Site-specific manipulation of *Arabidopsis* loci using CRISPR-Cas9 SunTag systems

Ashot Papikian1,2, Wanlu Liu1,3, Javier Gallego-Bartolomé1 & Steven E. Jacobsen1,4

Understanding genomic functions requires site-specific manipulation of loci via efficient protein effector targeting systems. However, few approaches for targeted manipulation of the epigenome are available in plants. Here, we adapt the dCas9-SunTag system to engineer targeted gene activation and DNA methylation in *Arabidopsis*. We demonstrate that a dCas9-SunTag system utilizing the transcriptional activator VP64 drives robust and specific activation of several loci, including protein coding genes and transposable elements, in diverse chromatin contexts. In addition, we present a CRISPR-based methylation targeting system for plants, utilizing a SunTag system with the catalytic domain of the *Nicotiana tabacum* DRM methyltransferase, which efficiently targets DNA methylation to specific loci, including the *FWA* promoter, triggering a developmental phenotype, and the *SUPERMAN* promoter. These SunTag systems represent valuable tools for the site-specific manipulation of plant epigenomes.
Genotype transcription, and thus function, can be controlled in trans, via the binding of transcription factors to promoters, or in cis, via epigenetic modifications. Functional analyses of plant genomes have relied mainly on the indirect reactivation of genes or transposable elements (TEs) through the use of mutants. However, the biological outcomes of these manipulations represent an aggregate view of gene expression changes. Examining epigenetic regulation of gene expression, such as by DNA methylation, faces similar issues, where indirect and widespread changes in epigenetic mutants complicate the exploration of locus-specific effects.

Mutant analysis, hairpin-mediated targeting of small interfering RNAs (siRNAs), and zinc-finger-mediated targeting have been used to assess the function of genes and DNA methylation in plants. For example, fusion of a zinc-finger protein to the RNA-directed DNA methylation effector SUVH9 fused to the methyltransferase domain of the Arabidopsis FWA promoter. The fwa-4 (fwa) epiallele in Arabidopsis plants displays a loss of FWA promoter methylation, leading to FWA activation and a late flowering phenotype. SUVH9-mediated de novo methylation of the FWA promoter in fwa plants restored FWA silencing and an early flowering phenotype, indicating that promoter methylation was sufficient to regulate FWA expression. Although zinc-finger fusions are an effective tool, they are laborious to design, difficult to verify, and often display broad, off-target binding activity.

CRISPR-Cas approaches enable targeted manipulation of specific loci. Synthetic transcriptional activators, for instance consisting of deactivated versions of Cas9 (dCas9) fused to transcription activation domains, can specifically activate genes in both plants and mammals. Several other CRISPR-Cas9-based activation systems, such as the synergistic activation mediator (SAM) as well as a hybrid VP64-p65-Rta (VPR) activator, have been developed to further enhance dCas9-mediated transcriptional upregulation as well as to recruit multiple protein effectors. The dCas9-SunTag-VP64 system is a potent transcriptional activator in mammalian cell lines. This system consists of two modules: dCas9 fused to tandem GCN4 peptide repeats, and a single chain variable fragment (scFv) GCN4 antibody fused to superfolder-GFP (sfGFP) and VP64. Thus, multiple copies of the VP64 transcriptional activator associate with the GCN4 repeats and are recruited to a specific locus via dCas9-guide RNAs. This method has been adapted for site-specific DNA demethylation in mammals and plants, and for DNA methylation in mammals.

DNA methylation in plants exists in three different nucleotide contexts: CG, CHG, and CHH (where H = A, T, or C). Maintenance methylation is controlled by several pathways in Arabidopsis: CG methylation is maintained by DNA METHYLTRANSFERASE 1 (MET1), a homolog of DNMT1; CHG methylation is maintained by CHROMOMETHYLASE 3 (CMT3), a plant specific methyltransferase; and much of the CHH methylation is maintained by DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)-a homologue of DNMT3 methyltransferases-through the RNA-directed DNA methylation (RdDM) pathway. While DRM2 is responsible for CHH maintenance methylation in short euchromatic regions, short TEs, and the edges of long TEs, CHROMOMETHYLASE 2 (CMT2) is responsible for CHH methylation in pericentromeric heterochromatin and the bodies of long TEs. RdDM is also responsible for de novo methylation of all three sequence contexts.

Here, we have developed CRISPR-Cas9-SunTag-based targeting systems to site-specifically and efficiently manipulate gene methylation and expression in plants. We modified the SunTag system to recruit multiple copies of a methylation effector or of VP64 to distinct loci. Using the previously characterized Nicotiana tabacum DRM methyltransferase catalytic domain as our methylation effector, we found that SunTag NiDRMcd effectively targets methylation to specific loci. Importantly, at the FWA locus, this methylated state remains meiotically heritable through multiple generations in the absence of the targeting transgene.

**Results**

**Targeted transcriptional activation of the FWA locus.** We previously adapted the SunTag system for site-specific DNA demethylation in plants by targeting the human TET1 catalytic domain to Arabidopsis loci. To generate a transcriptional activator system, we used the Arabidopsis UBQUITIN10 (UBQ10) promoter to drive the expression of dCas9-10 × GCN4 and of scFv-sfGFP-VP64 (Supplementary Fig. 1a). Additionally, we added an SV40-type NLS to the scFv module to ensure proper nuclear import in plants. Imaging of the roots of T2 transgenic Arabidopsis plants expressing the SunTag VP64 construct showed clear nuclear localization of the antibody module (Supplementary Fig. 1b). In addition, dCas9-10 × GCN4 was stably expressed in T2 Arabidopsis plants (Supplementary Fig. 1c).

To test whether this system activates gene expression, we targeted the DNA methylated and silent FWA gene in Arabidopsis wild-type (Col-0) plants. We observed ectopic activation of FWA in numerous T1 lines containing a single guide RNA (gRNA4) that targets FWA, but not in control lines that lack a guide (nog) or that lack VP64 (Supplementary Fig. 2a, b). Strong activation of FWA was also observed in the next generation T2 plants (Supplementary Fig. 2b,c). RNA-seq of T2 gRNA4 plants confirmed that FWA was robustly upregulated (Fig. 1a and Supplementary Fig. 2d). In addition to gRNA4, we tested a guide (gRNA17) that targets a region further upstream in the promoter. ~170 base pairs upstream from gRNA4. We detected FWA upregulation with gRNA17, although to a lesser extent than with gRNA4, suggesting that gRNAs placed near the transcription start site may be more effective to manipulate gene expression, as previously suggested with the SunTag system in mammalian cell lines (Supplementary Fig. 2e).

To comprehensively profile the specificity of SunTag VP64-mediated activation, we examined differentially expressed genes (DEGs) in the gRNA4 RNA-seq dataset. All three profiled lines displayed highly specific activation of FWA with very few DEGs compared to a no guide control line (Supplementary Fig. 3a). To examine dCas9 binding at the FWA promoter, we performed ChiP-qPCR with T2 gRNA4 plants. We observed a strong enrichment of dCas9 at the FWA promoter compared to the control ACT7 locus, and as expected, no enrichment in Col-0 control plants (Supplementary Fig. 3b). ChiP-seq showed highly specific binding of dCas9 to the FWA promoter, with only one major off-target site (Supplementary Fig. 3c). This off-target site contains a PAM and 14 base pairs complementary to the gRNA sequence, spanning the previously reported seed region of the protospacer. Therefore, SunTag VP64-mediated gene activation is highly specific due to the highly specific binding properties of the Cas9/gRNA complex.

To test whether VP64-mediated FWA activation affected promoter methylation, we performed whole-genome bisulfite sequencing (WGBS) of T2 gRNA4 plants. Compared to Col-0 and no guide controls, T2 gRNA4 lines showed reduced CG methylation within the promoter, whereas gene body methylation downstream of the target site, as well as genome-wide methylation levels, remained unaffected (Fig. 1b and Supplementary Fig. 4a, b). Thus, targeted activation of silenced genes can reduce promoter methylation.
**Fig. 1** SunTag VP64-mediated FWA activation.  
(a) RNA-seq tracks depicting normalized reads at the *FWA* locus and flanking loci in 1 representative Col-0 replicate, 1 representative T2 SunTag VP64 nog-2 replicate, 1 representative *fwa* replicate, and 1 representative T2 SunTag VP64 g4 replicate for each of the 3 independent lines. The black triangle indicates the position of gRNA4.  
(b) ChIP-seq and WGBS tracks at the *FWA* promoter. The top track shows a ChIP peak corresponding to gRNA4-mediated SunTag recruitment. The position of gRNA4 is illustrated with a black bar. CG, CHG, and CHH methylation tracks for Col-0, T2 SunTag VP64 nog-3, and 2 independent T2 lines of SunTag VP64 g4.  
(c) RNA-seq tracks depicting normalized reads at the *FWA* locus and flanking loci in 1 representative Col-0 replicate, 1 representative T2 22aa SunTag VP64 nog-1 replicate, 1 representative *fwa* replicate, and 1 representative T2 22aa SunTag VP64 g4+g17 replicate for each of the 3 independent lines. The black triangles indicate the positions of gRNA17 and gRNA4.  
(d) WGBS tracks for all 3 sequence contexts at the *FWA* promoter for Col-0 and 3 independent T1 lines of 22aa SunTag VP64 g4+g17. The positions of gRNA17 and gRNA4 are illustrated with black bars.  
(e) WGBS tracks for all 3 sequence contexts at the *FWA* promoter for Col-0 and 3 independent T1 lines of 22aa SunTag VP64 g4+g17. The positions of gRNA17 and gRNA4 are illustrated with black bars.  
(f) qRT-PCR analysis of *FWA* expression levels in *fwa*, Col-0, and 4 different T2 or T2- plants from 22aa SunTag VP64 g4+g17-3. Expression fold change relative to *fwa* is plotted. Error bars represent the mean ± s.e. of 2 technical replicates. Source data of Fig. 1f are provided as a Source Data file.
Although T2 gRNA4 plants showed FWA upregulation, they did not display altered flowering time compared to controls. Therefore, we hypothesized that the levels of FWA mRNA in T2 gRNA4 plants might not be sufficient to affect flowering time. In order to increase FWA expression even further, we combined gRNA4 and gRNA17 (g4 + g17) in one construct to test whether targeting two regions with gRNAs we have verified proximal to the promoter might further enhance activation. The 10 × GCN4 epitopes within the SunTag construct are separated by linkers of 5 amino acids (aa). To allow for maximum mobility of VP64, we also utilized 22aa linkers, as previously reported. Furthermore, we added another SV40-type NLS within the coding sequence of dCas9-10 × GCN4. qRT-PCR analysis of T1 plants indicated that multiple lines with gRNA4 + gRNA17 had FWA expression levels similar to fwa epilatele plants, resulting in a late flowering phenotype (Supplementary Fig. 5a). RNA-seq in the T2 generation confirmed the upregulation of FWA expression, where, consistent with the qRT-PCR data, FWA transcript levels in line 3 were similar to those observed in fwa plants (Fig. 1c and Supplementary Fig. 5b). Analysis of DEGs showed that as with gRNA4, constructs with gRNA4 + gRNA17 were highly specific in activating FWA, with few other genes affected (Supplementary Fig. 5c).

We next tested how FWA promoter methylation was affected after activation with gRNA4 + gRNA17. We performed WGBS of T1 plants and observed a reduction of methylation in lines 1 and 2. Furthermore, promoter methylation in line 3 was completely abolished, correlating with expression data where FWA overexpression was similar to levels observed in fwa plants (Fig. 1c, d and Supplementary Fig. 5a, b). Thus, expression may be a key contributor that leads to this reduction in methylation. FWA gene body methylation remained unaffected in activated lines (Supplementary Fig. 6a). In the T2 generation, plants from line 3 that retained the transgene (+) and those that had it segregated away (−) both retained the demethylated state (Fig. 1e and Supplementary Fig. 6b). Consistently, we observed FWA expression levels similar to an fwa epilatele plant in both T2+ and T2− plants, correlating with the late flowering phenotype (Fig. 1f and Supplementary Fig. 6c).

**Activation of loci in different chromatin contexts.** We next wanted to explore if other methylated loci, such as transposable elements (TEs), could be targeted for ectopic activation. We used two gRNAs to target _Evadé_ (EVD), a member of the _ATCOPIA93_ family of LTR/COPIA transposable elements. Compared to control lines, 5aa SunTag VP64 T1 lines displayed retrotransposition activation by hundreds- to thousands-of-fold (Supplementary Fig. 7). We also confirmed the upregulation of _EVD_ in three independent T2 lines (Supplementary Fig. 7).

Next, we examined genome-wide effects by RNA-seq. The two gRNAs targeting the 5′ end of _EVD_ had perfect matches to two different _ATCOPIA93_ loci, one in euchromatin corresponding to the _EVD_ locus, and another in heterochromatin corresponding to the _Attrapé_ (ATR) locus. Both loci were highly activated, indicating that SunTag VP64 can manipulate gene expression in distinct chromatin contexts (Fig. 2a, b and Supplementary Fig. 8a, b). One neighboring TE of the same family adjacent to _ATR_ was also upregulated (Fig. 2b). This effect might reflect co-regulation of these two TE copies and/or the presence of regulatory regions at the 3′ end. We observed robust activation of _EVD_ and _ATR_, and few DEGs, in three independent T2 lines compared to a no guide control line (Supplementary Fig. 8c). Thus, SunTag VP64-mediated activation was highly specific.

We performed WGBS to monitor methylation levels proximal to the gRNA targets within _EVD_ and _ATR_. We observed a decrease in CG methylation, while genome-wide methylation levels remained unaffected, recapitulating the effects observed at the FWA promoter (Supplementary Fig. 9a–d and 10). The SunTag system can thus be used to determine the biological consequences of activating specific TEs or TE families, rather than evaluating stress experiments or mutants that globally upregulate the expression of TEs.

To test the generality of this activation system, we also targeted two unmethylated loci within the _Arabidopsis_ genome. A zinc-finger–VP64 fusion has been shown to target and upregulate the expression of the floral development gene _APETALA3_ (AP3) [30]. To test whether we could activate AP3 with our system, we designed two gRNAs to target the AP3 promoter. By qRT-PCR, we observed over 300-fold and 500-fold upregulation of AP3 in T1 and T2 plants, respectively, compared to controls (Supplementary Fig. 11a). However, we did not observe the previously described AP3 overexpression phenotype [30], perhaps due to tissue-specific differences in transgene expression.

We also targeted the unmethylated stem cell regulator _CLAVATA3_ (CLV3) with two gRNAs [31]. We observed strong upregulation of _CLV3_ in several T1 lines where some exhibited the previously reported _wuschel_ mutant phenotype—where the generation and maintenance of stem cells are perturbed in the meristem—arising from _CLV3_ overexpression [31], which persisted in T2 plants (Fig. 2c, d and Supplementary Fig. 11b). Thus, SunTag VP64 can site-specifically activate genes with methylated or unmethylated promoters.

**A SunTag-based methylation targeting system.** Several reports have shown that dCas9 can be adapted to site-specifically modify DNA methylation in mammalian cells [19,32–36]. However, no such system yet exists to target DNA methylation in plants. We replaced VP64 in our SunTag system with the _Nicotiana tabacum_ DRM methyltransferase catalytic domain (NtDRMcd) to evaluate methylation targeting activity in vivo. We chose NtDRMcd because this fragment was previously shown to be well-folded, well-expressed, and could be crystallized [24]. We utilized the _fwa_ background, which has lost FWA promoter methylation, and established T1 plants that co-express gRNA4 to target the FWA promoter and 5aa SunTag NtDRMcd. These T1 plants showed some establishment of CHH methylation at the FWA promoter by WGBS, but very limited CG and CHG methylation (Supplementary Fig. 12a). The initial establishment of CHH methylation by NtDRMcd is consistent with the previously reported preference of tobacco DRM for non-CG methylation [37]. Ectopic methylation of the FWA promoter has been shown to generate early flowering plants [3]. However, we did not observe any flowering time differences between T1 plants and controls, likely due to the lack of CG methylation, which is required for _FWA_ silencing [8]. Similarly, T2 plants showed slightly higher levels of CHH methylation but did not exhibit early flowering (Supplementary Fig. 12b).

To improve the efficiency of ectopic methylation targeting, we used our 22aa SunTag construct to accommodate optimum recruitment of NtDRMcd by mitigating the potential effects of steric hindrance. T1 22aa SunTag NtDRMcd gRNA4 transgenic lines showed enhanced FWA methylation compared to the 5aa T1 lines, including the establishment of CHH and CHG methylation, and minimal amounts of CG (Supplementary Fig. 12b). However, these plants also did not display the early flowering phenotype, suggesting that _FWA_ expression was not silenced.

To further enhance targeted methylation, we utilized two additional gRNAs targeting the FWA promoter. Together, these three gRNAs spanned the wild-type methylation patch observed in Col-0 plants. WGBS and McrBC analysis of T1 plants...
expressing 22aa SunTag NtDRMcd with gRNA4, gRNA10, and gRNA18 (g4 + g10 + g18) displayed efficient methylation establishment in all three sequence contexts within the FWA promoter (Fig. 3a and Supplementary Fig. 12c), which led to FWA silencing (Supplementary Fig. 13a) and early flowering plants. T2 plants that retained the SunTag NtDRMcd transgene (T2+) displayed FWA methylation in both lines we followed (Fig. 3b, c). We identified T2 plants lacking the transgene (T2-) in line 2 that retained FWA promoter methylation, indicating that the targeted methylation was meiotically heritable (Fig. 3b). In line 1, FWA promoter methylation was lost in most T2- plants, which led to the reactivation of FWA (Fig. 3c and Supplementary Fig. 13a). Thus, the methylation established in T1 line 1 plants was insufficient to confer stable methylation and silencing in most T2- plants. In contrast, RNA-seq of line 2 T2 early flowering plants showed that FWA expression was silenced to wild-type levels in both T2+ and T2- plants, (Fig. 3d and Supplementary Fig. 13b), resulting in early flowering (Supplementary Fig. 13c).

We followed the T2+ early flowering plants of SunTag DRMcd g4 + g10 + g18 line 1 to the T3 generation. Both T3+ and T3- displayed robust FWA methylation and silencing (Supplementary Fig. 13d, e), as well as the early flowering phenotype (Fig. 3e). Thus, two generations in the presence of the SunTag NtDRMcd transgene were required for most plants to induce heritable methylation and silencing in this line. Furthermore, T4- plants derived from T3- plants continued to maintain
FWA promoter methylation and the early flowering phenotype (Supplementary Fig. 13d, f). Although more independent events need to be characterized for further studies of methylation heritability, these findings are in line with the known inter-generational silencing effects associated with hairpins and at FWA and other loci27,38–40.

In addition to targeted DNA methylation, the scFv-sfGFP-NtDRMcd fusion induced background methylation throughout the genome, mainly in the CHH context, consistent with the reported preference of DRM37 (Supplementary Figs. 14 and 15a,b). We also observed chloroplast methylation in the presence of scFv-sfGFP-NtDRMcd (Supplementary Fig. 16a).
Neither the genome-wide CHH hypermethylation nor the chloroplast methylation were retained in plants that had segregated away the transgene (Supplementary Figs. 15a, b, and 16a). Moreover, we profiled the methylation levels of fwa plants expressing the SunTag NtDRMcd transgene without a guide. T1 plants expressing this transgene showed no targeted methylation at FWA (Supplementary Fig. 16b), but again showed widespread background methylation that arises from the non-specific methyltransferase activity of NtDRMcd, as has been seen for targeting with mammalian methyltransferases 41.

To gain a better understanding of NtDRMcd off-target activity, we profiled genome-wide methylation levels in multiple generations of SunTag NtDRMcd g4 + g10 + g18 plants. Genome-wide methylation profiling of T1 plants showed hypermethylation throughout all chromosomes, mainly in the CHH context, with the CHG context being less affected and almost no effect on global CG methylation levels (Fig. 4a). In the T2 + generation, SunTag DRMcd g4 + g10 + g18 line 1 showed excessive hypermethylation in the CHH and CHG contexts. In contrast, T2 + line 2 showed minimal amounts of hypermethylation, and only in the CHH context (Supplementary Fig. 17a). These data emphasize that multiple lines should be evaluated in order to avoid those with excessive off-target hypermethylation. Importantly, segregating away the transgene reduced global hypermethylation to similar levels as the fwa control plants (Supplementary Fig. 17a). These findings were reiterated in T3+/− and T4− plants (Supplementary Fig. 17b).
We hypothesized that the SV40-type NLS in the scFv-sfGFP-NtDRMcd fusion may contribute to high levels of NtDRMcd in the nucleus leading to off-target DNA methylation. To test this hypothesis, we removed the SV40-type NLS, reasoning that nuclear localization of the scFv-sfGFP-NtDRMcd fusion would mainly occur only upon binding to the dCas9-NLS-10 x GCN4 fusion. Indeed, WGBS analysis of three independent T1 lines revealed that the SunTag NtDRMcd construct lacking the SV40-type NLS induced methylation at FWA, leading to early flowering, with limited effects on the surrounding regions (Fig. 4b and Supplementary Fig. 17c). Further, metaplots of genome-wide methylation showed that global CHH methylation levels in plants expressing the noNLS-SunTag NtDRMcd construct were similar to the fwa epiallele (Supplementary Fig. 17c and Fig. 4c). However, the noNLS-SunTag NtDRMcd construct was still able to access and methylate chloroplast DNA (Supplementary Fig. 18a). Thus, removal of the SV40-type NLS reduces nuclear off-target methylation of the SunTag NtDRMcd system.

To test the generality of the SunTag NtDRMcd methylation targeting system, we used two gRNAs to target the floral development gene SUPERMAN (SUP) in the ecotype Landsberg erecta (Len)42,43. We profiled four independent T1 lines and observed efficient establishment of non-CG methylation (Fig. 5a), consistent with minimal CG sites in this region42. As with FWA, we observed off-target CHH methylation in the regions surrounding SUPERMAN (Supplementary Fig. 18b). We next utilized our noNLS version of the SunTag NtDRMcd construct to avoid genome-wide hypermethylation. Methylation was successfully targeted to the SUPERMAN promoter (Fig. 5b), with the surrounding regions now showing methylation profiles similar to the Ler control (Supplementary Fig. 18c). Chromosome-wide metaplots showed that contrary to SunTag NtDRMcd, the noNLS version of the construct had global methylation levels similar to those observed in Ler controls (Fig. 5c, d).

Discussion
We have established SunTag systems to induce site-specific expression or methylation in Arabidopsis. SunTag VP64 is a highly efficient transcriptional activator that can be used to study the effects of overexpressing specific endogenous loci. In addition, conditional expression of VP64 could be used in the future to illuminate the tissue- and cell type-specific functions of genes. Compared to zinc fingers or TAL effectors fused to transcriptional activators, CRISPR-Cas9-based systems are more specific and efficient for multiplexing44,45, and simpler to engineer for new targets by simply designing new guide RNAs5,46. Because DNA methylated genes can be activated, this system provides an alternative to studying DNA methylation mutants, which can have indirect effects.

We also discovered an interesting phenomenon in which the promoter methylation of FWA was decreased or abolished as a result of activation, and also reduced at the 5′-end of EVD and ATR (Fig. 1b, d, e and Supplementary Fig. 9a–d). This observation suggests that gene expression plays an important role in reducing promoter methylation levels, through a mechanism that is either directly or indirectly perturbing methylation maintenance in promoter regions. Thus, gene activation and DNA methylation pathways are likely competing to regulate gene expression at promoters. Activation-mediated DNA demethylation further underscores that SunTag VP64 can be used to study the epigenetic regulation of methylated loci without altering global DNA methylation levels.

SunTag NtDRMcd-mediated DNA methylation targeting could be a valuable tool for agriculture for creating meiotically heritable epialleles without changing the DNA sequence47. As one example, plant genes required to support pathogenic bacteria or viruses could be targeted for promoter methylation and silencing48. Pleiotropic effects of silencing could potentially be avoided by fine-tuning the levels of repression caused by different levels of targeted methylation. Thus, the SunTag system is a versatile tool for site-specific manipulation of the epigenome in plants, and may have broad applications as a biotechnology tool.

Methods

Plant material and selection. The Columbia-0 (Col-0) ecotype of Arabidopsis thaliana was used in this study, along with the fwa-4 epiallele, which was isolated from a recombinant progeny49. The Ler ecotype was used for targeting methylation to the SUPERMAN locus. To obtain transgenic lines, plants were transformed using the agrobacterium floral dip method50. Transgenic lines were obtained by selecting for hygromycin resistant plants on MS plates containing 0.9% Phytoagar (plantMedia), 1/2 Murashige and Skoog Basal Medium (MP Biomedicals, LLC), and 35 μg/ml Hygromycin B (Invivogen). For microscopy, root tip was used for obtaining confocal microscope images (Zeiss). Images were processed using Fiji50.

SunTag cloning and design. Constructs used for activation and methylation targeting were cloned into either the pMOA binary vector51 or the pEG binary vector52. SunTag constructs were adapted from those described in Tanenbaum et al.51, and in order to develop a plant-specific SunTag system, we redesigned the targeting system in the constructs presented in this work were either PCR amplified from Addgene plasmid numbers 60903 and 60904 (gifts from Dr. Ron Vale, University of California, San Francisco) or were synthesized by GenScript.

The SunTag system consists of two main modules: dCas9 + epitope (10×GCN4) tagged to a single chain antibody (scFv) fused to sfGFP and either VP64 or NtDRMcd. The epitope tail has a defined number of repeats separated by linker regions. In this study we employed 10 repeats of the GCN4 epitope, dCas9 + epitope tail expression was driven by the Arabidopsis UBQ10 promoter that was followed by an omega translesional enhancer. scFv module expression was also driven by the UBQ10 promoter in the antisense orientation relative to UBQ10. dCas9 + epitope tail, gRNAs were cloned downstream from the scFv module and expressed using the U6 promoter. In order to express multiple gRNAs, each was cloned in tandem along with their independent U6 promoters. All cloning reactions were performed using In-Fusion (Takara) and all necessary components for activation and methylation targeting are obtainable. In order to improve the DNA methylation targeting ability of the targeted NtDRMcd, we made a 22aa linker version of the dCas9 + epitope tail module in addition to the 5aa version, which represents the linker length in between epitope repeats. This was done to avoid the effects of steric hindrance since NtDRMcd is larger than the VP64 fusion. The 22aa linker version of dCas9 + epitope tail was also used for targeting VP64 to FWA with gRNA4 + gRNA17. In addition to adding this linker, we added an extra SV40-type NLS in between the 1xHA tag and the flexible linker separating dCas9 and the epitope tail. An SV40-type NLS was also added to the scFv module for effective nuclear import in Arabidopsis. Plasmids related to this study have been deposited to Addgene for the scientific community (Plasmid numbers: 115480–115490, 117168, 119554, 119672, and 120249–120252).

We designed numerous guides in the FWA promoter region. The designed guides were numerically numbered and specific ones were chosen for either activation or methylation targeting. The use of specific guides does not indicate that others did not work. For example the use of gRNA4 does not indicate that gRNAa13 did not work. It was chosen for its position in the FWA promoter.

Western blotting. To extract total protein, leaf tissue was boiled at 95 °C with 2 x SDS buffer for 5 min. Raw protein extracts were then run with 3-8% Tris-acetate gels (NuPAGE) at 180-200 V. An iBlot gel transfer instrument (Thermo Fisher) was then used to transfer proteins to a PVDF membrane. Ponceau staining was used for visualizing loading controls, anti-HA Peroxidase, High Affinity Nickel Metal Chelate (Roche, catalog #12 013 819 001, clone BMG-3F10) were used for blotting.

qRT-PCR analysis. For all qRT-PCR experiments, RNA was first extracted from leaf tissue using the Direct-zol RNA Miniprep kit (Zymo). 500 ng–1 μg of total RNA was then used for cDNA synthesis using the SuperScript III First-Strand Synthesis Supermix (Invitrogen). qPCR analysis was then done using the iQ SYBR Green Supermix (Bio-Rad) to detect transcript expression levels. FWA transcripts were detected using oligos 5′-TTTAGATCCTAAACGGTGACATCAAAG-3′ and 5′-CTTGTGACCAGCGCGAGA-3′. EVD transcripts were detected using the previously reported oligos 5′-GGATAGGAGAGGAGGACATCAAAG-3′ and 5′-CTTGTGACCAGCGCGAGA-3′. AP3 transcripts were detected using the previously reported oligos 5′-TTTTGACGAGCTGCGATTCACG-3′ and 5′-GGATAGGAGAGGAGGACATCAAAG-3′. CD-V3 transcripts were detected using the previously reported oligos 5′-GGATAGGAGAGGAGGACATCAAAG-3′ and 5′-CGTCTCTGGTGGATCTACATTGCAAC-3′. All transcripts were

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normalized to the housekeeping gene ISOPENTENYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2 (IPP2). IPP2 transcripts were detected using oligos 5′-GTATGAGTTGCTTCTCCAGCAAAG-3′ and 5′-GAGGATGGCTGCAACAAGTGT-3′.

McrBC assay. To detect differences in methylation using the endonuclease McrBC (NEB), genomic DNA was first extracted using either a CTAB-based method or the DNeasy Plant Mini Kit (Qiagen). 100 ng of genomic DNA was then digested for 4 h at 37 °C. Water was added instead of McrBC for undigested control samples. After digestion, qPCR analysis was done using the iQ SYBR Green Supermix (Bio-Rad) to quantify differences in methylation. FWA promoter region sequences were detected using oligos 5′-TTGGGTTAGTGTTTACTTG-3′ and 5′-GAATGTTGATGGGATAAGGTA-3′.

RNA-seq library preparation and analysis. For transcriptome analyses, RNA was first extracted from leaf tissue using the Direct-zol RNA MiniPrep kit (Zymo). 1 μg of total RNA was used to prepare libraries for sequencing using the TruSeq Stranded mRNA-seq kit (Illumina). Libraries for SunTag VP64 targeting EVD were prepared using the TruSeq Stranded Total RNA with Ribo-Zero kit (Illumina). For sequencing of SunTag VP64 targeting FWA and SunTag NtDRMcd targeting FWA,
we obtained single-end 50 bp reads. For sequencing of SunTag VP64 targeting EVD, we selected for larger fragment sizes and obtained paired-end 100 bp reads. Sequencing reads were aligned to the TAIR10 genome annotation dataset using TopHat (2 mismatches allowed)\(^34\). We used the one max multihits option (-g 1) and --no-cover-search. If reads did not map to genes, they were aligned to the TAIR10 genome. Read counts were obtained by implementing HTSeq\(^35\) and subsequent differential expression analyses were done using DESeq (Bioconductor) and custom R scripts.

**ChIP.** T2 SunTag VP64 gRNA and Col-0 control plants were first grown on MS plates for 2 weeks and 2 grams of tissue were then collected per sample. After grinding the tissue, samples were crosslinked in 1% formaldehyde, chromatin was extracted, and then sonicated using Biourator Plus (diagenode). Immunoprecipitations were performed using mouse monoclonal anti-HA.11 epitope tag antibodies (clone 16B12, Covance catalog #MMMS-101R). Chromatin-protein complexes were isolated with a 1:1 mix of Protein A and Protein G Dynabeads (Invitrogen) for 3 h at 4 °C. Beads were washed with low salt buffer (2x), high salt buffer, LiCl buffer, and TE buffer, and complexes were eluted with elution buffer (2x) at 65 °C. DNA-protein complexes were reversed crosslinked overnight at 65 °C followed by proteinase K treatment at 45 °C for 5 h. DNA was purified using phenolchloroform, followed by NaOAc/EtOH precipitation along with GlycoBlue (Invitrogen) overnight at -20 °C. DNA was washed with 70% EtOH and resuspended with water. For ChIP-qPCR, the ACT7 promoter was deleted from leaves and in different experiments we obtained single-end 50 bp reads. For sequencing of SunTag VP64 targeting FWA, a targeted DNA demethylation was carried out using dsCas9–peptide repeat and scFv–TET1 catalytic domain fusions. Nat. Biotechnol. 34, 1060–1065 (2016).

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**Author contributions**

A.P. and S.E.J. designed the study. A.P. designed and performed experiments. A.P. and W.L. designed and performed bioinformatic analyses. J.G.-B. performed experiments. A.P. and S.E.J. wrote the manuscript.

**Additional information**

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**Competing interests:** A.P., J.G.-B., and S.E.J. have filed patents related to this work (US2016017348A1, WO2017205837, and WO2018136783). S.E.J. is a scientific co-founder of Inari Agriculture and a member of its scientific strategy board. The remaining author declares no competing interests.

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