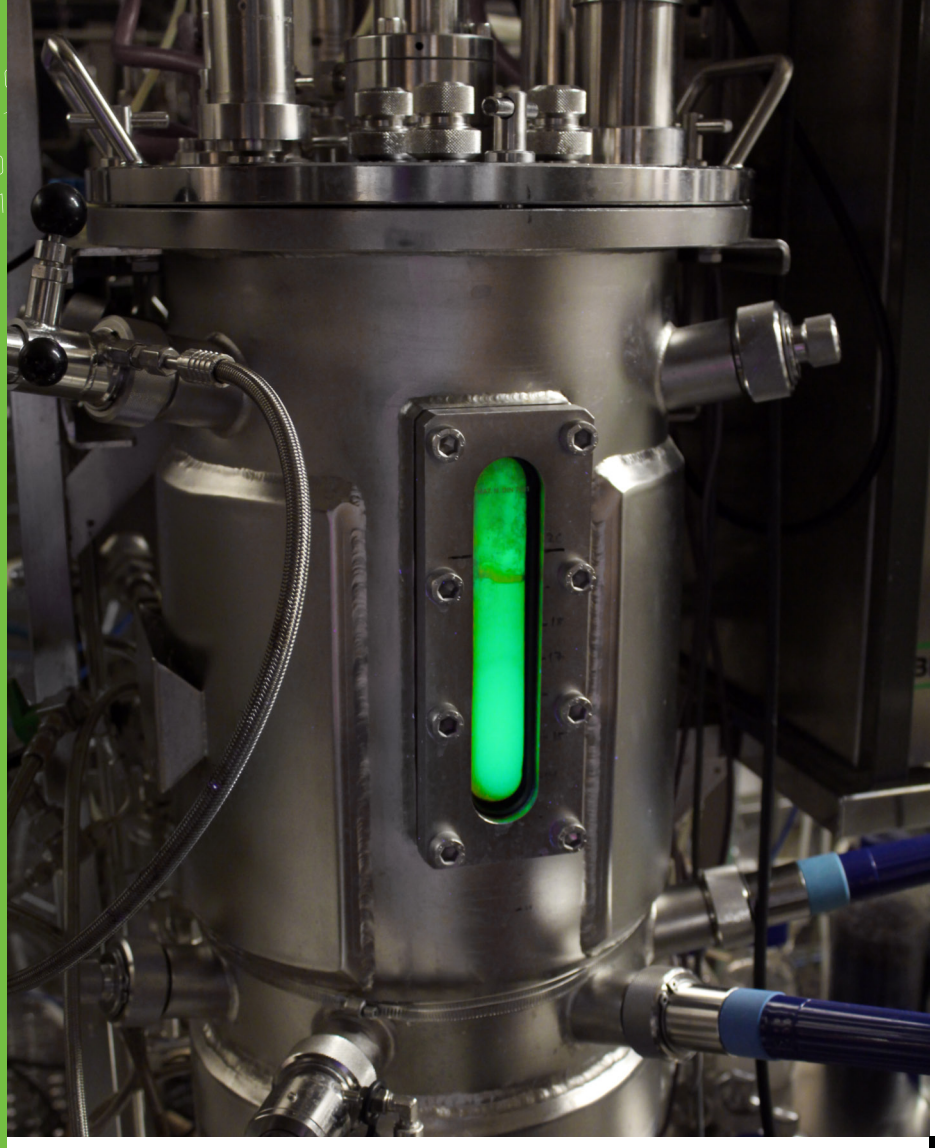
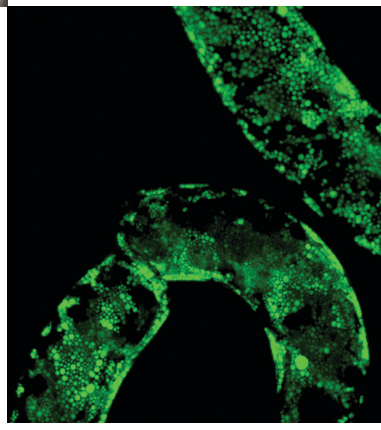


SCIENCE • TECHNOLOGY • RESEARCH HIGHLIGHTS • VISIONS

Dissertation
144



Plant cell factories

Production of hydrophobin fusion proteins in plant cell cultures

Lauri Reuter

VTT

VTT SCIENCE 144

Plant cell factories

Production of hydrophobin fusion proteins in plant cell cultures

Lauri Reuter

Thesis for the degree of Doctor of Philosophy to be presented with due permission of the Faculty of Agriculture and Forestry for public examination and criticism in auditorium PIII in Porthania, at the University of Helsinki, on the December 9th 2016 at 12 o'clock.

ISBN 978-951-38-8482-6 (Soft back ed.)
ISBN 978-951-38-8481-9 (URL: http://www.vttresearch.com/impact/publications)

VTT Science 144

ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8481-9

JULKAISIJA – UTGIVARE – PUBLISHER

Teknologian tutkimuskeskus VTT Oy
PL 1000 (Tekniikantie 4 A, Espoo)
02044 VTT
Puh. 020 722 111, faksi 020 722 7001

Teknologiska forskningscentralen VTT Ab
PB 1000 (Teknikvägen 4 A, Esbo)
FI-02044 VTT
Tfn +358 20 722 111, telefax +358 20 722 7001

VTT Technical Research Centre of Finland Ltd
P.O. Box 1000 (Tekniikantie 4 A, Espoo)
FI-02044 VTT, Finland
Tel. +358 20 722 111, fax +358 20 722 7001

Juvenes Print, Tampere 2016

Preface

This work was carried out at VTT Technical Research Centre of Finland Ltd during the years 2012–2016. The research was funded by the Academy of Finland. The Chancellor's Travel Grant and the Dissertation Completion Grant from the University of Helsinki and the grant for a Short-Term Scientific Mission from the COST Action were highly appreciated. I consider myself fortunate for having had the opportunity to conduct my research at VTT, in an exciting intersection of application driven and academic research, surrounded by talented and inspiring people.

My deepest gratitude goes to my supervisors, docent Anneli Ritala and docent Jussi Joensuu. Your commitment and guidance throughout these years have been irreplaceable. Thank you for making me part of the team and placing trust in me from day one, for the support through rough days and evenings and for all those long discussions leading to either inspiration or solution – often both. Thank you for always having your door open for me and for always having by back.

My sincere thanks goes to my team leader docent Heiko Rischer, head of research area docent Kirsi-Marja Oksman-Caldentey and the management of VTT for providing me with outstanding facilities and working environment. Beyond that, I want to thank Kirsi-Marja for always finding the time to stop by for a chat and for all the wise advice. I want to express my gratitude to my supervising professor Teemu Teeri for guiding my studies and showing tremendous flexibility and friendly understanding with my schedules and plans.

I owe a lot to all my co-authors professor Markus Linder, docent Heldér Santos, Katri Kurppa, Michael Bailey, Mohammad-Ali Shahbazi, Jarno Salonen and Ermei Mäkilä, who gave me valuable insights into academic writing and dissemination of knowledge. Without you, this work would have never been completed. I also wish to thank the pre-examiners of this dissertation, professor Eva Stöger and professor Hely Häggman.

In the framework of this study, I had the privilege to visit two of the top research institutes in the field. The visits gave me tremendous perspective – and not only on science. Thus I want to express my gratitude to professor Stefan Schillberg, Andreas Schiermeyer and Helga Schinkel at the Fraunhofer Institute for Molecular Biology and Applied Ecology, professor Rima Menassa and Reza Saberianfar at the Agriculture and Agri-Food Canada and all the others who made me feel so welcome.

This work succeeded only with the extremely valuable assistance in the laboratory from Tuuli Teikari, Jaana Rikkinen, Siv Matomaa, Mari Lämsä, Karita Viitaaho, Riitta Suihkonen, Juha Tähtiharju, Merja Aarnio, the whole plant biotechnology (and metabolomics) team and so many other talented people at VTT. Eva Fredriksson, without you nothing would have worked out, ever. You all are the true heroes of this story.

The peer support and game changing enthusiasm of so many VTT Young Professionals have been an invaluable resource during these years. Thank you Amir, Hannu, Marloes, Helena, Miriam, Katsu, Katariina, Ilkka, Colm, Outi and Outi, Ronny, Jenni, Jouni and Niko, to name just a few.

I extend my gratitude to my dear friends. You have been there through ups and downs; you have shared my excitement and supported me through difficult moments. You mean the world to me.

Last, I want to thank my family, my clan, for the love, care and playful creativity that you have surrounded me with. Especially I want to thank my parents, Eeva-Liisa and Hartwig, for raising me in a word where nothing seemed impossible or too odd to at least give it a try, and my brother Aapo for all the inspiring discussions going beyond ordinary.

Espoo, November 2016

Lauri Reuter

Academic dissertation

Supervising professor, Custos	Prof. Dr. Teemu Teeri Faculty of Agriculture and Forestry University of Helsinki Helsinki, Finland
Thesis advisors	Docent, Dr. Anneli Ritala VTT Technical Research Centre of Finland Ltd Espoo, Finland
	Docent, Dr. Jussi Joensuu VTT Technical Research Centre of Finland Ltd Espoo, Finland
Pre-examiners	Prof. Dipl.-Ing. Dr.rer.nat.Eva Stöger Department of Applied Genetics and Cell Biology The University of Natural Resources and Life Sciences Vienna, Austria
	Prof. Dr. Hely Häggman Department of Biology University of Oulu Oulu, Finland
Opponent	Prof. Dr. Karen Mc Donald Department of Chemical Engineering University of California Davis, USA

List of publications

This thesis is based on the following original publications which are referred to in the text as I–IV. Some additional unpublished results are included.

I Reuter, L., Bailey, M., Joensuu, J., Ritala, A. 2014. Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. *Plant Biotechnology Journal* 12, 402–410

II Reuter, L., Ritala, A., Linder, M., Joensuu, J. 2016. Novel hydrophobin fusion tags for plant-produced fusion proteins. *PLoS ONE 11(10): e0164032*

III Kurppa, K., Reuter, L., Ritala, A., Linder, M., Joensuu, J. Antibody harvesting with a plant-produced hydrophobin- Protein A fusion. *Manuscript*

IV Reuter, L., Shahbazi, M., Mäkilä, E., Salonen, J., Santos, H., Joensuu, J., Ritala, A. Coating nanoparticles with plant produced transferrin-hydrophobin fusion enhances uptake in cancer cells. *Manuscript*

Author's contributions

I The author carried out the experimental work in laboratory scale and was in charge of the pilot scale experiments. The author interpreted the results together with the co-authors and had the main responsibility for writing the article.

II The author carried out the experimental work, except the initial expression experiments. The author interpreted the result with the co-authors, had the main responsibility for writing the article and is the corresponding author.

III KK and LR shared equal contributions as first authors. The author generated and characterized the BY-2 cell lines, performed the pilot scale production experiments, conducted the microscopy experiments and studied the two phase separation of fusion proteins from plant leaf extract. The author conducted the experiments on antibody purification in solution together with KK and wrote the final version of the article.

IV The author planned the work together with the co-authors and conducted the experiments except generation and analysis of the nanoparticles. The author interpreted the result with co-authors, had the main responsibility for writing the article and is the corresponding author.

Contents

List of abbreviations

ATPS	Aqueous two phase separation
BY-2	Bright yellow 2
CHO	Chinese hamster ovary
DO	Dissolved oxygen
DSP	Downstream processing
GFP	Green fluorescent protein
GRAS	Generally recognized as safe
HFB	Hydrophobin
NP	Nanoparticle
Psi	Porous silicon
QCM-D	Quartz crystal microbalance with dissipation monitoring
RT	Room temperature
TEM	Transmission electron microscope
Tf	Transferrin
TSP	Total soluble protein

1. Introduction

Proteins are the nanoscale machines and building blocks of the living world. We are built of proteins according to the instructions of our genetic code, and functions of our bodies are carried out by proteins. We need proteins as an essential part of our diet, but the modern society is dependent on proteins also in many other ways. Enzymes are e.g. used in production process of food and feed and in removing stains from laundry. Production of new biofuels depends on enzymatic processing of biomass and many materials we use, such as silk, are composed of proteins. Importantly, a large part of the modern medicine is dependent on biopharmaceuticals: proteins such as hormones, antibodies and vaccine antigens. The market value of biopharmaceuticals reached 140 billion USD in 2013 and has been increasing steadily [1].

Proteins are large and complex molecules and are difficult to produce synthetically. Instead, they are either purified from their natural sources or produced biotechnologically by inserting a gene encoding for the protein to an appropriate living production organism. The production host can be a bacteria, a yeast, filamentous fungi, cultured insect, plant or animal cells, or in some cases whole animals or plants. *Escherichia coli* is by far the most used microbial production host followed by eukaryotic *Saccharomyces cerevisiae* (baker´s yeast) and *Pichia pastoris* [1]. Chinese Hamster Ovary cells (CHO) are the most used mammalian cells [2]. Total production of biopharmaceuticals in 2010 was 26 400 kg of pure protein of which 68% was produced in microbes and 32% in mammalian cells. All other platforms represented only a small fraction of the production [1]. The prominence of mammalian systems is increasing due to increasing demand of highly complex molecules such as monoclonal antibodies.

Unlike animals or fungi, plants are primary producers. That is, they produce biomolecules from simple resources: water and elements in the soil, carbon dioxide from the air and energy from the sun light. Other forms of life, like mankind, rely on plants as the source of those molecules. With the tools of modern biotechnology plants can be harnessed to produce valuable recombinant proteins - literally from thin air. This new domain of exploiting plants is known as “molecular farming” or, in case of pharmaceuticals, “molecular pharming” [3].

Hydrophobins are a peculiarity in the protein world. Their extraordinary properties have inspired numerous applications ranging from foam stabilizing food additives

to coating of nanoparticles and biosensors even to use as anti-freezing agent on aircrafts [4]. Difficulty of the production of hydrophobins in large scale is a major hurdle in commercialization of many applications. Recently, plants have merged as a promising alternative for manufacturing recombinant hydrophobin fusion proteins [5].

In this literature review I will first formulate an overview on the concept of molecular farming in general (1.1) and then focus specifically to various aspects of the use of tobacco cell cultures for protein production (1.2). The next part (1.3) will introduce hydrophobins and their use as components of fusion proteins with fascinating properties. Finally the target proteins, used as case studies in this work, will be introduced in short (1.4).

1.1 Plant-based protein production

Plants and plant cell cultures can offer several advantages over the more traditional platforms of protein production including low cost of manufacturing, capability to process very complex proteins and inherent safety [3]. While some of the advantages are common to all forms of plant-based production, some are specific to manufacturing strategy: open field (1.1.1), contained greenhouses (1.1.2) or fully controlled bioreactors (1.1.3).

Complex proteins and glycosylation

As higher eukaryotes, plants are capable of correctly processing complex molecules, assemble multiunit proteins, such as virus like particles [6] and antibodies [7] and perform most post-translational modifications. Plants, unlike bacteria, are also capable of adding complex glucans on recombinant proteins similarly to animal cells [3]. This is important because the solubility, stability, immunogenicity, pharmacokinetic properties and biological function of many proteins are dependent on the asparagine (N) linked glycan structures.

Plant N-glycosylation is similar to animals, but not identical. The glycosylation process starts in the endoplasmic reticulum (ER) simultaneously with translation by attachment of an oligosaccharide precursor. During protein maturation in ER the oligosaccharide precursor is trimmed to a high mannose type glycoform, which is identical to animal glycans. The glycans are further crafted by enzymes in the Golgi apparatus. Finally the plant glycoproteins contain core α1,3-fucose, instead of mammalian α1,6-fucose, and core β1.2-xylose residues that are not found on mammalian proteins. Additionally mammalian proteins carry β1,4-galactose and terminal sialic acid residues that are absent on plant glycans. Differences in glycosylation have been recently reviewed in [8].

The plant-like glycosylation has raised concerns of potential immunogenicity and negative effects on the activity of pharmaceutical proteins. However, the concern has remained mostly theoretical. In contrast, the plant-like glycosylation has been proven beneficial in some cases [9,10]. Plant glycosylation machinery also results in rather small variation in glycan structures in contrast to larger variation in

CHO cells, which is an advantage from regulatory point of view [8]. Furthermore, the glycosylation in plants can be controlled by targeting the protein accumulation to either ER or other subcellular compartments like vacuoles [9,10]. Plants are also amenable to modifications of the glycosylation machinery without compromising viability. Thus enzymes along the secretory pathway can be removed or added to obtain more human-like glycosylation [8].

Safety

Mammalian cell cultures can be infected by viruses hampering the production of pharmaceuticals or even posing risk to patients. Such a contamination occurred in 2008–2010 at Genzyme, a company producing biopharmaceuticals in CHO cells for rare diseases. The company struggled with repeated vesivirus contaminations and as a result the global stocks of these particular drugs was exhausted forcing authorities to prioritise patients who would get the treatment [11]. In contrast to mammalian cells, plants do not harbour any know human pathogens [12].

Plants also do not contain endotoxins like bacteria or hyper glycosylated proteins like yeast. However, the secondary metabolites in some plants, such as nicotine in tobacco, may be harmful and need to be considered in design of the purification process [12,13]. Many plants are staple foods and have the GRAS status (generally recognized as safe). This means they could be directly used as a vehicle for administering edible vaccines or therapeutics for both human and animal use [14]. For example Protalix Biopharmaceuticals is developing a form of glucocerebrosidase to be delivered orally within the matrix of carrot cells [15].

Finally, plant-based production of proteins is completely void of animal components. This is of a great interest as industries are looking for alternatives for extraction of proteins from their native animal sources, such as human transferrin from blood or for producing vaccines in immunized eggs.

1.1.1 On field

Cultivating plants on field is inherently scalable. Vast amounts of biomass can be cultivated with existing agricultural infrastructure with low cost and upfront investments. Therefore producing pharmaceutical proteins and industrial enzymes on field could respond the hugely growing demand of recombinant proteins. The scalability is especially important now, as many blockbuster biopharmaceuticals have lost, or will lose, patent protection during 2015 and 2016 [1]. Both old and new players in the field will be looking for alternative and economical production systems for biosimilars. Also new uses of pharmaceuticals, such as topical application of antibodies for prevention of HIV, require huge amounts of protein [7]. Production of sufficient amounts in current systems would surpass the whole global fermenter capacity and be prohibitively expensive. In plants the antibodies for topical applications could be produced in immense scale and applied as crude plant extract without expensive purification [7,16]. Production of recombinant proteins in plant seeds has also the benefits of providing homogenous material that can be safely stored in ambient temperatures [17].

To date there are no field produced proteins accepted to pharmaceutical use in humans. However, plant-produced industrial proteins have entered the market. The US based biotechnology company ProdiGene developed production of i.e. avidin and β-glucuronidase in transgenic maize already in 90´s. Production of the biomass took place on field and the proteins were found to be stable in the seed for months after harvesting. The products were functionally equal to proteins extracted from natural sources and moreover – economically viable. Both products were marketed by Sigma-Aldrich (reviewed in [12].

Use of food crops as production hosts for pharmaceutical components has, however, raised questions of potential harm to consumers through accidental mixing in the food chain. More over the open use of genetically modified plants in general has encountered public and political opposition, especially in Europe [16]. The strict regulations ended the story of Prodigene Inc. Volunteer maize plants were found in 2001 growing in a field the season following a field trial of a maize crop producing a veterinary vaccine. The breach of extremely strict environmental regulations resulted in massive fines and clean-up costs eventually forcing the company to cease trading [14].

Still, another US company, Ventria Bioscience, uses transgenic rice and barley to produce i.e. recombinant human transferrin (D. Zhang, 2013; discussed in more detail in 1.4) on field to be used as an animal-free cell culture supplement. The same company is developing an orally delivered lactoferrin for prevention of antibiotic-associated diarrhea. To be economically viable, it is estimated that the manufacturing cost cannot be greater than 3.75 USD /g of protein. This could only be achieved using plants cultivated on field [1].

In addition to agricultural regulations, production of pharmaceuticals also have to follow the guide lines of good manufacturing practise (GMP) [12]. The same regulation applies to compounds used in the process of manufacturing pharmaceuticals. The GMP has been developed to guarantee the safety and consistent quality of pharmaceuticals produced in contained fermentation based processes using microbes or animal cells. The GMP requires that the host organism is cultivated under precise and controlled conditions to ensure batch-to-batch consistency. A field is everything but that. Varying weather conditions, soil composition, pests and use of agrochemicals create an environment where adherence to current GMP is not possible [12].

1.1.2 In greenhouse

In contrast to field, greenhouses provide a contained environment with much more control over the growth conditions. Especially growth chambers equipped with automated cultivation systems and LED technology are getting close to controlled environment of traditional bioreactors [12]. The development of vertical farming is improving the economy of such systems rapidly. The advantage of immense scalability and process economy in the agricultural system is partly lost when production is moved indoors. However, contained cultivation alleviates the concerns associated with open cultivation of transgenic plants and builds a barrier to

isolate the product from food chain. Controllable environment has also enabled formulation of GMP guidelines accommodating molecular pharming [12]. First in-human clinical trial with an antibody produced in tobacco took place in 2011, in framework of the academic Pharma Planta project [7]. The study defined the complete path for GMP manufacturing of antibodies in transgenic plants grown in containment, from seed to syringe.

Already today ORF Genetics, an Islandic company, produces recombinant proteins in transgenic barley grown in contained greenhouses. The products are not aimed for pharmaceutical use, yet, but to research and cosmetic use instead.

Transient expression

Contained environment also enables large scale protein production using transient expression in tobacco plants [19]. Most commonly the genetic construct is delivered to plant cytoplasm using *Agrobacterium tumefaciens*. The expression cassette is not integrated to the genome, but is translated directly to messenger RNA and further to protein. The product can be harvested in few days. The method has been utilized widely in research for fast production of small amounts of proteins. However, the development of automated culture systems, new viral vectors and spray application has made transient expression a potential alternative for industrial protein production [19]. Most significant advantage of transient expression is the speed and therefore the ability to respond to urgent need of vaccines. For example influenza antigens have been produced by transient expression in matter of weeks from receiving the gene sequence [16].

The field is flourishing in US [16] and various pharmaceutical proteins have been produced in GMP-compliant processes using transient expression system [12]. Recently an antibody based drug, produced by transient expression in tobacco, was accelerated through clinical phase to provide emergency care during the Ebola outbreak in 2014. An influenza vaccine, likewise produced in transient mode, is in phase III clinical trials and is expected to enter the market in 2018 [20]. Transient expression in plants will reduce the time needed for production of the vaccine significantly in comparison to immunization of eggs. The months saved in production phase are off from the time for the virus to evolve.

Although the GMP guidelines have now been established in Europe for production of antibodies in transgenic plants [7], the European drug agency (EMA) does not yet recognise transient expression platforms.

1.1.3 In bioreactors

Due to regulatory hurdles encountered with field and greenhouse grown plants for molecular pharming, the spotlight is back on bioreactors [21]. Plant-based manufacturing in bioreactors is compatible with the current GMP as such [12]. Therefore it was no surprise that the first plant derived recombinant protein approved by FDA for pharmaceutical use in human was produced in plant cell culture [1,15]. In addition to plant cell cultures, plant-based protein production in bioreactors can be

done using hairy roots, microalgae, moss cultures or floating plant cultures [3]. In this work, however, I will focus on dedifferentiated plant cells alone.

Plant cell cultures have been used for decades for commercial production of a number of pharmaceutical plant secondary metabolites, such as shikonin, berberine, sanguinarine and skopolamin [22]. For example Phyton Biotech has produced a sustainable supply of paclitaxel in 75 000 L bioreactors from 90´s [22]. First recombinant protein, human serum albumin, was expressed in plant cell cultures already in the beginning of nineties [23], but it took another two decades before the first protein produced in plant cells gained FDA approval for use in human in 2012 [9].

Propagation and protein expression

Plant cell cultures are initiated by wounding plant tissue and inducing callus formation by application of growth factors. The totipotent, dedifferentiated cells are further maintained as axenic cultures. In liquid media the cells grow as individual cells or, more commonly, as small clusters or threads (Figure 1). As primary producers the plant cells do not have large requirements for the media, although most cell cultures require sugars as carbon source. In comparison to the complex, rich and expensive media required by animal cells, plant cell media is cheap, chemically defined and contains no animal derived components.

Heterologous protein expression in plant cell culture can be achieved in two ways. The cell culture can be generated from a transgenic plant or an already established cell culture can be transformed. The first option is often preferred when transgenic plants, are available as it requires very little effort in screening for good clones. However, generation of transgenic plants is tedious and time consuming. Transformation of plant cell cultures is significantly faster. Well established cell cultures, such as tobacco BY-2, also grow much faster than cultures established freshly from transgenic plants [24].

Plant cell cultures can be propagated in classical stirred tank bioreactors, although minor modifications are required due to the more viscose cell suspension, sensitivity to shear stress and tendency to foam [21,22,25]. Plant suspension cells have also been cultivated in several other types of bioreactors such as airlift, bubble columns, orbitally shaken vessels [26] and wavebag bioreactors [27]. In practise however, large scale commercial propagation has taken place in stirred tank bioreactors in volumes of tens of cubic meters [25].

Single use bioreactors are gaining ground also in propagation of plant cells. Pre-sterilized culture bags ease adherence to GMP, allow flexibility and reduce investments in sterilization facilities [25,26,28]. Protalix Biotherapeutics relies on their proprietary bioreactors based on pneumatically run flexible plastic bags. The bags are used for few consecutive culture cycles and allow horizontal scalability simply by increasing the number of bioreactors [9].

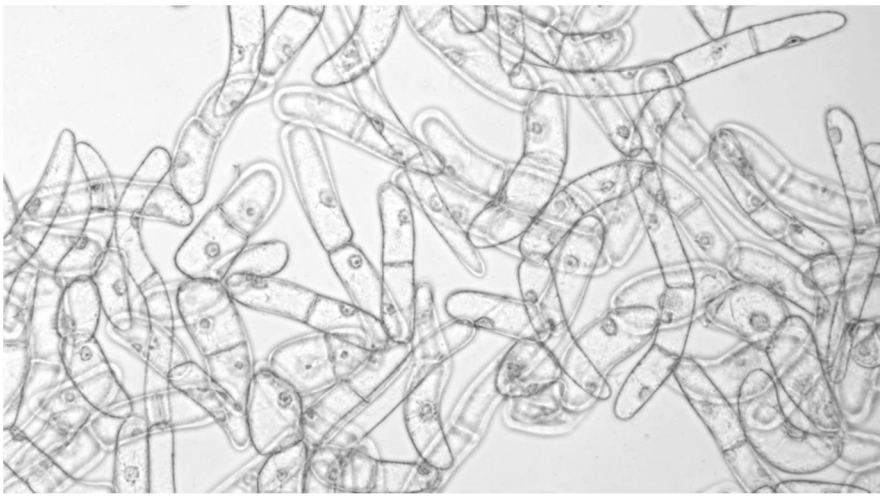


Figure 1. Tobacco BY-2 cells in suspension culture.

Plant species

Cell cultures have been established from numerous plant species. This can be seen as a disadvantage from regulatory point of view [12] or a resource, as the expression levels of the same protein vary between species [29]. The most used cell cultures for protein expression are derived from tobacco, rice and carrot [21]. Using food crops for molecular pharming on field is controversial (1.1.1), but in contained systems they may have a regulatory advantage due to GRAS status. Encapsulation of a pharmaceutical products in the cellulose capsule of plant cells could be utilized for oral administration [29]. For example a glucocerebrosidase product delivered orally within the matrix of carrot cells is being developed by Protalix Biotherapeutics [15].

Benefits of plant cells

From protein production point of view plant cell cultures combine the benefits of plants as a host organism to fermentation processes developed for more traditional platforms. Grown in bioreactors, the plant cell cultures are fully compatible with the existing GMP and similar process control to microbial or animal cell cultures [12]. Still, plant cells are inherently safe and capable of producing complex proteins. The benefit of scalability of field or greenhouse grown plants is lost when propagation is moved to bioreactors requiring large capital investments. However the cost of up-scaling is not fully comparable to propagation of mammalian cells. The complex and expensive media for mammalian cell cultures is a major cost and it increases directly with culture volume. The media for plant cells in turn, is cheap. While the plant cells do require similar facilities as mammalian cells, increasing the culture volume or operating continuous cultures are not a significant cost factors [26].

In comparison to intact plants, generation of transgenic cell lines and the growth of plant cell cultures is fast, which makes the production cycles significantly shorter [30]. A batch cultivation of tobacco cells takes approximately one week.

Product recovery from plant cell cultures is simple when the product is secreted into the medium, like in most other production platforms. As plant cells do not secrete much native proteins and media contains only few minerals, the purification is rather straight forward. Even when the product remains in the cells, requiring cell disruption, the plant cell material is easier to handle than intact plants as it lacks most of the fibres, waxes, oils and residues of agrochemicals present in whole plants. Cultured plant cells used for protein production also contain lower amounts of secondary metabolites in comparison to intact plants. Tobacco suspension cells for example contain only very low concentrations of nicotine. Downstream processing is discussed more in detail in 1.2.3

1.1.4 Future of molecular farming

It is unlikely that plant molecular farming will replace the traditional platforms for manufacturing recombinant proteins. It may however play an important part in manufacturing of niche products where its advantages are clear in comparison to other platforms in terms of quality, speed or economics. With the selected products, the focus needs to shift from proof of principle studies towards improving aspects important for commercial production: quality, purity and yield [31].

At the moment plant molecular farming covers several very different platforms: field and greenhouse grown plants, transient expression in plants, plants and moss cultivated in bioreactors and plant suspension cell cultures. In future it becomes more important to focus on and consolidate a small number of platforms [32]. One of those platforms will be the tobacco BY-2 cell line.

1.2 Tobacco Bright Yellow 2 suspension cells

The tobacco *Bright Yellow 2* (BY-2) cell line was originally generated in 1968 at Hatano Tobacco Experimental Station of the Japan Tobacco company [21]. It is fast growing, easy to propagate and can be readily transformed using *Agrobacterium tumefaciens* [33]. Especially, the possibility to efficiently synchronize the cell cycle and the morphology that supports microscopy have made the cell line a tool of choice for many laboratories studying the fundamentals of plant cell biology [34]. Due to its central role in fundamental research, the BY-2 cell line has been referred to as the “HeLa cell in the biology of higher plants” [33].

In 1982 another cell line was separated from BY-2. The sibling line, known as NT-1, has been maintained mainly in North America [35]. In principle the cell lines are genetically identical. However, decades of continuous passages have accumulated mutations and chromosomal rearrangements in both lines [36]. Thus neither of the lines is genetically identical to the original tobacco cultivar [33,36]. The BY-2 cell line has been more utilized in fundamental research (involving i.e.

cell cycle synchronization) which requires maintenance of the culture in optimal conditions for growth. It has been hypothesized that due to this selection pressure the BY-2 line may have retained its faster growth rate [35].

This chapter gives an overview on use of tobacco BY-2 cell line in protein production, its propagation, methods for downstream processing and ongoing platform development.

1.2.1 Use as a protein production platform

Rapid accumulation of biomass and easy transformation has made the BY-2 cells a good host for expression of recombinant proteins. Since the technology for heterologous protein expression in plant cells merged in early 90´s [23], a growing number of proteins have been successfully expressed in BY-2 and NT-1 cells (Table 1). By now, BY-2 has become the most frequently used plant cell line for protein production [29].

Protein yields in BY-2 cells are low, typically around 1% of total soluble protein (TSP), as in plant cell cultures in general. Reported yields range from hardly detectable to rare cases of hundreds of milligrams per litre (Table 1). In comparison, the product titres in mammalian CHO cells can reach levels of 10 g/l [2].

The first registration for a plant made pharmaceutical was obtained by Dow AgroSciences in January 2006 for a NT-1 produced vaccine against Newcastle disease in chicken [3]. However, the company made a strategic decision to end the product development [3]. Still to date no products produced in cultured tobacco cells are on market. However, recently Protalix Biopharmaceuticals has announced that all its new products are developed in tobacco BY-2 cells instead of carrot cells used earlier [9].

Table 1. Recombinant proteins expressed in tobacco cell cultures. Reporter proteins are not included.

Protein class	Protein	Cell line	Expression level (up to)	Reference
Antibodies	Mouse mAb	BY-2	N.A.	[37]
	Mouse IgG	BY-2	10 mg/l	[38]
	Mouse Fab	-	10mg/l	[39]
	mAb24	-	1% of TSP	[40]
	M12 (hIgG1) human scFv	BY-2	107 mg/l	[26,41,42]
	Lo-BM2 (hIgG1κ) Anti-IgM	BY-2	0.2 mg/l	[43]
	Human IgG	BY-2	35 mg/l	[38]
	Guy´s 13 (mIgG1k)	-	7.5 mg/l	[44]
	CL4 anti-HBsAg	BY-2	5 mg/l	[45]
	Anti-rabies mAb	-	0.5 mg/l	[24]
	Anti-phytochrome single-chain antibody	-	0.5% of TSP	[46]
	2G12 anti HIV	BY-2	5.5 µg/l	[47]
	2F5	BY-2	2.9 µg/g FW	[30,48]
	14D9 (mIgG1κ)	-	2 mg/l	[49]
Antigens	Norwalk virus capsid protein	NT-1	1.2% of TSP	[50]
	Newcastle disease virus eHN protein	BY-2	0.4% of TSP	[51]
	Influenza hemagglutinin	NT-1	1.4 mg/l	[52]
	Host cell-binding domain of E. coli O157:H7 intimin	NT-1	13 µg/g FW	[53,54]
	Hepatitis B surface antigen	NT-1	8 ug/g FW	[55,56]
	Dust mite allergens	BY-2		[57]
	Dengue virus envelope glycoprotein	BY-2	0.71 mg/l	[58]
	Allergen of Artemisia vulgaris pollen, Art v 1	-	<1µg/l	[59]
Enzymes	Transglutaminase	BY-2		[60]
	SEAP	NT-1	27 mg/l	[61,62]
	PRX-102 (a-Galactosidase-A)	BY-2		[63]
	Laccases (loblolly pine)	BY-2		[64]
	Laccase (lcc1 from lentinula edodes)	BY-2	250 U/l	[65]
	Human α-l-iduronidase	BY-2	10 mg/l	[66]
	Human α1-antichymotrypsin	BY-2		[67]
	Desmodus rotundus salivary plasminogen activator a1	BY-2	1.5 ug/g FW	[68]
	Carrot invertases	BY-2	3 mg/g FW	[69]
	β-Glucuronidase	NT-1	40 000 U/l	[70]
	ADA adenosine deaminase	BY-2	16 mg/l	[71]

Table 1. Continues.

Protein class	Protein	Cell line	Expression level (Up to)	Reference
Interferons and hormones	Viral IL-10	BY-2	5.3 ug/g FW	[72]
	Murine IL-10	BY-2	9.3 µg/g FW	[72]
	Human IL-10	BY-2	3% of TSP	[73]
	Interferon a2b - arabinogalactan –fusion	BY-2	28 mg/l	[74]
	Human IL-4	NT-1	180 µg/l	[75]
	Human IL-2	NT-1	100 µg/l	[75]
	Human IL-18	-	166 µg/l	[76]
	Human IL-12	-	175 µg/l	[77]
	Human growth hormone	BY-2	5 mg/l	[78]
	Human growth hormone - arabinogalactan – fusion	BY-2	35mg/l	[79]
	Human granulocyte–macrophage colony-stimulating factor	BY-2	17.89 mg/l	[80–83]
	Human granulocyte colony-stimulating factor	-	105µg/l	[84]
Others	Thrombomodulin	BY-2	27 ug/g Fw	[85]
	Human serum albumin	BY-2	22 mg/l	[86,87]
	Human lactoferrin	BY-2	4.3% of TSP	[88,89]
	EPO	BY-2	low	[90,91]

1.2.2 Expression systems

Transcription of heterologous genes in BY-2 cells is almost exclusively driven by constitutive cauliflower mosaic virus 35S promoter and either 35S, nopaline synthase (nos) or soybean vegetative storage protein (vsp) polyadenylation signal (references within Table 1). A constitutive *En2pPMA4p* promoter has been used with similar success to 35S promoter [38,43]. An oxidative stress-induced peroxidase (SWPA2) promoter drives strong gene expression during the stationary growth phase and has been used to express human lactoferrin in BY-2 cells [88]. Inducible expression of GFP has been reported using a 35S promoter-based tetracycline-specific derepressible expression system [92]. Bortesi and co-workers [72] applied the system to decouple accumulation of viral IL-10 from clone generation and growth phase. The inducible system allowed 3.5 fold increase in accumulation of the target, which has been shown to be harmful to tobacco plants and presumably also to BY-2 cells [72]. Estradiol induced expression of GFP, driven by tomato mosaic virus replication system has also been reported for BY-2 cells [93]. Similar approach was utilized in alcohol inducible expression of Norwalk virus capsid protein in NT-1 cells [50]. Nevertheless, none of the alternative expression systems have proven significantly better in BY-2 cells than the 35S promoter based system.

Transient expression of reporter protein GUS in BY-2 cells has been reported [92]. Regardless of the ongoing research efforts, transient expression mode has not yet been broadly utilized in protein production in plant suspension cells.

1.2.3 Generating production lines

The BY-2 cells can be transformed by nuclear bombardment or, more commonly, using agrobacteria (references within Table 1). Transformation of plant cells using agrobacteria results in insertion of the gene in random location and in variable numbers to the host genome [94]. The genomic location plays a pivotal role in regulating the gene expression by chromatin structure and proximity of regulatory elements. Larger copy number may also increase the expression significantly [3]. Therefore finding an elite line or a “jackpot clone” may increase the productivity by orders of magnitude [3], but requires tedious screening of a large number of transformants [38,42,56].

The tendency of plant cells to grow in cluster or cell files makes it difficult to pick a single transformation event to be sub-cultured. This may result in lines that are in fact heterogenic populations of cells with variable productivities [95]. This phenomenon has been observer with cell lines expressing fluorescent proteins as a mosaic or sectorial pattern of protein accumulation [42,72,94]. With time the metabolic burden of high expression of a recombinant protein may result in lower expressing cells to outcompete the more productive part of population and thus loss of overall productivity over time. The problem can be alleviated by generating monoclonal cell lines. This can be achieved by diluting cells, or protoplasts, into wild type “feeder” cells and re-generating cell lines from individual transgenic cells [94,95]

Co-expression of a fluorescent protein in the same expression cassette with a target protein as a surrogate marker and visual selection of calli with high and homogenous expression has been suggested as a tool for initial screening of the clones [72,94]. Correlation in accumulation level of a fluorescent marker and target protein has been reported [72]. The same principle was applied by Kirchoff and co-workers [42] for flow cytometric sorting of the most fluorescent individual cells of the population for sub-cloning. This approach resulted in significant increase in productivity and relatively long term stability of the clones. Similar techniques are commonly used for clone selection with mammalian cells [2].

In vitro cultured plant cells are affected by somaclonal variation: drifting of the phenotype due to genetic and epigenetic changes, including changes in chromatin states and methylation [96]. Genetic and genomic instability is very characteristic to dedifferentiated plant cell lines and karyotype variation has also been confirmed in BY-2 cell line [36]. Thus variation in expression levels are expected to arise during prolonged cultivation [95] and the selection steps discussed above may need to be repeated on regular basis.

Cryopreservation

Cryopreservation of the production lines in master and working cell banks is essential in order to avoid gradual loss of productivity over excessive passages [3]. Due to high water content, freezing plant cells is far from trivial. Nevertheless, robust protocols have been developed [97] and the productivity of a cell line has been shown to remain similar after regeneration of the suspension [86]. This has been a major step towards fulfilling the requirements of industrial standards [3,21].

Targeted insertions

Random insertion of the transgenes has two sides. On the other hand it can be seen as a tool to create diverse libraries of transformed lines where to select elite clones [31], but on the other hand it forces to tedious screening of the clones after every transformation. Vector-based site-specific integration and use of endonucleases allow insertion of the genes in known favourable hot-spots in genome and are a common practices with mammalian cells [2], but not with plants. However, new technologies may offer a shortcut.

The new genome editing technology based on clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) has shaken the fields of molecular biology as well as plant breeding in the past couple of years [98]. Targeted mutagenesis has been achieved in tobacco plants [99] and in BY-2 cells [100]. These advances pave road for targeted gene insertions through homologous recombination by homology-directed repair – a system that plant biotechnology is still missing. Next challenge will be to map and characterize so called “safe harbour loci” that would promote strong and stable expression of any inserted transgene. Standardised loci and precise tools to insert the heterologous genes will provide a shortcut to avoid excessive screening. Establishment of such a standardised expression platform may also facilitate the regulatory approval of the products and production lines [98].

Systems for gene amplification are another advantage of mammalian cells [2,21]. Multiplication of the copy number results in significant yield improvements, but the technology is still not developed for plant cells [21].

1.2.4 Propagation

The BY-2 cells are maintained as a callus culture on solid media or as cell suspension, normally in shake flasks. Requirements of the cell culture are rather simple: sufficient mixing, aeration and temperature of 26^{o}C to 28^{o}C. Unlike plants, the cultured BY-2 cells are not autotrophic: They grow in dark and need sugars as source of energy. A suspension culture of BY-2 cells is routinely maintained by weekly sub-culturing. Duration of the cell cycle is between 18.6 and 20.2 hours [34] and in ideal conditions a well maintained cell line can increase its biomass 100-fold in one week [35]. However, in many laboratories suspension cultures are maintained by diluting the mature culture only 20 to 30 times, instead of 100 [34]. At the end of the culture cycle the cell suspension reaches high cell density, rep-

resenting up to 70% of the suspension volume, causing high viscosity. This high viscosity needs to be considered in selection of bioreactor systems for up-scaling.

Selection of bioreactor

For larger volumes the BY-2 cells have been propagated in various types of bioreactors from simple wavebags, orbitally shaken vessels and bubble column bioreactors to standard stirred tank bioreactors originally designed for microbial fermentations and cultivation of animal cells. Most experimental data on propagation of BY-2 suspension cells has been generated, in addition to shake flasks, in stirred tank bioreactors (Table 2). The stirred tank bioreactors are also the established standard in the industry. Already a report from 1977 describes successful propagation of BY-2 cell suspension in 15 500 l culture volume in a steel tank bioreactor with accumulation of biomass in comparison to shake flask cultures [101].

Single use bioreactors are an emerging trend. Pre-sterilized culture bags or linings of the culture vessel lower significantly the needed facility investments. BY-2 suspension cells have been propagated in a 50 l single use stirred tank bioreactor [102] and recently also in 200 l volume in orbitally shaken single use vessel [26]. Simple cultivation systems, such as the air mixed polyethylene bags (up to 440 L) used by Protalix [9] are perfectly suitable to full fill the requirements of BY-2 cells. Additional benefit in these simple systems is their horizontal scalability: instead of increasing the culture volume the number of the bioreactors can be increased with low additional cost.

Large steel tank bioreactors require big capital investments. However, once the investments have been made, the industry may favour the already established systems over alternative processes. Thus the focus of this work will be on stirred tank bioreactors.

Table 2. Parameters used in some tobacco BY-2 suspension cell cultures in stirred tank bioreactors. N.A. stands for not available.

Vessel vol. (l)	Culture vol. (l)	Inoc. (%)	Aeration (vvm)	Treshold level of dissolved oxygen	Impeller type	Mixing (rpm)	Temp. (°C)	Antifoam	Reference
3	2	5	0.1	>20%	Pitched blade	N.A.	26	Pluronic L-61 (0.01%)	[103]
3	2	5	0.1	>20%	Pitched blade	125	26	Pluronic L-61 (0.01%)	[47]
3	1.75	10	0.1	>20%	Pitched blade	200	26	Pluronic L-61 (0.01%)	[86]
10	10	N.A.	N.A.	>30%	Turbines	120	25	Silbione 70414 (50mg/l)	[69]
N.A.	40	10	0.1		Three-bladed impeller	50	N.A.		[40]
3	2	20	0.43		Marine impeller	100	27		[104]
5	3	5	0.1-1.1		Four-flat-blade impeller	150	30		[70]
20 000	N.A.	N.A.	N.A.		Dual angled four-blade paddles	10-40	N.A.		[25]
2 000	N.A.	N.A.	N.A.		Dual disk turbine	10-100	N.A.		[25]
20 000	15 500	10	0.3		N.A.	N.A.	28		[101]

Culture parameters

The key parameters to consider in propagation of BY-2 suspension cells are aeration and mixing. In shake flask cultures gas exchange takes place passively through the flask mouth loosely covered with tin foil [70]. In stirred tank bioreactors aeration is normally supplied through different types of spargers in the bottom of the vessel or by aerating the headspace. Sufficient aeration is generally reached by sparging of 0.1 vvm (Table 2). Insufficient supply of air result in oxygen limited linear growth [25,70]. On the other hand, excessive sparging of air may cause uncontrolled foaming [70] and stripping the media of carbon dioxide and ethylene, key regulators of cell growth [25].

Transfer of oxygen from supplied air to the media is facilitated by agitation, which also provides efficient dispersal of nutrients and gases through the entire propagation vessel and prevents sedimentation [25]. In flask cultures sufficient mixing is provided by agitating the entire vessel on a rotary shaker, whereas stirred tank bioreactors are mixed with a rotating impeller. The efficiency of mixing depends on mixing speed and the used impeller type. Although a whole variety of different impeller installations have been reported for propagation of plant cells, the Rushton turbines, pitched blade impellers and marine impellers are most commonly used in steel tank bioreactors [25](Table 2). Rushton turbines are the industrial standard for microbial fermentations of low viscosity and with high oxygen demand. They generate predominantly radial and horizontal flow, but are not very efficient in generating circulatory flow. Pitched blade and marine impellers instead have better pumping efficiency and can mix viscose suspension more efficiently, especially when operated in upward pumping mode [25]. They also inflict less damage to cells than Rushton turbines when operated with same speed [25]. Insufficient mixing results in poor gas dispersion and oxygen limited conditions either locally or in the whole culture. High mixing speed, on the other hand, causes increased shear stress and cell damage [104]. Stress may induce secretion of polysaccharides and proteins in the media which may contribute to formation of foam [25].

Mixing speed and aeration rate can be adjusted during propagation based on inline measurements of dissolved oxygen and exhaust gases and pH of the media as well as off line measurements of accumulating biomass. However, real-time inline measurements of biomass development by radio frequency impedance spectroscopy (RFIS) may provide more accurate information of the process and development of response loops without off line sampling [103]

Although propagation of BY-2 cells in stirred tank bioreactors have been reported in several publications (Table 2), no general guidelines have been established.

Media

BY-2 cells are typically cultivated in media developed by Murashige and Skoog [105]. The media is composed of sucrose, mixture of salts and few plant growth factors and vitamins. The BY-2 cells have a distinctively high requirement on phosphate. The concentration of KH_2PO_4 has been increased three fold in modified MS commonly used to date [33].

The MS media was developed in the sixties in extensive trial and error based experiments and only to optimize growth. Recent research has focused specifically in improving accumulation of recombinant proteins.

Nitrogen is an essential building block of amino acids and proteins. Nitrate metabolism is also directly linked to carbon metabolism. The MS medium provides nitrogen in form of nitrate and ammonium [105]. A systematic analysis of the media showed that supplementing the media with additional nitrogen increased the yield of secreted antibody up to 150-fold, although with cost of the growth rate [47]. The increase may have been at least partly due to more efficient secretion of the protein in the stationary phase facilitated by larger osmotic pressure of the medium [3]. Later Vasilev and co-workers used factorial design to optimize the media for a cell line expressing a secretory antibody resulting in significant increase in yield and reduced packed cell volume [41]. The key changes here included increasing the nitrate/ammonium ratio and the amount of $CaCl_2$. Another experiment indicated that adding ammonium instead of nitrate, while reducing level of potassium, increased accumulation of biomass, amount of total protein and doubled the yield of intracellular ER-retained GFP [52]. Thus media composition seems to play a pivotal role in yield of recombinant proteins and different modifications may be required for secretory and intracellular proteins.

1.2.5 Downstream processing

Downstream processing can account for up to 80% of the total costs of manufacturing a recombinant protein [26,106]. A major part of the costs are product specific, such as chromatographic purification, and are not dependent on the production platform [12]. Only the first steps of the extraction process are host specific and therefore important to consider here.

Recombinant proteins can be either secreted to the culture medium or retained in the cell. Extraction and purification are easier if the product is secreted in the media. However, retaining the proteins in the cell often results in higher yield and desired post-transcriptional modifications [31]. When the product is retained in the cell, disruption of the cell walls, clarification of the extract and removal of contaminating soluble material are required. Efficient cell disruption can be achieved by pressure homogenization, sonication, enzymatic digestion or wet milling [27]. However, sonication and wet milling are difficult to scale-up and enzymatic maceration, although bulk enzymes could be used [40], may become cost prohibitive in large volumes [27]. Therefore pressure homogenization seems the most promising approach for large scale processes.

In addition to the product, cell disruption releases native plant proteins to the extract, including proteases. Product degradation can be prevented by cooling down the extract or using protease inhibitors. However, cooling may be hard to arrange and inhibitors prohibitively costly in large scale. The susceptibility to degradation depends on the target and preventive measures might not always be necessary [29].

Several methods have been developed to reduce the complexity of the extract before final purification, including heat precipitation of host cell proteins, flocculants to remove small particulates and generic chromatography steps [31]. High product concentrations are advantageous as cost of purification is proportional to the volumes of extract. Therefore the extracts are often concentrated using cross-filtration before chromatographic purification.

A significant advantage in plant cells is that in some cases expensive extraction of the protein is not necessary at all. For example a vaccine for Newcastle virus, produced in tobacco cells, was administered to chicken as crude extract by injection [3]. A protein product can also be administered orally even without disrupting the cells, as discussed in 1.1.

1.3 Hydrophobins

Hydrophobins are some of the most fascinating proteins in nature. They are detrimental to fungal life style and thus abundant in the environment and in food. In future the curious properties of these small and rigid proteins can be used e.g. in formulation of precisely targeted cancer drugs and in manufacturing of new biocomposite materials and in making of fluffier ice creams. The following chapters will give a brief overview on the biological role of HFBs, their structure and properties and applications including the use as a fusion partner for plant-produced recombinant proteins.

1.3.1 Biological role of hydrophobins

The hypha of filamentous fungi grow in moist substrates but reach in to the air to form reproductive structures. Some of those structures we call mushrooms. In order to reach air, the hypha needs to penetrate the water-air interphase where surface tension builds a barrier. The fungus secretes hydrophobins which assemble to the interphase dramatically lowering the surface tension (Figure 2). When in the air, the hypha keeps on producing hydrophobins which now assemble to the interphase between the cell wall and air. Normally the cell wall is hydrophilic and wets easily, but the amphiphilic hydrophobin layer inverts it hydrophobic instead (Figure 2d). This prevents the wettability of the aerial hypha and the fruiting body preventing them from falling back into the moist substrate and keeps water out of the gas channels. The layer of hydrophobins also covers the spores enabling their efficient dispersal by wind, water and insects. The coating also masks spores of some fungi, such as the opportunistic pathogen *Aspergillus fumigatus* from human immune system [107]. Hydrophobins also facilitate the attachment of the hypha and spores on hydrophobic surfaces, such as plant leafs or even Teflon. These properties are especially important for pathogenic organisms. Biological roles of HFBs are extensively reviewed in [4,108].

Most fungal species carry several genes for hydrophobins that are spatially and temporally regulated [109,110]. It seems that HFBs are adapted for specific roles.

However, all biological functions of hydrophobins are based on their extraordinary surface activity, ability to self-assemble to interphases and capability to reverse hydrophobicity of a given surface. These functions are all derived from the extraordinary structure of the proteins.

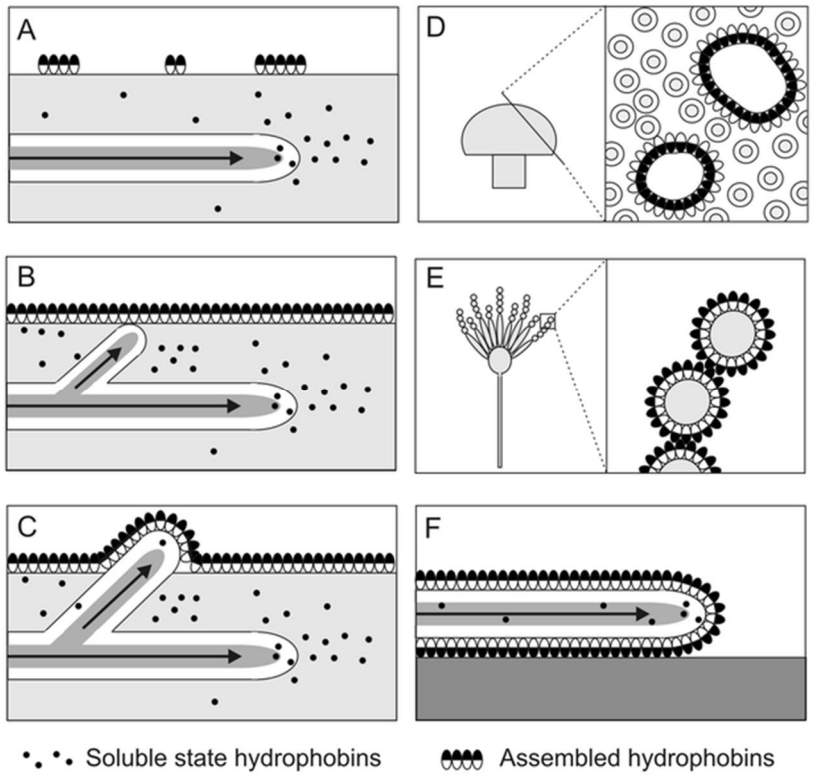


Figure 2. Illustration of some biological roles of hydrophobins during fungal lifecycle [4]. (**a**) Hypha of filamentous fungus growing in moist substrate secretes HFBs. (**b**) The HFBs assemble in to a membrane in water-air interphase and lower the surface tension. (**c**) Hypha breaches the interphase and continues producing HFBs that now assemble on the interphase of cell wall and air, rendering hydrophobic properties to the cell wall. (**d**) HFBs coat the fruiting bodies keeping water off the gas channels and (**e**) the spores facilitating their dispersal. (**f**) HFBs may also enable strong adhesion of the hypha to hydrophobic surfaces.

1.3.2 Structure and properties of hydrophobins

Hydrophobins are small globular proteins with diameter of about 2 to 3 nm and molecular mass around 10 kDa [108,111]. The proteins typically consist of 70-130 amino acids including a signal sequence for secretion. Although the primary structures of HFBs are very diverse they all share highly conserved sequence of eight

cysteine residues in a characteristic pattern of C-X-CC-X-C-X-C-X-CC-X-C (Figure 3). This suggests that all hydrophobins would share a common disulphide network and therefore also similar fold.[108].

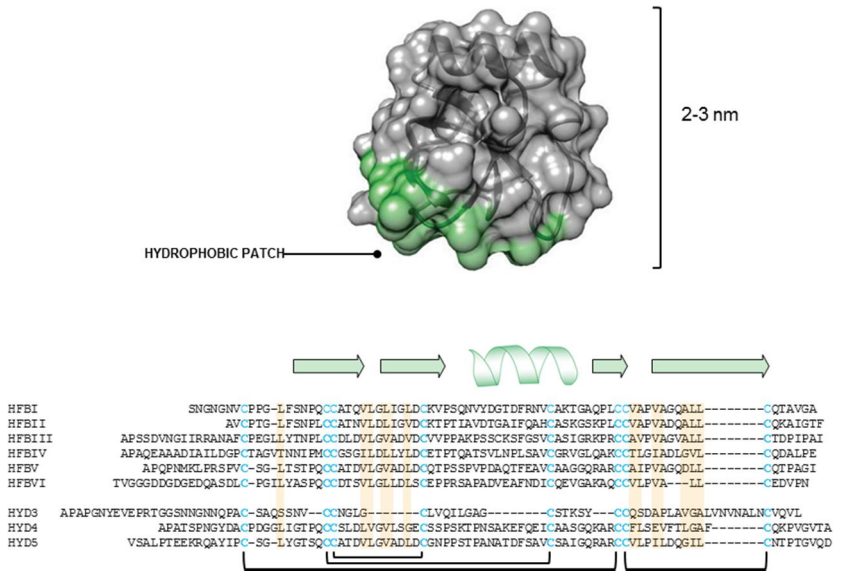


Figure 3. Tertiary (modified from Mathias S. Grunér, 2015), secondary and primary structure of some hydrophobins. The conserved cysteines (blue), disulphide bridges (black lines) and amino acids constituting the hydrophobic patch (orange) are highlighted. Extended N-terminal sequence has been removed from HFBVI.

The HFBs have been divided in two classes based on the hydropathy patterns in their sequence and spacing between the cysteine residues. In this work I will focus mainly on class II HFBs, which are found only in fungi of the phylum Ascomycota [108]. Especially, the focus will be on *Trichoderma reesei*, a filamentous fungus commonly used in production of both native and recombinant enzymes. *T. reesei* has an exceptionally broad arsenal of six class II HFBs [113]

The 3D structures of class II hydrophobins HFBI [111,114] and HFBII [110,115] from *T. reesei* have been resolved in detail and much of the structure of the other hydrophobins have been deduced from them. HFBI and HFBII consist of four β-sheets forming two β hairpins that further interlock in to a small barrel and one α-helix (Figure 3). The cysteine residues are linked with four di-sulphide bridges that stabilize the core of the protein. Two of the disulphide bridges are located inside the barrel stabilizing its structure. One disulphide bridge connects the N-terminal loop and another connects the α-helix to the barrel. The structure of hydrophobins is extremely stable and rigid [111,115].

Normally in soluble proteins the hydrophobic side chains are turned inside to form a stable hydrophobic core. However, the core of the hydrophobins is stabilized by the disulphide bridges and about half of the hydrophobic residues are exposed on the surface of the protein. Hence the hydrophobins can be thought to have turned inside-out. This peculiar fold makes hydrophobins stand out from all other known proteins [108]. The hydrophobic aliphatic residues are arranged into a large, flat hydrophobic patch near the loops of the two β hairpins on outer side of the barrel (Figure 3) [111]. When the sequences of HFBI and HFBII were aligned according to the conserved cysteines, the residues forming the hydrophobic patch were found to be the same [111] and highly conserved among all class II HFBs [108].

The hydrophobic patch makes the one end of the protein hydrophobic, while the other end remains hydrophilic. As whole, the hydrophobins are not particularly hydrophobic and thus should be better named as *amphiphilins*.

In very low concentrations the class II hydrophobins HFBI and HFBII are found in solutions as monomers [116]. However, when concentration increases dimer and tetramer forms become dominant to cover the energetically unfavourable exposure of the hydrophobic patch [115,116]. Despite oligomerization, the class II hydrophobins are highly soluble in water (up to 100 mg/ml) [111]. The oligomers dissociate when they come in contact with a hydrophilic-hydrophobic interphase, such as air-water, and the HFBs quickly self-assemble in to a highly structured monolayer to the boundary [117,118]. Orientation of the HFBs in the layer depends on the nature of the surface so that the hydrophobic patch is oriented towards the hydrophobic environment and vice versa. Thus the formed layer reverts the original nature of the surface [119]. The assemblages are in general extremely stable. In fact, the classification of HFBs was originally based on the solubility of these structures: Layers formed by class I HFBs can be normally dissolved with trifluoroacetic acid and layers of class II HFBs with 60% ethanol.

Differences between HFBs

Six class II hydrophobins have been identified in *T. reesei* [113,120] of which HFBI and HFBII are structurally very similar [111,115]. HFBI interacts more efficiently with non-ionic surfactant than HFBII (M. Linder et al., 2001; discussed in detail in 1.3.3). on the other hand HFBII lowers the surface tension of water faster than HFBI [122]. Yet HFBI was found to stabilize oil droplets in water over longer period of time [122]. Both form stable films on various hydrophobic and hydrophilic surfaces [123]. However, HFBI film on Teflon seems more repellent towards washing with water [122].

HFBIII is assumed to be structurally very similar to HFBI and HFBII based on the similarities in amino acid sequence, dimensions and functional characteristics. It does contain a ninth cysteine residue, which does not however seem to affect its behaviour in solution or interphases [118].

The amino acid sequence and the hydropathy profile of HFBIV are distinctly different from HFBI or HFBII and most substitutions occur on the surface of the protein possibly also influencing its properties [124]. Regulation of the gene appears

also different suggesting different biological role [120]. Nevertheless, HFBIV appears to bind tightly to both polar and hydrophobic surfaces, similar to HFBI and HFBII, altering their hydrophobicity [124].

HFBV and HFBVI have been identified from the *T. reesei* genome, but not characterized in detail [113]. The sequence data suggests that HFBVI has an extended N-terminal sequence [120]. The extended N-terminus is not expected to influence the core functions of HFBVI as hydrophobin, but could be hypothesized to be involved in further stabilization of HFB multimers [120].

1.3.3 Protein bodies

Plants store proteins in seeds, where they remain stable over long periods of time. The storage proteins accumulate in specialized storage compartments, such as ER derived protein bodies (PB) in cereals [17,125]. The PBs are membrane enveloped spherical structures, with diameter of approximately 1–2 µm, derived from the rough endoplasmic reticulum (ER) [126,127]. In plants the formation of PBs has been attributed to high concentrations of self-assembling prolamin storage proteins, such as zeins in maize ER. When reached their full size the PBs may bud off the ER and remain as terminally stored organelles in cytosol, integrate with other storage organelles or remain within the ER [127]. Final faith of the PBs depends on cereal species [17]

PBs can be artificially induced also in vegetative plant tissues by over expressing the storage proteins, or peptides derived from them, in the ER [128,129]. Interestingly the same plant derived proteins can induce PBs also in animal, fungal and insect cells suggesting that the phenomenon is not dependent on factors in plant ER, but the mechanism is conserved over all eukaryotes [126,129]. Moreover some proteins derived from animal origin, such as elastin-like polypeptides (ELP), are able to induce PBs in plants [130]. Fungal HFBs also accumulate in protein bodies when expressed as fusion proteins in plants, insect cells or filamentous fungus and targeted to ER [5,131–134].

The mechanism of PB formation appears to be extremely conserved, yet not completely understood. Accumulating data indicates that formation of these protein storage structures is due to interaction of eukaryotic ER with proteins of distinct characteristics [126,135]. Although originating from very different background, the most studied PB inducing recombinant proteins, ELP [130], zein derived Zera™ [128,129] and HFBs all share hydrophobic characteristics or amphiphilicity, and tendency to self-assemble [135]. High local concentration and subsequent aggregation of proteins in ER, beyond a certain threshold level, seems to play a central role in formation of PBs [127,131,132]. In some cases ER retention alone may be sufficient to trigger PB formation [17]. For example, expression of recombinant ER-retained GFP has been repeatedly observed to trigger formation of small PBs [5,130]. However, multimerisation of ELP, ZERA and HFBs may significantly increase the local concentration and thus trigger PB formation even with lower total expression levels [131].

Enhanced accumulation levels have been obtained when various proteins have been expressed as fusion with ELP [130], ZERA [129] and HFB (discussed in detail in 1.3.6). Accumulation of the protein in storage organelles has been though to improve the yields by shielding the proteins from physiological turnover, protecting the host from toxic effect an providing a sink to avoid overcrowding the ER [17,135]. Localization of recombinant proteins in PBs may also function as bioencapsulation, enabling oral administration of drugs and vaccines [125]. The PBs induced by recombinant proteins have been thought to bud off the ER and to be stored terminally in the cytosol as discrete compartments [5,130]. However, recent research indicates that they remain in contact with the ER, surrounded by the ER membrane and material can be transported between the PBs via ER lumen [136]. Also Zera™ induced protein bodies have been observed to remain in or surrounded by the ER lumen [128].

1.3.4 Applications of hydrophobins

The peculiar properties of hydrophobins have inspired numerous applications in food processing, biocomposite materials and medical technology that have been reviewed elsewhere [4,109,137]. This part will highlight some interesting prospects and focus in the most relevant applications in context of this work: purification by aqueous two-phase separation (ATPS) and use as coating of nanoparticles.

By assembling to elastic layers in the interphases HFBs stabilize foams to extreme extends already in minute concentrations [138]. This tendency has disastrous effects on beer. Gushing, the uncontrolled over foaming of beer, is caused by fungal contamination on the grain used for malting and residual amounts of hydrophobins [139]. The foam stabilizing characteristics can, however, be used in food industry in manufacturing of ice cream and other aerated products [138]. The fact that HFBs are already part of normal diet, in form of mushrooms, should facilitate the acceptance for use in food applications.

Self-assembly on surfaces as amphiphilic layers has been utilized in coatings of biosensors and biomaterials [140]. The protein layer improves biocompatibility and facilitates growth of mammalian cells and tissue on artificial support, such as Teflon [4,109]. Coating of medical instruments such as catheters and wires with hydrophobins can also radically reduce the friction and therefore also tissue damage [109]. Furthermore the HFB film on surfaces can be used to recruit and immobilize other proteins such as enzymes through non-covalent interactions [124,141].

Surfactant-based aqueous two phase separation

Hydrophobins HFBI and HFBII interact with non-ionic surfactants and can be purified through surfactant-based aqueous two-phase separation (ATPS; M. Linder et al., 2001). In aqueous solutions several non-ionic surfactants form a separate phase in appropriate temperature. The surfactant phase consisting of hydrophobic micelles and surrounded by aqueous environment accommodates well the amphiphilic hydrophobins. Separation of hydrophobins in to the surfactant phase is extremely efficient [121]. Recovery step with i.e. isobutanol replaces water in the

surfactant phase forcing the hydrophobins to the now energetically more favourable aqueous phase [121].

Fusing a hydrophobin to another protein allows them being used as purification tags for ATPS [142,143]. The size and properties of the fusion partner influence the separation, which is not as high as with HFB alone. Larger fusion proteins also seem to separate better in less hydrophobic surfactants [142]. The HFBI has been used as a tag to successfully purify recombinant proteins from fungi [133,143,144], insect cells [134] and plants (Joensuu et al., 2010) by ATPS. The system is also readily scalable up to volumes of 1200 l [144].

The exact mechanism of surfactant-based ATPS is not completely understood and development of the technology has relied heavily on trial and error. Different surfactants give very variable results [121,142]. However, commercial surfactants such as Agrimul NRE 1205 and Triton X-114 have been shown to be quite robust [143]. Larger amount of surfactant (8–10% w/v) gives better recovery, but lower amounts (2–4%) concentrate the product more effectively [5]. Additionally the separation temperature and ionic strength of the solution affect the separation [121,142].

Most significant benefits of the ATPS are its scalability and low cost in comparison to column chromatography. The whole process can be done in a single vessel and requires only liquid handling and cheap technical grade surfactants. These aspects are especially relevant in manufacturing of bulk products.

Nanoparticles

Delivering drugs to their targets is one of the biggest challenges in modern medicine. Especially cytotoxic drugs used in treatment of cancer, such as paclitaxel and doxorubicin, are harmful for all the cells in the body making treatment dose limiting [145]. Therefore the doses used have to be carefully adjusted and are often suboptimal for efficient treatment. Furthermore, increasing amount of new drugs are “brickdust drugs”: hardly soluble in water and exhibit poor bioavailability. New formulation methods are needed. Encapsulating drugs in to nanosize carriers, nanoparticles, is a potential solution for increasing solubility and controlling the delivery of the drugs to their targets. The abilities of hydrophobins to coat and solubilize solid particles and hide fungal spores from human immune system [107] have inspired their use as functional coating of nanoparticles.

HFBI and HFBII have been used to formulate spherical and stable nanoparticles from poorly soluble drugs beclomethasone dipropionate and itraconazole [146,147]. Coating with HFBI and HFBII have turned out to be the first environmentally safe method to produce monodispersed poly(vinylidene fluoride) nanoparticles that could also be freeze dried and re-dispersed without losing their properties [148].

Porous silicon (PSi) nanoparticles can be used as carriers for oral and intravenous drug delivery. Coating with HFBII improved the solubility and biocompatibility of the PSi particles still allowing release of the payload [149]. The coated particles were relatively stable in simulated gastrointestinal fluids and oral administration for rats increased the transit time from stomach to intestine due to improved mucoad-

hesion [150]. The HFBII coating also influences the distribution of PSi nanoparticles when administered to rats intravenously [151]. A major problem in parenteral administration of nanoparticle drugs is the adsorption of plasma proteins around the nanoparticles as a corona, causing aggregation and loss of activity. However, the HFBII coated PSi particles recruited significantly less plasma proteins than naked particles [151]. Avoidance of corona formation has been observed similarly with HFBII coated polystyrene nanoparticles [152].

1.3.5 Engineering bi-functional fusion proteins

By now it is clear that hydrophobins are extraordinarily versatile molecules with vast application potential in various industries. Moreover, the amphipathic properties HFBs can be carried to other proteins by rational design of chimeric fusion proteins. This brings about a completely new level of diversity to the applications.
For example, fusing active enzymes genetically to HFBI has enabled immobilization of endoglucanase on glass, Teflon and gold surfaces [123] and directing laccase activity to water-air interphase [153]. HFBI has also been utilized to immobilize peptides on graphene biosensors [140] and, as a fusion with two cellulose binding domains, to fabricate a novel and extremely durable nanocomposite by crosslinking nanocellulose with graphene [154]. With similar logic, the itraconazole nanoparticles (discussed in 1.3.5) were further functionalized by coating with HFBI genetically fused to a cellulose binding domain. The fusion protein allowed formulation of the nanoparticles, just like non-fused proteins, but also bound the nanoparticles tightly to cellulose nanofibrils within cellulose hydrogel [147]. The formulation in nanocellulose matrix stabilized the nanoparticles, but did not disturb fast release of the drug molecule or availability *in vivo* [147].

Most HFB-fusion proteins thus far have been constructed by linking the HFB-block from its N-terminus, which is thought not to interfere with the core functions of the proteins [120]. However, fusions to both N- and C-terminus have retained the functionality of the HFBI [123]. In contrast, fusing another protein to the N-terminus of HFBII seemed to reduce with hydrophobins ability to adhere on surfaces and to form multimers in solution [123]. In most published studies the HFB block has not interfered with the activity of the fusion partner and vice versa. Thus the HFBs appear as a versatile building block for bi-functional fusion proteins.

1.3.6 Hydrophobins as fusion tags in plant-based protein production

Utilization of HFBs as fusion tags for protein expression in plants serves two purposes. First, the induction of the protein bodies has been thought to enhance the yield of the recombinant proteins (discussed in 1.3.3). Second, the hydrophobins transfer some of their functional properties to the fusion proteins and thus enable use of ATPS a scalable method for first step purification (discussed in 1.3.4).

The first experiments on expression of GFP-HFBI fusion protein transiently in *N. benthamiana* did indeed result in doubled yield in comparison to non-fused GFP

and enabled efficient purification through ATPS [5]. However, since then only few target proteins, besides fluorescent markers, have been expressed and purified using HFB fusion technology (Table 3).

Table 3. Hydrophobin fusion proteins expressed in plants. C-terminally fused *T. reesei* HFBI was used in all experiments. Fluorescent proteins are excluded. N.A. stands for not available.

Target protein	Host	Effect on yield	Protein bodies	ATPS	Reference
Bacteriophage tailspike protein (Gp9)	*N.benthamiana*	Not clear	N.A.	N.A.	[155]
Xylanase (xyn11A)	*N.benthamiana*	Increase 10-fold	yes	N.A.	[132]
Influenza hemagglutinin (H5)	*N. tabacum* leaf and seed	Small increase	0.4±0.1 µm	N.A.	[156]
Polygalacturonase I	*N.benthamiana*	Reduced	N.A.	N.A.	[157]
Influenza hemagglutinin (H1)	*N.benthamiana*	Increased 2.5 -fold	N.A.	Yes	[158]
Influenza virus hemagglutinin (H1)	BY-2	N.A.	0.2-0.5 µm	N.A.	[158]
Glucose oxidase	*N.benthamiana*	N.A.	N.A.	Yes	[5]

The influenza hemagglutinins H1 and H5 were both found to accumulate in PBs when fused to HFBI, but both proteins were also found to form small PBs also without the fusion tag in BY-2 cells and transgenic tobacco plants, respectively [156,158]. Also HFBI fused xylanase was found to accumulate in PBs [132]. The fusion to HFBI only increased the yields of xylanase and H1, but not of any other fusion protein listed in Table 3. This strongly suggests that the yield enhancing effect of the HFB fusion tag is largely dependent on the target protein itself. Purification of plant-produced HFB-fusion proteins through ATPS has been earlier demonstrated only with glucose oxidase and H1, of which both separated with efficiency comparable to GFP-HFBI [5,158].

The enzymatic activity of HFBI fused glucose oxidase was similar to non-fused proteins and the HFB fused H1 protein was found to be immunogenic, indicating that the HFB-fusion tag did not hamper the biological function of either target proteins [5,158].

It is noteworthy that, until now, the HFB fusion technology in plants has relied solely on C-terminal fusion of *T. reesei* HFBI, leaving large variety of hydrophobins of different biological functions (discussed in 1.3.1 and 1.3.2 and reviewed in [109]) completely unexplored.

1.4 Target proteins

This work explores the use of hydrophobins as functional fusion partners for plant-based production of recombinant proteins. Two very different target proteins have been selected for two very different applications: *Stafylococcus aureus* Protein A and Human transferrin. This part gives a brief overview on their properties.

1.4.1 Protein A

Protein A in a cell wall bound pathogenicity factor of *Staphylococcus aureus.* The 42 kDa protein consists of five homologous immunoglobulin binding domains E, D, A, B and C and a cell wall binding domain [159]. Each of the IgG binding domains consist of a α-helical bundle stabilized by hydrophobic core [160]. They are capable of independently binding the constant regions of IgG1, IgG2 or IgG4 antibodies with high affinity, yet reversibly. The D and E domain also show affinity to Fab part of some antibodies. The affinity of protein A to antibodies is pH dependent. Affinity chromatography based on protein A is the industrial standard for purification of antibodies. Typically the antibodies are released from sepharose bound Protein A by decreasing the pH.

The antibody binding domains of Protein A have been previously expressed in plants as a fusion to oleosin in safflower seeds [161] and as fusion to tobamovirus coat protein in *N. benthamiana* [162]. Both studies aimed at low cost harvesting of recombinant antibodies from plant tissue.

1.4.2 Transferrin

Transferrin is an approximately 80 kDa, 679 amino acid monomeric glycoprotein. Transferrin contains as many as 19 intramolecular disulphide bridges making it challenging to produce in microbial systems [163].

The main function of transferrin is iron sequestration and transport in serum [18,145]. When free transferrin, apo form, binds two atoms of ferric iron turning in to holo form its conformation changes. The affinity of the transferrin receptor to holotransferrin is 10 to 500-fold higher than to the apo form. Uptake of transferrin by endocytosis and subsequent change in pH releases the iron and the protein is recycled back to bloodstream [18,145]. Transferrin has numerous therapeutic applications and its role in iron transport makes transferrin an essential component in growth media for mammalian cell cultures [18].

Transferrin receptor is ubiquitously expressed on normal cell types, but is up-regulated several hundred fold on various metastatic and drug resistant tumours reflecting increased consumption of iron [145]. This, combined with the capacity of transferrin to cross the blood brain barrier, has made it an utmost interesting molecule for active and selective drug targeting by direct conjugation to the active molecule or to nanocarriers [18,145].

Plant-based production of recombinant transferrin is a safe and scalable alternative to extraction from human plasma or expression in animal cells [18]. Production of transferrin in transgenic tobacco accumulated to 0.25% of TSP [163], but has reached yields of 1% of seed dry weight in rice [164]. Plant derived recombinant transferrin has been shown to be structurally and functionally similar to native human protein although it appears to be not glycosylated [18,163,164]. Human lactoferrin is an iron binding protein closely related to transferrin that shares 61.4% sequence homology and highly similar structure [163]. Active lactoferrin has been successfully expressed in BY-2 cell cultures yielding up to 4.3% of TSP [88,89].

Recombinant transferrin is produced commercially in rice seeds by InVitria under trade name Optiferrin™ and in yeast under name DeltaFerrin™ by Delta Biotechnology Ltd.

2. Aims

Hydrophobins have gained a lot of attention for their wide application potential in biotechnology and in material science. However, production of HFBs and HFB-fusion proteins has been challenging. Plants are emerging as an alternative platform for production of complex proteins. The overall aim of this work was to evaluate the suitability of plant-based production systems for manufacturing of HFB-fusion proteins. More specifically the aims were to:

1. Study a range of different HFBs as potential fusion partners (II)

2. Develop further the concept of bi-functional hydrophobin fusion proteins (III, IV)

3. Study expression of HFB-fusion proteins in BY-2 suspension cells (I, III, IV)

4. Develop large scale propagation of the BY-2 suspension cells and create practical solutions for clone selection and downstream process (I, III, IV)

3. Materials and methods

3.1 Cell cultures: maintenance and transformation

All experiments were conducted using *Nicotiana tabacum* Bright Yellow 2 cell line [33], received from University of Gent. Transformation of the BY-2 cells was performed as described earlier [165]. The cell lines were maintained as calli on modified MS-medium [35] supplemented with 25 mg/l kanamycin and were sub-cultured every 3–4 weeks by visually selecting the most fluorescent fractions under UV-light, when a fluorescence marker was used. Suspension cultures were maintained in 50 ml of the modified MS-medium supplied with 50 mg/l kanamycin and sub-cultured weekly by transferring 5% (v/v) of the culture to fresh media.

The stirred tank bioreactors and culture parameters used for propagation of BY-2 suspension cells in pilot scale are presented in Table 4.

3.2 Determination of biomass

It should be noted that the fresh weight (FW) of suspension cells was determined in this work by sampling 10 ml of culture suspension in a pre-weighed 15 ml tube, pelleting the cells by centrifugation (10 min, at RT, 3220 *g*, Eppendorf Centrifuge 5810R) and weighing the pellet after pouring off the supernatant. The FW obtained like this corresponds to packed cell volume (PCV) reported in many other studies: FW of 600 g/l here corresponds to PCV of 60%.

The dry weight (DW) reported here was determined by weighing the same pellet after freezing and freeze drying. This value should correspond well to the DW reported elsewhere, determined after drying the sample for example at 60 °C.

Table 4. Parameters for all BY-2 propagations in stirred tank bioreactors. (IF 40, IF400, BioFlo 510 and BioFlo PRO: New Brunswick Scientific, Enfield; Braun C-20: Sartorius Stedim Biotech) *Airflow was divided manually between the sparger and headspace. (N.A. stands for not available.)

	Bioreactor	Cell line	Volume (l)	Agitation	Tip speed (m/s)	Air flow (vvm)	Temp. (°C)	DO aim (%)	Pressure (bar)
1	Braun C-20	GFP-HFBI	20	75-150	0.4 - 0.8	0.1-0.2	28	20	N.A.
2	IF 40	GFP-HFBI	30	75-200	0.4 - 1	0.1-0.3	28	20	0.2
3	BioFlo PRO	GFP-HFBI	600	40-120	0.75 - 2.25	0.13 - 0.25	28	30	0.2 - 0.3
4	BioFlo 510	HFBI-ProtA	30	100-300	0.56 - 1.67	0.1-0.3	28	20	0.1 - 0.2
5	BioFlo 510	HFBIV-Tf	33	100-210	0.56 - 1.17	0.1-0.2	28	20	N.A.
6	BioFlo 510	WT	30	100-320	0.56 - 1.78	0.1-0.15	26	20	0.2
7	BioFlo 510	WT	30	100-320	0.56 - 1.78	0.1-0.15	26	20	0.2
8	IF400	WT	300	60-155	0.72 – 1.86	0.05-0.5*	26	20	0.2 - 0.6
9	BioFlo 510	WT	30	100-260	0.56 - 1.44	0.1	26	20	0.2 - 0.3
10	BioFlo PRO	WT	600	40-120	0.75 - 2.25	0.1-0.2	26	20	0.2 - 0.3

3.3 Protoplast preparation and immunolabeling

Agro-infiltrated leaves of *Nicotiana benthamiana*, harvested 6 days post infiltration, were cut into strips and digested in enzyme solution (1.5% w/v cellulaseR10 (Serva Germany), 0.4% w/v macerozymeR10 (Serva, Germany), 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM CaCl2, 5 mM β-mercaptoethanol) at RT overnight. Protoplasts were sieved through a 100 µm mesh and centrifuged (10 min, 80 g, 4 °C) and washed twice with WI buffer (0.5 M mannitol, 4 mM MES pH 5.7, 20 mM KCl). Protoplasts were prepared from BY-2 cells by digesting cell walls in PNT buffer (MS salts, 27.384 g/l sucrose, 0.5 mg/l NAA, 1.0 mg/l BAP) supplemented with 1% w/v cellulaseR10 and 0.3% w/v macerozymeR10 overnight at RT. Protoplasts were separated by centrifugation (100 g for 5 min) washed 3 times with PNT and pelleted in buffer (9 g/l NaCl, 18.38 g/l CaCl2, 0.375 g/l KCl, 1 g/l glucose) by centrifugation (100 g, 10 min).

The protoplasts were fixed in 4% w/v paraformaldehyde (Sigma-Aldrich, USA) in WI for 1 h at RT followed by incubation in 3% IGEPAL CA-630 (Sigma-Aldrich) and 10% DMSO (Merck, Germany) in PBS for 5 min at RT. Non-specific binding was blocked by incubation in 2% w/v BSA (Sigma-Aldrich) in PBS for 1 hour at RT.

Primary antibody against the c-Myc tag (mouse, A00864, GenScript, USA) was applied in PBS (1:100) and incubated at 4 °C overnight. Secondary antibody, conjugated with Alexafluor®568 (donkey anti mouse, A10037, Life Technologies, USA), was applied in PBS (1:100) and incubated for 2 hours at 38 °C. Between each step the protoplasts were washed three times with PBS.

3.4 Other methods

All other methods used in this work are described in detail in the original publications as listed in Table 5.

Table 5. Methods used in this work.

Method	Used and described in
Gene constructs and cloning	I, II, III, IV
Transient expression in *Nicotiana benthamiana*	II, III, IV
Aqueous two phase separation (ATPS)	I, II, III, IV
Protein Extraction and analysis	I, II, III, IV
Confocal microscopy	I, II, III, IV
Transmission electron microscopy	IV
Quartz crystal microbalance with dissipation monitoring	III
Preparation and analysis of porous silicon nanoparticles	IV
MDA-MB-231 cells and assays	IV
Transferrin iron binding assay	IV

4. Results and discussion

4.1 Hydrophobins to improve protein yields

The *Tricoderma reesei* HFBI fusion tag has been shown to increase yields of some fusion proteins when transiently expressed in plants [5]. Goal in this study was to evaluate whether other HFBs would also have an effect on the yield of recombinant fusion proteins and whether they could also be used for purification of the fusion proteins by ATPS.

4.1.1 Various hydrophobin tags influence the yields of respective fusion proteins in *Nicotiana benthamiana*

A library of HFB fusion proteins was created by fusing eight different HFBs to GFP both N- and C-terminally (II). The library covers HFBII [110], HFBIII [118], HFBIV [124], HFBV and HFBVI from *T. reesei* and HYD3, HYD4 and HYD5 from *Fusarium verticillioides* [166]. Transient expression of the library in *N. benthamiana* plants resulted in variable levels of protein accumulation (II). Later on HFBI and HFBII were fused to Protein A and HFBI (III), HFBII and HFBIV to transferrin (Tf) (IV). In this work a variant of Protein A was used that contains all five binding domains, but not the cell wall anchor. In addition, five glycosylation sites have been mutated to avoid N-glycosylation (III).

Of the new HFB tags, HFBII placed in the N-terminus of GFP showed highest accumulation: up to 21% GFP of TSP. Head to head comparison to HFBI-GFP revealed a two-fold increase in yield of GFP. Here the genes coding for both HFBII and HFBI were codon optimized. Thus the results are not fully comparable to previous reports of non-codon optimized and differently oriented GFP-wtHFBI accumulating up to 38% GFP of TSP [5]. Yet, in line with the experiments with GFP, a HFBII-Protein A fusion accumulated better than wtHFBI-Protein A (III). This supports the conclusion that HFBII-tag placed in the N-terminus of the fusion may indeed provide yields superior to those of HFBI-fused proteins. Curiously, HFBII placed in the C-terminus of the GFP-fusion yielded less than 1% GFP of TSP (II). Same trend was observed with other fusion partners. Both Protein A (III) and Tf (IV) accumulated significantly better when HFBII was connected to the N-terminus of the fusion instead of C-terminus. Comparatively low accumulation of

fusion proteins where HFBII is connected to the C-terminus of the target protein was hypothesized to relate to the short N-terminal amino acid sequence of the HFBII (II). Only two amino acids separate the linker from the first di-sulphide linked cysteine residue. With the linkers used in this work, the short C-terminal peptide chain may not provide sufficient space for proper folding. Thus the N-terminally fused fusion proteins may be prone to misfolding and directed to degradation pathway. In line with the results here, an early experiment with HFBI and HFBII fused endoglucanase indicated that the HFBII fusion protein partly lost its surface activity and ability to form multimers in solution [123]. A longer and more flexible linker could better allow folding and thus improve the yields.

Orientation of the HFBI-tag did not influence accumulation of Protein A (III), but the Tf-HFBI accumulate significantly less than HFBI-Tf (IV). Significant (p<0.05) differences in yield between the orientations of the GFP fusion protein were observed also with HYD4 and HYD5 (II). With both proteins accumulation was higher when the HFB was placed in the C-terminus. In contrast to HFBII, the differences here cannot be explained by short C-terminus of the HFBs. Hypothesis of the role of the short N-terminus of the HFBII has also been tested by replacing the short N-terminal sequence of HFBII with the N-terminus of HFBI. This did not solve the poor accumulation suggesting that the reason for low expression levels may be more complex (personal communication: Dr. Joensuu, 2016).

The most interesting question was: Do HFB-tags improve accumulation of target proteins? Protein A was expressed in *N. benthamiana* as fusions with HFBI and HFBII. In line with the experiments on GFP (II), both HFBI-Protein A and HFBII-Protein A accumulated significantly better than non-fused Protein A, HFBII performing slightly better with approximately 35-fold increase (III). However, in contrast to GFP and Protein A, accumulation of Tf was not improved by fusion to HFBs (IV). There were no statistically significant differences between accumulation levels of fusion proteins where HFBI, HFBII or HFBIV were connected to the N-terminus of transferrin. However, placing HFBI of HFBII in the C-terminus instead of N-terminus lowered the yields. In line with results obtained with GFP, fusions to HFBIV accumulated similarly in both orientations.

4.1.2 Accumulation of HFB-fusion proteins in BY-2 cells

Stably transformed tobacco BY-2 cells represent, in many respects, a very different expression platform in comparison to transient expression in *N. benthamiana*. Therefore validating the results of transient expression assays in BY-2 platform is necessary. Expression levels of different gene constructs in transient expression assays can be compared with relatively good reliability and reproducibility. However, the differences between BY-2 cell lines transformed with the same construct are vast (discussed more in 4.3.1) making detailed analysis challenging.

GFP-HFBI was the first hydrophobin fusion protein expressed in BY-2 cells (I). Although the HFBI fusion tag had doubled the accumulation of GFP in transient assays [5], only a slight increase could be observed in BY-2 expression system (Figure 4).

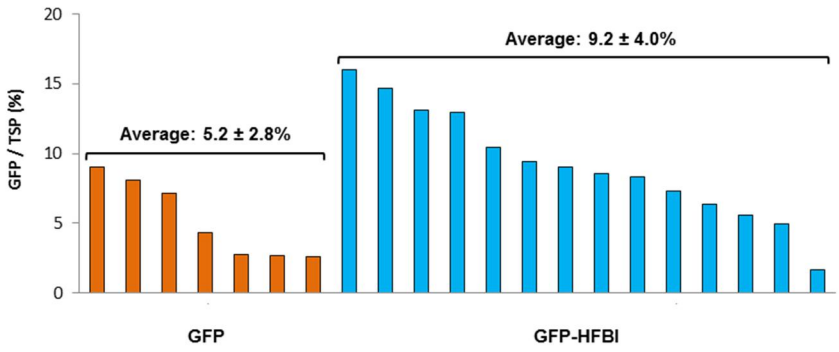


Figure 4. Accumulation of GFP in callus lines expressing either non-fused GFP or GFP-HFBI. (Unpublished data).

In line with transient expression in *N. benthamiana,* accumulation of Protein A in BY-2 calli, was clearly improved by both N-terminal HFBI and HFBII tags (III). In contrast to transient expression, no apparent difference was observed between accumulation of Protein A fused to HFBI or HFBII. However, the sample size did not allow detailed comparison of the constructs. A callus line with good expression level of HFBI-Protein A and favourable growth characteristics as a suspension culture was selected for further experiments. In suspension the cell line reached yield of 36 mg/l, which in respect to general yields in BY-2 suspension cultures (Table 1) represents a good level of protein accumulation.

Relying now on good correlation of expression levels in *N. benthamiana* and BY-2, Tf was expressed in BY-2 cells as Tf-HFBIV fusion (IV). The construct was not the best accumulating one in transient assays (IV), but the novelty of using HFBIV was a tempting selection criteria. Some cleavage of fusion proteins with HFBs place in the C-terminus suggested that N-terminal fusion may prove to be more stable. A suspension culture of a BY-2 cell line expressing Tf-HFBIV reached a good yield of 25 mg/l (IV).

The cell lines for both HFBI-Protein A and Tf-HFBIV were selected based on the expression levels determined in calli. However, the characteristics of cell lines as callus and in suspension culture are often not related and therefore it would be advisable to do the last screening steps in suspensions [21,26].

The results obtained with Protein A-fusions as well as preliminary analysis of calli expressing Tf and other target proteins fused to HFBI, HFBII and HFBIV (data not shown) indicate that screening of constructs in *N. benthamiana* may indeed give a relatively good estimation of protein accumulation in BY-2 cells.

4.1.3 Formation of protein bodies

The ability of HFBI to increase the yield of respective fusion partners has been attributed to the tendency to induce formation of protein bodies in the host cells [5,135]. The data on protein body formation has been generated largely using GFP as a target molecule. In this work the HFB-library (4.1.1), was utilized to study the localization of various HFB-fusion in *N. benthamiana* (II).

Consistent formation of protein bodies, similar to GFP-HFBI, was observed only with fusion proteins that accumulated to relatively high levels (II), such as HFBII-GFP. Fusion proteins with lowest accumulation levels were observed predominantly in the reticulate pattern of ER. These results are well in line with the previous reports suggesting that a threshold level of accumulation is essential for PB formation [131] and that higher concentration of recombinant protein in ER correlates with larger and more consistent PBs [132].

This work describes, for the first time, formation of protein bodies also in BY-2 cells (I). Non-fused, ER-targeted GFP was located predominantly in the reticulate structure of ER, while some spherical structures resembling protein bodies were observed in older cells with high concentration of GFP. However, GFP-HFBI accumulated in protein bodies even though the accumulation level was lower than with non-fused GFP, 30% in comparison to 17% of TSP, respectively (I). The central space of the cells was occupied by large vacuolar compartments and the protein bodies as well as the whole ER network were pushed in the periphery of the cells (I).

Research on protein body formation in plants has focused mostly on fluorescent proteins, such as GFP, that can be easily imaged without extensive sample preparation. This raises a concern of the potential role of the target proteins themselves in localization of the fusion proteins. Here the subcellular localisation of Protein A (III) and transferrin fusions (Figure 5) was investigated by immunofluorescent microscopy. The method was validated by immunolabeling GFP-HFBI expressing protoplasts of *N. benthamiana* with antibodies against the c-Myc-tag on the protein. Similar pattern of protein bodies could be imaged capturing the fluorescence either from GFP or the fluorescent label (III). This confirmed that the treatment of the protoplasts did not disrupt the subcellular localization pattern significantly. Small spherical structures, similar to those induced by GFP-HFBI, could be observed also in protoplasts prepared from leafs expressing Protein A, HFBI-Protein A and HFBII-Protein A (III). No apparent difference could be observed between localisation in any of the proteins, although the PBs induced by Protein A – constructs seemed to be less abundant than the GFP-HFBI induced PBs. This result is surprising as the yields of both HFB-Protein A and HFBII-Protein A were generally significantly higher than the yields of non-fused Protein A.

Tf, in contrast to Protein A, does not seem to accumulate in protein bodies in *N. benthamiana* regardless of the fusion to HFBIV, but is observed in the ER lumen (Figure 5). Co-expressed GFP-HFBI (discussed in detail in 4.3.1) accumulates in familiar protein bodies. Interestingly, when co-expressed with GFP-HFBI, both fused and non-fused Tf seemed to localize around or within the protein bodies

formed by the GFP-HFBI. Similar localization pattern was also observed in protoplasts prepared from BY-2 cells co-expressing transferrin-HFBIV and GFP-HFBI. These observations pose two interesting suggestions. First, the formation of protein bodies may be dependent not only of the properties of HFBs and protein concentration in ER, but also of the respective fusion partner: GFP itself may play a central role. Second, the observation supports the conclusion of Saberianfar and co-workers [136] that the HFBI induced protein bodies do not bud off the ER, but remain within the membrane system surrounded by other ER proteins, such as recombinant Tf here.

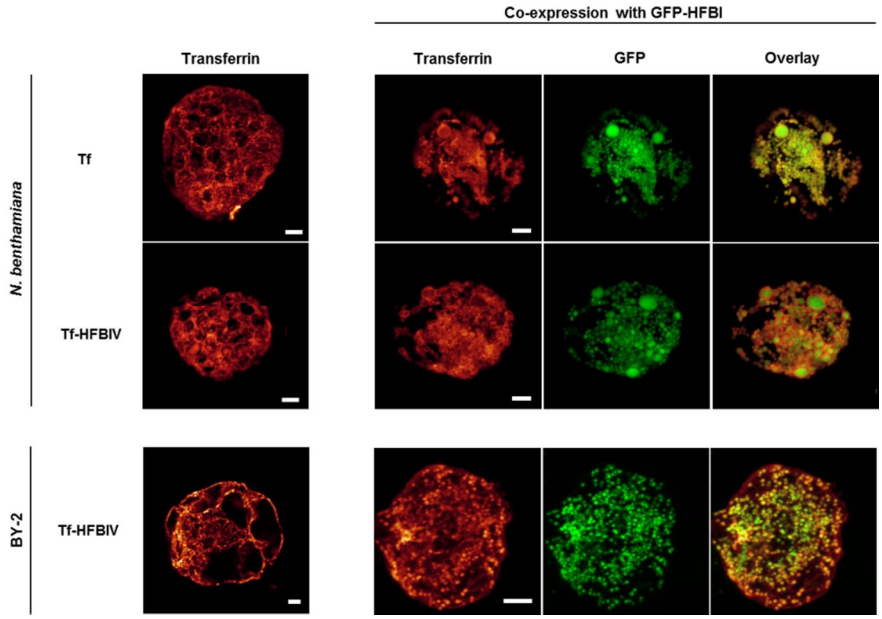


Figure 5. Immunolocalization of Tf, Tf-HFBIV and GFP-HFBI in protoplasts prepared from BY-2 suspension cells and agro infiltrated *N. benthamiana* leafs. Tf-HFBIV was detected with a primary anti- c-myc antibody (mouse) and secondary donkey anti mouse IgG labelled with Alexafluor568. Fluorescence signal derived from Alexafluor568 is presented as a heatmap: red indicates low intensity and yellow high intensity. Fluorescence from GFP is presented in green. Scale bars correspond to 10 µm. (unpublished data).

It is important to note that in imaging of Protein A or Tf constructs, the protein levels were not quantified from the same samples used for imaging. This undermines the reliability of conclusions, considering that PB formation appears to be a concentration dependent phenomenon. In general, the cell to cell variation in protein accumulation in both cell cultures and plant leaves can also be significant.

Altogether, the results presented here suggest that HFBs do enhance formation of protein bodies when fused to GFP, but also a certain threshold level of accumulation is required. However, when fused to either Protein A or Tf the HFBs had no apparent effect on localization of the proteins. This raises the question on the role of GFP. Some earlier studies on HFBI fused to other targets than GFP have reported formation of PBs [156,158]. However, the structures have been small, irregular and by far not as abundant as with GFP, similarly to the images obtained here with Protein A fusions. Round and consistent PBs have been imaged in relation to expression of a xylanase-HFBI, but only with co-expressed GFP [132]. All in all, it appears that clear connection of PB formation and increase in protein accumulation has not yet been shown with any other target proteins than GFP.

Besides GFP, fusion to HFBI has significantly improved the accumulation of two target proteins, a xylanase [132] and Protein A (III), but not of any others (Table 3). In both cases the role of PB has not been clear suggesting that formation of PBs may be an artefact. This leaves open the question of other possible yield-increasing mechanisms of the HFB fusions, which may apply only for certain fusion partners.

4.1.4 Attempts to express proteins that are harmful for the host cell

One of the expected advantages in using HFB fusion has been that accumulation of the respective fusion proteins in discrete envelopes of PBs would protect the host cell from toxic effects of the product [5,135]. Thus the HFB fusion technology has been speculated to enable expression of proteins that are otherwise difficult to produce.

Here three such proteins were selected as targets: phytocystatin Act d 4 [167], a *Nicotiana tabacum* cysteine protease [48] and glucose oxidase [5]. HFBI was fused to the C-terminus of each protein. BY-2 suspension cells were transformed with the constructs and transgenic calli was recovered. However, most of the calli were lost after few passages and no protein expression could be detected in the remaining clones (unpublished data). This may suggest that expression of the target proteins was indeed lethal to the host cells, at least in large concentrations [72].

As discussed above (in 4.1.2.), it seems that the protein bodies may in fact not leave the ER after all and thus cannot be regarded as discrete organelles. This is supported by recent findings of Saberianfar and co-workers indicating that the content of HFB induced protein bodies is connected to ER and is exchanged with the neighbouring, connected, protein bodies [136]. Therefore the hypothesis of protective effect of the protein bodies may need to be revised.

4.2 Bi-functional HFB fusion proteins

In light of the results discussed in the previous section, the initial idea of this work, suggesting that HFB-tags could be used as a general tool to improve yields in

plant-based protein production, may need to be re-considered. It is time for a frame shift. When used as fusion tags for recombinant proteins, HFBs carry some of the functionalities to the respective fusion protein. Hence the activities of recombinant proteins can be combined with properties of HFBs in a single molecule in a “click and go” fashion. The target proteins selected for this work provided an excellent opportunity to explore the potential of the fusion strategy for construction of bi-functional designer molecules.

4.2.1 New HFBs as purification tags for ATPS (II)

ATPS is an excellent method for first step purification of HFB fusion proteins from plants and especially from BY-2 suspension cells due to the very low background in the recovered fraction (I, II, III, and IV). Majority of the native plant proteins remain in the aqueous residue and only few co-separate in to the surfactant phase. The titre of the recombinant proteins is often low in plants and especially in in plant suspension cell platforms. Thus a “one pot” method requiring only liquid handling and providing a degree of concentration would be beneficial. The ability of HFBI and HFBII to interact with non-ionic surfactants and efficiently separate in ATPS has been studied widely, but only HFBI has been utilized for purification of recombinant fusion proteins.

The eight new HFB´s tested for protein expression in *N. benthamiana* (discussed in 4.1.) were also evaluated for purification of respective GFP fusion proteins from plant leaf extract. Only HFBII and HFBIV were able concentrate the fusion proteins in to the surfactant phase, while all other fusion proteins distributed evenly between the phases (II).

To exclude the effect of plant matrix and protein concentration [5] a head to head comparison of HFBII and HFBIV with the well-established HFBI was performed by spiking the purified proteins to buffer in equal concentrations. Recovery rate of HFBI-GFP was 83±4%, which is comparable with previous findings [5]. The recovery rates of HFBII-GFP and HFBIV-GFP were only slightly lower, 71±7% and 75±4%, respectively. The results are in line with early experiments indicating that HFBI separates more efficiently in ATPS than HFBII [121].

The results were not tied to GFP. When fused to Protein A, both HFBI and HFBII were capable of separating the fusion into the surfactant phase in ATPS whereas the non-fused Protein A remained mostly in the residue (III). The separation efficiency was comparable to that of respective GFP fusion proteins (II). The Tf-HFBIV fusion also migrated to surfactant phase with similar efficiency to GFP-HFBIV (IV) and HFBIV-GFP. The recovery rate for Tf-HFBIV was 88±2%, while only 9±1% of the non-fused Tf (Optiferrin) was recovered from the surfactant phase. To my knowledge, the separation of HFBIV or HFBIV-fusion proteins in two phase system has not been studied before.

The two-phase separation method has been optimized for HFBI [5] and thus further optimization of the system may balance out the observed differences between different HFB´s. Nonetheless, these experiments suggest that in addition to

HFBI also HFBII and HFBIV can be used as building blocks for fusion proteins capable to interact with non-ionic surfactants.

It is important to note, however, that the ability to interact with non-ionic surfactants seems to be decoupled from other properties of HFB´s, such as surface activity [168]. Therefore the HFB´s that did not perform in the initial ATPS experiments may turn out as useful fusion partners for other applications.

4.2.2 Fishing antibodies: HFBI-Protein A (III)

Purification of recombinant HFB-fusion proteins by ATPS (4.2.1) requires in many cases removal of the HFB tag after purification. This may not be economically feasible or may interfere with the integrity of the target protein. The aim here was to create a bifunctional fusion protein combining the properties of HFBs and the antibody binding domain of Protein A (III). On the other hand the molecule would interact with non-ionic surfactants in ATPS and on the other hand it would reversibly bind antibodies, thus concentrating and purifying antibodies in two-phase system as illustrated in Figure 6.

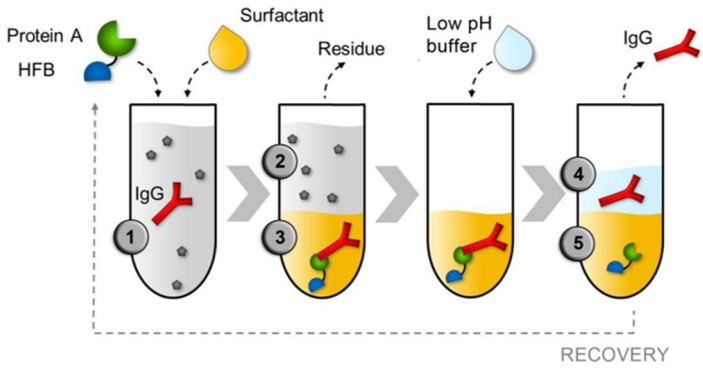


Figure 6. The concept of in-solution antibody harvesting using a HFB-Protein A fusion protein. The Protein A block (green) binds to the IgG (red) when added to the antibody-containing cell extract (1). Addition of a surfactant (tan) results in a two-phase system. The HFB block (blue) guides the HFB-Protein A/IgG complex to the surfactant phase. The aqueous residue (2) is discarded. The IgG is released by addition of acidic buffer and recovered from the aqueous phase (4). The HFB-Protein A carrier remains in the surfactant phase (5) and can be recycled for a new round of antibody harvesting. (III)

The HFB-component enabled the fusion protein to separate in ATPS (III), as describe above (4.2.1). The functionality of the antibody binding domain was confirmed by quartz crystal microbalance with dissipation monitoring (QCM-D) (III). The antibody binding capacities of HFBI-Protein A and HFBII-Protein A was compared to non-fused commercial Protein A. One immobilized HFBI-Protein A mole-

cule bound 1.5±0.3 (mean ± SD, n=3) antibodies. The corresponding number for HFBII-Protein A was slightly lower, 1.2±0.5, while commercial Protein A bound 1.2±0.3 antibodies. Subsequent rinsing of the surfaces with acidic buffer resulted in instant and complete release of the antibodies. After raising the pH back to 8.0 the protein layers were able to bind antibodies again without significant loss of efficiency. Based on these results it is safe to conclude that both fusion proteins retained antibody-binding capacity of the Protein A block (III).

Only HFBI-Protein A was tested for harvesting antibodies from *N. benthamiana* leaf extract. The extract was spiked with 0.2 g/l of Rituximab IgG to simulate purification of recombinant antibodies from transgenic plants. The HFBI-Protein A was added to the extract in molar ratio of 3:1 in relation to the antibody. After addition of Triton X-114 and phase separation the fusion proteins and antibodies were found in the surfactant phase while most native proteins remained in the aqueous residue. The antibodies were recovered directly from the surfactant phase by adding acidic buffer and letting the phases separate again. Acidic conditions efficiently released antibodies from the fusion protein, as already shown with QCM-D experiments and the antibodies migrated to the aqueous phase while the fusion protein remained in surfactant phase. By using the HFBI-Protein A fusion protein 28% of the antibody could be recovered. Passive distribution of the antibody alone resulted in recovery rate of 12% (III). The amount of recovered antibody is clearly lower than what would be expected based on the good recovery of HFB-Protein A alone in ATPS. The HFBI-Protein A/antibody complex is larger and of relatively hydrophilic nature, hence a single hydrophobin tag may not have been sufficient to drag the whole complex in to surfactant phase. On the other hand, the recovery rate of the HFB-Protein A was not influenced by the presence of the antibody. This suggests that the factor limiting the recovery of HFBI-Protein A/antibody complex is the affinity of the Protein A domain to the antibody, not the separation efficiency of the HFB domain. Thus the efficiency of antibody harvesting could be enhanced by improving the binding affinity of the fusion protein or using an antibody binding moiety that could attach several HFB fusion proteins to a single antibody.

The fusion protein remained in the surfactant phase after recovery of antibodies in acidic aqueous phase. In these experiments part of the fusion protein was degraded, probably due to the acidic conditions and recovery of the intact fusion protein was only approximately one fifth of the original amount. If the degradation issues could be solved, for example by re-engineering the protein, the HFBI-Protein A could be recycled and used for another round of antibody purification. The commercial Protein A based chromatography columns are re-used for several purification rounds and the reusability is one of the most important economic factors.

Although the recovery of antibodies was poor, these results confirm that, in principle, the bi-functional fusion protein can be used to harvest antibodies, or other non-covalently bound molecules, in ATPS. A re-usable system for protein harvesting involving only liquid handling could provide a very desirable solution for emerging industry of generic antibody drugs.

4.2.3 Arming nanoparticles: Transferrin-HFBIV (IV)

Targeted drug delivery is one of the grand challenges of modern medicine. The aim here was to combine the surface active properties of hydrophobins with human transferrin to create a bi-functional coating for nanoparticles enabling both formulation and targeting with a single molecule (Figure 7). Tf-HFBIV produced in BY-2 suspension cells was used in these experiments (IV).

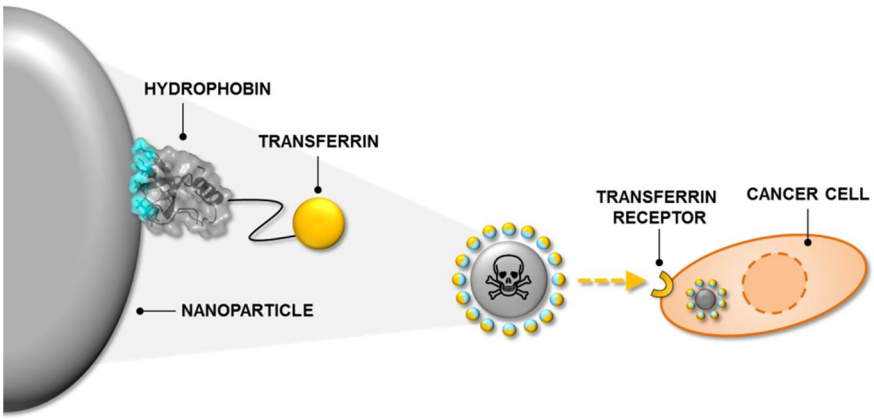


Figure 7. Overview of the concept of Tf-HFBIV fusion protein for functional coating of nanoparticles.

The HFBIV domain remained functional and enabled the whole fusion to interact with non-ionic surfactants (IV) as described above (4.2.1). The primary function of Tf requires sequestering iron. To test if HFB-fused transferrin retained its functionality, the fusion protein was subjected to a series of treatments that first removed all bound iron and subsequently re-saturated it with iron. The conformational changes indicated that HFBIV fused Tf binds iron reversibly, in similar manner to commercial Tf [164]. Before treatments the Tf-HFBIV was divided in both holo and apo forms indicating that the protein had sequestered some iron already in the production host. Removal of iron turned all protein in to the apo form. In iron saturated conditions most apo Tf-HFBIV protein obtained again the holo form. Some Tf-HFBIV did, however, remain in the apo form. Although the experiment did not provide quantitative measures of the iron binding capacity it did confirm that the Tf block of the fusion protein retained its ability to reversibly bind iron.

An *in vitro* experiment was set up in order to test if the Tf-HFBIV would be able to interact with the Tf-receptor and carry nanoparticles into human cells (IV). Porous silicon (Psi) nanoparticles were coated with Tf-HFBIV, HFBI and commercial Tf. None of the coated NPs caused toxic effects to MDA-MB-231 breast cancer cells (IV).

Interactions of the Psi nanoparticles with MDA-MB-231 breast cancer cells were studied by Transmission electron microscope (TEM) and confocal microscopy (Figure 8). Coating the nanoparticles with HFBI alone resulted in increased accumulation of the particles in close vicinity of the cell membrane in comparison to naked NPs, but did not lead in to internalization. However, nanoparticles coated with Tf-HFBIV or commercial Tf (Optiferrin) were observed also inside the cells in high numbers. Although not quantitative, these results suggest that the Tf-HFBIV fusion protein retained the biological function of Tf to interact with Tf-receptor and facilitated active up-take of nanoparticles in human cancer cells. These results present the first proof-of-concept for functionalization of HFB coating with Tf as targeting ligand.

The HFBIV was selected as fusion partner mostly out of academic interest (see 4.1.1 and 4.1.2). Only limited information is available on the properties of the *T. reesei* HFBIV [124], whereas the surface active properties of HFBI and HFBII are well documented [4]. The HFBIV did enable the fusion to interact with surfactant, but self-assembly on the nanoparticle surface was left out of the scope of this work. This work provides a proof-of-concept for combining the function of Tf to function of a HFB, but further experiments are required to test whether HFBIV is suitable for nanoparticle formulation. The HFBI and HFBII have been applied in coatings before and may indeed prove to be better alternatives or the whole HFB-library (I) could be screened for even better alternatives.

The bi-functional coating molecule may offer the best advantage in formulation of poorly soluble drugs like dipropionate and itraconazole [146,147]. In such an application both HFB and Tf would play a key role; the first in solubilisation and formulation and the other in targeting.

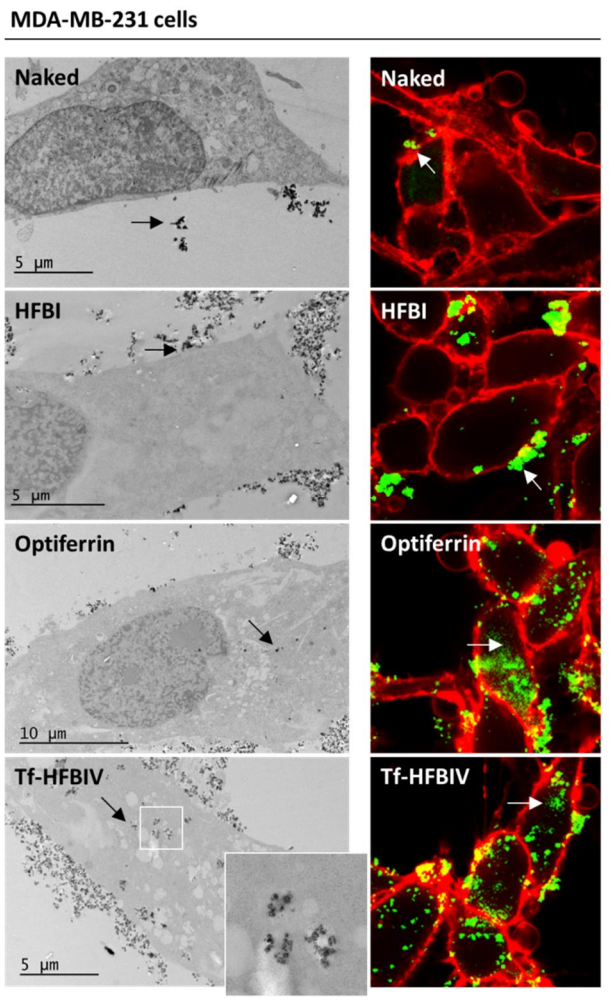


Figure 8. Interaction of the coated PSi nanoparticles with human MDA-MB-231 breast cancer cells. Left panel shows representative TEM images and the right panel are confocal fluorescence microscope images of non-coated and coated PSi nanoparticles with the cancer cells. Cell membranes were stained with RED Cell Mask® and the nanoparticles with FITC-green. Arrows indicate the respective PSi nanoparticles. (IV)

4.3 The platform: Tobacco bright yellow 2

The BY-2 suspension cells appear as a promising production platform for complex HFB-fusion proteins, such as Tf-HFBIV. However, several aspects including ge-

netic instability, low yields, scalability and cost of propagation and downstream processing need to be addressed in order to make BY-2 cells a commercially feasible platform. The aim of this part of the work was to evaluate methods for cell line screening (4.3.1 and 4.3.2) and to develop practices for large scale propagation (4.3.3), biomass harvesting (4.3.4) and protein purification through ATPS (4.3.5).

4.3.1 Visual selection of calli

Main challenges with the BY-2 platform are the tedious screening, heterogeneity and genetic instability of the transformed line. In this work a visual selection marker was studied as a potential solution (I and IV).

GFP was co-expressed in the same T-DNA with the target protein and the fluorescent signal was considered as an indication for simultaneous expression of Tf-HFBIV (IV). The surrogate marker allowed selection of only the most fluorescent fragments of transformed calli to create homogenous clones (I and IV) (Figure 9). Similar approach has been taken by Bortesi and co-workers using Ds-Red with encouraging results [72]. The procedure is to some extent analogous with the FACS-based method described by Kirchhoff and co-workers [42].

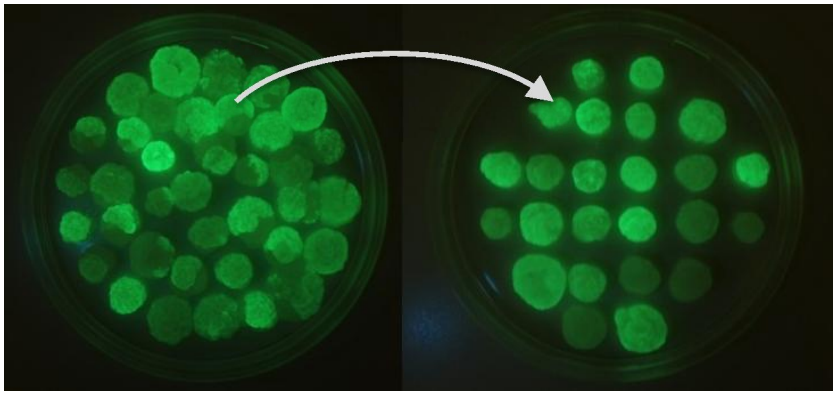


Figure 9. Calli expressing GFP as a surrogate marker. Selection of the most fluorescent fragments from the heterogeneous calli resulted in homogeneous lines. (Unpublished).

Here three different co-expression strategies were applied for the visual marker: targeting to plastid by rubisco transit peptide or targeting to ER with or without HFBI fusion tag. Targeting the expression of GFP to plastids was predicted to result in least interference with processing of the target protein in ER. Expression of HFBI fused GFP, on the other hand, was hypothesized to enhance formation of

protein bodies and thus improve accumulation of target proteins with otherwise low yields by “bandwagon effect” [132].

Targeting GFP to plastids resulted in very low expression levels and thus weak fluorescent signal was too hard to detect with naked eye in order to help manual selection (unpublished results). Nevertheless, the fluorescent signal could be detected with confocal microscope (Figure 10) and was located to plastids. The plastids in BY-2 cells are undeveloped, significantly smaller than chloroplasts in tobacco leafs and do not accumulate chlorophyll [169,170]. Presumably the protein storage capacity of the plastids was not sufficient to accumulate amounts of GFP that would have been required for visual selection. Previously, however, expression of plastid-targeted dsRed has been used successfully for clone selection [42,72].

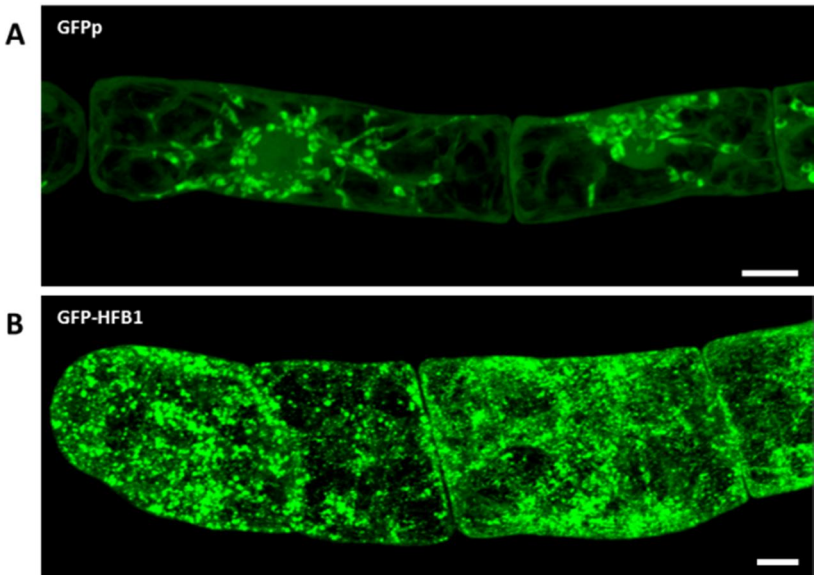


Figure 10. Z-stacs of confocal microscope images showing localization of the GFP in either plastids (A) or small PBs in ER. (Unpublished)

Both ER-targeted GFP and GFP-HFBI provided good fluorescence signals for manual selection. Over few passages, the originally mosaic pattern of fluorescence in the calli became homogenous (Figure 9).

GFP-HFBI was applied as a marker to assist expression of Tf-HFBIV in BY-2 (IV). Transient assay in *N. benthamiana* indicated that the surrogate marker had no significant influence on accumulation of Tf-HFBIV (IV). Accumulation of the fluorescent marker and Tf-HFBIV was evaluated in BY-2 calli after several passages on solid media. In contrast to previous reports [72] we found no clear correlation in the amount of GFP with the accumulation of transferrin (IV). This could be an artefact due to small sample size (n=16), but it does indicate that the link be-

tween co-expressed fluorescence marker and target protein might not be as direct as previously reported [72,171]. Several factors could uncouple the accumulation of a surrogate fluorescence marker and a target protein, such as multiple or partial T-DNA insertions and transgene rearrangements during prolonged cultivation [72]. Furthermore the expression cassettes used here for Tf and GFP-HFBI in the dual construct were identical. It is possible that the repetitive sequences may have caused elimination of one of the cassettes from the genome.

A surrogate fluorescent marker may be a feasible tool for initial screening of transformed calli and generation of homogenous lines. However, the actual screening for good producers needs to be done according to the actual target protein.

4.3.2 The Hulk-story

Although expression of a visual marker does not seem to correlate well with a co-expressed target protein, repeated selection did result in some stable lines with extremely high expression of the fluorescent proteins themselves (I). Two years after transformation and repeated selection of the calli, a BY-2 suspension cell line yielded up to 0.3 g GFP-HFBI per litre of culture volume (I). After three more years of selection of the calli another line carrying the same construct yielded 1.1±0.2 g/l (mean±SD, n=3) GFP-HFBI in suspension culture corresponding to 50.1±8.5% of TSP or 8.3±1.6% of the dry weight (Figure 11). This is the largest titre of recombinant protein ever reported in BY-2 or any other plant suspension cell cultures and is comparable to the yields generally reached in yeasts or animal cells. The cell line was renamed as HULK. The growth of the cell suspension was comparable to wild type BY-2 cells (Figure 11). Accumulation of GFP-HFBI was followed over a period of 19 passages after growing as suspension culture for approximately 5 months. The yield remained relatively stable through the whole period between 44% and 56% GFP of TSP, even though no visual selection was applied anymore (unpublished results). The cell line has not been analysed in more detail and the factors causing the high expression levels remain to be discovered. One possibility is that a superior transformation event has been selected during the process. Several copies of the gene or a hit to a “transcriptional hot spot” could explain the result. The expression level could also result from gradual mutations or somaclonal variation that may have accumulated over time under selective conditions.

However, it is clear that continuous manual selection over long periods of time is not a feasible method for generating production lines, and real life target proteins cannot be directly selected according to fluorescence. The next step forward is to discover a shortcut. The cell line offers a valuable lead for reverse engineering. Further research should aim to characterize the cell line thoroughly to identify any anomalies and pinpoint the genomic location of the insert(s). The new gene editing tools enable exploring the role of location effect by placing the same genes to same genomic locations in WT cells. The role of gradual changes in the cell line itself can be explored by replacing the gene for GFP-HFBI with another marker.

Protein yields in tobacco cell cultures typically vary from 0.005 to 200 mg/l depending on the product (Table 1). Generally protein yield of in range of 10 mg/l is considered satisfactory for starting commercial product development and range of 100 mg/l should provide the desired profit margin for plant-based recombinant protein expression [172]. Thus, this work proofs that the BY-2 cells can, in practise, reach productivity of economic interest.

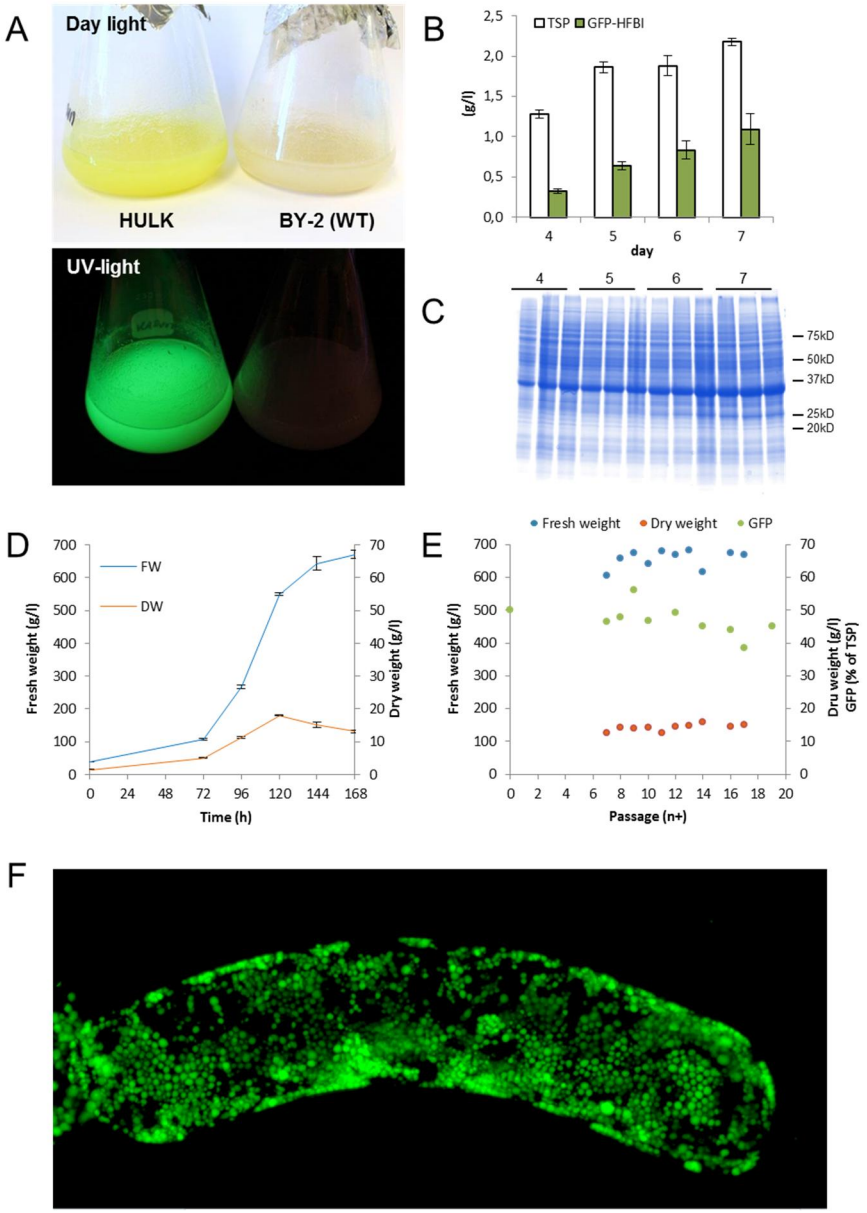


Figure 11. (Previous page) A BY-2 cell line (HULK) expressing high levels of GFP-HFBI. (A) The accumulating GFP-HFBI is visible in the suspension cells with naked eye in daylight. (B) Protein accumulation over one week of cultivation. Error bars represent standard deviations (n=3) (C) A Coomassie stained SDS-PAGE shows that GFP-HFBI (approximately 35 kD) builds the bulk of total protein. The parallel lanes represent 3 replicas. Same amount DW is loaded on the gel. (D) Growth of the cell line is similar to WT BY-2 cells. (E) Accumulation of fresh biomass, dry weight and GFP over follow-up period of 19 passages. (F) A z-stack of confocal microscope images showing accumulation of GFP-HFBI in protein bodies. (Unpublished)

4.3.3 Propagation of BY-2 cell suspension in pilot scale stirred tank bioreactors

In frame of this work the cultivation of BY-2 suspension cells was scaled-up from 50 ml shake flask cultures up to 600 l culture volume in standard stirred tank bioreactors (I). Different cell lines were propagated in various size pilot scale bioreactors all together ten times (Table 4).

Culture cycle

Figure 12 illustrates a representative batch cultivation of BY-2 suspension cells (accumulation of FW in all bathes is presented in Appendix 1). Culture circle started with a lag phase of two (48h) to three days (72h). During this phase the biomass did not accumulate significantly, but the oxygen usage increased steadily indicated by reduced amount of dissolved oxygen (DO) and increased concentration of carbon dioxide (CO_2) in exhaust air. Sucrose was hydrolysed to glucose (and fructose) catalysed by invertases located in the cell walls or secreted in the medium [52]. The culture pH dropped within the first day of the culture from 5.8 to 4.2–4.5, but rose quickly back to 4.7–4.8. This trend of pH change is characteristic to BY-2 suspension cells and is presumably caused by uptake of nutrients, such as ammonium, and release of ions [52,86].

After lag phase the cultures started exponential growth. The fresh weight (FW) and dry weight (DW) accumulated rapidly. Use of oxygen increased further and both mixing and aeration were increased to maximum settings (Table 4) to keep DO above level of 20% (or 30%). The mixing and aeration rates used in this work were not sufficient to maintain the oxygen levels in most of the cultivations: towards the end of the growth phase maximum aeration and mixing were reached and DO dropped close to zero. However, the apparent oxygen limitation did not seem to influence the biomass accumulation dramatically. Peaking of the dissolved CO_2 or pH indicated the end of active growth. Accumulation of DW at 16–19 g/l coincided with peaking CO_2 and exhaustion of glucose in the media during fifth (120h) or sixth day of cultivation (144h), although some fructose may still be left in the media [52]. After the drop in the beginning, the culture pH remained stable throughout the cultivation until it started to increase in middle of the growth phase and peaked at the end of growth, when all ammonia was taken up from the medium [52]. After exhaustion of sugar and peaking of the DW, the FW still in-

creased for a day or two up to 540–690 g/l. This is due to growth of the cells by elongation and increasing volume of the central vacuole by up-take of water. Detailed in-line measurements by radio frequency impedance spectroscopy indicate that the specific growth rates in this elongation stage may be even higher than during growth primarily by cell division [103].

Accumulation of biomass

Growth of BY-2 suspension cells in stirred tank bioreactors appears similar to growth in shake flasks (I and III) [26]. However the FW to DW ratio seems slightly larger in shake flasks. This was probably due to the vigorous agitation in the bioreactor that may keep the cell aggregates smaller or even reduce the cell size [25]. Changes in morphology would reflect in smaller volume of cell pellet that was used to determine the FW (3.2). The generated dry biomass 16–19 g/l was in agreement with consumed carbohydrates (30g/l). Exhaustion of the carbon source would imply that additional carbohydrates may further improve the yield of biomass. However, the extremely large cell volume (up to 70% of the cultivation volume) poses a physical limitation to growth. Thus reducing the water uptake seems critical for increasing the volumetric productivity. This could be achieved, at least to some extent, by adjusting the osmolarity of the culture media [103].

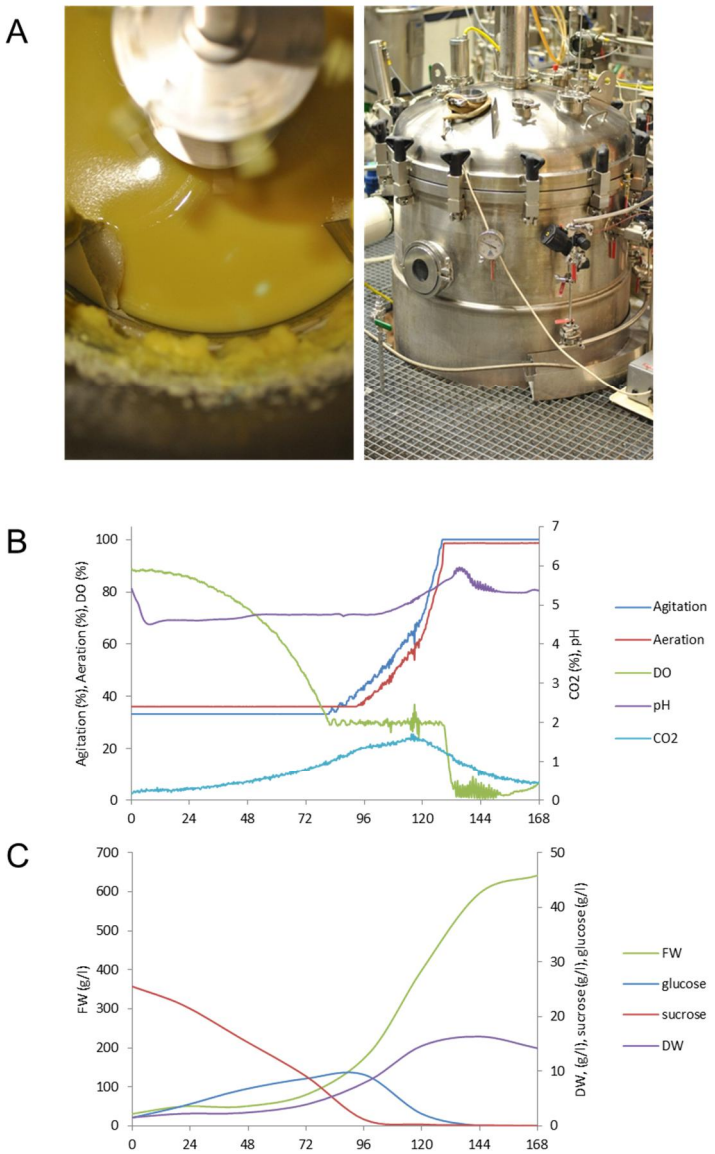


Figure 12. Representative 600l batch cultivation of BY-2 cell suspension. (A) Photographs from inside and outside the BioFlo PRO pilot bioreactor. (B) In-line and (C) off-line measurements. (I)

Inoculum

All in all BY-2 cells are very robust and can be propagated successfully in various bioreactors and with varying parameters, but based on the results here, sufficient inoculum seems to be a critical factor for successful batch. Cultures started with approximately 30 g/l FW (or 1–2 g/l DW) cells grew well and reached stationary phase in six days. A culture started with approximately 20 g/l FW (0.8 g/l DW) failed to grow to high density within 7 days (Appendix 1). On the other hand increasing the inoculum size to approximately 45 g/l FW seemed to reduce the cultivation time so that stationary phase was reached in 5 days (Appendix 1, batches 6, 7, 9 and 10). These results are in line with observations of Raven and co-workers that increase of inoculum size from 5% (corresponds to approximately 32.5 g/l FW) to 8% (52 g/l FW) may reduce cultivation time up to 48h [26].

Mixing and aeration

With the propagation parameters used in this work the oxygen does not seem to be a limiting factor for growth of BY-2 suspension cells in stirred tank bioreactors. Nevertheless, oxygen limitation before exhaustion of the carbohydrates may slow down the linear growth (cultivation bio1920 in supplement)[25,70]. The cultivation parameters were adjusted throughout this work in a practical rather than academic manner. Increasing the agitation and aeration helped to maintain the level of DO above 20% in the last bioreactor operations. Alternative way to maintain sufficient level of DO is to supplement the input gas flow with oxygen [53]. Oxygen transfer could also be improved by using a fine sparger producing smaller air bubbles [25]. However, the agitation and aeration parameters used here did not cause apparent limitation of culture growth and thus oxygen supplementation would only pose an unnecessary complication of the process.

Mixing in all bioreactor cultivations was done with Rushton turbines and controlled by a cascade (Table 4) to maintain DO above a set threshold level. Rushton turbines generate a radial flow pattern that induces a relatively high turbulence and specific power input, which may cause shear damage to the cells. Pitched blade impellers, generating an axial flow pattern and thus less shear damage [173], have been used in many other reports on bioreactor propagation of BY-2 cells (Table 2). However, no apparent shear related growth inhibition could be observed in any of the bioreactor runs in this work with tip speeds up to 2.25 m/s (supplement). It may still be possible to even increase the maximum mixing parameters as the impeller systems inducing axial flow pattern are generally considered suitable for plant cells with tip speeds up to 2.5 m/s [173]. However, another report indicated that cell damage may inhibit growth already when tip speed of marine impeller exceeds 0.24 m/s [104]. Lot of research effort is being put in developing more gentle agitation systems for plant cells in stirred tank bioreactors [173]. However, it appears that moderate bubbling combined with low speed mixing with conventional Ruston turbine system is sufficient for propagating BY-2 suspension cells in large scale.

Foaming

No anti-foaming agents were use during the pilot scale cultivations. Foaming of the culture broth caused problems only with some runs, but not the others. Foaming is generally most intensive at the beginning of the exponential growth and is reduced towards the end of the culture. It appears that in larger cultivation vessels the foam forming on top of the culture is more efficiently mixed in, than in smaller culture vessels. In 1000 l pilot scale bioreactor formation of foam was hardly observed (Figure 12 A). On the other hand, in 40 l cultivation vessel formation of foam did cause some practical problems in form of filter clogging during some cultivations, but not others. As a precaution the vessels were not filled to maximum working volume. Anti-foaming agents, such as Pluronic® L61 (BASF, Mount Olive, New Jersey) and Silbione 70414 have been used with BY-2 cells [47,69,86]. Also in our set-up use of anti-foaming agents would be recommendable.

Cultivation mode

All cultivations were performed in batch mode where setting up, sterilization and cleaning the bioreactors build up a major part of the costs. Repeated, or semi-continuous culture, where part of the suspension is left in the bioreactor and fresh media is added for another round of cultivation may reduce the total cost per obtained biomass tremendously [21,26,27]. Tobacco cells have also been propagated for extended duration in perfusion or chemostat cultures, where fresh media in continuously pumped in to the bioreactor while spent media or cell suspension is pumped out [82]. This mode of cultivation would suit well a process where the down-stream processing can also be operated in continuous mode.

Suggested parameters

Based on the results of this work following parameters are suggested as a starting point for future bioreactor operations: DO maintained above a threshold of 20% by agitation as in table 6 and aeration cascade 0.1–0.2 vvm, temperature 26°C and pressure 0.2 bar. At starting point the cell density should be 30 to 45 g/l FW to reach stationary phase in six to five days, respectively.

Table 6. Suggested parameters for bioreactor operations

Bioreactor	Working volume	Mixing cascade (min-max)	Tip speed (min-max)
BF510	30 l	100-300 rpm	0.56-1.78 m/s
IF400	300 l	60-150 rpm	0.70-1.80 m/s
BioFlo PRO	600 l	40-120 rpm	0.75-2.25 m/s

4.3.4 Biomass harvesting and cell disruption

One purpose of this work was to evaluate practical solutions for downstream processing of the BY-2 suspension cells. HFB-fusion proteins were retained to ER and therefore the product had to be harvested from the biomass. A major challenge in harvesting recombinant proteins from BY-2 suspension cells is their extremely high water content. The dry weight of the cell mass is at highest approximately 5% of the fresh weight in the beginning of the exponential growth phase (see 4.3.3). At the peak of cell volume dry weight represents only approximately 3% of the fresh weight. In other words, the harvested biomass consists of 95 to 97% water. Two approaches were evaluated for extracting the recombinant proteins: harvesting and freeze drying the cell mass prior to dry milling and extraction (I) or direct homogenization of the whole culture broth followed by clarification (III).

The biomass was recovered from the culture suspension in large scale by using two different filtration systems: pressurized filtration (Seitz) or filter press (Larox) (Figure 13). In the Seitz device the culture suspension was pumped in to a filter system with a filtration area of 7x 0.16 m^2. The filter chambers were filled with the biomass and the permeate flowed through. In the Larox system the cell suspension is filtered in the bowl of the filter press with slight pressure. When the bowl is filled the pressure is released and bowl emptied for subsequent round of filtration. Of the two filtration methods the Larox filter press yielded in drier cell mass and was easier to handle in pilot scale. The filtered cell mass was freeze dried and homogenized by milling. GFP-HFBI was found to be stable in the dry powder at RT for at least a month (I). No apparent degradation of the host cell proteins was observed either suggesting that also other recombinant proteins could be stored in the dried powder at ambient temperatures. Protein extraction was performed by mixing the powder with extraction buffer. Solubility of GFP-HFBI was good, thus a very small volume of buffer could be used in order to gain high protein concentration in the extract (I). Freeze drying large amounts of plant cell material is expensive and time consuming. When dried cell powder can be used as such, for example as oral vaccine for animals [53], it may be a good solution. However, for protein extraction the freeze drying step is not necessary, when disrupting the cells directly in the culture suspension is also possible.

Direct homogenization of cells in the culture broth was done by high-pressure homogenizer (III). Before homogenization concentrated extraction buffer (10X) was added to the cell suspension to improve the protein stability. Two subsequent rounds of homogenization (ca. 600 bar) seemed to break most of the cells, but not all. The homogenate was clarified by centrifugation. This step could be replaced by dead-end filtration in large scale. Approximately 80% of the TSP was recovered by high-pressure homogenization in comparison to extraction from freeze dried material. The smaller recovery is probably due to the incomplete disruption of the cells. The clarified extract was applied directly to ATPS. Regardless of the slightly lower recovery and dilution of the product, this approach suits large scale operations much better than harvesting the cell mass separately.

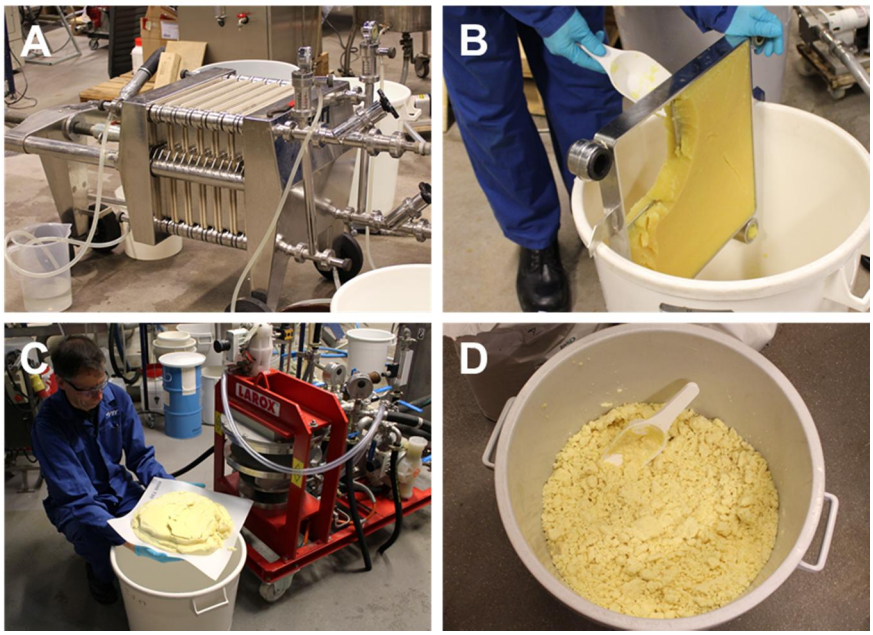


Figure 13. Filtration systems used for harvesting cells. (A) Seitz pressurized filtration. (B) A single filter unit of Seitz and filtered cell mass. (C) Larox filter press and (D) cell mass harvested with Larox.

4.3.5 Scaling up ATPS

ATPS has been previously performed in large, up to cubic meter scale [144], but not with plant-based material. In this work recombinant proteins from BY-2 extract were purified using ATPS in up to 20 litre volume (I and III). Experiments with GFP-HFBI showed that the volume of ATPS does not influence the efficiency of the separation dramatically (I). Approximately 50 to 60% of the fusion protein was recovered in all volumes using 3% (w/v) Triton X-114, which is comparable to experiments conducted in small scale. The time required for the phase separation increases with the volume: in 100 ml two phase system is established in 15 min, in 1 l in 30 min and in 20 l in 1 h and 30 min (I).

The recovered surfactant phase was washed with isobutanol. The subsequent phase separation poses a bottle neck. A floating precipitate supposedly composed of host cell proteins forms in the aqueous phase and disturbs the recovery of the phase in e.g. separation funnels. When the two phase system is centrifuged, even shortly, the precipitate forms a solid “raft” between the two phases that does not any more hinder recovery of the lower phase (Figure 14). Therefore, after mixing the surfactant phase with isobutanol, the mixture was aliquoted in 2 l bottles and

centrifuged to separate the phases and the precipitate. The centrifugation step was feasible in the scale of this work, but not in larger set ups. Nevertheless, it may be that in separation vessels with different dimensions the formation of the precipitate would not pose a problem.

Figure 14. Phase separation by centrifugation after washing the surfactant phase with isobutanol. A “raft” is clearly visible between the two phases. The product (GFP-HFBI) was recovered in the lower aqueous phase by pumping.

The recovered phase containing the concentrated and partially purified HFB-fusion protein also contains residual amounts of isobutanol that may interfere with following affinity chromatography steps. Therefore a buffer exchange step was required. In this work the buffer exchange was performed in pilot scale using a Millipore Pellicon 2 cross filtration unit. A 3.6 l volume of recovered phase from ATPS purification of Tf-HFBIV was washed with 10 l of buffer and concentrated to 1.1 l volume. After cross filtration the solution had only a faint smell of isobutanol and no problems were encountered during subsequent purification by affinity chromatography.

5. Conclusions

The extraordinary surface active characteristics of fungal HFBs have been utilized in a range of experimental applications from food additives to coating of biosensors and nanoparticles. The applications have been further broadened by combining the functions of HFBs to other proteins by genetic fusions. Yet manufacturing of HFBs and complex HFB-fusion proteins remains a challenge. The initial studies showed that HFB-fusion proteins accumulated in exceptionally high yields in plants [5]. The main purpose of this work was to extend the HFB-fusion technology beyond HFBI to other HFBs, evaluate suitability of tobacco BY-2 suspension cells for manufacturing of HFB-fusion proteins and develop practical solutions for scaling up the production.

The HFB-fusion technology, in plants, has relied solely on fusing target proteins to C-terminus of *Trichoderma reesei* HFBI. The results of this work suggest that also HFBII and HFBIV are suitable alternatives for fusion partners. All three HFBs retained their capability to interact with non-ionic surfactants also when fused to target proteins. HFBI and HFBIV may be fused from either termini, but the yield of HFBII fusions drops significantly when fused from its C-terminus. The literature suggests that all HFBI, HFBII and HFBIV all have somewhat different properties and biological roles, which builds diversity to the toolkit of HFB fusion technology. When fused to GFP or Protein A, the HFBII fusion tag accumulated higher yields than HFBI-tag. The improved accumulation has been attributed to formation of protein bodies and results of this work also indicate correlation between high accumulation and formation of PBs. However, it remains unclear if the PBs are a causing factor of the high yield, or merely a phenomenon resulting from it. In general HFB-fusion tags seem to increase yield of some proteins, but not others. The expectations for using HFBs as a production and purification tags may need to be revised. Relatively modest increases in yield or purification through ATPS are not enough to justify the extra effort, especially if application of the protein requires cleaving off the tag after purification. Thus the focus was changed to the interesting prospects of using the HFBs as functional components of new bi-functional fusion proteins.

In this work the HFBs were built in two bi-functional fusion proteins, HFBI-Protein A and Tf-HFBVI, for in-solution harvesting of antibodies and functional coating of nanoparticles, respectively. Proof-of-concept was shown for both appli-

cations. Hydrophobin fusion proteins have been purified using ATPS before, but this work describes for the first time harvesting of non-covalently bound antibodies using a bi-functional HFB fusion proteins. Bare hydrophobins have improved the properties of various nanoparticles, but this is the first time a targeting ligand has been incorporated in the same coating molecule. Most promising applications for HFB-fusion proteins may indeed be in immobilization of new activities on surfaces of nanoparticles and biosensors.

The tobacco BY-2 cells were found to be a suitable production platform for HFBI-GFP, HFBI-Protein A and Tf-HFBIV. Use of a surrogate fluorescence marker appears as a promising approach for preliminary line selection, although the correlation between the yields of the marker and target protein was not clear. Curiously, similar visual selection of a cell line expressing HFBI-GFP resulted in few years' time in exceptional yields of 1 g/l. It is not clear if the extraordinarily high protein accumulation was due to selection of a superior transformation event or selection of gradually improved expression caused by somaclonal variation. Nevertheless the outcome is a tangible showcase for the potential of BY-2 cells. The cell line will be used as a lead to discover shortcuts for development of production lines in the future.

Here the cultivation of BY-2 suspension cells was scaled up in standard stirred tank bioreactors up to culture volumes of 600l. The results show clearly that the cell line can be propagated in standard microbial fermentation facilities without any significant modifications or additional investments. In some respects the standard instrumentation may be even unnecessarily complex. The cultivation could probably be done in simple single use bioreactors such as the CellEx bioreactors used by Protalix Biotherapeutics. Although the up-scaling experiments here have been conducted in rather practical than systematic manner, they did result in guiding parameters for cultivation of BY-2 cells in stirred tank bioreactors at 30 l, 300 l and 600 l scale. Downstream processing of the biomass was studied here from the perspective of extracting intracellular proteins. For that aim high-pressure homogenization of culture broth, followed by clarification by filtration and ATPS to recover the product proved to be the most practical approach.

Overall, the results of this work indicate that tobacco BY-2 suspension cells are a potential platform for manufacturing complex high-value recombinant proteins, such as HFB-fusion proteins. Nevertheless, the platform cannot challenge the established eukaryotic production systems directly. From productivity point of view the yeast or animal cell platforms may still be better alternatives. A new product is required to change the game plan: a niche product for which the plant cell platform provides a clear advantage. Complete lack of animal derived components in plant cell cultures may be seen as such an advantage for products aimed for coating nanoparticles or biosensors. If such a product would fuel the development of the plant cell platform, it can build on three decades of work done with animal cell platforms and become commercially viable in relatively short time. If so - BY-2 cell line is the prime candidate for becoming the chassis to build the next generation plant cell factory.

References

1. Walsh G. Biopharmaceutical benchmarks 2014. Nat Biotechnol. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014 Oct;32(10):992–1000.
2. Kim JY, Kim Y-G, Lee GM. CHO cells in biotechnology for production of recombinant proteins: current state and further potential. Appl Microbiol Biotechnol. 2012 Feb;93(3):917–30.
3. Schillberg S, Raven N, Fischer R, Twyman RM, Schiermeyer A. Molecular farming of pharmaceutical proteins using plant suspension cell and tissue cultures. Curr Pharm Des. 2013 Jan;19(31):5531–42.
4. Wösten H a. B, Scholtmeijer K. Applications of hydrophobins: current state and perspectives. Appl Microbiol Biotechnol. 2015;99(4):1587–97.
5. Joensuu JJ, Conley AJ, Lienemann M, Brandle JE, Linder MB, Menassa R. Hydrophobin fusions for high-level transient protein expression and purification in Nicotiana benthamiana. Plant Physiol. 2010 Feb 1;152(2):622–33.
6. Marsian J, Lomonossoff GP. Molecular pharming-VLPs made in plants. Curr Opin Biotechnol. 2016 Feb;37:201–6.
7. Ma JK-C, Drossard J, Lewis D, Altmann F, Boyle J, Christou P, et al. Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. Plant Biotechnol J. Blackwell Publishing Ltd; 2015 Oct 3;13(8):1106–20.
8. Loos A, Steinkellner H. Plant glyco-biotechnology on the way to synthetic biology. Front Plant Sci. Frontiers; 2014 Jan 8;5:523.
9. Tekoah Y, Shulman A, Kizhner T, Ruderfer I, Fux L, Nataf Y, et al. Large-scale production of pharmaceutical proteins in plant cell culture-the protalix experience. Plant Biotechnol J. 2015 Oct;13(8):1199–208.
10. Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, et al. Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system. Plant Biotechnol J. 2007 Sep;5(5):579–90.
11. Allison M. As Genzyme flounders, competitors and activist investors swoop in. Nat Biotechnol. Nature Publishing Group; 2010 Jan;28(1):3–4.
12. Fischer R, Schillberg S, Hellwig S, Twyman RM, Drossard J. GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv. 2012 Jan;30(2):434–9.
13. Menassa R, Nguyen V, Jevnikar A, Brandle J. A self-contained system for the field production of plant recombinant interleukin-10. Mol Breed. Kluwer Academic Publishers; 2001;8(2):177–85.
14. Naqvi S, Ramessar K, Farré G, Sabalza M, Miralpeix B, Twyman RM, et al. High-value products from transgenic maize. Biotechnol Adv. 2011 Jan;29(1):40–53.
15. Shaaltiel Y, Gingis-Velitski S, Tzaban S, Fiks N, Tekoah Y, Aviezer D.

Plant-based oral delivery of β-glucocerebrosidase as an enzyme replacement therapy for Gaucher's disease. Plant Biotechnol J. 2015 Oct;13(8):1033–40.

16. Fischer R, Schillberg S, Buyel JF, Twyman RM. Commercial aspects of pharmaceutical protein production in plants. Curr Pharm Des. 2013;19(31):5471–7.

17. Khan I, Twyman RM, Arcalis E, Stoger E. Using storage organelles for the accumulation and encapsulation of recombinant proteins. Biotechnol J. 2012 Sep;7(9):1099–108.

18. Zhang D. Plant seed-derived human transferrin: expression, characterisation and applications [Internet]. OA Biotechnology. 2013 [cited 2015 Sep 17]. p. 17. Available from: http://www.oapublishinglondon.com/images/article/pdf/1379515504.pdf

19. Gleba Y, Klimyuk V, Marillonnet S. Viral vectors for the expression of proteins in plants. Curr Opin Biotechnol. 2007 Apr;18(2):134–41.

20. Landry N, Ward BJ, Trépanier S, Montomoli E, Dargis M, Lapini G, et al. Preclinical and Clinical Development of Plant-Made Virus-Like Particle Vaccine against Avian H5N1 Influenza. Fouchier RAM, editor. PLoS One. Public Library of Science; 2010 Dec 22;5(12):e15559.

21. Santos RB, Abranches R, Fischer R, Sack M, Holland T. Putting the Spotlight Back on Plant Suspension Cultures. Front Plant Sci. Frontiers; 2016 Mar 11;7.

22. Wilson SA, Roberts SC. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. Plant Biotechnol J. 2012 Apr;10(3):249–68.

23. Sijmons PC, Dekker BMM, Schrammeijer B, Verwoerd TC, van den Elzen PJM, Hoekema A. Production of Correctly Processed Human Serum Albumin in Transgenic Plants. Bio/Technology. Nature Publishing Company; 1990 Mar;8(3):217–21.

24. Girard LS, Fabis MJ, Bastin M, Courtois D, Pétiard V, Koprowski H. Expression of a human anti-rabies virus monoclonal antibody in tobacco cell culture. Biochem Biophys Res Commun. 2006 Jun 30;345(2):602–7.

25. Flickinger MC. Encyclopedia of Industrial Biotechnology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2009.

26. Raven N, Rasche S, Kuehn C, Anderlei T, Klöckner W, Schuster F, et al. Scaled-up manufacturing of recombinant antibodies produced by plant cells in a 200-L orbitally-shaken disposable bioreactor. Biotechnol Bioeng. 2014;112(2):1–44.

27. Xu J, Ge X, Dolan MC. Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. Biotechnol Adv. 2011 Jan;29(3):278–99.

28. Lehmann N, Dittler I, Lämsä M, Ritala A, Rischer H, Eibl D, et al. Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Paek K-Y, Murthy HN, Zhong J-J, editors. Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Dordrecht: Springer Netherlands; 2014. 17-46 p.

29. Doran PM. Therapeutically important proteins from in vitro plant tissue culture systems. Curr Med Chem. 2013 Jan;20(8):1047–55.

30. Sack M, Paetz A, Kunert R, Bomble M, Hesse F, Stiegler G, et al. Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. FASEB J. 2007 Jun;21(8):1655–64.

31. Fischer R, Vasilev N, Twyman RM, Schillberg S. High-value products from plants: the challenges of process optimization. Curr Opin Biotechnol. 2015 Apr;32:156–62.

32. Paul MJ, Teh AYH, Twyman RM, Ma JK-C. Target product selection - where can Molecular Pharming make the difference? Curr Pharm Des. 2013;19(31):5478–85.

33. Nagata T, Nemoto Y, Hasezawa S. Tobacco BY-2 Cell Line as the “HeLa” Cell in the Cell Biology of Higher Plants. Int Rev Cytol. 1992;132:1–30.

34. Srba M, Černíková A, Opatrný Z, Fischer L. Practical guidelines for the characterization of tobacco BY-2 cell lines. Biol Plant. 2015 Dec 17;60(1):13–24.

35. Nagata T, Kumagai F. Plant cell biology through the window of the highly synchronized tobacco BY-2 cell line. Methods Cell Sci. 1999 Jan;21(2–3):123–7.

36. Kovarik A, Lim K-Y, Soucková-Skalická K, Matyasek R, Leitch AR. A plant culture (BY-2) widely used in molecular and cell studies is genetically unstable and highly heterogeneous. Bot J Linn Soc. 2012 Nov 19;170(3):459–71.

37. Fujiyama K, Furukawa A, Katsura A, Misaki R, Omasa T, Seki T. Production of mouse monoclonal antibody with galactose-extended sugar chain by suspension cultured tobacco BY2 cells expressing human beta(1,4)-galactosyltransferase. Biochem Biophys Res Commun. 2007 Jun 22;358(1):85–91.

38. Magy B, Tollet J, Laterre R, Boutry M, Navarre C. Accumulation of secreted antibodies in plant cell cultures varies according to the isotype, host species and culture conditions. Plant Biotechnol J. 2014 May;12(4):457–67.

39. Yano A, Takekoshi M, Morita E, Imai S, Nisizawa T, Hanada N. Production of Fab fragment corresponding to surface protein antigen of Streptococcus mutans serotype c-derived peptide by Escherichia coli and cultured tobacco cells. J Biosci Bioeng. 2006 Mar;101(3):251–6.

40. Fischer R, Liao Y-C, Drossard J. Affinity-purification of a TMV-specific recombinant full-size antibody from a transgenic tobacco suspension culture. J Immunol Methods. 1999 Jun;226(1–2):1–10.

41. Vasilev N, Grömping U, Lipperts A, Raven N, Fischer R, Schillberg S. Optimization of BY-2 cell suspension culture medium for the production of a human antibody using a combination of fractional factorial designs and the response surface method. Plant Biotechnol J. 2013 Sep;11(7):867–74.

42. Kirchhoff J, Raven N, Boes A, Roberts JL, Russell S, Treffenfeldt W, et al. Monoclonal tobacco cell lines with enhanced recombinant protein yields

can be generated from heterogeneous cell suspension cultures by flow sorting. Plant Biotechnol J. 2012;10(8):936–44.

43. De Muynck B, Navarre C, Nizet Y, Stadlmann J, Boutry M. Different subcellular localization and glycosylation for a functional antibody expressed in Nicotiana tabacum plants and suspension cells. Transgenic Res. 2009 Jun;18(3):467–82.

44. Sharp JM, Doran PM. Characterization of monoclonal antibody fragments produced by plant cells. Biotechnol Bioeng. 2001 Jun 5;73(5):338–46.

45. Yano A, Maeda F, Takekoshi M. Transgenic tobacco cells producing the human monoclonal antibody to hepatitis B virus surface antigen. J Med Virol. 2004 Jun;73(2):208–15.

46. Firek S, Draper J, Owen MRL, Gandecha A, Cockburn B, Whitelam GC. Secretion of a functional single-chain Fv protein in transgenic tobacco plants and cell suspension cultures. Plant Mol Biol. 1993 Nov;23(4):861–70.

47. Holland T, Sack M, Rademacher T, Schmale K, Altmann F, Stadlmann J, et al. Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture. Biotechnol Bioeng. 2010;107(2):278–89.

48. Mandal MK, Fischer R, Schillberg S, Schiermeyer A. Inhibition of protease activity by antisense RNA improves recombinant protein production in Nicotiana tabacum cv. Bright Yellow 2 (BY-2) suspension cells. Biotechnol J. 2014;9(8):1065–73.

49. López J, Lencina F, Petruccelli S, Marconi P, Alvarez MA. Influence of the KDEL signal, DMSO and mannitol on the production of the recombinant antibody 14D9 by long-term Nicotiana tabacum cell suspension culture. Plant Cell, Tissue Organ Cult. 2010 Jun 29;103(3):307–14.

50. Zhang X, Mason H. Bean Yellow Dwarf Virus replicons for high-level transgene expression in transgenic plants and cell cultures. Biotechnol Bioeng. 2006 Feb 5;93(2):271–9.

51. Lai KS, Yusoff K, Mahmood M. Functional ectodomain of the hemagglutinin-neuraminidase protein is expressed in transgenic tobacco cells as a candidate vaccine against Newcastle disease virus. Plant Cell, Tissue Organ Cult. 2012 Jul 27;112(1):117–21.

52. Ullisch DA, Müller CA, Maibaum S, Kirchhoff J, Schiermeyer A, Schillberg S, et al. Comprehensive characterization of two different Nicotiana tabacum cell lines leads to doubled GFP and HA protein production by media optimization. J Biosci Bioeng. 2012 Feb;113(2):242–8.

53. O'Neill KM, Schilthuis AM, Leiter C a., Neihaus KM, Judge N a., Twiddy E, et al. Scale-up of transgenic tobacco cells that express intimin of enterohemorrhagic Escherichia coli O157:H7 for use as an oral vaccine in cattle. Vitr Cell Dev Biol - Plant. 2014;submitted.

54. Judge NA, Mason HS, O'Brien AD. Plant cell-based intimin vaccine given orally to mice primed with intimin reduces time of Escherichia coli O157:H7 shedding in feces. Infect Immun. 2004 Jan;72(1):168–75.

55. Sojikul P, Buehner N, Mason HS. A plant signal peptide-hepatitis B

surface antigen fusion protein with enhanced stability and immunogenicity expressed in plant cells. Proc Natl Acad Sci U S A. 2003 Mar 4;100(5):2209–14.

56. Smith ML, Mason HS, Shuler ML. Hepatitis B surface antigen (HBsAg) expression in plant cell culture: Kinetics of antigen accumulation in batch culture and its intracellular form. Biotechnol Bioeng. 2002 Dec 30;80(7):812–22.

57. Lienard D, Tran Dinh O, van Oort E, Van Overtvelt L, Bonneau C, Wambre E, et al. Suspension-cultured BY-2 tobacco cells produce and mature immunologically active house dust mite allergens. Plant Biotechnol J. 2007 Jan;5(1):93–108.

58. Martínez CA, Giulietti AM, Rodríguez Talou J. Expression of a KDEL-tagged dengue virus protein in cell suspension cultures of Nicotiana tabacum and Morinda citrifolia. Plant Cell, Tissue Organ Cult. 2011 May 6;107(1):91–100.

59. Siegert M, Pertl-Obermeyer H, Gadermaier G, Ferreira F, Obermeyer G. Expression of the major mugwort pollen allergen Art v 1 in tobacco plants and cell cultures: problems and perspectives for allergen production in plants. Plant Cell Rep. 2012 Mar;31(3):561–71.

60. Sorrentino A, Schillberg S, Fischer R, Rao R, Porta R, Mariniello L. Recombinant human tissue transglutaminase produced into tobacco suspension cell cultures is active and recognizes autoantibodies in the serum of coeliac patients. Int J Biochem Cell Biol. 2005 Apr;37(4):842–51.

61. Becerra-Arteaga A, Mason HS, Shuler ML. Production, secretion, and stability of human secreted alkaline phosphatase in tobacco NT1 cell suspension cultures. Biotechnol Prog. 2006 Jan;22(6):1643–9.

62. Nakashima K, Shibasaki-Kitakawa N, Miyamoto T, Kubo M, Yonemoto T, Shuler ML. Production of human secreted alkaline phosphatase in suspension and immobilization cultures of tobacco NT1 cell. Biochem Eng J. 2013 Aug;77:177–82.

63. Kizhner T, Azulay Y, Hainrichson M, Tekoah Y, Arvatz G, Shulman A, et al. Characterization of a chemically modified plant cell culture expressed human α-Galactosidase-A enzyme for treatment of Fabry disease. Mol Genet Metab. 2015 Feb;114(2):259–67.

64. Sato Y, Whetten RW. Characterization of two laccases of loblolly pine (Pinus taeda) expressed in tobacco BY-2 cells. J Plant Res. 2006 Nov;119(6):581–8.

65. Sakamoto Y, Nakade K, Yano A, Nakagawa Y, Hirano T, Irie T, et al. Heterologous expression of lcc1 from Lentinula edodes in tobacco BY-2 cells results in the production an active, secreted form of fungal laccase. Appl Microbiol Biotechnol. 2008 Jul;79(6):971–80.

66. Fu LH, Miao Y, Lo SW, Seto TC, Sun SSM, Xu Z-F, et al. Production and characterization of soluble human lysosomal enzyme α-iduronidase with high activity from culture media of transgenic tobacco BY-2 cells. Plant Sci. 2009 Dec;177(6):668–75.

67. Benchabane M, Saint-Jore-Dupas C, Bardor M, Faye L, Michaud D,

Gomord V. Targeting and post-translational processing of human alpha1-antichymotrypsin in BY-2 tobacco cultured cells. Plant Biotechnol J. 2009 Feb;7(2):146–60.

68. Schiermeyer A, Schinkel H, Apel S, Fischer R, Schillberg S. Production of Desmodus rotundus salivary plasminogen activator alpha1 (DSPAalpha1) in tobacco is hampered by proteolysis. Biotechnol Bioeng. 2005 Mar 30;89(7):848–58.

69. Verdelhan Des Molles D, Gomord V, Bastin M, Faye L, Courtois D. Expression of a carrot invertase gene in tobacco suspension cells cultivated in batch and continuous culture conditions. J Biosci Bioeng. 1999 Jan;87(3):302–6.

70. Gao J, Lee JM. Effect of oxygen supply on the suspension culture of genetically modified tobacco cells. Biotechnol Prog. American Chemical Society; 1992 Jan;8(4):285–90.

71. Singhabahu S, George J, Bringloe D. High-yield production of apoplast-directed human adenosine deaminase in transgenic tobacco BY-2 cell suspensions. Biotechnol Appl Biochem. 2015 Jan;62(1):87–93.

72. Bortesi L, Rademacher T, Schiermeyer A, Schuster F, Pezzotti M, Schillberg S. Development of an optimized tetracycline-inducible expression system to increase the accumulation of interleukin-10 in tobacco BY-2 suspension cells. BMC Biotechnol. 2012;12(1):40.

73. Kaldis A, Ahmad A, Reid A, McGarvey B, Brandle J, Ma S, et al. High-level production of human interleukin-10 fusions in tobacco cell suspension cultures. Plant Biotechnol J. 2013 Jun;11(5):535–45.

74. Xu J, Tan L, Goodrum KJ, Kieliszewski MJ. High-yields and extended serum half-life of human interferon alpha2b expressed in tobacco cells as arabinogalactan-protein fusions. Biotechnol Bioeng. 2007 Aug 1;97(5):997–1008.

75. Magnuson NS, Linzmaier PM, Reeves R, An G, HayGlass K, Lee JM. Secretion of biologically active human interleukin-2 and interleukin-4 from genetically modified tobacco cells in suspension culture. Protein Expr Purif. 1998 Jun;13(1):45–52.

76. Sharma N, Kim T-G, Yang M-S. Production and secretion of human interleukin-18 in transgenic tobacco cell suspension culture. Biotechnol Bioprocess Eng. 2006 Apr;11(2):154–9.

77. Kwon TH, Seo JE, Kim J, Lee JH, Jang YS, Yang MS. Expression and secretion of the heterodimeric protein interleukin-12 in plant cell suspension culture. Biotechnol Bioeng. 2003 Mar 30;81(7):870–5.

78. Xu J, Kieliszewski M. Enhanced accumulation of secreted human growth hormone by transgenic tobacco cells correlates with the introduction of an N-glycosylation site. J Biotechnol. 2011 Jun 10;154(1):54–9.

79. Xu J, Okada S, Tan L, Goodrum KJ, Kopchick JJ, Kieliszewski MJ. Human growth hormone expressed in tobacco cells as an arabinogalactan-protein fusion glycoprotein has a prolonged serum life. Transgenic Res. 2010 Oct;19(5):849–67.

80. Nair NR, Chidambareswaren M, Manjula S. Enhanced heterologous

expression of biologically active human granulocyte colony stimulating factor in transgenic tobacco BY-2 cells by localization to endoplasmic reticulum. Mol Biotechnol. 2014 Sep;56(9):849–62.

81. Lee J-H, Kim N-S, Kwon T-H, Jang Y-S, Yang M-S. Increased production of human granulocyte-macrophage colony stimulating factor (hGM-CSF) by the addition of stabilizing polymer in plant suspension cultures. J Biotechnol. 2002 Jul;96(3):205–11.

82. Lee S-Y, Kim Y-H, Roh Y-S, Myoung H-J, Lee K-Y, Kim D-I. Bioreactor operation for transgenic Nicotiana tabacum cell cultures and continuous production of recombinant human granulocyte-macrophage colony-stimulating factor by perfusion culture. Enzyme Microb Technol. 2004 Dec;35(6–7):663–71.

83. James EA, Wang C, Wang Z, Reeves R, Shin JH, Magnuson NS, et al. Production and characterization of biologically active human GM-CSF secreted by genetically modified plant cells. Protein Expr Purif. 2000 Jun;19(1):131–8.

84. Hong S-Y, Kwon T-H, Lee J-H, Jang Y-S, Yang M-S. Production of biologically active hG-CSF by transgenic plant cell suspension culture. Enzyme Microb Technol. 2002 May;30(6):763–7.

85. Schinkel H, Schiermeyer A, Soeur R, Fischer R, Schillberg S. Production of an active recombinant thrombomodulin derivative in transgenic tobacco plants and suspension cells. Transgenic Res. 2005 Jun;14(3):251–9.

86. Schmale K, Rademacher T, Fischer R, Hellwig S. Towards industrial usefulness - cryo-cell-banking of transgenic BY-2 cell cultures. J Biotechnol. 2006 Jun 25;124(1):302–11.

87. Sun Q-Y, Ding L-W, Lomonossoff GP, Sun Y-B, Luo M, Li C-Q, et al. Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture. J Biotechnol. 2011 Sep 10;155(2):164–72.

88. Choi SM, Lee OS, Kwon SY, Kwak SS, Yu DY, Lee HS. High expression of a human lactoferrin in transgenic tobacco cell cultures. Biotechnol Lett. 2003;25(3):213–8.

89. Mitra A, Zhang Z. Expression of a human lactoferrin cDNA in tobacco cells produces antibacterial protein(s). Plant Physiol. 1994 Nov;106(3):977–81.

90. Pires AS, Rosa S, Castanheira S, Fevereiro P, Abranches R. Expression of a recombinant human erythropoietin in suspension cell cultures of Arabidopsis, tobacco and Medicago. Plant Cell, Tissue Organ Cult. 2012 Mar 13;110(1):171–81.

91. Matsumoto S, Ikura K, Ueda M, Sasaki R. Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. Plant Mol Biol. 1995 Mar;27(6):1163–72.

92. David KM. Characterization of a Tobacco Bright Yellow 2 Cell Line Expressing the Tetracycline Repressor at a High Level for Strict Regulation of Transgene Expression. PLANT Physiol. 2001 Apr 1;125(4):1548–53.

93. Dohi K, Nishikiori M, Tamai A, Ishikawa M, Meshi T, Mori M. Inducible

virus-mediated expression of a foreign protein in suspension-cultured plant cells. Arch Virol. 2006 Jun;151(6):1075–84.

94. Nocarova E, Fischer L. Cloning of transgenic tobacco BY-2 cells; an efficient method to analyse and reduce high natural heterogeneity of transgene expression. BMC Plant Biol. 2009;9:44.

95. James E, Lee JM. Loss and recovery of protein productivity in genetically modified plant cell lines. Plant Cell Rep. 2006 Jul;25(7):723–7.

96. Miguel C, Marum L. An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond. J Exp Bot. 2011 Jul 1;62(11):3713–25.

97. Ogawa Y, Sakurai N, Oikawa A, Kai K, Morishita Y, Mori K, et al. High-throughput cryopreservation of plant cell cultures for functional genomics. Plant Cell Physiol. 2012 May;53(5):943–52.

98. Bortesi L, Fischer R. The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv. 2014 Dec 20;33(1):41–52.

99. Gao J, Wang G, Ma S, Xie X, Wu X, Zhang X, et al. CRISPR/Cas9-mediated targeted mutagenesis in Nicotiana tabacum. Plant Mol Biol. 2015 Jan;87(1–2):99–110.

100. Mercx S, Tollet J, Magy B, Navarre C, Boutry M. Gene Inactivation by CRISPR-Cas9 in Nicotiana tabacum BY-2 Suspension Cells. Front Plant Sci. 2016 Jan;7:40.

101. Matsumoto T, Hirata Y, Yamamoto K, Katsuyama A, Kato A. Improvement of ' Growth Rates of Plant Cell Cultures. 1977;

102. Eibl R, Werner S, Eibl D. Disposable bioreactors for plant liquid cultures at Litre-scale. Eng Life Sci. 2009 Jun;9(3):156–64.

103. Holland T, Blessing D, Hellwig S, Sack M. The in-line measurement of plant cell biomass using radio frequency impedance spectroscopy as a component of process analytical technology. Biotechnol J. 2013 Oct;8(10):1231–40.

104. Ho C-H, Henderson KA, Rorrer GL. Cell damage and oxygen mass transfer during cultivation of Nicotiana tabacum in a stirred-tank bioreactor. Biotechnol Prog. American Chemical Society; 1995 Mar;11(2):140–5.

105. Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiol Plant. 1962 Jul;15(3):473–97.

106. Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE. Considerations for the recovery of recombinant proteins from plants. Biotechnol Prog. 2004 Jan;20(4):1001–14.

107. Aimanianda V, Bayry J, Bozza S, Kniemeyer O, Perruccio K, Elluru SR, et al. Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature. Macmillan Publishers Limited. All rights reserved; 2009 Aug 27;460(7259):1117–21.

108. Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME. Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev. The Oxford University Press; 2005 Nov 1;29(5):877–96.

109. Zampieri F, Wösten HAB, Scholtmeijer K. Creating Surface Properties Using a Palette of Hydrophobins. Materials (Basel). 2010 Sep 6;3(9):4607–25.

110. Nakari-Setala T, Aro N, IlmeN M, Munoz G, Kalkkinen N, Penttila M. Differential Expression of the Vegetative and Spore-Bound Hydrophobins of Trichoderma Reesei Cloning and Characterization of the Hfb2 Gene. Eur J Biochem. 1997 Sep;248(2):415–23.

111. Hakanpää J, Szilvay GR, Kaljunen H, Maksimainen M, Linder M, Rouvinen J. Two crystal structures of Trichoderma reesei hydrophobin HFBI--the structure of a protein amphiphile with and without detergent interaction. Protein Sci. 2006 Sep;15(9):2129–40.

112. Grunér MS. Molecular interactions of hydrophobin proteins with their surroundings. Aalto-yliopisto; 2015.

113. Neuhof T, Dieckmann R, Druzhinina IS, Kubicek CP, Nakari-Setälä T, Penttilä M, et al. Direct identification of hydrophobins and their processing in Trichoderma using intact-cell MALDI-TOF MS. FEBS J. 2007 Feb;274(3):841–52.

114. Nakari-Setälä T, Aro N, Kalkkinen N, Alatalo E, Penttilä M. Genetic and biochemical characterization of the Trichoderma reesei hydrophobin HFBI. Eur J Biochem. 1996;235:248–55.

115. Hakanpää J, Paananen A, Askolin S, Nakari-Setälä T, Parkkinen T, Penttilä M, et al. Atomic resolution structure of the HFBII hydrophobin, a self-assembling amphiphile. J Biol Chem. 2004 Jan 2;279(1):534–9.

116. Szilvay GR, Nakari-Setälä T, Linder MB. Behavior of Trichoderma reesei hydrophobins in solution: interactions, dynamics, and multimer formation. Biochemistry. American Chemical Society; 2006 Jul 18;45(28):8590–8.

117. Szilvay GR, Paananen A, Laurikainen K, Vuorimaa E, Lemmetyinen H, Peltonen J, et al. Self-assembled hydrophobin protein films at the air-water interface: structural analysis and molecular engineering. Biochemistry. 2007 Mar 6;46(9):2345–54.

118. Kisko K, Szilvay GR, Vuorimaa E, Lemmetyinen H, Linder MB, Torkkeli M, et al. Self-assembled films of hydrophobin protein HFBIII from Trichoderma reesei. J Appl Crystallogr. International Union of Crystallography; 2007 Feb 3;40(s1):s355–60.

119. Grunér MS, Szilvay GR, Berglin M, Lienemann M, Laaksonen P, Linder MB. Self-assembly of class II hydrophobins on polar surfaces. Langmuir. 2012 Mar 6;28(9):4293–300.

120. Kubicek CP, Baker S, Gamauf C, Kenerley CM, Druzhinina IS. Purifying selection and birth-and-death evolution in the class II hydrophobin gene families of the ascomycete Trichoderma/Hypocrea. BMC Evol Biol. BioMed Central; 2008 Jan 10;8(1):4.

121. Linder M, Selber K, Nakari-Setälä T, Qiao M, Kula M-R, Penttilä M. The Hydrophobins HFBI and HFBII from Trichoderma r eesei Showing Efficient Interactions with Nonionic Surfactants in Aqueous Two-Phase Systems. Biomacromolecules. American Chemical Society; 2001 Jun;2(2):511–7.

122. Askolin S, Linder M, Scholtmeijer K, Tenkanen M, Penttilä M, de Vocht

ML, et al. Interaction and comparison of a class I hydrophobin from Schizophyllum commune and class II hydrophobins from Trichoderma reesei. Biomacromolecules. American Chemical Society; 2006 Apr;7(4):1295–301.

123. Linder M, Szilvay GR, Nakari-Setälä T, Söderlund H, Penttilä M. Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from Trichoderma reesei. Protein Sci. 2002 Sep;11(9):2257–66.

124. Espino-Rammer L, Ribitsch D, Przylucka A, Marold A, Greimel KJ, Herrero Acero E, et al. Two novel class II hydrophobins from Trichoderma spp. stimulate enzymatic hydrolysis of poly(ethylene terephthalate) when expressed as fusion proteins. Appl Environ Microbiol. 2013 Jul 15;79(14):4230–8.

125. Arcalis E, Ibl V, Peters J, Melnik S, Stoger E. The dynamic behavior of storage organelles in developing cereal seeds and its impact on the production of recombinant proteins. Front Plant Sci. Frontiers; 2014 Sep 3;5.

126. Schmidt SR. Protein bodies in nature and biotechnology. Mol Biotechnol. 2013 Jun;54(2):257–68.

127. Ibl V, Stoger E. The formation, function and fate of protein storage compartments in seeds. Protoplasma. Springer Vienna; 2012 Apr 26;249(2):379–92.

128. Llop-Tous I, Madurga S, Giralt E, Marzabal P, Torrent M, Ludevid MD. Relevant elements of a maize gamma-zein domain involved in protein body biogenesis. J Biol Chem. 2010 Nov 12;285(46):35633–44.

129. Torrent M, Llompart B, Lasserre-Ramassamy S, Llop-Tous I, Bastida M, Marzabal P, et al. Eukaryotic protein production in designed storage organelles. BMC Biol. 2009 Jan;7:5.

130. Conley AJ, Joensuu JJ, Menassa R, Brandle JE. Induction of protein body formation in plant leaves by elastin-like polypeptide fusions. BMC Biol. BioMed Central; 2009 Jan 7;7(1):48.

131. Gutiérrez SP, Saberianfar R, Kohalmi SE, Menassa R. Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. BMC Biotechnol. BioMed Central Ltd; 2013 Jan 10;13(1):40.

132. Saberianfar R, Joensuu JJ, Conley AJ, Menassa R. Protein body formation in leaves of Nicotiana benthamiana: a concentration-dependent mechanism influenced by the presence of fusion tags. Plant Biotechnol J. 2015 Sep;13(7):927–37.

133. Mustalahti E, Saloheimo M, Joensuu JJ. Intracellular protein production in Trichoderma reesei (Hypocrea jecorina) with hydrophobin fusion technology. N Biotechnol. Elsevier B.V.; 2013;30(2):262–8.

134. Lahtinen T, Linder MB, Nakari-Setälä T, Oker-Blom C. Hydrophobin (HFBI): A potential fusion partner for one-step purification of recombinant proteins from insect cells. Protein Expr Purif. 2008 May;59(1):18–24.

135. Conley AJ, Joensuu JJ, Richman A, Menassa R. Protein body-inducing fusions for high-level production and purification of recombinant proteins in

plants. Plant Biotechnol J. 2011 May;9(4):419–33.

136. Saberianfar R, Sattarzadeh A, Joensuu JJ, Kohalmi SE, Menassa R. Protein Bodies in Leaves Exchange Contents through the Endoplasmic Reticulum. Front Plant Sci. Frontiers; 2016 May 23;7:693.

137. Khalesi M, Gebruers K, Derdelinckx G. Recent Advances in Fungal Hydrophobin Towards Using in Industry. Protein J. 2015 Aug;34(4):243–55.

138. Cox AR, Aldred DL, Russell AB. Exceptional stability of food foams using class II hydrophobin HFBII. Food Hydrocoll. 2009 Mar;23(2):366–76.

139. Sarlin T, Kivioja T, Kalkkinen N, Linder MB, Nakari-Setälä T. Identification and characterization of gushing-active hydrophobins from Fusarium graminearum and related species. J Basic Microbiol. 2012 Apr;52(2):184–94.

140. Soikkeli M, Kurppa K, Kainlauri M, Arpiainen S, Paananen A, Gunnarsson D, et al. Graphene biosensor programming with genetically engineered fusion protein monolayers. ACS Appl Mater Interfaces. American Chemical Society; 2016 Mar 10;

141. Wang Z, Lienemann M, Qiau M, Linder MB. Mechanisms of protein adhesion on surface films of hydrophobin. Langmuir. American Chemical Society; 2010 Jun 1;26(11):8491–6.

142. Linder MB, Qiao M, Laumen F, Selber K, Hyytiä T, Nakari-Setälä T, et al. Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. Biochemistry. American Chemical Society; 2004 Sep 21;43(37):11873–82.

143. Collén A, Persson J, Linder M, Nakari-Setälä T, Penttilä M, Tjerneld F, et al. A novel two-step extraction method with detergent/polymer systems for primary recovery of the fusion protein endoglucanase I–hydrophobin I. Biochim Biophys Acta - Gen Subj. 2002 Jan;1569(1–3):139–50.

144. Selber K, Tjerneld F, Collén A, Hyytiä T, Nakari-Setälä T, Bailey M, et al. Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. Process Biochem. 2004 Mar;39(7):889–96.

145. Tortorella S, Karagiannis TC. Transferrin receptor-mediated endocytosis: a useful target for cancer therapy. J Membr Biol. 2014 Apr;247(4):291–307.

146. Valo HK, Laaksonen PH, Peltonen LJ, Linder MB, Hirvonen JT, Laaksonen TJ. Multifunctional hydrophobin: toward functional coatings for drug nanoparticles. ACS Nano. American Chemical Society; 2010 Mar 23;4(3):1750–8.

147. Valo H, Kovalainen M, Laaksonen P, Häkkinen M, Auriola S, Peltonen L, et al. Immobilization of protein-coated drug nanoparticles in nanofibrillar cellulose matrices--enhanced stability and release. J Control Release. 2011 Dec 20;156(3):390–7.

148. Pigliacelli C, D'Elicio A, Milani R, Terraneo G, Resnati G, Baldelli Bombelli F, et al. Hydrophobin-stabilized dispersions of PVDF nanoparticles in water. J Fluor Chem. 2015 Sep;177:62–9.

149. Bimbo LM, Mäkilä E, Raula J, Laaksonen T, Laaksonen P, Strommer K, et al. Functional hydrophobin-coating of thermally hydrocarbonized porous silicon microparticles. Biomaterials. 2011 Dec;32(34):9089–99.

150. Sarparanta MP, Bimbo LM, Mäkilä EM, Salonen JJ, Laaksonen PH, Helariutta AMK, et al. The mucoadhesive and gastroretentive properties of hydrophobin-coated porous silicon nanoparticle oral drug delivery systems. Biomaterials. 2012 Apr;33(11):3353–62.

151. Sarparanta M, Bimbo LM, Rytkönen J, Mäkilä E, Laaksonen TJ, Laaksonen P, et al. Intravenous delivery of hydrophobin-functionalized porous silicon nanoparticles: stability, plasma protein adsorption and biodistribution. Mol Pharm. 2012 Mar 5;9(3):654–63.

152. Grunér MS, Kauscher U, Linder MB, Monopoli MP. An environmental route of exposure affects the formation of nanoparticle coronas in blood plasma. J Proteomics. 2015 Nov 9;137:52–8.

153. Paananen A, Ercili-Cura D, Saloheimo M, Lantto R, Linder MB. Directing enzymatic cross-linking activity to the air–water interface by a fusion protein approach. Soft Matter. Royal Society of Chemistry; 2013 Jan 2;9(5):1612–9.

154. Laaksonen P, Walther A, Malho J-M, Kainlauri M, Ikkala O, Linder MB. Genetic engineering of biomimetic nanocomposites: diblock proteins, graphene, and nanofibrillated cellulose. Angew Chem Int Ed Engl. 2011 Sep 5;50(37):8688–91.

155. Miletic S, Simpson DJ, Szymanski CM, Deyholos MK, Menassa R. A Plant-Produced Bacteriophage Tailspike Protein for the Control of Salmonella. Front Plant Sci. Frontiers; 2015 Jan 8;6:1221.

156. Phan HT, Hause B, Hause G, Arcalis E, Stoger E, Maresch D, et al. Influence of elastin-like polypeptide and hydrophobin on recombinant hemagglutinin accumulations in transgenic tobacco plants. PLoS One. Public Library of Science; 2014 Jan 10;9(6):e99347.

157. Pereira EO, Kolotilin I, Conley AJ, Menassa R. Production and characterization of in planta transiently produced polygalacturanase from Aspergillus niger and its fusions with hydrophobin or ELP tags. BMC Biotechnol. 2014;14(1):59.

158. Jacquet N, Navarre C, Desmecht D, Boutry M. Hydrophobin Fusion of an Influenza Virus Hemagglutinin Allows High Transient Expression in Nicotiana benthamiana, Easy Purification and Immune Response with Neutralizing Activity. PLoS One. 2014;9(12):e115944.

159. Moks T, Abrahmsén L, Nilsson B, Hellman U, Sjöquist J, Uhlén M. Staphylococcal protein A consists of five IgG-binding domains. Eur J Biochem. 1986 May 2;156(3):637–43.

160. Tashiro M, Tejero R, Zimmerman DE, Celda B, Nilsson B, Montelione GT. High-resolution solution NMR structure of the Z domain of staphylococcal protein A. J Mol Biol. 1997 Oct 3;272(4):573–90.

161. McLean MD, Chen R, Yu D, Mah KZ, Teat J, Wang H, et al. Purification of the therapeutic antibody trastuzumab from genetically modified plants using safflower Protein A-oleosin oilbody technology. Transgenic Res.

2012;21(6):1291–301.

162. Werner S, Marillonnet S, Hause G, Klimyuk V, Gleba Y. Immunoabsorbent nanoparticles based on a tobamovirus displaying protein A. Proc Natl Acad Sci U S A. National Academy of Sciences; 2006 Nov 21;103(47):17678–83.

163. Brandsma ME, Diao H, Wang X, Kohalmi SE, Jevnikar AM, Ma S. Plant-derived recombinant human serum transferrin demonstrates multiple functions. Plant Biotechnol J. 2010;8(4):489–505.

164. Zhang D, Nandi S, Bryan P, Pettit S, Nguyen D, Santos MA, et al. Expression, purification, and characterization of recombinant human transferrin from rice (Oryza sativa L.). Protein Expr Purif. Elsevier Inc.; 2010;74(1):69–79.

165. Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, et al. Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J. Blackwell Science Ltd; 2005 Nov 24;44(6):1065–76.

166. Fuchs U, Czymmek KJ, Sweigard JA. Five hydrophobin genes in Fusarium verticillioides include two required for microconidial chain formation. Fungal Genet Biol. 2004 Sep;41(9):852–64.

167. Popovic MM, Milovanovic M, Burazer L, Vuckovic O, Hoffmann-Sommergruber K, Knulst AC, et al. Cysteine proteinase inhibitor Act d 4 is a functional allergen contributing to the clinical symptoms of kiwifruit allergy. Mol Nutr Food Res. 2010;54(3):373–80.

168. Lienemann M, Gandier J-A, Joensuu JJ, Iwanaga A, Takatsuji Y, Haruyama T, et al. Structure-function relationships in hydrophobins: probing the role of charged side chains. Appl Environ Microbiol. 2013 Sep 15;79(18):5533–8.

169. Langbecker CL, Ye G-N, Broyles DL, Duggan LL, Xu CW, Hajdukiewicz PTJ, et al. High-frequency transformation of undeveloped plastids in tobacco suspension cells. Plant Physiol. 2004 May 1;135(1):39–46.

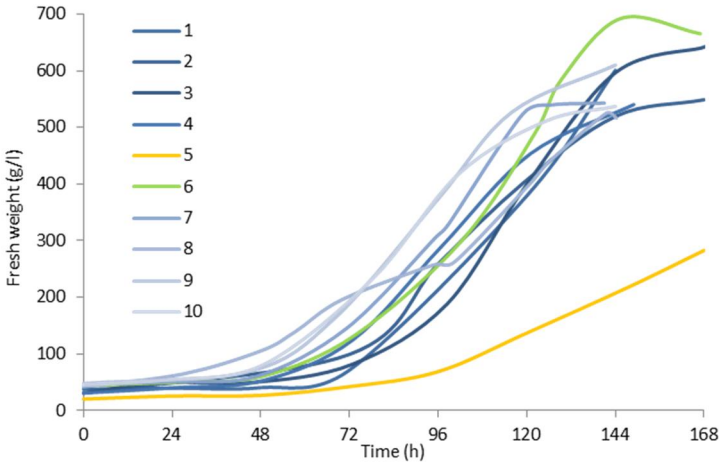
170. Hoppmann V, Fiore SD i., Zimmermann S, Emans N, Rademacher T, Fischer R, et al. The potato granule bound starch synthase chloroplast transit peptide directs recombinant proteins to plastids. J Plant Physiol. 2002 Jan;159(10):1061–7.

171. Kirchhoff J. Generation of highly productive polyclonal and monoclonal tobacco suspension lines from a heterogeneous transgenic BY-2 culture through flow cytometric sorting. 2012;

172. Hellwig S, Drossard J, Twyman RM, Fischer R. Plant cell cultures for the production of recombinant proteins. Nat Biotechnol. Nature Publishing Group; 2004 Nov;22(11):1415–22.

173. Huang T-K, McDonald KA. Bioreactor systems for in vitro production of foreign proteins using plant cell cultures. Biotechnol Adv. 2012 Jan;30(2):398–409.

Appendix 1. Accumulation of biomass in bioreactor cultivations



Accumulation of fresh weight in all 10 bioreactor baches. Batches 1 to 4 were started with smaller inoculum. Batch 5 (orange) was inoculated with only approximately 20 gFW/l. Batches 6 to 10 were inoculated with more than 40 gFW/l.

PUBLICATION I

Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells

Plant Biotechnology Journal 12, 402–410.

Plant Biotechnology Journal

Plant Biotechnology Journal (2014) **12**, pp. 402–410 doi: 10.1111/pbi.12147

Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells

Lauri J. Reuter, Michael J. Bailey, Jussi J. Joensuu and Anneli Ritala*

VTT Technical Research Centre of Finland, Espoo, Finland

Received 8 October 2013;
accepted 23 October 2013.
**Correspondence* (Tel +358 400 838 631;
fax +358 207 227 071;
email anneli.ritala@vtt.fi)

Keywords: aqueous two-phase separation, bioreactor, hydrophobin fusion, scale-up, suspension cells, tobacco bright yellow 2.

Summary

Plant suspension cell cultures are emerging as an alternative to mammalian cells for production of complex recombinant proteins. Plant cell cultures provide low production cost, intrinsic safety and adherence to current regulations, but low yields and costly purification technology hinder their commercialization. Fungal hydrophobins have been utilized as fusion tags to improve yields and facilitate efficient low-cost purification by surfactant-based aqueous two-phase separation (ATPS) in plant, fungal and insect cells. In this work, we report the utilization of hydrophobin fusion technology in tobacco bright yellow 2 (BY-2) suspension cell platform and the establishment of pilot-scale propagation and downstream processing including first-step purification by ATPS. Green fluorescent protein-hydrophobin fusion (GFP-HFBI) induced the formation of protein bodies in tobacco suspension cells, thus encapsulating the fusion protein into discrete compartments. Cultivation of the BY-2 suspension cells was scaled up in standard stirred tank bioreactors up to 600 L production volume, with no apparent change in growth kinetics. Subsequently, ATPS was applied to selectively capture the GFP-HFBI product from crude cell lysate, resulting in threefold concentration, good purity and up to 60% recovery. The ATPS was scaled up to 20 L volume, without loss off efficiency. This study provides the first proof of concept for large-scale hydrophobin-assisted production of recombinant proteins in tobacco BY-2 cell suspensions.

Introduction

Plants and suspension cultures of dedifferentiated plant cells are emerging as an alternative to mammalian cell cultures as eukaryotic production platforms for complex recombinant proteins. Plant cell suspensions combine some of the benefits of molecular farming in whole plants and cultivation of mammalian cells (Doran, 2013; Hellwig *et al.*, 2004; Xu *et al.*, 2011). In comparison with field- or greenhouse-grown plants, cell suspensions enable propagation in standardized bioreactors, thereby offering control over the culture conditions and higher batch to batch consistency. Full containment also enables adherence to current good manufacturing practise throughout the production chain (Fischer *et al.*, 2012) and avoids the concern of gene flow to the environment (Doran, 2013). Furthermore, downstream processing of cell suspensions is significantly facilitated due to the lack of fibres, waxes, many secondary metabolites and possible residues of agrochemicals. In comparison with mammalian cells, plant suspension cells grow rapidly in very simple and inexpensive, chemically defined media and most importantly are devoid of any known human pathogens (Doran, 2013). Plant cells can also be used to produce proteins that require plant-like post-transcriptional modifications or proteins that would be harmful or toxic for mammalian host cells.

Tobacco bright yellow 2 (BY-2) cell line has been referred to as the 'HeLa-cells in the biology of higher plants' (Nagata *et al.*, 1992) due to its various applications in fundamental research. However, it would be equally justified to refer to BY-2 as the 'CHO-cells of molecular farming'. The cell line has been utilized as an expression host for numerous recombinant proteins (Bortesi *et al.*, 2012; Kaldis *et al.*, 2013; Kirchhoff *et al.*, 2012; Sack *et al.*, 2007; Schiermeyer *et al.*, 2005; Schinkel *et al.*, 2005; Sun *et al.*, 2011) and exhibits an exceptional growth rate, multiplying 80- to 100-fold over 1 week in optimal conditions (Nagata *et al.*, 1992). Moreover, the propagation has been established in simple bioreactor systems (Holland *et al.*, 2010; Schmale *et al.*, 2006; Xu *et al.*, 2011). However, the yields of recombinant proteins produced in the BY-2 cell line are still generally low, only occasionally reaching levels of 0.1 to 0.5 g/l (Hellwig *et al.*, 2004; Kaldis *et al.*, 2013; Xu *et al.*, 2011), whereas yields of 5 g/l are common in mammalian cell cultures (Walsh, 2010). Thus, further development is needed in order to meet the general industrial demand of grams per litre product titre.

Recently, several fusion tags, including elastin-like polypeptides (ELP; Conley *et al.*, 2009; Kaldis *et al.*, 2013), zein-derived peptides (Joseph *et al.*, 2012; Torrent *et al.*, 2009) and hydrophobins (HFB; Joensuu *et al.*, 2010), have been introduced as alternative strategies to increase yields of recombinant proteins in plants by stabilizing the fusion partner and directing accumulation of the fusion protein in discrete storage structures (Conley *et al.*, 2011; Khan *et al.*, 2012). HFBs, ubiquitously produced by filamentous fungi, are small (7–15 kD) globular proteins with amphiphilic properties (Hakanpää *et al.*, 2006). Interestingly, when expressed as fusion protein, the hydrophobin 1 (HFBI) of *Trichoderma reesei* has been shown to induce the formation of protein bodies in plant leaves (Gutiérrez *et al.*, 2013; Joensuu *et al.*, 2010) and in filamentous fungi (Mustalahti *et al.*, 2013). Protein bodies are structures typically present in developing seeds, but the detailed mechanism of protein body formation in vegetative tissues induced by foreign proteins remains to be clarified. Nevertheless, the phenomenon appears to be comparable to the formation of similar structures by ELP (Conley *et al.*, 2009; Kaldis *et al.*, 2013) and zein-derived peptides (Torrent *et al.*, 2009). Previously, transient expression of green fluorescent

protein-hydrophobin fusion (GFP-HFBI) in *Nicotiana benthamiana* leaves (Joensuu *et al.*, 2010) as well as stable expression in tobacco plants (Gutiérrez *et al.*, 2013) has resulted in twofold yields in comparison with free GFP.

In addition to the potential increase in yield, the HFB-fusion enables a simple and efficient non-chromatographic method for recovering recombinant protein products by aqueous two-phase separation (ATPS; Linder *et al.*, 2004; Penttilä *et al.*, 2008). In this process, HFB-fusion proteins are captured in micellar structures and concentrated in a surfactant phase, while most of the native proteins remain in the aqueous phase. Subsequently, the HFB-fusion is recovered by removing the surfactant with isobutanol back extraction. ATPS has previously been reported as a potential method for first-step purification and concentration of recombinant proteins from *T. reesei* cultures (Linder *et al.*, 2004; Mustalahti *et al.*, 2013), insect cells (Lahtinen *et al.*, 2008) and *N. benthamiana* leaf material (Joensuu *et al.*, 2010).

Here, we report incorporation of HFB-fusion technology in large-scale tobacco BY-2 suspension cell culture, formation of protein bodies and efficient purification of GFP-HFBI fusion by ATPS.

Results

Generation of transgenic callus lines

To investigate the function of the *T. reesei* HFBI fusion tag, two expression vectors (Joensuu *et al.*, 2010), carrying expression constructs for endoplasmic reticulum (ER-) targeted free GFP and ER-targeted HFBI-fused GFP, respectively, were introduced to tobacco BY-2 cells through *Agrobacterium tumefaciens*-mediated transformation. A total of 29 transgenic callus lines were recovered: 10 lines expressing GFP and 19 lines expressing GFP-HFBI. Many of the callus lines exhibited heterogenic GFP expression, observed as sectorial or mosaic patterns of visible fluorescence under UV-light. To obtain high and consistent expression levels, the calli were subcultured by visually selecting fragments with the most intensive fluorescence, but after multiple rounds of fluorescence-based selection, the expression levels remained inconsistent. However, suspension cultures were prepared from several callus lines and growth, and GFP accumulation levels were screened. Two cell lines, each carrying one of the two constructs, were selected for further experiments on the basis of good expression levels as well as suspension morphology and growth. In these lines, the GFP and GFP-HFBI represented up to 30% and 17% of TSP, respectively (Figure 1f).

HFBI fusion induces protein bodies in BY-2 suspension cells

Laser scanning confocal microscopy was applied to confirm the formation of hydrophobin-induced protein bodies in BY-2 suspension cells. ER-targeted free GFP was localized in the typical reticulate structure of ER (Figure 1a,c, Movie S1). By contrast, hydrophobin-fused GFP was found to induce formation of dense spherical protein bodies (Figure 1b,d, Movie S2). The protein bodies as well as the ER network were located in the periphery of the cells, whereas the central space was occupied by large vacuolar compartments (Figure 1e). During the late phase of the suspension culture, both free GFP and GFP-HFBI were located in large, sometimes irregularly shaped structures in some cells (data not shown). Fluorescence was also observed in vacuoles at this time.

Microscopic observation of the cell suspensions confirmed the heterogeneity of the population with respect to the fluorescence intensity of individual cells: high, moderate and nonexpressing cells were observed.

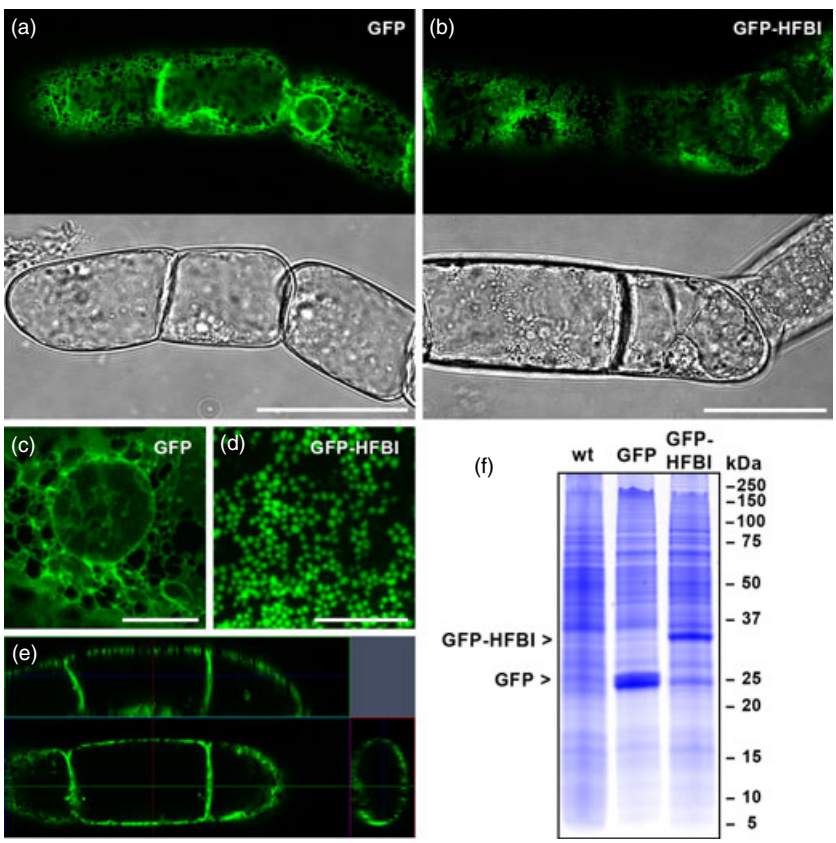


Figure 1 Expression of endoplasmic reticulum (ER)-targeted hydrophobin-fused GFP induces the formation of protein bodies in tobacco bright yellow 2 cells. (a,c) Free GFP is distributed in the typical web-like structure of ER, whereas (b,d) green fluorescent protein-hydrophobin fusion (GFP-HFBI) is located in small spherical protein bodies. A digital dissection of the Z-stack image (e) reveals the large vacuolar compartments restricting the ER and cytoplasm to the periphery of the cells (data shown only for a GFP-HFBI expressing cell). Scale bars represent 50 μm in (a) and (b) and 10 μm in (c) and (d). (f) Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis from the corresponding cell suspensions. TSP from equal amounts of dry cell mass were loaded on the gel. Free GFP at 25 kD and GFP-HFBI at 35 kD. A cleavage product slightly smaller than 25 kD is visible in both lanes.

Growth of cell suspension in bioreactors

To evaluate scalability of the BY-2 cell suspension platform, propagation of the GFP-HFBI line was scaled up from 50 mL culture volumes in shake flasks first to 20 and finally to 600 L culture volumes in stirred tank bioreactors. Accumulation of biomass was comparable in all culture volumes (Figure 2a). Thus, detailed data are presented only for the 600 L cultivation. During the initial lag phase in growth, sucrose was hydrolysed to glucose (and fructose, not measured) and the level of dissolved oxygen (DO) decreased steadily, while the concentration of dissolved carbon dioxide (CO_2) increased (Figure 2b). Characteristically for the BY-2 cells, a drop in pH was observed during the first hours (Figure S1). The lag phase changed into exponential growth after 72 h. The agitation, aeration and vessel overpressure gradually increased according to the output of the DO cascade controller to maintain DO >30%, reaching their maximum values at 125 h (Figure S1). Thereafter, the level of DO decreased close to 0%, which may or may not have correlated with oxygen deficiency in the culture (see Discussion). Approximately at the same time, the carbon sources were exhausted and cell growth reached a plateau, with culture dry weight (DW) peaking at 16–18 g/L (Figure 2a). Fresh weight (FW) and packed cell volume (PCV), however, continued to increase until termination of the culture, probably due to continuing uptake of water into the cell vacuoles.

Accumulation of GFP-HBFI fusion protein

Accumulation of GFP-HFBI fusion protein and total soluble protein (TSP) was monitored by offline sampling during the bioreactor operation. The proportion of TSP of the cell DW peaked during the phase of exponential growth and decreased after the stationary phase was reached (Figure 2c). However, the titre of GFP-HFBI in the culture suspension continued to increase until termination of the culture at 168 h. In the 600-L cultivation, GFP-HFBI titre reached a level of 0.30 ± 0.018 g/l, corresponding to 16.5% of TSP. Only minor cleavage of the fusion protein was observed from 120 h onwards (Figure 2d).

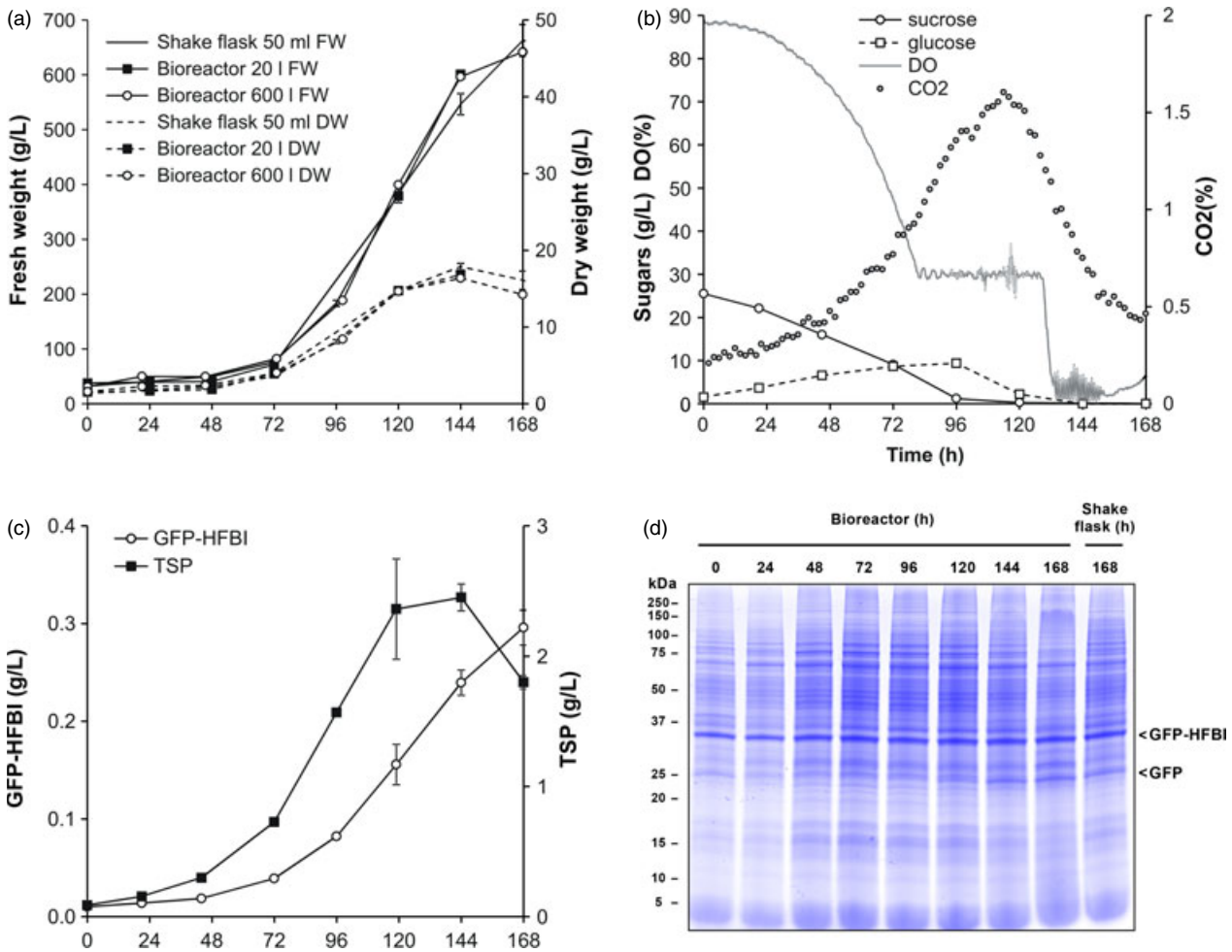


Figure 2 Suspension culture of tobacco bright yellow 2 cells is scalable in standard stirred tank bioreactors. (a) Accumulation of fresh and dry biomass in 20 and 600 L culture volumes in bioreactors (n = 1) compared with growth in a 50-mL culture volume in shake flask (n = 3, mean ± standard deviation). (b) Online measurements of dissolved oxygen (DO) and carbon dioxide (CO_2) and offline measured levels of sucrose and glucose in the 600 L culture. (c) Accumulation of intracellular green fluorescent protein-hydrophobin fusion (GFP-HFBI) and total soluble protein (TSP) in 600 L culture (technical repeats: n=3; mean ± standard deviation). (d) A Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicating the stability of the fusion protein in 600 L culture in comparison with shake flask culture. The fusion protein (approximately 35 kD) and cleaved GFP fraction (25 kD), clearly visible at time points 144 and 168 h, are indicated with arrows. TSP from equal amounts of dry cell mass was loaded on the gel.

Biomass harvesting, lyophilization and protein extraction

The biomass propagated in the 600-L bioreactor was harvested from culture suspension using a filter press and lyophilized for storing. The water removal efficiency of the filter press was rather good: the biomass after filtration, amounting to 34% of original suspension mass, was quite dry to the touch. Because of the very high bulk density of the culture (centrifuged PCV 65%), centrifugation was not suitable as a means of separation.

The option to store dehydrated BY-2 biomass safely at room temperature and the effect of drying on protein extraction were evaluated by lyophilizing BY-2 suspension cells expressing GFP-HFBI, storing a sample at RT for 1 month and extracting the soluble proteins. The final DW of the biomass was only approximately 3.5% of the FW, reflecting the very high vacuolar volume of BY-2 cells. Neither the freeze drying procedure *per se* nor subsequent storage at RT for 1 month caused measurable degradation or cleavage of the target protein (Figure 3a).

A range of buffer volumes was tested for extraction of soluble proteins from the freeze-dried and powdered cell material (Figure 3d). High concentration, up to 4.4 ± 0.7 mg/mL of GFP-HFBI in crude extract, was reached using 5 mL extraction buffer for 1 g of dry cell powder without apparent reduction in recovery rate. This indicates good solubility of the fusion protein. However, low buffer volumes resulted in thick suspensions that were difficult to handle. Therefore, subsequent extractions were made using 40 volumes of extraction buffer per dry cell powder weight.

Scaled up purification by ATPS

Aqueous two-phase separation was applied to capture the GFP-HFBI fusion protein from cell extract. To assess scalability of the process, the initial surfactant extraction was conducted in volumes of 100 mL, 1 and 20 L using Triton X-114 (3% w/v) (Joensuu *et al.*, 2010, 2012). Clear separation of the heavier surfactant phase carrying the GFP-HFBI was observed under UV-light after approximately 15 min in 100 mL, 30 min in 1 L and 1 h 30 min in 20 L volume (Figure 4a). In all separation volumes, the heavier phase comprised 35%–38% of the total volume.

The heavier phase was collected and the surfactant was removed by extraction with isobutanol, leaving the fusion protein in the buffer phase (back extract). The volume of the recovered back extract was 76% of that of the surfactant phase and <30% of the initial crude extract volume.

Visual observation of GFP-HFBI partitioning in the surfactant phase and subsequently to back extract (Figure 4a) was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorometry (Figure 4b). The fusion protein was found to selectively concentrate in the back extract, and only residual amounts remained in the aqueous residual phase with most of the native proteins (Figure 4b,c). Concentration of the product was doubled in comparison with crude extract, representing more than 70% of TSP. Although the time required for phase separation increased with separation volume, efficiency of the ATPS was found not to be dependent on the volume (Figure 4c). The overall recovery rate after the ATPS was approximately 50%–60% of the total soluble GFP-HFBI in all extraction volumes (Figure 4c). Only minimal cleavage of the fusion protein was observed, although the whole process was carried out in room temperature.

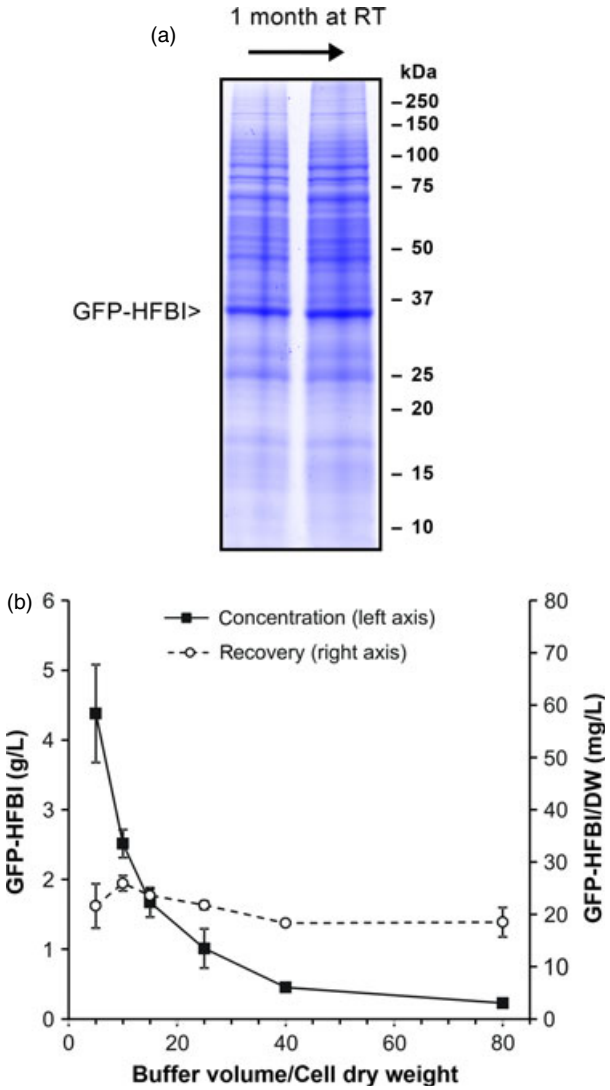


Figure 3 Extraction of the hydrophobin fusion protein from freeze-dried cell material. (a) Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis of crude protein extract after lyophilization, and after storing, the dry material for 1 month at RT does not indicate apparent cleavage of the fusion protein. (b) Effect of the volume of extraction buffer on product concentration and recovery (n = 3, mean ± standard deviation). On the x-axis: volumes of buffer (mL)/cell material (g).

Discussion

Several issues including improvement in production rates, batch to batch consistency and efficient purification still need to be addressed before plant suspension cell platforms for production of high-value recombinant proteins can compete with already established systems on an industrial level. We set out to investigate the potential of hydrophobin fusion technology as a tool to facilitate both large-scale production and purification of recombinant proteins in tobacco BY-2 cell suspension.

Genetic instability and inconsistent yields are recognized as a major drawback in plant cell cultures (Doran, 2013; Xu *et al.*, 2011). In this study too, no definite values could be given for expression levels due to the inconsistency in productivity. The

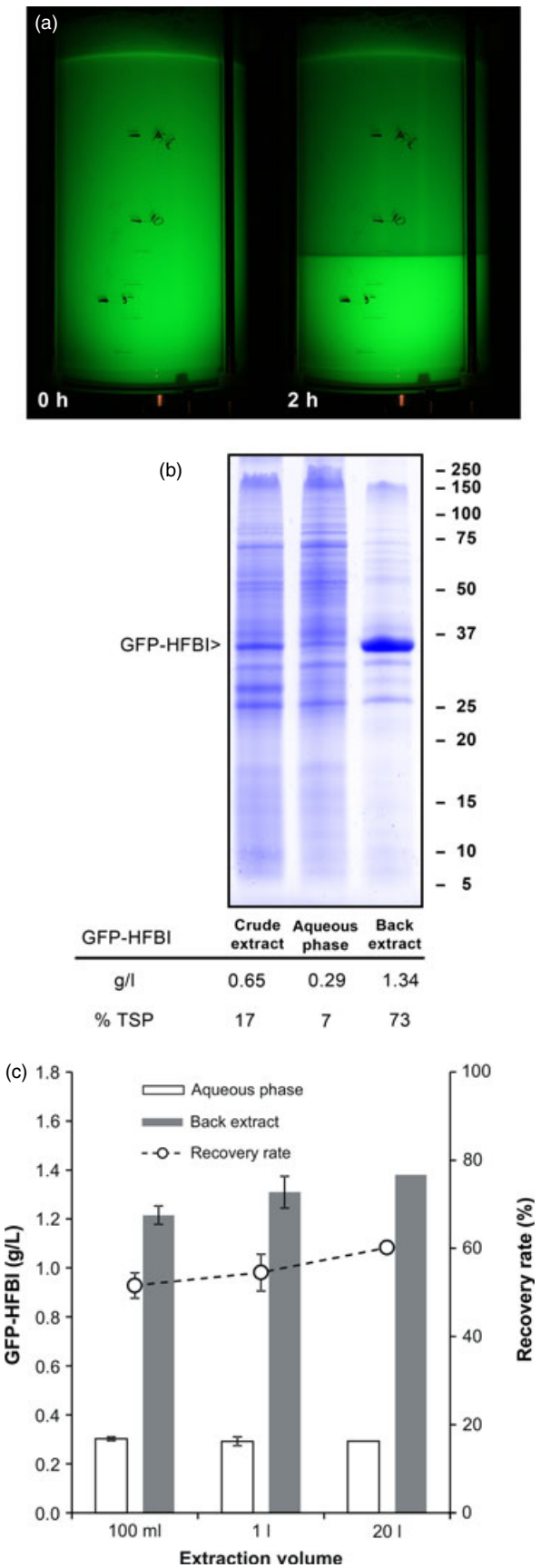


Figure 4 Purification of hydrophobin-fused green fluorescent protein (GFP) using surfactant-based aqueous two-phase separation (ATPS). (a) ATPS conducted in a volume of 20 L. Photographs were taken under UV-light to illustrate migration of the target protein to the surfactant phase. (b) Coomassie-stained sodium dodecyl sulphate-polyacrylamide gel electrophoresis showing selective recovery of the green fluorescent protein-hydrophobin fusion (GFP-HFBI) in the back extract, when most of the native proteins remain in the water phase. (c) The recovery rate and GFP-HFBI concentration in aqueous phase (residue) and in back extract in different separation volumes (100 mL: $n = 3$, mean ± standard deviation; 1 L: $n = 4$, mean ± standard deviation; 20 L: $n = 1$).

majority of the transgenic calli exhibited a mosaic or sectorial pattern of heterogenic intensity of visual fluorescence despite several passages manually selecting only the most fluorescent fractions. Microscopic examination of the cell suspensions derived from the calli confirmed this observation, revealing a heterogenic population of cells with varying fluorescence intensities. Furthermore, the levels of GFP expression decreased gradually over continuous passages in suspension culture. Similar heterogeneity has commonly been encountered in BY-2 cell lines expressing fluorescent proteins (Kirchhoff *et al.*, 2012; Nocarova and Fischer, 2009). Both genetic and epigenetic factors have been proposed as the cause for this variation (Doran, 2013). Preparation of monoclonal lines from the primary callus has been shown to reduce the heterogeneity significantly (Nocarova and Fischer, 2009) and improve the yield (Kirchhoff *et al.*, 2012). Although the expression levels of the monoclonal cell lines remained consistent over several months (Nocarova and Fischer, 2009) or even for up to 1 year (Kirchhoff *et al.*, 2012), they were not devoid of subsequent somaclonal variation or epigenetic changes, that is, gene silencing. Thus, the selection may ultimately need to be repeated. Nevertheless, generation of defined monoclonal cultures is required in order to meet the industrial demand for sufficient batch to batch consistency and specific characterization of production lines.

This is the first report confirming the formation of HFBI-induced protein bodies in BY-2 cells. The HFBI-fused GFP was localized in dense spherical structures closely resembling the protein bodies earlier reported in relation to HFBI in *N. benthamiana* (Joensuu *et al.*, 2010), in tobacco plants (Gutiérrez *et al.*, 2013) and on the other hand induced by ELP in BY-2 cells (Kaldis *et al.*, 2013).

Targeting of recombinant proteins to specific storage organelles using fusion tags, such as HFB, ELP or the zein-derived ZERA-peptide, has been a promising strategy to improve overall yields (Conley *et al.*, 2011; Khan *et al.*, 2012; Schmidt, 2013). Increased protein accumulation has been attributed to encapsulation of the recombinant protein from the proteolytic environment of the cytosol, thus protecting it from normal physiological turnover. Moreover, the encapsulation may also protect the host cell from the potentially toxic effects of the over-expressed protein (Joensuu *et al.*, 2010; Torrent *et al.*, 2009). However, the beneficial effect of the protein body formation on the yields in BY-2 cells could not be assessed reliably in this work due to the heterogeneity of the cell lines. Although brightly fluorescent protein bodies were observed in some of the GFP-HFBI-expressing suspension cells, some of the cells exhibited only weak or no fluorescence at all, and in these cells, the formation of protein bodies was not detected. As the

yield was measured from the bulk suspension, it does not reliably represent that of the cells with obvious protein body formation. Furthermore, the yields of the suspension cultures fluctuated over time, hampering comparison between the lines. In order to reliably assess the effect of hydrophobin fusion on the yield, stable monoclonal cell lines need to be generated and compared as discussed above.

The detailed mechanism of protein body formation in relation to ELP, zein-derived peptides or HFB is not well understood, although it appears to be conserved in all eukaryotes (Torrent *et al.*, 2009). All three fusion tags share hydrophobic or amphipathic properties and the tendency to self-assembly into stable aggregates in the ER (Conley *et al.*, 2011; Khan *et al.*, 2012). The variable levels of GFP-HFBI expression and consequently variable formation of protein bodies gives reason to assume that the concentration of the fusion protein in the ER may influence the self-assembly and that a certain threshold level of the fusion protein is required for protein body formation. Gutiérrez *et al.* (2013) reported similar conclusions in relation to HFBI-induced protein bodies in tobacco plants.

To assess the potential scalability of hydrophobin fusion technology-assisted protein production in BY-2 suspension cell platform, both pilot-scale suspension cultures and downstream processing were performed. Although several novel bioreactor systems have been proposed for propagation of plant suspension cells (Huang and McDonald, 2012; Kieran *et al.*, 1997; Xu *et al.*, 2011), we set out to evaluate the possibility of scaling up the propagation in standard steel stirred tank bioreactors designed for microbial cultivations.

The growth kinetics in various culture volumes and different bioreactors were found to be strikingly similar and well comparable to growth of the same cell line in shake flasks. Furthermore, the results obtained here are in line with the smaller-scale cultivations conducted with BY-2 suspension cells and reported previously (Holland *et al.*, 2010; Schmale *et al.*, 2006). Although the DO level of the pilot-scale culture was controlled by stirring speed, vessel overpressure and airflow, the level of DO decreased close to 0% at 125 h, coinciding with reaching the plateau of DW accumulation and peaking of pH. From this time point on, the culture might have suffered from lack of DO, possibly limiting the productivity. The equipment maxima of the DO cascade parameters (agitation, aeration, pressure) were far higher than those used in this cultivation of possibly mechanically sensitive plant cells. Another consideration was that it was not certain whether the observed low level of DO was in fact representative of the situation in the culture as a whole, or whether the DO probe was partially or completely covered by cell overgrowth in the conditions of low agitation. The fact that exhaust gas CO_2 was already decreasing before the start of the observed steep decrease in DO indicates that the latter explanation may be correct. Furthermore, on the basis of the offline curves, carbon source exhaustion also occurred at about the same time as the observed decrease in DO level, which is rather illogical from a biological perspective.

In this study, no growth inhibition due to shear stress was observed even in large culture volumes, even though the cell suspensions were cultured in bioreactors originally designed for microbial cultivations and equipped with Rushton turbines. This confirms the robustness of the BY-2 cell line and is in contrast to the general view of plant cells being highly sensitive to shear forces. Neither did the long cooling time of the media in pilot-scale bioreactor after sterilization cause any growth retardation.

In our experiments, the volumetric productivity of the recombinant protein was very high, reaching up to 0.3 g/L. However, the high yields cannot be directly attributed to the stabilizing function of the fusion partner, as the free GFP also accumulated in high levels. Even further improvement in the productivity could be obtained by selection of elite monoclonal lines, as discussed above, and by media optimization: Holland *et al.* (2010) reported 10- to 20-fold increase in antibody yields by the use of extra nitrate in the culture media.

Pilot- or large-scale cultivations of BY-2 suspension cells for production of recombinant proteins have not been reported in the literature. Thus, we regard the results presented here as important evidence of robustness and scalability of the BY-2 platform for recombinant protein production in conventional stirred tank bioreactors. In fact, the very same laboratory that originally generated the tobacco BY-2 cell line in the early 1970s at Japan Tobacco and Salt Co. reported successful propagations of the wild-type cell suspension in bioreactors as large as 20 000 L (Noguchi *et al.*, 1977). Despite the immense culture volume, the growth kinetics were similar to those in shake flask cultures and to the cultivations reported here.

Lyophilization of the filtered cell mass was found to allow convenient storage of the harvested material at room temperature as well as efficient cell disruption by milling. Removal of the intracellular water also provides significant concentration of the product. Although promising for small scale (<30 L) processing of high-value products, this approach may not be economical for handling large quantities of material due to limited capacity and the high cost of lyophilization in pilot scale. However, industrial-scale possibilities exist, for example, in the food industry, and in the case of high-value products, lyophilization may be considered feasible.

Surfactant-based ATPS is a convenient and low-cost method for first-step purification of hydrophobin fusion proteins from fungal cultures (Linder *et al.*, 2004; Mustalahti *et al.*, 2013; Selber *et al.*, 2004), insect cells (Lahtinen *et al.*, 2008) and plant tissues (Joensuu *et al.*, 2010). Here, we applied ATPS in purification of hydrophobin-fused GFP from BY-2 cell extract. Separation in ambient room temperature (21–24 °C) using 3% (w/v) Triton X-114 (Bordier, 1981) resulted in a heavy phase comprising 35%–38% of the total volume. After the back extraction, the final volume was further reduced to <30% of the original volume. With these parameters, both good volume reduction and recovery rate (50–60%) were obtained. Purification of the same fusion protein from tobacco leaf extracts using Agrimul NRE 1205 as surfactant was reported to result in comparable levels of recovery (Joensuu *et al.*, 2010). The work of Joensuu *et al.* (2010) further showed that optimization of the concentration of surfactant can be made to reach specific goals: more surfactant results in a better recovery rate, up to 90%, whereas less leads to higher product concentration.

The principles of how different sized hydrophobic molecules migrate in phases formed by different surfactants are not well understood. Thus, unpredictability and the requirement for empirical optimization remain the greatest challenges to development of the ATPS (Hatti-Kaul, 2001). By contrast, removal of surfactant by extracting with isobutanol has been shown to be very robust and has not been observed to cause denaturation of the target proteins (Joensuu *et al.*, 2010; Linder *et al.*, 2004). However, this step may also require optimization for less stable

products. Capturing hydrophobin-fused proteins from BY-2 cell lysate by surfactant-based ATPS appears to be readily scalable for large separation volumes. Our results indicate that the separation volume has significant impact only on the time needed for phase separation, not on the resulting concentrations, fraction volumes or product concentration. These findings support previous reports of the scalability of ATPS. Selber *et al.* (2004) as well as Penttilä *et al.* (2008) showed that scaling a surfactant-based two-phase separation of recombinant proteins from fungal cultures from 10 mL to 1200 L volume did not change the yield or partitioning efficiency.

Conclusions

This study provides for the first time a proof of concept for applying hydrophobin fusion technology in tobacco BY-2 suspension cell platform. Hydrophobin-fused GFP accumulated in ER-derived protein bodies, and the recombinant protein was captured from the cell lysate by surfactant-based ATPS. Furthermore, we have shown that propagation of BY-2 suspension cells is readily scalable in standard stirred tank bioreactors. Further investigations have been initiated to assess the feasibility of the BY-2-hydrophobin platform for various other target proteins.

Experimental procedures

Constructs

The expression vectors for ER-targeted GFP and GFP-HFBI were previously described by Joensuu *et al.* (2010). Briefly, the coding sequences were placed under control of the dual-enhancer *Cauliflower mosaic virus* 35S promoter and *A. tumefaciens* nos terminator. A TEV protease cleavage site was located in between the GFP and HFBI moieties.

Transformation and maintenance of the BY-2 cultures

Transformation of the BY-2 cells was performed as described by De Sutter *et al.* (2005). The stock cultures were maintained as calli on modified MS-medium (Nagata and Kumagai, 1999) containing 1% agar and 25 mg/L kanamycin and were subcultured every 3–4 weeks by visually selecting the most fluorescent fractions under UV-light. Suspension cultures were maintained in 50 mL of the modified MS-medium supplied with 50 mg/L kanamycin and subcultured weekly by transferring 5% (v/v) of the culture to fresh media.

Confocal microscopy

Subcellular localization of GFP and GFP-HFBI and formation of protein bodies were visualized in suspension cells 7 days after subculturing. A Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 40× or a 63× water immersion objective lens was used. Excitation was performed with a 488-nm argon laser, and fluorescence was detected at 493–598 nm.

Bioreactor cultivations

All bioreactor operations were conducted in batch cultivation mode by inoculating at 5% (v/v) with 7-day-old suspension. The 20- and 30-L cultivations (Biostat C, Sartorius AG, Goettingen, Germany and IF 40, New Brunswick Scientific, Enfield, CT) were inoculated from bulked shake flask cultures. For the 600 L cultivation (BioFlo PRO, New Brunswick Scientific, Enfield, CT), the inoculum was grown in an intermediate step in the 30-L bioreactor. The modified MS-medium (Nagata and Kumagai, 1999) was prepared and sterilized in the bioreactor for all cultivations. All cultivations were carried out in dark at 28 °C. Culture DO was controlled by stirring speed, airflow and vessel overpressure to maintain the DO concentration above a threshold of 30%; agitation was with standard Rushton turbines in all the bioreactors. In the pilot-scale cultivation (600 L), maximum agitation was set to 120 rpm (=2.3 m/s tip speed) and aeration to 200 L/min. The pH was monitored, but not controlled. No antifoam agent was added to the medium or during the cultivation procedure. All bioreactor cultivations were performed only once.

Packed cell volume was determined by sampling 10.0 mL of culture suspension in a conical tube and centrifuging at 3220 ***g*** for 10 min. The cell pellet was weighed to obtain FW and subsequently freeze-dried to obtain DW. The culture supernatant was stored at −20 °C and analysed later with a YSI 2900 Biochemistry Analyzer (YSI Life Sciences, Yellow springs, OH) to determine glucose and sucrose in the culture medium.

Downstream processing

In laboratory scale, the biomass was harvested by centrifugation and freeze-dried before cell disruption using steel beads and a Retsch mill (MM301, Haan, Germany). For pilot-scale downstream processing, the biomass was separated from the culture medium using a Larox filter press (PF 0.1 H2) and Aino T30 filter cloths, applying *ca.* 3–5 bar pressure. After primary filtration, the biomass cake was dried by applying a pressure of 8–10 bar via a rubber membrane over the biomass and removing the intercellular liquid thus released. The filtered cell mass was frozen and lyophilized. Cell disruption was performed with a Hosokawa Alpine (100 UPZ-lb) mill at 18 000 rpm.

Protein extraction and analysis

Disrupted cell powder was thoroughly mixed with extraction buffer (1 × phosphate buffered saline; 12 mM $Na_2HPO_4{\cdot}2H_2O$, 3 mM $NaH_2PO4{\cdot}H_2O$, 150 mM NaCl), 1 mM EDTA, 100 mM sodium ascorbate and 0.4 μM leupeptine hemisulfate (Sigma-Aldrich, St. Louis, MO), and insoluble material was removed by centrifugation (Eppendorf Centrifuge 5810R, 3220 ***g***, 10 min, RT). In pilot scale, centrifugation was carried out in 2-L bottles (Sorvall RC12BP, *ca*. 4000 ***g***, 15 min, RT).

Concentration of TSP was measured using the Bradford assay (1976) with Bio-Rad reagent (Bio-Rad, Hercules, CA) and bovine serum albumin (BSA; Sigma-Aldrich) as standard. Extracted proteins were separated by SDS-PAGE on Bio-Rad Criterion-TGX and Mini-PROTEAN precast gels. The GFP concentration in TSP and ATPS samples was determined by fluorometry. Dilutions of 1 : 50, 1 : 100 or 1 : 200 were prepared in black microtiter plates (Microfluor 2; Thermo Fisher Scientific, Waltham, MA) as triplicates by addition of PBS containing 1% (w/v) BSA. The fluorescence of the diluted samples was determined at 485/527 nm using a VICTOR^2 plate reader (Perkin Elmer, Waltham, MA) at 12 nm bandwidth and 100 ms measurement time. Sample dilutions were compared to a standard curve constructed with purified GFP (BioVision, Milpitas, CA).

ATPS

For ATPS, the cell extract (100 mL, 1 or 20 L) was thoroughly mixed with TritonX-114 (3% w/v; Sigma-Aldrich) and left to separate at RT in a separation funnel or in a 20-L cylindrical glass vessel. When the phases were clearly separated, the

surfactant phase was collected through the bottom valve and its volume was determined. The surfactant phase was mixed with an equal volume of isobutanol (Merck KGaA, Darmstadt, Germany), and phase separation was facilitated by centrifugation: in 100 mL scale in 50-mL tubes, in 1 L scale in 100-mL flasks (Eppendorf Centrifuge 5810R, 3220 ***g***, 5 min, RT) and in 20 L scale in 2-L flasks (Sorvall RC12BP, *ca*. 4000 ***g***, 5 min, RT). The product was recovered in the heavier aqueous phase. 100-mL separations were performed with three replicates, 1-L separations with four replicates and the 20-L separation only once. The recovery rate was calculated by dividing the total amount of GFP-HFBI in back extract by the total amount of GFP-HFBI in initial cell extract.

Acknowledgements

The authors gratefully thank Juha Tähtiharju, Merja Aarnio, Tuija Kössö and Riitta Suihkonen for their excellent assistance in pilot cultivations and downstream processing and Sirkka Kanervo, Tuuli Teikari, Jaana Rikkinen and Annika Majanen for technical assistance in cell culture work. This research was funded by Finnish Academy Grant 252442 and the VTT BizF_BY2UpScale 81598 funding. Support from COST action FA0804 Molecular Farming: plants as production platform for high-value proteins is also acknowledged.

References

Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**, 1604–1607.

Bortesi, L., Rademacher, T., Schiermeyer, A., Schuster, F., Pezzotti, M. and Schillberg, S. (2012) Development of an optimized tetracycline-inducible expression system to increase the accumulation of interleukin-10 in tobacco BY-2 suspension cells. *BMC Biotechnol.* **12**, 40 doi:10.1186/1472-6750-12-40.

Conley, A.J., Joensuu, J.J., Menassa, R. and Brandle, J.E. (2009) Induction of protein body formation in plant leaves by elastin-like polypeptide fusions. *BMC Biol.* **7**, 48.

Conley, A.J., Joensuu, J.J., Richman, A. and Menassa, R. (2011) Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. *Plant Biotechnol. J.* **9**, 419–433.

De Sutter, V., Vanderhaeghen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Karimi, M., Inzé, D., Goossens, A. and Hilson, P. (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. *Plant J.* **44**, 1065–1076.

Doran, P.M. (2013) Therapeutically Important Proteins From In Vitro Plant Tissue Culture Systems. *Curr. Med. Chem.* **20**, 1047–1055.

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R.M. and Drossard, J. (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnol. Adv.* **30**, 434–439.

Gutiérrez, S.P., Saberianfar, R., Kohalmi, S.E. and Menassa, R. (2013) Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. *BMC Biotechnol.* **13**, 40. doi:10.1186/1472-6750-13-40.

Hakanpää, J., Szilvay, G.R., Kaljunen, H., Maksimainen, M., Linder, M. and Rouvinen, J. (2006) Two crystal structures of *Trichoderma reesei* hydrophobin HFBI—The structure of a protein amphiphile with and without detergent interaction. *Protein Sci.* **15**, 2129–2140.

Hatti-Kaul, R. (2001) Aqueous two-phase systems: a general overview. *Mol. Biotechnol.* **19**, 269–277.

Hellwig, S., Drossard, J., Twyman, R.M. and Fischer, R. (2004) Plant cell cultures for the production of recombinant proteins. *Nat. Biotechnol.* **22**, 1415–1422.

Holland, T., Sack, M., Rademacher, T., Schmale, K., Altmann, F., Stadlmann, J., Fischer, R. and Hellwig, S. (2010) Optimal nitrogen supply as a key to increased and sustained production of a monoclonal full-size antibody in BY-2 suspension culture. *Biotechnol. Bioeng.* **107**, 278–289.

Huang, T.K. and McDonald, K.A.. (2012) Bioreactor systems for in vitro production of foreign proteins using plant cell cultures. *Biotechnol. Adv.* **30**, 398–409.

Joensuu, J.J., Conley, A.J., Lienemann, M., Brandle, J.E., Linder, M.B. and Menassa, R. (2010) Hydrophobin fusions for high-level transient protein expression and purification in *Nicotiana benthamiana*. *Plant Physiol.* **152**, 622–633.

Joensuu, J.J., Conley, A.J., Linder, M.B. and Menassa, R. (2012) Bioseparation of recombinant proteins from plant extract with hydrophobin fusion technology. *Methods Mol. Biol.* **824**, 527–534.

Joseph, M., Ludevid, M.D., Torrent, M., Rofidal, V., Tauzin, M., Rossignol, M. and Peltier, J.B.. (2012) Proteomic characterisation of endoplasmic reticulum-derived protein bodies in tobacco leaves. *BMC Plant Biol.* **12**, 36. doi:10.1186/1471-2229-12-36.

Kaldis, A., Ahmad, A., Reid, A., Mcgarvey, B., Brandle, J., Ma, S., Jevnikar, A., Kohalmi, S.E. and Menassa, R. (2013) High-level production of human interleukin-10 fusions in tobacco cell suspension cultures. *Plant Biotechnol. J.* **11**, 535–545.

Khan, I., Twyman, R.M., Arcalis, E. and Stoger, E. (2012) Using storage organelles for the accumulation and encapsulation of recombinant proteins. *Biotechnol. J.* **7**, 1099–1108.

Kieran, P.M., MacLoughlin, P.F. and Malone, D.M. (1997) Plant cell suspension cultures: some engineering considerations. *J. Biotechnol.* **59**, 39–52.

Kirchhoff, J., Raven, N., Boes, A., Roberts, J.L., Russell, S., Treffenfeldt, W., Fischer, R., Schinkel, H., Schiermeyer, A. and Schillberg, S. (2012) Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. *Plant Biotechnol. J.* **10**, 936–944.

Lahtinen, T., Linder, M.B., Nakari-Setälä, T. and Oker-Blom, C. (2008) Hydrophobin (HFBI): a potential fusion partner for one-step purification of recombinant proteins from insect cells. *Protein Expr. Purif.* **59**, 18–24.

Linder, M.B., Qiao, M., Laumen, F., Selber, K., Hyytiä, T., Nakari-Setälä, T. and Penttilä, M.E. (2004) Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. *Biochemistry*, **43**, 11873–11882.

Mustalahti, E., Saloheimo, M. and Joensuu, J.J. (2013) Intracellular protein production in *Trichoderma reesei* (Hypocrea jecorina) with hydrophobin fusion technology. *New Biotechnol.* **30**, 262–268.

Nagata, T. and Kumagai, F. (1999) Plant cell biology through the window of the highly synchronized tobacco BY-2 cell line. *Methods Cell Sci.* **21**, 123–127.

Nagata, T., Nemoto, Y. and Hasezawa, S. (1992) Tobacco BY-2 Cell Line as the "HeLa" Cell in the Cell Biology of Higher Plants. *Int. Rev. Cytol.* **132**, 1–30.

Nocarova, E. and Fischer, L. (2009) Cloning of transgenic tobacco BY-2 cells; An efficient method to analyse and reduce high natural heterogeneity of transgene expression. *BMC Plant Biol.* **9**, 44, doi:10.1186/1471-2229-9-44.

Noguchi, M., Matsumoto, T., Hirata, Y., Yamamoto, K., Katsuyama, A., Kato, A., Azechi, S. and Kato, K. (1977) Improvement of growth rates of plant cell cultures. In *Plant Tissue Culture and Its Bio-technological Application*, (Barz, W., Reinhard, E. and Zenk, M.H., eds), pp. 85–94. Springer-Verlag, Berlin.

Penttilä, M., Nakari-Setälä, T., Fagerström, R., Selber, K., Kula, M.R., Linder, M. and Tjerneld, F.. (2008) *Process for partitioning of proteins*. *United States Patent* 11/252,753.

Sack, M., Paetz, A., Kunert, R., Bomble, M., Hesse, F., Stiegler, G., Fischer, R., Katinger, H., Stoeger, E. and Rademacher, T. (2007) Functional analysis of the broadly neutralizing human anti-HIV-1 antibody 2F5 produced in transgenic BY-2 suspension cultures. *FASEB J.* **21**, 1655–1664.

Schiermeyer, A., Schinkel, H., Apel, S., Fischer, R. and Schillberg, S. (2005) Production of Desmodus rotundus salivary plasminogen activator a1 (DSPAa1) in tobacco is hampered by proteolysis. *Biotechnol. Bioeng.* **89**, 848–858.

Schinkel, H., Schiermeyer, A., Soeur, R., Fischer, R. and Schillberg, S. (2005) Production of an active recombinant thrombomodulin derivative in transgenic tobacco plants and suspension cells. *Transgenic Res.* **14**, 251–259.

Schmale, K., Rademacher, T., Fischer, R. and Hellwig, S. (2006) Towards industrial usefulness—cryo-cell-banking of transgenic BY-2 cell cultures. *J. Biotechnol.* **124**, 302–311.

Schmidt, S.R. (2013) Protein Bodies in Nature and Biotechnology. *Mol. Biotechnol.* **54**, 257–268.

Selber, K., Tjerneld, F., Collén, A., Hyytiä, T., Nakari-Setälä, T., Bailey, M., Fagerström, R., Kan, J., Van Der Laan, J., Penttilä, M. and Kula, M.R. (2004) Large-scale separation and production of engineered proteins, designed for facilitated recovery in detergent-based aqueous two-phase extraction systems. *Process Biochem.* **39**, 889–896.

Sun, Q.Y., Ding, L.W., Lomonossoff, G.P., Sun, Y.B., Luo, M., Li, C.Q., Jiang, L. and Xu, Z.F. (2011) Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture. *J. Biotechnol.* **155**, 164–172.

Torrent, M., Llompart, B., Lasserre-Ramassamy, S., Llop-Tous, I., Bastida, M., Marzabal, P., Westerholm-Pavinen, A., Saloheimo, M., Heifetz, P.B. and Ludevid, M.D. (2009) Eukaryotic protein production in designed storage organelles. *BMC Biol.* **7**, 5. doi:10.1186/1741-7007-7-5.

Walsh, G. (2010) Biopharmaceutical benchmarks 2010. *Nat. Biotechnol.* **28**, 917–924.

Xu, J., Ge, X. and Dolan, M.C. (2011) Towards high-yield production of pharmaceutical proteins with plant cell suspension cultures. *Biotechnol. Adv.* **29**, 278–299.

Supporting information

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

Figure S1 Online measurements of 20 L (a) and 600 L bioreactor cultures of green fluorescent protein-hydrophobin fusion (GFP-HFBI) expressing BY-2 cell line.

Movie S1 Video clip compiled of Z-stack confocal microscope images illustrates the accumulation of free green fluorescent protein in the reticular structure of endoplasmic reticulum in a BY-2 cell.

Movie S2 Video clip compiled of Z-stack confocal microscope images illustrates accumulation of green fluorescent protein-hydrophobin fusion fusion protein in protein bodies in a BY-2 cell.

PUBLICATION II

Novel hydrophobin fusion tags for plant-produced fusion proteins

PLoS ONE 11(10): e0164032.

PLOS ONE

RESEARCH ARTICLE

Novel Hydrophobin Fusion Tags for Plant-Produced Fusion Proteins

Lauri Reuter[1]*, Anneli Ritala[1], Markus Linder[1,2], Jussi Joensuu[1]

1 VTT Technical Research Centre of Finland Ltd., Espoo, Finland, **2** Aalto University, Department of Biotechnology and Chemical Technology, Espoo, Finland

* lauri.reuter@vtt.fi

OPEN ACCESS

Citation: Reuter L, Ritala A, Linder M, Joensuu J (2016) Novel Hydrophobin Fusion Tags for Plant-Produced Fusion Proteins. PLoS ONE 11(10): e0164032. doi:10.1371/journal.pone.0164032

Editor: Monika Schmoll, Austrian Institute of Technology, AUSTRIA

Received: June 4, 2016

Accepted: September 19, 2016

Published: October 5, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: We have received funding from Academy of Finland Grant number 252442 (AR LR JJ) and Centres of Excellence Programme HYBER (JJ ML) (http://www.aka.fi/) and Eurostars grant Hydropro 5320 (JJ) (www.eurostars-eureka.eu). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. All authors are, or have been, employed by VTT Technical research Centre of Finland. VTT Technical Research Centre of Finland Ltd., provided support in the form of salaries for

Abstract

Hydrophobin fusion technology has been applied in the expression of several recombinant proteins in plants. Until now, the technology has relied exclusively on the *Trichoderma reesei* hydrophobin HFBI. We screened eight novel hydrophobin tags, *T. reesei* HFBII, HFBIII, HFBIV, HFBV, HFBVI and *Fusarium verticillioides* derived HYD3, HYD4 and HYD5, for production of fusion proteins in plants and purification by two-phase separation. To study the properties of the hydrophobins, we used N-terminal and C-terminal GFP as a fusion partner. Transient expression of the hydrophobin fusions in *Nicotiana benthamiana* revealed large variability in accumulation levels, which was also reflected in formation of protein bodies. In two-phase separations, only HFBII and HFBIV were able to concentrate GFP into the surfactant phase from a plant extract. The separation efficiency of both tags was comparable to HFBI. When the accumulation was tested side by side, HFBII-GFP gave a better yield than HFBI-GFP, while the yield of HFBIV-GFP remained lower. Thus we present here two alternatives for HFBI as functional fusion tags for plant-based protein production and first step purification.

Introduction

Hydrophobins (HFB) are small, secretory proteins found in filamentous fungi with diverse biological functions [1,2]. The compact globular structure is stabilized by four disulphide bonds between conserved cysteine residues. A hydrophobic patch, exposed on the surface of the protein, gives HFBs their hydrophobic and extraordinarily surface active properties [1]: They assemble in aqueous solutions, interact with non-ionic surfactants and self-assemble into monolayers at liquid-air interfaces and on hydrophobic surfaces [3,4]. HFBs are divided into classes I and II based on differences in their hydrophobicity plots, solubility and spacing of the conserved cysteine residues [1,2].

The HFBs have several applications in biotechnology ranging from food additives [5] to functional coatings in nanomedicine [6]. When used as fusion partners for recombinant proteins, HFBs convey some of their functionalities to the respective fusion protein. This approach has been applied e.g. in immobilization of bioactive proteins on biosensors [7] or recruiting cellulose nano-fibrils into films to air-water or oil-water interfaces [3]. HFB fusion technology has been

authors [LR, AR, ML, JJ], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Competing Interests: All authors are, or have been, employed by VTT Technical research Centre of Finland Ltd. VTT owns a patent (WO 2000058342 A1, Process for partitioning of proteins) related to hydrophobin fusion technology. There are no further patents, products in development or marketed products to declare. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

further applied to purification of recombinant proteins using aqueous two-phase separation (ATPS)[8,9]. ATPS is a low-cost and scalable method for first step purification of recombinant proteins in fungal [8,9], insect [10], plant [11,12] and plant cell based production platforms [13].

When expressed as a fusion protein in plants or plant cell cultures, the *Trichoderma reesei* hydrophobin I (HFBI)[14] induces formation of protein bodies (PB) [12,13,15,16]. PBs are dense, spherical structures derived from the endoplasmic reticulum (ER). The mechanism of PB formation remains unclear, but it is thought to relate to the self-assembly and interaction of the fusion proteins in the ER. HFBI as a fusion tag improves accumulation of GFP in plants significantly [12,15] and has also increased the yield of some other target proteins [17], but not all [18,19]. The use of HFBI and other PB inducing tags has been reviewed earlier [20].

Thus far, several HFB fusion proteins have been expressed in plants [12,16–19,21]. However the application of HFB fusion technology in plants has relied solely on HFBI, a class II HFB. Aside from the conserved cysteine residues (Fig 1A), HFBs share little homology in amino acid sequences [1,2]. The genes coding for HFBs are found in small families and they are expressed differentially, both spatially and temporally [1,22,23]. This indicates that HFBs may have different roles throughout the fungal lifecycle and therefore also different functional properties. To explore the diversity of other HFBs as potential fusion tags, we have created a library of eight HFBs fused to both termini of GFP. The library covers rest of the characterized HFBs of *T. reesei*, HFBII [22], HFBIII [24], HFBIV [25], HFBV and HFBVI and additionally HYD3, HYD4 and HYD5 from *Fusarium verticillioides* [23] (Fig 1A). While HYD3 is a class I HFB, all others belong to the class II. In this study, we transiently expressed the HFB library in *Nicotiana benthamiana* to evaluate the accumulation levels and tested the applicability of the novel fusion tags for protein purification through ATPS.

Materials and Methods

Cloning

Sequences coding for HFBs (S1 Table) without native signal sequence were codon optimized and ordered from Genscript (S2 Fig). The coding sequences for HFBs, a GS-linker (amino acid sequence: GGGSGGGSGGGSENLYFQG) and eGFP (Uniprot: A0A076FL24) were assembled in the pJJJ178 vector (S3 Fig). The HFBI-GFP, HFBII-GFP and HFBIV-GFP constructs for comparison with HFBI-GFP contained another linker (amino acid sequence: GAGGGSGGGSGGGSA). Expression vectors were introduced to *Agrobacterium tumefaciens* strain EHA105.

Protein expression and extraction

Transient expression was done as described earlier [12]. In brief, the optical density of *A. tumefaciens* cultures were adjusted to 1.0 and the suspension was mixed (1:1) with Agrobacterium carrying an expression vector for post transcriptional gene silencing inhibitor p19 [26]. Agro-infiltrated *N. benthamiana* leaves from 8 plants were harvested 6 dpi and homogenized with extraction buffer (6:1 v/w; PBS [12 mM $Na_2HPO_42H_2O$, 3 mM $NaH_2PO_4H_2O$, 150 mM NaCl), 1 mM EDTA, pH 7.4]). The extract was clarified by centrifugation (Eppendorf Centrifuge 5424R, 21130 g, 10 min, 4°C for expression analysis or Eppendorf Centrifuge 5810R, 3220 g, 10 min, RT for ATPS). Samples were analysed by fluorometry [13], for total soluble protein (TSP) by Bradford analysis (Bio-Rad, USA) and on SDS-PAGE.

Aqueous two phase separation (ATPS)

ATPS was performed as described before [13] in 8 ml volume at RT with 4% Triton X-114 (w/v; Sigma-Aldrich). To recover the fusion proteins, the surfactant phase was extracted with 3.2

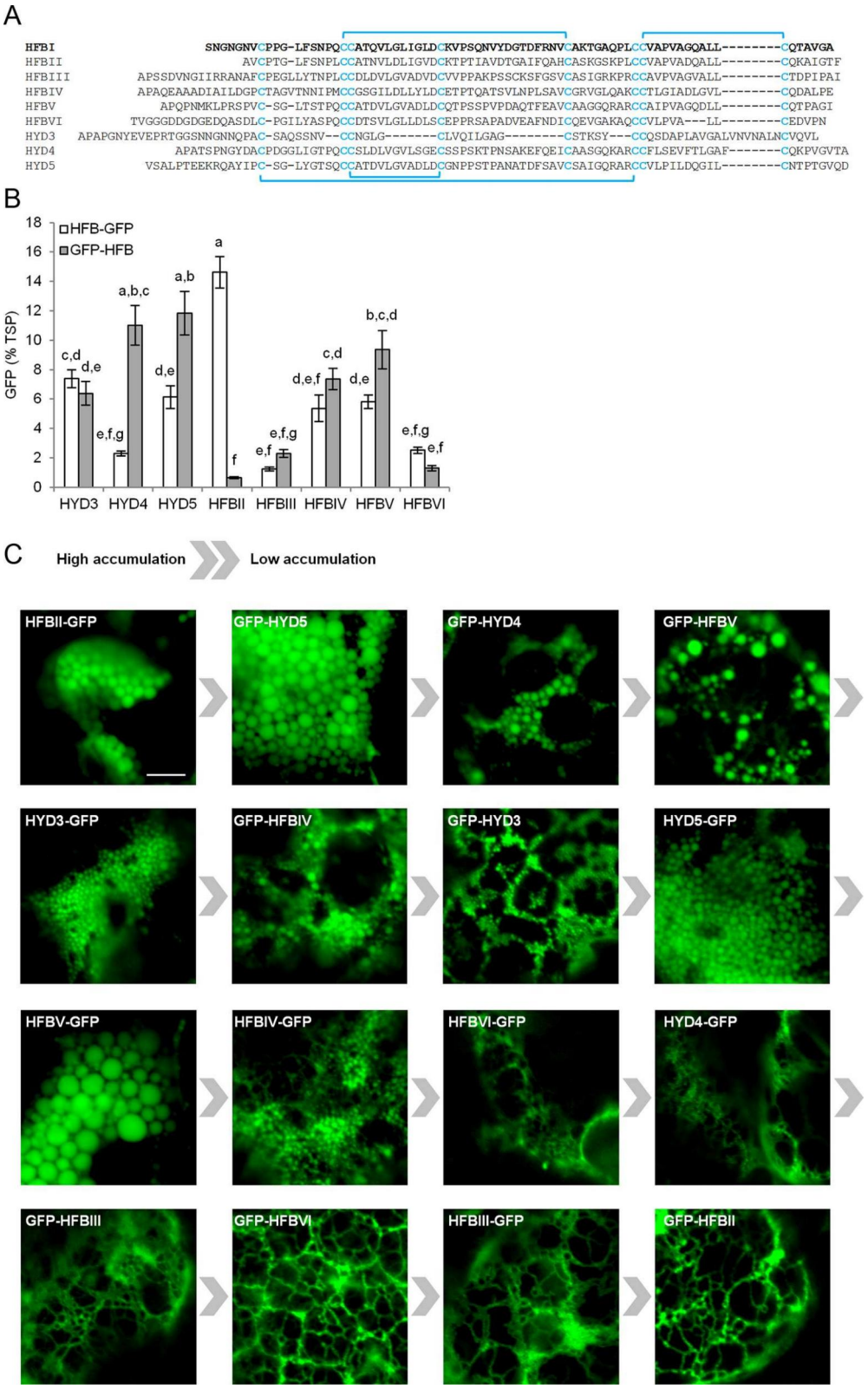

A
HFBI SNGNGNVCPPG-LFSNPQCCATQVLGLIGLDCKVPSQNVYDGTDFRNVCAKTGAQPLCCVAPVAGQALL--------CQTAVGA
HFBII AVCPTG-LFSNPLCCATNVLDLIGVDCKTPTIAVDTGAIFQAHCASKGSKPLCCVAPVADQALL--------CQKAIGTF
HFBIII APSSDVNGIIRRANAFCPEGLLYTNPLCCDLDVLGVADVDCVVPPAKPSSCKSFGSVCASIGRKPRCCAVPVAGVALL--------CTDPIPAI
HFBIV APAQEAAADIAILDGPCTAGVTNNIPMCCGSGILDLLYLDCETPTQATSVLNPLSAVCGRVGLQAKCCTLGIADLGVL--------CQDALPE
HFBV APQPNMKLPRSPVC-SG-LTSTPQCCATDVLGVADLDCQTPSSPVPDAQTFEAVCAAGGQRARCCAIPVAGQDLL--------CQTPAGI
HFBVI TVGGGDDGDGEDQASDLC-PGILYASPQCCDTSVLGLLDLSCEPPRSAPADVEAFNDICQEVGAKAQCCVLPVA---LL--------CEDVPN
HYD3 APAPGNYEVEPRTGGSNNGNNQPAC-SAQSSNV--CCNGLG-------CLVQILGAG--------CSTKSY---CCQSDAPLAVGALVNVNALNCVQVL
HYD4 APATSPNGYDACPDGGLIGTPQCCSLDLVGVLSGECSSPSKTPNSAKEFQEICAASGQKARCCFLSEVFTLGAF--------CQKPVGVTA
HYD5 VSALPTEEKRQAYIPC-SG-LYGTSQCCATDVLGVADLDCGNPPSTPANATDFSAVCSAIGQRARCCVLPILDQGIL--------CNTPTGVQD
B
HFB-GFP
GFP-HFB
GFP (% TSP)
18
16
14
12
10
8
6
4
2
0
c,d
d,e
e,f,g
a,b,c
d,e
a,b
a
f
e,f
e,f,g
d,e,f
c,d
d,e
b,c,d
e,f,g
e,f
HYD3
HYD4
HYD5
HFBII
HFBIII
HFBIV
HFBV
HFBVI
C
High accumulation
Low accumulation
HFBII-GFP
GFP-HYD5
GFP-HYD4
GFP-HFBV
HYD3-GFP
GFP-HFBIV
GFP-HYD3
HYD5-GFP
HFBV-GFP
HFBIV-GFP
HFBVI-GFP
HYD4-GFP
GFP-HFBIII
GFP-HFBVI
HFBIII-GFP
GFP-HFBII

Fig 1. Expression of the HFB library in *N. benthamiana*. (A) Amino acid sequences of the HFBI and the 8 novel hydrophobin fusion tags studied here. Conserved cysteine residues and disulphide bridges are highlighted. (B) Expression levels of fusion proteins determined by fluorometry. Letters indicate groups with significant difference ($p<0.05$, $n = 8$ individual plants). Error bars indicate standard deviation. (C) Confocal microscopy images illustrate subcellular localization of the fusion proteins. A control sample infiltrated with only p19 showed no signal (image not show). The scale bar represents 5μm.

doi:10.1371/journal.pone.0164032.g001

ml isobutanol (Merck). For the second round of ATPS, comparing HFBI-GFP, HFBII-GFP and HFBIV-GFP, purified (ATPS and a Strep-Tactin® column [IBA, Germany]) proteins were added to PBS to a concentration of 30 μg/ml. ATPS was performed in 1.5 ml volume with 3% Triton X-114 and recovered with 0.45 ml isobutanol. The partition coefficient (k) was calculated by dividing the protein concentration in the surfactant phase (protein amount in recovered phase divided by volume of the surfactant phase) by the concentration in the residue [9].

Confocal microscopy

Localization of GFP-fusion proteins was visualized in leaf disks harvested 7 dpi using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) with a 63× water immersion objective (excitation at 488-nm and detection at 493–598 nm).

Statistical analysis

Statistical analyses were done with SPSS Statistic 22.0 (IBM, Armonk, NY). A one way ANOVA test, followed by the Tukey HSD test were performed, with a significance level of 95%.

Results and Discussion

Accumulation of GFP and protein body formation are dependent on the HFB-fusion tag

To compare the accumulation levels, we transiently expressed GFP fused to eight different HFBs (Fig 1A), in both orientations, in *N. benthamiana*. Accumulation of GFP was quantified according to the fluorescence signal (Fig 1B). These results are in agreement with an SDS-PAGE analysis (S4 Fig).

The HFBII-GFP gave a yield of up to 15% GFP of TSP, but curiously the GFP-HFBII gave a yield of only less than 1%. We have observed the same trend with other target proteins fused to HFBII (unpublished data). The low accumulation of GFP-HFBII could be related to the short N-terminal sequence before the first cysteine residue (Fig 1A), which, with the linker used here, may not have been sufficient to provide space for proper folding. In addition to HFBII, the differences in accumulation levels between C-and N-terminal fusions were significant ($p<0.05$) with HYD4 and HYD5.

To investigate whether the novel fusion tags induce formation of PBs, as described earlier with GFP-HFBI [12,13,15,16], we examined the leaves expressing the proteins under confocal microscope (Fig 1C). Consistent formation of PBs, similar to GFP-HFBI [12,15,16], was apparent only with HFB-fusions with relatively high accumulation levels. Fusion proteins with lower yields, such as GFP-HFBII and both HFBIII and HFBVI fusion proteins were observed predominantly in reticulate ER. These observations are in agreement with earlier reports indicating that a threshold level of accumulation is essential for PB formation [15] and that higher concentration of protein in the ER correlates with larger and more consistent PBs [16]. It remains unclear whether the formation of PBs is a result of high concentration of protein, or a causing factor of it.

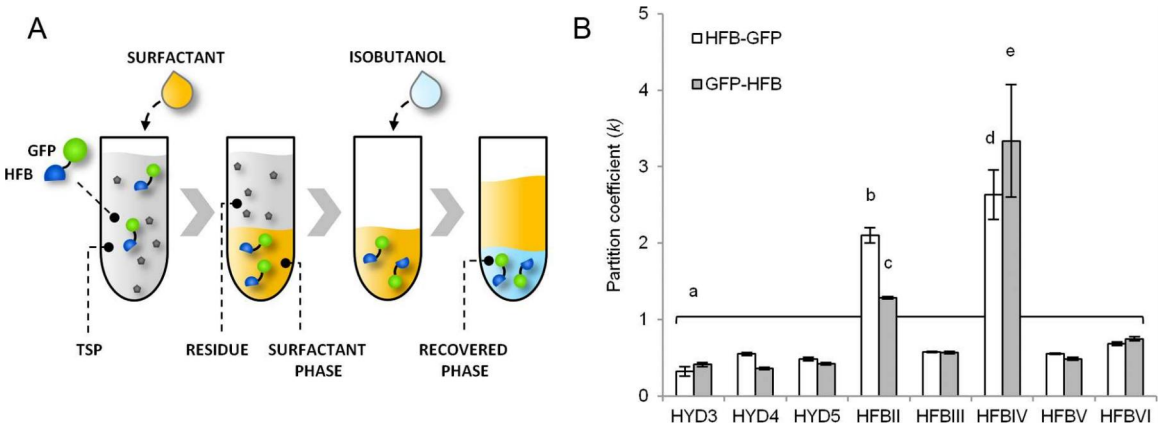


Fig 2. Purification of HFB-fusion proteins by ATPS. (A) A schematic illustration of the process. (B) Purification from plant extract. The partition coefficient is determined by dividing concentration in surfactant phase by concentration in residue. Letters indicate significant difference ($n = 4$, $p<0.05$) and error bars indicate standard deviation.

doi:10.1371/journal.pone.0164032.g002

Only fusion proteins with HFBII and HFBIV show partitioning in ATPS

We set up ATPS experiments to study the performance of the HFB tags for protein purification. First we examined whether the HFB fusion proteins would separate from leaf extract (TSP) into a surfactant phase and further to the recovered fraction (Fig 2A). The partition coefficient (k) [9] describes the ratio of the protein concentration between surfactant phase and residue (Fig 2B).

Most fusion proteins show low partitioning to the surfactant ($k<1$) with no significant differences between the constructs. Only HFBII and HFBIV concentrate the fusion proteins into the surfactant phase (Fig 2 and S5 Fig). The k-values for HFBII-GFP and GFP-HFBII were 2.1 ±0.1 and 1.3±0.0, respectively, and for HFBIV-GFP and GFP-HFBIV 2.6±0.3 and 3.3±0.7 (mean±SD, $n = 3$), respectively. Although not functional in ATPS, other HFBs may turn out useful for other applications, such as surface adhesion. This was, however, out of our scope here.

Efficient separation does not correlate with high accumulation

The ability of the HFB fusion proteins to interact with the non-ionic surfactant (Fig 2B), does not correlate ($R^2 = 0.0168$, S6 Fig) with accumulation levels (Fig 1B). Thus the expression level of a given HFB fusion protein, or tendency to accumulate in PBs, cannot be used to predict functionality in ATPS. The characteristics enabling HFBs to interact with surfactants and/or to induce formation of PBs are not well known. However, these results indicate that the fundamental properties responsible for the two phenomena are not the same.

Comparison to HFBI

Finally we compared HFBII and HFBIV side by side with HFBI (Fig 3). We used only N-terminally fused HFBs due to the low yield of GFP-HFBII.

The yields of HFBII-GFP and HFBIV-GFP (Fig 3A) were in line with the initial screening (Fig 1B). The yield of HFBI-GFP reached only 11.1±1.5% GFP of TSP (mean±SD, $n = 8$), which is significantly less than HFBII-GFP (21.3±2.3%), but more than HFBIV-GFP ($p<0.05$). The yield of HFBI-GFP here was much lower than the yield of 38% GFP of TSP that has been previously reported for the non-codon optimized and differently oriented GFP-HFBI [12].

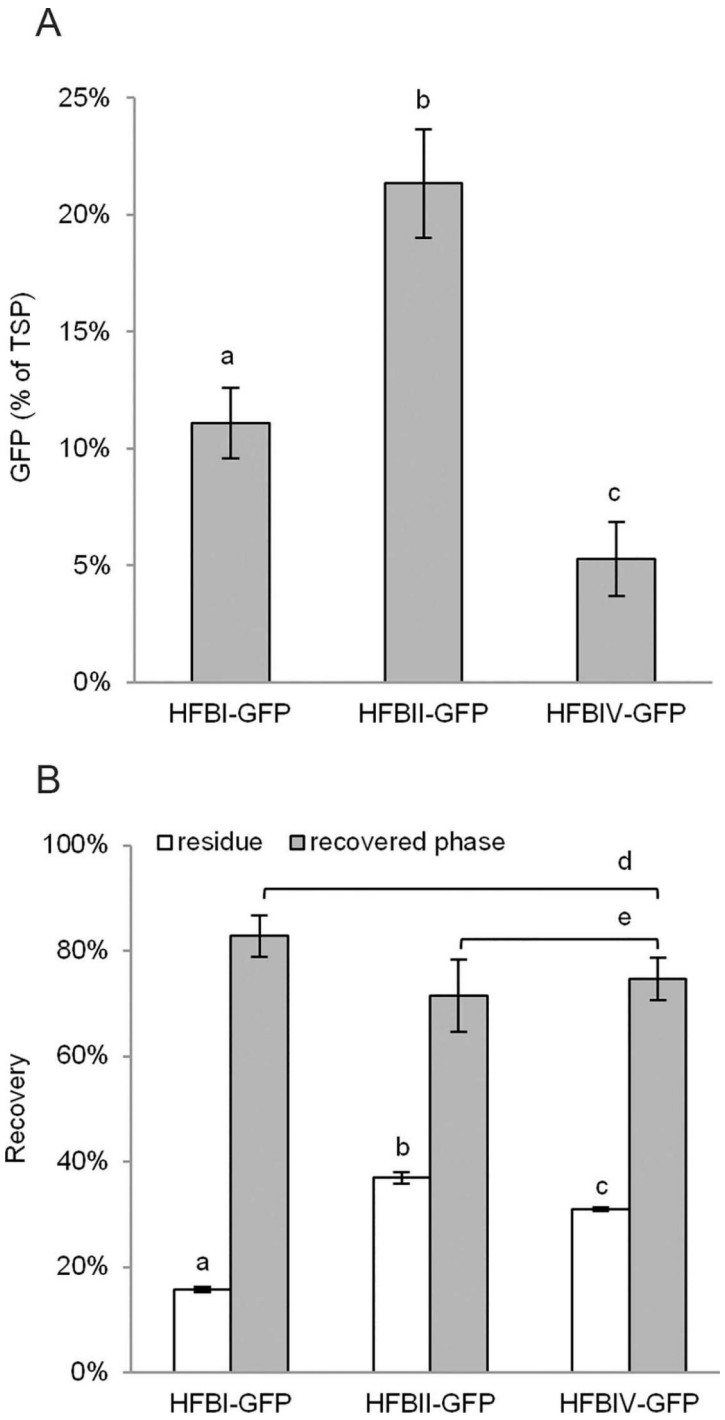


Fig 3. Comparison of HFBII and HFBIV to HFBI. (A) Accumulation in *N. benthamiana* ($n = 8$ individual plants). (B) ATPS with purified proteins to compare recovery rates ($n = 3$). Letters indicate significant difference ($p<0.05$). Error bars indicate standard deviation.

doi:10.1371/journal.pone.0164032.g003

Next, we set up an ATPS experiment (Fig 3B) by adding purified fusion proteins into buffer in equal concentrations, because the protein concentration and the matrix of the plant extract

may influence the separation efficiency [12]. The recovery yield of HFBI-GFP was 83±4% in the surfactant phase giving a *k*-value of 15.7±0.6 (mean±SD, $n = 3$). This is comparable with previous findings [12,13]. The recovery yields of HFBII-GFP and HFBIV-GFP were only slightly lower, 71±7% and 75±4%, respectively. However, the *k*-values for HFBII-GFP 5.7±0.5 (mean±SD, $n = 3$) and HFBIV-GFP 7.4±0.5 indicate significantly less efficient ($p<0.05$) separation. The slightly larger portions of the HFBII and HFBIV fusion proteins remaining in the residue were also visible on immunoblot (S6 Fig). The larger *k*-values in the later ATPS experiment reflect the smaller amount of surfactant used (3% vs. 4%) [12]. The difference in *k*-values between HFBII-GFP and HFBIV-GFP was significant ($p<0.05$), but there was no significant difference in the recovery yields. Similar behaviour of structurally similar HFBI and HFBII in the two phase system was expected based on previous reports [9]. However, the amino acid sequence and hydropathy profile of HFBIV are very different from HFBI and HFBII. In addition, most of the differences occur on the surface of the protein presumably influencing its properties [25]. To our knowledge this is the first report on separation of HFBIV in a two phase system based on a non-ionic surfactant. The two-phase separation method has been optimized for HFBI [12] and thus further optimization with other HFB´s may balance out the observed differences.

Conclusions

Until now, only HFBI has been applied in HFB-fusion technology in plants. In this study we screened eight novel HFBs for plant based-production of fusion proteins. Accumulation of most fusion proteins remained modest, only HFBII-GFP reaching similar or even higher yields than HFBI-GFP. With some HFBs, such as HFBII, the orientation of the fusion had a significant impact on the yield. In ATPS both HFBII and HFBIV performed well, being only slightly less efficient than HFBI. It appears that the tendency to separate in surfactant based aqueous two phase systems is a property shared only by few HFBs. Although the results need to be confirmed with other target proteins than GFP, it can be concluded that, in addition to HFBI, at least HFBII and HFBIV are potential fusion partners for plant-based production of fusion proteins capable of interaction with non-ionic surfactants.

Supporting Information

S1 Fig. Expression cassettes and amino acid sequences. (A) A schematic presentation of the expression cassettes. Genes for HFB, linker and GFP were cloned in the vector between BsaI restriction sites using Golden gate assembly. (B) The full nucleotide and amino acid sequences of the coding region of the expression cassette for representative construct: HFBII-GFP. (C) Sequence data for HFBIII, (D) HFBIV, (E) HFBV, (F) HFBVI, (G) HYD3, (H) HYD4 and (I) HYD5.
(PDF)

S2 Fig. Expression vector pJJJ178.
(PDF)

S3 Fig. Pooled leaf samples. (A) A Coomassie stained SDS-PAGE of pooled leaf samples (n = 8) showing accumulation of HFB fusion proteins (expected size indicated by arrows) in Nicotiana benthamiana. (B) Immunoblot analysis with anti StrepII-tag antibody indicates some degradation of the fusion proteins. Equal amounts of total soluble protein were loaded on all gels. A leaf infiltrated with only a construct for P19 was used as a negative control.
(PDF)

S4 Fig. SDS-PAGE illustrating aqueous two phase separation. (A) Coomassie stained SDS-PAGE gels of representative samples from ATPS (Fig 1B). Lane numbering refers to: 1) TSP; 2) residue; 3) recovered phase. (B) Concentrations of fusion proteins in residue and recovered phase in comparison to initial concentration in plant leaf extract (TSP). Error bars indicate standard deviation (n = 4).
(PDF)

S5 Fig. Protein accumulation levels (means, Fig 1B) blotted against k-values (means, Fig 2B).
(PDF)

S6 Fig. SDS-PAGE and immunoblot illustrating aqueous two-phase separation with purified proteins. (A) Coomassie stained SDS-PAGE and (B) an immunoblot of pooled samples (n = 3) from starting solution, residue phase and recovered phase in ATPS comparing HFBI-GFP, HFBII-GFP and HFBIV-GFP (Fig 3B). Detection was performed with anti-c-Myc tag primary antibody (rabbit, A00172, GeneScript) and a secondary antibody for IR-detection (goat anti-rabbit, IR Dye® 680RD, LI-COR Biosciences, Germany).
(PDF)

S1 Table. Genbak ID numbers for original gene sequences and Uniprot ID numbers for amino acid sequences used in this study. The sequence coding for the extended N-terminal part of HFBVI, presumably a cell wall binding domain (amino acids 1–179), was not included in the coding sequence.
(PDF)

Acknowledgments

We are grateful to Tuuli Teikari and Riitta Suihkonen for technical assistance. Academy of Finland Grant #252442 and Centres of Excellence Programme HYBER (2014–2019) as well as Eurostars Hydropro 5320 grant are acknowledged for the financial support.

Author Contributions

Conceptualization: ML JJ LR AR.

Formal analysis: LR JJ.

Funding acquisition: AR JJ.

Investigation: LR JJ.

Methodology: JJ AR LR.

Supervision: AR JJ.

Validation: LR JJ.

Visualization: LR JJ.

Writing – original draft: LR.

Writing – review & editing: LR AR JJ ML.

References

1. Sunde M, Kwan AHY, Templeton MD, Beever RE, Mackay JP. Structural analysis of hydrophobins. Micron. 2008 Oct; 39(7):773–84. doi: 10.1016/j.micron.2007.08.003 PMID: 17875392

2. Linder MB, Szilvay GR, Nakari-Setälä T, Penttilä ME. Hydrophobins: the protein-amphiphiles of filamentous fungi. FEMS Microbiol Rev. The Oxford University Press; 2005 Nov 1; 29(5):877–96. doi: 10.1016/j.femsre.2005.01.004 PMID: 16219510
3. Varjonen S, Laaksonen P, Paananen A, Valo H, Hähl H, Laaksonen T, et al. Self-assembly of cellulose nanofibrils by genetically engineered fusion proteins. Soft Matter. Royal Society of Chemistry; 2011 Mar 7; 7(6):2402. doi: 10.1039/c0sm01114b
4. Lienemann M, Gandier J- A, Joensuu JJ, Iwanaga A, Takatsuji Y, Haruyama T, et al. Structure-function relationships in hydrophobins: probing the role of charged side chains. Appl Environ Microbiol. 2013 Sep 15; 79(18):5533–8. doi: 10.1128/AEM.01493-13 PMID: 23835172
5. Cox AR, Aldred DL, Russell AB. Exceptional stability of food foams using class II hydrophobin HFBII. Food Hydrocoll. 2009 Mar; 23(2):366–76. doi: 10.1016/j.foodhyd.2008.03.001
6. Valo HK, Laaksonen PH, Peltonen LJ, Linder MB, Hirvonen JT, Laaksonen TJ. Multifunctional hydrophobin: toward functional coatings for drug nanoparticles. ACS Nano. American Chemical Society; 2010 Mar 23; 4(3):1750–8. doi: 10.1021/nn9017558 PMID: 20210303
7. Linder M, Szilvay GR, Nakari-Setälä T, Söderlund H, Penttilä M. Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from Trichoderma reesei. Protein Sci. 2002 Sep; 11 (9):2257–66. doi: 10.1110/ps.0207902 PMID: 12192081
8. Linder MB, Qiao M, Laumen F, Selber K, Hyytiä T, Nakari-Setälä T, et al. Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. Biochemistry. American Chemical Society; 2004 Sep 21; 43(37):11873–82. doi: 10.1021/bi0488202 PMID: 15362873
9. Linder M, Selber K, Nakari-Setälä T, Qiao M, Kula M-R, Penttilä M. The Hydrophobins HFBI and HFBII from Trichoderma r eesei Showing Efficient Interactions with Nonionic Surfactants in Aqueous Two-Phase Systems. Biomacromolecules. American Chemical Society; 2001 Jun; 2(2):511–7. doi: 10.1021/bm0001493 PMID: 11749214
10. Lahtinen T, Linder MB, Nakari-Setälä T, Oker-Blom C. Hydrophobin (HFBI): A potential fusion partner for one-step purification of recombinant proteins from insect cells. Protein Expr Purif. 2008 May; 59 (1):18–24. doi: 10.1016/j.pep.2007.12.014 PMID: 18267368
11. Joensuu JJ, Conley AJ, Linder MB, Menassa R. Bioseparation of recombinant proteins from plant extract with hydrophobin fusion technology. Methods Mol Biol. 2012 Jan; 824:527–34. doi: 10.1007/978-1-61779-433-9_28 PMID: 22160918
12. Joensuu JJ, Conley AJ, Lienemann M, Brandle JE, Linder MB, Menassa R. Hydrophobin fusions for high-level transient protein expression and purification in Nicotiana benthamiana. Plant Physiol. 2010 Feb 1; 152(2):622–33. doi: 10.1104/pp.109.149021 PMID: 20018596
13. Reuter LJ, Bailey MJ, Joensuu JJ, Ritala A. Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. Plant Biotechnol J. 2014; 12(4):402–10. doi: 10.1111/pbi.12147 PMID: 24341724
14. Nakari-Setälä T, Aro N, Kalkkinen N, Alatalo E, Penttilä M. Genetic and biochemical characterization of the Trichoderma reesei hydrophobin HFBI. Eur J Biochem. 1996; 235:248–55. doi: 10.1111/j.1432-1033.1996.00248.x PMID: 8631337
15. Gutiérrez SP, Saberianfar R, Kohalmi SE, Menassa R. Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. BMC Biotechnol. BioMed Central Ltd; 2013 Jan 10; 13(1):40. doi: 10.1186/1472-6750-13-40 PMID: 23663656
16. Saberianfar R, Joensuu JJ, Conley AJ, Menassa R. Protein body formation in leaves of Nicotiana benthamiana: a concentration-dependent mechanism influenced by the presence of fusion tags. Plant Biotechnol J. 2015 Sep; 13(7):927–37. doi: 10.1111/pbi.12329 PMID: 25640969
17. Jacquet N, Navarre C, Desmecht D, Boutry M. Hydrophobin Fusion of an Influenza Virus Hemagglutinin Allows High Transient Expression in Nicotiana benthamiana, Easy Purification and Immune Response with Neutralizing Activity. PLoS One. 2014; 9(12):e115944. doi: 10.1371/journal.pone.0115944 PMID: 25541987
18. Phan HT, Hause B, Hause G, Arcalis E, Stoger E, Maresch D, et al. Influence of elastin-like polypeptide and hydrophobin on recombinant hemagglutinin accumulations in transgenic tobacco plants. PLoS One. Public Library of Science; 2014 Jan 10; 9(6):e99347. doi: 10.1371/journal.pone.0099347 PMID: 24914995
19. Pereira EO, Kolotilin I, Conley AJ, Menassa R. Production and characterization of in planta transiently produced polygalacturanase from Aspergillus niger and its fusions with hydrophobin or ELP tags. BMC Biotechnol. 2014; 14(1):59. doi: 10.1186/1472-6750-14-59 PMID: 24970673
20. Conley AJ, Joensuu JJ, Richman A, Menassa R. Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. Plant Biotechnol J. 2011 May; 9(4):419–33. doi: 10.1111/j.1467-7652.2011.00596.x PMID: 21338467

21. Miletic S, Simpson DJ, Szymanski CM, Deyholos MK, Menassa R. A Plant-Produced Bacteriophage Tailspike Protein for the Control of Salmonella. Front Plant Sci. Frontiers; 2015 Jan 8; 6:1221. doi: 10.3389/fpls.2015.01221 PMID: 26779243

22. Nakari-Setala T, Aro N, Ilmen M, Munoz G, Kalkkinen N, Penttila M. Differential Expression of the Vegetative and Spore-Bound Hydrophobins of Trichoderma Reesei Cloning and Characterization of the Hfb2 Gene. Eur J Biochem. 1997 Sep; 248(2):415–23. doi: 10.1111/j.1432-1033.1997.00415.x PMID: 9346297

23. Fuchs U, Czymmek KJ, Sweigard JA. Five hydrophobin genes in Fusarium verticillioides include two required for microconidial chain formation. Fungal Genet Biol. 2004 Sep; 41(9):852–64. doi: 10.1016/j.fgb.2004.04.004 PMID: 15288021

24. Kisko K, Szilvay GR, Vuorimaa E, Lemmetyinen H, Linder MB, Torkkeli M, et al. Self-assembled films of hydrophobin protein HFBIII from Trichoderma reesei. J Appl Crystallogr. International Union of Crystallography; 2007 Feb 3; 40(s1):s355–60. doi: 10.1107/s0021889807001331

25. Espino-Rammer L, Ribitsch D, Przylucka A, Marold A, Greimel KJ, Herrero Acero E, et al. Two novel class II hydrophobins from Trichoderma spp. stimulate enzymatic hydrolysis of poly(ethylene terephthalate) when expressed as fusion proteins. Appl Environ Microbiol. 2013 Jul 15; 79(14):4230–8. doi: 10.1128/AEM.01132-13 PMID: 23645195

26. Silhavy D, Molnár A, Lucioli A, Szittya G, Hornyik C, Tavazza M, et al. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. EMBO J. 2002 Jun 17; 21(12):3070–80. doi: 10.1093/emboj/cdf312 PMID: 12065420

PUBLICATION III

Antibody harvesting with a plant-produced hydrophobin-Protein A fusion

Manuscript.

Antibody harvesting with a plant-produced hydrophobin-protein a fusion

AUTHORS
*Katri Kurppa[1Δ], Lauri Reuter[1Δ], Anneli Ritala[1], Markus Linder[1,2], Jussi Joensuu[1]**

AFFILIATIONS
[1] VTT Technical Research Institute of Finland Ltd., Tietotie 2, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

[2] Aalto University, Department of Biotechnology and Chemical Technology, Kemistintie 1, FI-00076, Espoo, Finland

*Corresponding author , [Δ] Equal contribution as first authors

SUMMARY

Purification is a bottleneck and a major cost factor in the production of antibodies. We set out to engineer a bi-functional fusion protein from two building blocks, Protein A and a hydrophobin, aiming at low-cost and scalable antibody capturing in solutions. Immunoglobulin-binding Protein A is widely used in affinity-based purification. The hydrophobin fusion tag, on the other hand, has been shown to enable purification by two-phase separation. Protein A was fused to two different hydrophobin tags, HFBI or II, and expressed transiently in *Nicotiana benthamiana*. The hydrophobins enhanced accumulation up to 35-fold, yielding up to 25% of total soluble protein. Both fused and non-fused Protein A accumulated in protein bodies. Hence the increased yield could not be attributed to HFB-induced protein body formation. We also demonstrated production of HFBI-Protein A fusion protein in tobacco BY-2 suspension cells in 30 l scale, with a yield of 35 mg/l. Efficient partitioning to the surfactant phase confirmed that the fusion proteins retained the amphipathic properties of the hydrophobin block. The reversible antibody binding capacity of the Protein A block was found to be similar to that of non-fused Protein A. The best-performing fusion protein was tested in capturing antibodies from plant leaf extract with two phase separation. The fusion protein was able to carry Rituximab antibodies to the surfactant phase and subsequently release them back to the aqueous phase after a change in pH. This report demonstrates the interesting potential of hydrophobin fusion proteins for novel applications, such as harvesting antibodies in solutions.

KEYWORDS
antibody, hydrophobin, *Nicotiana benthamiana*, Protein A, purification, tobacco BY-2 suspension cells

INTRODUCTION

Antibodies are essential in modern medicine as diagnostic agents and in targeted drug delivery. Being the fastest growing area of the pharmaceutical industry, monoclonal antibodies (mAbs) are estimated to reach a total market size of 125 billion US$ by 2020 (Ecker *et al.*, 2015). MAbs are mainly produced in animal cell cultures, where they are secreted to the culture media. The industrial standard for harvesting mAbs involves an initial Protein A-based affinity chromatography step. Despite their widespread use, chromatographic methods suffer from difficulties in scalability. The system relies on batch operation, and transfer to continuous mode is not possible. It is a multistep, labour-intensive process that represents a major part of the overall production costs. Alternative procedures include two-phase extraction using conventional salt-polymer systems, for example polyethylene glycol (Azevedo *et al.*, 2009). The drawback of these rather simple two-phase systems is often poor reproducibility due to sensitivity to e.g. temperature, contaminants or salt concentration (Collen *et al*., 2002).

Here we describe a novel bi-functional fusion protein, produced in plants, which may enable a novel, low cost and easily scalable strategy for antibody harvesting in solutions. Our approach is inspired by two proteins with specific properties: *Trichoderma reesei* hydrophobins (HFBs) and *Staphylococcus aureus* Protein A.

HFBs are small globular proteins which display extreme surface activity due to their unique amphipathic structure (Linder, 2009; Wessels, 1994; Wosten and Scholtmeijer, 2015). They are found exclusively in filamentous fungi, where they fulfil a broad range of biological functions. Secreted HFBs facilitate penetration of water-air interfaces by decreasing surface tension, and coat the hypha and spores thereby decreasing wettability, improving dispersion and providing surface adhesion. The versatile biological roles of HFBs have generated a multitude of potential uses in biotechnology, from structure-enhancing food additives to coating of sensors, nanoparticles and medical instruments (Wosten and Scholtmeijer, 2015).

HFBs are grouped into two classes according to their hydropathy plots. In this work, we focus on the class II hydrophobins HFBI and HFBII. HFBs show a distinct structure comprising a hydrophobic patch at one end of the molecule and a hydrophilic surface at the other (Hakanpää *et al.*,2006a; Hakanpää *et al.*, 2006b). Due to this unique structure, the hydrophobins self-assemble at liquid-liquid, liquid-solid or liquid-air interfaces to form monolayers (Linder, 2009; Liner *et al.*, 2002; Szilvay *et al.*,2007). Their amphipathic nature also allows hydrophobins to interact with small molecule surfactants. This property is commonly used in the purification of hydrophobins and hydrophobin fusion proteins by aqueous two-phase separation (ATPS) (Collen *et al.*, 2002; Joensuu *et al.*, 2010; Linder *et al.*, 2001).

Protein A is an antibody-binding protein widely used in affinity chromatography during recent decades. It reversibly binds antibodies of the IgG class (IgG1, IgG2, IgG4,

IgG3). Based on the number of binding sites, a Protein A molecule can bind up to five IgG molecules (Uhlen *et al.,* 1984). However, experimental data suggests that the ratio of Protein A to IgG is closer to 1:2 (Yang *et al.,* 2003). In most applications the Protein A is chemically bound to a solid chromatography matrix. The antibodies are released from Protein A by decreasing the pH.

We set out to engineer a fusion protein combining two active blocks, HFB and the immunoglobulin binding domain of Protein A, in the same polypeptide chain. We expected the novel bi-functional protein to bind mAbs effectively in solution, but also to be separated in a water-surfactant two-phase extraction system. Hence, the fusion protein may be used to capture antibodies from solution and concentrate them to the surfactant phase. The phase separation can be performed in a single vessel, by addition of the antibody-capturing fusion protein and a surfactant. The whole process requires only liquid handling and is therefore easily scalable and avoids the need for complex equipment. A similar two-phase system utilizing the Protein A – IgG interaction was recently reported by McLean *et al.* (2012). Whereas their two-phase system was formed intrinsically by an oleosin-tag fused to the Protein A moiety, we chose a strategy utilizing external two-phase systems based on non-ionic surfactant to allow case-sensitive optimization of purification conditions in a more flexible manner. Moreover, the hydrophobin-tag unit can be cleaved or modified without sacrificing surface-active functionality. Bound antibodies can also be guided to chosen liquid-solid interfaces via hydrophobin self-assembly.

HFB-fusion proteins have been produced in filamentous fungi (Linder *et al.,* 2004; Mustalahti *et al*., 2013), insect cell cultures (Lahtinen *et al*., 2008), plants (Gutiérrez *et al*., 2013; Jacquet *et al.,* 2014; Joensuu *et al*., 2010; Phan *et al*., 2014; Pereira *et al*., 2014; Saberianfar *et al*., 2015) and in plant cell cultures (Reuter *et al*., 2014). Whereas production of HFB-fusion proteins has been challenging in some other hosts, plants have shown to be an especially suitable production platform. The HFB-fusion strategy has, in some cases, significantly enhanced accumulation of the recombinant proteins (Joensuu *et al.,* 2010; Jacquet *et al.,* 2014). This effect has been attributed to HFB-induced formation of protein bodies in the host cells (Conley *et al.,* 2011; Joensuu *et al.,* 2010). In plants the fusion proteins are not only accumulated in high yields, but are also correctly folded. In addition, plants contain very few native proteins that would be co-purified in ATPS lowering the product purity (Joensuu *et al.,* 2010; Reuter *et al.,* 2014). Furthermore, field grown transgenic plants may provide an ideal low-cost production platform for commodity proteins aimed at biotechnological applications outside the pharma industry (Fischer *et al.,* 2013). However, contained production might be necessary for some applications, and regulatory issues may apply. Both transient expression systems and plant cell cultures may be contained and provide adherence to cGMP requirements (Fischer *et al.,* 2012; Ritala *et al.,* 2014). Considering the downstream processing, suspension cell cultures may provide better overall cost efficiency.

Our goal in this study was to demonstrate a proof of principle for in-solution antibody harvesting using a novel bi-functional fusion protein. We also evaluated production of the fusion proteins both in *Nicotiana benthamiana* plants and in tobacco BY-2 suspension cells.

RESULTS

Screening for a hydrophobin fusion strategy

We used agro-infiltrated *Nicotiana benthamiana* plants to screen for the best hydrophobin fusion strategy. Protein A was constructed in the same polypeptide chain with HFBI or HFBII in both N- and C-terminal orientations (Figure 1a and Figure S1).The yield of both N- and C-terminal HFBI fusions reached 1.7±0.3 and 1.3±0.5 mg/g of fresh leaf material (mean±SE, n=6) (Figure 1b). The HFBII-Protein A accumulated better than the HFBI fusions, 2.4±0.6 mg/g fresh leaf material or 24.3±6.9% of TSP. This represented an approximately 35-fold increase in yield in comparison to non-fused Protein A. However, the yield of Protein A-HFBII remained on a similar level to that of the non-fused Protein A. Due to consistent expression levels, we used only the N-terminal fusions, HFBI-Protein A and HFBII-Protein A, in further experiments.

Subcellular localization

We studied the subcellular localization of the ER-targeted recombinant proteins by immunofluorescent microscopy of protoplasts prepared from agro-infiltrated *N. benthamiana* leaves (Figure 2). GFP-HFBI fusion protein, which is known to accumulate in protein bodies (Joensuu *et al.,* 2010) served as a positive control. The GFP-HFBI-induced protein bodies were visible both in intact leafs (not shown) and in the fixed protoplasts (Figure 2). The protein bodies were visualized equally well by the GFP as by the signal derived from the fluorescent probe binding to c-Myc tag. Protein A, both fused and non-fused, aggregated similarly into protein body-like structures. We observed no apparent difference between the constructs. However, the bodies were less abundant and slightly more scattered than the GFP-HFBI induced protein bodies.

Aqueous two-phase separation

Next, we examined the amphipathic properties of the HFB blocks by performing ATPS using two fusion constructs, HFBI-Protein A and HFBII-Protein A (Figure 3). The partition coefficient (*k*) describes the ratio of the protein concentration between surfactant phase and residue. Both HFBI-Protein A and HFBII-Protein A displayed regular hydrophobin-like partitioning in the two-phase system resulting in *k*-values of 4.8±0.9 and 2.4±0.6, respectively (mean±SD, n=3), whereas the non-fused Protein A did not partition into the surfactant (*k*=0.4±0.1). The overall recovery rate of HFBI-Protein A (62±5%) was significantly better than that of HFBII-Protein A (47±4%) or non-fused Protein A (25±1%). Volumes of the phases are given in Figure S2.

Antibody binding capacity of the hydrophobin-Protein A fusion proteins

Having confirmed that the fusion proteins could be separated in ATPS, we set out to study the antibody binding capacity of the Protein A block. Antibody binding was measured using a quartz crystal microbalance with dissipation monitoring (QCM-D). The QCM-D technique measures the change in oscillation frequency as a substance is bound to the surface of a quartz crystal oscillating at its resonance frequency. The frequency change is related to the mass of the bound thin layer via the Sauerbrey equation (Höök *et al.,* 2001). The surface-bound layer dampens the oscillation frequency of the freely oscillating crystal. This effect is described by the dissipation factor and depicts the structure of the bound layer. Commercially available Protein A (Sigma Aldrich, USA) served as a reference for HFBI-Protein A and HFBII-Protein A. All three proteins formed reproducible and stable thin layers on the polystyrene surface (Figure 4b, bottom bars). In order to evaluate the IgG binding capacity of the fusion proteins, a solution of the Rituximab antibody was applied to the protein layers. Addition of the antibody resulted in a mass increase that was similar in the case of all three proteins (Figure 4b, top bars). The molar ratios of Rituximab bound to the immobilized fusion proteins were estimated on the basis of the Sauerbrey masses obtained from the QCM-D data. One mole of immobilized HFBI-Protein A bound 1.5±0.3 (mean ± SD, n=3) moles of Rituximab. The corresponding figure for HFBII-Protein A was slightly lower, 1.2±0.5. The molar ratio of the commercial Protein A to Rituximab was 1.2±0.3. No specific antibody binding was observed on layers of non-fused HFBI (data not shown) or BSA (Figure S3). The results confirmed that both fusion proteins retained the immunoglobulin-binding capacity of the Protein A block.

In order to demonstrate the release of antibodies and regeneration of the antibody-binding layer, we performed two successive rounds of IgG binding and release using commercial IgG λ antibodies. Release of the bound IgG λ from the HFBI-Protein A and HFBII-Protein A layers was accomplished by decreasing the pH by rinsing the layer with acidic buffer (Figure 4a). When glycine buffer at pH 2.2 was introduced to the surface-bound HFB-Protein A/IgG λ complex, the mass decreased instantly. The released mass corresponded to the amount of antibody initially bound. After elevating the pH to 8 the layer was capable of re-binding the IgG λ without a significant decrease with respect to the initial amount. We also noted that the HFB-Protein A layers remained stable and capable of binding IgG λ after overnight incubation in buffer (data not shown).

Antibody capture from plant leaf extract with hydrophobin-Protein A fusion protein

After confirming the bi-functionality of the fusion proteins, the IgG binding capacity of the Protein A block and the amphipathic properties of the HFB block, we proceeded to demonstrate the principle of antibody capture in ATPS (Figure 5a). In this experiment

we used only HFBI-Protein A, as it outperformed HFBII-Protein A in the initial ATPS and IgG binding experiments. We spiked *N. benthamiana* leaf extract with Rituximab IgG and HFBI-Protein A, either separately or both together. After establishing a two-phase system, the residual aqueous phase was removed and acidic buffer added to release the antibodies from the Protein A block and the surfactant phase.

Most of native plant proteins remained in the aqueous residue phase (figure 5 b, lane 2) and only little background was observed in surfactant phase (lane 3). The acidic buffer (lane 4) contains purified antibody. With HFBI-Protein A 28±1% (mean±SD, n=3) of the antibody was recovered while the recovery rate without the fusion protein was significantly lower, 12±2%. Volumes of the phases are given in Table S1.

Binding to IgG had no effect to the separation of the HFBI-Protein A into the surfactant phase: there was no significant difference in recovery rates in presence or absence of the antibody (figure 5 c). Recovery of the fusion protein after release of the antibody and second ATPS was poor, only a fifth of the initial amount (figure 5c). This could be partly due to degradation in acidic conditions as shown on the SDS-PAGE (figure 5b, lane 5).

Contained protein production in BY-2 suspension cells

Having established the good expression levels in *N. benthamiana* and demonstrated the functionality of the HFBI-Protein A, we decided to evaluate the possibility to produce the fusion proteins in transgenic BY-2 cells. After preliminary screening of callus lines, protein accumulation was quantified for the 10 best clones expressing Protein A, HFBI-Protein A and HFBII-Protein A (Figure 6). Non-fused Protein A yielded on average approximately 2 µg/g of fresh callus, whereas both HFBI and HFBII fusions boosted the average accumulation approximately tenfold to 20 to 30 µg/g fresh callus (Figures 6a and b). It should be noted however, that the accumulation levels between the best 10 clones of each line showed considerable variation (Figure 6c). This is most probably due to random insertion sites in the genome and effect of the location to the transcriptional activity.

Based on favourable growth characteristics and homogeneity of the callus,we selected a clone expressing HFBI-Protein A to be grown in suspension culture in shake flasks and subsequently in a stirred tank bioreactor in 30 l scale. The accumulation of biomass (dry weight) in the bioreactor was comparable to that in shake flasks (Figure 7a). The yield of HFBI-Protein A reached 30±6 mg/l (mean±SD, n=3) and 36±3 mg/l in shake flasks and bioreactor, respectively (Figure 7c). In order to establish a streamlined downstream process suitable for large scale production, the whole culture suspension was homogenized in a high pressure homogenizer and clarified by centrifugation. The clarified extract was directly applied to two phase separation with 2% surfactant, resulting in partially purified protein extract with HFBI-Protein A concentrated to 44±2

mg/l with recovery rate of 49±10% (mean±SD, n=3). Thus the total yield after first purification was approximately 18 mg HFBI-Protein per litre of culture volume.

DISCUSSION

Monoclonal antibodies have a key role in modern medicine, research and diagnostics. In many cases however, the high costs of production are limiting their use. The production cost becomes an issue especially now as the first generic antibody drugs are entering the market. Harvesting and initial purification of antibodies using chromatographic methods poses a major bottle-neck and represents a large part of the overall production cost (Farid, 2007; Raven *et al.*, 2015). The aim of this study was to show that the use of a HFB tag can be broadened to include not only purification of fusion proteins themselves, but also of non-covalently bound target molecules, such as antibodies. We constructed a bi-functional fusion protein from two blocks: Protein A and either HFBI or HFBII. The fusion proteins were produced in *Nicotiana benthamiana* plants and in BY-2 suspension cells. The best performing fusion protein was finally tested for capturing Rituximab antibodies from solution.

HFB-fused Protein A reached excellent yields in *N. benthamiana*. Both N-terminal HFB fusion-tags improved accumulation in comparison to non-fused Protein A up to 35 fold. We observed the same trend later in BY-2 calli, although the accumulation levels varied between the clones. HFBI fused to either the N- or C-terminus of the Protein A improved the accumulation to similar levels in *N. benthamiana*. HFBII, however, enhanced the accumulation of Protein A only as an N-terminal fusion, whereas the C-terminal fusion accumulated to levels similar to those observed with non-fused Protein A. The N-terminus of the HFBII, before the first disulphide bridge, is four amino acids shorter than that of HFBI (Sunde *et al.*, 2008). This may cause a steric hindrance for correct folding of the Protein A-HFBII and thus limit its accumulation. The HFBI fusion has previously been reported to enhance the accumulation of some fusion proteins in plants (Gutiérrez *et al.*, 2013; Jacquet *et al.*, 2014; Joensuu *et al.*, 2010). However, this effect has not been consistent and several studies have shown no improvement in yields (Pereira *et al.*, 2014; Phan *et al.*, 2014). This is the first report on improved product accumulation in BY-2 cells using a HFB tag.

The yield-enhancing effect of HFB fusion tags has been attributed to the formation of protein bodies (Conley *et al.*, 2011; Joensuu *et al.*, 2010). We examined the sub-cellular localization of the fusion proteins by immunofluorescent confocal microscopy of protoplasts prepared from agro-infiltrated *N. benthamiana* leaves. Interestingly, we found that all Protein A constructs accumulated in protein body-like structures, regardless of the HFB fusion. When compared to GFP-HFBI-induced protein bodies, the Protein A induced bodies appeared to be less abundant, but were similar in size. We observed no apparent differences in localization of fused or non-fused Protein A. Previous reports have suggested that protein bodies would form independently of the

presence of HFBs when the recombinant proteins accumulate in levels higher than 0.2% of TSP (Gutiérrez *et al.,* 2013; Saberianfar *et al.,* 2015). In our experiments the yields of all recombinant proteins exceeded that threshold. Thus the results here support the conclusions of the previous studies that formation of protein body-like structures may indeed be largely a concentration-dependent phenomenon. However, in our experiment even the ca. 20-fold difference in accumulation levels of fused and non-fused Protein A did not result in apparent differences in number or size of the protein bodies. Therefore the formation of protein bodies alone may not be the only reason for increased accumulation. This challenges the previous assumption and leaves open the question of other possible yield-increasing mechanisms of the HFB fusion. However, this question was outside the scope of this study.

In the future, transgenic plants grown in the field may provide an ideal low-cost production platform for HFBI-Protein A and other commodity proteins. However, contained production might be a necessity for some applications, especially in the case of pharmaceutical targets (Fischer *et al.,* 2012; Ritala *et al.,* 2014). In comparison to *N. benthamiana*-based transient production systems, plant suspension cells may prove to be a useful alternative. As demonstrated here and in previous studies, BY-2 cell lines can be propagated in conventional industrial scale bioreactors and the downstream processing is readily scalable (Raven *et al.,* 2015; Reuter *et al.,* 2014). Low productivity is nevertheless an issue. Yields in plant cell cultures typically vary from 0.005 to 200 mg/l and a yield in range of 10 mg/l is generally considered satisfactory for starting commercial product development (Hellwig *et al.,* 2004). Thus the intrinsic productivity of the suspension culture here was on a good level (36 mg/l). Nevertheless, an approximate calculation indicates that the 30 litre culture volume correlated in yield to only ca. 40 *N. benthamiana* plants. However, it should be noted that the yield of HFBI-Protein A in transient expression was very high, whereas the potential to increase productivity of the BY-2 suspension culture remains vast. We have previously reported tenfold increase in productivity with a stable model protein GFP-HFBI in BY-2 suspension cells (Reuter *et al.,* 2014). Several means for improving the productivity of BY-2 suspension cells have been published recently, including improved culture media (Holland *et al.,* 2010), FACS-based clone screening (Kirchhoff *et al.,* 2012), protease knockout lines (Mandal *et al.,* 2014) and development of culture systems (Raven *et al.,* 2015). However, improving the yield in the BY-2 suspension cells was not the aim of this study.

We expected the fusion proteins to exhibit two functions. First, they should demonstrate the amphipathic properties of hydrophobins and be efficiently separated into a surfactant phase from aqueous solution. Second, they should reversibly bind immunoglobulins. The initial ATPS experiment showed that both HFBI-Protein A and HFBII-Protein A partitioned well to surfactant phase. The HFBI-Protein A, however, partitioned slightly better than HFBII-Protein A (Figure 3).In order to examine the antibody binding capacity of the fusion proteins, we used a quartz crystal microbalance

with dissipation monitoring (QCM-D). Both fusion proteins bound IgG with similar efficiency to that of commercial Protein A. According to the literature, the wild type Protein A could theoretically bind to five immunoglobulins (Uhlen *et al.,* 1984), but the experimental data, as well as information from chemical providers, suggest that the real rate is close to 1:2. Although this potential rate was not reached in this experiment, we conclude that the HFB block does not hinder the antibody binding capacity of the fusion proteins. The fusion proteins also retained the capability of Protein A to repeated rounds of antibody binding and release by adjusting the pH. Thus the fusion protein could be potentially re-used in a recyclable system, thus lowering the purification costs.

Having separately confirmed the two functions of the fusion protein, we put the HFBI-Protein A to a final test to see whether it could be used to harvest antibodies from plant leaf extract. The ATPS experiments demonstrated that the antibody was bound by the Protein A block and carried to the surfactant phase by the HFB block of the fusion protein. Furthermore, the antibody could be recovered back to the aqueous phase by decreasing the pH. This would enable recycling of the HFBI-Protein A for another round of harvesting, if issues with degradation and low recovery can be solved. Use of other variants of Protein A may allow milder elution conditions and better stability in comparison to the wild type protein used here (Pabst, *et al.,* 2014). The recovery rate of the antibody was clearly lower than would have been expected on the basis of separation of HFB-Protein A alone. The vastly larger size and relatively hydrophilic nature of the HFBI-Protein A/IgG complex in comparison to the smaller and sufficiently amphipathic fusion protein alone may have hindered the separation of the complex. However, the presence of the IgG did not influence the recovery of HFB-Protein A. This suggests that it is the formation of the complex, or the binding of Protein A block to the antibody, rather than the separation efficiency of the fusion protein that limits the recovery rate of IgG. Binding of the fusion protein to the IgG could also have been hindered by multimerization of the fusion protein due to self-assembly tendency of the HFB block (Linder *et al.,* 2002). Further work to improve the affinity of the fusion protein and the purification conditions is ongoing.

A fraction of the antibody (12%) was recovered from the ATPS also without HFBI-Protein A. Some of the antibody may have migrated to the surfactant phase due non-specific hydrophobic interactions with the surfactant or passive distribution between the phases. Similarly the antibody may have migrated back to the acidic buffer. Nevertheless, the difference to recovery rate using HFB-Protein A was sufficient for proof of concept.

Whereas the experiments yielded merely a qualitative demonstration of the phenomenon, further optimization of the process could result in a feasible, recyclable antibody purification system. Options for tuning and optimization of the system are versatile with respect to choice of surfactant, additives and buffer composition.

This report makes a case for novel applications of HFBs beyond their use as a fusion tag simply to aid production and purification of recombinant proteins. The bi-functional fusion protein, inspired by the unique properties of the HFBs, may open novel applications for antibody harvesting and purification. However, the applications are not limited to that. Recently the surface active and self-assembling properties of HFBs and HFB-fusion proteins have been utilized for example in functional coatings of nanoparticles (Sarparanta *et al.,* 2012) and surfaces (Kurppa *et al.,* 2014). With the emerging interest in material technology, HFBs can be seen as very interesting building blocks for a host of novel fusion proteins.

EXPERIMENTAL PROCEDURES

Construct design

A codon optimized coding sequence for the immunoglobulin binding domain (amino acids 27-325) of *Staphylococcus aureus* Protein A (accession 1314205A) was synthesised at Genscript (USA). Four potential N-glycosylation sites were removed (N to Q) (Figure S1). The coding sequence was connected to *HFBI* (accession XM_006964119.1) or *HFBII* (accession P79073) of *Trichoderma reesei* by a $(GGGS)_3$ linker as described in Figure 1. The sequence for HFBII was codon optimized. The constructs were assembled and placed in a plant binary expression vector pCaMterX (Harris and Gleddie. 2001) under the control of the dual-enhancer cauliflower mosaic virus 35S promoter (Kay *et al.,* 1987), tcup translational enhancer (Wu *et al*., 2001) and the soybean (*Glycine max*) vspB (Mason *et al.,* 1988) terminator using Golden Gate cloning (Engler *et al.,* 2009). The vector incorporates a c-Myc-tag and a signal sequence for secretory pathway (Prb1) in the N-terminus and StrepII-tag and ER-retention signal (KDEL) in the C-terminus of the open reading frame. See Figure S1 for complete nucleotide sequence. The expression vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.,* 1993).

Transient expression in Nicotiana benthamiana plants, tissue sampling and protein extraction

A. tumefaciens cultures were grown in liquid LB-media overnight. The optical density at 600 nm was adjusted to 0.8 with infiltration buffer (1mM MES, 1mM $MgSO_4$). The suspension was mixed with (ratio 2:1) a suspension of *Agrobacterium* carrying an expression vector for p19 (Silhavy *et al.,* 2002). Leaves from six different 5 to 6 weeks old *N. benthamiana* plants were infiltrated using a syringe and sampled six days post infiltration (dpi) by collecting four leaf discs (Ø 7.1 mm) for each construct.

The leaf discs were stored frozen at -80 C and homogenized using a Retsch mill (MM301, Haan, Germany). Ice-cold extraction buffer (phosphate buffered saline, PBS;137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.8 mM KH2PO4 , 2 % sodium ascorbate, 1 mM EDTA, 1 mM PMSF, 1.25 ug/ml leupeptin pH 7.4) was added (300 ul)

and the leaf powder was mixed to a slurry. The protein extract was clarified by centrifugation at 16 873 **g** for 2x5 min at +4 ^{o}C; Eppendorf 5418R, Germany). The replicates were either analysed separately to obtain data for statistical analysis or pooled together to show representative sample on SDS-PAGE and western blot.

Protoplast preparation and imaging

Agro-infiltrated leaves (6 dpi) were cut into thin strips and digested in enzyme solution (1.5% cellulaseR10 (Serva Germany), 0.4% macerozymeR10 (Serva, Germany), 0.4 M mannitol, 20 mM KCl, 20 mM MES (pH 5.7), 10 mM $CaCl_2$, 5 mM β-mercaptoethanol) in the dark at RT overnight. Protoplasts were sieved through a 100 µm mesh and centrifuged for 10 min at 60 **g** at 4 ^{o}C (Eppendorf 5810R). After washing twice with WI buffer (0.5 M mannitol, 4 mM MES (pH 5.7), 20 mM KCl), the protoplasts were fixed in 4% paraformaldehyde (Sigma-Aldrich, USA) in WI for 1 hour at RT. The membranes were permeated by incubation in 3% IGEPAL CA-630 (Sigma-Aldrich) and 10% DMSO (Merck, Germany) in PBS for 5 min at RT. Non-specific binding was blocked by incubation in 2% BSA (Sigma-Aldrich) in PBS for 1 hour at RT. Primary antibody against the c-Myc tag (mouse, A00864, GenScript, USA) was applied in PBS (1:100) and incubated at 4 ^{o}C overnight. Secondary antibody, conjugated with Alexafluor®555 (goat anti mouse, A21422, Life Technologies, USA), was applied in PBS (1:100) and incubated for 2 hours at 38 ^{o}C. Between each step the protoplasts were washed 3x with PBS.

Z-stack images were acquired with a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss, Germany) equipped with a 63X water immersion objective. Excitation with a 488-nm agron laser was used for GFP and fluorescence was detected at 495-550 nm. Alexafluor®555 was excited with a 543-nm HeNe laser and fluorescence was detected at 550-630 nm.

ATPS and protein purification

Proteins were extracted for purification by homogenizing snap-frozen agro-infiltrated leaves in cold extraction buffer (4x buffer volume/leaf weight). The homogenate was clarified by centrifugation (10 min at 3220 **g** at 4 ^{o}C; Eppendorf 5810R). To precipitate host cell proteins, particularly Rubisco, the supernatant was set up on magnetic stirrer plate and the pH was adjusted to 4.8 by adding HCl. After two minutes the supernatant was tittered back to pH 7.2 with NaOH and clarified with a second centrifugation step. For the ATPS, the supernatant was warmed to 24^{o}C and mixed with Triton X-114 (6% w/v, Sigma Aldrich, USA). After mixing the phases were allowed to separate in a separation funnel. The lower (detergent-rich) phase was collected and washed with isobutanol (Sigma Aldrich, USA; 10-fold volume with respect to detergent mass). The aqueous phase was collected and the buffer was changed to 100 mM Tris-HCl, 150 mM NaCl and 1 mM EDTA (pH 8.0) with 10DG gel filtration columns (Biorad, USA).

Finally the extract was purified by affinity chromatography using a Streptactin macroprep column according to the manufacturers' protocol (IBA, Germany)

Transformation and maintenance of BY-2 cell cultures

Transformation of the BY-2 cells was performed as described earlier (De Sutter *et al.*, 2005). After two passages on selective media, 48 two weeks old calli were screened for product accumulation. Ten lines were selected for further experiments. After 3 weeks the lines were sampled again for quantitative analysis. The lines were further maintained by sub-culturing at 3 week intervals on modified MS media (Nagata and Kumagai, 1999) supplemented with 50 ppm kanamycin. Three lines with good expression levels of HFBI-Protein A were grown in suspension cultures of which one was selected for scaling-up according to product accumulation and growth characteristics. Suspension cultures were maintained in liquid modified MS media supplemented with 50 ppm kanamycin and sub-cultured weekly.

Bioreactor cultivation

Bioreactor (New Brunswick Scientific IF 40) cultivation was conducted in a total culture volume of 30 l in batch mode by inoculating at 5% (v/v) with a 7 days old suspension from shake flask cultures. The medium, without antibiotics, was prepared and sterilized in the bioreactor. Cultivation was carried out at 28^{o}C. Dissolved oxygen (DO) was controlled by stirring speed, airflow and vessel overpressure to maintain DO concentration above 20%. The pH was monitored, but not controlled. As a control, the same line was propagated in 50 ml volume in shake flasks.
The fresh weight was determined by sampling 10.0 ml of culture suspension in a conical tube and weighing the cell pellet after centrifugation for 10 min at 3220 **g** (Eppendorf 5810R). The pellet was freeze dried to obtain dry weight.

Protein extraction from BY-2

Callus samples were stored at -20 ^{o}C. For protein extraction ice-cold buffer (PBS, 1mM EDTA) was added 1:2 v/w to callus samples thawed on ice and subsequently homogenized using the Retsch mill. For protein extraction from freeze dried cell material from suspension cultures, extraction buffer was added to powdered cell material (40:1 v/w) and homogenized using the Retsch mill. The protein extracts were clarified by centrifugation for 10 min at 21130 **g** at 4 ^{o}C (Eppendorf 5424R).

For the scaled up downstream process 10x extraction buffer (10x PBS, 10mM EDTA) was added 1:10 to cooled (+4^{o}C) cell suspension. The broth was homogenized in a high pressure homogenizer (Rannie LAB 12.15 H, Maskinfabriken Rannie A/S, Denmark) two times at 500 to 600 bar and clarified by centrifugation in 2 litre bottles (Sorvall RC12BP, *ca*. 4000 **g**, 15 min, RT). The ATPS was done in a 20 l glass vessel with 2 % w/v Triton X-114.

Protein Analysis

Concentration of TSP was measured using the Bradford analysis (1976) with Bio-Rad reagent (Bio-Rad, USA). Protein separation was performed by SDS-PAGE on Bio-Rad Criterion-TGX and Mini-PROTEAN precast gels and stained using GelCode® Blue Stain Reagent (Thermo Scientific, USA). Protein quantifications were performed either from SDS-PAGE or by western blot analysis after transferring proteins on nitrocellulose membrane using the Trans-Blot® Turbo™ system (Biorad, USA). Proteins were visualized with anti-c-Myc tag primary antibody (rabbit, A00172, GeneScript) and a secondary antibody for detection (anti-rabbit-AP, 170-6518, BioRad) For quantification (Figure 1d) and work in BY-2 a fluorescently labelled secondary antibody (goat anti-rabbit, IR Dye® 680RD, LI-COR Biosciences, Germany) was used. Detection was done with Odyssey CLX densitometer (LI-COR Biosciences, Germany) and Image Studio 2.1 software. Protein quantities were assessed against known concentrations of purified HFBI-Protein A or commercial Rituximab (Oriola, Finland).

QCM-D

Protein adsorption was measured by QCM-D (E4 Biolin Scientific). Polystyrene crystals (Biolin Scientific) were cleaned according to supplier's protocol. Protein solutions were diluted in buffer M (0.1 M sodium phosphate, pH 7) and pumped for 5 min. Adsorbed surfaces were stabilized 45-60 min and rinsed with buffer M.

Protein samples were diluted as follows: HFB-Protein A 2 µM, IgG1 λ antibodies 0.05 mg/ml, 0.3 µM (Sigma Aldrich, USA). In Figure 4 a 1/3 molar equivalents of wild type HFBI was used together with HFBI-Protein A to enhance surface packing. Antibodies were released by rinsing with glycine-HCl buffer (pH 2.2) for 5 min, followed by buffer M (pH 8).

Three replicate binding experiments were conducted (Fig. 4b). HFBI-Protein A, HFBII-Protein A and commercial Protein A (Sigma Aldrich, USA) were diluted to 0.1 mg/ml (ca. 2 µM). Rituximab IgG was added (82 nM, 0.01 mg/ml) to the adsorbed protein surfaces for 5-7 min. The bound mass was calculated using the Sauerbrey equation $\Delta m = -C \cdot \Delta f / n_5$, where C = 17.7 $ngHz^{-1}cm^{-2}$ for a 5 MHz quartz crystal and n_5 = 5, the overtone number. The values for bound mass were obtained at the buffer rinsing steps by averaging the data over 100 time points (260 s). Dissipation D was used to examine the viscoelastic properties of the bound protein layer. D is defined as $E_{lost}/2\pi E_{stored}$, where E_{lost} is the energy lost during one oscillation cycle and E_{stored} is the total energy stored in the oscillator. Molar ratios were calculated using the Saurbrey mass values and molecular weights of 44 kDa (HFBI-Protein A and HFBII-Protein A) and 50 kDa (commercial Protein A).

Antibody capture by two-phase extraction

HFBI-Protein A (0.1 mg/ml) and Rituximab (0.2 mg/ml) were mixed with *N. benthamiana* leaf extract, incubated at RT for 45min and mixed with Triton X-114 (4% w/v). The total volume was 1.8 ml. Phases were allowed to separate at RT for 2 h and centrifuged at 16 873 **g** for 2 min (Eppendorf 5418R, Germany). The residue phase was removed. 1x volume acidic buffer (0.1M glycine-HCl, pH 2.3) was mixed into the surfactant phase and incubated for 5 min. After 2 min centrifugation at 16 873 **g** (Eppendorf 5418R) the aqueous top phase (containing the released antibodies) was recovered and neutralized by adding 70µl 1M Tris-HCl (pH 8.5). Prior to the analysis on SDS-PAGE gels the phases containing surfactant were extracted with isobutanol and centrifuged.

Statistical analysis

Statistical analyses were done with SPSS Statistic 22.0 (IBM, Armonk, NY) using Two-tailed Student's independent samples T test for two samples and one way ANOVA test followed by Tukey HSD for three or more samples, with significance level of 95%.

ACKNOWLEDGEMENTS

We thank the Academy of Finland, Centres of Excellence Programme (2014-2019) and Grant 252442 for the support. Also the support from Eurostars project E!5320 is acknowledged. We thank Tuuli Teikari, Juha Tähtiharju, Riitta Suihkonen and Karita Viita-aho for assistance with protein production and purification.

REFERENCES

Azevedo, A.M., Rosa, P.A.J., Ferreira, I.F. and Aires-Barros, M.R. (2009) Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. *Trends in Biotechnology*, **27**, 240-247.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.

Collén, A., Persson, J., Linder, M., Nakari-Setälä, T., Penttilä, M., Tjerneld, F., Sivars, U. (2002) A novel two-step extraction method with detergent/polymer systems for primary recovery of the fusion protein endoglucanase I–hydrophobin I. *Biochimica et Biophysica Acta*, **1569**, 139-150

Conley, A.J., Joensuu, J.J., Richman, A. and Menassa, R. (2011) Protein body-inducing fusions for high-level production and purification of recombinant proteins in plants. *Plant Biotechnology Journal*, **9**, 419-433.

De Sutter, V., Vanderhaeghen, R., Tilleman, S., Lammertyn, F., Vanhoutte, I., Karimi, M., Inzé, D., Goossens, A. and Hilson, P. (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. *Plant Journal,* **44**, 1065-1076.

Ecker, D.M., Jones, S.D. and Levine, H.L. (2015) The therapeutic monoclonal antibody market. *mAbs,* **7**, 9-14.

Engler, C., Gruetzner, R., Kandzia, R. and Marillonnet, S. (2009) Golden gate shuffling: A one-pot DNA shuffling method based on type ils restriction enzymes. *PLoS ONE,* **4**.

Farid, S. (2007) Process economics of industrial monoclonal antibody manufacture. *Joural of Chromatography B,* **848**, 8-18

Fischer, R., Schillberg, S., Buyel, J.F. and Twyman, R.M. (2013) Commercial aspects of pharmaceutical protein production in plants. *Curr. Pharm. Des.*, **19**, 5471-5477.

Fischer, R., Schillberg, S., Hellwig, S., Twyman, R.M. and Drossard, J. (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. *Biotechnol. Adv.,* **30**, 434-439.

Gutiérrez, S.P., Saberianfar, R., Kohalmi, S.E. and Menassa, R. (2013) Protein body formation in stable transgenic tobacco expressing elastin-like polypeptide and hydrophobin fusion proteins. *BMC Biotechnology*, **13**.

Hakanpää, J., Linder, M., Popov, A., Schmidt, A., Rouvinen, J. (2006a) Hydrophobin HFBII in detail: ultrahigh-resolution structure at 0.75Å. *Acta Cryst,* **62**, 356-367

Hakanpää, J., Szilvay, G.R., Kaljunen, H., Maksimainen, M., Linder, M., Rouvinen, J. (2006b) Two crystal structures of Trichoderma reesei hydrophobin HFBI—The structure of a protein amphiphile with and without detergent interaction. *Protein Science,* **15**, 2129-2140

Harris, L.J. and Gleddie, S.C. (2001) A modified Rp13 gene from rice confers tolerance of the Fusarium graminearum mycotoxin deoxynivalenol to transgenic tobacco. *Physiol. Mol. Plant Pathol.,* **58**, 173-181.

Hellwig, S., Drossard, J., Twyman, R.M., Fischer, R. (2004) Plant cell cultures for the production of recombinant proteins. *Nature biotechnology,* **22**, 1415-1422

Holland, T., Sack, M., Rademacher, T., Schmale, K., Altmann, F., Stadlmann, J., Fischer, R. and Hellwig, S. (2010) Optimal nitrogen supply as a key to increased and

sustained production of a monoclonal full-size antibody in BY-2 suspension culture. *Biotechnol. Bioeng.,* **107**, 278-289.

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New Agrobacterium helper plasmids for gene transfer to plants. *Transgenic Res.,* **2**, 208–218

Höök, F., Kasemo, B., Nylander, T., Fant, C., Sott, K. and Elwing, H. (2001) Variations in coupled water, viscoelastic properties, and film thickness of a Mefp-1 protein film during adsorption and cross-linking: A quartz crystal microbalance with dissipation monitoring, ellipsometry, and surface plasmon resonance study. *Anal. Chem.*, **73**, 5796-5804.

Jacquet, N., Navarre, C., Desmecht, D. and Boutry, M. (2014) Hydrophobin fusion of an influenza virus hemagglutinin allows high transient expression in Nicotiana benthamiana, easy purification and immune response with neutralizing activity. *PLoS ONE*, **9**.

Joensuu, J.J., Conley, A.J., Lienemann, M., Brandle, J.E., Linder, M.B. and Menassa, R. (2010) Hydrophobin Fusions for High-Level Transient Protein Expression and Purification in Nicotiana benthamiana. *Plant Physiol.,* **152**, 622-633.

Kay, R., Chan, A., Daly, M. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. *Science,* **236**, 1299-1302.

Kirchhoff, J., Raven, N., Boes, A., Roberts, J.L., Russell, S., Treffenfeldt, W., Fischer, R., Schinkel, H., Schiermeyer, A. and Schillberg, S. (2012) Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. *Plant Biotechnology Journal*, **10**, 936-944.

Kurppa, K., Hytönen, V. P., Nakari-Setälä, T., Kulomaa, M.S., Linder, M.B. (2014) Molecular engineering of avidin and hydrophobin for functional self-assembling interfaces. *Colloids and Surfaces B: Biointerphases*, **120**, 102-109

Lahtinen, T., Linder, M.B., Nakari-Setälä, T. and Oker-Blom, C. (2008) Hydrophobin (HFBI): a potential fusion partner for one-step purification of recombinant proteins from insect cells. *Protein Expr. Purif.* **59**, 18–24.

Linder, M., Selber, K., Nakari-Setälä, T., Qiao, M., Kula, M-R., Penttilä, M. (2001) The hydrophobins HFBI and HFBII from Trichoderma reesei showing efficient interactions with nonionic surfactants in aqueous two-phase systems. *Biomacromolecules*, **2**, 511-517

Linder, M., Szilvay, G.R., Nakari-Setälä, T., Söderlund, H., Penttilä, M. (2002) Surface adhesion of fusion proteins containing the hydrophobins HFBI and HFBII from Trichoderma reesei. *Protein Sci.*, **9**, 2257-2266

Linder, M.B., Qiao, M., Laumen, F., Selber, K., Hyytiä, T., Nakari-Setälä, T. and Penttilä, M.E. (2004) Efficient purification of recombinant proteins using hydrophobins as tags in surfactant-based two-phase systems. *Biochemistry*, **43**, 11873–11882.

Linder, M.B. (2009) Hydrophobins: Proteins that self assemble at interfaces. *Current Opinion in Colloid & Interface Science*, **14**, 356-363.

Mandal, M.K., Fischer, R., Schillberg, S. and Schiermeyer, A. (2014) Inhibition of protease activity by antisense RNA improves recombinant protein production in Nicotiana tabacum cv. Bright Yellow 2 (BY-2) suspension cells. *Biotechnology Journal*, **9**, 1065-1073.

Mason, H.S., Guerrero, F.D., Boyer, J.S. and Mullet, J.E. (1988) Proteins homologous to leaf glycoproteins are abundant in stems of dark-grown soybean seedlings. Analysis of proteins and cDNAs. *Plant Mol. Biol.*, **11**, 845-856.

McLean, M.D., Chen, R.J., Yu, D.Q., Mah, K.Z., Teat, J., Wang, H.F., Zaplachinski, S., Boothe, J. and Hall, J.C. (2012) Purification of the therapeutic antibody trastuzumab from genetically modified plants using safflower Protein A-oleosin oilbody technology. *Transgenic Research*, **21**, 1291-1301.

Mustalahti, E., Saloheimo, M. and Joensuu, J.J. (2013) Intracellular protein production in Trichoderma reesei (Hypocrea jecorina) with hydrophobin fusion technology. *New Biotechnol*. **30**, 262–268.

Nagata, T. and Kumagai, F. (1999) Plant cell biology through the window of the highly synchronized tobacco BY-2 cell line. *Methods in Cell Science*, **21**, 123-127.

Pabst, T.M., Palmgren, R., Forss, A., Vasic, J., Fonseca, M., Thompson, C., Wang, W.K., Wang, X., Hunter, A. (2014) Engineering of novel Staphylococcal Protein A ligands to enable milder elution pH and high dynamic binding capacity. *Journal of Chromatography A*, **1362**, 180-185

Pereira, E., Kolotilin, I., Conley, A. and Menassa, R. (2014) Production and characterization of in planta transiently produced polygalacturanase from Aspergillus niger and its fusions with hydrophobin or ELP tags. *BMC Biotechnology*, **14**, 59.

Phan, H.T., Hause, B., Hause, G., Arcalis, E., Stoger, E., Maresch, D., Altmann, F., Joensuu, J. and Conrad, U. (2014) Influence of elastin-like polypeptide and

hydrophobin on recombinant hemagglutinin accumulations in transgenic tobacco plants. *PLoS ONE,* **9**.

Raven, N., Rasche, S., Kuehn, C., Anderlei, T., Klöckner, W., Schuster, F., Henquet, M., Bosch, D., Büchs, J., Fischer, R. and Schillberg, S. (2015) Scaled-up manufacturing of recombinant antibodies produced by plant cells in a 200-L orbitally-shaken disposable bioreactor. *Biotechnol. Bioeng.,* **112**, 308-321.

Reuter, L.J., Bailey, M.J., Joensuu, J.J. and Ritala, A. (2014) Scale-up of hydrophobin-assisted recombinant protein production in tobacco BY-2 suspension cells. *Plant Biotechnology Journal,* **12**, 402-410.

Ritala, A., Häkkinen, S. and Schillberg, S. (2014) Molecular pharming in plants and plant cell cultures: a great future ahead? *Pharm. Bioprocess.,* **2 (3)**, 223-223-226.

Saberianfar, R., Joensuu, J.J., Conley, A.J. and Menassa, R. (2015) Protein body formation in leaves of Nicotiana benthamiana: a concentration-dependent mechanism influenced by the presence of fusion tags. *Plant Biotechnology Journal,* n/a-n/a.

Sarparanta, M., Bimbo, L.M., Rytkoänen, J., Mäkilä, E., Laaksonen, T.J., Laaksonen, P., Nyman, M., Salonen, J., Linder, M.B., Hirvonen, J., Santos, H.A. and Airaksinen, A.J. (2012) Intravenous delivery of hydrophobin-functionalized porous silicon nanoparticles: Stability, plasma protein adsorption and biodistribution. *Molecular Pharmaceutics,* **9**, 654-663.

Silhavy, D., Molnar, A., Lucioli, A., Szittya, G., Hornyik, C., Tavazza, M. and Burgyan, J. (2002) A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs. *EMBO J.,* **21**, 3070-3080.

Szilvay G.R., Paananen, A., Laurikainen, K., Vuorimaa, E., Lemmetyinen, H., Peltonen, J., Linder, M.B. (2007) Self-assembled hydrophobin protein films at the air-water interface: structural analysis and molecular engineering. *Biochemistry,* **46**, 2345-2354

Sunde, M., Kwan, A.H.Y., Templeton, M.D., Beever, R.E. and Mackay, J.P. (2008) Structural analysis of hydrophobins. *Micron,* **39**, 773-784.

Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. and Lindberg, M. (1984) Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *The Journal of biological chemistry,* **259**, 1695-702.

Wessels, J.G.H. (1994) Developmental Regulation of Fungal Cell-Wall Formation. *Ann. Rev. Phytopathol.,* **32**, 413-437.

Wosten, H.A.B. and Scholtmeijer, K. (2015) Applications of hydrophobins: current state and perspectives. *Applied Microbiology and Biotechnology*, **99**, 1587-1597.

Wu, K., Malik, K., Tian, L., Hu, M., Martin, T., Foster, E., Brown, D., Miki, B. (2001) Enhancers and core promoter elements are essential for the activity of a cryptic gene activation sequence from tobacco, tCUP. *Mol Genet Genomics*, **265**, 763–770

Yang, L., Biswas, M.E., Chen, P. (2003). Study of binding between protein A and immunoglobulin G using a surface tension probe. *Biphys J.*, **84**, 509-522.

FIGURES

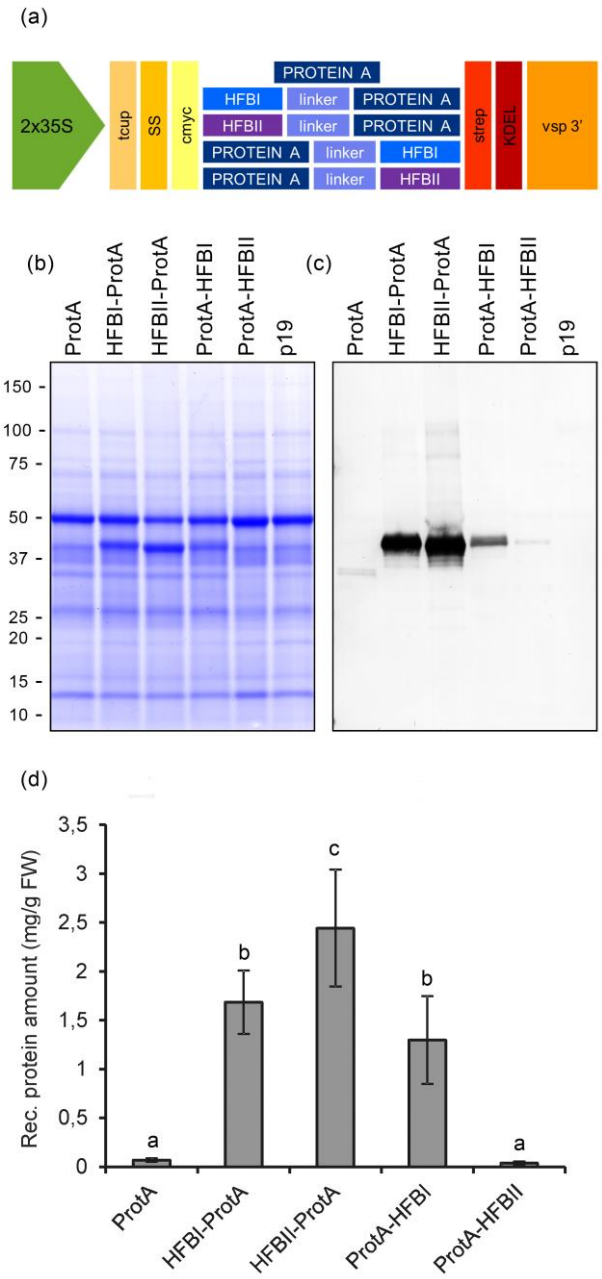


Figure 1. Transient expression of Protein A and HFB-fusions in *N. benthamiana.* a) Schematic presentation of gene constructs of Protein A and fusions with HFBI or HFBII. b) Pooled samples analyzed on Coomassie stained SDS-PAGE and c) on western blot. d) Recombinant protein yields analyzed as band intensities from western blots. Error bars indicate standard error of mean (n=6). The letters indicate significant difference (p<0.05).

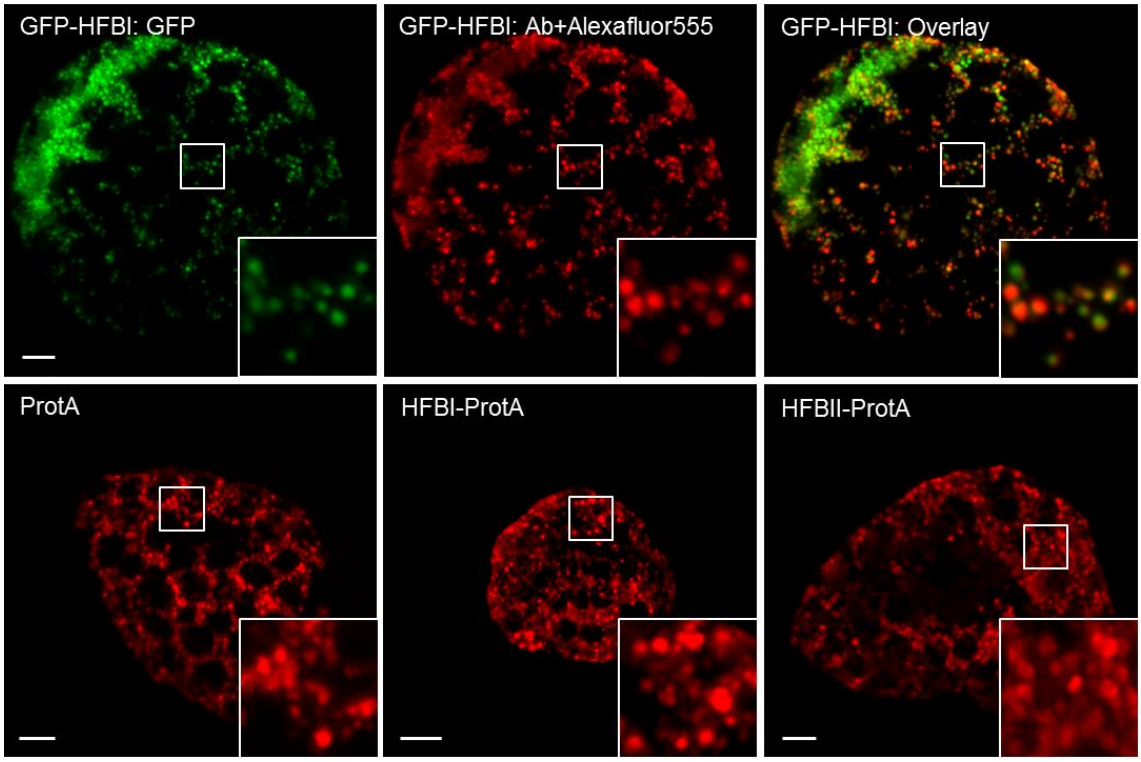


Figure 2. Immunostained confocal microscopy images of *N. benthamiana* protoplasts showing subcellular localization of recombinant proteins. Upper panel: GFP-HFBI was used as a positive control. On the left, GFP-derived signal shows a typical morphology of HFBI-induced protein bodies. In the midle, the same cell immunostained with anti-c-Myc primary antibody and Alexafluor®555 conjugated secondary antibody. On the right, an overlay image. No signal was detected from the same sample treated without the primary antibody. Lower panel: representative images of protoplasts expressing Protein A (left), HFBI-Protein A (middle) and HFBII-Protein A (right). Protein body-like structures, similar in size and shape, can be seen in all samples. All images are maximum intensity projections of z-stack images. Scalebars indicate 5µm.

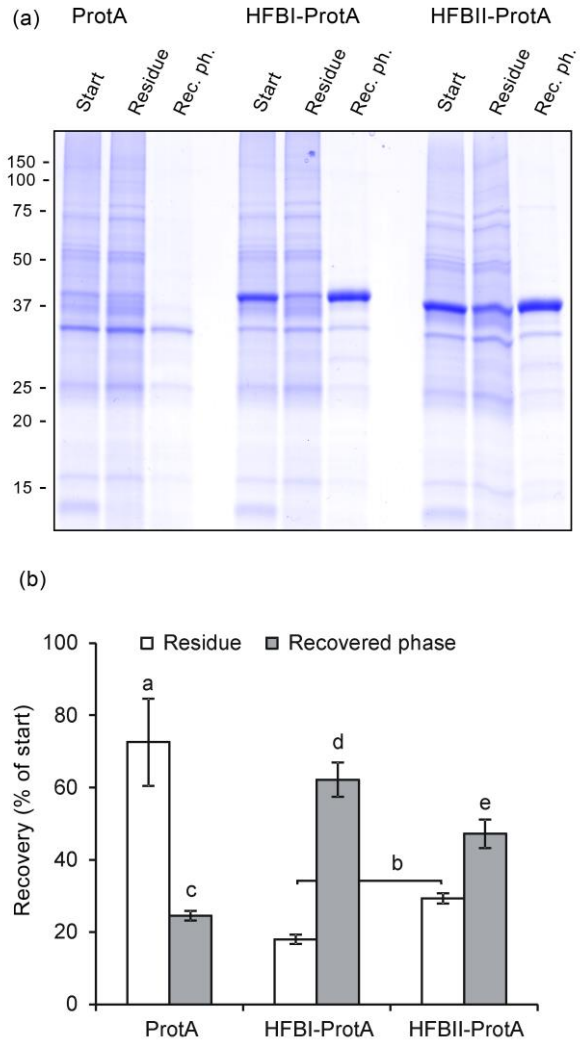


Figure 3. Fusion proteins retain the amphipathic properties of the HFB block. a) A Coomassie stained SDS-PAGE of pooled samples from three replicates shows that both fusion proteins partitioned to the surfactant and were found in recovered phase, whereas the non-fused Proten A remained mainly in the residue as did most native plant proteins. Equal volumes of samples were loaded on gel. Fraction volumes are presented in Figure S2. b) Recovery rate of the proteins in residue and in the recovered phase analyzed on a western blot. Letters indicate significant difference (n=3, p<0.05). Error bars indicate standard deviation.

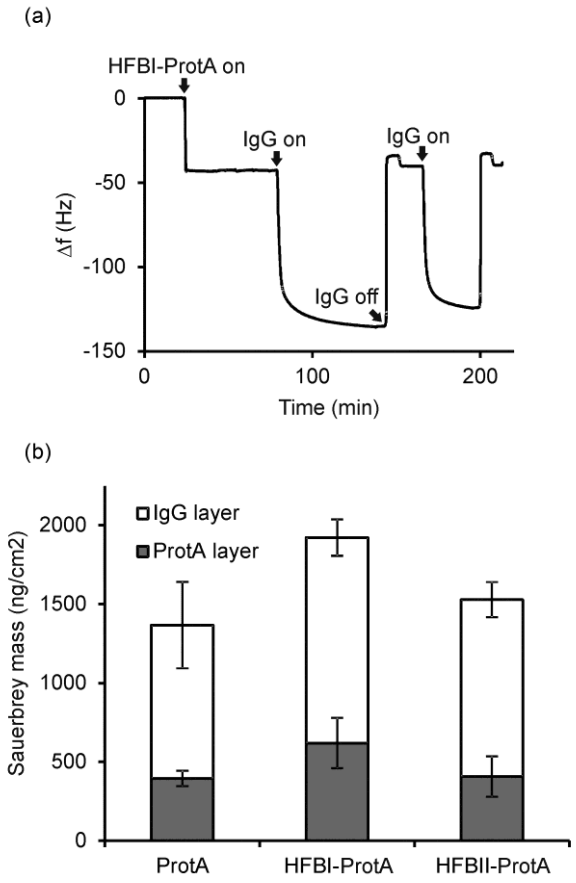


Figure 4. Fusion proteins retain the reversible antibody binding capacity of the Protein A block. a) The QCM-D experiment showed reversible antibody binding to the HFBI-Protein A layer, represented as a function of time and oscillation frequency. Protein binding reduced the oscillation frequency of the polystyrene-coated quartz crystal. The curve shows binding of HFBI-Protein A (20 min time point) and of IgG (80 min), and release of IgG by decreasing buffer pH to 2.2 (140 min). The procedure was repeated twice. b) A similar experiment shows that surface-bound Protein A, HFBI-Protein A and HFBII-Protein A (grey bars) all bind Rituximab with similar capacities (white bars). The error bars indicate standard deviation between repeated measurements.

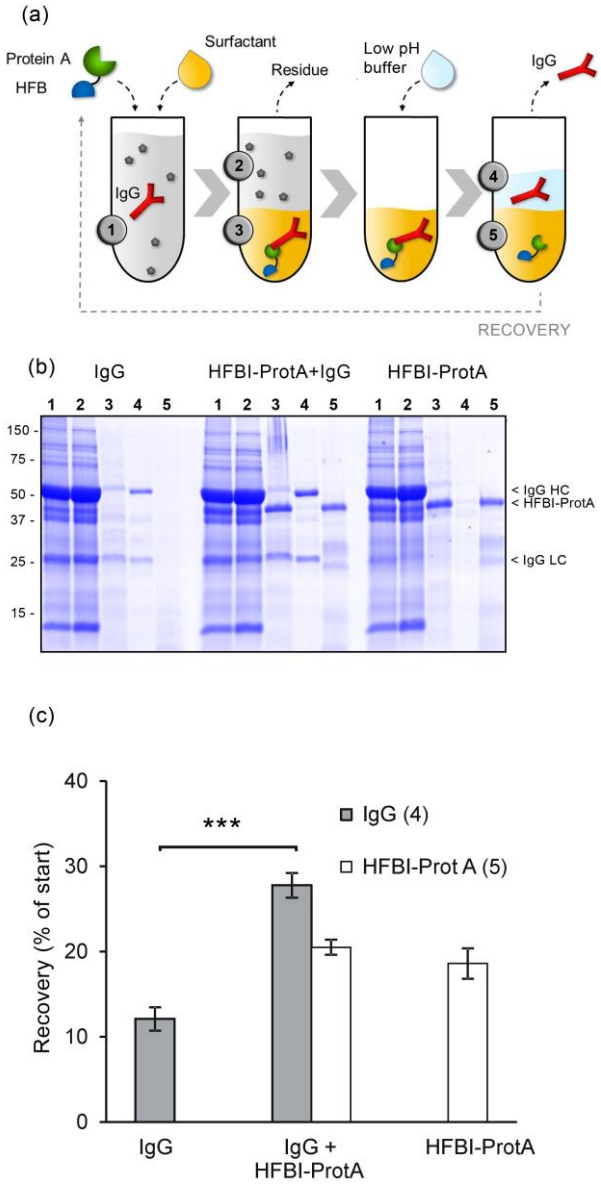


Figure 5. The HFBI-Protein A fusion protein can capture antibodies in solutions. a) The concept of the in-solution antibody harvesting. The Protein A block (green) binds to the IgG (red) when added to the antibody-containing plant leaf extract (1). Addition of a surfactant (tan) results in a two-phase system. The HFB block (blue) guides the HFBI-Protein A/IgG complex to the surfactant phase. The aqueous residue (2) is discarded. The IgG is released by addition of acidic buffer and recovered from the aqueous phase (4). The HFBI-Protein A carrier remains in the surfactant phase (5) and can be recycled for a new round of antibody harvesting. b) SDS-PAGE showing the partition of the IgG in ATPS with the HFBI-Protein A (middle) and without (left) and HFBI-Protein A alone (right). Lane numbering corresponds to the illustration on top (a). Volumes of the collected phases are given in Table S4. c) Overall recovery of IgG and HFBI-Protein A. The error bars represent standard deviation of the mean (n=3). The asterisks indicate significant difference (p>0.001).

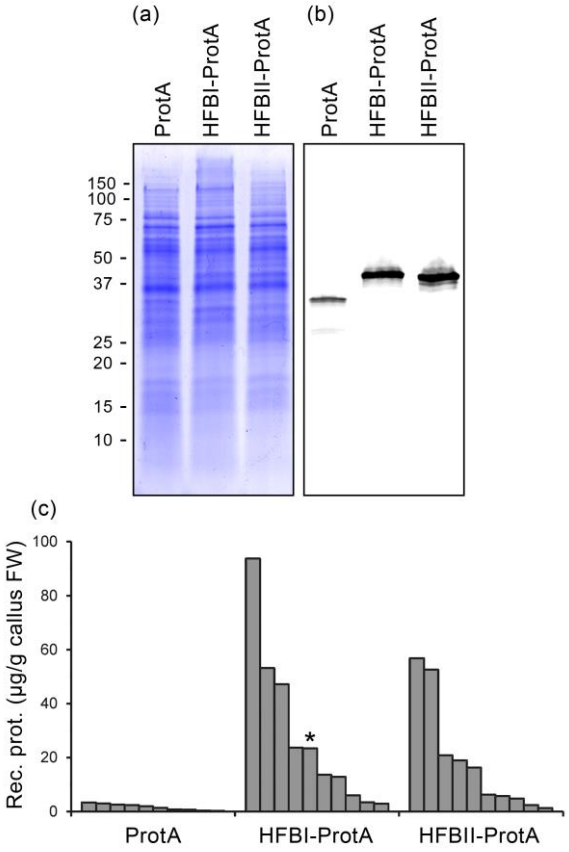


Figure 6. Accumulation of Protein A, HFBI-Protein A and HFBII-Protein A in tobacco BY-2 cell cultures. a) A Coomassie stained SDS-PAGE and b) a western blot illustrating the accumulation of the recombinant proteins in samples pooled from 10 callus clones for each construct. The western blot is visualised using anti-c-Myc antibodies. c) Amount of recombinant proteins in the 10 best callus clones for each construct determined from western blots. The line used to initiate a suspension culture is indicated with an asterisk.

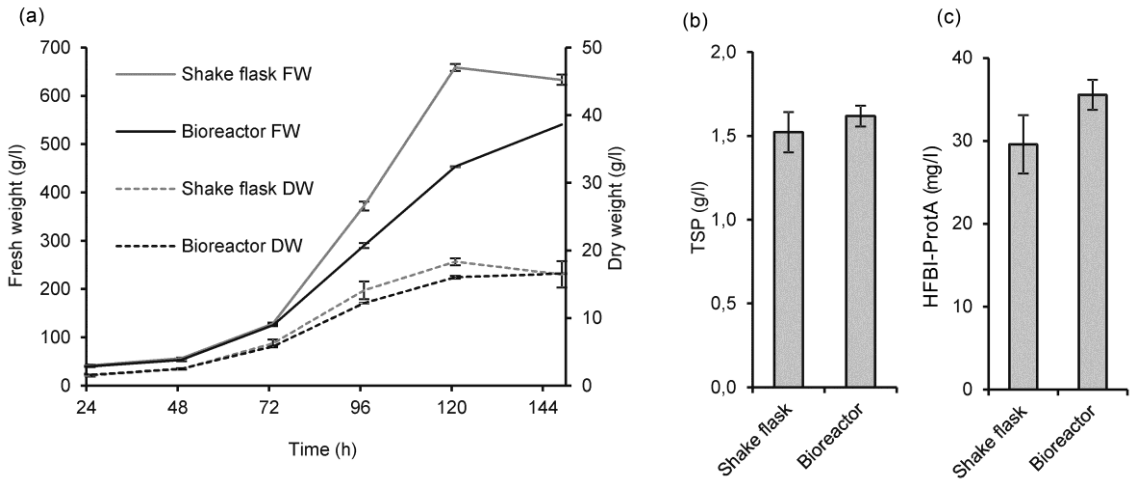


Figure 7. HFBI-Protein A producing BY-2 suspension cell culture propagated in 30 litre culture volume. a) Accumulation of dry mass was similar in shake flasks and in the bioreactor. The error bars represent standard deviation between three biological replicates in shake flasks and three technical replicates in the bioreactor. b) The accumulation of total soluble protein, analysed by Bradford-assay, and c) the recombinant protein, analysed from western blots, was comparable in the bioreactor and shake flask cultivations. The error bars represent standard deviation between three technical replicates.

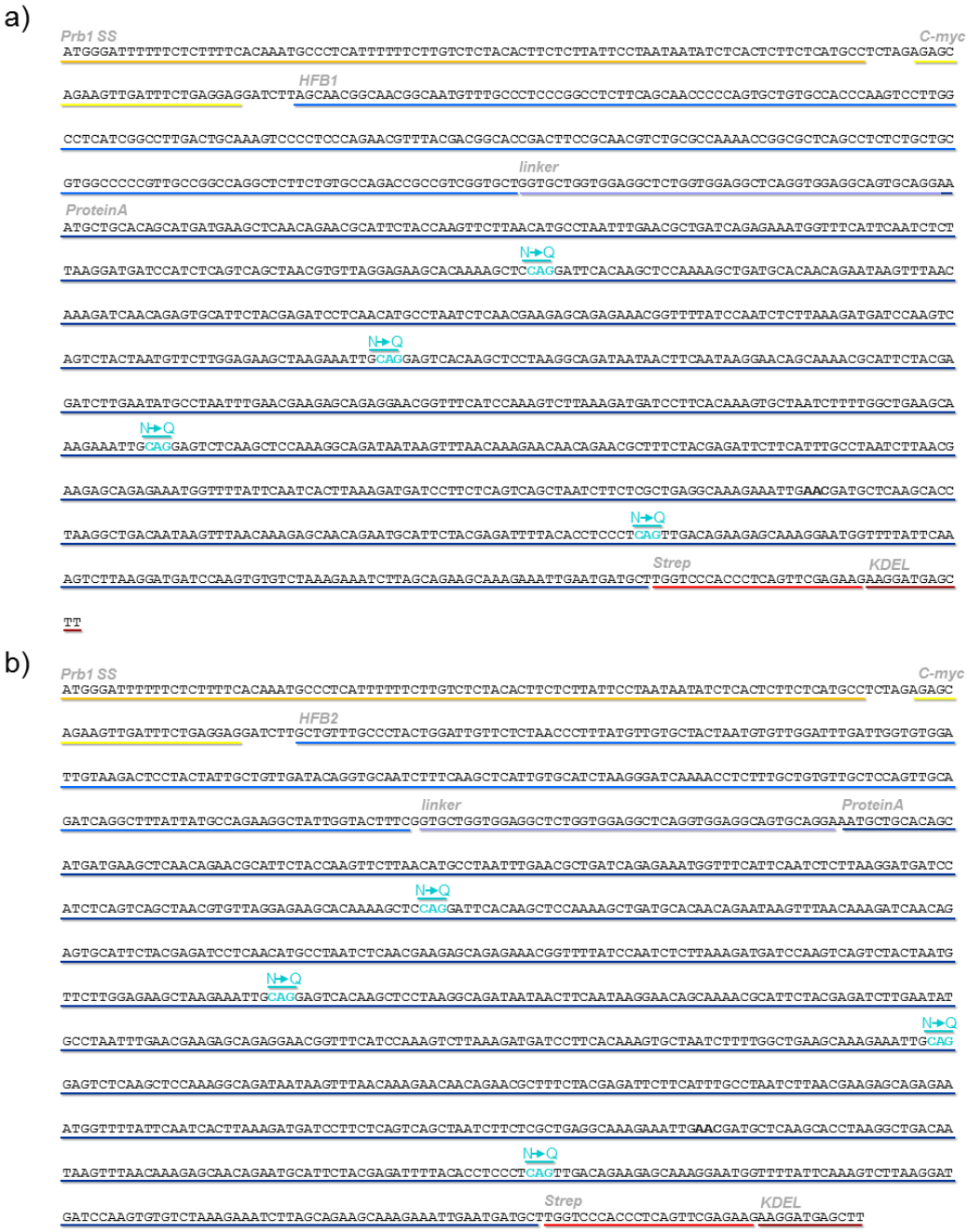


Figure S1. Nucleotide sequences of expression cassettes for a) HFBI-ProteinA and b) HFBII-ProteinA. Genes for HFB, linker and ProteinA were cloned in the vector between BsaI restriction sites using Golden gate assembly. The gene of interest is placed under control of double the 35S promoter and the vsp terminator. A Pr1b signal sequence (MGFFLFSQMPSFFLVSTLLLFLIISHSSHASR) directs the protein to secretory pathway and a KDEL-signal retains it in the ER. The vector also introduces a codon optimized C-myc-tag (GAGCAGAAGTTGATTTCTGAGGAGGATCTT) in the N-terminus and a codon optimized StrepII-tag (TGGTCCCACCCTCAGTTCGAGAAG) in the C-terminus of the amino acid sequence.

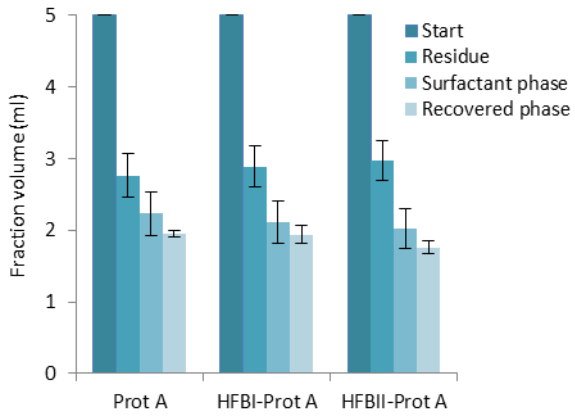


Figure S2. Volumes of fraction recovered from ATPS in Figure 3. Error bars indicate standard deviation (n=3).

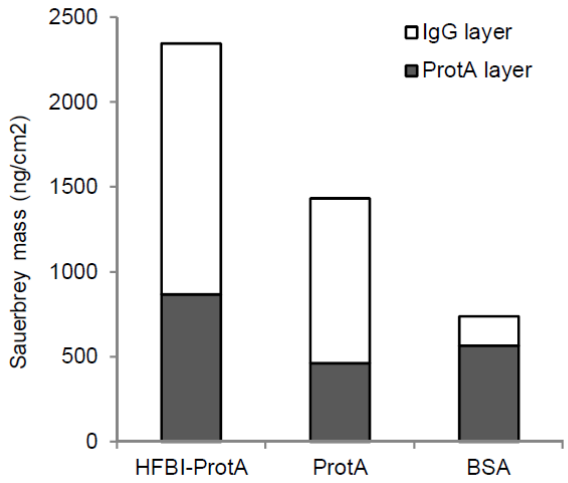


Figure S3. QCM-D experiment showing antibody binding to HFBI-Protein A layer, Protein A layer and BSA as negative control.

Table S1. Volumes of fractions recovered from ATPS in Figure 5b. Mean± (n=3). Numbers in brackets refer to labelling in figure 5b.

	start (1) (ml)	residue (2) (ml)	surfactant phase (3) (ml)	acid phase (4) (ml)
IgG	1.86±0.02	1.05±0.03	0.81±0.03	0.96±0.05
HFBI-ProtA+IgG	1.85±0.02	1.03±0.04	0.83±0.02	0.98±0.05
HFBI-ProtA	1.86±0.03	1.00±0.01	0.86±0.01	1.07±0.01

PUBLICATION IV

Coating nanoparticles with plant produced transferrin-hydrophobin fusion enhances uptake in cancer cells

Manuscript.

Coating nanoparticles with plant-produced transferrin-hydrophobin fusion protein enhances uptake in cancer cells

AUTHORS

*Lauri J. Reuter[1], Mohammad-Ali Shahbazi[2,3], Ermei M. Mäkilä[4], Jarno J. Salonen[4], Hélder A. Santos[2], Jussi J. Joensuu[1], Anneli Ritala[1]**

AFFILIATIONS

[1] VTT Technical Research Centre of Finland Ltd., Finland

[2] Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, Finland

[3] Current address: Department of Micro- and Nanotechnology, BioLabChip, Technical University of Denmark

[4] Laboratory of Industrial Physics, Department of Physics and Astronomy, University of Turku, Finland

ABSTRACT

Encapsulation of drugs to nanoparticles may offer a solution for targeted delivery. Hydrophobins act as self-assembling functional coating of porous silicon nanoparticles to improve stability and biodistribution. Since human transferrin is widely utilized as a targeting ligand, we set out to engineer a fusion protein combining the functionalities of the two. We showed that transferrin can be expressed in *Nicotiana benthamiana* plants as a fusion with *Trichoderma reesei* hydrophobins HFBI, HFBII or HFBIV. Transferrin-HFBIV was further expressed in tobacco BY-2 suspension cells. Both partners of the fusion protein retained their functionality: The hydrophobin moiety enabled migration to a surfactant phase in an aqueous two-phase system and the transferrin moiety was able to reversibly bind iron. Coating porous silicon nanoparticles with the fusion protein resulted in uptake of the nanoparticles in human cancer cells. This study provides a proof-of-concept for functionalizing hydrophobin coatings with transferrin as a targeting ligand.

KEYWORDS

hydrophobin, hydrophobin fusion protein, porous silicon nanoparticle, tobacco BY-2 cells, drug targeting

INTRODUCTION

Drug delivery is one of the biggest challenges in modern medicine, especially for cytotoxic drugs used in the treatment of cancer due to their severe off-target effects[1] and poor bioavailability. Thus, new formulations are needed to enhance the delivery to specific target cells. Encapsulating drugs in nanoparticles is a potential solution for both increasing their solubility and controlling their delivery and release, thus diminishing the unwanted side effects. However, nanoparticles need to be stable, degradable, non-immunogenic and reach specifically their targets[2]. In this regard, porous silicon (PSi) nanoparticles have been used extensively as carriers in oral[3] and intravenous drug delivery[4] for different biochemical applications.

Fungal hydrophobins (HFB) are small, globular proteins with extraordinary surface active properties due to their unique amphipathic structure[5,6]. HFBs are highly soluble in water, but form multimers at high concentrations[7,8]. When in contact with a hydrophilic hydrophobic interphase, HFBs self-assemble into a monolayer (Kisko et al., 2007; Szilvay et al., 2007). The self-assembly of Trichoderma reesei HFBI and HFBII has been successfully utilized to formulate nanoparticles from poorly water-soluble drug compounds (Valo et al., 2010; Valo et al., 2011). When applied on PSi nanoparticles, the HFB coating improved the solubility and biocompatibility of the particles, while allowing the controlled release of the payload (Bimbo et al., 2011). The coated particles were stable in simulated gastrointestinal fluids and the oral administration to rats increased the transit time from stomach to intestine (Sarparanta et al., 2012b). The HFB coating also influenced the biodistribution of the PSi nanoparticles when administered intravenously to rats (Sarparanta et al., 2012a). A major problem in parenteral administration is the adsorption of plasma proteins around the nanoparticles as a corona, causing aggregation and loss of activity (Shahbazi et al., 2014a; Shahbazi et al., 2014b). However, HFB-coated PSi nanoparticles and polystyrene nanoparticles recruited significantly less plasma proteins than naked particles (Grunér et al., 2015; Sarparanta et al., 2012a).

When linked to other proteins, HFBs convey some of their properties to the respective fusion partner. Several HFBI fusion proteins have been expressed in plants (Jacquet et al., 2014; Joensuu et al., 2010; Miletic et al., 2015; Pereira et al., 2014; Phan et al., 2014; Saberianfar et al., 2016). Recently, we have shown that also HFBII and HFBIV are potential candidates as fusion partners (Reuter et al., in press). While HFBI and HFBII are structurally similar (Hakanpää et al., 2004; Hakanpää et al., 2006), the amino acid sequence and the hydropathy profile of HFBIV are distinctly different, possibly also influencing its properties (Espino-Rammer et al., 2013). Nevertheless, HFBIV appears to bind to both polar and hydrophobic surfaces, similarly to HFBI and HFBII (Espino-Rammer et al., 2013).

The HFB fusion technology has been applied for purification of fusion proteins using surfactant based aqueous two-phase separation (ATPS) in fungal (Linder et al., 2001; Linder et al., 2004), insect (Lahtinen et al., 2008), plant (Joensuu et al., 2010) and plant cell based production platforms (Reuter et al., 2014). A fusion protein combining HFBI and a dual cellulose binding domain has also been used for adding functionality to HFB-coated nanoparticles. The fusion protein enabled formulation of the nanoparticles, similar as non-fused HFB, but also

bound the nanoparticle to cellulose nanofibrils within cellulose hydrogel allowing improved formulation (Valo et al., 2011). In this study, we took one step further and investigated whether a HFB fusion protein could be used for active targeting of nanoparticles utilizing transferrin receptor-mediated endocytosis. Transferrin (Tf) is an 80 kDa glycoprotein with 19 intramolecular disulphide bridges. Challenges in production of the complex molecule in bacterial systems (Brandsma et al., 2010) and risks involved in purification from human plasma have encouraged search of alternative production platforms, such as plants and yeasts. Human Tf was first expressed in tobacco (Brandsma et al., 2010) and is currently produced commercially in rice under trade name Optiferrin (Zhang, 2013). Plant-derived recombinant Tf has been shown to be structurally and functionally similar to native human protein although it appears not to be glycosylated (Brandsma et al., 2010; Zhang et al., 2010; Zhang, 2013).

The main function of Tf is iron sequestration and transport in serum (Tortorella and Karagiannis, 2014; Zhang, 2013). When free transferrin (apo form) binds ferric iron, its conformation changes (holo form) and the affinity to transferrin receptor increases. The Tf/Tf-receptor complex is taken-up by the cells through endocytosis and dissociation of iron occurs at low pH in the endosomal compartments. Subsequently, the protein is recycled back to the bloodstream (Tortorella and Karagiannis, 2014; Zhang, 2013). The transferrin receptor is ubiquitously expressed on normal cell types, but is upregulated on various tumors (Tortorella and Karagiannis, 2014). This, combined with the capacity of transferrin to cross the blood-brain barrier, has made it an interesting molecule for drug targeting via direct conjugation to the active molecule (Brandsma et al., 2010; Brandsma et al., 2011; Kratz et al., 1998), or to nanocarriers (van der Meel et al., 2013; Pei-Hui Yang et al., 2005; Tortorella and Karagiannis, 2014).

The aims of this study were to engineer a fusion protein that exhibits the functional characteristics of both HFB and transferrin, to produce the protein in plant cell culture and to test whether it can be used to facilitate targeting and uptake of nanoparticles in cancer cells.

MATERIALS AND METHODS

Construct design

Codon optimized coding sequences for human Tf (Uniprot: P02787) without the native signal peptide, HFBI (Uniprot: P52754), HFBII (Uniprot: P79073) and HFBIV (Uniprot: 0RHN0) were synthesised at Genscript (USA). The coding sequences were connected by a (GGGS)3 linker (Figure 1A) and placed in a plant binary expression vector pCaMterX (Harris and Gleddie. 2001) under the control of the dual-enhancer cauliflower mosaic virus 35S promoter (Kay et al., 1987) and the soybean (Glycine max) vspB (Mason et al., 1988) terminator using Golden Gate cloning (Engler et al., 2009). The vector incorporates a c-Myc-tag and a signal sequence for secretory pathway (Prb1) in the N-terminus and StrepII-tag and ER-retention signal (KDEL) in the C-terminus of the open reading frame. See Figure S1 for complete nucleotide sequence. The expression vectors were transformed into Agrobacterium tumefaciens strain EHA105 (Hood et al., 1993).

Transient expression in Nicotiana benthamiana, tissue sampling and protein extraction

Transient expression was done as described earlier (Joensuu et al., 2010). In brief, the optical density of A. tumefaciens cultures were adjusted to 1.0 and the suspension was mixed (2:1) with Agrobacterium carrying an expression vector for post transcriptional gene silencing inhibitor p19 (Silhavy et al., 2002). Four N. benthamiana plants were infiltrated using a syringe and sampled six days post infiltration. The samples were stored at -80 oC and homogenized (Retsch mill MM301, Haan, Germany). Extraction buffer (phosphate buffered saline, PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.8 mM KH2PO4, 2 % sodium ascorbate, 1 mM EDTA, 1 mM PMSF, 1.25 ug/ml leupeptin pH 7.4) was added (6:1 v/w) and the leaf powder was mixed to a slurry. The extract was clarified by centrifugation (Eppendorf Centrifuge 5424R, 21130 g, 10 min, 4oC). The replicates were either analyzed separately for statistical analysis or pooled together to show representative sample on SDS-PAGE and immunoblot.

Transformation and maintenance of BY-2 cell cultures

Transformation and maintenance of the BY-2 cells was performed as described earlier (Reuter et al., 2014). After two passages on selective media, 48 two weeks old calli were screened for product accumulation. Ten lines were selected for further experiments. Yield and growth of the suspension culture were evaluated after 31 weekly passages.

Protein extraction from BY-2

Callus samples were stored at -20 oC. Buffer (PBS, 1mM EDTA) was added 1:2 v/w to callus samples thawed on ice and subsequently homogenized using the Retsch mill. Samples from suspension culture were freeze dried and extraction buffer was added to powdered material (40:1 v/w) and homogenized by milling. The protein extracts were clarified by centrifugation for 10 min at 21130 g at 4oC (Eppendorf 5424R).

Protein Analysis

Concentration of TSP was measured using the Bradford analysis (1976) with Bio-Rad reagent (Bio-Rad, USA). Protein separation was performed by SDS-PAGE on Bio-Rad Criterion-TGX and Mini-PROTEAN precast gels and stained using GelCode® Blue Stain Reagent (Thermo Scientific, USA). Protein quantifications were performed either from SDS-PAGE or by western blot analysis after transferring proteins on nitrocellulose membrane using the Trans-Blot® Turbo™ system (Biorad, USA). Proteins were visualized with anti-c-Myc tag primary antibody (rabbit, A00172, GeneScript) and a secondary antibody for detection (anti-rabbit-AP, 170-6518, BioRad or in BY-2 work goat anti-rabbit, IR Dye® 680RD, LI-COR Biosciences, Germany). Detection was done with Odyssey CLX densitometer (LI-COR Biosciences, Germany) and Image Studio 2.1 software. Protein quantities were assessed against known concentrations of purified Tf-HFBIV or commercial Optiferrin (Sigma-Aldrich).

Aqueous two phase separation

ATPS experiments were conducted as described in Reuter et al., (in press) in 5 ml volume with 3% Triton-X 114 (Sigma-Aldrich).

Iron binding experiments

Purified Tf-HFBIV and Optiferrin were dialyzed over night against 50 mM NaOAc, 5 mM EDTA, pH 4.9, 2×1.5 h against water and 3×2 h against 25 mM Tris-HCl, pH7,5 at 4 oC. Iron saturation was done by incubating the proteins in 1mM Fe-NTA, 20 mM NaHCO3 at RT for 2 h. The mobility of non-treated, iron depleted and iron saturated proteins were analysed on Criterion™ TBE-Urea gels (Biorad, USA) and on SDS-PAGE.

Preparation and characterization of Alkyne-THCPSi nanoparticles

Preparation of Alkyne-terminated THCPSi (AlkyneTHCPSi) nanoparticles is described in SI1. For the surface absorption of HFBI, Tf-HFBIV and Tf, 1 mg of the Alkyne-THCPSi nanoparticles were dispersed in 125 µL of the Hanks' Balanced Salt Solution (HBSS)□4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.4 and added to 1 mL of the coating compounds (1 mg/mL) and gently vortexed for 30 sec until the nanoparticles were in suspension, followed by incubation at room temperature for 2 h. The solution was then centrifuged at 15000 rpm for 5 min and the supernatant removed. The particles were washed three times with 1 mL of Milli-Q water with subsequent centrifugation cycles at 15000 rpm for 5 min for every wash and re-suspended in HBSS buffer. To determine the hydrodynamic diameter (Z-average), polydispersity index (PdI) and surface zeta-potential of the nanoparticles, bare and surface modified PSi nanoparticles were centrifuged and re-dispersed in aqueous solution with a final concentration of 20 µg/mL prior to the measurements using Zetasizer Nano ZS (Malvern Instruments Ltd, UK). All measurements were performed in triplicate.

Cell−nanoparticle interaction studies

The cell culture conditions for MDA-MB-231 breast carcinoma cells (American Type Culture Collection) and viability studies are described in SI1. To evaluate the intracellular uptake and localization of the modified nanoparticles, the MDA-MB-231 cells were treated with AlkyneTHCPSi, AlkyneTHCPSi-HFBI, AlkyneTHCPSi-Tf-HFBIV or AlkyneTHCPSi-Tf nanoparticles and imaged with TEM and laser scanning confocal microscope.

For TEM imaging, the MDA-MB-231 cells (105 in 1 mL of RPMI 1640 media) were allowed to attach overnight on round coverslips (13 mm) placed at the bottom of 24-well plates (Corning Inc. Life Sciences, USA). The cell culture media was then replaced with 500 µL of nanoparticle suspension (100 µg/mL) and the samples were incubated at 37 °C for 6 h. Afterwards, the particle suspension was removed and the coverslips were washed twice with HBSS–HEPES

before fixing the cells with 2.5% glutaraldehyde in 0.1 M PBS solution (pH 7.4) for 1 h RT. After fixing, the coverslips were rinsed twice with HBSS–HEPES (pH 7.4) and sodium cacodylate buffer (NaCac) for 3 min prior post-fixation with 1% osmium tetroxide in 0.1 m NaCac buffer (pH 7.4). The cells were finally embedded in epoxy resin after dehydration of the cells with 30–100% ethanol for 10 min each. Ultrathin sections (60 nm) were cut parallel to the coverslip, post-stained with uranyl acetate and lead citrate, and observed by TEM.

For confocal microscopy Lab-Tek® 8-Chamber Slides (Thermo Fisher Scientific, USA) were seeded with the cells at a density of 5×104 per well. After overnight incubation at 37 °C, the cell medium was replaced with 250 µL of the fluorescein isothiocyanate (FITC)-loaded nanoparticles (50 µg/mL). The cells were incubated for 6 h before washing three times with HBSS–HEPES (pH 7.4). The plasma membrane of the cells was stained by 3 min incubation with 200 µL of the RED CellMask® (5 µg/mL; Invitrogen, USA) at 37 °C followed by washing twice with HBSS–HEPES buffer. The cells were fixed with 2.5% glutaraldehyde for 15 min. The intercellular localization of FITC-labelled nanoparticles was observed with a Leica SP5 inverted confocal microscope (Leica Microsystems, Germany), equipped with argon (488 nm) and DPSS (561 nm) lasers and HCX Plan Apochromate 63□/1.2-0.6 oil immersion objective.

Statistical analysis

Statistical analyses were done with SPSS Statistic 22.0 (IBM, Armonk, NY) using a two-tailed Student's independent samples t-test for two samples and one way ANOVA test followed by Tukey HSD for three or more samples, with significance level of 95%.

RESULTS AND DISCUSSION

Construct screening in Nicotiana benthamiana

We have recently reported that in addition to *T. reesei* HFBI, also HFBII and HFBIV are potential candidates for expression of fusion proteins in plants [9]. To find a suitable fusion strategy, we built six fusion constructs where human Tf was connected by a linker either N- or C-terminally to T. reesei hydrophobins HFBI, HFBII or HFBIV (Figure 1A). The constructs and a non-fused Tf were transiently expressed in N. benthamiana and accumulation levels were determined by immunoblot analysis (Figure 1B and C). All fusion constructs resulted in lower accumulation in comparison to non-fused Tf (43±19% of TSP, mean±SD, n=4). There was no statistically significant difference ($p<0.05$) in accumulation of fusions where different HFBs were placed in the N-terminus of the fusion protein. However, yields of fusions where HFBI or HFBII were placed in the C-terminus were lower. This observation was in line with our previous experiments [9]. The HFBIV fusion proteins accumulated to same levels in both orientations, similarly to previously reported GFP-HFBIV and HFBIV-GFP [9]. In general, it remains unclear why the HFB-tags seems to improve accumulation of some fusion partners [10–13], but not others [14].

It is not clear why HFBII-Tf migrated faster than Tf-HFBII and FHBIV-Tf faster than Tf-HFBIV on SDS-PAGE. Immunoblot analysis also indicated that all constructs were degraded to some extent (Figure 1B). Detection of the degraded fragments with antibody recognizing the C-terminus of the proteins suggests cleavage to take place close to the N-terminus. Based on the good accumulation (21±9% of TSP), relatively low degradation and out of interest to further explore HFBIV, we selected Tf-HFBVI to be used in following experiments requiring stable transformation.

An additional expression cassette for GFP-HFBI was included in the T-DNA (Figure 1A) as a surrogate marker to facilitate visual selection of homogeneous transgenic BY-2 calli [15–17]. Co-expression of GFP-HFBI has also been reported to increase accumulation some target proteins in *N. benthamiana* [13]. In *N. benthamiana* the dual construct resulted in high accumulation of GFP-HFBI (Figure 1B), but reduced the yield of Tf-HFBIV to 10±2% of TSP. This reduction was, however, not statistically significant.

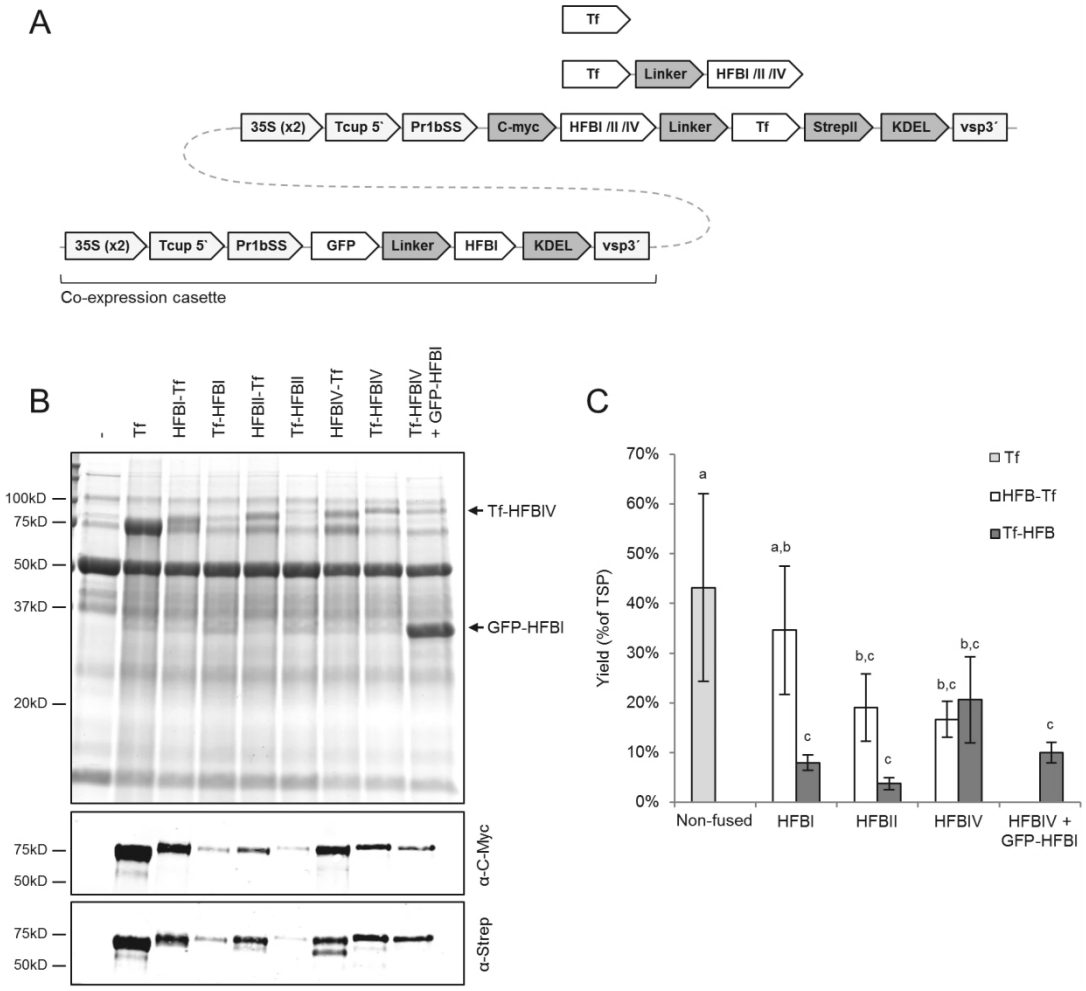


Figure 1. (A) A schematic presentation of the used constructs. **(B)** Pooled samples from four plants on SDS-PAGE and on immunoblots. Cleavage of the fusion protein is apparent with HFBIV-Tf, but also with HFBI-Tf and HFBII-Tf. **(C)** Accumulation levels of Tf, Tf-HFBs and co-expression of Tf-HFBIV with HFBI in *N. benthamiana* quantified from immunoblots detected based on Strep-tag (mean±SD, *n*=4 individual plants in the same experiment). The letters indicate statistically significant difference ($p<0.05$).

Expression in BY-2 cells

We generated BY-2 cell lines co-expressing the Tf-HFBIV fusion protein simultaneously with GFP-HFBI as visual selection marker. Transgenic calli were sub-cultured based on the intensity and homogeneity of GFP fluorescence in order to co-select for high accumulation of Tf-HFBIV and sixteen independent callus lines were screened for protein expression (Figure 2A). The visual selection resulted in homogeneous callus morphology, but the accumulation of GFP-HFBI did not correlate with the yield of Tf-HFBIV (R2 = 0.0464). This could be an artefact due to small sample size (n=16), but it does indicate that the link between co-expressed fluorescence

marker and target protein might not be as direct as previously reported [16,17]. The callus line with highest accumulation of Tf-HFBIV was grown in suspension.

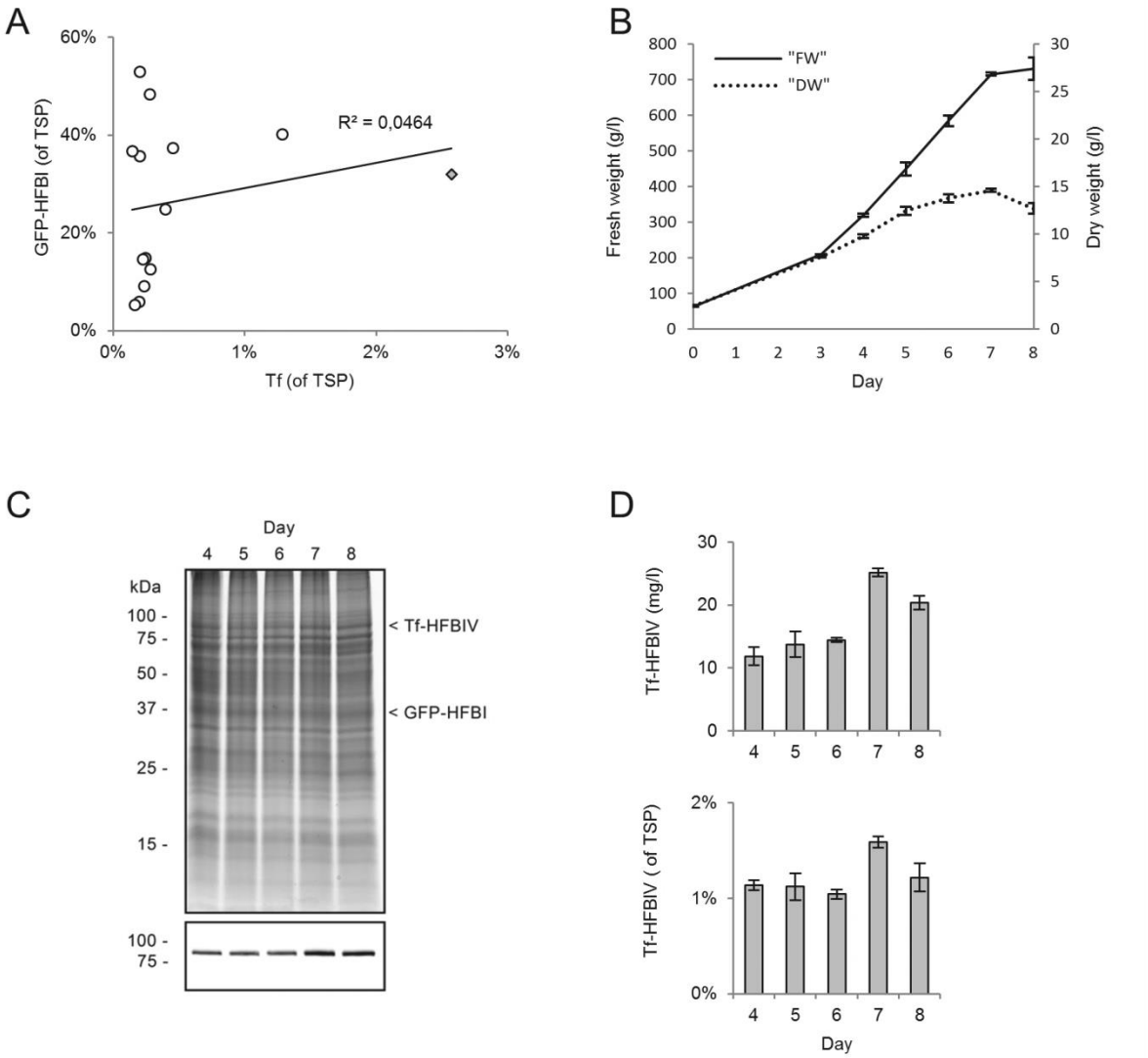


Figure 2. Expression of Tf-HFBIV in tobacco BY-2 cells. **(A)** Accumulation of Tf-HFBIV and GFP-HFBI in 16 callus lines. The line grown in suspension is marked as a diamond. **(B)** Growth of Tf-HFBIV expressing BY-2 cell suspension in shake flask. **(C)** TSP on SDS-PAGE and Tf-HFBIV detected on immunoblot. Arrows indicate the expected sizes of Tf-HFBIV (87 kDa) and GFP-HFBI (36 kDa). **(D)** Accumulation levels of Tf-HFBIV in suspension culture (n=3).

After 31 passages in suspension culture, the growth of the Tf-HFBIV-expressing cell line was comparable to wild type cells: the dry weight peaked at 14.6±0.2 g/L at day 7 (Figure 2B). Accumulation of Tf-HFBIV reached 25.2±0.7 mg/L or 1.6±0.1% of TSP at day 7, while the accumulation of GFP-HFBI was approximately two-fold higher (51.0±1.6 mg/L). The

accumulation of both GFP-HFBI and Tf-HFBIV were lower in the cell suspension than in the original screened calli. However, changes in protein expression are normal when moving cells from solid to liquid media. Thus, screening for a production line should be done in suspension culture.

Overall, plant suspension cells may provide a good production platform for a complex fusion protein, such as Tf-HFBIV. We have shown here that BY-2 cells are capable of folding correctly a protein with altogether 23 disulphide bridges providing a significant advantage over bacterial platforms. Although production in plant seeds, like commercial Optiferrin, is readily scalable and economical, bioreactor-based production is more amenable to the current regulations for manufacturing of pharmaceutical components. The yield reported here (25 mg/L), although good for BY-2 cells, is low in comparison to other production hosts [18,19]. We have previously reported recombinant protein (GFP-HFBI) yields ten-fold higher, up to 0.3 g/L in BY-2 suspension cells [15], and we have further improved that to the level of 1 g/L (unpublished data). Broader clone screening and optimizing the production process further can increase the target protein yields still tremendously.

The Tf-HFBIV fusion protein retains the amphiphilic properties of HFBIV

We setup an aqueous two-phase separation (ATPS) experiment to test whether the HFBIV confers to Tf-HFBIV the capability of interacting with non-ionic surfactants. Tf-HFBIV separated efficiently to the surfactant phase showing no significant difference ($p > 0.05$) to GFP-HFBIV used as a positive control (Figure 3A and B). The recovery rate of Tf-HFBIV was 88.3±1.8%. Commercial recombinant human Tf (Optiferrin), in turn, remained mostly in the aqueous phase and only 9.1±0.7% of the protein was detected in the surfactant phase. The separation efficiencies correspond to our earlier experiments with HFBIV-GFP and HFBI-GFP [9]. The difference in size of the fusion partner (GFP *vs.* Tf) does not appear to influence the performance in ATPS. It is worth noting that we did not test the surface binding properties to the fusion protein here, although HFBIV has been previously shown to adhere on glass and PET surfaces [20]. A variety of HFB fusion tags can be expressed in plants [9] and may provide different affinity to surfaces. This valuable resource should be explored to identify the best suitable HFB-fusion partner for coating purposes.

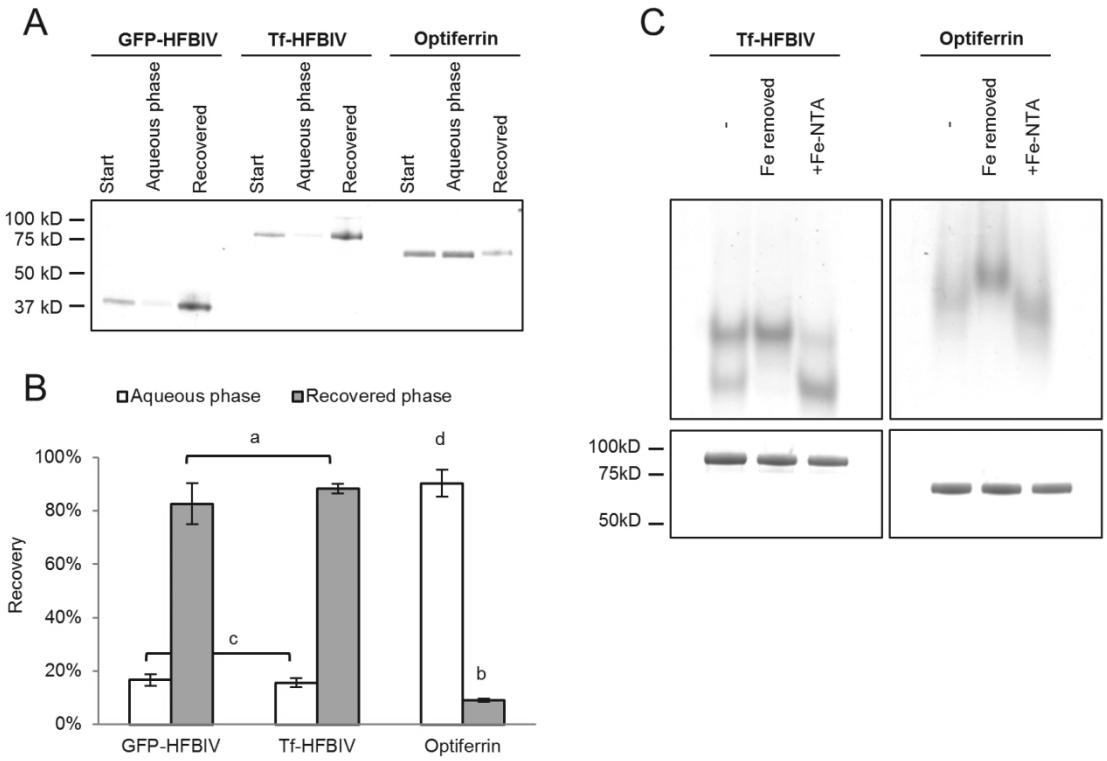


Figure 3. Both fusion partners of the Tf-HFBIV protein remained functional. **(A and B)** ATPS experiment showed separation of purified Tf-HFBIV into non-ionic surfactant phase, similarly to GFP-HFBIV. Commercial recombinant human Tf (Optiferrin) was used as control. The letters indicate significant difference between groups ($p<0.05$) **(C)** Conformational change upon binding and release of ferric iron was analyzed on Coomassie stained Urea-PAGE. Below the same samples on Coomassie stained SDS-PAGE.

HFBIV fused transferrin retains its capability to reversibly bind iron

To test whether HFBIV fused Tf would retain its ability to sequester iron, we subjected the fusion protein to a series of treatments removing all bound iron and subsequently resaturating the protein with iron. Binding and releasing iron resulted in conformational changes which were distinguished on urea-PAGE (Figure 3C). Before treatment, Tf (Optiferrin) was in the holo form. Removal of iron caused a reduction in mobility (apo form) while the treatment with excess ferric iron returned the protein back into the holo form. Before the treatments the Tf-HFBIV fusion protein was present in both holo and apo forms but removal of iron converted all the protein to the apo form. In iron saturated conditions most of the apo Tf-HFBIV converted to the holo form. Some Tf-HFBIV did, however, remain in the apo form. Nonetheless, this indicated that the BY-2 cell derived Tf-HFBIV did retain its capability to reversibly bind iron, although the used method did not allow quantitative conclusion. The capability to adopt the iron saturated holo-form is essential as it has 500-fold higher affinity to the Tf receptor in comparison to the iron depleted apo-form [21].

Surface coating of PSi nanoparticles with Tf-HFBIV

Having independently confirmed that both partners of the TF-HFBIV fusion protein are functional, we coated PSi nanoparticles with Tf-HFBIV, but also with Tf (Optiferrin) or HFBI (as HFBIV was not available). The nanoparticles showed an increase in size upon adsorption of HFBI, Tf or Tf-HFBIV molecules on their surface (Figure 4A). The polydispersity index (PdI) of all prepared nanoparticles was less than 0.3, indicating high monodispersity (Figure 4B). The zeta-potential of the naked nanoparticles became more negative after adsorption of HFBI on the surface due to high negative charge of the molecule (Figure 4C). In contrast, the conjugation of Tf and Tf-HFBIV resulted in a gradual increase in the surface charge of the particles, owing to the intrinsic positive charge of Tf. None of the coated nanoparticles showed toxic effects in MDA-MB-231 breast cancer cells after exposure for 6 and 24 h at concentrations of 25, 50, 100 or 150 µg/mL (Figure 4D).

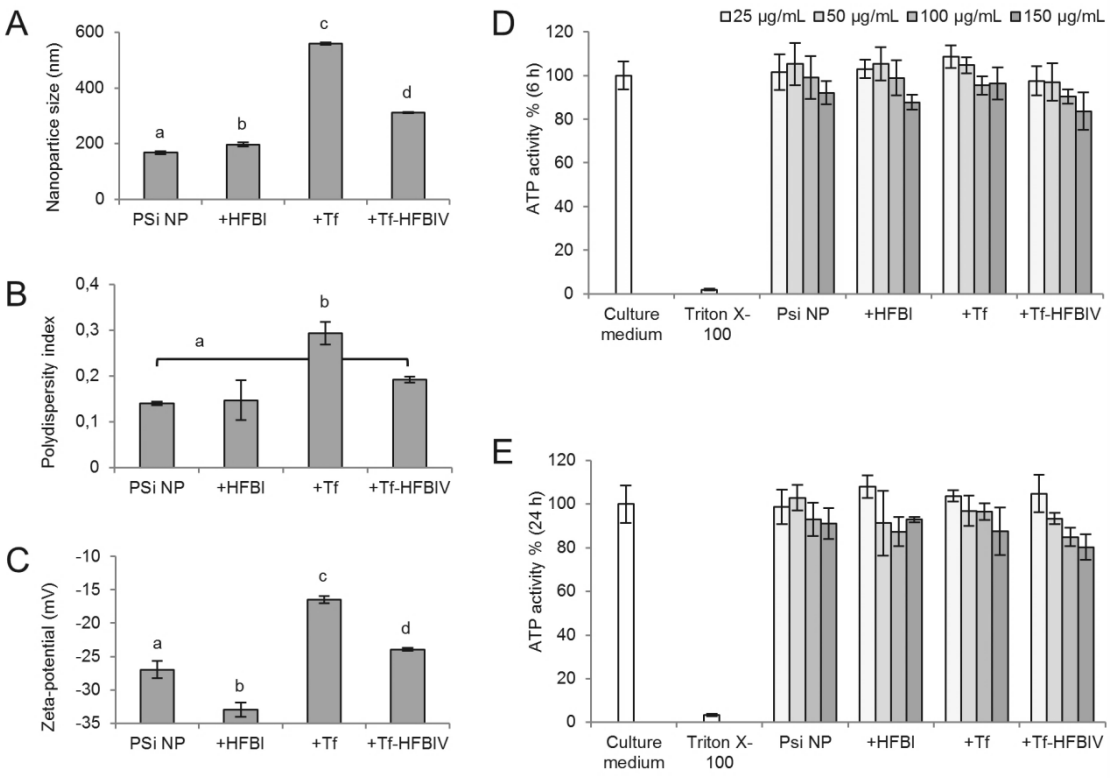


Figure 4. Characterization of PSi nanoparticles coated with HFBI, Tf (Optiferrin) or Tf-HFBIV. **(A)** Nanoparticle size, **(B)** PdI and **(C)** zeta-potential changes after surface modifications. **(D and E)** Coated and naked PSi nanoparticles showed no toxicity to MDA-MB-231 cells during 6 h and 24 h treatments at 37 °C. Culture medium (HBSS) and Triton X-100 were use as controls. Error bars represent standard deviations ($n \geq 3$). The letters indicate significant difference between groups ($p<0.05$).

Cellular uptake of Tf-HFBIV coated nanoparticles

Next, we tested whether the fusion protein retained its capability to interact with the transferrin receptor. A functional Tf domain was expected to facilitate the uptake of Tf-HFBIV-coated PSi nanoparticles into human cancer cells. Transmission electron microscope (TEM) images and confocal fluorescence microscopy confirmed a very limited interaction between naked PSi nanoparticles and MDA-MB-231 cells (Figure 5). In agreement with an earlier report [22], HFBI coating highly increased the accumulation of the nanoparticles in the close vicinity of the cell membrane, probably through hydrophobic interactions with the membrane phospholipids. However, HFBI coating did not lead to internalization of the nanoparticles in the tested conditions. Coating with Tf or Tf-HFBIV also resulted in accumulation of PSi nanoparticles around the cells, but more importantly also led to effective up-take inside the cells. We did not observe any apparent differences between behaviour of nanoparticles coated with Tf of Tf-HFBIV. The most plausible explanation for the observation is interaction with Tf receptors on the surface of the cells, mediating the cellular uptake [23,24].

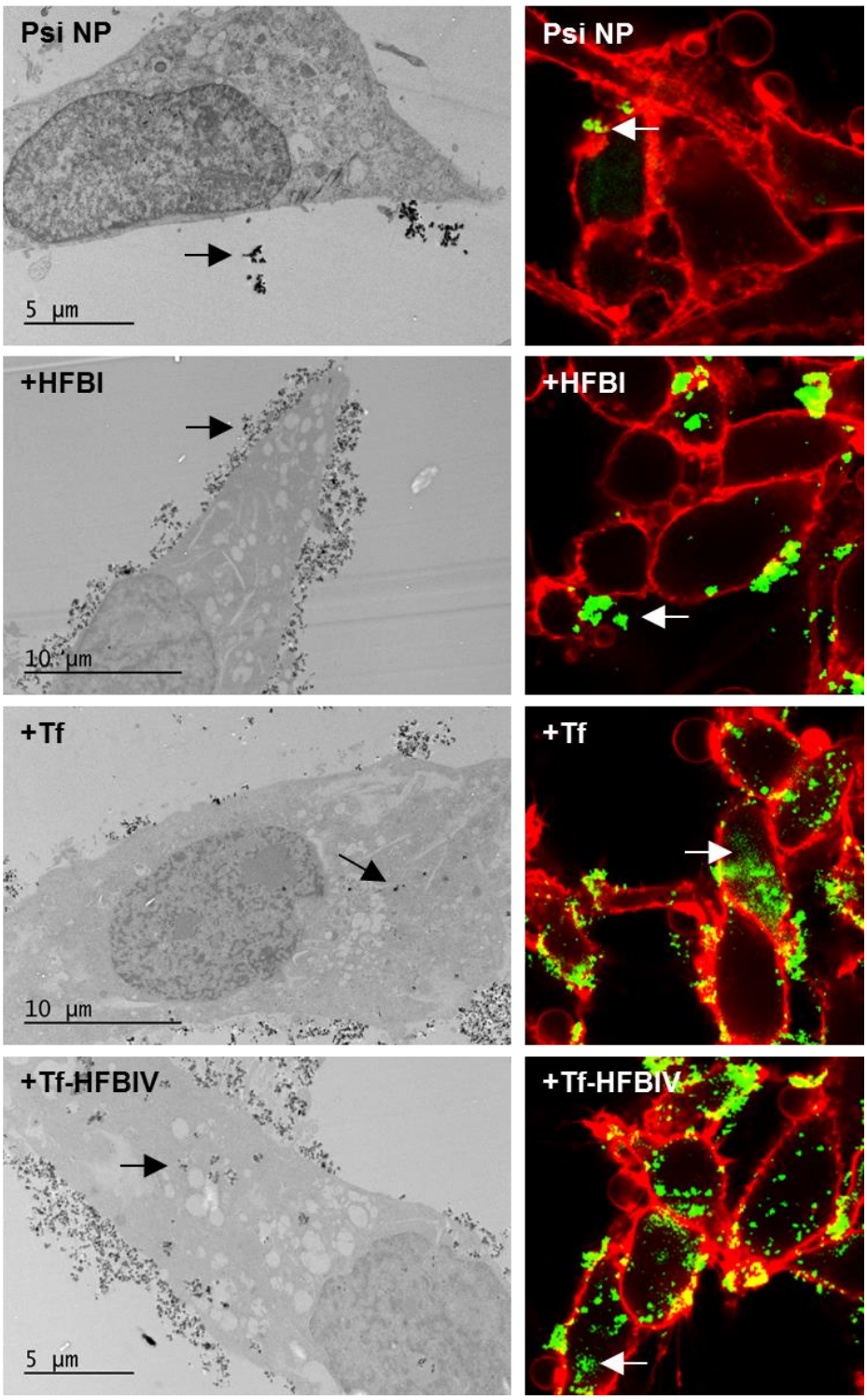


Figure 5. Interaction of the coated PSi nanoparticles with human MDA-MB-231 breast cancer cells. Left panel shows representative TEM images and the right panel are confocal fluorescence microscope images of non-coated and coated PSi nanoparticles with the cancer cells. Cell membranes were stained with RED Cell Mask® and the nanoparticles with FITC-green. Arrows indicate the respective PSi nanoparticles.

CONCLUSIONS

This work provides a proof-of-principle on the suitability of a plant produced Tf-HFB fusion protein for functional coating of nanoparticles. The results here indicate that fusing Tf to a HFB did not abolish its ability to interact with the transferrin receptor. However, further research is required to evaluate whether HFB-mediated adhesion to nanoparticle surface allows better interaction with the receptor than direct chemical conjugation of Tf [23]. Nevertheless, it could be hypothesized that immobilization via HFB and a flexible linker would allow presentation of Tf in a favorable position and would avoid conformational changes associated with chemical conjugation. The bio-stability, drug release, distribution and capability of the fusion protein to target nanoparticles in cells still needs to be tested *in vivo* and compared to other methods of Tf conjugation.

SUPPORTING INFORMATION

SI1 Methods for preparation of alkyne THCPSi nanoparticles, cell lines and culture conditions and cell viability studies (file type, PDF)

SI2 Sequence data for expression constructs (file type, PDF)

ACKNOWLEDGMENTS

Rima Menassa and Reza Saberianfar are thanked for valuable discussions and commenting the manuscript and Tuuli Teikari and Jaana Rikkinen for excellent technical support. We acknowledge financial support from the Academy of Finland (decision nos. 252215, 252442 and 281300), the University of Helsinki Research Funds, the Biocentrum Helsinki, and the European Research Council under the European Union's Seventh Framework Program (FP/2007–2013, Grant No. 310892). The Electron Microscopy Unit of the Institute of Biotechnology, University of Helsinki, is thanked for providing the laboratory facilities and assistance.

REFERENCES

(1) Tortorella, S.; Karagiannis, T. C. Transferrin Receptor-Mediated Endocytosis: A Useful Target for Cancer Therapy. *J. Membr. Biol.* **2014**, *247* (4), 291–307.

(2) Almeida, P. V.; Shahbazi, M.-A.; Mäkilä, E.; Kaasalainen, M.; Salonen, J.; Hirvonen, J.; Santos, H. A. Amine-Modified Hyaluronic Acid-Functionalized Porous Silicon Nanoparticles for Targeting Breast Cancer Tumors. *Nanoscale* **2014**, *6* (17), 10377.

(3) Shrestha, N.; Shahbazi, M.-A.; Araújo, F.; Mäkilä, E.; Raula, J.; Kauppinen, E. I.; Salonen, J.; Sarmento, B.; Hirvonen, J.; Santos, H. A. Multistage pH-Responsive Mucoadhesive Nanocarriers Prepared by Aerosol Flow Reactor Technology: A Controlled Dual Protein-Drug Delivery System. *Biomaterials* **2015**, *68*, 9–20.

(4) Shahbazi, M.-A.; Shrestha, N.; Mäkilä, E.; Araújo, F.; Correia, A.; Ramos, T.; Sarmento, B.; Salonen, J.; Hirvonen, J.; Santos, H. A. A Prospective Cancer Chemo-Immunotherapy

Approach Mediated by Synergistic CD326 Targeted Porous Silicon Nanovectors. *Nano Res.* **2015**, *8* (5), 1505–1521.

(5) Hakanpää, J.; Szilvay, G. R.; Kaljunen, H.; Maksimainen, M.; Linder, M.; Rouvinen, J. Two Crystal Structures of Trichoderma Reesei Hydrophobin HFBI--the Structure of a Protein Amphiphile with and without Detergent Interaction. *Protein Sci.* **2006**, *15* (9), 2129–2140.

(6) Linder, M. B.; Szilvay, G. R.; Nakari-Setälä, T.; Penttilä, M. E. Hydrophobins: The Protein-Amphiphiles of Filamentous Fungi. *FEMS Microbiol. Rev.* **2005**, *29* (5), 877–896.

(7) Hakanpää, J.; Paananen, A.; Askolin, S.; Nakari-Setälä, T.; Parkkinen, T.; Penttilä, M.; Linder, M. B.; Rouvinen, J. Atomic Resolution Structure of the HFBII Hydrophobin, a Self-Assembling Amphiphile. *J. Biol. Chem.* **2004**, *279* (1), 534–539.

(8) Szilvay, G. R.; Paananen, A.; Laurikainen, K.; Vuorimaa, E.; Lemmetyinen, H.; Peltonen, J.; Linder, M. B. Self-Assembled Hydrophobin Protein Films at the Air-Water Interface: Structural Analysis and Molecular Engineering. *Biochemistry* **2007**, *46* (9), 2345–2354.

(9) Reuter, L.; Ritala, A.; Linder, M.; Joensuu, J. Novel Hydrophobin Fusion Tags for Plant-Produced Fusion Proteins. *PLoS One* **2016**, *11* (10), e0164032.

(10) Jacquet, N.; Navarre, C.; Desmecht, D.; Boutry, M. Hydrophobin Fusion of an Influenza Virus Hemagglutinin Allows High Transient Expression in Nicotiana Benthamiana, Easy Purification and Immune Response with Neutralizing Activity. *PLoS One* **2014**, *9* (12), e115944.

(11) Joensuu, J. J.; Conley, A. J.; Lienemann, M.; Brandle, J. E.; Linder, M. B.; Menassa, R. Hydrophobin Fusions for High-Level Transient Protein Expression and Purification in Nicotiana Benthamiana. *Plant Physiol.* **2010**, *152* (2), 622–633.

(12) Phan, H. T.; Hause, B.; Hause, G.; Arcalis, E.; Stoger, E.; Maresch, D.; Altmann, F.; Joensuu, J.; Conrad, U. Influence of Elastin-like Polypeptide and Hydrophobin on Recombinant Hemagglutinin Accumulations in Transgenic Tobacco Plants. *PLoS One* **2014**, *9* (6), e99347.

(13) Saberianfar, R.; Joensuu, J. J.; Conley, A. J.; Menassa, R. Protein Body Formation in Leaves of Nicotiana Benthamiana: A Concentration-Dependent Mechanism Influenced by the Presence of Fusion Tags. *Plant Biotechnol. J.* **2015**, *13* (7), 927–937.

(14) Pereira, E. O.; Kolotilin, I.; Conley, A. J.; Menassa, R. Production and Characterization of in Planta Transiently Produced Polygalacturanase from Aspergillus Niger and Its Fusions with Hydrophobin or ELP Tags. *BMC Biotechnol.* **2014**, *14* (1), 59.

(15) Reuter, L. J.; Bailey, M. J.; Joensuu, J. J.; Ritala, A. Scale-up of Hydrophobin-Assisted

Recombinant Protein Production in Tobacco BY-2 Suspension Cells. *Plant Biotechnol. J.* **2014**, *12* (4), 402–410.

(16) Bortesi, L.; Rademacher, T.; Schiermeyer, A.; Schuster, F.; Pezzotti, M.; Schillberg, S. Development of an Optimized Tetracycline-Inducible Expression System to Increase the Accumulation of Interleukin-10 in Tobacco BY-2 Suspension Cells. *BMC Biotechnol.* **2012**, *12* (1), 40.

(17) Kirchhoff, J.; Raven, N.; Boes, A.; Roberts, J. L.; Russell, S.; Treffenfeldt, W.; Fischer, R.; Schinkel, H.; Schiermeyer, A.; Schillberg, S. Monoclonal Tobacco Cell Lines with Enhanced Recombinant Protein Yields Can Be Generated from Heterogeneous Cell Suspension Cultures by Flow Sorting. *Plant Biotechnol. J.* **2012**, *10* (8), 936–944.

(18) Hellwig, S.; Drossard, J.; Twyman, R. M.; Fischer, R. Plant Cell Cultures for the Production of Recombinant Proteins. *Nat. Biotechnol.* **2004**, *22* (11), 1415–1422.

(19) Santos, R. B.; Abranches, R.; Fischer, R.; Sack, M.; Holland, T. Putting the Spotlight Back on Plant Suspension Cultures. *Front. Plant Sci.* **2016**, *7*.

(20) Espino-Rammer, L.; Ribitsch, D.; Przylucka, A.; Marold, A.; Greimel, K. J.; Herrero Acero, E.; Guebitz, G. M.; Kubicek, C. P.; Druzhinina, I. S. Two Novel Class II Hydrophobins from Trichoderma Spp. Stimulate Enzymatic Hydrolysis of Poly(ethylene Terephthalate) When Expressed as Fusion Proteins. *Appl. Environ. Microbiol.* **2013**, *79* (14), 4230–4238.

(21) Daniels, T. R.; Delgado, T.; Rodriguez, J. A.; Helguera, G.; Penichet, M. L. The Transferrin Receptor Part I: Biology and Targeting with Cytotoxic Antibodies for the Treatment of Cancer. *Clin. Immunol.* **2006**, *121* (2), 144–158.

(22) Bimbo, L. M.; Mäkilä, E.; Raula, J.; Laaksonen, T.; Laaksonen, P.; Strommer, K.; Kauppinen, E. I.; Salonen, J.; Linder, M. B.; Hirvonen, J.; Santos, H. A. Functional Hydrophobin-Coating of Thermally Hydrocarbonized Porous Silicon Microparticles. *Biomaterials* **2011**, *32* (34), 9089–9099.

(23) Pei-Hui Yang, †,§; Xuesong Sun, †; Jen-Fu Chiu, ‡; Hongzhe Sun, *,† and; Qing-Yu He*, †. Transferrin-Mediated Gold Nanoparticle Cellular Uptake. **2005**.

(24) Kratz, F.; Beyer, U.; Roth, T.; Tarasova, N.; Collery, P.; Lechenault, F.; Cazabat, A.; Schumacher, P.; Unger, C.; Falken, U. Transferrin Conjugates of Doxorubicin: Synthesis, Characterization, Cellular Uptake, and in Vitro Efficacy‖. *J. Pharm. Sci.* **1998**, *87* (3), 338–346.

Series title and number

VTT Science 144

Title	**Plant cell factories** Production of hydrophobin fusion proteins in plant cell cultures
Author(s)	Lauri Reuter
Abstract	Recombinant proteins are used e.g. as pharmaceuticals, enzymes and components of nanotechnology. The exceptional characteristics of fungal hydrophobins make them interesting for many of those uses. They also transfer their surface active properties to fusion proteins enabling completely new applications. In general, plants are a potential platform for manufacturing recombinant proteins even in agricultural scale. This work explores production of hydrophobin fusion proteins in a plant cell factory: the tobacco bright yellow 2 suspension cells (BY-2). The hydrophobin fusion technology has been mainly based on a single hydrophobin molecule the Trichoderma reesei HFBI. This work expanded the toolkit with several new molecules. When expressed in plants, the hydrophobins fused to fluorescent marker (GFP) induced formation of protein bodies. In addition to HFBI, only HFBII and HFBIV could selectively separate fusion proteins in surfactant based two phase separation. HFBII-fusion improved accumulation of GFP and Protein A in comparison to both HFBI-fused and non-fused proteins. However, HFBI-, HFBII- and HFBIV-fusions all slightly reduced the yield of transferrin. Both HFBI-Protein A and transferrin-HFBIV were produced in BY-2 suspension cells with good yields. Furthermore, continuous selection resulted also in a cell line yielding 1.1 g/l GFP-HFBI. This is the first report on a plant cell culture reaching gram per litre yields of a recombinant protein. In pilot scale experiments the BY-2 suspension cells were grown in 600 litre culture volume in classical stirred tank bioreactors and the aqueous two phase separation from plant cell extract was successfully scaled to 20 l volume. The fusion proteins retained functional properties from both fusion partners. The HFBI-Protein A enabled harvesting of antibodies in solution using aqueous two phase separation. The HFBIV fused transferring retained its capability to bind iron and interact with the transferrin receptor. Coating with transferrin-HFBIV resulted in uptake of the silicon nanoparticles in human cancer cells. This work builds foundation for utilization of BY-2 suspension cells in industrial manufacturing of recombinant proteins and on the other hand opens interesting new applications for bi-functional hydrophobin fusion proteins.
ISBN, ISSN, URN	ISBN 978-951-38-8482-6 (Soft back ed.) ISBN 978-951-38-8481-9 (URL: http://www.vttresearch.com/impact/publications) ISSN-L 2242-119X ISSN 2242-119X (Print) ISSN 2242-1203 (Online) http://urn.fi/URN:ISBN:978-951-38-8481-9
Date	December 2016
Language	English, Finnish abstract
Pages	82 p. + app. 74 p.
Name of the project	
Commissioned by	
Keywords	Plant cell culture, hydrophobin, tobacco, BY-2, fusion protein
Publisher	VTT Technical Research Centre of Finland Ltd P.O. Box 1000, FI-02044 VTT, Finland, Tel. 020 722 111

Julkaisun sarja ja numero

VTT Science 144

Nimeke	**Kasvisolutehtaita** Hydrofobiinifuusioproteiinien tuottaminen kasvisoluviljelmissä
Tekijä(t)	Lauri Reuter
Tiivistelmä	Rekombinanttiproteiineja käytetään mm. lääkkeinä, entsyymeinä ja nanomateriaalien komponentteina. Sieniperäisten hydrofobiinien poikkeukselliset ominaisuudet tekevät niistä kiinnostavia moniin näistä käyttökohteista. Esimerkiksi pinta-aktiiviset ominaisuudet siirtyvät myös fuusioproteiineille, mikä mahdollistaa uudenlaisia sovelluksia. Kasvit ovat lupaava tapa tuottaa rekombinanttiproteiineja jopa maatalouden suuressa mittakaavassa. Tässä tutkimuksessa selvitettiin hydrofobiinifuusioproteiinien tuottamista kasvisolutehtaissa – tupakan BY-2-soluviljelmissä. Hydrofobiinifuusioteknologia on perustunut pääasiassa Trichoderma reesei -homeen HFBI-molekyyliin. Tämä tutkimus laajentaa käyttömahdollisuuksia useilla molekyyleillä. Kasvisoluissa hydrofobiinit muodostivat fluoresoivaan malliproteiiniin (GFP) liitettyinä pyöreitä proteiinijyväsiä. HFBI:n lisäksi vain HFBII ja HFBIV mahdollistivat fuusioproteiinien puhdistamisen kaksifaasiuutolla. HFBII-fuusio paransi sekä GFP:n että proteiini A:n tuottotasoja verrattuna HFBI-fuusiopartneriin tai fuusioimattomiin proteiineihin. Kuitenkin sekä HFBI-, HFBII- että HFBIV-fuusiot huononsivat transferriinin saantoa. Sekä HFBI-Proteiini A:ta että transferriini-HFBIV-fuusioproteiineja tuotettiin BY-2-soluviljelmässä hyvin saannoin. Huolellisella valinnalla kehitettiin lisäksi solulinja, joka tuotti 1,1 g/l GFP-HFBI-proteiinia. Tämä on ensimmäinen kerta, kun kasvisoluissa on saavutettu grammaluokan rekombinanttiproteiinisaantoja. Tuotantopiloteissa BY-2-soluja kasvatettiin fermentorissa 600 litran tilavuudessa ja kaksifaasiuutto kasvisolumateriaalista tehtiin 20 litran tilavuudessa. Fuusioproteiinit säilyttivät toiminnallisia ominaisuuksia molemmilta partnereilta. HFBI-Proteiini A mahdollisti vasta-aineiden puhdistamisen liuoksista kaksifaasiuutolla. HFBIV:hen liitetty transferriini taas säilytti kykynsä sitoa rautaa ja sitoutua reseptoriinsa. Nanopartikkelien pinnoittaminen transferriini-HFBIV-proteiinilla mahdollisti partikkelien kuljettamisen syöpäsoluihin. Tämä tutkimus luo pohjaa BY-2-solujen käytölle teollisessa rekombinanttiproteiinien tuotannossa ja toisaalta avaa mielenkiintoisia sovelluksia hydrofobiinifuusioproteiinien käytölle.
ISBN, ISSN, URN	ISBN 978-951-38-8482-6 (nid.) ISBN 978-951-38-8481-9 (URL: http://www.vtt.fi/julkaisut) ISSN-L 2242-119X ISSN 2242-119X (Painettu) ISSN 2242-1203 (Verkkojulkaisu) http://urn.fi/URN:ISBN:978-951-38-8481-9
Julkaisuaika	Joulukuu 2016
Kieli	Englanti, suomenkielinen tiivistelmä
Sivumäärä	82 s. + liitt. 74 s.
Projektin nimi	
Rahoittajat	
Avainsanat	Kasvisoluviljelmä, hydrofobiini, tupakka, BY-2, fuusioproteiini
Julkaisija	Teknologian tutkimuskeskus VTT Oy PL 1000, 02044 VTT, puh. 020 722 111

VTT SCIENCE 144

Plant cell factories

Plant cell factories

Production of hydrophobin fusion proteins in plant cell cultures

The exceptional surface active properties of fungal hydrophobins have inspired numerous applications in biotechnology ranging from coatings of medical instruments and nanoparticles to formulation of food products. Plant cells, cultivated in contained and controlled environment of bioreactors, represent an alternative production platform for complex recombinant proteins needed in medicine, biotechnological processes and nanotechnology.

This dissertation examined the manufacturing of hydrophobin fusion proteins in tobacco Bright Yellow 2 suspension cells in up to 600 litre scale. This work also introduces eight novel hydrophobin fusion tags for expression of fusion proteins in plants. The hydrophobins were further used to engineer bi-functional fusion proteins with Protein A for in-solution harvesting of antibodies and with human transferrin for active targeting of nanocarriers to cancer cells.

ISBN 978-951-38-8482-6 (Soft back ed.)
ISBN 978-951-38-8481-9 (URL: http://www.vttresearch.com/impact/publications)
ISSN-L 2242-119X
ISSN 2242-119X (Print)
ISSN 2242-1203 (Online)
http://urn.fi/URN:ISBN:978-951-38-8481-9