A centrifugation-assisted peptide-mediated gene transfer method for high-throughput analyses

Mitsuhiro Kimura*, Takeshi Yoshizumi², Keiji Numata**

Biomacromolecules Research Team, RIKEN Center for Sustainable Resource Science, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan

*E-mail: mitsuhiro.kimura@riken.jp  Tel: +81-48-467-9525  Fax: +81-48-462-4664
**E-mail: keiji.numata@riken.jp  Tel: +81-48-467-9525  Fax: +81-48-462-4664

Received August 15, 2018; accepted November 15, 2018 (Edited by S. Ogita)

Abstract  A peptide-mediated DNA delivery system for several plant species has been recently developed. This system uses ionic complexes of DNA and fusion peptides containing several domains, such as DNA-binding and cell-penetrating peptides. Although the peptide-DNA complexes are capable of penetrating into plant cells through the cell wall by mechanical pressure using a syringe, sample throughput is limited. Here, we describe a Centrifugation-Assisted Peptide-mediated gene Transfer (CAPT) method for improving sample throughput with reproducible gene transfer efficiency. We optimized the parameters of CAPT for transient gene transfer efficiency by using Nicotiana tabacum cotyledons as a model plant material. The optimal parameters for centrifugation were 10,000×g for 60 s. Furthermore, we successfully transferred the peptide–DNA complex into rice cotyledons using the optimized CAPT method. Thus, the CAPT method is superior to the previous syringe-mediated infiltration method in terms of sample throughput in multiple plant species.

Key words: Centrifugation-Assisted Peptide-mediated gene Transfer (CAPT) method, peptide–DNA complex, rice, tobacco.

Since the mid-1990s, genetically modified (GM) crops have been widely used in commercial agriculture production (Raman 2017). For the production of GM crops, Agrobacterium- and biolistic-mediated methods for gene transfer to plant cells have been developed to produce the desired trait. Recently, we succeeded in the delivery of dsDNA, dsRNA, and protein using cell penetrating peptides (CPPs) fused with a polycation sequence as a gene carrier into plant cells (Lakshmanan et al. 2013, 2015; Ng et al. 2016; Numata et al. 2014). CPPs can serve as a cargo carrier for macromolecules across cellular membranes into cells (Numata 2015; Rádis-Baptista et al. 2017). Additionally, we succeeded in the selective delivery of plasmid DNA into chloroplasts or mitochondria using polycationic peptides with chloroplast or mitochondrial targeting signals (Chuah et al. 2015; Yoshizumi et al. 2018). Yoshizumi et al. developed a syringe-based vacuum system for efficient delivery of peptide–DNA complexes into plant cells (Yoshizumi et al. 2018). However, the drawbacks of this system include its low throughput and the dependence of gene transfer efficiency on the experimenter’s ability.

Here, we developed a Centrifugation-Assisted Peptide-mediated gene Transfer (CAPT) method for various high-throughput analyses, overcoming the drawbacks of the previous method.

We used tobacco (Nicotiana tabacum cv. Petit Havana SR1) and rice (Oryza sativa cv. Nipponbare) in this study. Sterilized seeds of tobacco were placed on germination (GEM) medium composed of one-half Murashige–Skoog medium (Murashige and Skoog 1962) with 10 g L⁻¹ sucrose and 2.5 g L⁻¹ Phytagel. For gene transfer efficiency, a (KH)₉-BP100 fusion peptide (Amino acid sequence: KHKKHKKHKHKHKHKKKLFKKKILKYL) (Lakshmanan et al. 2013, 2015) and a CaMV 35S promoter-NanoLuc Luciferase construct (pGWB-Nluc) (England et al. 2016) were mixed by the method described previously (Yoshizumi et al. 2018). The detached cotyledons of 7-day-old seedlings were added to the peptide–DNA complex solutions and were centrifuged using a Tomy MX-307 centrifuge (Tomy Seiko Co., Tokyo, Japan). Cotyledons that were added to the complex solutions were harvested and analyzed for luciferase activity.

Abbreviations: CAPT, Centrifugation-Assisted Peptide-mediated gene Transfer; CPP, cell penetrating peptide; GM, genetically modified; Nluc, NanoLuc Luciferase; RMOP, revised medium for organogenesis (shoot regeneration) of Nicotiana plumbaginifolia.

*Present address: Takasaki University of Health and Welfare, 37-1 Nakaorui-machi, Takasaki, Gunma 370-0033, Japan. E-mail: m-kimura@takasaki-u.ac.jp  Tel: +81-27-352-1290  Fax: +81-27-353-2055

This article can be found at http://www.jspcmj.jp/

Copyright © 2019 The Japanese Society for Plant Cell and Molecular Biology

Advance Publication by J-STAGE
DOI: 10.5511/plantbiotechnology.18.1115a
Gene transfer to plant cells by centrifugation

The cotyledons of 7-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct using different centrifugation times (30, 60 and 180 s) at 10,000×g. Non-centrifugal treatment was used as a negative control. Relative light units (RLU) indicates the luciferase activity. The bars represent the mean±SE (n=19). Asterisks indicate significant differences between the control and treated explants (*: p<0.05, **: p<0.01) by Kruskal–Wallis t-test.

Without centrifugation were used as negative controls for all the experiments in the current study. After centrifugation, the explants were placed on GEM media. After 1 day, the explants were ground in Reporter Lysis 5X Buffer (Promega, Wisconsin, USA) with a pestle. The homogenized solutions were centrifuged and luciferase activity of the supernatants was measured by luciferase activity using the Nano-Glo® Luciferase Assay System (Promega) using GloMax® 20/20 Luminometer (Promega). A total protein amount in each sample was quantified by Bradford assay (XL-Bradford KY-1040, Apro Science Inc, Tokushima, Japan). The luciferase activities were evaluated in the relative light units (RLU) per mg of proteins. In a syringe-based vacuum system, the detached cotyledons of 7-day-old tobacco seedlings were transformed using the method described previously (Yoshizumi et al. 2018). For shoot induction efficiency, the detached cotyledons of 7-day-old tobacco seedlings were placed on revised medium for organogenesis (shoot regeneration) of Nicotiana plumbaginifolia (RMOP) medium and transferred to fresh RMOP medium every 2 weeks (Svab and Maliga 1993). After incubation for 4 weeks, the efficiency was examined. In experiments with rice, the detached cotyledons of 8-day-old seedlings were used in the methods described above. All statistical analyses were performed based on a Kruskal–Wallis t-test or Mann–Whitney U-test by EZR software (Kanda 2013).

We investigated the effect of centrifugation time on transient gene transfer efficiency by measuring NanoLuc Luciferase (Nluc) reporter activity in tobacco cotyledons. The gene transfer efficiencies by treatments for 60 s (p=0.0147) and 180 s (p=0.0070) at 10,000×g were higher than that of control (Figure 1). Treatment for 30 s showed no significant difference in gene transfer efficiency from treatment for 60 s and 180 s (Figure 1). We also examined the effect of centrifugal force on transient gene transfer efficiency. We found that the efficiency at 3,000×g (p=0.0141), 10,000×g (p=0.0104) and 20,000×g (p=0.0032) for 60 s were significantly higher than the negative control (Figure 2). The efficiency at 20,000×g was relatively lower than that at 10,000×g, implying that 20,000×g treatment might induce cellular damages of the cotyledons. Therefore, we concluded that the optimal conditions for the CAPT method were 10,000×g for 60 s, which were then used in the experiments described below.

To assess the advantages of CAPT, we compared the gene transfer efficiency of the optimized CAPT method with the syringe-based vacuum system in tobacco. We found that the efficiency of CAPT was approximately 150
Table 1. Effect of CAPT on the shoot regeneration efficiency of tobacco cotyledon explants ($n=25\times4$)

| Treatment type | Experiment No. | Total |
|---------------|---------------|-------|
|               | 1  | 2  | 3  | 4  |       |
| Control       | 23/25  | 25/25  | 25/25  | 25/25  | 98/100 |
| Syringe       | 25/25  | 21/25  | 22/25  | 23/25  | 91/100 |
| CAPT          | 25/25  | 24/25  | 24/25  | 23/25  | 96/100 |

Figure 4. CAPT mediates gene transfer in rice. The cotyledon segments of 8-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct by centrifugation at 10,000×g for 60 s. How was gene transfer efficiency measured? The bars represent the means±SE (n=10). Asterisks indicate significant differences between the control and treated explants (***: $p<0.001$) by Mann–Whitney U-test.

RLU mg⁻¹ protein on average, which was significantly higher in comparison to the control ($p=0.0037$) and the results by syringe-based vacuum system ($p=0.0283$) (Figure 3). The efficiency of the syringe-based vacuum system ranged widely from approximately 2 to 4800 RLU mg⁻¹ protein (Figure 3).

In addition to efficient gene transfer, plant regeneration is essential for efficient genetic transformation (Anami et al. 2013). Therefore, we analyzed the effect of CAPT on shoot regeneration efficiency in tobacco. The shoot regeneration efficiency of the optimized CAPT method was similar to those of the control and syringe-based vacuum system (Table 1), indicating that CAPT is suitable for plant transformation in terms of gene transfer efficiency, reproducibility and regeneration efficiency.

Moreover, to analyze the capability of CAPT for gene transfer in monocots, the gene transfer efficiency of CAPT was evaluated in rice cotyledons. We found that Nluc activity of the samples treated by centrifugation at 10,000×g for 60 s was higher than that of control (Figure 4). Therefore, CAPT could be useful for gene transfer to monocotyledonous as well as dicotyledonous plant species.

In this study, we optimized and developed the CAPT method for peptide-mediated gene transfer (Figures 1, 2) for various high-throughput analyses. Previous studies developed an infiltration–centrifugation method to isolate apoplastic fluid (Klement 1965; O’Leary et al. 2014). In this method, the appropriate buffers were infiltrated into the apoplastic air spaces with decompression and depressurization by infiltration–centrifugation. In addition, transient gene transfer of centrifuged banana suspension cells with Agrobacterium showed a 3- to 4-fold increase compared with non-centrifuged cells (Khanna et al. 2004). The improved efficiency may result from more Agrobacterium colonization on the plant cell surface. Based on these previous studies with Agrobacterium, CAPT might accelerate the infiltration of the solution, including the peptide–DNA complex, through the cellular barriers, resulting in high accumulation of the peptide–DNA complex in plant cells.

However, high-pressure treatment has been reported to cause cell damage to several plant species (Rajasekhar and Lafta 1996), and cell damage affects shoot regeneration efficiency (Rasco-Gaunt et al. 1999). Our optimized CAPT method did not affect shoot regeneration efficiency in tobacco (Table 1). Additionally, the gene transfer efficiency of CAPT was more consistent than the syringe-based vacuum system (Figure 3). Moreover, CAPT worked to introduce the peptide-DNA complex into rice cells (Figure 4). Considering previous reports on the peptide-mediated gene transfer of dsRNA and proteins (Ng et al. 2016; Numata et al. 2014), CAPT may improve sample throughput for efficient peptide-mediated transfer of various biological cargos in addition to DNA.

Acknowledgements

The authors would like to thank Dr. Yutaka Kodama for providing the pGWB-35S: Nluc vector. This work was supported by JSPS KAKENHI Grant Number JP18K05638 to M. K., NEDO Grant to T. Y and ERATO Grant Number JPMJER1602 to K. N.

References

Anami S, Njuguna E, Coussens G, Aesaert S, Van Lijssebetts M (2013) Higher plant transformation: Principles and molecular tools. Int J Dev Biol 57: 483–494

Chuah J-A, Yoshizumi T, Kodama Y, Numata K (2015) Gene introduction into the mitochondria of Arabidopsis thaliana via peptide-based carriers. Sci Rep 5: 7751

England CG, Ehlerding EB, Cai W (2016) NanoLuc: A small luciferase is brightening up the field of bioluminescence. Bioconjug Chem 27: 1175–1187

Kanda Y (2013) Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. Bone Marrow Transplant 48: 452–458

Khanna H, Becker D, Kleidon J, Dale J (2004) Centrifugation Assisted Agrobacterium tumefaciens-mediated Transformation (CAAT) of embryogenic cell suspensions of banana (Musa spp. Cavendish AAA and Lady finger AAB). Mol Breed 14: 239–252

Klement Z (1965) Method of obtaining fluid from the intercellular spaces of foliage and the fluid’s merit as substrate for
Gene transfer to plant cells by centrifugation

phytobacterial pathogens. *Phytopathology* 55: 1033–1034

Lakshmanan M, Kodama Y, Yoshizumi T, Sudesh K, Numata K (2013) Rapid and efficient gene delivery into plant cells using designed peptide carriers. *Biomacromolecules* 14: 10–16

Lakshmanan M, Yoshizumi T, Sudesh K, Kodama Y, Numata K (2015) Double-stranded DNA introduction into intact plants using peptide–DNA complexes. *Plant Biotechnol* 32: 39–45

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Ng KK, Motoda Y, Watanabe S, Sofiman Othman A, Kigawa T, Kodama Y, Numata K (2016) Intracellular delivery of proteins via fusion peptides in intact plants. *PLoS One* 11: e0154081

Numata K (2015) Poly(amino acid)s/polypeptides as potential functional and structural materials. *Polym J* 47: 537–545

Numata K, Ohtani M, Yoshizumi T, Demura T, Kodama Y (2014) Local gene silencing in plants via synthetic dsRNA and carrier peptide. *Plant Biotechnol J* 12: 1027–1034

O’Leary BM, Rico A, Mccraw S, Fones HN, Preston GM (2014) The infiltration–centrifugation technique for extraction of apoplastic fluid from plant leaves using phaseolus vulgaris as an example.

*Ràdis-Baptista G, Campelo IS, Morlighem J-ÉRL, Melo LM, Freitas VJF (2017) Cell-penetrating peptides (CPPs): From delivery of nucleic acids and antigens to transduction of engineered nucleases for application in transgenesis. J Biotechnol* 252: 15–26

Rajashekar CB, Lafta A (1996) Cell-wall changes and cell tension in response to cold acclimation and exogenous abscisic acid in leaves and cell cultures. *Plant Physiol* 111: 605–612

Raman R (2017) The impact of genetically modified (GM) crops in modern agriculture: A review. *GM Crops Food* 8: 195–208

Rasco-Gaunt S, Riley A, Barcelo P, Lazzeri PA (1999) Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. *Plant Cell Rep* 19: 118–127

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene. *Proc Natl Acad Sci USA* 90: 913–917

Yoshizumi T, Oikawa K, Chuah J-A, Kodama Y, Numata K (2018) Selective gene delivery for integrating exogenous DNA into plastid and mitochondrial genomes using peptide–DNA complexes. *Biomacromolecules* 19: 1582–1591