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Production of biologically active recombinant human factor H in *Physcomitrella*

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Summary

The human complement regulatory serum protein factor H (FH) is a promising future biopharmaceutical. Defects in the gene encoding FH are associated with human diseases like severe kidney and retinal disorders in the form of atypical haemolytic uremic syndrome (aHUS), membranoproliferative glomerulonephritis II (MPGN II) or age-related macular degeneration (AMD). There is a current need to apply intact full-length FH for the therapy of patients with congenital or acquired defects of this protein. Application of purified or recombinant FH (rFH) to these patients is an important and promising approach for the treatment of these diseases. However, neither protein purified from plasma of healthy individuals nor recombinant protein is currently available on the market. Here, we report the first stable expression of the full-length human FH cDNA and the subsequent production of this glycoprotein in a plant system. The moss *Physcomitrella patens* perfectly suits the requirements for the production of complex biopharmaceuticals as this eukaryotic system not only offers an outstanding genetical accessibility, but moreover, proteins can be produced safely in scalable photobioreactors without the need for animal-derived medium compounds. Transgenic moss lines were created, which express the human FH cDNA and target the recombinant protein to the culture supernatant via a moss-derived secretion signal. Correct processing of the signal peptide and integrity of the moss-produced rFH were verified via peptide mapping by mass spectrometry. Ultimately, we show that the rFH displays complement regulatory activity comparable to FH purified from plasma.

Introduction

The human complement system provides the first defence line of innate immunity, protecting the human body against invading organisms. factor H (FH) is the key regulator of the alternative pathway of complement activation, which acts in solution and on the surface of host cells, where it functions as a cofactor for factor I-mediated degradation of C3b (Kühn *et al.*, 1995). FH displays decay acceleration activity (Kühn and Zipfel, 1996) and interacts directly with surface-bound C3b and/or cell surface constituents (Pangburn *et al.*, 1991; Jokiranta *et al.*, 1996), to sum up—FH possesses several functions to protect cells of the human body from complement-mediated lysis.

FH mutations are associated with severe human renal and retinal diseases (Zipfel *et al.*, 2006) including atypical haemolytic uremic syndrome (aHUS) (Heinen *et al.*, 2007), membranoproliferative glomerulonephritis II (MPGN II) (Smith *et al.*, 2007; Pickering and Cook, 2008) or age-related macular degeneration (AMD) (Skerka *et al.*, 2007;

Pickering and Cook, 2008). With over 50 million people affected worldwide, AMD is the main cause of irreversible loss of central vision in the developed world. In contrast, aHUS and MPGN II are rare but extremely severe diseases (Noris and Remuzzi, 2005; Smith *et al.*, 2007) so that FH has recently been registered as an orphan drug by the European Commission Register of Designated Orphan Medical Drugs (http://ec.europa.eu/enterprise/pharmaceuticals/register/o425.htm). Presently, therapies for the above mentioned diseases are limited (Noris and Remuzzi, 2008). Substitution with FH is an option for patients with defects of the FH gene or with autoantibodies against FH. It is performed by plasma transfusion or exchange as neither protein purified from blood nor recombinantly produced factor H (rFH) is currently available. Thus, the establishment of a production system for rFH is considered highly desirable (Licht *et al.*, 2007; Smith *et al.*, 2007). Expression of rFH on a laboratory scale was shown in insect and in COS-7 cells (Sharma and Pangburn, 1994; Sanchez-Corral *et al.*, 2002), but production platforms devoid of the risk of contaminations with human pathogens in combination with cost-effective up-scaling capacities are still needed. At this point, plants offer a powerful alternative.

The moss *Physcomitrella patens* perfectly meets the requirements for a safe and flexible biopharmaceuticals production platform: for this plant model organism, excellent genetic resources and well-developed molecular tool boxes are available (Lang *et al.*, 2008; Rensing *et al.*, 2008), and the predominantly haploid life cycle combined with an outstanding high rate of homologous recombination in mitotic cells makes custom-designed, targeted modifications straightforward (Strepp *et al.*, 1998; Kamisugi *et al.*, 2006; Gitzinger *et al.*, 2009). Like other plants, *Physcomitrella* performs post-translational protein N-glycosylation preferentially of the complex type (Koprivova *et al.*, 2003; Vietor *et al.*, 2003), closely resembling those of human diantennary N-glycans. However, the two plant-specific sugar residues, a xylose unknown from human glycans and a fucose linked to the core glycan structure differently from its human counterpart, were shown to be immunogenic thus contradicting the use of plant-based systems for recombinant pharmaceutical glycoprotein production (van Ree *et al.*, 2000; Jin *et al.*, 2008; Karg and Kallio, 2009). By targeted gene replacements, the responsible moss glycosyltransferases have been destroyed, resulting in plants with an adapted N-glycosylation pattern (Koprivova *et al.*, 2004; Huether *et al.*, 2005). Moreover, moss can be grown axenically in scalable photobioreactors (Lucumi and Posten, 2006; Perner-Nochta *et al.*, 2007) and may secrete the protein of interest into a simple mineral medium thus facilitating downstream processing (Decker and Reski, 2007, 2008). Biopharmaceuticals like the human vascular endothelial growth factor, erythropoietin (EPO) (Baur *et al.*, 2005; Weise *et al.*, 2007) or IgG antibodies (Gorr and Jost, 2005; Schuster *et al.*, 2007) have been successfully produced in *P. patens*.

The production of rFH is a particular challenge with regard to size and complexity of the protein. FH is a 155 kDa single-chain glycoprotein exclusively composed of 20 repetitive protein domains, each of which consists of around 60 amino acids, termed short consensus repeat (SCR) (Ripoche *et al.*, 1988). Indeed, the molecular mass of FH is similar to that of IgG antibodies, which have been already generated in *P. patens*, but its tertiary structure entirely differs. Unlike IgGs, which are assembled from four independent chains subsequently connected by four disulphide bridges, FH is a single-chain molecule linked by 40 intrachain disulphide bridges. These are critical to maintain the higher order structure of the SCR domains, which are essential to retain the protein's biological activity (Discipio and Hugli, 1982; Ault *et al.*, 1997). Overall, the intricacy of FH requires an eukaryotic production machinery. In this proof-of-concept study, we present the successful stable production of intact, full-length, biologically active rFH in a plant-based system.

Results and discussion

Stable production of recombinant factor H

We generated moss lines that stably express rFH via co-transfection of *P. patens* protoplasts with the expression vector pFH, which encodes the full-length FH cDNA together with a plasmid encoding a selective marker (Figure 1a). Efficient secretion of the recombinant protein into the culture supernatant was allowed by replacing the human signal peptide of FH against an endogenous one, *Pp*AP1 (*P. patens* aspartic protease 1) signal sequence (Schaaf *et al.*, 2004, 2005). In addition, a C-terminal 6xHis-tag was added to simplify downstream processing.

After regeneration of the transformed cells for 2 weeks on medium without selection pressure, selection on antibiotics for at least 3 weeks led to 116 surviving plants. After screening of 94 plants by direct PCR (Schween *et al.*, 2002) (Figure S1), stable integration of the FH cDNA was confirmed for 43 plants. FH mRNA expression analysis performed for 12 of these plants revealed four lines with strong transgene expression (Figure 1b). These lines,

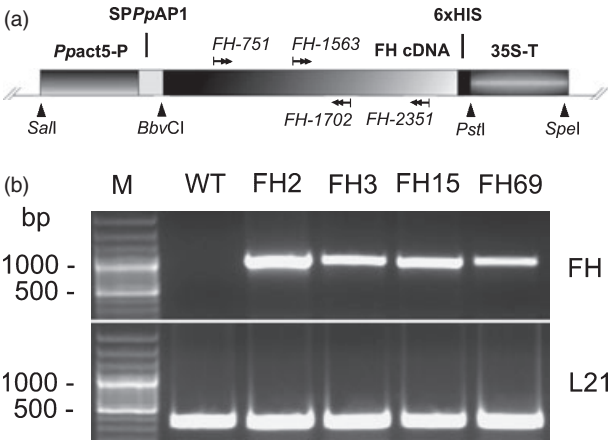

Figure 1 Human factor H is recombinantly expressed in stable transgenic moss lines. (a) Schematic representation of the expression vector pFH. The plasmid contains the cDNA coding for FH under the control of the *P. patens* actin5 gene promoter (Ppact5-P) and the 35S terminator (35S-T). Use of the endogenous signal peptide of aspartic protease 1 (SP*Pp*AP1) ensures subsequent secretion of the recombinant protein. To simplify downstream processing, a His-tag (6xHis) was added. Arrows indicate the primers used for direct PCR (FH-1563 and FH-2351) or RT-PCR (FH-751 and FH-1702). Restriction sites used for cloning (*Bbv*CI and *Pst*I) or linearization of the construct (*Sal*I and *Spe*I) prior to stable transformation are indicated. (b) Transgene expression analysis. After preselection by means of direct PCR (cf. Figure S1), four stable transformants (FH2, FH3, FH15, FH69) with highest transgene expression were identified using primers specific to FH cDNA (see Figure 1a); wild type (WT) was included as a negative control. In parallel, as a control for mRNA levels, RT-PCR was performed with primers corresponding to the constitutively expressed gene for the ribosomal protein L21.

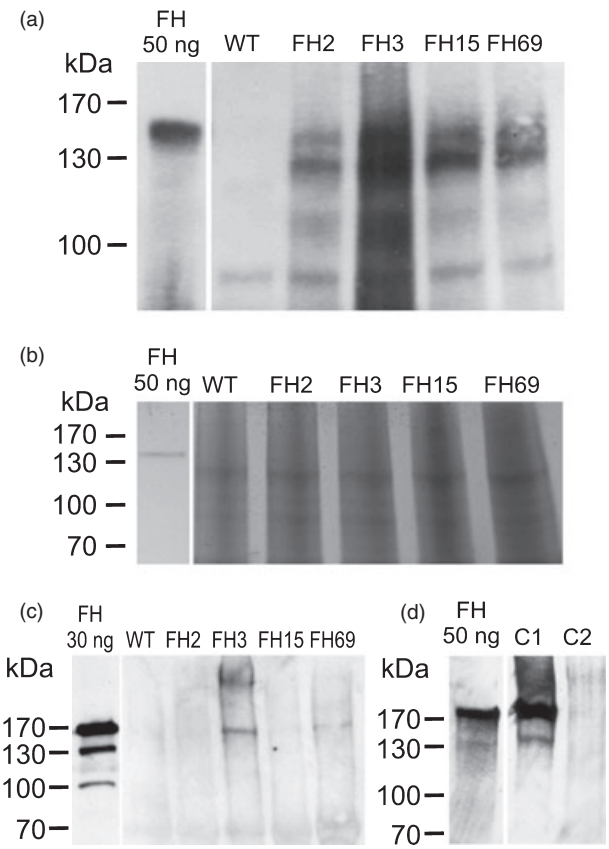

Figure 2 Transgenic line FH3 shows highest recombinant factor H accumulation. Immunodetection of rFH was performed via Western blot. (a) rFH extracted from cells; total protein extracts from 8 mg (fresh weight) moss tissue of transgenic plants FH2, FH3, FH15 and FH69 and wild type (WT) were loaded under nonreducing conditions (b) Coomassie blue-stained SDS-PAGE of the extracts indicated in (a) as a loading control for equal amounts of protein. (c) Recombinant factor H in moss culture supernatants; proteins were precipitated from the medium after 6 days of cultivation and loaded under reducing conditions. (d) Human factor H is greatly lost from culture medium within 13 days of cultivation; FH = human factor H; C1 = WT culture supernatant with 50 ng factor H, added after harvest; C2 = WT culture supernatant, supplemented with 100 ng factor H at the beginning of the culture period.

termed FH2, FH3, FH15 and FH69, were compared with regard to their intracellular and extracellular recombinant protein levels by immunodetection using FH-specific antibodies. In Western blot analyses, two specific bands for rFH were detected in the intracellular fraction (Figure 2a). Roughly estimated in comparison with the size marker they migrated at approximately 120 and 140 kDa. Because of the use of the reducing agent beta-mercaptoethanol, the rFH-specific band migrated slower in the extracellular samples (Figure 2c). This was demonstrated earlier for FH and FH-related proteins and is caused by conformational changes because of the reduction of the disulphide bonds within the FH molecule (Kühn *et al.*, 1995). The size differences between rFH and the FH standard can be explained by different terminal sugars in human or plant N-glycans, respectively. The rFH was produced in a moss strain, which was not manipulated in its glycosylation machinery. Fenaille *et al.* (2007) showed that out of nine potential N-glycosylation sites within the FH amino acid sequence eight sites are occupied with predominantly diantennary disialylated glycans of 2204 Da. Considering that the most abundant N-glycan in moss is the mature complex-type glycan GnGnXF with a MW of 1618 Da (Koprivova *et al.*, 2003), the theoretical shift obtained for the fully glycosylated rFH compared to human FH standard would be about 5 kDa, which is consistent with our results. The band detected in the extracellular samples as well as the highest FH-specific band in the cellular fraction migrated slightly below the (highest) band of the human FH standard (Figure 2a,c). Only partial or a lack of glycosylation of a portion of rFH may contribute to the reduction in apparent size, which could explain the band at around 120 kDa in the cellular samples. Deglycosylated FH has been shown before to display an altered mobility of around 120 kDa in

Western blot analysis (Jouvin *et al.*, 1984) although the calculated molecular weight of unglycosylated FH is 137 kDa. Partial degradation of FH has to be considered as a reason for a third band above 100 kDa in the cellular fraction (Figure 2a). The human FH standard sometimes also showed bands of reduced sizes, which may result from different glycosylation and partial degradation, respectively (Figures 2, 4 and S2). The two bands above 130 kDa of the human FH standard were subjected to mass spectrometry analysis , and both were shown to contain the complete protein as peptides over the whole length of the FH amino acid sequence were detected (Figure 6 and Data S1). As a loading control for comparable protein amounts in all lanes, Coomassie staining of an SDS-PAGE from the same extracts was performed (Figure 2b). Highest overall recombinant protein levels in the cellular fraction were detected for the line FH3 (Figure 2a). Also, in the culture medium, rFH was strongest in line FH3 while a weaker band was detected in FH69 (Figure 2c). For additional rFH-expressing lines, extracellular rFH was only detectable after heavy over-exposition of the films (data not shown). Therefore, plant FH3 was subsequently used for further investigations.

Secretion of a recombinant protein to the culture medium offers great advantages for downstream processing as the absence of most contaminating proteins and other metabolites in the supernatant allows easier protein purification. However, the stability of a secreted protein in the culture medium might be affected in the relatively simple plant tissue culture media, thus objecting the potential cost benefits (Doran, 2006). Therefore, to obtain further information about the stability of FH in the moss culture supernatant, we analysed the recovery of the protein standard applied to the medium. Even if in the set of proteins naturally secreted by the moss no protease activity was detected (reviewed in Decker and Reski (2004)), in the presence of moss tissue, the standard vanished from the medium within 13 days of cultivation (Figure 2d, lane C2 compared to day 0 in lane C1), indicating a loss of the product under standard cultivation conditions. Using a monoclonal capture antibody directed against an epitope in SCRs 15-18 (Manuelian *et al.*, 2003), the amounts of rFH produced by plant FH3 were quantified via ELISA. After 1 week of cultivation in a 5l photobioreactor, recombinant FH in the cellular fraction reached a concentration above 20 μg/g dry weight (25.8 μg/g DW). A similar concentration (23.2 μg/g DW) was measured for secreted rFH after 6 days cultivation in flasks at pH 7.4 supplemented with 0.001% BSA. These concentrations have to be further improved by setting up a FH-specific production process. However, they are similar to other recombinant proteins produced in *Physcomitrella* like human serum albumin (Baur *et al.*, 2005), while moss-produced hEPO was tenfold higher concentrated (Weise *et al.*, 2007). Anyhow, the production rate of rFH obtained in this work is in the range of biopharmaceuticals expressed via the nuclear genomes in other plant-based systems (Daniell *et al.*, 2009).

Transgenic moss shows wild-type phenotype

Alterations in plant morphology and development in the context of recombinant mammalian protein production were reported before (Oh *et al.*, 2003; Cheon *et al.*, 2004). Therefore, we investigated a potential impact of FH production on the moss phenotype. Small-scale suspension cultures of wild-type (WT) moss and the transgenic line FH3 were started in parallel at a density of 180 mg/L DW. Biomass gain and protonema differentiation were monitored over a time period of 14 days by regular dry weight measurements and microscopic analysis. The plants showed a comparable growth rate and development and, after 2 weeks, both WT and transgenic line FH3 had quintupled their biomass to about 1 g/L DW (Figure 3a). In summary, we did not observe any obvious phenotypic deviations between WT moss and the rFH-producing line under standard growth conditions.

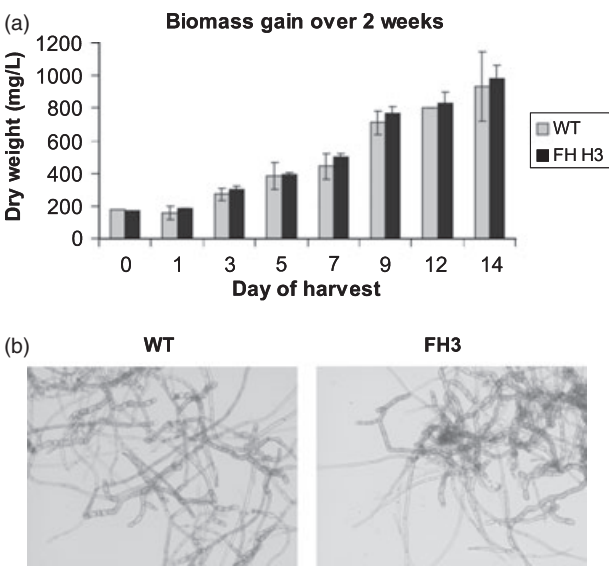

Figure 3 Morphology and development of transgenic moss are unimpaired by rFH expression. (a) Biomass determination via dry weight measurement over a period of 14 days. (b) Morphology of moss tissue from wild-type (WT) and transgenic line FH3 after 14 days of cultivation.

Full-length rFH is correctly assembled and displays complement regulatory activity

Recombinant FH was successfully concentrated via a C-terminal 6xHis-tag (Figure 4) and showed a similar mobility upon SDS-PAGE separation and Western blotting as the extracted protein analysed before (see Figure 2). Two distinct band sizes were detected with a polyclonal factor H antibody (Figure 4a) from transgenic FH3 extracts but not from WT extracts. These bands most probably display alternative glycosylation forms as discussed earlier. Again, a third smaller band (of approx. 90 kDa) occurred which may be a degradation product formed during the Ni-NTA purification. The occurrence of different glycosylation patterns even on the same protein is a common feature in mammalian as well as in plant cells (Cumming, 1991; Lerouge *et al.*, 1998). The precise function of the sugar moieties on FH is not clarified yet. Jouvin *et al.* (1984) showed that the ability of FH to interact with surface-bound C3b was unimpaired after desialylation and even complete deglycosylation. However, heterogeneity in glycosylation may derive from partial occupation of only some of the potential nine FH N-glycosylation sites or from only partial maturation of the individual glycans. Therefore, we incubated the elution fractions from the Ni-NTA enrichment with an anti-alpha1,3-fucose antibody (Figure 4b). The two larger rFH bands could clearly be detected in the elution fractions of line FH3 but not in WT fractions. As alpha1,3 fucosylation occurs late in the maturation of complex type but not on premature oligomannosidic glycans (Gorr and Altmann, 2006), we assume that the observed size differences in the larger bands resulted from a different grade of occupation of the glycosylation sites rather than from incomplete processing of the attached glycans. As it was suggested that the roles of FH N-glycans may be structural rather than functional (Fenaille *et al.*, 2007), we had chosen WT moss as a genetic background for rFH expression in our study but not a glyco-engineered strain lacking plant-specific xylosylation and fucosylation (Koprivova *et al.*, 2004). However, to avoid immune reactions against these residues (Bencurova *et al.*, 2004; Gorr and Altmann, 2006) which may occur when a recombinant protein with native plant glycosylation would be applied to patients, the rFH will have to be expressed in a glyco-engineered moss production strain.

Native FH displays several complement regulatory functions (Zipfel *et al.*, 1999). A major biological function is the regulation of the amplification loop of the alternative pathway of complement in fluid phase by acting as a cofactor for factor I-dependent C3b cleavage. Therefore, the cofactor activity of rFH was assayed and compared to the purified native human protein. Moss-produced rFH displayed cofactor activity for factor I-mediated cleavage of C3b similar to the native human protein as revealed by the appearance of the typical α68 and α43 kDa cleavage products (Figure 5). In conclusion, the functional activity of rFH produced in *P. patens* emphatically verifies the proper processing and folding of this complex protein.

As the N-terminal domains, SCRs 1-4, mediate this cofactor activity (Kühn *et al.*, 1995), efficient C3b cleavage by the moss-produced FH indicated an intact N-terminus of the recombinant protein. However, as rFH is expressed as a fusion of a moss-derived secretion signal with the human FH sequence, we aimed to confirm the precise processing of rFH by *Physcomitrella* signal peptidases. Fusions of a signal peptide to a recombinant protein with a different amino acid sequence adjacent to the fusion site not necessarily guarantee proper processing. An imprecise cleavage of the signal sequence may affect product activity and may raise concerns about product safety (Streatfield, 2007). By mass spectrometry analysis following tryptic digestion, we detected several FH peptides including the C-terminus as well as the N-terminal peptide (EDCNELPPRR) of the mature FH protein. This finding confirms the correct cleavage of the signal peptide in all

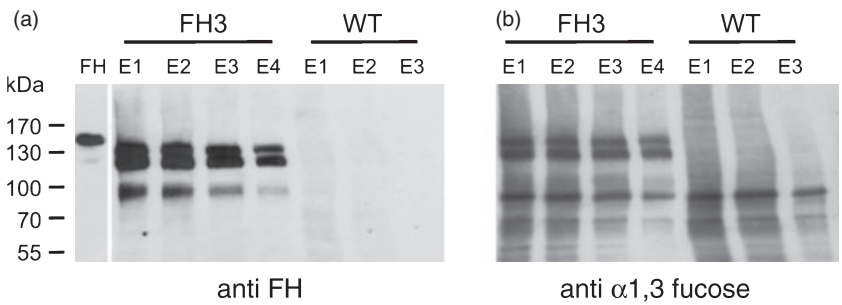

Figure 4 Specific detection of recombinant factor H concentrated via C-terminal His-tag. Western blot showing Ni-NTA eluates (E1-E4) with rFH from cell lysates of transgenic plant line FH3 and wild-type (WT) eluates (E1-E3) as a negative control. Signals corresponding to rFH could be detected using a polyclonal FH antibody (a) or a serum directed against alpha1,3-fucose (b); nonreducing conditions.

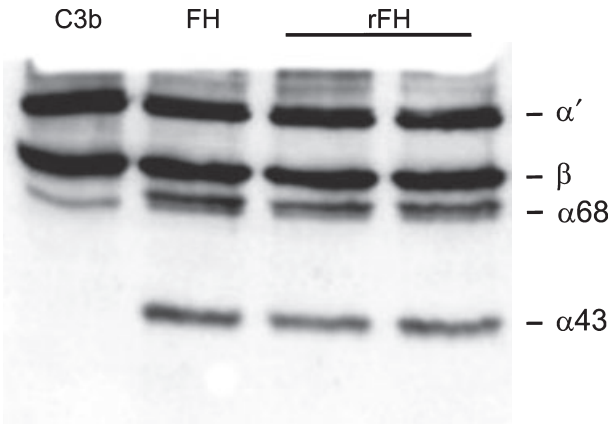

Figure 5 Recombinant factor H is active in a fluid-phase complement cofactor assay. Recombinant factor H (rFH) shows cofactor activity for factor I comparable to the wild-type human protein (FH) as evidenced by the appearance of the α68 and α43 kDa cleavage products of the C3b alpha-chain (α′). The same reaction setup without the addition of factor H served as a control (C3b).

samples analysed (human FH standard, purified from blood plasma and rFH (Figure 6)). Hence, the utilization of the moss signal peptide resulted in accurate processing of rFH.

Here, we demonstrated the successful stable expression of the full-length FH cDNA in a recombinant plant-based system, the moss *Physcomitrella patens*. As complement factor H is an important regulator of an essential part of the innate immune response, FH deficiency or defects are linked with several severe diseases. This makes FH a promising target for biopharmaceutical production. Despite the complex structure of the 155 kDa protein, which requires the correct assembly of 40 disulphide bridges, the moss-produced protein was correctly processed and secreted to the culture supernatant and showed a biological activity comparable to that of plasma-derived FH. This study represents the first successful production of recombinant factor H in a plant, thus highlighting the flexibility and potential of the moss system for production of complex biopharmaceuticals.

Experimental procedures

Design of the expression vector pFH

To create the expression construct pFH, the *Physcomitrella* actin-5 promoter (act5P) (Weise *et al.*, 2006) and the signal peptide coding sequence of PpAP1 (AP1-SP) (Schaaf *et al.*, 2004) were fused in a first step by splicing by overlap extension (SOE). Therefore, act5P was amplified from gDNA with the primers pFH_A (5′-TTGTCGATACTGGTACTAATG-3′) and pFH_C (5′-ACTCCTCGATGCCCCCATCTTCTATTTAATACGGACCTGCACA-3′) from *Pp*-gDNA. AP1-SP was amplified from *Pp*-cDNA using the primers pFH_D (5′-TGTGCAGGTCCGTATTAAATAGAAGATGGGGGCATCGAGGAGT-3′) and pFH_E (5′-CTTCCAATTTACCTGAATTATATGCCTCAGCTAAGGCTGC-3′). Both amplificates served as a template in a third PCR-step where pFH_B (5′-ACGCGTCGACTCCTTGTGACTTTTGTGCATT-3′) and pFH_E (5′CTTCCAATTTACCTGAATTATATGCCTCAGCTAAGGCTGC-3′) were added in normal concentration, and pFH_C and pFH_D served as bridging oligos and were added at 1/50th the concentration of the outer primers. The resulting product was ligated in pCR4-TOPO and termed pActP-SP.

The human FH cDNA was amplified without its endogenous signal sequence from human adult normal liver cDNA (BioCat) with the primers FH BbvCI for (5′-TGCTGAGGCAGAAGATTGCAATGAACTTCCTCC-3′) and FH HIS rev (5′-TCTGCAGTCAATGATGATGATGATGATGTCTTTTTGCACAAGTTGGATAC-3′). Subsequently, the introduced *Bbv*CI- and *Pst*I-restriction sites (underlined in primer sequence) were used to insert the cDNA in pActP-SP. Finally, the 35S-terminator sequence was amplified from the Plasmid pFIX1 (Schaaf *et al.*, 2004) with the primers 35STPHf (5′-GTGCAGAAGCTTCTAGAGTCCGCAAAAATCACCA-3′) and 35STr (5′-GACTAGTGGTCACTGGATTTTGGTTTTAGG-3′) and cloned in pCR4-TOPO. By making use of *Pst*I- and *Spe*I-restriction sites, the terminator was transferred into the beforehand described construct to yield the final expression vector pFH.

Moss cultivation

Physcomitrella patens (Hedw.) B.S. was cultured under standard conditions in liquid or on solid Knop medium as described previously (Frank *et al.*, 2005). Suspension cultures of WT and transgenic moss lines were subcultured in parallel weekly by disruption of the tissue with an ultraturrax (IKA, Staufen, Germany) with a rotational speed of 19000/min for 1 min and afterwards transferred into fresh Knop medium.

When moss was used for rFH production analyses in small scale, i.e. in 500 -mL flasks, subculturing was carried out without prior disruption of the tissue, and moss was transferred to Knop medium at pH 5.8 or Knop medium with 0.1% N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) at pH 7.4, in both cases supplemented with 0.001% bovine serum albumin (BSA; Sigma-Aldrich, Deisenhofen, Germany, fraction V, A3059). In addition, for stability experiments, WT cultures were supplemented with standard FH as control (#341274, Calbiochem, San Diego, CA, USA).

To gain a suitable amount of rFH to be used in the cofactor assay, a 5 -L photobioreactor culture was run as described previously (Hohe and Reski, 2005), but with more gentle stirring (320 instead of 500 rpm), increased aeration (0.45 instead of 0.3 vvm) and without CO_2 supplementation.

The dry weight (DW) of liquid cultures was determined by filtrating 10 mL of the culture suspension through gauze (Miracloth; Calbiochem) over a Buechner funnel by application of vacuum. The moss tissue was then dried for 2 h at 105 °C and its weight determined.

Transformation and transgenic plant screening

Protoplasts were isolated, and transformation was performed according to Strepp *et al.* (1998). A total number of 3×10^5

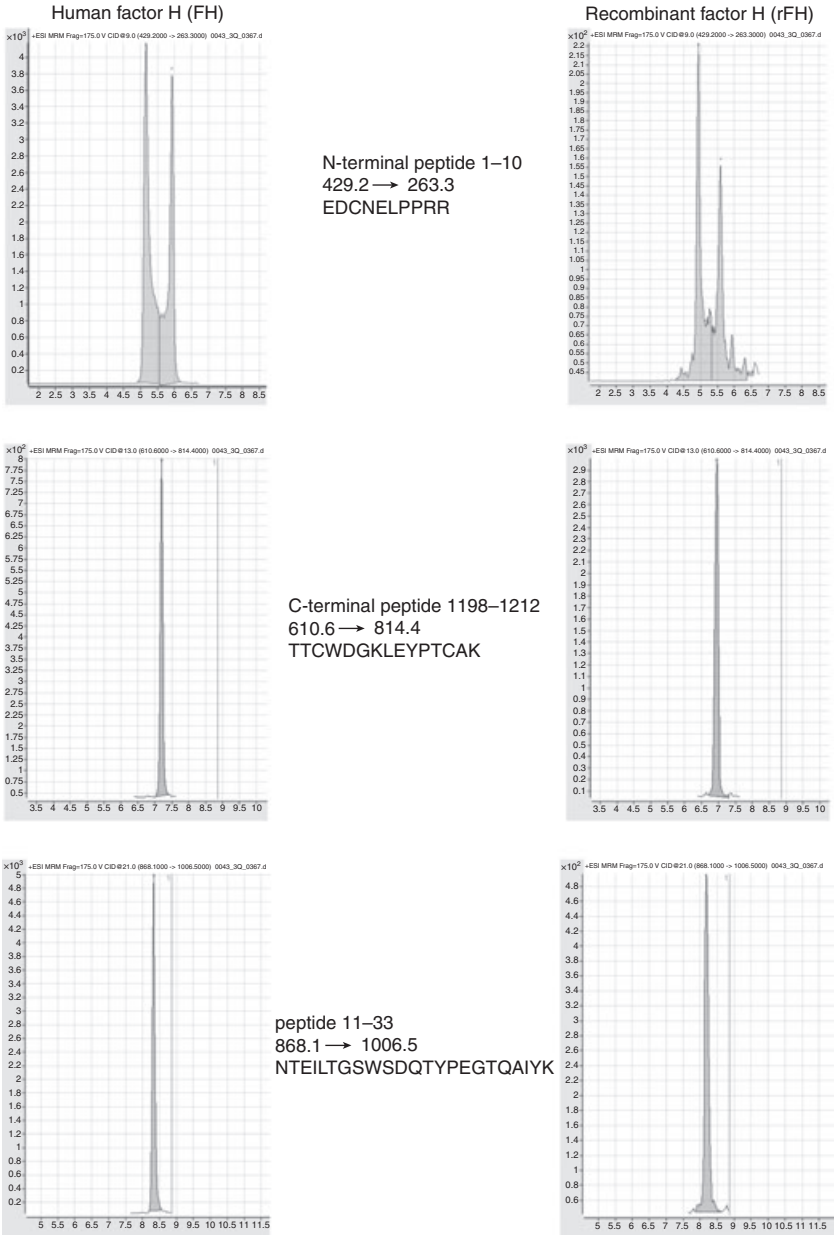

Figure 6 LC-MS/MS analyses confirming the completeness of recombinant factor H. Plasma-derived FH and moss-produced rFH were analysed by data dependent nanoLC-MS/MS on a Q-Tof instrument. Among the identified peptides, the N-terminal peptide (1-10, EDCNELPPRR), the peptide 11-33 (NTEILTGSWSDQTYPEGTQAIYK) as well as the C-terminal peptide (TTCWDGKLEYPTCAK) were identified with high confidence and from the obtained MS/MS data a multiple reaction monitoring (MRM) method was set up for the selective and highly sensitive detection of these tryptic peptides. All three peptides were clearly detectable in both protein samples, the plasma-derived FH and the recombinant FH, verifying the presence of the correct N- and C-terminal ends of the moss-derived rFH.

protoplasts were co-transfected with 20 μg DNA of pFH, which had been linearized by digestion with *Spe*I and *Sal*I, and 10 μg of a circular plasmid containing a nptII- cassette or hpt-cassette. Subsequently, selection of stable transformants on solidified Knop medium containing 25 μg/mL G418 or 12.5 μg/mL hygromycin respectively followed as described previously (Frank *et al.*, 2005). Screening of transformants by direct PCR (Schween *et al.*, 2002) was carried out by incubation of small pieces of gametophore tissue in 50 μL 1× Taq E buffer (Genaxxon, Biberach, Germany) for 15 min at 44 °C and subsequent freezing over night at –20 °C. Five microliters of the extract was used as template in a standard PCR with 0.4 mM of the primers FH-1563 (5′-ATGATGGTTATGAAAGCAATACTGGAAGCAC-3′) and FH-2351 (5′- TTGTATTTGTGCCATTGAGCAGTTCACTTC-3′). As stabilizing reagents, 1.5% PVP-40 and 0.25 mM spermidine were added. A negative control without template or a positive control with the plasmid pFH, respectively, was run in parallel.

For transgene expression analysis via RT (reverse transcription)-PCR, 50–100 mg (fresh weight) moss material was ground in liquid nitrogen. The frozen tissue was resuspended in 1 mL TRIzol® Reagent (Invitrogen, Karlsruhe, Germany), and RNA was isolated according to the manufacturer's instructions. Two micrograms of DNase-treated RNA was used for First Strand synthesis with Superscript Reverse Transcriptase II (Invitrogen, Karlsruhe, Germany). The quality of cDNA was checked in a subsequent standard PCR with the primers c45for (5′-GGTTGGTCATGGGTTGCG-3′) and c45rev (5′-GAGGTCAACTGTCTCGCC-3′) corresponding to the constitutively expressed gene for the ribosomal protein L21. Transgene expression was verified with the primers FH-751 (5′-ATGTGATAATCCTTATATTCCAAATGGTGACTACT-3′) and FH-1702 (5′- ATTTCAACACCTCTCCAACTTTATACTGGTCTTTC-3′).

The transgenic plant lines referred to in this study (FH2, FH3, FH15 and FH69) are stored in the International Moss Stock Center (http://www.moss-stock-center.org; IMSC #40313-40316).

Recombinant protein detection and purification

Recombinant FH production was monitored by either Western blotting or quantitatively using a sandwich ELISA.

For ELISA measurements, the wells of a microtiter plate (NUNC Maxisorb, Wiesbaden, Germany) were coated over night at 4 °C with the first antibody (monoclonal, T13 anti-factor H, mouse) (Manuelian *et al.*, 2003) diluted 1 : 1000 in coating buffer (50 mM *carbonate/bicarbonate buffer*, pH 9.6). The plate was blocked for 90 min with buffer A (2% BSA in phosphate-buffered saline (PBS)) with 0.5% Tween-20 at 37 °C. Samples and standard (1–50 ng/mL) FH (Calbiochem, Cat.No.341274) were diluted in buffer A with 0.5% Tween-20, the incubation was performed for 90 min at room temperature (RT), and the plate was washed with buffer A w/o Tween. The second (polyclonal anti-factor H from rabbit) and third (anti-rabbit horseradish peroxidase (HRP), NA934; GE Healthcare, München, Germany) antibodies were diluted 1 : 8000 in buffer A with 0.5% Tween-20 or buffer A with 0.05% Tween, respectively, and incubation took place for 60 min at RT with washing steps in between using buffer A with 0.05% Tween. After incubation with tetramethylbenzidine (TMB) (Calbiochem), the development reaction was stopped with 1 M sulphuric acid, and final read out was performed at 450 nm.

For Western blot analyses, 7.5% SDS-PAGE (Ready Gel Tris-HCl, Bio-Rad, Munich, Germany) was run at 150 V for 1 h and blotted to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA) in a Trans-Blot SD Semi-Dry Electrophoretic Cell (Bio-Rad) for 1.5 h with 1.8 mA/cm^2 membrane. Immunoblotting was performed using the *ECL™ Advance* Western Blotting Detection *Kit* (GE Healthcare) following the manufacturer's instructions using for the anti-FH Western blot the ab3613 (polyclonal anti-factor H, from goat) as primary and ab6667 (anti-goat HRP) as secondary antibody (both Abcam, Cambridge, MA, USA) in a 1 : 8000 and 1 : 100 000 dilution resp. For the anti-fucose immunoblot, a dilution 1 : 2000 from an anti-α1,3-fucose fraction purified from an anti-HRP rabbit serum (Faye *et al.*, 1993) and an anti-rabbit (NA934; GE Healthcare) 1 : 50 000 were used.

Prior to SDS-PAGE, rFH was either precipitated from culture supernatant, extracted from moss cells directly in sample buffer (Laemmli, 1970) or purified by nickel-chelate affinity chromatography. Culture supernatant was harvested by filtration, and proteins were precipitated by the addition of 100% (w/v) TCA (trichloracetic acid; Sigma-Aldrich, Deisenhofen, Germany) to a final concentration of 10% (w/v). After incubation at 4 °C overnight, samples were centrifuged for 2 h at 4500 ***g*** at 4 °C, and the supernatant was discarded. The precipitate was washed twice with cold (–20 °C) acetone and, after air drying, resuspended in 50–100 μL sample buffer (Laemmli, 1970) with or without addition of reducing agent (5% beta-mercapto-ethanol). For staining of proteins in SDS-gels, the PageBlue (Fermentas, St. Leon-Rot, Germany) ready-to-use staining solution containing colloidal Coomassie dye was used following the manufacturer's instructions.

Ni-NTA purification was performed in batch procedure with whole moss extracts from wild-type and transgenic line FH3, respectively. Total protein was extracted by resuspension of 300 mg moss tissue, grounded in liquid nitrogen, in 800 μL binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, 0.05% (v/v) Tween-20) under vigorous vortexing. After centrifugation at 20 800 ***g***, the supernatant was mixed with 50 μL Ni-NTA agarose (Qiagen, Hilden, Germany), which was beforehand equilibrated in binding buffer, and incubated overnight at 4 °C on a rotary shaker. The supernatant was discarded, and the Ni-NTA matrix was washed three times with 500 μL washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol). Finally, rFH containing fractions were eluted 2–4 times with 50 μL elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.5–1 M imidazol).

For the MS/MS analysis, elution fractions were pooled and four times the sample volume of cold (–20 °C) acetone was added. Protein precipitation was performed overnight at –20 °C. After centrifugation at 14 000 ***g,*** the supernatant was carefully discarded. The protein pellet was shortly air dried at room temperature and resuspended in NuPAGE LDS sample buffer (Invitrogen, Karlsruhe, Germany).

Complement cofactor assay

For determining the regulatory activity of rFH, the purified recombinant protein (0.5 μg/mL) was added with C3b (0.5 μg/mL) and factor I (0.2 μg/mL) in PBS (50 μL) and incubated for 30 min at 37 °C. The mixture was separated by SDS-PAGE transferred to a nitrocellulose membrane (Protran BA 83, Whatman, Dassel, Germany), and C3 degradation products were identified by Western blotting using a polyclonal goat C3 antiserum (1 : 2000, Merck, Whitehouse Station, NJ, USA) and rabbit anti-goat HRP (DAKO, Carpinteria, CA, USA) as secondary antibody.

Sample preparation, LC-MS/MS and data analysis

Proteins were alkylated using iodoacetamide before separating on a SDS-PAGE. For in-gel digestion, the excised gel bands were destained with 30% acetonitrile (ACN), shrunk with 100% ACN, and dried in a Vacuum Concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with trypsin (Promega, Madison, USA) were performed overnight at 37 °C in 0.05 M NH_4HCO_3 (pH 8). About 0.1 μg of protease was used for one gel band. Peptides were extracted from the gel slices with 5% formic acid.

LC-MS/MS analyses were performed on an Agilent 6460 triple quadrupole mass spectrometer and on an Agilent 6520 Q-Tof mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Both instruments were coupled to a 1200 Agilent nanoflow system via a HPLC-Chip cube ESI interface. Peptides were separated on a HPLC-Chip with an analytical column of 75 μm i.d. and 40 mm length and a 40-nL trap column, both packed with Zorbax 300SB C-18 (5 μm particle size). Peptides were eluted with a linear acetonitrile gradient with 5%/min at a flow rate of 300 nL/min (starting with 0% acetonitrile). Dynamic MRM (multiple reaction monitoring) analyses were performed for the following three tryptic peptides: EDCNELPPRR (N-terminal peptide (1-10), Cys was carbamidomethylated), NTEILTGSWSDQTYPEGTQAIYK (11-33) and TTCWDGKLEYPTCAK (C-terminal peptide, both Cys residues were carbamidomethylated) (for details see supplementary data).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Direct-PCR.

Figure S2 Human factor H standard migrates with different sizes.

Data S1 LC-MS/MS.

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