

Article

# I-SceI Endonuclease-Mediated Plant Genome Editing by Protein Transport through a Bacterial Type III Secretion System

Yuki Yanagawa <sup>1</sup>, Kasumi Takeuchi <sup>1</sup>, Masaki Endo <sup>1</sup>, Ayako Furutani <sup>2</sup>, Hirokazu Ochiai <sup>1</sup>, Seiichi Toki <sup>1</sup> and Ichiro Mitsuhashi <sup>1,\*</sup>

<sup>1</sup> Institute of Agrobiological Sciences, NARO, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan; yyana@affrc.go.jp (Y.Y.); kasumit@affrc.go.jp (K.T.); mendo@affrc.go.jp (M.E.); ochiaih@affrc.go.jp (H.O.); stoki@affrc.go.jp (S.T.)

<sup>2</sup> Gene Research Center, Ibaraki University, 3-21-1 Ami, Inashiki, Ibaraki 300-0393, Japan; ayako.furutani.asano@vc.ibaraki.ac.jp

\* Correspondence: mituhara@affrc.go.jp; Tel.: +81-29-838-7440

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**Abstract:** *Xanthomonas campestris* is one of bacteria carrying a type III secretion system which transports their effector proteins into host plant cells to disturb host defense system for their infection. To establish a genome editing system without introducing any foreign gene, we attempted to introduce genome editing enzymes through the type III secretion system. In a test of protein transfer, *X. campestris* pv. *campestris* (*Xcc*) transported a considerable amount of a reporter protein sGFP-CyaA into tobacco plant cells under the control of the type III secretion system while maintaining cell viability. For proof of concept for genome editing, we used a reporter tobacco plant containing a luciferase (*LUC*) gene interrupted by a meganuclease *I-SceI* recognition sequence; this plant exhibits chemiluminescence of *LUC* only when a frameshift mutation is introduced at the *I-SceI* recognition site. Luciferase signal was observed in tobacco leaves infected by *Xcc* carrying an *I-SceI* gene which secretes *I-SceI* protein through the type III system, but not leaves infected by *Xcc* carrying a vector control. Genome-edited tobacco plant could be regenerated from a piece of infected leaf piece by repeated selection of *LUC* positive calli. Sequence analysis revealed that the regenerated tobacco plant possessed a base deletion in the *I-SceI* recognition sequence that activated the *LUC* gene, indicating genome editing by *I-SceI* protein transferred through the type III secretion system of *Xcc*.

**Keywords:** bacterial type III secretion system; *Xanthomonas campestris*; *I-SceI*; protein transportation; genome editing

## 1. Introduction

Genome editing technology offers an attractive new approach to plant breeding. Genome editing involves making functional changes in endogenous genes by mutation and deletion with genome editing enzymes composed of protein or protein/RNA complex such as Zinc finger nucleases (ZFNs), Transcription activator-like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats (CRISPR)/Crispr associated protein 9 (Cas9) [1–4].

In mammalian cells, genome editing has been achieved by introducing Cas9 and guide RNA (gRNA) ribonucleoprotein complex by lipofection or electroporation [5]. In addition, microinjection was used for CRISPR/Cas9-mediated genome editing in murine zygotes [5]. Nanoparticles also worked to deliver the Cas9/gRNA complex for correcting gene mutation of genetic disease [6]. The foreign protein and RNA are degraded in the cells; therefore, genome editing is expected to accomplish functional changes without introducing any foreign gene. However, plants contain structures such

as cuticular layers, wax layers, and cell walls that prevent introduction of protein and RNA directly into the cells. Thus, these methods above used in animals cannot work efficiently in intact plant cells. Due to these barriers, genome editing enzyme genes are often transformed into plant cells and removed from regenerated plants in the next generation to obtain null segregants. However, plants with long life cycles such as trees take a long time to reproduce. Moreover, crop plants cultivated by vegetative propagation such as potato and strawberry are not fixed varieties, producing a different variety to parent one in the next generation. Thus, in such kinds of plants, transformed foreign genes cannot be removed from genome-edited plants. For this reason, it has been expected to develop effective techniques to introduce genome editing enzymes as proteins and RNAs in plants.

Genome editing in tobacco BY-2 protoplasts has been achieved by using lipofection to introduce Cas9/gRNA [7]. Cell-penetrating peptides are also useful to transfer proteins into plant cells using infiltration to leaves or stem cells, although no genome-edited plant has been produced yet [8]. We previously developed a technique to introduce protein directly into plant cells by using an atmospheric-pressure plasma [9]. In this work, we suggest a new technique for protein transfer into plant cells that uses phytopathogenic bacteria. The technique was also applied to make genome-edited plants. Since bacteria can proliferate on plant tissues until suppressed by plant resistance, during proliferation, the bacteria supply genome editing enzymes to the cells of inoculated tissues while enlarging the area of infection, implying efficient genome editing. In addition, protoplast preparation is not required for bacterial infection, indicating that it is easy handling for further experiments to produce genome-edited plants.

Several pathogenic bacteria in plants and animals have delivery systems of their effector proteins into host cells named type III secretion systems to make their surroundings comfortable by controlling physiological functions of the hosts such as interfering with host immune response. If we can control the secretion system for protein carrier apparatus to the cells, it will be a useful tool for direct delivery of target proteins such as genome editing enzymes into the cells. The genus *Xanthomonas* is known as consisting of a large group of pathogenic bacteria that use the type III secretion system in plants [10,11]. Within the genus, *X. campestris* can infect a variety of host plants, implying that its secretion system could be a good candidate of protein carrier apparatus. Therefore, we tried to develop genome editing method based on protein transport through *X. campestris* pv. *campestris* (*Xcc*) into plant cells.

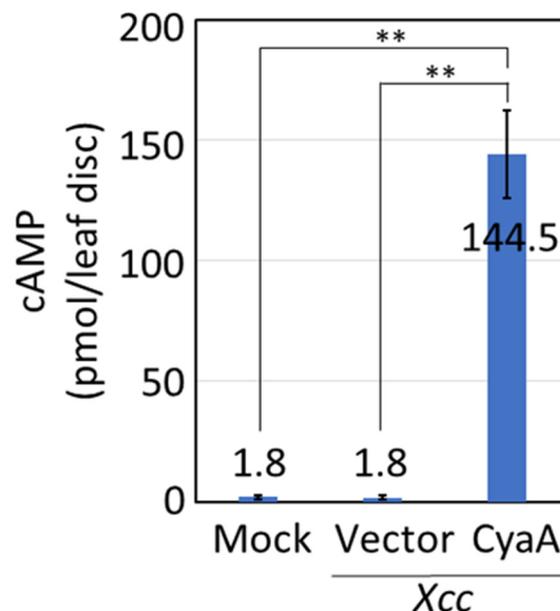
The purpose of this study is to develop a protein transfer method without introducing any foreign nucleic acid that can be used to produce genome-edited plants. I-SceI is a homing endonuclease often used in experimental models of genome editing systems. In this work, we showed that I-SceI protein was transported into tobacco leaf cells through the type III secretion system of *X. campestris* (*Xcc*) and genome-edited plants were regenerated by the *Xcc* infected leaf cells. We performed I-SceI-mediated genome editing in a tobacco reporter plant in which a luciferase gene is interrupted by a I-SceI recognition site. Luciferase expression occurs only following I-SceI digestion. We found that *Xcc* could transport a model protein into the cells of tobacco leaves by quantitative analysis. It was also showed that infected *Xcc* was sterilized from tobacco cells on a medium plate containing suitable antibiotics. Using this system, we successfully showed that a reporter gene in the tobacco plant was genome edited by the infection of *Xcc* carrying the I-SceI gene, indicating that I-SceI was transported through the type III secretion system of *Xcc* into the cells of reporter tobacco leaves. This technique will be useful for plant genome editing without introducing any foreign nucleic acid, by enabling the transfer of genome editing enzymes having the abilities of both DNA binding and digestion such as TALENs.

## 2. Results

### 2.1. Protein Transport into Plant Leaf Cells by *Xcc*, and its Sterilization with Antibiotics

We first examined whether the type III secretion system of *Xcc* could be used to transfer proteins into plant cells. We used bacterial adenylate cyclase (CyaA) as a reporter system. CyaA, which does not exist in plants, is an enzyme to catalyze the conversion of ATP to cAMP together with endogenous

calmodulin in eukaryotic cells. The amount of cAMP provides an indicator of the amount of CyaA introduced into living cells. We have previously identified 16 effector proteins in *X. oryzae* pv. *oryzae* MAFF311018 [12]. A XOO4208 ortholog named XopQ, which is one of representative effectors in *Xanthomonas*, is conserved in *Pseudomonas* and *Ralstonia*. We identified homologues of these effector proteins in the genome of *Xcc* MAFF106712 [13], and accordingly *Xcc1\_31290* was found to display a high level of homology (62% percent identity) to XOO4208. We decided to use a promoter and signal peptide sequence for the type III secretion system of *Xcc1\_31290* as an expression cassette for protein transport. Tobacco (*Nicotiana tabacum* cv. Samsun NN) leaves were infected by infiltration of *Xcc* carrying *Xcc1\_31290::CyaA*, and cAMP levels were measured by an enzyme immunoassay. Tobacco leaf cells infected by *Xcc* carrying *Xcc1\_31290::CyaA* contained approximately 80.3 times greater cAMP than leaf cells infected by *Xcc* carrying empty vector (Figure 1). The level of cAMP in leaf cells treated with only buffer (mock treatment) was low, similar to that in leaf cells treated with *Xcc* carrying an empty vector. These results indicate that *Xcc* transferred CyaA into the plant leaf cells through its type III secretion system.



**Figure 1.** cAMP amount in tobacco leaves infected by infiltration of *Xcc*. Tobacco leaves were infected by the infiltration of *Xcc* carrying the *Xcc1\_31290::CyaA* gene whose value of OD600 became 0.05. Buffer (Mock) and *Xcc* carrying an empty vector (Vector) used for *Xcc* were infiltrated as negative controls. Leaf discs were prepared after inoculation for 20 h. The amount of cAMP was measured and calculated per leaf disc. Asterisks indicate significant differences analyzed using Student's *t*-test compared with the negative controls at  $p < 0.01$  (\*\*).

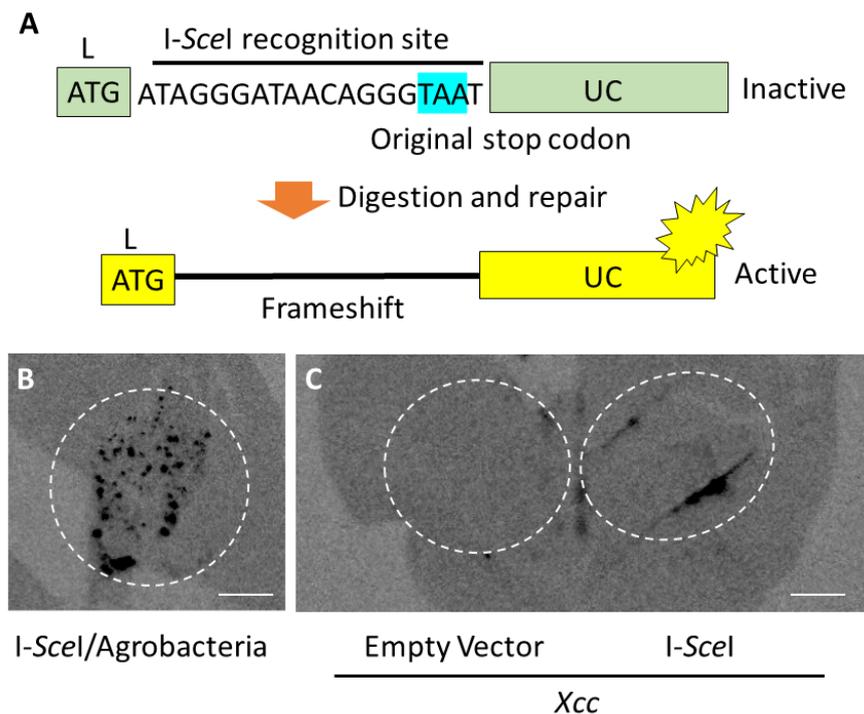
To use *Xcc* for protein transport in plant genome editing, *Xcc* must be sterilized following infection to obtain regenerated plants. To determine whether *Xcc* could be sterilized while leaving plant cells alive, *Xcc*-infected leaves were placed on regeneration medium plates containing antibiotics (200  $\mu\text{g}/\text{mL}$  cefotax and 50  $\mu\text{g}/\text{mL}$  rifampicin) (see Materials and Methods for detail). As shown in Figure 2, a green callus occurred from a part of the *Xcc*-infected leaves on the plate, indicating that the plant cells were alive by treatment with these antibiotics and could dedifferentiate even once *Xcc* infected them.



**Figure 2.** Callus from a *Xcc*-infected tobacco leaf piece. *Xcc*-infected leaf pieces were maintained on regeneration medium plates for 2 weeks. A living callus (light green) occurred from a part of a *Xcc*-infected leaf piece. Bar: 0.5 mm.

## 2.2. Genome Editing through *I-SceI* Protein Transport by *Xcc* into Plant Leaf Cells

To evaluate *I-SceI*-mediated genome editing, a transgenic tobacco plant carrying a *LUC* gene interrupted by an *I-SceI* recognition site just downstream of the 1st ATG was produced as a reporter plant (Figure 3A). Due to this insertion, translation from the ATG codon terminates at a stop codon inside of the *I-SceI* recognition site. If *I-SceI* digests the DNA at the recognition site and a frameshift mutation is introduced by misrepair, the reporter gene can be repaired, leading to *LUC* expression.



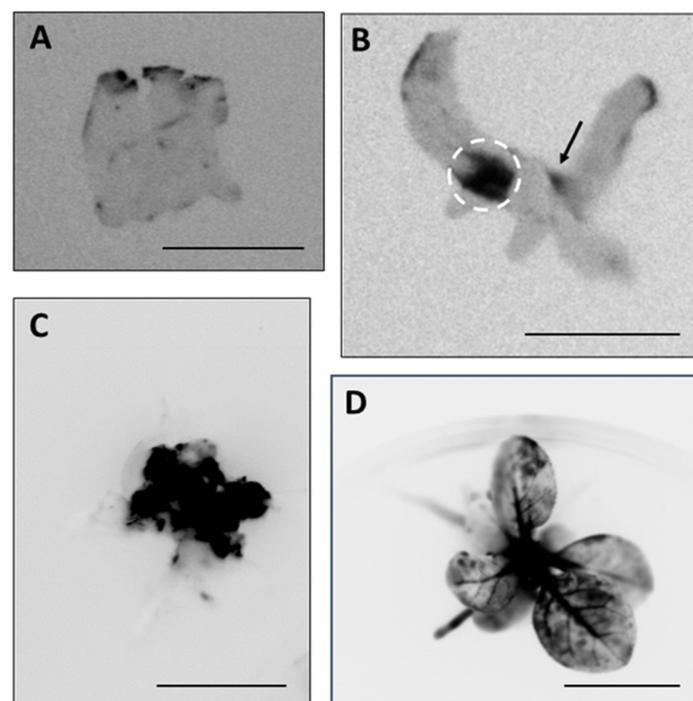
**Figure 3.** Genome editing through *I-SceI* protein transport by *Xcc* in tobacco leaf cells. (A) Schematic of luciferase (*LUC*) activation in the leaves of an L-(*I-SceI*)-UC reporter tobacco plant. *I-SceI* recognition site was inserted to divide the *LUC* gene with original stop codon. (B) Genome editing by infiltration of *Agrobacterium* carrying an *I-SceI* gene. The reporter plant with agroinfiltrated leaves was maintained for 3 days after agroinfiltration before chemiluminescence imaging. (C) Genome editing by infiltration of *Xcc* carrying an *I-SceI* gene or an empty vector as a negative control. The reporter plant with *Xcc* infiltrated leaves was maintained for 3 days after its infiltration before chemiluminescence imaging. Genome-edited areas are shown by dotted circles. Bars: 1 cm.

We first examined whether this reporter system works to evaluate *I-SceI*-mediated genome editing by *Agrobacterium*-mediated gene transfer of the *I-SceI* gene. Luciferase chemiluminescence was

observed in the agroinfiltrated region (Figure 3B), indicating that the reporter system could be used to evaluate I-SceI-mediated genome editing. Next, we infected reporter tobacco leaves by *Xcc* carrying the type III secretable *I-SceI* gene or an empty vector. Luciferase chemiluminescence was observed in the area infiltrated by *Xcc* carrying the *I-SceI* gene, but not in the area infected by *Xcc* carrying the empty vector (Figure 3C). This result indicates that I-SceI protein produced in *Xcc* was transported into the reporter plant leaf cells, and then in-frame shift occurred in the I-SceI recognition site after digestion of the target sequence by I-SceI.

### 2.3. Production of Genome-Edited Tobacco Plants Through I-SceI Protein Transport by *Xcc*

To produce genome-edited plants, leaf pieces inoculated with *Xcc* containing the *I-SceI* gene were cultivated *in vitro* for regeneration. *Xcc* infiltrated leaves that showed strong LUC chemiluminescence was excised and cultured on callus induction medium plates containing antibiotics cefotax and rifampicin in addition to suitable plant hormones for 1 month as described in Materials and Methods. During the dedifferentiation and differentiation from the leaf piece, areas with strong chemiluminescence were selected manually to concentrate the genome-edited cells (Figure 4A). After repeated dedifferentiation and differentiation, while concentrating area with strong chemiluminescence, on the regeneration medium plates containing cefotax, regenerated plants with genome-edited cells were produced on medium plates containing cefotax and suitable plant hormones (Figure 4B,C). By image analysis, the LUC chemiluminescence appeared patchy in the regenerated plants, indicating that they possessed genome-edited cells as mericlinal chimeras (Figure 4D).



**Figure 4.** Production of genome-edited tobacco plants through I-SceI protein transport by *Xcc*. (A) Luciferase chemiluminescence was observed in leaf pieces of an L-(I-SceI)-UC reporter tobacco plant inoculated with *Xcc* carrying the *I-SceI* gene after cultivation for 3 days on regeneration medium. (B) A regenerated plant was grown by selectively culturing regions that showed strong chemiluminescence. Genome-edited areas (indicated by a circle and an arrow) in B were cut out and further cultured to dedifferentiate (C) and differentiate (D). Bars: 1 cm.

Next, we examined the sequence in the I-SceI recognition site in the reporter gene of the regenerated plant shown in Figure 4D. A fragment of the reporter gene containing the I-SceI recognition site was



host plant species for genome editing. Other bacteria that possess the type III secretion system such as *Rhizobium* and *Pseudomonas* could also be used instead of *Xcc*.

This work suggests that the bacterial type III secretion system will be an important technique for genome editing in plants by direct introduction of genome editing enzymes into plant cells. Using this system, the type III secretion system of *Xcc* worked for I-SceI mediated-genome editing by transferring I-SceI protein into plant cells without introducing any foreign nucleic acid. Our model system of genome editing that used I-SceI can be easily adapted to deliver artificial nucleases such as TALENs. We will try to produce genome-edited plants using TALENs by our *Xcc* system in the near future. The reporter system used in this study is useful to concentrate genome-edited cells. However, the endogenous genome does not have any selection marker, implying that increasing efficiency of genome editing is needed to produce regenerated plants carrying mutations in endogenous genome. We plan to improve our *Xcc* system to make it an effective method of plant genome editing.

## 4. Materials and Methods

### 4.1. Plant and Bacterial Materials

The *L-(I-SceI)-UC* reporter gene [14] was introduced into a tobacco plant (*Nicotiana tabacum* cv. Samsun NN). The plant was grown at 25 °C with cycles of 16 h light/8 h dark.

*Xanthomonas campestris* pv. *campestris* (*Xcc*) or *Agrobacterium tumefaciens* strain C58C1 was grown in LB medium with or without suitable antibiotics with shaking at 28 °C.

### 4.2. Construction of Plasmids and Transformation into Bacteria

A plasmid expressing a fusion of the *Xcc* effector candidate and *CyaA* was generated as follows. We designed a pair of primers “*Xcc1\_31290 cya S*” (5'-aagaattcgccacagaagtactgggaagg-3') and “*Xcc1\_31290 cya AS*” (5'-ttggtaccctttgcgagccgggcgctcc-3') including restriction enzyme sites to amplify a promoter region of *Xcc1\_31290*. The PCR products were digested with *EcoRI* and *KpnI* and were subcloned into pBluescript II KS+, and the sequence was confirmed. The fusion fragment was then cloned into pHMCyA [12] to obtain a plasmid containing the *Xcc1\_31290::CyaA* fusion, and the plasmid was introduced into *Xcc* MAFF106712 by electroporation.

To construct a plasmid expressing the *Xcc1\_31290::I-SceI* fusion protein, the *Xcc1\_31290* gene including the promoter region was amplified by PCR using the primers “*Xcc31290FowBamI*” (5'-acgtgatccacagaagtactgggaagg-3') and “*Xcc31290Rev*” (5'-gttgaacacgccctttgcga-3'). *I-SceIAtNt* was amplified by PCR using the primers “*I-SceIAtNtFow*” (5'-tcgcaaagggcgtgttaacatgaagaagaagagaaagg-3') and “*I-SceIAtNtRevKpnI*” (5'-acgtgtactctattcaaaaaggtctcac-3'). Both amplified fragments were mixed as templates for PCR using the primers “*Xcc31290FowBamI*” and “*I-SceIAtNtRevKpnI*”. The resulting *Xcc1\_31290::I-SceIAtNt* fragment was inserted into Zero Blunt™ TOPO™ PCR Cloning vector (Thermo Fisher Scientific, Waltham, MA, USA). The *Xcc1\_31290::I-SceIAtNt* fragment was digested with *BamHI* and *KpnI* and inserted into pME6031. The resulting plasmid was introduced into *Xcc* as described above.

*pCAMBIA1390-sGFP* [15] was digested with *Sal I* and *BsrG I* to remove an open reading frame of the *sGFP* gene, and a synthesized *I-Sce I* codon-optimized for Arabidopsis and tobacco was inserted between the *Sal I* and *Bsr G1* sites to produce *pCa-HPT-I-SceIAtNt* plasmid. This plasmid was introduced into *A. tumefaciens* by electroporation.

### 4.3. Infection of Bacteria and cAMP Enzyme Immunoassay

Infection of *Agrobacterium* was performed as described previously [16]. *Xcc* containing pHMCyA or a control vector was cultivated overnight, and then harvested and suspended in A buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, pH 5.6) at OD<sub>600</sub> = 0.05 (measured using SmartSpec™ spectrophotometer, Bio-Rad, CA, USA). The suspension was infiltrated into tobacco leaves by using a needleless syringe.

To evaluate protein transport into plant cells, the amount of cAMP was measured with an Amersham cAMP Biotrak Enzymeimmunoassay System according to the manufacturer's instruction (GE Healthcare, Tokyo, Japan). Preparation of assay sample and assay method were performed as described previously [9]. Briefly, two leaf discs of 13-mm diameter from each infected leaf were prepared with a cork borer. After the discs were ground into a powder with a mortar and a pestle with liquid nitrogen, the leaf powder was dissolved in 340  $\mu$ L of 6% (*w/v*) trichloroacetic acid (TCA). Two-hundred microliters of assay buffer supplied by the kit were added to the dried extracts dissolved in 200  $\mu$ L of the TCA homogenate. Ten microliters of each dissolved extract were used for the cAMP enzyme immunoassay. Three independent samples were used for a statistical test.

#### 4.4. Genome Editing by *I-SceI*, Detection of LUC Chemiluminescence, and Sequencing of *I-SceI* Recognition Site

*Xcc* carrying an *I-SceI* gene was cultured overnight as described above. The bacteria were washed with A buffer (10 mM MES, 10 mM MgCl<sub>2</sub>, pH 5.6) once and diluted to OD<sub>600</sub> = 0.5 with A buffer. *Agrobacterium* carrying an *I-SceI* gene was washed with A buffer once and diluted to OD<sub>600</sub> = 0.05 with A buffer containing 150  $\mu$ M 3,5'-dimethyl-4'-hydroxy-acetophenone. They were infiltrated into the leaves of L-(*I-SceI*)-UC tobacco plants by using a needleless syringe. One millimolar of luciferin in 10 mM NaPO<sub>4</sub>, pH 7.0 was applied to the leaves to detect LUC chemiluminescence with a Fujifilm LAS-3000 imager (Fujifilm, Tokyo, Japan).

LUC signal was used to select genome-edited leaf regions and produce genome-edited plants. After infiltration of *Xcc* carrying the *I-SceI* gene, infected L-(*I-SceI*)-UC leaves were sterilized by soaking in 1% sodium hypochlorite for 1 min, and then washed twice with sterilized water. The leaves were cut into 0.5–1.0 cm pieces and placed on regeneration plates containing 1  $\times$  Murashige and Skoog (MS), 1  $\times$  MS vitamin (0.1  $\mu$ g/mL thiamine hydrochloride, 0.5  $\mu$ g/mL pyridoxine hydrochloride, 0.5  $\mu$ g/mL nicotinamide, 2  $\mu$ g/mL glycine, 100  $\mu$ g/mL myo-inositol), 0.1  $\mu$ g/mL  $\alpha$ -Naphthaleneacetic acid, 1  $\mu$ g/mL 6-Benzylaminopurine, and 200  $\mu$ g/mL cefotax, and 8.5 g/L agar, pH 8.5, and were maintained at 28  $^{\circ}$ C in cycles of 16 h light/8 h dark for 3 days. The leaves were transferred onto the regeneration plates supplemented with 100  $\mu$ g/mL kanamycin, 50  $\mu$ g/mL rifampicin, and 30% sucrose, and leaf pieces and/or callus exhibiting strong LUC signals were transferred onto the new plates every 1 week. After 1 month, callus with strong LUC signals were transferred onto the regeneration plates with 100  $\mu$ g/mL kanamycin and 30% sucrose to form shoots. The regenerated shoots were transferred onto root formation plates containing 1  $\times$  MS, 1  $\times$  MS vitamin, 30% sucrose, 200  $\mu$ g/mL cefotax, and 8.5 g/L agar, pH 8.5.

To examine the sequence of the *I-SceI* recognition site in the reporter gene of regenerated plants, a fragment of the reporter gene containing the *I-SceI* recognition site was amplified by PCR and cloned using the primers "P35S-90" (5'-atctccactgacgtaagggatgacg-3') and "eLUC241R" (5'-ctgcacagatgacacgac-3') and cloned with a Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific).

## 5. Patents

A patent titled "Method for producing genome-edited plant, WO2018123938" partially resulted from the work reported in this manuscript.

**Author Contributions:** Conceptualization, Y.Y. and I.M.; methodology, I.M.; validation, Y.Y., and I.M.; formal analysis, Y.Y.; investigation, Y.Y., K.T., M.E. and I.M.; resources, H.O. and I.M.; data curation, Y.Y. and I.M.; writing—original draft preparation, Y.Y.; writing—review and editing, K.T., A.F., H.O., S.T., and I.M.; visualization, Y.Y. and I.M.; supervision, I.M.; project administration, I.M.; funding acquisition, I.M. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Choo, Y.; Sanchez-Garcia, I.; Klug, A. In vivo repression by a site-specific DNA-binding protein designed against an oncogenic sequence. *Nature* **1994**, *372*, 642–645. [[CrossRef](#)] [[PubMed](#)]
2. Boch, J.; Scholze, H.; Schornack, S.; Landgraf, A.; Hahn, S.; Kay, S.; Lahaye, T.; Nickstadt, A.; Bonas, U. Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **2009**, *326*, 1509–1512. [[CrossRef](#)] [[PubMed](#)]
3. Moscou, M.J.; Bogdanove, A.J. A simple cipher governs DNA recognition by TAL effectors. *Science* **2009**, *326*, 1501. [[CrossRef](#)] [[PubMed](#)]
4. Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J.A.; Charpentier, E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **2012**, *337*, 816–821. [[CrossRef](#)] [[PubMed](#)]
5. Jacobi, A.M.; Rettig, G.R.; Turk, R.; Collingwood, M.A.; Zeiner, S.A.; Quadros, R.M.; Harms, D.W.; Bonthuis, O.J.; Gregg, C.; Ohtsuka, M.; et al. Simplified CRISPR tools for efficient genome editing and streamlined protocols for their delivery into mammalian cells and mouse zygotes. *Methods* **2017**, *15*, 121–122. [[CrossRef](#)] [[PubMed](#)]
6. Lee, K.; Conboy, M.; Park, H.M.; Jiang, F.; Kim, H.J.; Dewitt, M.A.; Mackley, V.A.; Chang, K.; Rao, A.; Skinner, C.; et al. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. *Nat. Biomed. Eng.* **2017**, *1*, 889–901. [[CrossRef](#)] [[PubMed](#)]
7. Liu, W.; Rudis, M.R.; Cheplick, M.H.; Millwood, R.J.; Yang, J.-P.; Ondzighi-Assoume, C.A.; Montgomery, G.A.; Burris, K.P.; Mazarei, M.; Chesnut, J.D.; et al. Lipofection-mediated genome editing using DNA-free delivery of the Cas9/gRNA ribonucleoprotein into plant cells. *Plant Cell Rep.* **2020**, *39*, 245–258. [[CrossRef](#)] [[PubMed](#)]
8. Guo, B.; Itami, J.; Oikawa, K.; Motoda, Y.; Kigawa, Y.; Numata, K. Native protein delivery into rice callus using ionic complexes of proteins and cell-penetrating peptides. *PLoS ONE* **2019**, *14*, e0214033. [[CrossRef](#)]
9. Yanagawa, Y.; Kawano, H.; Kobayashi, T.; Miyahara, H.; Okino, A.; Mitsuhashi, I. Direct protein introduction into plant cells using a multi-gas plasma jet. *PLoS ONE* **2017**, *12*, e0171942. [[CrossRef](#)]
10. Frank, F.; Potnis, N.; Jones, J.B.; Jiebbuj, R. The type III effectors of *Xanthomonas*. *Mol. Plant Pathol.* **2009**, *10*, 749–766.
11. Büttner, D. Behind the lines—actions of bacterial type III effector protein in plant cells. *FEMS Microbiol. Rev.* **2016**, *40*, 894–937. [[CrossRef](#)] [[PubMed](#)]
12. Furutani, A.; Takaoka, M.; Sanada, H.; Noguchi, Y.; Oku, T.; Tsuno, K.; Ochiai, H.; Tsuge, S. Identification of novel type III secretion effectors in *Xanthomonas oryzae* pv. *oryzae*. *Mol. Plant Microbe Interact.* **2009**, *22*, 96–106. [[CrossRef](#)] [[PubMed](#)]
13. Takeuchi, K.; Mitsuhashi, I. Complete genome sequences of two strains of *Xanthomonas campestris* pv. *campestris* isolated in Japan. *Microbiol. Resour. Announc.* **2020**, *9*, e0123919. [[CrossRef](#)] [[PubMed](#)]
14. Chujo, T.; Yoshikawa, M.; Ariga, H.; Endo, M.; Toki, S.; Ishibashi, K. A removable virus vector suitable for plant genome editing. *Plant J.* **2017**, *91*, 558–561. [[CrossRef](#)] [[PubMed](#)]
15. Toki, S.; Hara, N.; Ono, K.; Onodera, H.; Tagiri, A.; Oka, S.; Tanaka, H. Early infection of scutellum tissue with *Agrobacterium* allows high-speed transformation of rice. *Plant J.* **2006**, *47*, 969–976. [[CrossRef](#)] [[PubMed](#)]
16. Kawazu, K.; Wasano, N.; Konno, K.; Ohashi, Y.; Mochizuki, A.; Mitsuhashi, I. Evaluation of anti-herbivory genes using an *Agrobacterium*-mediated transient expression system. *Plant Biotech.* **2012**, *29*, 495–499. [[CrossRef](#)]



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