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LETTER TO THE EDITOR

Positional effects on efficiency of CRISPR/Cas9-based transcriptional activation in rice plants

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Abstract The nuclease-dead Cas9 (dCas9) has been reprogrammed for transcriptional activation by fusing dCas9 to a transcriptional activation domain. In the presence of a guide RNA (gRNA), the dCas9 fusions specifically bind to regions of a promoter to activate transcription. Significant amount of effort has been directed toward the identification and optimization of the fusions of dCas9-activation domain, but very little is known about the impact of gRNA target positions within a promoter in plants on transcriptional activation efficiency. The dCas9–6TAL–VP128 system (dCas9-TV) has been optimized to activate transcription in plants. Here we use the dCas9-TV to activate transcription of *OsWOX11* and *OsYUC1*, two genes that cause dramatic developmental phenotypes when overexpressed. We designed a series of gRNAs targeting the promoters of the two genes. We show that gRNAs that target regions within 350 bp upstream of the transcription start site were most effective in transcriptional activation. Moreover, we show that using two gRNAs that simultaneously target two discrete sites in a promoter can further enhance transcription. This work provides guidelines for designed transcriptional activation through CRISPR/dCas9 systems.

DEAR EDITOR,

Designed transcriptional activation of genes not only provides an effective tool for defining gene functions, but also is a foundation for commercial production of agriculturally and medically valuable products. In plants, genes can be activated when the *Cauliflower mosaic virus 35S* enhancers (*CaMV 35S*) are inserted near a gene, which is the basis of the widely used activation-tagging screens (Weigel et al. 2000). However, because gene targeting in plants is still very inefficient, currently it is very difficult to precisely place

transcriptional enhancers near a target gene using homology-directed recombination (HDR)-based gene targeting technologies (Li et al. 2019a). A gene can also be overexpressed using transgenic approaches by placing the genomic DNA or cDNA under the control of a strong promoter such as *UBIQUITIN*, *ACTIN*, or *CaMV35S* promoters. But the overexpression constructs are often randomly inserted into a genome, rendering it necessary to characterize multiple transgenic lines to rule out positional effects. There are several inherent obstacles in using transgenic approaches. Multiplexing overexpression of genes is difficult because placing several overexpression cassettes in the same plasmid is time consuming and may render the plasmid unstable. Moreover, large plasmids may also make plant transformation less efficient (Park et al. 2000). It is difficult to obtain a complete cDNA for some large genes such as *BIG* in *Arabidopsis*, which has a coding region of 15 kb (Gil et al. 2001).

Xiaoyu Gong and Tao Zhang contributed equally to this work.

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Cas9 nuclease can be brought to a specific target DNA locus through a guide RNA molecule (gRNA), which forms a duplex with the DNA target through base pairing. Cas9 then generates a double-stranded break (DSB) in the target sequence, providing opportunities for editing the target sequence using HDR or non-homologous end-joining DNA repair mechanisms (Mali et al. 2013). Recently, the CRISPR/Cas9 gene editing system has been repurposed for genomic activation and repression (Gilbert et al. 2013). When the key catalytic residues in Cas9 are mutated, the nuclease-dead Cas9 (dCas9) still retains the ability to bind DNA in a gRNA-dependent manner, but can no longer generate DSB (Qi et al. 2013). The dCas9 has been repurposed by fusing it with various functional domains for transcriptional activation, repression, visualization of cis-elements, epigenetic modification, and other applications (Adli 2018). When dCas9 is fused to a transcriptional activation domain, the fusion protein can be brought to a specific promoter in a genome through a gRNA. Subsequently, transcription of the gene can be activated, leading to strong expression of a target gene (Li et al. 2017; Lowder et al. 2015; Lowder et al. 2018; Piatek et al. 2015; Vazquez-Vilar et al. 2016). CRISPR/Cas9-mediated transcriptional activation offers several advantages. It is relatively straightforward to activate multiple genes using dCas9 because several gRNA multiplexing systems have been well established (He et al. 2017; Ma et al. 2015; Xie et al. 2015). The dCas9/gRNA activation system probably does not cause co-suppression, a phenomenon that is often encountered when transgenic approaches are used (Napoli et al. 1990).

Single transcriptional activation domain and combinations of various activation domains have been fused to dCas9 and the fusion proteins have been evaluated in animal cells and in plants for their efficiency in gRNA-dependent gene activation (Li et al. 2017; Lowder et al. 2015; Piatek et al. 2015; Vazquez-Vilar et al. 2016). The dCas9-6TAL-VP128 system (dCas9-TV), which consists of the combination of VP128 with up to six copies of the TALE transactivation motif, has emerged as a very potent and programmable transcriptional activator (Li et al. 2017, 2019c). It is known that the locations of gRNA targets within the regulatory sequences of a gene have a profound effect on dCas9-based transcriptional activation. In animal cells, it was reported that the optimal window for gRNA targeting is within 400 bp upstream of the transcription start site (TSS) (Gilbert et al. 2014; Konermann et al. 2015). However, it is not clear whether similar regions are also optimal for plant cells. Another consideration is whether to use several gRNAs targeting the same promoter simultaneously.

Several gRNAs simultaneously targeting the same promoter in plants using dCas9-VP64 displayed synergistic effects (Lowder et al. 2015; Lowder et al. 2018; Piatek et al. 2015; Vazquez-Vilar et al. 2016). Here, we used the potent dCas9-TV (Li et al. 2017) to activate the transcription of rice *OsWOX11* and *OsYUC1*, whose overexpression phenotypes in rice have been well documented (Yamamoto et al. 2007; Zhang et al. 2018; Zhao et al. 2009).

OsWOX11 plays essential roles in crown root development and the expression levels of *OsWOX11* determine the number and length of crown roots (Zhang et al. 2018; Zhao et al. 2009). We designed five gRNAs targeting the sequences within 700 bp upstream of the TSS (Fig. 1A). We also designed two gRNA molecules that target a region more than 2 kb upstream of the TSS and a region between the TSS and the start codon, respectively (Fig. 1A). We observed that gRNA target sequence (TG2) downstream of the TSS (TG2 in Fig. 1A) or 2 kb upstream of the TSS (TG7 in Fig. 1A) did not result in transcription activation of *OsWOX11* (Fig. 1A, B, Supplemental Table 1). The gRNAs that target sequences within the 350 bp upstream of the TSS (TG1, TG3, and TG4) led to a dramatic increase of *OsWOX11* expression (Fig. 1A, B, Supplemental Table 1). The gRNA3 activated *OsWOX11* expression to almost 6000-fold (Fig. 1B). Consistent with the observed *OsWOX11* overproduction, the transgenic plants developed more crown roots (Fig. 1C). The target regions 500 bp upstream of the TSS (TG6) also led to slight increases of *OsWOX11* expression, but the transgenic plants did not display the typical *OsWOX11* overexpression phenotypes (Supplemental Table 1). Our results suggest that the regions within 350 bp upstream of the TSS are preferred for designing gRNAs for dCas9-mediated transcriptional activation. The underlying reason for the preferred region is not clear; however, the close proximity of the transcriptional activation domain to transcription initiation complex may partially account for the observation.

We tested whether additional gRNAs can enhance transcriptional activation. We added a second gRNA to the plasmids that can express either *OsWOX11* gRNA1 or gRNA3, which displayed the most efficient transcriptional activation (Fig. 1B, D). As shown in Fig. 1, the addition of gRNA8, which targeted a region around − 600 bp (Fig. 1A), led to more than tenfold increase of *OsWOX11* expression compared to gRNA1 alone (Fig. 1B, D). The gRNA6 and gRNA10 only led to a slight increase of *OsWOX11* expression (Fig. 1B). Without a gRNA that targeted the core region (within 350 bp upstream of the TSS), even two gRNAs were not effective to induce *OsWOX11* expression (Fig. 1A, B).
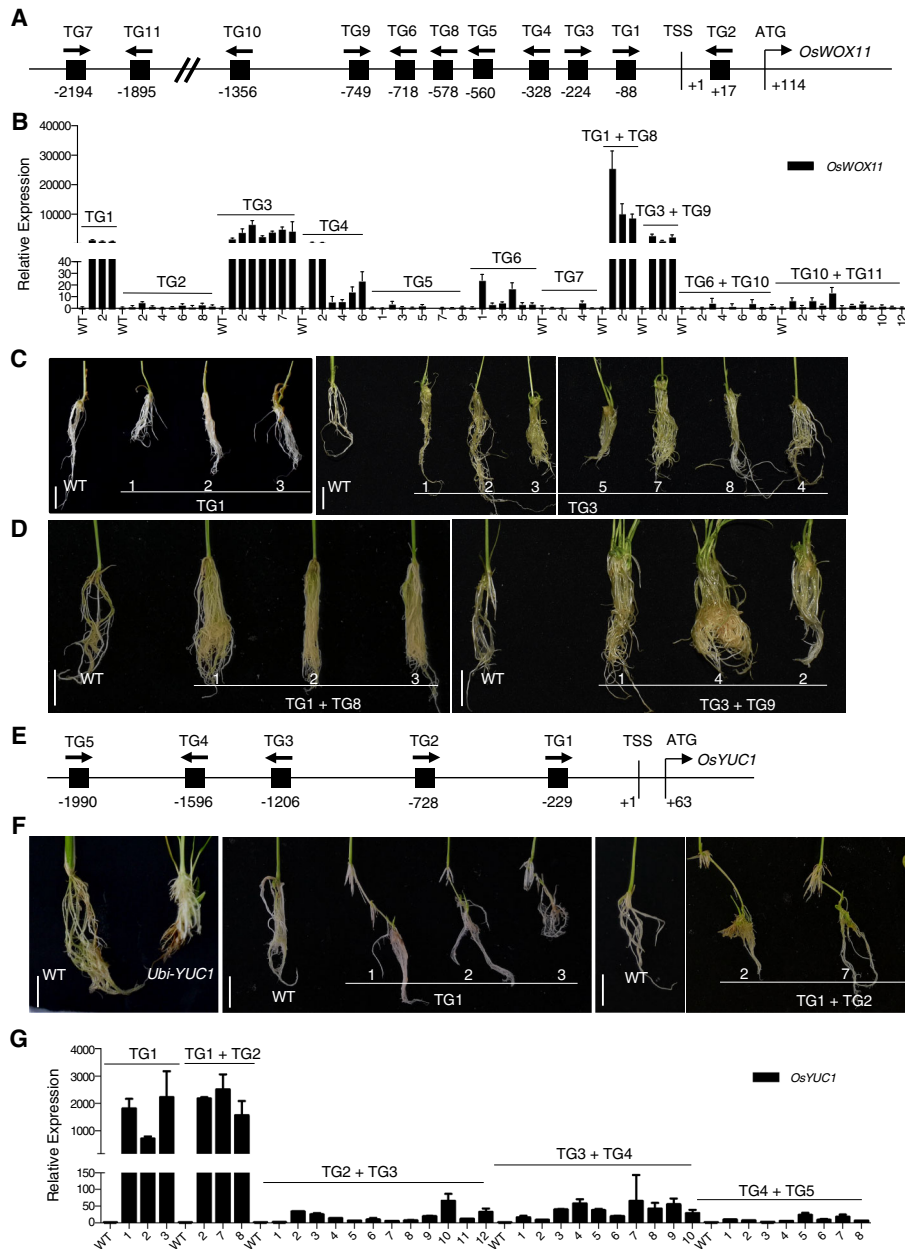
Fig. 1 Transcriptional activation of *OsWOX11* and *OsYUC1* by CRISPR/dCas9. **A** A schematic presentation of guide RNA (gRNA) target sites in the *OsWOX11*. "TG" refers to transcriptional gRNA target. "ATG" is the start codon. "TSS" represents the transcription start site. Arrows indicate the direction of the gRNA sequence. **B** Relative expression of *OsWOX11* in plants transformed with various *CRISPR/dCas9* constructs. Plants are transformed either with a single gRNA cassette (TG1, TG2, TG3, TG4, TG5, TG6, and TG7) or with dual gRNA cassettes (TG1 + TG8, TG3 + TG9, TG6 + TG10, and TG10 + TG11). Note that some gRNAs dramatically induced *OsWOX11* expression. Data represent the results of three technical replicates. Error bars represent standard error (SE). **C** Overexpression of *OsWOX11* stimulates crown root development. Both gRNA1 and gRNA3 (match TG1 and TG3, respectively) caused *OsWOX11* overexpression and led to proliferation of crown roots. **D** Dual gRNAs led to crown root proliferation. Plants that express either gRNA1 + gRNA8 or gRNA3 + gRNA9 developed more crown roots. **E** A schematic presentation of the position and directions of gRNA target sites in the promoter of *OsYUC1*. **F** The phenotypes of plants that overexpress *OsYUC1*. The first picture in the left shows more adventitious root development in plants that overexpress *OsYUC1* under the control of the *UBIQUITIN* promoter. The other pictures show ectopic root development induced by overexpressing *OsYUC1* using CRISPR/dCas9 system. The gRNA1 alone or gRNA1 + gRNA2 caused overexpression of *OsYUC1*. **G** Relative expression levels of *OsYUC1* in plants that express various *dCas9* constructs with gRNAs targeting the *OsYUC1* promoter. Data represent the results of three technical replicates. Error bars are standard error (SE)

We also targeted the promoter of *OsYUC1*, which encodes a flavin-containing monooxygenase and which plays a key role in auxin biosynthesis (Yamamoto et al. 2007; Zhao et al. 2001). We designed five gRNAs that target regions in the *OsYUC1* promoter (Fig. 1E). Over-expression of *OsYUC1* is known to promote adventitious root formation out of coleoptiles (Yamamoto et al. 2007; Zhang et al. 2018) (Fig. 1F). When *OsYUC1* was over-expressed, many hairy roots were initiated from the coleoptile (Fig. 1F). It was also clear that overexpression of *OsYUC1* inhibited the primary root elongation (Fig. 1F). Introduction of dCas9-TV system and the gRNA1, which targets the TG1 (Fig. 1E), into rice led to development of adventitious roots out of coleoptiles (Fig. 1F). Note that the TG1 plants had two clusters of roots and two apparent shoots (Fig. 1F). The root-like structure that links the two clusters of roots was actually coleoptile, from which adventitious roots were also developed (Fig. 1F). Both TG1 plants and *Ubi-OsYUC1* plants developed more adventitious roots. Interestingly, the TG1 plants displayed root phenotypes that were different from *Ubi-OsYUC1* plants (Fig. 1F), which was likely caused by differences in timing and magnitude of *OsYUC1* overexpression. Consistent with the observed phenotypes, we found that the gRNA1 caused more than 1000-fold increases in *OsYUC1* expression (Fig. 1F, G). Similar to our observations with *OsWOX11* constructs, the gRNA that targeted the region within 350 bp upstream of the TSS of *OsYUC1* was the most effective (Fig. 1E–G). Interestingly, adding another gRNA (gRNA2) did not significantly affect the expression of *OsYUC1* (Fig. 1F, G). Again, without a gRNA targeting the core region, even two gRNAs were not effective for activating *OsYUC1* expression (Fig. 1E, F).

We have shown clearly that dCas9-TV could effectively activate gene transcription in stable transgenic rice plants. Our work suggests that the most effective target regions are within the 350 bp upstream of the TSS. Traditionally, gRNAs for dCas9-TV-based transcriptional activation are first optimized using plant protoplast systems (Li et al. 2019b). Our findings that regions 350 bp upstream of the TSS are most effective for dCas9-TV-based transcriptional activation indicate that it can be productive by focusing on the small region without using the protoplast systems. Moreover, we demonstrated that simultaneously target two regions within the same promoter may be beneficial. At least, we did not observe strong detrimental effects when two gRNAs are simultaneously employed. Analyzing additional gRNA targets and tiling the entire promoters of *OsWOX11* and *OsYUC1* will help refine the regions and sequences optimal for dCas9-mediated transcriptional activation.

MATERIALS AND METHODS

Construction of transcriptional activation vectors

The *pCAMBIA-dCas9-TV* plasmid from Prof. Jian-Feng Li at the Sun Yat-Sen University provides the backbone of all the plasmids used in this study. For gRNA constructs, the gRNA expression cassette was inserted into the *S-wa*I site of the *dCas9-TV* vector. For plasmids that express two gRNAs, one gRNA was driven by the *OsU6* promoter and the other was under the control of the *OsU3* promoter. The location and sequences of gRNAs used in this study are listed in the Supplementary Table 2. The CRISPR P 2.0 (http://crispr.hzau.edu.cn/cgi-bin/CRISPR2/CRISPR) was used for selecting gRNA targets (Liu et al. 2017).

Rice transformation

Wild-type rice (*Oryza sativa* ssp. *japonica*) "Zhonghua 11" (ZH11) was used in this study. ZH11 was transformed via *Agrobacterium tumefaciens* (EHA105)-mediated callus transformation as previously described (Hiei et al. 1994).

RNA extraction and RT-PCR analysis

Young leaves of plants were collected and total RNA was extracted using TRIzol reagent (Invitrogen). Complementary DNA was synthesized according to the manufacturer's instructions (Invitrogen). RT-qPCRs were performed using gene-specific primers (Supplementary Table 3) and SYBR Premix Ex-Taq in a real-time PCR 7500 system (Applied Biosystems). Data were collected using the ABI PRISM 7500 sequence detection system following the manufacturer's instruction. The rice *ACTIN1* gene was used as the internal control. At least three technical repeats were conducted.

Accession numbers

Sequence data of rice genes in this article are accessible at the TIGR (Rice Genome Annotation Project http://rice.plantbiology.msu.edu) with the following accession numbers: *OsYUC1*, LOC_Os01g45760; *OsWOX11*, LOC_Os07g48560.

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