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Transgenesis of *Physcomitrella patens*

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ABSTRACT

The moss *Physcomitrella patens* has a simple life cycle, relatively few cell types during gametophytic growth, similar responses to plant growth regulators and environmental factors as seed plants, high regeneration capacity after homogenization, and dominant haploid generation, and as such, is an excellent experimental system. In addition to being able to use most molecular and biochemical methodologies, one can apply efficient gene targeting techniques to study gene function. For transformation, polyethyleneglycol-mediated protoplast transformation and particle bombardment-mediated biolistic delivery method are both efficient methods to introduce genes into gametophytic tissue. Finally, the genome of *P. patens* genome has been sequenced and assembled.

Keywords: homologous recombination, *Physcomitrella patens*, transformation
Abbreviations: **ABA**, abscisic acid; ***ABI3***, *ABSCISIC ACID INSENSITIVE 3*; ***Act*1**, *actin* I; **Arp2/3**, actin-related protein2/3; ***CaMV***, *Cauliflower mosaic virus*; **EM**, early-methionine-labeled; ***fuc-t***, *fucosyltransferase*; **HR**, homologous recombination; **NOS**, nopaline synthetase; **PEG**, polyethyleneglycol; **RNAi**, RNA interference; ***xyl-t***, *xylosyltransferase*

CONTENTS

INTRODUCTION

Phylogenetic studies indicate that bryophytes may have formed a sister clade with vascular plants, therefore making *P. patens* an ideal plant for comparative studies of biological processes in land plants (Schaefer and Zryd 2001). *P. patens* is a monoecious moss and requires very simple growth conditions for the completion of its life cycle (Cove and Knight 1993). The dominant haploid gametophyte (**Fig. 1**) is composed of a filamentous protonema and a leafy gametophore tissue (Reski 1999; Cove 2000), both of which are good resources for cell biological studies. In addition, dominant haploidy makes mutant isolation and genetic analysis easier than in other species with dominant diploidy. *P. patens* possess the ability to regenerate protonemal tissue from any tissues within a week after blending the tissues with a homogenizer (Cove 2000), which makes it easier to obtain sample for transformation and regenerants after transformation. Like higher plants, *P. patens* is regulated by plant hormones such as auxin (Imaizumi *et al.* 2002), cytokinin (Schulz *et al.* 2001), and abscisic acid (ABA) (Knight *et al.* 1995; Kamisugi and Cuming 2005) and environmental signals (Schumaker and Dietrich 1998; Cove *et al.* 2006). Furthermore, the transcription factor *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), which is involved with regulation of a subset of ABA-regulated genes during seed development, was shown to be functional in *P. patens* (Marella *et al.* 2006). The genome sequence of *P. patens* is about 487 Mbp, which is about three fold bigger than the *Arabidopsis* genome. The most unique advantage of *P. patens* among plant species is its high frequency homologous recombination (HR), which enables gene targeting for studying the functions of genes (Schaefer and Zryd 1997; Schaefer 2001; Decker and Reski 2004; Reski and Frank 2005; Cove 2006; Kamisugi *et al.* 2006).

Since transformation is an essential component of studying gene function in this system, our review focuses on this topic.

TRANSFORMATION OF *P. patens*

Protonemal tissue is exclusively used for transformation of *P. patens*, because it grows very fast, regenerates efficiently, and provides facilitated protoplast isolation. It is not necessary to supplement any additive or hormone other than the minimal mineral medium which is used for normal growth (Ashton and Cove 1977), for preparation of tissue before transformation (Cove 2000). Transformation of *P.*

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Invited Mini-Review

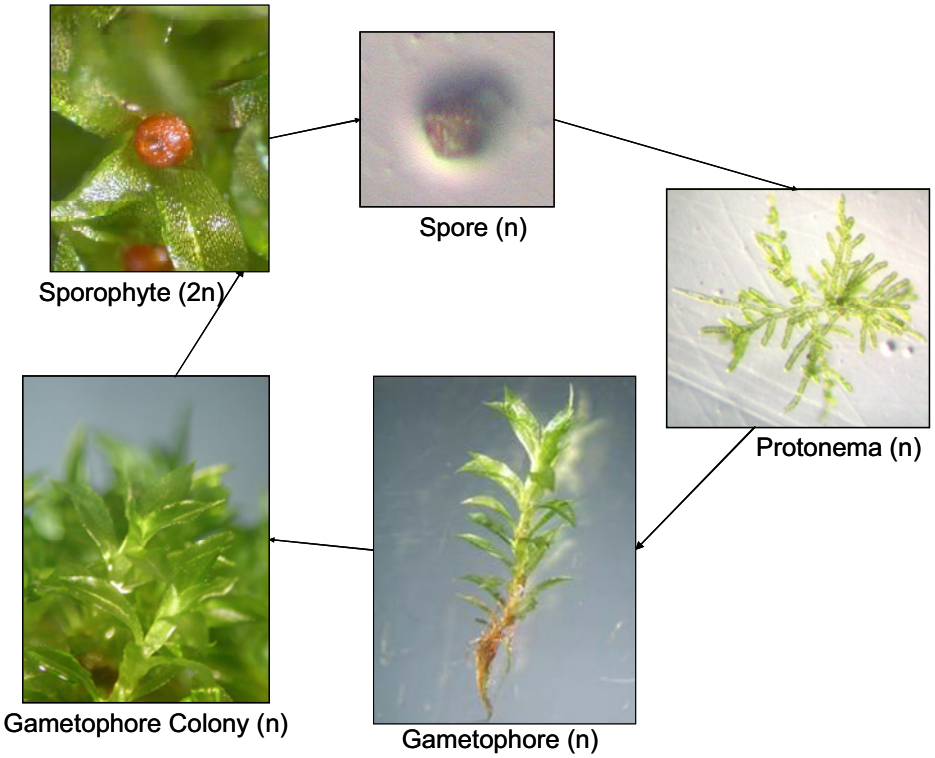

Fig. 1 The life cycle of *P. patens*. The development of *Physcomitrella* starts with the germination of spores, resulting in the formation of a filamentous structure, protonema, which is the juvenile gametophyte. Protonema grows by apical cell division, which develops into leafy adult gametophytes, gametophore. Gametophore generates the sex organs, resulting in the diploid sporophyte by fertilization. "n" and "2n" mean haploidy and diploidy, respectively.

patens commonly uses polyethyleneglycol (PEG)-mediated protoplast transformation (Schaefer *et al.* 1991) and microprojectile particle bombardment (Sawahel *et al.* 1992; Cho *et al.* 1999), the former being used more frequently (Frank *et al.* 2005; Cove 2006). For PEG-mediated transformation, protoplasts are isolated by digestion of the cell wall of protonemal tissue with Driselase enzyme. Single-stranded DNA by restriction enzymes makes integration and targeting rate higher. Particle bombardment is carried out by shooting protonemal tissue grown on a cellophane-overlaid medium (Cho *et al.* 1999). Denaturation of the secondary structure of plasmids before bombardment increases the efficiency of transformation.

Both methods of transformation produce three types of regenerants after selection for plasmid-encoded antibiotic resistance (Cove and Knight 1993; Cove 2000, 2006). The first type expresses transgenes transiently, so that it cannot be sub-cultured to subsequent selective medium. The second type shows resistance to antibiotics. However, it retains resistance only while selection is maintained. Once the selection is relaxed, the transgenes are lost rapidly. These transgenics are likely to replicate plasmid DNA extrachromosomally, and are thus considered unstable. The last type retains resistance even after relaxing selection, and molecular analysis identifies integration of DNA at a genomic locus, i.e. stable transformation. In order to obtain stably transformed transgenic lines, at least two rounds of selection and selection relax cycles are necessary. Stably transformed transgenics usually have multiple numbers of integrated transgenes in the genome, with average of 11 copies per plant (Kamisugi *et al.* 2006).

Agrobacterium tumefaciens-mediated transformation was reported unsuccessful because *P. patens* is not a host (Baur *et al.* 2005). However, recently a new virulent strain has been tried (Cove 2006) and may result in the higher rates of stable transformation compared with PEG or particle bombardment-mediated methods.

VECTOR CONSTRUCTS

Selection cassettes

Commonly used selection markers are also applicable for the selection of transformants in *P. patens*, e.g. geneticin *npt*II (Sakakibara *et al.* 2003), hygromycin *hph* (Kaewsuwan *et al.* 2006), and zeocin *zeo* (Cove 2006). These genes are normally driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter (Mittmann *et al.* 2004) and terminated by nopaline synthetase (NOS) terminator or the CaMV 35S terminator.

Overexpression vector

Although the CaMV 35S promoter is used widely, various promoters are also applicable in *P. patens* depending on desirable level of expression required. When activities of various promoters were compared to CaMV 35S promoter (Horstmann *et al.* 2004), the complete rice *actin* I (*Act1*) gene promoter showed the highest level of activity, 10-fold more than the normal CaMV 35S promoter, followed by a tandemly duplicated CaMV 35S promoter with 6-fold more activity. A single long version of the CaMV 35S promoter and the truncated version of the rice *Act1* promoter showed about 4-fold activity. In comparison, a lower level induction was achieved with two endogenous promoters of *P. patens*, α1,3-*fucosyltransferase* (*fuc-t*) or β1,2-*xylosyltransferase* (*xyl-t*), which exhibited about 2-fold and one third of the expression level, respectively. Promoters of maize ubiquitin (Bezanilla *et al.* 2003) and the wheat early-methionine-labeled (EM) gene (Knight *et al.* 1995) were also reported as strong promoters, even though there was no direct comparison with the CaMV 35S promoter. In particular, the EM promoter was strongly induced by 10 μM ABA treatment.

A chemical inducible system based on bacterial quorum-sensing system is also available for *P. patens* (You *et al.* 2006). When the traR protein binds 3-oxooctanyl-L-homoserine lactone (OOHL), it specifically recognizes the *tra* box in a promoter. The traR protein induced the reporter gene expression by 7-fold in the presence of 100 μM OOHL for 24 h in *P. patens*, compared to CaMV 35S promoter.

Targeting vector

P. patens exhibits a high rate of gene targeting by homologous recombination (HR), which allows for the study of genes (Cove 2006). Since the first report on HR in *P. patens* (Schaefer and Zryd 1997), a number of studies have reported gene targeting via HR (Strepp *et al.* 1998; Girod *et al.* 1999; Sakakibara *et al.* 2003; Mittmann *et al.* 2004; Lee *et al.* 2005; Kaewsuwan *et al.* 2006; Perroud and Quatrano

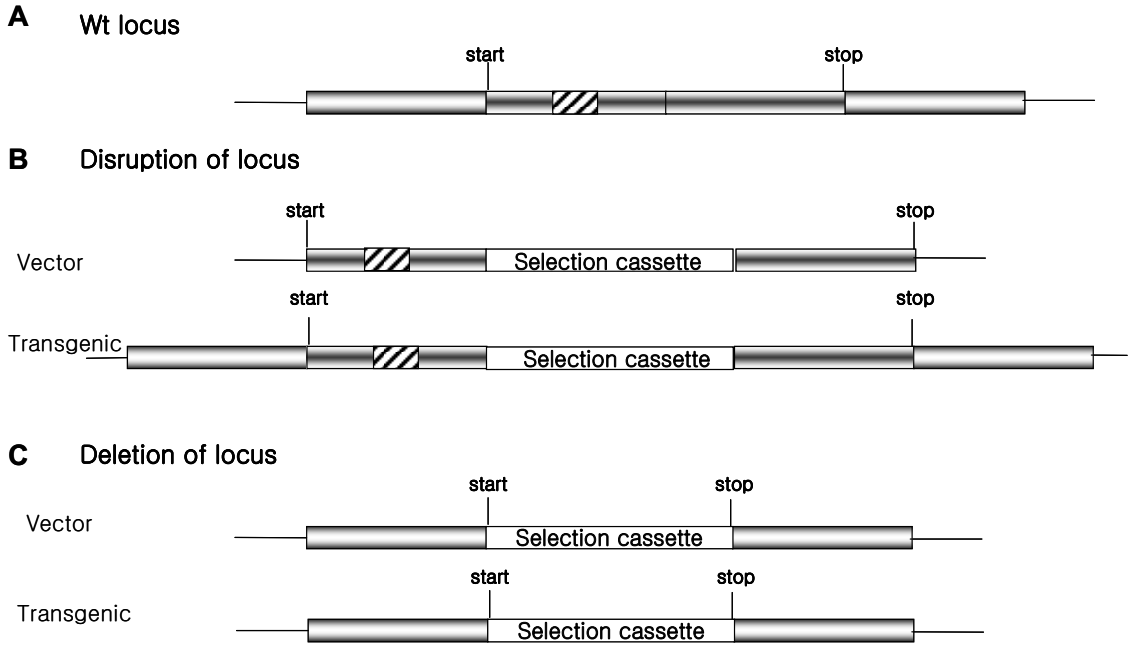

Fig. 2 Vector construction for gene knock-out by allele replacement. (**A**) Wild type (Wt) locus. (**B**) Vector for an inactivation of locus and the structure of recombined locus in transgenic lines. (**C**) Vector for a complete deletion of locus and the structure of recombined locus in transgenic lines. The hatched boxes in A and B indicate a functional domain. "start" and "stop" indicate start and stop codon of a gene, respectively. "Selection cassette" indicates a cassette for the selection of transgenic lines on selection media, for example, CaMV 35S promoter::selection marker::terminator.

2006). Although there are many proposed mechanisms and models of the targeting event (Quatrano *et al.* 2007), we describe the practical factors to be considered for targeting vector construction. In general, allele replacement has been tried to disrupt the function of a locus by HR, and two approaches have been utilized (**Fig. 2**). The first is a disruption of the locus (Strepp *et al.* 1998), and the other is a complete deletion of the locus (Kaewsuwan *et al.* 2006). The disruption of the locus (**Fig. 2B**) has been used by many studies, in which the cDNA (Strepp *et al.* 1998; Girod *et al.* 1999) or genomic DNA (Sakakibara *et al.* 2003) sequence of the gene were cloned into the vector. However, the efficiency of HR may be lowered by the use of cDNA because unmatched exon-intron structure may hamper the integration of the cDNA fragment. Relevant restriction enzyme sites in the gene have to be identified for insertion of a selection cassette. The restriction site should be in the middle of a gene and within an exon to ensure the similar length of flanking sequences and functional deletion of the gene. The deletion of short genomic DNA fragment by two restriction enzymes for insertion of selection cassette enhances the reliability (Frank *et al.* 2005). However, an uncertainty of the final phenotype exists in this approach, because the intact 5′ targeting region within the targeting vector may result in a truncated version of target protein, which may cause a phenotype. In particular, if certain functional domains or motifs locate at the 5′ targeting arm (the hatched boxes in **Fig. 2A, 2B**), it may lead to an incorrect interpretation for the function of gene. In contrast, the deletion of locus makes more reliable result (**Fig. 2C**), because the 5′-upstream and 3′-downstream regions from open reading frame (ORF) which are cloned into targeting construct ensure the complete deletion of the locus by HR (Kaewsuwan *et al.* 2006; Perroud and Quatrano 2006). This approach can also target tandemly-located loci on the genome by a single transformation. In addition, a promoter region can be completely deleted by this way without leaving any *cis*-element. Furthermore, generated mutants can be complemented with constructs which have various versions of modification of the gene for studying roles of functional domains as well as function of gene. Nevertheless, previously, it required more steps to obtain the sequences of 5′-upstream and 3′-downstream regions such as the inverse PCR and genomic DNA library screening. However, the genome sequencing of *P. patens* has facilitated to clone by a round of PCR.

Various approaches resulted in successful gene targeting. The efficiency of HR tends to be increased by linearized DNA exposing the flanking sequences at both ends of transforming DNA using restriction enzyme digestions or PCR (Cove 2006). The length of the flanking region for HR was also important for HR. The size of one flanking region should be bigger than 300 bp, and the flanking regions should be symmetric. In general, the size of each of the two flanking regions should be at least 600-800 bp, which results in about 50% of stable transformants with HR.

RNAi vector

Gene targeting by HR is limited to a single locus at any one time. Considering that many genes exist as multiple copies and their functions are redundant, several rounds of trans-

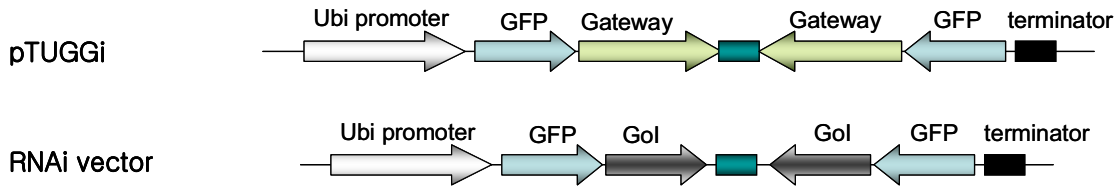

Fig. 3 RNAi vector construction (adapted from Bezanilla *et al.* 2005). The pTUGGi vector is a destination vector having Gateway cassettes. Ubi promoter, ubiquitin promoter; GFP, green fluorescent protein; GoI, gene of interest.

formation to obtain desirable knock out mutants is necessary. However, RNA interference (RNAi) makes it possible to overcome this obstacle (Shimomura *et al.* 2006; Mei *et al.* 2007; Ueno *et al.* 2007). RNAi was reported in *P. patens* using a stable line (NLS-4) expressing GFP in its nuclei by a fusion with nuclear localization signal (Bezanilla *et al.* 2003). A specially designed vector (pTUGGi) containing GFP sequence has been utilized for rapid screening of GFP silencing as an indicative of positive RNAi transformation in the NLS-4 line (Bezanilla *et al.* 2005). The pTUGGi vector takes advantage of the Gateway technology (Invitrogen, USA) in order to shuttle sequences between plasmids containing corresponding recombination sites (**Fig. 3**). After transformation into the NLS4 line, positive transformants can be screened under a fluorescent microscope by GFP silencing. Using this RNAi approach, ARPC1, a component of the actin-related protein2/3 (Arp2/3), was demonstrated to play a critical role in polarized growth and cell division patterning (Harries *et al.* 2005).

CHLOROPLAST TRANSFORMATION

Chloroplasts have their own genome, replicate independently and inherit traits maternally. Chloroplast transformation was shown to be advantageous over nuclear transformation, since maternal inheritance avoided possible transgene contamination to other plants, as well as the high copy chloroplast genome possibly resulting in a higher level of protein expression (Daniell *et al.* 1998; Kota *et al.* 1999; Maliga 2002; Daniell 2006). In addition, the function of chloroplast genes can be investigated by a targeted knockout using chloroplast transformation thorough HR. However, successful chloroplast transformation has been limited to several plant species such as tobacco (Svab and Maliga 1993), *Arabidopsis* (Sidorov *et al.* 1999), rice (Lee *et al.* 2006), and tomato (Ruf *et al.* 2001). *P. patens* is one of the plants available for chloroplast transformation (Sugiura and Sugita 2004). The arginine tRNA gene, *trnR-CCG* is transcribed at a very low level, and the codon usage of CGG is very low in chloroplast-coding proteins, suggesting that is *trnR-CCG* non functional. This was confirmed by chloroplast transformation with PEG-mediated transformation using a selection cassette with a spectinomycin-resistance gene (*aadA*) which is commonly used in chloroplast transformation in seed plants. In the future, this locus may be able to be used as a target for over production of foreign protein in terms of molecular farming.

TAGGED-MUTANT LIBRARIES

In order to generate a collection of mutants, a gene-trap system is often utilized. In a gene-trap system, a reporter gene without a promoter is randomly inserted into the genome, and tissue-specific genes expressing the reporter are identified (Hiwatashi *et al.* 2001). The gene-trap system was also applied to *P. patens* with genomic DNA (Nishiyama *et al.* 2000; Hiwatashi *et al.* 2001) or cDNA libraries (Egener *et al.* 2002) of *P. patens* inserted by mini transposon-tagged trap elements, which facilitated integration of constructs via HR. Higher phenotypic deviations were observed when a cDNA library was used (16.2%) (Egener *et al.* 2002) compared to a genomic DNA library (3.9%) (Nishiyama *et al.* 2000). The difference in efficiency might be due to the use of the cDNA library which is the collection of expressed genes. This technique was expanded to give rise to 73,329 mutant lines transformed with various sets of disruption cDNA libraries (Schween *et al.* 2005). Gametophyte libraries were constructed from protonema and gametophore tissues with various treatments and time points. In case of the sporophyte library, samples from different parts of a tissue and of time points were harvested. The phenotypic deviations from wild type were standardized with 16 categories such as plant structure, color, leaf shape, cell shape, and uniformity of leaves. These collections should be valuable sources for functional study of genes.

If *Agrobacterium*-mediated transformation is easily available in *P. patens* in the future (Cove 2006), a T-DNA tagging mutant library should provide another valuable source of mutant collection.

CONCLUSION

The moss *P. patens* has been increasingly used as a model plant during the last ten years (Cove 2006), mainly because of efficient gene targeting through HR. With the public release of the genome sequence, the genes of *P. patens* have become more easily accessible (Quatrano *et al.* 2007). Therefore, for the functional studies of genes of interest, every tool is available and the method straightforward in *P. patens*. Simply, one just finds homologues in the *Physcomitrella* genome, clones the genomic sequences, constructs knock-out vectors, and targets the genes. All of these processes can be finished within three to five months depending on the target and procedures. This will provide plant researchers an excellent alternative in the post genome-era.

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