



Molecular cloning of *Sdr4*, a regulator involved in seed dormancy and domestication of rice

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Seed dormancy provides a strategy for flowering plants to survive adverse natural conditions. It is also an important agronomic trait affecting grain yield, quality, and processing performance. We cloned a rice quantitative trait locus, *Sdr4*, which contributes substantially to differences in seed dormancy between *japonica* (Nipponbare) and *indica* (Kasalath) cultivars. *Sdr4* expression is positively regulated by *OsVPI1*, a global regulator of seed maturation, and in turn positively regulates potential regulators of seed dormancy and represses the expression of postgerminative genes, suggesting that *Sdr4* acts as an intermediate regulator of dormancy in the seed maturation program. *Japonica* cultivars have only the Nipponbare allele (*Sdr4-n*), which endows reduced dormancy, whereas both the Kasalath allele (*Sdr4-k*) and *Sdr4-n* are widely distributed in the *indica* group, indicating prevalent introgression. *Sdr4-k* also is found in the wild ancestor *Oryza rufipogon*, whereas *Sdr4-n* appears to have been produced through at least two mutation events from the closest *O. rufipogon* allele among the accessions examined. These results are discussed with respect to possible selection of the allele during the domestication process.

domestication | preharvest sprouting | quantitative trait locus

Plant seeds are major sources of human nutrition, either directly or indirectly, and are the major means of crop propagation. Consequently, various seed traits, including dormancy, have been selected through crop domestication (1, 2). Seed dormancy has both advantages and disadvantages for plants—especially crops—in terms of cultivation and utilization, because weak dormancy leads to uniform germination, whereas deep dormancy prevents preharvest sprouting but inhibits germination. Preharvest sprouting often occurs under favorable temperature and humidity at maturity, resulting in reduced grain quality and germinability. Thus, controlling seed dormancy is a very important goal in the breeding of rice and other cereals.

Seed dormancy is genetically controlled by the genotypes of both the mother plant and the embryo. The former affects the nature of the tissues surrounding the embryo, such as the seed coat (testa). These tissues impose dormancy and act as physical barriers to radicle growth on imbibition. Whereas this “coat-imposed” dormancy depends on the anatomy of the seed, which varies among species, embryonic dormancy is controlled more finely by the developmental program. Embryonic dormancy is acquired during seed maturation, in which the plant hormone abscisic acid (ABA) plays a fundamental role (3). Studies in *Arabidopsis* have revealed the framework of the regulatory network for seed maturation, which is controlled by several master transcription factors, including *ABI3*, which is orthologous to maize *VPI* and rice *OsVPI* (4–6). Mutations in these genes profoundly affect the acquisition of seed dormancy. In addition to these maturation-related regulatory genes, mutants have been isolated by direct screening for reduced-dormancy phenotypes in *Arabidopsis*. Among these, *REDUCED DORMANCY4* (*RDO4*), renamed *HISTONE MONOUBIQUITINATION1* (*HUB1*), has

been molecularly identified, and the results of this identification point to the importance of chromatin modification in seed dormancy (7). Studies of natural variations in seed dormancy in *Arabidopsis* have led to the cloning of a quantitative trait locus (QTL), *DELAY OF GERMINATION 1* (*DOG1*), which affects embryonic dormancy (8). *DOG1* encodes a member of a plant-specific protein family with a domain shared by the D class bZIP DNA-binding proteins.

Despite such remarkable findings, our knowledge of the molecular mechanisms underlying seed dormancy is far from complete. Information about the molecular identities of the genes controlling dormancy is even more limited in crops than in the model plant *Arabidopsis*. Particularly in cereals, there is a need to identify the genes contributing to natural variations in dormancy, to improve crops and better understand the history of domestication. Many studies have been performed to detect QTLs for seed dormancy in cereals, but thus far no genes have been molecularly identified (9–12).

In previous work, we detected five QTLs for dormancy in backcross inbred lines (BILs) derived from crosses between the *japonica* cultivar Nipponbare and the *indica* cultivar Kasalath (9). We have continued to perform map-based cloning of these QTLs. Here we report the molecular cloning of one of these QTLs, *Seed dormancy 4* (*Sdr4*). *Sdr4* encodes a novel protein with an amino acid sequence that has no similarity to proteins with known functions; it may act as a seed dormancy-specific regulator that is under the control of the seed global regulator *OsVPI*. Furthermore, haplotype analysis of the *Sdr4* region has revealed that *Sdr4* acts as an important determinant of seed dormancy in rice cultivars and might have been involved in rice domestication.

Results

Isolation and Characterization of the Kasalath Allele of *Sdr4*, Which Confers Preharvest Sprouting Resistance and Deeper Dormancy. We developed a nearly isogenic line of *Sdr4* (NIL[*Sdr4*]) with a 7.5-Mb Kasalath segment in a Nipponbare background (Fig. S1A). The germination rate of NIL seeds collected 6 weeks after heading (WAH) was very low (2%), even lower than that of Kasalath (4%), whereas Nipponbare had a high germination rate (58%), indicating that the Kasalath allele of *Sdr4* (*Sdr4-k*) had a strong positive effect on seed dormancy (Fig. 1A and B). From a

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AB506455 (*Sdr4-k*) and AB510199 (*Sdr4-n*)].

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population of 2,515 plants, we identified 8 plants with recombination in the vicinity of *Sdr4* (Fig. S1 B–D). Progeny testing delimited the candidate region to <8.7 kb (Fig. 1C). By transforming Nipponbare with subfragments of this region, we found that a 3.3-kb Kasalath fragment, which contained a single gene, *Os07g0585700*, substantially decreased germination compared with the control (Fig. 1D and Fig. S2 A and B). Furthermore, RNAi knockdown of *Os07g0585700* in both Nipponbare and NIL [*Sdr4*] increased germination rates to above that of the control Nipponbare (Fig. 1E). In addition, seeds of two independent mutants (M25 and M100) carrying the same seven-aa deletion in the *Os07g0585700*-encoded protein were less dormant than those of wild-type Nipponbare seeds (see below). Thus, we conclude that *Os07g0585700* is *Sdr4*. The results also indicate that *Sdr4-n* remained functional, although it was not as effective as *Sdr4-k* in conferring dormancy.

Sdr4 contained no intron, and the deduced amino acid sequence of the encoded protein was not homologous with those sequences of proteins with known functions (Fig. S3A). A putative bipartite-type nuclear localization signal (13) was found in the N-terminal region. Nuclear localization of *Sdr4* was confirmed by expression of *Sdr4-k*-GFP (green fluorescent protein) fusion protein in cultured rice cells (Fig. 2).

Prediction of Functional Nucleotide Polymorphisms of *Sdr4-k*. From the results of complementation analysis and genetic mapping, we expected to find functional nucleotide polymorphisms (FNPs) to explain the functional difference between *Sdr4-n* and *Sdr4-k*

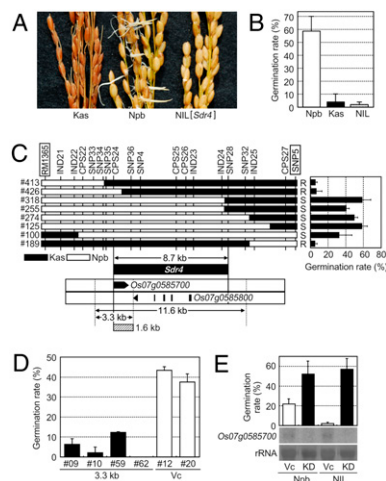


Fig. 1. Genetic effects, delimitation of candidate genomic region, and genetic complementation of *Sdr4*. (A) Germination of Kasalath (Kas), Nipponbare (Npb), and NIL [*Sdr4*] (NIL). (B) Germination rates of seeds from panicles sampled 6 weeks after heading. (C) Delimitation of *Sdr4*. Graphical genotypes of the *Sdr4* region in eight plants in which recombination occurred between RM1365 and SNP5 (Fig. S1) are shown at the left. Black and white regions represent chromosomal segments homozygous for the Kas and Npb alleles, respectively. The bar graph at the right shows the germination rates of recombinants. Preharvest sprouting resistance is denoted by “R,” and sensitive is denoted by “S.” Genes predicted by RAP-DB (<http://rapdb.dna.affrc.go.jp/>) (52) in the Npb genomic sequence around the *Sdr4* candidate region are indicated by closed arrows and closed boxes. Two genes were annotated in the region. The 8.7-kb *Sdr4* candidate region was defined by linkage analysis. The 3.3-kb fragment was used for complementation analysis. The 1.6-kb region with FNP is indicated by a hatched box. (D) Bar graph showing germination rates of T₃ seeds harboring the Kas fragment (3.3 kb) or empty vector (Vc). (E) RNA gel blot analysis of *Os07g0585700* gene expression in knockdown (KD) and Vc lines. Knockdown transgenic lines were generated from Npb and NIL. rRNA stained with methylene blue shows equal loading of RNAs (rRNA). Germination rates 7 days after imbibition are shown as averages of three biological repeats with standard deviation.

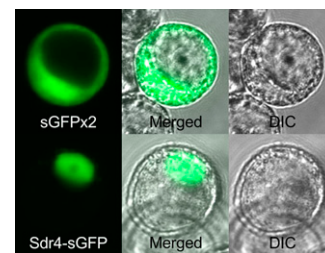


Fig. 2. Subcellular localization of *Sdr4*. The 35S promoter-driven dimer of sGFP and the *Sdr4-k*-sGFP fusion were transiently expressed in oc cells, and the localizations were investigated under a confocal laser scanning microscope (Left). Differential interference contrast images (Right) and merged images (Middle) are shown.

located within a 1.6-kb region (Fig. 1C) corresponding to the protein-coding region and 3'-UTR. In addition to a SNP in the 3'-UTR, the coding region contained clustered sequence changes corresponding to one InDel and three amino acid substitutions, along with four simple SNPs, three of which resulted in amino acid changes (Fig. 3A). The presence of an 18-bp direct repeat in *Sdr4-n*, which could have been created by double-strand cleavage and repair in the corresponding region of *Sdr4-k*, as is seen in maize *waxy* mutants (14), suggests that the clustered sequence changes resulted from a single event (Fig. 3B). Such polymorphisms were associated with a change in the amino acid sequence from $-(K)VIS_{270-274}$ (*Sdr4-k*) to $R(K)LLE_{270-275}$ (*Sdr4-n*). The dipeptide sequence Val-Ile (VI) in *Sdr4-k* was well preserved in a conserved sequence block among related proteins across species, including dicots, whereas other amino acids associated with simple SNPs were not well conserved (Fig. 3C and Fig. S4); thus, the polymorphisms most likely were FNPs.

Expression and Localization of *Sdr4*. *Sdr4* mRNA was detected preferentially in the seed tissues 14 days after flowering (DAF) (Fig. S5). Promoter-*GUS* transgenics and in situ hybridization suggest that *Sdr4* mRNA was expressed throughout the embryo but preferentially in the radicle; the in situ hybridization signals suggest that it

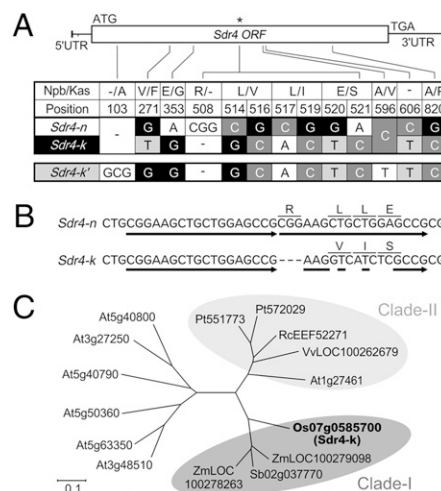


Fig. 3. InDels and SNPs between *Sdr4-n* and *Sdr4-k*, and amino acid sequence comparison among *Sdr4* and its homologs. (A) Haplotypes derived from 11 SNPs and two InDels of the *Sdr4* coding region. (B) Sequences of two alleles around the SNP cluster, indicated by the asterisk in A. The 18-nt direct repeats are indicated by arrows. (C) *Sdr4* homologs were searched by BLAST. Aligned sequences by Genetyx version 9 (Genetyx) are shown by unrooted phylogenetic tree using MEGA4 (53).

also was expressed in the shoot (Fig. 4A). *Sdr4* mRNA began to accumulate in the seed at 7 DAF, and its level increased as the seed matured (Fig. 4B). These expression patterns during the course of seed maturation were in accordance with the set of *cis*-elements predicted in the *Sdr4* promoter; there were seven RY repeats (CATGCA), which are important for seed-specific gene expression and are the target of the VP1/ABI3 subfamily of B3 domain transcription factors (15), along with an ABA response element [ABRE; ACGTGG/T(C)] and an ABRE-related coupling element (CE) (Fig. S3B) (16–18). One RY repeat is closely linked to an ABRE, and another is closely linked to an ABRE-CE and an ABRE. The combination of these elements and the close linkages are frequently seen in seed maturation-related genes (19).

Characterization of the Loss-of-Function Mutant of *Sdr4*. The seeds of two independent *sdr4* mutant lines, M25 and M100, contained embryos larger than those of the wild type (Fig. 4C and D) and were completely nondormant (nearly 100% germination at 4 WAH; Fig. 4E). The loss of dormancy was associated with severely reduced ABA sensitivity. Germination of *sdr4* seeds at 6 WAH was not inhibited by ABA at 100 μ M, whereas that of Nipponbare seeds was completely inhibited (Fig. 4F); however, no significant differences in the ABA content of seeds sampled at 6 WAH were seen (83 ng/g fresh weight for Nipponbare, 87 ng/g for NIL(*Sdr4*), 83 ng/g for M25, and 78 ng/g for M100).

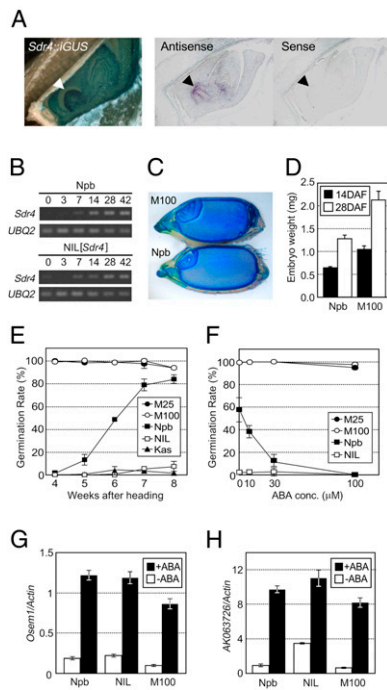


Fig. 4. Morphological and physiological analysis of *sdr4* mutant. (A) GUS staining of sliced seed of Nipponbare (Npb) with *Sdr4*:*n::GUS*. The radicle is indicated by an arrowhead. For tissue-specific expression of *Sdr4* mRNA, in situ hybridization used *Sdr4*-specific antisense and sense probes on sections of Npb seed. The radicle is indicated by an arrowhead. (B) Temporal changes in level of *Sdr4* mRNA during the ripening period, based on semiquantitative RT-PCR, at 0, 3, 7, 14, 28, and 42 days after heading. The *UBQ2* was used as a control to show equal loading. (C) Longitudinal slices of the seeds of Npb and the *sdr4* mutant were stained by toluidine blue. (D) Embryos were harvested ($n = 50$) and weighed, with three repeats. (E) Germination rates of Npb, NIL[*Sdr4*] (NIL), two *sdr4* mutants, and Kasalath (Kas) at various time points after heading were determined. (F) Germination rates just after harvest (6 WAH) of fresh seeds treated with different concentrations of ABA. (G) Embryoless half-seeds were treated with 30 μ M ABA or left untreated for 24 h; expression levels of *Osem1* were determined by real-time PCR. (H) *AK063726* expression levels in the embryoless half-seeds were measured.

Despite the ABA insensitivity of seed germination, the primary ABA-signaling mechanism, which leads to ABA-induced gene expression, was not affected by the *sdr4* mutation. Expression of two genes for the ABA-inducible late-embryogenesis-abundant proteins (LEA) *Osem1* and *AK063726*, having ABREs and a CE, was normally induced by 30 μ M ABA in embryoless half-seeds of the M100 mutant (Fig. 4G and H) (20, 21).

Analysis of Upstream (*Sdr4* Regulator) and Downstream Genes. The presence of ABREs and RY repeats in the *Sdr4* promoter, together with an expression pattern typical of that of maturation-related genes, prompted us to investigate whether *Sdr4* expression is regulated by *OsVP1*, a global regulator of seed maturation (6). *Sdr4* expression in embryos at 28 DAF were substantially reduced in *Osvp1* mutant embryos (Fig. 5B and Fig. S6B). This finding suggests that the regulation exerted by *Sdr4* was, at least in part, integrated into the global seed maturation program directed by *OsVP1*.

To relate *Sdr4* more closely to known seed dormancy and germination mechanisms, we examined the effects of *sdr4* mutation on the expression of several genes potentially related to dormancy and germination. Although little is known about regulators of embryonic dormancy other than those of seed maturation and ABA signaling in cereals, we examined the expression of the three closest rice homologs of Arabidopsis *DOG1* (8) (a QTL identified as a positive regulator of seed dormancy) as potential positive regulators of seed dormancy. The levels of expression of two of the three *OsDOG1-like* genes were lower in *sdr4* mutant embryos than in wild-type Nipponbare or NIL[*Sdr4*] (Fig. 5A and Fig. S7A and B). In addition, *OsDOG1-like-1* (*OsDOGIL-1*) expression was significantly higher in NIL[*Sdr4*] than in Nipponbare. It was difficult to judge this gene to be orthologous to Arabidopsis *DOG1* from the phylogenetic relationships, due to the presence of three highly related *DOG1-like* genes in Arabidopsis, some of whose mutations were reported to not affect dormancy. However, the observed down-regulation in the mutant and up-regulation in NIL[*Sdr4*] suggest that *OsDOGIL-1* is a positive regulator of dormancy in rice, and that *Sdr4*, at least in part, controls seed dormancy via the regulation of these *OsDOG1-like* genes.

In Arabidopsis nondormant mutants with mutations in seed maturation regulators such as *abi3*, germinative and postgerminative programs operate prematurely in the developing seed (22). Consequently, we examined the expression of several germination-related genes: a gibberellin biosynthesis gene (*OsGA20ox-1*) (23), aquaporin gene (*PIP1;3* and *PIP2;2*) (24), and an expansin gene (*OsEXPB3*)

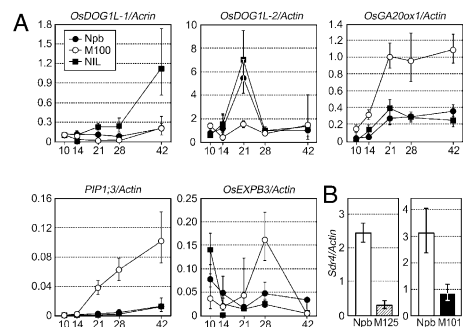


Fig. 5. Expression analysis of dormancy and germination related genes in *sdr4* and *Osvp1* mutants. (A) Temporal changes (DAF) in mRNA levels of *OsDOG1-like* genes, gibberellin biosynthesis gene (*OsGA20ox-1*), aquaporin gene (*PIP1;3*), and expansin gene (*OsEXPB3*) in embryos were monitored by real-time PCR in Nipponbare (Npb), the *sdr4* mutant (M100), and NIL[*Sdr4*] (NIL), with three biological repeats. Expression levels are shown as ratios to *Actin-1* gene expression. (B) *Sdr4* gene expression in wild-type (Npb), *Osvp1-1* (M125), and *Osvp1-2* (M101) at 28 DAF was determined.

(25) (Fig. 5A and Fig. S7C). Expression of these genes is induced in nondormant seeds on imbibition (26). Expression levels of all of these genes were significantly higher in the *sdr4* mutant than in the wild-type Nipponbare or NIL[*Sdr4*], consistent with the positive regulation of *Sdr4* by OsVP1.

The foregoing expression analysis results suggest that Sdr4 plays a regulatory, rather than a structural or metabolic, role in the promotion of dormancy and inhibition of germination, as also suggested by the nuclear localization of the gene product.

Natural Variations in *Sdr4* in Cultivars and Wild Rice. Sequence analysis of 59 cultivars from the world rice core collection revealed the existence of only three haplotypes, *Sdr4-n*, *Sdr4-k*, and *Sdr4-k'*, in the coding sequences (Figs. 3A and 6A) (27). Interestingly, all *japonica* cultivars had only *Sdr4-n*, whereas both *Sdr4-n* and *Sdr4-k* were found in *indica* In-2, and all three haplotypes were found in *indica* In-1 (Fig. 6B). SNP analysis of the chromosomal regions containing *Sdr4-n* in *indica* cultivars revealed that in most cases, the *japonica* haplotypes with *Sdr4-n* were flanked by *indica* haplotypes, although some complex haplotypes were found as well (Fig. S8). Therefore, we considered the *Sdr4-n* in *indica* cultivars to be the result of introgression from *japonica* sources. The seeds of cultivars with *Sdr4-n* had lower dormancy than those of cultivars with *Sdr4-k* ($P = 0.012$ by the F test). These results strongly suggest that the functional differences in *Sdr4* contribute substantially to the variations in dor-

mancy of Asian rice accessions, although the involvement of other loci is suggested by the presence of some *indica* cultivars with *Sdr4-k(k')* showing high germination rates and some *indica* cultivars with *Sdr4-n* showing low germination rates (Fig. 6A).

We also identified the sequences of *Sdr4* in 46 accessions of *Oryza rufipogon*, the wild ancestor of *O. sativa* (28, 29) (Table S1). Our analysis revealed 23 variations, mostly with sequences closer to the sequence of *Sdr4-k* than to the sequence of *Sdr4-n*. In particular, two of these variations (W1685 and W0610) were identical to *Sdr4-k*, and one variation (W2264) was identical to *Sdr4-k'*. Thus, *Sdr4-k* and *Sdr4-k'* in *indica* were inherited from these subgroups of the wild ancestor. In contrast, the *Sdr4-n* sequence was not found in any of the 46 accessions.

Discussion

We isolated and characterized the preharvest-sprouting-resistance gene *Sdr4*, which encodes a protein with no similarity to proteins of known function. However, in light of the findings that Sdr4 was located in the nucleus, was transcriptionally regulated by OsVP1 (the global regulator of seed maturation), and was involved in the regulation of gene expression related to seed dormancy and germination, Sdr4 might be involved in the gene expression machinery as, for example, a transcription factor. The *sdr4* mutant had a nondormant phenotype, as well as a larger embryo. In addition, the *sdr4* embryo precociously expressed germinative or postgerminative genes. Precocious expression of these genes in the mutant resembled the mutant expression of seed maturation regulators, including ABI3/VP1 (30, 31), and was consistent with the result that expression of *Sdr4* was under the control of OsVP1. The ABA insensitivity of *sdr4* seeds with respect to germination also was in accordance with OsVP1 control of *Sdr4* expression. In addition, ABA levels and basic ABA signaling as monitored by LEA gene induction in response to this hormone were not significantly affected in the mutant. This suggests that Sdr4 is a specialized regulator in the seed maturation program responsible for the dormancy pathway. Furthermore, the *Sdr4*-dependent expression of *OsDOGIL-1*, a rice homolog of Arabidopsis *DOG1*, is in line with this suggestion, although the role of OsDOG1L-1 in dormancy remains to be determined.

In addition to the seed coat, such tissues as the endosperm and coleorhiza surrounding the radicle are known to play important roles in the imposition of seed dormancy. These tissues act as barriers and need to be broken for the radicle to emerge—that is, to germinate. It has been recently shown that in barley, the coleorhiza undergoes a reduction in ABA metabolism and content during after-ripening; these changes ultimately allow the imbibed radicle to grow (32). It remains to be elucidated whether Sdr4 plays a role in the establishment of such a function of the coleorhiza. The preferential expression of *Sdr4* in the radicle and shoot tissues suggests that this gene plays a role in regulating the growth potential of the radicle and shoot, which counteracts the imposed barriers. In light of the aforementioned functions of the coleorhiza in dormancy in rice, it should be noted that a recent study found that *qLTG3-1*, which limits germination at low temperatures, is preferentially expressed in the coleorhiza and epiblast (33).

In crop plants, such as rice, wheat, and barley, seed dormancy must be finely controlled, because an excessively high or low level of dormancy can cause severe problems with seed germination and preharvest sprouting, respectively (34). Thus, cereals require appropriate levels of dormancy to maintain quality and yield. Genetic approaches have been used in wheat, barley, and several cereals, and numerous QTLs have been reported (35, 36). Rice chromosome 7 demonstrates synteny with wheat chromosome 2D (37). Four dormancy QTLs (including *Sdr4*) have been identified on rice chromosome 7, (9, 10). Because QTLs related to seed dormancy have been reported on wheat chromosome 2DS (35, 38), determining the locus of the wheat ortholog of *Sdr4* is of interest.

Most of the genes involved in seed dormancy that have been isolated so far are involved in ABA synthesis and ABA signal

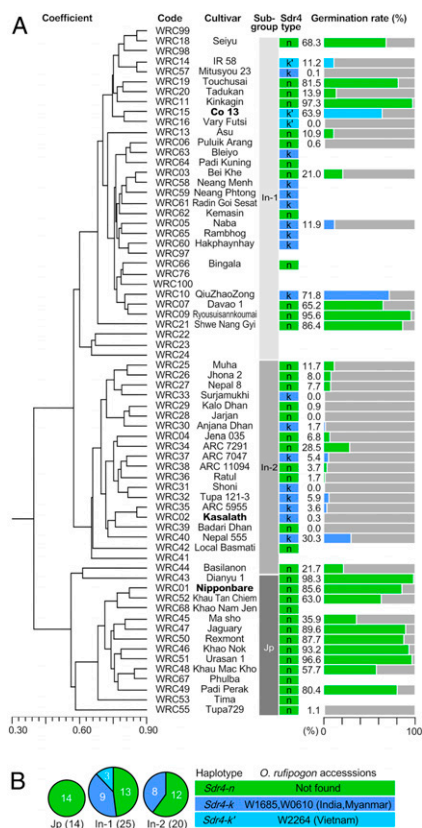


Fig. 6. Natural variations in the *Sdr4* coding region. (A) SNPs and germination rates at 6 WAH in cultivars in the world rice core collection. Cultivars, ordered according to the genetic distance based on RFLPs, are shown. Germination rates at 6 WAH are indicated in mauve-blue or sky-blue bars (cultivars with *Sdr4-k* or *Sdr4-k'*, respectively) or green bars (cultivars with *Sdr4-n*); the 100% scale is indicated by the gray bars. (B) The frequency of each haplotype in *japonica* and *indica* (In-1 and In-2) lines is summarized, and wild relatives showing the same haplotypes as the cultivars are shown.

transduction (34, 39, 40). Thus, modulation of these genes is predicted to have pleiotropic effects on seed maturation events, such as desiccation tolerance and storage material accumulation (41). In contrast, we can expect modification of *Sdr4* to not be accompanied by effects other than those on seed dormancy, because *Sdr4* is a seed dormancy-specific regulator of the seed maturation program. Cloning of *Sdr4* in rice will provide a unique opportunity to explore the genetic control and modification of seed dormancy in these crops.

The recent identification of FNPs in several cloned genes for agronomically important traits, including those for grain shattering (*Sh4*, *qSHI*), pericarp color (*Rc*), seed width (*qSW5*), and amylose content (*Waxy*), has allowed us to speculate on the rice domestication process (42–45). One current view of the process of domestication of rice from *O. rufipogon* proposes that the *japonica* and *indica* subspecies arose from independent subgroups of wild ancestors (1). It has been suggested that W1943, an *O. rufipogon* accession, is a close relative of the subpopulation from which the *japonica* group originated; this suggestion is based on the patterns of short interspersed nuclear transposable elements (46) and cDNA sequences (47). In fact, three accessions from China and India, including W1943, have *Sdr4* sequences closer than those of other accessions to *Sdr4-n*. The differences between *Sdr4-n* and *Sdr4-W1943* appear to have been generated by two events, a single nucleotide substitution and clustered substitutions of the potential FNP caused by a double-strand break and repair (see above). Because dormancy is considered a vital domestication trait in rice, we can reasonably expect that these mutations were selected during the domestication of *japonica*. We can speculate that, in their wisdom, ancestral farmers would not have failed to use the rare mutation events that produced a useful degree of reduction in function of *Sdr4*, rather than loss of function, which would have led to a problem of vivipary. The prevalent introgression of *Sdr4-n* in *indica* cultivars supports the profitability of this allele in cultivation, similar to that of the *rc* allele and *wx* (44, 48). Obviously, the *Sdr4* allele is not the only dormancy-related domestication gene. A recent transcriptomic study in Arabidopsis indicates that the mechanisms for establishing dormancy and controlling after-ripening potential can be differentiated (22). Our results suggest that *Sdr4* is involved in the former mechanism, although the possibility of participation in both mechanisms has not been excluded. Reduced seed dormancy during domestication could have been achieved by the selection of alleles of genes related to either type of mechanism. Presumably, selection was made based on both germination synchronization and the duration of the after-ripening period. Therefore, genes related to after-ripening potential may well be identified as domestication genes in the future.

Our molecular identification of *Sdr4* should not only provide clues to rice domestication, but also shed light on the molecular mechanisms of seed dormancy. More specifically, our results placing *Sdr4* in the central regulatory network of seed maturation provide opportunities for a more comprehensive understanding of seed development, which will be further enhanced by the cloning of other identified dormancy QTLs.

Methods

Plant Materials for Mapping. We crossed Nipponbare (a *japonica* cultivar) and Kasalath (an *indica* cultivar) and then performed repeated backcrossing with Nipponbare as the recurrent parent. BC₄F₂ plants in which the *Sdr4* region was heterozygous were selected to produce a mapping population. We used 100 and 2,515 self-pollinated progeny plants (OOF2#53) for coarse-resolution and high-resolution mapping of *Sdr4*, respectively. With this population, *Sdr4* was mapped in the interval between markers SNP1 and SNP8 (Fig. S1C). Twenty-eight BC₄F₂ plants in which recombination occurred in the interval between IND3 and IND8 were selected (Fig. S1D). The self-pollinated progeny of these plants (BC₄F₃) were used for progeny testing. The germination rates of three plants fixed for each allele were determined. The molecular markers used for high-resolution mapping are listed in Table S2A.

Germination Testing. Three panicles sampled at 4, 5, 6, 7, or 8 WAH were wrapped with paper towels and dipped in water. After the water was briefly drained off, the panicles were incubated in the dark at 30 °C for 1 week, and seed germination was scored.

Isolation of *sdr4* and *Osvp1* Mutants. The sequences of *Sdr4* and *Osvp1* were determined in 16 mutant lines exhibiting vivipary. The plants were selected from a mutant panel (49), a library of rice mutants created by tissue culture of Nipponbare. The same deletion of 21 nucleotides causing a 7-aa deletion in the C-terminal conserved-sequence block of *Sdr4* was found in three lines—M25, M26, and M100—derived from independent mutagenesis. This phenotype of the M100 line was complemented using a 3.3-kb fragment (Fig. 1C and Fig. S6A). Mutations in *Osvp1* were found in two lines, one line with a 32-nt deletion resulting in a frame shift generating a truncation before the B3 domain (M125; *Osvp1-1*), and the other line with an amino acid substitution in the B3 domain (M101; *Osvp1-2*) (Fig. S6B). Genetic analysis found that the phenotype and the *Osvp1-2* mutation were colocalized within a 131-kb region. This result supports the hypothesis that the viviparous phenotype was due to this substitution (Fig. S6C). Mutant lines M25, M26, M100, M101, and M125 corresponded to ND2054, ND2126, NE2331, NE2465, and NE5113, respectively, in the *Tos17* mutant panel database (<http://tos.nias.affrc.go.jp/>).

Transgenic Complementation and Knockdown. A BAC library of Kasalath genomic DNA was screened by PCR for a clone with the *Sdr4* region, from which the *NruI-NruI* 11.6-kb fragment was subcloned into the *EcoRV* site of pBluescript II SK(+) (Agilent Technologies), resulting in pBS-*Sdr4*KN. The 11.6-kb *KpnI-SmaI* fragment, the 11.2-kb *KpnI-SmaI* fragment with internal deletion of a 0.4-kb *BamHI-BamHI* fragment, or the 3.3-kb *BamHI-ApaI* fragment of pBS-*Sdr4*KN was inserted into pPZP2Hlac, yielding pPZP-*Sdr4*KN, pPZP-*Sdr4*KNd, or pPZP-*Sdr4*KNa, respectively. To produce a construct for knockdown, a fragment containing the 3'-UTR (nt 1,104–1,294 of cDNA) and part of the *GUS* gene (345 nt) as a spacer were amplified using primers with the created restriction sites, digested with the appropriate enzymes, and then inserted immediately into pPZP-Ha3(+), resulting in pPZP-355-*Sdr4*i. These plasmids were introduced into *Agrobacterium* (EHA101) and transformed into Nipponbare, M100 mutant, or NIL[*Sdr4*] by an *Agrobacterium*-mediated rapid method (50).

RNA Preparation and Real-Time PCR. RNAs were purified using the RNeasy Plant Kit with on-column DNaseI treatment (Qiagen). cDNA obtained by reverse-transcription reaction using a ReverTra Ace alpha kit (Toyobo) was amplified by conventional PCR or subjected to real-time PCR using an SYBR Green-based kit (QPK-212; Toyobo) and an ABI 7900HT system (Applied Biosystems). Primer sequences for the amplifications are listed in Table S2B.

Measurement of ABA. Seeds (500 mg) harvested at 6 WAH were extracted in aqueous methanol and purified by HPLC. Their ABA content was quantified by GC-MS as described by Iuchi et al. (51).

Subcellular Localization of *Sdr4*. The *Sdr4* coding sequence was inserted in front of the 5' end of the GFP (sGFP) in pCaMV35S-sGFP(S65T)-nos3', resulting in p35S-*Sdr4*n-GFP. This plasmid was introduced into the protoplasts of cultured rice cells (oc cells) by electroporation, and localization of the GFP fluorescence was observed under a confocal laser scanning microscope (FluoView; Olympus).

Sequencing of *Sdr4* in Rice Accessions. The genomic sequences around the *Sdr4* ORF were amplified by PCR with the primer sets 87535F (5'-ccg ccc acg cct tct taa cc-3') and 88688R (5'-aaa gtt tgc tcc ggc ttg atg c-3'). The PCR products were purified with a MagExtractor PCR and Gel Extraction Kit (Toyobo) and used directly for sequence reaction.

Statistical Methods. Association of the *Sdr4* allele with germination rate was tested using a general linear model. The significance of associations between the allele and traits was based on the *F* test. All statistical analyses were done with SPSS version 15 J for Windows (SPSS Inc.).

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