Possible role of small secreted peptides (SSPs) in immune signaling in bryophytes

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Abstract

Plants utilize a plethora of peptide signals to regulate their immune response. Peptide ligands and their cognate receptors involved in immune signaling share common motifs among many species of vascular plants. However, the origin and evolution of immune peptides is still poorly understood. Here, we searched for genes encoding small secreted peptides in the genomes of three bryophyte lineages—mosses, liverworts and hornworts—that occupy a critical position in the study of land plant evolution. We found that bryophytes shared common predicted small secreted peptides (SSPs) with vascular plants. The number of SSPs is higher in the genomes of mosses than in both the liverwort *Marchantia polymorpha* and the hornwort *Anthoceros sp*. The synthetic peptide elicitors—AtPEP and StPEP—specific for vascular plants, triggered ROS production in the protonema of the moss *Physcomitrella patens*, suggesting the possibility of recognizing peptide ligands from angiosperms by moss receptors. Mass spectrometry analysis of the moss *Physcomitrella patens*, both the wild type and the Δ*cerk* mutant secretomes, revealed peptides that specifically responded to chitosan treatment, suggesting their role in immune signaling.

Keywords Plant immunity · Small secreted peptides · *Physcomitrella patens* · Peptide signaling

Introduction

Plants cope with constantly changing environmental conditions and numerous threats, such as phytopathogens, throughout their life. Because of their ability to sense different molecules, pattern-recognition receptors (PRRs) are considered to be a part of the innate immune system (Couto and Zipfel 2016; Boutrot and Zipfel 2017). PRRs consist of receptor-like kinases (RLKs) with an intracellular kinase domain and receptor-like proteins (RLPs) that comprise only extracellular and transmembrane domains. To date, different ectodomains binding the ligands have been described,

such as leucine-rich repeats (LRRs), lysine motifs, lectin-like motifs as well as epidermal growth factor (EGF)-like domains (Couto and Zipfel 2016; Boutrot and Zipfel 2017). LRR-RLKs are the most abundant RLK family. Over two hundred of LRR-RLKs that regulate various processes from growth and development to immune response were found in *Arabidopsis thaliana* (Smakowska-Luzan et al. 2018). It was shown that the canonical RLK architecture consisting of kinase domain, transmembrane domain and extracellular domain was present after the divergence of land plants (Han 2019). It was proposed that RLK receptors participating in plant-microorganism symbiotic interactions have been already present in the most recent common ancestor of extant land plants and green algae (Delaux et al. 2015).

Genomes of bryophytes also contain LRR-RLK genes; however, their average number is lower compared with that of angiosperms (Ponce de León and Montesano 2013; Bressendorff et al. 2016). As such, 134 and 81 LRR-RLKs genes have been identified in moss and spikemoss, respectively (Dufayard et al. 2017). In particular, *Physcomitrella patens* encodes LysM-RLKs CERK1 homolog involved in the perception of fungal chitin (Bressendorff et al. 2016; Galotto et al. 2020). It is assumed that CERK1 was probably

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involved in plant immune signaling in the last common ancestor of all land plants (Fürst-Jansen et al. 2020). However, FLS and EFR receptors, responsible for the detection of proteaceous ligands, such as bacterial flagellin and elongation factor Tu, have not been found in *P.patens* (Bressendorff et al. 2016).

Another class of receptors that are able to detect pathogens inside plant cells are named nucleotide-binding and leucine-rich repeat domain (NBS-LRR) proteins. They are fairly studied in angiosperms, but for bryophytes the number of corresponding studies is much lower (Han 2019; Gao et al. 2018). The NBS-LRR proteins have been found in both some algae and bryophytes, suggesting the probable presence of such immune system components already in the earliest land plants (Gao et al. 2018; de Vries et al. 2018).

Pathogen-associated molecular patterns (PAMPs) that are sensed by plant PRRs trigger defense response cascades and the release of the plant's own damage-associated immune signals (DAMPs). The known PAMPs include peptides flg22 from flagellin (Felix et al. 1999), elf18 from EF-Tu (Kunze et al. 2004), specific for rice RaxX (Luu et al. 2019), percepted by the first predicted pattern recognition receptor XA21 (Song et al. 1995), as well as liposaccharides, lipoproteins, nucleic acids and chitin, percepted by a LysM RLK chitin-elicitor receptor kinase 1 (CERK1) (Miya et al. 2007; Wan et al. 2008; Bigeard et al. 2015).

To date, several dozens of DAMP peptides that are involved in the immune signaling have been identified. For example, the CAP-derived Peptides (CAPE) are derived from functional precursor protein PR-1b and are involved in pathogen defense pathways (Chen et al. 2020). One of the most well-studied and diverse groups of DAMP peptides is the plant elicitor peptides (PEP) family. PEPs have been identified in various families of angiosperms and are generated from inactive protein precursors. For example, in *Arabidopsis thaliana*, a 23-aa AtPEP1 is released from a 92-aa inactive protein precursor and is recognized by PEPR1 and PEPR2, which trigger immune signaling cascades (Huffaker et al. 2006; Yamaguchi et al. 2010). An increase in reactive oxygen species (ROS) levels in response to PEP treatment has been shown in several studies on *Arabidopsis* (Huffaker et al. 2006; Ma et al. 2013). There are 8 PEPs identified in *Arabidopsis thaliana*, and PEPs within plant families are highly conserved. Although a comparison of PEPs from dicots and monocots (AtPEP1 and ZmPEP1, respectively) revealed a sequence identity of 20.8 %, which is the percentage of the PEPs sequence conservation among different plant families (Lori et al. 2015). PEPs have not been identified in bryophytes and whether this DAMP signaling pathway is conserved among different plant lineage is unknown (Lori et al. 2015).

Antimicrobial peptides provide the first barrier against pathogenic bacteria and fungi, but a number of regulatory peptides are also involved in the immune responses in plants. Antimicrobial and signaling immune peptides in both plants and animals activate cascades of biochemical reactions in cells and induce expression of the corresponding defense genes (Czyzewicz et al. 2013; Oh et al. 2018). Such peptides have been found in many plant species, their precursor sequences contain conserved regions and are predicted from a number of genomes (Lease and Walker 2006; Butenko et al. 2009). These peptides belong to a group of small secreted peptides (SSPs) that usually represent sequences of preproteins of approximately 100 to 250 amino acids. SSPs are usually processed into bioactive peptides consisting of up to 50 amino acids (Lease and Walker 2006; Breiden and Simon 2016; Bang et al. 2017) and often work at nanomolar physiological concentrations (Murphy et al. 2012). Based on their origin and structure, SSPs are classified as Cys-rich, non-Cys-rich, post-translationally modified (PTM), non-PTM, functional precursor and encoded by short open reading frames (sORFs). The characterization and annotation of the SSPs in plant genomes is still ongoing. Several approaches have been developed to identify SSPs gene candidates in plant genomes in recent years (Ohyama et al. 2008; Pan et al. 2013; Zhou et al. 2013; Li et al. 2014; Ghorbani et al. 2015; Bang et al. 2017). Because there are similarities between the structure and sequences of functional peptides, the pipelines based on hidden Markov models (HMMs) of known families have been used to scan genome assemblies for SSP genes (Pan et al. 2013; Zhou et al. 2013). Recent evidence suggests that of all the tools used for prediction of potential SSP SPADA is currently the most efficient computational pipeline, which combines multiple approaches to identify new small peptides in plants (Zhou et al. 2013, 2020; Bang et al. 2017). Databases of known SSPs for various plant species are being created; the most recent one is MtSSPdb (https://mtsspdb.noble.org/) (Bang et al. 2017; Boschiero et al. 2020). Another database, PlantSSP, contains more than 39,000 small proteins derived from 32 plant species (Ghorbani et al. 2015). Machine learning has also been shown to be an efficient method for predicting genes of signaling peptides with conserved domains such as Clavata/Embryo Surrounding Region (CLE) (Zhang et al. 2020b). Recent research also shows that analysis of both the transcriptome and proteome followed by bioinformatic screening improves the identification accuracy (Wang et al. 2020) on per with a direct peptidomic approach because mature signaling peptides are often post-modified, and in silico analysis alone is not enough (Luo et al. 2019). The role of predicted new SSPs in plant defense responses has also been verified using a microarray assay (Zhou et al. 2020).

The knowledge gap surrounding immune system components of bryophytes such as *P.patens* or *Marchantia polymorpha* began to fill in recent studies (Bressendorff et al. 2017; Carella et al. 2019; Peñuelas et al. 2019; Galotto et al.

2020). Recently, it was reported that *M.polymorpha* has the components of the whole jasmonic acid (JA)-signaling cascade (Peñuelas et al. 2019; Monte et al. 2019). It was also reported that *A.thaliana* and *M.polymorpha* both have a functionally conservative jasmonoyl-isoleucine (JA-Ile) receptor COI1, although they recognize different ligands (Monte et al. 2018). In addition, a comparison between a liverwort *M.polymorpha* and an angiosperm *Nicotiana benthamiana* has shown a number of shared defense genes activating a phenylpropanoid metabolic pathway upon oomycetes infection (Carella et al. 2019). The key participants in this pathway have also been found in streptophyte algae (de Vries et al. 2017). There is also a clear antagonism of JA and salicylic acid (SA) pathways in *M.polymorpha* (Matsui et al. 2019). These findings indicate the functional conservation of key components of plant immunity across a wide range of plant lineages. However, the evolution of peptide immune signals as well as their signaling cascades components is still poorly studied. It is unclear if there are conserved immune peptide signaling pathways among non-vascular and vascular plants. The *Physcomitrella patens* secretome comprises hundreds of endogenous peptides and stress hormones treatment results in changes in peptide pools (Fesenko et al. 2019b; Filippova et al. 2019). It is possible that some of these peptides may be involved in immune signaling and are recognized by LRR-RLK receptors. Through many experiments, it was shown that the moss *P.patens* respond to attack from a wide range of phytopathogens such as oomycetes, bacteria and fungi (Overdijk et al. 2016; Ponce de León et al. 2007, 2012; Oliver et al. 2009; Ponce de León and Montesano 2013; Reboledo et al. 2015; Bressendorff et al. 2016, 2017). Bacterial pathogens trigger cell death, cytoplasmic shrinkage, chloroplast browning, induction of defense genes such as PR-1, PAL, CHS and to a lesser extent LOX in moss *P.patens* gametophores, similar to vascular plants (Ponce de León et al. 2007; Ponce de León and Montesano 2013). Cell wall reinforcement is accompanied by an accumulation of different ROS types, fungal infection, in particular, induces production of H_2O_2 and hydroxyl radicals in moss cells, as well as promotes defense genes expression (Ponce de León and Montesano 2013; Lehtonen et al. 2009, 2012). In angiosperms ROS accumulation is associated with stress signaling. Respiratory Burst Oxidase Proteins (RBOHs) catalyze the production of superoxide anion free radical (O_2^-) from oxygen and considered the main source of ROS in the apoplast under biotic stress. Then, superoxide dismutase catalyzes the dismutation of O_2^- radical into the most long-lived ROS - hydrogen peroxide (H_2O_2) (Qi et al. 2017; Shapiguzov et al. 2012). Apoplastic peroxidases can also lead to the production of H_2O_2 under biotic stress. It was shown that H_2O_2 from the apoplast might enter the cell through aquaporins (Qi et al. 2017; Shapiguzov et al. 2012). The cellular organelles - chloroplasts, mitochondria, and peroxisomes

can also produce ROS that regulate the immune response (Vellosillo et al. 2010; Shapiguzov et al. 2012).

Aside from fundamental studies of immune response mechanisms, a vast majority of research is focused on practical application of that knowledge for growing sustainable crops. Targets of such studies are usually pattern-recognition receptors; gene transfer of pattern-recognition receptors from donor plants, including model plants such as *Arabidopsis*, to recipients that lack such receptors leads to resistance of plants to pathogens to which they were initially susceptible (Lacombe et al. 2010; Schoonbeek et al. 2015; Schwessinger et al. 2015; Hao et al. 2016; Saur et al. 2016). In addition, genes of existing receptor-like kinases that recognize pathogen effectors within plant cells have been modified at the binding site to improve ligand recognition (De la Concepcion et al. 2019). Thus, these studies reveal a common principle of the immune response among a wide range of phylogenetically distant plant species. The gradual complication of the pathways and the addition of new specific components of the immune system in the process of evolution leads to differences in the ways different plants resist the pathogen attacks (Han 2019). However, the aforementioned examples indicate that the study of the conservative components of plant's immune system on non-vascular plant models is of significant importance for understanding and regulating defense responses in vascular plants.

The genomes of the bryophyte model organisms are fully annotated, as three high-quality genomes of *Anthoceros* hornworts have also been recently analyzed, thus broadening the availability of suitable model systems in the bryophyte lineage (Zhang et al. 2020a). In this study, we searched for possible homologs of receptor-like kinases and plant SSPs in the genomes of several bryophytes using bioinformatic tools such as SPADA (https://github.com/ZhaoBioinformaticsLab/PlantSSPProtocols) and HMMER (http://hmmer.org). We also conducted a comparative analysis of secreted endogenous peptide pools from a wild type model plant moss *Physcomitrella patens* and mutants with knocked-out *cerk* gene upon chitosan treatment. The data obtained were compared with our previous data from experiments with salicylic acid and methyl jasmonate (Fesenko et al. 2019b; Filippova et al. 2019). We also found the increase of ROS release upon the treatment of wild-type moss and Δ*cerk* mutants with chitosan, as well as the well-known plant peptide elicitors and our signal peptide candidates.

Materials and methods

Plant cultivation and elicitor treatment

Protonemata of the moss *Physcomitrella patens* subsp. patens Gransden 2004 (Freiburg, Germany) wild type and

mutant lines were grown in 200 mL liquid Knop medium containing 500 mg/L ammonium tartrate under white light with a photon flux of 61 μmol/m^2 s under a 16-hour photoperiod at 24°C. Moss Δ*cerk* mutant lines were provided by Dr Bressendorff (Bressendorff et al. 2016). For mass spectrometry analysis, 5-day-old protonemata were treated with 0.1 mg/mL chitosan (Mw = 50,000–190,000 Da, 75–85 % deacetylated; Sigma-Aldrich, USA) and incubated for 3 h. Chitosan was dissolved in water with 0.001 % glacial acetic acid and pre-filtered through a 0.50 μm membrane filter (Millipore). To analyze ROS accumulation, 5-day old protonemata were treated with 1 mg/mL chitosan for 1 min or synthetic 5 μM peptides for 15 min. For qRT-PCR analysis, moss protonemata were treated by 5 μM synthetic peptides for 2 and 4 h. All experiments were performed in three independent biological replicates.

Peptide pools extraction

Secreted peptides were extracted from 400 mL protonemata culture medium (Knop medium containing 500 mg/L ammonium tartrate). The culture medium was filtered through a 0.22 μm membrane filter (Millipore), lyophilized and resuspended in 700 μL 5 % acetonitrile (ACN) aqueous solution containing 0.1 % trifluoroacetic acid (TFA), followed by centrifugation at 10,000×*g* for 10 min, then the supernatant was transferred into clean tubes, cooled and centrifuged at 15,000×*g* for 30 minutes to remove any residual chitosan, and the pellet was discarded. For mass-spectrometry analysis, peptides were isolated from the culture medium by solid-phase extraction on reverse-phase DSC-18 cartridges (Discovery DSC-18, Supelco, USA) using a 50 % ACN/0.1 % TFA aqueous solution as eluent. The eluted peptides were concentrated in a SpeedVac and resuspended in 5 % ACN/0.1 % TFA. The evaporated precipitate was dissolved in aqueous solution containing chloracetomide (CAA), tris(2-carboxyethyl)phosphine (TCEP) and 8.5 pH Tris-HCl, heated to 90°C for 10 min and then cooled to restore S-S bridges in amino acids. The pool of peptides was isolated by solid-phase extraction on microtips with three SDB-RPS membranes using a 50 % ACN/0.1 % TFA aqueous solution as the eluent. Eluted peptides were concentrated in a Speed-Vac and resuspended in 5 % ACN/0.1 % TFA, followed by mass spectrometry analysis of the prepared samples.

ROS detection and measurement

To detect intracellular ROS, we used the fluorescent dye 2′,7′-Dichlorofluorescin Diacetate (DCFH-DA, Sigma-Aldrich, USA). Protonema was treated with 5 μM synthetic peptides (Table 1) and incubated with 10 μM DCFH-DA for 15 min. 6 pmol retention time (RT) peptides mix (Biognosys, Switzerland) was used as negative control. 1 mg/

Table 1 List of synthesized peptides

Name	Sequence
AtPEP1	ATKVKAKQRGKEKVSSGR-PGQHN
StPEP1	ATERRGRPPSRPKVGSGPPPQNN
pep3 (Pp3c17_15750V3.1.p)	LILPGELAK
pep4 (Pp3c13_3880V3.1.p)	VAEDVKSEDGGAQESTGADAAKI
pep7 (Pp3c21_4350V3.1.p)	EAAPAPVAEVEAPKAEE
pep8 (Pp3c14_22870V3.1.p)	INIINAPLQGFKIA

mL chitosan was added up to 1 mL of total volume directly before the detection on the fluorescent microscope Axio Imager M2 (Zeiss) with an AxioCam 506 mono digital camera and filter units. The No. 44 filter (λex BP 475 nm/40 nm; λem BP 530 nm/50 nm) was used for DCFH-DA fluorescence detection. The exposure was set to 300 msec, FITC and Brightlight channels were used. Data on the fluorescence intensity were obtained from the related Zeiss software Zen.

To detect the accumulation of extracellular ROS a deesterified DCFH was obtained from DCFH-DA via hydrolysis in NaOH (10 mM) as described (Smirnova et al. 2009). 1 mL liquid medium containing protonemata filaments, 10 μM dye and elicitors were centrifuged at 15,000×*g* for 1 min, the precipitate then discarded. The level of extracellular ROS was detected in supernatant using a multifunction reader Varioskan Flash (Thermo Scientific, USA). The fluorescence intensity was measured at λ 485/535 nm (excitation/emission wavelength) at 25°C. The ROS accumulation intensity was finally reported as relative fluorescence units (RFU).

Luminol (Sigma-Aldrich, USA) was used to detect the accumulation of extracellular ROS. The measurements were made in 500 μL protonemata with 20 μL luminol solution (1-2 M stock solution), 5 μL horseradish peroxidase solution (10 mg/mL stock solution) and elicitors. The measurements were carried out on a Lum1200 chemiluminometer (MSU, Russia) in a single-channel mode with an interval of 1 s at room temperature for 30 min. The result was estimated as the area under the curve after adding a mixture of peroxidase with luminol.

At the end of the measurements, H$_2$O$_2$ (5 μL from a 9 mM solution) was added and the chemiluminescence (CL) outbreak was recorded in the same mode as the maximum CL value. All measurements were made in three replicates. All control samples were treated with water.

LC-MS/MS analysis and peptide identification

Mass spectrometry analysis of endogenous peptides was performed in three biological repeats. LC-MS analysis was carried out on an Ultimate 3000 RSLCnano HPLC system connected to a Q Exactive Plus mass spectrometer

(ThermoFisher Scientific). Samples were loaded to a home-made trap column 20×0.1 mm, packed with Inertsil ODS3 3 µm sorbent (GLSciences), in the loading buffer (2 % ACN, 98 % H_2O, 0.1 % TFA) at 10 µL/min flow and separated at RT in a home-packed fused-silica column 500×0.1 mm packed with Reprosil PUR C18AQ 1.9 (Dr. Maisch) into the emitter prepared with P2000 Laser Puller (Sutter, USA) (Kovalchuk et al. 2019). Samples were eluted with a linear gradient of 80 % ACN, 19.9 % H_2O, 0.1 % formic acid (FA) (buffer B) in 99.9 % H_2O, 0.1 % FA (solvent A) from 4 to 36 % of solvent B in 1 h at 0.44 µL/min flow at RT.

MS data was collected in DDA mode. MS1 parameters were as follows: 140K resolution, 350–2000 scan range, max injection time 50 ms, AGC target 3×106. Ions were isolated with 1.4 m/z window and 0.2 m/z offset targeting the 10 highest intensity peaks of +1 to +6 charge, 8×103 minimum AGC, preferred peptide match and isotope exclusion. Dynamic exclusion was set to 40 s. MS2 fragmentation was carried out in HCD mode at 17.5K resolution with 27 % NCE. Ions were accumulated for max 50 ms with target AGC 1×105.

Mass-spectrometry data were searched with PEAKS Studio (version 8.0, Bioinfor Inc., CA, USA) against a database containing the protein sequences from Phytozome v12.0 merged with chloroplast and mitochondrial proteins (33,053 entries) and sequences predicted by SPADA. The search was done with the following parameters: precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.05 Da; the "digest mode" was set as "unspecific"; fixed modifications - Carbamidomethylation (+57.02); variable modifications - Oxidation (M) - +15.99 and Acetylation (N-term) - +42.01. The instrument setting was set to „orbi-orbi" (orbitrap was used for both precursor and fragment ion m/z detection). A false discovery rate of 1 % was used.
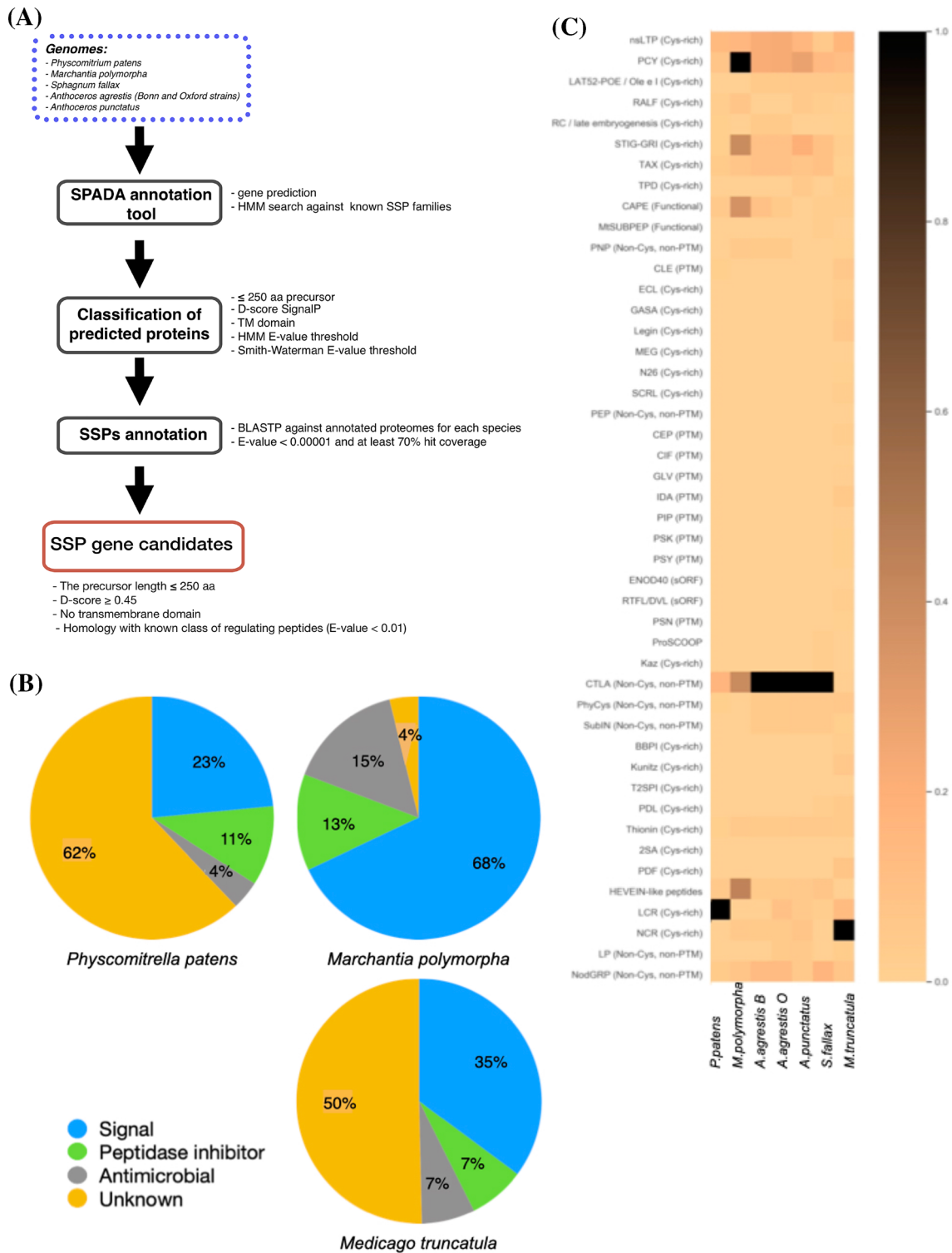
Bioinformatic analysis

SSP genes prediction

SPADA bioinformatics approach (Small Peptide Alignment Discovery Application) was applied for the search of peptide signals in the genomes of *P.patens* (Lang et al. 2018), *S.fallax* (Shaw et al. 2016), *M.polymorpha* (Bowman et al. 2017), *Anthoceros agrestis* (Bonn and Oxford strains) and *A.punctatus* (Zhang et al. 2020a) (Zhou et al. 2013; Boschiero et al. 2020). The pipeline of the bioinformatics algorithm is publicly available at https://github.com/ZhaoBioinformaticsLab/PlantSSPProtocols. The genomes of *P.patens*, *S.fallax* and *M.polymorpha* in FASTA format were downloaded from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html), the

genomes of representatives of *Anthoceros* were downloaded from the database at https://www.hornworts.uzh.ch/en.html. The SPADA algorithm was launched using the Linux command line. The Augustus package within the SPADA pipeline was run using *Arabidopsis thaliana* as a reference genome. A docker image (CRP_PlantSSPv1_Noble) containing all currently known SSPs was used for the HMM search within the SPADA pipeline. For the gene model prediction, an E-value < 0.001 threshold was used to exclude false-positive results. As a result, new gene model prediction annotations in GFF3 format were obtained. For further functional annotation and classification of the predicted genes, potentially encoding SSPs proteins (< 250 aa) were generated. The potential SSP precursors were then classified as Known SSP, Likely Known SSP, Putative SSP and Non-SSP (Boschiero et al. 2020). The Known SSP (encoding known SSP) meets the following criteria: D-score SignalP > 0.45; HMM homology E-value < 0.01; Smith-Waterman homology E-value < 0.01 and precursor length < 200 aa. The Likely Known SSP genes have HMM homology value E < 0.01 and Smith-Waterman homology E-value < 0.01; precursor length < 250. Putative SSP genes have no or little sequence similarity with previous SSPs, precursor size < 230 aa; SignalP D-score > 0.25; and no presence of TM domains. Non-SSP genes did not fit the above categories. Predicted SSPs were BLASTed against the corresponding protein databases to obtain the protein ID.

Multiple sequence alignments and HMMER search

Overall, 43 angiosperms, 1 gymnosperm and 1 lycophyte species were selected to analyze SSPs diversity. Protein sequences of CAPE, TAX, thionin and hevein SSP families were downloaded from NCBI database (https://www.ncbi.nlm.nih.gov/) and RALF sequences were obtained from Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html) in FASTA format. The list of known PEPRs in angiosperms was obtained from the paper Lori et al. (2015). The ClustalW package was applied to generate multiple alignments with default parameters (Larkin et al. 2007). After each alignment representative sequences were manually selected. The visualization of multiple alignments was performed using the Jalview2 software package (Waterhouse et al. 2009). The HMMERv3.3 (http://hmmer.org) package was applied to search for PROPEP homologs in the genome of *P.patens*. HMM profiles of all known PROPEP in angiosperms were generated using hmmbuild. Both per-sequence and per-domain thresholds E-value < 0.001 was set for the console jackhmmer

(A)

Genomes:
- *Physcomitrium patens*
- *Marchantia polymorpha*
- *Sphagnum fallax*
- *Anthoceros agrestis* (Bonn and Oxford strains)
- *Anthoceros punctatus*

SPADA annotation tool
- gene prediction
- HMM search against known SSP families

Classification of predicted proteins
- ≤ 250 aa precursor
- D-score SignalP
- TM domain
- HMM E-value threshold
- Smith-Waterman E-value threshold

SSPs annotation
- BLASTP against annotated proteomes for each species
- E-value < 0.00001 and at least 70% hit coverage

SSP gene candidates

- The precursor length ≤ 250 aa
- D-score ≥ 0.45
- No transmembrane domain
- Homology with known class of regulating peptides (E-value < 0.01)

(B)

Physcomitrella patens: Signal 23%, Peptidase inhibitor 11%, Antimicrobial 4%, Unknown 62%

Marchantia polymorpha: Signal 68%, Peptidase inhibitor 13%, Antimicrobial 15%, Unknown 4%

Medicago truncatula: Signal 35%, Peptidase inhibitor 7%, Antimicrobial 7%, Unknown 50%

Legend:
- Signal (blue)
- Peptidase inhibitor (green)
- Antimicrobial (grey)
- Unknown (yellow)

(C)

algorithm run. The console tool of the HMMER program, jackhmmer, searched for PROPEPs in the 6-frame translated *P.patens* genome using previously generated HMM profiles of 75 known amino acid sequences of PROPEPs identified in both monocots and dicots.

GO term analysis

GO terms list was obtained from the Phytozome tool PhytoMine (https://phytozome.jgi.doe.gov/phytomine/begin.do). The enrichment analysis was performed using online tool g:Profiler (https://biit.cs.ut.ee/gprofiler/gost).

◀**Fig. 1 a** A workflow of SSP prediction with SPADA tool. **b** Diagrams showing a distribution of SSP precursors groups in different plant taxons according to in silico prediction with SPADA. Percentage corresponds to: *Physcomitrella patens* «Signal» group − 31 precursors (23%), «Peptidase inhibitor» group − 14 precursors (11%), «Antimicrobial» group − 5 precursors (4%), «Unknown» group − 82 precursors (62%); *Marchantia polymorpha* - «Signal» group − 53 precursors (68%), «Peptidase inhibitor» group − 10 precursors (13%), «Antimicrobial» group − 12 precursors (15%), «Unknown» group − 3 precursors (4%); *Medicago truncatula* - «Signal» group − 694 precursors (35%), «Peptidase inhibitor» group − 147 precursors (7%), «Antimicrobial» group − 139 precursors (7%), «Unknown» group − 994 precursors (50%) - of the total number. **c** A heatmap showing a normalized distribution of SSP gene groups in different plant taxons according to *in silico* prediction with SPADA on a log2 scale. Colors in the heatmap correspond to a scaled number of SSP genes

Total RNA isolation and qRT-PCR

Total RNA from protonemata was isolated as previously described (Fesenko et al. 2019b). Quality and quantity were evaluated using electrophoresis on agarose gel with ethidium bromide staining. Total RNA concentration of samples was precisely measured using the Quant-iT™ RNA Assay Kit, 5–100 ng on a Qubit 3.0 (Invitrogen, US) fluorometer. cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Russia) according to the manufacturer's recommendations. OligoDT primers were used to prepare cDNA from 2 µg total RNA after DNase treatment. Primers were designed using the PrimerQuest Tool (http://eu.idtdna.com/Primerquest/Home/Index). Real-time PCR was performed using the qPCRmix-HS SYBR (5×) system fluorescent probes (Evrogen, Russia) on a LightCycler® 96 (Roche, Mannheim, Germany). qRT-PCR was carried out in three biological and three technical replicates. cDNA representation was normalized using stably transcribed reference gene actin 5 (Pp1s381_21V6.1). The 2-ddCT values were obtained using the LightCycler® 96 software. Control samples were used as a calibrator.

Statistical analysis

Statistical analysis was performed across at least three biological repeats. Statistical analyses and boxplots were made in Python v. 3.7.5 [G. van Rossum, Python tutorial, Technical Report CS-R9526, Centrum voor Wiskunde en Informatica (CWI, Amsterdam, May 1995)] using module scipy (Virtanen et al. 2020). The one-way analysis of variance (ANOVA) with a paired independent t-test were applied to determine which pairwise comparisons were statistically significant. Differences were considered to be significant at p-value < 0.05.

Peptide synthesis

P. patens endogenous signal peptides were chemically synthesized at Shanghai Ruifu Chemical Co., Ltd. (Shanghai, China), angiosperm plant elicitor peptides were chemically synthesized at the genetic engineering laboratory of the FRCC PCM (Moscow, Russia). The purity of the lyophilized peptides was > 95%, their molecular weight was confirmed by mass spectrometric analysis. The synthesized peptides were dissolved in sterile water and stored at − 80 °C.

Results

Identification of established SSP families in bryophyte genomes

Comprehensive bioinformatic analysis of potential SSPs in non-vascular plants has not been provided to date. Therefore, we used the SPADA pipeline tool (Zhou et al. 2013), to predict genes encoding SSPs in the genomes of five bryophyte species (Supplementary Table S1, Supplementary Fig. S1). The general workflow of SSPs prediction is depicted in Fig. 1a. SPADA utilizes the in-built Hidden Markov Models of SSP families included in the PlantSSP database and scans any genome for them. However, the prediction of potential SSPs is limited to the availability of HMM models of known families identified in angiosperms. Therefore, one caveat of our approach is the inability to identify bryophyte-specific SSPs.

Overall, we applied the SPADA pipeline to two moss species - *Physcomitrella patens* (v3.3) and *Sphagnum fallax* (v0.5); the liverwort *Marchantia polymorpha* (v3.1); three recently assembled hornwort genomes – *Anthoceros agrestis* (Bonn and Oxford strains) and *A. punctatus* (https://www.hornworts.uzh.ch/en.html).

At the first step, genes that potentially encode SSPs were predicted and classified (Table 2; Supplementary Table S5-7).

Additionally, to understand whether the predicted SSP precursor genes were previously annotated, we performed BLASTP analysis of the predicted precursors against annotated proteomes of studied species (E-value 0.00001 cut-off and at least 70% hit coverage) (Supplementary Table S2-7).

We further focused on the detailed characterization of genes, which were predicted as 'known SSP' in each bryophyte species.

"Signal" group

Potential immune signaling peptides CAPE In tomato, the CAPE peptide is derived from the C-terminal end of the tomato PR1b preproprotein of the pathogenesis-related

Table 2 The number of predicted SSP precursors

	P.patens	M.polymorpha	A.agrestis B	A.agrestis O	A.punctatus	S.fallax
Known	132	78	55	56	61	83
Likely known	5	4	4	3	6	9
Non-SSP	375	278	120	110	112	265
Putative	404	442	421	401	405	695
Total	916	802	600	570	584	1052

1 proteins (CAP) (Chen et al. 2014). Among the potential SSP predicted by SPADA in *P.patens*, we found four proteins that are potentially precursors of CAPE peptides: Pp3c18_21170V3.1.p - CYSTEINE-RICH SECRETORY PROTEIN-RELATED; Pp3c18_21090V3.1.p - Defense-related protein containing SCP domain; Pp3c25_120V3.1.p - ALLERGEN V5/TPX-1-RELATED FAMILY PROTEIN-RELATED; Pp3c25_500V3.1.p - PR-1. We also found possible CAPE precursors in other examined species – nine genes in *M.polymorpha* (Supplementary Table S4); two proteins and one protein in *A.agrestis* Bonn and Oxford strains, respectively (Supplementary Table S5-6); one predicted precursor in *S.fallax* (Supplementary Table S3). There were no genes predicted as potential CAPE precursors in *A.punctatus.*

We manually performed multiple sequence alignments of predicted precursors of CAPE peptides from other plants (Supplementary Fig. S2). This analysis showed that the conserved motif of the CAPE peptide (P*GN*****PY) is present in almost all potential precursors (Fig. 2a, S2). We then analyzed publicly available data of moss *P.patens* gene expression (https://peatmoss.online.uni-marburg.de/) and revealed that the expression of four genes predicted as CAPE precursors is upregulated upon OPDA treatment (Supplementary Table S12).

Peptides involved in developmental processes The SPADA pipeline has enabled us to predict a handful of protein precursors of known peptide families involved in the regulation of plant development processes. This might indicate the importance of peptide regulation in adaptation to terrestrial environments (Whitewoods et al. 2018). Our analysis has revealed several SSPs (RALF, EPFL, CLE), which have been already identified in *P.patens* and also several SSPs (RALF), which have been annotated in *M.polymorpha.* We considered this result as a positive control of the SPADA prediction tool application.

RALF peptides RALF peptides belong to the group of Cys-rich peptides and are identified in all angiosperms (Campbell and Turner 2017). Their primary function is connected with the regulation of root architecture; however, they have been also found in *P.patens, M.polymorpha* and *S.fallax*, which lack root systems (Campbell and Turner 2017; Bowman et al. 2017). Our analysis identified two RALF-like genes in *P.patens*, one of them was already annotated (Pp3c6_7200V3.1.p). There were also two genes predicted in *M.polymorpha* as RALF-like, which both were already annotated (Mapoly0076s0067.1, Mapoly0040s0047.1). One gene was predicted to be RALF-like in *S.fallax* (Supplementary Table S3) and one gene was predicted in *A.punctatus*, but none were found in both strains of *A.agrestis* (Supplementary Table S7, S5-6). Multiple sequence alignment also revealed the specific conserved motifs in predicted bryophyte precursors (Supplementary Fig. S3).

Plantcyanin/chemocyanin (PCY) PCY peptides belong to the group of Cys-rich peptides and are involved in regulation of pollen tube growth in tracheophytes (Chae and Lord 2011). We identified 9 genes classified as Known or Likely Known SSP, which potentially encode protein precursors of plantcyanins in *P.patens* (Pp3c3_25110V3.1.p, Pp3c5_5180V3.1.p, Pp3c5_23940V3.1.p, Pp3c16_22330V3.1.p, Pp3c8_8380V3.1.p, Pp3c20_17730V3.1.p, Pp3c23_9820V3.1.p, pcy_Chr04_13M_1, Pp3c7_7010V3.1.p). PCY peptides were also predicted in all other examined species. The most number of precursors were identified in *M.polymorpha* (Supplementary Table S3-7). However, the functions of this family of secreted peptides require the further elucidation.

TAX peptides TAX peptides belong to the group of Cys-rich peptides and are involved in the process of taxanes biosynthesis and synthesis of nicotinic alkaloids (Onrubia et al. 2014). Three genes (Pp3c5_22950V3.1.p, Pp3c3_16420V3.1.p, Pp3c4_17790V3.1.p) were predicted to encode the precursors of TAX peptides in *P.patens*. All of these protein precursors lacked any domain annotation in the current genome version v3.3 of *P.patens* and there was not any evidence of TAX presence in bryophytes. Two genes each were predicted to encode TAX peptides in *M.polymorpha*, *A.punctatus* and in both strains of *A.agrestis* (Supplementary Table S4-7). Four genes were predicted as TAX in *S.fallax* (Supplementary Table S3).

A multiple alignment of the bryophyte potential precursors with TAX protein precursors from other plants revealed

strong sequences similarity (Fig. 2d, Supplementary Fig. S4).

Peptides involved in regulation of other processes Non-specific lipid transfer protein (nsLTP) NsLTP peptides represent a large group of Cys-rich peptides, which are involved in lipid transfer, defense responses and plant development in most vascular plants (Liu et al. 2015). Eleven genes were predicted to encode protein precursors of nsLTP in *P.patens* (Supplementary Table S2). Nine of these protein precursors were already annotated as Probable lipid transfer (LTP_2) and two were annotated *de novo*. In both strains of *A.agrestis* five nsLTP genes were identified (Supplementary Table S5-6). Four nsLTP genes were predicted in *A.punctatus* and *M.polymorpha* (Supplementary Table S7, S4). Two of predicted nsLTP were already annotated in *M.polymorpha* (Mapoly0120s0024.1.p; Mapoly0955s0001.1.p). Two proteins were predicted as precursors of nsLTP peptides in *S.fallax* and they were also already annotated (Sphfalx0002s0328.1.p; Sphfalx0005s0219.1.p).

The SPADA tool also predicted the already annotated "Signal" group peptides, such as Epidermal Patterning Factor-Like (EPFL) protein precursors and Clavata/Embryo Surrounding Region (CLE) protein precursors in *P.patens* (Supplementary Table S2) and potential precursors of EPFL peptides in the three *Anthoceros* genomes (Supplementary Table S5-7). We were also able to detect Root_Cap/Late_Embryogenesis protein precursors belonging to "Signal" group in *P.patens* and in both strains of *A.agrestis* (Supplementary Table S2, S5-6). Precursors of plant natriuretic peptides (PNPs) were predicted in *M.polymorpha* and both strains of *A.agrestis*, but were not identified in *P.patens*, *S.fallax* and *A.punctatus* (Supplementary Table S2-7).

"Peptidase inhibitor" group

Cytotoxic T-lymphocyte antigen-2 alpha (CTLA) CTLA peptides are involved in regulation of Cys proteases activity and can inhibit protease activity (Sugita et al. 2011). Our analysis revealed 13 genes to encode potential CTLA peptides in *P.patens* (Pp3c2_30400V3.1.p and etc.; Supplementary Table S2). GO enrichment analysis showed that these protein precursors are involved in oxidation-reduction processes (Supplementary Table S13). CTLA peptide precursors were also predicted in all other examined species. The most number of precursors were identified in *S. fallax* (Supplementary Table S3-7).

"Antimicrobial" group

Antimicrobial peptides provide the first line of defense in pathogen attacks and have been found in all plant species (Czyzewicz et al. 2013; Oh et al. 2018).

Hevein-like peptides Hevein-like peptides belong to the group of antimicrobial peptides and are identified in various monocot and dicot plants (Slavokhotova et al. 2017). In our data four genes were predicted to encode possible candidates of hevein-like peptide precursors in *P.patens* (Supplementary Table S2). All of the above-mentioned protein precursors participate in plant defense responses; they bind and degrade chitin of the fungal cell walls, according to the gene description in the publicly available annotations. However, some of them also encompass hevein-like peptide sequences according to the formula of known hevein peptides ($C1 \times 4$ $5 C 2 \times 4 C3 C4 \times 5 C 5 \times 6 C6$, in which C stands for cysteine and X stands for any amino acid in between). The potential precursors of these peptides were also found in all examined bryophyte species (Tables S3-S7). Our manual multiple sequence alignment also revealed shared motifs among bryophytes and other plants protein precursors of known hevein peptides (Fig. 2b, Supplementary Fig. S5).

Thionin-like (THL) Thionin-like peptides are cysteine-rich peptides that have both toxic and antimicrobial functions by disrupting the pathogenic membrane (Plattner et al. 2015). They have been identified in both monocots and eudicots. To date, there is no annotation of genes encoding thionin-like peptides in *P.patens* or other bryophytes. However, we predicted two genes as possible THL SSPs (Pp3c22_12110V3.1, Pp3c19_18370V3.1) in *P.patens*. These genes are non-annotated in Phytozome v12. One gene each was predicted as possible THL in *M.polymorpha* and three *Anthoceros* genomes (Supplementary Table S4-7). Two proteins were predicted as THL in *S.fallax* (Supplementary Table S3). We also performed a multiple alignment of those protein precursors with known THL precursors from other plants (Fig. 2c, Supplementary Fig. S6).

"Unknown" group We identified Nodule-specific Glycine-rich Protein (NodGRP) precursors in all examined bryophyte species (Supplementary Table S2-7). The functions of this group of SSPs in bryophytes require further investigation. In addition, the SPADA pipeline predicted a number of low-molecular weight Cys-rich (LCR) peptides in *P.patens*, most of those were annotated *de novo* by SPADA (Supplementary Table S2). Precursors of LCRs were also predicted in *S.fallax*, *A.punctatus* and *A.agrestis* Oxford strain, but not in *M.polymorpha* and *A.agrestis* Bonn strain (Supplementary Table S3-7). The SPADA tool also predicted precursors of Nodule-specific Cysteine Rich Group
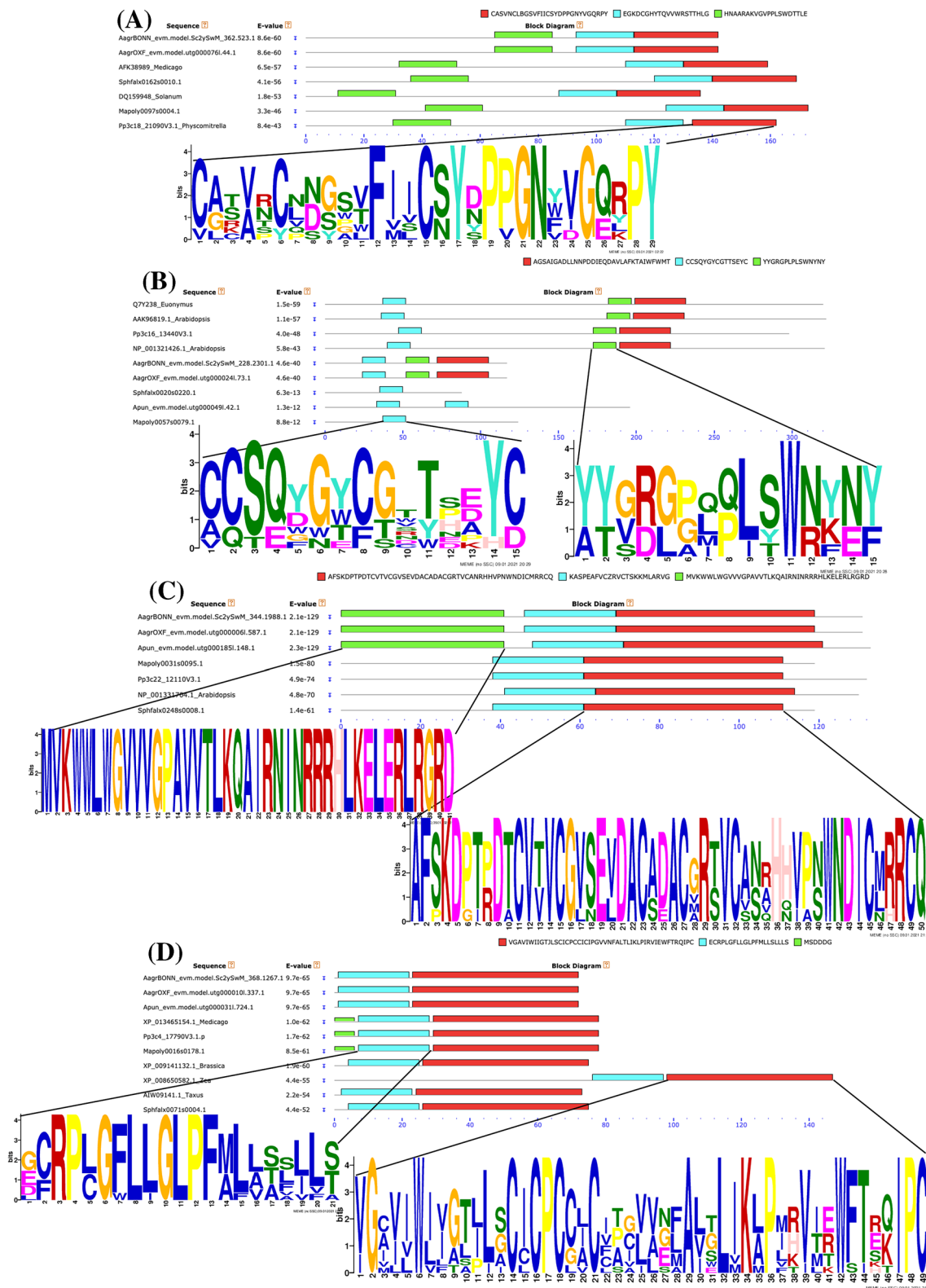
(NCR) peptides in *M.polymorpha*, both strains of *A.agrestis* and in *A.punctatus*, but they were not identified in *P.patens* and *S.fallax* (Supplementary Table S2-7).

There were also precursors predicted as known SSP from different groups exclusively in several species: LEED.PEED (LP) precursors in *A.agrestis* Oxford strain and *A.punctatus*; Subtilisin inhibitor (SubIN) precursors in three *Anthoceros*

◄**Fig. 2** Logo for **a** a known motif of CAPE peptides; **b** a known motif of hevein peptides; **c** shared motifs of thionin peptide; **d** shared motifs of TAX peptide from some vascular plants and bryophytes candidates generated by the online tool MEME (http://memesuite.org/tools/meme). Representative sequences of CAPE peptides and candidates: DQ159948 from *Solanum lycopersicum*, AFK38989 from *Medicago truncatula* (downloaded from NCBI database), Pp3c18_21090V3.1 from *Physcomitrella patens*, Mapoly0097s0004.1 from *Marchantia polymorpha*, Sphfalx0162s0010.1 from *Sphagnum fallax* (downloaded from Phytozome database), AagrBONN_evm.model.Sc2ySwM_362.523.1 from *Anthoceros agrestis* Bonn strain and AagrOXF_evm.model.utg000076l.44.1 from *Anthoceros agrestis* Oxford strain (downloaded from https://www.hornworts.uzh.ch/en.html database). Representative sequences of hevein peptides and candidates: Q7Y238 from *Euonymus europaeus*, AAK96819.1 and NP_001321426.1 from *Arabidopsis thaliana* (downloaded from NCBI database), Pp3c16_13440V3.1 from *Physcomitrella patens*, Mapoly0057s0079.1 from *Marchantia polymorpha*, Sphfalx0020s0220.1 from *Sphagnum fallax* (downloaded from Phytozome database), AagrBONN_evm.model.Sc2ySwM_228.2301.1 from *Anthoceros agrestis* Bonn strain, AagrOXF_evm.model.utg000024l.73.1 from *Anthoceros agrestis* Oxford strain, Apun_evm.model.utg000049l.42.1 from *Anthoceros punctatus* (downloaded from https://www.hornworts.uzh.ch/en.html database). Representative sequences of thionin peptides and candidates: NP_001331704.1 from *Arabidopsis thaliana* (downloaded from NCBI database), Pp3c22_12110V3.1 from *Physcomitrella patens*, Sphfalx0248s0008.1 from *Sphagnum fallax*, Mapoly0031s0095.1 from *Marchantia polymorpha* (downloaded from Phytozome database), AagrBONN_evm.model.Sc2ySwM_344.1988.1 from *Anthoceros agrestis* Bonn strain, AagrOXF_evm.model.utg000006l.587.1 from *Anthoceros agrestis* Oxford strain, Apun_evm.model.utg000185l.148.1 from *Anthoceros punctatus* (downloaded from https://www.hornworts.uzh.ch/en.html database). Representative sequences of TAX peptides and candidates: AIW09141.1 from *Taxus baccata*, XP_013465154.1 from *Medicago truncatula*, XP_008650582.1 from *Zea mays*, XP_009141132.1 from *Brassica rapa* (downloaded from NCBI database), Pp3c4_17790V3.1.p from *Physcomitrella patens*, Sphfalx0071s0004.1 from *Sphagnum fallax*, Mapoly0016s0178.1 from *Marchantia polymorpha* (downloaded from Phytozome database), AagrBONN_evm.model.Sc2ySwM_368.1267.1 from *Anthoceros agrestis* Bonn strain, AagrOXF_evm.model.utg000010l.337.1 from *Anthoceros agrestis* Oxford strain, Apun_evm.model.utg000031l.724.1 from *Anthoceros punctatus* (downloaded from https://www.hornworts.uzh.ch/en.html database)

genomes and in *S.fallax*; a Tapetum Determinant 1 (TPD) precursor in *A.punctatus*; and several others (Supplementary Table S2-7).

We found that the pattern of SSPs identified in *S.fallax* differed from *P.patens* and *M.polymorpha*, rather resembling that of hornworts (Supplementary Fig. S1, Supplementary Table S1). In the genome of *S.fallax* were identified several unique SSP precursors, such as MtSUBPEP, ProSCOOP, Kazal family inhibitors (Kaz) and Plant Defensin-like (PDL) (Supplementary Table S3).

In conclusion, the number of predicted genes encoding SSPs is higher in mosses than in both hornworts and liverworts but still lower than in angiosperms such as *Medicago truncatula* (Fig. 1b, c, Supplementary Fig. S1, Supplementary Table S1).

Identification of endogenous peptides induced by chitosan treatment

Taking into account that immune signalling peptides are often species-specific and cannot be predicted based on similarity search, we then performed mass-spectrometry analysis of the *P.patens* secretomes (Supplementary Table S8-11). Since the moss *P.patens* is unable to perceive the known bacterial elicitors such as flagellin or elf18, we induced the release of potential signalling peptides by chitosan (Supplementary Table S8, S9). First, we concentrated on the analysis of peptides and precursors from which peptides were identified in at least 2 biological repeats. In moss secretome treated by chitosan, 1187 unique peptides from 297 precursors were identified. A large portion of the precursors in chitosan-treated samples belong to chloroplastic, transport and proteolytic proteins, including proteins participating in amino acid transmembrane transport, and slightly fewer are translation factors, subunits of ribosomes and chaperones. We found that 6 % of precursors in chitosan-treated peptidomes were either uncharacterized or had an unclear function, with a half of them below 250 aa in length. Because of the huge variability of cell peptidomes, we discarded peptides derived from precursors also identified in control samples. This resulted in a filtered list of 52 peptides from 38 precursors, appearing only in chitosan-treated secretome. These precursors included stress-related proteins such as Pp3c2_12150 (molecular chaperone DnaK), Pp3c20_21140 (calcium binding protein); cell wall proteins such as Pp3c14_18450 (EXPANSIN-A5), Pp3c24_15950 (Leucine-rich repeat receptor-like protein kinase family protein), Pp3c5_23400 (Pectinesterase). Manual checking of the precursors revealed that 30 % of them were smaller than 250 aa, including Pp3c1_19210V3 (Protein of unknown function (DUF1118)), ribosomal proteins L28e and S19e and Pp3c26_5890V3 (peroxidase).

P.patens encodes four homologs of the *Arabidopsis* chitin receptor AtCERK1, but only one knock-out line (Δ*cerk1*) showed insensitivity to chitin response (Bressendorff et al. 2016). To determine the peptides specifically induced by chitosan, we performed peptidome analysis of a Δ*cerk1* mutant secretome (Supplementary Table S9, S11). It should be noted that we cannot rule out the possibility that this mutant line senses chitin to some extent. Using peptides, identified in Δ*cerk1* as a filter, we identified 14 peptides, specifically appearing only in at least two chitosan-treated secretomes from wild type. Two peptides were derived from the C-terminus of the 165 aa peroxidase (Pp3c26_5890). Peroxidases are involved in numerous cellular processes such as development and stress responses.

The detection of bioactive peptides by mass-spectrometry might be difficult because of their low concentration and rapid degradation in cells. Therefore, we also

looked at all peptides and precursors in chitosan-treated secretomes. First, we filtered off peptides found in control samples. Among the precursors of 5320 peptides from chitosan-treated samples were calmodulin (Pp3c10_21490), Chitinase-related (Pp3c1_30410), Probable lipid transfer (LTP_2) (Pp3c19_2080), Lactoperoxidase (Pp3c26_5890), cystatin-C (Pp3c9_24090) - inhibitor of cysteine proteinase, several actin proteins (Pp3c3_33410) and Cucumisins (Pp3c22_10010). Then, we selected 430 small (up to 250 aa) precursors appearing in chitosan-treated samples.

After discarding peptides that also appeared in Δ*cerk* mutants upon chitosan treatment, we identified 9 peptides from small precursors with unknown function specifically induced by chitosan.

We then compared the identified peptides with our previously published moss secretomes, treated with salicylic acid (SA) (Filippova et al. 2019) and methyl jasmonate (MeJA) (Fesenko et al. 2019b). We found that peptidomes treated with methyl jasmonate and salicylic acid overlapped the most with each other (Supplementary Fig. S7). Peptides common to all three elicitor treatments were cleaved from precursors, most of which take part in photosynthesis; in addition, peptides were cleaved from Cucumisin (Pp3c2_4520) as well as from several uncharacterized proteins. Among the common precursors of induced peptides, we also found precursors of our signaling peptide candidates selected and synthesized in previous studies (ININIINAPLQG-FKIA and EAAPAPVAEVEAPKAEE) (Filippova et al. 2019; Fesenko et al. 2019b). Several peptides induced only in wild type upon chitosan treatment and found previously in peptidomes treated with other stress factors were synthesized for further study. These were peptides derived from precursors Pp3c14_22870V3.1.p, Pp3c21_4350V3.1.p (already selected in our previous works (Filippova et al. 2019; Fesenko et al. 2019b)) and also from Pp3c13_3880V3.1.p and Pp3c17_15750V3.1.p (Table 1). However, the role of all these peptides as DAMP signals requires further elucidation.

Next, we searched for endogenous peptides derived from protein precursors, which were predicted as Known or Likely Known SSP in extracellular peptidomes. We detected hevein-like peptides in cerk-mutant lines upon treatment with chitosan (Pp3c1_30410V3.1.p). Three unique peptides contained a motif of Cys-rich peptides, which form disulfide bridges between the following cysteine residues: C1–C4, C2–C5 and C3–C6. Thus, these peptides are very likely to be hevein peptides. Although we were not able to detect such peptides in wild type samples treated with chitosan. The low concentration and the rapid degradation of the endogenous peptides in cells and extracellular space can make the isolation and identification of certain peptides very difficult. Thus, we cannot confirm that certain peptides are not produced in the plants using only LC-MS analysis.

Also, we detected a short sequence of the RALF peptide in MeJA-treated samples. In each stress-treated and control sample we identified potential PCY peptides derived from Pp3c5_23940V3.1.p, Pp3c16_22330V3.1.p, pcy_Chr04_13M_1 (SPADA-predicted gene). Interestingly, chitosan upregulated the potential Cys-rich PCY peptides in extracellular peptidomes.

Treatment with known peptide elicitors resulted in ROS accumulation

PEPs and their receptors—plant elicitor peptide receptors (PEPRs)—are found in almost all angiosperms, but not in bryophytes. PEPRs amino acid sequences show high inter-family diversity and are mostly represented by one form in angiosperms (Lori et al. 2015). Using the sequences list of known PEPRs in monocots and dicots, we performed a BLASTp similarity search for PEPR homologs in *Physcomitrella patens*. As a result, we identified 14 potential PEPR-like homologs and performed a multiple alignment using the ClustalW algorithm (Supplementary Fig. S8). According to the analysis of key amino acid residues involved in the interaction between ligand and its receptors, 7 sequences were excluded from the analysis (Supplementary Fig. S9). Surprisingly, the key amino acid residues essential for interaction between PEP and PEPRs, such as R487, D441, F419, were the most conserved ones in PERP sequences in both angiosperms and *P.patens*. The highest level of similarity between angiosperm PEPRs and *P.patens* PEPR-like homologs was shown by genes Pp3c3_12560V3.1 and Pp3c2_18570V3.1, as they seem to have the key amino acid residues in the exact positions required for efficient interaction between the PEP-PEPR-BAK complex (Supplementary Fig. S9). Thus, we suggest that *P.patens* might sense peptide elicitors derived from angiosperms due to similar structures in potential PEPR-like homologs receptor-like kinases. We then used HMMER and its in-built jackhmmer search tool to search for PROPEP homologs in *P.patens*. We identified 12 potential protein transcripts, which might encompass PEP sequences. We then performed multiple sequence alignments of known PROPEP sequences and our potential candidates, however we did not find any shared motifs. We next treated moss *P.patens* with synthetic PEPs - AtPEP1 and StPEP1 (plant elicitor peptide from *Solanum tuberosum*), and analyze whether the treatment lead to ROS accumulation and changes at the transcriptional level of selected genes. We choose these PEPs because AtPEP1 is a commonly use in studies of plant immunity (Kadota et al. 2014; Huffaker et al. 2006; Roux et al. 2011) and StPEP1 comes from agriculturally important plant (*Solanum tuberosum*).

The release of ROS is a common indicator of stress reaction in plants (Shapiguzov et al. 2012). Therefore, we measured intra- and extracellular ROS accumulation in *P.patens*

Fig. 3 A comparison between ROS accumulation after treatment with 5 μM AtPEP1 and StPEP and with 1 mg/mL chitosan of wild type and cerk-mutant *P.patens* showed in a **a** boxplot and **b** fluorescence microscopy. All experiments had at least three biological repeats (a paired t-test: *P-value < 0.05; **P-value < 0.005; ***P-value < 0.0005). Control samples were treated with water
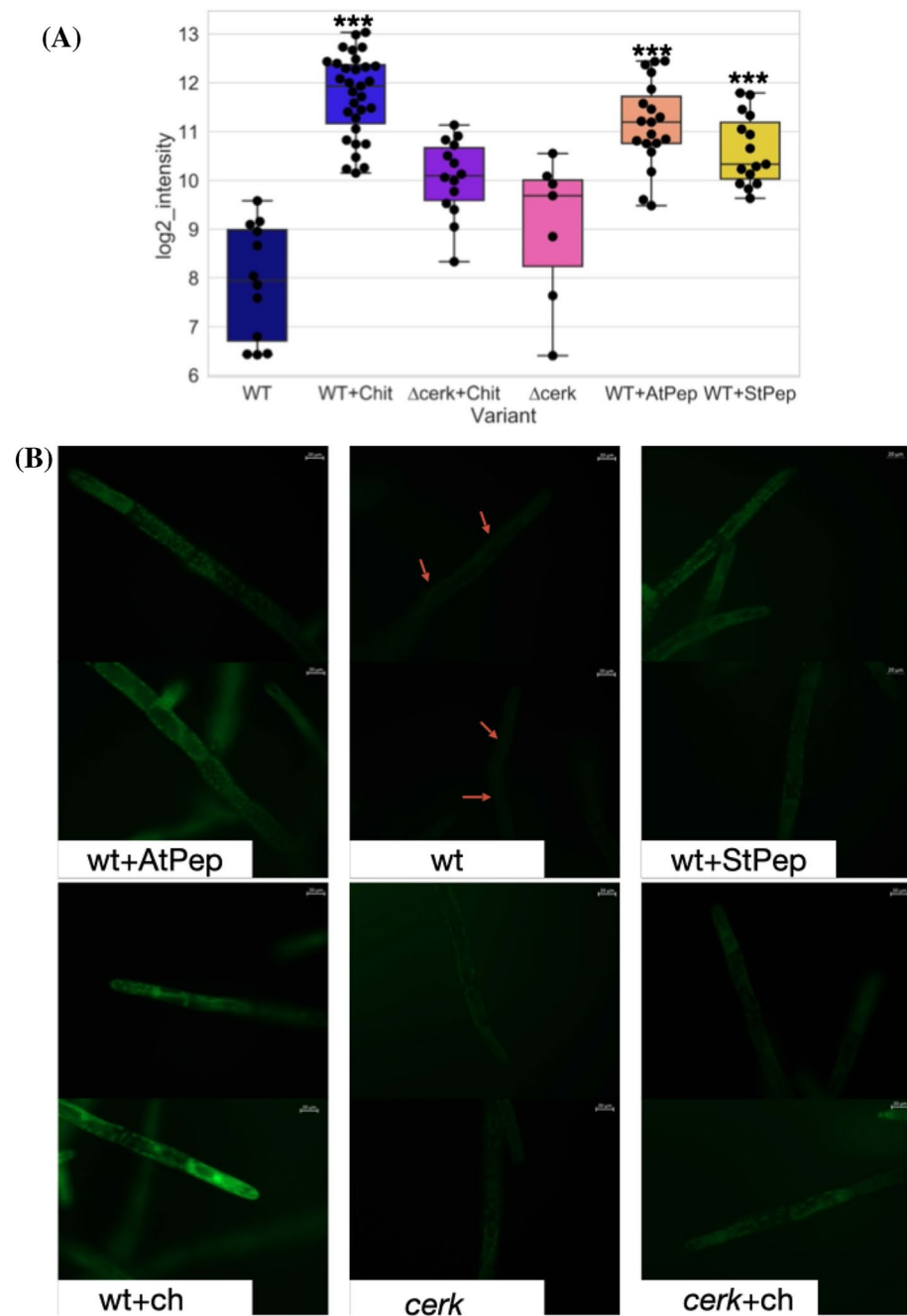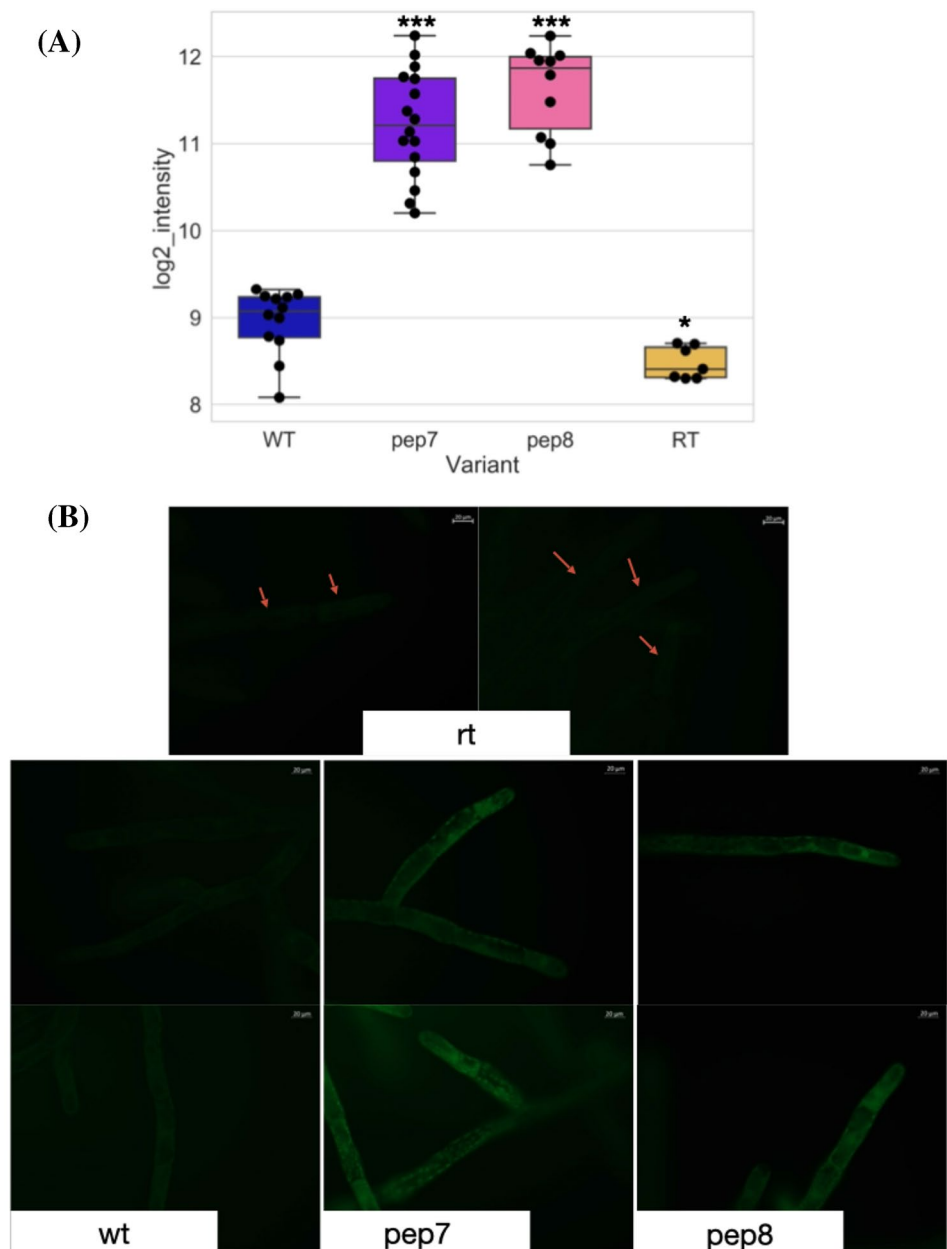

protonemata treated with synthetic PEPs and chitosan as a control (Fig. 3a and b; Supplementary Fig. S10). As expected, the chitosan treatment induced the release of ROS in wild-type cells, whereas aΔ*cerk* mutant line did not show significant changes in ROS accumulation (Fig. 3a). In wild-type plants, fluorescent signal was mainly detected in the cytoplasm and near the cell walls (Fig. 3b). Synthetic PEPs treatment also resulted in an increase of intracellular ROS in chloroplasts and in the cytoplasm (Fig. 3b). In addition, AtPEP1 and chitosan induced extracellular ROS accumulation, whereas StPEP1 did not (Supplementary Fig. S10). We

also analyzed the expression of some pathogenesis-related genes, such as allene oxide synthase (AOS), OPDA reductase (OPR), chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL4) in *P.patens* protonemata. The quantitative RT-PCR analysis (qRT-PCR) showed that the transcriptional level of CHS and PAL4 significantly increased upon treatment with 5 μM AtPEP1 (Supplementary Fig. S11a).

Then, we investigated peptide candidates identified in our previous experiments (Filippova et al. 2019; Fesenko et al. 2019b) and confirmed by mass-spectrometry analysis

in this study. We found a significant increase in ROS accumulation upon treatment with synthetic candidate peptides - pep7(EAA) EAAPAPVAEVEAPKAEE and pep8 INIINAPLQGFKIA (Fig. 4a). These peptides were also found in peptidomes treated with stress hormones salicylic acid (Filippova et al. 2019) and methyl jasmonate (Fesenko et al. 2019b). The level of intracellular ROS accumulation was comparable with chitosan, suggesting the probable potential of the selected peptides as immune signals. The treatment with synthetic pep7 also resulted in an increase of extracellular ROS accumulation (Supplementary Fig. S10).

We used qRT-PCR to check the ability of synthetic candidate peptides to induce the transcription of known pathogenesis-related genes as well. In our previous study we reported the ability of pep8 to alter the expression of OPR and AOS genes in a similar way to MeJA (Fesenko et al. 2019b). In this study, the qRT-PCR analysis showed that the expression of some pathogenesis-related genes was influenced in the time-dependent manner upon treatment with 5 μM pep7 peptide (Supplementary Fig. S11b). However, whether these peptides play a role as DAMPs requires further elucidation.

Our results suggest that mosses might be able to detect PEPs and the downstream signals are similar to those in angiosperms. Also moss itself may secrete a number of

peptide signals that are able to trigger general stress reactions such as accumulation of ROS.

Discussion

Because of their specific features such as small size or specific patterns of expression, SSPs often remain unannotated. Our study showed that the number of SSPs in annotated genomes is underestimated and further re-annotation efforts are needed.

It is unclear whether SSPs from angiosperms are similar to those from non-vascular plants. To shed light on this, we conducted a bioinformatic screening of potential homologs of well-known plant SSPs in the genome of the moss *Physcomitrella patens* as well as in the genomes of some other bryophytes, such as a liverwort *M.polymorpha*, moss *S.fallax* and three species of hornworts *Anthoceros* ssp. It is worth noting that the branching order of non-vascular plants is still uncertain (Delaux et al. 2019). Three main hypotheses support the branching order of the bryophytes: mosses and liverworts are monophyletic, whereas hornworts sister to tracheophytes (Puttick et al. 2018; Qiu et al. 2006); mosses and liverworts monophyletic with hornworts sister to all land plants (Wickett et al. 2014) and mosses, liverworts and hornworts form a monophyletic group (Wickett et al. 2014; Frangedakis et al. 2021). The recent analysis of 1KP plant transcriptomes recovered extant bryophytes as monophyletic, with hornworts sister to a moss and liverwort clade (Leebens-Mack et al. 2019). The hypothesis that liverworts are sister to all other extant land plant lineages was rejected (Leebens-Mack et al. 2019). Until very recently, the genomes of *Anthoceros* spp. had not been analyzed, but now the availability of three high-quality genomes of *Anthoceros* hornworts make it possible to perform comparative studies among other lineages of extant bryophytes (Zhang et al. 2020a). Hornworts are organisms of special interest as they share common traits with both green algae and other land plants (Zhang et al. 2020a). Therefore, understanding whether they share peptide signals among other lineages of Bryophytes seems a promising approach in evolutionary studies.

We observed a contrast pattern of predicted SSPs among studied bryophyte species. SSPs were grouped by mode of action as in MtSSPdb, in which the "Signal" group includes peptides involved in development and growth processes and immune signaling, such as CLE, PEP, RALF, CAPE and SCOOP; the "Peptidase inhibitor" group includes peptides involved in regulation of proteolysis, such as phytocystatin and CTLA; the "Antimicrobial" group includes Cys-rich peptides such as thionin and plant defensin and also CTLA; the "Unknown" group refers to peptides with unidentified

mode of action, such as LCR and NCR. Our analysis showed that the "Peptidase Inhibitors" group was the most abundant group of SSPs in *S.fallax* and *Anthoceros* spp., whereas in other analyzed species it is not as pronounced. The "Signal" SSPs were the most abundant in *M.polymorpha* in comparison to other bryophytes. The "Signal" group in three *Anthoceros* species had a similar size, suggesting low variability of these SSPs in related species. A substantial portion of predicted SSPs belongs to the "Unknown" group in *M.truncatula* and *P.patens*, whereas in all other species they constitute only a small part. The smallest group is the "Antimicrobial" group of peptides in all species. However, the largest number of them are represented in *M.polymorpha*.

Surprisingly, the pattern of SSP activity groups distribution was very similar for *P.patens* and *M.truncatula*. The patterns of SSPs in the liverwort *M.polymorpha* was quite unique compared with the rest of the analyzed species. For example, *M.polymorpha* has the largest number of signaling SSPs PCY, STIG-GRI and CAPE genes and antimicrobial Hevein-like peptide genes compared with other bryophyte species. The functions of these peptides in non-vascular plants are still unknown. Plastocyanin (PCY) and STIG-GRI have been reported to affect pollen tube growth in angiosperms (Chae and Lord 2011; Huang et al. 2014). At the same time, the number of predicted SSPs having a similarity to Cytotoxic T-lymphocyte antigen-2 alpha (CTLA) peptides was lower in *M.polymorpha*, as well as in *P.patens* and *M.truncatula*, than in hornworts and *S.fallax*. Cytotoxic T-lymphocyte antigen-2 alpha (CTLA) peptides were first found in mammals. This peptide class inhibits Cathepsin Cys proteases (Sugita et al. 2011). Taken together, our findings suggest that small peptides, probably controlling the proteolysis, form the largest group of SSPs in studied species. As expected, the number of NCR genes, which are associated with selection of symbiotic partners in nodules (Mergaert et al. 2003; Nallu et al. 2013), was much greater in *M.truncatula* then in the bryophytes, which seems logical as bryophytes do not have structures such as nodules for which these peptides are specific.

Surprisingly, the SSP patterns significantly differ in two studied mosses—*P.patens* and *S.fallax*. The reasons for this difference might be related to the conditions of their growth and external threats (Beike et al. 2015) as well as the quality of genome assembly and annotation.

We also found that *P.patens* has the largest number of nsLTP genes and LCR in comparison with other bryophyte species. NsLTPs participate in plant defense responses and various development pathways (Liu et al. 2015). *P.patens* also has SSP genes with high homology to known angiosperm SSPs, for example, antimicrobial Hevein-like and Thionin-like peptides, signaling CAPE and TAX peptides (Figs. 1c and 2; Supplementary Fig. S2-6). The other moss from our study—*S.fallax*—also has a homolog of signaling

peptide SUBPEP, cleaved out of subtilisin protease in *M.truncatula*, and also a homolog of the precursor of the recently discovered peptide SCOOP. These findings raise a question about the origin of functional SSPs from non-specific genes during evolution.

It is known that upon the transition from water to land and from unicellular to multicellular organisms, plants have acquired new signaling mechanisms that have allowed them to adapt to new environmental conditions (Bowles et al. 2020). For example, CLE peptides have been identified in bryophytes and in green alga *Chlamydomonas reinhardtii*, which lack vasculature (Oelkers et al. 2008). This raises a question whether the ancestral function of the CLE peptide was initially involved in developmental processes and why the peptide started to act as a regulator of vascular development in seed plants. In this context, our knowledge about peptide signaling in extant land plants as well as the origin of most phytocytokines is even more limited. It have been shown that immunity-related receptors evolved before the transition to land, but only the genomes of bryophytes encode a large repertoire of PRR proteins with all associated domains (> 320 in *Physcomitrella patens*, > 600 in *A.thaliana* and > 1100 in *Oryza sativa*) (Shiu and Bleecker 2003; Shiu et al. 2004; Han 2019). In this regard, the question remains as to when the peptide signaling system appeared, and also what should appear earlier: a peptide signal or a receptor recognizing it?

The study of immune signaling peptides evolution is a challenging task because of their species-specific or even family-specific nature (Lori et al. 2015). Therefore, we can only hypothesize on the existence of functional homologs of immune signaling peptides in different plant taxa. For example, PEPs are thought to be the missing functional homologs of systemin outside of the Solanaceae (Huffaker et al. 2013).

The PEP recognition induces phosphorylation of RBOHD (Respiratory Burst Oxidase Protein D), the NADPH oxidase, which is responsible for the production of ROS in ETI. It has also been shown in soybean that GmPEP treatment enhances RBOHD gene expression. In addition, it also induced expression of GmPEP precursor gene together with other genes associated with a defense response against nematodes (Lee et al. 2018). In our study, we detected the response of moss protonemata to exogenously applied AtPEP1 and StPEP1. Treatment with AtPEP and StPEP induced the production of intracellular ROS and change the transcription of selected pathogenesis-related genes, which suggested that moss genomes encode a receptor that can perceive PEP. Recent studies also showed that taxonomically distant species can effectively perceive non-self-danger peptides. There are a few studies that support the perception of systemin (SYS) in plant species lacking its orthologs. For example, constitutive expression of the tomato prosystemin gene in tobacco affected the host proteome, with the up-regulation of proteins involved in defense responses against pathogens in leaves (Rocco et al. 2008; Zhang and Hu 2017). It was also reported that external application of 5 and 10 μM tomato systemin led to inhibition of *Arabidopsis* seedling root growth and the expression of the plant defensin PDF1.2 (Zhang et al. 2018). Finally, *Arabidopsis thaliana* perceives signals upon exogenous treatment with systemins and Hyp-SyS, which are specific for solanaceous species. Moreover, these treatments induced resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Pastor-Fernández et al. 2020). These findings support the hypothesis that plants may have a common receptor's repertoire and receptor-mediated intracellular signaling pathway in response to peptides. It is interesting to note that signaling cascades are shared among different species, while the main domain of the PEPRs have evolved with the PEPs resulting in interfamily incompatibility but high intrafamily compatibility (Huffaker et al. 2013; Lori et al. 2015; Ruiz et al. 2018).

The number of studies used fluorescent dye DCFH-DA to detected intracellular ROS accumulation under biotic stress in moss *P.patens* (Oliver et al. 2009; Ponce de León 2011; Ponce de León et al. 2012; Ponce de León and Montesano 2013). However, the mechanisms of extracellular ROS generation in bryophytes are poorly studied. In our study, we detected both intracellular and extracellular ROS in *P.patens* using DCFH-DA and DCFH as well as luminol. Despite its wide use, luminol is not suitable for the detection of NO, $ONOO^-$, H_2O_2 or $OH^.$ in living systems (Pavelescu 2015; Halliwell and Whiteman, 2004). Moreover, because luminol can interfere with different phenolic compounds (Cui et al. 2004), the analysis of extracellular ROS in mosses may be difficult (Klavina et al. 2015). Therefore, we additionally measured the extracellular ROS accumulation by DCFH. This dye is a non-selective probe for specific types of ROS, as it can interact with H_2O_2, $OH^{.-}$ and even $ONOO^-$ (Halliwell and Whiteman 2004; Gomes et al. 2005).

It is interesting to note that, in contrast to the ROS level that was observed after treatment with flg22 and elf18, the ROS level after treatment with chitin and PEP was significantly lower (approximately two times), whereas it was comparable between chitin and PEP (slightly higher for chitin) (Ma et al. 2013). In our analysis, the levels of activated ROS in response to treatment with AtPEP1 and chitosan were comparable. Taking into account that chitin is an established immune elicitor, this may indicate quite strong response to PEP treatment in *P.patens* protonemata. The level of extracellular and intracellular ROS in response to StPEP treatment were contrasted. This result could be due to the release of compounds with antioxidant activity into the extracellular space.

Declarations

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