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Design and Characterization of a Modular Membrane Protein Anchor to Functionalize the Moss *Physcomitrella patens* with Extracellular Catalytic and/or Binding Activities

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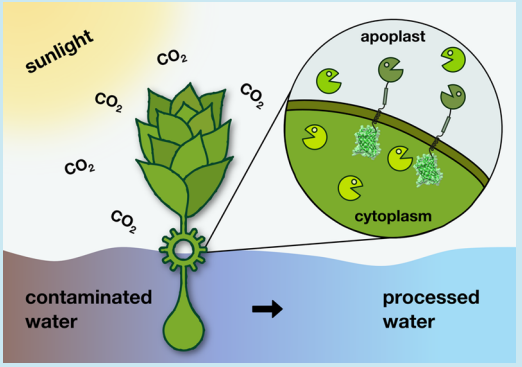
Supporting Information

ABSTRACT: Heterologous enzymes and binding proteins were secreted by the moss *Physcomitrella patens* or anchored extracellularly on its cell membrane in order to functionalize the apoplast as a biochemical reaction compartment. This modular membrane anchoring system utilizes the signal peptide and the transmembrane segment of the somatic embryogenesis receptor-like kinase (SERK), which were identified in a comprehensive bioinformatic analysis of the *P. patens* genome. By fusing the soluble enzyme NanoLuc luciferase to the signal peptide, its secretion capability was confirmed *in vivo*. The membrane localization of hybrid proteins comprising the SERK signal peptide, NanoLuc or other functional modules, the SERK transmembrane anchor, and a C-terminal GFP reporter was demonstrated using fluorescence microscopy as well as site-specific proteolytic release of the extracellular enzyme domain. Our membrane anchoring system enables the expression of various functional proteins in the apoplast of *P. patens*, empowering this photoautotrophic organism for biotechnological applications.



The (bio)chemical conversion or degradation of noxious solutes in an aqueous environment is a challenging task in biotechnology, for example, in order to neutralize micropollutants—especially antibiotics, hormones, and toxins—in contaminated water. An immobile organism such as a plant, in particular a mechanically resistant mesh-like moss, appears attractive as a self-renewable carrier for biocatalytic functions. The bryophyte model organism *Physcomitrella patens* is already in use for biotechnological applications and offers several advantages over other model plants, thus providing a convenient biological chassis.[1]

P. patens' beneficial features include the photoautotrophic and sedentary life-style, facile cultivation in minimal liquid culture media, availability of an efficient PEG-based transformation procedure, and ease of genetic manipulation with the ability for homologous recombination. From a practical perspective, the cellular metabolism of this moss can accomplish autonomous regeneration of relevant effector proteins, and it may be grown directly in contaminated water in a self-sustaining manner as only sunlight and carbon dioxide are required. Knowledge on the biology of *Physcomitrella* has greatly increased recently, and in fact, *P. patens* was the first bryophyte whose genome sequencing was completed already in 2007.[2]

So far, biotechnological applications of *P. patens* were confined to the secretion of therapeutic proteins.[1] However, for use as a potential biological water filter the excretion of recombinant proteins is not the preferred route; in contrast,

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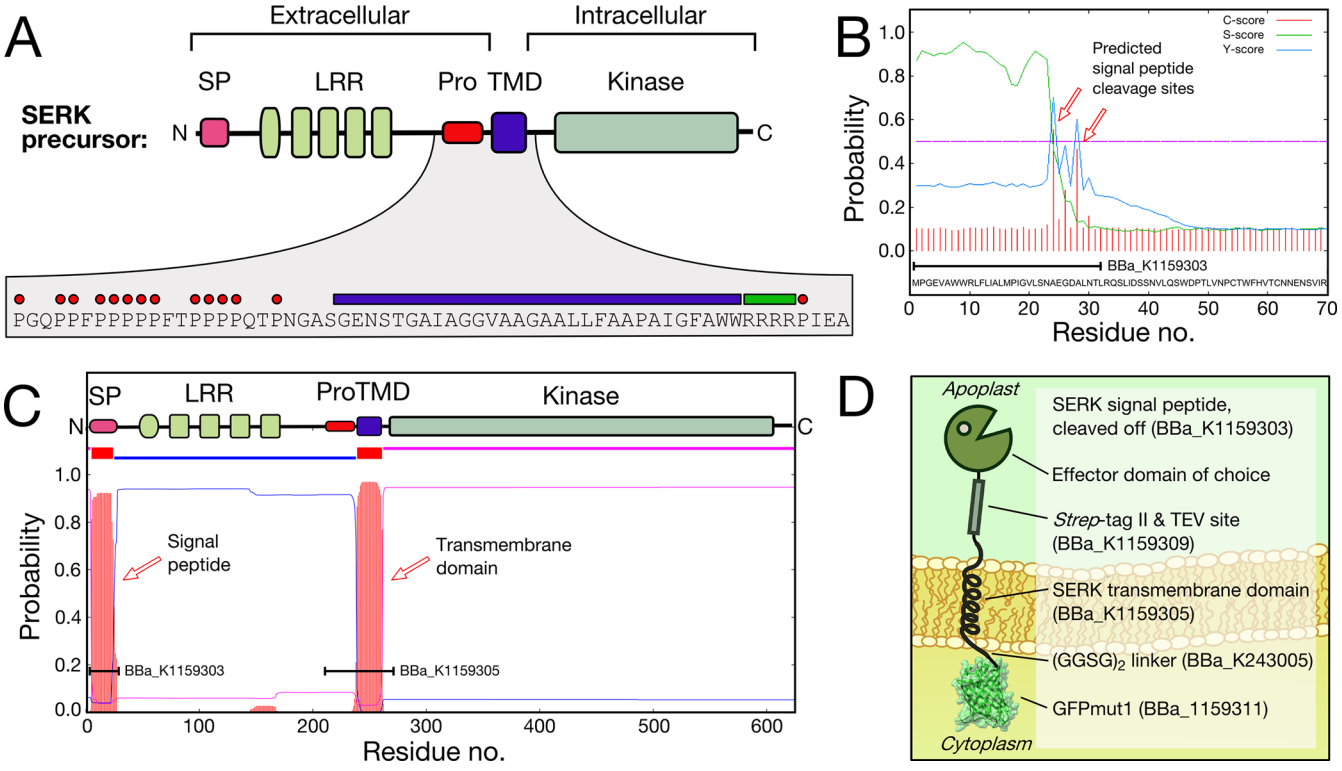

Figure 1. Bioinformatic analysis of receptor sequences from *P. patens* and design of new BioBricks for a modular membrane protein anchor. (A) Parts of the endogenous somatic embryogenesis receptor-like kinase (SERK) receptor were identified as suitable modules to develop a plant cell membrane protein anchor. The SERK precursor comprises a signal peptide (SP) preceding several leucine-rich repeat (LRR) extracellular domains, a proline-rich stalk region (Pro) and a transmembrane domain (TMD) followed by an intracellular protein kinase domain. The TMD is characterized by a hydrophobic segment (blue bar) that is flanked on the N-terminal side by a region with a high proportion of proline residues (red dots) and on the C-terminal side by a stop transfer signal of four strongly basic arginine residues (green bar). (B) At the N-terminus of SERK a signal peptide was identified using the SignalP 4.1 Server. Plotted probabilities represent the raw cleavage site "C-score", the signal peptide "S-score" and the combined cleavage site "Y-score". (C) A single transmembrane domain was identified in SERK using the TMHMM Server v. 2.0. (D) The design of hybrid synthetic membrane proteins to be expressed in the moss comprises the SERK SP, the effector domain (i.e., enzyme or binding protein), a TEV protease cleavage site, the SERK TMD, and a cytosolic GFP reporter module.

immobilization of biochemically functional domains on the plant cell membrane constitutes the most appropriate strategy. Examples of suitable enzymes or binding proteins include laccases, esterases, or Anticalins.[3] Each of these effector proteins possesses a simple fold with a single polypeptide chain, which makes them amenable to fusion with a transmembrane anchor domain; some of them even benefit from the oxidative milieu of the extracellular space for proper folding and disulfide bond formation.

In this study, we have investigated targeting of corresponding model proteins to the apoplast of *P. patens*, which is only surrounded by the permeable plant cell wall, and we present a modular membrane anchoring system that can be used to localize functional protein modules at the moss cell membrane. One advantage of this particular localization is that the diffusion of reactants across the plasma lipid bilayer is not a limiting factor, contrasting with enzymes that reside in the cytoplasm. Using a combination of existing BioBricks[4] that encode either enzymes with hydrolytic or oxidizing activities or engineered binding proteins (for difficult to degrade pollutants) with novel ones that enable anchoring of these functional modules on the plant cell membrane, a transgenic moss has been created that, in principle, may act as a biologically active and self-renewing water filter for applications in environmental biotechnology.

RESULTS AND DISCUSSION

Choice of an Appropriate Membrane Anchor Template from *P. patens*. Based on the annotated genome of *P. patens*,[2] all predicted cell surface receptors (Gene Ontologies "membrane" and "receptor activity"; http://www.cosmoss.org) were inspected for expressed sequences tags (EST), potential signal peptides and single-pass transmembrane regions (see Supporting Information) to choose promising modules for construction of functional hybrid membrane proteins (Figure 1). Using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP) and the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM), the somatic embryogenesis receptor-like kinase (SERK; COSMOSS entry Pp1s35_219 V6.1) was identified in this manner as the most appropriate template for a generic membrane anchor (Supporting Information Table 1).

SERK belongs to the receptor-like kinase family, whose members are responsible for signal transduction into the cell. The *P. patens* SERK precursor corresponds to a type I transmembrane receptor and comprises an N-terminal signal peptide (SP), several leucine-rich repeat (LRR) domains, a proline-rich region (Pro), a transmembrane domain (TMD), and a cytosolic protein kinase responsible for intracellular signal propagation (Figure 1A).

Identification and Experimental Validation of the Signal Peptide. Analysis of the primary sequence of the SERK

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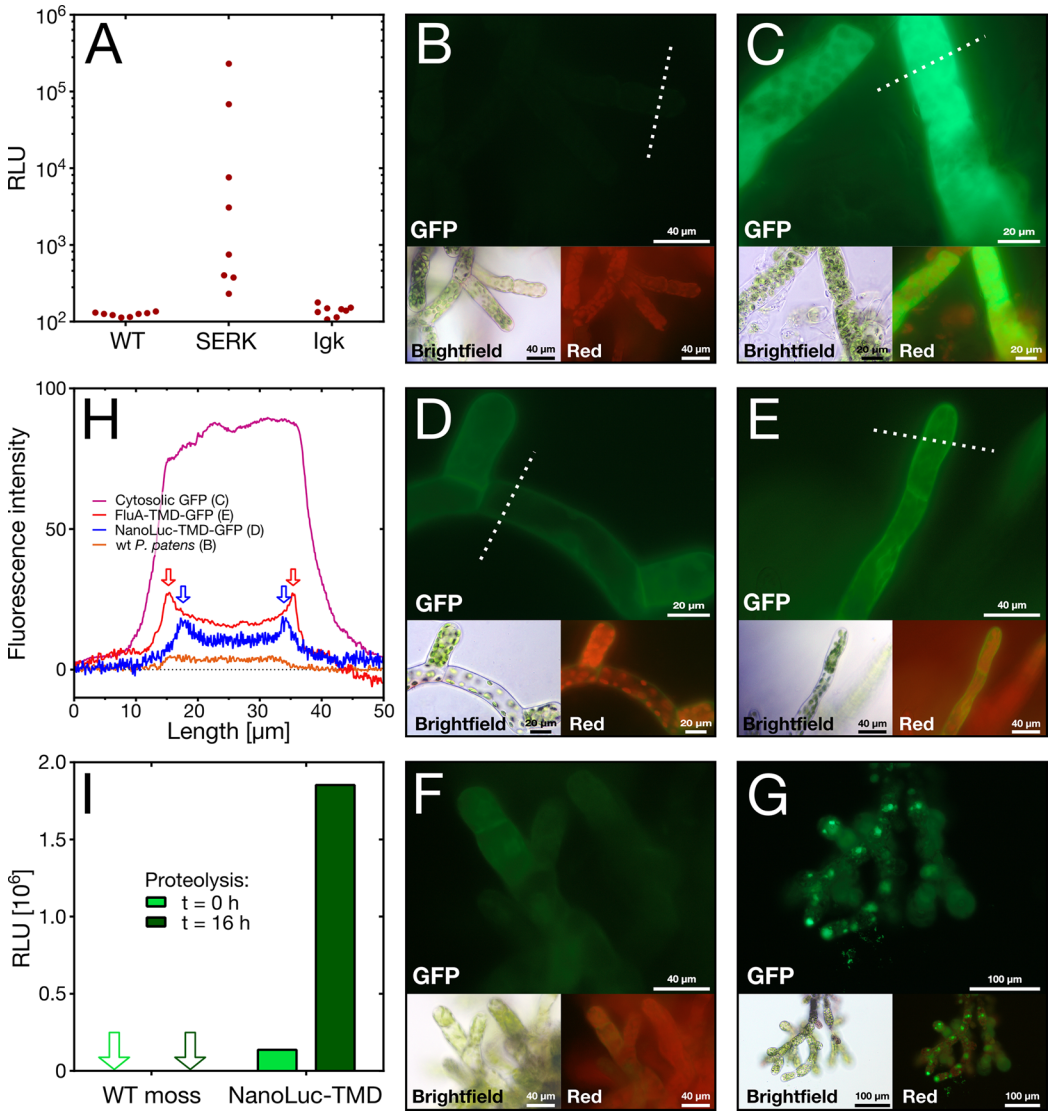

Figure 2. Demonstration of recombinant protein secretion as well as subcellular localization of the modular membrane protein anchor in *P. patens*. (A) The functionality of the SERK signal peptide was confirmed by investigating secretion of NanoLuc luciferase in stably transfected moss clones (N = 8). Nontransgenic wild type (WT) plants did not show detectable luciferase activity in the supernatant, whereas a mammalian Igκ signal peptide did not lead to secretion. (B–G) The subcellular localization of the GFP reporter module as part of the hybrid synthetic membrane protein (cf. Figure 1) was investigated by fluorescence microscopy on an Olympus BX61 microscope equipped with a Colorview camera (exitation at 488 nm, specific GFP detection at 500–530 nm shown in the large picture; lower right, both GFP and red chloroplast fluorescence detected in the wavelength range of 575–675 nm; lower left, brightfield illumination). Untransformed control plants did not show green fluorescence but chloroplast autofluorescence (red) (B), whereas GFP expression without signal peptide led to an intense cytosolic stain of the plant cell (C). Moss expressing hybrid membrane proteins with the NanoLuc luciferase (D), the Anticalin FluA (E), or laccase (F) as extracellular effector domains revealed enhanced fluorescence at the cellular plasma membrane. When using the human protein phosphatase 1 (PP1) as extracellular module, GFP was detected in cytosolic vesicles (G), most likely due to aggregation of PP1. (H) Fluorescence intensity of cross sections (dotted lines in the microscopic images) was analyzed using ImageJ, confirming highest GFP fluorescence at cellular boundaries (indicated by arrows) for NanoLuc luciferase and the Anticalin FluA. (I) To demonstrate extracellular localization of the luciferase effector module, transgenic moss shown in (D) was incubated with TEV protease for 16 h and NanoLuc activity before and after protease treatment was quantified in the supernatant. Whereas WT moss only showed negligible background signals (indicated by hollow arrows), the transgenic NanoLuc-TMD moss exhibited clearly increased luminescence after proteolytic treatment.

with SignalP indicated a potential signal peptide comprising residues 1 to 27 (Figure 1B). The predicted signal peptidase cleavage site between Gly[27] and Asp[28], with Ala[25] at position −3, follows the (−1/−3) rule.[5] Our SP BioBrick [BBa_K1159303] was designed to cover amino acid residues 1 to 32 and was implemented as a type RFC[25] N-part, also containing a plant consensus sequence (AACAATG) upstream of the translational start codon. The functionality of the SERK SP in comparison with the Igκ SP from *M. musculus* [BBa_K1159304] was investigated using a luciferase reporter system. For this purpose, *P. patens* was stably transformed by standard procedures[6] with an expression cassette composed of an actin promoter (PpAct5) from *P. patens*,[7] the secretable NanoLuc luciferase,[8] the CaMV 35S terminator and a neomycin phosphotransferase II (nptII) cassette to allow selection of transformants using Geneticin (G418). Stable transgenic moss lines were analyzed using fluorescence microscopy and luciferase reporter assays (Figure 2).

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From each transformation, 8 clones (at age 6−8 weeks) were inoculated in liquid media, and after 24 h 50 μL of supernatant was mixed with 50 μL Nano-Glo solution (Promega, Mannheim, Germany) in LumiNunc F96 microwell plates (Nalgene Nunc International, Rochester, NY). Bioluminescence was quantified with a BioTek II plate reader (BioTek, Bad Friedrichshall, Germany) with a 460 ± 40 nm filter set (Figure 2A). High bioluminescence values were detected in the supernatant, ranging from 231 to 230775 relative luminescence units (RLU), in the case of NanoLuc luciferase fused to the SERK SP. In contrast, the luciferase equipped with the Igκ SP only showed very low bioluminescence, even though this signal peptide was reported to be functional in *P. patens*.[9] Thus, our results demonstrate efficient protein secretion in *P. patens* using the SERK signal peptide. The considerable variance in expression level can be explained by different genomic integration sites.

Bioinformatic Analysis of the Transmembrane Domain. Analysis of the primary sequence of SERK with the TMHMM server indicated a single transmembrane domain (TMD) comprising the 23 residues Ile^{239} to Trp^{261}, also predicting α-helical secondary structure (Figure 1C). Notably, on the extracellular side of the SERK TMD a 31 residue proline-rich region became evident. Clusters of proline residues tend to form rods, which could provide a useful additional feature of the SERK TMD by functioning as a stiff spacer between the lipid bilayer and an extracellular protein domain. On the cytosolic side of the TMD a stop-transfer sequence composed of four consecutive basic residues ($Arg^{262-265}$) was apparent, most likely responsible for correct positioning of the TMD within the plasma membrane in line with the "positive-inside rule" (Figure 1A). The TMD BioBrick [BBa_K1159305] was again designed according to the RFC[25] standard, comprising the amino acid sequence stretch Pro^{208} to Ile^{267} of SERK.

Assembly and Characterization of Hybrid Membrane Proteins. Novel recombinant membrane proteins were assembled in a modular fashion according to the RFC[25] standard from the following BioBricks: (i) the SERK SP, (ii) an extracellular effector protein of choice, (iii) a linker domain containing the *Strep*-tag II and a TEV cleavage site [BBa_K1159309], (iv) the SERK TMD including its proline-rich extracellular stalk region, (v) a flexible $(GGSG)_2$ linker, and (vi) a cytosolic green fluorescent protein (GFP) as reporter moiety [BBa_K1159311] to detect expression and subcellular localization (Figure 1D). The following extracellular effector modules were investigated: NanoLuc luciferase,[8] the fluorescein-binding Anticalin FluA,[3] laccase from *Bacillus pumilus*, and the human protein phosphatase 1 (PP1).

The moss was stably transformed with these constructs and resulting clones were analyzed by fluorescence microscopy to investigate the subcellular localization of the GFP reporter as part of the membrane-anchored proteins (Figure 2B−G). While wild type plants emitted red chloroplast autofluorescence but no GFP signal (Figure 2B), transformed moss just expressing cytosolic GFP gave rise to an intense green staining spread evenly across the entire cell (Figure 2C). Notably, *P. patens* cells expressing the hybrid membrane proteins showed a distinct localization of the fluorescence with enhanced signal intensity at the cell boundaries for the constructs comprising NanoLuc luciferase (Figure 2D), the Anticalin (Figure 2E), and, to a lower degree, also the laccase (Figure 2F). In contrast, human PP1, which is a cytosolic protein containing 13 unpaired cysteine residues, showed localized fluorescence in cytosolic vesicles, potentially indicating aggregation upon entering the oxidizing milieu of the endoplasmic reticulum (Figure 2G). Locally elevated fluorescence intensity at the cellular boundaries was confirmed for NanoLuc-TMD-GFP and FluA-TMD-GFP by analyzing cross sections of the microscopy images (Figure 2H) using ImageJ 1.45 (http://rsb.info.nih.gov/ij). Thus, efficient membrane-anchoring of functional protein domains within the apoplast was demonstrated for a series of secretable protein modules.

Proteolytic Release of the Extracellular Functional Domain. The orientation of the hybrid membrane protein within the lipid bilayer was investigated using recombinant TEV protease, which was applied to a moss culture expressing NanoLuc fused to the membrane anchor. This protease specifically cleaves the TEV recognition site placed between the effector domain and the TMD, thus liberating the luciferase, which subsequently can diffuse through the cell wall and accumulate in the culture supernatant (Figure 2I). Finally, NanoLuc luciferase was quantified using a biochemical assay as described above. A high increase in luminescence after 16 h of incubation with the TEV protease was seen for the transgenic moss, thus indicating the extracellular accessibility of the NanoLuc luciferase, and, consequently, confirming the anticipated localization and orientation of the hybrid membrane protein.

Discussion and Conclusions. Bryophytes are an interesting class of plants with a promising future in biotechnology and synthetic biology. The anchoring of functional proteins in the lipid bilayer is essential to localize effector proteins at the extracellular (or, alternatively, intracellular) side of the cell membrane for various applications. Corresponding systems have been described for several types of host cells, including bacteria, yeast, and even mammalian cell lines. Cell surface anchoring of enzymes or binding proteins on the plant *P. patens* generally offers potential for the biotransformation of noxious solutes present in the culture medium, for example, for removal of toxic compounds from wastewater.

In order to specifically enable apoplast localization, the endogenous SERK of *P. patens* was identified as a suitable template to design functional hybrid membrane proteins. Applying a modular approach according to the BioBrick RFC[25][4] standard, recombinant genes for such membrane proteins were assembled from the signal peptide and transmembrane segment of SERK as well as from degrading enzymes or binding proteins, together with a fluorescent reporter protein. These genetic constructs were used for transformation of *P. patens*, followed by selection of stable transgenic moss lines. The apoplastic expression of the hybrid proteins was confirmed by fluorescence microscopy for three different functional protein modules. Using the sequence-specific TEV protease for cleavage, evidence for the extracellular localization of the effector module was obtained.

While recombinant membrane proteins have already been described for *A. thaliana*[10] and employed for basic research purposes, such an approach has not been reported for *P. patens* to date. Thus, to our knowledge, the set of BioBricks that were constructed in this work constitutes the first cell membrane localization tool kit developed for the functionalization of this moss. Our approach may boost a broader biotechnological application, prompting its wider use as a “chassis” organism for synthetic biology, beyond the secretion of therapeutic proteins.[1]

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More specifically, the generation of self-renewable and inexpensive moss-based biofilters seems feasible. Indeed, the concept of a live plant filter device, dubbed PhyscoFilter, that may allow specific degradation or scavenging of organic substrates was proposed by the TU-Munich team in frame of a project for the international Genetically Engineered Machines competition 2013 (iGEM; http://igem.org). We believe that such a system should provide an economically more affordable and environmentally friendly solution than current technologies for wastewater treatment.

■ ASSOCIATED CONTENT

Supporting Information

Details of the bioinformatic analysis of the *P. patens* genome and a list of identified templates for the construction of recombinant membrane proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

PEG, polyethylene glycol; RFC[25], Requests for Comments No. 25 of the BioBrick Foundation; SERK, somatic embryogenesis receptor-like kinase; TMD, transmembrane domain

■ REFERENCES

(1) Decker, E. L., and Reski, R. (2012) Glycoprotein production in moss bioreactors. *Plant Cell Rep. 31*, 453−460.

(2) Zimmer, A. D., Lang, D., Buchta, K., Rombauts, S., Nishiyama, T., Hasebe, M., Van de Peer, Y., Rensing, S. A., and Reski, R. (2013) Reannotation and extended community resources for the genome of the non-seed plant *Physcomitrella patens* provide insights into the evolution of plant gene structures and functions. *BMC Genomics 14*, 498.

(3) Beste, G., Schmidt, F. S., Stibora, T., and Skerra, A. (1999) Small antibody-like proteins with prescribed ligand specificities derived from the lipocalin fold. *Proc. Natl. Acad. Sci. U.S.A. 96*, 1898−1903.

(4) Smolke, C. D. (2009) Building outside of the box: iGEM and the BioBricks Foundation. *Nat. Biotechnol. 27*, 1099−1102.

(5) von Heijne, G. (1985) Signal sequences. The limits of variation. *J. Mol. Biol. 184*, 99−105.

(6) Hohe, A., Egener, T., Lucht, J. M., Holtorf, H., Reinhard, C., Schween, G., and Reski, R. (2004) An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss *Physcomitrella patens*. *Curr. Genet. 44*, 339−347.

(7) Weise, A., Rodriguez-Franco, M., Timm, B., Hermann, M., Link, S., Jost, W., and Gorr, G. (2006) Use of *Physcomitrella patens* actin 5′ regions for high transgene expression: Importance of 5′ introns. *Appl. Microbiol. Biotechnol. 70*, 337−345.

(8) Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., and Wood, K. V. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem. Biol. 7*, 1848−1857.

(9) Gitzinger, M., Parsons, J., Reski, R., and Fussenegger, M. (2009) Functional cross-kingdom conservation of mammalian and moss (*Physcomitrella patens*) transcription, translation, and secretion machineries. *Plant Biotechnol. J. 7*, 73−86.

(10) Albert, M., Jehle, A. K., Mueller, K., Eisele, C., Lipschis, M., and Felix, G. (2010) *Arabidopsis thaliana* pattern recognition receptors for bacterial elongation factor Tu and flagellin can be combined to form functional chimeric receptors. *J. Biol. Chem. 285*, 19035−19042.