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RESEARCH ARTICLE

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An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss, *Physcomitrella patens*

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Abstract The moss *Physcomitrella patens* is the only land plant known to date with highly efficient homologous recombination in its nuclear DNA, making it a unique model for plant functional genomics approaches. For high-throughput production of knockout plants, a robust transformation system based on polyethylene glycol-mediated transfection of protoplasts was developed and optimised. Both the DNA conformation and pre-culture of plants used for protoplast isolation significantly affected transformation efficiencies. Employing a newly developed PCR high-throughput method, the gene-targeting efficiency in more than 1,000 plants transformed with different cDNA-based knockout constructs was determined and analysed with regard to the length and intron/exon structure of the homologous gene locus. Different targeting constructs, each containing an identical selectable marker gene, were applied as batch DNA in a single transformation experiment and resulted in double-knockout plants. Thus, the fast and efficient generation of multiple targeted gene-knockouts is now feasible in *Physcomitrella*.

Keywords Ammonium tartrate · Bioreactor culture · Functional genomics · Gene-targeting · Homologous recombination

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Introduction

The moss *Physcomitrella patens* is able to integrate foreign DNA at high frequency by homologous recombination, which is a unique feature among plants. The rate of homologous recombination in the nuclear DNA of *Physcomitrella* is several orders of magnitude higher, compared with seed plants, including *Arabidopsis*. Accordingly, *Physcomitrella* has emerged as the only plant system that allows targeted knockouts with high precision and frequency, creating a new platform for plant functional genomics (Holtorf et al. 2002a). After the first reports on homologous recombination between transgenic and nuclear DNA (Schaefer and Zryd 1997; Strepp et al. 1998), a growing number of targeted gene-knockouts were published in various topics like sulfur metabolism (Koprivova et al. 2002), plant development (Imaizumi et al. 2002), fatty acid biosynthesis (Girke et al. 1998) and plastid division (Strepp et al. 1998). Because most of these reports deal with only a few targeted knockout plants in order to elucidate the gene function, these reports often lack quantitative and standardised transformation data to deduce an optimal protocol for gene-targeting (GT) in *Physcomitrella*.

The method mostly used for the stable transformation of *Physcomitrella* is polyethylene glycol

(PEG)-mediated DNA transfer to protoplasts. There are also reports on the production of stable transformants by particle bombardment (Sawahel et al. 1992; Cho et al. 1999), but so far no generation of targeted knockout mutants has been reported using this method. Thus, PEG-mediated DNA transfer was chosen for the establishment of a high-throughput transformation system, which had to be improved in order to obtain a high and reproducible number of transgenic plants. Interestingly, the protocols for PEG-mediated DNA transfer are quite consistent for both higher plants and mosses (Schaefer et al. 1991; Potrykus and Spangenberg 1995; Zeidler et al. 1999). Several factors of the transformation procedure itself have already been optimised for *Physcomitrella* by Schaefer et al. (1994). However, those authors started from protonema grown on Petri dishes, which is laborious and not well suited for a high-throughput system. In our group, large-scale cultures for mass production of protoplasts have been established (Hohe et al. 2001, 2002a; Hohe and Reski 2002) using automated bioreactor culture systems producing large amounts of uniform plant material.

Another crucial factor for a stable transformation output of *Physcomitrella* is DNA conformation (linear or circular), since Schaefer et al. (1991) described a heterogenous class of unstable transformants and assumed that the plasmid conformation might affect transformation frequency. Additionally, Ashton et al. (2000) reported on the replication of extrachromosomal elements in *Physcomitrella*. For the previous production of targeted knockout mutants, only linear DNA (Girke et al. 1998; Strepp et al. 1998; Hofmann et al. 1999; Imaizumi et al. 2002) or PCR products (Girod et al. 1999) have been used. Linear DNA has also been used for the production of a mutant collection (Nishiyama et al. 2000). Only Schaefer et al. (1991) compared linearised and supercoiled plasmids, regarding the number of stably transformed plants. Those authors obtained stable transformants with both linear and circular DNA, but their output (1–3 transgenics transformation^{-1}) was too low to compare efficiencies.

At the molecular level, GT by homologous recombination depends on the presence and extent of homologous sequences in the DNA molecules. GT can be limited by the overall length of the cDNA and by the intron/exon structure of the genomic locus. In our present study, we used partial cDNAs of different lengths from three genes with different genomic organisations, to compare transformation efficiencies and GT rates in protoplasts derived from different pre-culture conditions. Thus, a reliable high-throughput transformation system for *Physcomitrella* was established, yielding a rate of knockout plants uniquely high amongst transgenics.

We were further interested in the effects of batch transformations, i.e. using more than one DNA species per transformation, and how homologous integration would be affected by multiple-target DNAs. Batch transformations are currently used to create saturated mutant collections of *P. patens* (Nishiyama et al. 2000; Egener et al. 2002). With respect to targeted gene-knockouts, double or multiple gene disruptions are largely used for epistatis group genes like *RAD52* (Morrison and Takeda 2000; Sung et al. 2000) or to assess the function of highly similar genes that may complement each other for a single cellular function (Casal 2000). Consecutive gene-knockout in one transgenic line is often time-consuming and requires different marker genes for individual screens and selections. In this study, we demonstrate the generation of double gene-knockouts in *P. patens* in one transformation approach, using a single selection marker. For a transgenic mutant collection, this provides a tool for even larger phenotype deviations originating from more than one gene disruption per mutant.

Materials and methods

Physcomitrella cell and bioreactor culture

Protonema of *P. patens* (Hedw.) B.S.G. was grown in suspension culture as described by Reski and Abel (1985) in Knop medium containing (per litre):1,000 mg $Ca(NO_3)_2{\cdot}4H_2O$, 250 mg KCl, 250 mg KH_2PO_4, 250 mg $MgSO_4{\cdot}7H_2O$ and 12.5 mg $FeSO_4{\cdot}7H_2O$ (pH 5.8 before autoclaving). Suspension cultures (200 ml) were grown in 500-ml Erlenmeyer flasks on a rotary shaker, at 120 rpm and 25 °C with a light intensity of 50 μmol s^{-1} m^{-2} (light/dark regime of 16/8 h; Philips TLD 25).

Flask cultures for protoplast isolation were prepared following a modified procedure of Rother et al. (1994). The plant material from a culture as described above was transferred to 200 ml of a modified Knop medium containing all compounds as described above, but only 100 mg $Ca(NO_3)_2{\cdot}4H_2O$ ml^{-1} (10% of the original concentration). The dry weight of the inoculum (determined by drying samples of 10 ml at 105 °C for 2 h) was 200 mg l^{-1}; and, after transfer, the protonema was blended with an Ultra-Turrax blender (IKA, Staufen, Germany). After 4 days, the plant material was again transferred to 200 ml of fresh medium with a reduced amount of $Ca(NO_3)_2$. After another 3 days, the flask cultures were used for protoplast isolation.

Bioreactor cultures were performed semi-continuously in standard stirred-tank glass bioreactors with a working volume of 5 l or 10 l, respectively, as described in detail by Hohe et al. (2001) and Hohe and Reski (2002). The cultures were either grown in Knop medium or in Knop medium supplemented with 460 mg ammonium tartrate l^{-1}. In the case of Knop medium, the pH value of the cultures was controlled by automatic titration of 0.5 N HCl, with a setpoint of pH 4.5. When the cultures reached a dry weight of at least 100 mg l^{-1}, the semi-continuous culture mode was started, with an average dilution rate of 0.28 day^{-1}, corresponding to a daily harvest of 1.4 l. This material was directly used for protoplast isolation. Bioreactors with different treatments were always run in parallel, using identical inocula.

Protoplast isolation and transformation

From both flask and bioreactor material, protoplasts were isolated following a modified protocol of Rother et al. (1994), as described in detail by Holtorf et al. (2002b). The cell material from 200 ml suspension culture was digested by incubation in 2% Driselase (Sigma, Taufkirchen, Germany) and the resulting protoplast suspension adjusted to a density of $1.2{\times}10^6$ protoplasts ml^{-1} in 3M medium [15 mM $MgCl_2$, 0.48 M mannitol, 0.1% 2-(*N*-morpholino)ethanesulfonic acid buffer, pH 5.6]. For transformation, 250 μl

protoplast solution were mixed with 350 µl PEG solution (40% PEG 4000 in 3M medium, pH 6.0) and 100 µl 0.1 M $Ca(NO_3)_2$ containing 20 µg or 50 µg DNA. After 30 min incubation with occasional gentle mixing, the transformation solution was diluted with 3M medium every 5 min, adding 1, 2, 3 and 4 ml, respectively. This suspension was centrifuged for 10 min (45 *g*) and the pellet resuspended in 3 ml regeneration medium (medium B of Schween et al. 2003) based on Knop medium, but containing a number of additional supplements that might be necessary for regeneration of metabolic mutants. In 3-cm Petri dishes, the suspension was incubated at 25 °C for 1 day in the dark, followed by 10 days in the light (70 µmol s^{-1} m^{-2}, light/dark regime 16/8 h; Philips TLD 25). Afterwards, the suspension was plated onto cellophane covered agar dishes (1 ml per 9-cm Petri dish, using regeneration medium lacking mannitol, osmolarity not adjusted). After another 3 days, the cellophane with the cultures was transferred to selection medium containing 25 µg G418 ml^{-1} (Promega, Mannheim, Germany) for 2 weeks, followed by a release period of 2 weeks on medium without G418 and a second selection period of 2 weeks. Plants surviving the second round of selection were counted as stable transformants, since it was proven by PCR analysis and a third round of selection that more than 98% of these plants had stably integrated the transgene into the genome (Schween et al. 2002).

Molecular cloning of knockout constructs

Targeted knockout constructs were designed from protonema cDNA libraries cloned into pBSK− and tagged by the insertion of a *nptII*-disruption cassette (Bevan 1984; Yenofsky et al. 1990). This cassette, covering the *nptII* gene under the control of the *nos* promoter and terminator, was inserted centrally and in the same orientation as the cDNA (Fig. 1). pRKO25.2 (accession number AX155059), containing a 1,920-bp cDNA fragment with similarities to genes coding for Δ5 fatty acid desaturase, was cloned into the *Eco*RI site of pBSK− (reconstituting only one of the flanking *Eco*RI sites) and the *nptII* gene was inserted into the *Nru*I site. pRKO32.2 (accession number AJ583524), a 1,639-bp cDNA fragment coding for a homologue of glycosyl transferase, was cloned using *Eco*RI/*Xho*I in pBSK− and the *nptII* cassette was inserted into *Sna*BI/*Bse*RI, creating a short deletion of 157 bp. pRKO42.2 (952 bp, accession number AJ493273) codes for a homologue of bacterial maltose-*o*-acetyl transferase and was cloned using *Eco*RI/*Xho*I in pBSK− with the *nptII* cassette inserted into the *Esp*I site. pRKO45.2 (accession number AJ493276) was cloned using *Eco*RI/*Xho*I in pBSK− and *nptII* was inserted into the *Pff*MI site. This 1,945-bp cDNA shows homologies to phosphatidylcholine sterol acetyltransferases. Cloning was confirmed by restriction and sequence analysis. Plasmid DNA was amplified in *Escherichia coli* strain XL1-blue, isolated using Qiagen columns (Qiagen, Hilden, Germany) and digested with *Not*I and *Acc*65I (restriction sites located in the vector sequence), resulting in two equimolar fragments, one containing the cloning vector and the second containing the disrupted cDNA. The DNA was subsequently diluted to final concentrations ranging between 0.2 µg $µl^{-1}$ and 0.5 µg $µl^{-1}$, always in 0.1 M $Ca(NO_3)_2$, depending on the experimental setup.

Other contructs in mixes of batch transformations were derived from: (1) a myoinositol-1-phosphate synthase (accession number AJ583520), (2) a glycerol-3-phosphate acyltransferase (accession number AJ583521), (3) an acyltransferase (AJ583522), (4) an acetyl-CoA-thiolase (AJ583523), (5) a phosphohydrolase (AX155067), (6) a tocopherol-methyltransferase (AJ583525) and (7) a mevalonate diphosphate decarboxylase (AJ583526). All putative functions were assigned based on homology.

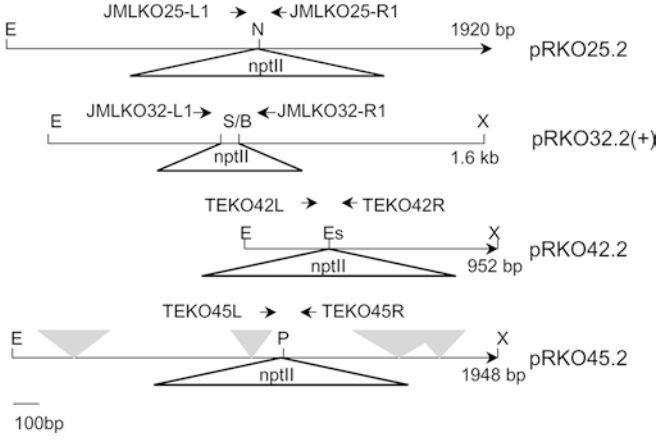


Fig. 1 Structure of *Physcomitrella* cDNA fragments encoding Δ5 fatty acid desaturase, glycosyl-transferase, maltose-*o*-acetyl transferase and phosphatidylcholine-sterol-acetyltransferase. Schematic maps are provided for the targeted knockout constructs pRKO25.2, pRKO32.2, pRKO42.2 and pRKO45.2, indicating cDNA length, insertion site of the *nptII* cassette (*triangle*), primers JMLKO25-L1/JMLKO25-R1, JMLKO32-R1/JMLKO32-L1, TEKO42L/TEKO42R and TEKO45L/TEKO45R for knockout PCR (*arrows*) and restrictions sites: *B Bse*RI, *E Eco*RI, *Es Esp*I, *EV Eco*RV, *N Nru*I, *P Pff*MI, *S Sna*BI, *X Xho*I. Introns in gene pRKO45 are indicated by *grey triangles*

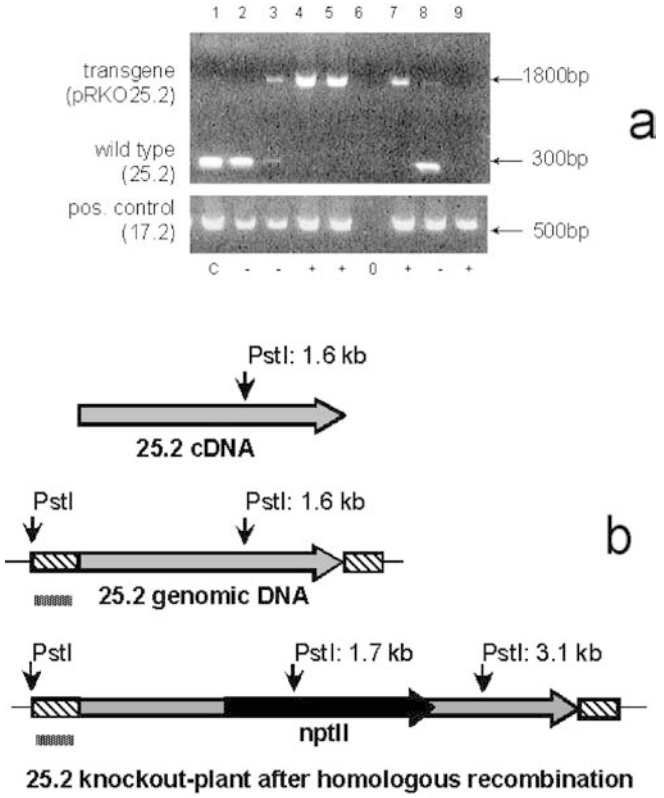
Transient transformation with a green fluorescent protein-fusion construct

For transient transformations, the DNA construct *Ppfts*Z1 (1-93)::GFP (green fluorescent protein; Kiessling et al. 2000) was transfected into protoplasts. The fusion protein contains the FtsZ1 chloroplast transit peptide at its N-terminus. Targeting of the GFP fusion protein to chloroplasts was monitored 3 days after transformation. Transformed and non-transformed protoplasts were counted with an inverted microscope, using a special filter combination (excitation filter D436/20, beam splitter 455 DCLP, longpass filter HQ 485 LP; AHF Analysetechnik, Tuebingen, Germany). Protoplast survival was determined by performing mock transformations using 0.1 M $Ca(NO_3)_2$ without DNA. Surviving cells with developing protonema were counted 10 days after protoplast isolation (total number of cells counted per transformation > 1,000).

PCR and Southern blot analysis

For the rapid analysis of targeted knockout events in *Physcomitrella*, a crude extract PCR protocol was established. Plant tissues (5 mg protonema or gametophore) were transferred to 100 µl 10× PCR buffer [0.75 M Tris-HCl, pH 8.8, 0.2 M $(NH_4)_2SO_4$, 0.1% Tween 20], incubated at 45 °C for 30 min and then heated to 68 °C for 10 min. PCR reactions were carried out according to Schween et al. (2002), using 5 µl plant extract as template in a 50-µl reaction. Primers for pRKO25.2 [JMLKO25-R1 (5′- CTC CAG TTG TCG GAG AAG GCG A-3′), JMLKO25-L1 (5′-ACT CTG GAG CAG CTT CAT GGC G-3′)], pRKO32.2 [JMLKO32-R1 (5′-ACA GTG CAC CCA CTC ATG GGC T-3′), JMLKO32-L1 (5′-AGC AGG TTC GGA TCT GAC GCT G-3′)], pRKO42.2 [TEKO42R (5′-ACG GGC CTT TCG TCT TTG CC-3′), TEKO42L (5′-AGG GGA TGG CCG GGA CAA TA-3′)] and pRKO45.2 [TEKO45L (5′-TTT TCT GGG CGT GCC CAA AG-3′), TEKO45R (5′-TTT GGC CCT TCA CCA ACC CA-3′)] were chosen to produce a specific and small product of approximately 300 bp in wild-type plants containing the insertion site of the *nptII* cartridge in the knockout constructs. Positive controls were performed by amplification of the single-copy gene 17.2 [TEKO17R (5′-XYZ-3′),TEKO17L (5′-ABC-3′)].

Southern blot analysis was carried out according to standard procedures (Egener et al. 2002). Genomic DNA (20 µg) was *Pst*I-digested and hybridised with a probe shown in Fig. 2b. DNA



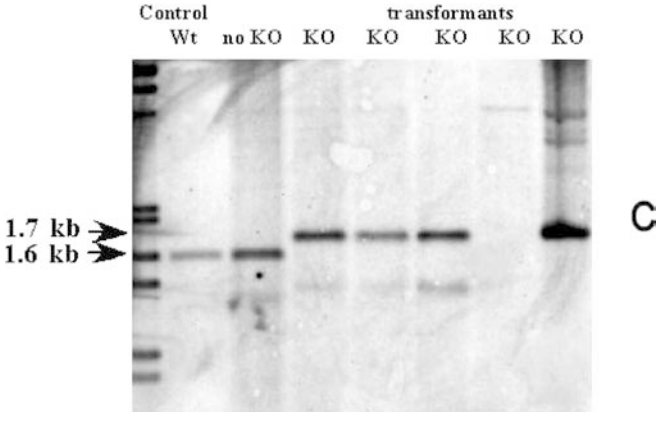


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Fig. 2a–c Molecular analysis of pRKO25.2 knockout plants. **a** Crude extract PCR analysis. Targeted knockout plants (+) show a shifted (*lanes 4*, *5*, *7*) or completely absent (*lane 9*) wild-type band, while wild type plants (−) show either only the wild-type band (*lane 2*), or both wild-type and transgene bands (*lane 3*, *8*), indicating transgene integration at a non-homologous site. Wild-type *Physcomitrella* was used as a control (*lane 1*). Extracts yielding no PCR product for either control and target DNA (*0*) were repeated (*lane 6*). Primers JMLKO25-L1/JMLKO25-R1 were used for knockout analysis of pRKO25.2. Primers TEKO17L/TE-KO17R were used for positive control amplification of the single-copy gene 17.2. **b** Schematic map of the cDNA used for transformation, the genomic locus of the wild-type plant and the genomic locus of knockout plants, indicating the recognition sites of enzyme *Pst*I (which was used for digestion of the DNA), the probe (*short grey bar below maps*, fragment length of 240 bp at the 5′ end of the genomic locus) used for Southern analysis and the expected fragment lengths (*above maps*) after hybridisation. **c** Southern analysis on knockout of gene pRKO25.2. The wild-type control plant (*Wt*) and a transformed plant that was classified as a non-knockout plant (*no KO*) by PCR analysis show a band with a size of 1.6 kb, indicating an intact wild-type locus of gene 25.2., whereas none of the knockout plants (*KO*) show this band. Four out of five plants that were classified to be knockout plants by PCR show a shifted band with a size of 1.7 kb, corresponding to integration of the knockout construct including the *nptII* cassette at the wild-type locus. One knockout plant (*lane 6*) shows neither the wild-type nor the shifted band, indicating more a complex rearrangement of the DNA at the 25.2 locus

probes were synthesised and digoxigenin-labelled by PCR, using primers JMLKO25-R3 (5′-CGC GAC TGG CTA TCT ATA GAC ACG A-3′) and JMLKO25-L3 (5′-CGC CTC ACG GTT AAG GCG AT-3′). Hybridisation and detection was carried out according to the supplier's manual (Roche Applied Science, Mannheim, Germany).

Results

High-throughput PCR is useful for rapid analysis of GT efficiencies

Molecular analysis of candidate knockout plants produced by transformation with insertion constructs is usually performed by PCR, using two sets of primers derived both from the genomic sequence of the targeted locus and from a part of the newly integrated DNA not occurring in the wild-type genomic sequence (e.g. the selection cassette). This results in two specific amplification products spanning the 3′ and the 5′ integration site only in case of proper integration of the foreign DNA by homologous recombination (see e.g. Strepp et al. 1998). Another possibility for molecular analysis is Southern blotting, using combinations of enzymes for digestion and probes that produce specific bands for wild-type and knockout plants (see e.g. Schaefer and Zryd 1997).

However, the described PCR analysis requires information on the genomic sequence of the targeted locus. In case of the production of knockout plants by transformation with cDNA-derived constructs, this information is lacking. Southern analysis, in contrast, requires a considerable amount of plant material for DNA isolation. Since our intention was to produce many different knockout plants on a large scale, neither method described above was amenable. Therefore, we developed a PCR method using primers producing a specific and small product containing the insertion site of the nptII cartridge in the knockout construct. While this primer combination in wild-type plants results in a product of about 300 bp, it shift ups or disappears in plants that carry a targeted knockout of this specific gene (Fig. 2a). For verification of this method, we performed a Southern analysis of a wild-type plant and six plants transformed with knockout construct pRKO25.2 (a *Physcomitrella* gene with similarity to Δ5 fatty acid desaturase). As shown in Fig. 2b, c, Southern analysis of the wild-type plant and a transformed plant that was

classified by PCR analysis to be non-knockout resulted in a band of 1.6 kb. In contrast, this band disappeared in all five transformed plants classified to be knockout plants by PCR analysis. From these, four plants additionally showed a band of 1.7 kb, as expected for plants in which the wild-type locus has been replaced by the knockout construct including the *nptII* cassette. Thus, the described PCR analysis was regarded as a reliable high-throughput method for large-scale molecular analysis of knockout plants.

Linear DNA is crucial for stable transformation of *P. patens*

It is known that intact bacterial plasmids are replicated in *Physcomitrella* cells without integration into the genome (Ashton et al. 2000) and that, due to homologous recombination, transformation efficiency is considerably higher when homologous DNA is used (Schaefer et al. 1991). These factors were investigated in an experiment with the aim of getting a high number of stably transformed plants. Protoplasts isolated from bioreactor-grown material with pH control were transformed in parallel either with undigested plasmid DNA pRKO25.2 or with the same plasmid cut into two equimolar fragments, representing the vector backbone and the cDNA plus *nptII* cassette. As a control, undigested plasmid DNA carrying only the *nptII* cassette without any target cDNA was used for transformation (always 20 μg DNA per transformation). Transformation with circular plasmids, either with or without target cDNA, resulted in a very high number of unstable transformants after the first selection, while on average only 0.2% of these plants survived the second round of selection, indicating that no stable integration into the genome had occurred in the majority of regenerating plants (Fig. 3). In contrast, 16% of the plants that survived the first selection after transformation with linear DNA were actually stable transgenics. Interestingly, the amount of stable transgenics obtained from transformations with circular DNA remained the same for knockout construct pRKO25.2 and the control plasmid without homologous *Physcomitrella* cDNA (Fig. 3).

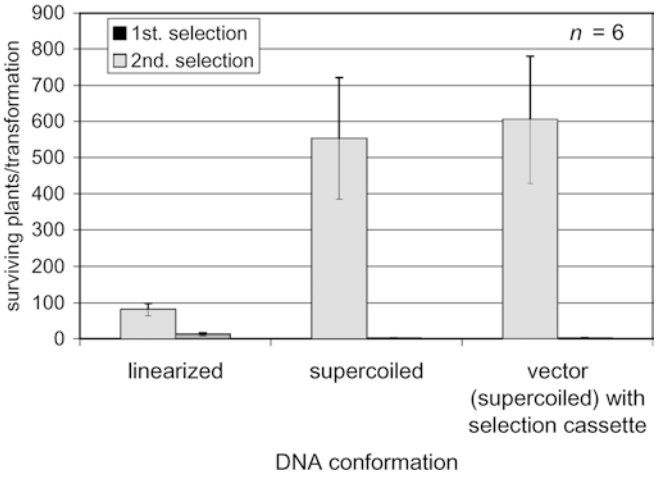

Fig. 3 Comparison of the number of transgenics after transfection with pRKO25.2 in different plasmid conformations, showing the number of surviving plants derived from protoplast transformation with different plasmid conformations of the knockout construct pRKO25.2 after the first and second selections (mean ± SD)

Pre-culturing of plant material influences transformation output

As the pre-culturing of plant material and the transformation protocol had to fit large-scale requirements, a semi-continuous bioreactor culture was developed (Hohe et al. 2001; Hohe and Reski 2002) instead of the formerly used Erlenmeyer flask cultures (Rother et al. 1994) to harvest homogenous plant material for protoplast isolation. We developed two methods for bioreactor cultures, each yielding an equally high numbers of protoplasts. In one method, the basic Knop medium was supplemented with 460 mg ammonium tartrate l^{-1} (no pH control, resulting in pH 4.4 during semi-continuous culture mode; Hohe and Reski 2002); and, in the other, cultures were grown in Knop medium with the pH value controlled at 4.5. Material from these two bioreactor cultures (5-l bioreactors, started in parallel with identical inocula) and from standard flask cultures were compared regarding the transformation efficiency by performing transformations with 50 μg DNA, using protoplasts isolated from each material in parallel. This experiment was repeated six times, using the three different targeted knockout constructs, pRKO25.2, pRKO42.2 and pRKO45.2, representing three different *Physcomitrella* genes. The knockout constructs pRKO25.2 and pRKO45.2 are comparable in length (1.9 kb), but the 45.2 genomic locus is disrupted by introns, while the 25.2 gene is not. pRKO42.2 corresponds to a short intronless genomic segment (approx. 1 kb, Fig. 1). Despite their structural differences (lengths and/or organisation of genomic locus), all cDNA clones showed a similar effect in transformation efficiency with respect to the plant material used for protoplast isolation (Table 1). Pre-culture in medium supplemented with ammonium tartrate reduced the transformation efficiencies of all targeted knockout constructs at least 4-fold, when compared with plant material pre-cultured in basic Knop medium, either in flasks (control) or in bioreactors.

In order to test whether these differences were caused by differences in protoplast survival rather than by differences in the transformation efficiency itself, the survival of protoplasts isolated from a bioreactor culture in medium supplemented with ammonium tartrate and from a pH-controlled bioreactor culture in Knop medium was investigated after mock transformations [i.e. following the transformation procedure, but using 0.1 M $Ca(NO_3)_2$ without DNA]. However, irrespective of the pre-culture, 10.4–13.7% of the cells developed

Table 1 Transformation yield and gene-targeting (GT) frequency in different culture conditions. The transformation yield (number of stably transformed plants per transformation) was tested for three different cDNAs and three different culture conditions, i.e. flask culture, bioreactor with pH control (pH 4.5) and bioreactor supplemented with ammonium tartrate. Data are means ±SD; $n = 6$. GT rate was determined by PCR analysis and calculated as the percentage of targeted knockouts per transformation

cDNA	Flask culture, Knop medium		Bioreactor, Knop medium, pH control		Bioreactor, Knop medium, ammonium tartrate	
	Yield	GT (%)	Yield	GT (%)	Yield	GT (%)
pRKO25.2	41.5 ± 18.0	42.4 ± 5.1	48.5 ± 13.0	45.6 ± 10.1	5.8 ± 3.1	30.0 ± 15.5
pRKO42.2	5.3 ± 2.2	34.8 ± 10.8	20.5 ± 10.2	31.1 ± 8.6	4.2 ± 4.0	8.5 ± 12.0
pRKO45.2	8.2 ± 2.3	18.3 ± 9.5	15.8 ± 5.3	16.6 ± 5.3	3.8 ± 3.1	11.1 ± 19.2

protonema filaments 10 days after protoplast isolation, indicating that protoplast regeneration was comparable for both culture conditions.

In a further set of experiments, differences in DNA uptake were investigated by transient transformation with a GFP-fusion construct and subsequent microscopic analysis for transformation efficiency. Here, 3 days after transformation, 2.3–3.1% of the protoplasts isolated from either culture type exhibited green fluorescence, showing a successful transformation. Thus, again, no difference in DNA uptake was detected, although we cannot exclude that differences in DNA uptake were compensated by different intensities of GFP expression.

The rate of homologous recombination was determined by scoring targeted knockout events via crude extract PCR analysis. Altogether, 734 transgenic plants were screened to monitor GT efficiencies. Protoplasts isolated from material grown in bioreactor with pH control showed targeted knockout rates comparable with flask culture for all three cDNAs tested (Table 1). In contrast, GT in bioreactor cultures supplemented with ammonium tartrate proved to be highly variable and not as reproducible as in bioreactor cultures with pH control or in flasks. Compared with the yield of stably transformed plants, GT efficiencies were only slightly affected by plant pre-culture. However, the strongly increased transformation efficiency plus the equal or better GT rates achieved in bioreactor culture with pH control combine to give an improvement in the targeted knockout production in *Physcomitrella* of up to 10-fold (Fig. 4).

Multiple gene-knockouts by single transformation

Batch transformations are currently used to generate saturated mutant collections of *Physcomitrella* by targeted gene-knockout via homologous recombination. In order to elucidate whether batch transformation is also suited to generate double knockouts in *Physcomitrella*, controlled batch transformations were performed with batches of five and ten cDNAs. The batches were compiled by mixing equal amounts of the different cDNAs prior to transformation. Total DNA concentration was always 20 μg per transformation; and experiments were repeated nine times. Two of the cDNAs fragments, pRKO25.2 and pRKO32.2, were analysed for targeted knockout events in the resulting transgenics. As a control, single gene transformations were performed for pRKO25.2 and pRKO32.2 in parallel, to compare the GT frequency in single gene transformations and batch transformations. In all, 49 plants were evaluated by PCR for targeted knockout events in single gene transformations of pRKO25.2 and 43 plants for pRKO32.2. Also, 124 plants from batch transformations with five cDNAs were analysed, as were 24 plants for transformations with ten cDNAs.

Single-gene transfections of pRKO25.2 and pRKO32.2 exhibited similar transformation outputs (approx. ten transformants per transformation) for both constructs but different GT frequencies. Of the transformants derived from transformations with pRKO25.2, 35% were targeted knockouts in gene 25.2, while 15% of the transformants generated with pRKO32.2 showed a gene disruption by targeted knockout (Fig. 5). These data correlated well with prior transformation experiments, which resulted in 40% targeted knockouts for pRKO25.2 and 12% for pRKO32.2 (data not shown). Of the 124 plants derived from batch transformation with mixes of five different cDNAs and analysed for

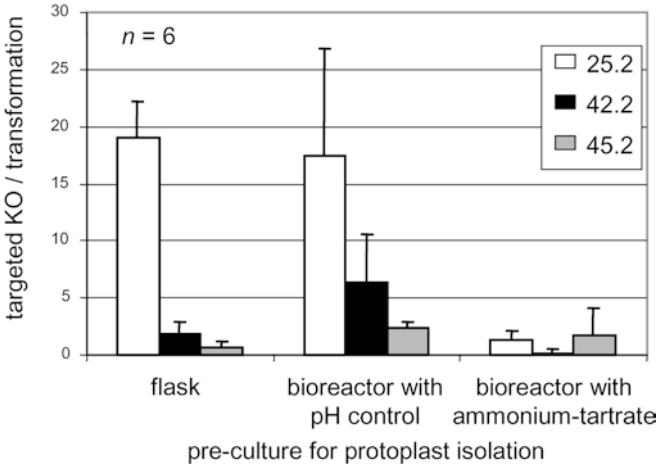

Fig. 4 Transformation efficiency in protoplasts derived from different plant pre-culture conditions, showing the number of targeted knockout mutants derived from plant material pre-cultured either in agitated Erlenmeyer flasks or in semi-continuous bioreactor cultures after transformation with knockout constructs pRKO25.2, pRKO42.2 and pRKO45.2 (mean ±SD)

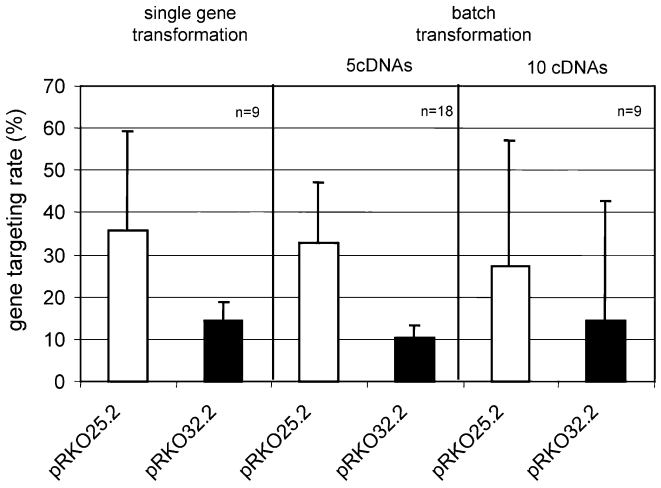

Fig. 5 Gene-targeting frequency for knockout constructs pRKO25.2 (*white columns*) and pRKO32.2 (*black columns*) in single-gene transformations and batch transformations (mean ± SD). Batch transformations were carried out with batches of either five or ten individual cDNAs. The number of replications was $n = 9$ and $n = 18$, respectively

knockout events by PCR in both loci, 40 transformants carried targeted knockouts in pRKO25.2 (32.3%) and 13 transformants in pRKO32.2 (10%). These ratios are similar to those determined for the single-gene transformation, indicating that the different cDNA fragments in the transformation do not interfere with each other's homologous integration. Using ten different cDNAs in a batch transformation, this tendency is still detectable, with 27.2% GT for pRKO25.2 and 14.2% for pRKO32.2, although the transformation output of 24 plants was very low and the analysis may be error-prone. Assuming that cDNAs in a batch transformation can create individual targeted knockouts, double knockouts in both genes should occur. Indeed, six plants generated by transformation with the mix of five different cDNAs had double knockouts in both genes 25.2 and 32.2, showing that independent GT of the different cDNAs occurs and double knockouts are possible using cDNA batches.

Discussion

PEG-mediated protoplast transformation is the transformation method of choice for *Physcomitrella* and the only method for which the generation of knockout mutants via homologous recombination has been proven (e.g. Girke et al. 1998; Strepp et al. 1998; Hofmann et al. 1999). It was first published by Schaefer et al. (1991), who also optimised various factors of the protocol, e.g. duration, PEG concentration and DNA amount (Schaefer et al. 1994).

Here, we report on improvements of this transformation protocol necessary for the production of targeted knockout plants on a large scale. Crucial factors proved to be DNA conformation and plant pre-culture. Only transformation with linear DNA yielded sufficiently high numbers of stably transformed plants. The transformation output was 10-fold higher for linear DNA molecules, compared with circular DNA. Furthermore, only linear DNA seemed to represent a suitable substrate for homologous integration. Undigested DNA was not integrated into the genome at high rates and even the presence of homologous DNA did not enhance stable DNA integration, if the transformation was performed with circular plasmid molecules. This indicates that, even if homologous DNA is offered as a circular molecule, it is not used for GT as efficiently as a linear copy.

Even more remarkable was the extremely high number of unstable transformants counted after the first selection step, if transformation was performed with circular plasmids. This is consistent with the data of Ashton et al. (2000) who showed that *Physcomitrella* is able to replicate bacterial plasmids. The high number of survivors of the first selection may be due to extrachromosomally replicating plasmids that are lost during the release period between the first and second rounds of selection.

The plant material used for protoplast isolation and subsequent transformation in previously published protocols was either grown on Petri dishes (Schaefer et al. 1991; Schaefer and Zryd 1997; Nishiyama et al. 2000) or in Erlenmeyer flasks (Reutter et al. 1998; Strepp et al. 1998), which is laborious and not suitable for a high-throughput system. Therefore, we developed a semi-continuous bioreactor culture for the large-scale production of plant material for protoplast isolation (Hohe et al. 2001; Hohe and Reski 2002). Two cultivation procedures resulted in high protoplast yields from bioreactor-grown material: either the supplementation of Knop medium with ammonium tartrate (Hohe et al. 2001), which is also widely used for cultures on solid medium in Petri dishes (first reported by Ashton and Cove; 1977), or pH control of the culture in plain Knop medium with a setpoint of 4.5 (Hohe and Reski 2002). The performance of these bioreactor cultures in transformation and GT was tested using three *Physcomitrella* genes of different length and genomic structure. Compared with the standard protocol, i.e. protoplasts isolated from standard flask cultures, plant material from bioreactors with pH control yielded a comparably high number of transformants. In contrast, this number was markedly reduced when plant material grown in a bioreactor with medium supplemented with ammonium tartrate was used for protoplast isolation and transformation. Our results show that this was not an effect of protoplast survival or DNA uptake, as shown by transient transformation with *Ppfts*Z1(1-93)::GFP. Furthermore, this material resulted in highly variable GT yields and it is therefore not well suited for the reproducible generation of targeted loss-of-function mutants in *Physcomitrella*. These effects, transformation efficiency and GT rate, add up to a much lower output of targeted knockout plants per transformation for medium supplemented with ammonium tartrate.

Ammonium tartrate supplementation is widely used for *Physcomitrella* growth media. Whether ammonium tartrate itself interferes with DNA or homologous integration cannot be explained by our data, although it is more likely that indirect influences on the cellular metabolism are involved. An indirect effect of the pH of the culture medium can be ruled out, since pH 4.4 (Hohe and Reski 2002) in the bioreactor culture supplemented with ammonium tartrate is almost identical with pH 4.5. It is known that ammonium tartrate blocks the development of *Physcomitrella* in the chloronema stage (Ashton and Cove 1977; Jenkins and Cove 1983), by providing an easily available nitrogen source. This was also confirmed for bioreactor cultures by Hohe and Reski (2002). Moreover, it has been shown here that, in contrast to bioreactor cultures supplemented with ammonium tartrate, protonema grown in a bioreactor at pH 4.5 develops caulonema. Assuming that further differentiation to caulonema and gametophores, up to the development of sexual organs, is triggered by unfavourable growth conditions (Hohe et al. 2002b), growth in the chloronema state might indicate the presence of an optimal stress-free growth environment. Several kinds of stress are known to promote recombination processes in higher plants (Lebel et al. 1993; Puchta et al. 1995; Lucht et al. 2002). Thus, in our case, the absence of nutritional stress might counteract active homologous recombination.

Interestingly, different knockout constructs reveal strong and reproducible differences, both in the number of transformants per transformation and in the GT rate. GT by homologous recombination depends on the availability and extent of homologous sequences in the DNA molecules. Exemplary for *Physcomitrella* cDNA, we used three clones of different length and genomic organisation. pRKO25.2 is not disrupted by introns on the chromosome and therefore represents a perfectly homologous sequence for recombination. pRKO45.2 is comparable with 25.2 in cDNA length but is disrupted by introns in the genomic target locus, while 42.2 has a short cDNA (1 kb). All genes are expressed in protonema tissue (data not shown). The length of homology between cDNA and genomic locus is reflected in the transformation output and the GT rate. Highest results, with up to 20 targeted knockout plants per transformation and more than 40% GT frequency, were obtained for pRKO25.2 which has the longest stretch of homology to its genomic locus. For cDNAs of comparable length but with introns in the genomic locus, GT rates were lower, as shown for pRKO45.2, with 17–18% GT frequency, yielding up to three targeted knockouts per transformation. Although shorter in length, pRKO42.2 shows a higher GT rate (more than 30%), with up to six targeted knockouts per transformation. These data correlate well with earlier *Physcomitrella* GT studies reporting eight transformants per transformation with 2,012 bp genomic DNA (Girke et al. 1998) and 14% GT frequency with 905 bp *Ppfts*Z1 cDNA (Strepp et al. 1998). These frequencies largely surpass single reports of GT in the seed plant *A. thaliana* with frequencies of 0.13% or less (Kempin et al. 1997; Puchta 2002; Hohe and Reski 2003). Obviously, the intron/exon structure of a gene is crucial for GT by cDNA and specifically influences the GT rate and transformation output of every cDNA transfection.

From these data, we deduced a protocol using plant material grown in a bioreactor without ammonium tartrate at pH 4.5 for protoplast isolation and linear DNA for high-throughput transformation of *Physcomitrella*. Using this protocol, 800 transgenic plants per week are currently produced by transformation, using transposon-tagged cDNA to generate a saturated *Physcomitrella* mutant collection with a high yield of targeted knockout plants. In this approach, transformations are performed with mixed batches of DNA (Egener et al. 2002). However, few data are available about the generation of targeted knockouts by batch transformation. Will both the transformation efficiencies and the GT rates of the single constructs be independent when transfected in a mixture, or will there be interactions or competition phenomena? Another very important question is whether multiple-knockout plants will be generated. By performing controlled batch transformations of known cDNAs with defined integration sites of the marker gene, PCR screens of the transformants for targeted knockout plants are possible. The screen for targeted knockout events and GT frequency of two cDNA fragments, pRKO25.2 and pRKO32.2, during batch transformation revealed that cDNAs transfected in batch approaches resulted in similar numbers of targeted knockout mutants, compared with single gene transformations (one targeted knockout in three transformants for pRKO25.2; one in ten for pRKO32.2). This indicates that homologous integration can occur independently at several loci on the genome and no competition for homologous integration occurs in the cell.

The generation of single-gene knockouts is well established in *P. patens*. For some approaches, double- or multiple-gene knockouts in a single cell line are required to assess complex networks or functional relations between proteins (Avery and Wassermann 1992; Casal et al. 2000; Imaizumi et al. 2002). Successive single-gene knockouts and screens in one line are often time-consuming. Using different cDNAs in one transformation, double-knockout mutants can be generated directly with only one marker gene. In batch transformations of five different cDNAs, six transformants carried double gene-knockouts of genes pRKO25.2 and pRKO32.2, giving a double-knockout rate of 4.8%. Even closely related genes like *Physcomitrella porA* and *porB* can be specifically targeted in one transformation step (unpublished data). In future work, a broader set of data will be generated, including several combinations of different knockout contructs in various numbers.

The fact that different genomic loci can be targeted simultaneously in one transformation may allow faster gene–function correlations of gene families or may reveal functional protein networks which will further

accelerate the progress of *Physcomitrella* functional genomics.

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