A deeply conserved protease, acylamino acid-releasing enzyme (AARE), acts in ageing in Physcomitrella and Arabidopsis

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Abstract

35   Reactive oxygen species (ROS) are constant by-products of aerobic life. In excess, ROS lead to

36   cytotoxic protein aggregates, which are a hallmark of ageing in animals and linked to age-related

37   pathologies in humans. Acylamino acid-releasing enzymes (AARE) are bifunctional serine

38   proteases, acting on oxidized proteins. AARE are found in all domains of life, albeit under different

39   names, such as acylpeptide hydrolase (APEH/ACPH), acylaminoacyl peptidase (AAP), or

40   oxidized protein hydrolase (OPH). In humans, AARE malfunction is associated with age-related

41   pathologies, while their function in plants is less clear. Here, we provide a detailed analysis of

42   *AARE* genes in the plant lineage and an in-depth analysis of AARE localization and function in

43   the moss Physcomitrella and the angiosperm Arabidopsis. *AARE* loss-of-function mutants have

44   not been described for any organism so far. We generated and analysed such mutants and

45   describe a connection between AARE function, aggregation of oxidized proteins and plant ageing,

46   including accelerated developmental progression and reduced life span. Our findings complement

47   similar findings in animals and humans, and suggest a unified concept of ageing may exist in

48   different life forms.

49

Introduction

Reactive oxygen species (ROS) are by-products of O_2 metabolism and represent a challenge to all aerobic life. ROS play a dual role as potentially lethal oxidants and as signalling molecules[1]. Therefore, aerobic organisms possess sophisticated redox systems to scavenge and detoxify excess ROS. The major sources of ROS in plant cells are the electron transport chains in mitochondria and plastids, but also in peroxisomes and at the plasma membrane[2]. Environmental stresses such as heat, drought or intense light are factors for increasing ROS production to detrimental levels. Plants possess a repertoire of detoxifying enzymes such as catalases, superoxide dismutases, ascorbate peroxidases, glutathione peroxidase-like proteins and peroxiredoxins. Electrons for reduction are largely provided *via* non-enzymatic components such as ascorbic acid, glutathione and NADPH[3,4,5,6,7]. In addition, a range of heat-shock proteins assist in disaggregation or refolding of damaged proteins[8,9,10].

Despite conversion into non-toxic derivatives, the continuous exposure to ROS results in oxidation of DNA, lipids, and proteins[5]. On the protein level, ROS lead to irreversible cysteine oxidation, advanced glycation end-products, adducts with ROS-generated reactive aldehydes, and carbonylation of amino-acid side-chains[11,12]. If not cleared *via* proteolysis, an excess of oxidized proteins accumulates to cytotoxic protein aggregates. Plant antioxidant systems and the role of ROS as signalling molecules in abiotic stress responses are well studied[13]. Yet, factors involved in a plant cell's last line of defence, such as the proteolytic systems for the clearance of irreversibly oxidized proteins, are still underexplored.

A class of serine proteases is evolutionary deeply conserved as their activity is found in bacteria, archaea, animals and plants, and can degrade irreversibly oxidized proteins[14,15,16,17]. These proteases have different names in different organisms, e.g. acylamino acid-releasing enzyme (AARE), acylpeptide hydrolase (APEH/ACPH), acylaminoacyl peptidase (AAP), or oxidized protein hydrolase (OPH)[18] but are collectively addressed as AARE here. AARE acts in multimeric complexes[14,17] as a bifunctional protease as it cleaves N^α-acetylated amino acids from oligopeptides *via* an exopeptidase mode, but also cleaves oxidized proteins *via* an endopeptidase mode[16,19,20,21,22]. AARE isoforms from various organisms show different specificities towards N^α-acetylated amino-acids, with bacterial and archaeal enzymes preferring AcLeu and AcPhe substrates[15,23,24,25] and plant and animal isoforms preferring AcAla, AcMet or AcGly substrates[16,17,26,27]. Besides substrate specificities, their subcellular localization appears to be conserved among eukaryotes, as human (HsACPH) and Arabidopsis (AtAARE) AAREs are reported as cytosolic enzymes[20,21].

83   In humans, AARE malfunction is linked to different types of cancer[26,28,29] and sarcoma cell

84   viability[30]. Moreover, AARE and proteasomal activity correlate and cooperatively prevent cytotoxic

85   aggregate formation[31,32,33]. Several selective inhibitors have been identified[34,35] and blocking of

86   AARE function is considered as anti-cancer treatment[28]. Despite an increasing number of studies

87   on AARE functionality in humans, AAREs in plants are far less characterized.

88   AARE from *Arabidopsis thaliana* (AtAARE) and from cucumber have endo- and exopeptidase

89   functions[17], and silencing of *AtAARE* increased the levels of oxidized proteins[21]. AtAARE activity

90   was also detected in plastid stroma fractions, although a fusion with a fluorescent reporter did not

91   co-localize with chloroplasts[21]. Suppression of *AtAARE via* RNAi resulted in an enhanced

92   accumulation of oxidized proteins in roots and enhanced electrolyte leakage in leaves, but a

93   further impact on plant physiology was not described[21].

94   Moreover, the complete loss of function of this protease has not yet been reported for any

95   organism, neither bacterium, archaeon, animal, or plant. Thus, although the deep evolutionary

96   conservation of AARE suggests its pivotal role in all major life forms, experimental evidence is far

97   from optimal.

98   In a proteomics study on protein arginylation we identified an AARE homolog from the moss

99   Physcomitrella[36]. Our further analysis revealed altogether three Physcomitrella AARE homologs

100 (PpAARE1-3). Here, we analysed the subcellular localization of these PpAARE isoforms and of

101 their homolog from the angiosperm Arabidopsis (AtAARE). We show that an alternative splicing

102 event is targeting PpAARE1 to chloroplasts, mitochondria and the cytosol. We provide evidence

103 that an alternative translation initiation is sufficient to localize AtAARE to the same three

104 subcellular compartments. Bioinformatic analyses of several genomes suggest that the

105 localization of AARE in chloroplasts and mitochondria is conserved across the plant lineage.

106 Employing combinatorial gene knockouts and protein co-immunoprecipitation we found distinct

107 interactions between these three isoforms and their concerted action on progressive ageing in

108 Physcomitrella. Likewise, an Arabidopsis *AARE* loss-of-function mutant exhibits enhanced levels

109 of oxidized proteins and accelerated bolting, as a hallmark of plant ageing.

110

Results

AARE gene family expansion and splice variants

Previously, PpAARE1 (Pp1s619_3V6.1) was identified as the most prominent target for N-terminal arginylation in Physcomitrella[36,37]. N-terminal arginylation mediates poly-ubiquitination *via* the N-degron pathway, thus triggering subsequent proteasomal degradation[38]. Simultaneously, two homologs (PpAARE2: Pp1s108_134V6.1 and PpAARE3: Pp1s97_68V6.1) were identified, although those were not proven arginylation targets[36]. Meanwhile, a new Physcomitrella genome version with chromosome assembly and updated gene versions was released[39]. Consequently, the gene accessions used here are *PpAARE1* (Pp3c2_30720V3.1), *PpAARE2* (Pp3c12_21080V3.1), and *PpAARE3* (Pp3c7_25140V3.1). According to *OrthoMCL*[40,41], all three proteins are homologs of the *Arabidopsis thaliana* acylamino acid-releasing enzyme (AtAARE: AT4G14570). According to publicly available data (https://peatmoss.online.uni-marburg.de)[42] *PpAARE1-3* are expressed in all major Physcomitrella tissues and developmental stages, although at varying levels (Fig. S1a). Except for leaves (phylloids) and spores, *PpAARE1* is the most strongly expressed gene of this family (between 4 and 20 times). In contrast, *PpAARE2* is expressed considerably stronger than *PpAARE1* and *PpAARE3* in spores (Fig. S1a). Likewise, *AtAARE* is expressed in all major Arabidopsis tissues (Fig. S1b, data utilized from Mergner et al. (2020)[43] and downloaded from http://athena.proteomics.wzw.tum.de/). These data indicate a strong, positive correlation between transcript level and protein abundance across all tissues (Fig. S1b). Stress conditions decrease *AARE* expression in Arabidopsis shoots and in Physcomitrella protonemata (Fig. S1c-e).

To investigate whether other plants also possess multiple AARE homologs and to infer their phylogenetic relation, we conducted *BLASTP*[44] searches against protein models from selected species using the protein sequence of AtAARE as a query. We selected the alga *Chlamydomonas reinhardtii*[45], the liverwort *Marchantia polymorpha*[46], the peat moss *Sphagnum fallax* (*Sphagnum fallax* v1.1, DOE-JGI, http://phytozome.jgi.doe.gov/)*,* the lycophyte *Selaginella moellendorffii*[47], the monocot *Oryza sativa*[48] and the dicot *Populus trichocarpa*[49], all available at the Phytozome12 database (https://phytozome.jgi.doe.gov). Additionally, we performed a NCBI *BLASTP* search against the charophyte *Klebsormidium nitens* proteome[50], and identified a single homolog (GAQ80280.1) in this species. We also included proteins of *Funaria hygrometrica*[51], a close relative to Physcomitrella from the Funariacea family[52], in our search. Finally, the AtAARE sequence was again searched against the Physcomitrella[39] and Arabidopsis[53] proteomes.

143 Homology of the resulting *BLAST* hits was confirmed if the reciprocal best *BLAST* hit against *A.*
144 *thaliana* was again AtAARE.

145 In *P. trichocarpa*, we identified a single homolog for which three distinct splice variants are
146 annotated (Potri.008G160400.1, Potri.008G160400.2, Potri.008G160400.3). These encode
147 different protein isoforms, but two variants seem to encode non-catalytic proteins. AARE enzymes
148 are prolyl-oligopeptidases with a conserved catalytic triad (Ser/Asp/His) in the C-terminal
149 peptidase domain[54,55]. In splice variant 2 (Fig. S2, Potri.008G160400.2) alternative splicing results
150 in the deletion of the catalytic Asp whereas the whole catalytic triad is lacking in splice variant 3
151 (Potri.008G160400.3). Hence, we consider these splice variants as non-active and disregard
152 them from further discussion.

153 In rice, we identified two homologs (LOC_Os10g28020.3, LOC_Os10g28030.1), with an
154 additional splice variant (LOC_Os10g28020.1) at one locus which encodes an N-terminal
155 extension.

156 In *C. reinhardtii, M. polymorpha*, *S. fallax* and *S. moellendorffii*, we identified a single ortholog
157 each. In *M. polymorpha*, three distinct splice variants are annotated (Mapoly0111s0036.1,
158 Mapoly0111s0036.2, Mapoly0111s0036.3). The latter two are UTR (untranslated region) splice
159 variants, thus resulting in the same protein sequence, whereas Mapoly0111s0036.1 encodes an
160 N-terminal extension of 97 aa compared to the other two variants. In *F. hygrometrica* we identified
161 three distinct isoforms.

162 Finally, our *BLASTP* searches using the latest Physcomitrella protein models[39] confirmed three
163 homologs of AtAARE (Pp3c2_30720V3.1, Pp3c12_21080V3.1, Pp3c7_25140V3.1). Additionally,
164 this search revealed another hit, Pp3c1_2590V3.1. This gene is composed of a single exon and
165 encodes a 131 aa protein which harbours the AARE N-terminal domain (PF19283). However, it
166 lacks a catalytic peptidase domain and is hardly expressed across different culture conditions and
167 tissues[56]. We also did not find any proteomics evidence across several Physcomitrella
168 analyses[36,57,58,59] for this protein. Therefore, we excluded this gene from further analysis.

169 We then used phylogenetic reconstruction to investigate the origin of gene duplications within the
170 gene family. As an outgroup, we included the well-characterized rat[16] and human[26] AARE and
171 two isoforms of the Antarctic icefish *Chionodraco hamatus*[27]. Physcomitrella and *F. hygrometrica*
172 share three distinct pairs of orthologs hinting at an expansion in the common ancestor of the two
173 species. Our phylogenetic analysis did not resolve AARE subfamilies across kingdoms (Fig. 1a)

174   and we conclude that the gene family expansions observed in rice and in the Funariaceae are

175   lineage-specific events.

176   In addition, this analysis reveals a closer relationship between *PpAARE1* and *PpAARE3*, which

177   presumably originate from a more recent gene duplication event, compared to *PpAARE2*. This is

178   supported by the fact that the open reading frames (ORFs) of *PpAARE1* and *PpAARE3* are

179   represented by a single exon whereas the ORF of *PpAARE2* is split across 17 exons, similar to

180   *AtAARE* (Fig. 1b). This is in congruence with a more recent emergence of intron-poor genes in

181   intron-rich families linked to stress response and developmental processes[60] and in line with

182   intron-less orphan Physcomitrella genes as earliest responders to abiotic stress[61].

183   For the three *PpAARE* genes, several splice variants are annotated, but only two splice variants

184   of *PpAARE1* give rise to distinct protein isoforms (Fig. 1b; PpAARE1_1, PpAARE1_2). Both splice

185   variants are present in Physcomitrella protonema (Fig. 1b, c), according to RT-PCR with splice

186   variant-specific primers (Supplementary Table S1). Likewise, for *AtAARE* two different ORF

187   definitions exist. With Araport11[62], a new version of the gene model was introduced exhibiting a

188   longer ORF at the 5' end (Fig. 1b). We detected the full-length transcript *via* reverse transcription

189   polymerase chain reaction (RT-PCR, Fig. 1c).

190   For *PpAARE1*, alternative splicing in the 5' end results in an N-terminal truncated variant whereas

191   the longer non-spliced variant encodes an N-terminal plastid transit peptide (cTP) according to

192   *TargetP2.0*[63]. A cleavage of the transit peptide at the predicted cleavage site (Ala^{72}-M^{73},

193   Supplementary Table S2) of PpAARE1 would release exactly the protein encoded by the short

194   splice variant. In contrast, PpAARE2 and PpAARE3 do not harbour any predicted N-terminal

195   targeting signals. Moreover, PpAARE3 is also lacking the WD40 domain that is present in

196   PpAARE1 and PpAARE2 (Fig. 1d).

197   The extension of the originally annotated ORF of *AtAARE* also encodes a plastid transit peptide

198   (Fig. 1d). To our knowledge, the longer variant of AtAARE has not yet been investigated, whereas

199   the short variant of AtAARE localizes to the nucleus and the cytosol[21]. In agreement with the latter

200   findings, we could predict a nuclear localization sequence (NLS, KKKK) with *LOCALIZER*[64]. Thus,

201   targeting of AtAARE to the cytosol and the nucleus, but also to plastids could be enabled by

202   alternative translation initiation. Likewise, PtAARE harbours a plastid transit peptide and a

203   potential alternative translation initiation site downstream of the predicted cTP cleavage site.

204   Accordingly, we checked for NLS in PpAARE isoforms and found one (KRRP, Supplementary

205   Table S2) in PpAARE1 and PpAARE3, whereas PpAARE2 has none, further supporting our

7

206 hypothesis that PpAARE1 and PpAARE3 originate from a relatively recent gene duplication event.
207 Accordingly, alternative splicing also generates two distinct transcripts for AARE1 in rice
208 (OsAARE1, Supplementary Table S2), where one variant encodes a potential plastid transit
209 peptide.

210 For all other plant species, no plastid targeting sequence was predicted, while the *C. reinhardtii*
211 AARE harbours a mitochondrial targeting sequence (Supplementary Table S2).

212 **PpAARE1 and AtAARE in mitochondria, chloroplasts and cytoplasm**

213 Organellar targeting of AARE has not yet been reported, although AARE activity was observed in
214 plastid-enriched fractions of cucumber[17]. However, chloroplasts, peroxisomes and mitochondria
215 are major hubs of ROS generation[65], and thus are likely organelles with elevated levels of oxidized
216 proteins. Thus, we investigated whether PpAARE1 and AtAARE would localize to chloroplasts *in*
217 *vivo*.

218 We generated fusion constructs of the PpAARE isoforms and of AtAARE with eGFP for transient
219 expression in Physcomitrella protoplasts. Due to the presence of a predicted plastid targeting
220 peptide for PpAARE1, eGFP was fused in frame to the 3' end of all coding sequences (CDS).
221 Since also peroxisomes are ROS-producing organelles, we used *PlantPredPTS1*[66,67] to check for
222 the presence of C-terminal positioned peroxisomal targeting signals. None of the selected AARE
223 isoforms were predicted to localize to peroxisomes (Supplementary Table S2). Although AtAARE
224 has a C-terminal CKL tripeptide, which is experimentally verified to mediate peroxisomal
225 targeting[66,68], the properties of its other C-terminal amino acids most likely prevent peroxisomal
226 targeting. A more recent prediction approach for PTS1-mediated targeting for Arabidopsis
227 proteins[69] further supports this conclusion. Peroxisomal targeting can also be mediated *via* N-
228 terminal nona-peptides[70,71], but these motifs are also not present within the first 100 aa in any of
229 the selected AARE sequences. In agreement with these predictions eGFP was fused to the 3'
230 end of the CDSs.

231 For PpAARE1 three different fusion constructs were assembled. Among these, a fusion of the
232 CDS of the short splice variant (Pp3c2_30720V3.1) and eGFP was cloned, as well as a fusion of
233 the CDS of the longer splice variant (Pp3c2_30720V3.2) and eGFP. Additionally, we cloned a
234 fusion of eGFP and the sequence part in which both variants differ (M^1-A^{72}, Pp3c2_30720V3.2).
235 This part harbours the plastid transit peptide predicted by *TargetP2.0*. All fusion constructs were
236 expressed under the control of the Physcomitrella Actin5 promoter[57,72] in a pMAV4 plasmid
237 backbone[73].

238   The PpAARE1 isoform derived from the short splicing variant (PpAARE1_1, Fig. 1b) clearly
239   localized to the cytoplasm (Fig. 2). The same localization was observed for PpAARE2 and
240   PpAARE3 (Fig. 2). Despite a predicted NLS, we did not observe a nuclear localization, either for
241   PpAARE1 or for PpAARE3.

242   The isoform encoded by the longer splice variant of *PpAARE1* (PpAARE1_2, Fig. 1b) localized to
243   chloroplasts and surprisingly also to mitochondria (Fig. 2). In contrast to the diffuse cytosolic
244   distribution of PpAARE1, specific foci were observed in chloroplasts. To investigate whether the
245   N-terminal sequence differing between the two PpAARE1 variants (M^1-A^{72}, Pp3c2_30720V3.2) is
246   sufficient to confer dual targeting, we fused this N-terminal sequence 5' to eGFP and observed
247   again dual localization (Fig. 2, PpAARE1_Nt). Full-length PpAARE1 was necessary to localize
248   eGFP to foci within chloroplasts, as the PpAARE1_Nt:eGFP fusion led to a uniform distribution.
249   However, full-length PpAARE1 was also homogeneously distributed throughout the cytoplasm.
250   This indicates the presence of interactors that recruit PpAARE1 to specific sites or complexes
251   within the chloroplasts. Further, we conclude that the N-terminal extension of PpAARE1_2
252   encodes an ambiguous targeting signal for import into chloroplasts and mitochondria as it is
253   capable of directing the fusion protein simultaneously to both organelles.

254   Simultaneous localization of proteins to chloroplasts and mitochondria can be mediated *via*
255   ambiguous targeting signals which are recognized by both translocation machineries. We
256   evaluated whether *ATP2*[75], a tool for the prediction of ambiguous targeting, would recognize
257   PpAARE1 but this was not predicted to be dually targeted. In contrast, AtAARE was predicted to
258   be dually targeted *via* an ambiguous signal. Thus, we cloned the analogous three fusion
259   constructs for AtAARE and investigated the subcellular localization of their encoded proteins
260   accordingly.

261   The AtAARE isoform translated from the shorter ORF (AtAARE, Fig. 1b) localized to the
262   cytoplasm (AtAARE_SV, Fig. 2), as observed for PpAARE1_1. This result is partially in agreement
263   with Nakai et al. (2012)[21] since we could not observe nuclear localization. Using the fusion
264   construct of the longer AtAARE variant, we observed clear dual targeting of the protein to
265   chloroplasts and mitochondria (AtAARE_LV, Fig. 2), as observed for PpAARE1_2. Here, the
266   eGFP signal was distributed homogeneously in the chloroplasts, in contrast to the foci of
267   PpAARE1:eGFP.

268   Next, we cloned only the N-terminal sequence differing between both variants (M^1-A^{55}, longer
269   ORF definition, Fig. 1b) and fused it to eGFP. In order to investigate whether the exact N-terminal

270   difference between the two AtAARE variants would be sufficient for targeting, the M^{56} (same as

271   M^1 in the shorter variant), which is the P1 aa at the predicted cleavage site, was deleted. Using

272   this construct, the eGFP signal localized to chloroplasts and mitochondria (AtAARE_Nt, Fig. 2).

273   The signal within chloroplasts was homogeneously distributed, similar to the longer AtAARE

274   variant. Thus, we conclude that the N-terminal extension of both long PpAARE and AtAARE

275   variants is sufficient for dual targeting of proteins *in vivo*. Intriguingly, the longer variant of AtAARE

276   localized exclusively to chloroplasts and mitochondria although alternative translation initiation

277   should be possible. This is interesting as alternative translation initiation is also possible in the

278   longer splice variant of *PpAARE1* (PpAARE1_2). In the latter also, the fusion protein localizes

279   exclusively to chloroplasts and mitochondria, which excludes the possibility of an alternative

280   translation initiation, at least in protoplasts. There are numerous transcripts in mammals where

281   translation of an upstream positioned ORF suppresses the translation of the downstream main

282   ORF[76]. A similar scenario is conceivable in Physcomitrella. However, it remains unclear how and

283   if translation from the internal start codons is controlled. It is also possible that factors controlling

284   alternative translation initiation of AtAARE are absent in Physcomitrella, at least in a

285   spatiotemporal manner, or they might only be triggered in specific physiological situations.

286   According to our data, the translation of the two variants of PpAARE1 is mainly controlled by

287   alternative splicing and not by alternative translation initiation.

288   In summary, PpAARE1 and AtAARE localize to three subcellular compartments *via* an ambiguous

289   targeting signal. In contrast, PpAARE2 and PpAARE3 localize solely to the cytoplasm.

290

291   **Double knockout of *PpAARE1/2* reduces lifespan**

292   Null mutants of *AARE* have not been described in any organism, and the biological role of this

293   protease apart from its catalytic function remained unclear. Hence, we created *AARE* knockouts

294   in Physcomitrella by deleting the full CDS of each gene *via* gene targeting (Fig. 3a) according

295   to[77]. To enable subsequent knockout (KO) of other AARE genes, different selection markers were

296   used. Since three distinct AARE genes exist in Physcomitrella which result in cytosolic proteases,

297   we generated all possible combinations of double KOs and triple KOs to avoid potential

298   compensation of the loss of function.

299   Plants surviving the selection procedure were screened *via* PCR for the absence of the respective

300   CDS and correct integration of the KO construct in the target locus (Fig. S4a). Additionally, the

301   number of genomic integrations of the KO construct was measured *via* quantitative PCR (qPCR)

302   as described[78] (Fig. S4b). At least three independent lines were identified for all single, double

303   and triple KOs (Fig. S4c-i) and a line with only a single integration in the genome was detected

304   for each KO (j-p, line numbers with stars). Further, haploidy of all lines was confirmed *via* flow

305   cytometry (Fig. S5a-c) as described[79]. These precautions were made as the transformation

306   procedure may generate plants with multiple integrations[80], possibly leading to off-target effects.

307   Further, the transformation procedure may lead to diploid plants with altered gene expression[81].

308   We used haploid lines with a single integration of the KO construct (Fig. S4) for subsequent

309   experiments.

310   Typically, AARE exopeptidase activity is assayed *via* N^{α}-acetylated amino acids like AcAla or

311   AcMet coupled to a reporter such as para-nitro-anilide (pNA) or 7-amido-4-methylcoumarin

312   (AMC). From these, AcAla-pNA was tested for several eukaryotic AAREs[22,26], including

313   AtAARE[17]. Here, we analysed the impact of AARE loss of function on the activity towards AcAla-

314   pNA and AcLeu-pNA. The latter is a substrate of bacterial and archaeal AARE isoforms but also

315   eukaryotic isoforms exhibit cleavage activity on this substrate[16,22,23,24,25]. On the single KO level,

316   the exopeptidase activity on both substrates was significantly reduced in the ΔPpAARE1 mutant

317   whereas the single KO of the other isoforms did not affect the activity (Fig. 3b). This strong impact

318   of PpAARE1 on the exopeptidase activity was consistent across all transgenic mutant lines (Fig.

319   S6).

320   One important step in Physcomitrella development is the transition from protonema to

321   gametophores, a developmental progression regulated among others by plant age and the

322   nutrient status[82]. The single KOs were phenotypically inconspicuous on the gametophore level

323   (Fig. 3c). In contrast, gametophores of Δ*PpAARE1/2* and Δ*PpAARE1/3/2* were severely stunted

324   and colonies were denser compared to wild type (WT) or the other mutants (Fig. 3c). This growth

325   effect is restricted to gametophores since protonema growth on solid medium did not differ

326   between WT and the KOs (Fig. 3d, e). Intriguingly, Δ*PpAARE1/2* and Δ*PpAARE1/3/2* mutants

327   showed accelerated developmental transition, as they developed gametophores from protonema

328   earlier (Fig. 3d). Since other KO lines did not show this effect, we attributed this to the double KO

329   of *PpAARE1/2* and performed a quantitative comparison with WT. Here, gametophores were

330   already observed after 6 days in the double KO of *PpAARE1/2*, while in WT a similar number of

331   gametophores per colony was observed only after 13 days (Fig. 3f). Consequently, the double

332   KO of *PpAARE1/2* causes accelerated developmental progression but gametophores remained

333   ultimately smaller than in WT (Fig. 3c). These effects are not linked to AARE exopeptidase activity

334   since the exopeptidase activity in gametophores was significantly reduced in all lines with a KO

335   of PpAARE1 (Fig. 3g), which mimics the activity profile in protonema (Fig. S6).

336   To analyse AARE endopeptidase activity, we assessed the total levels of oxidized proteins in

337   gametophores. In this assay, protein carbonyl groups derived from oxidation are derivatized with

338   2,4-dinitrophenylhydrazine (DNPH) to 2,4-dinitrophenylhydrazone (DNP). This irreversible

339   modification is then recognized on Western blots by a primary anti-DNP antibody. Since DNPH

340   can also react with oxidation states of cysteine side chains[83], this assay detects not only protein

341   carbonylation but general protein oxidation.

342   With this assay we found that PpAARE2 had the strongest impact on the level of oxidized proteins

343   in gametophores (Fig. 3h) and thus is not linked to exopeptidase activity. This was consistently

344   observed in three independent analyses (Fig. 3h, Fig. S7). Apparently, PpAARE3 does not have

345   any impact on exopeptidase and endopeptidase activity in gametophores under standard

346   conditions (Fig. 3g, h).

347   Taken together, PpAARE1 predominantly acts as exopeptidase, while PpAARE2 predominantly

348   acts as endopeptidase, and only the simultaneous loss of both activities in the double knockout

349   mutants has the severest phenotypical consequences.

350   We found another remarkable difference between WT and mutants with a double KO of

351   *PpAARE1/2* in older plants. After 5 months of cultivation, Δ*PpAARE1/2* and Δ*PpAARE1/3/2* were

352   only viable at the tip of the gametophores (Fig. 4a), whilst most of the colony was already dead.

353   In contrast, gametophores of WT and the other KOs were fully viable. After 8 months,

354   Δ*PpAARE1/2* and Δ*PpAARE1/3/2* were already dead, in contrast to WT and the respective

355   parental lines, which only showed some dead gametophores (Fig. 4b, c).

356   In summary, mutants with a double KO of *PpAARE1/2* exhibit accelerated developmental

357   transition from protonema to gametophore (Fig. 3f), while size and life span of gametophores is

358   strikingly reduced (Fig. 3c, Fig. 4a-c). In contrast, these effects are not visible in Δ*PpAARE1/3*.

359   Therefore, these ageing phenotypes are linked to the concurrent loss of major AARE

360   endopeptidase and exopeptidase activity.

361   **Distinct *in vivo* interactions of PpAARE isoforms**

362   In different organisms, AARE forms different homomeric complexes such as dimers[14],

363   tetramers[17], or hexamers[24]. Thus, we analysed whether the PpAARE isoforms can interact with

364   each other. Previously, all three isoforms were identified, although the protein modification used

365   for pulldown (N-terminal arginylation) was only identified on PpAARE1[36]. This gave rise to two

366   hypotheses: First, PpAARE2 and PpAARE3 are also targets for N-terminal arginylation, but

367   modified peptides were not identified for these isoforms. Second, the isoforms interact in

368   complexes which were pulled down due to the N-terminal arginylation of PpAARE1. We generated

369   Citrine fusion lines for each isoform *via* in-frame tagging at the native locus (knock-in, Fig. 5a).

370   The original stop codons of the respective PpAARE CDS were deleted. The Citrine-tag was

371   intended for two different analyses: First, it should enable *in vivo* monitoring of PpAARE isoforms

372   expressed from the native promoter, and second, it is a valid tag for co-immunoprecipitation (Co-

373   IP) *via* commercially available trap-beads.

374   In plants with a detectable fusion transcript (Fig. S8a-c) the presence of the target protein was

375   checked *via* IP and subsequent MS analysis. For *PpAARE3:Citrine* lines, we detected transcripts

376   in one line and obtained only insufficient coverage and intensity at the MS across several Co-IPs.

377   Thus, these Physcomitrella lines were excluded from further analysis. The co-precipitation of

378   other PpAARE isoforms with the respective bait isoforms was observed in test IPs (Fig. S8d-e)

379   confirming previous MS-Data[36]. All plants harbouring the Citrine fusion were phenotypically

380   inconspicuous (Fig. S8f) and haploid (Fig. S8g). Although the fusion proteins were detected in

381   two independent lines for each of the two isoforms (PpAARE1, PpAARE2), we could not observe

382   any Citrine signal within Physcomitrella protonemata or gametophores, probably due to the low

383   abundance of the PpAARE isoforms. Nevertheless, MS intensities and sequence coverage

384   enabled quantitative Co-IPs. The MS data have been deposited in PRIDE with the accession

385   codes PXD033854 and PXD038742[84,85].

386   When targeting PpAARE1:Citrine, both other isoforms appeared as significant interacting

387   partners (Fig. 5b, $p < 0.01$, FDR = 0.01). In a reciprocal Co-IP targeting PpAARE2:Citrine only,

388   PpAARE1 appeared as significant interacting partner. PpAARE3 was not detected in this

389   pulldown. Although lacking a reciprocal IP targeting PpAARE3:Citrine, the data show that

390   PpAARE1 interacts with PpAARE2 and PpAARE3, whereas PpAARE2 only interacts with

391   PpAARE1. Consequently, there are distinct interactions of PpAARE1 with PpAARE2 and

392   PpAARE3 *in vivo*, possibly resulting in cytosolic heteromeric AARE complexes in Physcomitrella.

393   **AARE affects bolting time in Arabidopsis**

394   In Physcomitrella three *AARE* genes exist, and the concerted action and interaction of the

395   enzymes affect plant ageing. To evaluate if this is an evolutionary conserved function, we

396   analysed the situation in Arabidopsis. Here, it was known that silencing of the single *AARE* gene

397   leads to an accumulation of oxidized proteins, whereas overexpression did not affect their levels[21].

398   To gain deeper insights, we screened for available Arabidopsis T-DNA mutants.

399   We identified two T-DNA insertion lines (SALK_071125 and GK-456A10) in the T-DNA Express

400   database at the SIGnAL website (http://signal.salk.edu). SALK_071125 (s68) has a T-DNA

401   insertion in an intron at the 5´UTR, whereas the T-DNA insertion in GK-456A10 (GK) maps to an

402   intron in the region encoding the catalytic domain (Fig. 6a). We obtained these mutants, identified

403   homozygous populations of GK-456A410 containing the T-DNA insertion by their resistance to

404   sulfadiazine, and confirmed their genotype by PCR. In the case of the Salk line (s68), homozygous

405   plants had lost their resistance to kanamycin, but we confirmed their genotype by PCR.

406   Homozygous plants of both genotypes were back-crossed with WT Col-0, and brought to

407   homozygosis for subsequent experiments. Primers for screening and validation of both T-DNA

408   lines are listed in Supplementary Table S3. Additionally, we analysed *AARE* gene expression *via*

409   RT-PCR. The transcript was still detectable in both lines (Fig. 6a), although very reduced in s68,

410   while the protein was not detectable *via* Western blot (Fig. 6b). Surprisingly, in WT AARE was

411   detected at around 100 kDa, although the estimated molecular weight is approximately 84 kDa

412   (90 kDa for the longer ORF variant without cleavage of the plastid targeting peptide) which

413   indicates the presence of posttranslational modifications. Phenotypically, neither seedlings nor

414   adult mutant plants showed obvious deviations from WT (Col, Fig. S9)

415   Next, we assayed AARE exopeptidase function in Arabidopsis WT and the two mutants. The

416   exopeptidase activity on AcAla-pNA was significantly reduced in both T-DNA mutants. In contrast,

417   the activity on AcLeu-pNA did not change significantly, although a slight reduction was observed

418   in the GK line (Fig. 6c). Thus, in agreement with Western blot and activity data, the remaining

419   transcripts detected *via* RT-PCR (Fig. 6a) are not translated to functional enzymes, suggesting

420   complete loss of AARE function in the mutants. Based on this characterization, we concentrated

421   on T-DNA mutant s68.

422   Subsequently, we assayed the AARE endopeptidase activity in Arabidopsis. The most striking

423   feature reported were increased levels of oxidized proteins in AARE-silenced Arabidopsis plants[21]

424   which is in line with our findings in Physcomitrella AARE mutants. To corroborate this, we

425   investigated levels of oxidized proteins in the Arabidopsis T-DNA mutant (s68) in comparison to

426   WT (Col) cultivated under short day conditions. In both genotypes, the distribution of oxidized

427   proteins was different to Physcomitrella gametophores (Fig. 6d). Using the same extraction

428   protocol, oxidized proteins in Arabidopsis were mainly of lower molecular weight, whereas in

14

429 Physcomitrella they were mainly at higher molecular weight (Fig. 3h). Despite these size
430 differences, the level of oxidized proteins was higher in the Arabidopsis mutant than in WT (Fig.
431 6d), mimicking the situation in Physcomitrella. Together, we found AtAARE exopeptidase and
432 endopeptidase activity to be reduced in our experiments.

433 Oxidized proteins accumulate during vegetative growth of Arabidopsis and are depleted at the
434 transition to bolting[86]. It was not clear from this study whether the level of oxidized proteins is a
435 signal for bolting, or if the reset of protein oxidation at the bolting transition is a side-effect of
436 enhanced protein turnover during this process. To address this, we assessed the bolting
437 behaviour of Arabidopsis WT and mutant plants and found that bolting in mutant plants differed
438 significantly from WT. In WT, bolting started at 24±1 rosette leaves whereas it started at 21±2
439 rosette leaves in the mutants (Fig. 6e). Consequently, this accelerated developmental transition
440 in the mutants correlates with enhanced levels of oxidized proteins.

441

Discussion

443 A universal definition of ageing is difficult due to strongly differing characteristics between and
444 within the domains of life. In humans, the term ageing is inherently linked to degenerative diseases
445 whereas plant ageing is considered as the progression of developmental transitions from seeds
446 to senescence. Nevertheless, a common feature of ageing is the execution of a genetic program
447 that controls growth, development and maturation. In turn, the progression of this genetic program
448 depends on the metabolic state and environmental factors. Among the molecules responsible for
449 ageing are ROS. Their involvement in ageing and diseases was first postulated by Harman[87] and
450 was extended by considering mitochondria as central sources[88]. In humans, mitochondrial
451 malfunction in line with increased ROS levels is a central determinant of ageing and associated
452 pathologies[89]. In plants, mitochondrial ROS increase during ageing of seeds[90] and are major
453 determinants of germination capacity[91]. In photosynthetic tissues chloroplasts are the major
454 source of ROS and their levels increase during ageing[92,93,94]. Plants cannot escape from stress
455 situations that increase ROS production to detrimental levels, and despite several layers of ROS
456 defence, oxidized proteins constitute the major share of modified molecules under stress.
457 Consequently, protein oxidation and subsequent aggregate deposition are hallmarks of
458 ageing[95,96]. The degradation of artificially oxidized proteins by AARE has been demonstrated
459 repeatedly, and silencing of *AtAARE* in turn increased the levels of oxidized proteins[19,20,21,22].

460   However, the contribution of AARE to the progress of ageing remained elusive, although several

461   studies associate AARE-function to age-related diseases[97,98,99]. Here, we identified three *AARE*

462   genes in Physcomitrella. This gene family expansion is an outstanding feature of the moss family

463   Funariaceae, as most organisms we analysed contain only a single *AARE* gene, and only a few

464   have two. We analysed these three isoforms in Physcomitrella and compared selected features

465   to the single isoform in Arabidopsis. Our data reveal specific functions in age-related

466   developmental transitions and life span determination.

467   Surprisingly, we found triple localization of AtAARE and PpAARE1 to chloroplasts, mitochondria

468   and the cytosol *in vivo*, suggesting a functional role for AARE in these cellular compartments. In

469   Physcomitrella, the triple localization is mediated *via* alternative splice variants and in Arabidopsis

470   likely *via* alternative translation initiation. Although there have been indications of AtAARE being

471   associated to chloroplasts[17,21], there was no clear evidence for organellar targeting of this

472   protease until now. It is remarkable that the triple localization of AARE is evolutionary conserved

473   between Arabidopsis and Physcomitrella, although likely executed *via* different molecular

474   mechanisms. This suggests an essential and evolutionary conserved function of AARE activity in

475   the cytoplasm, chloroplasts and mitochondria. As mosses and seed plants diverged more than

476   500 million years ago[100], this is a deep evolutionary conservation.

477   Previously, AARE exopeptidase activity was observed in cucumber chloroplasts[17], and AARE

478   peptides were found in proteomes of Arabidopsis chloroplasts[101] and mitochondria[102]. In contrast,

479   AARE was not identified in plastid or mitochondrial proteomes of Physcomitrella[57,59]. We found

480   AARE exopeptidase activity in chloroplasts and mitochondria of Physcomitrella. Chloroplasts are

481   a major source of ROS in photosynthetic tissues exposed to light, whereas mitochondria are the

482   major source of ROS in non-photosynthetic tissues or in the dark[93,94,103,104]. Until now, it remained

483   unresolved how plants deplete oxidized proteins from these organelles.

484   In yeast and mammals, the ATP-dependent LON and AAA proteases are involved in clearance

485   of misfolded and oxidized mitochondrial proteins. Intriguingly, mutants of plant homologs of LON

486   proteases did not show clear effects on the levels of oxidized proteins, but AAA-type FTSH

487   proteases may play a role[105]. Nevertheless, stressors such as heat, drought or intense light

488   compromise photosynthesis[106,107] and mitochondrial respiration[108], leading to a depletion of ATP

489   and to mitochondrial dysfunction[109]. In turn, energy supply for ATP-dependent defence systems

490   such as heat-shock proteins and AAA-type proteases is severely compromised, leaving the

491   question unanswered how oxidized proteins in chloroplasts and mitochondria can be cleared.

492   Because AARE is an ATP-independent protease, our data suggest that organellar-targeted AARE

493 may act as an ATP-independent defence to prevent or attenuate protein aggregation in the major
494 ROS-producing organelles.

495 Based on localization prediction, 70% of our selected plant species possess one AARE isoform
496 that localizes to either plastids or to mitochondria. Whether dual targeting *via* ambiguous targeting
497 also occurs in these species remains to be experimentally validated. Further, we do not exclude
498 that the remaining species also have organellar AARE isoforms, because our predictions may be
499 compromised by incomplete gene models. Such incomplete AARE gene models without a transit
500 peptide were present in earlier genome annotations of Arabidopsis and Physcomitrella[41,53], whilst
501 complete gene models with transit peptides were only introduced with later versions[39,62].

502 It is not yet clear how translation of both variants in Arabidopsis is regulated, but a recent study
503 highlights the importance of alternative translation initiation in shaping different subcellular
504 localizations or functions of proteoforms[110]. This mechanism is also present in Physcomitrella,
505 where dual targeting of FtsZ isoforms to the cytoplasm and chloroplasts is enabled *via* alternative
506 translation initiation[111]. Thus, localization of PpAARE1 to the cytoplasm is also possible from the
507 longer splice variant. Alternative translation initiation of *AtAARE* is further evidenced by
508 proteomics data (www.peptideatlas.org/builds/arabidopsis/)[112]. Here, we found evidence for N-
509 terminal acetylation of M^{56} which is the initiator methionine of the short variant (PXD012708[113]).

510 A reporter fusion of the shorter *AtAARE* ORF was observed in the cytoplasm and in the nucleus[21].
511 We did not detect a nuclear localization of any AARE isoform. Using *LOCALIZER*[64] we identified
512 an NLS in AtAARE, in PpAARE1 and PpAARE3, but not in PpAARE2. In contrast, the human
513 AARE homolog HsACPH does not have a predictable NLS, but nuclear import is mediated *via*
514 interaction with XRCC1 under stress, where it acts in DNA-damage repair[114]. Similarly, a nuclear
515 localization of AARE might also occur under stress *in planta*.

516 In Physcomitrella, AARE1 is the dominant exopeptidase, whereas AARE2 acts predominantly as
517 endopeptidase, and the operation mode of AARE3 remains unresolved. Crystal structures from
518 bacterial and archaeal AAREs revealed two possible entrances for substrates to the catalytic
519 centres[14,115] but the mode of substrate entry is not fully understood. Although the quaternary
520 arrangements of subunits differ between species[115,116], the secondary structure arrangement is
521 conserved across kingdoms and specific subunit interactions (multimerization) are likely a
522 mechanism to mediate substrate specificity and modulate activity. This could be further used as
523 a switch between endopeptidase and exopeptidase activity and additionally enable access of the

524 catalytic centre *via* structural re-arrangements. Accordingly, the interaction between the distinct
525 PpAARE isoforms may modify substrate specificity and activity.

526 Our data indicate a partial compensation between PpAARE1 and PpAARE2. Both single KO
527 mutants are phenotypically inconspicuous under normal conditions, although a significant
528 reduction in exopeptidase activity was observed in Δ*PpAARE1*. PpAARE2 had the strongest
529 effect on the accumulation of oxidized proteins in gametophores on the single isoform KO level,
530 whereas the double KO of *PpAARE1* and *PpAARE2* showed the most striking effect in all lines
531 as accelerated transition from protonema to gametophores. This phenotype is similar to mutants
532 with disturbed auxin transport[117]. However, colony growth in these mutants was reduced which is
533 different from our double KO mutant (*PpAARE1/2*). Intriguingly, this double KO results in stunted
534 gametophores and a reduced life span. This phenotype partially resembles a loss-of-function
535 mutant of a central component of autophagy (PpATG3) in Physcomitrella[118]. In Δ*PpATG3*,
536 gametophores exhibit a reduced life span and colonies are smaller than in WT. In contrast,
537 gametophore size was not affected. Further, photosynthetic capacity in Δ*PpATG3* was also
538 reduced, an effect which is apparently not caused by AARE depletion[21] and *PpAARE* genes were
539 not differentially expressed in Δ*PpATG3*[118]. We conclude that the reduced life span observed in
540 Δ*PpAARE1/2* and Δ*PpAARE1/3/2* is not due to an impaired autophagy system causing nitrogen
541 starvation. This is in line with data which opposes autophagy at the onset of senescence in
542 Arabidopsis[119].

543 In mammals, elevated levels of oxidized proteins are associated with age-related pathologies,
544 such as Alzheimer´s disease, diabetes and different types of carcinomas[120]. If proteolytic
545 clearance fails, further accumulation of oxidized proteins causes protein aggregation, which is a
546 hallmark of ageing in animals[96,121,122]. A connection between protein oxidation and ageing was
547 less well studied in plants. Plastid ROS levels increase during ageing[92], which is in line with strong
548 oxidation of plastid proteins in ageing leaves[86]. Likewise, protein oxidation marks the
549 developmental transition between vegetative growth and flowering in Arabidopsis[86].
550 Physcomitrella mutants Δ*PpAARE1/2* and Δ*PpAARE1/3/2* showed accelerated developmental
551 transition from protonema to gametophores, reduced life span and increased levels of oxidized
552 proteins as signs of accelerated ageing. This is supported by the fact that gametophore tips, which
553 is younger tissue, are viable longer than the older stems in both mutants. In the Arabidopsis *AARE*
554 T-DNA mutants we found increased levels of oxidized proteins under normal cultivation conditions
555 and an accelerated developmental transition, in this case premature bolting. These findings
556 suggest an evolutionary conserved connection between protein oxidation and ageing.

557   We provide here a detailed analysis of *AARE* genes in the plant lineage and an in-depth analysis

558   of AARE localization and function in the moss Physcomitrella and the annual angiosperm

559   Arabidopsis. *AARE* loss-of-function mutants have not been described for any organism so far.

560   We generated and analysed such mutants and describe a connection between AARE function,

561   aggregation of oxidized proteins and plant ageing, including accelerated developmental

562   progression and reduced life span. Our findings complement similar findings in humans and

563   animals where AARE malfunction is associated with protein aggregation and age-related

564   pathologies.

565   To solidify the role of AARE in ageing in different life forms, particularly in plants with contrasting

566   maximum life spans and in animals of different complexity, loss-of-function mutants should be

567   established and analysed in selected model species. Likewise, a deeper understanding of AARE-

568   function in human diseases is desirable. Together, such analyses may contribute to a unified

569   concept of ageing in different life forms.

570

571 **Methods**

572 Cultivation of Physcomitrella

573 Physcomitrella WT (new species name: *Physcomitrium patens* (Hedw.) Mitt.[123]); ecotype
574 "Gransden 2004" and AARE KO lines were cultivated in Knop medium[124] supplemented with
575 microelements. Knop medium (pH 5.8) containing 250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L
576 $MgSO_4$ x 7 H_2O, 1,000 mg/L $Ca(NO_3)_2$ x 4 H_2O and 12.5 mg/L $FeSO_4$ x 7 H_2O supplemented with
577 10 mL per litre of a microelement (ME) stock solution[125,126] (309 mg/L H_3BO_3, 845 mg/L
578 $MnSO_4$ x 1 H_2O, 431 mg/L $ZnSO_4$ x 7 H_2O, 41.5 mg/L KI, 12.1 mg/L Na_2MoO_4 x 2 H_2O, 1.25 mg/L
579 $CoSO_4$ x 5 H_2O, 1.46 mg/L $Co(NO_3)_2$ x 6 H_2O). For cultivation on solid medium, 12 g/L Agar was
580 added to the KnopME medium. Moss suspension cultures were disrupted weekly with an ULTRA-
581 TURRAX (IKA) at 18,000 rpm for 90 s. If not indicated otherwise, moss was grown under standard
582 light conditions (55 µmol photons/m^{2}s) at 22°C in a 16h/8h light/dark cycle.

583 Hydroponic Physcomitrella gametophore cultures were assembled as described[36,127]. Here, a thin
584 layer of protonema from suspension was distributed on gauze mesh (PP, 250 m mesh, 215 m
585 thread, Zitt Thoma GmbH, Freiburg, Germany) capped on a glass ring. The glass rings with
586 protonema-covered mesh gauze were placed in Magenta®Vessels (Sigma-Aldrich, St. Louis,
587 USA) and KnopME medium was added until the protonema-covered gauze mesh was moist. The
588 medium was exchanged every 4 weeks. Gametophores were harvested after 12 weeks.

589 Gametophore colonies on Agar plates (KnopME) were generated by transplanting single
590 gametophores to new plates. Plates were sealed with Parafilm®.

591 Generation of AARE knockout lines

592 Knockout constructs for each *PpAARE* gene were generated by amplifying genomic regions as
593 homologous flanks. The PCR products were fused to a neomycin (*PpAARE1*), hygromycin
594 (*PpAARE2*) and zeocin (*PpAARE3*) resistance cassettes, respectively, employing triple template
595 PCR as described[128] with primer sequences listed in Supplementary Table S1 using the *Q5*
596 polymerase (New England Biolabs, Ipswich, USA). The knockout constructs were released from
597 their vector backbones with XhoI (*PpAARE1*), BglII (*PpAARE2*) and DraI (*PpAARE3*),
598 respectively. Enzymes were purchased from Thermo Fisher Scientific. Digested plasmids were
599 precipitated and sterilized prior to transfection using standard ethanol precipitation method[129].
600 Transfection of Physcomitrella WT or KO (for consecutive knockouts) protoplasts was conducted
601 via PEG-mediated procedure[77,130]. The WT strain as well as the identified *PpAARE* KO lines are

602 accessible via the International Moss Stock Center (IMSC, www.moss-stock-center.org). IMSC

603 accession numbers for the mutant and WT are available in Supplementary Table S4.

604 Screening of plants surviving antibiotics selection was done by PCR. KO mutant plants surviving

605 the antibiotics selection were checked for the presence of a genomic region which should be

606 removed upon homologous recombination-based integration of the knockout construct (Fig. S4a).

607 In case of the absence of this WT signal, plants were further checked for correct 5' and 3'

608 integration of the respective knockout construct using primers listed in Supplementary Table S1.

609 Protonema growth and gametophore induction

610 Suspension cultures were started at the same day and disrupted weekly as described. Dry weight

611 was measured in triplicates and suspension density was adjusted to 440 dry weight (DW) per litre

612 (mg DW/L) as described[131]. Droplets of 15 µL were distributed on solid medium (Knop ME, 0.02%

613 glucose). Sealed plates were cultivated as described above. Three droplets each per line were

614 distributed on one plate and all lines were grown on the same plate. 3 plates (12 colonies) were

615 used per assay. Colony areas were measured with *ImageJ*. White pixels counted from binarized

616 colony images were used as quantitative values. Gametophores were counted upon visibility of

617 the first leafy structure on buds.

618 Generation of PpAARE-Citrine knock-in lines

619 Knock-in constructs to fuse the coding sequence of Citrine to the C-terminus of PpAARE isoforms

620 via an 18 bp linker[128] at the endogenous genomic locus were cloned via Gibson assembly[132]. All

621 necessary parts were amplified using primers listed in Supplementary Table S1. Additionally, XhoI

622 (*PpAARE1*), SalI (*PpAARE2*) and BamHI (*PpAARE3*) restriction sites were added to the 5' and 3'

623 ends of the respective knock-in construct. All parts were assembled into pJet1.2 vector backbone

624 (Thermo Scientific) using the Gibson Assembly®Cloning Kit from New England Biolabs (Ipswich,

625 Massachusetts, USA). Transfection of Physcomitrella WT protoplasts were conducted as

626 described[77,130] by co-transfecting a plasmid containing a neomycin phosphotransferase

627 resistance (nptII) cassette as transient selection marker (pBSNNNEV, Mueller et al., 2014)[57]. The

628 linearized plasmid and the co-transfection vector were purified and sterilized *via* ethanol

629 precipitation[129] prior to transfection.

630 The presence of Citrine was checked with primers listed in Supplementary Table S1 and resulting

631 positive lines were further checked for correct 5' and 3' integration by PCR using the Plant Phire

632 Kit with primers listed in Supplementary Table S1. All identified fusion lines are available *via* the

633   International Moss Stock Center (IMSC, www.moss-stock-center.org). IMSC accessions are

634   listed in Supplementary Table S4.

635   qPCR analysis

636   The copy number of the integrated KO constructs was determined using a qPCR-based method[78].

637   Genomic DNA was extracted from 100 mg frozen protonema using the GeneJEt Plant Genomic

638   DNA Purification Kit (Thermo Scientific, Waltham, USA). DNA concentrations were adjusted to 3

639   ng/µl for each sample and qPCR was performed with primers for the 5' and 3' flanks as well as

640   with primers for the corresponding selection cassette. Additionally, primers for the single copy

641   gene *CLF* (Pp3c22_2940V3) were used as internal control. Reference lines were WT as well as

642   in-house lines with known single integrations of the used selection cassettes. Primers are listed

643   in Supplementary Table S5. PCR reactions were done using 2x SensiFAST Mix (Bioline, London,

644   UK) and analysed in a Lightcycler 480 II (Roche, Basel, Schweiz).

645   cDNA preparation

646   RNA was extracted using the innuPREP Plant RNA Kit (Analytik Jena, Jena, Germany). The

647   extracted RNA was treated with DNAse I (Thermo Scientific) and subsequently reverse

648   transcribed into cDNA using Superscript III Reverse Transcriptase (Life Technologies, Carlsbad,

649   USA).

650   Fusion constructs for subcellular localization

651   All constructs were generated using Gibson assembly[132] and integrated into a

652   PpAct5:Linker:eGFP-MAV4 vector backbone[73]. Integration of the different coding sequences was

653   done in frame in front of the Linker:eGFP. All parts for the Gibson assembly (inserts and

654   corresponding vector backbones) were amplified either with Phusion™ polymerase (Thermo

655   Fisher Scientific) or with HiFi polymerase (PCR Biosystems Ltd) according to the manufacturer´s

656   instructions. The primers were designed to have a 25 bp overlap to the corresponding fragment

657   to be fused with. All primers and combinations are listed in Supplementary Table S6. In the case

658   of the N-terminal difference of AtAARE (M^1-A^{55} of AT4G14570.1, gene model provided by TAIR

659   (https://www.arabidopsis.org/)) the Actin5 promoter was replaced by the CaMV35S promoter[133]

660   previously validated in Physcomitrella[134].

661   Cloned plasmids were purified using the PureYield™ Plasmid Midiprep kit (Promega, Wisconsin,

662   USA) according to the manufacturer´s instructions. The plasmids were purified and sterilized *via*

663   ethanol precipitation[129].

664   Confocal microscopy

665   Confocal imaging was performed on transiently transfected live protoplasts using Leica TCS SP8
666   (Leica Microsystems, Wetzlar, Germany). Immediately before microscopy, MitoTracker™ Orange
667   CMTMRos (ThermoFisher Scientific) was added to protoplast suspensions to a final concentration
668   of 100 nM. For all imaging experiments, an HC PL APO CS2 63x/1.40 OIL objective was used
669   with a zoom factor of 4. The pinhole was set to 35.4 µm. For excitation, a WLL laser (70%) was
670   used. In a sequential acquisition setup, eGFP and chlorophyll were excited with the same laser
671   beam (488 nm, 2%) and their signals were detected simultaneously, whereas MitoTracker™ was
672   excited with a different laser beam (550 nm, 2%) and its signal was detected separately. The
673   detection ranges were specified as 502 nm - 546 nm for eGFP, 662 nm - 732 nm for chlorophyll
674   and 597 nm - 636 nm for MitoTracker™. The images were acquired as z-stacks with the number
675   of optical sections varying with the protoplast size. The voxel sizes of the resulting z-stacks were
676   0.0903, 0.0903, 0.239 µm in the x-y-z order. For visual representation and analysis, single slices
677   with the best signal-to-noise ratio were selected and extracted from each z-stack using FIJI
678   software.

679   Cultivation of Arabidopsis

680   Seeds were surface-sterilized for 4 min. in 80% ethanol and subsequently for 1 min. in 100%
681   ethanol. Seeds were placed on plates containing ½ MS supplemented with 1% (D+) sucrose and
682   0.8% Agar. Alternatively, seeds were directly placed on sterilized soil. Seeds were incubated at
683   8°C for 2-4 days in the dark for stratification before placing them in growth chambers. Plants were
684   further cultivated under short day conditions at 22°C and 70 µmol photons/m^{2}s in an 8h/16h
685   light/dark cycle. For bolting assays, plants were placed in a phyto-chamber at 22°C and 70 µmol
686   photons/m^{2}s in a 16h/8h light/dark cycle (long day condition). Rosette leaves and days since
687   sowing were counted upon appearance of the shoot in the middle of the rosette.

688   Screening of Arabidopsis AARE mutants

689   *Arabidopsis thaliana* lines with T-DNA insertions in the At4G14570 locus were identified from the
690   public T-DNA Express database at the SIGnAL website (Salk Institute Genomic Analysis
691   Laboratory). Lines GK-456A10, SALK_080653C, SALK_071125C, and SALK_205137C, were
692   obtained from the Nottingham Arabidopsis Stock Centre. Homozygous mutant alleles were
693   verified   by   PCR   using   the   following   primers: forward   LB   GK   5´-
694   ATATTGACCATCATACTCATTGC-3´ and reverse GK-456 5´-CTTCAAAGAAACACCAATCAG-
695   3´for the GK-456A10 line, and forward LB-pROK 5´-GCGTGGACCGCTTGCTGCAACT-3´ and

23

696 reverse Salk_53 5´-TCTTTAGCCGAATCAGTTCCAGA-3´for the SALK_080653C,

697 SALK_071125C, and SALK_205137C lines. Identified homozygous mutant plants were back-

698 crossed with Arabidopsis Col-0. The F2 generation was screened for homozygous mutant plants

699 using the above listed primer-sets to identify the mutant allele or substituting the forward primers

700 with forward WT-GK-456 5´-AAGATGCTTTGCAGTCTCTA-3´ and forward WT-Salk 5´-

701 ACTGCCTTATGATCCATTGTCTC-3´, to identify the GK and SALK lines WT alleles, respectively.

702 RT-PCR was additionally performed to check for the presence of AtAARE transcripts using Taq

703 polymerase on cDNA prepared as described above with primers At1-At4. All primer combinations

704 are listed in Supplementary Table S4.

705 AARE exopeptidase activity

706 The enzyme activity assay according to [17] was modified. Here, tissue (80-100 mg) was

707 homogenized in liquid nitrogen and dissolved in 1 mL extraction buffer (50 mM PBS, 1 mM EDTA,

708 2 mM DTT). After centrifugation at 20,000 x g for 20 min at 4°C, 300 µl supernatant was mixed

709 with 700 µL reaction buffer (50 mM HEPES-KOH, pH 7.5 containing 1 mM Ac-Ala-pNA (Bachem,

710 Bubendorf, Switzerland) or 50 mM HEPES-KOH, pH 7.5, 10% DMSO containing 1mM AcLeu-

711 pNA (Bachem) and incubated at 37°C for 30-120 min. The reaction was stopped by the addition

712 of 500 µL 30% acetic acid. Absorbance was measured at 410 nm in a photospectrometer. This

713 approach was used to generate the data of Fig. 3b and Fig. S6a-d. Later the method was modified

714 (Fig. 3g, Fig. S6e-g). Here, tissue was homogenized in liquid nitrogen and dissolved in 100 µL

715 extraction buffer (50 mM PBS, 1 mM EDTA, 2 mM DTT) per 10 mg FW. After centrifugation at

716 20,000 x g for 20 min at 4°C, 5 µL supernatant was mixed with 195 µL reaction buffer (50 mM

717 HEPES-KOH, pH 7.5 containing 1 mM Ac-Ala-pNA (Bachem, Bubendorf, Switzerland)) in a 96

718 well micro titer plate and incubated at 37°C for 30-120 min. In the case of Ac-Leu-pNA as

719 substrate, 50 mM HEPES-KOH, pH 7.5 with 10% DMSO containing 1mM AcLeu-pNA (Bachem)

720 was used as reaction buffer. Every biological sample was measured in three technical replicates.

721 Absorbance was measured at 410 nm. Activity was calculated using a molar absorbance

722 coefficient[21] of 8.8 mM*cm^{-1} and represents the release of pNA [µmol] per minute normalized to

723 the total protein concentration of the sample. The protein concentration was determined using the

724 A_{280} method of a NanoDrop^{TM} (Thermo Fisher Scientific) or with a Bradford assay[135].

725 Western Blots

726 Western Blots were performed as described[74] using the *ECL Advance detection kit* (GE

727 Healthcare). The primary antibody against *A. thaliana* AARE was kindly provided by Dr. Yasuo

728 Yamauchi[17]. The primary antibody was diluted 1:10,000 in TBST-Buffer with 2% Blocking (GE

729 Healthcare) and incubated on the membrane for 2 h. As secondary antibody anti-Guinea pig

730 antibody, coupled to a horseradish peroxidase (Agrisera, AS 10 1496) diluted 1:10,000 in 2%

731 TBST with 2% Blocking (GE Healthcare), was applied for 1 h.

732 Detection of oxidized proteins

733 Plant tissues were homogenized in liquid nitrogen and proteins were extracted in 50 mM PBS, 50

734 mM DTT, 1 mM EDTA. 3-6 µg total protein was derivatized with DNPH and subsequently detected

735 with an anti-DNP antibody according to the manufacturer's instruction of the *OxyBlot Protein*

736 *Oxidation Detectio*n Kit (S7150, Sigma-Aldrich). Equal amounts of the derivatized protein samples

737 were employed as loading control on a separate SDS-gel and stained with Coomassie or silver

738 staining.

739 Flow cytometry

740 Flow cytometry analysis was performed as described[79]. Here, protonemata were chopped with a

741 razor blade in a small petri dish (6 cm diameter) in 2 mL of DAPI-buffer containing 0.01 mg/L 4′,6-

742 Diamidin-2-phenylindol (DAPI), 1.07 g/L $MgCl_2$ x 6 H_2O, 5 g/L NaCl, 21.11g/L Tris, 0.1% Triton,

743 pH 7. The solution was filtered using 30 µm pore size filters and the fluorescence intensity was

744 measured using a Cyflow®Space flow cytometry system (Partec, Munich, Germany).

745 Computational predictions

746 Predictions for the presence of cleavable targeting peptides were performed with *TargetP2.0*[63].

747 Additional predictions of subcellular localizations were performed with *LOCALIZER*[64]. The

748 presence of peroxisomal targeting signals was predicted with *PredPlantPTS1*[66,67]. Prediction of

749 protein domains was performed using *InterProScan*[135] and protein domain annotations according

750 to PFAM[137] were used.

751 Co-Immunoprecipitation

752 Co-immunoprecipitation was performed using GFP-Trap Magnetic Particles M-270 (Chromotek,

753 Planegg-Martinsried, Germany) as recommended by the manufacturer with modifications. 300

754 mg protonema was homogenized in a 2 mL reaction tube using a tungsten and a glass bead. For

755 each line three biological replicates were realized. The extraction buffer was chosen according to

756 the manufacturer's recommendations for plant samples and contained 25 mM HEPES-KOH,

757 pH7.5, 2 mM EDTA, 100 mM NaCl, 200 nM DTT, 0.5% Triton X-100, 1% plant protease inhibitor

758 cocktail (PPI, P9599, Sigma Aldrich). Ground plant material was dissolved in a final volume of 2

759   mL ice-cold extraction buffer and incubated for 10 min in a water quench ultrasonic device.

760   Samples were centrifuged at 4°C at 20,000xg for 30 min. For each sample 25 µL magnetic particle

761   slurry was washed with 500 µL extraction buffer. The sample supernatant was transferred to the

762   cleaned beads and incubated, rotating for 1 h at 6°C. Subsequently, beads were washed with 1

763   mL extraction buffer without Triton and PPI and again with 500 µL. Beads were then dissolved in

764   500 µL wash buffer (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 0.5 mM EDTA), transferred to a new

765   reaction tube and washed again in 500 µL wash buffer. A RapiGest solution (0.2% in 50 mM Tris-

766   HCl, pH7.5; RapiGest SF Surfactant (Waters, Milford, MA, USA) was mixed 3:1 with 5 mM DTT

767   in 50 mM Tris-HCl, pH7.5. 30 µL of the resulting mixture was applied to each sample. Samples

768   were incubated at 95°C for 5 min under continuous shaking. Samples were cooled down to RT

769   and 5 µL of a trypsin (V5117, P) solution (0.025 µg/µL in 50 mM Tris-HCl, pH 7.5) were added to

770   each sample. Digestion on the beads was performed for 30 min at 32°C under continuous

771   shaking. Supernatants were transferred to new reaction tubes and the remaining beads were

772   washed twice with 50 µL 5 mM Iodoacetamide solution (in 50 mM Tris-HCl, pH7.5). The wash

773   supernatants were combined with the trypsin-containing supernatant and incubated over night at

774   30°C under continuous shaking. Acid-catalysed cleavage of the RapiGest surfactant was

775   performed as recommended by the manufacturer. Samples were purified using C18-STAGE-Tips

776   as described[138] and eluted from the Tip in 30% ACN in 0.1% FA.

777   Mass spectrometry measurement and data analysis

778   MS analysis was performed on an Orbitrap Q-Exactive Plus instrument (Thermo Fisher Scientific)

779   coupled to an UltiMate 3000 RSLCnano system (Dionex LC Packings/Thermo Fisher Scientific)

780   as described[139]. Database search and label-free quantitation was performed using *MaxQuant*

781   software V 1.6.0.16[36][140]. For each Co-IP a specific database was employed containing all V3.3

782   proteins of Physcomitrella[39] as well as the sequence of the respective fusion protein. Additionally,

783   the contaminant list provided by the software was included. Decoys were generated on the fly by

784   reverting the given protein sequences. Variable modifications were formation of pyro Glu (N term

785   Q, −17.026549 Da), oxidation (M, +15.994915 Da), acetylation (N-term, +42.010565 Da) and

786   deamidation (N, +0.9 84016 Da). Carbamidomethylation (C, +57. 021464 Da) was specified as

787   fixed modification. Enzymatic specificity was set to tryptic with semi-specific free N-terminus. An

788   FDR of 0.01 was set for protein identification. LFQ values[141] were used as quantitative values.

789   Interaction analysis was performed in Perseus V 1.6.12.0[142]. Missing values were imputed from

790   a normal distribution with a down-shift of 1.8 and distribution width of 0.3. Interaction partners

791   were accepted at an FDR of 0.01 and a p-value less than 0.01.

792   Raw files of the test-IP measurements (Fig. S8e) were processed using Mascot Distiller V2.7.10

793   and searched against all Physcomitrella protein models V3.3[39] using Mascot Server V2.7.0

794   (Matrix Science).Processed mgf files from immunoprecipitation experiments targeting N-terminal

795   arginylation (PXD003232[36,143]) and the test-IPs were searched again against all Physcomitrella

796   protein models V3.3[39] using Mascot Server V2.7.0 (Matrix Science). The precursor mass

797   tolerance was 5 ppm and the fragment mass tolerance was 0.02 Da. Variable modifications were

798   formation of pyro Glu (N term Q, −17.026549 Da), oxidation (M, +15.994915 Da), acetylation (N-

799   term, +42.010565 Da) and deamidation (N, +0.9 84016 Da). Carbamidomethylation (C, +57.

800   021464 Da) was specified as fixed modification. Enzymatic specificity was set to tryptic with semi-

801   specific free N-terminus. Search results were loaded in Scaffold™ 5 (V5.0.1, Proteome Software)

802   and proteins were accepted at an FDR = 1 and peptides at an FDR = 0.5. A table of identified

803   proteins is accessible in Supplementary Table S7.

804   Multiple sequence alignment and phylogenetic reconstruction

805   Homologous protein sequences were aligned with UPP[144] (version 4.4.0) using default

806   parameters and subsequently translated into a codon-aware CDS alignment with PAL2NAL[145]

807   (version 1.4). Based on this multiple coding sequence alignment we reconstructed a maximum

808   likelihood tree with RAxML[146] (version 8.2.12) using the GTRCAT model with 1000 rapid bootstrap

809   samples. The tree was rooted at the split between animal and plant sequences and plotted in R[147]

810   using the packages ape[148] and ggtree[149].

811   Statistics and reproducibility

812   Statistical differences in datasets were analysed with one-way Anova with subsequent post-hoc

813   test if different lines at same conditions were compared. Two-way Anova with subsequent post-

814   hoc test was performed to analyse differences between lines at different conditions. Sample sizes

815   of biological replicates are specified in the figure legends. Anova and post-hoc analysis was

816   performed in R[147]. Statistical significance was accepted at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$

817   (***).

818   **Data availability**

819   The authors confirm that all relevant data supporting the findings of this study are available within

820   the paper and its supplementary files. The mass spectrometry proteomics data have been

821   deposited at the ProteomeXchange Consortium via the PRIDE partner repository[84,85] with the

822   dataset identifier PXD033854 and 10.6019/PXD038742. Plant lines used in this study are

823 available upon reasonable request from the corresponding author (R.R.) or *via* the International
824 Moss Stock Center (IMSC, www.moss-stock-center.org). IMSC accessions are listed in
825 Supplementary Table S4. Full Blot images for Fig. 6b and Fig. S3b are available in Fig. S10.
826 Supplementary Table S8 contains all numeric source data used to generate the graphs and charts
827 in this study. Plasmids generated in this study are available from the International Moss Stock
828 Center IMSC (https://www.moss-stock-center.org) with the accession numbers P1519 (*PpAARE1*
829 KO), P1841 (*PpAARE2* KO (pJet)), P1520 (*PpAARE3* KO (pJet)), P1655 (*PpAARE1:Citrine* KI
830 (pJet)), P1813 (*PpAARE2:Citrine* KI (pJet)), P1814 (*PpAARE3:Citrine* KI), P1833
831 (*PpAARE1_1:eGFP* (pMAV4)), P1834 (*PpAARE1_2:eGFP* (pMAV4)), P1853
832 (*PpAARE1_Nt:eGFP* (pMAV4)), P1855 (*PpAARE3:eGFP* (pMAV4)), P1856 (*PpAARE3:eGFP*
833 (pMAV4)), P1854 (*AtAARE_SV:eGFP* (pMAV4)), P1881 (*AtAARE_LV:eGFP* (pMAV4)) and
834 P1862 (*AtAARE_Nt:eGFP* (pMAV4)).

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Author contributions

845 S.N.W.H. designed research, performed experiments, analysed data, and wrote the manuscript.
846 B.Ö. and N.v.G. analysed data and helped writing the manuscript. A.A.M. analysed data. B.R.v.B.,
847 L.N., J.S.F., R.K., S.G., T.W., and F.S. performed experiments. M.R.F. and S.J.M.S. designed
848 research and helped writing the manuscript. R.R. designed and supervised research, acquired
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Competing interests

851 All authors declare to have no competing interests.

852

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1228

1229 **Supplementary Information**

1230 **Figure S1** Expression levels of PpAARE genes and AtAARE in different tissues and under stress
1231 conditions.

1232 **Figure S2** Sequence alignment of AtAARE and PtAARE isoforms.

1233 **Figure S3** AARE activity in Physcomitrella plastids and mitochondria.

1234 **Figure S4** Identification of PpAARE single, double and triple KO mutants by PCR on genomic
1235 DNA.

1236 **Figure S5** FCM analysis of all confirmed PpAARE KO lines to analyse the ploidy.

1237 **Figure S6** AARE exopeptidase activity protonema of KO mutants.

1238 **Figure S7** Plate overview and additional OxyBlots from Physcomitrella gametophore colonies
1239 with loading controls.

1240 **Figure S8** Screening for haploid Physcomitrella lines with a citrine fusion transcript and overview
1241 on identification of the three PpAARE isoforms in different IP experiments.

1242 **Figure S9** Phenotypes of seedlings and adult AtAARE T-DNA mutant lines (GK, s68) and WT
1243 (Col).

1244 **Supplementary Table S1** List of primers used to assemble KO and KI constructs and primers
1245 used to screen for transgenic plants in Physcomitrella.

1246   **Supplementary Table S2** List of gene accession numbers for all sequences used for

1247   phylogenetic analysis and corresponding subcellular localization predictions.

1248   **Supplementary Table S3** List of primers used to screen possible T-DNA mutant plants in

1249   Arabidopsis.

1250   **Supplementary Table S4** List of accession numbers for the International Moss Stock Center

1251   (IMSC) of all transgenic Physcomitrella lines.

1252   **Supplementary Table S5** List of primers used for qPCR on genomic DNA to determine copy

1253   numbers of KO constructs in transgenic Physcomitrella lines.

1254   **Supplementary Table S6** List of primers used to assemble the constructs for transient

1255   localization analysis in Physcomitrella protoplasts.

1256   **Supplementary Table S7** Spectrum report of the revised database search of the anti-Arg[(Nt)] IP

1257   published in[36].

1258   **Supplementary Table S8** This table contains all numeric source data used to generate the

1259   graphs in Fig. 3b, Fig. 3e-g, Fig. 5b, Fig. 6c, Fig. 6e, Fig. S1, Fig. S3c, Fig. S4j-p, Fig. S5, Fig.

1260   S6, Fig. S8d, Fig. S8e and Fig. S8g.

1261

1262 **Fig. 1 Phylogenetic tree for AARE isoforms from selected organisms, gene structures and**
1263 **proteins domains of *P. patens* and *A. thaliana* AARE isoforms.** (a) Maximum likelihood tree
1264 based on an alignment of multiple *AARE* coding sequences. Duplication events in several species
1265 appear to be lineage-specific. Node values reflect percentage confidence values based on 1000
1266 bootstrap replicates. Species abbreviations: At: *Arabidopsis thaliana*; Pt: *Populus trichocarpa*; Os:
1267 *Oryza sativa*; Pp: *Physcomitrium patens*; Fh: *Funaria hygrometrica*; Sf: *Sphagnum fallax*; Mp:
1268 *Marchantia polymorpha*; Sm: *Selaginella moellendorffii*; Kn: *Klebsormidium nitens*; Cr:
1269 *Chlamydomonas reinhardtii*; Rn: *Rattus norvegicus*; Hs: *Homo sapiens*; Ch: *Chionodraco*
1270 *hamatus.* (b) Gene structure of *PpAARE1-3* and *AtAARE*. For *PpAARE1* two splice variants exist.
1271 For *AtAARE* two different 5' UTR definitions are present (upper: Araport11[62]; lower: TAIR10[53]).
1272 (c) Left: Both splice variants of *PpAARE1* were amplified from complementary DNA (cDNA; +:
1273 with cDNA template; -: without reverse transcriptase). Expected amplicon size: *PpAARE1_1*:
1274 2512 bp; *PpAARE1_2*: 2596 bp. Primers were designed to be specific for the splice variants
1275 (Table S1). Right: The longer open reading frame of *AtAARE* was amplified from cDNA. Expected
1276 amplicon size: 2457 bp (Table S3). (d) Protein structures showing PFAM-domains for PpAARE1-
1277 3 and AtAARE. All isoforms contain an AARE N-terminal domain (PF19283) and a catalytic
1278 Peptidase S9 domain (PF00326). PpAARE1, PpAARE2 and AtAARE additionally contain a WD40
1279 domain (PF07676). The long splice variant of PpAARE1 and the longer open reading frame of
1280 AtAARE encode a predicted N-terminal plastid transit peptide (cTP). AA: amino acid. Cleavable
1281 N-terminal sequences were predicted by *TargetP2.0*[63].

1282 **Fig. 2 Confocal microscopy images showing the localization of PpAARE isoforms and**
1283 **AtAARE with C-terminal fused eGFP in Physcomitrella protoplasts.** Fusion proteins with
1284 domain structures are depicted. PpAARE1_1, PpAARE2 and PpAARE3 localize to the cytoplasm.
1285 PpAARE1_2 localizes to specific foci in plastids (white arrows) and to mitochondria. The N-
1286 terminal extension of PpAARE1_2 encoding a predicted plastid transit peptide (PpAARE1_Nt)
1287 directs eGFP to plastids and mitochondria. The short variant of AtAARE (SV) localizes to the
1288 cytoplasm. The long variant (LV) localizes to plastids and mitochondria. The N-terminal extension
1289 of the long variant of AtAARE localizes to plastids and mitochondria. Left image: chlorophyll
1290 autofluorescence (red) and eGFP (green). Middle image: chlorophyll autofluorescence (red) and
1291 MitoTracker™ (magenta). Right image: chlorophyll autofluorescence (red), eGFP (green),
1292 Mitotracker™ (magenta) and co-localization of eGFP and MitoTracker™ (white). Bars = 7 μm.

1293 Based on this data, PpAARE1 is targeted to three organelles: chloroplasts, mitochondria and the
1294 cytosol. To independently scrutinize these findings, we measured AARE enzyme activity in the

1295   organelles. Using organelle fractionation as previously described[74], we detected AARE activity in

1296   chloroplasts, the cytosol, and mitochondria, although to a lesser extent in the latter (Fig. S3).

1297   Thus, *in vivo* localization of fusion proteins and enzyme measurements after cell fractionation

1298   independently confirm the predicted triple localization of AARE in Physcomitrella.

1299   **Fig. 3 Phenotype analyses, AARE activity and level of oxidized proteins in Physcomitrella**

1300   **WT and KO mutants.** (a) Each isoform was knocked out using a different selection marker. The

1301   selection markers confer resistance against G418 (npt), hygromycin (hpt) and zeocin (zeo),

1302   respectively. Homologous regions (HR) were chosen in order to remove the full CDS of the

1303   corresponding gene upon integration of the selection marker. (b) Mean AARE activity on AcAla-

1304   pNA in Physcomitrella protonema with standard deviation (n= three biological replicates).

1305   Significance levels are based on a one-way Anova and subsequent post-hoc test (***: p<0.001)

1306   and indicate differences compared to WT. (c) Phenotypes of gametophores of WT and the

1307   different KOs cultivated on KnopME for 4 months. All colonies were grown on the same plate.

1308   Upper panel: Bar = 0.5 mm; lower panel: Bar = 2mm. (d) Protonema colonies grown on KnopME

1309   with 0.02% glucose taken from suspension culture. Bar = 1 mm. Images were taken 9 days after

1310   spotting. (e) Box plot showing the distribution of colony areas (n = 12 colonies per boxplot; colony

1311   images taken 7 days after spotting). No significant difference of colony areas between WT and

1312   the KOs was observed (tested via one-way Anova). (f) Box plot showing the number of

1313   gametophores per colony and day of WT and Δ*PpAARE1/2*. Counting was performed at indicated

1314   days after spotting protonema suspension culture on solid medium (KnopME with 0.02% glucose).

1315   Indicated significance levels are based on a two-way Anova with subsequent post-hoc test (***:

1316   p<0.001). The boxplots (e, f) depict the mean (horizontal bold line) of the data, the interquartile

1317   range (box) and the 1.5x interquartile range (whiskers). (g) Mean AARE exopeptidase activity on

1318   AcAla-pNA in gametophores. Depicted is the mean of three independent colonies per line with

1319   standard deviations. All colonies were cultivated on the same plate (Fig. S7a). Significance levels

1320   are based on one-way Anova and indicate differences compared to WT (***: p<0.001). (h) Levels

1321   of oxidized proteins in gametophores of WT and the different KOs (Coomassie-stained loading

1322   control is shown in Fig. S7b). The analysis was repeated three times (additional Blots available in

1323   Fig. S7c, d). Detection of oxidized proteins was performed with the OxyBlot™ *Protein Oxidation*

1324   *Detection Kit* (Merck).

1325   **Fig. 4 Physcomitrella gametophore colonies of varying age.** (a) Colonies of all representative

1326   knockout mutants after 5 months on solid medium (KnopME). Bar = 5 mm. Gametophores of

1327   Δ*PpAARE1/2* and Δ*PpAARE1/3/2* are only viable at the tip, whereas plant material at the base

1328 (red arrow) is dead. (b) Colonies after 8 months on solid medium. Δ*PpAARE1* is the parental line

1329 for Δ*PpAARE1/2*. Gametophores of WT and Δ*PpAARE1* are still viable whereas Δ*PpAARE1/2*

1330 gametophores are mostly dead. Bar = 2 mm. (c) Colonies after 8 months on solid medium.

1331 Δ*PpAARE1* is the parental line for Δ*PpAARE1/3* and Δ*PpAARE1/3* is the parental line for

1332 Δ*PpAARE1/3/2*. Colonies of WT, Δ*PpAARE1* and Δ*PpAARE1/3* still have viable gametophores,

1333 whereas Δ*PpAARE1/3/2* gametophores are dead. Bar = 2 mm.

1334 **Fig. 5 Reciprocal co-immunoprecipitation (Co-IP) with Citrine-tagged PpAARE isoforms.**

1335 (a) Tagging of PpAARE isoforms was realized by in-frame fusion with a linker-*Citrine* CDS at the

1336 respective native locus *via* homologous recombination. Original stop codons were deleted. (b)

1337 Volcano plots showing the result of the Co-IPs against each of the PpAARE:Citrine fusion

1338 proteins. Left panel: Pulldown of PpAARE1:Citrine. Right panel: Pulldown of PpAARE2:Citrine.

1339 Co-IP was performed with GFP-Trap Magnetic Particles M-270 (Chromotek) and protonema from

1340 suspension culture. Log_2 ratios of normalized label-free quantitation values (LFQ) are plotted

1341 against $-log_{10}$ of adjusted p-values. Proteins significantly enriched in the Citrine tagged pulldown

1342 are shown in blue circles (p < 0.01, FDR = 0.01). Significantly enriched PpAARE isoforms are

1343 depicted as red crosses.

1344 **Fig. 6 Molecular and phenotypical characterization of *A. thaliana* T-DNA mutant lines.** (a)

1345 Structure of the AtAARE gene and positions of T-DNA insertions (red arrows indicate positions of

1346 T-DNA insertion, s68 (SALK_071125) and GK (GK-456A10)) and primers (At1, At2) used for RT-

1347 PCR (below). Transcription analysis of *AtAARE* was performed by RT-PCR in WT (Col), s68, and

1348 GK plants. Negative controls without reverse transcriptase (-), a water control (H_2O) and genomic

1349 DNA control (gDNA) are indicated. Expected amplicon sizes: *AtAARE*: cDNA 739 bp, gDNA: 1204

1350 bp; *PP2AA3* (reference gene): cDNA: 517 bp, gDNA: 911 bp. Primers are listed in Table S3. (b)

1351 Western blot analysis on extracts of the two T-DNA mutants and WT probed with a polyclonal

1352 @AARE antibody. In both T-DNA lines AtAARE is not detectable. Full blot images are available

1353 in Fig. S10. (c) Mean exopeptidase activity in *A. thaliana* extracts on AcAla-pNA and AcLeu-pNA

1354 with standard deviation (n = 3 biological replicates). Significance levels are based on one-way

1355 Anova and subsequent post-hoc test (***: p<0.001) and indicate differences compared to WT

1356 (Col). (d) Detection of oxidized proteins in WT (Col) and *AtAARE* mutant (s68). From three

1357 independent plants one exterior rosette leaf was taken after cultivation for 8 weeks in short-day

1358 conditions (8h light/16h dark) Bars correspond to 2 cm. Protein loading control by Coomassie

1359 staining is depicted below. (e) Boxplot of the bolting analysis of WT (Col, n = 14) and *AtAARE*

1360 mutant plants (s68, n=13). The boxplot (e, f) depicts the mean (horizontal bold line) of the data,

1361   the interquartile range (box) and the 1.5x interquartile range (whiskers). Outliers are depicted as

1362   white dots. Significance level is based on a one-way Anova and subsequent post-hoc test (***:

1363 $p < 0.001$). Exemplary plants (45-day-old plants grown in long-day conditions, 16h light/8h dark)

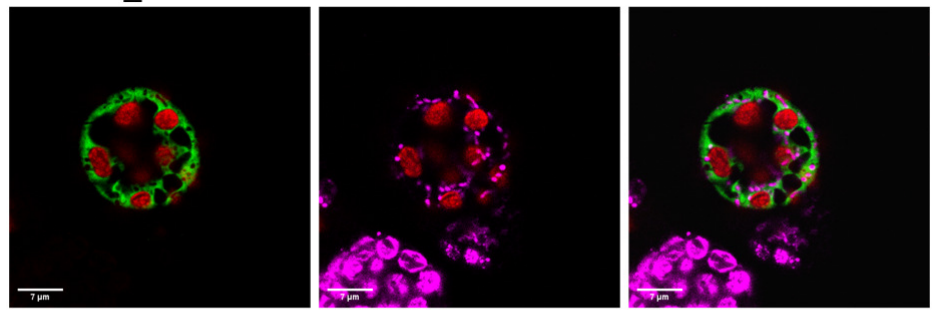1364   are depicted below the boxplot. Bars correspond to 1 cm.
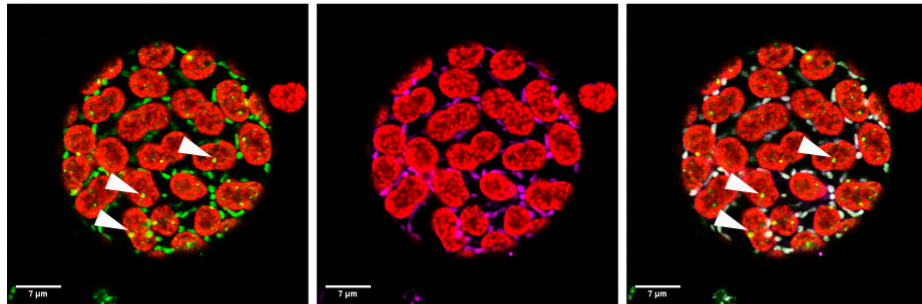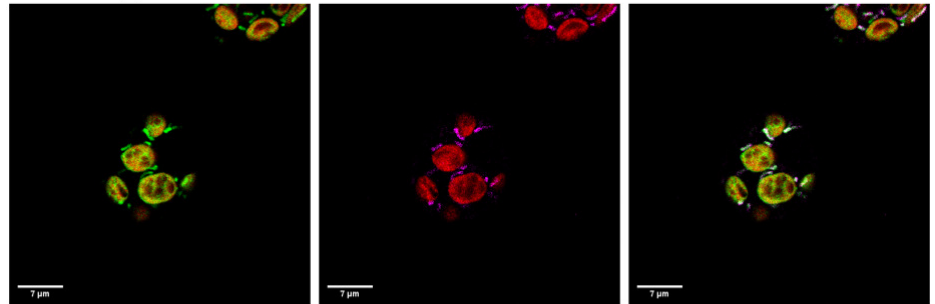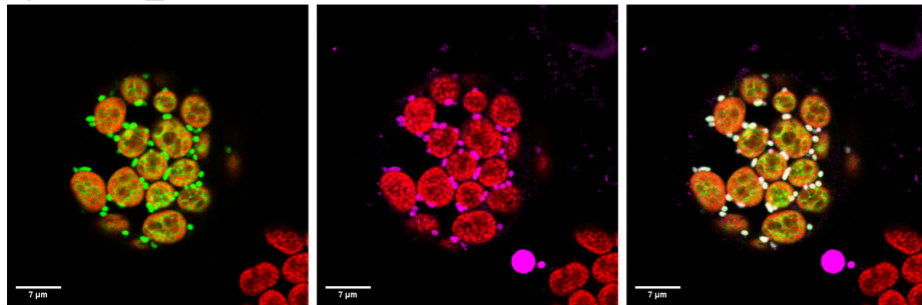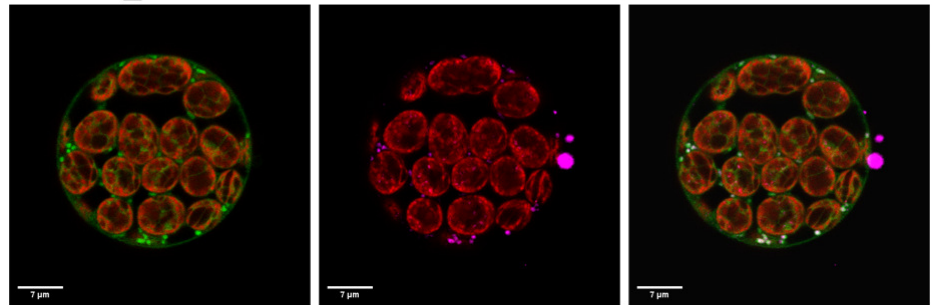
1365
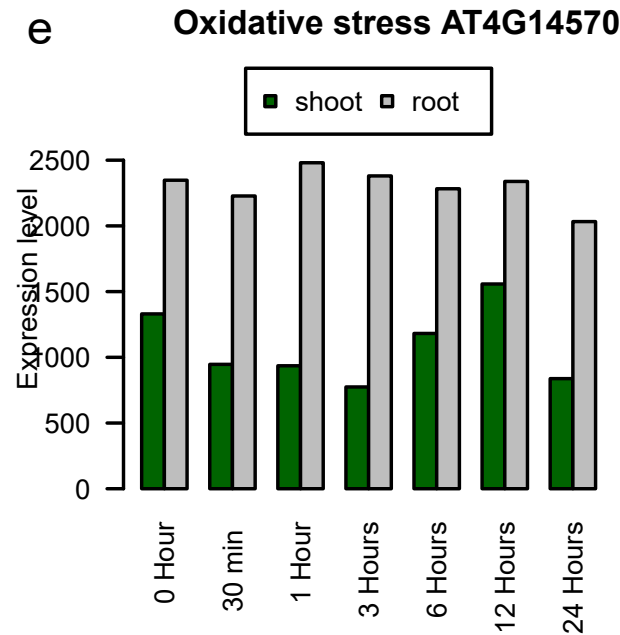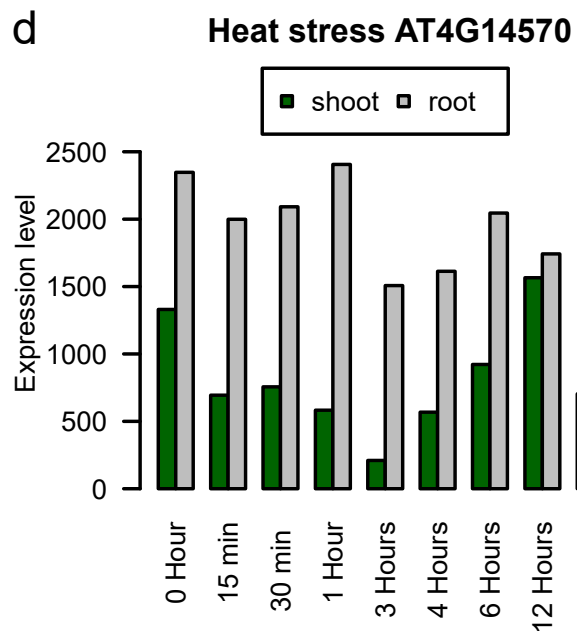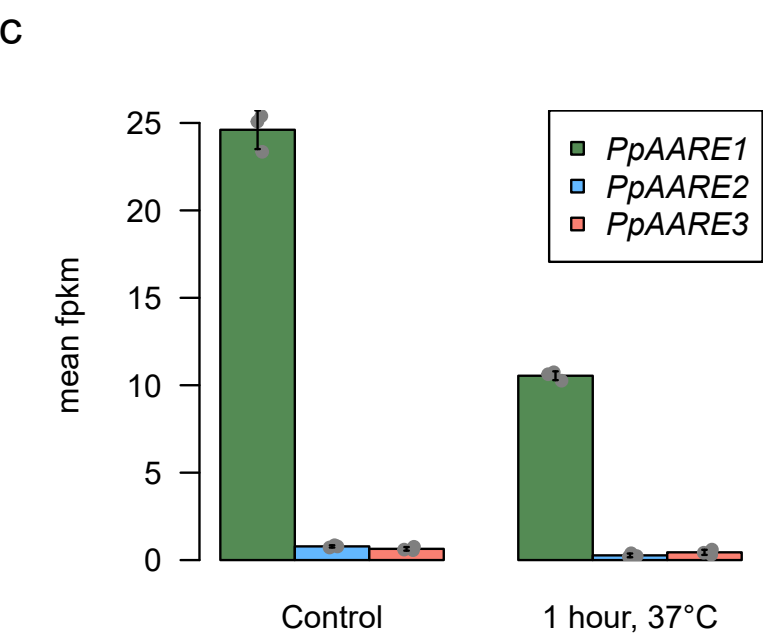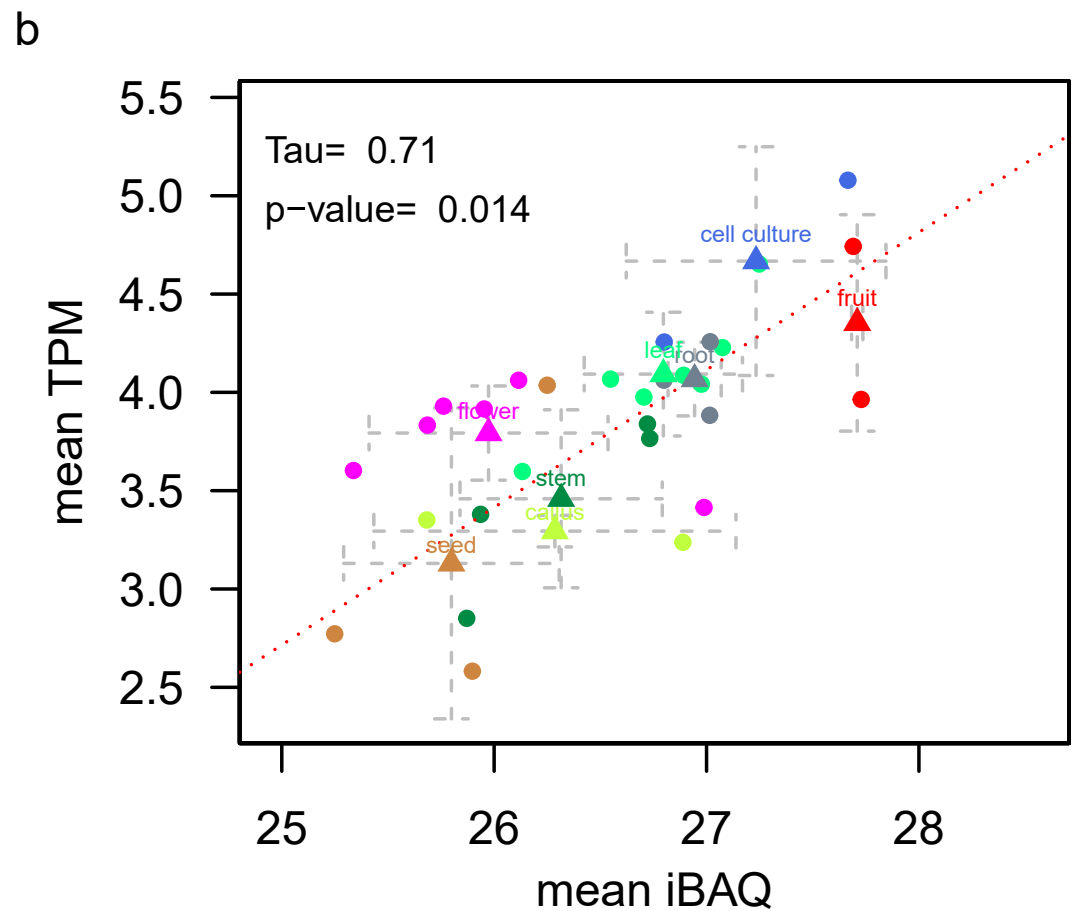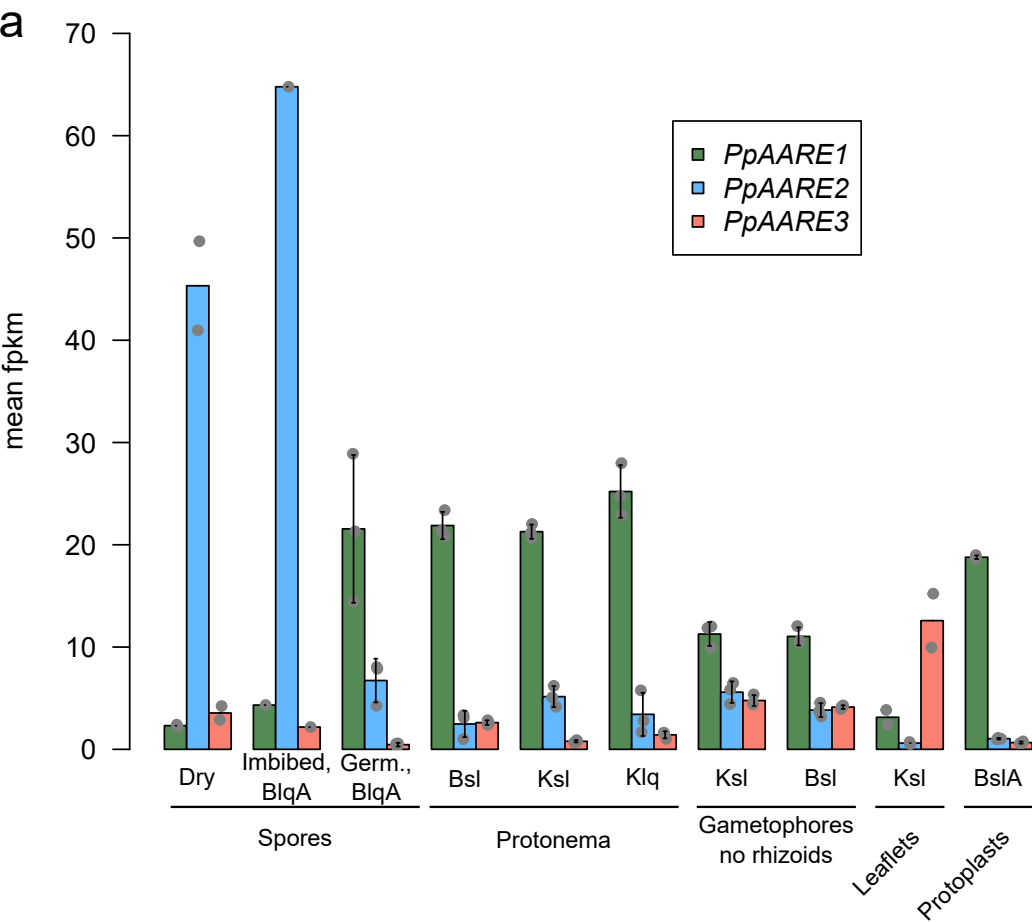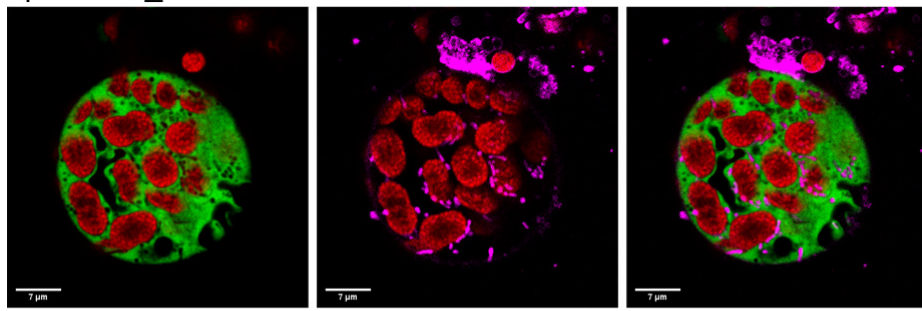
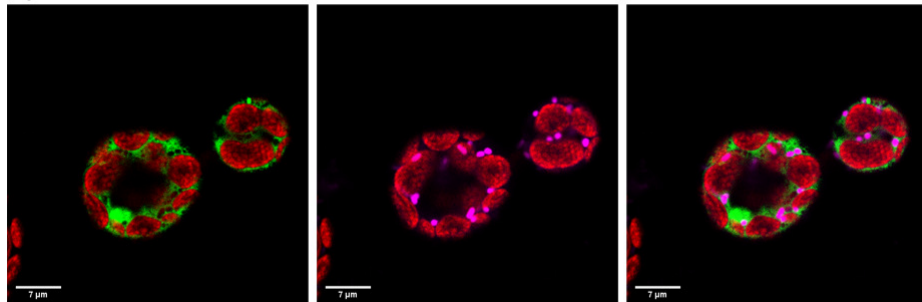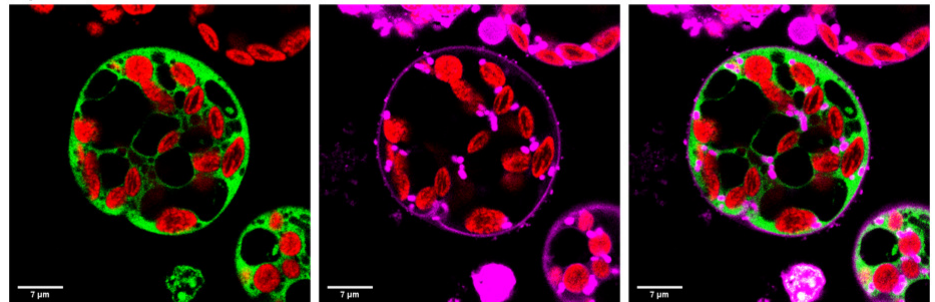| PpAARE1_1:eGFP | AtAARE_SV:eGFP |
PpAARE1_2:eGFP	AtAARE_LV:eGFP
PpAARE1_Nt:eGFP	AtAARE_Nt:eGFP
PpAARE2:eGFP	PpAARE3:eGFP

■ AARE N-terminal domain ■ WD40 domain ■ S9 Peptidase ■ Plastid transit peptide ■ GFP ├───┤ 50 AA

a

PpAARE1 (Pp3c2_30720V3.1)

UTR
Exon
Intron
Intergenic

5' HR | npt | 3' HR
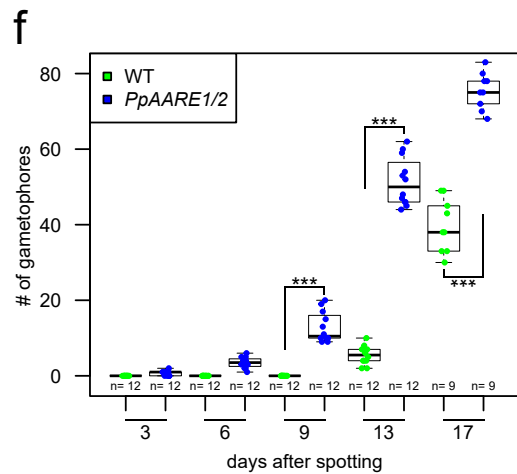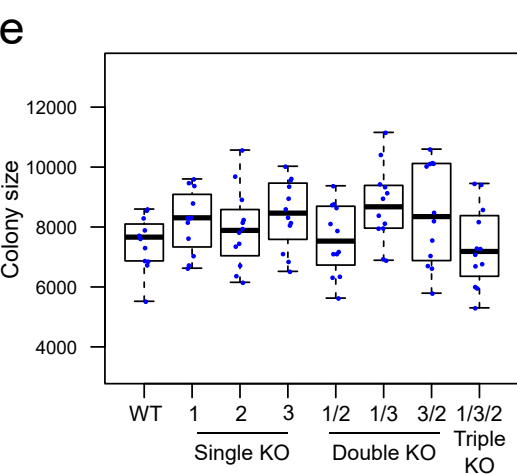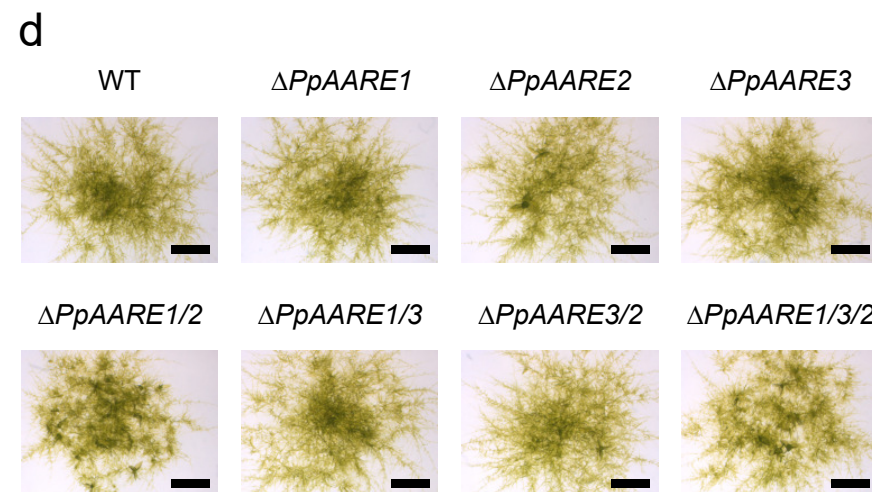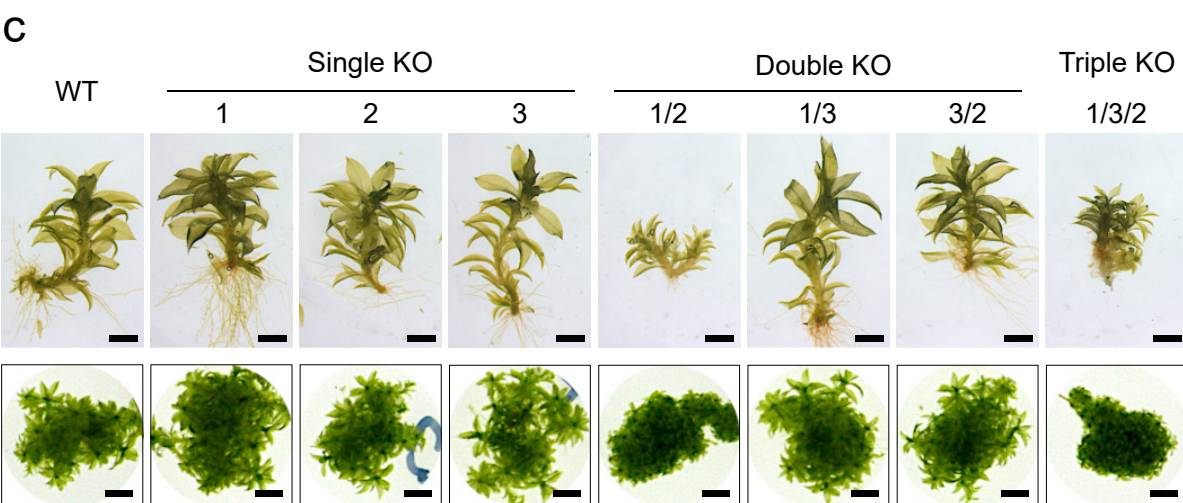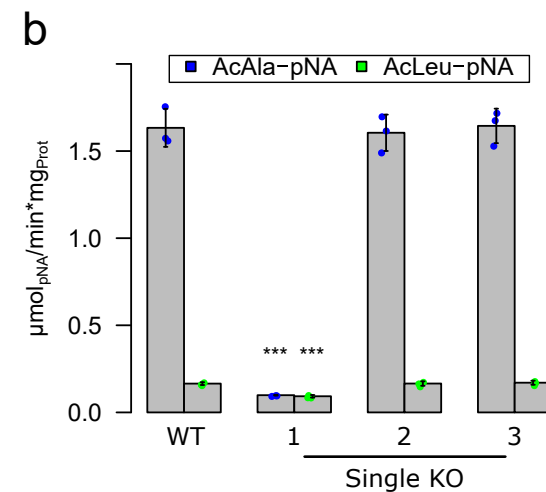
1 kb

PpAARE3 (Pp3c7_25140V3.1)

5' HR | zeo | 3' HR

PpAARE2 (Pp3c12_21080V3.1)

5' HR | hpt | 3' HR

b

■ AcAla-pNA   ■ AcLeu-pNA

$\mu mol_{pNA}/min*mg_{Prot}$

WT   1   2   3
      Single KO

*** ***

c

WT | Single KO 1 2 3 | Double KO 1/2 1/3 3/2 | Triple KO 1/3/2

d

WT | ΔPpAARE1 | ΔPpAARE2 | ΔPpAARE3
ΔPpAARE1/2 | ΔPpAARE1/3 | ΔPpAARE3/2 | ΔPpAARE1/3/2

e

Colony size

WT 1 2 3 1/2 1/3 3/2 1/3/2
    Single KO   Double KO   Triple KO

f

■ WT   ■ PpAARE1/2

# of gametophores

n= 12 ...

3   6   9   13   17
days after spotting

***

g

$\mu mol_{pNA}/min*mg_{Prot}$

WT 1 2 3 1/2 1/3 3/2 1/3/2
    Single KO   Double KO   Triple KO

***   *** ***   ***

h

Single KO | Double KO | Triple KO

WT 1 2 3 1/2 1/3 3/2 1/3/2

180
130
100
70
55
40
35
25
15

a

Single KO | Double KO | Triple KO

WT   1   2   3   1/2   1/3   3/2   1/3/2

Plate 1

b

WT   ΔPpAARE1   ΔPpAARE1/2

Plate 2

c

WT   ΔPpAARE1   ΔPpAARE1/3   ΔPpAARE1/3/2

Plate 3

a

| UTR | Exon | Intron | Intergenic | ⊢———⊣ 1 kb |

PpAARE1

PpAARE3

PpAARE2

b

a

s68   GK

At1   At2

Peptidase S9

M   Col   GK   s68 H_2O Col gDNA

    +   -   +   -   +   -

1 kb
0.75 kb
0.5 kb

AtAARE

1 kb
0.75 kb
0.5 kb

PP2AA3

b

Col   GK

100

Col   s68

100

αAtAARE

c

$\mu mol_{pNA}/min*mg_{Prot}$

■ AcAla-pNA
■ AcLeu-pNA

Col   GK   s68

***   ***

d

Col   s68

180
130
100
70
55
40
35
25
15

180
130
100
70
55
40
35
25
15

e

Nr. of leaves

26
25
24
23
22
21
20
19

Col   s68

***