LETTERS
https://doi.org/10.1038/s41477-017-0046-0
nature plants

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

Zhenxiang Li[1], Dandan Zhang[1], Xiangyu Xiong[1], Bingyu Yan[1], Wei Xie[1,2], Jen Sheen[3] and Jian-Feng Li[1,2,4*]

Overexpression of complementary DNA 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 labour 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 the 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 and DNA, outperforms zinc-finger proteins and transcription activator-like effectors, 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-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. dCas9–TV confers far stronger transcriptional activation of 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 with zinc-finger protein–TADs and transcription activator-like effector (TALE)–TADs because synthetic guide RNAs (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 genes 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 and 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 twofold to more than fivefold 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 (hereafter referred to 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 (hereafter referred to 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 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

[1]Key Laboratory of Gene Engineering of Ministry of Education, State Key Laboratory of Biocontrol, Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou, China. [2]Guangzhou Key Laboratory of Healthy Aging Research, School of Life Sciences, Sun Yat-sen University, Guangzhou, China. [3]Department of Molecular Biology and Centre for Computational and Integrative Biology, Massachusetts General Hospital, and Department of Genetics, Harvard Medical School, Boston, MA, USA. [4]Collaborative Innovation Center of Genetics and Development, School of Life Sciences, Sun Yat-sen University, Guangzhou, China. Zhenxiang Li and Dandan Zhang contributed equally to this work.
*e-mail: lijfeng3@mail.sysu.edu.cn

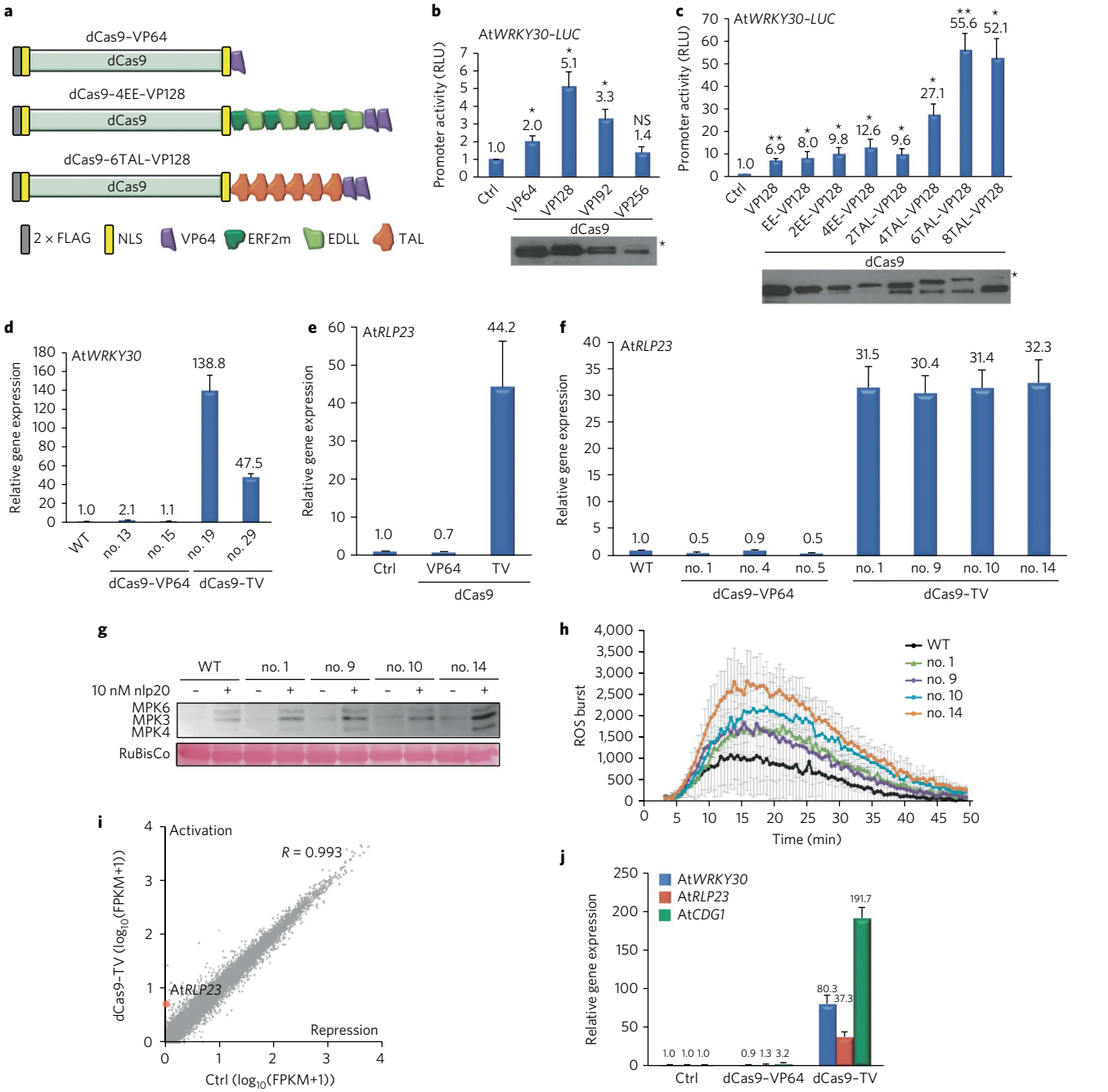

Fig. 1 | dCas9-TV-mediated gene activation in *Arabidopsis* **a**, Diagram of three representative dCas9 gene activators tested in this study. FLAG, FLAG tag; NLS, nuclear localization signal. **b**, dCas9-VP128 exhibits the highest activity among dCas9-$(VP64)_n$ ($n=1$–4) activators in *Arabidopsis* 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 *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 reactive oxygen species (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. At*RLP23* is marked by a red dot close to the *y* axis as a highly activated gene. **j**, Co-expression of dCas9-TV and three 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 above the bar) ± s.d. ($n=3$). $^*P<0.05$, $^{**}P<0.01$ (Student's *t*-test). NS, not significant; WT, wild type; RLU, relative luciferase unit; At, *Arabidopsis thaliana*.

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 reverse

transcription and quantitative PCR (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), because this dCpf1 has been recently reported to outperform its homologues in targeted gene suppression[21]. When dCpf1–TV and dCas9–TV were designed to target overlapping or proximal sequences within the *WRKY30* promoter, we detected only 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 homologues 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 conferred only 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 (such as 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).

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 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. As *RLP23* encodes an *Arabidopsis* immune receptor that is activated only following 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 (more than six-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-kilobase 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 *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 (hereafter referred to as the SAM sgRNA) that is able to recruit a chimaeric 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 following 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 *RLP23–LUC* induction compared with the two-component system of dCas9–TV and normal sgRNAs (Fig. 2b and 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 endeavour to upgrade the transcriptional activation of target genes 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 (Fig. 2,d). 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 cell. Furthermore, since TAL is known as an active TAD in yeast[20], we

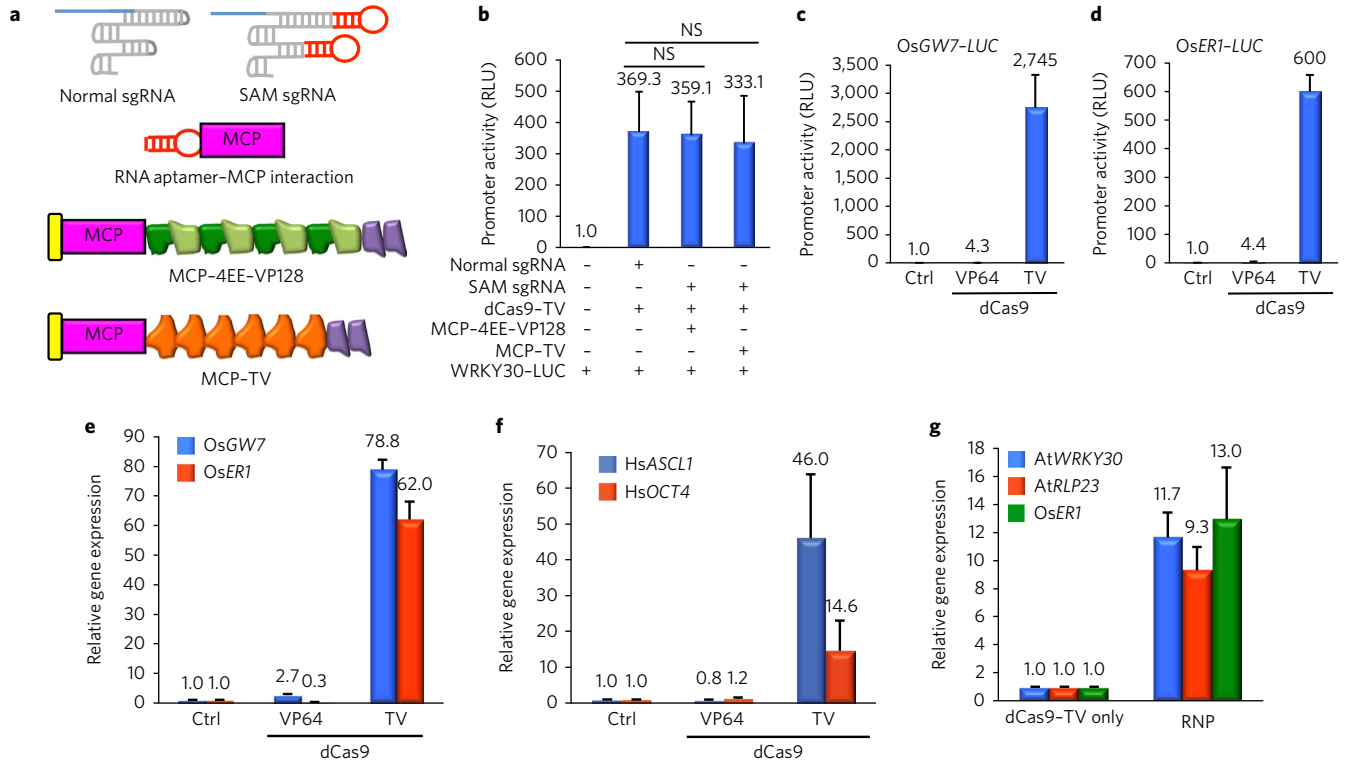

Fig. 2 | Modified strategies of dCas9–TV-mediated gene activation in *Arabidopsis* and other cells. a, Diagram of the SAM sgRNA, MCP activators and their interactions. **b**, SAM sgRNA-associated MCP activators fail to strengthen dCas9–TV-mediated *WRKY30* activation in *Arabidopsis* protoplasts. **c**,**d**, Rice protoplasts co-expressing dCas9–TV and sgRNAs targeting *GW7* (**c**) or *ER1* (**d**) exhibit strong target gene induction. **e**, Co-expression of dCas9–TV and two sgRNAs leads to simultaneous induction of *GW7* and *ER1* in rice protoplasts. **f**, HEK 293T cells co-expressing dCas9–TV and sgRNAs targeting *ASCL1* or *OCT4* exhibit clear induction of these genes. **g**, dCas9–TV 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 above the bar) ± s.d. ($n = 3$). NS, not significant; Os, *Oryza sativa*; Hs, *Homo sapiens*.

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 *Escherichia coli*-expressed dCas9–TV and in vitro transcribed sgRNA (Supplementary Fig. 12) targeting *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: 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 signalling pathway of interest using a promoter–LUC/GFP reporter as a signalling readout; it is useful for generating a synthetic plant transcriptome[25] to study the functions of transcription regulators; 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; 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 (for example, GC content or target site location; Supplementary Table 2) and their gene activation efficiencies has not been established. Meanwhile, as dCas9–TV is prone to protein degradation due to its high sequence repetition, future studies will address the possibility of whether genetic fusion of XTEN[26], 30Kc19α[27] or other protein-stabilizing polypeptides to the amino or carboxy terminus of dCas9–TV may mitigate its degradation issue and further potentiate the dCas9–TV-mediated transcriptional activation.

Methods

Plant growth. Wild-type *Arabidopsis thaliana* Col-0 plants were grown on Jiffy soil (Jiffy Group, Netherlands) in a plant growth room under photoperiods of 12 h light (75 μmol m^{-2} s^{-1}) at 23 °C and 12 h dark at 21 °C with a constant humidity of 65%. Wild-type Zhonghua 11 rice (*Oryza sativa*) plants were grown on Jiffy soil in a plant growth chamber under photoperiods of 12 h light (200 μmol m^{-2} s^{-1}) at 30 °C and 12 h dark at 27 °C with a constant humidity of 70%.

Plasmid construction. Routine molecular cloning procedures were followed for plasmid construction. To express the nuclease dead Cas9 (dCas9) in protoplasts, two point mutations (D10A and H840A) were introduced into *pcoCas9* (*plant codon-optimized SpCas9*) by PCR-based mutagenesis using the HBT-pcoCas9 plasmid[28] as template. The VP64 coding sequence was fused to the 3' end of *dCas9* by PCR as part of a mega-primer to obtain the HBT-dCas9-VP64 plasmid and an *Avr*II restriction site was introduced between *dCas9* and *VP64*. To express the dCas9 fusion with increasing copies of VP64, the VP64 coding sequence was synthesized as a gBlock dsDNA (Integrated DNA Technologies, USA) flanked by *Avr*II and *Nhe*I sites. Taking advantage of the compatibility between the DNA overhangs after *Avr*II and *Nhe*I digestion and the disruption of both restriction sites after ligation, the *Avr*II/*Nhe*I-digested *VP64* gBlock fragment could be sequentially inserted into the *Avr*II site of HBT-dCas9-VP64 to create HBT-dCas9-VP64n ($n = 2$, 3, or 4), where a single *Avr*II site could always be maintained at the junction between *dCas9* and (*VP64*)n. An EE (ERF2m-EDLL) coding sequence or a double-TAL (2TAL) coding sequence was also synthesized as a gBlock dsDNA flanked by *Avr*II and *Nhe*I sites, and was digested and sequentially inserted between *dCas9* and *VP128* to desired copy numbers. To express dCas9-TV (dCas9-6TAL-VP128) or dCas9-VP64 *in planta*, the expression cassette of *35SPPDK:dCas9-TV:NOS* or *35SPPDK:dCas9-VP64:NOS* was amplified by PCR and inserted into the *Stu*I site of the pFGC-RCS binary plasmid[29] to obtain pFGC-dCas9-TV and pFGC-dCas9-VP64, respectively. For sgRNA expression in Arabidopsis protoplasts, the *U6-1:sgRNA:TTTTTTT* or *U6-26:sgRNA:TTTTTTT* expression cassette was assembled by overlapping PCR as previously described[28], and was inserted into the *Sac*I site of the pUC119-RCS plasmid[29]. The *U6-26:sgRNA(WRKY30 #2):TTTTTTT* expression cassette was cut out from pUC119 by *Asc*I digestion and was inserted into the *Asc*I site of pFGC-dCas9-TV or pFGC-dCas9-VP64. For sgRNA expression in rice protoplasts, the *OsU6a:sgRNA: TTTTTTT* expression cassette was assembled by overlapping PCR and was inserted into the *Kpn*I and *Hind*III window of pUC119-RCS. To co-express sgRNAs targeting *ER1* and *GW7* in rice protoplasts, the *OsU6a:sgRNA GW7:TTTTTTT* and *OsU6b:sgRNA ER1:TTTTTTT* expression cassettes were inserted into the same pUC119-RCS plasmid at *Kpn*I/*Hind*III and *Sac*I sites, respectively.

HBT-dCpf1-TV was constructed by replacing the *Nco*I and *Avr*II fragment of *dCas9* with the synthesized plant codon-optimized *dCpf1* fragment (IGE Biotechnology LTD, China) digested with the same enzymes. To express crRNA for dCpf1, three strategies[21,30,31] were tested: a mega-primer containing the sequence of mature crRNA was used to amplify the *U6-26* promoter to directly generate a *U6-26:crRNA:TTTTTTT* expression cassette by PCR, and the resultant PCR product was cloned into *Sac*I site of the pUC119 plasmid; the pre-crRNA[31] and HH-crRNA-HDV[21] sequences were obtained by gene synthesis at IGE Biotechnology LTD (China). The *U6-26:pre-crRNA:TTTTTTT* expression cassette was assembled by overlapping PCR, and was inserted into pUC119 using *Kpn*I and *Pst*I. The synthesized HH-crRNA-HDV fragment was digested by *Sma*I and *Pst*I, and was ligated to the *AtUBQ10* promoter in a HBT plasmid to generate the *AtUBQ10:HH-crRNA-HDV:NOS* expression cassette.

To express the SAM sgRNA, the normal sgRNA plus two MCP-binding RNA aptamers[2] was synthesized as a gBlock dsDNA and was used to replace the normal sgRNA in overlapping PCR to assemble the *U6:SAM sgRNA:TTTTTTT* expression cassette. The *MCP* gene (encoding nuclear-localized MCP) was synthesized at IGE Biotechnology LTD, digested by *Bam*HI and *Pst*I, and inserted into the *Bam*HI/*Pst*I window of the HBT vector. The TV or 4EE-VP128 coding sequence was cut out by *Avr*II and *Pst*I from HBT-dCas9-TV and HBT-dCas9-4EE-VP128, respectively, and was inserted into the *Avr*II/*Pst*I window of the HBT-MCP plasmid to obtain HBT-MCP-TV and HBT-MCP-4EE-VP128 plasmids. For experiments in human cells, the TV or VP64 coding sequence was PCR amplified, digested by *Avr*II and *Xba*I, and was inserted into the *Xba*I-digested pcDNA3.1-dCas9 plasmid (Addgene plasmid #47106). The human *U6:sgRNA:TTTTTTT* expression cassette was assembled by overlapping PCR as described above. All recombinant plasmids (Supplementary Table 3) were subjected to DNA sequencing for sequence verification.

Protoplast isolation and transfection. Four-week-old Arabidopsis or ten-day-old rice plants were used for protoplast isolation according to the procedure as previously described[24,32]. Briefly, Arabidopsis leaves or rice seedlings were cut into 0.5 mm strips and were digested in enzyme solution containing 1.5% Cellulase R10, 0.4% macerozyme R10, 0.4 M mannitol, 20 mM MES (pH5.7), 20 mM KCl, 10 mM $CaCl_2$ and 0.1% BSA for 3 h. After mixing with 10 ml W5 solution containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES (pH 5.7), the digestion mixture was filtered through FALCON cell strainer. Cells were collected by centrifugation for 2 min at 100 *g* for Arabidopsis or at 150 *g* for rice, and were resuspended in 10 ml W5 solution and rested on ice for 30 min. After centrifugation for 1 min, the cell pellet was resuspended in the MMg solution containing 0.4 M mannitol, 15 mM $MgCl_2$ and 4 mM MES (pH 5.7) to a concentration of 2×10^5 cells per ml. DNA transfection was carried out in a 2 ml round-bottom microcentrifuge tube, where 200 µl protoplasts were mixed with 20 µl DNA (2 µg/µl) and 220 µl PEG solution containing 40% PEG4000 (v/v), 0.2 M mannitol and 0.1 M $CaCl_2$. The mixture was incubated at room temperature for 5 min for Arabidopsis or 15 min for rice before the transfection was quenched by adding 800 µl W5 solution. Transfected cells were harvested by centrifugation for 3 min at 100 *g* for Arabidopsis or at 150 *g* for rice and were incubated in 1 ml WI solution containing 0.5 M mannitol, 20 mM KCl and 4 mM MES (pH 5.7) in the dark for 12 h for dCas9-TAD and sgRNA expression.

Human cell transfection. HEK 293T cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin at 37 °C with 5% CO_2. For DNA transfection, 160,000 cells were seeded into 24-well plates the day before transfection. 500 ng of sgRNA-expressing plasmid, 500 ng of plasmid encoding dCas9-TV or dCas9-VP64 and 30 ng GFP-expressing plasmid were co-transfected with Lipofectamine LTX with PLUS (Thermo Fisher) according to the manufacturer's instruction.

LUC reporter assay. 200 µl Arabidopsis or rice protoplasts were transfected with a total of 21 µl DNA (2 µg/ µl) containing (1) 8 µl HBT-dCas9-TAD (or empty vector as control), 8 µl pUC119-sgRNA, 4 µl promoter-LUC reporter construct and 1 µl UBQ10-GUS (transfection internal control) or (2) 6 µl HBT-dCas9-TAD, 6 µl HBT-MCP-TAD, 6 µl pUC119-SAM sgRNA, 2 µl promoter-LUC reporter construct and 1 µl UBQ10::GUS. Cells were incubated in the dark for 12 h for gene expression and were harvested by centrifugation at 100 *g* for 2 min. Cells were lysed by adding 100 µl lysis buffer containing 25 mM Tris-HCl (pH 7.8), 2 mM DTT, 2 mM trans-1,2-diaminocyclohexane-N'N'N'N'-tetraacetic acid, 10% (v/v) glycerol and 1% (v/v) Triton X-100 and vortexing vigorously. The resultant lysate of 10 µl was mixed with 100 µl luciferase assay reagent containing 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM Coenzyme, 0.47 mM D-luciferin sodium salt, 0.53 mM ATP, pH 7.8, in a 96-well plate with white wells and black walls. Measurement of luminescence was conducted in a Varioskan LUX microplate reader (Thermo Scientific) with an integration time of 1 sec. In parallel, 10 µl protoplast lysate was added to 50 µl MUG reagent containing 10 mM Tris-HCl (pH 8.0), 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG), 2 mM $MgCl_2$ in a 96-well plate with black wells. The mixture was incubated at 37 °C for 30 min and the reaction was quenched by ice-water bathing. GUS activity was measured with the Varioskan LUX microplate reader. The promoter activity was quantified by dividing the LUC readout with the GUS readout of the same sample.

The effect of *RLP23* overexpression in Arabidopsis protoplasts was analyzed by co-transfecting 200 µl protoplasts with 36 µg (18 µl) of the HBT-RLP23-FLAG construct or empty vector, 4 µg (2 µl) of the *FRK1-LUC* reporter construct and 2 µg (1 µl) of the UBQ10-GUS plasmid. After incubation at room temperature for 12 h, protoplasts were treated with 10 nM nlp20 peptide (MoonBiochem) or mock for 4 h. The cells were harvested and the LUC reporter assay was carried out as described above.

Western blot. Protoplast lysate of 40 µl after the LUC assay was mixed with 10 µl 6×SDS loading buffer and was denatured at 95 °C for 5 min. Total proteins were resolved in an 8% SDS-PAGE gel and dCas9-TAD proteins were immunoblotted with anti-FLAG HRP-conjugated antibodies (Sigma-Aldrich) at 1: 10,000 dilution.

RNA extraction and RT-qPCR analysis. A total of 8×10^4 (400 µl) Arabidopsis or rice protoplasts co-transfected with 40 µg (20 µl) HBT-dCas9-TAD and 40 µg (20 µl) pUC119-sgRNA plasmids or 20-25 mg rosette leaves from 6-week-old Arabidopsis plants were used for RNA extraction. Total RNA was extracted from protoplasts or leaf tissues using RNAiso Plus reagent (TaKaRa) according to the manufacturer's instructions. DNA was removed by treatment with RNase-free DNase I (New England Biolabs). Total RNA of 1 µg was subjected to reverse transcription using the anchored-oligo$(dT)_{18}$ primer and Transcriptor First Strand cDNA Synthesis Kit (Roche). RT-qPCR was performed in LightCycler 96 Instrument (Roche) using FastStart Essential DNA Green Master (Roche). For RNA extraction from human HEK293T cells, total RNA was obtained using an RNeasy Plus RNA isolation kit (Qiagen) followed by RT-qPCR using a One Step SYBR PrimeScript RT-PCR kit (TaKaRa) in LightCycler 480 Instrument (Roche). Primers used for RT-qPCR are listed in the Supplementary Table 4. Targeted gene induction levels were normalized to the expression level of *AtACT2* (Arabidopsis), *OsACT1* (rice) or *HsGAPDH* (human), and fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Generation of transgenic plants. The recombinant pFGC binary plasmids were introduced into *Agrobacterium tumefaciens* GV3101 cells through electroporation, which were in turn used for floral dip-mediated Arabidopsis transformation[33]. Transgenic plants were selected on the soil soaked with 1: 1000 diluted Basta herbicides (Amersco) containing glufosinate ammonium.

MAPK activation assay. Protoplasts were isolated from 6-week-old wild-type or transgenic Arabidopsis plants expressing dCas9-TV and sgRNA RLP23. 6×10^4 (300 µl) protoplasts were pelleted, resuspended in 100 µl W5 solution and transferred to 1 ml WI solution in a 6-well plate. After resting at room temperature for 3 h, cells were treated with 10 nM nlp20 peptide or mock for 10 min. Cells were

collected and lysed by adding SDS-PAGE loading buffer and heated at 95 °C for 3 min. Total proteins were resolved in a 10% SDS-PAGE gel and western blot was conducted by using anti-phospho ERK antibodies (Cell Signaling Technology) as the primary antibodies and HRP-conjugated anti-rabbit antibodies (Cell Signaling Technology) as the secondary antibodies.

ROS burst assay. Leaf discs with a diameter of 5 mm were cut out with a punch from leaves of 6-week-old wild-type or transgenic Arabidopsis plants expressing dCas9-TV and sgRNA RLP23. Four pieces of leaf discs were taken from each plant for ROS burst detection using a luminol-HRP-based chemiluminescence assay. Briefly, each leaf disc was floated on 200 µl sterile water overnight in individual wells of a 96-well plate. On the next day, after removal of the liquid, 100 µl HRP-luminol reaction mixture containing HRP, luminol and 1 µM nlp20 was added into each well. ROS burst was immediately measured in a time-course manner using a Varioskan LUX microplate reader. Luminescence of each well was recorded one by one with an integration time of 1 sec for each reading. As a result, each well was measured every 30 sec for 100 times in total.

RNP complex preparation. His-tagged dCas9-TV proteins were expressed in *E. coli* and purified using the Ni-NTA affinity purification. Briefly, Rosetta cells (Transgen Biotech) harboring the dCas9-TV-6His plasmid were cultured in 2YT medium at 37 °C until OD600 reaches 0.6-0.8, and then incubated at 18 °C for 16 h after adding 0.2 mM IPTG. Cells were harvested and lysed by sonication in the Lysis buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, 20 mM imidazole and 10 mM Tris-HCl (pH 8.0). Lysate was cleared by centrifugation for 15 min at 4 °C at 7000 *g* and the supernatant was filtered through a 0.45 µm syringe-driven filter. The filtered supernatant was subjected to a nickel column (Transgen Biotech). The bound proteins were washed with the Washing buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, 50 mM imidazole and 10 mM Tris-HCl (pH 8.0) and eluted with the Elution buffer containing 300 mM NaCl, 50 mM NaH_2PO_4, 200 mM imidazole, 10 mM Tris-HCl (pH 8.0) and 10% (v/v) glycerol. Eluted proteins were dialyzed with the Cas9 Storage buffer containing 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT and 5% (v/v) glycerol and were concentrated using a 50 K centrifugal filter (Millipore). The final protein concentration was measured by the Bradford protein assay and was adjusted to 750 µg/ml. sgRNAs were synthesized by *in vitro* transcription. Briefly, transcription templates containing a T7 RNA polymerase promoter followed by a 20-nt guide sequence and 76-nt sgRNA scaffold were PCR amplified using the pUC119-sgRNA plasmid as template. The resultant PCR products were run on an agarose gel to confirm the correct amplicon size and were purified with an E.Z.N.A Gel Extraction Kit (OMEGA). *In vitro* transcription of sgRNAs was performed with 75 ng of purified PCR products as template using a HiScribe Quick T7 High Yield RNA Synthesis Kit (New England Biolabs). The synthesized sgRNAs were treated with RNase-free DNase I (New England Biolabs) to remove the template DNA and then purified with RNA Clean & Concentrator-25 (Zymo Research). Prior to the RNP complex formation, the size and integrity of *in vitro* transcribed sgRNAs were confirmed by electrophoresis in a denaturing urea-polyacrylamide gel, and the concentration of sgRNAs was determined by measuring UV absorbance at 260 nm in a Varioskan LUX microplate reader. To prepare the RNP complex, 30 µg (40 µl) dCas9-TV protein and 25 µg (10 µl) sgRNA or equal volume of nuclease-free water were mixed gently and incubated at room temperature for 10 min. The resulting RNP complex was immediately delivered into 8×10^4 (400 µl) Arabidopsis or rice protoplasts by PEG-mediated transfection and gene activation was conducted in the dark for 5 h.

RNA-seq analysis. Protoplasts expressing dCas9-TV/sgRNA RLP23 or empty vector in three biological repeats were pooled for RNA extraction with RNAiso Plus reagent (TaKaRa). A total amount of 3 µg RNA per sample was used for the RNA-seq library construction. RNA-seq and data analyses were carried out on the Illumina HiSeq X platform with 150 bp paired-end reads at Novogene (Beijing, China).

Statistical analysis. Three biological repeats were conducted for each experiment. Statistical significance between two samples was analyzed by two-tailed Student's *t* test, and asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$).

Life Sciences Reporting Summary. Futher information on experimental design is available in the Life Sciences Reporting Summary.

Data availability. The data generated or analysed during the current study are included in this published article (and its Supplementary Information) or are available from the corresponding author on reasonable request.

Received: 30 August 2017; Accepted: 11 October 2017;
Published online: 20 November 2017

References

1. Qi, L. S. et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173–1183 (2013).
2. Konermann, S. et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature* **517**, 583–588 (2015).
3. Tanenbaum, M. E., Gilbert, L. A., Qi, L. S., Weissman, J. S. & Vale, R. D. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. *Cell* **159**, 635–646 (2014).
4. Gilbert, L. A. et al. Genome-scale CRISPR-mediated control of gene repression and activation. *Cell* **159**, 647–661 (2014).
5. Chavez, A. et al. Highly efficient Cas9-mediated transcriptional programming. *Nat. Methods* **12**, 2–6 (2015).
6. Chavez, A. et al. Comparison of Cas9 activators in multiple species. *Nat. Methods* **13**, 563–567 (2016).
7. Piatek, A. et al. RNA-guided transcriptional regulation in planta via synthetic dCas9-based transcription factors. *Plant Biotechnol. J.* **13**, 578–589 (2015).
8. Lowder, L. G. et al. A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiol.* **169**, 971–985 (2015).
9. Vazquez-Vilar, M. et al. A modular toolbox for gRNA-Cas9 genome engineering in plants based on the GoldenBraid standard. *Plant Methods* **12**, 10 (2016).
10. Didovyk, A., Borek, B., Tsimring, L. & Hasty, J. Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications. *Curr. Opin. Biotechnol.* **40**, 177–184 (2016).
11. Beerli, R. R., Segal, D. J., Dreier, B. & Barbas, C. F. III 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 (1998).
12. Mali, P. et al. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* **31**, 833–838 (2013).
13. Perez-Pinera, P. et al. RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat. Methods* **10**, 973–976 (2013).
14. Maeder, M. L. et al. CRISPR RNA-guided activation of endogenous human genes. *Nat. Methods* **10**, 977–979 (2013).
15. Cheng, A. W. et al. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* **23**, 1163–1171 (2013).
16. Farzadfard, F., Perli, S. D. & Lu, T. K. Tunable and multifunctional eukaryotic transcription factors based on CRISPR/Cas. *ACS Synth. Biol.* **2**, 604–613 (2013).
17. Braun, C. J. et al. Versatile in vivo regulation of tumor phenotypes by dCas9-mediated transcriptional perturbation. *Proc. Natl Acad. Sci. USA* **113**, 3892–3900 (2016).
18. Tiwari, S. B. et al. The EDLL motif: a potent plant transcriptional activation domain from AP2/ERF transcription factors. *Plant J.* **70**, 855–865 (2012).
19. Li, J. et al. Activation domains for controlling plant gene expression using designed transcription factors. *Plant Biotechnol. J.* **11**, 671–680 (2013).
20. Zhu, W., Yang, B., Wills, N., Johnson, L. B. & White, F. F. The C terminus of AvrXa10 can be replaced by the transcriptional activation domain of VP16 from the herpes simplex virus. *Plant Cell* **11**, 1665–1674 (1999).
21. Tang, X. et al. A CRISPR-Cpf1 system for efficient genome editing and transcriptional repression in plants. *Nat. Plants* **3**, 17018 (2017).
22. Zipfel, C. & Oldroyd, G. E. D. Plant signalling in symbiosis and immunity. *Nature* **543**, 328–336 (2017).
23. Hu, J. et al. Direct activation of human and mouse *Oct4* genes using engineered TALE and Cas9 transcription factors. *Nucleic Acids Res.* **42**, 4375–4390 (2014).
24. Li, J. F., Zhang, D. & Sheen, J. Epitope-tagged protein-based artificial miRNA screens for optimized gene silencing in plants. *Nat. Protoc.* **9**, 939–949 (2015).
25. Puchta, H. Using CRISPR/Cas in three dimensions: towards synthetic plant genomes, transcriptomes and epigenomes. *Plant J.* **87**, 5–15 (2016).
26. Schellenberger, V. et al. A recombinant polypeptide extends the *in vivo* half-life of peptides and proteins in a tunable manner. *Nat. Biotechnol.* **27**, 1186–1190 (2009).
27. Ryu, J. et al. Protein-stabilizing and cell-penetrating properties of α-helix domain of 30Kc19 protein. *Biotechnol. J.* **11**, 1443–1451 (2016).
28. Li, J. F. et al. Multiplex and homologous recombination–mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. *Nat. Biotechnol.* **31**, 688–691 (2013).
29. Li, J. F. et al. Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. *Plant Cell* **25**, 1507–1522 (2013).
30. Zetsche, B. et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* **163**, 759–771 (2015).
31. Zetsche, B. et al. Multiplex gene editing by CRISPR–Cpf1 using a single crRNA array. *Nat. Biotechnol.* **35**, 31–34 (2016).
32. Zhang, Y. et al. A highly efficient rice green tissue protoplast system for transient gene expression and studying light/chloroplast-related processes. *Plant Methods* **7**, 30 (2011).

33. Clough, S. J. & Bent, A. F. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743 (1998).

Acknowledgements
We thank F. Ausubel and Z. Cheng for critical reading of this manuscript. This work was supported by the National Natural Science Foundation of China grant 31522006 and start-up funds from China's Thousand Young Talents Program to J.-F.L. and the NIH grant R01GM70567 to J.S. This work was partially supported by the Guangzhou Science and Technology Project grant 201605030012.

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 HEK 293T 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.

Competing interests
The authors have filed a patent application based on some results reported in this paper.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41477-017-0046-0.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to J.-F.L.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

nature research

Corresponding author(s): Jian-Feng Li

☐ Initial submission ☒ Revised version ☐ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.	Three biological repeats are generally considered sufficient for the experiments in this work.

2. Data exclusions

Describe any data exclusions.	No data were excluded from the analyses.

3. Replication

Describe whether the experimental findings were reliably reproduced.	All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.	Samples were randomly allocated into experimental groups.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.	The investigators were completely blinded to group allocation during data collection and/or analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	No software was used in this study.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	All unique materials used in this study are readily available from the authors or from standard commercial sources.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	The anti-FLAG M2-HRP antibodies used were from Sigma (Cat# A8592); The anti-ERK antibodies used were from Cell Signaling & Technology (Cat# 9101).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	The HEK293 cells used in this study were purchased from the company Procell, China
b. Describe the method of cell line authentication used.	The authentication of HEK293 cells was confirmed by morphological analysis under microscope.
c. Report whether the cell lines were tested for mycoplasma contamination.	The HEK293 cells used in this study were tested negative for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No commonly misidentified cell lines were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	This study did not involve human research participants.