Protocols

High-Throughput-PCR Screen of 15,000 Transgenic *Physcomitrella* Plants

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Abstract. An easy-to-handle protocol was developed for the rapid screening of a large number of *Physcomitrella* plants. Genomic DNA was isolated without liquid nitrogen or mechanical disruption of plant material and subsequently screened by PCR. This protocol has been applied successfully to approximately 15,000 mutants (800-1000 plants per wk). About 90% of the plants could be identified as either transgenic or nontransgenic in a single PCR reaction. The results of the PCR-based identification were verified by an additional round of selection of mutants on antibiotic-containing medium.

Key words: DNA extraction, functional genomics, moss, *Physcomitrella patens*, transformants

Abbreviations: PEG, polyethylene glycol, PVP, polyvinylpyrrolidone

Introduction

The moss *Physcomitrella patens* (Hedw.) B.S.G. is the only plant that shows high rates of homologous recombination in its nuclear DNA (Reski, 1998). This makes it possible to identify gene function by targeted knockouts (e.g., Strepp et al., 1998). In a functional genomics approach, we produced a saturated mutant collection of *Physcomitrella*, using a transposon-based shuttle mutagenesis system for moss cDNA libraries with a nos-regulated npt-II selection cassette (Egener et al., submitted). After transformation and regeneration, all plants surviving 2 rounds of selection on medium containing kanamycin were tested by PCR for the presence of the npt-II-marker gene to identify transformants with stable integration into the genome.

In the cases in which no PCR product was detected, the plants were subjected to another round of selection to rule out the possibility that the DNA extraction failed.

A method to test 800-1000 moss transformants per wk needed to be developed for high-throughput PCR analysis. Using a minimum amount of plant material for DNA extraction was a prerequisite because some developmental mutants showed retarded growth during the regeneration process.

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Material and Methods

Plant material

Although *Physcomitrella patens* can be cultured photoauxotrophically on Knop medium (Reski and Abel, 1985), which contains only macroelements (250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L $MgSO_4\times7H_2O$, 1000 mg/L $Ca(NO_3)_2\times4H_2O$, and 12.5 mg/L $FeSO_4\times7H_2O$), metabolic mutants may need supplementation of the medium. Thus, a full medium has been developed with macroelements according to Knop and the addition of 50 µM H_3BO_3, 50 µM $MnSO_4\times H_2O$, 15 µM $ZnSO_4\times7H_2O$, 2.5 µM KJ, 0.5 µM $Na_2MoO_4\times2H_2O$, 0.05 µM $CuSO_4\times5H_2O$, 0.05 µM $CoCl_2\times6H_2O$, 22 µM myo-inositol, 20 µM choline chloride, 8 µM nicotinic acid, 1.5 µM thiamine-HCl, 1.2 µM pyridoxine-HCl, 0.04 µM biotin, 1.8 µM p-aminobenzoic acid, 4 µM Ca-D-pantothenate, 0.04 µM riboflavine, 50 µM adenine, 13.8 µM Na-palmitic acid, 0.25 gL^{-1} peptone, 5 mM ammonium tartrate, and 50 gL^{-1} glucose.

After PEG-mediated transformation of protoplasts, the regeneration (for 14 d), first selection (14 d culture on medium containing 25 µg/L G 418 sulfate solution, Promega), release (14 d on medium without selection), and second selection (14 d on antibiotic-containing medium) were performed on full medium. Plants were tested by PCR 11 wk after protoplast isolation. Transformation and DNA isolation were performed 3 wk after transferring the plants to a fresh medium without antibiotics.

When no PCR product was detected, the plants went through a third round of selection to rule out the possibility that the DNA extraction failed.

To determine optimal conditions for PCR analysis of the mutants, experiments with wild-type plants were performed, using primers for a wild-type gene. The results were confirmed with transgenics.

DNA extraction

Chemicals:

Extraction buffer: 90.86 g/L Tris, 26.43 g/L $(NH_4)_2SO_4$, 1 mL/L Tween 20, pH 8.8 (use a 10-fold dilution).

Protocol:
- Place 1-5 mg moss (1 gametophore or 2-3 protonema-filaments) into 50 µL of extraction buffer.
- Incubate 96-well plate for 15 min at 45°C in a thermocycler.
- Use 10 µL of the moss-buffer mixture directly as template for PCR.
- Store samples at -20°C before or after incubation. This does not affect the PCR reaction.

PCR amplification:

The total PCR reaction volume was 50 µL, and it contained each of the following:
- Template: 10 µL of the moss-buffer mixture
- 5 µL $(NH_4)_2SO_4$ buffer (10-fold, Fermentas)
- 4 µL spermidine (3 mM, Sigma)
- 6 µL $MgCl_2$ (25 mM, Promega)

- 5 µL dNTP (2 mM, Fermentas)
- 1 µL primer (20 pmol, Genescan; Primer: 5'-TGAATGAACTGCAGGACGAG-3' and 5'-AGCCAACGCTATGTCCTGAT-3')
- 0.5 µL Taq DNA polymerase (5 U/µL, Promega)
- 18.5 µL 3%-PVP-solution (3 g Polyvinylpyrrolidone in 100 mL H_2O, PVP-10 from Sigma)

The thermocycler (PTC-100; MJ Research Inc.) program was set to 94°C for 5 min and was followed by 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. It then was set at 72°C for 10 min and 4°C. Alternatively, the annealing temperature was decreased by 0.3°C per cycle (beginning the first cycle with 62°C and running the last cycle with 50°C). Amplified products were run in a 2% agarose gel, which was stained with ethidium bromide and photographed using an image analyser (Image master VDS; Pharmacia Biotech).

Results and Discussion

Different temperatures and duration of heating were tested for optimal DNA extraction. The DNA extraction was insufficient at a temperature of 37°C. There were no differences among 45°C, 65°C, and 100°C, independent of the duration of the heating (15-60 min). All the samples yielded a PCR product (n=5). The lowest temperature and shortest incubation time possible were 45°C and 15 min.

The effect of the amount of template (1 µL, 5 µL, 10 µL, 15 µL, and 20 µL) on efficiency of the PCR was tested. Between 60% and 70% of the samples yielded a product, using a 1-µL or 5-µL template. An increase of the template volume improved the efficiency, with a maximum reached at 10 µL. The npt-II-marker gene was detected in 90% of the samples.

The PCR reaction of moss DNA cultivated on a full medium did not yield a PCR product unless PVP was added. A concentration of 3% PVP was most efficient.

When moss DNA was cultivated on a minimal medium, addition of PVP was not needed. Instead, 18.5 µL of sterile water was used.

Plants might contain polysaccharides and phenolic compounds that interfere with polymerases, ligases, and restriction enzymes. In *Physcomitrella*, the medium may have affected the occurrence of these inhibiting factors by changing metabolic pathways (auxotrophic growth against heterotrophic). Polyvinylpyrrolidone absorbs polyphenols, thereby preventing their interaction with DNA (Loomis, 1974). In other protocols, PVP is added during the extraction procedure of DNA and then removed before starting the PCR reaction (John, 1992; Kim et al., 1997; Pich and Schubert, 1993). In our samples, the use of PVP gave more pronounced PCR bands than those produced by adding the compound to the extraction buffer before and during the heating process (to 45°C).

In living cells, linear multivalent polyamines, like spermidine, play a key role in maintaining cellular DNA in a compact state. This probably is driven by nonspecific electrostatic binding, resulting in charge stabilization and macromolecular condensation (Deng et al., 2000). When testing different polyamines and their influence on PCR, positive effects of spermidine were found using crude extracts from a liverwort (Fiedorow et al., 1997). Addition of spermidine in a

Table 1. Agreement between results of PCR and third round of selection of transformants for *Physcomitrella patens* (n= 224).

Treatment	Transgenic Plants (%)
Third round of selection + PCR +	89.3
Third round of selection – PCR +	1.8
Third round of selection + PCR –	6.7
Third round of selection – PCR –	2.2

Plus sign (+): PCR product/survival of plants on third round of selection
Minus sign (–): no PCR product/dying of plants on third round of selection

range of concentrations (2-10 µL spermidine, 3 mM) improved the results of the PCR in *Physcomitrella patens* (results not shown).

Efficiency

The efficiency of the PCR first was tested on 224 transformants. In 91.5% of the samples, the results of PCR and third selection matched, and stable and unstable transformants could be identified correctly (Table 1). A very small percentage of transformants gave a positive PCR result but died during a third round of selection. These probably were metabolic mutants that were not able to survive on the Knop medium used. To avoid this, a third round of selection was performed on a full medium supplemented with antibiotics. A total of 8.9% of the plants did not yield a PCR product, and, in 2.2% of these cases, the plants died during the third round of selection. These plants were instable transformants. The loss of transformed phenotypes after 1 or 2 selection passages previously was reported for *Physcomitrella* (Ashton et al., 2000). This was explained by the existence of extrachromosomal transgenic elements, which were lost by dilution. Cell division outstrips replication and/or partitioning of the extrachromosomal elements upon alleviation of selective pressure.

In 6.7% of the cases, transgenic plants did not yield a PCR product but survived a third round of selection. We assume that these plants were not identified correctly by the PCR-based method because the DNA extraction failed.

For routine identification of stable transformants, plants were tested by PCR. Only plants without a PCR band were subjected to a third round of selection. On average, 13.7% of the 15,000 plants needed to be retested (i.e., negative PCR result) and were transferred to a third round of selection. Furthermore, 11.9% of the plants survived on antibiotic-containing medium, which indicates that PCR did not produce the expected result (npt-II-cassette was present but was not detected by PCR). Thus, the efficiency (correct identification of transgenic/nontransgenic plants) of the PCR, on average, was 88.1%.

Using this method, a PCR test of three to four 96-well plates can be performed per day (including sample preparation, DNA extraction, PCR run, gel

run). DNA extraction takes approximately 60 min (45 min for sample preparation, and 15 min for heating the 96-well plate).

Up to 3 independent PCR reactions can be performed with 1 sample, and the extract can be stored for weeks in the freezer (-20°C) before or after DNA extraction.

Thus, we have developed a PCR screening procedure that can be used in a functional genomics project, as well as in subsequent in-depth analyses of the model plant *Physcomitrella*.

Acknowledgments

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