

A metastable *DWARF1* epigenetic mutant affecting plant stature in rice

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Epigenetic mutations confer heritable changes in gene expression that are not due to changes in the underlying sequence of the DNA. We identified a spontaneous rice mutant, *Epi-d1*, that shows a metastable dwarf phenotype. The phenotype is mitotically and meiotically inheritable and corresponds to the metastable epigenetic silencing of the *DWARF1* (*D1*) gene. The silenced state is correlated with repressive histone and DNA methylation marks in the *D1* promoter region but is not associated with DNA sequence alterations. Compared with other known epigenetic silenced loci in plants such as paramutable maize alleles and silent *Arabidopsis* genes, the *Epi-d1* silencing phenomenon shows a high level of bidirectional metastable mutability. Epigenetic alleles such as *Epi-d1* could thus provide for rapid adaptation under selective conditions.

Epigenetic alleles are defined as those carrying heritable differences in gene expression that are not due to changes in the underlying sequence of the DNA (1). Although epigenetic changes in gene expression are likely to play an important role in evolution, very few epigenetic alleles have thus far been described. In plants, a well-described set of epialleles exist at maize loci that undergo paramutation. Paramutation is defined as an interaction between 2 alleles of a single locus, resulting in a heritable change of one allele that is induced by the other allele (2). Recent studies have suggested that small RNA-mediated gene silencing is important in the maintenance and establishment of the repressed state in paramutation (3, 4). Other epigenetic alleles have been studied in the model plant *Arabidopsis thaliana*, including at the *SUPERMAN*, *PAI*, *FWA*, and *BONSAI* loci, and studies of these have contributed to our understanding of gene-silencing mechanisms (5–10).

In this study, we report genetic analysis of a rice epigenetic mutant that affects plant stature. This mutant, named *Epi-d1* shows a metastable dwarf phenotype. It was originally isolated as spontaneous dwarf mutant and has been maintained as a breeding material in Kyusyu University >90 years. As a result of genetic analysis on its metastability, we determined the target gene for epigenetic regulation and found that its epigenetic state is bidirectionally mutable, from active to repressed and from repressed to active. *Epi-d1* is an epiallele described in rice and also has unique epigenetic characteristics.

Results

Character of *Epi-d1* Mutant. The *Epi-d1* mutant is often chimeric, producing both dwarf and normal tillers (vegetative branch shoots) on the same plant (Fig. 1A). Dwarf tillers have short dark-green leaves, compact panicles (inflorescences), and small round grains. On the other hand, the normal tillers have normal features (Fig. 1A). In addition, different *Epi-d1* plants show a wide variety of dwarf and normal features, from completely dwarf to completely normal (Fig. 1B). This strongly suggests that the cell fate to be dwarf or normal type is not genetically fixed but can vary epigenetically in the developing plant. The completely dwarf and normal type plants were named *Epi-dwarf* and *Epi-normal*, respectively (Fig. 1B Left and Right plants). Al-

though most dwarf tillers have small round grains, and most normal tillers have normal grains, we can find chimeric features also in the panicles (Fig. 1C). These results indicate that cell type (normal or dwarf) frequently changes from the vegetative to reproductive stage.

To clarify the epigenetic regulation of *Epi-d1*, phenotypic segregation ratios were determined for the progenies of independent chimeric *Epi-d1* plants [supporting information (SI) Table S1]. Although we always found a range of phenotypes in the progeny, we observed a strong tendency for seeds collected from normal tillers to give rise to a higher proportion of normal plants and seeds collected from dwarf tillers to give rise to more dwarf plants. As one example, the progeny from normal tillers of chimeric plant 1 segregated 44 normal, 127 chimeric, and 16 dwarf plants, whereas the progeny from the dwarf tillers on this same plant produced 3 normal, 44 chimeric, and 59 dwarf plants (Table S1). We also observed a tendency for small seeds of dwarf tillers to produce a higher proportion of dwarf plants, and normal seed in normal tillers to give rise to a higher proportion of normal plants. We also studied the progenies of 4 independent *Epi-normal* plants and 4 *Epi-dwarf* plants (Table S2). The progenies from *Epi-normal* had 86.2% normal, 9.3% chimeric, and 4.5% dwarf phenotypes (Table S2). In contrast, the progenies from *Epi-dwarf* had 0.8% normal, 26.6% chimeric, and 72.6% dwarf phenotypes (Table S2). Together, these results show that although the *Epi-d1* phenotypes are strongly heritable, the phenotypic transmission is metastable, and the conversion between the 2 phenotypic states occurs bidirectionally.

We next tested whether *Epi-d1* showed allelic interactions similar to those of known maize epialleles that undergo paramutation. We first performed reciprocal crosses between *Epi-normal* and *Epi-dwarf* plants. All 7 F₁ plants had the normal phenotype. In the F₂ generation, the progenies of both reciprocal crosses produced normal and dwarf plants in a ratio of ≈3:1 (Table S3). This suggests that the alleles of *Epi-normal* and *Epi-dwarf* do not affect each other and are thus not paramutable. These results also show that *Epi-normal* is dominant, and *Epi-dwarf* is a recessive allele. To confirm these results, *Epi-dwarf* plants were further reciprocally crossed with 2 wild-type rice cultivars, IR24 (an *indica* cultivar) and Taichung65 (a *japonica* cultivar). The F₂ progenies of these crosses also segregated in a ratio of 3:1 (Table S3), suggesting that the *Epi-dwarf* allele is recessive and that the *Epi-dwarf* allele does not significantly affect wild-type alleles. These genetic analyses suggest that the metastable phenomenon of *Epi-d1* is different from paramutation.

To understand the molecular mechanism of *Epi-d1*, we performed mapping analysis using 16,000 F₂ plants from crosses

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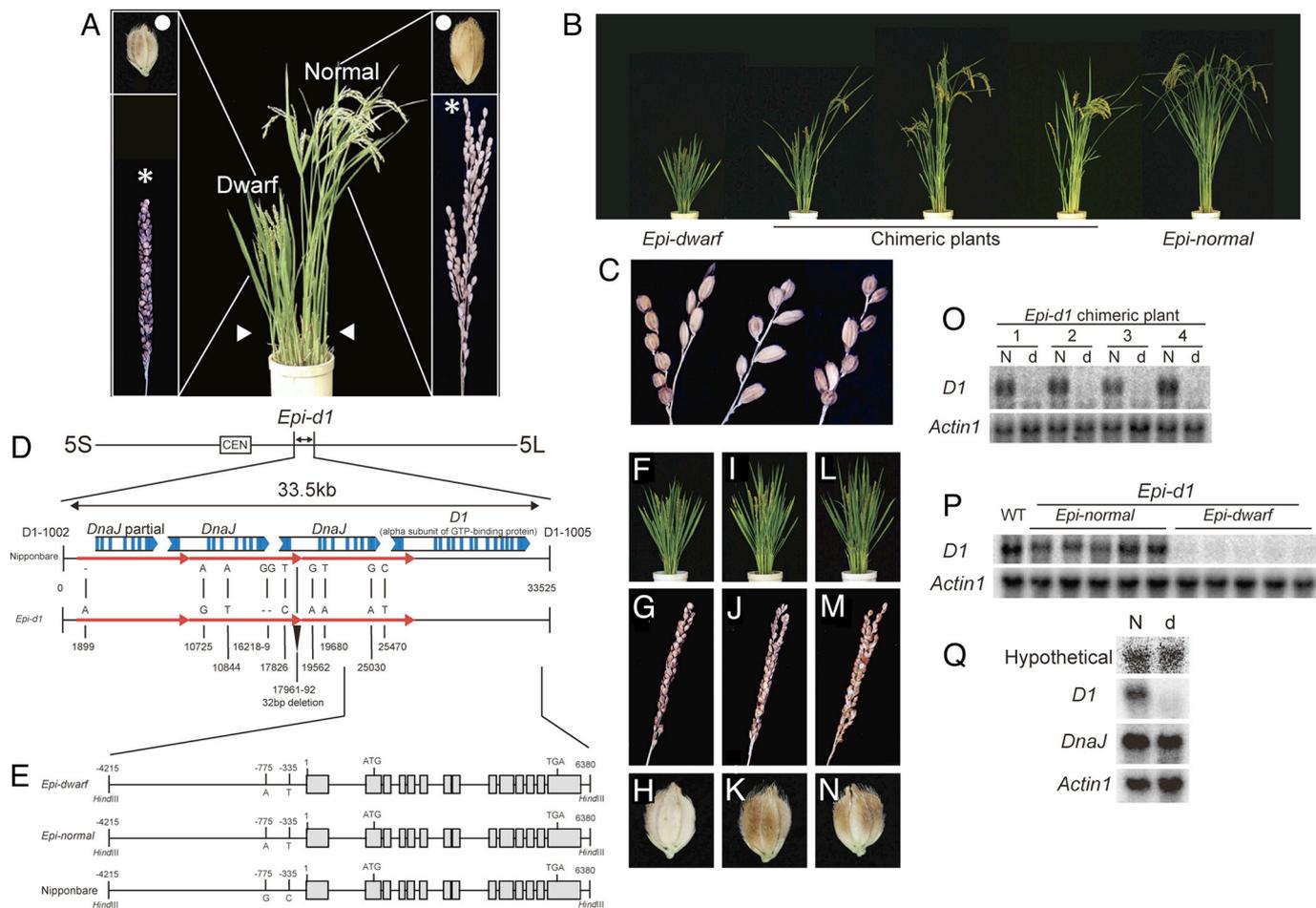


Fig. 1. Mapping analysis of *Epi-d1*. (A) Gross morphology of *Epi-d1* mutant (Center) and magnified view of its dwarf panicle and seed in dwarf tillers (Left) and normal panicle and seed in normal tillers (Right). Arrowheads indicate tillers (vegetative branch shoots), asterisks indicate panicles (inflorescences), and white circles indicate grains. (B) *Epi-d1* shows varying phenotypes from completely dwarf to completely normal. (C) Chimeric phenotype in panicle. A panicle showing 2 separate rachises with either small round or normal grains (Left). A panicle showing small round and normal grains arranged alternately on the rachis (Center). A panicle showing a random distribution of the 2 grain phenotypes (Right). (D) *Epi-d1* was mapped into 59 cM of chromosome 5, then narrowed down to a 33.5-kb region between 2 molecular markers, D1-1002 and D1-1005. In this region, there are 3 8.6-kb tandem repeats (red arrows) and 4 ORFs are predicted by RiceGAAS. There are 11 polymorphisms in this 33.5-kb region between Nipponbare and *Epi-d1* mutant. The numbers indicate the positions of polymorphisms in this region. (E) Comparison of sequence of *D1* gene between *Epi-normal*, *Epi-dwarf*, and Nipponbare. (F–H) The phenotypes of *d1* loss-of-function mutant are shown. (I–K) The phenotypes of *Epi-dwarf* plant are shown. (L–N) The phenotypes of *F*₁ plant derived from cross between *Epi-dwarf* and *d1* are shown. The gross morphologies are shown in F, I, and L; panicles in G, J, and M, and seeds in H, K, and N. (O) Total RNAs were extracted from 4 independent chimeric plants by separating tissues with different phenotypes. RNA blots were hybridized with *D1* probe. N and d indicate RNA extracted from normal and dwarf tillers, respectively. (P) Total RNAs were extracted from *Epi-normal* and *Epi-dwarf* plants and hybridized with *D1* probe. (Q) Total RNAs were extracted from *Epi-normal* and *Epi-dwarf* plants and hybridized with probes of *D1* and its neighbor genes. Hypothetical and *DnaJ* gene are located 7 kb downstream and 4 kb upstream of the *D1* gene, respectively. N and d indicate RNA extracted from *Epi-normal* and *Epi-dwarf*, respectively. *Actin1* is hybridized as a control (O–Q).

between *Epi-d1* and Kasalath (an *indica* rice cultivar). This mapping revealed that the *Epi-d1* gene was located between molecular markers D1-1002 and D1-1005 within a 33.5-kb region of rice chromosome 5 (Fig. 1D). This candidate region contains 3 8.6-kb tandem repeats, and 4 ORFs encoding 3 *DnaJ* genes and the *DWARF1* (*D1*) gene, encoding the α -subunit of a GTP-binding protein (Fig. 1D). The recessive loss-of-function of *dwarf1* (*d1*) shows a dwarf phenotype with short dark-green leaves, compact panicles, and small round grains (Fig. 1F–H) (11, 12). The dwarf characters of *Epi-d1* are very similar to that of *d1* (Fig. 1I–K). From the phenotypic resemblance and mapping analysis, we suspected that the dwarf phenotype of *Epi-d1* would be caused by defects in the *D1* gene. To clarify this, we tested allelism between *Epi-dwarf* and *d1* using complementation crosses. Of 150 *F*₁ plants; 6 showed a chimeric dwarf phenotype, and 144 showed a full dwarf phenotype (Table S4 and Fig. 1L–N). This strongly indicates that the *Epi-dwarf* is allelic to *d1*.

Metastable Phenotype of *Epi-d1* Is Due to Metastable Expression of *D1* Gene. Next, we compared sequences of the *D1* gene among *Epi-normal*, *Epi-dwarf*, and dwarf and normal tillers in a chimeric plant. Sequencing of the *D1* locus between –4,215 bp relative to the transcription initiation site showed that there were no differences in the *Epi-normal* and *Epi-dwarf* DNA sequence (Fig. 1E). In addition, we compared the *D1* sequences between *Epi-d1* and Nipponbare (a *japonica* rice cultivar). Although 2 SNPs were detected in the promoter region (Fig. 1E), no mutation was found in the *D1* coding region of *Epi-d1* (Fig. 1E). To determine whether these SNPs cause the metastable phenotype of *Epi-d1*, we transformed 3 versions of the 10.5-kb *D1* locus corresponding to the *Epi-dwarf*, *Epi-normal*, and Nipponbare alleles into a *d1* loss-of-function mutant (Fig. 1E). Transformants of all 3 constructs showed a fully normal phenotype (Fig. S1). This indicates that the *D1* gene of *Epi-d1* encodes a functional allele and that the 2 SNPs detected in the

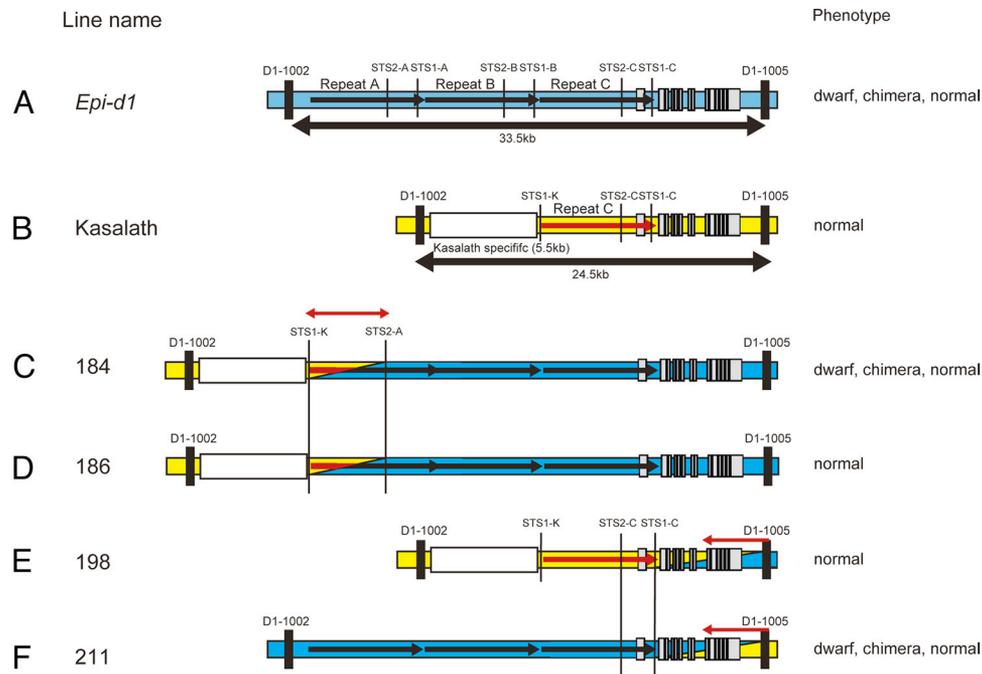


Fig. 2. Graphical genotypes of recombinant plants. Blue and yellow bars indicate chromosome of *Epi-d1* and Kasalath respectively. (A) Graphical genotype of *Epi-d1*. (B) Graphical genotype of Kasalath. (C–F) Graphical genotypes of recombinant lines 184, 186, 198, and 211 are shown. The phenotypes of these lines are shown on the right of the chromosome.

upstream region (–775 and –335) of *D1* are not mutations controlling the metastable phenotype of *Epi-d1*. We also investigated whether a wild-type *D1* locus could complement the dwarf phenotype of *Epi-dwarf*. We transformed the *D1* gene cloned from Nipponbare into the *Epi-dwarf* plants and found that its dwarf phenotypes were fully rescued (Fig. S1). These results strongly confirm that the defect in *Epi-d1* is due to a loss of function of the *D1* gene. Finally, we sequenced the 33.5-kb candidate region defined by our recombination mapping. Although there were several polymorphisms between *Epi-d1* and Nipponbare (Fig. 1D and Fig. S2), when we transformed the fragment containing the 33.5-kb candidate region cloned from *Epi-dwarf* into a *d1* mutant, the transformants also completely rescued the *d1* phenotype (Fig. S1). These results suggest that *Epi-d1* contains a potentially functional DNA sequence for the *D1* gene but that the *D1* gene in *Epi-dwarf* plants is not active.

To determine the cause of the metastable phenotype of *Epi-d1*, we performed expression analysis of the *D1* gene. Northern blot analysis revealed dramatically different expression of *D1* in *Epi-d1*. Although *D1* was highly expressed in normal tillers of chimeric plants, the *D1* transcript was not detected in dwarf tillers of these same plants (Fig. 1O). We also examined *D1* expression in *Epi-dwarf* and *Epi-normal* plants and found that the *D1* gene was highly expressed in *Epi-normal* but not in *Epi-dwarf* (Fig. 1P). These results indicate that the epigenetic phenomenon in *Epi-d1* is due to the switching on or off of RNA expression of *D1*. We conclude that the *Epi-d1* mutant is an epigenetic allele of the *D1* locus that we have named Epiallele of *d1* (*Epi-d1*).

To determine whether the difference in expression in *Epi-d1* occurs in a narrow or broad genomic region around *D1*, we investigated transcripts of genes located around the *D1* gene in *Epi-d1*. The transcript level of *DnaJ* located 4 kb upstream of *D1* and a hypothetical gene located 7 kb downstream of *D1* were equal between dwarf tillers and normal tillers (Fig. 1Q). These results suggest that epigenetic regulation in *Epi-d1* only affects the *D1* gene. Finally, to determine whether the *D1* locus,

narrowed down by mapping analysis, is sufficient for the metastable expression of *D1*, we used introgression to produce a nearly isogenic line (NIL) carrying only the *D1* locus of the *Epi-d1* chromosome segment in an otherwise Kasalath genome background (Fig. S3a). Resultant NIL-*Epi-d1* shows dwarf, chimeric, and normal phenotypes similar to the original *Epi-d1* plants (Fig. S3b). This indicates that the *D1* locus of *Epi-d1* is sufficient to confer the epigenetic *D1* expression instability.

During the mapping analysis, we obtained recombinant plants containing recombination breakpoints within the *D1* gene or upstream repeat region (Fig. 2 C–F). In these recombinant plants, lines 184 and 186 have a recombination point between the markers STS1-K and STS2-A on repeat A (Fig. 2 C and D). Although line 184 shows 3 kinds of phenotypes, dwarf, chimera, and normal phenotypes, line 186 shows only normal phenotype. These lines indicate the candidate region of metastability is located within repeat A. On the other hand, lines 198 and 211 have a recombination point between the markers, STS1-C and D1-1005 (Fig. 2 E and F). Because line 198 shows only normal phenotype, and line 211 shows dwarf, chimera, and normal phenotypes, the candidate epigenetic region of *Epi-d1* is located upstream from the marker D1-1005, which is also consistent with the interpretation from lines 184 and 186 that the metastability element maps to repeat A. From these results, repeat A seems to be required for phenotypic metastability.

Metastable *D1* Expression Is Caused by Epigenetic Modification. To determine the possible epigenetic marks that regulate the expression state of the *D1* gene, we analyzed DNA methylation and histone modification. In the continuous selection of *Epi-dwarf* plants from *Epi-dwarf* over several generations, we could obtain *Epi-dwarf* line 8 and 20, which rarely show normal tissues. We treated seeds derived from these *Epi-dwarf* lines with 5-aza-2'-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation (13). Although seeds not treated with 5-aza-dC showed no chimeric or normal plants, the 5-aza-dC-treated seeds produced 12–53% chimeric plants (Fig. 3A). This suggests that DNA demethylation

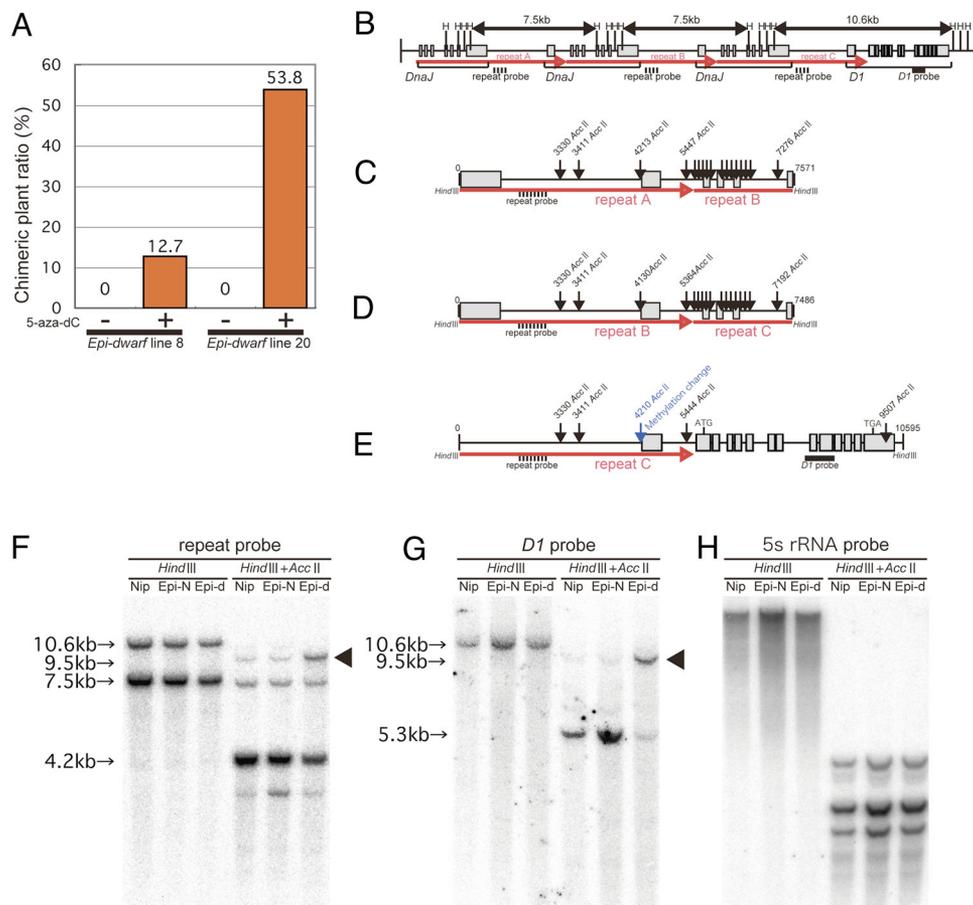


Fig. 3. DNA methylation analysis of *Epi-d1*. (A) The appearance of chimeric plants from *Epi-dwarf* seeds treated with inhibitor of DNA methylation, 5-aza-2'-deoxycytidine. Values in the graph indicate the ratio of chimeric plants from *Epi-dwarf* (percentage). (B) Restriction map of the candidate region of *Epi-d1* gene. HindIII sites are described as H. (C and D) The restriction map of first and second 7.5-kb region digested by 2 HindIII sites. (E) The restriction map of 10.6-kb region contains the *D1* gene. (F and G) The probes used are indicated as broken line and simple line, respectively. (C–E) Arrows indicate the sites of DNA methylation sensitive restriction enzyme, AccII (CGCG). (F–H) The genomic DNA of Nipponbare (Nip), *Epi-normal* (Epi-N), and *Epi-dwarf* (Epi-d) were digested by HindIII or double digested by HindIII and AccII. Digested DNAs were hybridized with repeat probe (F) and *D1* probe (G). 5s rRNA probe was also hybridized as a control (H).

promotes the transition from dwarf to normal in *Epi-d1* plants. Next, we investigated the DNA methylation level around the *D1* locus. When the genomic DNAs were double digested by HindIII and AccII (a methylated DNA-sensitive restriction enzyme that recognizes CGCG but cannot digest methylated CGCG), Nipponbare and normal tillers in *Epi-d1* had a 5.3-kb band, whereas a 9.5-kb band was observed in dwarf tillers in *Epi-d1* (Fig. 3G). When 5s rRNA and 168bp centromere repeat were hybridized as a probe, there were no difference in Nipponbare and dwarf and normal tillers of *Epi-d1* (Fig. 3H and Fig. S4), suggesting that these changes in DNA methylation are specific to the *D1* locus. There are 5 AccII sites in the 10.6-kb genomic DNA region between the 2 HindIII sites (Fig. 3E). The detection of a 5.3-kb signal in normal plants indicates that the DNA fragment was digested at the 4210 AccII site and the 9507 AccII site (Fig. 3E). The detection of a 9.5-kb signal in dwarf tillers indicates that the DNA fragment was digested with the 0 HindIII site and the 9507 AccII site (Fig. 3E and G). The methylation status at the 4210 AccII site therefore differs between dwarf and normal tillers (Fig. 3E). The 4210 AccII site is at position -2 bp relative to the *D1* transcription initiation site, and thus may be in a region critical for *D1* promoter activity. DNA methylation of the upstream repeat region was also examined by using a repeat probe for analysis (Fig. 3B–D). Southern hybridization using this probe showed 2 bands with HindIII, 10.6 kb and 7.5 kb (Fig. 3F). The 10.6-kb fragment contains the *D1* gene, and the 7.5-kb

fragment corresponds to both repeat A and B (Fig. 3B–D). When the genomic DNA was double digested by HindIII and AccII, a 9.5-kb band was enriched in dwarf tillers by using the repeat probe (Fig. 3F), again consistent with hypermethylation of the 4210 AccII site. Consistently, a 4.2-kb band was strongly detected in all plants, although it was weaker in dwarf plants than in normal plants (Fig. 3F), consistent with hypermethylation of the 4210 AccII site. The observation that the 4.2-kb band was present in all genotypes (Fig. 3F), indicates that the 3330 and 3411 AccII sites in both 7.5-kb fragments from junctions between repeat A to B and between repeat B to C (Fig. 3C and D) are consistently hypermethylated. These results suggest that variation in methylation at the AccII site in the promoter of the *D1* gene strongly correlate with *D1* silencing.

To investigate the relationship between metastable *D1* expression and chromatin modification, we performed ChIP analysis using acetylated K9 of histone H3 (Ac-H3K9), a mark usually associated with active genes, and dimethylated K9 of histone H3 (dime-H3K9), a mark usually associated with silent genes (14). We could successfully use primers designed over the *D1* gene (Fig. 4A); however, because of the highly conserved repeat sequence, we could not design primers specific for the upstream region of *D1*. We detected accumulation of Ac-H3K9 and reduced dime-H3K9 at the *D1* locus in *Epi-normal* (Fig. 4B). Conversely, accumulation of dime-H3K9 and reduction of Ac-H3K9 were detected at the *D1* locus in *Epi-dwarf* (Fig. 4B). These

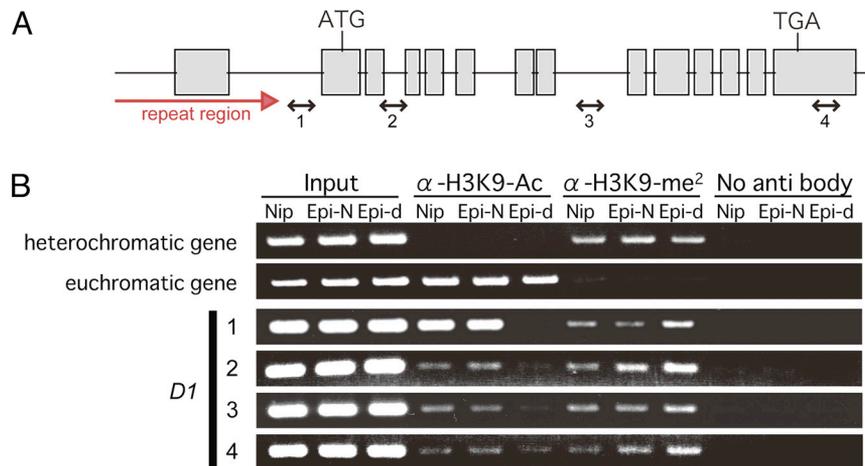


Fig. 4. ChIP assay of *D1* gene. (A, 1–4) The regions amplified to detect the chromatin state are indicated as arrows. (B) Nucleosomes were precipitated by anti-acetylated histone H3K9 (α -H3K9-Ac) and anti-dimethylated histone H3K9 (α -H3K9-me²). Input and no antibody were also amplified as positive and negative controls, respectively. The precipitated nucleosomes of Nipponbare (Nip), *Epi-normal* (Epi-N), and *Epi-dwarf* (Epi-d) were amplified by using primers of heterochromatic gene, euchromatic gene, and *D1* gene.

results are consistent with the expression pattern and DNA methylation of *D1*, and suggest that repressive chromatin marks are correlated with the silent *Epi-d1* state.

Discussion

In summary, *Epi-d1* represents a metastable epigenetic mutant of the *D1* locus that is associated with DNA methylation and repressive histone marks. *Epi-d1* is not only an epigenetic allele described in rice but also exhibits unique qualities compared with previously described epigenetic loci. Given that both the silent and active state of *D1* show metastability, and given that allelic interactions are not observed between alleles in these different expression states, the *Epi-d1* phenomenon is unlike paramutation. Furthermore, we did not observe allelic interactions between the silent *Epi-d1* alleles and incoming transgenic alleles (Fig. S1). This is different from *Arabidopsis* epigenetic alleles of the *FWA* locus, where silencing can be transmitted from the silent endogenous locus to cause efficient silencing of incoming *FWA* transgenes (15). Most strikingly, the transcriptional initiation site of *D1* was differentially hypermethylated in *Epi-d1*, even though identical sequences in the upstream repeats did not show methylation variation between Nipponbare, *Epi-normal*, and *Epi-dwarf* epialleles (Fig. 3). Thus, the factor(s) repressing *D1* expression in *Epi-dwarf* do not affect DNA sequences sharing homology with the target of repression. These observations suggest that *Epi-d1* silencing is a uniquely *cis*-limited epigenetic phenomenon, which may be controlled by unique epigenetic mechanisms. Finally, the observations that very stable *Epi-dwarf* plants can be selected over multiple generations suggests that epigenetic phenotypes such as *Epi-d1* could provide plants with a repertoire of quickly selectable traits that change much more quickly than those caused by difference in DNA sequence.

Materials and Methods

Mapping and NIL Production. To map *Epi-d1*, F₂ plants from cross between *Epi-dwarf* and the *indica* strain Kasalath were used. \approx 16,000 F₂ plants from this cross were used for positional cloning of *Epi-d1*. To create NILs of *Epi-d1*, F₁ plants (*Epi-dwarf*/Kasalath) were back-crossed with Kasalath 4 times. NIL were selected from the BC₄F₂ generation (i.e., *Epi-dwarf*/Kasalath/Kasalath/Kasalath/Kasalath/Kasalath) by using MAS (16, 17). The phenotypes of all plants were determined after grains ripened. Genomic DNA was extracted from each F₂ population and NIL by using the TPS method for mapping and NIL production. For the TPS method, \approx 2-cm lengths of rice leaf tips were harvested and ground by using a MultiBead Shocker (Yasui Kikai) in TPS buffer [100 mM Tris-HCl (pH 8.0), 1 M KCl, 10 mM EDTA]. After centrifugation, the supernatant

was recovered, and an equal volume of isopropyl alcohol was added. Isopropyl alcohol-insoluble material was recovered by centrifugation, and the pellet was washed with 75% ethanol. The pellet was then dried and dissolved in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. The purified DNA samples were then genotyped by using molecular markers. PCR-based markers, including simple-sequence repeat (SSR) markers (www.gramene.org/) (18, 19), cleaved amplified polymorphic sequence (CAPS) markers (20), and SNPs, which were identified by comparing the genomic DNA sequences of each parent, were used for mapping and NIL production.

Transgenic Analysis. A construct containing the *D1* gene of Nipponbare or *Epi-d1* was introduced to a *d1* loss-of-function mutant line, HO541, and *Epi-dwarf*. The *D1* gene in the BAC clone constructed from Nipponbare genomic DNA, B022G02, and from *Epi-d1* genomic DNA, DC15F12 (constructed by the National Institute of Agrobiological Sciences) were digested with HindIII and inserted into the HindIII site of a pBluescript vector. The clone was redigested with HindIII. The fragment of \approx 10.6 kb was fused into the HindIII site of the binary vector pBI-Hm12, which contains a hygromycin-resistance gene, kindly provided by Hiroyuki Hirano (Tokyo University, Tokyo). The 33.5-kb candidate *D1* locus was constructed from DC15F12 DNA partially digested by BamHI and cloned into BamHI site of binary vector pYLTA7 (provided by RIKEN BioResource Center, Ibaraki, Japan). The binary vector was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation, and rice plants were transformed with this strain.

Construction of a BAC Clone and Sequencing. To determine the sequence of the candidate region of *Epi-d1*, a rice BAC library was constructed from young leaves of *Epi-d1* based on the protocol reported by the Arizona Genomics Institute (www.genome.arizona.edu/information/protocols/index.html). The library (host: *Escherichia coli* strain DH10B; vector: plndigoBAC5; cloning site: BamHI) contains 19,439 clones with an average insert size of 157 kb, which is estimated to cover the haploid genome \approx 7 times. In total, 5 markers (d1–8, d1–17, D1-exon9–10, d1–11, and d1–12) were applied for screening of BAC clones with the PCR method using pooled DNA from the whole BAC library. Each positive BAC clone was used for end-sequencing using an ABI 3730xl DNA Analyzer (Applied Biosystems) according to a previously described method (23).

RNA Extraction and Expression Analysis. Total RNAs (10 μ g) isolated from leaves by using the TRIzol reagent (Invitrogen) were separated on a 1% agarose gel and transferred to Hybond *n* + membranes (Amersham Pharmacia). The membrane was hybridized with a ³²P-labeled partial *D1* cDNA fragment of 929 bp, which was amplified by using the primers D1-cDNA-probe-U: 5'-TTTGATGAGGCAGAACTTAG-3' and D1-cDNA-probe-L: 5'-AGCGTCTCAT-GCTCTCATC-3', which correspond to regions in exon 5 and the 3' untranslated region, respectively. The probes were ³²P-labeled by using the Bca BEST Labeling Kit (Takara). The membrane was hybridized at 65 °C in a hybridization solution of 5 \times SSC (1 \times SSC = 0.15 M NaCl and 0.015 M sodium citrate), 5 \times Denhardt's solution (1 \times Denhardt's solution = 0.02% ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 0.5% SDS, 10% dextran sulfate, and 0.1

mg/mL denatured salmon sperm DNA. DNA probes were labeled with ^{32}P -dCTP. The membrane was washed twice with $2\times$ SSC and 0.1% SDS at 65 °C for 20 min and once with $0.2\times$ SSC and 0.1% SDS at 65 °C for 10 min. The membranes were exposed to a Fuji imaging plate, and the image was visualized by using a BAS2000 imaging analyzer (Fuji Photo Film).

5-Aza-2'-Deoxycytidine Treatment. Seeds were sterilized with 2.5% NaClO for 30 min and washed 5 times in sterile distilled water. The seeds were then placed on MS medium [4.6 g/L MS plant salt mixture (cat. no. 392–00591; Nihon Pharmaceutical), 5 mg/L nicotinic acid, 10 mg/L pyridoxine hydrochloride, 10 mg/L thiamine hydrochloride, 2 mg/L glycine, 100 mg/L myo-inositol and 1% agar] with or without 70 mg/L 5-aza-2'-deoxycytidine (5-aza-dC) and grown under dark conditions at 30 °C for 1 week. After treatment with 5-aza-dC, seeds were transferred to water and grown under light conditions at 30 °C for 3 days and then transferred to the nursery bed and grown in greenhouse for 1 month. The seedlings were then transplanted to the paddy field.

DNA Gel Blot Analyses. Rice genomic DNA was isolated from leaf tissue by using the ISOPLANT DNA isolation kit (Nippon Gene), and 1 μg was digested with suitable restriction enzymes, transferred onto Hybond *n* + membranes (Amersham) under alkaline conditions, and analyzed. Hybridization was performed at 65 °C in 0.25 M Na_2HPO_4 , 1 mM EDTA, and 7% SDS. Filters were washed twice in $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65 °C for 15 min and once in $0.2\times$ SSC and 0.1% SDS at 65 °C for 15 min. Isotope probes were amplified and ^{32}P -labeled by using the Bca Best Labeling kit (Takara). The centromere-specific repeat probes were adopted from the centromere-specific sequences, RCS2, designed by Wu et al. (24).

ChIP Assay. Approximately 50 g of 2-week-old etiolated rice seedlings were ground to fine powder with liquid nitrogen, resuspended in 40 mL of TBS [0.01 M Tris-HCl (pH 7.5), 3 mM CaCl_2 , 2 mM MgCl_2 with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Complete; Roche Molecular Biochemicals)] with 0.5% Tween 40. The suspension was filtered through Miracloth (cat. no. 475855; Calbiochem) on ice. Nuclei were pelleted by centrifugation at $600\times g$ for 10 min at 4 °C and resuspended in $1\times$ TBE with 25% sucrose. Nuclei were further purified by centrifugation at $1,500\times g$ for

20 min at 4 °C through a $1\times$ TBE with a 25%/50% discontinuous sucrose gradient. Purified nuclei were then digested with 0.9 units of micrococcal nuclease (USB Corp.) in 4.5 mL of digestion buffer [0.32 M sucrose, 50 mM Tris-HCl (pH 7.5), 4 mM MgCl_2 , 1 mM CaCl_2 , 0.1 mM PMSF] at 37 °C for 10 min. After digestion was stopped by adding 45 μL of 0.5 M EDTA, the nucleosome was purified by centrifugation at $15,000\times g$ for 10 min at 4 °C, and the first supernatant was kept on ice. The pellet was further incubated with lysis buffer [1 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 0.2 mM PMSF, and protease inhibitors] on ice for 1 h and centrifuged as before, and the 2 supernatants were pooled. The resulting oligonucleosome suspension was precleared by incubation with a 1:1,000 dilution of preimmune serum and 10% protein A-Sepharose (cat. no. 17-1279-02GE Healthcare) at 4 °C for 4 h, and centrifuged at $250\times g$ for 5 min at 4 °C. The supernatant was used immediately for immunoprecipitation. Equal volumes of the input fraction and incubation buffer [50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1 mM PMSF, and protease inhibitors] were incubated with 5 μg of antibodies at 4 °C overnight.

Normal rabbit serum was then added as no antibody. Immune complexes were captured by incubation with 25% vol/vol protein A-Sepharose at 4 °C for 2 h. At the end of the incubation, the protein A-Sepharose was washed stepwise in buffer A [50 mM Tris-HCl (pH 7.5), 10 mM EDTA] containing 50, 100, and 150 mM NaCl 3 times. Bound immune complexes (bound fraction) were eluted with 2 vol of incubation buffer containing 1% SDS. DNA was extracted from the supernatant by phenol/chloroform extraction and resuspended in TE buffer (pH 8.0). The antibodies and primers used in this assay were anti-dimethyl histone H3 K9 antibody (cat. no. ab7312-100; Abcam), anti-acetyl histone H3 K9 antibody (cat. no. ab4441-50; Abcam).

The primers of C-kinase substrate and 30 designed by Nagaki et al. (25) were adopted as control for euchromatic and heterochromatic genes, respectively. For additional materials and methods, see *SI Text*.

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