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Heritable epigenetic mutation of a transposon-flanked *Arabidopsis* gene due to lack of the chromatin-remodeling factor DDM1

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Epigenetically silent transposons and repeats constitute a substantial proportion of eukaryotic genomes, but their impact on cellular gene function remains largely unexplored. In *Arabidopsis*, transposons are silenced by DNA methylation, and this methylation is often abolished by mutations in a chromatin-remodeling gene *DDM1* (*DECREASE IN DNA METHYLATION 1*). The *ddm1* mutation induces various types of developmental abnormalities through de-repression of transposons and repeats. Here, we report a novel mechanism for a *ddm1*-induced syndrome, called *bonsai* (*bns*). We identified the gene responsible for the *bns* phenotypes by genetic linkage analysis and subsequent transcriptional analysis. The *bns* phenotypes are due to silencing of a putative Anaphase-Promoting Complex (APC) 13 gene. The *BNS* gene silencing was associated with DNA hypermethylation, which is in contrast to the *ddm1*-induced hypomethylation in the other genomic regions. This paradoxical *BNS* hypermethylation was reproducibly induced during self-pollination of the *ddm1* mutant, and it was mediated by a long interspersed nuclear element (LINE) retrotransposon flanking the *BNS* gene. We discuss possible molecular mechanisms and the evolutionary implications of transposon-mediated epigenetic changes in the *BNS* locus.

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Introduction

Methylation of cytosine is a heritable epigenetic mark involved in several important biological processes, including genomic imprinting and transposon silencing (Jaenisch and Bird, 2003; Rangwala and Richards, 2004; Chan *et al*, 2005; Zilberman and Henikoff, 2005). Transposons are methylated in diverse organisms, and loss of cytosine methylation leads to activation of transposons (Yoder *et al*, 1997; Walsh *et al*, 1998; Miura *et al*, 2001; Singer *et al*, 2001; Kato *et al*, 2003; Selker *et al*, 2003). Genome-wide mapping of DNA methylation in the flowering plant *Arabidopsis* demonstrated that a majority of cytosine methylation is concentrated in heterochromatic regions, where transposons and repetitive sequences accumulate (Lippman *et al*, 2004; Zhang *et al*, 2006; Zilberman *et al*, 2007). Unexpectedly, however, recent high-resolution mapping studies revealed that ~20–30% of expressed genes have methylation within their transcribed regions, although the methylation level is generally lower than that in transposons (Zhang *et al*, 2006; Zilberman *et al*, 2007). Interestingly, the proportion of those methylated genes increases toward heterochromatic pericentromeric regions, possibly reflecting direct or indirect interaction(s) of epigenetic states between euchromatic genes and heterochromatic sequences (Zilberman *et al*, 2007). In the large genomes of plants and vertebrates, transposons and repeats are also scattered among and within genes. However, the impact of such local heterochromatin on activity of cellular genes remained largely unexplored.

The impact of epigenetic changes on transposon activity can be directly examined using *Arabidopsis* mutants with defective genomic DNA methylation. In plants, cytosine methylation is found in both CG and non-CG contexts. In *Arabidopsis*, methylation at CG sites is maintained by DNA methyltransferase MET1, an ortholog of Dnmt1 in mammals, while methylation at non-CG sites depends on DNA methyltransferase genes, *CMT3* and *DRM2* (Finnegan *et al*, 1996; Ronemus *et al*, 1996; Bartee *et al*, 2001; Lindroth *et al*, 2001; Cao *et al*, 2003; Kankel *et al*, 2003). Another gene involved in maintenance of methylation and silencing of heterochromatin loci is a chromatin-remodeling ATPase gene *DDM1* (*DECREASE IN DNA METHYLATION 1*), which is involved in both CG and non-CG methylation (Vongs *et al*, 1993; Jeddeloh *et al*, 1998). In addition, chromatin and RNAi components involved in *de novo* DNA methylation have recently been identified using several reporter transgene systems (Aufsatz *et al*, 2002; Kanno *et al*, 2004, 2005; Chan *et al*, 2005; Herr *et al*, 2005; Onodera *et al*, 2005; Pontier *et al*, 2005; Pontes *et al*, 2006). Notably, many of the putative endogenous targets of this pathway are located near transposon sequences, which might epigenetically regulate adjacent genes (Huettel *et al*, 2006).

Several examples of developmental variants were recovered in both *met1* and *ddm1* mutant lines (Finnegan *et al* 1996; Kakutani *et al*, 1996, 2004; Ronemus *et al*, 1996; Kankel *et al*, 2003; Saze *et al*, 2003). Genetic analysis of some of these *ddm1*-induced developmental variants revealed that each of the abnormalities is due to a heritable change in a locus other than *DDM1* (Kakutani *et al*, 1996). For example, a *ddm1*-induced dwarf phenotype named *bal* is produced by the overexpression of a cluster of disease resistance genes (Stokes *et al*, 2002). Another *ddm1*-induced developmental variation, characterized by a delay in flowering onset, is due

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to ectopic expression of the imprinted homeobox gene *FWA* (Kakutani, 1997; Soppe *et al*, 2000; Kinoshita *et al*, 2004). Although these abnormalities behave as dominant traits, some of the *ddm1*-induced abnormalities behave as heritable recessive traits, suggesting that a different mechanism is responsible (Kakutani *et al*, 2004).

Here, we report the identification of the target gene of a *ddm1*-induced loss-of-function epigenetic abnormality called *bns* (Kakutani, 1997; Kakutani *et al*, 2004). The loss of *BONSAI* gene function was due to gene silencing associated with DNA hypermethylation and small RNA accumulation. The *de novo* methylation of the *BONSAI* gene was induced reproducibly in independent *ddm1* mutant lines. This ectopic methylation depends on the presence of a long interspersed nuclear element (LINE) retrotransposon insertion within the 3′ non-coding region. The LINE insertion, which is found in the majority of natural accessions, generates a potential trigger for epigenetic variation with strong developmental effects.

Results

Repeated self-pollination of a ddm1 mutant induced a combination of phenotypes named bns

Repeated self-pollination of the DNA hypomethylation mutant *ddm1* results in a variety of developmental abnormalities (Kakutani *et al*, 1996). Genetic analyses of some of the phenotypes have revealed that they are caused by gain-of-function alleles, which reflect overexpression of the responsible genes (Soppe *et al*, 2000; Stokes *et al*, 2002). However, not all of the developmental abnormalities are gain-of-function alleles. An example is a *ddm1*-induced developmental syndrome that we named *bns*.

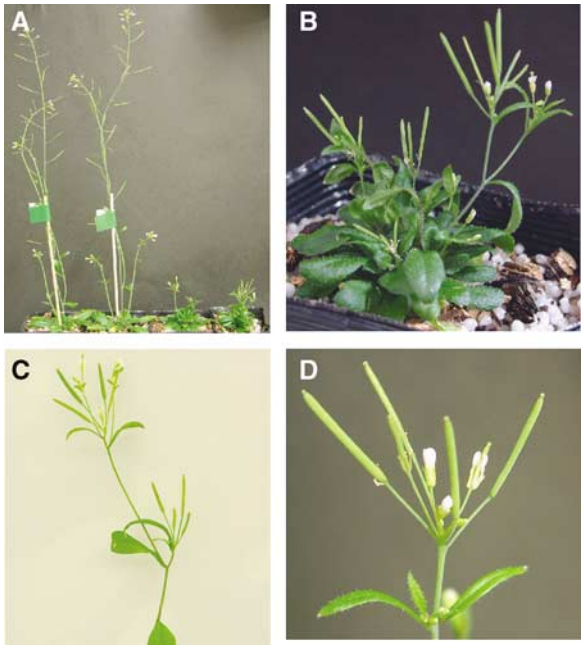


Figure 1 The *bns* phenotypes. (**A**) WT Col plants (two on the left) and *bns* plants in a *DDM1/DDM1* background (two on the right; hereafter referred to as *bns*). Both are six weeks old. (**B**) A close-up image of *bns*. (**C**) Inflorescences of *bns*. (**D**) A cluster of flowers produced in *bns*.

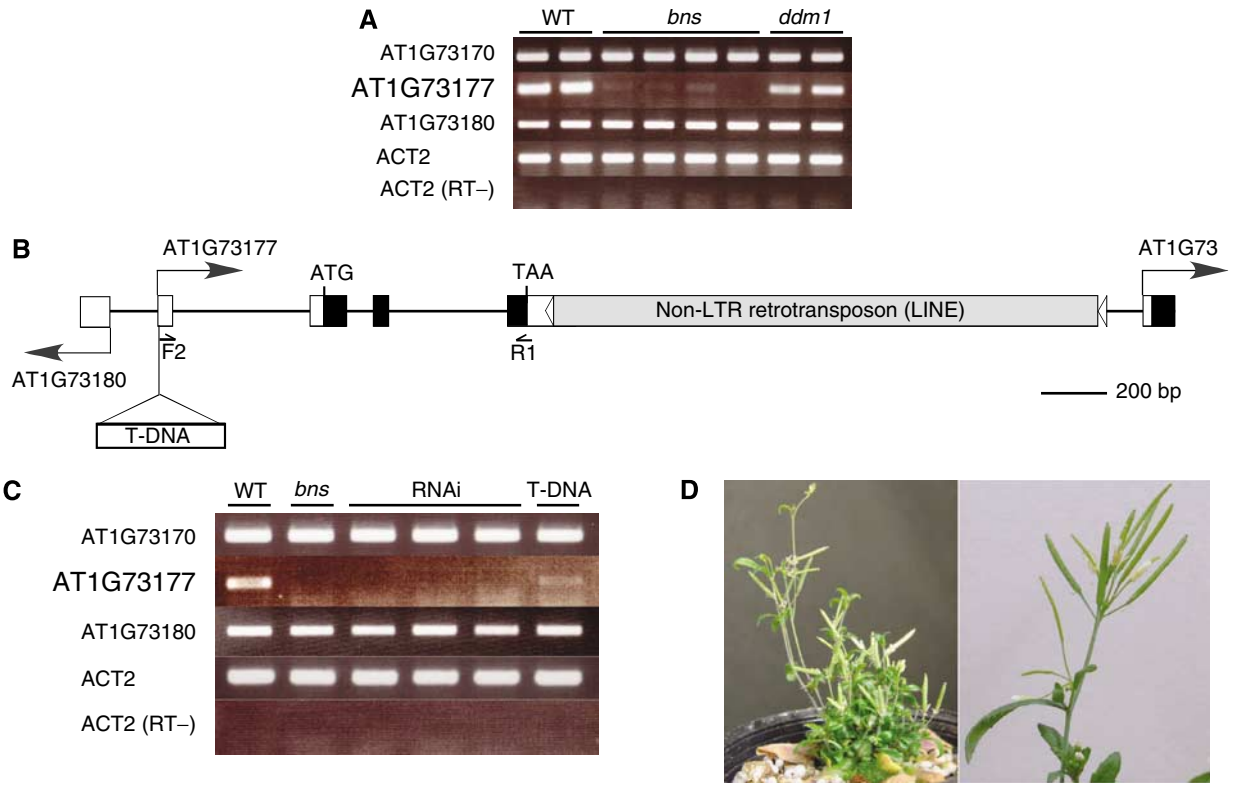
The *bns* phenotypes were characterized by short, compact inflorescence, resulting in reduced plant height (Figure 1A and B). The *bns* variant showed disrupted phyllotaxis, reduced apical dominance and production of clusters of bracts and flowers at the apex of the inflorescence (Figure 1C and D). These phenotypes seem to reflect the inhibition of internode elongation and the termination of shoot growth at the apical meristems (Figure 1; Kakutani, 1997; Kakutani *et al*, 2004).

After backcrossing to the parental wild-type (WT) Columbia (Col), the *bns* phenotype was not detectable in the F_1 population, suggesting that the abnormal phenotypes are not due to a gain-of-function mutation. In the self-pollinated progeny of an F_1 plant, we recovered F_2 plants showing the *bns* phenotype. The phenotypic plants included both *ddm1/ddm1* and *DDM1/-* genotypes. This observation suggests that the *bns* phenotypes are produced by a heritable change in a locus (or loci) other than *DDM1*.

Identification of the BNS gene

To further understand the basis of the heritable *bns* phenotypes, we examined their inheritance in the F_2 progeny from a cross of a *ddm1* plant with *bns* phenotypes (Col) to a WT Landsberg *erecta* (L*er*) plant. The genotype was determined for 531 F_2 plants with clear *bns* phenotypes, which comprised about 10% of the F_2 population. Characterization of Col/L*er* polymorphisms throughout the genome revealed that all of the phenotypic plants were homozygous for the Col haplotype in one locus in the bottom arm of chromosome 1, suggesting that a loss-of-function allele in this locus is responsible for the *bns* trait. This locus was narrowed to an interval between genetic markers NGA111 and BW54 (five recombinants and two recombinants, respectively, out of the 1062 chromosomes examined). We compared the transcript levels of 54 predicted genes in this genetically defined *BNS* region between WT and *bns* plants (backcrossed to *DDM1/DDM1*), using a reverse transcription (RT)–PCR assay. We found that one gene (AT1G73177) showed a severe reduction in its expression in *bns DDM1* compared to WT plants (Figure 2A). The AT1G73177 transcript was also reduced in the self-pollinated *ddm1* plants with the *bns* phenotypes (data not shown). The identified gene consists of four exons and encodes a predicted 63-amino acid (aa) protein (Figure 2B), and this annotation is supported by the presence of full-length cDNA in nucleotide sequence databases (GenBank: AY088589). A truncated non-LTR-type retrotransposon (LINE, long interspersed nuclear elements) sequence (AT1G73175) was found in the 3′UTR in the WT Col genome (Figure 2B and see below). Two flanking genes, AT1G73170 and AT1G73180, did not show a detectable reduction in their transcript level in *bns* plants (Figure 2A).

To test whether the *bns* phenotypes are due to the repression of AT1G73177, this gene was knocked down by RNAi in WT Col plants by transformation with a transgenic construct producing double-stranded RNA (dsRNA) of the gene sequence (Figure 2C). The transgenic lines showed the *bns*-like phenotypes (i.e., reduced plant height and clustered flowers) associated with a reduction in AT1G73177 transcript abundance (Figure 2C and D). In addition, we analyzed the effect of a T-DNA insertion in the upstream non-coding region in the first exon (SALK_027397). In the insertion mutant, a transcript was still detectable by RT–PCR, but the level was



E

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Hs_APC13    MDS----EVQRDGRILDLIDDAWREDKLPYEDVAI-----PLNELPEP--EQDNG---GTTESVKEQEMKWTDLALQYLHENVPPIGN 74
Mm          MDS----EVQRDGRILDLIDDAWREDKLPYEDVAI-----PLSELPEP--EQDNG---GTTESVKEQEMKWTDLALQGLHENVPPAGN 74
Os          MGGVEQEQLLSLGVLIDIVDEQWMRDTLPADDVPV-----PPAMAVKT--EEAEDPAPANQESQPAQGDVWRDFTLENL--------- 72
Zm          MSGLELELGLSLGVLIDVVDEQWMRDTLPADDIPV-----PPAMAVKT--EDAEDPAPANQESQPAQGDVWRDFALENL--------- 72
BNS         MA------EVSLGMLIDIVDEEWMRDTLPDDDLPL-----PPVLAVKT--DDTEE---TNQETQQADAETWRDLALDTQ--------- 63
Gm          MA------ELSLGILIDIVDEEWMRDTLPDDDLPL-----PPTLVVRT--DDTED---SNQETQQVNLDAWHDLAFGQE--------- 63
Sp_APC13    MDSNYNYVHMNKPGVV-LFASDWLKDRLPVDDVEVRVEHLPPVTEDEMTIQHSSANLILMKNKQLRHEPAWKDLELEDLVNAFAFIQ- 86
            *             :: :. . * .* ** :*: :     *     .   :. .       :.   .   * *: :
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Figure 2 Identification of the *BNS* gene. (**A**) RT–PCR for the *BNS* gene (AT1G73177) and neighboring genes (AT1G73170 and AT1G73180). Total RNA isolated from wild-type Col (WT), *bns* (backcrossed to *DDM1*) and *ddm1* plants (before repeated self-pollination) was used. The *BNS* transcript was also reduced in *ddm1* lines after repeated self-pollination (not shown). *Actin2* (*ACT2*) was used as a control. (**B**) A schematic representation of the *BNS* locus. Boxes represent exons (coding sequences in black and UTRs in white for *BNS* and the neighboring genes, and in gray for the LINE sequence). Black arrows indicate the annotated transcription start sites and transcript orientation (www.arabidopsis.org). Horizontal white arrowheads represent the target site duplications of the LINE insertion. The position of the T-DNA insertion in the first exon of *BNS* in SALK_027397 line is also indicated. The positions of primer pair, F2 and R3, used for RT–PCR of AT1G73177, are also shown. (**C**) Knockdown of *BNS* transcripts in the RNAi lines and in the T-DNA insertion line. RT–PCR was performed with total RNA from wild-type Col (WT), *bns*, transgenic plants expressing dsRNA of *BNS* gene (RNAi) and SALK_027397 line homozygous for the T-DNA insertion (T-DNA). (**D**) Phenotypes of a *BNS* RNAi line (left panel), and an inflorescence in a plant homozygous for the T-DNA insertion (right panel). (**E**) Multiple aa sequence alignment of BNS (*Arabidopsis thaliana*; AT1G73177) and APC13 homologs in *Homo sapiens* (Hs, NP_056206), *Mus musculus* (Mm, NP_852059), *Oryza sativa* (Os, NP_001060376), *Zea mays* (Zm, AY105005), *Glycine max* (Gm, CX701269) and *Schizosaccharomyces pombe* (Sp, NP_595754). The sequences were aligned using the ClustalW program that highlights the identical and conserved aa with asterisks and dots, respectively.

less than that observed in WT plants (Figure 2B and C). The plants homozygous for the T-DNA insertion showed similar phenotypes, although they were much milder (Figure 2D), further confirming that the reduction in AT1G73177 transcript induces the *bns* phenotypes. From these results, together with the recessive nature of the *bns* mutation, we concluded that the loss or reduction in AT1G73177 function is most likely to be responsible for the *bns* phenotypes.

The BNS gene product has similarity to a subunit of the Anaphase-Promoting Complex/Cyclosome (APC/C)

The predicted BNS protein has a high similarity to the mammalian Swm1/Apc13, a subunit of Anaphase-Promoting Complex/Cyclosome (APC/C) (Figure 2E). The APC/C is a large ubiquitin–protein ligase complex that regulates cell cycle progression in eukaryotic cells (Castro *et al*, 2005). Swm1/Apc13 was originally identified for its role in spore wall assembly in *Saccharomyces cerevisiae* (Ufano *et al*, 1999), and was later found to be a core subunit of the APC/C (Yoon *et al*, 2002; Hall *et al*, 2003). The protein is evolutionarily conserved in a wide range of organisms (Schwickart *et al*, 2004) (Figure 2E). We detected *BNS* expression in all tissues examined in WT plants (Supplementary Figure 1).

bns is an epigenetic mutation associated with DNA hypermethylation

Despite the marked reduction in *BNS* expression in the *bns* line, the nucleotide sequence of the *BNS* gene in the *bns* line

was identical to that in the WT progenitor strain, Col (from −825 to +946; data not shown). These results suggested that the silencing of the *BNS* gene has an epigenetic basis. We therefore examined the level of DNA methylation in this region.

To detect DNA methylation, we used the following two methods: digestibility by methylation-sensitive restriction enzymes and bisulfite genomic sequencing. Bisulfite genomic sequencing revealed that the *BNS* gene in the WT Col genome was almost free of DNA methylation (WT Col in Figure 3A). On the other hand, the flanking LINE sequence was heavily methylated, especially at CG sites (Figure 3A and B). In the *bns* line, the *BNS* gene region was also heavily methylated (bottom diagram in Figure 3A), which is in contrast to the situation in WT Col plants. The hypermethylation at the *BNS* locus was found at both CG and non-CG sites.

The methylation status of the *BNS* region was confirmed by digestion with methylation-sensitive restriction enzymes and subsequent PCR. WT Col samples did not show a PCR signal, reflecting the complete digestion of the genomic DNA (left panel in Figure 3C). Samples derived from *bns* plants showed bands reflecting incomplete digestion due to methylation. These results are consistent with the results of the bisulfite sequencing.

Repeated self-pollination of ddm1 mutant reproducibly induced de novo DNA methylation in the BNS gene

The hypermethylation in the *BNS* gene contrasts with the global DNA hypomethylation induced by the *ddm1*. We examined whether this paradoxical DNA hypermethylation in the *BNS* locus reflected one single purely stochastic event, or *BNS* hypermethylation could be reproducibly induced in a *ddm1* mutant background.

In order to see the initial effect of the *ddm1* mutation, *ddm1* homozygotes were selected from progeny derived by self-pollination of a *DDM1/ddm1* heterozygote. This *DDM1/ddm1* parent was generated by backcrossing a *ddm1* mutant six times to WT Col parent, in order to remove heritable effects from the original *ddm1/ddm1* mutant (Kakutani *et al*, 1996, 1999). *ddm1/ddm1* plants segregated in the self-pollinated progeny of the backcrossed *DDM1/ddm1* parent did not show signs of *BNS* gene hypermethylation (*ddm1*(1stG) in Figure 3). In order to see the effect of repeated self-pollination of the *ddm1* mutant, seven *ddm1* homozygotes in the segregating family were independently self-pollinated seven times. The hypermethylation of the *BNS* gene was detected in all seven independent *ddm1* lines (*ddm1*(8thG) in Figure 3). The *ddm1* mutation reproducibly induced *BNS* methylation, but this process was slow and required multiple generations. As a control, *BNS* methylation was also examined in four *DDM1/DDM1* sibling lines segregated from the same *DDM1/ddm1* parent and self-pollinated seven times in parallel (*DDM1* (8thG) in Figure 3). *BNS* methylation was not detected in any of the four *DDM1* control lines. The lack of methylation in the *DDM1* sibling families further confirmed that the *ddm1* mutation was responsible for the *de novo* methylation of the *BNS* gene.

The flanking LINE sequence showed a reduction in DNA methylation in the self-pollinated *ddm1* plants (*ddm1* (8thG) in Figure 3A and B). This result is consistent with the previous observations that *DDM1* activity is required for the maintenance of DNA methylation and silencing of endogenous transposons (Miura *et al*, 2001; Singer *et al*, 2001; Lippman *et al*, 2004). The hypomethylation of the LINE was found only after repeated self-pollination, which is similar to the situation for the SINE-related sequence in the *FWA* promoter, which remains methylated in the initial generations of *ddm1* inbreeding, but loses methylation stochastically in subsequent inbred generations (Soppe *et al*, 2000). Interestingly, the LINE sequence was methylated to the WT level in the *bns* mutant line backcrossed into a *DDM1/DDM1* background (Figure 3A and B), suggesting *de novo* methylation in the *DDM1* background.

The hypermethylation and silencing of BNS gene is associated with small RNAs

Epigenetic silencing of transposons and repeats are frequently associated with the production of small RNAs, which could be involved in RNA-directed DNA methylation (Zilberman *et al*, 2003; Chan *et al*, 2004; Matzke and Birchler, 2005). Because the *BNS* gene was methylated *de novo* in a *ddm1* background, we examined small RNAs corresponding to this region. As shown in Figure 4, *BNS* gene silencing was associated with the accumulation of small RNA in the size of 24–25 nt, the length of small RNA species often detected for heterochromatic sequences (Hamilton *et al*, 2002; Xie *et al*, 2004; Henderson *et al*, 2006; Pontes *et al*, 2006). Small RNAs (24–25 nt) were also detected in the *ddm1* mutant sample. A hybridization probe covering the 3′ region of the *BNS* gene near the boundary with the LINE (BNS 3′ probe) detected a weak but significant signal in the WT Col sample (Figure 4B and C), although the signal increased in the sample carrying a silent *BNS* allele.

As is the case for many other silent transposons, small RNA was also detected for the LINE sequence (Figure 4B) in the WT Col sample. Interestingly, the amount of the small RNA for this family of LINE increased in the *ddm1* and *bns* plants. The increase in the small RNA signal might mediate the *de novo* methylation of this LINE element, and that may explain the partial methylation of the LINE in the *ddm1* mutant, and the *de novo* methylation of the element after introduction into a background with a WT *DDM1* allele (Figure 3A and B).

The LINE insertion was found at the BNS locus in majority of Arabidopsis natural accessions

The LINE (AT1G73175) at the *BNS* locus belongs to a previously uncharacterized subfamily of LINE sequences in *Arabidopsis* (Wright *et al*, 1996; Noma *et al*, 2000, 2001). The presence of 16-bp target site duplication (TSD) followed by a 9-bp poly(A) sequence proximal to *BNS* indicates that the LINE sequence is inserted in a tail-to-tail orientation relative to the *BNS* gene (Figure 3A). In WT Col, *BNS* mRNA extends into the LINE sequence over the TSD and poly(A) sequences (Supplementary Figure 2). The Col genome contains two other members of this LINE subfamily, which share more than 97% nucleotide sequence identity (AT1G17390 and AT5G36935; Figure 5A). The copy on chromosome 5 (AT5G36935) is likely to be the full-length copy (Figure 5A). This copy encodes three open reading frames, with a structure similar to *ATLN-L* class LINEs (Noma *et al*, 2001). The presence of these three copies in the Col genome was confirmed by Southern blot analysis (Supplementary Figure 3).

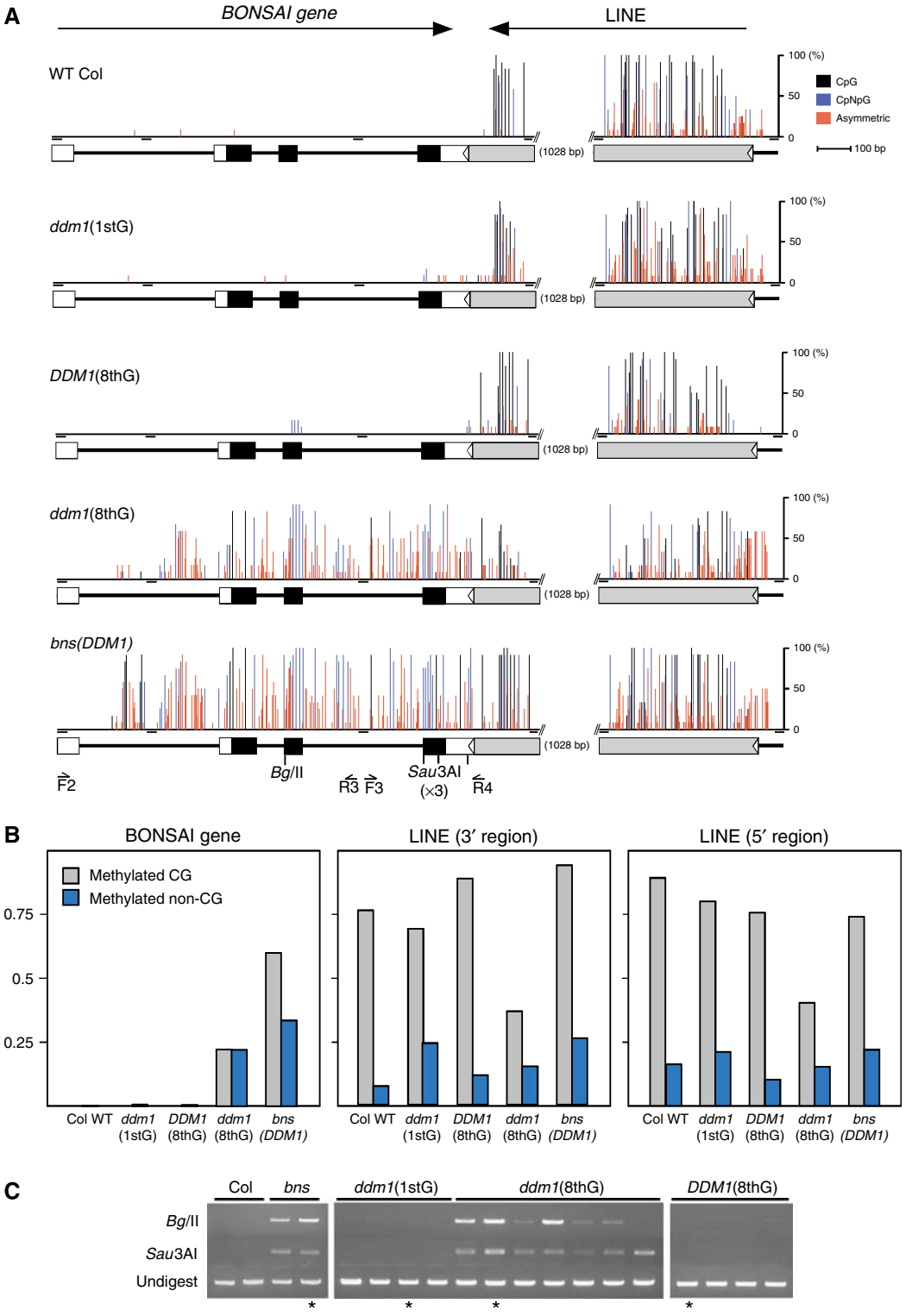

Figure 3 DNA methylation pattern in the *BNS* locus. (**A**) Schematic representations of the *BNS* locus and cytosine methylation level analyzed by bisulfite sequencing. After treatment with bisulfite, DNA fragments were amplified using four pairs of primers separately (the positions indicated as short horizontal black bars), and cloned for sequencing (12 clones for each amplified region). The percentage of methylated cytosine is indicated by vertical bars (black, CG; blue, CNG; red, asymmetric cytosine). Boxes below represent exons (coding sequences in black and UTRs in white for *BNS*, and in gray for the *LINE* sequence). (**B**) Proportion of methylated cytosines in the *BNS* locus, which is based on the results shown in panel A. (**C**) Methylation of the *BNS* region detected by restriction digestion. Genomic DNA was digested by methylation-sensitive restriction enzymes *Bgl*II (5′-AGATCT-3′) or *Sau*3AI (5′-GATC-3′) (http://rebase.neb.com), and was subsequently used as template for PCR amplification. The positions of the restriction sites and primers used for the PCR are indicated in the bottom of panel A; primer pairs F2 + R3 and F3 + R4 were used after *Bgl*II and *Sau*3AI digestion, respectively. Asterisks (*) indicate the samples used for bisulfite sequencing in panels A and B.

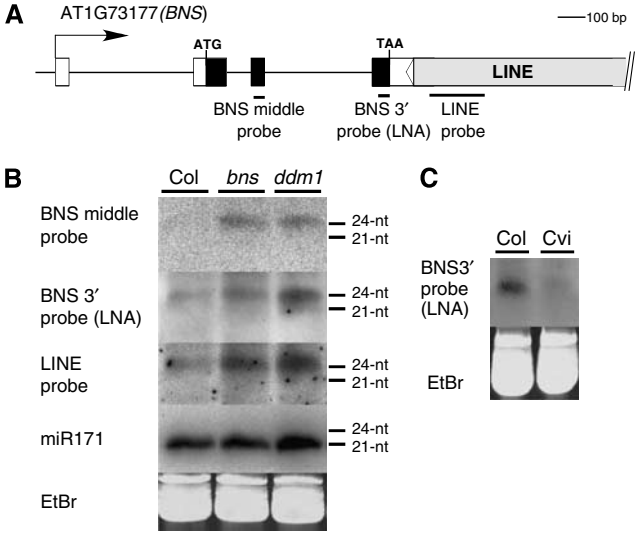

Figure 4 Small RNA northern analysis of the *BNS* locus. (**A**) The positions of three hybridization probes used are indicated. (**B**) Small RNA was examined in WT Col, *bns* in *DDM1* background and self-pollinated *ddm1* plants with reduced *BNS* expression. The same membrane was used for hybridization with each of the three probes and for the control miR171 probe. Ethidium bromide staining of the major RNA is shown as a control (EtBr). (**C**) WT Col and Cvi samples on a different membrane.

In order to evaluate the impact of the LINE insertion in natural populations, we examined the presence of the LINE insertion at the *BNS* locus in 96 natural accessions of *Arabidopsis thaliana*. Among them, 83 accessions have the LINE insertion in the *BNS* 3′UTR, while 13 did not have the insertion (Figure 5B). This was confirmed by Southern analysis of the 96 natural accessions (data not shown). Among the 13 accessions without the LINE insertion, five contain sequences almost identical to Col apart from the LINE insertion (Figure 5C). In those accessions, the TSD sequence remained intact, suggesting that these are ancestral alleles before the LINE insertion. Presence of the LINE in the majority of natural accessions suggests that the LINE insertion *per se* does not have deleterious effects in natural populations.

Dependence of the BNS hypermethylation on the flanking LINE sequence

Using Cvi, which does not have the LINE insertion at the *BNS* locus (Figure 5C), we tested whether the LINE sequence is necessary for the *ddm1*-induced *de novo* methylation at the *BNS* locus. WT Cvi was crossed to a *ddm1* heterozygote, which had already been backcrossed six times in the heterozygous state (Kakutani *et al*, 1996). A *DDM1/ddm1* heterozygote originating from this cross was self-pollinated, and from the progeny, we selected *ddm1* homozygotes with the *BNS* allele from genome of Col (BNS^{LINE}/BNS^{LINE}; homozygous for the LINE insertion) or Cvi (BNS^{-}/BNS^{-}; without the LINE insertion) (Figure 6A). After three rounds of self-pollination, DNA methylation of the *BNS* locus in these *ddm1* plants was examined using methylation-sensitive restriction enzymes. All of seven independent *ddm1* lines homozygous for the BNS^{LINE} allele (from Col) showed *de novo* DNA methylation of the *BNS* locus, whereas none of the five *ddm1* lines homozygous for the BNS^{-} allele (from Cvi) showed ectopic DNA methylation (Figure 6B). This result suggests that *ddm1*-induced *de novo* methylation at the *BNS* gene depends on the presence of the LINE insertion in the 3′UTR.

Discussion

Mechanism for BNS gene hypermethylation triggered by the ddm1 mutation

Here, we report the identification and characterization of a loss-of-function epigenetic developmental abnormality *bns*. The most striking feature of the *bns* trait is that the local hypermethylation of the *BNS* gene was induced in a background of global DNA hypomethylation. The hypermethylation of the *BNS* gene was not evident in newly segregated *ddm1* homozygous plants (1stG in Figure 3), but it was reproducibly induced in the self-pollinated progeny of *ddm1* mutants (8thG in Figure 3). These observations suggest that *BNS* hypermethylation may be due to an indirect effect of the globally hypomethylated *ddm1* background. Similar observations have been previously reported for *SUPERMAN* (*SUP*) and *AGAMOUS* (*AG*) sequences; these sequences are stochastically hypermethylated in the absence of *DDM1* or *MET1* activity (Jacobsen and Meyerowitz, 1997; Jacobsen *et al*, 2000). In both *SUP* and *AG*, pyrimidine-rich sequences such as CT dinucleotide repeats are found in the hypermethylated target sequences, and the possible involvement of this simple-sequence motif has been proposed (Jacobsen *et al*, 2000). However, a pyrimidine-rich sequence was not found in the *BNS* locus, suggesting that it is not the basis for *BNS* hypermethylation (data not shown). Instead, our results suggest that *BNS* hypermethylation is mediated by the pre-existing LINE insertion in non-coding region of the *BNS* gene (Figure 6).

The ectopic hypermethylation at *BNS* in *ddm1* background occurred in a spreading manner from the LINE into the *BNS* region (Figure 3A). Spread of heterochromatin into genic regions is also known in position-effect-variegation in *Drosophila* (Talbert and Henikoff, 2006) and telomeric silencing in budding yeast (Grunstein, 1997). Although the *BNS* locus resides in a euchromatic chromosomal arm, the dense DNA methylation on the LINE sequence at this locus suggests that the LINE sequence can function as local heterochromatin, which is maintained without affecting adjacent genes in the WT background. The *DDM1* gene is necessary for the maintenance of the heterochromatic characteristics of LINE and other transposons (Gendrel *et al*, 2002; Lippman *et al*, 2003, 2004). DDM1 might also be necessary to define a heterochromatin boundary (Figure 7A). In mammals, the chromatin insulator CTCF has a barrier function that blocks the extension of heterochromatin. The CTCF-dependent insulator activity was abolished by loss of an SNF2-like chromodomain helicase/ATPase protein, leading to a decrease in euchromatic histone modifications and DNA hypermethylation around the boundary sequences (Ishihara *et al*, 2006).

Interestingly, the spreading of DNA methylation was not found in the other side (opposite from the *BNS* gene) of the LINE sequence. On that side, the expression of the gene AT1G73170, which has the transcription start site approximately 150 bp away from the TSD of the LINE, was not affected in *bns* and self-pollinated *ddm1* lines (Figure 2A and B and data not shown). These results suggest that the

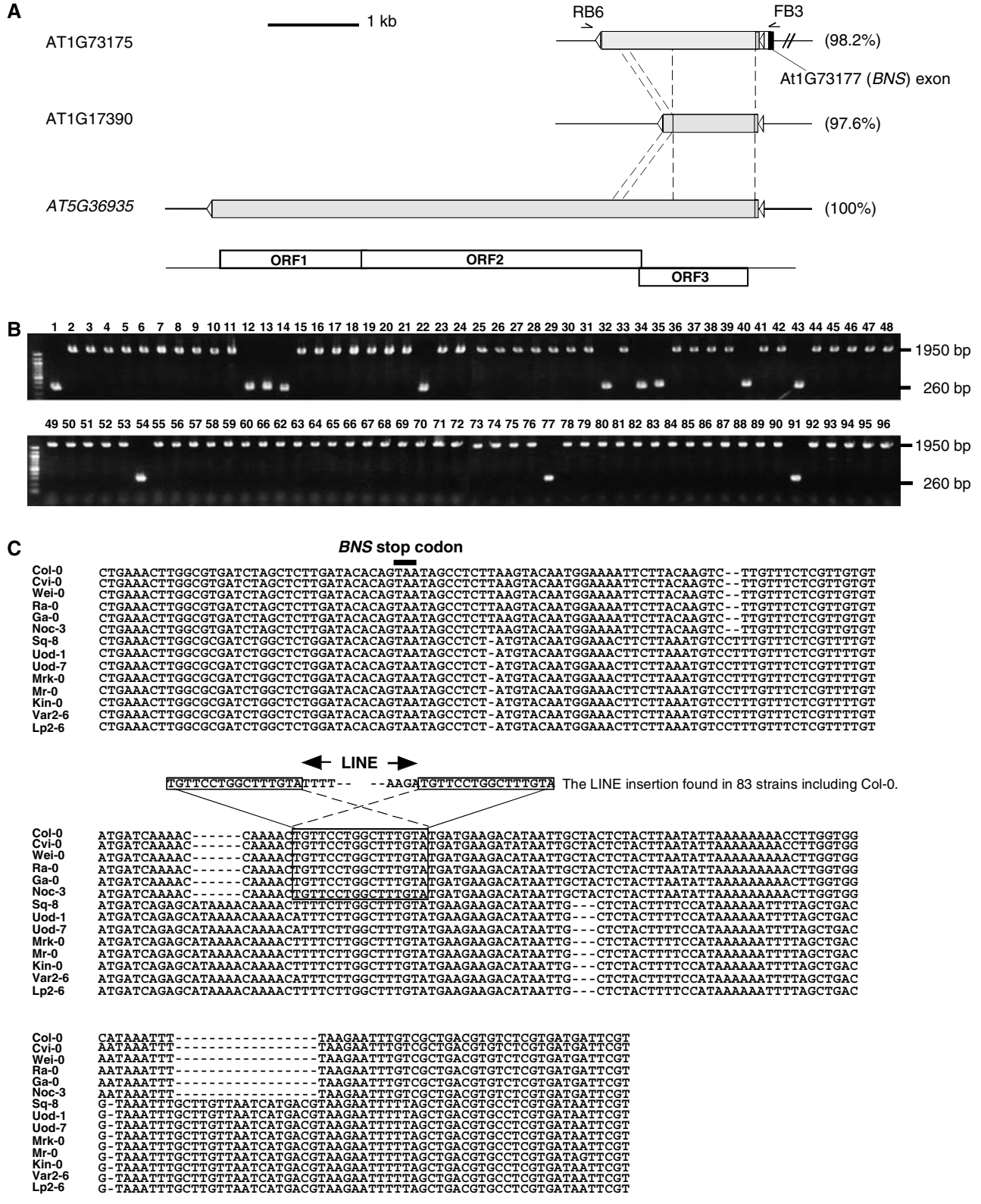

Figure 5 The *AT5G36935* LINE family in the *Arabidopsis* genome. (**A**) Schematic representations of the LINE sequence at the *BNS* locus, and two other related sequences in the Col genome. Sequence identities are indicated in the parentheses. Exons of LINEs are shown as gray boxes (coding sequences in black and UTRs in white for the *BNS* gene). White triangles indicate target site duplications of the LINE insertion. *AT1G17390* lacks a part of the internal sequence, as indicated by dashed lines. Predicted ORFs in *AT5G36935* are shown at the bottom. (**B**) The LINE insertion at the 3′UTR of *BNS* in 96 natural strains of *Arabidopsis thaliana*. Presence of the LINE insertion was examined by PCR using BNS FB3 and LINE RB6 primers indicated in panel A. The PCR using genomic DNA isolated from 96 *Arabidopsis* strains amplified either ~1950 bp (LINE+) or ~260 bp (LINE−) fragment. Names of the strains used are shown in Supplementary data. (**C**) An alignment of the nucleotide sequences of the *BNS* 3′UTR in 13 strains that do not have the LINE insertion. Col sequence is also shown. Note that the 13 strains are classified into two groups based on similarity, and only one group has an identical target site sequence to that found in the Col genome (indicated by box). The stop codon of the *BNS* gene is indicated by the black bar.

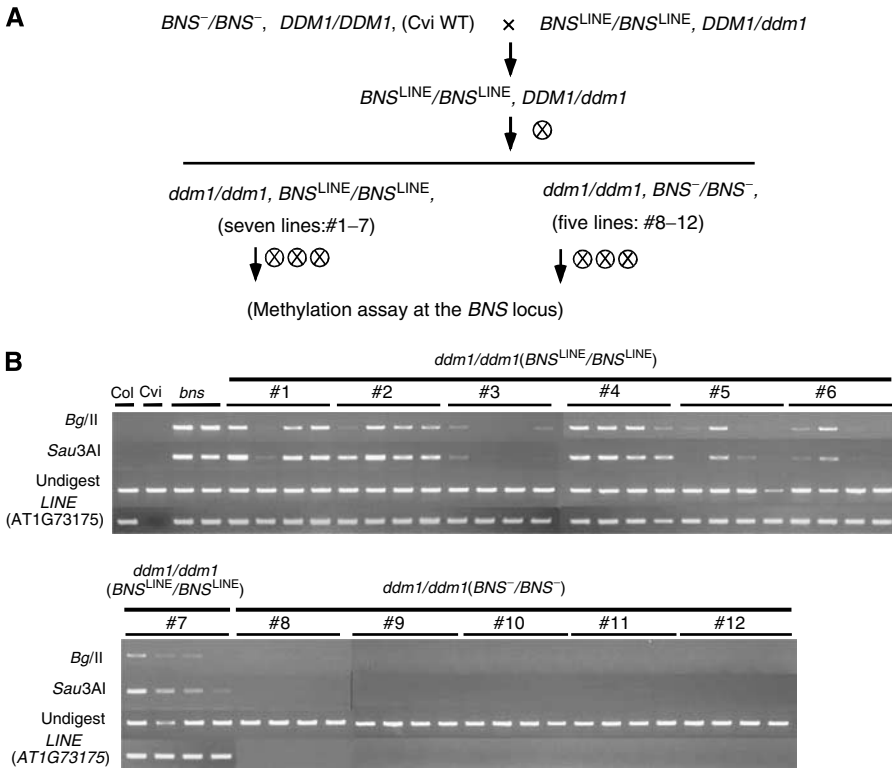

Figure 6 The *ddm1*-induced *de novo* methylation at the *BNS* locus depends on the presence of the LINE insertion. (**A**) Genetic scheme to generate *ddm1* plants with or without the LINE insertion at the *BNS* locus. X and encircled X indicate outcross and self-pollination, respectively. BNS^{LINE}: *BNS* allele from Col with LINE insertion. BNS^{-} : *BNS* allele from Cvi without the LINE insertion. DNA methylation was examined after three generations of self-pollination. (**B**) DNA methylation at the *BNS* gene in twelve independent lines. Methylation was analyzed as described in Figure 3C.

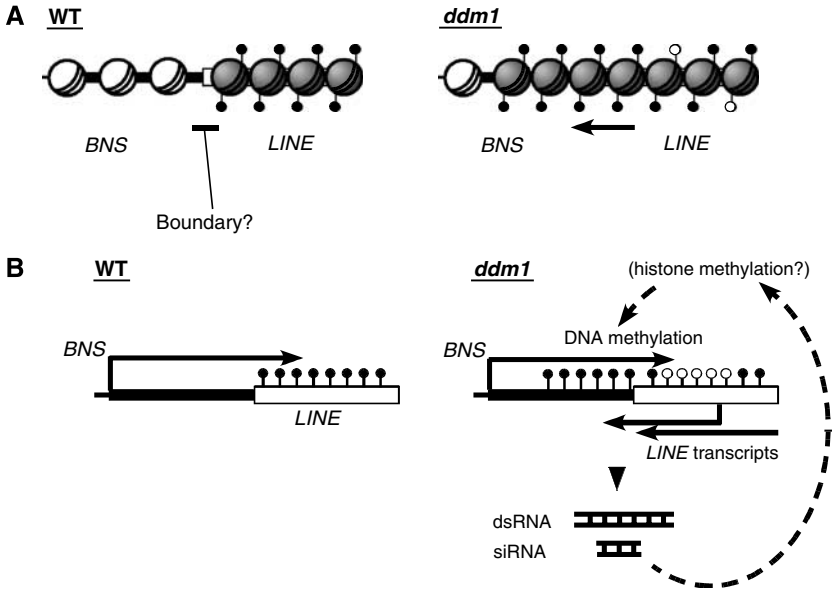

Figure 7 Two models for *ddm1*-induced *de novo* methylation at the *BNS* locus. (**A**) Heterochromatin spreading model. (**B**) RNA-directed DNA methylation model. Filled lollipops indicate DNA methylation. See Discussion for details.

spreading of cytosine methylation from the LINE was unidirectional. In addition, we could detect small RNA from the *BNS* coding sequence in the *ddm1* mutant plants (Figure 4), which correlates with the ectopic methylation. These features raise the possibility that LINE transcripts induced in the *ddm1* background form dsRNA with the *BNS* mRNA through the complementary sequence in the 3′UTR of *BNS* mRNA (Figure 7B; Supplementary Figures 2 and 3). It is also possible that the LINE at the *BNS* locus has a cryptic promoter within the element. Readout transcripts originating from cryptic

promoters in LTR-retrotransposons and LINEs can affect transcription of adjacent genes in both mammals and plants (Michaud *et al*, 1994; Nigumann *et al*, 2002; Kashkush *et al*, 2003). Indeed, the plant promoter database (PlantProm; Shahmuradov *et al*, 2003) predicted the presence of a putative bidirectional promoter sequence at the 3′ end of the LINE sequence (data not shown). The promoter could be activated through the loss of DNA methylation in self-pollinated *ddm1* lines (8thG in Figure 3A), and produce antisense transcripts of *BNS* mRNA, which subsequently result in the formation of dsRNA of *BNS* mRNA.

A low level of small RNA was also found in WT Col plants at the 3′ region of the *BNS* gene (Figure 4B and C). This situation might have parallels with the maize B paramutation system, in which small RNA are detected for the region controlling the epigenetic state of the B gene, even when this gene is expressed (Chandler, 2007). Similarly, small RNA was detected for the *Arabidopsis FWA* promoter even when it is unmethylated (Chan *et al*, 2006). Thus, the presence of small RNA correlates with the potential for the epigenetic silencing, which, in the case of the *BNS* gene, also correlates with presence of the flanking LINE sequence (Figure 4C).

Phenotypic effects of BNS gene silencing

The predicted product of the *BNS* gene shares similarity with APC13, a subunit of the Anaphase-Promoting Complex APC/C that regulates the metaphase–anaphase transition and exit from mitosis through the degradation of cell cycle regulators (Castro *et al*, 2005). In budding yeast, loss of Swm1/APC13 leads to slow growth and an accumulation of G2/M cells (Hall *et al*, 2003; Schwickart *et al*, 2004). The observed *bns* phenotypes—inhibition of internode elongation and termination of shoot growth at shoot apical meristems (Figure 1)—might result from defects in APC-dependent cell cycle events. Null mutants of other single-copy *Arabidopsis* APC/C components, *APC2* and *APC6*, exhibit female gametophytic lethality due to cell cycle arrests at an early stage of embryo sac development (Capron *et al*, 2003; Kwee and Sundaresan, 2003). Although *BNS* is present as a single-copy gene, *bns* phenotypes (Figure 1) are distinct from that of *apc2* or *apc6*. It is possible that the *bns* epiallele does not cause a complete loss-of-function, and the remaining activity circumvents the cell cycle arrest that occurs in *apc2* or *apc6* gametophytes.

Once the *bns* phenotypes were induced in the *ddm1* background, these phenotypes were inherited by their progeny even in the presence of a WT *DDM1* allele. However, phenotypic variability was observed in *bns DDM1* lines (data not shown), suggesting that the epigenetically silent state may be unstable in a *DDM1* background. Similarly, the loss-of-function epigenetic alleles of *sup* and *ag* are unstable (Jacobsen and Meyerowitz, 1997; Jacobsen *et al*, 2000).

The role of transposons as potential triggers for heritable epigenetic developmental variation

When a transposon is inserted near a cellular gene in maize, expression of that gene is often affected by the epigenetic state of the transposon (McClintock, 1965; Fedoroff, 1989). Similarly, in mouse, insertion of a retroviral element within a gene can form an allele that shows epigenetic variation, which is heritable over multiple generations (Whitelaw and Martin, 2001; Rakyan *et al*, 2003). In an evolutionary context, an important question is whether the newly generated allele, which is under the transposon control, survives within natural populations or not.

The presence of the LINE insertion at the *BNS* locus in a majority of *Arabidopsis* natural accessions suggests that the insertion of the LINE *per se* did not have a deleterious effect in natural populations. Considering that, it is striking that the LINE mediates changes in epigenetic state of the *BNS* gene that lead to strong developmental variation. It is often the case that alleles with transposon insertions are indistinguishable from the original allele in term of expression pattern, as long as the transposon is silent (Fedoroff, 1989; Martienssen, 1996). Such hidden phenotypic variability, generated by a transposon insertion, may broaden the potential for evolution, as implied from 'canalization' phenomena in *Drosophila* (Waddington, 1959; Flatt, 2005). A systematic survey of polymorphisms in transposon insertion sites in natural populations might reveal beneficial impact of transposon insertions as a source for epigenetic variability, which is heritable but reversible.

Materials and methods

Plant materials and growth conditions

The identification and isolation of the *bns* strain was described in the previous study (Kakutani, 1997). Sources of the 96 natural accessions (Figure 5B) are described in Nordborg *et al* (2005). Seeds of these strains (CS22660) and the *BNS* T-DNA insertion line (SALK_027397) (Figure 2) were obtained from the *Arabidopsis* Biological Resource Center. Plant seeds were allowed to germinate and grow on a medium containing 0.5 × MS salts (SIGMA), 2% sucrose and 0.8% agar (pH 5.7), for 2 weeks under long-day conditions (16 h, light; 8 h, dark) at 22°C. The seedlings were subsequently transferred and grown on vermiculite under the conditions described above. The *ddm1-1* mutant and the WT *DDM1* alleles were distinguished by PCR, as described by Kato *et al* (2003). The LINE insertion in the *BNS* locus (Figure 5B) was detected by PCR with primers BNS FB3 (5′-CAG GAA ACT CAG CAA GCA GAT G-3′) and LINE RB6 (5′-GAG CCG TTT GCC AAC CAC GTG G-3′).

RT–PCR

Total RNA from *Arabidopsis* leaves was isolated with the RNeasy Plant Mini kit (QIAGEN) and was treated with DNase I (TAKARA). cDNA was synthesized using the TAKARA RNA PCR kit (AMV) Ver.3.0 (TAKARA) and an oligo-(dT) primer. A total of 500 ng RNA in the RT reaction mixture (total 10 μl) was reverse transcribed at 42–50°C for 1 h, followed by heat inactivation at 95°C for 5 min. A one-fifth portion of the RT reaction was used as a template for PCR (total 20 μl). PCR conditions were as follows: 94°C for 2 min, 26–30 cycles at 94°C for 15 s, 60°C for 30 s, 72°C for 45 s and 72°C for 5 min. Control reactions without RT were carried out as described above. The primer pairs used for RT–PCR were as follows: the *BNS* gene (BNS F2: 5′-GCT AGA GGT TTT TAG TTC TCT G-3′ and BNS R1: 5′-TGT ACT TAA GAG GCT ATT ACT G-3′); AT1G73170 (AT1G73170 F1: 5′-GCG ATA CGG GCA TTA CTA ACA G-3′ and AT1G73170 R1: 5′-TAA TCA GG CAA TAG AGG TAA CC-3′); AT1G73180 (AT1G73180 F1: 5′-GGC GAA GGT CCT TAT AAC ACT C-3′ and AT1G73180 R1: 5′-TGA TTT CTT CAA TCA GGC GTT G-3′); LINE (LINE FB5: 5′-AAA TTA CAC TTG AAC GTT CCG G-3′ and LINE RD: 5′-AGT GGG GAG GAG ACA ATT CTA CAC-3′) and Actin2 (ACT2F: 5′-CTA AGC TCT CAA GAT CAA AGG C-3′ and ACT2R: 5′-AAC ATT GCA AAG AGT TTC AAG G-3′). cDNA synthesized from total RNA isolated from WT Col plants was used for 3′ RACE of the *BNS* gene. The initial PCR was performed using the BNS F2 primer and the M13 Primer M4 supplied with the kit, and then nested with BNS F1 primer (5′-TGT GTG GAG TAC GGC TGC ATT G-3′) and M13 primer M4. Amplified fragments were cloned and sequenced.

Transgenic plants

To prepare the RNAi construct (Figure 2), the *BNS* genomic sequence was amplified from genomic DNA, using the 177 12attB1 F (5′-AAA AAG CAG GCT TGT GTG GAG TAC GGC TGC AT-3′) and

177 12attB2 R (5′-AGA AAG CTG GGT AGA GGC TAT TAC TGT GTA TC-3′) primers, and cloned by a GATEWAY BP reaction (Invitrogen) into the binary vector pHELLSGATE 2 (Wesley *et al*, 2001). Plants were transformed by the standard floral dip method (Clough and Bent 1998).

DNA methylation analysis

Genomic bisulfite sequencing was performed as described by Paulin *et al* (1998). Detail is shown in Supplementary data.

DNA methylation was also analyzed by restriction enzymes *Bgl*II and *Sau*3AI (Figure 3C). A 100 ng weight of genomic DNA was digested with *Bgl*II and *Eco*RI, or *Sau*3AI and *Eco*RI, in 40 μl reaction mix. Control 'undigest' sample was digested with *Eco*RI alone. After digestion, PCR was performed by using 1 μl of the digested sample as a template. BNS F2 and BNS R3 (5′-TTC CTT ATG ACA TTT CAA GGT C-3′) primers were used for *Bgl*II-digested DNA, and BNS F3 (5′-GTA ATG GAG ACA CAT ACG TCA C-3′) and BNS R4 (5′-TAC AAA GCC AGG AAC AGT TTT G-3′) were used for *Sau*3AI-digested DNA.

Small RNA Northern analysis

Small RNA was isolated from mature leaves using the mirVana miRNA isolation kit (Ambion). RNA (25∼30 μg) was resolved on denaturing polyacrylamide/urea gels (15%). Electroblotting and hybridization were performed as described (Llave *et al*, 2002). Hybridization was performed overnight at 38°C using PerfectHyb Plus buffer (Sigma). Blots were washed at 42°C in 2× SSC, 0.2% SDS for 10 min, and in 0.5× SSC, 0.1% SDS for 60 min, and analyzed by BAS-2500 (Fuji film). BNS middle probe was 5′-TGG TTT CTT CAG TAT CAT CAG TTT TAA CAG CAA GCA CTG G-3′ and BNS 3′ probe was 5′-A+AG A+GC T+AG A+TC A+CG C+CA A+GT T+TC A+GC A+TC T+GC T+TG C+TG A-3′ (+ indicates LNA-modified bases). For the LINE probe, a PCR fragment amplified from genomic DNA by using LINE FB5 and LINE RD primers was used.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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