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## Epigenetic control of T-DNA during transgenesis and pathogenesis

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### Abstract

Mobile elements known as T-DNAs are transferred from pathogenic *Agrobacterium* to plants and reprogram the host cell to form hairy roots or tumors. Disarmed noncogenic T-DNAs are extensively used to deliver transgenes in plant genetic engineering. Such T-DNAs were the first known targets of RNA silencing mechanisms, which detect foreign RNA in plant cells and produce small RNAs that induce transcript degradation. These T-DNAs can also be transcriptionally silenced by the deposition of epigenetic marks such as DNA methylation and the dimethylation of lysine 9 (H3K9me2) in plants. Here, we review the targeting and the roles of RNA silencing and DNA methylation on T-DNAs in transgenic plants as well as during pathogenesis. In addition, we discuss the crosstalk between T-DNAs and genome-wide changes in DNA methylation during pathogenesis. We also cover recently discovered regulatory phenomena, such as T-DNA suppression and RNA silencing-independent and epigenetic-independent mechanisms that can silence T-DNAs. Finally, we discuss the implications of findings on T-DNA silencing for the improvement of plant genetic engineering.

### Introduction

Foreign nucleic acids can be naturally integrated into the genomes of land plants. Indeed, DNA sequences from a wide range of organisms (viruses, bacteria, fungi, and even other plant species) have been detected in plant genomes (Aubin et al. 2021; Ma et al. 2022). This process, whereby genetic information is transmitted across reproductive barriers between species, is called horizontal transfer. Foreign DNA can also be artificially introduced in the form of transgenes for biotechnological purposes (Basso et al. 2020).

This is illustrated by *Agrobacterium* spp., which are soil bacteria that have evolved the ability to transfer DNA to the host plant (Pitzschke and Hirt 2010; Soucy et al. 2015; Quispe-Huamanquispe et al. 2017; Aubin et al. 2021). The transferred DNA (T-DNA) is injected into plant cells through a specific type IV secretion system, T4SS<sub>T-DNA</sub>, and is integrated into the plant genome (Fig. 1). Bacterial genes encoded by the T-DNA are then transcribed to produce virulence factors, including enzymes such as indoleacetamide hydrolase (IaaH), tryptophan monooxygenase (IaaM), and isopentenyl transferase (Ipt). These enzymes catalyze the synthesis of auxins and cytokinins, which reprogram host cells into proliferating tissues (galls or hairy roots) (Fig. 1). The T-DNA also encodes enzymes that divert plant metabolism for the synthesis of opines (condensations of amino and organic acids and sugars), which are perceived as signals and used as nutrients by the *Agrobacterium* (Gelvin 2003; González-Mula et al. 2018; De Saeger et al. 2021).

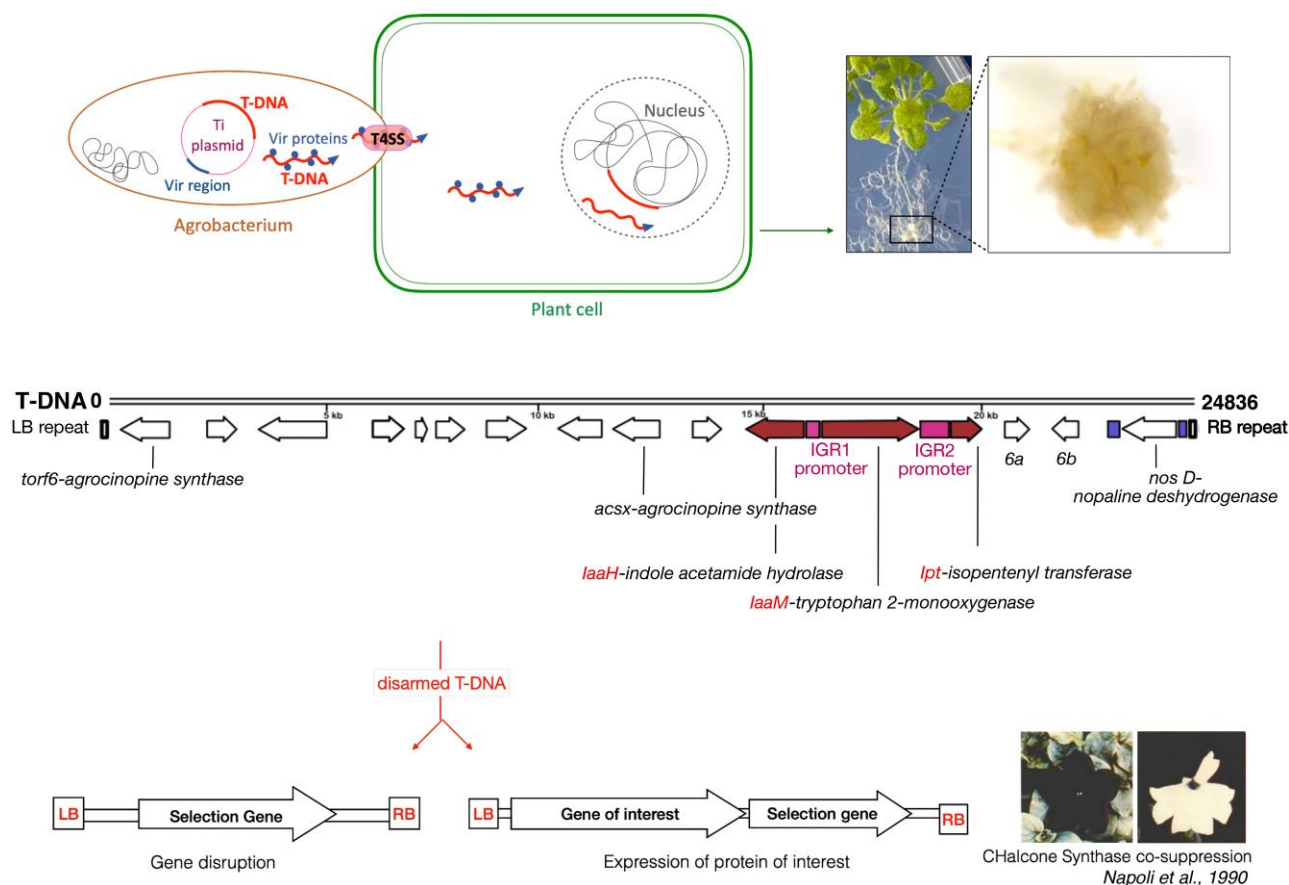
The length of T-DNA varies depending on the gene content; for example, in *Agrobacterium tumefaciens* strain C58, the T-DNA is a 20 kb element (Otten 2021). The T-DNA is delimited between the so-called 25-bp “left” and “right” borders (LB and RB, respectively)

(Fig. 1). The ability of the T-DNA to undergo horizontal transfer has made several *Agrobacterium* isolates powerful resources in plant biotechnology. Multiple *A. tumefaciens* C58-derived strains have been engineered with a disarmed T-DNA that no longer contains the pathogenic regions and instead carries gene(s) of interest (Fig. 1). These modified T-DNAs have enabled the production of transgenic plants for both fundamental research and crop biotechnology (Herrera-Estrella et al. 1983; Hwang et al. 2017; De Saeger et al. 2021). Their use for transgenesis has provided a powerful alternative to particle bombardment (also referred to as biolistics), which often results in fragmented and incomplete transgene insertions, more copies and/or larger arrays of transgenes, and potential cellular damage (Anami et al. 2013; Altpeter et al. 2016; Liu et al. 2019; Su et al. 2023).

Since the early days of plant genome modification, the introduction of transgenes has been associated with the induction of silencing (Matzke et al. 1989). When two homologous T-DNAs are inserted or when a plant carries a transgenic copy of a given endogenous gene, one or both are silenced; this phenomenon is referred to as cosuppression (Finnegan and McElroy 1994; Jorgensen 1995) (Fig. 1). Transgenes inserted by particle bombardment were also reported to undergo silencing (Kohli et al. 1999). These observations opened an entirely new field of research investigating the underlying mechanisms targeting T-DNAs in particular and foreign nucleic acids in general: RNA silencing and DNA methylation. In both the context of viral infection (Baulcombe 1996; Ratcliff et al. 1997; Jin et al. 2022) and in transgenic plants, exogenous RNA is recognized as “aberrant” by the RNA interference (RNAi) machinery, leading to the production of small interfering RNAs (siRNAs). The small RNAs can subsequently program an effector complex that acts on the RNA (via cleavage

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**Figure 1.** Integration of agrobacterial T-DNA into the plant cell and genome. Following the excision of the T-DNA and expression of the Vir region, the Vir-T-DNA complex passes through a T4SS and is transported to the plant nucleus, where it integrates into the plant genome. As a result, the plant cells are reprogrammed into tumors. Tumor formation can be induced on the roots of *A. thaliana* grown in vitro (right). The natural T-DNA from wild-type *A. tumefaciens* strain C58 contains 3 particularly important oncogenes (*iaah*, *ipt*, and *iaam*) responsible for phytohormone synthesis that result in tumorigenesis. The T-DNA genes can be removed to generate disarmed T-DNA used for gene disruption and the expression of proteins of interest. A T-DNA-based transgene initially designed to overexpress chalcone synthase (which produces anthocyanin pigments) can lead to the “cosuppression” of both transgenes and endogenous genes (right) (image from Napoli et al. 1990).

or inhibition of translation), leading to post-transcriptional gene silencing (PTGS). Small RNAs can also act on DNA by establishing epigenetic silencing marks, such as DNA methylation, leading to transcriptional gene silencing (TGS). Thus, potent surveillance mechanisms exist that hamper transgene expression. Therefore, many studies have focused on transgene silencing, as well as their induction, yet these mechanisms remain ill defined. Aside from transgenesis, the epigenetic regulation of T-DNA during pathogenesis has been understudied despite the profound impact of T-DNA expression during plant-pathogen interactions and tumorigenesis.

Here, we provide an update on the mechanisms that lead to transgene silencing, including RNA silencing and the associated epigenetic silencing mechanisms (in particular DNA methylation). We attempt to clarify the features of T-DNA-based transgenes that underlie the recruitment of these mechanisms and their implications for T-DNA suppression. We also discuss the DNA methylation-independent silencing mechanisms that have recently emerged. Finally, we review what is known about silencing and changes in methylation when natural T-DNAs are transferred into the plant host as part of pathogenesis and how our

knowledge of T-DNA silencing could be used to improve plant genetic engineering.

## Mechanisms leading to the silencing of T-DNA-based transgenes

### Post-transcriptional gene silencing

PTGS, also referred to as RNA silencing or RNAi, was first discovered in transgenic plants, where the RNAs produced by both the transgene and the homologous endogenous gene copy are degraded (Van Der Krol et al. 1990; Napoli et al. 1990). Since then, several transgenic *Arabidopsis* (*Arabidopsis thaliana*) lines have been shown to undergo PTGS, some of which have been extensively studied to decipher the molecular basis of this phenomenon. Among these is the *Arabidopsis* GxA line, which carries 2 transgenes under the control of the strong ubiquitous CaMV 35S promoter: one encoding the potato virus X fused to GFP (35S-PVX::GFP referred to as the “amplicon” or “A”) and one encoding GFP alone (“G”). This line shows both high-level transcription of the PVX gene and silencing of GFP at the post-transcriptional level (Dalmay et al. 2000). Importantly, the *Arabidopsis* L1 line carrying the GUS transgene under the control of the 35S promoter

control was shown to trigger spontaneous sensing of PTGS. A detailed report of the mechanisms of transgene PTGS was recently published (Vaucheret 2023); thus, it will not be extensively covered here.

Briefly, PTGS can be triggered by sense transgenes that produce an “aberrant RNA” that is recognized by SUPPRESSOR OF GENE SILENCING 3 (SGS3)/RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) and converted into dsRNA (S-PTGS). Alternatively, dsRNAs can originate from antisense transgenes (AS-PTGS)/inverted repeat transgenes (IR-PTGS) that encode self-complementary transcripts (Fig. 2). dsRNAs are processed by type III RNases of the Dicer family into 21- and 22-nt siRNAs. These siRNAs are further associated with ARGONAUTE (AGO) proteins, forming a complex that targets homologous mRNAs and mediates either their cleavage and degradation or translational inhibition (Hung and Slotkin 2021; Vaucheret 2023). In plants, core components of RNA silencing have diversified and specialized in different silencing pathways. RNA silencing of endogenous genes can be mediated by microRNAs (produced by DICER-LIKE1) or trans-acting siRNAs (produced by DICER-LIKE 4 and 2 via derivatives of microRNA-cleaved transcripts). On the other hand, RNA silencing of viruses, transposable elements (TEs), and transgenes shares a similar mechanism involving siRNAs that are produced by DICER-LIKE 4 and 2 and incorporated into AGO1 or AGO2 (Fig. 2).

## DNA methylation and transcriptional gene silencing

In transcriptional gene silencing (TGS), siRNAs produced in the cytoplasm act on foreign DNA by guiding the deposition of DNA methylation and histone 3 lysine 9 dimethylation (H3K9me2). Together, these two epigenetic marks ensure the stable repression state of the foreign DNA sequence (Zhang et al. 2018). While T-DNA silencing was observed to be associated with DNA methylation early on (Finnegan and McElroy 1994), the signals that initiate de novo methylation were unknown at the time. An early hint that the small RNAs involved in PTGS are the signals for establishing DNA methylation was derived from the analysis of plants carrying a GFP transgene. These GFP-expressing plants were subjected to virus-induced gene silencing (using viruses engineered with GFP) and showed sequence-specific de novo DNA methylation of the transgene (Jones et al. 1999). In addition, the insertion of inverted repeat transgenes corresponding to either coding sequences or promoters resulted in DNA methylation and transcriptional inactivation in tobacco (*Nicotiana tabacum*) (Mette et al. 1999), Arabidopsis (Luff et al. 1999), or *Petunia* (Sijen et al. 2001). The abovementioned L1 line was later reported to produce siRNAs in the central and 3' regions of the *GUS* sequence (Parent et al. 2015): these regions were shown to acquire DNA methylation in the same timeframe as PTGS (i.e. 4 to 10 d after germination). Thus, 21- to 22-nt siRNAs produced during PTGS can drive transgene DNA methylation (Fig. 2). When these siRNAs are targeted to the transgene promoter, they may cause TGS. This constitutes a robust feedback mechanism for stabilizing and perpetuating the silencing of the foreign sequence.

From a mechanistic point of view, 21- to 22-nt siRNAs are derived from the transcripts of transgenes that are converted into dsRNA by RDR6 (Parent et al. 2015). These siRNAs are thought to be incorporated into AGO6 to direct this effector to homologous DNA through base pairing with transcripts generated by the plant-specific PolV (also referred to as NRPE1) (Fig. 2). Methylation of the DNA template strand is catalyzed by the de novo methyltransferase DOMAINS

REARRANGED METHYLASE 2 (DRM2) in all sequence contexts (Fig. 2). This pathway is thus referred to as RDR6/PolV-RNA-directed DNA methylation (PolV-RdDM or RDR6-RdDM or “noncanonical” RdDM). This preestablishment phase transitions to canonical PolIV-RdDM via a TE-derived noncoding transgene known as “the FWA repeats” (Chan et al. 2004): this is the stabilization phase, when single-stranded RNAs (ssRNAs) are produced by PolIV (also referred to as NRPD1), another plant-specific polymerase that transcribes methylated DNA. The RNA-dependent RNA polymerase 2 (RDR2) presumably uses these ssRNAs as templates to produce dsRNAs that are processed into 24-nt siRNAs by DICER-LIKE 3 (DCL3); 24-nt siRNAs are then loaded into AGO4, AGO6, or AGO9. PolV transcripts interact with AGO4/6/9-loaded homologous siRNAs, and DNA methylation of the PolV DNA template strand is catalyzed by DRM2 (and possibly DRM1) through its binding to AGO4/6/9 (Deleeris et al. 2016). Of note, when DCL4 is saturated, DCL3 has been proposed to process the cytoplasmic dsRNA coming from expressed TEs, leading to the production of 24-nt siRNAs directly and to a similar PTGS-TGS transition (Marí-Ordóñez et al. 2013). Such a mechanism could also take place in establishing DNA methylation at transgenes although this has not been formally tested.

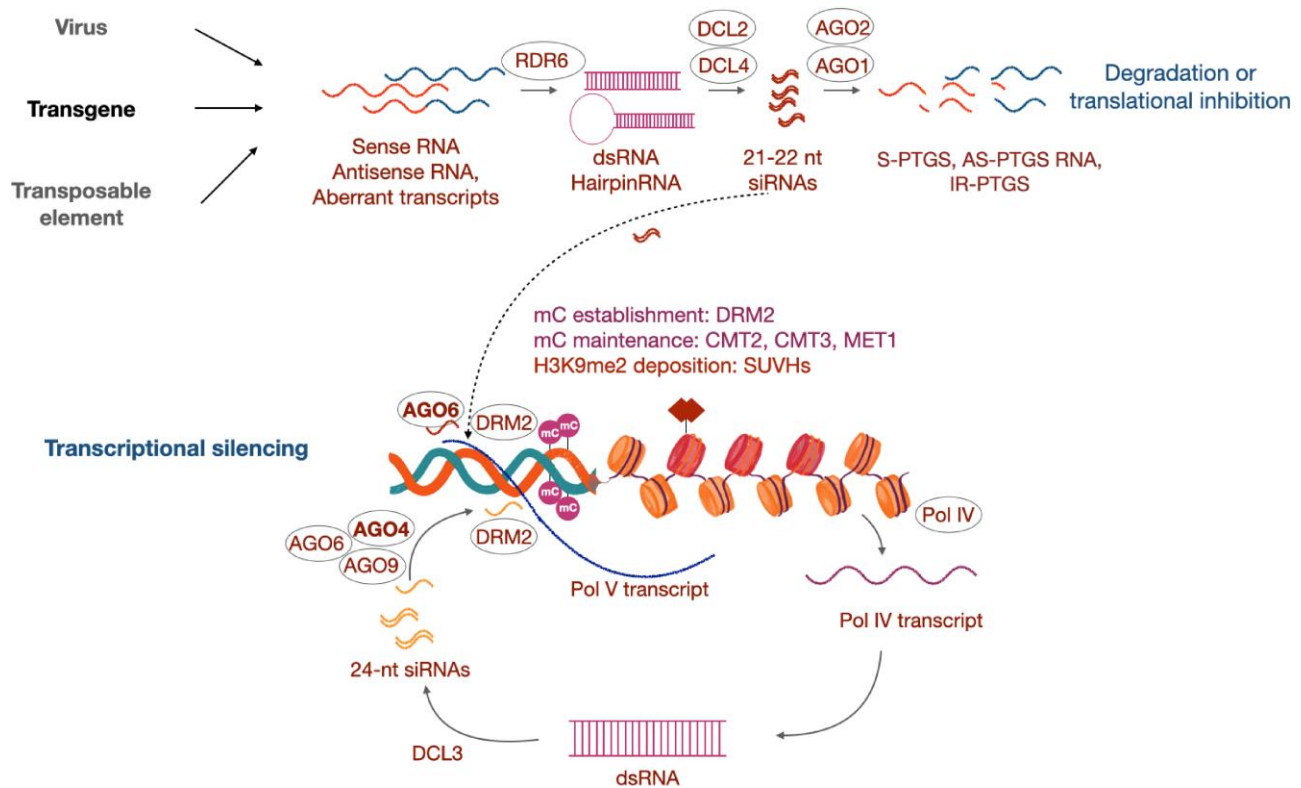
Four different pathways then amplify the established DNA methylation patterns and maintain them stably over time. CG, CHG, and CHH DNA methylation patterns are typically maintained by MET1 (Kankel et al. 2003), CMT3 (Lindroth et al. 2001), and CMT2 and RdDM (Stroud et al. 2014), respectively (Fig. 2). In addition, mechanistic loops have been described between DNA methylation and H3K9me2 deposition, which reinforce the silent state. A more detailed description of these pathways, which are primarily involved in the silencing of TEs, has been presented elsewhere (Zhang et al. 2018).

## T-DNA features that lead to RNA silencing and DNA methylation

In this section, we describe the molecular features that trigger RNA silencing and DNA methylation.

### Repetitiveness, configuration, and sequence homology of the transgene

A major feature that underlies transgene silencing is the repetitive nature of the sequence, which can be either present (i) as multiple copies at one insertion site or (ii) as dispersed copies within the genome. The silencing of transgenic lines containing multiple copies of a given insert was first reported in *Petunia* (*Petunia integrifolia*), tobacco (*N. tabacum*), and Arabidopsis (Hobbs et al. 1990; Linne et al. 1990; Scheid et al. 1991). A drug resistance gene inserted in arrays at one locus also presented decreased mRNA and increased DNA methylation in Arabidopsis; its silencing was proposed to be due to chromatin condensation because of homologous DNA pairing (Assaad et al. 1993) (Fig. 3, top right). This hypothesis has not yet received any experimental validation. In transformed plants derived from *Petunia* calli, a lack of transgene expression or expression variability was proposed to be due to the configuration of the transgene as an array composed of multiple copies arranged as head-to-tail repeats in the antisense orientation (Jones et al. 1987). This hypothesis was supported by the discovery of the RNA silencing pathway induced by sense-antisense RNAs, which generate double-stranded RNA and small RNAs as described above (Fig. 3, top left). Interestingly, the truncation of transgenes, rather than their copy number, was shown to be a



**Figure 2.** Simplified scheme for the PTGS and TGS mechanisms. First, some transgene-derived transcripts are converted into dsRNA by RDR6. The dsRNA is then processed into 21- and 22-nt siRNAs by DCL4 and DCL2 proteins. The siRNAs are loaded into AGO1/2 and target complementary RNAs for cleavage or translational inhibition. These siRNAs involved in PTGS can also be incorporated into AGO6 and target homologous DNA, leading to a transition from PTGS to RdDM. In the first phase, 21- to 22-nt siRNA-loaded AGO6 guides DRM2 onto DNA with the help of PolV transcripts to catalyze DNA methylation (lollipops). In the second phase, PolIV transcribes DNA-methylated loci; PolIV transcripts are converted into dsRNA by RDR2, and the dsRNA is processed into 24-nt siRNAs by DCL3; 24-nt siRNAs are loaded into AGO4/6 to bind PolV transcripts and guide DRM2 at the targeted locus (of note, when DCL4 is saturated, DCL3 has been proposed to process the cytoplasmic dsRNA leading to the production of 24-nt siRNAs directly and similarly leading to a PTGS-TGS transition, which is not represented here). The maintenance of DNA methylation is ensured by MET1, DRM2, CMT2, and CMT3. SUVH4/5/6 proteins recognize methylated CHG and deposit H3K9me2 marks (red lozenges).

major factor in the insertion of transgenes by particle bombardment (Kohli et al. 1999), indicating that transgene integrity could also play a role in the expression of T-DNA-based transgenes.

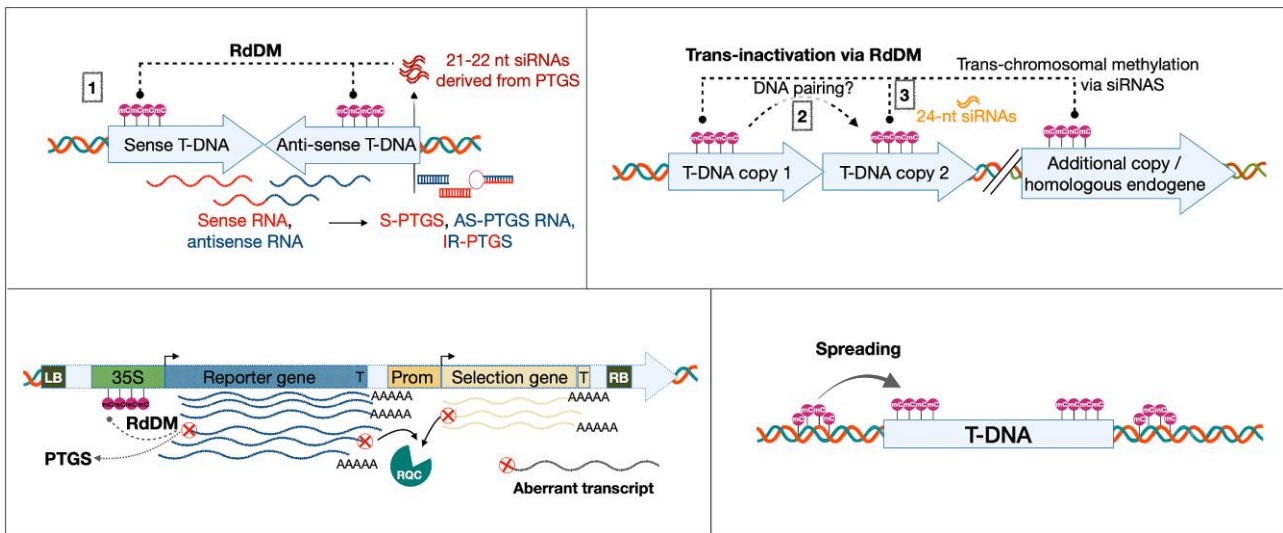
Regarding the silencing of dispersed copies, the phenomenon of trans-inactivation was proposed to describe the silencing of a transgene by the introduction of a second homologous transgene (Matzke and Matzke 1995; Matzke et al. 2000). Trans-inactivation was also shown to occur after crossing a 35S::GUS transgenic line with T-DNA insertional mutants from the SALK or GABI collections, where the T-DNAs comprise marker genes under the control of the 35S promoter. This resulted in GUS silencing and was proposed to be due to sequence homology. Accordingly, siRNAs were detected at the 35S promoter (Mourrain et al. 2007; Daxinger et al. 2008; Mlotshwa et al. 2010). (Fig. 3, top right). Silencing mechanisms induced by trans-inactivation vs. multiple copy numbers are not necessarily mutually exclusive, and these mechanisms could contribute to each other.

### Promoter strength (35S) and level of expression

Since silencing mechanisms can be activated in the absence of homologous sequences, the level of expression of gene units in transgenes is an important aspect to consider for TGS. The hypothesis that a threshold of mRNA accumulation could trigger PTGS was first proposed in the 1990s (Smith et al. 1994; Elmayan et al. 1998). Accordingly, the presence of multiple transgenic

copies or specific configurations is not always sufficient to induce silencing, which is triggered only in plants with highly expressed transgenes with high copy numbers (Lechtenberg et al. 2003). In addition, transgene silencing was shown to occur when multicopy transgene expression surpassed a certain threshold (Schubert et al. 2004; Kanazawa et al. 2007). A study in the wild tobacco *Nicotiana attenuata* suggested that although DNA methylation could be triggered by the presence of repetitive copies and increased during vegetative development, the loss of resistance of the selectable marker gene was revealed only in the T3 generation when plants were homozygous for the transgene; under this condition, a transcription threshold was surpassed, leading to the initiation of robust silencing (Weinhold et al. 2013).

A possible molecular link between the abundance of transcription, improper mRNA turnover, and the onset of RNA silencing was provided by the detection of aberrant, truncated non-polyadenylated transcripts at a 35S::NOSpro (NOS promoter) transgene targeted by DNA methylation (Mette et al. 1999). In fact, when the XRN ribonuclease machinery is saturated or mutated, RDR6 can use transgenic RNA as a template (Gazzani et al. 2004). Accordingly, improperly terminated, nonpolyadenylated transgene mRNAs, either due to transgene configuration (direct repeats) and/or high transcription, can trigger RDR6-mediated RNA silencing (Luo and Chen 2007). Highly expressed transgenes may generate aberrant RNAs because the RNA quality control (RQC) pathway is saturated and is thus predisposed to trigger RNA silencing and subsequent TGS (Vaucheret



**Figure 3.** Features that induce transgene silencing. Sequence homology (top). Homologous sequences can trigger the silencing of transgenes. (1, top left) Transgene integration and orientation can drive the production of sense, antisense, or hairpin transcripts that lead to dsRNA formation and the production of 21- to 22-nt siRNAs, resulting in PTGS. These siRNAs can also mediate the transition to the RdDM pathway to methylate the DNA sequence of the transgene (DNA methylation is represented by pink lollipops). (2, top right) T-DNA insertions in tandem may drive DNA methylation, possibly via non-mutually exclusive mechanisms: DNA pairing (has not yet been evidenced), increasing transgene expression (see bottom left), or RdDM-mediated trans-inactivation. (3, top right) Multiple genomic insertions in tandem, at different loci or homologous with endogenous genes, may also drive silencing by trans-chromosomal methylation via siRNAs and RdDM (trans-inactivation). High expression (bottom left). A strong promoter and high expression lead to the production of aberrant transcripts that escape the RQC pathway and enter the PTGS and RdDM pathways, which in turn lead to DNA methylation at the transgene promoter and subsequent TGS. Positional effects (bottom right). The genomic environment of the transgene insertion site may be important, since the spreading of methylation from adjacent sequences is possible.

2023) (Fig. 3, bottom left). Recently, Oberlin et al. (2022) reported that ribosome stalling coincides with unconventional 5'OH mRNA fragments from active Ty1/Copia retrotransposons. These modified mRNAs are not accessible to the RQC pathway and trigger their RDR6-mediated conversion into dsRNA and subsequent silencing (Oberlin et al. 2022). This mechanism may also apply to transgenes, which is an exciting possibility to test in the future.

### Position effect and chromosomal environment

T-DNA insertion can take place throughout the entire plant genome, but whether there is a bias in their insertion site has been a matter of debate. T-DNAs were initially thought to be preferentially inserted into active chromatin regions, 5' and 3' regulatory regions, AT-rich regions, gene-rich regions, and regions outside centromeres (Koncz et al. 1989; Brunaud et al. 2002; Szabados et al. 2002; Alonso et al. 2003; Chen et al. 2003; Sallaud et al. 2004). A caveat of these observations is that they were made after selection based on marker genes expression and were thus biased toward expressed transgenes.

The analysis of Arabidopsis transformants without selection led to the following estimation: approximately 30% of the insertions would be transcriptionally silenced due to their insertion into “cold spots of expression” and thus the marker gene would not be expressed and the transformants not recovered after selection (Francis and Spiker 2005). Analyses of the chromatin structure of the T-DNA in transformed Arabidopsis suspension cells without selection bias confirmed the random distribution of T-DNAs into heterochromatic or euchromatic regions (Kim and Veena 2007). In addition, a high-throughput analysis of T-DNA junctions under non-selective vs. selective conditions in root tissue at 6 and 24 h postinfection with *Agrobacterium* (Shilo et al. 2017) further revealed that (i) both selected and unselected T-DNAs display a preference for T-DNA microhomologies and AT-rich motifs, and (ii) the T-DNA junctions of

unselected events are more frequently found in regions with high-nucleosome occupancy and H3K27me3 marks, whereas the T-DNA junctions recovered after selection are located in regions with low-nucleosome occupancy, low DNA methylation, and low H3K27me3 (valleys) (Shilo et al. 2017). Finally, whole-genome resequencing of 21 T-DNA lines with single insertions recovered after selection revealed their distribution in all chromatin states described by Sequeira-Mendes et al. (2014) (Pérez-González and Caro 2019). Perhaps the differences among the results of these studies can be explained by the different cell types in which the T-DNA integrates (roots vs. premeiotic tissues for stable T-DNA insertions induced by the floral dip method) and their different epigenetic states.

These studies suggest that the heterochromatic status of the integration site does not impede T-DNA insertion but negatively affects its expression. This finding agrees with the earlier observation that transgene activity is influenced by the methylation pattern of the integration site (Pröls and Meyer 1992; Day et al. 2000). The underlying mechanism for this positional effect likely involves the spreading of endogenous DNA methylation into the nearby sequence, as proposed for the spreading of TE methylation into genes (Martin et al. 2009; Ahmed et al. 2011) (Fig. 3, bottom right). The impact of the insertion site on transgene silencing has been corroborated by several studies that identified synthetic sequences able to insulate transgenes from neighboring regions, which results in an increase in transgene transcription (Kishimoto et al. 2013; Pérez-González and Caro 2019). Interestingly, when transgenes were integrated into cotton (*Gossypium hirsutum*) via biolistics, as single copies and at the same gene-rich insertion site by customized COT-5/6 meganucleases, they presented different methylation patterns between independent lines. Differential double-strand break induction and repair (Verkest et al. 2019) was proposed to account for these differences and was suggested as an additional factor in the onset of silencing, which remains to be demonstrated.

## Intronic T-DNA methylation and “T-DNA suppression”

The large majority of *Arabidopsis* loss-of-function mutants are derived from publicly available collections of plant lines whose T-DNA fragments (containing resistance genes) are randomly integrated into the genome (such as the SALK, SAIL, or GABI collections). Both exonic and intronic T-DNA insertions can produce nonfunctional transcripts, as they disrupt ORFs or cause splicing, respectively. However, a phenomenon called “T-DNA suppression,” where gene function is unaffected by the presence of a T-DNA insertion, has been reported specifically for intronic T-DNA insertions (Gao and Zhao 2013; Sandhu et al. 2013; Jia et al. 2015; Osabe et al. 2017; Wouters et al. 2023). This primarily occurs upon crossing with another T-DNA mutant (intronic or not). Interestingly, the suppressed state can be maintained in the absence of the T-DNA that initially triggered suppression following its removal by crossing (Gao and Zhao 2013; Sandhu et al. 2013). While the epigenetic nature of this phenomenon is known (Gao and Zhao 2013), the molecular basis of the establishment and maintenance of T-DNA suppression has only recently been uncovered.

Genetic experiments in which various mutations were introduced into the double T-DNA mutant system revealed that intronic T-DNA suppression was caused by (i) DNA methylation/heterochromatinization of the intronic insertion due to trans-inactivation induced by the second T-DNA, providing it is itself DNA methylated, and (ii) the targeting of the intronic T-DNA by the IBM2-EDM2 machinery, which was previously shown to target heterochromatic introns to allow Pol II-mediated transcription and subsequent splicing (Osabe et al. 2017) (Fig. 4). Thus, central to the induction of this phenomenon is the epigenetic composition of the second T-DNA, i.e. the propensity of the second T-DNA to be a target of silencing mechanisms—because of the features mentioned in the previous paragraph—as well as the degree of similarity with the intronic T-DNA. To date, the phenomenon of T-DNA suppression has mostly been observed for genes whose loss of function leads to a developmental phenotype (AGAMOUS, LEAFY, GLABRA2) or metabolic genes (CINNAMOYL-CoA REDUCTASE 1, BRI1-5 ENHANCED-1). Perhaps there is also a variable propensity of the intronic insertion to achieve DNA methylation, which may vary depending on the endogenous gene and its initial epigenetic status, but this has not yet been explored. To test this hypothesis, future studies should explore additional T-DNA intronic mutants, which could lead to predictions about the preferential targets of T-DNA suppression.

## Alternative T-DNA silencing mechanisms independent of RNA silencing/DNA methylation

### KNOT-linked silencing

The silencing of resistance genes in the T-DNA used in mutational SALK/SAIL lines has often been associated with small RNAs and DNA methylation (Daxinger et al. 2008). However, this is not always the case in many other SALK T-DNA lines whose resistance genes are silent. In fact, some silenced SALK T-DNAs specifically interact with 3D chromatin structures referred to as KNOTs (Grob and Grossniklaus 2019) (Fig. 5, left). KNOTs are genome-interacting regions that are enriched in TEs and small RNAs (Grob et al. 2014). In addition, when silencing was due to transgene-KNOT interactions, the transcriptional state of one transgene was transferred to another transgene (Grob and Grossniklaus 2019), which is reminiscent of paramutation. Whether KNOT-linked silencing (KLS) is associated with specific

chromatin types or epigenetic marks other than DNA methylation is currently unknown.

## Gene Silencing based on ENcoded protein’s Intracellular localization (GESENI)

More recently, a transgene silencing phenomenon was reported in *Arabidopsis* sperm cells: transgenes encoding proteins with no specific intracellular localization (cytoplasmic proteins) were silenced transcriptionally or posttranscriptionally, in contrast to transgenes encoding proteins with targeted cellular localizations, such as the plasma membrane. This phenomenon was termed GEne Silencing based on ENcoded protein’s Intracellular localization (GESENI) (Fig. 5, right) (Ohnishi and Kawashima 2023). GESENI does not seem to be dependent on DNA methylation since transgene silencing is maintained in *met1* and *drm2* mutants. This process was also proposed to be independent of H3K27me<sub>3</sub>-mediated silencing or RNA silencing (PTGS) (Ohnishi and Kawashima 2023); however, additional mutants in these pathways should be tested to confirm this notion. Hence, the mechanism underlying GESENI has yet to be discovered.

### Possible alternative epigenetic silencing

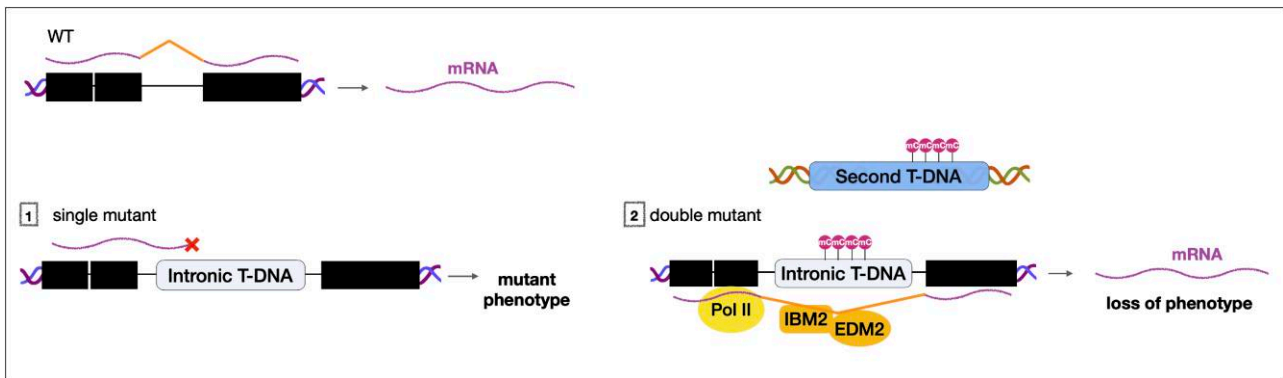
Regardless of whether epigenetic silencing is independent of these phenomena, the possibility that transgenes could be targeted by chromatin/epigenetic marks other than those mentioned above exists but has not been extensively investigated. In support of this idea, a histone deacetylase (Sir2-type) was shown to cause transgene silencing in the green alga *Chlamydomonas*, indicating that transgenes can be targeted by repressive chromatin. Thus, algae possess efficient systems of protection against foreign nucleic acid invasion, to which they may be particularly vulnerable in aquatic environments (Neupert et al. 2020).

Interestingly, TEs have been increasingly found to be decorated by H3K27me<sub>3</sub>, a silencing epigenetic mark usually associated with protein-coding genes and deposited by Polycomb-group proteins (Délérís et al. 2021). This mark was also recently shown to target horizontally T-DNA sequences from fungi to wheat (*Triticum aestivum*) (Kobayashi et al. 2023). Thus, one could envision that the T-DNA is similarly targeted by this alternative epigenetic silencing system in certain contexts, which have yet to be revealed. In support of this notion, ChIP-seq analyses of histone marks in various SALK and SAIL lines revealed that the deposition of H3K27me<sub>3</sub> chromatin marks (as well as H2AZ histone variants often associated with H3K27me<sub>3</sub>) can increase in the neighboring regions of the insertions (Jupe et al. 2019), perhaps providing indirect evidence that, in some instances, the T-DNA is modified with these chromatin marks. Interestingly, a mutation in lysine 27 of H3 histones, leading to a genome-wide decrease in H3K27me<sub>3</sub> marks, resulted in an increase in transformation efficiency (based on the recovery of primary transformants after selection) using *Agrobacterium* (Iwakawa et al. 2017). This could indicate that T-DNAs and their resistance genes are sometimes targeted and silenced by H3K27me<sub>3</sub>.

Together, these observations suggest T-DNAs could be targeted by alternative epigenetic marks, such as H3K27me<sub>3</sub>. This could, in a nonexclusive manner, underlie KNOT-based silencing, an exciting possibility that remains to be tested in the future.

## T-DNA silencing and DNA methylation during *Agrobacterium*-mediated tumorigenesis

In a pathogenic context, the T-DNA enters the plant cell as single-stranded DNA, presumably in a “naked” state—i.e. free of plant



**Figure 4.** T-DNA suppression. (1) Insertion of a T-DNA into an intron disrupts normal transcription and splicing, resulting in a mutant phenotype. (2) The integration of a second T-DNA into the genome of the intronic T-DNA mutant can result in T-DNA suppression. This occurs if the second T-DNA is DNA methylated, which results in trans-inactivation and de novo DNA methylation of the first intronic T-DNA via RdDM. The IBM2-EDM2 complex recognizes the newly heterochromatinized intron, resulting in Pol II transcription throughout the intron, proper splicing, and recovery of the wild-type (WT) phenotype. The black boxes represent exons, and lines represent introns.

DNA modifications. Following its integration in the genome and during tumorigenesis, the T-DNA is likely chromatinized and could thus be controlled by the host defense mechanisms that usually target foreign DNA, including DNA methylation. In this section, we review studies that have investigated this poorly explored topic, as well as the impact of T-DNAs on the plant methylome.

### DNA methylation of the T-DNA

Early studies aimed to address the methylation status of T-DNAs at discrete sites in flax (*Linum usitatissimum* L.) and tobacco (*N. tabacum*) tumors using Southern blot-based, methylation-sensitive assays. This approach revealed heterogeneity of methylation patterns between T-DNA copies (Hepburn et al. 1983) and between T-DNA molecules: some were methylated at discrete sites in the tumor, but some were unmethylated, consistent with the detected oncogene expression (Ooms et al. 1982; Gelvin et al. 1983). Accordingly, treating tumors with demethylating agents increased the expression of T-DNA-methylated genes (Hepburn et al. 1983). Together, these findings indicate that DNA methylation of natural T-DNAs can take place and result in their silencing. The heterogeneity of T-DNA methylation patterns between different plants regenerated from tumors could also indicate heterogeneity within the tumor (Amasino et al. 1984). In agreement with T-DNA expression and tumorigenesis, DNaseI and MNase analysis of a T-DNA in *N. tabacum* tumors revealed an open nucleosome conformation (Coates et al. 1987). Thus, if T-DNA methylation exists, it may be present only in some molecules and at a low enough average level so that T-DNA expression can be observed. More recent studies using bisulfite sequencing of discrete T-DNA genes (*Ipt*, *IaaH*, and *IaaM*) during *Agrobacterium*–*Arabidopsis* interactions revealed that these genes are not methylated, which is in agreement with their expression (Gohlke et al. 2013). Perhaps at early time points, the T-DNA is methylated, yet the cells with an unmethylated T-DNA would be selected throughout the proliferative process, leaving only a few molecules methylated, as shown in early studies. This finding is in accordance with reports showing that siRNAs can accumulate at oncogenes in *Nicotiana benthamiana*, but only at the beginning of infection and not in tumors (Dunoyer et al. 2006).

One hypothesis is that suppressed silencing in tumors is caused by *Agrobacterium* proteins. In support of this notion, the T-DNA-encoded 6b protein was found to interact with AGO1 and

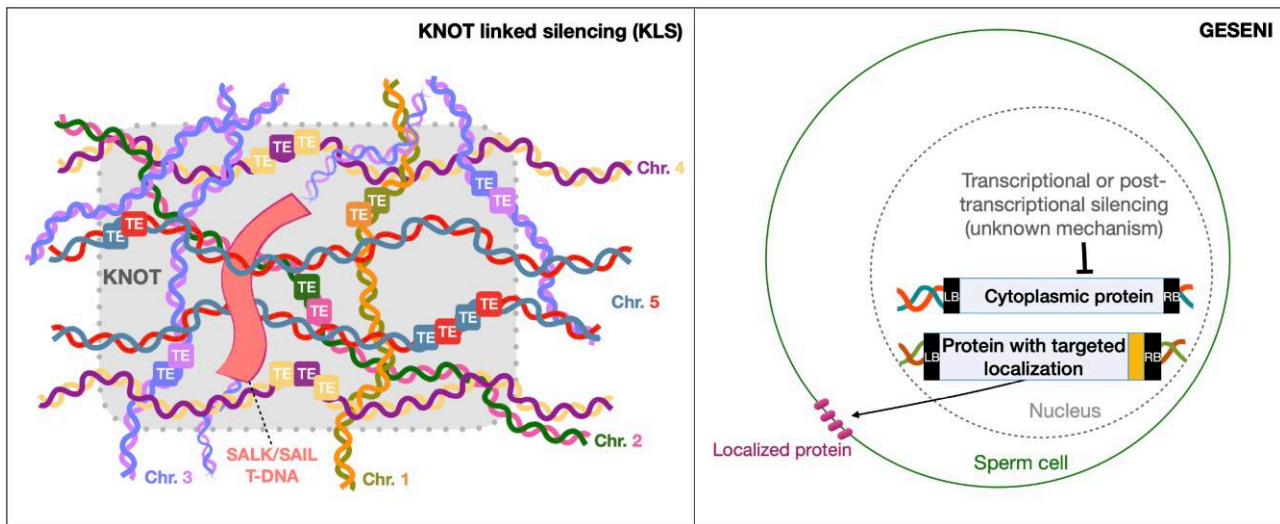
other RNA silencing factors (which leads to reduced microRNA accumulation) and with histone H3 (Wang et al. 2011; Ito and Machida 2015). In addition, the 6b gene is not present in the T-DNA of the Ri plasmid of *Agrobacterium rhizogenes*, and T-DNA-derived small RNAs are highly abundant during hairy root formation and target both oncogenes and host genes for PTGS (Peláez et al. 2017). Thus, the 6b protein is a good candidate for suppressing T-DNA silencing. A second (nonexclusive) hypothesis is that suppressed silencing occurs due to the proliferating state in the tumor. In support of this notion, potato (*Solanum tuberosum*) lines with strong silencing phenotypes showed suppressed RNA silencing during callus induction (Corrêa et al. 2004). The finding that T-DNA-derived small RNA accumulates in hairy roots induced by *A. rhizogenes* infection also supports this hypothesis, because hairy roots are differentiated, nonproliferative tissues, in contrast to tumors.

It would be interesting to test whether, during the initial hours after *A. tumefaciens* infection (before proliferation), siRNAs can be produced by T-DNAs in *Arabidopsis*, at which genes, and whether they are associated with DNA methylation. The mechanism underlying the lack of silencing of T-DNA genes, which are critical for the tumorigenesis process, also deserves investigation.

### Changes in DNA methylation induced by the T-DNA in the rest of the plant genome

Following T-DNA insertion, and during the process of tumorigenesis, the plant genome must undergo several structural and epigenetic changes. Differential analysis of DNA methylation in 35-day-old tumors compared with stems revealed global hypermethylation, mainly in protein-coding genes, in the crown gall genome. This analysis was accomplished via immunoprecipitation of methylated DNA and hybridization on tiling arrays covering only 22% of the *Arabidopsis* genome (mCIP) (Gohlke et al. 2013). Thus, these data may underestimate context-specific genome-wide changes in DNA methylation, particularly for cytosines in the CHH context, which were shown to undergo changes during infection by other types of bacteria (Deleris et al. 2016). Notably, during the transition from leaf to callus, under non-infected conditions, CG methylation is unchanged, CHG methylation increases, and CHH is notably lower than that of TEs, resulting in the regulation of cell cycle genes and pluripotency regulators (Shim et al. 2022). This finding is also consistent with the changes in DNA methylation during cell proliferation in cell culture





**Figure 5.** Methylation-independent mechanisms. KLS (left). T-DNA interacts with KNOT 3D structures composed of regions from the 5 chromosomes that are enriched in TEs and small RNAs. High KNOT interaction frequencies are associated with transgene silencing, which is not associated with DNA methylation or PTGS. The silencing mechanism is yet unknown. GESENI (right). In *Arabidopsis* sperm cells, a transgene without an encoded sequence for intracellular localization is silenced transcriptionally or posttranscriptionally, whereas a transgene with an encoded localization sequence in the cell is expressed normally. The mechanism, which is yet unknown, seems to be independent of the known epigenetic pathways, at least the ones tested.

(Borges et al. 2021). Furthermore, at an early timepoint after infection (up to 48 h), exposure to different *Agrobacterium* factors (Vir proteins, oncogenes, and T-DNAs) led to an increase or decrease in DNA methylation at the promoters of plant genes involved in the DNA repair pathway (Joseph et al. 2021).

Thus, changes in non-CG methylation during *Agrobacterium*-induced proliferation are expected, but this remains to be tested. Future studies should profile DNA methylation at a single-base resolution and over the entire genome during the *Arabidopsis*–*Agrobacterium* interaction, at early time points after infection, and in fully formed tumors. This would help determine the extent to which genome-wide changes in DNA methylation take place. It also remains to be investigated whether these changes are due to innate immunity and/or developmental reprogramming and whether T-DNA-encoded proteins and/or bacterial effectors can limit the loss of DNA methylation.

Most transcriptome studies of *Agrobacterium*-infected tissues at early time points and after tumorigenesis have revealed the regulation of phytohormone signaling, cellular biosynthesis, nucleic acid metabolism, and defense responses (Deeken et al. 2006; Ditt et al. 2006; Lee et al. 2009; Duan et al. 2018; Nathoo et al. 2019; Tkachenko et al. 2021). Interestingly, several studies have also pointed to the transcriptional dynamics of epigenetic modifiers and histones. For example, the levels of histones H2A, H2B, H3, and H4 decrease at early timepoints (3 to 12 h) but increase at later time points (18 to 36 h) of infection (Veena et al. 2003). These observations point to a reduced density of nucleosomes and increased DNA accessibility for transcription factors at the beginning of infection. DNA methyltransferases were shown to accumulate at the beginning of infection (Duan et al. 2018; Nathoo et al. 2019), whereas the accumulation of a methyl CpG-binding protein required for the incorporation of H2A.Z decreased in 10-d-old tumors (Tkachenko et al. 2021). Perhaps the plant initiated a defense response against T-DNAs in the early hours of infection that could subsequently be suppressed by proteins that antagonize DNA methylation. Hence, bacterial effectors might have evolved to interact with and modify the host epigenome to prepare for more permissive proliferation.

Interestingly, the disruption of 24 H2A and H3 histone genes resulted in the *rat* phenotype (*Arabidopsis* mutants resistant to *Agrobacterium* transformation), suggesting that epigenetic information can influence the efficiency of T-DNA integration (Zhu et al. 2003; Magori and Citovsky 2011). Moreover, histone deacetylases/acetyltransferases seem to be necessary for transformation (Ma Crane and Gelvin 2007). Another example is the role of the VirE2 bacterial factor and the plant VIP1 protein in mediating T-DNA–nucleosome interactions. Such dynamics have been described in different plant–pathogen interactions, whereby bacterial effectors can affect histone marks directly (such as direct chromatin modifiers) or indirectly by altering the host pathways that modify the epigenome (Hanford et al. 2021). Whether the dynamic regulation of epigenetic modifiers has a functional impact on the outcome of *Agrobacterium* infection should be formally tested in the future.

In conclusion, while the T-DNA is a foreign DNA that is expected to be detected and controlled by the host genome, bacterial adaptations presumably allow the T-DNA to be present in an active transcriptional state. Perhaps the plant triggers epigenetic defense mechanisms during the early hours of infection, but cells containing T-DNA insertions that are resistant to silencing mechanisms become dominant in the tumor. Another possibility is that bacterial suppressors of silencing are injected into the host and expressed during the course of infection. How bacterial effectors interact with plant chromatin and/or epigenetic modifiers in this context has yet to be determined. More comprehensive studies on the plant epigenome and transcriptome at different stages of tumorigenesis, together with functional assays, are necessary to answer these questions.

## Implications of studies on T-DNA silencing for plant engineering and research

The T-DNA is a powerful tool for generating mutants or expressing genes of interest for both fundamental research and crop improvement. The recent development of CRISPR-based technologies has enabled precise genome editing to create targeted

mutations or the insertion of exogenous constructs at a given location. However, even these technologies rely to some extent on *Agrobacterium*-mediated delivery of T-DNA-based transgenes encoding Cas9 and guide RNAs. For this reason, T-DNA silencing can limit the optimal implementation of CRISPR-based technologies, a topic that deserves further attention. While epigenetic T-DNA silencing can simply be overcome in unicellular green algae via the use of inhibitors of epigenetic silencing factors in cell culture (Beauchemin et al. 2024), this is not possible for other plants. Thus, understanding the features and mechanisms that silence transgenes remains a major goal in order to establish an exhaustive list of rules for transgene design and a roadmap to enable robust T-DNA expression.

First, during plasmid design, sequence repetitiveness and homology to preexisting sequences in the genome should be avoided, and promoter strength should be optimized. The opportunity to control the temporal and spatial expression of transgenes via synthetic promoters, which are tissue-specific, stress-specific, or species-specific promoters, is also expanding (Ali and Kim 2019; Atella et al. 2023; Khan et al. 2023). In addition, the recoding of transgene coding sequences via high-GC and high-GC3 codons (with G or C at the third position) has been shown to substantially increase heterologous transgene transcript and protein accumulation in both *Arabidopsis* and maize (*Zea mays*) (Sidorenko et al. 2017). This finding is in line with observations of increased transgene expression for high-GC transgenes in mammalian cells (Kudla et al. 2006) and *Chlamydomonas* (Barahimipour et al. 2015, 2016). The molecular bases for this phenomenon are unknown, yet some hypotheses related to reduced transgene silencing have been proposed (Sidorenko et al. 2017) and deserve to be tested. Moreover, empirical evidence justifies the inclusion of this parameter in studies aimed at high transgene expression. Future insights into the factors that prevent the onset of RNA silencing-independent mechanisms, such as 3D-based chromatin structures (KNOT) and GENESI, should further improve transgene design.

Depending on the purpose of transgenesis (if long-term expression is needed, for instance), an important step after transformation should be a detailed molecular characterization of the insertion site, copy number, and complexity of the transgene structure (insertions in tandem, in an inverted configuration, truncated copies, and so on). Such analysis should benefit from recently developed long-read sequencing technologies (Jupe et al. 2019; Giraldo et al. 2021), which further enable the direct detection of DNA methylation on single molecules (Ni et al. 2019). Likewise, the features of a secondarily introduced T-DNA should also be determined when trying to avoid the suppression of T-DNA mutations caused by intronic insertions (when exonic T-DNA mutations cannot be isolated). CRISPR-Cas9-mediated genome editing was recently used to create combinatorial loss-of-function mutants, which circumvented these issues very efficiently (Tayengwa et al. 2024) and is therefore an attractive alternative.

Position effects can only be predicted by better resolutions of T-DNA insertion sites. However, these effects can be limited using genomic insulators, which protect the transgenic cassette from the spread of neighboring epigenetic marks (Pérez-González and Caro 2019). Targeted insertion in a desired location via CRISPR-based genome editing was recently shown to be an exciting avenue for avoiding position effects completely. The low efficiency of the homologous recombination pathway still limits the use of this technology in many plants, including crops (Miki et al. 2018; Wolter et al. 2018; Merker et al. 2024). However, an alternative, powerful strategy based on Cas9/12-fused transposons was recently developed to engineer the genome at will and circumvent

this issue (Liu et al. 2024). As mentioned above, the expression of these Cas9-based constructs still mostly relies on the delivery and expression of the T-DNA, although sustained T-DNA expression throughout generations is less of a requirement in this case.

A more comprehensive understanding of the features associated with successful T-DNA expression could come from the analysis of T-DNA insertions during natural plant-*Agrobacterium* interactions. For example, the epigenetic regulation of natural T-DNA insertions remains understudied and poorly understood. This knowledge could also provide new insights into the plant[-] pathogen arms race. In fact, fighting crown gall disease might be improved by reinforcing plant defenses against putative bacterial effectors yet to be discovered, which could allow efficient T-DNA expression, particularly during the early hours of infection (Gohlke and Deeken 2014). Interestingly, many wild and domesticated dicots were recently shown to contain relics of T-DNAs in their genomes, indicating that T-DNA transfer can permanently modify the plant genome (Matveeva and Otten 2019). Thus,

#### Advances

- Many factors affect RNA silencing and DNA methylation of T-DNA-based transgenes: copy number, sequence homology, promoter strength and integration sites.
- During T-DNA suppression, a mutation induced by an intronic T-DNA insertion restores a wild-type phenotype when a second T-DNA is introduced, leading to heterochromatinization of the intronic T-DNA and splicing.
- Recently discovered transgene silencing mechanisms include long-range interactions of T-DNAs with 3D chromatin-based silencing structures called KNOTs, as well as GESENI.
- While genes with disarmed T-DNAs are often silenced, genes with natural T-DNA insertions are efficiently expressed during natural *Agrobacterium* infection. These genes do not appear to be subjected to RNA silencing or global epigenetic silencing, but genome defense mechanisms might be deployed during early infection.

#### Outstanding questions

- Is there a link between the complexity of a T-DNA insertion and the propensity for a transgene to be silenced?
- What is the mechanism of 3D KNOT-based silencing mechanism? Is it mediated by alternative epigenetic marks other than DNA/H3K9 methylation?
- Do alternative epigenetic marks contribute to T-DNA silencing?
- Are natural T-DNAs targeted by the same mechanisms described for transgenes? Does this occur during early infection or in T-DNA genes with specific features?
- Are RNA silencing and epigenetic modifications modulated or suppressed by bacterial effectors, cell proliferation, or both, during successful infection?
- Does natural T-DNA regulation have global epigenetic consequences for the host genome?
- How are T-DNA-derived relics in wild plants regulated?
- Can we learn from features of natural T-DNA insertions during *Agrobacterium* infection to improve T-DNA-based transgene expression?

beyond pathogenesis, T-DNAs may make a greater contribution than expected to plant genome dynamics and evolution. This highlights the importance of studying T-DNA regulation during *Agrobacterium*–*Arabidopsis* interactions as well as the regulation of T-DNA-derived relics that may have been domesticated for regulatory purposes over evolutionary time (see Outstanding questions).

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