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30. We gratefully acknowledge D.S. Howland (Molecular Genetics, Wyeth Research, Princeton, NJ 08543–8000) for the QPCR protocol; M. Mallat (U495, IFR70, INSERM UPMC, Paris, France) and V. Zujovic (UMR 546, INSERM UPMC, Paris, France) for advice on microglial cell culture; D. Swing for pronuclear injection; J. Folmer for tissue sections; and T. Jessel, G. Martin, and P. Soriano for Islet1-Cre, ZP3-Cre, and Rosa26 reporter mice, respectively. This work was supported by grants from NIH (NS 27036), the Center for ALS Research at Johns Hopkins, the ALS Association, and the European Commission (LSHG-CT-2005-005203). S.B. is a recipient of a Fondation pour la Recherche Medicale fellowship and an INSERM fellowship. K.Y. is a recipient of a Uehara Memorial Foundation fellowship and a Development grant from the Muscular Dystrophy Association.

Supporting Online Material
www.sciencemag.org/cgi/content/full/312/5778/1389/DC1
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6 December 2005; accepted 27 April 2006
10.1126/science.1123511

An SNP Caused Loss of Seed Shattering During Rice Domestication

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Loss of seed shattering was a key event in the domestication of major cereals. We revealed that the *qSH1* gene, a major quantitative trait locus of seed shattering in rice, encodes a BEL1-type homeobox gene and demonstrated that a single-nucleotide polymorphism (SNP) in the 5′ regulatory region of the *qSH1* gene caused loss of seed shattering owing to the absence of abscission layer formation. Haplotype analysis and association analysis in various rice collections revealed that the SNP was highly associated with shattering among *japonica* subspecies of rice, implying that it was a target of artificial selection during rice domestication.

Cultivation of major cereals likely started about 10,000 years ago (*1*–*4*). During this domestication, ancient humans subjected several key events to selection. These included increase in the number of seeds, improvement of fertility, change in plant architecture, change in seed shape, adaptation of flowering time to local areas, loss of seed color, and loss of seed shattering.

Recent studies in rice have revealed that several independent domestication events might have occurred to establish cultivated rice (*3*, *5*–*7*). The archaeological record reveals that *japonica* rice, a subspecies of *Oryza sativa*, was bred about 10,000 years ago in the upstream regions of the Yangtze River in southwest China (*3*, *8*, *9*).

The loss of seed-shattering habit is thought to be one of the most important events in rice domestication, because the "easy-to-shatter" trait in wild relatives results in severe reduction in yield. Over the course of human history, distinct grain-threshing systems have been developed in several different eras in local areas of the world, in accordance with the degree of seed shattering. In current rice-breeding programs, this seed-shattering habit is still a target, especially in the construction of new *indica* (another subspecies of *O. sativa*) cultivars. Thus, seed-shattering habit is one of

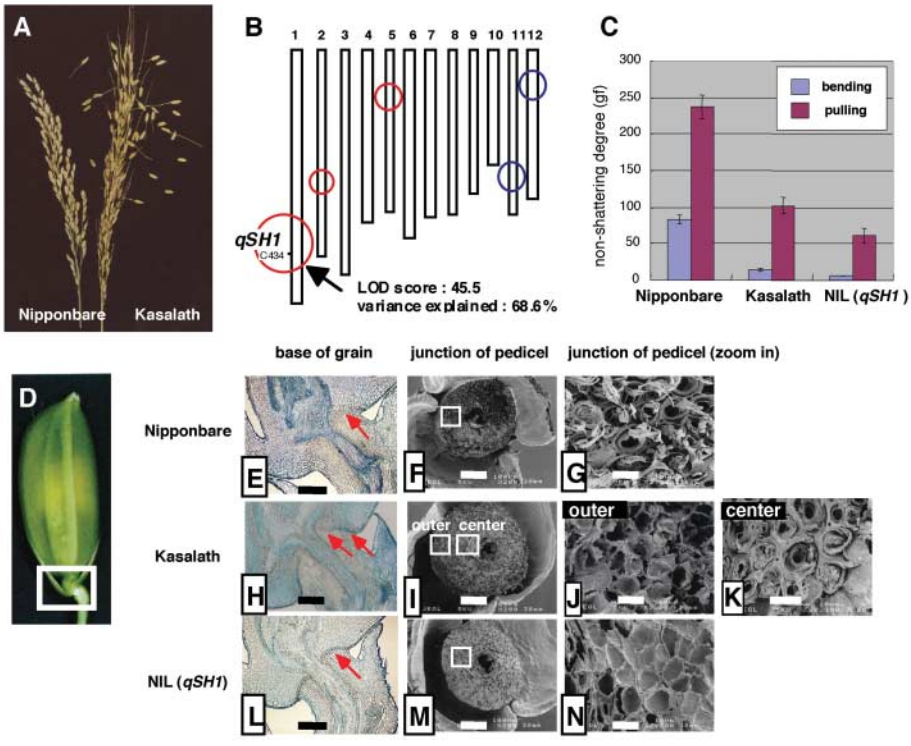

Fig. 1. *qSH1* is required for formation of the abscission layer at the base of the rice grain. (**A**) Seed-shattering habits of rice panicles. Photos taken after grabbing rice panicles. (Left) Nonshattering-type cultivar, Nipponbare. (Right) Shattering-type cultivar, Kasalath, in which the seed has shattered. (**B**) Chromosomal locations of QTLs for seed-shattering degree, based on an F_2 population from a cross between Nipponbare and Kasalath. Positions of circles indicate positions of QTLs, and circle size indicates the relative contribution of each QTL. Red circles, Nipponbare alleles contributing to non-shattering habit; blue circles, Kasalath alleles contributing to nonshattering. *qSH1* is marked on chromosome 1 with the nearest DNA marker (C434). (**C**) Non–seed-shattering habits of Nipponbare, Kasalath, and NIL(*qSH1*). Breaking tensile strength upon detachment of seeds from the pedicels by bending and pulling was measured (*10*). Increase in value indicates loss of shattering. NIL(*qSH1*), a nearly isogenic line carrying a Kasalath fragment at the *qSH1* locus in the Nipponbare background, as shown in fig. S1A. (**D**) Photo of a rice grain. White box indicates position of abscission layer formation. (**E** to **G**) Nipponbare. (**H** to **K**) Kasalath. (**L** to **N**) NIL(*qSH1*). (E), (H), and (L) Longitudinal sections of positions corresponding to white box in (D). Arrows point to the partial abscission layer of Kasalath in (H), the complete abscission layer of NIL(*qSH1*) in (L), and the corresponding region of Nipponbare in (E). (F), (I), and (M) Scanning electron microscope (SEM) photos of pedicel junctions after detachment of seeds. (G), (J), (K), and (N) Close-up SEM photos corresponding to white boxes in (F), (I), and (M). (G) Broken and rough surface of Nipponbare when forcedly detached. (N) Peeled-off and smooth surface of NIL(*qSH1*) upon spontaneous detachment. In Kasalath, rough center surface (K) and smooth outer surface (J) are observed. Scale bars: 500 μm in (E), (H), and (L); 100 μm in (F), (I), and (M); 10 μm in (G), (J), (K), and (N).

the most important agronomic traits in rice cultivation and breeding.

It is difficult to obtain shattering-related mutants and reveal the underlying molecular mechanisms, because most rice cultivars have somehow lost the seed-shattering habit. We therefore used natural variations in seed shattering among cultivars. Generally, *indica* cultivars exhibit relatively strong seed shattering, whereas some *japonica* cultivars do not exhibit it at all (Fig. 1A). We first performed a QTL (quantitative trait locus) analysis between a shattering-type *indica* cultivar, Kasalath, and a nonshattering-type *japonica* cultivar, Nipponbare. The seed-shattering degree (breaking tensile strength) of each grain was measured (*10*), and the average value was scored for QTL analysis.

Five QTLs were detected on five chromosomes of rice in an F_2 population of a cross between Kasalath and Nipponbare (Fig. 1B). Nipponbare alleles at three QTLs on chromosomes 1, 2, and 5, and Kasalath alleles at two other QTLs on chromosomes 11 and 12, all contributed to shattering reduction, suggesting that loss of seed shattering may occur independently in *japonica* and *indica*.

The QTL with the largest effect, termed *QTL of seed shattering in chromosome 1* (*qSH1*), explained 68.6% of the total phenotypic variation in the population (Fig. 1B). We therefore made a near-isogenic line (NIL) (fig. S1A) that contained a short chromosomal segment from Kasalath at the *qSH1* region in a Nipponbare genetic background. The NIL exhibited the formation of a complete abscission layer between pedicel and spikelet at the base of the rice seed (Fig. 1, D and L to N) and had a stronger seed-shattering phenotype than either Kasalath or Nipponbare (Fig. 1C). In contrast, no abscission layer was observed in Nipponbare at all (Fig. 1, E to G). This indicated that a mutation in the *qSH1* gene alone resulted in complete loss of the abscission layer in the Nipponbare genetic background and that the Kasalath allele of *qSH1* could rescue it. Kasalath could form a partial abscission layer only at the peripheries in the transverse plane (Fig. 1, H to K), perhaps because of the presence of the minor QTLs (Fig. 1B).

A large-scale linkage analysis of 10,388 plants segregating at the *qSH1* region (fig. S1, B and C) was performed for the fine mapping of *qSH1*. We finally succeeded in mapping the

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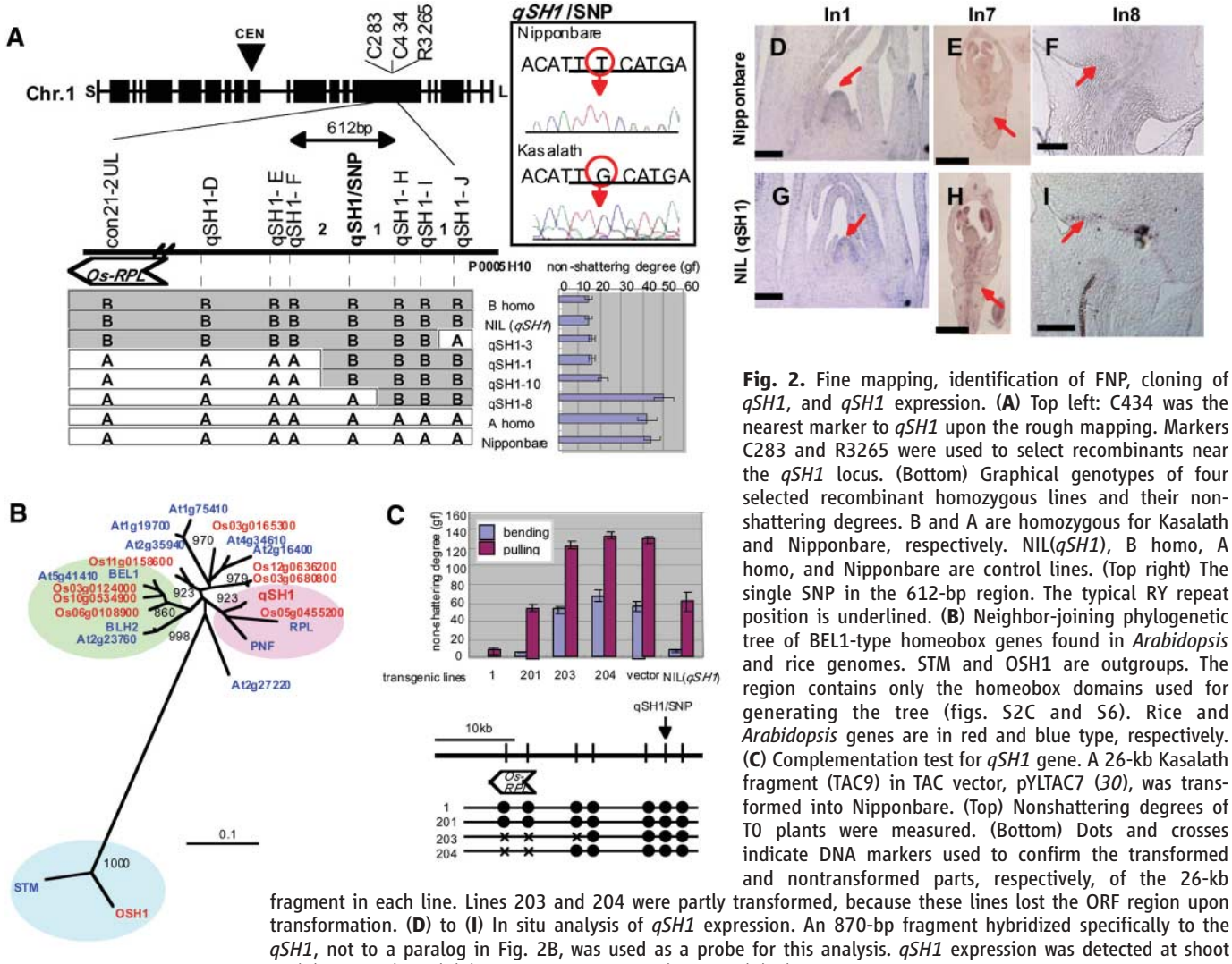
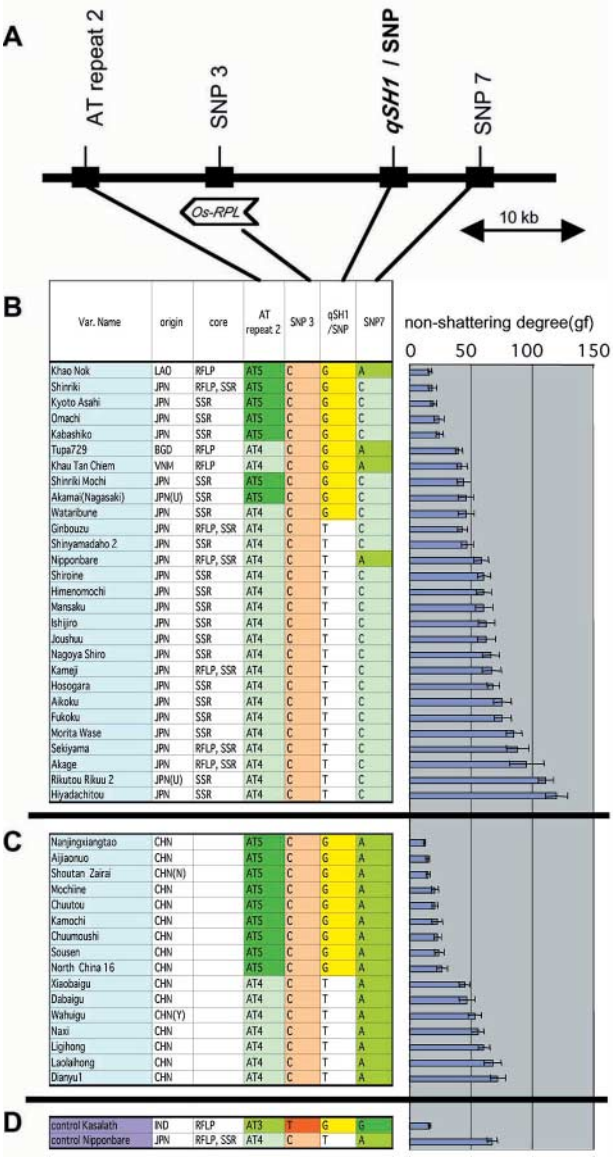


Fig. 2. Fine mapping, identification of FNP, cloning of *qSH1*, and *qSH1* expression. (**A**) Top left: C434 was the nearest marker to *qSH1* upon the rough mapping. Markers C283 and R3265 were used to select recombinants near the *qSH1* locus. (Bottom) Graphical genotypes of four selected recombinant homozygous lines and their non-shattering degrees. B and A are homozygous for Kasalath and Nipponbare, respectively. NIL(*qSH1*), B homo, A homo, and Nipponbare are control lines. (Top right) The single SNP in the 612-bp region. The typical RY repeat position is underlined. (**B**) Neighbor-joining phylogenetic tree of BEL1-type homeobox genes found in *Arabidopsis* and rice genomes. STM and OSH1 are outgroups. The region contains only the homeobox domains used for generating the tree (figs. S2C and S6). Rice and *Arabidopsis* genes are in red and blue type, respectively. (**C**) Complementation test for *qSH1* gene. A 26-kb Kasalath fragment (TAC9) in TAC vector, pYLTAC7 (*30*), was transformed into Nipponbare. (Top) Nonshattering degrees of T0 plants were measured. (Bottom) Dots and crosses indicate DNA markers used to confirm the transformed and nontransformed parts, respectively, of the 26-kb fragment in each line. Lines 203 and 204 were partly transformed, because these lines lost the ORF region upon transformation. (**D**) to (**I**) In situ analysis of *qSH1* expression. An 870-bp fragment hybridized specifically to the *qSH1*, not to a paralog in Fig. 2B, was used as a probe for this analysis. *qSH1* expression was detected at shoot apical meristems in both Nipponbare (D) and NIL(*qSH1*) (G) upon floral transition (stage In1) (*16*). At the flower-formation stage, *qSH1* expression was detected at the anther regions in both NIL(*qSH1*) and Nipponbare (E, H) and at the provisional abscission layer position only in NIL(*qSH1*) (H for stage In7, I for stage In8) and not in Nipponbare (E for stage In7, F for stage In8). Scale bars: 100 μm in (D), (F), (G), and (I); 200 μm in (E), and (H). Arrows point to the meristems in (D) and (G) and to the (provisional) abscission layers in (E), (F), (H), and (I).

functional natural variation in 612 bp between the flanking markers qSH1-F and qSH1-H and found only one single-nucleotide polymorphism (SNP) within this region (Fig. 2A). We confirmed this result using several recombinant homozygous plants in the progeny (Fig. 2A). Gene prediction for the *qSH1* region in both Nipponbare (*11*) and Kasalath genome sequences showed no distinct open reading frame (ORF) in the SNP region. However, located 12 kb away from the SNP, we found one ORF (locus ID Os01g0848400 in the Rice Annotation Project DataBase) for a rice ortholog of the *Arabidopsis REPLUMLESS* (*RPL*) (*12*, *13*) gene (Fig. 2B and fig. S2). The *RPL* gene encodes a BEL1-type homeobox (*14*, *15*) and is involved in the formation of a dehiscence zone (or abscission layer) alongside the valve in the *Arabidopsis* fruit (silique). Because the fruit originates from the carpels in *Arabidopsis*, the botanical origin of the dehiscence zone in *Arabidopsis* fruit does not correspond to that of the abscission layer in rice seeds. However, it was still possible that this *RPL* ortholog was the *qSH1* gene. To confirm this, we introduced ten 10- to 26-kb Kasalath genomic fragments scanning the predicted ORF and the SNP regions into the nonshattering Nipponbare cultivar (Fig. 2C and figs. S3 and S7). Only transgenic lines that contained the Kasalath fragment with both the ORF and the SNP exhibited complete seed shattering, although one fragment (termed sub51), which contained a full ORF region but not the SNP, partly complemented the phenotype (fig. S3). The other fragments were not able to complement it, even if they contained the entire ORF region or the SNP region. These results indicated that both the ORF and the SNP regions were required for full shattering function.

In situ hybridization analysis revealed that in the NIL the ORF was expressed at the inflorescence meristem in the stage of rachis meristem establishment [inflorescence stage 1 (In1)] (*16*) (Fig. 2G). It was also expressed at both the anther region and the provisional abscission layer at the base of the spikelet in the stage of floral organ differentiation (In7) (Fig. 2H) and in the stage of rapid elongation of the rachis and branches (In8) (Fig. 2I). The abscission layer was not yet observable in In7. On the other hand, in Nipponbare, the ORF was expressed in the same way as in the NIL (Fig. 2, D to F), except that it was not expressed at the provisional abscission layer in either In7 or In8 (Fig. 2, E and F).

These results, together with the complementation results, led us to conclude that this *RPL* ortholog was the *qSH1* gene and that the identified SNP affected only the spatial mRNA expression pattern of *qSH1* at the abscission layer. A quantitative RT-PCR for RNA samples of developing panicles supported this conclusion (fig. S8). Consistently, a *cis*-element search re-



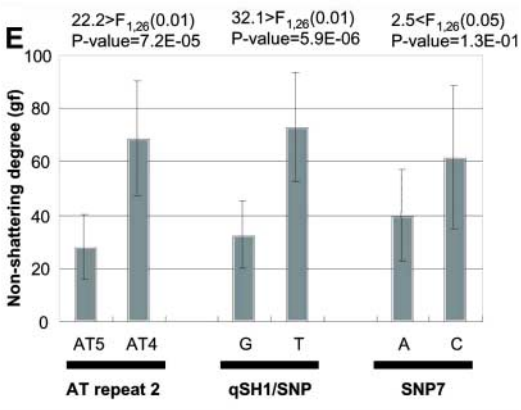


Fig. 3. Association of *qSH1* haplotypes with degree of shattering. (**A**) The four genomic regions with DNA polymorphisms at the *qSH1* locus are shown as thick black rectangles. *Japonica* has two subgroups, *tropical japonica* and *temperate japonica* (*28*). Only polymorphisms found in the population of *temperate japonica* cultivars are presented in (B), (C), and (D), with the exception of SNP3. SNP3 is present to show the lack of polymorphism at this site in all *japonica* cultivars tested, although the SNP3 found in Kasalath caused one amino acid change in *qSH1*, which was the sole amino acid change found between the ORFs of Nipponbare and Kasalath (fig. S5). SNP3 was not a target for human selection during rice cultivation. (**B**) Cultivars in *temperate japonica* core collections selected by genome-wide RFLP (*27*) and/or SSR (simple repeat sequence) analysis. U, upland-type cultivars. (**C**) *Temperate japonica* cultivars of Chinese origin. N and Y, North and Yunnan, respectively. These cultivars were assigned to *temperate japonica* by genome-wide RFLP analysis. No indications of RFLP and/or SSR in the core column mean the cultivars were not selected as core collections. (**D**) Nipponbare and Kasalath controls. At right in (B), (C), and (D), nonshattering degrees were also examined. (**E**) Statistical analysis of the association of seed shattering with genotype. ANOVA analysis was done with data shown in (B). AT repeat 2 and *qSH1*/SNP, but not SNP7, showed significant associations with seed shattering in *temperate japonica* cultivars. Standard errors are also shown in the graph.

vealed that the Kasalath *qSH1* allele contained a typical RY-repeat (*17*) that was a binding site of the *ABI3* (*VP1*)–type (*18*, *19*) transcription factor at the SNP site (Fig. 2A and fig. S4). Therefore, the change in the transcriptional control of key genes such as *RPL* and *qSH1* could explain the difference in abscission layer formation between rice and *Arabidopsis*. Several genes downstream of *RPL* have been identified, such as *SHP1/2* genes (*20*) belonging to the *AG*-clade MADS box genes in *Arabidopsis*. Phylogenetic analysis has revealed that *SHP* genes evolved after the eu-dicots separated from the common ancestors of eu-dicot and monocot plants (*21*), and all the *Arabidopsis AG*-clade MADS box genes are expressed in the carpel regions (*22*). In addition, it has been recently shown that two *AG* orthologs have evolutionally conserved functions with *AG* in rice (*23*). Thus, it is very likely that no functionally related ortholog of *SHP1/2* exists in the rice genome. Therefore, it is also possible that *qSH1* expression may lead to formation of the abscission layer at the base of the seed by a mechanism distinct from that of dehiscence zone formation in *Arabidopsis* fruit.

Hence, we believe that, like the *RPL* in *Arabidopsis* (*24*), *qSH1* may have pleiotropic functions in the spikelet development and plant architecture of rice, as well as in abscission layer formation. Therefore, null or severe mutations in the ORF may cause serious defects in rice development to establish it as a cultivar. This type of SNP, which caused loss of *qSH1* mRNA expression only at the abscission layer, could be survived during rice domestication. Similarly, it has been proposed that several maize domestication genes contain critical polymorphisms that have resulted from prehistoric artificial selection in the 5′ regulatory regions, although the functional nucleotide polymorphisms have not yet been identified (*25*, *26*).

To address how the SNP in *qSH1* prevailed during rice domestication, we next analyzed rice core collections (*27*) (Fig. 3, A and B). The results revealed that the SNP was highly associated with the degree of seed shattering among *temperate japonica* rice cultivars (a subgroup of *japonica*) (*28*) and implied that this SNP had been a target of artificial selection for nonshattering habit during rice domestication (Fig. 3E). All tested *indica* cultivars exhibited strong seed shattering; this result was consistent with the fact that they all contained the functional SNP (fig. S5C). Other QTLs need to be considered to explain the differences among *tropical japonica* cultivars (the other subgroup of *japonica*) (fig. S5D).

Rice cultivation likely started about 10,000 years ago; paddy-style rice cultivation is believed to have started in the Yangtze River region of China about 7000 years ago and to have been imported into Japan about 3000 years ago (*3*, *8*). We therefore analyzed rice cultivars of Chinese origin (Fig. 3C) and found that the nonshattering SNP at *qSH1* might have been used in ancient China 3,000 to 10,000 years ago, most likely about 7,000 years ago upon the establishment of paddy-style rice cultivation.

Crop domestication might have proceeded during relatively short periods (less than 10,000 years) through the occurrence of nucleotide polymorphisms, such as by spontaneous mutation, recombination, and fixation in populations. Because rice is a self-pollinated plant, such newly occurring nucleotide polymorphisms would have easily become fixed in individuals. If such individuals propagated and contributed to the establishment of cultivated rice, we should be able to follow step by step the haplotype changes that occurred during rice domestication. Therefore, we examined the haplotypes around the *qSH1* gene in the rice collections (Fig. 4A). The identified SNP was likely to be assigned as a mutation that occurred in early domesticates of *japonica* subspecies (Fig. 4B) but not as a preexisting natural variation. In the hypothetical process of evolution of *qSH1*, the SNP distribution clearly revealed a strong selection by ancient humans for the SNP during rice domestication (Fig. 4B). In addition, the

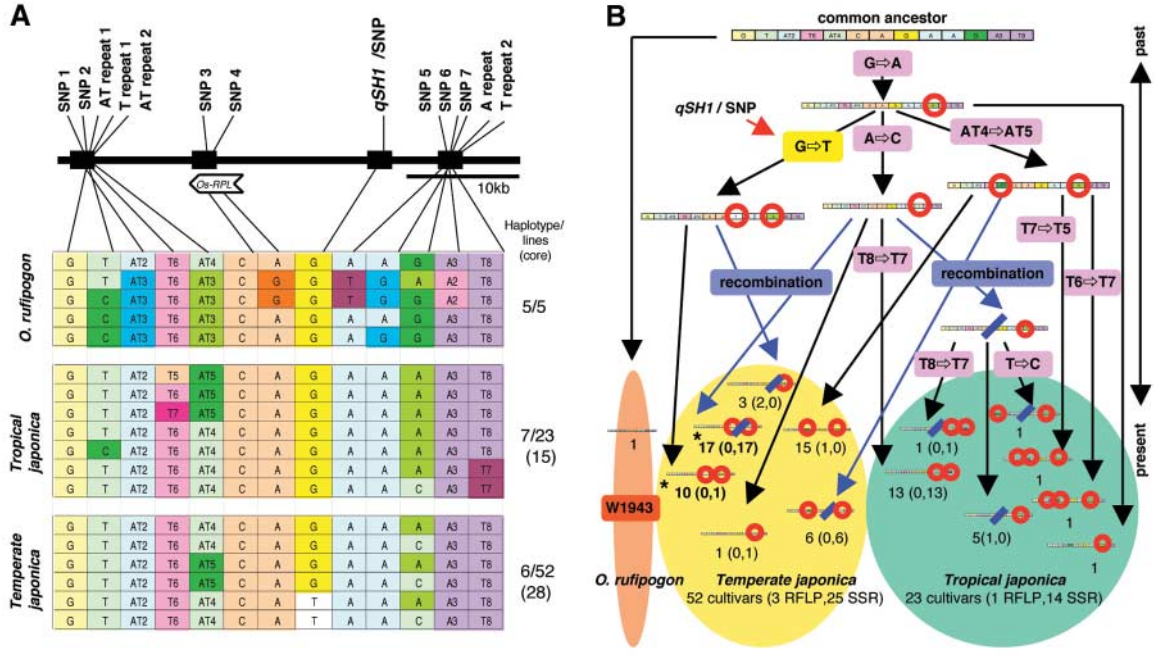


Fig. 4. Haplotypes at *qSH1* and hypothetical evolutionary process in *japonica*. (**A**) Eight SNPs (including the *qSH1*/SNP) and five SSRs from four genomic fragments were examined. On the basis of the results in 80 lines (including 43 cultivars from core collections), 18 haplotypes found at *qSH1* were presented (fig. S5). Results including the *indica* cultivars are shown only in fig. S5; we did not examine accurate haplotypes of some *indica* cultivars because of a lack of PCR amplification fragments. (**B**) Hypothetical process of evolution at *qSH1* during *japonica* rice domestication. Nine mutations and two recombination events are enough to explain the natural variations at *qSH1* in *japonica*. Among 75 cultivars tested (52 *temperate japonica* and 23 *tropical japonica*), 27 contained the nonshattering T allele at *qSH1*/SNP but represented only two haplotypes (asterisks) among 13 haplotypes found in the 75 cultivars, suggesting strong selection by humans during domestication. Numbers under haplotypes indicate corresponding numbers of rice accessions. Numbers of accessions in RFLP and SSR core collections, in that order, are indicated in parentheses. Red circles highlight the mutation position in the haplotypes.

estimated haplotype of the common ancestor at the *qSH1* locus was found in a wild rice accession, W1943 (Fig. 4B), which is closely related to the *japonica* subspecies (*6*). We could therefore follow how domestication proceeded at the level of DNA sequence change, from ancestors to cultivated rice. Many agronomic traits are related to domestication events and could have been the targets of artificial selection during domestication. Therefore, this type of evolutionary analysis may give us some insights into the domestication process and could reveal practical, useful allele information for future breeding in cereals (*29*). For instance, introgression of the Nipponbare *qSH1* allele into *indica* cultivars would reduce the seed-shattering degree and could improve yield.

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31. We thank T. Murakami for assistance with histology; T. Inoue and K. Tsuchihara for technical help with SEM; J. Kyozuka, T. Kurakawa, and A. Tagiri for assistance with in situ analysis; and K. Shu, F. Aota, and R. Matsuzaki for technical assistance. This work was supported in part by the Mutant Panel Project of the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan to M.Y. (MP1113a) and in part by the Diversified Genome Project of MAFF to S.K. and T.I. (GD2008). Data have been deposited into GenBank with accession numbers AB071330 to AB071333.

Supporting Online Material

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Materials and Methods
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References

21 February 2006; accepted 6 April 2006
Published online 13 April 2006;
10.1126/science.1126410
Include this information when citing this paper.

Outer Membrane Active Transport: Structure of the BtuB:TonB Complex

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In Gram-negative bacteria, the import of essential micronutrients across the outer membrane requires a transporter, an electrochemical gradient of protons across the inner membrane, and an inner membrane protein complex (ExbB, ExbD, TonB) that couples the proton-motive force to the outer membrane transporter. The inner membrane protein TonB binds directly to a conserved region, called the Ton-box, of the transporter. We solved the structure of the cobalamin transporter BtuB in complex with the C-terminal domain of TonB. In contrast to its conformations in the absence of TonB, the Ton-box forms a β strand that is recruited to the existing β sheet of TonB, which is consistent with a mechanical pulling model of transport.

In addition to an inner (plasma) membrane, Gram-negative bacteria have an outer membrane that affords additional environmental protection to the organism. Porins, which are β barrel proteins that typically function as diffusion pores, permit passive transport across the outer membrane of molecules with molecular weights ~600 daltons or less (*1*). However, bacteria, like other organisms, also require molecules that are larger and/or are present in the extracellular milieu at low concentration. Specifically, Gram-negative bacteria require iron, which is often taken up in the form of iron-siderophore complexes (*2*), as well as other organometallic compounds such as cobalamins (e.g., cyanocobalamin, vitamin B_{12}) (*3*). Because a reduction of iron uptake correlates with a decrease in bacterial virulence (*4*), these transport systems are an attractive target for antibacterial drug discovery.

The uptake of scarce nutrients across the outer membrane is performed by a specialized active transport system that requires three components: specialized outer membrane transport proteins, an inner membrane multiprotein complex, and the inner membrane proton-motive force (pmf) to drive active transport (*5*). The outer membrane transporters have a common architecture of a 22-stranded β barrel situated in the membrane, long extracellular loops, short periplasmic turns, and a distinctive luminal domain (*6*). This luminal domain, composed of the N-terminal portion of the transporter, forms a globular-like domain that occludes the barrel. The inner membrane protein complex consists of the proteins ExbB, ExbD, and TonB. ExbB and ExbD are homologous to the MotA and MotB "stator" proteins of the bacterial flagellar motor (*7*). The protein TonB—which has a single putative transmembrane helix, a proline-rich linker region, and a periplasmic C-terminal domain—couples the inner membrane pmf to the outer membrane transporter. Multiple structures of the mixed α helical/β sheet C-terminal domain of TonB have been determined (*8*). TonB-dependent outer membrane transporters have a conserved motif, the Ton-box (*9*, *10*), that interacts with TonB during the active transport cycle. Deletion (or certain mutations) of the Ton-box abrogate transport but do not affect substrate binding (*3*). The molecular mechanism of TonB-dependent outer membrane active transport is not known. Conformational change of the luminal domain to open a permeation path for substrate must occur, but whether the domain remains within the barrel or undergoes partial or full removal is not known. The presence of protein components in both bacterial membranes is suggestive of an "action-at-a-distance" mechanical pulling model, but compelling experimental evidence is lacking. In order to obtain additional information on the nature of TonB-dependent outer membrane active transport, we solved the structure of the C-terminal domain of TonB in complex with the cobalamin transporter BtuB.

BtuB and a C-terminal domain of TonB (residues 147 to 239) from *Escherichia coli*, were separately expressed and purified; the complex was made by combining BtuB and TonB in a molar ratio of ~1:5 in the presence of the substrate cyanocobalamin (vitamin B_{12}) and excess calcium. The structure was solved to 2.1 Å by molecular replacement using the structure of substrate-bound BtuB [Protein Data Bank (PDB) accession number 1NQH] (*11*) as the

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