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Note

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

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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: KHKHKHKHKHKHKHKHKKLFKKILKYL) (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 at various *g*-forces and times by a Tomy MX-307 centrifuge (Tomy Seiko Co., Tokyo, Japan). Cotyledons that were added to the complex solutions

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*.

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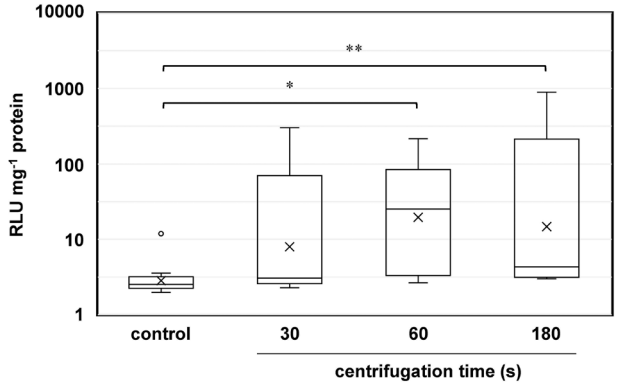

Figure 1. Effect of centrifugation time on gene transfer in tobacco. The cotyledons of 7-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct using different centrifugation times (30, 60 and 180 s) at 10,000×*g*. Non-centrifugal treatment was used as a negative 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.

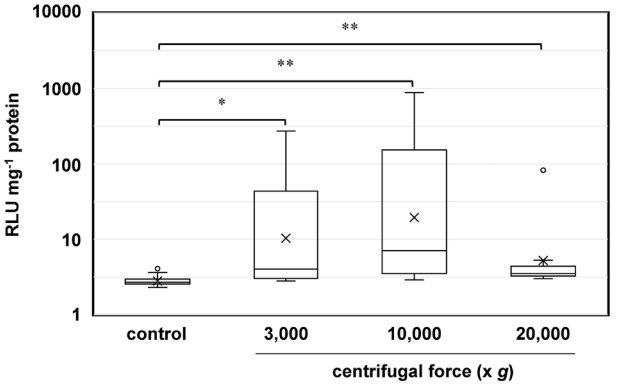

Figure 2. Effect of centrifugal force on gene transfer in tobacco. The cotyledons of 7-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct using different centrifugal forces (3,000, 10,000 and 20,000×*g*) for 60 s. Non-centrifugal treatment was used as a negative control. 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

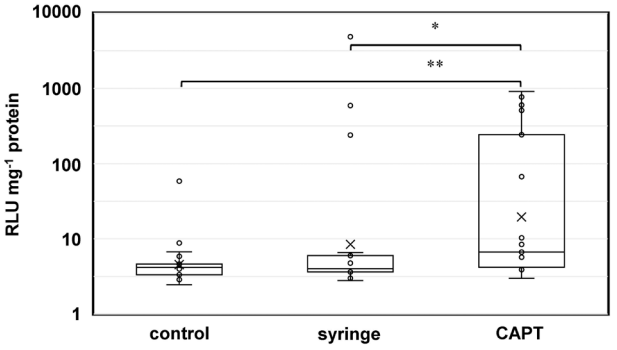

Figure 3. CAPT increases the efficiency of gene transfer over the syringe-based vacuum system in tobacco. For CAPT, the cotyledon segments of 7-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct by centrifugation at 10,000×*g* for 60 s. The syringe-based vacuum system was performed as described previously (Yoshizumi et al. 2018). The bars represent the means±SE (n=23). Asterisks indicate significant differences between the control and treated explants (*: p<0.05, **: p<0.01) by Kruskal–Wallis t-test.

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=25×4).

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

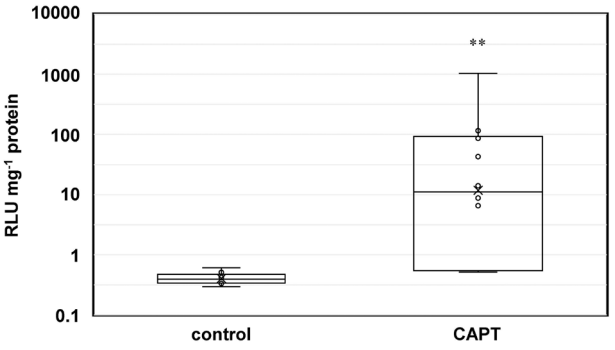

Figure 4. CAPT mediates gene transfer in rice. The cotyledon segments of 8-day-old seedlings were transformed with a DNA–peptide complex harboring a luciferase construct by centrifugation at 10,000×g for 60 s. How was gene transfer efficiency measured?] The bars represent the means±SE (n=10). Asterisks indicate significant differences between the control and treated explants (**: p<0.01) 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 (Rajashekar 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 protein (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.

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