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TRANSGENE-FREE GENOME EDITING IN TOBACCO MODELS CAN BE FACILITATED BY THE GENE ENCODING D-AMINO ACID OXIDASE, A CONDITIONAL NEGATIVE SELECTION MARKER

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SUMMARY

Although CRISPR/Cas9-mediated genome editing has become a common technology in gene manipulation, its application to commercial crop species still needs the development of methodology for generating transgene-free genome-edited plants. The transient expression of morphogenesis inducer, *ipt*, typically stimulates shoot formation and, thus, serves as a positive selection marker in different plant species. Employing a conditional negative selection maker, *DAO1*, combined with *ipt*, sought to streamline the screening of transgene-free genome-edited plants. We first generated transgenic tobacco lines expressing *DAO1* encoding D-amino acid oxidase, which reportedly conditions the resistance to a few toxic D-amino acids and the sensitivity to some non-toxic D-amino acids, and chose appropriate selection agents. The negative selection condition optimization with different *DAO1* tobacco lines became applicable to test the efficiency of genome editing mediated by the transient expression of CRISPR/Cas9. The negative selection with 10 mM D-valine reduced the number of vigorously growing shoots, resulting in enriching transgene-free shoots. This method allowed for the isolation of transgene-free, genome-edited plants with 1.5 times greater efficiency. Our strategy, which combines *ipt*-mediated shoot formation with the conditional negative selection marker *DAO1*, can be functional in other crop species to enhance the precision and applicability of genome editing in various plant species.

Keywords: CRISPR/Cas9, transgene-free, genome editing, morphogenesis inducer, negative selection marker

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Key findings: We optimized a methodology for generating transgene-free genome-edited plants using CRISPR/Cas9 by combining the transient expression of the morphogenesis inducer *ipt* with the conditional negative selection marker *DAO1*. The introduction of *DAO1* in tobacco lines allowed us to identify and apply the appropriate selection agents, with 10 mM D-valine effectively reducing the number of vigorously growing shoots. This approach successfully enriched the population of transgene-free shoots, demonstrating the efficiency of the developed selection system.

INTRODUCTION

CRISPR/Cas9 technology has become a common tool for genetic modification in plants. Expectations for it to solve various problems in agricultural production heightened through revolutionary breeding technology. CRISPR/Cas9 enables researchers to modify target genes precisely, creating favorable plant traits (Nerkar *et al*., 2022). This technology has long improved different crop species. However, the conventional CRISPR/Cas9 technique often employs transgenic technology, in subjection to public concerns over potential impacts on food security and the natural environment (Woźniak-Gientka *et al*., 2022). Moreover, a strict constraint arose on the practical or commercial use of transgenic plants in the field (Turnbull *et al*., 2021). Sidestepping such social and regulatory issues, a growing number of studies have developed methods or protocols of transgene-free technology to manipulate crop traits (Gu *et al*., 2021). As far as annual and genetically fixed crop species are concerned, segregating transgenes from the desired trait gene is possible by genetic cross after generating it in CRISPR/Cas9 transgenic plants. However, genetic cross alters the gene architecture of heterozygous or genetically unfixed crops, which include many important crops, such as, fruit trees or tuber crops. Therefore, genetic crosses are inapplicable to remove the transgenes after genome editing for such crops. Thus, requiring efficient and solid technology for genome editing of those crops.

Transient expression-mediated genome editing (TEGE) can be one of the most versatile technologies for transgene-free genome editing because of the wide applicability of Agrobacterium-mediated gene transfer. Improving the methodology of TEGE led the study to use tobacco models, which have been widely used in plant genetic engineering studies and are efficient in transformation and regeneration, and therefore, suitable for technological development. A previous report shared enhancing TEGE in the tobacco model can occur by the combined treatment of 3-day antibiotic selection and 24-hour heat treatment at 37 °C (Jamaluddin *et al*., 2024). In the study, scientists used the *ipt* gene, a morphogenic inducer, which also serves as a positive selection marker. Although the combined treatment significantly improved the efficiency of obtaining transgene-free genome-edited shoots, still many transgenic shoots emerged, accounting for more than 50%.

The reduction of transgenic shoots in our technology would further improve the TEGE efficiency. Reports declared several genes served as a negative selection maker to remove cells with the gene from a cell population (Shimatani *et al*., 2015). One such gene codes for D-amino acid oxidase (DAAO). DAAO converts toxic D-alanine and D-serine to non-toxic forms, serving as a positive selection marker in plants. Likewise, it converts non-toxic D-asparagine, D-isoleucine, and D-valine to toxic forms, serving as a negative selection marker (Erikson *et al*., 2004). In addition to functioning as a nuclear gene, it appeared to function as a chloroplast gene (Gisby *et al*., 2012). Its application eliminated transgenic progenies from CRISPR/Cas9-transgenic Arabidopsis to enrich transgene-free mutants.

Further improving the procedure prompted the research to examine the effect of negative selection on the efficiency of isolating transgene-free, genome-edited plants in the presented study (Figure 1). Researchers employed D-amino acid oxidase as a negative selection marker. The addition of the negative selection after the combined treatment above and subsequent non-selective culture decreased the ratio of transgenic shoots. The

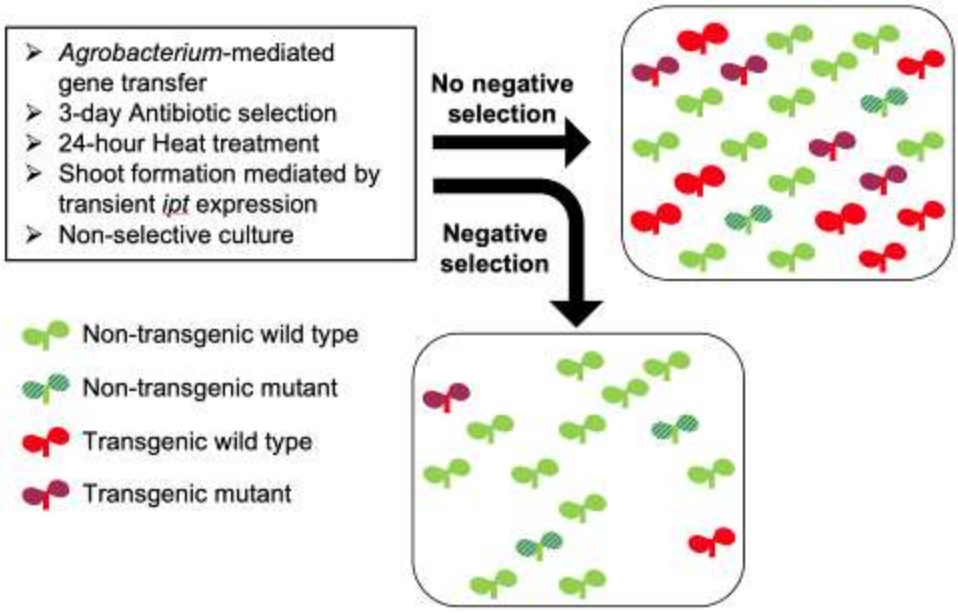

Figure 1. Scheme of the effect of negative selection. The expected effect of negative selection in transient expression-mediated genome editing is schematized.

CRISPR/Cas9-mediated mutation rates within non-transgenic shoots showed unaffected. Thus, the negative selection improved the isolation efficiency of transgene-free, genome-edited plants.

MATERIALS AND METHODS

DNA constructs

This study utilized two different binary vectors: pRI-PcUBI-OsADH-SpCas9-Hyg (Kaya *et al.*, 2016) to generate *DAO1* transgenic plants and pMKUA2iSpCas9 (Jamaluddin *et al*. 2024), a derivative of pMKV057 (Addgene #133312) (Maher *et al.*, 2020) for TEGE experiments. The coding sequence of *DAO1* from *Rhodosporidium toruloides* underwent custom-synthesis by Eurofins Genomics (Tokyo, Japan), based on the nucleotide sequence from the Genbank accession No. AF003339. The coding sequence, excised by XbaI and SacI, proceeded introducing into pRI-PcUBI-OsADH-SpCas9-Hyg, as digested with the same enzymes, resulting in pRIU-DAAO-Hyg for generating *Dao1* transgenic plants. The coding sequence amplification by PCR ensued with PrimeSTAR GXL DNA polymerase (TakaraBio, Kusatsu, Japan), using a primer set: NOSp-Dao1-F (GCTAGGTCACAGAAGCACCATGCACTCTCAGAA GCG) with Dao1-St_PI_II-polyA-R (CTCCTATGCCTGATGCTTACTACAACTTCGACTC CCGC). The fragment introduction into pMKUA2iSpCas9 digested with AfeI and SpeI resulted in pMDUA2iSpCas9 for expressing the *DAO1* along with *ipt* and *Cas9*. The pMDUA2iSpCas9 bore insertion with the sgRNA sequences for MAR1 and PDS1 targets, as described previously (Jamaluddin *et al*. 2024). The introduction of plasmids continued into Agrobacterium tumefaciens strain EHA105 (hereafter referred to as EHA105).

Chemicals

All purchased D-amino acids (D-alanine, D-asparagine, D-serine, D-isoleucine, and D-valine; hereafter, D-Ala, D-Asn, D-Ser, D-Ile, and D-Val, respectively) came from Tokyo Chemical Industry (Tokyo, Japan). MS salts mixture and other plant culture media components were procurements from Fuji film Wako Pure Chemicals (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan).

Plant growth and transformation

The experiment had tobacco plants (*N. tabacum* cv. Petit Havana SR1) grown aseptically on the MS-VHf media (MS basal medium containing 3% sucrose, 5 mM K-MES, 0.8% agar, and pH 5.7) and subjected to transformation as described previously (Jamaluddin *et al*., 2024). The leaf pieces' inoculation continued with EHA105 harboring pRIU-DAAO-Hyg, co-cultured for three days on a non-selective regeneration medium RMOP (Rahman *et al*., 2022) and transferred to selective plates containing 30 mg L^{-1} hygromycin until shoot regeneration. Leaves inoculated with EHA105 harboring pMDUA2iSpCas9 derivatives underwent co-culturing for three days on non-selective phytohormone-free MS medium (MS-HF), and then, applied to short-term selection on MSHF medium containing 50 mg L^{-1} kanamycin for three days. Afterward, applying to one-day heat treatment had a temperature at 37 °C on the second day of the selection, and then culture on a non-selective MSHF until shoot regeneration (Jamaluddin *et al*., 2024). The transfer of some leaf pieces with shoots proceeded to negative selection media.

Evaluation of selective conditions

The positive selection agents, D-Ala and D-Ser, dissolved in pure water and filter-sterilized, proceeded to add at 1, 3, 5, and 10 mM for D-Ala and 10 mM for D-Ser to RMOP after autoclaving. Transgenic and non-transgenic control plant leaves, cut into small pieces, continued placement onto selective plates to evaluate the selection efficacy. The addition of negative selection agents, 10, 30, and 50 mM of D-Asn, D-Ile, and D-Val, ensued while preparing the media, followed by autoclaving. Setting these concentrations employed the method of Gisby *et al*. (2012), who found that 6 mM D-Val suppressed shoot regeneration of *DAO1* transplastomic plants. Transgenic and non-transgenic control leaf pieces preceded culturing on a non-selective RMOP. Leaf pieces with tiny shoots' transfer to the negative selection media followed, monitoring for shoot growth and survival. For screening transgene-free shoot isolation, we used 50 mM D-Ile and 10 mM D-Val. Transgenic plants application for 2 weeks on negative selection transpired, with some of them grown further on MSHf without D-amino acid and served as a control.

Detection of transgenes

DNA extraction from selected shoots occurred, as described previously (Jamaluddin *et al*., 2024). The *DAO1* transgene detection used PCR with GoTaq master mix (Promega, Madison, WI, USA) using the primer set of DAAO-SF (CTGCCAGTACCTTGCAAGA) and DAAO-SR (CCTGGTCGGGGAATGATGTA). Transgenes in pMDUA2iSpCas9 derivatives reached detection, as described previously with PCR primers GemSF1 (GTGTCGTCCGAGACTTCTA) and GemSR4 (CCCAAGTGCTCATCTCAAA) (Jamaluddin *et al*., 2024). The PCR conditions were as follows: 94 °C, 2 min; 94 °C, 20 s; 60 °C, 20 s; 72 °C, 1 min; 72 °C, 5 min; and 35 cycles.

Detection of CRISPR/Cas9-mediated mutations

The mutations in MAR1 targets' detection came by PCR and CAPS analysis, with those in PDS1 targets detected by the heteroduplex mobility assay (HMA) using MCE-202 MultiNA (SHIMADZU, Kyoto, Japan) with a DNA-500 kit, as described previously (Jamaluddin *et al*., 2024). For the analysis of TEGE, researchers confirmed whether each shoot had the transgene or not and tested both transgenic and non-transgenic shoots for the mutation in the target sites. Each of the plants belonged to one of the categorized four groups: transgenic wild types, transgenic mutants, non-transgenic wild types, and non-transgenic mutants.

RESULTS AND DISCUSSION

Experimental design

We have designed the protocol for the improvement by *DAO1* of the transient expression-mediated genome editing as follows (Figure 1). First, plant materials' subjection to

Agrobacterium-mediated gene transfer by three-day co-culture, followed by three-day antibiotic selection with 24-hour heat treatment on the second day. During these steps, the expression of the *ipt* gene induces shoot formation and that of CRISPR/Cas9 system genes makes the genome edits. Then, culturing the materials under non-selective conditions allowed the growth of both transgenic and non-transgenic shoots with a history of gene transfer. Under the protocols, we have demonstrated more than half of the regenerated shoots were transgenic (Jamaluddin *et al*., 2024). Negative selection expectantly reduced the number of transgenic shoots and facilitated the screening of non-transgenic, genome-edited plants. With the residual expression of *DAO1* having deleterious effects on shoot regeneration, the time for starting negative selection is critical for establishing an efficient protocol.

Evaluation of D-amino acids

First, testing the toxic effects of D-Ala and D-Ser on non-transformed tobacco shoot formation commenced because of their known ability to serve as a positive selection agent. We found that D-Ala has a more rapid effect on tobacco shoot formation than D-Ser, which allowed a visible growth of tobacco leaf pieces (Figure 2A). After transformation with pRIU-

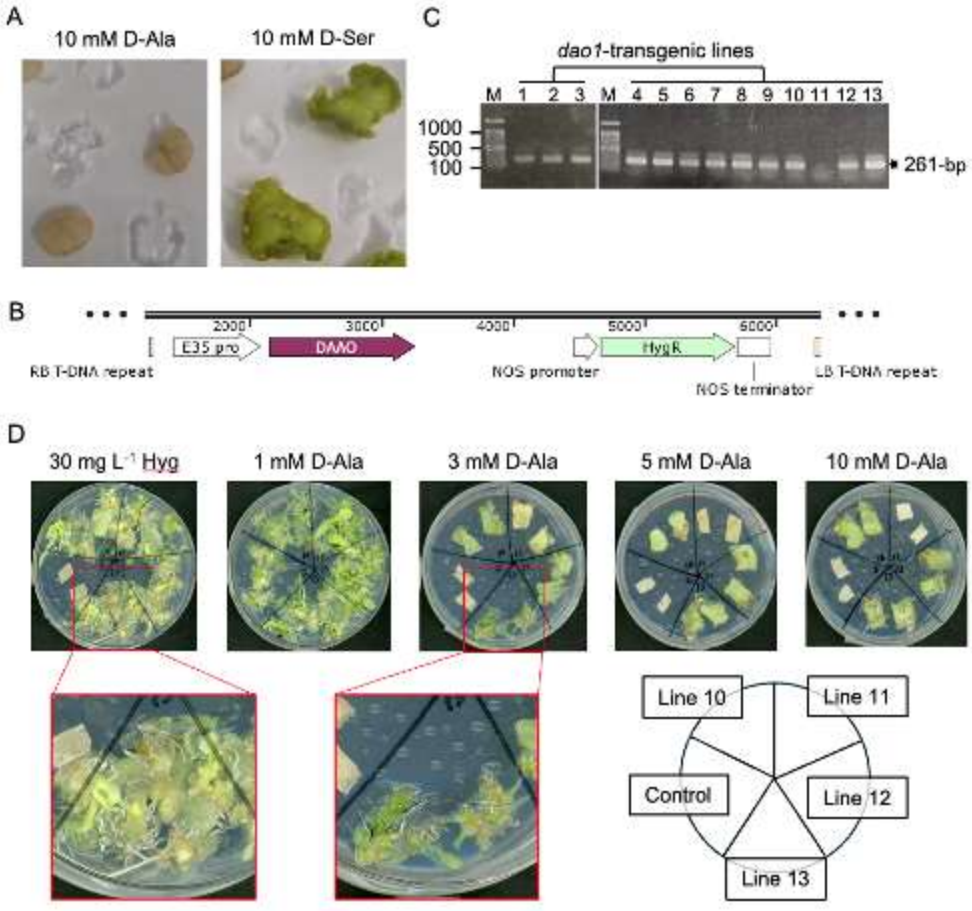

Figure 2. Evaluation of D-amino acids as positive selection agents. (A) Comparison of toxicity of D-Ala and D-Ser on non-transformed tobacco shoot formation. (B) Map of pRIU-DAAO-Hyg T-DNA region. (C) PCR detection of *DAO1* gene segments in transgenic lines. (D) Effect of different concentrations of D-Ala on shoot formation in transgenic lines. Positions in the plates of control non-transformation and transgenic lines are visible at the bottom right.

DAAO-Hyg (Figure 2B) and hygromycin selection, the study obtained 13 independent lines of hygromycin-resistant shoots, one without the *DAO1* transgene (Figure 2C, line 11). The transgenic lines grown further continued to the test to optimize D-Ala concentration for positive selection.

All transgenic lines, including line 11 lacking the *DAO1* transgene, produced many shoots on the regeneration media containing hygromycin, but non-transformed control plants did not (Figure 2D). Although all materials, including line 11 and non-transformed control, produced shoots in the presence of 1 mM D-Ala, shoot formation in line 11 and control incurred suppression by 3 mM or higher concentration of D-Ala (Figure 2D). The results suggest that 3 mM D-Ala could be suitable for positive selection. However, the shoot formation in the presence of 3 mM D-Ala was much less active than that on hygromycin media. The findings reveal *DAO1* is not suitable for positive selection, although earlier reports indicated it as an efficient positive selection agent (Erikson *et al*., 2004; Wang *et al*., 2024). The inconsistency may reflect the difference in selective agents or materials and processes: seed germination vs. shoot formation from leaf pieces.

Evaluation of D-amino acids for negative selection of *DAO1* transgenic tobacco

Before starting the genome editing research, we first determined the appropriate concentration of D-amino acids for the negative selection. Setting up the experimental conditions primarily followed a study by Gisby *et al.* (2012), which reported 6 mM D-Val suppressed shoot regeneration from leaf pieces of *DAO1* transplastomic tobacco lines. In our protocol, however, we anticipated the suppression when starting negative selection before shoot initiation because of a residual transient expression of *DAO1*, as mentioned above. Therefore, first inducing shoots of *DAO1* transgenic tobacco lines transpired, with the preformed shoots evaluated for the effect of negative selection agents also at higher concentrations of D-amino acids.

For the control experiment, we cultured the preformed shoots on RMOP containing hygromycin without D-amino acid. The small shoots of transgenic lines grew well in the control experiment (Figure 3A). We tested D-Asn, D-Ile, and D-Val at 10, 30, and 50 mM. The D-Asn had only a little effect on shoot growth, even at 50 mM. However, D-Ile and D-Val suppressed shoot growth in a dose-dependent manner (Figure 3A). Notably, D-Val hindered the shoot growth even at 10 mM. The 50 mM D-Ile and 10 mM D-Val did not affect the shoot growth of non-transformed control plants (Figure 3B). The results suggest a higher concentration of D-amino acids is necessary for suppressing the growth of preformed shoots than for suppressing the shoot initiation.

Effect of negative selection with D-amino acids on transgene-free shoot isolation

Next, we implemented the results from the earlier experiments to the transient expression-mediated genome editing protocol. Plant materials inoculated with Agrobacterium harboring pMDUA2iSpCas9 (Figure 4A) received the combined treatment of three-day antibiotic selection and 24-hour heat treatment. We applied a two-week negative selection with 50 mM D-Ile and 10 mM D-Val after a three-week non-selective culture (Figure 4B). The transgenic shoots, cultured on media without D-amino acids as the control group, grew well during the two weeks. Treating with 50 mM D-Ile showed no apparent effects on shoot growth. By contrast, 10 mM D-Val treatment was effective: some shoots failed to grow (red arrows), and others grew well (white arrows). Furthermore, some new shoots (blue arrow) emerged during the negative selection culture.

Tests for transgene highlighted the effect of the negative selection. Although the transgene-positive ratios were more than 60% in the control without D-amino acid and 50 mM D-Ile groups, those of 10 mM D-Val group were 38% (Figure 4C, and 4D Tg). The results demonstrate the negative selection with 10 mM D-Val can enrich transgene-free shoots to 1.5-fold, from less than 40% to more than 60%.

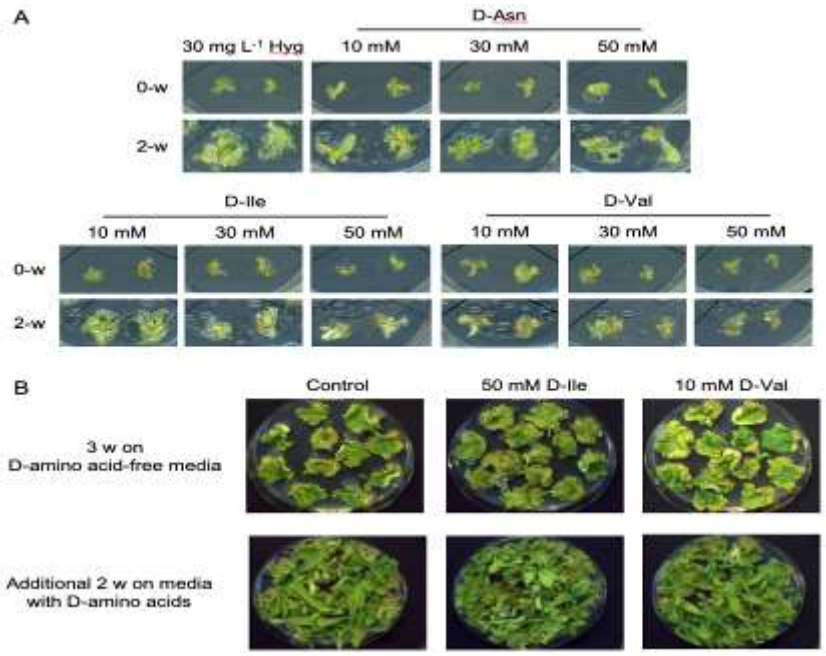

Figure 3. Evaluation of D-amino acids as negative selection agents. (A) Effect of D-Asn, D-Ile, and D-Val on transgenic shoot growth. (B) Effect of D-Ile and D-Val on non-transgenic shoot growth.

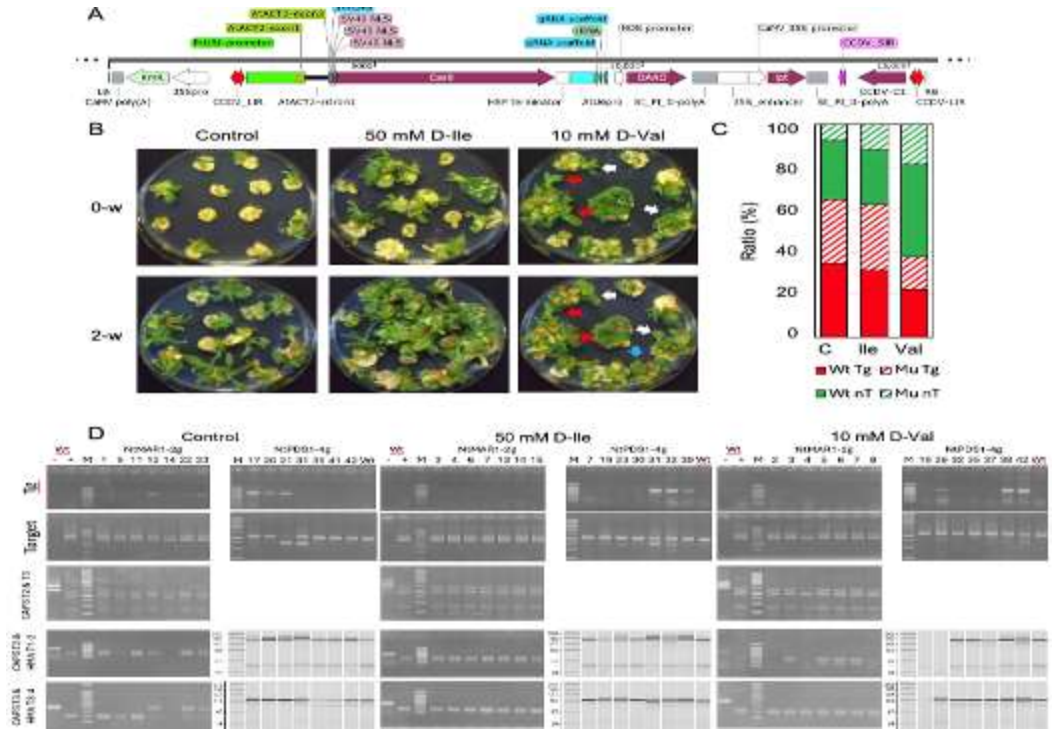

Figure 4. Effect of negative selection on screening of transgene-free, genome-edited plants. (A) Map of pMDUA2iSpCas9 T-DNA region. (B) Shoot growth in 2-week negative selection in transient expression-mediated genome editing. (C) The ratios of wild-type transgenic (Wt Tg), mutant transgenic (Mu Tg), wild-type non-transgenic (Wt nT), and mutant non-transgenic (Mu nT) shoots. C = control without D-Amino acid, Ile = D-Ile, Val = D-Val. (D) Representative results of transgene and mutation detection.

For the mutation analysis of the MAR1 and PDS1 targets, we analyzed 90 shoots from each group (Figure 4D, representative results). The control group showed the lowest mutation rates: only 10 mutants in non-transgenic shoots (Figure 4D, sample nos. 31, 35, 41, and 42). The 50 mM D-All treatment group displayed comparable results with 12 mutant shoots (Figure 4D, sample nos. 7, 19, 23, and 30). In contrast, the 10 mM D-Val group revealed higher mutant counts: 19 shoots of non-transgenic mutation found (Figure 4D, sample nos. 4, 32, 35, and 37). In control and 50 mM D-Ile groups, 10%–12% of shoots were transgene-free and genome-edited (Figure 4C). The ratios were slightly higher than in the previous study (Jamaluddin *et al*., 2024). In the 10 mM D-Val group, the ratio of transgene-free and genome-edited shoots increased to 21%, representing a 1.5-fold improvement as in the transgene-free ratios (Figure 4C).

In the experiments above, we found negative selection with 10 mM D-Val improved the efficiency of the transient expression-mediated genome editing by enriching the transgene-free shoots. However, the negative selection condition failed to eliminate transgenic plants: 38% of shoots were transgenic. The survival of transgenic shoots might have resulted from lower expression levels of *DAO1* in pMDUA2iSpCas9 than in pRIU-DAAO-Hyg, with *DAO1* driven by a weak Nos promoter and a strong ubiquitin promoter, respectively. In earlier studies with *DAO1* transplastomic plants or transgenic plants (Gisby *et al.*, 2012), *DAO1* as driven by a strong 35S promoter (García-Almodóvar *et al*., 2014), the *DAO1* expression levels must have been higher than in our TEGE system, and those plants showed high sensitivity to *DAO1*.

This study presents a method to enhance the TEGE, resulting in mutant plants without foreign genes and mitigating potential long-term and off-target effects associated with stable integration of foreign genes. Various studies, including our previous one, have explored techniques for producing genome-edited plants without integrated foreign genes. Chen *et al.* (2018) utilized an Agrobacterium-mediated transient expression system and a high-throughput screening method based on Illumina sequencing and high-resolution melt (HRM) analysis. Although Chen's approach effectively generated non-transgenic mutant plants with an overall non-transgenic mutation rate of 8.2%, our research achieved higher efficiency, making it a better approach to generating plants free of transgenes. Our research offers advantages in selecting transgene-free plants by combining a positive selection marker, *ipt*, and a conditional negative selection marker, *DAO1*. Optimizing negative selection conditions with different *DAO1* transgenic tobacco lines led us to a solid negative selection, leading to a 1.5 times increase in isolating transgene-free and genome-edited plants (21% of all plants examined).

The research utilized the *ipt* gene as a positive selection marker. Umemoto *et al.* (2023) also found the transient expression of the *ipt* gene promoted regeneration and shoot induction in potatoes. They intended to edit the potato genome through a procedure similar to ours. However, they found only nine mutants out of more than 1000 shoots. Although, in a different plant species, our methodology yielded transgene-free genome-edited shoots up to 21% of the shoots. The advantage of our method is likely due to two key technologies: one is the combined treatment with short-term antibiotic selection and heat treatment described before, and the other is the dual selection strategy presented in this report. As they successfully induced potato shoots by a transient expression of the *ipt* gene, our strategy could apply to potatoes and, possibly, other crop plant species.

This study successfully applied the *DAO1* gene as a negative selective marker in the T_0 population to enrich shoots induced by the transient expression of *ipt*. The same gene has been functional in different studies, including one by García-Almodóvar *et al*. (2014), applying the negative selection to T_0 transformants to obtain marker-free transgenic plants. However, the study described its application to TEGE here for the first time. A recent study by Wang *et al.* (2024) used *DAO1* as a dual selection marker to ensure the genome editing and remove the *Cas9*-containing progenies in Arabidopsis. They

applied the positive selection in T_1 generation after floral-dip transformation, which is equivalent to T_0 generation in tissue culture-mediated transformation. However, applying negative selection continued to the next generation after segregation. By contrast, our method applies the negative selection during shoot growth of T_0 generation. Therefore, our method can be suitable to plant species, such as trees or tuber crops, where sexual reproduction abolishes the existing haplotype combination or is difficult.

Our approach facilitated the generation of non-transgenic genome-edited plants without worries about genetic stability and off-target effects. The prolonged expression of Cas9 can cause genetic instability and off-target mutation. Therefore, their reduction is possible by using transiently transfected plasmid DNA or non-integrating viral vectors to minimize nuclease exposure (Yee, 2016). A study used a virus vector to express split-SaCas9 to facilitate the alleviation of prolonged Cas9 expression (Kaya *et al.*, 2017). The use of negative selection presented in this study could be an alternative way to avoid genetic instability and off-target mutations caused by CRISPR/Cas9.

The strategy the study developed can be appropriate to other crop species where *ipt* can induce shoot formation and sensitive to toxic compounds converted by *DAO1*. This approach addresses concerns and regulations related to transgenic plants by enabling the use of genome-edited crops without foreign DNA. This is especially beneficial for fruit trees and tuber crops, where traditional genetic crosses are not possible. It improves the precision and versatility of genome editing across different plant types.

CONCLUSIONS

The presented study examined the negative selection condition with D-amino acids and *DAO1* transgene and found selection with 10 mM D-Val can enrich transgene-free shoots induced by the transient expression of *ipt* morphogenic inducer. This negative selection facilitated the isolation of transgene-free, genome-edited plants, with 1.5-fold efficiency.

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