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Original Paper

Efficient plastid transformation in tobacco using small gold particles (0.07–0.3 μm)

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Abstract Plastid transformation methods have been developed for 20 plant species. However, only a few plant species, such as tobacco and lettuce, have been used in applied studies because transformation efficiencies are extremely low in other species. Plastid transformation has been mainly performed by particle bombardment using 0.6-μm gold particles as microcarriers of the transformation vector. Because the target materials in some plant species are undeveloped proplastids rather than fully developed chloroplasts, optimizing microcarrier size for the target size is a major consideration. In this study, we evaluated the availability of gold particles (0.07, 0.08, 0.1, 0.2, and 0.3 μm) that were smaller than those used for plastid transformation in previous studies. We obtained stable plastid transformants of tobacco with sufficient efficiency using all the tested small gold particles as the same level as 0.6-μm gold particles, even the smallest (0.07 μm). The average number of transformants obtained with 0.3-μm particles (9.3±4.6 per plate) was the highest among the tested gold particles. Because small gold particles were revealed to be sufficient for plastid transformation in a model tobacco plant, it is suggested that choosing appropriate small-sized gold particles which have never been used before will improve plastid transformation in many plant species.

Key words: Plastid transformation, gold particles, particle bombardment, chloroplasts, tobacco.

Plastid transformation is a powerful tool for producing useful proteins in plants and is also an important technique for investigating the function of genes encoded in plastid DNA (Bock 2007; Daniell et al. 2002; Maliga 2003; Wang et al. 2009). Extra genes integrated into plastid DNA are difficult to spread by pollen due to the maternal inheritance of plastid DNA, although low-level paternal inheritance of plastid DNA was detected in large screening of F_1 seeds obtained by artificial crossing (Ruf et al. 2007). In contrast, small proportion of plastid DNA were transferred to nuclear DNA (Lloyd and Timmis 2011a) and transferred gene could rarely be activated (Lloyd and Timmis 2011b). Although considering these recent findings, it is necessary to use appropriate containment methods, plastid transformants appeared to have an advantage of preventing introgression of extra gene compared with nuclear transformants. Following the development of plastid transformation in *Chlamydomonas* (Boynton et al. 1988), tobacco (*Nicotiana tabacum*) became the first higher plant in which stable plastid transformants were successfully produced (Svab et al. 1990). Since then, tobacco has been used as a model in many studies (Daniell et al. 2005, 2009; Maliga and Bock 2011). Although plastid transformation methods have been developed for 20 plant species (Khan 2012), most reports have involved the integration of a selectable marker or visual marker genes, such as the spectinomycin-resistance gene *aadA* and the green fluorescent protein gene *GFP*. Other than the model tobacco plant, a few plant species have been used in applied studies. For example, agronomic or pharmaceutically important proteins have been produced in plastids of tomato (Zhou et al. 2008), carrot (Kumer et al. 2004), and lettuce (Davoodi-Semiromi et al. 2009; Lelivelt et al. 2005; Lim et al. 2011; Ruhlman et al. 2007). Unfortunately, because plastid transformation efficiencies are low and only heteroplasmic plants have been obtained, it is currently difficult to produce useful protein in plastids of agronomically important plants, such as rice (Khan and Maliga 1999; Lee et al. 2006) and oilseed rape (Cheng et al. 2010; Hou et al. 2003). Therefore, improved plastid transformation methods are needed for these and many other plant species.

Gene integration into plastids is usually performed

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by the particle bombardment method developed by Klein et al. (1987). Although plastid transformation of protoplasts by the polyethylene glycol method has been reported (O'Neill et al. 1993; Koop et al. 1996; Lelivelt et al. 2005), it appears that protoplast regenerative methods have not been well developed or are not useful for most plants. Developing or improving particle bombardment transformation protocols requires optimizing factors for each plant species. Although several parameters can be modified, such as selectable markers, selection agents, or the plastid DNA integration region, selecting the particle size on the basis of the target size should be a major consideration (Sanford et al. 1993). Most plastid transformation protocols have used tungsten particles with an average diameter of 0.7 μm as microcarriers for delivering DNA into plant cells by accelerating helium pressure (Boynton et al. 1988; Svab et al. 1990). However, Kikkert (1993) reported that gold particles are rounder and relatively uniform in size compared with tungsten particles and are also less toxic to the cells. Randolph-Anderson et al. (1997) demonstrated that 0.6-μm gold particles are more useful than 1-μm gold particles or tungsten particles. Similarly, the use of 0.6-μm gold particles instead of 1-μm particles increased nuclear transformation efficiency in maize (Randolph-Anderson et al. 1997) and soybeans (Khalafalla et al. 2005). Because tobacco leaves used as target materials have fully developed chloroplasts of approximately 6–8 μm in diameter in our observation, 0.6-μm gold particles are appeared to be sufficient for delivering DNA. However, tobacco suspension cells have small, undeveloped plastids called proplastids that are approximately 2 μm in diameter, and their efficient plastid transformation has been achieved using 0.4-μm gold particles (Langbecker et al. 2004). In rice calli, target plastids are undeveloped proplastids of approximately 1.5 μm in diameter (Nakamura et al. 2009); thus, it is expected that a particle size of 0.6 μm would be unsuitable for delivering DNA into these organelles (Khan and Maliga 1999; Lee et al. 2006). However, it seems possible that using gold particles of less than 0.4 μm diameter would increase plastid transformation efficiency in rice and other plant species in which the target cells have undeveloped plastids. To date, the use of gold particles of less than 0.4 μm diameter for plastid transformation has not been reported. Therefore, we produced a series of small gold particles and evaluated whether these particles were suitable for plastid transformation. In order to evaluate the practical utility of small gold particles, we applied them to our improved tobacco plastid transformation system (Okuzaki and Tabei 2012). We also assessed the appropriate acceleration pressure for the small gold particles.

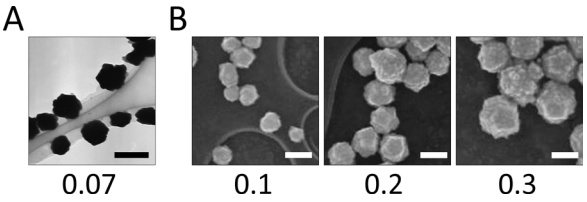

Figure 1. Micrographs of gold particles. A, TEM of 0.07-μm gold particles; bar = 0.1 μm. B, FE-SEM of 0.1-, 0.2-, and 0.3-μm gold particles; bars = 0.2 μm.

Materials and methods

Preparation of small gold particles

Gold particles were prepared according to a report by Kida et al. (2013) using a 100-ml agitated crystallizer that consisted of 100-ml three-necked round-bottom flask (SIBATA, Saitama, Japan), digital tubing pump DSP-100SA (AS ONE, Osaka, Japan), teflon PTFE stirring wing (FLON INDUSTRY, Tokyo, Japan), and three-one motor FBL3000 (TGK, Tokyo, Japan). The conditions of producing each size gold particles were as follows. 0.07 and 0.08 μm gold particles were obtained with a $HAuCl_4$ solution (1 mM) feed rate of 5.0 ml min^{-1} into 10 ml of ascorbic acid solution (10 mM) in a 100-ml agitated crystallizer. The agitation rate was maintained at 150 rpm. After feeding 20 ml $HAuCl_4$ solution, gold particle solution was collected from the crystallizer. Using the same method, 0.1-μm gold particles were obtained with a $HAuCl_4$ solution feed rate of 1 ml min^{-1}. Gold particles of 0.2 and 0.3 μm diameters were produced by the following steps. $HAuCl_4$ solution (1 mM) and ascorbic acid solution (10 mM) were fed simultaneously at a feed rate of 1 ml min^{-1} and 0.5 ml min^{-1}, respectively. Gold particles of 0.2 μm were obtained after 20 min of reduction, and 0.3-μm particles were obtained after 40 min of reduction. Average diameters of the gold particles were calculated by measuring the diameters of 450–500 individual gold particles using a transmission electron microscope (TEM; JEM-100CX; JOEL, Tokyo, Japan) or a field emission scanning electron microscope (FE-SEM; S-4500S; HITACHI, Tokyo, Japan) according to a protocol reported by Mikami et al. (2011). The gold particles of five sizes used for further studies were 0.07 μm (0.067 $\pm$ 0.016 μm), 0.08 μm (0.082 $\pm$ 0.017 μm), 0.1 μm (0.11 $\pm$ 0.02 μm), 0.2 μm (0.22 $\pm$ 0.033 μm), and 0.3 μm (0.32 $\pm$ 0.045 μm) (Figure 1; 0.08-μm particles not shown) in diameter.

DNA coating of gold particles

Solutions of the small gold particles (0.07, 0.08, 0.1, 0.2, and 0.3 μm) were centrifuged at 2000 $\times$ *g* for 2 min and resuspended in 99.5% ethanol after removing ascorbic acid. Powders of 0.6-μm and 1.0-μm gold particles (Bio-Rad Laboratories) were soaked into 99.5% ethanol. All gold particle solutions were maintained in low-retention 1.5-ml tubes (BM Equipment, Tokyo, Japan). Ethanol was removed from each solution after centrifugation at 2000 rpm for 2 min, and the particles were resuspended in sterile water at final concentrations of 40 mg ml^{-1}.

The transformation vector pNtag (Okuzaki and Tabei 2012), which contains the spectinomycin-resistance selectable marker *aadA* and the visual marker *GFP*, was extracted using the Qiagen HiSpeed Plasmid Maxi Kit (Qiagen KK, Tokyo, Japan) and used to coat the gold particles as follows. A stock solution of each gold particle (40 mg ml^{-1}) was sonicated (40 kHz) for 1–2 min before coating by floating the 1.5-ml tubes on water in an ultrasonic cleaner (USD-1R; AS ONE, Osaka, Japan). To prepare mixtures for two shots, a 20-μl stock solution of each gold particle was mixed with 4 μl of pNtag vector DNA (1 μg μl^{-1}), 20 μl of 2.5 M $CaCl_2$, and 1 mg ml^{-1} protamine sulfate from salmon (Wako Pure Chemical Industries) in a low-retention 1.5-ml tube, 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 at 2000×*g* for 5 s for the 0.6-μm and 1.0-μm gold particles, for 1 min for the 0.1-μm, 0.2-μm and 0.3-μm particles and for 2 min for the 0.08-μm and 0.07-μm particles. Each DNA/gold mixture was then washed with 100 μl of 70% ethanol, and the supernatants were removed after centrifugation under the above conditions. Finally, 22 μl of >99.5% ethanol were added to the DNA/gold pellets, which were gently suspended by pipetting. After DNA coating, the particles were resuspended in ethanol. Although the 0.6-μm gold particle precipitate could be resuspended by pipetting and gentle vortexing, the smaller gold particles were difficult to resuspend due to aggregation; however, they could be resuspended by sonicating for 2–4 s. Therefore, all the DNA/gold particles were resuspended by sonication.

Plastid transformation

The DNA/gold mixtures (10 μl) were applied onto a macrocarrier, dried, and bombarded on a sample plate containing 50 pieces of tobacco leaf (5×5 mm) as described by Okuzaki and Tabei (2012). Two shots per plate were performed using a 1100-psi (7.6 MPa) or 1350-psi (9.3 MPa) rupture disc under partial vacuum (711 mmHg) in a PDS-1000/He system (Bio-Rad Laboratories). The distance between the stopping screen and sample plate was 6 cm. With two shots, a total of 4 μg of the pNtag vector and 0.8 mg of gold particles was delivered per plate. Preparation of tobacco (*N. tabacum* L. cv. Petit Havana SR1) and spectinomycin (500 mg l^{-1}) selection culturing were performed according to the modified tobacco plastid transformation protocol (Okuzaki and Tabei 2012).

Analysis of plastid transformants

GFP fluorescence of spectinomycin-resistant shoots or plants was observed by microscopy (Leica Microsystems, Wetzlar, Germany) with a GFP2 filter (480-nm excitation filter/510-nm barrier filter). PCR analysis was performed as follows. Total DNAs were extracted from 1×1 cm tobacco leaves by simple DNA preparation methods (Edwards et al. 1991) and dissolved in 100 μl of distilled water. The inserted fragment between *aadA* and *GFP* and *rps3*, which was used as a control for detection of plastid DNA, were amplified from 2 μl of DNA solution with the specific primer sets aadA-GFP (5′-gtgatcgccgaagtatcgac-3′/5′-cgtatgttgcatcaccttcac-3′) or rps3 (5′-ggggaaccctaccttctctg-3′/5′-ccgaaaactgaacattgctg-3′). The PCR reactions were performed using ExTaq polymerase under the following conditions: 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 60°C for 10 s, and 72°C for 30 s, and ending with 72°C for 3 min. Amplified PCR products were detected by electrophoresis with 1% agarose Me (IWAI Chemicals Company Ltd., Tokyo, Japan). A 100-bp ladder (Takara Bio, Shiga, Japan) was used as the size marker.

Southern blot analysis

Total DNAs were extracted from the tobacco leaves using ISOPLANT II (Nippon Gene Co., Ltd., Tokyo, Japan). Total DNA (2.5 μg was digested with *Cla*I (New England Biolabs Japan Inc., Tokyo Japan), fractionated on a 0.8%-agarose gel, and transferred to a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland). Detections were performed according to standard protocols with a DIG Luminescent Detection Kit (Roche Diagnostics). Specific DNA probes for the 5′ region of *trnI* (probe I, 5′-gatcagccacactgggactgag-3′/5′-gaggattcgcggcatgtcaagc-3′; Figure 3A) and *aadA* (probe II, 5′-gtgatcgccgaagtatcgac-3′/5′-ccttggtgatctcgcctttc-3′; Figure 3a) were prepared using the PCR DIG Labeling Mix (Roche Diagnostics) and Primestar GXL DNA polymerase (Takara Bio).

Statistics

The statistical analysis was performed with JMP®8 (SAS Institute Inc., Cary, NC, USA). Comparisons between groups were made with the paired Student's *t*-test. Statistical significance was defined as $p<0.05$.

Results

Stable plastid transformants were obtained with 0.07-μm gold particles

We evaluated whether a general bombardment pressure with a rupture disk of 1100 psi was appropriate with small gold particles. We used the smallest gold particles (0.07 μm, Figure 1A) to compare helium pressures of 1100 psi and 1350 psi using specific rupture discs, with the 0.6-μm and 1.0-μm gold particles as normal and larger size controls, respectively. Two plates were bombarded for each combination of particle size and acceleration pressure. Each experiment with two plates was repeated thrice. Plastid transformants were obtained from all treatment combinations. Plastid transformants were analyzed by GFP fluorescence (Figure 2A) and PCR analysis (Figure 2B). Three independent plants containing pNtag-integrated plastid transformants were analyzed by Southern blotting, and the results indicated that the insert region of pNtag had integrated at the target site (data not shown). The number of plastid

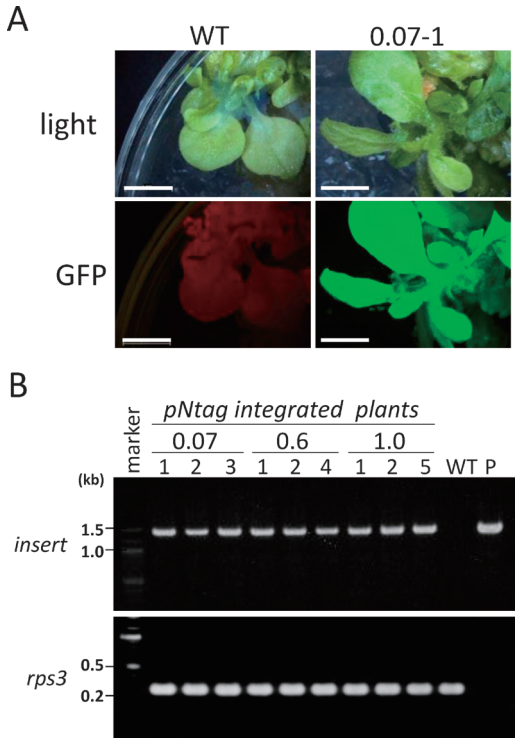

Figure 2. GFP fluorescence and PCR analysis of pNtag-integrated plants. A, Plants under the light (upper panels) and under the GFP2 filter (bottom panels) are shown. The wild-type (WT) shows the red autofluorescence signals of chlorophyll, and a plant (0.07–1) transformed with pNtag-coated 0.07-μm gold particles at 1100 psi shows strong green GFP signals. Bars=5 mm. B, To detect insertion of pNtag, a 1.48-kb fragment between the *aadA* and *GFP* expression cassettes was amplified by PCR. A 0.28-kb fragment of *rps3* was amplified for detecting plastid DNA. The upper numbers above the lanes indicate the size of the gold particles (0.07, 0.6, and 1.0 μm) used for the transformation, and the lower numbers indicate the transformants. P, pNtag plasmid DNA; marker, 100-bp ladder.

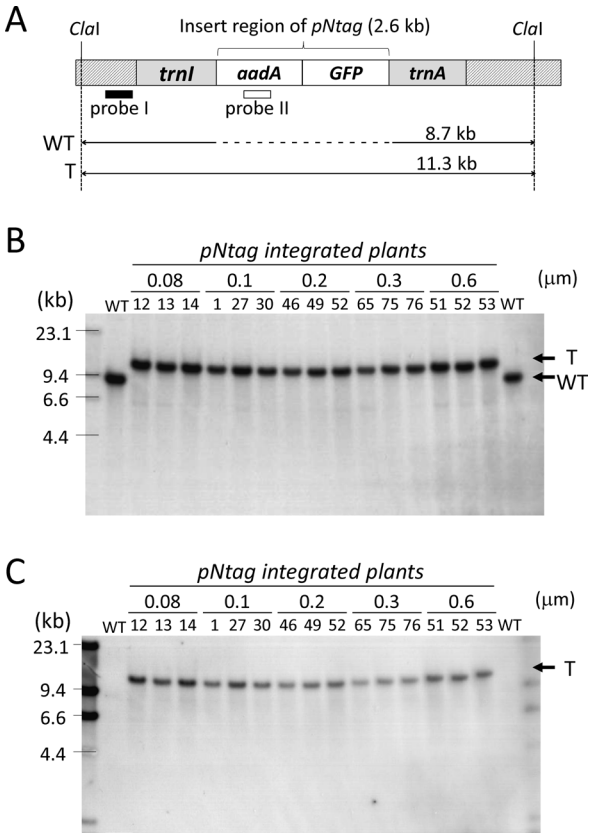

Figure 3. Southern blot analysis of pNtag-integrated plants. A, Plastid DNA map after integration of the pNtag insertion region at the target site (between *trnI* and *trnA*). Striped box, native DNA; grey box, homologous region of native DNA; white box, pNtag insertion region; *trnI* and *trnA*, homologous regions of the native DNA and pNtag vector; *aadA* and *GFP*, gene-expression cassettes of pNtag. The expected band sizes on Southern blot analysis are shown at the bottom of the map. B, Southern blot analysis of pNtag-integrated transformants obtained with five sizes of gold particles (0.08, 0.1, 0.2, 0.3, and 0.6 μm) with probe I. C, Southern blot analysis with probe II. As expected, the wild-type (WT) yielded an 8.7-kb band with probe I and no bands with probe II, and the transformants (T) yielded 11.3-kb bands with probe I and probe II.

transformants was counted for each repetition of each treatment. Using 0.07-μm particles, an average of 3.3±1.2 and 2.7±0.5 plastid transformants were obtained with acceleration pressures of 1100 psi and 1350 psi, respectively. With 0.6-μm particles, 7.0±2.2 and 4.3±0.9 transformants were obtained with 1100 psi and 1350 psi, respectively, and with 1-μm particles, 3.7±0.9 and 0.7±0.9 transformants were obtained with 1100 psi and 1350 psi, respectively (Table 1). The result with 1100 psi was significantly higher than that with 1350 psi for 1-μm particles ($p<0.05$); however, there was no significant difference between 1100 psi and 1350 psi for 0.07-μm and 0.6-μm particles. The combination of 0.6-μm particles and 1100 psi yielded the most transformants; however, there was no significant difference between the result for 0.6-μm particles and result for 0.07-μm or 1-μm gold particles with 1100 psi.

Application of small gold particles (0.08–0.3 μm) for plastid transformation

Next, we evaluated 0.08-μm, 0.1-μm, 0.2-μm, and 0.3-μm gold particles for plastid transformation, with 0.6-μm gold particles as the control. DNA/gold particles were introduced with one plate per experiment using 1100 psi acceleration pressure, and each experiment was repeated four times with 0.1-μm, 0.2-μm, and 0.3-μm particles and six times with 0.08-μm and 0.6-μm particles. Plastid transformants expressing GFP fluorescence were obtained with all sizes of gold particles. Gene integration into the plastid DNA target site was confirmed by GFP fluorescence and PCR analysis (data not shown). Three plastid transformants obtained with each size of gold particle (transformants 12, 13, 14 from 0.08-μm particles; 1, 27, and 30 from 0.1-μm; 46, 49, and 52 from 0.2-μm; 65, 75, and 76 from 0.3-μm; 51, 52, and 53 from 0.6-μm particles) were analyzed by Southern blotting (Figure 3B, 3C). As shown in Figure 3B, all the analyzed plants had the pNtag insertion at the target site and were

Table 1. Plastid transformants obtained with 1100- or 1350-psi acceleration pressure in gold particles of three sizes.

Gold particles (μm)	Rapture disc (psi)	No. of plastid transformants			No. of plastid transformants per experiment
		ex1	ex2	ex3	
0.07	1100	2	5	3	3.3±1.2
	1350	3	2	3	2.7±0.5
0.6	1100	6	5	10	7.0±2.2
	1350	3	5	5	4.3±0.9
1	1100	5	3	3	3.7±0.9
	1350	0	2	0	0.7±0.9

pNtag were bombarded on a sample plate. Two plates were treated as one experiment. Average data are presented as the mean±SD of the number of plastid transformants obtained per experiment.

Table 2. Plastid transformants obtained with small gold particles.

Gold particles (μm)	No. of plastid transformants						No. of plastid transformants per plate
	P1	P2	P3	P4	P5	P6	
0.08	3	4	6	7	3	13	6.0±3.5
0.1	13	6	8	2	n.t.	n.t.	7.3±4.0
0.2	8	1	0	10	n.t.	n.t.	7.3±4.3
0.3	12	3	7	15	n.t.	n.t.	9.3±4.6
0.6	7	5	5	6	1	5	4.8±1.9

pNtag were bombarded on a sample plate with 1100 psi rapture disc. One plate was treated as one experiment. Experiments were repeated 4 or 6 in each size gold particles. Average data are presented as the mean±SD of the number of plastid transformants obtained per plates. n.t.=not tested.

homoplasmic.

Transformation efficiencies were compared by the mean numbers of transformants per experiment. The transformation efficiencies with 0.08-, 0.1-, 0.2-, 0.3-, and 0.6-μm gold particles were 6.0±3.5, 7.3±4.0, 7.3±4.3, 9.3±4.6, and 4.8±1.9, respectively (Table 2). The average number of transformants obtained with 0.3-μm particles was the highest among all the tested particles; however, there was no statistical difference compared with the other particles.

Discussion

To the best of our knowledge, this is the first evaluation of the applicability of gold particles that are 0.07–0.3 μm in average diameter to plastid transformation. Because optimizing the size of the microcarrier is the first important step for particle bombardment methods, some DNA microcarrier materials and particle sizes have been evaluated in previous reports. Randolph-Anderson et al. (1997) reported that plastid transformation efficiency with 0.6-μm gold particles was 3–4 times higher than that with 1-μm gold particles or M10 tungsten (approximately 1 μm) in *Chlamydomonas*. Therefore, 0.6-μm gold particles have been used in most plant species. However, undeveloped plastids have been difficult to transform even with 0.6-μm gold particles (Lee et al. 2006). Langbecker et al. (2004) increased the transformation efficiency of tobacco cell suspensions, which have undeveloped 2-μm proplastids, using 0.4-μm gold particles. Although the transformation efficiency with 0.07-μm gold particles was lower than that with 0.6-μm particles under both pressure conditions (Table 1), there was no statistical difference. Since approximately 1.5 plastid transformants per plate were obtained under 1100 psi, we considered that 0.07-μm gold particles, which are the smallest size to be ever used, are sufficient for obtaining a practical number of plastid transformants in tobacco. Moreover, the transformation efficiency of 0.1-μm, 0.2-μm, and 0.3-μm gold particles tended to increase compared with 0.6-μm gold particles (Table 2), although statistical analysis revealed that the effectiveness of these small gold particles were the same as that of 0.6-μm gold particles. Because the particle diameter range in the Bio-Rad 0.6-μm gold powder has been reported to be 0.1–0.9 μm (Randolph-Anderson et al. 1997), Bio-Rad 0.6-μm gold powder also contains small gold particles of 0.1–0.3 μm. However, the size of the particles effective for plastid transformation was not evaluated. Small gold particles less than 0.3 μm were as effective as gold particles of 0.6-μm; therefore, it is suggested that a smaller diameter range of gold particles in 0.6-μm gold powder may be preferred for plastid transformation.

We considered that the proper acceleration pressure was important for integration of small gold particles and that higher acceleration pressure may be required for small-mass gold particles to penetrate plastids. Sanford et al. (1993) suggested that approximately 1000 psi is optimal for most applications because damage to the biological target increased markedly as the pressure

increased above 1000 psi, and Langbecker et al. (2004) showed that an acceleration pressure of 1100 psi was better than 1350 psi for plastid transformation of cell suspensions with 0.4-μm gold particles. Therefore, we compared the normal acceleration pressure of 1100 psi with a higher pressure of 1350 psi for the integration 0.07-μm gold particles. In contrast to our expectation, both 1100 psi and 1350 psi acceleration pressure was determined to be sufficient to produce plastid transformants in 0.07-μm and 0.6-μm gold particles but not in 1-μm gold particles (Table 1).

Recently, not only nanosized gold particles, but also gold-coated mesoporous silica nanoparticles (Torney et al. 2007) and gold-coated carbon nanoparticles (Vijayakumar et al. 2010) have been developed and evaluated as carriers for delivering DNA, proteins, and chemical substances to plant target cells (Ghormade et al. 2011; Moaveni et al. 2011). Nanoparticles have also been assessed for delivering siRNA (Song et al. 2010), vaccines, or chemical substances to mammalian cell (Rana et al. 2011). O'Brien and Lummis (2011) reported the integration of DNA into human cells with 40-nm gold particles with the same efficiency as that with 1-μm gold particles. However, unlike mammalian cells that have only a plasma membrane, plant cells have cell walls, and plastids have their own double membranes. Thus, it is predicted that transformation efficiency in tobacco with gold particles of less than 0.07 μm diameter, which were the smallest in our study, will gradually be reduced because of the difficulty in penetrating plastids. Silica and carbon nanoparticles would not have adequate density for penetrating plant cells without the gold surface coating (Torney et al. 2007; Vijayakumar et al. 2010). From our results, the 0.08–0.3-μm gold particles, a sufficient size range for plastid transformation, appear to be a suitable balance between sufficient density for penetrating plastids and minimal damage to the plastids.

For technical aspects of the particle bombardment method, it is preferable that DNA/gold solutions be well suspended and uniformly coated on the microcarrier film without aggregation for dispersing the particles to the target cells. Kausch et al. (1995) reported that DNA/gold particles formed extremely loose aggregates and were finely dispersed compared with tungsten particles. In our previous report, the DNA/gold solution with 0.6-μm gold particles was easily suspended by pipetting and low vortexing (Okuzaki and Tabei 2012). However, in our examination of DNA coating of small gold particles, the particles with aggregated DNA, and the aggregate could not be resuspended by pipetting and vortexing (data not shown). In order to resuspend the gold particles in ethanol, the aggregated pellet in ethanol was briefly (2–4 s) sonicated in the 1.5-ml tube. We were concerned that sonication would partially fragment the DNA. However, protamine, which is in the DNA/gold particles in our protocol, has been reported to improve the protection of condensed DNA from degradation by DNase compared with spermidine (Sivamani et al. 2009). Moreover, Wu et al. (2009) reported that condensed plasmid DNA was stabilized and remained more than 80% intact after a 30 s of sonication, whereas naked plasmid DNA was fragmented by 5 s of sonication. In our results, transformation efficiency with the control 0.6-μm pNtag-coated gold particles with protamine and treated by brief sonication was at the same level as that in our previous report, in which the 0.6-μm pNtag-coated gold particles with spermidine were not sonicated (Okuzaki and Tabei 2012). Therefore, condensing DNA with protamine is suggested to protect plasmid DNA on gold particles from fragmentation during brief sonication.

The decreased transformation efficiency with the smallest particles in this study appeared to be caused by less DNA coating efficiency on the particles due to the size of the condensed DNA. Hurst et al. (2006) demonstrated that the amount of DNA on gold particles decreased significantly with decreasing surface area. Delivered DNA is usually condensed by a polyamine, such as spermidine or protamine, for coating the surface of gold particles. In our protocol, plasmid DNA was condensed using protamine in the DNA-coating step because protamine improved nuclear transformation efficiency by providing resistance to DNase (Sivamani et al. 2008). Because protamine condenses 7.5 kb of DNA to approximately 40 nm in diameter (Allen et al. 1997), our 9.1-kb vector pNtag is estimated to be condensed to a little more than 40 nm. Balancing the surface area of the gold particles with the size of the condensed DNA, gold particles more than 0.08 μm in diameter would be considered suitable for efficiently carrying and integrating plasmid DNA into plastids.

In conclusion, we have shown that small gold particles are effective for plastid transformation. Since particle size is not only one transformation parameter, it may be necessary to adjust additional parameters, such as the particle amount or bombardment pressure, for each plant species, depending on the type of target cell. However, the application of small gold particles should encourage plastid transformation by improving DNA integration efficiency in many plant species, both those with small proplastids and those with fully developed chloroplasts in the target cells.

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