Targeted knockout in *Physcomitrella*

The high efficiency of homologous recombination found in *Physcomitrella* allows manipulation of the nuclear genome on the basis of single gene knockouts. The targeted knockout of a particular gene is achieved by the transformation of *Physcomitrella* protoplasts using DNA fragments harboring the gene of interest which has been disrupted by the integration of a selection marker cassette. For the generation of these knockout constructs either cDNA fragments or stretches of genomic DNA can be used. The efficiency of homologous recombination events may in some cases be higher using constructs based on genomic sequences, because unfavorable exon-intron structures may interfere with the integration of cDNA fragments. As mentioned above the preparation of knockout constructs can be performed using cDNA or genomic DNA stretches. The approach of the generation of gene disruption constructs is depicted in Figure 1. Based on the sequence information suitable restriction sites have to be identified for the integration of the selection marker cassette. The site of integration should be located approximately in the central region of the gene of interest creating flanking regions which are roughly of similar size. When using genomic DNA the selection marker cassette has to be cloned into an exon sequence to ensure the functional deletion of the gene after the mRNA processing events. It is possible to integrate the cassette into a single restriction site present in the sequence, however the deletion of short stretches using two restriction enzymes for sub-cloning also leads to reliable results. The regions flanking the integrated selection marker cassette should preferably be more than 300 bp in size since increased recombination efficiencies were observed with larger constructs . The orientation of the cassette should be in conformity with the orientation of the respective target gene. Before transformation it is recommended to release the knockout construct from the vector backbone since transformation with linear constructs results in higher yield of stable transformants compared to transformations using supercoiled DNA (Schaefer et al., 1991; Hohe et al., 2004). The knockout construct can be transferred into *Physcomitrella* protoplasts following the standard transformation protocol described above. When using multiple knockout constructs for the transformation of *Physcomitrella* protoplasts it is also possible to generate double and triple targeted knockout lines (Hohe et al., 2004). This may be helpful when redundant genes have to be analyzed.

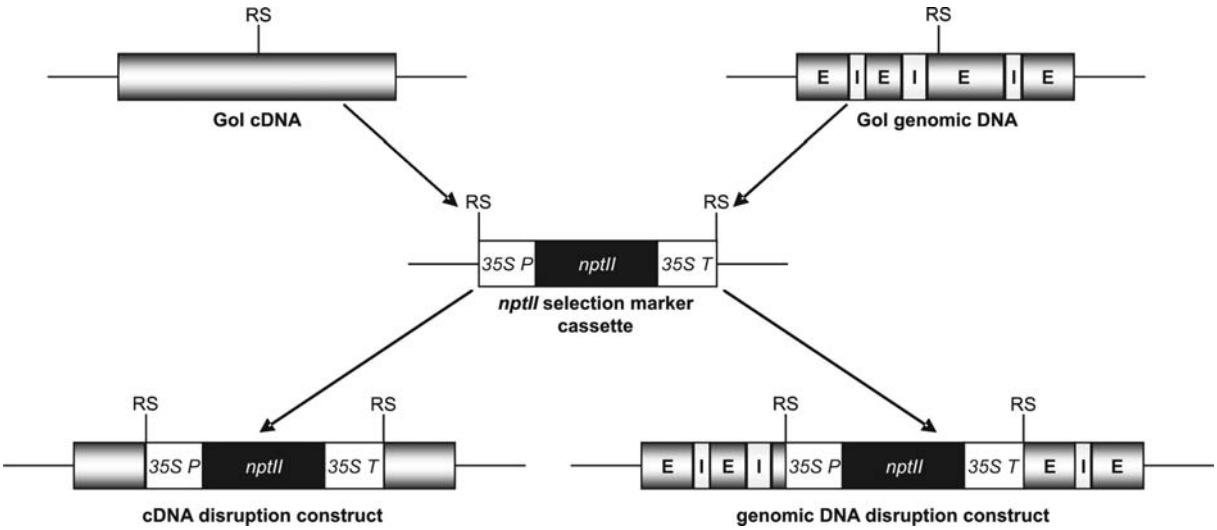

Figure 1:

Generation of targeted knockout constructs. Gene disruption constructs for the generation of targeted knockout plants can either be obtained by the insertion of a selection marker cassette (e.g. the *nptII* cassette) into cDNA sequences or by insertion into genomic DNA stretches of the gene of interest (GoI). By choosing a suitable restriction site (RS) the marker cassette is cloned into the cDNA or genomic sequence, respectively. The orientation of the *nptII* gene should comply with the orientation of the target gene. Beside using a single restriction site for cloning of the marker cassette two restriction sites can be chosen for the integration of the cassette into the gene of interest. This will result in a deletion of a short stretch of cDNA or genomic DNA, respectively (not shown). *35S P*: CaMV 35S promoter; *nptII*: neomycin phosphotransferase gene; *35S T*: CaMV 35S terminator; E: exon; I: intron. The selection marker cassette is not drawn to scale.

References

Hohe, A., Egener, T., Lucht, J. M., Holtorf, H., Reinhard, C., Schween, G., and Reski, R. (2004) An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss, *Physcomitrella patens*. Current Genetics 44, 339-347.

Schaefer, D., Zryd, J. P., Knight, C. D., and Cove, D. J. (1991) Stable transformation of the moss *Physcomitrella patens*. Molecular and General Genetics 226, 418-424.