Spatio-temporal patterning of arginyl-tRNA protein transferase (ATE) contributes to gametophytic development in a moss

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Summary

- The importance of the arginyl-tRNA protein transferase (ATE), the enzyme mediating post-translation arginylation of proteins in the N-end rule degradation (NERD) pathway of protein stability, was analysed in Physcomitrella patens and compared to its known functions in other eukaryotes.
- We characterize ATE:GUS reporter lines as well as ATE mutants in P. patens to study the impact and function of arginylation on moss development and physiology.
- ATE protein abundance is spatially and temporally regulated in P. patens by hormones and light and is highly abundant in meristematic cells. Further, the amount of ATE transcript is regulated during abscisic acid signalling and downstream of auxin signalling. Loss-of-function mutants exhibit defects at various levels, most severely in developing gametophores, in chloroplast starch accumulation and senescence. Thus, arginylation is necessary for moss gametophyte development, in contrast to the situation in flowering plants.
- Our analysis further substantiates the conservation of the N-end rule pathway components in land plants and highlights lineage-specific features. We introduce moss as a model system to characterize the role of the NERD pathway as an additional layer of complexity in eukaryotic development.

Introduction

Post-translational modifications alter protein specificity, activity and/or stability and are therefore critical for almost every physiological process (Mann & Jensen, 2003; Saha & Kashina, 2011). While phosphorylation/dephosphorylation and glycosylation are broadly studied mechanisms, other modifications are less well understood (Mann & Jensen, 2003). Among these is the tRNA-dependent addition of arginine to the N-terminus of target proteins. This protein modification is catalysed by the arginyl-tRNA protein transferase (ATE) which is conserved among eukaryotes (Varshavsky, 2011; Gibbs et al., 2014a). Post-translational arginylation affects protein stability via the N-end rule pathway degradation (NERD), originally proposed by Bachmair et al. (1986). Here, proteins bearing N-terminal arginine or other primary destabilizing residues (H, K, Y, W, F, L, I) are ubiquitinated by E3 ubiquitin ligases, the so-called N-recognins, and finally degraded by the proteasome (Graciet et al., 2006; Ciechanover & Stanhill, 2014). Additionally, in yeast and mouse, unacylated N-terminal methionine can be recognized by the corresponding ubiquitin ligases of the N-end rule pathway (Kim et al., 2014). Secondary and tertiary destabilizing residues can be converted to primary destabilizing residues. Tertiary destabilizing residues such as Gln or Asn can be deamidated to Glu or Asp by the N-terminal Gln/Asn amidohydrolases (NTAQ, NTAN) (Graciet & Wellmer, 2010). Further, N-terminal cysteine residues can be oxidized by cysteine oxidases in an oxygen and nitric oxide dependent manner (Gibbs et al., 2014b; Weits et al., 2014). N-terminal oxidized cysteine as well as Glu and Asp are secondary destabilizing residues that can be conjugated with arginine by ATE to primary destabilizing residues. The N-recognin that recognizes aromatic N-termini in Arabidopsis thaliana is PROTEOLYSIS 1 (PRT1, Stary et al., 2003), while the A. thaliana protein PRT6 is specific for the recognition of N-terminal arginine (Garzon et al., 2007). It has been suggested that additional N-recognins in plants exist, as not all N-terminal amino acids with destabilizing effects in plants are recognized by either PRT1 or PRT6 (Graciet & Wellmer, 2010). A schematic overview of this pathway and the involved proteins is depicted in Fig. 1.

Although the N-end rule pathway is conserved among eukaryotes, several differences do exist (Graciet & Wellmer, 2010). For
example, NTAN is found in A. thaliana, while yeast has only one bifunctional enzyme for the deamidation of both, Gln and Asn (Baker & Varshavsky, 1997). In Drosophila melanogaster and in mouse, loss of ATE function leads to embryonic lethality (Kwon et al., 2002). Interestingly, functions of arginylation besides proletosomal targeting via the N-end rule pathway are present in mouse, where internal amino acids (Wong et al., 2007) as well as side chains of glutamate residues (Wang et al., 2014) are arginylated. In contrast to mouse and Drosophila, ATE null mutants of yeast are viable (Balzi et al., 1990). The A. thaliana genome encodes two ATE genes, AtATE1 and AtATE2 (Graciet et al., 2009). Their loss-of-function mutants are viable but show abnormal shoot and leaf development and delayed senescence (Yoshida et al., 2002; Graciet et al., 2009). In addition, atate1ate2 double null mutants are defective in stress- and hormone-related responses to hypoxia, gibberellin and abscisic acid (ABA) (Licauzi et al., 2013). Reoxygenation from hypoxia in A. thaliana is recognized via arginylation-dependent degradation of VII ERF transcription factors subsequent to cysteine oxidation (Gibbs et al., 2011, 2014b; Licauzi et al., 2011). Interesting parallels between oxygen sensing and arginylation were also drawn for mammals (Gibbs et al., 2014a). However, although several attempts in plants were made (Majovsky et al., 2014; Zhang et al., 2015), direct identification of further arginylation targets was mainly achieved in mouse (Wong et al., 2007; Saha & Kashina, 2011; Wang et al., 2011).

During the last years the function of ATE and other NERD pathway components in plant development and physiology received more and more attention (Graciet & Wellmer, 2010; Mendiondo et al., 2015; Riber et al., 2015), whereas its understanding is merely in its infancy, compared with the state of knowledge in mammals (Kashina, 2014).

Here, we employed the moss Physcomitrella patens to investigate ATE function and regulation. This plant has become a model organism for the study of developmental processes and gene functions due to its simple morphology and ability for targeted gene knockout/knock in that is facilitated by a high rate of homologous recombination and the dominant haploid gametophyte (Hohe et al., 2004; Cove et al., 2006; Vidali & Bezanilla, 2012; Bennet et al., 2014). As moss is evolutionarily situated halfway between simple green algae and complex flowering plants (Kenrick & Crane, 1997; Lang et al., 2008), it also offers an evolutionary perspective on gene function. Here, we identify the single-copy PpATE gene, investigate the subcellular localization of the ATE protein as well as tissue-specific patterning in response to different treatments and generate and characterize loss-of-function mutants. Our results suggest the conservation of PpATE function in the dominant generation of land plants and reveal an as yet undescribed function of ATE in starch accumulation.

### Material and Methods

**Plant material and growth conditions**

*Physcomitrella patens* We used Physcomitrella patens (Hedw.) B.S. ecotype ‘Gransden 2004’, the strain that was used for genome sequencing and that was deposited at the International Moss Stock Centre (IMSC, http://www.moss-stock-center.org) under accession number 40001. Plants were grown axenically in agitated liquid Knop medium (Reski & Abel, 1985: 250 mg l\(^{-1}\) KH\(_2\)PO\(_4\), 250 mg l\(^{-1}\) KCl, 250 mg l\(^{-1}\) MgSO\(_4\) \(\times\) 7 H\(_2\)O, 1 g l\(^{-1}\) Ca(NO\(_3\))\(_2\) \(\times\) 4 H\(_2\)O and 12.5 mg l\(^{-1}\) FeSO\(_4\) \(\times\) 7 H\(_2\)O, pH 5.8). Moss cultures were cultivated in a growth chamber under controlled conditions (25°C) with a 16 h : 8 h, light : dark regime at 70 µmol m\(^{-2}\) s\(^{-1}\).

Gametophores were grown in Petri dishes sealed with Parafilm (Roth, Karlsruhe, Germany). For the induction of sporophores, the Petri dishes were transferred to inducing conditions according to Hohe et al. (2002). For dark treatment, the gametophores grew for 6 wk under standard conditions. Subsequently the Petri dishes were placed in a light-tight box in a growth chamber. For red-light treatment, the Petri dishes were subjected to continuous red light (650 nm) at 25°C. For treatment with cytokinin (5 µM 2iP), auxin (1 µM NAA) or ABA (25 µM) (all purchased from Sigma-Aldrich) gametophores grew for 6 wk under standard conditions. Subsequently they were transferred into liquid Knop medium containing the respective substance and cultivated for the stated time under standard conditions.

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**Fig. 1** Schematic overview of the N-end rule pathway in plants (*Arabidopsis thaliana*). Proteins with tertiary destabilizing residues like asparagine (N) or glutamine (Q) have to be deamidated by amidohydrolases (NTAN/NTAQ) to aspartate (D) or glutamate (E) before being recognized by arginyl-tRNA synthetases (PRT1/PRT6) and subsequently degraded via the proteasome. N-terminal cysteine can be oxidized via plant cysteine oxidases (PCO) which can then also be recognized and arginylated by ATE.
**Arabidopsis thaliana** Arabidopsis thaliana (L.) Heynh. wild-type and *ate1ate2* double mutant lines (both Col ecotype) were grown on a mixture of substrate and vermiculite (4:1) for 15 days at 21°C in a long-day (16 h:8 h, light:dark) or a short-day (8 h:16 h, light:dark) phytochamber. First true leaves of the seedlings were taken for transmission electron microscopy (TEM) ultrastructural analysis.

**Construct generation**

For generation of the knockout construct (d:Pp1s333:374-226..376576|ate; in text, d:ate) the homologous parts of *ATE* were amplified from *P. patens* genomic DNA. The homologous parts of *ATE* and the nptII resistance cassette were assembled by triple template PCR (for primers see Supporting Information Table S1). The date mutants were deposited in the IMSC with the accession numbers: 40691 (d:ate no. 1), 40692 (d:ate no. 2), 40693 (d:ate no. 3).

To generate the PpATE:GUS fusion (i:ate:GUS|ate; in text, ATE:GUS), genomic fragments of *PpATE* were amplified from *P. patens* genomic DNA. While amplifying the 5′ homologous fragment the original stop codon of PpATE was omitted. The homologous parts of *PpATE* and the coding sequence (CDS) of β-glucuronidase (GUS) were subsequently assembled by triple template PCR (for primer see Table S1). This assembly inserted the GUS CDS replacing the original PpATE stop codon. The ATE:GUS fusion strains were deposited in the IMSC with the accession numbers: 40964 (ATE:GUS no. 3), 40965 (ATE:GUS no. 9), 40966 (ATE:GUS no. 14).

To generate the PpATE:GFP fusion, the coding sequence of *PpATE* (without stop codon) was amplified from *P. patens* cDNA (for primer see Table S1). The sequence was cloned into a plasmid containing the endogenous act5-promoter (Weise et al., 2003, 2014) and the sequence of green fluorescent protein (GFP) (Fig. S1).

**Protoplast transformation**

Transformation was carried out as described previously (Hohe & Reski, 2002; Hohe et al., 2004). Plates containing protoplasts were kept for 24 h in the dark and subsequently transferred to normal culture conditions for 9 days. After this, protoplasts were distributed on cellophane-covered Knop-agar plates at 1 ml per plate and incubated for 3 days. The cellophane sheets were then transferred to Knop-agar containing 12.5 mg l−1 geneticin (G418) for 2 weeks. Afterwards the cellophane sheets were transferred to Knop-agar plates without antibiotics for 2 weeks. Subsequently the sheets were transferred on Knop-agar plates containing G418 for 2 weeks. Surviving plants were transferred to Knop-agar plates without cellophane and screened by PCR. Confirmed deletion mutants were grown under standard conditions and compared to wild-type.

The PpATE:GUS fusion construct does not contain a resistance cassette and was therefore co-transfected with a plasmid containing an nptII resistance cassette (pBSNNEV) (Mueller et al., 2014). The protoplasts were treated as described earlier but were subsequently subjected to a continuous selection of 4 weeks.

**Reverse transcriptase PCR**

After RNA-isolation using the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany) and cDNA synthesis from 2 μg total RNA using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA), reverse transcriptase (RT)-PCR was performed to analyse the presence or absence of the *PpATE* transcript in the transgenics (for primer see Table S1).

**Quantitative RT-PCR analysis**

RNA was extracted from three biological replicates of wild-type (WT) protonema, gametophores or hormone-treated ATE:GUS gametophores using the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany), treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA), and reverse-transcribed into first-strand cDNA by Taq Man Reverse Transcription Reagents (Life Technologies) using random hexamer primer. Real-time PCR was performed on a Roche 480 Light Cycler using gene-specific primer and Light Cycler 480 SYBR Green I Master (Hoffmann-La Roche, Basel, Switzerland) according to the manufacturer’s instructions. The constitutively expressed genes alpha tubulin 1 (TUA, Pp1s341_23V6.1) and ‘flower pigmentation protein’ (LWD, Pp1s92_5V6.1) were used for normalization (Kubo et al., 2013). Expression levels of *PpATE* in protonema and gametophores were calculated in relation to the transcript abundance of a plasmid containing *PpATE* employing relative and absolute quantification with efficiency correction (Whelan et al., 2003; Lee et al., 2006). Quantitative (q)RT was performed using biological and technical triplicates. Primer sequences are listed in Table S1.

Relative expression levels of *PpATE* in the hormone-treated ATE:GUS line were calculated according to Livak & Schmittgen (2001). TUA and LWD (see earlier) were used as references to calculate ΔCp values for each sample. Each 2−ΔΔCp value was calculated for every treatment using the ΔCp values of the treatment and the mock-treated control (200 mM KOH) which was inoculated and harvested in parallel to each treatment time point. The primers for *Dehydrin* (Pp1s442_22V6.1) and *IAA2* (Pp1s73_11V6.1) were according to Lavy et al. (2012), Prigge et al. (2010) respectively, and are listed in Table S1.

**Light microscopy**

Bright-field microscopy was performed using the inverse microscope Axioplan (Carl Zeiss AG, Oberkochen, Germany) or the stereomicroscope SZX7 (Olympus, Shinjuku, Japan). The images were taken with a charge-coupled device (CCD) camera (AxioCam MrC5 or Icc1; Zeiss).
Confocal laser scanning microscopy

Images were taken on a Zeiss LSM 510 with inverted microscope Axiosvert 200. Water immersion objectives LD LCI Plan-Apochromat ×25/0.8 DIC Imm Korr (UV) VIS-IR and the C-Apochromat ×63/1.2W Korr. were used. GFP and chlorophyll were excited with an argon laser (488 nm). Chlorophyll autofluorescence was detected above 650 nm, GFP fluorescence between 505 and 530 nm.

Fluorescence signals are false-coloured in red (chlorophyll) and green (GFP). Three-dimensional (3D)-reconstructions of z-stacks were performed with the Imaris software (Bitplane, Zurich, Switzerland). Screenshots were taken from 3D reconstructions using IrfanView (http://www.irfanview.de). Confocal planes were exported from the ZEN2010 software (Zeiss) and further processed with GIMP (http://www.gimp.org/).

Transmission electron microscopy

Plants were embedded, stained and analysed according to Abel et al. (1989) with modifications. Tissues were fixed for 3 h in 100 mM cacodylate buffer containing 2.5% glutaraldehyde (Sigma-Aldrich), and post-fixed with 1% OsO4 for 2 h at 4°C. Dehydration through a graded series of ethanol was performed before embedding in Epon 812 resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM10 (80 kV) electron microscope equipped with a Bioscan Camera Model 792. Images were recorded with the Digital Micrograph software (Gatan, Pleaston, CA, USA).

Flow cytometry analysis

Suspensions of intact nuclei were prepared from c. 5–30 mg of fresh plant material by chopping with a razor blade in a Petri dish with 2 ml of a 4',6-diamidino-2-phenylindole (DAPI)-containing buffer (0.01 mg l⁻¹ DAPI, 1.07 g l⁻¹ MgCl₂ 6H₂O, 5 g l⁻¹ NaCl, 21.11 g l⁻¹ trisaminomethane and 1 ml Triton) (Schween et al., 2003). The solution was filtered through a sieve of 30 μm pore size before measuring the fluorescence intensity with a Cyflow® Space flow cytometry system (Partec, Münster, Germany).

Histochemical GUS-assay

Tissues were incubated in 100 μl of X-gluc solution (1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, 100 mM sodium phosphate, pH 7.0) at 37°C for 12–16 h (Jefferson, 1987). Fixation was done in 200 μl 5% formalin for 10 min followed by incubation for 10 min in 200 μl 5% acetic acid. Pigments were removed by serial incubations in 30%, 50%, 70%, and finally 99% ethanol.

Chlorophyll content measurement

Chlorophylls of 50–100 mg FW moss were extracted in 2 ml 80% acetone for 15 min (Wiedemann et al., 2007). After centrifugation for 5 min and 1:10 dilution, the absorption of Chl a+b was measured at 664 and 647 nm, respectively. The chlorophyll content (mg Chl g⁻¹ DW) was calculated using the formula: \((A_{664} \times 0.00802 + A_{647} \times 0.0202) \times \text{ml acetone g}^{-1} \text{DW}\).

Starch quantification and staining

Protonema was grown for 7 d. Samples were harvested and frozen in liquid nitrogen. Extraction and quantification of starch was performed using a starch assay kit (Sigma-Aldrich; SA-20). Starch was extracted using the DMSO/HCl method described by the manufacturer but with 1/4 of the volume in the protocol. The assay was performed according to the manufacturer’s protocol.

A protonema sample was transferred to 1 ml of Lugol’s solution (Smith & Zeeman, 2006). After 2 min of incubation, the sample was washed once with water and was subsequently examined by bright-field microscopy.

Staining of oil bodies

Staining of oil bodies was performed using saturated Sudan Black B in 70% propylene glycol for 20 min as described in Huang et al. (2009).

Comparative and phylogenetic analysis of NERD pathway components

Based on gene family definitions established previously (Zimmer et al., 2013), protein sequences of family members were aligned using MAFFT L-INSI (Katoh & Standley, 2013), mapped to CDS using a custom Perl script and used to infer species-tree informed maximum likelihood phylogenies using PHYML as implemented by the TreeBest algorithm (http://treesoft.sourceforge.net/treebest.shtml) with subsequent tree reconciliation analysis to infer duplication events based on a species tree established previously (Lang et al., 2008). Taxonomic profiles of all families were analysed using the web BLAST interfaces of the UniProt and OneKP databases using the BLASTP algorithm (Altschul et al., 1997). In addition, the existence of the NTAN family in Physcomitrella was probed by scanning all predicted proteins (http://www.cosmoss.org) using the respective PFAM (PF14736) and PANTHER (PTHR12498) HMM domain profiles with the hmmssearch algorithm implemented in HMMER3 (Eddy, 2011).

Statistical analysis

Statistical analysis of chlorophyll contents (mg chlorophyll/g dry weight) was performed using R programming language. Resulting chlorophyll ratios were tested for deviation from normality using the Shapiro–Wilks test (Royston, 1982). As no significant deviation from normality was observed, data were analysed using the generalised linear model (glm/lm) and parametric factorial ANOVA (aov) with subsequent Tukey’s range tests (Tukey-HSD).
Statistical analysis of the measurements of starch content, cell width as well as cell length was performed using an unpaired t-test (http://www.graphpad.com/quickcalcs/ttest1/).

Results

The *Physcomitrella ATE* gene and NERD components

A BLAST search using the *Arabidopsis ATE1* (AT5G05700.1) and *ATE2* (AT3G11240.1) genes against the *P. patens* genome V1.6 (Zimmer et al., 2013) revealed a single gene coding for *ATE* (Pp1s333_56V6.1) and a highly decayed, pseudogenic locus on scaffold_235 (positions 30416–31530, reverse strand), covering c. 65% of the *ATE* CDS split across multiple nonconsecutive alignment blocks at an average sequence identity of 51%. The intact *P. patens* *ATE* gene comprises 10 exons, eight of which constitute the CDS (Fig. S2). This gene model was verified by amplification of the *ATE* CDS using cDNA from protonema and gametophore RNA (Fig. S3). The deduced ATE protein is composed of 704 amino acids and has a predicted molecular weight of 77.76 kDa.

Using the all-versus-all protein homology clustering of 27 sequenced Archaeplastida species published recently (Zimmer et al., 2013) we found homologues for *ATE* as well as for other NERD pathway components such as NTAQ, PRT1 and PRT6 in all other surveyed land plants. However, no homologues were identified for NTAN in *P. patens*. We then inferred phylogenetic trees for the members of the *ATE* (Fig. S4), NTAN (Fig. S5), NTAQ (Fig. S6), PRT6 (Fig. S7) and PRT1 (Fig. S8) families in *Chlamydomonas reinhardii*, *P. patens*, *Selaginella moellendorfii*, *Oryza sativa*, *A. thaliana* and *Populus trichocarpa*. We confirm that NTAQs are encoded by single-copy genes (the event in *Selaginella* represents two alleles of the sequenced haplotypes) and that NTAN is not present in *P. patens* in agreement with the findings of Graciet et al. (2010). Our BLAST searches against the OneKP transcriptomic database (Matasci et al., 2014) revealed NTAN homologues only from liverworts (Ptilidiaceae, Porellaceae, Metzgeriaceae, Marchantiaceae) and two peat mosses (*Sphagnum palustre* and *Sphagnum leucórum*) in the paraphyletic group of bryophytes (mosses, liverworts and hornworts), suggesting a loss of NTAN in the moss lineage, subsequent to the divergence of peat mosses. No hits were observed for hornworts. For the PRT1, PRT6 and ATE gene families our phylogenetic analysis proposes evidence of lineage-specific duplication events.

Subcellular and tissue-specific localization

To evaluate the subcellular localization of the ATE protein, *P. patens* protoplasts were transfected with a *PpATE:GFP* fusion construct driven by the moss *ACTIN5* promoter (Mueller et al., 2014). Confocal microscopy analysis revealed the GFP signal in the cytoplasm and in the nucleus (Fig. 2a).

To study tissue-specific patterning of ATE protein abundance, reporter lines were generated by gene targeting. A knock-in construct was generated in order to insert the CDS of GUS in frame at the end of the coding sequence of the endogenous *ATE* gene via homologous recombination, replacing the original stop codon (Fig. S9). Following protoplast transformation, three independent stable reporter lines were obtained. FCM analysis confirmed the genomic integrity and haploidy of these lines (Fig. S10). All subsequent analyses were carried out using all three lines and showed consistent results (Figs S11, S12).

No GUS signal was detectable in the filamentous protonema of the reporter lines. Instead, staining was observed in developing buds, that is, after the developmental switch from tip growth to three-dimensional growth with a three-faced apical cell (Fig. 2b). These buds developed into leafy shoots that showed GUS staining both in the lower part of the stem and in the apical cells (Fig. 2c). While no GUS signal was detectable in all leaves of younger gametophores (3–4 wk), most leaves of older

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Fig. 2 Intracellular and tissue-specific localization of arginyl-tRNA protein transferase (ATE) in *Physcomitrella patens*. (a) Intracellular localization of ATE in transiently transfected *P. patens* protoplast using Act5:ATE:GFP. Left panel, chlorophyll autofluorescence; middle panel, green fluorescent protein fluorescence; right panel, merged picture. Bar, 5 µm. (b–d) ATE:GUS no. 9 reporter line. (b) Stained bud with first leaflet, bud initial (arrowhead) and younger bud (insets). β-Glucuronidase (GUS) staining is present in buds forming first leaf initials. Bar, 10 µm (insets: bar, 30 µm). (c) Staining in a 4-wk-old gametophore; staining is visible in the apex and the lower part of the stem; no staining is visible in the leaflets (inset). Bar, 500 µm. (d) Staining in a 12-wk-old gametophore; staining is visible in the apex, in the stem and additionally in the leaflets (inset). Bar, 500 µm.
gametophores showed a clear signal (Fig. 2d). Thus, ATE-GUS accumulation displays a tissue-specific and an age-dependent pattern in *P. patens*.

To validate these findings, ATE transcript levels in protonema and gametophores of WT plants were determined. According to quantitative RT-PCR protonema contains 0.24 ± 0.07 copies of ATE per 15 ng cDNA, while shoots contain c. 10 times more transcript, that is, 2.53 ± 1.10 copies of ATE per 15 ng cDNA (*P* < 0.03).

**Physiological control of ATE protein abundance and patterning**

As the *A. thaliana* *ate1*ate2 double mutants show delayed senescence in darkness (Yoshida *et al.* 2002) and are hypersensitive to ABA (Holman *et al.*, 2009), gametophores of the three PpATE:GUS reporter lines were subjected to darkness and were treated with ABA, respectively (Fig. 3a–c). When left in darkness for 3 d the GUS signal increased markedly. Compared with untreated controls (Fig. 3a), not only the lower part of the stem but the whole stem was stained dark blue. The extent of staining increased from 3 to 7 d in darkness and also included the leaves (Fig. 3c). Incubation with 25 μM ABA for 3 d resulted in no change in the staining of the shoot apical cells but the GUS staining of the lower part of the stem vanished completely. After 7 d of ABA treatment strong GUS staining was visible in the upper part of the shoots and the lower part of the stem also showed a dark blue signal (Fig. 3b).

In plants grown in red light for 3 d the GUS signal increased markedly. Similar to the pattern in dark-treated plants, the staining covered the whole stem. After 7 d in red light the leaves began to display blue staining (Fig. 3c,d). Neither 3 nor 7 d of incubation in cytokinin (5 μM 2iP) altered the staining pattern compared with untreated shoots (Fig. 3e). Incubation with auxin (1 μM NAA) for 3 d resulted in a shoot elongation (Ben-net *et al.*, 2014), which was most pronounced at the apex. This segment showed a weak GUS staining in addition to the staining in the lower part of the stem. After 7 d the staining of the elongated apex increased further. In addition, leaves at the apex began to develop a light blue staining (Fig. 3f). Thus, spatio-temporal ATE:GUS patterning in moss shoots is modulated by light, auxin and ABA, but probably not by cytokinin.

As the response of the ATE protein level to the developmentally important hormones ABA, auxin (NAA) and cytokinin (2iP) was in the range of days, we additionally investigated the responsiveness of the *ATE* transcript to these treatments, including shorter treatment periods via qRT-PCR (Fig. 4). We used *Dehydrin* (Pp1s442_22V6.1, ABA responsive, Lavy *et al.*, 2012) and *IAA2* (responsive to auxin and cytokinin, Pp1s73_11V6.1, Prigge *et al.*, 2010) as treatment controls. Here, *Dehydrin* was >50-fold induced after 6 h treatment with ABA but then decreased again to the level of the untreated control until day 7 of the treatment (Fig. 4a). Similarly, *ATE* expression level was induced three-fold after 6 h and then decreased also to the level of the untreated control. *IAA2* transcript was increased almost constantly two-fold in response to the auxin treatment but was almost unaffected by cytokinin treatment (Fig. 4b,c), whereas *ATE* expression was two-fold induced after 3 d and seven- to eight-fold after 7 d of auxin application. In concordance with the results of the ATE:GUS reporter lines, *ATE* expression was unaffected by the application of cytokinin (Fig. 4b).

**Fig. 3** Different treatments influence abundance and localization of arginyl-tRNA protein transferase (ATE) in Physcomitrella patens. The ATE-GUS no. 9 line was subjected to different treatments for 3 d and 7 d, as indicated. Bars, 500 μm. (a) Untreated control, the apex and the lower part of the stem are stained. (b) Abscisic acid (ABA) treatment: after a 3 d treatment with 25 μM ABA, β-glucuronidase (GUS) staining is visible in the apex, but not in the stem. After 7 d of ABA treatment intense staining is visible in the upper part of the gametophore and also in the lower part of the stem. (c) Grown in darkness for 3 or 7 d: the staining is visible all over the gametophore. (d) Grown in red light (650 nm) for 3 or 7 d: the staining is visible all over the gametophore. (e) Cytokinin (5 μM 2iP) treatment: no visible changes. (f) Auxin (1 μM NAA) treatment for 3 or 7 d: staining is visible in the elongated part of the apex and in the lower part of the stem.
Fig. 4 Quantitative real-time PCR results of hormone-treated gametophores of *Physcomitrella patens*. The bars represent the mean of the $2^{-\Delta\Delta C_T}$ values (calculated using $\Delta C_T$ values from treatment and mock-treated control samples) for the biological triplicates for every time point. Error bars represent the ± SD. Dehydrin (Pp1s442_22V6.1) and IAA2 (Pp1s73_11V6.1) represent treatment controls for abscisic acid (ABA) (Lavy et al., 2012), auxin and cytokinin respectively (Prigge et al., 2010). (a) $2^{-\Delta\Delta C_T}$ values for ATE and Dehydrin in the ABA (25 μM) treatment. (b) $2^{-\Delta\Delta C_T}$ values for ATE and IAA2 in the Cytokinin (5 μM 2iP) treatment. (c) $2^{-\Delta\Delta C_T}$ values for ATE and IAA2 in the auxin (1 μM NAA) treatment.

Fig. 5 Phenotypical analysis of *Physcomitrella patens* d|ate mutant plants. (a–d) *P. patens* grown for 4 months on Knop medium. (a) Wild-type (WT); (b–d) d|ate mutant plants no. 1–3. (e) Gametophores of *P. patens* wild-type (left panel) and d|ate (middle and right panels). Bar, 500 μm. (f) Leaf of d|ate (bar, 200 μm) and wild-type (inset). Bar, 200 μm. Leaves were selected from comparable positions of the leaf series. (g) d|ate and wild-type (inset, same scale) chloronema cells. Bar, 20 μm. (h) d|ate and wild-type (inset) caulonema cell. Bars, 20 μm. (i) Comparison between WT and d|ate chloronema cell size (n = 60). Error bars indicate the ± SD. Asterisks mark significant differences (*, P < 0.01). (j) Comparison between WT and d|ate caulonema cell size (n = 20). Error bars indicate the ± SD. Asterisks mark significant differences (*, P < 0.01). (k, l) Unusual cell division planes in d|ate protonema filaments. Bars, 10 μm.
Generation of ATE null mutants

Null mutants were generated to infer ATE function in P. patens. For this, a knockout construct was created to disrupt the target gene and to confer resistance to neomycin (Fig. S13a). From all antibiotic-resistant plants screened, three independent knockout lines were isolated. The homologous integration of the gene disruption cassette at the target locus was confirmed. The plants consequently lack the ATE transcript (Fig. S13b). FCM analysis confirmed haploidy of these three mutant lines (dATE no. 1, dATE no. 2, dATE no. 3), that were subsequently examined (Fig. S10).

ATE is necessary for normal growth and development

Targeted knockout of the ATE gene severely affected development and morphology. In contrast to WT the mutants were developmentally arrested in the protonema stage for several months (Fig. 5a–d). Although bud-like structures were formed after 2 wk, mutants developed shoots only after 4 months. These shoots were dwarfed and malformed (Fig. 5e). Compared with WT, all mutant leaves were nonserrated and lack the midrib (Fig. 5f). While WT plants had already completed their life cycle, mutants grown under inducing conditions (Hohe et al., 2002) for several months showed no formation of sexual organs.

Furthermore, mutant protonema filaments did not only grow horizontally along the surface of the medium, but also vertically upwards. This partially negative gravitropic growth resulted in an irregular 3D-morphology of the colonies (Fig. 5). However, according to a classical test with dark-grown caulonema, gravitropism was not affected by the loss of ATE function (Fig. S14). Interestingly, WT plants lost their chlorophyll completely after 2 wk growth in the dark, whereas the dATE plants remained green, indicating delayed senescence in the mutants. It was also evident that the mutants grew considerably slower than WT (Fig. S14), both under a normal light : dark cycle and in the dark.

Cell division and expansion

Inspection of mutant protonema by light microscopy revealed significantly altered chloronema (Fig. 5g) and caulonema (Fig. 5h) cells compared with the wild-type. On average, dATE chloronema cells were significantly shorter but wider than WT cells (Fig. 5i). Mutant caulonema cells were longer and wider than WT cells (Fig. 5j).

Besides, unusual cell division planes occurred in some mutant filaments. Instead of perpendicular or oblique cell walls, a mixture of division planes occurred, resulting in an unstructured pattern of small cells with a triangular or rhomboid-like shape (Fig. 5k,l).

Chloroplast morphology, chlorophyll content and starch accumulation

Cells of ATE null mutants appeared more densely packed with chloroplasts and these often showed altered morphology, varying from altered internal structures to highly distorted chloroplasts (Fig. 6a–c). Furthermore, chlorophyll contents of WT and dATE protonema cells differed significantly (Fig. 6d). Statistical analysis showed that the dATE mutants have a 3.22 ± 0.08 (SD) times lower chlorophyll content than WT (Fig. 6d).

Analysis of chloroplast ultrastructure by TEM revealed a massive accumulation of starch in mutant plastids (Fig. 7). The biochemical quantification of starch showed that WT plants contained 0.83 ± 0.29 mg starch g⁻¹ FW. Mutant plants had a twelve times higher starch content at 9.96 ± 1.13 mg starch g⁻¹ FW (Fig. S15). The abundance of starch was dependent on the age of the cell with younger protonema cells, for example actively dividing tip cells, containing less starch than older cells (Fig. S16). We additionally investigated whether an accumulation of oil bodies was present in dATE plants but we did not observe any abnormality (Fig. S17).

The addition of sugars (0.15 M glucose or sucrose) did not improve the growth rate or the formation of gametophores in dATE plants, either in a light : dark environment (Fig. S18a), or in continuous light (Fig. S18b).

The descriptions of the A. thaliana dATE mutants did not mention a similar starch overaccumulation (Graciet et al., 2009). Therefore, we reanalysed the chloroplast ultrastructure of the A. thaliana dATEdATE double mutant and found no difference in chloroplast ultrastructure between WT and mutants (Fig. S19).
In the work presented here, two distinct patterning in the dominant generation ATE localization and conservation of spatio-temporal Discussion

ATE localization and conservation of spatio-temporal patterning in the dominant generation

In the work presented here, two distinct ATE genes were identified in moss, one of which represents a highly fragmentary locus without transcript evidence. Thus, either the ATE gene duplicated in the past and the second functional copy was lost, or the gene fragment resulted from a recombination event. From the full-length ATE gene (Pp1s333_56V6.1), we validated full-length transcripts encoding a 704 amino acid protein with a predicted molecular weight of 77.76 kDa.

Fluorescence of an ATE:GFP fusion protein was detectable in nuclei and in the cytoplasm, but not in other organelles, a finding that is in agreement with observations on ATE in mouse (Wang et al., 2011). Although three independent prediction tools (NucPred, NLS (nuclear localization signal) Mapper and NLS-tradamus: Brameier et al., 2007; Ba et al., 2009; Lin & Hu, 2013) did not predict an NLS, we exclude passive diffusion of the 104 kDa ATE:GFP fusion protein into the nucleus as this is limited to c. 40 kDa (Gerace, 1992).

The presence of ATE in the cytosol and the nucleus as well as the low gene copy number (Fig. S4) further substantiate and extend the current knowledge about conservation of ATE between plants and mammals (Graciet et al., 2006; Graciet & Wellmer, 2010).

Graciet et al. (2009) reported on the tissue-specific localization of ATE in A. thaliana. Here, we established and analysed PpATE:GUS knock-in moss reporter lines and demonstrated a tissue-specific and dynamic localization of the fusion protein in P. patens. During protonema development GUS staining was visible after the developmental switch from tip growth to the growth from a three-faced apical cell. We found a high PpATE:GUS accumulation in developing buds and in the shoot apex. This pattern is consistent with that of AtATE:GUS in root and shoot apices of A. thaliana (Graciet et al., 2009) and thus points to an evolutionary conserved role of ATE in meristematic and differentiating tissues. This implied conserved action of ATE in meristematic cells of both the P. patens gametophyte and the A. thaliana sporophyte further fuels the discussion about conserved molecular networks acting in meristematic cells in gametophytes and sporophytes (Yasumura et al., 2005; Menand et al., 2007; Mosquna et al., 2009; Landberg et al., 2013; Sakakibara et al., 2013; Noy-Malka et al., 2014). It is common belief that meristematic cells of both generations are governed by distinct underlying developmental mechanisms, requiring the action of KNOX1 genes in sporophytes, but not in gametophytes (Sakakibara et al., 2013). Interestingly, the A. thaliana ate1ate2 mutants show ectopic expression of the KNOX1 gene BREVIPEDICELLUS in leaves (Graciet et al., 2009), hinting at a possible involvement of arginylation in this key developmental pathway in the sporophyte of flowering plants. In moss, gametophyte development is severely affected in P. patens date mutants, an effect that is presumably not mediated via an effect on KNOX1 pathways (Sakakibara et al., 2008).

Additionally, ATE involvement in age-related processes might be conserved between gametophytes of moss and sporophytes of flowering plants, as the PpATE:GUS protein accumulated with increasing age in the leafy moss shoots, similar to an increased AtATE1:GUS staining in mature leaves of A. thaliana (Graciet et al., 2009). Further, while the overall delay of development observable in the moss mutants is more severe than that of the A. thaliana mutants, the ATE loss-of-function mutants in both A. thaliana and P. patens result in altered leaf morphology.

These findings support the concept of a conserved role of ATE in meristematic tissues of the dominant generation in the life cycle of embryophytes, but hint at the existence of distinct target proteins or distinct timing or directionality of these targets in different species.

ATE affects growth and the juvenile-to-adult gametophyte transition

According to the ATE:GUS reporter line, ATE was hardly detected in protonema, in agreement with the qRT-PCR analysis of the ATE transcript indicating a level of only 10% of that in shoots. Nevertheless, the date null mutants showed abnormal protonema cell elongation and orientation of division planes indicating that ATE function is present in protonema and crucial for normal protonema growth. In animal cells, arginylation occurs on nuclear and on cytosolic proteins, including actin and tubulin (Wong et al., 2007; Saha & Kashina, 2011). Interestingly, P. patens mutants with defects in actin cytoskeleton organization (e.g. ARP3a and BRICK1 mutants) show a phenotype...
(Finka et al., 2008; Perroud & Quatrano, 2008) similar to our observed d|ate protonema phenotype, raising the question whether altered tip growth and cell elongation in the d|ate mutants might be caused by a defective arginylation of the actin cytoskeleton.

An important switch in moss development is the transition from tip growth to growth with a three-faced apical cell, the so-called bud (Reski, 1998; Harrison et al., 2009). These buds mark the transition from the juvenile to the adult gametophyte (Saleh et al., 2011) and further develop into leafy shoots. This developmental transition is accompanied by profound metabolic changes (Erxleben et al., 2012; Beike et al., 2013). Bud formation can be induced by application of cytokinin (Reski & Abel, 1985). Compared with protonema the amount of PpATE:GUS was enhanced in buds, resulting in a visible GUS staining. Consistent with the increased abundance of PpATE:GUS during this transition, the loss of the protein in the d|ate mutants resulted in the elimination of normal shoot development. Few and very stunted shoots with altered leaf morphology occurred on mutant colonies only after a prolonged cultivation of several months. These shoots never developed sexual organs and thus remained sterile. As the developmental arrest could be partially overcome by ageing, age-related factors may to some extent bypass the loss of PpATE function. Because this loss probably leads to the accumulation of proteins due to reduced proteasomal degradation (Graciet & Wellmer, 2010), protein levels in the mutants might additionally be adjusted by changes in gene expression, or might follow an alternative degradation pathway. The importance of protein degradation for development is supported by loss-of-function P. patens mutants of another component of the protein degradation system, the MCB1 of the 26S proteasome, which have developmental defects (Girod et al., 1999) similar to the d|ate mutants described here.

ATE gene expression and protein abundance are independently regulated

Various treatments affected PpATE:GUS accumulation and patterning in P. patens. The most drastic effect occurred after cultivation in darkness. We suggest that arginylation is involved in the various metabolic changes that plants undergo in order to adapt to the absence of light. Bryophytes grown in darkness accumulate less chlorophyll than light-grown controls (Takio et al., 1988) implicating a downregulation of photosynthesis in the dark. In addition, moss shoots show a certain level of etiolation including elongation in the dark (Bierfreund et al., 2004; Possart & Hiltbrunner, 2013).

Three major hormone classes (ABA, auxin, cytokinin) have distinct functions in moss development (Decker et al., 2006). Related to the severe developmental phenotype caused by the knockout of ATE, we were interested if ATE protein abundance and ATE expression level was influenced by the application of these hormones.

We found that cytokinin had no, auxin had little and ABA had the most pronounced effect on PpATE:GUS protein accumulation, although only after several days of hormone stimulation. In agreement with the PpATE:GUS pattern in the cytokinin treatment, ATE expression was also unaffected. Although the control gene for the cytokinin response was also almost unduced (Fig. 4b), it is obvious that the cytokinin application was perceived as several stained buds on the protonema (Fig. 3e) were present after 7 d of hormone treatment.

Six hours after ABA application the ATE transcript accumulation was induced but then decreased again which is the opposite pattern to the observed GUS staining. This suggests the existence of further post-transcriptional regulatory mechanisms for ATE, as well as an involvement of ATE in the early response to ABA during signalling, and a late response and repatterning of the ATE protein under ABA treatment. By contrast, ATE expression was first increased after 3 d of auxin application and further increased after 7 d which is in agreement with the GUS staining, although the increase of GUS staining towards 7 d is not as pronounced as the increase of the ATE transcript. Interestingly, Graciet et al. (2009) showed that ATE in A. thaliana acts independently of auxin which is in contrast to our findings. We found that ATE transcript and protein abundance are altered in response to auxin application. Further, ATE function might be important during the late response involving morphological changes, as GUS staining was increased in the elongated apex after 3 and 7 d.

In summary, these results suggest that the reactions to hormone treatments which lead to specific growth responses and cellular differentiation, involve a remodulation of ATE abundance. This is especially interesting, as ATE is present in the apex of moss gametophores. Strikingly, this hormone-responsive pattern is consistent with the relevance of the ubiquitin-proteasome system (UPS) for the perception of hormone signals. While auxin and ABA signals are perceived via the UPS, cytokinin signals are not (Santner & Estelle, 2010), raising the question whether ATE function and thus the N-end rule pathway provides an additional layer of complexity in plant hormone signalling networks. It is also worthwhile pointing out that according to our findings ATE transcript and protein abundance do not necessarily correlate but can be regulated independently during different stimuli.

Regulation of energy homeostasis by ATE

Besides the strong developmental defects of d|ate plants, we observed an extreme overaccumulation of starch in plastids. In flowering plants, starch granules are formed during the day and are degraded during the night (Stitt & Zeeman, 2012). In A. thaliana, mutants with defects in starch metabolism display a delayed juvenile-to-adult transition, putatively due to reduced carbohydrate availability (Matsoukas et al., 2013). The P. patens d|ate mutants described here also show a delayed juvenile-to-adult transition (although in the gametophyte) and an overaccumulation of starch. This abnormally high starch content was most pronounced in differentiated subapical cells, as evidenced by a staining gradient in protonema filaments. Neither the exogenous supply with sugars nor the cultivation in continuous light could complement this mutant phenotype, indicating that the developmental delay is not caused by an insufficient availability of carbohydrates, but rather by a deregulation in energy usage.
This is further substantiated by the delayed senescence phenotype of the mutants, as a low growth rate may be beneficial under starvation conditions in darkness. Notably, several connections between developmental and metabolic regulators and N-end rule pathway mutants exist in plants (Graciet et al., 2009; Geigenberger, 2014).

Although we could not detect any abnormality in starch formation in the A. thaliana ate1ATE2 plants, a recently described PRT6 mutant greening after extended darkness 1 (ged1) as well as the prt6 mutants in A. thaliana show an increased starch accumulation compared with the WT after 24 h submergence in the dark (Riber et al., 2015). Hence, in A. thaliana the N-end rule pathway is linked to the metabolic adjustments made under submergence. Sugar availability under hypoxic conditions such as submergence is coordinated by the highly conserved SNF1-related kinase SnRK1 (Geigenberger, 2014). Whereas SnRK1 repression in pea lead to the concomitant downregulation of genes for starch synthase as well as the starch branching enzyme (Radchuk et al., 2010), an overexpression of SnRK1 in potato tuber lead to increased starch accumulation (McKibbin et al., 2006). However, overexpression of SnRK1 in A. thaliana did not result in enhanced starch accumulation (Jossier et al., 2009) which is in agreement with the fact that an increased starch content in A. thaliana seems to be linked to specific conditions, such as hypoxia. Connections between starch accumulation and development are also known for A. thaliana mutants overexpressing the key metabolic regulatory transcription factor LEAFY COTYLEDON2 (LEC2) (Feeney et al., 2013). Besides an increased starch accumulation, the overexpression of LEC2 induced accumulation of oil bodies, an effect that was also observed in ate1ATE2 plants of A. thaliana (Holman et al., 2009) but not in the ATE moss mutants described here (Fig. S17). However, there is no known homologue of LEC2 in P. patens (Zimmer et al., 2013). Hence, metabolic control differs between tissues and species, but has been repeatedly linked to the N-end rule pathway.

Notably, there are SNF1-related protein kinase mutants in moss which show an opposite phenotype to the moss dAte mutants. Double knockout of the two homologous protein kinases (SNF1a and SNF1b) in P. patens resulted in mutants that did not accumulate starch and showed premature senescence and were unable to grow without external energy (Thelander et al., 2004). This was interpreted as being continuously trapped in a ‘high energy growth mode’. We therefore suggest that the observed overaccumulation of starch in the moss dAte mutants might be the result of an altered SnRK1 abundance via direct or indirect control by the arginylation branch of the N-end rule pathway and thus the dAte mutants might be trapped in a ‘low energy growth mode’, comparable to the hxk1 mutants, also identified in P. patens (Olsson et al., 2003; Thelander et al., 2005).

In addition to a role during metabolism under submergence, a role of the N-end rule pathway in the recovery from hypoxia is well characterized, as the only known arginylation targets in plants are ethylene responsive (ERF) group VII transcription factors. In A. thaliana the hypoxic response is turned off by rapid proteasomal degradation of these group VII transcription factors, due to the oxidation of their N-terminal cysteine followed by arginylation mediated by ATE (Gibbs et al., 2011, 2014a; Licausi et al., 2011). In fact, the role of arginylation in sensing oxygen and nitric oxide seems to be conserved among eukaryotes (Gibbs et al., 2014b). Compared with A. thaliana, P. patens is much more resistant to submergence-derived hypoxia, as it is able to survive several months of complete submergence (Yasumura et al., 2012). Further, gene expression analysis of different cultivation forms such as liquid or solid media in P. patens revealed that gene expression patterns are related to the developmental stage and not to the cultivation condition (Hiss et al., 2014). Although the major components of ethylene signalling are conserved among land plants, specific differences exist. Based on phylogenetic reconstruction of the ERF super-families (Yasumura et al., 2012; Hiss et al., 2014), there are no clear group VII orthologues in P. patens, although this group of transcription factors seems to be conserved among vascular plants (Licausi et al., 2011).

Conclusively, we suggest that the arginylation branch of the N-end rule pathway may represent a general regulator of energy storage and availability in plants, and speculate that SNF kinases and key transcription factors are candidate targets of the N-end rule pathway.

Taken together, our analysis further substantiates the evolutionary conservation of the N-end rule pathway in eukaryotes (Graciet et al., 2010), but reveals additional lineage-specific features like the loss of NTAN with concomitant expansion of the PRT1/PRT6 families in the moss lineage.

Experimental evidence substantiates these findings: among others, (1) the conserved subcellular location of the ATE protein in nuclei and cytoplasm of moss and mouse are indicative for evolutionary conservation, while (2) the starch overaccumulation phenotype in Physcomitrella but not in Arabidopsis highlight lineage-specific differences.

While the key players responsible for the phenotypes of dAte plants in moss await identification, we infer that ATE is a main regulator of energy homeostasis and development in the gametophyte of basal land plants. During land-plant evolution, ATE targets may have changed, or the general importance of ATE function for the development of the dominant generation may have declined, leading to the moderate phenotypes of Arabidopsis ATE mutants. It may therefore be feasible that targets of ATE also changed the routing for degradation within the NERD pathway during evolution. Although the hierarchical organization of the NERD pathway is conserved among eukaryotes (Graciet & Wellmer, 2010), differences between species originate either at the enzymatic level (strong expansions of the PRT1, Fig. S8, and PRT6 families, Fig. S7, and the loss of NTANs in mosses, Fig. S5), or by the spatio-temporal patterning of ATE under different stimuli.

Based on the findings of our work, we suggest that the N-end rule pathway provides an additional layer of complexity in the developmental and metabolic control in plants that is linked to hormonal cross-talk and dependent on environmental conditions such as light. These hypotheses have to be challenged by the identification of target proteins in forthcoming experiments.
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Author contributions

G.L.I. and R.R. planned and designed the research. C.S., S.N.W.H., S.J.M., M.R.F., T.L. and D.L. performed experiments and analysed data. C.S., S.N.W.H., S.J.M. and R.R. wrote the manuscript. All authors discussed data and approved the final version of the manuscript.

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**Supporting Information**

Additional supporting information may be found in the online version of this article.