



Extremely simplified cut-dip-budding method for genetic transformation and gene editing in *Taraxacum kok-saghyz*

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Dear Editor,

Genetic transformation is an intricate and resource-intensive process that is a critical bottleneck in generating GMO (genetically modified organism) or gene-edited crops.¹ Many researchers have explored the use of hormones and plant development regulatory genes to bolster plant regeneration, thereby augmenting the efficiency of tissue culture-dependent genetic transformation.² Previously, we developed the tissue culture-free "cut-dip-budding" (CDB) method for genetic transformation, utilizing *Agrobacterium rhizogenes* to induce and transform hairy roots from the cut sites of explants.³ Genetically transformed plants are then grown from the transformed hairy roots that possess shoot-forming ability. The CDB method greatly simplifies the experimental workflow for genetic transformation and gene editing of plants including *Taraxacum kok-saghyz* Rodin (TKS). Here, we developed an extremely simplified procedure in TKS based on CDB method by omitting the process of hairy root formation, which significantly saves labor and time.

TKS, a latex-producing plant known for its high natural rubber yield,⁴ has gained popularity as an ideal model for studying natural rubber synthesis due to its relatively simple genome (~1.29 Gb, 2N=2X=16 chromosomes) and adaptability to various environments.⁵ In our previous work, using *Agrobacterium rhizogenes* to infect the wounds of TKS roots near the aboveground parts, we firstly obtained transformed hairy roots; after these hairy roots were grown to a suitable size, they were cut and cultivated in soil, so that transformed or gene edited buds could grow from the root segments.³ In the present work, we hypothesized that it may be possible to bypass the hairy root induction and cultivation processes to achieve genetic transformation and gene editing of TKS. To test this, we obtained roots from the TKS variety TKS-H, which had been grown in soil until the main root reached an average diameter of more than 2 mm. We cut the roots into 2-3 cm segments (Figure 1A) and coated the two cut ends of the segments with *Agrobacterium* cells grown on solid agar in Petri dishes. The *Agrobacterium*-coated root segments were then placed on the surface of moist vermiculite (Figure 1A and 1B). After about two weeks of cultivation, we found that new shoots regenerated from nearly all of the TKS root segments (Figure 1B).

We tried the new protocol using nine different *Agrobacterium* strains, including four *Agrobacterium tumefaciens* strains and five *Agrobacterium rhizogenes* strains. Following infection and cultivation of the TKS root segments, PCR test of the regenerated shoots revealed that *Agrobacterium tumefaciens* strains AGL1 and EHA105 and *Agrobacterium rhizogenes* strains MUS440, Ar 1193, C58C1, Ar Qual, and K599 were capable of inducing transgenic shoots when directly applied to the root segments. Notably, the *Agrobacterium rhizogenes* strain K599 exhibited superior efficiency, yielding 13 transgenic shoots from 61 infected root segments (Figure 1C). Subsequently, the transgenic shoots were transplanted to potted soil for growth to maturity (Figures 1A & D).

We used the protocol to introduce a construct designed for editing the TKS phytoene desaturase gene *TKPDS*, a target chosen for easy identification of gene-edited plants since its knockout results in an albino phenotype.^{3,6} We inoculated the cut ends of the TKS root segments with K599 strain carrying the gene editing construct. After approximately two weeks of cultivation,

shoots emerged from the ends of the TKS root segments (Figure 1E). As expected, among the regenerated shoots, we observed albino ones (Figure 1E). The editing of the *TKPDS* gene was examined through TA cloning and Sanger sequencing (Figure 1F). We conducted three independent experiments, achieving editing efficiencies ranging from 17.1% to 19.6% (19/111, 21/107, 26/133) (Figure 1G). Homozygous edited seedlings were obtained at high efficiency (Figure 1G). These results suggest that our improved CDB method is effective for gene editing in TKS.

The efficiencies of current tissue culture-dependent methods of genetic transformation all exhibit genotype-dependence.¹ We tested our improved CDB method on three additional TKS varieties. For all the three varieties, namely 20112, 1151, and TKS-L, the editing of the *TKPDS* gene was successfully accomplished (Figures 1H & I). Within approximately two weeks, we obtained homozygous albino TKS seedlings. In the 20112 variety, 8 transgenic seedlings were generated from 26 root segments, with 3 exhibiting editing. For the 1151 variety, 6 transgenic seedlings were obtained from 19 root segments, with 2 exhibiting editing. Similarly, in the TKS-L variety (which has three *PDS* alleles), 10 transgenic seedlings were obtained from 25 root segments, with 3 exhibiting editing (Figure 1J).

We attempted to further improve the TKS transformation method by simplifying the *Agrobacterium* infection step. Instead of individually coating the cut ends of each root segment, we immersed the root segments into *Agrobacterium* suspension for 30 minutes and then placed them onto moist vermiculite for cultivation. Albino gene edited TKS plants were generated from these *Agrobacterium*-soaked root segments (Figure 1K).

These findings collectively show that our improved CDB method can be used to effectively and rapidly generate stable gene-edited TKS plants. We termed this improved method an Extremely Simplified CDB (ES-CDB) method. The CDB method we have published does not require the labor-intensive and lengthy tissue culture process and does not even need sterile conditions, making it faster and much simpler compared to the conventional tissue culture-dependent plant transformation method.³ Compared to the published CDB method, the ES-CDB method here is even faster and simpler for TKS plant transformation and gene editing. Firstly, the ES-CDB method is significantly faster, requiring only ~2 weeks to obtain transformed or gene edited shoots compared to the ~14 weeks needed for the CDB method. Secondly, the ES-CDB method is further simplified. The ES-CDB method bypasses the steps of hairy root induction and cultivation of the CDB method, involving only root cutting and infecting the root segments with *Agrobacterium*. The root infection can be made extremely easy and high throughput by simply immersing the root segments in *Agrobacterium* suspension.

In summary, we have developed an extremely simple and fast method for TKS plant transformation and gene editing. TKS has significant economic value and complete genome information.⁵ Our extremely simple and fast ES-CDB method could make TKS transformation commonplace in laboratories, thus facilitating molecular genetics research and genetic improvement on this plant, just as the very simple floral dip plant transformation method has greatly facilitated research on *Arabidopsis*.⁷

The plasmid construction were based on previous research,¹ and the sgRNA sequence is as follows: 5'-TAATCCCAACTCTCCAAAT-3'. *Agrobac-*

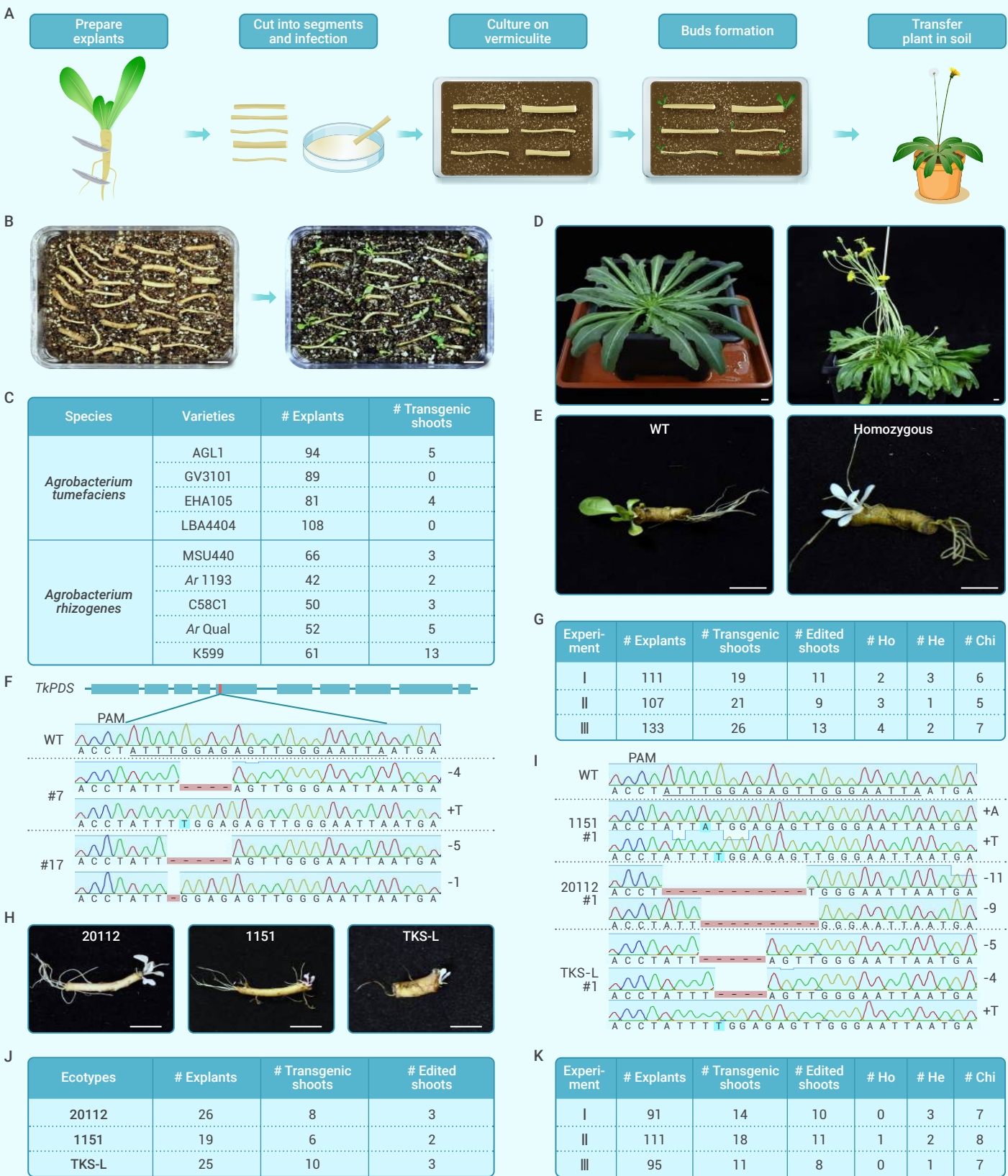


Figure 1. Gene editing in TKS through ES-CDB (A) Schematic diagram of the ES-CDB protocol. (B) Photograph of root segment cultivation and regenerated buds after *Agrobacterium* infection. (C) Statistics of transformation efficiency of nine *Agrobacterium* strains through ES-CDB. (D) Transgenic shoots were transplanted to potted soil for growth. (E) Photograph of wild type (WT) and homozygous *TkPDS* gene edited seedlings of TKS-H. (F) Schematics of the target site (underlined) of *TkPDS* gene and sequencing results of homozygous *TkPDS* edited seedlings of TKS-H. (G) The editing efficiency of TKS-H using ES-CDB. (H) Photograph of homozygous *TkPDS* edited seedlings of three TKS varieties. (I) Sequencing results of homozygous *TkPDS* edited seedlings of the varieties 20112, 1151 and TKS-L. Targeted site was underlined. (J) Numbers of transgenic and edited shoots for three TKS varieties. (K) The editing efficiency of TKS-H by immersing TKS root segments in *Agrobacterium* suspension. PAM: protospacer adjacent motif. Ho: homozygotes. He: heterozygotes. Chi: chimeras. Bar = 1cm.

terium tumefaciens carrying the constructed vector was cultured in LB medium containing the appropriate antibiotics (20 mg/L rifampicin and 50 mg/L kanamycin). *Agrobacterium rhizogene* was cultured in TY medium supplemented with suitable antibiotics (50 mg/L streptomycin and 50 mg/L kanamycin). Individual transformed colonies were picked and inoculated into 3-5 mL liquid medium, and cultured until turbid. Three hundred microliters of the cultured bacteria were spread evenly onto solid medium plates with appropriate antibiotics and incubated overnight. The culture was ready for plant infection once the bacterial growth covered the entire solid medium plate.

The TKS material was grown in a climate chamber with temperatures set at 26/22°C and a light-dark cycle of 16/8 hours. After approximately one month of growth, when the main root reached an average thickness of at least 2 mm, the main roots were cut into 2-3 cm segments. The TKS latex was gently wiped off, and bacterial culture was immediately applied to wounds of segments (or the cut TKS root segments were immersed into the *Agrobacterium* suspension for 30 minutes). The infected TKS root segments were placed on the surface of moist vermiculite, and the containers were covered to maintain humidity. They were then cultivated in a climate chamber with temperatures set at 26/22°C and a light-dark cycle of 16/8 hours for approximately 1-2 weeks. During this period, water was added as needed based on the moisture of the vermiculite. The regenerated buds were examined for transgene presence through the extraction of leaf DNA and PCR amplification using three pairs of primers. Editing was confirmed through Sanger sequencing of the amplified target sites. The PCR primer sequences are as follows: TkPDS-F (5'-TTGTTGAAGTTGGAGCTTACCC-3'), TkPDS-R (5'-TACCATTCAATGGTGAGGC-3'), Cas9-F (5'-CAGAAAGAGCGAGGAAACCA), Cas9-R (5'-CCTCAAACAGTGTGAGGGTCA-3'), GFP-F (5'-ATGGTGAGCAAGGGCGAGGAGCTG-3'), GFP-R (5'-TTACTTGTACAGCTCGTCCATGCCGAGAG-3'), TU6-F (5'-GCGATTAAGTTGGGTAACGCC-3'), TU6-R (5'-CGGACTAGCCTTATTTTAACTTGC-3').

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DECLARATION OF INTERESTS

J.-K.Z is an Editorial Board member of The Innovation Life. He was blinded from reviewing or making final decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board member and their research groups. The other authors declare that they have no conflicts of interest.

ETHICAL STATEMENT AND PATIENT CONSENT

Not Applicable.

DATA AND CODE AVAILABILITY

Data generated or analyzed during this study are included in the published article. Any additional information in this paper is available from the lead contact upon request.