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Functional cross-kingdom conservation of mammalian and moss (*Physcomitrella patens*) transcription, translation and secretion machineries

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Summary

Plants and mammals are separated by a huge evolutionary distance. Consequently, biotechnology and genetics have traditionally been divided into 'green' and 'red'. Here, we provide comprehensive evidence that key components of the mammalian transcription, translation and secretion machineries are functional in the model plant *Physcomitrella patens*. Cross-kingdom compatibility of different expression modalities originally designed for mammalian cells, such as native and synthetic promoters and polyadenylation sites, viral and cellular internal ribosome entry sites, secretion signal peptides and secreted product proteins, and synthetic transactivators and transrepressors, was established. This mammalian expression portfolio enabled constitutive, conditional and autoregulated expression of different product genes in a multicistronic expression format, optionally adjusted by various trigger molecules, such as butyrolactones, macrolide antibiotics and ethanol. Capitalizing on a cross-kingdom-compatible expression platform, we pioneered a prototype biopharmaceutical manufacturing scenario using microencapsulated transgenic *P. patens* protoplasts cultivated in a Wave Bioreactor. Vascular endothelial growth factor 121 ($VEGF_{121}$) titres matched those typically achieved by standard protonema populations grown in stirred-tank bioreactors. The full compatibility of mammalian expression systems in *P. patens* further promotes the use of moss as a cost-effective alternative for the manufacture of complex biopharmaceuticals, and as a valuable host system to advance synthetic biology in plants.

Introduction

The moss *Physcomitrella patens*, unique for its high rate of homologous recombination, generic codon usage, haploidy, simple body plan, physiologic properties and exclusive phylogenetic position (Quatrano *et al*., 2007; Rensing *et al*., 2008), is gathering momentum for the biopharmaceutical manufacture of protein therapeutics because of favourable bioprocess and downstream processing economics (Decker and Reski, 2007). *In vitro* cultivation of *P. patens* throughout its complete life cycle (Frank *et al*., 2005), transgenic protonema and transient protoplast cultures (Baur *et al*., 2005a), stirred-tank and tubular photo-bioreactors (Decker and Reski, 2007), the generation of moss mutants devoid of immunogenic product protein glycosylation (Huether *et al*., 2005), and the production of human antibodies with improved antibody-dependent cellular cytotoxicity (ADCC) activity (Nechansky *et al*., 2007) have been important milestones in establishing the moss as a promising biopharmaceutical manufacturing platform (Decker and Reski, 2007).

Transgenic mammalian cell cultures are currently the most successful platform for the production of biopharmaceuticals, as most of the protein therapeutics on the market originate from mammalian cell bioprocesses (Wurm, 2004). However, as the global product pipeline exceeds the worldwide manufacturing capacity, alternative host cell systems for

biopharmaceutical manufacturing are on the increase (Hamilton *et al*., 2006; Decker and Reski, 2007). Since the mid-1980s, the productivity of mammalian cells cultivated in bioreactors has reached the gram per litre range, an over 100-fold yield improvement over titres achieved for the first commercial bioprocesses (Wurm, 2004). Part of this success is based on the development of sophisticated expression technologies and metabolic engineering strategies (Umana *et al*., 1999; Hartenbach and Fussenegger, 2005). The latest generation of expression vectors harbour: (i) compact strong constitutive promoters for the high-level transcription of product genes (Hartenbach and Fussenegger, 2006); (ii) multicistronic expression units enabling one-vector-based selection and expression of multiprotein complexes (Fux *et al*., 2004); and (iii) regulated expression systems for the production of difficult-to-express protein therapeutics (Weber and Fussenegger, 2007).

In mammalian cells, the initiation of translation is typically managed by a cap structure which is post-transcriptionally attached to the 5′ end of mRNAs (Kozak, 1989). Alternatively, internal ribosome entry sites (IRESs), which adopt a specific secondary RNA structure triggering ribosome assembly and translational initiation, have evolved to ensure a minimal level of protein synthesis for survival during cap-compromising physiological emergency situations (co-ordination of viral defence; cellular IRES) (Gan and Rhoads, 1996) or to redirect the cellular translation machinery to the production of virus proteins (viral IRES) (Kaufman *et al*., 1991; Dirks *et al*., 1993). The tandem arrangement of different transgenes, each preceded by an IRES element, enables the transcription of a multicistronic mRNA producing stoichiometric levels of various proteins. Recently, a sophisticated vector platform (pTRIDENT) has been designed for the multicistronic expression of up to three different transgenes (Fux *et al*., 2004).

Heterologous mammalian transcription control modalities have been designed in two different configurations: ON-type systems, which are induced following the addition of a trigger molecule, and OFF-type systems, which are repressed after the administration of a regulating compound (Weber and Fussenegger, 2007). ON-type systems typically consist of a transrepressor (optionally containing a silencing domain), which binds to specific (tandem) operator sequences and blocks transcription from upstream constitutive promoters until the transrepressor is released after interaction with the inducer (Weber *et al*., 2002, 2005). Transactivators, which only bind to their operator modules in the presence of the inducer, are also classified as ON-type systems (Weber *et al*., 2004; Hartenbach and Fussenegger, 2005). OFF-type systems usually consist of a chimeric transactivator, which binds a specific (tandem) operator sequence and triggers transcription from an adjacent minimal promoter until it is released by interaction with the inducer (Weber *et al*., 2002, 2003). A variety of these transcription control systems have been used for basic and applied research (Weber and Fussenegger, 2007).

The protein production machineries of mammalian cells and plants are known to be largely incompatible, which requires mammalian expression technology to be specifically modified for use in plant cells and plants (Frey *et al*., 2001; Mayfield *et al*., 2003). The availability of cross-kingdom-compatible protein expression technology would significantly improve the use of plant cells for biopharmaceutical manufacturing. We provide comprehensive evidence that the transcription, translation and secretion machineries of mammalian cells and the non-seed plant *P. patens* are compatible, pioneer a novel protoplast-based fermentation technology for the production of human glycoproteins, and thus establish *P. patens* as a valuable host system for synthetic biology, in particular to functionally understand the most conserved molecular devices controlling biological signalling in the different kingdoms.

Results

Profiling of mammalian promoter activities in *P. patens*

The swapping of expression units between mammalian and plant cell platforms for gene function analysis has been hampered by incompatibilities in the transcription/translation/secretion machineries. These systems require exclusive genetic elements (promoters, reporter genes, polyadenylation sites) for the expression of transgenes (Frey *et al*., 2001). In order to measure the activity of mammalian promoters in *P. patens*, isogenic, all-mammalian expression vectors were designed harbouring the human placental secreted alkaline phosphatase (SEAP), an easy-to-assay reporter gene, a polyadenylation site derived from simian virus 40 and various mammalian promoters (P_{hCMV}, P_{SV40}, P_{GTX}, $P_{hEF1\alpha}$), including the smallest synthetic promoter P_{GTX} (Hartenbach and Fussenegger, 2006). The polioviral IRES ($IRES_{PV}$), known to be devoid of any promoter activity, was used as a negative control. Of the promoters tested, only $P_{hEF1\alpha}$ was not functional in *P. patens*. Interestingly, the world's smallest synthetic promoter (182 bp) was fully functional, reaching P_{hCMV}-driven expression levels in the moss. The SEAP expression profiles reached using mammalian expression vectors were compared with those of an isogenic plant expression vector encoding SEAP under the control of the cauliflower mosaic virus 35S promoter ($P_{CaMV35S}$) (Figure 1).

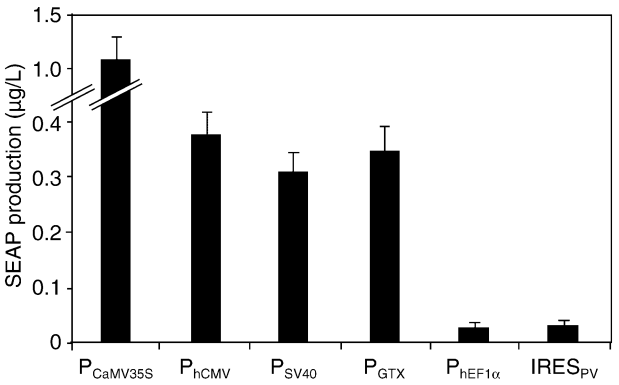

Figure 1 Comparative expression performance of different constitutive mammalian and plant promoters in *Physcomitrella patens*. Isogenic secreted alkaline phosphatase (SEAP) expression vectors (P_{hCMV} [pSS173], P_{SV40} [pMG31], P_{GTX} [pSH17], $P_{hEF1\alpha}$ [pMG32], -SEAP-pA_{SV40}; $P_{CaMV35S}$ [pMG65], -SEAP-$pA_{CaMV35S}$) were transfected into *P. patens* protoplasts. SEAP production was scored after 5 days. The polioviral internal ribosome entry site ($IRES_{PV}$), which lacks promoter activity in mammalian cells, was used as a control (pSH49, $IRES_{PV}$-SEAP-pA_{SV40}). Error bars indicate the standard deviation of at least three independent experiments.

The secretory machineries of mammalian cells and *P. patens* are compatible

Previous studies on mammalian promoter compatibility in the moss revealed that human placental SEAP could be secreted by *P. patens* protoplasts (Figure 1). In order to assess whether product genes containing mammalian secretion signals are generically secreted by *P. patens*, several product genes, including SEAP, the *Bacillus stearothermophilus*-derived secreted α-amylase (SAMY) containing an immunoglobulin G (IgG)-derived secretion signal, human vascular endothelial growth factor 121 ($VEGF_{121}$) and human erythropoietin (EPO), were cloned into isogenic P_{GTX}-driven mammalian and isogenic $P_{CaMV35S}$-driven plant expression vectors. The product levels were profiled in the supernatant of transfected moss protoplast cultures, as well as in the cytosol, in order to assess the overall product secretion efficiency (Table 1). All of the mammalian product proteins were efficiently secreted by moss protoplasts, whereas the control protein α-amylase (AMY) lacking the IgG secretion signal sequence could only be detected in the plant cytosol (Table 1). In order to characterize the processing of secreted mammalian proteins in *P. patens*, we N-terminally sequenced human $VEGF_{121}$ purified from moss culture supernatants. The finding that the first 10 amino acids of secreted $VEGF_{121}$ were A(OH-Pro)MAEGGGQN suggests that secreted mammalian proteins are identically processed in *P. patens*.

Mammalian cap-independent translation initiation is functional in *P. patens*

Internal ribosome entry sites are capable of managing cap-independent translation initiation under physiological conditions which compromise classical cap-mediated translation (Pestova *et al*., 2001). Non-limiting examples of IRES-mediated translation include: (i) virus infection, during which the virus interferes with the cellular translation machinery and redirects it to translation of its IRES-tagged transcripts (viral IRES) (Kaufman *et al*., 1991; Dirks *et al*., 1993), and (ii) hijacked cells may co-ordinate a molecular defence by translating a set of IRES-containing transcripts (cellular IRES) (Gan and Rhoads, 1996). With the functionality of mammalian promoters and protein secretion established in *P. patens*, mammalian cell- and virus-derived IRESs were

Table 1 Expression levels of mammalian reporter constructs in *Physcomitrella patens*

Expression vector	Expression level (supernatant)	Expression level (intracellular)
P_{GTX}-SEAP-pA (pSH17)	0.36 ± 0.02 µg/L	0.06 ± 0.007 µg/L
$P_{CaMV35S}$-SEAP-pA (pMG65)	1.02 ± 0.09 µg/L	0.19 ± 0.01 µg/L
P_{GTX}-SAMY-pA (pSH102)	9.0 ± 0.54 µmol/s/L	2.1 ± 0.02 µmol/s/L
$P_{CaMV35S}$-SAMY-pA (pMG66)	18.6 ± 2.3 µmol/s/L	4.1 ± 0.3 µmol/s/L
P_{GTX}-AMY-pA (pMG60)	1.65 ± 0.05 µmol/s/L	6.73 ± 0.34 µmol/s/L
$P_{CaMV35S}$-AMY-pA (pMG67)	2.5 ± 0.1 mol/s/L	13.2 ± 1.23 µmol/s/L
P_{GTX}-$VEGF_{121}$-pA (pSH100)	4.0 ± 0.1 ng/mL	0.42 ± 0.07 ng/mL
$P_{CaMV35S}$-$VEGF_{121}$-pA (pMG68)	13.5 ± 0.5 ng/mL	1.1 ± 0.1 ng/mL
P_{GTX}-EPO-pA (pMG61)	48 ± 10.6 mU/mL	5.2 ± 0.9 mU/mL
$P_{CaMV35S}$-EPO-pA (pMG69)	159 ± 8.1 mU/mL	22 ± 0.95 mU/mL

AMY, *Bacillus stearothermophilus*-derived α-amylase; EPO, human erythropoietin; $P_{CaMV35S}$, cauliflower mosaic virus promoter 35S; P_{GTX}, synthetic promoter derived from the GTX homeodomain protein; SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase; SEAP, human placental secreted alkaline phosphatase; $VEGF_{121}$, human vascular endothelial growth factor 121.

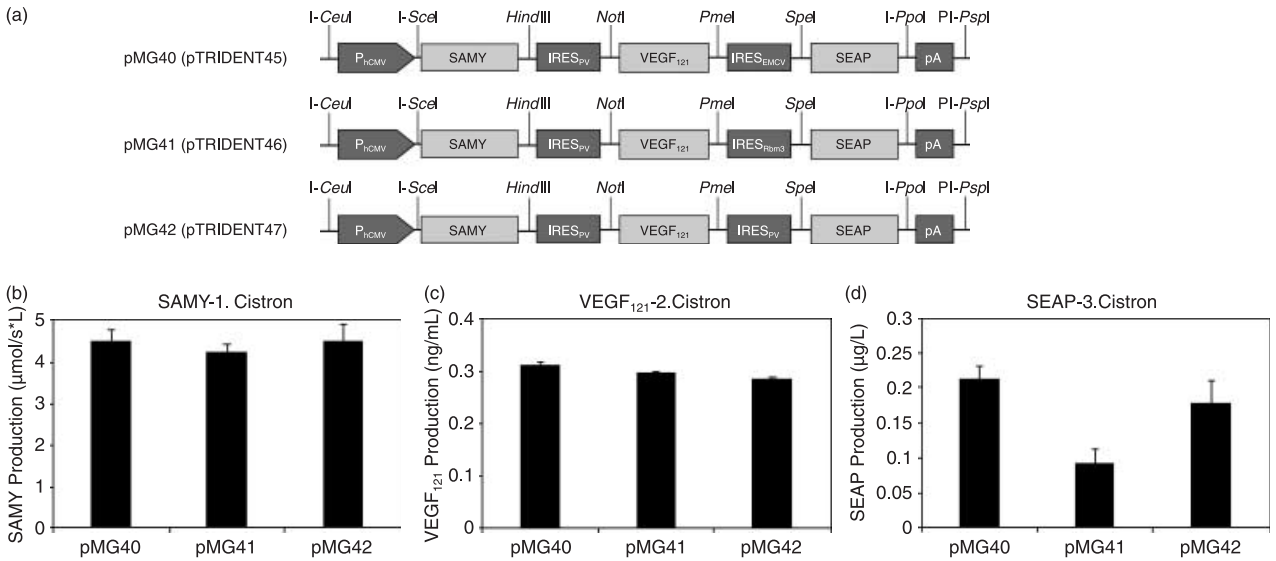

Figure 2 Internal ribosome entry site (IRES)-mediated translation initiation in *Physcomitrella patens*. (a) Schematic representation of tricistronic mammalian expression cassettes encoding a P_{hCMV}-driven multicistronic expression unit harbouring the *Bacillus stearothermophilus*-derived secreted α-amylase (SAMY), vascular endothelial growth factor 121 ($VEGF_{121}$) and secreted alkaline phosphatase (SEAP) in cistrons 1, 2 and 3, respectively. SAMY is translated in a classic cap-dependent manner, $VEGF_{121}$ requires cap-independent translation initiation by the poliovirus internal ribosome entry site ($IRES_{PV}$), and the translation of SEAP is mediated by either $IRES_{PV}$, the encephalomyocarditis virus IRES ($IRES_{EMCV}$) or the IRES element derived from the human RNA-binding motif protein 3 ($IRES_{Rbm3}$). SAMY (b), $VEGF_{121}$ (c) and SEAP (d) expression levels of moss protoplast cultures transfected with pTRIDENT45, pTRIDENT46 and pTRIDENT47. Reporter protein production was scored 5 days after transformation. Error bars indicate the standard deviation of at least three independent experiments.

evaluated in *P. patens* to determine whether they could trigger translation initiation and enable multicistronic expression.

We designed a variety of latest generation pTRIDENT vectors containing: (i) a constitutive P_{hCMV} driving the transcription of multicistronic mRNAs; (ii) an artificial poly-adenylation site (apA) signalling the terminus of the multicistronic transcript (Hartenbach and Fussenegger, 2005); (iii) two tandem IRES elements of poliovirus ($IRES_{PV}$) (Dirks *et al*., 1993) or encephalomyocarditis virus ($IRES_{EMCV}$) (Kaufman *et al*., 1991) and $IRES_{Rbm3}$, derived from the human RNA-binding motif protein 3 (Rbm3) (Chappell and Mauro, 2003); (iv) vast multiple cloning sites (MCSs) flanking each IRES element (many of which are targets for rare-cutting, 8-bp-recognizing restriction endonucleases) for complication-free sequential insertion of (v) product genes, including SAMY, $VEGF_{121}$ and SEAP; and (vi) P_{hCMV}, SAMY-IRES-$VEGF_{121}$-IRES-SEAP and apA, flanked by rare-cutting homing endonucleases (I-*Ceu*I, I-*Sce*I, I-*Pop*I, PI-*Psp*I), which enable, together with MCSs, straightforward exchange/swapping of expression modules and transgenes among different members of the pTRIDENT vector family (Fux *et al*., 2004).

Following the transfection of pTRIDENT45 (P_{hCMV}-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-apA), pTRIDENT46 (P_{hCMV}-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{Rbm3}$-SEAP-apA) and pTRIDENT47 (P_{hCMV}-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{PV}$-SEAP-apA) into *P. patens* protoplasts, significant levels of product protein were produced from all positions within the vectors, indicating that mammalian cell/virus-derived IRES elements are functional and enable multicistronic transgene expression in the moss (Figure 2).

Tunable product gene expression in *P. patens* using mammalian transgene control technology

Transcription control of specific genes by small trigger molecules is essential for gene function analysis (Malleret *et al*., 2001), drug discovery (Weber *et al*., 2008), the design of complex artificial gene circuits (Kramer and Fussenegger, 2005), precise and timely molecular interventions in gene therapy (Gersbach *et al*., 2006), engineering of preferred cell phenotypes for tissue engineering (Niwa *et al*., 2000) and biopharmaceutical manufacturing (Fussenegger *et al*., 1998). Although a variety of transgene control systems are available for fine-tuning transgene transcription in mammalian cells (Weber and Fussenegger, 2007), the choice for controlling transgene expression in plant cells, in particular in *P. patens*, is limited (Saidi *et al*., 2005). Recently, mammalian transcription control circuits have been designed which are responsive to the butyrolactone 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide (SCB1) (QuoRex; Q-ON, Q-OFF)

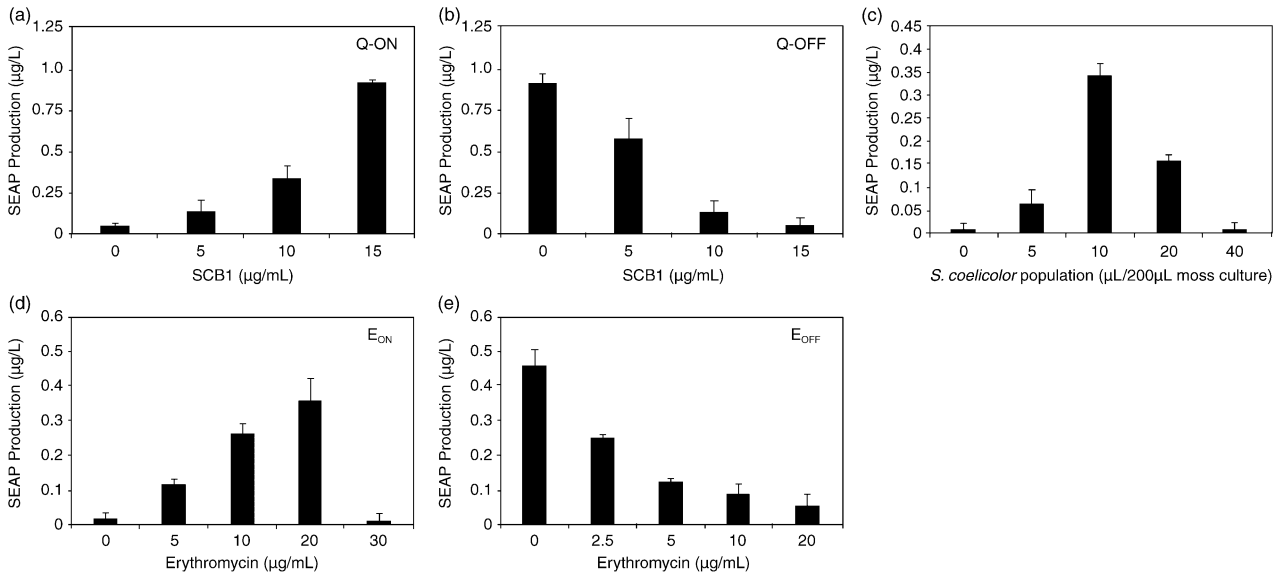

Figure 3 Quorum-sensing control of transgene expression in *Physcomitrella patens*. The mammalian Q-ON (a) and Q-OFF (b) systems, which are induced and repressed, respectively, by the *Streptomyces coelicolor* quorum-sensing butyrolactone 2-(1′-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide (SCB1), were transfected into moss protoplasts, and dose–response profiles of secreted alkaline phosphatase (SEAP) were recorded after 5 days. (c) Co-cultivation of Q-ON transgenic moss protoplasts with SCB1-producing *St. coelicolor* reveals quorum-sensing-based cross-species communication, resulting in a correlation between plant-based SEAP production and *St. coelicolor* population size. (d, e) Macrolide-responsive transgene expression in moss protoplasts. SEAP expression profiles of moss protoplast cultures transfected with the mammalian E_{ON} (d) and E_{OFF} (e) systems and cultivated for 5 days in the presence of varying erythromycin concentrations. Error bars indicate the standard deviation of at least three independent experiments.

(Weber *et al*., 2003, 2005), the macrolide antibiotic erythromycin (E.REX; E_{ON}, E_{OFF}) (Weber *et al*., 2002) and acetaldehyde or ethanol (AIR) (Weber *et al*., 2004, 2007). AIR-controlled transgenes are induced by acetaldehyde/ethanol, whereas E.REX and QuoRex are available in two different design versions, which can either be induced (E_{ON}, Q-ON) or repressed (E_{OFF}, Q-OFF) by the addition of the regulating molecule (Weber *et al*., 2002, 2003, 2005).

All mammalian transgene control systems were optimized for regulated SEAP expression and were transfected into *P. patens*, which was grown in the presence and absence of different trigger molecules at various concentrations. SCB1 was well tolerated by the moss (toxic only above 20 µg/mL, data not shown) and mediated adjustable, up to 15-fold induction (Q-ON; pWW504, P_{SV40}-*scbR*-KRAB-pA; pWW162, P_{SCA}ON8-SEAP-pA) and repression (Q-OFF; pWW122, P_{SV40}-*scbR*-VP16-pA; pWW124, P_{SPA}-SEAP-pA) of SEAP expression within a concentration range of 0–15 µg/mL (Figure 3a,b). The Q-ON system is so sensitive in *P. patens* that the moss senses the presence of co-cultivated SCB1-producing *Streptomyces coelicolor*, and participates in *St. coelicolor's* quorum-sensing cross-talk by adjusting SEAP production in response to the size of the bacterial population (Figure 3c).

The E_{ON} (pWW43, P_{SV40}-E-KRAB-pA; pWW56, P_{ETR}ON8-SEAP-pA) and E_{OFF} (pWW35, P_{SV40}-E-VP16-pA; pWW37, P_{ETR2}-SEAP-pA) systems were able to induce or repress SEAP expression up to 13-fold using erythromycin levels not exceeding 20 µg/mL (toxic above 30 µg/mL) (Figure 3d,e). The AIR system produced 20-fold SEAP expression (AIR; pWW195, P_{SV40}-*alcR*-pA; pWW192, P_{AIR}-SEAP-pA) in the moss when induced by 20 µL/mL ethanol (Figure 4a). This compares favourably with the regulated performance of plant-specific, ethanol-mediated transgene regulation in tobacco (Caddick *et al*., 1998), *Arabidopsis thaliana* (Roslan *et al*., 2001), potato and oilseed rape (Sweetman *et al*., 2002). The AIR-controlled system is incredibly sensitive in *P. patens*, such that SEAP production can be induced by *Saccharomyces cerevisiae* populations cultivated at a distance. As part of its metabolism, *Sa. cerevisiae* converts ethanol into gaseous acetaldehyde, which reaches moss cultures 'over the air' and induces SEAP production in a distance-dependent manner (Figure 4b).

The combination of AIR-based transcription control with multicistronic expression technology (pTRIDENT42; P_{AIR}-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-apA) enabled the co-ordinated induction of three different transgenes after the addition of 10 µL/mL ethanol (Figure 4c–e).

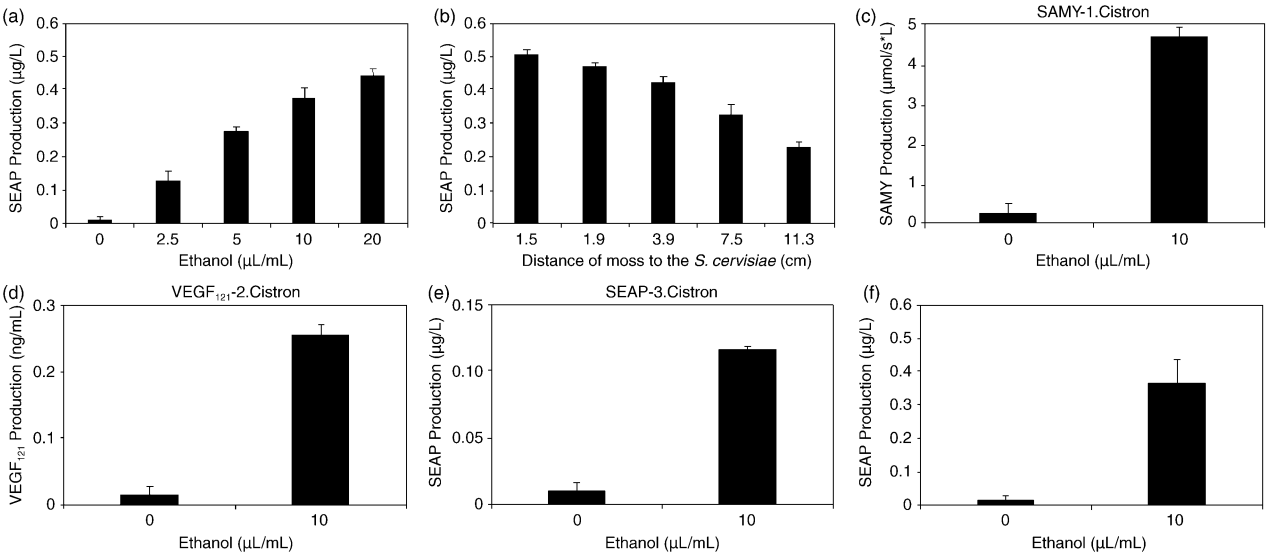

Figure 4 Ethanol- and gas-inducible transgene expression in *Physcomitrella patens*. (a) Ethanol-inducible secreted alkaline phosphatase (SEAP) expression in moss protoplasts transfected with the mammalian acetaldehyde or ethanol (AIR) system. (b) *Saccharomyces cerevisiae* producing gaseous acetaldehyde as part of its native metabolism triggers SEAP expression 'over-the-air' in distant *P. patens* cultures harbouring the mammalian AIR system. (c–e) Ethanol-controlled tricistronic gene expression in moss protoplasts with the *Bacillus stearothermophilus*-derived secreted α-amylase (SAMY) encoded in the first (c), vascular endothelial growth factor 121 ($VEGF_{121}$) in the second (d) and SEAP in the third (e) cistron. Protein production was scored 5 days after transformation of moss protoplasts with pWW195 (P_{SV40}-*alcR*-pA) and pSH3 (P_{AIR}-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-apA) and cultivation in the presence or absence of ethanol. (f) Autoregulated transgene expression in *P. patens*. Moss protoplasts transfected with the ethanol-inducible autoregulated expression vector pSH28 (P_{AIR}-SEAP-$IRES_{PV}$-*alcR*-apA) were cultivated for 5 days in the presence and absence of ethanol before SEAP expression levels were determined in the culture supernatant. Error bars indicate the standard deviation of at least three independent experiments.

Autoregulated transgene expression in *P. patens*

Classic transgene control systems consist of two expression vectors, one harbouring the transrepressor/transactivator and the other encoding the transgene driven by the trigger-inducible promoter (Weber and Fussenegger, 2007). Such a two-vector design is more complex to engineer compared with the latest generation autoregulated one-vector configurations (Hartenbach and Fussenegger, 2005). Capitalizing on the functionality of IRES elements in *P. patens*, protoplasts were transfected with the ethanol-controlled autoregulated SEAP expression vector pAutoRex8 (P_{AIR}-SEAP-$IRES_{PV}$-*alcR*-apA; (Hartenbach and Fussenegger, 2005). pAutoRex8 contains a P_{AIR}-driven dicistronic expression unit encoding SEAP in the first cistron and the acetaldehyde-dependent transactivator *alcR* in the second cistron. Leaky P_{AIR}-driven transcripts provide sufficient AlcR to kick start maximum SEAP expression in the presence of inducing ethanol concentrations. In the absence of exogenous ethanol, the autoregulated circuit remains silent. The autoregulated AIR-controlled system reaches SEAP induction factors of up to 36-fold when transfected into *P. patens* (Figure 4f).

$VEGF_{121}$-based biopharmaceutical manufacturing using microencapsulated moss protoplasts

The use of *P. patens* for the biopharmaceutical manufacture of protein therapeutics has been established, but remains challenging. The moss needs to be constantly blended in order to enable mixing in custom-designed, stirred-tank bioreactors (Decker and Reski, 2007), and the plant cell wall potentially compromises the efficient secretion of larger product proteins. As plant protoplasts lack any cell wall and can be grown in single-cell suspension cultures, they would be the ideal plant cell system for biopharmaceutical manufacturing. However, protoplasts are too fragile and shear force-sensitive for use in state-of-the-art bioprocesses.

We have pioneered a process to microencapsulate *P. patens* protoplasts in coherent alginate beads. Alginate bead polymerization is compatible with W5 culture medium, which was also used for the bioprocess. $tWT11.51_{VEGF}$-derived protoplasts (4×10^7) (Baur *et al.*, 2005b) were microencapsulated in 500-µm capsules (165 protoplasts per capsule) using state-of-the-art encapsulation technology, and cultivated for 9 days in a 2-L Wave Bioreactor operated at a

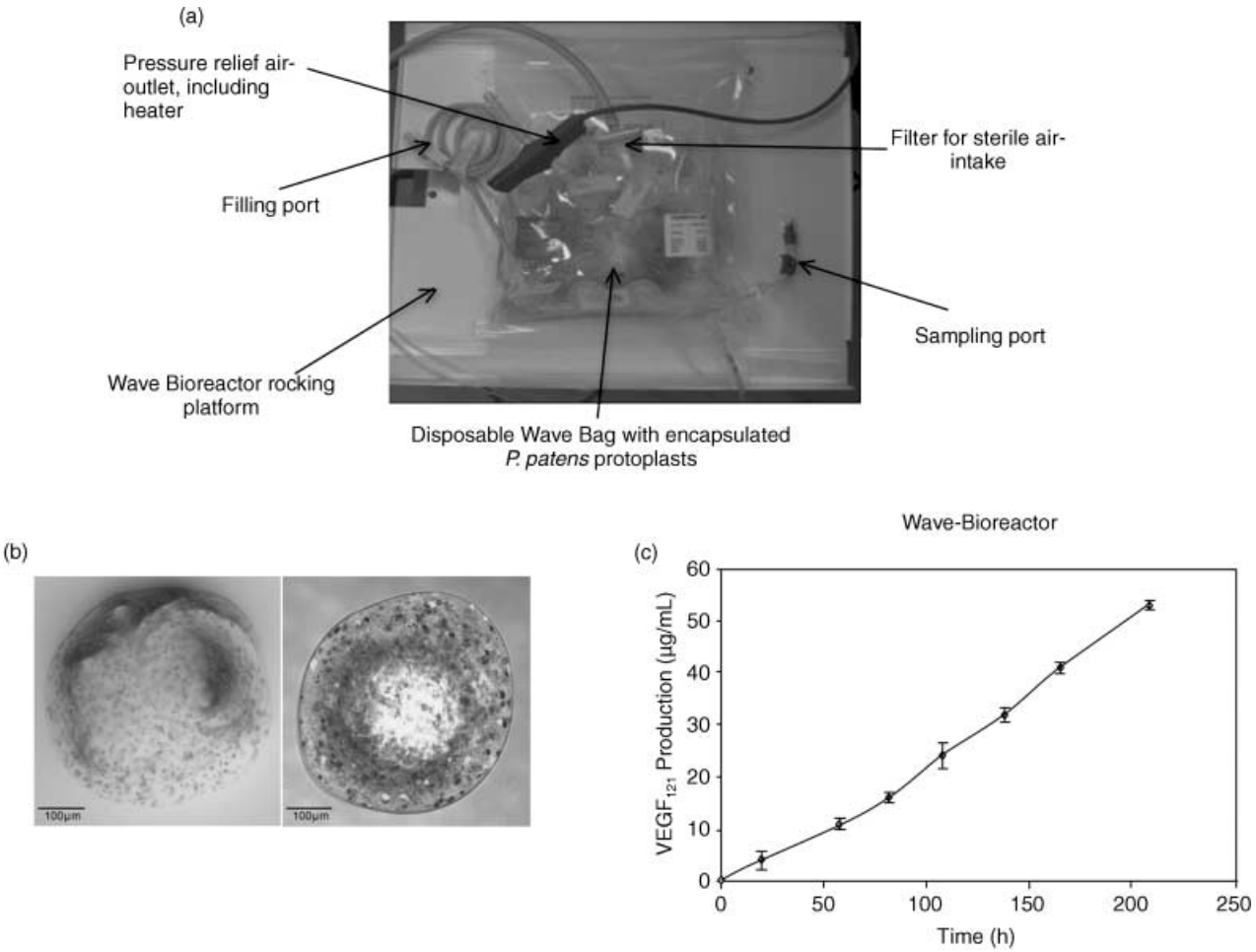

Figure 5 Prototype biopharmaceutical manufacturing of vascular endothelial growth factor 121 ($VEGF_{121}$) using *Physcomitrella patens* protoplasts microencapsulated in alginate beads and cultivated in a Wave Bioreactor. (a) Bioreactor set-up. (b) Light micrographs of moss protoplasts encapsulated in alginate beads. (c) $VEGF_{121}$ production profiles of microencapsulated transgenic moss protoplasts. Error bars indicate the standard deviation of three measurements of the samples.

culture volume of 1 L (Figure 5). $VEGF_{121}$ production reached 53 mg/L in a 9-day process, which compares well with forefront bioprocesses using moss protonema. A fluorescein/trypan blue-based live/dead staining revealed that microencapsulated protoplasts cultivated for 9 days in a Wave Bioreactor were still 74.8% ± 7.2% viable, which represents only a 5% viability decrease compared with a freshly prepared protonema-derived protoplast population.

Discussion

The complete functionality of the central mammalian expression portfolio, which includes various promoters, mRNA processing signals, transcription factors, translation elements and secretion peptides (Table 2), in the moss *P. patens* suggests that mammalian expression vectors and product proteins are generically compatible with this evolutionary old and simple plant. Interestingly, not only the functionality, but also the relative performance profiles, of different genetic elements in the moss matched those of mammalian cells. Examples of this include: (i) P_{hCMV} being a stronger promoter than P_{SV40}, with comparable strength to the smallest synthetic promoter P_{GTX} (Hartenbach and Fussenegger, 2006); (ii) $IRES_{PV}$ and $IRES_{EMCV}$ being equally efficient in the triggering of translation initiation and outperforming $IRES_{Rbm3}$ (Fux *et al.*, 2004); (iii) terminal IRES-driven translation units showing lower expression levels from multicistronic mRNAs compared with cap-dependent translation initiation; (iv) the AIR-controlled system responsiveness to gaseous acetaldehyde or ethanol being the most sensitive transgene control modality (Weber *et al.*, 2004); and (v) a one-vector-based, autoregulated expression configuration providing superior regulation performance.

Table 2 Mammalian genetic elements functional in *Physcomitrella patens*

Genetic element	Name	Description	Reference or source
Promoter	P_{hCMV}	Human cytomegalovirus immediate early promoter	Invitrogen
	P_{SV40}	Simian virus 40 promoter	Clontech
	P_{GTX}	Synthetic promoter derived from the GTX homeodomain protein	Hartenbach and Fussenegger (2006)
	$P_{hCMVmin}$	Minimal version of P_{hCMV}	Clontech
Operator	O_{alcA}	Operator of the *Aspergillus nidulans* alcohol dehydrogenase promoter	Weber *et al*. (2004)
	ETR	Operator of the *Escherichia coli* 2′-phosphotransferase 1 (*mph(A)*) promoter	Weber *et al*. (2002)
	O_{scbR}	Operator of the *Streptomyces coelicolor* butyrolactone-specific quorum-sensing receptor (ScbR)	Weber *et al*. (2003)
Transactivator	*AlcR*	Transcription factor co-ordinating ethanol metabolism in *Aspergillus nidulans*	Weber *et al*. (2004)
Repressor	E	Repressor of the *E. coli* macrolide-resistance gene (*mph(A)*)	Weber *et al*. (2002)
	ScbR	Butyrolactone-sensitive quorum-sensing receptor of *Streptomyces coelicolor*	Weber *et al*. (2003)
Transactivation domain	VP16	Herpes simplex virus-derived transactivation domain	Gossen and Bujard (1992)
Transrepression domain	KRAB	Human Kruppel-associated box-protein	Moosmann *et al*. (1997)
Internal ribosome entry site	$IRES_{PV}$	Poliovirus-derived IRES	Dirks *et al*. (1993)
	$IRES_{EMCV}$	Encephalomyocarditis virus-derived IRES	Kaufman *et al*. (1991)
	$IRES_{Rbm3}$	IRES derived from the human RNA-binding motif protein 3 (Rbm3)	Chappell *et al*. (2001)
Polyadenylation site	pA	Simian virus 40-derived polyadenylation site	Clontech
	apA	Artificial polyadenylation site	Fux *et al*. (2004)
Product proteins	SEAP	Human placental alkaline phosphatase	Dirks *et al*. (1993)
	SAMY	*Bacillus stearothermophilus*-derived secreted α-amylase	Schlatter *et al*. (2002)
	AMY	*Bacillus stearothermophilus*-derived α-amylase	Schlatter *et al*. (2002)
	$VEGF_{121}$	Human vascular endothelial growth factor 121	Weber *et al*. (2003)
	EPO	Human erythropoietin	Donation by P. Aebischer
Secretion signal	$S_{Ig\kappa}$	Murine Igκ secretion signal	Schlatter *et al*. (2002)

This cross-kingdom conservation of mammalian and moss protein production machineries is phylogenetically profound, and has several implications for basic and applied research. Comparative genomics, as well as functional studies, have recently established major differences in metabolic pathways and gene function between flowering plants and *P. patens*, and have suggested that a substantial moss gene pool is more closely related to mammals than to flowering plants (Frank *et al*., 2007; Rensing *et al*., 2008). In combination with the functional data presented here, these findings may expand our classical view on the molecular division between plants and animals (e.g. in Yamamoto *et al*., 2007).

This differentially expressed gene pool may reveal unique cross-kingdom functionalities useful for future advances in agriculture and human health. With the discovery that two fundamentally different living systems, such as the moss and mammalian cells, can utilize each other's gene expression and protein production machineries may expand the way in which we perceive ecosystems containing different coexisting species, and could, at least theoretically, lead to the exchange of a compatible gene pool.

Synthetic ecosystems have recently established the principle of cross-kingdom communication between *Sa. cerevisiae* or *E. coli* and mammalian cells, which replicated coexistence patterns as complex as oscillating predator–prey population dynamics (Weber *et al*., 2007), thus expanding our view on quorum sensing between bacteria (Keller and Surette, 2006). We have shown here that *P. patens* harbouring mammalian gene circuits is responsive to quorum-sensing communication initiated by co-cultivated *St. coelicolor*, as well as to 'over-the-air' signalling triggered by *Sa. cerevisiae* cultivated adjacently. Rational interventions into the quorum-sensing networks may foster unprecedented advances in agriculture, replicating the progress achieved in attenuating host–pathogen interaction in human therapy (Benghezal *et al*., 2006). Moreover, our recent findings have established *P. patens* as a promising host system for synthetic biology, a novel approach in the life sciences that relies on iterative cycles between analysis and synthesis (Benner and Sismour, 2005), utilizing devices of signalling networks in a cross-kingdom manner (e.g. Khandelwal *et al*., 2007).

Several biopharmaceutical production platforms, including *E. coli* (Georgiou and Segatori, 2005), (glyco-engineered) *Sa. cerevisiae* (Hamilton *et al*., 2006), mammalian cells (Wurm, 2004) and transgenic animals (Larrick and Thomas, 2001), are competing for the industrial production of protein therapeutics

(Fussenegger and Hauser, 2007). Mammalian cells have become the dominant system for the production of recombinant protein pharmaceuticals, partly as a result of the availability of a highly advanced portfolio of expression vectors and engineering strategies (Umana *et al*., 1999; Wurm, 2004; Hartenbach and Fussenegger, 2005). With the global mammalian cell-based biopharmaceutical manufacturing capacity plateauing into a bottleneck, this compromises the availability of drugs to patients. Alternative easy-to-implement bioprocessing concepts are urgently needed (Fussenegger and Hauser, 2007). The moss *P. patens* has recently come into the limelight as an easy-to-handle/engineer organism which could be cultivated in scale-up-compatible bioreactors, and was able to produce ADCC-optimized therapeutic IgGs in a Good Manufacturing Practice (GMP)-approved bioprocess (Decker and Reski, 2007).

Utilizing a compatible mammalian expression and engineering toolbox, the moss, as an emerging biopharmaceutical manufacturing platform, could be propelled to an *ex-aequo* competitor of mammalian cell-based production systems. Major bioprocess advantages of *P. patens* include the use of an inexpensive salt solution as production medium, which reduces downstream processing challenges and costs, and the availability of an efficient homologous recombination toolkit that provides stable and predictable production cultures (Kamisugi *et al*., 2006). Best-in-class production systems include transient protoplast cultures for the rapid evaluation of bioprocess parameters and a scalable stirred-tank photo-bioreactor that uses stable moss protonema.

Moss protonema tissue needs to be constantly blended to avoid complications in bioreactor operation, which may hinder large-scale biopharmaceutical manufacturing, and the established cell wall may compromise the secretion of larger proteins. Protoplasts could be an alternative (Baur *et al*., 2005a), but they are not sufficiently robust to survive long-term bioreactor operation. The microencapsulation protocol established during this study is compatible with the W5 medium, and enables the cultivation of encapsulated protoplasts in a proliferation-inhibited and cell wall-free state. Being protected by a physiologically inert alginate shell, the protoplasts are able to devote all of their metabolic energy to the production of heterologous protein rather than biomass, and, being devoid of any secretion-limiting cell wall, microencapsulated protoplasts cultivated in a standard Wave Bioreactor, equipped with a photosynthesis kit, were able to produce the human growth factor $VEGF_{121}$ at titres comparable with those of the highly optimized best-in-class protonema cultures. The use of Wave Bioreactor systems, which can be easily upscaled to 500-L cultures, has recently gathered momentum for use in the pilot production of proteins for clinical trials (Haldankar *et al*., 2006).

The combination of a novel protoplast-based bioprocess with powerful mammalian expression technology will further enhance the use of *P. patens* as a complementary and competitive platform for the biopharmaceutical manufacturing of protein therapeutics, and establishes this evolutionary old and simple plant as a valuable host for synthetic biology.

Experimental procedures

Expression vector design

Table 3 lists all the plasmids used in this study and provides detailed information about their construction.

Cultivation and transformation of *P. patens*

Physcomitrella patens (Hedw.) B.S.G. was grown axenically in Erlenmeyer flasks or in modified stirred-tank bioreactors (5 L; Applikon, Schiedam, the Netherlands) using a 10% modified Knop salt solution (100 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 25 mg/L KCl, 25 mg/L KH_2PO_4, 25 mg/L $MgSO_4{\cdot}7H_2O$ and 1.25 mg/L $FeSO_4{\cdot}7H_2O$; pH 5.8) (Reski and Abel, 1985). Protoplasts of *P. patens* were generated by incubation for 2 h in 0.5 M mannitol containing 4% Driselase (Sigma, Buchs, Switzerland), followed by two centrifugation steps (10 min, 50 ***g***) and resuspension of the protoplast-containing pellet at a desired cell density in 3M medium [87.5 g/L mannitol, 3.1 g/L $MgCl_2{\cdot}6H_2O$, 1 g/L 2-(*N*-morpholino)ethanesulphonic acid hydrate (MES); Sigma; pH 5.6 and 580 mOsm]. Protoplasts (300 000) were chemically transfected with 50 μg/mL DNA (80 μg/mL for transgene control systems), as described previously (Jost *et al*., 2005), and cultivated in Knop's regeneration medium [1 g/L $Ca(NO_3)_2{\cdot}4H_2O$, 250 mg/L KCl, 250 mg/L KH_2PO_4, 250 mg/L $MgSO_4{\cdot}7H_2O$, 12.5 mg/L $FeSO_4{\cdot}7H_2O$, 5% glucose, 3% mannitol, pH 5.7, 540 mOsm].

Protein production

Protein production was measured 5 days after transformation using standardized assays: (i) human placental SEAP: a *p*-nitrophenylphosphate-based light-absorbance time course (Berger *et al*., 1988; Schlatter *et al*., 2002); (ii) SAMY and AMY: a blue starch Phadebas® assay (cat. no. 10-5380-32; Pharmacia Upjohn, Peapack, NJ, USA) (Schlatter *et al*., 2002); quantification of intracellular reporter proteins required lysis of the plant cells by four freeze–thaw cycles and elimination of cell debris by centrifugation (2 min at 12 000 ***g***); (iii) human $VEGF_{121}$: using a $VEGF_{121}$-specific enzyme-linked immunosorbent assay (ELISA) (cat. no. 900-K10, lot. no. 1006010; Peprotech, Rocky Hill, NJ, USA); (iv) human EPO: using an EPO-specific ELISA (Quantikine® IVD®, cat. no. DEP00, lot. no. 243030; R & D Systems, Minneapolis, MN, USA).

Transgene regulation

All regulating agents were administered at the indicated concentrations immediately after transformation. The butyrolactone SCB1 was

Table 3 Expression vectors designed and used in this study

Plasmid	Description	Reference or source
pEF4/Myc-His	Mammalian expression vector containing $P_{hEF1\alpha}$	Invitrogen
pSEAP2-Basic	Mammalian SEAP expression vector	Clontech
pCF292 (pTRIDENT37)	P_{SV40}-driven tricistronic expression vector: (I-*Ceu*I)-P_{SV40}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{Rbm3}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	Fux *et al*. (2004)
pCF297 (pTRIDENT36)	P_{SV40}-driven tricistronic expression vector: (I-*Ceu*I)-P_{SV40}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	Fux *et al*. (2004)
pMF208	Streptogramin-repressible SEAP expression vector: P_{PIR3}-SEAP-pA	Fussenegger *et al*. (2000)
pMF242	P_{hCMV}-driven EPO expression vector: P_{hCMV}-EPO-pA	Fussenegger *et al*. (2000)
pMG31	P_{SV40}-driven SEAP expression vector. The PIP-specific operator was excised from pMF208 (*Hind*III/*Eco*RI), and the Klenow-polished vector backbone was re-ligated: P_{SV40}-SEAP-pA	This work
pMG32	$P_{hEF1\alpha}$-driven SEAP expression vector. $P_{hEF1\alpha}$ was excised from pEF4/*Myc*-His (*Nru*I/*Eco*RI) and cloned into pSEAP2-Basic (Clontech) (*Nru*I/*Eco*RI): $P_{hEF1\alpha}$-SEAP-pA	This work
pMG40 (pTRIDENT45)	P_{hCMV}-driven tricistronic expression vector. P_{hCMV} was PCR-amplified from pSS173 using oligonucleotides OMG23/OMG24 and cloned (I-*Ceu*I/I-*Sce*I) into pCF297: (I-*Ceu*I)-P_{hCMV}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	This work
pMG41 (pTRIDENT46)	P_{hCMV}-driven tricistronic expression vector. P_{hCMV} was PCR-amplified from pSS173 using oligonucleotides OMG23/OMG24 and cloned (I-*Ceu*I/I-*Sce*I) into pCF292: (I-*Ceu*I)-P_{hCMV}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{Rbm3}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	This work
pMG42 (pTRIDENT47)	P_{hCMV}-driven tricistronic expression vector. P_{hCMV} was PCR-amplified from pSS173 using oligonucleotides OMG23 (5′-gatcgacgt*ctaactataacggtcctaaggtagcga*TAGTAATCAATTACGGGGTCATTAGTTCATAGC-3′) and OMG24 (5′-gatcgaattc*attaccctgttatccct*aCTGACGGTTCACTAAACCAGCTCTGC-3′) and cloned (I-*Ceu*I/I-*Sce*I) into pMG43: (I-*Ceu*I)-P_{hCMV}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{PV}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I) (capital letters, annealing sequence; lower case italic, I-*Ceu*I and I-*Sce*I for OMG23 and OMG24, respectively)	This work
pMG43	P_{SV40}-driven expression vector. $IRES_{PV}$ was excised from pSAM241 (*Asc*I/*Spe*I) and cloned into pCF292: (I-*Ceu*I)-P_{SV40}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{PV}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	This work
pMG60	P_{GTX}-driven AMY expression vector. AMY was excised from pSS188 (*Hind*III/*Xba*I) and cloned into pSH17 (*Hind*III/*Xba*I): P_{GTX}-AMY-pA	This work
pMG61	P_{GTX}-driven EPO expression vector. EPO was excised from pMF242 (*Eco*RI/*Xba*I) and cloned into pSH17 (*Eco*RI/*Xba*I): P_{GTX}-EPO-pA	This work
pMG65	$P_{CaMV35S}$-driven SEAP expression vector. SEAP was PCR-amplified from pSH17 using OMG53 (5′-ccg*ctcgag*ggcccaccATGCTGCTGCTGCTGCTGCTG-3′) and OMG54 (5′-ttgc*tctaga*gctcagtggtgatggtgatgatgTGTCTGCTCGAAGCGGCCGGCCGCCCCGACCCTAGAGTAAC-3′) and cloned (*Xho*I/*Xba*I) into pRT101neo: $P_{CaMV35S}$-SEAP-his-pA (capital letters, annealing sequence; lower case italic, restriction enzymes)	This work
pMG66	$P_{CaMV35S}$-driven SAMY expression vector. SAMY was PCR-amplified from pSH102 using OMG55 (5′-ccg*ctcgag*ggcccaccATGGAGA-CAGACACACTCCTG-3′) and OMG56 (5′-ttgc*tctaga*gctcagtggtgatggtgatgatgAGGCCATGCCACCAACCGTGGTTCG-3′) and cloned (*Xho*I/*Xba*I) into pRT101neo: $P_{CaMV35S}$-SAMY-his-pA	This work
pMG67	$P_{CaMV35S}$-driven AMY expression vector. AMY was PCR-amplified from pMG60 using OMG57 (5′-ccg*ctcgag*ggcccaccATGGCCGCACCGTTTAACGGC-3′) and OMG58 (5′-ttgc*tctaga*gctcagtggtgatggtgatgatgAGGCCATGCCACCAACCGTGGTTCGGTCC-3′) and cloned (*Xho*I/*Xba*I) into pRT101neo: $P_{CaMV35S}$-AMY-his-pA	This work
pMG68	$P_{CaMV35S}$-driven VEGF expression vector. VEGF was PCR-amplified from pSH100 using OMG59 (5′-ccg*ctcgag*ggcccaccATGAACTTTCTGCTGTCTTGG-3′) and OMG60 (5′-ttgc*tctaga*gctcagtggtgatggtgatgatgCCGCCTCGGCTTGTCACATTTTTCTTGTCTTGC-3′) and cloned (*Xho*I/*Xba*I) into pRT101neo: $P_{CaMV35S}$-VEGF-his-pA	This work
pMG69	$P_{CaMV35S}$-driven EPO expression vector. EPO was PCR-amplified from pMG61 using OMG61 (5′-ccg*ctcgag*ggcccaccATGGGGGTGCCCGAACGTCCCACCC-3′) and OMG62 (5′-ttgc*tctaga*gctcagtggtgatggtgatgatgCCTGTCCCCTCTCCTGCAGACC-3′) and cloned (*Xho*I/*Xba*I) into pRT101neo: $P_{CaMV35S}$-EPO-his-pA	This work
pRT101neo	$P_{CaMV35S}$-driven expression vector for plant cells, carrying the nptII cassette for neomycin resistance: $P_{CaMV35S}$-nptII-pA (pA, 35s-Terminator)	Huether *et al*. (2005)

Table 3 *Continued*

Plasmid	Description	Reference or source
pSAM241 (pTFT1)	$P_{hCMV*-1}$-driven tricistronic expression vector. $P_{hCMV*-1}$-ECFP-$IRES_{PV}$-RFP-$IRES_{PV}$-EYFP-pA	Moser *et al*. (2000)
pSH3 (pTRIDENT42)	P_{AIR}-driven tricistronic expression vector: (I-*Ceu*I)-P_{AIR}-(I-*Sce*I)-SAMY-$IRES_{PV}$-$VEGF_{121}$-$IRES_{EMCV}$-SEAP-(I-*Ppo*I)-apA-(PI-*Psp*I)	Hartenbach and Fussenegger (2005)
pSH12	$VEGF_{121}$-encoding control vector: $IRES_{PV}$-$VEGF_{121}$-pA	Hartenbach and Fussenegger (2006)
pSH17	P_{GTX}-driven expression vector: P_{GTX}-SEAP-pA	Hartenbach and Fussenegger (2006)
pSH28 (pAutoRex8)	Autoregulated acetaldehyde-inducible tricistronic expression vector. (I-*Ceu*I)-P_{AIR}-(I-*Sce*I)-SEAP-$IRES_{PV}$-*alcR*-(I-*Ppo*I)-apA-(PI-*Psp*I)	Hartenbach and Fussenegger (2005)
pSH49	SEAP-encoding control vector. SEAP was excised from pSS173 (*Eco*RI/*Not*I) and cloned into pSH12 (*Eco*RI/*Not*I): $IRES_{PV}$-SEAP-pA	Unpublished (Hartenbach)
pSH100	P_{GTX}-driven $VEGF_{121}$ expression vector: P_{GTX}-$VEGF_{121}$-pA	Hartenbach and Fussenegger (2006)
pSH102	P_{GTX}-driven SAMY expression vector: P_{GTX}-SAMY-pA	Hartenbach and Fussenegger (2006)
pSS173	P_{hCMV}-driven SEAP expression vector: P_{hCMV}-SEAP-pA	Schlatter *et al*. (2002)
pSS188	P_{hCMV}-driven AMY expression vector: P_{hCMV}-AMY-pA	Schlatter *et al*. (2002)
pWW35	P_{SV40}-driven expression vector encoding the macrolide-dependent transactivator ET1: P_{SV40}-ET1-pA; ET1, E-VP16	Weber *et al*. (2002)
pWW37	Erythromycin-repressible, P_{ETR2}-driven SEAP expression vector: P_{ETR2}-SEAP-pA	Weber *et al*. (2002)
pWW43	P_{SV40}-driven expression vector encoding the macrolide-dependent transrepressor ET4: P_{SV40}-ET4-pA; ET4, E-KRAB	Weber *et al*. (2002)
pWW56	Erythromycin-inducible, P_{ETR}ON8-driven SEAP expression vector: P_{ETR}ON8-SEAP-pA	Weber *et al*. (2002)
pWW122	P_{SV40}-driven expression vector encoding the butyrolactone-dependent transactivator SCA: P_{SV40}-SCA-pA; SCA, *scbR*-VP16	Weber *et al*. (2003)
pWW124	Butyrolactone-repressible, P_{SPA}-driven SEAP expression vector: P_{SPA}-SEAP-pA	Weber *et al*. (2003)
pWW162	Butyrolactone-inducible, P_{SCA}ON8-driven SEAP expression vector: P_{SCA}ON8-SEAP-pA	Weber *et al*. (2005)
pWW504	P_{SV40}-driven expression vector encoding the butyrolactone-dependent transrepressor SCS: P_{SV40}-SCS-pA, SCS, *scbR*-KRAB	Weber *et al*. (2005)
pWW192	Acetaldehyde-inducible, P_{AIR}-driven SEAP expression vector: P_{AIR}-SEAP-pA	Weber *et al*. (2004)
pWW195	P_{SV40}-driven *alcR* expression vector: P_{SV40}-*alcR*-pA	Weber *et al*. (2004)

alcR, *Aspergillus nidulans* acetaldehyde-dependent transactivator (1521 bp); AMY, *Bacillus stearothermophilus*-derived α-amylase (1551 bp); apA, artificial polyadenylation site (91 bp); E, *Escherichia coli*-derived macrolide-dependent repressor (585 bp); ECFP, enhanced cyan fluorescent protein (720 bp); EPO, human erythropoietin (579 bp); ET1, macrolide-dependent transactivator (E-VP16) (972 bp); ET4, macrolide-dependent transrepressor (E-KRAB) (1044 bp); EYFP, enhanced yellow fluorescent protein (720 bp); $IRES_{EMCV}$, encephalomyocarditis virus internal ribosome entry site (502 bp); $IRES_{PV}$, poliovirus internal ribosome entry site (635 bp); $IRES_{Rbm3}$, internal ribosome entry site derived from the human RNA-binding motif protein 3 (Rbm3) (732 bp); KRAB, human Kruppel-associated box-protein (450 bp); nptII, neomycin phosphotransferase II (921 bp); pA, polyadenylation site (145 bp); P_{AIR}, acetaldehyde-responsive promoter (456 bp); $P_{CaMV35S}$, cauliflower mosaic virus 35S promoter (384 bp); P_{ETR2}, erythromycin-repressible promoter (200 bp); P_{ETR}ON8, erythromycin-inducible promoter (530 bp); P_{GTX}, synthetic promoter derived from the GTX homeodomain protein (182 bp); P_{hCMV}, human cytomegalovirus immediate early promoter (663 bp); $P_{hCMV*-1}$, tetracycline-responsive promoter (156 bp); $P_{hEF1\alpha}$, human elongation factor 1α promoter (1185 bp); PIP, pristinamycin-induced protein (867 bp); P_{PIR3}, streptogramin-repressible promoter (534 bp); P_{SCA}ON8, butyrolactone-inducible promoter (576 bp); P_{SPA}, butyrolactone-repressible promoter (194 bp); P_{SV40}, simian virus 40 promoter (308 bp); RFP, red fluorescent protein; SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase (1611 bp); SCA, butyrolactone-dependent transactivator (ScbR-VP16) (1035 bp); *scbR*, *Streptomyces coelicolor* butyrolactone-dependent repressor (648 bp); SEAP, human placental secreted alkaline phosphatase (1560 bp); $VEGF_{121}$, human vascular endothelial growth factor 121 (444 bp); VP16, herpes simplex virus-derived transactivation domain (387 bp).

synthesized and purified as described previously (Weber *et al*., 2003). Erythromycin (cat. no. 45673, lot. no. 1195447; Fluka, Buchs, Switzerland) was prepared as a stock solution of 1 mg/mL in ethanol. The AIR system was induced by the addition of the indicated volumes of 100% ethanol.

Microencapsulation of *P. patens* protoplasts

Protoplasts (4×10^7) generated from transgenic $VEGF_{121}$-producing moss $tWT11.51_{VEGF}$ (Baur *et al*., 2005b) were resuspended in 8 mL of 3M medium and stirred gently with 40 mL of 1.5% sodium alginate

solution (cat. no. IE1010, lot. no. 060125B1; Inotech Biotechnologies Ltd., Basle, Switzerland). Protoplasts were encapsulated in 500-μm alginate capsules (165 protoplasts per capsule) using an Inotech Encapsulator Research IE-50R (Inotech Biotechnologies Ltd., Basle, Switzerland) set at the following parameters: 0.5 mm nozzle; 853 unit flow rate using a 50-mL syringe; 1250 s^{-1} nozzle vibration frequency; 1.4 kV for bead dispersion. W5 medium (18.4 g/L $CaCl_2$, 8 g/L $NaCl_2$, 0.99 g/L glucose, 0.75 g/L KCl; pH 5.8, 600 mOsm) was used as a precipitation solution. It contains sufficient $CaCl_2$ for the precipitation of alginate beads and enables the direct cultivation of microencapsulated protoplast populations without the need for medium exchange. Microencapsulated $tWT11.51_{VEGF}$-derived (a high $VEGF_{121}$ producer line harbouring the product gene under the control of the moss actin 5 5′ region) protoplasts (240 000 capsules, 1 L W5 medium) were cultivated in a BioWave 20SPS-F bioreactor (Wave Biotech, Tagelswangen, Switzerland), equipped with 2-L Wave Bags and set at the following parameters: aeration, 100 mL/min sterilized air; rocking rate, 19 min^{-1}; rocking angle, 10°. The Wave Bioreactor was placed in an ISF-1-W incubator equipped with a photosynthesis kit set to 25 °C and a day/night cycle of 16 h/8 h (Kuehner, Birsfelden, Switzerland).

Edman sequencing

$VEGF_{121}$ was precipitated from $tWT11.51_{VEGF}$ protoplast culture supernatants for 10 min at 4 °C with 100% w/v trichloroacetic acid (TCA, Sigma) (supernatant : TCA, 9 : 1). The samples were centrifuged for 5 min at 12 000 ***g***, and the $VEGF_{121}$-containing pellet was washed twice in ice-cold acetone, dried and resuspended in 2 × sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer [50% glycerol, 250 mM tris(hydroxymethyl)aminomethane (Tris), 10% SDS, 500 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8]. The samples were then denatured for 5 min at 50 °C, and the proteins were size-fractionated on a 12% SDS-polyacrylamide gel and blotted on to a polyvinylidene fluoride membrane (cat. no. IPVH20200; Millipore Corporation, Bedford, MA, USA). $VEGF_{121}$ (35 kDa) was N-terminally sequenced on an Applied Biosystems (Foster City, CA, USA) model 492cLC Procise protein/peptide sequencer with an on-line Perkin-Elmer (Waltham, MA, USA) Applied Biosystems Model 140C PTH Amino Acid (phenylthiohydantonin amino acid) Analyser. The PTH amino acids were automatically transferred to a reverse-phase C-18 column (0.8 mm inside diameter) for detection at 269 nm, and identified by comparison with individual runs with a standard mixture of PTH amino acids.

Cultivation of *Sa. cerevisiae* and *St. coelicolor*

Saccharomyces cerevisiae [wild-type strain W303, BMA 64, European *Sa. cerevisiae* archive for functional analysis (EUROSCARF), Frankfurt, Germany] was cultivated on yeast–peptone–dextrose agar (YPD; 1% yeast extract, 2% peptone, 2% dextrose, 1% agar), and *St. coelicolor* MT1110 (kindly provided by Marc Folcher) was cultivated on mannitol–soy agar (2% soy flour, 2% mannitol, 1.5% agar). For the co-cultivation of *St. coelicolor* and *P. patens*, *St. coelicolor* were pre-cultured in Luria–Bertani (LB) medium to an optical density at 600 nm (OD_{600}) of 340, and the indicated volumes were then transferred to *P. patens* maintained in regeneration medium.

Viability profiling of microencapsulated *P. patens* protoplasts

The viability of microencapsulated moss protoplasts was determined by scoring live protoplasts, stained with fluorescein diacetate (FDA), and dead protoplasts, stained with trypan blue, using (fluorescence) microscopy (Leica DM-RB fluorescence microscope; Leica, Heerbrugg, Switzerland). Twenty protoplast-containing alginate beads were incubated for 10 min in a staining solution including 200 μL W5 medium, 20 μL phosphate-buffered saline (PBS; Dulbecco's phosphate-buffered saline, cat. no. 21600-0069; Invitrogen, Basle, Switzerland) containing 0.01% FDA (Sigma) and 40 μL of a 0.4% trypan blue stock solution (lot. no. 1230532; Fluka).

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