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Relationship between allelic state of T-DNA and DNA methylation of chromosomal integration region in transformed *Arabidopsis thaliana* plants

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Abstract

T-DNA insertions are currently used as a tool to introduce, or knock out, specific genes. The expression of the inserted gene is frequently haphazard and up to now, it was proposed that transgene expression depends on the site of insertion within the genome, as well as the number of copies of the transgene. In this paper, we show that the allelic state of a T-DNA insertion can be at the origin of epigenetic silencing. A T-DNA insertional mutant was characterized to explore the function of *AtBP80a′*, a vacuolar sorting receptor previously associated with germination. Seeds homozygous for the T-DNA do not germinate, but this can be overcome by a cold treatment and maintained by the following generations. The non-germinating phenotype is only observed in homozygous seed produced by heterozygous plants indicating that it is correlated with the allelic state of the T-DNA in parental lines. Analysis of the region between the T-DNA insertion and the ATG codon of *atbp80a′* showed that cytosine methylation is highly enhanced in chromatin containing the T-DNA. Data presented here show that an unpaired DNA region during meiosis could be at the origin of a *de novo* cytosine methylation mechanism.

Abbreviations: KAN^R, kanamycin resistant; T-DNA, transfer DNA; VSR, Vacuolar Sorting Receptor; WT, wild type

Introduction

Epigenetic silencing causes problems for the agricultural use of transgenic plants, since the silencing of gene expression by a DNA fragment present in excess in the genome has often been reported (Bourque, 1995). However, plants take advantage of the silencing mechanisms to protect their own genome integrity against proliferation of mobile elements such as transposons and viruses (Kumpatla *et al.*, 1998). Epigenetic phenomena are not fully understood, but both methylation of cytosine residues in DNA, and chromatin remodeling appeared to play a key function in epigenetic control in various organisms (Li *et al.*, 2002). These modifications do not alter DNA sequence, but result in changes of DNA accessibility for regulatory proteins (Meyer, 2001). These mechanisms would allow stable propagation of gene activity states from one generation to the next. Knowledge of *de novo* methylation mechanisms mainly comes from the characterization of transgenic plants. It was reported that transcriptional gene silencing is often associated to DNA

methylation and chromatin remodeling (Meyer, 2000; Paszkowski and Whitham, 2001; Verbsky *et al*., 2001). It can be induced by DNA–DNA pairing mechanism (Vaucheret, 1993) and experimental evidence also indicates that RNA–DNA coupling can also be at the origin of *de novo* DNA methylation (Wassenegger *et al*., 1994; Matzke *et al*., 2001). In *Caenorhabditis elegans*, it was observed that DNA lacking a partner during meiosis, such as the X chromosome in males, could be silenced by histone methylation (Bean *et al*., 2004) indicating that the genetic imprinting of individual loci or whole chromosomes was established during gametogenesis. In the haploid fungus *Neurospora crassa*, it was shown that a gene unpaired from its homologue during meiosis, could induce the silencing of all homologous sequences by a process named Meiotic Silencing by Unpaired DNA (MSUD) (Shiu *et al*., 2001; Shiu and Metzenberg, 2002).

In this work, to explore the function of *AtBP80a′*, a vacuolar sorting receptor from *Arabidopsis thaliana* previously associated with germination (Cao *et al*., 2000; Laval *et al*., 2003), a T-DNA insertional mutant containing a kanamycin resistance gene (KAN^R) was used to evaluate the role of *AtBP80a′ in planta*. We show that the T-DNA insertion located in the promoter region of the VSR gene is responsible for seed germination inhibition through an epigenetic mechanism. To gain new insight into epigenetic mechanisms, the *atbp80a′* mutant was then used as a tool to explore the existence of a relationship between the allelic state of the T-DNA and DNA methylation.

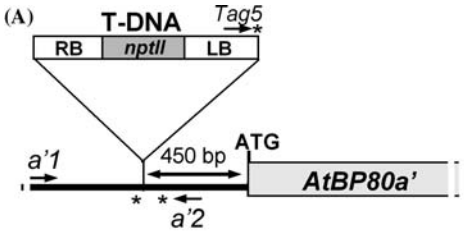
Materials and methods

Plant material

Arabidopsis thaliana ecotype Wassilewskija (Ws-4) was used for the wild type (WT) and the mutant. The *atbp80a′ A. thaliana* mutant came from the FLAGdb FST database (http://genoplante-info.infobiogen.fr/FLAGdb) (Samson *et al*., 2002).

DNA analysis

Genomic DNA was extracted as reported (Laval *et al*., 2003). PCR assays performed to evaluate the allelic state of *atbp80a′* used primers complementary to the left border of the T-DNA (*Tag5*: 5′ctacaaattgccttttcttatcgac3′) and sequences located upstream (*a′1*: 5′tcacatgcattccatgaatgtg3′) or downstream (*a′2*: 5′ataacgctatcaagccacgtg3′) of the T-DNA insertion site (Figure 1A).



(B)

T4	Non germinating seeds (%)	Kan resistant plants (%)	Kan sensitive plants (%)
WT	2.6 ± 0.7	0 ± 0	97.4 ± 0.7
T4.1	21.4 ± 0.06	67.9 ± 0.1	32.5 ± 1.9
T4.2	29.4 ± 3.9	69.7 ± 5.7	30.3 ± 5.7
T4.3	21.6 ± 3.4	75.1 ± 4.4	24.6 ± 4.2
T4.4	26.1 ± 2.6	76.3 ± 1.2	23.6 ± 1.2
T4.5	29 ± 1.9	71.2 ± 2.0	28.7 ± 2.0
T4.6	22.8 ± 2.5	73.6 ± 5.2	26.3 ± 5.7
T4.7	16.8 ± 3.0	72.9 ± 2.7	27 ± 2.7
T4.8	28.5 ± 4.1	76.6 ± 4.2	23.3 ± 4.2
T4.9	28.9 ± 4.7	73.4 ± 3.2	26.5 ± 3.1
T4.10	25.2 ± 5.8	78.9 ± 8.0	20.7 ± 7.6

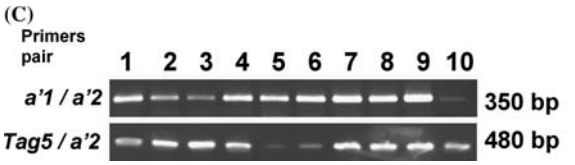


Figure 1. Molecular analyses. (A) Schematic representation of the insertion zone of the T-DNA in the *atbp80a′* gene. Primers used for the molecular characterization of the mutant lines are indicated by arrows (*Tag5*; *a′1*; *a′2*) and annealing region of primers used for methylation analysis by bisulfite sequencing are indicated by asterisk. (B) Results of germination tests performed on several lots of T4 seeds (T4.1 to T4.10). Results of germination and of kanamycin-resistant and -sensitive plants are given as percentages of total seed number sowed on MS medium containing a selective agent (kanamycin). Results are the means of three independent experiments. (C) PCR analysis carried-out on DNA extracted from KAN^R plants with different primer combinations, corresponding either to the T-DNA left border (*Tag5*) or to regions upstream (*a′1*) and downstream (*a′2*) of the insertion area.

Viability tests and germination assays

To estimate embryo viability, integuments from seeds were removed and embryos were incubated in a solution containing 1% fluorescein diacetate for 30 min, washed in distilled water and observed under fluorescence microscopy at 470–490 nm excitation. Germination assays were performed using different seed batches. Each of them was sown in triplicate (80–100 seeds per Petri dish) on water-soaked filter paper and incubated in a growth chamber (23 °C, 16 h light). For kanamycin segregation analysis, germination was done on selective medium containing 75 μg/ml kanamycin. Germination was scored every day during a 5-day period after imbibition. For stratification, sown seeds were placed for 48 h at 4 °C before transfer to 23 °C.

Mucilage detection and electron microscopy analyses

Seeds were imbibed for 24 h in water before being transferred to an aqueous solution of 0.03% (w/v) ruthenium red for mucilage detection at room temperature for 10 min (Western *et al.*, 2000). They were rinsed with distilled water before observation under stereomicroscope. For scanning electron microscopy, mature dried seeds were coated with gold and examined with an electron microscope (Hitachi C450) at an acceleration voltage of 15 kV.

DNA methylation analyses by bisulfite genomic sequencing

Genomic DNA (500 ng) was mixed with 1 μg of salmon sperm DNA and digested at 37 °C with *Xho*I before alkaline denaturation with 0.6 M NaOH. The bisulfite method (Grunau *et al.*, 2001) was used. For DNA prepared from *atbp80a′* mutant, PCR amplification was performed using a primer complementary to the left border of the T-DNA (5′ggtaataggatactgggattcgttttggat3′) and a primer anchored to the ATG initiation translation region (5′aaataacacagaaactgcttcatcttcaaa3′). For DNA obtained from WT, PCR was carried-out using a primer located 650 bp upstream of the ATG codon (5′aattaattgttttgttatatggaaagaggt3′) and the same primer corresponding to ATG region. PCR products were cloned in pGEM-T (Promega) and sequenced.

Results

Analysis of atbp80a′ mutant phenotype

T3 seeds corresponding to the mutant were sown on selective medium containing kanamycin to discriminate WT and transformed lines. PCR was performed on DNA from resistant plants, using the primers indicated in Figure 1A. We found that all the T3 plants analyzed were hemizygous for the transgene. PCR products resulting from *Tag5*/*a′2* primer amplification were sequenced to confirm the T-DNA location 450 nucleotides upstream of the ATG translation initiation codon (Figure 1A). Different southern blots using a radiolabeled probe corresponding to the complete T-DNA or to *nptII* gene indicated that the genome of the *atbp80a′* mutant contained a single T-DNA insertion as suggested by kanamycin segregation analyses (not shown). To check the presence of repeated sequences in the T-DNA, several PCR experiments were performed using different primer combinations corresponding to either T-DNA, or to the insertion flanking region. All the results indicated that a single T-DNA was inserted. Germination assays of T4 seeds on selective medium indicated that 25% of seeds failed to germinate, 25% were kanamycin sensitive and 50% were KAN^R (Figure 1B). This suggests that T4 germinating KAN^R seeds were hemizygous for the T-DNA, as confirmed by PCR analysis (Figure 1C).

Scanning electron microscopy was used to compare seeds of the WT with that of the *atbp80a′* mutant (Figure 2A–D). The surface of *Arabidopsis* WT seed consists of polygonal structure with a central elevation named *columella* (Figure 2A, B). Hexagonal structures were easily distinguishable by their thickened radial cell walls. However, about 1/4 of the seeds produced by plants hemizygous for the T-DNA, were irregularly shaped, with thinner cell walls (Figure 2C, D). Seeds also excrete a layer of mucilage upon imbibition and can be visualized by staining with ruthenium red. In all the WT seeds, a classical pink-stained halo is observed (Figure 2E, F) indicating the presence of acidic pectic components (Western *et al.*, 2000). In around 1/4 of the seeds derived from plants hemizygous for the T-DNA, the ruthenium red staining was not observed (Figure 2G, H). Collectively, these data indicated that seeds homozygous

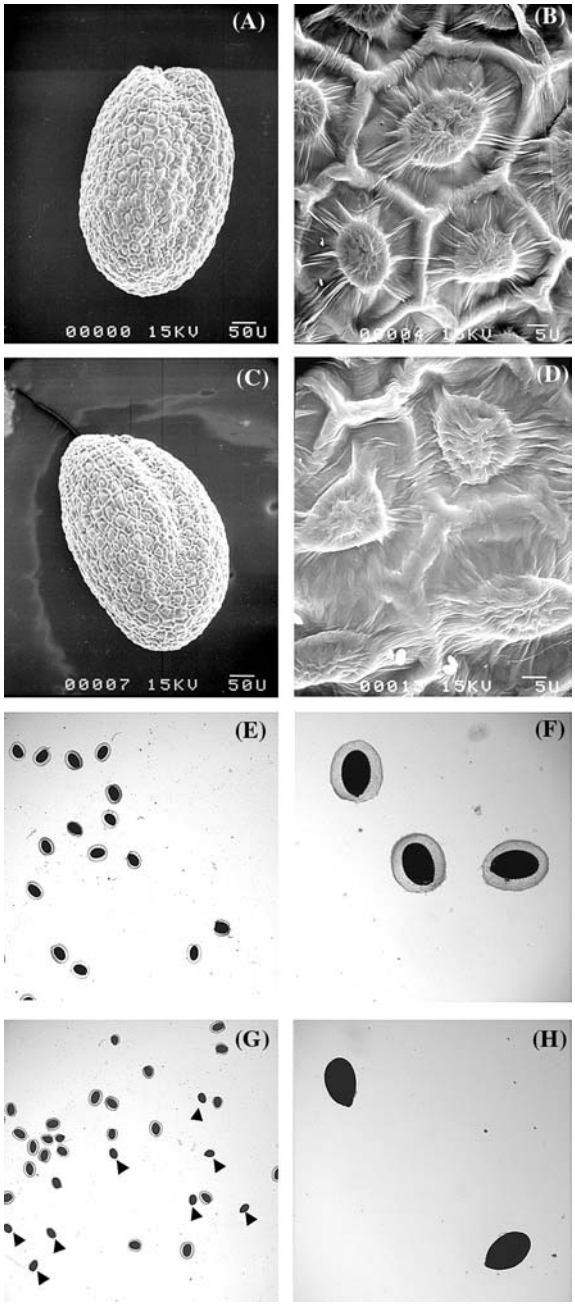


Figure 2. Scanning electron micrographs showing whole seeds (A, C) and magnifications (B, D) of WT (A, B) and *atbp80a′* (C, D). Ruthenium red staining of WT (E, F) and seed produced by hemizygous T3 plants (G, H). In G, arrows show seeds without ruthenium red coloration.

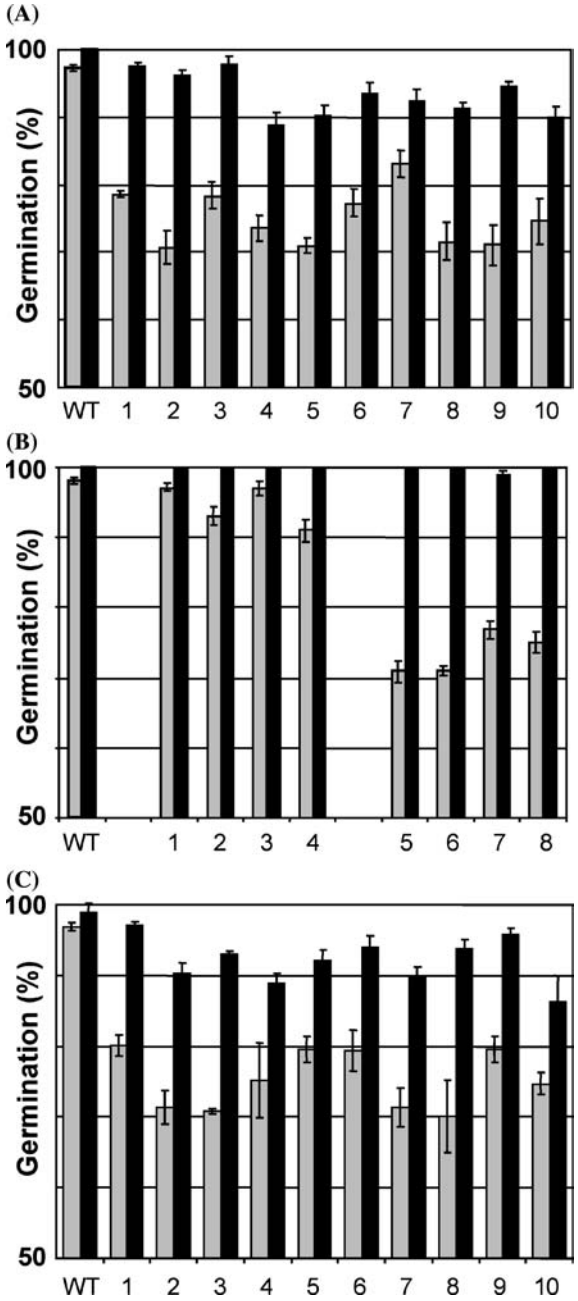
for the T-DNA insertion showed alterations in seed coat structure and lacked mucilage upon imbibition compared to the WT.

Stratification provokes atbp80a′ seed germination

Failure to germinate may be due to various external or internal factors such as seed conservation, seed viability, dormancy or seed-coat-imposed inhibition (Koornneef *et al*., 2002). Since seed germination can be influenced by a large number of parameters, all the analyses were performed on several independent lines to avoid misinterpretations. Seed viability tests indicated that in all the seeds from WT and hemizygous plants, the embryo was alive and various treatments were applied to induce germination of homozygous seeds. Since 25% of seeds produced by hemizygous plants showed seed coat alterations compared to the wild type, a germination test was carried-out on seeds without seed coats. No change in germination was observed indicating that the failure to germinate was not due to seed-coat inhibition. Only a cold treatment known as stratification (48 h at 4 °C) induced germination of all seeds derived from hemizygous T3 plants (Figure 3A). PCR analyses indicated the presence of plants homozygous for the T-DNA among the resistant plants, showing that the failure to germinate could be overcome by cold treatment. The seeds produced by plants homozygous for the T-DNA were tested for germination. Whatever the germination conditions (with or without stratification treatments), all the seed lots tested fully germinated (Figure 3B). None of those seeds showed alteration in seed coat structure and mucilage production. In order to evaluate the heritability of this character, the germination capacity of T5 seeds, obtained from homozygous T4 plants, was assessed. T5 seeds showed the same germination level as WT.

Figure 3. Germination analyses. Germination was scored after 5 days imbibition on seeds directly placed in growth chambers at 23 °C (gray bars) or after a stratification treatment 48 h at 4 °C (black bars). Results are the means of three independent experiments. (A) Effect of the stratification treatment on WT and T4 seed germination. T4 seeds were obtained from plants T3 hemizygous for the T-DNA. WT, wild type; lanes 1–10 correspond to T4 seeds from individual parents. (B) Effect of stratification on WT and T4 seed germination. WT, wild type; lanes 1–4 correspond to homozygous T4 seeds, and lanes 5 to 8 to seeds produced from hemizygous T3 plants. (C) Effect of stratification on WT and F2 seeds produced after back-cross between WT and homozygous *atbp80a′* mutant. WT, wild type; lanes 1–10 correspond to F2 seeds from individual parents.

In conclusion, the non-germination of seeds homozygous for the T-DNA was suppressed by a cold treatment, and the ability to germinate was heritable by the following homozygous generations. Only homozygous seeds produced by a hemizygous parent are defective in germination. Since these observations cannot be explained by classical Mendelian genetics, the existence of an epigenetic control was assumed.



Germination of T-DNA homozygous seeds is linked to the T-DNA allelic state of the parental lines

To evaluate the existence of an epigenetic control mechanism, linked to the allelic state of the plants, and to explain the reversible phenotype observed for the *atbp80a′* mutant, T-DNA homozygous plants were backcrossed with WT. Hemizygous F1 plants obtained were self-pollinated and F2 seeds were harvested and tested for germination (Figure 3C). Without stratification, about 75% of F2 seeds obtained from the backcross, germinated and 50% were KAN^R as previously observed for the original hemizygous parental line. Stratification produced germination of all the F2 seeds. These data show that the non-germinating phenotype can only be observed in the absence of stratification with seeds produced by parent hemizygous for the T-DNA.

The T-DNA insertion zone is highly methylated in non-germinating homozygous seeds

In *atbp80a′*, the T-DNA was located 450 bp upstream of the ATG initiation codon. According to EST available data, the T-DNA insertion occurred into the promoter region, 313 bp upstream the initiation site of transcription. Among the mechanisms involved in epigenetic gene silencing, methylation of cytosines is a well-known DNA modification observed in plants and other eukaryotes. It provides heritable genetic mark linked to gene transcription (Bender, 2004). Mapping of methylated cytosines in the T-DNA insertion region was carried-out to estimate the degree of cytosine methylation on the region upstream of the ATG translation initiation codon of *AtBP80a′*. This was performed on genomic DNA prepared from WT seeds, from *atbp80a′* homozygous seeds able to germinate, and from seeds produced by a hemizygous plant from the backcross experiment. In WT and the T-DNA tagged mutant homozygous seeds able to germinate, none of the cytosines present in the 650 bp analyzed were methylated (results not shown). In contrast, for hemizygous seeds, all the cytosines of the 593 bp upstream the ATG codon in the T-DNA containing chromatids, were methylated except for four residues that presented a variable methylation state (Figure 4).

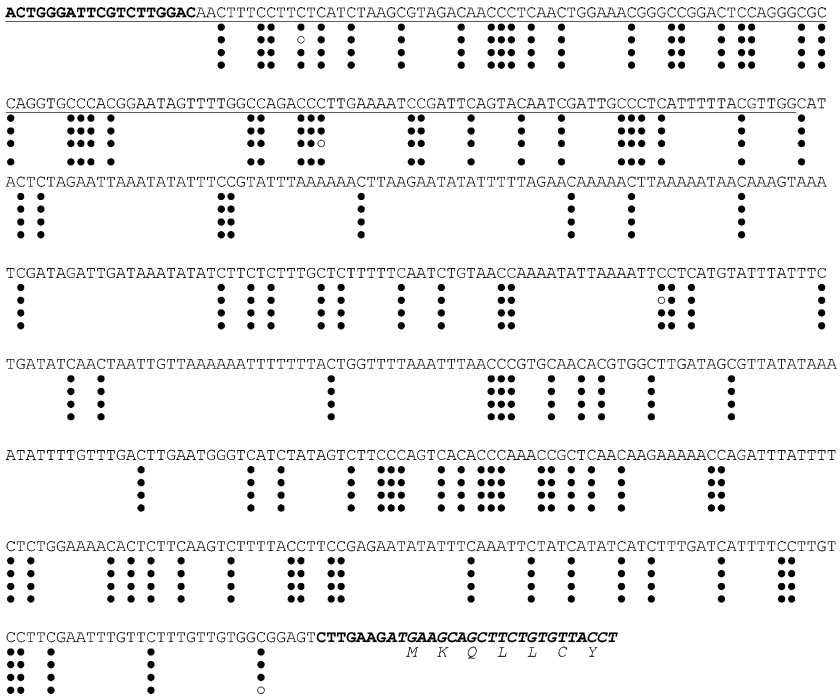


Figure 4. DNA methylation analysis of the T-DNA insertion zone in seeds produced by hemizygous plants by bisulfite sequencing. T-DNA sequence is underlined. The coding sequence and the deduced amino acid sequence of *AtBP80a′* is in italics. Sequences in bold correspond to PCR primers used. Each line shows the result of the sequencing of a different clone. Black circles indicate the methylated cytosines and empty circles indicate the non-methylated residues. Cytosine methylation was observed at symmetrical as well as non-symmetrical positions.

Discussion

Results presented here show that a T-DNA insertion in the promoter region of the *AtBP80a′* gene in the hemizygous state blocks the germination of homozygous seeds in the progeny, as well as induces alteration of seed coat. In a previous work, using antisense strategy, we showed that the extinction of all the *AtBP80* genes was associated with seed germination failure (Laval *et al*., 2003). The *atbp80a′* mutant also exhibits unexpected behavior: stratification allowed complete germination of homozygous seeds, and the progeny of homozygous parents was able to germinate without cold treatment. In addition, those seeds did not show the integument and mucilage abnormalities. The mutant phenotype was only inherited when the parental lines were hemizygous for the T-DNA. These data indicate that different phenotypes can be observed for seeds having the same genotype and points to the existence of an epigenetic mechanism (Wolffe and Matzke, 1999). In addition, homozygous seeds produced by a hemizygous parent contain heavily methylated cytosines, at symmetrical as well as non-symmetrical positions, in the region between the T-DNA insertion and the ATG codon. At the hemizygous state, the T-DNA would be unpaired during the prophase of meiosis and constitute a target for modifications. The T-DNA and neighboring regions would then carry heritable epigenetic information. The resulting homozygous seeds would be unable to germinate because of the modifications carried on their two chromatids. In the case of homozygous plants, two T-DNAs would be paired during the prophase of meiosis and not subjected to epigenetic modifications and hence resulting homozygous seeds are able to fully germinate (Figure 5). We showed that the homozygous seeds are unable to germinate only when produced by a hemizygous parent, as it was corroborated by the backcross experiment where 25% of the progeny from hemizygous parent did not germinate. Similar observations were previously reported (Klöti *et al*., 2002) using transgenic rice plants carrying a beta-glucuronidase reporter

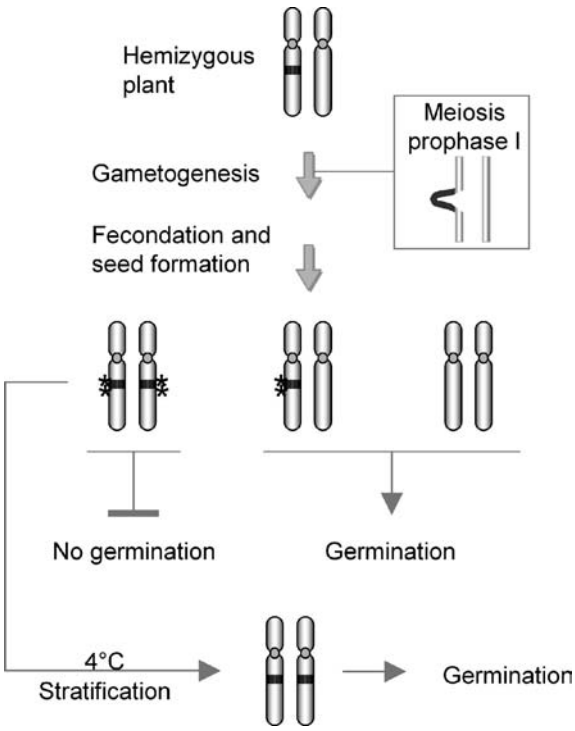


Figure 5. A model for activation of silencing mechanisms in *atbp80a′* T-DNA insertional mutant. In this model, only one of the two sister chromatids is present. In hemizygous plants, during meiosis, the T-DNA (black) forms an unpaired DNA loop that may be responsible for cytosine methylation (*) in the T-DNA insertion region. Two types of gametes were then produced, sharing either an epigenetic mark or not. After fecundation and seed formation, seeds having at least one WT gamete are able to germinate while those homozygous for the T-DNA cannot. Stratification treatment (4 °C) can suppress the epigenetic mark and allow seed germination.

gene under the control of the rice tungro bacilliform virus promoter. The silencing of homozygous lines was observed when those lines were produced from hemizygous parents. The silencing was correlated with increase of methylation of the respective promoter region. This expression pattern was maintained only in homozygous lines and backcrossing experiments with non-transformed rice resulted in F1 plants with reappearance of beta-glucuronidase expression. Collectively, these data indicate that gene expression might be epigenetically controlled by DNA pairing mechanisms as previously proposed (Della Vedova and Cone, 2004; Grant-Downton and Dickinson, 2004). *NptII* gene could also be a target of de novo methylation but surprisingly, kanamycin resistance behaves as expected. *NptII* gene was under the control of the 35S promoter and it was shown that 35S promoter is one of the simplest promoters that can be modified without effect on its efficiency (Benfey and Chua, 1990; Kuhlemeier, 1992). However, the efficiency of 35S promoter is sensitive to cytosine methylation level (Park *et al*., 1996; Mittelsten Scheid *et al*., 1998). In the experiment described here, a possible explanation is that this methylation can be transient and occurs only at particular developmental stages such as during germination (Zluvova *et al*., 2001). It was proposed that cytosine methylation pattern is very dynamic and data about the mechanisms that establish, maintain and modify this pattern are still scarce (Richard and Elgin, 2002). During germination process and seedling growth, methylation of the 35S promoter can be either reduced or erased allowing transcription of kanamycin resistance gene.

In the haploid *Neurospora crassa*, a gene not paired with its homologue in prophase I of meiosis forms a DNA loop, initiating Meiotic Silencing by Unpaired DNA (MSUD) (Shiu *et al*., 2001; Lee *et al*., 2004). This loop is detected and leads to a transient silencing by a post-transcriptional mechanism of all homologous sequences, including those that are themselves paired. In the *atbp80a′* mutant, it would be attractive to propose that MSUD mechanisms, recently described in *Neurospora*, are at the origin of the observed phenomenon. In *Neurospora*, it was now established that gene silencing depends on post-transcriptional mechanisms and recent data using *Arabidopsis* T-DNA transformants indicate that large variability in transgene expression is correlated to RNA sensing mechanisms (Schubert *et al*., 2004). Using systematic approach to analyze transgene expression in *Arabidopsis*, it was showed that difference in expression level couldn't be attributed to position effects but to post-transcriptional control of gene silencing. In our work, even if a chimeric aberant RNA was detected by RT-PCR analysis between the T-DNA and the *atbp80a′* gene (data not shown), this RNA cannot be considered as the silencing signal. In such case, we would expect that this RNA acts dominantly in *trans* by silencing the other *AtBP80* genes present in the *Arabidopsis* genome and all hemizygous seed produced from a hemizygous parent should not germinate. So we propose that silencing involves a *cis*-acting mechanism *via* methylation spreading in *cis* from the T-DNA. Similar observations were previ-

ously reported, showing that cytosine methylation can spread from the T-DNA or a foreign DNA to the host genome (Pelissier *et al*., 1999). It was also reported that the methylation pattern of a chromosomal region strongly influences gene activity of T-DNA by imposing DNA methylation at the borders of the integrated DNA (Pröls and Meyer, 1992). It is reasonable to propose that methylation spreading over the T-DNA into the *AtBP80a′* remaining promoter causes gene silencing.

Seed stratification is commonly used to break seed dormancy and to synchronize seed germination (Bewley and Black, 1994). However, the molecular mechanisms affected by the cold treatment are still unclear. In this work, we established on one hand that T-DNA homozygous seeds unable to germinate present a high level of methylated cytosines in the promoter region of *AtBP80a′*. On the other hand, the T5 seeds homozygous for the T-DNA produced by cold treated T4 homozygous plants did not showed methylation in the same region. We propose that the cold phase was at the origin of demethylation in the promoter region, leading to germination. It was already demonstrated that plant-virus interactions depend on epigenetic mechanisms and that low temperature modifies gene silencing, leading to plant hypersensitivity to viruses (Szittya *et al*., 2003). This suggests a relationship between DNA methylation level and environmental conditions, such as cold period. We cannot exclude that in natural conditions, maintenance of seed dormancy involves epigenetic mechanisms such as DNA methylation. Similar mechanisms have proposed that a cold treatment produced an extensive DNA demethylation that was essential for flower promotion (Burn *et al*., 1993). In the same way, DNA de-methylation was observed during breaking of dormancy in potato bud (Law and Suttle, 2003; Horvath *et al*., 2003). In summary, germination of the *atbp80a′* mutant is correlated to the presence of methylated cytosines in the promoter region, inhibition of germination, and alteration of seed coat. A cold treatment probably induces of DNA demethylation allowing normal seeds production and germination.

Acknowledgments

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