Running Head:
Identification of an Epiallele of RAV6 in rice

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Title
Epigenetic Mutation of RAV6 Affects Leaf Angle and Seed Size in Rice

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One-sentence Summary
Epigenetic modification of rice RAV6, which encodes a B3 transcription factor, alters rice leaf angle via modulation of brassinosteroid homeostasis.
Footnotes

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X.Z designed and performed experiments, J.S analysed data and modified the paper.
X.C designed experiments and modified the papers. X.S designed and performed experiments and wrote the paper.
ABSTRACT

Heritable epigenetic variants of genes, termed epialleles, can broaden genetic and phenotypic diversity in eukaryotes. Epialleles may also provide a new source of beneficial traits for crop breeding, but very few epialleles related to agricultural traits have been identified in crops. Here, we identified Epi-rav6, a gain-of-function epiallele of rice RELATED TO ABI3 AND VP1 6 (RAV6), which encodes a B3 DNA binding domain-containing protein. The Epi-rav6 plants show larger lamina inclination and smaller grain size; these agronomically important phenotypes are inherited in a semi-dominant manner. We did not find nucleotide sequence variation of RAV6. Instead, we found hypomethylation in the promoter region of RAV6, which caused ectopic expression of RAV6 in Epi-rav6 plants. Bisulfite analysis revealed that cytosine methylation of 4 CG and 2 CNG loci within a continuous 96-bp region plays essential roles in regulating RAV6 expression; this region contains a conserved MITE transposon insertion in cultivated rice genomes. Overexpression of RAV6 in wild type phenocopied the Epi-rav6 phenotype. The brassinosteroid (BR) receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1) and BR biosynthetic genes EBISU DWARF (D2), DWARFII1 (D11), and BR-DEFICIENT DWARF1 (BRD1) were ectopically expressed in Epi-rav6 plants. Also, treatment with a BR biosynthesis inhibitor restored the leaf angle defects of Epi-rav6 plants. This indicates that RAV6 affects rice leaf angle by modulating BR homeostasis and demonstrates an essential regulatory role of epigenetic modification on a key gene controlling important agricultural traits. Thus, our work identifies a novel rice epiallele, which may represent a common phenomenon in complex crop genomes.

Keywords: rice, epiallele, DNA methylation, B3 DNA binding protein, brassinosteroid
INTRODUCTION

Epigenetic gene variants (epialleles) carry heritable changes in gene expression that do not result from alterations in the underlying DNA sequence (Kakutani, 2002). In eukaryotes, cytosine DNA methylation, a conserved epigenetic mark, plays essential roles in the silencing of transposable elements (TEs) and genes (Law and Jacobsen, 2010). In higher plants, the few known epialleles involve alterations in DNA methylation, indicating that this epigenetic marker makes a large contribution to epigenetic diversity. The Arabidopsis thaliana clark kent (clk) epiallele, which has hypermethylated cytosines at the SUPERMAN locus, causes increased numbers of stamens and carpels (Jacobsen and Meyerowitz, 1997). Also, DNA hypomethylation at two direct repeats in the promoter region of FLOWERING WAGENINGEN (FWA) (Soppe et al., 2000) causes the late-flowering phenotype in Arabidopsis plants carrying the fwa epiallele. Plants carrying the natural epiallele hypermethylated at Linaria cyc-like (Lcyc) show altered floral symmetry, from bilateral to radial, in Linaria vulgaris (Cubas et al., 1999). In tomato, the Colorless non-ripening (Cnr) phenotype results from hypermethylation at the promoter of SQUAMOSA promoter binding protein-like (SBP-box) (Manning et al., 2006). In melon (Cucumis melo L.J), the transition from male to female flowers results from DNA hypermethylation in the promoter of CmWIP1, as mediated by a transposon insertion in gynoecious varieties (Martin et al., 2009).

Work in rice has found only two epialleles, Epi-d1 and Epi-df, both of which show a dwarf phenotype (Miura et al., 2009; Zhang et al., 2012). Epi-d1 is a spontaneous epiallele that shows a metastable dwarf phenotype caused by DNA hypermethylation in the promoter region of DWARF 1 (Miura et al., 2009). Epi-df is a gain-of-function epiallele caused by hypomethylation in the 5’ region of FERTILIZATION-INDEPENDENT ENDOSPERM1 (FIE1). The ectopic expression of FIE1 in Epi-df results in dwarf and various floral defects that are inherited in a dominant manner (Zhang et al., 2012).

In Arabidopsis, DNA methylation occurs in three sequence contexts: CG, CHG, and CHH (where H= A, C, or T) catalyzed by the de novo DNA methyltransferase DRM2 (Cao and Jacobsen, 2002). In the symmetrical contexts, DNA methyltransferases 1 (MET1) and
CHROMOMETHYLASE3 (CMT3) maintain methylation in the CG and CHG contexts, respectively (Law and Jacobsen, 2010; Lindroth et al., 2001). Small interfering RNAs (siRNAs) trigger \textit{de novo} methylation in all sequence contexts and also trigger the maintenance of CHH methylation, via RNA-directed DNA methylation predominately mediated by DRM2 (Cao and Jacobsen, 2002; Law and Jacobsen, 2010).

DNA methylation may be more prevalent and important in rice than in Arabidopsis, owing to the large numbers of TEs in the rice genome. In fact, Arabidopsis mutants of various DNA methyltransferases or \textit{DDM1} show few or no developmental defects (Cao and Jacobsen, 2002; Lindroth et al., 2001; Saze et al., 2003; Vongs et al., 1993). By contrast, the rice \textit{met1a} null mutant shows either viviparous germination or early embryonic lethality (Hu et al., 2014; Yamauchi et al., 2014). Moreover, impairment of the RNA-directed DNA methylation pathway proteins DCL3a and DRM2 causes drastic and pleiotropic developmental phenotypes (Moritoh et al., 2012; Wei et al., 2014).

Leaf angle is an important agronomic trait that directly affects crop architecture and grain yields (Sinclair and Sheehy, 1999). Crops with erect leaves capture more light for photosynthesis and are suitable for dense planting, all of which increase yields (Sakamoto et al., 2006). In rice, the brassinosteroid (BR) phytohormones participate in the determination of leaf angle (Tong and Chu, 2012; Zhang et al., 2014). BR-deficient or BR-insensitive mutants display erect leaves, while overexpression of BR biosynthesis genes or signaling components results in less-erect leaves with large leaf inclination (Bai et al., 2007; Hong et al., 2005; Yamamuro et al., 2000). For example, loss-of-function mutants of rice \textit{OsBRI1} and \textit{EBISU DWARF} (\textit{D2}), which encode the rice BR receptor kinase and a BR synthesis enzyme, respectively, show erect leaves (Hong et al., 2003; Yamamuro et al., 2000). In rice, RAV-LIKE 1 (RAVL1), a B3 DNA binding domain-containing protein, maintains BR homeostasis via the coordinated activation of BRI1 and BR biosynthetic genes, \textit{D2}, \textit{DWARF11} (\textit{D11}), and \textit{BR-DEFICIENT DWARF1} (\textit{BRD1}) (Je et al., 2010). The ravl1 mutant and RAVL1 overexpression lines showed erect leaves and large leaf angles, respectively (Je et al., 2010).

Here, we identified a natural epiallele of rice \textit{RAV6} and found that plants carrying this epiallele show increased leaf angle and small grains, as well as hypomethylation in the \textit{RAV6}
promoter region and ectopic expression of RAV6. Our work revealed that epigenetic modification of RAV6 plays an essential role in the regulation of important agricultural traits in rice and found that RAV6 acts via BR homeostasis.
RESULTS

A Semi-dominant Mutant Shows Large Lamina Inclination and Small Seed Size

A spontaneously occurring rice mutant with large leaf angle and small seeds was isolated from the japonica variety Zhonghua 11 and was named Epi-rav6 based on our subsequent characterization (see below). Compared with the wild type (Zhonghua11), almost all leaf angles throughout all developmental stages were larger in Epi-rav6 plants (Fig. 1A). The mutant had almost the same amount of rachis as wild type but had obviously smaller grains (Supplemental Fig. S1; Fig. 1B-D). Compared with the wild type, the Epi-rav6 seeds were markedly smaller, by 12.8% in length (average 7.8 mm in wild type to 6.8 mm in Epi-rav6) (Fig. 1C and 1D), 25% in width (average 3.6 mm in wild type to 2.7 mm in Epi-rav6) (Fig. 1B and 1D) and 20% in thickness (average 2.4 mm in wild type to 1.9 mm in Epi-rav6) (Fig. 1E). Thus, the 1,000-grain weight of Epi-rav6 seeds was only 57% of that of wild type (Fig. 1E). Together, these findings indicate that Epi-rav6 influences leaf angle and grain size.

To determine the inheritance of Epi-rav6, we examined the phenotypes of 260 progeny of self-pollinated Epi-rav6 plants. Three phenotypes segregated in these progeny: 62 were like wild type, 143 were like Epi-rav6, and 55 had severe defects, including fewer tillers, very large leaf angles, and sterility (Fig. 1A). The ratio of wild-type to Epi-rav6-like to severe phenotypes was 1:2.3:0.89 ($\chi^2=2.98$, P>0.05). In addition, 27 F1 plants derived from the cross between Epi-rav6 and Zhonghua 11 showed nearly a 1:1 ratio of wild-type progeny and Epi-rav6-like progeny (Supplemental Fig. S2). This genetic evidence demonstrated that the mutant is controlled by a single, semi-dominant locus and the derived allele is heterozygous. Thus, we regarded Epi-rav6-like progeny as heterozygotes and refer to them as Epi-rav6 (+/-); we also regarded the progeny with severe defects as homozygotes and refer to them as Epi-rav6 (-/-).

Cloning and Characterization of RAV6

To explore the molecular mechanism responsible for the Epi-rav6 phenotype, we used map-based cloning to isolate the causal gene. Using an F2 population derived from a cross between the Epi-rav6 mutant and Huajingxian74 (indica), we mapped Epi-rav6 to an 18.5-kb
region on chromosome 2. Based on the MSU7.0 annotation (http://rice.plantbiology.msu.edu/), this region contains only the promoter region of a putative gene, *Os02g45850* (Fig. 2A). However, we found no nucleotide sequence difference between the mutant and wild type in this region. We next investigated the expression level of *Os02g45850*, the only annotated gene in the mapped region. Compared with its low expression level in wild-type leaves, *Os02g45850* showed much higher expression in *Epi-rav6 (+/−)* and *Epi-rav6 (−/−)* leaves (Fig. 2B and 2C). Moreover, the degree of up-regulation was much higher in homozygous than heterozygous lines (Fig. 2B and 2C).

To further confirm that the ectopic expression of *Os02g45850* is responsible for the developmental defects in *Epi-rav6*, we over-expressed *Os02g45850* in wild type under the control of the maize (*Zea mays*) *Ubiquitin* promoter. All the positive transformants with high expression of *Os02g45850* displayed large leaf inclination and small grains (Fig. 3). Thus, we concluded that the abnormal phenotype in this mutant results from high expression of
Os02g45850 encodes a B3 DNA binding domain-containing protein, which belongs to the related to ABI3/VP1 (RAV) family and was named RAV6 (Romanel et al., 2009). Therefore, we named the allele Epi-rav6.

RAV6 Regulates Leaf Angle via Mediating brassinosteroid Homeostasis

The plant-specific RAV family belongs to the B3 transcription factor superfamily and consists of 12 members in rice, including RAV6 (Romanel et al., 2009). Phylogenetic analysis showed that the RAV6 (encoded by Os02g45850) is more closely related to RAVL1 (encoded by Os04g49230) than to other members of the RAV family (Fig. 4A). Comparison of deduced amino acid sequences suggested that RAV6 exhibits a high degree of sequence identity with RAVL1, especially in the B3 DNA binding domain (Fig. 4B). These observations indicate that RAV6 and RAVL1 may share similar functions. RAVL1 maintains BR homeostasis via the
coordinated activation of BR synthetic and receptor genes in rice (Je et al., 2010). In addition, plants over-expressing RAVL1 showed increased lamina inclination, like the Epi-rav6 mutants (Je et al., 2010), suggesting that RAV6 may also be involved in the BR pathway via activation of BR biosynthetic and signaling genes. To confirm this, we measured expression of representative genes encoding BR biosynthesis enzymes and the BR receptor, known RAVL1 targets, in Epi-rav6 mutants. As expected, expression of BR synthetic genes including D2, D11, and BRD1, was dramatically up-regulated in the Epi-rav6 mutant (Fig. 5A). Similarly, we also observed higher levels of induction for the BR receptor gene BRI1 in Epi-rav6 mutants. These data suggest that RAV6 affects the expression of genes involved in BR signaling and BR biosynthesis.

To further confirm that the large lamina inclination of rav6 mutant results from BR overdose, we postulated that treatment with a BR inhibitor would restore, at least partially, the mutant phenotype. As expected, in the presence of Propiconazole (Pcz), a BR biosynthesis inhibitor (Hartwig et al., 2012), the lamina inclination of Epi-rav6 mutant was restored (Fig. 5B). Taken together, these results suggest that RAV6 is involved in BR-mediated developmental processes.
Hypomethylation of the Promoter Region of RAV6 in Epi-rav6 plants

We found no nucleotide sequence difference between the wild type and Epi-rav6, but we did observe alteration of RAV6 expression levels in the Epi-rav6 plants, suggesting that the mutation may result from an epigenetic modification. We therefore investigated the DNA methylation status of the RAV6 locus. The promoter region of RAV6 contains many TEs including an adh-5-like MITE, a SINE, two unclassified retrotransposons, and a Snabo-like MITE (Fig. 6A; Supplemental Fig. S3A). TEs, especially those proximal to genes, can act as epigenetic mediators to influence nearby gene expression (Hollister and Gaut, 2009; Hollister et al., 2011; Wei et al., 2014). So we performed bisulfite sequencing to analyze methylation in a 2,358-bp genomic region consisting of 573-bp of gene body, 1,321-bp of upstream region
and 464-bp of 5’ distal retrotransposon sequence (Supplemental Fig. S3A). In wild type and homozygous Epi-rav6 mutants, we found no DNA methylation in all three sequence contexts in the 573-bp gene body of RAV6. However, we observed higher CG and CHG but not CHH DNA methylation in proximal and distal upstream regions of RAV6 in wild type compared with the homozygous Epi-rav6 mutant. All the changed methylation sites occurred in a contiguous 96-bp region at -600-bp to -504-bp relative to the transcriptional start site including the closest MITE (adh5-like MITE). This region is hypermethylated in the wild type but hypomethylated in Epi-rav6, containing four CG sites and two CHG sites (Fig. 6B; Supplemental Fig. S3B). To further confirm that the ectopic expression of RAV6 in Epi-rav6 results in hypomethylation of the promoter region, we treated the wild-type seeds with 5-aza-2’ deoxycytidine (5-aza-dC), an inhibitor of DNA methylation (Chang and Pikaard, 2005). The expression levels of RAV6 were measured in 7-day-old seedlings with or without 5-aza-dC treatment. Treatment with 5-aza-dC up-regulated RAV6 expression to varying degrees in three cultivated rice strains, including two japonica (Zhonghua11 and Nipponbare) and one indica...
(Kasalath) accessions (Figure 6C). These three cultivated accessions also contain the MITE 259
(see below); therefore, these results indicate that the DNA methylation mediated by the TE in
the 5′ upstream sequence of RAV6 plays an essential role in the regulation of RAV6
expression.

The effect of MITE-mediated DNA methylation on RAV6 expression is conserved in
cultivated rice.

MITEs are short (less than 600-bp), nonautonomous DNA transposons. As the TEs with the
highest copy numbers in the rice genome, MITEs mainly occur in the chromosomal arms,
especially in the vicinity of genes (Jiang et al., 2004). According to the Plant Repeat Database,
the O. sativa L. spp. japonica cv. Nipponbare genome contains 2984 copies of the adh5-like
MITE (Ouyang and Buell, 2004). The adh5-like MITE in the RAV6 promoter region is 100-bp
long and located 198-bp upstream of the RAV6 gene body.

To further explore the evolutionary significance of the epigenetic regulation of RAV6, we
investigated the natural variation of the MITE in the promoter region of the RAV6 locus in 40
accessions of cultivated rice, which represent all of the major groups of Asian cultivated rice
The results showed that the TE insertion in the promoter region of \( RAV6 \) is conserved in all cultivated rice accessions (data not shown). To further confirm this, we aligned the sequences of the MITEs, as well as the 5’ region of \( RAV6 \), in four known assembled rice genomes including three cultivated rice strains, Nipponbare, 93-11, Kasalath, and wild rice \textit{Oryza brachyantha} (Chen et al., 2013; Rice, 2005; Sakai et al., 2014; Yu et al., 2002). The MITE insertion was conserved in the cultivated rice genomes but was absent in \( RAV6 \) locus in different rice accessions. A. Comparative sequence analysis of the \( RAV6 \) locus in 4 assembled rice genomes. The synteny of the \( RAV6 \) locus in Nipponbare, 93-11, Kasalath, and \textit{O. brachyantha} is shown in the top panel. The bottom panel shows an alignment of the DNA sequence 258-bp upstream and the 5’ gene body of \( RAV6 \) in four assembled rice genomes. The MITE region and 5’ gene body are underlined by green and black lines, respectively. The ‘*’ indicates the cytosine region, whose degree of methylation was reduced in \textit{Epi-rav6} (-/-), as shown in Figure 6B. B. Expression analysis of \( RAV6 \) by RT-PCR (top) and real-time RT-PCR (bottom) in cultivated rice strains and \textit{Epi-rav6} (-/-). Zhonghua11, Nipponbare, Koshihikari and Koshikari are japonica cultivars; Kasalath is an indica cultivar. OsEFTa was used as a control. Values are means ± SD of three biological replicates. C, DNA methylation status of the bisulfite-sequenced region (as indicated in Figure 6A) in WT (Zhonghua11), \textit{Epi-rav6} (-/-) and four cultivated rice strains. Histograms represent the percentage of methylation in the CG (red), CNG (blue), and CHH (green) contexts.
*Oryza brachyantha* (Figure 7A). Consistent with this, the sequence of the 5’ promoter region of *RAV6* is also more conserved in cultivated rice, especially the 6 cytosine methylation sites, which are essential for regulation of *RAV6* expression (Figure 7A).

To further determine the conserved effect of the MITE-mediated DNA methylation on *RAV6* expression in cultivated rice accessions, we measured *RAV6* transcript levels and DNA methylation patterns in four cultivated rice strains, including three *japonica* (Nipponbare, Kongyu131, and Koshihikari) and one *indica* (Kasalath) accessions. Compared with the high expression of *RAV6* observed in *Epi- rav6*, we observed little or no *RAV6* expression in leaves at the tillering stage in all cultivated rice strains (Figure 7B). Like the pattern of wild-type Zhonghua11, the other four cultivated rice accessions also showed higher DNA methylation in the contiguous 96-bp region of *RAV6* promoter, in contrast with very low DNA methylation in *Epi- rav6* (Figure 7C; Supplemental Fig. S4). Thus, it is reasonable to predict that the TE insertion occurred prior to divergence of *indica* and *japonica* rice and that the TE-mediated epigenetic regulation of *RAV6* expression is evolutionarily conserved in cultivated rice.
**DISCUSSION**

In this work, we identified an epiallele of rice *RAV6*; this epiallele shows hypomethylation of the *RAV6* promoter region, causing ectopic *RAV6* expression, larger lamina inclination, and smaller grains. We found that *RAV6* expression is associated with extensive methylation of a MITE along with the nearby sequence in the 5’ region of this gene. *RAV6* encodes a B3 DNA binding domain-containing protein that has the most sequence similarity to RAVL1, which can maintain BR homeostasis to control rice development (Je et al., 2010). Functional analysis revealed that RAV6 coordinately activates the BR receptor BRI1 and BR biosynthetic genes to control rice leaf angle. This suggests that RAV6 performs a similar function to its homolog, RAVL1.

The Rice Mutant *Epi-rav6* Shows Hypomethylation of DNA at *RAV6*

Ectopic expression of *RAV6* in *Epi-rav6* mutants indicates that it is a gain-of-function allele of *RAV6*. Although we found no DNA sequence changes in *RAV6*, we did find that *Epi-rav6* shows a loss of cytosine DNA methylation in a MITE along with the nearby sequence located in its 5’ promoter region (Fig. 6A; Supplemental Fig. S3A). Thus, *Epi-rav6* has similar features to the Arabidopsis *fwa* mutant, the first DNA hypomethylated mutant identified in plants, with hypomethylation at two direct repeats in the 5’ region of *FWA* and ectopic expression of *FWA* (Soppe et al., 2000). In rice, only two epialleles, *Epi-d1* and *Epi-df*, were identified previously, with hyper- and hypomethylation at the causal genes, respectively (Miura et al., 2009; Zhang et al., 2012). Although both *Epi-df* and *Epi-rav6* are hypomethylated, the methylation sites of the two epi-alleles differ. *Epi-df* shows hypomethylation mainly in the coding region and not associated with repeated sequences (Zhang et al., 2012). However, the gain of methylation at the *D1* locus in *Epi-d1* is associated with the upstream repeat sequences (Miura et al., 2009), similar to *fwa* and *Epi-rav6*. Therefore, these repeat elements around the causal genes in *Epi-d1*, *fwa*, and *Epi-rav6* might function as epigenetic mediators to influence nearby gene expression, further confirming previous findings (Hollister et al., 2011; Wei et al., 2014).

The phenotypes of epi-alleles generally show metastable inheritance with a low
percentage of revertants, such as in *Epi-d1* and *Epi-df*. *Epi-rav6* is a semi-dominant mutant and the homozygous lines are sterile. Therefore, it is impossible to identify authentic revertants between generations (Fig. 1).

**RAV6 Controls Rice Lamina Inclination via Mediating BR Homeostasis**

The B3 DNA binding domain-containing proteins are plant-specific transcription factors that play important roles in growth, development, flowering time, seed development, and seed maturation (Romanel et al., 2009; Swaminathan et al., 2008). The B3 domain can bind DNA in a sequence-specific or non-specific manner and activate or repress the transcription of specific target genes (Je et al., 2010). Both RAV6 and RAVL1 belong to the RAV (Related to ABI3/VP1) family and have higher sequence similarity to each other than to other members of the RAV family (Figure 4). RAVL1 functions as a transcription factor, directly binding BR biosynthetic and receptor genes and thus maintaining BR homeostasis (Je et al., 2010). In *Epi-rav6*, the direct targets of RAVL1, including *BRI1*, *D2*, *D11*, and *BRD1*, showed increased expression (Fig. 5A), indicating that RAV6 might also target these genes. The lines overexpressing *RAVL1* showed larger lamina inclination and hypersensitivity to BR (Je et al., 2010). Consistent with this, increased leaf angles were observed in *Epi-rav6* mutants and RAV6 overexpression lines (Fig. 3A). Moreover, treatment with BR inhibitor fully rescued the larger lamina inclination in *Epi-rav6* (Fig. 5B). All these results indicated that both RAV6 and RAVL1 control leaf angles via maintaining BR homeostasis.

Besides leaf angle, BRs also influence rice grain size (Zhang et al., 2014). Generally, BR-deficient and BR-insensitive mutants, such as *d2*, *d11*, and *brd1*, showed small grains, while overexpression lines such as *Increase Leaf Inclination 1* (*OsILII*) and *BRII-SUPPRESSOR 1* (*OsBU1*) showed large lamina joints and increased grain size (Hong et al., 2002; Hong et al., 2003; Tanabe et al., 2005; Tanaka et al., 2009). The *Epi-rav6* plants showed activation of BR signaling and large leaf angles, but small grains, which differs from typical mutants affecting BR. We proposed that decreased grain size in *Epi-rav6* may result from an integrated effect of changes in expression of multiple target genes. Since RAV6 encodes a B3 DNA binding domain-containing protein, lots of target genes involved multiple developmental processes might be influenced by the gain-of-function change of RAV6 in...
Epi-rav6. This also can be confirmed as the Epi-rav6 homozygotes showed pleiotropic developmental defects (Fig. 1A).
MATERIALS AND METHODS

Plant Materials and Growth Conditions

A spontaneously occurring rice mutant Epí-rav6 was isolated from a Zhonghua 11 (Oryza sativa L. ssp. japonica) population in Guangzhou, China in 2006. Transgenic plants overexpressing RAV6 were produced by introducing the respective genes driven by the maize (Zea mays) Ubiquitin promoter into wild-type plants. Briefly, the 1,239-bp coding sequence of RAV6 was amplified with the primers OsRAV-1BXF and OsRAV-1239RNS (Supplemental Table S1) and the PCR product was inserted into the BamHI/SpeI sites of pCUbi1390, resulting in a plant expression vector driven by the Ubiquitin promoter, which was then transformed into rice. Plants were grown in the field under natural conditions.

Map-Based Cloning

For map-based cloning of OsRAV6, 725 recessive individual plants showing normal leaf inclinations were selected from an F2 population derived from a cross between the Epí-rav6 mutant and indica var Huajingxian74. Simple sequence repeat (SSR) markers and insertion/deletion (InDel) markers on chromosome 2 were used for fine mapping. The RAV6 gene was selected from an approximately 18-kb region as the candidate gene. To find out the mutation site, we amplified the corresponding fragments from the Epí-rav6 mutant and wild-type plants, respectively. Primers used in the map-based cloning are listed in Supplemental Table S1.

RT-PCR and Real-Time PCR

Real-time PCR analysis was performed using the CFX96 Real-Time PCR System (Bio-Rad) and SYBR Green I (S-7567; Invitrogen). PCR was performed using hot-start Taq DNA polymerase (DR007B; Takara Bio Inc., http://www.takara-bio.com/). For each sample, quantifications were made in triplicate. Melting curves were read at the end of each amplification by steps of 0.3°C from 65°C to 95°C to ensure that the quantifications were derived from real PCR products, and not primer dimers. The primers used for Fig. 5A are listed in Supplemental Table S1.
Bisulfite Sequencing

Genomic DNAs were isolated from 3-week-old rice seedlings or the leaf at tillering stage. Bisulfite treatment of genomic DNA was conducted using the Methylation-Gold kit according to the manufacturer’s instructions (ZYMO Research Corporation). Treated DNAs were then used for PCR amplification using hot-start Taq DNA polymerase (DR007B; TaKaRa). PCR conditions were as follows: 95°C, 5 min; 35 cycles of (95°C, 30 s; 50°C, 30 s; 72°C, 40 s); 72°C, 10 min. The PCR products were cloned into the pEASY-T1 cloning vector (TransGen Biotech Co., Ltd.), and individual clones were sequenced. The analysis of sequencing data was performed using KISMETH (Gruntman et al., 2008). The primers used for bisulfite sequencing are listed in Supplemental Table S1.

5-Aza-2′Deoxycytidine Treatment

Rice seeds were soaked in water at 37°C for 16 h. The seeds were then immersed in 20 mM Tris–HCl, pH 7.5, with or without 0.3 mM 5-aza-2′deoxycytidine (A3656, Sigma) at room temperature for 48 hours in the dark. After washing, seeds were planted in soil. 7-day-old seedlings were sampled for further analysis.

Phylogenetic Analysis and Alignment

Full-length protein sequences were used for phylogenetic analysis. The sequence of OsRAV6 was downloaded from TIGR, release 7 (http://rice.plantbiology.msu.edu/) and used to search the proteome database of rice available at TIGR using BLAST. Alignments of protein sequences were performed using MUSCLE (Edgar, 2004) with default parameters (gap opening, -2.9; gap extension, 0; hydrophobicity multiplier, 1.2; max iteration, 8). The maximum likelihood (ML) tree was constructed using MEGA6 (Tamura et al., 2013). Forty-eight different amino acid substitution models were tested and the JTT+G+F model (Jones et al., 1992) was considered as the best model with the lowest BIC (Bayesian Information Criterion) scores. All sites of the alignment were used for model testing. The –Ln likelihood was 10996.08. Nonparametric bootstrap analyses (Sanderson and Wojciechowski, 2000) consisted of 1000 replicates. All 4 known genome sequences of Oryza were collected.
including *Oryza sativa* ssp. *japonica* cv. Nipponbare (Rice, 2005), *O. sativa* ssp. *indica* cv. 93-11 (Yu et al., 2002), *O. sativa* ssp. *aus* cv. Kasalath (Sakai et al., 2014), and *O. brachyantha* (wild rice) (Chen et al., 2013). The genomic DNA sequence of the *OsRAV6* gene body and 230 bp upstream region was used as a query sequence, and homologous regions were searched in the other 3 genomes of *Oryza* using FASTA (v36.3.5d) (Pearson and Lipman, 1988). Multiple sequence alignment was performed using ClustalW (v2.1) (Larkin et al., 2007; Thompson et al., 1994).

**Propiconazole Treatment**

In this study, propiconazole (Pcz), a brassinosteroid biosynthesis inhibitor (Hartwig et al., 2012) was used to measure the mutants’ growth and morphological responses to BR by observing inclination of lamina joints after exposure to Pcz. For Pcz treatment, the plants were grown in the fields after incubating seeds for 3 days at 30°C to ensure synchronized germination. The 30-d-old seedlings were sprayed with 10 mM Pcz (Banner Maxx, Syngenta) every three days. After 15 days, plants were transplanted into pots and photographed. Control plants were grown and not treated with Pcz.

**Supplemental Data**

**Supplemental Figure S1.** The panicle and seeds of WT and *Epi-rav6 (+/-)* plants.

**Supplemental Figure S2.** The WT, *Epi-rav6 (+/-)*, and hybrid F1 plants at the heading stage.

**Supplemental Figure S3.** Analyses of DNA methylation levels at *RAV6* in WT and mutants by bisulfite sequencing.

**Supplemental Figure S4.** Analyses of DNA methylation levels at *RAV6* in WT, *Epi-rav6 (-/-)* and four cultivated rice strains by bisulfite sequencing.

**Supplemental Table S1.** List of primers used in this paper.

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Figure legends

**Figure 1.** Characterization of a semi-dominant rice mutant with larger leaf angle and smaller seeds. A, The wild-type (WT), heterozygous Epi-rav6 (+/-) and homozygous Epi-rav6 (-/-) plants at tilling stage. Bar = 20cm. B and C, Comparison of grain and seed width (B) and length (C) between WT and Epi-rav6 (+/-). Bar = 1 cm. D, Characterization of seed weight, seed length, seed width, and seed thickness in WT and the Epi-rav6 mutant. Values are means ± SD. In each graph, statistically significant differences are indicated by double asterisks (**P<0.01).

**Figure 2.** Molecular cloning and expression analysis of RAV6. A, Map-based cloning of RAV6. The RAV6 locus was mapped to the long arm of rice chromosome 2 between markers RM3512 and RM318. The gene was further delimited to an 18-kb genomic region between the markers ID8-26 and ID5-3 within the BAC clones AP004178 and AP004255. The number of recombinants is marked corresponding to the molecular markers. This 18-kb interval contains the candidate gene Os02g45850. Grey and black boxes indicate the untranslated regions and the coding sequence, respectively. B and C, Expression analysis of RAV6 by RT-PCR (B) and real-time RT-PCR (C) in WT, Epi-rav6 (+/-) and Epi-rav6 (-/-). OsEF1a was used as a control. Values are means ±SD of three biological replicates.

**Figure 3.** Over-expression of RAV6 phenocopies the Epi-rav6 mutant phenotype. A, The morphologies of WT, Epi-rav6 (+/-), and transgenic plants overexpressing (OV) RAV6 at seedling stages. Bar = 1 cm. B, Relative expression analysis of RAV6 in WT, Epi-rav6 (+/-), and the RAV6-overexpressing (OV) plants at seedling stage. C and D, The grain morphologies of the transformed WT plants. OV-1, 2: transgenic plants overexpressing RAV6. Bars = 2 cm.

**Figure 4.** Evolutionary relationship between RAV6 and RAVL1 in rice. A, Phylogenetic relationship of the RAV subfamily of B3 DNA binding domain-containing proteins in rice. The bar indicates substitutions per site. B, Amino acid sequence alignment of rice RAV6 and RAVL1. Identical and similar amino acids are boxed in black and grey, respectively. The
signature B3 binding domain is underlined.

**Figure 5.** *RAV6* regulates leaf angle by controlling genes affecting BR homeostasis. A, Real-time RT-PCR analyses of *BRD1, D2, D11, and BRI1* in WT, *Epi-rav6 (+/−)*, and *Epi-rav6 (−/−)* plants. 25S rRNA was used as a control. Values are means ± SD of three biological replicates. B, Lamina inclination of 45-day-old seedlings in WT, *Epi-rav6 (+/−)*, and *Epi-rav6 (−/−)* plants subjected to propiconazole (Pcz) treatment for 15 days. Bar = 20 cm.

**Figure 6.** DNA methylation analysis of the *RAV6* locus. A, Schematic representation of *RAV6* with putative MITE, MITE-adh-5-like DNA transposon. Boxes indicate exon (black), UTR regions (white) and MITE (green). The red line shows the region used for analysis of DNA methylation by bisulfite sequencing (B). B, DNA methylation status of bisulfite-sequenced region (as indicated in A) in WT and *Epi-rav6 (−/−)* plants. Histograms represent the percentage of CG (red), CNG (blue), and CHH (green). C, Real-time RT-PCR analyses of *RAV6* expression in 7-day-old seedlings treated with (+) or without (−) 5-aza-2’ deoxycytidine, an inhibitor of DNA methylation. *OsEF1a* was used as a control. Values are means ± SD of three biological replicates.

**Figure 7.** Genome sequence-, gene expression- and DNA methylation analysis of the *RAV6* locus in different rice accessions. A, Comparative sequence analysis of the *RAV6* locus in 4 assembled rice genomes. The synteny of the *RAV6* locus in Nipponbare, 93-11, Kasalath, and *O. brachyantha* is shown in the top panel. The bottom panel shows an alignment of the DNA sequence 258-bp upstream and the 5’ gene body of *RAV6* in four assembled rice genomes. The MITE region and 5’ gene body are underlined by green and black lines, respectively. The “*” indicates the cytosine region, whose degree of methylation was reduced in *Epi-rav6 (−/−)*, as shown in Figure 6B. B, Expression analysis of *RAV6* by RT-PCR (top) and real-time RT-PCR (bottom) in cultivated rice strains and *Epi-rav6 (−/−)*. Zhonghua11, Nipponbare, Kongyu131 and Koshihikari are *japonica* cultivars; Kasalath is an *indica* cultivar. *OsEF1a* was used as a control. Values are means ± SD of three biological replicates. C, DNA methylation status of the bisulfite-sequenced region (as indicated in Figure 6A) in WT
(Zhonghua11), *Epi-rav6 (−/−)* and four cultivated rice strains. Histograms represent the percentage of methylation in the CG (red), CNG (blue), and CHH (green) contexts.


