Phytohormones as Regulators of Mitochondrial Gene Expression in *Arabidopsis thaliana*

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Article

Phytohormones as regulators of mitochondrial gene expression in *Arabidopsis thaliana*

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Abstract: Coordination of activities between nuclei and organelles in plant cells involves information exchange, in which phytohormones may play an essential role. Therefore, dissection of the mechanisms of hormone-related integration between phytohormones and mitochondria is an important and challenging task. Here, we found that inputs from multiple hormones may cause changes in transcript accumulation of mitochondrial-encoded genes and nuclear genes encoding mitochondrial (mt) proteins. In particular, treatments with exogenous hormones induced changes in *GUS* expression in *the* reporter line possessing a 5'-deletion fragment of the *RPOTmp* promoter. These changes corresponded in part to up- or downregulation of *RPOTmp* in wild-type plants, which affected the transcription of mt-encoded genes, implying that promoter fragments of the *RPOTmp* gene are functionally involved in responses to IAA (indole-3-acetic acid), ACC (1-aminocyclopropane-1-carboxylic acid), and ABA (abscisic acid). Hormone-dependent modulations in the expression of mt-encoded genes can also be mediated through mitochondrial transcription termination factors 15, 17, and 18 of the mTERF family and genes for tetratricopeptide repeat proteins that are coexpressed with *mTERF* genes, in addition to *SWIB5* encoding a mitochondrial SWI/SNF (nucleosome remodelling) complex B protein. These genes specifically responded to hormone treatment, displaying both negative and positive regulation in a context-dependent manner. According to bioinformatic resources, their promoter regions possess putative *cis*-acting elements involved in responses to phytohormones. Alternatively, hormone-related transcriptional activity of these genes may be modulated indirectly, which is especially relevant for brassinosteroids (BS). In general, the results of the study indicate that hormones are essential mediators that are able to cause alterations in the transcript accumulation of mt-related nuclear genes, which in turn trigger the expression of mt genes.

Keywords: *Arabidopsis thaliana;* gene expression; mitochondria; phytohormones; *RPOTmp*

1. Introduction

The endosymbiotic organelles of eukaryotic cells, plastids and mitochondria, are tightly integrated into cellular signaling networks as inseparable parts of the plant cell needed for photosynthesis and ATP production [1]. The coordinated expression of the organellar and nuclear genomes is achieved by a variety of signals, among which phytohormones make essential contributions. The effects of various hormones on the expression of chloroplast genes are well documented. Exogenously supplied methyl jasmonate (MJ), IAA, ABA and gibberellic acid (GA) repressed the transcription and transcript accumulation of plastid genes, while cytokinins (CKs) counteracted their action [2]. However, the molecular mechanisms underlying the transduction of hormonal signals to plastids are still poorly understood. Despite the fact that chloroplasts are sites for the production of a number of hormones or their precursors (CK, ABA, SA, IAA, jasmonic acid and ethylene) [3], the plastid genome does not retain the genes responsible for the perception and

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transduction of hormonal signals. Therefore, all stages in the hormone-dependent expression of plastid genes are determined primarily by anterograde signals coming from the nucleus.

Research into interactions between phytohormones and mitochondria has mainly focused on stress-related aspects [4], and despite some progress, the mechanisms of such interactions are far from being fully understood. The Arabidopsis mitochondrial genome contains 58 genes encoding tRNAs, rRNAs, ribosomal proteins and subunits of the respiratory chain, in addition to 42 noncoding genes [1]. They are transcribed by two nuclear-encoded page-type RNA polymerases: RPOTm, which is exclusively targeted to mitochondria, and RPOTmp, which is found only in dicots and is bidirected to mitochondria and chloroplasts. RPOTm is vital for plant development, as its disruption was found to be lethal [5]. RPOTmp is needed to optimally transcribe a subset of mitochondrial genes, including those for respiratory chain complexes I and IV [5]. Lack of this enzyme causes mitochondrial dysfunction, resulting in a strongly reduced mitochondrial respiratory chain and a compensatory upregulation of alternative oxidase (AOX)-dependent respiration. In addition to functions in mitochondria, RPOTmp was shown to transcribe the *rrn* operon from the *PC* promoter in plastids during seed imbibition [6].

The effects of hormones on organellar gene expression (OGE) can at least partially be transduced through the genes for transcription machinery. In our previous works, we showed that CK-induced changes in the expression of genes encoding chloroplast RNA polymerases and polymerase-associated proteins (PAPs) resulted in modulated expression of chloroplast genes, suggesting a role for the transcription apparatus in their hormone-dependent regulation [7,8], However, the exact way in which components of the transcription apparatus induce or suppress the transcription of plastid genes is not known. Hormone-related shifts can also be regulated by organellar-specific import of transcription factors, providing direct binding to transcription initiation sites and conferring promoter specificity in organellar transcription. To date, a convincing example has been presented for ABA-dependent transcription of the chloroplast *psbD* gene from the blue light responsive promoter (*BLRP*) *via* ABA-dependent stimulation of SIG5–PEP-dependent transcription [9].

Among OGE regulators affected by hormones (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) are proteins of the mitochondrial transcription termination factor family (mTERF) and tetra- and pentatricopeptide repeat proteins (TPR and PPRs), which are coexpressed with *mTERF* genes of the mitochondrial cluster [10], 2012). Another potential candidate is a mitochondrial SWI/SNF (nucleosome remodelling) complex B protein, SWIB5, which is capable of associating with mitochondrial DNA (mtDNA) and influencing mtDNA architecture in *Arabidopsis thaliana* [11]. Whether these regulatory proteins are engaged in the transduction of hormonal signals to mitochondria and directing the expression of mt genes remains to be seen.

Accumulating data suggest that in addition to anterograde signaling, the coordinated expression of organellar and nuclear genomes can be achieved through mechanisms of hormone-dependent retrograde signaling. In particular, Wang and Auwerx [4] established that proteotoxic stress in mitochondria caused by the accumulation of unassembled or unfolded proteins culminates in a systemic hormone response mainly reliant on ethylene signaling but also involving auxin and jasmonate. Blocking ethylene signaling partially repressed mitochondria-to-nucleus signaling, most likely independently of the transcription factor ANAC017, a key regulator of mitochondrial proteotoxic stress responses in plants [12]. Contrary to these data, Meridino et al [1] showed that a mutation in *RPOTmp* that caused defects similar to the triple response in the dark needed ANAC017. These contradictory results are explained as a result of a weak positive feedback loop linking ethylene and ANAC017-dependent regulation of mitochondrial retrograde signaling. Anyway, these data indicate that hormones are integral factors in regulating the coordinated expression of the organellar and nuclear genomes. However, there is only limited information regarding their exact functions in this process.

In this work, we found that inputs from multiple hormones may cause context-dependent alterations in transcript accumulation of genes for mt RNA polymerases as well as MTERF and SWIB family genes, which play a role in modulating expression changes of mt genes.

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2. Results

2.1. Plant hormones trigger alterations in mitochondrial transcript abundance

Changes in the expression of nuclear-encoded RNA polymerases (NEP) through direct promoter-binding interactions with hormone-dependent transcription factors may affect the transcription of mitochondrial genes. To test the putative role of RPOTmp in hormone-dependent mitochondrial transcription, we first analysed the accumulation of mitochondrial transcripts in Columbia 0 and RPOTmp-deficient *rpotmp-2*. Twelve genes were selected for the analysis, which represent the main functional groups of the mitochondrial genome. The analysis included genes for subunits of complexes I (*nad3* and *nad6*), III (*cob*), IV (*cox1*) and V (*atp6-1*) of the electron transport chain, genes for cytochrome C biogenesis (*ccmC* and *ccmFc*), ribosomal and transport proteins (*rps4* and *mttB*), rRNA (*rrn26*), and the maturase gene (*matR*).

Some of the selected genes (*nad6, cox1, ccmC, mttB), rps4, matR*) are preferentially transcribed by RPOTmp, and their transcription levels were reduced in *rpotmp-2* (**Figure 1, Table S1**), which is consistent with the data of Kuhn et al. [5]. Steady-state levels of some RPOTmp-independent transcripts (*rnn26*, *ccMFc, nad3*) were even enhanced in the absence of RPOTmp. These alterations are thought to be associated with elevated levels of cellular mtDNA caused by general energy constraints in the mutant [5].

To address hormone-induced changes, we performed qRT–PCR analysis. Differentially expressed genes with a ratio of transcript change of 1.5 in at least two tests were classified as regulated [13]. According to the results obtained, all selected mitochondria-encoded genes were strongly repressed by MJ and induced by CK (except for *rrn26*) in wild-type (WT) seedlings (**Figure 1, Table S1**). The response to other hormones was gene specific, with certain mt genes exhibiting expression shifts, but others remaining unaltered. Thus, *cox1* and *nad6* displayed a 2-fold increase in transcript abundance following BL, IAA, ACC, GA_3 and SA treatments, while none of these hormones affected *atp6* expression. The *rrn4* gene was upregulated by BL and IAA, but induction was not observed in response to ACC, GA_3 and SA. In this regard, it should be noted that some hormone responses may be near saturation due to optimal endogenous concentrations.

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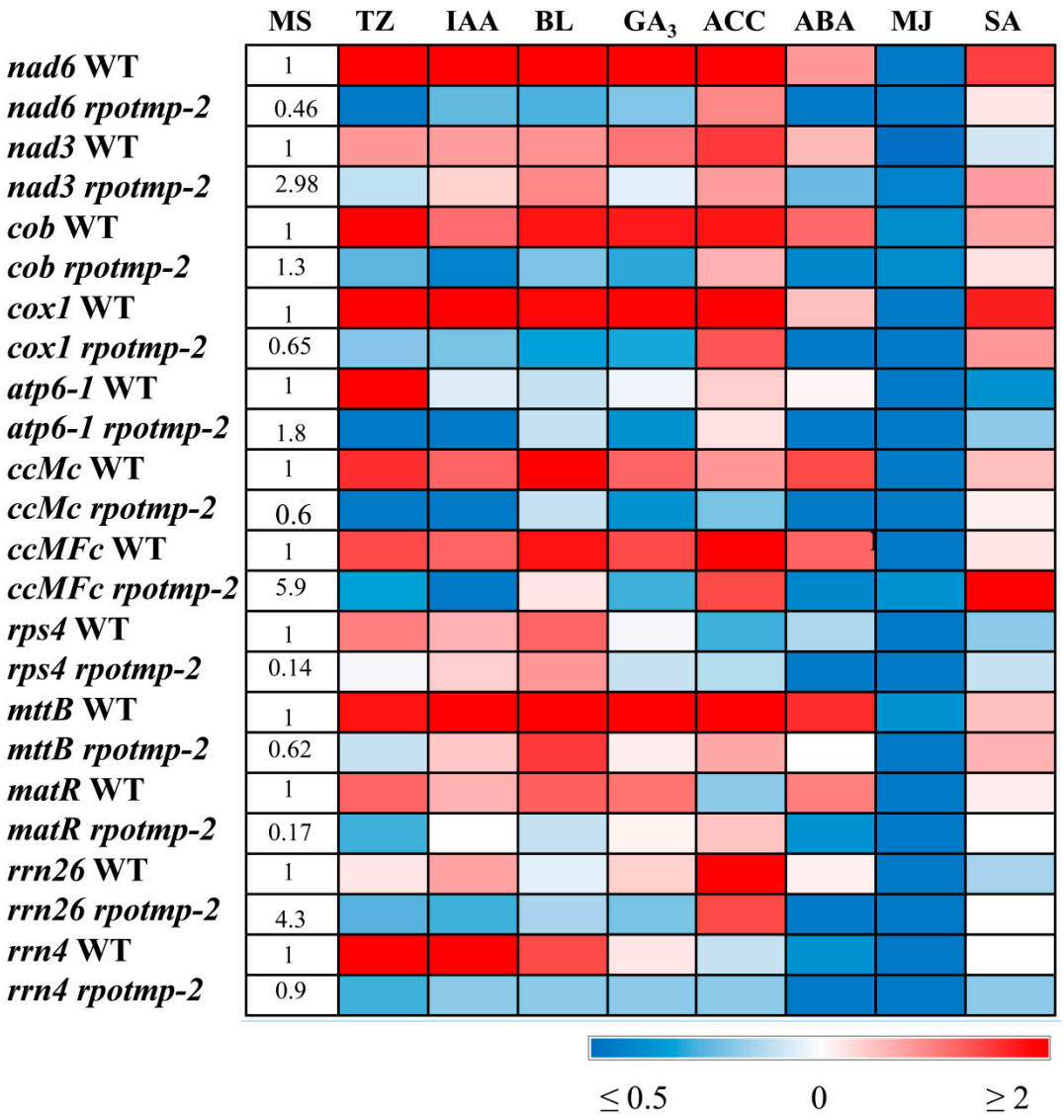

Figure 1. Effect of phytohormone treatment on relative gene expression values of mitochondrial genes. *A. thaliana* wild-type and *rpotmp* mutant plants were grown on MS medium in Petri dishes at an illumination of 50 μmol·m^{-2}·s^{-1} and a temperature of 23°C with a 16 h photoperiod. Ten-day-old seedlings were treated with hormone solutions and collected after 3 h of exposure. Total RNA was isolated from seedlings and analysed by relative quantitative RT–PCR using *UBQ10* and *PP2A* as internal standards. The numbers in the "MS" column indicate the baseline ratio of expression of each gene in the wild type and mutant without treatments. Full numerical data are presented in Table S1.

Another potential complication in assessing the sensitivity of mt-encoded genes to hormones is associated with the phases of ontogenesis. Depending on the age of a plant, gene expression changes resulting in either an increase or decrease in transcript levels can be triggered by the same treatment. Thus, the expression of mt-encoded genes sharply decreased when wild-type seedlings were grown in the dark for 4 days on medium containing *trans*-zeatin (TZ, 1 μM) compared with seedlings cultivated on medium without hormone (**Table S2**). Hence, the changes in mt gene expression in response to hormonal treatment may represent the outcome of completely different effects, reflecting opposite pathways of their regulation.

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2.2. Disruption of RPOTmp alters sensitivity to hormone treatment

Shifts in the transcript abundance of mt-encoded genes were abolished in *the rpotmp-2* background following hormone treatment, with the exception of BL-induced accumulation of *mttB* matrices (**Figure 1**). Moreover, several genes that were upregulated in WT were even repressed in *rpotmp-2*. It thus appears that the loss of RPOTmp blocks or attenuates hormone-dependent responses of mt-encoded genes. Strikingly, similar responses were observed for both RPOTmp-dependent and RPOTmp-independent genes when the magnitude of their fluctuations in the mutant was assessed relative to basal values. Moreover, such a response was also found for hormones, the corresponding regulatory elements of which were absent in the promoters of mt polymerases. These results suggest that the modulation in mt gene regulation may be the result of an altered hormonal status of *rpotmp-2*. The changes may also reflect modified expression in the mutant of the second mitochondrial polymerase RPOTm, which is the only active one in the *rpotmp-2* mitochondria, since hormone-related changes in *RPOTm* transcript levels were mitigated in the *rpotmp* background (**Figure 2**).

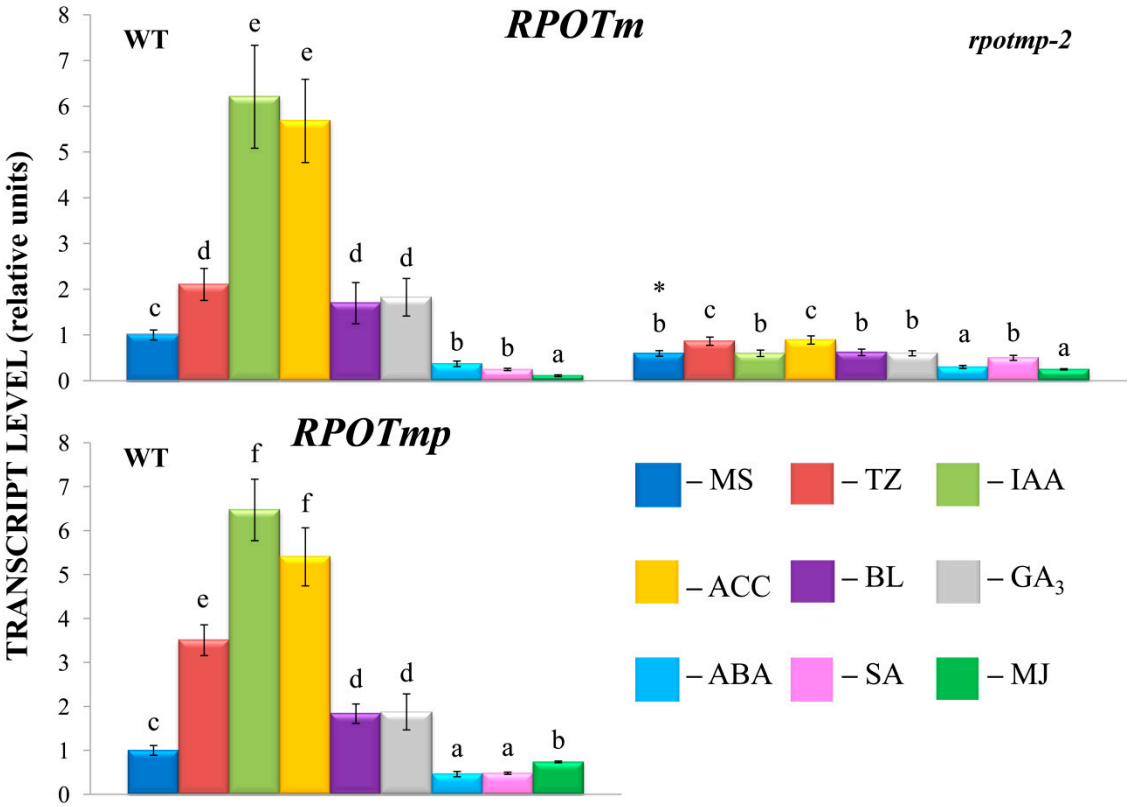

Figure 2. Effect of phytohormone treatment on relative gene expression values of nuclear-encoded page-type RNA polymerases RPOTmp and RPOTm. *A. thaliana* wild-type and *rpotmp* mutant plants were grown on MS medium in Petri dishes at an illumination of 50 μmol·m^{-2}·s^{-1} and a temperature of 23°C with a 16 h photoperiod. Ten-day-old seedlings were treated with hormone solutions and collected after 3 h of exposure. Total RNA was isolated from seedlings and analysed by relative quantitative RT–PCR using *UBQ10* and *PP2A* as internal standards. Different letters denote statistically significant differences among variants within the same genotype at $p < 0.05$ (ANOVA with post hoc Tukey's multiple-comparison test).

To experimentally evaluate the sensitivity of *rpotmp-2* to hormone treatment, we first examined hormone reporters. The mutant and WT had similar levels of the auxin marker *IAA19* and BS marker *DWF4* in 2-week-old seedlings. At the same time, the mutant exhibited elevated steady-state expression values of ethylene and the MJ marker *ERF1*. In parallel, the *rpotmp-2* mutant was less sensitive to ACC in root elongation and dark detached assays than Col0 (**Figure 3).** We further found that mutant leaves were less sensitive to MJ in the dark detached leaf assay, consistent with a higher

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transcript abundance of the MJ marker gene *ERF1* **(Figure 3)**. In contrast, the expression levels of the ABA reporter RD29 were reduced in *rpotmp-2* and were upregulated by the hormone two times higher than those in the wild type. The mutant was more sensitive to ABA treatment in the root elongation assay, especially in germination tests **(Figure 3),** and had expectedly reduced levels of the gibberellic acid synthesis gene *GA3*.

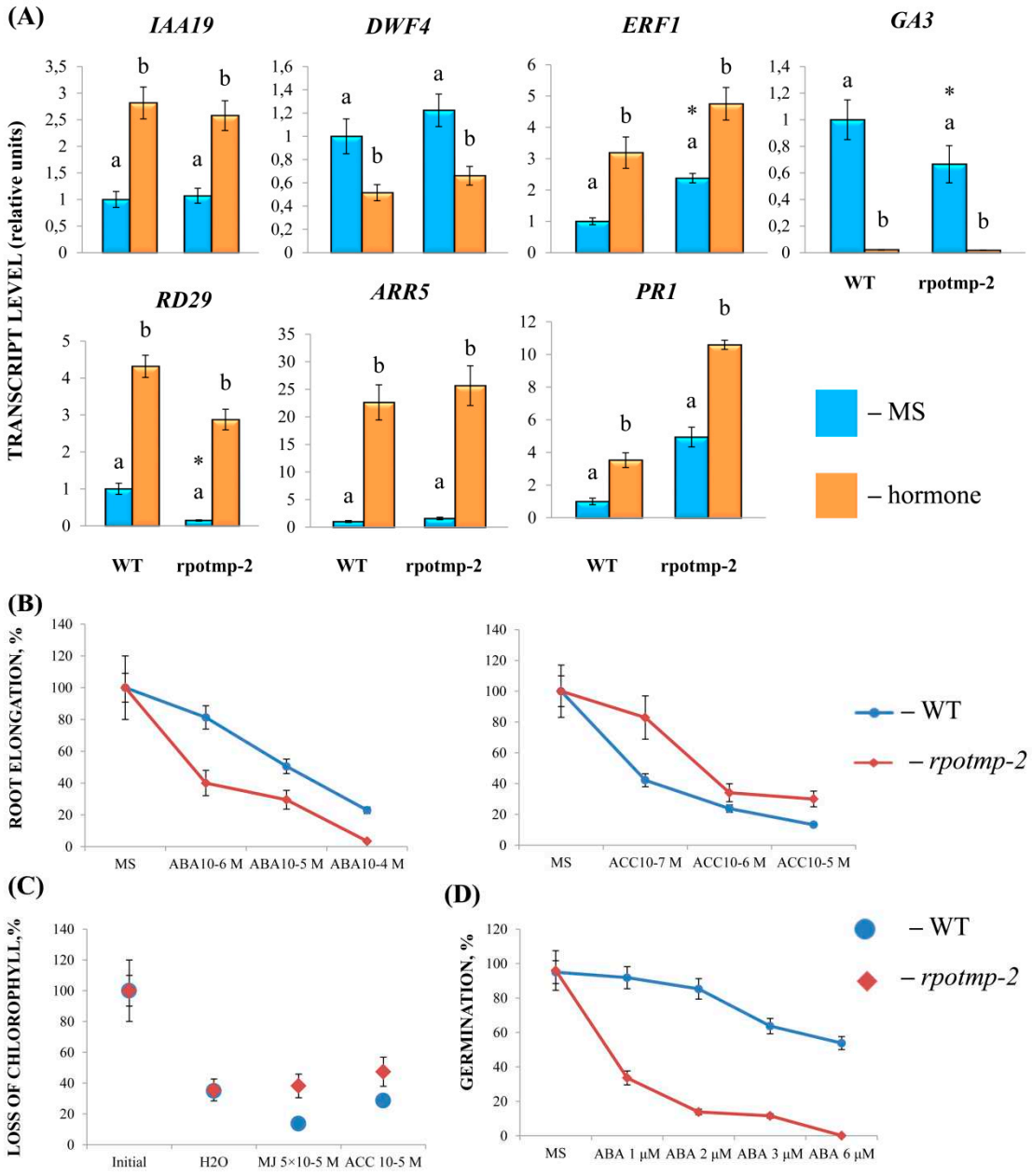

Figure 3. Sensitivity to hormone treatment of *A. thaliana* wild-type and *rpotmp* mutant. A. Relative expression values of phytohormone reporter genes. B. Inhibition of root elongation by ABA and ACC treatment. Ten-day-old seedlings were transferred to 1/2 MS plates with a range of hormone concentrations and grown vertically for 4 days under 16 h light/8 h dark conditions. C. Sensitivity to hormone treatment during dark-induced leaf senescence. Total chlorophyll content (chlorophyll *a*+ chlorophyll *b*) was measured in detached 5th and 6th rosette leaves excised from the soil-grown 5-week-old plants and incubated in the dark at 23°C on water, MJ (5×10^{-5}) or ACC (10^{-5} M) solutions for 3 days. The loss of chlorophyll is presented as a percentage of the initial value. D. Inhibition of seed germination by ABA. The percentage of fully expanded cotyledons was estimated after 6 days of germination on MS media with a range of ABA concentrations.

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In addition, we found that the mutant exhibited higher expression levels of the CK marker gene *ARR5* and SA reporter *PR1*, suggesting a possibly elevated content of corresponding hormones. However, there were no significant differences in their responses to CK and SA treatments in physiological tests (**Figure 3**).

In summary, it can be concluded that disruption of *RPOTmp* may alter the hormonal status of the mutant and its response to treatment with exogenous hormones. Furthermore, the changes in mt transcript levels in the mutant background may be the result of multifactor events, when impaired RPOTmp function and altered hormonal metabolism are superimposed on the modified RPOTm activity and, possibly, on the activity of additional transcription factors that bind directly to promoter regions of mitochondrial genes.

2.3. The RPOTmp promoter has potential cis-regulatory elements that respond to phytohormones

In silico analysis highlighted a number of consensus sequences in the 1.2 kb promoter region upstream of the RPOTmp coding sequence recognized by potential *cis*-regulatory elements (CREs) that may be involved in the response to phytohormones. The most significant differences are listed in **Table 1.** Putative motifs at positions –297/–287 bp and –1081/–1073 bp (AGATCCTC) and –966/–958 bp (AAAGATTCGA) relative to ATG (**Figure S1**) are well aligned with the consensus sequence 5'-(AGATHY, H(a/t/c), Y(t/c))-3' [14] for cytokinin-sensitive type B response regulators (ARR-B) in a direct strand. Reverse complement sequences of ARR-B (TCGAATCTTT and GAGGATCTTA) were also found.

Two to four putative auxin-responsive elements (AuxRE) were predicted within the *RpoTmp* promoter. However, only two of them, GGGTCGGGTA for ARF3 at position –305/–315 bp in the direct strand and TCAGACAAAA for ARF5 at –799/–808 bp in the complementary strand, contained the canonical motif AuxREs 5'-(TGTCNC, N(a/t/c/g))-3' for auxin-responsive factor (ARF) [15].

We did not detect the classical G-box with ABA-responsive elements: ABRE 5'-((c/g/t)ACGTG(g/t)(a/c))-3' and coupling element 3 (CE3) ACGCGTGTC), characteristic of ABA-regulated genes. At the same time, a 1.2-kb region of *RPOTmp pro* is abundant with *cis*-regulatory elements for ABA-regulated genes, including MYB (5'-c/tAACNA/G)-3'), MYC (5'-CANNTG-3'), WRKY (5'-(T)(T)TGAC(C/T)-3') and RAV (5'-CAACA-3'; 5'-CACCTG-3') family transcription factors **(Table 1)** [16]. In parallel, the DPBF1&2 binding site motif at –923/–927 bp (ACACCTG) in a complementary strand could be indirectly responsible for the reactions to ABA treatment [17].

Table 1. Putative *cis*-regulatory elements identified in the 1,200 bp Arabidopsis *RPOTmp* promoter sequence relative to ATG *in silico* (based on the results of analysis by AGRIS, PlantRegMap/PlantTFDB v5.0, PLACE and PlantCARE programs).

Putative *cis*-regulatory element	**Consensus motif**	**Sequence in *RPOTmp* (5′->3′)**	**Putative transcriptional factors based on PlantRegMap/Plant TFDB v5.0**
Auxin response factor transcriptional factor ARF B3 family protein / ARF AUX/IAA-like protein	5'-(TGTCNC, N(a/t/c/g))-3'	TCAGACAAAA GGGTCGGGTA ATTGTTGACCAAAAAAATAAA	ARF5 (AT1G19850) ARF3 (AT2G33860) ARF16 (AT4G30080)
Cytokinin-responsive type B response regulators ARR-B	5'-(AGATHY, H(a/t/c), Y(t/c)-3'	AAAGATTCGA AGATCCTC GAGGATCTTA AGATTCGA	ARR11 (AT1G67710) ARR10 (AT4G31920) ARR2 (AT4G16110) ARR14 (AT2G01760)

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ABA-responsive element AtMYC2 BS in RD22	5'-(CANNTG, N(a/t/c/g))-3'	CATGTG	
W-box promoter motif	5'-(T)(T)TGAC(C/T)-3'	ATTGTTGACCAAAAAA	WRKY2 (AT5G56270) WRKY20 (AT4G26640) WRKY23 (AT2G47260) WRKY38 (AT5G22570) WRKY60 (AT2G25000) WRKY62 (AT5G01900) WRKY63 (AT1G66600)
Related to ABI3 and VP1 (RAV1-A / RAV1-B) motif	5'-(CANNTG/A)-3'	CCAACGAAGATCACTCG	RAV1(AT1G13260)
DPBF1&2 binding site motif		ACACCTG	
		Light-responsive elements	
SORLIP1AT EveningElement promoter motif	(A/T)GATA(G/A)[1] C(A/C/G)ACA(N)$_{2-8}$(C/A/T)ACCTG[1]	GCCAC AAAATATCT	
GATA promoter motif	AAAATATCT	AGATAA TGATAG	
GCC-box	5'-GCCGCCGCC-3'	GGTTTAAGGCGGCTTCGTCGT	DREB2 (AT3G11020)

The 5'UTR of *RPOTmp pro* also contains various CREs (Figure S1). including AuxRE, two sequences specific for REF6 (RELATIVE OF ELF6) with the consensus motif 5'-CTCTGYTY-3', which may play a role in ethylene and brassinosteroid signaling, and ethylene response elements (EREs) or GCC boxes with the 5'-GCCGCCGCC-3' core sequence [18].

In addition, cross-regulation of *RPOTmp* gene expression by different phytohormones can be achieved by numerous MYB or MYB-related factors even in the absence of characteristic CREs (**Table S3).**

2.4. Hormone treatments induce changes in GUS expression in the RPOmp::GUS reporter strain

To assess the functional properties of the identified motifs, a genetic construct based on the pCAMBIA1381z vector containing a 5'-deletion fragment (–958 bp) upstream of the start codon of *RPOTmp* was fused to the open reading frame of the β-glucuronidase reporter gene and used to generate the *RPOTmp pro::GUS* line (**Figure 4A**). β-Glucuronidase staining of 10-day-old seedlings of transgenic P958 (T2 and T3 generations) grown on half MS showed stable blue colouration in the vascular tissues of roots, cotyledons and primary leaves as a result of *RPOTmp pro::GUS* expression (**Figure 4B**), while plants expressing the reporter GUS gene without a promoter remained virtually unchanged.

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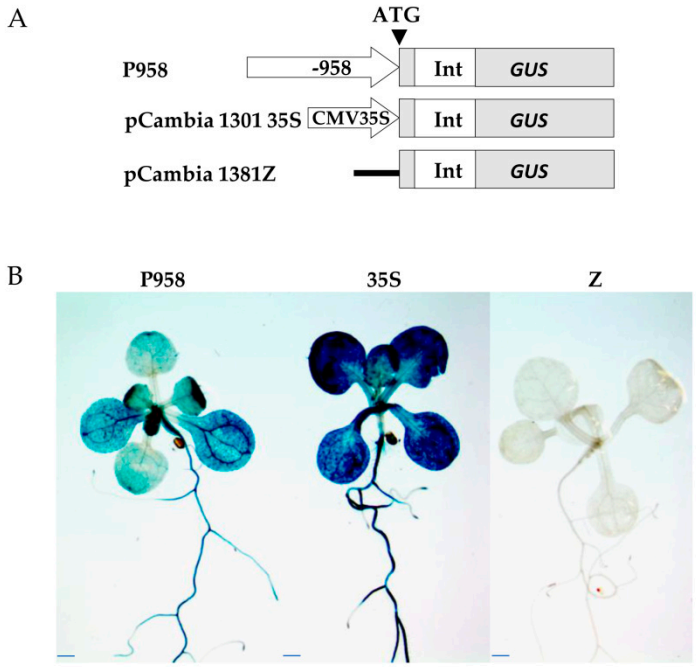

Figure 4. GUS staining images of 2-week-old plants expressing GUS activity under the *RPOTmp* promoter containing a 5'-deletion fragment (–958 bp) upstream of the start codon (P958), under the -35S CAMV promoter (35S positive control) and a negative control of the reporter GUS gene without a promoter (1381Z, negative control).

To examine whether the expression patterns of the construct change in a hormone-dependent manner, 10-day-old plants were treated with solutions of hormones for 24 hours at 22°C under a 16-h light regime, and GUS staining was performed. Plants expressing GUS activity under the 35S CAMV promoter were used as a positive control, whereas plants expressing the reporter GUS gene without a promoter were used as a negative control (**Figure 5**).

We found that GUS staining was obviously darker after ACC and IAA treatment in plants containing the -958 bp fragment than in the control samples. There were also no pronounced differences from the control variants when the reporter strain was treated with CK, GA, BL or SA. In parallel, the signal decreased when the seedlings containing the 958 bp fragment were exposed to ABA. From these observations, we conclude that the -958 bp promoter fragment of the *RPOTmp* gene is functionally involved in responses to IAA, ACC, and ABA.

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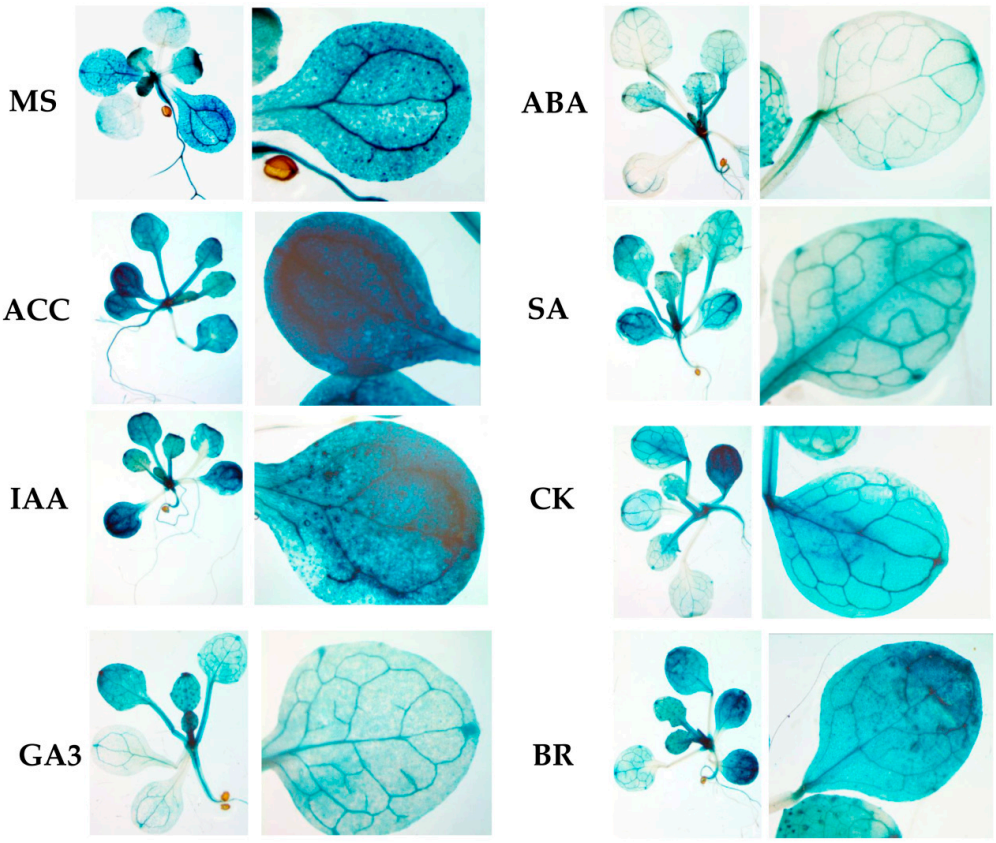

Figure 5. GUS staining images of 2-week-old plants expressing GUS activity under the *RPOTmp* promoter (P958) after 24 h of incubation in half MS supplemented with the appropriate phytohormone or without (control) at 22°C with a 16 h photoperiod.

GUS activity in the *RPOTmp::GUS* reporter strain only partially corresponded to *RPOTmp* transcript abundance under hormone treatment of WT plants. According to our qRT–PCR tests, *RPOTmp,* as well as *RPOTm*, were reproducibly induced by CK, IAA, ACC, and BL and downregulated by ABA in WT plants (**Figure 2**). They were also downregulated by SA, although no reliable changes in GUS activity were observed when *RPOmp::GUS* reporter strains were treated with these hormones.

We therefore conclude that changes in the expression of *RPOTmp* may affect the transcription of mitochondrial genes both directly through promoter-binding interactions with hormone-dependent transcription factors and indirectly.

2.5. Exogenous hormone treatment modulated the MTERF and SWIB family genes

Hormone-related shifts in the expression of organellar genes can also be induced by organellar-specific transcription factors *via* direct binding to transcription initiation sites of mt genes. Proteomic studies revealed the presence of a large number of proteins containing DNA-binding motifs in plant mitochondria, which are expected to play key roles in mtDNA expression [19]. Some of these factors may represent facilities for hormonal regulation of mt gene expression. Among them is a diversified mTERF family that includes 35 members targeted to chloroplasts and/or mitochondria, where they have been shown to function in OGE at the transcriptional or posttranscriptional level [20]. Although the members of the “mitochondrial” and “mitochondrion-associated” clusters respond weakly to physiological perturbations [10], at least some of them were up- or downregulated more than 2-fold in response to exogeneous hormones according to the microarray data provided on the resource server (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

To test whether they could represent instruments for the transduction of hormonal signals to mitochondria, we focused on mTERF15,17 and 18 since their promoter regions are predicted to

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possess binding site motifs for hormone-regulated transcription factors (https://agris-knowledgebase.org). mTERF15, with an experimentally confirmed function, is naturally induced during germination [10] and is needed for *nad2* intron 3 splicing [21]. Its dysfunction disrupts formation and decreases its activity of complex I. MTERF18 transcript levels were shown to fall upon exposure to heat and during germination [10].

In 10-day-old light-grown seedlings, transcripts of all three genes of interest increased in abundance by 2-4-fold in response to IAA or CK and decreased by approximately 2-5 times after treatment with ABA, SA, and MJ. This finding correlates well with the data obtained for *RPOTm* and *RPOTmp* and with hormone-mediated expression of mt-encoded genes. It is worth noting that while *mTERF15* and *18* were upregulated by ACC, *mTERF17* was even slightly repressed. A unique response of *mTERF17* was also observed in etiolated seedlings grown on medium containing CK: While its expression was upregulated, the expression of *mTERF15* and *18* was suppressed in accordance with the repressive regulation of mt-encoded genes by cytokinin in this experimental setup. These results suggest that members of the mTERF family may have overlapping but also specific functions in hormone-mediated regulation of mt-encoded genes depending on development status and/or environmental conditions.

The effects of hormonal application could also be regulated through tetra- and pentatricopeptide repeat proteins (TPR and PPR) targeted to mitochondria and coexpressed with mTERFs of the mitochondrial cluster. We have shown that two such genes, *At1g09190* and *At2g37320*, encoding TPR-like superfamily proteins, followed *mTERF15* and *mTERF18* in their hormone-mediated expression patterns (**Figure 6, Table S1**) and corresponded to the expression patterns of some mt-encoded genes.

In addition, modulations in the expression of mt-encoded genes could be attributed to the activity of the mitochondrial nucleoid-associated protein SWIB5, a member of the SWIB (ATP-dependent multisubunit switch/sucrose nonfermentable multiprotein complex B) family, which is implicated in DNA binding and remodelling. SWIB5 associates with mtDNA and participates in the regulation of mitochondrial gene expression [11](Blomme et al, 2017). According to our tests, the relative expression values of *SWIB5* were increased by 3-10-fold after CK, ACC and IAA application and nearly 6-fold following BL and GA_3 treatment (**Figure 6**). Interestingly, the response of mTERF genes and the genes for mt RNA polymerases to the last two hormones was considerably weaker and barely exceeded 1.5 times, despite significant induction of some mt-encoded genes.

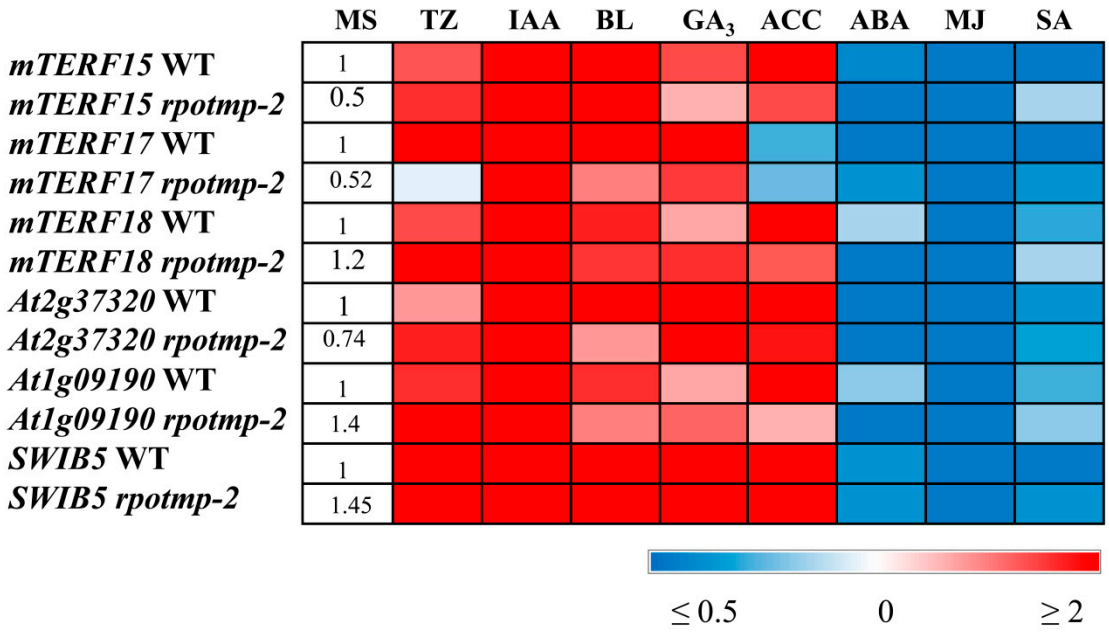

Figure 6. Effect of phytohormone treatment on relative gene expression values of nuclear-encoded genes. *A. thaliana* wild-type and *rpotmp* mutant plants were grown on MS medium in Petri dishes at

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an illumination of 50 μmol·m^{-2}·s^{-1} and a temperature of 23°C with a 16 h photoperiod. Ten-day-old seedlings were treated with hormone solutions and collected after 3 h of exposure. Total RNA was isolated from seedlings and analysed by relative quantitative RT–PCR using *UBQ10* and *PP2A* as internal standards. The numbers in the "MS" column indicate the baseline ratio of expression of each gene in the wild type and mutant without treatments. Full numerical data are presented in Table S1.

As expected, stress-related hormones (ABA, MJ and SA) downregulated all aforementioned nuclear genes. However, it is worth noting that several mt-encoded genes (*nad6, mttB, matR, cob* and *ccMc*) were significantly activated by ABA treatment, suggesting the involvement of alternative regulatory pathways.

In summary, these data indicate a function for MTERF and coexpressing TPR proteins in hormone-regulated expression of mt-encoded genes in addition to organelle RNA polymerases and mitochondrial nucleoid-associated proteins.

3. Discussion

In general, the results obtained indicate that the hormone-mediated responses of mt genes in Arabidopsis seedlings can be attributed to the activity of mitochondrial RNA polymerases able to bind hormone-dependent transcription factors and, possibly, to supplementary transcription factors that can bind directly to the promoter regions of mitochondrial genes. This assumption is at least partially supported by hormone-induced changes in GUS expression in the *RPOmp::GUS* reporter strain and coordinated transcription responses of *RPOTmp* and/or *RPOTm* with some mitochondrial-encoded genes following hormone treatment. The most regular fluctuations occurred upon treatment with CK and MJ, which promoted up- or downregulation of all tested mt genes in a context-specific manner. The responses of selected mt genes to other hormones were gene-specific and did not always follow the expression patterns of RNA polymerase genes, consistent with the idea that nuclear and mitochondrial transcription may be independently regulated [22].

In particular, side-by-side comparison showed that downregulation of *RPOTmp* by ABA corresponded to upregulation of a number of mt genes, including *nad6, cob, ccMc, ccMfc* and *mttB* (**Figure 1, 2**). Enhanced mitochondrial activity induced by ABA treatment may be a consequence of increased energy consumption in response to stressful situations, which are usually accompanied by an increase in ABA levels. Growth effects documented for *RPOTmp* transcripts following IAA treatment were not observed for *atp6* or *rnn 26,* although the expression of other selected mt genes was significantly increased. This specificity could provide a means to fine-tune the activity of certain genes in response to multiple challenges.

While our tests clearly linked CK-induced changes in *RPOTmp* and *RPOTm* transcript abundance to the modulations of mt-encoded gene expression, there was one unexpected finding. GUS activity in *RPOmp::GUS* reporter strains showed no changes following CK treatment, contrary to the upregulation of *RPOTmp* transcript abundance. Such results clearly contradicted the presence of putative CK-regulated *cis*-elements in *the RPOTmp* promoter, as predicted by bioinformatic resources. It should be noted, however, that the accessibility of such binding sites is doubted by ChiP studies of Zubo et al. [23] and Xie et al. [14], who found no binding locations for type B ARRs in *the RPOTmp* promoter. Overall, these data indicate that CK-dependent regulation of *RPOTmp* may be indirect.

In addition to mt RNA polymerases, transcription factors directly binding to the promoter regions of mitochondrial genes could be implicated in mediating phytohormone signals to mt genes. Among them, the genes of the mTERF family and the genes for tetra- and pentatricopeptide repeat proteins (TPR and PPRs) coexpressed with *mTERF* genes are of particular interest since they are regulated by hormone treatment and are capable of binding nucleic acids [20]. In our tests, MTERF and TPR protein genes specifically responded to hormone treatment, displaying both negative and positive regulation in a context-dependent manner. According to bioinformatic resources, the promoter regions of these genes possess putative *cis*-acting elements involved in responses to a number of phytohormones, including ABF, ABRE, GATA, W-box, etc. (Agris). However, additional

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experiments are needed to confirm physical interactions between MTERFs and hormone-induced TFs. Of note, mTERF17 and 18 have been shown to bind type B response regulators ARR12 and ARR1,10 and 12, respectively [14], thus presenting direct targets for cytokinin signaling.

Binding motifs for ARR1 and 10 were also revealed in the promoter region of *SWIB5* [14,23]. In our experiments, this gene was strongly upregulated by CK in light-grown seedlings and downregulated in etiolated seedlings grown on CK-containing medium in the dark, which was consistent with the changes in transcript abundances of selected mt-encoded genes. The protein encoded by *SWIB5* belongs to the nucleosome remodelling complex of mitochondria, similar to the bacterial nucleoid. It is essential for cell proliferation and stress responses and is believed to adjust the accessibility of mtDNA for RNA polymerases, linking hormone responses with chromatin remodelling [24]. SWIB5OE displayed a significant downregulation of *CRF6* (CYTOKININ RESPONSE FACTOR6) [11], encoding a cytokinin responsive AP2/ERF transcription factor that plays a key role in the inhibition of dark-induced senescence and oxidative stress as a negative regulator of the CK-associated module. [25]. Furthermore, CRF6 refers to a stimulator of mitochondrial dysfunction (MDS) induced by mitochondrial perturbation. Since both cellular proliferation and stress responses are associated with CK-mediated modulations, we suggest that CK-dependent regulation of SWIB5 may be one of the mechanisms underlying the expression of mitochondrial genes. Additionally, the involvement of SWIB5 in responses to several other plant hormones suggests pleiotropic functions in the regulation of the mt genome. However, this suggestion must be further addressed in future experiments.

Since any biological function is usually implemented by several independent mechanisms, both organelle RNA polymerases and mitochondrial transcription factors, as well as mitochondrial nucleoid-associated proteins acting redundantly, can be direct targets for hormone-regulated transcription factors. They can form a core regulatory module that acts in the direct transduction of hormonal signals to mitochondria. Alternatively, hormone-related transcriptional activity of these genes may be modulated indirectly, suggesting that additional factors are needed for their regulation. This is especially relevant for brassinosteroids, since the promoters of *RPOTmp* (and of selected genes for mTERF and PPR proteins) do not contain consensus binding sites for BS-induced transcription factors BZR and BES.

Strikingly, upregulation of mt genes by BS (except for *mttB*) was dampened in the *rpotmp-2* background in the same way as for hormones whose transcription factors can directly interact with the *RPOTmp* promoter. Therefore, loss of RPOTmp has far-reaching implications for the activity of hormone-related genes. According to the data of Meredino et al. [1], impaired function of RPOTmp caused changes reminiscent of the triple response of seedlings exposed to ethylene and could therefore contain increased levels of ethylene. In accord with extensive crosstalk and signal integration among growth-regulating hormones, plants with reduced or increased content of one hormone can show altered responses to another [26]. Thus, *rpotmp* exhibited increased steady-state levels of transcripts for the CK marker gene ARR5, as well as elevated transcript accumulation of the CK synthesis genes *IPT3* and *IPT5* and a reduced level of hormone catabolism gene *CKX3* expression, which implies a possible increase in the content of endogenous cytokinins [27]. In parallel, the expression of reporter genes for GA (*GA3*) and SA (*PR1*) was changed in the *rpotmp* compared to WT (**Figure 3**). These results suggest that disruption of RPOTmp may induce a hormonal imbalance in concerted hormonal synthesis and signaling and, as a result, a differential mitochondrial response to hormonal treatment. It thus appears that knockout or overexpression of genes regulating organellar proteins can provoke indirect effects that cast doubt on the validity of corresponding mutants in the elucidation of naturally occurring hormone-dependent responses.

On the other hand, a change in the hormonal status of the *rpotmp* mutant may be a consequence of retrograde signaling from dysfunctional mitochondria. It has been suggested that ethylene boosts mitochondrial respiration and restores mitochondrial function upon mitochondrial proteotoxic stress (mitochondrial unfolded protein response) as the anterograde arm of a feedback loop [4]. This was accompanied by MAPK6 activation and an increase in the transcription of the ethylene synthesis gene

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ACS6. Similarly, altered ethylene levels in the *rpotmp* mutant may be a means to recover mitochondrial functionality under reduced levels of respiratory complexes I and IV.

Other hormone responses to mitochondrial proteotoxic stress included the induction of auxin, ABA, and jasmonate signaling; a decrease in cytokinin signaling; and unchanged salicylic acid signaling [4]. Notably, in line with these results, detached *rpotmp* leaves were more resistant to MJ treatment (**Figure 3C**), suggesting increased steady-state levels of this hormone in the case of mitochondrial dysfunction.

The role of auxin is especially significant. Two independent works revealed that mitochondrial stress stimuli caused a suppression of auxin signaling, and conversely, auxin treatment repressed mitochondrial stress [28,29]. According to the results of our analyses, ethylene and IAA reproducibly induced transcript accumulation of *RPOTmp* and RPOTmp::GUS fusion activity, which correlated with the enhanced levels of mitochondrial RNAs. This implies a direct signaling interaction between these two hormones and RNA polymerase in the transduction of hormone operational signals from nuclei to mitochondria.

In general, the results of the study indicate that hormones are essential mediators that regulate mitochondrial gene expression in a context-dependent manner. Inputs from multiple hormones can cause/induce alterations in transcript accumulation of mt-related nuclear genes, which in turn trigger the expression of mt genes.

4. Materials and Methods

4.1. Plant material, growth conditions, and hormone treatment

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia 0 and *rpotmp-2* (NASC N6328420; Salk 132842) were used in our experiments. *RPOTmp::GUS* and *35S::GUS* were generated in this work. The seeds were soaked in a 30% bleach solution for 10 min, rinsed 3 times with sterilized water and stratified for 2 days in darkness. Seedlings were grown in half strength Murashige and Skoog (MS) medium (pH 5.7) containing 1% sucrose and 0.5% phyto agar (Duchefa Biochemie) in a growth chamber at 22°C with a 16-h light/8-h dark cycle. Ten-day-old seedlings were treated with the hormone solutions and collected after 3 h of exposure, unless otherwise stated. The compounds assayed included abscisic acid (ABA, 5×10^{-5} M), gibberellic acid (GA_3, 10^{-6} M), indole-3-acetic acid (IAA, auxin, 10^{-6} M), 1-aminocyclopropane-1-carboxylic acid (ACC; ethylene precursor 10^{-5} M), *trans*-zeatin (CK; 5×10^{-6} M), brassinolide (BL, 10^{-7} M), salicylic acid (SA, 10^{-5} M) and methyl jasmonate (MJ, 5×10^{-5} M), in addition to a mock treatment. The concentrations of active reagents and treatment time were selected in preliminary experiments. The effectiveness of hormonal treatment was confirmed by expression analysis of marker genes specific for each hormone.

For tests with mature plants, the seedlings were transferred into the soil and grown until the age of 5 weeks. CK-dependent effects were also studied in a model system designed by Cortleven et al. [30]. Seeds were germinated in the dark for 4 days on full MS medium with or without cytokinin (1 μM *trans*-zeatin).

4.2. Hormone sensitivity assays

For the seed germination assay, seeds were grown on MS medium supplemented with different concentrations (0, 1, 2, 3 or 6 μM) of ABA at 22°C with a 16-h light/8-h dark photoperiod. The number of fully expanded cotyledons was estimated after 6 days in triplicate of 50 seeds for each experiment.

For the root elongation assay, 10-day-old seedlings were transferred to ½ MS plates with different concentrations of phytohormones and grown vertically for another 4 days under 16 h light/8 h dark conditions. Measurements were performed in triplicate with 20 seedlings for each experiment.

A chlorophyll retention assay was performed with the 5th and 6th rosette leaves excised from the soil-grown plants. The leaves were placed on filters moistened with hormone solutions or water with a solvent and kept for 3 days in darkness. The chlorophyll content (chlorophyll *a*+ chlorophyll *b*) was measured as described by Lichtenthaler [31] and was related to the leaf area (mg/cm^2). Three samples

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each containing 3 leaf discs were measured for each test. The chlorophyll content at the start of the experiment was taken as a reference and set at 100%.

4.3. RNA extraction and quantitative RT–PCR (qRT–PCR)

Total RNA was isolated from hormone-treated and control 10-d-old seedlings using the TRIzol (Thermo Fisher Scientific) method. DNA contamination was removed with DNAse I (Thermo Fisher Scientific) treatment. cDNA synthesis and RT–PCR were performed using a LightCycler 96 (Roche, Switzerland) with the hot start SYBR Green I technology and gene-specific primers as described previously [32]. The nucleotide sequences of primers for qRT–PCR analysis are given in **Table S4**. The qPCR program included initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 25 s and melting curve analysis. *UBQ10* (*At5g53300*) and protein phosphatase 2A (PP2A) regulatory subunit A2 (*At3g25800*) genes were used as internal controls for qRT–PCR data normalization.

4.4. Prediction of cis-acting elements in the RpoTmp promoter

Using the bioinformatics resources of the Arabidopsis Information Resource (TAIR; https://www.arabidopsis.org/), the promoter sequence of *RPOTmp* (1,200 bp upstream of the translation initiation site ATG) was obtained. The *cis*-acting elements were predicted using Plant *cis*-acting regulatory DNA elements (PLACE, http://www.dna.affrc.go.jp/htdocs/PLACE/) [33]; Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [34]; PlantRegMap/PlantTFDB v5.0 (http://plantregmap.gao-lab.org/binding_site_prediction.php) [35]; and the Arabidopsis Gene Regulatory Information Server (AGRIS) (https://agris-knowledgebase.org) [36] databases. The gene transcription start site (TSS) and promoter region were indicated using the TSSPlant and TSS tools PlantProm DB database [37]. Nucleotide sequence analysis, primer design and localization of regulatory elements were performed with Vector NTI Advance 9.0 (Invitrogen) and visualized (**Figure S**1). The results of the search obtained with PlantRegMap are summarized in Supplementary **Table S3.**

4.5. Construction of the RPOTmp promoter::GUS reporter gene fusion and Agrobacterium transformation

Arabidopsis genomic DNA was extracted using the CTAB method. A–958 bp (–958/–1) upstream of the translation start codon of *RPOTmp* was amplified from the genomic DNA by polymerase chain reaction (PCR) using a single reverse primer and different forward primers carrying PstI and BamHI restriction sites, respectively. The primers used for plasmid construction and sequencing are listed in **Table S4**. The resulting BamHI/PstI fragment was cloned upstream of the promoterless *uidA* reporter gene (*GUS*) of the binary vector pCambia-1381Z (Clontech, USA), producing pCambia *RPOTmp pro*::*GUS* constructs, namely, P336, P530 and P958. After sequencing to exclude possible point mutations, the resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101 (C58) (GoldBio, USA) using the freeze–thaw method [38]. Positively transformed *A. tumefaciens* cells resistant to kanamycin (50 μg/μL) and rifampicin (100 μg/μL) were selected on LB plates at 28°C for two days.

4.6. Generation of RPOTmp pro::GUS Arabidopsis transgenic plants

A. tumefaciens cells harboring the P958 plasmids were used to transfect 4- to 5-week-old, wild-type Arabidopsis plants in the Col-0 background by the floral dip method in the presence of 5% sucrose and 0.01% (v/v) Silwet L-77, as previously described by Clough and Bent [39]. The original genetic constructs of pCambia1381Z and pCambia1301-35S (Clontech, USA) were used to generate negative and positive control plants, respectively, based on GUS activity. T1 transgenic seeds from each transformant plant were tested for germination on half MS medium containing 0.8% (w/v) phyto agar (Duchefa Biochemie) and 30 mg/L hygromycin (Hyg). The Hyg-resistant seedlings were then grown in soil or on ½ MS medium at 22°C under long-day conditions for further analysis. (**Figure S2**). To improve the integration of the P958 construct into Col-0 plants, genomic DNA was extracted

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from rosette leaves of transgenic T1 plants and used for PCR analysis with *GUS*-specific primers (**Figure S4**). The T2 and T3 generations were used for the subsequent analysis of GUS activity.

4.7. GUS staining

GUS activity was assayed in *Hyg*-resistant P958 seedlings after 24 hours of incubation in half MS supplemented with appropriate phytohormone or without (control) at 22°C with a 16-h day light. All treatments were performed in similar time and daylight periods. Seedlings of the *pCambia1381Z* and *pCambia1301-35* transgenic lines were used as control plants. GUS staining was performed according to Gallagher [40]. Samples were collected and immediately fixed in ice-cold acetone (90%) for 30 min at 4°C in the dark, rinsed 2 times for 15 min with 50 mM phosphate buffer (pH 7.0), and immersed in a GUS assay solution containing 0.5 mM X-Gluc (5-bromo-4-chloro-3-indoly-β-D-GlcA) in 50 mM phosphate buffer, 0.1% (v/v) Triton X-100, 1 mM EDTA, 0.5 mM potassium ferrocyanide and 0.5 mM potassium ferricyanide. The staining was carried out between 4 and 16 h at 37°C. GUS-colored tissues were washed gradually with 25 to 75% (v/v) ethanol to remove chlorophyll. The results were recorded photographically using a stereomicroscope MSP-2 (LOMO, Russia).

4.8. Statistical Data Processing

All experiments were performed in three biological replicates. The data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's method using an online calculator (astatsa.com/OneWay_Anova_with_Tukey HSD/). All data are presented as the means ± their standard errors (SE).

5. Conclusions

Dynamic communication between the nucleus and mitochondria requires acute coordination of various aspects of their activities in which phytohormones play a vital role. This process is predominantly regulated by the nuclear genome, as over the course of symbiotic evolution, the majority of mitochondrial genes migrated into the nuclear genome, leaving a set of essential genes that encode the subunits of the respiratory chain, heme and cytochrome assembly, and mitochondrial ribosomes. Here, we have shown that the hormone-related expression of the mitochondrial genome is at least in part regulated *via* the genes encoding nuclear-encoded RNA polymerases ROTmp and RPOTm, as well as MTERF and SWIB family members. According to our RT–PCR tests, these genes are reproducibly induced by CK, IAA, ACC, and BL and downregulated by ABA in WT plants, modulating the transcript accumulation of mt genes in a context-dependent manner. Disruption of *RPOTmp* blocks or attenuates hormone-dependent responses of mt-encoded genes and alters the sensitivity of the *rpotmp-2* mutant to hormone treatment. It should be noted, however, that the mutation may induce a hormonal imbalance in concerted hormonal synthesis and signaling and, as a result, a differential mitochondrial response to hormonal treatment. *In silico* analysis highlighted a number of consensus sequences in the regions of the selected nuclear-encoded mt genes recognized by *cis*-regulatory elements that may be involved in response to phytohormones. Moreover, the functional properties of the potential motifs were verified with a construct possessing the *GUS* gene fused to the -958 bp promoter fragment of the *RPOTmp* gene. GUS activity in the *RPOTmp::GUS* reporter strain was upregulated in response to IAA and ACC and downregulated by ABA. In parallel, hormone-related changes in the transcriptional activity of mt genes may be modulated indirectly, suggesting that additional factors are needed for their regulation.

Supplementary Materials: The following supporting information can be downloaded at Preprints.org.

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