Plant Cell Rep (2009) 28:213–221
DOI 10.1007/s00299-008-0636-4

GENETIC TRANSFORMATION AND HYBRIDIZATION

Protamine-mediated DNA coating remarkably improves bombardment transformation efficiency in plant cells

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Received: 23 July 2008 / Revised: 24 October 2008 / Accepted: 26 October 2008 / Published online: 18 November 2008

Abstract We have developed a method by which remarkably higher efficiencies of transient and stable transformation were achieved in bombardment transformation of plants. Over fivefold increase in transient *gus* gene expression was achieved when rice or maize suspension cells were bombarded with gold particles coated with plasmid DNA in the presence of protamine instead of the conventional spermidine. A 3.3-fold improvement in stable transformation efficiency was also observed using rice suspension cells with the new coating approach. The coated protamine-plasmid DNA complex resisted degradation by a DNase or by rice cell extract much longer than the spermidine-plasmid DNA complex. The results from this study suggest that protamine protects plasmid DNA longer than spermidine when being delivered inside the cells, probably by forming a nano-scale complex, and thus helps improve the efficiency of particle bombardment-mediated plant transformation.

Communicated by P. Ozias Akins.

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Keywords DNA coating · DNA delivery · GUS · Plant transformation · Particle bombardment · Protamine

Abbreviations

GUS	β-Glucuronidase
hyg B	Hygromycin B
LUC	Luciferase
MU	Methylumbelliferone
MUG	4-Methylumbelliferyl-β-D-glucuronide
NOS	Nopaline synthase
pDNA	Plasmid DNA
X-Gluc	5-Bromo-4-chloro-3-indolyl β-D-glucuronic acid

Introduction

Plant transgenic technology has undergone key changes in the past 30 years. Major technological developments have been sustained by breakthrough studies by investigators all over the world (Vain 2007). However, there are still a number of bottlenecks in the technology used to transform plant species. *Agrobacterium*-based technology is the choice for nuclear transformation in plants, whenever applicable, because of its relatively simple procedure and the low number of transgene copies inserted (Vain 2007). However, *Agrobacterium*-mediated transformation cannot be applied to some plant species or genotypes due to poor infectivity and/or other related physiological parameters. For example, *Agrobacterium* transformation of major cereal crops, other than rice, still lags significantly behind (Cheng et al. 2004). Sorghum is one such crop species that is the least successful in *Agrobacterium*-mediated transformation (Shrawat and Lorz 2006). In that case, particle bombardment-mediated gene delivery (Klein et al. 1987)

offers the best alternative solution. Particle bombardment is more versatile and allows transformation of plants and other organisms that are not amenable to gene transfer using other methods. In addition, particle-mediated gene delivery is the only method reported so far to introduce foreign genes into cell organelles such as chloroplasts (Grevich and Daniell 2005). Moreover, particle bombardment is very often exclusively employed for DNA delivery in transient gene expression studies to investigate many aspects of plant gene expression for its ability to introduce DNA directly into various tissues. For example, analysis of tissue specific promoters in the tissues of interest (Rogers 1991) or to deliver DNA molecules carrying a marker gene and a chemical that is needed for transgene expression into plant cells simultaneously and release the encapsulated chemical in a controlled manner to trigger the expression of co-delivered transgenes in the cell (Torney et al. 2007).

Various devices for accelerating small particles (microprojectiles) have been designed and reported to successfully deliver DNA into plant cells (Christou et al. 1991; Finer et al. 1992; Klein et al. 1987; McCabe and Christou 1993; Sanford et al. 1993). Traditionally in all these devices, gold or tungsten particles are coated with DNA in the presence of calcium chloride and spermidine (a cationic polyamine) (Sanford et al. 1993). Although the coating process has been a standard one used by most researchers in the past 20 years, improved transformation efficiency by altering the coating process has been reported (Perl et al. 1992). One of the critical steps in the coating process, the addition of spermidine to the DNA/microprojectile mixture, was used in view of its involvement in condensation of DNA and/or DNA-gold binding mechanism (Klein et al. 1987; Perl et al. 1992; Thomas et al. 1996; Vasil et al. 1991). To some extent spermidine is also considered an agent to protect DNA from degradation by the cellular nucleases (Brune et al. 1991).

To our knowledge, the use of other cationic polyamines to substitute for spermidine in DNA coating has not been reported in plant transformation, whereas various types of cationic polymers have been employed as a vehicle to increase the efficiency of transfection in animal cells (Li and Huang 1997; Wagner et al. 1991). One such cationic polyamine is protamine. Protamines are small peptides (~31 AA, MW 4,000–4,250), which are very basic due to their high arginine content. They are naturally occurring substances found only in animal sperms and are usually purified from the mature testes of certain fishes, such as salmon and herring. Protamine's role in sperm is to bind with DNA, in place of histone as in other cells, assisting in forming a compact structure and delivering the DNA to the nucleus of the egg after fertilization (Bode and Lesemann 1977; Willmitzer and Wagner 1980). Protamine has been successfully used for efficient delivery of DNA in transfection studies of mammalian cells (Sorgi et al. 1997). Furthermore it can build complexes (e.g., nanoparticles) with DNA and RNA via electrostatic interaction (Kerkmann et al. 2006). In a report for delivery of a DNA vaccine, plasmid DNA coated on gold particles using protamine had much higher percentage of supercoiled DNA than those coated with spermidine or other reagents tested (Knowle et al. 2006). In another study, Park and coworkers explored the possibility and potential of utilizing a low molecular weight protamine (LMWP, 14 AA containing 10 arginine residues) as an effective carrier for gene delivery in human embryonic kidney cells (Park et al. 2003). The in vitro capability of LMWP in cell trafficking, condensing DNA, translocating DNA inside the cells, protecting DNA from DNase I degradation, transfecting cells, and inducing cytotoxicity of the cells was thoroughly investigated in their study. They concluded that the LMWP can form a stable complex with plasmid DNA to efficiently deliver DNA into cells and to protect plasmid DNA from nuclease attack. They also reported that the LMWP yielded a higher transfection efficiency and had lower toxicity than any existing polycationic carriers (Park et al. 2003). In a similar approach, a multifunctional envelope type nano device (MEND) was developed as a non-viral system to deliver plasmid DNA, oligonucleotides, or siRNA using an octa-arginine (R8) peptide (Kogure 2007).

All these reports prompted us to investigate the potential of protamine for coating DNA onto microprojectiles in plant transformation experiments. Here we report the results using protamine to deliver DNA in rice and maize suspension cells by particle bombardment. The system reported here yielded over fivefold increases in transient expression of both species and had 3.3-fold higher efficiency in stable transformation in rice cells over the conventional spermidine coating process. We also show evidence that plasmid DNA in the protamine complex is better protected against degradation by a pure DNase or by endogenous DNase(s) present in rice cell extract than that of the spermidine complex. The longer presence of more intact plasmid DNA inside the nucleus could be a critical cause for the improved transformation efficiency.

Materials and methods

Plant materials

Suspension cell lines of rice (*Oryza sativa* cv. Nipponbare) established from mature embryo-derived callus (Sivamani and Qu 2006) were used in the transient and stable transformation assays. The maize (*Zea mays* L.) BMS (Black Mexican Sweet) suspension cell line was maintained in liquid MS medium (Murashige and Skoog 1962)

containing 2 mg/l 2,4-D with periodic subcultures every 14 days. All suspension cells were grown in Erlenmeyer flasks at 25°C in dark on a rotary shaker set at 150 rpm.

Plasmid DNA

The Plasmid DNA pRESQ70 (GenBank accession EU503043) was derived from pRESQ48 (Sivamani and Qu 2006) in which the nine nt leader sequence was removed so that the first start codon of the *gus* gene was fused directly to the *rubi3* intron 3′ splice site. The plasmid construct pRESQ74 (Fig. 3c) was made by inserting the *gus* expression cassette from pRESQ70 in the MCS region of pCAMBIA1300 (http://www.cambia.org) in order to link it with a cassette for hygromycin B selection for stable transformation experiments. In transient GUS assay experiments, the pRESQ70 plasmid DNA was co-transformed with pJD313 containing a *LUC* expression cassette to serve as an internal control to normalize the *gus* expression data (Sivamani and Qu 2006). All the plasmids used for transformation experiments were purified using the QIAGEN® plasmid midi kit (Qiagen, Valencia, CA, USA) according to the manufacturer's procedure.

Particle coating and bombardment

Gold particles (1.0 μm in diameter; Bio-Rad, Hercules, CA, USA) were coated with plasmid DNA in the presence of spermidine (Catalog No. S0266, Sigma-Aldrich, St Louis, MO, USA), or clupeine, a protamine from herring sperm (in the form of sulfate salt, Catalog No. P4505, Sigma-Aldrich) using the procedure described by Sivamani et al. (1996) and Knowle et al. (2006) with modifications. Briefly, 10 μl of freshly prepared gold particles (100 mg/ml), 10 μl of DNA (containing 2 μg total of equimolar quantities of pRESQ70 and pJD313), and 10 μl of freshly prepared 0.1 M spermidine or freshly prepared protamine solutions of various concentrations in water were mixed in sequential order. While gently vortexing, 10 μl of 2.5 M $CaCl_2$ solution was slowly added. The mixture was incubated at room temperature for 10 min and pelleted at 13,000*g* in a microfuge for 1 min. The supernatant was carefully removed, and the pellet was resuspended in 100 μl of ice cold 100% ethanol. The suspension was gently vortexed, and an aliquot of 25 μl of the suspension was drawn while vortexing and dispensed onto the center of a macrocarrier membrane. The membrane was allowed to dry completely for 2–5 min, and used immediately. Four shots were performed from each coating preparation, and each experiment included three replicates (preparations of coating). For stable transformation experiments, the procedure was the same except that plasmid pRESQ74 DNA was used (2 μg per preparation of 4 shots as mentioned before). The Petri plates containing the calluses were bombarded once with 900-psi rupture disk at a distance of 9 cm from the rupture disk. Particle bombardment was performed using the PDS-1000 He biolistic particle delivery system (Bio-Rad).

Transient GUS assays

The pDNA-coated gold particles were bombarded on rice suspension cells or maize BMS suspension cells to evaluate the efficiencies of protamine and spermidine coating. All the procedures related to the culture conditions, and transient GUS assays, etc., were as previously described (Sivamani and Qu 2006). Approximately 0.5 ml packed cell volume (PCV) rice or BMS suspension cells were evenly distributed over the surface of a circular 15 mm Whatman No. 2 filter paper disc on culture medium for particle bombardment. After 72 h incubation at 25°C, the bombarded cells from each filter were transferred into a microfuge tube and homogenized with an equal volume of 2 × GUS extraction buffer (Leckie et al. 1994) for both GUS and luciferase assays. Cell homogenates were centrifuged at 10,000*g* for 5 min at 4°C, and the supernatant was collected and assayed. The protein concentration of the supernatant was determined using the Bio-Rad protein assay kit (Bio-Rad). For GUS enzyme assay, 50 μl of the supernatant were assayed with MUG as substrate according to the method described by Gallagher (1992) using a FLUO-star BMG fluorometer (BMG Lab Technologies, Durham, NC, USA). For each bombardment, triplicate samples were assayed and expressed as pmole MU released min^{-1} mg^{-1} total protein. For luciferase activity measurement, 25 μl of the supernatant were assayed with a luciferase assay system (Promega, Madison, WI, USA) and quantified using a LUMIstar BMG luminometer (BMG Lab Technologies). Normalization of the GUS data was performed as in the following: in an experiment, the treatment with the highest LUC activity was used as the reference. GUS activities in other treatments were adjusted proportionally based on the ratio of the LUC activities in a treatment and in the reference.

Stable transformation of rice cells

Transformation of rice suspension cells (cv. Nipponbare) with the vector pRESQ74 was performed using particle bombardment described by Sivamani et al. (1996). The plasmid pRESQ74 has the *hph* gene under control of the CaMV 35S promoter for stable transformation selection against hygromycin B (hyg B). Approximately 500 μl PCV of the rice suspension cells were used for each shot. Eighteen hours after bombardment, the cells on each filter disc was resuspended in 10 ml of AA liquid medium (Sivamani and Qu 2006) containing 0.04% phytagel

(Sigma-Aldrich). After a brief vortexing, a volume of 3 ml was evenly plated on AA solid medium containing 2.5 g/l phytagel and 30 mg/l hyg B (A.G. Scientific, San Diego, CA, USA) and incubated for 4 weeks in dark at 25°C. The hyg B resistant calluses were picked and further selected and maintained in AA solid medium containing 50 mg/l hyg B.

PCR and Southern analysis

Genomic DNA from transgenic rice suspension cells was isolated using a modified procedure of Dellaporta et al. (1983). For detecting the *hph* and the *gus* gene in the transgenic callus lines, the following primers were used in PCR reactions with approximately 100 ng of genomic DNA as template.

Hyg2	5′ GTCAGGACATTGTTGGAG 3′
Hyg4	5′ TCTCGTGCTTTCAGCTTC 3′
GUS1F	5′ CAACGAACTGAACTGGCAGA 3′
GUS1R	5′ TTTTTGTCACGCGCTATCAG 3′

A standard 35 cycle PCR was performed with ACCUPOWER™ PCR premix (Bioneer Corporation, Daejeon, Korea) at annealing temperature of 60°C. The PCR products were electrophoresed in a 1% TAE agarose gel.

For Southern blot analysis, genomic DNA (5 µg) from transgenic rice callus lines was digested with *Hin*dIII, which cut at an end of the *gus* construct, and resolved by 1.0% agarose gel electrophoresis. The fractionated DNA was transferred onto Hybond-N+® nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). The blot was probed with a 759 bp PCR-generated DNA fragment of the *gus* coding sequence using the primers GUS1F and GUS1R. The probes were labeled with [^{32}P]-dCTP using the PRIME-IT II® random priming kit (Stratagene, Cedar Creek, TX, USA) according to the manufacturer's protocol. Hybridization and washings of filters were performed using procedures provided by Amersham. Washed filters were exposed to BIOMAX MS film (Eastman Kodak, Rochester, NY, USA).

Time course assay with RQ1 DNase

Multiple tubes containing 10 µl aliquots of the DNA-protamine (or spermidine)-gold ethanol suspension in each were centrifuged at 16,000*g* for 30 s, the supernatant was removed and the DNA coated gold particles were allowed to air dry for 1 min. To the dried gold particles (which contained approximately 300 ng of plasmid DNA), 20 µl of RQ1 DNase solution in its own buffer (Promega) containing approximately 0.25 Kunitz unit was added. After mixing, the samples were incubated at 37°C for 0, 15, 30, 60 min, and 3, 6, 12, 24, 48, or 72 h, respectively. At each time point, a tube containing the reaction was collected, with 200 µl of 100% ethanol added, and the sample was immediately stored at −80°C until use. For longer incubation, after every 24 h, the tubes were briefly spun in a microfuge to bring down the condensate accumulated at the top of the tubes. After all the samples were collected, they were spun in a microfuge for 10 s, the supernatant was discarded and the pellet was air dried, and then added with 30 µl of extraction buffer which contained 50 mM Tris–HCl, pH 7.5, and 0.1% SDS (Knowle et al. 2006), and incubated at 37°C for 30 min to extract the bound DNA from the gold particles. The samples were spun in a microfuge for 10 s. The supernatant was mixed with 5 µl of 5× DNA loading buffer (Maniatis et al. 1982), and 50% of the volume was loaded in a 1% TAE agarose gel and electrophoresed using standard conditions to visualize the plasmid DNA.

Rice cell extract preparation

The cell extracts were prepared fresh for the DNA degradation assays. About 500 mg of freshly grown rice suspension cells in AA liquid medium (Sivamani and Qu 2006) were collected from the medium and blotted on a piece of sterile Whatman No. 1 filter paper disc to remove excessive medium. Cells were transferred into a microfuge tube containing four glass beads of 3 mm in diameter (Macalaster Bicknell Co., New Haven, CT, USA). The tube was then placed in a SILAMAT® S5 amalgamator (Ivoclar Vivadent AG, FL9494 Schaan Liechtenstein, Austria) and cells homogenized for 5 s. The tube was centrifuged for 2 min at 9,000*g* in a microfuge at 4°C. The supernatant was collected and re-centrifuged again for 2 min at 9,000*g* at 4°C to remove all cell debris, and was stored on ice until further use.

Time course DNA degradation assay with rice cell extract

To demonstrate DNase activity in rice cell extract, 10 µl of rice cell extract containing approximately 30 µg of total protein was added to 100 ng of plasmid DNA in 10 µl sterile distilled water. The mixture was incubated at 25°C for 0, 10, 20, 30, 40 and 80 min. The reactions were collected at each time point and immediately stored in liquid nitrogen until all the samples were ready. The entire content of each tube was mixed with 5 µl 5× DNA loading buffer and electrophoresed in a 1% TAE agarose gel using standard conditions to view the plasmid DNA.

To evaluate any protection effect of protamine for plasmid DNA in rice cell extract, 60 µl suspension (in ethanol) of DNA-coated gold particles in the presence of protamine or spermidine, prepared as described above, was

centrifuged at 9,000*g* for 30 s, the supernatant was removed and the DNA-coated gold particles were allowed to air dry for 1 min. To the dried gold particles (which contained approximately 2 μg of plasmid DNA), 200 μl of cell extract (containing approximately 300 μg total protein) was added. After mixing, samples were incubated at 25°C and an aliquot of 20 μl was taken at 0, 15, 30, 60, 180, 360, 720 min time points and immediately stored in liquid nitrogen. After all the samples were collected, they were spun in a microfuge for 10 s, the supernatant was discarded and the pellet was then added with 30 μl of extraction buffer and incubated as described above to extract the bound DNA from the gold particles. The samples were spun in a microfuge for 10 s. The supernatant was mixed with 5 μl of 5× DNA loading buffer, and 50% of its content was electrophoresed in a 1% TAE agarose gel using standard conditions to visualize the plasmid DNA.

Statistical analysis

Statistical analyses were performed using an online *t* test calculator from the following website: http://www.graphpad.com/quickcalcs/ttest1.cfm.

Results

Protamine coating enhances GUS activity in transient assays

In mammalian cells, clupeine (a protamine from herring) exhibited a better DNA coating efficiency over salmine (a protamine from salmon) (Knowle et al. 2006), and thus clupeine was used throughout the experiments reported here as the choice of protamine. Various concentrations of protamine were tested first. Concentrations of 0.5, 1 and 2 mg/ml yielded similar results in transient *gus* expression assays, which were approximately fourfold higher than 0.25 mg/l (Fig. 1a). Concentrations higher than 2 mg/ml of protamine resulted in excessive clumping of the gold particles and could not be used in the experiments. All the coating procedures, however, still included $CaCl_2$ (Sanford et al. 1993; Knowle et al. 2006). Protamine concentration of 1 mg/ml was then used to compare with three different concentrations of spermidine in the coating process [i.e., 100 mM, the standard concentration of spermidine used in most bombardment experiments; 1 mg/ml, equal w/v concentration to that of protamine; and 0.209 mM (0.03 mg/ml), equimolar concentration to 1 mg/ml of protamine]. Among the three spermidine concentrations, the highest GUS activity was achieved at 100 mM (Fig. 1b). Thus, in all future experiments, 1 mg/ml protamine and 100 mM spermidine were used in coating for comparison.

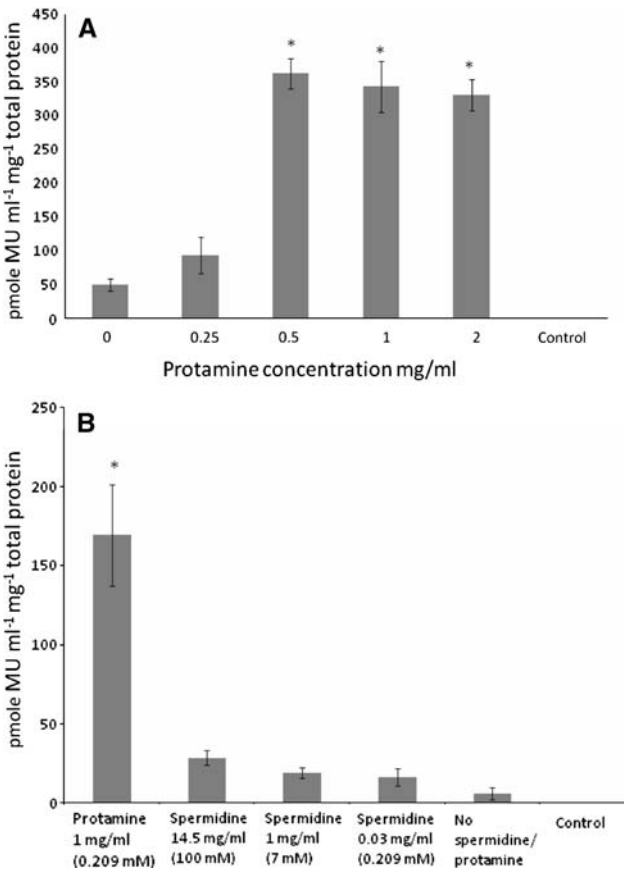

Fig. 1 Effects of concentration of protamine and spermidine on transient *gus* expression. **a** Optimum protamine concentration**.** Transient MUG assays performed on rice suspension cells with various concentrations of protamine to determine the optimum concentration to be used in this study. The *columns* represent GUS enzyme activities as normalized by LUC activities. The standard deviation bars are shown. *c* Control, cells bombarded with pJD313 only. Significant at $^*P < 0.01$, when compared with that of control (0 mg/ml) and 0.25 mg/ml concentration. **b** Comparison of various concentrations of spermidine against the optimal protamine concentration used in this study. The plasmids used and the method of normalization was the same as in **a**. Three different spermidine concentrations, namely, 100 mM (14.5 mg/ml), 7 mM (1 mg/ml) and 0.209 mM (0.03 mg/ml) were used. The 100 mM spermidine concentration is widely used in most bombardment experiments. The 7 mM concentration is equal to 1 mg/ml protamine in w/v concentration. The 0.209 mM spermidine concentration is equal in molar concentration of 1 mg/ml protamine. *Error bars* represent the standard deviation of three replicates. Control, cells bombarded with pJD313 only. Significant at $^*P < 0.001$, when compared with those of 100 mM (14.5 mg/ml), 7 mM (1 mg/ml) and 0.209 mM (0.03 mg/ml) concentrations of spermidine

The rice and maize suspension cells bombarded with the protamine-pDNA-gold particle mixture showed remarkably enhanced transient GUS enzyme activity as measured by MUG assays when compared with that of the spermidine-pDNA-gold particle mixture (Fig. 2a). A 5.7-fold increase in rice and 5.6-fold elevation in maize suspensions in transient *gus* gene expression were observed. The MUG

assay results were supported by the strikingly darker and larger blue spots in the protamine treatment (Fig. 2b). On the other hand, the cells bombarded with the spermidine-coated gold particles showed obviously less intense *gus* expression (Fig. 2b).

Protamine coating improved stable transformation efficiency

An increase in stable transformation efficiency was also observed when protamine was used to coat plasmid DNA onto gold particles. A total of 23 independent hyg B-resistant callus lines were obtained when 24 discs were bombarded with the protamine-pDNA-gold particle mixture. Using the same amount of cells, only seven hyg B-resistant callus lines were recovered when bombarded with the spermidine-pDNA-gold particle mixture. All the hyg B-resistant callus lines obtained from both the treatments were PCR positive for the *hph* gene, indicating the transgenic nature of the lines. All the seven callus lines obtained with the spermidine treatment and 22 of the 23 callus lines obtained in the protamine treatment were PCR positive for the presence of the *gus* gene. In the GUS histochemical assay, 12 of the 23 transgenic callus lines derived from the protamine treatment and five of the seven transgenic callus lines from the spermidine treatment were positive for the *gus* gene expression in the histochemical assays. Southern blot hybridization from six randomly picked callus lines derived from each experiment is shown in Fig. 3a. The *gus* probe hybridized with the transgenic callus genomic DNA revealed patterns unique for each line. Overall, stable transformation efficiency using protamine coating was 3.3-fold greater than the one with spermidine coating.

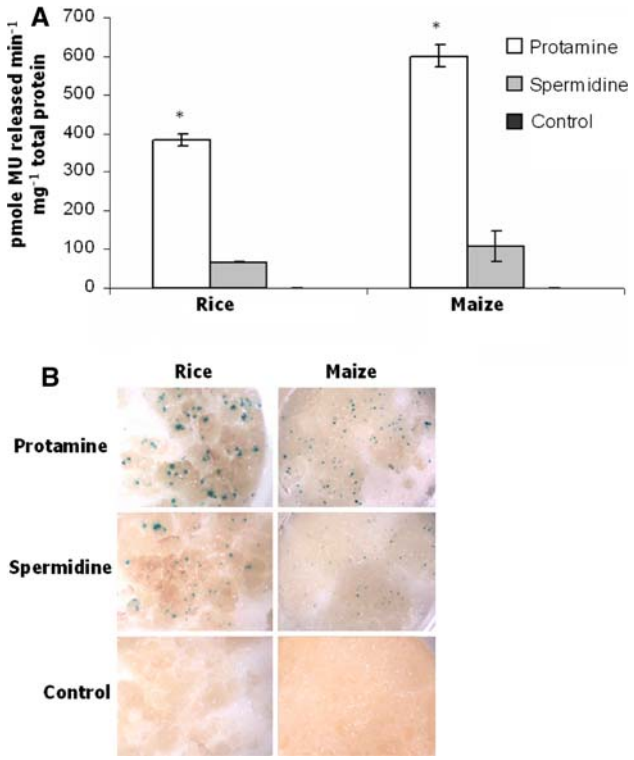

Fig. 2 Protamine coating improves transient *gus* expression in rice and maize suspension cells. **a** Fluorescence assay of transient *gus* gene expression in rice or maize suspension cells bombarded with gold particles with protamine coating at a concentration of 1 mg/ml (0.209 mM) or spermidine coating at 14.5 mg/ml (100 mM). The MUG assay data were normalized with a *LUC* gene construct co-bombarded. The *white* (protamine) and *gray* (spermidine) columns in the chart indicate the mean of pmoles of MU released min^{-1} mg^{-1} total protein in two independent experiments in rice and maize suspension cells, respectively. The standard deviation *bars* are shown. Significant at $*P < 0.001$, when compared with those of spermidine treatments. **b** Histochemical assay of transient *gus* expression of rice or maize suspension cells bombarded with protamine or spermidine coated plasmid DNA-gold particles. The control tissue was bombarded with pJD 313 plasmid only

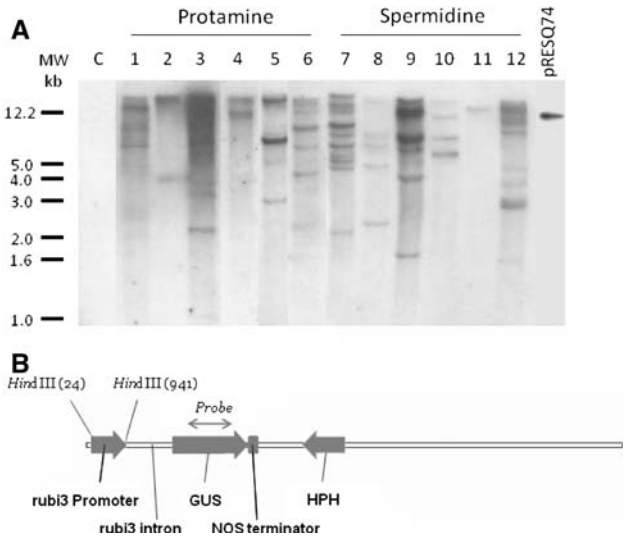

Fig. 3 Protamine coating improves stable transformation efficiency in rice suspension cells. **a** Southern blot hybridization of genomic DNA from stably transformed callus lines. The *lines* were obtained from transformation experiments using pRESQ74 plasmid DNA coated on gold particles in the presence of spermidine or protamine. Five micrograms genomic DNA from representative callus lines from each group was digested with *Hin*dIII and hybridized with ^{32}p-labeled *gus* gene probe. The *lane* marked as pRESQ74 was loaded with 1 ng of the pRESQ74 plasmid DNA digested with *Hin*dIII to serve as a positive control. The *Hin*dIII-digested genomic DNA from non-transgenic rice callus is loaded in the lane marked as *C*. Approximate marker (1 kb DNA ladder from Invitrogen, Carlsbad, CA, USA) positions are presented as *horizontal bars* on the *left* of the panel. **b** Schematic representation of the plasmid pRESQ74. The double headed *arrow* marked above the *gus* gene represents the 759 bp region of the probe used in the hybridization. The *hph* gene is in a cassette with the CaMV 35S promoter and the terminator

Protamine coating protects the intact plasmid DNA longer from degradation than spermidine coating

To understand the possible mechanism of the improved transformation efficiency with protamine coating, protection of plasmid DNA by the protamine was studied with a pure DNase as well as rice cell extract containing endogenous DNase(s). The gel analysis of plasmid DNA recovered from the gold particles after treatment with RQ1 DNase of bovine pancreas in a time course study is presented in Fig. 4. A good portion of the pDNA coated in the presence of protamine was still intact even after 12 h of incubation with ~0.25 Kunitz unit of the DNase, whereas the plasmid DNA coated with spermidine was quickly degraded and there was no trace of intact plasmid DNA detected after 60 min (Fig. 4).

In another experiment, the endogenous DNase activity in rice cell extract was demonstrated. After 10 min exposure to the cell extract, the plasmid DNA was completely degraded (Fig. 5a). The pDNA coated on the gold particles with protamine or spermidine was then examined for its resistance to degradation by rice cell extract in a time course study. From Fig. 5b, it can be seen that the pDNA coated with either reagent was protected better when compared with the naked pDNA shown in Fig. 5a. There

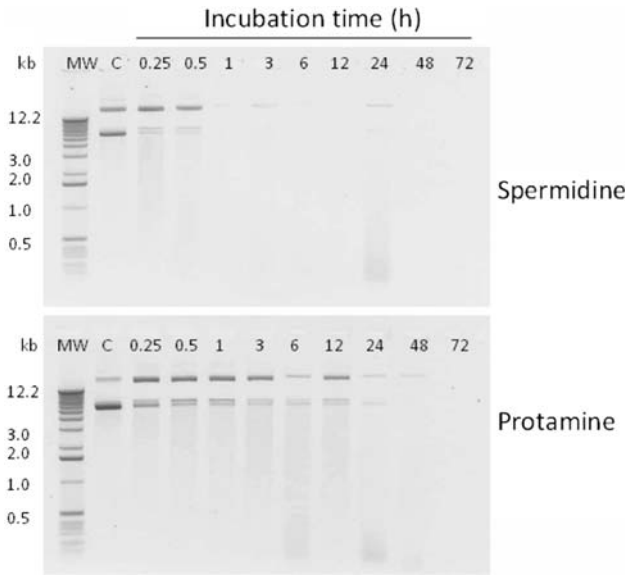

Fig. 4 Degradation of pDNA by RQ1 DNase. A time course comparison of spermidine/protamine coated gold particle plasmid DNA complexes degraded by pure RQ1 DNase from bovine pancreas. Samples were collected at various time points as indicated and the plasmid DNA was recovered from the gold particles and subjected to gel electrophoresis using a 1% TAE agarose gel stained with ethidium bromide. *Lane C* was loaded with plasmid DNA extracted from gold particles without DNase treatment to serve as a control. The 1 kb DNA ladder (Invitrogen) was loaded in the lane marked MW as a size standard in the gel

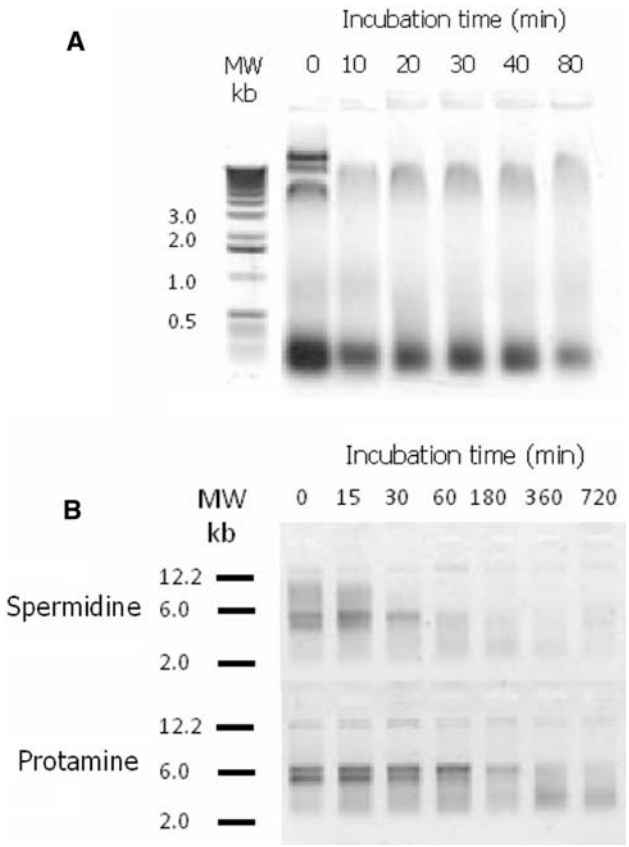

Fig. 5 Degradation of pDNA by rice cell extract. **a** Assay of DNase activity in rice cell extract. A 10 µl sample containing 100 ng of plasmid DNA was incubated at 25°C with an equal volume of cell extract (containing about 30 µg total protein). Samples were incubated for 0, 10, 20, 30, 40 and 80 min and electrophoresed in 1% TAE agarose gel. A molecular weight (MW) standard is shown in the *first lane*. **b** A time course of degradation assay of plasmid DNA coated with protamine or spermidine on gold particles after incubation with rice cell extract

was still some intact pDNA left in the spermidine treatment 30 min after incubation. However, it was clear that protamine coating was superior to spermidine coating in terms of pDNA protection. The amount of intact pDNA was almost unchanged in the first 30 min while about half of it still remained intact by 60 min in the protamine treatment when the DNA from spermidine coating was completely degraded. By 180 min of incubation, intact DNA was still visible in the protamine treatment. The data show that protamine coating protects the intactness of the plasmid DNA much longer from degradation by the endogenous cellular DNase(s).

Discussion

Particle bombardment for delivering DNA into cells is a multi-stage process involving various physical, chemical

and biological parameters. Coating of the plasmid DNA on the microprojectile carriers is one of the most important steps in this process that determines the extent of success in DNA delivery. Several investigators have worked on optimizing these parameters and conditions for various host species (Klein et al. 1987; Perl et al. 1992; Rasco-Gaunt et al. 1999). In bombardment experiments in plants, spermidine, a cationic polyamine, is conventionally used to protect the DNA from degradation and to participate in the DNA binding mechanism (Klein et al. 1987; Perl et al. 1992; Vasil et al. 1991). However, there were contradictory reports on the use of spermidine in the coating process of plasmid DNA with gold particles. Rasco-Gaunt et al. (1999) found that absence of spermidine in the DNA/gold preparation resulted in inefficient precipitation and subsequent loss of DNA through the washes. On the contrary, Perl et al. (1992) reported that spermidine had no effect on DNA precipitation onto gold particles.

Our results clearly demonstrate that increased transformation efficiencies could be achieved by coating DNA onto the microprojectiles with protamine. To our knowledge, this is the first report using an alternative reagent to coat gold particles with plasmid DNA for improved transformation by bombardment in plants. Remarkable increase in transformation efficiency was observed in both transient expression and stable transformation experiments with protamine treatment as compared to spermidine treatment. In addition to rice suspension cells, we demonstrated that similar improvement can be achieved in maize cells (Fig. 2), suggesting that this approach could be applied to nuclear transformation of other plant species too. Despite much advancement in the *Agrobacterium*-mediated transformation of plants, DNA transfer via particle bombardment is still a widely used method for nuclear transformation of many monocot and dicot species. Moreover, it is the only means used so far for chloroplast transformation (Daniell et al. 1990; Rasco-Gaunt et al. 1999). It is very likely that protamine coating will help improve chloroplast transformation efficiency as well. In addition, enhancement of gene expression by protamine coating in transient assays, which are widely used for gene expression studies, would likely improve the accuracy of the data especially in the cases when the expression levels are low.

Our experiments demonstrate that the plasmid DNA was rapidly degraded by nucleases in rice cell extract even when protected by spermidine, which likely reflected the fate of the pDNA when being delivered into the cells. Instability of pDNA is also a concern for delivery of the therapeutic genes in mammalian cells (Midoux and Monsigny 1999; Zauner et al. 1996). DNase I is known to be a major nuclease present in the serum or cytosol of mammals (Park et al. 2003). When the DNA coated on the gold particles enters the cells, it has to be protected for certain time of period in order for the DNA to have an opportunity to be integrated into the plant genome. The whole process of DNA delivery, release from microprojectiles, and integration into the plant genome is still poorly understood. There has been no report on the extent of damage the plant cell could do to the plasmid DNA coated on the microcarriers. Our results clearly showed that the DNase(s) present in plant cells degrades naked pDNA rapidly (Fig. 5a). We demonstrated pDNA was much better protected by protamine coating than spermidine when treated with either a pure DNase or rice cell extract, suggesting that the survival of pDNA of the endogenous DNase attack after being delivered into plant cells is a critical factor for transformation efficiency. Similar observation was reported by Park et al. (2003) that pDNA-LMWP complex was protected from DNase I degradation in 80 min of incubation. Knowle et al. (2006) reported that protamine retained a significantly higher amount of supercoiled pDNA when compared with other reagents including spermidine. In our experiments, extended protection of pDNA did not seem to have a substantial impact on the transgene copy number (Fig. 3a).

It was reported that the complexes protamine formed with DNA are biodegradable nanoparticles. Kerkmann et al. (2006) observed round-shaped nanoparticles, with 200–300 nm in diameter, formed after mixing protamine with 24-nt oligonucleotides in water through electrostatic interaction. They found the protamine nanoparticles strongly enhanced the immunostimulatory effects of the CpG oligonucleotides vaccine in certain cell lines. Knowle et al. (2006) studied the payload amount of plasmid DNA on the gold particles and found protamine significantly retained more supercoiled plasmid DNA than other reagents tested, and reported formation of nanoparticles between protamine and plasmid DNA on the surface of gold particles. In addition, a DNA delivery system using liposome-protamine-DNA nanoparticles in mammalian cells was also achieved (Cui et al. 2004). In plants, successful delivery of plasmid DNA coated on mesoporous silica nanoparticles (MSN), facilitated by particle bombardment, was reported, and a controlled release of a chemical packed inside the MSN was accomplished (Torney et al. 2007). Moreover, direct gene transfer by incubating plant suspension cells with DNA-nanoparticles was recently reported (Pasupathy et al. 2008). Together with the results we present here, it appears that nanotechnology may have wide applications in gene delivery and expression study in plants.

Acknowledgments The Plasmid DNA construct pJD313 from Dr. J. Sheen, and technical assistance from S. Srilaxmi are gratefully acknowledged. The authors are also thankful to Dr. A. Weissinger for helpful discussion.

References

Bode J, Lesemann D (1977) Anatomy of cooperative binding between protamines and DNA. Hoppe Seylers Z Physiol Chem 358:1505–1512

Brune B, Hartzell P, Nicotera P, Orrenius S (1991) Spermine prevents endonuclease activation and apoptosis in thymocytes. Exp Cell Res 195:323–329

Cheng M, Lowe BA, Spencer TM, Ye XD, Armstrong CL (2004) Factors influencing Agrobacterium-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40:31–45

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and Japonica varieties via electric-discharge particle-acceleration of exogenous DNA into immature zygotic embryos. Biotechnology 9:957–962

Cui ZG, Han SJ, Huang L (2004) Coating of mannan on LPD particles containing HPV E7 peptide significantly enhances immunity against HPV-positive tumor. Pharm Res 21:1018–1025

Daniell H, Vivekananda J, Nielsen BL, Ye GN, Tewari KK, Sanford JC (1990) Transient foreign gene-expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. Proc Natl Acad Sci USA 87:88–92

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 14:19–21

Finer JJ, Vain P, Jones MW, Mcmullen MD (1992) Development of the particle inflow gun for DNA delivery to plant-cells. Plant Cell Rep 11:323–328

Gallagher SR (1992) GUS protocols: using the GUS gene as a reporter of gene expression quantitation of GUS activity by fluorometry. Academic Press, New York, pp 47–59

Grevich JJ, Daniell H (2005) Chloroplast genetic engineering: recent advances and future perspectives. Crit Rev Plant Sci 24:83–107

Kerkmann M, Lochmann D, Weyermann J, Marschner A, Poeck H, Wagner M, Battiany J, Zimmer A, Endres S, Hartmann G (2006) Immunostimulatory properties of CpG-oligonucleotides are enhanced by the use of protamine nanoparticles. Oligonucleotides 16:313–322

Klein TM, Wolf ED, Wu R, Sanford JC (1987) High-velocity microprojectiles for delivering nucleic-acids into living cells. Nature 327:70–73

Knowle R, Werner A, DeLong RK (2006) R4 peptide-pDNA nanoparticle coated HepB vaccine microparticles: sedimentation, partitioning, and spray freeze dry bioprocesses. J Nanosci Nanotechnol 6:2783–2789

Kogure K (2007) Development of a novel artificial gene delivery system multifunctional envelope-type nano device for gene therapy. Yakugaku Zasshi J Pharm Soc Jpn 127:1685–1691

Leckie F, Devoto A, Delorenzo G (1994) Normalization of GUS by luciferase activity from the same cell extract reduces transformation variability. Biotechniques 17:52–55

Li S, Huang L (1997) Protamine sulfate provides enhanced and reproducible intravenous gene transfer by cationic liposome/DNA complex. J Liposome Res 7:207–219

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor

McCabe D, Christou P (1993) Direct DNA transfer using electric-discharge particle-acceleration (accell(Tm) technology). Plant Cell Tissue Organ 33:227–236

Midoux P, Monsigny M (1999) Efficient gene transfer by histidylated polylysine pDNA complexes. Bioconjug Chem 10:406–411

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

Park YJ, Liang JF, Ko KS, Kim SW, Yang VC (2003) Low molecular weight protamine as an efficient and nontoxic gene carrier: in vitro study. J Gene Med 5:700–711

Pasupathy K, Lin S, Hu Q, Luo H, Ke PC (2008) Direct plant gene delivery with a poly(amidoamine) dendrimer. Biotech J 3:1078–1082

Perl A, Kless H, Blumenthal A, Galili G, Galun E (1992) Improvement of plant-regeneration and gus expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures. Mol Gen Genet 235:279–284

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

Rogers SG (1991) Free DNA methods for plant transformation. Curr Opin Biotechnol 2:153–157

Sanford JC, Smith FD, Russell JA (1993) Optimizing the biolistic process for different biological applications. Methods Enzymol 217:483–509

Shrawat AK, Lorz H (2006) Agrobacterium-mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnol J 4:575–603

Sivamani E, Qu R (2006) Expression enhancement of a rice polyubiquitin gene promoter. Plant Mol Biol 60:225–239

Sivamani E, Shen P, Opalka N, Beachy RN, Fauquet CM (1996) Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. Plant Cell Rep 15:322–327

Sorgi FL, Bhattacharya S, Huang L (1997) Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther 4:961–968

Thomas TJ, Kulkarni GD, Greenfield NJ, Shirahata A, Thomas T (1996) Structural specificity effects of trivalent polyamine analogues on the stabilization and conformational plasticity of triplex DNA. Biochem J 319:591–599

Torney F, Trewyn BG, Lin VSY, Wang K (2007) Mesoporous silica nanoparticles deliver DNA and chemicals into plants. Nat Nanotechnol 2:295–300

Vain P (2007) Thirty years of plant transformation technology development. Plant Biotechnol J 5:221–229

Vasil V, Brown SM, Re D, Fromm ME, Vasil IK (1991) Stably transformed callus lines from microprojectile bombardment of cell-suspension cultures of wheat. Biotechnology 9:743–747

Wagner E, Cotten M, Foisner R, Birnstiel ML (1991) Transferrin polycation DNA complexes—the effect of polycations on the structure of the complex and DNA delivery to cells. Proc Natl Acad Sci USA 88:4255–4259

Willmitzer L, Wagner KG (1980) The binding of protamines to DNA—role of protamine phosphorylation. Biophys Struct Mech 6:95–110

Zauner W, Kichler A, Schmidt W, Sinski A, Wagner E (1996) Glycerol enhancement of ligand-polylysine/DNA transfection. Biotechniques 20:905–913