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1 **Title: Retrograde signals from mitochondria reprogram skoto-morphogenesis in**  
2 ***Arabidopsis thaliana* via Alternative Oxidase 1a**

3

4 Authors:

5 Livia Merendino<sup>1,2,3\*</sup>, Florence Courtois<sup>1</sup>, Björn Grübler<sup>1</sup>, Olivier Bastien<sup>1</sup>, Vera  
6 Straetmanns<sup>1</sup>, Fabien Chevalier<sup>1</sup>, Silva Lerbs-Mache<sup>1</sup>, Claire Lurin<sup>2,3</sup>, Thomas  
7 Pfannschmidt<sup>1,4\*</sup>

8

9 <sup>1</sup> Univ. Grenoble-Alpes, CNRS, CEA, INRA, BIG-LPCV, 38000, Grenoble, France

10 <sup>2</sup> Institute of Plant Sciences Paris-Saclay IPS2, CNRS, INRA, Université Paris-Sud,  
11 Université Evry, Université Paris-Saclay, Bâtiment 630, Rue Noetzlin, 91405 Orsay, France

12 <sup>3</sup> Institute of Plant Sciences Paris-Saclay IPS2, Paris Diderot, Sorbonne Paris-Cité, Bâtiment  
13 630, Rue Noetzlin, 91405, Orsay, France

14 <sup>4</sup>Present address: Institute for Botany, Plant Physiology, Leibniz University Hannover,  
15 Herrenhäuser Str. 2, 30419 Hannover, Germany

16

17 \*To whom correspondence may be addressed:

18 Livia Merendino, E-mail: [livia.merendino@u-psud.fr](mailto:livia.merendino@u-psud.fr); ORCID: 0000-0003-1593-5442

19 Thomas Pfannschmidt, E-mail: [t.pfannschmidt@botanik.uni-hannover.de](mailto:t.pfannschmidt@botanik.uni-hannover.de); ORCID: 0000-  
20 0002-7532-3467

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25 **Summary**

26 The early steps in germination and development of angiosperm seedlings often occur in the  
27 dark inducing a special developmental programme called skoto-morphogenesis. Under these  
28 conditions photosynthesis cannot work and all energetic requirements must be fulfilled by  
29 mitochondrial metabolization of storage energies. Here, we report the physiological impact of  
30 mitochondrial dysfunctions on the skoto-morphogenic programme by using the *Arabidopsis*  
31 *rpoTmp* mutant. This mutant is defective in the T7-phage-type organellar RNA polymerase  
32 shared by plastids and mitochondria. Lack of this enzyme causes a mitochondrial dysfunction  
33 resulting in a strongly reduced mitochondrial respiratory chain and a compensatory up-  
34 regulation of the alternative-oxidase (AOX)-dependent respiration. Surprisingly, the mutant  
35 exhibits a triple-response-like phenotype with a twisted apical hook and a shortened  
36 hypocotyl. Highly similar phenotypes were detected in other respiration mutants (*rug3* and  
37 *atphb3*) or in WT seedlings treated with the respiration inhibitor KCN. Further genetic and  
38 molecular data suggest that the observed skoto-morphogenic alterations are specifically  
39 dependent on the activity of the AOX1a enzyme. Microarray analyses indicated that a  
40 retrograde signal from mitochondria activates the ANAC017-dependent pathway that controls  
41 the activation of *AOX1A* transcription. In sum, our analysis identifies AOX as a functional  
42 link that couples the formation of a triple-response-like phenotype to mitochondrial  
43 dysfunction.

44

45 **Key words:** *Arabidopsis*; Mitochondrial respiration; Alternative oxidase; Retrograde signal;  
46 Organellar RPOTmp RNA polymerase; Skoto-morphogenesis.

## 47 **Introduction**

48 Mitochondria are essential cell organelles that convert organic matter into chemical energy in  
49 form of ATP and reducing power through the process of respiration [1]. To this end in seeds  
50 of oilseed plants as *Arabidopsis*, the triacylglycerol lipids present in the oil bodies are  
51 oxidatively degraded into citrate which is fed into the tricarboxylic acid (TCA) cycle within  
52 the mitochondrial matrix [2]. Here the carbon skeleton is completely oxidized and CO<sub>2</sub>, ATP  
53 and reducing equivalents are released by the cycle. The reducing power then is used to drive  
54 an electron transport through the mitochondrial KCN-sensitive electron transport chain (ETC)  
55 within the inner mitochondrial membrane towards oxygen as end acceptor. ETC consists of  
56 three large multi-subunit protein complexes (complexes I, III and IV) that are connected by  
57 mobile electron carriers. The electron transport through this chain is coupled to a proton-  
58 translocation across the mitochondrial membranes, which is finally used by the ATP synthase  
59 to build ATP. The oxidized forms of the reducing equivalents produced by the ETC are then  
60 available again for the TCA cycle as electron sink. By this way TCA cycle and ETC are  
61 functionally coupled and any restriction in metabolic flux in one of them affects also the other  
62 [1].

63 Plant mitochondria display an extraordinary plasticity in their respiratory pathways as they  
64 possess additional oxidoreductases that can release excess levels of reducing power by  
65 bypassing parts of the ETC without concomitant ATP production [1,3]. At the ETC input site  
66 electrons can be donated to the alternative type II NADH dehydrogenases that bypass  
67 complex I. A second shunt is found at the end of the ETC where electrons can be transferred  
68 to oxygen by the alternative oxidase (AOX), which bypasses complexes III and IV. The role  
69 of both the alternative NADH dehydrogenases and AOX is still a matter of debate, but they  
70 are proposed to avoid generation of dangerous reactive oxygen species (ROS) and to stabilize  
71 the NAD<sup>+</sup>/NADH balance by preventing a metabolic feedback inhibition of the TCA cycle  
72 [1]. In addition, these enzymes are also suggested to serve as safety valves for photosynthesis  
73 under various stress conditions. Experimental evidences support the function of AOX as a  
74 releasing enzyme of excess reducing power transferred from the chloroplast under high light  
75 [3]. The additional enzymes also provide high flexibility to the ETC of plant mitochondria  
76 allowing genetic studies. Mutants in nuclear genes, that are necessary for the proper  
77 expression of mitochondrial-encoded proteins, are defined as surrogate mutants [4]. The  
78 surrogate mutants *rpoTmp* and *rug3* present a defective expression of ETC complex subunits  
79 but they are viable in that they maintain residual complexes. Even if they display delayed

80 growth phenotypes, they still generate rosettes that are comparable in size to wildtype and  
81 produce seeds [5–7]. These mutants accumulate high levels of AOX protein. In *rpoTmp*  
82 plants, the AOX pathway was shown to be activated [8].

83 Recent studies have shown that stress induced by block of mitochondrial translation or  
84 respiration as well as by environmental constraints activates retrograde signaling pathways  
85 from mitochondria towards the nucleus [9,10]. One of the target genes is *AOX1a*  
86 (*ALTERNATIVE OXIDASE 1a*) that is now commonly used as a marker to investigate  
87 mitochondrial retrograde signaling [7,11,12]. Also, recently identified regulators of *AOX1A*  
88 expression (e.g. RAO1/CDKE1 and RAO2/ANAC017) seem to respond to mitochondrial as  
89 well as to chloroplast retrograde signals [13,14]. Furthermore, chloroplast redox signals from  
90 photosynthetic electron transport impact the expression of mitochondria-located genes [15].  
91 These last studies investigating the signaling functions of the two energy-converting  
92 organelles have been all performed on plants grown in the light where both chloroplasts and  
93 mitochondria are active. Because of the multiple connections between these two organelles a  
94 clear separation of mitochondrial and chloroplast retrograde signals, therefore, remains  
95 difficult and partial overlaps and interactions occur in green tissues [14,16]. How a  
96 mitochondrial dysfunction would affect plant growth and development when chloroplasts and  
97 energy supply from photosynthesis are not available, i.e. during germination in the dark, is  
98 however, not well investigated. Angiosperms such as *Arabidopsis* undergo a special  
99 developmental program when they germinate and grow in the dark, the skoto-morphogenesis  
100 [17]. When a germinating seed is buried by humus or soil the seedling re-allocates all storage  
101 energy into shoot elongation while the cotyledons remain small, being directed down-wards  
102 and forming an apical hook that protects the sensitive apical meristem. In this phase the  
103 seedling relies completely on its storage energies and their metabolization by mitochondria.  
104 As soon as the cotyledons breach the surface and perceive light, photo-morphogenesis is  
105 initiated and chloroplasts are formed [18]. Mutants with mitochondrial dysfunction, therefore,  
106 should be expected to show major defects in the performance of skoto-morphogenesis.

107 *Arabidopsis rpoTmp* mutants are defective in a phage-type single-subunit RNA polymerase  
108 that is bi-localized to chloroplasts and mitochondria [19,20]. This RNA polymerase is  
109 required for the proper expression of the organelle genomes, however, it was shown that its  
110 impact in chloroplasts is restricted to the recognition of the PC promoter in front of the *16S*  
111 *rRNA* operon [21], while it displays a major deficiency in the transcription of several  
112 mitochondrial genes including those for complex I and IV [5,22]. The mutant exhibits

113 strongly reduced complex I and complex IV accumulation, but displays only a mild  
114 phenotype in the light with a delayed growth and curly leaves in the fully developed rosette  
115 [5].

116 In this manuscript, we analyze the impact of restrictions of respiration on early seedling  
117 development in the dark using the respiration deficient *rpoTmp* mutant from *Arabidopsis*. We  
118 found that limitations of the ETC lead to drastic changes in the early developmental program  
119 of etiolated seedlings and that this reprogramming of skoto-morphogenesis is triggered by the  
120 AOX1a enzyme.

121 **Material and Methods**

122 *Plant material*

123 *Arabidopsis thaliana* wildtype (WT) (ecotype Columbia) and mutant lines (*rpoTmp-2*  
124 SALK\_132842 and *rpoTmp-3* SALK\_086115 [21][5], *rug3* SALK\_092071 [6], *atphb3*  
125 SALK\_020707 [23], *rpoTmp* SALK\_132842/*anac017* (*anac017*, *rao2-1*) [7,24], *aox1A*  
126 SALK\_084897 [8] and *rpoTmp* SALK\_132842/*aox1A* SALK\_084897 [8]) were used in this  
127 study. Seeds were surface-sterilized and spread on Murashige and Skoog (MS) agar plates  
128 supplemented with sucrose (1%). Activated charcoal (in powder, SIGMA-ALDRICH, 0.08%)  
129 was added to the plant culture medium in order to improve image quality of etiolated plants  
130 by increasing background contrast. Seeds were stratified for 72 hours (at 4°C in darkness),  
131 exposed to light (100 µE white light) for 6 hours and then grown at 23°C in darkness. Plants  
132 were harvested after 3 days. For microarray, qRT-PCR and western-immuno-blot analyses the  
133 etiolated plants were harvested in the dark under a green safe-light, immediately frozen in  
134 liquid nitrogen, ground in a mortar and subjected to further techniques as described below.  
135 The germination percentage was variable according to the seed lot and the drug treatment. For  
136 chemical treatments MS agar plates were supplemented with 1-aminocyclopropane-  
137 carboxylic acid (ACC, SIGMA-ALDRICH A3903, 20 µM), KCN (1 mM) or  
138 salicylhydroxamic acid (SHAM, SIGMA-ALDRICH S7504, 1 mM in DMSO). In case of  
139 KCN or SHAM treatments, seeds were first spread on black nitrocellulose membrane filters  
140 (Whatman 10409770, 0.45 µm) on MS agar plates, stratified for 72 hours and light-exposed  
141 for 6 hours, and then transferred (on filter) to KCN/SHAM-containing MS agar plates for  
142 growth in the dark. DMSO-containing MS agar plates were used as mock-control for the  
143 SHAM treatment.

144

145 *Measurements of phenotypic parameters*

146 Phenotypic appearance of plantlets was analyzed with a dissection microscope (Olympus  
147 SZX12) using the ACT-1C for DXM1200C software. Digital images of individual plants  
148 were taken and the angles formed in the apical hook between hypocotyl and cotyledons were  
149 subsequently determined using the ImageJ program. In absence of any curvature (cotyledons  
150 straight and in the same axis as the hypocotyl but oppositely oriented from the roots) the angle  
151 was considered as 0°. In a hook where cotyledons were in the same axis as the hypocotyl, but  
152 oriented towards the roots, the angle was considered as 180°. Any hook angle larger than 180°

153 was considered as twisted. The length of the hypocotyls was measured by determining the  
154 shoot length between the origins of roots and cotyledons.

155

#### 156 *Statistical analyses of phenotypic parameter measurements*

157 All statistical tests were performed using the computing environment R [25], with a  
158 significance level of 0.05. Details about the statistical analyses are found in the supplementary  
159 methods.

160

#### 161 *Microarray analysis*

162 Total RNA purification and microarray analysis were performed as indicated in [26]. Total  
163 RNA was isolated using RNeasy Plant Mini Kit (Qiagen) with on-column DNase I (Quiagen)  
164 treatment. Affymetrix whole transcriptome microarray analysis was performed on three  
165 biological replicates by the commercial service Kompetenz-Zentrum für Fluoreszente  
166 Bioanalytik (KFB) (Regensburg, Germany). cDNAs were prepared using the Ambion®  
167 Whole Transcriptome (WT) Expression Kit and fragmented and labeled using the Affymetrix  
168 GeneChip® WT Terminal Labeling Kit. Expression analyses were performed using the  
169 “GeneChip® Arabidopsis Gene 1.0 ST Array”. Since the “Ambion® WT Expression Kit”  
170 uses a mixture of oligo-dT and random hexamer primers for the generation of the first strand  
171 cDNA, the resulting hybridization signals reflect also the organellar transcript accumulation  
172 besides the one from the nucleus.

173

#### 174 *Analysis of microarray data*

175 Analysis of microarray data was performed as indicated in [26]. The cel files containing the  
176 scanned chips received from KFB were analyzed with RobiNA [27] with the background  
177 correction method RMA (robust multi-array average expression measure) [28], p-value  
178 correction method Benjamini-Hochberg [29] and the analysis Strategy Limma for the quality  
179 control [30]. For the main analysis the normalization method RMA with p-value correction  
180 method after BH and a multiple testing strategy “nestedF” was chosen. In the analysis using  
181 Excel (MS-Office), data with the description “Multiple Hits” were ignored and only results  
182 with one-to-one correspondence to a given gene identity were regarded for further analysis.

183



184 *RT-qPCR analysis of RNA levels*

185 Ground plant material was resuspended in 3 volumes of solution A (10 mM Tris-HCl pH8.0;  
186 100 mM NaCl; 1 mM EDTA; 1% SDS) and 2 volumes of phenol/chloroform/isoamyl alcohol  
187 (25:24:1; v/v/v). After centrifugation, RNAs in the aqueous phase were again extracted twice  
188 with phenol-chloroform and finally once with chloroform. After over-night precipitation in 2  
189 M LiCl at 4°C, RNAs were then precipitated in ethanol, washed in ethanol 70% and  
190 resuspended in water. 300 ng of DNase (DNase Max Kit, Quiagen) treated-RNAs were  
191 retro-transcribed using random hexo-nucleotides and the Super-Script II enzyme (Invitrogen)  
192 according to the manufacturer's protocol. The PCR reaction was performed in the Biorad  
193 CFX384™ Real Time System PCR machine in presence of forward and reverse gene-  
194 specific primers (0.5 μM, **Table S1**) using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus)  
195 from Takara Bio. Data were analyzed using the CFX Manager Software. The expression  
196 levels of the transcript of interest were normalized with the levels of *ACTIN 2-8* expression,  
197 used as reference gene. The mean values of biological and/or technical replicates were plotted  
198 together with the error bars corresponding to standard errors.

199

200 *Protein immuno-western analysis*

201 Total protein extracts were prepared by re-suspending 100 mg of ground plant material in 100  
202 μl of protein lysis buffer (50 mM Tris pH 8.0; 2% SDS; 10 mM EDTA; protease inhibitors  
203 (Roche (04693159001) cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail)) and  
204 incubating at room temperature for 30 min. Extracts were cleared by centrifugation for 30 min  
205 at 12000 × g at 4°C and dilutions of the supernatant were quantified with Bradford. Samples  
206 were then denatured for 5 min at 95 °C in 4X reducing electrophoresis sample buffer.  
207 Increasing amounts of wild-type and mutant protein extracts (10, 20, 40 μg) were fractionated  
208 on a 12% gel by SDS-PAGE, electro-blotted on a nitrocellulose or PVDF membranes and  
209 immuno-decorated with specific polyclonal rabbit antisera against mitochondrial protein  
210 NAD9 [31]; mitochondrial AOX1/2 plant alternative oxidase 1 and 2 (Agrisera, AS04 054);  
211 plastidial RBCL (Agrisera, AS03 037); vacuole V-ATPase (Agrisera, AS07 213). The blots  
212 were developed with Clarity Western ECL substrate (Bio-Rad, Hercules, USA). Images of the  
213 blots were obtained using a CCD imager (Chemidoc MP, Bio-Rad) and the Image Lab  
214 program (Bio-Rad, Hercules, USA). Image analysis for AOX protein quantification was  
215 performed using the Image Lab program and normalized with the levels of V-ATPase.

216

217 *In planta O<sub>2</sub> consumption measurements*

218 Etiolated plantlets were harvested from Petri dishes and immediately soaked in the dark with  
219 1 mL of oxygenated phosphate buffer (10 mM sodium phosphate buffer pH 7.2; 10 mM KCl;  
220 10 mM glucose) in the measurement cell of a Clark oxygen electrode (Hansatech). For  
221 electron transport inhibition experiments, KCN (0.1 M stock, inhibitor of KCN-sensitive  
222 chain at the level of complex IV) and SHAM (0.1 M stock in DMSO, inhibitor of the AOX-  
223 dependent pathway) were added directly into the electrode chamber to a final concentration of  
224 1 mM once the recorded signal reached a constant regime. The capacity (or maximum  
225 activity) of the AOX-dependent pathway (corresponding to KCN-insensitive pathway) and of  
226 the COX- dependent pathway (corresponding to KCN-sensitive pathway) was calculated as  
227 ratio of O<sub>2</sub> consumption rate upon addition of KCN or SHAM and total O<sub>2</sub> consumption rate,  
228 respectively. The contribution of extra mitochondrial O<sub>2</sub> consumption to mitochondrial  
229 respiration was evaluated at the end of each measurement after addition of both KCN and  
230 SHAM and systematically subtracted from O<sub>2</sub> consumption rates.

## 231 **Results**

### 232 ***rpoTmp* mutants display a triple-response-like phenotype**

233 To investigate the impact of mitochondrial dysfunction on skoto-morphogenesis we compared  
234 3-day-old dark-grown (etiolated) WT with *rpoTmp* mutant plants grown under identical  
235 conditions (**Fig.1a**). WT seedlings displayed a typical skoto-morphogenesis with elongated  
236 hypocotyl, apical hook and yellow cotyledons. In contrast, seedlings of two allelic *rpoTmp*  
237 mutant lines (*rpoTmp-2* SALK\_132842 and *rpoTmp-3* SALK\_086115) developed a  
238 significant exaggeration of apical hook curvature (twist) and a marked shortening of the  
239 hypocotyls. The phenotype of both alleles was identical indicating that indeed the genetic  
240 defect in *rpoTmp* causes the massive change in the seedling developmental program. The  
241 observed mutant phenotype was reminiscent of morphological changes reported for etiolated  
242 seedlings exposed to high levels of ethylene, commonly known as triple-response [32,33]. To  
243 investigate this in more detail we compared the *rpoTmp* phenotype to that of WT plants  
244 grown in presence of 20  $\mu$ M 1-aminocyclopropane-1-carboxylic acid (ACC), a biosynthetic  
245 precursor of ethylene known to induce the triple-response [34] (**Fig. 1a**). We observed  
246 comparable values for the increased apical hook curvature (**Fig. 1b**) and the hypocotyl  
247 shortening (**Fig. 1c**) in the two plant samples. Only the widening of the hypocotyl diameter in  
248 response to ACC treatment was not observable in *rpoTmp* (**Fig. 1a**) suggesting that the  
249 genetic defect in the *RPOTmp* locus causes a strong but not perfect phenocopy of the ACC-  
250 treatment. Therefore, we termed the observed phenotypic response a triple-response-like  
251 phenotype.

252

### 253 **The triple-response-like phenotype is dependent on an active AOX1a enzyme**

254 In order to find a potential link between the genetic inactivation of the *RPOTmp* gene and the  
255 morphological changes, we performed a transcriptomic profiling of etiolated mutant seedlings  
256 in comparison to WT (**Table S2**) and compared these data to previous expression analyses  
257 performed in light-grown plants. An overview of the gene expression profiles in etiolated  
258 plants in relation to the corresponding cellular metabolic pathways using the MapMan tool  
259 indicated that the general impact of the mutation in *RPOTmp* was relatively weak (Fig. S1).  
260 Only a small number of genes displayed a strong expression change of more than log<sub>2</sub> fold  $\geq 2$   
261 (29 genes) or  $\leq -2$  (60 genes, **Table S2**). These data suggest that the observed triple-response-  
262 like phenotype is rather a specific effect than the result of pleiotropic disturbance in the

263 mutant. By analyzing the mitochondrial transcriptome, we found that from the twenty  
264 mitochondrial genes that were presenting the highest degree of transcriptional variations in  
265 previous analyses in light-grown *rpoTmp* adult plants [7], 17 were also differentially  
266 expressed in etiolated *rpoTmp* seedlings and, here, in the same direction of expression change,  
267 even though to a lower extent (**Table S3**). More particularly, the subset of mitochondrial  
268 genes encoding ETC complex subunits that was previously described as RPOTmp-dependent  
269 [5] was also down-regulated in etiolated *rpoTmp* seedlings (**Fig. 2a**). Analysis of the  
270 accumulation of the mitochondrial-encoded NAD9 protein as marker for complex I  
271 accumulation indicated that these gene expression defects were also reflected at the protein  
272 level (**Fig. 2b**). While clearly present in WT, NAD9 was almost undetectable in *rpoTmp*  
273 mutant plants in contrast to the control RBCL, the plastid-encoded large subunit of Ribulose-  
274 1.5-bisphosphate-carboxylase/oxygenase. This indicated strong deficiency of complex I in  
275 *rpoTmp* plants also in the dark, which was accompanied by a decrease in the capacity of the  
276 KCN-sensitive pathway in *rpoTmp* seedlings (**Fig. S2**). By analyzing the transcriptomic  
277 profile in more detail, we found that more than half of the 20 most up-regulated genes encode  
278 proteins that are localized in the mitochondria (**Fig. 2c**). Functional sub-grouping of these  
279 genes identified four groups: one class containing genes corresponding to markers of  
280 mitochondrial stress and targets of the retrograde-mitochondrial pathway, as *AT12CYS* and its  
281 co-expression partner *NDB4* [11] and three *DUF295 Organellar B* genes [35]; one tRNA  
282 class including one nucleus- and three mitochondria-encoded *tRNAs*; one class corresponding  
283 to general mitochondrial functions, including *TOM7-2* (coding for a member of the family of  
284 the TOM7 translocases of outer membrane), *SPL2* phosphatase [36], ATP synthase or an  
285 undefined gene; and a final group including genes coding for proline- and glycine-rich  
286 proteins, often involved in stress response (**Fig. 2c, d**). Functional sub-grouping of the 20  
287 most down-regulated genes also identified a group of genes coding for glycine rich proteins  
288 and/or factors involved in response to stress. An additional group of genes was also defined  
289 corresponding to the response to hormonal *stimuli*, and a final class of genes related to  
290 reduction/oxidation activities (**Fig. 2e, Table S4**).

291 We have compared our transcriptomic data (threshold 1.5) concerning dark-grown *rpoTmp*  
292 seedlings with previous analyses performed by Van Aken and colleagues with light-grown  
293 adult plants [7]. We found 33 up DEGs (Differentially Expressed Genes) and 40 down DEGs  
294 in common (**Table S5**). Of these genes, four are among the top 20 up DEGs in both studies  
295 (highlighted in yellow and by an asterisk), eight are in the list of our top 20 up and seven are

296 among our top 20 down DEGs (highlighted in yellow). These data emphasize the strong  
297 similarity in the gene-expression profiles of *rpoTmp* plants that differ for developmental  
298 stages and growth conditions. Remarkably, among the common up DEGs, 20 genes encode  
299 mitochondrial localized proteins and at least nine genes are induced by mitochondrial  
300 dysfunction and transcriptionally regulated by mitochondrial retrograde signaling, as  
301 *AT12CYS2* [11], *NDB4* [11], five different *DUF 295 organellar genes* [35] and *UPOX* that is  
302 an hallmark of oxidative stress [37]. All these data pointed to the presence of a strong  
303 mitochondrial stress in *rpoTmp* plants and prompted us to explore the possibility that defects  
304 in mitochondrial respiration are at the origin of the observed skoto-morphogenetic alteration.  
305 In order to test this hypothesis by an independent experimental line we chose to grow  
306 etiolated WT plants on a medium containing 1 mM KCN, an inhibitor of complex IV in the  
307 ETC. Here, a statistically significant increment in apical hook bending could be detected (**Fig.**  
308 **3a** and **Fig. S3**). This indicates that limitation of KCN-sensitive ETC results in the same  
309 phenotype as in the *rpoTmp* mutant suggesting that respiration restriction is indeed at the  
310 origin of the phenotypic deviations. To obtain independent proof for this assumption we  
311 | observed the phenotype of dark-grown *rug3* and *atphb3 Arabidopsis* mutant seedlings (**Fig.**  
312 **3b**). The *rug3* mutant is disturbed in complex I biogenesis [6] and *atphb3* mutant is defective  
313 in the mitochondrial membrane protein PROHIBITIN 3, that was suggest to be a structural  
314 support for membrane protein complexes, such as the electron transport chain [23]. Both  
315 mutants possess a fully functional RPOTmp enzyme, but like in *rpoTmp* mutants or KCN-  
316 treated WT we found a twisted apical hook. This supports our hypothesis of ETC limitation as  
317 cause of the reprogramming of skoto-morphogenesis and excludes also any side-effect from  
318 the defective RPOTmp enzyme in the plastid.

319 Up-regulation of the AOX pathway was already reported in light-grown *rpoTmp* plants [8]. In  
320 etiolated *rpoTmp* plants *AOX1a* expression was also strongly activated both at transcript and  
321 protein levels (**Fig. 4a, b**). Together with the *AOX1a* gene, also the levels of two other  
322 markers of the cellular response to oxidative stress, *AT12CYS* [11,38] and *UPOX* [37,38],  
323 were highly induced in *rpoTmp* plants (**Fig. S4a**). Transcriptional activation of *AOX1a* and  
324 partially of *AT12CYS* mRNAs was impaired by the inactivation of the mitochondrial  
325 retrograde master regulator ANAC017 [7] (**Fig. 4a, Fig. S4b**). The activation of the *AOX1a*  
326 gene expression corresponded to an increase in the AOX-dependent respiration capacity that  
327 is KCN-insensitive (**Fig. 4c, Fig S5**). To investigate whether or not the AOX-dependent  
328 pathway is involved in the triple-response-like phenotype we grew *rpoTmp* plantlets in

329 presence of 1 mM SHAM, a chemical inhibitor of the AOX-dependent pathway. Under this  
330 condition a statistically significant decrease of the twisting of the apical hook angle could be  
331 detected (**Fig. 3a, Fig. S3 right panel**).

332 To further prove independently the involvement of the AOX-pathway, we aimed to  
333 characterize the *rpoTmp/aox1a* double mutant plants in the dark in which the AOX1a isoform  
334 is lacking. The AOX enzyme is known to be expressed in various isoforms that potentially  
335 could replace each other. In western-blot analyses of *rpoTmp/aox1a* protein extracts using an  
336 antiserum detecting the five AOX isoforms no signal was detected (**Fig. 4b**, right panel)  
337 indicating that AOX1a is the main AOX isoform in the *rpoTmp* genetic background in the  
338 dark. Analysis of *rpoTmp/aox1a* mutant seedlings, thus, should provide conclusive evidence  
339 about the involvement of the AOX1a enzyme in the phenotypic appearance of *rpoTmp*  
340 mutants. A strong decrease in the AOX-capacity was observed in *rpoTmp/aox1a* double  
341 mutants when compared to *rpoTmp* plants (**Fig. 4c, Fig. S5**). Importantly, we observed a  
342 suppression of the twisting of the apical hook in *rpoTmp/aox1a* mutants (**Fig. 4d, Fig. S6**).  
343 However, the hypocotyl elongation was not restored suggesting that it is regulated by a  
344 different pathway.

345

#### 346 **The triple-response-like phenotype requires a retrograde signal from mitochondria**

347 While the genetic defect in *rpoTmp* mutant lines causes a dysfunction in mitochondria, the  
348 up-regulated AOX1a enzyme is encoded in the nucleus. The up-regulation for involvement in  
349 the observed developmental change, therefore, requires a mito-nuclear retrograde signal.  
350 Recently, the ER-bound transcription factor ANAC017 was shown to be responsible for the  
351 retrograde activation of the *AOX1a* gene [7,24]. We, thus, proposed that the double mutant  
352 *rpoTmp/anac017*, which keeps the basal *AOX1a* expression but lacks the capacity to  
353 upregulate it to its full extent, should also show a reduction of the triple-response-like  
354 phenotype. Indeed, we found intermediate levels of AOX proteins and AOX capacity in the  
355 double mutant *rpoTmp/anac017* plants when compared to *rpoTmp* and *rpoTmp/aox1a* mutant  
356 plants (**Fig. 4b, c, S5**). As expected, we detected a partial suppression of the twisting of the  
357 apical hook in the *rpoTmp/anac017* double mutant when compared to *rpoTmp* plants (**Fig. 4d,**  
358 **e, S6**) indicating that the ANAC017-dependent pathway is required for the induction of the  
359 triple-response-like phenotype.

360 In sum, the results with the *rpoTmp/aox1a* and *rpoTmp/anac017* double mutants provide  
361 compelling evidence that the hook exaggeration in *rpoTmp* requires AOX1a. A strong  
362 decrease in the AOX capacity was also observed in *aox1a* single mutants (**Fig. 4c, S5**), but  
363 these plants displayed a normal skoto-morphogenesis without any aberrations (**Fig. 4d**). We  
364 concluded that a lack of AOX1a becomes critical for skoto-morphogenesis only under  
365 conditions when ETC is limited.

## 366 Discussion

367 In the light, *Arabidopsis rpoTmp* mutants display defects in ETC but only mild phenotypes  
368 with slightly retarded growth and few macroscopic changes such as curled leaves [5,21]. In  
369 the dark we expected stronger impact on the phenotype as no energy compensation from  
370 photosynthesis can occur. Here we report that the expression of the mitochondrial sub-set of  
371 genes that were previously identified as RPOTmp-dependent [5] was highly impacted in  
372 *rpoTmp* plants also in the dark (**Fig. 2a, b; Table S3**). In addition, the strong activation of  
373 stress marker genes indicates the presence of a severe mitochondrial stress in etiolated mutant  
374 plants (**Fig. 2c, d, 4a** and **Fig. S4a**). Comparison of our transcriptomic data obtained from  
375 dark-grown seedlings with previous analyses done on light-grown adult plants [7] identified  
376 high similarities at the level of the response to mitochondrial dysfunction (**Table S5**). These  
377 data underline the robustness of the plant stress reaction to the genetic defect in the *RPOTMP*  
378 gene locus that is largely independent of the developmental stage and the light regime.  
379 However, to our surprise we did not observe any retardation in development of the dark-  
380 grown seedlings (as observed in the light), but instead we found a strong modification of the  
381 skoto-morphogenic programme (**Fig. 1**).

382 We could confirm that limitation of the ETC leads to shortened hypocotyls and twisted apical  
383 hooks during the development of the seedlings by both chemical means (in KCN-treated  
384 plants, **Fig. 3a** and **Fig. S3** left panel) and independent genetic tools (in *rug3* and *atphb3*  
385 mutant plants, **Fig. 3b**). This phenotype is highly reminiscent of the triple response of  
386 seedlings exposed to ethylene (or precursors of ethylene such as ACC) (**Fig.1**). The third  
387 parameter, a widened hypocotyl, could not be significantly determined. Therefore, we termed  
388 the observed phenotypic response a triple-response-like phenotype. In the *rpoTmp/aox1a*  
389 double mutant the twisting of the hook was prevented, however, the hypocotyl shortening was  
390 maintained (**Fig. 4d**). These observations suggest that the three phenotypic traits may be  
391 controlled by different specific pathways and/or different hormone thresholds.

392 A recent study reported that limitation of mitochondrial translation causes proteotoxic stress  
393 and activates an unfolded protein response in mitochondria by MAP kinase cascades which  
394 leads to shortened hypocotyls [10]. Partial inhibition of mitochondrial translation, however,  
395 leads also to a general down-regulation of mitochondrial respiration and, concomitantly,  
396 respiration stress does occur. This stress was apparently not as strong as in our system since  
397 an exaggerated hook was not observed in that study. Interestingly, an up-regulation of AOX1a



398 could be observed also in that study, but its potential involvement in the signaling of the  
399 proteotoxic stress was not investigated.

400 Here, we show that limitation of the ETC leads to drastic changes in skoto-morphogenesis  
401 and that the increased capacity of the AOX-pathway is the critical parameter that triggers the  
402 hook exaggeration (**Fig. 5**). Both pharmaceutical interference of AOX enzyme activity (by  
403 SHAM) or genetic impairment of the AOX1a isoform resulted in a significant suppression of  
404 the twisting phenotype in *rpoTmp* plants (**Fig. 3a, S3 right panel, 4d, S6**). Our western-  
405 analyses indicate also, that AOX1a appears to be the sole isoform being expressed in the dark  
406 (**Fig. 4b**). Recently, it was reported that the AOX1a enzyme expression is induced at the  
407 transcriptional level by the action of the ANAC017-dependent retrograde pathway in response  
408 to mitochondrial stress [7,24]. In our study the genetic impairment of the ANAC017-  
409 dependent transcriptional activation of the *AOX1a* gene (**Fig. 4a**) affects moderately both the  
410 increase of the accumulation (**Fig. 4b**) and the enzymatic capacity of AOX proteins (**Fig. 4c**)  
411 and the induction of the twisting phenotype (**Figs.4d and e, S6**) in *rpoTmp* seedlings. These  
412 data highlight the dependence of the triple-response-like phenotype on the AOX1a protein  
413 levels and the mitochondrial retrograde pathway. It is important to note that in the  
414 *rpotmp/anac017* double mutant seedlings, the basal *AOX1a* expression is not reduced (as in  
415 an *aox1a* knock out line). The plants just lack the ability to upregulate *AOX1a* expression to  
416 its full extent. As such the phenotypical effect is expected to be less pronounced than in  
417 *rpotmp/aox1a* mutants (**Fig. 4d, S6**). This observation suggests that transcriptional activation  
418 of *AOX1a* by the ANAC017-dependent retrograde pathway has not an exclusive role in the  
419 dark and likely additional translational or post-translational regulation of the AOX1a enzyme  
420 might be also implemented. Based on *in vitro* experiments, it was proposed that AOX activity  
421 might be regulated at the post-translational level, at the redox state and by the interaction with  
422 TCA cycle intermediates, leading to a very tight and rapid metabolic control of the  
423 mitochondrial respiration process [39,40].

424 We conclude that the limitation of respiration, therefore, induces the triple-like response by  
425 promoting the abundance and capacity of the AOX1a enzyme (**Fig. 5**). Our study strongly  
426 suggests that the AOX1a enzyme is not just a biomarker for stress [3,41] that accumulates as  
427 a side effect, but it represents a key component of the stress reaction network that triggers  
428 subsequent cellular responses to mitochondrial dysfunction. How precisely the AOX1a  
429 enzyme induces the reprogramming remains to be elucidated, but one can imagine that

430 especially under oxygen limitation high capacities of AOX1a activity could reduce alternative  
431 substrates generating products that may serve as signaling molecules such as nitric oxide [42].

432 A physiological role for the triple response was obscure for a long time until recently a study  
433 uncovered that the triple response can be triggered by the soil overlay of buried seeds [43].  
434 The triple morphogenic change provides higher resistance to soil friction and allows the  
435 seedling to breach the surface while protecting the apical meristem. A recent study  
436 investigated the influence of hypoxic conditions on skoto-morphogenesis and uncovered that  
437 a low oxygen tension applied after the apical hook maintenance phase prevented the opening  
438 of the hook in subsequent light conditions [44]. The study identified that seedlings are able to  
439 sense the oxygen tension *via* the N-end rule pathway-controlled stability of ERFVII  
440 transcription factors. However, the hypoxic condition in that study was imposed to the  
441 seedlings after the apical hook was formed and, thus, represents a phase later than that in our  
442 study. An attractive hypothesis is that dense soil overlay may lead to hypoxic conditions that  
443 in turn induce respiration stress (due to limitation of the final electron acceptor of the KCN-  
444 sensitive ETC, the oxygen) and a subsequent triple response-like phenotype. How soil and O<sub>2</sub>  
445 sensing mechanisms relate to our proposed model will be subject of future research.

446 Our proposed model (**Fig. 5**) is based on respiration deficiency generated in the laboratory by  
447 genetic or pharmaceutical blocking. However, it would fit also with other natural scenarios in  
448 which the final electron acceptor for KCN-sensitive electron transport (i.e. oxygen) becomes  
449 limiting for instance in response to water flooding, drought or acute ozone exposure [9]. In  
450 addition, rapid responses to touch and wounding stress involve changes in the expression of  
451 genes for mitochondria-localized proteins that are responsible for the availability of the  
452 substrates required for mitochondrial energy metabolism [14]. It will be of great interest to  
453 investigate whether the proposed model becomes active also in these conditions and which  
454 developmental effects could be triggered.

455

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473 authors. All authors read and approved the manuscript.

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- 604
- 605

606 **Supporting Information**

607 **Fig. S1: Mapman representation of the relative gene expression profile and related**  
608 **metabolic processes in *rpoTmp* dark-grown seedlings**

609 **Fig. S2: Capacity of the KCN-sensitive pathway in etiolated seedlings**

610 **Fig. S3: Images of etiolated inhibitor-treated plants**

611 **Fig. S4: Expression changes of oxidative stress marker genes in etiolated mutant plants**  
612 **compared to WT**

613 **Fig. S5: Total O<sub>2</sub> consumption rate in etiolated mutant plants**

614 **Fig. S6: Images of etiolated *rpoTmp*, *rpoTmp/anac017* and *rpoTmp/aox1a* mutant plants.**

615 **Table S1: Primers used in qPCR for expression analyses**

616 **Table S2: Microarray-based relative expression profiling of etiolated *rpoTmp* seedlings**

617 **Table S3: Microarray-based relative mitochondrial expression profiling of etiolated**  
618 ***rpoTmp* seedlings and comparison with heatmap of mitochondrial-encoded DEGs of**  
619 **light-grown adult plants [7]**

620 **Table S4: List of the 20 most down-regulated genes in etiolated *rpoTmp* seedlings versus**  
621 **WT**

622 **Table S5: List of the common up and down DEGs (threshold 1.5) between *rpoTmp***  
623 **etiolated seedlings (this work) and light-grown adult plants [7]. The genes that are**  
624 **among the top 20 DEGs in *rpoTmp* etiolated seedlings are underlined in yellow; the**  
625 **genes that are among the top 20 DEGs in *rpoTmp* light-grown plants are marked with an**  
626 **asterisk**

627

628 **Supplemental information to Methods.**

629



630 **Figure Legends**

631 **Fig. 1 Etiolated *rpoTmp* mutant plantlets exhibit a triple-response-like phenotype. a)**  
632 Images of etiolated *rpoTmp* mutant (two mutant alleles, *rpoTmp-2* SALK\_132842 and  
633 *rpoTmp-3* SALK\_086115) and WT plants (grown in presence or in absence of 20  $\mu$ M ACC)  
634 were taken under a dissection microscope. Scale bar corresponds to 1 mm. **(b)** Top panel:  
635 Median values of apical hook angle measurements were box-plotted for WT plants grown in  
636 absence or in presence of 20  $\mu$ M ACC and for *rpoTmp* plants. The number of pooled  
637 individuals (N) that were measured in 7 independent hook angle analyses corresponds to 304  
638 for WT plants; N (measured in 3 independent analyses) corresponds to 130 for WT + ACC  
639 plants; N (measured in 5 independent analyses) corresponds to 211 for *rpoTmp* plants. Data  
640 are box-plotted using versions of the first and third quartiles [25] with median values  
641 (horizontal lines inside the box). The external horizontal lines show the largest or smallest  
642 observations that fall within a distance of 1.5 times the box size from the nearest hinge.  
643 Additional points are extreme value observations. The asterisks (\*) indicate significant  
644 difference between two samples. Bottom panel: Principle of angle determination. Magnified  
645 images of the hook from WT, *rpoTmp* and ACC-treated WT. The apical hook curvature was  
646 measured as the angle (blue line) that is formed by the two straight lines (red) passing through  
647 the hypocotyl and the cotyledon axes. Any hook angle larger than 180° (blue horizontal line)  
648 was considered as twisted. Images were analyzed with the ImageJ programme. **c)** Median  
649 values of hypocotyl length measurements were box-plotted for WT plants grown in absence  
650 or in presence of 20  $\mu$ M ACC and for *rpoTmp* plants. N (measured in 2 independent analyses)  
651 corresponded to 92 for WT plants; to 75 for ACC-treated WT plants; to 98 for *rpoTmp* plants.  
652 Data are box-plotted as in **Fig.1b**.

653 **Fig. 2 Relative gene expression profiles of *rpoTmp* mutants versus WT etiolated plants.**  
654 **a)** The relative expression values for the RPOtmp-dependent sub-set of mitochondrial genes  
655 are indicated in the middle panel together with the corresponding P-values, which are derived  
656 from a t-test adjusted for false discovery rate (FDR) after the Benjamini-Hochberg (BH)  
657 procedure. Gene identities are indicated in the left, and protein identities in the right panels.  
658 Given values represent log<sub>2</sub>-fold changes (FC). **b)** Analysis of accumulation of NAD9 protein  
659 used as marker for complex I accumulation. Protein extracts (10, 20, 40  $\mu$ g) from etiolated  
660 WT and *rpoTmp* plants were fractionated and immunoblotted with specific antisera against  
661 the mitochondrial proteins NAD9 (mitochondrial-encoded) and the plastidial proteins RBCL  
662 (plastidial-encoded). **c)** Relative expression values for the 20 most up-regulated genes in

663 *rpoTmp* plants. Details as in **a**). Mitochondrial (mt) localization and protein function are also  
664 indicated on the right side of the table. “Mt stress” stands for “response to mitochondrial  
665 stress”. **d**) Functional sub-grouping of the 20 most up-regulated genes listed in **c**). The four  
666 groups identified were color-coded as in **c**). For details see text. **e**) Functional sub-grouping of  
667 the 20 most down-regulated genes (**Table S4**). The four groups identified were color-coded as  
668 in **c**). The terms “hormones” and “stress” for protein functions stand for “response to  
669 hormonal stimuli” and “response to stress”, respectively.

670 **Fig. 3 Plants that are defective in respiration present a triple-response-like phenotype. a)**  
671 Median values of apical hook angle measurements were box-plotted for WT plants that were  
672 grown in presence of 1 mM KCN or H<sub>2</sub>O as mock-control and mutant *rpoTmp* plants that were  
673 grown in presence of 1 mM SHAM or DMSO as mock-control. N (measured in four  
674 independent analyses) corresponds to 178 for H<sub>2</sub>O-treated WT plants and to 128 for KCN-  
675 treated WT plants. N (measured in two independent analyses) corresponds to 148 for DMSO-  
676 treated *rpoTmp* plants and to 85 for SHAM-treated *rpoTmp* plants. Details of box-plot  
677 representation are explained in **Fig. 1b**. **b**) Images of etiolated mutant *rug3* and *atphb3* plants  
678 were taken under a dissection microscope. Scale bar corresponds to 1 mm.

679 **Fig. 4 The triple-response-like phenotype in *rpoTmp* mutant plants requires AOX1a. a)**  
680 Linear fold change (FC) induction of *AOX1a* mRNAs in mutant *rpoTmp* and  
681 *rpoTmp/anac017* plants *versus* WT determined by RT-qPCR. The *AOX1a* expression levels  
682 were normalized with the mean of *ACTIN2-8* expression, used as reference gene. The mean  
683 values of two biological replicates are plotted. Error bars correspond to standard errors. **b**)  
684 Protein extracts from etiolated WT, *rpoTmp*, *rpoTmp/anac017* plants (left panel) and WT,  
685 *rpoTmp/aox1A*, *rpoTmp* plants (right panel) were fractionated and immunoblotted with  
686 specific antisera against the mitochondrial AOX isoforms and the vacuolar protein V-ATPase  
687 as loading control. Linear fold change variation of AOX protein levels in mutant *rpoTmp*  
688 plants *versus* WT using V-ATPase for normalization is described in the table (bottom panel).  
689 **c**) Capacity of the AOX-dependent pathway in etiolated mutant plants. AOX capacity was  
690 calculated upon addition of 1 mM KCN into the measurement cell (final concentration). The  
691 figure presents the ratio in percentage of the KCN-insensitive O<sub>2</sub> consumption rate to the total  
692 consumption rate. Median values (thick horizontal lines) of N independent measurements (full  
693 circles) were scatter-plotted for WT (N=19), *rpoTmp* (N=12), *rpoTmp/aox1a* (N=5), *aox1a*  
694 (N=4) and *rpoTmp/anac017* plants (N=4). **d**) Images of etiolated plants: WT, *aox1a*, *rpoTmp*,  
695 *rpoTmp/aox1a*, *rpoTmp/anac017* were taken under a dissection microscope. Scale bar

696 corresponds to 1 mm. e) Median values of apical hook angle measurements are box-plotted  
697 for WT, mutant *rpoTmp* and *rpoTmp/anac017* plants. Values were measured in 2 independent  
698 analyses. N =53 for WT plants; N =57 for *rpoTmp* plants; N= 57 for *rpoTmp/anac017* plants.  
699 Details of box-plot representation are as given in **Fig. 1b**.

700 **Fig. 5 Scheme of the proposed functional link between the respiratory chain capacities**  
701 **(KCN-sensitive and AOX-dependent) and the skoto-morphogenic programme.** In  
702 mitochondria the KCN-sensitive transport of electrons (ETC) from NADH is used for O<sub>2</sub>  
703 reduction and ATP production. Only a limited fraction of electrons is diverted to the AOX  
704 enzyme for reduction of O<sub>2</sub> without concomitant ATP production. In etiolated *rpoTmp*, *rug3*,  
705 *atphb3* or KCN-treated WT plants, the KCN-sensitive electron flux is strongly decreased  
706 inducing a strong dysfunctional stress (red indication). This stress activates the canonical  
707 (ANAC017-dependent) mitochondrial retrograde pathway that activates *AOX1a* expression. It  
708 may be supported by yet unknown factors (represented by a yellow oval with question mark).  
709 Additional AOX regulation by dysfunctional stress at the level of protein accumulation and  
710 modification might be possible (thick back arrow). As ultimate effect the capacity of the  
711 AOX-dependent chain is up-regulated and more electrons can be diverted by this pathway  
712 releasing the stress (green indication). The dysfunctional stress in combination with the highly  
713 increased AOX capacity, however, sends a signal *via* an unknown transmitter (represented by  
714 a grey circle with question mark) that triggers the formation of the triple-like-response (or  
715 parts of it).