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Engineering of Synthetic Mammalian Gene Networks and their Cross-Kingdom Conservation

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Summary

The engineering and the refinement of novel trigger-induced gene expression systems are an ongoing quest for biological research fields including gene therapy, drug discovery, functional genomics and proteomic analysis, and biopharmaceutical manufacturing. The cross-kingdom transferability, the design and the application of precisely tailored systems enabling advanced biopharmaceutical manufacturing and prototype gene therapy scenarios are the topic of this work.

The moss *Physcomitrella patens* could serve in the future as a valid organism for high yield and high quality biopharmaceutical manufacturing. We were able to show the functional cross-kingdom conservation of mammalian and *P. patens* transcription, translation and secretion machineries, by transferring several genetic building blocks without extra modifications directly to the moss. Employing these molecular building blocks, we were able to establish constitutive, conditional and autoregulated expression of different product genes within the moss. Furthermore, we were able to run a prototype biopharmaceutical production using microencapsulated protoplasts in a Wave Bioreactor, rather than protonema cultures in a glass stirred-tank bioreactor.

Beside the cross-kingdom transferability of a synthetic biology toolbox to moss, we have pioneered the first gene regulation system that can be induced in a completely non-invasive manner, via the skin. Taking advantage of the *Pseudomonas putida* DOT-TE1 TtgABC efflux pump response regulator TtgR, we have engineered a synthetic mammalian phloretin responsive control element (PEACE), which was responsive to the apple metabolite phloretin. PEACE showed excellent regulation performance in a multitude of mammalian cells and phloretin, when formulated within a skin lotion enabled tight regulation of product gene expression via the skin of mice.

Employing the *Caulobacter crescentus* derived VanR response regulator, we engineered a second synthetic mammalian gene regulation system with excellent regulatory behavior, showing high maximal expression and tight repression of product gene expression. This system is responsive to the licensed food additive vanillic acid, thus enabling physiologically inert adjustability of gene expression.
Zusammenfassung


Unter Verwendung des aus *Caulobacter crescentus* stammenden Repressors VanR, konnten wir ein zweites synthetisches Regulationssystem zur Verwendung in Säugetierzellen konstruieren. Auch dieses System zeigte sehr gute Regulations-
Introduction
General Introduction

The large amount of data generated by classical biological research has resulted in great advances of understanding the complexity of molecular interactions within living organisms. Current interdisciplinary research fields like bioinformatics, biophysics, systems biology and medicine are combining this knowledge striving towards unprecedented industrial applications, such as the affordable production of complex to produce biopharmaceuticals, more efficient drug discovery and next generation gene therapy approaches. Engineering of complex genomic circuits, which have the potential to achieve these goals, require a large toolbox of well-characterized genetic modules that can be assembled according to the desired output function. In recent years, precise transcription control of specific transgenes formed the functional basis for the design of such synthetic gene networks and heterologous transcription control circuits represent the fundamental biological parts for their construction, no matter what complexity. From this panoply of biological building blocks an impressive set of functional outputs, such as synthetic biologic gates, bistable expression networks, such as toggle- or hysteretic switches, transcriptional cascades, time-delay circuits or cellular oscillators, have been engineered recently and are expected to provide solutions for novel cell-based therapeutic strategies, drug discovery, biotechnology and environmental protection.

In this work we have shown (i) the cross-kingdom conservation of essential genetic tools required for efficient transgene regulation systems between mammalian cells and the moss *Physcomitrella patens* and we have constructed two novel synthetic circuits enabling (ii) transdermal control of transgene expression and (iii) transcription control via a physiological inert food additive.
The moss *Physcomitrella patens* and its role in modern biotechnology

The production of biopharmaceuticals is currently still dominated by mammalian production cell cultures, as most of the protein therapeutics on the market originate from mammalian cell bioprocesses (Wurm 2004). New solutions need to be evaluated though, as broader product pipelines and an increasing demand for biopharmaceuticals drive the manufacturing capacities to their limits. Hence, researchers are evaluating alternative host cell systems for efficient, cost-effective and high quality biopharmaceuticalal manufacturing (Decker and Reski 2007; Hamilton et al. 2006).

Plants play a pivotal role in closing this gap, as they combine advantages of microbial- and mammalian- production systems. They eliminate the risk of product contamination by human pathogens possibly hidden within mammalian cell lines or in their complex organic production media and yet, as higher eukaryotes, they have the ability to synthesize complex multimeric proteins with post-translational modifications closely resembling the ones from mammalian cells (Decker and Reski 2008).

In particular, the moss *Physcomitrella patens*, brings along a panoply of interesting traits that put this small land plant in a leading position to become an alternative high through-put production organism in biotechnology. Other than in seed plants, in mosses the dominating generation is not formed by the diploid sporophyte, but rather by the haploid gametophyte (Reski and Frank 2005) (Fig. 1 a and b). As there are no dominant / recessive traits in haploids, there is no back-crossings needed to obtain isogenic lines and even more important is the high rate of homologous recombination within *P. patens* (Schaefer 2001), as this feature allows for fast forward reverse genetics approaches and for targeted integration of any gene of interest for production purposes. (Decker and Reski 2007; Reski and Frank 2005). Most importantly, the moss can be cultivated *in vitro* under fully controlled conditions as intact plant with differentiated cells in the form of so called protonema (Decker and Reski 2007). In small scale, moss plants are grown in agitated glass flasks, while a scale up is achieved in either 15 L highly controllable photo-bioreactors or in up to
100 L tubular moss bioreactors (Decker and Reski 2007; Lucumi et al. 2005) (Fig. 1 c and d).

**Fig. 1:** *In vitro* cultivation of a moss, *Physcomitrella patens* for production of biopharmaceuticals. a) Life cycle of mosses with haploid (1n) and diploid (2n) stages. R!: meiosis b) Storage of transgenic moss lines on multi-well agar plates. c and d) *In vitro* propagation of moss protonema in stirred glass-tank and tubular photobioreactors, respectively. Photographs courtesy of Andreas Schaaf (b) and Clemens Posten (d). (Decker and Reski 2007).

In recent years, the increasing genetic toolbox for mammalian cells has considerably improved the production capacity of mammalian-cell biopharmaceutical production (Wurm 2004). Consequently, it would significantly ease the introduction of a novel production organism if these genetic tools were transferable without labor intensive adjusting of codon usage, re-cloning of the genes of interest into adapted high expression vectors and re-designing molecular tools, such as *Internal Ribosome Entry Sites* (IRES) and trigger induced gene regulation modalities. We have therefore shown in chapter I of this work the cross compatibility of the transcription, translation and secretion machineries of mammalian cells and the non-seed plant *P. patens*.
**Introduction**

**Synthetic Biology**

Biochemistry and molecular biology focus mainly on investigating individual genes or proteins as a basis to move on to single individual pathways, one at the time. Genomic and proteomic research continue to unveil the inventory of life. However, these more classical disciplines remain largely reductive sciences that deduce the operation of living systems by mutating them or breaking them apart with the limitation that the whole cell remains beyond the reach of the methodologies and cannot be studied as a whole. Nonetheless, the breakthroughs from these disciplines enabled researchers to start studying whole cells and larger parts within an organism as interconnected systems. Describing larger structures and the processes within cells was the starting point for systems biology (Ideker et al. 2001). To fully understand the general principles that govern the structures and their interconnected networks, it is necessary though, to start with synthetic biology, which start connecting the experimental and the theoretical work in order to reconstruct biological systems by assembly of minimal functional building blocks, rather than a stepwise devolution of the organism (Hartwell et al. 1999; Hasty et al. 2001). Synthetic biology is a highly interdisciplinary field that unites engineers, biologists, mathematicians and chemists in order to design and build novel biomolecular components, networks and pathways to rewire and reprogram organisms (Khalil and Collins 2010). The three major goals within this ambitious field are defined as (Gibbs 2004):

- To learn about life by building it (or at least some modular parts), rather than breaking it apart. Accomplishments range from simple building blocks such as toggle switches (Gardner et al. 2000; Kramer et al. 2004b), to synthetic oscillators (Atkinson et al. 2003; Elowitz and Leibler 2000; Tigges et al. 2009; Tigges et al. 2010) up to the creation of synthetic viral genomes (Cello et al. 2002; Smith et al. 2003) and recently even to the first synthetic bacterial genome (Gibson et al. 2010).

- Advance genetic engineering to a real engineering discipline that builds systems from well-defined standardized modules, in order to improve previous creations and to recombine such modular building blocks to higher order systems (Brent 2004).
To re-write the genetic program of a cell to obtain novel functions or behavior and eventually yield truly controllable organisms. This involves the linking of fully synthetic gene networks to the host organism (Kemmer et al. 2010; Kobayashi et al. 2004; Kramer et al. 2005), the engineering of cell-to-cell communication (Chen and Weiss 2005; Weber et al. 2007b; You et al. 2004) and eventually the creation of life (Gibson et al. 2010).

As the applications of synthetic networks and even whole organisms slowly rise from prototype devices towards real applications within human gene therapy, the production of biofuels, pharmaceuticals and novel biomaterials, the requirements for the underlying modular building parts constantly raise in terms of environmental safety, long term applicability, versatility and to be physiologically inert towards interacting host organisms.

In the next section we will give an introduction to the general principle of the underlying regulatory building blocks, which are essential for synthetic biology applications.

**Trigger-Inducible Gene Regulation Systems**

Fine-tuning of gene expression via synthetic transgene regulation systems is of paramount importance for a variety of research areas and applications, including functional genomics (Baumgartel et al. 2008), biopharmaceutical manufacturing of complex to produce proteins (Ulmer et al. 2006; Weber and Fussenegger 2007), drug discovery (Sharpless and Depinho 2006; Weber et al. 2008), tissue engineering (Greber and Fussenegger 2007; Sanchez-Bustamante et al. 2006; Weber and Fussenegger 2006), the design of complex synthetic networks (Deans et al. 2007; Kramer and Fussenegger 2005; Tigges et al. 2010; Tigges et al. 2009), gene therapy (Gersbach et al. 2006; Kemmer et al. 2010; Weber and Fussenegger 2006) and the design of functional materials (Ehrbar et al. 2008).

The vast amount of applications that directly rely on the precise expression-control of a gene of interest, reaches from basic research to applied fields such as synthetic biology. This broad applicability accounts for the high demand of ever-improving synthetic transgene control systems, which enable intricate regulation of
either a single gene or complex synthetic networks. Genetic circuits enabling such precise expression management upon specific external or intrinsic stimuli have first been described within prokaryotes, like upon the response to nutrients (Jacob and Monod 1961), temperature (Hurme et al. 1997; Servant and Mazodier 2001), toxic agents (Folcher et al. 2001; Schumacher et al. 2002) or for communication purposes (Prithiviraj et al. 2003; Takano et al. 2001).

In this work we will focus on synthetically engineered circuits, which enable heterologous expression control within eukaryotic cells that are based on similar

![Molecular configuration of OFF-type (A) and ON-type (B) mammalian gene regulation systems.](image)

**Fig. 2:** Molecular configuration of OFF-type (A) and ON-type (B) mammalian gene regulation systems. (A) OFF-type systems. A bacterial DNA-binding response regulator (DBR), fused to a mammalian transactivation domain (TA) binds to a specific operator module and induces polymerase (Poly)-mediated transcription of the gene of interest (goi) from a minimal promoter (P_min). DPR-TA only binds to its cognitive operator in the absence of the regulating trigger molecule (white diamond). (B) ON-type systems. The DPR, fused to a mammalian transcription repressor domain (TR), binds to the specific operator sequence located downstream of a constitutive promoter, and thus represses its transcription. Upon addition of the regulating trigger molecule (represented by the diamond), repression is abolished and the gene of interest (goi) is expressed.
design principles as their prokaryotic predecessors. The majority of currently available eukaryotic transgene control systems benefit from a generic design principle comprising a prokaryotic DNA-binding response regulator (DBR) fused to a mammalian transactivation (TA) (transrepression/TR) domain, thus forming a synthetic transactivator (transrepressor) with the ability to activate (repress) a hybrid promoter engineered from a specific operator in close proximity to an eukaryotic minimal (constitutive) promoter (Urlinger et al. 2000; Weber et al. 2002a; Weber et al. 2002b). Employing these modular building blocks offers the possibility of creating two output functions depending on the ability of an inducer molecule to either (i) activate (ON-type system) (Fussenegger et al. 2000; Gossen et al. 1995; Neddermann et al. 2003; Weber et al. 2004) or repress (OFF-type system) (Fussenegger et al. 2000; Gossen and Bujard 1992) gene expression upon binding to the transactivator (transrepressor) protein (Fig 2).

The first generation of mammalian gene regulation systems resulted in an array of various inducer molecules enabling transcription control, ranging from clinically approved small-molecule drugs, such as antibiotics (Fussenegger et al. 2000; Gossen et al. 1995; Weber et al. 2002a), immunosuppressives or antidiabetic drugs (Pollock and Clackson 2002; Rollins et al. 2000), steroid hormones and hormone analogs (Nordstrom 2002; Palli et al. 2005), to quorum sensing molecules (Neddermann et al. 2003; Weber et al. 2006). All of these established gene regulation systems fulfill the basic requirements for transcriptional control with a low level of leakage and high maximum transgene expression levels. However, due to several drawbacks, such as (i) limited pharmacokinetic and pharmacodynamic properties (ii) the risk of pleiotropic side effects and (iii) the risk to develop new bacterial antibiotic-resistant strains (Alanis 2005; Lautermann et al. 2004; Sanchez et al. 2004), the broad adaptability of these trigger molecules to more sophisticated synthetic biology applications is constricted. Gene therapy approaches rely on traceless inducer molecules with no side effects (Ehrbar et al. 2008; Kemmer et al. 2010), complex synthetic networks need interference-free regulation within the host organism (Kramer et al. 2004a; Tigges et al. 2010; Tigges et al. 2009) and drug screening approaches need very specific response patterns (Weber et al. 2008). Hence, researchers are striving to create the
second generation of synthetic molecular building blocks, which are responsive to inducer molecules that are directly derived from endogenous metabolites, such as amino acids (Hartenbach et al. 2007), vitamins (Weber et al. 2007a; Weber et al. 2009), gaseous acetaldehyde (Weber et al. 2004), pathological signals, such as urate (Kemmer et al. 2010), or which are already licensed as food additives (Weber et al. 2008). Employing these trigger molecules in the most recent synthetic biology networks resulted in unprecedented advances in the field and could result, in the not-too-distant future, in the first human gene therapy trials capitalizing on synthetic biology (Aubel and Fussenegger 2010; Khalil and Collins 2010; Tigges and Fussenegger 2009; Weber and Fussenegger 2009). In this work, we showed in Chapters II and III two important contributions towards the second generation of trigger inducible-synthetic gene circuits.
Contributions of this work

Chapter I: Functional cross-kingdom conservation of mammalian and moss (Physcomitrella patens) transcription, translation and secretion machineries

Biopharmaceutical manufacturing of complex to produce proteins plays an ever-increasing role in modern healthcare. Transgenic mammalian cell cultures are currently the most successful production platform, though the global demand for biopharmaceuticals exceeds already the worldwide manufacturing capacity. Hence, alternative production organisms are being assessed for their potential to produce high quality mammalian proteins at high yields and at low costs. One of the most interesting plant organisms to fulfill these requirements is the moss Physcomitrella patens. This non-seed land plant unifies some unique features, like homologous recombination and glycosylation patterns similar to mammals, which put it in a favorite position to become a valid alternative to mammalian cell culture production. We have shown here that on top of these features, P. patens has the ability to translate, transcribe and secrete mammalian genetic modules and proteins without further modification. This significantly simplifies the transfer of well-established expression machineries from mammalian production to the moss. Furthermore, we have introduced native and synthetic promoters and polyadenylation sites, viral and cellular internal ribosome entry sites, secretion signal peptides and secreted product proteins, and synthetic transactivators and transrepressors, which were all designed for mammalian cells, to the moss. Using these molecular building blocks, we were able to establish constitutive, conditional and autoregulated expression of different product genes within P. patens, which resulted in a completely new genetic toolbox for researches in this field. As an alternative to the standard protonema stirred-tank bioreactors, we have also been able to show a prototype biopharmaceutical production scenario using microencapsulated transgenic P. patens protoplasts producing human vascular endothelial growth factor 121 (VEGF121) at similar rates as the standard
method. This provides yet another option for biopharmaceutical production within the moss *P. patens*.

**Chapter II: Controlling transgene expression in subcutaneous implants using a skin lotion containing the apple metabolite phloretin**

For future gene therapy applications to be accepted by patients and thus be broadly applicable, there are certain requirements to be fulfilled. On top of the clear medical indication and a therapeutic benefit, new technologies always need to have a good patient compliance and ideally traceless, side effect free mode of action. In this chapter, we designed a novel synthetic gene network for mammalian cells that was able to precisely regulate transgene expression via the skin of mice, by simply applying a cream containing the apple metabolite phloretin. This novel transgene regulation system did thus not only show excellent regulation performance, but also serves as a prototype gene therapy approach where implanted cells producing a therapeutic protein can be controlled in an absolutely non-invasive manner. On top of that the system shows interference free regulation, when applied in parallel with other gene regulation systems, broad applicability within a large set of mammalian cells and enables a timed production start within a prototype bioreactor setup. Taken these features together, the system also offers a valid alternative to existing gene regulation systems for the synthetic biology community.

**Chapter III: Transcription control in mammalian cells and mice via the food additive vanillic acid**

The requirements for second generation gene regulation systems are clearly set towards improving the inducer molecules, as the precise functionality, adjustability and reversibility of synthetically engineered transcription control elements was already covered by the first generation and is thus “a given”. However the inducer molecules employed in the first generation accounted for their drawback within highly sophisticated applications of synthetic biology. In brief, inducer molecules need to be physiological inert to ensure long term applicability and side effect free efficacy, alongside with interference free regulation of the regulation circuit. We present here,
vanillic acid as an inducer for a novel gene regulation system. Vanillic acid is licensed as a food additive by the U.S. Food and Drug Administration (FDA) and thus has a great chance to fulfill, at physiological concentrations all safety requirements for future gene therapy applications. The vanillic acid responsive system (VAC) was engineered by taking advantage of the *Caulobacter crescentus* VanR-response regulator, which showed very high specificity towards vanillic acid, as we tested a library of several chemical derivatives of vanillic acid within mammalian cell culture for their ability to interact with the VAC-system. None of the tested chemicals showed the ability to modulate the activity of VAC. This specificity puts the VAC-system to the forefront of synthetic building blocks, when engineering complex synthetic networks, where interference free regulation is the main requirement. Taken together with its broad applicability within different mammalian cell lines and the excellent functionality within a prototype *in vivo* scenario in mice, the VAC-system adds a precise gene regulation tool to the synthetic biology community with broad spectrum of implementations, such as gene therapy, complex synthetic networks and precise gene expression control.
References


Introduction


Introduction


Chapter I

Functional cross-kingdom conservation of mammalian and moss
(Physcomitrella patens) transcription, translation and secretion machineries

Marc Gitzinger, Juliana Parsons, Ralf Reski and Martin Fussenegger

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Summary

Plants and mammals are separated by a huge evolutionary distance. Consequently, biotechnology and genetics have traditionally been divided into “green” and “red”. Here, we provide comprehensive evidence that key components of the mammalian transcription, translation and secretion machineries are functional in the model plant Physcomitrella patens. Cross-kingdom compatibility of different expression modalities originally designed for mammalian cells such as (i) native as well as synthetic promoters and polyadenylation sites, (ii) viral and cellular internal ribosome entry sites, (iii) secretion signal peptides and secreted product proteins, and (iv) synthetic transactivators and transrepressors, was established. This mammalian expression portfolio enabled constitutive, conditional as well as autoregulated expression of different product genes in a multicistronic expression format optionally adjusted by various trigger molecules such as butyrolactones, macrolide antibiotics and ethanol. Capitalizing on a cross-kingdom-compatible expression platform we pioneered a prototype biopharmaceutical manufacturing scenario using microencapsulated transgenic P. patens protoplasts cultivated in a Wave Bioreactor. Vascular endothelial growth factor 121 (VEGF_{121}) titers matched those typically achieved by standard protonema populations grown in stirred-tank bioreactors. The full compatibility of mammalian expression systems in P. patens further promotes the use of moss as a cost-effective alternative for the manufacturing of complex biopharmaceuticals and as a valuable host system to advance synthetic biology in plants.

Introduction

The moss Physcomitrella patens, unique for its high rate of homologous recombination, generic codon usage, haploidy, simple body plan, physiologic properties and its exclusive phylogenetic position (Quatrano et al., 2007; Rensing et al., 2007), is gathering momentum for biopharmaceutical manufacturing of protein therapeutics due to favorable bioprocess and downstream processing economics (Decker and Reski, 2008). In-vitro cultivation of P. patens throughout its complete life cycle (Frank et al., 2005), transgenic protonema and transient protoplast cultures
(Baur et al., 2005a), stirred-tank and tubular photo-bioreactors (Decker and Reski, 2007), the generation of moss mutants devoid of immunogenic product protein glycosylation (Huether et al., 2005) and the production of human antibodies with improved ADCC activity (Nechansky et al., 2007) have been important milestones in establishing the moss as a promising biopharmaceutical manufacturing platform (Decker and Reski, 2007).

Transgenic mammalian cell cultures are currently the most successful platform for the production of biopharmaceuticals since most of the protein therapeutics on the market originate from mammalian cell bioprocesses (Wurm, 2004). However, since the global product pipeline exceeds the worldwide manufacturing capacity, alternative host cell systems for biopharmaceutical manufacturing are on the rise (Decker and Reski, 2007; Hamilton et al., 2006). Since the mid-1980s the productivity of mammalian cells cultivated in bioreactors has reached the gram per liter range, an over 100-fold yield improvement over titers achieved for the first commercial bioprocesses (Wurm, 2004). Part of this success is based on the development of sophisticated expression technologies and metabolic engineering strategies (Hartenbach and Fussenegger, 2005; Umana et al., 1999). The latest generation of expression vectors harbor (i) compact strong constitutive promoters for high-level transcription of product genes (Hartenbach and Fussenegger, 2006), (ii) multicistronic expression units enabling one-vector-based selection and expression of multiprotein complexes (Fux et al., 2004) and (iii) regulated expression systems for production of difficult-to-express protein therapeutics (Weber and Fussenegger, 2007).

In mammalian cells, initiation of translation is typically managed by a cap structure which is posttranscriptionally attached to the 5’ end of mRNAs (Kozak, 1989). Alternatively, internal ribosome entry sites (IRES), which adopt a specific secondary RNA structure triggering ribosome assembly and translational initiation, have evolved to ensure a minimal level of protein synthesis for survival during cap-compromising physiologic emergency situations (coordination of viral defense; cellular IRES) (Gan and Rhoads, 1996) or to redirect the cellular translation machinery to the production of virus proteins (viral IRES) (Dirks et al., 1993; Kaufman et al., 1991). Tandem arrangement of different transgenes, each preceded by
an IRES element, enables transcription of a multicistronic mRNA producing stoichiometric levels of various proteins. Recently, a sophisticated vector platform (pTRIDENT) has been designed for multicistronic expression of up to three different transgenes (Fux et al., 2004).

Heterologous mammalian transcription control modalities have been designed in two different configurations: ON-type systems, which are induced following addition of a trigger molecule and OFF-type systems, which are repressed after administration of the regulating compound (Weber and Fussenegger, 2007). ON-type systems typically consist of a transrepressor (optionally containing a silencing domain), which binds to specific (tandem) operator sequences and blocks transcription from upstream constitutive promoters until the transrepressor is released after interaction with the inducer (Weber et al., 2002; Weber et al., 2005). Transactivators, which only bind to their operator modules in the presence of the inducer are also classified as ON-type systems (Hartenbach and Fussenegger, 2005; Weber et al., 2004). OFF-type systems usually consist of a chimeric transactivator, which binds a specific (tandem) operator sequence and triggers transcription from an adjacent minimal promoter until it is released by interaction with the inducer (Weber et al., 2002; Weber et al., 2003). A variety of these transcription control systems have been used for basic and applied research (Weber and Fussenegger, 2007).

The protein production machineries of mammalian cells and plants are known to be largely incompatible which requires mammalian expression technology to be specifically modified for use in plant cells and plants (Frey et al., 2001; Mayfield et al., 2003). Availability of cross-kingdom-compatible protein expression technology would significantly improve the use of plant cells for biopharmaceutical manufacturing. We provide comprehensive evidence that transcription, translation and secretion machineries of mammalian cells and the non-seed plant *P. patens* are compatible, pioneer a novel protoplast-based fermentation technology for the production of human glycoproteins, and thus establish *P. patens* as a valuable host system for synthetic biology, in particular, to functionally understand the most conserved molecular devices controlling biological signaling in different kingdom.
Results

Profiling of mammalian promoter activities in *P. patens.* Swapping of expression units between mammalian and plant cell platforms for gene-function analysis has been hampered by incompatibilities in the transcription/translation/secretion machineries. These systems require exclusive genetic elements (promoters, reporter genes, polyadenylation sites) for expression of transgenes (Frey et al., 2001). In order to measure the activity of mammalian promoters in *P. patens* isogenic, all-mammalian expression vectors were designed harboring the human placental alkaline phosphatase (SEAP), an easy-to-assay reporter gene, a polyadenylation site derived from the simian virus 40, and various mammalian promoters (*P*~hCMV~, *P*~SV40~, *P*~GTX~, *P*~hEF1~) including the smallest synthetic promoter *P*~GTX~ (Hartenbach and Fussenegger, 2006). The polioviral internal ribosome entry site (IRES<sub>PV</sub>), known to be devoid of any promoter activity, was used as a negative control. Of the promoters tested, only *P*~hEF1~ was not functional in *P. patens.* Interestingly, the world’s smallest synthetic promoter (182bp) was fully functional reaching *P*~hCMV~-driven expression levels in the moss. The SEAP expression profiles reached using mammalian expression vectors were compared with those of an isogenic plant expression vector encoding SEAP under the control of the cauliflower mosaic virus 35S promoter (P<sub>CaMV35S</sub>) (Figure 1).

![Figure 1](image_url)

*Figure 1.* Expression performance of different constitutive mammalian promoters in *P. patens* compared to P<sub>CaMV35S</sub>. Isogenic SEAP expression vectors (*P*~hCMV~ [pSS173], *P*~SV40~ [pMG31], *P*~GTX~ [pSH17], *P*~hEF1~ [pMG32], -SEAP-pA<sub>SV40</sub>) were transfected into *P. patens* protoplasts and compared to a standard plant expression vector (pMG65, P<sub>CaMV35S</sub>-SEAP-
SEAP production was scored after five days. The polioviral internal ribosome entry site (IRES<sub>PV</sub>), which lacks promoter activity in mammalian cells, was used as control (pSH49, IRES<sub>PV</sub>-SEAP-pASV<sub>40</sub>). Error bars indicate the standard deviation of at least three independent experiments.

The secretory machineries of mammalian cells and <i>P. patens</i> are compatible. Previous studies on mammalian promoter compatibility in the moss revealed that the human placental secreted alkaline phosphatase (SEAP) could be secreted by <i>P. patens</i> protoplasts (Figure 1). In order to assess whether product genes containing mammalian secretion signals are generically secreted by <i>P. patens</i> several product genes including SEAP, the <i>Bacillus stearothermophilus</i>-derived secreted α-amylase (SAMY) containing an IgG-derived secretion signal, human vascular endothelial growth factor 121 (VEGF<sub>121</sub>) and human erythropoietin (EPO) were cloned into isogenic P<sub>GTX</sub>-driven mammalian and isogenic P<sub>CaMV35S</sub>-driven plant expression vectors. The product levels were profiled in the supernatant of transfected moss protoplast cultures, as well as in the cytosol, in order to assess the overall product secretion efficiency (Table 1). All of the mammalian product proteins were efficiently secreted by moss protoplasts, while the control protein AMY lacking the IgG secretion signal sequence (AMY) could only be detected in the plant cytosol (Table 1). In order to characterize the processing of secreted mammalian proteins in <i>P. patens</i>, we N-terminally sequenced human VEGF<sub>121</sub> purified from moss culture supernatants. The finding that the first 10 amino acids of secreted VEGF<sub>121</sub> were A(OH-Pro)MAEGGGQN suggests that secreted mammalian proteins are identically processed in <i>P. patens</i>
Table 1: Expression levels of mammalian reporter constructs in *Physcomitrella patens*

<table>
<thead>
<tr>
<th>Expression vector</th>
<th>Expression level (supernatant)</th>
<th>Expression level (intra-cellular)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_{GTX-SEAP-pA} (pSH17)</td>
<td>0.36 ± 0.02 µg/L</td>
<td>0.06 ± 0.007 µg/L</td>
</tr>
<tr>
<td>P_{CaMV35S-SEAP-pA} (pMG65)</td>
<td>1.02 ± 0.09 µg/L</td>
<td>0.19 ± 0.01 µg/L</td>
</tr>
<tr>
<td>P_{GTX-SAMY-pA} (pSH102)</td>
<td>9.0 ± 0.54 µmol/s/L</td>
<td>2.1 ± 0.02 µmol/s/L</td>
</tr>
<tr>
<td>P_{CaMV35S-SAMY-pA} (pMG66)</td>
<td>18.6 ± 2.3 µmol/s/L</td>
<td>4.1 ± 0.3 µmol/s/L</td>
</tr>
<tr>
<td>P_{GTX-AMY-pA} (pMG60)</td>
<td>1.65 ± 0.05 µmol/s/L</td>
<td>6.73 ± 0.34 µmol/s/L</td>
</tr>
<tr>
<td>P_{CaMV35S-AMY-pA} (pMG67)</td>
<td>2.5 ± 0.1 µmol/s/L</td>
<td>13.2 ± 1.23 µmol/s/L</td>
</tr>
<tr>
<td>P_{GTX-VEGF121-pA} (pSH100)</td>
<td>4.0 ± 0.1 ng/mL</td>
<td>0.42 ± 0.07 ng/mL</td>
</tr>
<tr>
<td>P_{CaMV35S-VEGF121-pA} (pMG68)</td>
<td>13.5 ± 0.5 ng/mL</td>
<td>1.1 ± 0.1 ng/mL</td>
</tr>
<tr>
<td>P_{GTX-EPO-pA} (pMG61)</td>
<td>48 ± 10.6 mU/mL</td>
<td>5.2 ± 0.9 mU/mL</td>
</tr>
<tr>
<td>P_{CaMV35S-EPO-pA} (pMG69)</td>
<td>159 ± 8.1 mU/mL</td>
<td>22 ± 0.95 mU/mL</td>
</tr>
</tbody>
</table>

Abbreviations: AMY, *Bacillus stearothermophilus*-derived α-amylase; EPO, human erythropoietin; P_{CaMV35S}, cauliflower mosaic virus promoter 35s; P_{GTX}, synthetic promoter derived from the GTX homeodomain protein; SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase; SEAP, human placental secreted alkaline phosphatase; VEGF_{121}, human vascular endothelial growth factor 121.

**Mammalian cap-independent translation initiation is functional in *P. patens***. Internal ribosome entry sites (IRES) are capable of managing cap-independent translation-initiation under physiological conditions which compromise classical cap-mediated translation (Pestova et al., 2001). Non-limiting examples of IRES-mediated translation include (i) virus infection during which the virus interferes with the cellular translation machinery and redirects it to translation of its IRES-tagged transcripts (viral IRES) (Dirks et al., 1993; Kaufman et al., 1991) and (ii) hijacked cells may coordinate a molecular defense by translating a set of IRES-containing transcripts (cellular IRES) (Gan and Rhoads, 1996). With the functionality of mammalian promoters and protein secretion established in *P. patens*, mammalian cell-
and virus-derived IRES’s were evaluated in *P. patens* to determine if they could trigger translation-initiation and enable multicistronic expression.

We have designed a variety of latest-generation pTRIDENT vectors, which contain (i) a constitutive PhCMV driving transcription of multicistronic mRNAs, (ii) an artificial polyadenylation site (apA) signaling the terminus of the multicistronic transcript (Hartenbach and Fussenegger, 2005), (iii) two tandem IRES elements of poliovirus (IRES<sub>PV</sub>) (Dirks et al., 1993) or encephalomyocarditis virus (IRES<sub>EMCV</sub>) (Kaufman et al., 1991) and IRES<sub>Rbm3</sub>, derived from the human RNA-binding motif protein 3 (Rbm3; Chappell and Mauro, 2003), (iv) vast multiple cloning sites (MCS) flanking each IRES element (many of which are targets for rare-cutting 8bp-recognizing restriction endonucleases) for complication-free sequential insertion of (v) product genes including SAMY, VEGF<sub>121</sub> and SEAP. (vi) PhCMV, SAMY-IRES-VEGF<sub>121</sub>-IRES-SEAP and apA are flanked by rare-cutting homing endonucleases (I-CeuI, I-SceI, I-PpoI, PI-PspI), which enable, together with the MCS, straightforward exchange/swapping of expression modules and transgenes among different members of the pTRIDENT vector family (Fux et al., 2004).

Following transfection of pTRIDENT45 (PhCMV-SAMY-IRES<sub>PV</sub>-VEGF<sub>121</sub>-IRES<sub>EMCV</sub>-SEAP-apA), pTRIDENT46 (PhCMV-SAMY-IRES<sub>PV</sub>-VEGF<sub>121</sub>-IRES<sub>Rbm3</sub>-SEAP-apA) and pTRIDENT47 (PhCMV-SAMY-IRES<sub>PV</sub>-VEGF<sub>121</sub>-IRES<sub>PV</sub>-SEAP-apA) into *P. patens* protoplasts significant levels of product protein were produced from all positions within the vectors indicating that mammalian cell/virus-derived IRES elements are functional and enable multicistronic transgene expression in the moss (Figure 2).
**Figure 2.** IRES-mediated translation-initiation in *P. patens.* (A) Schematic representation of tricistronic mammalian expression cassettes encoding a PhCMV-driven multicistronic expression unit harboring SAMY, VEGF_{121} and SEAP in cistrons 1, 2 and 3, respectively. SAMY is translated in a classic cap-dependent manner, VEGF_{121} requires cap-independent
translation-initiation by the polioviral internal ribosome entry site (IRES_{PV}) and translation of SEAP is mediated by either IRES_{PV}, the encephalomyocarditis virus IRES (IRES_{EMCV}) or the IRES element derived from the human RNA-binding motif protein 3 (IRES_{Rbm3}). (B) SAMY, (C) VEGF_{121} and (D) SEAP expression levels of moss protoplast cultures transfected with pTRIDENT45, pTRIDENT46 and pTRIDENT47. Reporter protein production was scored five days after transformation. Error bars indicate the standard deviation of at least three independent experiments.

**Tunable product gene expression in P. patens using mammalian transgene control technology.** Transcription control of specific genes by small trigger molecules is essential for gene-function analysis (Malleret et al., 2001), drug discovery (Weber et. al. submitted), design of complex artificial gene circuits (Kramer and Fussenegger, 2005), precise and timely molecular interventions in gene therapy (Gersbach et al., 2006), engineering of preferred cell phenotypes for tissue engineering (Niwa et al., 2000) and biopharmaceutical manufacturing (Fussenegger et al., 1998). While a variety of transgene control systems are available for fine-tuning transgene transcription in mammalian cells (Weber and Fussenegger, 2007), the choice for controlling transgene expression in plant cells, in particular in *P. patens*, is limited (Saidi et al., 2005). Recently, mammalian transcription control circuits were designed which are responsive to the butyrolactone 2-(1’-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide (SCB1) (QuoRex; Q-ON, Q-OFF, (Weber et al., 2005; Weber et al., 2003), the macrolide antibiotic erythromycin (E.REX; E_{ON}, E_{OFF}; (Weber et al., 2002)) and to acetaldehyde or ethanol (AIR; (Weber et al., 2007; Weber et al., 2004)). AIR-controlled transgenes are induced by acetaldehyde/ethanol whereas E.REX and QuoRex are available in two different design versions, which could either be induced (E_{ON}, Q-ON) or repressed (E_{OFF}, Q-OFF) by addition of the regulating molecule (Weber et al., 2002; Weber et al., 2005; Weber et al., 2003).

All mammalian transgene control systems were optimized for regulated SEAP expression and were transfected into *P. patens* which were grown in the presence and absence of different trigger molecules at various concentrations. SCB1 was well tolerated by the moss (toxic only above 20μg/ml, data not shown) and mediated adjustable, up to 15-fold induction (Q-ON; pWW504, P_{SV40-scBR-KRAB-pA}; pWW162, P_{SCAON8-SEAP-pA}) as well as repression (Q-OFF; pWW122, P_{SV40-scBR-}
VP16-pA; pWW124, P_{SPA}-SEAP-pA), of SEAP expression within a concentration range of 0-15μg/ml (Figure 3A and B). The Q-ON system is so sensitive in _P. patens_ that the moss senses the presence of co-cultivated SCB1-producing _S. coelicolor_, and participates in _S. coelicolor_'s quorum-sensing crosstalk by adjusting SEAP production in response to the size of the bacterial population (Figure 3C).

**Figure 3.** Quorum-sensing control of transgene expression in _P. patens_. The mammalian Q-ON (A) and Q-OFF (B) systems, which are induced and repressed, respectively, by the _S. coelicolor_ quorum-sensing butyrolactone SCB1, have been transfected into moss protoplasts and dose-response profiles of SEAP were recorded after five days.
Figure 3: (C) Co-cultivation of Q-ON-transgenic moss protoplasts with SCB1-producing *S. coelicolor* reveals quorum-sensing-based cross-species communication resulting in a correlation between plant-based SEAP production and *S. coelicolor* population size. (D and E) Macrolide-responsive transgene expression in moss protoplasts. SEAP expression profiles of moss protoplast cultures transfected with the mammalian E<sub>ON</sub> (D) and E<sub>OFF</sub> (E) systems.
and cultivated for five days in the presence of varying erythromycin concentrations. Error bars indicate the standard deviation of at least three independent experiments.

The $E_{ON}$ ($p$WW43, $P_{SV40}$-E-KRAB-pA; $p$WW56, $P_{ETRON8}$-SEAP-pA) and $E_{OFF}$ ($p$WW35, $P_{SV40}$-E-VP16-pA; $p$WW37, $P_{ETR2}$-SEAP-pA) systems were able to induce or repress SEAP expression up to 13-fold using erythromycin levels not exceeding $20\mu g/ml$ (toxic above $30\mu g/ml$) (Figure 3D and E). AIR-controlled SEAP expression attained a 20-fold SEAP expression (AIR; $p$WW195, $P_{SV40}$-$alcR$-pA; $p$WW192, $P_{AIR}$-SEAP-pA) in the moss when induced by $20\mu l/ml$ ethanol (Figure 4A). This compares favorably with the regulated performance of plant-specific ethanol-mediated transgene regulation in tobacco (Caddick et al., 1998), $Arabidopsis thaliana$ (Roslan et al., 2001), potato and oilseed rape (Sweetman et al., 2002). The AIR-control system is incredibly sensitive in $P. patens$ that SEAP production can be induced by $S. cerevisiae$ populations cultivated at a distance. As part of its metabolism, $S. cerevisiae$ converts ethanol into gaseous acetaldehyde which reaches moss cultures “over the air” and induces SEAP production in a distance-dependent manner (Figure 4B).

The combination of AIR-based transcription control with multicistronic expression technology ($p$TRIDENT42; $P_{AIR}$-SAMY-IRESPV-VEGF$_{121}$-IRESEMCV-SEAP-apA) enabled coordinated induction of three different transgenes after addition of $10\mu l/ml$ ethanol (Figure 4C-E).
Figure 4. Ethanol and gas-inducible transgene expression in P. patens. (A) Ethanol inducible SEAP expression in moss protoplasts transfected with the mammalian AIR system. (B) S. cerevisiae producing gaseous acetaldehyde as part of their native metabolism trigger SEAP expression “over-the-air” in distant P. patens cultures harboring the mammalian AIR system. (C-E) Ethanol-controlled tricistronic gene expression in moss protoplasts with SAMY encoded in the first (C), VEGF\textsubscript{121} in the second (D) and SEAP in the third cistron (E). Protein production was scored five days after transformation of moss protoplasts with pWW195 (P\textsubscript{SV40-alcR-pA}) and pSH3 (P\textsubscript{AIR-SAMY-IRESPV-VEGF\textsubscript{121}-IRESEMCV-SEAP-apA}) and cultivation in the presence or absence of ethanol. (F) Autoregulated transgene expression in P. patens. Moss protoplasts transfected with the ethanol-inducible autoregulated expression vector pSH28 (P\textsubscript{AIR-SEAP-IRESPV-alcR-apA}) were cultivated for five days in the
presence and absence of ethanol before SEAP expression levels were determined in the culture supernatant. Error bars indicate the standard deviation of at least three independent experiments.

**Autoregulated transgene expression in *P. patens***. Classic transgene control systems consist of two expression vectors, one harboring the transrepressor/transactivator and the other encoding the transgene driven by the trigger-inducible promoter (Weber and Fussenegger, 2007). Such two-vector design is more complex to engineer compared to latest-generation autoregulated one-vector configurations (Hartenbach and Fussenegger, 2005). Capitalizing on the functionality of IRES elements in *P. patens*, protoplasts were transfected with the ethanol-controlled autoregulated SEAP expression vector pAutoRex8 (P\textsubscript{AIR}-SEAP-IRES\textsubscript{PV}-alcR-apA; (Hartenbach and Fussenegger, 2005). pAutoRex8 contains a P\textsubscript{AIR}-driven dicistronic expression unit encoding SEAP in the first and the acetaldehyde-dependent transactivator *alcR* in the second cistron. Leaky P\textsubscript{AIR}-driven transcripts provide sufficient AlcR to kickstart maximum SEAP expression in the presence of inducing ethanol concentrations. In the absence of exogenous ethanol the autoregulated circuit remains silent. The autoregulated AIR control system reaches SEAP induction factors of up to 36-fold when transfected into *P. patens* (Figure 4F).

**VEGF\textsubscript{121}-based biopharmaceutical manufacturing using microencapsulated moss protoplasts**. The use of *P. patens* for biopharmaceutical manufacturing of protein therapeutics has been established but remains challenging. The moss needs to be constantly blended in order to enable mixing in custom-designed stirred-tank bioreactors (Decker and Reski, 2007) and the plant cell wall potentially compromises efficient secretion of larger product proteins. Since plant protoplasts lack any cell wall and can be grown in single-cell suspension cultures, they would be the ideal plant cell system for biopharmaceutical manufacturing. However, protoplasts are too fragile and shear force-sensitive for use in state-of-the-art bioprocesses.

We have pioneered a process to microencapsulate *P. patens* protoplasts in coherent alginate beads. Alginate bead polymerization is compatible with W5 culture
medium which was also used for the bioprocess. 4x10^7 tWT11.51VEGF (Baur et al., 2005b) -derived protoplasts were microencapsulated in 500µm capsules (165 protoplasts per capsule) using state-of-the-art encapsulation technology and cultivated for nine days in a 2L Wave Bioreactor operated at a culture volume of 1L (Figure 5). VEGF_{121} production reached 53µg/L in a nine-day process which compares with forefront bioprocesses using moss protonema. A fluorescein/trypan blue-based live/dead staining revealed that microencapsulated protoplasts cultivated for nine days in a Wave Bioreactor were still 74.8% ± 7.2% viable, which represent only a 5% viability decrease compared to a freshly prepared protonema-derived protoplast population.

**Figure 5.** Prototype biopharmaceutical manufacturing of VEGF_{121} using *P. patens* protoplasts microencapsulated in alginate beads and cultivated in a Wave Bioreactor. (A) Bioreactor set-up. (B) Light micrographs moss protoplasts encapsulated in alginate beads.
Figure 5: (C) VEGF$_{121}$ production profiles of microencapsulated transgenic moss protoplasts. Error bars indicate the standard deviation of between three measurements of the samples.

Discussion

The complete functionality of the central mammalian expression portfolio which includes various promoters, mRNA processing signals, transcription factors, translation elements and secretion peptides (Table 2) in the moss *P. patens* suggests that mammalian expression vectors and product proteins are generically compatible with this evolutionary old and simple plant. Interestingly, not only the functionality but also the relative performance profiles of different genetic elements in the moss matched those of mammalian cells. Examples of this include: (i) $P_{hCMV}$ being a stronger promoter than $P_{SV40}$, with comparable strength to the smallest synthetic promoter $P_{GTX}$ (Hartenbach and Fussenegger, 2006), (ii) $IRES_{PV}$ and $IRES_{EMCV}$ being equally efficient in triggering translation initiation and outperforming $IRES_{Rbm3}$ (Fux et al., 2004), (iii) terminal IRES-driven translation units showing lower expression levels from multicistronic mRNAs compared to cap-dependent translation initiation, (iv) the AIR control system responsiveness to gaseous acetaldehyde or ethanol being the most sensitive transgene-control modality (Weber et al., 2004), and (v) one-vector-based auto-regulated expression configuration providing superior regulation performance.
### Table 2. Mammalian genetic elements functional in *Physcomitrella patens*

<table>
<thead>
<tr>
<th>Genetic element</th>
<th>Name</th>
<th>Description</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>P_{hCMV}</td>
<td>Human cytomegalovirus immediate early promoter</td>
<td>Invitrogen</td>
</tr>
<tr>
<td></td>
<td>P_{SV40}</td>
<td>Simian virus 40 promoter</td>
<td>Clontech</td>
</tr>
<tr>
<td></td>
<td>P_{GTX}</td>
<td>Synthetic promoter derived from the GTX homeodomain protein</td>
<td>Hartenbach and Fussenegger, 2006</td>
</tr>
<tr>
<td></td>
<td>P_{hCMVmin}</td>
<td>Minimal version of P_{hCMV}</td>
<td>Clontech</td>
</tr>
<tr>
<td>Operator</td>
<td>O_{alcA}</td>
<td>Operator of the <em>Aspergillus nidulans</em> alcohol dehydrogenase promoter</td>
<td>Weber et al. 2004</td>
</tr>
<tr>
<td></td>
<td>ETR</td>
<td>Operator of the <em>E. coli</em> 2’-phosphotransferase 1 (mph(A)) promoter</td>
<td>Weber et al. 2002</td>
</tr>
<tr>
<td></td>
<td>O_{scbR}</td>
<td>Operator of the <em>Streptomyces coelicolor</em> butyrolactone-specific quorum-sensing receptor (ScbR)</td>
<td>Weber et al. 2003</td>
</tr>
<tr>
<td>Transactivator</td>
<td>AlcR</td>
<td>Transcription factor coordinating ethanol metabolism in <em>Aspergillus nidulans</em></td>
<td>Weber et al. 2004</td>
</tr>
<tr>
<td>Repressor</td>
<td>E</td>
<td>Repressor of the <em>E. coli</em> macrolide-resistance gene (mph(A))</td>
<td>Weber et al. 2002</td>
</tr>
<tr>
<td></td>
<td>ScbR</td>
<td>Butyrolactone-sensitive quorum-sensing receptor of <em>Streptomyces coelicolor</em></td>
<td>Weber et al. 2003</td>
</tr>
<tr>
<td>Transactivation domain</td>
<td>VP16</td>
<td><em>Herpes simplex</em> virus-derived transactivation domain</td>
<td>Gossen et al. 1992</td>
</tr>
<tr>
<td>Internal ribosome entry site</td>
<td>IRES_{PV}</td>
<td>Poliovirus-derived IRES</td>
<td>Dirks et al. 1993</td>
</tr>
<tr>
<td></td>
<td>IRES_{EMCV}</td>
<td>Encephalomyocarditis virus-derived IRES</td>
<td>Kaufman et al. 1991</td>
</tr>
<tr>
<td></td>
<td>IRES_{Rbm3}</td>
<td>IRES derived from the human RNA-binding motif protein 3 (Rbm3)</td>
<td>Chappell et al. 2001</td>
</tr>
<tr>
<td>polyadenylation site</td>
<td>pA</td>
<td>Simian virus 40-derived polyadenylation site</td>
<td>Clontech</td>
</tr>
<tr>
<td></td>
<td>apA</td>
<td>Artificial polyadenylation site</td>
<td>Fux et al. 2001</td>
</tr>
<tr>
<td>Product proteins</td>
<td>SEAP</td>
<td>Human placental alkaline phosphatase</td>
<td>Dirks et al. 1993</td>
</tr>
<tr>
<td></td>
<td>SAMY</td>
<td><em>Bacillus stearothermophilus</em>-derived secreted α-amylase</td>
<td>Schlatter et al. 2002</td>
</tr>
<tr>
<td></td>
<td>AMY</td>
<td><em>Bacillus stearothermophilus</em>-derived α-amylase</td>
<td>Schlatter et al. 2002</td>
</tr>
<tr>
<td></td>
<td>VEGF_{121}</td>
<td>Human vascular endothelial growth factor 121</td>
<td>Weber et al. 2003</td>
</tr>
<tr>
<td></td>
<td>EPO</td>
<td>Human erythropoietin</td>
<td>Donation by P. Aebischer</td>
</tr>
</tbody>
</table>
This cross-kingdom conservation of mammalian and moss protein production machineries is phylogenetically profound and has several implications for basic and applied research. Comparative genomics as well as functional studies have recently established major differences in metabolic pathways and gene function between flowering plants and *P. patens* and suggested that a substantial moss gene pool is more closely related to mammals than to flowering plants (Frank et al., 2007; Rensing et al., 2007). In combination with the functional data presented here, these findings may expand our classical view on the molecular division between plants and animals (e.g., in (Yamamoto et al., 2007).

This differentially expressed gene pool may reveal unique cross-kingdom functionalities useful to future advances in agriculture and human health. With the discovery that two fundamentally different living systems such as the moss and mammalian cells can utilize the other’s gene expression and protein production machineries may expand the way of how we perceive ecosystems in which different species co-exist and could, at least theoretically, exchange a compatible gene pool.

Synthetic ecosystems have recently established the principle of cross-kingdom communication between *S. cerevisiae* or *E. coli* and mammalian cells which replicated co-existence patterns as complex as oscillating predator-prey population dynamics (Weber et al., 2007), thus expanding our view on quorum-sensing between bacteria (Keller and Surette, 2006). We have shown here that *P. patens* harboring mammalian gene circuits were responsive to quorum-sensing communication initiated by co-cultivated *S. coelicolor* as well as to “over-the-air” signaling triggered by *S. cerevisiae* cultivated adjacently. Rational interventions into the quorum-sensing networks may foster unprecedented advances in agriculture replicating the progress achieved in attenuating host-pathogen interaction in human therapy (Benghezal et al., 2006). Moreover, our recent findings establish *P. patens* as a promising host system for synthetic biology, a novel approach in the life sciences that relies on iterative cycles between analysis and synthesis (Benner and Sismour, 2005), utilizing devices of signaling networks in a cross-kingdom approach (e.g., (Khandelwal et al., 2007).
Several biopharmaceutical production platforms including *E. coli* (Georgiou and Segatori, 2005), (glyco-engineered) *S. cerevisiae* (Hamilton et al., 2006), mammalian cells (Wurm, 2004) and transgenic animals (Larrick and Thomas, 2001) are competing for industrial production of protein therapeutics (Fussenegger and Hauser, 2007). Mammalian cells have become the dominant system for the production of recombinant protein pharmaceuticals in part due to availability of a highly advanced portfolio of expression vectors and engineering strategies (Hartenbach and Fussenegger, 2005; Umana et al., 1999; Wurm, 2004). With the global mammalian cell-based production capacity plateauing into a bottleneck, this compromises the availability of drugs to patients. Alternative easy-to-implement bioprocessing concepts are urgently needed (Fussenegger and Hauser, 2007). The moss *P. patens* has recently come into the limelight as an easy-to-handle/engineer organism which could be cultivated in scale-up-compatible bioreactors and was able to produce ADCC-optimized therapeutic IgGs in a GMP-approved bioprocess (Decker and Reski, 2007).

Utilizing a compatible mammalian expression and engineering toolbox, the moss as emerging biopharmaceutical manufacturing platform could be propelled to an *ex-aequo* competitor of mammalian cell-based production systems. Major bioprocess advantages of *P. patens* include the use of an inexpensive salt solution as production medium, which reduces downstream processing challenges and cost, and the availability of an efficient homologous recombination toolkit that provides stable and predictable production cultures (Kamisugi et al., 2006). Best-in-class production systems include transient protoplast cultures for rapid evaluation of bioprocess parameters and a scalable stirred-tank photo-bioreactor that uses stable moss protonema.

Moss protonema tissue needs to be constantly blended to avoid complications in bioreactor operation, which may hinder large-scale biopharmaceutical manufacturing and the established cell wall may compromise secretion of larger proteins. Protoplasts could be an alternative (Baur et al., 2005a) but they are not sufficiently robust to survive long-term bioreactor operation. The microencapsulation protocol established during this study is compatible with the W5 medium and enables
cultivation of encapsulated protoplasts in a proliferation-inhibited and cell wall-free state. Being protected by a physiologically inert alginate shell, the protoplasts are able to devote all of their metabolic energy to the production of heterologous protein rather than biomass. And being devoid of any secretion-limiting cell wall, microencapsulated protoplasts cultivated in a standard Wave Bioreactor, equipped with a photosynthesis kit were able to produce the human growth factor VEGF₁₂₁ at titers comparable to the highly optimized best-in-class protonema cultures. The use of Wave Bioreactor systems, which can be easily upscaled to 500L cultures, has recently gathered momentum used in pilot production of proteins for clinical trials (Haldankar et al., 2006).

The combination of a novel protoplast-based bioprocess with powerful mammalian expression technology will further enhance the use of *P. patens* as a complementary and competitive platform for the biopharmaceutical manufacturing of protein therapeutics and establishes this evolutionary old and simple plant as a valuable host for synthetic biology.

**Material and Methods**

**Expression vector design.** Table 3 lists all plasmids used in this study and provides detailed information about their construction.

**Table 3.** Expression vectors designed and used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEF4/Myc-His</td>
<td>Mammalian expression vector containing P&lt;sub&gt;hEF1&lt;/sub&gt;-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSEAP2-Basic</td>
<td>Mammalian SEAP expression vector.</td>
<td>Clontech</td>
</tr>
<tr>
<td>pCF292 (pTRIDENT3 7)</td>
<td>P&lt;sub&gt;SV40&lt;/sub&gt;-driven tricistronic expression vector. (I-CeuI)-P&lt;sub&gt;SV40&lt;/sub&gt;-(I-SceI)-SAMY-IRE&lt;sub&gt;PV&lt;/sub&gt;-VEGF&lt;sub&gt;121&lt;/sub&gt;-IRE&lt;sub&gt;Rhm3&lt;/sub&gt;-SEAP-(I-P&lt;sub&gt;pol&lt;/sub&gt;)-apA-(PI-P&lt;sub&gt;spI&lt;/sub&gt;).</td>
<td>Fux et al. 2004</td>
</tr>
<tr>
<td>pCF297 (pTRIDENT3 6)</td>
<td>P&lt;sub&gt;SV40&lt;/sub&gt;-driven tricistronic expression vector. (I-CeuI)-P&lt;sub&gt;SV40&lt;/sub&gt;-(I-SceI)-SAMY-IRE&lt;sub&gt;PV&lt;/sub&gt;-VEGF&lt;sub&gt;121&lt;/sub&gt;-IRE&lt;sub&gt;EMCV&lt;/sub&gt;-SEAP-(I-P&lt;sub&gt;pol&lt;/sub&gt;)-apA-(PI-P&lt;sub&gt;spI&lt;/sub&gt;).</td>
<td>Fux et al. 2004</td>
</tr>
<tr>
<td>pMF242</td>
<td>P&lt;sub&gt;hCMV&lt;/sub&gt;-driven EPO expression vector. P&lt;sub&gt;hCMV&lt;/sub&gt;−EPO-pA</td>
<td>Fussenegger et al. 2000</td>
</tr>
<tr>
<td>pMG31</td>
<td>P&lt;sub&gt;SV40&lt;/sub&gt;-driven SEAP expression vector. The PIP-specific</td>
<td>This work</td>
</tr>
</tbody>
</table>
operator was excised from pMF208 (*Hind*III/*Eco*RI) and the Klenow-polished vector backbone was religated. \( P_{SV40} \)-SEAP-pA.

| **pMG32** | \( P_{hEF1} \)-driven SEAP expression vector. \( P_{hEF1} \) was excised from pEF4/Myc-His (*Nru*I/*Eco*RI) and cloned into pSEAP2-Basic (Clontech) (*Nru*I/*Eco*RI). \( P_{hEF1} \)-SEAP-pA. This work |
| **pMG40** (pTRIDENT4 5) | \( P_{hCMV} \)-driven tricistronic expression vector. \( P_{hCMV} \) was PCR-amplified from pSS173 using oligonucleotides OMG23/OMG24 and cloned (I-*Ceul/I-SceI*) into pCF297. (I-*Ceul*)-\( P_{hCMV} \)-(I-*SceI*)-SAMY-\( IR_{SPV} \)-VEGF121-\( IR_{SPV} \)-SEAP-(I-*PpoI*)-apA-(PI-*PspI*). This work |
| **pMG41** (pTRIDENT4 6) | \( P_{hCMV} \)-driven tricistronic expression vector. \( P_{hCMV} \) was PCR-amplified from pSS173 using oligonucleotides OMG23/OMG24 and cloned (I-*Ceul/I-SceI*) into pCF292. (I-*Ceul*)-\( P_{hCMV} \)-(I-*SceI*)-SAMY-\( IR_{SPV} \)-VEGF121-\( IR_{Rbm3} \)-SEAP-(I-*PpoI*)-apA-(PI-*PspI*). This work |
| **pMG42** (pTRIDENT4 7) | \( P_{hCMV} \)-driven tricistronic expression vector. \( P_{hCMV} \) was PCR-amplified from pSS173 using oligonucleotides OMG23 (5’ gatecagcttaaattgctatcgcgtacctagACTGTAATCAATTACGGGGTCATTAGTTCATAGC 3’) and OMG24 (5’ gatecagcttaaattgctatcgcgtacctagCTGACGCTTGATCAATACCCAGCTCTGC 3’). \( P_{hCMV} \) was cloned (I-*CeuI/I-SceI*) into pMG43. (I-*CeuI*)-\( P_{hCMV} \)-(I-*SceI*)-SAMY-\( IR_{SPV} \)-VEGF121-\( IR_{SPV} \)-SEAP-(I-*PpoI*)-apA-(PI-*PspI*). (Upper case, annealing sequence, lower case italic I-*CeuI* and I-*SceI* for OMG23 and OMG24 respectively) This work |
| **pMG43** | \( P_{SV40} \)-driven expression vector. \( IR_{SPV} \) was excised from pSAM241 (*Ascl/Spel*) and cloned into pCF292. (I-*Ceul*)-\( P_{SV40} \)-(I-*SceI*)-SAMY-\( IR_{SPV} \)-VEGF121-\( IR_{SPV} \)-SEAP-(I-*PpoI*)-apA-(PI-*PspI*). This work |
| **pMG60** | \( P_{GTX} \)-driven AMY expression vector. AMY was excised from pSS188 (*Hind*III/*XbaI*) and cloned into pSH17 (*Hind*III/*XbaI*). \( P_{GTX} \)-AMY-pA. This work |
| **pMG61** | \( P_{GTX} \)-driven EPO expression vector. EPO was excised from pMF242 (*EcoRI/XbaI*) and cloned into pSH17 (*EcoRI/XbaI*). \( P_{GTX} \)-EPO-pA. This work |
| **pMG65** | \( P_{CaMV35S} \)-driven SEAP expression vector. SEAP was PCR-amplified from pSH17 using OMSG53 (5’ ccgcttgagggcccaATGCTGCTGCT-GCTGCTGCTG 3’) and OMSG54 (5’ tgctctagagctcatgatggtagtgatgatgatgatgatgagtagttgtagtaggcagcaccatagggccagctccctgacagcactactctg 3’) and cloned \( XhoI/XbaI \) into pRT101neo. \( P_{CaMV35S} \)-SEAP-his-pA. (Upper case, annealing sequence; lower case italic, restriction enzymes) This work |
| **pMG66** | \( P_{CaMV35S} \)-driven SAMY expression vector. SAMY was PCR-amplified from pSH102 using OMSG55 (5’ ccgcttgagggcccaATGAGGAGACGACACACTCTCTG 3’) and OMSG56 (5’ tgctctagagctcatgatggtagtgtagtagcagcaccatagggccagctccctgacagcactactctg 3’) This work |
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and cloned XhoI/XbaI into pRT101neo. $P_{CaMV35S}$-SAMY-his-pA.

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<th>pMG67</th>
<th>$P_{CaMV35S}$-driven AMY expression vector. AMY was PCR-amplified from pMG60 using OMG57 (5’ cccgctcgagggcccccacATGGGCCACCGT3’ and OMG58 (5’ tgctctagagctcgggtggtatgtagtcatATGGCCACCGTGGTCC 3’) and cloned XhoI/XbaI into pRT101neo. $P_{CaMV35S}$-AMY-his-pA</th>
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<td>$P_{CaMV35S}$-driven VEGF expression vector. VEGF was PCR-amplified from pSH100 using OMG59 (5’ cccgctcgagggcccccacATGAACTTTCT-GCTGTCTTGG 3’) and OMG60 (5’ tgctctagagctcgggtggtatgtagtcatATGGCCACCGTGGTCC 3’) and cloned XhoI/XbaI into pRT101neo. $P_{CaMV35S}$-VEGF-his-pA</td>
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<td>pMG69</td>
<td>$P_{CaMV35S}$-driven EPO expression vector. EPO was PCR-amplified from pMG61 using OMG61 (5’ cccgctcgagggcccccacATGGGGGTGCCCGACGTCCCACCC 3’) and OMG62 (5’ tgctctagagctcgggtggtatgtagtcatATGGCCACCGTGGTCC 3’) and cloned XhoI/XbaI into pRT101neo. $P_{CaMV35S}$-EPO-his-pA</td>
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<td>pRT101neo</td>
<td>$P_{CaMV35S}$-driven expression vector for plant cells, carrying the nptII cassette for neomycin resistance. $P_{CaMV35S}$-nptII-pA (pA, 35s-Terminator).</td>
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<td>VEGF121-encoding control vector. IRES$_{PV}$-VEGF121-pA</td>
<td>Hartenbach and Fussenegger 2006</td>
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<td>pSH49</td>
<td>SEAP-encoding control vector. SEAP was excised from pSS173 (EcoRI/NotI) and cloned into pSH12 (EcoRI/NotI). IRES$_{PV}$-SEAP-pA.</td>
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<td><strong>P</strong><em>{SV40}-driven expression vector encoding the butyrolactone-dependent transactivator SCA. <strong>P</strong></em>{SV40}-SCA-pA; SCA, scbR-VP16.</td>
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<td>Butyrolactone-repressible, <strong>P</strong><em>{SPA}-driven SEAP expression vector. <strong>P</strong></em>{SPA}-SEAP-pA.</td>
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<td>Butyrolactone-inducible, <strong>P</strong><em>{SCAON8}-driven SEAP expression vector. <strong>P</strong></em>{SCAON8}-SEAP-pA.</td>
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<td><strong>P</strong><em>{SV40}-driven expression vector encoding the butyrolactone-dependent transrepressor SCS. <strong>P</strong></em>{SV40}-SCS-pA; SCS, scbR-KRAB.</td>
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Abbreviations: **alcR**, *Aspergillus nidulans* acetaldehyde-dependent transactivator (1521bp); **AMY**, *Bacillus stearothermophilus*-derived α-amylase (1551bp); **apA**, artificial polyadenylation site (91bp); **E**, *E.coli*-derived macrolide-dependent repressor (585bp); **ECFP**, enhanced cyan fluorescent protein (720bp); **EPO**, human erythropoietin (579bp); **ET1**, macrolide-dependent transactivator (E-VP16) (972bp); **ET4**, macrolide-dependent transrepressor (E-KRAB) (1044bp); **EYFP**, enhanced yellow fluorescent protein (720bp); **IRESEMCV**, encephalomyocarditis virus internal ribosome entry site (502bp); **IRESPV**, poliovirus internal ribosome entry site (635bp); **IRESRbm3**, internal ribosome entry site derived from the human RNA-binding motif protein 3 (Rbm3) (732bp); **KRAB**, human kruppel-associated box protein (450bp); **NptII**, neomycin phosphotransferase II (921bp); **pA**, polyadenylation site (145bp); **P**_{AIR}, acetaldehyde-responsive promoter (456bp); **PCaMV35S**, cauliflower mosaic virus 35s promoter (384bp); **PETR2**, erythromycin-repressible promoter (200bp); **PETRON8**, erythromycin-inducible promoter (530bp); **PGTX**, synthetic promoter derived from the GTX homeodomain protein (182bp); **PhCMV**, human cytomegalovirus immediate early promoter (663bp); **PhCMV*-1, tetracycline-responsive promoter (156bp); **PhEFla**, human elongation factor 1α promoter (1185bp); **PIP**, pristinamycin-induced protein (867bp); **PPIR3**, streptogramin-repressible promoter (534bp); **PSCAON8**, butyrolactone-inducible promoter (576bp); **PSPA**, butyrolactone-repressible promoter (194bp); **PSV40**, simian virus 40 promoter (308bp); **RFP**, red fluorescent protein;
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SAMY, *Bacillus stearothermophilus*-derived secreted α-amylase (1611bp); SCA, butyrolactone-dependent transactivator (ScbR-VP16) (1035bp); *scbR*, *Streptomyces coelicolor* butyrolactone-dependent repressor (648bp); SEAP, human placental secreted alkaline phosphatase (1560bp); *VEGF_{121}* , human vascular endothelial growth factor 121 (444bp); VP16, *Herpes simplex* virus-derived transactivation domain (387bp).

**Cultivation and transformation of *P. patens***. *P. patens* (Hedw.) B.S.G. was grown axenically in Erlenmeyer flasks or modified stirred-tank bioreactors (5L, Applikon, Schiedam, The Netherlands) using a 10% modified Knop salt solution (100mg/L Ca(NO$_3$)$_2$·4H$_2$O, 25mg/L KCl, 25mg/L KH$_2$PO$_4$, 25mg/L MgSO$_4$·7H$_2$O and 1.25mg/L FeSO$_4$·7H$_2$O; pH5.8) (Reski and Abel, 1985). Protoplasts of *P. patens* were generated by incubation for 2h in 0.5M mannitol containing 4% Driselase (Sigma, Buchs, Switzerland), followed by two centrifugation steps (10min, 50xg) and resuspension of the protoplast-containing pellet at a desired cell density in 3M-medium (87.5g/L mannitol, 3.1g/L MgCl$_2$·6H$_2$O, 1g/L MES (2-(N-morpholino)ethansulfonic acid hydrate; Sigma); pH5.6 and 580mOsm). 300,000 protoplasts were chemically transfected with 50μg/ml DNA (80μg/ml for transgene control systems) as previously described (Jost et al., 2005) and cultivated in Knop’s regeneration medium (1g/l Ca(NO$_3$)$_2$·4H$_2$O, 250mg/l KCl, 250mg/l KH$_2$PO$_4$, 250mg/l MgSO$_4$·7H$_2$O, 12.5 mg/l FeSO$_4$·7H$_2$O, 5% glucose, 3% mannitol, pH 5.7, 540mOsm).

**Protein production.** Protein production was measured five days after transformation using standardized assays: (i) human placental secreted alkaline phosphatase (SEAP), a p-nitrophenylphosphate-based light-absorbance time course (Berger et al., 1988; Schlatter et al., 2002); (ii) *Bacillus stearothermophilus*-derived secreted (SAMY) and intracellular (AMY) α-amylase, a blue starch Phadebas® assay (Pharmacia Upjohn, Peapack, NJ, cat. no. 10-5380-32) (Schlatter et al., 2002); quantification of intracellular reporter proteins required lysis of the plant cells by four freeze-thaw cycles and elimination of cell debris by centrifugation (2min at 12000 g); (iii) human vascular endothelial growth factor 121 (*VEGF_{121}*), using a *VEGF_{121}*-specific ELISA (Peprotech, Rocky Hill, NJ, USA, cat. no. 900-K10, lot. no. 1006010); (iv) human erythropoietin (EPO), using an EPO-specific ELISA.
Transgene regulation. All regulating agents were administered at indicated concentrations immediately after transformation. The butyrolactone 2-(1’-hydroxy-6-methylheptyl)-3-(hydroxymethyl)-butanolide (SCB1) was synthesized and purified as described previously (Weber et al., 2003). Erythromycin (cat. no. 45673, lot. no. 1195447; Fluka, Buchs, Switzerland) was prepared as a stock solution of 1mg/ml in ethanol. The AIR system was induced by the addition of indicated volumes of 100% ethanol.

Microencapsulation of P. patens protoplasts. 4x10⁷ protoplasts generated from transgenic VEGF₁₂₁-producing moss tWT11.5₁VEGF (Baur et al., 2005b) were resuspended in 8ml 3M-medium and stirred gently with 40ml 1.5% sodium-alginate solution (Inotech Biotechnologies Ltd, Basel, Switzerland, cat. no. IE1010, lot. no. 060125B1). Protoplasts were encapsulated in 500µm alginate capsules (165 protoplasts per capsule) using an Inotech Encapsulator Research IE-50R (Inotech Biotechnologies Ltd, Basel, Switzerland) set at the following parameters: 0.5mm nozzle, 853 unit flow rate using a 50ml syringe, 1250s⁻¹ nozzle vibration frequency, 1.4kV for bead dispersion. W₅ medium (18.4g/l CaCl₂, 8g/l NaCl₂, 0.99g/l Glucose, 0.75g/l KCl; pH5.8, 600mOsm) was used as a precipitation solution. It contains sufficient CaCl₂ for precipitation of alginate beads and enables direct cultivation of microencapsulated protoplast populations without the need for a medium exchange. Microencapsulated tWT11.5₁VEGF (high expressing P. patens plant containing the VEGF₁₂₁-encoding cDNA under the control of the moss actin 5′ region) protoplasts (240,000 capsules, 1L W₅ medium) were cultivated in a BioWave 20SPS-F bioreactor (Wave Biotech, Tagelswangen, Switzerland), equipped with 2L Wave Bags and set at the following parameters: aeration, 100ml/min sterilized air; rocking rate, 19 min⁻¹; rocking angle, 10°. The Wave Bioreactor was placed in an ISF-1-W incubator equipped with a photosynthesis kit set to 25°C and a day/night cycle of 16/8h (Kuehner, Birsfelden, Switzerland).

Edman sequencing. VEGF₁₂₁ was precipitated from tWT11.5₁VEGF protoplast culture supernatants for 10 min at 4°C with 100% w/v trichloroacetic acid (TCA,
Sigma) (supernatant: TCA 9 : 1). The samples were centrifuged for 5 min at 12000 g, and the VEGF121-containing pellet was washed twice in ice-cold acetone, dried and resuspended in 2 x sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer [50% glycerol, 250 mM tris(hydroxymethyl)aminomethane (Tris), 10% SDS, 500 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8]. The samples were then denatured for 5 min at 50 °C, and the proteins were size-fractionated on a 12% SDS-polyacrylamide gel and blotted on to a polyvinylidene fluoride membrane (cat. no. IPVH20200; Millipore Corporation, Bedford, MA, USA). VEGF_{121} (35 kDa) was N-terminally sequenced on an Applied Biosystems (Foster City, CA, USA) model 492cLC Procise protein/peptide sequencer with an on-line Perkin-Elmer (Waltham, MA, USA) Applied Biosystems Model 140C PTH Amino Acid (Phenylthiohydantoin amino acid) Analyser. The PTH amino acids were automatically transferred to a reverse-phase C-18 column (0.8 mm inside diameter) for detection at 269 nm, and identified by comparison with individual runs with a standard mixture of PTH amino acids.

**Cultivation of *Saccharomyces cerevisiae* and *Streptomyces coelicolor*.** *S. cerevisiae* (wild type strain W303, BMA 64, European *S. cerevisiae* archive for functional analysis [EUROSCARF], Frankfurt, Germany) was cultivated on yeast-peptone-dextrose agar (YPD; 1% yeast extract, 2% peptone, 2% dextrose, 1% agar) and *Streptomyces coelicolor* MT1110 (kindly provided by Marc Folcher) was cultivated on mannitol-soy agar (2% soy flour, 2% mannitol, 1.5% agar). For co-cultivation of *S. coelicolor* and *P. patens*, *Streptomyces* were pre-cultured in Luria Bertani (LB) medium to an OD$_{600}$ of 340 and indicated volumes were then transferred to *P. patens* maintained in regeneration medium.

**Viability profiling of microencapsulated *P. patens* protoplasts.** Viability of microencapsulated moss protoplasts was determined by scoring live protoplasts, stained with fluorescein diacetate (FDA), and dead protoplasts, stained with trypan blue using (fluorescence) microscopy (Leica DM-RB fluorescence microscope; Leica Heerbrugg, Switzerland). 20 protoplast-containing alginate beads were incubated for 10 min in a staining solution containing 200μl W5 medium, 20μl phosphate-buffered saline (PBS, Dulbecco’s Phosphate-Buffered Saline, Invitrogen, Basel, Switzerland,
cat. no. 21600-0069) containing 0.01% FDA (Sigma) and 40µl of a 0.4% trypan blue stock solution (Flucka, Buchs, Switzerland; lot. No. 1230532).

Acknowledgements

We thank Wilfried Weber, Eva Decker and Marcel Tigges for productive discussions, Dr. Gilbert Gorr, Greenovation Biotech GmbH, for the *P. patens* strain tWT11.51_{VEGF} used in this study, Peter Hunzicker, Functional Genomics Center Zurich, for the help with the Edman sequencing, and Domenick Grasso and Wilfried Weber for critical comments of the manuscript. This work was supported by the Swiss National Science Foundation (grant no. 3100AO-112549) and the Excellence Initiative of the German Federal and State Governments (EXC 294).
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Controlling Transgene Expression In Subcutaneous Implants Using A Skin Lotion Containing The Apple Metabolite Phloretin

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Abstract

Adjustable control of therapeutic transgenes in engineered cell implants following transdermal and topical delivery of non-toxic trigger molecules would increase convenience, patient compliance and elimination of hepatic first-pass effect in future therapies. *Pseudomonas putida* DOT-T1E has evolved flavonoid-triggered TtgR operon which controls expression of a multisubstrate-specific efflux pump (TtgABC) to resist plant-derived defense metabolites in its rhizosphere habitat. Taking advantage of the TtgR operon, we have engineered a hybrid *P. putida*-mammalian genetic unit responsive to phloretin. This flavonoid is contained in apples and, as such, or as dietary supplement, regularly consumed by humans. The engineered mammalian phloretin-adjustable control element (PEACE) enabled adjustable and reversible transgene expression in different mammalian cell lines and primary cells. Due to the low half-life of phloretin in culture PEACE could also be used to program expression of difficult-to-produce protein therapeutics during standard bioreactor operation. When formulated in skin lotions and applied to the skin of mice harboring transgenic cell implants, phloretin was able to fine-tune target genes and adjust heterologous protein levels in the bloodstream of treated mice. PEACE-controlled target gene expression could foster advances in biopharmaceutical manufacturing as well as gene- and cell-based therapies.

Introduction

Synthetic mammalian expression systems which enable reversible and adjustable transgene expression have been essential for recent advances in (i) functional genomic research (1), (ii) drug discovery (2, 3), (iii) manufacturing of difficult-to-produce protein therapeutics (4, 5), (iv) the design of synthetic gene networks replicas reaching the complexity of electronic circuits (6-9) and gene therapy applications (10-12).

To date, a multitude of heterologous transgene expression systems, for use in mammalian cells and transgenic animals, have been described (4). The prevailing design consists of a heterologous small molecule-responsive transactivator engineered
by fusing a prokaryotic repressor to a eukaryotic transactivation domain and a transactivator-specific promoter containing the matching prokaryotic operator linked to a minimal eukaryotic promoter. Inducer-triggered modulation of the transactivator’s affinity to its cognate promoter results in adjustable and reversible transcription control of the specific target gene (13-16). In recent years, a panoply of such heterologous transcription control modalities have been developed which are responsive to a variety of inducer molecules such as antibiotics (13, 14, 17), steroid hormones and their analogs (18, 19), quorum-sensing molecules (20, 21), immunosuppressive and anti-diabetic drugs (22, 23), biotin (24), L-arginine (25) as well as volatile acetaldehyde (16). Apart from gaseous acetaldehyde which can simply be inhaled, all other inducers need to be either taken up orally or be administered by injection in any future gene therapy application. Transdermal and topical delivery of inducer molecules, which would provide advantages over conventional injection-based or oral administration such as convenience, improved patient compliance and elimination of hepatic first-pass effect, have not yet been developed.

Phloretin is mainly found in the root bark of apple trees and in apples where it acts as a natural antibacterial plant defense metabolite (26). Phloretin has been studied as a possible penetration enhancer for skin-based drug delivery (27-31), attenuates inflammation by antagonizing prostaglandins (32), protects the skin from UV light-induced photodamage (33, 34) and is currently evaluated as a chemopreventive agent for cancer treatment (35). Since the plant rhizosphere is one of the natural habitats of *Pseudomonas putida* (strain DOT-T1E), this prokaryote has evolved the RND family transporter TtgABC with multidrug recognition properties which is controlled by its cognate repressor TtgR binding to a specific operator, (O_{TtgR}) in the TtgR promoter (P_{TtgR}). Phloretin has been shown to bind to the TtgR-operator complex at a stoichiometric ratio of one effector molecule per TtgR-dimer and to release TtgR from O_{TtgR} which results in induction of TtgABC production and effective pump-mediated efflux of the flavonoid from *P. putida* (26, 36).

Capitalizing on the phloretin-responsive TtgR-O_{TtgR} interaction of *P. putida* DOT-T1E we have assembled a synthetic mammalian phloretin-adjustable control element (PEACE), which was able to reversibly adjust product gene expression of
transgenic cells grown in culture, standard bioreactors or implanted into mice following addition of pure phloretin or topical administration of a phloretin-containing skin lotion.

Results

**Design of a synthetic mammalian phloretin-adjustable control element (PEACE).** With a half-life of 70h in culture and no negative influence on viability, growth or production of CHO-K1 cells, phloretin is a valid flavonoid candidate for trigger-inducible transcription control in mammalian cells [supporting information (SI) Text and Fig.S1]. Living in the plant rhizosphere, *Pseudomonas putida* DOT-T1E has evolved resistance to a variety of plant-derived antimicrobials (37, 38) which is triggered by phloretin-induced release of TtgR from the operator (O_{TtgR}) of its target promoter and subsequent induction of a broadly specific TtgABC efflux pump (26, 36). By fusing TtgR (36) to the *Herpes simplex*-derived transactivation domain VP16 (39) we created a synthetic mammalian transactivator (TtgA_{1}), which is able to bind and activate transcription from chimeric promoters (P_{TtgR1}) harboring O_{TtgR} linked to a minimal human cytomegalovirus immediate early promoter (P_{hCMVmin}), in a phloretin-responsive manner (Fig. 1A and B). Co-transfection of the constitutive TtgA_{1} expression vector pMG11 (P_{SV40-TtgA_{1}-pA}) and pMG10 (P_{TtgR1-SEAP-pA}) encoding a TtgA_{1}-specific P_{TtgR1}-driven SEAP expression unit, resulted in high-level SEAP expression (23.6±3.1U/L), which compares with an isogenic vector containing a constitutive P_{SV40}-driven SEAP expression cassette (pSEAP2-Control; 21.4±1.0U/L). Addition of increasing concentrations of phloretin (0-70µM) to a culture of pMG10- and pMG11-co-transfected CHO-K1 cells resulted in dose-dependent reduction of SEAP expression up to complete repression (Fig. 1C). These data suggest that PEACE-controlled transgene expression is adjustable and enables complete repression within a non-toxic phloretin concentration range. We have also designed P_{TtgR1} variants with different tandem O_{TtgR} modules and TtgA_{1} variants harboring various transactivation domains and provide a detailed combinatorial performance analysis in different cell lines and different expression configurations including auto-regulated
that are known to be essential for the assembly of complex synthetic gene networks (6, 9, 40) (SI Materials and Methods, Fig. S2 and S3, Table S1).

Fig. 1. Design and functionality of phloretin-adjustable control element (PEACE). The *Pseudomonas putida* DOT-T1E-derived bacterial repressor TtgR was fused to the VP16 transactivation domain of *Herpes simplex* virus and the resulting transactivator TtgA1 (TtgR-VP16) was cloned under control of the constitutive simian virus 40 promoter (P_{SV40}) (pMG11). The phloretin-responsive promoter (P_{TtgR1}; O_{TtgR-P_{hCMVmin}}) contains a chimeric TtgR-specific operator sequence (O_{TtgR}, CAGTATTTACAAACAACCATGAATGTA...
AGTATATTC; TtgR binding sites in italic), which is located 5' of a minimal human cytomegalovirus immediate early promoter (P_{CMVmin}) and was set to drive expression of the human placental secreted alkaline phosphatase (SEAP) (pMG10). (A) ON status; TtgA1 is constitutively expressed and binds to P_{TtgR1} in the absence of phloretin thereby inducing SEAP expression. (B) OFF status; Addition of phloretin releases TtgA1, from P_{TtgR1}, which switches SEAP expression off. (C) SEAP expression profiles of CHO-K1 transiently transfected with pMG11 (P_{SV40-TtgA1-pA}) and pMG10 (P_{TtgR1-SEAP-pA}) and cultivated for 48h in the presence of different phloretin concentrations (0-70μM).

**PEACE control by phloretin and other flavonoids.** Since TtgR of *P. putida* was shown to bind several plant-derived flavonoids with high affinity (26) we profiled their PEACE-controlling capacities in mammalian cells. CHO-K1 were transiently (co-)transfected with either pMG10 (P_{TtgR1-SEAP-pA}) and pMG11 (P_{SV40-TtgA1-pA}), to score regulation performance, or with pSEAP2-Control (P_{SV40-SEAP-pA}), to assess compound-related cytotoxicity, and then cultivated for 48h in medium containing different concentrations (0, 25, 50μM) of specific flavonoids (berberine, butylparaben, genistein, luteolin, β-naphthol, naringenin, phloretin, phloridzin or quercetine) before SEAP production was profiled (Fig. 2A). Whereas genistein, luteolin, β-naphthol, naringenin and quercetine were cytotoxic within the tested concentration range (genistein only at 50μM) berberine, butylparaben, phloridzin and phloretin did not reduce cell viability. However, berberine failed to control PEACE and butylparaben as well as phloridzin were able to regulate but not fully repress SEAP production (Fig. 2B). Therefore, phloretin which enabled maximum expression levels as well as full transgene repression was chosen as the ideal PEACE inducer for all further experiments.

**Phloretin-controlled transgene expression is functional in different mammalian cell lines and human primary cells.** To assess its versatility we tested PEACE in several immortalized mammalian cell lines as well as in human primary cells. Therefore, pMG10 (P_{TtgR1-SEAP-pA}) and pMG11 (P_{SV40-TtgA1-pA}) were co-transfected into BHK-21, COS-7, HaCaT, HEK-293, HT-1080 and NIH/3T3 cell lines as well as into primary human fibroblasts and keratinocytes, and cultivated for 48h in the presence (50μM) and absence of phloretin, followed by scoring of SEAP levels.
(Table 1). PEACE-controlled transgene expression was functional in all tested cell lines suggesting that this technology will be broadly applicable.

![Graph A](image1.png)

**Fig. 2.** PEACE responsiveness to different flavonoids. (A) CHO-K1 cells transiently expressing all PEACE components (pMG10 and pMG11) were cultivated in the presence of different flavonoids and SEAP expression was profiled after 48h. (B) Toxicity of flavonoids. CHO-K1 were transiently transfected with pSEAP2-Control, cultivated in medium supplemented with different flavonoids (0, 25, 50µM) SEAP levels were scored after 48h.

**Table 1:** PEACE-controlled transgene expression in different mammalian cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 µM Phloretin</th>
<th>50 µM Phloretin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>14.4 ± 0.4 U/L</td>
<td>1.5 ± 0.2 U/L</td>
</tr>
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</table>
SEAP production was quantified 48h after co-transfection pMG10 (P_TtgR1-SEAP-pA) and pMG11 (P_SV40-TtgA1-pA) into indicated cell lines and primary cells.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>SEAP Production (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COS-7</td>
<td>1.3 ± 0.02</td>
</tr>
<tr>
<td>HaCaT</td>
<td>0.9 ± 0.03</td>
</tr>
<tr>
<td>HEK-293</td>
<td>27.3 ± 0.8</td>
</tr>
<tr>
<td>HT-1080</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>8.5 ± 0.3</td>
</tr>
<tr>
<td>Primary human fibroblasts</td>
<td>1.0 ± 0.04</td>
</tr>
<tr>
<td>Primary human keratinocytes</td>
<td>1.5 ± 0.05</td>
</tr>
</tbody>
</table>

Expression kinetics, adjustability and reversibility of PEACE-controlled transgene expression in a stable transgenic CHO-K1 cell line. We have generated 5 double-transgenic cell lines (CHO-PEACE) by sequential transfection and clonal selection of pMG11 and pMG10 into CHO-K1. All of these PEACE-transgenic cell lines showed phloretin-regulated SEAP expression but differed in their overall regulation performance (maximum and leaky expression levels) as a result of differences in transgene copy number and integrations sites which remains beyond control using standard transfection technology (41) (Fig. S4). CHO-PEACE8, which was considered the best cell line and was therefore used in all follow-up experiments, showed (i) unchanged maximum SEAP expression levels and unaffected regulation performance in long-term cultures over 60 days (day 0, ON, 70.9±3.1U/L, OFF, 1.8±0.1U/L; day 60, ON, 67.6±2.7U/L, OFF, 2.1±0.2U/L; OFF at 50µM phloretin), (ii) excellent adjustability (Fig. 3A), (iii) exponential SEAP expression kinetics (Fig. 3B), (iv) full reversibility of transgene expression (Fig. 3C) and (v) optimal compatibility with other transgene regulation systems (SI Materials and Methods and Table S2 A and B).
Fig. 3. Design and characterization of the stable CHO-PEACE\textsubscript{8} cell line transgenic for phloretin-responsive SEAP expression. (A) Dose-response profile of CHO-PEACE\textsubscript{8}. (B) SEAP expression kinetics of CHO-PEACE\textsubscript{8} cultivated for 72h in the presence and absence of phloretin. (C) SEAP expression kinetics of CHO-PEACE\textsubscript{8} cultivated for 72h in the presence and absence of phloretin.
50µM phloretin. (C) Reversibility of CHO-PEACE₈-based SEAP production. 2x10⁵ CHO-PEACE₈ were cultivated for 144h in the presence or absence of 50µM phloretin. Every 48h the cell density was readjusted to 2x10⁵ and the phloretin status of the culture was reversed.

**Time-delayed induction of product gene expression in a prototype biopharmaceutical manufacturing scenario.** Since phloretin is a non-toxic fruit component, it could be an ideal product gene inducer for biopharmaceutical manufacturing scenarios which require precise timing or dosing of difficult-to-express protein pharmaceuticals (4, 42-44). Also, since phloretin has a determined half-live of 70h in mammalian cell culture systems (SI Results) any production culture can be programmed to start product gene expression at a predefined point in time as phloretin concentrations drop below a repressing threshold level. We have inoculated a 1L BioWave® bioreactor with 2x10³ CHO-PEACE₈ and different transgene-repressing phloretin doses of 60, 80 or 100µM. While CHO-PEACE₈ grew exponentially from the start of the bioprocess, SEAP production gradually increased once phloretin was degraded to non-repressive levels (40µM; Fig. 4). Using PEACE mammalian production cultures could indeed be programmed for timely induction of product gene expression without any process intervention.

![Graph](image-url)

**Fig. 4.** Automatically programmed product gene expression in bioreactors using well-defined phloretin degradation profiles. 2x10³ cells/mL CHO-PEACE₈ were cultivated in a bioreactor containing 1L culture medium supplemented with either 60, 80 or 100µM phloretin and SEAP production was profiled for 211h. Owing to a defined phloretin degradation profile in
culture and a precise induction threshold of 40μM phloretin, the onset of SEAP production can be programmed to occur at a very precise point in time by defining the cell density and the phloretin concentration at production start.

**Phloretin-mediated transdermal gene expression in subcutaneous implants in mice.** Since phloretin has been suggested as a penetration enhancer for transdermal drug delivery (27-31) and was shown to propagate systemically in rodents following local skin-based administration (28), we have evaluated phloretin as a potential transdermal therapeutic transgene expression inducer. Therefore, we have microencapsulated CHO-PEACE8 in coherent alginate-PLL-alginate capsules and implanted them subcutaneously into mice. The back of treated mice was partially shaved and vaseline-based skin lotions (200μL) containing different amounts of phloretin (0-42mg) were put on every day. The SEAP levels detected in treated mice 72h after implantation showed phloretin-dependent dose-response characteristics akin to the ones observed with the same microencapsulated implant batch cultivated and exposed to phloretin *in vitro* (Fig. 5A and B). Control mice receiving CHO-K1 cells transgenic for constitutive SEAP expression were insensitive to any treatment with phloretin-containing skin lotion (0mg phloretin: 6.2±0.43U/L; 42mg phloretin: 6.02±0.58U/L).
Fig. 5. PEACE-controlled transgene expression in mice. (A) Microencapsulated CHO-PEACE\textsubscript{8} were implanted subcutaneously into female OF1 mice (2x10\textsuperscript{6} cells/mouse). 200\textmu L of a cream containing different amounts of phloretin (0, 5.25, 10.5, 21 and 42mg) was applied to a shaved skin area near the implant site. SEAP serum levels were quantified 72h post implantation. (B) SEAP expression profiles of the microencapsulated CHO-PEACE\textsubscript{8} implant batch cultivated \textit{in vitro} for 72h at different phloretin concentrations.

**Discussion**

Flavonoids such as phloretin are polyphenols widely distributed in the plant kingdom and are present in fruits and vegetables regularly consumed by humans. The recent findings that phloretin and some of its derivatives have potent anti-inflammatory (32), antioxidative (33, 45) and even some anti-cancer (35, 46) activities form the scientific basis for the common saying “an apple a day keeps the doctor
away”. Phloretin has also been successfully evaluated as a penetration enhancer for transdermal drug delivery (27-31) and as skin protectant reducing oxidative stress resulting from external insults, such as UV irradiation which triggers skin cancer and photo aging (33, 34, 47). Recent studies in rodents have confirmed that dermal administration of phloretin will systemically spread in the animal (28) and that phloretin has a rather short half-life in vivo (48). All of the aforementioned characteristics corroborate phloretin to be a non-toxic natural compound with high metabolic turnover, which could be ideal for reversible transdermal induction of therapeutic transgenes. Transdermal and topical delivery of drugs and regulating molecules provide advantages over conventional oral or injection-based administrations, such as convenience, improved patient compliance and elimination of hepatic first-pass effect. However, most molecules are not applicable to dermal administration due to the excellent barrier properties of the skin which requires penetrating molecules to pass the stratum corneum with its compact keratinized cell layers and the viable epidermis before reaching the papillary dermis and crossing the capillary walls into systemic circulation. Phloretin-containing skin lotions put on the skin of mice containing cell implants harboring a synthetic phloretin-responsive expression system were able to precisely fine-tune target gene expression in the animal. This pioneering transdermal transcription control system may enable precise patient-controlled dosing of protein pharmaceuticals that are produced in situ by cell implants contained in clinically licensed devices (49-52). There should be no risk of nutrition-based interference of PEACE-controlled therapeutic transgene expression in transgenic cell implants since a patient (70kg) would need to eat over 2000 apples to reach PEACE-modulating phloretin levels in his bloodstream (48, 53). Besides this gene therapy-focused in-vivo scope of phloretin-responsive transgene expression the system showed excellent regulation performance including adjustability and reversibility in vitro. Owing to its short half-life in culture, phloretin-responsive production cultures grown in bioreactors could be pre-programmed for timely production initiation by inoculation with excessive phloretin concentrations. While the production cell cultures grow and phloretin levels decrease following precise kinetics, production will be initiated at a defined point in time, which is a function of the
inoculum and the initial phloretin concentration. Such a time-delayed production concepts would be particularly valuable for difficult-to-express protein therapeutics like those, which impair growth or are cytotoxic (4,42-44). The fact that phloretin is a natural fruit component regularly consumed by humans and licensed for use in skin lotions (34) may facilitate approval of such biopharmaceutical manufacturing protocols by governmental agencies. Also, since the PEACE system has been assembled following a standard binary transactivator/promoter design it is conceivable that its performance can easily be adapted to specific control requirements using an established refinement program (54, 55).

Considering all facts, the pioneering phloretin-based transgene control technology is unique and may foster advances in the production of difficult-to-express protein pharmaceuticals as well as in cell implant-based therapeutic applications.

**Material and Methods**

**Expression vector design.** pMG10 (P’TtgR1-SEAP-pA) harbors a phloretin-responsive SEAP expression unit and pMG11 (P SV40-TtgA1-pA) encodes constitutive expression of the phloretin-dependent transactivator. Detailed information on expression vector design and plasmids used in this study is provided in SI Table S3.

**Cell culture and transfection.** Wild-type Chinese hamster ovary cells (CHO-K1, ATCC: CCL-61) and their derivatives were cultivated in standard medium: ChoMaster® HTS (Cell Culture Technologies, Gravesano, Switzerland) supplemented with 5% (v/v) fetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany, Cat. No. 3302, Lot No. P251110) and 1% (v/v) penicillin/streptomycin solution (Sigma, St Louis, MO, USA, Cat. No. P4458). Human embryonic kidney cells (HEK-293, (56)), African green monkey kidney cells (COS-7, ATCC: CRL-1651), baby hamster kidney cells (BHK-21, ATCC: CCL10), human fibrosarcoma cells (HT-1080, ATCC: CCL-121), the human keratinocyte cell line HaCaT (57) and mouse fibroblasts (NIH/3T3, ATCC: CRL-1658) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Cat. No. 52100-39) supplemented with 10% FCS (v/v) and 1% (v/v) penicillin/streptomycin solution. Primary human foreskin fibroblasts were cultivated in DMEM supplemented with 20% FCS (v/v) and 1% (v/v) penicillin/streptomycin
solution and primary human foreskin keratinocytes were cultured in chemically defined serum-free keratinocyte medium (Invitrogen, Cat. No. 10744019) (all kindly provided by Sabine Werner). All cell types were cultivated at 37°C in a 5% CO₂-containing humidified atmosphere. For transient transfection of CHO-K1, 1µg of total plasmid DNA (for co-transfection an equal amount of each plasmid) was transfected into 50,000 cells per well of a 24-well plate according to an optimized calcium phosphate protocol, which resulted in typical transfection efficiencies of 35±5% (58). Plasmid DNA was diluted to a total volume of 25µL 0.5M CaCl₂ solution, which was mixed with 25µL 2x HBS solution (50mM HEPES, 280mM NaCl, 1.5mM Na₂HPO₄, pH7.1). After incubation for 15min at room temperature, the precipitates were immediately added to the well and centrifuged onto the cells (5min at 1,200xg) to increase transfection efficiency. After 3h, the cells were treated with 0.5mL glycerol solution (ChoMaster® HTS medium containing 15% glycerol) for 60s. After washing once with phosphate-buffered saline (PBS, Dulbecco’s Phosphate-Buffered Saline; Invitrogen, Cat. No. 21600-0069), cells were cultivated in 0.5mL standard ChoMaster® HTS medium in the presence or absence of different concentrations of phloretin. For transfection of BHK-21, COS-7 and HEK-293, plasmid DNA-Ca₃(PO₄)₂ precipitate was prepared and applied to the cells as described above. HEK-293 and COS-7 cells were washed once with PBS after 3h incubation with the DNA-Ca₃(PO₄)₂ precipitate and subsequently cultivated in standard DMEM, while BHK-21 cells were incubated overnight with the precipitates and then cultivated in DMEM medium after being washed once with PBS. HaCaT, HT-1080, NIH/3T3 as well as primary human fibroblasts and keratinocytes were transfected with Fugene™ 6 (Roche Diagnostics AG, Basel, Switzerland, Cat. No. 11814443001) according to the manufacturer’s protocol and cultivated in the cell culture medium specified above. After transfection, all cells were cultivated in DMEM supplemented with different concentrations of phloretin and reporter protein levels were profiled 48h after transfection, unless otherwise indicated.

**Construction of stable cell lines.** The stable CHO-PEACE₈ cell line, transgenic for phloretin-controlled SEAP expression, was constructed in two steps: (i) CHO-K1 cells were co-transfected with pMG11 (PV₄0-TtgA₁-pA) and pSV2neo
(Clontech, Cat. No. 6172-1) at a ratio of 20:1 and clonal selection resulted in the cell line CHO-TtgA. (ii) CHO-TtgA was co-transfected with pMG10 (P_{TtgR1}-SEAP-pA) and pPur (Clontech, Cat. No. 6156-1) (ratio of 20:1) and the phloretin-responsive SEAP-producing double-transgenic cell line CHO-PEACE₈ was chosen after clonal selection. Phloretin-dependent dose-response characteristics of CHO-PEACE₈ were analyzed by culturing 100,000 cells/mL for 48h in standard ChoMaster® HTS medium at different phloretin concentrations ranging from 0 to 70µM. Reversibility of phloretin-mediated SEAP production was assessed by cultivating CHO-PEACE₈ (100,000 cells/mL) for 144h while alternating phloretin concentrations from 0 to 50µM every 48h.

**Quantification of reporter protein production.** Production of the human placental secreted alkaline phosphatase (SEAP) was quantified using a p-nitrophenylphosphate-based light absorbance time course (59).

**In vivo methods.** CHO-PEACE₈ and CHO-SEAP₁₈ (60) were encapsulated in alginate-poly-(L-lysine)-alginate beads (400µm; 200 cells/capsule) using an Inotech Encapsulator Research IE-50R (Recipharm, Basel, Switzerland) according to the manufacturer’s instructions and the following parameters: 0.2mm nozzle, 20mL syringe at a flow rate of 405 units, nozzle vibration frequency of 1024Hz and 900V for bead dispersion. The back of female OF1 mice (oncins France souche 1, Charles River Laboratories, France) was shaved and 300µL of ChoMaster® HTS containing 2x10⁶ encapsulated CHO-PEACE₈ were subcutaneously injected. Control mice were injected with microencapsulated CHO-K1. Shaving ensured direct contact of the phloretin-containing cream with the skin of the animal. One hour after implantation 200µL of the phloretin-containing cream was applied to the skin around the injection site. The phloretin amounts in creams ranged from 0 to 42mg. The cream was applied once a day for up to three days. Thereafter, the mice were sacrificed, blood samples collected and SEAP levels were quantified in the serum which was isolated using microtainer SST tube according to the manufacturer’s instructions (Beckton Dickinson, Plymouth, UK). All the experiments involving mice were performed according to the directives of the European Community Council (86/609/EEC),
approved by the French Republic (No. 69266310) and performed by Marie Daoud El-Baba at the Université de Lyon, F-69622 Lyon, France.

**Bioreactor operation.** CHO-PEACE₈ (inoculum of 2x10³ cells/mL) were cultivated in a BioWave 20SPS-F bioreactor (Wave Biotech AG, Tagelswangen, Switzerland) equipped with a 2L Wave Bag® optimized for optical pH and DO (dissolved oxygen concentration) control of the 1L culture. The bioreactor was operated at a rocking rate of 15min⁻¹, a rocking angle of 6° and an aeration rate of 100mL/min with inlet gas humidification (HumiCare® 200, Gruendler Medical, Freudenstadt, Germany) to prevent evaporation of the medium. The medium (ChoMaster® HTS, 5% FCS, 1% penicillin/streptomycin) was supplemented with 60, 80 or 100µM phloretin.

**Inducer compounds and formulation of the skin lotion.** Berberine (Acros, Geel, Belgium, Cat. No. 20425-0100) and luteolin (Alfa Aesar, Karlsruhe, Germany, Cat. No. L14186) were prepared as 10mM stock solutions in 1:5 (v/v) DMSO/H₂O. Butylparaben (ABCR, Karlsruhe, Germany, Cat. No. AV14043), genistein (Axonlab, Baden, Switzerland, Cat. No. A2202.0050), β-naphthol (Sigma, Cat. No. 185507), naringenin (Sigma, Cat. No. N5893), phloretin (Sigma, Cat. No. P7912), phloridzin (Sigma, Cat. No. P3449) and quercetin (Sigma, Cat. No. Q0125) were prepared as 50mM stock solution in DMSO and were used at a final concentration of 50µM unless indicated otherwise. The vaseline-based phloretin-containing creams were professionally formulated (Pharmacy Hoengg Ltd., Zurich, Switzerland) and contained 25, 12.5, 6.25 and 3.125% (wt/wt) of phloretin. 200 µl skin lotion were topically applied per mouse and treatment which corresponds to a respective total phloretin amount per dose of 42, 21, 10.5 and 5.25mg.

In order to quantify phloretin in cell culture medium, the samples were added to 5x10⁴ CHO-PEACE₈ and incubated for 48h prior to SEAP quantification. Phloretin levels were calculated by comparing SEAP production with a calibration curve (Fig. 3A), established using the same parameters and defined phloretin concentrations. Similarly, the half-life of phloretin was estimated based on the degradation dynamics observed in cell culture (61, 62).
Acknowledgements. We thank Martine Gilet for skilled technical assistance, Sabine Werner (Swiss Federal Institute of Technology Zurich, Zurich, Switzerland) for providing primary human fibroblasts and keratinocytes and Marcia Schoenberg, Marcel Tigges and William Bacchus for critical comments on the manuscript. This work was supported by the Swiss National Science Foundation (grant no. 3100A0-112549) and in part by the EC Framework Program 7 (PERSIST).
References

Chapter II


Chapter II


Supporting Information:

Results

Stability and impact of phloretin on mammalian cell cultures. To evaluate the stability of phloretin in culture medium and assess its impact on mammalian cells, we have supplemented standard ChoMaster® HTS medium with increasing concentrations of phloretin (0, 25, 50, 75, 100µM) and incubated this medium in the presence and absence of CHO-K1 engineered for constitutive SEAP expression (pSEAP2-Control; P_{SV40}-SEAP-pA, Clontech). The SEAP levels in samples taken from the cultures after 48h indicated that phloretin had no negative impact on heterologous protein production which is an indicator for metabolic integrity and viability (1). Parallel scoring of viable cell density confirmed that the apple flavonoid did not compromise CHO-K1 proliferation up to 100µM (Fig. S1). Phloretin levels determined over a period of 64h in cell-free culture medium established a functional half-live of 70h for this flavonoid.

![Fig. S1. Impact of phloretin on cell proliferation and protein production. CHO-K1 engineered for constitutive SEAP expression were cultivated in culture medium supplemented with different phloretin concentrations (0-100 µM) and SEAP production (bars) as well as maximum cell density were assessed after 48h (line).](image-url)

Engineering and validation of different phloretin-dependent transactivator variants. Previous studies have suggested that transactivators equipped with different transactivation domains can influence the overall regulation
performance (inducer range, leakiness, maximum expression levels) of a particular transgene control system in specific cell types (2). We have therefore designed three different transactivators harboring either the VP16 (TtgA1; pMG11, TtgR-VP16) (3), the p65 (TtgA2; pMG18, TtgR-p65) (4) or the human E2F4 (TtgA3; pMG19, TtgR-E2F4) (5) transactivation domains. pMG11, pMG18 or pMG19 were co-transfected with pMG10 (P_{TtgR1-SEAP-pA}) into CHO-K1, HEK-293 as well as primary human fibroblasts and keratinocytes, and SEAP expression was profiled after 48h of cultivation in the presence or absence of 50μM phloretin. The maximum levels of transgene production varied significantly among different transactivators: TtgA1 enabled the highest transactivation in all cell lines, TtgA2 showed the best performance in human primary cells and TtgA3 activity was inferior in all situations (Table S1).

**Table S1**: Combinatorial profiling of different PEACE transactivators and promoters in various cell types

<table>
<thead>
<tr>
<th>SEAP Production (U/L)</th>
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<table>
<thead>
<tr>
<th>Phloretin (50μM)</th>
<th>pMG10/pMG11</th>
<th>pMG10/pMG18</th>
<th>pMG10/pMG19</th>
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<tbody>
<tr>
<td>Phloretin (50μM)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>23.6 ± 3.1</td>
<td>0.2 ± 0.02</td>
<td>15.1 ± 0.1</td>
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<td>HEK-293</td>
<td>27.3 ± 0.8</td>
<td>0.2 ± 0.04</td>
<td>29.4 ± 1.6</td>
</tr>
<tr>
<td>HaCaT</td>
<td>0.9 ± 0.03</td>
<td>*</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Primary human fibroblasts</td>
<td>1.0 ± 0.04</td>
<td>*</td>
<td>2.6 ± 0.1</td>
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<tr>
<td>Primary human keratinocytes</td>
<td>1.5 ± 0.05</td>
<td>*</td>
<td>11.9 ± 1.3</td>
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</table>

SEAP production was quantified 48 h after transient co-transfection of pMG10 (P_{TtgR1-SEAP-pA}) and either pMG11 (P_{SV40-TtgA1-pA}; TtgR-VP16), pMG18 (P_{SV40-TtgA2-pA}; TtgR-p65) or pMG19 (P_{SV40-TtgA3-pA}; TtgR-E2F4).

*Undetectable

All transactivators mediated similar basal expression levels when phloretin was present in the medium, but due to their cell-type specificity and graded maximum
transcription-initiation capacities, the three transactivators offer a selection of defined expression windows.

**Phloretin-responsive promoter variants that differ in the distance between O\textsubscript{TtgR} and minimal promoter.** The torsion angle and the distance between operator-bound transactivator and minimal promoter play central roles in the efficient assembly of the transcription-initiation machinery (6, 7). With the goal of achieving the optimal design for P\textsubscript{TtgR} configurations we engineered linkers of 2bp increments, ranging from 0 to 10bp, between O\textsubscript{TtgR} and P\textsubscript{hCMVmin} and generated SEAP expression vectors which are isogenic to pMG10 (P\textsubscript{TtgR1}, pMG10, O\textsubscript{TtgR}-0bp-P\textsubscript{hCMVmin}; P\textsubscript{TtgR2}, pMG20, O\textsubscript{TtgR}-2bp-P\textsubscript{hCMVmin}; P\textsubscript{TtgR3}, pMG21, O\textsubscript{TtgR}-4bp-P\textsubscript{hCMVmin}; P\textsubscript{TtgR4}, pMG22, O\textsubscript{TtgR}-6bp-P\textsubscript{hCMVmin}; P\textsubscript{TtgR5}, pMG23, O\textsubscript{TtgR}-8bp-P\textsubscript{hCMVmin}; P\textsubscript{TtgR6}, pMG24, O\textsubscript{TtgR}-10bp-P\textsubscript{hCMVmin}) (see Table S3). Each of the SEAP expression vectors harboring phloretin-responsive promoter variants were co-transfected with pMG11 (P\textsubscript{SV40-TtgA1-pA}), pMG18 (P\textsubscript{SV40-TtgA2-pA}) or pMG19 (P\textsubscript{SV40-TtgA3-pA}) into CHO-K1 and SEAP production was profiled after cultivation for 48h in medium containing 0, 25 and 50\mu M phloretin (Fig. S2A-C). P\textsubscript{TtgR1} harboring 0bp between O\textsubscript{TtgR} and P\textsubscript{hCMVmin} drove maximum SEAP expression and showed the tightest repression. pMG20, pMG21 and pMG24, with increments of 2, 4 and 10bp exhibited similar regulation performance. However, 6 and 8bp increments (pMG22 and pMG23) had a strong negative effect on maximum transgene expression and repression. All promoter variants showed similar TtgA-specific expression profiles indicating that performance of transactivators and chimeric promoters can independently be optimized (Fig. S2A-C).

![Bar chart showing SEAP production profiles for different promoter configurations.](image-url)
Fig. S2. Design and combinatorial characterization of different PEACE transactivator and promoter configurations. PEACE transactivators harboring different transactivation domains (A, TtgA1, VP16; pMG11) (B, TtgA2, p65; pMG18) (C, TtgA3, E2F4; pMG19) were co-transfected with SEAP-driving PEACE promoter variants containing 0 (P_TtgR1; pMG10), 2 (P_TtgR2; pMG20), 4 (P_TtgR3; pMG21), 6 (P_TtgR4; pMG22), 8 (P_TtgR5; pMG23) and 10 (P_TtgR6; pMG24) base pair linkers between the TtgR operator and the minimal promoter into CHO-K1 and SEAP expression was profiled after 48h of cultivation in medium supplemented with different phloretin concentrations.

**Autoregulated phloretin-inducible transgene expression.** In addition to the classical two-vector design (transactivator and responsive promoter encoded on separate plasmids) we constructed an autoregulated version of the synthetic phloretin control system, which enables simultaneous P_TtgR1-driven expression of the transactivator (TtgA1) and the transgene (SEAP) in a single-vector format. This set-up has been shown to overcome undesirable variation in the expression of transient transfectionsx and is suitable for the design of noise-resistant gene networks (8).
Following transfection of pMG13 (P_{TtgR1}-SEAP-IRESPV-TtgA1-pA) in CHO-K1, leaky P_{TtgR1}-driven transcripts lead to cap-independent translation of initial TtgA1, mediated by the polioviral internal ribosome entry site (IRES_{PV}), which triggers, in an auto-regulated positive feedback loop, full expression of TtgA1 along with co-cistronically encoded SEAP. In the presence of 50\textmu M phloretin the positive feedback loop is interrupted as TtgA1 no longer binds P_{TtgR1} and SEAP is completely repressed (Fig. S3). The maximum SEAP production achieved by the auto-regulated PEACE configuration is comparable to levels reached by the binary system with constitutive transactivator expression (Fig. 1C) indicating that both arrangements produce sufficient transactivator to fully saturate the inducible promoter.

![Fig. S3. Autoregulated PEACE-controlled SEAP expression in CHO-K1. pMG13 encoding a P_{TtgR1}-driven dicistronic expression unit harboring SEAP in the first and TtgA1 in the second cistron was transfected into CHO-K1 and SEAP expression was assessed 48h after cultivation in the presence (50\textmu M) and absence of phloretin.](image)

**Compatibility of PEACE control with other transgene regulation systems.**

The design of complex synthetic mammalian transgene networks such as the recently reported mammalian oscillator requires availability of several control modalities which are compatible and co-operate in an interference-free manner (9-12). To evaluate the functional compatibility of PEACE with the established tetracycline-[TETOFF; (13)] and macrolide- [EOFF; (14)] responsive expression systems, we transiently transfected CHO-PEACE8, transgenic for phloretin-responsive SEAP expression, with either the TETOFF- (pSAM200, P_{SV40-tTA-pA}; pBP99, P_{hCMV*-1}-
SAMY-pA) or the E\textsubscript{OFF}- (pWW35, P\textsubscript{SV40-ET1-pA}; pBP100, P\textsubscript{ETR3-SAMY-pA}) system set to control the *Bacillus stearothermophilus* -derived secreted α-amylase (SAMY) in response to tetracycline and erythromycin, respectively. SEAP and SAMY expression were scored 48h after cultivation of the transfected populations in the presence (50\mu M; 2\mu g/mL) or absence of the inducer molecules (phloretin, tetracycline, erythromycin). Analysis of cross-regulation showed no interference between PEACE, TET\textsubscript{OFF}, and E\textsubscript{OFF} systems (Table S2 \textit{A} and \textit{B}).

\textbf{Table S2:} Compatibility of phloretin-, erythromycin- and tetracycline-responsive transgene control systems

\textbf{A:} CHO-PEACE\textsubscript{8} transiently transfected with the tetracycline-responsive control system

<table>
<thead>
<tr>
<th>Inducer</th>
<th>- Tet / - Phl</th>
<th>- Tet / + Phl</th>
<th>+ Tet / - Phl</th>
<th>+ Tet / + Phl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative SEAP Production (%)</td>
<td>100 ± 4.3</td>
<td>2.2 ± 0.2</td>
<td>93.2 ± 3.8</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Relative SAMY Production (%)</td>
<td>100 ± 5.2</td>
<td>98.1 ± 2.8</td>
<td>3.0 ± 0.4</td>
<td>4.0 ± 1.0</td>
</tr>
</tbody>
</table>

\textbf{B:} CHO-PEACE\textsubscript{8} transiently transfected with macrolide-responsive control system

<table>
<thead>
<tr>
<th>Inducer</th>
<th>- EM / - Phl</th>
<th>- EM / + Phl</th>
<th>+ EM / - Phl</th>
<th>+ EM / + Phl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative SEAP Production (%)</td>
<td>100 ± 6.0</td>
<td>2.0 ± 1.2</td>
<td>102.7 ± 5.3</td>
<td>1.6 ± 0.9</td>
</tr>
<tr>
<td>Relative SAMY Production (%)</td>
<td>100 ± 5.2</td>
<td>100.6 ± 7.0</td>
<td>1.5 ± 1.8</td>
<td>1.7 ± 2.0</td>
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</tbody>
</table>

CHO-PEACE\textsubscript{8} were co-transfected with (\textit{A}) pSAM200 (P\textsubscript{SV40-tTA-pA}) and pBP99 (P\textsubscript{6CMV*-1-SAMY-pA}) or (\textit{B}) pWW35 (P\textsubscript{SV40-ET1-pA}) and pBP100 (P\textsubscript{ETR3-SAMY-pA}) and grown for 48h in the presence and absence of phloretin (Phl, 50\mu M), erythromycin (EM, 2\mu g/mL) or tetracycline (Tet, 2\mu g/mL) before SEAP and SAMY production was assessed.
Fig. S4. Design and characterization of stable CHO-PEACE cell lines transgenic for phloretin-responsive SEAP expression. CHO-K1 was stably co-transfected with pMG11 (P_{SV40-TtgA1-pA}) and pMG10 (P_{TtgR1-SEAP-pA}) and individual clones were assessed for phloretin-modulated SEAP expression after a cultivation period of 48h.

**Material and Methods**

**Expression vector design.** Table S3 lists all plasmids used in this study and provides detailed information about their construction.

**Table S3. Expression vectors and oligonucleotides designed and used in this study**

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or Source</th>
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<td>pBP10</td>
<td>Vector encoding a P_{ETR5}-driven SEAP expression unit (P_{ETR5-SEAP-pA}; P_{ETR5, ETR-2bp-PhCMVmin})</td>
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<td>pBP11</td>
<td>Vector encoding a P_{ETR6}-driven SEAP expression unit (P_{ETR6-SEAP-pA}; P_{ETR6, ETR-4bp-PhCMVmin})</td>
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<td>pBP12</td>
<td>Vector encoding a P_{ETR7}-driven SEAP expression unit (P_{ETR7-SEAP-pA}; P_{ETR7, ETR-6bp-PhCMVmin})</td>
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<td>pBP13</td>
<td>Vector encoding a P_{ETR8}-driven SEAP expression unit (P_{ETR8-SEAP-pA}; P_{ETR8, ETR-8bp-PhCMVmin})</td>
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<td>pBP14</td>
<td>Vector encoding a P_{ETR9}-driven SEAP expression unit (P_{ETR9-SEAP-pA}; P_{ETR9, ETR-10bp-PhCMVmin})</td>
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<td>pBP99</td>
<td>Vector encoding a tetracycline-responsive SAMY expression unit (P_{hCMV*-1-SAMY-pA}). pCF59 was restricted with BprPl/EcoRV and religated.</td>
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<td>pBP100</td>
<td>Vector encoding an erythromycin-responsive SAMY expression unit (P_{ETR3-SAMY-pA})</td>
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<td>pCF59</td>
<td>Vector encoding P_{PIR}-driven SAMY expression (P_{hCMV*-1-pA-IRES-P_{PIR}-SAMY-pA}). SAMY was excised from pSS158 using SpeI/BgII and ligated into pMF187 (SpeI/BgII).</td>
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<td>pMF111</td>
<td>Vector encoding a P_{hCMV*-1}-driven SEAP expression unit (P_{hCMV*-1-SEAP-pA})</td>
<td>(16)</td>
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Chapter II

<table>
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<th>pMF187</th>
<th>Dual-regulated expression vector (P&lt;sub&gt;CMV*1&lt;/sub&gt;-MCSI-ires-MCSII-pAI-P&lt;sub&gt;PIR&lt;/sub&gt;-MCSIII-pAII)</th>
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<td>pMG9</td>
<td>Vector encoding O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-ET1-pA; O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt; was PCR-amplified from pRevTRE using OMG21: 5'-gatacaagcttgacgctcCAGTA TITACAAACACATTGAATTATCTCCTGAGTCGAC TCGGTACCCGGGTC-3' and OW22: 5'-gctagattcCGCGGAGGCTGGA TCGG-3' (upper case, annealing sequence; lower case italics, restriction sites; upper case italics, O&lt;sub&gt;TtgR&lt;/sub&gt;), digested with AatII/EcoRI and ligated into pWW35 (AatII/EcoRI).</td>
<td>This work</td>
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<td>pMG10</td>
<td>Vector encoding a P&lt;sub&gt;TtgR1&lt;/sub&gt;-driven SEAP expression unit (P&lt;sub&gt;TtgR1&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR1&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt; was excised from pMG9 (SspI/EcoRI) and ligated into pMF111 (SspI/EcoRI).</td>
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<td>pMG11</td>
<td>Constitutive TtgA&lt;sub&gt;1&lt;/sub&gt; expression vector (P&lt;sub&gt;SV40&lt;/sub&gt;-TtgA&lt;sub&gt;1&lt;/sub&gt;-pA). TtgR was excised from pUC19-TtgR (EcoRI/BssHII) and ligated into pWW35 (EcoRI/BssHII).</td>
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<td>pMG13</td>
<td>Autoregulated phloretin-controlled SEAP expression vector (P&lt;sub&gt;TtgR1&lt;/sub&gt;-SEAP-ires&lt;sub&gt;P&lt;/sub&gt;-TtgA&lt;sub&gt;1&lt;/sub&gt;-pA). TtgA&lt;sub&gt;1&lt;/sub&gt; was excised from pMG11 (SspI/NotI) and ligated into pMG10 (SspI/NotI).</td>
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<td>pMG18</td>
<td>Constitutive TtgA&lt;sub&gt;2&lt;/sub&gt; expression vector (P&lt;sub&gt;SV40&lt;/sub&gt;-TtgA&lt;sub&gt;2&lt;/sub&gt;-pA). p65 was excised from pWW42 (BssHII/BamHI) and ligated into pMG11 (BssHII/BamHI).</td>
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<td>pMG19</td>
<td>Constitutive TtgA&lt;sub&gt;3&lt;/sub&gt; expression vector (P&lt;sub&gt;SV40&lt;/sub&gt;-TtgA&lt;sub&gt;3&lt;/sub&gt;-pA). The E2F4 transactivation domain was excised from pWW64 (BssHII/BamHI) and ligated into pMG11 (BssHII/BamHI).</td>
<td>This work</td>
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<td>pMG20</td>
<td>Vector encoding a P&lt;sub&gt;TtgR2&lt;/sub&gt;-driven SEAP expression unit (P&lt;sub&gt;TtgR2&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR2&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). 2bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-SEAP was excised from pBP10 (SspI/EcoRI) and ligated into pMG10 (SspI/EcoRI).</td>
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<td>Vector encoding a P&lt;sub&gt;TtgR3&lt;/sub&gt;-driven SEAP expression unit (P&lt;sub&gt;TtgR3&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR3&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). 4bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-SEAP was excised from pBP11 (SspI/EcoRI) and ligated into pMG10 (SspI/EcoRI).</td>
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<td>pMG22</td>
<td>Vector encoding a P&lt;sub&gt;TtgR4&lt;/sub&gt;-driven SEAP expression unit (P&lt;sub&gt;TtgR4&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR4&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). 6bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-SEAP was excised from pBP12 (SspI/EcoRI) and ligated into pMG10 (SspI/EcoRI).</td>
<td>This work</td>
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<td>pMG23</td>
<td>Vector encoding a P&lt;sub&gt;TtgR5&lt;/sub&gt;-driven SEAP expression unit (P&lt;sub&gt;TtgR5&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR5&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-0bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). 8bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-SEAP was excised from pBP13 (SspI/EcoRI) and ligated into pMG10 (SspI/EcoRI).</td>
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<td>pMG24</td>
<td>Vector encoding a P&lt;sub&gt;TtgR6&lt;/sub&gt;-driven expression unit (P&lt;sub&gt;TtgR6&lt;/sub&gt;-SEAP-pA; P&lt;sub&gt;TtgR6&lt;/sub&gt;, O&lt;sub&gt;TtgR&lt;/sub&gt;-10bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;). 10bp-P&lt;sub&gt;CMVmin&lt;/sub&gt;-SEAP was excised from pBP14 (SspI/EcoRI) and ligated into pMG10 (SspI/EcoRI).</td>
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<td>pPur</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
<td>Supplier</td>
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<td>Selection vector conferring neomycin resistance</td>
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<td>pUC19-TtgR</td>
<td>Cloning vector containing the TtgR (E&lt;sub&gt;coRI&lt;/sub&gt;-TtgR-BssHII)</td>
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<td>pWW35</td>
<td>Constitutive ET1 expression vector (P&lt;sub&gt;SV40&lt;/sub&gt;-ET1-pA)</td>
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<td>pWW64</td>
<td>Constitutive ET3 expression vector (P&lt;sub&gt;SV40&lt;/sub&gt;-ET3-pA)</td>
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</table>

Abbreviations: E2F4, transactivation domain of the human E2F transcription factor 4; ET1, macrolide-dependent transactivator (E-VP16); ET2, macrolide-dependent transactivator (E-p65); ET3, macrolide-dependent transactivator (E-E2F4); ETR, operator specific for macrolide-dependent transactivators; IRES<sub>PV</sub>, polioviral internal ribosome entry site; NF-κB, human transcription factor; OTtgR, TtgR-specific operator; p65, transactivation domain of NF-κB; pA, polyadenylation site; P<sub>ETR3-9</sub>, macrolide-responsive promoters containing different spacers between ETR and P<sub>hCMVmin</sub>; P<sub>hCMV</sub>, human cytomegalovirus immediate early promoter; P<sub>hCMVmin</sub>, minimal P<sub>hCMV</sub>; P<sub>hCMV*-1</sub>, tetracycline-responsive promoter; P<sub>PTR3</sub>, streptogramin-responsive promoter; P<sub>SV40</sub>, simian virus 40 promoter; P<sub>TtgR1-6</sub>, phloretin-responsive promoters containing different spacers between OTtgR and P<sub>hCMVmin</sub>; SEAP, human placental secreted alkaline phosphatase; SAMY, Bacillus stearothermophilus-derived secreted α-amylase; TtgR, repressor of the Pseudomonas putida DOT-T1E ABC multi-drug efflux pump; TtgA<sub>1</sub>, phloretin dependant transactivator (TtgR-VP16); TtgA<sub>2</sub>, phloretin dependant transactivator (TtgR-p65); TtgA<sub>3</sub>, phloretin dependant transactivator (TtgR-E2F4); VP16, Herpes simplex virus-derived transactivation domain.

**Quantification of reporter protein production.** Bacillus stearothermophilus-derived secreted α-amylase (SAMY) levels were assessed using the blue starch Phadebas® assay (Pharmacia Upjohn, Peapack, NJ, USA; Cat. No. 10-5380-32) (15).

**Inducer compounds.** Tetracycline (Sigma, Cat. No. T7660) was prepared as a 1mg/mL stock solution in H<sub>2</sub>O and erythromycin (Fluka, Buchs, Switzerland, Cat. No. 45673) as a stock solution of 1mg/mL in ethanol. Both antibiotics were used at a final concentration of 2µg/mL.
SI References


Chapter III

Transcription Control In Mammalian Cells and Mice Via The Food Additive Vanillic Acid

Marc Gitzinger, Christian Kemmer, David A. Fluri, Marie Daoud El-Baba, Wilfried Weber and Martin Fussenegger

Submitted
Abstract

Heterologous transgene expression systems in mammalian cells are essential tools for successfully studying metabolic pathways, producing difficult-to-express proteins and, most importantly, they constitute a toolbox for mammalian synthetic biology and novel gene therapy approaches. To exploit the full potential of such synthetic regulation assemblies, the access to response regulators that become activated by either (i) physiologically inert and ideally already licensed inducers such as food additives and cosmetics or, (ii) which directly respond to mammalian-derived metabolites is crucial. Living in oligotrophic freshwater habitats, the Gram-negative bacterium *Caulobacter crescentus* adapted to metabolize vanillic acid, a phenolic acid that results from lignin degradation in plant decay. The VanR-response regulator of *Caulobacter crescentus* regulates the metabolic pathway enabling the usage of vanillic acid as a carbon source. Employing the VanR-operon, we engineered a synthetic mammalian gene regulation system responsive to vanillic acid (VAC), which is a licensed food additive regularly consumed by humans via vegetables and fruits and thus can be considered as safe at physiological concentrations. The VAC system was distinguished by its unprecedented specificity towards its inducer and by enabling adjustable and reversible gene expression control in several mammalian cell lines and in mice with high maximum expression levels. The VAC system has the potential to endorse advances in building complex synthetic networks and in gene- and cell-based therapies.

Introduction

Fine-tuning of gene expression via synthetic mammalian transgene regulation systems has been playing a pivotal role in functional genomics research (1), the biopharmaceutical manufacturing of complex proteins, even across species (2-4), drug discovery (5,6), tissue engineering (7-9), the design of complex synthetic networks (10-13), gene therapy (9,14,15) and the design of functional materials (16). The sheer amount of basic and applied research areas directly relying on the adjustability and reversibility of gene activity explains the high demand for ever-improving synthetic transgene control systems, which enable intricate regulation of a single gene of
interest up to complex synthetic networks. The majority of currently available mammalian transgene control systems benefit from a generic design principle comprising a prokaryotic DNA-binding response regulator fused to a mammalian transactivation (transrepression) domain, thus forming a synthetic transactivator (transrepressor) with the ability to activate (or repress) a hybrid promoter engineered from a specific operator in close proximity to a eukaryotic minimal (constitutive) promoter (17-19). Employing these modular building blocks offers the possibility of creating two output functions depending on the ability of an inducer molecule to either (i) activate (ON-type system) (20-23) or (ii) repress (OFF-type system) (20,24) gene expression upon binding to the transactivator (transrepressor) protein. The first wave of mammalian transcription control systems resulted in an array of various inducer molecules enabling gene regulation, ranging from antibiotics (18,20,21), to steroid hormones and their analogues (25,26), quorum sensing molecules (22,27) and immunosuppressive and anti-diabetic drugs (28,29). Most of these established gene regulation systems display reliable regulatory behaviours with a low level of leakage and high maximum transgene expression levels in vitro and in animals. However, recent advances in the field of synthetic biology demand physiologically inert and traceless inducer molecules in order to minimize potential side effects when applied in sophisticated gene therapy approaches (15,16), and no interference with host organisms within complex synthetic networks (12,13,30) or drug screening approaches (6). Therefore, the second generation of synthetic mammalian transgene regulation systems strives for inducer molecules that are directly derived from endogenous metabolites, such as amino acids (31), vitamins (32,33), gaseous acetaldehyde (23), pathologic signals, such as urate (15), or which are already licensed as cosmetic and food additives (6,34). Utilizing these trigger molecules in the most recent synthetic biology networks resulted in unprecedented advances in the field and could result, in the not too distant future, in the first human gene therapy trials capitalizing on synthetic biology (35-37).

Vanillic acid (or vanillate) is a naturally occurring compound which belongs to the class of phenolic acids and is primarily found in vanilla beans (38,39). As a flavouring and scent agent that produces a pleasant, creamy odour, vanillic acid is
registered by the Joint Food and Agriculture Organization (FAO) as a food additive (FAO/WHO Expert Committee on Food Additives, JECFA no. 959). In the chemical synthesis of vanillin, vanillic acid is an intermediate between ferulic acid and vanillin (40). Furthermore, it is also a metabolic by-product of several natural compounds regularly consumed by humans, such as caffeic acid, and therefore often occurs in human urine (41). Vanillic acid has also been evaluated as a pharmacophore for several applications and was shown to act as a specific inhibitor of the snake venom 5’-nucleotidase, which targets 5’-AMP (42,43), and of carcinogenesis (44), where it suppressed cell apoptosis induced by methylglyoxal in Neuro-2A cells (45,46). Vanillic acid also exhibits a hepatoprotective effect by suppressing immune-mediated liver inflammation in mice (47). Therefore, vanillic acid would be a desirable, physiologically inert inducer molecule for expanding the portfolio of available second-generation gene regulation systems.

_Caulobacter crescentus_ is a Gram-negative, oligotrophic freshwater bacterium that plays an important role in the carbon cycle by disposing of the soluble phenolic intermediates that are released from the fungal oxidative cleavage of lignin, derived from decaying vascular plant material (48-50). Vanillic acid is a predominant component of these phenolic intermediates. _Caulobacter crescentus_ has the ability to utilize vanillic acid as its sole carbon source, which implies it has the capability of converting vanillic acid into protocatechuate in order to cleave the aromatic ring to finally yield succinyl-CoA and acetyl-CoA, which can directly enter the citric acid cycle (51,52). In many bacteria, O-demethylation in the first step of the aforementioned metabolic pathway is carried out by a two-component monooxygenase encoded by the VanAB gene cluster, which is regulated by a transcriptional repressor, VanR (51,53-56).

Capitalizing on the vanillic acid-responsive transcriptional repressor VanR derived from _C. crescentus_, we engineered a synthetic vanillic acid-responsive mammalian expression system (VAC) which exhibits excellent regulation performance in mammalian cell cultures and when implanted in mice.
Methods

Plasmid construction. Table 1 lists all the plasmids used in this study and provides detailed information about their construction.

Table 1. Expression vectors and oligonucleotides designed and used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or Source</th>
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<tbody>
<tr>
<td>pBP99</td>
<td>Vector encoding a tetracycline-responsive SAMY expression unit (P_{PlCMV*-1}-SAMY-pA). pCF59 was restricted with BprPl/EcoRV and religated.</td>
<td>unpublished</td>
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<tr>
<td>pBP100</td>
<td>Vector encoding an erythromycin-responsive SAMY expression unit (P_{Estr3}-SAMY-pA)</td>
<td>Weber et al. 2002b</td>
</tr>
<tr>
<td>pCF59</td>
<td>Vector encoding a P_{Plr}-driven SAMY expression unit (P_{hCMV*-1}-pA-IRES-P_{Plr}-SAMY-pA). SAMY was excised from pSS158 using SpeI/BglII and ligated into pMF187 (SpeI/BglIII).</td>
<td>unpublished</td>
</tr>
<tr>
<td>pMG10</td>
<td>Vector encoding a P_{TgrR1}-driven SEAP expression unit (P_{TgrR1}-SEAP-pA; P_{TgrR1}, O_{TgrR-0bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG18</td>
<td>Constitutive TtgA2 expression vector (P_{SV40-TtgA2-pA}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG19</td>
<td>Constitutive TtgA3 expression vector (P_{SV40-TtgA3-pA}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG20</td>
<td>Vector encoding a P_{TgrR2}-driven SEAP expression unit (P_{TgrR2}-SEAP-pA; P_{TgrR2}, O_{TgrR-2bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG21</td>
<td>Vector encoding a P_{TgrR3}-driven SEAP expression unit (P_{TgrR3}-SEAP-pA; P_{TgrR3}, O_{TgrR-4bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG22</td>
<td>Vector encoding a P_{TgrR4}-driven SEAP expression unit (P_{TgrR4}-SEAP-pA; P_{TgrR4}, O_{TgrR-6bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG23</td>
<td>Vector encoding a P_{TgrR5}-driven SEAP expression unit (P_{TgrR5}-SEAP-pA; P_{TgrR5}, O_{TgrR-8bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG24</td>
<td>Vector encoding a P_{TgrR6}-driven expression unit (P_{TgrR6}-SEAP-pA; P_{TgrR6}, O_{TgrR-10bp-P_{hCMVmin}}).</td>
<td>Gitzinger et al. 2009</td>
</tr>
<tr>
<td>pMG250</td>
<td>Constitutive VanA1 expression vector (P_{SV40-VanA1-pA}). VanR was PCR-amplified from Caulobacter crescentus genomic DNA using ODF150: 5'-cggacttccacATGGACATGCGCGCATAAAGCCGGGCGC-3' and ODF151: 5'-gtacgaacctggctggaGGCAGATGCGCGCGATAAGCCGGGCGC-3' (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pWW35 (EcoRI/BssHII).</td>
<td>This work</td>
</tr>
<tr>
<td>pMG252</td>
<td>Vector encoding a P_{1VanO2}-driven SEAP expression unit (P_{1VanO2}-SEAP-pA; P_{1VanO2}, VanO2-0bp-P_{hCMVmin}). VanO2 was created by annealing Oligos OMG65 (5'-phosphate-</td>
<td>This work</td>
</tr>
</tbody>
</table>
Chapter III

| pMG256 | Constitutive VanA\textsubscript{2} expression vector (P\textsubscript{SV40}-VanA\textsubscript{2}-pA). VanR was PCR-amplified from \textit{Caulobacter crescentus} genomic DNA using ODF150: 5’-cgagaattccac\textit{ATGGACATGCGCGC\textsc{G}AT\textsc{G}CGC\text{ATAA}\textsc{G}CGCGG}\textsc{G}G\textsc{C}-3’ and ODF151: 5’-gtacgacgc\textit{GGCGCGCAATGCT CCAGCGCGCGC}-3’ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pMG18 (EcoRI/BssHII). |
|        | This work |

| pMG257 | Constitutive VanA\textsubscript{3} expression vector (P\textsubscript{SV40}-VanA\textsubscript{3}-pA). VanR was PCR-amplified from \textit{Caulobacter crescentus} genomic DNA using ODF150: 5’-cgagaattccac\textit{ATGGACATGCGCGC\textsc{G}AT\textsc{G}CGC\text{ATAA}\textsc{G}CGCGG}\textsc{G}G\textsc{C}-3’ and ODF151: 5’-gtacgacgc\textit{GGCGCGCAATGCT CCAGCGCGCGC}-3’ (lower case italics, restriction sites; upper case italics, annealing), digested with EcoRI/MluI and ligated into pMG19 (EcoRI/BssHII). |
|        | This work |

| pMG262 | Vector encoding a P\textsubscript{1VanO1}-driven SEAP expression unit (P\textsubscript{1VanO1}-SEAP-pA; P\textsubscript{1VanO1}, VanO\textsubscript{1}-0bp-PhCMV\textsc{m}in). pMG252 was digested using either NruI/HindIII or Eco47III/HindIII. The resulting fragments were ligated to result in pMG262 harboring one VanO-operator element. |
|        | This work |

| pMG263 | Vector encoding a P\textsubscript{1VanO3}-driven SEAP expression unit (P\textsubscript{1VanO3}-SEAP-pA; P\textsubscript{1VanO3}, VanO\textsubscript{3}-0bp-PhCMV\textsc{m}in). pMG252 was digested using either EcoRV/HindIII or Eco47III/HindIII. The resulting fragments were ligated to result in pMG263 harboring three VanO-operator elements. |
|        | This work |

| pMG264 | Vector encoding a P\textsubscript{1VanO4}-driven SEAP expression unit (P\textsubscript{1VanO4}-SEAP-pA; P\textsubscript{1VanO4}, VanO\textsubscript{4}-0bp-PhCMV\textsc{m}in). pMG252 was digested using either EcoRV/HindIII or NruI/HindIII. The resulting fragments were ligated to result in pMG264 harboring four VanO-operator elements. |
|        | This work |

| pMG265 | Vector encoding a P\textsubscript{2VanO2}-driven SEAP expression unit (P\textsubscript{2VanO2}-SEAP-pA; P\textsubscript{2VanO2}, VanO\textsubscript{2}-2bp-PhCMV\textsc{m}in). 2bp-PhCMV\textsc{m}in-SEAP was excised from pMG20 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI) |
|        | This work |

| pMG266 | Vector encoding a P\textsubscript{3VanO2}-driven SEAP expression unit (P\textsubscript{3VanO2}-SEAP-pA; P\textsubscript{3VanO2}, VanO\textsubscript{2}-4bp-PhCMV\textsc{m}in). 4bp-PhCMV\textsc{m}in-SEAP was excised from pMG21 (SbfI/XhoI) and ligated into pMG252 (SbfI/XhoI) |
|        | This work |
### Abbreviations:

- **E2F4**, transactivation domain of the human E2F transcription factor 4;
- **ET1**, macrolide-dependent transactivator (E-VP16);
- **ETR**, operator specific for macrolide-dependent transactivators;
- **IRESPV**, polioviral internal ribosome entry site;
- **NF-κB**, human transcription factor;
- **OTtgR**, TtgR-specific operator;
- **p65**, transactivation domain of NF-κB;
- **pA**, polyadenylation site;
- **P ETR3**, macrolide-responsive promoter;
- **PhCMV**, human cytomegalovirus immediate early promoter;
- **PhCMVmin**, minimal PhCMV;
- **PhCMV*-1**, tetracycline-responsive promoter;
- **PSV40**, simian virus 40 promoter;
- **PTtgR1-6**, phloretin-responsive promoters containing different spacers between O TtgR and PhCMVmin;
- **P1-6VanO2**, vanillic acid-responsive promoters containing different spacers between VanO and PhCMVmin;
- **P1VanO1-4**, vanillic acid-responsive promoters harboring 1, 2, 3 or 4 VanO-operator repeats in front of PhCMVmin;
- **SEAP**, human placental secreted alkaline phosphatase;
- **SAMY**, Bacillus stearothermophilus-derived secreted α-amylase;
- **TtgR**, repressor of the *Pseudomonas putida* DOT-T1E ABC multi-drug efflux pump;
- **TtgA2**, phloretin-dependent transactivator (TtgR-p65);
- **TtgA3**, phloretin-dependent transactivator (TtgR-E2F4);
- **VanAB**, gene cluster within *Caulobacter crescentus* that plays a role within the vanillic acid metabolism;
- **VanO**, VanR specific operator;
- **VanR**, repressor of the *Caulobacter crescentus* VanAB gene cluster;
- **VP16**, Herpes simplex virus-derived transactivation domain.

### Document Content

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>pMG267</strong></td>
<td>Vector encoding a P_{4VanO2}-driven SEAP expression unit (P_{4VanO2}-SEAP-pA; P_{VanO2}, VanO_{-6bp-PhCMVmin}). 6bp-PhCMVmin-SEAP was excised from pMG22 (SbfI/Xhol) and ligated into pMG252 (SbfI/Xhol).</td>
<td>This work</td>
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<tr>
<td><strong>pMG268</strong></td>
<td>Vector encoding a P_{5VanO2}-driven SEAP expression unit (P_{5VanO2}-SEAP-pA; P_{VanO2}, VanO_{-8bp-PhCMVmin}). 8bp-PhCMVmin-SEAP was excised from pMG23 (SbfI/Xhol) and ligated into pMG252 (SbfI/Xhol).</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pMG269</strong></td>
<td>Vector encoding a P_{6VanO2}-driven SEAP expression unit (P_{6VanO2}-SEAP-pA; P_{VanO2}, VanO_{-10bp-PhCMVmin}). 10bp-PhCMVmin-SEAP was excised from pMG24 (SbfI/Xhol) and ligated into pMG252 (SbfI/Xhol).</td>
<td>This work</td>
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<tr>
<td><strong>pMG270</strong></td>
<td>Autoregulated vanillic acid-controlled SEAP expression vector (P_{1VanO2-SEAP-IRESPV-VanA1-pA}). VanA1 was excised from pMG250 (SspI/NotI) and ligated into pMG252 (SspI/NotI).</td>
<td>This work</td>
</tr>
<tr>
<td><strong>pPur</strong></td>
<td>Selection vector conferring puromycin resistance</td>
<td>Clontech, Palo Alto, CA, USA</td>
</tr>
<tr>
<td><strong>pSAM200</strong></td>
<td>Constitutive tTA expression vector (P_{SV40-tTA-pA})</td>
<td>Fussenegger et al. 1997</td>
</tr>
<tr>
<td><strong>pSEAP2-Control</strong></td>
<td>Constitutive SEAP expression vector (P_{SV40-SEAP-pA})</td>
<td>Clontech, Palo Alto, CA, USA</td>
</tr>
<tr>
<td><strong>pSS158</strong></td>
<td>P_{hCMV}-driven SAMY expression vector (P_{hCMV-SAMY-pA})</td>
<td>Schlatter et al. 2002</td>
</tr>
<tr>
<td><strong>pSV2neo</strong></td>
<td>Selection vector conferring neomycin resistance</td>
<td>Clontech, Palo Alto, CA, USA</td>
</tr>
<tr>
<td><strong>pWW35</strong></td>
<td>Constitutive ET1 expression vector (P_{SV40-ET1-pA})</td>
<td>Weber et al. 2002b</td>
</tr>
</tbody>
</table>
Cell culture. Wild-type Chinese hamster ovary cells (CHO-K1, ATCC: CCL-61) and their derivatives were cultivated in standard medium: ChoMaster® HTS (Cell Culture Technologies, Gravesano, Switzerland) supplemented with 5% (v/v) foetal calf serum (FCS, PAN Biotech GmbH, Aidenbach, Germany, Cat. No. 3302, Lot No. P251110) and 1% (v/v) penicillin/streptomycin solution (Biowest, Nuaillé, France, Cat. No. L0022-100, Lot No. S07497L0022). Human embryonic kidney cells, transgenic for the Simian virus 40 (SV40) large T-antigen [(HEK-293T, (57)], human cervical carcinoma cells (HeLa, ATCC: CCL-2), African green monkey kidney cells (COS-7, ATCC: CRL-1651), baby hamster kidney cells (BHK-21, ATCC: CCL10), human fibrosarcoma cells (HT-1080, ATCC: CCL-121) and mouse fibroblasts (NIH/3T3, ATCC CRL-1658) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS and 1% penicillin/streptomycin solution. All cell lines were cultivated at 37 °C in a humidified atmosphere containing 5% CO2.

Transfection. The CHO-K1 cells were transiently transfected using 0.5 µg of total plasmid DNA (an equal amount of each plasmid was used for co-transfections) according to an optimized calcium phosphate-based protocol which resulted in typical transfection efficiencies of 35% ± 5% (58). In brief, 50,000 CHO-K1 cells were seeded into each well of a 24-well plate and cultured overnight. The plasmid DNA was then diluted to a total volume of 25 µl with 0.5 M CaCl₂ solution, which was mixed with 25 µl 2× HBS solution (50 mM HEPES/280 mM NaCl/1.5 mM Na₂HPO₄, pH 7.1). This mixture was incubated for 15 min at room temperature before the precipitates were directly added into the well and centrifuged onto the cells (5 min at 1200 × g). After 3 h, the cells were treated with 0.5 ml glycerol solution (ChoMaster® HTS medium containing 15% glycerol) for 60 s. After washing once with phosphate-buffered saline (PBS, Dulbecco’s phosphate-buffered saline; Invitrogen, Basel, Switzerland, Cat. No. 21600-0069), the cells were cultivated in 0.5 ml standard ChoMaster® HTS medium in the presence or absence of different concentrations of the inducer molecule (vanillic acid). For the transient transfection of BHK-21, COS-7 and HEK293-T cells, a plasmid DNA-Ca₂PO₄ precipitate was
prepared and applied to the 50,000 cells cultivated per well of a 24-well plate, as described above. The HEK293-T and COS-7 cells were washed once with PBS after 3 h incubation with the DNA-Ca\textsubscript{2}PO\textsubscript{4} precipitate and subsequently cultivated in standard DMEM, while BHK-21 and HeLa cells were incubated overnight with the precipitates and then cultivated in DMEM medium after being washed once with PBS. The HT-1080 and NIH/3T3 cell lines were transfected with Fugene™ 6 (Roche Diagnostics AG, Basel, Switzerland, Cat. No. 11814443001) according to the manufacturer’s protocol and cultivated in the cell culture medium specified above. After transfection, all cells were cultivated in DMEM supplemented with different concentrations of vanillic acid and reporter protein levels were profiled 48 h after transfection, unless otherwise indicated.

**Construction and characterization of the stable cell line CHO-VAC.** The CHO-VAC\textsubscript{12} cell line, transgenic for vanillic acid-controlled SEAP expression, was constructed by sequential co-transfection and clonal selection of (i) pMG250 (P\textsubscript{SV40}-VanA\textsubscript{1}-pA) and pSV2\textsubscript{neo} (Clontech, Cat. No. 6172-1) at a ratio of 20:1 to result in the cell line CHO-VanA; (ii) CHO VanA was subsequently co-transfected with pMG252 (P\textsubscript{1VanO2}-SEAP-pA) and pPur (Clontech, Cat. No. 6156-1) (ratio of 20:1), and the resulting double-transgenic cell line CHO-VAC\textsubscript{12}, showing vanillic acid-responsive SEAP production, was chosen after clonal selection. To assess the vanillic acid-mediated transgene regulation characteristics, 100,000 cells/ml of CHO-VAC\textsubscript{12} were cultivated for 48 h in standard ChoMaster® HTS medium supplemented with increasing concentrations of vanillic acid, ranging from 0 – 1000 µM. Reversibility of the vanillic acid-mediated SEAP production was assessed by culturing CHO-VAC\textsubscript{12} (100,000 cells/ml) for 144 h while alternating vanillic acid concentrations from 0 to 500 µM every 48 h.

**Quantification of reporter gene expression.** Production of the human placental secreted alkaline phosphatase (SEAP) was quantified using a \textit{p}-nitrophenylphosphate-based light absorbance time course (59,60).

**Inducer compounds: vanillic acid and its derivatives.** The following were prepared as 50 mM stock solutions in 70% (v/v) EtOH and the pH level was adjusted using 2.5 M NaOH when required: 2-vanillic acid (2-hydroxy-3-methoxybenzoic acid,
ABCPR, Karlsruhe, Germany, Cat. No. AB177480), 2-vanillin (2-hydroxy-3-methoxybenzaldehyde, ABCR, Cat. No. AB117268), acetovanillone (4’-hydroxy-3’-methoxyacetophenone, ABCR, Cat. No. AB125832), benzaldehyde (Acros, Geel, Belgium, Cat. No. 378361000), benzoic acid (ABCR, Cat. No. AB113879), benzyl acetate (ABCR, Cat. No. AB131641), benzyl alcohol (ABCR, Cat. No. AB171491), ethyl-vanillate (ABCR, Cat. No. AB178082), ethyl-vanillin (3-ethoxy-4-hydroxybenzaldehyde, ABCR, Cat. No. AB126381), eugenol (ABCR, Cat. No. AB111881), homovanillic acid (Sigma, St Louis, MO, USA, Cat. No. H1252-1G), isovanillic acid (3-hydroxy-4-methoxybenzoic acid, ABCR, Cat. No. AB117271), isovanillin (3-hydroxy-4-methoxybenzaldehyde, ABCR, Cat. No. AB117270), methyl-vanillate (ABCR, Cat. No. AB132603), protocatechualdehyde (3,4-dihydroxybenzaldehyde, ABCR, Cat. No. AB110948) and vanillin (ABCR, Cat. No. AB117415). Vanillic acid (Fluka, Buchs, Switzerland, Cat. No. 94770-10G) was prepared as a 50 mM stock solution in water and the pH level was adjusted with 2.5 M NaOH. All solutions were used at a final concentration of 250 µM unless indicated otherwise. Tetracycline (Sigma, Cat. No. T7660) was prepared as a 1 mg/ml stock solution in H2O, and erythromycin (Fluka, Cat. No. 45673) as a stock solution of 1 mg/ml in ethanol. Both antibiotics were used at a final concentration of 2 µg/ml.

**In vivo methods.** The CHO-K1 cells, engineered for vanillic acid-controlled SEAP expression (CHO-VAC12) and for constitutive SEAP expression [(CHO-SEAP18 (61)], were encapsulated into 400 µm alginate-poly-(L-lysine)-alginate beads (400 cells/capsule) using an Inotech Encapsulator Research IE-50R (EncapBioSystems Inc., Greifensee, Switzerland) according to the manufacturer’s instructions and the following parameters: 0.2 mm nozzle, 20 ml syringe at a flow rate of 405 units, nozzle vibration frequency of 1116 Hz and 950 V for bead dispersion. Female OF1 mice (oncins France souche 1, Charles River Laboratories, France) were injected intraperitoneally with 700 µl of FCS-free ChoMaster® HTS containing 4 × 10⁶ encapsulated CHO-VAC12 cells. Control mice were injected with microencapsulated CHO-K1 or CHO-SEAP18. One hour after implantation, vanillic acid was administered at doses ranging from 0 to 500 mg/kg. The injections of vanillic acid were repeated twice daily during the next three days. For in vivo administration
of vanillic acid, a stock solution was prepared at 50 mg/ml in PBS and adjusted to pH 7.0 using a 2.5 M NaOH solution. The vanillic acid stock solution was diluted in PBS, accordingly. After 72 h, the mice were sacrificed, blood samples were collected and SEAP levels were quantified in the serum which was isolated using a microtainer SST tube according to the manufacturer’s instructions (Beckton Dickinson, Plymouth, UK). All the experiments involving mice were performed according to the directives of the European Community Council (86/609/EEC), approved by the French Republic (No. 69266310) and performed by Marie Daoud El-Baba at the Université Claude Bernard-Lyon 1, F-69622 Villeurbanne, France.

Results

Design of a vanillic acid (VAC)-responsive mammalian expression system. We designed a mammalian synthetic gene regulation circuit, which is induced by a well-tolerated food additive, vanillic acid (vanillate). This system capitalizes on the Caulobacter crescentus-derived VanR repressor that binds a distinct perfect inverted repeat operator (VanO; ATTGGATCCAAT) upstream of the vanAB promoter region and regulates the vanAB genes which play a key role in vanillic acid metabolism (51). The design of this mammalian vanillic acid-responsive system (VAC) is built on two components, (i) a synthetic mammalian transactivator (VanA1), created by fusing VanR to the Herpes simplex-derived transactivation domain VP16 (62), and (ii) by a chimeric promoter (P1VanO2) harbouring two VanO-operator sequences, separated only by an Eco47III restriction site (VanO2, 5’-ATTGGATCCAATgcgctATTGGATCCAAT-3’, predicted VanO-boxes upper case) at the 5’-end of a minimal version of the human cytomegalovirus immediate-early promoter (P_{hCMVmin}) (Figure 1A and B). Co-transfection of pMG250 (P_{SV40-VanA1-pA}), constitutively expressing VanA1, and pMG252 (P_{1VanO2-SEAP-pA}), encoding the mammalian reporter gene SEAP (human placental alkaline phosphatase) under the control of a VanA1-responsive promoter (P_{2VanO}), resulted in high-level reporter protein expression of 20.3 ± 0.5 units per liter (U/l), which was comparable to the constitutively expressing SEAP control vector (pSEAP2-Control; P_{SV40-SEAP-pA}) (33.9 ± 0.2 U/l). Upon adding increasing concentrations of vanillic acid (0 – 250 µM)
to the cultures of CHO-K1 cells transiently co-transfected with pMG250 and pMG252, a dose-dependent reduction of SEAP expression, up to complete repression, was observed, suggesting that vanillic acid is able to regulate VanA₁-binding to its cognate operator site VanO₂ (Figure 1C).

Figure 1. Diagram and functionality of the VAC system. The bacterial transrepressor VanR from *Caulobacter crescentus* was fused to the VP16 transactivation domain of the *Herpes simplex* virus resulting in VanA₁ (VanR-VP16), which was expressed by the constitutive Simian virus 40 promoter (PSV40) (pMG250). The vanillic acid-responsive promoter P₁VanO₂ contains two VanO operator sites (ATTTGGATCCAAATAGCGCTATTGGATCCAAAT; VanR binding sites in italics) immediately 5’ of a minimal human cytomegalovirus immediate-early promoter (PhCMVmin), which was set to drive the human placental secreted alkaline phosphatase (SEAP) (pMG252). (A) ON status: VanA₁ is constitutively expressed and, in the absence of vanillic acid, binds to P₁VanO₂ and activates SEAP expression. (B) OFF status: in the presence of vanillic acid, VanA₁ is released from P₁VanO₂ and thus SEAP expression is repressed.
Assessing the impact of vanillic acid on mammalian cell cultures. To assess the impact of a broad concentration range of vanillic acid on reporter gene production, viability and cell proliferation of mammalian cell cultures, we exposed CHO-K1 cells, transfected with pSEAP2-control ($P_{SV40}$-(SEAP)-pA), to increasing concentrations of vanillic acid (0, 25, 50, 100, 150, 200, 250, 500 and 1000 µM). The SEAP levels and cell numbers were scored 48 h after transfection. It was observed that even the highest concentration of vanillic acid (1000 µM) had no impacts on heterologous protein production or cell proliferation, both indicators of metabolic integrity and viability (6) (Figure 2). Thus, the licensed food additive vanillic acid does not impair mammalian cells up to a concentration of 1000 µM.

VAC-regulated transgene expression in various mammalian cell lines. Versatility of the VAC-system was assessed by co-transfection of pMG250 ($P_{SV40}$-VanA$_1$-pA) and pMG252 ($P_{1VanO2}$-SEAP-pA) into different immortalized mammalian cell lines (BHK-21, COS-7, HEK293-T, HeLa, HT-1080 and NIH/3T3) and cultivation for 48 h in the presence and absence of 250 µM vanillic acid. The samples were analysed for SEAP production levels (Table 2), which indicated that VAC-controlled transgene regulation was functional in all tested cell lines, revealing a broad applicability of this technology.

**Fig. 1.** (C) CHO-K1 cells were transiently transfected with pMG250 ($P_{SV40}$-VanA$_1$-pA) and pMG252 ($P_{1VanO2}$-SEAP-pA) and SEAP-expression profiles were assessed 48 h after cultivation of the cells in different concentrations of vanillic acid (0 – 250 µM).
Figure 2. Impact of vanillic acid on cell proliferation and protein production. CHO-K1 cells were transiently transfected with pSEAP2-control (pSV40-SEAP-pA) and exposed to vanillic acid concentrations ranging from 0 to 1000 µM. SEAP production (bars) and maximum cell densities (line) were assessed after 48 h.

Table 2: Vanillic acid-controlled transgene expression in different mammalian cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>0 µM Vanillic acid</th>
<th>250 µM Vanillic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>0.52 ± 0.02 U/l</td>
<td>0.05 ± 0.01 U/l</td>
</tr>
<tr>
<td>COS-7</td>
<td>17.95 ± 0.55 U/l</td>
<td>0.45 ± 0.02 U/l</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>484.96 ± 54.24 U/l</td>
<td>20.85 ± 1.24 U/l</td>
</tr>
<tr>
<td>HeLa</td>
<td>7.89 ± 0.99 U/l</td>
<td>1.38 ± 0.11 U/l</td>
</tr>
<tr>
<td>HT-1080</td>
<td>0.65 ± 0.11 U/l</td>
<td>0.07 ± 0.01 U/l</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>7.41 ± 0.16 U/l</td>
<td>0.28 ± 0.12 U/l</td>
</tr>
</tbody>
</table>

SEAP production was quantified 48h after co-transfection pMG250 (P_{SV40-VanA1-pA}) and pMG252 (P_{1VanO2-SEAP-pA}) into indicated cell lines.

Optimizing the VAC-responsive system I – assessment of promoter variants containing varying numbers of VAC operator modules. Altering the number of operator modules in front of an inducible promoter impacts the regulation performance regarding (i) maximal expression levels, as an increasing number of operator sequences can recruit more transactivators, and (ii) basal expression of the system’s repressed state (33,63). To evaluate the optimal number of VanO-operator sites for VAC-responsive gene regulation, we constructed VanA1-responsive promoter variants harbouring either one (pMG262, P_{1VanO1-SEAP-pA}; P_{1VanO1}, NruI-VanO-0bp-P_{hCMVmin}), two (pMG252, P_{1VanO2-SEAP-pA}; P_{1VanO2}, NruI-VanO-Eco47III-VanO-0bp-P_{hCMVmin}), three (pMG263, P_{1VanO3-SEAP-pA}; P_{1VanO3}, NruI-VanO-Eco47III-
VanO-6bp-VanO-0bp-P_{hCMVmin}) or four (pMG264, P_{1VanO4}-SEAP-pA; P_{1VanO4}, NruI-VanO-Eco47III-VanO-6bp-VanO-Eco47III-VanO-0bp-P_{hCMVmin}) operators immediately 5’ of the minimal promoter and consequently co-transfected them with pMG250 (P_{SV40-VanA1-pA}) in CHO-K1, HEK293-T and BHK-21 cells to assess their transcriptional performances. The expression of SEAP was profiled after 48 h of cultivation in the presence or absence of 250 µM vanillic acid. In all cell lines, it was observed that an increasing number of operator modules resulted in higher maximum expression levels but also in higher basal expression of the repressed status of the VAC-system. The dimeric configuration (pMG252, P_{1VanO2}-SEAP-pA) resulted in the best regulation performance with a regulation factor of 72-fold in CHO-KI cells and 23-fold in HEK293-T cells, while the trimeric configuration showed the best performance in BHK-21 cells with a regulation factor of 11 (Figure 3A-C).
Figure 3. Validation of vanillic acid-responsive promoter variants containing different numbers of VanO operator modules. Vectors encoding SEAP expression driven by a vanillic acid-responsive promoter harbouring monomeric (pMG262), dimeric (pMG252), trimeric (pMG263) or tetrameric (pMG264) operator modules were co-transfected with pMG250 (P_{SV40-VanA1-pA}) into (A) CHO-K1, (B) HEK-293T and (C) BHK-21 cells and SEAP production was scored after 48 h.

**Optimizing the VAC-responsive system II – engineering of different vanillic acid-dependent transactivator variants.** It has been previously shown that the performance of synthetic mammalian gene regulation systems is influenced by the kind of mammalian transactivator domain, which is fused to the bacterial DNA binding protein, in terms of leakiness and maximum expression (19,64). We therefore characterized different transactivation domains by designing vectors containing VanR fused to the *Herpes simplex*-derived VP16 domain (pMG250, P_{SV40-VanA1-pA}; VanA1, VanR-VP16), the human nuclear factor ‘kappa light chain enhancer’ of activated B-cells- (NF-κB) derived transactivation domain p65 (pMG256, P_{SV40-VanA2-pA}; VanA2, VanR-p65) or the transactivation domain of the human E2F transcription factor (E2F4) (pMG257, P_{SV40-VanA3-pA}; VanA3, VanR-E2F4), and consequently co-transfected them with pMG252 into CHO-K1, HEK293-T, BHK-21, HT-1080 and HeLa cells. After 48 h of cultivation in the presence or absence of 250 μM vanillic acid, SEAP production was profiled. The maximum expression levels varied considerably amongst the different transactivation domains, but also the basal expression level of the repressed status showed altering performances depending on
the transactivator chosen. In general, E2F4 showed the weakest maximum expression levels within all tested cell lines and could therefore not compete with the best-in-class regulation performance of VP16 and p65. In CHO-K1, VP16 exhibited the highest maximum expression paired with the lowest leakiness, making it the transactivator of choice for this cell line (Figure 4 and Table 3). Peak expression in HEK293-T cells was achieved by p65, although the high basal expression again rendered VP16 the best choice in terms of the regulation factor. In HeLa cells, only VP16 provided a significant transactivation capacity, while in BHK-21 and HT-1080 cells p65 gave the best performance (Table 3). Due to their cell-type specificity, their graded maximum transcription-initiation capacities and their basal expressions in the repressed status, the three transactivators offered a selection of different expression windows and regulation factors depending on the cell line and application chosen.

**Table 3:** Combinatorial profiling of different VAC transactivators and promoters in various cell types

<table>
<thead>
<tr>
<th></th>
<th>SEAP Production (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pMG252/pMG250</td>
</tr>
<tr>
<td>Vanillic Acid (250μM)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>BHK-21</td>
<td>0.52 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>CHO-K1</td>
<td>27.06 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>2.13 ± 0.17</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>484.96 ± 54.24</td>
</tr>
<tr>
<td></td>
<td>54.2 ± 4.00</td>
</tr>
<tr>
<td>HeLa</td>
<td>7.89 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>1.49 ± 0.08</td>
</tr>
<tr>
<td>HT-1080</td>
<td>0.65 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>0.13 ± 0.02</td>
</tr>
</tbody>
</table>

SEAP production was quantified 48 h after transient co-transfection of pMG252 (P1VanO2-SEAP-pA) and either pMG250 (PSV40-VanA1-pA; VanR-VP16), pMG256 (PSV40-VanA2-pA; VanR-p65) or pMG257 (PSV40-VanA3-pA; VanR-E2F4).
Optimizing the VAC-responsive system III – promoter variants that differ in the distance between VAC operator modules and the minimal promoter.

Maximum transcription levels and minimum leakiness are not only influenced by the number of operator modules recruiting the transactivator molecules, but also by the relative spacing of the operator modules to the minimal promoter and the resulting torsion angle of the operator-bound transactivator (65,66). To further assess the optimal design of \( P_{VanO} \) configurations, we engineered spacers of 2 bp increments between the two VanO modules (VanO\textsubscript{2}) and \( P_{hCMVmin} \), resulting in SEAP expression vectors, isogenic to pMG252 (\( P_{1VanO2}-SEAP-pA \)) which harbours the default 18 bp between VanO\textsubscript{2} and \( P_{hCMVmin} \), while pMG265 (\( P_{2VanO2}, \text{VanO}_2-2bp-P_{hCMVmin} \)), pMG266 (\( P_{3VanO2}, \text{VanO}_2-4bp-P_{hCMVmin} \)), pMG267 (\( P_{4VanO2}, \text{VanO}_2-6bp-P_{hCMVmin} \)), pMG268 (\( P_{5VanO2}, \text{VanO}_2-8bp-P_{hCMVmin} \)) and pMG269 (\( P_{6VanO2}, \text{VanO}_2-10bp-P_{hCMVmin} \)) contain an additional 2, 4, 6, 8 or 10 bp between VanO\textsubscript{2} and \( P_{hCMVmin} \). Any of the vectors comprising the vanillic acid-responsive promoter variants were co-transfected with pMG250 (\( P_{SV40-VanA1-pA} \)), pMG256 (\( P_{SV40-VanA2-pA} \)) or pMG257 (\( P_{SV40-VanA3-pA} \)) into CHO-K1 cells to evaluate the optimal promoter configuration independently of the transactivator variant. The expression of SEAP was scored 48 h after cultivation of the transfected cells in media containing 0, 50 or 250 µM of vanillic acid (Figure 4 A-C). Generally, \( P_{1VanO2} \) exhibited the best regulation performance in terms of maximal expression and minimal leakiness. The promoter variants with 2, 4 and 10 bp spacers (pMG265, pMG266 and pMG269) showed expression performances, which were comparable to the best-in-class configuration harbouring no spacer (pMG252). However, pMG267 and pMG268 (6 and 8 bp increments) resulted in a much lower maximal expression. All promoter variants displayed similar expression profiles for all VanA transactivator variants, implying that chimeric promoters and transactivators can be independently optimized.
Figure 4. Combinatorial validation of the VAC-system in different transactivator and promoter configurations. VAC transactivators employing different transactivation domains (A: VanA1, VanR-VP16; pMG250) (B: VanA2, VanR-p65; pMG256) (C: VanA3, VanR-...
E2F4; pMG257) were co-transfected with different vanillic acid-responsive promoter variants containing 0 (P\textsubscript{1VanO2}; pMG252), 2 (P\textsubscript{2VanO2}; pMG265), 4 (P\textsubscript{3VanO2}; pMG266), 6 (P\textsubscript{4VanO2}; pMG267), 8 (P\textsubscript{5VanO2}; pMG268) and 10 (P\textsubscript{6VanO2}; pMG269) base-pair linkers between VanO and the minimal promoter into CHO-K1 cells. All promoter variants drove SEAP expression and the production was profiled 48 h after cultivation of the cells in media containing different concentrations of vanillic acid (0, 50 and 250 µM).

**Design of an autoregulated version of the VAC-responsive transgene expression system.** Besides the conventional two-vector design, employing a vector for constitutive expression of a transactivator and a second vector encoding the responsive promoter element followed by a gene of interest, we also designed an autoregulated single vector setup for vanillic acid-inducible gene regulation. Capitalizing on the polioviral internal ribosome entry site (IRES\textsubscript{PV}), the configuration mediates the simultaneous, cap-independent expression of transactivator and reporter proteins, both driven by the inducible P\textsubscript{1VanO2}-promoter. Such an autoregulated design is convenient for overcoming undesired expression variations in transient transfections and is beneficial for the design of noise-resistant gene networks (67). Transfection of pMG270 (P\textsubscript{1VanO2}-SEAP-IRES\textsubscript{PV}-VanA\textsubscript{1}-pA) in CHO-K1 cells resulted in a leaky expression of VanA\textsubscript{1}, which then in an autoregulated feedback initiated full activation of the VanA-responsive promoter P\textsubscript{1VanO2} to reach maximum levels of SEAP production and co-cistronic production of VanA\textsubscript{1}. When 250 µM vanillic acid was added to the culture, the autoregulated induction was interrupted and SEAP expression remained in the fully repressed state (0 µM vanillic acid: 9.58 ± 0.29 U/l; 250 µM vanillic acid: 0.30 ± 0.04 U/l). The SEAP levels for this experiment were scored after 48 h.

**Expression kinetics, adjustability and reversibility of VAC-controlled transgene expression in a stably transgenic CHO-K1 cell line.** A detailed characterization regarding long-term expression, adjustability and reversibility of the VAC-system requires the creation of a stable cell line. We therefore established stable CHO-K1-derived VAC-expressing cell lines (CHO-VAC) by sequential transfection of pMG250 (P\textsubscript{SV40}-VanA\textsubscript{1}-pA) and pMG252 (P\textsubscript{1VanO2}-SEAP-pA) and subsequent clonal selection. The expression of SEAP was profiled after 48 h for five single clones from cultures grown in the absence and presence of 500 µM vanillic acid. All clones
showed similar basal expression levels, but varied substantially in maximum expression allowing for clonal variation (Figure 5A). Out of the five single clones, CHO-VAC_{12} showed the best performance in terms of maximum transgene expression levels and a regulation factor of 92-fold repression. Furthermore, CHO-VAC_{12} revealed precise adjustability according to the level of vanillic acid administered to the medium (Figure 5B) and displayed unchanged maximal expression and repression levels in long-term cultures of up to 90 days (day 0, ON: 109.13 ± 3.80 U/l, OFF: 1.19 ± 0.04 U/l; day 90, ON: 104.36 ± 5.75 U/l, OFF: 1.45 ± 0.09 U/l). Besides excellent adjustability, rapid response kinetics and reversibility are indispensible for mammalian gene regulation systems. When cultivating CHO-VAC_{12} for 72 h, the system displayed exponential SEAP expression kinetics without vanillic acid in the medium, whereas upon addition of 500 µM of the inducer, SEAP expression levels did not significantly exceed the background levels (Figure 5C). Full reversibility of the VAC-system was monitored when cultivating CHO-VAC_{12} for 144 h while alternating the vanillic acid concentration every 48 h between 0 and 500 µM (Figure 5D).

![Figure 5](image-url)

**Figure 5.** Characterization of a stably transgenic vanillic acid-responsive CHO-K1 cell line. CHO-K1 was stably co-transfected with pMG250 (P_{SV40-VanA1-pA}) and pMG252 (P_{1VanO2-SEAP-pA}) and the resulting CHO-VAC cell lines transgenic for vanillic acid-responsive SEAP expression were characterized. (A) After clonal expansion, individual clones were assessed for their vanillic acid-responsive regulation performance. SEAP levels were profiled after 48 h of cultivation.
Figure 5. (B) The dose-response profile of CHO-VAC\textsubscript{12} was profiled after 48 h of cultivation with increasing concentrations of vanillic acid (0 – 500µM). (C) SEAP expression kinetics of CHO-VAC\textsubscript{12} cultivated for 72 h in the presence and absence of 250 µM vanillic acid. (D)
Reversibility of vanillic acid-mediated transgene expression following periodic addition and removal of the inducer. CHO-VAC\textsubscript{12} (80,000 cells/ml) were cultivated for 144 h in the presence and absence of 250 µM vanillic acid. Every 48 h, the cell density was re-adjusted to 80,000 cells/ml and the cells were cultivated in fresh medium with reversed vanillic acid concentrations.

Compatibility of the VAC-system with other transgene regulation systems. The broad applicability of mammalian transgene regulation systems within complex synthetic networks is also determined by their ability to function interference-free alongside other regulation systems that capitalize on different inducers (11,12,36,68). To assess this important requirement, we transiently transfected CHO-VAC\textsubscript{12} with the established components of the tetracycline- (TET\textsubscript{OFF}) (24) or the erythromycin- (E\textsubscript{OFF}) (18) responsive expression systems. Both, the TET\textsubscript{OFF} (pSAM200, P\textsubscript{SV40}-tTA-pA; pBP99, P\textsubscript{hCMV}*-1-SAMY-pA) and the E\textsubscript{OFF} (pWW35, P\textsubscript{SV40}-ET1-pA; pBP100, P\textsubscript{ETR3}-SAMY-pA) systems drove the expression of the heat-stable \textit{Bacillus stearothermophilus}-derived secreted α-amylase [SAMY, (60)] under a tetracycline, an erythromycin-responsive promoter. The levels of SEAP and SAMY were scored 48 h after cultivation of the transiently transfected CHO-VAC\textsubscript{12} cell line in the presence (500 µM; 2 µg/ml) or absence of the different inducers (vanillic acid/tetracycline/erythromycin) and an interference-free and fully functional regulation performance was demonstrated for the VAC, TET\textsubscript{OFF} and E\textsubscript{OFF} systems (Table 4A and B).

Table 4: Compatibility of vanillic acid-, erythromycin- and tetracycline-responsive transgene control systems

\textbf{A:} CHO-VAC\textsubscript{12} transfected with the tetracycline-responsive regulation system

<table>
<thead>
<tr>
<th>Inducer</th>
<th>- Tet / - Vac</th>
<th>- Tet / + Vac</th>
<th>+ Tet / - Vac</th>
<th>+ Tet / + Vac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative SEAP Production (%)</td>
<td>100 ± 5.62</td>
<td>2.18 ± 0.31</td>
<td>101.04 ± 6.21</td>
<td>2.07 ± 0.29</td>
</tr>
<tr>
<td>Relative SAMY Production (%)</td>
<td>100 ± 5.03</td>
<td>99.06 ± 4.53</td>
<td>4.53 ± 0.52</td>
<td>5.01 ± 1.61</td>
</tr>
</tbody>
</table>
B: CHO-VAC\textsubscript{12} transfected with macrolide-responsive regulation system

<table>
<thead>
<tr>
<th>Inducer</th>
<th>- EM / - Vac</th>
<th>- EM / + Vac</th>
<th>+ EM / - Vac</th>
<th>+ EM / + Vac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative SEAP Production (%)</td>
<td>100 ± 6.31</td>
<td>2.56 ± 0.09</td>
<td>102.19 ± 7.08</td>
<td>1.95 ± 0.59</td>
</tr>
<tr>
<td>Relative SAMY Production (%)</td>
<td>100 ± 5.67</td>
<td>98.97 ± 7.73</td>
<td>5.20 ± 0.68</td>
<td>4.83 ± 1.22</td>
</tr>
</tbody>
</table>

CHO-VAC\textsubscript{12} were co-transfected with pSAM200 (P\textsubscript{SV40-tTA-PA}) and pBP99 (P\textsubscript{hCMV*-1-SAMY-PA}) (A) or pWW35 (P\textsubscript{SV40-ET1-PA}) and pBP100 (P\textsubscript{ETR3-SAMY-PA}) and grown for 48h in the presence and absence of vanillic acid (Vac, 250\textmu M), erythromycin (EM, 2\mu g/mL) or tetracycline (Tet, 2\mu g/mL) before SEAP and SAMY production was assessed.

Specificity of the VAC system. VanR plays a key role in the lignin biodegradation of Caulobacter crescentus. One of the commonly produced compounds in this pathway is vanillic acid, but closely related compounds, such as vanillin, were also suggested as being able to directly interact with VanR (51). To assess the specificity of the VAC system and the capability of isomeric compounds of vanillic acid to interact with the synthetic mammalian-adapted VanA\textsubscript{1} transactivator, we cultivated CHO-VAC\textsubscript{12} for 48 h in media containing 0, 250 and 500 \textmu M of a comprehensive set of compounds closely related to vanillic acid (2-vanillic acid, 2-vanillin, acetovanillone, benzaldehyde, benzoic acid, benzyl acetate, benzyl alcohol, ethyl-vanillate, ethyl-vanillin, eugenol, homovanillic acid, isovanillic acid, isovanillin, methyl-vanillate, protocatechualdehyde and vanillin). In parallel, we assessed the toxicity of these compounds on a stable CHO-K1-derived cell line constitutively expressing SEAP (CHO-SEAP\textsubscript{15}). Some of the compounds were toxic when administered to CHO cells at concentrations of 500 \textmu M (2-vanillin, eugenol, isovanillin, methyl-vanillate and protocatechualdehyde), but none of the 16-tested structures had the ability to regulate the VAC system, which implies an extraordinary inducer specificity of this mammalian transgene expression system (Table 5).
Table 5: Responsiveness of the VAC-system to a variety of molecules chemically closely related to vanillic acid

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>SEAP Production (U/l)</th>
<th>CHO-VAC (_{12})</th>
<th>CHO-SEAP (_{18})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0µM</td>
<td>250µM</td>
<td>500µM</td>
</tr>
<tr>
<td>2-vanillic acid</td>
<td><img src="image" alt="Structural formula" /></td>
<td>132.23 ± 14.45</td>
<td>129.83 ± 10.69</td>
<td>116.39 ± 8.75</td>
</tr>
<tr>
<td>2-vanillin</td>
<td><img src="image" alt="Structural formula" /></td>
<td>126.27 ± 10.22</td>
<td>60.35 ± 5.38</td>
<td>19.33 ± 2.89</td>
</tr>
<tr>
<td>Aceto-vanillone</td>
<td><img src="image" alt="Structural formula" /></td>
<td>130.96 ± 7.61</td>
<td>117.31 ± 0.86</td>
<td>74.73 ± 6.51</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td><img src="image" alt="Structural formula" /></td>
<td>124.08 ± 13.11</td>
<td>125.74 ± 7.21</td>
<td>124.55 ± 7.05</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td><img src="image" alt="Structural formula" /></td>
<td>131.83 ± 11.36</td>
<td>127.29 ± 7.04</td>
<td>133.41 ± 5.00</td>
</tr>
<tr>
<td>Benzyl acetate</td>
<td><img src="image" alt="Structural formula" /></td>
<td>126.72 ± 5.96</td>
<td>122.42 ± 7.17</td>
<td>128.65 ± 8.32</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td><img src="image" alt="Structural formula" /></td>
<td>129.11 ± 11.95</td>
<td>134.79 ± 3.76</td>
<td>132.51 ± 4.72</td>
</tr>
<tr>
<td>Ethyl-vanillate</td>
<td><img src="image" alt="Structural formula" /></td>
<td>130.68 ± 1.86</td>
<td>131.39 ± 8.19</td>
<td>105.82 ± 4.84</td>
</tr>
<tr>
<td>Ethyl-vanilline</td>
<td><img src="image" alt="Structural formula" /></td>
<td>124.86 ± 4.84</td>
<td>123.87 ± 5.15</td>
<td>110.68 ± 2.75</td>
</tr>
</tbody>
</table>
CHO-VAC\textsubscript{12} and CHO-SEAP\textsubscript{18} were cultivated in medium with and without different derivatives of vanillic acid. SEAP production was quantified 48 h after addition of the chemicals.

**Vanillic acid - transgene expression in mice is mediated by a food additive.**

For subsequent applications in functional genomics research or future gene therapy settings, it is essential that state-of-the-art gene regulation systems are functional within entire organisms. To validate the vanillic acid-induced gene regulation system *in vivo*, we injected microencapsulated CHO-VAC\textsubscript{12}-containing cell implants intraperitoneally into mice. The treated mice were given a dose of vanillic acid within the range of 0 – 500 mg/kg twice daily. The SEAP levels were quantified 72 h after implantation and showed comparable vanillic acid-dependant dose response.
characteristics to the control experiment using the same batch of microencapsulated CHO-VAC_{12} cells exposed to vanillic acid in an in vitro setting (Figure 6A and B). The serum SEAP levels of control mice, encapsulated with constitutively expressing CHO-SEAP_{18}, were unresponsive to vanillic acid treatment of a twice-daily dose of 500 mg/kg and thus showed similar expression levels as untreated mice (0 mg/kg vanillic acid: 15.37 ± 1.57 U/l; 500 mg/kg vanillic acid: 16.61 ± 1.33 U/l).

**Figure 6.** Vanillic acid-controlled SEAP expression in mice. (A) CHO-VAC_{12} cells were microencapsulated in alginate-poly-(L-lysine)-alginate beads and implanted intraperitoneally into female OF1 mice (4 × 10^6 cells per mouse). The implanted mice were injected twice daily with different concentrations of vanillic acid. Seventy-two hours after implantation, the level of SEAP in the serum of the mice was determined. (B) SEAP expression profiles of the microencapsulated CHO-VAC_{12} implant batch were cultivated in vitro for 72 h at different vanillic acid concentrations.
Discussion

Heterologous transgene expression control by non-toxic small molecule inducers remains one of the major challenges for gene therapy scenarios and biopharmaceutical manufacturing of difficult-to-produce protein therapeutics. The employed inducers must meet high medical standards and also need to be physiologically inert for long-term applications in humans. Antibiotics, steroid hormones, immunosuppressive drugs and a multitude of other regulating molecules fail to meet these requirements due to their high levels of side-effects, particularly when given over a long period of time (69-71).

Phenolic acids are a class of compounds that are naturally produced by many plants, such as vegetables and fruits, and thus are widely distributed throughout human dietary products, like coffee, wine, beer and vanilla (72). In general, phenolic acids are said to possess many physiological and pharmacological functions (73) and vanillic acid, in particular, was successfully evaluated as a suppressor of a potent snake venom (42), cell apoptosis in Neuro-2A cells (45,46), immune-mediated liver inflammation in mice (47) and carcinogenesis (44). Being a licensed food additive with a very agreeable smell (which also enables vanillic acid to be used in fragrances), this specific phenolic acid combines the ideal properties for functioning as a physiologically inert inducer molecule in future gene regulation systems. This judgment is supported by the reported LD50 value of 5.02 g/kg, which was tested intraperitoneally in rats (74).

Combining the elements of the Caulobacter crescentus VanR-regulated VanAB gene cluster and mammalian transactivation domains, we created a novel mammalian heterologous transgene regulation system which responds to the licensed food additive vanillic acid. The generic design of the VAC system allows for several configurations using (i) a varying numbers of operators, (ii) different mammalian transactivation domains and (iii) varying the distance between the specific operator site and the minimal promoter, to provide ideal setups for different applications within several mammalian cell lines. Due to the high modularity of the VAC system, we were able to provide a specific configuration for all of the tested cell types, which exhibited an optimal regulation performance with high maximal expression and full
reversibility. Furthermore, the VAC system demonstrated interference-free regulation characteristics when employed in parallel settings with the TET_{OFF} (24) and the E_{OFF} systems (18). Owing to its unprecedented specificity (VanA is only responsive to vanillic acid) the presented regulation unit is likely to be an ideal building block for complex synthetic networks operating with various inducer inputs. The fact that vanillic acid is a natural plant component which is licensed as food additive may facilitate its approval by governmental agencies for applications in the biopharmaceutical production of difficult-to-express proteins and in gene therapy approaches, driving the VAC system to the forefront of second generation gene regulation systems.

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References


Conclusion

Latest advances in the control of synthetic mammalian gene expression have led to a multitude of unprecedented advances in the field of synthetic biology. Meanwhile applications of synthetically engineered circuits within gene therapy approaches and biopharmaceutical production come closer to reality. Although extensive research was performed on the optimization of protein production levels within mammalian cells, the increasing demand for biopharmaceuticals sets the challenge for industry to overcome the current bottleneck within biopharmaceutical production of difficult-to-produce proteins by mammalian cells. One potential alternative to handle this bottleneck could be the introduction of alternative production organisms. We have shown here the potential of the moss *Physcomitrella patens* to function as an alternative production organism, being able to grow in fully contained and controlled environments of bioreactors and most importantly being able to functionally employ a whole set of ready to use mammalian synthetic molecular tools without any additional modifications. We were able to show the cross kingdom conservation of the mammalian and moss transcription, translation and secretion machineries giving way to a whole set of constitutive, conditional and autoregulated expression possibilities for the production any genes of interest. Still there are hurdles to be taken, before the moss can function as a full alternative to mammalian cell culture production, one of them being the relatively low yield of moss heterologous protein production. However, the synthetic biology toolbox we provided in this work, taken together with the prototype biopharmaceutical production in microencapsulated protoplasts will help researchers to overcome these hurdles and potentially enable the high quality and yield production at low cost of complex to produce biopharmaceuticals within a moss.

Besides the transfer of a synthetic biology toolbox to the moss *Physcomitrella patens*, we were able to establish two novel gene regulations systems, which are responsive to second-generation inducer molecules (phloretin and vanillic acid), which are physiological inert and provide interference free, precise product gene adjustability. The described PEACE-system is the first ever regulation system, which enables expression control via the skin. We have tested this system in a prototype
study using a phloretin containing skin lotion, which we applied to the shaved skin of mice that carried microencapsulated cells engineered with the PEACE-system under their skin. The system shows excellent regulation performance and could potentially become a valid gene therapy application for in the treatment of patients with growth hormones or other therapeutic proteins. The next steps would include the long-term fine-tuning of a therapeutic protein to treat mice containing a corresponding disease model. The VAC-system provides similar potential for future gene therapy approaches, being responsive to a licensed food additive and thus not expecting any pleiotropic side effects from the inducer molecule. To add to the patient compliance, vanillic acid has pleasant smell and taste for potential oral applications. Similar to the PEACE-system, the next step would be to test the system together with an industry partner for the applicability in an animal disease model. Other than for gene therapeutic applications, both systems will add to the versatility of the available synthetic biology building blocks to construct complex synthetic networks, enable biopharmaceutical production of complex-to-produce proteins and be employed in drug discovery, as both systems equally have excellent regulation capabilities with high maximal expression, very low leakage and fast reaction times.
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Oct. 1999 – May 2004 Albert Ludwigs University Freiburg i. Br. / Germany
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WORK EXPERIENCE

Sep. 2008 – ongoing BioVersys AG, Innovative Antibacterial Drugs
Co-Founder and CEO; ETHZ Spin-Off that focuses on the R&D of new antibacterial drugs; www.bioversys.com

Associate Intern; Project within the Pharma/ Healthcare Sector: “Market Penetration and Business Strategy for a new medical device”

Jan. 2001 – March 2003 Assistant at the Socrates / Erasmus – exchange office, Faculty of Biology at the Albert Ludwigs University Freiburg i. Br.
“Managing of exchange agreements with the European Partner Universities; Counseling of incoming foreign- and the outgoing-students”

“Structuring patents; Writing patent applications; Counseling and 0 recruiting of a new client; Trade Mark Right”
Curriculum Vitae

**August 2001**

**Internship at the patent agency Ernest Freylinger S.A.**

**Luxembourg**

“Translation of patents from English to French and German; Learning the basics of patent structuring; Writing a patent application”

**GRANTS AND AWARDS**

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<td>2009</td>
<td>3 years personal coaching of Genilem; <a href="http://www.genilem-suisse.ch">www.genilem-suisse.ch</a></td>
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<td>2008</td>
<td>Part of the Suisse Venture Leaders 2008; <a href="http://www.venturelab.ch">www.venturelab.ch</a></td>
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<td>2003</td>
<td>Scholar of the “Landesstiftung Baden-Württemberg” as part of the UNIVERSITAS 21 exchange program</td>
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**LANGUAGES AND COMPUTER SKILLS**

**Languages:**

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<td>French</td>
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**Computer Skills:**

General Office Applications (Word, Excel, Power Point); Specialized Biological Software (microarray analysis, cloning, FACS)

**EXTRA CURRICULAR ACTIVITIES**

- Working for Promotions for several agencies and companies in Germany and Australia
- Snowboard teacher at the “Skischule Hochschwarzwald, Feldberg”
- Member of the committee of Luxembourg’s student association in Freiburg i. Br.
- Member of the cycling club “RV Schwalbe Trier” (former youth representative)
Publications


Patent applications
