Expression of a human cDNA in moss results in spliced mRNAs and fragmentary protein isoforms

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ABSTRACT

Production of biopharmaceuticals relies on the expression of mammalian cDNAs in host organisms. Here we show that the expression of a human cDNA in the moss *Physcomitrella patens* generates the expected full-length and four additional transcripts due to unexpected splicing. This mRNA splicing results in non-functional protein isoforms, cellular misallocation of the proteins and low product yields. We integrated these results together with the results of our analysis of all 32,926 protein-encoding *P. patens* genes and their 87,533 annotated transcripts in a web application, physCO, for automatized codon-optimization. A thus optimized cDNA results in about eleven times more protein, which correctly localizes to the ER. An analysis of codon preferences of different production hosts suggests that similar effects also occur in non-plant hosts. We anticipate that the use of our methodology will prevent so far undetected mRNA heterosplicing resulting in maximized functional protein amounts for basic biology and biotechnology.

KEYWORDS

alternative splicing / cryptic introns / intron engineering / biopharmaceutical production / blood coagulation factor IX / complement regulator / factor H / heterologous expression systems / aberrant mRNA-splicing / *Physcomitrella patens* / heterosplicing

INTRODUCTION

The majority of eukaryotic genes comprise multiple exons interspersed by noncoding introns, the number and length of which vary between species. The human genome size (GRCh38.p13, Genome Reference Consortium Human Build 38 patch release 13) is 3,099,706,400 bp comprising 20,444 protein-encoding genes, with 8.8 exons and 7.8 introns per gene on average¹. Generally, human genes have many short exons (average 50 codons) separated by long introns (up to >10 kbp)². It is widely accepted that such an exon-intron gene structure emerged during evolution and lead to complex gene regulation and diversification of multicellular eukaryotes^{2,3}. Introns that may contain regulatory elements affecting gene expression are removed from pre-mRNAs by RNA splicing. Subsequently, exons are ligated together to generate functional mature mRNAs⁴. Over 30 years of research on pre-mRNA splicing elucidated an underlying complex regulation, which includes exon versus intron recognition⁵, co-transcriptional splicing⁶, alternative splicing⁷, exonic and intronic splicing enhancers and repressors^{8,9}, and mRNA export from the nucleus¹⁰.

Alternative splicing (AS) is a fundamental regulatory process that contributes to proteome expansion¹¹. AS can produce multiple mRNAs from the same gene through the preference of splice sites during pre-mRNA splicing. It is modulated based on sequence motifs in the pre-mRNA, the interactions between RNA-binding proteins and splice sites, different cell types, and environmental signals¹². Splicing is carried out by a large ribonucleoprotein (RNP) complex, the spliceosome, which brings selected exons together by two transesterification reactions¹³. During splicing, uridine (U)-rich small nuclear RNPs (snRNPs) together with non-snRNP splicing factors, and serine/arginine-rich (SR) proteins participate in the recognition of 5'- and 3'-splice sites as well as the branch site¹⁴. The core of the spliceosome is highly conserved in all well-characterized eukaryotes^{3,15}. The key requirement for splicing is the recognition of a donor splice site by the U1 snRNP¹⁶. On the other hand, the acceptor splice site, downstream of the polypyrimidine tract, is recognized by the U5 snRNP¹⁷. Another important splicing element containing the reactive adenosine, the branch point signal, is located upstream of the acceptor splice signal and is important for the formation of the lariat-like intermediate structure¹⁷. While the majority of the introns have "GT" at the donor splice site and "AG" at the acceptor splice site, in rare cases introns can have unusual dinucleotides: for instance "AT" at the donor site and "AC" at the acceptor site¹⁸. This class of introns is spliced out of the pre-mRNA by the minor spliceosome U12¹⁹.

Besides its role in proteome expansion, AS controls transcript levels by generating unstable mRNA isoforms that may activate nonsense-mediated mRNA decay (NMD)²⁰. Generation of alternative functional mRNAs due to the introduction of premature termination codons (PTC), intron retention, and alternative use of the 5'- and 3'-splice sites causes changes in the localization, stability and/or function of a protein. While in humans more than 95% of genes encode alternatively spliced mRNAs²¹, in an early study 6,556 out of 39,106 predicted genes showed AS in the basic land plant *Physcomitrella patens* (moss) with a strong bias towards intron retention²². Many PTC-containing alternatively spliced transcripts are NMD targets in *P. patens*²³. Furthermore, about half of the AS events altered coding sequences and resulted in 2,380 distinct proteins. In these studies, analysis of AS relied on expressed sequence tags (ESTs) and the alignment of ESTs and cDNA sequences to the genome. The percentage of AS events in *P. patens* is estimated as 58%, but large-scale and/or tissue-dependent profiling of AS in moss by the use of improved sequencing methods may result in a higher percentage²⁴.

Two models of splice-site recognition exist²⁵: In the intron-definition model, U1 binds to the upstream donor splice site and U2AF/U2 to the downstream acceptor splice site and branch site of the same intron^{26,27}. The length of introns, which probably remain short under evolutionary selection³, limits the efficiency of splicing. In lower eukaryotes where introns are shorter, this mechanism might be dominant¹⁵. In the exon-definition model, the splicing machinery recognizes splice sites flanking the

same exon^{28,29}. This is the major mechanism in higher eukaryotes, where introns are generally longer. Due to these differences, the correct splicing of transgene transcripts has to be considered to maximize the production of a functional protein. For instance, it was necessary to remove a cryptic intron in the coding sequence (CDS) of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* to function in the flowering plant *Arabidopsis thaliana*³⁰.

The use of plants as alternative pharmaceutical protein production hosts has been on the rise for the past two decades, and some plant-made products have reached the market³¹. However, the percentage of plant-based biopharmaceuticals in the pharmaceutical market is still minute, mainly because of lower yields compared to prokaryotic and mammalian systems³². Nevertheless, they offer alternative solutions in niche areas³³. Production of blood clotting factors could be such a niche³¹. The human blood-clotting factor IX (FIX), a member of the intrinsic pathway of coagulation, is required for normal hemostasis and its absence or abnormal levels causes hemophilia B³⁴. Current treatment is restricted to protein replacement therapy and factor concentrates costs are between \$100,000 and \$200,000 per patient and year³⁵. Thus, there is a need for an economic production of FIX, which makes us attempting to produce it in the moss system. The moss *P. patens*, a model organism for evolutionary and functional genomics approaches, is an established host for the production of complex recombinant biopharmaceuticals and proven its suitability for large-scale production^{36,37}. The first moss-made drug candidate, moss-aGal for enzyme replacement therapy of Fabry disease, has successfully completed phase I clinical trials³⁸. Additionally, a variety of other potential biopharmaceuticals is being produced in moss^{39–41}.

In the present study, we report the expression of a human blood clotting factor IX-encoding cDNA in *P. patens*. We show that specific sequence motifs in the FIX mRNA were recognized by the splicing machinery as donor and acceptor sites and produced several different transcripts, which resulted in different protein isoforms. We named this new phenomenon heterosplicing, and were able to prevent it by changing the splice sites and optimizing codons without affecting the FIX aa sequence (optiFIX). Expression of optiFIX resulted in full-length transcripts only, and an eleven-fold increase of protein accumulation. In addition, we found heterosplicing also in transgenic moss lines expressing the CDS of human factor H (FH) CDS. By optimizing the sequence, we generated optiFH, which showed no heterosplicing and yielded significantly enhanced FH protein abundances *in vivo*. We suggest that our methodology of CDS optimization will provide a better quantification of transcript levels, and precise detection, purification, and quantification of functional proteins. It may reduce the bioenergetic costs of heterologous cDNA expression by preventing the formation of non-functional products and maximizing functional recombinant protein production, major bottlenecks in plant-based systems.

RESULTS

Predicted miRNA activity is inhibited by mutating miRNA binding sites

MicroRNAs can regulate gene expression at the posttranscriptional level, either via target mRNA degradation or translational inhibition. The possible actions of *P. patens* miRNAs on the RNA sequence for human Factor IX (NCBI reference NM_000133) were investigated using psRNATarget (http://plantgrn.noble.org/v1_psRNATarget/). Thus, two miRNAs were identified (miR1028c-3p and miR1044-3p), that might interfere with FIX expression in this moss. The respective binding sites on the FIX RNA sequences were modified in the DNA construct without a change in the FIX aa sequence. The potential binding site of ppt-miR1028c-3p, which might inhibit translation, was changed into ATCATGTGAGCCAGCTGTACCC between positions 495 and 516. The binding site of ppt-miR1044-3p, which might lead to cleavage of the FIX RNA, was mutated into

AGGGTAAGTACGGCATCTAT between positions 1304 and 1323. The sequence of miRNAs and their binding sites as well as the maximum energy to unpair the target site (UPE) and expectation value are shown in Supplementary Table 1.

Splicing of FIX mRNA occurs in transgenic plants and in transiently transfected cells

The native FIX signal peptide was replaced by the moss aspartic proteinase signal peptide of *PpAP1* to target the protein to the secretory pathway for its proper posttranslational modifications⁴². After transfection of the parental moss line $\Delta xt/ft^{41}$ with the human FIX-encoding, cDNA-based expression construct that comprises the complete FIX coding sequence (CDS) of 1401 bp without any introns; 49 transgenic moss lines were regenerated and screened. The presence and integration of the full-length FIX CDS in the moss genome was confirmed via PCR (Supplementary Fig. 1). In order to validate the completeness of the FIX transcripts, RT-PCR was performed with primers spanning the complete CDS. In addition to the full-length transcript, several smaller products occurred (Fig. 1). In cDNA from the juvenile moss protonemal tissue, PCR products of discrete sizes were detected ranging from ~700 to 1370 bp (Fig. 1a). Sequencing of the PCR products revealed that five of the observed bands were FIX-specific products with a length of 1371 bp, 1240 bp, 895 bp, 769 bp, and 691 bp, respectively. Apparently, unexpected splicing of the FIX mRNA produced the smaller aberrant transcripts. To analyze this phenomenon across different tissues, we repeated the same procedure with RNA isolated from adult plants (gametophores). FIX transcripts detected in gametophores were consistent with those from protonema (Fig. 1b).

The sequencing of different PCR products revealed that specific motifs along the human FIX mRNA were recognized as splice signals in *P. patens*. The spliced transcripts were identical in all 49 transgenic lines and in 2 different moss tissues. Hence, we conclude that different transcripts were exclusively derived from unexpected splicing of the mRNA transcribed from the complete FIX CDS, and not by a partial integration of the FIX expression construct into the *P. patens* genome. PCRs from genomic DNA support this conclusion (Supplementary Fig. 1).

In order to scrutinize the presence of spliced FIX CDS variants in transiently transfected cells (protoplasts), total RNA was extracted from cells 14 days after transfection. After RT-PCR, FIX CDS variants with varying lengths occurred. Like in the RT-PCR from transgenic lines, 5 FIX CDS variants (1 full-length and 4 aberrant spliced FIX transcripts) were confirmed in these protoplasts (Fig. 1c). Thus, we found an unexpected splicing of an mRNA transcribed from a human full-length cDNA in moss, a phenomenon we henceforth name heterosplicing.

Spliced FIX transcripts and splice-site analysis

Splice junctions within the FIX mRNA were revealed by sequencing. The splicing of the primary transcripts from human FIX cDNA in moss caused the production of four alternative products (Fig. 2a): i. deletion of a 44 aa long domain in the part coding for the heavy chain (Fig. 2a.ii), ii. large deletion in the part coding for the light chain (115 aa) together with the absence observed in the heavy chain (Fig. 2a.iii), iii. nearly complete absence of the sequence coding for the light chain and parts of the activation peptide domain (157 aa) and the heavy chain (Fig. 2a.iv), iv. nearly complete lack of the sequence encoding the light chain, complete absence of the activation peptide domain (186 aa) and the sequence representing 41 aa of the heavy chain (Fig. 2a.v). The heavy chain harbors a serine protease domain, which is responsible for the activation of Factor X in the presence of Factor VIII, calcium and phospholipid surfaces following removal of the activation peptide. Any deletion in this domain will interfere with the activity of the FIX protein. The light chain consists of the Gla domain

followed by two EGF domains. Any loss in these domains will adversely affect the properties of FIX. Therefore, any alterations caused by the heterosplicing will result in the loss of FIX function.

The GT-AG splicing rule applies for almost all eukaryotic genes⁴³ and especially *P. patens* accepts control elements originally optimized for mammalian expression systems without a need for adapting these elements⁴⁴. Here, we inferred four different motifs for acceptor and donor sites based on sequencing data. All FIX heterosplice sites followed the GT-AG rule. Moreover, the data revealed that a CAGGT motif is present in the exon-intron junction, i.e. the donor site (Fig. 3a). In the intron-exon junction, the acceptor site, the motif CAG is conserved in 3 out of 4 cases, but the adjacent two nucleotides do not follow any trend.

To analyze whether donor and acceptor sites and their neighboring nucleotides are conserved in *P. patens*, the genomic vicinity of all splice sites of all 87,533 annotated transcripts corresponding to the 32,926 protein-encoding genes of the current *P. patens* genome release v3.3⁴⁵ were retrieved (Fig. 3b, c). In alignment with our experimental findings, an *in silico*-analysis of these transcripts showed that the CAG|GT motif is the most abundant one in both donor and acceptor sites with a fraction of 23% and 18%, respectively. The full list of splice sites is compiled in Supplementary Tables 2 and 3.

Subsequently, we analyzed the FIX CDS with a moss splice-site prediction tool⁴⁶, but it largely failed to predict the experimentally verified splicing motifs. This might be due to the fact that this tool has been developed several years ago using only 368 donor and acceptor sites⁴⁶ and has not been updated since then.

Detection of truncated mossFIX protein variants and validation by mass spectrometry

The analyses at RNA level suggested that *P. patens* would produce a full-length FIX protein and four aberrant fragmentary FIX isoforms. To validate this inference, the culture supernatants of transiently transfected cells were precipitated and analyzed with a polyclonal anti-FIX antibody via immunoblot after reduction and alkylation (Fig. 4a). Full-length FIX was expected at a molecular mass of around 52 kDa and spliced FIX variants were calculated based on the heterospliced transcripts with molecular masses of 47, 34, 29, and 27 kDa, respectively. Observed bands on the immunoblot could be attributed to these isoforms together with a band at around 40 kDa (potentially a degradation product) and a band at around 70 kDa (potentially dimers of fragmentary protein isoforms). Additionally, we used the other half of the supernatants for mass-spectrometric (MS) determination of FIX-variant peptide sequences. For the MS-database search, protein models for FIX variants derived from the sequenced splice variants were employed.

We detected the peptide VVGGEDAKPGEHNIEETEHTQKR (Supplementary Fig. 2) in MS analysis after trypsin digestion, which resulted from splicing in the CDS for the heavy chain that led to the loss of 44 aa between the peptides VVGGEDAKP and GEHNIEETEHTQKR (Fig. 4b; variants II, III, and IV in Fig. 2a). This result confirmed the presence of a predicted FIX variant caused by heterosplicing on an additional level.

Prevention of heterosplicing by codon optimization

To analyze the effectiveness of mutating donor and acceptor sites on heterosplicing of the FIX mRNA, we created two different FIX CDS versions. In the first one, aspFIX, the codons neighboring the detected splice junctions were modified. *P. patens* was transiently transfected with the resulting aspFIX plasmid. On day 14, the cells were collected and RNA was isolated. RT-PCR and sequencing of the PCR products revealed that there were only two bands representing FIX variants: the full-length FIX CDS and a shorter variant (~700 bp) (Fig. 5a). The donor and acceptor sites of the shorter variant were identified (Fig. 5b) and this shorter variant was lacking the nucleotides encoding the light chain,

AP and parts of the heavy chain (Fig. 5c). Moreover, splicing would cause a frameshift mutation resulting in early stop codons. Although such nonsense mRNAs may be degraded via nonsense-mediated mRNA decay (NMD), not all nonsense mRNAs undergo NMD, which was reported e.g. for *P. patens*²³ and *A. thaliana*^{47,48}. In the second attempt, we performed a complete codon optimization of the FIX CDS in the aspFIX construct according to previous studies^{49,50} resulting in the optiFIX sequence with an overall increased GC content. This was used to transiently transfect moss cells. Subsequent RT-PCR analysis and sequencing revealed that the full-length CDS of FIX was the only specific band; formation of any heterospliced FIX variant was not detected (Fig. 5d). The presence of exclusively the full-length CDS was confirmed on protein level via immunoblot analysis (Fig. 5e).

Next, we compared the codon usage bias in *P. patens* to biases of other pharmaceutical production platforms. The codon usage frequencies of under- and over-represented codons for Cys, Glu, Phe, His, Lys, Asn, Gln, and Tyr in *P. patens* were compared with *Spodoptera frugiperda* (insect cells), *Homo sapiens* (HEK cells), *Cricetulus griseus* (CHO cells), *Nicotiana tabacum* (tobacco) and *Oryza sativa* (rice). For instance, the over-represented codon for Cys, TGC, in *P. patens* (9.8 in thousands) is also an over-represented codon in *S. frugiperda*, *H. sapiens*, *C. griseus*, and *O. sativa*. On the other hand, this codon is under-represented in *N. tabacum* and TGT is preferred over TGC for Cys. The synonymous codon usage bias in *P. patens* is similar to codon usage biases in *S. frugiperda*, *H. sapiens*, *C. griseus* (CHO cells), and *O. sativa* (rice), but different from *N. tabacum* (tobacco) (Table 1).

Comparison of protein levels of FIX and optiFIX *in vivo*

In order to analyze whether the protein amounts of FIX and optiFIX show any difference in live moss cells, we cloned two fusion constructs, FIX-Citrine and optiFIX-Citrine. Subsequently, we transiently transfected moss protoplasts with these constructs, and performed confocal laser scanning microscopy (CLSM) on day 3 after transfection. A visual analysis of the images revealed that the FIX-Citrine signal shows a diffuse localization pattern, which might be due to intracellular mislocalization of aberrant protein isoforms (Fig. 6a). In contrast, optiFIX-Citrine exhibited a complex network-like organization, indicating a proper localization of the protein in the ER (Fig. 6b). Furthermore, the optiFIX-Citrine signal intensity was higher than that of FIX-Citrine. Comparison of mean voxel intensities calculated for FIX-Citrine and for optiFIX-Citrine (3.88 and 41.73, respectively) shows a nearly eleven-fold higher fluorescence intensity after the codon optimization, which had prevented undesired heterosplicing (Fig. 6c).

Automatic codon-optimization of transgenic coding sequences

In order to automatize the process of *P. patens*-specific codon optimization, we compiled our findings into a JavaScript-based web application: physCO, and made it available at www.plant-biotech.uni-freiburg.de. physCO takes as input coding sequences in FASTA format, inspects them codon-wise, substitutes codons where possible according to our findings on codon usage (Table 2) and finally outputs them in FASTA format. In addition, physCO checks the input for consistent lengths, valid start and stop codons and internal, premature stop codons and raises warnings in case it detects irregularities.

Splicing of FH mRNA like FIX occurs in transgenic plants

In order to test whether heterosplicing is a common phenomenon in *P. patens* upon transgene expression, we analyzed already existing recombinant human complement factor H (FH)-producing moss lines⁴⁰. Our RT-PCR analysis revealed that expression of the human FH CDS in moss generated two transcript variants, the full-length FH transcript of 3,639 bp and a 1,215 bp FH variant, which could lead to a truncated FH isoform with a molecular mass of 45.6 kDa (Supplementary Fig. 3). Therefore, we generated with the help of physCO an optimized version of hFH, and named it optiFH.

These changes affected 386 of 1213 codons, and increased the GC% content from 33.9% to 49.8% (Supplementary Fig. 4).

To investigate the effects of these alterations in live moss cells, we cloned two fusion constructs, FH-Citrine and optiFH-Citrine. Subsequently, we transiently transfected moss protoplasts with these constructs, and performed CLSM on day 3 after transfection. A visual analysis of the images acquired for FH and optiFH revealed that while an FH-Citrine signal was not detectable, optiFH-Citrine signal intensity was high and filled the secretory pathway (Supplementary Figure 5). Analysis on FH showed that heterosplicing is not an effect specific for FIX, but a general phenomenon in moss. Therefore, transgene sequences have to be modified using physCO prior to transfection for proper transcription and translation.

DISCUSSION

Heterologous gene expression is of fundamental importance for basic biology and for industrial production. In order to optimize transgene transcription and translation as well as transcript stability, many factors have to be taken into account. Engineering of upstream and downstream regulatory sequences, replacing original signal peptides with host-suitable ones, and optimizing codons according to host codon-usage pattern can be used to improve protein production.

Splicing/alternative splicing is another important factor that alters expression rates by the generation of various transcripts, which create different protein isoforms. Generation of these protein isoforms can affect fundamental characteristics of the protein such as function, activity, intracellular localization, interaction with other molecules, and stability. Moreover, splicing can create a transcript with an early stop codon, which may be degraded by NMD. Thus, it can be hypothesized that modulation of splicing, a neglected point in transgene expression, can improve protein production. For instance, the expression of GFP from the jellyfish *Aequorea victoria* in *A. thaliana* failed due to aberrant mRNA processing³⁰. To avoid AS, generally cDNAs derived from already spliced mRNAs are introduced into the genome of the production host.

We introduced the human FIX cDNA under the control of the *PpActin5* promoter and CaMV 35S terminator into the *P. patens* genome. RNA was isolated from protonemata of transgenic lines and RT-PCR analysis revealed unexpectedly that a fraction of human FIX mRNA was spliced into four variants in addition to the unspliced full-length transcript, resulting in five mRNAs of different lengths derived from one cDNA. According to RT-PCR analyses, this phenomenon occurred not only in juvenile protonema, but also in adult moss plants. We sequenced these mRNAs and surprisingly found that reading frames had not changed in the four smaller variants in comparison to the mature full-length transcript. This may have happened by a pure coincidence. Alternatively, we may not have been able to detect such putative minor transcripts because they were either too low abundant for RT-PCR, or they have been eliminated by NMD.

The outcome of this heterosplicing was detectable on four levels, by RT-PCR from mRNAs, immunoblot, mass spectrometry of the protein isoforms, and *in vivo* by confocal imaging showing deviant localization of Citrine reporter fusions. These findings were also reflected in RT-PCR analysis from transiently transfected moss cells. Our observations revealed that heterosplicing of the human FIX mRNA in moss is consistent across different tissues and not dependent on developmental stages. Furthermore, the consistency of heterosplicing in episomally (transiently) FIX-expressing cells indicates that heterosplicing of the FIX mRNA in transgenic lines was neither due to illegitimate or partial integration of the construct into the moss genome nor a locus-specific effect. Moreover, results of PCRs from genomic DNA of transgenic lines supported this conclusion. Unlike a previously reported

case, in which expression of full-length GFP transcript and fluorescence were not detectable³⁰, we report here for the first time heterosplicing of a human cDNA in a plant. It caused the generation of various FIX mRNA variants and protein isoforms in addition to the complete transcript and protein. Therefore, a possible occurrence of heterosplicing should be analyzed on RNA level, even if a full-length protein can be detected in heterologous cells.

All splice junctions in the FIX CDS were determined by sequencing. In addition, we identified consensus motifs of donor and acceptor splice sites in the latest *P. patens* genome version⁴⁵ using all 87,533 annotated transcripts that originate from all 32,926 protein-coding genes, and compared them with our experimentally verified motifs. This revealed that *P. patens*, like most eukaryotes, follows the GT-AG splicing rule. The same was true for the cryptic additional introns recognized in the FIX mRNA; obviously, the *P. patens* spliceosome interprets parts of the FIX CDS as intronic sequences. Presumably, moss cells recognize motifs in the human FIX CDS as *cis* exonic and intronic splicing enhancers. Little is known about exonic and intronic regulatory sequences in plants^{51,52}. Moreover, plant introns are different from animal introns in terms of UA- or U-richness, which is crucial for splicing efficiency⁵³. Due to these characteristic differences, it is likely that the GC content can affect the characteristics of splice site recognition¹⁵. The GC content is correlated with many features including gene density⁵⁴, intron length⁵⁵, meiotic recombination⁵⁶, and gene expression⁵⁷. It varies among species, and even along chromosomes⁵⁸. It was shown before that the average GC content in *P. patens* coding sequences is 50%⁴⁶. The GC content of the human FIX CDS recognized as cryptic additional introns ranged from 38% to 40%. This remained the same after exclusively mutating the splice sites' environment in aspFIX; even though mutations in the splicing motifs decreased the number of transcripts from five to two, the GC content of the cryptic alternative intron was still only 40%. The shorter transcript was not in frame, but still detectable by RT-PCR. Therefore, we created the fully codon-optimized plasmid optiFIX to check the effect of overall codon optimization on pre-mRNA splicing. A closer comparison of the aspFIX and optiFIX constructs revealed that there was no change in the donor and acceptor sites of the newly emerged cryptic intron. There was, however, an increase of the GC content within the cryptic intron sequence. The GC contents of the sequences previously defined as introns in the FIX and aspFIX constructs increased to 46% - 50% in optiFIX. Hence, we conclude that the increase of GC content introduced by the overall codon optimization together with mutating splice sites' environment prevented the heterosplicing, the mis-interpretation of any sequence as intron by the moss spliceosome. As a result, a nearly eleven-fold increase in protein amounts was achieved in transiently transfected moss protoplasts *in vivo*. As heterosplicing did not cause detectable frameshift mutations, fluorescence signals obtained from the FIX-Citrine construct were the sum of all FIX protein isoforms, full-length and fragmentary, fused to Citrine. Based on this inference and proteins, which were harvested from transient transfection with the FIX construct, and detected on the immunoblot (Fig. 4), we conclude that the prevention of heterosplicing from the optiFIX construct has improved full-length protein amounts *in vivo* even more than eleven-fold.

Two mechanisms, namely boosted translation rates and the prevention of mRNA degradation via NMD, may contribute to this result. In addition, the protein from the optiFIX construct was solely detectable in the ER, the compartment of choice for correct post-translational modifications and subsequent secretion of the protein. A similar phenomenon was observed in the expression of another transgene in *P. patens*. One example of heterosplicing and its prevention by our methodology is the expression of human complement factor H (FH). FH is an important regulator of the alternative pathway in the human complement system. Currently, a recombinant FH is not available on the market, although it has potential as a biopharmaceutical in the treatment of severe human diseases like atypical hemolytic uremic syndrome (aHUS), age-related macular degeneration (AMD) or C3 glomerulopathies (C3G). The recombinant production of FH, devoid of plant-specific N-linked sugar residues, in *P. patens* resulted in a range of promising biological activities⁴⁰. Moreover, moss-derived FH is currently being examined for use in Covid-19 treatment (www.elevabiologics.com). RT-PCR

analysis in FH-producing stable moss lines revealed that the expression of human FH CDS generated 2 transcript variants, the full-length FH transcript of 3,639 bp and a 1,215 bp FH variant that could lead to the production of an FH isoform with a calculated molecular mass of 45.6 kDa (Supplementary Fig. 3). We optimized the FH CDS using our bioinformatics tool physCO, described here, to analyze whether the protein amounts of FH and optiFH show any difference in live moss cells. A visual analysis of the images acquired for FH and optiFH from transiently transfected moss cells revealed that while FH-Citrine signal intensity is not detectable, optiFH-Citrine signal intensity is high (Supplementary Fig. 5). Thus, an optimization of the currently used CDS of human FH in plants is advantageous for plant-based production.

These findings suggest that the splicing of heterologous mRNAs should be taken into account on a routine basis. In addition, our analysis of synonymous codon usage bias suggests that our methodology to prevent heterosplicing can be directly implemented in organisms, which follow similar codon usage patterns as *P. patens*, such as *O. sativa*, insect or mammalian cells. Our methodology might still be vital in organisms that have different codon usage patterns, such as the species of the genus *Nicotiana*, e.g. *N. tabacum* and *N. benthamiana*. Mutating donor and acceptor sites and their neighboring nucleotides together with replacing codons ending with A or T to G or C may prevent the generation of heterosplice variants.

Why should splice site mutation and codon optimization be employed at the same time rather than codon optimization alone? Although differences between GC-rich and GC-poor genes were not reported in moss, analyses of various plant species can explain why codon optimization might not be sufficient. The GC content of GC-poor CDS in *A. thaliana*, soybean, pea, tobacco, tomato, and potato ranges from 40-46% while GC content of introns is 26-31%⁵⁹. A similar trend is visible in GC-rich genes: 46-49% in CDS and 29-31% in introns. On the other hand, in maize, the GC content is higher: 56% in GC-poor CDS and 67% in GC-rich CDS. The GC content of introns in maize is also elevated: 40% in GC-poor genes and 48% in GC-rich genes⁵⁹. This shows that the intron/exon definition is not solely dependent on the GC content. Hence, increasing the GC content of cryptic introns to a certain level without mutating splice site motifs might not be sufficient to prevent aberrant mRNA processing.

In general, the analysis of spliced transcripts is beneficial for at least five reasons. (i) It facilitates the reliable quantification of gene expression by designing primers that differentiate between full-length transcripts and shorter variants. (ii) It enables the precise detection and quantification of heterologous proteins by antibodies that differentiate functional isoforms from non-functional ones. (iii) It helps to better purify proteins either by specific antibodies or by size exclusion chromatography. (iv) Optimized cDNAs may result in better detectable reporter proteins *in vivo*. (v) Aberrant protein isoforms due to heterosplicing may show mislocalization in cells of the production host and thus may contribute to poorly modified glycoproteins. Prevention of heterosplice variants eventually will improve recombinant protein production and decrease downstream processing costs significantly. Hence, plant-based systems can become an alternative to traditional production platforms. Moreover, it may have implications for basic biology as well, because here the use of heterologous reporter constructs is of vital importance. For these purposes, we developed physCO, an automated *P. patens*-specific codon-optimization tool, which allows the optimization of transgene expression by the prevention of undesired mRNA splicing. Moreover, our analysis of codon usage patterns indicates that this tool can also be used for insect (*S. frugiperda*), HEK (*H. sapiens*), and CHO (*C. griseus*) cells as well as rice (*O. sativa*). Besides that, we cannot exclude the possibility, that the phenomenon of mRNA splicing here described as heterosplicing is a novel gene regulatory mechanism occurring in eukaryotes in general.

MATERIALS AND METHODS

Design of the expression vectors pFIX, aspFIX, and optiFIX

The coding DNA sequence for human Factor IX without the native signal peptide was synthesized as N-terminal translational fusion to the signal-peptide coding sequence of PpAP1 (Pp3c5_19520V3.1)⁴² by GeneArt (Thermo Fisher Scientific, Waltham, MA, USA). It was cloned into the pAct5-MFHR1 plasmid⁴¹ to generate the final FIX expression vector, pFIX. Its expression is driven by the promoter of the *PpActin5* gene⁶⁰ and the CaMV 35S terminator.

Nucleotides involved in splicing were replaced without a change of the FIX aa sequence in the aspFIX plasmid which was synthesized by GeneArt. For codon optimization, two sources, codon usage bias calculated by Hiss *et al.*⁴⁹ and Kazusa codon usage database⁵⁰ (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=145481>), were used. Unlike a recent report⁴⁹, we decided to exchange the eight under-represented codons for Cys, Glu, Phe, His, Lys, Asn, Gln and Tyr. Under-represented codons were replaced with the over-represented alternative codons (Table 2) via physCO. The optiFIX CDS was synthesized by GeneArt. See Fig. 7 for a multiple sequence alignment of FIX, aspFIX, and optiFIX.

Design of the expression vectors pFH and optiFH

The previously generated Factor H expression plasmid pFH⁴⁰ contains the same promoter and terminator as pFIX. It was used in transient expression assays. The optiFH CDS was deduced via physCO and synthesized by GeneArt. It was cloned into the same vector as FH. See Supplementary Fig. 4 for a sequence alignment of FH and optiFH.

Transfection and screening of transgenic plants

The *Physcomitrella patens* (Hedw.) B.S. $\Delta xtf/ft$ moss line, a double knockout line in which the $\alpha 1,3$ fucosyltransferase and the $\beta 1,2$ xylosyltransferase genes had been abolished⁶¹ (IMSC accession number 40828), was cultured in liquid Knop medium (pH 4.5) one week prior to transfection as described previously⁶² to obtain cells (protoplasts). Protoplast transfection was performed as described⁶³ using 50 μ g of linearized pFIX DNA per transfection. In addition to the generation of transgenic lines, mossFIX was produced transiently by an upscaled transfection protocol based on the proportion of 0.7-1 μ g DNA of pFIX for 1×10^5 cells. Stable transfectants were selected on solidified Knop medium containing 25 μ g/mL hygromycin as described⁶². Cells transiently expressing mossFIX were grown in special regeneration medium (7.5 mM $MgCl_2 \times 6H_2O$, 2.6 mM MES, 200 mM D(-)-Mannitol, 62.6 mM $CaCl_2 \times 2H_2O$, 68.4 mM NaCl, 2.8 mM D(+)-Glucose monohydrate, 5 mM KCl; pH: 5.6; osmolarity: ~550–570 mOsm) and harvested for RNA and protein analyses two weeks after transfection. To analyze whether splicing of FIX was prevented on RNA level, WT cells were also transfected with the aspFIX and optiFIX constructs, respectively, and harvested for RNA isolation two weeks after transfection.

Plant cell culture

After selection on solidified Knop medium, transgenic lines were cultured under standard conditions in liquid Knop medium⁶⁴. They were subcultured periodically by disrupting the tissue with the use of an Ultra-Turrax (IKA, Staufen, Germany) at a rotational speed of 16,000 – 18,000/min for 1 min. Subsequently, they were transferred to fresh medium.

Molecular characterization of transgenic lines

Following the hygromycin selection process, stable lines were characterized by the presence of the FIX transcript. To validate the presence of the transgene in the moss genome, 80-100 mg (fresh weight) moss material from protonema cultures were disrupted with the use of a stainless steel ball in TissueLyser II (Qiagen, Hilden, Germany) for 1 min with an impulse frequency set to 30. Genomic DNA was extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Transgene presence and completeness were verified with a standard PCR with the primers FIXfwdB (5'-GGAGTGTTCGATTGGCTTTCT-3') and FIXrevB (5'-TGGTGGTGGTGGGTTAGTTT-3') that amplifies almost the full-length FIX CDS. For transgene expression analysis via reverse transcription (RT)-PCR, 50-100 mg (fresh weight) moss material from protonema cultures were disrupted with the use of a stainless steel ball in TissueLyser II for 2 min with an impulse frequency set to 30. The material was resuspended in 1 mL TRIzol® Reagent (Thermo Fisher Scientific), and RNA was isolated according to the manufacturer's instructions. For analysis of gametophores, 5-6 gametophores were collected and RNA isolation was performed as described above. Six micrograms of DNase-treated RNA were used for first strand synthesis with Superscript Reverse Transcriptase III (Thermo Fisher Scientific). The quality of cDNA was controlled with a standard PCR with the primers c45for (5'-GGTTGGTCATGGGTTGCG-3') and c45rev (5'-GAGGTCAACTGTCTCGCC-3') corresponding to the gene coding for the ribosomal protein L21. Transgene expression was verified with the primers FIXfwdB and FIXrevB that amplifies almost the full-length FIX CDS. PCR reactions were performed with Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific). The PCR products were examined by standard agarose gel electrophoresis with visualization of DNA by ethidium bromide fluorescence.

Molecular characterization of transiently transfected cells

Following the transfection, cells were grown in regeneration medium for two weeks⁶⁵. Transiently transfected cells (nearly 3.6 million cells for FIX and aspFIX; 7.2 million cells for optiFIX) were used for transgene expression analysis via RT-PCR. RNA was isolated using the TRIzol® Reagent and RNA was purified through spin columns of Direct-zol™ RNA MicroPrep kit (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. After DNase-treatment and first-strand synthesis (for the cDNA synthesis: 0.4 µg RNA were used for FIX and aspFIX; 2 µg RNA were used for optiFIX), the quality of cDNA was controlled with primers c45for and c45rev. Transgene expression was verified again with primers FIXfwdB and FIXrevB.

Analysis of FIX cDNA products

Amplified products were excised from the gel and purified using QIAEX II Gel Extraction Kit (Qiagen). Sequencing was done by Eurofins Genomics (GATC Sanger sequencing service). In the sequencing reactions, in addition to FIXfwdB and FIXrevB, FIX6deepseq1F (ATGGGGGCGATCGAGGAGTGTTCGAT), FIX6deepseq1R (TTCCCCAGCCACTTACATAGCCAGA), and FIXseq6 (CACCAACAACCCGAGTGAAG) were used. The remaining purified products were separately cloned into the pJET vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Clones with aberrant insert sizes were selected for plasmid isolation with the GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific). Sequencing was done as described above.

Splicing motif search

The most recent release of the *Physcomitrella* patens v3.3 genome annotation⁴⁵, which is available from <https://phytozome.jgi.doe.gov/>, was used. The splice sites of all 87,533 annotated transcripts

from all 32,926 protein-coding genes were used as anchors to extract surrounding genomic sequences with a fixed window size of ± 9 bp. Consensus sequence logos of all extracted donor and acceptor sites were created using R and the package ggseqlogo^{66,67}.

Codon usage bias among different species

Synonymous codon usage biases in *P. patens*, *S. frugiperda*, *H. sapiens*, *C. griseus*, *O. sativa*, and *N. tabacum* were obtained from the Codon Usage Database (<https://www.kazusa.or.jp/codon/>) and biased codons in moss were compared with the codon usage pattern of other species.

Confocal microscopy imaging and image analysis

All images were taken with a Leica TCS SP8 microscope (Leica Microsystems, Wetzlar, Germany) using HCX PL APO 63x/1.40 oil objective with a zoom factor of 4.5. For the excitation of Citrine, an WL laser was applied at 2% with an excitation wavelength of 514 nm. The voxel sizes were 0.080 μm on the X-Y dimensions and 0.300 μm on the Z dimension. The pinhole was adjusted to 1 Airy Unit (95.5 μm).

The image processing consists of the following steps: i) denoising, ii) edge enhancement, iii) Richardson-Lucy restoration, iv) local intensity equalization, v) segmentation. In the first step, a median filter with a window of (3,3,3) was applied to the images in order to remove the salt-and-pepper noise. In the second step, the denoised images were subjected to an unsharp-mask operation^{68,69}, which involves blurring the image with a Gaussian filter, subtracting the blurred image from the input image, and adding the resulting difference back to the input image, a process that sharpens the edges in the images. The edge-enhanced images were then subjected to the Richardson-Lucy restoration algorithm, as implemented in the scikit-image package⁷⁰ for three iterations, assuming an averaging filter in a window of (3,5,5) as point spread function (here the aim was to smooth the image without losing the thin structures, rather than a true deblurring). The code for the Richardson-Lucy algorithm was adapted from scikit-image package⁷⁰ using Python programming language. Subsequently, a spatial intensity equalization step was implemented on the images using a home-built algorithm. In short, each image was divided into boxes with dimensions of (7,7,7) voxels. The voxel values within these boxes were rescaled by multiplying the values in each box with weights that are calculated based on the skewness value corresponding to the same box. This step corrected for the intensity gradients in the image in order to minimize the loss of some low-intensity foreground voxels. In the last step, the images were segmented by applying an adaptive thresholding method, where the thresholds were determined locally by applying Otsu's method to local windows of (7,19,19) voxels throughout the image volume. The code for the adaptive Otsu thresholding algorithm was developed based on the Otsu threshold function of the scikit-image package⁷⁰ and the Numba package⁷¹. To suppress over-segmentation of noisy background areas, a low global threshold was also applied by first calculating Otsu's threshold for whole image and multiplying it with 0.66. The adaptive thresholding operation yielded the binary masks that specified the foreground voxels, which in turn were used to calculate the mean voxel intensity for each original image. Three replicates were used for analysis of each of the FIX-Citrine and optiFIX-Citrine constructs. The same is true for FH-Citrine and optiFH-Citrine but the quantification was not possible due to undetectable/very low signals in FH-Citrine images. All six FIX images were processed and quantified in a single run of a Python script by using the exact same parameters to avoid any possible bias. Other packages used to develop the code are: NumPy^{70,72}, Pandas⁷³ and SciPy⁷⁴. The code used for the image processing and quantification can be obtained from www.plant-biotech.uni-freiburg.de. The procedure is shown in Supplementary Fig. 6.

Recombinant protein extraction and detection

Proteins from the culture supernatant were precipitated as described⁷⁵. After air drying, the precipitate was resuspended in 50 mM HEPES, 2% SDS (pH 7.5) and disulfide bonds within proteins were reduced with 25 mM dithiothreitol (DTT), followed by alkylation of the thiol groups with 60 mM iodoacetamide (IAA). For Western blot analyses, 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE Ready Gel® Tris-HCl Precast Gels, BioRad, CA, USA) was run at 100 V for 1h 30min and blotted to polyvinylidene fluoride (PVDF) membranes (Amersham Hybond P 0.45 µm pore size PVDF blotting membrane, GE Healthcare) in a Trans-Blot® SD Semi-Dry Transfer Cell (BioRad) for 2 h with 1.8 mA/cm² membrane. The membrane was blocked for 1 h at room temperature in TBS containing 4% Amersham ECL Prime Blocking Reagent (GE Healthcare) and 0.1% Tween 20. It was then incubated with polyclonal anti-factor IX antibody (F0652, SIGMA) (1:3000) overnight at 4°C. Afterwards, the membrane was washed three times with TBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-linked anti-rabbit IgG (NA934, GE Healthcare) at a dilution of 1:10,000 for 1 h. The blot was washed again and proteins were detected using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer's instructions.

Sample preparation and MS analysis

The reduced and alkylated protein extracts were subjected to SDS-PAGE. Appropriate gel bands showing FIX fractions were excised and processed for in-gel digestion: Following the destaining of the gel bands with 30% acetonitrile (ACN), they were treated with 100% ACN and dried completely in a vacuum concentrator. Tryptic in-gel digestion of gel slices was performed overnight at 37°C in 50 mM ammonium bicarbonate using 0.1 µg trypsin (Promega) per gel band. Afterwards, peptides were extracted from the gel with 5% formic acid. Analyses were performed using the UltiMate 3000 RSLCnano system (Dionex LC Packings/Thermo Fisher Scientific) coupled online to a QExactive Plus instrument (Thermo Fisher Scientific). The UHPLC systems was equipped with a C18-precursor column (Ø: 0.3 mm, 5 mm; PepMap, Thermo Fisher Scientific) and an Acclaim® PepMap analytical column (ID: 75 µm, 500 mm, 2 µm, 100 Å, Dionex LC Packings/Thermo Fisher Scientific).

MS analyses were performed using a binary solvent system consisting of 0.1% formic acid (FA, solvent A, "A") and 0.1% FA/86% ACN (solvent B, "B"). Samples were washed and concentrated on a C18 pre-column with 0.1% TFA for 5 min before switching the column in line with the analytical column. Peptide separation was performed applying a 45-min gradient at a flow rate of 250 nl/min. Peptide samples were eluted with a gradient of 4–40% B in 30 min and 40–95% B in 5 min. After each gradient, the analytical column was washed with 95% B for 5 min and re-equilibrated for 15 min with 4% B. The MS instrument was externally calibrated using standard compounds and equipped with a nanoelectrospray ion source and distal coated SilicaTips (FS360–20-10-D, New Objective, Woburn, MA, USA), and MS/MS analyses were performed on multiply charged peptide ions. The measurement was performed using UltiMate 3,000 RSLCnano system (Dionex LC Packings/Thermo Fisher Scientific, Dreieich, Germany) coupled online to a QExactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and mass spectrometer was operated in the data-dependent mode to automatically switch between MS (max. of 1x10 ions) and MS/MS. Each MS scan was followed by a maximum of 12 MS/MS scans using HCD with a normalized collision energy of 35%. The mass range for MS was $m/z = 375-1,700$ and resolution was set to 70,000. MS parameters were as follows: spray voltage 1.5 kV; ion transfer tube temperature 200°C. Raw data were analyzed using Mascot Distiller V2.5.1.0 (Matrix Science, USA) and the peak lists were searched with Mascot V2.6.0 against an in-house database containing all *P. patens* V1.6 protein models²² as well as human FIX (complete and alternative ones based on splicing). The peptide mass tolerance was set to 8 ppm and the fragment mass tolerance was set to 0.02 Da. carbamidomethylation (C, +57.021464 Da) was specified as fixed

modification. Variable modifications were Gln->pyro-Glu (-17.026549 Da), oxidation/hydroxylation (M, P, +15.994915 Da) and deamidation (N, +0.984016 Da). A total of two missed cleavages was allowed as well as semitryptic peptides. Search results were loaded into Scaffold4 software (Version 4.11.0, Proteome Software Inc., Portland, OR) and an additional database search using X!Tandem⁷⁶ implemented in the software on the loaded spectra was performed.

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AUTHOR CONTRIBUTIONS

O.T., E.L.D. and R.R. designed research; O.T., S.W.L.M., B.Ö., and N.v.G. performed research and together with S.N.W.H analyzed data; O.T., E.L.D., and R.R. wrote the paper.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this article and its supplementary information.

CODE AVAILABILITY

JavaScript-based web application, physCO, as well as the code used for the image processing and quantification are available at www.plant-biotech.uni-freiburg.de.

COMPETING INTERESTS

The authors are inventors of patents and patent applications related to the production of recombinant proteins. R.R. is a founder of Greenovation Biotech (now eleva). He currently serves as advisory board member of this company. Eleva develops and markets moss-based biopharmaceuticals.

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FIGURES AND FIGURE LEGENDS

Fig. 1: Expression of FIX in *Physcomitrella patens*. **a** Total RNA from protonema cultures of 4 FIX-transgenic lines (F) and the parental line $\Delta xt/ft$ was prepared and used for RT-PCR using the primers FIXfwdB and FIXrevB. **b** RT-PCR of FIX using the primers FIXfwdB and FIXrevB from gametophores of the same lines. **c** RT-PCR of FIX with mRNA extracted from transiently transfected (FIX) as well as non-transfected ($\Delta xt/ft$) cells. M: 1 kb Marker (Thermo Fisher Scientific), -: without reverse transcription, +: with reverse transcription, CO: Water control.

Fig. 2: Schematic representation of FIX transcripts and multiple sequence alignment of predicted FIX protein isoforms based on experimentally verified transcripts detected in *Physcomitrella patens*. **a** Full-length FIX transcript (a.i), FIX variant with the deletion of a 44 aa long domain in the CDS for the heavy chain (a.ii), FIX variant with deletions in the CDS for both light and heavy chain (a.iii), FIX variant with deletions in the CDS for light chain, activation peptide and heavy chain (a.iv), FIX variant missing the CDS completely for the activation peptide, nearly full-length of light chain and 41 aa long domain in the heavy chain (a.v). The star indicates the usage of a donor site 9 bp earlier than previously identified donor site in the heavy chain. SP: PpAP1 signal peptide, PP: propeptide, AP: activation peptide, H: 8x His tag. **b** Alignment was performed and formatted for publication with MUSCLE via the "MSA" package for R^{49,50}.

Fig. 3: Schematic representation of donor and acceptor sites within the FIX sequence and consensus sequence motifs of donor and acceptor splice sites recovered from all 87,533 *P. patens* transcripts. **a** Donor and acceptor sites. Four different splicing motifs were identified after sequencing FIX variants. Left side shows donor site and right side shows acceptor site. “-1” represents last nucleotide of exon in the donor site (left side) or last nucleotide of intron in the acceptor site (right side). **b, c** Consensus genomic sequence motifs of donor (b) and acceptor splice sites (c) extracted from all 32,926 *P. patens* protein-coding genes. Probability and height of individual letters correspond to base frequencies at each position.

Fig. 4: Analysis on moss-produced FIX protein isoforms. a Immunodetection of extracellular FIX produced by two transfections (anti-FIX Ab 1:5,000). Culture supernatant of non-transfected cells was used as negative control (Neg). T1, T2: Two different transient transfections with the FIX cDNA-based expression plasmid. **b** Full-length mossFIX amino acid sequence. Amino acids marked in yellow were identified in the MS analysis. Blue boxes mark the parts of the peptide VVGGEDAKPGEHNIEETEHTEQKR which was identified in MS analysis and represents a heterosplice product.

Fig. 5: Heteroslicing of the human FIX CDS in *Physcomitrella patens* was successfully prevented by codon optimization. **a** RT-PCR of FIX RNA from cells that were transiently transfected with aspFIX and non-transfected cells (WT). M: 1 kb Marker (Thermo Fisher Scientific), -: without reverse transcription, +: with reverse transcription, Pos: PCR with aspFIX plasmid used as positive control. **b** Splicing motifs identified after sequencing the FIX variant. The left side shows the donor site and the right side the acceptor site. "-1" represents the last nucleotide of the exon at the donor site or the intron at the acceptor site, respectively. **c** Schematic representation of complete FIX (upper) and the additional variant (lower). SP: signal peptide, PP: propeptide, AP: activation peptide. **d** RT-PCR of FIX RNA from cells that were transiently transfected with optiFIX and non-transfected cells (WT), respectively. **e** Immunodetection of extracellular FIX protein with anti-FIX Ab (1:5,000). Culture supernatant of non-transfected cells was used as negative control (Neg). T1, T2: Two different transient transfections with the optiFIX plasmid. M: 1 kb Marker (Thermo Fisher Scientific).

Fig. 6: Comparison of FIX and optiFIX protein levels using confocal microscopy and quantitative image analysis. **a** Transient overexpression of FIX-Citrine. The slice with the brightest mean intensity was extracted from a Z-stack and its voxel intensity range was adjusted to (0-36) for illustration. Scale bar is 5 μm. **b** Transient overexpression of optiFIX-Citrine. The slice with the brightest mean intensity was extracted from a Z-stack and its voxel intensity range was adjusted to (0-36) for illustration. Scale bar is 5 μm. **c** Comparison of the mean signal intensities of the Z-stacks for FIX-Citrine and optiFIX-Citrine (n=3).

TABLES AND THEIR LEGENDS

Table 1: The codon usage frequencies of under- and over-represented codons for eight amino acids in different protein expression systems. Numbers represent the frequency of codon per thousand. Data are from <http://www.kazusa.or.jp/codon/>.

Codon frequency in thousands

		<i>Physcomitrella patens</i>	<i>Spodoptera frugiperda</i>	<i>Homo sapiens</i> (HEK cells)	<i>Cricetulus griseus</i> (CHO cells)	<i>Oryza sativa</i>	<i>Nicotiana tabacum</i>	
<i>under-represented</i>	TGT	7.0	8.5	10.6	9.1	6.2	9.8	Cys
<i>over-represented</i>	TGC	9.8	13.2	12.6	10.3	12.4	7.2	
<i>under-represented</i>	GAA	24.1	27.6	29.0	28.4	21.6	36.0	Glu
<i>over-represented</i>	GAG	37.4	33.1	39.6	41.1	38.6	29.4	
<i>under-represented</i>	TTT	17.2	10.1	17.6	19.6	13.1	25.1	Phe
<i>over-represented</i>	TTC	23.6	27.5	20.3	22.0	22.4	18.0	
<i>under-represented</i>	CAT	11.1	8.7	10.9	10.2	11.3	13.4	His
<i>over-represented</i>	CAC	11.6	15.6	15.1	12.9	13.8	8.7	
<i>under-represented</i>	AAA	18.9	26.8	24.4	24.6	16.0	32.6	Lys
<i>over-represented</i>	AAG	34.7	49.2	31.9	38.4	32.3	33.5	
<i>under-represented</i>	AAT	17.8	13.4	17.0	17.4	15.1	28.0	Asn
<i>over-represented</i>	AAC	20.4	28.8	19.1	21.2	18.5	17.9	
<i>under-represented</i>	CAA	16.7	16.1	12.3	10.3	13.5	20.7	Gln
<i>over-represented</i>	CAG	22.0	21.6	34.2	33.4	20.8	15.0	
<i>under-represented</i>	TAT	10.5	10.0	12.2	13.1	10.0	17.8	Tyr
<i>over-represented</i>	TAC	17.2	24.4	15.3	16.4	15.1	13.5	

Table 2: Under-represented codons were changed into the over-represented ones based on the codon usage tables^{49,50} to optimize FIX sequence using aspFIX as a template.

Amino acid	Under-represented codons in aspFIX	For optiFIX, changed into
Cys	TGT	TGC
Glu	GAA	GAG
Phe	TTT	TTC
His	CAT	CAC
Lys	AAA	AAG
Asn	AAT	AAC
Gln	CAA	CAG
Tyr	TAT	TAC

Supplementary Data

Supplementary Fig. 1: The presence of complete FIX CDS in transgenic lines. Genomic DNA from protonema cultures of 4 FIX-transgenic lines (F), the parental line $\Delta xt/ft$, and WT was prepared and used for PCR using the primers FIXfwdB and FIXrevB. M: 1 kb Marker (Thermo Fisher Scientific), CO: Water control.

Supplementary Fig. 2: Identification of the peptide VVGGEDAKPGEHNIEETEHTQKR by MS. a HCD fragment ion spectrum of the identified peptide. **b** Fragment mass error distribution of the b- and y-ion series.

Spliced_FH	1	EDCNELPPRRNTEILTGSWSDDQTYPEGTQAIYKCRPGYRSLGNVIMVCRKGEWVALNPLR
FH	1	EDCNELPPRRNTEILTGSWSDDQTYPEGTQAIYKCRPGYRSLGNVIMVCRKGEWVALNPLR
consensus	1	*****
Spliced_FH	61	KCQKRPCGHPGDTPFGTFTLTGGNVFEYGVKAVYTCNEGYQLLGEINYRECDTDGWTNDI
FH	61	KCQKRPCGHPGDTPFGTFTLTGGNVFEYGVKAVYTCNEGYQLLGEINYRECDTDGWTNDI
consensus	61	*****
Spliced_FH	121	PICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSGYKIEGDEEMHCSDDGFW
FH	121	PICEVVKCLPVTAPENGKIVSSAMEPDREYHFGQAVRFVCNSGYKIEGDEEMHCSDDGFW
consensus	121	*****
Spliced_FH	181	SKEKPKCVEISCKSPDVINGSPISQKIIYKENERFQYKCNMGYEYSERGDAVCTESGWRP
FH	181	SKEKPKCVEISCKSPDVINGSPISQKIIYKENERFQYKCNMGYEYSERGDAVCTESGWRP
consensus	181	*****
Spliced_FH	241	LPSCE-----
FH	241	LPSCEEKSCDNPYIPNGDYSPLRIKHRTGDEITYQCRNGFYPATRGNTAKCTSTGWIPAP
consensus	241	*****
Spliced_FH	246	-----
FH	301	RCTLKPDCDYPDIKHGGLYHENMRRPYFPVAVGKYYSYCDHFETPSGSYWDHIHCTQDG
consensus	301	
Spliced_FH	246	-----
FH	361	WSPAVPCLRKCYFPYLENGYNQNHGRKFVQGKSIDVACHPGYALPKAQTTVTCMENGWSP
consensus	361	
Spliced_FH	246	-----
FH	421	TPRCIRVKTCCKSSIDIENGFISESQYTYALKEKAKYQCKLGYVTADGETSGSIRCGKDG
consensus	421	
Spliced_FH	246	-----
FH	481	WSAQPTCIKSCDIPVFMNARTKNDFTWFKLNDTLDYECHDGYESNTGSTTGSIVCGYNGW
consensus	481	
Spliced_FH	246	-----
FH	541	SDLPICYERECLEPKIDVHLPDRKKDQYKVGVLKFSCKPGFTIVGPNSVQCYHFGLSG
consensus	541	
Spliced_FH	246	-----
FH	601	DLPICKEQVQSCGPPPELLNGNVKEKTKEEYGHSEVVEYYCNPRFLMKGPNKIQCVDGEW
consensus	601	
Spliced_FH	246	-----
FH	661	TTLPVCIVEESTCGDIPELEHGWAQLSSPPYYGDSVEFNCSESFTMIGHRSITCIHGVW
consensus	661	

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Spliced_FH 246 -----
FH          721 TQLPQCVAIDKLLKKCKSSNLIILEEHLKNKKEFDHNSNIIRYRCRGKEGWIHTVCINGRWD
consensus   721 .....

Spliced_FH 246 -----
FH          781 PEVNCSMAQIQLCPPPPQIPNSHNMTTTLNYRDGEKVSVLCQENYLIQEGEEITCKDGRW
consensus   781 .....

Spliced_FH 246 -----
FH          841 QSIPLCVEKIPCSQPPQIEHGTINSSRSSQESYAHGTKLSYTCEGGFRISEENETTCYMG
consensus   841 .....

Spliced_FH 246 -----
FH          901 KWSSPPQCEGLPCKSPPEISHGVVAHMSDSYQYGEEVTYKCFEGFGIDGPAIAKCLGEKW
consensus   901 .....

Spliced_FH 246 -----
FH          961 SHPPSICKTDCLSLPSFENAI PMGEKKDVYKAGEQVTTYTCATYYKMDGASNVTCINSRWT
consensus   961 .....

Spliced_FH 246 -----
FH          1021 GRPTCRDTSCVNPPTVQNAY IVSRQMSKYPSEGERVRYQCRSPYEMFGDEEVMCLNGNWTE
consensus   1021 .....

Spliced_FH 246 -----DSTGKCGPPPIDNGDITSFPLSVYAPASSVEYQCQNLYQLEGNKRITCRNGQWS
FH          1081 PPQCKDSTGKCGPPPIDNGDITSFPLSVYAPASSVEYQCQNL YQLEGNKRITCRNGQWS
consensus   1081 .....*****

Spliced_FH 301 EPPKCLHPCVISREIMENYNIALRWTAKQKLYSRTGESVEFVCKRGYRLSSRSHTLRTTC
FH          1141 EPPKCLHPCVISREIMENYNIALRWTAKQKLYSRTGESVEFVCKRGYRLSSRSHTLRTTC
consensus   1141 *****

Spliced_FH 361 WDGKLEYPTCAKRHHHHHH
FH          1201 WDGKLEYPTCAKRHHHHHH
consensus   1201 *****

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Supplementary Fig. 3: Pairwise sequence alignment of full-length and predicted FH isoform based on experimentally verified transcript detected in RT-PCR.

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FH      ATGGCTTTCTATAAGATTTTCATCTGTTTTTTTCATCTTCTGTTTCTTCTTGATTGCCCTT  60
optiFH  ATGGCTTTCTACAAGATTTTCATCTGTTTTCTTCATCTTCTGCTTCTTCTTGATTGCCCTT  60
          *****
FH      CCGTTCCACTCGTACGCCGAAGATTGCAATGAACTTCCTCCAAGAAGAAATACAGAAATT  120
optiFH  CCGTTCCACTCGTACGCCGAGGATTGCAACGAGCTTCCTCCAAGAAGAAACACAGAGATT  120
          ***** **
FH      CTGACAGGTTCTGCTGACCAAACATATCCAGAAGGCA CCCAGGCTATCTATAAATGC  180
optiFH  CTGACAGGTTCTGCTGACCAAGACATACCCAGAGGGCACCCAGGCTATCTACAAGTGC  180
          ***** **
FH      CGCCCTGGATATAGATCTCTTGAAATGTAATAATGGTATGCAGGAAGGGAGAATGGGTT  240
optiFH  CGCCCTGGATACAGATCTCTTGAAACATCATCATGGTATGCAGGAAGGGAGAGTGGGTT  240
          ***** * **
FH      GCTCTTAATCCATTAAGGAAATGTCAGAAAAGGCCCTGTGGACATCCTGGAGATACTCCT  300
optiFH  GCTCTTAACCCATTAAGGAAGTGCCAGAAGAGGCCCTGCGGACACCC TGGAGATACTCCT  300
          ***** **
FH      TTTGGTACTTTTACCCTTACAGGAGGAAATGTGTTTGAATATGGTGTAAAAGCTGTGTAT  360
optiFH  TTCGGTACTTTTACCCTTACAGGAGGAAACGTGTTTCGAGTACGGTGTAAAGGCTGTGTAC  360
          ** *****
FH      ACATGTAATGAGGGGTATCAATTGCTAGGTGAGATTAATTACCGTGAATGTGACACAGAT  420
optiFH  ACATGCAACGAGGGGTACCAGTTGCTAGGTGAGATTAACTACCGTGAGTGCACACAGAT  420
          ***** **
FH      GGATGGACCAATGATATTCCTATATGTGAAGTTGTGAAGTGTTTACCAGTGACAGCACCA  480
optiFH  GGATGGACCAACGATATTCCTATCTGCGAGGTTGTGAAGTGCTTACCAGTGACAGCACCA  480
          ***** **
FH      GAGAATGGAAAAATTGTCAGTAGTGCAATGGAACCAGATCGGGAATACCATTTTGGACAA  540
optiFH  GAGAACGGAAAGATTGTCAGTAGTGCAATGGAGCCAGATCGGGAGTACCACTTCGGACAG  540
          *****
FH      GCAGTACGGTTTGTATGTAACCTCAGGCTACAAGATTGAAG GAGATGAAGAAATGCATTGT  600
optiFH  GCAGTACGGTTCGTATGCAACTCAGGCTACAAGATTGAGGGAGATGAGGAGATGCACTGC  600
          ***** **
FH      TCAGACGATGGTTTTTGGAGTAAAGAGAAACCAAAGTGTGTGGAAATTTTCATGCAAAATCC  660
optiFH  TCAGACGATGGTTTTCTGGAGTAAGGAGAAGCCAAAGTGCCTGGAGATTTTCATGCAAGTCC  660
          *****
FH      CCAGATGTTATAAATGGATCTCCTATATCTCAGAAGATTATTTATAAGGAGAATGAACGA  720
optiFH  CCAGATGTTATCAACGGATCTCCTATCTCTCAGAAGATTATTTACAA GGAGAACGAGCGA  720
          ***** **
FH      TTTCAATATAAATGTAACATGGGTTATGAATACAGTGAAAGAGGAGATGCTGTATGCACT  780
optiFH  TTCCAGTACAAGTGCAACATGGGTTACGAGTACAGTGAGAGAGGAGATGCTGTATGCACT  780
          ** **
FH      GAATCTGGATGGCGTCCGTTGCCTTCATGTGAAGAAAAATCATGTGATAATCCTTATATT  840
optiFH  GAGTCTGGATGGCGTCCGTTGCCTTCATGCGAGGAGAAGTCATGCGATAACCCTTACATT  840
          ** *****
FH      CCAAATGGTGACTACTCACCTTTAAGGATTAAGACACAGAAGTGGAGATGAAATCACGTAC  900
optiFH  CCAAACGGTGACTACTCACCTTTAAGGATTAAGCACAGAAGTGGAGATGAGATCACGTAC  900
          *****
FH      CAGTGTAGAAATGGTTTTTATCCTGCAACCCGGGGAAATACAGCAAAATGCACAAGTACT  960
optiFH  CAGTGCAGAAACGGTTTTCTACCCTGCAACCCGGGGAAACACAGCAAAGTGCACAAGTACT  960
          *****

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FH      GGCTGGATACCTGCTCCGAGATGTACCTTGAAACCTTGTG ATTATCCAGACATTA AACAT 1020
optiFH  GGCTGGATCCCTGCTCCGAGATGCACCTTGAGCCTTGCGATTACCCAGACATTAAGCAC 1020
          *****
FH      GGAGGTCTATATCATGAGAATATGCGTAGACCATACTTTCCAGTAGCTGTAGGAAAATAT 1080
optiFH  GGAGGTCTATACCACGAGAATATGCGACGGCCTTATTTTCCGGTAGCTGTAGGAAAGTAC 1080
          ***** ** ***** * ** ** *****
FH      TACTCCTATTACTGTGATGAACATTTTGAGACTCCGTCAGGAAGTTACTGGGATCACATT 1140
optiFH  TACTCCTACTACTGCGATGAGCACTTCGAGACTCCGTC AGGAAGTTACTGGGATCACATT 1140
          *****
FH      CATTGCACACAAGATGGATGGTCGCCAGCAGTACCATGCCTCAGAAAATGTTATTTTCCT 1200
optiFH  CACTGCACACAGGATGGATGGTCGCCAGCAGTACCATGCCTCAGAAAGTGCTACTTCCCT 1200
          ** *****
FH      TATTTGGAAAATGGATATAATCAAAATCATGGAAGAAAGTTTGTACAGGGTAAATCTATA 1260
optiFH  TACTTGGGAGAACGGATACAACCAGAACTACGGAAGAAAGTTTCGTACAGGGTAAGTCTATC 1260
          ** ***** ** ***** ** ** * *****
FH      GACGTTGCCTGCCATCCTGGCTACGCTCTTCCAAAAGCGCAGACCACAGTTACATGTATG 1320
optiFH  GACGTTGCCTGCCACCTGGCTACGCTCTTCCAAAGGCGCAGACCACAGTTACATGCATG 1320
          *****
FH      GAGAATGGCTGGTCTCCTACTCCCAGATGCATCCGTGTCAAAACATGTTCCAAATCAAGT 1380
optiFH  GAGAACGGCTGGTCTCCTACTCCCAGATGCATCCGTGTCAAGACATGCTCCAAGTCAAGT 1380
          *****
FH      ATAGATATTGAGAATGGGTTTATTTCTGAATCTCAGTA TACATATGCCTTAAAAGAAAAA 1440
optiFH  ATCGATATTGAGAACGGGTTTATTTCTGAGTCTCAGTACACATACGCCTTAAAGGAGAAG 1440
          ** *****
FH      GCGAAATATCAATGCAAACCTAGGATATGTAACAGCAGATGGTGAAACATCAGGATCAATT 1500
optiFH  GCGAAGTACCAGTGCAAGCTAGGATACGTAACAGCAGATGGTGAGACATCAGGATCAATT 1500
          ***** ** ** *****
FH      ACATGTGGGAAAGATGGATGGTCAGCTCAACCCACGTGCATTAAATCTTGTGATATCCCA 1560
optiFH  ACATGCGGGAAGGATGGATGGTCAGCTCAGCCACGTGCA TTAAGTCTTGCATATCCCA 1560
          *****
FH      GTATTTATGAATGCCAGAACTAAAAATGACTTCACATGGTTTAAAGCTGAATGACACATTG 1620
optiFH  GTATTCATGAACGCCAGAACTAAGAACGACTTCACATGGTTCAAGCTGAACGACACATTG 1620
          *****
FH      GACTATGAATGCCATGATGGTTATGAAAGCAATACTGGAAGCACCCTGGTTCCATAGTG 1680
optiFH  GACTACGAGTGCCACGATGGTTACGAGAGCAACACTGGAAGCACCCTGGTTCCATCGTG 1680
          ***** ** *****
FH      TGTGGTTACAATGGTTGGTCTGATTTACCCATATGTTATGAAAGAGAATGCGAACTTCCT 1740
optiFH  TGCGGTTACAACGGTTGGTCTGATTTACCCATCTGCTACGAGAGAGAGTGCGAGCTTCCT 1740
          ** *****
FH      AAAATAGATGTACACTTAGTTCCTGATCGCAAGAAAGACCAGTATAAAGTTGGAGAGGTG 1800
optiFH  AAGATCGATGTACACTTAGTTCCTGATCGCAAGAAAGACCAGTACAAGTTGGAGAGGTG 1800
          ** ** *****
FH      TTGAAATTCTCCTGCAAACCAGGATTTACAATAGTTGGACCTAATTCCGTTTCAGTGCTAC 1860
optiFH  TTGAAGTTCTCCTGCAAGCCAGGATTCACAATCGTTGGACCTAACTCCGTTTCAGTGCTAC 1860
          *****

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FH	CACTTTGGATTGTCTCCTGACCTCCCAATATGTAAAGAGCAAGTACAATCATGTGGTCCA	1920
optiFH	CACTTCGGATTGTCTCCTGACCTCCCAATCTGCAAGGAGCAGGTACAGTCATGTGGACCA	1920

FH	CCTCCTGAACTCCTCAATGGGAATGTTAAGGAAAAAACGAAAGAAGAATATGGACACAGT	1980
optiFH	CCACCAGAACTTCTCAACGGGAACGTTAAGGAGAAGACGAAGGAGGAGTACGGACACAGT	1980
	** ** *	
FH	GAAGTGGTGGAAATATTATTGCAATCCTAGATTTCTAATGAAGGGACCTAATAAAATTCAA	2040
optiFH	GAGGTGGTGGAGTACTACTGCAACCCTAGATTCCTAAT GAAGGGACCTAACAAGATTTCAG	2040
	** *****	
FH	TGTGTTGATGGAGAGTGGACAACCTTTACCAGTGTGTATTGTGGAGGAGAGTACCTGTGGA	2100
optiFH	TGCGTTGATGGAGAGTGGACAACCTTTACCAGTGTGCATTGTGGAGGAGAGTACCTGCGGA	2100
	** *****	
FH	GATATACCTGAACTTGAACATGGCTGGGCCAGCTTTCTTCCCCTCCTTATTACTATGGA	2160
optiFH	GATATCCCTGAGCTTGAGCACGGCTGGGCCAGCTTTCTTCCCCTCCTTACTACTACGGA	2160

FH	GATTCAGTGGAAATTC AATTGCTCAGAATCATTTACAATGATTGGACACAGATCAATTACG	2220
optiFH	GATTCAGTGGAGTTCAACTGCTCAGAGTCATT CACAATGATTGGACACAGATCAATTACG	2220

FH	TGTATTCATGGAGTATGGACCCCACTTCCCCAGTGTGTGGCAATAGATAAACTTAAGAAG	2280
optiFH	TGCATTACGGAGTATGGACCCAGCTTCCCCAGTGC GTGGCAATCGATAAGCTTAAGAAG	2280
	** *****	
FH	TGCAAATCATCAAATTTAATTATACTTGAGGAACATTT AAAAAACAAGAAGGAATTCGAT	2340
optiFH	TGCAAGTCATCAAACCTTAATTATCCTTGAGGAGCACTTAAAGAACAAGAAGGAGTTCGAT	2340

FH	CATAATTCTAACATAAGGTACAGATGTAGAGGAAAAGAAGGATGGATACACACAGTCTGC	2400
optiFH	CACAACCTCTAACATTAGATATCGGTGTCTGTGGAAGGAGGGATGGATCCACACAGTCTGC	2400
	** ** *	
FH	ATAAATGGAAGATGGGATCCAGAAGTGAAGTCTCAATGGCACAAATACAATTATGCCCCA	2460
optiFH	ATCAACGGAAGATGGGATCCAGAGGTGAAGTCTCAATGG CACAGATCCAGTTATGCCCCA	2460
	** ** *	
FH	CCTCCACCTCAGATTCCCAATTCTCACAATATGACAACCACACTGAATTATCGGGATGGA	2520
optiFH	CCTCCACCTCAGATTCCCAACTCTCACAACATGACAACCACACTGAATTATCGAGACGGT	2520

FH	GAAAAAGTATCTGTTCTTTGCCAAGAAAATTATCTAATTCAGGAAGGAGAAGAAATTACA	2580
optiFH	GAAAAAGTTTCAGTTCTTTGCCAGGAGAACTACCTAATTCAGGAGGGAGAGGAGATTACA	2580

FH	TGCAAAGATGGAAGATGGCAGTCAATACCACTCTGTGTTGAAAAAATTCCATGTTTACAA	2640
optiFH	TGCAAGGATGGAAGATGGCAGTCAATCCCACTCTGCGTTGAGAAGATTCCATGCTCACAG	2640

FH	CCACCTCAGATAGAACACGGAACCATTAATTCATCCAGGTCTTCACAAGAAAGTTATGCA	2700
optiFH	CCACCTCAGATCGAGCACGGAACCATTAACCTCATCCAGGTCTTCACAGGAGAGTTACGCA	2700

FH	CATGGGACTAAATTGAGTTTACTTGTGAGGGTGGTTT CAGGATATCTGAAGAAAATGAA	2760
optiFH	CACGGGACTAAGTTGAGTTACACTTGCGAGGGTGGTTT CAGGATCTCTGAGGAGAACGAG	2760
	** *****	

FH	ACAACATGCTACATGGGAAAATGGAGTTCTCCACCTCAGTGTGAAGGCCTTCCTTGTA	2820
optiFH	ACAACATGCTACATGGGAAAGTGGAGTTCTCCACCTCAGTGCGAGGGCCTTCCTTGCAAG	2820

FH	TCTCCACCTGAGATTTCTCATGGTGTGTAGCTCACATGTCAGACAGTTATCAGTATGGA	2880
optiFH	TCTCCACCTGAGATTTCTCACGGTGTGTAGCTCACA TGTCAGACAGTTACCAGTACGGA	2880

FH	GAAGAAGTTACGTACAAATGTTTTGAAGGTTTTGGAATTGATGGGCCTGCAATTGCAAAA	2940
optiFH	GAGGAGTTACGTACAAGTGTCTCGAGGGTTTCGGAATTGATGGGCCTGCAATTGCAAAAG	2940
	** * *	
FH	TGCTTAGGAGAAAAATGGTCTCACCTCCATCATGCATAAAAAACAGATTGTCTCAGTTTA	3000
optiFH	TGCTTAGGAGAGAAGTGGTCTCACCTCCATCATGCATCAAGACAGATTGCCTCAGTTTA	3000

FH	CCTAGCTTTGAAAATGCCATACCCATGGGAGAGAAGAAGGATGTGTATAAGGCGGGTGAG	3060
optiFH	CCTAGCTTCGAGAACGCCATCCCCATGGGAGAGAAGAAGGATGTGTACAAAGCGGGTGAG	3060

FH	CAAGTGACTTACACTTGTGCAACATATTACAAAATGGATGGAGCCAGTAATGTAACATGC	3120
optiFH	CAGGTGACTTACACTTGCGCAACATACTACAAGATGGATGGAGCCAGTAACGTAACATGC	3120
	** *	
FH	ATTAATAGCAGATGGACAGGAAGGCCAACATGCAGAGACACCTCCTGTGTGAATCCGCCC	3180
optiFH	ATTAACAGCCGATGGACTGGTCGTCTACGTGCAGAGACACCTCCTGCGTGAACCCGCCC	3180

FH	ACAGTACAAAATGCTTATATAGTGTGCGAGACAGATGAGTA AATATCCATCTGGTGAGAGA	3240
optiFH	ACAGTACAGAACGCTTACATCGTGTGCGAGACAGATGAGTAAGTACCCATCTGGTGAGAGA	3240

FH	GTACGTTATCAATGTAGGAGCCCTTATGAAATGTTTGGGGATGAAGAAGTGATGTGTTTA	3300
optiFH	GTACGTTACCAGTGCAGGAGCCCTTACGAGATGTTTGGGGATGAGGAGGTGATGTGCTTA	3300

FH	AATGGAAACTGGACGGAACCACTCAATGCAAAGATTCTACAGGAAAATGTGGGCCCCCT	3360
optiFH	AACGGAAACTGGACGAGCCACCTCAGTGCAAGACTCGACA GGAAAGTGGGGCCCCCT	3360
	** *	
FH	CCACCTATTGACAATGGGGACATTACTTCATTCCCCTTGTGTCAGTATATGCTCCAGCTTCA	3420
optiFH	CCACCTATTGACAACGGGGACATTACTTCATTCCCCTTGTGTCAGTATACGCTCCAGCTTCA	3420

FH	TCAGTTGAGTACCAATGCCAGAACTTGTATCAACTTGAGGGTAACAAGCGAATAACATGT	3480
optiFH	TCAGTTGAGTACCAAGTGCAGAACTTGTATCAACTTGAGGGTAACAAGCGAATCACATGC	3480

FH	AGAAATGGACAATGGTCAGAACCAACAAAATGCTTACATCCGTGTGTAATATCCCGAGAA	3540
optiFH	AGAAACGGACAGTGGTCAGAGCCACCAAGTGTGTAACCCGTGCGTAATCTCCCGAGAG	3540

FH	ATTATGGAAAATTATAACATAGCATTAAAGGTGGACAGCCAAACAGAAGCTGTATTCGAGA	3600
optiFH	ATTATGGGAACTACAACATCGCATTAAAGGTGGACAGCCAAAGCAGAAGCTGTACTCGAGA	3600

FH	ACAGGTGAATCAGTTGAATTTGTGTGTAAACGGGGATATCGTCTTTTCATCACGTTCTCAC	3660
optiFH	ACAGGTGAGTCAGTTGAGTTTCGTGTGCAAGCGGGGATACCGTCTTTTCATCACGTTCTCAC	3660

FH	ACATTGCGAACAACATGTTGGGATGGGAACTGGAGTATCC AACTTGTGCAAAAAGATAA	3720
optiFH	ACATTGCGAACAACATGCTGGGATGGGAAGCTGGAGTACCAACTTGCACAAAGAGATAA	3720

Supplementary Fig. 4: Pairwise sequence alignment of FH and optiFH

Supplementary Fig. 5: 3D rendered confocal Z-stacks of moss protoplasts transfected with FH-Citrine (A) and optiFH-Citrine (B). Scale bar is 3 μ m.

Supplementary Fig. 6: The workflow of the image processing steps demonstrated with an exemplary single slice from a FIX-Citrine cell. The original, raw image from the microscope (A) was first processed with a median filter (B). The resulting image was subjected to an unsharp-mask operation (C). Afterwards, a Richardson-Lucy restoration algorithm was implemented (F). The pre-processing was completed with a local intensity equalization operation (E). The final version of the image was then thresholded using a local adaptive Otsu thresholding method, leading to the binary masks (D) that were subsequently used for selection of the voxels for quantification. Scale bar is 5 μm .

Supplementary Table 1: Moss microRNAs targeting human FIX CDS. Analysis was performed using psRNAtarget (http://plantgrn.noble.org/v1_psRNATarget/). The miRNAs as well as their binding sites, expectation score, maximum energy to unpair the target site (UPE) and mode of action of miRNAs are shown.

MicroRNA	Alignment				Expectation	UPE	Mode of action
miR1044-3P	miRNA	20	UUUUGUUUUAUACGUGAUGUU	1	5	13.996	Cleavage
	Target	1304	AAGGCAAUAUGGAAUAUAU	1323			
miR1028c-3p	miRNA	21	CGAGA-AUUUGGAUGUUACGGU	1	5	21.847	Translational inhibition
	Target	495	GUCCUGUGAACCAGCAGUGCCA	516			

Supplementary Table 2: The donor splice sites for all 87,533 annotated transcripts corresponding to the 32,926 protein-encoding genes of the current *P. patens* genome release v3.3.

<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>	<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>	<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>
CAGGT	113,285	22.5460	TAAGT	1,610	0.3204	AGAGC	44	0.0088
AAGGT	84,769	16.8708	AGTGT	1,530	0.3045	GCTGC	44	0.0088
GAGGT	57,630	11.4695	TGAGT	1,492	0.2969	ATCAT	41	0.0082
CTGGT	20,698	4.1193	AAGGC	1,487	0.2959	CGGGC	41	0.0082
ATGGT	18,574	3.6966	CTCGT	1,385	0.2756	GATGC	41	0.0082
TTGGT	15,064	2.9980	GGAGT	1,330	0.2647	TTTGC	40	0.0080
TAGGT	12,835	2.5544	ATCGT	1,273	0.2534	TCGGC	39	0.0078
CAAGT	12,206	2.4292	GACGT	1,273	0.2534	GCGGC	36	0.0072
GTGGT	9,443	1.8793	GTTGT	1,259	0.2506	GGGGC	36	0.0072
ACGGT	9,246	1.8401	TTCGT	985	0.1960	GGAGC	35	0.0070
TGGGT	9,034	1.7980	GAGGC	981	0.1952	CATGC	34	0.0068
AGGGT	8,314	1.6547	ACCGT	914	0.1819	TCAGC	33	0.0066
TCGGT	7,588	1.5102	TACGT	908	0.1807	CCGGC	32	0.0064
CGGGT	6,757	1.3448	CTAGT	879	0.1749	TTCGC	32	0.0064
AAAGT	6,677	1.3289	CGTGT	870	0.1731	ATTGC	31	0.0062
CCGGT	6,223	1.2385	ATAGT	856	0.1704	CTTGC	31	0.0062
GCGGT	6,059	1.2059	CCCGT	804	0.1600	GGTGC	30	0.0060
AATGT	5,632	1.1209	TCCGT	742	0.1477	TCTGC	30	0.0060
CATGT	5,495	1.0936	TGCGT	673	0.1339	AGTGC	28	0.0056
GGGGT	5,371	1.0689	AGCGT	665	0.1323	TGTGC	26	0.0052
GAAGT	4,718	0.9390	GCCGT	516	0.1027	CTCGC	23	0.0046
GATGT	4,086	0.8132	TTAGT	506	0.1007	TGCGC	23	0.0046
CTTGT	3,916	0.7794	GTCGT	406	0.0808	ACTGC	22	0.0044
ATTGT	3,638	0.7240	CGCGT	396	0.0788	GAAAT	22	0.0044
AGAGT	2,987	0.5945	GGTGT	336	0.0669	CCTGC	21	0.0042
CCTGT	2,979	0.5929	GTAGT	300	0.0597	GGCGC	19	0.0038
ACTGT	2,904	0.5780	GGCGT	244	0.0486	TACGC	19	0.0038
CAGGC	2,860	0.5692	CTGGC	221	0.0440	ACAGC	18	0.0036
GCTGT	2,830	0.5632	ATGGC	199	0.0396	GTTGC	18	0.0036
TCTGT	2,772	0.5517	TAGGC	181	0.0360	ATCGC	17	0.0034
TTTGT	2,553	0.5081	TTGGC	89	0.0177	TTAGC	17	0.0034
ACAGT	2,426	0.4828	CAAGC	65	0.0129	TGAGC	16	0.0032
CCAGT	2,286	0.4550	AAAGC	55	0.0109	AACGC	15	0.0030
CACGT	2,273	0.4524	GAAGC	55	0.0109	CCAGC	15	0.0030
AACGT	1,965	0.3911	TGGGC	54	0.0107	GACGC	15	0.0030
GCAGT	1,948	0.3877	AATGC	52	0.0103	GTCGC	15	0.0030
TATGT	1,894	0.3769	GTGGC	52	0.0103	GTCAT	14	0.0028
TCAGT	1,882	0.3746	ACGGC	47	0.0094	TATGC	14	0.0028
CGAGT	1,876	0.3734	AGGGC	46	0.0092	GTAGC	13	0.0026
TGTGT	1,656	0.3296	GCAGC	46	0.0092	TCCGC	13	0.0026

<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>	<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>
AAGAT	12	0.0024	TGCAT	4	0.0008
AGCGC	12	0.0024	AGAAT	3	0.0006
ATAGC	12	0.0024	ATGAT	3	0.0006
CGTGC	12	0.0024	GCTAT	3	0.0006
CTAGC	12	0.0024	TAATG	3	0.0006
CGAGC	11	0.0022	TTGAT	3	0.0006
AAAAT	10	0.0020	ATAAT	2	0.0004
CAAAT	10	0.0020	CAACT	2	0.0004
CACGC	10	0.0020	CCAAT	2	0.0004
CCCGC	10	0.0020	GAATA	2	0.0004
GCCGC	10	0.0020	GATAT	2	0.0004
ACCAT	9	0.0018	TAATA	2	0.0004
TAAGC	9	0.0018	TGAAA	2	0.0004
TCAAT	9	0.0018	TGGAT	2	0.0004
ATTAT	8	0.0016	CTGAT	1	0.0002
GCAAT	8	0.0016	CTGTG	1	0.0002
TCTAT	8	0.0016	CTTAT	1	0.0002
CATAT	6	0.0012	GGTAA	1	0.0002
TAGAT	6	0.0012	TAAAT	1	0.0002
TATAT	6	0.0012	TAACA	1	0.0002
ACCGC	5	0.0010	TAAGA	1	0.0002
CAGAT	5	0.0010	TAGAG	1	0.0002
CGCGC	5	0.0010	TGATG	1	0.0002
TCGAT	5	0.0010	TGCTT	1	0.0002
TTCAT	5	0.0010	TTGTC	1	0.0002
TTTAT	5	0.0010	TTTCG	1	0.0002
TAAGG	4	0.0008	TTTTA	1	0.0002
TGAGG	4	0.0008	TTTTT	1	0.0002

Supplementary Table 3: The acceptor splice sites for all 87,533 annotated transcripts corresponding to the 32,926 protein-encoding genes of the current *P. patens* genome release v3.3.

<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>	<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>
CAGGT	90,834	18.0778	AAGTG	669	0.1331
CAGGA	54,132	10.7734	TAGCG	609	0.1212
CAGAT	45,474	9.0503	AAGAG	591	0.1176
CAGGG	36,713	7.3066	AAGAC	547	0.1089
CAGGC	36,350	7.2344	AAGCA	440	0.0876
CAGAA	22,672	4.5122	GAGGT	365	0.0726
CAGAG	21,613	4.3014	AAGCC	315	0.0627
TAGGT	19,555	3.8918	AAGTA	285	0.0567
CAGTT	19,239	3.8290	AAGTC	273	0.0543
CAGCT	18,854	3.7523	AAGCG	238	0.0474
CAGTG	15,897	3.1638	GAGGA	185	0.0368
CAGAC	14,770	2.9395	GAGAT	182	0.0362
CAGCA	11,526	2.2939	GAGGG	168	0.0334
TAGGA	9,029	1.7970	GAGGC	160	0.0318
CAGCC	8,266	1.6451	GAGAA	126	0.0251
CAGTC	7,702	1.5329	GAGTG	108	0.0215
TAGGG	7,378	1.4684	GAGAG	100	0.0199
TAGAT	7,164	1.4258	GAGCT	80	0.0159
CAGTA	6,745	1.3424	GAGTT	77	0.0153
TAGGC	6,040	1.2021	GAGAC	69	0.0137
CAGCG	6,004	1.1949	GAGCA	67	0.0133
AAGGT	4,063	0.8086	CACAT	51	0.0102
TAGAG	2,667	0.5308	GAGTC	48	0.0096
TAGAA	2,624	0.5222	GAGTA	46	0.0092
TAGTT	2,554	0.5083	GAGCG	39	0.0078
TAGTG	2,225	0.4428	GAGCC	37	0.0074
TAGCT	2,183	0.4345	CACGC	26	0.0052
TAGAC	2,119	0.4217	CACAA	16	0.0032
AAGGA	1,792	0.3566	TACCT	14	0.0028
AAGGC	1,483	0.2951	CACAC	11	0.0022
TAGCA	1,480	0.2946	CACGG	10	0.0020
AAGAT	1,342	0.2671	TACAC	10	0.0020
AAGGG	1,107	0.2203	CACGT	8	0.0016
TAGTC	967	0.1925	TACAT	8	0.0016
TAGCC	916	0.1823	CACAG	6	0.0012
AAGAA	784	0.1560	TACAA	6	0.0012
TAGTA	757	0.1507	TACGA	6	0.0012
AAGTT	685	0.1363	TACGC	6	0.0012
AAGCT	671	0.1335	AACGT	5	0.0010

<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>	<i>Motif</i>	<i>Count</i>	<i>Percentage (%)</i>
AGAAT	5	0.0010	ACGAT	1	0.0002
CACCT	4	0.0008	AGCAT	1	0.0002
AACAA	3	0.0006	ATAAT	1	0.0002
AGGAT	3	0.0006	CATCT	1	0.0002
CACCA	3	0.0006	CTAAT	1	0.0002
CACTA	3	0.0006	CTGGC	1	0.0002
TACGT	3	0.0006	GATAT	1	0.0002
TACTC	3	0.0006	GCTAT	1	0.0002
AACAG	2	0.0004	GCTTC	1	0.0002
AAC TT	2	0.0004	GGAAG	1	0.0002
ACTAA	2	0.0004	GGTAT	1	0.0002
CACTG	2	0.0004	GTGAT	1	0.0002
CACTT	2	0.0004	GTTAT	1	0.0002
GAAAT	2	0.0004	NAGGA	1	0.0002
GAACT	2	0.0004	NAGGG	1	0.0002
GACAA	2	0.0004	NAGTG	1	0.0002
GACAT	2	0.0004	TACCG	1	0.0002
GCAAT	2	0.0004	TACTA	1	0.0002
GCCAT	2	0.0004	TACTG	1	0.0002
GGCAT	2	0.0004	TGCGT	1	0.0002
GGGAT	2	0.0004	TGTGA	1	0.0002
AACAT	1	0.0002	TTATT	1	0.0002
ACCAT	1	0.0002	TTTAC	1	0.0002