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Protoplast: A more efficient system to study nucleo-cytoplasmic interactions

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ABSTRACT

Agrobacterium tumefaciens-mediated genetic transformation is a powerful tool for plant research, but it can be labor-intensive and time-consuming. Here, we report a protoplast-based approach to study nucleo-cytoplasmic interactions, such as cytoplasmic male sterility/fertility restoration (CMS/Rf) and organellar RNA editing. To test the system, we transfected the fertility restorer gene *Rf5*, which is involved in the rice HL-CMS/Rf system, into rice protoplasts prepared from the HL-CMS line. As the Rf5 protein accumulated in the transformed protoplasts, the CMS-associated transcripts were endonucleolytically cleaved. There were much lower levels of the CMS-associated protein ORFH79 in the transfected protoplasts than in the mock-transfected protoplasts. Next, we used a dsRNA-mediated gene silencing approach to down-regulate the pentatricopeptide protein gene *MPR25*, which participates in RNA editing of the organellar transcript *nad5*. The editing efficiency of mitochondrial transcripts of *nad5* at nucleotide 1580 was much lower in the transfected protoplasts than in the mock-transfected protoplasts. Together, these results show that protoplast is a simple and efficient system to study interactions between the nucleus and organelles.

1. Introduction

Genetic transformation is a powerful tool to functionally characterize a gene of interest through up- or down-regulating its expression. In plants, the most commonly used approach for genetic transformation is *Agrobacterium tumefaciens*-mediated transformation, because of its relatively high transformation efficiency [1]. However, this method is labor-intensive and time-consuming, and there is a risk of microbial/ fungal contamination during the callus culture stage. Another promising approach is protoplast-based genetic transformation. This method can be used in diverse areas of plant cell biochemistry and physiology research, including research on cell wall synthesis, cell division, embryogenesis, and dedifferentiation [2]. Compared with stable genetic transformation, protoplast-based genetic transformation represents a faster, less expensive, and more efficient tool for plant biology research.

The antagonism between cytoplasmic male sterility (CMS) and fertility restoration (Rf) in plants is a classic example of a nucleo-cytoplasmic interaction. CMS is defined as the maternally inherited inability to produce functional pollen (male gametes), whereas the female gametes are viable. A previous study revealed that mitochondrial defects are responsible for all of the CMSs characterized so far. It is thought that aberrant mitochondria give a retrograde signal to the nucleus and trigger the programmed cell death (PCD). However, the CMS phenotype can be counteracted by a class of nuclear-encoded fertility restorer (Rf) genes [3]. In China, there are three widely distributed CMS rice varieties: "Honglian" (HL-CMS), "Wild Abortive" (WA-CMS), and "Chinsurah Boro II/Taichung native 65" (BT-CMS). These three CMS lines show differences in inheritance patterns, in the morphology of abortive pollen, and in their restoration-maintenance relationships [4–6]. The HL-CMS rice was selected by backcrossing red-awned wild rice (*Oryza rufipogon*) with the *indica* variety Lian Tangzao in the 1970s. At the molecular level, the HL-CMS is caused by an abnormal open reading frame (*orfH79*), which is chimeric to *atp6*. The product of *orfH79* impairs mitochondrial function via interacting with a subunit of electron transport chain complex III [7]. A recent study has shown that the product of the nuclear-encoded fertility restorer gene *Rf5* forms a complex with the glycine-rich protein GRP162. The complex catalyzes the cleavage of the CMS-associated transcripts *atp6-orfH79* and *orfH79(s)*. Ultimately, this suppresses the expression of *orfH79* [4]. The function of mitochondria has been shown to recover in the absence of the detrimental peptide ORFH79.

Organellar RNA editing is another typical example of a nucleo-cytoplasmic interaction in higher plants. After transcription,

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specific cytidines in organellar RNAs are modified to uridines. This generally causes the coding information to differ from that predicted from the DNA sequence [8]. Research using forward and reverse genetics approaches has revealed that nuclear-encoded pentatricopeptide repeat (PPR) proteins play important roles in RNA editing in plastids and mitochondria. The PPR proteins are characterized by a tandem array of a degenerate 35-amino-acid repeat. There is a very large family of genes encoding these proteins in land plants. This gene family has 450 members in *Arabidopsis* and 477 members in rice [9,10]. PPR proteins are almost targeted to organelles and are involved in various organellar RNA metabolism, including RNA editing [11–14], RNA splicing [15,16], RNA processing [17–19], RNA stabilization [20], and translational activation [21,22]. According to the features of their C terminus, members of the PPR protein family can be classified into three groups: E, E+, and DWY [23]. A previous study showed that in rice, *MPR25*, which encodes an E-type PPR protein, is essential for editing at nucleotide 1580 of *nad5* transcripts in the mitochondria [24]. Mutation of *MPR25* failed to modify the editing site, and resulted in growth retardation and pale green leaves at the early stages of plant development. Therefore, organellar RNA editing is one of the critical nucleo-cytoplasmic interactions.

To develop a fast, simple, and more efficient system, we explored whether protoplasts could be used to study nucleo-cytoplasmic interactions. Here, we focused on genes with roles in nucleo-cytoplasmic interactions, such as the CMS/Rf system or organellar RNA editing. Overexpression of a fertility restorer gene in protoplasts derived from a cytoplasmic male sterile (CMS) line could result in the endonucleolytic cleavage of the CMS-associated transcripts and suppression of CMS gene expression in the mitochondria. Also, silencing of the RNA editing factor *MPR25* in rice protoplasts resulted in changes in organellar *nad5* RNA editing. Taken together, these results suggest that the nucleo-cytoplasmic interactions that normally occur in protoplasts can be investigated using a simple and convenient transfection assay. Therefore, protoplast represents a simple, rapid, inexpensive, and efficient system to study interactions between nucleus and organelles.

2. Materials and methods

2.1. Plant materials and growth conditions

Dehusked rice seeds of the HL-CMS line (YtA) or Nipponbare were sterilized in 75% ethanol for 30 s, then in 0.15% $Hgcl_2$ for 15 min. Finally, the seeds were washed with sterile water and sown on ½ MS medium to germinate. The rice plants were grown in the dark at 28 °C for 2 weeks.

2.2. Rice protoplast isolation and transfection

Rice protoplasts were isolated as described previously, with some modifications. The middle region of young seedlings was cut into 0.5 mm pieces using a fresh razor blade. The pieces of plant tissue were submerged in 10 mL enzyme solution [10 mM MES, pH 5.7, 0.6 M mannitol, 0.1% BSA, 1.5% (w/v) cellulase R10 (Yakult Honsha, Tokyo, Japan), 0.75% (w/v) macerozyme R10 (Yakult Honsha), 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 0.25 g/ml carbenicillin]. The strips were vacuum infiltrated for 20 min and then incubated for 4 or 5 h in the dark at 28 °C with gentle shaking at 80 rpm. After incubation, the enzyme/protoplast solution was filtered through a 35-μm nylon mesh. The filtered solution was centrifuged in a round-bottomed tube for 5 min at 100*g* to pellet the protoplasts. The protoplasts were resuspended in 10 mL cool W5 solution (2 mM MES, pH 5.8, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 5 mM glucose) and then centrifuged at 100*g* for 5 min. The cells were resuspended in 1 mL MMG solution (4 mM MES, pH 5.6, 0.6 M mannitol, and 15 mM $MgCl_2$) to a final concentration of 2×10^5 cells/ml.

For transformation, 100 μl protoplast solution was gently and thoroughly mixed with 10 μl plasmid DNA solution (containing 10–20 μg plasmid DNA/dsRNA) and 110 μl PEG solution (40% w/v PEG-4000, 0.6 M mannitol, 100 mM $CaCl_2$). The mixture was incubated for 15–25 min at room temperature, and then the cells were washed once in 440 μl W5 solution. The protoplasts were incubated in 1 mL WI solution (4 mM MES, pH 5.7, 0.6 M mannitol, 20 mM KCl) in the dark for 12–48 h. The protoplasts were harvested and stored at −80 °C until use.

2.3. Cell viability assessment

Evans blue dye was added to the protoplasts in W5 solution to a final concentration of 0.1%. After incubation for 10 min at room temperature, the protoplasts were collected and washed three times in W5 solution. Then, the viability of protoplasts was assessed by light microscopy.

2.4. Plasmid construction

The *Rf5* cDNA coding region was amplified, and the correct amplicon was cloned into a pUC18-based vector with a GFP tag under the control of the UBI (*Zea mays* ubiquitin) promoter.

2.5. In vitro synthesis of double-stranded dsRNA

The DNA templates were generated by PCR using primers containing the T7 promoter sequence at both ends. Then, the dsRNAs were produced by *in vitro* transcription (TranscriptAid T7 High Yield Transcription Kit, Waltham, MA USA). RNA was purified by phenol–chloroform extraction, precipitated with ethanol, and then dissolved in nuclease-free water.

2.6. Quantitative real time PCR analyses

Briefly, 1 μg total RNA was digested by DNaseI (Invitrogen, Carlsbad, CA, USA) and then used for first-stand cDNA synthesis using a SuperScript III Reverse Transcriptase kit (Invitrogen), according to the manufacturer's instructions. Quantitative real time (qRT)-PCR was performed using a LightCycler 480 detection system (Roche, Basel, Switzerland) using an SYBR Green kit (Roche). Primers for qRT-PCR analysis of CMS or *MPR25* transcripts are listed in Supplementary Table S1. The rice *Actin1* gene (LOC_Os03g50890) was used as an internal reference to normalize the transcript levels of tested genes. Three biological repeats were analyzed.

2.7. Protein extraction and Western-blot analysis

The rice protoplast pellet was resuspended in lysis buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EGTA, 5 mM EDTA, 10 mM DTT, and 0.5% [v/v] Triton X-100). Proteins were separated by 10% SDS–PAGE and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA). Primary antibodies were used at the following dilutions: anti-GFP 1:2000; anti-ORFH79 1:3000. Secondary antibody goat anti-mouse IgG was used at a 1:2000 dilution. Signals were visualized by chemiluminescence (Bio-Rad).

2.8. Analysis of RNA editing site

Total RNA was isolated using TRIZOL reagent (Invitrogen) and treated with DNaseI (Invitrogen). The DNA-free RNA was reverse transcribed using random primers and high-fidelity reverse

transcriptase SuperScript III (Invitrogen). The fragment of *nad5* containing the editing site was amplified by RT-PCR and then directly sequenced. Three biological replicates were analyzed.

3. Results

3.1. Isolation of rice protoplasts and protoplast transfection efficiency

Preparation of high-quality rice protoplasts is essential for successful transformation and subsequent procedures and analyses. Firstly, it is important to prepare protoplasts from fresh, well-grown seedlings. We usually used 2-week-old rice seedlings germinated on ½ MS medium. These seedlings were superior to those cultivated on wet gauze and produced more protoplasts with a large, round shape. Secondly, the middle region of the seedlings, rather than the whole seedlings, gave the best results (Fig. 1A). Isolation of rice protoplasts was performed as described with the changes noted in the Section 2. Evans blue staining of the prepared protoplasts, in which dead cells stained blue, showed that almost all of the protoplasts were viable (Fig. 1B). We used a simple visual assay to assess transfection efficiency after transfecting protoplasts with a GFP expression plasmid (HBT-sGFP). More than 70% of protoplasts showed strong GFP fluorescence (Fig. 1C and D). These results showed that we had prepared high-quality protoplasts and achieved high transfection rates.

3.2. Gene overexpression or silencing in protoplasts

To visualize overexpression or silencing of a gene in the protoplasts, a GFP expression plasmid (HBT-sGFP) was introduced/cointroduced with the $dsRNA_{GFP}$ corresponding to CDS of the GFP or the $dsRNA_{actin1}$ corresponding to CDS of the *Actin1*, into the rice protoplasts through a PEG-calcium-medicated transfection approach. Fluorescence microscopy analysis showed that most of the protoplasts displayed a strong GFP signal after transfection with the HBT-sGFP plasmid alone (Fig. 2A). However, most of the protoplasts cotransfected with the HBT-sGFP plasmid and $dsRNA_{GFP}$ did not display green florescence (Fig. 2B). As a control, the protoplasts cotransfected with the HBT-sGFP plasmid and $dsRNA_{Actin1}$ displayed strong fluorescence, similar to that in the HBT-sGFP-transfected protoplasts (Fig. 2C). These results showed that the delivery of an *in vitro*-synthesized dsRNA could specifically suppress the expression of its target in rice protoplasts. Based on these observations, we concluded that overexpression or silencing of a gene in rice protoplasts was feasible and efficient.

3.3. Protoplast is an efficient system to study the CMS/Rf system

In plants, the conflict/reconciliation between mitochondria and nuclei manifested by the cytoplasmic male sterility (CMS) and nucleus-controlled fertility restoration (Rf) is a striking example of a nucleo-cytoplasmic interaction. We focused on a gene involved in the HL-CMS/Rf system as a target to study this nucleo-cytoplasmic interaction in protoplasts. The protoplasts derived from the CMS line (YtA) were transformed with a recombinant construct, which was designed to over-express the GFP-tagged restorer gene *Rf5* in the protoplasts. Under normal culture conditions, the fusion protein Rf5-GFP gradually accumulated in the transfected protoplasts (Fig. 3A). It has been proposed that the fusion protein exclusively localizes in the mitochondria, and functions with other factors to mediate endonucleolytic cleavage of

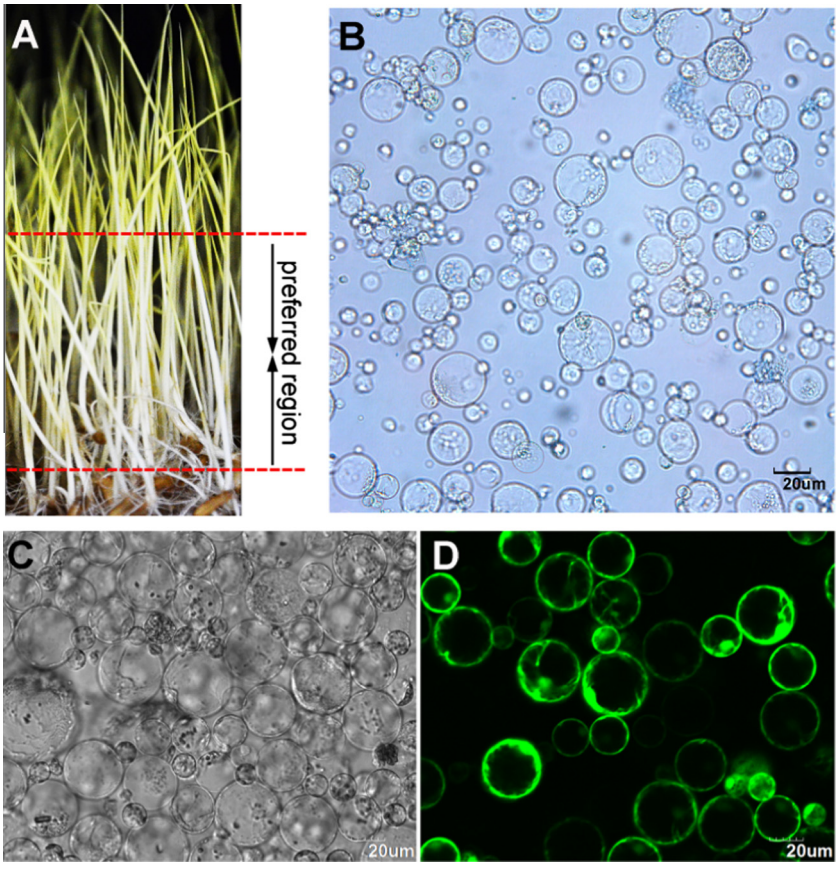

Fig. 1. Preparation and transfection of rice protoplasts. (A) Preferred region of etiolated seedlings for preparation of protoplasts. (B) Protoplasts stained with Evans blue dye. (C and D) Simple assay to evaluate transfection efficiency by a GFP expression plasmid. Bright-field (C, Bright) and fluorescent microphotographs (D, EGFP) were obtained using an FV1000 confocal system (400×).

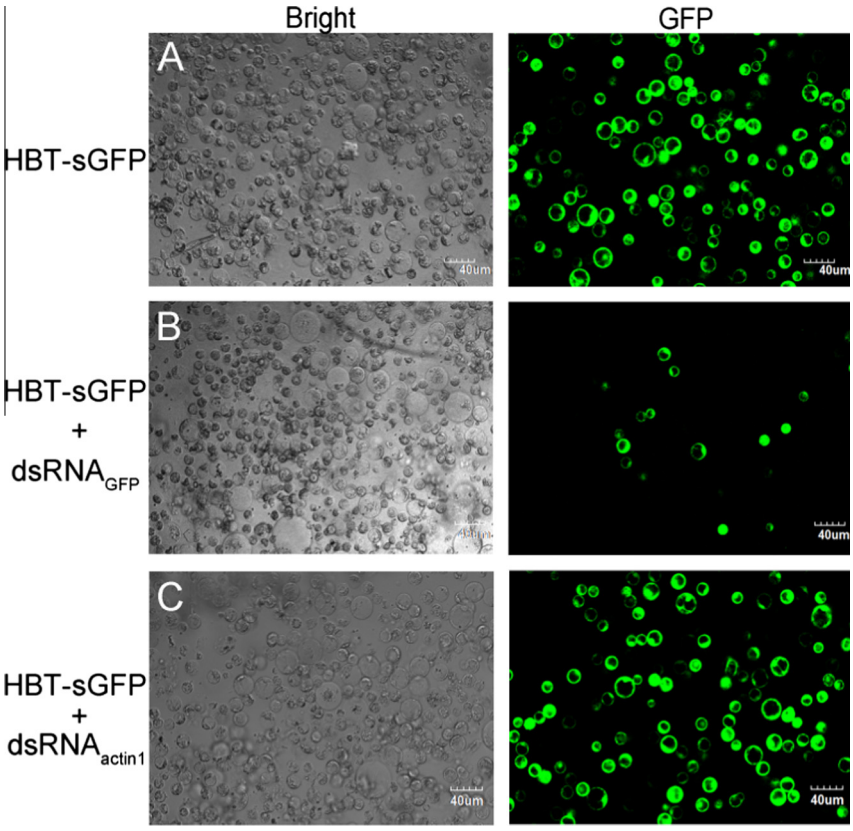

Fig. 2. Overexpression and silencing of GFP in rice protoplasts. Bright-field (Bright, left) and fluorescent microphotographs (EGFP, right) were obtained using an FV1000 confocal system. (A) Rice protoplasts transfected with HBT-sGFP plasmid only. (B) Silencing of GFP in protoplasts cotransfected with HBT-sGFP plasmid and dsRNA$_{GFP}$. (C) Protoplasts cotransfected with HBT-sGFP plasmid and dsRNA$_{actin1}$ (control).

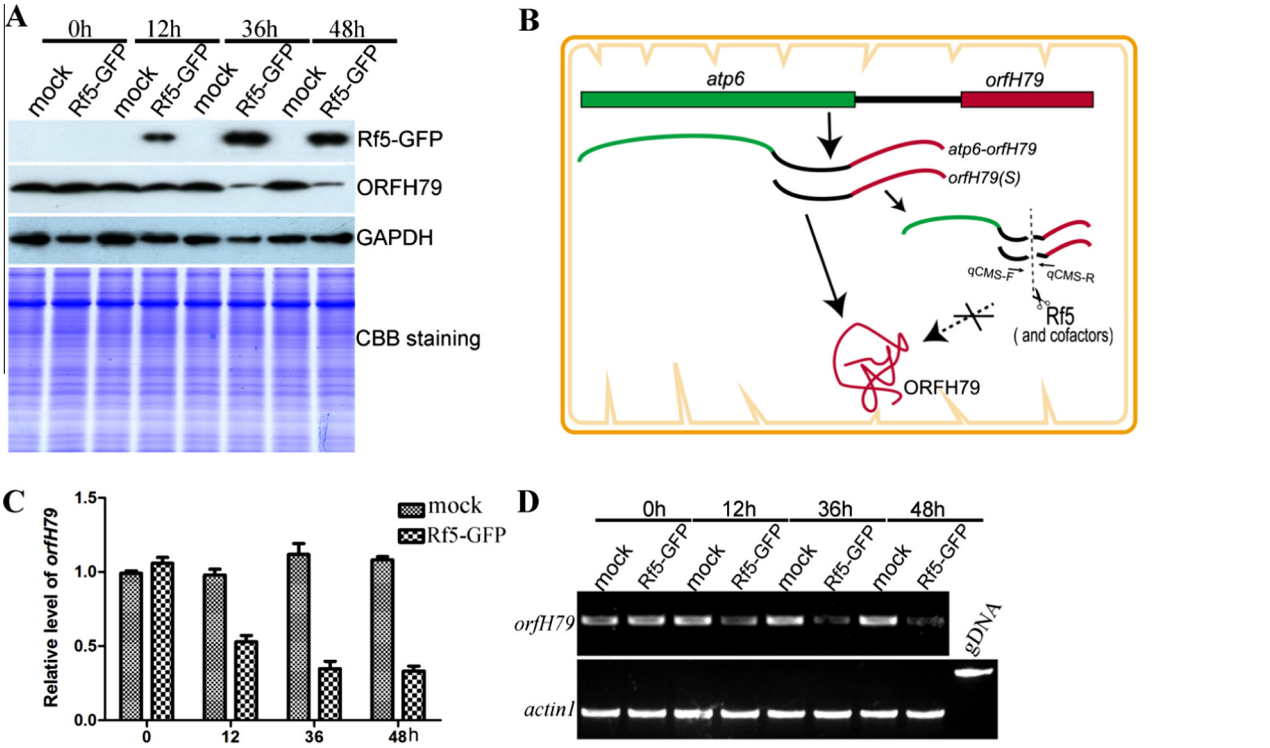

Fig. 3. Overexpression of fertility restorer gene *Rf5* in CMS-derived protoplasts. (A) Western blotting assay to detect amounts of fusion protein Rf5-GFP and CMS protein ORFH79 accumulated in protoplasts during culture period. (B) Schematic diagram of mechanism of fertility restoration in mitochondria. (C and D) qRT-PCR and RT-PCR assays to detect products of cleavage process. Primers spanning cleavage site were used in these analyses.

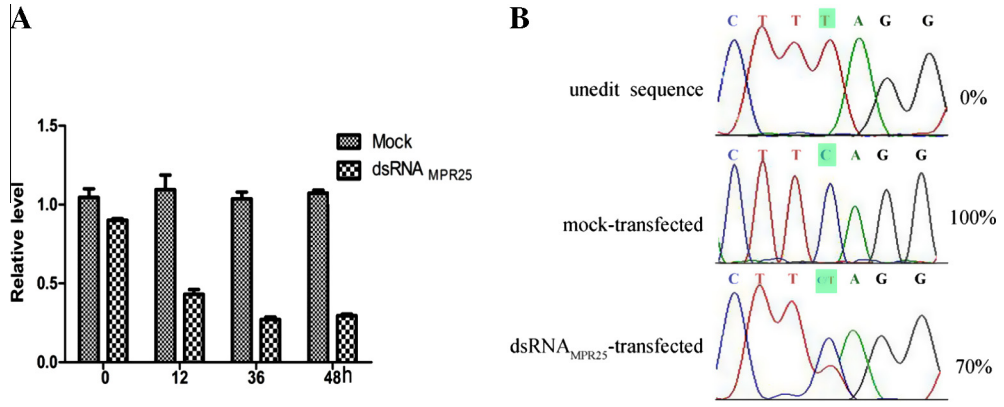

Fig. 4. Silencing of organellar RNA editing gene *MPR25* in rice protoplasts. (A) qRT-PCR analysis to quantify *MPR25* transcripts after transfection of protoplasts with $dsRNA_{MPR25}$. (B) Bulk sequencing analysis of *nad5* cDNA from mock- and $dsRNA_{MPR25}$-transfected protoplasts. Upper panel: unedited sequence. Middle and lower panels: sequences of RT-PCR products reverse-transcribed and amplified from RNA extracted from mock- (water) and transfected ($dsRNA_{MPR25}$) protoplasts.

CMS-associated transcripts (Fig. 3B, and Supplementary Fig. S1). To validate this cleavage process, we conducted qRT-PCR and RT-PCR assays using primers spanning the cleavage site. The abundance of the unprocessed CMS transcripts in Rf5-GFP-transfected protoplasts gradually reduced to one-third of the level in mock-transfected protoplasts during the culture period (Fig. 3C and D). Consistent with the results of qRT-PCR and RT-PCR analyses, a western blot analysis showed that amount of ORFH79 protein was also decreased in the Rf5-GFP-transfected protoplasts (Fig. 3A). These results confirmed that the nuclear-encoded *Rf* gene was over-expressed and correctly targeted to the mitochondria in the transformed protoplasts, and was able to cleave mitochondrial CMS transcripts and suppress the expression of the detrimental peptide ORFH79.

3.4. Protoplast represents an efficient system to study organellar RNA editing

Organellar RNA editing in flowering plants is another example of a nucleo-cytoplasmic interaction. It has been reported that the C → U RNA editing at nucleotide 1580 in *nad5* is conserved among diverse plants and algae including rice, Arabidopsis, *Brassica napus*, *Physcomitrella patens*, and *Chlamydomonas reinhardtii*. Here, we chose MPR25 as the target. This protein is encoded by the nuclear genome and is involved in RNA editing of *nad5* in mitochondria. To investigate whether the editing state of *nad5* was affected by silencing the expression of *MPR25* in rice protoplasts, we transfected *in vitro*-synthesized $dsRNA_{MPR25}$ corresponding to the CDS of *MPR25* (550-849nt) into rice protoplasts. A qRT-PCR analysis revealed that the abundance of *MPR25* in $dsRNA_{MPR25}$-transfected protoplasts gradually decreased, compared with that in mock-transfected protoplasts (Fig. 4A). Bulk sequencing of RT-PCR products prepared from mock- and $dsRNA_{MPR25}$-transfected protoplasts after incubation for 48 h showed that the editing state of *nad5* at nt 1580 was partially altered in the $dsRNA_{MPR25}$-transfected protoplasts (Fig. 4B). These results implied that the dsRNA-mediated gene silencing of nuclear-encoded *MPR25* in protoplasts directly affected the mitochondrial RNA editing state.

4. Discussion

Protoplasts, which are single plant cells, have been used in research in diverse areas of plant biology. Compared with time-consuming stable genetic transformation, the protoplast transformation system represents a more efficient and convenient approach. However, a recent study showed that protoplasts prepared from different tissues showed some major differences. It was reported that the DFRa promoter was active only in transiently transformed protoplasts derived from the petal epidermis, and not those derived from petal mesophyll cells. Also, proteins involved in the acidification of the vacuole in epidermal petal cells, such as the tonoplast pumps PH5 and PH1 and vacuolar SNAREs, showed highly cell-specific subcellular localization patterns [25]. These observations suggest that protoplasts derived from particular tissues retain their tissue- and cell-specific features within the time frame of a transient expression assay. Consequently, more attention should be paid to the experimental material used as the source of protoplasts.

Mitochondria and chloroplasts are semiautonomous organelles that have retained their own genomes and gene expression apparatuses. However, because the majority of organellar proteins are nuclear-encoded, a degree of genome coordination between the nucleus and organelles is essential to maintain cell function. Both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals are important in the coordination of the nuclear and organellar genomes. So far, it is challenging to identify and characterize proteins involved in nucleo-cytoplasmic interactions. Until now, *A. tumefaciens*-mediated genetic transformation has been used as a powerful tool to screen for important genes (e.g., using T-DNA mutants) and validate candidate proteins that may play roles in nucleo-cytoplasmic crosstalk. However, this approach is time consuming and labor intensive. In contrast, the protoplast system is more efficient for research on nucleo-cytoplasmic interactions. In this work, we successfully over-expressed and silenced nuclear-encoded genes with roles in nucleo-cytoplasmic interactions in protoplasts. As expected, changes in the appropriate organelles occurred as a result of the genetic transformation. Together, these results show that protoplast represents a promising system to identify and characterize proteins involved in cross-talk between the nucleus and organelles.

Author contribution statement

C. Yu, L. Wang, J. Hu designed research and conducted experiments.

C. Chen, C. He contributed new reagents and analytical tools.

C. Yu analyzed data and wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.043.

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