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Review Article

Marchantia polymorpha L.: An Emerging Model Plant System to Study Contemporary Plant Biology – A Review

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Abstract

The liverwort, *Marchantia polymorpha* L., one of the species of first land plants is a promising model plant system for the analysis of diverse facets of contemporary plant biology. The unique characteristics of the plant such as dominant haploid gametophytic generation enables the isolation and disruption of mutant for genetic analysis, rapid sexual and asexual reproduction can be induced under controlled conditions which leads to the formation of genetically homogenous lines and also the complete organelle genome sequence of chloroplast and mitochondria has been established. In addition, the ongoing whole genome sequencing of *M. polymorpha* by the community sequencing plan at the Joint Genome Institute specifies the conservation of several mechanisms of biological science that are instituted in other terrestrial plants in a smaller extent of intricacy. Thus, with the development of several feasible and reliable genetic transformation strategies, *in vitro* cell culture, gene silencing, targeted gene modification and its critical evolutionary position make this plant as a potential model plant to study evolutionary and developmental biology in detail.

Keywords
Bryophyta; Evolution; Liverworts; Molecular Genetics; Transformation

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Introduction

For nearly 200 years, *Marchantia polymorpha* L. is intensively used as an investigational organism to study several physiological and morphological changes in a response to environmental factors (Shimamura, 2015; Bowman, 2016). But now it is gaining importance as a model plant system for contemporary plant science due to availability of several molecular genetics tools such as transformation techniques which can be utilized to understand the several aspects of evolutionary and developmental biology of plants in detail.

Based on the morphological, fossil record and molecular analysis, liverworts are thought as the earliest land plant to grow and inhabit the primordial landscape (Mishler and Churchill, 1984; Kenrick and Crane, 1997a; Qui *et al.*, 2006; Qiu *et al.*, 2008; Kato, 2010). Further, the genome analysis showed that the a lot of the regulatory gene families are conserved (Floyd and Bowman, 2007; Rensing *et al.*, 2008; Banks *et al.*, 2011; Nystedt *et al.*, 2013). Therefore, along with mosses and hornworts, liverworts are a key group in comparative genomics to understand the genetic basis of evolutionary and developmental biology of land plants.

M. polymorpha is dioecious in sexuality. The gametophytic (haploid) generation is dominant in the life cycle, which gives remarkable benefits over

diploid higher plants for genetic study. The reproduction strategies are also appealing as it reproduces sexually and asexually (by gemmae) both (Barnes and Land, 1908; Hughes, 1971), which permits speedy proliferation of isogenic biomass which is very handy for molecular and biochemical experiments. Reproductive phases and plant growth can be tempted at the preferred times under *in vitro* conditions, which aids genetic investigation. The developmental progression from a haploid spore (single-celled) to a multi-cellular composite body can be experiential straightforwardly in full detail. Recently, the taxonomy, phylogeny and morphology of *M. polymorpha* have been described in detail by Shimamura *et al.* (2015).

M. polymorpha has been chosen as an experimental plant for genetic analysis from bygone time. In addition, the chloroplast (Ohyama *et al.*, 1986) and mitochondrial genome (Oda *et al.*, 1992) of *M. polymorpha* were foremost to be entirely sequenced amid all plants. But the utility of this plant as a model plant system has been neglected due to unavailability of genetic and genomic tools for the study (Shimamura *et al.*, 2015; Ishizaki *et al.*, 2015). The genome sequence of sex chromosomes 'Y' was also sequenced in *M. polymorpha*, which add to the understanding of the structure and evolution of sex chromosomes in plants with the haploid genome (Yamato *et al.*, 2007). Moreover, a continuing project for *M. polymorpha* genome sequencing under the Community Sequencing Program (CSP) at the Joint Genome Institute designates that various biological events found in higher land plants are conserved in a very uncomplicated form. In recent times, the development of transformation techniques for *M. polymorpha* enables the utilization of other genetic tools such as gene targeting, gene silencing, homologous recombination and gene editing (Kajikawa *et al.*, 2003; Ishizaki *et al.*, 2008; 2013a; 2013b). The central criteria required for versatile model organisms include forward genetics, reverse genetics, genetic transformation and genome-wide studies. In the past few years, techniques have been established in *M. polymorpha* that promote this species as a member of the model organism group. The molecular tools and techniques used for research in *M. polymorpha* have been reviewed in detail by Ishizaki *et al.* 2015.

Over the past decade, the plant molecular biologist are paying incredible consideration only on few plant species *viz. Arabidopsis thaliana* (flowering plant) and the *Physcomitrella patens* (moss). But in recent times the thalloid liverwort *M. polymorpha* has greatly attracted the contemplation of molecular biologists as a model plant because of its decisive evolutionary position, and advances in the various genetic tools such as transformation techniques (Ishizaki *et al.*, 2008; Kubota *et al.*, 2013; Tsuboyama and Kodama, 2014; Tsuboyama and Kodama, 2015), forward and reverse genetics, homologous recombination and targeted genome modification (Ishizaki *et al.*, 2013a; Sugano *et al.*, 2014) (Fig 1). Thus, *M. polymorpha* emerges as potential model plant of choice to study evolution, cellular and developmental biology in detail.

Life cycle of *Marchantia polymorpha* L.

The life cycle of *M. polymorpha* comprises of two alternating generations with haploid gametophytic phase alternates with diploid sporophytic phase. The gametophyte generation dominates the life cycle and produces the thallus (Glime, 2013). The unicellular spores germinate to form short filamentous protonema with rhizoids. This protonema further develops by regulating cell division of apical cell and form complex thalloid plant body. On the dorsal side of mature thallus, gemma cups harbouring numerous multicellular gemmae are produced. The thallus can reproduce asexually through these gemmae. *M. polymorpha* is a dioecious species so the male and female gamentangia known as archegonium and antheridium are produced on the umbrella like sexual branches of the male and female thalli respectively. Within antheridia motile male gametes are formed which move in the presence of water and fertilize the single egg cell present in archegonuim. After fertilization, the zygote develops into a sporophyte inside the archegonium. As the sporophyte grows, the venter of the archegonium also grows and becomes a calyptra, which protects the growing young sporophyte. The sporophytes hang upside down beneath the archegoniophore. Following meiotic divisions in the capsule, numerous spores are discharged. Detailed morphological and developmental descriptions of each organ and tissue are reviewed by Shimamura *et al.* (2015). The dominant haploid generation of *M. polymorpha* allows the development of phenotypic mutant (Burgeff, 1943). It is evident that genetically uniform lines in the haploid generation can be recognized effortlessly and proliferated well through the asexual reproduction, which assists greatly in diverse genetic and biochemical experiments. Due to its dioecious nature, male and female reproductive organs are produced in separate thalli, which facilitates crossing in a completely controlled way. Likewise, reproductive growth can also be encouraged at the preferred time of life cycle under laboratory setting, which facilitates somewhat unproblematic genetic analysis.

Aspects of plant evolution: *M. polymorpha* as first land plant

Various workers of past and contemporary biologist considered liverworts as first land plant which grew and inhabited on the primeval landscape (Goffinet, 2000; Wellman *et al.*, 2003; Shaw and Renzaglia, 2004). Because of its crucial

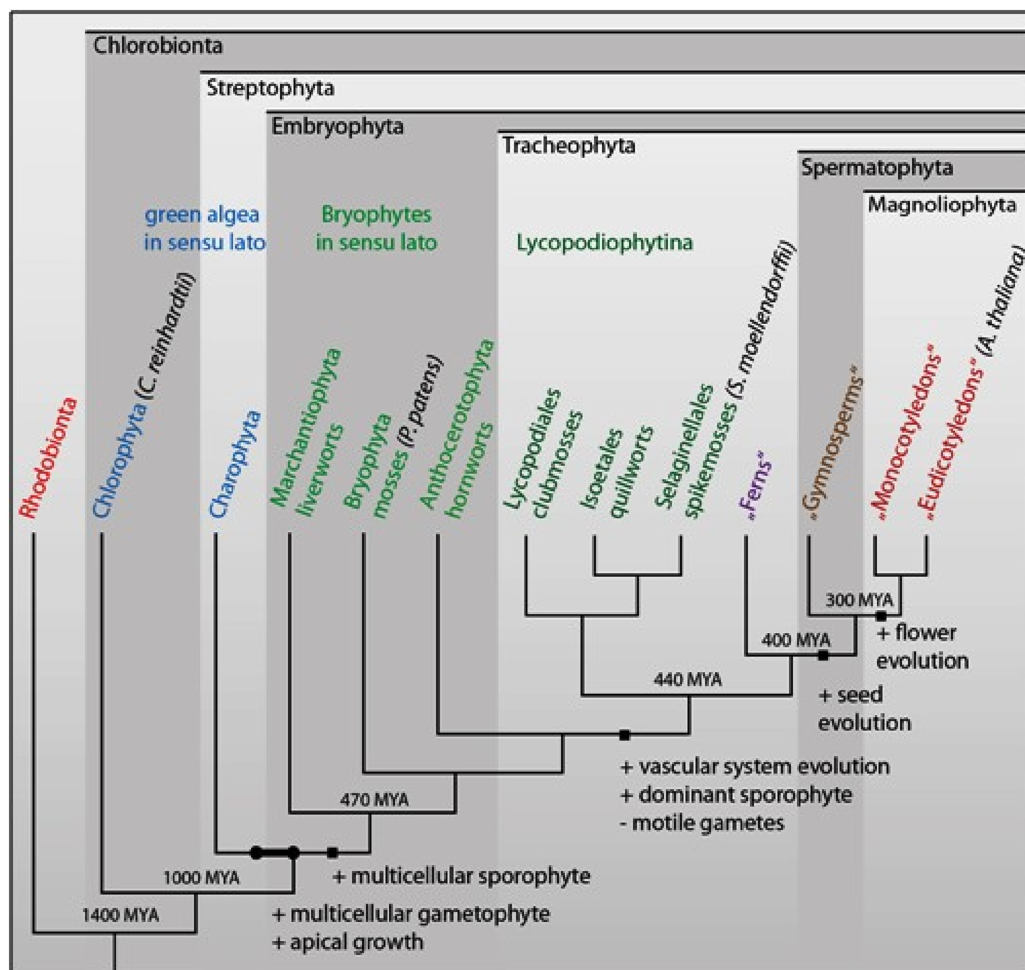

Figure 1: Phylogenetic Tree. Phylogenetic tree representing the relationship between algae, bryophytes and angiosperms. Selected model species representing taxa with phylogenetic key positions in land plant evolution are in parentheses (adopted from Pires and Dolan, 2012 with slight modification)

position in the evolution of land plants, liverwort particularly *M. polymorpha* has drawn attention as a model plant to study the genetic basis of the major cause and events that occurred during the transition of plants from aquatic to terrestrial life, the genetic changes during its development are accountable for the moderate to complex multicellularity, and the alterations in body plan within the terrestrial plants (Bowman *et al.*, 2007). The first land plant evolved about 1 billion years ago from a common algal ancestor among which charophytes are more closely related to land plants (Graham, 1993; Sanderson *et al.*, 2004).The transition of embryophytes from aquatic life approximately 470 MYA (Wicket *et al.*, 2014), was made possible by plentiful innovations, including parental safeguard for the emergent embryo, egg and sperm production in multicellular protective structures, and an undulation of phases between sporophytic and gametophytic generations. Further subsequent innovations led to the development of vascular tissue, seed, flowers and also the shift of life cycle from gametophytic to sporophytic dominance. The beginning of embryophytes was a key event in evolutionary record that produced an incredible diversity in ecological, reproductive, morphological and physiological traits of land plants. The position of liverworts in the evolution of land plants has been reviewed in detail by several authors (Bennici, 2008; Kato, 2010; Ligrone, 2012; Shimamura, 2015; Bowman, 2016). However, the plant phylogeny study based on the fossil records (Kato 2010) and molecular data including plastid DNA (Nishiyama, 2004; Chang and Graham, 2011), mitochondrial genes (Qui *et al.*, 1998; Qui *et al.*, 2007), ribosomal genome sequence (Nickrent, 2000; Shaw *et al.*, 2011) and nuclear genome sequence (Sanderson *et al.*, 2004; Floyd and Bowman, 2007; Rensing *et al.*, 2008; Banks *et al.*, 2011; Nystedt *et al.*, 2013) of the representative model species of the respective clade to study the events during plant evolution. Thus, *M. polymorpha* occupying critical positions in plant evolution can be used as a model plant to study the events occurred during the transition of aquatic to terrestrial life. By comparative analysis of gene function in the species representing different evolutionary steps, it is possible to differentiate between gene families that emerged recently in the course of evolution (such as by adaptation) and conserved gene families encoding proteins with fundamental functions.

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Specific features of *M. polymorpha* as a model plant

The model plant should have three significant characteristic features:

1) *In vitro* cultivation and physical manipulation.
2) The availability of genome sequence information.
3) The availability of techniques to genetically modify the plant.

In vitro cultivation and growth of *M. polymorpha*

The isogenic individuals of *M. polymorpha* can be effortlessly proliferated from vegetative tissues (thallus, gemma/gemmaling and gametangiophore) or spores in an asexual manner using synthetic growth media, such as Gamborg's B5 basal medium, without vitamin supplements. The germination of spores *in vitro* can be initiated by photo induction or the photosynthesis derived sugar, such as glucose (Nakazato *et al.*, 1999). The spore cells undergo mitosis and form sporelings which further develop into thallus. The cell division and branching in sporeling can be promoted by the red light via activation of the Pfr form of phytochrome (Nishihama *et al.*, 2015b). Thus, by altering the culture conditions such as light and photosynthetic sugar at different developmental stage, the process involved in the formation of multicellular body from spore can be studied in detail.Similarly the thallus can be upholded and proliferated by transferring apical explants containing meristematic cell to the suitable growth media. Further, the gemmae cup formation can be induced by the addition of 1% glucose or sucrose in growth media. As the gemmae cups developed from single epidermal cells, isogeneic cell line can be established through subcultures of gemmae cups. The *M. polymorpha* has the exceptional regenerative ability (Vochting, 1885), thus, any protoplasm containing cell can be regenerated into a new thallus (Goebel, 1908).This ability of *M. polymorpha* has been exploited to obtain intact thallus using a basal thallus as explants and suitable growth medium (Kubota *et al.*, 2013; Nishihama *et al.*, 2015b). The regeneration of the thallus is encouraged by an R signal, mediated by a phytochrome, which is rate limited by the availability of sucrose (Nishihama *et al.*, 2015b). In addition to these culture techniques *M. polymorpha* can also be regenerated through callus culture (Oho, 1973) and protoplast culture (Ono *et al.*, 1979). The efficient isolation of protoplast and its subsequent regeneration are of particular significance because genetic transformation of protoplast is an imperative method of transformation (Hohe and Reiske, 2005). Once an axenic culture has been started, *M. polymorpha* also can be cultivated in liquid media by the inoculation of plant material and regular mechanical disruption. Earlier two major efforts have been made in this direction by Ohta *et al.* (1977), who reported the successful establishment of a cell suspension culture of *M. polymorpha*, and Katoh (1983) who published a comprehensive study on the growth kinetics of suspension cultures of *M. polymorpha.*

The sexual reproduction of *M. polymorpha* can be entirely handled easily under laboratory conditions by scheming the photoperiod. One such study showed that the long day conditions induce the formation of archegoniophores and antheridiophores in male and female individuals, respectively (Wann, 1925; Benson-Evans, 1961). Recently, the development of knockout of GI or FKF1 gene responsible for photoperiodic growth transition puts an end to the dependency of growth phase transition on photoperiod (Kubota *et al.*, 2014). In addition, the FR is indispensable to induce the transition in *M. polymorpha* (Chiyoda *et al.*, 2008), signifying the involvement of a phytochrome in the regulatory process. So the further crossing and the development of spores can be initiated any time in controlled laboratory conditions for genetic analysis.

Tools and Techniques for genetic analysis

Homologous Recombination

Homologous recombination mediated gene targeting is a potent tool to develop knockout for the functional analysis of genes (Terada *et al.*, 2007). The efficient gene targeting via HR is difficult in most of the plants (Terada *et al.*, 2002; Puchta, 2002) because the integration of targeted gene *via* HR is lower as compared to the random integration (10^{-4}- 10^{-6}) via non homologous recombination (Risseeuw *et al.*, 1995; Paszkowski *et al.*, 1988; Hanin *et al.*, 2001; Hanin and Paszkowski, 2003, Iida and Terada, 2004). However, moss *Physcomitrella patens* is an exception which shows a highly efficient rate of homologous recombination mediated gene targeting (Schaefer and Zryd, 1997).A reproducible gene-targeting method was developed in the monocot rice using a positive (the hygromycin resistance gene, hpt) or negative (the diphtheria toxin A fragment gene, DT-A) selection system (Terada *et al.*, 2002; Terada *et al.*, 2007) was successfully implemented for the gene targeting in *M. polymorpha* (Ishizaki *et al.*, 2013a). The highly efficient transformation system is prerequisite to exploit this positive/negative selection for efficient gene targeting (Terada *et al.*, 2004). Thus, the *Agrobacterium*-mediated sporeling transformation method (100 of transformants per sporangium) (Ishizaki *et al.*, 2008) is valuable for efficient gene targeting based on positive/negative selection system. The homologous recombination efficiency is evaluated by using NOP1 as model target gene because a visible but non-selective phenotype is shown on the gene mutation. The result shows that

the frequency of HR is approximately 2% of transformants that passed the positive/negative selection. This method is also proved to be useful in the gene targeting of other genetic loci such as MpGI, MpFKF (Kubota *et al.*, 2014), MpPHOT (Komatsu *et al.*, 2014) and MpTAA (Eklund *et al.*, 2015). The knockout of essential gene may lead to lethality in haploid system and this show the inadequacy of HR mediated gene targeting in haploid *M. polymorpha*.Thus, to overcome this problem, conditional knockout strategy based on an inducible site-specific recombination system was developed in *M. polymorpha* by Nishihama *et al.* (2015a). The heat- and dexamethasone-controllable gene expression/deletion system was developed by expressing the P1-phage Cre site-specific recombinase fused with the ligand-binding site of the rat glucocorticoid receptor, under the control of an endogenous heat-inducible promoter. Thus, providing an efficient system for the development of conditional knockout mutants of essential genes in *M. polymorpha*.

RNA interference (RNAi)

The knockout strategy is not applicable for the analysis of function of redundant gene and the essential gene. As the knockout of the essential gene may lead to lethality and thus the gene function cannot be analysed. The single knockout of one of the redundant genes will not cause significant change in phenotype. So, multiple knockout mutants need to be generated to shut down redundancy, which is a time consuming process (Strotbek *et al.*, 2013). So, to overcome the limitations offered by knockout strategies, the mechanism of RNAi is used to knock down the gene of interest. This method has been extensively used for functional study of genes in plants, animals and fungi (Voinnet, 2001). The use of the RNAi method for the functional suppression of gene involved in plant development and metabolism is also reported in other model organisms such as *Arabidopsis thaliana* (Chuang *et al.*, 2000; Stautjesdijk *et al.*, 2002) and moss *Physcomitrella patens* (Bezanilla *et al.*, 2003; Nakaoka *et al.*, 2012).

In this method of knockdown development, the invert repeated cDNA region of the gene of interest is cloned into an expression cassette which after transcription form hairpin structure containing partially double-stranded RNA region perfectly matching the sequence of the gene of interest. The dsRNA is further recognized by the endogenous RNA interference machinery that cleave the long dsRNA into siRNA (small interfering RNA) which unwound into ssRNAs (single stranded RNA) and the target transcript is degraded, resulting in post transcriptional gene silencing (Smith *et al.*, 2000; Kanno *et al.*, 2000, Wiedenheft et al 2012). By the selection of a conserved cDNA region shared by different members of a gene family, this approach allows the simultaneous silencing of an entire gene family. In 2003, RNA interference mechanism was used for functional analysis of β keto-acyl synthase gene (MpFAE2) in liverwort, *M. polymorpha* (Kajikawa *et al.*, 2003). The RNA interface is a useful method for analysis of phenotype appeared due to the loss of function of genes during biosynthetic pathway (Kajikawa *et al.*, 2003).

Gene knockdown by artificial micro RNA

The major drawback of using homologous recombination (Ishizaki *et al.*, 2013)andCRISPR-CAS- (clustered regularly interspaced short palindromic repeats) mediated genome modification (Sugano *et al.*, 2014) for development of gene-targeted transformants is the appearance of lethality in haploid system due to disruption of some endogenous gene (Ishizaki *et al.*, 2013a). Thus to circumvent this negative effect, specific and endogenous micro RNA has been exploited to create inducible and conditional alleles for genetic analysis. The miRNA is encoded by primary miRNA transcript (pri-miR) that is transcribed by RNA polymerase II (Reinhart *et al.*, 2002; Dugas and Bartel, 2004; Jones-Rhoades *et al.*, 2006). This pri-miRNA transcript via several step process fold back into precursor-miRNA hairpin which further cleaved to form a 21 nucleated miR/miR* duplex. The artificial substitution of this duplex with different target specific genes creates an artificial miR(amiR). The artificial miR has been widely used in several species, including the green alga, *Chlamydomonas reinhardtii* (Molnar *et al.*, 2009), the bryophyta (moss) *Physcomitrella patens* (Khraiwesh *et al.*, 2008) and a varied range of angiospermic plant species, together with monocots (Warthmann *et al.*, 2008) and several eudicots (Schwab *et al.*, 2005; Alvarez *et al.*, 2006). Sandoval *et al.* (2016) reported efficient and inducible use of the artificial micro RNA in *M. polymorpha* to study gene function. In this method two conserved sequences, including MpMIR160 and SkMIR166, derived from *Arabidopsis thaliana* and *Selaginella kraussiana* respectively, are used as backbone to construct artificial miR. The miRNA regions of these conserved sequences were replaced by the sequence designed to target the three loci *i.e.* MpARF1, MpRR-B and PRC2 of *M. polymorpha*. The transformed plants produced by knockdown of transcription factor genes such as MpRR-B and MpARF1 resulted in morphological and physiological defects in *M. polymorpha*, thus helpful in interpretation of gene functions. Similarly, the knockdown of gene encoding a component of the Polycomb recessive complex 2, MpE(z) results in sporeling lethality due to constitutive expression of this gene in construct. Thus, the conditional loss-of-function allele can be produced using an estrogen-inducible system (Zuo *et al.*, 2000; Brand *et al.*, 2006) which allowed the analysis of the phenotypic effects of induction of this armoire during other stages of the life cycle.

Table 1. Trends in *M. polymorpha* transformation through different genetic transformation methods

Explant	Method	Promoters	Plasmid	Transgene	Marker Gene	Transgenic Analysis	Reference
Suspension cultured cell	Gene gun	CaMV 35S	pBI221, pCH	hpt, uidA	Hyg	Southern, HPT enzyme activity, GUS assay	Irifune *et al.*, (1996)
Cutured cell	Agrobacterium mediated	CaMV 35S	pBIl21	GUS	G418	GUS assay Southern	Nasu *et al.*, (1997)
thallus	Gene gun	CaMV 35S	pMT	hpt	Hyg	Southern blot	Takenaka *et al.*, (2000)
Suspension cultured cell	Gene gun	rRNA operon	pCS31	aadA	Spe	PCR, DNA gel-blot	Chiyoda *et al.*, (2007)
Sporeling	Gene gun		pKI101	aadA	Hyg	PCR and DNA gel-blot	Chiyoda *et al.*, (2008)
Sporeling	Agrobacterium mediated	CaMV 35S	pIG121Hm	uidA	Hyg	GUS activity, southern, TAIL-PCR	Ishizaki *et al.*, (2008)
thallus	Agrobacterium mediated	ProGH3	pMN-GH3GUS	uidA	GUS	GUS activity	Ishizaki *et al.*, (2012)
Regenerating thalli	Agrobacterium mediated	CaMV 35S	pIG121Hm	uidA	Hyg	GUS activity	Kubota *et al.*, (2013)
Sporeling	AgarTrap	CaMV 35S	pMpGWB103-Citrine	hpt	Spe	PCR analysis, Southern blot	Tsuboyama et., al (2013)
Gemmae/ gemmaeling	Agartrap	CaMV 35S	*pMpGWB103-Citrine*	hpt	Hyg	PCR analysis	Tsuboyama, & Kodama 2015

Genetic Transformation Methods

Various methods being used in the genetic transformation of *M. polymorpha* are described below (Table 1).

Particle Bombardment

Particle bombardment also known as the biolistic or the particle gene gun method (Sanford *et al.*, 1987). This method of genetic transformation involves the plant tissues or cells being bombarded with microscopic particles (gold or tungsten particles) coated with the DNA fragment containing the genes to be transferred using a device called gene gun. The DNA fragment coated on particles is incorporated into the host genome through a recombination and repair process (Sah *et al.*, 2014). The microprojectile method helps to conquer some of the insufficiencis of *Agrobacterium* mediated method such as bacterial contagion, less-efficiency of transfer to cereal crops, and discrepancy of results (Strobek *et al.*, 2014). This technique has successfully been used for transformation of *M. polymorpha* cultured cells or thallus (Irifune *et al.*, 1996; Takenaka *et al.*, 2000; Chiyoda *et al.*, 2007; Chiyoda *et al.*, 2008). Irifune *et al.* 1996 have used particle bombardment to transform cultured cells. Thallus could not be generated from those transformed cells, which is necessary to study cell differentiation, morphogenesis and other developmental processes. However the use of thallus for particle bombardment allows the regeneration of thallus from transformed cell (Takenaka *et al.*, 2000). The study reported that the use of immature thalli grown from spores (Chiyoda *et al.*, 2008) instead of gemmae (Takenaka *et al.*, 2000) increases the particle bombardment transformation efficiency. This process is the most well-organized approach to attain plastid transformation in plants and is the lone method which is so far used to accomplish mitochondrial transformation (Johnston *et al.*, 1988; Sah *et al.*, 2014). Plastid transformation of *M. polymorpha* suspension cultured cells was done using particle bombardment (Chiyoda *et al.*, 2007; Chiyoda *et al.*, 2014). It is interesting that the genetic composition of chloroplast genome is extremely conserved between liverwort and higher plants (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986). Thus, the system stated here can give important insights that will be helpful in improving our perceptive concerning fundamental genetic mechanisms in plastids.

Particle bombardment has many advantages than other techniques like rapid gene transfer, efficient, non-specific to tissue, complex cloning

strategies with no biological limit or host restrictions and simultaneous multiple gene transfer. There is no intrinsic vector necessities, so transgenes of any magnitude and array can be introduced, and multiple gene co-transformations are straightforward. Also the delivered DNA can be maneuvered to persuade the quality and structure of the consequential transgene loci. This strategy can be used for the transfer of more than one gene concurrently in a host plant (Sah *et al.*, 2014).

Gene-gun based transformation was used for the functional study of genes by over-expression, silencing of genes and mutagenesis (Kajikawa *et al.*, 2003; Yamaoka *et al.*, 2004; Kajikawa *et al.*, 2008). Nevertheless, physical DNA delivery repeatedly results in a huge number of self-regulating insertions and enormously intricate transgene rearrangements (Kohli *et al.*, 2003), which make difficult to analyse further genetics of such mutants. Ishizaki *et al.* (2012) developed transgeneic *M. polymorpha* using particle bombardment to study the role of auxin in the growth and development. The result suggests that auxin signaling pathway is conserved among plants. Therefore the progression of the auxin signaling coordination in land plants can be assessed by using liverworts as a model plant (Cooke *et al.*, 2002; Lau *et al.*, 2008). Yamaoka *et al.* (2004) have developed a mutant of *M. polymorpha* by means microprojectile mediated mutagenesis that forms sexual organs under short day conditions constitutively compared to the wild-type, which develops sexual organs only under long-day conditions. Genetic investigation exposed that the phenotype was caused by a mutation in a single autosomal locus which possibly controls signal transduction in the changeover to sexual reproduction.

Agrobacterium mediated transformation

A. tumefaciens is able to transfer foreign DNA *via* the defined T-DNA region of its Ti-plasmid into plant cells, resulting in a non-directed random integration of the DNA into the plant genome (Van-Haute *et al.*, 1983).*Agrobacterium* approach is the most widely used methods for gene delivery in plant cells. This method of gene delivery offers various advantages over other methods such as clean insertion, low-copy number of the inserted genes, easy to handle, higher efficiency, more predictable pattern of foreign DNA integration. Studies on *A. tumefaciens* provided the basis that has made this soil bacterium as a dominant customer for plant transformation (Sah *et al.*, 2014). Nasu *et al.* (1997) used *Agrobacterium*-mediated transformation method to develop a transgenic plant of *M. polymorpha* but the transgenic plant developed was not intact. The first efficient and reproducible *Agrobacterium*-mediated transformation proctocol for *M. polymorpha* was developed using immature thalli developed from spores (Ishizaki *et al.*, 2008). The use of immature thalli developed from spores eliminates the protoplast preparation and regeneration step and also have high transformation efficiency. DNA examinations confirmed the random integrations of 1–5 copies of intact T-DNAs with the right (R) and the left (L) borders into the *M. polymorpha* genome in 7 transformants studied (Ishizaki *et al.*, 2008). Sporeling transformation is predominantly handy for experiments entailing a large number of transformants (10^4-10^5), for instance, screening of T-DNA-tagged mutants (Ishizaki *et al.*, 2013b) and homologous recombination (HR)-mediated gene targeting (Ishizaki *et al.*, 2013a). However, the methods using sporeling for transformation resulted in a large number of transformants, but the major drawback is a heterogenous genetic background of transgenic sporelings, which may affect the uniformity among resulting transgenic plant. Thus, to obtain the transformant with identical genetic background, the *Agrobacterium*-mediated transformation protocols using either tissue regenerated from a thallus from which the apical regions have been surgically removed (Kubota *et al.*, 2013), or using gemmalings (Tsuboyama and Kodama, 2015) have been developed. The further improvement of this protocol, called ‘agartrap’ method is widely used to obtain independent transgenic plants suitable for the molecular analysis (Tsuboyama and Kodama, 2014). *Agrobacterium*-mediated transformation of thallus has been also utilized for the secondary transformation of existing transgenic lines (Ishizaki *et al.*, 2013a; Komatsu *et al.*, 2014; Kubota *et al.*, 2014).

Forward and Reverse genetics

Reverse genetics are widely used for the functional gene studies in *M. polymorpha.* In this approach the functional analysis of gene is done by analysing the phenotypic effect of specific engineered gene sequences. The selection of genes of interest that will be disrupted is based on bioinformatic predictions or experimental indications. In reverse genetic, the gene of interest is disrupted by gene targeting, gene silencing or genome editing and then the effect of the disrupted gene on phenotypic trait is used for analysis of the function of genes. Gene targeting mediated by HR is a powerful tool for functional analysis in reverse genetics. Homologous recombination based gene targeting has been used for targeted disruption of many genetic loci in *M. polymorpha*, such as, NOP1 (Ishizaki *et al.*, 2013a),MpGI, MpFKF (Kubota *et al.*, 2014), MpPHOT (Komatsu *et al.*, 2014) and MpTAA (Eklund *et al.*, 2015). However, HR-mediated gene targeting is suitable for the single mutation (Ishizaki *et al.*, 2013a; Ishizaki *et al.*, 2013b). Thus, to obtain multiple mutations in single genes, CRISPR/Cas9-mediated genome editing technology

was developed (Cong *et al.*, 2013; Mali *et al.*, 2013). This technology is based on a prokaryote-specific adaptive immune system (Wiedenheft *et al.*, 2012) and has been developed for eukaryotic model organisms, including plants (Belhaj *et al.*, 2013). CRISPR/Cas9-based targeted mutagenesis has been established in *M. polymorpha* (Sugano *et al.*, 2014). In this method two types of expression vector *i.e.* gRNA containing endogenous U6 promoter-driven RNA designed to disrupt the gene encoding ARF1 and hCas9 vector containing Cas9 gene under control CaMV 35S or MpEF1a promoter are used. These expression vectors were used to co-transformed the sporeling using *Agrobacterium*-mediated transformation method (Ishizaki *et al.*, 2008). The mutant alleles thus obtained after selection showed the auxin resistant phenotype.

In addition to these techniques, RNA interference (Kajikawa *et al.*, 2003) and artificial miR (Sandoval *et al.*, 2016) inducible gene silencing has also been reported in *M. polymorpha* for functional analysis in reverse genetics.

Forward genetics approaches seek to identify the gene function by the screening of the mutants with altered phenotypes and the subsequent mapping of the allele within the genome. The model plant, *Physcomitrella patens* has a high rate of gene integration (Schaefer and Zryd, 1997) and genome duplication (Strotbek *et al.*, 2013) which impose limitation for its use in forward genetics. However, *M. polymorpha* has a noteworthy prospective as a model plant with haploid genome for forward genetics, because of its low genetic redundancy.

A gene tagging approach to create mutants has been successfully applied in *M. polymorpha* (Yamaoka *et al.*, 2004; Ueda *et al.*, 2013; Ishizaki *et al.*, 2013b) by using the efficient *Agrobacterium*-mediated transformation protocol (Ishizaki *et al.*, 2008) or particle bombardment method (Takenaka *et al.*, 2000). A morphological mutant showing impaired air-chamber formation (nop1) (Ishizaki *et al.*, 2013b) and constitutive organ development (hpt2040) (Yamaoka *et al.*, 2004) were created using the *Agrobacterium*-mediated gene transformation and particle bombardment respectively.

Chemical or physical mutagenesis is also used to develop mutants in the *M. polymorpha.* Millar *et al.* (1962) developed 12 nutritionally deficient mutant of *M. polymorpha* by using physical mutagen (X-Ray). However, the successful identification of mutations responsible for respective phenotypic variation is not reported till date. A promising alternative method to identify new genes by forward genetic approaches exploit next-generation sequencing technology for the complete sequencing of nuclear DNA from the wild type and mutants and subsequent advanced bioinformatic analysis (Nordstrom *et al.*, 2013). This method allows the simultaneous detection of mutations and sequence recovery of the corresponding loci. With decreasing sequencing costs, next-generation sequencing can turn into a high-throughput technology to analyse existing or new *M. polymorpha* mutants generated by diverse forward genetics approaches. Thus, forward genetic strategy in *M. polymorpha* is useful to understand the molecular systems that are difficult to study in other model plants, due to presence of genetic redundancy.

Conclusion

The liverwort *M. polymorpha* now emerges as the potential model plant system to study evolution, cellular and developmental biology in detail. In addition to critical evolutionary position, it has several other characteristic features that make *M. polymorpha* suitable model plant system in plant research. The comparative genetic analysis of conserved regulatory gene of bryophytes and vascular plant has increased the understanding of land plant evolution. Also, the development of several molecular tools and techniques such as transformation, large scale cell culture, gene silencing and gene targeting has increased the recognition of *M. polymorpha* as a model plant among researchers to solve many evolutionary enigmas. Moreover, it is also accepted that with the accomplishment of continuing whole genome sequencing project, which is well equipped with recent technology, our understanding of the genome will greatly enhance in the near future. With improved perceptive, the developmental and evolutionary biology of land plants can be better realize with the backing of one of the first land plants, *M. polymorpha*.

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