Plant Biotechnology Journal (2005) **3**, pp. 331–340 doi: 10.1111/j.1467-7652.2005.00127.x

Enhanced recovery of a secreted recombinant human growth factor using stabilizing additives and by co-expression of human serum albumin in the moss *Physcomitrella patens*

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Received 13 September 2004;
revised 8 December 2004;
accepted 22 December 2004.
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Keywords: co-expression, human serum albumin, moss, recombinant protein production, stabilizer, vascular endothelial growth factor

Summary

The production of pharmaceutical proteins in plants provides a valuable alternative to other traditional eukaryotic expression systems from economic and safety perspectives. The moss *Physcomitrella patens* allows the expression and secretion of complex target proteins into a simple aqueous maintenance medium, which facilitates downstream processing by rendering it less complex. To address the question of whether the addition of protein-stabilizing substances enhances the recovery of a target protein secreted into the culture medium, several additives at different concentrations were tested in a small-scale screening system. Although polyvinylpyrrolidone (PVP) and human serum albumin (HSA) showed a significant impact on protein levels, supplementation of the medium with these substances was accompanied by certain limitations in upstream processes, such as foam formation (HSA), and in downstream processes, such as reduced binding efficiency on chromatography columns (PVP), respectively. In order to reap the benefit of the enhancing effect and to avoid the given negative aspects, we developed a new strategy based on the recombinant expression of HSA in plants that are already capable of expressing a target protein. First, we analysed the expression and secretion of recombinant HSA in transiently and stably transformed wild-type (WT) plants. HSA was then co-expressed in *Physcomitrella* plants transgenic for human vascular endothelial growth factor (VEGF). Even with high expression levels of recombinant human VEGF (rhVEGF), the co-expression of recombinant HSA (rHSA) resulted in 48%–102% higher recovery of the target protein without concomitant negative effects on the upstream process. This strategy enables the enhanced recovery of target protein and does not require the addition of foreign components directly to the culture medium.

Introduction

The rapidly growing number of complex therapeutic proteins in clinical development indicates that the pharmaceutical industry faces a potentially enormous manufacturing shortfall in terms of commercial production capacity. Traditional production systems that use mammalian cell cultures possess drawbacks in terms of cost and capacity. Newly developed expression systems, such as insect cell cultures or transgenic animals, give rise to additional problems with respect to product safety and authenticity. However, plants provide a particularly efficient manufacturing system for the rapidly growing market and will help to alleviate production bottlenecks. Transient and stable expression systems in plants have already been used to produce many recombinant proteins in various plant species (for reviews, see Ma *et al*., 2003; Fischer *et al*., 2004).

Targeting the protein of interest to intracellular compartments gives rise to further complications downstream in protein purification procedures. As a consequence, some plant-based production systems that allow the secretion of target protein

into the medium have been developed (Magnuson *et al*., 1996; Wongsamuth and Doran, 1997; Borisjuk *et al*., 1999; Lee *et al*., 2002). In such systems, the protein production phase is extended and is largely independent of the growth period as the medium containing the secreted target protein is harvested and plant cells are therefore retained for further cultivation and production.

Of these plant-based systems, transgenic moss plants of *Physcomitrella patens* offer a unique production system for biopharmaceuticals (Decker and Reski, 2004). Economic photoautotrophic growth is accomplished in a contained system (Hohe *et al*., 2002); easy downstream processing of the target protein from a simple mineral medium and unique genetic manipulation through homologous recombination (Schaefer and Zryd, 1997; Hohe and Reski, 2003) allow the expression of complex humanized proteins in a safely controlled environment. Recently, gene disruption by homologous recombination has been used to design a double knockout production strain that lacks the two plant-specific sugar residues, fucose and xylose (Koprivova *et al*., 2004), which are implicated in allergic immune responses (Lerouge *et al*., 2000). Thus, by eliminating the addition of plant-specific sugar residues to target protein, the major limitation to the production of plant-made glycosylated biopharmaceuticals can be avoided.

Another important aspect relates to product yield. Recently, it has been shown that recombinant expression of human vascular endothelial growth factor 121 (rhVEGF) in *Physcomitrella* can be enhanced dramatically by the use of moss-derived regulatory sequences, e.g. promoters and introns (Jost *et al*., 2004; A. Weise, M. Rodriguez-Franco, B. Timm, M. Hermann, S. Liuk, W. Jost and G. Gorr, in preparation). In addition to such improvements at the molecular level, optimization of the culture conditions forms the basis of a further strategy for acquiring high protein yield in production systems for proteins that are secreted into the medium.

Some efforts have been attempted to enhance the stabilization of secreted target proteins in suspension cultures of higher plants. Additives such as gelatin and polyvinylpyrrolidone (PVP) appear to be effective tools (Magnuson *et al*., 1996; LaCount *et al*., 1997; Lee *et al*., 2002). The favoured candidate is PVP because of its non-human and non-animal source. The stabilizing effect of PVP has been reported by Magnuson *et al*. (1996), who demonstrated the successful enhanced recovery of a secreted mammalian protein from a suspension culture of genetically modified tobacco cells. In the case of heavy-chain monoclonal antibody production in tobacco suspension culture, LaCount *et al*. (1997) could only stabilize the target protein with the presence of PVP (0.075%) in the medium. Elsewhere, Lee *et al*. (2002) described a 4.6-fold increase in human granulocyte–macrophage colony-stimulating factor (hGM-CSF) concentration in the medium by the addition of 5% gelatin. However, in that study, no stabilizing effect on hGM-CSF was achieved by the addition of PVP. In conclusion, it appears that different additives have unpredictable stabilizing effects depending on the secreted protein of interest, and each additive needs to be tested thereon.

In suspension cultures of fully differentiated transgenic moss tissue, high levels of secreted rhVEGF have been observed without the use of any stabilizing additive (Jost *et al*., 2004; A. Weise, M. Rodriguez-Franco, B. Timm, M. Hermann, S. Liuk, W. Jost and G. Gorr, in preparation). $VEGF_{121}$ is a small homodimeric glycoprotein of 28 kDa that induces the proliferation of endothelial cells and therefore plays an important role in angiogenesis (Ferrara and Davis-Smith, 1997). It is an interesting pharmaceutical target protein for the therapy of wound healing in diabetics and is now being evaluated by Genentech in phase I trials.

Although high yields of rhVEGF secreted into the medium of transgenic moss lines have been achieved without any stabilizer, we were interested in investigating further improvements in recovery. In addition to PVP, we tested several protein-based stabilizers as, for VEGF, bovine serum albumin (BSA) and human serum albumin (HSA) are used as stabilizing additives in enzyme-linked immunosorbent assay (ELISA) kits and for product formulation. HSA is a non-glycosylated monomeric globular protein with a molecular mass of 66.5 kDa. It is also widely used as a stabilizing agent for therapeutic proteins and vaccines and for the production of complex cell culture media. With the aim of commercial production, recombinant expression of HSA has been shown in yeast (Ballance, 1999) and higher plants (Sijmons *et al*., 1990; Farran *et al*., 2002; Fernández-San Millán *et al*., 2003). In contrast, the use of HSA as a stabilizing additive does not appear to have been demonstrated in the scientific literature.

In this study, we analysed the impact of different additives on the recovery of secreted rhVEGF in transgenic moss cultures. Moreover, we demonstrated the feasibility of a new strategy for enhancing protein recovery: the co-expression of recombinant HSA (rHSA) together with the target protein rhVEGF, which appears to represent an outstanding alternative to conventional medium supplementation technologies.

Results

Comparative test of stabilizing additives in a small-scale screening system

To test different putative stabilizing substances in a fast and efficient manner, we established a small-scale screening

Table 1 Impact of stabilizer additives used at different concentrations on recombinant human vascular endothelial growth factor (rhVEGF) recovery relative to unsupplemented medium after 10 days

Additive	Concentration (%)	rhVEGF (%)
Control	–	100
PVP	0.1	262
	0.075	367
	0.05	344
HSA	1	447
	0.1	367
	0.01	240
	0.001	105
StabilZyme Select	1	227
	0.4	104
	0.2	98
Prionex	5	341
	1	97
	0.5	102

Preliminary small-scale screening in 48-well plates with the transgenic plant tWTP31VEGF expressing up to 1 ng/mL. Control, 1/10 Knop with no additive; HSA, human serum albumin; PVP, polyvinylpyrrolidone.

system in 48-well plates enabling us to perform experiments in parallel. Culture volumes of 300 μL were sufficient for ELISA analysis of the target protein rhVEGF. Several common stabilizers, such as PVP360, HSA, StabilZyme Select and Prionex, were tested at different concentrations using the transgenic moss plant $tWTP31_{VEGF}$ (Gorr, 1999), which contains the complementary DNA (cDNA) for $VEGF_{121}$ and its human signal peptide under the control of the 35S promoter. The most suitable concentration of each additive (PVP, 0.075%; HSA, 1%; StabilZyme Select, 1%; Prionex, 5%) resulted in two very promising candidates: HSA and the synthetic polymer PVP performed best and increased the yield up to 4.5-fold, whereas StabilZyme Select and Prionex showed an enhancing effect up to 3.4-fold (Table 1). Higher concentrations of StabilZyme Select and Prionex showed a lethal effect on the moss.

Medium supplementation studies performed in flask and bioreactor suspension cultures

Experiments with transgenic *Physcomitrella* plants in shaking flasks were performed with PVP and HSA to transfer the conditions from the small-scale screening system to normal culture conditions, resulting in a comparable improvement in recovery (data not shown). Additionally, moss bioreactor cultures were started and the medium was supplemented with 0.075% PVP from the start of the culture, and with 0.01% HSA on day 10 respectively. Compared with standard culture conditions, no differences during cultivation were observed in bioreactor cultures performed in medium supplemented with 0.075% PVP. In contrast with the unchanged upstream process, supplemented medium had a negative influence on the downstream purification process. According to Kondo *et al*. (1995), medium supernatants of transgenic mosses without any supplements were adjusted to pH 5.2. When using fast performance liquid chromatography (FPLC) for the purification of rhVEGF, up to 82% of the target protein was bound to the column. With the addition of 0.075% PVP to the medium, the binding efficiency of the target protein was reduced to 2.5%–34% even with different pH adjustments.

The supplementation of the bioreactor medium with HSA immediately resulted in a breaking off of cultivation due to massive foam formation and agglutination of the cells.

In the case of HSA, effective stabilization could be confirmed in shaking flasks with the high-expressing plant $tWT11.51_{VEGF}$ containing the VEGF-encoding cDNA under control of the moss actin5 5′ region. Recovery was increased up to three-fold from 300 to 900 μg rhVEGF/g dry weight (dw).

Expression of rHSA in *Physcomitrella*

To analyse whether *Physcomitrella* is capable of rHSA expression, we cloned the cDNA for HSA into two different expression vectors for transient expression and secretion experiments (Figure 1). Expression vector $pRT101\_HSA_{CDS}$ containing a 35S

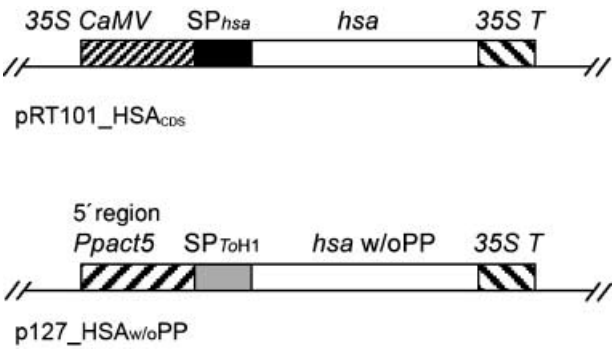

Figure 1 Schematic representation of the plant expression vectors and sequence of the signal peptides employed. The $pRT101\_HSA_{CDS}$ expression vector containing the human cDNA for human serum albumin (HSA) with propeptide (*hsa*) was under the control of the 35S cauliflower mosaic virus promoter (35S CaMV) and the 35S terminator (35S T). The signal peptide of HSA (SPhsa) is used for secretion into the medium. The p127_HSAw/oPP expression vector containing the human cDNA for HSA without propeptide (*hsa* w/oPP) under control of the 5′ region of the *Physcomitrella patens* actin5 gene (5′ region *Ppact5*) is composed of the act5 promoter, 5′ untranslated region (UTR) and 5′ intron. For secretion, the signal peptide of the thaumatin-like protein H1 from *Thuja occidentalis* (SP*To*H1) was used. Both plasmids additionally contain an ori and ampicillin resistance gene.

promoter was used to express rHSA with the human signal peptide and the propeptide that is later cleaved in the Golgi vesicles prior to the secretion of the mature protein. The second expression vector p127_HSAw/oPP contained the HSA-encoding cDNA without propeptide (w/oPP) and the plant signal peptide of the thaumatin-like protein H1 from *Thuja occidentalis* (*To*H1) for secretion under the control of the *P. patens* actin5 5′ region. This construct was selected because Sijmons *et al*. (1990) showed that rHSA, containing a plant signal peptide instead of a native signal peptide and propeptide, was correctly processed and secreted in transgenic plants.

In transiently transformed protoplasts derived from wild-type (WT) plants, rHSA was successfully expressed and secreted using both constructs. The amount secreted was determined by HSA ELISA. p127_HSAw/oPP, containing the regulatory sequence from moss and the plant signal peptide, resulted in an average yield of 0.3 μg/mL, which was 10-fold higher than pRT101_HSA_{CDS} (Figure 2a). Analysis of rHSA transiently expressed and secreted in moss protoplasts showed the same molecular weight of the processed protein compared with control HSA, as confirmed by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or non-reducing conditions followed by Western blot analysis (Figure 2b).

Plants stably transformed with the construct p127_HSAw/oPP were generated for control experiments and to examine the effect of recombinantly expressed HSA on moss culture. In stably transformed plants, rHSA was successfully expressed and secreted, resulting in levels of up to 40 μg/g dw. In addition, no changes were observed in the growth characteristics of the transgenic strains.

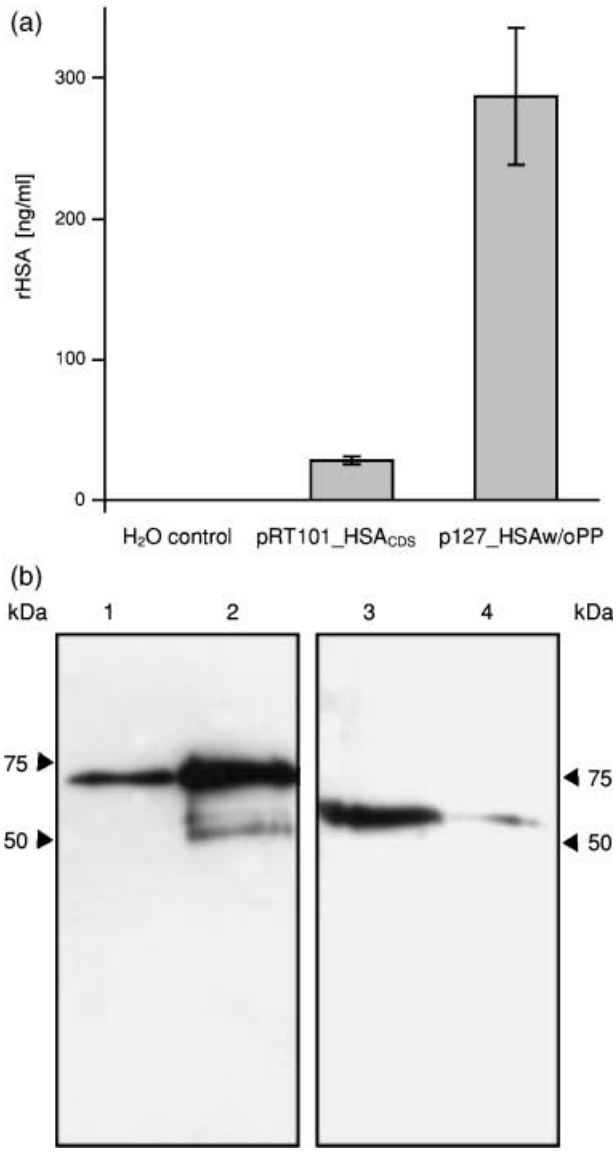

Figure 2 Transient expression of recombinant human serum albumin (rHSA) in wild-type (WT) plants. (a) Quantification of secreted rHSA in supernatants of transiently transformed WT plants after 96 h was performed by enzyme-linked immunosorbent assay (ELISA). Expression vectors pRT101_HSA_{CDS} and p127_HSAw/oPP were used for transformation. The mean of triplicates with deviation is displayed. (b) Western blot analysis of rHSA transiently expressed and secreted after 96 h using expression vector p127_HSAw/oPP. Supernatant samples were directly loaded on to the gel without prior purification or concentration. rHSA was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (1, control 10 ng HSA; 2, moss-derived rHSA) and non-reducing (3, control 10 ng HSA; 4, moss-derived rHSA) conditions.

Generation of transgenic co-transformed rHSA plants

Based on the results obtained for rHSA expression in transformed WT plants, the stably transformed plant tWT11.51_{VEGF} expressing high levels of rhVEGF was transformed with the construct p127_HSAw/oPP. After selection on hygromycin, putative transgenic plants were screened by polymerase chain reaction (PCR) for the insertion of p127_HSAw/oPP, followed by a preliminary HSA ELISA screening in 48-well plates (data not shown). Three plants, positive in antibiotic resistance selection, PCR analysis (Figure 3a) and HSA ELISA screening (data not shown), were further analysed in flask suspension culture. Doubly transformed plants were investigated further for any differences in growth or morphology due to the second transformation. Growth was compared with WT and tWT11.51_{VEGF} plants in 1/10 Knop medium after 5 days. The dry weights of all co-transformed plants varied only in the range of the standard deviation and were comparable with that of the WT plants. The morphology was reviewed microscopically (Zeiss, Axiovert 200) and found to be unchanged (data not shown).

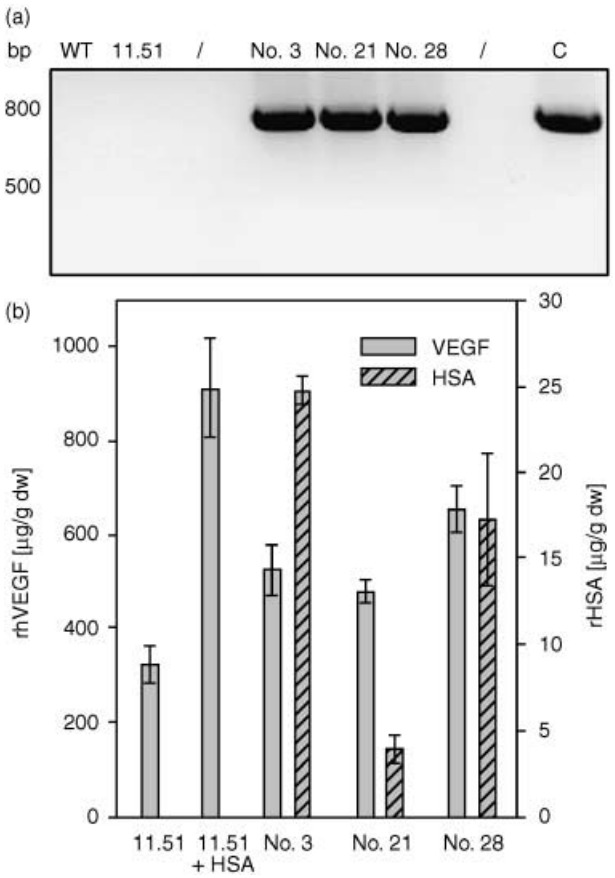

Figure 3 Analysis of stably co-transformed t11.51 HSA plants. (a) Polymerase chain reaction (PCR) analysis of co-transformed t11.51 HSA plants (No. 3, 21 and 28) after three rounds of antibiotic selection, tWT11.51$_{VEGF}$ (11.51) and wild-type (WT). Plasmid DNA of p127_HSAw/oPP was used as positive control (C). (b) Enzyme-linked immunosorbent assay (ELISA) analysis of secreted recombinant human proteins in supernatants of stably co-transformed t11.51 HSA and tWT11.51$_{VEGF}$ plants cultivated for 5 days in suspension culture. Recombinant human vascular endothelial growth factor (rhVEGF) is displayed as grey bars and recombinant human serum albumin (rHSA) as hatched grey bars. 11.51, tWT11.51$_{VEGF}$; 11.51 + HSA, medium supplemented with 0.01% HSA; No. 3, 21, 28: t11.51 HSA. The mean of triplicates with deviation is displayed.

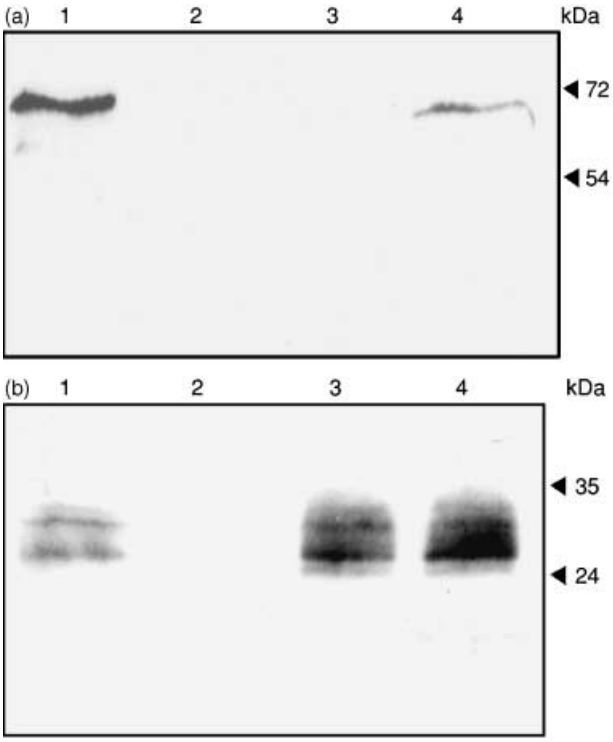

Figure 4 Western blot analysis of stable recombinant human vascular endothelial growth factor (rhVEGF) and recombinant human serum albumin (rHSA) in transgenic plants. Supernatant samples containing rHSA and rhVEGF were directly loaded on to the gel without prior purification or concentration. (a) Western blot analysis of rHSA: 1, control 20 ng HSA; 2, control wild-type (WT); 3, tWT11.51$_{VEGF}$; 4, t11.51 HSA No. 28 under reducing conditions. (b) Western blot analysis of rhVEGF: 1, control 10 ng VEGF$_{121}$; 2, control WT; 3, tWT11.51$_{VEGF}$; 4, t11.51 HSA No. 28 under non-reducing conditions.

In flask cultures, the expression of secreted rHSA varied from 4.5 to 25 µg/g dw [corresponding to a (0.3–2) × 10^{-7}% solution]. The rHSA co-transformed plants showed an increase of rhVEGF recovery of 480–656 µg/g dw, which corresponds to 48%–102% (Figure 3b). Additionally, Western blot analysis showed appropriate processing of rHSA (Figure 4a) and rhVEGF (Figure 4b). Finally, experiments were carried out with HSA as a medium supplement at very low concentrations (0.3 × 10^{-7}% and 2 × 10^{-7}%) corresponding to the amount of co-expressed HSA in the co-transformed plants. Compared with the unsupplemented control, no significant increase in product yields was observed with the low HSA concentrations used (control, 368 ± 13 µg/g dw; 2 × 10^{-7}% supplemented HSA, 375 ± 37 µg/g dw; 0.3 × 10^{-7}% supplemented HSA, 390 ± 28 µg/g dw).

Moreover, the plant t11.51 HSA No. 28 was cultivated in a bioreactor. The improved rhVEGF recovery was comparable with that determined in flask cultures and resulted in up to 450 µg/g dw without concomitant foam formation.

To rule out the possibility that enhanced rhVEGF expression and recovery are a result of genome doubling through protoplast fusion, all transformants were analysed by flow cytometry (FCM). FCM analysis of DAPI (4′,6-Diamidino-2-phenylindole)-stained nuclei of the transgenic plants was compared with that of the WT, and demonstrated that all plants were haploid and that no protoplast fusion had occurred (Figure 5).

Discussion

In different plant expression systems that are based on the secretion of recombinant protein into the medium, enhanced protein recovery may be achieved by supplementation of the culture medium with stabilizing substances. Lee *et al*. (2002)

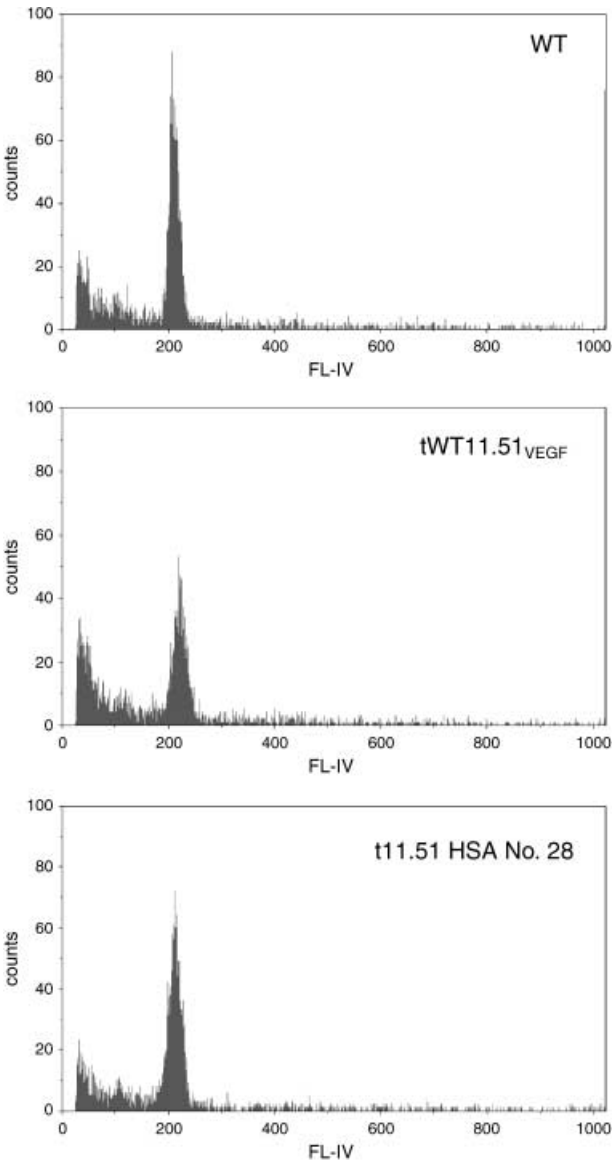

Figure 5 Flow cytometry analysis of transgenic plants. Histograms of isolated protoplasts of *Physcomitrella* wild-type (WT), tWT11.51$_{VEGF}$ and an example of the co-transformed HSA plants t11.51 HSA No. 28, summarizing representative flow cytometry analysis of DAPI-stained isolated nuclei. The abscissa represents the channel numbers corresponding to the relative fluorescence intensities of analysed particles in the linear mode. The ordinate indicates the number of events counted.

showed an increased production of rhGM-CSF by the addition of stabilizing polymers in plant suspension cultures. Although the addition of PVP (0.5–2 g/L) and polyethyleneglycol (PEG) (1%–5%) did not significantly increase the extracellular rhGM-CSF yield, gelatin (5%) had a significant effect on stabilization of the protein, but only over a short period of time (5 days). This improvement of protein recovery was accompanied by a negative effect on cell growth and physiology. Furthermore, Lee *et al*. (2002) determined an increased protease activity during suspension culture, and argued that this may be due to the accelerating release of protease or enhanced activity in the presence of stabilizing gelatin. In contrast with these results, in suspension culture, *Physcomitrella* naturally secretes only a few endogenous proteins extracellularly that accumulate in very low amounts in the medium. None of these extracellular proteins displays protease activity (Decker and Reski, 2004).

The use of gelatin for the production of biopharmaceuticals represents a high risk with respect to safety aspects, and therefore it was not included in this study.

Whereas PVP had only minor effects on rhGM-CSF levels, in the case of heavy-chain antibody fragments expressed and secreted with tobacco cells, supplementation of the medium with PVP resulted in a major improvement in protein recovery (Magnuson *et al*., 1996). PVP is a well-known inert substance of non-human, non-animal origin (included in the Food and Drug Administration inactive ingredient list; Anderson *et al*., 1979), and does not influence the culturing and growth characteristics of tobacco cell cultures (Magnuson *et al*., 1996). We achieved comparable results to Magnuson *et al*. (1996), in that by adding PVP to the culture medium of *Physcomitrella*, we also observed no changes in the growth parameters. Furthermore, in bioreactor cultures of *Physcomitrella*, which were normally stirred at 500 r.p.m. (Hohe *et al*., 2002), the addition of 0.075% of PVP did not cause any foam formation.

Although HSA is a stabilizing additive in diagnostic kits and in the formulation of biopharmaceuticals, there appears to be no information about its use in plant cell culture in the scientific literature. Nevertheless, we were interested in this additive and introduced it into our studies. In addition, we also tested the two commercially available protein-based stabilizers Prionex and StabilZyme Select.

A small-scale screening system was established allowing the parallel testing of different concentrations of each stabilizer under suspension culture conditions for mosses in 48 wells. The tested protein-based stabilizers Prionex and StabilZyme Select showed a clear enhancing effect on protein recovery up to 3.4-fold, but increased concentrations of both substances showed a negative effect on the viability of *Physcomitrella*, which may be due to the preservative additives in these stabilizers. Higher recovery rates of rhVEGF, up to 4.5-fold, were achieved with HSA and PVP without an observable effect on growth. For PVP, its effect is thought to be due to its excellent complexing, stabilizing and colloidal properties, while it is metabolically and physiologically inert (Anderson *et al*., 1979). In the case of HSA, the prevention of adsorption to surfaces, resulting in the stabilization of diluted allergens, has been discussed by Naerdal and Vilsvik (1983).

The determined optimal concentrations for the two favoured candidates, PVP and HSA, were then transferred to flask cultures. Although the cultivation conditions of moss in small volumes in wells without shaking are quite different from those of generally used flask cultures, the results obtained in both types of cultivating conditions were comparable, and indicated that the fully differentiated moss tissue can be readily adapted to different cultivating conditions. This is a very useful biological property of moss, illustrating its versatility and apparent robustness as a protein-producing system, because additional testing in flask cultures does not need to be conducted, thus opening up the possibility for great savings in terms of man-hours and space required for experimental work. Therefore, the small-scale screening system offers a good tool for preliminary tests on different culture conditions or medium supplementations. It would provide an interesting method for further medium component optimization and for the screening of transgenic plants, post-transformation.

Despite the many advantages of PVP, as described above, it caused serious problems during the downstream purification process when using different pH adjustments for ion-exchange chromatography. Binding of the target protein to the column material was inhibited because PVP somehow masks the protein or column material and prevents interaction.

In contrast with PVP, in bioreactor cultures of *Physcomitrella*, the addition of 0.01% HSA resulted in major problems under standard cultivation parameters, namely the agglutination of cells and their ascent to the medium surface. Furthermore, massive foam formation completely filled the space between the surface and the head plate of the bioreactor. Thus, cultivation had to be aborted due to the wetting of the air outlet filter and the increased risk of contamination. These effects caused by supplementation of the medium with HSA could be circumvented by adding antifoaming substances. However, this, in turn, would result in negative effects on cell viability and the downstream purification process (Li *et al*., 1995).

Taken together, the promising results achieved with PVP and HSA addition to the medium for the improvement of rhVEGF recovery from transgenic mosses were accompanied by major disadvantages. Therefore, we decided to try a new approach: the co-expression of rHSA together with the target protein rhVEGF.

The first step was to express rHSA transiently and stably in WT plants to confirm the possibility of rHSA secretion in moss. rHSA has been expressed and secreted in seed plants (Sijmons *et al*., 1990). Nevertheless, HSA is a non-glycosylated protein, and the recombinant expression and secretion of such a protein within moss tissue has not been shown previously.

After the successful expression and secretion of rHSA in transiently and stably transformed WT plants without any apparent negative effect on growth parameters, the next issue was to look at the co-expression of rHSA as a second recombinant protein and whether it would influence the expression of the target protein rhVEGF.

In other plant suspension cultures that did not include added stabilizers, levels of up to 10 μg/L (corresponding to 0.75–1 μg/g dw) in the case of a secreted mammalian protein (Magnuson *et al*., 1996; LaCount *et al*., 1997) and 180 μg/L (corresponding to 36 μg/g dw) in the case of hGM-CSF (Lee *et al*., 2002) were achieved. We showed that high levels (up to 300 μg/g dw) of secreted rhVEGF in suspension cultures of fully differentiated transgenic moss tissue of $tWT11.51_{VEGF}$ could be obtained. Therefore, the improvement in rhVEGF recovery (up to three-fold) achieved by supplementation with HSA was very promising.

Co-expression of rHSA in $tWT11.51_{VEGF}$ was performed by transformation with a construct containing the coding sequence for the processed HSA with a plant signal peptide under control of the moss-derived actin5 5′ region. Despite the rather low rHSA levels determined in all analysed plants (4.5–25 μg/g dw), an improvement in rhVEGF recovery in the range 48%–102% was observed. This was a very exciting result, because at the beginning of these experiments the effect of this approach was not predictable. The additional expression of a second recombinant protein might also have resulted in a decrease in the high expression rate of rhVEGF due to the additional burden on the transcription and secretion machinery of the cell. These results are promising for the expression of other independent protein combinations.

In some cases, protoplast fusions can occur (Rother *et al*., 1994) during the transformation procedure. The fusion of protoplasts derived from $tWT11.51_{VEGF}$ would result in the doubling of the VEGF cassettes by doubling of the genome. In Southern blot analysis, it is generally difficult to distinguish between a haploid and a diploid moss strain derived by fusion of protoplasts of the same strain. Nevertheless, the enhancing effect described here could have resulted from increased transcription of VEGF as a result of genome doubling. We performed FCM analysis of transgenic plants co-expressing rHSA and rhVEGF and compared the results with those of WT plants and $tWT11.51_{VEGF}$. No diploid transgenic lines were found, indicating that enhanced recovery of rhVEGF was not due to a doubling of the rhVEGF expression cassettes as a result of protoplast fusion.

In addition to the enhanced recovery of the target protein rhVEGF, no negative effect on growth and morphology was

observed. In contrast with the effect of adding HSA as a supplement to the medium, no foaming was observed in the bioreactor culture of the co-transformed plants. The rather small amounts of co-expressed rHSA in transformed $tWT11.51_{VEGF}$ plants seemed to be sufficient to increase the product yield in bioreactor cultures, but did not influence the culture conditions and did not result in enhanced foam formation or cell agglutination. To further examine the possible mechanism of enhanced rhVEGF recovery, experiments with supplemented HSA at very low concentrations (0.3×10^{-7}% and 2×10^{-7}%), corresponding to the amounts of secreted rHSA in the co-transformed plants, were carried out. However, no significant effect was achieved in these supplementation experiments, indicating that the enhanced recovery is not a direct consequence of the amount of added HSA supplement. Perhaps the stabilizing effect of co-expressed rHSA is due to an interaction of the two proteins during the secretory pathway.

HSA is associated with different functions in the human blood. It has an important role in transport, e.g. of fatty acids, steroids, etc., and contributes to the maintenance of osmotic balance. However, the mechanism of enhanced recovery by the co-expression of rHSA and the target protein remains unclear and requires further investigation.

In conclusion, we have demonstrated the successful expression and secretion of rHSA in the moss *Physcomitrella patens.* We highlight that the co-expression of rHSA in transgenic plants already expressing high yields of rhVEGF results in an enhanced recovery of the target protein. This approach may be further improved by the optimization of rHSA expression by the use of other promoters or signal peptides. Studies with other transgenic plants expressing different target proteins may provide more information about the applicability of the approach described here.

Experimental procedures

Plant cell culture

Physcomitrella patens (Hedw.) B.S.G. was grown axenically under sterile conditions in Erlenmeyer flasks containing 1/10 of inorganic liquid modified Knop medium (100 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 25 mg/L KCl, 25 mg/L KH_2PO_4, 25 mg/L $MgSO_4 \cdot 7H_2O$ and 1.25 mg/L $FeSO_4 \cdot 7H_2O$; pH 5.8). Subculturing of liquid cultures was performed as described in Reski and Abel (1985).

Bioreactor cultures were grown in 1/10 of modified Knop medium in stirred-tank glass bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 5 L, as described in Hohe *et al*. (2002).

Small-scale screening system for stabilizers

Plant material was taken from flask cultures, filtered (100 μm sieve) and washed with 1/10 Knop medium. Material corresponding to 15 mg dry weight was transferred to 48-well plates (Greiner Bio-one, Frickenhausen, Germany) in triplicate and the medium was adjusted to 300 μL in each case. Medium was exchanged once after 24 h and samples were taken after 10 days. The following stabilizers diluted in 1/10 Knop medium were tested: PVP360 (Sigma, Steinheim, Germany), HSA (Sigma, Germany), Prionex® Solution 10% (Pentapharm, Basel, Switzerland) and StabilZyme Select® solution (SurModics, Eden Prairie, MN, USA).

Analysis of recombinant proteins

VEGF

Samples from culture medium or transient expression were analysed by sandwich ELISA (anti-VEGF, capture, #AF-293-NA, 1 : 500; anti-VEGF, detection, #BAF-293, 1 : 1000; $rhVEGF_{121}$, standard, #298-VS, R&D, Wiesbaden, Germany; streptavidin-POD conjugate, #1089153, Roche, Mannheim Germany; TMB substrate, #TMBUS, Moss Inc., Pasadena, MD, USA; Nunc-Immunosorb plates, Roskilde, Denmark). Dilutions were made in 1 × phosphate-buffered saline (PBS)/0.1% BSA (Serva, Heidelberg, Germany).

Western blot was performed using anti-human VEGF (#AF-293-NA, R&D, Germany, 1 : 500), anti-goat IgG (#A5420, Sigma, St Louis, MO, USA, 1 : 8000) and rhVEGF (#298-VS, R&D, Germany) as standard.

The purification of rhVEGF from medium supernatants was performed by FPLC (ÄKTA Explorer, Amersham Biotech, Frieburg, Germany) using a HiTrap SP FF 1 mL column (Amersham Biotech, Germany) according to Kondo *et al*. (1995). For equilibration, 10 mM of potassium acetate was used.

HSA

Samples from stable plants and transient expression were analysed by HSA ELISA (Cygnus Technologies, Delaware Water Gap, PA, USA) according to the manufacturer's instructions. Dilutions were made in 0.05 M Tris-HCl (Roth, Karlsruhe, Germany), pH 7.2, 0.15 M NaCl (Merck, Darmstadt, Germany) and 8 mg/mL of high-grade BSA (Serva, Germany).

Western blot was performed using anti-HSA (A6684, Sigma, USA, 1 : 2500), anti-mouse IgG (A4416, Sigma, USA, 1 : 6000) and HSA (Sigma, Germany) as standard.

SDS-PAGE of rhVEGF and rHSA was performed according to Laemmli (1970). For Western blot, the reinforced nitrocellulose membrane Optitran (Schleicher & Schuell, Dassel,

Germany) and Nova blot (Pharmacia Biotech, Frieburg, Germany) were used for semidry protein transfer. Blocking of the membrane and dilutions of the antibodies were made in 1 × HS-TBST (high salt Tris buffered saline-Tween) containing 2.5% skimmed milk powder. Washing was performed three times with 1 × TBST in between each step. An ECL Advance Western Blotting Detection Kit (Amersham, Freiburg, Germany) was used for visualization. Supernatant samples were directly separated by SDS-PAGE without concentration or purification.

Construction of rHSA expression vectors

The cDNA for HSA was cloned from I.M.A.G.E. cDNA clone 4734617, pDNR-LIB-HSA (#97002RG, Invitrogen, Karlsruhe, Germany). For construction of pRT101_HSA_{CDS}, the pDNR-LIB-HSA was digested *Eco*RI/*Xho*I. The obtained fragment containing the cDNA for HSA was blunted (T4 DNA-Polymerase, Fermentas, St Leon-Roth, Germany) and ligated (T4-DNA-Ligase, Fermentas, Germany) into *Eco*RI/*Xba*I digested and blunted pRT101 (Toepfer *et al*., 1987). In this expression vector, the cDNA for HSA is under the control of the 35S promoter. For construction of p127_HSAw/oPP, the cDNA for HSA without propeptide (HSAw/oPP) was amplified by PCR (5′ primer MOB 906 GATGCACACAAGAGTGAGG and 3′ primer MOB 907 ATCGGATCCTTATAAGCCTAAGGCAGC containing a *Bam*HI restriction site). After digestion of the PCR fragment, it was ligated into the *Bam*HI/*Pdi*I-digested expression vector p127.

p127 consists of the 5′ region of the *Physcomitrella patens* actin5 gene, including the 5′ intron and 5′ untransformed region (UTR) (GenBank accession no. AY745190), and the coding sequence for the signal peptide of the thaumatin-like protein *To*H1 from *Thuja occidentalis* (GenBank accession no. AY95849) to control the expression (Weise *et al.* in preparation). Both inserts of the resulting plasmids were sequenced and found to be identical with validated HSA precursor (ABHUS GI:2144898).

Transient expression of rHSA

Transient transformation of rHSA constructs was accomplished as described by Koprivova *et al*. (2004) and performed in WT plants. The amount of plasmid DNA for HSA was 50 μg per transformation. Instead of regeneration medium, the volume was adjusted to 350 μL of 3M medium (15 mM $MgCl_2 \cdot 7H_2O$, 0.1% MES (N-morpholinoethanesulphonic acid), 0.48 M mannitol, pH 5.6, 580 mOsm) in 48-well plates (Greiner Bio-one, Germany); 200 μL of the medium was replaced after 24 h and supernatants were taken after 96 h for further analysis.

Generation of stably transformed plants

Transformation was performed with WT and $tWT11.51_{VEGF}$, respectively, as described by Strepp *et al*. (1998). Plasmid DNA of p127_HSAw/oPP was adjusted to 1 μg/μL and linearized by digestion for 2 h at 37 °C with *Sca*I. DNA was purified using GFX columns (Amersham Biosciences, Germany). The amount of plasmid DNA for HSA was 15 μg for each transformation and, for antibiotic selection with hygromycin, 3 μg of pCambia1305 (CAMBIA, Canberra, Australia) was added. After transformation, protoplasts were kept under half-light conditions (4.6 $\mu mol/s/m^2$) in six-well plates for 3 days and then transferred to Knop agar dishes with and without antibiotic (30 mg/mL). Plants remained for 7–10 days alternately on selection and release for three selection rounds.

PCR analysis of stable plants

DNA from putative stably transformed plants was prepared as follows. Plant material was minced in 2 mL Eppendorf tubes containing one tungsten carbide bead for 30 s with a frequency of 30 in a Mixer Mill (Retsch, Haan, Germany). After the addition of 500 μL of DNA extraction buffer (250 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% SDS) and centrifugation (2 min/16 050 ***g***), 300 μL of supernatant was precipitated with isopropanol for 2 min at room temperature. After another centrifugation step (5 min/16 050 ***g***), the supernatant was discarded, and the pellet was resuspended in 50 μL of H_2O. One microlitre of this suspension was used as template during PCR. For a positive control, 1 μL of 1 : 100 diluted miniprep DNA of p127_HSAw/oPP was used. The following primers (MWG, Ebersberg, Germany) were used for $t11.51_{VEGF}$:p127_HSAw/oPP: MOB 906 GATGCACACAAGAGTGAGG and MOB 976 GCAGTGCACATCACATCAACC.

FCM analysis

FCM analysis was carried out as described in Schween *et al*. (2003) with a cell counter analyser (CCA; Partec, Muenster, Germany).

Acknowledgements

We thank Gabriele Schween for the introduction to FCM analysis and Franz Kaufmann for the introduction to FPLC. We are also indebted to Lionel Scott for correcting the manuscript linguistically. This work was supported by a grant from the German Federal Ministry of Education and Research (BMBF) no. 0312624.

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