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COMBINED EFFECT OF SHORT-TERM ANTIBIOTIC SELECTION AND HEAT TREATMENT ON TRANSIENT EXPRESSION-MEDIATED GENOME EDITING IN A MODEL PLANT TOBACCO

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SUMMARY

CRISPR/Cas9-mediated genome editing has become a common technology for gene manipulation in plant gene research and crop improvement. Studies have developed technologies with *Agrobacterium*-mediated transient expression of genome-editing machinery to generate transgene-free plants with an edited locus. This study examined the combined effect of short-term (three-day) antibiotic selection and heat treatment (24 hours at 37 °C) on genome editing efficiency in two different systems. Both systems targeted the same two genes, *PDS* and *MAR1*, in tobacco. The first system employed developmental regulator genes (DRs) inducing shoot formation to select plants with transient foreign gene expression. The other relied on phytohormone-induced shoot formation. The DRs, including the cytokinin-producing *ipt* gene, induced tobacco shoot formation after *Agrobacterium*-mediated transient expression. The combined treatment and heat treatment alone reduced the shoot formation, but the three-day selection alone did not. Interestingly, the combined treatment elevated the percentage of transgene-free mutant shoots to 7.6% as compared to 1.2% with heat treatment alone in *ipt*-induced shoots. In the shoots induced by the externally added phytohormone, the percentages of transgene-free mutant shoots were 2.8%, 5%, and 7.7% after three-day antibiotic selection alone, heat treatment alone, and the combined treatment, respectively.

Keywords: Genome editing, transgene-free, mutation, heat treatment, antibiotic selection

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Key findings: The study investigated the impact of combining three-day antibiotic selection and 24-hour heat treatment on CRISPR/Cas9-mediated genome editing in a model plant, tobacco. The combined treatment significantly increased the percentage of transgene-free mutant shoots compared with individual treatments, regardless of shoot induction modes: transient expression of morphogenic inducer or optimized phytohormones in a medium.

INTRODUCTION

CRISPR/Cas9 technology has been revolutionizing plant sciences, offering solutions to various challenges in agriculture. CRISPR/Cas9 enables precise modification of target genes, offering unprecedented control over plant traits (Gao, 2021). By harnessing this technology, researchers aim to improve different crop species efficiently. However, conventional CRISPR/Cas9 genome editing has used transgenic technology. Thus, concerns regarding integrating foreign genes into plant genomes and their potential environmental impacts also accompany genome editing technology (Kuzma *et al*., 2023). In this context, the development of transgene-free methods for crop improvement has garnered considerable attention (Gu *et al*., 2021). Researchers have explored innovative approaches leveraging CRISPR/Cas9 genome editing technology to address this issue.

Plant genome editing involves three steps: introducing and functioning genome-editing machinery into plant cells, regeneration of plants from the genome-edited cells, and selection of genome-edited cells or plants before or after plant regeneration. Efficient transgene-free genome editing requires some innovation in all these three steps. Heat treatment enhanced CRISPR/Cas9-mediated mutagenesis efficiency significantly (Kurokawa *et al.*, 2021; LeBlanc *et al.*, 2018), most likely through enhancing the Cas9 enzymatic activity. The use of developmental regulators (DRs), such as transcription factors involved in meristem maintenance, such as maize *WUSCHEL* 2 (*Wus2*) (Lowe *et al*., 2016) and *SHOOT MERISTEMLESS* (*STM*) (Aida *et al*., 1999), or cytokinin biosynthetic isopentenyl transferase gene (*ipt*; Smigocki and Owens, 1988; Ebinuma *et al*., 1997) has been shown to enhance shoot regeneration and served as a selective marker. A study has shown the use of DRs facilitates side-stepping tissue culture (Maher *et al*., 2020). The transient expression of *ipt* has appeared to induce shoot formation in potatoes (Umemoto *et al*., 2023). Moreover, a short-term antibiotic selection was shown to help transient expression-mediated genome editing (Bánfalvi *et al*., 2020).

This study tested the combined effect of three-day antibiotic selection and heat treatment to enhance transgene-free genome editing in tobacco (*Nicotiana tabacum*) in two different transformation/regeneration systems in tobacco. One relied on its shoot induction by the transient expression of *Wus2* and *ipt* on phytohormone-free media and is hereafter referred to as the DRs system. The other did not involve the DRs and is henceforth called the non-DR system. Here, a report states the combined treatment of three-day antibiotic selection and 24-hour heat treatment enhanced the transient expression-mediated genome editing in both systems. The findings from this research hold significant implications for the development of sustainable and environmentally friendly crop improvement strategies, paving the way for the broader adoption of transgene-free technologies in agriculture.

MATERIALS AND METHODS

DNA constructs

In the DRs system, researchers used pRNUA2iSpCas9 and pMKUA2iSpCas9 (Figure 1), the derivatives of pRN227 (Addgene #127222) and pMKV057 (Addgene #133312) [generous gifts from Dan Voytas via Addgene] for the expression of *Wus2* and *STM* (*Wus2*+*STM*) and *Wus2* and *ipt* (*Wus2*+*ipt*), respectively (Maher *et al*., 2020). In the non-DR system, researchers used pRIUA2iSp-Cas9-Hyg and its derivatives (Figure 1), constructed

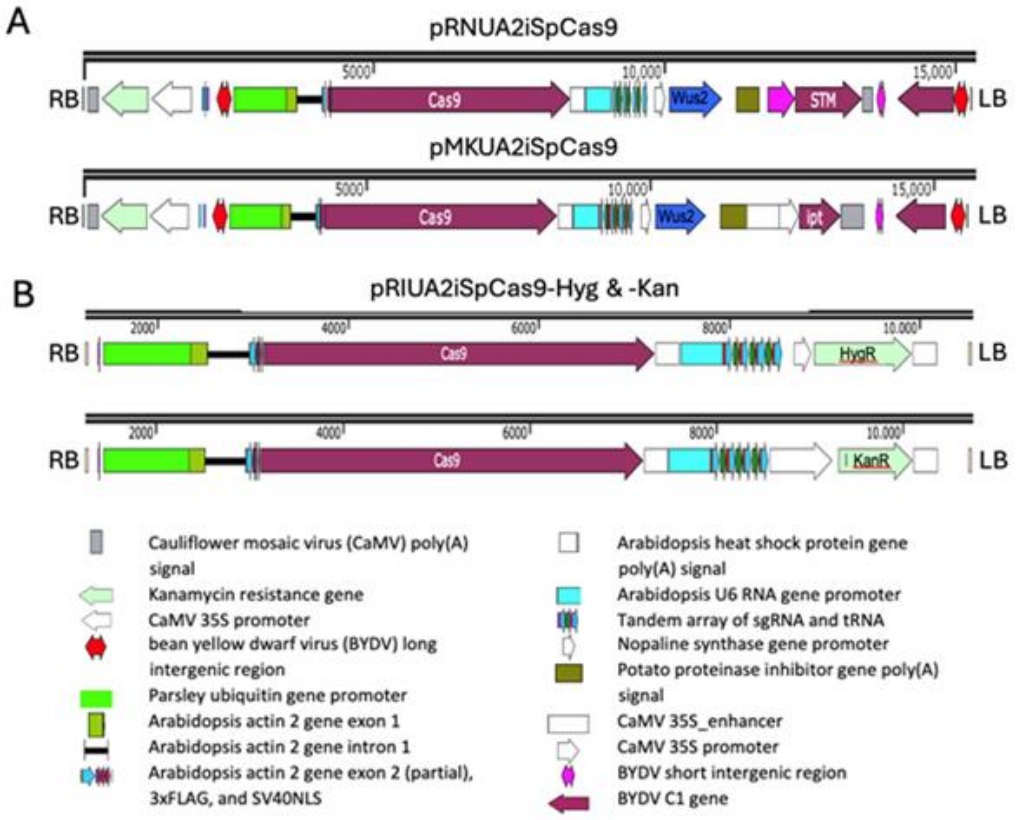

Figure 1. Schematic representation of the plasmids used in this study. A: Plasmids for the DR system. The plasmids pRNUA2iSpCas9 and pMKUA2iSpCas9 differ in encoding DRs, *Wus2,* and *STM* or *Wus2* and *ipt*, respectively. B: Plasmids for the non-DR system. The plasmids pRIUA2iSpCas9-Hyg and pRIUA2iSpCas9-Kan differ in selective markers, hygromycin, and kanamycin resistance genes, respectively. Other components are listed at the bottom.

from pRI-PcUBI-OsADH-SpCas9-Hyg described previously (Kaya *et al*., 2016). The modification of the Cas9 expression unit, UA2iSpCas9, had the OsADH, rice alcohol dehydrogenase 5'-untranslated region of pRI-PcUBI-OsADH-SpCas9-Hyg replaced with Arabidopsis actin 2 sequence containing intron 1/enhancer (An *et al*., 1996) and introduced into all the constructs.

Two target sequences for the *N. tabacum* homologs of Arabidopsis *MAR1* (NtMAR1; Figure 2A) had previous reports (Rahman *et al*., 2022), assembled with a sgRNA scaffold and a tRNA sequence as described by Xie *et al*. (2015) using primers NtMar1-F and -R (Table 1) to obtain NtMAR1-2gRNA. Four target sequences for the *N. tabacum* phytoene desaturase (*PDS*) gene (NtPDS1; Figure 2B), including one reported before (Chen *et al*., 2018), bore assembling to obtain NtPDS1-4gRNA. Afterward, its amplification used primer pairs of HiFi-U6pro-sgR-F and -R or Bbs-NtPDS1-1F and NtPDS1-4-Bbs-R (Table 1) before introducing into Cas9 expression vectors. The DRs system constructs have a kanamycin resistance selective marker gene (Km^R), and the original non-DR system constructs contain hygromycin resistance marker gene (Hyg^R). The Km^R segment amplified with primers HiFi-KmF and HiFi-KmR (Table 1) replaced the Hyg^R to obtain pRIUA2iSp-Cas9-Km to compare the effect of different selective agents in the three-day selection.

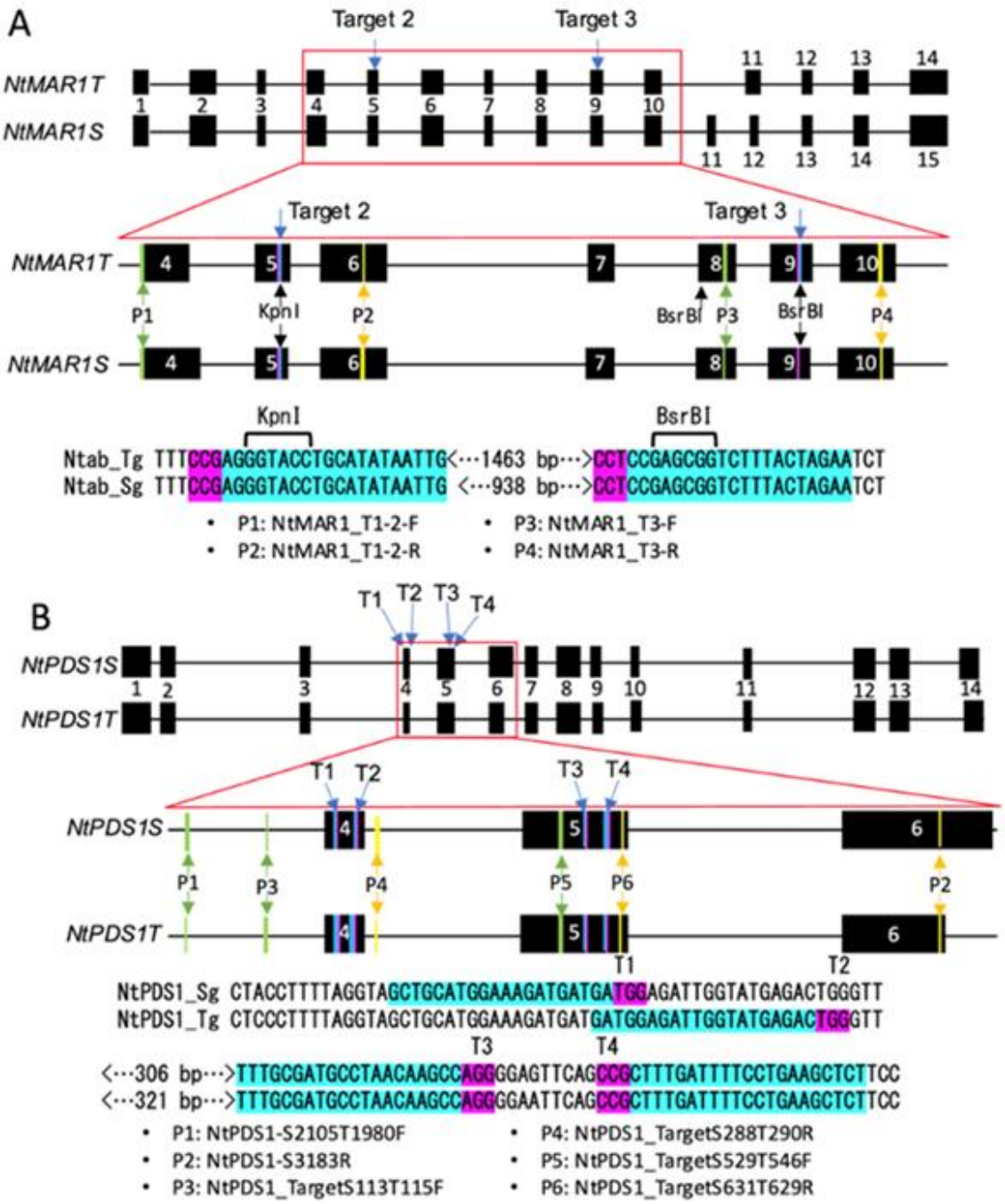

Figure 2. The genome editing targets in tobacco. Schemes and alignments of target sequences in *NtMAR1* (A) and *NtPDS1* genes (B). Exons are shown in filled boxes, and introns are shown with lines. The target sequences and their locations are denoted in blue or cyan and PAMs in magenta. The target sequences in S- (_Sg) and T-genome (_Tg) copies are aligned. The forward and reverse primers for mutation analyses (Table 2) are shown in green and yellow, respectively. The black arrows denote the restriction sites of enzymes used for CAPS analyses. Their recognition sites are shown above in the alignments. T1, T2, T3, and T4, Targets 1-4 in *NtPDS1*. T1 is from a previous study (Chen *et al.*, 2018), while the others were newly designed in this study. T1 and T2 are highlighted in the alignment in either of the S- and T-genome copies because they overlap with each other.

Table 1. List of primers for constructing plasmids.

Primer Name	5'→3' Primer Sequences	Usage
NtMar1 F	'GTGGTGGTGAAGACACATTGCAATTATATG-CAGGTACCCTGTTTTAGAGCTAGAAATAGC'	sgRNA for *MAR1*
NtMar1 R	'GGAGGAGGAGAAGACACAAACCCGAGC-GGTCTTTACTAGAATGCACCAGCCGGG'	
HiFi-U6pro-sgR-F	GCTAGAGTCGAAGTAGTGATTG	sgRNA for PDS1
HiFi-NtPDS4g-R	CTTGCTATTTCTAGCTCTAAAACCT	
Bbs-NtPDS1-1F	GTGGTGGTGAAGACACATTGGCTGCA	Non-DR construct
NtPDS1-4-Bbs-R	GGAGGAGGAGAAGACACAAACCTTTG	
HiFi-KMF	GGTGCTTTTTTTCCCGGGCCCTGAGACTTTTCA	Kanamycin
HiFi-KmR	CAAATGTTTGAACGATCGGGGCCCTCAGAAGAACTC	expression unit

Plant transformation

Transferring all plasmids into *Agrobacterium tumefaciens* strain EHA105 received culturing overnight at 28 °C in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose) containing 20 mg L^{-1} rifampicin and 25 mg L^{-1} kanamycin. The cultures' dilution comprised nine volumes of sterile deionized water. *N. tabacum* cv. Petit Havana SR1 plants proceeded growing aseptically on the MS-VHf media (MS basal medium containing 3% sucrose, 5 mM K-MES, 0.8% agar, and pH 5.7), with the leaves cut into pieces of about 1 cm^2. The leaf pieces sustained immersing in the diluted *Agrobacterium* suspension for 20 min. In the DRs system, the leaves culturing on phytohormone-free MS media (MSHF; MSVHf containing 1 mg L^{-1} thiamine HCl and 100 mg L^{-1} myo-inositol) continued for three days. Some inoculated leaf pieces' transfer to selective media (MSHF containing 50 mg L^{-1} Meropenem and 50 mg L^{-1} Kanamycin) ensued, cultured for three days, before transferring to non-selective media (MSHF containing 50 mg L^{-1} Meropenem) and culturing for one month. The culture of others on the non-selective media served as a control. In the non-DR system, the culture of inoculated leaf pieces occurred on RMOP media (Rahman *et al*., 2022) for three days. Some leaf pieces' transfer to selective media (RMOP containing 50 mg L^{-1} Meropenem and 50 mg L^{-1} Kanamycin or 30 mg L^{-1} Hygromycin) transpired, then cultured for three days before moving to non-selective media (RMOP containing 50 mg L^{-1} Meropenem) for one month culturing. Others reached non-selective media culturing as a control. In both systems, few leaf pieces gained continuous culturing on selective media for about a month to obtain stable transformants.

Detection of transgene

DNA extraction used a one-tube method (Hu and Lagarias, 2020), with a slight modification to include 70% ethanol wash after isopropanol precipitation. The transgenes detection utilized PCR primers CAS9519-F3 and CAS9832-R3 for the *Cas9* gene, GemSF1 and GemSR4 for the DRs system constructs-specific sequences (GemDRs), and Act2-77F and Act2-633R for the Arabidopsis actin 2 intron/enhancer (AtACT2i/e) in the *Cas9* expression unit (Table 2). 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.

Mutation detection

For both *MAR1* targets, using NtMAR1_T1_F and _R (Table 2) with PrimeSTAR GXL DNA Polymerase (Takara Bio, Kusatsu, Japan) had the PCR conditions at 94 °C for 2 min, 30 cycles of 95 °C for 10 s, 55 °C for 5 s, and 68 °C for 30 s, and 68 °C for 7 min. For individual targets of *MAR1*, researchers used primers NtMAR1_T1_F and NtMAR1_T1_R for target 1 and NtMAR1_T2_F and NtMAR1_T2_R for target 2 (Table 2), as above. Using CAPS analysis with restriction enzyme KpnI for target 1 and BsrBI for target 2 (Figure 3) helped detect mutations in NtMAR1 targets 1 and 2. For detecting deletion between four *PDS* targets, the amplification of fragments

Table 2. List of primers for detecting transgene, target genes, and mutations.

Primer Name	5′→3′ Primer Sequences	Amplicon sizes (bp)
CAS9519-F3	F: GGTGGCATACCACGAGAAGT	313-bp
CAS9832-R3	R: GGAGCTGAGCAATGAGGTTC	
GemSF1	F: GTGTCGTCCGAGACTTCTA	716-bp
GemSR4	R: CCCAAGTGCTCATCTCAAA	
Act2-77F	F: GTGCAGCTTGTCTCGTTGTC	556-bp
Act2-633R	R: GCTTTCTGTTCAACGTACGACA	
NtPDS1_TargetS2105T1980F	F: CCYGTCCTGTTGGKTGYATTTCTC	1079-bp
NtPDS1_S3183R	R: TCACGCACTTGCTTTCTCATCCAG	
NtPDS1_TargetS113T115F	F: AGCAGTCACCAAGAATCTAGYT	178-bp
NtPDS1_TargetS288T290R	R: YGCATTCTTGAGGAGTCAAACT	
NtPDS1_TargetS529T546F	F: GRTTGCAGTGGAAGGAACAT	103-bp in Sg &
NtPDS1_TargetS631T629R	R: CCATTTAATGGCGCAGGAAG	84-bp in Tg
NtMAR1_T1-2_F	F: TGCATTCGAACAGCTTTATATGCT	1511-bp in Sg &
NtMAR1_T3_R	R: TGCTTGCTGGAAGAACAGGT	2026-bp in Tg
NtMAR1_T1-2_F	F: TGCATTCGAACAGCTTTATATGCT	597-bp
NtMAR1_T1-2_R	R: CAACAGCTCCCGCTAGAACA	
NtMAR1_T3_F	F: CGGTTTGATGATTGGGGCAC	381-bp
NtMAR1_T3_R	R: TGCTTGCTGGAAGAACAGGT	

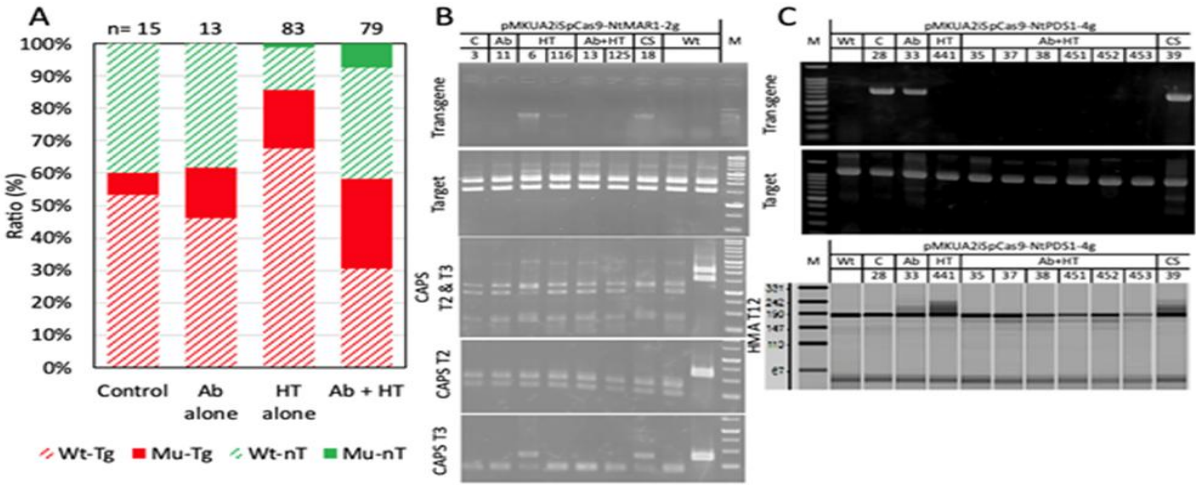

Figure 3. Transgene-free genome editing in the DR system. A: transgene status and targeted mutations in DRs-induce shoots. Percentages of transgenic and transgene-free shoots are shown in red and green, respectively, and those of wild-type and mutant shoots are shown by hatched and solid bars, respectively. The total number of shoots analyzed and the transgene-free shoots in each group are shown above and on the bars, respectively. Wt-Tg, transgenic shoots without mutations in target genes; Mu-Tg, transgenic shoots with mutations; Wt-nT, non-transgenic shoots without mutations; Mu-nT, non-transgenic shoots with mutation. B: detection of transgene and mutation analysis in NtMAR1 targets by PCR and CAPS analyses. C: detection of transgene and mutation analyses in NtPDS1 targets by PCR and HMA analyses. Control/C, without antibiotic selection or heat treatment; Ab, antibiotic selection alone; HT, heat treatment alone; Ab+HT, combined treatment; CS, continuous antibiotic selection; Wt, wild type; M, molecular weight marker; Transgene, BYDV sequence amplified with primers Gem-SF1 and -SR4; Target, PCR amplification of target gene fragment encompassing all target sequences; CAPS T2 and T3, CAPS analysis in DNA fragment encompassing targets 2 and 3; CAPS T2 and CAPS T3, CAPS analyses of target 2 alone and those of target 3 alone, respectively; HMA T12, HMA of DNA fragment encompassing targets 1 and 2.

encompassing all targets utilized a primer pair of a 1-to-1 mixture of NtPDS1-S2105F and NtPDS1-T1980F and NtPDS1-S3183R (Table 2) with GoTaq® Green Master Mix enzyme by Promega and under the PCR conditions: 94 °C for 2 min, 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 1 min 10 s, and 72 °C for 5 min. For detecting small insertion/deletion (InDel) mutations, amplifying the fragments encompassing targets 1 and 2 and targets 3 and 4 applied NtPDS1_TargetS113T115F and NtPDS1_TargetS288T290R and NtPDS1_TargetS529T546F and NtPDS1_TargetS631T629R, respectively (Table 2). PCR application with KOD FX Neo enzyme by TOYOBO had the PCR condition: 94 °C for 15 s, 40 cycles of 98 °C for 15 s, 60 °C for 20 s, and 68 °C for 20 s. The amplified fragments underwent the heteroduplex mobility assay (HMA) using MCE-202 MultiNA (SHIMADZU, Kyoto, Japan) with a DNA-500 kit.

RESULTS

Three-day selection and heat treatment effect on DRs-mediated induction

First, scientists tested the three DRs, *Wus2+STM* and *Wus2+ipt*, in the induction of tobacco shoot formation on MSHF. After one month, *Wus2+STM* did not induce shoots at all, but *Wus2+ipt* induced shoots in more than 97% of leaf pieces, with an average of 7–9 shoots/leaf disc. Therefore, they focused on these DRs, *Wus2+ipt*, in further experiments.

They hypothesized that in the DRs system, the three-day antibiotic selection could select shoots derived from cells with a higher transient expression of DRs and *Cas9*. Therefore, they tested the effect of three-day selection on shoot induction in the DRs system. The leaf pieces inoculated with the *Agrobacterium* harboring *Wus2+ipt* bore culturing on selective media for three days before transferring to non-selective media. As compared with the control group cultured on non-selective media throughout the experiments, the three-day selection only slightly reduced the shoot formation from 8.8 to 7.5 shoots/leaf disc. The results indicate the three-day selection has the least negative effect on DRs-mediated tobacco shoot formation.

Next, testing the effect of heat treatment on the DRs-mediated shoot formation took place. Preliminary experiments revealed that heat treatment on the second day of three-day antibiotic selection showed the highest CRISPR/Cas9-mediated mutation rates in transgenic plants. Therefore, applying the heat treatment on the day and evaluating shoot formation continued. The heat treatment reduced the DRs-dependent shoot formation from 17.7 shoots/leaf pieces without heat treatment to 8.04 shoots/leaf pieces. The results suggest that the heat treatment affects the DRs-mediated shoot induction.

Transgene-free genome editing in DRs system

The experiments above demonstrated that the heat treatment reduced the DRs-induced shoot formation. However, the study evaluated the total but not the transient expression-mediated shoot formation. Therefore, researchers randomly selected shoots and tested them for the presence of transgenes. The detection of three different transgenes gave consistent results, with results for GemDRs shown in Figure 3B and C. A report stated the *ipt* has served as a selective marker for tobacco transformation (Endo *et al*., 2001). This study consistently found a significant proportion (60%) of transgenic shoots even in the control experiments without either treatment (Figure 3A, shown in red). The three-day antibiotic selection did not alter the percentage of transgenic shoots (62%), suggesting that three-day selection does not select stable transformants (Figure 3A). The heat treatment alone increased the percentage of transgenic shoots to 86% (Figure 3A), signifying that the expression of DRs or plant responses to them incurs effects from high temperature. The combined treatment resulted in a percentage of transgenic shoots (58%) comparable to those of the control and three-day selection alone (Figure 3A).

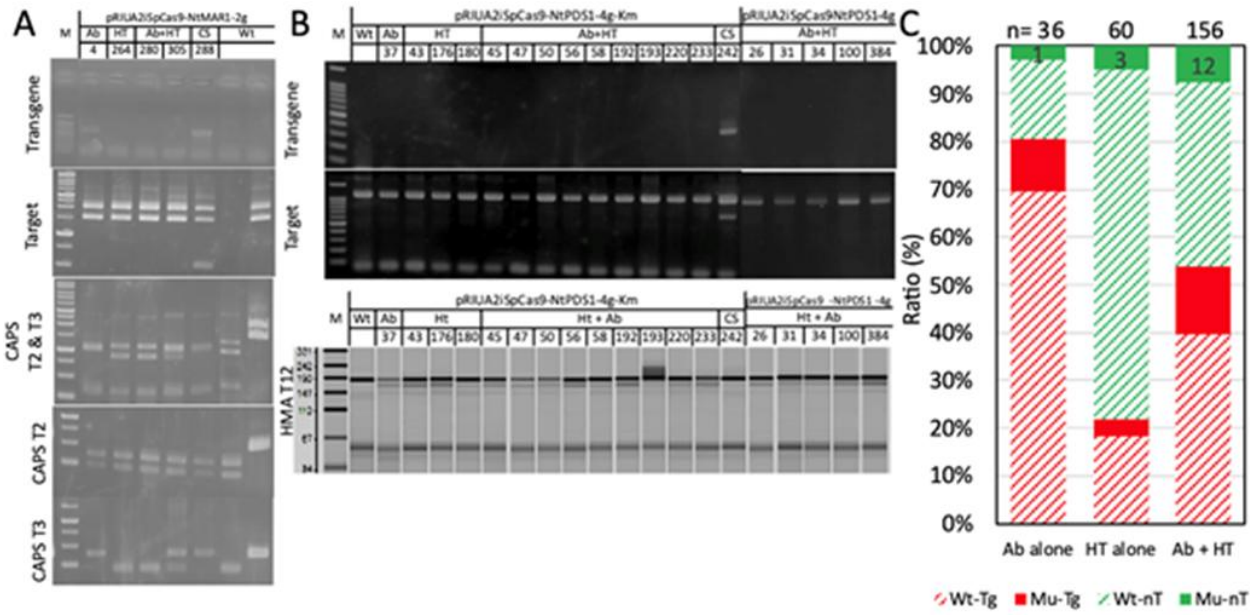

Figure 4. Transgene-free genome editing in the non-DR system. A: detection of transgene and mutation analysis in NtMAR1 targets by PCR and CAPS analyses. B: detection of transgene and mutation analyses in NtPDS1 targets by PCR and HMA analyses. C: transgene status and targeted mutations in shoots induced by externally added phytohormones. Labels are as in Figure 4.

The CRISPR/Cas9-mediated mutations' detection in NtMAR1 targets resulted from the CAPS analysis with the lack of complete digestion by restriction enzymes (Figure 4B). Likewise, detecting the mutations in NtPDS1 targets emerged from the HMA with shifted bands, which were not evident in the wild type (Figure 4C). Transgenic lines 18 (Figure 4B) and 39 (Figure 4C) appeared through continuous antibiotic selection and served as a control for mutant shoot detection. Scientists did not find any transgene-free shoots with mutations in the control and three-day selection groups, although some transgenic shoots with mutations were notable (Figure 4C, lines 28 and 33). The mutation detection rates were higher in the three-day selection group (Figure 4A). In contrast, researchers found one and six transgene-free shoots with mutations in the heat treatment group alone (Figure 4C, line 441) and combined treatment group (Figure 4C, lines 35, 37, 38, 451, 452, and 453), respectively, in addition to more transgenic shoots with mutations (Figure 4A). The percentages of mutant shoots were 1.2% and 7.6% in the heat treatment group and combined treatment group, respectively (Figure 4A). The results suggest that the combined treatment can make the screening for transgene-free mutants more efficient. It could refer to the enrichment of the shoots derived from cells with high transient expression levels.

Transgene-free genome editing in non-DR system

The non-DR system relies on the shoot induction by the externally added phytohormones and, therefore, produces a lot of transgene-free shoots. However, most of them are mainly derivatives from cells without transient foreign gene expression. Thus, the study evaluated the effect of the combined treatment on the transient expression-mediated genome editing in this system by comparing with groups with either the three-day antibiotic selection or heat treatment. The detection of sequences of *Cas9* and AtACT2i/e gave consistent results (Figure 4A and B). A large fraction (78%) of shoots from the heat treatment group were transgene-free, as

anticipated (Figure 4C). In contrast, only 19% of shoots from the three-day selection group were transgene-free, suggesting that the selection only for three days enhances the stable transformation (Figure 4A). About half (46%) of shoots from the combined treatment group were transgene-free (Figure 4C). It is noteworthy that they contained more transgene-free shoots than the three-day selection group, indicating that the heat treatment affects the transgene integration into the host genome.

As in the DRs system, transgenic lines 288 (Figure 4A) and 242 (Figure 4B) came through continuous antibiotic selection and served as a control for mutant shoot detection. In the three-day selection group, scientists found a single shoot with the NtMAR1 mutation comprising 2.8% of all shoots tested in the group (Figure 4A, line 4, and C). In the heat treatment group, no NtMAR1 occurred but three NtPDS1 mutant shoots (Figure 4B, lines 43, 176, and 180) were prominent, which accounted for 5.0% of tested shoots (Figure 4C). For the combined treatment group, detecting one NtMAR1 (Figure 4A, line 305) and 11 NtPDS1 mutant shoots (Figure 4B, lines 45, 47, 50, 56, 58, 192, 193, 220, 233, 26, 31, 100, and 384) comprised 7.7% of tested shoots (Figure 4C). The comparison of kanamycin and hygromycin selection in non-DR systems ensued. The three-day kanamycin and hygromycin selection yielded 10% and 5% of non-transgenic mutants, respectively, when combined with the heat treatment, suggesting that kanamycin has an advantage over hygromycin in the short-term selection-mediated enhancement of transgene-free genome editing. Overall, the results suggest that the combined treatment is effective in enhancing the transient expression-mediated genome editing also in non-DR systems.

DISCUSSION

In the presented study, we found that the combined treatment facilitated the identification of transgene-free shoots with targeted mutations in both DRs and non-DR systems. The percentages of transgene-free mutant shoots were 7.6% and 7.7% in DRs and non-DR systems, respectively. They were higher than those in the heat treatment groups (1.2% and 5% in DRs and non-DR systems, respectively) and the three-day antibiotic selection groups (0% and 2.8% in DRs and non-DR systems, respectively). Thus, the promising study highly suggests the efficacy of the combined treatment in transient expression-mediated genome editing.

In the DRs system, the heat treatment significantly reduced the shoot induction. On the contrary, we did not find any negative effects of the heat treatment on the shoot formation in the non-DR system, wherein the shoot formation relies on an externally added phytohormone. The differences in shoot induction between these systems suggest that the heat treatment reduced the *ipt* gene expression or functioning and subsequently reduced the shoot formation in the DRs system. There is a trade-off between the shoot induction and *Cas9* functioning in the genome editing in the DRs system. Nonetheless, the combined treatment has proven to enhance the transient expression-mediated genome editing. Improving the thermostability of DRs function and expression could further enhance the transient expression-mediated genome editing.

Reports declared heat treatment has enhanced the CRISPR/Cas9-mediated genome editing in different plant species (Kurokawa *et al.*, 2021; Lee *et al.*, 2024; Poddar *et al.*, 2023). In addition to the suppression of *Wus2*+*ipt*-mediated shoot induction, we found another effect of the heat treatment. Heat treatment in the non-DR system reduced the number of transgenic shoots in the combined treatment group versus the three-day selection group, implying that heat treatment reduces transgene integration in this system (Figure 4C). Furthermore, heat treatment alone remarkably increased the transgenic shoots in the DRs system (Figure 3A). However, this observation can be because of the suppression of DRs-mediated shoot induction by heat treatment and the ability of transgenic cells to initiate shoot induction after the heat treatment. A study reported the enhanced transformation of grasses by heat treatment before the *Agrobacterium*-mediated

transformation (Wang et al., 2023). However, little is known about the effect of heat treatment during the early stages of transformation when the T-DNA integration should take place. Nonetheless, the above comparison in the non-DR system also suggests that heat treatment has another role in the enhancement of transient expression-mediated genome editing: the suppression of transgene integration.

A study stated short-term antibiotic selection can enhance transient expression-mediated genome editing in potatoes (Bánfalvi *et al*., 2020). We expected that in the DRs system, the short-term selection could suppress the shoot induction in bystander cells that did not express DRs. However, the study observed no significant difference in transgene-free shoot formation within groups with neither treatment, three-day selection, nor combined treatment. Therefore, it is likely that transient expression of DRs produced all the transgene-free shoots in this system (Figure 3A). Nonetheless, the combined treatment significantly enhanced transient expression-mediated genome editing. It could be due to the selection of cells with higher transient expression, which is supported by the observation that the average shoot number/leaf piece was less in the combined treatment (7.27 shoots/leaf pieces) than in the heat treatment alone (8.75 shoots/leaf pieces).

In the non-DR system, the short-term antibiotic selection naturally reduced the shoots derived from non-transformed cells with a significant proportion of stable transformants (Figure 4C), which supports the effectiveness of the antibiotic selection. Thereby, the origin of the transgene-free shoots to be transiently transformed cells. This effectiveness may be a disadvantage for obtaining transgene-free shoots. As discussed above, however, the combination with the heat treatment could reduce the T-DNA integration and promote transgene-free shoot formation. The results also suggested that kanamycin selection is more effective in generating transgene-free mutants in the combined treatment than hygromycin selection.

CONCLUSIONS

The presented study demonstrated the combined treatment of three-day antibiotic selection and 24-hour heat treatment improved the efficiency of transgene-free genome editing in tobacco. This finding, although gained in a model plant, provides a clue for improving the efficiency of transgene-free genome editing in different crop species. Refinement of the combined treatment condition for each crop species would enable us to develop efficient genome editing, thereby promoting crop improvement.

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