Plant Cell Reports (2024) 43:227
https://doi.org/10.1007/s00299-024-03316-6

FOCUS ARTICLE

# Orthogonal genome editing and transcriptional activation in tomato using CRISPR-Combo systems

**Innocent Byiringiro[1] · Changtian Pan[2] · Yiping Qi[1,3]**

Received: 20 July 2024 / Accepted: 26 August 2024 / Published online: 5 September 2024


## Abstract

***Key message*** **The CRISPR-Combo systems (Cas9-Combo and CBE-Combo) are designed for comprehensive genetic manipulation, enabling Cas9-based targeted mutagenesis or cytosine base editing with simultaneous gene activation in tomato stable lines.**

CRISPR-Combo systems are versatile tools for plant genome engineering, which allows for simultaneous genome editing and gene activation. Initially demonstrated in rice, these systems not only support hormone-free regeneration of edited lines but also significantly boost genome editing efficiency, resulting in a higher rate of heritable mutations (Pan et al. 2022). Combo systems are based on two distinct functionalities facilitated by either Cas9 or Cas9n-CBE (cytosine base editor) and single guide RNA (sgRNA). CRISPR-Cas9 is used for targeted mutagenesis by non-homologous end-joining (NHEJ) repair of DNA double strand breaks (DSBs), that results in the insertions or deletions (indels) at the target site. In contrast, Cas9n-CBE achieves precise C-to-T base change at the target site without inducing DSBs(Pan et al. 2022). Beyond genome editing, CRISPR systems have been adapted for genome modification at the transcriptional level. A highly efficient CRISPR-Act3.0 for gene activation in plants was developed. This system uses a tailored sgRNA2.0 scaffold for recruiting transcriptional activators (Pan et al. 2021). Combining the functionalities of Cas9/Cas9n-CBE with sgRNA2.0 thus creates a dual orthogonal system capable of simultaneous gene editing and gene activation, named "CRISPR-Combo" (Pan et al. 2022)(Pan and Qi 2023). CRISPR-Combo systems have previously been demonstrated in stable plants of rice, Arabidopsis, and poplar (Pan et al. 2022). Given the importance of tomato as a model crop for agriculture research and food production, demonstrating the CRISPR-Combo systems in stable tomato plants will facilitate their adoption to advance tomato breeding.

Based on our previously developed CRISPR-Combo system, we created Combo system tailored for tomato targeted mutagenesis and gene activation. The CRISPR-Combo for tomato was created by first replacing the Cas9 expression promoter, poll II promoter Zmubi with AtUBQ10, then subsequently with 2 × 35S promoter. Each sgRNA was expressed under control of *Arabidopsis* Ubiquitin 3(AtU3) promoter and terminated with the AtU3 terminator. Beside the promoter, Combo system is facilitated by the co-expression of sgRNA1.0 (gR1.0) and sgRNA2.0 (gR2.0) scaffolds with 20-nt protospacers and 15-nt protospacers, respectively. gR1.0 directs Cas9 to the target DNA sequence and induce double strand break (Fig. 1a). gR2.0 is unable to cause mutations, therefore, repurposed for activation. The gR2.0 activation is based on the SunTag system. It is modified to carry MS2 hairpins that are recognized by MS2 bacteriophage coat protein (MCP). The MCP is fused to a tandem repeat of ten GCN4's. Each GCN4 recruits a single chain fragment variant (scFV) through antigen–antibody interaction mechanism. The scFV is fused to two TAL effector activation domain (TAD) and stabilized by the super folder GFP (Pan et al. 2021) (Fig. 1a, b). For target genes, we selected the *SlPSY* (phytoene synthase) gene for editing due to its distinct phenotype (Cui et al. 2024) and *SlSFT* (single flower truss) gene (Krieger et al. 2010) for activation.

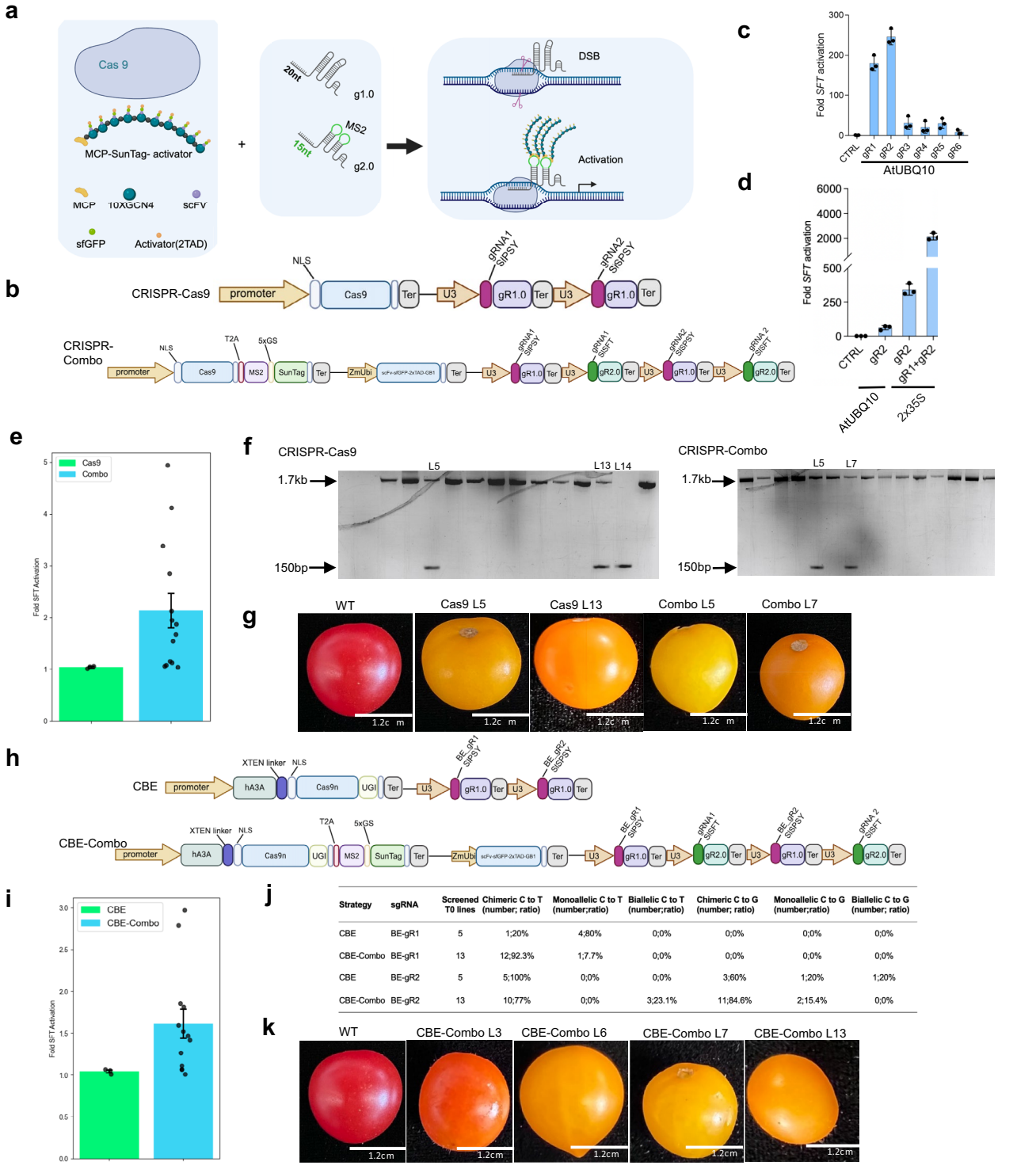
Communicated by Günther Hahne.

✉ Yiping Qi
yiping@umd.edu

[1] Department of Plant Science and Landscape Architecture, University of Maryland, College Park, MD, USA

[2] Department of Horticulture, College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, China

[3] Institute for Bioscience and Biotechnology Research, Rockville, MD, USA




| Strategy | sgRNA | Screened T0 lines | Chimeric C to T (number; ratio) | Monoallelic C to T (number;ratio) | Biallelic C to T (number;ratio) | Chimeric C to G (number; ratio) | Monoallelic C to G (number;ratio) | Biallelic C to G (number; ratio) |
|---|---|---|---|---|---|---|---|---|
| CBE | BE-gR1 | 5 | 1;20% | 4;80% | 0;0% | 0;0% | 0;0% | 0;0% |
| CBE-Combo | BE-gR1 | 13 | 12;92.3% | 1;7.7% | 0;0% | 0;0% | 0;0% | 0;0% |
| CBE | BE-gR2 | 5 | 5;100% | 0;0% | 0;0% | 3;60% | 1;20% | 1;20% |
| CBE-Combo | BE-gR2 | 13 | 10;77% | 0;0% | 3;23.1% | 11;84.6% | 2;15.4% | 0;0% |

◂**Fig. 1** Simultaneous genome editing and gene activation in tomato by CRISPR-Combo systems. **a** Diagram of the Cas9-Combo system. The Cas9-Combo system consists of a catalytically active Cas9 nuclease, and a complex formed by MS2 bacteriophage coat protein (MCP), SunTag, and an activator. It employs two types of single guide RNA (sgRNA) scaffolds: gR1.0 and gR2.0. Each SunTag peptide recruits ten copies of the 2xTAL activation domain (TAD) through a single-chain variable fragment (scFV) of the GCN4 antibody fused to a super-folder GFP (sfGFP). The gR2.0 scaffold, which includes two MS2 RNA aptamers, binds to the MCP-SunTag-2xTAD transcriptional activation complex to activate gene expression without inducing double-strand breaks (DSBs). Simultaneously, the gR1.0 scaffold induces DSBs using a ~ 20-nucleotide protospacer and the Cas9 nuclease. **b** Schematic illustration of Cas9 and Cas9-Combo expression vectors. Cas9 is used for targeted gene editing, and Cas9-Combo is used for simultaneous genome editing and gene activation. **c** Prescreening sgRNAs in tomato protoplasts for gene activation using the Cas9-Combo driven by the Arabidopsis Ubiquitin 10 promoter (AtUBQ10) to identify the most effective sgRNAs for activating the *SlSFT* gene. **d** Comparing the efficiency of gene activation between two different promoters with varying sgRNA configurations in tomato protoplasts. **e** *SlSFT* was activated by Cas9-Combo in tomato stable lines, quantified by RT-qPCR. Each dot represents an average of three technical repeats measured for each plant. **f** Comparison of Cas9 and Cas9-Combo induced large deletions in $T_0$ lines using agarose gel electrophoresis. **g** The phenotypic outcomes in tomato fruits resulting from the editing of the *SlPSY* gene using the Cas9 and Cas9-Combo systems. **h** Schematic illustration of CBE and CBE-Combo expression vectors. CBE is used for targeted base editing, and CBE-Combo is used for targeted base editing and gene activation. **i** *SlSFT* was activated by CBE-Combo in tomato stable lines, quantified by RT-qPCR. Each dot represents an average of the three technical repeats measured for each plant. **j** Comparing CBE and CBE-Combo zygosity of mutations for both CBE-gR1 and CBE-gR2. Zygosity is based on editing efficiency measured by NGS based on this criterion: 0–30% = Chimeric, 30–70% = Monoallelic, > 70% = biallelic. **k** The phenotypic outcomes in tomato fruits resulting from the base editing of the *SlPSY* gene using the CBE and CBE-Combo systems. **l** Genotypes of four representative base edited lines by CBE-Combo. Note, the base editing frequencies at both target sites were measured by NGS

The system was initially assessed in tomato protoplasts to prescreen sgRNAs for transcriptional activation of *SlSFT*, which is recommended to ensure optimal gene activation. Two top-performing sgRNAs (gR1 and gR2) out of six were identified (Fig. 1c). To further increase the activation level of these two selected sgRNAs, we compared two strong promoters driving the Cas9 (AtUBQ10 and 2 × 35S) and explored the strategy of multiplexing both sgRNAs. This led to the identification of the optimal gene activation cassette, based on two multiplexed sgRNAs (gR1 and gR2) with Cas9 driven by 2 × 35S. This cassette achieved up to 2000-fold activation in tomato protoplasts (Fig. 1d).

Having chosen two sgRNAs for *SlSFT* activation, final T-DNA vectors were constructed to include two sgRNAs for *SlPSY* editing. The two sgRNAs were designed spanning ~ 1700 bp region in the gene body, which would induce both large deletions and small indels. Transgenic tomato lines were obtained for CRISPR–Cas9 and CRISPR-Combo constructs. With RT-qPCR, increased expression levels of *SlSFT* were detected for the CRISPR-Combo lines as opposed to the CRISPR-Cas9 control group (Fig. 1e). To test the editing efficiency, first we amplified the entire region spanning outside the target sites. This analysis revealed that CRISPR-Combo induced large deletions as was with CRISPR-Cas9. Among the 15 $T_0$ lines examined for each construct, CRISPR-Combo had two large DNA dropout events, while CRISPR–Cas9 had three dropout events (Fig. 1f, Supplementary Fig. 1), suggesting that the Cas9 in the CRISPR-Combo system maintains the wildtype Cas9 catalytic activity. Second, stable lines that showed large deletions on the agarose gel were further investigated through long read sequencing. Aligning the raw reads to reference sequence showed similar patterns of mutations for Cas9 and Combo systems (Supplementary Fig. 2). Third, we found that the allelic variation correlates with the phenotype variation. The resulting tomato mutants displayed yellow to orange color because the disruption of the *SlPSY* gene that is involved in the carotenoid biosynthesis pathway in tomatoes (Fig. 1g, Supplementary Figs. 3 and 4).

We then pursued to develop a CBE-Combo system for simultaneous C-to-T base editing and gene activation in tomato stable lines. The CBE-Combo system was based on the previously developed CBE-Cas9n-Act3.0 (Pan et al. 2022). As done with CRISPR-Combo, we swapped the CBE-Cas9n expression promoter from ZmUbi to 2 × 35S. CBE-Combo is made of the highly efficient codon optimized human APOBEC3A(hA3A)-Y130F deaminase fused to Cas9 nickase (Cas9n) on its N terminus, and UGI fused to its C terminus, plus the SunTag activation system (Fig. 1h, Supplementary Fig. 5). The same two sgRNAs for *SlSFT* activation was used for testing gene activation, and two sgRNA spanning ~ 1700 bp in the *SlPSY* gene body (BE-gR1 and BE-gR2) were used for testing base editing. The CBE-Combo also tested in tomato stable lines, with CBE as the control (Fig. 1h). RT-qPCR analysis of $T_0$ lines showed increased expression levels of the *SlSFT* gene as an indication of successful activation by CBE-Combo (Fig. 1i). We compared base editing efficiency between CBE and CBE-Combo. At the BE-gR1 target site, one $T_0$ CBE line carried chimeric base edits, with four lines having monoallelic C-to-T base editing (Fig. 1j). Majority of the CBE-Combo carried chimeric edits (12 out of 13; 92.3%) while one line carried monoallelic C-T edit (Fig. 1j). At the BE-gR2 target site, base editing efficiency appeared to be higher. All five $T_0$ CBE lines were edited. Among them, one line carried monoallelic C-to-G edit and one line carried biallelic C-to-G edit. It is not uncommon to obtain C-to-G editing with the hA3A-Y130F deaminase (Ren et al. 2021). Among the 13 CBE-Combo $T_0$ lines, 10 lines carried chimeric C-to-T edits and 3 lines carried bialleic C-to-T edits (Fig. 1j). These data suggest that CBE-Combo displayed comparable or higher

base editing efficiency than CBE at these two target sites. We also analyzed the phenotypes of the base edited lines (Fig. 1k), which showed consistency with the genotypes (Fig. 1l). For example, CBE-Combo L3 is chimeric at both BE-gR1 and BE-gR2; therefore, it did not exert a strong yellow phenotype as a result of *SlPSY* knockout (Fig. 1k, Fig. 1l). In contrast, the fruits of CBE-Combo L6, CBE-Combo L7 and CBE-Combo L13 exhibited *SlPSY* knockout phenotype (Fig. 1k). Overall, the genotypes and phenotypes for the CBE and CBE-Combo tomato lines are consistent (Supplementary Figs. 6 and 7).

Although we initially hypothesized that activation of *SlSFT* by CRISPR-Combo would promote earlier flowering, we did not observe this phenotype clearly. This could be partly due to the relative low level of gene activation of *SlSFT* in stable tomato plants as observed in somatic leaf tissues (Fig. 1e, i), as compared to the high levels of activation in the protoplasts (Fig. 1c, d). For promoting early flowering, further research may try alternative gene activation targets such as *SlSP* (Soyk et al. 2017) or *SlTFL1* (Wang et al. 2022).

In summary, we demonstrated the capabilities of the CRISPR-Combo and CBE-Combo systems in stable tomato plants. These Combo systems can perform nuclease-based targeted mutagenesis and base editing equally well (if not better) than the Cas9 and CBE counterparts. The added capability of simultaneous gene activation could be advantageous to promote tomato breeding and trait improvement. Validation of the functionality in tomato stable lines in this study will open door for a wide range of applications of these CRISPR-Combo systems in this important crop.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00299-024-03316-6.

**Acknowledgements** This work was supported by the NSF Plant Genome Research Program (award no. IOS-2029889 and IOS-2132693) and FFAR Next Generation Crops Program (award no. 21010111).

**Author contributions** I.B., C.P. and Y.Q. designed the experiments. I.B., and C.P. conducted the experiments and data analysis. I.B. and Y.Q. wrote the manuscript. C.P. revised the manuscript. All authors read and approved the final manuscript.

**Funding** Directorate for Biological Sciences, IOS-2029889, Yiping Qi, IOS-2132693, Yiping Qi, Foundation for Food and Agriculture Research, 21010111, Yiping Qi.

**Data availability** The raw data for all the Next Generation Sequencing were deposited to the NCBI-SRA with the BioProject accession number PRJNA1135222.

## Declarations

**Conflict of interest** The authors have no conflict interests to declare.

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