Impact of differential DNA methylation on transgene expression in cotton (Gossypium hirsutum L.) events generated by targeted sequence insertion

Aurine Verkest, Stephane Bourout, Jurgen Debaveye, Kristine Reynaert, Bernadette Saey, Ilse Van den Brande and Kathleen D’Halluin*

BASF Agricultural Solutions Belgium NV, Gent, Belgium

Summary
Targeted Genome Optimization (TGO) using site-specific nucleases to introduce a DNA double-strand break (DSB) at a specific target locus has broadened the options available to breeders for generation and combination of multiple traits. The use of targeted DNA cleavage in combination with homologous recombination (HR)-mediated repair, enabled the precise targeted insertion of additional trait genes (2mepsps, hppd, axmi115) at a pre-existing transgenic locus in cotton. Here we describe the expression and epigenome analyses of cotton Targeted Sequence Insertion (TSI) events over generations. In a subset of events, we observed variability in the level of transgene (hppd, axmi115) expression between independent but genetically identical TSI events. Transgene expression could also be differential within single events and variable over generations. This expression variability and silencing occurred independently of the transgene sequence and could be attributed to DNA methylation that was further linked to different DNA methylation mechanisms. The trigger(s) of transgene DNA methylation remains elusive but we hypothesize that targeted DSB induction and repair could be a potential trigger for DNA methylation.

Introduction
Targeted Sequence Insertion in cotton has been achieved by the introduction of a targeted DNA DSB and its repair by HR-mediated insertion of trait genes. By using a customized meganuclease, we were able to precisely insert two herbicide tolerance (HT) genes (2mepsps, hppd) in close vicinity to a pre-existing transgenic locus in cotton and demonstrated that the resulting molecular stack was transmitted as a single locus to further generations (D’Halluin et al., 2013). We have also used this approach to introduce a HT gene (2mepsps) combined with an insect control (IC) gene (axmi115) at the same pre-existing transgenic locus in cotton. Originally, we selected the transgene integration position with the objective to limit transcriptional interference between the transgenes of the molecular stack and to obtain more predictable expression of the newly added transgenes at the pre-existing good performing transgenic locus. Somewhat unexpectedly, we observed expression variability of the newly added hppd or axmi115 genes in a subset of the TSI events.

It has already been reported that targeted insertion events in tobacco, generated by Cre-lox mediated site-specific transgene integration into a specific chromosomal location can produce alleles that express at a predictable level, as well as alleles that are differentially silenced while the alleles were identical at the DNA sequence level. Transcriptional gene silencing via DNA methylation was attributed as a trigger of the variation in transgene expression (Day et al., 2000).

DNA methylation is linked to regulation of gene expression, genomic imprinting, transposon silencing and chromatin structure in plants. In plants, methylation occurs at cytosines in CG, CHG and CHH contexts (where H is any nucleotide except G). It is established and maintained simultaneously by several DNA methyltransferases involved in distinct pathways. Establishment of plant DNA methylation in all sequence contexts is mediated by RNA-directed DNA methylation (RdDM) (Law and Jacobsen, 2010; Matzke and Mosher, 2014). RdDM involves small interfering RNAs (siRNAs), which target the de novo DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE DRM2 to genomic sites for DNA methylation. Once established, DNA methylation is maintained by distinct DNA methyltransferases that are responsible for maintaining methylation at either CG, CHG or CHH sites (Law and Jacobsen, 2010; Stroud et al., 2014). Methylation maintenance at CG sites relies on DNA METHYLTRANSFERASE 1 (MET1). Maintenance of CHG methylation occurs through a self-reinforcing loop that requires CHG methyltransferase CHROMOMETHYLASE 3 (CMT3) and histone 3 lysine 9 (H3K9) methyltransferases KRYPTONITE. Methylation at CHH sites occurs through two distinct mechanisms: H3K9me2 linked CHROMOMETHYLASE 2 (CMT2) or RdDM recruited DRM2. The functional consequences of DNA methylation on gene expression often depend on the location of the methylation relative to the gene. Many genes can tolerate substantial levels of methylation in flanking regions (Li et al., 2015a). The presence of gene body methylation seems to have minimal or no effect on gene expression (Bewick et al., 2016). By contrast, promoter and transcription start site methylation is often associated with gene silencing (Niederhuth et al., 2016).

To-date, there are only a few publications describing targeted HR-mediated transgene insertion in plants (Anil et al., 2013; Begemann et al., 2017; Li et al., 2015b; Shukla et al., 2009; Svirachev et al., 2015). Furthermore, there is no information available about the transgene expression of identical TSI events over generations. In this study, we report...
on the characterization of transgene expression over generations. We observed variability over several generations in expression levels of the \textit{hppd} or \textit{axmi115} transgenes in a subset of TSI events with identical DNA sequence and this was observed over generations in both, independently generated but genetically identical events and between sister plants from the same event. Further analyses demonstrated that the variation of transgene expression is mediated by DNA methylation and suggest that the trigger(s) for silencing might engage different pathways.

**Results**

**Cotton targeted sequence insertion events can show strong expression variation of the newly introduced transgenes**

Using the customized COT-5/6 meganuclease, we made targeted introduction of different transgene expression cassettes at a position located 2072 bp upstream of an existing cotton event that carries the \textit{cry2Ae} and the \textit{bar} genes (described in published patent application WO2008/151780). Besides the described homologous pCV211 donor DNA (D’Halluin et al., 2013) we used additional donor DNAs containing either the 2m\textit{epsps}\_\textit{hppd} or the 2m\textit{epsps}/\textit{axmi115} expression cassettes flanked by cotton genomic sequences corresponding to the target locus. Details about the donor DNAs are listed in Table S1. The \textit{hppd} and \textit{axmi115} genes are referred to as the genes of interest (GOI) hereafter. The \textit{hppd} gene conferring tolerance to 4-hydroxyphenylpyruvate dioxygenase (\textit{hppd}) inhibitors and the \textit{axmi115} gene conferring insect control were each linked to the selectable marker (SM) gene, the double mutant enol-pyruvylshikimate-3-phosphate synthase gene (2m\textit{epsps}), allowing selection of plants on glyphosate. Co-delivery, using particle bombardment, of the COT-5/6 meganuclease gene and the respective donor DNAs into embryogenic callus (EC) of the target cotton line and consecutive selection of glyphosate tolerant EC events followed by PCR analysis allowed the recovery of targeted sequence insertion (TSI) events at frequencies ranging from 1.8 to 7.5% (Table S1). Southern blot analysis, sequencing of the PCR amplicon covering the recombination sites upstream and downstream of the insertion site and capture-based target enrichment prior to Illumina MiSeq next-generation sequencing (NGS) on genomic DNA isolated from several TSI events belonging to the different expression classes (positive, negative and mixed; Table S3). These clean TSI events displayed a HR-mediated transgene insertion at the genomic cotton target site without additional random insertions of DNA from the donor DNAs and meganuclease vectors. Also, within these clean TSI events, we could identify events displaying variation or silencing of expression of the GOI (\textit{hppd/axmi115}). This variation in expression was not observed for 2m\textit{epsps} which was not surprising as this gene was used as SM gene to select for glyphosate tolerant events.

To summarize, these results show that TSI of 2m\textit{epsps}/\textit{hppd} or 2m\textit{epsps}/\textit{axmi115} at the same genomic location can give rise to genetically identical events that display variation in \textit{hppd} or \textit{axmi115} expression. Importantly, the variation in gene expression of the \textit{hppd} or \textit{axmi115} occurred independently of the expression of the linked 2m\textit{epsps} SM gene. In addition, the observed variation in expression appeared to occur independently from the donor DNA sequence and expression cassette design since it was observed with different donor DNAs with the GOI under control of different promoters and the GOI in different orientations compared to the SM gene. The expression variation was not due to any epistatic effect of additional vector sequences integrated elsewhere in the genome.

**Clean TSI events show differences in GOI expression over generations**

Progeny analysis was performed on a number of clean, independent TSI events. T1 progeny was generated most often through selfing and only through crossing with cotton line C312 for T0 plants with male sterility problems. T2 progeny and beyond were generated through selfing (see Table S4).

T1 up to T5 progeny were screened by real-time PCR (Ingham et al., 2001) for copy number determination of the four transgenes – two from the TSI event (2m\textit{epsps}/\textit{hppd} or 2m\textit{epsps}/\textit{axmi115}), and two from the original cotton event (\textit{cry2Ae}, \textit{bar}) where the TSI were inserted. Hemizygous and homozygous progeny plants carried 1 and 2 copies, respectively, of each of the four genes of the molecular stack, while null segregates were null for all four genes. As reported previously, all T1 progeny did show inheritance of the molecular stack (\textit{cry2Ae/ bar/hppd} or \textit{axmi115}/2m\textit{epsps}) in a Mendelian manner as a single genetic locus (D’Halluin et al., 2013).

HPPD/AXMI115 protein expression was analysed by ELISA on multiple plants from independent TSI events over several generations (Table S4).
For the pCV211 donor DNA containing the hppd gene, progeny from 2 sister plants derived from the event G4GH9000-023 (G4GH9000-023_1 and G4GH9000-023_2), were followed from T1 through T5 generations. In the T1 generation all G4GH9000-023_2 progeny plants expressed HPPD and the expression was maintained in 59 analysed plants up to generation T5 (‘stable’ expressed, green, Figure 2a). The T1 progeny of the sister plant G4GH9000-023_1 displayed ‘variable’ expression of HPPD, with one plant displaying expression and five plants silencing. In later generations, the progeny of the expressing plant maintained HPPD expression (‘variable’ expressed, blue, Figure 2b). For T1 plants without HPPD expression, silencing was lost over generations. Starting with over 80% of silenced plants in T1 (‘variable’ silenced, red), 34% displayed silencing in T2, 5% in T3 and only 1% in T4.
Finally, all progeny gained HPPD expression in T5 (Figure 2b). Progeny that reverted from non-expressing progenitors (‘variable’ silenced, red) to expressing descendants (‘reverted’ expressed, pale blue) continued to stably express HPPD in subsequent generations (‘variable’ expressed, blue).

When looking at events generated with donor DNA pCV260 with the axmi115 gene, several events were followed over maximum five generations (Table S4). Again, both stable (e.g. G4GH9029-065, green, Figure 3a) and variable (e.g. G4GH9044-025, Figure 3b and c) GOI expressing events were identified. In case of the variable AXMI115 expressing event G4GH9044-025, for the plant G4GH9044-02502_1, just as in the above example of the variable pCV211 G4GH9000-023_1 plant, loss of silencing over generations could be observed (Figure 3b, Table S4). However, in its sister plant G4GH9044-02502_2, silencing of AXMI115 was maintained and stable up to T4 (Figure 3c, Table S4).

Finally, axmi115 donor DNA pCV261 progeny from two different TSI events were analysed (Table S4). Stable AXMI115 expression was observed in all 35 pCV261 G4GH9041-166_2 progeny plants up to T5 (Figure 4a, green). In the event G4GH9057-110, different expression patterns were seen within the progeny of plant G4GH9057-110_3 (Figure 4b, Table S4). Some T1 progeny plants displaying AXMI115 expression (‘variable’ expressed, blue) gave rise to silenced progeny (‘reverted’ silenced, pink) and this established AXMI115 silencing remained stable (‘variable’ silenced, red) in its descendants while other T1 plants displaying AXMI115 silencing (‘variable’ silenced, red), showed partial loss of silencing in their progeny with 60% expressing descendants in T2 (‘reverted’ expressed, pale blue). In T3 re-establishment of AXMI115 silencing was seen in 50% of the progeny plants (‘reverted’ silenced, pink) of a reverted expressing T2 plant, and this silencing was maintained in the T4 progeny (‘variable’ silenced, red; Figure 4b, Table S4).

Quantitative RT-PCR was performed on several plants to confirm the ELISA results. GOI stable expressing (green), variable expressing (blue) and variable silenced (red) plants were analysed in different generations (Figure S1; Table S4). GOI RT-qPCR results were in line with the ELISA results and confirmed the variable GOI expression and silencing within some events (Figure S1). In contrast, examination of the expression level of the 2mepsps/axmi gene, corroborated the glyphosate tolerance selection and was relatively steady-state. Also, analysis of the mRNA levels of transgenes from the original cotton event (bar and cry2Ae), demonstrated stable and comparable expression in all tested lines (Figure S1).

In summary, the analysed clean TSI events could be grouped in two general categories: ‘stable’ events for which all plants show GOI expression over generations, and ‘negative’ or ‘mixed’ events, that show variability in GOI expression. The latter display different outcomes of GOI expression in their progeny with GOI expression remaining stable, or being lost or gained over generations. Remarkably, sister plants of the same event sometimes show a very different expression pattern.
methylation was observed outside the 3′ and red). In the stably expressing plants (green) no GOI expressing sequences in DNA methylation of the GOI between plants stably donor DNAs (Table S4).

To determine which hppd/axmi115 expression levels differ between and/or within independent TSI events, and vary over generations, we quantified DNA methylation levels of the inserted GOI sequences by targeted bisulfite sequencing of a subset of plants from selected expressing and variable TSI events of different donor DNAs (Table S4).

In pCV211 event G4GH9000-023 (Figure 5a) we saw differences in DNA methylation of the GOI between plants stably expressing hppd (G4GH9000-023, green) and plants whose progeny displayed variable expression (G4GH9000-023, blue and red). In the stably expressing plants (green) no GOI methylation was observed outside the 3′ UTR sequence. In the variable expressing (blue) plants methylation was restricted to the coding and 3′ UTR sequence while in the silenced (red) plants methylation spread along the coding sequence and the promoter (Figure 5a). The consistency of the DNA methylation distribution pattern within the three expression classes (stably expressed GOI, variable expressed GOI and silenced GOI) was confirmed in different plants over two generations (Figure S2A). Hypermethylation of the promoter was specific for plants silenced (red) for the GOI and reduced in progeny of silenced plants with regained hppd expression (reverted expressed, pale blue; Figure S2A).

In pCV260 TSI plants from a stable expressing event (G4GH9029-065, Figures 5b and S2B, green) limited GOI DNA methylation was observed in the promoter and the region close to the transcription start site. The pCV260 G4GH9044-025 event that express axmi115 (blue) showed weak methylation in regions of both the promoter and axmi115 coding sequence that became stronger and spread along the coding sequence and promoter in silenced (red) plants (Figures 5b and S2B, red).

Similarly, stable expressing pCV261 G4GH9041-166_2 progeny (green) appeared to have a hypomethylated (no DNA methylation) GOI sequence. Progeny of the ‘mixed’ pCV261 G4GH9057-110 event that express axmi115 (blue) showed no or little methylation whereas silencing appeared linked with heavy promoter and CDS hypermethylation. In variable expressing plants, the coding sequence (CDS) methylation shows GOI sequence specific distribution patterns.

GOI expression variability is associated with differences in promoter and coding sequence DNA methylation levels

To infer the molecular mechanisms responsible for DNA methylation in almost the entire coding sequence and discrete parts of the promoter in progeny showing axmi115 expression (Figures 5b and S2B, blue). In plants silenced for axmi115 expression, the DNA methylation pattern became denser and covered the complete GOI coding sequence and its upstream promoter region (Figures 5b and S2B, red).

Together these results reveal different methylation patterns between expressing and silenced plants showing a clear inverse correlation between DNA methylation and GOI expression levels. Irrespective of the inserted transgenes, stable expressing plants displayed no or little methylation whereas silencing appeared linked with heavy promoter and CDS hypermethylation. In variable expressing plants, the coding sequence (CDS) methylation shows GOI sequence specific distribution patterns.

To get more insight in the establishment and role of the DNA methylation signatures we looked at the methylation sequence contexts (Figures 6 and S3). Inspection of the methylation density in the CG, CHG and CHH contexts revealed specific differences among the different donor DNA events. For pCV211, the variable expressing progeny (blue) of plant G4GH9000-023_1 shows hppd gene body methylation in the CG context. In hppd silenced plants (red) CG, CHG and CHH gene body and promoter methylation is accumulating (Figures 6 and S3A). For pCV260 and pCV261 events gene body CG methylation cannot occur since the axmi115 CDS has no CGs. pCV260 variable expressing plants (blue) are mainly characterized by CHG CDS methylation and CG promoter methylation. Silencing requires additional promoter CG, CHG and CHH, and CDS CHG and CHH sequence methylation (Figures 6 and S3B, red). Axmi115 variable expressing (blue) pCV261 plants show different methylation patterns than pCV260 plants and display mainly promoter CG, CHG and CHH methylation. In silenced (red) pCV261 plants, promoter methylation intensifies and emerges/arises within the CDS (Figures 6 and S3C).

Together, the DNA methylation data showed transgene silencing being correlated with GOI promoter, transcription start site (TSS) and 5′ coding sequence (CDS) DNA methylation with differences in methylation contexts. Although these differences are likely GOI sequence context dependent, this indicates that alternative pathways might induce and maintain the methylation dependent variable transgene expression and stability in TSI events.

Variable contribution of sRNA and histone marks to GOI silencing

To infer the molecular mechanisms responsible for DNA methylation in the different TSI events, we looked whether the GOI sequences are associated with small RNAs (sRNA). sRNAs, in particular 24-nt sized sRNAs, are a hallmark of RdDM all context de novo and CHH maintenance DNA methylation (Law and Jacobsen, 2010). Illumina HiSeq of sRNAs was performed on stable expressing, variable expressing and silenced plants generated from TSI events of different donor DNAs. Profiling of the
sRNAs mapping to the different TSI was performed and their distribution and abundance on both the complete transgene insert and the GOI in stable expressing (green), variable expressing (blue) and silenced (red) plants was investigated (Figures 7 and S4–S6). Accumulation of GOI associated 24-nt sRNAs in silenced plants would indicate RdDM mediated methylation.

For the pCV211 TSI event no accumulation of *hppd* associated 24-nt sRNAs is seen in silenced plants (Figures 7a and S4). In contrast, we observed higher levels of *hppd* mapping sRNAs in expressing plants (see Discussion). Besides 24-nt sRNAs, also 21-nt GOI targeted sRNAs accumulate in expressing plants suggesting post-transcriptional regulation of gene expression (Figure S4).

In pCV260 TSI plants, very few sRNAs target the GOI and no evidence points to RdDM mediated silencing.

Finally, pCV261 TSI plants also displayed a low number of sRNAs dispatched along the GOI and the complete TSI sequence. However, a discrete accumulation of sRNAs targeting the *axmi*115 expression driving promoter is observed in variable expressing (blue) and silenced (red) plants (Figures 7c and S6). Whether these potentially initiate methylation through RdDM remains to be analysed.

Next, we examined distribution of H3K9me2 a hallmark of heterochromatin, associated with CHG and CHH methylation by CMT and DRM DNA methyltransferases (Law and Jacobsen, 2010). Also, H3K4me3 accumulation, a mark linked with active transcription, was tested. Chromatin immunoprecipitation (ChIP) and qPCR of promoter, TSS and/or 5’CDS regions of the GOI, associated with differential DNA methylation in expressing and silenced plants, was performed.

In pCV211 TSI events, accumulation of the repressive heterochromatin H3K9me2 mark was observed in silenced (red) plants in the regions comprising the promoter and the 5’CDS of the *hppd* gene (Figure 7a). In contrast, stable *hppd* expressing (green) plants failed to accumulate H3K9me2 in these regions but displayed accumulation of the transcription linked H3K4me3 mark at the *hppd* 5’CDS (Figure 7a).

*Axmi115* silencing (red) in pCV260 TSI plants was found to be associated with an enriched deposition of repressive H3K9me2 in the promoter region just upstream of the TSS, whereas stable *axmi115* expressors (green) accumulate H3K4me3 in their 5’CDS region (Figure 7b).

This higher H3K4me3 accumulation in the axmi115 5’CDS sequence was also observed in stable pCV261 TSI axmi115 expressing plants (green). However, pCV261 silenced plants (red) did not display enriched heterochromatin H3K9me2 deposition in the analysed promoter and TSS regions (Figure 7c).
Combined, these results illustrate that the contribution of sRNAs and H3K9me2 to GOI silencing appears variable, strengthening the variable DNA methylation context data and suggesting that alternative pathways trigger and/or maintain DNA methylation of the transgenes in the different TSI events.

**Discussion**

In this study, we analysed the transgene expression of a number of cotton plants generated from independent TSI events or derived from sister plants of the same TSI event. TGO based on the use of site-specific nucleases, combined with the increasing insight into genome sequences and gene functions, is expected to facilitate insertion of (trait) transgenes at predetermined integration sites, so called ‘safe harbours’. Targeted integration is perceived as advantageous over random integration because it could allow for the desired expression of the GOI while reducing or eliminating possible unintended effects due to disruption of native genes and regulatory elements associated with random transgene integration. However, site-specific integration of (trait) transgenes by HR in plants has been rarely reported and currently there is no information available on the stability/variability of gene expression from such targeted integration events.

Here, analysis of cotton TSI events generated via HR, demonstrated that for a subset of the events, considerable variation in GOI (hppd or axmi115) expression over generations exists between plants generated from independent TSI events and between sister plants derived of the same TSI event. Whereas variation in transgene expression between random integration events has been described frequently, and laid the basis of epigenetic research (Meyer, 2013), reports on targeted integration events are scarce. Day et al. (2000) described the cre-lox mediated generation of targeted transgene integrated tobacco events and attributed DNA methylation as a trigger of observed variable transgene expression. Using a mechanistically different approach for targeted integration of transgenes, also we identified transcriptional gene silencing through differential DNA methylation of the transgene promoter as at least one of the factors mediating variation in GOI expression in the analysed TSI events. Even gene targeting based on gene replacement for the targeted introduction of a few amino acid substitutions in an endogene could lead to changes in the DNA methylation profile as has been shown in *Arabidopsis* (Lieberman-Lazarovich et al., 2013). Methylation could be either completely lost in a region of the target gene, maintained with minor changes, or show variability in subsequent generations.

Transcriptional gene silencing can be affected by various factors. These include characteristics of the insert, the position of and nature of the integration site and plant developmental stages and/or environmental conditions. Our experimental strategy and the obtained observations exclude several well-established causes implicated in gene silencing.

As we observed variable GOI expression in clean TSI, we can eliminate the possibility of homology-dependent transgene...
silencing (HDGS) due to integration of small stretches of donor or vector backbone DNA (Selker, 1999). Also the occurrence of silencing triggered by the donor DNA sequence itself, is unlikely as variable transgene expression was observed in TSI events generated by using donor DNAs with different construct designs, promoters and GOIs. In case of the hppd GOI events, it is unlikely that the endogenous native hppd gene might trigger silencing of the hppd transgene as the level of DNA sequence homology between both genes is less than 40%. In TSI events where the GOI is under the control of the 35S promoter that also drives the expression of the cry2Ae gene from the original cotton event, the double presence of the 35S promoter might have influenced the level of GOI expression in some events. Especially since it has been reported that the 35S sequence is known to be susceptible to silencing by methylation (Okumura et al., 2016; Wang et al., 2017). However, we believe that another, more general trigger is at play since we observed silencing in TSI events with other GOI promoters while the expression of the 35S driven cry2Ae gene was stable and not affected in any of the plants tested.

Several reports describe a correlation between the incidence of gene silencing and high transgene copy number, but here single copy TSI events were selected and confirmed by Illumina MiSeq next-generation sequencing (NGS) after capture-based target enrichment, not to contain additional random insertions of DNA from the donor DNAs and meganuclease vectors. Similarly silencing originating from the allelic state of the sequence insertion (Masclaux et al., 2005) can be ruled out since no correlation was seen between GOI expression variability/stability in selfed, backcrossed, hemizygous or homozygous TSI progeny.

With regard to the integration site, position effects frequently have been associated with variable expression (Matzke and Matzke, 1998) although some studies suggest that these play minor roles (Nagaya et al., 2005). Nevertheless, transgene integration into heterochromatic regions has been shown to lead to silencing (Ahmed et al., 2011; Hollister and Gaut, 2009).

Figure 7 Variable transgene expressing TSI plants have different 24-nt sRNA and histone mark GOI accumulation patterns. sRNA sequencing and ChIP-qPCR on samples of stable expressing, variable expressing and silenced plants. Green, ‘stable’ expressed; dark blue, ‘variable’ expressed; red, ‘variable’ silenced; pale blue, ‘reverted’ expressed; pink, ‘reverted’ silenced. (a) pCV211 donor DNA, (b) pCV260 donor DNA and (c) pCV261 donor DNA TSI plants. Left, Mapping of the 24-nt sRNA sequencing reads to the GOI sequences. The y axes are scaled the same per TSI/GOI. Reads were normalized per 10 million of genome-matched (including the transgene sequence) 18-to-28 nucleotide sequences. Analyses on additional samples and accumulation of other sRNA size classes on the GOI (hppd/axmi) and complete TSI (hppdaxmi + epsps) sequence are shown in Figures S4–S6. Right, H3K9me2 and H3K4me3 ChIP. Enrichment was determined by qPCR and for each region normalized against the input. Analysed regions are indicated with a black line with digit 1 and 2 below it. Dots represent biological replicates. We indicate in Table S4 which plants have been analysed (ChIP column). Grey, background mock ChIP values.
targeted integration was done at an approximately 2000 bp distance from the locus of an existing cotton event which is positioned in a gene-rich genomic region without interrupting any functional annotated sequences.

Also, we have to take in account that cotton transformation is particularly challenging because of its strong genotype dependence for regeneration through somatic embryogenesis, its long timelines and its high level of somaclonal variation (Kumria et al., 2003). The duration of the tissue culture period including cell dedifferentiation and differentiation processes contributes to enhancing the rate of somaclonal variation and changes in DNA methylation. This process of tissue culture and regeneration was reported to induce consistent and stable epigenomic changes in both rice and maize (Stelpflug et al., 2014; Stroud et al., 2013a).

However, we observed high variability in GOI expression and methylation between independent TSI events, between sister plants of the same TSI event, and this over generations making the comparison of the foreign sequence to an epigenetic memory of previously expressed sequences. Maintenance of silencing requires chromatin factors and RdRP-generated small RNAs. Activating and silencing signals may compete in foreign versus non-foreign discrimination. Thus also in C.elegans genetically identical targeted insertion individuals can show remarkably opposite patterns of expression, very similar to what we have observed in cotton TSI events although epigenetic pathways may differ widely from C. elegans to plants (Seth et al., 2013; Shirayama et al., 2012).

The interplay of H3K9me2 accumulation with CHG and CHH DNA methylation in silenced plants was confirmed in pCV211 and pCV260, but not pCV261 events. This, in combination with the absence of massive GOI targeting by sRNAs in silenced plants, indicates an independence of the RdDM pathway. Also it suggests maintenance of silencing via different pathways in the different donor DNA TSI events. Maybe RdDM de novo DNA methylation was established in the T0 generation and only maintenance methylation pathways are acting in analysed progeny. This could explain part of the observed variability in GOI expression, namely stable silencing and loss of silencing over generations, but cannot account for re-established silencing in later generations. However, we cannot exclude the possibility that this de novo silencing, which was only observed in pCV261 events, is an additional distinct co-occurring silencing mechanism specific for these lines. Different silencing pathways often co-occur, confounding the interpretation of their mechanisms. Alternatively, de novo methylation might be induced by another mechanism utilizing the different DNA methyltransferases independently of RdDM (Ahmed et al., 2011; Singh et al., 2008).

Looking at open chromatin regions (using DNasel, ATAC-seq or NicE-seq techniques) between silenced and expressed transgenes could shed additional light on the mechanism leading to gene expression or repression (Chandler and Vaucheret, 2001; Ponnaluri et al., 2017; Wang et al., 2018).

The trigger responsible for variable silencing in different independent clean TSI events as such remains elusive. An intriguing possibility lies in the targeted DSB induction. Studies in mammalian cells have shown that targeted DNA methylation occurs upon homology-mediated repair of induced DSBs, resulting in variable gene expression among cells (Cuozzo et al., 2007; Morano et al., 2014; Russo et al., 2016). This is facilitated through recruitment of DNA methyltransferases and histone-modifying enzymes at the DSB during HR. Upon targeted DSB induction, the transcribed strand blocked by stalled RNA PolII may become preferential target for DNA methyltransferases and other proteins that regulate methylation (Morano et al., 2014). Whether this also occurs in plant cells is not known. A role for the DDB2 DNA
damage factor in DNA methylation through both RdDM and regulation of DNA methylcytosine glycosylase has been shown in Arabidopsis (Schalk et al., 2016). Also, interconnections between sRNAs and DNA damage repair have been reported (Schalk et al., 2017; Wei et al., 2012). DSBs were shown to trigger the induction of sRNAs required for mediating RdDM independent DSB repair (Wei et al., 2012). A link between DNA DSB repair and DNA methylation in plants remains to be further investigated and should give interesting insights, particularly for TGO applications.

Methylation in plants remains to be further investigated and (Wei et al., 2012). A link between DNA DSB repair and DNA methylation in plants remains to be further investigated and should give interesting insights, particularly for TGO applications.

Experimental procedures

Plant material

Targeted Sequence Insertion (TSI) plants were grown in controlled environment greenhouse compartments with a 16 h light period at 26 °C under lighting and 20 °C during darkness and 40%–60% relative humidity. Leaf material at the same developmental stage (2nd to 5th fully expanded leaf) was harvested from 5 to 6-week-old seedlings. Targeted insertion events with the hppd, 2mepsps or axmi115 genes were generated in an existing cotton event that carries the cry2Ae and the bar genes, conferring Lepidoptera resistance and glufosinate tolerance, respectively. The cry2Ae gene was cloned between the 35S promoter and the 3’35S terminator and the bar gene between the Csrmv promoter and the 3’nos terminator. The procedure for generation of targeted insertion events in cotton is described in (D’Halluin et al., 2013). The design of the repair DNAs is shown in Table S1.

RNA/DNA analyses

Genomic DNA was isolated using a DNeasy Plant Mini kit (Qiagen, Hilden, Germany). RNA was extracted with a Spectrum Plant Total RNA kit (Sigma, Saint-Louis, MO).

All qPCRs were performed with the Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA) and amplified on a Bio-Rad CFX 384.

For RT-qPCR, RNA was treated with HL-dsDNase (Articyzmes) and reverse transcribed with a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). qPCR results were normalized to Gh_pp2a1 (Artico et al., 2010) and are expressed as 2^(-ΔΔCq target-Cq Gh_pp2a1).

Protein analyses

The levels of 2mEPSPS, PAT, cry2Ae and HPPD protein were quantified using an enzyme-linked immunosorbent assay (ELISA) using Enviroligo kits with Enviroligo-catn® AP 084 NW V10, AP013, AP005CNTNW V10 and AP-126 NWV10, respectively. All quantitations were normalized to the protein concentration of the cotton leaf extract, as determined using the Coomassie Protein Assay Reagent Kit from Biorad.

Capture-based target enrichment and Illumina MiSeq next-generation sequencing (NGS)

Targeted sequence capture of cotton DNA samples and sequencing on Illumina MiSeq was done by Eurofins Genomics (https://www.eurofins.com). DNA probe library design and preparation, targeted capture, sequencing and data analysis were done as described in Kovalic et al. (2012) and Shearer et al. (2012).

Directed Bisulfite sequencing

Bisulfite conversion, PCR-based library generation, sequencing and analysis were performed by Active Motif (http://www.activemotif.com). Reads were aligned to the transgene sequences using the bowtie software. Alignment and methylation information was captured in BAM files, and CpG alignment coverage and percentage methylation at each C site was determined. The alignment and cytosine methylation details are shown in Table S5.

sRNA sequencing analysis

Custom libraries for sRNAs were constructed and sequenced by Fasteris SA (http://www.fasteris.com/). Small RNA reads were filtered to 18- to 28-nt reads and aligned to the Gossypium hirsutum genome (Li et al., 2015c) with the transgene DNA integrated at the targeted insertion. The following bowtie (version 0.12.9) parameters were used: -a, report all alignments per read; -v 1, report end-to-end hits with <=1 mismatch; –best hits, guaranteed best stratum; –strata, hits in sub-optimal strata aren’t reported. Reads were normalized to reads per million (RPM) of mapped reads. For visualization of transgene DNA directed sRNAs 20-to-22- and 23-to-25-nt sRNAs reads were size extracted.

ChiP-qPCR

Chromatin immunoprecipitation was performed as described by Luo et al. (2013) with some minor changes. 0.5 g ground frozen cotton leaf material was suspended in 45 ml nuclei isolation buffer I (10 m M Heps pH=7.6, 440 m M sucrose, 5 m M KCl, 5 m M MgCl2, 5 m M EDTA, 1% formaldehyde, 0.1% β-mercaptoethanol, 0.5% Triton X-100, 0.4 m M PMSF, Complete EDTA-free Protease Inhibitor Cocktail (1/50 mL) (Sigma-Aldrich, St Louis)) and incubated for 5 min at room temperature. Glycine was added to a final concentration of 125 m M and incubated at room temperature for 5 min to stop the crosslinking. The lysate was filtered twice over a 70-um nylon mesh (Fisher Scientific). Nuclei were pelleted by centrifugation at 2000 × g for 10 min at 4 °C and the pellet was suspended with 3 mL of nuclear isolation buffer II (10 m M Heps pH=7.6, 250 m M sucrose, 5 m M KCl, 5 m M MgCl2, 5 m M EDTA, 0.1% β-mercaptoethanol, Complete EDTA-free Protease Inhibitor Cocktail). The nuclei suspension was put onto a 30% Percoll nuclear isolation buffer II and centrifuged at 1500 × g for 20 min at 4 °C. The nuclei pellet was dissolved in 2 ml nuclei lysis buffer (50 m M Tris-Cl pH 7.5, 0.1% SDS, 10 m M EDTA, 50 m M NaCl, Complete EDTA-free Protease Inhibitor Cocktail), incubated at 4 °C during 1 h. The chromatin was sheared to 100–300 bp using milliTUBE 1 ml AFA Fiber tubes with a CovarisM220 sonicator (Covaris Inc., Brighton, UK).

Fragmented chromatin samples were cleared by centrifugation at 2000 × g for 5 min at 4 °C before being diluted in an equal volume of ChiP dilution buffer (50 m M Tris-Cl pH 7.5, 0.2% Triton X-100, 50 m M NaCl, 0.1 m M PMSF, Complete EDTA-free Protease Inhibitor Cocktail). 20 μL of Dynabeads Protein G (Invitrogen, Thermo Fisher Scientific, Waltham, MA) were washed twice and resuspended in 100 μL of Incubation buffer (20 m M Tris pH 7.5, 50 m M NaCl, 5 m M EDTA, 0.1% TritonX). Antibodies were added: H3K4me3 (1 μg of Millipore 07-473, http://www.merc kmillipore.com/) or H3K9me2 (2 μg of Millipore 07-441) and incubated for 2 h at 4 °C with gentle rotation on a wheel. Beads
were washed in Incubation buffer and loaded with 200 μL (H3K4me3) or 400 μL (mock and H3K9me2) diluted chromatin solution that was pre-cleared with 20 μL Dynabeads Protein G for 2 h at 4 °C. The chromatin/antibody/bead mix was incubated overnight at 4 °C with rotation. Beads were washed with 500 μL Incubation Buffer and 500 μL Wash Buffer (50 mM Tris-Cl pH 7.5, 10 mM EDTA) with 50 μL (Wash1), 100 μL (Wash2) and 150 μL NaCl (Wash3), respectively. Samples were washed in TE buffer (twice). The immunocomplexes were eluted twice with 150 μL of 1% SDS, 0.1 M NaHCO3 at 37 °C for 10 min. 12 μL 5 M NaCl was added and samples were incubated overnight at 37 °C. Samples were digested with 20 μg of proteinase K for 2 h at 42 °C followed with phenol/chloroform/IAA extraction and MinElute DNA purification (Qiagen) of the aqueous phase to obtain qPCR ready DNA. Results are expressed as % input.

Acknowledgements

Ana Atanassova, Frank Meulewaeter and Rene Ruiter are greatly acknowledged for critical reading of the manuscript, Manoj Jody Dubin for scientific discussions, Martine Bossut, Kristel D’Hont, Anouk Pennewaert and Jolien Van Hulle for the generation of targeted insertion events, Joanna Rosolowska and Chantal Vanderstraeten for molecular characterization of the targeted insertion events, Maaike Heyneman and the lab support team for excellent technical support. The authors declare no conflict of interest.

References

Ahmed, I., Sarazin, A., Bowler, C., Colot, V. and Queensville, H. (2011) Genome-wide evidence for local DNA methylation spreading from small RNA-targeted sequences in Arabidopsis. Nucleic Acids Res. 39, 6919–6931.

Alien, W.M., Sasty-Dent, L., Welter, M.E., Murray, M.G., Zeitler, B., Amora, R., Corbin, D.R. et al. (2013) Trait stacking via targeted genome editing. Plant Biotechnol. J. 11, 1126–1134.

Artico, S., Nardeli, S.M., Brilliante, O., Grossi-de-Sa, M.F. and Alves-Ferreira, M. (2010) Identification and evaluation of new reference genes in Gossypium hirsutum for accurate normalization of real-time quantitative RT-PCR data. BMC Plant Biol. 10, 49.

Begemann, M.B., Gray, B.N., January, E., Gordon, G.C., He, Y., Liu, H., Wu, X. and Begemann, M.B., Gray, B.N., January, E., Gordon, G.C., He, Y., Liu, H., Wu, X. et al. (2017) Precise isolation and guided editing of higher plant genomes using CpG1 CRISPR nucleases. Sci. Rep. 7, 11606.

Bewick, A.J., Ji, L., Niederhuth, C.E., Willing, E.-M., Hofmeister, B.T., Shi, X., Wang, L. et al. (2016) On the origin and evolutionary consequences of gene body DNA methylation. Proc. Natl Acad. Sci. 113, 9111–9116.

Chandler, V.L. and Vacheret, H. (2001) Gene activation and gene silencing. Plant Physiol. 125, 145–148.

Cubas, P., Vincent, C. and Coen, E. (1999) An epigenetic mutation responsible for natural variation in floral symmetry. Nature 401, 157–161.

Cuenda-Gil, D. and Slotkin, R.K. (2016) Non-canonical RNA-directed DNA methylation. Nat. Plants, 2, 16163.

Cuoozo, C., Porcellini, A., Angrisano, T., Morano, A., Lee, B., Di Pardo, A., Messina, S. et al. (2007) DNA damage, homology-directed repair, and DNA methylation. PLoS Genet. 3, e110.

Day, C.D., Lee, E., Kobayashi, J., Holappa, L.D., Albert, H. and Ow, D.W. (2000) Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. Genes Dev. 14, 2869–2880.

D’Halluin, K., Vanderstraeten, C., Van Hulle, J., Rosolowska, J., Van Den Brande, J., Pennewaert, A., D’Hont, K. et al. (2013) Targeted molecular trait stacking in cotton through targeted double-strand break induction. Plant Biotechnol. J. 11, 933–941.

Hollister, J.D. and Gaut, B.S. (2009) Epigenetic silencing of transposable elements: a trade-off between reduced transposition and deleterious effects on neighboring gene expression. Genome Res. 19, 1419–1428.

Ingham, D., Beer, S., Money, S. and Hansen, G. (2001) Quantitative Real-Time PCR assay for determining transgene copy number in transformed plants. BioTechniques. 31(1), 132–140.

Klein-Cosson, C., Chambrier, P., Rogowsky, P.M. and Vernoud, V. (2015) Regulation of a maize HD-ZIP IV transcription factor by a non-conventional RDR2-dependent small RNA. Plant J. 81, 747–758.

Kovalc, D., Garnaat, C., Guo, L., Yan, Y., Groat, J., Silianovich, A., Ralston, L. et al. (2012) The use of next generation sequencing and junction sequence analysis bioinformatics to achieve molecular characterization of crops improved through modern biotechnology. The Plant Genome. 5, 149–163.

Kumria, R., Leelavathi, S., Bhatnagar, R.K. and Reddy, V.S. (2003) Regeneration and genetic transformation of cotton: present status and future perspectives. Plant Tissue Cult. 13, 211–225.

Law, J.A. and Jacobsen, S.E. (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat. Rev. Genet. 11, 204–220.

Li, Q., Gent, J.J., Zynida, G., Song, J., Makarevitch, I., Hirsch, C.D., Hirsch, C.N. et al. (2015a) RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. Proc. Natl Acad. Sci. 112, 14728–14733.

Li, Z., Liu, Z.-B., Xing, A., Moon, B.P., Koelhoffel, J.P., Huang, L., Ward, R.T. et al. (2015b) Cas9-Guide RNA directed genome editing in soybean. Plant Physiol. 169, 960–970.

Li, F., Fan, G., Lu, C., Xiao, G., Zou, C., Kohel, R.J., Ma, Z. et al. (2015c) Genome sequence of cultivated Upland cotton (Gossypium hirsutum TM-1) provides insights into genome evolution. Nat. Biotechnol. 33, 524–530.

Lieberman-Lazovich, M., Melmed-Bessudo, C., de Pater, S. and Levy, A.A. (2013) Epigenetic alterations at genomic loci modified by gene targeting in Arabidopsis thaliana. PLoS ONE, 8, e65383.

Luo, C., Sidote, D.J., Zhang, Y., Kerstetter, R.A., Michael, T.P. and Lam, E. (2013) Integrative analysis of chromatin states in Arabidopsis identified potential regulatory mechanisms for natural antisense transcript production. Plant J. 73, 77–90.

Manning, K., Tör, M., Poole, M., Hong, Y., Thompson, A.J., King, G.J., Giovannoni, J.J. et al. (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat. Genet. 38, 948–952.

Marí-Ordóñez, A., Marchais, A., Etcheverry, M., Martin, A., Colot, V. and Voinnet, O. (2013) Reconstructing de novo silencing of an active plant retrotransposon. Nat. Genet. 45, 1029–1039.

Masclaux, F.G., Pont-Lezica, R. and Galaud, J.-P. (2005) Relationship between allelic state of T-DNA and DNA methylation of chromosomal integration in transformed Arabidopsis thaliana plants. Plant Mol. Biol. 58, 295–303.

Matzke, A.J.M. and Matzke, M.A. (1998) Position effects and epigenetic silencing of plant transgenes. Curr. Opin. Plant Biol. 1, 142–148.

Matzke, M.A. and Mosher, R.A. (2014) RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. Nat. Rev. Genet. 15, 394–404.

Meyer, P. (2013) Transgenes and their contributions to epigenetic research. Int. J. Dev. Biol. 57, 509–515.

Morano, A., Angrisano, T., Russo, G., Landi, R., Pezone, A., Bartollino, S., Zuchegna, C. et al. (2014) Targeted DNA methylation by homology-directed repair in mammalian cells. Transcription reshapes methylation on the repaired gene. Nucleic Acids Res. 42, 804–821.

Nagaya, S., Kato, K., Kinomiyaz, Y., Horie, R., Sekine, M., Yoshida, K. and Shinmyo, A. (2005) Expression of randomly integrated single complete copy transgenes does not vary in Arabidopsis thaliana. Plant Cell Physiol. 46, 438–444.

Niederhuth, C.E., Bewick, A.J., Ji, L., Alabady, M.S., Kim, K.D., Li, Q., Rohr, N.A. et al. (2016) Widespread natural variation of DNA methylation within angiosperms. Genome Biol. 17, 194–213.

Nuthikuttu, S., McCue, A.D., Panda, K., Fultz, D., Defraia, C., Thomas, E.N. and Slotkin, R.K. (2013) The Initiation of epigenetic silencing of active transposable elements is triggered by RDR6 and 21-22 nucleotide small interfering RNAs. Plant Physiol. 162, 116–131.

© 2018 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd, 17, 1236–1247.
Okumura, A., Shimada, A., Yamasaki, S., Morino, T., Iwata, Y., Koizumi, N., Nishihara, M. et al. (2016) CaMV-35S promoter sequence-specific DNA methylation in lettuce. Plant Cell Rep. 35, 43–51.

Ong-Abdullah, M., Ordway, J.M., Jiang, N., Ooi, S.-E., Kok, S.-Y., Sarpan, N., Azimi, N. et al. (2015) Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. Nature, 525, 533–537.

Ponnaluri, V.K.C., Zhang, G., Estrov, P., Spraklin, G., Sian, S., Xu, S., Benoukraf, T. et al. (2017) NICe-seq: high resolution open chromatin profiling. Genome Biol. 18, 122.

Russo, G., Landi, R., Pezone, A., Morano, A., Zucchella, C., Romano, A., Muller, M.T. et al. (2016) DNA damage and repair modify DNA methylation and chromatin domain of the targeted locus: mechanism of allele methylation polymorphism. Sci. Rep. 6, 33222.

Schäfl, C., Drevensek, S., Kramdi, A., Kassam, M., Ahmed, I., Cognat, V., Graindorge, S. et al. (2016) DNA DAMAGE BINDING PROTEIN2 Shapes the DNA Methylation Landscape. Plant Cell, 28, 2043–2059.

Schäfl, C., Cognat, V., Graindorge, S., Vincent, T., Voinnet, O. and Moliner, J. (2017) Small RNA-mediated repair of UV-induced DNA lesions by the DNA DAMAGE BINDING PROTEIN 2 and ARGONAUTE 1. Proc. Natl Acad. Sci. 114, E2965–E2974.

Selker, E.U. (1999) Gene silencing. Cell, 97, 157–160.

Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D. and Mello, C.C. (2013) The C. elegans CSR-1 argonaute pathway counteracts epigenetic silencing to promote germline gene expression. Dev. Cell, 27, 656–663.

Shearer, A.E., Hildebrand, M.S., Ravi, H., Joshi, S., Guiffre, A.C., Novak, B., Happe, S. et al. (2012) Pre-capture multiplexing improves efficiency and cost-effectiveness of targeted genomic enrichment. BMC Genom. 13, 618.

Shirayama, M., Seth, M., Lee, H.-C., Gu, W., Ishidate, T., Conte, D. and Mello, C.C. (2012) piRNAs initiate an epigenetic memory of non-self RNA in the C. elegans germline. Cell, 150, 65–77.

Shukla, V.K., Doyon, Y., Miller, J.C., DeKelver, R.C., Moehle, E.A., Worden, S.E., Mitchell, J.C. et al. (2009) Precise genome modification in the crop species Zea maps using zinc-finger nucleases. Nature, 459, 437–441.

Singh, A., Zubko, E. and Meyer, P. (2008) Cooperative activity of DNA methyltransferases for maintenance of symmetrical and non-symmetrical cytosine methylation in Arabidopsis thaliana. Plant J. 56, 814–823.

Stelflug, S.C., Eichten, S.R., Hermanson, P.J., Springer, N.M. and Kaeppler, S.M. (2014) Consistent and heritable alterations of DNA methylation are induced by tissue culture in Maize. Genetics, 198, 209–218.

Stroud, H., Ding, B., Simon, S.A., Feng, S., Bellizzi, M., Pellegrini, M., Wang, G.L. et al. (2013a) Plants regenerated from tissue culture contain stable epigenome changes in rice. Elife, 2, e00354.

Stroud, H., Greenberg, M.V.C., Feng, S., Bernatavichute, Y.V. and Jacobsen, S.E. (2013b) Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. Cell, 152, 352–364.

Stroud, H., Do, T., Du, J., Zhong, X., Feng, S., Johnson, L., Patel, D.J. et al. (2014) Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. Nat. Struct. Mol. Biol. 21, 64–72.

Svitash, S., Young, J.K., Schwartz, C., Gao, H., Falco, S.C. and Cigan, A.M. (2015) Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. Plant Physiol. 169, 931–945.

Wang, K., Liu, H., Du, L. and Ye, X. (2017) Generation of marker-free transgenic hexaploid wheat via an Agrobacterium-mediated co-transformation strategy in commercial Chinese wheat varieties. Plant Biotechnol. J. 15, 614–623.

Wang, J., Zibetti, C., Shang, P., Srirath, S.R., Zhang, P., Cano, M., Hoang, T. et al. (2018) ATAC-Seq analysis reveals a widespread decrease of chromatin accessibility in age-related macular degeneration. Nat. Commun. 9, 1364.

Wei, W., Ba, Z., Gao, M., Wu, Y., Ma, Y., Amiard, S., White, C.J. et al. (2012) A role for small RNAs in DNA double-strand break repair. Cell, 149, 101–112.

Zemach, A., Kim, M.Y., Hsieh, P.-H., Coleman-Derr, D., Eshed-Williams, L., Thao, K., Harmer, S.L. et al. (2013) The Arabidopsis nucleosome remodeler DDM1 allows DNA methyltransferases to access H1-containing heterochromatin. Cell, 153, 193–205.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 RT-qPCR in GOI stable expressing, variable expressing and silenced pCV211, pCV260, and pCV261 plants.

Figure S2 Targeted bisulfite sequencing.

Figure S3 Different methylation contexts invoke unstable or silenced expression in different TSI events.

Figure S4 Results sRNA sequencing from pCV211 TSI plants.

Figure S5 Results sRNA sequencing from pCV260 TSI plants.

Figure S6 Results sRNA sequencing from pCV261 TSI plants.

Table S1 List of donor DNAs and obtained TSI frequencies.

Table S2 Overview of ELISA on T0 plants.

Table S3 Summary of targeted DNA sequencing results.

Table S4 List of all TSI event plants/progeny analysed.

Table S5 Bismark targeted DNA methylation sequencing reports.