Distinct types of short open reading frames are translated in plant cells

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ABSTRACT

Genomes contain millions of short (<100 codons) open reading frames (sORFs), which are usually dismissed during gene annotation. Nevertheless, peptides encoded by such sORFs can play important biological roles, and their impact on cellular processes has long been underestimated. Here, we analyzed approximately 70,000 transcribed sORFs in the model plant *Physcomitrella patens* (moss). Several distinct classes of sORFs that differ in terms of their position on transcripts and the level of evolutionary conservation are present in the moss genome. Over 5000 sORFs were conserved in at least one of ten plant species examined. Mass spectrometry analysis of proteomic and peptidomic datasets suggested that 602 sORFs located on distinct parts of mRNAs and long non-coding RNAs (lncRNAs) are translated, including 74 conservative sORFs. Combined analysis of the translation of the sORFs and the main ORF from a single gene suggested the existence of bi- and poly-cistronic mRNAs with tissue-specific expression. Alternative splicing is likely involved in the excision of translatable sORFs from such transcripts. We identified a group of sORFs homologous to known protein domains and suggested they function as small interfering peptides. Functional analysis of a candidate lncRNA-encoded peptide showed it to be involved in regulating growth and differentiation in moss. The high evolutionary rate and wide translation of sORFs suggest that they may provide a reservoir of potentially active peptides and their importance as a raw material for gene evolution. Our results thus open new avenues for discovering novel, biologically active peptides in the plant kingdom.

INTRODUCTION

The genomes of nearly all organisms contain hundreds of thousands of short open reading frames (sORFs; <100 codons) whose coding potential has been the subject of recent reviews (Andrews and Rothnagel 2014; Couso 2015; Hellens et al. 2016; Couso and Patraquim 2017). However, gene annotation algorithms are generally not suited for dealing with sORFs because short sequences are unable to obtain high conservation scores, which serve as an indicator of functionality (Ladoukakis et al. 2011). Nevertheless, using various bioinformatic approaches, sORFs with high coding potential have been identified in a range of organisms including fruit flies, mice, yeast and *Arabidopsis thaliana* (Ladoukakis et al. 2011; Hanada et al. 2013; Aspden et al. 2014; Bazzini et al. 2014). The first systematic study of sORFs was conducted on baker's yeast, where 299 previously non-annotated sORFs were identified and tested in genetic experiments (Kastenmayer et al. 2006). Subsequently, 4561 conserved sORFs were identified in the genus *Drosophila*, 401 of which were postulated to be functional, taking into account their syntenic positions, favorable Dn/Ds values and transcriptional evidence (Ladoukakis et al. 2011). In a recent study, Mackowiak and colleagues predicted the presence of 2002 novel conserved sORFs (from 9 to 101 codons) in *H. sapiens*, *M. musculus*, *D. rerio*, *D. melanogaster* and *C. elegans* (Mackowiak et al. 2015). The first comprehensive study of sORFs in plants postulated the existence of thousands of sORFs with high coding potential in Arabidopsis (Lease and Walker 2006; Hanada et al. 2007; Hanada et al. 2013), including 49 that induced various morphological changes and had visible phenotypic effects.

Recent studies have pointed to the important roles of sORF-encoded peptides (SEPs) in cells (Magny et al. 2013; Nelson et al. 2016; D'Lima et al. 2017; Huang et al. 2017; Matsumoto et al. 2017). However, unraveling the roles of SEPs is a challenging task, as is their detection at the biochemical level. In animals, SEPs are known play important roles in a diverse range of cellular processes (Kondo et al. 2010; Magny et al. 2013). By contrast, only a few functional SEPs have been reported in plants, including POLARIS (PLS; 36 amino acids), EARLY NODULIN GENE 40 (ENOD40; 12, 13, 24 or 27 amino acids), ROTUNDIFOLIA FOUR (ROT4; 53 amino acids), KISS OF DEATH (KOD; 25 amino acids), BRICK1 (BRK1; 84 amino acids), Zm-908p11 (97 amino acids) and Zm-401p10 (89 amino acids) (Andrews and Rothnagel 2014; Tavormina et al. 2015). These SEPs help modulate root growth and leaf vascular patterning (Chilley et al. 2006), symbiotic nodule development (Djordjevic et al. 2015), polar cell proliferation in lateral organs and leaf morphogenesis (Narita et al. 2004), and programmed cell death (apoptosis) (Blanvillain et al. 2011).

To date, functional sORFs have been found in a variety of transcripts, including untranslated regions of mRNA (5′ leader and 3′ trailer sequences), lncRNAs, and microRNA transcripts (pri-miRNAs) (Andrews and Rothnagel 2014; Laing et al. 2015; Lauressergues et al. 2015; Couso and Patraquim 2017). Evidence for the transcription of potentially functional sORFs has been obtained in *Populus deltoides*, *Phaseolus vulgaris*, *Medicago truncatula*, *Glycine max* and *Lotus japonicus* (Guillen et al. 2013). The transcription of sORFs can be regulated by stress conditions and depends on the developmental stage of the plant (De Coninck et al. 2013; Hanada et al. 2013; Rasheed et al. 2016). Indeed, sORFs might represent an important source of advanced traits required under stress conditions. During stress, genomes undergo widespread transcription to produce a diverse range of RNAs (Kim et al. 2010; Mazin et al. 2014); therefore, a large portion of sORFs becomes accessible to the translation machine for peptide production. Stress conditions can lead to the transcription of sORFs located in genomic regions that are usually non-coding (Giannakakis et al. 2015). Such sORFs appear to serve as raw materials for the birth and subsequent evolution of new protein-coding genes (Couso and Patraquim 2017).

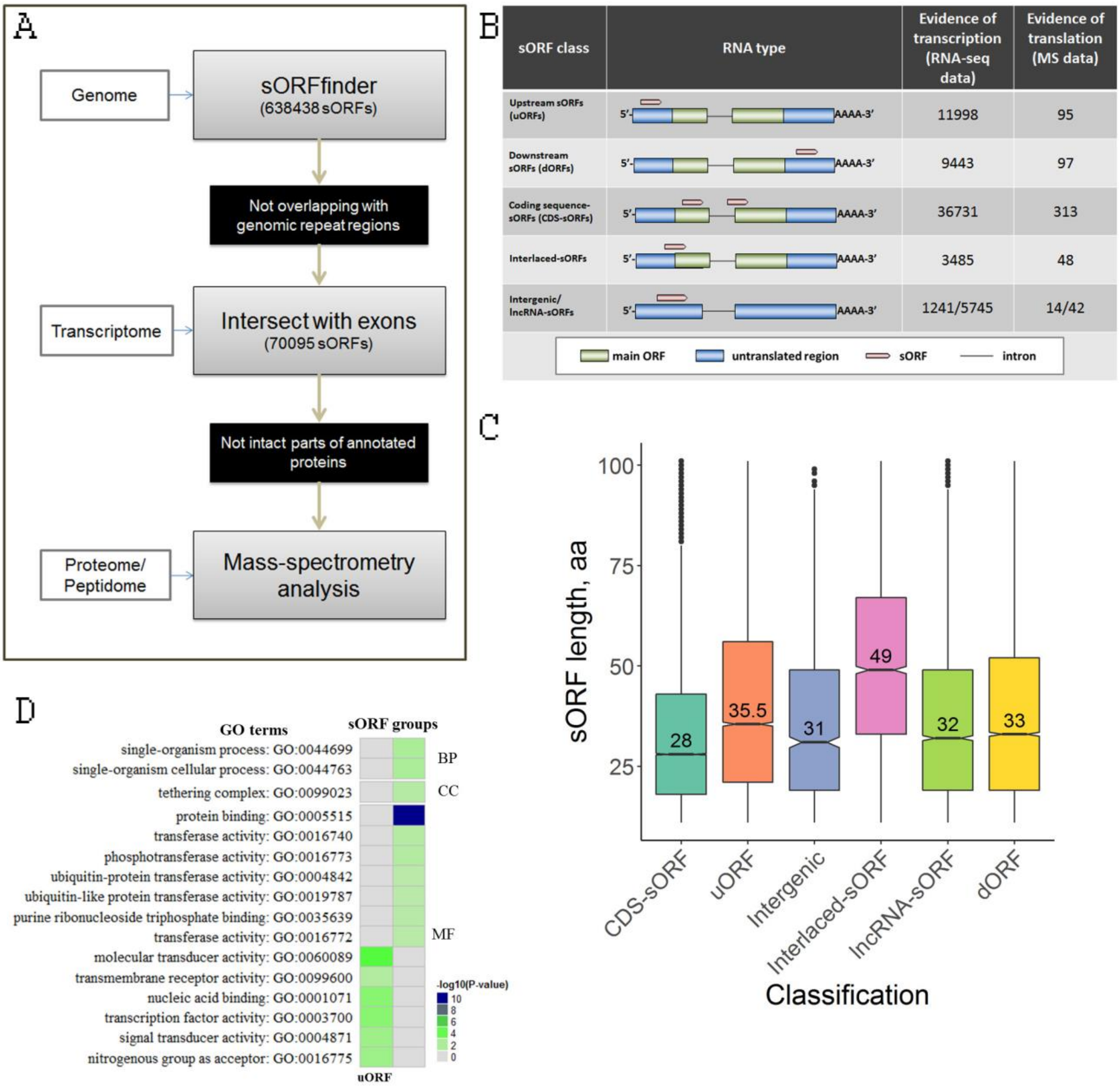
The transcription of an sORF does not necessarily indicate that it fulfills any biological role, as opposed to being a component of the so-called translational noise (Guttman et al. 2013). According to ribosomal profiling data, thousands of lncRNAs display high ribosomal occupancy in regions containing sORFs in mammals (Ingolia et al. 2011; Aspden et al. 2014; Bazzini et al. 2014). However, lncRNAs can have the same ribosome profiling patterns as canonical non-coding RNAs (e.g., rRNA) that are known not to be translated, implying that these lncRNAs are unlikely to produce functional peptides (Guttman et al. 2013). In addition, identification of SEPs via mass spectrometry analyses has found many fewer peptides than predicted sORFs (Slavoff et al. 2013; Aspden et al. 2014). Thus, the abundance, lifetime and other features of SEPs are generally unclear.

In this study, we performed comprehensive analysis of sORFs with canonical AUG start codons and high coding potential in the genome of the model moss *P. patens*. To identify candidate functional sORFs, we developed an integrated pipeline, including analysis of transcriptomic, proteomic and peptidomic data. We classified the sORFs based on their locations on transcripts and analyzed their features, such as evolutionary conservation, peptide-coding potential and possible functions. We determined that plant genomes contain hundreds of translatable sORFs, including those located in alternative frames in protein-coding genes. The speed of evolution depended on the type of sORF, with CDS-sORFs and lncRNA-ORFs under strong positive selection while uORFs and dORFs had a greater chance of being fixed in the genome. Moreover, the presence of some sORFs in the transcriptome depended on alternative splicing events. We also identified more than 200 sORFs sharing homology with known proteins, implying that they function as small interfering peptides. Finally, we selected a candidate lncRNA-encoded peptide for further analysis and provide evidence for its biological function.

RESULTS

Discovery and classification of potential coding sORFs in the moss genome

Our approach is summarized in Figure 1A. At the first stage of analysis, we used the sORFfinder tool (Hanada et al. 2010) to identify single-exon sORFs starting with an AUG start codon and less than 300 bp long. This approach resulted in the identification of 638,439 sORFs with high coding potential (CI index) in all regions of the *P. patens* genome.



sORF class	RNA type	Evidence of transcription (RNA-seq data)	Evidence of translation (MS data)
Upstream sORFs (uORFs)	5'- AAAA-3'	11998	95
Downstream sORFs (dORFs)	5'- AAAA-3'	9443	97
Coding sequence-sORFs (CDS-sORFs)	5'- AAAA-3'	36731	313
Interlaced-sORFs	5'- AAAA-3'	3485	48
Intergenic/ lncRNA-sORFs	5'- AAAA-3'	1241/5745	14/42

main ORF — untranslated region — sORF — intron

Figure 1. Several distinct types of sORFs are present in the moss genome. A – Pipeline used in this study to identify coding sORFs; **B** – Proposed classification of sORFs according to the types of encoding transcripts: upstream ORFs (uORFs) and downstream ORFs (dORFs) in the untranslated

regions (UTRs) of canonical mRNAs; CDS-sORFs, which overlap with protein-coding sequences in non-canonical +2 or +3 reading frames or are truncated versions of proteins generated by alternative splicing; interlaced-sORFs, which overlap both the protein-coding sequence and UTR on the same transcript; lncRNA-sORFs and intergenic sORFs, which are located on short non-protein coding transcripts.; **C** – Boxplot of the length distribution of sORFs in different groups; **D** – The results of GO enrichment analysis for genes possessing uORFs and CDS-sORFs. BP, CC and MF represent "Biological process", "Cellular component" and "Molecular function", respectively.

We selected 70,095 unique sORFs located on transcripts annotated in the moss genome (phytozome.jgi.doe.gov) and/or our dataset (Fesenko et al. 2015) for further analysis, as well as those on lncRNAs from two databases -CantataDB (Szczesniak et al. 2016) and GreenC (Paytuvi Gallart et al. 2016); sORFs located in repetitive regions were discarded (Supplemental Table S1). These selected sORFs, which were 33 to 303 bp long, were located on 33,981 transcripts (22,969 genes), with up to 28 sORFs per transcript (Supplemental Figure S1A).

We then classified the sORFs based on their location on the transcript: 63,109 "genic-sORFs" (located on annotated transcripts, but not on lncRNA), 1241 "intergenic-sORFs" (located on transcripts from our dataset and not annotated in the current version of the genome) and 5745 "lncRNA-sORFs" (located on lncRNAs from CantataDB (Szczesniak et al. 2016), GreenC (Paytuvi Gallart et al. 2016) or our data set (Fesenko et al. 2017); Figure 1B). The genic-sORFs include 11,998 upstream ORFs (uORFs; for 5'-UTR location), 9443 downstream ORFs (dORFs; for 3'-UTR location), 36,731 coding sequence-sORFs (CDS-sORFs; sORFs overlapping with major ORFs in non-canonical +2 and +3 reading frames) and 3485 interlaced-sORFs (overlapping with both the CDS and 5'-UTR or CDS and 3'-UTR on the same transcript) (Figure 1B, Supplemental Figure S1B).

As expected based on the sORFfinder search strategy (Hanada et al. 2010), the sORF set was enriched in CDS-sORFs (52%, Fisher's exact test, P-value = 1.736392e-285), whereas dORFs, uORFs and interlaced-sORFs were underrepresented (Fisher's exact test, P-value < 4.792689e-88) compared to a random exonic fragments (REF) set, which was used as a negative control.

On average, CDS-sORFs (median size of 22 codons) were shorter than uORFs (median size of 35 codons; Mann-Whitney P = 2.2e-151) and dORFs (median length 32 codons, Mann-Whitney P = 1.03e-43). The median size of interlaced-sORFs was 49 codons, which is significantly longer than other genic-sORFs (Mann-Whitney P = 0.0021) (Figure 1C).

We performed comparative GO enrichment analysis of four groups of genic-sORFs (dORF, uORF, CDS-sORF and interlaced). To exclude the possibility that differences between groups could be explained merely by structural differences in genes carrying sORFs (e.g., genes with longer 5'-UTRs have a greater chance of possessing uORFs), we also performed GO enrichment analysis of a set of genes with randomly selected exon fragments (REFs). GO terms that were enriched in both datasets were excluded. The analysis showed significant (adjusted P-value < 0.01) GO enrichment for genes possessing CDS-sORFs and uORFs. The patterns of GO enrichment differed between the two groups of genes: set of genes possessing CDS-sORFs were enriched in GO terms associated with protein binding and transferase activity, while genes possessing uORFs were involved in signal transduction and transcriptional regulation (Figure 1D). Such contrasting patterns in functions between genes with different sORF locations allude to the roles of sORFs and/or their peptides in different levels of cellular regulation.

Analysis of evolutionary conservation of sORFs

It is widely accepted that evolutionary conservation is a strong indicator of functionality (Ladoukakis et al. 2011). To estimate the number of conservative sORFs in the moss genome and the evolutionary pressure on their amino acid sequences, we performed a tBLASTn (e-value cutoff 0.00001) search of each sORF sequence against the transcriptomes of ten species including those that diverged from *P. patens* 177 (*Ceratodon purpureus*), 320 (*Sphagnum fallax*), 493 (*Marchantia polymorpha*), 532 (*Arabidopsis thaliana*, *Oryza sativa, Zea mays, Selaginella moellendorffii, Spirodela polyrhiza*) and 1160 (*Volvox carteri, Chlamydomonas reinhardtii*) Mya (Supplemental Figure S2).

We found 5034 conserved sORFs with detectable homologous sequences in at least one species: 4797 in *C. purpureus*, 1049 in *S. fallax*, 436 in *M. polymorpha*, 328 in *S. moellendorffii*, 297 in *S. polyrhiza*, 275 in *A. thaliana*, 282 in *Z. mays*, 274 in *O. sativa*, 86 in *V. carteri* and 89 in *C. reinhardtii*.

The number of conserved sORFs was negatively correlated with the time since divergence, with the fewest homologous sequences found in *V. carteri* and *C. reinhardtii*, which diverged more than 1000 Mya from a common ancestor. We found that lncRNA-sORFs were underrepresented among sORFs having homologs in the ten species examined (Figure 2A). We also found significantly fewer uORFs and dORFs in the two closest species, *C. purpureus* and *S. fallax*, whereas CDS-sORFs were significantly overrepresented in these species (Fisher's exact test, P<2.2e-16) (Figure 2B).

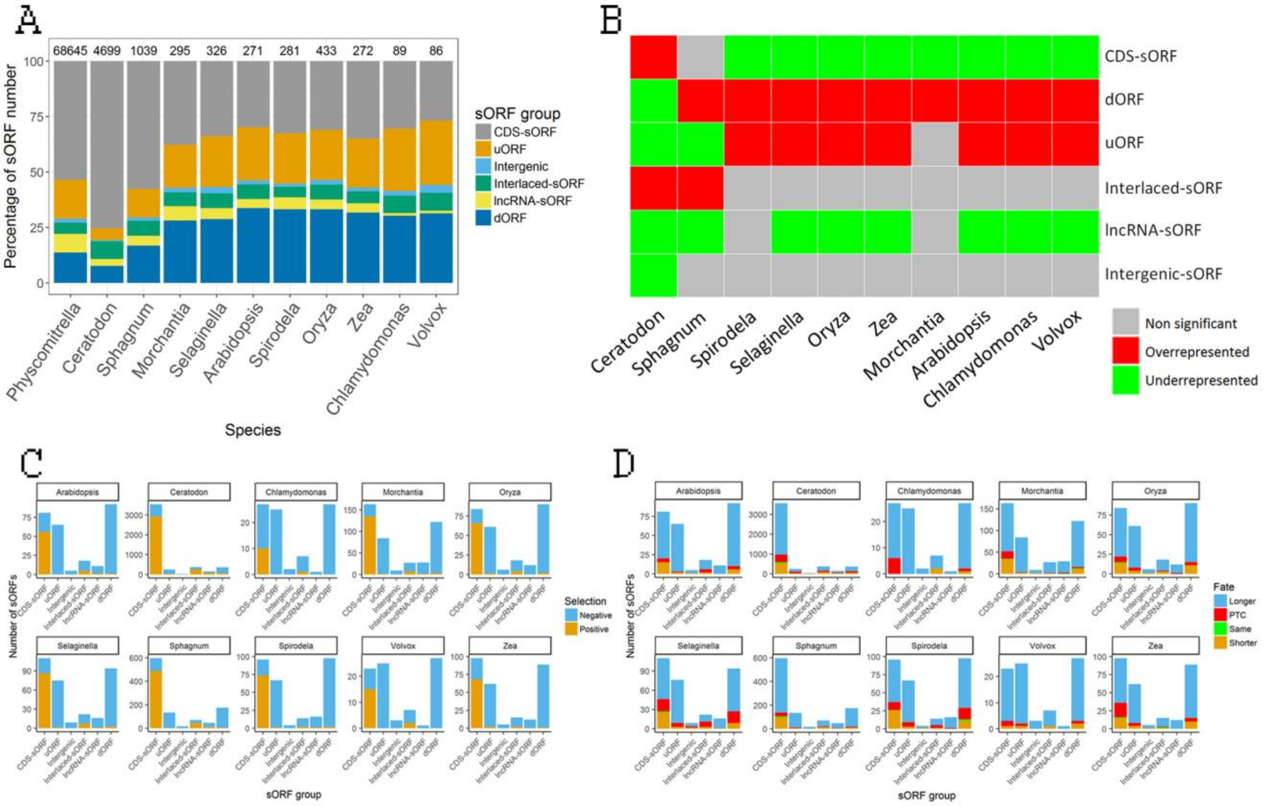


Figure 2. Analysis of the trends in sORFs evolution. A – The percentage of each type of sORF among sORFs having homologs in ten plant species. B – Statistical analysis (by Fisher's exact test) of differences between the number of conservative sORFs in each of ten species and the initial dataset; C – Pairwise Ka/Ks ratio distribution for each type of sORF conserved among ten plant species; D – Bar plot comparing the lengths of moss sORFs with the putative lengths of homologous sORFs in ten plant species. Homologous sORF sequences can be expanded (blue) in PTC – premature termination codon in homologous sORFs.

However, the portion of uORFs and dORFs found in the more distant species was increased relative to the initial dataset compared to CDS-sORFs, causing their significant overrepresentation (Fisher's exact test, P<0.0005). Thus, the relative enrichment of conserved CDS-sORFs and interlaced-sORFs found in the two closest species of *P. patens*, *C. purpureus* and *S. fallax*, resulted from a significant reduction in the number of uORFs and dORFs (Figure 2A).

Because upstream sORFs are capable of attenuating translation of the downstream main open reading frame, they can undergo strong selection and be eliminated from UTRs. As a control, we also investigated changes in the proportion of uREFs, dREFs and CDS-REFs in these ten species and obtained opposite results, with significant overrepresentation of CDS-REFs and underrepresentation of dREFs and uREFs in all species (Supplemental Figure S3). To compare this trend with protein coding genes, we selected 158 intronless small proteins (< 100 aa) from the *P. patens* genome annotation. The percentages of sORFs and these proteins showing homology with at least one species were significantly different (7.2% sORFs vs. 86% small proteins), pointing to high genome turnover of sORF sequences.

To better understand the large-scale trends of sORF evolution, we examined the differences in selection pressure at the amino acid level between different major groups of sORFs (CDS-sORFs, uORFs, dORFs, lncRNA-sORFs, interlaced-sORFs) using the criterion of Dn/Ds. This analysis showed that the highest portion of sORFs comprised CDS-sORFs, with Dn/Ds ratio > 1, implying ongoing positive selection of sORFs emerging in the CDS of protein-coding genes. This criterion for other sORF groups was < 1 in most cases, pointing to purifying selection for these sequences (Figure 2C).

The possible evolution of non-coding portions of the genome into protein-coding genes is a subject of intensive debate (Carvunis et al. 2012; McLysaght and Guerzoni 2015; Couso and Patraquim 2017). The ability of non-coding RNAs bearing sORF sequences to give rise to new genes is a controversial idea whose confirmation is a challenging task. To gain new insight into the process of gene birth, we assessed whether the lengths of homologous sORFs in other species were the same as those in moss or if they tended to change in size. According to our data, putative homologous sORFs tended to differ in length in most cases (Figure 2D). We found that most sORFs expanded during evolution, providing support for the notion that they function as raw materials for selection; however, this point requires further confirmation.

Thus, evolutionary analysis demonstrated that the conservation of an sORF on a large evolutionary scale differs from that of randomly selected exon sequences and depends on the location of the sORF, with a greater chance of being fixed for uORFs and dORFs, whereas CDS-sORFs and lncRNA-ORFs are under strong positive selection. A high rate of evolution is the driving force for the exclusion of an sORF from a coding sequence.

Experimental evidence for the translation of sORFs

Obtaining evidence for the translation of sORFs is an important step towards identifying functional SEPs. We verified the translation of our predicted sORFs using mass-spectrometry (MS) analysis, which is often considered to be the gold standard for detecting proteins or peptides in a cell. Taking into account the shortage of proteomic methods for identifying small proteins or peptides, in the current study, we used two datasets: the “peptidomic” dataset (endogenous peptides extracted from three types of moss cells: gametophores, protonemata and protoplasts) and the “proteomic” dataset (tryptic peptides generated in a standard proteomic pipeline). All datasets were mapped with MaxQuant against a custom database containing our sORFs together with nuclear, chloroplast and mitochondrial moss protein sequences (see details in the Methods). In total, we confirmed the translation of 602 sORFs: 205 in gametophores, 288 in protonemata and 196 in protoplasts (Figure 3A, Supplemental Table S2). The most prominent group of translatable sORFs consisted of CDS-sORFs (306, 51%) (Figure 3B). Interestingly, the translation of 42 sORFs located on lncRNAs was also detected by our analysis.

The length of translatable sORFs ranged from 11 to 100 amino acids (aa), which were generally longer than untranslatable sORFs (Mann-Whitney P = 4e-53) (Figure 3C). The length of interlaced-sORFs differed significantly from that of CDS-sORFs and lncRNA-sORFs (Mann-Whitney P = 0.002 and Mann-Whitney P = 0.001, respectively) but did not differ from uORFs (Mann-Whitney P = 0.06). We observed that PSMs (peptide spectrum matches) supporting SEP identifications had lower average quality than those mapped to the protein sequences of all datasets (Supplemental Figure

S4A and B). This finding is in agreement with data obtained for the animal kingdom (Slavoff et al. 2013; Mackowiak et al. 2015).

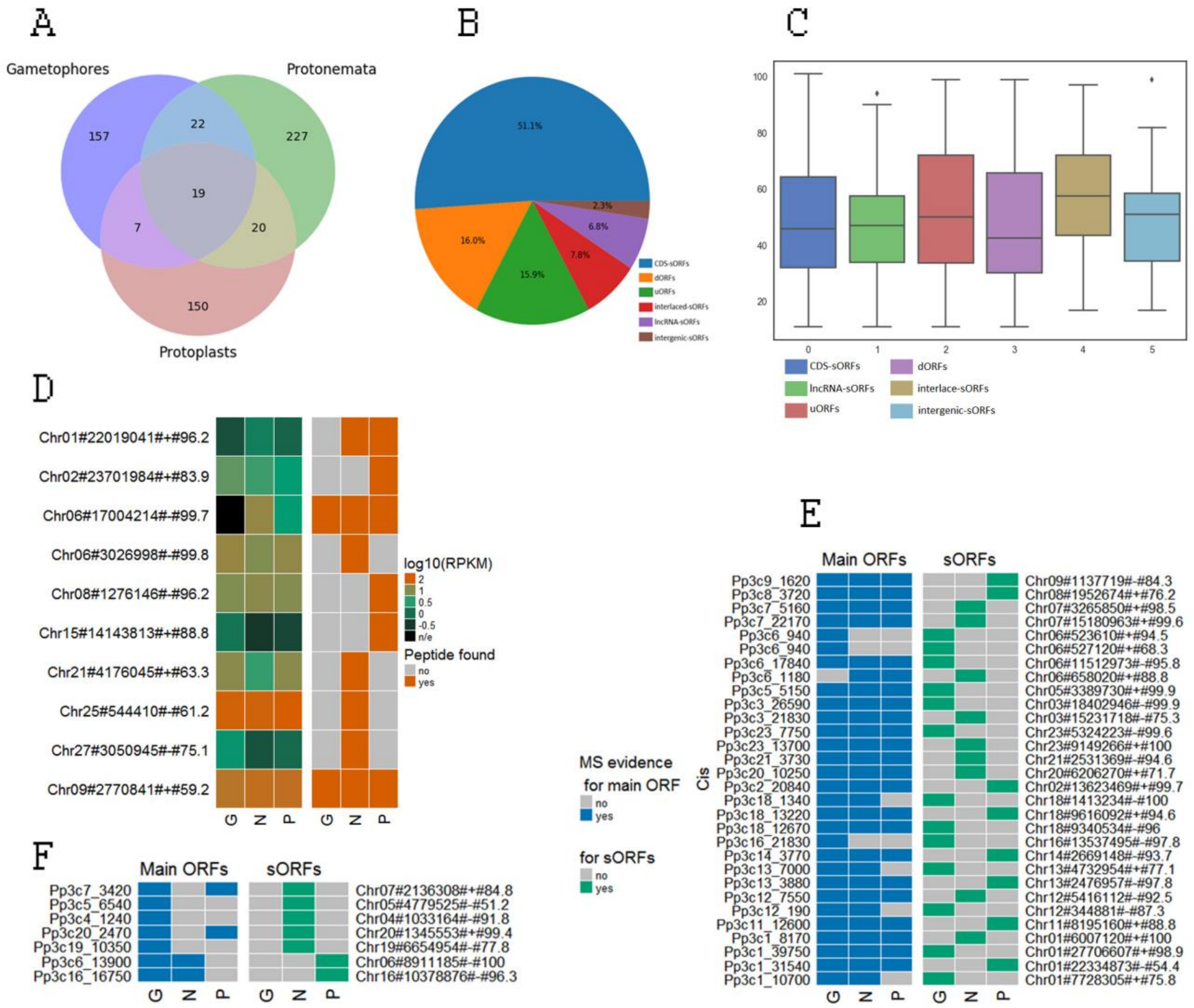


Figure 3. Moss contains hundreds of translatable sORFs. A – Venn diagram showing the distribution of the identified sORFs among three types of moss cells; B - Distribution of translatable sORFs based on the suggested classification; C - Length distribution of various groups of translatable sORFs; D - Heatmap of RPKM expression levels of transcripts carrying lnsRNA-sORFs and evidence of translation for three moss tissues; G, N and P correspond to gametophores, protonemata and protoplasts, respectively; E – Heatmap showing evidence of translation for sORFs and proteins in polycistronic genes in three moss tissues. G, N and P correspond to gametophores, protonemata and protoplasts, respectively. F - Examples of contrasting translational patterns of the main ORF and CDS-sORF. Only proteins confirmed by more than three unique tryptic peptides in the MS data are shown.

Interestingly, the quality of spectra and the values of PSMs supporting the expression of SEPs were better in the "peptidomic" dataset (Supplemental Figure S4C). Also, translatable sORFs were longer for those identified in the peptidomic dataset (Supplemental Figure S4D).

There were no significant dependencies between the level of expression of a transcript and the chance of finding peptides from sORFs located on this transcript (logistic regression, P-value >> 0.05). However, among the 19 sORFs with evidence of translation in all types of moss cells, lncRNA-sORFs were significantly overrepresented (Fisher's exact test, P-value = 0.001). Moreover, lncRNA transcripts were highly expressed and produced peptides that were also detected in gametophores, protonemata and protoplasts (Figure 3D). These data may point to biological significance for the peptides translated from these sORFs rather than the sORFs having regulatory functions in the translation of the main ORF. To investigate this notion, we explored the activity of one such SEP encoded by an lncRNA (see below).

Standard proteomic validation requires the presence of two non-overlapping tryptic peptides to confirm the translation of a protein. However, it is unlikely that more than one tryptic fragment will be detected in the case of an SEP. Nevertheless, we identified more than one unique peptide for six sORFs in the peptidomic dataset. Moreover, two of these SEPs, Chr09#2770841#+#59.21 (41aa) and Chr25#544410#-#61.2 (61aa), were common to all three cell types and were confirmed by 15 and 17 unique endogenous peptides, respectively (Figure 3C). In the proteomic dataset, we observed two non-overlapping tryptic peptides for only one 89-aa sORF (Chr20#14861199#+#85.5). Presumably, the analysis of endogenous peptide pools may be more suitable for detecting smaller SEPs.

sORFs can be translated together with proteins

Several reports provide evidence that eukaryotic mRNA can have more than one coding ORF (bi- and polycistronic genes) in both plants and animals (Blumenthal 1998; Rohrig et al. 2002; Pi et al. 2009; Tautz 2009). We analyzed our MS data to detect the translation of main ORFs and sORFs from the same gene (putatively polycistronic). We identified 144 genes for which at least two ORFs (one main ORF and one sORF) were translated, according to our MS data, including 82 connected to the translation of CDS-sORFs (Supplemental Table S3). Some of these were translated simultaneously with protein-coding ORFs in the same type of moss cell (Figure 3E), while others showed tissue-specific expression patterns (Figure 3F). This observation suggests that specific regulatory mechanisms may exist to fine-tune the translation of both sORFs and proteins situated in the same gene locus. We next sought to determine whether these sORFs encode some known protein domains. Interestingly, all ten sORFs that were identified in this analysis harbor intrinsically disordered regions (IDRs).

Taken together, our findings indicate that at least 27% of translatable CDS-sORFs are expressed simultaneously with main ORFs and that the moss genome has more than 100 putative bicistronic and three polycistronic genes with detectable translation products. Moreover, the translation of sORFs and proteins located together in the same locus might be regulated in a tissue-specific manner.

Most translatable sORFs are not evolutionarily conserved

Analysis of the evolutionary conservation of sORFs is often a key step in revealing biologically active sORFs (Andrews and Rothnagel 2014). To determine whether the translatable sORFs were more highly conserved than the other sORFs, we analyzed the intactness of these sORFs in the reconstructed genomes of three *P. patens* ecotypes, 'Villersexel', 'Reute' and 'Kaskasia', as well as the ten abovementioned species. We found that 19 (3.2%) of 602 translatable sORFs in the ecotypes either lost the start/stop codon or had a frameshift or premature termination codon (PTC). This number was not significantly different from the number (2.4%) occurring by chance suggesting that sORF translation does not disrupt trends of sORF elimination in these ecotypes.

To investigate whether the trend in translatable sORF evolution differs from that of the other sORFs, we estimated the age (number of species in which homologs can be found) and the selection pressure (Da/Ds) on translatable sORFs on an evolutionary timescale using the transcriptomes of the ten abovementioned species. Overall, we found 74 sORFs had evidence of translation and conservation in at least one species while only 11 were under negative selection (Ka/Ks << 1) (Supplemental Figure S5).

Sixty-four of these were CDS-sORFs or interlaced-sORFs. These results point to a high level of conservation of these sORFs, which is apparently connected to the conservation of overlapping protein-coding genes. Although conservative sORFs were significantly enriched in a set of translatable sORFs (Fisher's exact test, P = 2.716567e-05), we found that most translatable sORFs (525, 87.6%) were not conserved.

We next examined whether the translatable sORFs detected in this study share similarity with a recently defined set of 13,748 putative SEPs in the *A. thaliana* (Hazarika et al. 2017). We identified two sORFs (Chr20#13303500#-#88.2 (uORF), Chr11#14549091#+#97.9 (CDS-sORF)) with evidence of translation according to our MS analysis that shared similarity with ARA-PEP peptides (e-value < 0.01), implying that these sORFs are evolutionarily conserved and may produce peptides in *A. thaliana* cells.

Alternative splicing regulates the number of sORFs in protein-coding transcripts

Alternative splicing (AS) events may lead to the specific gain, loss or truncation of different groups of sORFs located on the transcripts of the same gene. For example, AS can generate sORFs that are truncated version of proteins (see below). We found 6092 alternatively spliced sORFs (AS-sORFs) belonging to transcripts from 4389 genes. CDS-sORFs were significantly overrepresented (Figure 4A), while interlaced-sORFs, uORFs and dORFs were significantly underrepresented among AS-sORFs compared to the control dataset (AS-REF). The number of translatable sORFs in a set of AS-sORFs did not significantly differ from that expected by chance (Fisher's exact test p-value=0.9423), suggesting that AS does not preferentially occur in peptide-encoding sORFs.

We randomly selected ten different translatable AS-sORFs and searched for the corresponding isoforms with/without sORFs in the transcriptomes of three types of moss cells. RT-PCR analysis revealed the transcription of these isoforms, confirming that they could indeed be translated (Supplemental Figure S6). Moreover, four sORFs contained isoforms showing tissue-specific transcription. These observations led to the hypothesis that the translation of sORFs is extensively regulated by AS.

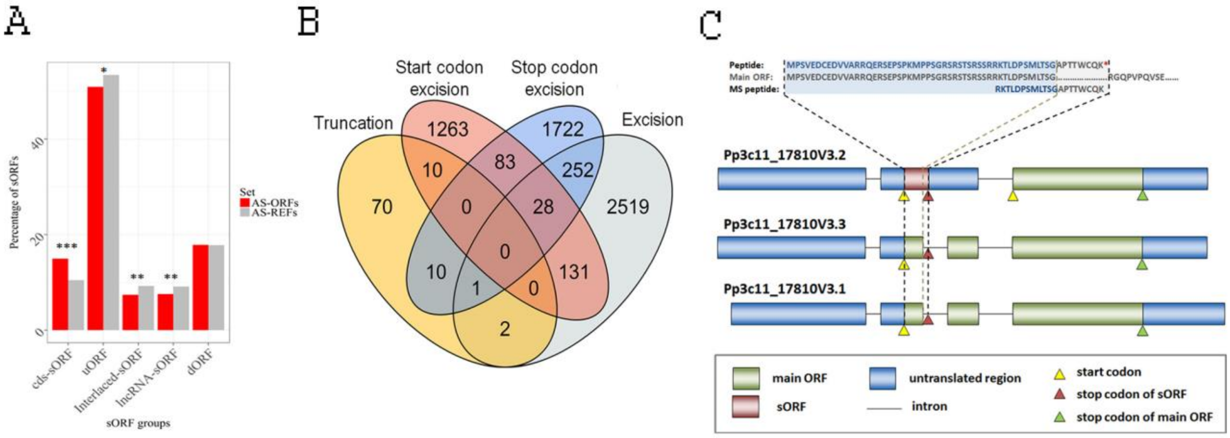


Figure 4. Alternative splicing regulates the expression of sORFs. A – Enrichment analysis of different sORF groups in a set of AS-sORFs and AS-REFs. P-value was calculated by Fisher's exact test. ***P < 1e-10; **P < 0.001; *P < 0.05; B – Venn diagram showing the number of AS-sORFs influenced by different AS events. C – Example of a translatable CDS-sORF, which was generated by an AS event and partially overlaps with the main ORF of Pp3c11_17810. Intron retention caused the formation of the isoform with the sORF, while splicing of this intron led to the excision of the sORF stop codon and its disruption. MS detection of the peptide located at the exon-AS-intron junction allowed the translation of the sORF to be unambiguously distinguished from the translation of the main ORF. Upper panel shows the amino acid sequence of the sORF-encoded peptide, MS detected peptide and (partial) protein translated from the main ORF. Black and gray dotted lines mark the borders of the sORF and the canonical intron start site, respectively. The intron-exon structure of three transcript isoforms of the gene was retrieved from Phytozome (v12).

We then performed GO enrichment analysis of the genes carrying AS-sORFs and found that they were significantly enriched (Fisher's exact test, p-value < 0.01) for 13 GO terms. Ten GO terms linked with nucleic acid binding (GO:0001071, GO:0003700), signal transducer activity (GO:0004871), aminopeptidase activity (GO:0004177), transferase activity (GO:0003950, GO:0016772, GO:0016775) and kinase activity (GO:0004672, GO:0004673, GO:0000155) were specifically enriched in a set of AS-sORF-carrying genes. These overrepresented GO terms demonstrate that alternative splicing does influence the sORF landscape for regulatory genes and suggest that sORFs play an important role in the regulation of their translation.

We then classified the events leading to changes in sORF sequences into four groups: 1) truncation, if the middle part of the sORF was excised; 2) stop codon excision; 3) start codon excision and 4) excision, if the complete sORF was removed from an isoform. We found that half of the sORFs (48%, 2933) had undergone complete excision from transcripts, whereas only 93 sORFs were truncated (Figure 4B). Moreover, the complete excision of sORFs occurred significantly more frequently in uORFs than in the other sORF groups (57% vs. 20–44%, Fisher's exact test P-value < 1e-05). In addition, in the set of AS-sORFs with complete excision, evolutionarily conserved sORFs (conserved in >1 species) were significantly underrepresented (6.76e-42) compared to the other sets of AS-sORFs ("Truncation", "Stop codon excision", "Start codon excision"). Thus, our analysis demonstrated that AS and evolutionary forces lead to the excision of sORFs from the transcriptome and genome of *P. patens*.

The role of sORFs in modulating protein–protein interactions

Protein–protein interactions (PPI) are critical for the formation of higher order protein complexes. Competitive inhibitors of PPI are referred to as MicroProteins (miPs) or small interfering peptides (siPEPs) (Seo et al. 2011; Eguen et al. 2015). These proteins, which are usually small, can be generated by alternative splicing or evolutionarily generated by domain loss (Staudt and Wenkel 2011; Eguen et al. 2015). We hypothesized that sORFs with similarity to known proteins could impair the functions of these proteins by mimicry or by regulating the activity of proteins translated from the main ORF. To identify such sORFs, we performed BLASTP (E-value < e-5) similarity searches between the encoded amino acid sequences of sORFs and the annotated proteins of *P. patens.* We identified 363 sORFs resulting from AS events that partially overlapped with the main ORF, thereby generating truncated versions of the proteins (cis-sORFs, see in Supplemental Table S4). First, we analyzed how many cis-ORFs contained known complete or incomplete protein domains, finding that 60 sORFs harbored IDRs, while 30 cis-sORFs contained parts of 28 different domains (Supplemental Table S4). Among these, we observed the protein kinase domain (PS50011, Chr13#1404821#+#28.4), protein tyrosine kinase (PF07714, Chr11#4429996#+#64.5) and MYB-like DNA-binding domain (TIGR01557, Chr19#1622814#+#55.3). Potential SEPs from these sORFs can be considered potential candidate microPeptides (Straub and Wenkel 2017). Interestingly, the genes containing cis-sORFs were enriched in kinase and kinase-like domains. GO enrichment analysis also revealed significant overrepresentation of terms associated with protein modifications, such as GO:0006468 (protein phosphorylation) and GO:0036211 (protein modification process).

Another group of peptides carrying similarity with DNA binding and protein-protein interaction domains of transcription factors is small interfering peptides. Because similarity with transcription factors (TFs) domains they can act as dominant-negative repressors of TFs. Among genes containing cis-sORFs, we found some moss small interfering peptides that had similarity to putative transcription factor genes such as genes encoding GROWTH-REGULATING FACTOR (e.g., Pp3c20_10590), C2H2 zinc finger domain containing (e.g., Pp3c1_16920)), BTB/POZ domain containing (e.g., Pp3c16_9230), B3 DNA binding domain containing (e.g., Pp3c7_7990) and MYB-CC type transcription factor (e.g., Pp3c21_2850).

To obtain evidence for the translation of these sORFs, we analyzed MS data and found at least two examples (Figure 4C). A few detected translatable cis-sORFs could be explained by a significant overlap with the protein sequences, whereas we filtered out the 'ambiguous' PSMs. Moreover, the formation of a premature termination codon (PTC), for example, as a result of intron retention events, leads to mRNA decay (Ge and Porse 2014; Karousis et al. 2016). Thus, we suggest that peptides from the cis-sORFs are produced by moss cells and accordingly, they might be involved in cis regulation of main ORF protein activity or have distinct functions.

We identified 272 sORFs that shared similarity with annotated proteins but were located on other transcripts (trans-sORFs, see in Supplemental Table S4). The translation of six trans-sORFs was confirmed by our MS data. We found 36 potential trans-SEPs with similarity to known protein domains (Supplemental Table S4). Trans-sORFs may have originated through the divergence of ancient paralogous genes, which occurred after the paleo duplication of the moss genome (Rensing et al. 2007; Rensing et al. 2008). In fact, 159 (58.5%) trans-sORFs shared similarity to genes from at least one species (age 1-10). In addition, all of these trans-sORFs are under strong purifying selection (dN/dS << 1). The low rate of evolution of trans-sORFs suggests that the encoded peptides have important biological functions and might be involved in proteomic networks based on their similarity to functional proteins. Interestingly, we observed significantly fewer AS-sORFs in the set of trans-sORFs, suggesting that mechanisms other than AS are responsible for their formation. We then investigated which trans-sORFs share similarity to large gene families. Several distinct clusters with sORF-encoded peptides sharing similarity with more than four proteins from distinct genes were detected (Supplemental Figure S7). Each cluster encompasses genes from different protein families, including one containing leucine-rich repeat and zinc-finger domains involved in protein–protein and protein–nucleic acid interactions, respectively. Potential SEPs from these clusters share similarity with the respective domains and are therefore considered to be potential candidate microPeptides (Straub and Wenkel 2017).

We then addressed two questions: 1) Are proteins with higher numbers of interactions overrepresented among those with BLASTP hits; and 2) Is there a connection between the expression of sORFs and similar proteins? To answer the first question, we identified orthologous genes in the *A. thaliana* genome and used data from the interactome database (AtPID) (Lv et al. 2017). However, we did not detect a significant difference from the set of randomly selected genes. To determine whether sORF-protein pairs more frequently coexist in a cell, we examined the coexpression data and compared the distribution of correlation coefficient values with those from randomly selected pairs (10 iterations) of genes. On average, these sORF-protein pairs had higher correlation coefficients than randomly selected gene pairs (Wilcoxon Rank Sum and Kolmogorov-Smirnov Tests P-value < 0.05), implying that sORF-bearing and target genes are frequently coexpressed.

SEPs regulate moss growth

Despite the recent finding that 10% of overexpressed intergenic sORFs have clear phenotypes in Arabidopsis (Hanada et al. 2013), the functions of most sORFs and SEPs in plants are generally unknown. Known bioactive SEPs in plants are encoded by sORFs located on short non-protein-coding transcripts, which can be referred to as lncRNAs (Rohrig et al. 2002; Chilley et al. 2006). In this context, it would be intriguing to determine how many plant lncRNAs encode peptides, as well as the biological functions of these SEPs. Our pipeline allowed us to identify hundreds of translated sORFs, including those encoded by lncRNAs. Some of these lncRNA-sORFs showed tissue-specific transcription and translation patterns, while others were expressed in all types of moss cells (Figure 3C). We reasoned that stably expressed lncRNA-sORFs can produce peptides that play fundamental roles in various cellular processes. To explore this hypothesis, we examined the impact of overexpression and knockout of these lncRNA-sORF sequences on moss morphology. Here, we present an example of such analysis using a 41-aa peptide (SEP1) encoded by the stably expressed lncRNA-sORF Chr09#2770841#+#59.21 (Figure 3C).

We obtained multiple independent mutant lines and confirmed the expression of the transgenes or the knockout of sORF sequences (Supplemenatl Figure S8). The overexpression as well as knockout of sORF sequences led to clear morphological changes, implying that these peptides play a role in regulating filamentous architecture in *P. patens* (Figure 5). The overexpression of *SEP1* induced the formation of long caulonema cells compared with the wild type and knockout lines (Figure 5B and 5C). Moreover, there was a significant difference between the growth rates of the wild type and *SEP1* mutant lines (Figure 5D).

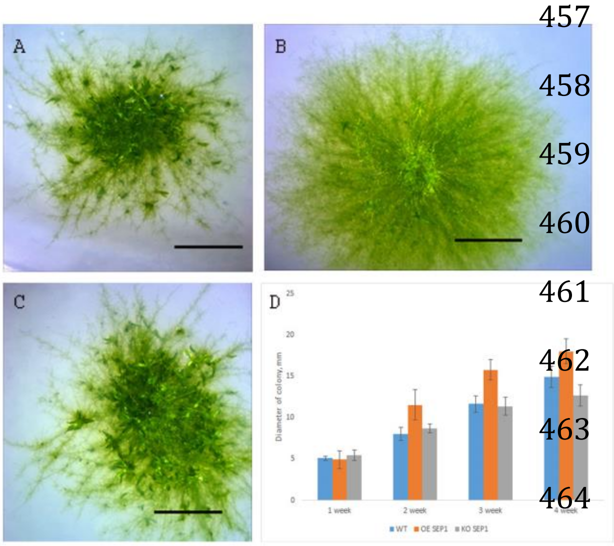


Figure 5. Morphology of wild type and mutants lines grown on BCD-medium. A – WT; B –overexpression mutant line; C – knockout mutant line; Scale bar: 50 mm; D – The diameter of WT and mutant colonies on BCD medium.

The knockout of the *SEP1* sequence led to moss protonemata with a reduced growth rate and a significant delay in senescence. Conversely, the *SEP1* overexpression lines were characterized by rapid growth and senescence compared to the wild type and knockout lines. Taken together, our findings suggest that lncRNA-sORFs can influence growth and development in moss and that our pipeline allows biologically active peptides to be identified.

DISCUSSION

Although functionally characterized SEPs have been shown to play fundamental roles in key physiological processes, sORFs are arbitrarily excluded during proteome annotation. Given the difficulty in identifying translatable, functional sORFs, we know little about their origin, evolution and regulation in the genome. In the present study, we investigated the abundance, evolutionary history and possible functions of sORFs in the genome of the model moss *Physcomitrella patens*. The use of an integrated pipeline that includes transcriptomics, proteomics and peptidomics data allowed us to identify hundreds of translatable sORFs in three types of moss cells. We propose that several distinct classes of sORFs that differ in terms of their position on transcripts, the level of evolutionary conservation and possible functions are present in the moss genome (Figure 6).

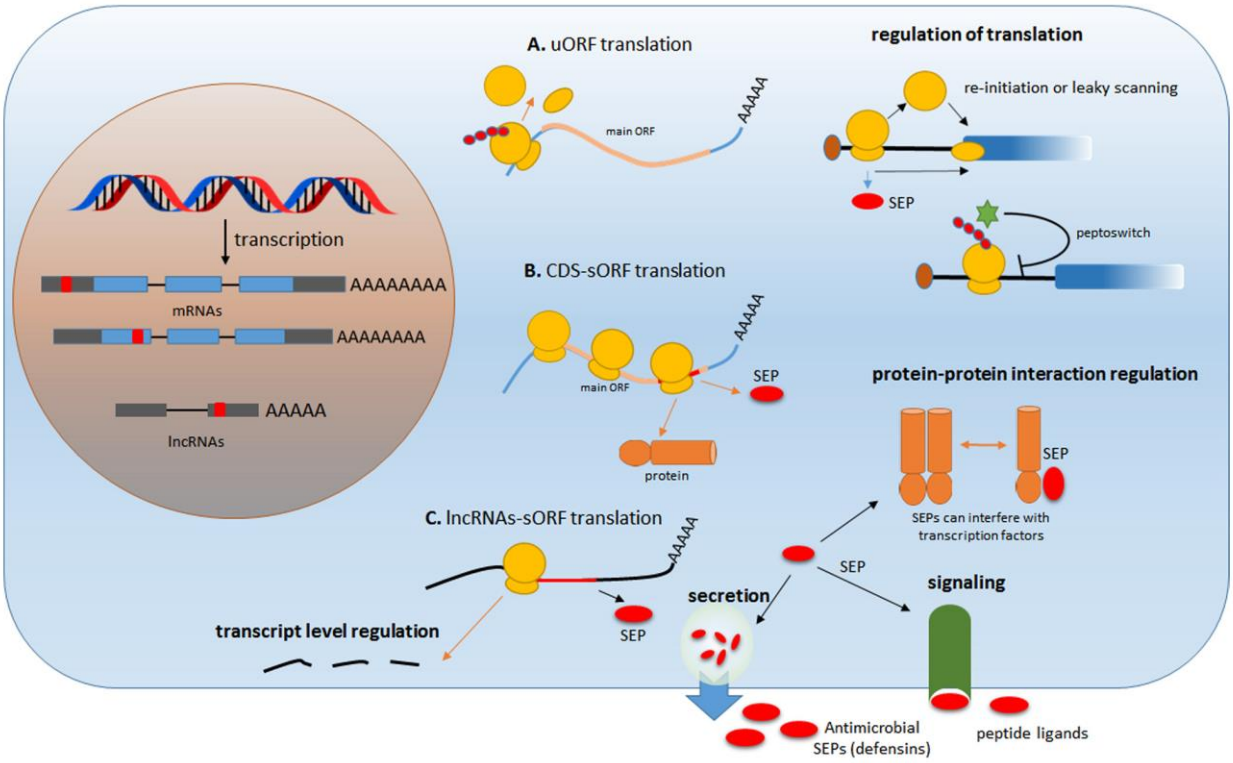

transcription
AAAAAAAA
mRNAs
AAAAAAAA
AAAAA
lncRNAs
A. uORF translation
main ORF
AAAAA
B. CDS-sORF translation
AAAAA
main ORF
SEP
protein
C. lncRNAs-sORF translation
AAAAA
SEP
transcript level regulation
regulation of translation
re-initiation or leaky scanning
SEP
peptoswitch
protein-protein interaction regulation
SEP
SEPs can interfere with transcription factors
SEP
secretion
signaling
Antimicrobial SEPs (defensins)
peptide ligands

Figure 6. Proposed functions of sORF-encoded peptides. A – uORFs can function in the regulation of translation of the downstream main ORF. The functions of peptides encoded by uORFs are unknown, and most are likely to represent "noise" from protein translation; B – CDS-sORF-encoded peptides can help regulate protein-protein interactions, and some interfere with the translation of the main ORF; C – long non-coding RNAs or intergenic-sORFs can produce biologically active peptides that perform signaling, defense or regulatory functions. In addition, the translation of sORFs

can activate the nonsense-mediated decay (NMD) mechanism, which leads to the degradation of the corresponding transcripts.

According to our MS data, the translation patterns of most sORFs tend to be tissue specific. Our results suggest that the evolutionary rates of various types of sORFs differ, with weak overall conservation and fast elimination from a genome. We also showed that alternative splicing is an additional mechanism to control sORF expression in plant cells, changing their sequences at the transcriptome level. Finally, our results suggest that stably expressed sORFs located on lncRNAs can play important roles in plant growth.

sORFs with high coding potential are not conserved among genomes

Even though the analysis of sequence conservation is somewhat biased against the detection of short sequences (Ladoukakis et al. 2011), this technique is widely used to select candidate functional sORFs. Although analyzing the conservation of short amino acid sequences is not trivial (Moyers and Zhang 2016), hundreds of conserved sORFs have recently been identified in plants, yeast and animals (Ladoukakis et al. 2011; Hanada et al. 2013; Mackowiak et al. 2015). The number of sORFs conserved in the plant kingdom is undoubtedly underestimated due to the low sensitivity of tools used for conservation analysis and the limited number of available sequenced genomes from closely related species. Our pipeline allowed us to identify 5034 conserved sORFs among the transcriptomes of ten different plant species, 74 of which showed evidence of translation according to our MS data. However, we suggest that the possibly functional sORFs might significantly outnumber the conserved ones.

Despite the observation that approximately 1% of uORFs and dORFs had evidence of translation, a significant “loss” of these sORF types was observed among the closest related species. We even detected rapid inactivation of uORFs and dORFs (756 sORFs) in the reconstructed genomes of three *P. patens* ecotypes due to the disruption of start or stop codons. As the occurrence of sORFs downstream or upstream of the main ORF can be deleterious to its translation, we cannot rule out the possibility that this may cause strong selection pressure and the rapid elimination of uORFs and dORFs (Iacono et al. 2005; Neafsey and Galagan 2007; Johnstone et al. 2016). Moreover, we observed significant depletion (Fisher's exact test P-value = 5.25e-13) of uORFs and dORFs in a set of translatable conservative sORFs. Taken together, these findings suggest that sORFs located in untranslated regions are evolving rapidly and may play a regulatory roles rather than encoding bioactive peptides.

In a recent study, more than 1000 alternative proteins were experimentally detected by mass-spectrometry in human cell lines (Vanderperre et al. 2013). In *P. patens*, we found tens of thousands of sORFs (CDS-sORFs) that overlapped with the CDS of protein-coding genes, 306 of which were translatable. The evolutionary origins, functions and mechanisms of translation of such alternative sORFs are still unclear. According to our results, a significant number of CDS-sORFs are under positive selection and are dramatically eliminated from the genomes of distant species. This process must not be stochastic, as opposite results were obtained for randomly selected CDS. The speed and direction of the evolution of protein-coding genes depends on their level of transcription, biological functions, protein-protein connectivity and functional redundancy (Hirsh and Fraser 2001; Zhang and Li 2004; Julenius and Pedersen 2006; Enard et al. 2014). Moreover, only specific domains or regions of protein-coding sequences can evolve under positive selection (Montoya-Burgos 2011).

The evolution of CDS-sORFs is undoubtedly an expensive process for the cell, as these elements may be located in regions encoding protein domains and influence the structure and function of the protein encoded by the main ORF (Cherry 2010). In addition to being located in regions encoding functional domains, CDS-sORFs can be generated in fast-evolving regions of genes (e.g., those encoding protein disordered regions). We found both CDS-sORFs originated from regions associated with known protein domains and CDS-sORFs from disordered regions. Indeed, a comparison among the conserved (homologs found in >1 species) sORFs of the number of the two types of CDS-sORFs in similar species revealed significant differences, with higher conservation for CDS-sORFs originated from protein domain-encoding regions. These results indicate that the evolution of CDS-sORFs depends on their locations insight main CDS sequence.

In the current study, we found that both the transcription and translation of CDS-sORFs occurred in a tissue-specific manner. Protein-coding genes with tissue-specific transcription patterns and functional redundancy of the gene product are often under positive selection (Zhang and Li

2004; Montoya-Burgos 2011). This finding, together with other properties of CDS-sORFs, such as their overlap with particular parts of protein-coding sequences, might explain the high turnover rate of CDS-sORFs. However, whether sORFs are preferentially generated in fast-evolving regions of proteins or whether the selective pressure on sORFs leads to changes in protein-coding sequences is still unknown.

Analysis of sORF translation: approaches that makes sense

Clear evidence of transcription and translation points to the biological significance of sORFs. Thus, identifying translatable sORFs is an important step that could lead to the discovery of new biological functions. Ribosome profiling provides a direct readout of the ribosome occupancy of different transcripts, thereby providing a measure of the level of translation. According to ribosome profiling data from a wide variety of species, translation appears to occur in a pervasive manner (Ingolia et al. 2011; Guttman et al. 2013; Bazzini et al. 2014; Couso and Patraquim 2017). These observations have led some researchers to conclude that the majority of non-coding RNAs (e.g., lncRNAs) in cells can be translated and produce peptides (Crappe et al. 2013; Bazzini et al. 2014; Housman and Ulitsky 2016). In addition to lncRNAs, the ribosome occupancy of short frames in the UTRs of mRNA has also been investigated (Weatheritt et al. 2016). However, ribosome-profiling data alone are not sufficient to classify transcripts as coding or noncoding (Guttman et al. 2013). Thus, alternative approaches such as proteomics and peptidomics should be used to investigate the translation of sORFs (Slavoff et al. 2013; Ma et al. 2016). Comparisons of ribosome profiling and mass spectrometry results have led to the conclusion that MS detects peptides arising from the most highly translated sORFs (Aspden et al. 2014; Bazzini et al. 2014). However, a recent study showed that there are no technical obstacles to the detection of lncRNA-encoded peptides by mass spectrometry (Verheggen et al. 2017). Nonetheless, peptide detection by MS analysis can be difficult, for example, due to the location of SEPs in cellular membranes (Andrews and Rothnagel 2014; Couso and Patraquim 2017). Mass-spectrometry studies have thus far confirmed the presence of a few dozen SEPs in the peptidomes of animal cells (Slavoff et al. 2013; Prabakaran et al. 2014; Mackowiak et al. 2015; Ma et al. 2016). In the present study, we found evidence for the translation of only a small portion of the predicted sORFs.

Moreover, we confirmed the presence of only a few sORFs in all three cell types. Therefore, it is difficult to estimate the full extent of the presence of sORFs in a cell.

In previous studies, only standard proteomics analysis was used to identify SEPs. We reasoned that analyzing endogenous peptide pools instead of tryptic peptides has several disadvantages in terms of SEP identification: 1) standard proteomic approaches are not suitable for the isolation and analysis of small and low-abundance peptide molecules; and 2) SEPs are shorter than standard proteins and it is unlikely that more than one tryptic fragment will be detected in a single proteomic experiment. Moreover, peptidomic approaches can theoretically be used to identify full-length SEPs in a cell. We firstly used endogenous peptides pools to detect SEPs and according to our data the values of PSMs, supporting expression of SEPs, were better in "peptidomic" dataset. Moreover, some SEPs were confirmed by several endogenous peptides (up to 17), that an increase the reliability of their detection. Notably, we did not observe any significant overlap between the sORFs detected using proteomic and peptidomic approaches. Thus, our study demonstrates the advantage of using complementary approaches for building a complete list of SEPs.

Functionality of SEPs

It was recently suggested that sORFs are randomly generated in a genome (Couso and Patraquim 2017). We detected more than 600,000 sORFs with high coding potential in the moss genome. Assuming the average length of an sORF is approximately 60 bp and distinct sORFs are not overlapping, these elements occupy a substantial portion of the moss genome. This raises the following question: to what extent are sORFs present in the transcriptome and (even more interesting) the proteome of a cell? Some of these sORFs are translated into peptides, suggesting they might contribute to physiological processes, but the extent of selective pressure on these elements has been unclear.

We identified hundreds of translatable sORFs of different types and suggested various functions for these types of sORFs (Figure 6). The average conservation of sORFs within 5′ leader sequences (uORFs) is low (Aspden et al. 2014); uORFs are thought to regulate the translation of the downstream ORF (Johnstone et al. 2016). Based on our conservation analysis and MS data, we suggest that the majority of uORFs and dORFs play regulatory roles instead of encoding peptides

(Figure 6A). By contrast, CDS-, interlaced- and lncRNA-sORFs have greater potential to encode bioactive peptides, as they are more highly conserved, frequently contain protein domains and, according to the MS data, often produce peptides. However, the functions of the peptides are unclear and require more detailed investigations. One possible function of sORF-encoded peptides that are similar to known proteins is mimicry of the main protein function.

MiPs (or siPEPs) are small, usually single domain proteins (Seo et al. 2011; Eguen et al. 2015). Some miPs are important modulators of protein–protein and protein–DNA interactions that, for example, prevent the formation of functional protein complexes (Seo et al. 2013; Graeff et al. 2016). Most known miPs/siPEPs are small proteins evolutionarily generated by domain loss (Eguen et al. 2015). We suggest that the potential for sORFs that overlap with the CDS of protein-coding genes to be a source of small interfering peptides is currently underestimated (Figure 6B). Based on the analogy of cis-miPs generated by alternative splicing events (Eguen et al. 2015), we refer to these SEPs as cis-SEPs. In the present study, 363 sORFs encompassing parts of the main ORFs and originating from AS were identified. According to the MS data, some of these sORFs have evidence of translation. Analyzing sequence similarity to known proteins or the presence of particular domains can be useful for predicting peptide function. We found that approximately 30% of cis-SEPs harbor protein domains such as protein kinase domains and MYB-like DNA-binding domain or IDRs. This finding points to the high regulatory potential of these SEPs resulting from their interference with functional proteins (Eguen et al. 2015). At the same time, it should be noted that protein domains or incomplete protein domains in isolation can have functions unrelated to those observed in multi-domain proteins (Kelley and Sternberg 2015).

We also found SEPs sharing homology with proteins produced from other genes that likely originated through the divergence of ancient paralogs. Perhaps SEPs with similarity to other proteins, such as those translated from CDS-sORFs, can perform functions like those of miPs/siPEPs and regulate the activity of protein or protein complexes. Indeed, we found that genes harboring CDS-sORFs were enriched in GO terms connected to protein binding and transferase activity. Also, some sORFs with disordered regions might mediate protein–protein or protein–nucleic acid interactions, as suggested previously (Mackowiak et al. 2015). Taken together, these findings suggest that sORFs may strongly interfere with protein interactions.

In this study, we explored several groups of sORFs, including those encoded by lncRNAs. The translation of peptides from lncRNAs is intriguing, and there is some evidence that these peptides play important biological roles in various processes (Kondo et al. 2010; Magny et al. 2013; Matsumoto et al. 2017). Nevertheless, the biological functions of most lncRNA-sORF-encoded peptides are currently unclear, especially those in the plant kingdom (Tavormina et al. 2015). For example, it is unknown whether there is a connection between the functions of lncRNAs and the peptides they encode. The transcription of the non-coding portions of the genome into lncRNAs is thought to give rise to the translation of sORFs located within them. In this case, some of these peptides would not be vital but may be important for survival under certain conditions by serving as a raw material for evolution (Figure 6C). According to our data, the number of sORFs located on single lncRNAs varied from 1 to 28. Thus, the translation of lncRNAs can potentially lead to the production of many peptides in a cell. However, the opportunity for the translation of lncRNAs and the subsequent stability of such peptides in the cell are under debate (Saric et al. 2004; Loose et al. 2007; Housman and Ulitsky 2016). There are only a few examples of lncRNA-encoded peptides involved in signaling and plant growth. For example, POLARIS (PLS), encoding a 36-amino acid peptide, is required for correct root growth and vascular development in Arabidopsis (Chilley et al. 2006). In the current study, we confirmed the translation of 42 SEPs encoded by lncRNAs. Plants overexpressing an lncRNA-encoded peptide (41 aa) showed clear phenotypic differences from wild-type plants, suggesting its possible role in regulating cell growth and development. Our results lay the groundwork for systematic analysis of functional peptides encoded by sORFs.

The conservation rate of lncRNA- sORFs is not high. Moreover, most lncRNA-encoded peptides are not conserved. In our view, they might serve as a reservoir of peptide activity (Bao et al. 2017) for adaptation processes and/or as a source for *de novo* generation of new genes, a topic that is currently under intensive debate (Couso and Patraquim 2017).

Our analysis led to several conclusions concerning the extent, evolution and possible biological functions of sORFs in plants. We discovered that most sORFs, including translatable ones, are not widely conserved and that the most slowly evolving group overlaps with the CDS of protein-coding genes. We demonstrated the role of alternative splicing in shaping the sORF landscape in terms of transcripts, as well as isoform-specific transcription of sORFs. We identified a group of sORFs homologous to known protein domains and suggested they function as small interfering peptides. Finally, we demonstrated the functional potential of one peptide encoded by an lncRNA. The high potential regulatory activity of peptides, high evolutionary rate and wide translation of sORFs suggest that they may provide a reservoir of potentially active molecules and that some of sORFs can give rise to new protein-coding genes. We provide a resource of putatively functional peptides for further analysis.

METHODS

Physcomitrella patens growth conditions

Physcomitrella patens protonemata were grown on BCD medium supplemented with 5 mM ammonium tartrate (BCDAT) under continuous white light at 25^{0}C in 9-cm Petri dishes (Nishiyama et al. 2000). For all analyses, the protonemata were collected every 5 days. The gametophores were grown on free-ammonium tartrate BCD medium under the same conditions, and 8-week-old gametophores were used for analysis. Protoplast was prepared from protonema as described previously (Fesenko et al. 2015).

For morphological analysis, samples of fresh protonemal tissue 2 mm in diameter were inoculated on BCD and BCDAT Petri dishes. For colony growth rate measurements, photographs were taken at 7 d intervals over 42 days. Colonies and protonema cells were photographed using a Microscope Digital Eyepiece DCM-510 attached to a Stemi 305 stereomicroscope or Olympus CKX41.

Identification of coding sORFs in the *P. patens* genome

To identify sORFs with high coding potential, the sORFfinder (Hanada et al. 2010) tool was utilized. Intron sequences and CDS were used as negative and positive sets, respectively. Intron sequences and CDS were extracted from the *P. patens* v3.3 genome (Phytozome v12) by parsing a gff3 file of the moss genome annotation using custom-made python scripts (available upon request), followed by DNA sequence extraction using the subcommand getfasta from bedtools (Quinlan and Hall 2010). These sequences were used to train sORFfinder as described in the user manual. Parameter -d was set to "b" for searching the sORF sequences in both direct and reverse orientation. The search for sORFs was performed using the whole genome sequence of *P. patens* (release 3.3, https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Ppatens). Of the 6,706,696 sORF sequences found in the genome, 786,439 had high coding potential according to the sORF finder. To eliminate sORFs that are transcribed, located in the exons of transcripts and have introns, a bed file was generated using a custom-made python script and intersected with exon positions extracted from a gff3 file of *P. patens* genome annotations. To identify intergenic-sORFs, the bed file was also intersected with transcribed regions determined based on our RNAseq data (Fesenko et al. 2017). Using an R script, sORFs fully overlapping with exons were selected; 75,685 sORFs remained after this step. Identical sORFs were removed from the dataset. In addition, sORFs overlapping repetitive regions identified by RepeatMasker, as well as sORFs comprising parts of annotated *P. patens* proteins, were also removed from the dataset, resulting in a final dataset of sORFs comprising 70,095 sequences.

sORF classification

The step-by-step procedure performed for sORF classification is illustrated in Supplemental Figure S9. In the first step, lncRNA-sORFs were identified by searching for identical sORFs in known lncRNA databases, including CantataDB (Szczesniak et al. 2016), GreenC (Paytuvi Gallart et al. 2016) and our previously published moss dataset (Fesenko et al. 2017). After this sORF bed file was intersected with moss genome annotation, the locations of the sORFs on transcripts were determined, resulting in the further classification of genic-sORFs into uORFs, dORFs, CDS-sORFs and interlaced-sORFs.

Because alternative splicing leads to inaccuracy in genome annotation, the locations of a subset of genic-sORFs cannot be unambiguously classified, as they can be located in different regions in different isoforms of the same gene. All sORFs located on transcripts that were not annotated in the *P. patens* genome but were identified using our RNAseq data were classified as intergenic-sORFs.

To detect alternatively spliced sORFs (AS-sORFs), a bed file with sORF locations was intersected with a bed file containing intron coordinates for all isoforms. Those sORFs that overlapped for both exons (see above) and introns were classified as AS-sORFs.

Evolutionary conservation analysis

The transcriptomes of nine plant species were downloaded from Phytozome v12: *Sphagnum fallax* (release 0.5), *Marchantia polymorpha* (release 3.1), *Selaginella moellendorffii* (release 1.0), *Spirodela polyrhiza* (release 2), *Arabidopsis thaliana* (TAIR 10), *Zea mays* (Ensembl-18), *Oryza sativa* (release 7), *Volvox carteri* (release 2.1) and *Chlamydomonas reinhardtii* (release 5.5). The transcriptome of *Ceratodon purpureus* was *de novo* assembled using Trinity (Haas et al. (2013)). To identify transcribed homologous sequences, tBLASTn (word size = 3) was performed using sORF peptide sequences as queries and the transcriptome sequences of the abovementioned species as subjects. The following cutoffs parameters were used to distinguish reliable alignments: E-value < e-5 and query coverage > 60%.

Pairwise Ka/Ks ratios (equivalent to dN/dS) were calculated using the codeml algorithm with PAML software (Yang 2007). The calculation procedure, which was facilitated using a custom-made python script (available under request), included alignment extraction from the tBLASTn output, PAL2NAL (Suyama et al. 2006) correction of the nucleotide alignment using the corresponding aligned protein sequences and calculation of Ka/Ks ratios using codeml. The script implements packages from biopython (Cock et al. 2009).

To estimate homologous sORF lengths, a python script (available under request) was designed. The script uses tBLASTn alignment output and estimates the presence of in-frame start and stop codons within (as well as downstream and upstream of) alignment regions. If a stop codon was found upstream of an alignment region, it was considered to be a premature termination codon (PTC). Otherwise, start and stop codons closest to the alignment region were used for homologous sORF length calculation.

GO enrichment analysis

GO enrichment analysis was performed using the topGO bioconductor R package using the Fisher's exact test in conjunction with the 'classic' algorithm (false discovery rate [FDR] < 0.05). Gene Ontology (GO) terms assigned to *P. patens* genes were downloaded from Phytozome. Only GO terms containing >5 genes in a background dataset were considered in the enrichment analysis. Redundant GO terms were removed using the web-based tool REVIGO (Supek et al. 2011).

Peptide and protein extraction

Endogenous peptide extraction was conducted as described previously (Fesenko et al. 2015). Proteins were extracted as described previously (Fesenko et al. 2016). Four biological repeats for gametophores, four for protonemal and four for protoplast samples were used.

Mass-spectrometry analysis and peptide identification

Mass-spectrometry analysis was performed using three biological and three technical repeats for the proteomic (Fesenko et al. 2017) and peptidomic datasets. Analysis was performed on two different mass spectrometers: a TripleTOF 5600+ mass spectrometer with a NanoSpray III ion source (ABSciex,Canada) and a Q Exactive HF mass spectrometer (Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer, Thermo Fisher Scientific, USA). For the Q Exactive HF mass spectrometer (Thermo Fisher Scientific, USA), peptide samples were separated by high-performance liquid chromatography (HPLC, Ultimate 3000 Nano LC System, Thermo Scientific, USA) in a 15-cm long C18 column with a diameter of 75 µm (Acclaim® PepMap™ RSLC, Thermo Fisher Scientific, USA). The peptides were eluted with a gradient from 5–35 % buffer B (80 % acetonitrile, 0.1 % formic acid) for 45 min at a flow rate of 0.3 µl/min. The total run time, including 10 min to reach 99% buffer B, flushing 5 min with 99% buffer B and 10 min re-equilibration to buffer A (0.1% formic acid) amounted to 70 min. Mass spectra were acquired at a resolution of 60,000 (MS) and 15,000 (MS/MS) in a range of 400–1500 m/z (MS) and 200–2000 m/z (MS/MS). An isolation threshold of 67,000 was determined for precursor selection and (up to) the top 10 precursors were chosen for fragmentation via high-energy collisional dissociation (HCD) at 25 NCE and 100 ms activation time. Precursors with a charged state of +1 were rejected, and all measured precursors were excluded from measurement for 20 s.

Mass-spectrometry analysis on a TripleTOF 5600+ mass spectrometer with a NanoSpray III ion source (ABSciex, Framingham, MA 01701, USA) coupled with a NanoLC Ultra 2D+ nano-HPLC system (Eksigent, Dublin, CA, USA) was performed as described (Fesenko et al, 2016).

All datasets were searched individually with MaxQuant v1.5.8.3 (Tyanova et al. 2016) against a custom database containing the protein sequences from Phytozome v12.0 merged with chloroplast and mitochondrial proteins, a database of common contaminant proteins and the set of predicted sORFs. MaxQuant's protein FDR filter was disabled, while 1% FDR was used to select high-confidence PSMs, and ambiguous peptides were filtered out. Moreover, any PSMs with Andromeda scores of less than 30 were discarded (to exclude poor MS/MS spectra). For dataset of endogenous peptides, the parameter "Digestion Mode" was set to "unspecific" and modifications were not allowed. All other parameters were left at default values. Features of the PSMs (length, intensity, number of spectra, Andromeda score, intensity coverage and peak coverage) were extracted from MaxQuant's msms.txt files.

RT-PCR analysis of AS-sORFs

Total RNA from gametophores, protonema and protoplasts was isolated as previously described (Cove et al. 2009). RNA quality and quantity were evaluated via electrophoresis in an agarose gel with ethidium bromide staining. The precise concentration of total RNA in each sample was measured using a Quant-iT™ RNA Assay Kit, 5–100 ng on a Qubit 3.0 (Invitrogen, US) fluorometer. The cDNA for RT-PCR was synthesized using an MMLV RT Kit (Evrogen, Russia) according to the manufacturer's recommendations employing oligo(dT)17 -primers from 2 μg total RNA after DNase treatment. The primers were designed using Primer-BLAST (Ye et al. 2012) (Supplementary Table). The minus reverse transcriptase control (-RT) contained RNA without reverse transcriptase treatment to confirm the absence of DNA in the samples. The RT-PCR products were resolved on an 1.5% agarose gel and visualized using ethidium bromide staining.

Generation of overexpression and knockout lines

For the overexpression experiments, the plant LIC vector (De Rybel et al. 2011) was used. PCR was carried out using genomic DNA isolated from *P. patens* as a template and PEP4f and PEP4r as primers (Supplemental Table S5). Amplicons were cloned into the pPLV27 vector (GenBank JF909480) using the Ligation-independent (LIC) procedure (Aslanidis and de Jong 1990). The resulting plasmid was named pPLV-Hpa-4FR. The nucleotide sequence of the cloned fragment was verified by sequencing using a BigDye Terminator Cycle Sequencing Kit (v. 3.1) and AbiPrism 3730xl (Applied Biosystems, USA). For moss transformation, pPLV-Hpa-4FR purified using a Qiagen Plasmid Maxi Kit (Qiagen, Germany) and linearized with SacI (pPLV-Hpa-4FR-SacI) was utilized.

Knockout lines were created using the CRISPR-Cas9 system (Collonnier et al. 2017). The plasmid containing the sgRNA expression cassette was generated with internal restriction enzyme sites that permit rapid, directional cloning of 20-mer guide sequences. This cassette consisted of the U6 promoter from *P. patens* and the tracrRNA scaffold with two BbsI sites between them (Supplemental Figure S10). The cassette was synthesized from oligonucleotides and cloned into the TA vector pTZ57R/T (Thermo Fischer Scientific, USA). The resulting plasmid was named pBB.

Coding sequences of *SEP1* were used to search for CRISPR RNA (crRNA) preceded by a PAM motif in *S. pyogenes* Cas9 (NGG) using the web tool CRISPR DESIGN (http://crispr.mit.edu/). The 3-1 crRNA closest to the translation start site (ATG) was selected for cloning.

The plasmid pBB with the guide RNA expression cassette was linearized by digestion with BbsI. Oligonucleotides were designed to contain compatible overhangs and a 20-mer guide sequence (targeting *SEP1*, Supplementary Table 1). The hybridized oligonucleotides (2G Top-2G Bottom) were ligated into the digested plasmid, yielding the final complete sgRNA expression cassette. The resulting plasmid was named pBB-3-1.

The plasmids pACT-CAS9 (for CAS9 expression) and pBNRF (resistance to G418) were kindly provided by Dr. Fabien Nogué. For moss transformation, the plasmids were purified using a Qiagen Plasmid Maxi Kit (Qiagen, Germany).

In the overexpression experiments, protoplasts were transformed with 20 µg pPLV-Hpa-4FR and circular DNA (7.5 µg each) from pBB-3-1, pACT-CAS9 and pBNRF and spread onto regeneration medium composed of BCDAT medium supplemented with 0.33 M mannitol, followed by 1 week of incubation before selection.

To select clones overexpressing of *SEP1*, the transformed protoplasts were planted on selective medium containing hygromycin, and knockout lines were selected on G418. Selection for hygromycin resistance was repeated twice, and after 1 week, the clones of survivors were placed in standard medium. The presence of the insert was determined by PCR with primer pairs p5-p4r, and HygF-HygR, and deletion was detected by sequencing the fragment with primers seqF and seqR (Supplemental Figure S8). Independent knockout and overexpression mutant lines showed similar phenotypes.

DATA ACCESS

All raw mass spectrometry data from this study have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al. 2016) partner repository with the dataset identifiers PXD005223, PXD007922, PXD007923, PXD007973.

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Authors’ contributions

IF and IK conceived and designed experiments. AK performed moss transformation experiments. IF, RK, VL, DK, EG, VZ, IB and AM performed the proteomics analyses. IF, IK and GA performed the statistical and bioinformatics analyses. IF, IK, VI and VG wrote the manuscript with input from all authors. IF supervised the project. All authors read and approved the final manuscript.

DISCLOSURE DECLARATION

The authors declare that they have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

REFERENCES

Andrews SJ, Rothnagel JA. 2014. Emerging evidence for functional peptides encoded by short open reading frames. *Nat Rev Genet* **15**(3): 193-204.

Aslanidis C, de Jong PJ. 1990. Ligation-independent cloning of PCR products (LIC-PCR). *Nucleic acids research* **18**(20): 6069-6074.

Aspden JL, Eyre-Walker YC, Phillips RJ, Amin U, Mumtaz MA, Brocard M, Couso JP. 2014. Extensive translation of small Open Reading Frames revealed by Poly-Ribo-Seq. *eLife* **3**: e03528.

Bao Z, Clancy MA, Carvalho RF, Elliott K, Folta KM. 2017. Identification of Novel Growth Regulators in Plant Populations Expressing Random Peptides. *Plant Physiol* **175**(2): 619-627.

Bazzini AA, Johnstone TG, Christiano R, Mackowiak SD, Obermayer B, Fleming ES, Vejnar CE, Lee MT, Rajewsky N, Walther TC et al. 2014. Identification of small ORFs in vertebrates using ribosome footprinting and evolutionary conservation. *The EMBO journal* **33**(9): 981-993.

Blanvillain R, Young B, Cai YM, Hecht V, Varoquaux F, Delorme V, Lancelin JM, Delseny M, Gallois P. 2011. The Arabidopsis peptide kiss of death is an inducer of programmed cell death. *The EMBO journal* **30**(6): 1173-1183.

Blumenthal T. 1998. Gene clusters and polycistronic transcription in eukaryotes. *BioEssays : news and reviews in molecular, cellular and developmental biology* **20**(6): 480-487.

Carvunis AR, Rolland T, Wapinski I, Calderwood MA, Yildirim MA, Simonis N, Charloteaux B, Hidalgo CA, Barbette J, Santhanam B et al. 2012. Proto-genes and de novo gene birth. *Nature* **487**(7407): 370-374.

Cherry JL. 2010. Expression level, evolutionary rate, and the cost of expression. *Genome biology and evolution* **2**: 757-769.

Chilley PM, Casson SA, Tarkowski P, Hawkins N, Wang KL, Hussey PJ, Beale M, Ecker JR, Sandberg GK, Lindsey K. 2006. The POLARIS peptide of Arabidopsis regulates auxin transport and root growth via effects on ethylene signaling. *Plant Cell* **18**(11): 3058-3072.

Cock PJ, Antao T, Chang JT, Chapman BA, Cox CJ, Dalke A, Friedberg I, Hamelryck T, Kauff F, Wilczynski B et al. 2009. Biopython: freely available Python tools for computational molecular biology and bioinformatics. *Bioinformatics* **25**(11): 1422-1423.

Collonnier C, Epert A, Mara K, Maclot F, Guyon-Debast A, Charlot F, White C, Schaefer DG, Nogue F. 2017. CRISPR-Cas9-mediated efficient directed mutagenesis and RAD51-dependent and RAD51-independent gene targeting in the moss Physcomitrella patens. *Plant Biotechnol J* **15**(1): 122-131.

Couso JP. 2015. Finding smORFs: getting closer. *Genome Biol* **16**.

Couso JP, Patraquim P. 2017. Classification and function of small open reading frames. *Nature reviews Molecular cell biology*.

Cove DJ, Perroud PF, Charron AJ, McDaniel SF, Khandelwal A, Quatrano RS. 2009. Isolation of DNA, RNA, and protein from the moss Physcomitrella patens gametophytes. *Cold Spring Harbor protocols* **2009**(2): pdb prot5146.

Crappe J, Van Criekinge W, Trooskens G, Hayakawa E, Luyten W, Baggerman G, Menschaert G. 2013. Combining in silico prediction and ribosome profiling in a genome-wide search for novel putatively coding sORFs. *Bmc Genomics* **14**.

D'Lima NG, Ma J, Winkler L, Chu Q, Loh KH, Corpuz EO, Budnik BA, Lykke-Andersen J, Saghatelian A, Slavoff SA. 2017. A human microprotein that interacts with the mRNA decapping complex. *Nat Chem Biol* **13**(2): 174-180.

De Coninck B, Carron D, Tavormina P, Willem L, Craik DJ, Vos C, Thevissen K, Mathys J, Cammue BP. 2013. Mining the genome of Arabidopsis thaliana as a basis for the identification of novel bioactive peptides involved in oxidative stress tolerance. *J Exp Bot* **64**(17): 5297-5307.

De Rybel B, van den Berg W, Lokerse A, Liao CY, van Mourik H, Moller B, Peris CL, Weijers D. 2011. A versatile set of ligation-independent cloning vectors for functional studies in plants. *Plant Physiol* **156**(3): 1292-1299.

Djordjevic MA, Mohd-Radzman NA, Imin N. 2015. Small-peptide signals that control root nodule number, development, and symbiosis. *J Exp Bot* **66**(17): 5171-5181.

Eguen T, Straub D, Graeff M, Wenkel S. 2015. MicroProteins: small size-big impact. *Trends Plant Sci* **20**(8): 477-482.

Enard D, Messer PW, Petrov DA. 2014. Genome-wide signals of positive selection in human evolution. *Genome Res* **24**(6): 885-895.

Fesenko I, Khazigaleeva R, Kirov I, Kniazev A, Glushenko O, Babalyan K, Arapidi G, Shashkova T, Butenko I, Zgoda V et al. 2017. Alternative splicing shapes transcriptome but not proteome diversity in Physcomitrella patens. *Scientific reports* **7**(1): 2698.

Fesenko I, Seredina A, Arapidi G, Ptushenko V, Urban A, Butenko I, Kovalchuk S, Babalyan K, Knyazev A, Khazigaleeva R et al. 2016. The Physcomitrella patens Chloroplast Proteome Changes in Response to Protoplastation. *Front Plant Sci* **7**: 1661.

Fesenko IA, Arapidi GP, Skripnikov AY, Alexeev DG, Kostryukova ES, Manolov AI, Altukhov IA, Khazigaleeva RA, Seredina AV, Kovalchuk SI et al. 2015. Specific pools of endogenous peptides are present in gametophore, protonema, and protoplast cells of the moss Physcomitrella patens. *Bmc Plant Biol* **15**: 87.

Ge Y, Porse BT. 2014. The functional consequences of intron retention: alternative splicing coupled to NMD as a regulator of gene expression. *BioEssays : news and reviews in molecular, cellular and developmental biology* **36**(3): 236-243.

Giannakakis A, Zhang J, Jenjaroenpun P, Nama S, Zainolabidin N, Aau MY, Yarmishyn AA, Vaz C, Ivshina AV, Grinchuk OV et al. 2015. Contrasting expression patterns of coding and noncoding parts of the human genome upon oxidative stress. *Scientific reports* **5**: 9737.

Graeff M, Straub D, Eguen T, Dolde U, Rodrigues V, Brandt R, Wenkel S. 2016. MicroProtein-Mediated Recruitment of CONSTANS into a TOPLESS Trimeric Complex Represses Flowering in Arabidopsis. *PLoS genetics* **12**(3): e1005959.

Guillen G, Diaz-Camino C, Loyola-Torres CA, Aparicio-Fabre R, Hernandez-Lopez A, Diaz-Sanchez M, Sanchez F. 2013. Detailed analysis of putative genes encoding small proteins in legume genomes. *Front Plant Sci* **4**.

Guttman M, Russell P, Ingolia NT, Weissman JS, Lander ES. 2013. Ribosome Profiling Provides Evidence that Large Noncoding RNAs Do Not Encode Proteins. *Cell* **154**(1): 240-251.

Hanada K, Akiyama K, Sakurai T, Toyoda T, Shinozaki K, Shiu SH. 2010. sORF finder: a program package to identify small open reading frames with high coding potential. *Bioinformatics* **26**(3): 399-400.

Hanada K, Higuchi-Takeuchi M, Okamoto M, Yoshizumi T, Shimizu M, Nakaminami K, Nishi R, Ohashi C, Iida K, Tanaka M et al. 2013. Small open reading frames associated with morphogenesis are hidden in plant genomes. *P Natl Acad Sci USA* **110**(6): 2395-2400.

Hanada K, Zhang X, Borevitz JO, Li WH, Shiu SH. 2007. A large number of novel coding small open reading frames in the intergenic regions of the Arabidopsis thaliana genome are transcribed and/or under purifying selection. *Genome Res* **17**(5): 632-640.

Hazarika RR, De Coninck B, Yamamoto LR, Martin LR, Cammue BP, van Noort V. 2017. ARA-PEPs: a repository of putative sORF-encoded peptides in Arabidopsis thaliana. *Bmc Bioinformatics* **18**(1): 37.

Hellens RP, Brown CM, Chisnal MAW, Waterhouse PM, Macknight RC. 2016. The Emerging World of Small ORFs. *Trends Plant Sci* **21**(4): 317-328.

Hirsh AE, Fraser HB. 2001. Protein dispensability and rate of evolution. *Nature* **411**(6841): 1046-1049.

Housman G, Ulitsky I. 2016. Methods for distinguishing between protein-coding and long noncoding RNAs and the elusive biological purpose of translation of long noncoding RNAs. *Biochimica et biophysica acta* **1859**(1): 31-40.

Huang JZ, Chen M, Chen, Gao XC, Zhu S, Huang H, Hu M, Zhu H, Yan GR. 2017. A Peptide Encoded by a Putative lncRNA HOXB-AS3 Suppresses Colon Cancer Growth. *Mol Cell* **68**(1): 171-184 e176.

Iacono M, Mignone F, Pesole G. 2005. uAUG and uORFs in human and rodent 5'untranslated mRNAs. *Gene* **349**: 97-105.

Ingolia NT, Lareau LF, Weissman JS. 2011. Ribosome Profiling of Mouse Embryonic Stem Cells Reveals the Complexity and Dynamics of Mammalian Proteomes. *Cell* **147**(4): 789-802.

Johnstone TG, Bazzini AA, Giraldez AJ. 2016. Upstream ORFs are prevalent translational repressors in vertebrates. *The EMBO journal* **35**(7): 706-723.

Julenius K, Pedersen AG. 2006. Protein evolution is faster outside the cell. *Molecular biology and evolution* **23**(11): 2039-2048.

Karousis ED, Nasif S, Muhlemann O. 2016. Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. *Wiley interdisciplinary reviews RNA* **7**(5): 661-682.

Kastenmayer JP, Ni L, Chu A, Kitchen LE, Au WC, Yang H, Carter CD, Wheeler D, Davis RW, Boeke JD et al. 2006. Functional genomics of genes with small open reading frames (sORFs) in S-cerevisiae. *Genome Res* **16**(3): 365-373.

Kelley LA, Sternberg MJ. 2015. Partial protein domains: evolutionary insights and bioinformatics challenges. *Genome Biol* **16**: 100.

Kim TS, Liu CL, Yassour M, Holik J, Friedman N, Buratowski S, Rando OJ. 2010. RNA polymerase mapping during stress responses reveals widespread nonproductive transcription in yeast. *Genome Biol* **11**(7): R75.

Kondo T, Plaza S, Zanet J, Benrabah E, Valenti P, Hashimoto Y, Kobayashi S, Payre F, Kageyama Y. 2010. Small Peptides Switch the Transcriptional Activity of Shavenbaby During Drosophila Embryogenesis. *Science* **329**(5989): 336-339.

Ladoukakis E, Pereira V, Magny EG, Eyre-Walker A, Couso JP. 2011. Hundreds of putatively functional small open reading frames in Drosophila. *Genome Biol* **12**(11).

Laing WA, Martinez-Sanchez M, Wright MA, Bulley SM, Brewster D, Dare AP, Rassam M, Wang D, Storey R, Macknight RC et al. 2015. An Upstream Open Reading Frame Is Essential for Feedback Regulation of Ascorbate Biosynthesis in Arabidopsis. *Plant Cell* **27**(3): 772-786.

Lauressergues D, Couzigou JM, Clemente HS, Martinez Y, Dunand C, Becard G, Combier JP. 2015. Primary transcripts of microRNAs encode regulatory peptides. *Nature* **520**(7545): 90-93.

Lease KA, Walker JC. 2006. The Arabidopsis unannotated secreted peptide database, a resource for plant peptidomics. *Plant Physiol* **142**(3): 831-838.

Loose CR, Langer RS, Stephanopoulos GN. 2007. Optimization of protein fusion partner length for maximizing in vitro translation of peptides. *Biotechnol Prog* **23**(2): 444-451.

Lv Q, Lan Y, Shi Y, Wang H, Pan X, Li P, Shi T. 2017. AtPID: a genome-scale resource for genotype-phenotype associations in Arabidopsis. *Nucleic acids research* **45**(D1): D1060-D1063.

Ma J, Diedrich JK, Jungreis I, Donaldson C, Vaughan J, Kellis M, Yates JR, 3rd, Saghatelian A. 2016. Improved Identification and Analysis of Small Open Reading Frame Encoded Polypeptides. *Analytical chemistry* **88**(7): 3967-3975.

Mackowiak SD, Zauber H, Bielow C, Thiel D, Kutz K, Calviello L, Mastrobuoni G, Rajewsky N, Kempa S, Selbach M et al. 2015. Extensive identification and analysis of conserved small ORFs in animals. *Genome Biol* **16**.

Magny EG, Pueyo JI, Pearl FM, Cespedes MA, Niven JE, Bishop SA, Couso JP. 2013. Conserved regulation of cardiac calcium uptake by peptides encoded in small open reading frames. *Science* **341**(6150): 1116-1120.

Matsumoto A, Pasut A, Matsumoto M, Yamashita R, Fung J, Monteleone E, Saghatelian A, Nakayama KI, Clohessy JG, Pandolfi PP. 2017. mTORC1 and muscle regeneration are regulated by the LINC00961-encoded SPAR polypeptide. *Nature* **541**(7636): 228-232.

Mazin PV, Fisunov GY, Gorbachev AY, Kapitskaya KY, Altukhov IA, Semashko TA, Alexeev DG, Govorun VM. 2014. Transcriptome analysis reveals novel regulatory mechanisms in a genome-reduced bacterium. *Nucleic acids research* **42**(21): 13254-13268.

McLysaght A, Guerzoni D. 2015. New genes from non-coding sequence: the role of de novo protein-coding genes in eukaryotic evolutionary innovation. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* **370**(1678): 20140332.

Montoya-Burgos JI. 2011. Patterns of positive selection and neutral evolution in the protein-coding genes of Tetraodon and Takifugu. *Plos One* **6**(9): e24800.

Moyers BA, Zhang J. 2016. Evaluating Phylostratigraphic Evidence for Widespread De Novo Gene Birth in Genome Evolution. *Molecular biology and evolution* **33**(5): 1245-1256.

Narita NN, Moore S, Horiguchi G, Kubo M, Demura T, Fukuda H, Goodrich J, Tsukaya H. 2004. Overexpression of a novel small peptide ROTUNDIFOLIA4 decreases cell proliferation and alters leaf shape in Arabidopsis thaliana. *Plant J* **38**(4): 699-713.

Neafsey DE, Galagan JE. 2007. Dual modes of natural selection on upstream open reading frames. *Molecular biology and evolution* **24**(8): 1744-1751.

Nelson BR, Makarewich CA, Anderson DM, Winders BR, Troupes CD, Wu F, Reese AL, McAnally JR, Chen X, Kavalali ET et al. 2016. A peptide encoded by a transcript annotated as long noncoding RNA enhances SERCA activity in muscle. *Science* **351**(6270): 271-275.

Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M. 2000. Tagged mutagenesis and gene-trap in the moss, Physcomitrella patens by shuttle mutagenesis. *DNA research : an international journal for rapid publication of reports on genes and genomes* **7**(1): 9-17.

Paytuvi Gallart A, Hermoso Pulido A, Anzar Martinez de Lagran I, Sanseverino W, Aiese Cigliano R. 2016. GREENC: a Wiki-based database of plant lncRNAs. *Nucleic acids research* **44**(D1): D1161-1166.

Pi H, Lee LW, Lo SJ. 2009. New insights into polycistronic transcripts in eukaryotes. *Chang Gung medical journal* **32**(5): 494-498.

Prabakaran S, Hemberg M, Chauhan R, Winter D, Tweedie-Cullen RY, Dittrich C, Hong E, Gunawardena J, Steen H, Kreiman G et al. 2014. Quantitative profiling of peptides from RNAs classified as noncoding. *Nature communications* **5**: 5429.

Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* **26**(6): 841-842.

Rasheed S, Bashir K, Nakaminami K, Hanada K, Matsui A, Seki M. 2016. Drought stress differentially regulates the expression of small open reading frames (sORFs) in Arabidopsis roots and shoots. *Plant signaling & behavior* **11**(8): e1215792.

Rensing SA, Ick J, Fawcett JA, Lang D, Zimmer A, Van de Peer Y, Reski R. 2007. An ancient genome duplication contributed to the abundance of metabolic genes in the moss Physcomitrella patens. *BMC evolutionary biology* **7**: 130.

Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud PF, Lindquist EA, Kamisugi Y et al. 2008. The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. *Science* **319**(5859): 64-69.

Rohrig H, Schmidt J, Miklashevichs E, Schell J, John M. 2002. Soybean ENOD40 encodes two peptides that bind to sucrose synthase. *Proc Natl Acad Sci U S A* **99**(4): 1915-1920.

Saric T, Graef CI, Goldberg AL. 2004. Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases. *The Journal of biological chemistry* **279**(45): 46723-46732.

Seo PJ, Hong SY, Kim SG, Park CM. 2011. Competitive inhibition of transcription factors by small interfering peptides. *Trends Plant Sci* **16**(10): 541-549.

Seo PJ, Park MJ, Park CM. 2013. Alternative splicing of transcription factors in plant responses to low temperature stress: mechanisms and functions. *Planta* **237**(6): 1415-1424.

Slavoff SA, Mitchell AJ, Schwaid AG, Cabili MN, Ma J, Levin JZ, Karger AD, Budnik BA, Rinn JL, Saghatelian A. 2013. Peptidomic discovery of short open reading frame-encoded peptides in human cells. *Nat Chem Biol* **9**(1): 59-+.

Staudt AC, Wenkel S. 2011. Regulation of protein function by 'microProteins'. *EMBO reports* **12**(1): 35-42.

Straub D, Wenkel S. 2017. Cross-Species Genome-Wide Identification of Evolutionary Conserved MicroProteins. *Genome biology and evolution* **9**(3): 777-789.

Supek F, Bosnjak M, Skunca N, Smuc T. 2011. REVIGO summarizes and visualizes long lists of gene ontology terms. *Plos One* **6**(7): e21800.

Suyama M, Torrents D, Bork P. 2006. PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic acids research* **34**(Web Server issue): W609-612.

Szczesniak MW, Rosikiewicz W, Makalowska I. 2016. CANTATAdb: A Collection of Plant Long Non-Coding RNAs. *Plant Cell Physiol* **57**(1): e8.

Tautz D. 2009. Polycistronic peptide coding genes in eukaryotes--how widespread are they? *Briefings in functional genomics & proteomics* **8**(1): 68-74.

Tavormina P, De Coninck B, Nikonorova N, De Smet I, Cammue BP. 2015. The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions. *Plant Cell* **27**(8): 2095-2118.

Tyanova S, Temu T, Cox J. 2016. The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc* **11**(12): 2301-2319.

Vanderperre B, Lucier JF, Bissonnette C, Motard J, Tremblay G, Vanderperre S, Wisztorski M, Salzet M, Boisvert FM, Roucou X. 2013. Direct Detection of Alternative Open Reading Frames Translation Products in Human Significantly Expands the Proteome. *Plos One* **8**(8).

Verheggen K, Volders PJ, Mestdagh P, Menschaert G, Van Damme P, Gevaert K, Martens L, Vandesompele J. 2017. Noncoding after All: Biases in Proteomics Data Do Not Explain Observed Absence of lncRNA Translation Products. *J Proteome Res* **16**(7): 2508-2515.

Vizcaino JA, Csordas A, Del-Toro N, Dianes JA, Griss J, Lavidas I, Mayer G, Perez-Riverol Y, Reisinger F, Ternent T et al. 2016. 2016 update of the PRIDE database and its related tools. *Nucleic acids research* **44**(22): 11033.

Weatheritt RJ, Sterne-Weiler T, Blencowe BJ. 2016. The ribosome-engaged landscape of alternative splicing. *Nature structural & molecular biology* **23**(12): 1117-1123.

Yang Z. 2007. PAML 4: phylogenetic analysis by maximum likelihood. *Molecular biology and evolution* **24**(8): 1586-1591.

Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *Bmc Bioinformatics* **13**: 134.

Zhang L, Li WH. 2004. Mammalian housekeeping genes evolve more slowly than tissue-specific genes. *Molecular biology and evolution* **21**(2): 236-239.