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CRISPR/dCas-mediated transcriptional and epigenetic regulation in plants

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The CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR Associated) system-mediated precise genome editing has revolutionized genome engineering due to ease of use and versatility of multiplexing. Catalytically inactivated Cas variants (dCas) further expand the usefulness of the CRISPR/Cas system for genetics studies and translational research without inducing DNA double-strand breaks. Fusion of diverse effector domains to dCas proteins empowers the CRISPR/dCas system as a multifunctional platform for gene expression regulation, epigenetic regulation and sequence-specific imaging. In this short review, we summarize the recent advances of CRISPR/dCas-mediated transcriptional activation and repression, and epigenetic modifications. We also highlight the future directions and broader applications of the CRISPR/dCas systems in plants.

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Introduction

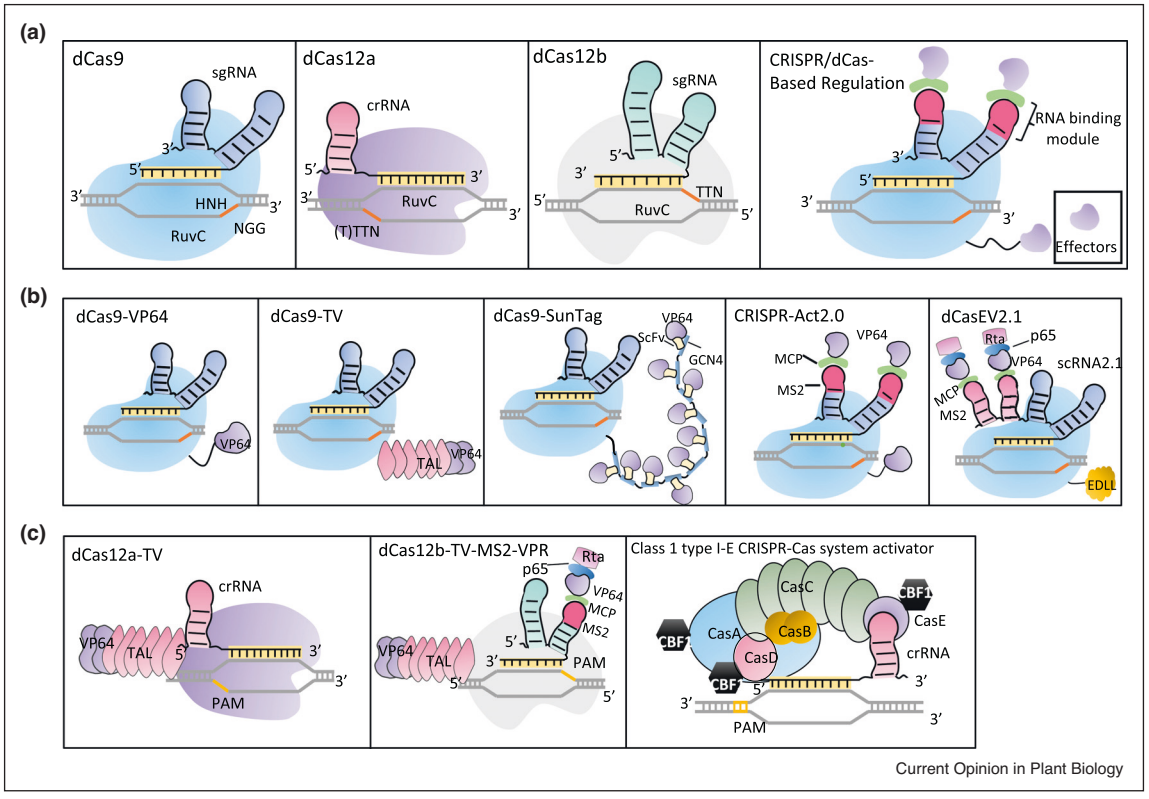
Recent advances in sequencing technology have substantially contributed to plant biology. The underlying mechanisms of plant growth and development are elucidated by high-throughput sequencing of genome-wide coding and non-coding RNA (ncRNA) transcripts. However, one of the greatest challenges in plants remains to be defining the causal relationships between gene expression and phenotypic features. Conventional reverse genetics approaches for investigating gene function aim to disrupt gene expression through transgenic overexpression or RNA interference (RNAi) in plants. However, they lack the flexibility and scalability to achieve simultaneous multigene modulations and epigenetic modifications [1]. Despite intense research efforts, the molecular functions of many coding RNAs and ncRNAs remain poorly understood. Transcription is a fundamental and dynamic step in the regulation of gene expression. Thus, genome engineering technologies that enable dynamic and precise regulation of individual or multiple transcripts at the transcriptional or epigenetic level provide a promising approach to investigate gene functions.

In the past few years, significant progress has been made in developing programmable genome engineering technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas systems that enable permanent DNA modifications in a designable and sequence-specific manner [2]. Because of its robustness and flexibility, the CRISPR/Cas systems represent an efficient and simple method to manipulate targeted genome sequences [3]. Beyond gene editing, CRISPR/Cas systems have been repurposed as a programmable platform for transcriptional and post-transcriptional regulation. This is aided by mutating the nuclease domains to create catalytically inactive Cas proteins (dCas), which remain competent for RNA-guided DNA binding but inadequate to induce DNA double-strand breaks [4]. The dCas proteins can be fused with effector proteins including transcriptional activators, repressors, and epigenetic modulators, enabling efficient gene-specific CRISPR-mediated activation (CRISPRa), interference (CRISPRi), and epigenome modifications, respectively [5••]. Here, we provide a succinct overview of the up-to-date advances of CRISPR/dCas based transcriptional and epigenetic regulation in plants.

Nuclease-deactivated Cas (dCas): a programmable platform beyond genome editing

Among class 2 CRISPR-Cas systems, type II Cas9, type V-A Cas12a (formerly Cpf1) and type V-B Cas12b systems act as RNA-guided endonucleases [6]. Both Cas9 and Cas12b require single guide RNAs (sgRNAs), fusions of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA), for precisely editing the genomic DNA, while Cas12a requires only a crRNA (Figure 1a) [6]. Cas9 recognizes G-rich protospacer adjacent motifs (PAMs), whereas Cas12a/b recognize T-rich PAMs, significantly increasing the targeting scope within the genome (Figure 1a) [7]. Beyond genome editing, CRISPR-Cas9 and Cas12a/b have been repurposed as multifunctional platforms for transcriptional regulation, chromatin engineering, and fluorescence based live-imaging using dCas9 and dCas12a/b fused to effector

Figure 1



Engineered CRISPR/dCas and CRISPRa systems in plants.
(a) A schematic representation of dCas9/12a/12b-mediated CRISPR complexes and their use for transcription regulation. The sgRNA consists of a trans-activating crRNA (tracrRNA) and CRISPR RNA (crRNA). The HNH and RuvC domains are mutated in dCas9, and RuvC domain is mutated in dCas12a and dCas12b. Effectors include activators, repressors or epigenetic modifiers. NGG, (T)TTN, TTN represent the PAM nucleotide sequences for Cas9, Cas12a, and Cas12b, respectively. **(b)** A schematic representation of the first and second generations of dCas9-based activators. In dCas9-VP64, the transcriptional activator VP64 is directly fused to dCas9; in dCa9-TV, a potent activator TV, consisting of six copies of the TALE TAD motif and two copies of VP64 is directly fused to dCas9; in dCas9-SunTag, ten tandem repeats of a small peptide GCN4 are utilized to recruit multiple copies of scFv fusion with the transcriptional activator VP64. scFv, single-chain variable fragment; in CRISPR-Act2.0, the sgRNA scaffold is modified to contain two MS2 RNA aptamers to recruit the MS2 bacteriophage coat protein (MCP) fusion with the transcriptional activator VP64 and the dCas9 is fused to VP64; in dCasEV2.1, two MS2 RNA aptamers are fused to the 3' end of sgRNA2.1 scaffold and MCP is fused to a combinatory transcriptional activator VP64–p65–Rta (VPR), and the dCas9 is fused to transcriptional activator EDLL. VP64, four copies of herpes simplex viral protein 16 (VP16); TAL, TAL effector transcription activation domain; p65, activator domain of nuclear factor kappa B; Rat, Epstein–Barr virus R transactivator; EDLL, a potent plant transcriptional activation domain from AP2/ERF transcription factors. **(c)** A schematic representation of dCas12a/b and Class 1 type I-E CRISPR-Cas-based activators. In dCas12a-TV, a potent activator TV is fused to a dCas12a; in dCas12b-TV-MS2-VPR, the sgRNA scaffold is modified to contain one MS2 RNA aptamer to recruit the MCP fusion with the transcriptional activator VPR and the dCas9 is fused to activator TV; in Class 1 type I-E CRISPR-Cas-based activator, the Class 1 type I-E CRISPR-Cas system from *Streptococcus thermophilus* DGCC7710 is consisted of *CasA*, *CasB*, *CasC*, *CasD*, *CasE* and crRNA, and a plant transcriptional activation domain CBF1 is fused to the C-terminus of *CasA*, *CasD*, and *CasE*.

domains (Figure 1a) [8]. Based on the similar concept, several CRISPR–dCas platforms for transcriptional or epigenome engineering have been engineered and demonstrated in plants [9].

CRISPR/dCas-mediated transcriptional activation

The first-generation CRISPRa systems

In 2013, Gilbert *et al.* fused four copies of the transcription activator VP16 (VP64) or a single copy of the p65 activation domain to dCas9 to generate the first-generation of CRISPRa, dCas9-VP64 and dCas9-p65, both achieving targeted reporter gene activation of 12-fold to 15-fold in eukaryotic cells [4]. Meanwhile, following a similar concept, activation domains including VP64, EDLL and transcriptional activator-like (TAL) effector were fused to the C-terminus of dCas9 to generate dCas9-VP64, dCas9-EDLL and dCas9-TAL in plants, respectively [10,11]. Although the first-generation CRISPRa systems were able to achieve target loci upregulation with a sgRNA or multiple sgRNAs, the activation potency typically remained low (up to 12-fold), indicating the needs for improvement.

The second-generation CRISPRa systems

To improve CRISPRa, a total of four second-generation CRISPRa systems have been developed in plants using different strategies (Figure 1b) [12••,13•,14••,15]. Li *et al.* developed and showcased a potent CRISPRa system, dCas9-TV, in rice and *Arabidopsis* [12••]. In the dCas9-TV system, the dCas9 is fused to six copies of the TALE transcription activation domain (TAD) and two copies of VP64 [12••]. Papikian *et al.* adapted the dCas9-SunTag system for targeted gene activation in *Arabidopsis* [14••]. In the dCas9-SunTag system, a tandem array of ten GCN4 peptides is fused to dCas9, and a single-chain variable fragment (scFv), the GCN4 antibody, is fused to the superfolder-GFP (sfGFP) and transcriptional activator VP64. Therefore, multiple copies of VP64 can be recruited to the target locus, through the CRISPR/dCas9 system [14••]. In addition to direct fusion to dCas9, the activation domains can also be recruited through the sgRNA scaffold [16]. The sgRNA2.0 scaffold containing two RNA aptamers, such as MS2 hairpins that specifically bind MS2 coat protein (MCP), was engineered for transcriptional activation in mammalian cells [17]. Based on the sgRNA2.0 strategy, Lowder *et al.* developed another effective second-generation CRISPRa system in plants, CRISPR-Act2.0, which is composed of sgRNA2.0, dCas9-–VP64, and MS2–VP64 [13•]. Additionally, using a novel sgRNA scaffold, scaffold RNA 2.1 (scRNA 2.1), which contains the anchoring sites for transcriptional activator VPR (VP64-p65-Rta), Selma *et al.* achieved high transcriptional activation in *N. benthamiana* [15]. Aforementioned four second-generation CRISPRa systems conferred significantly stronger transcriptional activation than dCas9-VP64. However, it remains ambiguous which system is the most potent across various plant species.

CRISPRa systems based on Cas12a/b

Recently, several groups have demonstrated dCas12a-based gene activation in mammalian cells by directly fusing activator to dCas12a or engineered split dCas12a [18•,19,20]. The split dCas12a activator induced greater activation efficiency than analogous dCas9-based activators [18•]. However, an attempt to engineer a potent plant dCas12a activator failed since the dCas12a-TV activator only resulted in a low activation of 4.7-fold in *Arabidopsis* cells (Figure 1c) [12••]. As a proof-of-concept experiment for developing dCas12b activator, Teng *et al.* tested the dAaCas12b fused to a VP64 or VPR activator for activation in mammalian cells, demonstrating only a low activation potency [21]. Ming *et al.* screened a total of 12 transcriptional activation configurations in rice protoplasts and revealed that the combination of dAaCas12b-TV, Aac.3 sgRNA scaffold, and MS2-VPR represents a strong dAaCas12b-based transcriptional activation system (Figure 1c), achieving fivefold to eightfold transcriptional activation in plants [7]. Notably, the activation potency of dCas12a/b activators was significantly lower compared to dCas9 [7,21]. This discrepancy may be caused by the considerable difference in their structures, components and mechanisms of action [22,23], suggesting further efforts on developing the dCas12-based gene activation platform is warranted.

CRISPRa based on Type I CRISPR systems

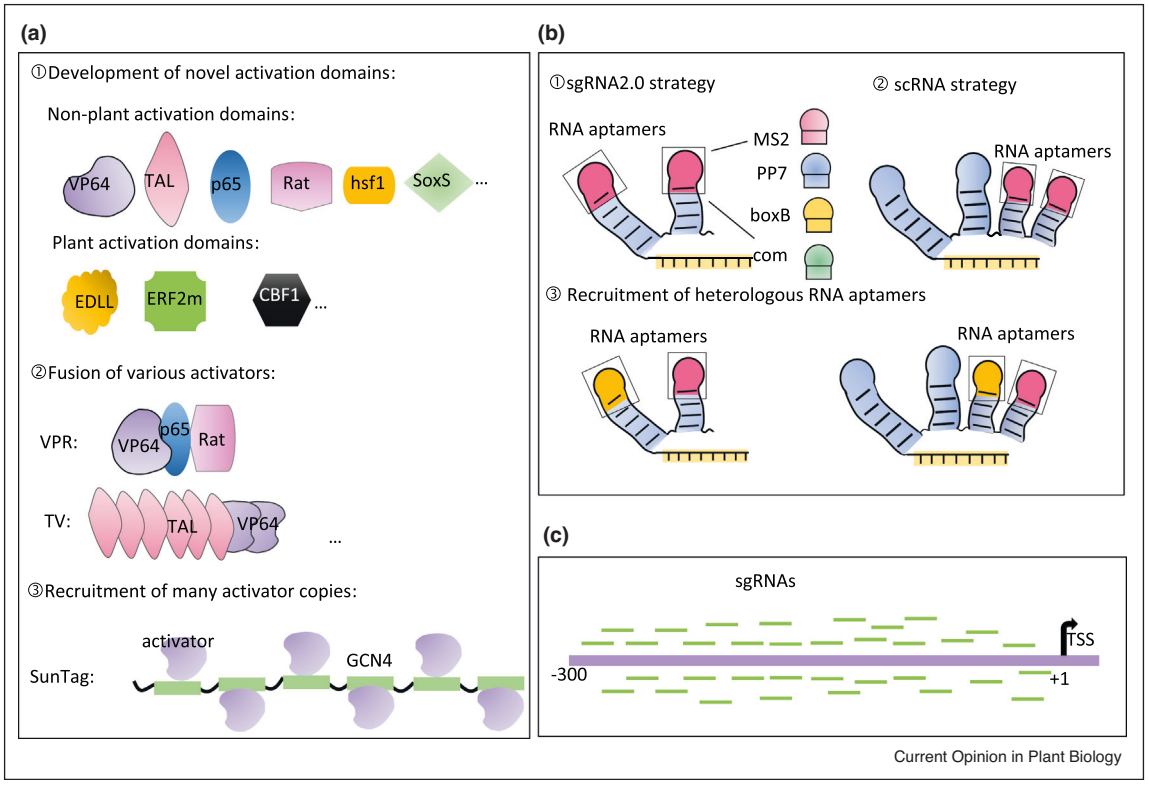
While most genome engineering systems are based on Type II CRISPR systems that contain single Cas proteins such as Cas9 and Cas12a/b, two recent works repurposed the complex Type I CRISPR system for gene activation in human cells [24] and in maize [25]. In the latter case, the class 1 type I-E CRISPR system from *S. thermophilus* was used for developing a plant CRISPRa system, consisting of five Cas proteins (CasA-E) (Figure 1c). Young *et al.* fused C-terminal acidic plant transcriptional activation domain from the *Arabidopsis* cold binding factor 1 (CBF1) to CasA, CasD and CasE individually and demonstrated activation of the reporter gene, *DsRed*, in each case (Figure 1c) [25]. Impressively, simultaneous recruitment of the activator by these three Cas proteins resulted in much higher gene activation (~100 fold), likely due to additive or synergistic effects [25]. Hence, the use of Type I CRISPR system to recruit multiple copy of the same transcriptional activator or different transcriptional activators represents another promising strategy for developing efficient CRISPRa systems in plants.

Strategies to improve CRISPRa efficiency

Development of robust transcriptional activators

The potency of CRISPRa can be significantly enhanced by recruiting effective transcriptional activators using different strategies (Figure 2). The first approach involves developing novel transcriptional activation domains. The herpes simplex viral protein 16-like activation domain (VP16), and multiple tandem copies of VP16 (VP64, VP128, and VP160) have been widely used in mammalian cells and plants (Figure 2a) [11,13•,14••,17,26]. Recently, Dong *et al.* screened a series of candidate transcriptional activation domains and demonstrated that SoxS activator can induce robust gene activation by recruiting the RNA polymerase in *E. coli* [27]. The second strategy to enhance CRISPRa activation potency is based on fusing various activators in tandem to dCas proteins (Figure 2a). The VPR activator, an improved activation module, is composed of a hybrid tripartite activator, VP64, p65AD, and Epstein–Barr virus R transactivator (Rta) [28]. The dCas9-VPR/-MS2:VPR can induce higher gene activation than dCas9-VP64 in both mammalian cells and plants [15,28,29]. TV activator is composed of six copies of the TALE TAD motif (TAL) and two copies of VP64 conferring significantly stronger transcriptional activation than VP64 activator [12••]. In addition, the enhancement of gene activation can be achieved by the recruitment of many activator copies (Figure 2a). In dCas9–SunTag system, multiple VP64 copies can be recruited by a single dCas9, resulting in strong activation of endogenous genes in mammalian cells and in *Arabidopsis* [14••,30]. It is

Figure 2



Strategies for enhancing the potency of CRISPRa.
(a) Developing robust transcriptional activators. The activation potency of CRISPRa could be significantly improved by recruiting potent activation domains. VP64, TAL, p65, Rat, hsf1 and SoxS are non-plant activation domains, and EDLL, ERF2m and CBF1 are plant derived activation domains. Fusion of dCas proteins with various activation domains in tandem and many activator copies based on SunTag can enhance CRISPRa activation potency. VP64, four copies of herpes simplex viral protein 16 (VP16); TAL, TAL effector transcription activation domain; p65, transactivator domain of nuclear factor kappa B; Rat, Epstein–Barr virus R transactivator; hsf1, human heat shock factor 1; EDLL, a potent plant transcriptional activation domain from AP2/ERF transcription factors; ERFm, a modified plant transcriptional activation domain from ethylene response factor; CBF1, a C-terminal acidic plant transcriptional activation domain from the *Arabidopsis* cold binding factor 1. **(b)** Optimization of CRISPR RNA scaffolds for CRISPRa. The sgRNA scaffolds with MS2, PP7, boxB or com RNA aptamer can recruit their cognate RNA-binding proteins fused to activation domains to activate gene expression. Multiple orthogonal RNA-binding modules can be adopted into one sgRNA scaffold, which allows for recruitment of the same or different activators. **(c)** Optimization of targeting location for CRISPRa. CRISPRa is affected by the position of the sgRNA relative to the transcription-start site (TSS) of the target gene. MS2, PP7, boxB, com represent RNA aptamers.

anticipated that CRISPRa in plants can be further improved when highly potent transcriptional activators are used.

Optimization of CRISPR RNA scaffolds

Previous studies have demonstrated that the efficiency of CRISPRa can be improved by engineering sgRNA scaffolds [16,17]. In sgRNA2.0 scaffold, two copies of MS2 RNA aptamer were added into the tetraloop and stem-loop 2 regions of the sgRNA sequence (Figure 2b), respectively, which protrude outside of the Cas9–sgRNA ribonucleoprotein complex [17]. Each MS2 aptamer can bind to bacteriophage MS2-coat protein (MCP) fused to an activator, indicating multiple activators can be recruited by the sgRNA2.0 scaffold (Figure 2b). The sgRNA 2.0-based CRISPR-Act2.0 system resulted in higher transcriptional activation than dCas9-VP64 [13•,17]. Besides MS2, other RNA aptamers such as PP7, boxB, and com can be introduced into sgRNA scaffolds for the recruitment of effector proteins (Figure 2b) [16,31•]. Zalatan *et al.* reported that a heterologous MS2-PP7 scRNA scaffold induced substantially stronger activation compared to a single type of RNA aptamer [16], demonstrating a promising approach to recruit multiple activation effectors by employing diverse RNA aptamers in a single sgRNA scaffold (Figure 2b). In addition, this strategy allows for orthogonal transcriptional regulation, such as simultaneous activation and repression of different target loci in the same cell or organism.

Optimization of targeting location

Previous studies have reported that the potency of CRISPRa is highly sensitive to the distance between the

sgRNA target site and transcription start site (TSS) (Figure 2c). Konermann *et al.* revealed that sgRNAs targeting the −200 bp to +1 bp window upstream of the TSS confer the highest activation level in mammalian cells [17]. In bacteria, effective gene activation requires sgRNAs to be situated in a narrower window of the 60–80 bp upstream of the TSS on the non-coding strand and 80–90 bases upstream on the coding strand [27]. Gong *et al.* demonstrated that sgRNAs within the 350 bp upstream region of the TSS are the most effective for dCas9-TV-based transcriptional activation in rice [32]. In humans, numerous prediction algorithms have been used to develop genome-wide sgRNA libraries for CRISPRa/i based on position, sequence features, and off-target activity of sgRNAs [33]. These prediction algorithms allow for the selection of highly specific and active sgRNAs in human cells. However, there is currently little guidance on how to design efficient sgRNAs for CRISPRa/i in plants, suggesting further efforts on developing such algorithms in plants are needed.

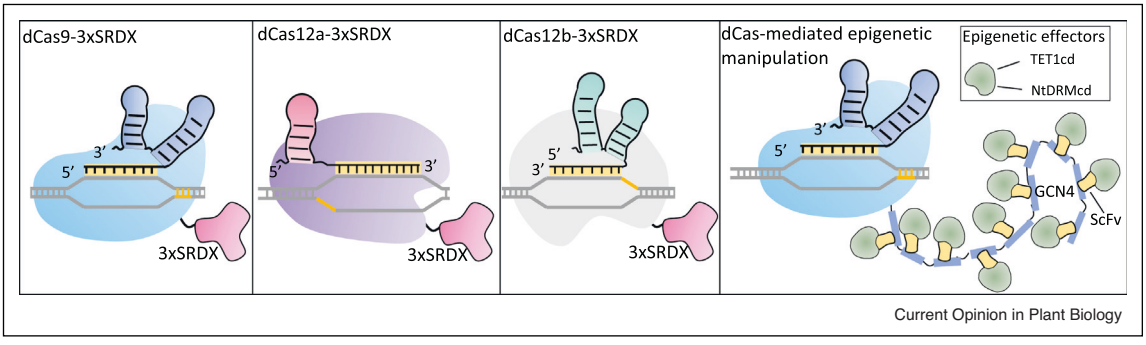
CRISPR/dCas-mediated transcriptional repression

CRISPR interference (CRISPRi) represents a new reprogrammable tool for targeted gene repression [34]. By binding to the promoter region proximal to the TSS, the dCas proteins or dCas proteins fused to transcriptional repression domains can interfere with transcription initiation or elongation by blocking RNA polymerase and transcription factor binding. In plants, only a few cases of CRISPRi application have been reported [7,10,11,12••]. dCas9-3xSRDX (SUPERMAN Repression Domain X) and dCas9-SRDX transcriptional repressors have been demonstrated in *Arabidopsis* and *N. benthamiana* (Figure 3), respectively, both leading to a transcript level reduction to approximately 40% of the control [10,11]. Based on the same strategy, dCas12a-mediated and dCas12b-mediated repressors have been demonstrated in *Arabidopsis* and rice (Figure 3), respectively [7,36]. However, the low efficiency of current CRISPRi tools limits the widespread use of CRISPRi for programmed gene repression in plants. The strategies for improving CRISPRa efficiency could be adopted to optimize the CRISPRi potency [35•,37–41].

CRISPR/dCas-mediated epigenetic manipulation

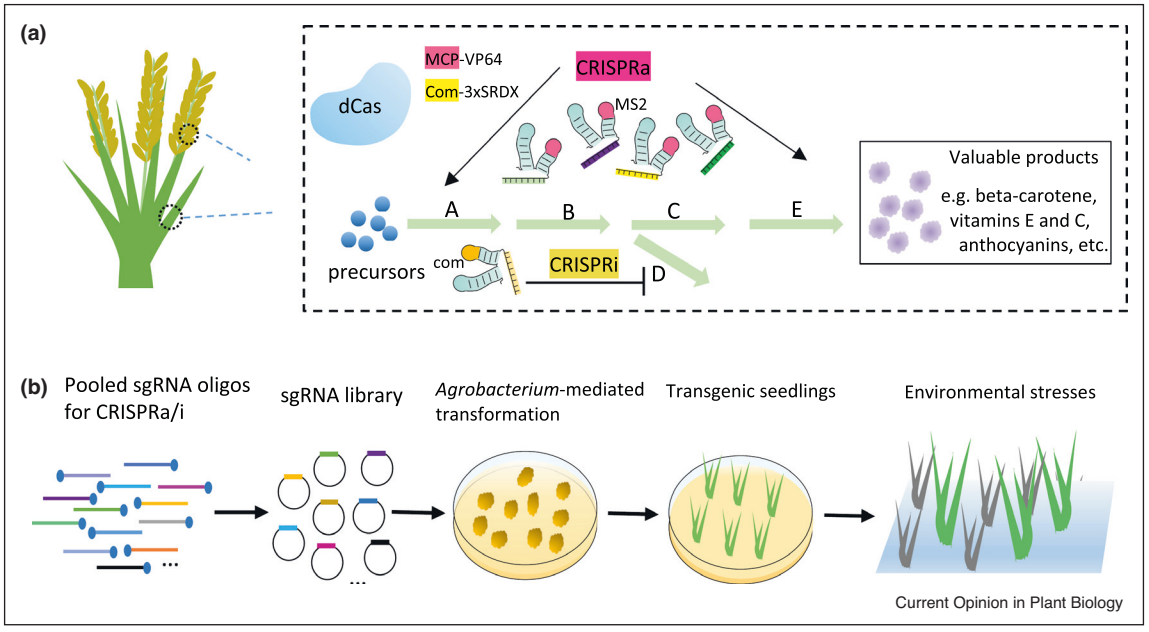
Epigenetic modifications such as histone modifications and DNA methylation are important means of transcriptional regulation [42]. CRISPR/dCas9-based epigenome editors have been developed to facilitate site-specific epigenetic modulation by recruiting a diverse array of epigenetic-effector domains [14••,43,44], offering an unprecedented opportunity for investigating the relationships between specific phenotypes and chromatin features. Gallego-Bartolomé *et al.* developed a site-specific DNA demethylation system in *Arabidopsis* based on the dCas9-SunTag-TET1cd (Figure 3), where the human demethylase TEN-ELEVEN TRANSLOCATION1 (TET1cd) was recruited by the SunTag [45]. By targeting the *FWA* promoter, the authors successfully achieved heritable site-specific DNA demethylation, resulting in a late-flowering phenotype [45]. Papikian *et al.* further applied the dCas9-SunTag system to develop a targeted DNA methylation tool (Figure 3) [14••]. By fusing to the *Nicotiana tabacum* DRM methyltransferase catalytic domain (NtDRMcd), the dCas9-SunTag system conferred efficient methylation within the *FWA* promoter and led to early flowering plants [14••]. Another study enabled manipulation of *Arabidopsis* flowering time by

Figure 3



CRISPR/dCas-mediated repression and epigenetic manipulation.
Schematic representation of dCas9/12a/12b-3xSRDX repressors and dCas-SunTag system-mediated epigenetic modifiers. 3xSRDX repressor can be recruited to the target site via the dCas/sgRNA complex. Transcription repression by dCas proteins can be improved by fusing dCas proteins with different repressor domains. Based on SunTag system, multiple copies of epigenetic modifiers, TET1cd or NtDRMcd, fused to scFv are recruited to a specific locus by the dCas/sgRNA complex. dCas, catalytically inactivated Cas; 3xSRDX, three copies of SUPERMAN Repression Domain X; TET1cd, TEN-ELEVEN TRANSLOCATION1 of demethylase; NtDRMcd, *Nicotiana tabacum* DRM methyltransferase catalytic domain; ScFv, single-chain variable fragment.

Figure 4



Future applications of the CRISPR–dCas technology in plants.
(a) Overview of rewiring metabolic engineering using CRISPR activation (CRISPRa) and CRISPR interference (CRISPRi) systems. CRISPR–dCas platform can be used to perturb numerous parts of a pathway simultaneously, thus redirecting flux and enhancing the output of the desired product from a branched metabolic pathway. Working together with dCas proteins, sgRNA constructs with MS2 hairpin recruit MCP fused to VP64 to activate the gene expression of *A*, *B*, *C* and *E*, sgRNA construct with com hairpin recruits Com fused to 3xSRDX to repress the gene expression of *D*. **(b)** CRISPRa/i-based high-throughput screen for key genes controlling plants development and environmental stress responses. The sgRNA library may target a whole genome, gene family, or cluster of genes with related functions. The sgRNAs can be transformed into plants along with a chosen dCas protein fused with an activator or repressor. The target sites of sgRNAs in the select population with interesting traits can be revealed using deep sequencing. dCas, catalytically inactivated Cas; MCP, MS2 coat protein; VP64, four copies of the transcription activator VP16; 3xSRDX, three copies of SUPERMAN Repression Domain X; Com, non-cognate-binding proteins of com; MS2, RNA aptamer binding to MCP; com, RNA aptamer binding to Com; sgRNA, single guide RNA.

altering the epigenetic status within the flowering time gene *FLOWERING LOCUS T (FT)* promoter based on the sgRNA 2.0 scaffold strategy [44]. However, these dCas9-based epigenome editors generally lead to global off-target effects by leaving epigenetic modification footprints [14••,43,46,47]. Future research is needed to improve targeting specificity of these epigenome editors.

Conclusions and future perspectives

In this review we have summarized the adoption of the CRISPR/dCas systems for transcriptional and epigenetic regulation, allowing for precise modification of gene expression and epigenetic marks. Importantly, the CRISPR/dCas platforms offer a simple method for manipulating the expression of multiple genes and is, thus, important for rewiring metabolic engineering and producing valuable metabolites in plants (Figure 4a). Furthermore, the CRISPR/dCas platforms, so far demonstrated in mammalian cells, enable genome-wide screening [48–50], which could allow for high-throughput screen of key genes controlling plant development and environmental stress responses (Figure 4b). Future efforts towards improvement of the repression and activation efficiency as well as the specificity of epigenetic manipulation are expected. The development of inducible and cell/tissue-type-specific CRISPR/dCas tools will further broaden the application scope of the CRISPR/dCas systems in plants.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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