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Epigenetic Suppression of T-DNA Insertion Mutants in *Arabidopsis*

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ABSTRACT T-DNA insertion mutants have been widely used to define gene functions in *Arabidopsis* and in other plants. Here, we report an unexpected phenomenon of epigenetic suppression of T-DNA insertion mutants in *Arabidopsis*. When the two T-DNA insertion mutants, *yuc1-1* and *ag-TD*, were crossed together, the defects in all of the *ag-TD* plants in the F2 population were partially suppressed regardless of the presence of *yuc1-1*. Conversion of *ag-TD* to the suppressed *ag-TD* (named as *ag-TD) did not follow the laws of Mendelian genetics. The *ag-TD** could be stably transmitted for many generations without reverting to *ag-TD*, and *ag-TD** had the capacity to convert *ag-TD* to *ag-TD**. We show that epigenetic suppression of T-DNA mutants is not a rare event, but certain structural features in the T-DNA mutants are needed in order for the suppression to take place. The suppressed T-DNA mutants we observed were all intronic T-DNA mutants and the T-DNA fragments in both the trigger T-DNA as well as in the suppressed T-DNA shared stretches of identical sequences. We demonstrate that the suppression of intronic T-DNA mutants is mediated by *trans*-interactions between two T-DNA insertions. This work shows that caution is needed when intronic T-DNA mutants are used.**

Key words: epigenetics; T-DNA mutant; genetic suppression; *trans*-interaction; YUC.

INTRODUCTION

Agrobacterium-mediated plant transformation is achieved when the T-DNA (Transfer DNA) fragment from the modified Ti plasmids is integrated into chromosomes in plant cells. T-DNA transformation can be used as a tool for insertional mutagenesis and also serves as an efficient vehicle for delivering target genes into plant cells. T-DNA fragments randomly insert into a plant genome during transformation and, when a T-DNA insertion is inserted in an exon or an intron, it often leads to the inactivation of the gene. As part of the different functional genomic initiatives in *Arabidopsis*, a number of T-DNA insertional mutagenesis have been conducted and currently we have access to large libraries of sequence-indexed T-DNA insertion lines in *Arabidopsis* (Samson et al., 2002; Sessions et al., 2002; Alonso et al., 2003). The T-DNA insertion mutants are tremendous resources for the determination of gene function and the elucidation of metabolic/signaling pathways. T-DNA mutants in *Arabidopsis* have become the first choice for many scientists because (1) the mutants are easily accessible through the *Arabidopsis* stock centers and (2) the mutants are often null alleles. T-DNA insertion mutants have been extensively used in reverse genetics and in studies of genetic interactions in *Arabidopsis*.

Studies on genetic interactions between two non-allelic mutants often provide insights into the functions of the two genes and the relative positions of the genes in a genetic pathway (Guarente, 1993; Hodgkin, 2005). Phenotypes of a mutant can be suppressed or enhanced by mutations in other genes. Synergistic genetic enhancement between two mutants often suggests that the two genes have overlapping functions or participate in parallel pathways (Guarente, 1993). If the two mutants are not null alleles and the two genes have no sequence homology, synergistic enhancement can also suggest that the two genes function in the same pathway (Cheng et al., 2008). Genetic suppression of the phenotypes of one mutant by a mutation in another gene could be achieved through several mechanisms (Hodgkin, 2005). A mutant could be rescued if the general machinery of transcription and/or translation is altered. For example, a mutation that converts a sense codon to the stop codon UAG in a gene can be suppressed if the anti-codon in Trp-tRNA is mutated from CCA to CUA. Additionally, genetic suppression could also take place if the suppressor removes toxic proteins or metabolic intermediates. A mutant can also be suppressed

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if protein interactions or gene dosages are altered. Genetic screens for enhancers and suppressors for mutants have led to the discoveries of the regulatory mechanisms of major signaling and metabolic pathways.

In general, the phenotypes of a mutation are suppressed when an extragenic suppressor is present. Removal of the suppressor leads to the restoration of the original mutant phenotypes. In this paper, we report an unexpected phenomenon that phenotypes of a T-DNA insertion mutant are partially suppressed by another T-DNA insertion at another locus. Remarkably, the suppressed phenotypes could be stably transmitted for generations even in the absence of the suppressor T-DNA insertion. We crossed an auxin biosynthesis mutant *yuc1-1* to a floral mutant *ag-TD* in order to generate the *yuc1-1ag-TD* double mutants for analyzing the roles of auxin in flower development. Both *yuc1-1* and *ag-TD* are T-DNA insertion mutants (Figure 1A) and both are loss-of-function, recessive mutants. The *YUC1* gene encodes a flavin-containing monooxygenase involved in auxin biosynthesis (Zhao et al., 2001; Cheng et al., 2006, 2007). The *yuc1-1* mutant has no obvious developmental defects because of the existence of other homologous *YUC* genes in *Arabidopsis* (Cheng et al., 2006). *AGAMOUS* (*AG*) is an essential gene for reproductive organ formation in *Arabidopsis* (Yanofsky et al., 1990). The *ag-TD* mutant displays the characteristic *ag* loss-of-function phenotypes including the transformation of stamens into petals, loss of floral meristem determinacy, and a lack of carpels and stamens (Yanofsky et al., 1990). Surprisingly, none of the *ag-TD* plants in the F2 population displayed the typical *ag-TD* phenotypes, regardless of the presences of *yuc1-1*. We demonstrate that suppression of *ag-TD* is mediated by *trans*-interaction between the T-DNA insertions in *yuc1-1* and *ag-TD*. Although gene silencing mediated by *trans*-interaction between two T-DNA insertions has been well documented (Daxinger et al., 2008), it has never been reported previously that such a *trans*-interaction among T-DNA insertions can lead to the restoration of gene functions inactivated by the same T-DNA insertions. We show that suppression of intronic T-DNA insertional mutants is frequently induced by other T-DNA insertions, suggesting that caution is needed when intronic T-DNA mutants are used in *Arabidopsis*.

RESULTS

Suppression of an *agamous* T-DNA Insertion Mutant by *yuc1-1*

To investigate the mechanisms of local auxin biosynthesis in specifying flower development, we combined the auxin biosynthesis mutant *yucca1* (*yuc1*) (Cheng et al., 2006) with a known floral homeotic mutant *agamous* (*ag*) (Yanofsky et al., 1990). We chose the recessive T-DNA insertion mutants *ag-TD* and *yuc1-1* because both mutants are in the Columbia background. In both *ag-TD* and *yuc1-1*, the T-DNA is inserted in an intronic region (Figure 1A). The *yuc1-1* does not show obvious developmental defects (Cheng et al., 2006), but *ag-TD* fails to produce any stamens and carpels (Figure 1). We crossed *ag-TD*$^{+/-}$ to *yuc1-1* and

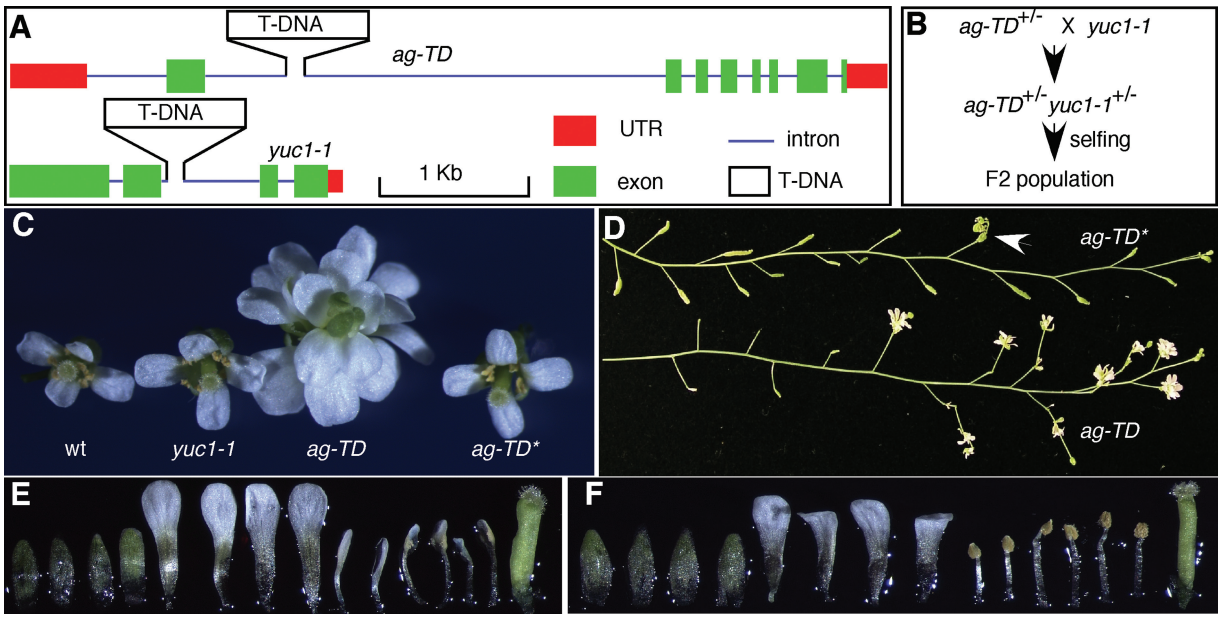


Figure 1. Suppression of *ag-TD* by Crossing *ag-TD*$^{+/-}$ to *yuc1-1*.
(A) A diagrammatic presentation of the two T-DNA insertion mutants: *yuc1-1* and *ag-TD*.
(B) The crossing scheme. The *ag-TD*$^{+/+}$ *yuc1-1*$^{+/-}$ F1 plants were discarded.
(C) Suppression of the floral defects in *ag-TD* in the F2 population. From left to right: WT, *yuc1-1*, *ag-TD*, and *ag-TD**.
(D) Phenotypic difference between *ag-TD* and *ag-TD** inflorescence.
(E) Production of petal-like stamens in *ag-TD**. **(F)** Normal floral organs in *ag-TD**.

genotyped the F1 plants to select the *ag-TD*$^{+/-}$ *yuc1-1*$^{+/-}$ plants (Figure 1B), which did not have obvious defects as expected. We let the F1 *ag-TD*$^{+/-}$ *yuc1-1*$^{+/-}$ plants self-pollinate and collected the F2 seeds (Figure 1B). We analyzed the F2 population in order to identify the *ag-TD yuc1-1* double mutants. Unexpectedly, none of the *ag-TD* plants displayed the typical *ag* phenotypes, indicating that *ag-TD* phenotypes were partially suppressed (Figure 1C). We named the plants with *ag-TD* genotype but without *ag* flower phenotypes as *ag-TD** (Figure 1C). Note that *ag-TD** is still homozygous for the T-DNA insertion as shown in Figure 1A, but the *AG* function is no longer inactivated by the T-DNA insertion in *ag-TD**. The *ag-TD** plants were fertile and produced viable seeds (Figure 1D). The suppression of *ag-TD* was only partial, because some *ag-TD** flowers still contained petal-like stamens (Figure 1E) and indeterminate flowers (Figure 1D). However, the majority of *ag-TD** flowers had flower with four sepals, four petals, and one gynoecium consisting of two fused carpels (Figure 1F).

The *yuc1-1* Is Not Required in the F2 Population for *ag-TD** Phenotypes

We genotyped the F2 plants from the cross between *ag-TD* and *yuc1-1* for the presence of *ag-TD* and *yuc1-1*. Among the 176 F2 individual plants, 56 were *ag-TD*, indicating that the T-DNA insertion at the *AG* locus segregated normally. Among the *ag-TD* plants, 43 did not contain T-DNA insertion at *YUC1*, 13 were *yuc1-1*$^{+/-}$, and zero were *yuc1-1*. Because both *AG* and *YUC1* are on chromosome IV and they are about 15 cM apart, it was expected that very few *ag-TD yuc1-1* would be observed in the F2 population. Floral defects in all of the *ag-TD* plants in the F2 population were partially suppressed. Overall, 80% of the *ag-TD YUC1* plants were suppressed well enough to be fertile. We noticed that all of the *ag-TD*$^{-/-}$ *yuc1-1*$^{+/-}$ plants were able to set seeds, suggesting that the presence of the *yuc1-1* mutation enhanced the suppression. However, the continued presence of the *yuc1-1* mutation was not required to suppress *ag-TD*.

The *ag-TD** Is Genetically Stable

To test whether *ag-TD** phenotypes could be stably transmitted, we let the *ag-TD** plants self-fertilize and studied the progeny for five generations. All of the progeny of *ag-TD** was fertile in every generation and set a good number of seeds. We also noticed that the later generation of *ag-TD** produced more seeds than the earlier generation of *ag-TD** (Figure 2). We concluded that, once *ag-TD* was converted to *ag-TD**, the *ag-TD** does not spontaneously revert to *ag-TD* over generations (Figure 2).

We crossed *ag-TD** to wild-type (WT) Columbia (Col) and let the F1 plants self-pollinate to generate an F2 population for analysis of the genotypes and phenotypes. Among the 98 plants analyzed, 23 were homozygous for the T-DNA insertion at the *AG* locus and all of the *ag-TD* plants displayed the *ag-TD** phenotypes, indicating that *ag-TD** is very stable.

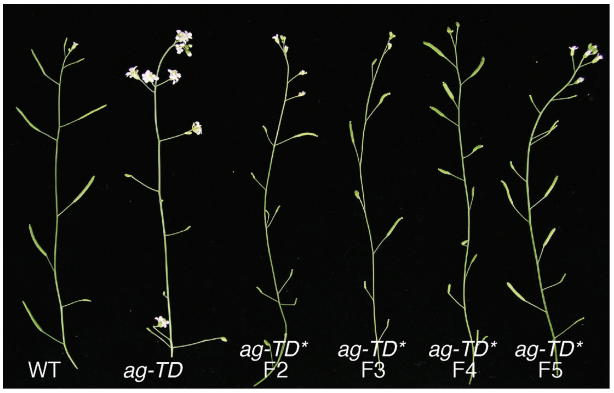


Figure 2. Inheritability of *ag-TD**. The *ag-TD** has been transmitted for five generations. Note that the fifth generation of *ag-TD** produced more seeds than the earlier generations of *ag-TD**.

The *ag-TD** Is Able to Convert *ag-TD* to *ag-TD**

We tested whether *ag-TD** could induce similar changes in *ag-TD*. We crossed *ag-TD** to *ag-TD*$^{+/-}$ plants and half of the resulting F1 plants were homozygous with the T-DNA insertion as expected. The F1 plants that presumably had the *ag-TD*/ag-TD* genotype were fertile and set a good number of seeds. We further analyzed the F2 plants generated from *ag-TD*/ag-TD* selfing. Among the 68 F2 plants analyzed, 66 plants behaved like *ag-TD**. Two plants had weak *ag-TD* phenotypes and did not set seeds. Our data suggest that *ag-TD** has the capacity to convert *ag-TD* into *ag-TD**.

The *ag-TD** Cannot Suppress Non-T-DNA *ag* Alleles

We have shown that *ag-TD** allele induced the conversion of *ag-TD* into *ag-TD**. We investigated whether *ag-TD** could also restore the *AG* functions in other non-T-DNA *ag* mutant alleles. We used the strong *ag-3* mutant and the weak *ag-4* mutant alleles for the experiments (Figure 3A). Both *ag-3* and *ag-4* carried point mutations at splice junction sites (Figure 3A)

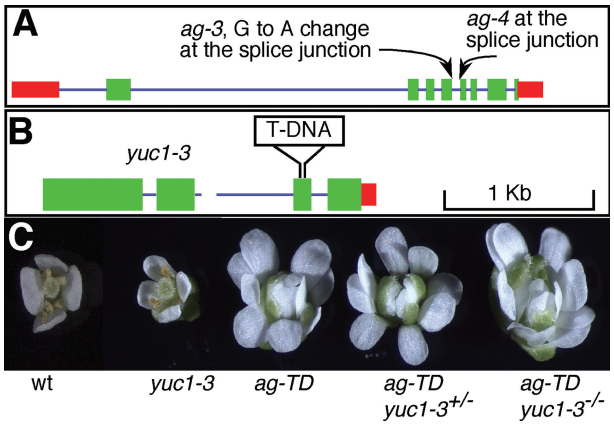


Figure 3. Suppression of *ag* Involves Special Alleles of *ag* and *yuc*.
(A) The two non-T-DNA alleles of *ag* used in this study.
(B) Allele of *yuc1-3*. *yuc1-3* has a T-DNA insertion in the third exon.
(C) The *ag-TD* was not suppressed by *yuc1-3*.

(Jack et al., 1997; Chen and Meyerowitz, 1999). We crossed *ag-TD** to *ag-3*$^{+/-}$, and the resulting F1 *ag-TD*/ag-3* plants still displayed the typical *ag* mutant phenotypes and were sterile, suggesting that *ag-TD** could not rescue *ag-3*. When we crossed *ag-TD** to the weak *ag-4*$^{+/-}$ plants, the *ag-TD*/ag-4* plants were partially fertile. Normally, the *ag-4* plants produce some stamens and carpel-like structures, but are sterile in our growth conditions. The *ag-TD*/ag-4* plants could set seeds and their phenotypes were intermediate when compared to *ag-TD** and *ag-4* plants. We further analyzed the F2 population produced from selfing the *ag-TD*/ag-4* plants. All of the homozygous *ag-TD* plants from the F2 population displayed the same phenotypes as those of *ag-TD**. The *ag-TD*/ag-4* plants in the F2 population were fertile, but all of the *ag-4* plants were sterile. Our data indicate that *ag-TD** could not rescue non-T-DNA *ag* mutants.

Conversion of *ag-TD* to *ag-TD** Depends on a Specific *yuc1* T-DNA Allele

The *ag-TD* mutant was rescued when it was crossed to *yuc1-1* (Figure 1). We tested whether other T-DNA insertion mutants in *yuc1* could also convert *ag-TD* to *ag-TD**. We crossed *ag-TD* to *yuc1-3* (Figure 3B). The *yuc1-3* contained a T-DNA insertion at the third exon in the *YUC1* gene (Figure 3B). Although both *yuc1-1* and *yuc1-3* were T-DNA insertion lines, they were generated using two different plasmids. The *yuc1-1* was generated using the plasmid pROK2, which renders kanamycin resistance in *Arabidopsis*. The *yuc1-3* was produced using a different plasmid that contains the SPM transposase gene and the *BAR* gene.

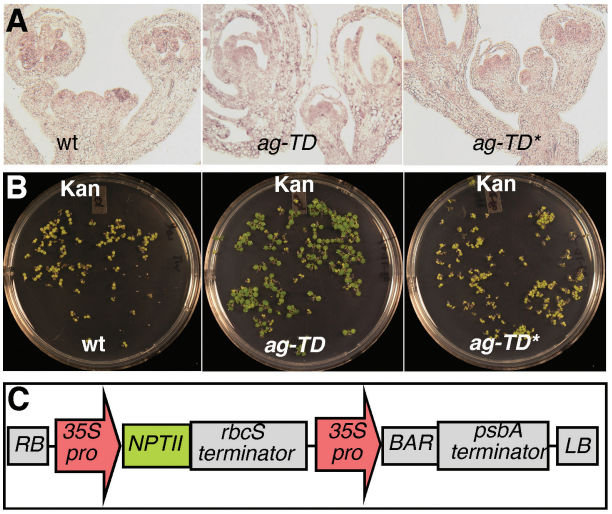
We genotyped the F2 population generated from selfing *ag-TD*$^{+/-}$ *yuc1-3*$^{+/-}$ to identify *ag-TD* plants. All of the *ag-TD* plants in the F2 population displayed the typical *ag-TD* phenotypes regardless of the existence of *yuc1-3* mutation and none of the *ag-TD* plants set any seeds (Figure 3C). We also isolated *ag-TD yuc1-3* plants from the progeny of a single *ag-TD*$^{+/-}$ *yuc1-3* plant and the double mutants behaved like *ag-TD*. These results suggest that inactivation of *YUC1* is not sufficient to trigger the suppression of *ag-TD* and that the suppressor and the suppressed T-DNA mutants need to be generated from similar plasmids.

Production of Full-Length *AG* cDNA Using mRNAs from *ag-TD* and *ag-TD**

We investigated whether the *ag-TD* to *ag-TD** conversion is caused by an increased expression of *AG* in *ag-TD**. We designed PCR primers to amplify the portion of *AG* cDNA starting from the start codon to the stop codon. To our surprise, *ag-TD* produced the full-length *AG* cDNA, suggesting that *ag-TD* is a partial loss-of-function mutant. We sequenced the *AG* cDNA from WT plants, *ag-TD*, and *ag-TD**, and discovered that there were no structural differences among the cDNAs from the analyzed genotypes. It is difficult to compare the expression levels of *AG* in WT and in *ag-TD* using RT–PCR or Northern blot because the floral structures are quite different for the two genotypes. We used RNA *in situ* hybridization to detect the expression levels of *AG* in WT, *ag-TD*, and *ag-TD**. The *AG* expression in *ag-TD* was weaker than that in WT, but *ag-TD** clearly had more *AG* expression than *ag-TD*, suggesting that the conversion of *ag-TD* to *ag-TD** correlates with an increased *AG* mRNA level in *ag-TD** (Figure 4A).

Kanamycin Resistance Gene Is Silenced in *ag-TD**

We hypothesized that perhaps the partial restoration of *AG* function in *ag-TD** might be caused by structural changes in DNA/chromatin in or near the T-DNA insertion. Such DNA/chromatin structural modifications might also alter the expression of the Neomycin phosphotransferase II (*NPT II*) gene, which renders plants resistant to kanamycin, within the T-DNA fragment. The *NPT II* gene in the T-DNA insertion made *ag-TD* plants resistant to kanamycin (Figure 4B) and, accordingly, about 25% of the progeny from *ag-TD*$^{+/-}$ plants were kanamycin-sensitive, suggesting that *ag-TD* contains a single T-DNA insertion. In contrast, all of the *ag-TD** plants were kanamycin-sensitive (Figure 4B), although the *NPT II* gene still existed in *ag-TD**. These data suggest that transcripts from the T-DNA fragment are also affected by the epigenetic modifications that suppressed *ag-TD*.



Phenotypes of T1 Plants	Number of plants (Total = 25)	Percentage
Strong *ag-TD* like	6	24
Weak *ag-TD** (no seeds)	8	32
Strong *ag-TD** (with seeds)	11	44

Figure 4. Suppression of *ag-TD* Is Probably Mediated by *Trans*-Interaction between Two -DNA Insertions.
(A) *In situ* analysis of *AG* expression in WT, *ag-TD*, and *ag-TD**.
(B) Conversion of *ag-TD* to *ag-TD** correlates with the loss of kanamycin resistance.
(C) Conversion of *ag-TD* to *ag-TD** can be achieved by the introduction of a T-DNA fragment that expresses the *NPT II* gene. About 75% of the T1 plants with *ag-TD* genotype did not show the typical *ag* phenotypes.

Suppression of *ag-TD* by *Trans*-Interactions between T-DNA Loci

The observation that kanamycin resistance was lost in *ag-TD** suggested that *trans*-interactions between the T-DNA fragment in *yuc1-1* locus and the T-DNA in *ag-TD* may be responsible for the suppression of *ag-TD*. To test this hypothesis, we transformed *ag-TD*$^{+/-}$ plants with a construct that expressed both the *NPT II* and the *BAR* gene (Figure 4C). Transformants were selected on basta-containing media. Among the 26 T1 plants with *ag-TD* genotype, 76% were partially suppressed and 44% were fertile (Figure 4C), demonstrating that introduction of another T-DNA insertion that expresses *NPT II* gene is sufficient to suppress *ag-TD*. The suppression of *ag-TD* is likely mediated by *trans*-interactions among T-DNA insertions.

Suppression of T-DNA Mutants by Other T-DNA Insertions Is Not Rare

We have demonstrated that *ag-TD* is suppressed by *yuc1-1* and also by transforming a T-DNA fragment into *ag-TD*. We investigated whether other T-DNA insertion mutants can also be suppressed by similar T-DNA interactions. We crossed *yuc1-1* to *cob-TD*, which also contains a T-DNA insertion in the large intron (Figure 5A). The *COB* gene encodes a glycosylphosphatidylinositol (GPI) anchored protein and plays an important role in cellulose microfibril orientation in *Arabidopsis* (Schindelman et al., 2001; Ko et al., 2006). Inactivation of *COB* by the T-DNA insertion led to very short roots and other defects (Figure 5B). However, all of the *cob-TD* plants in the F2 plants from the cross between *yuc1-1* and *cob-TD* had longer roots than the original *cob-TD* lines, indicating that *yuc1-1* also converted *cob-TD* to *cob-TD**, which was partially suppressed (Figure 5). The presence of *yuc1-1* made the suppression of *cob-TD* better (Figure 5B). This result is consistent with the observation that the *ag* phenotypes in *ag-TD yuc1-1*$^{+/-}$ were better suppressed than those in *ag-TD* alone. Interestingly, the conversion of *cob-TD* to *cob-TD** also led to the loss of kanamycin resistance (Figure 5D).

DISCUSSION

In this paper, we presented the analyses of an unexpected epigenetic phenomenon in *Arabidopsis*. We showed that some *Arabidopsis* T-DNA mutants were stably suppressed by T-DNA insertions in other non-homologous loci. We proposed that a T-DNA insertion in one locus could trigger undefined epigenetic modifications at a different T-DNA insertion site. The epigenetic modifications in the T-DNA mutants were heritable in the absence of the T-DNA suppressor. Because T-DNA mutants have been widely used in reverse genetics and in analyzing genetic interactions in *Arabidopsis*, this work suggests that we should be cautious about intronic T-DNA mutants.

Suppression Reported in this Work Violates Rules of Mendelian Genetics

When *ag-TD* was crossed to *yuc1-1*, all of the *ag-TD* plants in the F2 population were partially suppressed no matter whether *yuc1-1* was present or not, although the presence of *yuc1-1* rendered better suppression (Figure 1). The *ag-TD** not only could be stably transmitted for many generations in the absence of *yuc1-1* (Figure 2), but also had the ability to trigger new epigenetic suppressions in *ag-TD*. There are many similarities between the epigenetic suppression of T-DNA mutants and paramutation, a well-studied epigenetic phenomenon in Maize (Brink et al., 1968; Wolffe and Matzke,

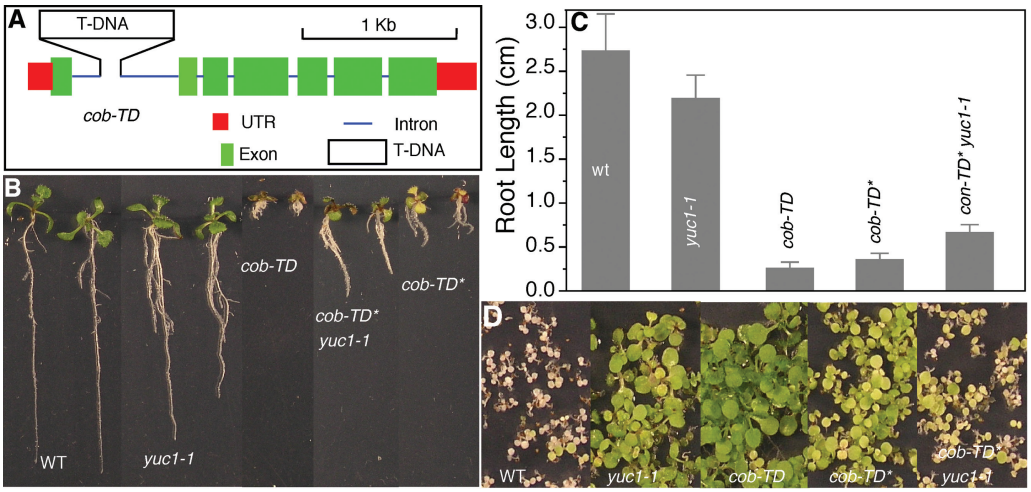


Figure 5. Partial Suppression of *cob-TD* by *yuc1-1*.
(A) The *cob-TD* mutant with a T-DNA insertion in the large intron.
(B) Suppression of *cob-TD* in the F2 population from a cross between *cob-TD* and *yuc1-1*.
(C) Increased root length in *cob-TD**.
(D) The conversion of *cob-TD* to *cob-TD** correlated with the loss of kanamycin resistance.

1999; Erhard et al., 2009; Chandler, 2010). In paramutation, one allele (*B′*) causes heritable changes in another allele (*B-I*) of the same locus. Both the conversions of *B-I* to *B′* and *ag-TD* to *ag-TD** were triggered by a cross. Once *B-I* is converted to *B′*, the new *B′* from *B-I* can be stably transmitted. We have shown that *ag-TD** could also be stable for many generations (Figure 2). The newly converted *B′* can convert *B-I* to *B′* and we showed that *ag-TD** can convert *ag-TD* to *ag-TD**. The end results of paramutation appear to be unidirectional because it is always that *B-I* is converted to *B′*. In the cases of T-DNA suppression that we have analyzed, the suppression appeared to be one-directional as well. When *ag-TD** was crossed with *ag-TD*, *ag-TD* was always converted to *ag-TD**.

Epigenetic Suppression of T-DNA Mutants Is Triggered by *Trans*-Interaction between T-DNA Insertions

Several lines of evidence support the hypothesis that the conversion of *ag-TD* to *ag-TD** is probably caused by *trans*-interaction between T-DNA insertions. First, *ag-TD* mutant displays strong kanamycin resistance whereas *ag-TD** is kanamycin-sensitive (Figure 4). It has been demonstrated that *trans*-inactivation between homologous genes causes the loss of antibiotic resistance in T-DNA insertion mutants (Daxinger et al., 2008). The T-DNA insertions in both *yuc1-1* and *ag-TD* are from the same plasmid; therefore, the *NPT II* transcripts from the T-DNA insertion at the *yuc1-1* locus have the capacity to induce the silencing of the *NPT II* gene in the T-DNA fragment at the *ag-TD* locus. Second, the conversion of *ag-TD* to *ag-TD** could also be achieved by transforming *ag-TD*$^{+/-}$ plants with a construct that expresses *NPT II* from the 35S promoter (Figure 4C).

We propose that transcripts such as the *NPT II* mRNA from the T-DNA insertions in *yuc1-1* and *ag-TD* interact *in trans* to cause the silencing of *NPT II*. It has been well documented that *trans* T-DNA interactions can lead to the silencing of homologous genes (Daxinger et al., 2008). What is very unusual is that the silencing of the genes located in the T-DNA fragments such as *NPT II* is correlated with the restoration of the gene function inactivated by the T-DNA insertion.

It is often hypothesized that intronic T-DNA insertions disrupt gene function because transcripts cannot be properly spliced. However, some genes have very large introns that are spliced out properly from the primary transcripts, suggesting that other factors may also contribute to the inactivation of gene function by intronic T-DNA insertions. When a T-DNA fragment is inserted into an intron of a gene, the primary transcript from the gene contains the entire intron plus the T-DNA fragment if the transcription is not prematurely terminated within the T-DNA region. Therefore, it is conceivable that transcripts from the T-DNA fragment such as the *NPT II* transcript may be able to form a partial duplex with the primary transcript when the *NPT II* gene is transcribed from the opposite direction. Such a duplex may affect proper processing of the primary transcript or even lead to degradation of the transcript. When those T-DNA-generated reverse transcripts are silenced by transcripts from another homologous T-DNA insertion (a process very similar to co-suppression), the duplex between the *NPT II* transcript and the primary transcript would be resolved. Consequently, the intronic T-DNA mutants are partially suppressed and the *NPT II* gene is silenced. We recognize that the *NPT II* transcript from the T-DNA insertion is similar to long intronic non-coding RNA, which causes epigenetic changes and affects gene expression levels (Heo and Sung, 2010)

Our findings indicated that intronic T-DNA insertion mutants can be easily suppressed by *trans*-interaction with another T-DNA. Therefore, the use of intronic T-DNA insertion mutants sometimes may lead to incorrect interpretations. We would like to point out that *trans*-interaction with another T-DNA insertion may not be the only trigger that is capable of causing the suppression of the phenotypes of an intronic T-DNA mutant. Environmental factors may also be able to cause the suppression of T-DNA mutants. For example, the intronic T-DNA insertion mutant *opr3* has long been recognized as a null allele and it produced no detectable Jasmonic acids (JAs) following wounding and looper infestation (Chehab et al., 2011). However, recently it was shown that the same *opr3* mutant became activated upon fungal infection and accumulated substantial levels of JAs. It was suggested that splicing of the T-DNA-containing intron might be responsible for the reactivation of *OPR3* (Chehab et al., 2011). In light of our findings, it is also possible that epigenetic modifications induced by fungal infection may play a role. Our study indicates that we should be careful about the use of intronic T-DNA mutants because some intronic T-DNA insertion mutants may undergo epigenetic changes that complicate interpretations of genetic interactions in *Arabidopsis*.

METHODS

The T-DNA insertion mutants *cob-TD* and *yuc1-3* were obtained from the ABRC at Ohio. The *ag-TD* was from Dr Yanofsky. The *yuc1-1* was previously described (Cheng et al., 2006; Won et al., 2011). For genotyping T-DNA mutants, we used PCR-based methods as previously described (Alonso et al., 2003). The gene-specific primers for genotyping *ag-TD* were 5′-ACGGCGTACCAATCGGAGCTAGGAGGA-3′ and 5′-TCTAGCTAGTTTCACCTTATTCACTCTC-3′.Primersforgenotyping *yuc1-1* and *yuc1-3* were 5′-GGTTCATGTGTTGCCAAGGGA-3′ and 5′-CCTGAAGCCAAGTAGGCACGTT-3′. Gene-specific primers for *cob-TD* were 5′-TCCACTCCTCCTTCAAGCAAAGC-3′ and 5′-CCATTTCATTGTAATGTTGCCTTC-3′. The T-DNA specific primer for genotyping *ag-TD*, *cob-TD*, and *yuc1-1* was JMLB1 (5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′). T-DNA primer for *yuc1-3* was Spm32 (5′-TACGAATAA GAGCGTCCATTTTAGAGTGA-3′). RNA *in situ* hybridization was performed as described previously (Cheng et al., 2006).

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