NRC Publications Archive Archives des publications du CNRC

Identification and characterization of interactions between abscisic acid and mitochondrial adenine nucleotide translocators

Kharenko, Olesya A.; Boyd, Jason; Nelson, Ken M.; Abrams, Suzanne R.; Loewen, Michele C.

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.

For the publisher's version, please access the DOI link below. / Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:

<https://doi.org/10.1042/BJ20101898>

Biochemical Journal, 437, pp. 117-123, 2011-04-07

NRC Publications Record / Notice d'Archives des publications de CNRC:

<https://nrc-publications.canada.ca/eng/view/object/?id=b68989bf-3d2f-4402-929d-00525b5e250d>

<https://publications-cnrc.canada.ca/fra/voir/objet/?id=b68989bf-3d2f-4402-929d-00525b5e250d>

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at

<https://nrc-publications.canada.ca/eng/copyright>

READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site

<https://publications-cnrc.canada.ca/fra/droits>

LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at

PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

Identification and characterization of interactions between abscisic acid and mitochondrial adenine nucleotide translocators

Olesya A. KHARENKO*, Jason BOYD*, Ken M. NELSON*, Suzanne R. ABRAMS*,[†], Michele C. LOEWEN*,^{‡,1}

* Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK., S7N 0W9, [†] Department of Chemistry & [‡] Department of Biochemistry, University of Saskatchewan.

¹ To whom correspondence should be addressed (michele.loewen@nrc.ca)

Abbreviations: Absciscic Acid, ABA; Adenine Nucleotide translocator, ANT; mitochondrial ATP synthase, ATPase;

SYNOPSIS

Abscisic acid (ABA) is a plant hormone involved in important processes including development and stress responses. Recent reports have identified a number of plant ABA receptors and transporters, highlighting novel mechanisms of ABA action. Herein we describe application of a chemical proteomics approach leading to the identification of mitochondrial adenine nucleotide translocators (ANTs) as ABA-interacting proteins. Initial *in vitro* studies confirmed inhibition of ANT-dependent ATP translocation by ABA. Further analysis demonstrated ANT-dependent uptake of ABA into both recombinant *Arabidopsis thaliana* ANT2-containing proteoliposomes and native isolated spinach mitochondria; the latter with a K_m of 3.5 μM and a V_{max} of 2.5 nmol/min/g protein. ATP was found to inhibit ANT-dependent ABA-translocation. Specificity profiles highlight the possibility of mechanistic differences in translocation of ABA and ATP. Finally, ABA was shown to stimulate ATPase activity in spinach mitochondrial extracts. ABA concentrations in plant cells are estimated to reach the low μM range during stress responses, supporting potential physiological relevance of these *in vitro* findings. Overall, this *in vitro* work suggests the possibility of as yet uncharacterized mechanisms of ABA action *in planta* related to inhibition of mitochondrial ATP-translocation and functional localization of ABA in the mitochondrial matrix.

Key words: phytohormone, mitochondria, adenine nucleotide translocator, inhibition, uptake

INTRODUCTION

Abscisic acid (ABA) is a plant hormone known to control many physiological responses including germination, stomata opening, desiccation tolerance, seed maturation, dormancy, growth, lateral root formation and modulation of plant responses to environmental stress [1]. This breadth of functionality suggests multiple modulatory mechanisms, a concept that is supported by the increasing number of reports highlighting ABA interacting/receptor proteins. In particular, a family of 14 START domain proteins (RCAR/PYR/PYL) [2, 3], two homologous GPCR-Type G-proteins: GTG1 and GTG2 [4] and the magnesium-protoporphyrin IX chelatase large subunit (CHLH) [5] have been shown to function as ABA receptors. These reports demonstrate that ABA signals through both extracellular and intracellular mechanisms and that these receptors are responsible for mediating at least some of the ABA associated physiological responses. At the same time, two ABC transport proteins were recently shown to mediate controlled import and export of ABA across cell membranes [6, 7]. This highlights a novel mechanism involving protein catalyzed ABA translocation and emphasizes that passive diffusion of ABA through membranes is likely not the primary mode of ABA translocation [6].

Now, in this report, through application of a chemical proteomics approach [8, 9] mitochondrial adenine nucleotide translocators (ANTs) as well as the F_1 -ATP synthase β subunit are identified as putative ABA-binding proteins. F_1 -ATP synthase β subunit is one of five subunits (α , β , γ , δ and ϵ) comprising the matrix localized F_1 domain of the plant mitochondrial H^+ driven F_1F_0 -ATP synthase complex [10, 11]. The β subunit, in particular contains the majority of the catalytic site residues comprising the active site where ATP is synthesized from ADP and P_i . ANT s on the other hand contribute to regulation of the rate of ATP-synthesis by performing ATP/ADP exchange across the mitochondrial inner membrane [12]. ANT s display a significant flexibility of nucleotide binding, and despite a recent crystal structure, the mechanism of translocation remains enigmatic [13]. The physiological importance of ANT s is highlighted by the observation that decreases in ANT activity leads to the inhibition of cellular metabolism [14].

The concept of a functional linkage between ABA and mitochondria functionalities is supported by a limited number of other reports. In one instance ABA was shown to protect the mitochondria of maize seedling from irreversible damage during chilling by inducing expression of antioxidant enzymes [15]. In another report the gene corresponding to the *Arabidopsis thaliana* (L.) Heynh. mitochondrial adenine nucleotide translocator 1 (ANT1, At3g08580, also known as AAC1) was significantly induced in response to ABA [16]. However, there are other reports linking mitochondrial function to regulation of plant bioenergetics during stress responses [17, 18]; and conversely, mitochondrial functionality and related ATP supply have been shown to be affected by abiotic stressors [19-22]. As well studies have implicated roles specifically for some ANT s and ATPases in these stress responses. For example, expression of the gene encoding a mitochondrial 6 kDa ATPase subunit was significantly induced in response to abiotic stress, while expression of mitochondrial ATP synthase α and β subunits were decreased in response to oxidative stress [23, 24]. In another report, activity of the mitochondrial F_1F_0 ATPase in aluminum-resistant wheat was induced in response to aluminum stress [25].

These studies linking ABA and stress and mitochondrial function, highlight associated mechanisms regulated primarily at the gene expression level. In contrast to this, through applying an *in vitro* system that precludes the possibility of regulation by gene expression, we demonstrate that ABA is transported by mitochondrial ANT s . We show that ABA directly inhibits ANT-mediated ATP translocation and vice versa. We demonstrate unique specificity

profiles that highlight the possibility of different binding sites and/or mechanisms of translocation for ABA compared to ATP. Finally we show that ABA can act as an activator of the matrix localized mitochondrial ATP synthesizing machinery. Together these findings support the novel possibility of functional modulation of mitochondrial proteins by direct interaction with ABA.

EXPERIMENTAL

Materials

All materials were from Sigma-Aldrich (Sigma-Aldrich, Oakville, Ontario) unless otherwise indicated. The desalting column (PD-10), HiTrap streptavidin column, Streptavidin-HRP conjugate, ECL biotinylated protein markers and ECLplus Western Blotting Detection Reagents and [³H] (±) ABA were all obtained from Amersham Biosciences, Baie d'Urfe, Quebec. (+) ABA was prepared as described previously [26]. PBI 686, PBI 410, and other ABA analogues were synthesized according to Nyangulu et al. [8, 27]. [³H] ATP was obtained from Sigma-Aldrich (Sigma-Aldrich, St.Louis, MO, USA). All plotted values are means with standard deviations.

Preparation of total cell protein extracts from *B. napus* cell cultures.

The microspore-derived suspension cell culture of *Brassica napus* L. cv. Jet Neuf was propagated for two weeks and maintained on NLN medium containing 6% sucrose [28, 29]. The cultures were induced with 10 µM ABA for 24 hours. Freshly harvested cells (100-200 g) were washed with de-ionized water and homogenized in a blender in the presence of 400-500 ml ice-cold homogenization buffer (0.33 M sucrose, 40 mM ascorbate, 20 mM EDTA, protease inhibitor cocktail (CompleteTM Roche, Mannheim, Germany), 0.1% n-octyl-β-D-glucopyranoside ((OG) Affymetrix, Santa Clara, CA), 2 mM dithiothreitol, 100 mM sodium phosphate buffer, pH 7.5) and one third of the total volume acid-washed glass beads (500 micron). Homogenates were centrifuged at 1,000 g for 10-15 min, the supernatant was collected and proteins were concentrated by precipitation with 75 % ammonium sulfate at 4 °C. The precipitated proteins were centrifuged at 10,000 g for 15 min and re-dissolved in 10-20 ml of 20 mM phosphate buffer (pH 7.6) containing 6 mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Excess ammonium sulfate was removed by application to a desalting column (PD-10) with buffer-exchange to 20 mM sodium phosphate, 150 mM NaCl, pH 7.5.

Chemical proteomic identification of putative ABA binding proteins

The resulting protein extract sample was transferred to Pyrex tubes and mixed with 10 µM PBI686, a photoactivable, biotinylated ABA mimetic probe [8]. The resulting samples were UV irradiated as described previously [8, 9]. Samples treated with UV but no PBI686, or with no PBI686 and no UV were used as negative controls. Tagged proteins were enriched by affinity chromatography using a HiTrap streptavidin column (1-3 ml matrix volume) and AKTATM Explorer FPLC system. The column was equilibrated with running buffer (20 mM sodium

phosphate, 150 mM NaCl, pH 7.5). Protein solutions were injected at a flow rate of 0.3 ml/min, washed with the running buffer for 20 column volumes and eluted with a gradient of 8M Guanidine-HCl at pH 2 (1 ml/min, 30 ml total). Collected fractions were desalted with the PD-10 desalting columns into 20 mM sodium phosphate, 0.15 M NaCl, pH 7.5, concentrated using AmiconTM Ultrafree centrifugal filters (Millipore, Carrigtwohill, Ireland). Protein samples were separated by application to 10 % acrylamide SDS-PAGE gels according to the method of Laemmli [30]. For Western blot analysis, after gel electrophoresis, the proteins were transferred to PVDF membrane for 1 hour at 100 V, blocked in a phosphate buffered saline solution (pH 7.5) containing 0.1 % v/v Tween (PBS-T) with 3 % w/v bovine serum albumin and further incubated with Streptavidin-HRP conjugate diluted 1:1000 in PBS-T for 1h at room temperature. ECLplus Western Blotting Detection Reagents were applied to the membrane and luminescence detected using a Storm Scanner (Amersham Biosciences, Baie d'Urfe, Quebec) system. ECL biotinylated protein marker was used as molecular weight reference.

Alternatively, gels were stained using FOCUS-FAST silver-stain kit (Genotech, St Louis, MO, USA) according to the manufacturer protocol. Protein bands were excised and placed in a 96-well microtiter plate (Sigma, Milwaukee WI). The resulting gel pieces were automatically de-stained, reduced with DTT, alkylated with iodoacetamide, and digested with porcine trypsin (sequencing grade, Promega, Madison WI) using a MassPREP protein digest station and recommended procedures (Waters, Manchester, UK). Peptides from tryptic digestion were analyzed using a capLC ternary HPLC system (Waters, Milford, MA) coupled to a Q-ToF Ultima Global (Waters, Manchester, UK). The method used for separation of the peptide digest samples and subsequent analysis using LC-MS/MS and Data Dependent Acquisition (DDA) has been described previously [31]. The LC/MS/MS data were processed using ProteinLynx software (Waters) and searched against databases using MASCOT Daemon and Mascot MS/MS ion search performed on a MASCOT server hosted by IBS-NRC (Ottawa, Canada).

Proteoliposome transport assays

Recombinant expression and purification of *A. thaliana* ANT2 (ANT2; At5g13490) was performed as described previously [32]. [³H] ATP transport assays were carried out based on the protocols in Genchi *et al.*, Palmieri *et al.*, and Thuswaldner *et al.*, as described in Kharenko *et al.*, with minor modifications for measurement of uptake of [³H] (±) ABA (DL-*cis*, *trans*-[G-³H] abscisic acid) as follows [17, 32-34]. Liposomes were preloaded internally with 20 mM (+) ABA instead of ATP, to insure that ABA transport would not be influenced by the presence of ATP. Transport was initiated by the addition of 100 nM of [³H] (±) ABA. Each experimental point corresponds to the average of six replicates. Externally added substrates (inhibitor, nucleotides and ABA analogues) were added to a final concentration of 200 μM together with [³H] substrate. Transport for these latter experiments was allowed to proceed at room temperature for 10 min and then terminated, with each data point representing the average of four experiments.

Mitochondrial transport assays

Mitochondria were extracted from spinach-leaf as described in Hamsur *et al.*[35]. Uptake of [³H] ATP or [³H] (±) ABA into mitochondria was characterized by the method of LaNoue, K.F., *et al.* [36]. Assays were initiated with the addition of 100 nM [³H] ATP or 188 nM [³H] (±) ABA to spinach mitochondria (50 μg/reaction) and terminated with the addition of the ANT specific

inhibitor pyridoxal 5'-phosphate. Each point represents the average of five replicates. To estimate the kinetic parameters for [³H] (±) ABA uptake, mitochondria were incubated with increasing concentrations of [³H] (±) ABA (as indicated) and transport terminated by the addition of pyridoxal 5'-phosphate after 2 minutes. Each resulting time course curve was fitted to first order kinetics using Origin software (OriginLab, Northampton, Massachusetts) from which a rate was obtained for the given concentration of ATP. Kinetic values were obtained from these rates by Michaelis–Menten analysis using ENZFITTER software (Biosoft, Cambridge, UK). Efficiency of translocation was calculated as k_{cat}/K_m , where k_{cat} is defined as $V_{max}/[\text{active site}]$ with [active site] assumed to be the same for both ABA and ATP uptake experiments. External substrates (inhibitor, nucleotides or ABA analogues) were added to a final concentration of either 200 or 400 μM for ATP and ABA uptake experiments respectively, with either 100 nM [³H] ATP or 400 nM [³H] (±) ABA respectively. Uptake was allowed to proceed for 5 min. Each point represents the average of three separate experiments.

F₁ ATPase assays.

Quantification of F₁ ATPase activity was performed as described previously using an enzyme coupled reaction and monitoring the disappearance of NADH by following absorbance at 340 nm [37, 38]. F₁ ATPase assays were performed in triplicate in 200 μL volumes and were composed of 65 mM MOPS-KOH pH 8.0, 2.5 mM MgCl₂, 20 mM KCl, 100 μM KCN, 134 μM NADH, 25 mM KHCO₃, and 500 μM ATP. The reaction was initiated with the addition of 10 μg of mitochondrial preparation, 500 μM phosphoenolpyruvate (PEP), 3U pyruvate kinase (PK), 2.5U lactate dehydrogenase (LDH) and NADH oxidation was measured at 20 °C for 30 sec. Kinetic values were obtained by Michaelis–Menten analysis using ENZFITTER software (Biosoft, Cambridge, UK). ABA concentration values were converted to the negative reciprocal of their log in order to plot all points of the Michaelis-Menton curve. Basal F₁ ATPase activity (recorded in the absence of (+) ABA) was subtracted from the (+) ABA stimulated assays to account for any background activity. The specific ATPase inhibitors oligomycin (10 μM; [39]) and ouabain (50μM; [40]) were applied as controls.

RESULTS AND DISCUSSION

Identification of novel putative ABA-binding proteins

Novel putative ABA-binding proteins were identified through the application of a bioactive ABA-mimetic photoactivable probe, PBI686 (Figure 1) [8, 9]. In addition to a photoactivable crosslinking ABA-mimetic tetralone domain, PBI686 also contains a biotin tag. Following UV irradiation, the biotin tag allows crosslinked proteins to be detected by Western analysis with a streptavidin-HRP conjugate, as well as enriched by streptavidin affinity chromatography. The aromatic ring of the tetralone in PBI686 has previously been shown to not interfere with the recognition of the compound by ABA binding proteins [8]. Following incubation and UV irradiation of cell lysates of ABA-induced *B. napus* Jet Neuf cultures containing PBI686, crosslinked proteins were enriched by application to and elution from a Streptavidin Sepharose affinity column. Visualization of enriched fractions by Western blot using a streptavidin-HRP conjugate revealed four biotin-tagged bands at ~ 65, 55, 48 and 35 kDa respectively (Figure 2A,

left panels). The corresponding bands/regions on silver-stained gels (Figure 2, right panels) were subjected to tryptic digest and the resulting peptides analyzed by an LC/MS/MS-Q-TOF coupled Mascot MS/MS ion search (Table 1 and Supplemental Figures S1-S3). The 65 kDa gel region was found to contain predominantly peptides homologous to the *A. thaliana* mitochondrial F₁ ATP synthase β subunit (ATP2; At5g08690), while the ~48 kDa region was comprised of protein with homology to mitochondrial ANT protein. The 35 kDa region also contained peptides derived from homologs of ATP2. With an expected molecular weight of ~65 kDa, the presence of ATP2-like protein on the gel at ~35 kDa most likely represents a degradation product. These results were all shown to be reproducible. The ~55 kDa region did not yield reproducible MS data and is not addressed further in this report.

ABA inhibits ANT-dependent ATP translocation

Mitochondrial ANT_s mediate ATP/ADP exchange across the inner membranes of mitochondria (reviewed in [12]). Toward initial evaluation of the relationship between ABA and ANT_s, the relative effect of 2000-fold molar excess of ATP, ABA and analogues of ABA respectively on [³H] ATP uptake into isolated spinach mitochondria was assessed and plotted relative to values obtained for the known ANT inhibitor, pyridoxal 5' phosphate [17, 32-34, 41]. ATP led to ~140 % relative inhibition (Figure 3, Supplemental Table S1). This observation of greater than 100% relative inhibition is most likely explained by the presence of mitochondrial ANT_s (or other translocators) in the native mitochondria that are less sensitive or insensitive to pyridoxal 5'-phosphate. (+)-ABA inhibited [³H] ATP uptake by ~ 60 %, while corresponding amounts of ABA analogs, including (-)-ABA and the non-biologically relevant (+)-trans-ABA showed very low relative inhibition (< 35%), emphasizing specificity of the biologically relevant form. In order to address the high standard deviations and high relative inhibitions, a purified reconstituted, recombinant *A. thaliana* ANT2 proteoliposome system was subsequently applied [32]. While deviations were still high in some instances, similar trends were observed (Figure 3, Supplemental Table S2). 2000-fold molar excesses of ATP showed the highest relative inhibition at ~ 70%, with (+)-ABA showing 60 % and related analogs < 50%. While not assessed for mitochondria, PBI686 and related (+/-)-tetralone PBI410 showed relative inhibition comparable to ATP and ABA in the proteoliposome assays. Overall these results show that ABA inhibits ANT-dependent translocation of ATP and that the inhibition is relatively specific for ABA. Unsuccessful attempts at determining a simple binding constant for purified ANT2-[³H] ABA interaction suggested that the effect of ABA on ATP-translocation might be the result of competition for translocation.

ABA is translocated by ANT_s

Using the reconstituted ANT2 proteoliposome system the ability of ANT2 to translocate ABA was assessed. With proteoliposomes preloaded with 20 mM (+)-ABA, external 100 nM [³H] ($\pm$) ABA was taken up, saturating at approximately 0.4 nmol [³H] ABA / g protein, in about 300 seconds (Supplemental Figure S4). This is comparable to values observed previously for ATP uptake in the same system (8 nmol [³H] ATP / g protein starting with 5 μ M external [³H] ATP) and highlight that ANT2 can specifically translocate ABA [32]. Toward biological relevance, and efficiency of preparation, we subsequently shifted back to spinach mitochondria for comparative quantitation of translocation. Spinach mitochondria were used because of the

intrinsically limited availability of *A. thaliana* leaf tissue for mitochondria preparation. [³H] (±) ABA (188 nM external concentration) uptake saturated at 0.3 nmol / g protein in approximately 300 seconds (Figure 4A). A Michaelis-Menten analysis of rates at increasing external [³H] ABA concentrations yielded a K_m of 3.5 μ M and V_{max} of 2.5 nmol / min / g protein (Figure 4B). For comparison values for [³H] ATP uptake were determined. Uptake was found to saturate at ~0.3 nmol [³H] ATP / g protein in less than 200 seconds (100 nM external [³H] ATP; Figure 4C), while a Michaelis-Menten analysis revealed a K_m of 0.4 μ M and V_{max} of 1.2 nmol / min / g protein (Figure 4D). These values for ATP uptake into mitochondria are in agreement with previously documented values [34, 42], and are somewhat lower than those observed for ABA. A comparison of the efficiency of translocation (see methods for a description of this calculation), suggests ABA is transported by mitochondrial ANT_s at one third the rate of ATP. Similarly, kinetic parameters reported for ABC transporter-catalyzed ABA uptake across cell membranes (K_m s ranging from 0.25 – 1.0 μ M and a V_{max} of 6.9 nmol / min / g protein) also highlight similar kinetic values compared to ANT_s [6, 7]. Documented ABA concentrations inside of guard cells as high as 13 μ M under stress conditions (reviewed in [43]), support the physiological relevance of the low μ M range K_m s for ANT-dependent ABA uptake. Overall, these findings emphasize that the mechanism of ABA-mediated inhibition of ATP-translocation is likely competition for translocation. At the same time, these results also raise the novel possibility that ABA may play a role inside the mitochondrial matrix.

ATP inhibits ANT-dependent ABA translocation

The specificity [³H] ABA uptake into mitochondria was assessed in the presence of 1000-fold molar excess of externally added ATP, ABA or ABA analogs and plotted relative to values obtained for the known ANT inhibitor, pyridoxal 5' phosphate [17, 32-34, 41]. (+) ABA inhibited uptake at a level comparable to pyridoxal 5'-phosphate, whereas ABA analogues caused only 50 % relative inhibition or less (Figure 5, Supplemental Table S3). (+)-trans-ABA showed very little effect again emphasizing specificity of the biologically relevant form. ATP was found to be a very potent inhibitor of ABA uptake. Again to address the high standard deviations of these mitochondrial results, specificities were further probed in the ANT2 reconstituted proteoliposomes system. [³H] ABA uptake was measured in the presence of 2000-fold molar excess of an expanded repertoire of substrates. Standard deviations were again still high in some instances, but similar results to those for mitochondrial uptake were obtained, supporting the general trends. (+) ABA was found to inhibit uptake at levels comparable to pyridoxal 5'-phosphate, and ABA analogues including the PBI686 and PBI410 caused only 50 % relative inhibition or less (Figure 5, Supplemental Table S4). ATP and most of the other tested nucleotides were found to be very potent inhibitors of ABA uptake. The observation that UTP and GTP show the highest levels of inhibition of ABA uptake is noteworthy in that these particular nucleotides act as only very weak inhibitors of ATP translocation [32]. This opposite effect is true also for PBI686 and PBI410, shown here to be only weak inhibitors of ABA uptake and relatively strong inhibitors of ATP uptake (Figure 5). These reciprocal activity relationships suggest the possibility of differences in binding/translocation sites and/or specific mechanisms of ANT translocation for ATP and ABA. The consistently observed potency of ATP as an inhibitor of ABA uptake is in agreement with the relative efficiency of translocation of ATP compared to ABA observed in kinetic analyses.

ABA modulates mitochondrial ATPase activity.

The possibility of uptake of ABA into mitochondrial matrix raises the question of the biological relevance of having ABA in the matrix. Toward addressing this and based on the identified interaction of the PBI686 probe with the catalytic β subunit of the F_1 -ATP synthase domain (Figure 2, Table 1), we assessed the effect of ABA on mitochondrial ATPase activity [37, 38]. By way of controls, oligomycin, a H^+ ATPase specific inhibitor was applied and found to reduce ATPase activity by ~50%. At the same time the Na^+/K^+ ATPase specific inhibitor ouabain was applied and found to have no effect, demonstrating that the measured ATPase activity is not arising from Na^+/K^+ driven ATPases. Upon application of ABA, a stimulation of the ATPase activity in isolated spinach mitochondrial extracts was observed, even at concentrations of ABA in the low pM range (Supplemental Figure S5). However, while kinetic analysis yielded a K_a of 1.56 μ M, only a 2-fold stimulation of activity was observed over the pM to mM range of ABA concentrations tested (Figure 6). Thus the K_a value is presented here only as an approximation.

In the context of an activating molecule, this limited concentration dependent stimulation of the ATPase activity could be considered an almost negligible effect, and we do not dismiss the possibility that biologically this observed stimulation of ATPase is not relevant, or only of secondary importance to the effect of ABA on modulation of ATP translocation. However, energetically, a 2 fold increase in ATPase activity is fairly significant and this stimulation is observed even at low pM concentrations of ABA. Physiologically, cytosolic ABA concentrations transiently reach a maximum of approximately 5-10 μ M under stress conditions, which correlates with the K_m of 3.5 μ M determined for ABA translocation into mitochondria. More speculatively, taking the demonstrated relative specificity of the ANT_s for ABA and ATP into consideration, concentrations of ABA accumulating in the mitochondrial matrix would not likely be very high; potentially on the order of pM to nM at best, correlating with the effect of pM ABA on ATPase activity described above. In further support of the general concept of ABA modulating membrane pumps, the β subunit of the chloroplast ATP synthase complex was also recently identified, although not characterized, as a putative ABA binding protein [9]. At the same time other reports have shown that ABA induces depolarization of the plasma membrane via the inhibition of the plasma membrane pumps in *A. thaliana* cell culture [44]. It was also shown that ABA has direct and indirect effects on plasma membrane and vacuolar H^+ -ATPases in response to salt stress [45, 46]. Overall, while not conclusive regarding biological relevance, the ABA-dependent ATPase stimulation demonstrated here does validate the initial interaction observed in the pull-down of F_1 -ATP synthase β subunit using PBI686 and suggests one possibility for a role for ABA inside the mitochondria.

The work described herein was completed entirely *in vitro* specifically to preclude the possibility of competing gene regulation effects. Indeed two earlier reports highlight gene regulation as an important mechanism of ABA action related to mitochondrial modulation. In one instance ABA was shown to stimulate mitochondrial activity through induction of the expression of antioxidant enzymes, while the second report highlights ABA gene induction of mitochondrial ANT1 [15, 16]. Less relevant, but in the same vein, a third study showed ABA to significantly increase expression of chloroplast ATP-synthase CF_1 α subunit [38]. Taking these

reports together with the findings described herein, we conclude that ABA potentially regulates mitochondrial functionality through a number of different mechanisms; including gene regulation as well as mechanisms based on direct interaction of ABA with ANT_s and possibly mitochondrial matrix localized proteins.

In Conclusion

In conclusion the *in vitro* results presented here highlight novel interactions between ABA and mitochondrial proteins, suggesting the possibility of as yet uncharacterized mechanisms of ABA action *in planta*. Whether the translocation of ABA by ANT_s is primarily directed at inhibiting ATP translocation, or toward enabling interactions with mitochondrial matrix localized proteins, or both, remains to be determined.

AUTHOR CONTRIBUTIONS

Olesya Kharenko designed and performed the ANT related experiments, analyzed the data and wrote the paper. Jason Boyd designed and performed the ATPase related experiments and analyzed the data. Ken Nelson synthesized and purified all ABA and related analogs. Sue Abrams conceived the idea. Michele Loewen designed experiments, analyzed and interpreted the data and wrote the paper.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. Andrew Ross and the Mass Spectroscopy facility of the National Research Council for mass spectrometry analysis, Dr. Michelle Alting-Meese and Dr. Marek Galka for discussions and Dr.'s Peter Loewen (U Manitoba), Pierre Fobert and Adrian Cutler (PBI/NRC) for critical reading of the manuscript. This manuscript represents NRCC# 50170.

FUNDING

This work was funded by a National Research Council of Canada - Genomics Health Initiative grant to M.C.L and S.R.A.

REFERENCES

- 1 Smet, I. D., Zhang, H., Inze, D. and Beeckman, T. (2006) A novel role for abscisic acid emerges from underground. *Trends Plant Sci.* **11**, 434-439
- 2 Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A. and Grill, E. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324**, 1064-1068
- 3 Park, S.-Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.-f. F., Alfred, S. E., Bonetta, D., Finkelstein, R., Provart, N. J., Desveaux,

- D., Rodriguez, P. L., McCourt, P., Zhu, J.-K., Schroeder, J. I., Volkman, B. F. and Cutler, S. R. (2009) Absciscic Acid Inhibits Type 2C Protein Phosphatases via the PYR/PYL Family of START Proteins. *Science* **324**, 1068-1071
- 4 Pandey, S., Nelson, D. C. and Assmann, S. M. (2009) Two Novel GPCR-Type G Proteins Are Absciscic Acid Receptors in Arabidopsis. *Cell* **136**, 136-148
- 5 Shang, Y., Yan, L., Liu, Z.-Q., Cao, Z., Mei, C., Xin, Q., Wu, F.-Q., Wang, X.-F., Du, S.-Y., Jiang, T., Zhang, X.-F., Zhao, R., Sun, H.-L., Liu, R., Yu, Y.-T. and Zhang, D.-P. (2010) The Mg-Chelatase H Subunit of Arabidopsis Antagonizes a Group of Transcription Repressors to Relieve ABA-Responsive Genes of Inhibition. *Plant Cell* **22**, 1909-1935
- 6 Kang, J., Hwang, J.-U., Lee, M., Kim, Y.-Y., Assmann, S. M., Martinoia, E. and Lee, Y. (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone absciscic acid. *Proc. Natl. Acad. Sci. U S A.* **107**, 2355-2360
- 7 Kuromori, T., Miyaji, T., Yabuuchi, H., Shimizu, H., Sugimoto, E., Kamiya, A., Moriyama, Y. and Shinozaki, K. (2010) ABC transporter AtABCG25 is involved in absciscic acid transport and responses. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 2361-2366
- 8 Nyangulu, J. M., Galka, M. M., Jadhav, A., Gai, Y., Graham, C. M., Nelson, K. M., Cutler, A. J., Taylor, D. C., Banowetz, G. M. and Abrams, S. R. (2005) An affinity probe for isolation of absciscic acid-binding proteins. *J. Am. Chem. Soc.* **127**, 1662-1664
- 9 Galka, M. M. (2009) Identification of Absciscic Acid Binding Proteins Using a Bioactive Photoaffinity Probe. In Department of Chemistry, University of Saskatchewan, Saskatoon
- 10 Devenish, R. J., Prescott, M. and Rodgers, A. J. (2008) The structure and function of mitochondrial F1F0-ATP synthases. *Int. Rev. Cell. Mol. Biol.* **267**, 1-58
- 11 Pedersen, P. L. (2007) Transport ATPases into the year 2008: a brief overview related to types, structures, functions and roles in health and disease. *J. Bioenerg. Biomembr.* **39**, 349-355
- 12 Klingenberg, M. (2008) The ADP and ATP transport in mitochondria and its carrier. *Biochim. Biophys. Acta* **1778**, 1978-2021
- 13 Nury, H., Dahout-Gonzalez, C., Trezeguet, V., Lauquin, G. J., Brandolin, G. and Pebay-Peyroula, E. (2006) Relations between structure and function of the mitochondrial ADP/ATP carrier. *Annu. Rev. Biochem.* **75**, 713-741
- 14 Neuhaus, H. E., Thom, E., Mohlmann, T., Steup, M. and Kampfenkel, K. (1997) Characterization of a novel eukaryotic ATP/ADP translocator located in the plastid envelope of Arabidopsis thaliana L. *Plant J.* **11**, 73-82
- 15 Prasad, T. K., Anderson, M. D. and Stewart, C. R. (1994) Acclimation, hydrogen peroxide, and absciscic acid protect mitochondria against irreversible chilling injury in maize seedlings. *Plant Physiol.* **105**, 619-627

- 16 Millar, A. H. and Heazlewood, J. L. (2003) Genomic and proteomic analysis of mitochondrial carrier proteins in Arabidopsis. *Plant Physiol.* **131**, 443-453
- 17 Palmieri, L., Santoro, A., Carrari, F., Blanco, E., Nunes-Nesi, A., Arrigoni, R., Genchi, F., Fernie, A. R. and Palmieri, F. (2008) Identification and characterization of ADNT1, a novel mitochondrial adenine nucleotide transporter from Arabidopsis. *Plant Physiol.* **148**, 1797-1808
- 18 Pastore, D., Trono, S., Laus, M. N., Fonzo, M. D. and Flagella, Z. (2007) Possible plant mitochondria involvement in cell adaptation to drought stress *J. Exp. Bot.* **58**, 195-210
- 19 Kaklewski, K., Nowak, J. and Ligocki, M. (2008) Effects of selenium content in green parts of plants on the amount of ATP and ascorbate-glutathione cycle enzyme activity at various growth stages of wheat and oilseed rape. *J. Plant Physiol.* **165**, 1011-1022
- 20 Kim, S.-H., Yang, S. H., Kim, T.-J., Han, J.-S. and Suh, J.-W. (2009) Hypertonic Stress Increased Extracellular ATP Levels and the Expression of Stress-Responsive Genes in Arabidopsis thaliana Seedlings. *Biosci. Biotech. Biochem.* **73**, 1252-1256
- 21 Sobczyk, E. and Kacperska-Palacz, A. (1979) Adenine Nucleotide Changes during Cold Acclimation of Winter Rape Plants. *Plant Physiol.* **62**, 875-878
- 22 Song, C. J., Steinebrunner, I., Wang, X., Stout, S. C. and Roux, S. J. (2006) Extracellular ATP Induces the Accumulation of Superoxide via NADPH Oxidases in Arabidopsis. *Plant Physiol.* **140**, 1222-1232
- 23 Sweetlove, L. J., Heazlewood, J. L., Herald, V., Holtzapffel, R., Day, D. A., Leaver, C. J. and Millar, A. H. (2002) The impact of oxidative stress on Arabidopsis mitochondria. *Plant J.* **32**, 891-904
- 24 Zhang, X., Takano, T. and Liu, S. (2006) Identification of a mitochondrial ATP synthase small subunit gene (RMtATP6) expressed in response to salts and osmotic stresses in rice (*Oryza sativa* L.). *J. Exp. Bot.* **57**, 193-200
- 25 Hamilton, C. A., Good, A. G. and Taylor, G. J. (2001) Induction of vacuolar ATPase and mitochondrial ATP synthase by aluminum in an aluminum-resistant cultivar of wheat. *Plant Physiol.* **125**, 2068-2077
- 26 Abrams, S. R., Reaney, M. J. T., Abrams, G. D., Mazurek, T., Shaw, A. C., Gusta, L. V. . (1989) Ratio of (S)- to (R)-abscisic acid from plant cell cultures supplied with racemic ABA. *Phytochem.* **28**, 2885-2889
- 27 Nyangulu, J. M., Nelson, K. M., Rose, P. A., Gai, Y., Loewen, M., Loughheed, B., Quail, J. W., Cutler, A. J. and Abrams, S. R. (2006) Synthesis and biological activity of tetralone abscisic acid analogues. *Org. Biomol. Chem.* **4**, 1400-1412
- 28 Lichter, R. (1981) Anther culture of Brassica napus in a liquid medium. *Z Pflanzenphysiol.* **103**, 229-237

- 29 Weselake, R., Byers, S., Davoren, J., Laroche, A., Hodges, D., Pomeroy, M. and Furukawa-Stoffer, T. (1998) Triacylglycerol biosynthesis and gene expression in microspore-derived cell suspension cultures of oilseed rape. *J. Exp. Bot.* **49**, 33-39
- 30 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
- 31 Sheoran, I. S., Olson, D. J., Ross, A. R. and Sawhney, V. K. (2005) Proteome analysis of embryo and endosperm from germinating tomato seeds. *Proteomics* **5**, 3752-3764
- 32 Kharenko, O. A. and Loewen, M. C. (2010) Characterization of the ATP-Translocating Properties of the Predicted Arabidopsis thaliana Mitochondrial Adenine Nucleotide Translocator 2. *Botany* **88**, 685-690
- 33 Genchi, G., Ponzone, C., Bisaccia, F., De Santis, A., Stefanizzi, L. and Palmieri, F. (1996) Purification and characterization of the reconstitutively active adenine nucleotide carrier from maize mitochondria. *Plant Physiol.* **112**, 845-851
- 34 Thuswaldner, S., Lagerstedt, J. O., Rojas-Stutz, M., Bouhidel, K., Der, C., Leborgne-Castel, N., Mishra, A., Marty, F., Schoefs, B., Adamska, I., Persson, B. L. and Spetea, C. (2007) Identification, expression, and functional analyses of a thylakoid ATP/ADP carrier from Arabidopsis. *J. Biol. Chem.* **282**, 8848-8859
- 35 Hamasur, B., Birgersson, U., Eriksson, A. and Glaser, E. (1990) Large-scale purification procedure of spinach leaf mitochondria —isolation and immunological studies of the F1–ATPase. *Physiologia Plantarum.* **78**, 367-373
- 36 LaNoue, K. F. and Schoolwerth, A. C. (1979) Metabolite transport in mitochondria. *Annu. Rev. Biochem.* **48**, 871-922
- 37 Fisher, R. J., Liang, A. M. and Sundstrom, G. C. (1981) Selective disaggregation of the H⁺-translocating ATPase. Isolation of two discrete complexes of the rutamycin-insensitive ATPase differing in mitochondrial membrane-binding properties. *J. Biol. Chem.* **256**, 707-715
- 38 Zhu, M., Simons, B., Zhu, N., Oppenheimer, D. G. and Chen, S. (2010) Analysis of abscisic acid responsive proteins in Brassica napus guard cells by multiplexed isobaric tagging. *J. Proteomics* **73**, 790-805
- 39 Devenish, R. J., Prescott, M., Boyle, G. M. and Nagley, P. (2000) The oligomycin axis of mitochondrial ATP synthase: OSCP and the proton channel. *J. Bioenerg. Biomembr.* **32**, 507-515
- 40 Lingrel, J. B. (2010) The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annu. Rev. Physiol.* **72**, 395-412
- 41 Bogner, W., Aquila, H. and Klingenberg, M. (1986) The transmembrane arrangement of the ADP/ATP carrier as elucidated by the lysine reagent pyridoxal 5-phosphate. *J. Biol. Chem.* **161**, 611-620

- 42 Weidemann, M. J., Erdelt, H. and Klingenberg, M. (1970) Adenine nucleotide translocation of mitochondria: identification of carrier sites. *Eur. J. Biochem.* **16**, 313-335
- 43 McCourt, P. and Creelman, R. (2008) The ABA receptors -- we report you decide. *Curr. Opin. Plant Biol.* **11**, 474-478
- 44 Brault, M., Zmiar, Z., Pennarun, A.-M., Monestiez, M., Zhang, Z., Cornel, D., Dellis, O., Knight, H., Bouteau, F. and Rona, J.-P. (2004) Plasma membrane depolarization induced by abscisic acid in *Arabidopsis* suspension cells involves reduction of proton pumping in addition to anion channel activation, which are both Ca^{2+} -dependent. *Plant Physiol.* **135**, 231-243
- 45 Fukuda, A. and Tanaka, Y. (2006) Effects of ABA, auxin, and gibberellin on the expression of genes for vacuolar H^{+} -inorganic pyrophosphatase, H^{+} -ATPase subunit A, and $\text{Na}^{+}/\text{H}^{+}$ antiporter in barley. *Plant Phys. Biochem.* **44**, 351-358
- 46 Janica-Russak, M. and Klobus, G. (2007) Modification of plasma membrane and vacuolar H^{+} -ATPase in response to NaCl and ABA. *J. Plant Phys.* **164**, 295-302

FIGURE LEGENDS

Figure 1 ABA and related ABA analogs

1. (+)-(S)-ABA, 2. 2-trans-ABA, 3. (-)-(R)-ABA, 4. (+)-PBI410, (+)-tetralone 5. (+)-PBI686 photoactive, bioactive ABA-mimetic biotinylated probe used to pull out putative ABA-binding proteins.

Figure 2 Streptavidin-affinity enriched PBI686-tagged protein extracts

Total protein extract, photocrosslinked with PBI686 was enriched using streptavidin sepharose affinity chromatography. Eluted proteins were desalted, concentrated and analyzed using far-Western blot with a streptavidin-HRP conjugate (left-panels) and silver-staining (right panels) techniques. Band regions that were excised are indicated with the symbol '] '.

Figure 3 Inhibition of ANT-mediated ATP translocation by ABA

Effect of ATP, ABA and ABA analogs on [^3H] ATP uptake into mitochondria (dark grey). Transport was initiated by the addition of 100 nM [^3H] ATP to mitochondria with 200 μM substrate or 20 mM pyridoxal 5'-phosphate. Values, mean with standard deviation ($n=3$), were plotted relative to inhibition by pyridoxal 5'-phosphate (set as 100% inhibition). Effect of external ABA and related substrates on [^3H] ATP uptake in proteoliposomes (light grey). ANT2 proteoliposomes contained 20 mM ATP. 100 nM [^3H] ATP was added with 200 μM of each of the indicated substrates at time zero. Mean values with standard deviation ($n=4$) are plotted relative to inhibition by pyridoxal 5'-phosphate (set as 100% inhibition).

Figure 4 ABA uptake into mitochondria.

A) Time-course of [^3H] ABA uptake into spinach mitochondria. Transport was initiated by adding 188 nM [^3H] ABA to 50 μg mitochondria and terminated by the addition of 20 mM pyridoxal 5'-phosphate. The difference plot was obtained by the subtraction of the corresponding values for control samples pretreated with inhibitor. Each point represents mean values ($n=5$; standard error 0.016; R-squared 0.991). **B)** Concentration dependence of [^3H] ABA uptake into spinach mitochondria. Transport was initiated by adding

the corresponding concentration of [³H] ABA to mitochondria. Mean values are plotted for each concentration tested (n=5; standard error 0.042; R-squared 0.986). The kinetic values were obtained using ENZFITTER software (Biosoft). **C)** Time-course of [³H] ATP uptake into spinach mitochondria. Transport was initiated by adding 100 nM [³H] ATP to mitochondria and terminated by the addition of 20 mM pyridoxal 5'-phosphate. The difference plot was obtained by subtraction of values obtained for control samples pretreated with inhibitor. Each point represents the mean (n=5; standard error 0.02; R-squared 0.974). **D)** Concentration dependence of [³H] ATP uptake into spinach mitochondria. Transport was initiated by the addition of [³H] ATP to mitochondria and terminated after 2 min by the addition of 20 mM pyridoxal 5'-phosphate. The difference plot was obtained by subtraction of values obtained for control samples pretreated with inhibitor. Mean values are plotted for each concentration (n=5; standard error 0.0196; R-squared 0.974). Kinetic values were obtained using ENZFITTER software (Biosoft).

Figure 5 Specificity of ABA translocation

Effect of ATP, ABA and ABA analogues on [³H] ABA uptake into mitochondria (dark grey). Transport was initiated by the addition of 400 nM [³H] ABA added together with 400 μ M substrates or 20 mM pyridoxal 5'-phosphate. Values represent means with standard deviation (n=3). Values, mean with standard deviation were plotted relative to inhibition by pyridoxal 5'-phosphate (set as 100% inhibition). Effect of ATP and ABA and their related analogs on [³H] ABA uptake into proteoliposomes (light grey). ANT2 proteoliposomes contained 20 mM ABA. Transport was terminated after 10 minutes. External substrates were added (final concentration 2.5 mM each) together with [³H] ABA (100 nM). Values, mean with standard deviation (n=4), were plotted relative to inhibition by pyridoxal 5'-phosphate (set as 100% inhibition).

Figure 6 ABA modulation of F₁-ATPase activity in mitochondrial extracts

Purified spinach mitochondrial extracts were assayed for F₁-ATPase activity in the presence of increasing concentrations of ABA (n=3). Basal activity in the absence of ABA was subtracted at all assayed time points to obtain activation rates for each ABA concentration tested. Michaelis-Menton analysis was used to evaluate the K_a of F₁-ATPase for ABA over concentrations ranging from pM to mM. ABA concentrations were plotted as the negative reciprocal of their log.

Table 1. Two Putative ABA-Binding Proteins Identified in *Brassica* Cell Lysates

Band/ Region	Protein Name	NCBI/Protein Data	Peptides Matched	Score
~65kDa	Mitochondrial F ₁ ATP Synthase β Subunit	gi 17939849	8	247 (Figure S1)
~48kDa	Mitochondrial ATP/ADP Carrier Protein	gi 944842	5	169 (Figure S2)
~35kDa	Mitochondrial F ₁ ATP Synthase β Subunit	gi 17939849	4	179 (Figure S3)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6