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A potent Cas9-derived gene activator for plant and mammalian cells

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Abstract

Overexpression of complementary DNA (cDNA) represents the most commonly used gain-of-function approach for interrogating gene functions and for manipulating biological traits. However, this approach is challenging and inefficient for multigene expression due to increased labor for cloning, limited vector capacity, requirement of multiple promoters and terminators, and variable transgene expression levels. Synthetic transcriptional activators provide a promising alternative strategy for gene activation by tethering an autonomous transcription activation domain (TAD) to an intended gene promoter at endogenous genomic locus through a programmable DNA-binding module. Among the known custom DNA-binding modules, the nuclease-dead *Streptococcus pyogenes* Cas9 (dCas9) protein, which recognizes a specific DNA target through base pairing between a synthetic guide RNA (sgRNA) and DNA, outperforms zinc finger proteins (ZFPs) and transcription activator-like effectors (TALEs), both of which target through protein-DNA interactions[1]. Recently, three potent dCas9-based transcriptional activation systems, namely VPR, SAM, and Suntag, have been developed for animal cells[2–6]. However, an efficient dCas9-

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Zhenxiang Li and Dandan Zhang contributed equally to this work.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

AUTHOR CONTRIBUTIONS
J.F.L. and J.S. conceived the study. J.F.L. designed the experiments and supervised the study. D.Z. conducted the protoplast-based screens of dCas9 activators. Z.L. conducted other dCas9-TV experiments in Arabidopsis protoplasts and transgenic plants. X.X. and Z.L. conducted the dCas9-TV experiments in rice protoplasts. B.Y. conducted the dCas9-TV experiments in human HEK293T cells. Z.L., X.X. and W.X. performed the RNP-mediated gene activation. J.F.L. wrote the manuscript with input from J.S. and all other authors.

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based transcriptional activation platform is still lacking for plant cells[7–9]. Here, we developed a new potent dCas9-TAD named dCas9-TV through plant cell-based screens, which confers far stronger transcriptional activation of a single or multiple target genes than the routinely used dCas9-VP64 activator in both plant and mammalian cells.

Among synthetic gene activators, dCas9-TADs potentially offer unparalleled simplicity and multiplexability compared to ZFP-TADs and TALE-TADs because sgRNAs can be easily modified to achieve new targeting specificities and dCas9 guided by multiple sgRNAs can simultaneously bind to several different target loci[10]. However, a dCas9 fusion with VP64, a frequently used TAD[11], only weakly activates target gene using a single sgRNA in plant and mammalian cells[7–9,12–15]. Using Arabidopsis protoplast-based promoter-luciferase (LUC) assays, we confirmed that dCas9-VP64 with a single sgRNA only weakly (maximally 2.4-fold) or ineffectively activated target genes (Supplementary Results; Supplementary Figs. 1 and 2). Interestingly, when the target sequence lacks a 5′ G, an extra G appended to the 5′ end of the sgRNA was found to enhance the promoter activation (Supplementary Fig. 1), presumably by promoting the transcription initiation of the sgRNA by the *U6* promoter. Therefore, we routinely add a G to the 5′ end of sgRNAs when the target sequences start with a non-G nucleotide.

Although multiple sgRNAs tiling the proximal promoter of the target gene can synergistically boost the dCas9-VP64-mediated gene activation[7–9,12–15], this strategy reduces the scalability of the system[2] and may increase the risk of dCas9-mediated transcriptional perturbation at off-target non-promoter loci[15–17]. Therefore, we sought to devise and screen for an improved dCas9-TAD (Fig. 1a) that would allow potent transcriptional activation of target genes using only a single sgRNA, thus maximizing the system's scalability. As the first step, we modified dCas9-VP64 by adding additional VP64 moieties (Supplementary Sequences). By using a *WRKY30* promoter-LUC reporter and a pre-screened optimal sgRNA (WRKY30#2, Supplementary Fig. 2a), we observed that dCas9-VP128 outperformed dCas9-VP64 by increasing the *LUC* activation from 2-fold to more than 5-fold relative to the basal level (Fig. 1b). In contrast, dCas9-VP192 and dCas9-VP256 exhibited a sharp decrease of protein production and severe protein degradation, leading to an overall reduced *LUC* activation (Fig. 1b). The fact that VP192 and VP256 correspond to 12 and 16 repeats of the VP16 motif[11], respectively, suggests that their expression and stability issues may be incurred by highly repetitive sequences.

As a second step to enhance the activity of dCas9-VP128, we incorporated other sequence-unrelated portable TADs, which included plant-specific EDLL[18] and ERF2m[19] (modified ERF2) motifs as well as a TAD from *Xanthamonas* TALEs[20]. To minimize potential interference between different TADs, flexible GGSGG linkers were used as spacers between TADs (Supplementary Sequences). We observed that the combination of VP128 with up to four copies of tandem ERF2m-EDLL motifs (thereafter simplified as EE) activated *LUC* expression by 12.6-fold relative to the basal level (Fig. 1c), and the combination of VP128 with up to six copies of the TALE TAD motif (thereafter simplified as TAL) exhibited a maximal activation of *LUC* expression by more than 55-fold (Fig. 1c). Of note, further insertion of TAL motifs triggered severe protein degradation presumably due to the high

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sequence repetition, resulting in an overall decreased *LUC* induction (Fig. 1c). Therefore, the cell-based screens identified dCas9-6TAL-VP128 as a potentially strong transcription activator, which was renamed dCas9-TV for simplicity. In addition, when we used an Arabidopsis *U6-26* rather than *U6-1* promoter to express the sgRNA-WRKY30#2, we detected a further boost of *LUC* expression to 201-fold relative to the basal level (Supplementary Fig. 3). Therefore, the *U6-26* promoter was used for sgRNA expression in the following experiments in Arabidopsis. To validate the data from cell-based assays, we generated transgenic Arabidopsis plants to co-express the sgRNA-WRKY30#2 along with dCas9-TV or dCas9-VP64. As quantified by RT-qPCR, the expression of endogenous *WRKY30* was strongly induced by dCas9-TV by 48- and 139-fold, respectively, in two independent T1 transgenic lines (Fig. 1d), whereas negligible *WRKY30* activation by dCas9-VP64 was observed in representative transgenic lines (Fig. 1d).

No Cpf1-derived gene activator has so far been explored in plant or mammalian cells. Therefore, we also evaluated the transcriptional activation activity of a nuclease-dead *Acidaminococcus sp. BV3L6* Cpf1 (dCpf1)-TV fusion (Supplementary Results), since this dCpf1 has been recently reported to outperform its homologs in targeted gene suppression[21]. When dCpf1-TV and dCas9-TV were designed to target overlapping or proximal sequences within the *WRKY30* promoter, we only detected minimal activation (maximally 4.7-fold) of the promoter by dCpf1-TV in contrast to a strong activation by dCas9-TV (about 215-fold, Supplementary Fig. 4). Although we cannot exclude the possibility that other dCpf1 homolog may be suitable for targeted transcriptional activation, we focused the following study only on dCas9-TV.

To generalize our finding about the potency of dCas9-TV, we pre-screened a single sgRNA to target each promoter of six more Arabidopsis genes (Supplementary Fig. 5), and then compared the transcriptional activation efficiencies of dCas9-TV and dCas9-VP64 side by side using cell-based promoter-LUC assays. Induction of *LUC* expression by dCas9-TV was detected for all six genes from 1.6- to 92-fold, whereas dCas9-VP64 only conferred marginal transcriptional upregulation or even suppression of target gene expression (Supplementary Fig. 6). Interestingly, the magnitudes of gene activation by dCas9-TV were roughly negatively correlated with the basal expression levels of these genes, as genes with weak basal expression (Supplementary Fig. 7), such as *RLP23*, *WRKY30* and *CDG1*, tended to be better induced than those already under vigorous transcription, such as *FLS2*, *EFR*, *AVP1* and *HDC1*. Similar trends have also been documented for other strong dCas9 activators (e.g., VPR and SAM) in mammalian cells[2,5,6], indicating the generality of this phenomenon. We further validated the contrasting transcriptional activation efficiencies between dCas9-TV and dCas9-VP64 by RT-qPCR on the endogenous expression of *RLP23*. While dCas9-TV activated *RLP23* expression by 44-fold in protoplasts (Fig. 1e), dCas9-VP64 slightly suppressed *RLP23* expression (Fig. 1e). Moreover, we generated transgenic Arabidopsis plants co-expressing sgRNA-RLP23 and dCas9-TV or dCas9-VP64. As quantified by RT-qPCR, multiple T2 transgenic lines co-expressing the sgRNA and dCas9-TV exhibited robust induction of endogenous *RLP23* by over 30-fold (Fig. 1f). In contrast, several T2 transgenic lines co-expressing the sgRNA and dCas9-VP64 showed compromised *RLP23* expression (Fig. 1f), consistent with what has been observed in protoplasts (Fig. 1e).

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Arabidopsis *RLP23* encodes a cell-surface immune receptor that perceives nlp20, a conserved eliciting peptide across bacteria, fungi, and oomycetes[22]. Overexpression of *RLP23* could sensitize plant cells to a low concentration of nlp20 (Supplementary Fig. 8), which otherwise cannot be efficiently detected due to the low basal expression level of *RLP23* (Supplementary Fig. 7). We found that those T2 transgenic plants with dCas9-TV-mediated *RLP23* activation all exhibited enhanced immune responses to nlp20, as exemplified by the elevated MAPK activation (Fig. 1g) and reactive oxygen species (ROS) burst (Fig. 1h). These results highlighted the competence of the dCas9-TV/single sgRNA system as a tool to rewire cellular responses.

A critical concern for a targeted gene activation system is its specificity. Because *RLP23* encodes an Arabidopsis immune receptor that is only activated upon binding to its ligand nlp20, we reasoned that dCas9-TV-mediated transcriptional upregulation of *RLP23* in cells without nlp20 stimulation should provoke minimal secondary transcriptional perturbation to confound the specificity analysis. Therefore, we assessed the genome-wide transcriptional activation specificity of *RLP23* by dCas9-TV in protoplasts using RNA-sequencing (RNA-seq). Very similar transcriptome profiles were detected between samples expressing or not expressing dCas9-TV and sgRNA-RLP23 (Pearson's correlation coefficient 0.993; Fig. 1i), suggesting that gene expression at the whole genome level is not broadly influenced by dCas9-TV. In addition, none of the six Arabidopsis genes with potential off-target binding sites (Supplementary Table 1) showed altered gene expression. However, as revealed by RNA-seq (Supplementary Database), whereas *RLP23* was induced by dCas9-TV/sgRNA-RLP23 by 129-fold as expected, five other genes also exhibited increased expression (> 6-fold), which might be due to the secondary effect of *RLP23* upregulation since no predictable binding sites of sgRNA-RLP23 with up to five mismatches could be identified within the 2 kb promoters of these genes (data not shown).

We also evaluated the multiplexability of our transcriptional activation system in cell-based assays by co-expressing dCas9-TV with three sgRNAs targeting to *WRKY30*, *RLP23* and *CDG1*. As quantified by RT-qPCR, the endogenous gene expression was induced by 80-fold for *WRKY30*, 37-fold for *RLP23*, and 192-fold for *CDG1* (Fig. 1j). These results demonstrated the robust multiplexability of the dCas9-TV system.

To further strengthen the dCas9-TV-based transcriptional activation, we adopted a modified sgRNA (thereafter referred to as the SAM sgRNA) that is able to recruit a chimeric TAD (Fig. 2a) consisting of the MS2 phage coat protein (MCP) and 4EE-VP128 or TV through internally embedded MCP-binding aptamers[2]. When using the SAM sgRNA-WRKY30#2 to target the *WRKY30* promoter, dCas9-VP64 paired with either MCP-4EE-VP128 or MCP-TV resulted in much stronger induction of *WRKY30-LUC* than the combination of dCas9-VP64 and a normal sgRNA (Supplementary Fig. 9), suggesting that MCP-4EE-VP128 and MCP-TV are both active transcription activators upon association with the target promoter. Notably, the combination of dCas9-TV and a normal sgRNA was still more active than that of dCas9-VP64, SAM sgRNA and MCP-TV (Supplementary Fig. 9). We reasoned that the combination of dCas9-TV with the SAM sgRNA and MCP-4EE-VP128 or MCP-TV may lead to additive or synergistic activation of the target gene. However, this three-component activation system failed to enhance the *WRKY30-LUC* induction and even led to decreased

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RLP23-LUC induction compared to the two-component system of dCas9-TV and normal sgRNAs (Fig. 2b; Supplementary Fig. 10). These results suggested that MCP-TADs piggybacked on the SAM sgRNA are unable to strengthen the dCas9-TV-mediated transcriptional activation in plant cells, presumably due to the saturation of local transcriptional machineries or steric incompatibility between MCP-TAD and dCas9-TV. Interestingly, a recent similar endeavor to upgrade the transcriptional activation of target gene by combining the robust dCas9-VPR activator with the SAM-based transcriptional activation strategy was also unsuccessful in mammalian cells[6].

In addition to Arabidopsis cells, which represent cells of dicotyledon species, we also investigated the transcriptional activation induced by dCas9-TV in cells of monocot species, such as rice. We targeted the proximal *GW7* or *ER1* promoter in rice cells using a single pre-screened sgRNA expressed by the rice *U6a* promoter (Supplementary Fig. 11), and found that dCas9-TV induced the target promoter activity two orders of magnitude stronger than dCas9-VP64 (Figs. 2c and 2d). Moreover, concurrent expression of both sgRNAs along with dCas9-TV in rice cells led to simultaneous induction of endogenous *GW7* by 79-fold and *ER1* by 62-fold (Fig. 2e), as quantified by RT-qPCR. By contrast, dCas9-VP64 only modestly activated *GW7* by 2.7-fold and suppressed *ER1* expression (Fig. 2e). These results suggested that dCas9-TV is broadly effective for targeted gene activation in various types of plant cells. Furthermore, since TAL is known as an active TAD in yeast[20], we speculated that the dCas9-TV-mediated transcriptional activation may also work for mammalian cells, which are distantly related to Arabidopsis and rice cells. Indeed, when we targeted *ASCL1* or *OCT4* in human embryonic kidney (HEK) 293T cells with a single sgRNA[2,23], we detected induction of endogenous *ASCL1* by 46-fold and *OCT4* by 14.6-fold, whereas dCas9-VP64 failed to enhance the expression of either gene (Fig. 2f).

Lastly, we explored the possibility of targeted gene activation in plant cells by dCas9-TV ribonucleoprotein (RNP) complexes. To this end, purified *E. coli*-expressed dCas9-TV and *in vitro* transcribed sgRNA (Supplementary Fig. 12) targeting to Arabidopsis *WRKY30* or *RLP23* or rice *ER1* were pre-assembled into RNP complexes and then delivered into Arabidopsis or rice protoplasts through polyethylene glycol (PEG)-mediated transfection. As quantified by RT-qPCR, induction of *WRKY30* by 11.7-fold, *RLP23* by 9.3-fold and *ER1* by 13-fold was detected at 5 h after transfection (Fig. 2g). The proof-of-concept of dCas9-TV RNP-mediated transcriptional activation promises a possible DNA-free strategy to achieve short bursts of gene activation with minimal cloning efforts, and leverages the easy availability of recombinant dCas9-TV proteins, synthetic sgRNAs and plant protoplasts[24].

In summary, dCas9-TV exhibits potent transcriptional activation activity in plant and mammalian cells, which can be utilized to efficiently and specifically activate single or multiple genes when these genes have modest basal expression levels. We envision that dCas9-TV will be particularly useful in basic and applied plant research, including but not limited to: (i) when combined with a genome-scale sgRNA library, dCas9-TV can be used in protoplast-based gain-of-function screens for regulatory genes in a signaling pathway of interest using a promoter-LUC/GFP reporter as a signaling readout; (ii) it is useful for generating a synthetic plant transcriptome[25] to study the functions of transcription

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regulators; (iii) it can be applied in metabolic engineering by multiplex activation of lowly expressed enzymes throughout a metabolic pathway to increase the production of valuable metabolites; (iv) it can also be applied to upregulate crop genes conferring beneficial traits such as biotic and abiotic resistances. Future efforts will be needed to improve the rational design of sgRNAs, as different sgRNAs targeting the same promoter can lead to variable transcriptional regulation by the same dCas9 activators[2,7,9,12–17] (Supplementary Figs. 2, 5 and 11), and a straightforward correlation between the parameters of sgRNAs (e.g., GC content or target site location, Supplementary Table 2) and their gene activation efficiencies has not been established. Meanwhile, since dCas9-TV is prone to protein degradation due to its high sequence repetition, future studies will address the possibility whether genetic fusion of XTEN[26], 30Kc19α[27] or other protein-stabilizing polypeptides to the N- or C-terminus of dCas9-TV may mitigate its degradation issue and further potentiate the dCas9-TV-mediated transcriptional activation.

METHODS

Methods and any associated references are available in the Supplementary Information in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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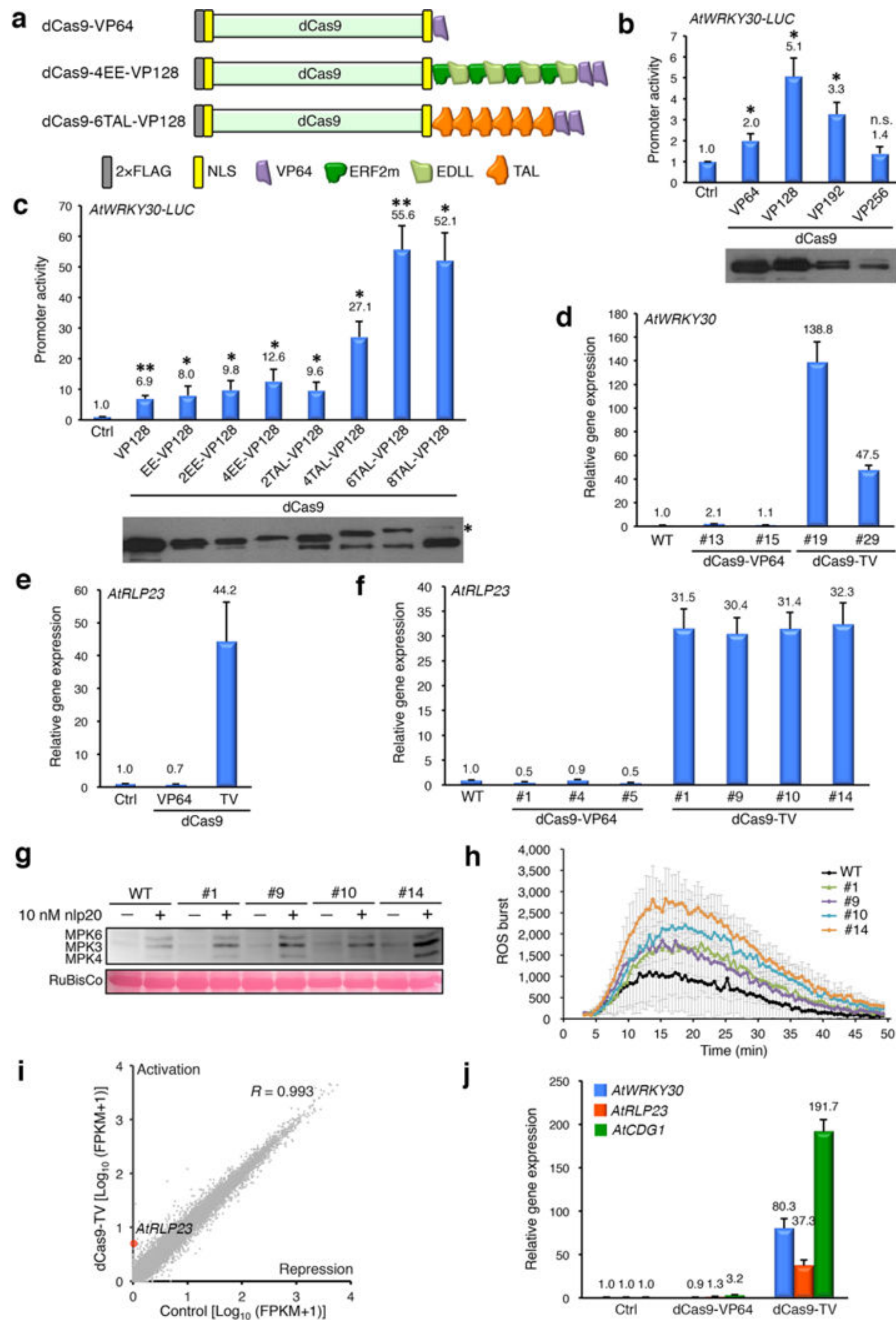

Figure 1.

dCas9-TV-mediated gene activation in Arabidopsis. (**a**) Diagram of three representative dCas9 gene activators tested in this study. (**b**) dCas9-VP128 exhibits the highest activity among dCas9-$(VP64)_n$ (n=1-4) activators in Arabidopsis protoplasts. dCas9-VP256 is marked by an asterisk in the blot. (**c**) dCas9-6TAL-VP128 (dCas9-TV) exhibits the highest activity among all screened dCas9 activators in Arabidopsis protoplasts. dCas9-8TAL-VP128 is marked by an asterisk in the blot. (**d**) Representative transgenic Arabidopsis T1 plants co-expressing dCas9-TV and sgRNA-WRKY30 show strong induction of endogenous

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WRKY30. (**e, f**) Arabidopsis protoplasts (**e**) or representative transgenic T2 plants (**f**) co-expressing dCas9-TV and sgRNA-RLP23 show robust induction of endogenous *RLP23*. (**g, h**) Arabidopsis transgenic T2 plants with *RLP23* induction accordingly exhibit enhanced MAPK activation (**g**) and ROS production (**h**) in response to the pathogen elicitor nlp20. RuBisCo in (**g**) indicates equal protein loading. (**i**) Evaluation of dCas9-TV specificity by RNA-seq. Arabidopsis protoplasts expressing or not expressing dCas9-TV and sgRNA-RLP23 exhibit very similar transcriptome profiles. *R* indicates Pearson's correlation coefficient. *RLP23* is marked by a red dot close to the y-axis as a highly activated gene. (**j**) Co-expression of dCas9-TV and 3 sgRNAs leads to simultaneous induction of *WRKY30*, *RLP23* and *CDG1* in Arabidopsis protoplasts. Empty vectors were used in Control (Ctrl) samples to replace constructs expressing dCas9 activator and sgRNAs. Gene activation was determined by promoter-LUC assays (**b**, **c**) or RT-qPCR (**d**-**f**, **j**), and data are shown as mean (indicated with a number on the top) ± sd (n=3). *$P < 0.05$, **$P < 0.01$ (student's *t*-test). n.s., not significant.

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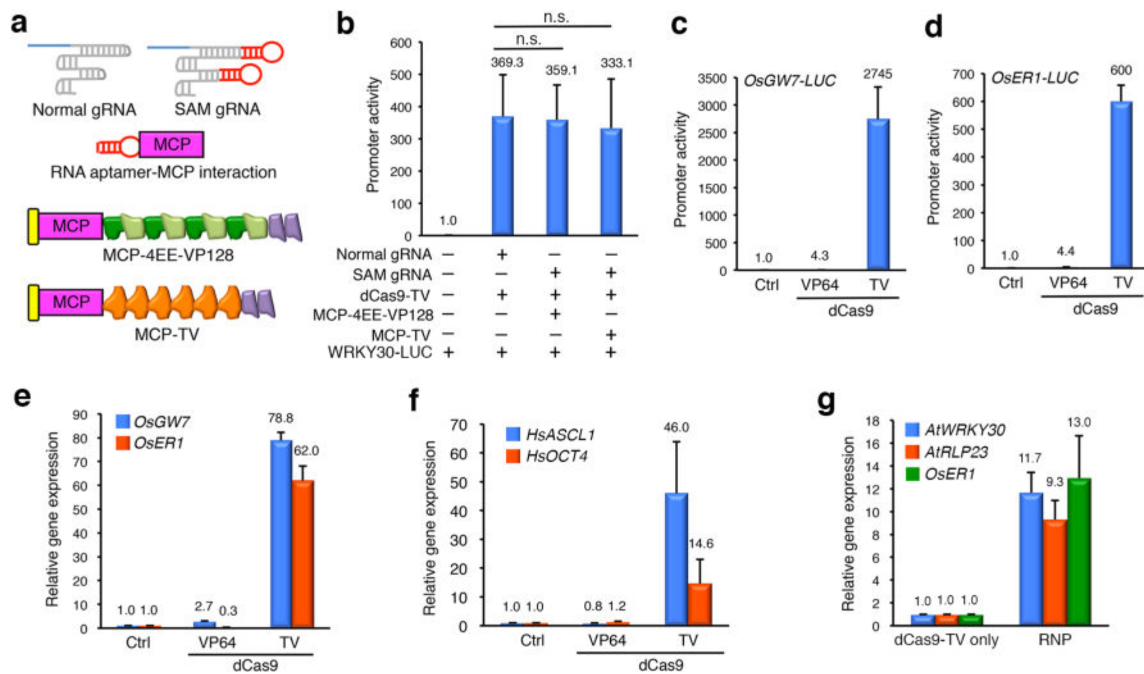

Figure 2.

Modified strategies of dCas9-TV-mediated gene activation in Arabidopsis and other cells. (**a**) Diagram of the SAM sgRNA, MCP activators and their interactions. (**b**) SAM sgRNA-associated MCP activators fail to strengthen dCas9-TV-mediated *WRKY30* activation in Arabidopsis protoplasts. (**c**, **d**) Rice protoplasts co-expressing dCas9-TV and sgRNAs targeting to *GW7* (**c**) or *ER1* (**d**) exhibit strong target gene induction. (**e**) Co-expression of dCas9-TV and 2 sgRNAs leads to simultaneous induction of *GW7* and *ER1* in rice protoplasts. (**f**) HEK 293T cells co-expressing dCas9-TV and sgRNAs targeting to *ASCL1* or *OCT4* exhibit clear induction of these genes. (**g**) dCas9-TV ribonucleoprotein (RNP)-mediated activation of *WRKY30* and *RLP23* in Arabidopsis protoplasts and *ER1* in rice protoplasts. Empty vectors were used in Control (Ctrl) samples to replace constructs expressing dCas9 activator and sgRNAs. Gene activation was determined by promoter-LUC assays (**b-d**) or RT-qPCR (**e-g**), and data are shown as mean (indicated by a number on the top) ± sd (n=3). n.s., not significant.