

The ERA-related GTPase AtERG2 associated with mitochondria 18S RNA is essential for early embryo development in *Arabidopsis*

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Running Title:

AtERG2 is essential for early embryo development

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33 Abstract

34 The ERA (*E. coli* RAS-like protein)-related GTPase (ERG) is a nuclear-encoded
 35 GTPase with two conserved domains: a GTPase domain and a K Homology domain.
 36 ERG plays a vital role in early seed development in *Antirrhinum majus*. However, the
 37 mechanism that regulates seed development remains unclear. Blasting the genome
 38 sequence revealed two homologies of ERG, AtERG1, and AtERG2 in *Arabidopsis*. In
 39 this study, we found that AtERG2 is localised in the mitochondria and binds
 40 mitochondrial 18S RNA. Promoter and transcript analyses indicated that *AtERG2* was
 41 mainly expressed in the leaf vein, trichome, mature pollen, and ovule. The mutants of
 42 *AtERG2* showed recessive lethal, gametophytic maternal effects, silique shortage, and
 43 early seed abortion, in which some seeds arrested in the zygotic stage at 1.5 days after
 44 pollination (DAP) and aborted at 2.0 DAP in *aterg2-1* +/- . Reactive oxygen species
 45 (ROS) accumulated at 1.5 DAP in the arrested seeds, and the transcription of several
 46 ROS-responsible genes, *WRKY40*, *ANAC017*, and *AOX1a*, was up-regulated in the
 47 *aterg2-1* +/- seeds which were arrested 1.5 and 2.0 DAP but not in wild-type (WT)
 48 and *aterg2-1* +/- seeds. The cell death-related gene *BAG6* was also transcriptionally
 49 activated in *aterg2-1* +/- seeds arrested at 2.0 DAP. Chloramphenicol treatment during
 50 pollination induced a similar phenotype and gene expression pattern but showed no
 51 transcriptional changes of *ANAC017* in WT. These results suggested that AtERG2
 52 promotes early seed development by affecting the maturation of the mitochondria
 53 ribosome small subunit and mitochondrial protein translation in *Arabidopsis*.

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56 Introduction

57 The small GTP-binding proteins (GTPases) are found in all domains of life and act as
 58 molecular switches that are “activated” by GTP and “inactivated” by the hydrolysis of
 59 GTP to GDP to regulate numerous cellular processes, such as signal transduction,
 60 reorganisation of the cellular cytoskeleton, regulation of transcription and translation,
 61 protein transport and vesicle trafficking (Takai et al., 2001; Vernoud et al., 2003; Shan,
 62 2016). Based on the sequences and structural signatures, the small GTPase superclass
 63 can be divided into two large classes: the translation factors (TRAFAC) class and the
 64 signal recognition particle (SRP), MinD-like ATPases, and BioD (SIMIBI) class. The
 65 TRAFAC class includes proteins involved in translation, signal transduction (in
 66 particular, the extended Ras-like family), and cell motility. The SIMIBI class is
 67 involved in protein localisation, chromosome partitioning, membrane transport, and a
 68 group of metabolic enzymes with kinase or related phosphate transferase activity
 69 (Leipe et al., 2002). In 1986, a yeast Ras-like protein that contains 316 amino acids
 70 and low GTP hydrolyse activity was found in *E.coli* and named *E.coli* Ras-like
 71 protein (ERA) (Ahnn et al., 1986). ERA is essential for cell growth and viability
 72 (Inada et al., 1989), cell division (Britton et al., 1997), and pleiotropic processes,
 73 including carbon metabolism, fatty acid metabolism, and adaptation to thermal stress
 74 (Lerner and Inouye, 1991; Voshol et al., 2015). ERA has two conserved domains: the
 75 GTPase domain, which is homologous to RAS and belongs to the TRAFAC class, and
 76 the K Homology (KH) domain, which binds to the *E.coli* 16S rRNA and the 30S
 77 ribosomal subunits (Meier et al., 1999). Tu *et al.* showed that the GTP-binding form
 78 of ERA is helpful for recognition and binding to the (1530)GAUCACCUCC(1539)
 79 sequence at the 3' end of 16S rRNA, and RNA recognition stimulates its
 80 GTP-hydrolysing activity and the switch to the GDP-binding form; the latter
 81 suggested that ERA acts as a chaperone for the processing and maturation of 16S
 82 rRNA to benefit the assembly of the 30S ribosomal subunit (Tu et al., 2009; Tu et al.,
 83 2011).

84 ERA is highly conserved among bacteria, archaea, and eukaryotes, and its
 85 homologue in humans is called Era-like 1 (ERAL1), which is nuclear-encoded and
 86 localised in the mitochondria matrix (Britton et al., 2000; Uchiumi et al., 2010).
 87 Previous studies showed that ERAL1 binds mitochondria 12S rRNA, but not
 88 cytoplasmic ribosome rRNA, to help the maturation of the mitochondria small
 89 ribosomal subunit, which is similar to Era (Dennerlein et al., 2010; Uchiumi et al.,
 90 2010; Uchiumi and Kang, 2012). Prior studies showed that knockdown of ERAL1 by
 91 siRNA inhibits mitochondrial protein translation and elevates mitochondrial reactive
 92 oxygen species (ROS) production, leading to cell death (Uchiumi et al., 2010; Xie et
 93 al., 2012). These results indicated that ERAL1 plays an important role in the small
 94 ribosomal constitution and is involved in cell viability.

95 In plants, *ERA-related GTPase* (*ERG*) was first found in *Antirrhinum majus* and is
 96 expressed in dividing or metabolically active cells. *ERG* was predicted to target to the
 97 mitochondria by amino-terminal sequences, and a deletion allele of *ERG* by
 98 site-selected transposon mutagenesis resulted in the arrested development of seeds

99 containing embryos and endosperm after fertilisation (Ingram et al., 1998). Genomic
100 data on the *ERG* genes in *Arabidopsis* showed that there are two homologues,
101 AtERG1, localised in chloroplast, and AtERG2, localised in the mitochondria
102 (Suwastika et al., 2014). Jeon *et al* (Jeon et al., 2014) showed that *Nicotiana*
103 *benthamiana* double Era-like GTPase (NbDER) contains two tandemly repeated
104 GTPase domains and a C-terminal KH-like domain involved in RNA binding.
105 NbDER was localised primarily to chloroplast nucleoids, possessed GTPase activity,
106 and bound to 23S and 16S ribosomal RNAs, which contributed to the maturation and
107 assembly of the chloroplast 50S ribosomal subunit. Depletion of NbDER impaired
108 processing of plastid-encoded ribosomal RNAs, resulting in accumulation of the
109 precursor rRNAs in the chloroplasts and a leaf-yellowing phenotype caused by
110 disrupted chloroplast biogenesis. However, the mechanism of AtERGs involved in the
111 organelle rRNA processing and ribosome biogenesis remains unclear. Here, we
112 showed that AtERG2 was localised in the mitochondria, dependent on its N-terminal
113 sequence, and bound mitochondria 18S RNA *in vivo*. The T-DNA insertion mutant of
114 *ATERG2* is recessive lethal, and some seeds arrest at the zygote stage at 1.5 DAP and
115 abort at 2.0 DAP in *aterg2-1 +/-*. We further found that ROS were accumulated, and
116 the transcription of several ROS-responsible genes, *WRKY40*, *ANAC017*, and *AOX1a*,
117 and the cell death-related gene, *BAG6*, were up-regulated in the *aterg2-1 +/-* arrested
118 seeds but not in WT and *aterg2-1 +/-* developed seeds; this is similar to CAP
119 treatment with WT. These results suggest that AtERG2 is involved in regulating seed
120 early development through mitochondria ribosome maturation and protein translation.

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122

123 **Materials and Methods :**

124 **Plant growth and chloramphenicol treatment**

125 *Arabidopsis thaliana* (Columbia-0) plants were grown in soil with a 16/8-h light/dark
126 cycle at 22°C in the plant growth room. Seeds were sterilised and plated onto 1/2
127 Murashige and Skoog (MS) basal salt medium with 0.8% agar and 1.5% (w/v)
128 sucrose in the dark for 2 days at 4°C. Then, the plates were grown in the growth
129 chamber (Panasonic, Japan) at a 16/8-h light/dark cycle at 22°C for 7 days, and the
130 seedlings were transplanted in the soil for continual growth. One week after stemming,
131 the pre-pollinated flowers were treated by dipping the whole flower into 500 µg /mL
132 Chloramphenicol (CAP) in 0.02% Sliwet-77 solution for 30 s. The treatment lasted
133 for 3 days, and the siliques were collected at the exact developmental stages for the
134 next analysis. The control was the same buffer without CAP.

135 **DNA and RNA extraction**

136 *Arabidopsis* genomic DNA was isolated from 4-week-old leaves using the extraction
137 buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) as
138 described (Edwards et al., 1991). Total RNA was extracted by TRIZOL Reagent
139 (Thermo Fisher Scientific, USA) from *Arabidopsis* seeds according to the
140 manufacturer's instructions (Thermo Fisher Scientific) and quantified using a
141 Nanodrop ND-1000 spectrophotometer (LabTech, USA). Total RNA (5 µg) was used
142 for cDNA synthesis with Superscript II RNase H-Reverse Transcriptase (Life
143 Technologies, USA) and oligo(dT)₁₈ primers according to the manufacturer's
144 instructions.

145 **Characterisation of T-DNA Insertion Mutants**

146 Two T-DNA insertion lines of *AtERG2*: *aterg2-1* (SALK_032115) and *aterg2-2*
147 (SALK_032124) were purchased from *Arabidopsis* stock centres
148 (<http://www.arabidopsis.org/>) (Figure 3A). The genotype of T-DNA lines was
149 confirmed by PCR (Figure S3A) using the gene-specific primers and a
150 T-DNA-specific primer (Table S1).

151 **Constructs and transgenic plants**

152 The coding sequence of *AtERG2* was amplified by PCR and cloned into the Gateway
153 Cloning vector pE6c or pE3c (Addgene, USA) with Bam HI and Not I sites. To
154 generate the various truncated *AtERG2* constructs, such as *AtERG2_N* (containing
155 only N-terminal), *AtERG2_C* (containing GTPase domain (GD) and KH domain
156 (KH)), *AtERG2_GD* (containing only GD), and *AtERG2_KH* (containing only KH),
157 the corresponding *AtERG2* fragments were amplified and subcloned into pE6c.
158 Construct maps containing different parts of *AtERG2* are shown in Figure S2. The
159 different constructs were used in combination with the destination vector pMDC32
160 (<http://www.arabidopsis.org/>) with the Gateway LR II kit (Life Technologies, USA) to
161 generate the plant expression vectors. A genomic fragment of 1.8 kb in the promoter

162 region of *AtERG2* was amplified by PCR and cloned into a GUS binary vector
163 pCambia 1305.1 with EcoR I and Bgl II sites. The primers used in these constructs
164 are listed in Table S1. All vectors were confirmed by sequencing.

165 The resulting 35S::AtERG2-6Xmyc and *P_{AtERG2}::GUS* constructs were
166 transformed into *Agrobacterium tumefaciens* GV3101. Transgenic plants were
167 generated by the floral dip method (Clough and Bent, 1998) and selected on MS
168 medium containing hygromycin B (25 mg/L) or kanamycin (50 mg/L). More than 100
169 AtERG2-6Xmyc or *P_{AtERG2}::GUS* lines were recovered. Independent homozygous
170 transformants carrying a single insertion in the T3 generation were further analysed.

171 **Transient transformation of *Arabidopsis* mesophyll protoplasts**

172 The vectors containing AtERG2-eYFPs were co-transformed with a mCherry-tagged
173 organelle marker gene and transiently expressed in *Arabidopsis* mesophyll protoplasts,
174 as described previously (Liang et al., 2015). After incubation for 16 h at 22°C,
175 fluorescence was visualised using an LSM 700 confocal microscope (Zeiss, Germany).
176 Observations were made using a 63× objective under oil immersion. eYFP
177 fluorescence was excited at 488 nm and collected at SP 550 IR. The mCherry
178 fluorescence was stimulated at 555 nm and collected at SP 630 IR.

179 **Immunoprecipitation of mitochondria 18S RNA with AtERG2**

180 The 2-week-old transgenic *Arabidopsis* seedlings overexpressing AtERG2-6Xmyc
181 were ground in liquid nitrogen, and the protein/RNA complexes were extracted using
182 equal volumes of lysis buffer (IP buffer (50 mM NaCl, 2 mM MgCl₂, 10 mM
183 HEPES pH 7.5, 2 mM DTT, 2 mM EDTA, 100 U/mL RNasin RNase inhibitor
184 [Promega, USA], and 100 U/mL protease inhibitor cocktail [Sigma-Aldrich, USA]
185 plus 1% TritonX-100 and 0.5% SDS) and incubated for 1 h on ice, then centrifuged
186 twice at 13,000 g for 10 min at 4°C to remove the insoluble debris. The supernatant
187 was diluted with nine volumes of IP Buffer and incubated with 10 µL anti-Myc
188 antibody (Sigma-Aldrich) for 4 h on ice with occasional gentle mixing. Next, the
189 anti-Myc-decorated extracts were incubated with 10 µL protein G agarose beads
190 pre-washed with IP buffer for 1 h at 4°C with rotation. Then, the beads were spun
191 down at 10,000 rpm in a microfuge for 1 min at 4°C, and washed eight times with 1
192 mL washing buffer (100 mM NaCl, 2 mM MgCl₂, 10 mM HEPES pH 7.5, 2 mM DTT,
193 2 mM EDTA, 100 U/mL RNasin RNase inhibitor, and 100 U/mL protease inhibitor
194 cocktail). After elution with TRIZOL reagent, co-immunoprecipitated RNA was
195 isolated and analysed by quantitative real-time RT-PCR (qRT-PCR).

196 **qRT-PCR**

197 qRT-PCR was performed on the 7500 Fast Real-Time PCR System (Applied
198 Biosystems, USA) using Power SYBR[®] Green PCR Master Mix (Applied
199 Biosystems). The thermal program was 2 min at 50°C, 10 min at 95°C, followed by
200 40 cycles of 15 s at 95°C and 60 s at 60°C. The data were normalised to the
201 expression of *Arabidopsis actin*. The dissociation curve program was used to confirm
202 the specificity of the target amplification product. All primers used in this study are

listed in Table S1. At least three independent biological replicates were performed for qRT-PCR analysis. The expression analysis was performed using the Data Processing System. A one-way analysis of variance (ANOVA) and Tukey's multiple range test were conducted to determine significant differences. $P < 0.05$ was considered statistically significant.

Microscopy analysis

Seeds were removed from siliques at different developmental stages, cleared in Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 mL glycerol in 30 mL water) and examined using a Zeiss Axio Observer A1 microscope equipped with DIC optics as described by Liu and Meinke (Liu and Meinke, 1998). The ROS detection was performed according to a modified version of a previously described method (Daudi et al., 2012) by staining with 1 mg/mL 3,3'-diaminobenzidine (DAB, Sigma) containing Triton X-100 (0.1% v/v) and 10 mM sodium phosphate buffer (pH 7.0). As previously described (Yu et al., 2005), the GUS staining was performed for 4 hours with seedlings and leaves or overnight with flowers and seeds at 37°C in GUS staining solution (50 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 1 mg/mL X-Gluc) before the clearing procedure.

223 Results

224 Phylogenetic relationship of AtERGs and its homologues

225 A previous study showed that there are two homologues of ERG in the *Arabidopsis*
 226 genome: AtERG1 (At5g66470), localised in the chloroplast, and AtERG2
 227 (At1g30960), localised in the mitochondria (Suwastika et al., 2014). We used the
 228 sequence of ERA, ERAL1, ERG, AtERG1, and AtERG2 to search NCBI public
 229 databases (<http://blast.ncbi.nlm.nih.gov>) at an E-value of 1e-10. The presence of
 230 conserved GTPase and KH domains is the exclusive criterion for confirmation of 16
 231 ERA homologous proteins in 14 species, which include MmERA from *Mus musculus*,
 232 DrERA from *Danio rerio*, DmERA from *Drosophila melanogaster*; ScERA from
 233 *Saccharomyces cerevisiae*, OsERG1 and OsERG2 from *Oryza sativa*, CsERG from
 234 *Cucumis sativus*, ZmERG from *Zea mays*, NtERG from *Nicotiana tabacum*, StERG
 235 from *Solanum tuberosum*, and SIERG from *Solanum lycopersicum*. Additionally, Jeon
 236 et al. (Jeon et al., 2014) found a chloroplast-localised double Era-like GTPase in
 237 *Nicotiana benthamiana* (NbDER), which binds to chloroplast 23S and 16S ribosomal
 238 RNAs. We further identified the homologues of NbDER, such as AtDER from
 239 *Arabidopsis thaliana*, EcDER from *E. coli*, ZmDER from *Zea mays*, and OsDER
 240 from *Oryza sativa*. Then, a phylogenetic tree was generated using the CDS (sequence
 241 coding for amino acids in protein) of 21 ERA homologues, and we found that
 242 members of the ERA homologues were separated into four distinct clades, designated
 243 I, II, III, and IV (Figure S1). Clade I included 12 members: ERG, AtERG2, CsERG,
 244 OsERG2, ZmERG, NtERG, SIERG, StERG, ERAL1, MmERAL1, DrERAL1, and
 245 DmERAL1, which were predicted and/or identified as mitochondria-localised. Clade
 246 II contained three members: ERA, AtERG1, and OsERG1; this suggested that ERA is
 247 most closely related to chloroplast-localised AtERG1 and OsERG1. Clade III
 248 included one member: ScERA. Clade IV had five DERs. These eukaryotic ERALs
 249 and ERGs were nuclear-encoded and organelle-localised genes, indicating that they
 250 were transferred to the nucleus after organelle symbiogenesis.

251 AtERG2 is dependent on its N-terminal sequence for localisation to the 252 mitochondria

253 To further investigate the subcellular localisation of AtERG2, we developed various
 254 constructs containing different parts of AtERG2 fused with yellow fluorescent protein
 255 (eYFP) at their C-terminal end (Figure S2) and transiently co-transformed cells with a
 256 mCherry-labelled organelle marker in the *Arabidopsis* mesophyll protoplasts. The
 257 full-length AtERG2 was localised in the mitochondria as previously reported (Figure
 258 1A) (Suwastika et al., 2014). We further found that the N-terminal end (1-150 aa) of
 259 AtERG2 was localised in the mitochondria. However, the C-terminal end, GTPase
 260 domain, and KH domain alone diffused into the cytoplasm (Figure 1A). These results
 261 indicated that the N-terminal end of AtERG2 is critical for its localisation to the
 262 mitochondria.

263 AtERG2 associates with mitochondria 18S rRNA *in vivo*

We next used RNA immunoprecipitation (RNA-IP) assays to detect AtERG2 binding ribosome rRNA. The AtERG2-Myc:rRNA complex was extracted from the transgenic *Arabidopsis* stably expressed AtERG2-Myc fusion protein, preincubated with Myc antibody, and purified by protein G agarose beads. Then, the rRNA was isolated with Trizol solution and detected by RT-PCR (Figure 1B). The results showed that AtERG2 binds mitochondria ribosome small subunit 18S rRNA but not large subunit component 26S rRNA, suggesting that AtERG2 plays the same role as its homologues ERA and ERAL1 for the maturation of 16S rRNA and assembly of ribosomes.

AtERG2 is mainly expressed in the leaf vein, trichome, mature pollen, and ovule.

To detect the transcription pattern of *AtERG2* in *Arabidopsis*, we first generated the independent transgenic *Arabidopsis* lines, which stably expressed *P_{AtERG2}::GUS* (Figure 2). In the F2 generation, GUS activity was observed in the leaf vein of 1-week-old seedling cytoledons (Figure 2A), trichomes of 3-week-old rosette leaves (Figure 2B and 2C), pollen grains inside the anthers (Figure 2D and 2E), and ovules during the period of pollination and early-stage seed development (Figure 2F-I). In anthers, GUS activity was relatively weak the day before pollination (Figure 2D), but it increased in mature pollen at 0.5 DAP (Figure 2E). We also found that GUS staining was detected at a low level in the pre-matured embryo sac before pollination (Figure 2F); it then reached the highest level in the ovule of 0.5- and 1.0-DAP seeds during the zygotic stage (Figure 2G and 2H) and quickly dropped in the 1.5-DAP seeds as soon as it reached the early-globe stage (Figure 2I). These results were further confirmed by qRT-PCR assays to evaluate the expression of *AtERG2* in different tissues and in the development of *Arabidopsis*. Figure 2J showed that *AtERG2* transcripts were highly expressed in the 0.5-, 1.0-, and 1.5-DAP seeds.

The T-DNA insertion mutant of *AtERG2* shows a recessive lethal and gametophytic maternal effect (GME) phenotype

To characterise the function of *AtERG2*, the two T-DNA insertion lines (SALK_032115, named as *aterg2-1*, and SALK_032124, named as *aterg2-2*, respectively, Figure 3A) were obtained from The *Arabidopsis* Information Resource (TAIR, <https://www.arabidopsis.org/>), and the genotype of the progeny from self-crossed plants was examined by PCR. There were no homozygous mutant seeds from the progeny of self-crossed *aterg2-1* or *aterg2-2* plants (Figure S3A), and the segregation ratio of WT to *aterg2-1* +/- was close to 1:1, which indicated that the mutant line was recessive lethal (Table 1). Then, we backcrossed *aterg2-1* +/- with WT plants and found that the segregation ratio of WT to *aterg2-1* +/- was 2:1 when using WT as a paternal provider, and the ratio of WT to *aterg2-1* +/- was almost 1:1 when using WT as the maternal provider (Table 1). These results showed that *aterg2-1* has a GME phenotype, indicating that *AtERG2* plays a vital role in regulating *Arabidopsis* seed development.

***aterg2-1* +/- shows defects in early embryo development**

During the whole growth period from germination to flowering, both *aterg2-1* and

305 *aterg2-2* showed similar phenotypes to WT, with the exception of silique length and
 306 seed number; also, the ratios of the developed seeds of mutant lines were lower than
 307 those of the WT (Figure 3B to 3D and Figure S3B and S3C). We found that the
 308 developed seeds per silique in *aterg2-1* and *aterg2-2* were about 30% of WT at 6 DAP
 309 until seed maturation (Figure S3B and S3C), which suggested that seed development
 310 was arrested and aborted at the early development of seeds in both *aterg2-1* and
 311 *aterg2-2*. Therefore, we used *aterg2-1 +/-* to perform the following experiments.

312 Next, we investigated the silique development of WT and *aterg2-1 +/-* from the
 313 pre-pollination stage (BP) to 4 days after pollination (DAPs) and found that the
 314 silique length was significantly shorter at 3 DAP in *aterg2-1 +/-* than in WT (Figure
 315 3E). Some seeds were small and arrested at 1 DAP, and the arrested ratio of seed
 316 development reached 65% at 3 DAP in *aterg2-1 +/-* (Figure 3F), which is almost the
 317 same as the ratio of seed development at 6 DAP. We further dissected the siliques and
 318 hyalinised seeds to observe the embryo development at BP, 0.5, 1.5 and 2.0 DAP to
 319 explore the impairment of early seed development in *aterg2-1 +/-*. Firstly, we found
 320 that there is no developmental difference of embryo sac between WT and *aterg2-1 +/-*
 321 plants at BP and 0.5 DAP (Figure S4). In addition, we showed that there are 50.7%
 322 and 64.2% small-sized seeds at 1.5 DAP and 2.0 DAP in *aterg2-1 +/-* silique,
 323 respectively, but only 2.4% were found in WT silique, although the silique length was
 324 the same between WT and *aterg2-1 +/-* plants at 2.0 DAP (Figure 4A and 4B). At 1.5
 325 DAP, there were two types of embryos in WT and *aterg2-1 +/-* siliques: 31.1% in the
 326 zygote stage and 68.9% in the early-globe stage for WT and 56.9% in the zygote stage
 327 and 43.1% in the early globe-stage embryos for *aterg2-1 +/-* siliques, respectively
 328 (Figure 4C). Furthermore, we found 9.8% heart-stage, 57.9% globe-stage (eight or
 329 more cells of embryos), and 32.2% early globe-stage embryos in 2.0-DAP WT seeds,
 330 and 1.3% heart-stage, 21.1% globe-stage, and 23.2% early globe-stage embryos were
 331 found in 2.0-DAP *aterg2-1 +/-* seeds. More interestingly, as much as 38% of seeds
 332 stayed in the zygote stage, and 16.5% of these seeds were collapsed seeds without
 333 embryos in 2.0-DAP *aterg2-1 +/-* siliques (Figure 4C). These results suggested that
 334 insertional inactivation of *AtERG2* causes delay in the development of embryos after
 335 pollination and arrested seeds from 1.5 DAP in *aterg2-1 +/-* plants.

336 **ROS accumulation and activated transcription of ROS-responsible and cell** 337 **death-related genes in *aterg2-1 +/-* arrested seeds**

338 Previous studies showed that the knockdown of ERAL1 in human cell lines by siRNA
 339 inhibits mitochondrial protein translation and elevates mitochondrial reactive oxygen
 340 species (ROS) production, leading to cell death (Uchiumi et al., 2010; Xie et al.,
 341 2012). Thus, ROS production and the expression of the ROS-related genes may cause
 342 the developmental stagnation of embryos and arrested seeds in *aterg2-1 +/-*. To test
 343 this hypothesis, we performed DAB staining to detect ROS content in *Arabidopsis*.
 344 We found that the arrested seeds in *aterg2-1 +/-* exhibited more pronounced increases
 345 in DAB staining than did the developed seeds in WT and *aterg2-1 +/-* (Figure 5A),
 346 suggesting higher ROS levels in the arrested seeds of *aterg2-1 +/-* than in the
 347 developed seeds of WT and *aterg2-1 +/-*. Furthermore, *Alternative oxidase1a*

(*AOX1a*), a nuclear gene encoding mitochondrial protein, is activated by ROS through ROS-responsible transcription factors WRKY40 and ANAC017 (Ng et al., 2013; Van Aken et al., 2013; Liu et al., 2014). Thus, these three genes can act as the marker genes for monitoring ROS levels. qRT-PCR showed that the transcription levels of these three genes was specifically higher at 1.5 and 2.0 DAP in the arrested seeds of *aterg2-1* +/- than in the developed seeds of WT and *aterg2-1* +/- (Figure 5B).

The BCL2-associated athanogene (BAG) family, as the calmodulin-binding proteins, serve as co-chaperones to recruit HSP70/HSC70 to a specific target protein regulating diverse cellular pathways, such as programmed cell death (PCD) and stress responses in eukaryotes (Hung et al., 2003; Yan et al., 2003). Kang *et al* (Kang et al., 2006) showed that overexpression of *AtBAG6* induced cell death phenotypes consistent with programmed cell death (PCD) in yeast and plants. Here, we found that *AtBAG6* is extremely up-regulated in 2.0-DAP seeds of *aterg2-1* +/- but not in WT plants (Figure 5B), which suggested that inactivation of *AtERG2* induces the higher ROS levels and the cell death of embryos after pollination to produce the arrested seeds in *aterg2-1* +/- plants.

Chloramphenicol treatment causes arrested development of the early embryo in *Arabidopsis*

To investigate whether the abortion of seeds in *aterg2-1* +/- is associated with mitochondria protein synthesis, we chose chloramphenicol (CAP), which prevents protein chain elongation by inhibiting the peptidyl transferase activity of the bacterial ribosome, to inhibit the translation of mitochondria-encoded genes and observe the phenotype of seed development in *Arabidopsis* WT plants. *Arabidopsis* flowers were treated with CAP solution before pollination until 2 DAPs using the floral dip method. We found that the silique length was shorter at 1.5 and 2.0 DAP with CAP treatment than in the control (Figure 6A), and the development rate of the embryo was 98.4% for the zygote period at 1.5 DAP and 62.5% for the zygote at 2.0 DAP with CAP treatment. However, the ratio of the zygote was 45.0% for 1.5 DAP and 8.2% for 2.0 DAP in the control (Figure 6B). Additionally, the transcription of *WRKY40*, *ANAC017*, *AOX1a*, and *AtBAG6* was induced with CAP treatment, and the transcription level of *AtERG2* did not change (Figure 6C). These results indicated that CAP treatment plays a role similar to that of insertional inactivation of *AtERG2* to trigger the ROS-related cell death through dysfunction of mitochondrial proteins.

383 Discussion

384 After double fertilisation in flowering plants, seed development starts with the
385 coordinated development of embryo and endosperm, as well as interactions between
386 endosperm and seed coat, which require both parental genomes. However, prior
387 studies showed that embryo development is dominantly affected by both the female
388 gametophyte and the sporophytic tissue of the parent plant, which is classified as the
389 gametophytic maternal effect (GME) (Grossniklaus et al., 1998; Pien and
390 Grossniklaus, 2007). Based on high-throughput sequencing, Autran *et al.* (Autran et
391 al., 2011) showed that maternal epigenetic regulation controls the parental
392 contributions in early embryogenesis in *Arabidopsis*. Additionally, mitochondria act
393 as another maternal inheritance factor that affects the early seed development,
394 although the ultrastructure observations implied that mitochondria could also be
395 transmitted from sperm to the egg cell in tobacco (Yu and Russell, 1994). Here, we
396 found that AtERG2 is a nucleus-encoded and mitochondria-localised ERA-like
397 GTPase in *Arabidopsis*, and the T-DNA insertion mutant lines of *AtERG2* show a
398 recessive lethal and GME phenotype after self-crossing or back-crossing with WT,
399 which suggested that *aterg2* +/- represents a distinct class of GME mutants affecting
400 early seed development.

401 The *Arabidopsis* genome contains three homologues of ERG: AtERG1 is localised
402 in the chloroplast, AtERG2 is localised in mitochondria (Suwastika et al., 2014), and
403 AtDER, similar to chloroplast-localised double Era-like GTPase in *Nicotiana*
404 *benthiana* (NbDER) (Jeon et al., 2014), is predicted to be localised in the
405 chloroplast. In this study, we further showed that AtERG2 is localised to the
406 mitochondria, dependent on its N-terminal sequence and is mainly expressed in the
407 leaf vein, trichome, mature pollen, and ovule. The heterozygous *aterg2* +/- plant
408 showed a severe abortion of seeds in siliques from 1.5 DAP, and more than 50% were
409 arrested in an early stage of development, but only 2.4% were arrested in WT silique.
410 After an additional 8 h of development, 16.5% of the seeds were severely crushed into
411 a group of dead cell aggregates, and only 45.5% of embryos could successfully split
412 out suspensors and develop into the globe stage. However, almost 100% of embryos
413 developed into globe-stage embryos in WT silique. This phenomenon is similar to
414 ERG function in *Antirrhinum majus* (Ingram et al., 1998), suggesting that
415 mitochondria-localised ERG are essential for early seed development in plants.

416 The mitochondria genome size ranges from <6 kilobase pairs (kbp) in *Plasmodium*
417 *falciparum* (the human malaria parasite) to >200 kbp in land plants (Gray et al., 1999),
418 which encode a limited number of mitochondria tRNAs, ribosomal proteins, essential
419 subunits of the different respiratory chain complexes, and some components involved
420 in cytochrome-c biogenesis (Marienfeld et al., 1999). Therefore, mitochondria play a
421 pivotal role in regulating cellular energy homeostasis and redox balance, and the
422 disabled respiratory chain may cause broken electron transport chain balance and
423 result in ROS accumulation in the matrix, possibly leading to cell death (Blackstone
424 and Green, 1999). Here, we showed that AtERG2 binds mitochondrial ribosome small
425 subunit component 18S rRNA, which benefits the maturation of 18S rRNA and the

426 assembly of the mitochondrial ribosome. Therefore, disruption of AtERG2 in the
 427 *aterg2-1* +/- mutant affected the translation of the respiratory chain-related proteins to
 428 trigger ROS accumulation; it followed the expression of the ROS-related genes
 429 WRKY40, ANAC017, and AOX1a and the cell death-related gene BAG6, finally
 430 resulting in arrest of embryos at the zygote stage or early-globe stage in the aborted
 431 seeds of a mature silique from an *aterg2-1* +/- mutant. These results were further
 432 confirmed by CAP treatment of WT seeds because CAP caused the translational
 433 dysfunction of mitochondrial-encoded protein (Xie et al., 2012). These results are in
 434 accordance with previous studies, which showed that nuclear- or
 435 mitochondria-encoded gene mutations affecting mitochondrial functions, such as
 436 transcription, protein synthesis, and oxidative phosphorylation, affected ROS levels in
 437 developing gametophytes, thereby adversely affecting gametophyte and polar nuclei
 438 fusion during seed development (Maruyama et al., 2016; Pratibha et al., 2017). Our
 439 results suggest that AtERG2 promotes early seed development by affecting the
 440 maturation of the mitochondria ribosome small subunit and the mitochondrial protein
 441 translation in *Arabidopsis*, which highlights the role of mitochondrial ROS
 442 homeostasis in earlier seed development.

443

444

445 Accession numbers

446 Sequence data for the genes in this study can be found in the GenBank database under
447 the following accession numbers: AtERG2 (At1g30970), ANAC017 (AT1G34190),
448 AOX1a (AT3G22370), WRKY40 (AT1G80840), BAG6 (AT2G46240), mitochondrial
449 ribosome large subunit component 26S rRNA (AtMG00020), and small subunit 18S
450 rRNA (AtMG01390).

451

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455 Higher Education (20090003110016)

456

457 Conflict of interest

458 The authors declare no conflict of interest.

459

460 Supplementary Material

461 Table S1. Primer sequences used in this study.

462 **Figure S1. Phylogenetic analysis of AtERGs and homologous proteins.**
463 Phylogenetic tree constructed using the predicted amino acid sequences of the 21
464 ERA homologous proteins in 14 species. ERA (NP_417061.1) and EcDER (double
465 Era-like GTPase, AAC75564) were from *E.coli*; ERAL1 (NP_005693.1) from *Homo*
466 *sapiens*; DrERA (NP_001122219.1) from *Danio rerio*; MmERA (NP_071708.2) from
467 *Mus musculus*; ScERA (NP_013736.1) from *Saccharomyces cerevisiae*; ERG
468 (O82626.1) from *Antirrhinum majus*; AtERG1 (At5g66470), AtERG2 (At1g30960),
469 and AtDER (At3g12080) from *Arabidopsis thaliana*; OsERG1 (Os05g49220),
470 OsERG2 (Os08g10649.1), and OsDER (XP_015628913.1) from *Oryza sativa*;
471 ZmERG (NP_001167959.1) and ZmDER (ACL53683) from *Zea mays*; StERG
472 (XP_006363190.1) from *Solanum tuberosum*; CsERG (XP_004134545.1) from
473 *Cucumis sativus*; NtERG (XP_016481550.1) from *Nicotiana tabacum*; SlERG
474 (XP_004232641.1) from *Solanum lycopersicum*; NbDER (KC846070) from
475 *Nicotiana benthamiana*.

476 **Figure S2. Schematic of different AtERG2-eYFP constructs.** The full-length and
477 different truncated AtERG2 constructs were constructed with eYFP (green rectangles)
478 attached to the C-terminal region. AtERG2 was divided into three different regions:
479 1–150 aa as the N-terminal of AtERG2 (AtERG2_N); 151–437 aa as the C-part of
480 AtERG2 (AtERG2_C), which contains 151–326 aa as GTPase domain (AtERG2_GD);
481 and 338–437 aa as the KH-domain (AtERG2_KH).

482 **Figure S3. Characterisation of T-DNA insertion mutant of AtERG2.** (A) Two

483 T-DNA insertion lines, *aterg2-1* (SALK_032115) and *aterg2-2* (SALK_032124), were
 484 identified by PCR. Wild-type (WT) *Arabidopsis* was used as the control. LP, LB, and
 485 RP primers are shown in Figure 1A and Table S1. (B) The mature seed numbers per
 486 silique of WT and mutant lines (n=60). (C) The ratio of developed and arrested seeds
 487 in WT and mutant lines at 6 DAP (number of siliques = 8).

488 **Figure S4. The ovule development in WT and *aterg2-1* +/- at Before pollination**
 489 **(BP) and 0.5 DAP.**

490 The ratio of developed ovules for WT and *aterg2-1* +/- at BP and 0.5 DAP,
 491 respectively (n > 150). The egg cell is indicated by ec; the synergid cells are indicated
 492 by sy; The two polar nuclei are indicated as pc; the central cell after the polar cell
 493 fused is indicated by cc; the early globe-stage embryos (with fewer than four cells in a
 494 single embryo) are indicated by ea. gl; the endosperm cell is indicated as end. Bar =
 495 0.1 mm.

496

497

Table 1. Genotype of the *aterg2* +/- mutant

Parental Genotype (Female x Male) ^a	<i>AtERG2</i>	<i>aterg2</i> +/-	<i>aterg2</i>	ratio ^b
<i>aterg2</i> +/- × <i>aterg2</i> +/-	153	166	0	0.921 : 1
<i>aterg2</i> +/- × <i>AtERG2</i>	21	10		2.1 : 1
<i>AtERG2</i> × <i>aterg2</i> +/-	47	36		1.306 : 1

^a Plants were crossed manually, and seeds of the resulting cross were collected and grown on MS plates. The germinated seedlings were transferred to soil, and their 4-week-old leaves were then collected for genomic DNA extraction. Their genotypes were determined by PCR.

^b The ratio is the number of plants exhibiting the WT genome: the number of plants with the *aterg2* +/- genome without the T-DNA insertion.

499

Figure legends

Figure 1. *AtERG2* is localised in mitochondria and associated with mitochondria 18S RNA.

(A) Localisation of various truncated *AtERG2* constructs in *Arabidopsis* mesophyll cells. The full-length *AtERG2* (*AtERG2*_FL-YFP) and *AtERG2* N-terminal part (*AtERG2*_N-YFP) were co-transformed with mCherry-labelled mitochondria marker; *AtERG2* C-part (*AtERG2*_C-YFP), *AtERG2* GTPase domain (*AtERG2*_GD-YFP), and *AtERG2* KH domain (*AtERG2*_KH-YFP) co-transformed with mCherry-labelled cytoplasm marker. All images in this figure were obtained from one optic section. Scale bars are equivalent to 20 μm. The schematic of different *AtERG2*-eYFP constructs were shown in Figure S3.

(B) RNA immunoprecipitation (RNA-IP) assays (top) showing the interaction between *AtERG2*-MYC and Mt 18S RNA in *Arabidopsis* were performed with (Lane 3 and Lane 5) or without (Lane 4) MYC antibody followed by RT-PCR detection. Mt 28S RNA served as a negative control (Lane 1 and Lane 2). The Western blot (WB, bottom) indicates the protein level in the transgenic plants, which was detected prior to the RNA-IP assay.

Figure 2. Differential Expression of *AtERG2* in pollen and early-stage ovule.

GUS staining of *P_{AtERG2}::GUS* transgenic *Arabidopsis* plants for 4 hours with seedlings and leaves or overnight with flowers and seeds. Bar = 2 mm in (A); 5 mm in (B); 0.2 mm in (C); 0.5 mm in (D) and (E); 0.05 mm in (F), (G), (H), and (I); and 0.02 mm in pollen of (D) and (E).

(A) *P_{AtERG2}::GUS* fusion staining in 1-week-old *Arabidopsis* seedling showing GUS activity in cotyledon and shoot apical meristem.

524 (B) and (C) *P_{AtERG2}::GUS* fusion staining in 4-week-old *Arabidopsis* leaves and leaf
525 trichome.

526 (D) and (E) *P_{AtERG2}::GUS* fusion staining in flowers and pollen at BP (before
527 pollination) and 0.5 DAP (day after pollination), respectively.

528 (F) to (I) *P_{AtERG2}::GUS* fusion staining in developed seeds at BP, 0.5, 1.0, and 1.5
529 DAP, respectively. The central polar nuclei are indicated by cn; the egg cells are
530 indicated by ec; the synergid cells are indicated by sc; the central triploid nucleus is
531 indicated by cc; the endosperm are indicated by end; the embryos at zygote are
532 indicated by zy; and the early globe-stage embryos (with fewer than four cells in a
533 single embryo) are indicated by ea gl.

534 (J) The transcription level of *AtERG2* is detected by qRT-PCR in different tissues of
535 *Arabidopsis*. UBI10 was used as internal control. Error bars indicate the SD of three
536 biological replicates. Different letters (a–e) indicate statistically significant differences
537 ($p < 0.05$) according to Tukey's multiple range test.

538 **Figure 3. *AtERG2* is essential for early seedling development.**

539 (A) The schematic diagram for two T-DNA insertions in the *Arabidopsis* genome of
540 *aterg2*. Two lines, SALK_032115 (*aterg2-1*) and SALK_032124 (*aterg2-2*), were
541 purchased from The *Arabidopsis* Information Resource (TAIR,
542 <http://www.arabidopsis.org/>). Open box, UTR; black box, exon; line, intron. Bar =
543 100 bp.

544 (B) The 6-week-old whole aerial parts of the wild-type (WT) and heterozygous
545 mutant *aterg2-1* +/- after planting in soil. Bar = 5 cm.

546 (C) The opened 6-DAP (day after pollination) siliques from WT and *aterg2-1* +/- . The
547 arrows point to the aborted seeds. Bar = 1 mm.

548 (D) The length of mature siliques from WT and *aterg2-1* +/- (n = 60). The values are
549 means $\pm$ SD. *** indicates extremely statistically significant values ($P < 0.01$).

550 (E) The silique length of WT and *aterg2-1* +/- measured from the day before
551 pollination to four DAP (n = 16). The values are means $\pm$ SD. *** indicates extremely
552 statistically significant values ($P < 0.01$).

553 (F) The seed abortion ratio of WT and *aterg2-1* +/- calculated from the day before
554 pollination to 4 DAP (silique number = 8).

555 **Figure 4. The development phenotypes of seeds and ovule in WT and *aterg2-1* +/-
556 at 1.5 and 2.0 DAP.**

557 (A) The opened 2.0-DAP siliques from WT (top) and *aterg2-1* +/- (bottom). The
558 arrows point to the aborted seeds. Bar = 1 mm.

559 (B) The seed abortion ratio of WT and *aterg2-1* +/- at 1.5 and 2 DAP. Silique
560 numbers = 8.

(C) The ratio of developed ovules for WT and *aterg2-1* +/- at 1.5 and 2.0 DAP, respectively (n > 200). The embryos at the zygote stage are indicated by zy; the early globe-stage embryos (with fewer than four cells in a single embryo) are indicated by ea. gl; globe-stage embryos are indicated by gl; heart-stage embryos are indicated by H; the collapsed stage is indicated by the arrested seeds without the embryo. Bar = 0.1 mm

Figure 5. ROS accumulation and expression of ROS-responsible and cell death-related genes in the arrested seeds of *aterg2-1*.

(A) DAB staining of 1.5-DAP seeds of WT and *aterg2-1*. The arrow points to the early globe-stage embryos (a and c) and the zygote embryos (b, d, and e). Bar=0.05 mm.

(B) The relative expression levels of ROS-responsible genes *WRKY40*, *ANAC017*, and *AOX1a*, and cell death-related genes *AtBAG6* were determined using qRT-PCR in 1.5- and 2.0-DAP seeds of WT and *aterg2-1*. The mRNA levels were normalised to that of *actin*. The dark bars represent the WT seeds, and the red and white bars represent the developed and arrested seeds of *aterg2-1*, respectively. Error bars indicate the SD of three biological replicates. Different letters (a–c) indicate statistically significant differences (p < 0.05) according to Tukey's multiple range test.

Figure 6. Chloramphenicol treatment causes seed abortion and cell death in *Arabidopsis*.

(A) The length of 1.5- and 2.0-DAP siliques of wild-type (WT) plants after treatment with Chloramphenicol (CAP). The orange bars represent treatment with CAP solution (500 µg /mL CAP in 0.02% Silwet-77), the gray bars represent treatment with 0.02% Silwet-77 (Control). The values are means ± SD (n = 8). * indicates statistically significant values (P < 0.05) *** indicates extremely statistically significant values (P < 0.01).

(B) The abortion ratio of 1.5- and 2.0-DAP seeds (n > 150) treated with CAP solution. The zygote embryo is indicated by zy; the early globular-stage embryo is indicated by ea gl; and the globular-stage embryo is indicated by gl. *** indicates extremely statistically significant values (P < 0.01).

(C) The relative expression levels of *AtERG2*; ROS-responsible genes *WRKY40*, *ANAC017*, and *AOX1a*, and cell death-related genes *AtBAG6* were determined using qRT-PCR in 1.5- and 2.0-DAP seeds treated with CAP. The mRNA levels were normalised to that of *actin*. The yellow bars represent the seeds treated with CAP solution, and the purple bars represent the control. Error bars indicate the SD of three biological replicates. *** indicates extremely statistically significant differences (p < 0.01) according to Tukey's multiple range test.

598

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712

713

Figure 1

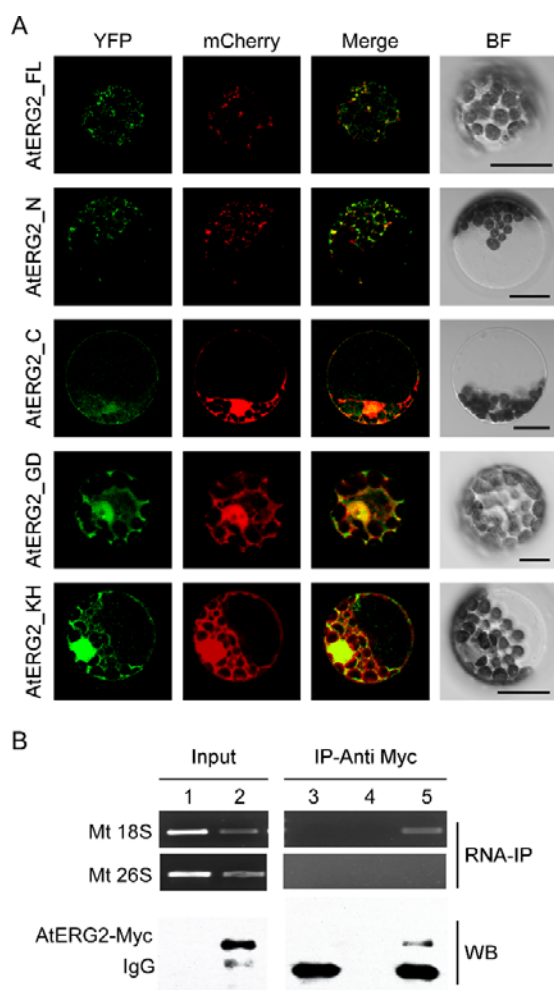


Figure 2

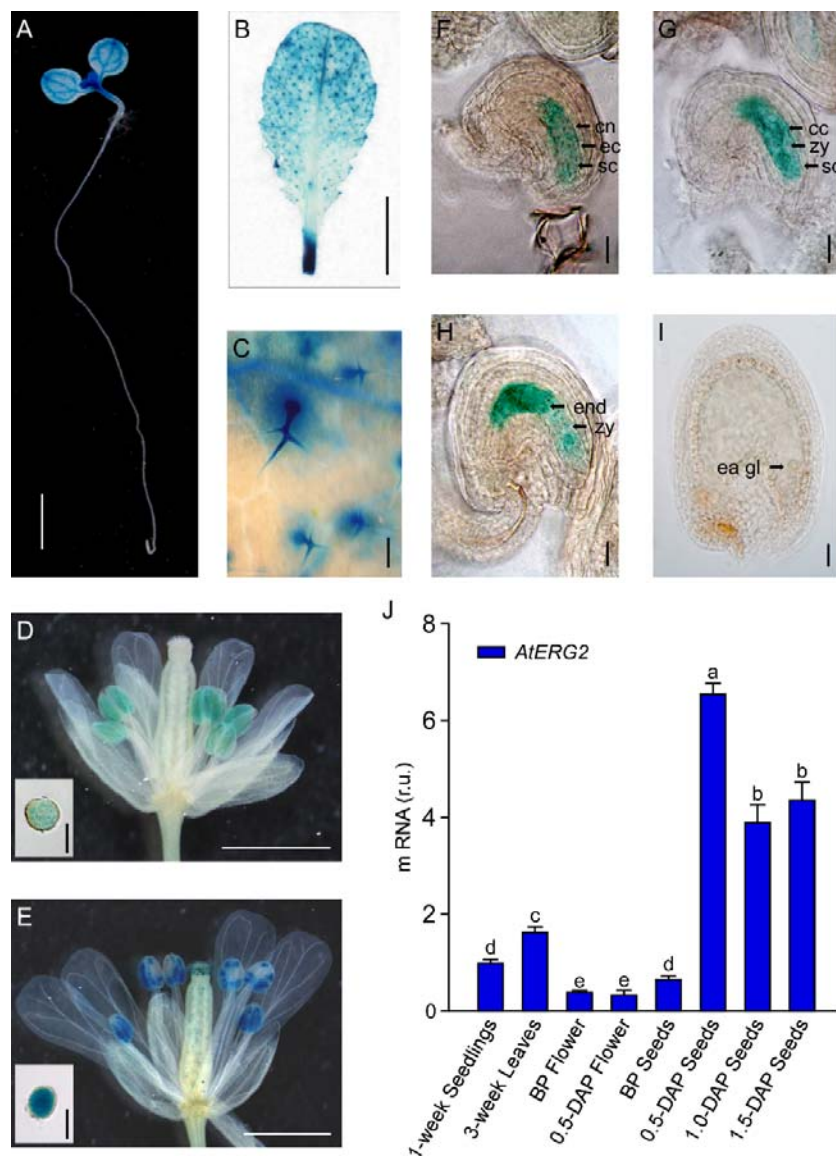


Figure 3

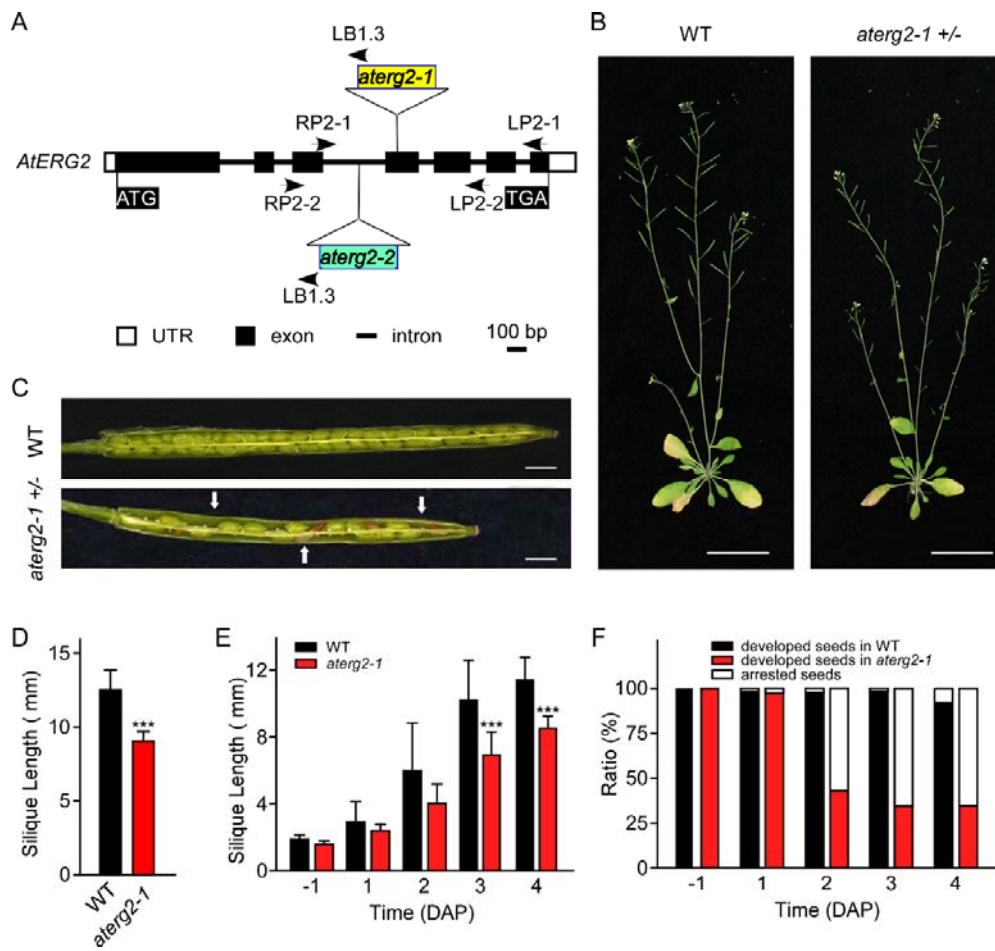


Figure 4

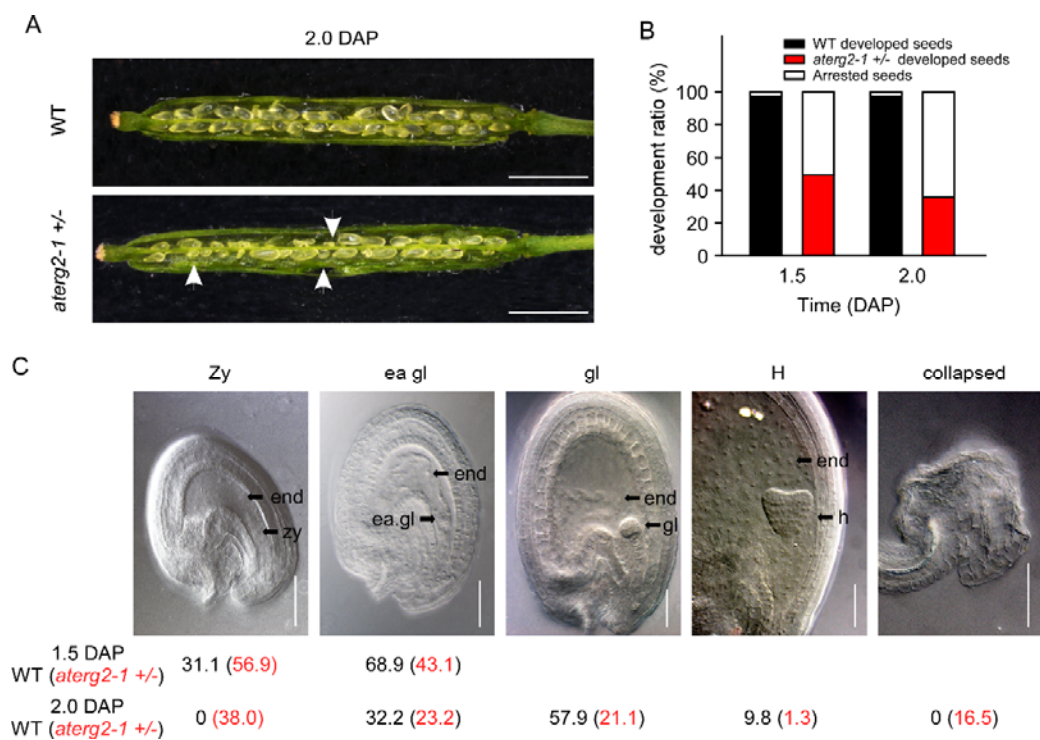


Figure 5

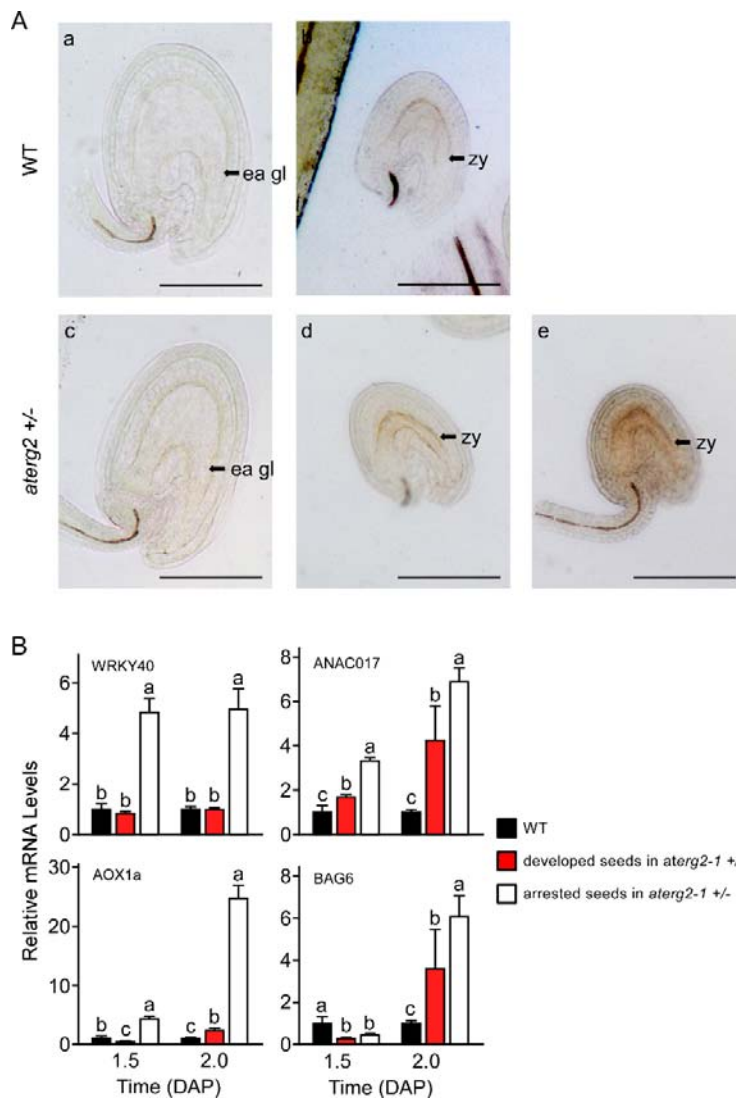


Figure 6

