly different domestication histories (4–6), we predicted the elucidation of two different domestication processes by comparing these subspecies. The mean CL of Kasalath was significantly longer than that of Nipponbare (116.0 ± 8.4 cm vs. 83.7 ± 4.4 cm; Fig. 1*A* and *B*). In the BILs, CL ranged continuously from 56.9 to 118.8 cm, and transgressive segregants were observed beyond the parental varieties (Fig. S1). These segregants indicated that this trait was controlled by multiple QTLs. We detected four QTLs, and focused further studies on a QTL designated *qCL1* (QTL for CL on chromosome 1), which explained 20.9% of the total phenotypic variation in the population (Fig. 1*C* and Table S1). The CL of lines introgressed with the Kasalath *qCL1* region in the Nipponbare background was significantly longer than that of Nipponbare (Fig. 1*D* and Fig. S2), confirming that the introgressed segment included the QTL. High-resolution mapping using ~5,000 plants segregating at the *qCL1* locus demonstrated that *qCL1* consisted of at least two loci, *qCL1a* and *qCL1b*. Because the genetic effect of *qCL1a* was larger than that of *qCL1b* (Fig. 1*D*), we chose *qCL1a* as a target for positional cloning. As a result of positional cloning, *qCL1a* was delimited within a 336-kb region between markers AS-147-1 and AS-151-1 (Fig. 1*D*). The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/) indicated that this region contained at least 40 genes. Of these, we focused on *GA20ox-2*, *SD1*, which encodes GA20 oxidase, an enzyme involved in gibberellin (GA) biosynthesis. Previously, several mutations that resulted in a semidwarf phenotype were identified in *SD1*, a trait that triggered the green revolution in rice (14–17). Comparison of the *SD1* sequence revealed two nonsynonymous SNPs at residue 100 in the first exon [glutamic acid

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See Commentary on page 10931.

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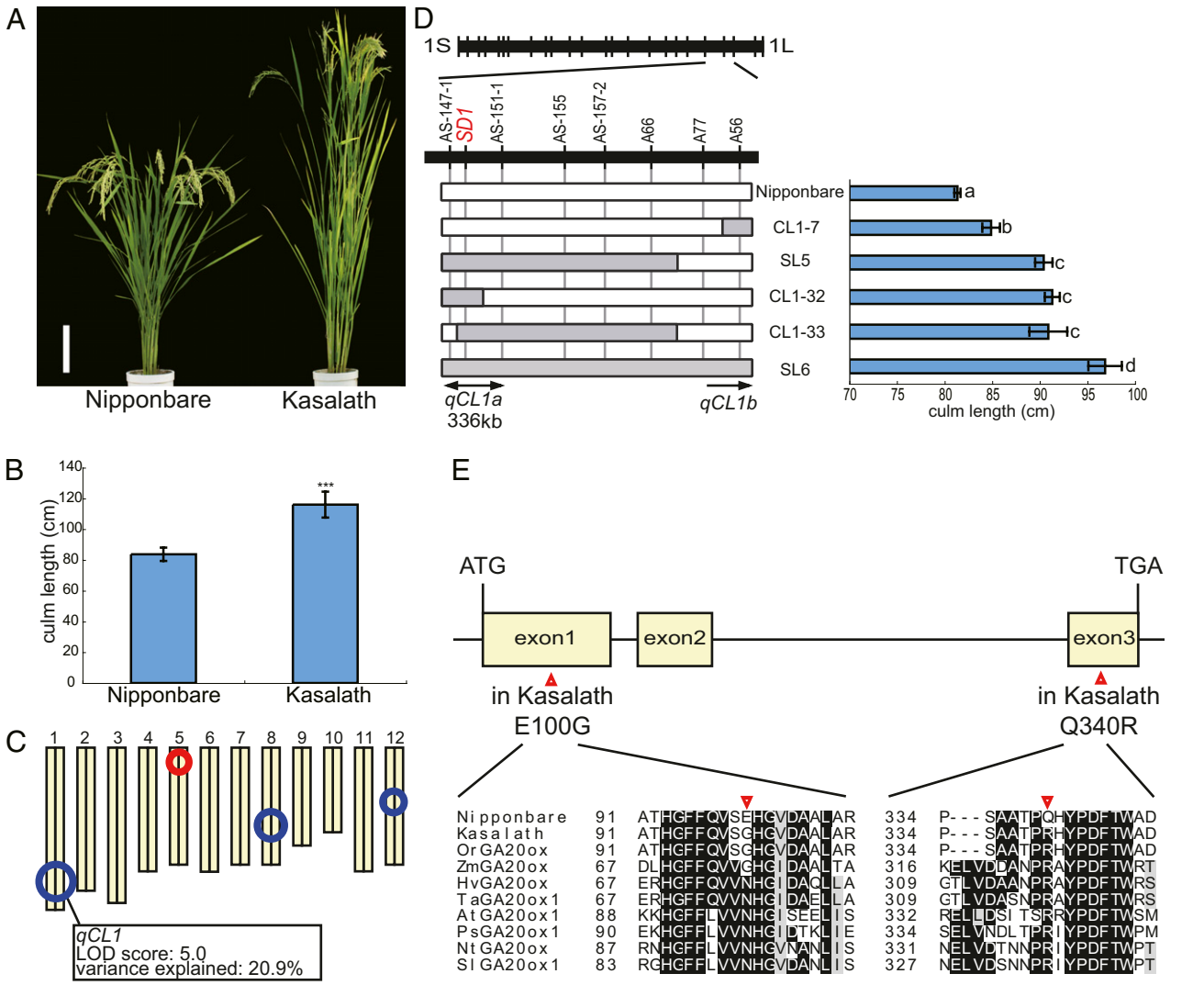

Fig. 1. QTL analysis for CL and isolation of *qCL1a*. (*A* and *B*) Gross morphology (*A*) and CL (*B*) of Nipponbare and Kasalath at the mature stage. (Scale bar in *A*: 20 cm.) Asterisks in *B* indicate a significant difference ($P < 0.001$) according to the *t* test. Error bars represent the SD from the mean ($n = 6$). (*C*) QTL analysis for CL in a BIL population. The circles indicate the positions of QTLs, and the circle sizes indicate the relative contribution of each QTL. The red and blue circles indicate QTLs that Nipponbare and Kasalath alleles contribute to the elongation of CL, respectively. *qCL1* is marked on chromosome 1. (*D*) High-resolution mapping of *qCL1*. (*Left*) Graphical genotypes of four selected recombinant homozygous lines. The horizontal lines represent chromosome 1, and a physical map is shown for the *qCL1* region of chromosome 1. The vertical bars represent the molecular markers. The white and gray bars indicate homozygous alleles of Nipponbare and Kasalath, respectively. (*Right*) CL for each recombinant homozygous line. Letters (a–d) denote statistically significant differences ($P < 0.05$) according to Tukey's test. Error bars indicate the SD from the mean ($n = 5$). (*E*) Comparison of SD1 amino acid sequences between Nipponbare and Kasalath. The yellow squares and horizontal lines denote the exons and introns of *SD1*, respectively. The amino acid sequences of GA20ox proteins from Nipponbare, Kasalath, *O. rufipogon*, maize, barley, wheat, *Arabidopsis*, pea, tobacco, and tomato were aligned using ClustalW, followed by manual alignment. The red triangles indicate amino acid substitutions between Nipponbare and Kasalath.

(E) and glycine residues (G)] and at residue 340 in the third exon [glutamine (Q) and arginine (R)] in Nipponbare and Kasalath, respectively (Fig. 1*E*).

Comparison of *SD1* Alleles from Nipponbare and Kasalath. To verify that *SD1* corresponds to *qCL1a*, we produced transgenic plants containing the entire *SD1* alleles from Nipponbare (*N-SD1*) and Kasalath (*K-SD1*) in the Nipponbare background. *K-SD1* showed significantly longer CL than *N-SD1* and the empty vector control (Fig. 2 *A* and *B*), demonstrating that *SD1* corresponds to *qCL1a*. To clarify the effect of the two amino acid differences, we compared the catalytic activity of N-SD1 (SD1-EQ), K-SD1 (SD1-GR), and intermediate types (SD1-GQ and SD1-ER) produced in *Escherichia coli*. SD1 catalyzes the pathway from GA_{53} to GA_{20}, the main GA synthesis pathway in rice leaves and stems (19). Although all SD1 proteins catalyzed the conversion of GA_{53} to GA_{20}, SD1-GR demonstrated significantly higher activity than the other three SD1 types (Fig. 2*C*). This indicates that two SNPs in *SD1-EQ* are functional nucleotide polymorphisms (FNPs) that are key natural variations in this gene.

Genetic Diversity Analysis in the *SD1* Region. We analyzed the prevalence of two FNPs in *SD1* in a set of 72 diverse rice accessions. To exclude the effects of modern breeding, we chose landraces that were considered primitive cultivars after domestication and that represented maximum genetic diversity within *O. sativa* (20) for the subsequent analyses. In our collection, all of the *japonica* landraces (including both *tropical* and *temperate japonica*) carried *SD1-EQ*, whereas most of the *indica* landraces carried *SD1-GR* (Tables S2 and S3), indicating that these FNPs

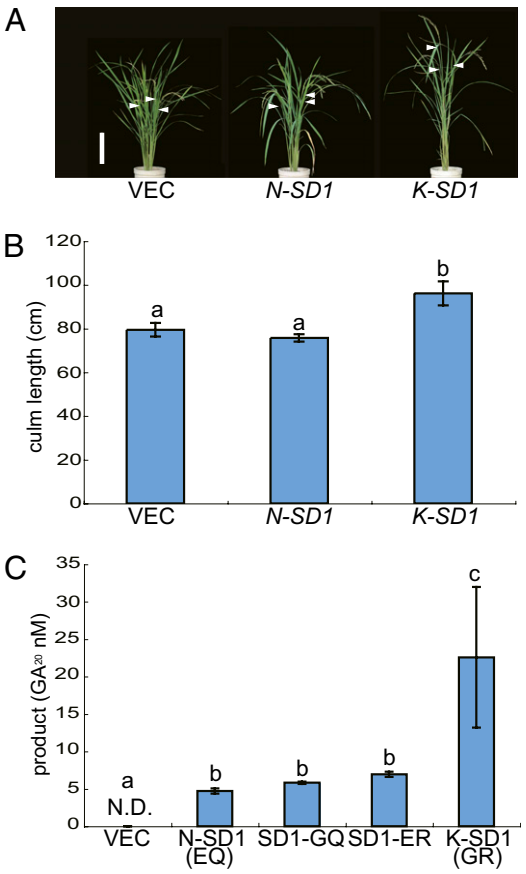

Fig. 2. Comparison of the *SD1* alleles from Nipponbare and Kasalath. (*A*) Phenotype of transgenic plants containing the additional *SD1* allele: from left to right, transgenic plants containing empty vector (VEC), the Nipponbare allele (*N-SD1*), and the Kasalath allele (*K-SD1*). The white arrowheads indicate the position of the panicle node. (Scale bar in *A*: 20 cm.) (*B*) CL in transgenic plants containing additional *SD1*. The letters a and b denote statistically significant differences ($P < 0.05$) according to Tukey's test. Error bars represent the SD from the mean of the longest culms ($n = 15$). (*C*) GA biosynthetic activity of SD1. N.D., not detected. The letters a–c denote statistically significant differences ($P < 0.05$) according to Tukey's test. Error bars represent the SD from the mean ($n = 3$).

differentiate *japonica* and *indica*. All 42 accessions of *O. rufipogon* from different origins carried *SD1-GR* (Tables S2 and S3), strongly suggesting that the two FNPs in *SD1-EQ* had been specifically selected during the *japonica* domestication process.

To determine whether *SD1-EQ* had undergone artificial selection during *japonica* domestication, we analyzed the genetic variation in a ~4.0-kb region encompassing the entire *SD1* sequence in *O. sativa* and *O. rufipogon*. Our representative samples included 16 landraces of *japonica*, 15 landraces of *indica*, and 16 accessions of *O. rufipogon* (Table S3). An apparent reduction in genetic variation was observed at the *SD1* locus in *japonica* landraces, but not in *indica* landraces or in *O. rufipogon*. The *SD1* nucleotide diversity in the *japonica* landraces ($\pi = 0.00013$) lost 98% of the diversity in the *O. rufipogon* sample ($\pi = 0.00568$), whereas the *indica* landraces ($\pi = 0.00424$) showed only a 25% reduction (Table S4). The nucleotide diversity in *japonica SD1* was 10-fold lower than that of 111 randomly chosen gene fragments ($\pi = 0.00111$) (21), suggesting that the low nucleotide diversity observed in *japonica SD1* cannot be explained by a population bottleneck alone, because that would have caused a reduction in nucleotide diversity throughout the genome.

Detection of Selective Sweep and Coalescent Simulation. If *SD1-EQ* had been selected during the process of *japonica* domestication, then a lower level of genetic diversity in the flanking region known as selective sweep (3, 10, 22, 23) should be observed. Thus, we compared the nucleotide diversity in 18 genes spanning a 664-kb region surrounding the *SD1* locus in *japonica*, *indica*, and *O. rufipogon*. The diversity of *japonica* in this region was apparently lower than that of *indica* and *O. rufipogon* across a ~404-kb region from genes 3–15 (Fig. 3). Coalescent simulations (24) demonstrated that the *japonica* genetic diversity seen in this region ($\pi = 0.0000536$) was significantly lower ($P < 0.01$) than that of *O. rufipogon* ($\pi = 0.00524$), supporting our hypothesis of selection for *SD1-EQ* during *japonica* domestication.

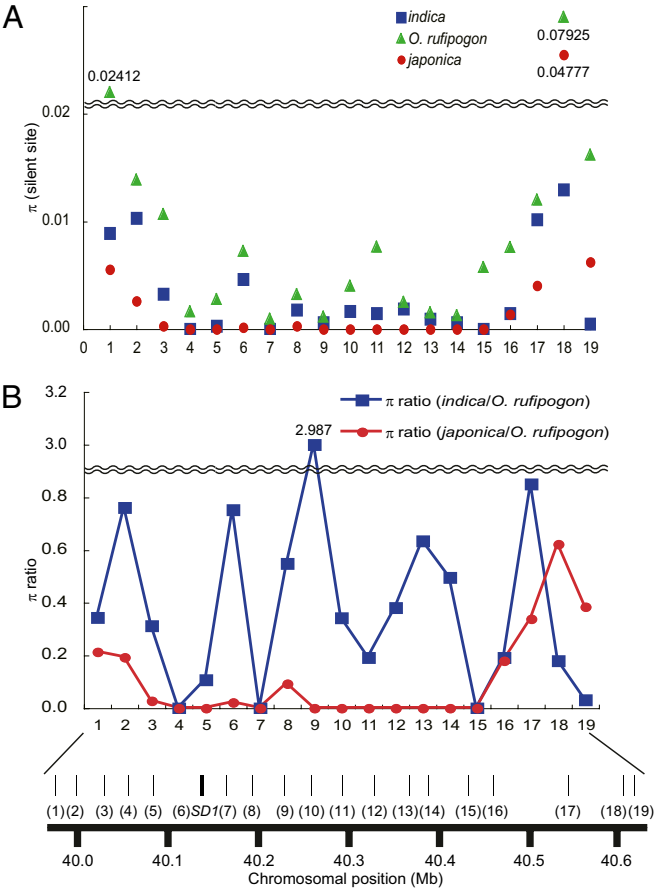

Fig. 3. Genetic diversity analysis around the *SD1* region. (*A*) Values of π for the silent site of *japonica* (red circle), *indica* (blue square), and *O. rufipogon* (green triangle) across the *SD1* genomic region of chromosome 1. (*B*) Nucleotide variation in *japonica* or *indica* relative to *O. rufipogon*. The blue and red lines indicate the ratios of all site nucleotide diversities in *indica* and *japonica* relative to *O. rufipogon*, respectively. In both *A* and *B*, ~180- to 880-bp portions of 18 flanking genes were sequenced, along with the entire *SD1* gene. The approximate genomic locations of the genes are indicated by solid bars, with the following gene identities: 1, hypothetical protein; 2, hypothetical protein; 3, leucine-rich repeat, cysteine-containing containing protein; 4, protein of unknown function, DUF803 family protein; 5, hypothetical protein; 6, *SD1*; 7, Armadillo-like helical domain containing protein; 8, conserved hypothetical protein; 9, conserved hypothetical protein; 10, similar to AGAMOUS homolog; 11, similar to EMB1879 (EMBRYO DEFECTIVE 1879); 12, hypothetical protein; 13, similar to NAC-domain containing protein 18 (ANAC018) (NO APICAL MERISTEM protein; AtNAM); 14, RIO-like kinase domain-containing protein; 15, similar to LOB domain protein 6 (ASYMMETRIC LEAVES2); 16, hypothetical conserved gene; 17, hypothetical protein; 18, hypothetical conserved gene, and 19, metallophosphoesterase domain-containing protein.

We found no evidence supporting selection for *indica SD1*, suggesting that this selection event could be specific to *japonica* domestication (*SI Methods* and Fig. S3).

Origin of *SD1-EQ*. To clarify the origin of *SD1-EQ*, we performed phylogenetic analyses based on genome-wide transposon insertion patterns and entire *SD1* genomic sequences. In *O. rufipogon*, five

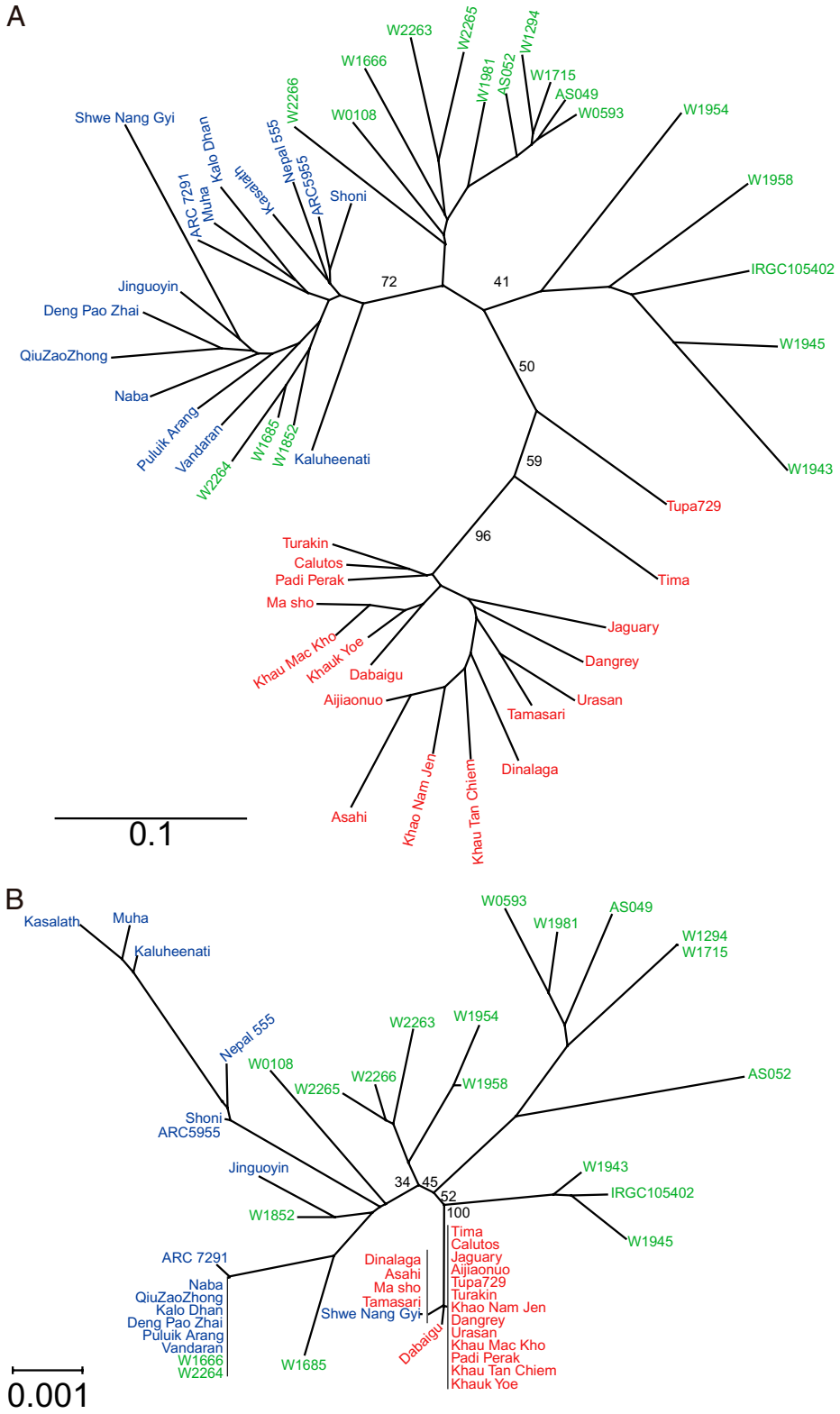

Fig. 4. Phylogenetic trees based on genome-wide transposon insertion patterns (*A*) and the *SD1* genomic sequence (*B*). Landraces and accessions are indicated by red for *japonica*, blue for *indica*, and green for *O. rufipogon*. Bootstrap values (%) were obtained by 1,000 bootstrap replicates.

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accessions (W1954, W1958, IRGC105402, W1945, and W1943) were closely related to *japonica* landraces on a genome-wide level (Fig. 4*A*). Three of these accessions (IRGC105402, W1945, and W1943) had *SD1* nucleotide sequences similar to those of *japonica* landraces even though these accessions had *SD1-GR* (Fig. 4*B* and Table S3). This finding suggests that *SD1-EQ* was derived from an ancestor related to the *japonica*-like *O. rufipogon* (Fig. 5). This finding also indicates that FNPs in *SD1* clearly differentiate between *O. rufipogon* and *japonica*.

Discussion

In this study, we have identified *SD1* as a domestication gene controlling CL and have demonstrated that FNPs in *SD1* were subjected to artificial selection during *japonica* domestication. Because *SD1* is a gene associated with rice plant architecture, this agronomic trait must be an essential target of artificial selection including both rice domestication and modern breeding. Our data suppose that ancient humans already used the green revolution gene; they took an interest in the height of rice plants as well as in seed shattering and size, and ultimately selected shorter plants with the *SD1-EQ* allele. On the other hand, in the rice green revolution during the 20th century, breeders favored the null allele of *SD1* from the *indica* population (14–17), indicating that distinct *SD1* alleles played an active role in the short plant height during rice evolution. Note that a clear reduction in genetic variation observed around *SD1* indicates selection for this region. These results do not completely exclude the possibility that selection acted on other genes in the region (i.e., the region between genes 3 and 15 in Fig. 3). However, no obvious phenotypic differences were observed between near-isogenic lines containing the *SD1-GR* region and Nipponbare, except for CL. In addition, there are no reports or annotations for agronomically important traits in this region except *SD1*. Thus, *SD1* would be the target for artificial selection.

Natural variation revealed the extant accessions with *SD1-EQ* (*japonica*) and *SD1-GR* (*O. rufipogon* and general *indica*). Mysteriously, no accession was found to have the intermediate alleles *SD1-GQ* and *SD1-ER* in either *O. sativa* or *O. rufipogon*. Two possible processes led to the eventual generation of *SD1-EQ*: mutations occurring twice and resulting in conversion of *SD1-GR* to *SD1-EQ*, or a mutation in *SD1-GR* resulting in *SD1-GQ* and *SD1-ER*, which subsequently underwent a recombination event. These intermediate alleles might have disappeared because of reasons such as genetic drift. Because there was little difference in enzymatic activity between SD1-EQ and the intermediates (Fig. 2*C*), which probably resulted in shorter CL, ancient humans could not distinguish plants with *SD1-EQ* and the intermediates. Whether the intermediates were targets for artificial selection before *SD1-EQ* was generated remains unclear from current data.

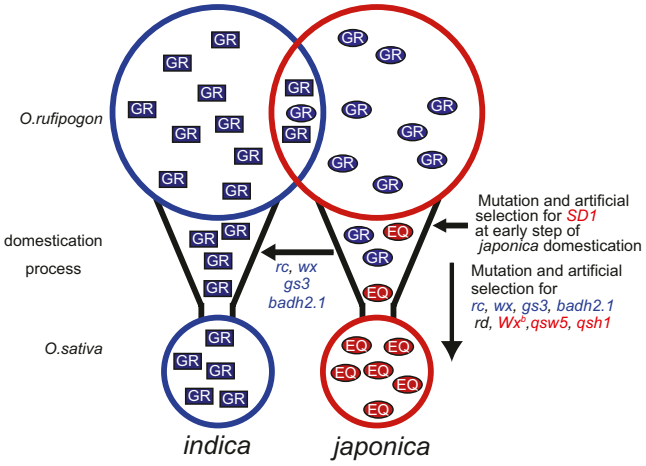

Fig. 5. Model of the domestication process in rice. The blue and red circles indicate two genetically distinct groups, *indica* and *japonica*, and their corresponding ancestor, *O. rufipogon*. "GR" enclosed by a blue rectangle or ellipse indicates a GR-type *SD1* allele. "EQ" enclosed by red ellipse indicate an EQ-type *SD1* allele. Rectangles and ellipses indicate *indica* and *japonica*-like alleles in relation to sequences outside of the FNPs of *SD1*, respectively. Genes indicated by blue and red letters are genes introgressed from *japonica* into *indica* and genes isolated into the *japonica* population, respectively.

We also have demonstrated that FNPs in *SD1* clearly differentiate between *O. rufipogon* and *japonica*. The *SD1-EQ* allele was probably fixed during *japonica* domestication before the speciation of *tropical* and *temperate japonica*. Such a distinct differentiation is rare in crops. Most FNPs in genes associated with domestication were not completely fixed between cultivated and wild relatives (7, 11, 13, 25–28), meaning that several cultivars still retain wild-relative alleles in domestication-related genes. These genes may be strongly affected by artificial selection during late stages of domestication; however, exceptions include FNPs in *sh4* for seed shattering in rice (29) and *tga1* for the loss of cupulate fruitcases in maize (30), which are fixed differences between cultivated and wild relatives. Thus, mutation and selection for these genes are considered critical steps in the domestication of these crops. Collectively, our results indicate that the *SD1-EQ* allele arose in a *japonica* ancestor in the early stages of *japonica* domestication, and that selection for these FNPs was a critical step in *japonica* domestication (Fig. 5).

Previous studies have suggested that hybridization between subpopulations and subsequent introgressions of domestication-related genes, combined with artificial and natural selection, gave rise to the present rice landraces (4–6, 11, 13, 25–28). In fact, many rice domestication-related genes, including *rc*, *wx*, *gs3*, and *badh2.1*, were presumably established during *japonica* domestication and subsequently introgressed into *indica* (11, 13, 25, 28). In the present study, despite the earlier prevalence of *SD1-EQ* in *japonica* landraces, *SD1-EQ* appeared to be introgressed into a small number of *indica* landraces (3 out of 52; Tables S2 and S3). These few exceptional landraces carry a defined region of *japonica*-like DNA flanking *SD1* in the *indica* background, likely the result of natural crossing and subsequent introgression during domestication after the emergence of *SD1-EQ* in *japonica*. This clear differentiation of *SD1* between *japonica* and *indica* might be attributed to the adaptation of *SD1* alleles in a specific region. An intriguing alternative possibility is the contribution of a reproductive barrier to prevent introgression of this locus. Relevant to this point, it is important to note that *qsh1*, a domestication-related gene for reduced seed shattering, originated in *japonica* and was also not introgressed into *indica* (7). Both *SD1-EQ* and *qsh1* are located on the long arm of chromosome 1, ~1.9 Mbp apart, and thus should behave as a linked locus. The existence of a reproductive barrier between *japonica* and *indica* has been reported in this region (31), and this barrier might prevent the introgression of these two genes from *japonica* into *indica*. It is noteworthy that two contradictory events, hybridization between subpopulations and a potential reproductive barrier, might play important roles in rice domestication.

Methods

Plant Material and Growth Conditions. Rice BILs, substitution lines, and accessions used for genetic diversity analysis were obtained from the National Institute of Agrobiological Sciences (18, 20). Other varieties used in this study were maintained at Nagoya University. All varieties were grown under natural field conditions in the research field. Seeds of all varieties were immersed in water for 2 d and then sown in a nursery bed. After 1 mo, the seedlings were transplanted to a paddy field.

QTL Analysis. The CL of 98 BILs from a cross between Nipponbare and Kasalath was evaluated for QTL analysis. The CL was defined as the length from the soil surface to the panicle node. The linkage map was constructed using MAPMAKER/EXP version 3.0 (32). QGene version 3.29 (33) was used to identify QTLs. Four loci were scored, and all had an LOD score >2.5.

Selection of Accessions Used for Genetic Diversity Analysis. Accessions used for genetic diversity analysis were chosen from a set of 72 landraces and 42 accessions of *O. rufipogon* to represent the genetic diversity of the entire set based on restriction fragment length polymorphism data for *O. sativa* (20) and *p-SINE1*, as well as the LTR retrotransposon insertion pattern for *O. rufipogon*.*

Sequencing of *SD1* and Surrounding Genes. Accessions were sequenced at *SD1* and portions of 18 flanking loci located at 11- to 82-kb intervals upstream and downstream of *SD1*. All primers were designed from the Nipponbare genomic sequence. For *SD1*, primers were designed to amplify seven partially overlapping portions of the gene. For flanking loci, primers were designed to amplify ~880-bp portions of genes with putative or known function. All primer pairs for flanking loci were designed on exons and spanned one or more intron regions (Table S5). All PCR primers were analyzed by BLAST against the Rice Annotation Project Database, to ensure amplification of only the targeted genomic region. Nucleotide sequences were determined by the dideoxynucleotide chain termination method using an automated sequencing system. Sequences were analyzed with GENETYX software.

Genetic Diversity Analysis. Population genetic analyses were conducted for *SD1* and the 18 flanking genes using DnaSP 5.1 (34). Levels of nucleotide diversity per site and silent site were estimated as π for each of the landrace groups of cultivated rice and *O. rufipogon*.

Phylogenetic Analysis. Phylogenetic trees were constructed using the neighbor-joining method in PHYLIP version 3.69 (35). To produce the phylogenetic tree based on genome-wide transposon patterns, we used the Cavalli–Sforza chord distance (36) calculated by Microsatellite Analyzer 4.05 (37), followed by the neighbor-joining method.

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