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学 位 論 文 要 旨
Dissertation Abstract

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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）

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Genome editing is a novel genetic manipulation technology recently adapted to a growing number of crops. Unlike conventional genetic manipulation through a genetic transformation using *Agrobacterium*-mediated or direct gene transfer techniques, genome-editing technology enabled us to make a minor modification in the existing crop genomes. Since 2013, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) from *Streptococcus pyogenes* (*SpCas9*) has been engineered to modify plant genomes. This system was applied to edit the genome of *Poncirus trifoliata* (L.) Raf. (hereafter Pt), a model plant of citrus lineage and a widely used rootstock, *Nicotiana tabacum*, and *Arabidopsis thaliana* in my study.

In the first part of my study, the closest Pt homolog of *A. thaliana Multi-Antibiotic Resistance 1*, referred to as *PtMAR1*, was edited at three different targets using *Agrobacterium*-mediated transformation of Pt epicotyl segments. Cleaved amplified polymorphic sequences (CAPS) analysis of transformed callus lines identified 4, 22, and 4 mutants, with mutation rates determined by amplicon sequencing ranging from 0.21 to 40.09%, 8.57 to 99.96%, and 73.36 to 99.96% in three targets, respectively. The results support that the Pt genome can be efficiently edited, albeit highly dependent on the sgRNA sequence. Although a classic Pt transformation protocol failed to regenerate shoots from those calluses, I successfully obtained multiple shoots from each callus line by combining a classic Pt shoot propagation protocol. Most shoots regenerated from callus lines with high mutation rates showed high biallelic mutation rates in the CAPS analysis, and the amplicon sequencing supported their near-clonal propagation. The successful regeneration and propagation of multiple shoots from each mutated callus line would provide a reliable phenotypic evaluation of the genome-edited Pt plants.

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. I selected 5 target regions in *PtPDS1* and targeted the gene in Pt plants. CAPS analysis of 195 Hygromycin-resistant calluses identified 7 mutants each in two *PtPDS1* targets, target 2 and 4 (hereafter T2 and T4, respectively), but not in the other three. 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. I could regenerate multiple shoots from only a single callus line (1^{st} transformation) with a high mutation rate in T4 through the protocol above. CAPS analysis of 201 regenerated shoots discovered 37 mutants from other transformations. Some of them were highly chimeric, unlike *PtMAR1*–edited shoots. Although they had a minor portion of wild-type alleles, none showed full albino phenotypes, suggesting a role of *PtPDS1* in shoot regeneration or growth distinct from other plant species.

In the third part, I explored the possible use of *MAR1* gene knockout 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). To this end, I knocked out the tobacco homologs of the *MAR1* gene, mutations of which confer aminoglycoside resistance. The RNP delivery to plant cells has yet been established in the lab, *Cas9* and sgRNA genes were introduced into *Nicotiana tabacum* and *Nicotiana sylvestris* by *Agrobacterium*-mediated gene transfer. *MAR1* homologs were successfully edited in both diploid *N. sylvestris* and tetraploid *N. tabacum* with high InDel mutation rates as manifested by CAPS and amplicon sequencing analyses. The transformants were evaluated in the shoot-induction media in the presence of aminoglycosides, but none of them showed resistance. By contrast, *MAR1*–knockout *N. tabacum* T_1 seedlings showed a limited resistance to a low concentration of kanamycin. The results indicate that the tobacco *MAR1* homologs have a role in plant sensitivity to aminoglycoside antibiotics like *Arabidopsis MAR1* and suggest the involvement of additional factor(s) in the aminoglycoside sensitivity in tobacco.

In the fourth part, a collaborative study on the improvement of plant genome editing efficiency is presented. Editing of the *Arabidopsis* genome is achieved through the introduction of *SpCas9* 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, which are subjected to a tissue culture step. To alleviate the problem, we selected a highly active parsley *UBIQITIN* promoter and examined the effect of a simple heat treatment on the mutation efficiency in *Arabidopsis*. Just one heat treatment at 37 °C for 24 h increased the mutation efficiency at all four targets examined from 3 to 42%, 43 to 62%, 54 to 75%, and 89 to 91%, without detectable off-target mutations. The results indicated that a simple heat treatment of plants at 37 °C for 24 h could increase the efficiency of CRISPR/Cas9-mediated mutagenesis in *Arabidopsis*, and possibly in other plant species.