Application of protoplast technology to CRISPR/Cas9 mutagenesis: from single-cell mutation detection to mutant plant regeneration

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Summary

Plant protoplasts are useful for assessing the efficiency of clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)-associated protein 9 (Cas9) mutagenesis. We improved the process of protoplast isolation and transfection of several plant species. We also developed a method to isolate and regenerate single mutagenized Nicotiana tabacum protoplasts into mature plants. Following transfection of protoplasts with constructs encoding Cas9 and sgRNAs, target gene DNA could be amplified for further analysis to determine mutagenesis efficiency. We investigated N. tabacum protoplasts and derived regenerated plants for targeted mutagenesis of the phytoene desaturase (NtPDS) gene. Genotyping of albino regenerants indicated that all four NtPDS alleles were mutated in amphiidiploid tobacco, and no Cas9 DNA could be detected in most regenerated plants.

Keywords: protoplast isolation, CRISPR/Cas9, protoplast regeneration, single-cell analysis.

Introduction

Genome engineering is an important component of the relatively novel field of synthetic biology. Gene editing, the directed change of a specific DNA sequence, is an important element of genome engineering. The clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system is a convenient genome-editing tool that requires only two reagents: Cas9 protein and a single guide RNA (sgRNA) (Feng et al., 2013; Gaj et al., 2013; Li et al., 2013; Nekrasov et al., 2013; Shan et al., 2013). Following CRISPR-mediated mutagenesis, integrated transgenes encoding gene editing reagents can often be removed from the genome through genetic segregation, mitigating public concerns regarding genetically modified organisms (Huang et al., 2016). CRISPR/Cas9 mutagenesis is therefore becoming an important technology for basic plant science and agriculture.

Numerous published studies have described various vectors to improve CRISPR-mediated target mutagenesis. These studies describe parameters such as promoters, different versions of Cas9, and the use of multiple sgRNAs (Ali et al., 2015, 2016; Belhaj et al., 2013; Bortesi and Fischer, 2015; Butt et al., 2017; Cermak et al., 2017; Eid et al., 2016; Kaya et al., 2016; Ma et al., 2015; Murovec et al., 2017; Shimatani et al., 2017; Wang et al., 2015b; Yan et al., 2015). Not only Cas9, but other endonucleases such as Cpf1 can induce mutations (Endo et al., 2016; Kim et al., 2017; Mahfouz, 2017; Xu et al., 2017). However, stable transformation to evaluate CRISPR mutagenesis efficacy can be time-consuming. Transient protoplast transfection is an alternative strategy to test multiple mutagenesis parameters rapidly. Protoplasts from at least five crop species (rice, wheat, maize, lettuce, and tomato), in addition to Arabidopsis and tobacco, have been used to evaluate gene editing reagents using CRISPR/Cas9-based systems (Cermak et al., 2015; Liang et al., 2014; Shan et al., 2014; Woo et al., 2015). Although several methods have been developed to obtain and transfect Arabidopsis protoplasts (Sheen, 2001; Wu et al., 2009), protoplast isolation remains a bottleneck to testing genome-editing reagents in many crop species.

Previously, DNA from pooled mutagenized protoplasts was used to determine target site mutagenesis efficiency (Cermak et al., 2015; Liang et al., 2014; Shan et al., 2014; Woo et al., 2015). The target region is amplified by PCR, and the resulting amplicons are further evaluated using restriction fragment length polymorphism (RFLP) (Feng et al., 2013; Nekrasov et al., 2013; Shan et al., 2013) or cleaved amplified polymorphic sequence analysis (Kaya et al., 2016; Mikami et al., 2015a,b; Shimatani et al., 2017), T7 endonuclease I (T7E1) analysis (Kim et al., 2017; Woo et al., 2015) or next-generation sequencing (NGS) (Kim et al., 2017; Woo et al., 2015).
Because the PCR amplicons constitute a mixture of wild-type and mutated DNA, mutagenesis efficiency is determined by calculating the gel image density (RFLP and T7E1 assays) or the per cent of mutant sequences (by NGS). It is often difficult to detect low target site mutagenesis efficiency. False-positive results can occur because of incomplete restriction endonuclease digestion or PCR errors. Although NGS can resolve these problems, the process can be time-consuming and costly. Because there are only two potential target site alleles in a diploid cell, single-cell DNA analysis can rapidly determine mutagenesis efficiency. Several single-cell isolation methods have been published (Brennecke et al., 2013; Efroni and Birnbaum, 2016; Efroni et al., 2015; Gierahn et al., 2017; Klein et al., 2015; Macosko et al., 2015; Yamamoto et al., 2016). Single-cell analyses have been applied to transcriptome and metabolome studies (Efroni et al., 2015; Yamamoto et al., 2016). However, these studies required expensive facilities or technically demanding protocols, including flow cytometry (Gierahn et al., 2017; Klein et al., 2015; Macosko et al., 2015) or microinjection (Yamamoto et al., 2016). A convenient and reliable single-cell isolation protocol would greatly benefit plant scientists conducting gene editing experiments.

Protoplasts can be used to determine target site mutagenesis efficiency and can be regenerated into plants (Woo et al., 2015). Furthermore, genome-editing reagents such as sgRNAs and Cas9 protein can be synthesized and assembled in vitro to form active ribonucleoprotein (RNP) complexes. These complexes can be delivered into protoplasts and mutagenize the target gene. Thus, target mutants can be obtained without the presence of exogenous DNA (Kim et al., 2017; Liang et al., 2017; Woo et al., 2015). Such DNA-free genome editing avoids stable introduction of transgenes. Although geminiviruses can be used in whole plants to deliver donor DNA for homology-directed repair (HDR; Wang et al., 2017), protoplast transfection is an alternative to deliver high amounts of DNA required for HDR. However, protoplast regeneration is difficult in most plant species.

In this report, we further develop protoplast isolation protocols for several crop and ornamental species, and the model plant Arabidopsis. We used these protoplasts to evaluate CRISPR/Cas9 mutagenesis efficiency. We describe a simple single-protoplast isolation protocol and use this protocol to edit the tobacco NtPDS gene. Multiple plants regenerated from single mutagenized tobacco protoplasts contain a variety of CRISPR-induced mutations.

Results

Improvement of protoplast isolation for transfection

We designed a tool to generate multiple longitudinal cuts in monocot seedlings. A razor blade was divided into four pieces, which were stacked in parallel on a scalpel handle (Figure 1a). In previous reports, seedlings were cut in cross section (Zhang et al., 2011; Figure 1b). In this report, seedlings were sorted and arranged in parallel (or affixed to clear tape) for longitudinal cutting (Figure 1c). When a seedling is cut in cross section, only the cells near the edge are digested (Figure 1d); cutting in longitudinal sections increases the release of protoplasts (Figure 1e). The protoplast yield from rice seedlings subjected to longitudinal cutting was higher than that from seedlings subjected to cross-cutting (4.8 x 10^6 protoplasts/g fresh weight (FW) vs 2.2 x 10^6 protoplasts/g FW). Longitudinal cutting permitted efficient cell wall digestion by cellulose R10 and mancerolase R10 enzymes (Table S1), which are less expensive than are cellulase RS and mancerolase RS enzymes used previously (Zhang et al., 2011). Longitudinal cutting was used successfully for five Poaceae species: rice, wheat, maize, millet and bamboo. Protoplasts derived from this protocol were transfected with a red fluorescence protein (RFP) gene by a PEG-mediated method. Total intact cell number was calculated, and the transfection efficiency was calculated as the percentage of intact protoplasts with RFP fluorescence/intact protoplasts. Transfection efficiencies of these protoplasts were >40% (Figure 1j and k, bamboo: 54%; millet: 51%; rice: 44%; maize: 47%; wheat: 41%; Figure S1).

We previously established an efficient ‘Tape-Arabidopsis Sandwich’ protoplast isolation protocol (Wu et al., 2009). This protocol can be applied to several Brassicaceae species, including Brassica oleracea, B. napus, Cleome spinosa, C. monophilla, and C. gynandra. Using 3M Scotch Tape, we peeled off the epidermal layer of leaves or cotyledons, facilitating protoplast release. Our results indicated that 2- to 3-week-old in vitro cotyledons from B. napus were more suitable for protoplast isolation than one 1-week-old cotyledons. For Cleome species, mature leaves from plants grown in a greenhouse were suitable. C. gynandra is a C4 plant, so there are two types of protoplasts, from mesophyll and bundle sheath cells. Protoplasts from all six Brassicaceae species isolated by the Tape Sandwich method were transfected using a PEG-mediated method with efficiencies >40% (Figure S1; Arabidopsis: 67%; broccoli: 43%; rapeseed: 63%; C. gynandra: 46%; C. spinosa: 79%; C. monophilla: 83%).

It was difficult to isolate high-quality protoplasts from tomato leaves using the Tape Sandwich method (data not shown). Instead, a suspension cell line was developed from tomato hypocotyls using the tomato cultivar ‘Micro-Tom’ (Figure 1f–i). Tomato hypocotyls were cut, callus cultures were developed, and the callus used to establish a suspension cell culture (Figure 1g,h). The proliferation rate of this tomato suspension line is similar to that of tobacco BY-2 cells. Protoplasts were isolated by incubating the suspension cells in a cell wall digestion buffer. Similar to BY-2 protoplasts, the tomato protoplasts were transfected using a PEG-mediated transfection method with 63% efficiency (Figures 1l,m, and S1).

CRISPR/Cas9-mediated mutagenesis of protoplasts

To evaluate mutagenesis efficiency, we targeted in most species the phytoene desaturase (PDS) gene. For each PDS gene, we chose the sgRNA such that a restriction enzyme site upstream of the protospecies adjacent motif (PAM; Figure 2a) sequence may be lost if target mutations occurred. If there were no suitable sgRNA targeting site (e.g. lacking a PAM sequence) or unique sequence for PDS, a published sgRNA for another gene was used.

Protoplasts from each species were transfected with a VirD2-NLS-mRFP plasmid (Lee et al., 2008) and pCAMBIA1300-OsU3 (Aarl)-Cas9 or pCAMBIA1300-OsU6(Aarl)-Cas9 carrying the species-specific sgRNA (Figures 2b and S2). After 24 or 48 h, DNA from total protoplasts was extracted and the target gene amplified by PCR. The PCR product was digested with a restriction enzyme whose site is adjacent to the PAM, and the products separated by electrophoresis through an agarose gel (Figure 2c). Undigested DNA appeared in the 48 h sample. This result suggests that some genomic DNA had been mutated, disrupting the restriction endonuclease site targeted by the sgRNA. The undigested PCR fragments were cloned into a T/A vector, transformed into Escherichia coli, and the insert subjected to colony PCR. The PCR fragment was digested by the appropriate restriction endonuclease, and PCR products not digested by the
restriction enzyme were sequenced (Figure 2d–f). Protoplast transfection and CRISPR/Cas9 editing were evaluated for each of the nine species. The results are summarized in Table 1.

CRISPR editing of Poaceae protoplasts

Bamboo

For the bamboo PDS gene, a target sequence (sg2) that contains a BsaI restriction site was chosen. Results of the DNA amplifications and digestions after the transfection are shown in Figure S3. After 48-h transfection, there was a minor undigested band indicating a mutation efficiency of only 6.6%. The intensity of the undigested band is 52.800 arbitrary units (au). The sum of the intensities of the digested bands is 1434.911 au. The transfection efficiency is 54%. Therefore, the mutation frequency is [52.8/(52.8 + 1434.911)]0.54 = 6.6%). The PCR products amplified by the first set of primers were cloned, and clones with putative mutated regions were sequenced. Five of 40 clones showed mutations (12.5%), which were either deletions (1–13 bp) or a 1-bp substitution (Figure S4a). Although base substitutions are relatively rare, several studies reported such CRISPR/Cas9-induced mutations (Cermak et al., 2015; Ikeda et al., 2016; Li et al., 2013; Liang et al., 2016; Mikami et al., 2015a,b; Schiml et al., 2014; Wang et al., 2016; Xu et al., 2014; Zhang et al., 2014). To reduce the cloning work and increase the sensitivity of mutant validation, we amplified DNA (using a second set of primers) from the restriction enzyme mixture and digested the amplicons with BsaI to detect mutations again. Meanwhile, the second PCR products were cloned into a T/A vector (Figure S4b). After enrichment of the mutated amplified DNA, 71% (5/7) of the clones carried mutations, all of which were deletions (3–8 bp).

Millet

The results in millet were similar to those of bamboo, with a more significant undigested band in protoplast DNA PCR products after 48 h (Figure S5). The scanned electrophoresis image indicated a mutation frequency of 10.2% after 48-h incubation (Figure S5a).

Figure 1 Improved protoplast isolation. (a) A razor blade was divided into four pieces and the pieces assembled in parallel in a scalpel handle. (b) Seedlings of rice were cut in cross section and placed in digestion solution. Bar = 1 cm. (c) Seedlings of rice were cut longitudinally (in the same direction as the veins) and placed in digestion solution. Bar = 1 cm. (d) Microscopic image of rice seedlings cut in cross section (perpendicular to vascular bundles) after 3-h digestion. Bar = 10 μm. (e) Microscopic image of rice seedlings cut in longitudinally (parallel to vascular bundles) after 3-h digestion. Bar = 10 μm. (f) Tomato hypocotyl sections were incubated in medium supplemented with 10 mg/L NAA and photographed after 1 month. Bar = 1 cm. (g) Microscopic image of tomato suspension cells. Bar = 40 μm. (h) Tomato suspension cells grown in 1 mg/L 2,4-D after 7 days. Bar = 1 cm. (i) Tomato suspension cells formed calli on solid medium supplemented with 1 mg/L 2,4-D. Bar = 1 cm. (j) mRFP-NLS plasmid DNA was delivered to rice protoplasts using a PEG-mediated method. Protoplasts were photographed after 24 h. Red colour indicates RFP epifluorescence. Bar = 50 μm. (k) Overlay of epifluorescence and bright field images of transfected rice protoplasts. Bar = 50 μm. (l) mRFP-NLS plasmid DNA was delivered into tomato Micro-Tom protoplasts using a PEG-mediated method. Protoplasts were photographed after 24 h. Red colour indicates RFP epifluorescence. Bar = 50 μm. (m) Overlay of epifluorescence and bright field images of transfected tomato protoplasts. Bar = 50 μm.

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(a) Forward primer

(b) incubated for 24-48 h

(c) PCR to amplify uncut fragment

(d) Sequencing detecting Frameshift mutation (SNP, insertion, deletion) in fourth bp from PAM

(e) TA cloning, PCR to amplify inserts, PstI digestion

(f) Deletion:

| gRNA | PAM |
|------|-----|
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| TACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 

Wild type

−1

−3

−5

−6

−7

−8

−14

−31

−43

Insertion:

| gRNA | PAM |
|------|-----|
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 
| ACTATGGTTGCTGTAATTGCCCTCTGACAGGCAGGCTGGTTGACAGAACCAT | 

Wild type

+1
one showing an insertion and one showing an insertion/deletion (line 7, the sequence with the grey colour code; Figure S5c).

Rice
The transfection of rice protoplasts with pCAMBIA1300-OsU3 (AarI)-OsPDS resulted in a minor undigested band after DNA amplification 24 h after transfection and a more visible band after 48 h (Figure S6). The scanned gel image indicated a mutation frequency of 7.3%. The second PCR product cloning and sequencing showed mutations at the predicted site (the fourth nucleotide before the PAM). Most mutations were deletions. There were two cases of insertions; one was a 1-bp insertion, and the other contained 175 nucleotides of vector sequence (Figure S6c).

Maize
The maize inositol phosphate kinase (IPK) gene was targeted using two sgRNA sequences (Liang et al., 2014). These sgRNAs were cloned individually into pCAMBIA1300-OsU3(AarI). SacI and BamHI were used to validate mutations effected by gRNA1 and gRNA2, respectively. After 48-h transfection, there were minor undigested bands in both gRNA1 and 2 treatments, indicating that the mutation efficiency was low. When individual gRNAs were used for transfection, gRNA2 was more efficient than was gRNA1 48 h after transfection (Figure S7, mutation frequency, gRNA1: 0.2%; gRNA2: 1.1%). Sequencing revealed mutations consisting of either deletions or insertions (Figures S7c and d). When both guide RNAs were co-transfected, the first PCR did not reveal any truncated product. However, there was a ~300-bp smaller PCR product after the second PCR (Figure S8a). These results confirmed that the DNA region between sgRNA1 and 2 was deleted (Figure S8b).

CRISPR editing of Brassicaceae protoplasts

Arabidopsis
Arabidopsis protoplasts were transfected with pCAMBIA1300-OsU3(AarI)-AtPDS3. Although the AtPDS3 sgRNA was driven by either a rice OsU3 or OsU6 promoter, CRISPR/Cas9-mediated mutations were detected. Using the construct carrying the OsU3 promoter, DNA extracted from protoplasts amplified with AtPDS3 primers and digested with Ncol showed faint undigested bands 24 h after transfection (Figure S9a). The results were similar when the OsU6 promoter was used (Figure S9b). After 48 h, more intense undigested bands were observed. The mutation frequency was 6.5% in the OsU6 treatment after 48-h incubation. The PCR products were cloned and sequencing indicated that mutations (deletions and insertions) initiated at or before the fourth nucleotide before the PAM (Figure S9d).

Table 1 Targeted mutations in protoplasts of nine plant species

| Species       | Transfection efficiency (%) | Target gene | Mutagenesis efficiency (%) | Sequenced mutations (bp) |
|---------------|-----------------------------|-------------|---------------------------|--------------------------|
| Poaceae       |                             |             |                           |                          |
| Bambusa oldhami  | 54                          | PDS         | 6.6                       | 1, 3, 4, 6, 8, 11, 13     |
| Setaria italica  | 51                          | PDS         | 10.2                      | 1, 3, 5, 6, 7, 8, 14, 31  |
| Oryza sativa    | 44                          | IPK         | 7.3                       | 1, 175                   |
| Zea mays       | 47                          |              | 0.2*                      | 1, 5                     |
| Brassicaceae   |                             |             |                           |                          |
| Arabidopsis thaliana  | 67                          | PDS         | 1.1*                      | 1, 1                      |
| Brassica oleracea | 43                          | GA4a        | 6.5                       | 1(A), 1(T), 2            |
| Brassica napus  | 63                          | GA4a        | 56.8                      | 2, 5, 6, 8, 10, 14       |
| Solanaceae     |                             |             |                           |                          |
| Nicotiana tabacum | 41                          | PDS         | 15.0                      | 1, 3, 6, 8, 12           |
| Solanum lycopersicum | 63                          | PDS         | 3.7                       | 1, 2, 3, 8, 9            |

*sgRNA1. †sgRNA2.
Broccoli

For broccoli, we targeted the sgRNA gene BoLG44.a at a HphI site (Lawrenson et al., 2015). Both rice promoters, U3 and U6, were tested. Despite the use of a monocot promoter, genome editing in B. oleracea had a 75.2% mutation efficiency with OsU6 treatment after 48-h incubation (Figure S10). To confirm that the undigested PCR product is mutated by CRISPR/Cas9, the first PCR product was cloned and sequenced. The results indicated that 64.1% (25/39) of the clones were mutated at or before the fourth nucleotide preceding the PAM (Figure S11). These results are similar to those of the gel image. Using these two methods, both mutagenesis efficiency and the mutated sequences could be obtained.

Rapeseed

Because the rapeseed G44.a gene is highly homologous to that of broccoli, the same construct was used for both species. The CRISPR/Cas9 editing efficiency was also high (in OsU3 treatment after 24-h incubation, 56.8%) in rapeseed protoplasts. The first PCR product was cloned and sequenced. Sequencing indicated both deletions and insertions (Figure S12).

CRISPR editing of Solanaceae protoplasts

To edit Solanaceous crop genomes, we used tobacco (BY-2) and tomato (Micro-Tom) suspension cells. The PDS homologs, which harbour a Mdy site, were targeted in both species. The OsU3 promoter was used to drive sgRNA expression in both cases. The results are shown in Figure S13 (for tobacco BY-2 cells, the mutation frequency was 15.0% after 48 h) and Figure S14 (for tomato Micro-Tom, the mutation frequency was 3.7% after 48 h).

Detecting CRISPR-mediated mutations in individual protoplasts

Transfection of protoplasts with CRISPR editing reagents usually results in a mixture of genotypes, including nonedited wild-type sequences and various differently edited genomes. Even when the protoplast transfection efficiency is high, frequently only a small percentage of cells contain edited genomes. It is often difficult to detect these rare edited genomes among a high background of nonedited genomes. We thus developed a protocol for analysing DNA from individual mutagenized protoplasts. Because the PCR template is from a single cell, it is easier to validate the electrophoresis results, including complete digestion of the amplicon DNA by restriction endonucleases. Mutations in the PCR product can be confirmed by Sanger sequencing.

We evaluated the parameters of transfection and single-cell analysis by targeting mutations in the tobacco PDS gene (NpPDS). Tobacco (N. tabacum) is an amphidiploid derived from N. sylvestris and N. tomentosiformis (Endo et al., 2016; Sierro et al., 2014). Specific primers were designed to amplify PDS genes from the N. sylvestris genome (S form) and the N. tomentosiformis genome (T form). We transfected N. tabacum protoplasts with various amounts of a mixture of a construct encoding the sgRNA which can target identical sequences in both genomes and the Cas9 protein (Kaya et al., 2016) and isolated more than 20 individual protoplasts for each treatment.

Table 2 shows that with increasing amounts of DNA, a higher percentage of individual protoplasts showed PDS mutations. Using 20 μg of DNA, on average 55.3% of the protoplasts showed S form target mutations in three experiments. Because the transfection efficiency in this experiment (Figure 3) was 43%, these results indicate that the S genome PDS target mutation efficiency was 83.7% (0.360.43 = 83.7%, Data S1). Although the target sequence was identical, only 29.7% of the T form PDS genes showed mutations (Table 2). Similar S/T mutation ratios were seen when protoplasts were incubated for 2–4 days following transfection (Figure 4, Data S2). Triplicate experiments yielded similar results (Figure S15, Data S2).

To apply this single-protoplast protocol to other species, we investigated CRISPR-mediated mutagenesis of the maize ZmIPK gene. Poaceae protoplasts are smaller than those of tobacco and Arabidopsis, but we could isolate individual maize protoplasts after transfection. Similar to the experiments described above, we used two sgRNA to delete a fragment of ZmIPK. Using a protoplast mixture, deletions could not be detected during the first round of PCR analysis, but could be observed using a second round of PCR after a restriction enzyme was used to digest the amplicons resulting from the first PCR product (Figure S8). Because of these two rounds of PCR, it is difficult to calculate target site mutagenesis efficiency using the scanned gel image. However, using a single protoplast, we could detect deletions in 23% of the protoplasts (Figure 5). Because the transfection efficiency was 55%, the calculated mutagenesis efficiency was 41%. PCR products were cloned and sequenced to confirm the deletions. The sequence data are shown in Data S3.

According to these results, single-cell analysis is a convenient method to detect mutagenesis efficiency and to determine mutated sequences. Using pooled protoplast DNA as the template, the mutagenesis efficiency could be measured by gel image analysis. In the ZmIPK two sgRNA experiment (Figure 5), single-cell analysis was more sensitive because the low amount of the PCR product containing deletions was not observed in the scanned gel image (Figure S7). To obtain the mutated sequences, the pooled DNA PCR products had to be cloned for sequencing. Using single-cell analysis, the PCR product could be sequenced without cloning. Heterozygous PCR products were identified using bioinformatics tools. The scanned gel image analysis plus PCR product cloning/sequencing, and the single-cell analysis which we present in this report are alternative methods to determine mutagenesis efficiency and mutated sequences. The results from these two methodologies were consistent.

| DNA (μg) | Gene* | 0 | 5 | 10 | 20 |
|---------|-------|---|---|----|----|
| **Experiment 1** | | | | | |
| S | 0 | 9 | 23 | 36 |
| T | 0 | 9 | 9 | 9 |
| **Experiment 2** | | | | | |
| S | 0 | 65 | 70 | 85 |
| T | 0 | 15 | 40 | 50 |
| **Experiment 3** | | | | | |
| S | 0 | 10 | 60 | 45 |
| T | 0 | 5 | 25 | 30 |
| **% Target mutation (±SE)** | | | | | |
| S | 0 | 28.0 ± 32.0 | 51.0 ± 24.8 | 55.3 ± 26.1 |
| T | 0 | 9.7 ± 5.0 | 24.7 ± 15.5 | 29.7 ± 20.5 |

*5 N. sylvestris gene; T, N. tomentosiformis gene. Bold-face type indicates the average ± s.d. among the multiple experiments.

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Regeneration of CRISPR-edited plants from single protoplasts

To show that CRISPR-mediated mutagenesis can be useful to generate genome-edited plants, we targeted the NtPDS gene of tobacco. The PDS gene is important for chlorophyll biogenesis (Qin et al., 2007), and homozygous PDS mutants are albino. Because N. tabacum contains four PDS genes (two each from the N. sylvestris and N. tomentosiformis progenitors), mutation of all four genes is necessary to obtain albino plants. Following transfection, protoplasts were transferred to growth medium to obtain calli. We obtained both albino and green calli (Figure 6a), which we regenerated into shoots. Green calli grew more quickly than did albino calli, but we were able to obtain shoots from both types of calli (Figure 6b–d). From three biological repeats, protoplasts were transferred to growth medium to obtain calli. We obtained both albino and green calli (Figure 6a), which we regenerated into shoots. 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one report claiming that in maize >90 candidate target sites must be screened to find a suitable sgRNAs (Zhu et al., 2016). Therefore, a CRISPR platform with a rapid and efficient evaluation protocol is needed. A protoplast transient transfection system with high transfection efficiency fulfills this need. The bottleneck of isolating high-quality protoplasts from different species has hindered this application.

For rice protoplast isolation, we made two modifications to previous methods (Chen et al., 2006; Zhang et al., 2011) to improve yield and reduce costs. First, instead of cutting the seedlings into cross section pieces, we cut the rice seedlings parallel to the veins. Early attempts to use random or cross section cutting resulted in fewer and lower quality protoplasts, likely because there are proportionally more cell walls in the same-sized cross-sectional area. Longitudinal cuts generated more damage to cells along the cut edge but allowed easier access of the maceration enzymes to the cells, resulting in more protoplasts released during digestion (Figure 1d,e). When seedlings were cut in cross section, more cells remained within the leaf sheath after digestion. We substituted the less expensive Cellulase R10 for the more expensive Cellulase RS (Zhang et al., 2011). We could apply this protocol to other Poaceae species, including wheat, bamboo, millet and corn. We believe this convenient method will benefit Poaceae crop research.

The Tape Sandwich method can be used to isolate protoplasts from Brassicaceae species (Arabidopsis (Wu et al., 2009), broccoli, Cleome, and rapeseed (Figure S1)) and from other family, such as Euphorbiaceae (Poinsettia, Pitzschke and Persak, 2012). However, this protocol could not be applied to tomato leaves because the epidermal cells could easily be pulled off by tape.

Although tomato mesophyll protoplasts can be isolated by leaf cutting (Niedz et al., 1985), this is often not convenient. Suspension cell lines are an alternative material for tomato protoplast isolation. These lines can be maintained in a controlled environment without variations caused by temperature or light (Nagata et al., 1992). The tobacco suspension cell line BY-2 has been used for several purposes, such as subcellular protein localization, BIFC vector validation (Lee et al., 2008) and CRISPR gene editing (Mercx et al., 2016). In addition to a ‘Micro-Tom’ tomato cell line, we also established a line from a different variety (CL5915; World Vegetable Center, Tainan, Taiwan) following this protocol. Other advantages of suspension cells include the ease of transgenic material containment and the ability to maintain good manufacturing practices for production of pharmacological proteins or antibodies, which can be secreted to the extracellular medium for easy purification (Mercx et al., 2016).

Validation of CRISPR/Cas9-mediated targeting mutagenesis in plant protoplasts

Bamboo

Bamboo is an economically and ecologically important vegetable and forest plant in Asia (Ma et al., 2015). Bamboo grows quickly and is a sustainable and environmentally friendly crop. Bamboo

Figure 4 Single-protoplast analysis of the effect of incubation times on NtPDS target mutagenesis. Tobacco protoplasts were transfected with 20 μg plasmid DNA (containing the expression cassette of NtPDS sgRNA and SaCas9; Kaya et al., 2016) and incubated for various number of days. The target mutation was analysed by RFLP. S, sylvestris form; T, tomentosiformis form. Green box, wild-type RFLP control; red box, albino mutant RFLP control. Numbers above the gel lanes indicate protoplast number.
Figure 5  Targeted mutagenesis of the ZmIPK gene in protoplasts. (a) The gene structure of ZmIPK. Blue, gRNA positions; green, two pairs of primers for single-cell PCR analysis; red, size of PCR product from the wild-type gene; orange, size between two gRNAs; purple, theoretical size after precise deletion. (b) Maize protoplasts were transfected with two sgRNAs that targeted different regions of the ZmIPK gene. DNA from several single protoplasts was amplified by PCR 72 h after transfection. P, pooled DNA PCR product; −, no plasmid DNA transfected. Numbers above the gel lanes indicate protoplast number.

Figure 6  NtPDS mutant plants derived from CRISPR mutagenesis of transfected protoplasts. (a) Growing calli were embedded in shooting medium for 1.5 months. (a*) Magnified view of one portion of the plate shown in (a). Bar = 1 cm. (b) Green shoot clusters were subcultured in shooting medium for 3 weeks. (b*) Magnified view of one portion of the plate shown in (b). Bar = 1 cm. (c) Albino calli were subcultured in shooting medium for 3 weeks. (c*) Magnified view of one portion of the plate shown in (c). Bar = 1 cm. (d) Albino calli were subcultured in shooting medium after 7 weeks. (d*) Magnified view of one portion of the plate shown in (d). Bar = 1 cm.
Table 3 Analysis of regenerated green and albino shoots from CRISPR-mutagenized tobacco protoplasts

| DNA (µg) | 0   | 5   | 10  | 20  |
|---------|-----|-----|-----|-----|
| Green shoots | 113 | 127 | 62  | 139 |
| (repeat 1) |     |     |     |     |
| Green shoots | 119 | 174 | 148 | 48  |
| (repeat 2) |     |     |     |     |
| Green shoots | 136 | 129 | 167 | 123 |
| (repeat 3) |     |     |     |     |
| Total green shoots | 368 | 430 | 377 | 310 |
| Albino shoots | 0   | 3   | 4   | 54  |
| (repeat 1) |     |     |     |     |
| Albino shoots | 0   | 11  | 6   | 28  |
| (repeat 2) |     |     |     |     |
| Albino shoots | 0   | 4   | 2   | 45  |
| (repeat 3) |     |     |     |     |
| Total albino shoots | 0   | 18  | 12  | 127 |
| % albino shoots | 0   | 4.0 ± 1.2 | 3.1 ± 1.5 | 29.1 ± 6.9 |

Mutated NtPDS genes in green shoots (%)
Repet 1 0 10 20 80
Repet 2 0 30 10 30
Repet 3 0 30 30 40
Total mutant 23.3 ± 11.5 20.0 ± 10.0 50.0 ± 26.5

Mutated NtPDS genes in all shoots (%)
Repet 1 0 12.1 24.8 85.6
Repet 2 0 34.1 13.5 55.8
Repet 3 0 32.1 30.8 56.1
Total mutant 26.1 ± 12.2 23.1 ± 8.8 65.8 ± 17.1

Bold-face type indicates the average ± S.D. among the multiple experiments.

protoplasts have been isolated from suspension cells and leaves (Hsiamoto and Kobayashi, 2010; Huang et al., 1989, 1990; Yeh et al., 2011). Protoplasts derived from in vitro propagated shoots have been transfected for subcellular protein localization studies (Yeh et al., 2011). In this report, the protoplast yield was increased using longitudinal cutting from in vitro material and immature leaves of greenhouse-grown plants. We demonstrated that the bamboo genome can be mutated by the CRISPR/Cas9 system.

**Millet**

Millet is an important feed and food crop in arid regions (Pan et al., 2015). Millet is a C4-photosynthesis plant. Because the genome size of millet is smaller than that of other grass crops (490 M), it has become a model plant for C4 photosynthesis (Doust et al., 2009). Because transformation of millet is inefficient (~5.5%; Wang et al., 2011), it is difficult to use a traditional transformation strategy to test sgRNAs for genome editing. Protoplasts can be used to screen for suitable sgRNA and Cas9 constructs for use in stable transformation. Xiang et al. (2004) developed a millet protoplast isolation protocol. Millet protoplasts have been used for protoplast transfection and for BIFC investigation (Liu et al., 2016). Using protoplast transfection, we demonstrate that the millet genome can be mutated using the CRISPR/Cas9 system.

**Rice**

Since the first report of CRISPR/Cas9-mediated genome editing in rice, there have been more than 30 articles published about rice genome editing using CRISPR/Cas9 (e.g. Feng et al., 2013; Mao et al., 2013; Miao et al., 2013; Shan et al., 2013; Xie and Yang, 2013). Compared with other crop plants, CRISPR technology in rice is mature. Modifications reported include single sgRNA knockouts and deletion of a large DNA fragment (>200 kb; Zhou et al., 2014). DNA-free genome editing has also been reported (Woo et al., 2015). CRISPR/Cas9 has been used in rice breeding to improve phenotypes to change leaf morphology (Ikeda et al., 2016), grain number, panicle architecture, grain size and plant architecture (Li et al., 2016a). CRISPR/Cas9 has also been used to induce male sterility (Li et al., 2016b), herbicide-tolerance (Sun et al., 2016) and pathogen resistance (Wang et al., 2016). Numerous studies used protoplasts to confirm the editing efficiency of the sgRNA and Cas9 protein (Jiang et al., 2013; Li et al., 2016b; Lowder et al., 2015; Shan et al., 2013, 2014; Wang et al., 2016; Woo et al., 2015; Xie and Yang, 2013; Xie et al., 2015). These articles indicate that a convenient and high transient transfection protoplast protocol is useful to determine the efficiency of designed gRNAs. We improved rice protoplast isolation and showed that these protoplasts can be used for transient transfection. Our convenient rice protoplast isolation

![Image](image-url)
Table 4 Analysis of regenerated green and albino shoots from CRISPR-mutagenized tobacco protoplasts which were transfected with 20 μg plasmid DNA

| Phenotypes of regenerated shoots | Experiment 1 | Experiment 2 | Experiment 3 | Average |
|----------------------------------|--------------|--------------|--------------|---------|
| Albino/Green (%)                 | 0.20 ± 0.07 (169/969) | 0.52 ± 0.20 (205/379) | 0.37 ± 0.11 (183/503) | 0.36 |
| Mutated NtPDS genes in green shoots (%) | 33.3 ± 2.9 | 46.7 ± 10.4 | 53.3 ± 2.9 | 44.4 |
| T                               | 15.0 ± 5.0 | 25.0 ± 10.0 | 36.7 ± 7.6 | 25.6 |
| Total mutant genes (±SE)         | 35.0 ± 0.0 | 46.7 ± 10.4 | 53.3 ± 2.9 | 45.0 |
| Mutated NtPDS genes in white shoots (%) | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100 |
| T                               | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100 |
| Total mutant genes (±SE)         | 100.0 ± 0.0 | 100.0 ± 0.0 | 100.0 ± 0.0 | 100 |
| Mutated NtPDS genes in all shoots (%) | 45.6 ± 23.0 | 64.9 ± 6.0 | 65.9 ± 3.3 | 58.8 |
| Shoots containing Cas9 gene (%)  | 1.7 ± 2.9 | 3.3 ± 5.8 | 1.7 ± 2.9 | 2.2 |
| Green                            | 15.0 ± 8.7 | 26.7 ± 15.3 | 17.2 |

Bold-face type indicates the average ± s.d. among the multiple experiments.

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Protocol will be useful to reduce the time to evaluate the efficiency of nucleases and sgRNAs.

**Maize**

Protoplasts isolated from some maize genotypes can be regenerated into plants (Rhodes et al., 1988; Sheen, 2001). There are several maize CRISPR/Cas9 studies that used protoplasts (e.g. Feng et al., 2016; Liang et al., 2014; Svitashev et al., 2015; Xing et al., 2014; Zhu et al., 2016). In this report, we targeted the IPK gene with RFLP validation in protoplasts. Using two sgRNAs, we could generate a large (300 bp) deletion within this gene.

**OsU3 and OsU6 application in dicots**

Most current CRISPR/Cas9 studies have focused on developing only monocot or dicot vectors (Jiang et al., 2013). In our study, we used the rice promoters OsU3 and OsU6 to express sgRNAs in a vector containing Cas9 and sgRNA genes. The results indicate that monocot promoters can be used efficiently in both monocot and dicot protoplasts.

**Broccoli**

Broccoli plants can be regenerated from mesophyll-derived protoplasts (Robertson and Earle, 1986). These protoplasts can be transfected using a PEG-mediated method (Nugent et al., 2006). We used the Tape Sandwich method to establish a broccoli protoplast transfection system using cotyledons as starting material. Lawrenson et al. (2015) established a broccoli CRISPR/Cas9 mutagenesis protocol. We used their sgRNA sequence in this study. In a broccoli GA4a CRISPR/Cas9 stable transformation study, 80% mutation frequency was achieved using the Arabidopsis U6 promoter to drive the same sgRNA (Lawrenson et al., 2015). We conclude that our high mutagenesis results from the chosen target gene and the high efficiency of protoplast isolation and transfection. The sgRNA mutation efficiency has been positively correlated with GC content, and the GA4a gene has a high GC ratio (65%; Ren et al., 2014). This speculation has to be proven by further sgRNA investigations.

**Rapeseed**

Rapeseed is cultivated mainly for its oil-rich seed, the third largest source of vegetable oil in the world (http://apps.fas.usda.gov/psd online/circulars/oilseeds.pdf). Kartha et al. (1974) described a rapeseed protoplast isolation protocol, and plantlets could be regenerated from these protoplasts. A rapeseed protoplast transient transfection system has also been established (Wang et al., 2015a). Our results indicate that the rapeseed protoplast genome can be mutated by CRISPR/Cas9.

**Solanaceae**

Tobacco is one of the first crops in which CRISPR/Cas9 genome editing was demonstrated (Jiang et al., 2013; Li et al., 2013; Nekrasov et al., 2013). There are many articles demonstrating that the tomato genome can be edited by CRISPR/Cas9 (Brooks et al., 2014; Cermak et al., 2015). In previous studies, the sgRNA was driven by an Arabidopsis U6 promoter. We showed that the same vectors used in monocots (pCAMBIA1300-OsU3(AarI)-Cas9 vectors) can be used in Solanaceae (tobacco and tomato), even though the promoters are derived from rice.

**Application of single-cell analysis to genome editing**

Numerous studies have demonstrated the utility of protoplasts to evaluate the efficiency of various CRISPR systems to mutate specific target genes (Feng et al., 2013; Li et al., 2013; Shan et al., 2013; Xie and Yang, 2013). Populations of protoplasts are transfected with DNA encoding Cas9 (or other editing proteins) and genes encoding sgRNAs. Alternatively, Cas9 protein/sgRNA complexes can be transfected into protoplasts (Woo et al., 2015). Total DNA from the protoplast population is isolated and assayed for mutagenesis frequency using RFLP or other methods of analysis, such as the T7E1 surveyor assay (Woo et al., 2015). When the mutation frequency is high, it is relatively easy to detect mutated molecules after electrophoresis through agarose gels. However, as we have shown above with the IPK gene of maize, the frequency of CRISPR-mediated mutagenesis can be low,
requiring a second round of PCR to amplify sequences from the small amount of nondigested DNA extracted from the gel.

Single-cell (protoplast) analysis is a sensitive and convenient method to evaluate the efficiency of various sgRNAs for CRISPR-mediated mutagenesis (Figure S18). Because only two copies of a given gene exist in a diploid cell (or four copies in an amphidiploid such as N. tabacum), edited copies of the gene will represent 50% of the gene molecules (for heterozygous cells) or 100% of the gene molecules (for homozygous or biallelic cells). PCR products of these genes can directly be sequenced without the need for prior cloning. Because of the small amount of DNA in a single protoplast, a gene of interest cannot be amplified by one step PCR directly, even if the protocol is increased to 50 cycles. Target sequences can be amplified in a second round of PCR (use 1 µL of the first step PCR product as a template). It is important to use nested primers for the second round of PCR. If the same primers are used for both PCR steps, only a smear of products is obtained.

In a previous protocol, transfected protoplasts had to be cultured in W5 solution (Wu et al., 2009), then transferred to liquid callus medium for further regeneration. Our new single-protoplast analysis protocol allows faster analysis using much less plant material.

Homzygous transgene-free mutant plants can be obtained from protoplasts in the first generation

One of the advantages of using protoplasts is that DNA-free genome editing can be conducted using preassembled RNP complexes consisting of Cas9 protein and sgRNAs. These complexes were introduced directly into protoplasts using a polyethylene glycol-based method (Woo et al., 2015) to generate mutagenized lettuce. RNP complexes can also be delivered to calli by particle bombardment (Liang et al., 2017). Generation of homozygous or biallelic mutants lacking transgenes encoding genome-editing reagents in the first generation is important for those plant species that cannot easily be crossed to eliminate transgenes. These include vegetatively propagated species, such as potato and banana, and species with long generation times, such as woody trees.

In this report, we show that we can generate transgene-free genome-edited plants by transfecting protoplasts with DNA encoding sgRNAs and Cas9 protein (Figure S19). Most regenerated N. tabacum plants containing edited PDS genes lacked the Cas9-encoding transgene DNA (Table 4). Homozygous transgene-free mutant plants could be regenerated in the T1 generation. Similar mutagenesis results were reported for wheat (Zhang et al., 2016) and lettuce (Woo et al., 2015). Compared to RNP complexes, DNA encoding the genome-editing reagents is less expensive and more convenient to use. One advantage of RNP complexes is their higher target mutation efficiency (Woo et al., 2015). If the efficiency of a given sgRNA were high, we recommend using DNA encoding genome-editing reagents. As we have shown, protoplast transfection can be highly efficient and up to 60% of mutagenized regenerated tobacco plants contained at least one mutated PDS gene (Tables 3 and 4). In hexaploid wheat, only 0.6% (67/10448) of the plants transformed by CRISPR constructs were homozygous mutants (Zhang et al., 2016). We have shown that for allotetraploid tobacco, ~30% of the plants regenerated from mutagenized protoplasts were homozygous/tetra-allelic mutants. In theory, we could obtain 154 transgene-free albino mutants from one transfection

\[\left\{169 + 205 + 183/3\right\} \times (1-0.172)\], a number much higher than can be obtained by particle bombardment (Liang et al., 2017; Zhang et al., 2016). Thus, CRISPR-based mutagenesis of protoplasts can be a useful technology for polyploid crops, especially horticultural crops that are propagated via asexual methods. Our studies suggest that this new technology can reduce breeding time significantly.

Protoplast regeneration remains a bottleneck for this platform. Especially for monocot species, high regeneration efficiency protocols are not available (Liang et al., 2017; Zhang et al., 2016). Improvements in protoplast regeneration protocols will be important for genome editing and the delivery of high editing efficiency reagents (RNP and donor DNA) to obtain transgene-free crops.

Materials and methods

Plant materials

Millet (Setaria italica), corn (Zea mays), rice (Oryza sativa), broccoli (Brassica oleracea), and rapeseed (B. napus) plants were grown on filter paper with drip irrigation in an environment-controlled chamber with a long photoperiod (16-h light/8-h dark) at 25 °C. Two-week-old plants were used for protoplast isolation. Bamboo (Bambusa oldhamii) shoots were incubated in Murashige and Skoog (MS) salts medium (Sigma-Aldrich, St. Louis, MO) supplemented with 0.1 mg/L TDZ (Lin et al., 2007). Arabidopsis seeds were grown in soil using a 16-h light/8-h dark cycle at 26 °C. Cleome spinosa, C. monophylla, and C. gynandra plants were grown in a greenhouse. Tobacco BY-2 cells were grown in MS salts supplemented with 1 mg/L thiamine-HCl, 370 mg/L KH2PO4, 30 g/L sucrose, and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.7 (Lee et al., 2008). A tomato Micro-Tom cell line was subcultured in MS medium supplemented with 1 mg/L 2,4-D, pH 5.7. Detailed information on this cell line is presented in Figure 1.

Protoplast isolation and PEG-mediated protoplast transfection

Protoplast isolation and transfection were performed following Wu et al. (2009) for Brassicaceae, Zhang et al. (2011) for Poaceae, and Lee et al. (2008) for Solanaceae, with modifications. Details are described in Table S1 for Poaceae, Table S2 for Brassicaceae, and Table S3 for Solanaceae. Following transfection, 1.5 mL W5 solution were added, the protoplasts were transferred into six-well plates (precoated with 1% BSA solution), and cultured in the dark at room temperature for 48 h.

Plasmid constructions

The primers OsU3-HindIII-f and OsU3-HindIII-r were used to amplify the gRNA cassette (OsU3 promoter, Aar restriction site and gRNA scaffold) from the plasmid pU3-gRNA which was ligated into HindIII digested pCAMBIA1300 to generate pCAMBIA1300-OsU3(Aar) (Figure S2). The Cas9 cassette fragment [355 promoter, human codon-optimized SpCas9 (hSpCas9) gene and terminator] was obtained from 355-Cas9-SK by digestion with SacI and EcoRI and ligated into SacI/EcoRI digested pCAMBIA1300-OsU3(Aar) to generate pCAMBIA1300-OsU3(Aar)-Cas9 (Accession no.: KX400856). The same strategy was used to generate pCAMBIA1300-OsU6(Aar)-Cas9 (Accession no.: KX388151). Detailed information is found in Supplemental Material and Methods.
Mutation validation in protoplasts

Genomic DNA was extracted from pooled protoplasts using a Mini GenoPlus Genomic DNA Extraction Kit (Viogene, New Taipei City, Taiwan). To amplify the genomic region targeted by the sgRNA, two pairs of primers were designed. The restriction enzyme and primer sequence information are shown in the Supplementary Figures. PCR conditions are 94 °C for 5 min; 35 cycles of 94 °C for 30 s, annealing (55–63 °C; detailed information is shown in the Supplemental Figures) for 30 s, polymerisation at 72 °C for 30 s, followed by 72 °C for 3 min. The PCR product was digested by the appropriate restriction enzyme and the products subjected to electrophoresis. Electrophoresis gel images were analysed by ImageJ (Schneider et al., 2012). The intensity of the undissected band was divided by the total intensity of all bands to generate the raw mutation frequency. The raw mutation frequency was divided by the transfection efficiency to obtain the final mutation frequency. Secondary PCR products were cloned into the T&A™ vector (FYC002-20P; Yeastern Biotech Co. LTD, New Taipei City, Taiwan). Colonies harbouring the edited DNA were screened by a PCR/restriction enzyme assay, and the insert DNA was sequenced. VirD2-NLS-mRFP plasmid DNA (Lee et al., 2008) was transfected into protoplasts by PEG-mediated methods as a positive control to determine transfection efficiency.

Single-protoplast isolation and target mutagenesis validation

Transfected protoplasts were centrifuged (300×g, 3 min, 25 °C, Eppendorf Centrifuge 5804R; Eppendorf, Hamburg, Germany) and transferred to liquid callus medium [1/2 MS medium supplemented with 0.4 m mannitol, 30 g/L sucrose, 1 mg/L 1-naphthaleneacetic acid (NAA) and 0.3 mg/L kinetin]. Protoplast concentration was measured using a hemocytometer and adjusted to 1 cell/μL. A single protoplast was isolated using a pipette (SelectPette, Genomics, New Taipei City, Taiwan), transferred to a slide, and the condition checked by microscopy (Olympus, Tokyo, Japan). A single protoplast was transferred to 20 μL PCR mixture containing the first pair of primers for the initial DNA amplification. One microliter of the PCR product was used as a template for a second round of PCR using a second pair of primers. Amplicons resulting from the second PCR were digested by the appropriate restriction endonuclease and analysed by electrophoresis to validate mutation of the target sequence. A schematic for single-cell analysis is shown in Figure S18.

Tobacco protoplast regeneration

We used a protocol modified from Liao (1990, Figure S19). Transfected protoplasts were incubated in a 5-cm-diameter Petri dish containing liquid callus medium. After 2- to 3-week incubation in the dark, the protoplasts proliferated and formed dust-like calli. The calli were embedded in solidified callus medium in a 9-cm-diameter petri dish and incubated at 25 °C for 3–4 weeks in the dark. Calli larger than 3 mm were embedded in shoot medium for shoot induction. After 1 month at 25 °C in the light (light/dark: 16/8 h; 3000 lux), shoot clusters containing leaves >5 mm were transferred to fresh shoot medium for 2–3 weeks for further shoot proliferation. Shoot clusters with leaves were then transferred to solidified root medium.

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Competing interests

The authors declare that they have no competing interests.

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Author contributions

CSL, LYL, SBG, and MCS conceived and designed the experiments. YZ, RW, WW, and WJC conducted vectors. CSL, LHY, CTH, and FHW performed targeted mutagenesis and protoplast-related experiments. CTH, CSL, and LIL conducted protoplast regeneration. CTH, QWC, FJY, CTY, LHY, and FHW analysed the data. CSL, LYL, LHY, CTH, SBG, and HCHW interpreted the data. CSL, LYL, SBG, and MCS wrote the manuscript.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** Protoplast transfection of various species.

**Figure S2** Construction of pCAMBIA1300-OsU3-Cas9.

**Figure S3** Targeted mutagenesis of *Bambusa oldhamii* protoplasts.

**Figure S4** The putative mutated *BoPDS* PCR products from *Bambusa oldhamii* protoplasts were cloned and validated by sequencing.

**Figure S5** Targeted mutagenesis of *Setaria italica* protoplasts.

**Figure S6** Targeted mutagenesis of *Oryza sativa* protoplasts.

**Figure S7** Targeted mutagenesis of *Zea mays* protoplasts.

**Figure S8** The PCR-RFLP-PCR results of *ZmIPK* mutagenesis.

**Figure S9** Targeted mutagenesis of *Arabidopsis thaliana* protoplasts using constructs carrying the OsU3 or OsU6 monoregulator promoter.

**Figure S10** Targeted mutagenesis of *Brassica oleracea* protoplasts using constructs carrying the OsU3 or OsU6 monoregulator promoter.

**Figure S11** The putative mutated *BoLGAl* PCR products from *Brassica oleracea* protoplasts were cloned and validated by sequencing.

**Figure S12** Targeted mutagenesis of *B. napus* protoplasts using constructs carrying the OsU3 or OsU6 monoregulator promoter.

**Figure S13** Targeted mutagenesis of *Nicotiana tabacum* protoplasts.

**Figure S14** Targeted mutagenesis of *Solanum lycopersicum* protoplasts.

**Figure S15** Effect of incubation times on *NtPDS* target mutagenesis analysed in single protoplasts in Experiments 2 and 3.

**Figure S16** Targeted mutagenesis of *NtPDS* in tobacco protoplast regenerants of Experiment 1.

**Figure S17** Effect of plasmid dosage on *NtPDS* mutagenesis in
tobacco protoplast regenerants.

**Figure S18** Schematic representation of single-cell isolation and validation of targeted mutagenesis.

**Figure S19** Schematic of tobacco protoplast regeneration.

**Table S1.** Protocol for protoplast isolation and PEG transformation of different Poaceae species.

**Table S2** Protocol for protoplast isolation and PEG transformation of different Brassicaceae species.

**Table S3** Protocol for protoplast isolation and PEG transformation of different Solanaceae species.

**Data S1** The *NtPDS* sequences of Figure 3 (Experiment 1) and Table 2.

**Data S2** The *NtPDS* sequences of Figure 4 (Experiment 1) and Figure S15 (Experiment 2 and 3).

**Data S3** The sequences of *Zea mays* single-cell *ZmIPK* genes in Figure 5.

**Data S4** The *NtPDS* sequences of Figure 7 (Exp2R1) and Table 4.

**Data S5** The *NtPDS* sequences of Figure S17.