



# Development of a Mesophyll Protoplast-Based System for Gene Editing of Papaya

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## Abstract

*Carica papaya* L. is an economically significant crop in tropical and subtropical regions, with a gross production value of  $\$6.2 \times 10^9$  in 2020. However, various biotic and abiotic stresses threaten crop productivity. To enhance stress resistance, genetic engineering and traditional breeding have been employed. Unfortunately, these methods are limited by the scarcity of innate disease resistance genes in the genome and the poor fertility of interspecific hybrids. Therefore, to circumvent these limitations, we developed a papaya protoplast-based gene editing system. By optimizing protoplast isolation, 28% higher yields were achieved from older ( $\geq 75$  d) plants at  $1.11 \times 10^8 \pm 0.069$  protoplasts per gram-fresh-weight. Protoplast viability was  $89.87 \pm 2.02\%$ . We established an efficient genetic transfection method and verified proper expression, cellular function and localization of GFP and PDI-mCherry fusions in the protoplasts. Using preassembled CRISPR-Cas9 ribonucleoprotein complexes, we successfully edited a mutant GFP transgene, resulting in a frame-shift restoration efficiency of  $27.88 \pm 1.65\%$ . Next, the *CpPDS* and *CpMLO6* genes were targeted, creating knockouts in three different papaya cultivars. The average *CpPDS* mutant frequency obtained was  $42.31 \pm 1.90\%$ , of which  $31.25 \pm 1.46\%$  were frame-shift knockouts, while  $11.05 \pm 1.37\%$  were in-frame protein variants. The average *CpMLO6* mutant frequency was  $16.20 \pm 1.53\%$ , of which  $13.71 \pm 1.67\%$  were frame-shift knockouts and  $2.50 \pm 0.26\%$  were in-frame variants. Taken together, a DNA-free CRISPR-Cas9 gene editing system was successfully demonstrated in papaya protoplasts on multiple target genes for use in papaya crop improvement.

**Keywords** DNA-free · Papaya protoplasts · Gene editing · CRISPR-Cas9 · Crop improvement

## Introduction

Papaya (*Carica papaya* L., family Caricaceae) is an economically important tropical and subtropical crop worldwide, with a global gross agricultural production value of  $\$US 6.2 \times 10^9$  in 2020 (FAOSTAT 2020; Evans *et al.* 2021). The unripe fruit and tree trunk are rich sources of the proteolytic enzyme papain, which aids digestion and is a meat tenderizer (Brocklehurst *et al.* 1981; Krishna *et al.* 2008). Fresh ripe papaya ranks first among fresh fruit for vitamins C and A, calcium, iron, potassium, folate, riboflavin, niacin, thiamin, and fiber (Krishna *et al.* 2008; Hewajulige and Dhekney 2016). Besides its dietary health benefits, the cosmetic, pharmaceutical, textile, and food processing industries use papaya and its by-products (Carlos-Hilario and Christopher 2015; Hewajulige and Dhekney 2016; Evans *et al.* 2021). The agricultural importance of papaya necessitates efforts to genetically improve its resistance to biotic and abiotic threats that undermine its productivity.

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The elucidation of the papaya genome sequence facilitated studies on gene identification, genetic engineering, and comparative genomics that are useful for crop improvement (Ming *et al.* 2008; Ming and Moore 2014). The relatively small papaya genome of 372 megabases is approximately 2.75 times larger than the *Arabidopsis thaliana* genome (Arumuganathan and Earle 1991). Papaya and *Arabidopsis* share a common ancestor, with both species belonging to the order Brassicales (Ming and Moore 2014). Although the *Arabidopsis* genome has helped identify papaya genes, papaya contains fewer homologous disease resistance genes than *Arabidopsis* (Ming *et al.* 2008).

The limited genetic resistance to diseases is of concern because disease-causing pathogens such as the pathogenic fungus, *Oidium caricae-papayae*, and the oomycete, *Phytophthora palmivora* (Nelson 2008; Cunningham and Nelson 2012) threaten the viability of the papaya industry. *Oidium caricae-papayae* causes powdery mildew on papaya, resulting in agricultural losses as infected fruit are unsuitable for the market (Cunningham and Nelson 2012). *Phytophthora* is a hemibiotrophic pathogenic oomycete that affects agronomically important food crops around the world (Lamour 2013). The *Phytophthora palmivora* species causes *Phytophthora* blight of papaya (Nelson 2008). Symptoms include fruit and root rot, stem canker, and severe structural damage, leading to plant death (Nelson 2008). Chemical and biological control agents have been used to control *Phytophthora* infection, with chemical fungicides proving the most effective (Agrios 2005; Nelson 2008); the latter have been associated with environmental health concerns (Zubrod *et al.* 2019). Therefore, developing eco-friendlier alternatives will be useful and welcomed for papaya. Genetic engineering and traditional breeding methods were used to moderately improve resistance to *P. palmivora* (Zhu *et al.* 2007a, b). However, the few innate disease resistance genes in its genome is compounded by F1 sterility of hybrids made with closely related species (Manshardt and Wenslaff 1989). In addition, genetic engineering with an interspecific resistance transgene led to progeny with low viability and vigor (Porter *et al.* 2014). Clearly, new approaches are needed for this crop. The evolving ability of pathogens to infect plants by overcoming resistance (R) gene-based immunity necessitates a gene editing system capable of developing new resistant varieties via the mutation of the susceptibility (S) loci (van Schie and Takken 2014).

A critical family of S-genes, which are involved in plant pathogen pathosystems, is the *MLO* gene family. The *MLO* gene is involved in host compatibility, playing a role in facilitating pathogen entry into the host. It also increases innate immunity upon disrupting its function (van Schie and Takken 2014). The *MLO* locus encodes a seven-transmembrane domain protein anchored in the plasma membrane whose exact biochemical function is unknown

(Büschges *et al.* 1997; Devoto *et al.* 1999). Naturally occurring loss-of-function *MLO* mutants provided broad-spectrum resistance to powdery mildew (Büschges *et al.* 1997; Bai *et al.* 2008). Actin remodeling is critical for determining whether pathogenic fungi successfully invade host cells, and *MLO* may influence this process (Opalski *et al.* 2004). *O. caricae* and *P. palmivora* enter the host cell by similar mechanisms during the early biotrophic infection stage, with the leaves of young barley *MLO5* mutant plants demonstrating improved resistance to *P. palmivora* (Le Fevre *et al.* 2016). The development of a gene editing method for papaya to generate *CpMLO6* mutant lines will help facilitate resistance studies in the future.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) are part of an innovative gene editing technology first identified as an adaptive immune response in bacteria against bacteriophage infection (Jinek *et al.* 2012; Sorek *et al.* 2013; Hsu *et al.* 2014). The type II CRISPR-Cas9 system comprises a Cas9 endonuclease bound with a transactivating CRISPR RNA (tracrRNA) fused to a CRISPR RNA (crRNA), to comprise a chimeric single-guide RNA (sgRNA) with a 20 nucleotide target sequence (Anzalone *et al.* 2020). The complex can target, bind, and cleave complementary DNA sequences, introducing double-strand breaks (DSB) into host DNA. Due to the cell's inaccurate non-homologous end joining (NHEJ) DNA repair mechanism, erroneous repairs are often made, introducing a diverse range of insertion and deletion (indel) mutations that can dramatically alter or terminate targeted protein function (Chen *et al.* 2019; Zhang *et al.* 2021a).

*Agrobacterium*-mediated CRISPR-Cas9 gene editing has been used in various crop improvement studies (Tian *et al.* 2017; Kaur *et al.* 2018; Zhang *et al.* 2019a; Zhang *et al.* 2019b; Navet and Tian 2020). Recently, this traditional DNA-based method (Brewer and Chambers 2022) was used to edit the phytoene desaturase (*CpPDS*) gene in papaya. However, extended time constraints are introduced while regenerating calli tissue suitable for transformation, segregating transgenic from non-transgenic material via Mendelian segregation (Zhang *et al.* 2019a), and navigating regulatory processes for commercialization (El-Mounadi *et al.* 2020). Developing a DNA-free method for gene editing in papaya will provide the tools necessary to bypass these constraints. Specifically, using papaya protoplasts instead of embryogenic calli reduces the time and costs required to generate and screen transformants, because protoplast isolation takes hours rather than weeks or months to produce large quantities of suitable target protoplasts. They are homogenous with similar physiological and physical characteristics. Finally, protoplasts provide numerous independent events in a single experiment, as each represents a single *in vivo* system (Nadakuduti *et al.* 2019).

A DNA-free method for performing CRISPR-Cas9 gene editing in plant cells is the use of CRISPR-Cas9 ribonucleoprotein (RNP) complexes. These RNP complexes can enter plant protoplasts, perform stable gene edits, and then are degraded through normal cell turnover activities. It is considered a transgene-free gene editing method because no transgenic material remains in the host genome (He *et al.* 2022). As such, regulatory concerns associated with traditional gene editing methods are diminished (Metje-Sprink *et al.* 2019). The CRISPR-Cas9 RNP complex is formed using all the same components necessary for traditional CRISPR-Cas9 gene editing, although the components are complexed *in vitro* rather than *in vivo*, then transfected into the plant protoplast using methods such as PEG-mediated transfection (Metje-Sprink *et al.* 2019).

Delivery of the CRISPR-Cas9 RNP complexes into plant protoplasts using the PEG-mediated transfection method aids in transfection efficiency, especially when combined with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , to increase phospholipid membrane permeability and improve transfection rates (Boss and Mott 1980; Maas and Werr 1989; Parray *et al.* 2020). This method has been used in several plant species, including *A. thaliana* (Woo *et al.* 2015), tobacco (Woo *et al.* 2015), rice (Woo *et al.* 2015), potato (Andersson *et al.* 2018; González *et al.* 2020), tomato (Lin *et al.* 2022), grapevine (Malnoy *et al.* 2016), apple (Malnoy *et al.* 2016), cabbage (Park *et al.* 2019), canola (Sidorov *et al.* 2021), and banana (Wu *et al.* 2020). Furthermore, complexing Cas9 endonuclease with a single-guide RNA (sgRNA), having a 2- to 10-fold molar excess of sgRNA, was effective for RNP-based gene editing in several plant species (Woo *et al.* 2015; Malnoy *et al.* 2016; Subburaj *et al.* 2016; Liang *et al.* 2017). The RNP delivery method has numerous advantages over traditional CRISPR-Cas9 delivery methods. Firstly, off-target effects are decreased, as there is no longer a transgenic CRISPR-Cas9 cassette continuously expressed in the host genome (Liang *et al.* 2017; He *et al.* 2022). Secondly, the time required for Cas9 transcription and translation prior to gene editing is eliminated because preassembled gene editors are delivered as an activated complex. Lastly, RNP delivery eliminates the concern of introducing insertion mutations that may occur using traditional stable transformation methods (Zhang *et al.* 2021b).

Considering these aforementioned advantages, we undertook a study to apply this DNA-free method to papaya protoplasts with the long-term goal of crop improvement. We improved the method to isolate intact viable protoplasts from papaya of various aged leaves and transfected them with reporter gene fusions for transient expression. Finally, DNA-free editing was conducted and confirmed by correcting a frame-shift mutant GFP transgene and deep amplicon sequence verification of targeted editing of endogenous papaya genes, *CpPDS*, and *CpMLO6*.

## Materials and Methods

**Growing Papaya Plants** Papaya Solo Sunrise and Sunset seeds were purchased from the University of Hawai'i Agricultural Diagnostic Service Center – Seed Program and Paramount Seeds Inc. ([paramountseeds.com/collections/papaya](http://paramountseeds.com/collections/papaya)). Papaya Solo Kapoho seeds were obtained by cleaning and drying the seeds from the fruit. Seeds were stored at 4°C. To germinate, the seeds were soaked for 24 h in 1 M  $\text{KNO}_3$  before transferring them into Sunshine Mix No. 4, mixed with 1/5 vol of perlite. Plants were grown under broad-spectrum light at 75–150  $\mu\text{mol m}^{-2} \text{s}^{-2}$  for a 16-h daily photoperiod. Germination occurred approximately 10 d after planting. The age of a plant was calculated from the day of seedling emergence from the soil after germination. Plants were well watered with care taken to ensure that they were free from drought or overwatering stress, as both conditions could affect the quality of isolated protoplasts. The leaves were visually inspected to ensure that the selected tissue was free of abnormalities and signs of biotic or abiotic stress. Leaves were harvested when plants were 50–71 d old for the young age category and 79–136 d for the old age category. New, fully expanded leaves were selected with petioles no longer than 1 inch and no reddening.

**Protoplast Isolation and Quantification** Leaves were collected and prepared according to methods previously described (Zhang *et al.* 2011), with a few modifications. Approximately 0.5 g of the newest fully expanded leaves from young or old plants was surface sterilized in a 50% EtOH solution for 30 s, and then transferred to a 20% Clorox solution for 7 min. Sterilized leaves were rinsed three times in ddH<sub>2</sub>O and then pat dried. A sharp scalpel was used to cut the leaves transversely into strips 1–2 mm wide. A sheet of white paper was used as a cutting surface to identify chlorophyll staining, indicating when the scalpel blades needed replacement. The protoplast isolation medium (1.3% Cellulase R-10, 0.3% Macerozyme R-10, 0.44 M D-mannitol, 20 mM MES at pH 5.8, 20 mM KCl, 10 mM  $\text{CaCl}_2$ , 0.5% w/v PVP) was heated in a 55°C liquid bath for 10 min to deactivate proteases and ensure the enzymes were fully dissolved. The isolation medium was cooled to RT, and then, polyvinylpyrrolidone (PVP) was added to 0.5% w/v, vortexed to dissolve the PVP thoroughly, and then sterilized using a 0.20- $\mu\text{m}$  filter. 0.5 g of leaf strips was added to 10 ml of isolation medium in a 50-ml conical tube, and a vacuum (10–12 mbar, 1.1–1.3 kPa) was applied for 30 min under low light. The vacuum was released every 5 min to resubmerge the leaf material in the solution and reapplied cyclically over the 30-min infiltration period. Vacuum-infiltrated leaves were poured onto a 100-mm Petri dish, spread evenly, sealed with micropore tape, placed in an orbital shaker, and incubated

at 26°C with 60 rpm for 13–15 h in the dark. After incubation, protoplasts were washed in an ice-cold modified W5 buffer solution (154 mM NaCl, 125 mM CaCl<sub>2</sub>, 5 mM KCl, 2 mM MES, pH 5.8). Ten milliliters of W5 buffer was added to the Petri dish and mixed gently. Protoplasts were filtered through a 30–70- $\mu$ m nylon mesh in a 50-ml conical tube. The protoplast solution was centrifuged for 5 min at 100  $\times$  g, at 4°C. The supernatant was carefully discarded, and the protoplast pellet was resuspended in 10 ml W5 buffer. The washes were repeated 2 $\times$  until the supernatant was free of debris. Finally, the protoplast pellet was resuspended in 10 ml of W5 buffer and incubated on ice for 30 min. Protoplasts were quantified using a hemocytometer. The total number of protoplasts was divided by leaf GFW to determine the total number of papaya protoplasts isolated per GFW leaves.

**Protoplast Viability Assay** The FDA hydrolysis assay (Larkin 1976) was used to determine the viability of freshly isolated papaya protoplasts. A 500- $\mu$ l aliquot of protoplasts in W5 buffer was added to a 2-ml round-bottom tube. Ten microliters  $\mu$ l of 0.5% FDA (5 mg/ml in acetone stock solution) was added to the protoplasts to obtain a final concentration of 0.01% FDA. After 5 min, the protoplasts were analyzed and imaged using brightfield and epifluorescence microscopy with an Olympus BX51 upright compound epifluorescence microscope equipped with a Leica DFC7000T 2.8-megapixel cooled CCD camera located in the Biological Electron Microscope Facility at the University of Hawai'i at Mānoa. A Chroma bandpass eGFP filter with excitation (EX 480/20 nm, BA 495LP) and emission (EM 510/20 nm) was used for epifluorescence imaging. The fluorescing to the non-fluorescing protoplasts were quantified using a hemocytometer.

**Transient Transfection of Papaya Protoplasts** Protoplasts were genetically transfected using the PEG-mediated transfection protocol (Yoo *et al.* 2007) with a few modifications. Freshly isolated protoplasts were centrifuged at 100  $\times$  g for 5 min. The protoplasts were resuspended in a modified MMg buffer solution (0.6 M Mannitol, 15 mM MgCl<sub>2</sub>, 4 mM MES at pH 5.8) to a concentration of 5  $\times$  10<sup>5</sup> protoplasts ml<sup>-1</sup>. A 200- $\mu$ l aliquot of protoplast in MMg was added to a 2-ml round bottom tube; then, 30–40  $\mu$ g of the plasmid construct enhanced GFP(S65T), also termed eGFP, was added and mixed gently and thoroughly. The GFP(S65T) construct is based on the vector pBluescript KS(+), with eGFP driven by a CaMV 35S promoter and flanked by a NOS terminator (35S-eGFP). The eGFP emits an increased signal intensity with reduced photobleaching by replacing serine at site 65 with a threonine (Chiu *et al.* 1996). The total vol of plasmid DNA did not exceed 10% of the total vol of added protoplasts. An equal vol of PEG-CaCl<sub>2</sub> (0.2 M D-mannitol, 0.1 M CaCl<sub>2</sub>, and 40% (w/v) polyethylene glycol 4000) was

added, and then mixed by inversion. The transfection mixture was incubated for 30 min at RT in the dark. Transfection was stopped by adding 800  $\mu$ l of W5 buffer at RT. The tube was gently inverted and then centrifuged for 3 min at 300  $\times$  g at RT. The supernatant was discarded, and the W5 buffer wash step was repeated twice. The protoplasts were then resuspended in 1 ml of WI buffer (0.6 M D-mannitol, 20 mM KCl, and 4 mM MES at pH 5.8) and incubated at RT in the dark for 16–18 h.

Brightfield microscopy, imaging, and quantification were performed as described above (“Growing Papaya Plants” section). Then, confocal images were obtained using a TCS Leica SP8 X confocal laser scanning microscope with a white light laser mounted on a Leica DF6 CFS upright microscope integrated with a high-speed DFC9000 GTC digital camera (Biological Electron Microscope Facility at the University of Hawai'i at Mānoa). Observations were made with a HC PL APO CS2 63 $\times$  1.4 NA oil immersion lens. Protoplasts were visualized using the eGFP excitation/emission spectrum of 488/505–525 nm with a time-gating of 0.3–6.0 ns to reduce chlorophyll autofluorescence. All images were acquired using Leica LAS X software.

**Transient Cotransfection and Colocalization Analysis** Papaya leaf mesophyll protoplasts were cotransfected as described in the “An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts” section with a few modifications. For cotransfection, 15  $\mu$ g of the construct PDI9:eGFP and 15  $\mu$ g of the construct ER:mCherry were added. PDI9:eGFP is in pBluescript KS(+) driven by the CaMV 35S promoter ending with a NOS terminator. PDI9 is fused at the C-terminus to GFP(S65T), with a KDEL ER retention motif of PDI9 added to the C-terminus of GFP(S65T) (Yuen *et al.* 2013). The construct ER:mCherry in pBluescript KS(+) is driven by the CaMV 35S promoter ending with a NOS terminator (Cho *et al.* 2011). Images were obtained as described above (“An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts” section) with a few modifications. Observations were made with a HC PL APO CS2 100 $\times$  1.4 NA oil immersion lens. PDI9-GFP and ER-mCherry were visualized using the excitation/emission spectra of 488/505–525 nm and 543/585–615 nm, respectively. A time-gating of 0.3–6.0 ns was used to reduce chlorophyll autofluorescence.

**Frame-Shift Restoration of the GFP Mutant (GFPm) Using CRISPR-Cas9 RNP** A recombinant Alt-R<sup>®</sup> SpCas9 nuclease containing a nuclear localization signal (NLS) and a C-terminal 6-His tag was purchased from Integrated DNA Technologies, Inc. (IDT; Coralville, IA). The custom design tool for IDT CRISPR-Cas9 gRNA (<http://www.idtdna.com/site/order/oligoentry/index/crispr>) was used to purchase a predesigned IDT Alt-R CRISPR-Cas9 sgRNA

oligo integrated with a custom 20 nt (5'-ACCATGGTGAGC AAGGGGCG-3') sequence targeting the GFP+1 frame-shift mutation site in GFPm (Liu *et al.* 2018). The GFPm gene is inserted in pUC19, driven by a CaMV 35S promoter and ended by a NOS terminator. The GFPm frame-shift mutant construct and the 20 nt sgRNA target sequence used in this study were provided by (Liu *et al.* 2018). Papaya leaf mesophyll protoplasts were cotransfected as described in the "An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts" section with several modifications. Ten microliters of RNP complex buffer (20 mM HEPES, 150 mM KCl, pH 7.5), 20 µg of SpCas9 enzyme, and 10 µg sgRNA were added to a 2-ml tube with sgRNA present in a molar excess of approximately 2.5× of SpCas9. The solution was mixed and then incubated at RT for 5 min to allow the RNP complexes to assemble. Forty micrograms of the GFPm construct and 200 µl of a  $5 \times 10^5$  protoplasts ml<sup>-1</sup> in MMg solution were added, and the solution was mixed gently by inversion. The transfection procedure then followed the "An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts" section, with an extended incubation time of 48 h. Protoplasts with GFP frame restored were quantified using a Leica SP8 X confocal laser scanning microscope and a hemocytometer using the excitation/emission spectrum of 488/505–525 nm.

**Western Blot Analysis** Immunoblot analysis was performed on protoplast samples transfected with the GFPm plasmid in the presence or absence of RNP complex, as described in Fig. 4D, scaled up to 5-fold. A sample transfected with the plasmid pUC19 was included as a negative control. Samples were centrifuged at 300 × g for 3 min, and then, the supernatant was discarded. The protoplast pellet was resuspended by adding 80 µl protoplast protein extraction buffer (PEB) (10 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8.0, 10% glycerol, 0.5% Triton X-100, 625 µM PMSF) to the 50-ml conical tube containing the pelleted protoplasts. The resuspended protoplasts in PEB were then transferred to a 2-ml round bottom tube, and samples were vortexed vigorously for 30 s. Lysate protein levels were quantified using the Bio-Rad DC™ Protein Assay Kit II (Bio-Rad;5000112). For immunoblot detection, protein samples were mixed with Non-Reducing Lane Marker 5× sample buffer from Thermo Scientific and 200 mM DTT, and then heated for 4 min at 90°C. The samples were quenched on ice, separated by SDS-PAGE (5% polyacrylamide stacking gel at pH 6.8, 10% polyacrylamide resolving gel at pH 8.8), and transferred to a nitrocellulose membrane. Each well was loaded with 10 µg of total protein while the positive control contained 1 µg. Immunoblot analysis was performed using Invitrogen A6455 rabbit anti-GFP primary antibody at 1:2000 dilution and Advansta R-05072 goat anti-rabbit IgG (H+L) HRP conjugate secondary antibody at 1:20,000

dilution, and then visualized by chemiluminescence using the Advansta WesternBright™ ECL Western blotting detection kit K-12045-D20.

**Editing of *CpMLO6* and *CpPDS* in papaya protoplasts using CRISPR-Cas9 RNP** A phylogenetic tree was constructed to determine which *MLO* homolog in papaya would most likely contribute to the susceptibility to powdery mildew. Sixteen annotated non-low-quality papaya *MLO*-like protein sequences were aligned with *MLO* homologs from seven plant species belonging to clade IV of monocots and clade V of dicots (Supplemental file 1), which were functionally characterized to be susceptibility (S) genes to powdery mildew (Pessina *et al.* 2014). The resulting alignment was used to construct a phylogenetic tree using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm with bootstrap resampling over 1000 replicates. Papaya *MLO*-like protein 6 (*CpMLO6*, XP\_021909409.1) was determined to be closely related to *MLO* homologs in dicot clade V (Supplemental file 1).

Candidate 20 nt sgRNA target sequences were identified using the EuPaGDT web server (<http://grna.ctegd.uga.edu>) with the mRNA sequences of *CpPDS* (NCBI Reference Sequence: XM\_022033216.1) and *CpMLO6* (NCBI Reference Sequence: XM\_022053717.1) as the queries and the SunUp Papaya genome (Ming *et al.* 2008) assembly 1.0 (NCBI GenBank Assembly Accession: GCA\_000150535.1) uploaded as a custom genome, for on- and off-target analyses, editing efficiency scores, and GC content. Candidate target sequences located in exons close to 5' end, with no off-targets, editing efficiency scores higher than 0.4, and GC content between 40 and 65%, were selected for prediction of RNA secondary structures using the RNAstructure web server (<http://rna.urmc.rochester.edu/RNAstructureWeb/>). The candidate target sequences coupled with a gRNA scaffold were used for RNA structure prediction. The ones predicted to have a secondary structure similar to other functionally active sgRNAs were selected and subjected to *in vitro* DNA cleavage activity assay (Gumtow *et al.* 2018; Navet and Tian 2020; Hasley *et al.* 2021).

*CpPDS*-sgRNA16 (20nt target sequence: 5'-AGTGTT TCTGCGGCGAGCTT-3') and *CpMLO6*-sgRNA254 (20nt target sequence: 5'-CGAAGTCAATTGGAGCCACG-3') targeting *CpPDS* and *CpMLO6*, respectively, were selected for *in vitro* cleavage assays. sgRNAs containing 20 nt target sequence followed by 80 nt sgRNA scaffold sequence were synthesized by IDT. The primers *CpMLO6*\_InVitro\_F1 (5'-CCTTCATATGTCCGTACTACTG-3'), *CpMLO6*\_InVitro\_R1 (5'-ACATCCATACGCTACGTACTTC-3'), *CpPDS*\_InVitro\_F1 (5'-CCAGATAGACATTACCCAGAATC-3'), and *CpPDS*\_InVitro\_R1 (5'-CTATGTCCTGGAATGAAC TTCAC-3') were used to produce 1250 bp amplicons as

the target DNA for *in vitro* cleavage assays and to obtain sequence information for *CpMLO6* and *CpPDS* papaya cultivars Sunset, Sunrise, and Kapoho. Sanger sequence data provided by Genewiz, Inc (South Plainfield, NJ) for *CpMLO6* and *CpPDS* were identical in all three cultivars. *In vitro* cleavage assays were performed using the GenCrispr sgRNA Screening Kit (GenScript, Piscataway, NJ) following the manufacturer's instructions.

Editing of *CpMLO6* and *CpPDS* in papaya protoplasts was performed with several modifications. Sixty micrograms of SpCas9 and 30  $\mu\text{g}$  of sgRNA were added to a 2-ml round bottom tube. RNP complexing buffer (20 mM HEPES, 150 mM KCl, pH 7.5) was added to reach a final volume of 40  $\mu\text{l}$ . Transfection protocols were performed as described in the "An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts" section with an extended incubation period of 48 h. DNA was extracted from gene edited protoplasts using the Macherey-Nagel, Inc (Allentown, PA) NucleoSpin Plant II Mini kit (Cat. No. 740770.50). PCR amplification using Phusion® High-Fidelity DNA Polymerase produced amplicons for Sanger and Illumina® next generation deep amplicon sequencing from Genewiz, Inc. Primers *CpMLO6\_sg254\_F1* (5'-GAGAGC TCTGTACGAATCACTTG-3') and *CpMLO6\_sg254\_R1* (5'-GCACATTTATCAGTAGAGGCA-3') amplified a 386 bp region spanning the *CpMLO6* sgRNA254 target site. Primers *CpPDS\_sg16\_F1* (5'-CCAGATAGACATTAC CCAGAATC-3') and *CpPDS\_sg16\_R1* (5'-GGATTCCT AACCCTAAATGC-3') amplified a 406 bp region spanning the *CpPDS* sgRNA16 target site. Amplicons were prepared according to Genewiz, Inc. sample submission guidelines for Amplicon-EZ® services. Amplicon-EZ results were processed using Geneious Prime software from Biomatters, Inc (Boston, MA). Illumina paired-end reads were paired and merged, and then processed using the Analyze CRISPR Editing Results tool. A minimum read frequency of 0.10% was applied, filtering mutant detection below the threshold. Mutants were screened against the WT amplicon to ensure they were exogenous to the WT genome.

## Results

**An Improved Method for Isolating High-Yield, Highly Viable Papaya Protoplasts** We initially focused on the protoplast isolation procedure to maximize the yield of viable mesophyll protoplasts. We examined the influence of leaf and plant age, the components of the isolation medium, enzyme concentration, incubation period, plant and leaf health, and vacuum infiltration during preliminary trials. The data shown here were obtained using the optimized conditions as described in the Methods. Plants were divided into two age categories: old ( $\geq 75$  d) and young ( $< 75$  d) (Fig. 1). The

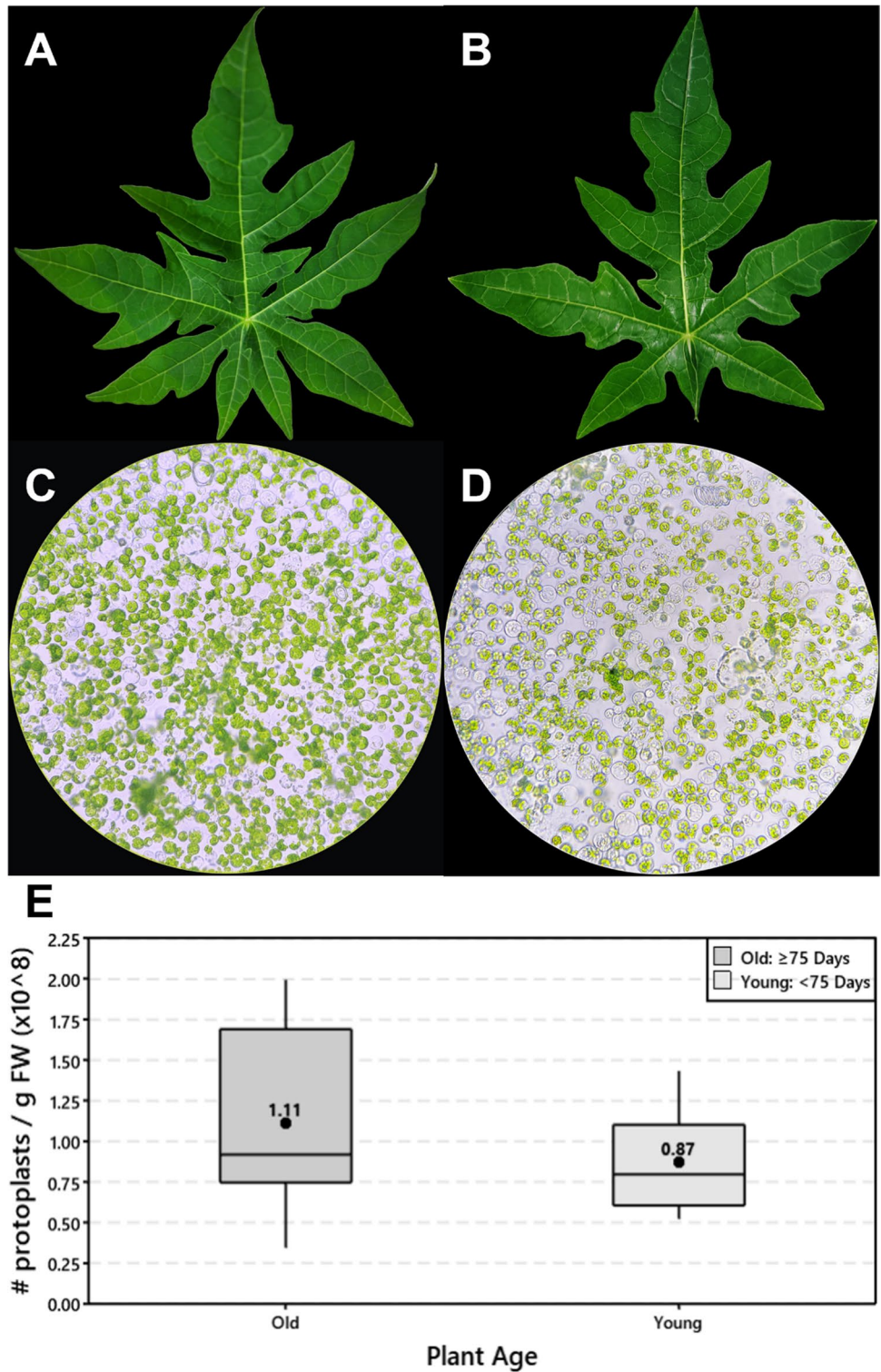
leaf tissue of old plants (Fig. 1A, C) provided an average yield of  $1.11 \times 10^8 \pm 0.069$  protoplasts per GFW (Fig. 1E), whereas the leaf tissue of young plants (Fig. 1B, D) yielded  $0.87 \times 10^8 \pm 0.079$  protoplasts per GFW (Fig. 1E). The yield differences between old and young plants were statistically significant, with  $t(37) = 2.28$ ,  $p = .028$ . Generally, both ages produced large numbers of intact protoplasts with minimal extraneous debris. However, protoplasts from older plants were more durable; they tolerated repeated centrifugation and washing steps without lysing during downstream procedures. Therefore, older plants were used for all subsequent protoplast isolations reported here. Interestingly, the leaf mesophyll protoplasts of papaya were relatively small at 15–25  $\mu\text{m}$ , compared to protoplasts from other plant species, such as *Arabidopsis*, which ranged from 30 to 50  $\mu\text{m}$  (Yoo *et al.* 2007).

**Freshly Isolated Papaya Protoplasts Maintain High Viability, as Assessed by the Fluorescein Diacetate Hydrolysis Assay** The viability of protoplasts was determined using fluorescein diacetate (FDA) staining (Larkin 1976). Immediately after protoplast isolation, protoplasts were treated with FDA and observed using bright field and epifluorescence microscopy (Fig. 2A, B). The viability of protoplasts from new leaf tissue of older plants was  $89.87 \pm 2.02\%$  (Fig. 2C). The consistently high percentage of viable protoplasts obtained here was used for downstream transfection and gene editing.

**An Optimized Method for Transient Transfection of Papaya Protoplasts Developed Using a Fluorescent Marker** We next focused on ensuring that isolated protoplasts could be genetically transfected to transiently express a reporter gene as a prerequisite for subsequent gene editing applications. The freshly isolated papaya protoplasts were transfected with plasmid DNA containing the 35S-eGFP construct. As expected, the successfully transfected protoplasts emitted green fluorescence throughout the cell (Cho *et al.* 2011) (Fig. 3A). No signal was detected from the untransfected protoplasts. Both transfected and non-transfected protoplasts were observed. The efficiency of protoplast transfection of the single construct was  $43.79 \pm 1.65\%$  (Fig. 3D). PEG concentrations ranging from 25 to 40% were used, with 40% providing optimal results. When using fragile protoplasts from young plants, 25% PEG minimized cell damage while maintaining transfection with reduced efficiency.

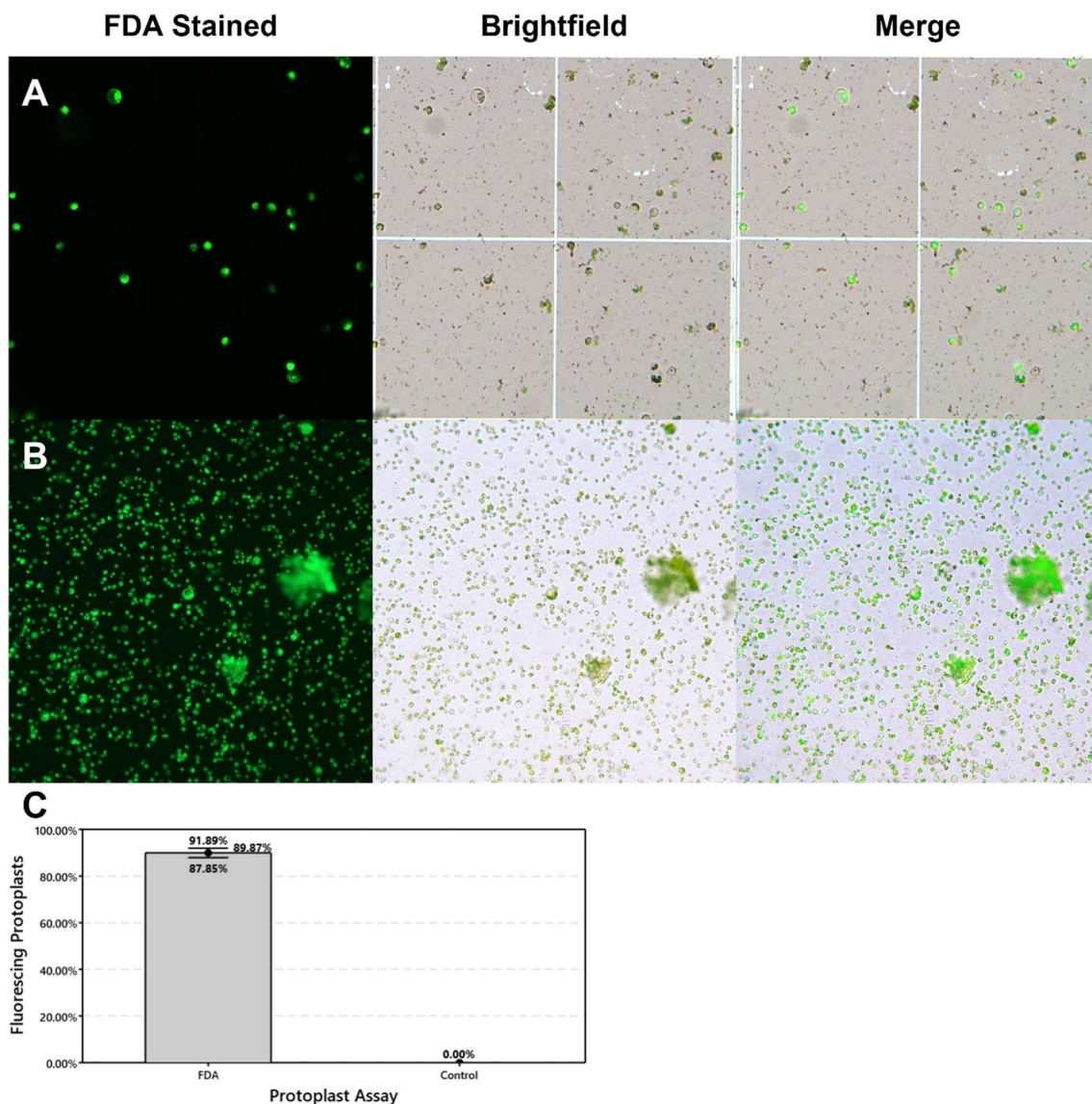
**Colocalization of the Fluorescent Fusion Proteins ER-mCherry and Arabidopsis PDI9-GFP Confirms Normal Cell Function in the Papaya Protoplasts** The ability to localize the resulting fusion proteins to the appropriate intracellular compartments was evaluated to verify normal cellular function and activity post-transfection. A fluorescent protein fusion, PDI9:eGFP

**Figure 1.** Enzymatic isolation of papaya leaf mesophyll protoplasts from old ( $\geq 75$  d) and young ( $< 75$  d) plants. (A) Representative leaf sample from an old plant and (B) a young plant. (C, D) Freshly isolated mesophyll protoplasts from old and young plants, respectively, viewed at 20 $\times$  magnification. (E) A two-sample *t*-test was used to evaluate mean protoplast yield per gm FW of leaf tissue. The *boxplot* represents quartiles of data points over 23 total replicates. The *horizontal lines* represent median values. Equal variances were not assumed.



plasmid, which contained the *Arabidopsis* protein disulfide isomerase 9 gene (*AtPDI9*) (Yuen *et al.* 2013), was delivered to protoplasts simultaneously with the plasmid containing the endoplasmic reticulum (ER) marker, ER-rk (Cho *et al.* 2011; Nelson *et al.* 2007). As in previous studies in *Arabidopsis* (Cho *et al.* 2011; Yuen *et al.* 2013), we observed

that the PDI9-GFP fusion was localized to the ER in papaya using confocal laser scanning microscopy (Fig. 3B). The analysis further showed that PDI9-GFP strongly colocalized with the ER-mCherry marker, indicating that PDI9-GFP is expressed and localized to the ER lumen in papaya (Fig. 3C). The overall percent efficiencies of transfection for individual



**Figure 2.** Fluorescein diacetate (FDA) viability assay of freshly isolated papaya mesophyll protoplasts. (A) Representative image of papaya protoplasts counted with a hemocytometer utilizing epifluorescence and brightfield microscopy merged into a single composite image. (B) Representative image of papaya protoplasts viewed on a

single-well concavity slide using epifluorescence and brightfield microscopy. Both images are merged into a single composite image and viewed at 10 $\times$  magnification. (C) An *interval plot* of average protoplast viability as determined by FDA fluorescence is shown. Papaya plants were  $\geq 75$  d old.

PDI9-GFP and ER-mCherry alone were  $46.21 \pm 1.22\%$ , which is similar to the efficiency of 35S:eGFP above. However, for double transfections with two different constructs, the efficiency decreased to  $27.33 \pm 1.83\%$ . In summary, plasmid DNA entered the nucleus of the host cell using the optimized transfection procedure, the reporter genes were properly expressed, and the resulting proteins were trafficked to the correct subcellular compartments. These results demonstrate proper cellular function post-transfection.

#### CRISPR-Cas9 Targeted Restoration of the GFP+1 Frame-Shift Mutation Using Preassembled

**Ribonucleoprotein Complexes** We then used the above-mentioned parameters for protoplast transfection to test whether the preassembled CRISPR-Cas9 RNP gene editing complex could perform targeted editing *in vivo*. The assay utilized a GFP mutant (GFPm), which contained a single nt frame-shift mutation that disrupted the reading frame for GFP mRNA, thereby preventing the formation of GFP protein and fluorescence, as previously described (Liu *et al.* 2018). Upon CRISPR-Cas9 editing of the mutation site, the error-prone NHEJ DNA repair mechanism corrects the reading frame, restoring GFP protein function and fluorescence, which is readily observable.



Papaya protoplasts were cotransfected with GFPm and Cas9 complexed with a sgRNA targeting the GFPm mutation site (Liu *et al.* 2018). The protoplasts containing the CRISPR-edited frame-shift of GFP that restored the correct reading frame emitted green fluorescence when analyzed under confocal laser scanning microscopy (Fig. 4A). Unedited protoplasts, or those that were edited but did not restore the correct reading frame, did not emit fluorescence (Fig. 4B). Three *in vivo* protoplast gene editing experiments were completed, resulting in a frame-shift restoration efficiency of  $27.88 \pm 1.65\%$  (Fig. 4C). The papaya-optimized PEG-mediated transient transfection method successfully introduced a CRISPR-Cas9 RNP-based gene editor that corrected the GFPm gene *in vivo*.

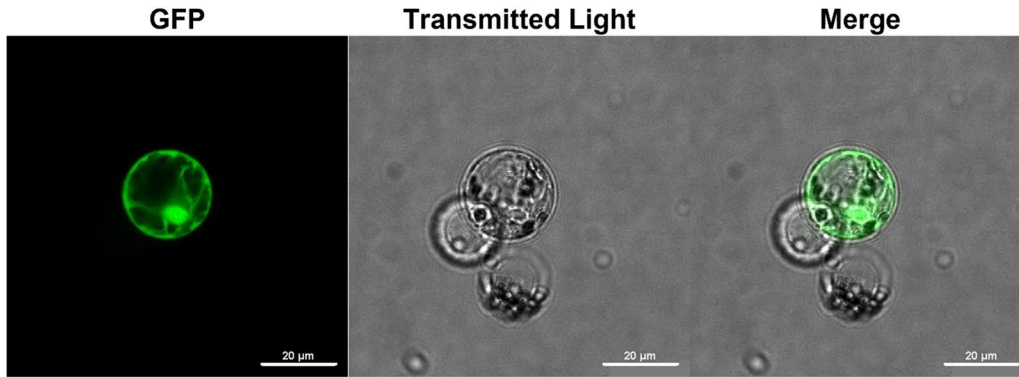
Immunoblot analysis was performed using a GFP-specific antibody to confirm and quantify the presence of the functional GFP in the protein extracts from transfected protoplasts. The frame-shift restored GFP protein was detected in the sample cotransfected with the GFPm plasmid and the Cas9-sgRNA RNP complex targeting the GFP+1 frame-shift mutation site (Fig. 4D). No observable bands were detected in the sample transfected with the empty vector control pUC19, or GFPm plasmid in the absence of Cas9-sgRNA RNP complex despite the equal loading of protein in each treatment (Fig. 4D, E). The results further confirmed that the proper editing of GFPm occurred, detecting functionally restored GFP protein in protoplasts.

**DNA-Free CRISPR-Cas9 Targeted Mutagenesis of Papaya Mildew Resistance Locus O-Like 6 and Phytoene Desaturase Gene** A critical next step in developing a DNA-free gene editing system was to target the papaya endogenous genes and edit them *in vivo*, producing knockout mutants. *CpPDS* (NCBI accession: XP\_021888908.1) and *CpMLO6* (NCBI accession: XP\_021909409.1) were selected to develop the DNA-free gene editing system. *CpPDS* was chosen because its mutation was expected to produce a readily observable albino phenotype (Arias *et al.* 2006), as described in banana (Kaur *et al.* 2018), tobacco (Lin *et al.* 2018), and rice (Wang *et al.* 2017). The *MLO6* locus was chosen to disrupt because the induced and naturally occurring *MLO* mutants display increased resistance to infection by powdery mildew-causing fungal pathogens (Opalski *et al.* 2004). In the papaya genome (Ming *et al.* 2008), 16 high-quality protein sequences were annotated as *MLO*-like proteins. We conducted a phylogenetic analysis of these sequences with representative *MLO* genes of Clade IV and Clade V from plants (Supplemental file 1), which contain known genes involved in susceptibility to powdery mildew in monocots and dicots, respectively (Pessina *et al.* 2014). We found that *CpMLO6* was clustered with Clade V *MLOs* (Supplemental file 1), suggesting that *CpMLO6* is a candidate S-gene *MLO* homolog in papaya and an ideal target for genome editing

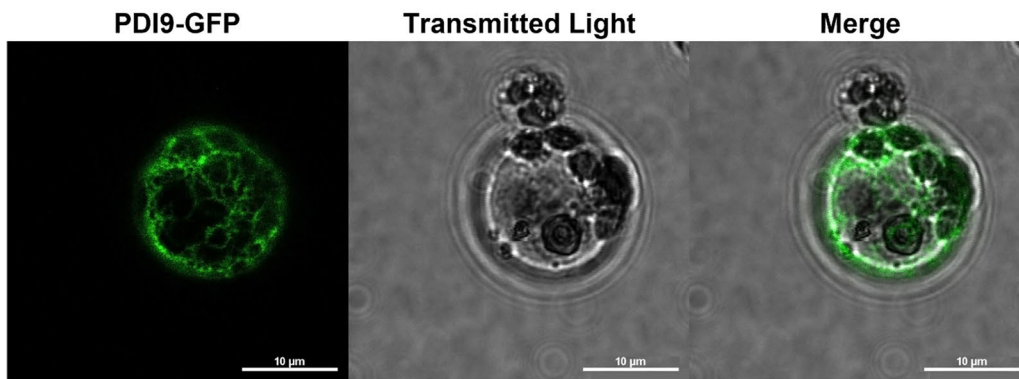
to disrupt susceptibility. For each gene, a functional sgRNA was selected based on the analyses of on- and off-targets in the genome, gene editing efficiency score, GC content, and predicted secondary structure (Supplemental file 2), followed by *in vitro* DNA cleavage assays (Supplemental file 3). *CpPDS*-sgRNA16 (20nt target sequence: 5'-AGTGTT TCTGCGGCGAGCTT-3') and *CpMLO6*-sgRNA254 (20nt target sequence: 5'-CGAAGTCAATTGGAGCCACG-3') targeting *CpPDS* and *CpMLO6*, respectively, were able to successfully guide the cleavage of the target DNA.

To specifically edit the papaya *CpPDS* gene using an RNP containing *CpPDS*-sgRNA16 and Cas9, two replicates were performed in papaya cv. Solo Sunrise, which produced an average mutant frequency of 45.09% (Fig. 5A). 32.08% of the generated mutants resulted in frame-shift knockouts, while in-frame protein variants represented 13.01% (Fig. 5A). Of the 32.08% knockout mutants, 19.80% consisted of premature stop codons (Fig. 5A). Indel types ranged from +5 to -13 bp (Fig. 5B). The deletion of -3 bp occurred with the highest frequency at 10.44%, followed by the addition of +1 bp at 9.51%, deletion of -1 bp at 8.66%, and deletions of -4 bp at 6.08% and -6 bp at 3.45% (Fig. 5B). The specific types of mutations detected from next generation sequencing (NGS) data targeting *CpPDS* in cv. Solo Sunrise are provided (Tables 1, 2, Supplemental file 4). This experiment was repeated on two other papaya cultivars, Sunset and Kapoho. In cv. Solo Sunset, a mutant frequency of 37.22% was produced, with 28.24% being frame-shift knockout mutants and 8.73% being in-frame protein variants. Of the total frame-shift mutants detected, 17.81% produced premature stop codons (Fig. 5C). Indel types ranged from +1 to -64 bp, with the deletion of -1 bp occurring with the highest frequency at 11.35% followed by the addition of +1 bp at 7.95% and deletions of -3, -4, and -2 bp at 6.89%, 5.34%, and 1.74%, respectively (Fig. 5D). Targeting *CpPDS* in papaya cv. Solo Kapoho produced a mutant frequency of 41.81%. Frame-shift mutants accounted for 32.36% of the total mutants detected, while 9.46% were in-frame protein variants (Fig. 5E). 20.81% of frame-shift knockout mutants were resultant of premature stop codons (Fig. 5E). Indel types ranged from +5 to -53 bp (Fig. 5F). The indel type produced with the highest frequency was the deletion of -1 bp at 12.99% followed by the addition of +1 bp at 7.98% and the deletions of -4, -3, and -6 bp at 6.09%, 5.98%, and 2.54%, respectively (Fig. 5F). More detailed results obtained targeting *CpPDS* in cv. Solo Sunset and Kapoho are provided (Tables 3, 4, Supplemental file 4). The average mutant frequency obtained targeting *CpPDS* in all three cultivars was  $42.31 \pm 1.90\%$  (Supplemental file 5).  $31.25 \pm 1.46\%$  of the generated mutants were frame-shift knockouts, while in-frame protein variants accounted for  $11.05 \pm 1.37\%$  (Supplemental file 5). Of the  $31.25 \pm 1.46\%$  frame-shift knockout mutants detected,  $19.56 \pm 1.64\%$  generated premature stop codons.

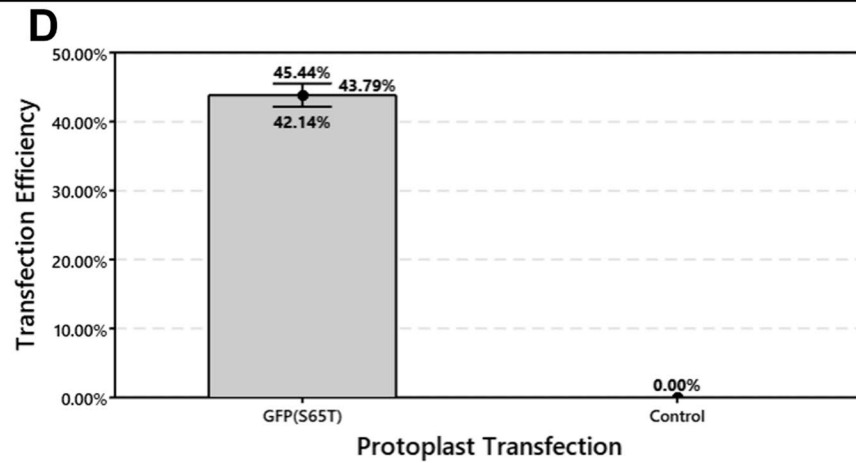
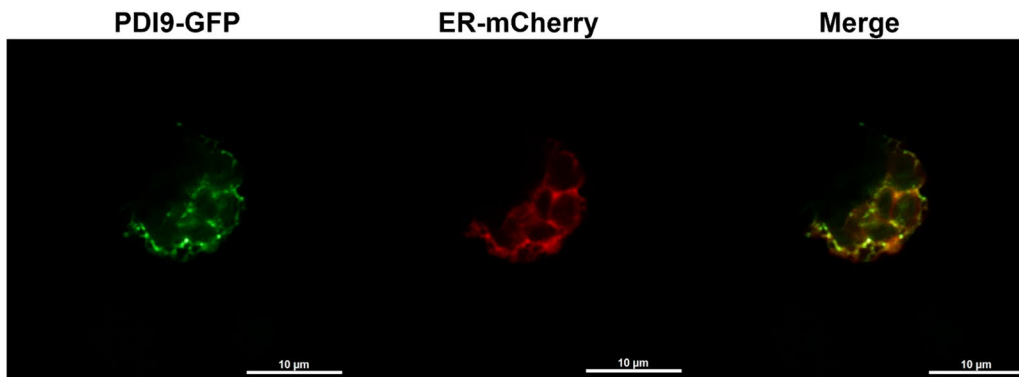
## A GFP Transfection



## B PDI9-GFP



## C PDI9-GFP ER-mCherry



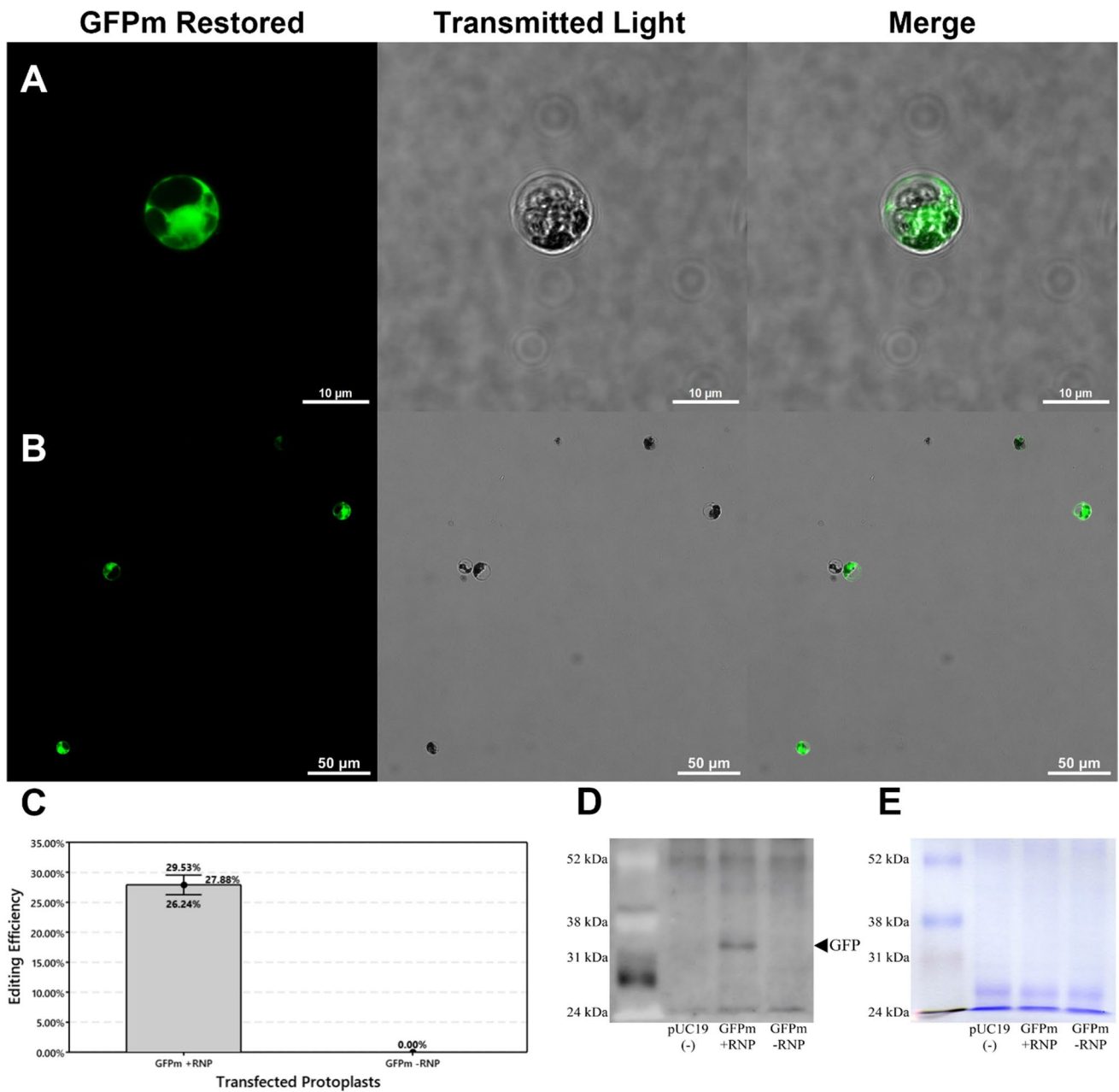
**Figure 3.** Confocal laser scanning microscopy analysis of transfected papaya mesophyll protoplasts. (A) Transient transfection of the protoplasts with the fluorescent marker GFP(S65T). The fluorescent signal is detected throughout the cell as expected. The protoplasts were imaged using excitation/emission spectra of 488/505–525 nm. (B) Transient transfection of the protoplasts with the fluorescent protein fusion PDI9-GFP, observed localized to the ER as imaged using the excitation/emission spectra, 488/505–525 nm. (C) Cotransfection of the protoplasts with fluorescent protein fusion constructs PDI9-GFP and ER-mCherry (the ER marker Er-rk) as imaged using the excitation/emission spectra of 488/505–525 nm and 543/585–615 nm, respectively. (D) Interval plot of mean transfection efficiency using GFP(S65T). Data represents a triplicate of experiments. Interval bars represent one standard error from the mean.

A CRISPR-Cas9 RNP targeting the papaya locus, *CpMLO6*, using *CpMLO6*-sgRNA254, performed gene editing at the specified site in papaya cv. Solo Sunrise producing an average mutant frequency of 18.84% (Fig. 6A). 16.54% of the generated mutants resulted in frame-shift knockouts, while 2.30% were in-frame protein variants (Fig. 6A). The indel types ranged from +2 to –15 bp (Fig. 6B). The deletion of –1 bp occurred with the highest frequency at 6.96%, followed by the addition of +1 bp at 4.40%, deletion of –2 bp at 1.91%, deletion of –3 bp at 1.70%, and deletion of –4 bp at 1.29% (Fig. 6B). Total mutants detected from the NGS data obtained targeting *CpMLO6* in cv. Solo Sunrise is provided (Tables 5, 6, Supplemental file 6). This process was repeated, targeting *CpMLO6* in cv. Solo Sunset and Kapoho. In cv. Solo Sunset, a mutant frequency of 13.60% was observed, with frame-shift mutants representing 10.37% of the total mutations detected, while 3.23% were in-frame protein variants (Fig. 6C). Detected indels ranged from +1 to –10 bp with the deletion of –1 bp observed at the highest frequency, followed closely by the addition of +1 bp at 3.69% and the deletion of –3 bp at 2.64% (Fig. 6D). While targeting *CpMLO6* in Solo Kapoho, a mutant frequency of 13.52% was produced (Fig. 6E). As a proportion of total mutants, 11.36% were frame-shift knockouts, while 2.16% were in-frame protein variants (Fig. 6E). Indels ranged from +1 to –10 bp with the deletion of –1 bp occurring with the highest frequency, followed by the addition of +1 bp at 2.47%, deletion of –3 bp at 1.19%, and deletion of –4 bp at 1.03% (Fig. 6F). Detailed results obtained targeting *CpMLO6* in cv. Solo Sunset and Kapoho are provided (Tables 7, 8, Supplemental file 6). The average mutant frequency obtained targeting *CpMLO6* in all three cultivars was  $16.20 \pm 1.53\%$  (Supplemental file 5).  $13.71 \pm 1.67\%$  of the generated mutants were frame-shift knockouts, while  $2.50 \pm 0.26\%$  were in-frame protein variants (Supplemental file 5).

## Discussion

**The Yield for Isolating Durable, Viable Papaya Leaf Mesophyll Protoplasts Has Substantially Improved** Protoplasts play an important role in plant research (Cocking 1972) with studies of protoplast fusion (Keller and Melchers 1973; Kao and Michayluk 1974; Zimmermann and Scheurich 1981) and hybridization (Kao *et al.* 1974; ; Melchers *et al.* 1978; Evans 1983; Dudits *et al.* 1987; Sihachakr *et al.* 1988), cell wall regeneration and cell division (Nagata and Takebe 1970; Kao *et al.* 1970; Vasil and Vasil 1972), nucleic acid delivery (Takebe and Otsuki 1969), cell signaling (Jang and Sheen 1994; Sheen 1996), stable transformation (Krens *et al.* 1983; Paszkowski *et al.* 1984), and transient transfection (Negrutiu *et al.* 1990; Abel and Theologis 1994). These early reports provide the basis for developing new uses of protoplasts, such as defining subcellular localizations of proteins (Yuen *et al.* 2013) and protein-protein interactions (Carrillo and Christopher 2022). Continuing along this line, we theorized that protoplasts offer an effective experimental system for gene editing of papaya. We demonstrated an improved method for isolating papaya mesophyll protoplasts for use in a DNA-free targeted gene editing system.

The results collected in 23 isolations reflect a 7-fold increase in the average protoplast yield of  $1.11 \times 10^8 \pm 0.069$  (Fig. 1E) compared to the previously published optimal protoplast yield of  $1.56 \times 10^7 \pm 0.100$  (Zhang *et al.* 2011). This increase in yield may be due to morphological or physiological differences between cultivars, or the extensive effort placed here in optimizing the protoplast isolation media. These optimizations include adjustments to enzyme concentration, incubation duration, temperature, pH, and osmolarity (Wu *et al.* 2017; Sangra *et al.* 2019; Adedeji *et al.* 2020; Xu *et al.* 2021). Plant tissues have been processed uniquely to remove the sometimes difficult to penetrate epidermal layer (Murata *et al.* 1994; Locatelli *et al.* 2003; Wu *et al.* 2009; Yuen *et al.* 2016). Vacuum infiltration is also widely used to enhance the removal of the formidable waxy cuticle on leaves (Newell and Luu 1985; Yoo *et al.* 2007; Yuen *et al.* 2013; Nanjareddy *et al.* 2016). The use of a cyclical vacuum infiltration and release method is believed to have improved protoplast yields. When the vacuum infiltration step was removed, the protoplast isolation failed. Additionally, when protoplasts were isolated under constant vacuum, protoplast yields were reduced. The cyclical vacuum infiltration method was critical for obtaining optimal results. Our papaya-optimized protoplast isolation method produces a significantly improved yield of highly viable and durable protoplasts suitable for research regardless of leaf age. However, protoplasts isolated from older plants

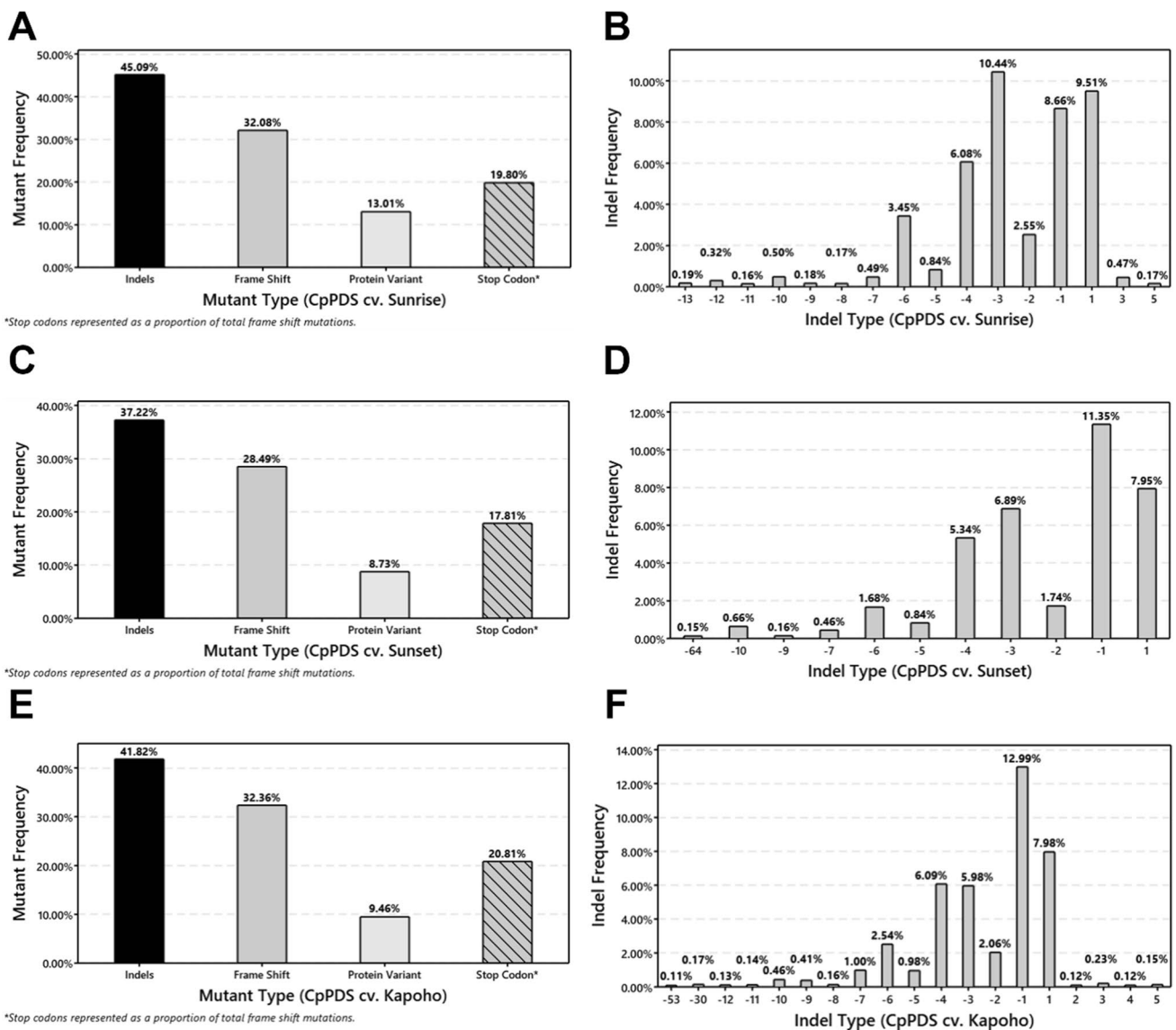


**Figure 4.** CRISPR-Cas9 RNP targeted gene editing and restoration of the GFP+1 (GFPm) frame-shift mutation *in vivo* in papaya mesophyll protoplasts. (A, B) Representative images of the reading frame corrected GFPm visualized using confocal laser scanning microscopy with the excitation/emission spectra, 488/505–525 nm. The untransfected, unedited, and non-frame-shift restored protoplasts do not emit a fluorescent signal. (C) An *interval plot* of mutant editing efficiency used protoplasts cotransfected with GFPm and a RNP complex targeting the GFP mutation site (+RNP) and without (-RNP). The data

represents triplicate experiments. (D) Immunoblot detection assay of the frame-shift restored GFP protein using a GFP-specific antiserum on protoplast protein lysates. The *arrowhead* denotes a GFP band in the GFPm+RNP lane only. Protoplasts transfected with the pUC19 empty vector (no RNP) and plasmid with GFPm with (+) and without (-) RNP. (E) A corresponding Coomassie-stained protein gel is shown as loading control of protoplast protein lysates. Protein markers are shown in kDa.

were more durable in downstream steps and are thus recommended. The resulting viability of the protoplasts was  $89.87 \pm 2.02\%$  (Fig. 2C), which is similar to the previously reported viability at  $89.96 \pm 2.89\%$  (Zhang *et al.* 2011). In summary, the optimal conditions for obtaining

viable papaya leaf mesophyll protoplast yields are 1.3% cellulase, 0.3% macerozyme, 0.44M mannitol, pH 5.8, 30-min vacuum infiltration with vacuum release every 5 min, and 13–15-h incubation period at 26°C and 60 rpm in the dark.



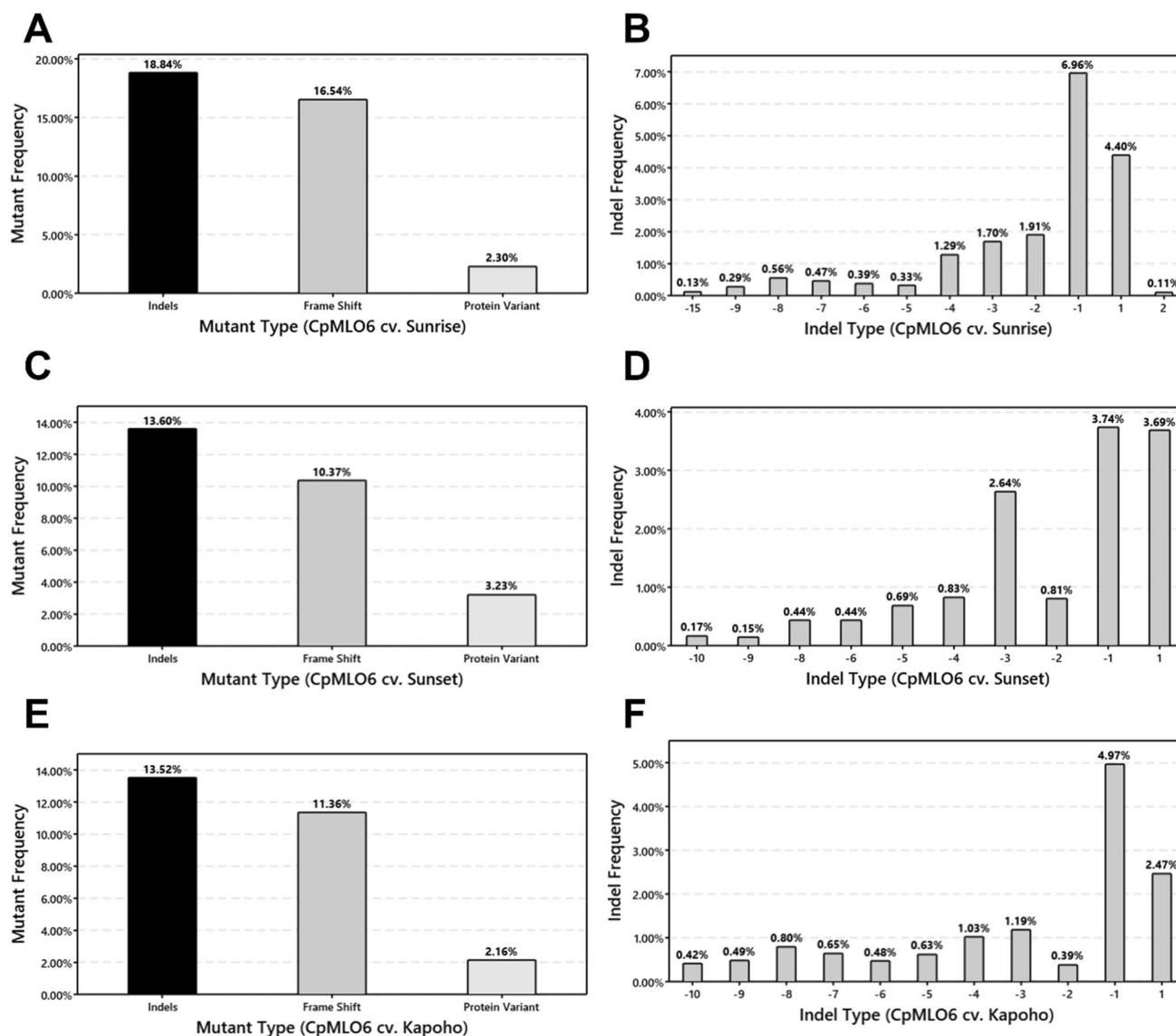
**Figure 5.** The measurement of DNA-free CRISPR-Cas9 gene editing efficiency in papaya protoplasts targeting the *CpPDS* gene as confirmed by NGS. The bar chart representations of total *CpPDS* CRISPR-Cas9 gene editing efficiency (indels—black) and proportion of editing events leading to frame-shift knockouts (gray) and in-frame protein variants (light gray). Premature stop codons are presented as a

proportion of total frame-shift knockouts (striped). The summary of mutants (A) and indels (B) generated for the *CpPDS* gene from the cv. Solo Sunrise. The summary of mutants (C) and indels (D) generated for the *CpPDS* gene from the cv. Solo Sunset. The summary of mutants (E) and indels (F) generated for the *CpPDS* gene from the cv. Solo Kapoho.

**PEG-Mediated Transfection and Subcellular Localization of Fluorescent Fusion Proteins Were Optimized in Papaya Mesophyll Protoplasts** We used the eGFP reporter construct GFP(S65T) (Chiu *et al.* 1996) by itself and as the PDI9-GFP fusion protein for the following purposes: (a) To develop an efficient method for transient transfection of the protoplasts; (b) To verify proper GFP gene expression *in vivo* as an additional criterion for protoplast viability; (c) To confirm that transiently expressed proteins are properly targeted to subcellular compartments as indicators of preserved normal

cellular functions. These results provided the foundation for subsequent gene editing experiments.

First, an optimized PEG-mediated method for transfection of protoplasts with reporter gene-plasmids was established for papaya. The fluorescent signal observed in transfected protoplasts confirmed plasmid uptake and viable expression of the construct (Fig. 3A). The efficiency of the PEG-mediated transient transfection method of 44%, reported here (Fig. 3D), is similar to or above the transfection efficiencies



**Figure 6.** The measurement of DNA-free CRISPR-Cas9 gene editing efficiency in papaya protoplasts targeting the *CpMLO6* gene as confirmed by NGS. Bar chart representations of total *CpMLO6* CRISPR-Cas9 gene editing efficiencies (indels—black) and proportions of editing events leading to frame-shift knockouts (gray) and in-frame

protein variants (light gray). The summary of mutants (A) and indels (B) generated for the *CpMLO6* gene from cv. Solo Sunrise. The summary of mutants (C) and indels (D) generated for the *CpMLO6* gene from the cv. Solo Sunset. The summary of mutants (E) and indels (F) generated for the *CpMLO6* gene from the cv. Solo Kapoho.

reported for some plant species, including potato at 49% (Konovalova *et al.* 2021), orchid hybrid ‘Ruili Beauty’ at 42% (Li *et al.* 2018), sugarcane at 41% (Wu *et al.* 2021), and cannabis at 23% (Matchett-Oates *et al.* 2021). Although efficiency results are below those reported for other plant species, including cassava at 71% (Wu *et al.* 2017), cabbage at 68% (Sivanandhan *et al.* 2021), cucumber at 57% (Huang *et al.* 2013), and the well-established *Arabidopsis* protoplast transfection method at 60–90% (Yoo *et al.* 2007). Interestingly, increased PEG concentrations and incubation times in the referenced plant species negatively affected transfection efficiency in some species, while positively affecting others,

indicating that PEG-mediated protoplast transfection optimization is highly species-dependent. Although the efficiency of our papaya-optimized transfection method is less than 50%, as required for some applications (Yoo *et al.* 2007), it falls within the range of results reported for a diverse range of plant species.

Second, the *Arabidopsis PDI9* gene encodes a protein folding chaperone of the ER secretory pathway (Yuen *et al.* 2013). We used cotransfection of *PDI9:eGFP* with the ER marker, ER:mCherry, to demonstrate that *PDI9-GFP* was clearly and correctly localized to the ER in papaya protoplasts as part of the secretory pathway (Fig. 3B). These

results confirmed normal cell function and the ability of transfected protoplasts to viably express constructs delivered using our papaya-optimized PEG-mediated transfection method (Fig. 3C).

**The CRISPR-Cas9 RNP-Based Gene Editing System in Papaya Corrected the GFP+1 Frame-Shift Mutation** The transient reporter assay used here containing the mutant frame-shift GFP (Liu *et al.* 2018) proved to be very effective in testing the competence of gene editing of papaya protoplasts. A CRISPR-Cas9 gene editing RNP complexed with a sgRNA (designed to target the GFPm mutation site) was able to edit the GFPm gene in the plasmid construct. A diverse range of indel mutations was introduced into the repaired GFPm sequence, some of which corrected the frame-shift and restored the wild-type (WT) GFP phenotype (Fig. 4A, B). The editing was confirmed using immunoblot analysis with a GFP-specific antibody to detect the fully translated GFP polypeptide.

The resulting GFPm editing efficiency of  $27.88 \pm 1.65\%$  (Fig. 4C) was higher than the 3.3% reported by Liu *et al.* (2018). Significant differences existed between the methods used to introduce the CRISPR-Cas9 complexes into protoplasts. In switchgrass, protoplasts were cotransfected with a construct carrying a gene editing cassette and a separate construct carrying GFPm. Both constructs must be expressed and available in sufficient quantities to form complexes in the cell to edit the gene (Dewitt *et al.* 2017). In contrast, our system used the CRISPR-Cas9 complexed with the guide RNA and no plasmid expression of the editing components was needed.

From days 1 to 3, protoplast viability was reduced from 90 to ~40% when suspended in a mannitol-based solution, as reported in other plants (Poddar *et al.* 2020). The window of viable expression is not a significant concern using an RNP-based gene editing system, such as used here, which can perform targeted edits almost immediately upon entry into the cell (Kim *et al.* 2014). We believe this RNP-based system positively influenced the increased GFPm editing efficiency observed in papaya. Developing these parameters by using the *in vitro* complexed CRISPR-Cas9 RNP editor acting on a GFPm plasmid was a prerequisite to targeting genes of interest in the papaya genome.

**The DNA-Free CRISPR-Cas9 RNP-Based System Successfully Edited Two Endogenous Genes, *CpPDS* and *CpMLO6*, of the Papaya Genome** The highest gene editing efficiencies for *CpPDS* and *CpMLO6* reported here (46% and 19.15%, respectively (Figs. 5C and 6C), were similar to or above those reported for other plants when evaluated under similar conditions. For example, the *PDS* editing efficiencies were 0%, 1.33%, and 24.51% in oilseed rape, cabbage, and

Chinese cabbage, respectively (Murovec *et al.* 2018), 1.8% in cabbage (Lee *et al.* 2020), and 0.92% in Cavendish banana (Wu *et al.* 2020). Results targeting *MLO*-like homologs were 11.3% in sweet pepper (Kim *et al.* 2020) and 0.1% in grapevine (Malnoy *et al.* 2016). The efficiency of RNP-based editing of various genes in plant protoplasts is highly variable between plant species and between gene targets (Zhang, *et al.* 2021). Such efficiencies include 60% in tomato (Lin *et al.* 2022), 2% in wild cabbage (Park *et al.* 2019), 9% in potato (Andersson *et al.* 2018), 45% in bread wheat (Liang *et al.* 2017), 7% in apple (Malnoy *et al.* 2016), and 44%, 19%, and 16% in tobacco, rice, and Arabidopsis, respectively (Woo *et al.* 2015). As described here, both mutant *CpPDS* and *CpMLO6* produced indels in protoplasts at rates similar to or above those of other plant species. Results obtained while targeting *CpPDS* and *CpMLO6* in the papaya cultivars Solo Sunrise, Sunset, and Kapoho were similar and consistent for their respective genes, with Sunrise consistently producing slightly higher editing efficiencies, while Sunset and Kapoho produced slightly reduced efficiencies. Therefore, an effective DNA-free gene editing system was established here to target the genome of papaya protoplasts, and the next step is the regeneration of these edited cells into whole plants.

## Conclusions

This study used DNA-free CRISPR-Cas9 ribonucleoprotein complexes for gene editing in papaya protoplasts. The long-term goal is to enhance disease resistance for crop improvement. We first optimized the isolation of intact viable protoplasts from papaya leaves of various ages, providing a consistent and reliable source of plant material for gene editing experiments. Next, we tested the utility of the DNA-free CRISPR-Cas9 RNP gene editing system by correcting a frame-shift mutant GFP transgene, providing evidence of the method's effectiveness. This approach involved preassembling Cas9 endonuclease with single-guide RNA complexes *in vivo*, then transfecting the protoplasts using PEG-mediated transfection. We then successfully employed this gene editing method to target the endogenous papaya genes, *CpPDS* and *CpMLO6*. We confirmed targeted editing of the endogenous papaya genes *CpPDS* and *CpMLO6* through deep amplicon sequence verification. This transgene-free method eliminates the introduction of transgenic material into the host genome, reducing regulatory concerns and providing a more rapid and efficient alternative for papaya improvement. Furthermore, several advantages over traditional gene editing methods are realized, such as reduced off-target effects, decreased Cas9 transcription and translation time, and elimination of insertion mutations associated with stable transformation. The next

step is to develop a regeneration protocol for the protoplasts, from different tissue sources, containing the edited *CpPDS* and *CpMLO6* genes. The successful application of this method in papaya paves the way for future research and development to improve crop resistance to pathogens and other environmental threats.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s11627-023-10373-1>.

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