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A fast and flexible PEG-mediated transient expression system in plants for high level expression of secreted recombinant proteins

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Abstract

Plant expression systems offer a valuable alternative to traditional systems for the production of recombinant biopharmaceuticals. A highly efficient polyethyleneglycol (PEG)-mediated transient expression system for secreted recombinant proteins in plants has been developed. The human vascular endothelial growth factor 121 (rhVEGF) has been successfully expressed and efficiently secreted into the culture medium by transiently transformed moss protoplasts. In order to obtain secretion efficiency data, different expressed signal peptides were analysed and time course studies were performed with expression constructs containing different promoters. The transformation procedure was optimised for high level expression (up to 10 μg/ml) and successfully performed even with a transgenic glyco-engineered strain lacking plant-specific immunogenic sugar residues in *N*-glycans. The amount of rhVEGF was produced in such quantity that it allowed for the analysis of biological activity, silver-staining and Western blotting, revealing the correct formation and processing of the homodimer. This fast and flexible transient expression system enables feasibility studies and construct optimisation to be concluded within a few days, thus avoiding the time consuming step of having to generate stably transformed lines.

Keywords: Transient expression; PEG-mediated transformation; Signal peptide; Recombinant human vascular endothelial growth factor; *Physcomitrella patens*

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GUS, beta-glucuronidase; HUVEC, human umbilical vein endothelial cells; PEG, polyethyleneglycol; rhVEGF, recombinant human vascular endothelial growth factor; RELIDA, receptor ligand detection assay; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; SP, signal peptide; wt, wild type; Δxyl-t/Δfuc-t, Δxyl-t/Δfuc-t double knockout strain of *Physcomitrella patens*

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1. Introduction

In recent years, plant-based expression systems have become an attractive alternative to conventional non-plant-based expression systems for the production of recombinant biopharmaceuticals. In addition to advanced safety aspects, they offer benefits relating to cost and commercial scale-up. It has been shown several times that plants are capable of expressing complex proteins. Different approaches utilising plant expression systems use diverse strategies to optimise product yield (for reviews see Fischer et al., 2004; Horn et al., 2004; Ma et al., 2003).

For large-scale production, the generation of stable transgenic lines is the method of choice. To achieve important information about the optimal expression of a target protein, transient gene expression has several advantages over so-called stably incorporated gene expression systems. No selection procedure is required to identify transformed cells since the cells are used only for temporary (i.e. transient) protein production. Such transient gene expression systems allow for the rapid expression and analysis of a recombinant protein within weeks and they have the further advantage of being suitable for verifying the functionality of a gene expression construct.

The production of sufficient amounts of the target protein enables confirmation of product quality to be carried out, for example, in the processing of the primary polypeptide chain, and in any additional post-translational modifications, such as glycosylation (Mokrzycki-Issartel et al., 2003).

Additionally, expression constructs may be compared at the molecular level by employing different regulatory sequences and targeting peptides that may influence product yield and product quality.

Different methods have been used in transient expression systems in plants. Infiltration of cells with recombinant *Agrobacterium* can be performed on a small scale and also in suspension culture (Fuentes et al., 2004). Alternative methodologies that have been reported for transient expression in plants include the use of modified viral vectors for plant infection (Fischer et al., 1999; Porta and Lomonossoff, 2002). Both the above-mentioned systems are limited to species suitable for such types of DNA delivery. Transfer methods using naked DNA, such as biolistic transformation are generally not thought to be efficient enough for expression of recombinant target proteins (Fischer et al., 1999). As a consequence, biolistic methods and polyethyleneglycol (PEG)-mediated DNA transfer are limited to studies with reporter genes, such as beta-glucuronidase (*gusA*) and luciferase (*luc*) (Basu et al., 2004; Godon et al., 1993).

Mosses, such as *Physcomitrella patens* are discussed as an attractive alternative to existing mammalian systems for the production of biopharmaceuticals (Decker and Reski, 2004). The moss bioreactor is based on the secretion of the target protein in bioreactor cultures of the filamentous moss tissue, the protonema. The moss *Physcomitrella patens* is the only known plant today that shows a high frequency of homologous recombination that can be used for the construction of transgenic knockout plants (Hohe and Reski, 2003; Schaefer and Zyrd, 1997). Recently, gene disruption by homologous recombination was used to design a double knockout production strain that lacks the two plant-specific sugar residues, fucose and xylose (Koprivova et al., 2004); these sugar residues have the potential to cause allergic reactions (Garcia-Casado et al., 1996).

The above notwithstanding, transient expression in mosses can only be performed by transfer of naked DNA, since moss tissue is not suitable for viral infiltration or *Agrobacterium* transfection. Furthermore, biolistic DNA transfer has been reported as resulting in very low transformation efficiencies (Sawahel et al., 1992). In contrast, it has been demonstrated that in the case of PEG-mediated transient transformation in *Physcomitrella*, the transformation efficiency relating to surviving protoplasts has been measured at about 10–20% (Schaefer, 2002). This PEG-system has been used inter alia to characterise promoters by measuring GUS or luciferase intracellulary (Zeidler et al., 1999; Horstmann et al., 2004).

In this study, we describe the optimisation of a novel PEG-mediated transient expression system based on the secretion of the target protein into the medium. We used the human vascular endothelial growth factor 121 (hVEGF), a small homodimeric glycoprotein of 28 kDa, which induces proliferation of endothelial cells and therefore plays an important role in angiogenesis (Ferrara and Davis-Smyth, 1997). This method was used recently for the characterisation of promoters (Jost et al., 2005; Weise et al., submitted for publication) and analysis of the secretion capacity of transgenic plants

in which the glycosylation pattern had been modified (Koprivova et al., 2004).

We have altered the conditions under which the transient expression system is employed by optimising the numbers of protoplasts required and the amount of medium volume to achieve higher yields of the secreted recombinant protein.

2. Experimental procedure

2.1. Plant material

Wild type strain and Δxyl-t/Δfuc-t double knock-out transgenic line (Koprivova et al., 2004) of *Physcomitrella patens* (*Hedw.*) *B.S.G.* were cultivated in a bioreactor as described by Hohe and Reski (2002) for the production of protoplasts.

2.2. PEG-mediated transient transformation of *Physcomitrella* protoplasts

Pre-cultivation and isolation of protoplasts were performed as described by Hohe and Reski (2002). Transformation of the protoplasts was carried out as described previously by Jost et al. (2005) using 30 μg of DNA per transformation.

For medium experiments protoplasts were re-suspended in 3 M medium (15 mM $MgCl_2 \cdot 7H_2O$, 0.1% MES, 0.48 M mannitol, pH 5.6, 540 mOsm), W5 medium (125 mM $CaCl_2 \cdot H_2O$, 137 mM NaCl, 5.5 mM glucose, 10 mM KCl, pH 5.6, 660–680 mOsm) or MMS medium (3 M medium containing sorbitol instead of mannitol). For regeneration, protoplasts were re-suspended in regeneration medium (Knop medium: 1000 mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 250 mg/l KCl, 250 mg/l KH_2PO_4, 250 mg/l $MgSO_4 \cdot 7H_2O$ and 12.5 mg/l $FeSO_4 \cdot 7H_2O$; pH 5.8, supplemented with 5% glucose and 3% mannitol. The pH was adjusted to 5.6–5.8 and osmolarity to ∼540 mOsm) after transformation.

For analysis of intracellular recombinant human vascular endothelial growth factor (rhVEGF), protoplasts were sedimented by centrifugation (1 min at 16,060 × *g*) and remaining supernatant was discarded. Protoplasts were re-suspended in 100 μl of MESII buffer [10 mM MES/NaOH pH 6.2; 10 mM EDTA; 10 mM $MgCl_2$; 2 mM Pefabloc SC-protease inhibitor (Roth, Germany); 0.1% NP-40 (Roche, Germany)] and disrupted with an ultrasonic device (Bandelin, Germany). Cell debris was sedimented by centrifugation (1 min at 16,060 × *g*) and supernatant was further analysed by VEGF ELISA.

2.3. Microscopy and protoplast analysis

Protoplasts were analysed using an Axiovert 200 microscope (Zeiss, Germany). Protoplasts were stained with cellulose specific Fluorescent Brightener 28 (Sigma, Germany, 5 μg/ml in H_2O, diluted in a protoplast sample 1:2, incubation for 5 min at room temperature) and visualised by fluorescence using filter set 02 (excitation: G 365 nm, emission: LP 420 nm) after 72 h.

2.4. Construction of expression vectors

The plasmid pRT101VEGF_C3 (Gorr, 1999) containing the cDNA for human VEGF without signal peptide (SP) was used as a starting point (we thank H. Weich for kindly providing the cDNA for hVEGF). The human VEGF signal peptide was amplified from pRT101VEGF_P21 (Gorr, 1999) with the primers MOB_323 (5′-ATACTCGAGGAAGATGAACTTTCTGCTGTCTTGG-3′) and MOB_349 (5′-CTGCCATGGGTGCAGCCTGGGACCAC-3′) and cut with *Xho*I/*Nco*I for ligation into pRT101VEGF_C3 resulting in pRT101TP_VEGF_C3.

The plant signal peptide of the thaumatin-like protein To32 (GenBank accession no. AY95850) from *Thuja occidentalis* (SPTo32) was amplified from plasmid pTo32 using primers 5′ MOB_249: ATACTCGAGGAAGATGGCCACAGTTTC and 3′ MOB_250: CTGCCATGGGTGCTGCTCCCGCCTCT. Using the restriction sites *Xho*I and *Nco*I, it was ligated into pRT101_C3_VEGF resulting in pRT101TPTo32_VEGF_C3. Amplification with primers 5′ MOB_249 and 3′ MOB_324: CTGCCATGGGTGCTCCCGCCTCTTGG resulted in the deletion construct pRT101TP-1To32_VEGF_C3.

The plant signal peptide of the thaumatin-like protein To34 (GenBank accession no. AY796073 from *Thuja occidentalis* (SPTo34) was amplified from plasmid pTo34 using primers 5′ MOB_260: ATACTCGAGGAAGATGGTATCCCAAAAAG and 3′ MOB_248: CTGCCATGGGTGCACCATCTGCAT-

GGTTTATGA G. Using the restriction sites *Xho*I and *Nco*I, it was ligated into pRT101_C3_VEGF resulting in pRT101TPTo34_VEGF_C3. Amplification with primers 5′ MOB_260 and 3′ MOB_372: CTGCCATGGGTGCATCTGCATGGTTTATGAG resulted in the deletion construct pRT101TP-1To34_VEGF_C3.

The plant signal peptide of the thaumatin-like protein ToH1 (GenBank accession no. AY795849) from *Thuja occidentalis* (SPToH1) was amplified from plasmid pToH1 using primers 5′ MOB_251: ATACTCGAGGAAGATGGCTTTCTATAAG and 3′ MOB_252: CTGCCATGGGTGCAGCATAGGAGTGG. Using the restriction sites *Xho*I and *Nco*I, it was ligated into pRT101_C3_VEGF resulting in pRT101TPToH1_VEGF_C3. Amplification with primers 5′ MOB_251 and 3′ MOB_286: CTGCCATGGTGCATAGGAGTGG resulted in the deletion construct pRT101TP-1ToH1_VEGF_C3.

Construct 5′ PpAct5::Vegf (Weise et al., submitted for publication) uses the 5′ region of the *Physcomitrella patens* actin5 gene (GenBank accession no. AY745190) to control the expression of rhVEGF.

2.5. ELISA analysis of recombinant VEGF

Intra- and extra-cellular rhVEGF samples from transient transformation experiments were analysed by sandwich enzyme-linked immunosorbent assay (ELISA) (anti-hVEGF, capture, #AF-293-NA, anti-hVEGF, detection, #BAF-293, $rhVEGF_{121,}$ standard, #298-VS, R&D, Germany; Nunc-Immunosorb plates, Denmark). Dilutions were made in 1× PBS/0.1% BSA (Serva, Germany). All samples were measured in duplicates.

2.6. RELIDA analysis of recombinant VEGF

Biological activity of transiently expressed rhVEGF was determined by VEGF-specific receptor ligand detection assay (RELIDA) (RELIATech, Germany) according to the manufacturer's instructions. All dilutions were made in 1× PBS containing 0.1% BSA (Serva, Germany). The assay recognises only free, uncomplexed and biologically active forms of VEGF that are not sequestered by soluble receptors.

2.7. SDS-PAGE and Western blot analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under non-reducing conditions according to Laemmli (1970). Western blot was carried out using a reinforced Optitran Nitrocellulose Membrane (Schleicher & Schuell, Germany) and the semi-dry transfer method with Nova Blot (Pharmacia Biotech, Germany). Anti-human VEGF (AF-293-NA, R&D, Germany, 1:500), anti-goat IgG (A5420, Sigma, USA, 1:8000) and $rhVEGF_{121}$ (298-VS, R&D, Germany) were used. Dilutions were made in 1× HS-TBST with 2.5% skimmed milk powder. ECL Advance Western Blotting Detection Kit (Amersham, Germany) was used for visualisation. Supernatant samples were directly separated on SDS-PAGE without concentration or purification.

2.8. Silver-staining of rhVEGF

Following protein separation on a 15% SDS-PAGE, gels were silver-stained using SilverQuest Silver-staining Kit (Invitrogen, Germany) according to the manufacturer's instructions.

2.9. Cell culture and HUVEC proliferation assay

Human umbilical vein endothelial cells (HUVEC) were purchased from Oligene (Berlin, Germany, Lot No. 077V190901) and cultivated in endothelial cell growth medium/FCS (Oligene, Berlin, Germany), supplemented with 100 μg/ml streptomycin and 100 IU/ml penicillin (Biochrom AG, Berlin, Germany), according to the manufacturer's protocol. For proliferation experiments endothelial cell growth medium/basal (Oligene, Berlin, Germany), supplemented with 1 μg/ml hydrocortisone, 50 ng/ml amphotericin, 50 μg/ml gentamicin, 100 μg/ml streptomycin, 100 IU/ml penicillin and 2% FCS (further on named BMF) was used. For the determination of proliferation, the cells were seeded into 96-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 5000 cells/cm^2. After an incubation of 24 h in endothelial cell growth medium/FCS, the supernatant was removed by gentle tapping and the cells were treated with 10 ng/ml transient VEGF in BMF. As a control BMF supplemented with the respective volume of sample vehicle (3 M medium) was used. After fur-

ther incubation for 48 h the cell proliferation was determined using the CellTiter® AQ_{ueous} One Solution Cell Proliferation Assay (Promega GmbH, Mannheim, Germany), according to the manufacturer's protocol. Measurement of the resulting formazan product was performed with a MRX microplate reader (Dynatech Deutschland GmbH, Denkendorf, Germany) at a wavelength of 490 nm.

3. Results

3.1. Protoplast cultivation and medium experiments

The long-term cultivation and regeneration ability of wild type (wt) *Physcomitrella* protoplasts was examined. Protoplasts of mosses that were cultivated in 3 M medium or W5 medium were viable over a period of from 2 to 6 months. Under such conditions they were not able to regenerate cell walls or to divide (Fig. 1a and b). However, transfer of 2-month-old protoplasts into regeneration medium resulted in regeneration into protonema. Regeneration of the cell wall 72 h after transfer was verified by using specific dyes for cellulose (Fluorescent Brightener 28) and visualised by microscopy (Fig. 1c–d).

In order to analyse the influence of different culture conditions on transient expression and the secretion of rhVEGF, wild type protoplasts were transformed with expression vector pRT101TPVEGF_C3. Expression of rhVEGF is driven by the 35S CaMV promoter. Protoplasts were cultivated in various media after transformation and heterologous protein levels were quantified by ELISA. Calculation of secretion rates of rhVEGF over 96 h in the standard 3 M medium was set to 100%. MMS medium that included sorbitol instead of mannitol in 3 M medium resulted in yields of 93%. Slightly

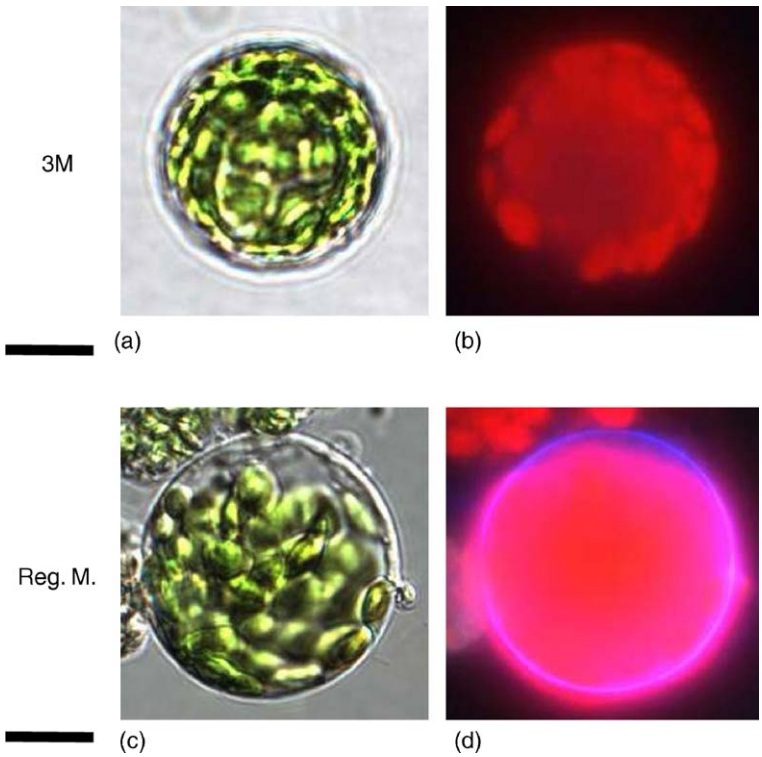

Fig. 1. Microscopic analysis of isolated wild type protoplasts. (a) Brightfield picture of a 2-month-old protoplast cultivated in 3 M medium (3 M). (b) Fluorescence picture of corresponding protoplast treated with Fluorescent Brightener 28 (cellulose-specific). (c) Brightfield picture of a 2-month-old protoplast transferred to regeneration medium (reg. m.) after 72 h. (d) The same protoplast under fluorescence light, treated with Fluorescence Brightener. Scale bars correspond to 10 μm.

higher yields (127%) were obtained with W5 medium that contained a low glucose concentration instead of the high mannitol concentration of 3 M medium.

3.2. Signal peptide analysis for secretion of rhVEGF

The efficiency of different signal peptides was tested for secretion of the target protein VEGF. Native human and plant-derived signal peptides were analysed in transient transformation experiments. In addition, deletion constructs missing the last amino acid of the signal peptides, which are located at the cleavage site were constructed to investigate the influence of this modification on secretion of the target protein. Secreted rhVEGF in the supernatant (Fig. 2a) and intracellular rhVEGF (Fig. 2b) were quantified by ELISA.

The control expression vector without a signal peptide (pRT101_VEGF_C3) showed almost no rhVEGF in the supernatant and very little intracellular rhVEGF. The construct that used the native human signal peptide showed rhVEGF levels of 25 ± 3 ng/ml in the supernatant fluid and very low intracellular rhVEGF levels. The secretion level achieved by the deletion construct was reduced to 2% in the supernatant and to one-third intracellularly. Signal peptide To32 reached the highest level of 50 ± 5 ng/ml in the supernatant. A reduction to 8.6% was observed in the case of the corresponding deletion construct. The signal peptide of To34 allowed the secretion of only 7.5 ± 1.02 ng/ml into the medium and that of the deletion construct was reduced to 5%. A yield of 40 ± 6 ng/ml was obtained with signal peptide ToH1. The corresponding deletion construct reached only 12.5% of that of the intact construct. Intracellular rhVEGF levels for all deletion constructs were also reduced.

3.3. Time course of transiently secreted rhVEGF using two different promoters

To examine the feasibility of time course studies in the PEG-mediated transient transformation described here, two different promoters that regulate the expression of rhVEGF were examined. Supernatants were sampled every 24 h over a time period of 120 h. Secreted rhVEGF in the supernatants was determined by ELISA (Fig. 3). When transferred to 48-well plates protoplasts quickly sedimented on the bottom of the wells and were not disrupted by the sampling procedure which was reviewed microscopically.

The first vector, pRT101TPVEGF_C3 expressed rhVEGF under control of the 35S CaMV promoter

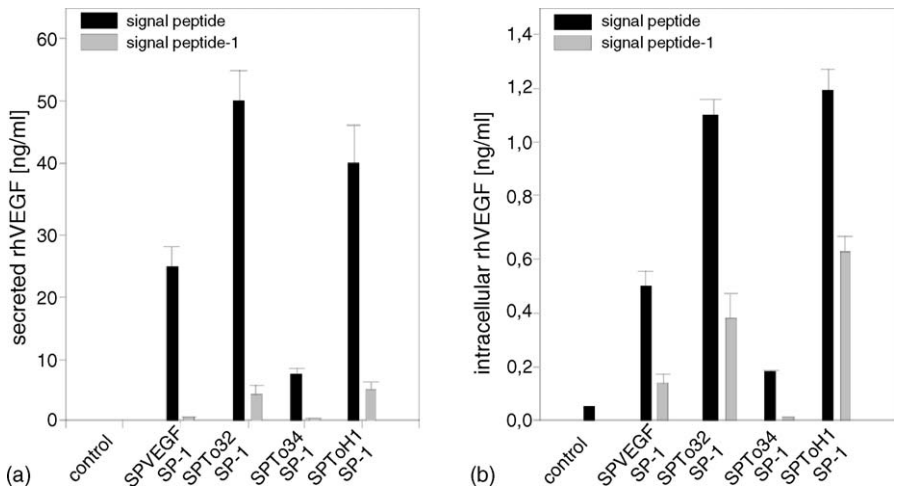

Fig. 2. Quantification of transiently expressed rhVEGF in wild type protoplasts by ELISA using different signal peptides for secretion. (a) ELISA analysis of secreted rhVEGF in the supernatant (3 M medium) after 72 h. (b) ELISA analysis of intracellular rhVEGF from disrupted protoplasts after 72 h. Control: expression vector pRT101_VEGF_C3 containing the cDNA for VEGF without signal peptide; SPVEGF: native human signal peptide of hVEGF; SPTo32: signal peptide of plant-derived To32 protein from *Thuja occidentalis*; SPTo34: signal peptide of plant-derived To34 protein from *Thuja occidentalis*; SPToH1: signal peptide of plant-derived ToH1 protein from *Thuja occidentalis*; SP-1: corresponding "deletion construct" of each signal peptide missing the last amino acid before the cleavage site.

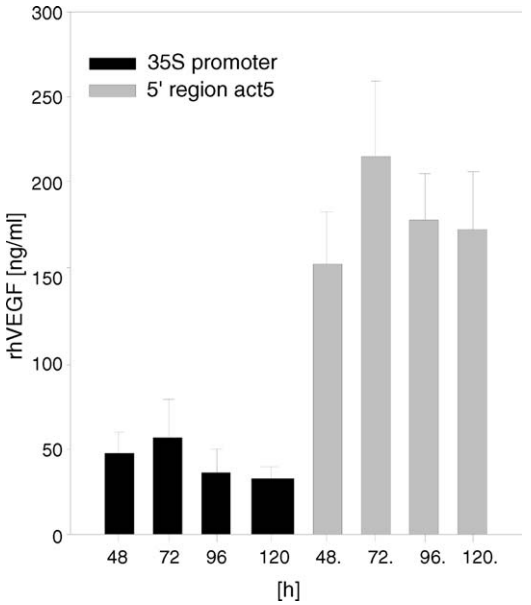

Fig. 3. Time course of promotor analysis in wild type protoplasts. Two expression vectors were analysed over a period of 120 h in 3 M medium. The first vector (pRT101TPVEGF_C3) expressed rhVEGF under control of the 35S CaMV promoter (35S). The second construct 5′ PpAct5::Vegf uses the 5′ region of *Physcomitrella patens* actin5 gene (5′ region act5) as regulatory sequence. Samples of 200 μl were collected after 48, 72, 96 and 120 h and quantified by VEGF ELISA. The removed volume was replaced by the same volume of fresh 3 M medium.

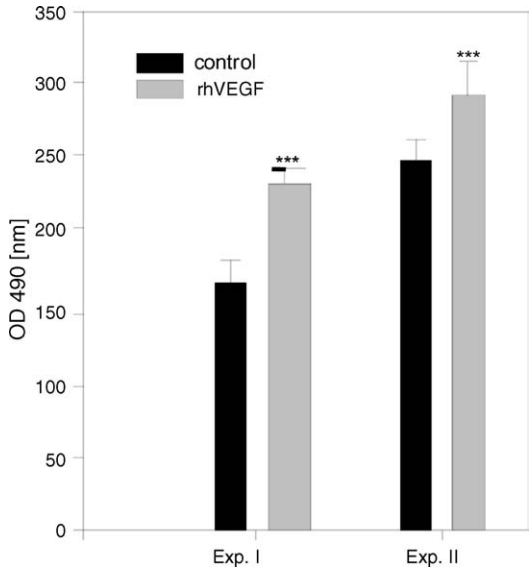

Fig. 4. Determination of the biological activity of transiently expressed rhVEGF in HUVEC proliferation assays. After incubation of the human umbilical vein endothelial cells (HUVEC) for 24 h in growth medium, the supernatant was removed and replaced with either 10 ng/ml transiently expressed rhVEGF in BMF medium (rhVEGF) or BMF medium supplemented with the respective volume of 3 M medium (control). Two independend experiments (Exp. I + II), with six values each are displayed. Formazan production of the cell proliferation assay was determined after 48 h at a wavelength of 490 nm.

(35S) and started with 47.5 ± 13 ng/ml after 48 h and reached its highest level of 57 ± 22 ng/ml after 72 h. After 96 h, a level of 36.8 ± 12.9 ng/ml was obtained that dropped slightly to 32.6 ± 7.2 ng/ml after 120 h. The second vector, 5′ PpAct5::Vegf uses the 5′ region of *Physcomitrella patens* actin5 gene to drive expression and showed an rhVEGF level of 151 ± 31 ng/ml after 48 h that rose to a maximum of 215 ± 44 ng/ml after 72 h. After 96 and 120 h, it was almost unchanged at a level of 177 ± 27 and 172 ± 33 ng/ml, respectively. Despite differences in target protein yields, the time course for the two promoters was similar over 120 h and allowed a non-invasive examination of the target protein.

3.4. HUVEC proliferation assay with rhVEGF expressed in wild type protoplasts

Biological activity of moss-derived rhVEGF was examined in a proliferation assay. Human umbilical vein endothelial cells (HUVEC) were used as specific target cells to analyse the influence on proliferation of the moss-derived growth factor. Two independent experiments with 10 ng/ml rhVEGF with six values each were performed. Moss-derived rhVEGF induced a significantly enhanced proliferation (corresponding to 130%) of HUVEC when compared to the control experiments where the corresponding volume of 3 M medium was applied (Fig. 4).

3.5. Optimised protocol for high yield expression and analysis of the target protein

To increase product yield the protocol was optimised by using 9×10^5 protoplasts per transformation event and 90 μg of expression construct DNA in a maximum volume of 150 μl. Seven hundred and fifty microlitres of PEG was applied to the protoplast suspensions and the incubation time was lengthened to 12 min. Subsequently, the transformation approach was gradually

diluted by adding 1.5, 3 and 6 ml of 3 M medium followed by the final centrifugation step. For transfer to 48-well plates the protoplasts were re-suspended in 400 μl 3 M medium.

Using the optimised protocol, transiently expressed and secreted rhVEGF by wild type and Δxyl-t/Δfuc-t double knockout protoplasts (Koprivova et al., 2004) was quantified by VEGF ELISA. Yields of 1.1–1.27 μg per transformation event were recorded. Maximum concentrations of up to 10 μg/ml were achieved in single experiments. RELIDA technology was used to determine the amount of biologically active rhVEGF in each sample. Quantification by VEGF-specific RELIDA recorded yields of 1.03–1.42 μg per transformation event were in complete agreement with the amount of rhVEGF determined by ELISA (Fig. 5).

In order to analyse whether *Physcomitrella* is capable of correctly processing rhVEGF and secreting

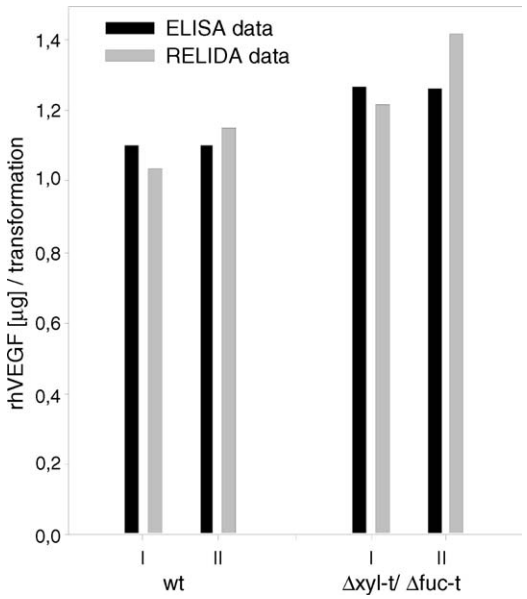

Fig. 5. Analysis of biological activity of rhVEGF by receptor-based VEGF RELIDA. Two independent transformations of wild type (wt) and Δxyl-t/Δfuc-t double knockout (Δxyl-t/Δfuc-t) protoplasts were analysed by VEGF ELISA for quantification of rhVEGF. Identical samples were measured in VEGF RELIDA, a receptor ligand detection assay that recognises only free, uncomplexed and biologically active forms of VEGF that are not sequestered. All samples were measured in duplicates. Intra-assay variation of the ELISA is quoted as 5.9 and 6.7% for the RELIDA according to the manufactures data. Protoplasts were cultivated in 3 M medium and samples were collected after 10 days.

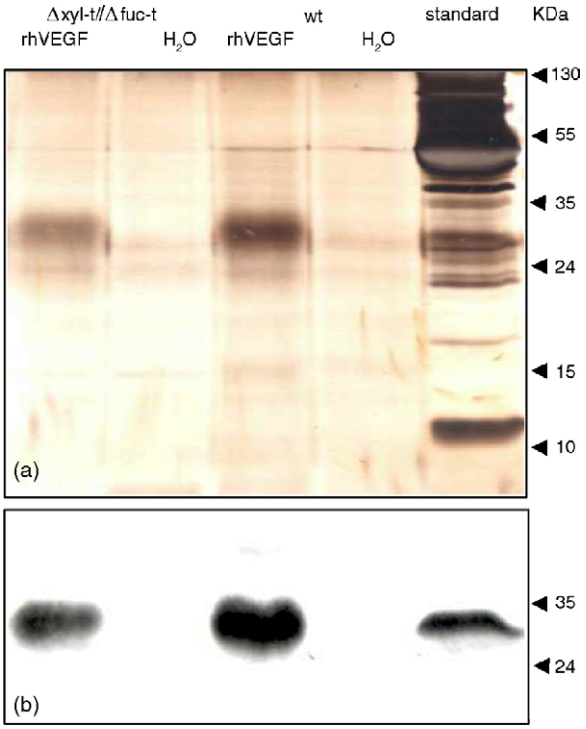

Fig. 6. Silver-staining and Western blot analysis of moss-derived rhVEGF separated on 15% SDS-PAGE under non-reducing conditions. 100 ng of transiently expressed and secreted rhVEGF from wild type (wt) and the Δxyl-t/Δfuc-t double knockout (Δxyl-t/Δfuc-t) protoplasts and the corresponding H_2O controls were visualised on silver-stained SDS-PAGE (a) and identified by VEGF Western blot analysis (b). The respective samples from transient transformation in 3 M medium were directly applied on SDS-PAGE without prior concentration or purification. Purchased standard rhVEGF is resuspended in 1× PBS/0.1% BSA buffer.

the target protein effectively into the medium even under the optimised conditions described here, we transiently transformed wild type and Δxyl-t/Δfuc-t double knockout protoplasts and visualised the target protein by silver-staining and Western blot (Fig. 6). rhVEGF was secreted into the supernatants which were directly applied to and separated on a 15% SDS-PAGE under non-reducing conditions. Silver-staining of the gel (Fig. 6a) visualised a dominant band in the case of the supernatant from wild type and Δxyl-t/Δfuc-t double knockout protoplasts transformed with the VEGF expression vector. Only a slight background containing a few bands could be seen that correspond to the bands of the control transformations.

Western blot analysis (Fig. 6b) demonstrated that rhVEGF is successfully processed by wild type and Δxyl-t/Δfuc-t double knockout protoplasts. Both signals showed the same molecular weight of the processed protein compared to control standard rhVEGF. Dominant bands from the silver-stained gel correspond well with the signals from Western blot analysis identifying correctly processed and secreted rhVEGF by both *Physcomitrella* strains. Moss-derived rhVEGF analysed under reducing conditions showed a double band of about 14 kDa (data not shown) that together with the signal of about 28 kDa under non-reducing conditions confirmed the ability of the moss to process disulfide-linked dimers.

4. Discussion

For large-scale production of recombinant proteins in plants stably transformed strains are required. In contrast, transient expression in plants, including transient expression in mosses, typically has been carried out using only reporter systems, for example, for promoter analysis (McElroy et al., 1990; Horstmann et al., 2004; Zeidler et al., 1999), analysis of protein targeting and trafficking (Schaaf et al., 2004), signal transduction and protein–protein interaction (Sheen, 2001). Recently, transient expression systems were developed for the production of sufficient amounts of recombinant proteins to perform more detailed characterisations using methodologies such as ELISA, SDS-PAGE and Western blot analysis. These expression systems are based on agro-infiltration (Mokrzycki-Issartel et al., 2003; Vaquero et al., 1999) or the use of viral vectors (Marillonnet et al., 2004; Verch et al., 1998) because PEG-mediated or biolistic direct DNA-transfer methods usually resulted in low transformed/untransformed ratios (Fuentes et al., 2004; for review see Fischer et al., 1999). Due to the lack of moss viruses or functional *Agrobacterium* strains (Schaefer, 2002), both the transient expression systems that have been successfully used in seed bearing plants have not been found suitable for mosses which are known to represent an alternative and attractive production system for biopharmaceuticals (Decker and Reski, 2004). Expression of recombinant proteins in stable transgenic moss strains is based on the secretion of the target protein into a simple mineral medium. However, a rapid transient expression method for mosses would be helpful to gather sufficient information about different expression tools for example optimal promoters and targeting signals. Survival rates of *Physcomitrella patens* protoplasts post-PEG-mediated DNA-transfer are described in the literature as being low and typically between 10 and 30% (Schaefer, 2002). Nevertheless, it has been shown recently that transient expression and secretion of rhVEGF could be used for promoter analysis (Jost et al., 2005) as well as for the comparison of the secretion capacities of different moss strains that have been modified in their glycosylation patterns (Koprivova et al., 2004).

In contrast to the low survival rates due to the transformation procedure, surviving moss protoplasts were viable under non-regenerating culture conditions in 3 M medium over a long time period and do not regenerate cell walls or undergo cell division.

As described earlier, moss protoplasts are obtained mainly from chloronema cells, resulting in a highly homogenous expression system with respect to cell type and cell cycle phase (Schween et al., 2003), which is especially important when considering the reliability of the system.

Reporter assays have already been used in transient transformation experiments with seed plant protoplasts for the analysis of signal peptides for protein secretion (De Loose et al., 1991; Denecke et al., 1990). Here, we analysed the impact of different signal peptides on secretion of rhVEGF in transiently transformed moss protoplasts. Two of the plant signal peptides resulted in improved secretion, but one resulted in lower secretion levels compared to the native rhVEGF signal peptide. For the control in our work, we used rhVEGF without signal peptide and rhVEGF in the supernatants was not detected. The drastically decreased secretion levels, achieved with the signal peptides in which the last amino acid was removed, also confirmed the active secretion of the mature rhVEGF into the medium. Moreover, this approach gave important hints as to the proper cleavage of the signal peptides, which may prove useful for the selection of optimal expression tools in stable transgenic strains.

In general, transient expression systems used for promoter studies are based on the analysis of a reporter protein, which is located intracellularly. These systems are limited in direct quantification of the target protein and/or in the possibility to quantify the reporter over

a time course. In contrast, secretion-based transient expression in moss protoplasts allowed time course experiments to be followed over a time interval of at least 120 h even at different expression levels.

Furthermore, investigations with moss-derived rhVEGF in the HUVEC-based proliferation assay confirmed the biological activity of the target protein underlining the potential of the moss to generate functional biopharmaceuticals.

The fact that secretion-based PEG-mediated transient transformation gave excellent results in the studies described in this paper was very promising for further possible improvements in protein yields. As a consequence we scaled up the number of protoplasts, amount of DNA used and volume of PEG-solution. In order to achieve high concentrations of the recombinant protein with transiently transformed protoplasts these were cultured in small volumes at the 48-well scale. High yield protoplast production as a prerequisite for such further optimisation can be easily achieved by large-scale pre-cultivation of moss tissue in bioreactors adjusted to pH 4.5 (Hohe and Reski, 2002).

Finally, high protein concentrations in the supernatant could be gained in both transformed wild type protoplasts and in protoplasts derived from transgenic mosses lacking both plant-specific *N*-glycan residues. Despite complex glyco-engineering the expression and secretion capacity of the Δxyl-t/Δfuc-t double knockout strain was comparable to that of the wild type and reached concentrations of up to 10 μg/ml rhVEGF. The entire amount of recombinant product from both genotypes was biologically active as confirmed by the receptor assay technology (RELIDA).

Taken together, a highly efficient PEG-mediated transient expression method based on secretion of the recombinant protein has been developed for mosses. It can be used prior to stable transformation to gain important information about optimal expression tools for the production of the target protein in a short time. Although this system was developed for bryophytes, we think that it may also be useful for other plant systems for which the use of viral vectors or agroinfiltration are not suitable.

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