

Analysis of transformants

1 Overview

After the regeneration of protoplasts and two rounds of selection on antibiotic-containing medium the transformants can be analyzed for stable integration of the transgene by PCR-based methods. Routinely a first PCR screen of the transformants is based on the detection of stable integration of the selection marker gene. To verify the integration of constructs containing an *nptII* selection marker cassette we have developed a one-step protocol to isolate genomic DNA from a large number of transformants which can be used for PCR analyses (Schween et al., 2002). Genomic DNA can be isolated from 1-5 mg of regenerated plant tissue by heating the samples at a moderate temperature in a detergent-containing extraction buffer. Aliquots of the resulting extract can be immediately used for PCR analysis without further purification. Beside the detection of the selection marker gene further analysis of the plants is dependent on the experimental approach. Transformants which have been regenerated from transformations using gene disruption constructs have to be screened for homologous integration of the construct into the gene of interest (Fig. 1). To verify integration of the disruption construct primers are derived from sequences of the selection marker cassette and from the gene of the interest. The latter ones have to be derived from a region of the gene which was not part of the disruption construct. When using these primer combinations PCR products are only obtained when the disruption construct was integrated at the genomic locus of interest by means of homologous recombination. Furthermore, primers derived from the gene of interest flanking the selection marker cassette can be used to screen the transformants for disruption of the genomic wild type locus. Using these primers the PCR products will either correspond to the predicted size of the genomic region indicating the wild type locus or the size of the PCR product will be shifted according to the size of the inserted selection marker cassette indicating a homologous recombination event. For further analyses e.g. Southern blot analyses genomic DNA can be prepared following a detailed protocol described before (Schlink and Reski, 2002).

2 One-step isolation of genomic DNA from *Physcomitrella*

2.1 Reagents and material

1. 10 x DNA extraction buffer: To prepare 1 l dissolve 90.86 g Tris, 26.43 g $(\text{NH}_4)_2\text{SO}_4$, 1 ml Tween 20 in 800 ml H_2O , adjust pH to 8.8 with HCl; before use dilute 1:10 with H_2O .
2. 96 well PCR plate for parallel isolation of genomic DNA from 96 transformants, 96 well thermocycler.

2.2 Method

1. Fill 50 µl of 1 x extraction buffer into each well of a 96 well PCR plate. Place 1 to 5 mg moss material (approximately corresponding to one gametophore or two to three protonema filaments) from each transformant into 50 µl of extraction buffer.
2. Cover the plate using self-adhesive aluminum foil and incubate for 15 min at 45°C in a thermocycler.
3. Directly use 10 µl of the extract for PCR analysis or store it at –20°C until needed.

3 PCR based detection of transgenes

3.1 Reagents and material

1. 10 x PCR buffer with (NH₄)₂SO₄: 750 mM Tris-HCl, pH 8.8, 200 mM (NH₄)₂SO₄, 0.1% Tween 20 (buffer available from Promega), 25 mM MgCl₂ (Promega), 2 mM dNTP mix (MBI Fermentas), Taq DNA Polymerase (Promega).
2. 3 mM spermidine solution: Dissolve 38.2 mg spermidine trihydrochloride (Sigma, S2501) in 50 ml H₂O, sterilize by filtration through a 0.22 µm filter.
3. PCR primers: The PCR primers which are used for the analysis of the transformants have to be designed according to the experimental approach. Different primer combinations have to be used to detect (1) the *nptII* transgene, and (2) a homologous recombination event when generating knockout plants. A schematic overview of the possible primers is given in Figure 1. (1) Primer sequences for the detection of the *nptII* gene: Nfwd 5' TGAATGAACTGCAGGACGAG 3'; Nrev 5' AGCCAACGTATGTCCTGAT 3'; the expected size of the product is 503 bp. (2) Primers to detect correct integration of a gene disruption construct: 35SP 5' TGTCGTGCTCCACCATGTT 3'; 35ST 5' GTTGAGCATATAAGAAACCC 3'; two gene specific primers (Gs5' and Gs3') derived from the gene of interest, which must not be present in the gene disruption construct. When using the primer combinations 35SP/Gs5' or 35ST/Gs3' only PCR products are obtained when the disruption construct integrated by homologous recombination at the genomic locus of interest.
4. Thermocycler

3.2 Method

For PCR amplification use

- 10 µl of moss extract
- 5 µl 10 x PCR buffer with (NH₄)₂SO₄
- 4 µl spermidine (3 mM)
- 6 µl MgCl₂ (25 mM)

- 5 µl dNTP mix (2 mM each)
- 1 µl forward primer (20 pmol/µl)
- 1 µl reverse primer (20 pmol/µl)
- 0.5 µl Taq DNA Polymerase (5U/µl; Promega)
- 18.5 µl H₂O

The first denaturing step of the PCR is carried out for 5 min at 94°C. In total 40 PCR cycles are recommended. The annealing temperature and the extension time are dependent on the primer sequences and the length of the PCR product.

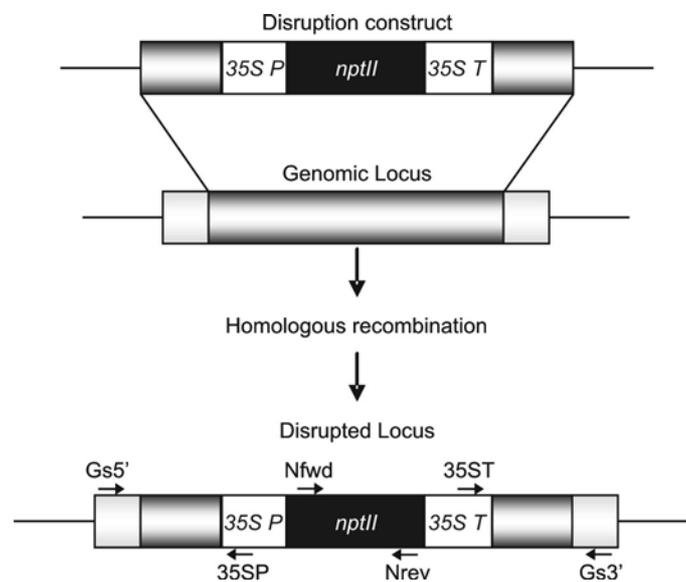


Figure 1:

PCR-based analysis of putative *Physcomitrella* knockout plants. A first PCR is carried out to confirm the integration of the selection marker gene, e.g. the *nptII* gene (primers Nfwd and Nrev). To detect homologous recombination and integration at the correct genomic locus primer combinations are used which consist of primers derived from the selection cassette (35SP and 35ST) and gene specific primers (Gs5' and Gs3') that have not been present in the disruption construct. For detection of correct 5' integration the combination Gs5' and 35SP is used whereas the combination 35ST and Gs3' confirms integration of the disruption construct at the 3' site of the gene. Furthermore, the primer combination Gs5' and Gs3' can be used to distinguish between the wild type and disrupted locus.

References

- Schlink, K., and Reski, R. (2002) Preparing high-quality DNA from moss *Physcomitrella patens*. *Plant Molecular Biology Reporter* 20, 423a-423f.
- Schween, G., Fleig, S., and Reski, R. (2002) High-throughput-PCR screen of 15,000 transgenic *Physcomitrella* plants. *Plant Molecular Biology Reporter* 20, 43-47.