***Agrobacterium rhizogenes*-Mediated Marker-Free Transformation and Gene Editing System Revealed that *AeCBL3* Mediates the** **Formation of Calcium Oxalate Crystal in Kiwifruit**

Pengwei Li1†, Yiling Zhang1†, Jing Liang1, Xufan Hu1, Yan He1, Tonghao Miao1, Zhiyin Ouyang1, Zuchi Yang1, Abdul Karim Amin1, Chengcheng Ling1, Yize Liu1, Heqiang Huo2, Yongsheng Liu1, Wei Tang1\*, Songhu Wang1\*.

1. Anhui Provincial Key Laboratory of Horticultural Crop Quality Biology, School of Horticulture, Anhui Agricultural University, Hefei 230036, China;
2. Mid-Florida Research and Education Center, University of Florida, Institute of Food and Agricultural Sciences, Apopka, FL 32703, USA.

† These authors contribute equally.

\*Corresponding authors: Songhu Wang (wangsonghu@ahau.edu.cn) and Wei Tang (tangwei@ahau.edu.cn)

**ABSTRACT**

The transformation and gene editing of the woody species kiwifruit are difficult and time-consuming. To date, the marker-free transformation and gene-editing system for kiwifruit has not been developed yet. Here, we establish a fast and efficient marker-free transformation and gene editing system mediatedby *Agrobacterium rhizogenes* for kiwifruit. The transformation of *GUS* and *eGFP* indicated that the hairy root induction efficiency is about 50% and 80% of induced hairy roots are transgenic. Moreover, a removing-root-tip method was developed to significantly increase the regeneration efficiency of transgenic hairy roots. Through *A. rhizogenes*-mediated CRISPR/Cas9 gene editing, the editing efficiencies of *CEN4* and *AeCBL3* achieved 55% and 50%, respectively. And several homozygous knockout lines for both genes were obtained. Our method has been successfully applied in the transformation and gene editing of two different species of kiwifruit. Meanwhile, calcium oxalate (CaOx) crystals are widely present in most photosynthetic organisms including kiwifruit and play multiple roles in excess calcium excretion, heavy metal detoxification, and protection against herbivory. However, little is known about how CaOx crystal is formed in plants. Our results indicated that *AeCBL3* overexpression enhanced CaOx crystal formation, but its knockout via CRISPR/Cas9 significantly impaired crystal formation in kiwifruit. Together, we developed a fast maker-free transformation and highly efficient CRISPR-Cas9 gene editing system for genetic modification of kiwifruit. Besides, our work revealed a novel gene mediating CaOx crystal formation and provides a clue to elaborate the underlying mechanisms.

**KEYWORD:** Marker-Free transformation; *Agrobacterium rhizogenes*; CRISPR/Cas9; Kiwifruit; *AeCBL3*