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Short Communication

Improvement of the plastid transformation protocol by modifying tissue treatment at pre- and post-bombardment in tobacco

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Abstract Plastid transformation in higher plants has been established in tobacco using particle bombardment, and the protocol has been used as a model in other plant species; however, as target materials, tobacco leaves may be partially exposed to unexpected abiotic stresses—drought stress during post-bombardment culture and damage due to cutting before selection culture. Plastid transformation efficiency may be increased by modification of leaf treatment to lessen such damage. In the modified protocol, tobacco leaves were cut into pieces (5×5 mm) and placed on the culture medium plate (approximately fifty pieces per plate) for one day before bombardment. After bombardment, these pieces were transferred onto the non-selection medium and cultured for three days; they were then transferred onto spectinomycin selection culture medium. A transformation vector containing an aminoglycoside 3′-adenylyltransferase gene and a green fluorescence protein gene were used for plastid transformation. Approximately four independent plastid transformants per bombarded plate were obtained on average using the modified protocol; the transformation efficiency was 1.6 times higher than that in a control experiment using the standard protocol. Modified leaf treatment improved the efficiency and stability of plastid transformation. This finding should aid plastid transformant production in tobacco and other plant species.

Key words: aadA, chloroplast, particle bombardment, plastid transformation, tobacco.

Plastid transformation is a useful tool for producing recombinant proteins in plants or investigating the function of plastid genes. Although plastid transformation was first accomplished in tobacco by particle bombardment, its efficiency was low, with only 0.01–0.03 transformants per bombarded plate (Svab et al. 1990). The use of spectinomycin-resistant *aadA* as a selectable marker markedly increased the transformation efficiency to 1–2.5 transformants per plate (Svab and Maliga 1993; Khan and Maliga 1999). Despite the difference in vector construction, such as homologous regions and selectable marker genes, plastid transformants were produced not only in tobacco (Daniell et al. 1998; Bock et al. 1996; Jeong et al. 2004) but also in other species (Day and Goldschmidt-Clermont 2011; Cui et al. 2011). However, plastid transformation efficiency in other species except tobacco was still very low, making it difficult to obtain one plastid transformant per bombarded plate. Although a combination of tobacco suspension cells and 0.4-μm-gold particles increased plastid transformation efficiency to approximately 4–5 events per plate (Langbecker et al. 2004), subsequent reports adopted a protocol using leaves as target materials in tobacco. The preparation of leaves as target material is thought to be easier and less time-consuming than suspension cells. We considered that the efficiency of plastid transformation using leaves as target materials may be further increased by modification of leaf treatment. We supposed that, under the conventional protocol, tobacco leaves as target materials may be exposed to unintended abiotic stress such as drought stress during post-bombardment culture and damage due to cutting before selection culture. Therefore, we investigated the advantage of a modified leaf treatment protocol.

We first constructed the plastid transformation vector *pNtag*, which has *aadA* and *gfp* expression cassettes between the homologous sequences of *trnI* region and *trnA* region derived from tobacco plastid DNA (Fig. 1a). The plasmid DNA of *pNtag* was coated on 0.6-μm-gold particles (DNA/gold) as follows. A stock solution of 0.6-μm-gold particles (40 mg ml^{-1}) was sonicated (40 kHz) for 1–2 min before preparing the DNA/gold mixture. To prepare a mixture for 8 shots, 80 μl of the

Abbreviations: GFP, green fluorescence protein gene; aadA, aminoglycoside 3′-adenylyltransferase gene.
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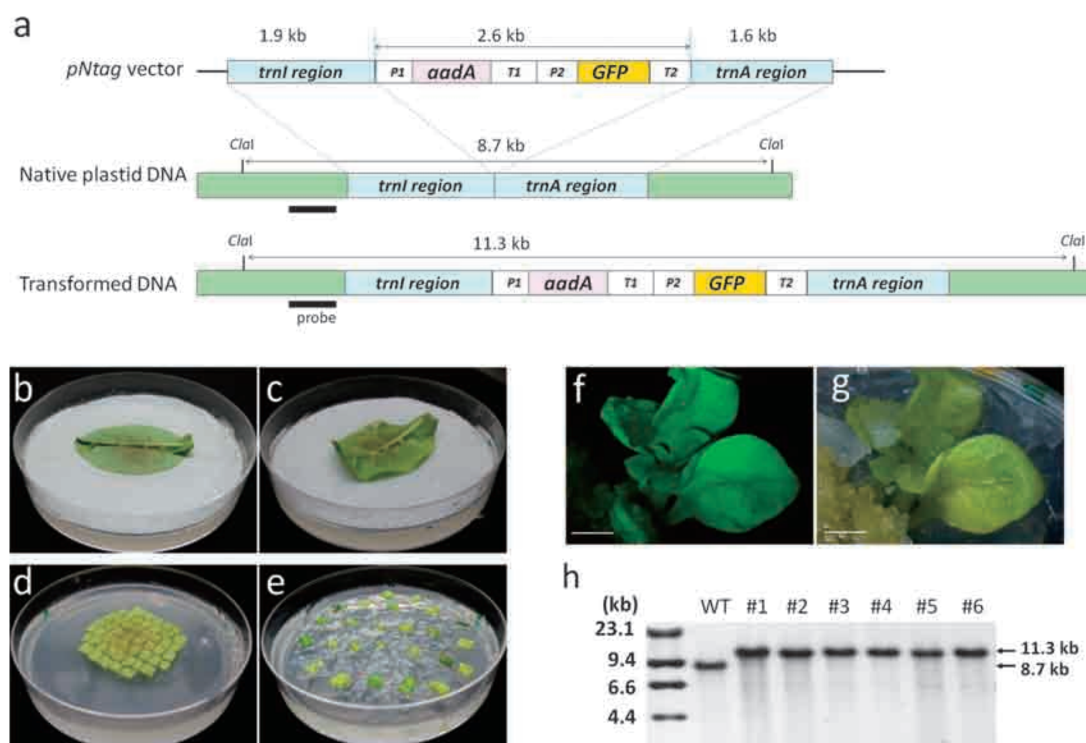

Figure 1. *pNtag* construct and *pNtag*-integrated tobacco. a: Construction of the *pNtag* vector, native DNA of tobacco plastid, and transformed plastid DNA is shown. *trnI region*, *a* flanking region around the *trnI* gene of tobacco plastid DNA (Accession No. Z00044: 103417-105335); *trnA region*, *a* flanking region around the *trnA* gene of tobacco plastid DNA (Accession No. Z00044: 105330–106944); P1, ribosomal RNA operon promoter connected with 5′UTR of gene 10 from T7 phage; T1, *rbcL* terminator; P2, psbA terminator; T2, rps16 terminator. A DNA fragment of *aadA*-T2-P2-*GFP*-T2 was cloned from *pLD200-6-GFP* (Kajiyama et al. 2008) and a ribosomal RNA operon promoter was cloned from 5′ region of the 16S ribosomal RNA gene of tobacco plastid DNA (Accession No. Z00044: 102550–102725). b: A tobacco leaf was placed on the filter paper covering the medium, and it was bombarded c: A leaf (Fig. 1b) was incubated for 48 h after bombardment. d: Leaf pieces bombarded according to the modified protocol. e: Cultured leaf pieces of Fig. 1d on the MSBN medium for three days after bombardment. f: Plastid transformants showed GFP fluorescence. g: Plastid transformants observed in light. h: Detecting the insertion of *pNtag* in the plastid DNA of transformants. Total DNA extracted from the leaves of the wild-type and transformants were digested with *Cla*I, and Southern blot analysis was performed according to a standard protocol. A specific DNA probe for the native plastid DNA (black bar in Fig. 1a) was prepared using the PCR DIG labeling Mix (Roche Diagnotics, Switzerland) with primer set for probe (5′-GATCAGCCACACTGGGACTGAG-3′/5′-GAGGATTCGCGGCATGTCAAGC-3′). Southern blot hybridization signals were detected using the DIG luminescent detection kit (Roche Diagnostics, Basel, Switzerland). A band of 8.7 kb was detected in the wild-type with the probe (black bar in Fig. 1a). On the other side, bands of 11.3 kb were detected in plastid transformants, indicating the integration of *pNtag* at the target site of tobacco plastid DNA. bar: 5 mm (Figs. 1f, g)

stock solution was mixed with 32 μl of *pNtag* vector DNA (1 μg μl^{-1}), 80 μl of 2.5 M $CaCl_2$, and 32 μl of 0.1 M spermidine in a low retention 1.5-ml tube (BM Equipment, Tokyo, Japan), and the mixture was gently vortexed with a microtube mixer (EM-36, TAITEC, Saitama, Japan) at approximately 300 rpm for 30 min at room temperature. The DNA/gold mixture was placed on a tube rack for 3 min and then centrifuged for 5 s at 2,000×***g***. This mixture was then washed with 500 μl 70% ethanol, and the supernatant was removed after centrifugation for 5 s at 2,000×***g***. Finally, 110 μl >99.5% ethanol was added to the DNA/gold mixture, and it was gently vortexed for 30–60 min to suspend gold particles well. DNA/gold mixture (10 μl) was applied onto a macrocarrier, dried, and then bombarded. Two shots were bombarded per plate using a 1,100 psi rupture disc under partial vacuum (711 mmHg) in a PDS-1000/He system (Bio-Rad Laboratories, Hercules, CA, USA). The distance between the stopping screen and sample plate was 6 cm. For each plate, 4 μg of the *pNtag* vector was delivered, with 0.8 mg gold particles in total. As target material, about thirty tobacco (*N. tabacum* L. cv. Petit Havana SR1) plantlets were grown aseptically in the MS medium (Murashige and Skoog, 1962) supplemented with 8 g l^{-1} agar (Wako Pure Chemical Industries, Osaka, Japan) in a magenta box (Iwaki Glass, Tokyo, Japan) (two or three plantlets per a box) for 6–8 weeks at 25°C and 16 h light. The shoot-inducing culture medium consisted of the MS medium supplemented with N^6-benzyladenine (1 mg l^{-1}), 1-naphthaleneacetic acid (0.1 mg l^{-1}) and 7 g l^{-1} agar (MSBN medium).

Tobacco leaves obtained from some independent plants of the same growing stage were used for preparing each plate by two ways: by the standard protocol of Svab et al. (1990) as a control experiment and by our modified protocol. According to the standard protocol, a fresh

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whole tobacco leaf (about 45×30 mm in length) was placed on the filter paper covering the MSBN medium; it was bombarded with *pNtag*/DNA on the same day. The bombarded leaf was contacted with the filter paper by wetting with the medium (Fig. 1b) and cultured for 2 days at 25°C in the dark. Leaves were then cut into small pieces (ca. 5×5 mm) and replaced on the MSBN medium with 500 mg l^{-1} spectinomycin (MSBN-SPC). In the modified protocol, tobacco leaves were prepared for bombardment as follows. Two pieces of autoclaved filter paper were placed on a plastic plate and wetted with 1 ml of sterile water. Leaves of tobacco (30–50 mm in length) were placed abaxial side up on the filter paper and cut into pieces (5×5 mm) without the costa. Approximately fifty leaf pieces were placed abaxial side up on the center part (a circle of 4 cm diameter) of the MSBN medium in a plastic plate (90×20 mm) (Fig. 1d) and pre-cultured for a day in an incubator at 25°C (16 h light). To avoid detaching the edges of leaf pieces from the medium because of swelling and being knocked together during pre-culture, leaf pieces were placed approximately 1 mm apart on the MSBN medium. After bombardment with *pNtag*/DNA (Fig. 1d), bombarded sample plates were incubated for 1–4 h at 25°C in the dark. Leaf pieces were placed abaxial side down on MSBN plates (15–20 pieces per plate) after the solid surface was prepared into a paste by sterile tweezers (about 5 mm in depth), and incubated for 3 days at 25°C in the dark (Fig. 1e). The solid surfaces of MSBN and MSBN-SPC media using later steps were also prepared into a paste before transferring leaf pieces in order to contact leaf pieces well with the medium. Leaf pieces prepared using both standard and modified protocols were replaced every 2 weeks for 4–6 weeks (25°C, 16 h light). Several spectinomycin-resistant primary shoots were regenerated at 4–6 weeks after bombardment. Some primary adventitious shoots were spontaneously mutated spectinomycin-resistant shoots (Svab et al. 1990). In order to eliminate these, GFP fluorescence in plastids was observed using a fluorescence microscope (Leica MZ 16FA) with a GFP2 filter (480 nm excitation filter/510 nm barrier filter). Primary adventitious shoots showing GFP fluorescence in plastids were assigned as putative plastid transformants. Most primary transformants were seen to be heteroplasmic, which had both wild-type plastids showing red autofluorescence signals of chlorophyll with the GFP2 filter and transformed plastids showing GFP fluorescence, as reported previously (Khan and Maliga 1999). Total DNA was extracted from the primary shoots showing GFP signal, and insertion of the *pNtag* vector into the target site of the plastid DNA was confirmed by PCR analysis (data not shown). Some homoplasmic plastid transformants (Fig. 1f, 1g) were obtained from the continuous propagation of primary adventitious shoots. These were transferred to rooting medium in a *magenta box*. Rooting medium consisted of the MS medium with 30 g l^{-1} sucrose, 500 mg l^{-1} spectinomycin, and 8 g l^{-1} agar (pH 5.8). Homoplasmic plants showed GFP fluorescence over the entire surface of leaves. Six homoplasmic plants produced by the modified protocol were subjected to Southern blot analysis (Fig. 1h), confirming that all had an insertion of *pNtag* at the specific target site of plastid DNA. Homoplasmic plants were transplanted to pots and cultivated in the greenhouse at 27/22°C (day/night) in the natural photoperiod. We examined and confirmed the maternal transmission of transformed plastid DNA by reciprocal hybridization (data not shown).

pNtag was introduced to target leaves prepared by both the control standard protocol and our modified protocol under identical bombardment conditions. In the control experiment, *pNtag* was introduced into two plates per experiment, and the experiments were replicated twice. Ten primary plastid transformants in total were obtained from four plates (Table 1). On average, 2.5 primary plastid transformants per bombarded plate were obtained, a result close to that previously reported (Khan and Maliga 1999). Under the modified protocol, twelve plates were bombarded with *pNtag*/*DNA*, with four plates per experiment, and the experiments triplicated. The triplicate experiments yielded 22, 11, and 16 primary plastid transformants (Table 1). Approximately four plastid transformants were obtained per bombarded plate by the modified protocol, or 1.6 times more than by the standard protocol. We observed that whole leaf samples prepared and bombarded by the standard protocol (Fig. 1b) were partially detached from the filter paper owing to the curvature of the leaf fringe on the medium after 48 h (Fig. 1c). This detachment of the leaves was observed even after 24 h (data not shown). We speculated that the detached part of the leaf may undergo marginal drought stress during the two days of culture after bombardment. In the modified protocol, the small pieces of the leaf seemed not to undergo drought stress because they were in direct contact with the medium, and the fringes of the leaf pieces did not detach from the medium during post-bombardment culture (Fig. 1d). Moreover, it may

Table 1. Plastid transformants integrated *pNtag*.

Leaf preparing	No. of experiment	Bombarded plates, no.	Plastid transformants, no.
Modified protocol	1	4	22
	2	4	11
	3	4	16
	total	12	49
Standard protocol (control)	1	2	6
	2	2	4
	total	4	10

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be better for the leaf pieces to transfer its bombarded side directly to the medium during post-bombardment culture (Fig. 1e). In contrast, cutting the leaves after bombardment in the standard protocol may damage cells in which plastids have successfully incorporated DNA/gold. In addition, costa of the leaves received DNA/gold in the former protocol using whole leaf as a material, but costa are not suitable for regeneration. In view of these considerations, it may be better to remove the costa of the leaves and prepare sample plates uniformly even using various shapes of leaves. Modifying leaf treatment in pre- and post-bombardment yielded more independent primary transformants effectively and stably in small scale treatment as per an examination. The transformation efficiency under the modified protocol was as high as that using suspension cells as material and 0.4-μm-gold particles as DNA carrier (Langbecker et al. 2004), although we used leaves and 0.6-μm-gold particles. Sample plates of suspension cells (Langbecker et al. 2004) were also thought to be prepared uniformly and without drought stress, and this absence of stress may be one of the key factors increasing transformation efficiency in both their protocol and our modified protocol. Langbecker et al. (2004) reported that 0.4-μm-gold particles were appropriate for transformation of proplastids in suspension cells because these proplastids are smaller than mature plastids in leaves. We expect that 0.4-μm-gold particles will also be effective for transforming mature plastids, and the combination of smaller particles and our modified transformation system may further increase transformation efficiency.

In plastid transformation, an additional cycle of regeneration is essential to produce homoplasmic plants because most primary regenerated plastid transformants are heteroplasmic. However, we observed that a nearly homoplasmic primary shoot tends to regenerate from 5 to 10 primary regenerated shoots, and homoplasmic plants easily regenerated from propagation of nearly homoplasmic primary shoots without leaf segmentation. Thus obtaining many primary shoots may help to obtain homoplasmic primary shoots immediately.

This report reaffirmed that treatment of target samples significantly affects transformation efficiency even in basic tobacco plastid transformation. Uniformly preparing target sample plates and decreasing unexpected abiotic stress may be crucial factors for plastid transformation not only using leaf as target material but also using other tissue. Although, in many plant species, it is difficult to regenerate adventitious shoots or somatic embryos from leaf, these plants use other tissues as targets, e.g., petioles in sugarbeet (Marchis et al. 2009), stems in eggplant (Singh et al. 2010), and calli in rice (Lee et al. 2006). Furthermore, the transformation efficiency of such plants is expected to increase by another modification of tissue treatment. We hope that our modified protocol helps in increasing plastid transformation efficiency not only in tobacco but also in other plant species.

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