Review

Genetic transformation of moss plant

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Bryophytes are among the simplest and oldest of the terrestrial plants. Due to the special living environment and characteristics, bryophytes have become attractive experimental tools for the elucidation of complex biological processes in plants. Mosses grow rapidly when cultured on simple salt media, thereby making them ideal materials for metabolic studies, particularly transformation and gene homologous recombination. Thus, in recent times mosses such as *Physcomitrella patens, Funaria hygrometrica, Ceratodon purpureus,* and T*ortula ruralis* are being developed for genetic engineering studies. Recently, the finding of efficient homologous recombination of *P. patens* and yeast and murine cells could be comparable. So, the moss, *P. patens* has become an extremely powerful organism for the functional analysis of plant genes. In this report, we reviewed recent research progress so far made on genetic transformation of the bryophytes with emphasis on *P. patens*. We also discussed the advantages and limitation of bryophytes transformation.

Key words: Bryophytes, moss, genetic transformation, homologous recombination.

INTRODUCTION

Bryophytes, small green land plants, are among the oldest terrestrial organisms and appeared 4.5-5 million years ago. It comprises three groups: Mosses, hornworts, liverworts. Bryophytes are higher non-vascular plants which show an alternation of generations. The life cycle can be divided into two phases: Sporophyte generation (diploid stage) and gametophyte generation (haploid stage), both of them are photosynthetic. The haploid gametophyte stage is dominant, while the Sporophyte is parasitic on the gametophyte. The protonemal stage of gametophyte development comprises chloronemal and caulonemal formation.

With the strong regeneration and short life cycle, mosses are ideal model systems for the study of plant. Any part of moss can be regenerated into protonemal, and then grow into gametophyte. The regeneration process does not need to go through dedifferentiation, but directly differentiate into protonemal. The diploid gametophyte, which is predominant in the life cycle, is also useful for mutant screening and genetic analysis, making mosses attractive for genetic studies. The moss species *Physcomitrella patens* is considered suitable for studying plant model system due to:

1. The relative simplicity of the species,
2. The common characteristics with higher plants, example phytohormones (Ashton et al., 1985), hormone response mechanisms (Knight et al., 1995), phytochrome

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Abbreviations: **PEG,** Polyethylene glycol-mediated transformation; **GUS,** β-D-glucuronidase (GUS) gene; **A.T.,** Agrobacterium tumefaciens-mediated transformation; **NM,** not mentioned; **G418,** geneticin; **APT,** adenine phosphoribosyl transferase reporter gene; **Hyg,** hygromycin; **Para,** paramomycin; **Spe,** spectinomycin; **GC–MS,** gas chromatography and mass spectroscopy; **Pr,** protein assay; **nptII,** neomicin phosphotransferase II gene; **uidA,** GUS gene; **aphIV,** a type IV hygromycin phosphotransferase gene; **hpt,** hygromycin phosphotransferase gene; **aadA,** streptomycin resistance gene; **TAIL-PCR,** thermal asymmetric interlaced PCR; **EYFP,** yellow fluorescence protein gene; **RAD51,** an eukaryote gene.

and phytochrome-controlled photomorphogenesis (Cove et al., 1978; Kolukisaoglu et al.,1993),
3. The simple axenic propagation under control conditions (Knight et al., 1988) and,
4. The amenableness to study using genetic (Ashton et al., 1988), physiological and cytological (Doonan and Duckett, 1988) methods (Ashton et al., 2000).

LIFE CYCLE

Mosses, like most plants, show an alternation of generations comprising of a free-living haploid gametophyte stage and a diploid Sporophyte stage, with the Sporophyte being parasitic on the gametophyte. The Sporophyte produces spores by meiosis that germinates to produce further gametophytes. In this cycle, spores germinate to form protonemal tissue, consisting of simple cell filaments. The protonema is formed by chloronemal filaments packed with large chloroplasts and extend by serial division of their apical cells, with a cell cycle of about 24 h while the sub-apical cells of chloronemal filaments undergo a limited number of divisions to form side branches. The apical cells can transform to divide to caulonemal filaments. This caulonemal filament is another protonemal, with fewer cells and less Chloroplasts like the chloronemal, caulonemal filaments extension also undergoes serial division of the apical cells, but has a short life cycle of only 6 h. Besides, the linear extension rate of the caulonemal cell is as rapid as 40 µm/h, but only 2 µm/h for chloronemal filaments, allowing for rapid growth in the soil and agar surface (Cove and Knight, 1993). The sub-apical cells of caulonemal filaments can produce side branches via limited division and may develop into chloronemal filaments under high light conditions, while others develop into caulonemal filaments or buds. Then, the buds produce leafy gametophytes with gametangia and gametes (Zhao et al., 2004*)*. In the genera *Funaria* and *Physcomitrella*, female and male gametes are produced by the same plant whereas; in *Ceratodon* there are separate sexes. Figure 1 summarizes the life cycle of mosses, illustrating some of the main stages in *Physcomitrella* (Cove, 2000)*.*

IMPORTANCE OF TRANSGENIC MOSS

The moss, especially *Physcomitrella patens,* has recently become a powerful genetically tractable model plant system. With its efficient gene targeting (Schaefer and Zryd, 1997; Strepp et al., 1998), the *P. patens* have been used to study the basic developmental mechanisms of land plants, particularly flowering plant (Prigge et al., 2010). It also applied to analyze gene function (Brucker et al., 2005; Schaefer et al., 1997; Strepp et al., 1998) and express many complex secreted eukaryotic proteins
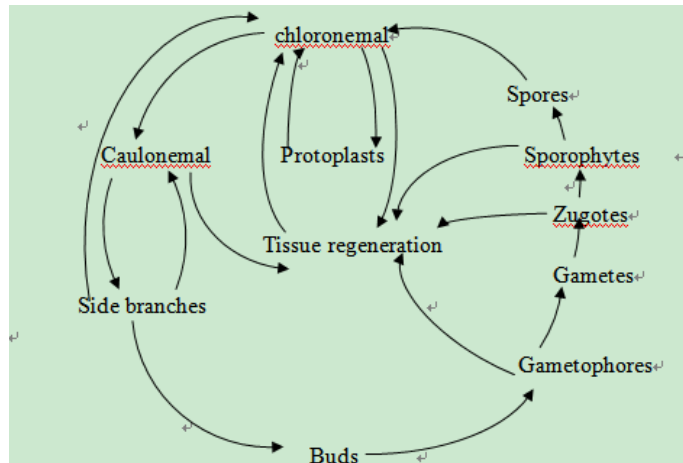

Figure 1. The life cycle of *P. patens.* All stages are haploid except the zygote and the Sporophyte.

(Decker and Reski, 2007). The transgenic plants have a low-cost and safe pathogen-free advantage of producing complex recombinant pharmaceutical proteins. However, the high plants specific protein N-glycosylation was reported to be immunogenic in biopharmaceutical production (Faye et al., 2005). The moss, with the strong regeneration and feasibility of targeted gene replacements, has non-immunogenic humanized glycan patterns for recombinant biopharmaceuticals (Decker et al., 2008).

TRANSGENIC STUDIES

Protoplasts used for the transformation

Plant protoplasts which had its cell walls removed offer a versatile cell-based experimental system (Yoo et al., 2007). The cell wall is degraded by digestion with different mixture of hydrolytic enzymes example cellulase, hemicellulase. Protoplasts are used to create hybrid cells via protoplast fusion. In some cases they are totipotent, and are thus capable of regenerating into plant on appropriate culture medium. Such protoplasts can be used in plant transformation. Mosses, unlike other plant protoplasts, do not need phytohormones for regeneration, nor do they form a callus during regeneration. Instead, they regenerate directly into the filamentous protonemal thus mimicking a germinating moss spore.

TRANSGENIC TECHNOLOGY OF MOSS

Polyethylene glycol (PEG)-mediated transformation

The first stable genetic transformation of plant cells was reported in tobacco by Krens et al. (1982), who used the

method of polyethylene glycol-mediated Ti-plasmid DNA uptake of protoplasts. The first successful transformation of moss was achieved by the transfer of resistance gene by polyethylene glycol (PEG)-mediated DNA uptake of protoplasts of the moss *P. patens* (Schaefer et al., 1991) (Table 1), a technique which is widely used for the transformation of seed plants. With slight modification, this technique could also be used for genetic transformation of the moss *Ceratodon purpureus.* Furthermore, the technique has also been used for the generation of targeted knockout mutants. Thus, PEG-mediated DNA transfer has been selected for the construction of a high-throughput transformation system for both higher plants and mosses (Schaefer et al., 1991; Potrykus and Spangenberg, 1995; Zeidler et al., 1999). However, the efficiency of transformation using this technique is low, about 0.5 -1 transformant per µg DNA. Besides this low efficiency, PEG-mediated transformation requires more extensive tissue and protoplast manipulation, complicated procedure and high efficiency regeneration of protoplasts which is obtained under scrupulously sterile conditions (Šmídková et al., 2010). Table 1 shows PEG-mediated transformation of some species of mosses.

Microprojectile bombardment transformation

An alternative method widely used for transformation of higher plants and mosses is particle bombardment. This technique has successfully being used for the transformation of *P. patens* (Sawahel et al., 1992) and *Marchantia polymorpha* cells (Irifune et al., 1996). Biolistic transformation has several advantages over PEG- mediated transformation mainly because it can shorten the procedure to a minimum thereby avoiding microbial contamination. The biolistic transformation efficiency depends to a large extent on the form of DNA. Šmídková et al. (2010) reported that particle bombardment of tissues with 1 µm gold particles generated about 20 to 40 stable transformants per 1 µg DNA. They further reported that transformation of circular plasmids yielded higher frequencies of random transgene integration, and linear plasmids are even more efficient in targeted transformation using particle bombardment.

Agrobacterium-mediated transformation

Although *Agrobacterium*-mediated transformation was the method of choice for the genetic transformation of seed plants, mosses are not often amenable to *Agrobacterium* infection. However, report on *Agrobacterium*-mediated transformation of the *Marchantia polymorpha* by - Nasue et al. (1997) did not obtain intact transgenic plants using *Agrobacterium* – mediated transformation. Contraily, Ishizaki et al. (2008)

established an efficient and reproducible *Agrobacterium*-mediated transformation protocol for *M. polymorpha* using immature thalli . In Ishizaki et al. (2008) infected immature thalli developed from spores directly by *Agrobacterium*, and achieved high transformation efficiency. The high transformation efficiency could be attributed to the direct infection of the immature thalli with the Agrobacterium without going through protoplast isolation and regeneration steps in the transformation process. They further reported that acetosyringone is essential for *Agrobacterium*-mediated T-DNA transfer into *M. polymorpha* cells. The duration of pre-culture and co-cultivation also have significant effects on the transformation efficiency of *M. polymorpha.*

TYPES OF REGENERANTS

All the methods used for transformation of moss yielded three types of regenerants after selection for plasmid-encoded antibiotic resistance. These are transient, weak, and strong resistance phenotypes. Some transformed cells lose resistance and were probably expressing resistance transiently. Although others continue to show resistance, the resistance is weak and are lost when the transgenic moss are transferred to nonselective medium after about 14 days of growth. These transgenics were therefore termed unstable and were likely to replicate plasmid DNA outside of the chromosome (Knight, 1994). In spite of these negative results, few transgenic mosses grew strongly on selective medium and when transferred to nonselective medium, they maintained their resistant phenotype with Mendelian pattern of inheritance (Schaefer et al., 1991). These transgenics were termed stable and confirmed the integration of the plasmid at a genomic locus (Ashton et al., 2000). According to Cove (2000) multiple copies of the plasmid DNA integrated at the same locus and in the same orientation.

TRANSFORMATION EFFICIENCIES

The transformation frequencies of moss as reported in literature varied according to the method employed. Both the PEG-mediated and particle bombardment transformation method yielded almost the same frequency of transgenic moss. It has been estimated that PEG-mediated DNA uptake procedure requires more DNA (routinely 20 µg per transformation) while the particle bombardment required only 2 shots per transformation and 1 µg per shot (Cove, 2000). Consequently, the transformation efficiencies, defined as transgenic per ug of DNA by biolistic bombardment are higher than PEG-mediated method (Cove, 2000). The frequency of transformation by *Agrobacterium tumefaciens* in the presence of acetosyringone was reported to be 10% in *M. polymorpha* (Nasue et al., 1997).

Table 1. Transformation of bryophyte.

Host	Gene	Method	Detection	Selection	Plasmid vector	Reference
P. patens	aphIV	PEG	Southern	G418 Hyg	pLGVneo1103 pLGVneo2103, pABD1, pHP23b ,pGL2, pBR322	Schaefer et al. (1991)
P. patens	GUS	Gene gun	GUS activity	NM	pBI221.23	Sawahel et al. (1992)
P. patens	nptII Em-GUS	Gene gun	Pr.,GUS activity, Gel Retardation, DNase I Footprinting	G418	pBM113kp, pBM200.15, pMG76.155, pBM233.3,pBMlaA , pLUG2	Knight et al. (1995)
M. polymorpha	hpt, uidA,	Gene gun	Southern, HPT enzyme activity, GUS assay	Hyg	pBI221, pCH	Irifune et al. (1996)
M. polymorpha	GUS	A.T.	GUS assay Southern	G418	pBII21	Nasue et al. (1997)
P. patens	smGFP, aadA	Gene gun	Southern blot, GUS and GFP assay	G418 Hyg	pJIT161, pCAMBIA1300-smGFP, pCAMBIA1303, pSBL-CtV3	Cho SH et al. (1999)
M. polymorpha	hpt	Gene gun	Southern blot	Hyg	pMT	Takenaka et al. (2000)
P. patens	GUS	PEG	GUS activity,PCR, Southern blot	G418	pBI426	Ashton et al. (2000)
P. patens	trnR	PEG	PCR, Southern, Northern	Spe	pCSΔtrnR	Sugiura et al. (2004)
P. patens	rhVEGF	PEG	ELISA ,RELIDA ,SDS-PAGE,Western, Silver-staining	NM	pRT101VEGF C3	Baur et al. (2005)
M. polymorpha	aadA	Gene gun	PCR, DNA gel-blot	Spe	pCS31	Chiyoda et al. (2007)
P. patens; C. purpureus	APT	NM	PCR	Hyg Para	PpAPT-KO, CpAPT-KO	Trouiller et al. (2007)
M. polymorpha	aadA	Gene Gun	PCR and DNA gel-blot	Hyg	pKI101	Chiyoda et al. (2008)
M. polymorpha	uidA	A.T.	GUS activity, southern, TAIL-PCR,	Hyg	pIG121Hm	Ishizaki et al. (2008)
P. patens	Taxadiene	PEG	PCR, Southern, western, GC–MS	Hyg	pTHUBI:TS	Anterola et al. (2009)
P. patens	Taxol	Gateway	PCR, GC-MS	NM	three plasmids(NM)	Hughes et al. (2010)
P. patens	EYFP	Gene Gun	Southern blot	G418	pKA127	Šmídková et al. (2010)
P. patens	RAD51	NM	PCR a Western blot	Hyg	pBNRF	Schaefer et al. (2010)
P. patens	NM	PEG	RT-PCR	Hyg	pBHRF,NM	Liu et al. (2011)
P. patens	PPO1	PEG	Enzyme activity, UPLC-MS/MS	NM	pET28a, pTrcHis2_TOPO	Richter et al. 2012

A study by Ishizaki et al. (2008) reported that hundreds of hygromycin-resistant plants per sporangium were obtained by co-cultivation of immature thalli of *liverwort Marchantia polymorpha* with *Agrobacterium* carried a binary vector.

HOMOLOGOUS RECOMBINATION

One of the most important characteristic of *P. patens* is its ability to integrate foreign DNA into its genome by homologous recombination at a very high rate than other plants (Schaefer et al., 1997). This characteristic which could make gene knockout possible could be used to analyze gene functions. Schaefer and Zryd (1997) demonstrated that the efficiency of gene targeting evaluated in their transformation experiments which designed to target three single-copy genomic loci in *P. patens* was above 90%, an observation similar to that of yeast. They also reported that the transformation efficiency was higher than previous reports on gene targeting in plants. These observation suggests that haplobiontic moss is the first example from the plant kingdoms which could be used to gene knock-out; previously this method was only applied in lower eukaryotes such as protozoa, yeasts and filamentous fungi.

Strepp et al. (1998) disrupted the *ftsZ* gene which was isolated from the eukaryote Physcomitrella patens. The *ftsZ* gene encoding a homolog of the bacterial cell division protein *FtsZ,* an ancestral tubulin. It controls the chloroplast division in the *P. patens.* Seven out of 51 transgenics obtained were knockout plants generated by homologous recombination; they were specifically impeded in plastid division with no detectable effect on mitochondrial division or plant morphology. So, this was proved the effect of the *FtsZ* gene in the chloroplast division.

The reduction of adenosine 5'-phosphosulfate (APS) to sulfite catalyzed by adenosine 5'-phosphosulfate reductase (APSase) is considered to be the key step to the understanding of sulfate assimilation in higher plants. However, analogous to enteric bacteria, an alternative pathway of sulfate reduction via phosphoadenosine 5'-phosphosulfate (PAPS) had been proposed in higher plants. To date, the presence of the corresponding enzyme, PAPS reductase, could neither be confirmed nor rejected in plants. Koprivova et al. (2002) disrupted the adenosine 5'-phosphosulfate reductase (APSase) single copy gene in *P. patens* by homologous recombination resulting in complete loss of the correct transcript and enzymatic activity.

The moss *P. patens* is thus the first plant species in which PAPS reductase had been confirmed on the molecular level; it is also the first organism where both APS- and PAPS-dependent sulfate assimilation co-exist (Koprivova et al., 2002).

THE EXTANT PROBLEMS AND PREDICTION

In general, the transformation frequencies of mosses have been reported to be higher than other plants, nevertheless they have not reached the levels achieved in eukaryotic microbes such as yeasts. Although, the frequency of transformants achieved is on the ascendancy, and the reliability of the transformation procedure is improving too, efforts should be made to continue to find perfect the conditions that will improve both frequency and reliability of the methods used to achieve transgenic moss (Cove, 2000). Now the research platform with mosses has not been completed. However, a genetic linkage map has been yielded by crossing a French ecotype to a British ecotype (Kamisugi et al., 2008). We need more time to establish the cDNA library and genome DNA, which could be identified by using next-generation sequencing technologies (Prigge et al., 2010).

The mechanism of homologous recombination rate with *P. patens* has not yet been researched enough. The *P. patens* genome can only represent the portion of the terrestrial plant, which has not been studied by gene knockout. Thus, the mechanism of homologous recombination rate should be investigated. Mosses, especially *P. patens*, are the most attractive to study due to their simplified molecular biology characteristics, compared to higher plants. The development of transgenic studies in moss could make them model plants for genetic studies and also play a catalytic role in molecular biology in plants similar to that of *Arabdopsis*.

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