EXPERT REVIEWS

The potential of *Physcomitrella patens* as a platform for the production of plant-based vaccines

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The moss *Physcomitrella patens* has a number of advantages for the production of biopharmaceuticals, including: i) availability of standardized conditions for cultivation in bioreactors; ii) not being part of the food chain; iii) high biosafety; iv) availability of highly efficient transformation methods; v) a haploid, fully sequenced genome providing genetic stability and uniform expression; vi) efficient gene targeting at the nuclear level allows for the generation of mutants with specific post-translational modifications (e.g., glycosylation patterns); and vii) oral formulations are a viable approach as no toxic effects are attributed to ingestion of this moss. In the light of this panorama, this opinion paper analyzes the possibilities of using *P. patens* for the production of oral vaccines and presents some specific cases where its use may represent significant progress in the field of plant-based vaccine development. The advantages represented by putative adjuvant effects of endogenous secondary metabolites and producing specific glycosylation patterns are highlighted.

Keywords: bioreactor • glycosylation • low cost vaccines • molecular farming • moss • oral immunization

After two decades of research, extensive proofs of the concept for plant-based vaccination have been provided, but these approaches have also faced different maturation steps. Improving expression levels and increasing biosafety were priorities. Expression levels have been improved substantially through platforms based on transplastomic technologies or transient expression mediated by viral elements [1,2]. Other relevant aspects were biosafety concerns, especially those related to the risk of undesired gene flow. Therefore, exploring in detail new plant expression platforms compatible with full containment are of special importance [3]. Although some plant species have been assessed in bioreactor-based biomass production, some disadvantages are identified. Dedifferentiated plant cells cultured in suspensions tend to be sensitive to shearing due to their tough cell wall and large size, which limits operating conditions [4]. In addition, these suspension cultures require the presence of plant growth regulators, being prone to induce genetic instability known as somaclonal variation [5].

Another trend in the field was changing the concept of 'eating transgenic fruits as vaccine' to the concept of using formulations obtained by processing the plant biomass. Therefore, new platforms would provide possibilities to expand the alternatives for the production of vaccines at low cost and under an environment-friendly process.

The moss life cycle is characterized by an alternation of two stages: a haploid gametophyte and a diploid sporophyte. Gametes are generated from the gametophyte by mitosis. Their fusion results in diploid zygotes, which give rise to embryos that develop into the sporophyte. Subsequently, sporophytes can produce spores through meiosis, which germinate to produce further gametophytes [6]. Following spore germination, most of the moss species develop a filamentous stage called protonema. In the case of *Physcomitrella patens*, this stage can be long-lived under lab culture and comprises only two distinct cell types, chloronema and caulonema.

Chloronema filaments are produced right after spore germination and possess densely

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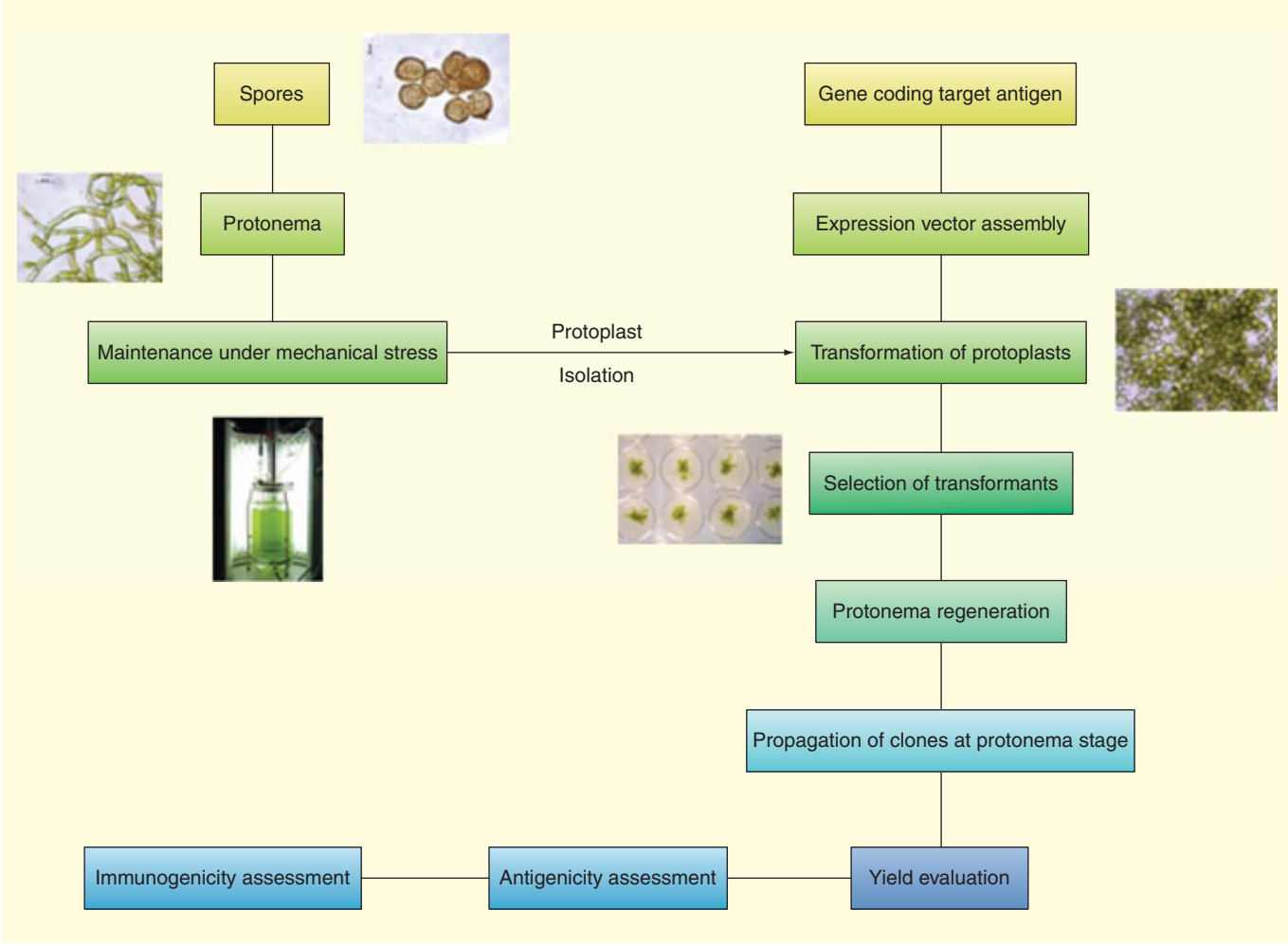

Figure 1. General workflow for developing moss-based vaccination models. The general strategy for developing moss-based candidate vaccines comprises: (i) establishing protonemal tissue from spores, and its propagation in a bioreactor, (ii) isolating and transforming protonema-derived protoplasts with a specific vector targeting the expression of the gene of interest, (iii) rescuing transformants under selective conditions and regeneration of protonema clones, (iv) assessing antigen production and evaluating the antigenic and immunogenic properties.
Spores and protonema pictures, courtesy of Nelly Horst.

packed cells containing large chloroplasts, while caulonema cells emerge later from the apical cells of chloronema filaments and present fewer spindle-shaped chloroplasts. Interestingly, protoplast regeneration can lead directly to the development of chloronema filaments, making this event a convenient intervening point for genetic transformation [7]. In addition, caulonema filaments present the production of buds as side branches, which develop into gametophores, the leafy shoots, which subsequently are able to produce gametes [8,9]. In particular, *Physcomitrella* produces female and male gametes in the same plant.

Over the last years, *P. patens* has been developed as a model organism for plant science [8,10,11]. Its genome has been fully sequenced and established community resources further facilitate its use [12]. In addition, the production of biopharmaceuticals using this organism as expression host has been explored, and provided attractive evidences of its usefulness as a low cost and convenient platform [13]. So far, moss has not been exploited as a vaccine biofactory despite its several advantages. The following sections summarize the features related to, not only using *P. patens* as an expression host for immunoprotective proteins, but also as a vehicle for delivering these molecules by the oral route, which would lead to a new trend in the field of plant-based vaccines (Figure 1) [14].

Moss biotechnology tools & the molecular farming field

Problems of social acceptance of biotechnology approaches can certainly hamper the adoption of new technologies. In particular, the risk that has been associated with the use of plants in the biopharmaceutical production field is significant. As undesired gene flow could lead to transferring antigen-encoding genes into food chains, it is highly desirable to have systems that are functional and efficient under full containment. Moss can be propagated in a simple medium of inorganic salts [15]

resulting in a very cost-effective bioprocess when compared with mammalian cell systems.

Physcomitrella is a very fast and stably growing plant culture that can be grown in standard stirred tank glass bioreactors without difficulty and as fully differentiated plants in inorganic media without any hormones, antibiotics or vitamins [16,17].

The protonema stage of gametophyte development comprises cell filaments that extend by the serial division of their apical cells [18] and is very convenient for biotechnology purposes, as it can be vegetatively propagated as a genetically stable system, where mechanical stress, rather than being a risk for tissue integrity, is a critical parameter to maintain this filamentous developmental stage and to prevent the development of the more complex adult tissues in the bioreactor [19].

Moreover, the growth rate of the photoautotrophic culture is markedly increased by aeration with CO_2-enriched air and continuous illumination. Various photobioreactor configurations have been assessed in the cultivation of moss, such as batch, semicontinuous and continuous cultures [20–22]. Among these systems, a stirred-tank glass bioreactor or a tubular glass bioreactor are functional for moss cultivation at a scale of up to 30 l. Although scale-up above that capacity is limited as the biomass itself limits the incidence of light at moderate and high cell density, some alternatives have been explored. For example, operating several bioreactors in parallel has been postulated as the easiest alternative to scaling up the process. Currently, the most advanced systems for moss cultivation are based on a 100-l tubular photobioreactor and a 500-l disposable wave-mixed bioreactor, established by Greenovation Biotech GmbH in Germany [23,24].

It is estimated that biomass production rates reported for moss yields, which range 0.5–4 g/l, are comparable with those reached under other emerging platforms in the biofarming field, such as *Chlamydomonas reinhardtii* [25]. Therefore, we consider that moss constitutes a potential platform for the production of competitive biomass amounts for vaccine evaluation in preclinical and clinical trials, although accumulation levels for specific immunogen candidates will determine this to an important degree. Performing initial experimental evaluations of this system on vaccine production is a critical factor to determine the feasibility of the platform, having expression levels and plant biomass yield as the central parameters to be measured. Indeed, the fact that a purification process for this approach is not needed seems to be a major cost-saving factor and may allow for the elicitation of immune responses, even at modest accumulation levels.

In terms of genetic engineering, the generation of stably transformed bryophytes was first documented in 1991 for *P. patens* [26] through protoplast transformation, which is still the method of choice. This method is based on the polyethylene glycol-mediated transformation of protoplasts generated from cultures of protonema tissue, which should be properly grown in suspension cultures to allow the successful isolation of protoplasts and high transformation rates [19].

In contrast to other plants examined so far, nuclear-encoded genes of *P. patens* can be specifically targeted and ablated by homologous recombination, minimizing the position effects and silencing associated with transgene copy number. Highly efficient gene targeting in moss relies on an expression vector design comprising, along with expression cassettes for transgene and gene markers, the flanking regions identical to those at the target genomic insertion point. The polyethylene glycol-mediated transformation of protoplasts is subsequently applied to rescue stable transformants through an antibiotic-mediated selection process. The regenerating protonema colonies are subsequently subjected to a transgene and protein expression screening [27,28]. Interestingly, not only plant-derived expression systems are functional in moss but also genetic elements known from mammalian cell culture [29].

A second method used for transforming bryophytes is particle bombardment. This has been applied to *P. patens* [30,31] and *Marchantia polymorpha* cells [32]. The principle of this method consists of using helium-accelerated gold particles coated with DNA which enter moss cells, thus delivering the foreign DNA. This technique is especially suitable for transient gene expression studies, but it can be also used to obtain stable transformants.

Based on early reports that describe the attachment of *Agrobacterium* to moss (*Phylaisiella selwynii*) cell walls [33,34], it is now increasingly clear that *P. patens* [35] as well as the liverwort *M. polymorpha* [36] can be transformed by *Agrobacterium*-mediated gene transfer.

This set of methods reflects the feasibility of generating genetically engineered moss clones [27,37]. It should also be mentioned that availability of efficient transformation protocols limits the exploitation of some plant species in the molecular farming field. In particular, routine efficient plastid transformation protocols are basically available for tobacco [38]. It is therefore encouraging to have a set of efficient genetic engineering tools for the species *P. patens*. Also, it is important to note that moss promoters have been isolated and used for the production of recombinant proteins in *Physcomitrella* [39,40]. These regulatory sequences can be exploited in the optimized expression of heterologous proteins.

Physcomitrella has been used as a bioreactor for the production of complex biopharmaceuticals and proved suitable for low cost and high volume production of recombinant proteins (Table 1). This system has the capability of extensively processing proteins at the posttranslational level, including the formation of disulfide bridges and complex glycosylation, as described in a further section. As no vaccines have been produced in moss, a representative example of a biopharmaceutical produced in moss is described in the following lines. The case of the human factor H (FH) illustrates the potential of *P. patens* as a convenient biopharmaceutical production platform. Complement FH is an important regulator of a key component of the innate immune response. Thus, FH deficiency or defects are associated with several diseases, identifying this protein as a target for biopharmaceutical production. However, FH has a complex structure requiring the correct formation of 40 disulfide bridges. In a report made by Buettner-Mainik *et al.* in 2011 [41], moss

Table 1. Comprehensive list of the biopharmaceuticals produced in moss to date.

Study (year)	Biopharmaceutical	Expression approach	Glycosylation profiles	Yields	Reported functional Features	Ref.
Parsons *et al.* (2012)	Asialo-hEPO	*Physcomitrella* ubiquitin gene-derived promoter for the expression of hEPO cDNA	Generation of single and double Δ-fuc-t Δ-gal-t mutant lines	NR	Disappearance of Lea residues on total intracellular *N*-glycans of moss plants lacking β1,3-GalT. Lea-free rhEPO	[52]
Weise *et al.* (2007)	Asialo-hEPO	*Physcomitrella* ubiquitin gene-derived promoter for the expression of hEPO cDNA	Generation of Δ-fuc-t Δ-xyl-t mutant lines	250 µg/g DW	Secretion of rhEPO through the cell wall to the culture medium	[68]
Schuster *et al.* (2007)	Ab IGN314 (tumor antigen specific antibody)	Expression-promoting functions of PpAct5 and PpAct7 5′ introns	Generation of Δ-xyl-t Δ-fuc-t double-knockout line	8.2 µg/ml 2 mg	Enhanced lytic ADCC potential	[64]
Baur *et al.* (2005)	hVEGF	Expression of rHSA containing a 35S promoter and signal peptide of the thaumatin-like protein H1		300 µg/g DW	Enhanced protein recovery by co-expression of rHSA with the target protein rhVEGF	[99]
Buettner-Mainik *et al.* (2011)	Factor H	Fusion of actin-5 promoter and the signal peptide coding sequence of PpAP [AP1-SP]		20 µg/g DW	Protein was correctly processed and secreted to the culture and showed biological activity comparable with that of plasma-derived F	[41]

ADCC: Antibody-dependent cellular cytotoxicity; DW: Dry weight; EPO: Erythropoietin; GalT: Galactosyltransferase; hVEFG: Human VEFG; Lea: Lewis A epitope; rhEPO: Recombinant human erythropoietin; rHSA: Recombinant human serum albumin; rhVEFG: Recombinant human VEFG; NR: Not reported.

lines that stably express recombinant FH (rFH) were generated by means of co-transfection of the vector carrying the gene of interest and a gene coding for an appropriate marker gene. Efficient secretion of the rFH into the culture supernatant was successfully induced by the inclusion of an endogenous signal peptide, *P. patens* aspartic protease 1 [42]. Secretion into the medium represents a substantial advantage, as downstream processing is greatly simplified. Immobilized-metal affinity chromatography was successfully applied as a purification strategy based on the inclusion of a C-terminal 6xHis-tag. Based on mRNA levels, four expression lines were selected from the 43 lines showing stable integration of the transgene. Importantly, the moss-produced rFH was correctly processed as it showed a biological activity comparable with that of plasma-derived human factor. In terms of yields, cultivation during 1 week in a 5 l photobioreactor resulted in about 25.8 µg/g dry weight, which is comparable with those levels reached when biopharmaceuticals are expressed via nuclear expression in conventional plant-based systems [43]. Importantly, no changes in growth rate and development of the production strains were observed when compared with wild-type *P. patens*. This case illustrates appropriately the advantages and the general strategies involved in using moss for the production of biopharmaceuticals. It is then considered that vaccines may follow this general procedure even if the immunogen is a complex and glycosylated protein.

Glycoengineering & immunological implications

It is well known that glycosylation patterns can alter the immunogenic properties of biopharmaceuticals, such as antibodies and vaccines [44–46]. Of particular interest is the fact that asparagine (Asn/N)-linked protein glycosylation in plants shares a common core structure with mammalian *N*-glycosylation, that is, di-antennary glycans of the complex type. Main differences consist in the lack of terminal β-1,4-galactose and sialic acid residues in plants, while present in mammals. By contrast, in plants complex-type *N*-glycans are modified by the enzymes β1,2-xylosyltransferase and α1,3-fucosyltransferase that are not present in mammals [47–50].

Modification of glycosylation processes in a specific host can be achieved through a set of molecular approaches, where deleting glycosyltransferases responsible for certain modifications has been the first-hand approach [51–53], although RNAi has also been used to decrease the amount of the target glycosyltransferases [54–56]. In addition, heterologous expression of the desired glycosylstransferases that are absent in the host constitute another important tool in this field [57–59].

Using these kinds of molecular strategies, both beneficial and adverse effects can be attributed to plant-specific glycosylation patterns. The effect of glycosylation on the immunogenic properties of biopharmaceuticals has been

mainly studied in higher plants. As no vaccines have been produced in moss so far, higher plants are considered the closer system that may serve as example on how glycosylation is an important aspect on vaccine production. Jin *et al.* [44] have reported that glycosylation in plants seems to confer enhanced immunogenicity of plant-specific *N*-glycans on a human monoclonal antibody (2G12) against HIV, compared with that produced in glycoengineered plant lines with the absence of β1,2-xylose and α1,3-fucose. Passive immunization of rabbits with the protein carrying these residues resulted in a humoral immune response to both types of *N*-glycan structures. Also, immunoblotting studies with sera from allergic patients showed binding to these monoclonal antibodies decorated with α1,3-fucose. One of the mechanisms that could be responsible for this effect is an increased lectin-mediated antigen uptake by the antigen-presenting cells, such as dendritic cells. It is well known that C-type lectin receptors usually recognize carbohydrates from self or non-self origin and internalize glycosylated antigens for further antigen presentation by MHC class II molecules. Some undesired effects are also a possibility as these fucose and xylose residues can mediate the development of allergies through the binding of anti-IgE, especially when considering the correlation between IgE levels responsible for immunoreactivity to carbohydrate compounds and allergic disorders [60,61].

Recently, therapeutic antibodies have become an interesting subject due to the high demand for the treatment of different types of diseases. Monoclonal antibodies produced in different plant systems [62,63] have exhibited reduced *in vivo* efficacy compared with their *in vitro* activity. Endogenous IgG competes with therapeutic antibodies preventing antibody-dependent cellular cytotoxicity in effector cells. Modification of glycosylation processes has shown to increase antibody-dependent cellular cytotoxicity activity [64] of fucose-deficient antibodies produced in *P. patens.* Moreover, the activity of these therapeutic IgGs is not impaired by normal human serum.

It is clear that these kind of posttranslational modifications are responsible for complex and diverse effects and therefore should be evaluated case by case in order to determine if interventions are required according to this particular goal.

In this context it deserves special consideration that some mosses offer a singular flexibility for achieving interventions on glycosylation as deleting specific glycosyltransferases or conferring the ability of expressing heterologous ones can lead to directed glycoengineering approaches. *P. patens* and *Ceratadon purpureus* are able to integrate foreign DNA in a site-directed manner, as homologous recombination occurs at high frequencies in the nuclear genome. This advantage is unique in mosses and *P. patens* in particular exhibits a higher frequency of gene targeting compared with *C. purpureus* [65]. This feature makes this moss a robust platform for functional genomics but also for developing strains with improved biosynthetic traits in terms of the production of heterologous proteins [66,67]. For example, double-knockout variants for β1,2-xylosyl-transferase and α1,3-fucosyl-transferase genes have been generated, in order to 'humanize' and optimize *N*-linked oligosaccharide structures [51]. The purpose of this humanization is to yield a heterologous protein lacking the potentially immune reactive α1,3-fucose and β1,2-xylose residues [59,64,68]. As shown in Table 1, a number of biopharmaceuticals have been produced successfully in moss knockouts to accomplish specific glycosylation patterns.

On the other hand, having specific glycosylation is especially desirable in those cases when vaccine targets are highly glycosylated proteins, in which this trait has a relevant role in immunogenicity. This is the case for some viral proteins, specifically those of enveloped viruses, such as HIV and influenza virus. In particular, gp120 from HIV is considered a crucial target in vaccine development as it contains well-characterized neutralizing epitopes, which elicit antibodies able to mediate the blockade of viral entrance. Interestingly, potential gp120 epitopes have shown differential immunogenic properties when changes on glycosylation patterns are induced. Half of the gp120 molecular mass is contributed by *N*-glycans that may be potential epitopes shielding an immune recognition, contributing to antibody reactivity to evasion from host immune responses [45,69–72]. Recent studies have evaluated the efficacy of 'deglycosylation' [45]. Some glycans from *Env* were devoided to allow a protective immune response against infection in macaque models. However, this resulted in a reduced vaccine efficacy and reduced immune responses, compared with wild-type *Env* immunized animals. By contrast, it was recently reported that the removal of an *N*-linked glycan enhanced *in vitro* antigenicity of some neutralizing epitopes in the V3 loop [73]. In addition, the conserved glycan N448 was identified, whose disruption induced changes in the structure of the C4 region of gp120, making this specific region more resistant to proteolytic processing [74]. It has also been demonstrated that the differences in glycan composition of gp120 produced by different host cells can affect recognition of *Env* [70]. Some site-specific deglycosylations have resulted in an enhancement of the elicitation of neutralizing humoral responses [75,76].

Since specific glycosylation patterns have been associated with differential immunogenic properties, it is of relevance having moss as a host that can be engineered to generate the desired glycosylation pattern. This is a tool that would allow the investigation of glycosylation structural and conformational features that may lead to novel HIV vaccine design approaches, and also to elucidate the impact of differential glycosylation on the quality of the immune response to HIV. Therefore, the development of HIV vaccines may be positively impacted by the use of immunogens with engineered glycans able to evoke broader effector immune responses. Since only a few pathogens are being analyzed from this point of view, it is considered a field of opportunity with a particular potential for the vaccine development area.

Expert commentary

The use of moss biomass for the production and delivery of vaccines remains unexplored. However, as plant cells have been

used over the past two decades as oral delivery vehicles for antigenic proteins, having as a result interesting perspectives for the vaccinology field, it is reasonable to expect the same application for moss. In higher plants, several antigens have been properly expressed yielding functional immunogenic proteins [77–79]. This immunization strategy has also been associated with increased immunogenicity when compared with that consisting of administering the pure recombinant protein alone [80–82]. This aspect is interesting and should be explored in more detail. It could be related to the delay of antigen degradation when it is accompanied by several complex molecules such as polysaccharides; the differential glycosylation of the target protein to those produced in bacteria; the presence of plant secondary metabolites with adjuvant activity, among other possible factors [83].

Interestingly, despite containing high amounts of phenolics, several bryophytes are edible and considered safe for oral consumption as they serve as supplements in food production. For example, *Sphagnum* sp. has been used as an ingredient in bread, as tea, as a flavoring agent for Scotch whisky and also as an animal feedstock component [84]. This species has been found to serve as a feed source for lepidopterans, showing similar digestibility as that of lettuce. Importantly, in a very preliminary assessment, *P. patens* has been consumed as a drink component with no apparent adverse effects in humans, although it has not yet been assigned to the category of Generally Recognized as Safe oral communication. It is expected that further studies will provide in detail data on the safety for the ingestion of moss biomass. Therefore, although *P. patens* has not been explored as a vehicle for oral delivery of biopharmaceuticals, it is considered that the development of moss-based oral vaccines is a potential field of opportunity. In spite of the limited knowledge on the safety of moss intake, a particular field where oral administration of moss biomass might have a straightforward influence is in the veterinary field as the regulatory system is less strict than those for use in humans [85].

As the failure of various vaccines are attributed to their inability to trigger a robust immune response able to protect when exposure to an infectious agent takes place, the use of adjuvants is necessary to achieve protective immunity. Despite major advances in vaccinology, the formulations basically still depend on aluminum salts as adjuvants. The secondary effects associated with these compounds as well as the necessity to direct the immune responses in a more precise manner raises the need for new adjuvants.

A number of plant extracts or fractions have significant if not strong immunomodulatory properties, such as inducing increase in cytokine production, enhancing the activation of CD4 and CD8 T cells or enhancing the activity of NK cells [86–88]. These plant metabolites can be administered along with the vaccine to elicit stronger immune response [89]. Plant-derived adjuvants are gaining attention as potential vaccine components. Recently, increased attention has been paid to the use of plant secondary metabolites as possible adjuvants in vaccine formulations. This trend is based on the fact that a number of secondary metabolites have shown adjuvant properties when co-administered in vaccine formulations [90–94].

Interestingly, *P. patens* produces a diverse set of secondary metabolites, including typical animal, algal and mushroom metabolites [95]. Although not widely discovered and characterized, among the metabolites produced by moss, one may identify interesting cases, such as the fact that moss produces important levels of eicosapentaenoic acid. This polyunsaturated fatty acid can affect inflammation and metabolism in humans, providing benefits in immunologic and metabolic disorders. These effects are believed to be mediated by modifying the production of inflammatory mediators and the suppression of inflammatory leukocytes [96].

As another example, polyphenols are known to exert immunomodulatory effects [97], and it is of interest to note that these kinds of compounds are present in *P. patens* and enzymes related to their metabolism are under characterization [98].

Therefore, the hypothesis of expecting immunomodulatory effects by the moss metabolites is of particular relevance and identifies in moss a particular potential of serving not only as a delivery vehicle but also as a source of adjuvant compounds that may enhance vaccine efficacy. We therefore propose that these compounds could have a potential adjuvant effect when co-administered with vaccines.

Five-year view

As the moss *P. patens* would represent an advantageous platform for both production and delivery of oral vaccines, it opens interesting perspectives for the field of plant-based vaccine development. These perspectives comprise: studying in detail the convenience of moss oral administration in terms of safety and efficacy as an oral delivery vehicle, exploring the adjuvant effect of the metabolites produced by this host, taking advantage of the low cost and environmentally friendly processes for moss propagation in order to facilitate approval by the regulatory agencies and exploring the production of antigens requiring specific glycosylation which can be produced easily by means of moss glycoengineering. Therefore, experimental approaches to evaluate these aspects would positively impact the development of advanced platforms for low-cost vaccine production in the following years. Vaccines where glycosylation is relevant for immunogen potency are priority targets for exploration. As no moss-derived vaccine has been produced yet, these interesting and unexplored aspects would constitute a relevant perspective for the field in the next years. We expect this trend to be of significant interest to vaccinologists and may lead to a next generation of plant-based vaccines.

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Key issues

- The moss *Physcomitrella patens* is an advantageous system for the production of biopharmaceuticals in terms of biosafety, genetic stability and versatility of genetic engineering.
- Oral vaccines produced in moss may represent a novel and convenient system for the formulation of low cost and orally administered vaccines.
- Future research should focus on: studying the putative adjuvant effects of the secondary metabolites produced by this host and the development of glycoengineered strains for the production of optimized vaccines.

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