Method for the production of proteinaceous substances

The present invention generally relates to the field of the production of proteinaceous substances in plant material. In particular, the invention relates to a novel method for the production of desired proteinaceous substances in mosses.

The exploitation of biotechnological methods for production purposes is an important possibility for man of producing substances which cannot be produced economically, if at all, by other routes, for example by chemical synthesis, and of which insufficient amounts are available naturally to act as raw materials. Even though over 10 000 plant secondary metabolites are known, only few of these compounds are produced on an industrial scale with the aid of plant cell cultures. These substances are predominantly pharmaceutically active secondary metabolites. Examples which may be mentioned are a) berberin (production on the 4 000 l scale), which has a bacteriostatic and fungicidal action (Y. Fujita and M. Tabata, in: Plant tissue and cell culture, plant science; Vol. 3, p. 169, C.E. Green et al. (Ed.), A.R. Liss Inc., New York (1987)), b) shikonin (750 l scale) which has an antibiotic and antiinflammatory action (M. Tabata and Y. Fujita, in: Biotechnology in plant science; p. 207-218, P. Day et al. (Ed.), Academic Press, Orlando (1985)), and c) paclitaxel (75 000 l scale), better known under the name taxol, which has antitumor action (M. Jaziri et al., *Taxus* sp. cell, tissue and organ cultures as alternative sources for taxoids production: a literature survey, Plant Cell Tiss. Org. Cult., **46**, pp. 59-75 (1996)).

A further important biotechnological method, in which plant cell cultures are exploited, is the biotransformation of digitoxin to digoxin, a cardiac and circulation drug. This stereospecific hydroxylation reaction is carried out successfully in bioreactor cultures of *Digitalis lanata* (E. Reinhard and W. Kreis, Kultivierung von pflanzlichen Zellen im Bioreaktor [Plant cell culture in the bioreactor], Bio. Engin., **5**, pp. 135-136 (1989))

in high yields. An up-to-date and extensive review of the use of plant cell cultures in biotechnology can be found in H.-P. Mühlbach, Use of plant cell cultures in biotechnology, Biotechnol. Annu. Rev., **4**, pp. 113-176 (1998).

The development of genetic transformation methods for higher plants at the beginning of the eighties made it possible considerably to increase the productivity of plants for specific secondary constituents by transforming the genes for specific key enzymes of the metabolic pathways in question. Not only transgenic intact plants but also plant cell cultures were exploited. Examples which may be mentioned are the overexpression of a bacterial lysine decarboxylase in transgenic root-hair cultures of *Nicotiana tabacum*, which increased the yields of the biogenic amines Cadaverin and Anabasin by a factor of up to 14 (J. Berlin et al., Genetic modification of plant secondary metabolism: Alteration of product levels by overexpression of amino acid decarboxylases, in: Advances in Plant Biology, Studies in Plant Science, Vol. **4**, pp. 57-81, D.D.Y. Ryu and S. Furasaki (Ed.), Elsevier, Amsterdam (1994)).

However, the possibility of transferring DNA into plants not only opened up quantitative and qualitative alterations of plant constituents; in addition, plants and plant cell cultures became more interesting for the production of heterologous proteins (A.S. Moffat, High-Tech plants promise a bumper crop of new products, Science **256**, pp. 770-771 (1992)), two different approaches being chosen in principle.

One approach comprises the production of heterologous proteins in transgenic intact plants. Besides the production of antibodies in transgenic tobacco plants (J.K.-C.Ma et al., Generation and assembly of secretory antibodies in plants, Science **268**, pp. 716-719 (1995)), the expression and correct processing of human serum albumin both in transgenic tobacco plants and in transgenic potato plants has been described (P.C.

Sijmons et al., Production of correctly processed human serum albumin in transgenic plants, Bio/Techn., **8**, pp. 217-221 (1990)). Human epidermal growth factor (hEGF) was also expressed in transgenic tobacco plants (A.-H. Salmanian et al., Synthesis and expression of the gene for human epidermal growth factor in transgenic potato plants, Biotechnol. Lett., 18, pp. 1095-1098 (1996)). However, other plants were also used. Leu-encephalin was produced successfully using *Arabidopsis thaliana* and *Brassica napus* (E. Krebbers and J. Vandekerckhove, Production of peptides in plant seeds, Tibtech., **8**, pp. 1-3. (1990)). Furthermore, transgenic *Vigna unguiculata* plants were used for the expression of chimeric viral particles which act as vaccines (K. Dalsgaard et al., Plant-derived vaccine protects target animals against a viral disease, Nat. Biotech., 15, pp. 248-252 (1997)).

A principal disadvantage when using intact plants as those described above by way of example is the necessity of growing them, which is time-consuming and expensive, and the large cultivation area which is required for industrial-scale production. Moreover, the isolation of the desired target substances from intact plants usually requires complex process steps, in particular when the consistency and quality of the products must meet high requirements, as is the case with substances to be employed for pharmaceutical or nutritional purposes.

In the second approach, transgenic tobacco cell cultures were exploited for the production of antibodies. Described are, for example, the expression of antibodies and their secretion into the medium (N.S. Magnuson et al., Enhanced recovery of a secreted mammalian protein from suspension culture of genetically modified tobacco cells, Prot. Expr. Pur., 7, pp. 220-228 (1996)). Since the purification of heterologous proteins from cells is complicated, secretion of the target protein into the medium constitutes a marked improvement. Moreover, the

production of recombinant pharmaceutically relevant proteins in cell cultures is also of interest from the safety point of view since the transgenic plant cells can be grown exclusively in bioreactors and need not be released. The necessary mass culture was made possible by the development of bioreactors for heterotrophic plant cell cultures on a larger scale (for example M.L. Shuler et al., Bioreactor engineering as an enabling technology to tap biodiversity: The case of taxol., Ann. N. Y. Acad. Sci., **745**, pp. 455-461 (1994)).

The principal disadvantages of this second approach, in which plant suspension cultures are used, are the low growth rate, the relatively slow formation of secondary metabolites, the inhibition of product formation at high cell densities and, as a consequence, low productivity per volume, the formation of aggregates and cell wall constituents, and the increased sensitivity of the cells to shear forces. It must also be taken into account that complex media with a multiplicity of constituents, some of which are expensive, must always be provided when using heterotrophic cell cultures. However, the most serious disadvantage to be mentioned is the occurrence of somaclonal variations in plant *in vitro* cell cultures, which bring about quantitative and qualitative changes in the production of heterologous proteins (see, for example, M.G.K. Jones and K. Lindsey, Plant biotechnology, in: Molecular biology and biotechnology, J.M. Walker and E.W. Gingold (Eds.), 2nd Ed., Royal Soc. of Chem., Burlington House, London (1988). Heterogeneity of the products formed and of their functional properties cannot be accepted, in particular in connection with the production of pharmaceuticals and other desired substances whose official approval demands reliable quality assurance and a standardized production method.

The object of the present invention is therefore to provide a method for the standardized production of heterologous proteinaceous substances in plant materials which essentially

eliminates not only the above-described disadvantages of using intact plants, but also the disadvantages of using cell culture systems.

This object is achieved in accordance with the invention by providing a novel method for the production of heterologous proteinaceous substances in plant material in which fully differentiated moss plants are grown under standard conditions and the proteinaceous substances produced are obtained from the culture medium essentially without disrupting the producing tissues or cells.

The term "proteinaceous substance" as used herein encompasses peptides, polypeptides and proteins and also fragments of these which are suitable in particular for diagnostic, clinical, pharmaceutical and nutritional purposes. Also encompassed are those molecules which have peptide bonds and which are translated by plant material.

In a preferred embodiment of the present invention, the desired heterologous proteinaceous substance is released into the culture medium in its biologically active form.

The term "biologically active" as used in the present description means that the target substances provided with this attribute have the functional properties desired or required for the respective purpose. If, for example, it is desired to produce antibodies, the protein produced, or a functional fragment thereof, is biologically active when it is capable of establishing the expected specific binding with the antigen. It is obvious to the skilled worker that the complete protein is not always required for such purposes, but that it is possible to search for epitopes or low-molecular-weight structures which ensure the desired biological activity or functionality. For example, an enzyme is biologically active when it is capable of converting its target substrate.

In a further preferred embodiment of the invention, the plant material is grown in the form of intact moss plants in a culture medium which is essentially free from sugars, vitamins and phytohormones or functional fragments of these.

The method according to the invention allows the possibility of growing intact and fully differentiated plants under photoautotrophic conditions which can be standardized, i.e. without requiring the addition of sugars, vitamins and phytohormones and the like, as is required in prior-art heterotrophic suspension cell culture systems. Because an inexpensive and simple culture medium is used, the steps of obtaining and purifying the desired target substances are facilitated considerably.

The plant material to be employed in the method according to the invention is preferably an intact moss plant selected from the group of the mosses, including liverworts, with species from the genera *Physcomitrella*, *Funaria*, *Sphagnum* and *Ceratodon*, and also *Marchantia* and *Sphaerocarpos* being especially preferably employed. The method according to the invention is most preferably carried out using the moss *Physcomitrella patens*.

In a further preferred embodiment, the nucleic acid construct used for the transformation encodes not only the desired proteinaceous substance, but also a transit peptide for secreting the substance from the host cell into the culture medium. Any of the autologous and heterologous nucleic acid sequences known to the skilled worker can be employed in accordance with the invention and can be used for generating an expression cassette for transforming the producer tissue. The use of signal peptides for the endoplasmic reticulum or cellular transport is especially preferred.

Work carried out for the present invention demonstrates that the above-described problem of somaclonal variation, which is encountered in cell cultures, does not exist in photoautotrophic liquid cultures of mosses. Furthermore, the mosses used in accordance with the invention have the advantage over other systems of a clear sequence of precisely defined differentiation steps (chloronema, caulonema, buds, gametophores), which can be influenced by adding plant hormones (indole-3-acetic acid induces caulonema development, isopentenyladenine induces the development of buds) (see, for example, N.W. Ashton et al., Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants, Planta, **144**, pp. 427-435 (1979)). Directed differentiation-specific expression of heterologous proteins in bioreactor cultures is therefore made possible, and a synchronously dividing, pure and thus homogeneous chloronema culture is especially preferably suitable in accordance with the invention owing to its controllable uniform protein production in the bioreactor and its suitability for the use of hormone-dependent or differentiation-specific promoters.

In addition to such an expression system, an inducible promoter system may also be used in accordance with the invention, in particular for the production of proteins which have a short half-life or which are cytotoxic, the *Agrobacterium tumefaciens* 1'-promoter being used especially preferably.

The cultivation of mosses proposed in accordance with the invention for the production of heterologous proteins under economical aspects can be effected for example by using *Physcomitrella* in volumes in the order of magnitude of from 20 ml to over 6 l up to 10 l and above in shake cultures or in aerated glass containers (see, for example, R. Reski, Zell- und molekularbiologische Untersuchungen der cytokinin-induzierbaren Gewebedifferenzierung und Chloroplastenteilung bei *Physcomitrella patens* (Hedw.) B.S.G., [Cell- and molecular-

biological studies of the cytokinin-inducible tissue differentiation and chloroplast division in *Physcomitrella patens* (Hedw.) B.S.G.], Ph.D. thesis, University of Hamburg (1990)). Since this is a culture of differentiated photoautotrophic plants, the medium needs neither supplementation with plant hormones nor vitamins nor sugars. In comparison with the complex media required, for example, for animal cell cultures, the costs are lower by a factor of 100. It has emerged in accordance with the invention that the yield of biologically active heterologous protein in the culture medium can be increased by a factor of 35 in the presence of PVP, which is why the use of PVP in the culture medium is preferred in the method according to the invention.

Detailed information on culturing further mosses which are suitable in accordance with the invention such as, for example, *Leptobryum pyriforme* and *Sphagnum magellanicum* in bioreactors is described in the prior art (see, for example, E. Wilbert, Biotechnologische Studien zur Massenkultur von Moosen unter besonderer Berücksichtigung des Arachidonsäurestoffwechsels [Biotechnological studies concerning the mass culture of mosses with particular consideration of the arachidonic acid metabolism], Ph.D. thesis, University of Mainz (1991); H. Rudolph and S. Rasmussen, Studies on secondary metabolism of Sphagnum cultivated in bioreactors, Crypt. Bot., **3**, pp. 67-73 (1992)). Especially preferred for the purposes of the present invention is the use of *Physcomitrella*, in particular since all of the usual molecular-biological techniques are established for this organism (for a review see R. Reski, Development, genetics and molecular biology of mosses, Bot. Acta, **111**, pp. 1-15 (1998)).

Suitable transformation systems were developed for the biotechnological exploitation of *Physcomitrella* for the production of heterologous proteins. For example, successful transformations were carried out by direct DNA transfer into

protonema tissue using the particle gun. The PEG-mediated DNA transfer into moss protoplasts was also successful. This transformation method has been described repeatedly for *Physcomitrella* and leads both to transient and to stable transformants (see, for example, K. Reutter and R. Reski, Production of a heterologous protein in bioreactor cultures of fully differentiated moss plants, Pl. Tissue culture, @ Biotech., **2**, pp. 142-147 (1996)).

Although the present invention is principally suitable for the production of any proteinaceous substance, the production of a pharmaceutically relevant protein will be demonstrated hereinbelow with reference to the human vascular endothelial growth factor (VEGF).

VEGF was first isolated by N. Ferrara and W.J. Henzel (Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, Biochem. Biophys. Res. Commun., **161**, pp. 851-858 (1989)) and characterized as regulatory factor for the controlled angiogenesis and endothelial cell division under normal physiological conditions (N. Ferrara et al., The vascular endothelial growth factor family of polypeptides, J. Cell. Biochem., **47**, pp. 211-218 (1991)). The authors also demonstrated that this growth factor acts highly specifically on vascular endothelial cells and is inactive for other cell types. VEGF is a homodimeric glycoprotein linked by disulphide bridges. Four different forms of human VEGF are known. The four isoforms are 121, 165, 189 and 206 amino acids in length and are formed by alternative splicing of VEGF RNA. $VEGF_{206}$ was only evidenced in a fetal liver cDNA, while transcripts of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ were evidenced in a number of tumor cells and tumor tissues. All VEGF isoforms have leader sequences for secretion, but only the two smallest forms are secreted efficiently (see, for example, G. Martiny-Baron and D. Marmé, VEGF-mediated tumor angiogenesis: A new

target for cancer therapy, Curr. Opin. Biotechnol., **6**, pp. 675-680 (1995)).

Suitable amounts of VEGF were and are still required both for the development and improvement of existing approaches for tumor therapy and for characterizing VEGF. During the early stages of work carried out in context with the present invention, all that was described was the recombinant production of VEGF in insect cells by means of the baculovirus expression system (for example B.L. Fiebich et al., Synthesis and assembly of functionally active human vascular endothelial growth factor homodimers in insect cells, Eur. J. Biochem., **211**, pp. 19-26 (1993)). *Saccharomyces cerevisiae* (S. Kondo et al., The shortest isoform of human vascular endothelial growth factor/vascular permeability factor (VEGF/VPF_{121}) produced by *Saccharomyces cerevisiae* promotes both angiogenesis and vascular permeability, Biochim. Biophys. Acta, **1243**, pp. 195-202 (1995)), the yeast *Pichia pastoris* (D. Mohanraj et al., Expression of biologically active human vascular endothelial growth factor in Yeast, Growth factors, **12**, pp. 17-27 (1995)) and *Escherichia coli* (G. Siemeister et al., Expression of biologically active isoforms of the tumor angiogenesis factor VEGF in *Escherichia coli*, Biochem. Biophys. Res. Commun., **222**, pp. 249-255 (1996)) followed as further production organisms. Biologically active VEGF was produced with all these recombinant systems. However, the *E. coli* expression system is complicated with regard to purification and reconstitution of the protein since the latter is packaged into inclusion bodies.

Examples

Summary

The establishment of controllable *Physcomitrella patens* mass cultures (Reutter and Reski, loc. cit.) and methods of transferring DNA into the moss *Physcomitrella patens* (K.

Reutter, Expression heterologer Gene in *Physcomitrella patens* (Hedw.) B.S.G. [Expression of heterologous genes in *Physcomitrella patens* (Hedw.) B.S.G.], Ph.D. thesis, University of Hamburg (1994)) created the basic prerequisites for the biotechnological exploitation of this plant.

Work carried out at the beginning demonstrated the long-term stability of the integration using transgenic *Physcomitrella*-lines originating from Reutter (loc. cit., 1994). Expression of the heterologous *npt* II and *gus* genes, which were employed by way of example for this purpose, was still detectable after four years.

The *Physcomitrella* bioreactor culture was optimized. A stirrer was developed which brings about comminution of the protonemata and thus ensures the required homogeneity of the culture at continuous speeds of 300-500 rpm. Standardized sampling was thus made possible. At the same time, the incoming air was distributed more uniformly in the liquid culture. Under these conditions it was demonstrated that biomass and protein development without external pH regulation proceed in the same manner as with pH regulation; surprisingly, the latter is therefore not necessary. A weekly biomass production of 500 mg of dry weight, or 22 mg of total protein, per litre was obtained under semi-batch conditions. This means an increase in biomass production by a factor of five over the conventional 5 l glass flask culture. Reducing the salt concentrations of the Knop medium to one tenth led to similar data and thus to reduced costs.

Addition of 5mM ammonium tartrate accelerated biomass development by reducing the lag phase. Simultaneously, the addition of ammonium tartrate gave cultures which comprised virtually exclusively chloronema cells. It was demonstrated with the aid of flow cytometry that virtually one hundred percent of the cells of these cultures were at the G2/M phase of the cell

cycle. This result was confirmed by further physiological studies with auxin and by studies with the differentiation-specific mutants *cal*112 and *cal*113, and it was concluded that caulonema cells are in the G1/G0 phase most of the time, while chloronema cells are predominantly in the G2/M phase.

A promoter was studied for possible inducibility in moss by using the agrobacterial 1'-promoter. The β-glucuronidase (*gus*) gene was used as marker gene. In transiently transformed moss protoplasts (transformation rate = $3x10^{-4}$), expression of the *gus* gene was observed following induction with 5 µM indole-3-acetic acid. No expression was observed in any of the controls.

The gene for the 121 amino acid splice form of the human vascular endothelial growth factor ($VEGF_{121}$) was transferred into *Physcomitrella* at transformation rates of $0.5x10^{-5}$ and $3.3x10^{-6}$. To this end, the gene was cloned behind the constitutive 35S promoter and into the transformation vector pRT99, which is suitable for plants. In a second approach, the sequence encoding the corresponding human ER transit peptide was additionally cloned. Integration of the heterologous DNA was confirmed and the type of integration described by subjecting the stable transformants obtained to Southern analysis. Northern analysis confirmed the existence of the nptII and the two VEGF transcripts in these transformants. $VEGF_{121}$ expression in the moss cells was demonstrated by indirect immunofluorescence. The protein was unambiguously localized in the cells with the aid of a confocal laser scanning microscope. These studies revealed for the transformants without transit peptide that the protein is localized in particular in the cytoplasm. In the transformants which additionally contain the ER transit peptide, the protein can be found in the nuclear regions and in the apical regions of the apical cells, regions with a very high ER content. The biological activity of the heterologous protein produced in accordance with the invention was verified by carrying out ELISA

assays and two functionality assays with the VEGF protein obtained from the culture medium.

Materials and methods:

Unless otherwise specified in the text, the chemicals used were analytical-grade quality and obtained from Fluka (Neu-Ulm), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) and Sigma (Deisenhofen).

The solutions were made with purified, pyrogen-free water, hereinbelow termed H_2O, from a Milli-Q water purification system (Millipore, Eschborn).

Restriction endonucleases, DNA-modifying enzymes and molecular biology kits were obtained from AGS (Heidelberg), Amersham (Braunschweig), Applied Biosystems (Weiterstadt), Biometra (Göttingen), Boehringer Mannheim GmbH (Mannheim), Genomed (Bad Oeynhausen), New England Biolabs (Schwalbach/Taunus), Novagen (Madison, Wisconsin, USA), Pharmacia (Freiburg), Qiagen (Hilden) and Stratagene (Heidelberg). Unless otherwise specified, they were used in accordance with the manufacturer's instructions.

Vectors and constructs

The plasmid pCYTEXP-$VEGF_{121}$ is a derivative of pCYTEXP1 (T.N. Belev et al., A fully modular vector system for the optimization of gene expression in *Escherichia coli*, Plasmid, **26**, pp. 147-150 (1991)), in which the cDNA of human $VEGF_{121}$ is integrated for expression in *E. coli*. The $VEGF_{121}$ cDNA is excized from pCYTEXP-$VEGF_{121}$ using the restriction endonucleases *Nde* I and *Sal* I, purified, made blunt-ended and cloned into the *Sma* I cleavage site of pRT101 (R. Töpfer et al., A set of plant expression vectors for transcriptional and translational fusions, Nucleic Acids Res., **15**, p. 5890 (1987)) between the 35S promoter and the polyadenylation sequence of CaMV. Using *Hin* dIII, the cassette thus obtained is again excized and cloned

into the *Hin* dIII restriction cleavage site of the transformation vector pRT99. pRT99 contains not only a multiple cloning site, but also the neomycin phosphotransferase gene under the regulation of the 35S promoter and the corresponding polyadenylation sequence of CaMV (R. Töpfer et al., Versatile cloning vectors for transient gene expression and direct gene transfer in plant cells, Nucleic Acids Res., **16**, p. 8725 (1988)). This gene confers resistance to the antibiotic G418 in stably transformed plants. The plasmids were replicated in the *Escherichia coli* strain DH5α (J. Sambrook et al., Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, New York (1989)).

Using the restriction enzymes *Bam* HI and *Bgl* II, the cDNA is excized from the vector pVE-121, which was originally constructed for expressing $VEGF_{121}$ in insect cells and which additionally to the $VEGF_{121}$ sequence comprises the DNA encoding the natural transit peptide which, in animal cell systems, mediates secretion into the medium via the endoplasmic reticulum (Fiebich et al., Synthesis and assembly of functionally active human vascular endothelial growth factor homodimers in insect cells, Eur. J. Biochem., **211**, pp. 19-26 (1993)), and, using pRT101, cloned into pRT99 and verified.

Plasmid pNA201 is a derivative of the binary vector pBI101 (A.R. Jefferson et al., Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol. Rep., **5**, pp. 387-405 (1987)). It contains the *nptII* gene under the nopalin synthase promoter as selection marker for plants. The *gus* gene, which is also present, is regulated by the 1'-promoter from *Agrobacterium tumefaciens*. pNA201 is suitable for the direct transformation of *Physcomitrella patens*.

Antibodies

Two different antibodies against the VEGF protein are used. The first antibody is a rabbit-anti-VEGF antibody and directed

against a synthetic peptide which corresponds to amino acids 1-20 of the native human VEGF (Fiebich et al., loc. cit. (1993)). The second antibody is a monoclonal mouse antibody directed against the human $VEGF_{121}$ protein (R&D Systems, Wiesbaden).

Plant material

The wild-type strain of the moss *Physcomitrella patens* (Hedw.) B.S.G., which originates from the collection of the Genetics Group at the Department of General Botany, University of Hamburg, is employed. Its origin is the strain 16/14, which had been collected by H.L.K. Whitehouse in Gransden Wood, Huntingdonshire (England) and had been subcultured from a spore.

The wild-type strain is grown either in liquid culture with Knop medium (R. Reski and W.O. Abel, Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine, Planta, **165**, pp. 354-358 (1985)) or on solid Knop medium with 1% oxoid agar (Unipath, Basingstoke, England). Liquid cultures were performed as described by Reski (loc. cit., 1990).

Bioreactor culture

For mass cultivation, plant material is introduced into a 7 l round-bottom glass flask bioreactor equipped with a double jacket (Applikon Biotek, Knüllwald). In this bioreactor system, waste air condenser, aeration tube, pH electrode (Conducta, Gerlingen), temperature sensor, stirring device, sampling tube and the inlets for acid, base and medium are introduced into the culture volume from above through bores in the lid. Cultures are performed at 25°C and are controlled by means of a suitably adjusted water bath which is connected to the double jacket. In experiments which are carried out with pH regulation, the pH is kept constant at 5.8 by means of the titration unit. Temperature measurements and pH regulation are carried out with the Biocontroller ADI 1030 (Applikon Biotek, Knüllwald). The stirrer

speed can be varied by means of the motor controller ADI 1012 (Applikon Biotek, Knüllwald). The culture medium is aerated constantly with 1 bar of air via a porous injection element. To ensure sterility in the culture vessel, all of the inlet and exhaust lines are provided with filter sterilization units (Midisart, 0.2 µm; Sartorius, Göttingen). The bioreactor culture is performed in a controlled-environment cabinet with lateral illumination (white light; Osram L 40 W/20; max. 100 $\mu mols^{-1}m^{-2}$). The cultures are inoculated with 0.5 - 1g wet weight plant material per litre of bioreactor culture under sterile conditions. The sampling tube is located at the same level as the stirrer, thus ensuring uniform sampling while stirring. Small sample quantities (< 100 ml) are taken using a sterile syringe via a Luer lock connection, while large sample volumes are removed using, for example, a peristaltic pump type 302/3A equipped with head 501 RI (Sartorius, Göttingen).

Chloronema cultures of the wild type are generated by supplementing the Knop medium with 5 mM ammonium tartrate.

The yield of biologically active heterologous protein which is secreted can be increased markedly when the stabilizer polyvinylpyrrolidone (PVP) is present in the culture medium.

Differentiation of the caulonema, which takes place during protonema development, can be induced and increased by exogenous addition of physiological amounts of auxin, suitable concentrations being, for example, 5 µmol/l of indole-3-acetic acid (IAA).

For performing the liquid culture of transformants under selection pressure, 50 mg/l of the antibiotic G418 (Calbiochem, Bad Soden) are added to the Knop medium. To this end, the cultures are removed by filtration over sterile 100 µm sieves (Wilson Sieves, Nottingham, England) every ten days immediately

prior to comminution and transferred into Erlenmeyer flasks filled with selection medium.

For the nutrient element experiments, the Knop medium is diluted 1:10 with H_2O.

Transformation

The chosen transformation method is the PEG-mediated direct DNA transfer into protoplasts as described by Reutter and Reski (loc. cit., 1996). 50 µg of plasmid DNA is employed for $3x10^5$ protoplasts in each transformation. Protoplast regeneration and selection for stable transformants is carried out as described by Reutter and Reski (loc. cit., 1996), unless otherwise specified.

Indirect immunofluorescence

Buffer: MSB: 100 mM PIPES; 5-10 mM EGTA, 5 mM $MgSO_4$, pH 6.8
F-MSB: MSB + 5% DMSO
E-MSB: MSB + 5% DMSO + 5% Nonidet
W-MSB: MSB diluted 1:2 with H_2O (wash buffer)

Enzyme solution: 1% cellulase, 1% pectinase, 2% Driselase in MSB, pH 5.6 (all from Sigma, Deisenhofen)

The moss protonemata are fixed in 1.25% glutaraldehyde in F-MSB (v/v) by incubation for no longer than 10 minutes and briefly washed in W-MSB. Then, the protonemata are incubated with 2% paraformaldehyde in MSB (v/v) for 40 minutes and washed 3x with W-MSB (rinse 1x; wash 2x for 5 min).

Free aldehydes which cannot be eliminated by washing are reduced by adding MSB and a spatula-tipful of solid boron hydride, with an incubation time of 10 minutes. The boron hydride is removed by three washes with W-MSB.

In the next step, the cell walls are made permeable by adding the enzyme solution for 10 minutes. The enzymatic reaction is quenched by changing the pH (MSB, pH 6.8). Again, the mixture is washed 3x with W-MSB.

Chlorophylls are extracted by incubation with a detergent solution over a period of 120 minutes. The solution is then removed by three washes with W-MSB.

After this preparation, the moss protonemata can be incubated with the primary antibody (anti-VEGF; dilution 1/50). This is done at 37°C for 45 minutes. After three washes with W-MSB, the labeled secondary antibody (anti-rabbit or anti-mouse; dilution 1/30; labeled with fluorescein isothiocyanate (FITC) (Molecular Probes, Leiden, Netherlands)) is added for 45 minutes at 37°C. In addition to the 3 wash steps as above, the mixture is washed once with W-MSB + 0.1% Triton. The protonemata are subsequently taken up in W-MSB and stored at 4°C at least overnight.

The material is evaluated with the aid of a confocal laser scanning microscope (CLSM) type TCS 4D (Leica Lasertechnik, Heidelberg) and the software Scanware 5.0 (Leica Lasertechnik, Heidelberg).

To analyze the samples under the CLSM, they are placed on a slide into the mounting solution (Dabco, Sigma, Deisenhofen). Excitation of the fluorescent dye FITC coupled to the secondary antibody is carried out with the aid of an argon-krypton laser at a wavelength of 488 nm. FITC emits the light at a wavelength of 528 nm.

ELISA assay

The VEGF protein in the culture medium, which is formed in suitably transformed moss plants, is determined qualitatively and quantitatively by conventional methods using ELISA assays

and the above-described antibodies. An amount of 200 µl of culture medium is used directly in the ELISA assay.

Functionality assays

The biological activity of the recombinantly formed VEGF obtained from the culture medium is checked in a mitogenic assay (Miyazono et al., Purification and properties of an endothelial cell growth factor from human platelets, J. Biol. Chem., **262**, pp. 4098-4103 (1987)) and in a 'Day-13 chorioallantoic-membrane angiogenesis assay' (Wilting et al., A morphological study of the rabbit corneal assay, Anat. Embryol., **183**, pp. 1167-1174 (1991)). The culture medium is subjected beforehand to ultrafiltration, then lyophilized and subsequently resuspended in buffer. If desired, a further purification step using a cation column may be carried out.

Induction of the 1'-promoter

The inducibility of the 1'-promoter by auxin is assayed with 5 µmol/l of indole-3-acetic acid (IAA). Five days after transformation, 100 µl protoplast aliquots of a transformation reaction with pNA201 are transferred into the wells of a 96-well microtiter plate (Nunc, Wiesbaden). The protoplast suspensions are incubated for five hours with IAA (end concentration = 5 µM). The induction experiments are evaluated directly after the incubation period with the aid of the qualitative β-glucuronidase assay.

Qualitative β-glucuronidase assay

The β-glucuronidase activity is determined in a qualitative assay (A.R. Jefferson, Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol. Rep., **5**, pp. 387-405 (1987)).

Substrate buffer:	50 mM $K_3Fe(CN)_6$	50 mM Na_2HPO_4
	50 mM $K_4Fe(CN)_6$	1% (v/v) Triton X-100
	50 mM NaH_2PO_4	10 mM EDTA, pH 7.0
	4 mg/ml PVP (MW 10 000)	

Staining solution: 12.5 mg of 5-bromo-4-chloro-3-indolylglucuronic acid (Biomol, Hamburg) dissolved in 250 µl of N,N-dimethylformamide/50 ml of substrate buffer

Moss protonemata and moss protoplasts in Knop or regeneration medium are incubated for up to 72 hours at 37°C in an equal volume of staining solution and evaluated immediately thereafter using a microscope.

Results

Homogeneity of the bioreactor culture; sampling

Only homogeneous cultures ensure that sampling from cell cultures is standardized. After prolonged culture, protonema growth into long cell filaments frequently leads to cell aggregates and thus to inhomogeneous distribution of the plant material in the liquid cultures. To avoid such aggregation, the protonemata are comminuted at specific intervals - in the bioreactor every other day from day 10 and in shake culture every 12 days - by using high-speed stirrers/homogenizers. To make possible continuous conditions in the bioreactor while simultaneously standardizing sampling even over a prolonged culture period, it is recommended to modify a turbine stirrer with three stirrer blades by grinding the edges of the stirrer blades, thus transforming them into shear blades. Constant "stirring" at 300-500 rpm thus makes it possible to operate homogeneous bioreactor cultures.

Biomass development (in DW [mg/l]) over a period of 35 days (840 h) at 500 rpm in the control cultures with the turbine stirrer is the same as in bioreactor cultures stirred with the shear-blade stirrer.

Culture homogeneity is assessed by comparing in each case six parallel samples. The dry weight of plant material from a sample

volume of 100 ml is determined as the comparison parameter. When using an unmodified turbine stirrer, the standard deviation increases with increasing biomass concentration. As a consequence of stirring with the shear-blade stirrer, the standard deviations of the samples taken remain low. This allows the conclusion that a uniformly homogeneous culture can be obtained with the modified stirrer over a period of 35 days.

Studies into the inducibility of the 1'-promoter

In the plasmid pNA201, the 1'-promoter is positioned upstream of the *gus* gene and acts as control element. The known β-glucuronidase assay is suitable as detection assay for induction experiments with the moss. Experiments with transgenic tobacco show that the 1'-promoter leads to expression of β-glucuronidase in tissue with a high auxin content, and it is therefore assumed that this promoter is auxin-dependent. The inducibility of the 1'-promoter by auxin in *Physcomitrella patens* is studied in transiently transformed protoplasts.

The transformation reactions are subjected to the β-glucuronidase assay with (5 h) and without incubation with 5 µM indole-3-acetic acid (IAA). In the controls without addition of IAA, evaluation under the microscope reveals no blue protoplasts in any of the reactions. In contrast, the evaluation of the protoplasts incubated with auxin confirms the expression of the *gus* gene. Based on the blue protoplasts, a transformation rate of $3x10^{-4}$ is achieved. This is a clear suggestion that the 1'-promoter is inducible by the plant hormone auxin in transiently transformed moss protoplasts.

Generation of the vectors for the VEGF transformations

To transform the cDNA of $VEGF_{121}$ without leader sequence, termed VEGFC hereinbelow, and the cDNA of $VEGF_{121}$ with leader sequence, termed VEGFP hereinbelow, into *Physcomitrella*, it is necessary to clone the sequences between a promoter/terminator unit which is suitable for plants. The 35S CaMV promoter and the

corresponding polyadenylation signal are chosen for this purpose. The suitably prepared VEGF cDNA sequences are cloned into the *Sma* I restriction cleavage site of the multiple cloning site of vector pRT101.

The resulting vectors (pRT101VEGFC 3 and VEGFP 21) are sequenced with a primer derived from the terminal region of the 35S promoter, and the correct integration between promoter and polyadenylation site is verified.

The resulting cassettes are excized with the restriction enzyme *Hin* dIII and cloned into the actual transformation vector pRT99 into the *Hin* dIII cleavage site (pRT99VEGFC 3 and VEGFP 21). The orientation of the cassettes relative to the NPTII cassette can be determined by restriction with *Sma* I and *Hinc* II. In the case of promoter-to-polyadenylation signal orientation, a 5250 (VEGFC) and a 5380 bp (VEGFP) fragment are obtained, while the reverse orientation gives a 1100 (VEGFC)/1230 (VEGFP) and a 4150 bp (VEGFC and P) fragment. The restriction analyses reveal only a 5250/5380 bp fragment, and the VEGFC/P cassettes have thus been incorporated in promoter-to-polyadenylation signal orientation relative to the *nptII* gene of pRT99.

VEGFC transformations into *Physcomitrella*

The absolute transformation rate for the transformation of the VEGFC construct in wild-type protoplasts after repeatedly changing from Knop medium to selection medium is 0.5×10^{-5}, with constant stability of the transformants.

Demonstration of the integration of the transformed plasmid

Successful integration into the plant genome is demonstrated by Southern hybridization.

Probes used are firstly an *Nco* I fragment of the *npt* II gene from pRT99 and secondly an *Nde* I/*Sal* I VEGFC fragment from pCYTEXP-$VEGF_{121}$.

The signals detected in the uncleaved total DNA of the transformants confirm successful integration of the plasmid DNA into the plant genome. Whether all of the 35S VEGFC PolyA cassette is obtained even after integration is tested by restriction with *Hin* dIII. If the cassette has remained intact upon integration, this restriction enzyme excizes a 1100 bp fragment from the total DNA.

The hybridization pattern with the VEGFC probe reveals a 1100 bp fragment for all transformants. The integration of the complete VEGFC expression unit, which is a prerequisite for the correct transcription and expression of $VEGF_{121}$ in the moss, has thus been demonstrated.

Demonstration of the transcription of the heterologous genes

The transcripts of the heterologous genes VEGFC and NPTII of the transformants are detected with the nonradioactive DIG detection system using the VEGF and the NPT II probes. With 760 nucleotides for the VEGFC transcript and 1100 nucleotides for the NPT II transcript, the sizes of the transcripts detected in the fluorogram are within the order of magnitude expected for each case. As expected, none of the two heterologous transcripts are detected in the WT control.

Analysis of the transformants with human transit peptide

The PEG-mediated DNA transfer of 50 µg of pRT99P 21 plasmid DNA per transformation reaction generates transformants which are permanently stable on selection medium. A stable transformation rate of 3.3×10^{-6} results.

Demonstration of the integration of the transformed plasmid

Integration of the above-described transformants with transit peptide is demonstrated as described above by Southern hybridization using the described probes, and hybridization of *Hin* dIII-cleaved total DNA with the VEGF probe reveals a 1230 bp

fragment: demonstration of the completeness of the integrated 35S VEGFP PolyA cassette.

Demonstration of the transcription of the heterologous genes

Both NPTII and VEGFP transcripts can be detected by the method outlined above.

Detection of human $VEGF_{121}$ in transgenic moss cells using the confocal laser scanning microscope

In this method, the test protein is labeled directly in mounted cells. The evaluation is done under the confocal laser scanning microscope, which has an improved resolution power compared with a normal light microscope.

The recombinant $VEGF_{121}$ protein - if detectable in the moss cells - should accumulate in the cytoplasm in VEGFC transformants. In the VEGFP transformants, it should be detectable in the ER system if the transit peptide is functional as signal in the moss.

The expression of human $VEGF_{121}$ in transgenic moss cells has been demonstrated successfully with the indirect immunofluorescence method and a computer-aided evaluation with the confocal laser scanning microscope. In addition, it is demonstrated that, in transgenic moss, $VEGF_{121}$ is successfully transported into the endoplasmic reticulum in the presence of the corresponding human transit peptide.

Assay for the presence of VEGF in the culture medium

A 200 µl aliquot of the culture medium in the presence of PVP is assayed by ELISA and shows that the moss plants transformed with the expression cassette including transit peptide encoding sequence are capable of releasing VEGF into the medium. The positive results allow the conclusion to be drawn that a functional VEGF protein is present.

Assay of the biological activity

Both assays employed for verifying the biological activity of the VEGF protein released into the culture medium give positive results and confirm that VEGF produced in accordance with the invention can be obtained from the culture medium with the desired biological activity.

Patent Claims

1. A method for the production of heterologous proteinaceous substances in plant material, characterized in that protonema moss tissue is used as plant material and that the proteinaceous substances produced are obtained from the culture medium without disrupting the producing tissues or cells.

2. The method according to claim 1, characterized in that the proteinaceous substance released into the culture medium is biologically active.

3. The method according to claim 1 or 2, characterized in that a culture medium is used which is free from sugars, vitamins and phytohormones or functional fragments thereof.

4. The method according to any of claims 1 to 3, characterized in that the moss tissue is selected from the group of the mosses including liverworts.

5. The method according to claim 4, characterized in that the moss tissue is selected from mosses of the group consisting of *Physcomitrella*, *Funaria*, *Sphagnum* and *Ceratodon*.

6. The method according to claim 4, characterized in that the moss tissue is selected from liverworts of the group consisting of *Marchantia* and *Sphaerocarpos*.