Building a strong Cas9 transcriptional activator for plants

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Plant molecular biology is currently constrained by the ability to rapidly test hypotheses. Generating a transgenic plant stably expressing a desired cassette requires significant time and effort, up to a year or more for some plant species. Further, the method of transgenesis via *Agrobacterium tumefaciens* will randomly integrate the DNA cassette into the genome, resulting in variable transgene expression. In addition, each gene in a multi-gene cassette will require a promoter with the desired expression pattern, making multi-gene casettes of 10 or more genes difficult to design. A method to robustly manipulate the expression of many genes at once in a single transgenic event would allow plant biologists to investigate the underlying genetics of desirable traits.

CRISPR-Cas9 has partially solved this problem, allowing scientists to simultaneously knockout multiple genes by expressing a single Cas9 protein with multiple sgRNAs targeting coding regions of interest[1]. While loss of function mutations are interesting to study, there is additional benefit to increasing the amount of a particular gene product. This can be accomplished with Cas9 transcriptional activators. A catalytically dead Cas9 (dCas9) was generated such that DNA will be bound but not cleaved by the Cas9 protein[2]. Transactivation domains (TADs), such as VP64, were subsequently fused to dCas9 to comprise a programmable transcriptional activator[3]. While the animal field has been able to utilize strong activators such as dCas9-VPR[4], the plant field has lagged considerably with the best dCas9 gene activation systems in plants still routinely utilizing VP64[5,6].

We set out to optimize our gene activation system in plants by searching for novel TADs from plant backgrounds. Through literature searches and homology to known TADs, we predicted 36 putative TADs from endogenous plant, bacterial, or viral genes. This library was then synthesized as dsDNA fragments containing Golden Gate overhangs for cloning into a CRISPR-Cas9 transcription activation system. Our activation system is adapted from Papikian et al[7], in which a dCas9 protein is fused to a 24x multimeric GCN4 epitope tail. Each GCN4 epitope can bind to an scFv fragment, which is translationally fused to a TAD. We designed our scFv plasmid such that a successful assembly resulted in an in frame scFv-TAD fusion.

To test the library, we utilized a dual luciferase assay in plant protoplasts to quantify the strength of each putative TAD. Protoplasts are isolated by treating plant tissue with an enzyme mixture containing macerozyme and cellulase, which will degrade the cell wall and allow for transformation of plasmid DNA into the plant cells. The cells survive for only 48h, but DNA,

RNA or protein can be isolated from these cells 24h after transformation for analysis. Following isolation, we transformed protoplasts from both Setaria viridis and Arabidopsis thaliana with dCas9-SunTag plasmids. This includes the dCas9-24xGCN and scFv-TAD under the expression of strong constitutive promoters We also included a dual luciferase plasmid, containing two luciferase genes. A *renilla* luciferase was driven by a constitutive promoter, while a downstream *firefly* luciferase gene was driven by a minimal promoter. This minimal promoter contained a transcription start site, TATA box, and six copies of the Lac Operator (LacO). The final plasmid transformed contains a U6 promoter driving a sgRNA targeting the LacO, targeting of six dCas9 proteins to the minimal promoter. Thus, *renilla* expression is a readout of transformation frequency and *firefly* expression is a readout of TAD strength.

The library was tested three times in *Setaria* protoplasts, with the results shown below (Figure 1). DREB1, DREB2, and HSFA6b consistently produce the greatest TAD strength in our assays. For *Arabidopsis* our results are preliminary, with HSFA6b showing the greatest TAD strength (Figure 2).

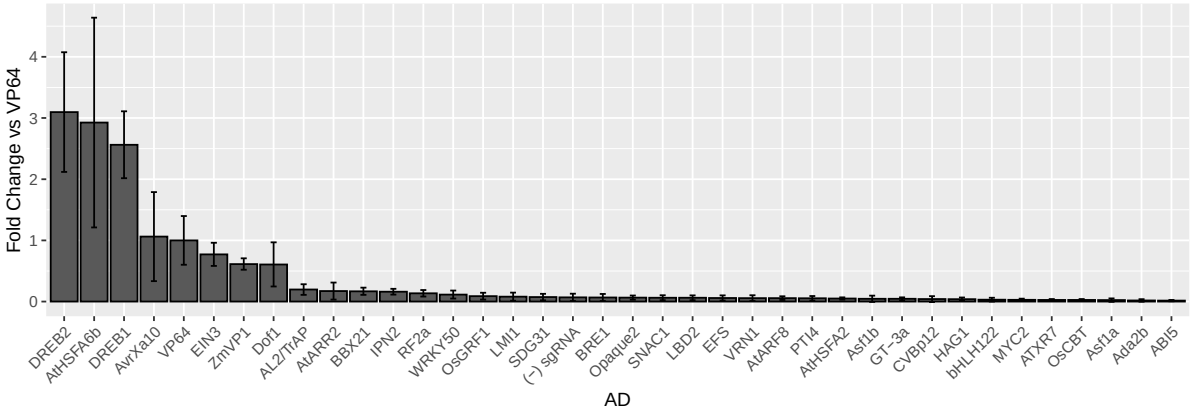

Figure 1: TAD strength in Setaria viridis protoplasts. Protoplasts were transformed with CMYLCV:dCas9-24xGCN4, LacO sgRNA, 35S:renilla, 6xLacO:min35S:firefly, and the indicated 35S:scFv-TAD. Fold Change is calculated relative to SunTag-VP64.

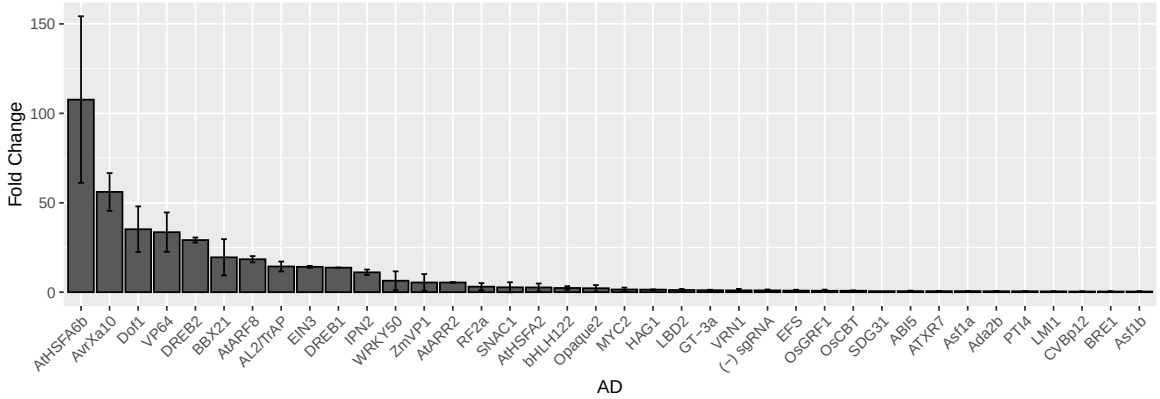

Figure 2: TAD strength in Setaria viridis protoplasts. Protoplasts were transformed with Ubi10:dCas9-24xGCN4, LacO sgRNA, 35S:renilla, 6xLacO:min35S:firefly, and the indicated 35S:scFv-TAD. Fold Change is calculated relative to a no sgRNA negative control.

sgRNAs targeting a single promoter are flanked by tRNA cleavage sequences and expressed under a strong pol II promoter[8]. This results in a single large transcript which is subsequently cleaved into four short fragments each containing a sgRNA. Three different multi-guide arrays were generated targeting *WUS, Lec1*, and *STM* in the Arabidopsis genome. Protoplasts were isolated from Arabidopsis thaliana and transformed with the multi-guide arrays, plasmids encoding dCas9 directly fused to VP64 or DREB2, or plasmids encoding SunTag-DREB2 or SunTag-VP64. Our results indicate that DREB2 is stronger than VP64 as both a direct fusion to dCas9, and in the SunTag system. It also demonstrates that DREB2 can consistently activate both reporter genes and endogenous loci.

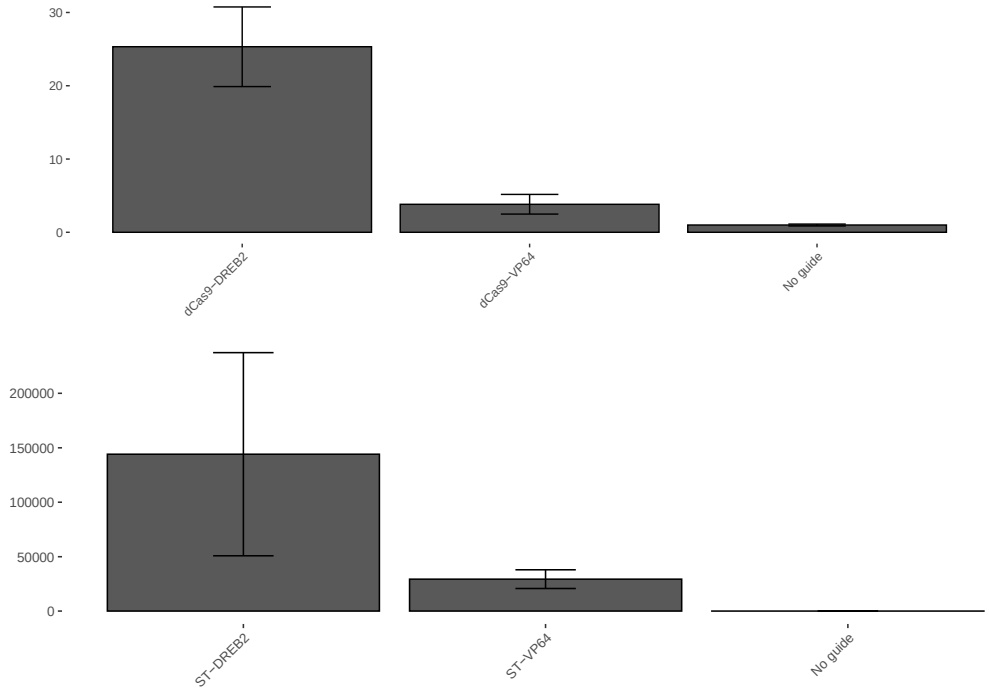

Figure 3 – DREB2 vs VP64 in Arabidopsis thaliana protoplasts. The top bar graph illustrates DREB2 and VP64 directly fused to dCas9 and their average fold change activation of three endogenous target genes in Arabidopsis. The bottom bar graph illustrates DREB2 and VP64 fused to scFv in the SunTag system and their average fold change activation of the same three endogenous target genes. Fold change is relative to a no sgRNA negative control.

Transgenic lines have been constructed and are currently being characterized to confirm the strength of the novel TADs as a stably integrated cassette. We hope that this work shows endogenous plant TADs offer a promising alternative for plant transcriptional activation over methods developed for animal systems, and that dCas9-based activators offer both a robust and scalable avenue toward activating many plant genes simultaneously.

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