Letters

Unexpected silencing effects from T-DNA tags in *Arabidopsis*

Lucia Daxinger[1], Ben Hunter[3], Mazhar Sheikh[3], Vincent Jauvion[2], Virginie Gasciolli[2], Hervé Vaucheret[2], Marjori Matzke[1] and Ian Furner[3]

[1] Gregor Mendel Institute of Molecular Plant Biology, Dr Bohr-Gasse 3, A-1030 Vienna, Austria
[2] Laboratoire de Biologie Cellulaire Institut Jean-Pierre Bourgin, INRA (Institut National de la Recherche Agronomique), 78026 Versailles, Cedex, France
[3] Department of Genetics, University of Cambridge, Cambridge, CB2 3EH, UK

The molecular resources available for studies of *Arabidopsis thaliana* are unparalleled in any higher plant. Perhaps the most valuable resource is a collection of >250 000 sequence-indexed T-DNA inserts in the *Arabidopsis* genome (http://signal.salk.edu/cgi-bin/tdnaexpress). This corresponds to ~10 T-DNA inserts per gene and for any specific gene a T-DNA insertion mutant can be found in >90% of genes (http://*Arabidopsis*.info/info/MASC_2007.pdf). T-DNA tagged mutants are central to reverse genetics in *Arabidopsis* and are used in a similar way to conventional mutants in crosses, phenotypic assessments and molecular analyses. The T-DNA tag typically causes a loss of gene expression and results in a monogenic recessive mutation in the gene. Here we report effects from commonly used T-DNA tagging lines that result in the silencing a variety of diverse constructs using the cauliflower mosaic virus 35S promoter.

Of the gene-silencing phenomena described in higher plants, a subset, including *trans*-inactivation, involves interactions between two (or more) homologous nucleic acid sequences. *Trans*-inactivation is a term originally used to describe silencing of a transgene by a second homologous transgene introduced by transformation [1]. The transgenes usually have common 3′ sequences or promoters derived from the original *Agrobacterium* T-DNA [such as the nopaline synthase promoter (NOS pro)] or viruses [such as the 35S promoter of cauliflower mosaic virus (CAMV)]. Typically one T-DNA exerts a dominant epigenetic silencing effect on another transgene on a second (unlinked) T-DNA in *trans* [2,3]. Silencing is often correlated with hypermethylation of the silenced gene, which can persist after removal of the silencing insert [1–3].

Constructs used to generate populations of tagged *Arabidopsis* lines frequently contain sequence elements with the potential to result in *trans*-inactivation, in particular the 35S promoter, which is present in the T-DNAs of the SALK, FLAG and GABI collections (Table 1). Of the frequently used collections, only the SAIL lines have no 35S promoter homology (Table 1). Thus, there is potential for *trans*-inactivation and unexpected silencing of 35S promoter-driven transgenes in experiments using SALK-, FLAG- or GABI-generated mutants. It was in experiments using T-DNA insertion mutants defective in genes involved in silencing that we noticed unexpected dominance relationships and segregations suggesting that the T-DNA tags were behaving as more than simple mutants.

DICER-LIKE3 (DCL3) processes double-stranded RNA precursors to generate 24-nucleotide siRNAs involved in DNA methylation and heterochromatin formation [4]. The *dcl3–1* allele is a T-DNA insertion mutant from the SALK collection (The SALK Institute http://signal.salk.edu/tdna_protocols.html) (SALK_005512) [4]. This type of

Table 1. *Arabidopsis* T-DNA tagging lines and the CAMV 35S promoter[a]

Collection name	Number of mapped inserts (thousands)	Vector name	CAMV35S promoter in tagging construct	Reference and construct detail address
SALK	151	pROK2	Yes	Alonso *et al.* [11] http://signal.salk.edu/tdna_protocols.html
SAIL	57	pCSA110 pDAP101	No	Sessions *et al.* [12] http://www.*Arabidopsis*.org/abrc/pCSA110.pdf
GABI	63	pAC161	Yes	Rosso *et al.* (2003) http://www.gabi-kat.de/
FLAG	31	pGKB5	Yes	Samson *et al.* (2002) http://www-ijpb.versailles.inra.fr/en/sgap/equipes/cyto/ressources/pGKB5.html
WISCDSLOX	10	pDs-Lox	Yes	http://www.hort.wisc.edu/krysan/2010/default.htm
Other	63	Varies	Varies	Various

Abbreviations: SALK, The Salk Institute; SAIL, Syngenta Arabidopsis Insertion Library; GABI, Genomanalyse im biologischen System Pflanze; FLAG, refers to real flags as indicators of where the insertions are; WISCDSLOX, University of Wisconsin collection (DS,maize transposable element 'Ds'; LOX,site-specific recombination site 'LoxP').
[a]Insert number is derived from the SIGnAL database (http://signal.salk.edu/cgi-bin/tdnaexpress). Homozygous SALK lines and the GABI-KAT (Kölner Arabidopsis T-DNA lines) confirmed lines have been omitted. The insert numbers are valid as of October 25th, 2007.

Corresponding author: Furner, I. (ijf@mole.bio.cam.ac.uk).

mutant might be expected to exert a recessive effect on siRNA production from double-stranded RNA. To examine this, a cross was made between a *dcl3–1* homozygote and a line containing a 35S promoter-driven hairpin construct designed to silence a chlorophyll biosynthetic gene. Although wild-type plants are green and silenced plants are yellow (Figure 1d,e), the double heterozygote showed a surprising reversion towards green coloration (Figure 1f), suggesting that the *dcl3–1* mutant exerted a dominant epistatic effect on the hairpin construct. To test whether this was because of *trans*-inactivation mediated by homology between the 35S promoters on the T-DNAs, the *dcl3–1* mutant was crossed with a line containing a 35S promoter-driven β-glucuronidase (GUS) gene [5]. The staining of F2 plants from the *dcl3–1* cross was greatly reduced (Figure 1b) compared with the control cross with wild-type (Figure 1a). Other SALK lines showed an even stronger suppression of GUS expression in the same cross (Figure 1c). To examine whether this is a general phenomenon the experiment was repeated with 21 randomly chosen SALK line homozygotes and 11 showed reduced GUS staining in the F_2 generation seedlings (data not shown).

In independent experiments, another T-DNA insertion mutant of *DCL3* (*dcl3–4,* GABI_327D02) was used to investigate DICER-LIKE (DCL) requirements for generation of siRNAs from a NOS promoter hairpin RNA, which is transcribed by the 35S promoter [6]. Although NOS pro siRNAs were not seen in the *dcl3–4*-hairpin background this was not because of a DCL3 deficiency but rather because of the absence of the hairpin RNA precursor (Figure 1g). Loss of NOS pro siRNAs was also observed in a T-DNA insertion mutant of *DCL2* (*dcl2–1*; SALK_064627) and this was again accompanied by a reduction in the precursor hairpin RNA (Figure 1h). These results suggest that the 35S promoter driving expression of the NOS pro hairpin is silenced by 35S promoter sequences in the T-DNA-tagged mutant lines.

Additional evidence of 35S-silencing mediated by SALK and GABI T-DNA-tagged mutants was obtained with the *Arabidopsis* line L1. This L1 line carries a 35S–GUS transgene and is a model for post-transcriptional gene silencing (PTGS) studies. L1 accumulates GUS 21-nucleotide siRNAs at high levels and GUS mRNA levels are low. A genetic screen identified mutants (*ago1, hen1, rdr6, sgs3*) that have high GUS mRNA levels and correspondingly low GUS siRNA levels [7]. An *ago7* EMS (ethyl methyl sulphonate) mutation (*zip-1*) has no effect on L1 PTGS [8] but an *ago7* T-DNA mutation (SALK_037458) resulted in the loss of GUS 21-nucleotide siRNAs without an increase in GUS mRNA accumulation (Figure 1i) suggesting transcriptional silencing of the 35S–GUS reporter by the *ago7* T-DNA tag. Similar results were obtained using the T-DNA mutations *dcl2–6* (SALK_079428), *dcl3–1* (SALK_005512), *dcl4–2* (GABI_160G05) and *drb4–1* (SALK-000736). Loss of GUS 21-nt siRNAs without restoration of GUS mRNA expression also was observed when confronting L1 to the T-DNA mutations *dcl2-6* (SALK_079428), *dcl3-1* (SALK_005512), *dcl4-2* (GABI_160G05) and *drb4-1* (SALK-000736) (Figure 1i). Thus, it is impossible to analyze the genetics of PTGS-L1 using T-DNA tagged mutants that contain a 35S promoter because of *trans*-inactivation.

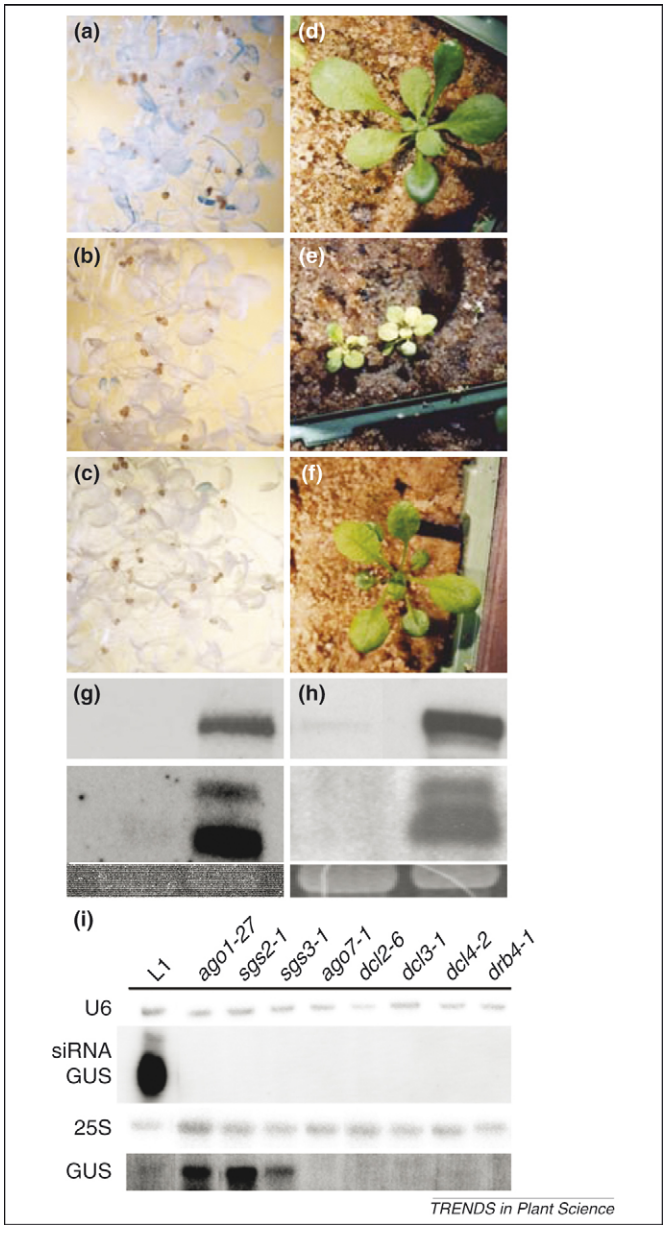

Figure 1. Unexpected silencing effects from T-DNA tags in *Arabidopsis thaliana*. **(a)** Plants segregating for an active 35S-driven *GUS* transgene. **(b)** Plants segregating for a 35S-driven *GUS* transgene and the *dcl3–1* mutant (SALK_005512). The majority of plants are not stained and staining is absent from most of the roots. **(c)** Plants segregating for a 35S-driven *GUS* transgene and the T-DNA tag SALK_112922. Only a few plants show very weak staining. **(d)** A wild-type plant. **(e)** Plants heterozygous for a hairpin construct with homology to the 3′ end of the *CH-42* gene, which encodes a subunit of magnesium chelatase that is required for chlorophyll biosynthesis. The silenced plants are yellow and small. **(f)** A plant heterozygous for the *CH-42* hairpin construct and *dcl3–1* (SALK_005512). Note the reversion to the green wild-type phenotype because of the relief of hairpin silencing of *CH-42*. **(g)** A NOSpro hairpin RNA (top) and dicer-generated siRNAs (middle) are observed in wild-type plants homozygous for a NOSpro hairpin construct (right lane) but neither is detectable in plants doubly homozygous for the NOSpro hairpin construct and *dcl3–4* (GABI_327D02) (left lane). The major band on the ethidium bromide-stained gel is shown as a loading control (bottom). The NOSpro silencing system is described in [6]. **(h)** Same labeling as in (g) except the mutant is *dcl2–1* (SALK_064627). **(i)** The PTG-silenced line L1 accumulates high levels of GUS 21-nt siRNAs and low levels of GUS mRNA, whereas the opposite is observed in the PTGS-deficient mutants *ago1–27, rdr6(sgs2–1)* and *sgs3–1*, which derive from line L1 by EMS mutagenesis. By contrast, both GUS siRNAs and GUS mRNA are below detectable levels in the T-DNA tagged mutants *ago7–1* (SALK_037458), *dcl2–6* (SALK_079428), *dcl3–1* (SALK_005512), *dcl4–2* (GABI_160G05) and *drb4–1* (SALK_000736) in which L1 was introduced by crossing, suggesting transcriptional silencing of the 35S-GUS reporter by these T-DNA tags. Standard for small RNA blot was U6 snRNA. Standard for mRNA blot was 25S rRNA.

Indeed, ~50% of SALK and GABI lines, but not SAIL lines, caused failure of L1 PTGS in the F1 generation.

Our results imply that gene silencing mediated by 35S promoter homology between transgenes and T-DNAs used for insertional mutagenesis is a common problem and occurs in tagged lines from different collections. Whether silencing is mediated by 35S promoter siRNAs produced from complex transgene inserts is not known. However, previous work has shown that the 35S promoter can be silenced and methylated by homologous siRNAs [9,10]. Care must be taken to control for unwanted silencing when using T-DNA insertional mutants to study expression of transgenes that also use the 35S promoter. It might be prudent to avoid using the 35S promoter in conjunction with any of the T-DNA-tagged mutant collections except for the SAIL lines.

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