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学位論文全文に代わる要約

Extended Summary in Lieu of Dissertation

氏名： Hafizur Rahman

Name

学位論文題目： Development of genome editing technologies in plants

Title of Dissertation (植物におけるゲノム編集技術の開発)

学位論文要約：

Dissertation Summary

Genome editing is a cornerstone of modern molecular biology research. It is routine practice to insert, delete, edit, or substitute DNA in the genomes of a wide range of organisms. These are used to develop model organisms for research and genetically modified plants and animals for resource production. The purpose of this research was to integrate the CRISPR/Cas9 system with *Agrobacterium*-mediated transformation to provide a new technique of genome editing tools for model plant development. World food production must increase by at least 70% to meet the growing global population, which is estimated to reach 9.1 billion people by 2050. Furthermore, abiotic, and biotic factors contribute to the loss of up to one-third of overall food production, resulting in approximately a billion people going hungry. We must explore diverse ways of increasing the food supply. To this end, the most important is to improve crop varieties/cultivars. We can't employ conventional plant breeding procedures since they have too many drawbacks; therefore, we'll have to rely on new approaches like molecular biology and biotechnology. Many molecular approaches to assisting and speeding up conventional breeding operations have recently emerged. People take fiber, beneficial metabolites, and nutrients from fruits and fruit juice, among other foods. As a source of revenue, many countries and areas worldwide rely largely on fleshy fruit-producing crops. The most popular and widely consumed fruits include citrus, apple, grape, banana, strawberry, watermelon, kiwifruit, and tomato. Fruit

production, disease resistance, and fruit quality features have all been the subject of extensive investigation. Traditional breeding approaches such as selective hybridization, progeny evaluation, and propagation were used to make significant advances in previous studies. These techniques are labor-intensive and time-consuming, and they are limited by the germplasm available. With the advent of new and exciting molecular genetic techniques, such as the recently developed CRISPR/Cas9 genome editing tools, the subject will likely advance and change quickly.

Genome editing is a novel genetic manipulation technology that has recently been adapted to a growing number of plant species, including *Arabidopsis*, tobacco, sorghum, rice, *Nicotiana benthamiana*, tomatoes, wheat, soybean, maize, potato, sweet orange, Populus, cucumber, cotton, and melon. Unlike conventional genetic manipulation approaches such as *Agrobacterium*-mediated or direct gene transfer, genome editing technology allowed us to make minor changes to existing crop genomes. Sexual recombination, induced random mutagenesis, and transgenic methods have all been used to secure genetic benefits for developing superior crop types. Targeted genome-editing technologies have recently emerged as a new avenue for introducing beneficial genetic modifications in the world's most significant agricultural crop species. Engineered nucleases are used in genome-editing technologies such as Zinc-Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to induce double-strand breaks (DSB) at known DNA sequences in the genome. The following repair at the target site, insertions, and deletions (InDels) are generated via error-prone non-homologous end joining (NHEJ), with infrequent nucleotide substitutions happening at the repair areas. In the presence of a homologous donor DNA template spanning the DSB, the error-free homologous recombination (HR) repair process can be initiated, resulting in targeted gene replacement. Protein-guided recognition processes underlying ZFNs and TALENs. The construction of vectors for targeting DNA sequences using these methods is a time-consuming and expensive operation. The CRISPR/Cas9 system, on the other hand, is based on DNA or RNA sequence homology, and it only requires a 17–23-bp complementary nucleotide sequence termed a guide RNA (gRNA) to target a specific DNA region. CRISPR/Cas9 is the most used of the three genome-editing technologies due to its versatility, high efficacy, and low cost. CRISPR/Cas9 has been used to disrupt, activate, and inhibit specified genes in human, animal, and plant cells. CRISPR/Cas9-induced mutations have been demonstrated to be heritable across sexual generations in some cases.

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Citrus is one of the most cultivated fruits in the world, and the global total citrus fruit production in 2015 has been estimated at 130947 thousand tons, which was about 20% of total fruit production. Breeding of citrus cultivars has been achieved through a conventional genetic cross as in other crop species. However, despite the accelerated scheme with DNA marker-assisted selection (MAS), it takes a long time to obtain a new useful cultivar because of the long lifespan of citrus species. Moreover, most citrus cultivars are heterozygous, and therefore, it is not easy to make a minor improvement by backcross-mediated introgression of a particular allele. Thus, genome editing is indispensable in citrus breeding. Although studies have shown that genome editing can be applied to different citrus cultivars, some technical issues are to be addressed. They include the chimerism of the genome-edited citrus plants and the difficulty of phenotypic evaluation: studies could analyze only a single plant from a single transformation event unless the plants are vegetatively propagated via grafting. In addition to conventional crossbreeding, gene manipulation technologies have been applied to improve crop plants. Different transgenic crops have been bred since the 1980s, but only a few are successful in commercial production. Many countries have legal regulations to constrain genetically modified (GM) crops to use. Some people have concerns or uncertainty about using GM crops as foods, which could be a limiting factor for popularizing GM crops. The transgenic technology introduces foreign genes into the crop genome to confer desired traits. By contrast, genome-editing technology can modify existing crop genes, as conventional mutagenesis can, but in a pinpointing manner. Therefore, the genome-editing has been expected as a significant crop breeding technology, which can enjoy higher public acceptance than transgenic technology (Fig. 1).

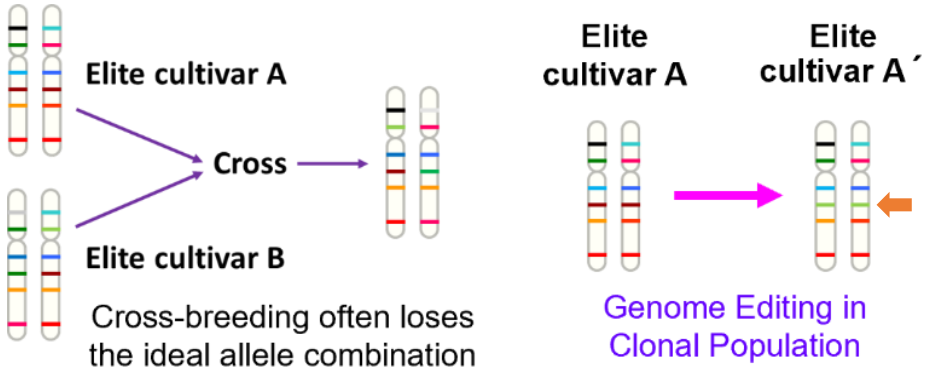

Fig. 1. A strategy of genome editing for improvement of the crop plants.

Poncirus is closely related to the genus *Citrus* and comprises only one species, *Poncirus trifoliata* (L.) Raf. (hereafter Pt). Pt is cultivated in different countries as a hedge and a medicinal crop. It is also used as a rootstock for most citrus trees in Japan because it results in a compact tree useful in different fields of citrus cultivation. It is commonly recognized that citrus trees grafted on Pt usually show high yields and good fruit quality in Japan. Although Pt is cold-hardy and resistant to citrus tristeza virus, it is susceptible to some soil-borne diseases such as satsuma dwarf or citrus mosaic virus infection. Because resistant rootstocks could help control such soil-borne diseases, the improvement of Pt is a valuable challenge of horticultural sciences for the promotion of citrus production. Genome-editing studies have applied many crops species but not yet to Pt plants. So, I used the Pt in my genome-editing study. As a result, CRISPR/Cas9-targeted genome editing opens many new possibilities for Pt improvement, production, and use.

In the first part of my study, I employed *Agrobacterium*-mediated transformation of Pt epicotyl segments to edit the closest Pt homolog of *A. thaliana MULTI-ANTIBIOTICS RESISTANCE 1*, *PtMAR1* (Ptrif.0005s1628.1). Both valine and alanine, which were mutated in antibiotics-resistant *Arabidopsis* mutants, are conserved in the *PtMAR1*. I aligned the genomic and cDNA sequences of *PtMAR1*, revealing *PtMAR1* has 13 exons. Three target sequences were designed, each of which perfectly matched both cDNA and genomic sequences. Pt epicotyl segments were transformed using binary vectors for three target sequences, T1, T2, or T3. Transformed calluses selected by hygromycin resistance were tested for mutations in *PtMAR1* using Cleaved amplified polymorphic sequence (CAPS) analysis (Fig. 2). Mutation events are indicated by partially/incompletely digested PCR products from the lines by restriction enzymes to varying degrees. Even though some plant lines produced more cleaved fragments than uncleaved ones, cleaved fragments were found in all plant lines, implying that the ribonucleoprotein complex of Cas9 and T1-sgRNA (Cas9/T1-sgRNA complex) triggered some mutation, albeit with less efficiency. In T2-targeted Pt transformants, the transformed lines displayed multiple bands in the PCR product before restriction digestion, indicating somehow larger InDels. Although the PCR products from some lines were entirely digested, most of the lines showed no or only partial digestion, implying that genome editing in T2 is very efficient. In T3-targeted Pt transformants, most lines yielded cleavable DNA fragments, implying that T3 editing is not as effective as T2.

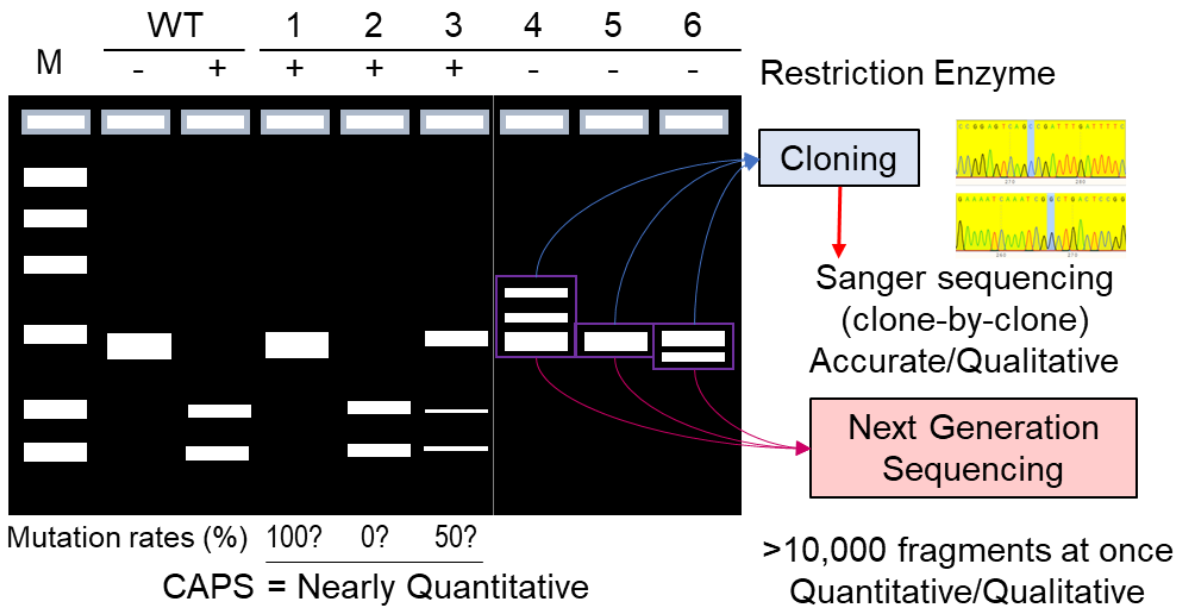

Fig. 2. A schematic presentation of detection of CRISPR/Cas9-mediated mutagenesis in Pt: CAPS and Amplicon-seq.

Amplicon sequencing was performed to obtain more precise mutation rates in the *PtMAR1*-edited plant lines (Fig. 2). The results revealed that T1 mutation rates range from less than 1% to about 41%; T2 mutation rates range from about 9% to more than 99%, and T3 mutation rates range from about 73% to more than 99%. T1 target has 1-82 bp deletion and 1-91 bp insertion, T2 target has 1-81 bp deletion and 1-13 bp insertion, and T3 target has 1-88 bp deletion and 1-72 bp insertion. In T1 targets, deletion is more frequent than insertion, whereas, in T2 and T3 targets, insertion is more frequent than deletion. The above findings indicated that the Pt genome could be efficiently edited by CRISPR/Cas9 system using epicotyl segments, albeit on sgRNAs.

Although I could induce high levels of mutation in the *PtMAR1* gene, I failed to induce the genome-edited shoots early in this study. By combining a modified protocol for Pt shoot propagation, I finally obtained numbers of genome-edited shoots (Fig. 3). In a classic protocol, transgenic shoot regeneration and growth of Pt were reported: Pt epicotyl segments placed on shoot induction (SI) medium, then transferred to shoot elongation (SE) medium for shoot growth before being transferred to rooting medium for root development. This classic protocol worked for untransformed Pt in my hands but did not for transformed Pt. After three days of co-culture, transformed epicotyl segments were placed on SI media, but no successful shoot induction occurred; instead, calluses formed, and the sub-culture

was continued to induce shoots, but unfortunately, no shoot induction occurred after a long time, while calluses grew continuously. Those calluses were placed on SE media and continued to be sub-cultured to stimulate shoot induction; however, no shoot induction happened after a long time, only callus growth and multiplication. After studying several pieces of works of literature, I decided to establish a new protocol by including some procedures described for the shoot propagation of Pt. In new protocol, I used woody plant (WP) media to induce shoot development. After 4 weeks of culture on WP media, we obtained shoot primordia, which were then transferred to Nitsch and Nitsch (NN) media. Regenerated shoots were then put to Murashige & Tucher (MT) media for elongation and multiplication, and thus, multiple shoots were grown well on the MT media.

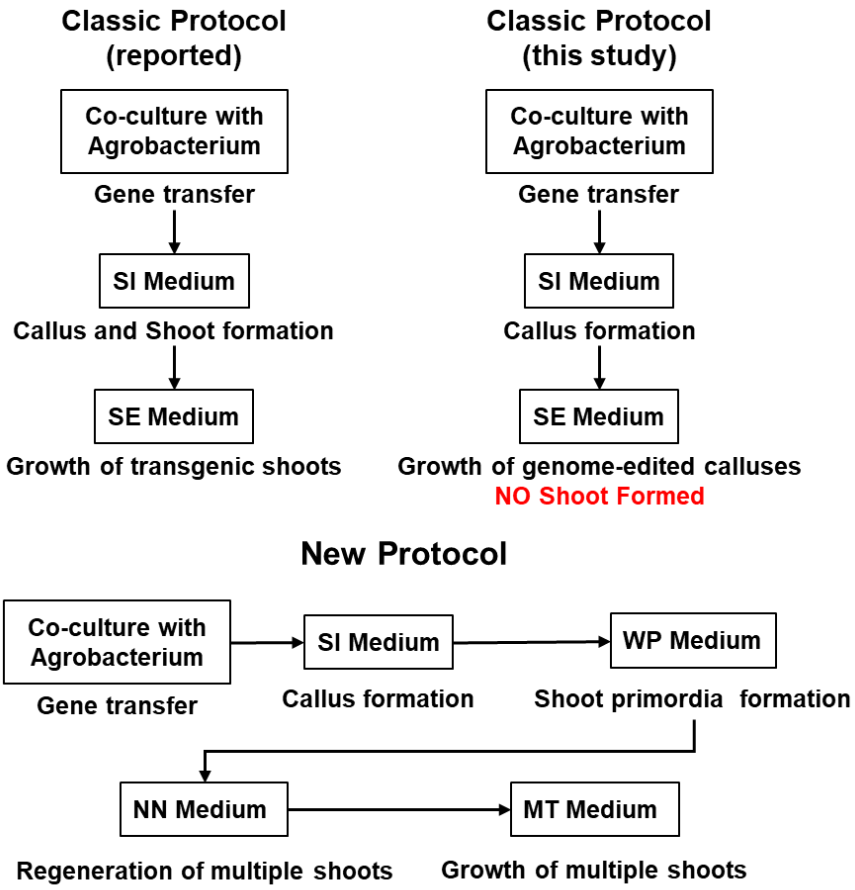

Fig. 3. A schematic representation of difficulty and breakthrough of shoots regeneration in genome-editing of Pt.

A few lines were chosen and subsequently studied using amplicon sequencing based on the findings of the CAPS investigation of regenerated *PtMAR1-T2* shoots. Mutation rates ranged from 99.83% to 100% in four shoots each from *PtMAR1-T2-9, 10, 12,* and *17* mutant lines. The following mutation patterns were detected in *PtMAR1-T2* regenerated shoots were 1-64 bp deletion, 1-2 bp

insertion, and very few portions of wild-type sequences. These results showed that multiple regenerated shoots from genome-edited Pt callus lines showed nearly clonal propagation.

As mentioned above, I established a protocol to edit the Pt genome and propagate the genome-edited shoots. This protocol will likely solve the technical issue of phenotypic evaluation of genome-edited Pt. For instance, resistance to multiple antibiotics can be evaluated in the near-clonally propagated population of shoots. It enables us to collect numerical data, such as biomass or chlorophyll contents, and perform statistical tests for validating the results. Thus, the protocol could contribute to the future breeding of Pt in disease resistance and other agriculturally important traits.

In the second part, I used the CRISPR/Cas9 system to modify the *PHYTOENE DESATURASE 1* (*PDS1*) gene of Pt (*PtPDS1*) through *Agrobacterium*-mediated transformation. The *PDS1* gene encodes a phytoene desaturase involved in carotenoid biosynthesis, and its disruption results in an albino phenotype. The ease of phenotypic validation has thus prompted researchers to target the *PDS1* in the early genome editing studies in different plants. The public database was used to identify the nucleotide and amino acid sequences of the *Phytoene Desaturase* (*PDS*) gene in citrus & other plant species. The genomic sequence of *PtPDS1* is 12,612 nucleotides in size, comprising 14 exons and 15 introns. To knock-out *PtPDS1*, five distinct sgRNAs were designed that had a perfect match in genomic and cDNA sequences as predicted by the CCTop-CRISPR/Cas9 target online predictor to have the highest efficacy. *AtPDS* (AT4G14210.1) and *SlPDS* are 84.88% and 83.27% identical at the protein level, respectively, suggesting a conserved function in Pt.

I initially transformed Pt using *Agrobacterium* clones harboring binary vectors with 5 different sgRNA with the classic protocol. Among the 5 target sequences tested, I failed to detect any mutation in T1, T3, and T5. CAPS analysis of 195 Hygromycin-resistant calluses identified 7 mutants each in two *PtPDS1* targets, T2 and T4. The combined use of gel electrophoresis, Sanger sequencing, and the next-generation sequencing of PCR-amplified fragments revealed the mutation rates less than 1% in T2 and ranged from 0.34 to 99.31% in T4. The large insertions of 269 & 302 bp match perfectly with the Pt genome. According to amplicon sequencing of selected mutant lines based on CAPS results, the modification rates in selected lines are consistent with the CAPS analysis. The above findings indicated that the Pt genome could be edited by CRISPR/Cas9 system. The new protocol was used for genome-

edited shoot regeneration and growth. I could regenerate shoots from only a single callus line with a high mutation rate in T4. CAPS analysis of 153 regenerated shoots of T4 discovered 33 mutants. The *PDS1* gene is involved in carotenoid biosynthesis, and its disruption results in an albino phenotype. Although CAPS indicated that a very small portion of DNA from the shoots had the wild-type sequence, none of them showed albino phenotypes. Those shoots were highly chimeric, unlike *PtMAR1*-edited shoots. The results would suggest a role of *PtPDS1* in shoot regeneration or growth distinct from other plants.

In the third part, I explored the possible use of *MAR1* gene knock-out as a recessive positive selective marker (RPSM) for future DNA-free genome editing by direct delivery of CRISPR/Cas9-single guide RNA (sgRNA) ribonucleoprotein complex (RNP). Nitab4.5 0001338g0110.1 and Nitab.5 0004525g0030.1 are the closest homologs of *Arabidopsis MAR1* locating on T- and S-genomes of tobacco, respectively. Both valine and alanine in *MAR1* that are substituted to methionine and valine in *rts3-1* and *mar1-1* mutants are conserved in these tobacco homologs. The CRISPR/Cas9 sequences of *NtMAR1T* and *NtMAR1S* were shown to have 14 and 15 exons, respectively. We next aligned the genomic and cDNA sequences of these sequences with the cDNA sequence of *N. sylvestris* homolog to confirm the sequence identity. *N. tabacum* and *N. sylvestris* plants were transformed using the binary vectors, and transformant shoots selected by hygromycin resistance were tested for the mutations in *MAR1/RTS3* homologs by CAPS analysis and the amplicon sequencing. The InDels mutation rates ranged from less than 1% to more than 99. Most of the InDels mutations were likely to knock-out the target genes through frameshifts, but some *NtMAR1S* genes in Nt-T3-13 had a single codon deletion, which may not abolish the gene function. In the transformed *N. sylvestris* plants, InDels mutation rates were higher than in transformed *N. tabacum* lines. As in *N. tabacum* plants, the mutation rates were not very high in T4-targeted *N. sylvestris* lines as compared to those targeted in T2 or T3.

I tested plants with high and low mutation rates in shoot regeneration capacity on different aminoglycoside antibiotics. *N. tabacum* and *N. sylvestris* lines formed shoots on a medium containing meropenem but no aminoglycoside. *N. tabacum* and *N. sylvestris* lines with high mutation rates did not form shoots in the presence of aminoglycosides. The results show knock-out of *N. tabacum* and *N. sylvestris MAR1* homologs unlikely useful to select genome-edited plants in tissue culture. I collected

T_1 seeds of T2-1, T3-11, and T3-13 lines and tested their aminoglycoside resistance. I observed tiny true leaves in Nt-T3-11 and Nt-T3-13, but not in Nt-T2-1 seedlings after 3 weeks on a kanamycin plate. The CAPS and sequencing analysis showed that Nt-T3-11 and Nt-T3-13 were knocked out in both *NtMAR1T* and *NtMAR1S*, and Nt-T2-1 had no mutation. The T_1 seedlings of the knock-out lines failed to form shoots on the regeneration medium containing kanamycin. A study has shown that disruption of *SlyMAR1*, the tomato homolog of the *MAR1* gene, leads to regeneration of kanamycin-resistant tomato shoots. In tobacco, my study has successfully knocked out the *MAR1* homologs and found that the knock-out plants show limited but evident kanamycin resistance, suggesting that the tobacco homologs have roles in aminoglycoside susceptibility. However, the knock-out of *NtMAR1T/NtMAR1S* is unlikely to be suitable as a selective marker for genome editing of tobacco.

In the fourth part, I took part in a collaborative study on the improvement of plant genome editing efficiency. Editing of the *Arabidopsis* genome is achieved through the introduction of *Cas9* and guide RNA genes by the floral dip method and mutation screening in the T_1 seed population. Although this method can easily produce genome-edited plants, mutation efficiency in *Arabidopsis* using *SpCas9* is not as high as that in rice and tobacco. Based on the higher optimal temperature for Cas9, we examined the effect of a simple heat treatment on the mutation efficiency in *Arabidopsis*. The strategy for improving genome editing efficiency in *Arabidopsis* are described here (Fig. 4).

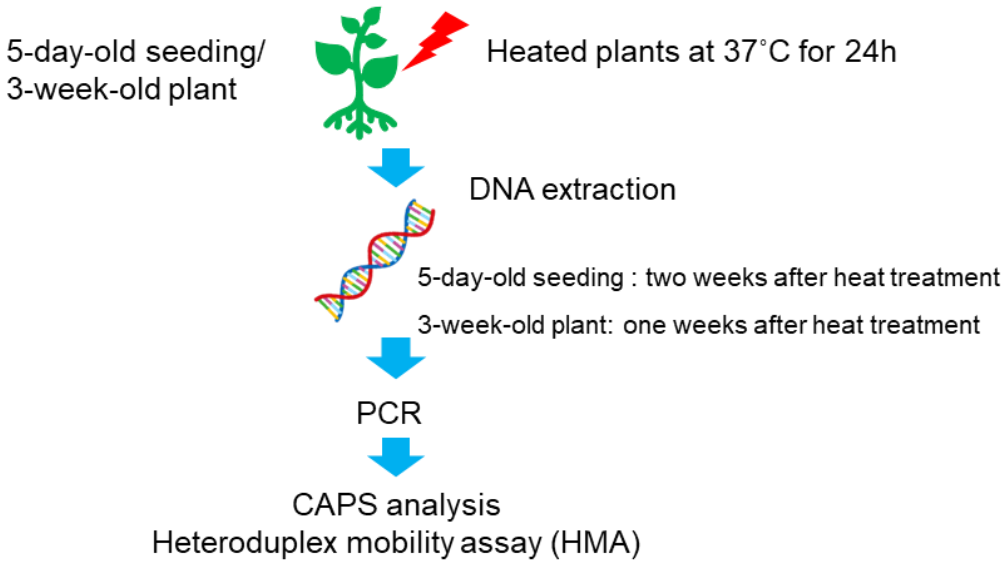

Fig. 4. A strategy for improving genome-editing efficiency in *Arabidopsis*.

CAPS analyses revealed mutation rates at the seedling stage (5-day-old) indicated striking enhancement with statistical significance ($p < 0.01$): from 53.8% to 74.5% for Site 1, from 42.7% to 61.9% for Site 2, from 88.9% to 91.0% for Site 3 and from 2.5% to 42.0% for Site 4. The results indicated a clear enhancement of mutagenesis efficiency in both seedlings and vegetative plants. A heat treatment at 37˚C for 24h increased the efficiency of CRISPR/Cas9-mediated mutagenesis in both the seedling (5-day-old) and vegetative growth (3-week-old). No statistically significant difference was found between the different growth stages, suggesting that heat treatment can be applied to increase the mutagenesis efficiency at any stage of plant growth. Sequencing revealed different insertions and deletions, regardless of the target site or heat treatment. Off-target mutation, a common problem in genome editing studies, was not enhanced by the heat treatment. The heat treatment at 37°C for 24 hours did not affect the expression levels of the *SpCas9* gene under the *PcUBI* promoter in *Arabidopsis*. In vitro cleavage assay strongly suggests that efficiency of mutagenesis in heat-treated *Arabidopsis* was enhanced by the activation of DNA cleavage activity of *SpCas9* protein, rather than by the increased expression of the protein.

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