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# Direct protein delivery into intact *Arabidopsis* cells for genome engineering

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**Intracellular delivery of biomolecules is a prerequisite for genetic techniques such as recombinant engineering and genome editing. Realizing the full potential of this technology requires the development of safe and effective methods for delivering protein tools into cells. In this study, we demonstrated the spontaneous internalization of exogenous proteins into intact cells and root tissue of whole plants of *Arabidopsis thaliana*. We termed this internalization phenomenon as protein Delivery Independent of Vehicles or Equipment (DIVE), which efficiently delivered genome engineering proteins including Cre recombinase and zinc-finger nucleases (ZFN) into plant cells. Using protein DIVE, we achieved less toxic protein delivery than electroporation with up to 94% efficiency in *Arabidopsis* cell culture and 19% genome modification in *Arabidopsis* plants that was maintained in regenerated tissue. This work illustrates the potential of protein DIVE for a wide range of applications, including genome engineering in plants.**

Intracellular delivery of biomolecules is an indispensable process for the application of genetic tools such as recombinant engineering and genome editing. The various techniques that facilitate intracellular delivery of biomolecules in plants include *Agrobacterium*-mediated gene transfer (AMGT), particle gun bombardment, protoplast fusion, and electroporation. Each of these methods have advantages over the others but they also have some limitations.

AMGT has been widely used to introduce foreign DNA into plant cells[1] and is popular for genetic transformation of *Arabidopsis thaliana* as transgenic seeds can be readily obtained using the floral dip technique[2]. However, transgenes derived from the AMGT vector are randomly integrated into the host genome[3,4]; this might result in the disruption of host gene expression. Such limitations of AMGT call for alternative physical or chemical methods to expand the scope of plant engineering. On the other hand, methods such as protoplast fusion facilitate intracellular delivery[5–8] of foreign DNA. Although cell wall removal greatly enhances the delivery efficiency of the resultant protoplasts, it causes severe cytotoxicity and cell damage due to biochemical and/or mechanical stress[9,10]. Thus, the regeneration of whole plants from protoplasts is either impossible or inefficient in many plant species. The physical perforation of cells during DNA delivery by particle gun bombardment causes unavoidable cell damage; however, transformants can be obtained by targeting mature embryos[11]. The problems associated with this technique include difficulty in targeting tissues, fragmentation of transgene, and requirement of expensive equipment[12]. Recently, certain research groups have attempted the delivery of exogenous substances into plant cells using electroporation[13–17].

Conventionally, DNA delivery using these methods has been dominant in genome engineering, however, there remain safety concerns including host DNA damage due to insertion of foreign DNA and side effects caused by long-term expression of genome-modifying enzymes. Direct protein delivery, free from these concerns, has therefore been attracting attention for biotechnology applications. We aim to develop a DNA-damage free and efficient protein delivery method for plants using Cre recombinase as a model. Cre recombinase is widely used in a variety of organisms to manipulate DNA both in vitro and in vivo[18–25]. Combined with consensus DNA sequences, the loxP, Cre/loxP recombination system facilitates DNA modifications including deletions, insertions, translocations, and inversions at specific DNA loci of cells[26–28]. Previous work in our laboratory involved the introduction of Cre protein into *A. thaliana* cells using electroporation where we demonstrated the removal of a specific gene fragment in the plant genome[14,15]. Using a suitable buffer, we succeeded in delivering

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Cre proteins into *Arabidopsis* cells with low cytotoxicity and a high efficiency of over 80% of cells electroporated in the presence of Cre protein[14,15].

Surprisingly, after our previous study, we found that Cre proteins are spontaneously internalized into the cells and modified the plant genome without electroporation, albeit with low efficiency (Supplementary Fig. 1). This is an indication that DNA-modifying proteins are capable of penetrating cell walls, cell membranes, and nuclear membranes to gain access to genomic DNA. Based on the above observations, we hypothesized that by optimizing experimental conditions, it could be an efficient method for protein delivery into intact cells in culture as well as whole plants. Here, we demonstrated a simple and efficient protein delivery method using Cre recombinase and ZFN using *Arabidopsis* as the host. By optimizing the various conditions including buffer types and incubation time, we achieved a delivery efficiency greater than electroporation.

## Results

### Protein internalization into plant culture cells

To investigate the spontaneous internalization of proteins by plant cells in culture, we utilized the experimental system of Cre protein and T87-xGxGUS reporter cells constructed in our electroporation study (Fig. 1a)[14]. While the Cre protein was incubated with the cells on ice in the electroporation protocol, our results indicated that the delivery efficiency could be further improved by incubating with an appropriate buffer at 23 °C without electric stimuli (Supplementary Fig. 2). In particular, Opti-MEM I or NT1HF medium (Murashige–Skoog-based

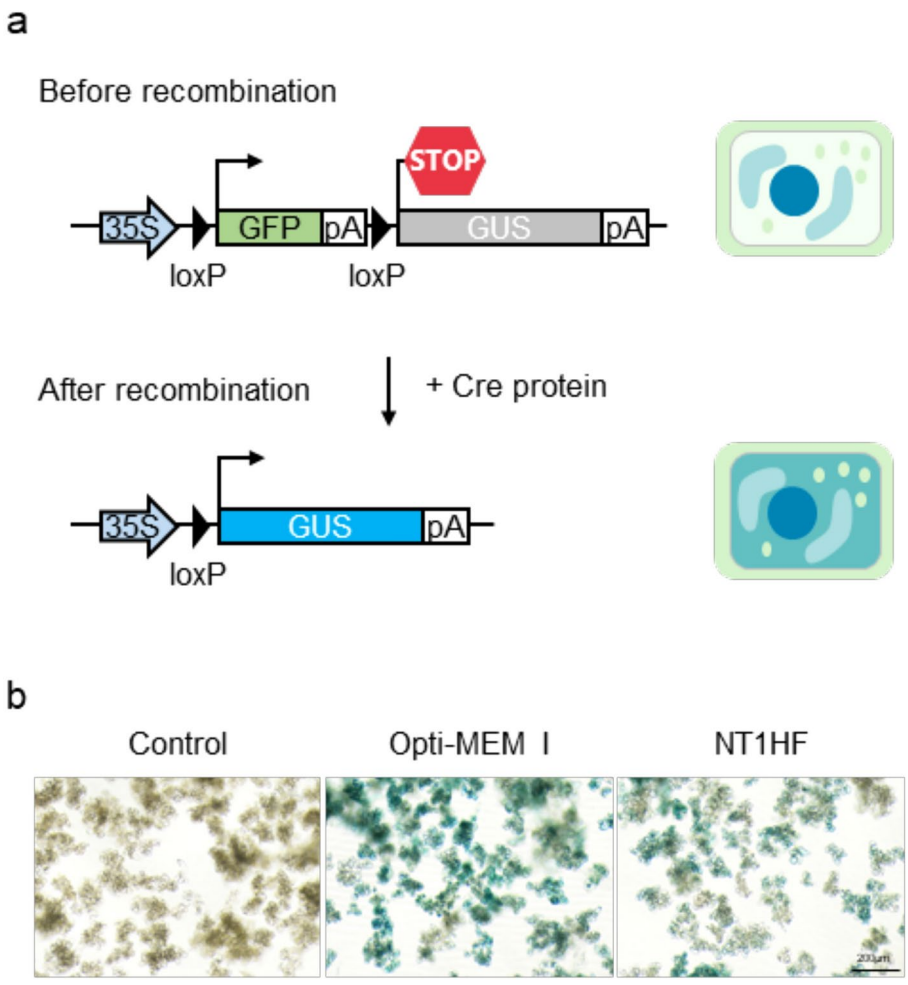


**Fig. 1**. Cre protein delivery into T87 cells. (**a**) Schematic diagram of Cre reporter design before and after Cre-mediated recombination constructed as described in our previous study[14]. The construct T87-xGxGUS includes an expression cassette encoding the gene for green fluorescence protein (GFP) followed by a transcription termination signal and the gene for ß-glucuronidase (GUS) into the genome of T87 cell line. After Cre-mediated recombination between the two loxP sites flanking the GFP gene, T87-xGxGUS cells, originally expressing GFP, expressed GUS and, thus, appeared blue in the presence of X-Glucuronide substrate. GFP, pA, and GUS indicate green fluorescent protein, terminator polyadenylation signal, and ß-glucuronidase, respectively. (**b**) GUS staining of T87-xGxGUS cells treated with 1 µM Cre protein using Opti-MEM I and NT1HF medium. GUS staining was performed 2 days after the treatment. Control indicates T87-xGxGUS cells without the DIVE treatment including the buffer and the Cre protein. Scale bar represents 200 µm.

medium without 2,4-dichlorophenoxyacetic acid) enabled Cre protein delivery with the lowest toxicity and the highest efficiency of the buffers tested (Fig. 1b and Supplementary Fig. 2). These buffers did not induce the GUS expression of T87-xGxGUS cells in the absence of Cre protein (Supplementary Fig. 3). This internalization phenomenon was temperature-dependent, similar to the energy-dependent endocytosis in other organisms and exhibited higher delivery efficiency at a normal culture temperature (23 °C) than at a low temperature (4 °C; Fig. 2). Surprisingly, the efficiency was much higher at 37 °C. At 42 °C, the cells were damaged and the number of delivered cells decreased compared with that at 37 °C (Supplementary Fig. 4). While the efficiency at 37 °C was the highest, we used incubation at 23 °C in the following experiments as it showed less cytotoxicity with good delivery efficiency. Moreover, the protein delivery efficiency also decreased with the endocytosis inhibitor, wortmannin (Supplementary Fig. 5). These results reveal that this protein internalization phenomenon is endocytosis-dependent.

## Protein Delivery Independent of Vehicles or Equipment (DIVE) method

In our experimental procedure, T87 cells were washed once with Opti-MEM I or NT1HF medium, and then, Cre proteins were added to the cells. After incubation for an hour, the cells were washed once and suspended in a culture medium. As no reagents were added and no physical stimuli were applied to the cells during the incubation, we named this method as protein Delivery Independent of Vehicles or Equipment (DIVE) to distinguish it from protein transduction[29], which delivers proteins into cells in combination with other materials. We assessed the effect of incubation time on DIVE efficiency. We observed DIVE phenomenon as early as 10 min of incubation (Supplementary Fig. 6). Next, we assessed the effect of Cre protein concentration on delivery efficiency. GUS expression was observed even at 0.2 μM Cre protein concentration, which increased with increasing Cre protein concentration of up to 5 μM (Supplementary Fig. 7a, b). However, the concentrations of Cre proteins also affected cell viability (Supplementary Fig. 7c). The viability difference between NT1HF and OptiMEM-I at lower Cre concentrations suggests Opti-MEM I itself has some toxicity to T87 cells. For a detailed quantification of delivery efficiency, we generated protoplasts using cellulase and pectinase to dissociate the cell aggregates just before GUS staining. GUS staining of protoplasts showed that more than 80% of cells expressed GUS (Fig. 3a, b). We also performed a genomic PCR analysis using primers that flanked the two loxP sites in the reporter cassette. As shown in Fig. 3c, the cells with DIVE treatment exhibited over 90% recombination rate. This is consistent with the results of GUS staining (Fig. 3a, b). Furthermore, protein DIVE-treated cells proliferated better than

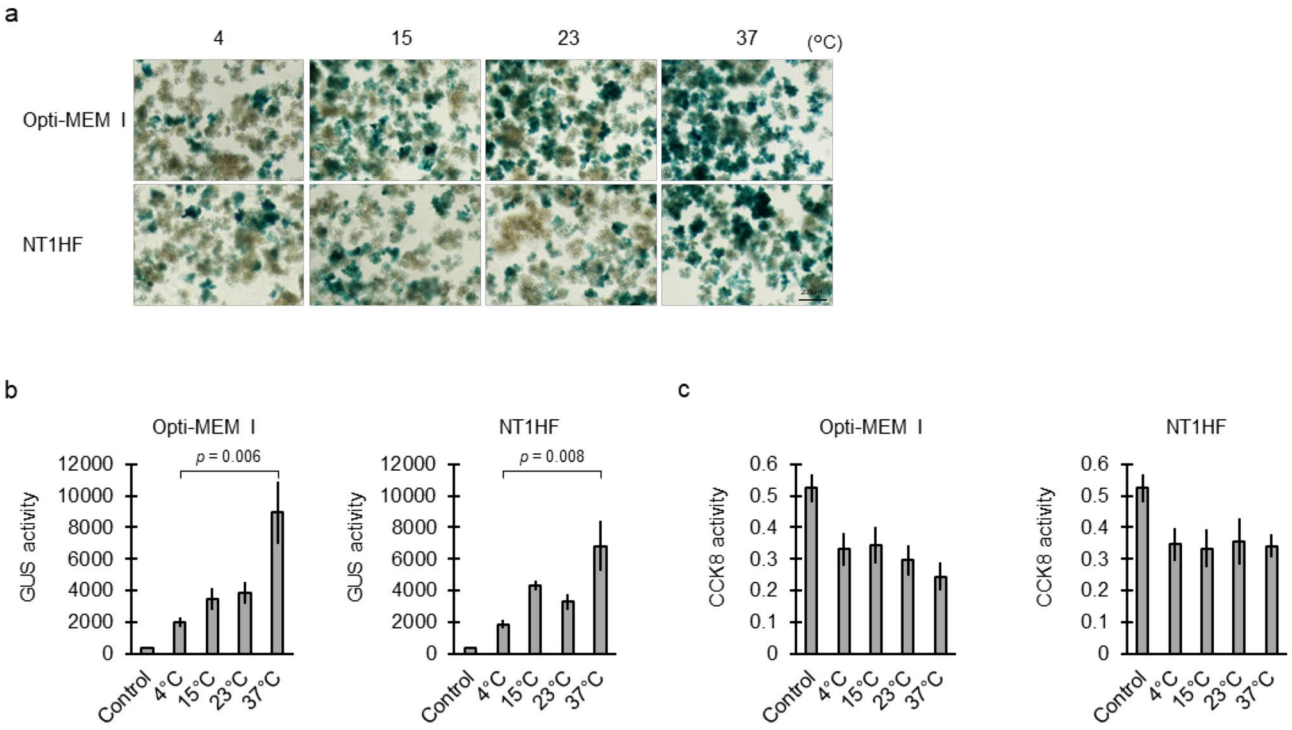


**Fig. 2**. Effect of incubation temperature on DIVE efficiency. (**a**) GUS staining of T87-xGxGUS cells incubated with 1 μM Cre protein at different temperatures. The cell number was normalized based on their volume. The cells were incubated with 1 μM Cre protein in Opti-MEM I or NT1HF at the indicated temperatures for 1 h. GUS staining was performed 2 days after Cre treatment. Scale bar represents 200 μm. (**b**) The total GUS activity of the cells in panel **a**. GUS activity was determined by the fluorescence level of catalyzed 6-chloro-4-methylumbelliferyl β-D-glucuronide 2 days after Cre treatment. *P* values were obtained using Student's t-test. (**c**) Viability of the cells in panel **a**. CCK8 assay was performed 2 days after incubation. CCK8 activity, i,e., absorbance at 450 nm, was indicated. Control indicates T87-xGxGUS cells without the DIVE treatment including the buffer and the Cre protein. Values shown are the mean ± SE of n = 3.

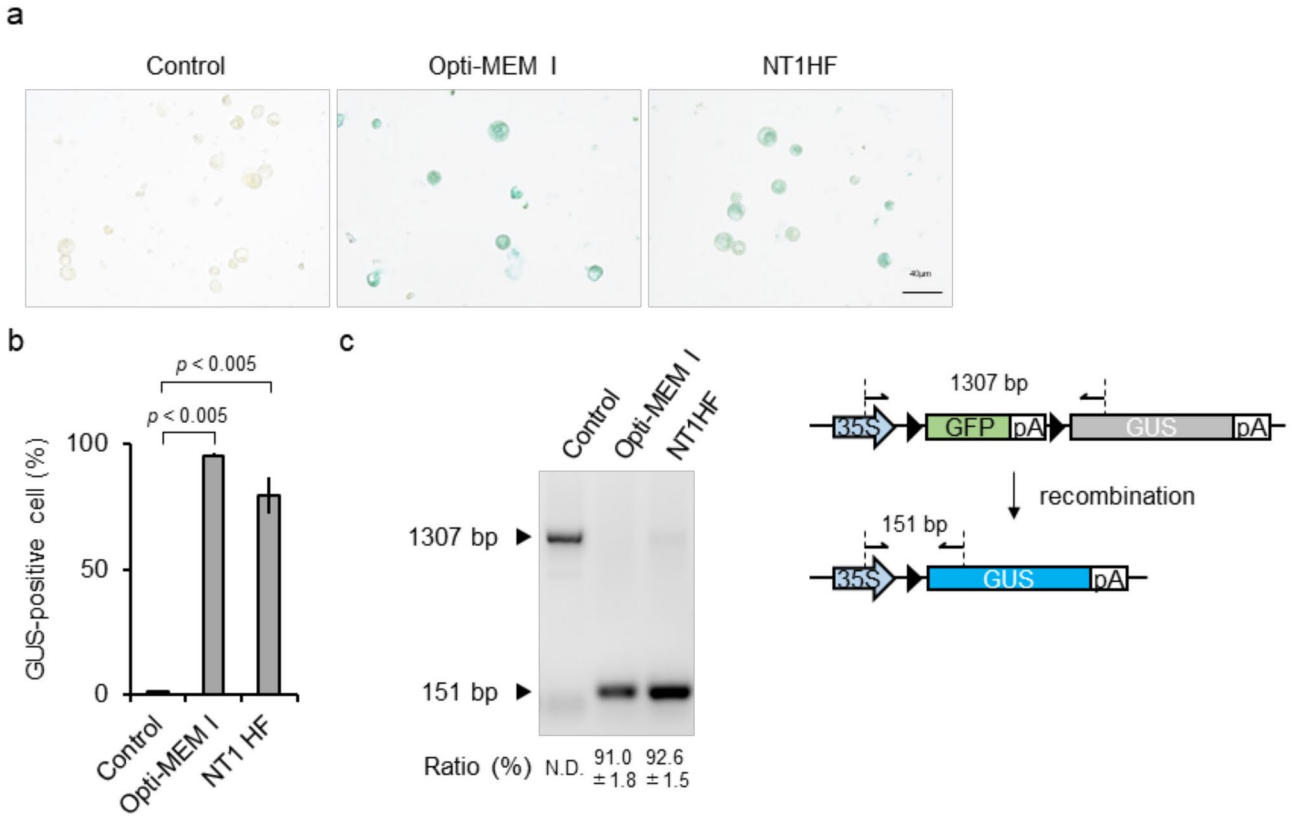


**Fig. 3**. Quantification of protein DIVE efficiency. (**a**) GUS staining of protoplasts generated from T87-xGxGUS cells with Cre treatment. Cells were incubated with 5 μM Cre protein in Opti-MEM I or NT1HF for 1 h. Protoplasts were constructed and stained 2 days after Cre treatment. Scale bar represents 40 μm. (**b**) Quantification of the percentage of GUS-positive cells in panel **a**. Values shown are the mean ± SE of n = 3. In total, 416, 402, and 450 cells were used for the quantification of control, Opti-MEM I, and NT1HF samples, respectively. *P* values versus control were determined by Tukey's test (n = 3 independent experiments each). (**c**) Agarose gel electrophoresis of genomic PCR products from T87-xGxGUS cells. The 1307-bp and 151-bp fragments represent the reporter gene cassette before and after Cre-mediated recombination, respectively. A half arrow indicates a primer binding site. Control indicates T87-xGxGUS cells without the DIVE treatment including the buffer and the Cre protein. Values shown are mean ± SE of the ratio of bands after recombination, n = 3. A full-length gel image is presented in Supplementary Fig. 18.

electroporated cells and showed a growth curve similar to that of non-treated control cells (Supplementary Fig. 8). Considering transduction efficiency and cell viability, we standardized the DIVE protocol for Cre protein: incubation with 1 μM Cre protein in Opti-MEM I or NT1HF for 1 h at 23 °C. These results indicate that protein DIVE enables efficient protein delivery and genome engineering, which requires only few days.

## Peptide tagging effect on DIVE efficiency

To investigate the effect of peptide tags which are known to enhance the protein delivery on protein DIVE, we additionally prepared Cre proteins to be fused with several peptide tags. We evaluated the effect of membrane translocation sequence (MTS) fusion on protein DIVE, which enhances protein transduction[30,31], but we did not observe any significant change (Supplementary Fig. 9). The effect of nuclear localization signal (NLS) fusion, which enhances macropinocytosis in mammalian cells[32], was also evaluated; the delivery efficiency of Cre protein without NLS (HCre) was lower than that with NLS (HNCre; Supplementary Fig. 10). Although this could be attributed to differences in nuclear translocation activity, the high basicity of NLS might contribute to Cre delivery. We also evaluated the fusion effect of Tat and R9 peptides, which are widely used to increase protein transduction[33,34]. However, Tat and R9 peptide tags did not enhance the delivery efficiency of Cre protein (Supplementary Fig. 11). The fusion of virulence proteins such as VirE2 or VirF, which improved the delivery efficiency of Cre protein during AMGT[35], did not show a notable increase in the delivery efficiency of the Cre protein (Supplementary Fig. 12). This is consistent with the report that the protein translocation effect of VirE2 and VirF is dependent on VirB/D4 T4SS generated during *Agrobacterium* infection[35]. These results indicate that the peptide tagging including membrane-permeable peptides except NLS has minimum impact on protein DIVE.

### Protein DIVE of ZFNs

We tested whether protein DIVE could be applied to other protein-based enzymes besides Cre. To this end, we chose the genome editing enzyme ZFN as a model protein. ZFN consists of a site-specific DNA-binding zinc-finger domain and a non-specific nuclease domain. To evaluate the delivery of ZFN, we established a new reporter cell line, T87-SSA500-ZFN(C5R), which expresses GUS only when the homodimeric ZFNs induce double-strand break at its target sequence, C5R, followed by single-strand annealing (SSA; Fig. 4a). We observed

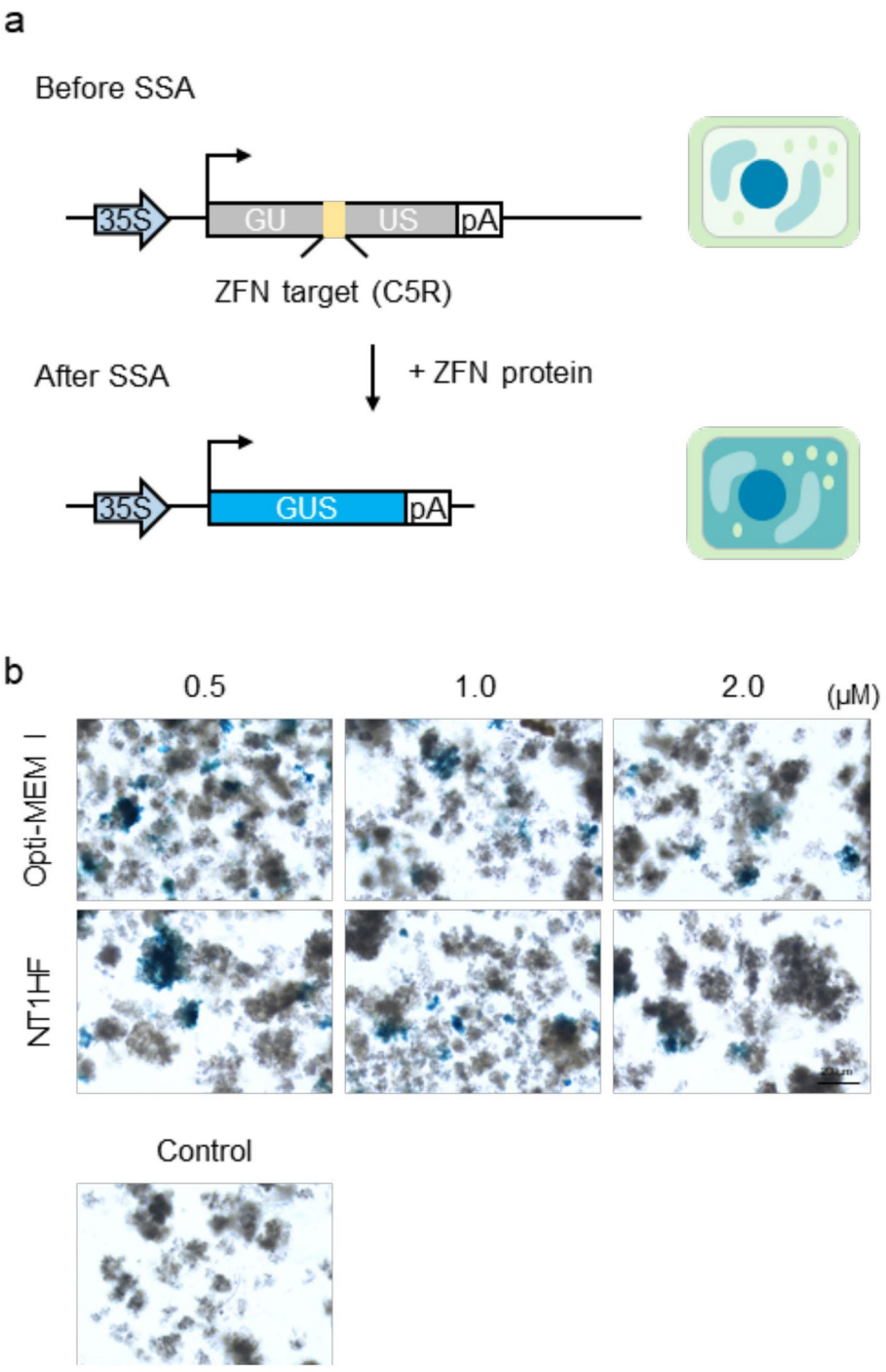


**Fig. 4**. ZFN protein delivery into T87 cells. (**a**) Schematic diagram of ZFN reporter design before and after ZFN-mediated SSA in T87-SSA500-ZFN(C5R) cell line. GUS, GU, US, and pA indicate ß-glucuronidase, N-terminal fragment of GUS, C-terminal fragment of GUS, and terminator polyadenylation signal, respectively. (**b**) GUS staining of T87-SSA500-ZFN(C5R) cells treated with 0.5, 1.0, and 2.0 μM ZFN protein using Opti-MEM I and NT1HF medium. GUS staining was performed 2 days after the treatment. Control indicates T87-SSA500-ZFN(C5R) cells without the DIVE treatment including the buffer and the ZFN protein. Scale bar represents 200 μm.

some cells expressing GUS after the delivery of the ZFN protein for C5R, indicating that ZFN proteins were successfully delivered into the cell nucleus (Fig. 4b). Moreover, we tested two types of nuclease domains for ZFN to improve the editing efficiency; one from a commonly-used FokI (*Flavobacterium okeanokoites*) and the other from a homolog of FokI (*Firmiculus* sp.), named FirmCut nuclease domain. We fused them with a zinc-finger domain targeting an artificial sequence ZFA36 and constructed another SSA reporter cell line with ZFA36 sequence. ZFN proteins were successfully delivered into the reporter cells (Supplementary Fig. 13). These results indicate that ZFN can also be internalized into plant cells and exhibit enzymatic functions in the nucleus and that protein DIVE can be applied to proteins other than Cre.

## Protein DIVE into *Arabidopsis* plants

To validate the effectiveness of protein DIVE in plant tissue, we generated transgenic *Arabidopsis* plants, namely, *A. thaliana* Col-0 xGxGUS, carrying the same reporter gene cassette as T87-xGxGUS cell line. We observed strong GUS staining, especially in the root tissue of *A. thaliana* seedlings when treated with 5 µM Cre protein (Fig. 5).

To investigate whether the root tissues treated with protein DIVE can maintain regenerative functions comparable to those of natural tissues, calli induction was performed (Fig. 6a). We successfully obtained the same number of calli from the root tissue with DIVE treatment as that from the untreated tissue. The efficiency of

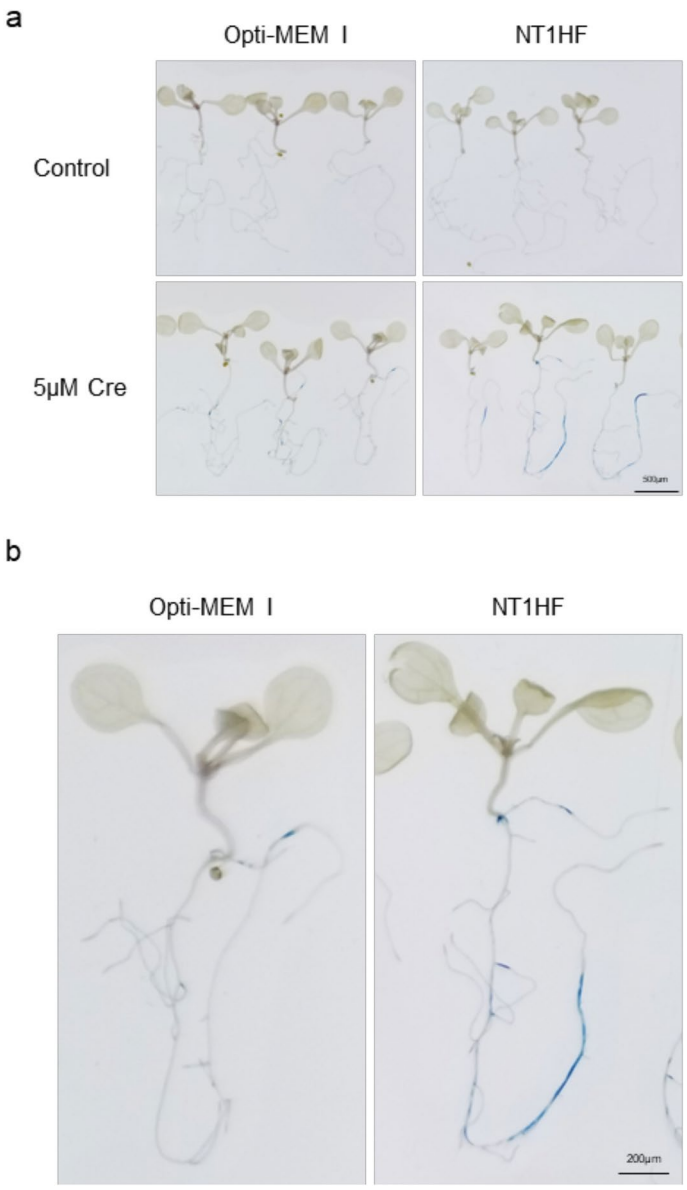


**Fig. 5**. Cre protein DIVE in *A. thaliana* plants. (**a**) GUS staining of *A. thaliana* Col-0 xGxGUS plants treated with 5 µM Cre protein using Opti-MEM I and NT1HF medium. GUS staining was performed 2 weeks after Cre treatment. Control indicates untreated plants. Scale bar represents 500 µm. (**b**) Close-up view of panel **a**. Scale bar represents 200 µm.

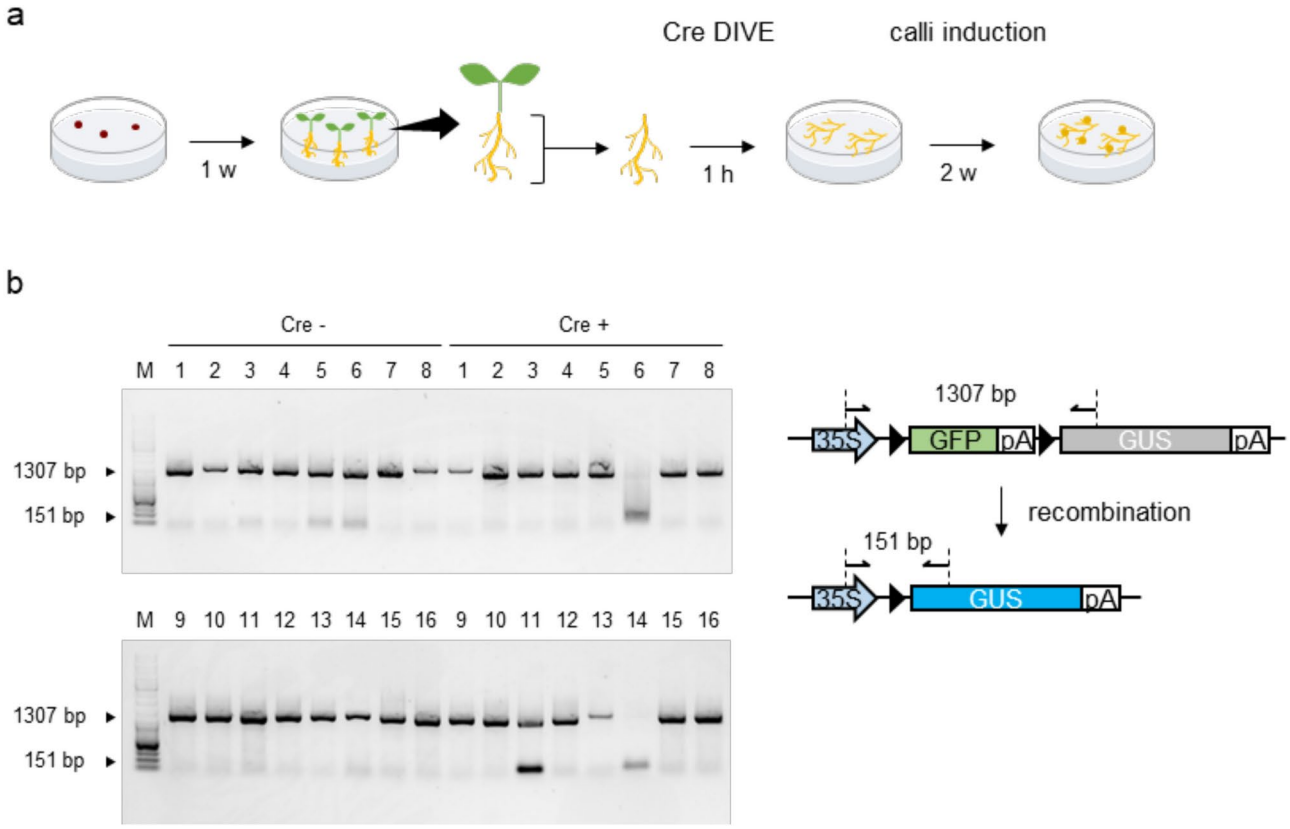


**Fig. 6**. Calli induction from *A. thaliana* root tissue after Cre-mediated recombination. (**a**) Schematic diagram of calli induction from *A. thaliana* Col-0 xGxGUS roots with Cre treatment. (**b**) Agarose gel electrophoresis of genomic PCR products from calli generated from *A. thaliana* Col-0 xGxGUS plants with Cre treatment. The 1307-bp and 151-bp fragments represent the reporter gene cassette before and after Cre-mediated recombination, respectively. M indicates DNA ladder marker. A half arrow indicates a primer binding site.

Cre-mediated recombination measured using genomic PCR analysis showed positive results in 3 out of 16 calli, with a recombination rate of approximately 19% (Fig. 6b). These results indicate that protein DIVE is effective when administered in planta, and that the genome modification is maintained in the regenerated tissues.

## Internalization dynamics in culture cells and plants

To further investigate how proteins pass through the plant cell wall and reach the cell nucleus, we developed a split luciferase complementation (SLC) assay. In the SLC assay, the green enhanced Nano-lantern (GeNL) is reconstituted resulting in luminescence only when its small fragment (HiBiT) interacts directly with its large fragment (GeLgBiT; Fig. 7a). Thus, using a Cre protein fused with HiBiT [HBNCre(wt)] and T87 cells expressing GeLgBiT in the cytoplasm (T87-GeLgBiT) and in the nucleus (T87-NLS-GeLgBiT), it is possible to make real time observations of the presence of added protein in the target cell compartment. When the purified HBNCre(wt) protein was added to GeLgBiT cells, the luminescence increased after a few minutes and reached the maximum after 20 min (Fig. 7b). The signal kinetics was almost the same with NLS-GeLgBiT cells (Fig. 7c). The maximum luminescence increased with an increase in Cre protein concentration. Background luminescence due to the leakage of GeLgBiT or NLS-GeLgBiT protein to the extracellular medium was negligible, compared to the intracellular luminescence (Supplementary Fig. 14).

We also performed SLC assay in planta using *A. thaliana* Col-0 plants subjected to Cre treatment. Strong luminescence was detected immediately after the addition of purified HBNCre(wt) protein and NanoLuc substrate to the seedlings expressing NLS-GeLgBiT (Fig. 7d). While the whole plants luminesced, the luminescence was stronger especially in the root tissue. This was consistent with the pattern of GUS staining as a result of recombination (Fig. 5). These results indicate that the protein-based genome engineering tools can pass through barriers of cell wall, membrane, and nuclear membrane and eventually into the cell nucleus of plant tissue without any additional intervention of physical/chemical inputs.

## Discussion

In this study, we introduced a protein delivery method called protein DIVE, which enables the introduction of target proteins with a very simple experimental procedure without the use of any special and expensive equipment, as shown in Supplementary Fig. 15. Direct protein delivery is a promising technology in plant research, especially in genome engineering. In the presence of genome engineering proteins delivered using

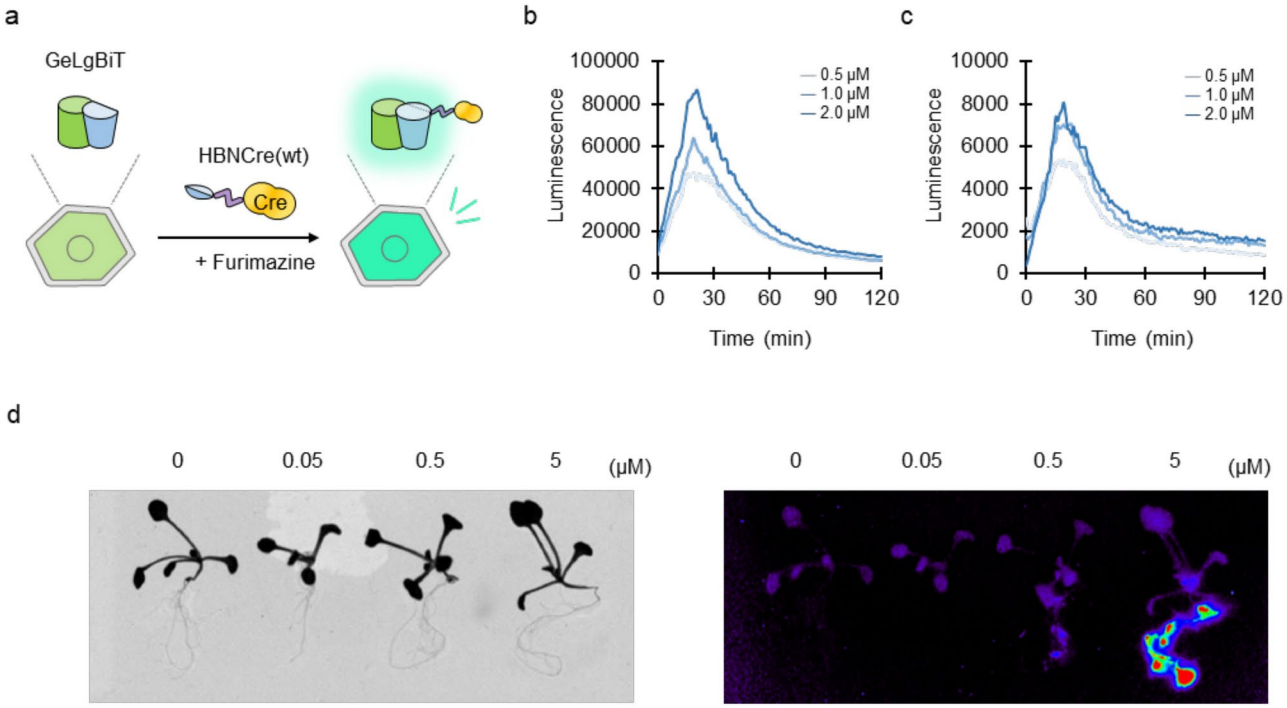


**Fig. 7**. Luminescence quantification of GeLgBiT-HiBiT complementation in vitro and in vivo. (**a**) Schematic diagram of GeLgBiT-HiBiT complementation assay. LgBiT and HiBiT are the large and small fragments of NanoLuc luciferase. Only when these two fragments are directly associated, an active luciferase is reconstituted resulting in oxidation of furimazine and luminescence[36]. In our SLC assay, mNeonGreen is fused to LgBiT (GeLgBiT) to shift the emission wavelength of bioluminescence suitable for plant cells[37]. This allows monitoring of the internalization kinetics of HBNCre(wt), a Cre protein fused to HiBiT, in GeLgBiT-expressing cells and plants with high sensitivity. (**b**, **c**) Luminescence kinetics of (**b**) T87-GeLgBiT and (**c**) T87-NLS-GeLgBiT cells with HiBiT-tagged Cre treatment. Cells were incubated with Nano-Glo Live Cell Substrate in NT1HF for 5 min. After incubation, HBNCre(wt) (final concentration of 0.5–2 μM) in NT1HF was added and luminescence was measured. (**d**) Luminescence image of *A. thaliana* Col-0 NLS-GeLgBiT plants after the addition of Nano-Glo Live Cell Substrate and HBNCre(wt) protein. *A. thaliana* Col-0 NLS-GeLgBiT plants were soaked in a mixture of the indicated concentration of HBNCre(wt) protein and Nano-Glo Live Cell Substrate solution. Luminescence imaging was performed immediately after soaking.

DNA-based conventional delivery methods, unintended genetic modifications such as integration of protein expression vectors into the host genome are likely to occur. Such problems are alleviated by using the direct protein delivery method. Even as genome engineering proteins function in cells for a relatively short period of time, DNA 'scars' last permanently as the cell proliferates.

Protein DIVE is also superior in terms of delivery efficiency and cytotoxicity compared to AMGT and protein electroporation. Our method does not include antibiotic selection as in the case of AMGT that eliminates undelivered cells, which means that the protein delivery efficiency must be high enough to screen positive clones with the desired genotype. With respect to the proteins tested in this study, at least, the delivery was efficient enough that there was no need for antibiotic selection or other enrichment steps (Figs. 1 and 4). To compare the delivery efficiency of protein DIVE with other methods, we performed a preliminary genomic PCR assay using the same reporter cells. The recombination efficiency in cells with protein DIVE treatment was comparable to that with electroporation and much higher than that with AMGT without drug selection (Supplementary Fig. 16). In general, a trade-off is observed in which increasing delivery efficiency increases toxicity. Considering tissue and whole plant regeneration, low toxicity is desirable. In this respect, our method is less toxic (Supplementary Fig. 8), simply because only the target protein and medium optimization are needed. However, our method has not yet been tested for proteins and plant species other than those described here.

Validation of protein internalization is essential to establish protein DIVE efficiency. Exogenous proteins are generally accumulated in the endosomes and enter the endocytosis pathway[38]. Endosomes are compartmentalized from the cell cytosol by membranes. Proteins are not freely shuttled through these organelles. Instead, most of the exogenous proteins are sorted to the lysosomes and digested. Thus, microscopic fluorescent observation may not be appropriate especially when we observe fluorescent-labeled proteins of interest. Even if the fluorescent signal is visible, it might be the result of digested protein fragments. Furthermore, fluorescent backgrounds owing to chloroplast or other fixation artifacts must be taken into account during microscopic imaging[39]. In this study, we established that many types of proteins are translocated to the cell cytosol and nucleus; we validated the delivery of proteins using genomic DNA reporter and SLC assays. These validation methods only rely on the

events in the cell cytosol or nucleus exclusively. We also demonstrated the protein internalization in plant tissues using these methods. We observed marked staining in the tip of the root (Figs. 4 and 7d), suggesting that the root tissue has the special feature to uptake biomolecules as its intrinsic nature of the nutrition intake and the fast proliferation.

Internalization of proteins into cells are found in several organisms. A peptide sequence Tat, derived from human immunodeficiency virus, enhanced protein internalization into animal cells[40,41]. Peptides such as Tat are called cell-penetrating peptides (CPPs) or protein transduction domains and are genetically fused with the protein of interest, including Cre recombinase. Since Tat-Cre fusion proteins only react with genomes having two loxP sequences, protein internalization is thus established with the reporter gene expression downstream of loxP[23,42,43]. CPPs, especially cationic CPPs such as Tat and polyarginines, have recently been used in plant transformation[44–46]. Although the peptide fusions did not show any major changes in our experiments, their effects may vary depending upon the protein used for fusion, the solvent, as well as the target cells (Supplementary Fig. 11). The molecular weight of proteins tested here ranged from 39.9 kDa (ZFN) to 63.7 kDa [HNCre-virF(1-202)] and the theoretical isoelectric point (pI) ranged from 9.6 (ZFN) to 9.8 (HNCre). These protein characteristics, with molecular weight < 60 kDa and high pI, are consistent with the results obtained for endocytosis in other organisms[32].

In most of the reports involving animal cells, proteins of interest are administered without serum components, which are essential for the nourishment for cells. However, the presence of serum dramatically reduces protein delivery efficiency[43], implying several mechanisms. It is possible that the cells are susceptible to alien protein internalization when they are deprived of nutrition and are weakened in homeostasis. This is also applicable to plant cells; hence, the delivery of proteins of interest may require unusual conditions rather than a nutrient-rich environment. Cre proteins differed in their delivery efficiency in T87 cells with respect to different culture media, suggesting the need for a conducive environment for efficient delivery of proteins (Fig. 1 and Supplementary Fig. 2). The use of varying conditions may be the key for protein internalization, which is yet to be understood in plants. We found that a shift in temperature from 23 °C, an optimal culture temperature, to 37 °C accelerated DIVE efficiency (Fig. 2). This finding indicates that protein DIVE is mediated by a temperature-dependent endocytosis process or that a higher temperature is conducive for protein delivery in one of the unusual culture conditions described above. Further studies are needed to determine the factors responsible for variations in protein delivery in plants.

Although we have taken initial steps towards a safe and efficient protein delivery into plants, this method is currently applicable to relatively small proteins with high PI, such as Cre and ZFN. We also attempted the delivery of Cas9-sgRNA complexes, however, reproducible results have not been obtained so far (data not shown). Future studies will reveal the relationship between the physicochemical properties of proteins and their delivery efficiency. It is also essential to systematically investigate the compatibility of protein DIVE with a range of target plant species as well as protein properties and to improve protein DIVE for expanding its scope. Furthermore, while we used protein DIVE for nuclear delivery in this study, this method should be easily applied to other cell organelles such as mitochondria, simply by changing the localization signal. This work illustrates the potential of protein DIVE for a wide range of applications, including genome engineering in plants.

## Methods

### Plasmid construct preparation

To construct pET-HCre, used for the preparation of HCre protein, deletion mutagenesis using an In-Fusion HD Cloning Kit (Takara Bio, Shiga, Japan) after inverse PCR was carried out with overlapping primers using pET-HNCre[47] as the template. To construct pET-HTNCre, used for the preparation of HTNCre protein, NCre fragment from pET-HNCre was PCR amplified and inserted into pET-HNCre at NdeI and SalI sites. To construct pET-HR9NCre, used for the preparation of HR9NCre protein, NCre fragment from pET-HNCre was PCR amplified and inserted into pET-HNCre at NdeI and SalI sites. To construct pET-HNCre-vir derivatives, used for the preparation of vir derivative fused HNCre proteins, each vir gene fragment was PCR amplified using LBA4404-associated Ti-plasmid as the template. The vir fragments were inserted directly after the Cre gene at the BsaI site of pHNCre. To construct pET-HNCre(wt), used for the preparation of HNCre (wt) protein, the wild-type NCre fragment from pFT-HNCre(wt)[14] digested with NcoI and SalI was cloned into pET vector. To construct pET-HNCre(wt)-MTS, used for the preparation of HNCre(wt)-MTS protein, insertion mutagenesis after inverse PCR was conducted with overlapping primers and pET-HNCre(wt) as a template. To construct pET-HBNCre(wt), used for the preparation of HBNCre(wt) protein, the pre-hybridized oligo DNAs encoding HiBiT were inserted into pET-HNCre(wt) at the NdeI site. To construct pCambiaNbar-GUS, used for the generation of bialaphos-resistant T87 reporter cells, synthetic DNA fragment encoding bialaphos-resistant gene (gBlocks, IDT Inc., IA, USA) was integrated into pCambiaN-GUS at AvrII and XhoI sites to replace the hygromycin-resistant gene. To construct the gene for GeLgBiT, mNeonGreen and LgBiT fragments were fused by PCR using pCambiaN-GeNL[37] and FRB-LgBiT (Promega, Madison, WI, USA) as templates. The resultant GeLgBiT fragment was PCR amplified and cloned into pCambiaNbar-GUS at NcoI and PmlI sites using an In-Fusion HD Cloning Kit to construct pCambiaNbar-GeLgBiT and pCambiaNbar-NLS-GeLgBiT, used for SLC assays, respectively. To construct pCAMBIA-SSA500-ZFN(C5R), used for the generation of ZFN(C5R)-responsive T87 cells, the internal GUS fragment was PCR amplified from pCAMBIA 1305.2 (Marker Gene Technologies Inc., Eugene, OR, USA). The resulting fragment was cloned using the In-Fusion kit into pCAMBIA 1305.2, which had been digested with MluI. To construct pCAMBIA-SSA500-ZFN(ZFA36), used for generation of ZFA36-responsive T87 cells, the internal GUS fragment was PCR amplified from pCAMBIA 1305.2. The resulting fragment was cloned using the In-Fusion into pCAMBIA 1305.2, which had been digested with MluI. All oligonucleotides used for each of the plasmid constructs are listed in Supplementary Table 1 and a schematic representation of each of the plasmid construct is given in Supplementary Fig. 17.

## Preparation of Cre and ZFN protein

The Cre proteins used in this study were prepared as described previously[14,15]. Briefly, Cre proteins were expressed using *Escherichia coli* strain BL21(DE3) (Nippon Gene, Tokyo, Japan) carrying the plasmid that encodes a hexa-histidine tag at the N-terminus followed by peptide tags and Cre recombinase (Supplementary Fig. 17). The Cre genes in HNCre, HCre, HTNCre, HR9NCre, and HNCre-vir constructs carry the A207T mutation, whose recombination activity is comparable to that of the wild-type[14]. HNCre(wt), HNCre(wt)-MTS, and HBNCre(wt) constructs have the wildtype A207 sequence. An overnight starter culture of *E. coli* BL21(DE3) cells was diluted 100-fold using LB Broth (Lennox; Merck, Darmstadt, Germany) supplemented with 10 μg/ml kanamycin and incubated until $A_{600}$ reached 0.6 at 37 °C. The cells were then incubated with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for another 2.5 h at 37 °C to induce protein expression, and then harvested. Cells were then lysed using a Digital Sonifier (250D Advanced, BRANSON, CT, USA) and purified using an Ni-NTA column with elution buffer (50 mM Tris-HCl, 500 mM NaCl, 10% glycerol, and 500 mM imidazole, pH 8.0). Cre proteins were further purified using a gel filtration column (HiPrep 16/60 Sephacryl S-200 HR; GE healthcare, Chicago, IL, USA) with Buffer A (20 mM HEPES, 500 mM NaCl, 10% glycerol, and 1 mM dithiothreitol, pH 7.4). The purified Cre proteins were flash-frozen in liquid $N_2$ and stored at −80 °C until further use. To prepare ZFN proteins, *E. coli* BL21(DE3) cells carrying the plasmid that encodes a hexa-histidine tag at the N-terminus followed by ZFN was used. The same protocol was followed for the preparation of ZFN proteins as that for Cre proteins, except the 2.5 h incubation at 37 °C after addition of IPTG; instead, the cells were incubated overnight at 18 °C.

## *A. thaliana* cell line and *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) culture

The cell line used for AMGT as well as electroporation and DIVE experiments was *A. thaliana* T87. The cell line was obtained from RIKEN Bio Resource Center (Ibaraki, Japan). T87-xGxGUS cells, the T87 cell line carrying Cre-responsive reporter cassette, was generated as described previously[14]. T87 and T87-xGxGUS cells were cultured in a liquid NT1 culture medium with the following composition: 30 g/L sucrose, 0.1 mM $KH_2PO_4$, 1x Murashige and Skoog Plant Salt Mixture (FUJIFILM Wako Pure Chemical, Osaka, Japan), 1x Murashige and Skoog Modified Vitamin Solution 1000X (FUJIFILM Wako Pure Chemical), and 2 μM 2,4-dichlorophenoxyacetic acid, pH 5.8 adjusted with KOH) at 23 °C while shaking (120 rpm) under continuous white light, unless otherwise specified. Cells were maintained using weekly 15-fold dilutions.

*A. tumefaciens* (*R. radiobacter*) strain GV3101 cells were cultured in LB medium (Merck) supplemented with 50 μg/mL rifampicin and 30 μg/mL gentamicin at 28 °C with agitation at 200 rpm using BR-43FL (TAITEC, Saitama, Japan). To prepare the transformants of GV3101 cells, cells were electroporated with the binary plasmids and cultured in LB medium supplemented with 50 μg/mL rifampicin, 30 μg/mL gentamicin, and 50 μg/mL kanamycin. Prior to infection, the GV3101 cells were grown overnight in LB medium supplemented with 50 μg/mL rifampicin, 30 μg/mL gentamicin, and 50 μg/mL kanamycin. The cells were then harvested using centrifugation (3300xg, 25 °C, 5 min) with MX-300 (TOMY SEIKO, Tokyo, Japan), washed thrice with liquid B5 medium (30 g/L sucrose, 0.5 g/L MES, 1x Gamborg's B5 Salt Mixture and Vitamins, and 1 μM 1-naphthaleneacetic acid) and resuspended to a final $A_{600}$ of 0.6. To culture *A. tumefaciens* strain LBA4404 cells, LB medium supplemented with 100 μg/ml streptomycin, instead of rifampicin and gentamicin, was used. The transformation and infection procedures were the same as those for GV3101 cells.

## *Agrobacterium*-mediated transformation of T87 cells

T87 cells were transferred into liquid B5 medium and cultured for 2 days. Using AMGT, we conducted Cre transformation of T87-xGxGUS cells, generated T87-SSA500-ZFN(C5R) and T87-SSA500-ZFN(ZFA36) cell lines for ZFN reporter assay, and generated T87-GeLgBiT and T87-NLS-GeLgBiT cell lines for SLC assays. To generate T87-SSA500-ZFN(C5R) and T87-SSA500-ZFN(ZFA36) cells, which express GUS in response to ZFNs, the T87 cells containing 30 μL of packed cell volume (PCV) were infected with 0.8 μL of GV3101 cells ($A_{600}$ of 0.6) harboring pCAMBIA-SSA500-ZFN(C5R) and pCAMBIA-SSA500-ZFN(ZFA36) in the presence of 200 μM acetosyringone. Cells were incubated at 23 °C with shaking (120 rpm) under constant white light. Two days post infection, the cells were washed thrice using liquid B5 medium supplemented with 200 μg/mL cefotaxime to eliminate GV3101 and were further cultured in liquid B5 medium supplemented with 200 μg/mL cefotaxime for 3 days. The cells were then transferred onto B5 agar (B5 medium with 0.6% agar) with 200 μg/mL cefotaxime and 20 μg/mL hygromycin and cultured at 23 °C under constant white light for 2 weeks. A single clone was then picked and cultured in liquid NT1 medium containing 10 μg/mL hygromycin. To generate T87-GeLgBiT and T87-NLS-GeLgBiT cell lines, which express GeLgBiT and NLS-GeLgBiT, LBA4404 cells harboring pCambiaNbar-GeLgBiT and pCambiaNbar-NLS-GeLgBiT, respectively, were used. Instead of hygromycin, 10 μg/mL bialaphos was used for selection of transformed T87 cells. The remaining procedures were the same as those for T87-SSA500-ZFN(C5R) cells. The cell lines generated were maintained under the same conditions as described for T87 cells, using liquid NT1 medium containing 10 μg/mL hygromycin or bialaphos.

## *Agrobacterium*-mediated transformation of *Arabidopsis* plants

*A. thaliana* (Col-0 strain; Inplanta Innovations, Kanagawa, Japan) plants were cultivated at 23 °C under constant white light. A flower bud was dipped in a culture of *Agrobacterium* cells harboring the binary reporter plasmid cell suspension containing 5% w/v sucrose, and 0.025% Silwet L-77 (Biomedical Science, Tokyo, Japan) for 3 min. After inoculation, the plant was covered using an anti-static film of oriented polypropylene and incubated overnight under light conditions and high humidity. Subsequently, the plant was cultivated at 23 °C under constant white light until seed formation. Seeds were collected and sown on a GeM agar plate (0.5% agar, 20 g/L glucose, 0.6 g/L MES, 1x Gamborg's B5 Salt Mixture, 1x Murashige–Skoog Vitamins, and 200 μg/mL cefotaxime, pH 5.7 adjusted with KOH) supplemented with 20 μg/mL hygromycin for xGxGUS plants (*A. thaliana* Col-0

xGxGUS) and 20 μg/mL bialaphos for NLS-GeLgBiT plants (*A. thaliana* Col-0 NLS-GeLgBiT). After germination of the rhizome, $T_1$ plants emitting green fluorescence were transferred onto soil (vermiculite). Plants of the $T_2$ generation were used for further experiments.

## Electroporation

Reporter T87 cells were 15-fold diluted into fresh NT1 medium 3–5 days before electroporation. Electroporation conditions including Cre protein concentration were set as described in the previous study[14]. On the day of electroporation, cells were collected in a tube and washed once with Opti-MEM I (Thermo Fisher Scientific, MA, USA). Cells with 20 μL of PCV were suspended in 200 μL of Opti-MEM I with 1 μM HNCre protein on ice and transferred into a 4-mm-wide cuvette (Nepa Gene, Chiba, Japan). The electroporation program was 375 V/cm (150 V setting/0.4 cm width), 10 ms, 5 times, and a 50-ms interval for poring pulse and 20 V, 50 ms, 20 times, and a 50-ms interval for transfer pulse. Electroporation was performed using NEPA21 Type II (Nepa Gene). After electroporation, the cells were washed once with NT1 medium and cultured in 2 mL of NT1 medium at 23 °C with shaking until they were used for further experiments.

## Protein DIVE in vitro

Reporter T87 cells were diluted 15-fold into fresh NT1 medium 3–5 days before protein delivery. On the day of protein delivery, cells were collected in a tube and washed once with one of the following solutions: phosphate-buffered saline (Thermo Fisher Scientific), HEPES-buffered saline (20 mM HEPES and 150 mM NaCl, pH 7.4), HEPES buffer (20 mM HEPES, pH 7.4), Opti-MEM I, NT1 medium, NT1HF (NT1 hormone free; NT1 medium without 2,4-dichlorophenoxyacetic acid) medium, B5 medium, B5HF (B5 hormone free; B5 medium without 1-naphthaleneacetic acid) medium, or JPL (Jouanneau and Péaud-Lenoël medium supplemented with 1 μM 1-naphthaleneacetic acid; RIKEN Bio Resource Center) medium. Cells with 20 μL of PCV were suspended in 200 μL of the same solutions used for washing and 1 μM Cre protein was added at 25 °C. Cells were then incubated for 1 h at 23°C with shaking under constant white light. After 1 h of incubation, cells were washed once with NT1 medium and cultured in 2 mL of NT1 medium at 23 °C with shaking under constant white light for 2 days and were used for further experiments. The delivery conditions were appropriately adjusted in each experiment as follows: To evaluate the temperature effect, cells were incubated with 1 μM Cre protein for 1 h at 4, 15, 23, 30, 37, or 42 °C with shaking under constant white light. For GUS staining of protoplasts and genomic PCR assay, cells were incubated with 5 μM Cre protein for 1 h at 23 °C with shaking under constant white light. For endocytosis inhibition, 0, 10, 33, or 100 μM wortmannin (FUJIFILM Wako Pure Chemical) was added with 1 μM Cre protein. To evaluate the effect of incubation time, cells were incubated with 1 μM Cre protein for 10, 30, 60, or 120 min at 23 °C with shaking under constant white light. To evaluate the effect of incubation time, cells were incubated with 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, or 10 μM Cre protein for 1 h at 23 °C with shaking under constant white light. To evaluate the effect of Tat and R9 tags, cells were incubated with 0.5, 1.0, or 2.0 μM Cre protein for 1 h at 23 °C with shaking under constant white light. To evaluate the effect of vir protein tag, cells were incubated with 1.0 or 2.0 μM Cre protein for 1 h at 23 °C with shaking under constant white light. To evaluate ZFN protein DIVE, cells were incubated with 0.5, 1.0, or 2.0 μM ZFN protein for 1 h at 23 °C with shaking under constant white light.

## Protein DIVE in planta

The delivery of Cre proteins using protein DIVE was performed in planta using *A. thaliana* Col-0 seedlings. For GUS reporter assay that indicates the delivery of Cre protein into the nuclei of the plant cells, *A. thaliana* Col-0 xGxGUS seeds were sown on a GeM agar plate and incubated at 23 °C for one week under constant white light. Plants 4–5 days after germination were soaked in the delivery solution supplemented with 5 μM HNCre protein for efficient delivery and incubated at 23 °C with shaking (120 rpm) for 1 h. After incubation, plants were washed once with ultrapure water and incubated on GeM agar plate for 2 weeks and were used for GUS staining.

For calli genomic PCR assay, *A. thaliana* Col-0 xGxGUS seeds were sown on a GeM agar plate at 23 °C for 1 week. The roots of plants 4–5 days after germination were excised and soaked in NT1HF supplemented with 5 μM Cre protein at 23 °C with shaking for 1 h. After 1 h of incubation, the roots were washed once with ultrapure water and cultivated at 23 °C on CIM agar plate (0.5% agar, 20 g/L glucose, 0.6 g/L MES, 1x Gamborg's B5 Salt Mixture, 1x Murashige–Skoog Vitamins, 0.5 μg/ml 2,4-dichlorophenoxyacetic acid, and 0.1 μg/ml kinetin, pH 5.7 adjusted with KOH) for 2 weeks in the dark and were used for genomic PCR assay.

For SLC assay, *A. thaliana* Col-0 NLS-GeLgBiT seeds were sown on a GeM agar plate and incubated at 23 °C for 9 days under constant white light. Plants 6–7 days post germination were transferred onto a glass slide and soaked in a mixture of 5 μL of 0, 0.05, 0.5, or 5 μM Cre protein dissolved in Buffer A and 5 μL of Nano-Glo LCS Dilution Buffer supplemented with 5% v/v Nano-Glo Live Cell Substrate (Nano-Glo Live Cell Assay System; Promega). Immediately after soaking, luminescence was imaged using the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

## Preparation of protoplasts

*A. thaliana* T87-xGxGUS cells were used for protoplast preparation. After DIVE treatment and 2 days incubation, T87-xGxGUS cells were washed with 400 mM mannitol. The supernatant was then discarded and the cells were treated with an enzyme solution comprising 20 mM MES (pH 5.7), 400 mM mannitol, 20 mM KCl, 10 mM $CaCl_2$, 1.5% (w/v) Onozuka RS (Serva Electrophoresis, Heidelberg, Germany), and 0.25% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Industry, Tokyo, Japan). The suspension of T87-xGxGUS cells in the enzyme solution was incubated at 23 °C with shaking in the dark for 4 h. After incubation, $CaCl_2$ (final concentration of 67 mM) was added to the mixture. Cells were then used for GUS staining.

*Cell Counting Kit-8 (CCK8) assay*
Cell viability was analyzed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). After 1 h of protein delivery unless otherwise specified, 100 µL each of the cell suspensions was transferred to a 96-well plate to which 10 µL of CCK8 solution was added. The cells were incubated for 4 h at 25 °C. Absorbance at 450 nm was measured using a microplate reader (Infinite 200 Pro; Tecan, Zurich, Switzerland) to evaluate the viability of cells.

*GUS staining*
To visualize GUS expression, cells or plants treated with Cre proteins or ZFN proteins were incubated with 0.5 mg/mL of X-Glucuronide (Carbosynth, Berkshire, United Kingdom) dissolved in staining buffer (20% (w/v) methanol and 50 mM $NaH_2PO_4$, pH 7.0) for 30 min at 37 °C. For the staining of protoplasts, mannitol (final concentration of 400 mM) was added to the GUS staining solution. To visualize GUS spots, samples were then transferred into 70% (w/v) ethanol and images were obtained using a phase contrast microscope (Nikon, Tokyo, Japan).

*Fluorescence quantification of GUS activity*
Two days after protein delivery, T87 cells were washed once with NT1 medium (pH 7.0, adjusted with KOH). Cells with 5 µL of PCV were then suspended in 100 µL of NT1 medium (pH 7.0) containing 10 µM 6-chloro-4-methylumbelliferyl β-D-glucuronide (Glycosynth, Cheshire, United Kingdom) and transferred to a black 96-well plate (Thermo Fisher Scientific). Fluorescence was measured using a microplate reader (Infinite 200 Pro; Tecan). The excitation and emission wavelengths were set at 355 and 460 nm, respectively.

*Genomic PCR of Cre-responsive reporter cassette*
T87 cells 2 days after protein delivery, AMGT or electroporation, or calli generated from root tissue of *A. thaliana* Col-0 plants 2 weeks after protein delivery were incubated with 10 µL of 250 mM NaOH and 0.1% Tween 20 at 98 °C for 10 min to extract genomic DNA[14]. The samples were then treated with 5 µL of 1 M Tris-HCl (pH 6.5) and centrifuged. Using the extracted genomic DNA, the integrated sequence of Cre-responsive reporter cassette in T87 genome was amplified via PCR using MightyAmp DNA Polymerase Ver.3 (Takara Bio; 98 °C for 2 min; 30 cycles each of 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 2 min) with the primers 5′-TCCTTCGCAAGA CCCTTCCTC-3′ and 5′-GGATGGCAAGAGCCAAATGCTTAG-3′. The PCR products were analyzed using agarose gel electrophoresis. Gels were stained with GelGreen (Biotium, Hayward, CA, USA) and imaged using a ChemiDoc XRS+ system (Bio-Rad). The band intensities were quantified and the background was subtracted using ImageLab software (Bio-Rad). The following equation was used to calculate the percent recombination by Cre protein: $100 \times (a/[a + b])$, where a or b is the background-subtracted intensity of the PCR product with or without recombination, respectively.

*Luminescence quantification in cells*
Cells were washed once with NT1 HF medium (pH 7.0, adjusted with KOH). Cells with 5 µL of PCV were then suspended in 50 µL of NT1 HF medium (pH 7.0) and transferred to a black 96-well plate (Thermo Fisher Scientific). Subsequently, cells were added with 12.5 µL of Nano-Glo Live Cell Substrate (Promega) and incubated at 25 °C for 5 min. After incubation, Cre protein (final concentration of 1 µM) was added and luminescence was measured using a microplate reader (Infinite 200 Pro; Tecan).

*Quantification and statistical analyses*
All measurements are presented as mean ± standard error (SE). *P* values were obtained using Student's t-test or Tukey's test. Sample sizes are indicated in the figure legends.

## Data availability
Sequence data of cDNA for HNCre protein is available at the DNA Data Bank of Japan (DDBJ) under accession number ID LC830482. You can access our data from the DDBJ getentry site below. If you input "LC830482", then the data will be appeared. https://getentry.ddbj.nig.ac.jp/top-e.html. The data of this study are available from Yoshio Kato upon request.

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## Author contributions

Y.F. and Y.K. designed the research; M.K. and T.Y.a generated A. thaliana Col-0 xGxGUS plants; T.S. and T.Y.b designed FokI nuclease domains; A.S., T.M., and E.E. purified the proteins; Y.F., M.K., A.S., T.M., and E.E. performed the experiments; Y.F., M.K., A.S., T.M., E.E., and Y.K. analyzed the data; Y.F. and Y.K. drafted the manuscript. All authors reviewed and edited the manuscript.

## Competing interests
The authors declare no competing interests.

## Additional information
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