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

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Summary

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

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

Introduction

Mitochondria are essential cell organelles that convert organic matter into chemical energy in form of ATP and reducing power through the process of respiration [1]. To this end in seeds of oilseed plants as *Arabidopsis*, the triacylglycerol lipids present in the oil bodies are oxidatively degraded into citrate which is fed into the tricarboxylic acid (TCA) cycle within the mitochondrial matrix [2]. Here the carbon skeleton is completely oxidized and CO_2, ATP and reducing equivalents are released by the cycle. The reducing power then is used to drive an electron transport through the mitochondrial KCN-sensitive electron transport chain (ETC) within the inner mitochondrial membrane towards oxygen as end acceptor. ETC consists of three large multi-subunit protein complexes (complexes I, III and IV) that are connected by mobile electron carriers. The electron transport through this chain is coupled to a proton-translocation across the mitochondrial membranes, which is finally used by the ATP synthase to build ATP. The oxidized forms of the reducing equivalents produced by the ETC are then available again for the TCA cycle as electron sink. By this way TCA cycle and ETC are functionally coupled and any restriction in metabolic flux in one of them affects also the other [1].

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

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

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

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

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

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

Material and Methods

Plant material

Arabidopsis thaliana wildtype (WT) (ecotype Columbia) and mutant lines (*rpoTmp-2* SALK_132842 and *rpoTmp-3* SALK_086115 [21][5], *rug3* SALK_092071 [6], *atphb3* SALK_020707 [23], *rpoTmp* SALK_132842/*anac017* (*anac017, rao2-1*) [7,24], *aox1A* SALK_084897 [8] and *rpoTmp* SALK_132842/*aox1A* SALK_084897 [8]) were used in this study. Seeds were surface-sterilized and spread on Murashige and Skoog (MS) agar plates supplemented with sucrose (1%). Activated charcoal (in powder, SIGMA-ALDRICH, 0.08%) was added to the plant culture medium in order to improve image quality of etiolated plants by increasing background contrast. Seeds were stratified for 72 hours (at 4°C in darkness), exposed to light (100 µE white light) for 6 hours and then grown at 23°C in darkness. Plants were harvested after 3 days. For microarray, qRT-PCR and western-immuno-blot analyses the etiolated plants were harvested in the dark under a green safe-light, immediately frozen in liquid nitrogen, ground in a mortar and subjected to further techniques as described below. The germination percentage was variable according to the seed lot and the drug treatment. For chemical treatments MS agar plates were supplemented with 1-aminocyclopropane-carboxylic acid (ACC, SIGMA-ALDRICH A3903, 20 µM), KCN (1 mM) or salicylhydroxamic acid (SHAM, SIGMA-ALDRICH S7504, 1 mM in DMSO). In case of KCN or SHAM treatments, seeds were first spread on black nitrocellulose membrane filters (Whatman 10409770, 0.45 µm) on MS agar plates, stratified for 72 hours and light-exposed for 6 hours, and then transferred (on filter) to KCN/SHAM-containing MS agar plates for growth in the dark. DMSO-containing MS agar plates were used as mock-control for the SHAM treatment.

Measurements of phenotypic parameters

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

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

Statistical analyses of phenotypic parameter measurements

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

Microarray analysis

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

Analysis of microarray data

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

RT-qPCR analysis of RNA levels

Ground plant material was resuspended in 3 volumes of solution A (10 mM Tris-HCl pH8.0; 100 mM NaCl; 1 mM EDTA; 1% SDS) and 2 volumes of phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v). After centrifugation, RNAs in the aqueous phase were again extracted twice with phenol-chloroform and finally once with chloroform. After over-night precipitation in 2 M LiCl at 4°C, RNAs were then precipitated in ethanol, washed in ethanol 70% and resuspended in water. 300 ng of DNAse (DNAse Max Kit, Quiagen) treated-RNAs were retro-transcribed using random hexo-nucleotides and the Super-Script II enzyme (Invitrogen) according to the manufacturer´s protocol. The PCR reaction was performed in the Biorad CFX384TM Real Time System PCR machine in presence of forward and reverse gene-specific primers (0.5 µM, **Table S1**) using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus) from Takara Bio. Data were analyzed using the CFX Manager Software. The expression levels of the transcript of interest were normalized with the levels of *ACTIN 2-8* expression, used as reference gene. The mean values of biological and/or technical replicates were plotted together with the error bars corresponding to standard errors.

Protein immuno-western analysis

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

In planta O_2 consumption measurements

Etiolated plantlets were harvested from Petri dishes and immediately soaked in the dark with 1 mL of oxygenated phosphate buffer (10 mM sodium phosphate buffer pH 7.2; 10 mM KCl; 10 mM glucose) in the measurement cell of a Clark oxygen electrode (Hansatech). For electron transport inhibition experiments, KCN (0.1 M stock, inhibitor of KCN-sensitive chain at the level of complex IV) and SHAM (0.1 M stock in DMSO, inhibitor of the AOX-dependent pathway) were added directly into the electrode chamber to a final concentration of 1 mM once the recorded signal reached a constant regime. The capacity (or maximum activity) of the AOX-dependent pathway (corresponding to KCN-insensitive pathway) and of the COX- dependent pathway (corresponding to KCN-sensitive pathway) was calculated as ratio of O_2 consumption rate upon addition of KCN or SHAM and total O_2 consumption rate, respectively. The contribution of extra mitochondrial O_2 consumption to mitochondrial respiration was evaluated at the end of each measurement after addition of both KCN and SHAM and systematically subtracted from O_2 consumption rates.

Results

rpoTmp mutants display a triple-response-like phenotype

To investigate the impact of mitochondrial dysfunction on skoto-morphogenesis we compared 3-day-old dark-grown (etiolated) WT with *rpoTmp* mutant plants grown under identical conditions (**Fig.1a**). WT seedlings displayed a typical skoto-morphogenesis with elongated hypocotyl, apical hook and yellow cotyledons. In contrast, seedlings of two allelic *rpoTmp* mutant lines (*rpoTmp-2* SALK_132842 and *rpoTmp-3* SALK_086115) developed a significant exaggeration of apical hook curvature (twist) and a marked shortening of the hypocotyls. The phenotype of both alleles was identical indicating that indeed the genetic defect in *rpoTmp* causes the massive change in the seedling developmental program. The observed mutant phenotype was reminiscent of morphological changes reported for etiolated seedlings exposed to high levels of ethylene, commonly known as triple-response [32,33]. To investigate this in more detail we compared the *rpoTmp* phenotype to that of WT plants grown in presence of 20 µM 1-aminocyclopropane-1-carboxylic acid (ACC), a biosynthetic precursor of ethylene known to induce the triple-response [34] (**Fig. 1a**). We observed comparable values for the increased apical hook curvature (**Fig. 1b**) and the hypocotyl shortening (**Fig. 1c**) in the two plant samples. Only the widening of the hypocotyl diameter in response to ACC treatment was not observable in *rpoTmp* (**Fig. 1a**) suggesting that the genetic defect in the *RPOTmp* locus causes a strong but not perfect phenocopy of the ACC-treatment. Therefore, we termed the observed phenotypic response a triple-response-like phenotype.

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

In order to find a potential link between the genetic inactivation of the *RPOTmp* gene and the morphological changes, we performed a transcriptomic profiling of etiolated mutant seedlings in comparison to WT (**Table S2**) and compared these data to previous expression analyses performed in light-grown plants. An overview of the gene expression profiles in etiolated plants in relation to the corresponding cellular metabolic pathways using the MapMan tool indicated that the general impact of the mutation in *RPOTmp* was relatively weak (Fig. S1). Only a small number of genes displayed a strong expression change of more than log2 fold ≥2 (29 genes) or ≤-2 (60 genes, **Table S2**). These data suggest that the observed triple-response-like phenotype is rather a specific effect than the result of pleiotropic disturbance in the

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

A physiological role for the triple response was obscure for a long time until recently a study uncovered that the triple response can be triggered by the soil overlay of buried seeds [43]. The triple morphogenic change provides higher resistance to soil friction and allows the seedling to breach the surface while protecting the apical meristem. A recent study investigated the influence of hypoxic conditions on skoto-morphogenesis and uncovered that a low oxygen tension applied after the apical hook maintenance phase prevented the opening of the hook in subsequent light conditions [44]. The study identified that seedlings are able to sense the oxygen tension *via* the N-end rule pathway-controlled stability of ERFVII transcription factors. However, the hypoxic condition in that study was imposed to the seedlings after the apical hook was formed and, thus, represents a phase later than that in our study. An attractive hypothesis is that dense soil overlay may lead to hypoxic conditions that in turn induce respiration stress (due to limitation of the final electron acceptor of the KCN-sensitive ETC, the oxygen) and a subsequent triple response-like phenotype. How soil and O_2 sensing mechanisms relate to our proposed model will be subject of future research.

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

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Author contribution. LM and TP designed research, LM, FC, BG, VS, FCh and SB performed experiments, OB performed statistical analyses, CL contributed analytical tools. LM, FC, SLM, TP analyzed data, LM and TP wrote the manuscript with the help of all co-authors. All authors read and approved the manuscript.

Competing interests. The authors declare no conflict of interest.

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Supporting Information

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

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

Fig. S3: Images of etiolated inhibitor-treated plants

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

Fig. S5: Total O_2 consumption rate in etiolated mutant plants

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

Table S1: Primers used in qPCR for expression analyses

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

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

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

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

Supplemental information to Methods.

Figure Legends

Fig. 1 Etiolated *rpoTmp* mutant plantlets exhibit a triple-response-like phenotype. **a**) Images of etiolated *rpoTmp* mutant (two mutant alleles, *rpoTmp-2* SALK_132842 and *rpoTmp-3* SALK_086115) and WT plants (grown in presence or in absence of 20 µM ACC) were taken under a dissection microscope. Scale bar corresponds to 1 mm. (**b**) Top panel: Median values of apical hook angle measurements were box-plotted for WT plants grown in absence or in presence of 20 µM ACC and for *rpoTmp* plants. The number of pooled individuals (N) that were measured in 7 independent hook angle analyses corresponds to 304 for WT plants; N (measured in 3 independent analyses) corresponds to 130 for WT + ACC plants; N (measured in 5 independent analyses) corresponds to 211 for *rpoTmp* plants. Data are box-plotted using versions of the first and third quartiles [25] with median values (horizontal lines inside the box). The external horizontal lines show the largest or smallest observations that fall within a distance of 1.5 times the box size from the nearest hinge. Additional points are extreme value observations. The asterisks (*) indicate significant difference between two samples. Bottom panel: Principle of angle determination. Magnified images of the hook from WT, *rpoTmp* and ACC-treated WT. The apical hook curvature was measured as the angle (blue line) that is formed by the two straight lines (red) passing through the hypocotyl and the cotyledon axes. Any hook angle larger than 180° (blue horizontal line) was considered as twisted. Images were analyzed with the ImageJ programme. **c**) Median values of hypocotyl length measurements were box-plotted for WT plants grown in absence or in presence of 20 µM ACC and for *rpoTmp* plants. N (measured in 2 independent analyses) corresponded to 92 for WT plants; to 75 for ACC-treated WT plants; to 98 for *rpoTmp* plants. Data are box-plotted as in **Fig.1b**.

Fig. 2 Relative gene expression profiles of *rpoTmp* mutants *versus* WT etiolated plants. **a**) The relative expression values for the RPOTmp-dependent sub-set of mitochondrial genes are indicated in the middle panel together with the corresponding P-values, which are derived from a t-test adjusted for false discovery rate (FDR) after the Benjamini-Hochberg (BH) procedure. Gene identities are indicated in the left, and protein identities in the right panels. Given values represent log2-fold changes (FC). **b**) Analysis of accumulation of NAD9 protein used as marker for complex I accumulation. Protein extracts (10, 20, 40 µg) from etiolated WT and *rpoTmp* plants were fractionated and immunoblotted with specific antisera against the mitochondrial proteins NAD9 (mitochondrial-encoded) and the plastidial proteins RBCL (plastidial-encoded). **c**) Relative expression values for the 20 most up-regulated genes in

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

Fig. 3 Plants that are defective in respiration present a triple-response-like phenotype. **a**) Median values of apical hook angle measurements were box-plotted for WT plants that were grown in presence of 1 mM KCN or H_20 as mock-control and mutant *rpoTmp* plants that were grown in presence of 1 mM SHAM or DMSO as mock-control. N (measured in four independent analyses) corresponds to 178 for H_2O-treated WT plants and to 128 for KCN-treated WT plants. N (measured in two independent analyses) corresponds to 148 for DMSO-treated *rpoTmp* plants and to 85 for SHAM-treated *rpoTmp* plants. Details of box-plot representation are explained in **Fig. 1b**. **b**) Images of etiolated mutant *rug3* and *atphb3* plants were taken under a dissection microscope. Scale bar corresponds to 1 mm.

Fig. 4 The triple-response-like phenotype in *rpoTmp* mutant plants requires AOX1a. **a**) Linear fold change (FC) induction of *AOX1a* mRNAs in mutant *rpoTmp* and *rpoTmp*/*anac017* plants *versus* WT determined by RT-qPCR. The *AOX1a* expression levels were normalized with the mean of *ACTIN2-8* expression, used as reference gene. The mean values of two biological replicates are plotted. Error bars correspond to standard errors. **b**) Protein extracts from etiolated WT, *rpoTmp, rpoTmp/anac017* plants (left panel) and WT, *rpoTmp/aox1A, rpoTmp* plants (right panel) were fractionated and immunoblotted with specific antisera against the mitochondrial AOX isoforms and the vacuolar protein V-ATPase as loading control. Linear fold change variation of AOX protein levels in mutant *rpoTmp* plants *versus* WT using V-ATPase for normalization is described in the table (bottom panel). **c**) Capacity of the AOX-dependent pathway in etiolated mutant plants. AOX capacity was calculated upon addition of 1 mM KCN into the measurement cell (final concentration). The figure presents the ratio in percentage of the KCN-insensitive O_2 consumption rate to the total consumption rate. Median values (thick horizontal lines) of N independent measurements (full circles) were scatter-plotted for WT (N=19), *rpoTmp* (N=12), *rpoTmp/aox1a* (N=5), *aox1a* (N=4) and *rpoTmp/anac017* plants (N=4). **d**) Images of etiolated plants: WT, *aox1a, rpoTmp, rpoTmp/aox1a, rpoTmp/anac017* were taken under a dissection microscope. Scale bar

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

Fig. 5 Scheme of the proposed functional link between the respiratory chain capacities (KCN-sensitive and AOX-dependent) and the skoto-morphogenic programme. In mitochondria the KCN-sensitive transport of electrons (ETC) from NADH is used for O_2 reduction and ATP production. Only a limited fraction of electrons is diverted to the AOX enzyme for reduction of O_2 without concomitant ATP production. In etiolated *rpoTmp, rug3, atphb3* or KCN-treated WT plants, the KCN-sensitive electron flux is strongly decreased inducing a strong dysfunctional stress (red indication). This stress activates the canonical (ANAC017-dependent) mitochondrial retrograde pathway that activates *AOX1a* expression. It may be supported by yet unknown factors (represented by a yellow oval with question mark). Additional AOX regulation by dysfunctional stress at the level of protein accumulation and modification might be possible (thick back arrow). As ultimate effect the capacity of the AOX-dependent chain is up-regulated and more electrons can be diverted by this pathway releasing the stress (green indication). The dysfunctional stress in combination with the highly increased AOX capacity, however, sends a signal *via* an unknown transmitter (represented by a grey circle with question mark) that triggers the formation of the triple-like-response (or parts of it).