CRISPR–Cas-mediated transcriptional control and epi-mutagenesis

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Abstract
Tools for sequence-specific DNA binding have opened the door to new approaches in investigating fundamental questions in biology and crop development. While there are several platforms to choose from, many of the recent advances in sequence-specific targeting tools are focused on developing Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR Associated (CRISPR-Cas)-based systems. Using a catalytically inactive Cas protein (dCas), this system can act as a vector for different modular catalytic domains (effector domains) to control a gene’s expression or alter epigenetic marks such as DNA methylation. Recent trends in developing CRISPR-dCas systems include creating versions that can target multiple copies of effector domains to a single site, targeting epigenetic changes that, in some cases, can be inherited to the next generation in the absence of the targeting construct, and combining effector domains and targeting strategies to create synergies that increase the functionality or efficiency of the system. This review summarizes and compares DNA targeting technologies, the effector domains used to target transcriptional control and epi-mutagenesis, and the different CRISPR-dCas systems used in plants.

Introduction
Over the past three decades, the development of tools that can bind to DNA in a sequence-specific manner has led to technologies that can specifically target and regulate gene transcription and epi-mutagenesis. Due to its ease of use, in the most recent wave of developments, tools based on the Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated system (CRISPR-Cas) using a catalytically inactive Cas protein (dCas) have come to the forefront with an array of attachments allowing for the targeted transcriptional control or epi-mutagenesis of a specific locus; pushing the functionality of CRISPR–Cas beyond gene editing.

These constructs rely on either the direct recruitment of basal transcription machinery or the targeting of epigenetic factors to manipulate transcription of nearby genes. While the recruitment of basal transcription machinery requires the presence of the targeting construct, changes in DNA methylation can in some cases be mitotically and meiotically inherited allowing for this targeted epi-mutagenesis to be maintained in the following generations in the absence of the targeting construct (Johnson et al., 2014; Gallego-Bartolomé et al., 2018; Papikian et al., 2019). The heritability of DNA methylation is well documented at a few loci; however, further work is needed to understand how frequently
In this review, we will cover the recent discoveries and advancements in targeted transcriptional control and epimutagenesis using CRISPR–dCas-based targeting technologies in plants.

**Epigenetics and plant gene expression**

Eukaryotic DNA is packaged into \( \sim 146 \) base pair (bp) DNA segments that wrap around a histone octamer known as a nucleosome (Luger et al., 1997; Richmond and Davey, 2003; Ou et al., 2017). Nucleosomes are the base units of chromatin and can adopt two main configurations: euchromatin, which is less compact and more accessible to transcription factors and other proteins, or heterochromatin, which is more compact and less accessible (Roudier et al., 2009) (Figure 1). This means that gene regulation depends not only on the presence or absence of transcription factors, but also on chromatin accessibility (Li et al., 2007). Chromatin state can be altered by chromatin remodeling complexes (CRCs), histone modifications, histone variants, and DNA methylation, which work together to activate or repress different transcriptional networks in eukaryotes (Li et al., 2007; Bannister and Kouzarides, 2011; Pikaard and Mittelsten Scheid, 2014; Zhong et al., 2021).

In Arabidopsis (Arabidopsis thaliana), CRCs, such as the SWITCHING DEFECTIVE 2/SUCROSE NON-FERMENTING 2 (SWI2/SNF2) proteins use ATP hydrolysis to alter the structure or positioning of nucleosomes, which in turn mediate the accessibility of the chromatin to transcription factors and other regulatory proteins (Corona and Tamkun, 2004; Jiang and Pugh, 2009). In addition to the CRCs, histone modifications and histone variants can affect transcriptional gene regulation by modulating histone–DNA interactions (Feng et al., 2010; Bannister and Kouzarides, 2011). The exposed N-terminal tails of the core histones are subjected to various post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, etc. (Pfluger and Wagner, 2007). The addition or removal of these histone modifications corresponds with activation or repression of transcription (Feng et al., 2010; Bannister and Kouzarides, 2011). For example, adding acetylation to the histone tails via histone acetyltransferases is associated with transcriptional activation, while the removal of acetylation through histone deacetylases leads to transcriptional repression (Pandey et al., 2002; Lawrence et al., 2004).

Unlike histone acetylation, which corresponds with the activation of transcription, histone methylation can be associated with activation or repression of transcription, depending on which lysine residues are methylated (Xiao et al., 2016). For example, methyl groups on the fourth lysine of the histone 3 tail (H3K4me3), H3K36me2, and H3K36me3 are associated with active transcription (Zhao et al., 2005; Xu et al., 2008; Zhang et al., 2009; Ding et al., 2012) (Figure 1). On the contrary, H3K27me1, H3K27me3, and H3K9me2 are associated with transcriptional repression (Goodrich et al., 1997; Gendall et al., 2001; Jackson et al., 2002; Malagnac et al., 2002; Johnson et al., 2007; Zhang et al., 2007; Bernatavichute et al., 2008; Jacob et al., 2009; Du et al., 2012).
DNA cytosine methylation, which in plants occurs in the CG, CHG, and CHH sequence contexts (H is any nucleotide other than G), has also been associated with gene regulation. Often, the presence or absence of DNA methylation is associated with repression and activation, respectively, of nearby genes or transposable elements. In plants, DNA methylation can be established de novo by the RNA-directed DNA methylation (RdDM) pathway (Law and Jacobsen, 2010). The canonical RdDM pathway can be split into two different arms: the Polymerase IV (Pol IV) arm, which is responsible for the generation of small interfering RNAs (siRNAs), and the Pol V arm, which provides an RNA scaffold for the recruitment of the DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) at the target site (Erdmann and Picard, 2020) (Figure 1). While the majority of the siRNAs used in RdDM are produced by the Pol IV–RDR2 complex (Herr et al., 2005; Raja et al., 2008; Stroud et al., 2013; Huang et al., 2021), a small amount are generated from other sources including inverted repeats, microRNA (miRNA) precursors, fragments of cleaved mRNA, or other non-coding dsRNAs that are processed by DCL proteins or a combination of AGO4 and exonucleases, loaded into AGO4/6/9, and directly recruit the POLV arm of the pathway to a target site. Removal of DNA methylation: DEMETER (DME) family of bifunctional glycosylase/lyases, consisting of DME and DME -LIKE 1/REPRESSOR OF SILENCING 1 (DML1/ROS1), DML2, and DML3, actively remove methylated cytosines which are then replaced with unmethylated cytosines by the base excision repair (BER) pathway. Created with BioRender.com.

**Figure 1** Overview of histone and DNA epigenetic modifications. Heterochromatin and euchromatin represent a more compact or less compact chromatin status, that can be manipulated by CRCs. The fundamental unit of chromatin is the nucleosome, which is composed of histones wrapped with DNA. Both histone tails and DNA cytosines can be epigenetically modified. Histone tail modifications: histone tails can be modified by various epigenetic marks, including methylation, acetylation, phosphorylation, SUMOylation, etc. Histone epigenetic marks are associated with transcriptional gene regulation. For example, histone acetylation and trimethylation of H3 Lysine 4 (H3K4me3) and H3K36me3 are associated with transcriptional activation. De novo DNA cytosine methylation: Cytosines can be de novo methylated through the RdDM pathway. During canonical RdDM, SAWADEE HOMEODOMAIN HOMOLOGUE 1 is recruited to sites containing methyl groups on the ninth lysine of the histone 3 tail (H3K9) and directly interacts with and recruits Polymerase IV (Pol IV) to these sites initiating transcription of short ~32 nt RNA transcripts. Pol IV then feeds this transcript directly into RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) that then converts the single stranded RNA transcripts into double-stranded RNAs, which are then digested into 24 nt small interfering RNAs (siRNAs) by RNase III endonuclease DICER-LIKE 3 (DCL3), and loaded into ARGONAUTE 4, 6, or 9 (AGO4/6/9). Concurrently, a complex consisting of SU(VAR) homologs (SUVH) 2 or 9, DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), RNA-DIRECTED DNA METHYLATION 1 (RDM1), and Pol V is brought to a locus through the interaction between DNA methylation and SUVH2/9. The RNA scaffold of Pol V transcripts can be recognized by the siRNA–AGO4/6/9 complex with help from SUPPRESSOR OF TY 5-LIKE (SPT5L). DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) is then recruited to sites recognized by siRNA-bound AGO4/6/9 through RDM1, allowing DRM2 to methylate the adjacent DNA. DRM2 adds methylation to the targeted DNA, SUVH4/5/6 binds the DNA methylation and deposits H3K9 methylation, which attracts the Pol IV arm of the pathway thus, creating a positive feedback loop that helps maintain the newly added methylation. siRNAs generated from sources other than Pol IV are sometimes incorporated into RdDM in a process called non-canonical RdDM. These siRNAs can be generated from inverted repeats, miRNA precursor’s, fragments of cleaved mRNA, or other non-coding dsRNAs that are processed by DCL proteins or a combination of AGO4 and exonucleases, loaded into AGO4/6/9, and directly recruit the POLV arm of the pathway to a target site. Removal of DNA methylation: DEMETER (DME) family of bifunctional glycosylase/lyases, consisting of DME and DME -LIKE 1/REPRESSOR OF SILENCING 1 (DML1/ROS1), DML2, and DML3, actively remove methylated cytosines which are then replaced with unmethylated cytosines by the base excision repair (BER) pathway. Created with BioRender.com.
the RdDM pathway to specific sites, and are important for pioneering sites of RdDM de novo (Allen et al., 2005; Slotkin et al., 2005; Vazquez et al., 2008; Chellappan et al., 2010; Khraiwesh et al., 2010; Wu et al., 2010, 2012; Garcia et al., 2012; Mari-Ordóñez et al., 2013; Nuthikattu et al., 2013; Creasey et al., 2014; Bond and Baulcombe, 2015; McCue et al., 2015; Panda et al., 2016; Yang et al., 2016; Ye et al., 2016; Sigman et al., 2021) (Figure 1).

DNA methylation is a mitotically and meiotically heritable mark (Law and Jacobsen, 2010). Maintenance of methylation by RdDM in euchromatic regions depends on siRNAs. DNA methylation in all sequence contexts is replicated on the newly formed daughter strands through the concerted effort of three additional DNA methyltransferases as well. Methylation at CG sites is maintained during replication by DNA METHYLTRANSFERASE 1 (MET1), through an interaction with VARIANT IN METHYLATION 1 (VIM1), VIM2, and VIM3 proteins that can bind to methylated cytosines in CG context on the parent strands (Woo et al., 2007, 2008). Non-CG methylation in heterochromatic regions is primarily maintained by the plant-specific CHROMOMETHYLASE 2 (CMT2) and CMT3 methyltransferases via a positive feedback loop with H3K9me2 histone modification and by RdDM in euchromatic regions (Lindroth et al., 2001; Du et al., 2012; Stroud et al., 2013, 2014; Erdmann and Picard, 2020). In plants, DNA methylation is actively removed by the DEMETER (DME) family of bifunctional glycosylase/lyases consisting of DME and DME-LIKE 1/REPRESSOR OF SILENCING 1 (DML1/ROS1), DML2, and DML3 (Choi et al., 2002; Gong et al., 2002; Agius et al., 2006; Gehring et al., 2006; Morales-Ruiz et al., 2006; Penterman et al., 2007; Ortega-Galisteo et al., 2008) (Figure 1).

Technologies to recruit factors to manipulate transcription

Targeted manipulation of epigenetic marks or gene expression requires a way to specifically and ectopically recruit molecular components capable of transcriptional control or epimutagenesis to the site of interest on the genome. Several targeting technologies are reviewed in the following section.

Small RNA-based targeting

Both siRNAs and miRNAs have been broadly applied for transcriptional and post-transcriptional gene silencing, not only because small RNA-mediated gene silencing is sequence specific and efficient, but also because they can cause partial loss-of-function alleles that can overcome the lethality of

Figure 2 Targeting systems A. The small interfering RNA (siRNA) targeting system which takes advantage of artificially designed inverted repeats or microRNAs (miRNA) to target specific sequences through RNA-directed DNA methylation (RdDM) or RNA interference mechanisms. DICER-LIKE (DCL); ARGONAUTE (AGO) B, The Zinc Finger targeting system where each artificially designed Zinc Finger domain is capable of recognizing a unique nucleotide triplet. C, The TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR (TALE) targeting system highlighting the RVDs, which give nucleotide binding specificity to each repeat unit. D, The CRISPR–Cas9 targeting system highlighting the CRISPR RNA (crRNA), trans-acting crRNA (tracrRNA) and protospacer adjacent motif (PAM). E, Direct fusion, SunTag, and MS2 based CRISPR–dCas9 systems and how these have been combined in the CRISPR ACT 3.0 system. MS2 coat protein (MCP), Single chain variable fragment (ScFv). Created with BioRender.com.
transcriptional activation in addition to repression. Dependent manner allow for more specific control of tran-
allowing for the targeting of effector domains in a sequence
targeted activation, and are prone to off-target effects (Xu
et al., 2006; Ossowski et al., 2008)( Table 1). Technologies
targeting systems include virus-induced
mediated approaches to silence genes include virus-induced
with the complementary sequence (Huang et al., 2019). One
of the most popular methods for the generation of synthetic
siRNAs or miRNAs is to express hairpin RNA or
mediated to target specific sequences (Boch et al., 2009;
rearranging these repeat units, designer TALEs can be cre-
transcriptional activation in addition to repression.

**Table 1 Comparison of targeting systems**

| Parameters | sRNA | TALE | Zinc Finger | CRISPR–dCas9 |
|------------|------|------|-------------|--------------|
| 1. Target sites | No limit | Occurs every ~35 bp | Occurs at every ~200–500 bp | Depends on PAM sites. New PAM-less CRISPR variants are available that increase available target sites |
| 2. Specificity | Less specific | More specific than other technologies | Less specific | Highly specific |
| 3. User friendly a. Cloning | Easy to clone constructs | Cloning is laborious and tedious | Cloning is laborious and tedious | Easy to clone constructs |
| b. Adaptability to target new sites | Highly adaptable—only need to modify the pre-cursor RNA sequence | Less adaptable—must design new protein for each new target; protein engineering can be unpredictable | Less adaptable—must design new protein for each new target; protein engineering can be unpredictable | Highly adaptable—only need to modify the guide RNA sequence |
| c. Cost | Targeting new site depends on manipulating the pre-cursor sequence, which is less expensive | Can be expensive as it requires testing of several TALEs to target new site | Can be expensive as it requires testing of several Zinc Fingers to target new site | Targeting new site depends on manipulating guide RNA sequence, which is less expensive |

certain null mutants (Ossowski et al., 2008). Endogenously,
siRNAs and miRNAs are the slicing products of DICER-LIKE (DCL) proteins from double-stranded RNAs (dsRNAs) and primary miRNA transcripts with a stem–loop structure, respectively (Kurihara and Watanabe, 2004; Xie et al., 2005)(Figure 2A). Both siRNAs and miRNAs are loaded into AGO proteins to form an RNA-induced silencing complex (Mallory and Vaucheret, 2010), which in turn silences the target gene with the complementary sequence (Huang et al., 2019). One of the most popular methods for the generation of synthetic siRNAs or miRNAs in plants is to express hairpin RNA or inverted repeats that are linked by an intron sequence. This hairpin RNA can be recognized and processed by plant DCLs into siRNAs and then cause the target gene silencing (Chuang and Meyerowitz, 2000; Wesley et al., 2001). While these are frequently designed to target exon regions to trigger post-transcriptional gene silencing, they can also be designed to target promoter regions to trigger RdDM-directed silencing (Mette et al., 2000; Dadami et al., 2014; Williams et al., 2015; Gallego-Bartolomé et al., 2019). Some other small RNA-mediated approaches to silence genes include virus-induced gene silencing (Wassenegger et al., 1994; Baulcombe, 1999; Burch-Smith et al., 2004), and artificial miRNA directed gene silencing (Schwab et al., 2006; Ossowski et al., 2008).

While synthetic siRNAs and miRNAs can target gene silencing, they cannot recruit other factors or be used for targeted activation, and are prone to off-target effects (Xu et al., 2006; Ossowski et al., 2008)(Table 1). Technologies allowing for the targeting of effector domains in a sequence dependent manner allow for more specific control of transcriptional activation in addition to repression.

**Sequence-dependent DNA binding modules**

**Zinc Fingers**

Zinc Fingers (ZFs) are one of the earliest and best characterized tools for targeting effector domains to specific regions of a genome. These proteins typically contain a classical C2H2 ZF structure with two β-sheets and one α-helix maintained by hydrophobic interactions and a zinc ion (Lee et al., 1989). Each finger primarily recognizes and binds to a unique 3-bp DNA sequence encoded in the amino acid residues of the α-helix (Pavletich and Pabo, 1991; Eldred-Erickson et al., 1996)(Figure 2B). This amino acid sequence can be manipulated and repeated to develop artificial ZF proteins with multiple finger domains capable of differentiating the DNA sequence of a target site from the rest of the genome (Liu et al., 1997; Segal et al., 1999; Dreier et al., 2001). These artificial ZFs can then be fused to an effector domain in order to activate or repress transcription. While ZF targeting systems have been widely used, they have several drawbacks relative to other targeting systems, such as a relative lack of specificity, which are outlined in Table 1 (Beerli et al., 1998; Johnson et al., 2014; Gallego-Bartolomé et al., 2019; Gardiner et al., 2020; Liu et al., 2021).

**Transcription activator-like effectors**

Like ZFs, TRANSCRIPTION ACTIVATOR-LIKE EFFECTORS (TALES) contain specific amino acid sequences that allow for the programmable recognition of specific DNA sequences (Figure 2C). The TALE DNA binding domain consists of around 18 units of repeats with each unit comprising ~34 amino acids containing two variable amino acids at positions 12 and 13, known as repeat-variable diresidues (RVDs), which direct the binding specificity of a unit (Boch et al., 2009; Moscou and Bogdanove, 2009)(Figure 2C). Thus, by rearranging these repeat units, designer TALEs can be created to target specific sequences (Boch et al., 2009; Morbitzer et al., 2010). Assembly of additional TALEs to target a unique site is mostly a matter of assembling these repeat units so that the RVD nucleotide preference matches the target site. Certain RVDs either only bind to or are unable to bind to methylated DNA (Bultmann et al., 2012; Deng et al., 2012; Valton et al., 2012; Tsuji et al., 2016), and can be used to build TALE constructs that can discriminate between methylated and unmethylated recognition sites (Deng et al., 2012; Valton et al., 2012; Tsuji et al., 2016).
While a powerful tool, TALEs are difficult to assemble due to the repetitiveness of the units, which only differ by two amino acids (Cermak et al., 2011; Morbitzer et al., 2011; Zhang et al., 2011) (Table 1).

**CRISPR–Cas direct fusion**

CRISPR–Cas systems can be used to target specific regions of a genome. These systems are naturally occurring in bacteria and archaea, and evolved as a type of adaptive immune system (Sorek et al., 2013). Unlike ZF or TALE systems that use the manipulation of amino acid sequences to specify the target site, CRISPR–Cas systems use non-coding RNAs (Figure 2D). The natural targeting system consists of an RNA sequence complementary to the target sequence known as the spacer or CRISPR RNA (crRNA) and a scaffold sequence that is bound by the Cas protein known as the trans-activating crRNA (tracrRNA) (Deltcheva et al., 2011) (Figure 2D). To simplify the tool, the crRNA and tracrRNA have been fused to form a single RNA molecule called a guide RNA (gRNA) (Jinek et al., 2012). The gRNA component is loaded into a Cas protein that is then able to identify and create a double-stranded break at the appropriate target site. Recognition of the target sequence also requires a protospacer adjacent motif (PAM) specific to the Cas protein being used limiting the number of possible targets (Figure 2D). However, recent advancements in the development of CRISPR–Cas systems have created systems that do not require this PAM motif (Walton et al., 2020; Ren et al., 2021). In addition to using Cas to trigger mutations at target sites, modified systems using catalytically inactive Cas (dCas) fused to an effector domain can be designed to cause a wide range of targeted effects, depending on the effector domain used (Jinek et al., 2012; Larson et al., 2013; Qi et al., 2013; Lowder et al., 2015; Platek et al., 2015; Liu et al., 2016; Li et al., 2017, 2020; Tang et al., 2017; Khakhar et al., 2018; Selma et al., 2019; Ghoshal et al., 2021).

The reduced expense, ease of construction, and high specificity of targeting has made CRISPR–dCas systems incredibly popular leading to the further development of more advanced systems beyond the simple direct fusion. To enhance the targeted effect of these systems, additional systems with the ability to synergistically target multiple effector domains to a single locus have been developed as discussed below.

**SunTag**

The SunTag system was originally developed in animals to recruit multiple copies of GREEN FLUORESCENT PROTEIN (GFP) to a single locus allowing for the visualization of the target (Tanenbaum et al., 2014). This was quickly incorporated into a dCas9-based system for other uses such as the activation of transcription or the addition/removal of DNA methylation in both plants and animals (Tanenbaum et al., 2014; Morita et al., 2016; Gallego-Bartolomé et al., 2018; Pflueger et al., 2018; Papikian et al., 2019; Tang et al., 2021). The SunTag-dCas9 system requires the coordinated expression of three different components: a dCas9 fused to a peptide tail containing an array of epitope repeats; a complementary single-chain variable fragment (scFv) fused to an effector domain and a gRNA (Figure 2E). This enables each dCas9 to recruit multiple copies of the effector domain via interactions between the scFv and the epitope tail. While this increases the complexity of the system, recruiting multiple copies of an effector domain to a single locus has proven more effective than targeting a single effector domain through a direct fusion to dCas9 (Morita et al., 2016; Pflueger et al., 2018). More recently, epitope tails containing a combination of two different epitopes bound by separate scFvs have enabled the co-targeting of two different unique scFv-effector fusions at the same time (Boersma et al., 2019). Recruiting multiple copies of an effector protein using the SunTag system raises the possibility that these systems can have a larger targeted epigenetic footprint than the directly fused dCas9-effector version. Although this might be true in certain cases, the targeted epigenetic footprint seems to be influenced by several factors. For example, in mammalian cell lines, targeting the mammalian de novo DNA methyltransferase DNA-methyltransferase 3 alpha (DNMT3A), fused directly to dCas9 resulted in a DNA methylation footprint of approximately 200 bp (McDonald et al., 2016; Vojta et al., 2016). While using the SunTag system with the DNMT3A resulted in a wider DNA methylation footprint of ~4 kb on the Homeobox A5 (HOXA5) gene, this was not observed when the DNMT3A-SunTag system targeted the Krüppel-like factor 4 gene, indicating a locus-specific effect (Huang et al., 2017). The influence of genomic context was also implied in plants (Ghoshal et al., 2021). For example, in Arabidopsis, identical targeted DNA methylation footprints were observed at the FWA gene when targeting a CG-specific bacterial methyltransferase using either a direct fusion with dCas9 or the SunTag system. This was most likely due to the targeted region being flanked by genomic regions lacking CG sites limiting the span of DNA methylation targeted by both tools. Thus, further research is required to directly assess the factors influencing the DNA methylation footprints induced by these multi-effector protein targeting tools.

**MS2**

Like the SunTag system, the MS2 system is a CRISPR–dCas9-based system that gives the user the ability to target multiple effector proteins to a specific locus (Konermann et al., 2015). However, unlike SunTag or direct fusions to dCas9, the MS2 system recruits effector proteins via interactions with a modified gRNA. The MS2 system takes advantage of the MS2 bacteriophage coat protein and its known RNA binding site. Like the SunTag system, this system also requires the coordinated expression of three components: a dCas9, a MS2 coat protein-effector domain fusion, and a gRNA scaffold including an MS2 binding site added to the tetraloop and/or stem loop 2 positions of the tracrRNA (Konermann et al., 2015) (Figure 2E). The MS2 system can be combined with dCas9 direct fusion (known as the Synergistic Activation Mediator system or SAM) or the SunTag system or both (known as CRISPR Act 3.0) to recruit
even more or different effector proteins to a target site via effector protein interactions with both the gRNA and the dCas9 (Konermann et al., 2015; Lowder et al., 2018; Pan et al., 2021) (Figure 2E).

Targeted epi-mutagenesis and transcriptional control

Effector domains used for transcriptional activation

There are an array of different effector domains that can be used to activate transcription in targeted systems. This can be achieved directly by attracting basal transcription factors, or indirectly by adding active histone marks or removing repressive marks. Targeting of the modular activating domain of the herpes simplex virus VP16 to a specific locus has been extensively described as an efficient way to activate transcription (Dreier et al., 2001; Sánchez et al., 2002; Stege et al., 2002; Morbitzer et al., 2010; Geißler et al., 2011; Miller et al., 2011; Zhang et al., 2011; Cheng et al., 2013; Maeder et al., 2013; Mali et al., 2013; Perez-Pinera et al., 2013; Qi et al., 2013; Liu et al., 2014; Tanenbaum et al., 2014; Vazquez-Vilar et al., 2016; Li et al., 2017; Park et al., 2017; Lee et al., 2019; Papikian et al., 2019; Selma et al., 2019). The VP16 activator domain is an acidic peptide that interacts with basal transcription factors and the mediator complex to facilitate the assembly of the pre-initiation complex at its target site (Hall and Struhl, 2002; Hirai et al., 2010). VP16 also interacts with histone acetyltransferases and the SWI/SNF ATPase complex to manipulate the surrounding chromatin structure into an active state (Hall and Struhl, 2002; Hirai et al., 2010). Creating a tetramer (VP64) or octamer (VP128) of the minimal VP16 activator domain can dramatically increase the potency of this activator (Beerli et al., 1998; Li et al., 2017). In addition to this, recruiting multiple copies of VP64 via systems like the SunTag can improve the activation of downstream targets compared to direct fusion systems (Tanenbaum et al., 2014; Lowder et al., 2018; Selma et al., 2019).

Directly targeting transcriptional activation can also be achieved through targeting the highly conserved plant-specific acidic-type activator domains found in the APETALA2 family of proteins, known as the EDLL domains. The EDLL domain of AtEFR98 is frequently used as a modular component to activate gene expression (Tiwari et al., 2012; Piatak et al., 2015; Selma et al., 2019). This EDLL motif is relatively small (24 amino acids) compared to other common activator domains like the VP16 (78 amino acids) making this activating domain an attractive option for development of compact synthetic targeted activation systems. However, at some target sites, multiple copies of EDLL were needed to achieve similar transcriptional activation as VP16 (Tiwari et al., 2012). Like VP16 and EDLL domains, TAL acidic-type activator domains, found in natural TALE systems, have also been used in a modular way to activate gene expression (Piatak et al., 2015; Li et al., 2017; Selma et al., 2019). This effector domain, when targeted using the TALE system, can activate genes upstream and downstream of its binding site regardless of which strand the effector is targeted to (Wang et al., 2017).

In addition to the direct activation of transcription by recruiting activator domains, another option is recruiting domains capable of adding active or removing repressive epigenetic marks, thereby activating gene expression indirectly. In plants, targeting transcriptional activation through H3K27 acetylation using the p300 domain from humans or the catalytic domain of the plant-specific ARABIDOPSIS HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1 (HAC1) can activate transcription of targeted genes; however, at least in the case of p300, a higher level of activation is achieved when VP64 is used (Lee et al., 2019; Roca Paixão et al., 2019; Selma et al., 2019). Removing repressive DNA methylation from a promoter using the human TEN-ELEVEN TRANSLOCATION1 (TET1) can also cause transcriptional activation (Maeder et al., 2013; Amabile et al., 2016; Choudhury et al., 2016; Liu et al., 2016; Morita et al., 2016; Xu et al., 2016; Lo et al., 2017; Okada et al., 2017; Gallego-Bartolomé et al., 2018; Li et al., 2020; Tang et al., 2021). In animals, the TET family of oxidases can oxidize methylated DNA, leading to either the passive removal of the methyl group through a lack of maintenance during DNA replication or the active removal by glycosylases such as thymine DNA glycosylase (Lio et al., 2020). Such oxidized variants have been detected in plants; however, at such low levels that its importance is called into question (Mahmood and Dunwell, 2019). While no proteins homologous to the TET family have been described in plants, ectopic expression of TET proteins, targeted or otherwise, in plants causes a loss of DNA methylation, suggesting the existence of a similar passive or active mechanism for the removal of oxidized DNA methyl groups in plants (Hollway et al., 2016; Gallego-Bartolomé et al., 2018; Ji et al., 2018).

Unlike recruiting the basal transcriptional machinery, which can cause unwanted overexpression, using epigenetic marks to control transcription only facilitates the accessibility of the target promoter to the transcriptional machinery. This highlights an advantage to the manipulation of epigenetic marks over the targeting of an activator domain. In addition to this, altering epigenetic marks can also facilitate the accessibility of targeted activator domains and thus, achieve a synergistic effect when they are combined (Roca Paixão et al., 2019).

Co-targeting Activation

While many studies have attempted to compare single activator domains to determine the best one to use for targeted activation, such studies will be heavily influenced by a number of unrelated factors, including the choice of targeting system and target site. While an activator domain might appear superior in a specific system or at a specific target site, this cannot be extrapolated to every possible scenario that exists in every plant genome, and thus a variety of tools are needed. However, a consistent trend seen in the development of these tools is that the ones that are capable of targeting multiple activator domains to a single locus have
consistently been shown to perform better than those targeting a single domain (Beerli et al., 1998; Tiwari et al., 2012; Li et al., 2017; Selma et al., 2019; Morita et al., 2020; Pan et al., 2021). While there are limits to the number of domain repeats that can be included in a single coding sequence due to protein instability, using multiple different activator domains to synergistically activate gene expression has been an immensely successful strategy (Li et al., 2017; Selma et al., 2019). Targeting direct fusions of VP128 to EDLL or VP128 to TAL activator domains leads to an increase in the activation of gene expression (Li et al., 2017). In addition the fusion of VP64 to P65 and Rta, two additional modular activator domains originally shown to work in animals (VPR), is also capable of activating gene expression in plants to a higher level than VP64 alone (Chavez et al., 2015; Li et al., 2017). Further, systems that allow the co-targeting of different activator domains can now push this further by combining even more activator domains such as the EDLL domain with the VPR fusion (Selma et al., 2019). However, quantitatively comparing these different co-targeting strategies is challenging and again depends on the target site and targeting system. For example, co-targeting the EDLL domain with multimers of the VP16 activator domain either through the SAM targeting system or direct fusion, has been shown to be inferior to fusions to other activator domains or simply by co-targeting multiple VP64 peptides to the target locus (Li et al., 2017; Lowder et al., 2018; Pan et al., 2021). However, when the EDLL domain is co-targeted along with the VPR fusion using the SAM targeting system, it has been shown to produce a higher gene activation than targeting multiple VP64 domains. Thus, if an activator domain is effective at one locus or with a particular targeting system, it does not mean it will be effective when used in a different system or at a different locus.

Further, in animal cells, when VP64 and TET1 were co-targeted to the same locus they worked synergistically to upregulate gene expression causing a greater fold change increase in expression than targeting either factor alone (Morita et al., 2020), suggesting that manipulating the epigenetic landscape to be more amenable to activation is synergistic with targeting activator domains. In plants, targeting multiple VP64 activator modules to the FWA promoter using the SunTag targeting system also resulted in the loss of DNA methylation, suggesting that simultaneously targeting mechanisms that remove DNA methylation and directly activate of gene expression could work synergistically in plants as it does in animals (Papikian et al., 2019; Morita et al., 2020).

**Effectors domains used for transcriptional repression**

Targeted transcriptional repression can be achieved by several mechanisms such as chromatin remodeling, adding repressive or removing active epigenetic marks on histones, adding DNA methylation, inhibiting RNA Polymerase II processivity, or by triggering degradation of mRNA.

The most widely used effector domains for targeted gene repression in plants are the ERF-associated amphiphilic repression (EAR) domains, such as those found in SUPERMAN or BODENLOS. Outside of the targeting systems discussed here, these domains have been used extensively as a way to study highly redundant genes, as these repressor domains are dominant over activator domains, including VP16, and can be used to turn a constitutive activator into a constitutive repressor (Hiratsu et al., 2002, 2004; Kagale and Rozwadowski, 2011). EAR domains have been found to recruit histone deacetylases such as HDA19 and co-repressors such as TOPLESS (Kagale and Rozwadowski, 2011). The EAR domain SUPERMAN-REPRESSIVE DOMAIN X, an optimized version of the SUPERMAN EAR domain, and the downstream co-repressor TOPLESS have been used together with the targeting systems discussed above to specifically target transcriptional repression (Lowder et al., 2015; Tang et al., 2017; Khakhar et al., 2018).

Targeted addition of DNA methylation to promoters using DNA methyltransferases has also been used successfully to repress transcription. The catalytic domain of the RdDM-based DNA methyltransferase from tobacco (Nicotiana tabacum), DOMAINS REARRANGED METHYLTRANSFERASE (DRM) can add DNA methylation to a target promoter, resulting in transcriptional repression (Papikian et al., 2019). The inheritance of the targeted DNA methylation and thus, the transcriptional repression is highly dependent on the levels of CG methylation established at the target site (Gallego-Bartolomé et al., 2019). To increase the heritability of the targeted methylation recent studies in plants have used the CG-specific bacterial *Mollicutes Spiroplasma* DNA methyltransferase MQ1 containing a Q147L mutation to increase the specificity of the effector domain (Ghoshal et al., 2021). In the absence of the RdDM pathway, the targeted gene was found to only have CG methylation, while in wild type plants non-CG methylation was also observed, indicating that MQ1 only installs CG methylation to the target site which then recruits other DNA methylations (Ghoshal et al., 2021).

Besides modifying the epigenome for transcriptional repression, a direct mechanism to repress gene expression is by hindering the movement of the RNA polymerase II, known as CRISPR interference (CRISPRi). This is achieved by targeting CRISPR–dCas-based systems to the TSS region or downstream of the transcriptional start site of a gene and has been well demonstrated in mammalian cells (Qi et al., 2013). However, reports of CRISPRi are limited in plants. Only one example has shown partial repression of a gene by CRISPRi in maize (*Zea mays*) (Gentzel et al., 2020). In plants, no studies have been reported on co-targeting different effector domains to repress transcription. However, in animals, recruiting a DNMT3a domain, a DNMT3-LIKE (DNMT3L) domain, and a Krüppel-associated box transcriptional repression domain to a target gene via direct fusion of all three to dCas9 has been demonstrated as a powerful gene repression strategy (Nuñez et al., 2021).

**Conclusions**

While a number of platforms have been developed for targeted transcriptional control and epi-mutagenesis in plants,
including small RNAs, ZFs, and TALEs, development of CRISPR-based systems have recently become the dominant focus due to their ease of use and flexibility. Recent developments in CRISPR–dCas systems have focused on increasing efficiency and functionality, including creating systems capable of recruiting multiple copies and multiple types of effectors to a target site. In addition to this, progress has also been made in the development of additional and optimized delivery systems for these tools (Box 1). The development of CRISPR–dCas systems for targeted transcriptional control and epi-mutagenesis is still in its infancy and there are many ways in which these tools can be improved (see Outstanding Questions). Their recent development means that these tools have only been utilized in a few studies outside of the ones creating or optimizing them; however, examples are available and demonstrate the usefulness of these systems in answering basic questions and bioengineering (Lee et al., 2021; Leydon et al., 2021). The further development of these tools provides us with additional ways to target specific transcriptional or epigenetic manipulations in plants, allowing us to collect more direct evidence for the function of epigenetic marks and genes which can then be applied to the benefit of agriculture.

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References

Agius F, Kapoor A, Zhu JK (2006) Role of the Arabidopsis DNA glycosylase/lyase ROS1 in active DNA demethylation. Proc Natl Acad Sci USA 103: 11796–11801

Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121: 207–221

Altpeter F, Springer NM, Bartley LE, Blechl AE, Brutnell TP, Citovsky V, Conrad LJ, Gelvin SB, Jackson DP, Kausch AP, et al. (2016) Advancing crop transformation in the era of genome editing. Plant Cell 28: 1510–1520

Amabile A, Migliara A, Capasso P, Biffi M, Cittaro D, Naldini L, Lombardo A (2016) Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. Cell 167: 219–232.e14

Bannister AJ, Kouzarides T (2011) Regulation of chromatin by histone modifications. Cell Res 21: 381–395

Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. Curr Opin Plant Biol 2: 109–113

Beerli RR, Segal DJ, Dreier B, Babars CF (1998) Toward controlling gene expression at will: Specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. Proc Natl Acad Sci USA 95: 14628–14633

Bernaertavichute Y V, Zhang X, Cokus S, Pellegrini M, Jacobsen SE (2008) Genome-wide association of histone H3 lysine nine methylation with CHG DNA methylation in Arabidopsis thaliana. PLoS One 3: e3156

Boch J, Scholze H, Schornack S, Landgraf A, Hahn S, Kay S, Boorsma S, Khuperkar D, Verhagen BMP, Sonneveld S, Grimm JB, Lin S, Kiani S, Guzman CD, Wiegand DJ, et al. (2012) Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. Cell 148: 1072–1085

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39: 734–746

Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Soma N V, Bogdanove AJ, Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res 39: 1–11

Chavez A, Scheiman J, Vora S, Pruitt BW, Tuttle M, P R Iyer E, Lin S, Kiani S, Guzman CD, Wiegand DJ, et al. (2015) Highly efficient Cas9-mediated transcriptional programming. Nat Methods 12: 326–328

Chellappan P, Xia J, Zhou X, Gao S, Zhang X, Coutino G, Vazquez F, Zhang W, Jin H (2010) siRNAs from miRNA sites mediate DNA methylation of target genes. Nucleic Acids Res 38: 6883–6894

Cheng AW, Wang H, Yang H, Shi L, Katz Y, Theunissen TW, Rangarajan S, Shivalila CS, Dadon DB, Jaenisch R (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res 23: 1163–1171

Choi Y, Gehring M, Johnson L, Hannon M, Harada JJ, Goldberg RB, Jacobsen SE, Fischer RL (2002) DEMETER, a DNA glycosylase domain protein, is required for endosperm gene imprinting and seed viability in Arabidopsis. Cell 110: 33–42

Choudhury SR, Cui Y, Lubecka K, Stefanska B (2016) CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. Oncotarget 7: 1–12

Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proc Natl Acad Sci USA 97: 4985–4990

Corona DFV, Tamkun JW (2004) Multiple roles for ISWI in transcription, chromosome organization and DNA replication. Biochim Biophys Acta Genet Struct Exp 1677: 113–119

Creasey KM, Zhai J, Borges F, Van Ex F, Regulski M, Meyers BC, Martienssen RA (2014) MiRNAs trigger widespread epigenetically activated siRNAs from transposons in Arabidopsis. Nature 508: 411–415

Damadi E, Dalakouras A, Zwiebel M, Krczal G, Wassenegger M (2014) An endogene-resembling transgene is resistant to DNA methylation and systemic silencing. RNA Biol 11: 934–941

Dalcheta E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pizzada ZA, Eckert MR, Vogel J, Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471: 602–607

Deng D, Yin P, Yan C, Pan X, Gong X, Qi S, Xie T, Mahfouz M, Zhu JK, Yan N, et al. (2012) Recognition of methylated DNA by TAL effectors. Cell Res 22: 1502–1504

Ding Y, Ndamukong I, Xu Z, Lapko H, Fromm M, Avramova Z (2012) ATX1-generated H3K4me3 is required for efficient elongation of transcription, not initiation, at ATX1-regulated genes. PLoS Genet 8: e1003111

Dreier B, Beerli RR, Segal DJ, Flippin JD, Babars CF (2001) Development of zinc finger domains for recognition of the 5′-ANN-3′ family of DNA sequences and their use in the construction of artificial transcription factors. J Biol Chem 276: 29466–29478

Du J, Zhong X, Bernaertavichute Y V., Stroud H, Feng S, Caro E, Vashisht AA, Terragni J, Ching HW, Tu A, et al. (2012) Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. Cell 151: 167–180

Elrod-Erickson M, Roald MA, Nekludova L, F, Zhang W, Jin H (2002) Efficient Cas9-mediated transcriptional programming. Nat Methods 12: 326–328

Feng S, Jacobsen SE, Reik W (2010) Epigenetic reprogramming in plant and animal development. Science 330: 622–627

Gallego-Bartolomé J, Gardiner J, Liu W, Papikian A, Ghoshal B, Kuo HY, Zhao JM-CC, Segal DJ, Jacobsen SE (2018) Targeted DNA demethylation of the Arabidopsis genome using the human TET1 catalytic domain. Proc Natl Acad Sci USA 8: 201716945

Gallego-Bartolomé J, Liu W, Kuo PH, Feng S, Ghoshal B, Gardiner J, Zhao JMC, Park SY, Chory J, Jacobsen SE (2019) Co-targeting RNA polymerases IV and V promotes efficient de novo DNA methylation in Arabidopsis. Cell 176: 1068–1082.e19

García D, García S, Pontier D, Marchais A, Renou JP, Lagrange T, Voisin D, Gardiner et al. (2012) Ago hook and RNA helicase motifs underpin dual roles for SDE3 in antiviral defense and silencing of non-conserved intergenic regions. Mol Cell 48: 109–120
Gardiner J, Zhao JM, Chaffin K, Jacobsen SE (2020) Promoter and terminator optimization for DNA methylation targeting in Arabidopsis. Epigenomes 4:9

Gehring M, Huh JH, Hsieh TF, Penterman J, Choi Y, Harada JJ, Goldberg RB, Fischer RL (2006) DEMETER DNA glycosylase establishes MEDEA polycomb gene self-imprinting by allele-specific demethylation. Cell 124:495–506

Geißler R, Scholze H, Hahn S, Streubel J, Bonas U, Behrens SE, Boch J (2011) Transcriptional activators of human genes with programmable DNA-specificity. PLoS One 6:1–7

Gendall AR, Levy YY, Wilson A, Dean C (2001) The VERNALIZATION 2 gene mediates the epigenetic regulation of vernalization in Arabidopsis. Cell 107:525–535

Gentzel IN, Park CH, Bellizzi M, Xiao G, Gadhave KR, Murphree C, Yang Q, Lamantia J, Redinbaugh MG, Ballest-Kurti P, et al. (2020) A CRISPR-dCas9 toolkit for functional analysis of maize genes. Plant Methods 16:1–9

Ghoshal B, Gardiner J (2021) CRISPR-dCas9-based targeted manipulation of DNA methylation in plants. In MT Islam, KA Molla, eds, Crispr-Cas Methods - (Springer Protocols Handbooks, Humana, New York, NY, pp 57–71

Ghoshal B, Picard CL, Vong B, Feng S, Jacobsen SE (2021) CRISPR-based targeting of DNA methylation in Arabidopsis thaliana by a bacterial CG-specific DNA methyltransferase. Proc Natl Acad Sci U S A 118:e2125016118

Ghoshal B, Vong B, Picard CL, Feng S, Tam JM, Jacobsen SE (2020) A viral guide RNA delivery system for CRISPR-Based transcriptional activation and heritable targeted DNA demethylation in Arabidopsis thaliana. Plant Cell 10:1–19

Gong Z, Morales-ruiz T, Ariza RR, Rolda T, David L, Zhu J, De Gene D (2002) ROS1, a Repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. Nature 411:803–814

Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Goodrich J, Puangsomlee P, Martin M, Long D, Meyerowitz EM, Coupland G (1997) A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature 386:44–51

Hall DB, Struhl K (2002) The VP16 activation domain interacts with multiple transcriptional components as determined by protein-protein cross-linking in vivo. J Biol Chem 277:46043–46050

Harris CJ, Scheibe M, Wongpalee SP, Liu W, Cornett EM, Vaughan RM, Li X, Chen W, Xue Y, Zhong Z, et al. (2018) A DNA methylation reader complex that enhances gene transcription. Science 362:1182–1186

Herr AJ, Jensen MB, Dalmay T, Baulcombe DC (2005) RNA polymerase IV directs silencing of endogenous DNA. Science 308:118–120

Hirai H, Tani T, Kikyo N (2010) Structure and functions of powerful transactivators: VP16, MyoD and FoxA. Int J Dev Biol 54:1589–1596

Hiratsu K, Mitsuda N, Matsui K, Ohme-Takagi M (2004) Identification of the minimal repression domain of SUPERMAN shows that the DELLRL hexapeptide is both necessary and sufficient for repression of transcription in Arabidopsis. Biochem Biophys Res Commun 321:172–178

Hiratsu K, Ohta M, Matsui K, Ohme-Takagi M (2002) The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flow- eres. FEBS Lett 514:351–354

Holloway E, Watson M, Meyer P (2016) Expression of the C-terminal domain of mammalian TET3 DNA dioxygenase in Arabidopsis thaliana induces heritable methylation changes at rDNA loci. Adv Biosci Biotechnol 7:243–250

Huang CY, Wang H, Hu P, Hamby R, Jin H (2019) Small RNAs – big players in plant-microbe interactions. Cell Host Microbe 26:173–182

Huang K, Wu XX, Fang CL, Xu ZG, Zhang HW, Gao J, Zhou CM, You LL, Gu ZX, Mu WH, et al. (2021) Pol IV and RDR2: a two-RNA-polymerase machine that produces double-stranded RNA. Science 374:1579–1586

Huang YH, Su J, Lei Y, Brunetti L, Gundry MC, Zhang X, Jeong M, Li W, Goodell MA (2017) DNA epigenome editing using CRISPR-Cas SunTag-directed DNMT3A. Genome Biol 18:1–11

Ichino L, Boone BA, Strausklage L, Harris CJ, Kaur G, Gladstone MA, Tan M, Feng S, Jami-Alahmadi Y, Duttke SH, et al. (2021) MBDS and MBD6 couple DNA methylation to gene silencing through the J-domain protein SILENZIO. Science 372:1434–1439

Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature 416:556–560

Jacob Y, Feng S, LeBlanc CA, Bernatavichute YV, Y, Stroud H, Cokus S, Johnson LM, Pellegrini M, Jacobsen SE, Michaels SD (2009) ATXRS and ATXR6 are FACT monomethyltransferases required for chromatin structure and gene silencing. Nat Struct Mol Biol 16:763–768

Ji L, Jordan WT, Shi X, Hu L, He C, Schmitz RJ (2018) TET-mediated epimutagenesis of the Arabidopsis thaliana thymine. Nature Commun 9:895

Jiang C, Pugh BF (2009) Nucleoside positioning and gene regulation: advances through genomics. Nat Rev Genet 10:161–172

Jinek M, Chylinski K, Fonfar A, Hauer M, Doudna JA, Charpentier E (2012) A Programmable Dual-RNA – Guided. 337:816–822

Johnson LM, Bostick M, Zhang X, Kraft E, Henderson I, Callis J, Jacobsen SE (2007) The SRA methyl-cytosine-binding domain links DNA and histone methylation. Curr Biol 17:379–384

Johnson LM, Du J, Hale CJ, Bischof S, Feng S, Chodavarapu RK, Zhong X, Marson G, Pellegrini M, Segal DJ, et al. (2014) SRA- and SET-domain-containing proteins link RNA polymerase V occupancy to DNA methylation. Nature 507:124–8

Kagale S, Rozwadowski K (2011) EAR motif-mediated transcriptional repression in plants: an underlying mechanism for epigenetic regulation of gene expression. Epigenetics 6:141–146

Khakhar A, Leydon AR, Lemmess AC, Klavins E, Nemhauser JL (2018) Synthetic hormone-responsive transcription factors can monitor and reprogram plant development. Elife 7:1–16

Khakhar A, Voytas DF (2021) RNA viral vectors for accelerating plant synthetic biology. Front Plant Sci 12:668580

Khraiwesh B, Arif MA, Seumel GI, Ossowski S, Weigel D, Reski R, Frank W (2010) Transcriptional control of gene expression by microRNAs. Cell 140:111–122

Konermann S, Brigham MD, Trevino AE, Joong J, Abudayeh OO, Barcena C, Hsu PD, Habib N, Gootenber G, Nishiuma H, et al. (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517:583–588

Kuruhara Y, Watanabe Y (2004) Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. Proc Natl Acad Sci USA 101:12753–12758

Larson MH, Gilbert LA, Wang X, Lim WA, Weissman JS, Qi LS (2013) CRISPR interference (CRISPRi) for sequence-specific control of gene expression. Nat Protoc 8:2180–2196

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11:204–220

Lawrence RJ, Earley K, Pontes O, Silva M, Chen ZJ, Neves N, Viegas W, Pikaard CS (2004) A concerted DNA methylation/histine methylation switch regulates rRNA gene dosage control and nucleolar dominance. Mol Cell 13:599–609

Lee DY, Hua L, Khoshvarav R, Giuliani R, Kumar I, Cousins A, Sage TL, Hibberd JM, Brutnell TP (2021) Engineering chloroplast development in rice through cell-specific control of endogenous genetic circuits. Plant Biotechnol J 19:2291–2303

Lee JE, Neumann M, Duro DI, Schmid M (2019) CRISPR-based tools for targeted transcriptional and epigenetic regulation in plants. PLoS One 14:1–17

Lee MS, Gippert GP, Soman K V., Case DA, Wright PE (1989) Three-dimensional solution structure of a single zinc finger DNA-binding domain. Science 245:635–637
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Panda K, Ji L, Neumann DA, Daron J, Schmitz RJ, Slotkin RK (2016) Full-length autonomous transposable elements are preferentially targeted by expression-dependent forms of RNA-directed DNA methylation. Genome Biol 17: 1–19

Pandey R, Mu A, Ller E, Napoli CA, Selinger DA, Pikaard CS, Richards EJ, Bender J, Mount DW, Jorgensen RA (2002) Analysis of histone acetyltransferase and histone deacetylase families of Arabidopsis thaliana suggests functional diversification of chromatin modification among multicellular eukaryotes. Nucleic Acids Res 30: 5036–5055

Papikian A, Liu W, Gallego-Bartolome J, Jacobsen SE (2019) Site-specific manipulation of Arabidopsis loci using CRISPR-Cas9 SunTag systems. Nat Commun 10: 1–11

Park JJ, Demepewolf E, Zhang W, Wang ZY (2017) RNA-guided transcriptional activation via CRISPR/dCas9 mimics expression phenotypes in Arabidopsis. PLoS One 12: 1–13

Pavletch NP, Pabo C (1991) Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science 252: 809–818

Penterman J, Zilberman D, Huh JH, Ballinger T, Henikoff S, Richmond TJ, Davey CA (2003) The structure of DNA in the nucleosome core. Nature 423: 145–150

Pentea A, Ali Z, Baazim H, Li L, Abulfaraj A, Al-Sharief S, Ali Z, Bahaj AH, Li L, Aoudia M, Mahfouz MM (2015) RNA-guided transcriptional regulation in plants via synthetic dCas9-based transcription factors. Plant Biotechnol J 13: 578–589

Pikaard CS, Mittelsten Scheid O (2014) Epigenetic regulation in plants. Cold Spring Harb Perspect Biol 6: a019315–a019315

Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA (2013) Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152: 1173–1183

Raja P, Sanville BC, Buchmann RC, Bisaro DM (2008) Viral genome methylation as an epigenetic defense against gemiviruses. J Virol 82: 8997–9007

Ren Q, Sretenovic S, Liu S, Tang X, Huang L, He Y, Liu L, Guo Y, Zhong Z, Liu G, et al. (2021) PAM-less plant genome editing using a CRISPR–SpRY toolbox. Nat Plants 7: 25–33

Richmond TJ, Davey CA (2003) The structure of DNA in the nucleosome core. Nature 423: 145–150

Roca Paixão JF, Gillet FX, Ribeiro TP, Bournaud C, Lourenço-Tessutti IT, Noriega DD, Melo BP de, de Almeida-Engler J, Grossi-de-Sa MF (2019) Improved drought stress tolerance in Arabidopsis: an epigenomic tale of tails and more. Trends Genet 35: 511–517

Sánchez JP, Ullman C, Moore M, Choo Y, Chua NH (2002) Regulation of gene expression in Arabidopsis thaliana by artificial zinc finger chimeras. Plant Cell Physiol 43: 103–114

Sandhya D, Jagom P, Allini VR, Abbagani S, Alok A (2020) The present and potential future methods for delivering CRISPR/Cas9 components in plants. J Genet Eng Biotechnol 18: 25 doi: 10.1186/s43141-020-00036-8

Schwab R, Ossowski S, Riester M, Warthmann N, Weigel D (2006) Highly specific gene silencing by artificial microRNAs in Arabidopsis. Plant Cell 18: 1121–1133

Segal DJ, Dreier B, Beerli RR, Barbas CF (1999) Toward controlling gene expression at will: Selection and design of zinc finger domains recognizing each of the 5′-GGN-3′ DNA target sequences. Proc Natl Acad Sci USA 96: 2758–2763

Selma S, Bernabé-Orts JM, Vazquez-Vilar M, Diego-Martin B, Ajenjo M, García-Carpintero V, Granell A, Orzaez D (2019) Strong gene activation in plants with genome-wide specificity using a new orthogonal CRISPR/Cas9-based programmable transcriptional activator. Plant Biotechnol J 17: 1703–1705

Sigman MJ, Panda K, Kirchner R, McLain LL, Payne H, Peasari JR, Husbands AE, Slotkin RK, McCue AD (2017) An siRNA-guided ARGONAUTE protein directs RNA polymerase V to initiate DNA methylation. Nat Plants 3: 1461–1474

Slotkin RK, Freeling M, Lisch D (2005) Heritable transposition silencing initiated by a naturally occurring transposon inverted duplication. Nat Genet 37: 641–644

Sorek R, Lawrence CM, Wiedenheft B (2013) CRISPR-mediated adaptive immune systems in bacteria and archaea. Annu Rev Biochem 82: 237–266

Stege JT, Guan X, Ho T, Beachy RN, Barbas CF (2002) Controlling gene expression in plants using synthetic zinc finger transcription factors. Plant J 32: 1077–1086

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

Stroud H, Greenberg MVC, Feng S, Bernatavichute Y V., Jacobsen SE (2013) Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. Cell 152: 352–364

Tenaenbaum ME, Gilbert LA, Qi LS, Weissman JS, Vale RD (2014) A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159: 635–646

Tang S, Yang C, Wang D, Deng X, Cao X, Song X (2021) Targeted DNA demethylation produces heritable epialleles in rice. Sci China Life Sci 1: 1–4

Tang X, Lowder LG, Zhang T, Malzahn AA, Zhong X, Voytas DF, Zhong Z, Chen Y, Ren Q, Li Q, et al. (2017) A CRISPR – Cpf1 system for ef fient genome editing and transcriptional repression in plants. Nat Plants 3: 17018

Tiwari SB, Belachew A, Ma SF, Young M, Ade J, Shen Y, Marion CM, Holtan HE, Bailey A, Stone JK, et al. (2012) The EDLL motif: a potent plant transcriptional activation domain from AP2/ERF transcription factors. Plant J 70: 855–865

Tsujii S, Futaki S, Imanishi M (2016) Sequence-specific recognition of methylated DNA by an engineered transcription activator-like effector protein. Chem Commun 52: 14238–14241

Valton J, Dupuy A, Daboussi F, Thomas S, Marechal A, Macmaster R, Meliandi K, Juillerat A, Duchateau P (2012) Overcoming transcription activator-like effector (TALE) DNA binding domain sensitivity to cytosine methylation. J Biol Chem 287: 38427–38432

Wesley VS, Hellwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoujtsdjik PA, et al. (2001) Present design for construct effective, efficient and high-throughput gene silencing in plants. Plant J 27: 581–590

Vazquez-Vilar M, Bernabé-Orts JM, Fernandez-del-Carmen A, Ziaroso P, Blanca J, Granell A, Orzaez D (2016) A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. Plant Methods 12: 1–12

Vazquez F, Blevins T, Ailhas J, Boller T, Meins F (2008) Evolution of Arabidopsis MIR genes generates novel microRNA classes. Nucleic Acids Res 36: 6429–6438

Voigt A, Dobrinic P, Tadic V, Bockor L, Korac P, Julg B, Kladic M, Zoldos V (2016) Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucleic Acids Res 44: 5615–5628

Walton RT, Christie KA, Whitaker MN, Kleinstiver BP (2020) Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. Science 368: 290–296
