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BRIEF COMMUNICATION

Efficient biolistic transformation of the moss *Physcomitrella patens*

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Abstract

High rates of homologous recombination (HR) in comparison to other plants make the moss *Physcomitrella patens* an attractive model organism for genetic studies as well as biotechnological applications. We describe a simple protocol for the efficient biolistic transformation of protonemal tissue with minimum tissue handling steps. The transformation efficiency depends on the biolistic conditions. The bombardment of tissue with 1 μm gold particles yielded between 20 and 40 stable transformants per 1 μg of DNA. Transformation with circular plasmids generates higher frequencies of random transgene integration, whereas linear plasmids are more efficient in generating gene-targeted insertions.

Additional key words: *Helios* biolistic gun, moss protonemal tissue, particle size, random and targeted integration, regeneration.

The moss *Physcomitrella patens* is an attractive plant model (Cove 2005) to study gene function as well as a biotechnologically useful production plant (Frank *et al.* 2005). Particularly attractive features are its efficient somatic homologous recombination (HR) enabling targeted gene modification, not possible in higher plants (Schaefer 2002), and its dominant haploid phase during most of the life cycle, which make genetic changes directly evident.

Several methods have been successfully tested for delivery of DNA into *Physcomitrella* cells including even *Agrobacterium* T-DNA transfer. Currently, the method most commonly used to transform *Physcomitrella* is PEG-mediated delivery of dsDNA into protoplasts (Schaefer *et al*. 1991). This approach has several drawbacks, including the requirement for extensive tissue and protoplast manipulation, the relatively complicated procedure of PEG-mediated DNA uptake and the need to regenerate protoplasts with high efficiency under scrupulously sterile conditions. An alternative possibility is biolistic delivery of transforming DNA into intact tissue on metal microparticles (Sawahel *et al.* 1992, Cho *et al.* 1999, Bezanilla *et al.* 2003). We decided to evaluate biolistic approach in detail when we experienced only low efficiency of transformation (0.5 - 1 transformant per μg DNA) mediated by PEG.

There are two biolistic systems routinely used for acceleration of DNA-coated microparticles to penetrate tissue. The system firstly developed uses an accelerated plug (macrocarrier) carrying DNA-coated microparticles. Newer systems accelerate microparticles directly by a burst of helium from a rapidly opened valve (Gray *at al.* 1994). Gal-On *et al.* (1997) later simplified Gray's approach by showing that microparticles accelerated by a helium burst do not need a vacuum for efficient penetration of plant tissue and designed a simple hand held instrument for *in planta* transformation.

The potential of biolistic transformation has stimulated significant technical improvement and commercialization of delivery devices in recent years. The *PDS-1000/He* model represents the macrocarrier/ microcarrier system, whereas the *Helios* gene gun (both made by *BioRad*, Hercules, USA) is an acceleration device for direct bombardment of a target. The *Helios* gun uses DNA-coated gold particles, deposited on the inner wall of a plastic tube that are flushed off and accelerated by a burst of pressurized helium. However, the procedure developed by *BioRad* for coating of plasic tubing with microparticles is a time-consuming and error-prone procedure that requires a dedicated "Tubing Prep Station".

Here we describe a modified, low-cost biolistic

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Abbreviations: dsDNA - double stranded DNA; PEG - polyethylene glycol; T-DNA - transfer DNA of Ti-plasmid.
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application for delivery of the DNA-gold particle suspension to *Physcomitrella* protonemal tissue with the *Helios* gene gun and show high yields of both, random and targeted transformants. Our simple transformation protocol circumvents the technical complexity of the PEG-mediated protoplasts transformation.

For construction of the random integration vector we used plasmids and cassettes of the *pGreen* system (www.pgreen.ac.uk). To express enhanced yellow fluorescence protein (EYFP) we first modified the multiple cloning site (MCS) of the 2 × 35S cassette of the *pJIT60* vector to contain a *Nco*I site upstream of the *Eco*RI site. The whole expression cassette was then recloned between the *Sac*I and *Eco*RV of pGreenI 0029, which already contains an *NPTII* selection cassette. The *NcoI*/*Eco*RI fragment from pEYFP-C1Kana (kind gift of Dr. Y. Eshed, WIS, Rehovot, Israel) containing the EYFP coding region was then directly inserted into the cloning site to produce binary pKA127 vector (Fig. 1*A*). For delivery of linear DNA, *Apa*I site was used to cut the vector between reporter and selection cassette.

Gene-targeting vector was based on the sequence derived from the moss dehydrin gene *PpLEA2* (Phypa1_1:173331; GenBank Accession XM_001785041). Into pBS::Lea2 vector (kindly provided by Dr. A. Cuming, CPS, University of Leeds, UK) containing genomic coding region from amino acid 9 *Lea2A* and 3'-untranslated termination region *Lea2B* (Fig. 1*B*) , there was cloned in frame with 5´-Lea2 region *NPTII* amplified by PCR from pGreenI 0029 . The *NPTII* 5'-end PCR primer was designed to contain an *Nco*I site and the fragment was made blunt ended at the 5'-end by cutting with *Nco*I, and fill-in with the Klenow fragment of DNA PolI. Digestion at the 3'-end with *Eco*RI generated a fragment that could be cloned in frame with *Lea2A* between the *SmaI* and *EcoRI* sites generating the plasmid pKA133 (Fig. 1*B*). In this vector *NPTII* should only be expressed if a correct targeting event occurs, because there are two introns upstream of the *NPTII* fusion. If these are not spliced out, they will not permit *NPTII* expression, since there are 4 in-frame termination codons (3 in the first and 1 in the second intron). Vector was linearized for biolistic transformation by digestion with *Xba*I at the 3'-end of the targeting cassette.

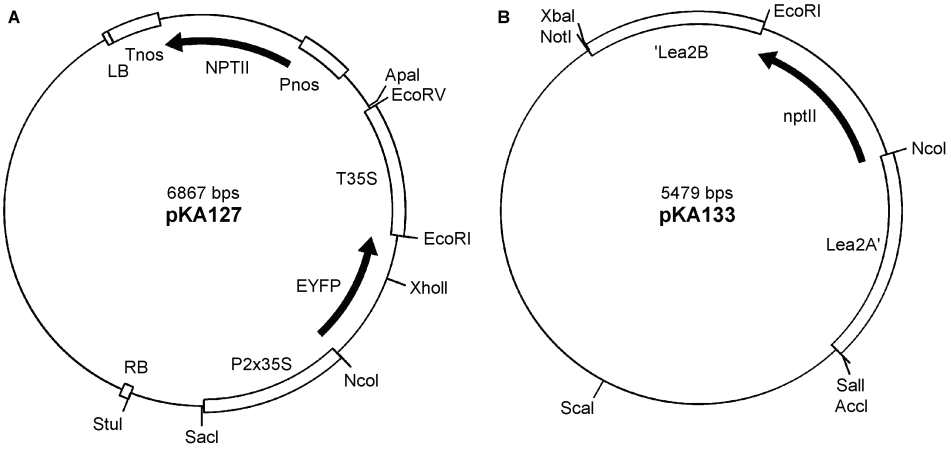

Fig. 1. Transformation vectors: *A* - pKA127 for random integration, B - pKA133 for targeted insertion into *Lea2* gene. For detail description see text.

The wild-type moss *Physcomitrella patens* Grandsen was grown on Petri plates with modified PPNH4 medium (Knight *et al.* 2002) with [g dm^{-3}] 0.8 $Ca(NO_3)_2 . 4 H_2O$, 0.25 $MgSO_4 . 7 H_2O$, 0.062 $FeSO_4 . 7 H_2O$, and 0.25 KH_2PO_4, pH 5.8 before autoclaving. Medium was enriched with 0.5 g dm^{-3} ammonium tartrate, *AltTES* macro- and microelements [μg dm^{-3}]: 55 $CuSO_4 . 5 H_2O$, 614 H_3BO_3, 55 $CoCl_2 . 6 H_2O$, 25 $Na_2MoO_4 . 2 H_2O$, 55 $ZnSO_4 . 7 H_2O$, 389 $MnCl_2 . 4 H_2O$, and 28 KI and solidified with 8 g dm^{-3} agar (*Duchefa*, Haarlem, The Netherlands) and overlaid with cellophane discs.

A *Helios* gun and microcarriers were used. The gun was operated according to the manufacturer's recommendation, although a new protocol for preparation of the DNA-coated microcarrier suspension and gun loading was developed. We tested four types of microcarriers, three gold particles (0.6, 1.0 and 1.6 μm), and *Tungsten M17*. The microcarriers (50 mg) were resuspended in 1 cm^3 of 100 % ethanol for 10 min to break particle clumps. Purified plasmid DNA was used either linearized or circular. The transforming DNA (2 μg in 2 mm^3) was added to the bottom of a 0.5 cm^3 Eppendorf tube and 5 mm^3 of 100 % ethanol were slowly pipetted dropwise down the side of the tube. Immediately 11 mm^3 of the particle/ethanol suspension were added to DNA/ethanol mixture, briefly vortexed and immediately used for delivery. A 5 mm^3 aliquot of DNA/ ethanol/microcarrier suspension was as a single droplet directly loaded into pieces of plastic tubing (cartridges) in the holder and the "charged" holder was then inserted into a gun. An armed *Helios* gun was then used for shooting moss tissue on Petri plates by 550 kPa helium bursts. One-week-old protonemal tissue on one plate

(equivalent of approximately 15 mg of moss dried tissue) was pooled into two "humps", approximately 3 cm in diameter and shot with particles from 3 cartridges (2 helium bursts through each cartridge). After shooting, tissue was roughly dispersed on the plate and allowed to recover on modified PPNH4 medium for 3 d without selection.

After 3 d of recovery, tissue was sheared by a homogenizer *T25* (*IKA*, Staufen, Germany) at 10 000 rpm in 8 cm^3 of distilled water and subcultured on four PPNH4 plates containing 50 μg cm^{-3} of G418 (*Gibco BRL*, Eggenstein, Germany) as needed for two or more weeks, when transformed foci were clearly visible. To select stable transformants individual foci were transferred for two-week release period onto medium without antibiotic and a second two-week selection period. Plants surviving the third round of selection were considered to be stable transformants. Transformants expressing EYFP were identified with *Leica* MZ16 fluorescence stereo-microscope equipped with a *Leica* YFP (excitation 510/20, barrier 560/40 nm) filter set.

DNA for analysis was isolated from 100 mg of 7-d-old protonema tissue using a *NucleoSpin* plant DNA kit (*Macherey-Nagel*, Düren, Germany). 7.5 μg of genomic DNA were digested with *Bam*HI, electrophoresed in a 0.7 % agarose gel and transferred to *Hybond-N*$^+$ nylon membrane (*GE-Healthcare*, Uppsala, Sweden) by alkali capillary transfer with 0.4 M NaOH and 0.6 M NaCl without UV fixation. Hybridization probe was an *Nco*I/*Xho*I fragment of pKA127 (Fig. 1*A*), labeled with digoxigenin-dUTP and used for hybridization at concentration 25 ng cm^{-3}. Labelling, hybridization and detection were carried out according to the manufacturer's instructions using a DIG DNA labeling and detection kit (*Roche*, Indianapolis, USA) and chemilumini-scence *CDP-Star* substrate (*Roche*). Chemiluminiscence signals were captured on *ChemiDoc* (*BioRad*).

To avoid damage of protonemal tissue we have tested the pressure range 400 - 800 kPa for direct shooting. The regular growth medium solidified with 8 g dm^{-3} agar shows severe signs of tissue damage when pressure over 550 kPa was used. To avoid a grouped impact pattern we found it necessary to use a diffusion screen (*BioRad*). With such a set up it was possible repeatedly to shoot plates from a distance of 5 cm and continue cultivation on the same plate. Two helium bursts were enough to remove all microcarriers from a single cartridge. During the first shot approximately 80 % of the microcarriers were flush out and remainder with the second shot.

The biolistic transformation was dependent on microcarrier size. The best yields of stable transformants per 1 μg of DNA were obtained with 1 μm spherical gold particles (20 transformants). *Tungsten M17* particles, a rod like crystals rated as 1 μm provided 9 transformants. Smaller particles 0.6 μm might not have sufficient momentum to effectively penetrate the tissue (3 transformants), whereas larger particles may disrupt tissue to an extent that prevents efficient recovery (8 transformants). In contrast to Cho *et al.* (1999) we have not detected any significant difference in transformation efficiency when using denatured DNA. In most transformants after the first round of G418 selection and in all stable transformants the yellow signal of expressed EYFP was positively detected in foci of 2 mm in diameter by fluorescence microscopy.

Construction of the targeting vector pKA133 does not allow selection without in frame intergration into the *Lea2* locus of the *Physcomitrella* genome. This is demonstrated by 5 - 10 time lower recovery of primary transformants in comparison to transformation with pKA127, where a substantial number of transient transformants expressing *NPTII* could be recovered (Table 1). The yield of transformants after the third round of G418 selection was only 30 % lower than the number of primary transformants for both, circular and linear form of the pKA133 vector.

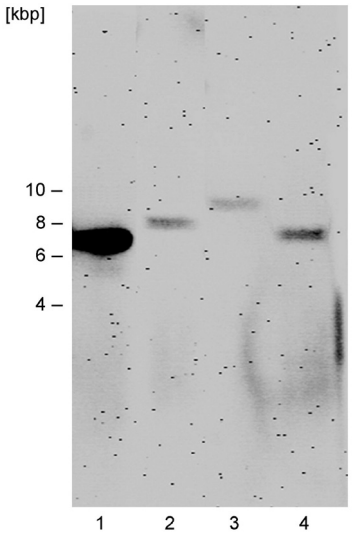

Fig. 2. Southern blot of *Physcomitrella* transgenic lines. Genomic DNA (7.5 μg) of two independent transformants was digested with BamHI (*lane* 2 and 3). *Eco*RI-linearized plasmid pKA127 at concentration 50 ng (*lane* 1) and 10× diluted in plant extract (*lane* 4) were positive controls. 502 bp digoxigenin-labeled *Nco*I/*Xho*II restriction fragment of pKA127 was used as hybridization probe.

To prove insertion into *Physcomitrella* genome several random transformants were analysed by Southern blot hybridization. For this purpose the moss genomic DNA was cut with *Bam*HI, which does not have a recognition site within transformation vector pKA127. Surprisingly in 10 transformants analyzed (Fig. 2) we found only single copy of the vector inserted in genome.

Biolistic transformation provides several advantages over PEG transformation mainly by reducing handling steps to a minimum and avoiding potential microbial contamination. Transformation is carried out on the same plate where *Physcomitrella* is grown until transfer to selective medium, and is completed within an hour. Optimized biolistic conditions, *e.g.*, the microcarrier size

and helium pressure, provide a high yield of stable transformants per μg of DNA. The limiting factor in the advantage of using the hand-held gun is the freedom of handling as well as a lower cost per shot. Direct loading of a microparticle suspension in the helium path is often used in custom-built systems (*e.g*. Gray *et al.* 1994, Gal-on *et al.* 1997) and also works well in the *Helios* gun, even though according to the manufacturer's protocol cartridges should be prepared in advance.

Table 1. Number of transformants during three cycles of G418 selection. Targeted transformation into *Lea2* locus was carried out with pKA133, whereas random transformation with pKA127.

	Circular			Linear		
	1	2	3	1	2	3
Integration to Lea 2	35	17	23	56	45	41
Random integration	322	55	40	236	46	22

The transformation efficiency depends on the form of the transforming DNA. Random integration is approximately twice as effective with a circular than with a linear vector. This suggests that random integration preferentially occurs as a single crossover event. In targeted transformation the situation is opposite and linearized pKA133 is nearly as twice as effective than circular, suggesting that linear form of transforming DNA promotes gene replacement by double crossover.

wider use of this approach is the need of a delivery device. We have adopted the *BioRad* hand-held *Helios* gene gun*,* instead of the *BioRad PDS-1000/He*, which is more widely used by the plant science community. The

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