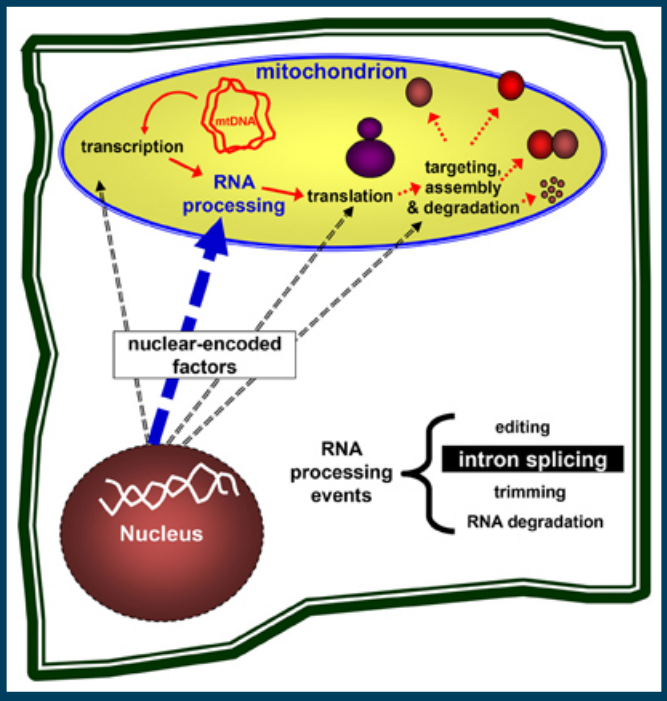
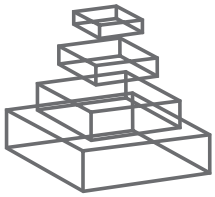
frontiers
RESEARCH TOPICS



BIOGENESIS OF THE OXIDATIVE PHOSPHORYLATION MACHINERY IN PLANTS. FROM GENE EXPRESSION TO COMPLEX ASSEMBLY

Topic Editors
Daniel H. Gonzalez and Philippe Giegé

frontiers in PLANT SCIENCE



frontiers

ISSN 1664-8714
ISBN 978-2-88919-278-6
DOI 10.3389/978-2-88919-278-6

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

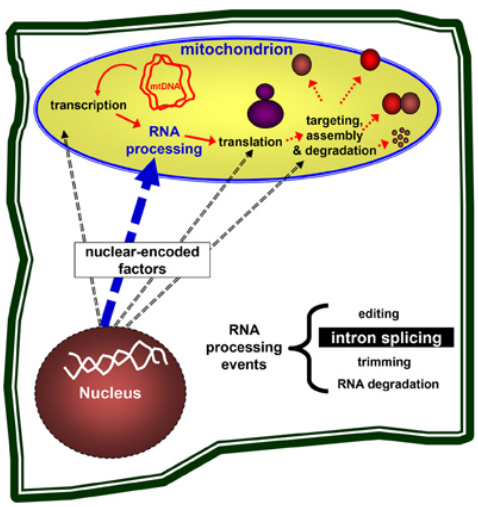
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

BIOGENESIS OF THE OXIDATIVE PHOSPHORYLATION MACHINERY IN PLANTS. FROM GENE EXPRESSION TO COMPLEX ASSEMBLY

Topic Editors:
Daniel H. Gonzalez, Universidad Nacional del Litoral, Argentina
Philippe Giegé, Centre National de la Recherche Scientifique, France



Mitochondrial biogenesis is an extremely complex process. Nuclear-encoded genes are necessary for the expression of mitochondrial genes, including transcription, pre-mRNA processing, translation of the mRNAs into mitochondrial proteins, the assembly of ribosomes and respiratory complexes, and are also required for the targeting and degradation of organellar subunits. This e-book presents a collection of articles dealing with different aspects of mitochondrial biogenesis in plants. Figure taken from: Brown GG, Colas des Francs-Small C and Ostersetzer-Biran O (2014) Group II intron splicing factors in plant mitochondria. *Front. Plant Sci.* 5:35. doi: 10.3389/fpls.2014.00035

Mitochondrial biogenesis is an extremely complex process. A hint of this complexity is clearly indicated by the many steps and factors required to assemble the respiratory complexes involved in oxidative phosphorylation. These steps include the expression of genes present in both the nucleus and the organelle, intricate post-transcriptional RNA processing events, the coordinated synthesis, transport and assembly of the different subunits, the synthesis and assembly of co-factors and, finally, the formation of supercomplexes or respirasomes. It can be envisaged, and current knowledge supports this view, that plants have evolved specific mechanisms for the biogenesis of respiratory complexes. For example, expression of the mitochondrial genome in plants has special features, not present in other groups of eukaryotes. Moreover, plant mitochondrial biogenesis and function should be considered in the context of the presence of the chloroplast, a second organelle involved in energetic and redox metabolism. It implies the necessity to discriminate between proteins destined for each organelle and requires the establishment of functional interconnections between photosynthesis and respiration. In recent years, our

knowledge of the mechanisms involved in these different processes in plants has considerably increased. As a result, the many events and factors necessary for the correct expression of proteins encoded in the mitochondrial genome, the cis acting elements and factors responsible for the expression of nuclear genes encoding respiratory chain components, the signals and mechanisms involved in the import of proteins synthesized in the cytosol and the many factors required for the synthesis and assembly of the different redox co-factors (heme groups, iron-sulfur clusters, copper centers) are beginning to be recognized at the molecular level. However, detailed knowledge of these processes is still not complete and, especially, little is known about how these processes are interconnected. Questions such as how the proteins, once synthesized in the mitochondrial matrix, are inserted into the membrane and assembled with other components, including those imported from the cytosol, how the expression of both genomes is coordinated and responds to changes in mitochondrial function, cellular requirements or environmental cues, or which factors and conditions influence the assembly of complexes and supercomplexes are still open and will receive much attention in the near future.

This Research Topic is aimed at establishing a collection of articles that focus on the different processes involved in the biogenesis of respiratory complexes in plants as a means to highlight recent advances. In this way, it intends to help to construct a picture of the whole process and, not less important, to expose the existing gaps that need to be addressed to fully understand how plant cells build and modulate the complex structures involved in respiration.

Table of Contents

frontiers in
PLANT SCIENCE

EDITORIAL
published: 23 May 2014
doi: 10.3389/fpls.2014.00225

Biogenesis of the oxidative phosphorylation machinery in plants. From gene expression to complex assembly

Daniel H. Gonzalez[1]* and Philippe Giegé[2]*

[1] *Instituto de Agrobiotecnología del Litoral (CONICET-UNL), Universidad Nacional del Litoral, Santa Fe, Argentina*
[2] *Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire des Plantes du CNRS (IBMP-CNRS), Université de Strasbourg, Strasbourg, France*
**Correspondence: dhgonza@fbcb.unl.edu.ar; philippe.giege@ibmp-cnrs.unistra.fr*

Edited and reviewed by:
Andreas Weber, Heinrich-Heine-Universität Düsseldorf, Germany

Keywords: cofactor assembly, coordinated expression, mitochondrion, mitoribosome, protein import translocase, respirasome, respiratory pathway, RNA editing

Mitochondrial biogenesis is an extremely complex process. A hint of this complexity is clearly indicated by the many steps and factors required to assemble the respiratory complexes involved in oxidative phosphorylation. These steps include the expression of genes present in both the nucleus and the organelle, intricate post-transcriptional RNA processing events, the coordinated synthesis, transport and assembly of the different subunits, the synthesis and assembly of co-factors and, finally, the formation of supercomplexes or respirasomes. Plants have evolved specific mechanisms for the biogenesis of respiratory complexes. For example, expression of the mitochondrial genome in plants has special features, not present in other groups of eukaryotes. Moreover, plant mitochondrial biogenesis and function should be considered in the context of the presence of the chloroplast, a second organelle involved in energetic and redox metabolism. This particularity implies the necessity to discriminate between proteins destined to each organelle and requires the establishment of functional interconnections between photosynthesis and respiration. In recent years, our knowledge of the mechanisms involved in these different processes in plants has considerably increased. As a result, the many events and factors necessary for the correct expression of proteins encoded in the mitochondrial genome, the *cis* acting elements and factors responsible for the expression of nuclear genes encoding respiratory chain components, the signals and mechanisms involved in the import of proteins synthesized in the cytosol and the many factors required for the synthesis and assembly of the different redox co-factors (heme groups, iron-sulfur clusters, copper centers) are beginning to be recognized at the molecular level (Carrie et al., 2013; Duncan et al., 2013; Garcia et al., 2014; Hammani and Giegé, 2014). However, detailed knowledge of these processes is still not complete and, especially, little is known about how these processes are interconnected. Regarding gene expression, key questions remain, e.g., the nature of the enzyme performing RNA editing remains elusive and the *cis* elements recruiting mitoribosomes are unknown. Other central questions regarding the post-translational fate of proteins are also unanswered, e.g., how the proteins, once synthesized in the mitochondrial matrix, are inserted into the membrane and assembled with other components, including those imported from the cytosol, how the expression of both genomes is coordinated and responds to changes in mitochondrial function, cellular requirements or environmental cues, or which factors and conditions influence the assembly of complexes and supercomplexes are still open and will receive much attention in the near future.

This Research Topic is aimed at establishing a collection of articles that focus on the different processes involved in the biogenesis of respiratory complexes in plants as a means to highlight recent advances. In this way, it intends to help to construct a picture of the whole process and, not less important, to expose the existing gaps that need to be addressed to fully understand how plant cells build and modulate the complex structures involved in respiration.

In the first article of this Topic, Schertl and Braun (2014) illustrate the complexity of plant mitochondrial electron pathways involved in respiration and describe the many dehydrogenases present in plant mitochondria and the entry points of electrons to the respiratory chain. Processes involved in the expression of the mitochondrial genome, like the role of maturases encoded in the organelle or the nucleus in the splicing of group II introns, RNA editing by PPR proteins and the translation of mitochondrial RNAs are discussed in the articles by Brown et al. (2014), Brehme et al. (2014) and Kazama et al. (2014), respectively. Welchen et al. (2014) present a survey of the current knowledge about the coordination of the expression of nuclear genes encoding mitochondrial components and how the expression is modulated by signals that arise in several compartments, including the organelle itself. In relation to this, Janska and Kwasniak (2014) discuss recent findings indicating the selective translation of sets of mitochondrial transcripts by mitoribosomes and the possible regulatory role of this phenomenon in the synthesis of mitochondrial proteins and the assembly of respiratory complexes, and Murcha et al. (2014) present new findings that mitochondrial Tim21-like proteins interact with both the protein import translocase of the inner membrane and respiratory chain complexes, reinforcing the idea that the functioning of the respiratory chain and the import and assembly of the corresponding subunits are tightly interconnected processes. In other review articles, Rigas et al. (2014) discuss the role of Lon proteases in mitochondrial biogenesis and analyze the evolutionary history of the Lon family and the processes that originated the current diversity of this family that includes a couple of dual-targeted (i.e., chloroplastic and mitochondrial) proteins, while Gehl and Sweetlove (2014) discuss on the role of Band-7 family proteins in mitochondrial biogenesis and the supramolecular organization and

functioning of the respiratory chain through the possible formation of lipid microdomains. Finally, Steinebrunner et al. (2014) present results about the divergent role of plant Sco proteins, involved in metal cofactor insertion into respiratory Complex IV in other organisms, in mitochondrial biogenesis and stress responses.

In summary, the collection of articles of this Research Topic highlights the complexity of the mechanisms involved in the biogenesis of the plant mitochondrial respiratory chain and the unique features of this process in plants. We thank all authors for their contribution to the assembly of this issue and hope that it will be useful for a broad readership of students and researchers interested in both plant sciences and organelle biology.

REFERENCES

Brehme, N., Zehrmann, A., Verbitskiy, D., Härtel, B., and Takenaka, M. (2014). Mitochondrial RNA editing PPR proteins can tolerate protein tags at E as well as at DYW domain termini. *Front. Plant Sci.* 5:127. doi: 10.3389/fpls.2014.00127

Brown, G. G., Colas des Francs-Small, C., and Ostersetzer, O. (2014). Group II intron splicing factors in plant mitochondria. *Front. Plant Sci.* 5:35. doi: 10.3389/fpls.2014.00035

Carrie, C., Murcha, M. W., Giraud, E., Ng, S., Zhang, M. F., Narsai, R., et al. (2013). How do plants make mitochondria? *Planta* 237, 429–439. doi: 10.1007/s00425-012-1762-3

Duncan, O., Murcha, M. W., and Whelan, J. (2013). Unique components of the plant mitochondrial protein import apparatus. *Biochim. Biophys. Acta* 1833, 304–313. doi: 10.1016/j.bbamcr.2012.02.015

Garcia, L., Welchen, E., and Gonzalez, D. H. (2014). Mitochondria and copper homeostasis in plants. *Mitochondrion* doi: 10.1016/j.mito.2014.02.011. [Epub ahead of print].

Gehl, B., and Sweetlove, L. J. (2014). Mitochondrial Band-7 family proteins: scaffolds for respiratory chain assembly? *Front. Plant Sci.* 5:141. doi: 10.3389/fpls.2014.00141

Hammani, K., and Giegé, P. (2014). RNA metabolism in plant mitochondria. *Trends Plant Sci.* doi: 10.1016/j.tplants.2013.12.008. [Epub ahead of print].

Janska, H., and Kwasniak, M. (2014). Mitoribosomal regulation of OXPHOS biogenesis in plants. *Front. Plant Sci.* 5:79. doi: 10.3389/fpls.2014.00079

Kazama, T., Yagi, Y., Toriyama, K., and Nakamura, T. (2014). Heterogeneity of the 5'-end in plant mRNA may be involved in mitochondrial translation. *Front. Plant Sci.* 4:517. doi: 10.3389/fpls.2013.00517

Murcha, M. W., Kubiszewski-Jakubiak, S., Wang, Y., and Whelan, J. (2014). Evidence for interactions between the mitochondrial import apparatus and respiratory chain complexes via Tim21-like proteins in Arabidopsis. *Front. Plant Sci.* 5:82. doi: 10.3389/fpls.2014.00082

Rigas, S., Daras, G., Tsitsekian, D., Alatzas, A., and Hatzopoulos, P. (2014). Evolution and significance of the Lon gene family in Arabidopsis organelle biogenesis and energy metabolism. *Front. Plant Sci.* 5:145. doi: 10.3389/fpls.2014.00145

Schertl, P., and Braun, H.-P. (2014). Respiratory electron transfer pathways in plant mitochondria. *Front. Plant Sci.* 5:163. doi: 10.3389/fpls.2014.00163

Steinebrunner, I., Gey, U., Andres, M., Garcia, L., and Gonzalez, D. H. (2014). Divergent functions of the Arabidopsis mitochondrial SCO proteins: HCC1 is essential for COX activity while HCC2 is involved in the UV-B stress response. *Front. Plant Sci.* 5:87. doi: 10.3389/fpls.2014.00087

Welchen, E., García, L., Mansilla, N., and Gonzalez, D. H. (2014). Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements. *Front. Plant Sci.* 4:551. doi: 10.3389/fpls.2013.00551

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 16 April 2014; accepted: 05 May 2014; published online: 23 May 2014.
*Citation: Gonzalez DH and Giegé P (2014) Biogenesis of the oxidative phosphorylation machinery in plants. From gene expression to complex assembly. Front. Plant Sci. **5**:225. doi: 10.3389/fpls.2014.00225*
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 29 April 2014
doi: 10.3389/fpls.2014.00163

Respiratory electron transfer pathways in plant mitochondria

Peter Schertl and Hans-Peter Braun*

Abteilung Pflanzenproteomik, Institut für Pflanzengenetik, Leibniz Universität Hannover, Hannover, Germany

Edited by:
Daniel H. Gonzalez, Universidad Nacional del Litoral, Argentina

Reviewed by:
Joshua L. Heazlewood, Lawrence Berkeley National Laboratory, USA
Nicolas L. Taylor, The University of Western Australia, Australia

***Correspondence:**
Hans-Peter Braun, Institut für Pflanzengenetik, Abteilung Pflanzenproteomik, Leibniz Universität Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany
e-mail: braun@genetik.uni-hannover.de

The respiratory electron transport chain (ETC) couples electron transfer from organic substrates onto molecular oxygen with proton translocation across the inner mitochondrial membrane. The resulting proton gradient is used by the ATP synthase complex for ATP formation. In plants, the ETC is especially intricate. Besides the "classical" oxidoreductase complexes (complex I–IV) and the mobile electron transporters cytochrome c and ubiquinone, it comprises numerous "alternative oxidoreductases." Furthermore, several dehydrogenases localized in the mitochondrial matrix and the mitochondrial intermembrane space directly or indirectly provide electrons for the ETC. Entry of electrons into the system occurs via numerous pathways which are dynamically regulated in response to the metabolic state of a plant cell as well as environmental factors. This mini review aims to summarize recent findings on respiratory electron transfer pathways in plants and on the involved components and supramolecular assemblies.

Keywords: plant mitochondria, electron transport chain, dehydrogenase, alternative oxidase, respiratory supercomplex

INTRODUCTION

During cellular respiration, organic compounds are oxidized to generate usable chemical energy in the form of ATP. The respiratory electron transport chain (ETC) of mitochondria is at the center of this process. Its core consists of four oxidoreductase complexes, the NADH dehydrogenase (complex I), the succinate dehydrogenase (complex II), the cytochrome c reductase (complex III) and the cytochrome c oxidase (complex IV), as well as of two mobile electron transporters, cytochrome c, and the lipid ubiquinone. Overall, electrons are transferred from the coenzymes NADH or $FADH_2$ onto molecular oxygen which is reduced to water. Three of the four oxidoreductase complexes (complexes I, III and IV) couple their electron transfer reactions with proton translocation across the inner mitochondrial membrane. As a result, a proton gradient is formed which can be used by the ATP synthase complex (complex V) for the phosphorylation of ADP. In its classically described form, cellular respiration is based on a linear ETC (from NADH via complexes I, III, and IV to molecular oxygen). However, electrons can enter and leave the ETC at several alternative points. This is especially true for the plant ETC system, which is highly branched. In this review we aim to integrate current knowledge on the ETC system in plants with respect to its components, electron transport pathways and supramolecular structure.

COMPONENTS OF THE PLANT ETC SYSTEM

The "classical" oxidoreductase complexes of the respiratory chain (given in dark blue in **Figure 1**) resemble their homologues in animal mitochondria but at the same time have some clear distinctive features (reviewed in Millar et al., 2008, 2011; Rasmusson and Moller, 2011; van Dongen et al., 2011; Jacoby et al., 2012). *Complex I* is especially large in plant mitochondria and includes nearly 50 different subunits (Braun et al., 2014). Compared to its homologs from bacteria and other eukaryotic lineages it has an extra domain which includes carbonic anhydrase-like proteins. The function of this additional domain is currently unclear but it has been suggested to be important in the context of an intracellular CO_2 transfer mechanism to provide mitochondrial CO_2 for carbon fixation in chloroplasts (Braun and Zabaleta, 2007; Zabaleta et al., 2012). *Complex II* is composed of four subunits in bacteria and mitochondria of animals and fungi. In plants complex II includes homologs of these subunits but additionally four extra proteins of unknown function (Millar et al., 2004; Huang and Millar, 2013). In contrast, the subunit composition of *complex III* from plants is highly similar to the ones in yeast and bovine mitochondria (Braun and Schmitz, 1995a). The two largest subunits of this protein complex, termed "core proteins" in animals and fungi, are homologous to the two subunits of the mitochondrial processing peptidase (MPP) which removes pre-sequences of nuclear-encoded mitochondrial proteins after their import into mitochondria. In animal mitochondria, the core proteins are proteolytically inactive. Instead, an active MPP is present within the mitochondrial matrix. In contrast, the core subunits of complex III from plants have intact active sites (Braun et al., 1992; Glaser et al., 1994). Indeed, complex III isolated from plant mitochondria efficiently removes pre-sequences of mitochondrial pre-proteins. The differing functional states of complex III in diverse eukaryotic lineages might reflect different evolutionary stages of this protein complex (Braun and Schmitz, 1995b). Also *complex IV* has some extra subunits in mitochondria of plants (Millar et al., 2004). Eight subunits are homologous to complex IV subunits from other groups of eukaryotes and another six putative subunits represent proteins of unknown functions.

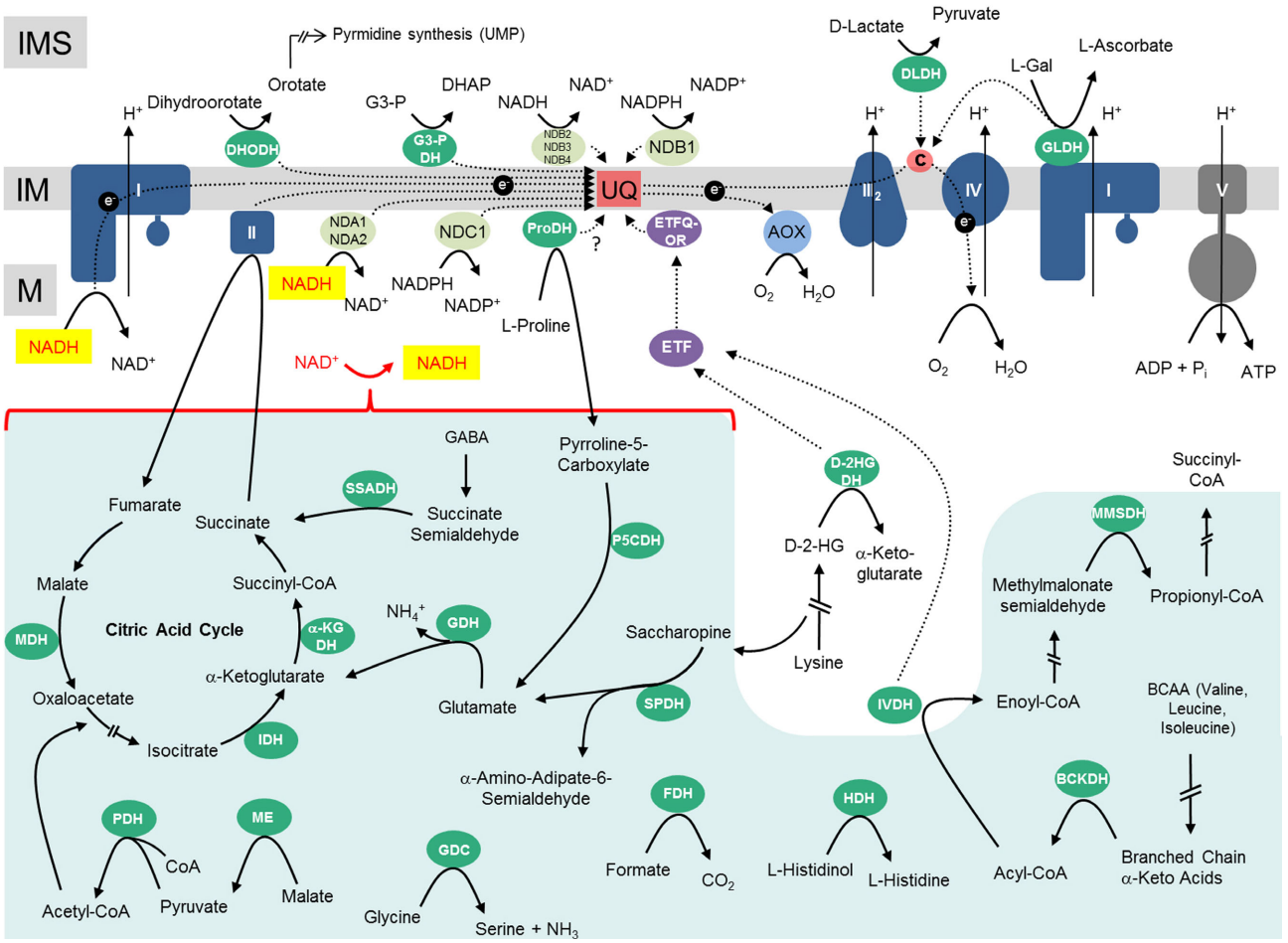


FIGURE 1 | Mitochondrial dehydrogenases and the respiratory chain. Within the mitochondrial matrix (M) numerous dehydrogenases generate NADH by oxidizing various carbon compounds. NADH subsequently is re-oxidized at the inner mitochondrial membrane (IM) by the respiratory electron transfer chain (ETC). The electrons of NADH can enter the ETC through complex I or at the ubiquinone level via alternative NAD(P)H-dehydrogenases. Besides, some dehydrogenases of the mitochondrial matrix transfer electrons to ubiquinone via the ETF/ETFQOR system. Proline dehydrogenase possibly directly transfers electrons onto ubiquinone. In the intermembrane space (IMS), electrons from NAD(P)H generated in the cytoplasm can be inserted into the ETC via alternative NAD(P)H dehydrogenases. Furthermore, some dehydrogenases of the IMS can directly transfer electrons onto ubiquinone or cytochrome c. Color code—dark blue, protein complexes of the ETC; blue, AOX; purple, ETF/ETFQQ system; light green, alternative NAD(P)H dehydrogenases of the ETC; green, dehydrogenases; red, ubiquinone and cytochrome c; yellow, NADH produced by dehydrogenases of the mitochondrial matrix/NADH re-oxidized by complex I or internal alternative NADH dehydrogenases; dark gray, ATP synthase complex; light green background, NADH producing dehydrogenases of the mitochondrial matrix. Abbreviations—*alphabetically ordered*. I, complex I; II, complex II; III, complex III; IV, complex IV; V, complex V; α-KGDH, α-ketoglutarate dehydrogenase; AOX, alternative oxidase; BCKDH, branched-chain α-ketoacid dehydrogenase complex; c, cytochrome c; D-2HGDH, D-2-hydroxyglutarate dehydrogenase; DHODH, dihydroorotate dehydrogenase; DLDH, D-lactate dehydrogenase; ETF, electron transfer flavoprotein; ETFQOR, electron transfer flavoprotein ubiquinone oxidoreductase; FDH, formate dehydrogenase; GDC, glycine dehydrogenase; GDH, glutamate dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; G3-PDH, glyceraldehyde 3-phosphate dehydrogenase; HDH, histidinol dehydrogenase; IDH, isocitrate dehydrogenase; IVDH, isovaleryl-coenzyme A dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; MMSDH, methylmalonate-semialdehyde dehydrogenase; NDA1/2, NDB2/3/4, alternative NADH dehydrogenase; NDC1, NDB1, alternative NADPH dehydrogenase; P5CDH, pyrroline-5-carboxylate dehydrogenase; PDH, pyruvate dehydrogenase; ProDH, proline dehydrogenase; SPDH, saccharopine dehydrogenase; SSADH, succinic semialdehyde dehydrogenase; UQ, ubiquinone. For further information of the enzymes see **Table 1**.

The ETC of plant mitochondria additionally includes several so-called "alternative oxidoreductases": the alternative oxidase (AOX; light blue in **Figure 1**) and several functionally distinguishable alternative NAD(P)H dehydrogenases (alternative NDs, light green in **Figure 1**). Findings on their functional roles have been reviewed recently (Rasmusson et al., 2008; Rasmusson and Moller, 2011; Moore et al., 2013). AOX directly transfers electrons from ubiquinol to molecular oxygen and therefore constitutes an alternative electron exit point of the ETC. As a result, complexes III and IV are excluded from respiratory electron transport. The alternative NAD(P)H dehydrogenases serve as alternative electron entry points of the plant ETC and may substitute complex I. They differ with respect to co-factor requirement and localization at the outer or inner surface of

Table 1 | Mitochondrial dehydrogenases in *Arabidopsis thaliana*[a].

Enzyme	**Accession no.**[b] subunits isoforms etc.	**Catalysed reaction**	**Oligomeric state** Native mass/monomer mass according to GelMap[c] (according to other data in the literature)	**Publication**[d] for Arabidopsis (for other plants)
Malate dehydrogenase	At1g53240 At3g15020	Malate + NAD^+ ⇔ Oxaloacetate + NADH	At1g53240: 89 kDa/42 kDa At3g47520: 157 kDa/38 kDa	Journet et al., 1981 Gietl, 1992 Krömer, 1995 Nunes-Nesi et al., 2005 Lee et al., 2008 Tomaz et al., 2010
Isocitrate dehydrogenase	At4g35260 At5g14590 At4g35650 At3g09810 At5g03290 At2g17130	Isocitrate + NAD^+ ⇔ α-Ketoglutarate + CO_2 + NADH	At4g35260: 89 kDa/42 kDa At5g14590: 140 kDa/53 kDa At3g09810: 138 kDa/40 kDa At5g03290: 138 kDa/40 kDa	Behal and Oliver, 1998 Lancien et al., 1998 Lin et al., 2004 Lemaitre and Hodges, 2006 Lemaitre et al., 2007
α-Ketoglutarate dehydrogenase complex	At3g55410 (E1) At5g65750 (E1) At4g26910 (E2) At5g55070 (E2) At3g17240 (E3) At1g48030 (E3) At3g13930 (E3)	α-Ketoglutarate + coenzyme A + NAD^+ ⇔ succinyl-CoA + CO_2 + NADH	At5g65750: 207 kDa/91 kDa At3g55410: 207 kDa/91 kDa (1.7 MDa complex)	Poulsen and Wedding, 1970 Wedding and Black, 1971a,b Dry and Wiskich, 1987 Millar et al., 1999 Araújo et al., 2008 Araújo et al., 2013
Glutamate dehydrogenase	At5g18170 At5g07440 At3g03910	Glutamate + H_2O + NAD^+ ⇔ α-Ketoglutarate + NH_4^+ + NADH	At5g18170: 209 kDa/48 kDa At5g07440: 209 kDa/48 kDa At3g03910: 209 kDa/48 kDa	Yamaya et al., 1984 Turano et al., 1997 Aubert et al., 2001 Miyashita and Good, 2008a,b Fontaine et al., 2012 Tarasenko et al., 2013 Fontaine et al., 2012
Malic enzyme	At2g13560 At4g00570 At1g79750	Malate + NAD^+ ⇔ Pyruvate + NADH + CO_2	At2g13560: 370 kDa/63 kDa At4g00570: 370 kDa/63 kDa	Jenner et al., 2001 Tronconi et al., 2008 Tronconi et al., 2010 Tronconi et al., 2012
Pyruvate dehydrogenase complex	At1g59900 (E1) At1g24180 (E1) At5g50850 (E1) At3g52200 (E2) At1g54220 (E2) At3g13930 (E3) At3g17240 (E3) At1g48030 (E3)	Pyruvate + coenzyme A + NAD^+ ⇔ Acetyl-CoA + CO_2 + NADH	At3g13930: 1500 kDa/54 kDa At1g24180: 470 kDa/41 kDa At5g50850: 150 kDa/39 kDa At1g59900: 138 kDa/44 kDa (9.5 MDa complex)	Luethy et al., 1994 Grof et al., 1995 Zou et al., 1999 Tovar-Méndez et al., 2003 Szurmak et al., 2003 Yu et al., 2012
Glycine dehydrogenase complex	At4g33010 (P) At2g26080 (P) At1g32470 (H) At2g35120 (H) At2g35370 (H) At1g11860 (T) At4g12130 (T) At3g17240 (L) At1g48030 (L)	Glycine + H_4 folate + NAD^+ ⇔ methylene-H_4 folate + CO_2 + NH_3 + NADH	At4g33010: 144 kDa/91 kDa At2g26080: 209 kDa/91 kDa At1g11860: 148 kDa/46 kDa (1.3 MDa complex)	Somerville and Ogren, 1982 Oliver et al., 1990 Oliver, 1994 Srinivasan and Oliver, 1995 Douce et al., 2001

(Continued)

Table 1 | Continued

Enzyme	**Accession no.**[b] subunits isoforms etc.	**Catalysed reaction**	**Oligomeric state** Native mass/monomer mass according to GelMap[c] (according to other data in the literature)	**Publication**[d] for Arabidopsis (for other plants)
Branched-chain alpha keto acid dehydrogenase complex	At5g09300 (E1) At1g21400 (E1) At1g55510 (E1) At3g13450 (E1) At3g06850 (E2) At3g13930 (E3) At3g17240 (E3) At1g48030 (E3)	Branched chain alpha keto-acids + CoA + NAD^+ ⇔ Acyl-CoA + NADH	At1g55510: 150 kDa/39 kDa (0.95 MDa complex)	Fujiki et al., 2000 Mooney et al., 2000 Fujiki et al., 2001 Fujiki et al., 2002 Taylor et al., 2004 Binder, 2010
Formate dehydrogenase	At5g14780	Formate + NAD^+ ⇔ CO_2 + NADH	(200 kDa complex)	Halliwell, 1974 Colas des Francs-Small et al., 1993 Hourton-Cabassa et al., 1998 Jänsch et al., 1996 Bykova et al., 2003 Baack et al., 2003 Olson et al., 2000 Alekseeva et al., 2011
Methylmalonate semialdehyde dehydrogenase	At2g14170	(S)-methylmalonate-semialdehyde + coenzyme A + NAD^+ + H_2O ⇔ propanoyl-CoA + bicarbonate + NADH	At2g14170: 200 kDa/59 kDa	Oguchi et al., 2004 Tanaka et al., 2005 Kirch et al., 2004
Isovaleryl-CoA dehydrogenase	At3g45300	Isovaleryl-CoA + acceptor (ETF) ⇔ 3-methylbut-2-enoyl-CoA + reduced acceptor (ETF) (also considerable activity with other acyl-CoA's)	At3g45300: 132 kDa/46 kDa (homodimeric complex)	Däschner et al., 1999 Reinard et al., 2000 Faivre-Nitschke et al., 2001 Däschner et al., 2001 Goetzman et al., 2005 Araújo et al., 2010
D-2-Hydroxyglutarate dehydrogenase	At4g36400	D-2-hydroxyglutarate + acceptor (ETF) ⇔ 2-oxoglutarate + reduced acceptor (ETF)	(homodimeric complex)	Engqvist et al., 2009 Araújo et al., 2010 Engqvist et al., 2011
Saccharopine dehydrogenase	At5g39410	Saccharopine + NAD^+ + H_2O ⇔ Glutamate + -Amino adipate semialdehyde + NADH	not known	Zhu et al., 2000 Heazlewood et al., 2003
Pyrroline-5-carboxylate dehydrogenase	At5g62530	Pyrroline-5-carboxylate + NAD^+ ⇔ Glutamate (Glutamate-5-semialdehyde) + NADH	At5g62530: 158 kDa/59 kDa	Forlani et al., 1997 Deuschle et al., 2001 Deuschle et al., 2004 Miller et al., 2009
Proline dehydrogenase	At3g30775 At5g38710	L-Proline ⇔ Pyrroline-5-Carboxylate	not known	Elthon and Stewart, 1981 Verbruggen et al., 1996 Kiyosue et al., 1996 Mani et al., 2002 Szabados and Savouré, 2010 Funck et al., 2010 Sharma and Verslues, 2010 Schertl et al., in press

(Continued)

Table 1 | Continued

Enzyme	Accession no.[b] subunits isoforms etc.	Catalysed reaction	Oligomeric state Native mass/monomer mass according to GelMap[c] (according to other data in the literature)	Publication[d] for Arabidopsis (for other plants)
L-Galactono-1,4-lactone dehydrogenase	At3g47930	L-Galactono-1,4-Lactone ⇔ L-Ascorbate	(420 kDa, 470 kDa, 850 kDa complexes)	Mapson and Breslow, 1958 Siendones et al., 1999 Leferink et al., 2008 Pineau et al., 2008 Leferink et al., 2009 Schertl et al., 2012
D-Lactate dehydrogenase	At5g06580	D-Lactate ⇔ Pyruvate	(homodimeric complex)	Bari et al., 2004 Atlante et al., 2005 Engqvist et al., 2009 Wienstroer et al., 2012
Glycerol-3-phosphate dehydrogenase	At3g10370	Glycerol 3-phosphate ⇔ Dihydroxyacetonephosphate	At3g10370: 160 kDa/65 kDa	Shen et al., 2003 Shen et al., 2006
Dihydroorotate dehydrogenase	At5g23300	Dihydroorotate ⇔ Orotate	At5g23300: 156 kDa/49 kDa	Ullrich et al., 2002 Doremus and Jagendorf, 1985 Miersch et al., 1987
Succinic semialdehyde dehydrogenase	At1g79440	Succinic semialdehyde ⇔ Succinate	At1g79440: 163 kDa/55 kDa	Busch and Fromm, 1999 Bouché et al., 2003 Kirch et al., 2004 Toyokura et al., 2011
Histidinol dehydrogenase	At5g63890	L-histidinol + NAD^+ ⇔ L-histidine + NADH	At5g63890: 115 kDa/51 kDa	Nagai and Scheidegger, 1991 Ingle, 2011
Alternative NAD(P)H dehydrogenases (NDA1, NDB4, NDA2, NDB2, NDB3, NDB1, NDC1)	At1g07180 At2g20800 At2g29990 At4g05020 At4g21490 At4g28220 At5g08740	NAD(P)H + UQ ⇔ $NAD(P)^+$ + UQH_2	At2g20800: 160 kDa/65 kDa At2g29990: 163 kDa/55 kDa At4g05020: 160 kDa/65 kDa	Escobar et al., 2004 Rasmusson et al., 2004 Rasmusson et al., 2008 Wulff et al., 2009 Wallström et al., 2014a,b

[a] *Mitochondrial dehydrogenases without complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) of the respiratory chain. This list corresponds to the dehydrogenases shown in* ***Figure 1****.*

[b] *Accession numbers in accordance with The Arabidopsis Information Resource (TAIR).*

[c] *Oligomeric state: native mass and monomer mass according to GelMap (https://gelmap.de/231).*

[d] *Key publications for Arabidopsis (other plants).*

the inner mitochondrial membrane (external alternative NDs, internal alternative NDs). Some of the genes encoding alternative NDs are activated by light (Rasmusson et al., 2008; Rasmusson and Moller, 2011). The latter enzymes are considered to be important during photorespiration and all alternative enzymes during various stress conditions. Since none of the alternative oxidoreductases couple electron transfer with proton translocation across the inner mitochondrial membrane, their enzymatic function is believed to be important in the context of an overflow protection mechanism for the ETC which is especially relevant during high-light conditions.

Finally, dehydrogenases (dark green in **Figure 1**; **Table 1**) can directly or indirectly insert electrons into the respiratory chain (Rasmusson et al., 2008; Rasmusson and Moller, 2011). Numerous dehydrogenases of the mitochondrial matrix generate NADH which is re-oxidized by complex I and the internal alternative NDs. However, some dehydrogenases directly transfer electrons onto ubiquinone [dihydroorotate dehydrogenase (DHODH), glyceraldehyde 3-phosphate dehydrogenase (G3-PDH) and possibly proline dehydrogenase (ProDH)] or onto cytochrome c [L-galactone-1,4-lactone dehydrogenase (GLDH) and D-lactate dehydrogenase (DLDH)]. Furthermore, at least two dehydrogenases [isovaleryl-coenzyme A dehydrogenase (IVDH) and D-2-hydroxyglutarate dehydrogenase (D-2HGDH)] transfer electrons onto ubiquinone via a short electron transfer chain composed of the "electron transfer flavoprotein" and the "electron transfer flavoprotein-ubiquinone oxidoreductase" (ETF and ETFQ-OR, purple in **Figure 1**) (Ishizaki et al., 2005, 2006; Araújo et al., 2010). IVDH is involved in the branched chain amino acid catabolism and D-2HGDH in the catabolism of lysine. In

plants, degradation of amino acids for respiration was shown to be especially important during carbon starvation conditions, e.g., extended darkness (Araújo et al., 2011). In contrast to animal mitochondria, fatty acid oxidation does not take place in plant mitochondria and the involved dehydrogenases consequently are absent. Instead, additional metabolic pathways occur in plants, e.g., the final step of an ascorbic acid biosynthesis pathway, which is catalyzed by GLDH. Electrons of L-galactono-1,4-lactone (GL) oxidation are inserted into the ETC via cytochrome c (Bartoli et al., 2000). Proline, besides being a building block for protein biosynthesis, is used as an osmolyte in plant cells. Proline is catabolized in mitochondria by a two-step process involving pyrroline-5-carboxylate dehydrogenase (P5CDH) and ProDH (Szabados and Savouré, 2010). P5CDH produces NADH, whereas ProDH represents a flavoenzyme which is assumed to transfer electrons directly or indirectly onto ubiquinone. Some additional dehydrogenases occur in plant mitochondria in the mitochondrial matrix and the intermembrane space which also contribute electrons to the ETC (**Figure 1**, **Table 1**). However, in some cases their mitochondrial localization is not entirely certain and should be further investigated by future research.

ELECTRON ENTRY PATHWAYS INTO THE ETC

All electrons enter the ETC via NAD(P)H (generated by a variety of dehydrogenases in the mitochondrial matrix or the intermembrane space/the cytoplasm) or via flavine nucleotides ($FADH_2$, $FMNH_2$), which generally are bound to proteins termed flavoproteins. Consequently, the following electron entry pathways into the ETC can be defined: (i) the Matrix NAD(P)H pathway, (ii) the Matrix-$FADH_2$ pathway, (iii) the Intermembrane-space-NAD(P)H pathway, and (iv) the Intermembrane-space-$FADH_2$/$FMDH_2$ pathway (**Figure 2**).

Different metabolic processes, which vary depending on the physiological state of the plant cell, contribute to the four electron entry pathways. During stable carbohydrate conditions, electrons

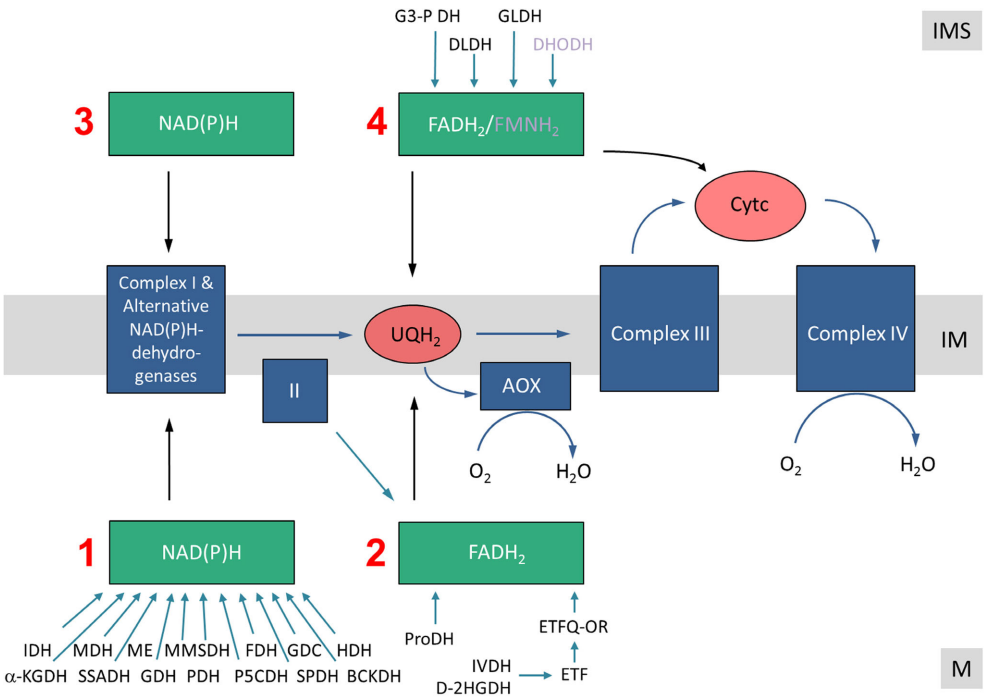


FIGURE 2 | Electron entry pathways into the mitochondrial electron transport chain in plants. Electrons enter the respiratory chain via four different pathways. **(1)** The Matrix-NAD(P)H pathway. Various dehydrogenases oxidize carbon compounds in the mitochondrial matrix. Electrons are transferred in the form of NADH to the ETC. NADH is re-oxidized by complex I or the internal alternative NAD(P)H dehydrogenases. **(2)** The Matrix-$FADH_2$ pathway. FAD-containing enzymes oxidize carbon compounds in the mitochondrial matrix and directly (ProDH?) or indirectly (via the ETF/ETFQQ system) transfer electrons to the ubiquinone pool. **(3)** The IMS-NAD(P)H pathway. Cytoplasmically formed NAD(P)H is re-oxidized via external alternative dehydrogenases. **(4)** The IMS-$FADH_2$ pathway. FAD/FMN-containing enzymes oxidize carbon compounds in the mitochondrial intermembrane space. Electrons are transferred either to the ubiquinone or the cytochrome c. M, matrix; IM, inner membrane; IMS, intermembrane space. Abbreviations—*alphabetically ordered*. I, complex I; II, complex II; III, complex III; IV, complex IV; α-KGDH, α-ketoglutarte dehydrogenase; AOX, alternative oxidase; BCKDH, branched-chain α-ketoacid dehydrogenase complex; Cytc, cytochrome c; D-2HGDH, D-2-hydroxyglutarate dehydrogenase; DHODH, dihydroorotate dehydrogenase; DLDH, D-lactate dehydrogenase; ETF, electron transfer flavoprotein; ETFQOR, electron transfer flavoprotein ubiquinone oxidoreductase; FDH, formate dehydrogenase; GDC, glycine dehydrogenase; GDH, glutamate dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase; G3-PDH, glyceraldehyde 3-phosphate dehydrogenase; HDH, histidinol dehydrogenase; IDH, isocitrate dehydrogenase; IVDH, isovaleryl-coenzyme A dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; MMSDH, methylmalonate-semialdehyde dehydrogenase; P5CDH, pyrroline-5-carboxylate dehydrogenase; PDH, pyruvate dehydrogenase; ProDH, proline dehydrogenase; SPDH, saccharopine dehydrogenase; SSADH, succinic semialdehyde dehydrogenase; UQH_2, ubiquinol.

for the respiratory chain can be supplied by NADH and $FADH_2$ provided by the tricarboxylic acid (TCA) cycle. This is believed to be the standard mode of cellular respiration in non-green plant tissues or green tissues at night and resembles the basic situation in animal cells. However, during photosynthesis, NADH generation of the TCA cycle is reduced because some of its intermediates are used for anabolic reactions (reviewed in Sweetlove et al., 2010). Furthermore, the pyruvate dehydrogenase (PDH) complex is deactivated in plant mitochondria in the light by phosphorylation (Budde and Randall, 1990). At the same time photorespiration leads to an increase in NADH formation in the mitochondrial matrix by the activity of the glycine dehydrogenase complex (GDC). Indeed, at high-light conditions, NADH formed by GDC is believed to be the main substrate of the ETC, and not the NADH formed by the enzymes of the TCA cycle. At the same time, plant cells might become over-reduced in the presence of high-light. In this situation alternative oxidoreductases can insert excess electrons into the respiratory chain without contributing to the proton gradient. Upon carbon starvation conditions (e.g., extended darkness) electrons from the breakdown of amino acids are provided to the ETC (Araújo et al., 2011). Especially after release of salt stress the amino acid proline is used as an electron source (Szabados and Savouré, 2010). In summary, electron entry into the ETC is a highly flexible process in plants which much depends on light, the metabolic state of the cell as well as environmental stress factors.

SUPRAMOLECULAR STRUCTURE OF THE ETC SYSTEM

The ETC is based on defined protein-protein interactions. Most stable interactions occur within the four "classical" oxidoreductase complexes of the respiratory chain. Indeed, complexes I to IV can be isolated in intact form by various biochemical and electrophoretic procedures. Furthermore, several lines of evidence indicate that complexes I, III and IV interact within the inner mitochondrial membrane forming respiratory supercomplexes (reviewed in Dudkina et al., 2008). Complex I as well as complex IV associate with dimeric complex III ($I + III_2$ and $IV_2 + III_2$ supercomplexes). An even larger supercomplex includes complexes I, III_2, and IV and was proposed to be called "respirasome" because it can autonomously catalyzes the overall ETC reaction in the presence of ubiquinone and cytochrome c. The alternative oxidoreductases of the plant ETC seem not to be part of the respiratory supercomplexes. However, alternative NDs were found to be part of other protein complexes of about 160 kDa (Klodmann et al., 2011) or 150–700 kDa (Rasmusson and Agius, 2001).

Experimental data also indicate that several of the mitochondrial dehydrogenases form protein complexes. TCA cycle

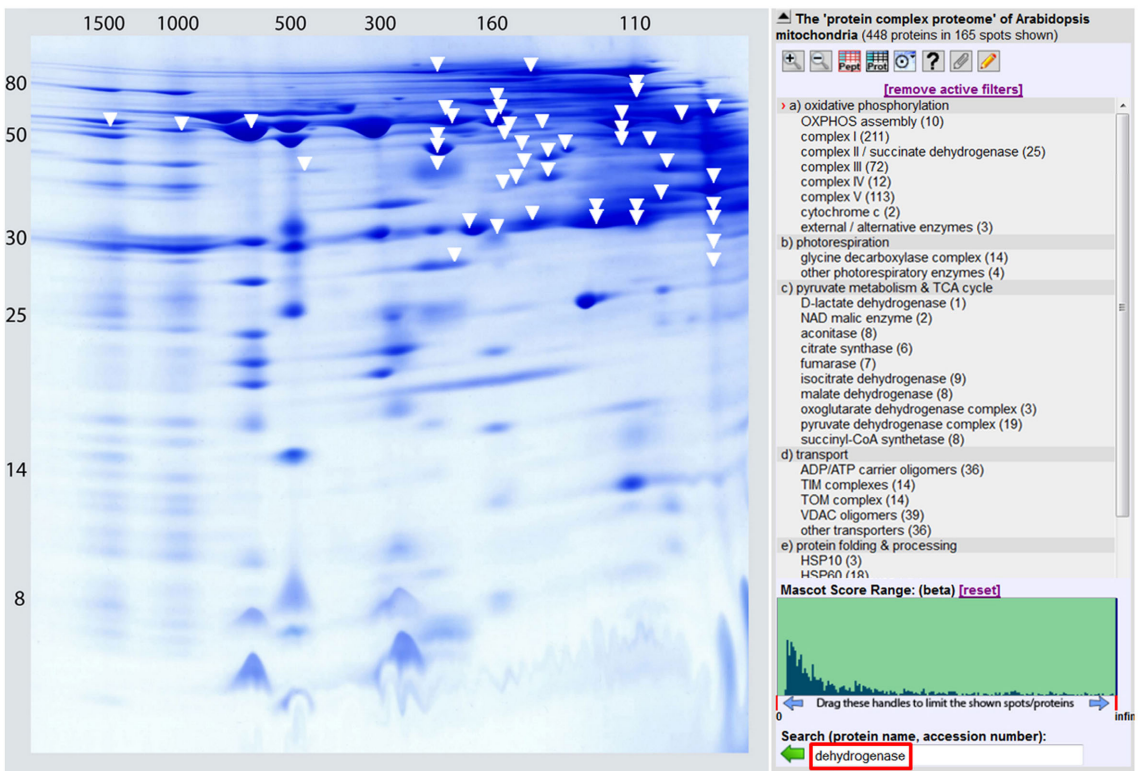


FIGURE 3 | The dehydrogenase subproteome of plant mitochondria. Mitochondrial proteins from *Arabidopsis thaliana* were separated by 2D Blue native/SDS PAGE and displayed via GelMap (https://gelmap.de/231#). Protein separation under native condition was from left to right, protein separation in the presence of SDS from top to bottom. Molecular masses of standard proteins are given to the left/above the 2D gel. All proteins annotated as "dehydrogenase" are indicated by white arrows. Exception: The subunits of complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) are not indicated on the figure.

enzymes can assemble forming multienzyme clusters (Barnes and Weitzman, 1986). In addition, some of the mitochondrial dehydrogenases interact with ETC complexes, e.g., malate dehydrogenase has been reported to interact with complex I in animal mitochondria (Fukushima et al., 1989; see Braun et al., 2014 for review). Information on the native state of mitochondrial dehydrogenases furthermore comes from the GelMap project (Klodmann et al., 2011). Using 2D Blue native/SDS PAGE and systematic protein identifications, various dehydrogenases were described (**Figure 3**, **Table 1**). Native molecular mass of the dehydrogenases in many cases much exceeds the molecular mass of the monomeric proteins (**Table 1**, column 3). This indicates that probably most mitochondrial dehydrogenases form part of defined higher order structures.

CONCLUSION AND OUTLOOK

Cellular respiration in plants is an especially dynamic system. The classical protein complexes of the ETC have extra functions and several alternative oxidoreductases occur. A network of mitochondrial dehydrogenases directly or indirectly supplies electrons for the respiratory chain. Insertion of electrons via various pathways is highly dependent on the metabolic state of the plant cell. The regulation of electron entry pathways into the respiratory chain is only partially understood and might, besides others, depend on the formation of supramolecular structures. Non-invasive experimental procedures will be necessary to physiologically investigate the function of these structures by future research.

ACKNOWLEDGMENTS

We thank Tatjana Hildebrandt and Holger Eubel, Leibniz University Hannover, for critically reading the manuscript. This research project was supported by the Deutsche Forschungsgemeinschaft (DFG), grant Br 1829/10-2. Furthermore we acknowledge support by the Open Access Publishing Fund of Leibniz Universität Hannover, which is funded by the DFG.

REFERENCES

Alekseeva, A. A., Savin, S. S., and Tishkov, V. I. (2011). NAD (+) -dependent formate dehydrogenase from plants. *Acta Nat.* 3, 38–54.

Araújo, W. L., Ishizaki, K., Nunes-Nesi, A., Larson, T. R., Tohge, T., Krahnert, I., et al. (2010). Identification of the 2-hydroxyglutarate and isovaleryl-CoA dehydrogenases as alternative electron donors linking lysine catabolism to the electron transport chain of Arabidopsis mitochondria. *Plant Cell* 22, 1549–1563. doi: 10.1105/tpc.110.075630

Araújo, W. L., Nunes-Nesi, A., Trenkamp, S., Bunik, V. I., and Fernie, A. R. (2008). Inhibition of 2-oxoglutarate dehydrogenase in potato tuber suggests the enzyme is limiting for respiration and confirms its importance in nitrogen assimilation. *Plant Physiol.* 148, 1782–1796. doi: 10.1104/pp.108.126219

Araújo, W. L., Tohge, T., Ishizaki, K., Leaver, C. J., and Fernie, A. R. (2011). Protein degradation - an alternative respiratory substrate for stressed plants. *Trends Plant Sci.* 16, 489–498. doi: 10.1016/j.tplants.2011.05.008

Araújo, W. L., Trofimova, L., Mkrtchyan, G., Steinhauser, D., Krall, L., Graf, A., et al. (2013). On the role of the mitochondrial 2-oxoglutarate dehydrogenase complex in amino acid metabolism. *Amino Acids* 44, 683–700. doi: 10.1007/s00726-012-1392-x

Atlante, A., de Bari, L., Valenti, D., Pizzuto, R., Paventi, G., and Passarella, S. (2005). Transport and metabolism of D-lactate in Jerusalem artichoke mitochondria. *Biochim. Biophys. Acta* 1708, 13–22. doi: 10.1016/j.bbabio.2005.03.003

Aubert, S., Bligny, R., Douce, R., Gout, E., Ratcliffe, R. G., and Roberts, J. K. (2001). Contribution of glutamate dehydrogenase to mitochondrial glutamate metabolism studied by (13)C and (31)P nuclear magnetic resonance. *J. Exp. Bot.* 52, 37–45. doi: 10.1093/jexbot/52.354.37

Baack, R. D., Markwell, J., Herman, P. L., and Osterman, J. C. (2003). Kinetic behavior of the *Arabidopsis thaliana* leaf formate dehydrogenase is thermally sensitive. *J. Plant Physiol.* 160, 445–450. doi: 10.1078/0176-1617-00995

Bari, R., Kebeish, R., Kalamajka, R., Rademacher, T., and Peterhänsel, C. (2004). A glycolate dehydrogenase in the mitochondria of *Arabidopsis thaliana*. *J. Exp. Bot.* 55, 623–630. doi: 10.1093/jxb/erh079

Barnes, S. J., and Weitzman, P. D. (1986) Organization of citric acid cycle enzymes into a multienzyme cluster. *FEBS Lett.* 201, 267–270. doi: 10.1016/0014-5793(86)80621-4

Bartoli, C. G., Pastori, G. M., and Foyer, C. H. (2000). Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol.* 123, 335–344. doi: 10.1104/pp.123.1.335

Behal, R. H., and Oliver, D. J. (1998). NAD(+)-dependent isocitrate dehydrogenase from *Arabidopsis thaliana*. Characterization of two closely related subunits. *Plant Mol. Biol.* 36, 691–698. doi: 10.1023/A:1005923410940

Binder, S. (2010). Branched-chain amino acid metabolism in *Arabidopsis thaliana*. *Arabidopsis Book* 8, e0137. doi: 10.1199/tab.0137

Bouché, N., Fait, A., Bouchez, D., Møller, S. G., and Fromm, H. (2003). Mitochondrial succinic-semialdehyde dehydrogenase of the gamma-aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. *Proc. Natl. Acad. Sci. U.S.A.* 100, 6843–6848. doi: 10.1073/pnas.1037532100

Braun, H. P., Binder, S., Brennicke, A., Eubel, H., Fernie, A. R., Finkemeier, I., et al. (2014). The life of plant mitochondrial complex I. *Mitochondrion*. doi: 10.1016/j.mito.2014.02.006. [Epub ahead of print].

Braun, H. P., Emmermann, M., Kruft, V., and Schmitz, U. K. (1992). The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. *EMBO J.* 11, 3219–3227.

Braun, H. P., and Schmitz, U. K. (1995a). The bifunctional cytochrome c reductase/processing peptidase complex from plant mitochondria. *J. Bioenerg. Biomembr.* 27, 423–436. doi: 10.1007/BF02110005

Braun, H. P., and Schmitz, U. K. (1995b) Are the "core" proteins of the mitochondrial bc1 complex evolutionary relics of a processing peptidase? *Trends Biochem. Sci.* 20, 171–175. doi: 10.1016/S0968-0004(00)88999-9

Braun, H. P., and Zabaleta, E. (2007) Carbonic anhydrase subunits of the mitochondrial NADH dehydrogenase complex (complex I) in plants. *Physiologia Plantarum* 129, 114–122. doi: 10.1111/j.1399-3054.2006.00773.x

Budde, R. J., and Randall, D. D. (1990). Pea leaf mitochondrial pyruvate dehydrogenase complex is inactivated *in vivo* in a light-dependent manner. *Proc. Natl. Acad. Sci. U.S.A.* 87, 673–676. doi: 10.1073/pnas.87.2.673

Busch, K. B., and Fromm, H. (1999). Plant succinic semialdehyde dehydrogenase. Cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. *Plant Physiol.* 121, 589–597. doi: 10.1104/pp.121.2.589

Bykova, N. V., Stensballe, A., Egsgaard, H., Jensen, O. N., and Moller, I. M. (2003). Phosphorylation of formate dehydrogenase in potato tuber mitochondria. *J. Biol. Chem.* 278, 26021–26030. doi: 10.1074/jbc.M300245200

Colas des Francs-Small, C., Ambard-Bretteville, F., Small, I. D., and Rémy, R. (1993). Identification of a major soluble protein in mitochondria from nonphotosynthetic tissues as NAD-dependent formate dehydrogenase. *Plant Physiol.* 102, 1171–1177. doi: 10.1104/pp.102.4.1171

Däschner, K., Couée, I., and Binder, S. (2001). The mitochondrial isovaleryl-coenzyme a dehydrogenase of arabidopsis oxidizes intermediates of leucine and valine catabolism. *Plant Physiol.* 126, 601–612. doi: 10.1104/pp.126.2.601

Däschner, K., Thalheim, C., Guha, C., Brennicke, A., and Binder, S. (1999). In plants a putative isovaleryl-CoA-dehydrogenase is located in mitochondria. *Plant Mol. Biol.* 39, 1275–1282.

Deuschle, K., Funck, D., Forlani, G., Stransky, H., Biehl, A., Leister, D., et al. (2004). The role of [Delta]1-pyrroline-5-carboxylate dehydrogenase in proline degradation. *Plant Cell* 16, 3413–3425. doi: 10.1105/tpc.104.023622

Deuschle, K., Funck, D., Hellmann, H., Däschner, K., Binder, S., and Frommer, W. B. (2001). A nuclear gene encoding mitochondrial Delta-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. *Plant J.* 27, 345–356. doi: 10.1046/j.1365-313X.2001.01101.x

Doremus, H. D., and Jagendorf, A. T. (1985). Subcellular localization of the pathway of de novo pyrimidine nucleotide biosynthesis in pea leaves. *Plant Physiol.* 79, 856–861. doi: 10.1104/pp.79.3.856

Douce, R., Bourguignon, J., Neuburger, M., and Rébeillé, F. (2001). The glycine decarboxylase system: a fascinating complex. *Trends Plant Sci.* 6, 167–176. doi: 10.1016/S1360-1385(01)01892-1

Dry, I. B., and Wiskich, J. T. (1987). 2-Oxoglutarate dehydrogenase and pyruvate dehydrogenase activities in plant mitochondria: interaction via a common coenzyme a pool. *Arch. Biochem. Biophys.* 257, 92–99. doi: 10.1016/0003-9861(87)90546-7

Dudkina, N. V., Sunderhaus, S., Boekema, E. J., and Braun, H.-P. (2008). The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. *J. Bioenerg. Biomembr.* 40, 419–424. doi: 10.1007/s10863-008-9167-5

Elthon, T. E., and Stewart, C. R. (1981). Submitochondrial location and electron transport characteristics of enzymes involved in proline oxidation. *Plant Physiol.* 67, 780–784. doi: 10.1104/pp.67.4.780

Engqvist, M., Drincovich, M. F., Flügge, U.-I., and Maurino, V. G. (2009). Two D-2-hydroxy-acid dehydrogenases in *Arabidopsis thaliana* with catalytic capacities to participate in the last reactions of the methylglyoxal and beta-oxidation pathways. *J. Biol. Chem.* 284, 25026–25037. doi: 10.1074/jbc.M109.021253

Engqvist, M. K. M., Kuhn, A., Wienstroer, J., Weber, K., Jansen, E. E. W., Jakobs, C., et al. (2011). Plant D-2-hydroxyglutarate dehydrogenase participates in the catabolism of lysine especially during senescence. *J. Biol. Chem.* 286, 11382–11390. doi: 10.1074/jbc.M110.194175

Escobar, M. A., Franklin, K. A., Svensson, A. S., Salter, M. G., Whitelam, G. C., and Rasmusson, A. G. (2004). Light regulation of the Arabidopsis respiratory chain. Multiple discrete photoreceptor responses contribute to induction of type II NAD(P)H dehydrogenase genes. *Plant Physiol.* 136, 2710–2721. doi: 10.1104/pp.104.046698

Faivre-Nitschke, S. E., Couée, I., Vermel, M., Grienenberger, J. M., and Gualberto, J. M. (2001). Purification, characterization and cloning of isovaleryl-CoA dehydrogenase from higher plant mitochondria. *Eur. J. Biochem.* 268, 1332–1339. doi: 10.1046/j.1432-1327.2001.01999.x

Fontaine, J.-X., Tercé-Laforgue, T., Armengaud, P., Clément, G., Renou, J.-P., Pelletier, S., et al. (2012). Characterization of a NADH-dependent glutamate dehydrogenase mutant of Arabidopsis demonstrates the key role of this enzyme in root carbon and nitrogen metabolism. *Plant Cell* 24, 4044–4065. doi: 10.1105/tpc.112.103689

Fontaine, J.-X., Tercé-Laforgue, T., Bouton, S., Pageau, K., Lea, P. J., Dubois, F., et al. (2013). Further insights into the isoenzyme composition and activity of glutamate dehydrogenase in *Arabidopsis thaliana*. *Plant Signal. Behav.* 8, e23329. doi: 10.4161/psb.23329

Forlani, G., Scainelli, D., and Nielsen, E. (1997). [delta]1-pyrroline-5-carboxylate dehydrogenase from cultured cells of potato (purification and properties). *Plant Physiol.* 113, 1413–1418.

Fujiki, Y., Ito, M., Itoh, T., Nishida, I., and Watanabe, A. (2002). Activation of the promoters of Arabidopsis genes for the branched-chain alpha-keto acid dehydrogenase complex in transgenic tobacco BY-2 cells under sugar starvation. *Plant Cell Physiol.* 43, 275–280. doi: 10.1093/pcp/pcf032

Fujiki, Y., Ito, M., Nishida, I., and Watanabe, A. (2001). Leucine and its keto acid enhance the coordinated expression of genes for branched-chain amino acid catabolism in Arabidopsis under sugar starvation. *FEBS Lett.* 499, 161–165. doi: 10.1016/S0014-5793(01)02536-4

Fujiki, Y., Sato, T., Ito, M., and Watanabe, A. (2000). Isolation and characterization of cDNA clones for the e1beta and E2 subunits of the branched-chain alpha-ketoacid dehydrogenase complex in Arabidopsis. *J. Biol. Chem.* 275, 6007–6013. doi: 10.1074/jbc.275.8.6007

Fukushima, T., Decker, R. V., Anderson, W. M., and Spivey, H. O. (1989). Substrate channeling of NADH in mitochondrial redox processes. *J. Biol. Chem.* 264, 16483–16488.

Funck, D., Eckard, S., and Müller, G. (2010). Non-redundant functions of two proline dehydrogenase isoforms in Arabidopsis. *BMC Plant Biol.* 10:70. doi: 10.1186/1471-2229-10-70

Gietl, C. (1992). Malate dehydrogenase isoenzymes: cellular locations and role in the flow of metabolites between the cytoplasm and cell organelles. *Biochim. Biophys. Acta* 1100, 217–234. doi: 10.1016/0167-4838(92)90476-T

Glaser, E., Eriksson, A., and Sjöling, S. (1994). Bifunctional role of the bc1 complex in plants. Mitochondrial bc1 complex catalyses both electron transport and protein processing. *FEBS Lett.* 346, 83–87. doi: 10.1016/0014-5793(94)00312-2

Goetzman, E. S., Mohsen, A.-W. A., Prasad, K., and Vockley, J. (2005). Convergent evolution of a 2-methylbutyryl-CoA dehydrogenase from isovaleryl-CoA dehydrogenase in *Solanum tuberosum*. *J. Biol. Chem.* 280, 4873–4879. doi: 10.1074/jbc.M412640200

Grof, C. P., Winning, B. M., Scaysbrook, T. P., Hill, S. A., and Leaver, C. J. (1995). Mitochondrial pyruvate dehydrogenase. Molecular cloning of the E1 alpha subunit and expression analysis. *Plant Physiol.* 108, 1623–1629. doi: 10.1104/pp.108.4.1623

Halliwell, B. (1974). Oxidation of formate by peroxisomes and mitochondria from spinach leaves. *Biochem. J.* 138, 77–85.

Heazlewood, J. L., Howell, K. A., Whelan, J., and Millar, A. H. (2003). Towards an analysis of the rice mitochondrial proteome. *Plant Physiol.* 132, 230–242. doi: 10.1104/pp.102.018986

Hourton-Cabassa, C., Ambard-Bretteville, F., Moreau, F, and Davy de Virville, J., Rémy, R., and Francs-Small, C. C. (1998). Stress induction of mitochondrial formate dehydrogenase in potato leaves. *Plant Physiol.* 116, 627–635. doi: 10.1104/pp.116.2.627

Huang, S., and Millar, A. H. (2013). Succinate dehydrogenase: the complex roles of a simple enzyme. *Curr. Opin. Plant Biol.* 16, 344–349. doi: 10.1016/j.pbi.2013.02.007

Ingle, R. A. (2011). Histidine biosynthesis. *Arabidopsis Book* 9, e0141. doi: 10.1199/tab.0141

Ishizaki, K., Larson, T. R., Schauer, N., Fernie, A. R., Graham, I. A., and Leaver, C. J. (2005). The critical role of Arabidopsis electron-transfer flavoprotein:ubiquinone oxidoreductase during dark-induced starvation. *Plant Cell* 17, 2587–2600. doi: 10.1105/tpc.105.035162

Ishizaki, K., Schauer, N., Larson, T. R., Graham, I. A., Fernie, A. R., and Leaver, C. J. (2006). The mitochondrial electron transfer flavoprotein complex is essential for survival of Arabidopsis in extended darkness. *Plant J.* 47, 751–760. doi: 10.1111/j.1365-313X.2006.02826.x

Jacoby, R. P., Li, L., Huang, S., Pong Lee, C., Millar, A. H., and Taylor, N. L. (2012). Mitochondrial composition, function and stress response in plants. *J. Integr. Plant Biol.* 54, 887–906. doi: 10.1111/j.1744-7909.2012.01177.x

Jänsch, L., Kruft, V., Schmitz, U. K., and Braun, H. P. (1996). New insights into the composition, molecular mass and stoichiometry of the protein complexes of plant mitochondria. *Plant J.* 9, 357–368. doi: 10.1046/j.1365-313X.1996.09030357.x

Jenner, H. L., Winning, B. M., Millar, A. H., Tomlinson, K. L., Leaver, C. J., and Hill, S. A. (2001). NAD malic enzyme and the control of carbohydrate metabolism in potato tubers. *Plant Physiol.* 126, 1139–1149. doi: 10.1104/pp.126.3.1139

Journet, E. P., Neuburger, M., and Douce, R. (1981). Role of glutamate-oxaloacetate transaminase and malate dehydrogenase in the regeneration of NAD for glycine oxidation by spinach leaf mitochondria. *Plant Physiol.* 67, 467–469. doi: 10.1104/pp.67.3.467

Kirch, H.-H., Bartels, D., Wei, Y., Schnable, P. S., and Wood, A. J. (2004). The ALDH gene superfamily of Arabidopsis. *Trends Plant Sci.* 9, 371–377. doi: 10.1016/j.tplants.2004.06.004

Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1996). A nuclear gene encoding mitochondrial proline dehydrogenase, an enzyme involved in proline metabolism, is upregulated by proline but downregulated by dehydration in Arabidopsis. *Plant Cell* 8, 1323–1335.

Klodmann, J., Senkler, M., Rode, C., and Braun, H.-P. (2011). Defining the protein complex proteome of plant mitochondria. *Plant Physiol.* 157, 587–598. doi: 10.1104/pp.111.182352

Krömer, S. (1995) Respiration during photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 46, 47–70. doi: 10.1146/annurev.pp.46.060195.000401

Lancien, M., Gadal, P., and Hodges, M. (1998). Molecular characterization of higher plant NAD-dependent isocitrate dehydrogenase: evidence for a heteromeric structure by the complementation of yeast mutants. *Plant J.* 16, 325–333. doi: 10.1046/j.1365-313x.1998.00305.x

Lee, C. P., Eubel, H., O'Toole, N., and Millar, A. H. (2008). Heterogeneity of the mitochondrial proteome for photosynthetic and non-photosynthetic Arabidopsis metabolism. *Mol. Cell Proteomics* 7, 1297–1316. doi: 10.1074/mcp.M700535-MCP200

Leferink, N. G. H., van den Berg, W. A. M., and van Berkel, W. J. H. (2008). l-Galactono-gamma-lactone dehydrogenase from *Arabidopsis thaliana*,

a flavoprotein involved in vitamin C biosynthesis. *FEBS J.* 275, 713–726. doi: 10.1111/j.1742-4658.2007.06233.x

Leferink, N. G. H., van Duijn, E., Barendregt, A., Heck, A. J. R., and van Berkel, W. J. H. (2009). Galactonolactone dehydrogenase requires a redox-sensitive thiol for optimal production of vitamin C. *Plant Physiol.* 150, 596–605. doi: 10.1104/pp.109.136929

Lemaitre, T., and Hodges, M. (2006). Expression analysis of *Arabidopsis thaliana* NAD-dependent isocitrate dehydrogenase genes shows the presence of a functional subunit that is mainly expressed in the pollen and absent from vegetative organs. *Plant Cell Physiol.* 47, 634–643. doi: 10.1093/pcp/pcj030

Lemaitre, T., Urbanczyk-Wochniak, E., Flesch, V., Bismuth, E., Fernie, A. R., and Hodges, M. (2007). NAD-dependent isocitrate dehydrogenase mutants of Arabidopsis suggest the enzyme is not limiting for nitrogen assimilation. *Plant Physiol.* 144, 1546–1558. doi: 10.1104/pp.107.100677

Lin, M., Behal, R. H., and Oliver, D. J. (2004) Characterization of a mutation in the IDH-II subunit of the NAD1-dependent isocitrate dehydrogenase from *Arabidopsis thaliana*. *Plant Sci.* 166, 983–988. doi: 10.1016/j.plantsci.2003.12.012

Luethy, M. H., Miernyk, J. A., and Randall, D. D. (1994). The nucleotide and deduced amino acid sequences of a cDNA encoding the E1 beta-subunit of the *Arabidopsis thaliana* mitochondrial pyruvate dehydrogenase complex. *Biochim. Biophys. Acta* 1187, 95–98. doi: 10.1016/0005-2728(94)90171-6

Mani, S., van de Cotte, B., van Montagu, M., and Verbruggen, N. (2002). Altered levels of proline dehydrogenase cause hypersensitivity to proline and its analogs in Arabidopsis. *Plant Physiol.* 128, 73–83. doi: 10.1104/pp.010572

Mapson, L. W., and Breslow, E. (1958). Biological synthesis of ascorbic acid: L-galactono-gamma-lactone dehydrogenase. *Biochem. J.* 68, 395–406.

Miersch, J., Grancharov, K., Krauss, G. J., Spassovska, N., Karamanov, G., Maneva, L., et al. (1987). Biological activity and mode of action of some dihydroorotic and dihydroazaorotic acid derivatives. *Biomed. Biochim. Acta* 46, 307–315.

Millar, A. H., Eubel, H., Jänsch, L., Kruft, V., Heazlewood, J. L., and Braun, H.-P. (2004). Mitochondrial cytochrome c oxidase and succinate dehydrogenase complexes contain plant specific subunits. *Plant Mol. Biol.* 56, 77–90. doi: 10.1007/s11103-004-2316-2

Millar, A. H., Hill, S. A., and Leaver, C. J. (1999). Plant mitochondrial 2-oxoglutarate dehydrogenase complex: purification and characterization in potato. *Biochem. J.* 343(Pt 2), 327–334. doi: 10.1042/0264-6021:3430327

Millar, A. H., Small, I. D., Day, D. A., and Whelan, J. (2008). Mitochondrial biogenesis and function in Arabidopsis. *Arabidopsis Book* 6, e0111. doi: 10.1199/tab.0111

Millar, A. H., Whelan, J., Soole, K. L., and Day, D. A. (2011). Organization and regulation of mitochondrial respiration in plants. *Annu. Rev. Plant Biol.* 62, 79–104. doi: 10.1146/annurev-arplant-042110-103857

Miller, G., Honig, A., Stein, H., Suzuki, N., Mittler, R., and Zilberstein, A. (2009). Unraveling delta1-pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation enzymes. *J. Biol. Chem.* 284, 26482–26492. doi: 10.1074/jbc.M109.009340

Miyashita, Y., and Good, A. G. (2008a). Glutamate deamination by glutamate dehydrogenase plays a central role in amino acid catabolism in plants. *Plant Signal. Behav.* 3, 842–843. doi: 10.4161/psb.3.10.5936

Miyashita, Y., and Good, A. G. (2008b). NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidopsis thaliana* during dark-induced carbon starvation. *J. Exp. Bot.* 59, 667–680. doi: 10.1093/jxb/erm340

Mooney, B. P., Henzl, M. T., Miernyk, J. A., and Randall, D. D. (2000). The dihydrolipoyl acyltransferase (BCE2) subunit of the plant branched-chain alpha-ketoacid dehydrogenase complex forms a 24-mer core with octagonal symmetry. *Protein Sci.* 9, 1334–1339. doi: 10.1110/ps.9.7.1334

Moore, A. L., Shiba, T., Young, L., Harada, S., Kita, K., and Ito, K. (2013). Unraveling the heater: new insights into the structure of the alternative oxidase. *Annu. Rev. Plant Biol.* 64, 637–663. doi: 10.1146/annurev-arplant-042811-105432

Nagai, A., and Scheidegger, A. (1991). Purification and characterization of histidinol dehydrogenase from cabbage. *Arch. Biochem. Biophys.* 284, 127–132. doi: 10.1016/0003-9861(91)90274-M

Nunes-Nesi, A., Carrari, F., Lytovchenko, A., Smith, A. M. O., Loureiro, M. E., Ratcliffe, R. G., et al. (2005). Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. *Plant Physiol.* 137, 611–622. doi: 10.1104/pp.104.055566

Oguchi, K., Tanaka, N., Komatsu, S., and Akao, S. (2004). Methylmalonate-semialdehyde dehydrogenase is induced in auxin-stimulated and zinc-stimulated root formation in rice. *Plant Cell Rep.* 22, 848–858. doi: 10.1007/s00299-004-0778-y

Oliver, D. J. (1994) The glycine decarboxylase complex from plant mitochondria. *Annu. Rev.Plant Physiol. Plant Mol. Biol.* 45, 323–338. doi: 10.1146/annurev.pp.45.060194.001543

Oliver, D. J., Neuburger, M., Bourguignon, J., and Douce, R. (1990). Interaction between the component enzymes of the glycine decarboxylase multienzyme complex. *Plant Physiol.* 94, 833–839. doi: 10.1104/pp.94.2.833

Olson, B. J., Skavdahl, M., Ramberg, H., Osterman, J. C., and Markwell, J. (2000). Formate dehydrogenase in *Arabidopsis thaliana*: characterization and possible targeting to the chloroplast. *Plant Sci.* 159, 205–212. doi: 10.1016/S0168-9452(00)00337-X

Pineau, B., Layoune, O., Danon, A., and Paepe, R., de (2008). L-galactono-1,4-lactone dehydrogenase is required for the accumulation of plant respiratory complex I. *J. Biol. Chem.* 283, 32500–32505. doi: 10.1074/jbc.M805320200

Poulsen, L. L., and Wedding, R. T. (1970). Purification and properties of the alpha-ketoglutarate dehydrogenase complex of cauliflower mitochondria. *J. Biol. Chem.* 245, 5709–5717.

Rasmusson, A. G., and Agius, S. C. (2001) Rotenone-insensitive NAD(P)H dehydrogenases in plants: immunodetection and distribution of native proteins in mitochondria. *Plant Physiol. Biochem.* 39, 1057–1066. doi: 10.1016/S0981-9428(01)01334-1

Rasmusson, A. G., Geisler, D. A., and Møller, I. M. (2008). The multiplicity of dehydrogenases in the electron transport chain of plant mitochondria. *Mitochondrion* 8, 47–60. doi: 10.1016/j.mito.2007.10.004

Rasmusson, A. G., and Moller, I. M. (2011). "Mitochondrial electron transport and plant stress," in *Plant Mitochondria*, ed F. Kempken (New York, NY: Springer), 357–381.

Rasmusson, A. G., Soole, K. L., and Elthon, T. E. (2004). Alternative NAD(P)H dehydrogenases of plant mitochondria. *Annu. Rev. Plant Biol.* 55, 23–39. doi: 10.1146/annurev.arplant.55.031903.141720

Reinard, T., Janke, V., Willard, J., Buck, F., Jacobsen, H. J., and Vockley, J. (2000). Cloning of a gene for an acyl-CoA dehydrogenase from *Pisum sativum* L. and purification and characterization of its product as an isovaleryl-CoA dehydrogenase. *J. Biol. Chem.* 275, 33738–33743. doi: 10.1074/jbc.M004178200

Schertl, P., Cabassa, C., Saadallah, K., Bordenave, M., Savouré, A., and Braun, H.P. (in press). Biochemical characterization of ProDH activity in Arabidopsis mitochondria. *FEBS J.* doi: 10.1111/febs.12821

Schertl, P., Sunderhaus, S., Klodmann, J., Grozeff, G. E. G., Bartoli, C. G., and Braun, H.-P. (2012). L-galactono-1,4-lactone dehydrogenase (GLDH) forms part of three subcomplexes of mitochondrial complex I in *Arabidopsis thaliana*. *J. Biol. Chem.* 287, 14412–14419. doi: 10.1074/jbc.M111.305144

Sharma, S., and Verslues, P. E. (2010). Mechanisms independent of abscisic acid (ABA) or proline feedback have a predominant role in transcriptional regulation of proline metabolism during low water potential and stress recovery. *Plant Cell Environ.* 33, 1838–1851. doi: 10.1111/j.1365-3040.2010.02188.x

Shen, W., Wei, Y., Dauk, M., Tan, Y., Taylor, D. C., Selvaraj, G., et al. (2006). Involvement of a glycerol-3-phosphate dehydrogenase in modulating the NADH/NAD+ ratio provides evidence of a mitochondrial glycerol-3-phosphate shuttle in Arabidopsis. *Plant Cell* 18, 422–441. doi: 10.1105/tpc.105.039750

Shen, W., Wei, Y., Dauk, M., Zheng, Z., and Zou, J. (2003). Identification of a mitochondrial glycerol-3-phosphate dehydrogenase from *Arabidopsis thaliana*: evidence for a mitochondrial glycerol-3-phosphate shuttle in plants. *FEBS Lett.* 536, 92–96. doi: 10.1016/S0014-5793(03)00033-4

Siendones, E., Gonzalez-Reyes, J. A., Santos-Ocana, C., Navas, P., and C rdoba, F. (1999). Biosynthesis of ascorbic acid in kidney bean. L-galactono-gamma-lactone dehydrogenase is an intrinsic protein located at the mitochondrial inner membrane. *Plant Physiol.* 120, 907–912. doi: 10.1104/pp.120.3.907

Somerville, C. R., and Ogren, W. L. (1982). Mutants of the cruciferous plant *Arabidopsis thaliana* lacking glycine decarboxylase activity. *Biochem. J.* 202, 373–380.

Srinivasan, R., and Oliver, D. J. (1995). Light-dependent and tissue-specific expression of the H-protein of the glycine decarboxylase complex. *Plant Physiol.* 109, 161–168. doi: 10.1104/pp.109.1.161

Sweetlove, L. J., Beard, K. F. M., Nunes-Nesi, A., Fernie, A. R., and Ratcliffe, R. G. (2010). Not just a circle: flux modes in the plant TCA cycle. *Trends Plant Sci.* 15, 462–470. doi: 10.1016/j.tplants.2010.05.006

Szabados, L., and Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends Plant Sci.* 15, 89–97. doi: 10.1016/j.tplants.2009.11.009

Szurmak, B., Strokovskaya, L., Mooney, B. P., Randall, D. D., and Miernyk, J. A. (2003). Expression and assembly of *Arabidopsis thaliana* pyruvate dehydrogenase in insect cell cytoplasm. *Protein Expr. Purif.* 28, 357–361. doi: 10.1016/S1046-5928(02)00712-X

Tanaka, N., Takahashi, H., Kitano, H., Matsuoka, M., Akao, S., Uchimiya, H., et al. (2005). Proteome approach to characterize the methylmalonate-semialdehyde dehydrogenase that is regulated by gibberellin. *J. Proteome Res.* 4, 1575–1582. doi: 10.1021/pr050114f

Tarasenko, V. I., Garnik, E. Y., and Konstantinov, Y. M. (2013). Rate of alternative electron transport in arabidopsis mitochondria affects the expression of the glutamate dehydrogenase gene gdh2. *Dokl. Biochem. Biophys.* 452, 234–236. doi: 10.1134/S1607672913050037

Taylor, N. L., Heazlewood, J. L., Day, D. A., and Millar, A. H. (2004). Lipoic acid-dependent oxidative catabolism of alpha-keto acids in mitochondria provides evidence for branched-chain amino acid catabolism in Arabidopsis. *Plant Physiol.* 134, 838–848. doi: 10.1104/pp.103.035675

Tomaz, T., Bagard, M., Pracharoenwattana, I., Lindén, P., Lee, C. P., Carroll, A. J., et al. (2010). Mitochondrial malate dehydrogenase lowers leaf respiration and alters photorespiration and plant growth in Arabidopsis. *Plant Physiol.* 154, 1143–1157. doi: 10.1104/pp.110.161612

Tovar-Méndez, A., Miernyk, J. A., and Randal,l D. D. (2003) Regulation of pyruvate dehydrogenase complex activity in plant cells. *Eur. J. Biochem*. 270, 1043–1049. doi: 10.1046/j.1432-1033.2003.03469.x

Toyokura, K., Watanabe, K., Oiwaka, A., Kusano, M., Tameshige, T., Tatematsu, K., et al. (2011). Succinic semialdehyde dehydrogenase is involved in the robust patterning of Arabidopsis leaves along the adaxial-abaxial axis. *Plant Cell Physiol.* 52, 1340–1353. doi: 10.1093/pcp/pcr079

Tronconi, M. A., Fahnenstich, H., Gerrard Weehler, M. C., Andreo, C. S., Flügge, U.-I., Drincovich, M. F., et al. (2008). Arabidopsis NAD-malic enzyme functions as a homodimer and heterodimer and has a major impact on nocturnal metabolism. *Plant Physiol.* 146, 1540–1552. doi: 10.1104/pp.107.114975

Tronconi, M. A., Gerrard Wheeler, M. C., Drincovich, M. F., and Andreo, C. S. (2012). Differential fumarate binding to Arabidopsis NAD+-malic enzymes 1 and -2 produces an opposite activity modulation. *Biochimie* 94, 1421–1430. doi: 10.1016/j.biochi.2012.03.017

Tronconi, M. A., Maurino, V. G., Andreo, C. S., and Drincovich, M. F. (2010). Three different and tissue-specific NAD-malic enzymes generated by alternative subunit association in *Arabidopsis thaliana*. *J. Biol. Chem.* 285, 11870–11879. doi: 10.1074/jbc.M109.097477

Turano, F. J., Thakkar, S. S., Fang, T., and Weisemann, J. M. (1997). Characterization and expression of NAD(H)-dependent glutamate dehydrogenase genes in Arabidopsis. *Plant Physiol.* 113, 1329–1341. doi: 10.1104/pp.113.4.1329

Ullrich, A., Knecht, W., Piskur, J., and Löffler, M. (2002). Plant dihydroorotate dehydrogenase differs significantly in substrate specificity and inhibition from the animal enzymes. *FEBS Lett.* 529, 346–350. doi: 10.1016/S0014-5793(02)03425-7

van Dongen, J. T., Gupta, K. J., Ramírez-Aguilar, S. J., Araújo, W. L., Nunes-Nesi, A., and Fernie, A. R. (2011). Regulation of respiration in plants: a role for alternative metabolic pathways. *J. Plant Physiol.* 168, 1434–1443. doi: 10.1016/j.jplph.2010.11.004

Verbruggen, N., Hua, X. J., May, M., and van Montagu, M. (1996). Environmental and developmental signals modulate proline homeostasis: evidence for a negative transcriptional regulator. *Proc. Natl. Acad. Sci. U.S.A.* 93, 8787–8791. doi: 10.1073/pnas.93.16.8787

Wallström, S. V., Florez-Sarasa, I., Araújo, W. L., Aidemark, M., Fernández-Fernández, M., Fernie, A. R., et al. (2014a). Suppression of the external mitochondrial NADPH dehydrogenase, NDB1, in *Arabidopsis thaliana* affects central metabolism and vegetative growth. *Mol. Plant* 7, 356–368. doi: 10.1093/mp/sst115

Wallström, S. V., Florez-Sarasa, I., Araújo, W. L., Escobar, M. A., Geisler, D. A., Aidemark, M., et al. (2014b). Suppression of NDA-type alternative mitochondrial NAD(P)H dehydrogenases in *Arabidopsis thaliana* modifies growth and metabolism, but not high light stimulation of mitochondrial electron transport. *Plant Cell Physiol.* doi: 10.1093/pcp/pcu021. [Epub ahead of print].

Wedding, R. T., and Black, M. K. (1971a). Evidence for tighter binding of magnesium-thiamine pyrophosphate to -ketoglutarate dehydrogenase when activated by adenosine monophosphate. *J. Biol. Chem.* 246, 4097–4099.

Wedding, R. T., and Black, M. K. (1971b). Nucleotide activation of cauliflower alpha-ketoglutarate dehydrogenase. *J. Biol. Chem.* 246, 1638–1643.

Wienstroer, J., Engqvist, M. K. M., Kunz, H.-H., Flügge, U.-I., and Maurino, V. G. (2012). D-Lactate dehydrogenase as a marker gene allows positive selection of transgenic plants. *FEBS Lett.* 586, 36–40. doi: 10.1016/j.febslet.2011.11.020

Wulff, A., Oliveira, H. C., Saviani, E. E., and Salgado, I. (2009) Nitrite reduction and superoxide-dependent nitric oxide degradation by Arabidopsis mitochondria: influence of external NAD(P)H dehydrogenases and alternative oxidase in the control of nitric oxide levels. *Nitric Oxide* 21, 132–139. doi: 10.1016/j.niox.2009.06.003

Yamaya, T., Oaks, A., and Matsumoto, H. (1984). Characteristics of glutamate dehydrogenase in mitochondria prepared from corn shoots. *Plant Physiol.* 76, 1009–1013. doi: 10.1104/pp.76.4.1009

Yu, H., Du, X., Zhang, F., Zhang, F., Hu, Y., Liu, S., et al. (2012). A mutation in the E2 subunit of the mitochondrial pyruvate dehydrogenase complex in Arabidopsis reduces plant organ size and enhances the accumulation of amino acids and intermediate products of the TCA cycle. *Planta* 236, 387–399. doi: 10.1007/s00425-012-1620-3

Zabaleta, E., Martin, M. V., and Braun, H.-P. (2012). A basal carbon concentrating mechanism in plants? *Plant Sci.* 187, 97–104. doi: 10.1016/j.plantsci.2012.02.001

Zhu, X., Tang, G., and Galili, G. (2000) Characterization of the two saccharopine dehydrogenase isozymes of lysine catabolism encoded by the single composite AtLKR=SDH locus of Arabidopsis. *Plant Physiol.* 124, 1363–1372. doi: 10.1104/pp.124.3.1363

Zou, J., Qi, Q., Katavic, V., Marillia, E. F., and Taylor, D. C. (1999). Effects of antisense repression of an *Arabidopsis thaliana* pyruvate dehydrogenase kinase cDNA on plant development. *Plant Mol. Biol.* 41, 837–849. doi: 10.1023/A:1006393726018

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 February 2014; accepted: 07 April 2014; published online: 29 April 2014.
Citation: Schertl P and Braun H-P (2014) Respiratory electron transfer pathways in plant mitochondria. Front. Plant Sci. ***5****:163. doi: 10.3389/fpls.2014.00163*
This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 18 February 2014
doi: 10.3389/fpls.2014.00035

Group II intron splicing factors in plant mitochondria

Gregory G. Brown[1], Catherine Colas des Francs-Small[2] and Oren Ostersetzer-Biran[3]*

[1] *Department of Biology, McGill University, Montreal, QC, Canada*
[2] *ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, Crawley, WA, Australia*
[3] *Department of Plant and Environmental Sciences, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Mamoru Sugita, Nagoya University, Japan
Benoît Castandet, Boyce Thompson Institute for Plant Research, USA

***Correspondence:**
Oren Ostersetzer-Biran, Department of Plant and Environmental Sciences, The Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Edmond J. Safra Campus, Givat Ram, Jerusalem 91904, Israel
e-mail: oren.ostersetzer@mail.huji.ac.il

Group II introns are large catalytic RNAs (ribozymes) which are found in bacteria and organellar genomes of several lower eukaryotes, but are particularly prevalent within the mitochondrial genomes (mtDNA) in plants, where they reside in numerous critical genes. Their excision is therefore essential for mitochondria biogenesis and respiratory functions, and is facilitated *in vivo* by various protein cofactors. Typical group II introns are classified as mobile genetic elements, consisting of the self-splicing ribozyme and its intron-encoded maturase protein. A hallmark of maturases is that they are intron specific, acting as cofactors which bind their own cognate containing pre-mRNAs to facilitate splicing. However, the plant organellar introns have diverged considerably from their bacterial ancestors, such as they lack many regions which are necessary for splicing and also lost their evolutionary related maturase ORFs. In fact, only a single maturase has been retained in the mtDNA of various angiosperms: the *matR* gene encoded in the fourth intron of the NADH-dehydrogenase subunit 1 (*nad1* intron 4). Their degeneracy and the absence of cognate ORFs suggest that the splicing of plant mitochondria introns is assisted by *trans*-acting cofactors. Interestingly, in addition to MatR, the nuclear genomes of angiosperms also harbor four genes (*nMat 1-4*), which are closely related to maturases and contain N-terminal mitochondrial localization signals. Recently, we established the roles of two of these paralogs in Arabidopsis, nMAT1 and nMAT2, in the splicing of mitochondrial introns. In addition to the nMATs, genetic screens led to the identification of other genes encoding various factors, which are required for the splicing and processing of mitochondrial introns in plants. In this review we will summarize recent data on the splicing and processing of mitochondrial introns and their implication in plant development and physiology, with a focus on maturases and their accessory splicing cofactors.

Keywords: group II intron, splicing, maturase, splicing factor, respiration, mitochondria, plant

PLANT MITOCHONDRIAL GENOMES (mtDNAs)

Mitochondria in plants house the oxidative phosphorylation (OXPHOS) machinery and many other essential metabolic pathways (for review see Millar et al., 2011). The vast majority of the proteins responsible for these processes, as well as those that participate in the biogenesis of the organelle (e.g., the translocons involved in protein import) are encoded in the nuclear genome. A small number of essential proteins, however, are encoded in the organelle's own genome. In vascular plants these genomes (mitochondrial DNAs, or mtDNAs) are much larger and more variable in size than the mtDNAs of other organisms and also display an array of other unique features (Knoop, 2012). The mtDNAs in plants encode tRNAs, rRNAs, ribosomal proteins, subunits of the respiratory machinery, including NADH:ubiquinone oxidoreductase (complex I), the cytochrome bc_1 complex (complex III), cytochrome *c* oxidase (complex IV), ATP-synthase (complex V), and several other proteins involved in cytochrome *c* biogenesis and the twin-arginine protein translocation (Unseld et al., 1997; Kubo et al., 2000; Adams et al., 2002; Notsu et al., 2002; Handa, 2003; Clifton et al., 2004; Ogihara et al., 2005; Sugiyama et al., 2005). The mitochondrial translation machinery and energy transduction complexes are composed of both nuclear and organellar encoded subunits, thus necessitating complex mechanisms for the coordination of the expression of these two physically distinct genomes.

The expression of mitochondrial genes in plants is regulated primarily at the post-transcriptional level (Finnegan and Brown, 1990; Binder and Brennicke, 2003). The primary transcripts in plant mitochondria undergo extensive processing, including the nucleolytic maturation of 5′ and 3′ termini, RNA editing (C-to-U changes in angiosperms), and the splicing of numerous introns which lie within genes encoding proteins required for both organellar gene expression and respiration (Gagliardi and Binder, 2007; Bonen, 2008; Takenaka et al., 2008). These processes are necessary for these RNAs to carry out their functions in protein synthesis and are accomplished largely by nuclear-encoded factors, which may also provide a means to link organellar functions with environmental and/or developmental signals (see **Figure 1**).

In this review, we will summarize recent progress on the splicing of mitochondrial group II introns in angiosperms, with emphasis on RNA maturases and several other accessory factors.

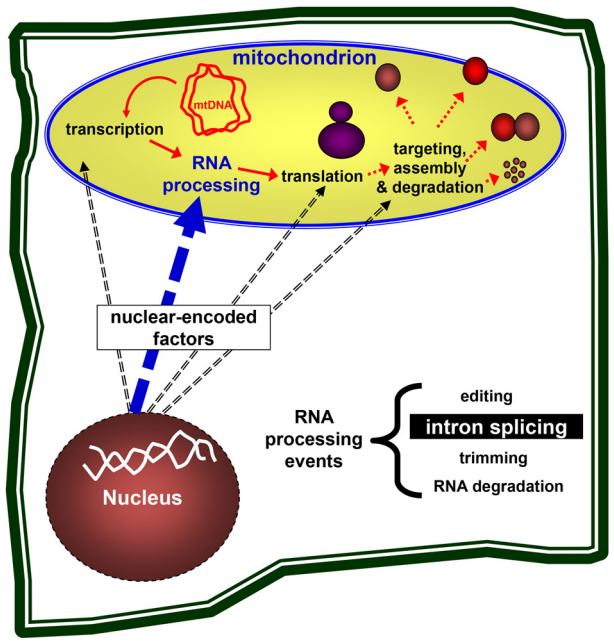


FIGURE 1 | Nuclear genes control the mitochondria biogenesis. Nuclear-encoded genes are necessary for the expression of mitochondrial genes, including transcription, pre-mRNA processing, translation of the mRNAs into mitochondrial proteins, the assembly of ribosomes and respiratory complexes, and are also required for the targeting and degradation of organellar subunits. Plant organelles are excellent systems to study these processes in eukaryotes.

GROUP II INTRONS

Introns within organellar genomes in plants belong to group I and group II (Michel et al., 1989; Bonen and Vogel, 2001; Zimmerly et al., 2001; Lambowitz and Zimmerly, 2004; Bonen, 2008). The vast majority of mitochondrial introns in angiosperms are classified as group II intron RNAs (Bonen, 2008). Introns in this class have been identified in prokaryotes, where they are relatively rare, and in the mitochondria of fungi, protists and a few primitive metazoans and in chloroplasts. Group II introns are particularly prevalent, however, in the mtDNAs in vascular plants (Malek and Knoop, 1998; Bonen and Vogel, 2001; Belfort et al., 2002; Bonen, 2008; Lambowitz and Zimmerly, 2011). These large introns are defined mainly by their capacity to fold into a conserved secondary structure of six domains (DI–DVI) extending from a central hub (Michel and Ferat, 1995; Qin and Pyle, 1998). Such intron structure models were later supported by a crystal structure of a self-spliced group II intron from *Oceanobacillus iheyensis*, further showing that multiple interactions between the different domains are indeed required to stabilize the tertiary structure of group II introns into their catalytically active forms (Toor et al., 2008). **Figure 2A** represents a secondary structure model of plant mitochondria group II intron RNAs.

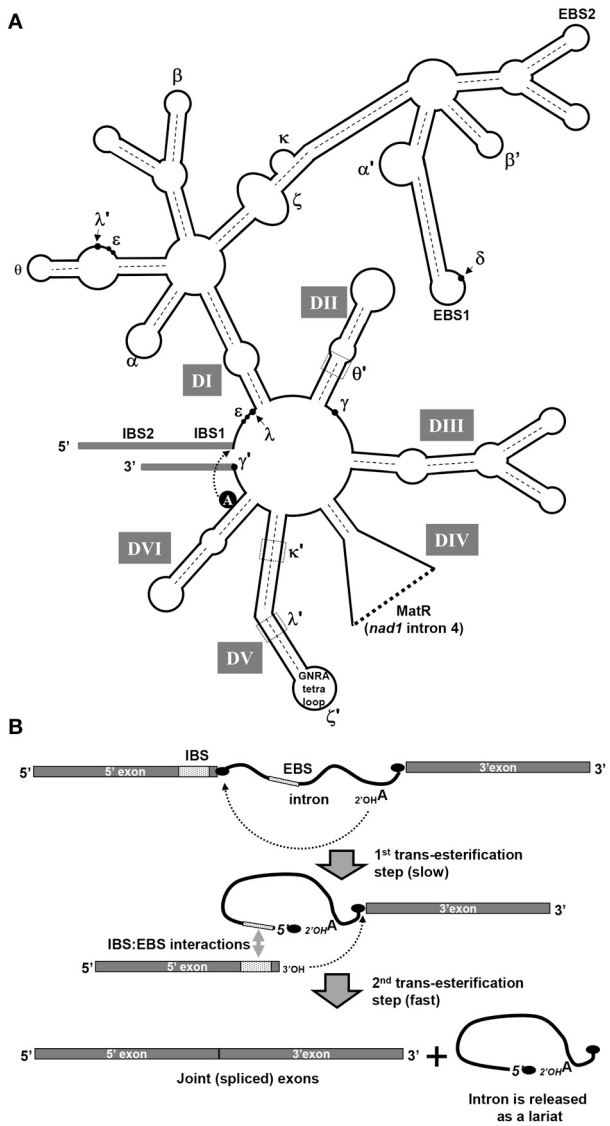


FIGURE 2 | Secondary structure model of plant mitochondrial group II introns. (A) The secondary structure of group II introns is characterized by six double-helical domains (I-VI), arising from a central hub. Each subdomain of DI and DII, DIII, DIV, DV, and DVI are outlined within the structure. All plant mitochondrial intron structures in angiosperms are classified as standard group IIA RNAs (Bonen, 2008). The conserved bulged-A residue in DVI, the exon-intron binding sites (i.e., EBS1/IBS1 and EBS2/IBS2), and tertiary interactions between different intron regions (indicated by roman letters) are shown in the model structure. The ORF encoding the MatR protein in *nad1* intron 4 is encoded in intron domain IV. **(B)** Splicing pathway of the autocatalytic group II introns occurs by a two-step trans-esterification pathway. In the first step of the branching pathway, the 2′-OH group of the branch point adenosine nucleophilically attacks the phosphate at the 5′-splice site. The 5′-exon is released and the attacking adenosine adopts a 2′,5′-branched structure that gives the intron a lariat form. Yet, in addition to this classical "branch-point" splicing reaction, some mitochondrial introns which lacks a DVI bulged A are excised as linear molecules which are generated by a "hydrolytic-pathway" of splicing (Li-Pook-Than and Bonen, 2006).

SPLICING MECHANISM

The splicing of group II introns is mechanistically identical to that of the nuclear spliceosomal introns. Splicing is initiated through a trans-esterification involving a nucleophilic attack on the 5′ splice site by the 2′ hydroxyl group of an adenosine residue in domain VI (see **Figure 2B**). This results in the formation of a free 3′ hydroxyl at the 5′ splice site and a lariat shaped intermediate. In a second trans-esterification step, the flanking exons are joined when the free 3′ hydroxyl at the 5′ splice site forms

a 5′-3′ phosphodiester bond with the nucleotide at the 3′ splice site; the intron is then finally released as a lariat. An alternative splicing reaction involves the "hydrolytic pathway," where a water molecule acts as the nucleophile in the first step and cleaves the 5′-exon from the intron without forming a branched structure (Daniels et al., 1996). The second step of splicing is identical to the "branched-point" reaction; the 3′-terminal OH group of the 5′-exon attacks the phosphate at the 3′-splice site, thereby splicing the exons and releasing the intron as a linear form. The presence of both linear and circular forms of excised intron molecules in plant mitochondria points to multiple novel group II splicing mechanisms *in vivo* (Li-Pook-Than and Bonen, 2006).

Because some group II introns can splice auto-catalytically, their splicing, and by extension the splicing of nuclear spliceosomal introns, can be considered to be an example of RNA-mediated catalysis. The conditions required for *in vitro* splicing are generally non-physiological and most, if not all, group II introns require proteins for splicing *in vivo.*

Few organellar introns, and the majority of prokaryotic group II introns, can function as "homing" retroelements that insert themselves into related genomic sites (Cousineau et al., 1998; Lambowitz and Zimmerly, 2004). This process is mediated through the association of the intron with a specific reverse transcriptase (RT) encoded within the domain IV of the intron itself, termed as maturases (see below and **Figure 2**). In general, the interaction between the maturase and its cognate group II intron is critical for both splicing and mobility (Lambowitz and Zimmerly, 2004).

Interestingly, many plant organellar group II introns lack an ORF capable of specifying a maturase, or they encode a degenerate maturase protein which is probably unable to promote intron mobility (Michel et al., 1989; Bonen and Vogel, 2001; Zimmerly et al., 2001; Barkan, 2004; Lambowitz and Zimmerly, 2004; Bonen, 2008). These introns must therefore rely on other cofactors to facilitate their splicing *in vivo* (see below). Group II introns have not yet been identified in the nuclear genomes of eukaryotes, but their predicted descendants (i.e., the spliceosomal introns and retrotransposons), are highly abundant in eukaryotes, comprising together more than half of the genome in mammals.

Cis- AND TRANS-SPLICING

In prokaryotes, group II introns are often found near genes or after *rho* elements (Dai et al., 2003), and are thus expected to be expressed at low levels and to have only little effect on bacteria fitness. By contrast, the mitochondrial introns in plants are commonly (if not exclusively) found within the coding regions of various genes required for both genome expression and energy transduction (Bonen and Vogel, 2001). Structure-wise, some of the organellar introns in plants have diverged considerably from their bacterial ancestors and have lost elements considered to be essential for splicing (Bonen and Vogel, 2001; Bonen, 2008). Accordingly, none of the mitochondrial introns in angiosperms has been found to self-splice *in vitro*. Moreover, several of the mitochondrial introns in plants (i.e., *nad 1* introns 1 and 3, *nad2* intron 2, and *nad5* introns 3 and 4; **Table 1**) are fragmented in that they are transcribed as separate RNAs, which must assemble *in trans* through base-pairing interactions to form a splicing-competent structure (Chapdelaine and Bonen, 1991; Knoop et al., 1991; Pereira de Souza et al., 1991; Malek and Knoop, 1998; Bonen and Vogel, 2001; Bonen, 2008). In addition to *nad1*, *nad2*, and *nad5*, fragmentation of group II introns can be also observed in various other introns, as in the cases of the fourth intron in *nad1* in tobacco and rice and *cox2* intron in onion (Bonen, 2008; Kim and Yoon, 2010). The *trans*-spliced group II introns in plants are typically bipartite in structure, with their fragmentation sites occurring within the introns domain IV. Interestingly, this situation is reminiscent of the *trans*-interaction of snRNAs of the spliceosome with substrate pre-mRNAs, which replicates certain features of the group II introns (Sharp, 1985; Pyle and Lambowitz, 1999). This is discussed in greater detail below.

Mis-SPLICING

The *nad5* gene in most angiosperms contains four group II introns, of which the second and third flank a small, 22 bp exon and splice in "*trans*." The formation of a mature *nad5* mRNA therefore involves the production of three independently transcribed transcripts and two distinct RNA-RNA associations through which the functional second and third introns assemble and splice. Analysis of partially spliced products generated when only one of the two trans-splicing events had occurred, revealed that these reactions must take place in a particular sequence (see **Figure 3**). When the splicing of the third intron precedes that of the second, a properly *trans*-spliced intermediate is formed that is competent to correctly engage in the other *trans*-splicing event. If the two portions of the second intron associate prior to the removal of the third intron, however, in a number of monocot and dicot plants, including plants of the *Arabidopsis, Brassica, Beta, Zea,* and *Triticum* genera, a variety of mis-spliced products are generated in which the correct 3′ splice site is joined to any of a number of sites present in exon b (Elina and Brown, 2010). A model to explain these surprising results was formulated based on the observation that the 3′ end of the portion of the third intron co-transcribed with exon c contained a sequence that was potentially capable of forming an extended duplex with the first exon. It was proposed that when the initial base-pairing interactions between the two portions of the second intron occurred, the sequences from the third intron and exon a annealed. This interaction sterically hindered the 3′ splice site from assuming a position where it could join the correct 5′ splice site; instead the 3′ splice site joined to a variety of alternative sites within the upstream exon.

In plants of the genus *Oenothera*, the third *nad5* intron is further split, and requires the assembly of three separate transcripts to form a splicing-competent form (Knoop et al., 1997). In this case, the portion of the third intron predicted to base pair with the first exon "a" is not present on the same RNA as the 3′ splice site of the second intron. Accordingly, mis-splicing of the second intron would be predicted to not take place, and this expectation was confirmed through the analysis of partially spliced products. Thus, the problem of intron 2 mis-splicing that was generated during the first evolutionary fragmentation of intron 3 was resolved through a second fragmentation. As discussed below, these findings may provide some insight into the evolutionary

Table 1 | Mitochondrial group II introns and their identified splicing factors in higher plants.

Intron	Comments[a]	Splicing factor(s)	References
(*nad1* intron 1)[b]	trans-spliced; lacks a bulged A residue in domain VI[c]	OPT43 nMAT1	Falcon de Longevialle et al., 2007 Keren et al., 2012
nad1 intron 2	degenerate; no clear bulged A residue in domain VI[c]	nMAT2 mCSF1	Keren et al., 2009 Zmudjak et al., 2013
(*nad1* intron 3)	trans-spliced	mCSF1	Zmudjak et al., 2013
nad1 intron 4			
nad2 intron 1	lacks a bulged A residue in domain VI[c]	nMAT1 mCSF1 mTSF1?[d]	Keren et al., 2012 Zmudjak et al., 2013 Haïli et al., 2013
(*nad2* intron 2)	trans-spliced	RUG3 mCSF1 mTSF1?[d]	Kühn et al., 2011 Zmudjak et al., 2013 Haïli et al., 2013
nad2 intron 3	degenerate intron	RUG3 ABO5 mCSF1	Kühn et al., 2011 Liu et al., 2010 Zmudjak et al., 2013
nad2 intron 4		mCSF1	Zmudjak et al., 2013
nad4 intron 1		NMS1[e]	Brangeon et al., 2000
nad4 intron 2	degenerate intron; lacks a bulged A residue in domain VI[c]	nMAT1	Keren et al., 2012
nad4 intron 3			
nad5 intron 1		mCSF1	Zmudjak et al., 2013
(*nad5* intron 2)	trans-spliced; degenerate intron	mCSF1	Zmudjak et al., 2013
(*nad5* intron 3)	trans-spliced; degenerate intron	mCSF1	Zmudjak et al., 2013
nad5 intron 4		mCSF1?[d]	Zmudjak et al., 2013
nad7 intron 1		BIR6	Koprivova et al., 2010
nad7 intron 2		nMAT2 mCSF1	Keren et al., 2009 Zmudjak et al., 2013
nad7 intron 3			
nad7 intron 4			
ccmFc		WTF9	Colas des Francs-Small et al., 2012
cox2		nMAT2 PMH2[f] mCSF1	Keren et al., 2009 Köhler et al., 2010 Zmudjak et al., 2013
rpl2	degenerate intron; no clear bulged A residue in domain VI[c]	WTF9	Colas des Francs-Small et al., 2012
rps3	degenerate intron; no clear bulged A residue in domain VI[c]	mCSF1	Zmudjak et al., 2013

[a] Data modified from Bonen (2008).

[b] Brackets indicate trans-spliced introns.

[c] Introns lacking a bulged adenosine (A) that acts as the nucleophile in the first trans-esterification step during splicing (see Bonen, 2008)

[d] Mutants are only partially affected in the splicing.

[e] A nuclear mutation in Nicotiana sylvestris; the identity of NMS1 is currently unknown.

[f] In addition to cox2, the efficiencies of many other splicing events and the steady-state levels of several mature mRNAs are reduced in the pmh2 mutants. Such non-specific RNA binding activity is concordant with RNA-chaperones, which facilitate transitions from non-functional to active conformations of structured RNAs (Köhler et al., 2010).

pressures that may have driven the evolution of the spliceosome from a group II intron in early evolving eukaryotes.

GROUP II INTRON SPLICING FACTORS

Proteins which facilitate the splicing of group II introns have probably originated in two ways (Lambowitz et al., 1999; Barkan, 2004; Fedorova et al., 2010): some are encoded within the introns themselves (Intron Encoded Proteins, or maturases) and have ancient relationships with their host introns, whereas others are derived "more recently" from the nuclear genome.

MATURASES

Maturases are characterized by three functional domains, which are required for both splicing and intron mobility activities: an N-terminal RT that is related to HIV-RTs, followed by an RNA-binding and splicing motif (domain X) and a site-specific endonuclease (D/En) motif at the C-terminus. Genetic and biochemical data show that the processing of group II introns in bacteria is facilitated by a single maturase-ORF encoded within DIV of the intron itself (see **Figure 2A**), although in some cases the maturase may also act on additional closely-related RNAs. The best characterized maturase to-date is LtrA protein, which binds with high affinity and specifically to its own intron, the self-splicing *Ll.LtrB* intron (Wank et al., 1999; Matsuura et al., 2001; Noah and Lambowitz, 2003). These assays indicated that the binding of LtrA facilitates *LtrB* splicing and folding under physiological conditions and is required for intron retrohoming.

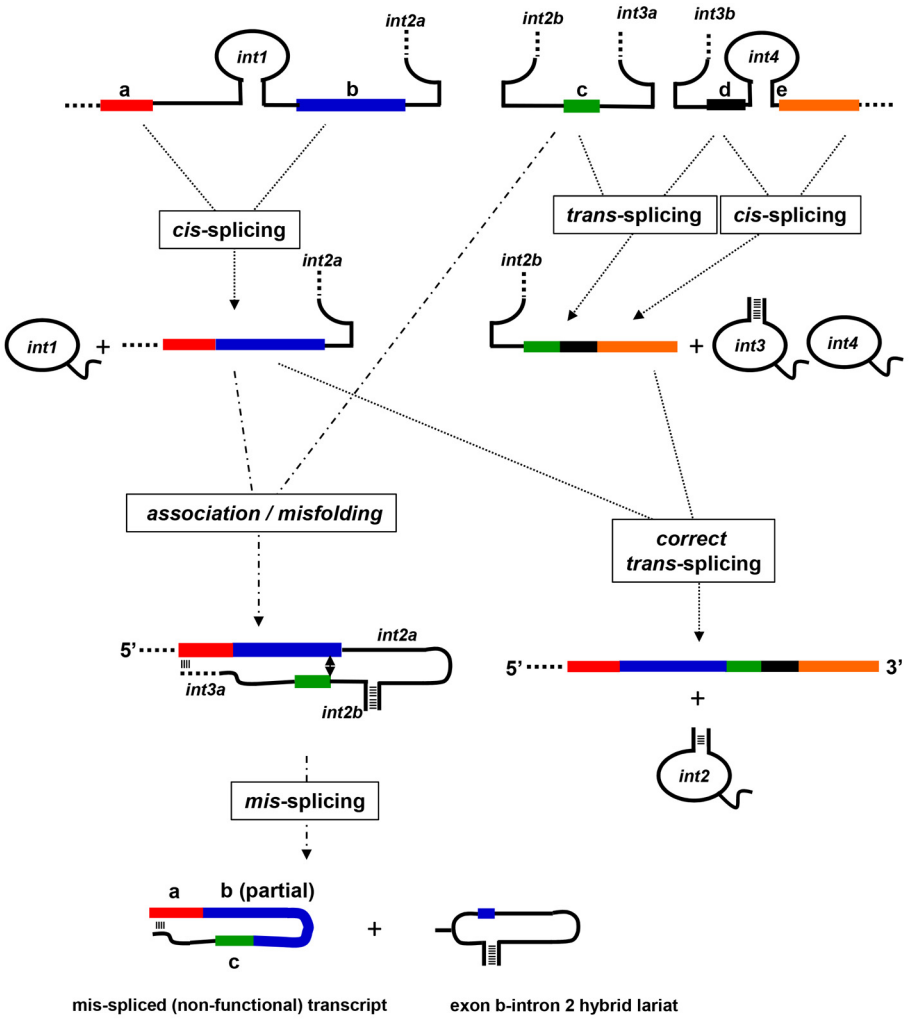


FIGURE 3 | Model for mis-splicing of mitochondrial group II introns. A schematic illustration of a model for the splicing and mis-splicing of angiosperm *nad5* transcripts based on the model of Elina and Brown (2010). The *cis*-splicing of introns 1 and 4 proceeds normally. If the *trans*-splicing intron 3 assembles prior to the assembly of the *trans*-splcing intron 2, it splices correctly, and intron 2 can assemble and splice correctly to generate a properly spliced and functional mRNA. If intron 2 assembles first, however, a sequence at the 3′ end of intron 3a (the portion of the intron co-transcribed with exon c) base pairs with a sequence within exon a, preventing correct positioning of the 5′ splice site of intron 2. This results in the joining of exon c to sites within exon b to form mis-spliced, non-functional transcripts composed of exon a, a portion of exon b, exon c, and intron 3a; these transcripts do not engage in further splicing. A hybrid lariat mis-splicing product composed of intron 2 and a portion of exon b is also formed.

MatR

As outlined above, group II introns have diverged considerably from bacteria and plant organellar genomes. These introns in plants sometime lack regions which are considered to be essential for their splicing. Also, the number of maturases has been dramatically reduced during the evolution of organellar genomes in plants. The mitochondrial genomes of bryophytes, as *Marchantia polymorpha* and *Physcomitrella patens*, contain a few maturase ORFs, while only a single intron has retained its maturase gene in the mtDNAs in angiosperms: the *matR* gene encoded within *nad1* intron 4 (Wahleithner et al., 1990) (**Figures 1**,**3**). MatR proteins contain a well-conserved domain X, but have degenerate RT motifs and lack the En domain (**Figure 4**). The high conservation of MatR across the plant lineage (Adams et al., 2002) and RNA-editing events which restore conserved amino-acids (Thomson et al., 1994) suggest that *matR* encodes a functional protein. Preliminary data suggest that MatR binds to several group II introns *in vivo*, but its putative roles in splicing are yet to be determined. Analogously, MatK protein, encoded within *trnK* gene in chloroplasts, is associated with numerous group II introns *in vivo* (Zoschke et al., 2010). Besides MatR there are no splicing factor candidates among the ORFs of angiosperm mitochondrial genomes (although some lower plants may contain a few maturase ORFs in the mtDNAs). The splicing of mitochondrial introns in plants is therefore expected to be facilitated by nuclear-encoded proteins. These are translated by cytosolic ribosomes and subsequently imported into the organelle.

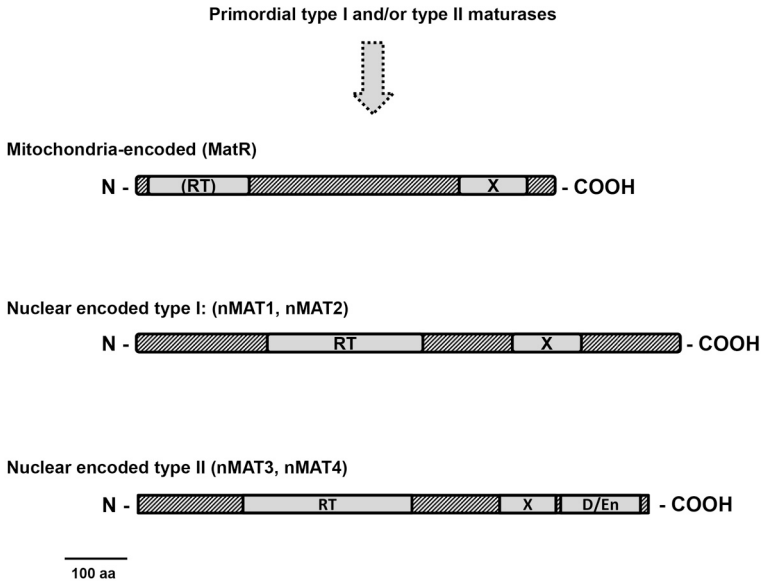


FIGURE 4 | Plant mitochondria maturases. Plant maturase ORFs are shown as rectangles, with different shadings indicating conserved regions. The three domains typical to group II intron encoded maturases are outlined in the maturase ORFs: A reverse transcriptase (RT); domain X, associated with maturase activity; D, DNA-binding and endonuclease domain (D/En). The organelle-encoded MatR and MatK proteins contain a well conserved domain X, but have degenerate RT motifs and lack the En domain. In angiosperms, four other maturase-related proteins, denoted as nMAT 1–4, are encoded by nuclear genes, but have N-terminal mitochondrial targeting sequences (Keren et al., 2009). Similarly to MatR, the four nMATs have a conserved domain X, but show deviations in the RT domain and lack (type I) or have deviations in the D/En domain (type II), suggesting loss of mobility functions.

Nuclear-encoded maturases (nMATs)

In addition to *matR*, plants also contain several genes designated *nMat* 1 to 4, which are closely related to group II intron-encoded maturases and exist in the nucleus as self-standing ORFs, out of the context of their evolutionary related group II intron hosts (Mohr and Lambowitz, 2003; Keren et al., 2009). These are all predicted to reside within mitochondria and are thus expected to function in the splicing of organellar introns in plants. GFP-fusion analyses further established the mitochondrial localization of the four nuclear-encoded maturases *in vivo* in Arabidopsis, but also indicated a possible dual localization (in chloroplasts and mitochondria) for nMAT4 (Keren et al., 2009). Based on their topology and predicted evolutionary origins, the four nMATs are classified into two main groups (**Figure 4**): nMAT1 and nMAT2 are classified as type-I maturases, which lack the D/En motif, while nMAT3 and nMAT4 belong to type-II and contain all three domains (i.e., RT, X, and En/D) typical to "model" group II intron maturases (Mohr and Lambowitz, 2003). Yet, while the RT regions in nMAT1 and nMAT2 are degenerate, the D/En domains in nMAT3 and nMAT4 have mutations that are expected to inactivate the endonuclease activity (Mohr and Lambowitz, 2003). Thus, although expected to retain their splicing activities, the four nuclear-encoded maturases in angiosperms seem to have lost their mobility-associated functions.

Genetic studies indicate that nMAT proteins function in the splicing of mitochondrial introns in angiosperms (Nakagawa and Sakurai, 2006; Keren et al., 2009, 2012) and **Table 1**). In Arabidopsis, they seem particularly important for the maturation of primary *nad1* transcripts (see **Table 1**). Homozygote *nmat* mutants show altered growth and developmental phenotypes, modified respiration and altered stress responses, which are tightly correlated with mitochondrial complex I defects (Keren et al., 2009, 2012). While nMAT1 is required in *trans*-splicing of *nad1* intron 1, *nad2* intron 1, and *nad4* intron 2 (Keren et al., 2012), nMAT2 functions in the efficient splicing of *nad1* intron 2, *nad7* intron 1 and the single intron in the cytochrome oxidase subunit 2 gene (*cox2* intron 1) (Keren et al., 2009). Interestingly, the three intron targets of nMAT1 are all lacking the canonical bulged A residue, which is required for the first trans-esterification step and the release of the 5′-exon (see above and **Figure 2**). The precise biochemical functions of nuclear-encoded maturases in the splicing process have not yet been established, but an intriguing possibility is that nMAT1 may function in the hydrolysis of the phosphodiester bond at the 5′ splice site, or recruit specific RNA nucleases required for the release of the 5′-exon. Similar to nMAT1 and nMAT2, studies in progress suggest that MatR nMAT3 and nMAT4 also play a role in the splicing of mitochondrial introns.

Maturase phylogeny

A recent study by Guo and Mower (2013) has provided new insights into the distribution and the evolution of land-plant maturases. The authors used known plant maturase sequences to conduct an extensive search of recently sequenced green algal and land-plant mitochondrial genomes, including those of the sequenced nuclear genomes of the lycophyte *Selaginella* and the moss *Physcomitrella*. The authors detected multiple, previously unrecognized plant sequences potentially cable of specifying

a protein with maturase function. These included seven new nuclear maturase loci in *Selaginella* (bringing the total number of maturases for this species to eight), and five new nuclear maturase loci in *Physcomitrella* (bringing its total to 12). Building upon this new information a comprehensive phylogeny was constructed to determine the evolutionary relationships among the different maturase sequences; a simplified version of this phylogeny, displaying only the relationships among the seed plant (six maturase genes), *Marchantia* (eight related genes) and *Selaginella* maturases, is shown in **Figure 5**. Most, but not all of the new nMATs are predicted to be mitochondrial proteins.

Several interesting features of plant maturase evolution are revealed by this phylogeny. In general, plant *mat* genes fall into two main categories (**Figure 5**): mitochondria-encoded maturases which have no homologs in other plants (group A) and orthologous maturase-genes, found in the mitochondrial and/or nuclear genomes of different plants (group B). These can be further divided into four groups (i.e., *matR* sequences in land plants, type I and type II maturases and plastidial *matK* ORF in *trnK* intron). It is likely, therefore, that the angiosperm type I nMat1 and nMat2 genes resulted from a nuclear gene duplication, following the translocation to the nucleus of a single mitochondrial maturase related to Marchantia maturase mat-Mpo1 (formerly designated rtl, for RT-like) (**Figure 5**). It is further likely that three gene duplication events in the Selaginella lineage following the same translocation event led to four of the Selaginella nMats. As many mitochondrial genes, which were copied to a nuclear genome, the "original" mitochondrion-encoded Mpo1 ORF probably degenerated rapidly, resulting in the gene persisting only in the nucleus. Similarly, the type II nMat3 and nMat4 genes in angiosperms likely arose from a gene duplication event following the transfer to the nucleus of a maturase resembling modern Marchantia mat-cobi84 (see **Figure 5**); this event also resulted in the single Selaginella nuclear maturase nMat6. Interestingly, the matR maturases appear most similar to the Marchantia mat-cox1i44, suggesting common matures ancestor for these two maturases. Of particular interest is the observation that matK, the single maturase encoded in the chloroplast genome, clusters with mitochondrial maturases from group B, suggesting the possible transfer of a mitochondrial intron to the chloroplast in an ancestor of modern land plants and charophyte algae. Although branch support for the position of the matK clade was considered weak (Guo and Mower, 2013), this possibility was also suggested in an earlier analysis of matK phylogeny (Hausner et al., 2006).

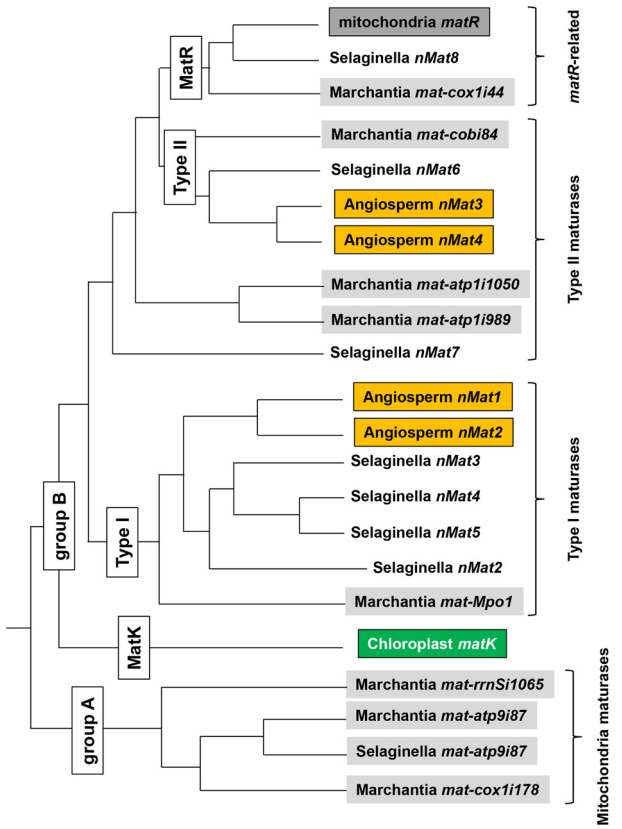


FIGURE 5 | A comparative sequence analysis of plant maturases. Phylogentic relationships among seed plant, *Selaginella* and *Marchantia* mitochondrial, chloroplast and identified nuclear maturases, based on Guo and Mower (2013). Nomenclature of Guo and Mower (2013) is used for mitochondrial maturases other than *matR* (designated by the authors as *mat-nad1i728*). Nomenclature for nuclear maturases (*nMats*) is identical with that employed in this review and previous analyses (Mohr and Lambowitz, 2003; Keren et al., 2009). Branch lengths, as illustrated, do not represent phylogenetic distances and are intended only to illustrate relationships among orthologous *mat* genes in plants. Mitochondrially encoded genes are shown as gray shaded boxes and chloroplast *matK* is in green, while orange shaded boxes represent plant maturase homologs.

HOST-ENCODED FACTORS IN PLANT MITOCHONDRIA

Different RNA-binding proteins were shown to function in the splicing and processing of introns in plant organellar genomes (see below). Some, such as a DEAD RNA-helicase (PMH2) and a CRM-domain protein (mCSF1) are required for optimal splicing of a large set of introns, whereas others (e.g., PPR, PORR, and RCC proteins) rather appear to be specific for a single, or few, individual introns. Although this review focuses mainly on maturases, this section summarizes the information available about other types of mitochondrial group II intron splicing factors.

CRM

CRM (Chloroplast RNA splicing and ribosome Maturation) proteins are characterized by a conserved RNA-binding domain of ∼10 kDa (Pfam-PF01985), which is related to a conserved bacterial ORF (YhbY) (Barkan et al., 2007). In *E.coli*, YhbY is mainly associated with the maturation of the pre-50S ribosomal subunit (Barkan et al., 2007). In eukaryotes, CRMs are restricted to the plant lineage, where they are found in a small family of proteins containing between 1 and 4 repeats of the conserved CRM domain (Barkan et al., 2007). Biochemical analyses indicated that CRM domains share structural similarities and RNA-binding characteristics with the RNA recognition motif (RRM) (Keren et al., 2008). The nuclear genomes in angiosperms contain ∼15 CRM proteins, of which the majority are predicted to be plastidial by different targeting prediction programs, although few may exist within the mitochondria and nucleus as well (Barkan et al.,

2007). Two of these paralogs in Arabidopsis, mCSF1 and mCSF2, are targeted to mitochondria *in vivo* (Zmudjak et al., 2013). Genetic and biochemical data indicate that mCSF1 encodes an essential polypeptide which is required for the processing of many of the mitochondrial introns in Arabidopsis (Zmudjak et al., 2013). Accordingly, *RNAi*-knockdown *mcsf1* lines show strong defects in the assembly of both complex I and complex IV (Zmudjak et al., 2013). No other mitochondrial CRM domain protein has been described so far, but other mitochondrial CRM members are expected to have important roles in RNA metabolism in higher plant mitochondria.

RNA DEAD-box helicases

RNA helicases from the DEAD-box family are widely distributed in both prokaryotes and eukaryotes and have essential roles in RNA processing. These include ATP-dependent RNA duplex unwinding and/or formation, displacement of proteins from RNA transcripts, formation of assembly platforms for larger ribonucleoprotein complexes, and also the sensing of bacterial metabolites (Putnam and Jankowsky, 2013). These processes are fundamental steps in RNA metabolism in all organisms. About 60 such DEAD-box encoding genes have been identified in plants, but most have not yet been characterized. One of these members in Arabidopsis, PMH2 (Putative Mitochondrial Helicase 2), is found in a large ribonucleoprotein complex *in vivo*, and is thought to be a hetero-multimeric splicing unit (Matthes et al., 2007; Köhler et al., 2010). Although the activities of PMH2 are not essential for the maturation of the mitochondrial pre-mRNAs, homozygous *pmh2* mutants in Arabidopsis are affected in the splicing efficiency of many organelle introns, as evident by increased levels of many pre-mRNAs in *pmh2* mutant mitochondria.

PPR proteins

PPR proteins (Small and Peeters, 2000) are found in most eukaryotes, but are in particularly abundant in plants, with nearly 500 different members the nuclear genomes of both monocot and dicot species (Lurin et al., 2004). Many, if not all, of the characterized PPR proteins in plants were shown to be involved in post-transcriptional RNA processing events in mitochondria and chloroplasts, including RNA editing, trimming, RNA stability, and more recently also in the removal of introns (Schmitz-Linneweber and Small, 2008). PPR proteins which function in the splicing of mitochondrial pre-mRNAs include OTP43 (Falcon de Longevialle et al., 2007), BIR6 (Koprivova et al., 2010), and ABO5 (Liu et al., 2010), all dealing with transcripts encoding subunits of the respiratory complex I (see **Table 1**).

Based on their topology and number of repeats, the PPR family is divided into two major classes, P and PLS (Lurin et al., 2004). In angiosperms, the splicing factors, together with several other PPR proteins which function in RNA stability and protection, belong to the P subclass and identified as "pure" PPR proteins, while editing factors are generally PLS domain proteins containing additional C-terminal domains (Schmitz-Linneweber and Small, 2008). Other P-class factors, which are predicted to reside within mitochondria, are therefore anticipated to function in the splicing of mitochondrial pre-mRNAs. However, PPR proteins belonging to the PLS class may also be involved in group II intron splicing in angiosperm mitochondria, as shown in Arabidopsis chloroplasts (OTP70, E subfamily) (Chateigner-Boutin et al., 2011) and the splicing of the mitochondrial *cox1* transcript in *Physcomitrella patens* by PpPPR_43 (a DYW subclass PPR protein) (Ichinose et al., 2012). Recently, the code of RNA recognition by PPR proteins was deciphered (Barkan et al., 2012; Yagi et al., 2013), and was further established by structural data (Yin et al., 2013). Accordingly, systematic bioinformatics studies provide a powerful tool for molecular characterization of the roles additional PPRs in mitochondria RNA metabolism in plants.

PORR domain family

In addition to known RNA-binding proteins, genetic studies also led to the identification of novel factors which function in the processing of group II introns in plant organelles. Among these is the PORR (Plant Organellar RNA Recognition) domain which is represented in a small family of proteins in angiosperms (15 in Arabidopsis and 17 in rice) (Kroeger et al., 2009). One of these members in maize is associated with different group II introns *in vivo* and was shown to promote the splicing of about half of the plastid introns (Kroeger et al., 2009). In contrast, a mitochondrial member of the family, WTF9, is more specific and was shown to act on two group II introns, namely the essential ribosomal *rpl2* subunit and $ccmF_C$ (encoding a subunit of the *c*-type cytochrome maturation system) (Colas des Francs-Small et al., 2012). Interestingly, homozygous Arabidopsis *wtf9* lines are viable, possibly because the *rpl2* transcript has been "split" during its evolution and is encoded in two parts, one of which is nuclear- encoded in Arabidopsis (Colas des Francs-Small et al., 2012). Thus, the lack of splicing of the mitochondrial *rpl2* transcript leads to a partially functional Rpl2 protein. The strong phenotypes of the *wtf9* mutants were therefore mainly attributed to the depletion of cyt*c* and $cytc_1$, and subsequently to complex III and complex IV defects (Colas des Francs-Small et al., 2012).

RCC proteins

Another factor involves a eukaryotic protein (RCC1, Regulator of Chromosome Condensation) which binds to chromatin and interacts with the nuclear Ran GTPase (Dasso, 1993). Similarly to PPRs, RCC proteins also contain tandem repeats of a conserved domain of about 50 amino acids. The association of RCC1 with Ran is postulated to be important in the regulation of nuclear gene expression. Only one member of the RCC1 domain family, RUG3, has been identified so far as a splicing factor. RUG3 is closely related to RCC1 and UV-B RESISTANCE 8, which were shown to function in chromatin modification. The RUG3 protein is involved in the splicing of *nad2* in Arabidopsis (Kühn et al., 2011). Similar to other complex I mutants, *rug3* knockout lines show slow growth and reduced size. As no RNA-binding was demonstrated for RCC1-like proteins, it has been proposed that these factors may recruit RNA-binding cofactors, such as the ABO5 protein (Liu et al., 2010; Kühn et al., 2011), to the splicing complex.

In addition to the identified factors, a nuclear mutation in *Nicotiana sylvestris* (*nms1*) was shown to disrupt the splicing of the first intron in the *nad4* transcript (Brangeon et al., 2000),

however the identity of this gene remains unknown. The specific roles of these factors in the splicing and processing of mitochondrial group II introns are illustrated in **Figure 6**.

PHYSIOLOGICAL CONSEQUENCES OF MITOCHONDRIAL GROUP II INTRON SPLICING DEFECTS

The challenges of maintaining prokaryotic-type structures and functions in the cell are common to all eukaryotes. The respiratory chain is composed of four major complexes, complexes I to IV, which are localized within the mitochondrial inner-membrane. These complexes contain subunits encoded by both nuclear and mtDNAs. A miscommunication between the organelles and their host cell (i.e., a breakdown in the nuclear-organellar "cross-talk") often results in severe developmental defects.

Plants possess some of the most complex organelle compositions of all known eukaryotic cells. As sessile organisms, land-plants must cope with multiple environmental stresses. During their evolution, plants have acquired complex regulatory mechanisms to coordinate cellular functions with environmental stresses. The participation of nuclear-encoded factors in organelle RNA metabolism may therefore provide means to control the biogenesis of the respiratory (and photosynthetic) machineries, and thus to link cellular metabolism and environmental and developmental signals. Following this idea, the splicing of both plastidial (Barkan, 1989; Kahlau and Bock, 2008) and mitochondrial introns (Li-Pook-Than et al., 2004; Dalby and Bonen, 2013) is developmentally and environmentally responsive. Likewise, the expression of group II intron splicing factors and other genes implicated in mitochondrial genome expression and RNA

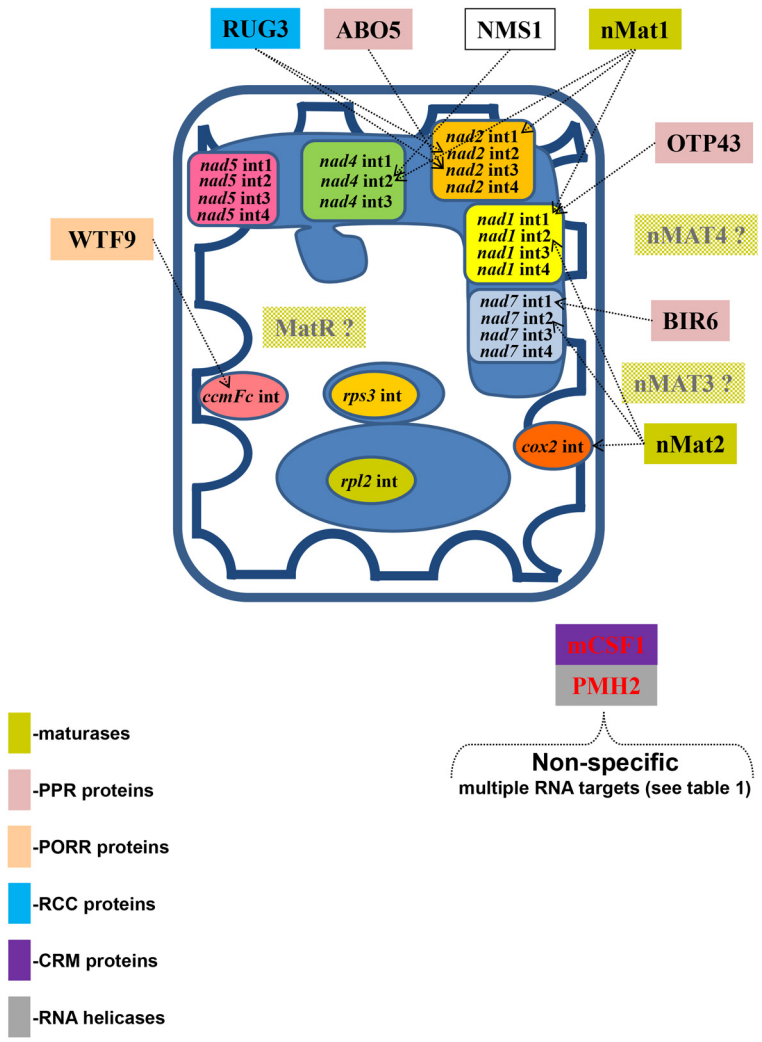


FIGURE 6 | The splicing of individual group II introns in Arabidopsis mitochondria is facilitated by multiple protein cofactors. Genetic and biochemical data indicate that mitochondrial splicing factors are essential for organellar functions and thus for plant survival. These analyses, involving selected mutants in various nuclear-encoded mitochondrial splicing factors, also indicate that the processing of individual mitochondrial introns in plant is facilitated by multiple protein cofactors. The different mitochondrial introns in angiosperms and their specific splicing factors are indicated in the figure. Colors refer to different protein families, while the shaded boxes indicate preliminary biochemical and genetic data in Arabidopsis.

metabolism in plants is tightly regulated during the early stages of germination (Narsai et al., 2011).

The importance of these stages is further reflected in plant mutants affected in genes required for the splicing of mitochondrial introns. Such mutants exhibit reduced germination and seedling establishment, and are often affected in growth and development (see Keren et al., 2012). As many of the introns in the mtDNAs in angiosperms are inserted in complex I subunit genes, but some also reside in *ccmFc*, *cox2* and the ribosomal *rpl2* and *rps3* genes, the splicing mutants are strongly affected in respiratory associated functions and cellular metabolism (Keren et al., 2012). These mutants are viable in plants, plausibly due to the presence of non-energy conserving respiratory pathways which allow electron transport from NAD(P)H to oxygen (O_2) (reviewed in Millar et al., 2011). These pathways also allow plant mitochondria to respire in the presence of rotenone and cyanide, inhibitors of complex I and complex IV, respectively. Thus, the analysis of nuclear-encoded factors required in pre-mRNA processing is expected to give new insights into these processes in plant organellar biology and to contribute to our overall understanding of complex regulatory pathways controlling the biogenesis of respiratory apparatus in plants and other eukaryotic systems (Meyer et al., 2011; Colas des Francs-Small and Small, 2013).

PERSPECTIVES

MITOCHONDRIAL GROUP II INTRONS—PROGENITORS OF THE NUCLEAR SPLICEOSOME MACHINERY?

Based on the structural features, the similarity of exon-intron boundaries and a common splicing-mechanism, group II introns are proposed to be ancestors of the eukaryotic spliceosomal introns (Sharp, 1985; Cech, 1986; Lynch and Kewalramani, 2003; Martin and Koonin, 2006). While bacterial group II introns are generally thought to be self-sufficient with respect to splicing, numerous different nuclear-encoded factors are required to support the splicing of organellar group II introns in angiosperms (de Longevialle et al., 2010; Barkan, 2011). While only a single intron maturase has been retained in the two organelle genomes of angiosperms, several other maturase genes have been transferred into the nucleus during the evolution of plants. These are all postulated to function in the splicing of organellar introns. Similarly, genetic screens in *Chlamydomonas reinhardtii* demonstrated that the *trans*-splicing of the two group II introns in its chloroplast genome, is also facilitated by a large set of nuclear-encoded RNA binding cofactors (Goldschmidt-Clermont et al., 1990; Perron et al., 2004). Recently, a group II intron in *Clostridium tetani*, was shown to undergo alternative splicing reactions resulting with five alternative mRNAs products, each encoding a distinct protein isoform (McNeil et al., in press). Together, these observations further suggested that a group II intron invader of the eukaryotic cell nucleus, possibly derived from the mitochondrial symbiont, served as the evolutionary precursor to the nuclear splicesomal introns. According to this view, the RNA structures required for group II catalysis began to function in "*trans*" in a primitive spliceosome, and the group II structural features of the target primordial introns degenerated. Interestingly, features of some plant mitochondrial introns reflect possible stages that may have occurred in such a process. Several undergo *trans*-splicing: different portions of the intron are assembled from two and in one case, three different transcripts. At least one *trans*-splicing intron undergoes a significant degree of mis-splicing, and this is lost in plants in which the intron is further fragmented, a process that may have played a role in the formation of the primordial spliceosome. Also, several introns lack key structural features, such as the bulged adenosine in the DVI stem, which serves as the nucleophile in the first splicing step (**Figure 2**) and may be reflective of an early stage in the structural degeneracy that took place in target introns of the evolving spliceosome.

Maturases and their accessory splicing factors in plant mitochondria, thus represent a highly versatile set of proteins which differ from their presumptive monospecific ancestors. These features may correspond to a further evolutionary link between mitochondrial group II introns and the spliceosomal machinery in nuclear genomes of most eukaryotic cells. Likewise, the recruitment of different protein cofactors to assist with the splicing of mitochondrial introns in plants (see **Figure 6**) may reflect processes analogous to those that took place during the evolution of the nuclear spliceosome. In particular, the transition of maturases from specific to versatile splicing factors, may have allowed them to serve as principal component in the evolution of the nuclear spliceosome with its ability to act on a distinct subset of introns in "*trans*." The homology of group II maturases to the core spliceosomal splicing factor (Dlakic and Mushegian, 2011) further supports this view. Future research will be aimed at determining whether plant maturases have gained the ability to act on multiple intron targets by acquiring versatility in intron recognition and intron structure modification.

ACKNOWLEDGMENTS

This work was supported by grants to Oren Ostersetzer-Biran from the Israeli Science Foundation (ISF grant no. 980/11) and from the Natural Sciences and Engineering Research Council, Canada, to Gregory G. Brown.

REFERENCES

Adams, K. L., Qiu, Y.-L., Stoutemyer, M., and Palmer, J. D. (2002). Punctuated evolution of mitochondrial gene content: high and variable rates of mitochondrial gene loss and transfer to the nucleus during angiosperm evolution. *Proc. Natl. Acad. Sci. U.S.A.* 99, 9905–9912. doi: 10.1073/pnas.042694899

Barkan, A. (1989). Tissue-dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. *Plant Cell* 1, 437–445. doi: 10.1105/tpc.1.4.437

Barkan, A. (2004). "Intron splicing in plant organelles," in *Molecular Biology and Biotechnology of Plant Organelles, Intron Splicing in Plant Organelles*, eds H. Daniell and C. Chase (Dordrecht: Kluwer Academic), 281–308. doi: 10.1007/978-1-4020-3166-3_11

Barkan, A. (2011). Expression of plastid genes: organelle-specific elaborations on a prokaryotic scaffold. *Plant Physiol.* 155, 1520–1532. doi: 10.1104/pp.110.171231

Barkan, A., Klipcan, L., Ostersetzer, O., Kawamura, T., Asakura, Y., and Watkins, K. P. (2007). The CRM domain: an RNA binding module derived from an ancient ribosome-associated protein. *RNA* 13, 55–64. doi: 10.1261/rna.139607

Barkan, A., Rojas, M., Fujii, S., Yap, A., Chong, Y., Bond, C., et al. (2012). A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. *PLoS Genet.* 8:e1002910. doi: 10.1371/journal.pgen.1002910

Belfort, M., Derbyshire, V., Parker, M. M., Cousineau, B., and Lambowitz, A. M. (2002). "Mobile introns: pathways and proteins," in *Mobile DNA II*, eds N. L. Craig, R. Craigie, M. Gellert, and A. M. Lambowitz (Washington, DC: ASM Press), 761–783.

Binder, S., and Brennicke, A. (2003). Gene expression in plant mitochondria: transcriptional and post-transcriptional control. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 181–188. doi: 10.1098/rstb.2002.1179

Bonen, L. (2008). Cis- and trans-splicing of group II introns in plant mitochondria. *Mitochondrion* 8, 26–34. doi: 10.1016/j.mito.2007.09.005

Bonen, L., and Vogel, J. (2001). The ins and outs of group II introns. *Trends Genet.* 17, 322–331. doi: 10.1016/S0168-9525(01)02324-1

Brangeon, J., Sabar, M., Gutierres, S., Combettes, B., Bove, J., Gendy, C., et al. (2000). Defective splicing of the first nad4 intron is associated with lack of several complex I subunits in the *Nicotiana sylvestris* NMS1 nuclear mutant. *Plant J.* 21, 269–280. doi: 10.1046/j.1365-313x.2000.00679.x

Cech, T. R. (1986). The generality of self-splicing RNA: relationship to nuclear messenger-RNA splicing. *Cell* 44, 207–210. doi: 10.1016/0092-8674(86)90751-8

Chapdelaine, Y., and Bonen, L. (1991). The wheat mitochondrial gene for subunit I of the NADH dehydrogenase complex: a trans-splicing model for this gene-in-pieces. *Cell* 65, 465–472. doi: 10.1016/0092-8674(91)90464-A

Chateigner-Boutin, A.-L., des Francs-Small, C. C., Delannoy, E., Kahlau, S., Tanz, S. K., de Longevialle, A. F., et al. (2011). OTP70 is a pentatricopeptide repeat protein of the E subgroup involved in splicing of the plastid transcript rpoC1. *Plant J.* 65, 532–542. doi: 10.1111/j.1365-313X.2010.04441.x

Clifton, S. W., Minx, P., Fauron, C. M.-R., Gibson, M., Allen, J. O., Sun, H., et al. (2004). Sequence and comparative analysis of the maize NB mitochondrial genome. *Plant Physiol.* 136, 3486–3503. doi: 10.1104/pp.104.044602

Colas des Francs-Small, C., Kroeger, T., Zmudjak, M., Ostersetzer-Biran, O., Rahimi, N., Small, I., et al. (2012). A PORR domain protein required for rpl2 and ccmFc intron splicing and for the biogenesis of c-type cytochromes in Arabidopsis mitochondria. *Plant J.* 69, 996–1005. doi: 10.1111/j.1365-313X.2011.04849.x

Colas des Francs-Small, C., and Small, I. (2013). Surrogate mutants for studying mitochondrially encoded functions. *Biochimie*. doi: 10.1016/j.biochi.2013.08.019. [Epub ahead of print].

Cousineau, B., Smith, D., Lawrence-Cavanagh, S., Mueller, J. E., Yang, J., Mills, D., et al. (1998). Retrohoming of a bacterial group II intron: mobility via complete reverse splicing, independent of homologous DNA recombination. *Cell* 94, 451–462. doi: 10.1016/S0092-8674(00)81586-X

Dai, L. X., Toor, N., Olson, R., Keeping, A., and Zimmerly, S. (2003). Database for mobile group II introns. *Nucleic Acids Res.* 31, 424–426. doi: 10.1093/nar/gkg049

Dalby, S. J., and Bonen, L. (2013). Impact of low temperature on splicing of atypical group II introns in wheat mitochondria. *Mitochondrion* 13, 647–655. doi: 10.1016/j.mito.2013.09.001

Daniels, D. L., Michels, W. J. Jr., and Pyle, A. M. (1996). Two competing pathways for self-splicing by group II introns: a quantitative analysis of *in vitro* reaction rates and products. *J. Mol. Biol.* 256, 31–49. doi: 10.1006/jmbi.1996.0066

Dasso, M. (1993). RCC1 in the cell cycle: the regulator of chromosome condensation takes on new roles. *Trends Biochem. Sci.* 18, 96–101. doi: 10.1016/0968-0004(93)90161-F

de Longevialle, A. F., Small, I. D., and Lurin, C. (2010). Nuclearly encoded splicing factors implicated in RNA splicing in higher plant organelles. *Mol. Plant* 3, 691–705. doi: 10.1093/mp/ssq025

Dlakic, M., and Mushegian, A. (2011). Prp8, the pivotal protein of the spliceosomal catalytic center, evolved from a retroelement-encoded reverse transcriptase. *RNA* 17, 799–808. doi: 10.1261/rna.2396011

Elina, H., and Brown, G. G. (2010). Extensive mis-splicing of a bi-partite plant mitochondrial group II intron. *Nucleic Acids Res.* 38, 996–1008. doi: 10.1093/nar/gkp994

Falcon de Longevialle, A., Meyer, E. H., Andres, C., Taylor, N. L., Lurin, C., Millar, A. H., et al. (2007). The pentatricopeptide repeat gene OTP43 is required for *trans*-splicing of the mitochondrial *nad1* intron 1 in *Arabidopsis thaliana*. *Plant Cell* 19, 3256–3265. doi: 10.1105/tpc.107.054841

Fedorova, O., Solem, A., and Pyle, A. M. (2010). Protein-facilitated folding of group II intron ribozymes. *J. Mol. Biol.* 397, 799–813. doi: 10.1016/j.jmb.2010.02.001

Finnegan, P. M., and Brown, G. G. (1990). Transcriptional and post-transcriptional regulation of RNA levels in maize mitochondria. *Plant Cell* 2, 71–83. doi: 10.1105/tpc.2.1.71

Gagliardi, D., and Binder, S. (2007). "Expression of the plant mitochondrial genome," in *Plant Mitochondria,* ed D. Logan (Ames, IA: Blackwell Publishing), 50–95. doi: 10.1002/9780470986592.ch3

Goldschmidt-Clermont, M., Girard-Bascou, J., Choquet, Y., and Rochaix, J. D. (1990). Trans-splicing mutants of Chlamydomonas reinhardtii. *Mol. Gen. Genet.* 223, 417–425. doi: 10.1007/BF00264448

Guo, W., and Mower, J. (2013). Evolution of plant mitochondrial intron-encoded maturases: frequent lineage-specific loss and recurrent intracellular transfer to the nucleus. *J. Mol. Evol.* 77, 43–54. doi: 10.1007/s00239-013-9579-7

Haïli, N., Arnal, N., Quadrado, M., Amiar, S., Tcherkez, G., Dahan, J., et al. (2013). The pentatricopeptide repeat MTSF1 protein stabilizes the nad4 mRNA in Arabidopsis mitochondria. *Nucl. Acids Res.* 41, 6650–6663. doi: 10.1093/nar/gkt337

Handa, H. (2003). The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. *Nucleic Acids Res.* 31, 5907–5916. doi: 10.1093/nar/gkg795

Hausner, G., Olson, R., Simon, D., Johnson, I., Sanders, E. R., Karol, K. G., et al. (2006). Origin and evolution of the chloroplast *trnK* (*matK*) intron: a model for evolution of group II intron RNA structures. *Mol. Biol. Evol.* 23, 380–391. doi: 10.1093/molbev/msj047

Ichinose, M., Tasaki, E., Sugita, C., and Sugita, M. (2012). A PPR-DYW protein is required for splicing of a group II intron of cox1 pre-mRNA in Physcomitrella patens. *Plant J.* 70, 271–278. doi: 10.1111/j.1365-313X.2011.04869.x

Kahlau, S., and Bock, R. (2008). Plastid transcriptomics and translatomics of tomato fruit development and chloroplast-to-chromoplast differentiation: chromoplast gene expression largely serves the production of a single protein. *Plant Cell* 20, 856–874. doi: 10.1105/tpc.107.055202

Keren, I., Bezawork-Geleta, A., Kolton, M., Maayan, I., Belausov, E., Levy, M., et al. (2009). AtnMat2, a nuclear-encoded maturase required for splicing of group-II introns in Arabidopsis mitochondria. *RNA* 15, 2299–2311. doi: 10.1261/rna.1776409

Keren, I., Klipcan, L., Bezawork-Geleta, A., Kolton, M., Shaya, F., and Ostersetzer-Biran, O. (2008). Characterization of the molecular basis of group II intron RNA recognition by CRS1-CRM domains. *J. Biol. Chem.* 283, 23333–23342. doi: 10.1074/jbc.M710488200

Keren, I., Tal, L., des Francs-Small, C. C., Araújo, W. L., Shevtsov, S., Shaya, F., et al. (2012). nMAT1, a nuclear-encoded maturase involved in the *trans*-splicing of *nad1* intron 1, is essential for mitochondrial complex I assembly and function. *Plant J.* 71, 413–426. doi: 10.1111/j.1365-313X.2012.04998.x

Kim, S., and Yoon, M.-K. (2010). Comparison of mitochondrial and chloroplast genome segments from three onion (Allium cepa L.) cytoplasm types and identification of a trans-splicing intron of cox2. *Curr. Genet.* 56, 177–188. doi: 10.1007/s00294-010-0290-6

Knoop, V. (2012). "Seed plant mitochondrial genomes: complexity evolving," in *Genomics of Chloroplasts and Mitochondria*, eds Bock and Knoop (East Lansing, MI: Springer), 175–195. doi: 10.1007/978-94-007-2920-9_8

Knoop, V., Altwasser, M., and Brennicke, A. (1997). A tripartite group II intron in mitochondria of an angiosperm plant. *Mol. Gen. Genet.* 255, 269–276. doi: 10.1007/s004380050497

Knoop, V., Schuster, W., Wissinger, B., and Brennicke, A. (1991). Trans splicing integrates an exon of 22 nucleotides into the nad5 mRNA in higher plant mitochondria. *EMBO J.* 10, 3483–3493.

Köhler, D., Schmidt-Gattung, S., and Binder, S. (2010). The DEAD-box protein PMH2 is required for efficient group II intron splicing in mitochondria of *Arabidopsis thaliana*. *Plant Mol. Biol.* 72, 459–467. doi: 10.1007/s11103-009-9584-9

Koprivova, A., Colas des Francs-Small, C., Calder, G., Mugford, S. T., Tanz, S., Lee, B.-R., et al. (2010). Identification of a pentatricopeptide repeat protein implicated in splicing of intron 1 of mitochondrial nad7 transcripts. *J. Biol. Chem.* 285, 32192–32199. doi: 10.1074/jbc.M110.147603

Kroeger, T. S., Watkins, K. P., Friso, G., van Wijk, K. J., and Barkan, A. (2009). A plant-specific RNA-binding domain revealed through analysis of chloroplast group II intron splicing. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4537–4542. doi: 10.1073/pnas.0812503106

Kubo, T., Nishizawa, S., Sugawara, A., Itchoda, N., Estiati, A., and Mikami, T. (2000). The complete nucleotide sequence of the mitochondrial genome of sugar beet (Beta vulgaris L.) reveals a novel gene for tRNA(Cys)(GCA). *Nucleic Acids Res.* 28, 2571–2576. doi: 10.1093/nar/28.13.2571

Kühn, K., Carrie, C., Giraud, E., Wang, Y., Meyer, E. H., Narsai, R., et al. (2011). The RCC1 family protein RUG3 is required for splicing of nad2 and complex I biogenesis in mitochondria of *Arabidopsis thaliana*. *Plant J.* 67, 1067–1080. doi: 10.1111/j.1365-313X.2011.04658.x

Lambowitz, A. M., Caprara, M. G., Zimmerly, S., and Perlman, P. (1999). "Group I and Group II Ribozymes as RNPs: clues to the past and guides to the future," in

The RNA World, eds R. Gesteland, T. Cech, and J. Atkins, (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), 451–485.

Lambowitz, A. M., and Zimmerly, S. (2004). Mobile group II introns. *Annu. Rev. Genet.* 38, 1–35. doi: 10.1146/annurev.genet.38.072902.091600

Lambowitz, A. M., and Zimmerly, S. (2011). Group II introns: mobile ribozymes that invade DNA. *Cold Spring Harb. Perspect. Biol.* 3, 1–19. doi: 10.1101/cshperspect.a003616.

Li-Pook-Than, J., and Bonen, L. (2006). Multiple physical forms of excised group II intron RNAs in wheat mitochondria. *Nucleic Acids Res.* 34, 2782–2790. doi: 10.1093/nar/gkl328

Li-Pook-Than, J., Carrillo, C., and Bonen, L. (2004). Variation in mitochondrial transcript profiles of protein-coding genes during early germination and seedling development in wheat. *Curr. Genet.* 46, 374. doi: 10.1007/s00294-004-0544-2

Liu, Y., He, J., Chen, Z., Ren, X., Hong, X., and Gong, Z. (2010). ABA overly-sensitive 5 (ABO5), encoding a pentatricopeptide repeat protein required for cis-splicing of mitochondrial nad2 intron 3, is involved in the abscisic acid response in Arabidopsis. *Plant J.* 63, 749–765. doi: 10.1111/j.1365-313X.2010.04280.x

Lurin, C., Andres, C., Aubourg, S., Bellaoui, M., Bitton, F., Bruyere, C., et al. (2004). Genome-wide analysis of arabidopsis pentatricopeptide repeat proteins reveals their essential role in organelle biogenesis. *Plant Cell* 16, 2089–2103. doi: 10.1105/tpc.104.022236

Lynch, M., and Kewalramani, A. (2003). Messenger RNA surveillance and the evolutionary proliferation of introns. *Mol. Biol. Evol.* 20, 563–571. doi: 10.1093/molbev/msg068

Malek, O., and Knoop, V. (1998). Trans-splicing group II introns in plant mitochondria: the complete set of cis-arranged homologs in ferns, fern allies, and a hornwort. *RNA* 4, 1599–1609. doi: 10.1017/S1355838298981262

Martin, W., and Koonin, E. V. (2006). Introns and the origin of nucleus-cytosol compartmentalization. *Nature* 440, 41–45. doi: 10.1038/nature04531

Matsuura, M., Noah, J. W., and Lambowitz, A. M. (2001). Mechanism of maturase-promoted group II intron splicing. *EMBO J.* 20, 7259–7270. doi: 10.1093/emboj/20.24.7259

Matthes, A., Schmidt-Gattung, S., Köhler, D., Forner, J., Wildum, S., Raabe, M., et al. (2007). Two DEAD-box proteins may be part of RNA-Dependent High-Molecular-Mass protein complexes in Arabidopsis Mitochondria. *Plant Physiol.* 145, 1637–1646. doi: 10.1104/pp.107.108076

McNeil, B. A., Simon, D. M., and Zimmerly, S. (in press). Alternative splicing of a group II intron in a surface layer protein gene in *Clostridium tetani*. *Nucleic Acids Res.* doi: 10.1093/nar/gkt1053

Meyer, E. H., Solheim, C., Tanz, S. K., Bonnard, G., and Millar, A. H. (2011). Insights into the composition and assembly of the membrane arm of plant complex I through analysis of subcomplexes in Arabidopsis mutant lines. *J. Biol. Chem.* 286, 26081–26092. doi: 10.1074/jbc.M110.209601

Michel, F., and Ferat, J. L. (1995). Structure and activities of group-II introns. *Annu. Rev. Biochem.* 64, 435–461. doi: 10.1146/annurev.bi.64.070195.002251

Michel, F., Umesono, K., and Ozeki, H. (1989). Comparative and functional anatomy of group II catalytic introns—a review. *Gene* 82, 5–30. doi: 10.1016/0378-1119(89)90026-7

Millar, A. H., Whelan, J., Soole, K. L., and Day, D. A. (2011). Organization and regulation of mitochondrial respiration in plants. *Annu. Rev. Plant Biol.* 62, 79–104. doi: 10.1146/annurev-arplant-042110-103857

Mohr, G., and Lambowitz, A. M. (2003). Putative proteins related to group II intron reverse transcriptase/maturases are encoded by nuclear genes in higher plants. *Nucleic Acids Res.* 31, 647–652. doi: 10.1093/nar/gkg153

Nakagawa, N., and Sakurai, N. (2006). A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II Intron maturase, causes impaired splicing of mitochondrial nad4 transcript and altered carbon metabolism in *Arabidopsis thaliana*. *Plant Cell Physiol.* 47, 772–783. doi: 10.1093/pcp/pcj051

Narsai, R., Law, S. R., Carrie, C., Xu, L., and Whelan, J. (2011). In depth temporal transcriptome profiling reveals a crucial developmental switch with roles for RNA processing and organelle metabolism that are essential for germination in *Arabidopsis thaliana*. *Plant Physiol.* 157, 1342–1362. doi: 10.1104/pp.111.183129

Noah, J. W., and Lambowitz, A. M. (2003). Effects of maturase binding and Mg2+ concentration on group II intron RNA folding investigated by UV cross-linking. *Biochemistry* 42, 12466–12480. doi: 10.1021/bi035339n

Notsu, Y., Masood, S., Nishikawa, T., Kubo, N., Akiduki, G., Nakazono, M., et al. (2002). The complete sequence of the rice (*Oryza sativa* L.) mitochondrial genome: frequent DNA sequence acquisition and loss during the evolution of flowering plants. *Mol. Genet. Genomics* 268, 434–445. doi: 10.1007/s00438-002-0767-1

Ogihara, Y., Yamazaki, Y., Murai, K., Kanno, A., Terachi, T., Shiina, T., et al. (2005). Structural dynamics of cereal mitochondrial genomes as revealed by complete nucleotide sequencing of the wheat mitochondrial genome. *Nucleic Acids Res.* 33, 6235–6250. doi: 10.1093/nar/gki925

Pereira de Souza, A., Jubier, M. F., Delcher, E., Lancelin, D., and Lejeune, B. (1991). A trans-splicing model for the expression of the tripartite nad5 gene in wheat and maize mitochondria. *Plant Cell* 3, 1363–1378. doi: 10.1105/tpc.3.12.1363

Perron, K., Goldschmidt-Clermont, M., and Rochaix, J.-D. (2004). A multiprotein complex involved in chloroplast group II intron splicing. *RNA* 10, 704–711. doi: 10.1261/rna.5237804

Putnam, A. A., and Jankowsky, E. (2013). DEAD-box helicases as integrators of RNA, nucleotide and protein binding. *Biochim. Biophys. Acta* 1829, 884–893. doi: 10.1016/j.bbagrm.2013.02.002

Pyle, A. M., and Lambowitz, A. M. (1999). "Group II Introns: ribozymes that splice RNA and invade DNA," in *The RNA World*, 3rd Edn., eds R. Gesteland, T. R. Cech, and J. Atkins (Cold Spring Harbor, NY: John Inglis), 469–505.

Qin, P. Z., and Pyle, A. M. (1998). The architectural organization and mechanistic function of group II intron structural elements. *Curr. Opin. Chem. Biol.* 8, 301–308. doi: 10.1016/S0959-440X(98)80062-6

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci.* 13, 663–670. doi: 10.1016/j.tplants.2008.10.001

Sharp, P. A. (1985). On the origin of RNA splicing and introns. *Cell* 42, 397–400. doi: 10.1016/0092-8674(85)90092-3

Small, I. D., and Peeters, N. (2000). The PPR motif - a TPR-related motif prevalent in plant organellar proteins. *Trends Biochem. Sci.* 25, 45. doi: 10.1016/S0968-0004(99)01520-0

Sugiyama, Y., Watase, Y., Nagase, M., Makita, N., Yagura, S., Hirai, A., et al. (2005). The complete nucleotide sequence and multipartite organization of the tobacco mitochondrial genome: comparative analysis of mitochondrial genomes in higher plants. *Mol. Genet. Genomics* 272, 603–615. doi: 10.1007/s00438-004-1075-8

Takenaka, M., Verbitskiy, D., van der Merwe, J. A., Zehrmann, A., and Brennicke, A. (2008). The process of RNA editing in plant mitochondria. *Mitochondrion* 8, 35–46. doi: 10.1016/j.mito.2007.09.004

Thomson, M. C., Macfarlane, J. L., Beagley, C. T., and Wolstenholme, D. R. (1994). RNA editing of mat-r transcripts in maize and soybean increases similarity of the encoded protein to fungal and bryophyte group-II intron maturases - evidence that mat-r encodes a functional protein. *Nucleic Acids Res.* 22, 5745–5752. doi: 10.1093/nar/22.25.5745

Toor, N., Keating, K. S., Taylor, S. D., and Pyle, A. M. (2008). Crystal structure of a self-spliced group II intron. *Science* 320, 77–82. doi: 10.1126/science.1153803

Unseld, M., Marienfeld, J. R., Brandt, P., and Brennicke, A. (1997). The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. *Nat. Genet.* 15, 57–61. doi: 10.1038/ng0197-57

Wahleithner, J. A., MacFarlane, J. L., and Wolstenholme, D. R. (1990). A sequence encoding a maturase-related protein in a group II intron of a plant mitochondrial nad1 gene. *Proc. Natl. Acad. Sci. U.S.A.* 87, 548–552. doi: 10.1073/pnas.87.2.548

Wank, H., SanFilippo, J., Singh, R. N., Matsuura, M., and Lambowitz, A. M. (1999). A reverse transcriptase/maturase promotes splicing by binding at its own coding segment in a group II intron RNA. *Mol. Cell* 4, 239–250. doi: 10.1016/S1097-2765(00)80371-8

Yagi, Y., Hayashi, S., Kobayashi, K., Hirayama, T., and Nakamura, T. (2013). Elucidation of the RNA recognition code for pentatricopeptide repeat proteins involved in organelle RNA editing in plants. *PLoS ONE* 8:e57286. doi: 10.1371/journal.pone.0057286

Yin, P., Li, Q., Yan, C., Liu, Y., Liu, J., Yu, F., et al. (2013). Structural basis for the modular recognition of single-stranded RNA by PPR proteins. *Nature* 504, 168–171. doi: 10.1038/nature12651

Zimmerly, S., Hausner, G., and Wu, X. C. (2001). Phylogenetic relationships among group II intron ORFs. *Nucleic Acids Res.* 29, 1238–1250. doi: 10.1093/nar/29.5.1238

Zmudjak, M., Colas des Francs-Small, C., Keren, I., Shaya, F., Belausov, E., Small, I., et al. (2013). mCSF1, a nucleus-encoded CRM protein required for the processing of many mitochondrial introns, is involved in the biogenesis of respiratory complexes I and IV in Arabidopsis. *New Phytol.* 199, 379–394. doi: 10.1111/nph.12282

Zoschke, R., Nakamura, M., Liere, K., Sugiura, M., Börner, T., and Schmitz-Linneweber, C. (2010). An organellar maturase associates with multiple group II introns. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3245–3250. doi: 10.1073/pnas.0909400107

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 December 2013; paper pending published: 07 January 2014; accepted: 27 January 2014; published online: 18 February 2014.

*Citation: Brown GG, Colas des Francs-Small C and Ostersetzer-Biran O (2014) Group II intron splicing factors in plant mitochondria. Front. Plant Sci. **5**:35. doi: 10.3389/fpls.2014.00035*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 03 April 2014
doi: 10.3389/fpls.2014.00127

Mitochondrial RNA editing PPR proteins can tolerate protein tags at E as well as at DYW domain termini

Nadja Brehme, Anja Zehrmann, Daniil Verbitskiy, Barbara Härtel and Mizuki Takenaka*

Molekulare Botanik, Universität Ulm, Ulm, Germany
**Correspondence: mizuki.takenaka@uni-ulm.de*

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Mareike Schallenberg-Rüdinger, Philipps-University of Marburg, Germany

Keywords: RNA editing, plant organelles, PPR protein, E domain, DYW domain

INTRODUCTION

In plastids and mitochondria of plants, RNA editing changes numerous cytidines to uridines. The nucleotides to be edited are selected by *trans*-acting proteins which are structurally characterized as pentatricopeptide repeat (PPR) proteins (Kotera et al., 2005; Schmitz-Linneweber and Small, 2008; Takenaka et al., 2013b). The approximately 35 amino acids long elements each attach to a specific nucleotide moiety and several tandemly arranged elements establish selective contact to a unique RNA sequence pattern. The PPR parameters defining the nucleotide specificity were identified by computational analysis and confirmed by experimental retargeting and by crystal structures (Barkan et al., 2012; Ke et al., 2013; Takenaka et al., 2013a; Yagi et al., 2013; Yin et al., 2013). Half of the PPR family of about 450 proteins in flowering plants consists only of these repeats and few further domains if any. This group of PPR proteins is involved in RNA processing events other than RNA editing (Schmitz-Linneweber and Small, 2008). The about 200 PPR proteins associated with RNA editing are C-terminally extended by the so-called E domains, the function of which is so far unknown. About half of these proteins contain an additional C-terminal domain with key features of deaminases in the form of characteristic amino acids which may bind an essential zinc atom (Hayes et al., 2013). However, so far no deaminase activity has been found, one of these domains rather shows an RNase activity (Nakamura and Sugita, 2008). This domain terminates in most instances with the name giving amino acid triplet DYW. In several such PPR RNA editing factors in plastids and mitochondria, the DYW domain can be deleted without compromising the function of the protein in editing (Okuda et al., 2009), while in others the DYW domain is required (Zehrmann et al., 2010). In some of the E class PPR RNA editing factors, addition of a DYW domain does not affect their competence in editing (Verbitskiy et al., 2012a). So far the functional parameters are unclear which distinguish those protein factors that require the C-terminal DYW extension beyond the E domain from those that do not. The E domain is in all of these RNA editing PPR proteins essential and cannot be removed.

To learn more about the function and structural features of these extension domains, we probed the importance of native C-termini of several PPR proteins in mitochondria by adding an additional protein domain. These chimeric proteins were assayed for their *in vivo* RNA editing competence to complement respective *Arabidopsis thaliana* mutants.

MOST E DOMAIN PPR EDITING PROTEINS ARE FULLY FUNCTIONAL WITH A C-TERMINAL GFP EXTENSION

As representatives for the PPR type site-specific mitochondrial RNA editing factors (MEF) terminating with an E domain, we selected MEF9, MEF18, MEF19, MEF20, and MEF21 (Takenaka, 2010; Takenaka et al., 2010). Their respective open reading frames were extended by adding a C-terminal GFP coding sequence (260 amino acids) in frame and cloned under control of the 35S promoter. The chimeric proteins were analyzed in *Arabidopsis thaliana* plants for their competence to complement respective mutants. Fusion proteins of MEF9, MEF18, MEF19, and MEF20 with GFP all showed full competence in RNA editing at their respective target sites and gave transformants fully recovered to wild type level editing (**Figure 1**). For MEF9 and MEF19, all twelve, and for MEF18 all 11 regenerated mutant plants complemented with the respective C-terminal GFP fusion protein regained wild type RNA editing levels. The MEF20-GFP chimera complemented fully in 1 of 12 stably transformed plants, more than 90% editing levels were seen in another five plants, and between 50 and 90% editing were recovered in another four plants. Two plants did not show any alteration of the absence of editing. Nine of the MEF21-GFP transformed mutant plants regained the ability for RNA editing which increased from 0 to 21–84%, 2 of 12 plants complemented with MEF21-GFP showed no editing, none of them fully recovered the apparently complete nucleotide conversion of the wild type. The variation of editing recovery between individual transformants can most likely be attributed to varying cosuppression effects and/or the individually differing nuclear integration locus of the transgene, which is known to strongly influence transgene expression levels. In addition, *in vivo* editing levels sustained by introduced PPR proteins have been found to correlate unpredictably with their RNA levels (Okuda et al., 2008) probably due to further posttranslational regulation, wherefore we did not analyze mRNA levels generated off the transgenes.

DYW DOMAIN PPR EDITING PROTEINS CAN ALSO TOLERATE A C-TERMINALLY ATTACHED GFP

Of the PPR type site-specific mitochondrial RNA editing factors terminating with a DYW domain, we tested MEF14 (Verbitskiy et al., 2011) and the rather

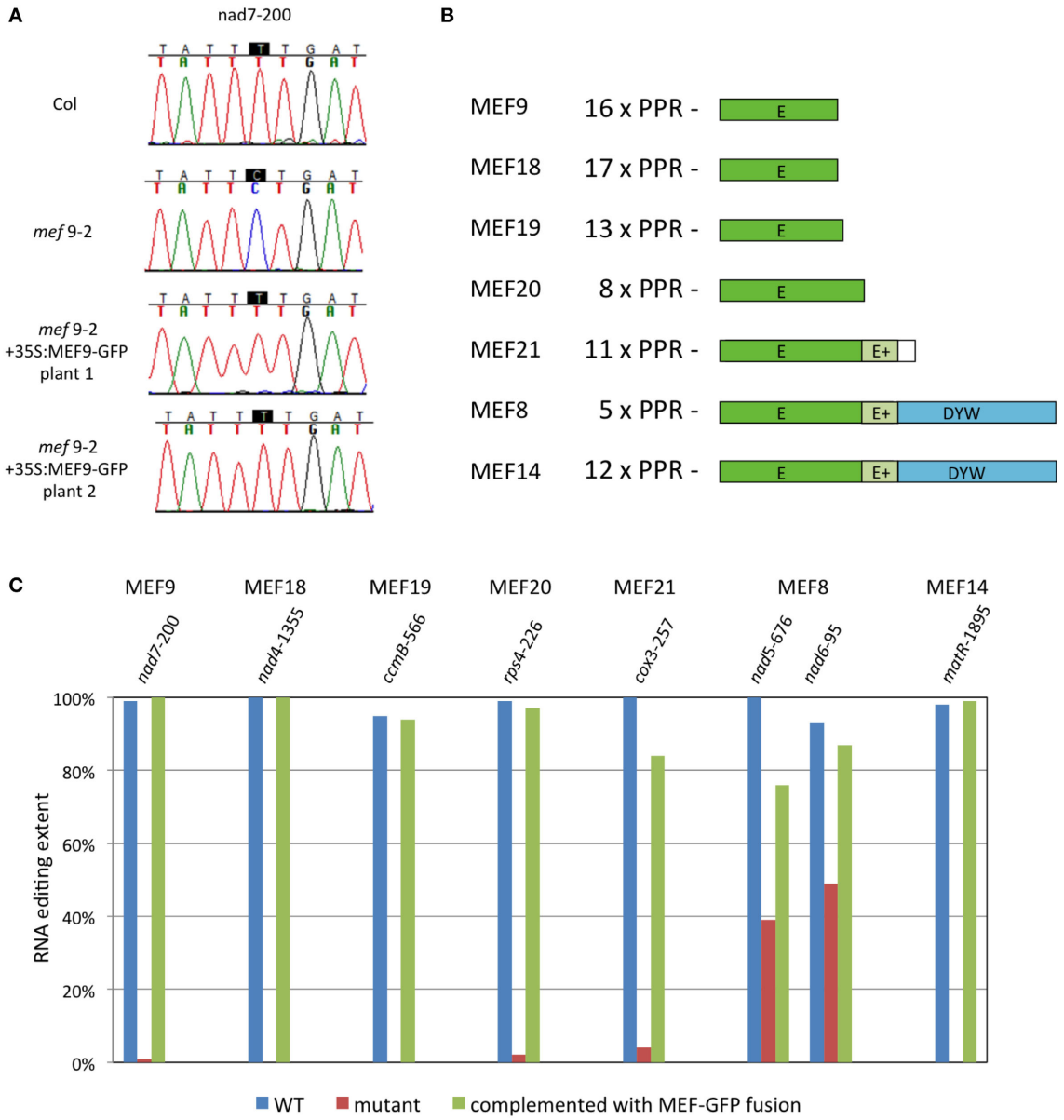


FIGURE 1 | RNA editing specific PPR-proteins terminating with E- or DYW-domains tolerate an additional protein tag. (A) Analysis of MEF9 is shown as a representative example. The single target site of MEF9 in the mitochondrial mRNA coding for subunit 7 of the respiratory chain complex I is nearly completely edited to U in wild type *Arabidopsis thaliana* plants of the Columbia ecotype (Col; Takenaka, 2010). In the *mef9-2* mutant plant, RNA editing at nucleotide *nad7*-200 (counted from the A in the AUG translational start codon) is below the background threshold of 10%. The chimeric protein of MEF9 with GFP attached to the C-terminus of the E domain fully recovers editing at this site (Plants 1 and 2). Color traces are: G, black; A, green; T, red; C, blue. **(B)** Structure of the PPR RNA editing proteins analyzed as chimeric MEF-GFP proteins. The number of the PPR elements are given and the approximate lengths of the E domains are depicted. Several proteins contain an additional extension termed E+. The white box in MEF21 indicates an extension of the open reading frame beyond the E/E+ domains but without similarity to the DYW region. **(C)** Bar graph of the complementation levels achieved at respective target sites in mutant plant lines transformed with the corresponding chimeric MEF-GFP proteins. Shown are the maximal editing levels observed in at least 1 of the 11 or 12 transgenic plant lines analyzed for each mutant. *Arabidopsis thaliana* plants were grown, transformed and analyzed as described (Zehrmann et al., 2009; Verbitskiy et al., 2012a). Chimeric gene constructs were cloned under control of the 35S promoter. Individual transformants were selected and propagated independently. RNA editing was verified by cDNA sequence analysis in each transgenic plant line, percentages of editing were calculated by relative peak heights of C and T sequence signals, respectively (Zehrmann et al., 2009).

short MEF8 protein (Verbitskiy et al., 2012b). With MEF14-GFP fusion proteins, 3 of 12 regenerants recovered the editing level of wild type plants, showing that a GFP can be tolerated at the C-terminus. A further four plants showed 80–90% recovery, up from no discernible editing in the mutant, another four regained 30–80% and one showed no recovery beyond the background of the sequence analysis.

The MEF8 protein contains only five recognizable PPR elements, which

would hardly be sufficient for sequence-specificity, yet does affect unique sites when mutated (Verbitskiy et al., 2012b). In *mef8-2* mutant plants, site *nad5*-676 is edited to only about 40% compared to the 100% of the wild type, and site *nad6*-96 is reduced to about 50% from about 95% editing. In 10 of 11 regenerated complemented plants the MEF8-GFP chimera raised the editing levels at both sites by 10–40%, indicating that the recombinant protein is functional in editing.

BIOCHEMICAL APPLICATION OF FUNCTIONAL E AND DYW PPR EDITING PROTEINS WITH A C-TERMINAL GFP TAG

The PPR type site-specific mitochondrial RNA editing factors are required for editing at one, or at most few, specific sites. It is presently not clear whether some of them are sufficient for editing their target site(s), since so far no successful *in vitro* editing assays with a clean full length PPR protein and its target RNA have been reported. So far, only *in vitro* assays showing the RNA-PPR protein connection (Okuda et al., 2009) and the nuclease activity of a partial PPR protein containing the DYW domain have been published (Nakamura and Sugita, 2008). It is more likely that at least *in vivo* a more complex editosome performs the editing reaction since a number of additional proteins are required for or at least influence RNA editing in either or both plant organelles. Foremost are the MORF proteins whose major function in the cell may be in RNA editing and which can directly contact specific editing PPR proteins (Bentolila et al., 2012; Takenaka et al., 2012). Other RNA binding proteins have been found to influence RNA editing such as ORRM and other RRM binding domain containing organellar proteins (Tillich et al., 2009; Sun et al., 2013). Recently, an enzyme of the tetrapyrrole biosynthesis pathway has been found to be required for RNA editing at selected sites in the chloroplast transcriptome (Zhang et al., 2014).

The here evaluated construction of MEF-GFP fusion proteins from two subgroups of PPR proteins, those terminating with the E and those with an additional DYW domain, will allow to detect interactions with other proteins. These transgenic mutant plants can be used for pull-down experiments to identify associated proteins. The confirmed functionality of the fusion proteins suggests that the added GFP moieties do not inhibit necessary interactions with other protein molecules. It seems prudent to first establish the functionality of PPR proteins modified by comparatively large tags as the entire GFP protein which may distort the *in vivo* relevance of protein-RNA or protein-protein interactions. The major obstacle hindering biochemical investigations, the very low abundance of the editing PPR proteins, can possibly be circumvented or alleviated by selecting transgenic plant lines with elevated MEF-GFP expression levels.

THE CONSERVED TERMINAL AMINO ACIDS CAN BE EXTENDED IN SOME, BUT NOT ALL DYW PPR EDITING PROTEINS

The DYW amino acid triplet is generally at the very C-terminus of this domain. However, several PPR mitochondrial RNA editing factors show variations of the DYW triplet to other triplets such as DFW in MEF7, DSW in OTP86, or EYW in MEF8S. Altogether, about a third of the mitochondrial and plastid "DYW" editing factors contain triplets other than DYW. Nevertheless, the C-terminal amino acid triplet being conserved in so many proteins implies a functional constraint. Similarly, some of the His and Cys residues implicated in the potential deaminase activity (Salone et al., 2007) are not found in all DYW domains. However, these variants may be explained if not all DYW domains are actually functional as suggested by the distinct requirement for this domain in individual PPR proteins.

In this context, the MEF11 mutant *mef11-2* shows that in this PPR protein, the DYW domain is essential for some targets, but not for all (Verbitskiy et al., 2010). The T-DNA insertion removes half of the domain, the DYW triplet and 56 upstream amino acids, but leaves the amino acid pattern of the potential deaminase intact. Surprisingly, deletion and complementation assays showed that this entire domain is not required and can be removed from the MEF11 protein (Zehrmann et al., 2011). Conversely, the DYW domain is essential in the MEF1 protein, truncated versions ending with the E domain cannot recover editing in the respective mutant. Concomitantly, the MEF1 protein does not tolerate a C-terminal extension by a His-tag, suggesting that some essential DYW termini have to be free and are not functional when extended (Zehrmann et al., 2010). The here observed partial complementation of the tagged MEF8 protein may also indicate that masking of the C-terminus by the GFP moiety does inhibit the activity and function of this DYW PPR protein. Similar differential results for several plastid editing PPR proteins support the inference that the DYW is essential in only some PPR proteins. The likely explanation is that the DYW function can be substituted by another protein containing this region. Prominent support for this surmise comes from the instance of the DYW1 and the CRR4 proteins, these proteins can be present in trans or can be linked into a single amino acid chain to edit a plastid site (Boussardon et al., 2012). By analogy, absent or non-functional DYW domains will be supplied by additional PPR proteins interacting with the site specific PPR protein possibly as a direct heteromer or with the support of one or more of the other factors contributing to the editosome complex. Evidence for direct dimer formation of PPR proteins has come from crystallization studies, in which the PPR10 protein forms a dimer, with or without bound target RNA (Ke et al., 2013; Yin et al., 2013).

PERSPECTIVE

In plant organellar RNA editing, specific sites are addressed by PPR proteins which all contain C-terminal extension domains. We here report that in *Arabidopsis thaliana*, several PPR editing proteins terminating with an E or with an additional DYW domain tolerate the addition of a GFP moiety. Since the DYW motif marks the C-terminus of the respective domain, it is surprising that a GFP protein can be added in frame to some DYW proteins without disturbing their apparent functionality.

ACKNOWLEDGMENTS

We thank Dagmar Pruchner, Angelika Müller and Bianca Wolf for excellent experimental help. This work was supported by grants from the Deutsche Forschungsgemeinschaft. Mizuki Takenaka is a Heisenberg fellow.

REFERENCES

Barkan, A., Rojas, M., Fujii, S., Yap, A., Chong, Y. S., Bond, C. S., et al. (2012). A combinatorial amino acid code for RNA recognition by pentatricopeptide repeat proteins. *PLoS Genet.* 8:e1002910. doi: 10.1371/journal.pgen.1002910

Bentolila, S., Heller, W. P., Sun, T., Babina, A. M., Friso, G., van Wijk, K. J., et al. (2012). RIP1, a member of an Arabidopsis protein family, interacts with the protein RARE1 and broadly affects RNA editing. *Proc. Natl. Acad. Sci. U.S.A.* 109, E1453–E1461. doi: 10.1073/pnas.1121465109

Boussardon, C., Salone, V., Avon, A., Berthomé, R., Hammani, K., Okuda, K., et al. (2012). Two interacting proteins are necessary for the editing of the NdhD-1 site in *Arabidopsis* plastids. *Plant Cell* 24, 3684–3694. doi: 10.1105/tpc.112.099507

Hayes, M. L., Giang, K., Berhane, B., and Mulligan, R. M. (2013). Identification of two pentatricopeptide repeat genes required for RNA editing and zinc binding by C-terminal cytidine deaminase-like domains. *J. Biol. Chem.* 288, 36519–36529. doi: 10.1074/jbc.M113.485755

Ke, J., Chen, R.-Z., Ban, T., Zhou, X. E., Gu, X., Tan, M. E., et al. (2013). Structural basis for RNA recognition by a dimeric PPR-protein complex. *Nat. Struct. Mol. Biol.* 20, 1377–1382. doi: 10.1038/nsmb.2710

Kotera, E., Tasaka, M., and Shikanai, T. (2005). A pentatricopeptide repeat protein is essential for RNA editing in chloroplasts. *Nature* 433, 326–330. doi: 10.1038/nature03229

Nakamura, T., and Sugita, M. (2008). A conserved DYW domain of the pentatricopeptide repeat protein possesses a novel endoribonuclease activity. *FEBS Lett.* 582, 4163–4168. doi: 10.1016/j.febslet.2008.11.017

Okuda, K., Chateigner-Boutin, A.-L., Nakamura, T., Delannoy, E., Sugita, M., Myouga, F., et al. (2009). Pentatricopeptide repeat proteins with the DYW motif have distinct molecular functions in RNA editing and RNA cleavage in Arabidopsis chloroplasts. *Plant Cell* 21, 146–156. doi: 10.1105/tpc.108.064667

Okuda, K., Habata, Y., Kobayashi, Y., and Shikanai, T. (2008). Amino acid variations in Nicotiana CRR4 orthologs determine the species-specific efficiency of RNA editing in plastids. *Nucl. Acids Res.* 36, 6155–6164. doi: 10.1093/nar/gkn629

Salone, V., Rüdinger, M., Polsakiewicz, M., Hoffmann, B., Groth-Malonek, M., Szurek, B., et al. (2007). A hypothesis on the identification of the editing enzyme in plant organelles. *FEBS Lett.* 581, 4132–4138. doi: 10.1016/j.febslet.2007.07.075

Schmitz-Linneweber, C., and Small, I. (2008). Pentatricopeptide repeat proteins: a socket set for organelle gene expression. *Trends Plant Sci.* 13, 663–670. doi: 10.1016/j.tplants.2008.10.001

Sun, T., Germain, A., Giloteaux, L., Hammani, K., Barkan, A., Hanson, M. R., et al. (2013). An RNA recognition motif-containing protein is required for plastid RNA editing in *Arabidopsis* and maize. *Proc. Natl. Acad. Sci. U.S.A.* 110, E1169–E1178. doi: 10.1073/pnas.1220162110

Takenaka, M. (2010). MEF9, an E subclass PPR protein, is required for an RNA editing event in the nad7 transcript in mitochondria of Arabidopsis thaliana. *Plant Physiol.* 152, 939–947. doi: 10.1104/pp.109.151175

Takenaka, M., Verbitskiy, D., Zehrmann, A., and Brennicke, A. (2010). Reverse genetic screening identifies five E-class PPR proteins involved in RNA editing in mitochondria of Arabidopsis thaliana. *J. Biol. Chem.* 285, 27122–27129. doi: 10.1074/jbc.M110.128611

Takenaka, M., Zehrmann, A., Brennicke, A., and Graichen, K. (2013a). Improved computational target site prediction for pentatricopeptide repeat RNA editing factors. *PLoS ONE* 8:e65343. doi: 10.1371/journal.pone.0065343

Takenaka, M., Zehrmann, A., Härtel, B., Kugelmann, M., Verbitskiy, D., and Brennicke, A. (2012). MORF family proteins are required for RNA editing in mitochondria and plastids of plants. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5104–5109. doi: 10.1073/pnas.1202452109

Takenaka, M., Zehrmann, A., Verbitskiy, D., Härtel, B., and Brennicke, A. (2013b). RNA-editing in plants and its evolution. *Annu. Rev. Genet.* 47, 353–370. doi: 10.1146/annurev-genet-111212-133519

Tillich, M., Hardel, S. L., Kupsch, C., Armbruster, U., Delannoy, E., Gualberto, J. M., et al. (2009). Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* 10, 6002–6007. doi: 10.1073/pnas.0808529106

Verbitskiy, D., van der Merwe, J. A., Zehrmann, A., Härtel, B., and Takenaka, M. (2012a). The E-class PPR Protein MEF3 of Arabidopsis thaliana can function in mitochondrial RNA editing also with an additional DYW domain. *Plant Cell Physiol.* 53, 358–367. doi: 10.1093/pcp/pcr182

Verbitskiy, D., Zehrmann, A., Härtel, B., Brennicke, A., and Takenaka, M. (2011). The DYW-E-PPR protein MEF14 is required for RNA editing at site matR-1895 in mitochondria of Arabidopsis thaliana. *FEBS Lett.* 585, 700–704. doi: 10.1016/j.febslet.2011.01.037

Verbitskiy, D., Zehrmann, A., Härtel, B., Brennicke, A., and Takenaka, M. (2012b). Two related RNA editing proteins target the same sites in mitochondria of Arabidopsis thaliana. *J. Biol. Chem.* 287, 38064–38072. doi: 10.1074/jbc.M112.397992

Verbitskiy, D., Zehrmann, A., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2010). The PPR-protein encoded by the lovastatin insensitive 1 gene is involved in RNA editing at three sites in mitochondria of Arabidopsis thaliana. *Plant J.* 61, 446–455. doi: 10.1111/j.1365-313X.2009.04076.x

Yagi, Y., Hayashi, S., Kobayashi, K., Hirayama, T., and Nakamura, T. (2013). Elucidation of the RNA recognition code for pentatricopeptide repeat proteins involved in organelle RNA editing in plants. *PLoS ONE* 8:e57286. doi: 10.1371/journal.pone.0057286

Yin, P., Li, Q., Yan, C., Liu, Y., Liu, J., Yu, F., et al. (2013). Structural basis for the modular recognition of single-stranded RNA by PPR proteins. *Nature* 504, 168–171. doi: 10.1038/nature12651

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2010). RNA editing competence of trans-factor MEF1 is modulated by ecotype-specific differences but requires the DYW domain. *FEBS Lett.* 584, 4181–4186. doi: 10.1016/j.febslet.2010.08.049

Zehrmann, A., Verbitskiy, D., Härtel, B., Brennicke, A., and Takenaka, M. (2011). PPR proteins network as site-specific RNA editing factors in plant organelles. *RNA Biol.* 8, 67–70. doi: 10.4161/rna.8.1.14298

Zehrmann, A., Verbitskiy, D., van der Merwe, J. A., Brennicke, A., and Takenaka, M. (2009). A DYW domain containing pentatricopeptide repeat protein is required for RNA editing at multiple sites in mitochondria of Arabidopsis thaliana. *Plant Cell* 21, 558–567. doi: 10.1105/tpc.108.064535

Zhang, F., Tang, W., Hedtke, B., Zhong, L., Liu, L., Peng, L., et al. (2014) Tetrapyrrole biosynthetic enzyme protoporphyrinogen IX oxidase 1 is required for plastid RNA editing. *Proc. Natl. Acad. Sci. U.S.A.* 111, 2023–2028. doi: 10.1073/pnas.1316183111

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 February 2014; paper pending published: 26 February 2014; accepted: 17 March 2014; published online: 03 April 2014.

*Citation: Brehme N, Zehrmann A, Verbitskiy D, Härtel B and Takenaka M (2014) Mitochondrial RNA editing PPR proteins can tolerate protein tags at E as well as at DYW domain termini. Front. Plant Sci. **5**:127. doi: 10.3389/fpls.2014.00127*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

OPINION ARTICLE
published: 17 December 2013
doi: 10.3389/fpls.2013.00517

Heterogeneity of the 5′-end in plant mRNA may be involved in mitochondrial translation

*Tomohiko Kazama[1], Yusuke Yagi[2], Kinya Toriyama[1] and Takahiro Nakamura[2]**

[1] Laboratory of Environmental Biotechnology, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan
[2] Faculty of Agriculture, Kyushu University, Fukuoka, Japan
*Correspondence: tnaka@agr.kyushu-u.ac.jp

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Thalia Salinas, Centre National de la Recheche Scientifique associé à l'Université de Strasbourg, France

Keywords: plants, mitochondria, translation, RNA processing, protein-RNA interaction

INTRODUCTION

Genomic organization and gene expression system of plant mitochondria are distinct from those of other eukaryotes, including animals, even though of chloroplast, another organelle in plants that contain its own genomes. Recent research has revealed the significance of the control of gene expression at the RNA level, including the formation of the 5′- and 3′-ends of mitochondrial mRNA. Although the details of gene expression in mitochondria are not the same as in chloroplasts, huge numbers of nuclear-encoded pentatricopeptide repeat (PPR) proteins are transported in both mitochondria and chloroplasts and control their gene expression. (See other articles in this issue.)

CMS/*Rf* SYSTEM

Research on mitochondrial gene expression in plants has focused on understanding cytoplasmic male sterility (CMS), which is caused by the incompatibility between the mitochondrial and nuclear genomes. The mitochondrial genome allows active recombination of genes to occur and easily generates new protein-coding genes. Expression of such a gene sometimes causes male sterility, and consequently the gene is called a CMS-associated gene. Pollen fertility can be restored by suppressing the expression of the CMS-associated gene with a nuclear-encoded *fertility restorer* (*Rf*) gene. The CMS/*Rf* system is useful in agriculture because it enables easy crossbreeding of varieties to produce hybrid seeds. It is also an excellent model in which to study the nuclear control of mitochondrial gene expression. Extensive research has been conducted to identify CMS-associated genes and *Rf* genes.

One organism that has been studied for this purpose is BT-CMS rice. The BT-CMS/*Rf* system contains the mitochondrial CMS gene *orf79* and the nuclear-encoded *Rf1a* gene, which codes for a PPR protein (Kazama and Toriyama, 2003). The *orf79* gene is co-transcribed with its upstream gene, *atp6*. The protein of *Rf1a* promotes the cleavage of *atp6–orf79* co-transcribed mRNAs. The cleavage prevents the translation of *orf79* (Kazama et al., 2008).

A similar system exists in a *Brassica* CMS of Ogura. The mitochondrial genome contains the CMS-associated gene *orf138*, which is co-transcribed as *orf138–atp8* (Bonhomme et al., 1992). The amount and processing pattern of the co-transcribed mRNA is not affected by the presence or absence of the *Rf* gene, which codes for a PPR protein (Koizuka et al., 2003). The RFo/PPR-B protein is known to be associated with the *orf138* gene containing RNA, suggesting that its function is direct suppression of the translation (Uyttewaal et al., 2008a). In the cases of both rice and the *Brassica* CMS, the translational step seems to be the critical step in the CMS/*Rf* system.

TRANSLATION IN PLANT MITOCHONDRIA

Hardly anything is known about the translational control of gene expression in plant mitochondria. Regarding the *cis*-regulatory element, the mitochondrial mRNAs do not follow the Shine-Dalgarno sequence that exists in prokaryotic organisms. An early informatics study found three conserved sequence blocks in the 5′ untranslated region (UTR) of mitochondrial RNAs (Pring et al., 1992): block I (GGGAGCAGAG), block II (AGUCUCCCUUUC), and block III [GU (n) CGUUGG]. These blocks generally occur within 100 bases of the 5′ flanking region of the start codons, suggesting that they are involved in mitochondrial translation. However, their functionality has not been evaluated, due to the lack of experimental techniques to study mitochondrial translation.

Recent advances in genetic studies have revealed, although sporadically, the protein factors that are involved in plant mitochondrial translation. The silencing of the nuclear-encoded *rps10* gene, which codes for the mitochondrial ribosomal protein S10, has induced differential translations of mitochondrial transcripts, including over-expression of ribosomal proteins and down-regulation of oxidative phosphorylation subunits (Kwasniak et al., 2013). The PPR protein of the *MPPR6* gene in maize has been shown to interact with the 5′ UTR of the *rps3* mRNA, encoding mitochondrial ribosomal protein S3. This protein may also be involved in 5′ maturation and translational initiation of the *rps3* mRNA. The loss of *MPPR6* results, consequently, in a considerable reduction of mitochondrial translation (Manavski et al., 2012). The loss of translation activity induces general down-regulation of mitochondrial RNA, in contrast with the silencing of the *rps10* gene (Kwasniak et al., 2013). Another PPR protein of the *PPR336* gene has been shown to associate with polysomes in the mitochondria. The mutant plant has unusual polysomal profiles, suggesting that

PPR336 could be involved in translation (Uyttewaal et al., 2008b), although the actual mechanism has not been elucidated.

PUTATIVE ROLE OF 5′-END HETEROGENEITY OF mRNA FOR TRANSLATION IN PLANT MITOCHONDRIA

Forner et al. (2007) reported that *Arabidopsis* mitochondrial RNAs tend to have heterogeneous 5′-ends but uniform 3′-ends. We conducted an analysis using circularized (CR) reverse transcriptase (RT) PCR to determine whether the heterogeneity of the 5′-end of mRNA is involved in translational efficiency. We compared the 5′-ends of mRNA derived from total mitochondrial RNA with those of mRNA during translation. The RNAs during translation (i.e., the RNAs associated with polysomes) were fractionated by centrifuging at 100,000 × *g* (**Figure 1A**). The purity of the mitochondria and the enrichment of ribosomes in the polysomal fraction used this study were verified by the western and northern blot analyses (**Figure 1B**). Next, the RNA termini were determined by CR-RT-PCR for several genes in rice mitochondria (*atp1*, *atp6*, *atp8*, and *atp9*; **Figure 1**).

The CR-RT-PCR analysis revealed the three major signals for *atp1* (**Figure 1C**; a, b, and c). Sequence analysis revealed that the 3′-ends are uniform (+141/+142 relative to the stop codon). The multiple 5′-ends were mapped at −390 to −453 relative to the start codon (**Figure 1C**; a), −167 to −207 (**Figure 1C**; b), and

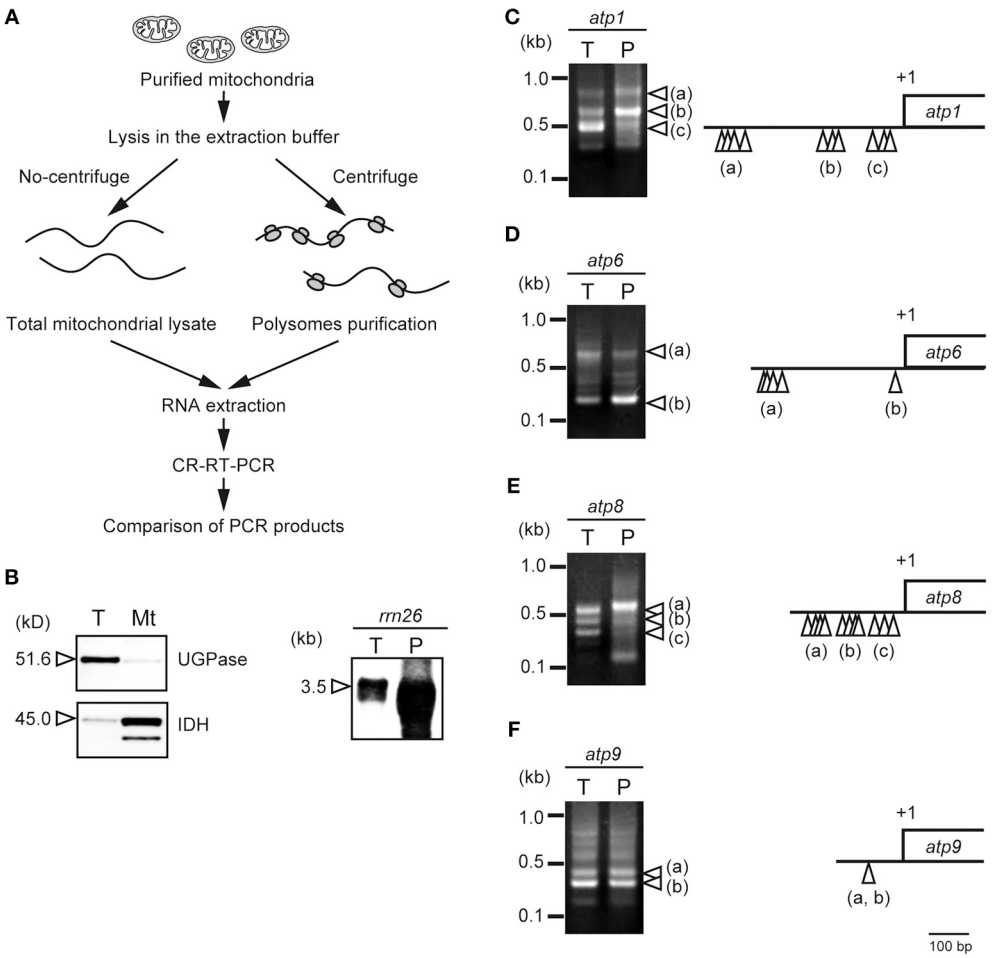


FIGURE 1 | Heterogeneity in the 5′-end of mRNA and its involvement in translational efficiency in plant mitochondria. (A) Overview of the CR-RT-PCR analysis compared mitochondrial total RNA with the polysomal RNA. The rice mitochondria were purified as described (Kazama et al., 2008). Purified mitochondria were homogenized in extraction buffer [0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, 35 mM $MgCl_2$, 1% triton X-100, 0.5 mg/ml heparin, 3 mM DTT, 0.5 mg/ml chloramphenicol, 25 mM EGTA, 0.2 M sucrose, 2% polyoxyethylene-10-tridecyl ether]. The suspension was centrifuged for 5 min at 20,000 × *g* and 4°C, twice to remove cell debris. The cell debris was further removed by centrifugation for 30 min at 30,000 × *g* and 4°C, twice. The resultant supernatant was transferred to a new tube and centrifuged for 3 hours at 100,000 × *g* and 4°C in TLA100 rotor (Beckman). The precipitate was used for polysomal fraction. **(B)** Purity of the isolated mitochondria was examined by a cytosolic marker protein of (UGPase, UDP-glucose pyrophosphorylase) and a mitochondrial marker protein (IDH, isocitrate dehydrogenase) (left panel). The western blot analyses were performed using total protein (T) and isolated mitochondria (Mt) using the specific antibodies (Agrisera). The enrichment of ribosomes in the polysomal fraction was also assessed by northern blot analysis in total mitochondrial RNA (T) and polysomal mitochondrial RNA (P) using *rrn26* probe (right panel). The images of CR-RT-PCR were shown for mRNAs of *atp1* **(C)**, *atp6* **(D)**, *atp8* **(E)**, and *atp9* **(F)**. The 5′ terminal of each transcript is indicated by white arrowheads in the CR-RT-PCR images mapped on the schematic gene structure (right panel).

−34 to −78 (**Figure 1C**; c). Interestingly, the CR-RT-PCR profile was different in the polysome-associated RNA. The *atp1*-b RNA was most enriched in the polysomal fraction, whereas the *atp1*-c RNA was predominant in the total mitochondrial RNA, suggesting that the heterogenity of the 5′-end could be involved in the translational efficiency of plant mitochondrial RNA.

Similar observations have been made in other mRNAs. The *atp6* RNA accumulated in two forms (**Figure 1D**; a and b). Their 5′-ends were mapped at around −300 for *atp6*-a and –24/–23 for *atp6*-b, relative to the start codon. The 3′-ends of all cDNAs for *atp6* have been mapped in the dense region (+27 to +29, relative to the stop codon). Polysomal analysis revealed that the shorter form of RNA (*atp6*-b) seems to be more enriched in polysome than the longer one, as shown by the distinct CR-RT-PCR profiles of total RNA vs. polysomal RNA. The *atp8* RNA was shown to accumulate in three different forms with the 5′-ends at −240 to −199, −171 to −113, and −76 to −19 (**Figure 1E**; a, b, and c, respectively). The 3′-ends were mapped at the same position in all the RNA species (+119/+121, relative to the stop codon). The longest RNA (**Figure 1E**; a) was concentrated in the polysomal fraction. The *atp9* contains two major isoforms with identical 5′ termini (−85/−84, relative to the start codon) but with different 3′ termini (+110/+111 and +6/+9) (**Figure 1F**; a and b, respectively). Polysome analysis suggested that the different 3′-end status is not involved in translation.

Together, these results suggested that the heterogeneity of 5′-ends could be involved in translational efficiency in plant mitochondria. Preliminary *in silico* searches have failed to find conserved motifs within the putative translational active RNAs.

MITOCHONDRIAL TRANSLATION IN YEASTS AND HUMANS

The status of the 5′ UTR differs among different species. As in plants, the mitochondrial mRNAs of the yeast *Saccharomyces cerevisiae* possess characteristic 5′ and 3′ UTRs. The *S. cerevisiae* would be regarded as the best system studying mitochondrial translation. Currently, tens of the translational activators have been identified for the several mitochondrial transcripts (Herrmann et al., 2013). For instance, PET309, which is a membrane-bound PPR protein, acts on the 5′ UTR of the *cox1* mRNA to activate translation and is required to stabilize the precursor of *cox1* RNAs (Manthey and McEwen, 1995). A series of mutations in PPR motifs within PET309 revealed that the PPR motifs are necessary for *cox1* mRNA translation, but not for stabilization (Tavares-Carreón et al., 2008). Thus, the PPR motifs of PET309 may induce a particular RNA conformation to attract and/or interact with the translational machinery. This evidence indicates that mitochondrial translation in *S. cerevisiae* is mainly controlled by the gene-specific translation activator through its association with the 5′ UTR in mRNA (Gruschke and Ott, 2013; Herbert et al., 2013).

Human mitochondrial genes are transcribed using three promoters. The RNAs are subsequently processed and polyadenylated to generate the stop codons (Rorbach and Minczuk, 2012). Mapping of the 5′-end of human mitochondrial mRNAs revealed that mRNAs start directly at or very near the start codon (Montoya et al., 1981). Thus, their mRNAs lack the ribosome-binding site at the 5′ UTR. The analysis of secondary structure at the 5′-ends indicated that the 5′-ends of all mRNA are highly unstructured (Jones et al., 2008). The mechanism of human mitochondrial translation is poorly understood (Koc and Koc, 2013).

Plant mitochondrial RNAs have long 5′ UTRs with no obvious conserved motifs, suggesting that gene-specific translational regulation occurs, as in *S. cerevisiae*. PPR proteins are believed to play a pivotal role in the translation regulation via the 5′ UTR in plant mitochondria.

PERSPECTIVE

Translation is a critical step that determines the final level of protein production. Recent research has suggested that mitochondrial gene expression is important in various plant phenomena, such as the pollen production, stress response, germination, and metabolite synthesis.

It is critical to develop techniques to analyze the mitochondrial translational system. The recently developed "genome editing" technology, which has been used to study the human mitochondrial genome, may be applicable to studies of transformation in plant mitochondria (Bacman et al., 2013). Alternatively, incorporation of exogenous RNA into mitochondria using the import pathway for tRNA or via PNPase may facilitate analysis of the *cis*-regulatory element (Wang et al., 2010; Mahato et al., 2011). The tRNA import pathway is applicable for plant as already shown (Sieber et al., 2011; Val et al., 2011). These approaches will facilitate elucidation of the plant mitochondrial translational system and understanding of the diverse methods of mitochondrial translation among different organisms.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid 22681028 and 22380008 (Takahiro Nakamura), and 2338002 (Kinya Toriyama) from the Ministry of Education, Culture, Sports, Science, and Technology; the Adaptable and Seamless Technology Transfer Program through Target-driven R&D, JST (Takahiro Nakamura); and Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry (Kinya Toriyama). Yusuke Yagi is a recipient of JSPS post-doctoral fellowship.

REFERENCES

Bacman, S. R., Williams, S. L., Pinto, M., Peralta, S., and Moraes, C. T. (2013). Specific elimination of mutant mitochondrial genomes in patient-derived cells by mitoTALENs. *Nat. Med.* 19, 1111–1113. doi: 10.1038/nm.3261

Bonhomme, S., Budar, F., Lancelin, D., Small, I., Defrance, M. C., and Pelletier, G. (1992). Sequence and transcript analysis of the Nco2.5 Ogura-specific fragment correlated with cytoplasmic male sterility in *Brassica* cybrids. *Mol. Gen. Genet.* 235, 340–348. doi: 10.1007/BF00279379

Forner, J., Weber, B., Thuss, S., Wildum, S., and Binder, S. (2007). Mapping of mitochondrial mRNA termini in *Arabidopsis thaliana*: t-elements contribute to 5′ and 3′ end formation. *Nucleic Acids Res.* 35, 3676–3692. doi: 10.1093/nar/gkm270

Gruschke, S., and Ott, M. (2013). "Mechanisms and control of protein synthesis in yeast mitochondria," in *Translation in Mitochondria and Other Organelles*, ed A.-M. Duchêne (Berlin; Heidelberg: Springer), 109–131 doi: 10.1007/978-3-642-39426-3_5

Herbert, C. J., Golik, P., and Bonnefoy, N. (2013). Yeast PPR proteins, watchdogs of mitochondrial gene expression. *RNA Biol.* 10, 1477–1494. doi: 10.4161/rna.25392

Herrmann, J. M., Woellhaf, M. W., and Bonnefoy, N. (2013). Control of protein synthesis in yeast mitochondria: the concept of translational activators. *Biochim. Biophys. Acta* 1833, 286–294. doi: 10.1016/j.bbamcr.2012.03.007

Jones, C. N., Wilkinson, K. A., Hung, K. T., Weeks, K. M., and Spremulli, L. L. (2008). Lack of secondary structure characterizes the 5′ ends of mammalian mitochondrial mRNAs. *RNA* 14, 862–871. doi: 10.1261/rna.909208

Kazama, T., Nakamura, T., Watanabe, M., Sugita, M., and Toriyama, K. (2008). Suppression mechanism of mitochondrial *ORF79* accumulation by Rf1 protein in BT-type cytoplasmic male sterile rice. *Plant J.* 55, 619–628. doi: 10.1111/j.1365-313X.2008.03529.x

Kazama, T., and Toriyama, K. (2003). A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. *FEBS Lett.* 544, 99–102. doi: 10.1016/S0014-5793(03)00480-0

Koc, E., and Koc, H. (2013). "Mechanism and regulation of protein synthesis in mammalian mitochondria," in *Translation in Mitochondria and Other Organelles*, ed A.-M. Duchêne (Berlin; Heidelberg: Springer), 29–53. doi: 10.1007/978-3-642-39426-3_2

Koizuka, N., Imai, R., Fujimoto, H., Hayakawa, T., Kimura, Y., Kohno-Murase, J., et al. (2003). Genetic characterization of a pentatricopeptide repeat protein gene, *orf687*, that restores fertility in the cytoplasmic male-sterile Kosena radish. *Plant J.* 34, 407–415. doi: 10.1046/j.1365-313X.2003.01735.x

Kwasniak, M., Majewski, P., Skibior, R., Adamowicz, A., Czarna, M., Sliwinska, E., et al. (2013). Silencing of the nuclear *RPS10* gene encoding mitochondrial ribosomal protein alters translation in *Arabidopsis* mitochondria. *Plant Cell* 25, 1855–1867. doi: 10.1105/tpc.113.111294

Mahato, B., Jash, S., and Adhya, S. (2011). RNA-mediated restoration of mitochondrial function in cells harboring a Kearns Sayre Syndrome mutation. *Mitochondrion* 11, 564–574. doi: 10.1016/j.mito.2011.03.006

Manavski, N., Guyon, V., Meurer, J., Wienand, U., and Brettschneider, R. (2012). An essential pentatricopeptide repeat protein facilitates 5′ maturation and translation initiation of *rps3* mRNA in maize mitochondria. *Plant Cell* 24, 3087–3105. doi: 10.1105/tpc.112.099051

Manthey, G. M., and McEwen, J. E. (1995). The product of the nuclear gene *PET309* is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial *COX1* locus of *Saccharomyces cerevisiae*. *EMBO J.* 14, 4031–4043.

Montoya, J., Ojala, D., and Attardi, G. (1981). Distinctive features of the 5′-terminal sequences of the human mitochondrial mRNAs. *Nature* 290, 465–470. doi: 10.1038/290465a0

Pring, D. R., Mullen, J. A., and Kempken, F. (1992). Conserved sequence blocks 5′ to start codons of plant mitochondrial genes. *Plant Mol. Biol.* 19, 313–317. doi: 10.1007/BF00027353

Rorbach, J., and Minczuk, M. (2012). The post-transcriptional life of mammalian mitochondrial RNA. *Biochem. J.* 444, 357–373. doi: 10.1042/BJ20112208

Sieber, F., Placido, A., El Farouk-Ameqrane, S., Duchene, A. M., and Marechal-Drouard, L. (2011). A protein shuttle system to target RNA into mitochondria. *Nucleic Acids Res.* 39:e96. doi: 10.1093/nar/gkr380

Tavares-Carreón, F., Camacho-Villasana, Y., Zamudio-Ochoa, A., Shingú-Vázquez, M., Torres-Larios, A., and Pérez-Martinez, X. (2008). The pentatricopeptide repeats present in Pet309 are necessary for translation but not for stability of the mitochondrial COX1 mRNA in yeast. *J. Biol. Chem.* 283, 1472–1479. doi: 10.1074/jbc.M708437200

Uyttewaal, M., Arnal, N., Quadrado, M., Martin-Canadell, A., Vrielynck, N., Hiard, S., et al. (2008a). Characterization of *Raphanus sativus* pentatricopeptide repeat proteins encoded by the fertility restorer locus for Ogura cytoplasmic male sterility. *Plant Cell* 20, 3331–3345. doi: 10.1105/tpc.107.057208

Uyttewaal, M., Mireau, H., Rurek, M., Hammani, K., Arnal, N., Quadrado, M., et al. (2008b). PPR336 is associated with polysomes in plant mitochondria. *J. Mol. Biol.* 375, 626–636. doi: 10.1016/j.jmb.2007.11.011

Val, R., Wyszko, E., Valentin, C., Szymanski, M., Cosset, A., Alioua, M., et al. (2011). Organelle trafficking of chimeric ribozymes and genetic manipulation of mitochondria. *Nucleic Acids Res.* 39, 9262–9274. doi: 10.1093/nar/gkr580

Wang, G., Chen, H. W., Oktay, Y., Zhang, J., Allen, E. L., Smith, G. M., et al. (2010). PNPASE regulates RNA import into mitochondria. *Cell* 142, 456–467. doi: 10.1016/j.cell.2010.06.035

Received: 14 November 2013; accepted: 02 December 2013; published online: 17 December 2013.

Citation: Kazama T, Yagi Y, Toriyama K and Nakamura T (2013) Heterogeneity of the 5′-end in plant mRNA may be involved in mitochondrial translation. Front. Plant Sci. ***4****:517. doi: 10.3389/fpls.2013.00517*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 08 January 2014
doi: 10.3389/fpls.2013.00551

Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements

Elina Welchen[1,2] *, Lucila García[1,2], Natanael Mansilla[1,2] and Daniel H. Gonzalez[1,2] *

[1] Instituto de Agrobiotecnología del Litoral–Consejo Nacional de Investigaciones Científicas y Técnicas-Universidad Nacional del Litoral, Santa Fe, Argentina
[2] Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Ján A. Miernyk, University of Missouri, USA
Hannetz Roschzttardtz, University of Wisconsin-Madison, USA

***Correspondence:**
Elina Welchen and Daniel H. Gonzalez, Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina
e-mail: ewelchen@fbcb.unl.edu.ar; dhgonza@fbcb.unl.edu.ar

Plant mitochondria are complex organelles that carry out numerous metabolic processes related with the generation of energy for cellular functions and the synthesis and degradation of several compounds. Mitochondria are semiautonomous and dynamic organelles changing in shape, number, and composition depending on tissue or developmental stage. The biogenesis of functional mitochondria requires the coordination of genes present both in the nucleus and the organelle. In addition, due to their central role, all processes held inside mitochondria must be finely coordinated with those in other organelles according to cellular demands. Coordination is achieved by transcriptional control of nuclear genes encoding mitochondrial proteins by specific transcription factors that recognize conserved elements in their promoter regions. In turn, the expression of most of these transcription factors is linked to developmental and environmental cues, according to the availability of nutrients, light–dark cycles, and warning signals generated in response to stress conditions. Among the signals impacting in the expression of nuclear genes, retrograde signals that originate inside mitochondria help to adjust mitochondrial biogenesis to organelle demands. Adding more complexity, several nuclear encoded proteins are dual localized to mitochondria and either chloroplasts or the nucleus. Dual targeting might establish a crosstalk between the nucleus and cell organelles to ensure a fine coordination of cellular activities. In this article, we discuss how the different levels of coordination of mitochondrial biogenesis interconnect to optimize the function of the organelle according to both internal and external demands.

Keywords: mitochondrial dynamics, mitochondrial biogenesis, retrograde signal, post-transcriptional gene regulation, site II, coordination

MITOCHONDRIA AS DYNAMIC ORGANELLES

New mitochondria arise from the fission of preexisting organelles. Mitochondria can also undergo fusion events, where two or more organelles converge into a single new one. Mitochondria are among the most plastic organelles of cells, alternating their architecture and distribution throughout the cytosol in order to carry out cellular functions. Moreover, mitochondrial number and morphology vary among different organisms and depending on the physiological and developmental conditions of the cell. Plant cells typically contain several hundred physically discrete mitochondria. For example, *Arabidopsis* mesophyll cells contain 200–300 discrete mitochondria, while tobacco mesophyll protoplasts contain 500–600 (Logan, 2010; Preuten et al., 2010). The processes related with changes in mitochondrial shape, size, and number are known as mitochondrial dynamics (Scott and Logan, 2010; **Figure 1**). This is a regulated process in plants, essential for the exchange of metabolic, genetic, and protein contents and to modulate mitochondrial bioenergetics, ATP production, autophagy, plant cell death (PCD), and connections with the cell cycle (Hyde et al., 2010; Westermann, 2012; Schwarzländer et al., 2012).

Besides the tight regulation required during changes associated with mitochondrial dynamics, it is evident that these changes imply coordinated responses of the many genes that encode organelle components. As proposed in mammals, the regulation of mitochondrial dynamics occurs at two interconnected levels. One of the coordination events is known as "organellar" control and is represented by the control that the mitochondrion exerts on itself. This local process, mainly represented by post-translational protein modifications, alters the microenvironment of the individual mitochondrion modifying its ability to fuse, divide, or move through the cell (Hyde et al., 2010). The "global" control is enforced by the cellular environment, when nuclear-encoded proteins, ions, and second messengers modify mitochondrial protein composition, thus remodeling mitochondrial populations. Changes in mitochondrial dynamics during the cell cycle are the most common examples of global control (Hyde et al., 2010).

The main goal of mitochondrial dynamics would be to optimize mitochondrial function according to the specific energetic needs of the cell. As mentioned, one of the first evidences relating mitochondrial dynamics and function comes from the observation of plant mitochondria at different stages of the cell cycle. The observation of electron micrographs of serial thin-sections prepared from *Arabidopsis* apical meristems at various developmental stages demonstrated the presence of large sheet-like mitochondria that undergo characteristic morphological and architectural

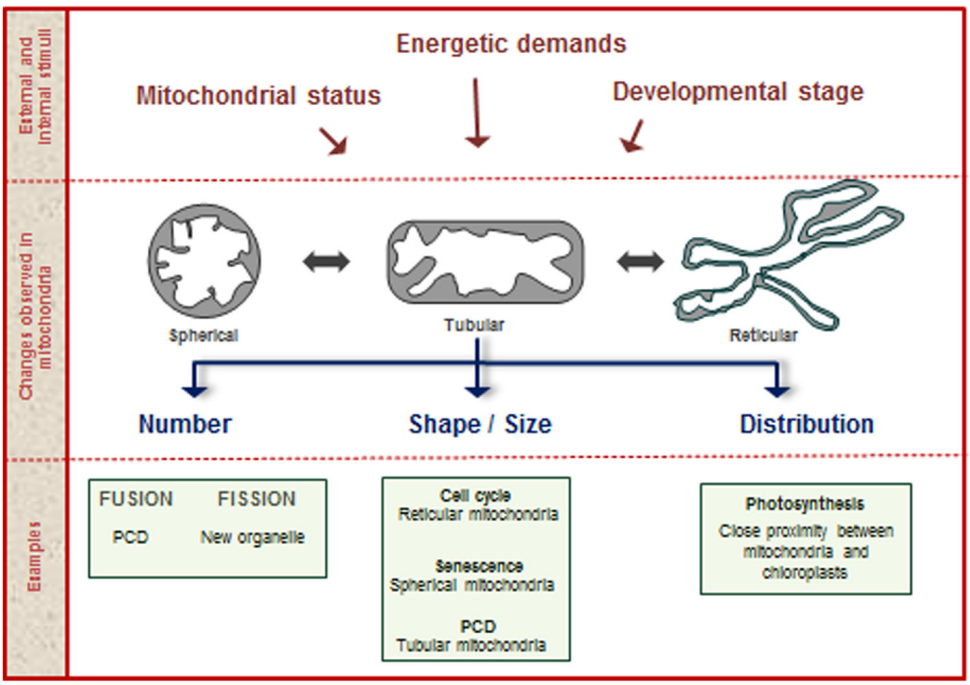


FIGURE 1 | Mitochondrial dynamics. During the plant life cycle, mitochondria change in shape, size, and number, according to numerous stimuli provided by internal and external factors. Examples of processes related with changes in mitochondrial structure are shown in the lower part of the figure.

changes during the cell cycle (Seguí-Simarro and Staehelin, 2009). The authors proposed that large, reticulate mitochondria provide an efficient means to deliver ATP for cell cycle and cytokinesis and enable efficient mixing and recombination of mitochondrial DNA (mtDNA; Seguí-Simarro et al., 2008). In this sense, it has been postulated that the plant chondriome is organized as a discontinuous whole and that there is a requirement for movement of the organelles, predominantly trough the actin cytoskeleton, to drive the meeting of discrete mitochondria in order to allow the exchange of mtDNA (Logan, 2006, 2010).

Another evidence linking dynamics to function was obtained in plant photosynthetic tissues through the observation of the co-localization or close proximity between mitochondria and chloroplasts (Logan and Leaver, 2000). More recently, Islam and Takagi (2010) showed the existence of changes in mitochondrial cellular location associated with chloroplast movements under different light regimes. In the dark, mitochondria were distributed randomly in palisade mesophyll cells. However, under different light intensities mitochondria moved coordinately with chloroplasts, suggesting that they either follow a specific signal or become physically associated with chloroplasts through the cytoskeleton. Although there is no specific evidence on this, it is assumed that this close association facilitates the exchange of gases and metabolites required for the maintenance of efficient photosynthesis (Islam and Takagi, 2010).

In addition, physical interactions between mitochondria and the endoplasmic reticulum (ER) were observed in mammalian cells. These contacts are established through proteins exposed in the surface of the organelles and allow the exchange of lipids and calcium (Kornmann, 2013). They also mark the sites where mitochondrial division will take place, thus suggesting a role of the ER in mitochondrial dynamics (Friedman et al., 2011).

Mitochondrial dynamics and morphology also change during different growth phases of cells in culture. Healthy, growing cells are characterized by a typical reticular arrangement of mitochondria. When cells enter senescence, the network disintegrates into very small mitochondria. Finally, giant mitochondria are observed when high levels of cell death are reached in the culture (Zottini et al., 2006). Other authors also demonstrated that one of the first evidences of the onset of PCD induced by reactive oxygen species (ROS) accumulation is the morphological change from tubular to spherical mitochondria in *Arabidopsis* (Yoshinaga et al., 2005). All these data reinforce the idea that mitochondria are dynamic organelles, changing their number, form, and position inside the cell according to developmental stage, type of tissue, cell cycle phase, energetic cell demand, external stimuli, and PCD. This dynamic behavior is likely a consequence of their central role in many cellular activities.

MITOCHONDRIAL BIOGENESIS

Usually, mitochondrial biogenesis is defined as the process by which "new" mitochondria are formed in the cell. Since "new" mitochondria arise from pre-existing ones, the term mitochondrial biogenesis refers rather to the processes involved in the synthesis and assembly of new mitochondrial components. As a consequence, mitochondrial biogenesis is closely associated with mitochondrial dynamics, since many of the processes referred in the previous section most likely imply changes in

mitochondrial composition and/or number. The synthesis and assembly of mitochondrial components is a complex process that requires the expression of two different genomes, the synthesis of proteins in two different compartments, the transport of proteins and/or their insertion into membranes and, frequently, the assembly of proteins and cofactors into multimeric complexes.

The complex nature of this process and of the functions performed by mitochondrial components suggests the idea that mitochondrial biogenesis must highly coordinated. In the following sections, we will try to summarize the knowledge available about the factors and mechanisms involved in the coordination of mitochondrial biogenesis in plants. The term coordination is used here in two different ways. One is to imply that the different components of the organelle must be coordinately synthesized to assemble "new" mitochondria. The other one refers to the fact that mitochondrial biogenesis must be coordinated with other cellular activities, considering the central role of these organelles in eukaryotic cells. While gene expression within mitochondria is essential for mitochondrial biogenesis, we will not refer to the expression mechanisms of mitochondrial genes, which have been extensively reviewed (Binder and Brennicke, 2003; Gagliardi and Binder, 2007; Liere et al., 2011). We will rather focus on the factors, either from outside or inside the organelle, that impact on the expression of nuclear genes encoding mitochondrial proteins and try to understand the mechanisms involved in coordinating gene expression and mitochondrial biogenesis both among different mitochondrial components and with the rest of the cell and the environment.

FROM OUTSIDE TO INSIDE: REGULATION OF MITOCHONDRIAL BIOGENESIS BY EXTERNAL FACTORS

Factors from outside the organelle that influence mitochondrial biogenesis

Mitochondria are not static and are constantly changing in form, volume, number, and composition inside cells (Logan, 2010; Preuten et al., 2010; Peters et al., 2012; Lee et al., 2013). These changes are not random, but rather respond to different factors, like plant organ, tissue and developmental stage, environmental stimuli, and cell energy and metabolic demands, among others (Howell et al., 2009; Law et al., 2012). In agreement with the central role of mitochondria in cellular metabolism and as energy suppliers during developmental processes and cellular differentiation, their biogenesis is highly regulated at different stages of development, and according to organ and tissue type. As an example of coordination, similar patterns of transcript accumulation for the nuclear encoded cytochrome *c* gene and genes encoded in the mitochondrial genome were reported during flower development in sunflower (Ribichich et al., 2001). This is in agreement with a wealth of information indicating that most components of the oxidative phosphorylation machinery show higher expression in flowers, particularly in anthers (Welchen et al., 2004; Gonzalez et al., 2007; Peters et al., 2012), maybe in relation with increased energy demand during pollen maturation. In addition, it has been shown that the relative abundance of the respiratory complexes varies according to tissue type. In this sense, Peters et al. (2012) showed that the ratio of protein components of Complexes I and II differ in photosynthetic tissues with respect to non-green organs such as roots and calli. Other authors found differences in respiratory rates with different substrates and in activities of specific enzymes in mitochondria isolated from non-photosynthetic cell cultures or from photosynthetic shoots (Lee et al., 2008, 2011).

Another process in which mitochondria play a crucial role is during germination. Studies performed in rice demonstrated that respiration increases rapidly during the first 24 h after imbibition. This is due to the execution of a program where the components of the protein import machinery are already present in promitochondrial structures to facilitate a rapid rate of mitochondrial biogenesis after imbibition (Howell et al., 2006; Taylor et al., 2010). In *Arabidopsis*, Law et al. (2012) made an exhaustive analysis to elucidate how mitochondrial biogenesis takes place during germination and early seedling growth. They showed the existence of changes in mitochondrial number, size, and morphology after 12 h of imbibition in continuous light, prompted by an increase of mitochondrial protein expression, and established a model for mitochondrial biogenesis during germination in which an early increase in the abundance of transcripts encoding proteins involved in transcription, translation and replication precedes a later cascade of expression of genes encoding bioenergetic and metabolic functions (Law et al., 2012). The examples mentioned above indicate that factors related with plant developmental or cell differentiation programs influence mitochondrial biogenesis either globally or specifically, most likely to adjust mitochondrial functions to cellular demands.

The expression of nuclear respiratory genes is also regulated by numerous external factors not directly related with plant developmental programs, such as nutrient availability (Welchen et al., 2002, 2004; Giegé et al., 2005), hormones (Comelli et al., 2009), light/dark conditions (Welchen et al., 2002), the diurnal cycle (Gibala et al., 2009; Lee et al., 2010), growth conditions generating oxidative stress (Ho et al., 2008; Obata et al., 2011; Tan et al., 2012; Van Aken and Whelan, 2012; Giraud et al., 2012; Lee et al., 2013), and abiotic or biotic stress conditions (Attallah et al., 2007; Livaja et al., 2008; Cvetkovska and Vanlerberghe, 2013). Due to the multiple interconnections of mitochondria with other organelles (peroxisomes, chloroplasts, ER, and the nucleus), it is difficult to identify if these external signals impact directly in mitochondrial biogenesis or act through changes in the cellular environment.

Molecular mechanisms of nuclear gene expression involved in mitochondrial biogenesis

Mitochondrial respiratory activity is carried out by a series of multimeric complexes consisting of subunits encoded either in the nucleus or in the organelle. Based on this, the assembly of these complexes is a finely regulated process that requires the coordination of the expression of multiple genes (Giegé et al., 2005; Gonzalez et al., 2007). Giegé et al. (2005), using *Arabidopsis* cells grown under starvation conditions, proposed a model suggesting that mitochondrial encoded proteins are always in excesses inside the organelle, while proteins encoded in the nucleus are the limiting factors. In this sense, it was shown that, for nuclear genes,

coordination takes place at the transcriptional level, most likely through the interaction of transcription factors with common regulatory elements present in the respective promoters (Welchen and Gonzalez, 2006; Welchen et al., 2009; Comelli et al., 2009; Comelli and Gonzalez, 2009). Coordinated expression of most nuclear genes encoding components of the different respiratory complexes was observed as a consequence of changes in carbohydrate supply or metabolism, photo-destruction of chloroplasts, inhibition of cellulose synthesis, and release from dormancy and germination (Gonzalez et al., 2007). Particularly, carbohydrate levels would operate to balance the biogenesis of the photosynthetic apparatus and the respiratory chain, which are involved in their synthesis and use, respectively. It has been demonstrated that the expression of a majority of nuclear genes for mitochondrial proteins is controlled by elements known as site II, which are either responsible for basal gene expression (Welchen and Gonzalez, 2006; Attallah et al., 2007) or modify the magnitude of the response under different growth conditions (Welchen et al., 2009; Mufarrege et al., 2009; Comelli et al., 2009, 2012). Indeed, over 80% of genes encoding constituents of Complexes I, III, IV, and V of the mitochondrial respiratory machinery in *Arabidopsis* and rice have site II elements in their promoter regions (Welchen and Gonzalez, 2006). It was postulated that site II elements are recognized by transcription factors from the TCP family (Trémousaygue et al., 2003; Martín-Trillo and Cubas, 2010; Uberti Manassero et al., 2013), which modulate processes related with cell proliferation and growth. This would provide a link for integrating gene expression with cellular demands (Gonzalez et al., 2007). Interestingly, site II elements are also enriched in genes encoding ribosomal proteins and are present in many nuclear genes encoding plastid and peroxisomal components (**Figure 2A**). This suggests that site II elements may be involved in the biogenesis of several cell components under conditions of active cell or tissue growth. This global mode of regulation may superimpose with more specific factors to modulate responses of subsets of genes. It is interesting that site II elements seem to accumulate closer to the transcription start site in genes encoding mitochondrial proteins than in those encoding components of other organelles (**Figure 2B**). In addition, other enriched elements, like those identified by Leister et al. (2011), may help to impose specificity to the response.

Lee et al. (2010) also showed that most genes that exhibit a strong response to changes in light/dark cycles have site II elements in their promoter regions. In contrast, genes without site II elements did not show clear cycling transcript abundance patterns. Giraud et al. (2010) showed that interactions between site II elements and TCP proteins are able to coordinate the expression of nuclear-encoded genes for mitochondrial proteins with the circadian clock. Recently, it was shown that one member of the TCP family in *Arabidopsis*, AtTCP8, is able to interact in the nucleus with the promoter region of *PNM1*, a gene that encodes a pentatricopeptide repeat (PPR) protein that localizes to the nucleus and mitochondria (Hammani et al., 2011a). Since PPR proteins participate in the expression of plant mitochondrial genes, it has been postulated that the AtTCP8-PNM1 interaction may operate to adjust the expression of both genomes during mitochondrial biogenesis (Hammani et al., 2011b).

Among other transcription factors involved in the expression of nuclear genes encoding mitochondrial components, we can mention transcription factors from the bZip, AP2/ERF, bHLH, and trihelix families. Specifically, the incorporation of the abscisic acid (ABA)-responsive element binding factor AREB2/ABF4 into complexes involved in the expression of the *COX5b-1* and *Cytc-2* genes may have allowed the diversification of regulatory

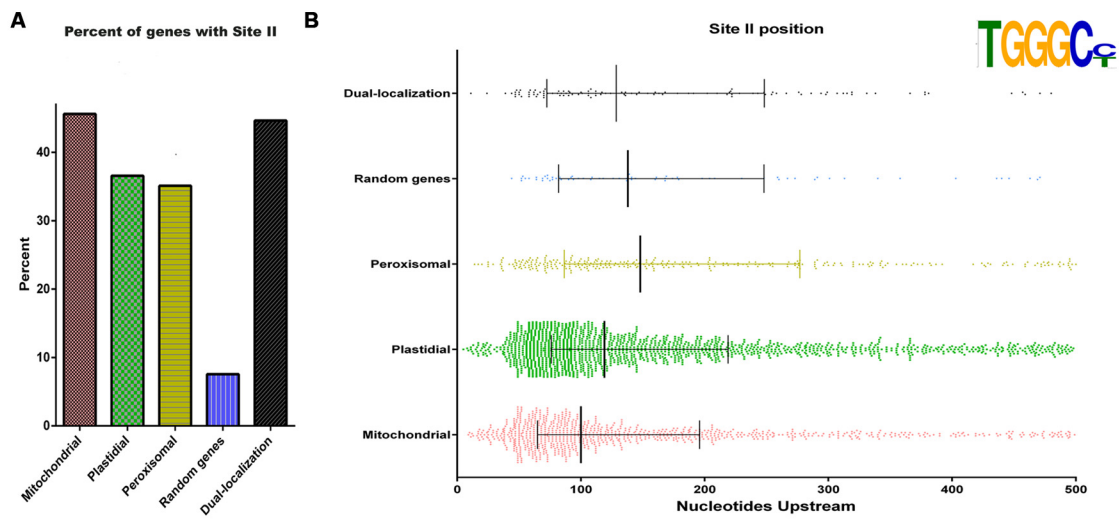


FIGURE 2 | Site II elements are enriched in genes encoding mitochondrial proteins and are present in many nuclear genes encoding plastid and peroxisomal components. (A) Proportion of genes with site II elements among *Arabidopsis* nuclear genes encoding proteins destined to different cell compartments. Gene sets were taken from Law et al. (2012). Dual targeted proteins were taken from Xu et al. (2013b). The term "Random genes" refers to a set of 490 genes picked at random. **(B)** Location of site II elements respective to the transcription start site in different sets of genes. Vertical lines represent the first, second, and third quartiles, respectively.

mechanisms (Comelli et al., 2009, 2012; Comelli and Gonzalez, 2009; Welchen et al., 2009). In an extreme case of diversification, a gene encoding an isoform of a succinate dehydrogenase (Complex II) subunit that is expressed specifically in seeds has been shown to be regulated by three B3 domain transcription factors involved in seed maturation (Elorza et al., 2004; Roschzttardtz et al., 2009).

As mentioned above, transcriptional regulation is a main regulatory point in the expression of nuclear encoded genes associated with respiration. However, there are several examples in which protein levels do not perfectly correlate with relative transcript abundance. One example of post-transcriptional regulation occurs during germination of *Arabidopsis* seeds and in responses to oxygen availability, where levels of import machinery components change markedly, while their transcript levels remain relatively stable (Howell et al., 2006, 2007; Law et al., 2012; Murcha et al., 2013). Interestingly, transcript abundance of the components of the electron transport chain display a high level of similarity with protein abundance, in marked contrast with the observation made for mitochondrial import components (Law et al., 2012). This example shows that the expression of different sets of proteins encoded by nuclear genes may be regulated at different levels even in response to the same process. In this case, it is logical to assume that post-transcriptional regulation of the import machinery reassures a rapid increase of its components that are required for the incorporation of other nuclear encoded proteins during mitochondrial biogenesis.

Even if initial studies suggested that mitochondrial-encoded subunits are present in excess and nuclear-encoded proteins are the limiting factors (Giegé et al., 2005), a recent study by Kwasniak et al. (2013) demonstrated that the synthesis of mitochondrial proteins may become a limiting factor under certain situations. These authors showed that mutations in the nuclear *RPS10* gene produce an increase in mitoribosomes but a decrease in the abundance of all oxidative phosphorylation complexes. This is due to an increased synthesis of mitochondrial encoded ribosomal proteins, while the opposite occurs for mitochondrial encoded respiratory chain subunits. Although these observations were made with a mutant plant, it can be envisaged that certain conditions may impose limitations to mitochondrial translation and this may impact in the differential synthesis of mitochondrial components. The findings highlight the importance of different levels of regulation in establishing the synthesis of mitochondrial protein complexes.

In animals, a recently proposed mechanism for the regulation of mitochondrial biogenesis involves the participation of microRNAs (miRNAs; Li et al., 2012; Yamamoto et al., 2012). These are a class of 20- to 24-nucleotide endogenous small non-coding RNAs that can bind to mRNAs, thereby inhibiting mRNA translation or promoting RNA degradation. This miRNA-mediated regulation is sequence-specific and occurs at the post-transcriptional level (Li et al., 2012). In plants, miRNAs control the expression of genes involved in many different processes (Rogers and Chen, 2013). Several animal miRNAs modulate the expression of mitochondrial proteins encoded by nuclear genes (Li et al., 2012; Tomasetti et al., 2013) and miRNAs have also been found in mitochondria, then receiving the name of "mitomiRs" (Bian et al., 2010). An example showing how mitomiRs may be involved in regulating mitochondrial functions comes from studies with miR181c. It has been reported that this miRNA encoded in the nucleus can enter mitochondria and target the genome, causing remodeling of Complex IV and mitochondrial dysfunction (Das et al., 2012).

In plants, there is little evidence on the involvement of miRNAs in mitochondrial biogenesis. By using a computational approach, Kamarajan et al. (2012) identified seven potential mitochondrial miRNA targets, but functional studies on the effect of these miRNAs were not provided. Regarding the expression of nuclear genes encoding mitochondrial proteins, an example of the involvement of miRNAs in regulation is provided by miR398, which binds to the 5′-UTR of the *Cox5b-1* transcript (Jones-Rhoades and Bartel, 2004). This miRNA is involved in regulating the expression of genes encoding copper binding proteins located in several cell compartments according to copper availability and may thus act to coordinate the biogenesis of Complex IV with other cellular processes. However, a clear demonstration that miR398 levels affect Complex IV assembly has not been provided.

FROM OUTSIDE TO MORE THAN ONE SITE: DUAL TARGETING OF PROTEINS

The term "dual targeting" refers to the phenomenon where a single protein can be distributed and/or localized in more than one subcellular compartment. This event may be a solution found through evolution to increase the number of cellular functions without increasing the number of genes. It can also be thought as a dynamic process that enables cells to adapt and coordinate their functions in a flexible way according to cellular and environmental demands (Yogev and Pines, 2011). The fact that sometimes both dual targeted and specific protein isoforms exist in the same organelle suggests that, rather than for economy, the cell may use in some cases this mechanism for regulatory purposes. Dual targeting can be achieved via two basic mechanisms: alternative transcription initiation or splicing and ambiguous targeting signals recognized by the organelle receptors and import machinery (Peeters and Small, 2001; Yogev and Pines, 2011).

Contrary to other eukaryotes, very few instances of nucleo-mitochondrial proteins have been described in plants. However, because the primary function of the mitochondrion is the respiratory activity performed by a series of multisubunit complexes whose expression must be coordinated between the nucleus and mitochondria, it seems obvious that the dual localization of proteins in both compartments may have an important role. A well described example is represented by PNM1, an *Arabidopsis* PPR protein identified in both mitochondria and the nucleus (Hammani et al., 2011a). PNM1 may be related to organellar translation processes because it is associated with mitochondrial polysomes (Hammani et al., 2011a). In addition, it interacts in the nucleus with the transcription factor TCP8 that belongs to the TCP family (Hammani et al., 2011b) whose members may participate in the expression of nuclear encoded mitochondrial proteins (Welchen and Gonzalez, 2006; Gonzalez et al., 2007). In the absence of PNM1, nuclear genes encoding mitochondrial proteins show increased transcript levels. This may suggest that PNM1 acts to coordinate the expression of genes for mitochondrial

components located in both compartments (Hammani et al., 2011b; Duchêne and Giegé, 2012).

While information about proteins present in mitochondria and the nucleus is scarce, more than 100 proteins were shown to be targeted to mitochondria and plastids (Carrie et al., 2009; Carrie and Small, 2013; Xu et al., 2013a). The number of proteins actually targeted to both organelles may be considerably higher than this, since a recent report has shown that even many proteins predicted to be targeted to a single organelle are imported into both *in vitro* and *in vivo* (Baudisch et al., 2014). Although it has not been clearly demonstrated, it can be speculated that the presence of proteins which are able to fulfill related functions in both organelles is a strategy acquired through evolution to coordinate processes related with energy homeostasis in the cell. There are also examples of proteins targeted to both mitochondria and peroxisomes, like type II NAD(P)H dehydrogenases and Mia40 (Xu et al., 2013b). Mia40 is a mitochondrial protein involved in the transport of CX_9C and CX_3C proteins in other organisms. Inactivation of *Arabidopsis* Mia40 produces changes in mitochondrial and peroxisomal proteins (Carrie et al., 2010), but there does not appear to be any effect on the assembly of Complex IV, that requires several CX_9C and CX_3C proteins for its biogenesis, suggesting that either a different import system is used by these proteins in plants or another protein fills the Mia40 function.

FROM INSIDE TO OUTSIDE: IMPACT OF MITOCHONDRIAL HOMEOSTASIS ON MITOCHONDRIAL BIOGENESIS AND NUCLEAR GENE EXPRESSION

FUNCTIONAL INTERCONNECTIONS BETWEEN THE MITOCHONDRIAL IMPORT MACHINERY AND THE RESPIRATORY CHAIN MODULATE MITOCHONDRIAL BIOGENESIS

An important step in mitochondrial biogenesis is the import of proteins encoded in the nucleus into the organelle. In addition to respiratory complexes, the mitochondrial inner membrane (IM) harbors the protein translocases required for the import of mitochondrial precursor proteins. The import of respiratory chain precursor proteins across and into the IM requires the membrane potential generated by the proton pumping complexes of the respiratory chain. In addition, certain components of the import machinery are physically linked to respiratory chain complexes. This physical interaction between components of both systems most likely produces also a functional connection. It can be envisaged that these interactions provide a mechanism for the coordination of mitochondrial biogenesis and function.

Among the examples of the importance of physical interconnections between the import machinery and the respiratory chain in plants, one of the best characterized is that of the α and β subunits of the mitochondrial processing peptidase (MPP), which are components of the cytochrome bc_1 complex (Complex III; Brumme et al., 1998). The second type of physical interaction between the import machinery and the respiratory chain is represented by the multiple interactions observed between the TIM17:23 complex and Complexes I, III, and IV in *Arabidopsis* and yeast. In yeast, the TIM17:23 complex has been shown to form dynamic supercomplexes with both the TOM40 channel and Complexes III and IV via Tim21. This interconnection would guarantee an efficient protein translocation process at contact sites by utilizing the membrane potential of the IM (Stuart, 2008; Duncan et al., 2013).

In *Arabidopsis*, Tim23-2 was shown to associate with Complex I. Independent Complex I knock-out lines show increased abundance of Tim23-2. In turn, Tim23-2 overexpressing lines show a decrease in Complex I abundance and a two to threefold increase in transcript levels of mitochondrial genes, organelle translation, and protein import. Nuclear genes encoding mitochondrial proteins also display increased expression, in particular genes encoding proteins involved in mitochondrial biogenesis (Wang et al., 2012; Murcha et al., 2012). In addition, overexpression of SD3, a protein with high similarity to yeast Tim21 which is part of the TIM23 complex, increases cotyledon size, ATP levels, and the expression of genes for several subunits of respiratory chain Complexes III and IV. This constitutes another example of the role of the import machinery in the control of respiratory activity and also suggests the existence of a link between mitochondrial activity and the control of organ size through cell growth and proliferation (Hamasaki et al., 2012).

MITOCHONDRIAL RETROGRADE SIGNALING: MITOCHONDRIAL STATUS MODULATES NUCLEAR GENE RESPONSES

Respiratory chain biogenesis implies the coordination between mitochondria and the nucleus since the different components are encoded either in the nuclear or the mitochondrial genome. In this coordinated network, mitochondria need to generate signals to modify nuclear gene expression according to organelle and cellular requirements. Signaling pathways from organelles to the nucleus are referred to as Retrograde Signaling pathways. While several relevant players in chloroplast retrograde signaling have been extensively characterized (for reviews see Leister et al., 2011; Kleine and Leister, 2013), relatively little is known about mitochondrial retrograde signaling (MRS). Even if a wealth of information published in the last 10 years has clearly shown that mitochondrial perturbations have a consequence in nuclear gene expression (Clifton et al., 2005; Giraud et al., 2009; Meyer et al., 2009; Shedge et al., 2010; Busi et al., 2011; Schwarzländer et al., 2012), the signals that operate these changes remain unclear. Considering that chloroplasts and mitochondria are closely connected, signaling pathways involved in chloroplast retrograde regulation need to be explored in the context of mitochondria to get new insights into MRS.

How mitochondria produce and transmit the signal is a critical step to clarify MRS pathways. Schwarzländer and Finkemeier (2013) extensively revised putative mitochondrial signals and focused in redox and bioenergetic processes housed in the mitochondrion as strategic sources of signals to transmit mitochondrial state. In line with this, metabolites like ascorbate and glutathione have been postulated as players in chloroplast retrograde signaling (Foyer and Noctor, 2011). These metabolites are considered as potential molecular signals for retrograde signaling due to their capacity to transfer redox information. In addition, an intermediate metabolite in the tetrapyrrole pathway (Mg-protoporphyrin IX) has been shown to be a signaling molecule from the chloroplast (Mochizuki et al., 2008). More recently, citrate has been proposed

as a signaling molecule that affects nuclear gene expression by unknown mechanisms (Finkemeier et al., 2013). Finding response elements in promoters of nuclear-encoded mitochondrial genes and, particularly, the discovery of specific marker genes for mitochondrial dysfunction are the first steps to get more insight into MRS in plants. In the next short sections we briefly revise the latest advances in MRS, considering two steps of this intricate process: downstream (nuclear gene expression) and upstream (signals triggered by mitochondria) components.

Downstream components: transcription factors involved in MRS

AOX1a, a gene encoding an isoform of the alternative oxidase, is a classic example of a gene regulated by mitochondrial signals. Chemical inhibition of electron transport through the respiratory chain induces the expression of AOX1a (Zarkovic et al., 2005; Vanlerberghe, 2013). Analysis of proteins that interact with the *AOX1a* promoter showed that the transcription factor ABA INSENSITIVE 4 (ABI4) may be an important player in regulating the expression of AOX1a (Clifton et al., 2005; Giraud et al., 2009). ABI4 acts as a repressor, since *AOX1a* expression is induced in *ABI4* mutant plants (Giraud et al., 2009). The fact that the respiratory chain inhibitor rotenone induces *AOX1a* expression in wild-type but not in *ABI4* mutant plants suggests that ABI4 mediates mitochondrial retrograde signals involved in *AOX1a* expression. This observation also provides a molecular link between mitochondrial and chloroplast retrograde signaling, since ABI4 was also shown to participate in at least two chloroplast retrograde signaling pathways (Koussevitzky et al., 2007). It was also shown that *AOX1a* shares six *cis*-acting regulatory elements that play a role in responses to rotenone and H_2O_2 with the external NAD(P)H-dehydrogenase *NDB2* gene (Ho et al., 2008).

A recent transcriptomic analysis considering 27 different mitochondrial and chloroplast perturbations identified a set of 12 nuclear-encoded mitochondrial genes that exclusively respond to mitochondrial perturbations (Van Aken and Whelan, 2012). Among them, the authors identified a new MRS marker, AtMSM1, which has a CHCH domain and resembles Mic17p, a yeast protein located in the mitochondrial intermembrane space. The characteristics of this protein and its putative location suggest that it may not only be a target, but also a transducer of MRS. Several *Arabidopsis* proteins that contain redox active cysteines related in structure to AtMSM1, like AtCOX17 and AtCOX19 among others, are present in the intermembrane space and are induced under several stress conditions (Attallah et al., 2007). This points to the intermembrane space as a site of sensing or transduction of signals that connect mitochondrial state with the rest of the cell.

WRKY transcription factors are involved in several biotic and abiotic stress responses. These factors recognize a responsive element known as the W box. The fact that W boxes are overrepresented in promoters of genes that specifically respond to mitochondrial dysfunction suggests that these factors may participate in MRS (Van Aken and Whelan, 2012). For example, three W-box motifs were identified in the *AOX1a* promoter (Dojcinovic et al., 2005). In addition, genes encoding mitochondrial proteins were overrepresented among genes repressed in plants overexpressing WRKY15, which was suggested as a player in MRS under salt stress, influencing mitochondrial responses regulated by calcium fluxes (Vanderauwera et al., 2012). Since WRKY transcription factors participate in multiple signaling pathways related to stress, the question remains if WRKY15 or any other member of the family is specific for MRS or has a more general role in stress responses. More recently, two other members of the family, WRKY40 and WRKY63, were identified as mediators of retrograde responses related with both chloroplast and mitochondrial perturbations (Van Aken et al., 2013).

Other players in MRS that were recently identified belong to the NAC transcription factor family. ANAC013 and ANAC017 are functionally associated with MRS and bind to a newly identified responsive element (named MDM) present in a set of MRS genes, like *AOX1a* (De Clercq et al., 2013; Ng et al., 2013). Interestingly, these factors are bound to the ER and, at least for ANAC017, its cleavage and migration to the nucleus seems to be related with induction of the *AOX1a* gene by MRS (Ng et al., 2013).

Upstream signaling: looking for the molecules that connect mitochondria with nuclear responses

Respiration is essentially a redox process. As a consequence, redox changes are likely involved in signaling mechanisms related with mitochondrial function (Finkemeier et al., 2005; Hicks et al., 2007; Collins et al., 2012). Inhibition or overreduction of the electron transport chain generates an increase in ROS production in mitochondria, mainly at the level of Complexes I and III (Turrenes, 2003; Dröse and Brandt, 2012). ROS are known as signaling molecules in many different cellular processes and may well also function in MRS (Møller and Sweetlove, 2010; Schwarzländer et al., 2012). Different H_2O_2-sensitive transcription factors were discovered in plants (Lindermayr et al., 2010; Shaikhali et al., 2008; Shaikhali and Baier, 2010; Viola et al., 2013). Particularly, TCP15, AtbZIP16 and Rap2.4a are involved in the control of nuclear gene expression mediated by redox state (Shaikhali et al., 2008; Shaikhali et al., 2012; Viola et al., 2013). However, there are two main questions about the role of ROS in MRS. The first one is related with the specificity of the response (i.e., how the information about the origin of the signal is retained and transmitted to the nucleus). The second one is related with the possibility that these molecules, once generated in mitochondria, travel through the cell to reach the nucleus and directly modify the activity of transcription factors. Recently, a model for H_2O_2 migration across the cell concludes that this type of signaling is unlikely to work in practice (Vestergaard et al., 2012). However, even if H_2O_2or other ROS could not be the signal *per se*, redox changes produced by these molecules in other compounds may act as signal relayers. Plant mitochondria contain many proteins with redox-active thiols that could sense H_2O_2 *in situ* and transduce ROS signaling. However, if this kind of redox regulation is involved in MRS is not fully understood. Another well characterized ROS is the superoxide anion (Zarepour et al., 2012). Superoxide is specifically reactive to iron-sulfur (Fe-S) clusters. In this way, aconitase is prone to suffer superoxidative inactivation (Morgan et al., 2008). Also, nitric oxide (NO) production was shown to inactivate aconitase and produce an increase in AOX1a expression (Polidoros et al., 2005; Gupta et al., 2012; Watanabe et al., 2010).

Other redox molecules that may be involved in MRS are glutathione and ascorbate. The last step of ascorbate synthesis is functionally and physically linked to the mitochondrial respiratory chain. Although ascorbate levels in plant cells are tightly controlled at the level of synthesis, recycling, degradation and transport (Green and Fry, 2005), chemical inhibition of respiration produces a strong decrease in total ascorbate levels (Millar et al., 2003). Perturbations in ascorbate metabolism lead to severe changes in gene expression, mostly related with plant-pathogen interactions, hormones and photosynthesis light reactions (Kiddle et al., 2003; Pastori et al., 2003; Kerchev et al., 2011). A role of ascorbate in MRS, though possible, has not been demonstrated. Interestingly, transcriptomic changes produced by mutations in ABI4, VTC2 and VTC4 proteins (the last two affect plant ascorbate levels) show a strong overlap suggesting the existence of a link between ascorbate and ABA signaling (Kerchev et al., 2011). Since ABI4 is involved in MRS, ascorbate levels may influence MRS acting on the ABI4 pathway. In the case of glutathione, no reports linking changes in its redox status with MRS are available. It should also be mentioned that, apart from redox molecules, changes in the levels of other metabolites, like carbon compounds or ATP, due to mitochondrial perturbations, may be used by the cell to adjust nuclear gene expression. The recently described effect of citrate in nuclear gene expression (Finkemeier et al., 2013) is an example of how changes in mitochondrial metabolism may be transduced to the nucleus through changes in metabolite levels.

How the specificity of the signal is transduced to the nucleus is difficult to envisage. In an extreme case, a molecule that is exclusively present in mitochondria must travel to the nucleus to induce MRS. In this sense, Møller and Sweetlove (2010) postulated that these specific molecules may be peptides derived from the degradation of mitochondrial proteins under stress conditions. Although appealing, evidence for the existence of this pathway is lacking. A different possibility is that there is not one specific signal, but rather a mitochondrial signature composed of many different unspecific signals and it is the integration of these signals in the nucleus that provides specificity to the response. This may have allowed the utilization and adaptation of preexisting signaling pathways to establish the communication between both compartments of the cell.

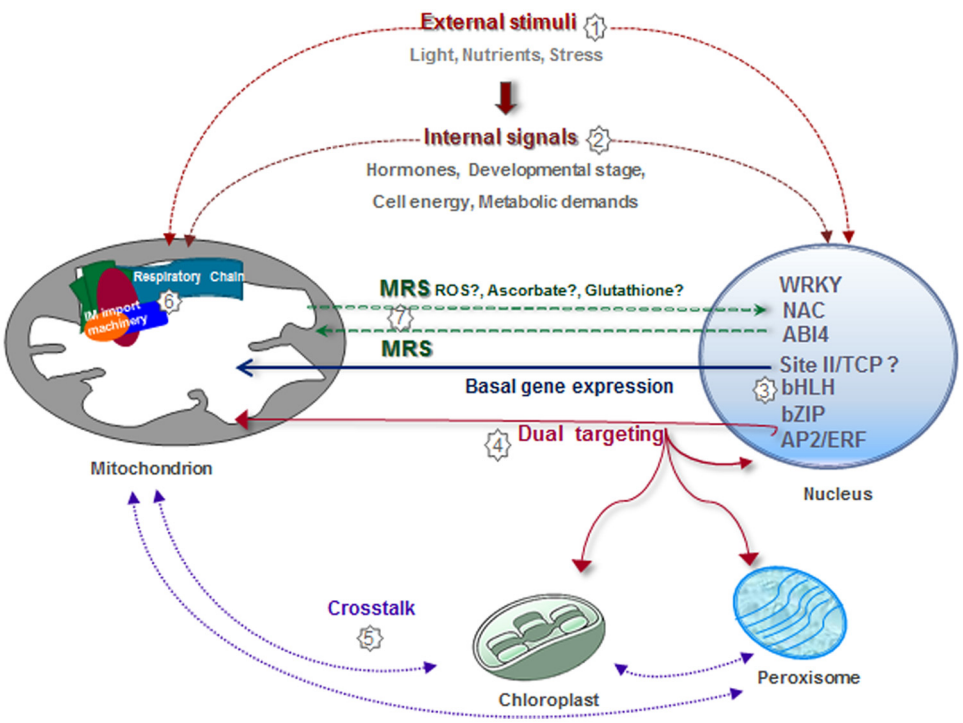


FIGURE 3 | Integrative view of different regulatory pathways involved in mitochondrial biogenesis. Mitochondrial biogenesis is regulated by numerous external factors, such as nutrient availability, environmental stimuli, light/dark conditions, diurnal cycle, and oxidative stress situations, among others **(1)**. Mitochondrial biogenesis is also internally regulated by different stages of development, organ/tissue type, hormones, and cell energy and metabolic demands **(2)**. The expression of a majority of nuclear genes for mitochondrial proteins is controlled by elements known as site II that are recognized by TCP transcription factors **(3)**. Site II elements are either responsible for basal gene expression or modify the magnitude of the response under different growth conditions. Other transcription factors involved in the expression of nuclear genes encoding mitochondrial components belong to the bZip, AP2/ERF, and bHLH families **(3)**. Evidence exists demonstrating the presence of proteins dual targeted to different organelles, which may act to coordinate the activities of these organelles **(4,5)**. Physical and functional interactions between the inner membrane (IM) import machinery and complexes I, III, and IV help to adjust gene expression and protein assembly **(6)**. Mitochondria generate signals to modify nuclear gene expression according to organelle demands **(7)**.

CONCLUSION

Mitochondria are among the most plastic organelles of cells, alternating their number, architecture, and distribution throughout the cytosol in order to optimize their function according to specific energetic needs. In agreement with the central role of mitochondria as energy suppliers for cellular processes, mitochondrial biogenesis is a highly regulated process. In **Figure 3**, we summarize the different regulatory pathways which converge for the correct assembly of functional mitochondria (**Figure 3**). Many nuclear genes encoding mitochondrial components, and especially those encoding components of the respiratory chain, are coordinately transcribed due to the presence of common regulatory elements in their promoters. This reassures the synthesis of a basic set of mitochondrial components and produces global changes associated with cellular requirements for growth and defined metabolic states. More specific interactions between certain transcription factors and defined genes help to fine-tune this general response. Superimposed on this, external (e.g., stress) or internal (i.e., mitochondrial status) signals impact on the expression of specific sets of genes involved in the adaptation to the new situation. Many of the changes in nuclear gene expression take place at the transcriptional level. However, although examples of other forms of regulation are not abundant, it is likely that post-transcriptional mechanisms also have a role. Once imported, many nuclear-encoded proteins have to assemble with their counterparts synthesized in mitochondria to constitute functional complexes. The final amount of assembled complexes will depend on the availability of the respective subunits. It can be envisaged that limiting factors may be different under different conditions and this will provide a complex way of regulation by multiple inputs. Assembly may be also regulated by post-translational modification of proteins, but limited information is available on this. Finally, supercomplexes may be assembled or disassembled according to specific demands. The molecules and signals involved in this complex regulatory network are still largely unknown.

ACKNOWLEDGMENTS

Work from our laboratory has been supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), and Universidad Nacional del Litoral. Elina Welchen and Daniel H. Gonzalez are members of CONICET; Lucila García and Natanael Mansilla are Fellows of the same Institution.

REFERENCES

Attallah, C. V., Welchen, E., Pujol, C., Bonnard, G., and Gonzalez, D. H. (2007). Characterization of *Arabidopsis thaliana* genes encoding functional homologues of the yeast metal chaperone Cox19p, involved in cytochrome c oxidase biogenesis. *Plant Mol. Biol.* 65, 343–355. doi: 10.1007/s11103-007-9224-1

Baudisch, B., Langner, U., Garz, I., and Klösgen, R. B. (2014). The exception proves the rule? Dual targeting of nuclear-encoded proteins into endosymbiotic organelles. *New Phytol.* 201, 80–90. doi: 10.1111/nph.12482

Bian, Z., Li, L. M., Tang, R., Hou, D. X., Chen, X., Zhang, C. Y., et al. (2010). Identification of mouse liver mitochondria-associated miRNAs and their potential biological functions. *Cell Res.* 20, 1076–1078. doi: 10.1038/cr.2010.119

Binder, S., and Brennicke, A. (2003). Gene expression in plant mitochondria: transcriptional and post-transcriptional control. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 181–189. doi: 10.1098/rstb.2002.1179

Brumme, S., Kruft, V., Schmitz, U. K., and Braun, H. P. (1998). New insights into the co-evolution of cytochrome c reductase and the mitochondrial processing peptidase. *J. Biol. Chem.* 273, 13143–13149. doi: 10.1074/jbc.273.21.13143

Busi, M. V., Gomez-Lobato, M. E., Rius, S. P., Turowski, V. R., Casati, P., Zabaleta, E. J., et al. (2011). Effect of mitochondrial dysfunction on carbon metabolism and gene expression in flower tissues of *Arabidopsis thaliana*. *Mol. Plant* 4, 127–143. doi: 10.1093/mp/ssq065

Carrie, C., and Small, I. (2013). A reevaluation of dual-targeting of proteins to mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1833, 253–259. doi: 10.1016/j.bbamcr.2012.05.029

Carrie, C., Giraud, E., Duncan, O., Xu, L., Wang, Y., Huang, S., et al. (2010). Conserved and novel functions for *Arabidopsis thaliana* MIA40 in assembly of proteins in mitochondria and peroxisomes. *J. Biol. Chem.* 285, 36138–36148. doi: 10.1074/jbc.M110.121202

Carrie, C., Kühn, K., Murcha, M. W., Duncan, O., Small, I. D., O'Toole, N., et al. (2009). Approaches to defining dual-targeted proteins in *Arabidopsis*. *Plant J.* 57, 1128–1139. doi: 10.1111/j.1365-313X.2008.03745.x

Clifton, R., Lister, R., Parker, K. L., Sappl, P. G., Elhafez, D., Millar, A. H., et al. (2005). Stress-induced co-expression of alternative respiratory chain components in *Arabidopsis thaliana*. *Plant Mol. Biol.* 58, 193–212. doi: 10.1007/s11103-005-5514-7

Collins, Y., Chouchani, E. T., James, A. M., Menger, K. E., Cochemé, H. M., and Murphy, M. P. (2012). Mitochondrial redox signalling at a glance. *J. Cell Sci.* 125, 801–806. doi: 10.1242/jcs.098475

Comelli, R. N., and Gonzalez, D. H. (2009). Divergent regulatory mechanisms in the response of respiratory chain component genes to carbohydrates suggests a model for gene evolution after duplication. *Plant Signal. Behav.* 4, 1179–1181. doi: 10.4161/psb.4.12.10045

Comelli, R. N., Viola, I. L., and Gonzalez, D. H. (2009). Characterization of promoter elements required for expression and induction by sucrose of the *Arabidopsis* COX5b-1 nuclear gene, encoding the zinc-binding subunit of cytochrome c oxidase. *Plant Mol. Biol.* 69, 729–743. doi: 10.1007/s11103-008-9451-0

Comelli, R. N., Welchen, E., Kim H. J., Hong, J. C., and Gonzalez, D. H. (2012). Delta subclass HD-Zip proteins and a B-3 AP2/ERF transcription factor interact with promoter elements required for expression of the *Arabidopsis* cytochrome c oxidase 5b-1 gene. *Plant Mol. Biol.* 80, 157–167. doi: 10.1007/s11103-012-9935-9

Cvetkovska, M., and Vanlerberghe, G. C. (2013). Alternative oxidase impacts the plant response to biotic stress by influencing the mitochondrial generation of reactive oxygen species. *Plant Cell Environ.* 36, 721–732. doi: 10.1111/pce.12009

Das, S., Ferlito, M., Kent, O. A., Fox-Talbot, A., Wang, R., Liu, D., et al. (2012). Nuclear miRNA regulates the mitochondrial genome in the heart. *Circ. Res.* 110, 1596–1603. doi: 10.1161/CIRCRESAHA.112.267732

De Clercq, I., Vermeirssen, V., Van Aken, O., Vandepoelie, K., Murcha, M. W., Law, S. R., et al. (2013). The membrane-bound NAC transcription factor ANAC013 functions in mitochondrial retrograde regulation of the oxidative stress response in *Arabidopsis*. *Plant Cell* 25, 3472–3490. doi: 10.1105/tpc.113.117168

Dojcinovic, D., Krosting, J., Harris, A. J., Wagner, D. J., and Rhoads, D. M. (2005). Identification of a region of the *Arabidopsis* AtAOX1apromoter necessary for mitochondrial retrograde regulation of expression. *Plant Mol. Biol.* 58, 159–175. doi: 10.1007/s11103-005-5390-1

Dröse, S., and Brandt, U. (2012). Molecular mechanisms of superoxide production by the mitochondrial respiratory chain. *Adv. Exp. Med. Biol.* 748, 145–169. doi: 10.1007/978-1-4614-3573-0_6

Duchêne, A. M., and Giegé, P. (2012). Dual localized mitochondrial and nuclear proteins as gene expression regulators in plants? *Front. Plant Sci.* 3:221. doi: 10.3389/fpls.2012.00221

Duncan, O., Murcha, M. W., and Whelan, J. (2013). Unique components of the plant mitochondrial protein import apparatus. *Biochim. Biophys. Acta* 1833, 304–313. doi: 10.1016/j.bbamcr.2012.02.015

Elorza, A., León, G., Gómez, I., Mouras, A., Holuigue, L., Araya, A., et al. (2004). Nuclear SDH2-1 and SDH2-2 genes, encoding the iron-sulfur subunit of mitochondrial complex II in *Arabidopsis*, have distinct cell-specific expression patterns and promoter activities. *Plant Physiol.* 136, 4072–4087. doi: 10.1104/pp.104.049528

Finkemeier, I., Goodman, M., Lamkemeyer, P., Kandlbinder, A., Sweetlove, L. J., and Dietz, K. J. (2005). The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of *Arabidopsis thaliana* under stress. *J. Biol. Chem.* 280, 12168–12180. doi: 10.1074/jbc.M413189200

Finkemeier, I., König, A. C., Heard, W., Nunes-Nesi, A., Pham, P. A., Leister, D., et al. (2013). Transcriptomic analysis of the role of carboxylic acids in metabolite signaling in *Arabidopsis* leaves. *Plant Physiol.* 162, 239–253. doi: 10.1104/pp.113.214114

Foyer, C. H., and Noctor, G. (2011). Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18. doi: 10.1104/pp.110.167569

Friedman, J. R., Lackner, L. L., West, M., DiBenedetto, J. R., Nunnari, J., and Voeltz, G. K. (2011). ER tubules mark sites of mitochondrial division. *Science* 334, 358–362. doi: 10.1126/science.1207385

Gagliardi, D., and Binder, S. (2007). "Expression of the plant mitochondrial genome," in *Annual Plant Reviews,* Vol. 31, *Plant Mitochondria*, ed. D. C. Logan (Hoboken, NJ: John Wiley & Sons, Inc.), 50–96.

Gibala, M., Kicia, M., Sakamoto, W., Gola, E. M., Kubrakiewicz, J., Smakowska, E., et al. (2009). The lack of mitochondrial AtFtsH4 protease alters *Arabidopsis* leaf morphology at the late stage of rosette development under short-day photoperiod. *Plant J.* 59, 685–699. doi: 10.1111/j.1365-313X.2009.03907.x

Giegé, P., Sweetlove, L. J., and Leaver, C. (2005). Coordination of nuclear and mitochondrial genome expression during mitochondrial biogenesis in *Arabidopsis*. *Plant Cell* 17, 1497–1512. doi: 10.1105/tpc.104.030254

Giraud, E., Ng, S., Carrie, C., Duncan, O., Low, J., Lee, C. P., et al. (2010). TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*. *Plant Cell* 22, 3921–3934. doi: 10.1105/tpc.110.074518

Giraud, E., Van Aken, O., Ho, L. H. M., and Whelan, J. (2009). The transcription factor ABI4 is a regulator of mitochondrial retrograde expression of alternative oxidase1a. *Plant Physiol.* 150, 1286–1296. doi: 10.1104/pp.109.139782

Giraud, E., Van Aken, O., Uggalla, V., and Whelan, J. (2012). REDOX regulation of mitochondrial function in plants. *Plant Cell Environ.* 35, 271–280. doi: 10.1111/j.1365-3040.2011.02293.x

Gonzalez, D. H., Welchen, E., Attallah, C. V., Comelli, R. N., and Mufarrege, E. F. (2007). Transcriptional coordination of the biogenesis of the oxidative phosphorylation machinery in plants. *Plant J.* 5, 105–116. doi: 10.1111/j.1365-313X.2007.03121.x

Green, M. A., and Fry, S. C. (2005). Vitamin C degradation in plant cells via enzymatic hydrolysis of 4-O-oxalyl-L-threonate. *Nature* 433, 83–87. doi: 10.1038/nature03172

Gupta, K. J., Shah, J. K., Brotman, Y., Jahnke, K., Willmitzer, L., Kaiser, W. M., et al. (2012). Inhibition of aconitase by nitric oxide leads to induction of the alternative oxidase and to a shift of metabolism towards biosynthesis of amino acids. *J. Exp. Bot.* 63, 1773–1784. doi: 10.1093/jxb/ers053

Hamasaki, H., Yoshizumi, T., Takahashi, N., Higuchi, M., Kuromori, T., Imura, Y., et al. (2012). SD3, an *Arabidopsis thaliana* homolog of TIM21, affects intracellular ATP levels and seedling development. *Mol. Plant* 5, 461–471. doi: 10.1093/mp/ssr088

Hammani, K., Gobert, A., Hleibieh, K., Choulier, L., Small, I., and Giegé, P. (2011a). An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. *Plant Cell* 23, 730–740. doi: 10.1105/tpc.110.081638

Hammani, K., Gobert, A., Small, I., and Giegé, P. (2011b). A PPR protein involved in regulating nuclear genes encoding mitochondrial proteins? *Plant Signal. Behav.* 6, 748–750. doi: 10.4161/psb.6.5.15148

Hicks, L. M., Cahoon, R. E., Bonner, E. R., Rivard, R. S., Sheffield, J., and Jez, J. M. (2007). Thiol-based regulation of redox-active glutamate-cysteine ligase from *Arabidopsis thaliana*. *Plant Cell* 19, 2653–2661. doi: 10.1105/tpc.107.052597

Ho, L. H. M., Giraud, E., Uggalla, V., Lister, R., Clifton, R., Glen, A., et al. (2008). Identification of regulatory pathways controlling gene expression of stress-responsive mitochondrial proteins in *Arabidopsis*. *Plant Physiol.* 147, 1858–1873. doi: 10.1104/pp.108.121384

Howell, K. A., Millar, A. H., and Whelan, J. (2006). Ordered assembly of mitochondria during rice germination begins with pro-mitochondrial structures rich in components of the protein import apparatus. *Plant Mol. Biol.* 60, 201–223. doi: 10.1007/s11103-005-3688-7

Howell, K. A., Millar, A. H., and Whelan, J. (2007). Building the powerhouse: what are the signals involved in plant mitochondrial biogenesis? *Plant Signal. Behav.* 2, 428–430. doi: 10.4161/psb.2.5.4464

Howell, K. A., Narsai, R., Carroll, A., Ivanova, A., Lohse, M., Usadel, B., et al. (2009). Mapping metabolic and transcript temporal switches during germination in rice highlights specific transcription factors and the role of RNA instability in the germination process. *Plant Physiol.* 149, 961–998. doi: 10.1104/pp.108.129874

Hyde, B. B., Twig, G., and Shirihai, O. S. (2010). Organellar vs cellular control of mitochondrial dynamics. *Semin. Cell Dev. Biol.* 21, 575–581. doi: 10.1016/j.semcdb.2010.01.003

Islam, S., and Takagi, S. (2010). Co-localization of mitochondria with chloroplasts is a light-dependent reversible response. *Plant Signal. Behav.* 5, 146–152. doi: 10.4161/psb.5.2.10410

Jones-Rhoades, M. W., and Bartel, D. P. (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell.* 14, 787–799. doi: 10.1016/j.molcel.2004.05.027

Kamarajan, B. P., Sridhar, J., and Subramanian, S. (2012). In silico prediction of MicroRNAs in plant mitochondria. *Int. J. Bioautomat.* 12, 251–262.

Kerchev, P. I., Pellny, T. K., Vivancos, P. D., Kiddle, G., Hedden, P., Driscoll, S., et al. (2011). The transcription factor ABI4 Is required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in *Arabidopsis*. *Plant Cell* 23, 3319–3334. doi: 10.1105/tpc.111.090100

Kiddle, G., Pastori, G. M., Bernard, S., Pignocchi, C., Antoniw, J., Verrier, P. J., et al. (2003). Effects of leaf ascorbate content on defense and photosynthesis gene expression in *Arabidopsis thaliana*. *Antioxid. Redox Signal.* 5, 23–32. doi: 10.1089/152308603321223513

Kleine, T., and Leister, D. (2013). Retrograde signals galore. *Front. Plant Sci.* 12:45. doi: 10.3389/fpls.2013.00045

Kornmann, B. (2013). The molecular hug between the ER and the mitochondria. *Curr. Opin. Cell Biol.* 25, 443–448. doi: 10.1016/j.ceb.2013.02.010

Koussevitzky, S., Nott, A., Mockler, T. C., Hong, F., Sachetto-Martins, G., Surpin, M., et al. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. *Science* 316, 715–719. doi: 10.1126/science. 1140516

Kwasniak, M., Majewski, P., Skibior, R., Adamowicz, A., Czarna, M., Sliwinska, E., et al. (2013). Silencing of the nuclear RPS10 gene encoding mitochondrial ribosomal protein alters translation in *Arabidopsis* mitochondria. *Plant Cell* 25, 1855–1867. doi: 10.1105/tpc.113.111294

Law, S. R., Narsai, R., Taylor, N. L., Delannoy, E., Carrie, C., Giraud, E., et al. (2012). Nucleotide and RNA metabolism prime translational initiation in the earliest events of mitochondrial biogenesis during *Arabidopsis* germination. *Plant Physiol.* 158, 1610–1627. doi: 10.1104/pp.111.192351

Lee C. P., Eubel H., and Millar A. H. (2010). Diurnal changes in mitochondrial function reveal daily optimization of light and dark respiratory metabolism in *Arabidopsis*. *Mol. Cell Proteomics* 9, 2125–2139. doi: 10.1074/mcp.M110.001214

Lee, C. P., Eubel, H., O'Toole, N., and Millar, A. H. (2008). Heterogeneity of the mitochondrial proteome for photosynthetic and non-photosynthetic *Arabidopsis* metabolism. *Mol. Cell Proteomics* 7, 1297–1316. doi: 10.1074/mcp.M700535-MCP200

Lee, C. P., Eubel, H., O'Toole, N., and Millar, A. H. (2011). Combining proteomics of root and shoot mitochondria and transcript analysis to define constitutive and variable components in plant mitochondria. *Phytochemistry* 72, 1092–1108. doi: 10.1016/j.phytochem.2010.12.004

Lee, C. P., Taylor, N. L., and Millar, A. H. (2013). Recent advances in the composition and heterogeneity of the *Arabidopsis* mitochondrial proteome. *Front. Plant Sci.* 4:4. doi: 10.3389/fpls.2013.00004

Leister, D., Wang, X., Haberer, G., Mayer, C. F. X., and Kleine, T. (2011). Intracompartmental and intercompartmental transcriptional networks coordinate the expression of genes for organellar functions. *Plant Physiol.* 157, 386–404. doi: 10.1104/pp.111.177691

Liere, K., Weihe, A., and Börner, T. (2011). The transcription machineries of plant mitochondria and chloroplasts: composition, function, and regulation. *J. Plant Physiol.* 168, 1345–1360. doi: 10.1016/j.jplph.2011.01.005

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010). Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide. *Plant Cell* 22, 2894–2907. doi: 10.1105/tpc.109.066464

Li, P., Jiao, J., Gao, G., and Prabhakar, B. S. (2012). Control of mitochondrial activity by miRNAs. *J. Cell Biochem.* 113, 1104–1110. doi: 10.1002/jcb.24004

Livaja, M., Palmieri, M. C., von Rad, U., and Durner, J. (2008). The effect of the bacterial effector protein harpin on transcriptional profile and mitochondrial proteins of *Arabidopsis thaliana*. *J. Proteomics* 71, 148–159. doi: 10.1016/j.jprot.2008.04.002

Logan, D. C. (2006). Plant mitochondrial dynamics. *Biochim. Biophys. Acta* 1763, 430–441. doi: 10.1016/j.bbamcr.2006.01.003

Logan, D. C. (2010). The dynamic plant chondriome. *Semin. Cell Dev. Biol.* 21, 550–557. doi: 10.1016/j.semcdb.2009.12.010

Logan, D. C., and Leaver, C. J. (2000). Mitochondria-targeted GFP highlights the heterogeneity of mitochondrial shape, size and movement within living plant cells. *J. Exp. Bot.* 51, 865–871. doi: 10.1093/jexbot/51.346.865

Martín-Trillo, M., and Cubas, P. (2010). TCP genes: a family snapshot ten years later. *Trends Plant Sci.* 15, 31–39. doi: 10.1016/j.tplants.2009.11.003

Meyer, E. H., Tomaz, T., Carroll, A. J., Estavillo, G., Delannoy, E., Tanz, S. K., et al. (2009). Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses *Arabidopsis* germination and growth and alters control of metabolism at night. *Plant Physiol.* 151, 603–619. doi: 10.1104/pp.109.141770

Millar, A. H., Mittova, V., Kiddle, G., Heazlewood, J. L., Bartoli, C. G., Theodoulou, F. L., et al. (2003). Control of ascorbate synthesis by respiration and its implications for stress responses. *Plant Physiol.* 133, 443–447. doi: 10.1104/pp.103.028399

Mochizuki, N., Tanaka, R., Tanaka, A., Masuda, T., and Nagatani, A. (2008). The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 39, 15184–15189. doi: 10.1073/pnas.0803245105

Møller, I. M., and Sweetlove, L. J. (2010). ROS signalling – specificity is required. *Trends Plant Sci.* 15, 370–374. doi: 10.1016/j.tplants.2010.04.008

Morgan, M. J., Lehmann, M., Schwarzländer, M., Baxter, C. J., Sienkiewicz-Porzucek, A., Williams, T. C., et al. (2008). Decrease in manganese superoxide dismutase leads to reduced root growth and affects tricarboxylic acid cycle flux and mitochondrial redox homeostasis. *Plant Physiol.* 147, 101–114. doi: 10.1104/pp.107.113613

Mufarrege, E. F., Curi, G. C., and Gonzalez, D. H. (2009). Common sets of promoter elements determine the expression characteristics of three *Arabidopsis* genes encoding isoforms of mitochondrial cytochrome c oxidase subunit 6b. *Plant Cell Physiol.* 50, 1393–1399. doi: 10.1093/pcp/pcp080

Murcha, M. W., Wang, Y., Narsai, R., and Whelan, J. (2013). The plant mitochondrial protein import apparatus – the differences make it interesting. *Biochim. Biophys. Acta* doi: 10.1016/j.bbagen.2013.09.026

Murcha, M. W., Wang, Y., and Whelan, J. (2012). A molecular link between mitochondrial preprotein transporters and respiratory chain complexes. *Plant Signal. Behav.* 7, 1594–1597. doi: 10.4161/psb.22250

Ng, S., Ivanova, A., Duncan, O., Law, S. R., Van Aken, O., De Clercq, I., et al. (2013). A membrane-bound NAC transcription factor, ANAC017, mediates mitochondrial retrograde signaling in *Arabidopsis*. *Plant Cell* 25, 3450–3471. doi: 10.1105/tpc.113.113985

Obata, T., Matthes, A., Koszior, S., Lehmann, M., Araújo, W. L., Bock, R., et al. (2011). Alteration of mitochondrial protein complexes in relation to metabolic regulation under short-term oxidative stress in *Arabidopsis* seedlings. *Phytochemistry* 72, 1081–1091. doi: 10.1016/j.phytochem.2010.11.003

Pastori, G. M., Kiddle, G., Antoniw, J., Bernard, S., Veljovic-Jovanovic, S., Verrier, P. J., et al. (2003). Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. *Plant Cell* 15, 939–951. doi: 10.1105/tpc.010538

Peeters, N., and Small, I. (2001). Dual targeting to mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1541, 54–63. doi: 10.1016/S0167-4889(01)00146-X

Peters, K., Nießen, M., Peterhänsel, C., Späth, B., Hölzle, A., Binder, S., et al. (2012). Complex I–complex II ratio strongly differs in various organs of *Arabidopsis thaliana*. *Plant Mol. Biol.* 79, 273–284. doi: 10.1007/s11103-012-9911-4

Polidoros, A. N., Mylona, P. V., Pasentsis, K., Scandalios, J. G., and Tsaftaris, A. S. (2005). The maize alternative oxidase 1a (Aox1a) gene is regulated by signals related to oxidative stress. *Redox Rep.* 10, 71–78. doi: 10.1179/135100005X21688

Preuten, T., Cincu, E., Fuchs, J., Zoschke, R., Liere, K., and Börner, T. (2010). Fewer genes than organelles: extremely low and variable gene copy numbers in mitochondria of somatic plant cells. *Plant J.* 64, 948–959. doi: 10.1111/j.1365-313X.2010.04389.x

Ribichich, K. F., Tioni, M. F., Chan, R. L., and Gonzalez, D. H. (2001). Cell-type-specific expression of plant cytochrome c mRNA in developing flowers and roots. *Plant Physiol.* 4, 1603–1610. doi: 10.1104/pp.125.4.1603

Rogers, K., and Chen, X. (2013). Biogenesis, turnover, and mode of action of plant microRNAs. *Plant Cell* 25, 2383–2399. doi: 10.1105/tpc.113.113159

Roschzttardtz, H., Fuentes, I., Vásquez, M., Corvalán, C., León, G., Gómez, I., et al. (2009). A nuclear gene encoding the iron-sulfur subunit of mitochondrial complex II is regulated by B3 domain transcription factors during seed development in *Arabidopsis*. *Plant Physiol.* 150, 84–95. doi: 10.1104/pp.109.136531

Schwarzländer, M., and Finkemeier, I. (2013). Mitochondrial energy and redox signaling in plants. *Antioxid. Redox Signal.* 18, 2122–2144. doi: 10.1089/ars.2012.5104

Schwarzländer, M., Logan, D. C., Johnston, I. G., Jones, N. S., Meyer, A. J., Fricker, M. D., et al. (2012). Pulsing of membrane potential in individual mitochondria: a stress-induced mechanism to regulate respiratory bioenergetics in *Arabidopsis*. *Plant Cell* 24, 1188–1201. doi: 10.1105/tpc.112.096438

Scott, I., and Logan, D. C. (2010). "Mitochondrial dynamics," in *Plant mitochondria, Advances of Plant Biology Series*, ed. F. Kempken (New York: Springer), 31–63.

Seguí-Simarro, J. M., Coronado, M. J., and Staehelin, L. A. (2008). The mitochondrial cycle of *Arabidopsis* shoot apical meristem and leaf primordium meristematic cells is defined by a perinuclear tentaculate/cage-like mitochondrion. *Plant Physiol.* 148, 1380–1393. doi: 10.1104/pp.108.126953

Seguí-Simarro, J. M., and Staehelin, L. A. (2009). Mitochondrial reticulation in shoot apical meristem cells of *Arabidopsis* provides a mechanism for homogenization of mtDNA prior to gamete formation. *Plant Signal. Behav.* 4, 168–171. doi: 10.4161/psb.4.3.7755

Shaikhali, J., and Baier, M. (2010). Ascorbate regulation of 2-Cys peroxiredoxin-A promoter activity is light-dependent. *J. Plant Physiol.* 167, 461–467. doi: 10.1016/j.jplph.2009.10.021

Shaikhali, J., Heiber, I., Seidel, T., Ströher, E., Hiltscher, H., Birkmann, S., et al. (2008). The redox-sensitive transcription factor Rap2.4a controls nuclear expression of 2-Cys peroxiredoxin A and other chloroplast antioxidant enzymes. *BMC Plant Biol.* 8:48. doi: 10.1186/1471-2229-8-48

Shaikhali, J., Norén, L., Barajas-López, J. D., Srivastava, V., König, J., Sauer, U. H., et al. (2012). Redox mediated mechanisms regulate DNA-binding activity of the G-group of bZIP transcription factors in *Arabidopsis*. *J. Biol. Chem.* 287, 27510–27525. doi: 10.1074/jbc.M112.361394

Shedge, V., Davila, Y., Arrieta-Montiel, M. P., Mohammed, S., and MacKenzie, S. A. (2010). Extensive rearrangement of the *Arabidopsis* mitochondrial genome elicits cellular conditions for thermotolerance. *Plant Physiol.* 152, 1960–1970. doi: 10.1104/pp.109.152827

Stuart, R. A. (2008). Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria. *J. Bioenerg. Biomembr.* 40, 411–417. doi: 10.1007/s10863-008-9168-4

Tan, Y. F., Millar, A. H., and Taylor, N. L. (2012). Components of mitochondrial oxidative phosphorylation vary in abundance following exposure to cold and chemical stresses. *J. Proteome Res.* 11, 3860–3879. doi: 10.1021/pr3003535

Taylor, N. L., Howell, K. A., Heazlewood, J. L., Tan, T. Y., Narsai, R., Huang, S., et al. (2010). Analysis of the rice mitochondrial carrier family reveals anaerobic accumulation of a basic amino acid carrier involved in arginine metabolism during seed germination. *Plant Physiol.* 154, 691–704. doi: 10.1104/pp.110.162214

Tomasetti, M., Neuzil, J., and Dong, L. (2013). MicroRNAs as regulators of mitochondrial function: role in cancer suppression. *Biochim. Biophys. Acta* doi:10.1016/j.bbagen.2013.09.002 [Epub ahead of print].

Trémousaygue, D., Garnier, L., Bardet, C., Dabos, P., Hervé, C., and Lescure, B. (2003). Internal telomeric repeats and 'TCP domain' protein-binding sites co-operate to regulate gene expression in *Arabidopsis thaliana* cycling cells. *Plant J.* 33, 957–966. doi: 10.1046/j.1365-313X.2003.01682.x

Turrenes, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol.* 15, 335–344. doi: 10.1113/jphysiol.2003.049478

Uberti Manassero, N. G., Viola, I. L., Welchen, E., and Gonzalez, D. H. (2013). TCP transcription factors: architectures of plant form. *Biomol. Concepts* 4, 111–127. doi: 10.1515/bmc-2012-0051

Van Aken, O., and Whelan, J. (2012). Comparison of transcriptional changes to chloroplast and mitochondrial perturbations reveals common and specific responses in *Arabidopsis*. *Front. Plant Sci.* 3:281. doi: 10.3389/fpls.2012.00281

Van Aken, O., Zhang, B., Law, S., Narsai, R., and Whelan, J. (2013). AtWRKY40 and AtWRKY63 modulate the expression of stress-responsive nuclear genes encoding mitochondrial and chloroplast proteins. *Plant Physiol.* 162, 254–271. doi: 10.1104/pp.113.215996

Vanderauwera, S., Vandenbroucke, K., Inzé, A., van de Cotte, B., Mühlenbock, P., De Rycke, R., et al. (2012). AtWRKY15 perturbation abolishes the mitochondrial stress response that steers osmotic stress tolerance in *Arabidopsis. Proc. Natl. Acad. Sci. U.S.A.* 109, 20113–20118. doi: 10.1073/pnas.1217516109

Vanlerberghe, G. C. (2013). Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *Int. J. Mol. Sci.* 14, 6805–6847. doi: 10.3390/ijms14046805

Vestergaard, C. L., Flyvbjerg, H., and Møller, I. M. (2012). Intracellular signaling by diffusion: can waves of hydrogen peroxide transmit intracellular information in plant cells? *Front. Plant Sci.* 3:295. doi: 10.3389/fpls.2012.00295

Viola, I. L., Güttlein, L. N., and Gonzalez, D. H. (2013). Redox modulation of plant developmental regulators from the class I TCP transcription factor family. *Plant Physiol.* 162, 1434–1447. doi: 10.1104/pp.113.216416

Wang, H., Wang, S., Lu, Y., Alvarez, S., Hicks, L. M., Ge, X., et al. (2012). Proteomic analysis of early-responsive redox-sensitive proteins in *Arabidopsis. J. Proteome Res.* 11, 412–424. doi: 10.1021/pr200918f

Watanabe, C. K., Hachiya, T., Takahara, K., Kawai-Yamada, M., Uchimiya, H., Uesono, Y., et al. (2010). Effects of AOX1a deficiency on plant growth, gene expression of respiratory components and metabolic profile under low-nitrogen stress in *Arabidopsis thaliana. Plant Cell Physiol.* 51, 810–822. doi: 10.1093/pcp/pcq033

Welchen, E., Chan, R. L., and González, D. H. (2002). Metabolic regulation of genes encoding cytochrome c and cytochrome c oxidase subunit Vb in *Arabidopsis. Plant Cell Environ.* 25, 1605–1615. doi: 10.1046/j.1365-3040.2002.00940.x

Welchen, E., Chan, R. L., and Gonzalez, D. H. (2004). The promoter of the *Arabidopsis* nuclear gene COX5b-1, encoding subunit 5b of the mitochondrial cytochrome c oxidase, directs tissue-specific expression by a combination of positive and negative regulatory elements. *J. Exp. Bot.* 55, 1997–2004. doi: 10.1093/jxb/erh223

Welchen, E., and Gonzalez, D. H. (2006). Overrepresentation of elements recognized by TCP-domain transcription factors in the upstream regions of nuclear genes encoding components of the mitochondrial oxidative phosphorylation Machinery. *Plant Physiol.* 141, 540–545. doi: 10.1104/pp.105.075366

Welchen, E., Viola, I. L., Kim, H. J., Prendes, L. P., Comelli, R. N., Hong, J. C., et al. (2009). A segment containing a G-box and an ACGT motif confers differential expression characteristics and responses to the *Arabidopsis* Cytc-2 gene, encoding an isoform of cytochrome c. *J. Exp. Bot.* 60, 829–845. doi: 10.1093/jxb/ern331

Westermann, B. (2012). Mitochondrial fusion and fission in cell life and death. *Nat. Rev. Mol. Cell Biol.* 11, 872–884. doi: 10.1038/nrm3013

Xu, L., Carrie, C., Law, S. R., Murcha, M. W., and Whelan, J. (2013a). Acquisition, conservation, and loss of dual-targeted proteins in land plants. *Plant Physiol.* 161, 644–662. doi: 10.1104/pp.112.210997

Xu, L., Law, S. R., Murcha, M. W., Whelan, J., and Carrie, C. (2013b). The dual targeting ability of type II NAD(P)H dehydrogenases arose early in land plant evolution. *BMC Plant Biol.* 13:100. doi: 10.1186/1471-2229-13-100

Yamamoto, H., Morino, K., Nishio, Y., Ugi, S., Yoshizaki, T., Kashiwagi, A., et al. (2012). MicroRNA-494 regulates mitochondrial biogenesis in skeletal muscle through mitochondrial transcription factor A and Forkhead box j3. *Am. J. Physiol. Endocrinol. Metab.* 303, 1419–1427. doi: 10.1152/ajpendo.00097.2012

Yogev, O., and Pines, O. (2011). Dual targeting of mitochondrial proteins: mechanism, regulation and function. *Biochim. Biophys. Acta* 1808, 1012–1020. doi: 10.1016/j.bbamem.2010.07.004

Yoshinaga, K., Arimura, S., Niwa, Y., Tsutsumi, N., Uchimiya, H., and Kawai-Yamada, M. (2005). Mitochondrial behaviour in the early stages of ROS stress leading to cell death in *Arabidopsis thaliana. Ann. Bot.* 96, 337–342. doi: 10.1093/aob/mci181

Zarepour, M., Simon, K., Wilch, M., Nieländer, U., Koshiba, T., Seo, M., et al. (2012). Identification of superoxide production by *Arabidopsis thaliana* aldehyde oxidases AAO1 and AAO3. *Plant Mol. Biol.* 80, 659–671. doi: 10.1007/s11103-012-9975-1

Zarkovic, J., Anderson, S. L., and Rhoads, D. M. (2005). A reporter gene system used to study developmental expression of alternative oxidase and isolate mitochondrial retrograde regulation mutants in *Arabidopsis. Plant Mol. Biol.* 57, 871–888. doi: 10.1007/s11103-005-3249-0

Zottini, M., Barizza, E., Bastianelli, F., Carimi, F., and Lo Schiavo, F. (2006). Growth and senescence of *Medicago truncatula* cultured cells are associated with characteristic mitochondrial morphology. *New Phytol.* 172, 239–247.doi: 10.1111/j.1469-8137.2006.01830.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 15 November 2013; paper pending published: 12 December 2013; accepted: 23 December 2013; published online: 08 January 2014.

*Citation: Welchen E, García L, Mansilla N and Gonzalez DH (2014) Coordination of plant mitochondrial biogenesis: keeping pace with cellular requirements. Front. Plant Sci. **4**:551. doi: 10.3389/fpls.2013.00551*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 05 March 2014
doi: 10.3389/fpls.2014.00079

Mitoribosomal regulation of OXPHOS biogenesis in plants

Hanna Janska* and Malgorzata Kwasniak

Molecular Biology of the Cell Department, Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Linda Bonen, University of Ottawa, Canada
Géraldine Bonnard, Centre National de la Recherche Scientifique, France

****Correspondence:***
Hanna Janska, Molecular Biology of the Cell Department, Faculty of Biotechnology, University of Wroclaw, F. Joliot-Curie 14A, 50-383 Wroclaw, Poland
e-mail: janska@ibmb.uni.wroc.pl

The ribosome filter hypothesis posits that ribosomes are not simple non-selective translation machines but may also function as regulatory elements in protein synthesis. Recent data supporting ribosomal filtering come from plant mitochondria where it has been shown that translation of mitochondrial transcripts encoding components of oxidative phosphorylation complexes (OXPHOS) and of mitoribosomes can be differentially affected by alterations in mitoribosomes. The biogenesis of mitoribosome was perturbed by silencing of a gene encoding a small-subunit protein of the mitoribosome in *Arabidopsis thaliana*. As a consequence, the mitochondrial OXPHOS and ribosomal transcripts were both upregulated, but only the ribosomal proteins were oversynthesized, while the OXPHOS subunits were actually depleted. This finding implies that the heterogeneity of plant mitoribosomes found *in vivo* could contribute to the functional selectivity of translation under distinct conditions. Furthermore, global analysis indicates that biogenesis of OXPHOS complexes in plants can be regulated at different levels of mitochondrial and nuclear gene expression, however, the ultimate coordination of genome expression occurs at the complex assembly level.

Keywords: OXPHOS, mitoribosome, mitochondrial translation, ribosome filter hypothesis, ribosome heterogeneity, coordination of gene expression

INTRODUCTION

It is a widely held belief that post-transcriptional control dominates in the regulation of mitochondrial gene expression in plants, but the experimental evidence was until recently limited to events affecting RNA quality and quantity (Binder and Brennicke, 2003). Translational regulation in plant mitochondria was expected but, until now (Kwaśniak et al., 2013), only hypothetical. The basic process of translation involves the decoding of the mRNA-encoded information into proteins by ribosomes. Therefore, historically, ribosomes were considered to have a constitutive rather than a regulatory function, and the efficiency of translation was believed to be determined either by features of the mRNA itself or by mRNA-binding factors. However, a number of observations indicated that ribosomes themselves could also differentially affect the translation of particular mRNAs (Mauro and Edelman, 2007). This mini-review summarizes current knowledge on ribosomal regulation of OXPHOS biogenesis.

RIBOSOME HETEROGENEITY AS BASIS FOR RIBOSOME FILTER HYPOTHESIS

All ribosomes consist of the large subunit (LSU) and small subunit (SSU) composed of both proteins and rRNAs. Originally, ribosomes of an organism were viewed as homogeneous entities showing no variation in their rRNA or protein composition despite the observation of a growth rate-dependent ribosome heterogeneity in *Escherichia coli* made over 40 years ago (Deusser, 1972). The earliest evidence demonstrating heterogeneity of ribosomes in a eukaryotic cell emerged in 1981 during studies on the social amoeba *Dictyostelium discoideum* (Ramagopal and Ennis, 1981). The ribosomes from the different developmental stages of *D. discoideum* varied in their *composition and* covalent modifications (Ramagopal, 1992). Since then ample evidence has accumulated supporting the view that heterogeneous pools of ribosomes exhibiting variations in the RNA and or the protein components are present in bacteria and in eukaryotic cells (Brygazov et al., 2013; Filipovska and Rackham, 2013). Since the focus of this review is the translational regulation in plant mitochondria the heterogeneity of plant mitoribosomes will be presented in more detail.

The plant mitoribosome contains three rRNA molecules encoded in the mitochondrial DNA, 26S and 5S for the LSU, and 18S for the SSU. The rRNAs undergo several important post-transcriptional modifications (pseudouridylation and methylation; Bonen, 2004), and an rRNA methyltransferase required for the dimethylation of two conserved adenines in the mitochondrial 18S rRNA of *Arabidopsis* was characterized (Richter et al., 2010). In contrast to the rRNAs, the mitoribosomal proteins are encoded by both the mitochondrial and nuclear genomes. The sets of proteins encoded by these two genomes vary between plant species (Salinas et al., 2013), and the exact protein composition of plant mitoribosomes has not been fully determined yet. A recent bioinformatics analysis identified 71 genes encoding mitoribosomal proteins in *A. thaliana*, among them eight in the mitochondrial genome (Sormani et al., 2011). It should be emphasized that these numbers do not represent unique genes, since 16 of mitochondrial ribosomal proteins are encoded by more than one copy of gene. Potato and broad bean proteomics found 68–80 mitoribosomal proteins (Pinel et al., 1986; Maffey et al., 1997). Thus, compared with the bacterial ribosomes with only 54 proteins (Wittmann, 1982), the plant mitoribosome is apparently more complex. The plant mitochondrial ribosomal proteins are of two evolutionarily distinct types, those with a direct bacterial origin and those

recruited during the evolution (Salinas et al., 2013). Additionally, in *Arabidopsis* two proteins have been identified to be associated with mitoribosomes, namely PNM1 and PPR336 (Uyttewaal et al., 2008; Hammani et al., 2011). They belong to the pentatricopeptide repeat (PPR) protein family that is particularly large in higher plants, but their role in mitochondrial translation is still unknown.

The first indication of a possible heterogeneity of plant mitoribosomes comes from a study on four paralogs of ribosomal protein L12 in potato (Delage et al., 2007). At the RNA level these paralogs are expressed simultaneously and at similar abundance. All four L12 variants were present in the mitochondrial ribosome fraction, but they showed a divergent tendency to dissociate upon treatments that affects ribosomes integrity. The presence of the four paralogs of slightly different proportion suggests either occurrence of heterogeneous L12 composition among each mitoribosome and/or a heterogeneous population of mitochondrial ribosomes in the plant cell.

A heterogeneity of plant mitoribosomes is also implied by several developmental phenotypes of mutants defective in a mitoribosomal protein (Schippers and Mueller-Roeber, 2010). Slightly different phenotypes connected with defects in leaf morphology have been reported for three *Arabidopsis* mutants. In all three, the leaves are smaller, but an irregular leaf shape is characteristic for the mutant with reduced expression of both *MRPS3* and *MRPL16* (Sakamoto et al., 1996) as well as the mutant with RNAi-dependent silencing of *MRPS10* (Majewski et al., 2009), but not for the mutant with a 90% reduction in *MRPL11* transcript (Pesaresi et al., 2006). The alteration in leaf morphology was observed also in maize *rps3-rpl16* mutants, which produces severely stunted plants with striations on the leaves (Hunt and Newton, 1991). The role of mitoribosomal proteins in other specific developmental processes is underlined by several specific *Arabidopsis* mutants with defects in genes: *MRPL14*, which is essential for ovule development (Schneitz et al., 1998; Skinner et al., 2001), *MRPL21* and *MRPS11* which are required during female gametophyte development (Portereiko et al., 2006), as well as *MRPS16* gene, in which transposon-induced knockout causes an embryo-defective lethal phenotype (Tsugeki et al., 1996). Thus, specific mitoribosomal proteins appear to influence selectively different phases of the plant development, hinting on the existence of specialized ribosomes with the translational activity critical at specific developmental stages. This view is strengthened by the finding that the expression of individual *A. thaliana* genes encoding mitochondrial ribosomal proteins is highly variable during leaf development (Schippers and Mueller-Roeber, 2010). A functional heterogeneity of ribosomes could also be generated by interactions with accessory proteins as well as posttranslational modifications of ribosomal proteins, but the significance, if any of these factors for plant mitoribosomes is unknown.

The apparent ribosome heterogeneity formed a basis for the ribosome filter hypothesis in which the ribosome acts like a filter selecting specific mRNAs and consequently modulating translation (Mauro and Edelman, 2007). The different subpopulations of ribosomes would vary in their ability to translate specific subsets of mRNA and thus the ribosomes would selectively control translation and, consequently, gene expression. In accordance with the ribosome filter hypothesis, it has recently been shown that the biogenesis of OXPHOS complexes in *A. thaliana* mitochondria is subject to translational control by mitoribosomes (Kwaśniak et al., 2013).

MITORIBOSOME STATUS SELECTIVELY AFFECTS EFFICIENCY OF TRANSLATION OF MITOCHONDRIAL mRNAs FOR OXPHOS AND RIBOSOMAL PROTEINS IN *Arabidopsis*

Mitochondrial translation is indispensable for the biogenesis of the OXPHOS machinery and mitoribosomes simply because several protein subunits of the both types of complexes are synthesized in mitochondria. Spice has been added to that simple story by recent data indicating that plant mitoribosomes do not synthesize proteins encoded in the mitochondrial genome non-selectively but rather execute a transcript-specific translational control (Kwaśniak et al., 2013).

This finding is fully consistent with the ribosome filter theory. RNAi-mediated silencing leads to the generation of unique, heterogeneous population of ribosomes. Ribosomes lacking the S10 protein coexisted with wild-type ones and an excess of free LSU (**Figure 1**). It should be underlined that the *rps10* mutant did not suffer from an insufficiency of wild-type mitoribosomes since the silencing provoked a compensatory response increasing the overall abundance of both small and LSUs. The polymorphic population of mitoribosomes turned out to translate two subsets of mRNAs, those encoding OXPHOS subunits and ribosomal proteins, with altered efficiency compared to the wild-type. The majority of the OXPHOS transcripts were translated less efficiently, whereas most of the mitoribosome protein transcripts were translated with an enhanced efficiency relative to the wild-type. It should be emphasized that in the *RPS10*-silenced plants the mitochondrial transcripts for both OXPHOS and ribosomal proteins were up-regulated. Thus, the altered translation was not correlated with the transcript level but was due to an altered efficiency of their binding by the mitoribosomes. As a consequence, the rate of synthesis of the OXPHOS proteins was below that observed in wild-type mitochondria despite the over-abundance of their mRNAs. Thus, the diversified mitochondrial translation efficiency modulated profoundly the outcome of the earlier steps of mitochondrial genome expression. The effect of the altered SSU and/or the excess of free LSU on the efficiency of protein synthesis in the *rps10* mitochondria was postulated to have resulted from the formation of unique interactions between those subunits and other components of the translation apparatus. It is tempting to speculate that similar translational regulation could occur in wild-type plants, namely under conditions that lead to heterogeneity of mitoribosome population in response to environmental or developmental signals.

According to existing reports plant mitochondrial RNAs have long 5′ untranslated regions (UTR), which could be involved in translational efficiency by interacting with altered SSU and/or the excess of free LSU. However, no obvious motifs have been found within the 5′ UTR sequences suggesting that these parts of mRNAs are involved in gene-specific translation regulation (Kazama et al., 2013), whereas in *rps10* rather group-specific translational regulation was observed. Future studies are awaited to understand how

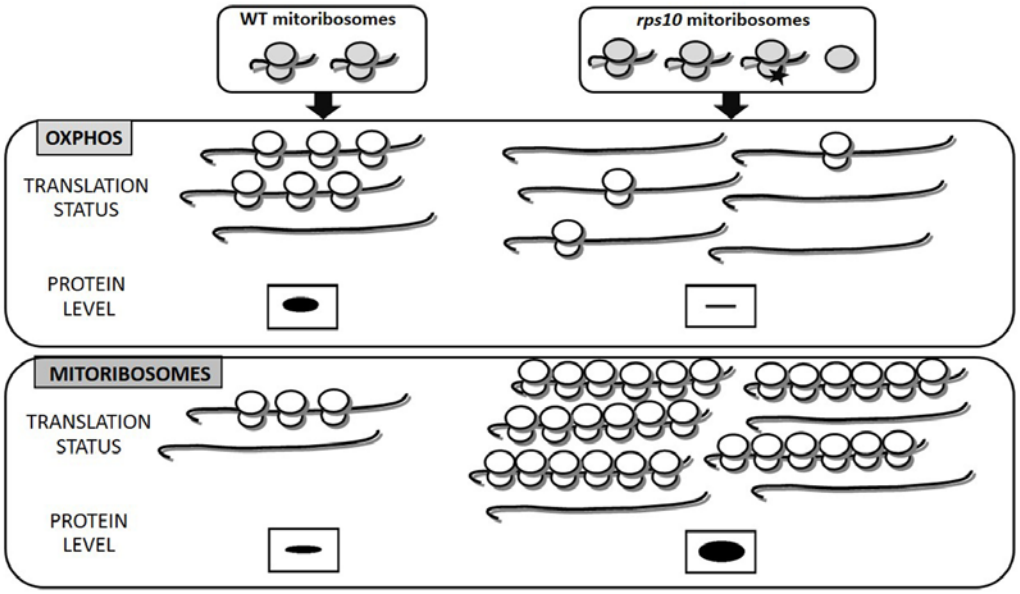


FIGURE 1 | Heterogeneity of mitoribosomes differentially affects the translation of mitochondrial OXPHOS and ribosomal proteins (based on Kwaśniak et al., 2013). In *rps10* protein synthesis is carried out by a heterogeneous population of mitoribosomes characterized by an excess of free LSU subunits and a portion of SSUs lacking the S10 protein (black asterisk). Decreased translation of OXPHOS in *rps10* results mainly from the reduced number of ribosomes per mRNA, whereas increased translation of mitoribosomal proteins results from enhanced ribosomal loading and also increased ribosomal density. In consequence, OXPHOS proteins are depleted, whereas mitoribosomal proteins over-abundant in *rps10* mitochondria compared to wild-type ones. The amount of proteins translated by mitoribosomes is represented by the size of the black bands.

differential translation of OXPHOS and mitoribosomal proteins is achieved in the *rps10* mutant.

The importance of mitochondrial translational regulation for OXPHOS complex I biogenesis has recently been suggested basing on analysis of an *A. thaliana* mutant with a depletion of the mitochondrial matrix iron-sulfur protein required for NADH dehydrogenase (INDH)(Wydro et al., 2013). INDH was shown not to be a complex I-specific translational regulator but rather to have a more general role in mitochondrial translation. Depending on the *A. thaliana* growth stage and the extent of INDH depletion diverse alterations in the mitochondrial translation rate and/or protein pattern were observed in the *indh* mutants. A strong depletion of INDH decreased the rate of mitochondrial translation, whereas the steady state levels of almost all mitochondrial transcripts examined were unaltered or increased.

The data indicating a heterogeneity of plant mitoribosomes combined with the finding that translation could be the overriding regulatory stage of mitochondrial gene expression regulation suggest that in plants the status of the mitoribosomal population could be an essential regulator of expression of mitochondrially encoded proteins in response to developmental or environmental signals. The postulated ribosome-mediated selectivity of mitochondrial translation should be reflected by a poor correlation between the steady-state abundance of mitochondrial transcripts and the encoded proteins. However, despite the recent intense studies of the plant mitochondrial transcriptome and proteome no enough quantitative data exist to substantiate such a claim. Relations between the transcript and the protein level are mainly known for the nuclear-encoded mitochondrial proteins (Law et al., 2012; Tan et al., 2012).

EXPRESSION OF NUCLEAR AND MITOCHONDRIAL GENES ENCODING OXPHOS SUBUNITS IS ULTIMATELY COORDINATED AT THE COMPLEX ASSEMBLY STAGE

Biogenesis of OXPHOS complexes requires balanced production of nuclear- and mitochondrially encoded subunits. Some reports indicate that in plants coordination of the expression of OXPHOS genes between the nuclear and mitochondrial genomes may occur at the transcript level (Topping and Leaver, 1990; Smart et al., 1994; Ribichich et al., 2001; Howell et al., 2006), but recent global analyses of expression of the mitochondrial and nuclear genomes in plants underscore the crucial role of the post-transcriptional coordination (Giegé et al., 2005; Leister et al., 2011; Law et al., 2012; Kwaśniak et al., 2013). Furthermore, Giegé et al. (2005) and Kwaśniak et al. (2013) studying biogenesis of OXPHOS complexes under sugar starvation conditions and in the *rps10* mutant, respectively, reached the same conclusion that the ultimate coordination of expression of the two genomes occurs at the level of protein complex assembly. The authors of both those papers assumed that an excess of unassembled subunits were degraded by nuclear-encoded ATP-dependent proteases. Apart from this similarity, there were also differences between the two experimental systems, as the *RPS10* silencing mainly down-regulated the biosynthesis of the mitochondrially encoded subunits whereas under sugar starvation the biogenesis of OXPHOS complexes was regulated mostly by curbing the expression of the respective nuclear genes. As a

consequence, the nuclear-encoded proteins in *rps10* and the mitochondrially encoded proteins under sugar starvation had to be degraded to bring their abundance down to the level of their partners. One is tempted to suggest that depending on the nature or the duration of the stress the direct response of the two collaborating genomes is in fact limited to one or the other and the expression of the second genome is adjusted ultimately at the complex assembly level.

In line with the above predictions, an up-regulation of mitochondrial ATP-dependent proteases was indeed observed in the *rps10* mutant. An induction of nuclear-encoded mitochondrial proteases and chaperones upon accumulation of unfolded or misfolded proteins in the mitochondrial matrix is a hallmark of the mitochondrial unfolded-protein response (UPR^{mt}) that has been reported for mammals and *Caernohabditis elegans* (Haynes and Ron, 2010; Haynes et al., 2013). Conceivably, also in plants UPR^{mt} is activated by the stoichiometric imbalance between the nDNA- and mtDNA-encoded OXPHOS proteins caused by the silencing of *RPS10* expression and restores the balance between the two sets of the subunits by selectively degrading the ones in excess.

MITOCHONDRIAL TRANSLATION REGULATION OF OXPHOS BIOGENESIS IN NON-PLANT SYSTEMS

In contrast to plants, where translational regulation has just emerged as an important step of the biogenesis of OXPHOS complexes, a diversity of mitochondrial translation control mechanisms have been reported in other organisms, particularly in *Saccharomyces cerevisiae*. One such mechanism is associated with translational activators which interact with mitochondrial mRNAs, in most cases at their 5′ UTR, to facilitate their translation (Smits et al., 2010). It is unclear whether all translational activators bind the mRNA directly or whether some of them interact with other components of the translation machinery (Herrmann et al., 2013). Ample data show that the translational activators can be part of a feedback mechanism coupling the rate of synthesis of mitochondrial proteins to the rate of their assembly into complexes, known as *control by epistasy of synthesis* (CES). This mechanism was first reported for chloroplasts (Choquet and Wollman, 2002). In the yeast mitochondria the best known such feedback loop is the one that controls the synthesis of Cox1. This regulation is achieved by the translational activator Mss51 which interacts with the *Cox1* mRNA, newly synthesized unassembled Cox1 protein, and other Cox assembly factors during Cox1 maturation/assembly (Fontanesi et al., 2010). Assembly defects lead to a sequestration of Mss51 and consequent, stoppage of Cox1 synthesis. A link between mitochondrial translation and complex assembly is also supported by another regulatory mechanism found in yeast, in which generation of assembly competent proteins during mitochondrial translation requires the mitoribosomal protein MPRL36 (Prestele et al., 2009). Mitoribosomes with a C-terminal domain-deficient MRPL36 protein still carry out translation, but the resulting polypeptides fail to be properly assembled into OXPHOS complexes and are rapidly degraded. The authors suggested that during translation, MRPL36 interacts with the ribosome at an as-yet unidentified step, which could be a critical for the production of assembly competent products.

A negative feedback mechanism for the regulation of mitochondrial translation based on an interplay between the translation and m-AAA protease, the key component of the mitochondrial quality control system has also been proposed for yeast (Nolden et al., 2005). The m-AAA protease not only degrades misfolded or damaged proteins, but also processes the mitoribosomal protein MRPL32. This maturation seems to be required for mitochondrial translation since the synthesis of mitochondrially encoded proteins was substantially impaired in cells lacking the m-AAA protease. Under certain conditions, misfolded, non-native respiratory subunits can compete with MRPL32 for binding to the m-AAA protease and thereby hamper the MRPL32 processing, ribosome assembly, and mitochondrial translation. MRPL32 processing and mitochondrial translation resume when the level of the respiratory subunits decreases as a consequence of their m-AAA-mediated degradation. It is worth mentioning in this context that plant m-AAA is able to process the yeast ribosomal protein MRPL32 (Kolodziejczak et al., 2002; Piechota et al., 2010). However, no direct evidence has been provided that plant m-AAA proteases can process a plant homolog of this mitoribosomal protein.

Given the mammalian mitochondrial transcripts contain no or only a short untranslated regulatory 5′ region (Koc and Spremulli, 2003), it seems unlikely that in mammals the synthesis of mitochondrial OXPHOS subunits is subject to CES, as it is in yeast. Instead, some reports indicate that members of the PPR protein family function as translational regulators of OXPHOS biogenesis in mammalian cells. One of them is leucine-rich PPR containing (LRPPRC) protein reported not only to coordinate mitochondrial translation but also to stabilize mitochondrial mRNAs and promote their polyadenylation (Sasarman et al., 2010). In the absence of LRPPRC the translation pattern is misregulated, with excessive translation of some transcripts and no translation of others. Interestingly, the two homologs of LRPPRC protein in fission yeast, Ppr4, and Ppr5, that also affect translation, play opposite roles in the expression of the *cox1* transcript (Kühl et al., 2011). Ppr4 specifically activates the translation of the *cox1* mRNA, whereas Ppr5 is a general negative regulator of mitochondrial translation. Furthermore, in humans another PPR protein – PTCD1 has been proposed to inhibit mitochondrial translation probably by decreasing the stability of the mitochondrial leucine tRNAs (Rackham et al., 2009). Other noteworthy human PPR proteins implicated in translation include PTCD2 involved in the maturation of cytochrome *b* transcripts (Xu et al., 2008) and PTCD3 associated with the SSU of mitochondrial ribosomes by binding 12S rRNA and necessary for efficient mitochondrial translation (Davies et al., 2009).

CONCLUDING REMARKS

In bacteria mRNA abundance is the primary factor controlling the rate of protein synthesis. Recent studies have shown that in plant mitochondria the synthesis of OXPHOS subunits is surprisingly insensitive to the accumulation of respective transcripts (Kwaśniak et al., 2013; Wydro et al., 2013). It seems that, similarly to the chloroplast system (Marin-Navarro et al., 2007), the expression of mitochondrial genes in plants is mainly regulated

at the level of translation. Compared with transcriptional regulation, translational regulation combined with selective degradation allows the organelles to modulate protein abundance more rapidly.

ACKNOWLEDGMENT

This work was supported by grant N N301 784940 from the Ministry of Science and Higher Education.

REFERENCES

Binder, S., and Brennicke, A. (2003). Gene expression in plant mitochondria: transcriptional and post-transcriptional control. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 358, 181–189. doi: 10.1098/rstb.2002.1179

Bonen, L. (2004). "Translational machinery in plant organelles," in *Molecular Biology and Biotechnology of Plant Organelles,* eds H. Daniell and C. Chase (Netherlands: Springer) 323–345. doi: 10.1007/978-1-4020-3166-3_12

Brygazov, K., Vesper, O., and Moll, I. (2013). Ribosome heterogeneity: another level of complexity in bacterial translation regulation. *Curr. Opin. Microbiol.* 16, 133–139. doi: 10.1016/j.mib.2013.01.009

Choquet, Y., and Wollman, F. A. (2002). Translational regulations as specific traits of chloroplast gene expression. *FEBS Lett.* 529, 39–42. doi: 10.1016/S0014-5793(02)03260-X

Davies, S. M., Rackham, O., Shearwood, A. M., Hamilton, K. L., Narsai, R., Whelan, J., et al. (2009). Pentatricopeptide repeat domain protein 3 associates with the mitochondrial small ribosomal subunit and regulates translation. *FEBS Lett.* 583, 1853–1858. doi: 10.1016/j.febslet.2009.04.048

Delage, L., Giegé, P., Sakamoto, M., and Maréchal-Drouard, L. (2007). Four paralogues of RPL12 are differentially associated to ribosome in plant mitochondria. *Biochimie* 89, 658–668. doi: 10.1016/j.biochi.2007.02.002

Deusser, E. (1972). Heterogeneity of ribosomal populations in *Escherichia coli* cells grown in different media. *Mol. Gen. Genet.* 119, 249–258. doi: 10.1007/BF00333862

Filipovska, A., and Rackham, O. (2013). Specialization from synthesis: how ribosome diversity can customize protein function. *FEBS Lett.* 587, 1189–1197. doi: 10.1016/j.febslet.2013.02.032

Fontanesi, F., Soto, I. C., Horn, D., and Barrientos, A. (2010). Mss51 and Ssc1 facilitate translational regulation of cytochrome c oxidase biogenesis. *Mol. Cell. Biol.* 30, 245–259. doi: 10.1128/MCB.00983-09

Giegé, P., Sweetlove, L. J., Cognat, V., and Leaver, C. J. (2005). Coordination of nuclear and mitochondrial genome expression during mitochondrial biogenesis in *Arabidopsis. Plant Cell* 17, 1497–1512. doi: 10.1105/tpc.104.030254

Hammani, K., Gobert, A., Hleibieh, K., Choulier, L., Small, I., and Giegé, P. (2011). An *Arabidopsis* dual-localized pentatricopeptide repeat protein interacts with nuclear proteins involved in gene expression regulation. *Plant Cell* 23, 730–740. doi: 10.1105/tpc.110.081638

Haynes, C. M., Fiorese, C. J., and Lin, Y. F. (2013). Evaluating and responding to mitochondrial dysfunction: the mitochondrial unfolded-protein response and beyond. *Trends Cell Biol.* 23, 311–318. doi: 10.1016/j.tcb.2013.02.002

Haynes, C. M., and Ron, D. (2010). The mitochondrial UPR – protecting organelle protein homeostasis. *J. Cell Sci.* 123, 3849–3855. doi: 10.1242/jcs.075119

Herrmann, J. M., Woellhaf, M. W., and Bonnefoy, N. (2013). Control of protein synthesis in yeast mitochondria: the concept of translational activators. *Biochim. Biophys. Acta* 1833, 286–294. doi: 10.1016/j.bbamcr.2012.03.007

Howell, K. A., Narsai, R., Carroll, A., Ivanova, A., Lohse, M., Usadel, B., et al. (2006). Mapping metabolic and transcript temporal switches during germination in rice highlights specific transcription factors and the role of RNA instability in the germination process. *Plant Physiol.* 149, 961–980. doi: 10.1104/pp.108.129874

Hunt, M. D., and Newton, K. J. (1991). The NCS3 mutation: genetic evidence for the expression of ribosomal protein genes in *Zea mays* mitochondria. *EMBO J.* 10, 1045–1052.

Kazama, T., Yagi, Y., Toriyama, K., and Nakamura, T. (2013). Heterogeneity of the 5'-end in plant mRNA may be involved in mitochondrial translation. *Front. Plant Sci.* 4:517. doi: 10.3389/fpls.2013.00517

Koc, E. C., and Spremulli, L. L. (2003). RNA-binding proteins of mammalian mitochondria. *Mitochondrion* 2, 277–291. doi: 10.1016/S1567-7249(03)00005-9

Kolodziejczak, M., Kolaczkowska, A., Szczesny, B., Urantowka, A., Knorpp, C., Kieleczawa, J., et al. (2002). A higher plant mitochondrial homologue of the yeast m-AAA protease. Molecular cloning, localization, and putative function. *J. Biol. Chem.* 277, 43792–43798. doi: 10.1074/jbc.M203831200

Kühl, I., Dujeancourt, L., Gaisne, M., Herbert, C. J., and Bonnefoy, N. (2011). A genome wide study in fission yeast reveals nine PPR proteins that regulate mitochondrial gene expression. *Nucleic Acids Res.* 39, 8029–8041. doi: 10.1093/nar/gkr511

Kwaśniak, M., Majewski, P., Skibior, R., Adamowicz, A., Czarna, M., and Janska, H. (2013). Silencing of nuclear RPS10 gene encoding mitochondrial ribosomal protein alters translation in *Arabidopsis* mitochondria. *Plant Cell* 25, 1855–1867. doi: 10.1105/tpc.113.111294

Law, S. R., Narsai, R., Taylor, N. L., Delannoy, E., Carrie, C., Giraud, E., et al. (2012). Nucleotide and RNA metabolism prime translational initiation in the earliest events of mitochondrial biogenesis during *Arabidopsis* germination. *Plant Physiol.* 158, 1610–1627. doi: 10.1104/pp.111.192351

Leister, D., Wang, X., Haberer, G., Mayer, K. F., and Kleine, T. (2011). Intracompartmental and intercompartmental transcriptional networks coordinate the expression of genes for organellar functions. *Plant Physiol.* 157, 386–404. doi: 10.1104/pp.111.177691

Maffey, L., Degand, H., and Boutry, M. (1997). Partial purification of mitochondrial ribosomes from broad bean and identification of proteins encoded by the mitochondrial genome. *Mol. Gen. Genet.* 254, 365–371. doi: 10.1007/s004380050428

Majewski, P., Woloszynska, M., and Janska, H. (2009). Developmentally early and late onset of Rps10 silencing in *Arabidopsis thaliana*: genetic and environmental regulation. *J. Exp. Bot.* 60, 1163–1178. doi: 10.1093/jxb/ern362

Marin-Navarro, J., Manuell, A. L., Wu, J., and Mayfield, S. (2007). Chloroplast translation regulation. *Photosynth. Res.* 94, 359–374. doi: 10.1007/s11120-007-9183-z

Mauro, V. P., and Edelman, G. M. (2007). The ribosome filter redux. *Cell Cycle* 6, 2246–2251. doi: 10.4161/cc.6.18.4739

Nolden, M., Ehses, S., Koppen, M., Bernacchia, A., Rugarli, E. I., and Langer, T. (2005). The m-AAA protease defective in hereditary spastic paraplegia controls ribosome assembly in mitochondria. *Cell* 123, 277–289. doi: 10.1016/j.cell.2005.08.003

Pesaresi, P., Masiero, S., Eubel, H., Braun, H. P., Bhushan, S., Glaser, E., et al. (2006). Nuclear photosynthetic gene expression is synergistically modulated by rates of protein synthesis in chloroplasts and mitochondria. *Plant Cell* 18, 970–991. doi: 10.1105/tpc.105.039073

Piechota, J., Kolodziejczak, M., Juszczak, I., Sakamoto, W., and Janska, H. (2010). Identification and characterization of high molecular weight complexes formed by matrix AAA proteases and prohibitins in mitochondria of *Arabidopsis thaliana. J. Biol. Chem.* 285, 12512–12521. doi: 10.1074/jbc.M109.063644

Pinel, C., Douce, R., and Mache, R. (1986). A study of mitochondrial ribosomes from the higher plant *Solanum tuberosum* L. *Mol. Biol. Rep.* 11, 93–97. doi: 10.1007/BF00364820

Portereiko, M. F., Sandaklie-Nikolova, L., Lloyd, A., Dever, C. A., Otsuga, D., and Drews, G. N. (2006). Nuclear fusion defective 1 encodes the *Arabidopsis* RPL21M protein and is required for karyogamy during female gametophyte development and fertilization. *Plant Physiol.* 141, 957–965. doi: 10.1104/pp.106.079319

Prestele, M., Vogel, F., Reichert, A. S., Herrmann, J. M., and Ott, M. (2009). Mrpl36 is important for generation of assembly competent proteins during mitochondrial translation. *Mol. Biol. Cell* 20, 2615–2625. doi: 10.1091/mbc.E08-12-1162

Rackham, O., Davies, S. M., Shearwood, A. M., Hamilton, K. L., Whelan, J., and Filipovska, A. (2009). Pentatricopeptide repeat domain protein 1 lowers the levels of mitochondrial leucine tRNAs in cells. *Nucleic Acids Res.* 37, 5859–5867. doi: 10.1093/nar/gkp627

Ramagopal, S. (1992). The *Dictyostelium* ribosome: biochemistry, molecular biology, and developmental regulation. *Biochem. Cell Biol.* 70, 738–750. doi: 10.1139/o92-113

Ramagopal, S., and Ennis, H. L. (1981). Regulation of synthesis of cell-specific ribosomal proteins during differentiation of *Dictyostelium discoideum. Proc. Natl. Acad. Sci. U.S.A.* 78, 3083–3087. doi: 10.1073/pnas.78.5.3083

Ribichich, K. F., Tioni, M. F., Chan, R. L., and Gonzalez, D. H. (2001). Cell-type-specific expression of plant cytochrome c mRNA in developing flowers and roots. *Plant Physiol.* 125, 1603–1610. doi: 10.1104/pp.125.4.1603

Richter, U., Kühn, K., Okada, S., Brennicke, A., Weihe, A., and Börner, T. (2010). A mitochondrial rRNA dimethyladenosine methyltransferase in *Arabidopsis. Plant J.* 61, 558–569. doi: 10.1111/j.1365-313X.2009.04079.x

Sakamoto, W., Kondo, H., Murata, M., and Motoyoshi, F. (1996). Altered mitochondrial gene expression in a maternal distorted leaf mutant of *Arabidopsis* induced by chloroplast mutator. *Plant Cell* 8, 1377–1390. doi: 10.1105/tpc.8.8.1377

Salinas, T., Remacle, C., and Maréchal-Drouard, L. (2013). "Mitochondrial translation in green algae and higher plant," in *Translation in Mitochondria and Other Organelles*, ed. A. M. Duchêne (Berlin: Springer Berlin-Heidelberg), 181–205. doi: 10.1007/978-3-642-39426-3_8

Sasarman, F., Brunel-Guitton, C., Antonicka, H., Wai, T., Shoubridge, E. A., and LSFC Consortium. (2010). LRPPRC and SLIRP interact in a ribonucleoprotein complex that regulates posttranscriptional gene expression in mitochondria. *Mol. Biol. Cell* 21, 1315–1323. doi: 10.1091/mbc.E10-01-0047

Schippers, J. H. M., and Mueller-Roeber, B. (2010). Ribosomal composition and control of leaf development. *Plant Sci.* 179, 307–315. doi: 10.1016/j.plantsci.2010.06.012

Schneitz, K., Baker, S. C., Gasser, C. S., and Redweik, A. (1998). Pattern formation and growth during floral organogenesis: HUELLENLOS and AINTEGUMENTA are required for the formation of the proximal region of the ovule primordium in *Arabidopsis thaliana*. *Development* 125, 2555–2563.

Skinner, D. J., Baker, S. C., Meister, R. J., Broadhvest, J., Schneitz, K., and Gasser, C. S. (2001). The *Arabidopsis* HUELLENLOS gene, which is essential for normal ovule development, encodes a mitochondrial ribosomal protein. *Plant Cell* 13, 2719–2730. doi: 10.1105/tpc.010323

Smart, C. J., Monéger, F., and Leaver, C. J. (1994). Cell-specific regulation of gene expression in mitochondria during anther development in sunflower. *Plant Cell* 6, 811–825.

Smits, P., Smeitink, J., and van den Heuvel, L. (2010). Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. *J. Biomed. Biotechnol.* 2010, 24. doi: 10.1155/2010/737385

Sormani, R., Masclaux-Daubresse, C., Daniele-Vedele, F., and Chardon, F. (2011). Transcriptional regulation of ribosome components are determined by stress according to cellular compartments in *Arabidopsis thaliana*. *PLoS ONE* 6:e28070. doi: 10.1371/journal.pone.0028070

Tan, Y. F., Millar, A. H., and Taylor, N. L. (2012). Components of mitochondrial oxidative phosphorylation vary in abundance following exposure to cold and chemical stresses. *J. Proteome Res.* 11, 3860–3879. doi: 10.1021/pr3003535

Topping, J. F., and Leaver, C. J. (1990). Mitochondrial gene expression during wheat leaf development. *Planta* 182, 399–407. doi: 10.1007/BF02411391

Tsugeki, R., Kochieva, E. Z., and Federoff, N. V. (1996). A transposon insertion in the *Arabidopsis* SSR16 gene causes an embryo-defective lethal mutation. *Plant J.* 10, 479–489. doi: 10.1046/j.1365-313X.1996.10030479.x

Uyttewaal, M., Mireau, H., Rurek, M., Hammani, K., Arnal, N., Quadrado, M., et al. (2008). PPR336 is associated with polysomes in plant mitochondria. *J. Mol. Biol.* 375, 626–636. doi: 10.1016/j.jmb.2007.11.011

Wittmann, H. G. (1982). Components of bacterial ribosomes. *Annu. Rev. Biochem.* 51, 155–183. doi: 10.1146/annurev.bi.51.070182.001103

Wydro, M. M., Sharma, P., Foster, J. M., Bych, K., Meyer, E. H., and Balk, J. (2013). The evolutionarily conserved iron-sulfur protein INDH is required for complex I assembly and mitochondrial translation in *Arabidopsis*. *Plant Cell.* 25, 4014–4027. doi: 10.1105/tpc.113.117283

Xu, F., Ackerley, C., Maj, M. C., Addis, J. B., Levandovskiy, V., Lee, J., et al. (2008). Disruption of a mitochondrial RNA-binding protein gene results in decreased cytochrome b expression and a marked reduction in ubiquinol-cytochrome c reductase activity in mouse heart mitochondria. *Biochem. J.* 416, 15–26. doi: 10.1042/BJ20080847

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 November 2013; paper pending published: 13 January 2014; accepted: 19 February 2014; published online: 05 March 2014.

*Citation: Janska H and Kwasniak M (2014) Mitoribosomal regulation of OXPHOS biogenesis in plants. Front. Plant Sci. **5**:79. doi: 10.3389/fpls.2014.00079*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 11 March 2014
doi: 10.3389/fpls.2014.00082

Evidence for interactions between the mitochondrial import apparatus and respiratory chain complexes via Tim21-like proteins in Arabidopsis

Monika W. Murcha[1]*, Szymon Kubiszewski-Jakubiak[1], Yan Wang[1] and James Whelan[2]

[1] *ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia, Perth, WA, Australia*
[2] *Department of Botany, ARC Centre of Excellence in Plant Energy Biology, School of Life Sciences, La Trobe University, Bundoora, VIC, Australia*

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Lee Sweetlove, University of Oxford, UK
Anne-Marie Duchene, Université de Strasbourg, France

***Correspondence:**
Monika W. Murcha, ARC Centre of Excellence in Plant Energy Biology, University of Western Australia, MCS Building M316, 35 Stirling Highway, Crawley, Perth, WA 6009, Australia
e-mail: monika.murcha@uwa.edu.au

The mitochondrial import machinery and the respiratory chain complexes of the inner membrane are highly interdependent for the efficient import and assembly of nuclear encoded respiratory chain components and for the generation of a proton motive force essential for protein translocation into or across the inner membrane. In plant and non-plant systems functional, physical, and evolutionary associations have been observed between proteins of the respiratory chain and protein import apparatus. Here we identify two novel Tim21-like proteins encoded by At2g40800 and At3g56430 that are imported into the mitochondrial inner membrane. We propose that Tim21-like proteins may associate with respiratory chain Complex I, III, in addition to the TIM17:23 translocase of the inner membrane. These results are discussed further with regards to the regulation of mitochondrial activity and biogenesis.

Keywords: protein import, Arabidopsis, mitochondrial biogenesis, Tim21, respiratory complexes

INTRODUCTION

Mitochondria are membrane bound organelles that play essential roles in metabolism, energy production, and biosynthesis of a variety of compounds in almost all eukaryotic cells. They are endosymbiotic in origin, and over time the majority of genes in the endosymbiont were lost or transferred to the host nucleus (Gray et al., 2001). Thus, the majority of the 1000 or more proteins located in the mitochondria are encoded by nuclear genes, translated in the cytosol, and imported via specialized components and protein complexes on the outer, intermembrane space, and inner membrane (Balsera et al., 2009).

The core components and mechanisms involved in mitochondrial protein import and assembly were established in the earliest eukaryotes with ancient origins of many components of the protein import apparatus (Albrecht et al., 2010; Delage et al., 2011; Hewitt et al., 2011). This is evidenced by the presence of many core and central components, such as the translocase of the outer membrane (TOM), sorting and assembly machinery (SAM), and the translocases of the inner membrane (TIM), in stramenophiles and hydrosomes (Lithgow and Schneider, 2010; Delage et al., 2011; Hewitt et al., 2011; Heinz and Lithgow, 2013). These ancient fundamental transporters originating from bacterial ancestors, exhibit a high level of conservation across the eukaryotic kingdoms from unicellular eukaryotes to plants, present in nearly all systems studied to date (Liu et al., 2011). Nevertheless, throughout the course of evolution, major adaptions have occurred to deal with the complexity of multi-cellular environments or in the case of plants the acquisition of an additional organelle, the plastid, and the adaptation to terrestrial environments. Therefore, the selective pressure to maintain protein targeting specificity and efficiency has led to the evolution of unique and specialized features for the regulation of mitochondrial biogenesis in plants (Duncan et al., 2013; Murcha et al., 2014).

Examples of the divergence and/or acquisition of functions within the plant mitochondrial machinery exist within nearly all protein complexes and compartments. Firstly, the cytosolic precursor protein is recognized by the outer membrane receptor Tom20, a cytosolic facing receptor anchored to the outer membrane. Plant Tom20 is evolutionary distinct from yeast Tom20 being identified via biochemical means (Werhahn et al., 2001), rather than homology, and is anchored to the outer membrane via its C-terminus. This is the opposite of what is observed in yeast and mammalian models with Tom20 being anchored via the N-terminal domain and thus plant Tom20 provides an example of functional convergence of two distinct genes (Perry et al., 2008; Rimmer et al., 2011). Several examples of disparities also exist on the inner mitochondrial membrane, such as with the TIM17:23 translocase channel, responsible for the import of the majority of the matrix located and certain inner membrane proteins (Rehling et al., 2001). A unique feature of plants is that it is Tim17 that links the inner and outer membranes, and not Tim23 as observed in yeast (Donzeau et al., 2000; Murcha et al., 2005a).

Whilst there are several unique features within the plant mitochondrial import apparatus it is interesting that to date only one plant specific import component has been identified, OM64 (outer membrane protein 64) (Chew et al., 2004; Lister et al., 2007). OM64 is actually paralog to a chloroplast import component TOC64 (translocase of the outer envelope of chloroplasts) but is localized to the mitochondrial outer membrane and shown to import a subset of precursor proteins (Chew et al., 2004; Lister et al., 2007). Whilst deletion of OM64 does not cause deleterious

effects, it can compensate to some degree for the loss of plant Tom20, as a quadruple mutant of all three *tom20* isoforms and *om64* is not viable (Lister et al., 2007; Duncan et al., 2013).

The functional divergence of the plant mitochondrial import machinery is partly due to the large expansion of gene family members, a good example is the Preprotein and Amino Acid Transporters family (PRAT) that constitute the inner membrane translocases (Rassow et al., 1999). This family of transporters originate from a single eubacterial ancestor and in yeast comprises the inner membrane translocases Tim17, Tim23, and Tim22 (Rassow et al., 1999). Conversely, in Arabidopsis this gene family has expanded to 17 members, including the inner membrane translocases along with the chloroplast outer envelope proteins (OEP16) with multiple isoforms encoding each transporter (Murcha et al., 2007; Pudelski et al., 2010). Sub-functionalization can also be observed within PRAT sub-groups, such as the TIM17's. Of the three isoforms that encode Tim17 in Arabidopsis, AtTim17-1, AtTim17-2 and AtTim17-3, isoforms 1 and 2 contain the C-terminal extension that was shown to link both membranes, whilst the third, is significantly shorter, and most similar to yeast Tim17 (Murcha et al., 2003, 2005a). Complementation studies further revealed that AtTim17-2 could only complement the yeast deletion strain when the carboxy-terminal extension was removed (Murcha et al., 2003). Additionally, each isoform of Tim17 exhibits differential expression profiles throughout development and in response to stress providing insights into their functional roles (Lister et al., 2004; Duncan et al., 2013).

Plant mitochondria lack direct orthologs to several yeast mitochondrial import components, such as Tim12, Tim54, Tim18, and Tom70 (Murcha et al., 2014), all of which are either essential or integral for yeast growth and viability (Hines et al., 1990; Kerscher et al., 1997, 2000; Sirrenberg et al., 1998). This emphasizes the likelihood that specialized novel proteins may evolve and only be present in a lineage specific manner.

The complexity of the mitochondrial import apparatus is further exemplified by cases of interactions between proteins of the import apparatus and components of the respiratory chain. The first example identified was the cytochrome bc_1/MPP (mitochondrial processing peptidase) complex. In plants the cytochrome bc_1 complex is bi-functional playing both roles in electron transfer and in the proteolytic removal of mitochondrial targeting sequences (Braun et al., 1992; Glaser et al., 1994). Further examples now exist in both plant and non-plant species of proteins which can have either a dual-function in respiration and protein import or a dual-location, i.e., associated with the protein import machinery and of the respiratory chain (Van der Laan et al., 2006; Saddar et al., 2008; Gebert et al., 2011; Kulawiak et al., 2012; Wang et al., 2012). Such inter-functional interactions are thought to be beneficial for the efficient import and assembly of respiratory chain subunits, and to maintain a membrane potential required via the inner membrane translocases (Kulawiak et al., 2012). Tim23 has also been shown to interact with the respiratory apparatus and is located within both respiratory Complex I and TIM17:23 in Arabidopsis (Wang et al., 2012). Furthermore, the Complex I subunit B14.7 (Meyer et al., 2008; Klodmann et al., 2010) is also associated with the TIM17:23 complex (Wang et al., 2012). It was proposed that the dual-location of protein in two complexes might be a mechanism to co-ordinate mitochondrial activity and biogenesis due to the inverse relationship between the abundance of Tim23 and the abundance of Complex I (Murcha et al., 2012; Wang et al., 2012).

Tim21 is an interesting example of dynamic interactions, initially identified as a subunit of the TIM17:23 translocation complex has also been shown to interact with the respiratory apparatus but also the TOM complex of the outer membrane. In yeast, Tim21's association with TIM17:23 forms the sorting and organization translocase (SORT) complex along with accessory protein Tim50 (Van der Laan et al., 2006). This dynamic configuration of TIM17:23 promotes the tethering of Tim21 to the outer membrane translocation pore and initiates the insertion of proteins into the inner membrane (Chacinska et al., 2005; Mokranjac et al., 2005). Further characterization of yeast Tim21 revealed its physical association with both components of the TIM17:23 complex respiratory subunits of complex III and IV (cytochrome c1, Rieske Fe/S, and cox4). In addition, it was shown to have a direct role in the import and insertion of proteins into the inner membrane and not in the translocation of matrix located proteins (Van der Laan et al., 2006).

In Arabidopsis, one gene encodes for Tim21 termed AtTim21 (At4g00026) (Carrie et al., 2010; Murcha et al., 2014). Similarly as observed with yeast Tim21, AtTim21 was shown to interact with TIM17:23 complex and Complex III (Wang et al., 2012). Furthermore, deletion of AtTim21 results in early seedling lethality (Hamasaki et al., 2012), which is in contrast to its non-essential nature in yeast (Chacinska et al., 2005). Moreover, an overexpression line of AtTim21 exhibited increased cell numbers, cell size and ATP production, whilst the transcript abundance of complex III, IV, and ATP synthase subunits was also up-regulated (Hamasaki et al., 2012). Therefore, both studies support the premise that AtTim21 may also be involved in the import and biogenesis of respiratory chain components.

In this study, we identify two additional Tim21-like proteins in Arabidopsis encoded by At2g40800 and At3g56430. Whereas they contain the conserved Tim21 protein domain (conserved in all Tim21 proteins), they are phylogenetically distinct from the Tim21 family and thus are termed as Tim21-like. Tim21-like proteins appear to be plant specific, originating in green algae and are identified in almost all angiosperms tested. As with AtTim21, AtTim21-like proteins are also targeted to the mitochondria and biochemical characterization suggests that AtTim21-like 1 and 2 have the ability to interact with the TIM17:23 complex, and Complex I and III.

MATERIALS AND METHODS

PHYLOGENETIC ANALYSES

AtTim21-like 1 (At2g40800) and AtTim21-like 2 (At3g56430) were selected for investigation due to the presence of the Tim21 protein domain (PF08294) recognized in the Conserved Domains Database (Marchler-Bauer et al., 2011). Orthologs to AtTim21 and AtTim21-like 1 and 2 were identified by sequence homology using BLASTN (Altschul and Koonin, 1998) within each respective non-plant species databases and using Phytozome 9.1 (Goodstein et al., 2012) for all plant species. Orthologs were manually curated to exclude proteins with a similarity threshold above

$1e^{-10}$. Alignments were performed using ClustalOmega (Sievers et al., 2011) (www.ebi.ac.uk) and the percentage identity and similarity scores were determined using MatGAT 2 (Campanella et al., 2003). Transmembrane domains were identified using TMHMM (Krogh et al., 2001) and targeting signals predicted using MitoProt II (Claros, 1995). The phylogenetic tree was analyzed and drawn using MEGA 5.2.2 (Tamura et al., 2011) using the maximum likelihood tree method and the Jones-Thornton-Taylor model after 1000 replications.

cDNA CLONES

Full-length cDNA was amplified using gene-specific primers flanked by Gateway recombination cassettes (**Supplemental Table 1**) and cloned into C-terminal GFP fusion vectors (Carrie et al., 2009) for GFP localization or pDEST14 (Invitrogen), for *in vitro* transcription and translation under a T7 promoter.

PLANT MATERIAL AND MITOCHONDRIAL ISOLATION

Mitochondria were isolated from 14-d old plate-grown Col-0 plants. Seeds were sterilized with chlorine gas and sown on MS media followed by 48 h stratification at 4°C and grown with a light intensity of 80 nmol quanta m^{-2} s^{-1} in a 16-h photoperiod.

GFP LOCALIZATION ASSAYS

Biolistic co-transformation of GFP and mt Cherry fusion vector (Nelson et al., 2007) was carried out on 5-d old Arabidopsis cell suspensions as previously described (Carrie et al., 2009). 5 μg of GFP and mt Cherry plasmids were co-precipitated onto gold particles and bombarded using the PDS-1000/He biolistic transformation system (Bio-Rad). GFP and mt Cherry expression was visualized and captured at 100X magnification using the BX61 Olympus microscope at 460/480 nM (GFP) and 570–625 nm (mt Cherry).

PROTEIN IMPORT AND BN-PAGE ANALYSIS

Precursor proteins were radiolabeled using the rabbit reticulocyte T_NT *in vitro* transcription/translation kit (Promega) accordingly to manufacturer's instructions. In small-scale *in vitro* import experiments, 100 ug of mitochondria were incubated in 90 μl of ice-cold import master mix (0.3 M sucrose, 50 mM KCl, 10 mM MOPS, 5 mM KH_2PO_4, 0.1% [w/v] BSA, 1 mM $MgCl_2$, 1 mM Met, 0.2 mM ADP, 0.75 mM ATP, 5 mM succinate, 5 mM DTT, 1 mM GTP, and 1 mM NADH, pH 7.5) with or without the addition of 1 μM valinomycin for 3 min on ice. Following incubation, 10 μl of [^{35}S]-labeled precursor protein was added and the import reaction was initiated by incubation at 26°C at 350 rpm. Following import, tubes were moved to ice and 3.2 μg of Proteinase K was added and incubated for a further 30 min. Proteolysis was inhibited by adding 1 μl of 100 mM PMSF. Reactions were centrifuged (20,000 *x* g, 3 min at 4C), supernatant was discarded and pellets were re-suspended in sample buffer and analyzed by SDS-PAGE. To investigate the intra-mitochondrial location of precursor proteins, mitoplasts were prepared following the import reaction and prior to the addition of PK, as described previously (Murcha et al., 2005b). For large-scale *in vitro* import experiments, for analysis by BN-PAGE, 250 μg of mitochondria were incubated in 360 μl of the import master mix with 50 μl of [35S]-labeled precursor protein. The experiments were performed as above (without the valinomycin or PK treatments) at 10, 20, and 40 min incubations and analyzed by BN-PAGE as described below.

BN-PAGE ANALYSIS

Mitochondrial complexes were solubilized in 5% (v/v) digitonin and separated by BN-PAGE as described previously (Meyer et al., 2009). Gels were stained with Coomassie Brilliant Blue, dried, and radiolabeled proteins were detected as outlined previously (Murcha et al., 1999).

YEAST 2-HYBRID ASSAYS

Coding regions of AtTim21-like 1, AtTim21-like 2, Tim44-2, AtTim50, AtTim22, AtTom5, AtTom9, and AtRISP, were amplified and cloned into both pGADT7 and pGBKT7 (Clontech) using primers containing restriction appropriate restriction sites at N- and C-terminus (**Supplemental Table 1**). Yeast vectors containing, AtTim21, AtTim17-2 AtTim23-2, AtB14.7, AtCyc1-1, and AtMPPα were used as described previously (Wang et al., 2012). The yeast 2-Hybrid screen was carried out by transforming bait (pGBKT7) and prey (pGADT7) vectors into mating compatible yeast strains, Y187, and AH109 respectively, and subsequent mating overnight. Diploid strains were plated onto selection media DDO (-Leu -Trp) for diploid selection, and QDO (-Leu -Trp -His -Ade) for indication of positive protein-protein interactions. pGBK 53 and pGAD SV40 was mated as a positive control. The plates were incubated for 5 days at 30°C.

ACCESSION NUMBERS

Sequence data from this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: AtTim21 (At4g00026), AtTim21-like 1 (At2g40800), AtTim21-like 2 (At3g56430), AtTim50 (At1g55900), AtTim17-2 (At2g37410), AtTim23-2 (At1g72750), AtB14.7 (At2g42210), AtTim22 (At3g10110/At1g18320), AtTim44-2 (At2g36070), AtRISP (At5g13430), AtCyc1-1 (At3g27240), AtTom5 (At5g08040), AtTom9 (At5g43970), MPPα (At3g16480), Cytochrome bd ubiquinol oxidase (At4g32470), and CAL1 (At5g63510).

RESULTS

Analysis of all Tim21 domain-containing proteins in Arabidopsis identifies AtTim21 (At4g00026) as the closest ortholog by sequence similarity and identity to yeast Tim21 (YGR033C). Two additional Tim21 domain containing proteins are also identified via the Conserved Domain Database (Marchler-Bauer et al., 2011), termed AtTim21-like 1 and AtTim21-like 2 encoded by At2g40800 and At3g56430 respectively (**Figure 1A**). Both are predicted to be significantly larger proteins of 414 and 434 aa compared to AtTim21 (269 aa) and ScTim21 (239 aa) and predicted to contain N-terminal mitochondrial targeting signals (**Figure 1A**). AtTim21-like 1 and 2 show low percentage similarity and identity scores of around 28–32% to ScTim21 and AtTim21 (**Figure 1B**), they are predicted to contain similar membrane spanning regions

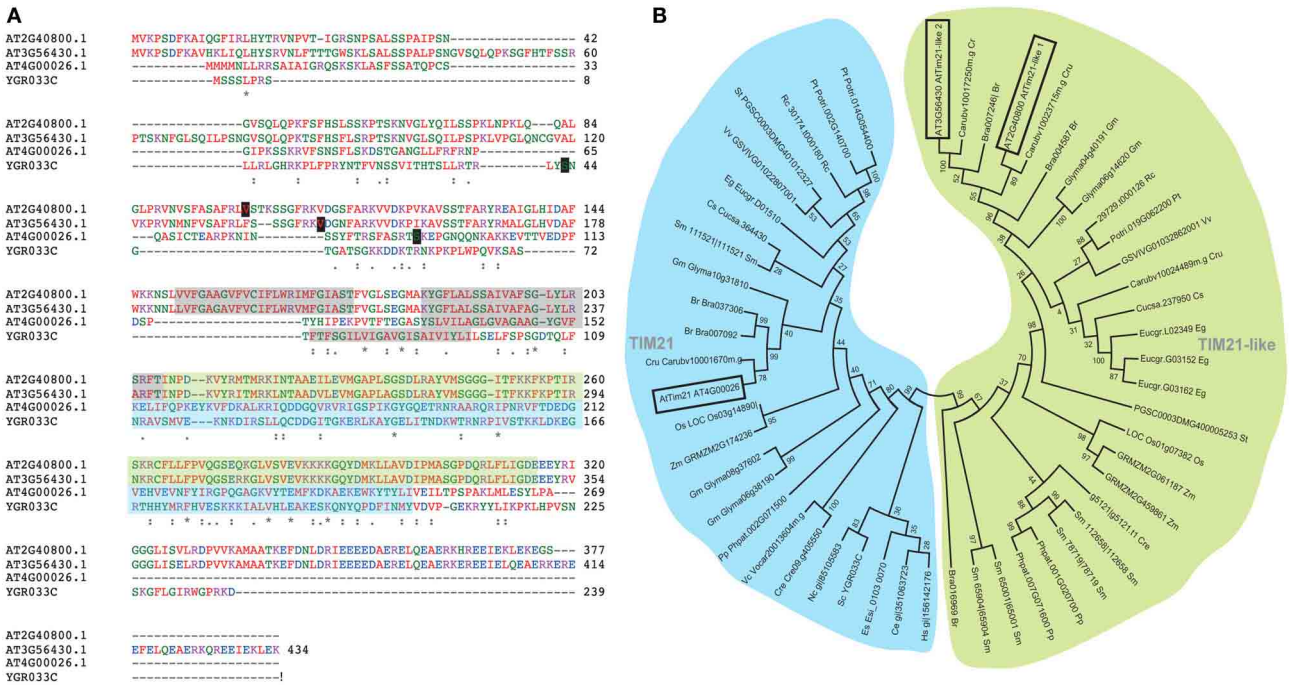


FIGURE 1 | Bioinformatic analysis of Tim21 and Tim21-like proteins from Arabidopsis. (A) Protein alignments of Tim21 from yeast (*Saccharomyces cerevisiae*, YGR033C), AtTim21 (At4g00026) and AtTim21-like 1 and 2 proteins from Arabidopsis (At2g40800 and At3g56430). The predicted mitochondrial processing sites are boxed in black and the predicted transmembrane domains are shaded in gray. The conserved Tim21 domain is shaded in blue and green. **(B)** Protein similarity and identity scores of ScTim21, AtTim21, and AtTim21-like 1 and 2. Similarity scores are shaded in light gray and identity scores are shaded in dark gray. **(C)** Phylogenetic analysis of all Tim21 and Tim21-like proteins from 24 species, the Tim21 clade is shaded in blue, and the Tim21-like clade is shaded in green. **(D)** The number of genes encoding Tim21 and Tim21-like proteins from each species. Abbreviations: Cm, *Cyanidioschyzon merolae*; Dd, *Dictyostelium discoideum*; Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; Sm, *Selaginella moellendorffii*; Zm, *Zea mays*; Os, *Oryza sativa*; St, *Solanum tuberosum*; Vv, *Vitis vinifera*; Eg, *Eucalyptus grandis*; Br, *Brassica rapa*; Cru, *Capsella rubella*; At, *Arabidopsis thaliana*; Gm, *Glycine max*; Rc, *Ricinus communis*; Pt, *Populus trichocarpa*; Cs, *Cucumis sativus*; Pp, *Physcomitrella patens*; Vc, *Volvox carteri*; Cre, *Chlamydomonas reinhardtii*; Es, *Ectocarpus siliculosus*. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties-scoring > 0.5 in the Gonnet PAM 250 matrix. A . (period) indicates conservation between groups of weakly similar properties-scoring ≤ 0.5 in the Gonnet PAM 250 matrix.

(shaded in gray) in the N-terminal portion and contain the consensus Tim21 superfamily motif (Pfam08294) as shaded in blue and green.

Phylogenetic analysis of Tim21 and Tim21-like proteins was carried out from 24 species ranging from yeast, fungi, algae (red, green and brown), and plant species representative of each evolutionary clade from *Physcomitrella patens* to *Eucalyptus grandis* (**Figure 1C**). Isoforms of AtTim21 and AtTim21-like proteins branch distinctly from each other suggesting that the may have diverged early as distinct lineages (**Figure 1C**). Tim21 proteins were identified in all species with the exception of *Cyanidioschyzon merolae* and *Dictyostelium discoideum*. Tim21-like proteins could only be identified in plants species, including the evolutionary precursors, green algae (*Chlamydomonas reinhardtii*), moss *(Physcomitrella patens)*, and spikemoss *(Selaginella moellendorffii)* (**Figure 1D**).

AtTim21-LIKE PROTEINS ARE LOCATED IN THE MITOCHONDRIA

Mitochondrial targeting predictions predict both isoforms of AtTim21-like 1 and AtTim21-like 2 to contain N-Terminal mitochondrial targeting peptides of 100 and 144 amino acids respectively. Mitochondrial import ability was tested using *in vitro* import assays of radiolabeled AtTim21-like 1 and AtTim21-like 2 into isolated Arabidopsis mitochondria. Translation of AtTim21-like 1 and AtTim21-like 2, produced radiolabeled proteins of an apparent molecular weight of 37 and 43 kDa respectively, compared to AtTim21 that has an apparent molecular mass of 27 kDa (Wang et al., 2012) Import of precursor protein into isolated Arabidopsis mitochondria produced a band of lower molecular mass of 27 and 30 kDa for AtTim21-like 1 and AtTim21-like 2, suggesting that upon import the predicted targeting peptide was cleaved (**Figure 2**, lanes 2 and 3). As this mature protein band was not produced in the presence of valinomycin and is Proteinase

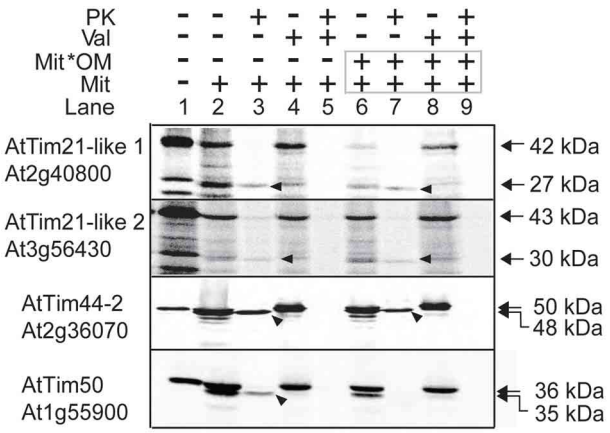


FIGURE 2 | ***In vitro*** **localization of AtTim21-like precursor proteins into isolated Arabidopsis mitochondria.** Radiolabeled precursor proteins incubated with isolated mitochondria under conditions that support import. Subsequently, mitochondria were treated with PK before or after rupture of the outer membrane via osmotic shock. Lane 1, precursor protein alone, lane 2, precursor protein incubated with mitochondria, lane 3, as lane 2 except with the addition of PK. Lane 4 and 5, as lane 2 and 3 except with the addition of valinomycin prior to import, lane 6–9, as lanes 2–5 except with rupture of the outer membrane following import and prior to PK treatment. The apparent molecular weights of the precursor and mature proteins are indicated on the right. Arrows indicate the mature processed protein. Abbreviations: Mit, mitochondria; Val, valinomycin; Mit*OM, mitoplasts; PK, Proteinase K.

K protected, it indicates that these mature proteins are a result of import and processing in mitochondria. The mature radiolabeled bands of AtTim21-like 1 and AtTim21-like 2 were resistant to Proteinase K digestion suggesting that the mature protein is protected within the inner membrane or matrix (**Figure 2**, lanes 6–9). Radiolabeled AtTim21, AtTim44-2, and AtTim50, known components of the TIM17:23 translocase were used as controls. As expected mature AtTim44-2 is insensitive to protease digestion as it is located in the inner membrane matrix facing side of TIM17:23, whilst AtTim50 also located in the inner membrane but with a large portion facing the inter membrane space was largely susceptible to PK degradation (**Figure 2**).

GFP targeting assays were also carried out to determine AtTim21-like 1 and AtTim21-like 2 targeting ability *in vivo*. Biolistic transformation was carried out using AtTim21-like 1 and AtTim21-like 2 constructs fused to GFP at the C-terminus. AtTim21-like 1 and AtTim21-like 2 were able to target GFP to the mitochondria (**Figure 3**). A mitochondrial control (mt cherry) was co-transformed as a mitochondrial control (Nelson et al., 2007). It should be noted that additional GFP signal is evident (mostly with AtTim21-like 1) that does not align to mt Cherry signal, which we suggest, results from heterogeneity in the mitochondrial population.

AtTim21-LIKE PROTEINS ASSOCIATE WITH THE PROTEIN IMPORT MACHINERY AND RESPIRATORY COMPLEXES

To determine the integration of AtTim21-like 1 and AtTim21-like 2 proteins within mitochondrial protein complexes, a large-scale import assay using radiolabeled AtTim21-like 1 and AtTim21-like 2 was carried out followed by BN-PAGE analysis. Import and assembly of AtTim21-like 1, AtTim21-like 2 and AtTim21 was evident within respiratory Complex III, along with Complex III subunits, MPPα (At1g51980) and ubiquinol-cytochrome C reductase (**Figure 4A**) (indicated by the arrow). Weak incorporation was also evident in smaller molecular weight complexes (indicated by *). Incorporation of radiolabeled AtTim21-2 was also evident at a small complex at ∼100 kDa, similar to the complex TIM17:23, as evidenced by the import and assembly of radiolabeled AtTim17-2 and AtTim23-2 proteins.

To confirm the incorporation of AtTim21-like 1 and AtTim21-like 2 within protein complexes was a result of specific import and accumulation, a time-course assay was carried out (**Figure 4B**). The incorporation of AtTim21-like 1 and AtTim21-like 2 within Complex III occurred in a time dependent manner (indicated by the arrow). Incorporation of both precursors was also evident within the monomeric form of Complex I and the supercomplex Complex I and III (**Figure 4B**). The import of AtTim21 was also carried out in a similar manner and similar incorporation was observed within Complex I, Complex III, and Complex I and III (**Figure 4B**). Time dependent labeling was also observed to unknown protein complexes (indicated by *) and incorporation within the TIM17:23 complex was only observed with AtTim21-like 2 (**Figure 4B**) in a time dependent manner. Complex I subunit, gamma carbonic anhydrase like 1 (CAL1), was used as a Complex I control that exhibited incorporation within Complex I and III and weaker labeling within a smaller unknown complex (**Figure 4B**). The alpha subunit of MPP was used as a Complex III control showing incorporation into Complex III, along with weak labeling at the position of Complex I (**Figure 4B**).

To further confirm the interactions of AtTim21-like 1 and AtTim21-like 2 with subunits of TIM17:23 complex and respiratory chain complexes, yeast 2-Hybrid assays were carried out (**Figure 5**). AtTim21-like 1 and AtTim21-like 2 were cloned into the bait vector and transformed into Y187 yeast strain were mated against import components and respiratory subunits cloned into the prey vector and transformed into a mating compatible strain (AH109). The mated strains were plated out on DDO (double drop out) media to select for diploids and QDO (quadruple drop out) to determine protein-protein interactions. Positive interactions were observed between AtTim21-like 1 and 2 and AtTim21, with TIM17:23 import component, AtTim17-2. Both AtTim21-like 2 and AtTim21 interacted with AtTim23-2 and its accessory protein AtTim50. AtTim21-like 2 showed a positive interaction with AtTim44-2 and all proteins exhibited an interaction with AtTim21. Interactions were also tested against the carrier protein translocase of the inner membrane, AtTim22, with only AtTim21-like 2 exhibiting positive colonies. The Complex I subunit, AtB14.7, showed positive interactions with both AtTim21-like 2 and AtTim21, whilst Complex III subunit ubiquinol-cytochrome c reductase cytochrome c1 subunit (AtCyc1-1), AtTim21 also exhibited positive interaction with Complex III subunits MPPα and rieske iron sulphur protein (RISP). AtTim21 was also seen to interact with the outer membrane complex subunits AtTom9 (Tom22 in yeast) and AtTom5 (**Figure 5**). No interactions were observed using AtTim21-like 1, AtTim21-like 2, and AtTim21 against the pGAD empty vector confirming positive interactions.

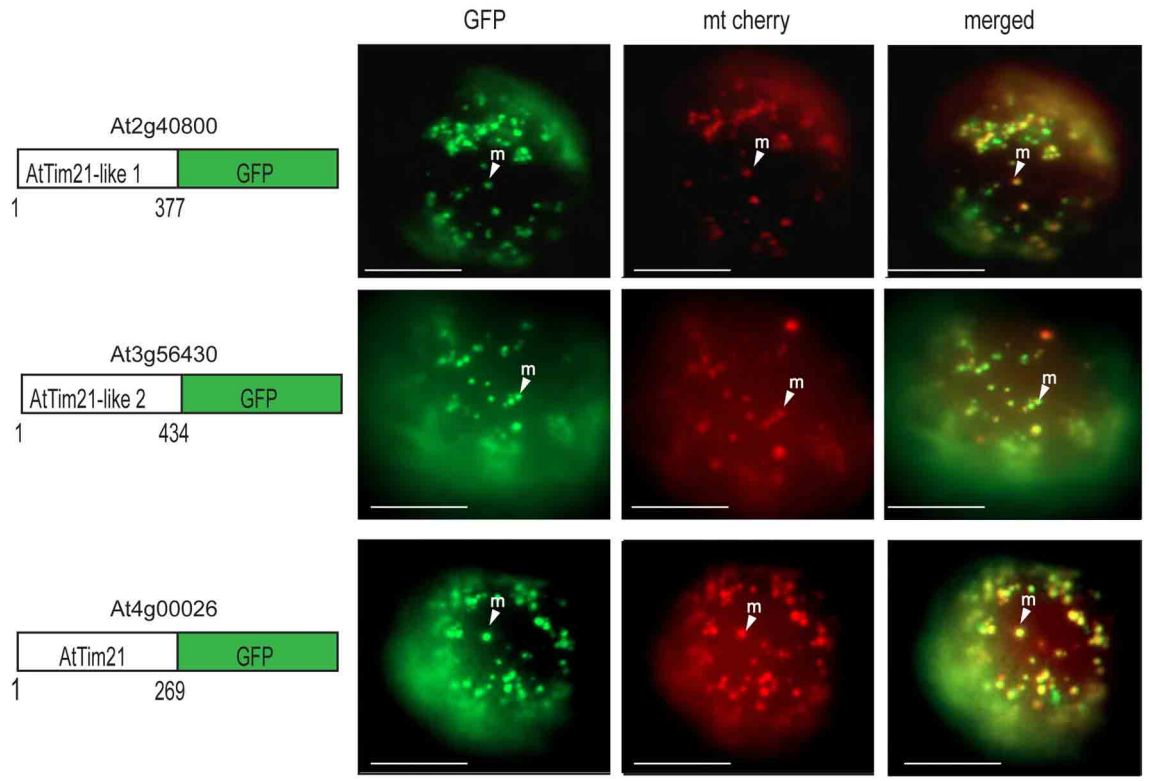


FIGURE 3 | Subcellular localizations of Arabidopsis Tim21-like proteins. GFP was fused to the carboxy-terminus of AtTim21 (At4g00026), AtTim21-like 1 (At2g40800), and AtTim21-like 2 (At3g56430). Targeting was analyzed in Arabidopsis cell suspension along with a mitochondrial cherry control. The location of the GFP and the number of amino acids for each construct are drawn. M, mitochondria; Scale bar indicates 10 μm.

DISCUSSION

In addition to Tim21, Arabidopsis mitochondria contain unique Tim21-like proteins. Whilst evolutionary distinct from each other, both contain the conserved Tim21 protein domain and phylogenetic analysis suggests that these Tim21-like proteins are plant specific. Tim21-like proteins most likely arose early in land plant evolution, as they can be identified in green algae, moss, and Selaginella. Both Tim21 and Tim21-like genes were conserved throughout all plants analyzed, containing at least one copy of each gene, suggesting that these proteins may have unique functions specific to plants.

For both AtTim21-like 1 and AtTim21-like 2, mitochondrial localization was determined by *in vitro* import assays and GFP targeting. GFP localizations showed distinct mitochondrial targeting. They were both determined to contain N-terminal targeting and assembled within the inner mitochondrial membrane. As no shift was observed following protease treatment of ruptured mitochondria we concluded that both AtTim21-like 1 and 2 are integrally located within the inner membrane. The radiolabeled band intensity of the mature protein is somewhat weak, though this is due to the fact that the native forms of AtTim21-like 1 and AtTim21-like 2, were cloned with three additional methionine's at the N-terminus for *in vitro* transcription and translation. Therefore, removal of the targeting peptide upon import results in a lower intensity band.

Import of AtTim21-like 1 and 2 and analysis via BN-PAGE revealed that these proteins may associate in the monomeric forms of Complex I and Complex III in addition to the supercomplex Complex I and III. Furthermore, AtTim21-like 2 was seen to incorporated within the Tim17:23 complex with these interactions further supported by yeast 2-Hybrid assays. This is in parallel with the previously observed interaction of AtTim21 (Wang et al., 2012) and yeast Tim21 (Van der Laan et al., 2006). AtTim21, was shown to similarly associate within Complex III using both radiolabeled proteins and immunodetection whilst interaction with the TIM17:23 complex was identified via protein interactions (Wang et al., 2012). This suggests that AtTim21 and AtTim21-like proteins may also play a role in tethering the import and respiratory chain complexes as with yeast Tim21 (Van der Laan et al., 2006). These associations are also supported by previous work involving the characterization of a AtTim21 overexpressing line, that showed alterations to mitochondrial activity and more importantly exhibited changes to the expression levels of respiratory complex subunits (Hamasaki et al., 2012).

AtTim21-like 1 and AtTim21-like 2 were also seen to associate into smaller unknown protein complexes, which remain to be identified. Although recent data showing that yeast Tim21 has the ability to associate with respiratory chain intermediates, termed MITRAC (mitochondrial protein translocation to respiratory chain assembly) containing several structural components

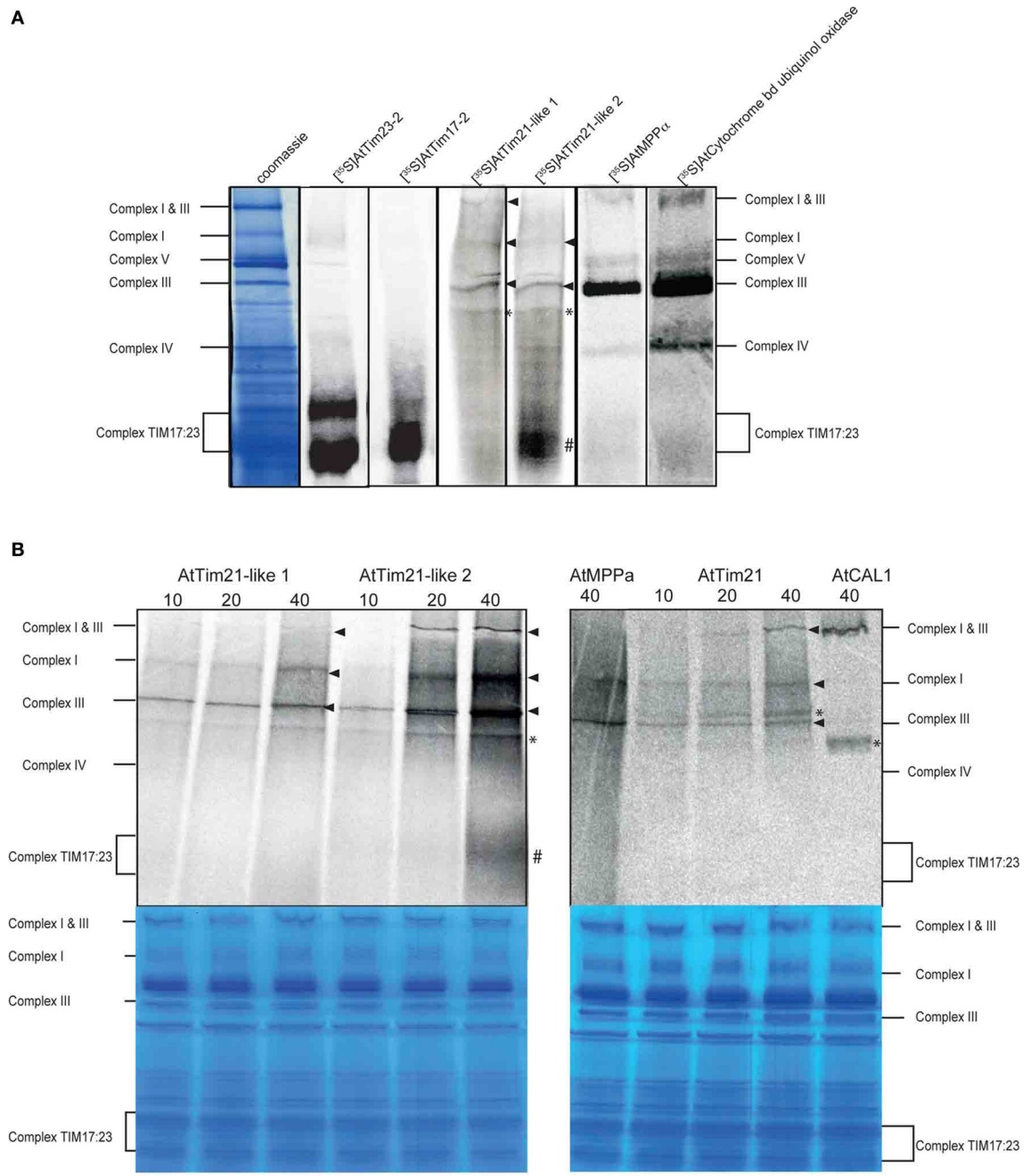


FIGURE 4 | Association of AtTim21-like proteins into mitochondrial protein complexes. (A) Radiolabeled precursor protein was incubated with isolated Arabidopsis mitochondria for 40 min and separated by BN-PAGE. The positions of the respiratory complexes and the TIM17:23 complex are indicated. **(B)** BN-PAGE of time-course analysis (min) of AtTim21-like 1, 2 and AtTim21 import into isolated mitochondria. The incorporation of radiolabeled proteins into respiratory chain complexes is indicated by the arrow. Incorporation of radiolabeled protein into TIM17:23 complex is indicated by # and into unknown protein complexes indicated by *. Corresponding coomassie stained gels are shown.

of Complex IV plus the assembly factors COX15 and COX16 (Mick et al., 2012). In our studies weak labeling of both Complex I and Complex III controls could also be observed within these unknown complexes and thus raises the possibility that these additional complex bands observed via BN-PAGE analysis may in fact be respiratory complex assembly intermediates. Differences were also observed within the positions of these possible intermediates between AtTIm21 and AtTim21-like proteins. These small differences observed may indicate that there are some variances to protein specificity between the AtTim21 and AtTim21-like proteins and raises the possibility that AtTim21-like may interact with plant specific subunits of the respiratory complexes.

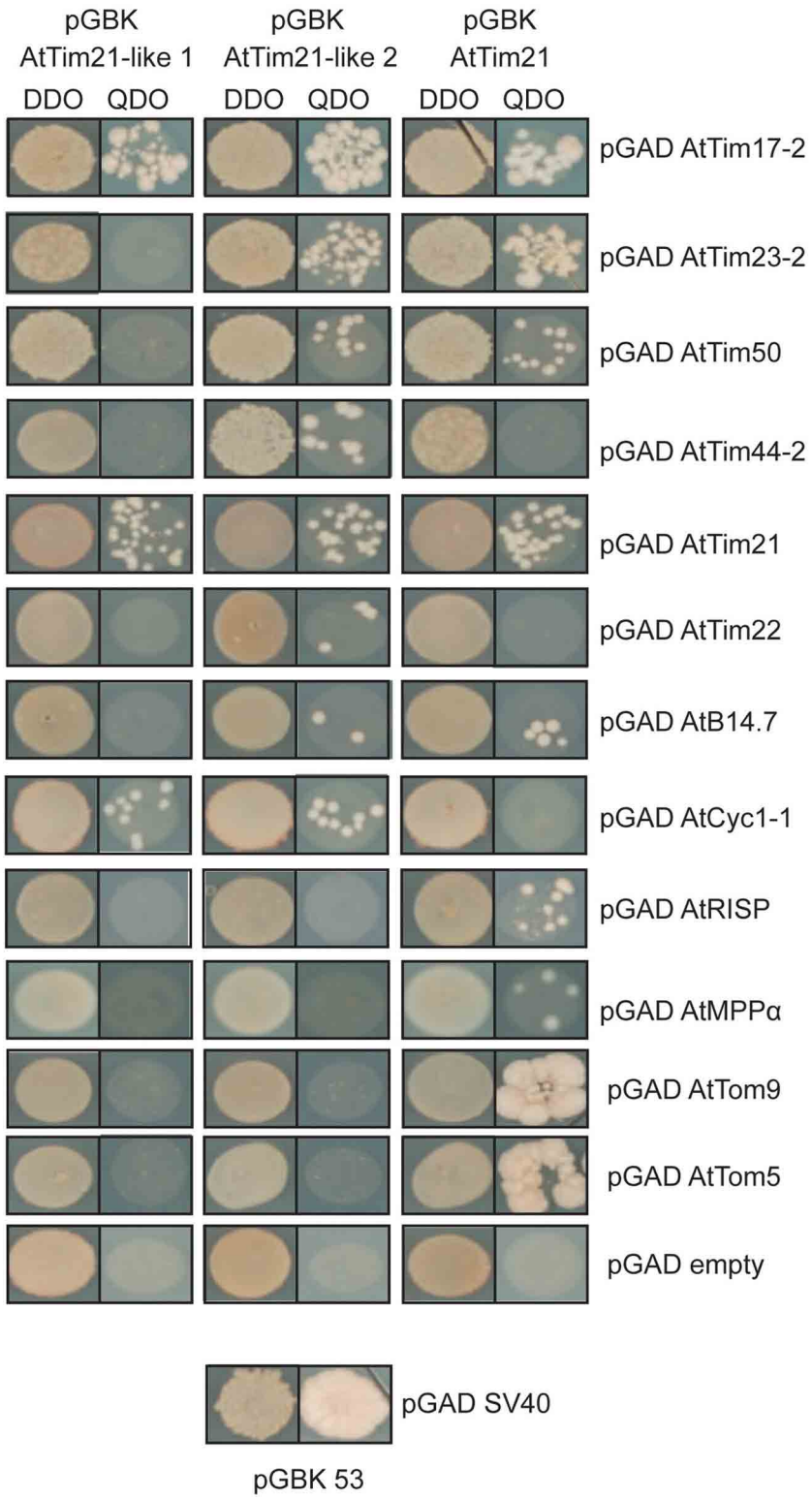


FIGURE 5 | Interactions between AtTim21-like 1, AtTim21-like 2 and components of the respiratory chain and import apparatus. Yeast 2-Hybrid interactions indicate a positive interaction between AtTim21-like 1 and 2 and AtTim21 and various components of the mitochondrial import apparatus (AtTim17-2, AtTim23-2, AtTim50, AtTim44-2, AtTim22), Complex I (AtB14.7), Complex III (AtCyc1-1, AtRISP, AtMPPα). AtTim21 was also able to interact with TOM subunits, AtTom9, and AtTom5. Positive diploids are indicated by growth on Double Drop out media (DDO) and protein-protein interactions are indicated by growth of diploid strains on Quadruple Drop Out media (QDO). No interactions of pGBK AtTim21-like 1 and 2 and pGBK AtTim21 were observed against the pGAD empty vector. SV40 and p53 was used as a positive control.

Further investigations on AtTim21 and AtTim21-like proteins will be required to determine their potential role in the assembly of respiratory complex intermediates in Arabidopsis mitochondria and to unravel the molecular mechanisms of these novel plant specific proteins involved in mitochondrial biogenesis.

CONCLUSION

Here we present the mitochondrial localization of two novel Tim21-like proteins from *Arabidopsis thaliana* and show that Tim21-like 1 and 2 have the ability to interact with the translocase of the inner membrane TIM17:23 and Complex I and III of the respiratory chain. These observed interactions suggest that as seen with Tim21, Tim21-like proteins may also be involved in the import and biogenesis of respiratory chain components.

AUTHOR CONTRIBUTIONS

Monika W. Murcha and James Whelan designed the experiments and carried out the data analysis, Monika W. Murcha, Yan Wang, and Szymon Kubiszewski-Jakubiak performed the experimental procedures and all authors contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

This work was supported by an Australian Research Council, Postdoctoral Fellowship Grant DP0878603 to Monika W. Murcha and an Australian Research Council Discovery Grant DP0664692 to James Whelan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.2014.00082/abstract

Supplemental Table 1 | Primer pairs used for cloning.

REFERENCES

Albrecht, V., Simkova, K., Carrie, C., Delannoy, E., Giraud, E., Whelan, J., et al. (2010). The cytoskeleton and the peroxisomal-targeted snowy cotyledon3 protein are required for chloroplast development in Arabidopsis. *Plant Cell* 22, 3423–3438. doi: 10.1105/tpc.110.074781

Altschul, S. F., and Koonin, E. V. (1998). Iterated profile searches with PSI-BLAST–a tool for discovery in protein databases. *Trends Biochem. Sci.* 23, 444–447. doi: 10.1016/S0968-0004(98)01298-5

Balsera, M., Soll, J., and Bolter, B. (2009). Protein import machineries in endosymbiotic organelles. *Cell. Mol. Life Sci.* 66, 1903–1923. doi: 10.1007/s00018-009-8644-2

Braun, H. P., Emmermann, M., Kruft, V., and Schmitz, U. K. (1992). The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain. *EMBO J.* 11, 3219–3227.

Campanella, J. J., Bitincka, L., and Smalley, J. (2003). MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. *BMC Bioinformatics* 4:29. doi: 10.1186/1471-2105-4-29

Carrie, C., Kuhn, K., Murcha, M. W., Duncan, O., Small, I. D., O'Toole, N., et al. (2009). Approaches to defining dual-targeted proteins in Arabidopsis. *Plant J.* 57, 1128–1139. doi: 10.1111/j.1365-313X.2008.03745.x

Carrie, C., Murcha, M. W., and Whelan, J. (2010). An *in silico* analysis of the mitochondrial protein import apparatus of plants. *BMC Plant Biol.* 10:249. doi: 10.1186/1471-2229-10-249

Chacinska, A., Lind, M., Frazier, A. E., Dudek, J., Meisinger, C., Geissler, A., et al. (2005). Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17. *Cell* 120, 817–829. doi: 10.1016/j.cell.2005.01.011

Chew, O., Lister, R., Qbadou, S., Heazlewood, J. L., Soll, J., Schleiff, E., et al. (2004). A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. *FEBS Lett.* 557, 109–114. doi: 10.1016/S0014-5793(03)01457-1

Claros, M. G. (1995). MitoProt, a Macintosh application for studying mitochondrial proteins. *Comput. Appl. Biosci.* 11, 441–447. doi: 10.1093/bioinformatics/11.4.441

Delage, L., Leblanc, C., Nyvall Collen, P., Gschloessl, B., Oudot, M. P., Sterck, L., et al. (2011). *In silico* survey of the mitochondrial protein uptake and maturation systems in the brown alga Ectocarpus siliculosus. *PLoS ONE* 6:e19540. doi: 10.1371/journal.pone.0019540

Donzeau, M., Kaldi, K., Adam, A., Paschen, S., Wanner, G., Guiard, B., et al. (2000). Tim23 links the inner and outer mitochondrial membranes. *Cell* 101, 401–412. doi: 10.1016/S0092-8674(00)80850-8

Duncan, O., Murcha, M. W., and Whelan, J. (2013). Unique components of the plant mitochondrial protein import apparatus. *Biochim. Biophys. Acta* 1833, 304–313. doi: 10.1016/j.bbamcr.2012.02.015

Gebert, N., Gebert, M., Oeljeklaus, S., von der Malsburg, K., Stroud, D. A., Kulawiak, B., et al. (2011). Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane. *Mol. Cell* 44, 811–818. doi: 10.1016/j.molcel.2011.09.025

Glaser, E., Eriksson, A., and Sjoling, S. (1994). Bifunctional role of the bc1 complex in plants. Mitochondrial bc1 complex catalyses both electron transport and protein processing. *FEBS Lett.* 346, 83–87. doi: 10.1016/0014-5793(94)00312-2

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., et al. (2012). Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* 40, D1178–D1186. doi: 10.1093/nar/gkr944

Gray, M. W., Burger, G., and Lang, B. F. (2001). The origin and early evolution of mitochondria. *Genome Biol.* 2:reviews1018. doi: 10.1186/gb-2001-2-6-reviews1018

Hamasaki, H., Yoshizumi, T., Takahashi, N., Higuchi, M., Kuromori, T., Imura, Y., et al. (2012). SD3, an Arabidopsis thaliana homolog of TIM21, affects intracellular ATP levels and seedling development. *Mol. Plant* 5, 461–471. doi: 10.1093/mp/ssr088

Heinz, E., and Lithgow, T. (2013). Back to basics: a revealing secondary reduction of the mitochondrial protein import pathway in diverse intracellular parasites. *Biochim. Biophys. Acta* 1833, 295–303. doi: 10.1016/j.bbamcr.2012.02.006

Hewitt, V., Alcock, F., and Lithgow, T. (2011). Minor modifications and major adaptations: the evolution of molecular machines driving mitochondrial protein import. *Biochim. Biophys. Acta* 1808, 947–954. doi: 10.1016/j.bbamem.2010.07.019

Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brutsch, H., and Schatz, G. (1990). Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO J.* 9, 3191–3200.

Kerscher, O., Holder, J., Srinivasan, M., Leung, R. S., and Jensen, R. E. (1997). The Tim54p-Tim22p complex mediates insertion of proteins into the mitochondrial inner membrane. *J. Cell Biol.* 139, 1663–1675. doi: 10.1083/jcb.139.7.1663

Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000). Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. *Mol. Biol. Cell* 11, 103–116. doi: 10.1091/mbc.11.1.103

Klodmann, J., Sunderhaus, S., Nimtz, M., Jansch, L., and Braun, H. P. (2010). Internal architecture of mitochondrial complex I from Arabidopsis thaliana. *Plant Cell* 22, 797–810. doi: 10.1105/tpc.109.073726

Krogh, A., Larsson, B., Von Heijne, G., and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* 305, 567–580. doi: 10.1006/jmbi.2000.4315

Kulawiak, B., Hopker, J., Gebert, M., Guiard, B., Wiedemann, N., and Gebert, N. (2012). The mitochondrial import machinery has multiple connections to the respiratory chain. *Biochim. Biophys. Acta* 1827, 612–626. doi: 10.1016/j.bbabio.2012.12.004

Lister, R., Carrie, C., Duncan, O., Ho, L. H., Howell, K. A., Murcha, M. W., et al. (2007). Functional definition of outer membrane proteins involved in preprotein import into mitochondria. *Plant Cell* 19, 3739–3759. doi: 10.1105/tpc.107.050534

Lister, R., Chew, O., Lee, M. N., Heazlewood, J. L., Clifton, R., Parker, K. L., et al. (2004). A transcriptomic and proteomic characterization of the Arabidopsis mitochondrial protein import apparatus and its response to mitochondrial dysfunction. *Plant Physiol.* 134, 777–789. doi: 10.1104/pp.103.033910

Lithgow, T., and Schneider, A. (2010). Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365, 799–817. doi: 10.1098/rstb.2009.0167

Liu, Z., Li, X., Zhao, P., Gui, J., Zheng, W., and Zhang, Y. (2011). Tracing the evolution of the mitochondrial protein import machinery. *Comput. Biol. Chem.* 35, 336–340. doi: 10.1016/j.compbiolchem.2011.10.005

Marchler-Bauer, A., Lu, S., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., Deweese-Scott, C., et al. (2011). CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res.* 39, D225–D229. doi: 10.1093/nar/gkq1189

Meyer, E. H., Taylor, N. L., and Millar, A. H. (2008). Resolving and identifying protein components of plant mitochondrial respiratory complexes using three dimensions of gel electrophoresis. *J. Proteome Res.* 7, 786–794. doi: 10.1021/pr700595p

Meyer, E. H., Tomaz, T., Carroll, A. J., Estavillo, G., Delannoy, E., Tanz, S. K., et al. (2009). Remodeled respiration in ndufs4 with low phosphorylation efficiency suppresses Arabidopsis germination and growth and alters control of metabolism at night. *Plant Physiol.* 151, 603–619. doi: 10.1104/pp.109.141770

Mick, D. U., Dennerlein, S., Wiese, H., Reinhold, R., Pacheu-Grau, D., Lorenzi, I., et al. (2012). MITRAC links mitochondrial protein translocation to respiratory-chain assembly and translational regulation. *Cell* 151, 1528–1541. doi: 10.1016/j.cell.2012.11.053

Mokranjac, D., Popov-Celeketic, D., Hell, K., and Neupert, W. (2005). Role of Tim21 in mitochondrial translocation contact sites. *J. Biol. Chem.* 280, 23437–23440. doi: 10.1074/jbc.C500135200

Murcha, M. W., Elhafez, D., Lister, R., Tonti-Filippini, J., Baumgartner, M., Philippar, K., et al. (2007). Characterization of the preprotein and amino acid transporter gene family in Arabidopsis. *Plant Physiol.* 143, 199–212. doi: 10.1104/pp.106.090688

Murcha, M. W., Elhafez, D., Millar, A. H., and Whelan, J. (2005a). The C-terminal region of TIM17 links the outer and inner mitochondrial membranes in Arabidopsis and is essential for protein import. *J. Biol. Chem.* 280, 16476–16483. doi: 10.1074/jbc.M413299200

Murcha, M. W., Huang, T., and Whelan, J. (1999). Import of precursor proteins into mitochondria from soybean tissues during development. *FEBS Lett.* 464, 53–59. doi: 10.1016/S0014-5793(99)01674-9

Murcha, M. W., Lister, R., Ho, A. Y., and Whelan, J. (2003). Identification, expression, and import of components 17 and 23 of the inner mitochondrial membrane translocase from Arabidopsis. *Plant Physiol.* 131, 1737–1747. doi: 10.1104/pp.102.016808

Murcha, M. W., Rudhe, C., Elhafez, D., Adams, K. L., Daley, D. O., and Whelan, J. (2005b). Adaptations required for mitochondrial import following mitochondrial to nucleus gene transfer of ribosomal protein S10. *Plant Physiol.* 138, 2134–2144. doi: 10.1104/pp.105.062745

Murcha, M. W., Wang, Y., Narsai, R., and Whelan, J. (2014). The plant mitochondrial protein import apparatus. The differences make it interesting. *Biochim. Biophys. Acta*. 1840, 1233–1245. doi: 10.1016/j.bbagen.2013.09.026

Murcha, M. W., Wang, Y., and Whelan, J. (2012). A molecular link between mitochondrial preprotein transporters and respiratory chain complexes. *Plant Signal. Behav.* 12, 1594–1597. doi: 10.4161/psb.22250

Nelson, B. K., Cai, X., and Nebenfuhr, A. (2007). A multicolored set of *in vivo* organelle markers for co-localization studies in Arabidopsis and other plants. *Plant J.* 51, 1126–1136. doi: 10.1111/j.1365-313X.2007.03212.x

Perry, A. J., Rimmer, K. A., Mertens, H. D., Waller, R. F., Mulhern, T. D., Lithgow, T., et al. (2008). Structure, topology and function of the translocase of the outer membrane of mitochondria. *Plant Physiol. Biochem.* 46, 265–274. doi: 10.1016/j.plaphy.2007.12.012

Pudelski, B., Kraus, S., Soll, J., and Philippar, K. (2010). The plant PRAT proteins—preprotein and amino acid transport in mitochondria and chloroplasts. *Plant Biol. (Stuttg.)* 12(Suppl. 1), 42–55. doi: 10.1111/j.1438-8677.2010.00357.x

Rassow, J., Dekker, P. J., van Wilpe, S., Meijer, M., and Soll, J. (1999). The preprotein translocase of the mitochondrial inner membrane: function and evolution. *J. Mol. Biol.* 286, 105–120. doi: 10.1006/jmbi.1998.2455

Rehling, P., Wiedemann, N., Pfanner, N., and Truscott, K. N. (2001). The mitochondrial import machinery for preproteins. *Crit. Rev. Biochem. Mol. Biol.* 36, 291–336. doi: 10.1080/20014091074200

Rimmer, K. A., Foo, J. H., Ng, A., Petrie, E. J., Shilling, P. J., Perry, A. J., et al. (2011). Recognition of mitochondrial targeting sequences by the import receptors Tom20 and Tom22. *J. Mol. Biol.* 405, 804–818. doi: 10.1016/j.jmb.2010.11.017

Saddar, S., Dienhart, M. K., and Stuart, R. A. (2008). The F1F0-ATP synthase complex influences the assembly state of the cytochrome bc1-cytochrome oxidase supercomplex and its association with the TIM23 machinery. *J. Biol. Chem.* 283, 6677–6686. doi: 10.1074/jbc.M708440200

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539. doi: 10.1038/msb.2011.75

Sirrenberg, C., Endres, M., Folsch, H., Stuart, R. A., Neupert, W., and Brunner, M. (1998). Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5. *Nature* 391, 912–915. doi: 10.1038/36136

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Van der Laan, M., Wiedemann, N., Mick, D. U., Guiard, B., Rehling, P., and Pfanner, N. (2006). A role for Tim21 in membrane-potential-dependent preprotein sorting in mitochondria. *Curr. Biol.* 16, 2271–2276. doi: 10.1016/j.cub.2006.10.025

Wang, Y., Carrie, C., Giraud, E., Elhafez, D., Narsai, R., Duncan, O., et al. (2012). Dual location of the mitochondrial preprotein transporters B14.7 and Tim23-2 in complex I and the TIM17:23 complex in Arabidopsis links mitochondrial activity and biogenesis. *Plant Cell* 24, 2675–2695. doi: 10.1105/tpc.112.098731

Werhahn, W., Niemeyer, A., Jansch, L., Kruft, V., Schmitz, U. K., and Braun, H. (2001). Purification and characterization of the preprotein translocase of the outer mitochondrial membrane from Arabidopsis. Identification of multiple forms of TOM20. *Plant Physiol.* 125, 943–954. doi: 10.1104/pp.125.2.943

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 January 2014; accepted: 21 February 2014; published online: 11 March 2014.

*Citation: Murcha MW, Kubiszewski-Jakubiak S, Wang Y and Whelan J (2014) Evidence for interactions between the mitochondrial import apparatus and respiratory chain complexes via Tim21-like proteins in Arabidopsis. Front. Plant Sci. **5**:82. doi: 10.3389/fpls.2014.00082*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

REVIEW ARTICLE
published: 11 April 2014
doi: 10.3389/fpls.2014.00145

Evolution and significance of the *Lon* gene family in *Arabidopsis* organelle biogenesis and energy metabolism

Stamatis Rigas, Gerasimos Daras, Dikran Tsitsekian, Anastasios Alatzas and Polydefkis Hatzopoulos*

Department of Biotechnology, Agricultural University of Athens, Athens, Greece

Edited by:
Daniel H. Gonzalez, Universidad Nacional del Litoral, Argentina

Reviewed by:
Diego Fabian Gomez-Casati, National University of Rosario – National Scientific and Technical Research Council, National Research Council of Argentine, Argentina
Chris Carrie, Ludwig-Maximilians-Universität, Germany

***Correspondence:**
Polydefkis Hatzopoulos, Department of Biotechnology, Agricultural University of Athens, Iera Odos 75, Athens 118 55, Greece
e-mail: phat@aua.gr

Lon is the first identified ATP-dependent protease highly conserved across all kingdoms. Model plant species *Arabidopsis thaliana* has a small *Lon* gene family of four members. Although these genes share common structural features, they have distinct properties in terms of gene expression profile, subcellular targeting and substrate recognition motifs. This supports the notion that their functions under different environmental conditions are not necessarily redundant. This article intends to unravel the biological role of Lon proteases in energy metabolism and plant growth through an evolutionary perspective. Given that plants are sessile organisms exposed to diverse environmental conditions and plant organelles are semi-autonomous, it is tempting to suggest that *Lon* genes in *Arabidopsis* are paralogs. Adaptive evolution through repetitive gene duplication events of a single archaic gene led to *Lon* genes with complementing sets of subfunctions providing to the organism rapid adaptability for canonical development under different environmental conditions. Lon1 function is adequately characterized being involved in mitochondrial biogenesis, modulating carbon metabolism, oxidative phosphorylation and energy supply, all prerequisites for seed germination and seedling establishment. Lon is not a stand-alone proteolytic machine in plant organelles. Lon in association with other nuclear-encoded ATP-dependent proteases builds up an elegant nevertheless, tight interconnected circuit. This circuitry channels properly and accurately, proteostasis and protein quality control among the distinct subcellular compartments namely mitochondria, chloroplasts, and peroxisomes.

Keywords: Lon, mitochondria, chloroplasts, protein dual-targeting, gene expression, gene evolution, molecular modeling, energy metabolism

NO GIFT WITHOUT A PRICE: LIFE IN AN AEROBIC WORLD IS NOT NECESSARILY PERFECT

The paradox of aerobic life or the "Oxygen Paradox" argues that organisms do not survive in oxygen depleted environments, yet oxygen is inherently dangerous to their existence. This "dark side" of oxygen is attributed to the damage of biomolecules (Davies, 1995). Life in an oxygenated environment contributed to the evolution of aerobic metabolic processes such as respiration and photosynthesis that unavoidably result in the production of molecular oxygen metabolites known as reactive oxygen species (ROS). Although increasing evidence indicates that ROS in plants could function as signaling molecules in regulating development and pathogen defense response, ROS have the capacity to stochastically cause oxidative damage to proteins, DNA, and lipids (Apel and Hirt, 2004; Møller et al., 2007).

Mitochondria, chloroplasts, and peroxisomes represent subcellular sources for ROS production and the principle targets for oxidative macromolecular damage. In particular, the electron transport chain of mitochondria transfers high energy electrons to oxygen through a series of inner membrane protein complexes. This process of electron transfer from NADH or $FADH_2$ to O_2 by the electron carriers, known as oxidative phosphorylation (OXPHOS), is leading to energy production in the form of ATP. However, through this process leakage of electron occurs, ultimately generating highly reactive species, causing severe cell damage. This side-effect in mitochondria led to the "free-radical theory," conceived in 1956, speculating that endogenous oxygen radicals were generated within cells and resulted in a pattern of cumulative damage (Harman, 1956). Nowadays, this theory is widely appreciated by an increasing number of scientists from an expanding circle of fields, including plant biologists, supporting the role of oxidants in cellular damage (Beckman and Ames, 1998; Finkel and Holbrook, 2000).

To cope with the hostile oxygenated environment, organisms have evolved sophisticated networks of defense (Finkel and Holbrook, 2000; Apel and Hirt, 2004; Friguet et al., 2008). The first line of defense against oxidative injury is composed of a complex array of ROS detoxifying enzymes and non-enzymatic antioxidants that counteract and regulate the overall ROS levels, maintaining physiological homeostasis. During physiological steady state conditions the cellular oxidants are efficiently scavenged by these antioxidative defense components that are often confined to particular compartments. However, under persisting adverse oxidative conditions the equilibrium between ROS production and scavenging is perturbed resulting in rapid intracellular accumulation of oxidants. These disturbances characterized as oxidative stress, induce modifications to

both the polypeptide backbone and amino acid side chains of proteins.

As plants are sessile organisms exposed to harsh environmental conditions, numerous abiotic conditions result in protein misfolding usually caused by ROS-mediated chemical modifications. These conditions include exposure to high light intensity, drought stress, low or high temperature and mechanical stress (Apel and Hirt, 2004; Møller et al., 2007). The misfolded proteins are particularly prone to oxidation (Dukan et al., 2000) leading to the formation of adducts that often bring in carbonyl groups and cross-links (Friguet et al., 2008). The carbonylated proteins are functionally impaired or completely inactive, creating toxic protein aggregates and cross-linked inclusion bodies that interfere with normal cellular function (Petropoulos and Friguet, 2006). Hence, the second line of defense against oxidative injury is composed of the protein quality control mechanisms that essentially ensure the proper level of functional proteins within the cell and eliminate non-functional proteins.

The ATP-dependent Lon protease is a key component of protein quality control highly conserved across the kingdoms of living organisms. This article presents important findings and the progress recently made in plants, whereas special emphasis is simultaneously given on major scientific breakthroughs regarding the Lon function in non-plant organisms. This comparative approach will contribute toward better understanding of Lon in organellar proteostasis and cellular homeostasis.

THE AAA$^+$ Lon PROTEASE IS A MAJOR COMPONENT OF PROTEIN QUALITY CONTROL MECHANISM

Protein fate depends on an elegant protein quality control system that precisely orchestrates protein complex assembly and degradation, thereby safeguarding cellular homeostasis especially under stress conditions. The role of protein quality control is biphasic, as it is composed of energy-dependent repair molecular chaperones and degradation machines. Chaperones and proteases represent two sides of the same coin, acting in opposing pathways to clear unfolded proteins from the cell (Voos, 2013). The molecular chaperones within the cell facilitate the folding of newly synthesized proteins into their native conformations, prevent aggregation and assist in the assembly of multiprotein complexes. Conversely, ATP-dependent proteases degrade irreparably damaged or improperly synthesized proteins. In the cytosol and nucleus of higher eukaryotes, the proteins to be removed are ubiquitylated and delivered to the 26S proteasome for degradation (Hershko and Ciechanover, 1998). The 26S proteasome is the most elaborate AAA$^+$ protease (ATPases associated with diverse cellular activities), consisting of a 20S protease core particle and two 19S regulatory caps modulating several aspects of plant development (Coux et al., 1996; Voges et al., 1999; Smalle and Vierstra, 2004). In contrast to these cellular compartments and as a legacy of their endosymbiotic heritage, eukaryotic organelles maintain independent AAA$^+$ protein degradation machineries categorized into the soluble Lon and Clp (caseinolytic protease) families and the membrane-integrated FtsH-class (filament-forming temperature-sensitive) proteases (also called as AAA-proteases; Adam et al., 2001; Sinvany-Villalobo et al., 2004; Sakamoto, 2006; Rigas et al., 2012; Janska et al., 2013). In the case of FtsH and Lon, the ATPase and proteolytic domains are formed by a single polypeptide, whereas in Clp these domains are separate proteolytic (ClpP) and chaperone-like (ClpX) subunits.

Protease La encoded by the *Lon* gene homolog in *Escherichia coli*, was the first discovered AAA$^+$ protease (Chung and Goldberg, 1981). As "La" is the sixth musical note of the solfège syllable, the nomenclature describes the order of Lon elution from the chromatographic analysis of *E. coli* soluble proteolytic enzymes (Swamy and Goldberg, 1981). Lon is an ubiquitous proteolytic machine present in unicellular and multicellular organisms. The Lon protease consists of three functional domains (Rotanova et al., 2006; Rigas et al., 2012). The long N-terminal domain that possibly together with the central AAA$^+$ module selectively interact with target proteins and the C-terminal proteolytic domain (P-domain) with a typical Serine–Lysine catalytic dyad at the active center (Botos et al., 2004). In plants, the N-domain and the P-domain exhibit the highest evolutionary conservation. On the contrary, the AAA$^+$ module that includes the Walker Box A and B motifs shows the highest degree of divergence in both amino acid composition and length, and is responsible for binding and hydrolysis of ATP (Rigas et al., 2009b). The orthologs of Lon are divided into two subgroups (Rotanova et al., 2006; Rigas et al., 2012): A type (A-Lons), which have a large multi-lobed N-terminal domain together with the ATPase and protease domains, and B type (B-Lons), which instead of the N domain have a membrane-anchoring region emerging from the ATPase domain. B-Lons are exclusively present in Archaea that lack FtsH and the Clp proteases and thereby B-Lons are the major ATP-dependent proteolytic machines in those cells. The soluble A-Lons are found in all bacteria and in eukaryotic cell organelles, such as mitochondria, chloroplasts, and peroxisomes (Lingard and Bartel, 2009; Rigas et al., 2009a,b, 2012). In the yeast *Saccharomyces cerevisiae*, Pim1 (proteolysis in mitochondria) the homologous Lon protease has a major role in mitochondrial proteostasis as this organism lacks Clp (Venkatesh et al., 2012).

As a chambered protease, the 26S proteasome degrades protein substrates that carry multiple ubiquitin moieties (Hershko and Ciechanover, 1998). Given that mitochondria do not exhibit a covalent tagging system for damaged proteins like the ubiquitin tag in the cytosol or nucleus, substrate selectivity of Lon ATP-dependent protease most likely depends on the intrinsic characteristics of the polypeptide to be degraded. Lon preferentially degrades to completion damaged or misfolded polypeptides having a 50–60 amino acid long unstructured and exposed protein segment with compromised conformational state (von Janowsky et al., 2005). Upon protein misfolding, specific sequences rich in aromatic and hydrophobic residues become accessible to be recognized by Lon (Gur and Sauer, 2008). Moreover, Lon can also degrade folded unassembled polypeptides that can be accommodated into the proteolytic central channel with surfaced-exposed hydrophobic residues located within a highly charged environment (Ondrovicová et al., 2005). Consequently, Lon selectively degrades untagged non-natively folded substrates or folded but unassembled subunits, ultimately protecting the functional integrity of the organellar proteome.

EXPRESSION AND PROTEIN TARGETING OF *Arabidopsis Lon* GENES

The protein isoforms of Lon are encoded by small nuclear gene families and predicted to be targeted to distinct subcellular organelles. In *Arabidopsis*, four nuclear genes have been identified that potentially encode for members of the *Lon* family (Sinvany-Villalobo et al., 2004; Janska et al., 2010; Rigas et al., 2012). On the basis of publicly available microarray data in the Genevestigator database and scientific reports (Rigas et al., 2009a) *Lon* genes in *Arabidopsis* are expressed in various cell types and tissues. Nevertheless, the *Lon* genes show distinct expression profiles (**Figure 1**). The expression of *Lon1* (At5g26860) is high in rapidly growing organs of embryonic origin and in high-energy dependent tissues, which have increased mitochondria population per cell to sustain increased energy requirements. *Lon1* is predominantly expressed in germinating seeds, embryonic organs, including cotyledons and primary roots, and in organs with high growth rates such as developing inflorescences, while it was hardly detected in mature roots or stems of adult plants (**Figure 1**; Rigas et al., 2009a). In comparison to *Lon1*, *Lon4* (At3g05790) shows the lowest level of expression, albeit *Lon4* gene response is qualitatively similar to *Lon1*. Among the members of the *Lon* gene family, *Lon2* (At5g47040) is highly expressed, while gene expression generally remains constant among the examined cell types and tissues without significant fluctuations. Due to the lack of experimental evidence to report the presence of gene transcripts, *Lon3* (At3g05780) is presumed to be a pseudogene (Ostersetzer et al., 2007; Rigas et al., 2009a). However, microarray data strongly support that *Lon3* expression dominates in sperm cells. This specific *Lon3* expression profile implies a potential role in plant reproduction and particularly in male gametes maturation and double fertilization. Apart from the sperm cells, the marginal level of *Lon3* expression detected in other tissues most likely represents experimental noise impossible to be filtered as *Lon3* and *Lon4* are almost identical.

Most of the nuclear-encoded proteins are specifically targeted to a single organelle. However, dual-targeting of proteins to mitochondria and chloroplasts has been surprisingly frequent due to their post-endosymbiotic evolution (Millar et al., 2006; Baudisch et al., 2014). Two types of dual-targeting configurations have been reported in plants: twin and ambiguous presequences (Peeters and Small, 2001; Silva-Filho, 2003; Mackenzie, 2005; Baudisch et al., 2014). The ambiguous presequence generates a single protein isoform with a targeting peptide recognized by the import apparatus of both mitochondria and chloroplasts. Although this configuration can be organized in domains determining targeting specificity to an individual organelle, the signals responsible for organellar targeting most frequently overlap (Berglund et al., 2009a). Hence, ambiguous presequences cannot be completely distinguished from organelle-specific targeting peptides and they have an intermediate amino acid composition using the same organellar import pathways as the organelle-specific proteins. Despite the fact that the determinants for dual-targeting are not fully understood, the physicochemical properties within the N-terminal of the ambiguous presequences including hydrophobicity, the charge of amino acids and secondary structure, modulate the double localization (Berglund et al., 2009b; Ge et al., 2014). The twin presequences include two distinct targeting domains arranged in tandem at

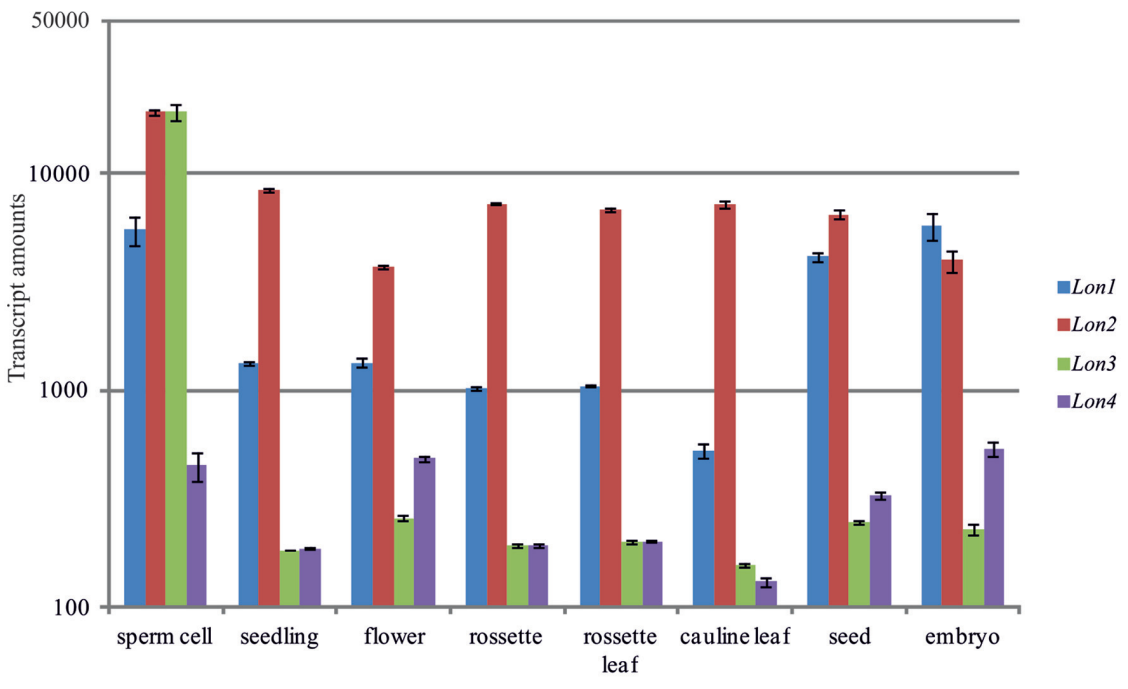


FIGURE 1 | Comparative analysis of the *Arabidopsis Lon* gene expression profiles in various cell types and tissues. Gene expression data were obtained from the Genevestigator server (Hruz et al., 2008). The vertical axis uses a base 10 logarithmic scale.

the N-terminus. In eukaryotes, twin presequences can confer dual-targeting to distinct subcellular compartments by employing two alternative in-frame translational initiation codons (Danpure, 1995; Silva-Filho, 2003; Carrie et al., 2009; Carrie and Small, 2013). Both ambiguous and twin presequences amplify the number of protein isoforms in subcellular compartments without affecting genome size. The majority of dual-targeted proteins in plants contain an ambiguous presequence showing an overall prevalence over twin presequences (Carrie et al., 2009).

The protein isoforms encoded by the nuclear *Lon* genes in *Arabidopsis* are scattered to plant cell organelles mainly involved in energy metabolism by utilizing different mechanisms of protein translocation (**Figure 2**). Lon4 is dual-targeted to mitochondria and chloroplasts displaying an ambiguous presequence (Sakamoto, 2006; Ostersetzer et al., 2007). The C-terminus of Lon2 bears a type 1 peroxisome-targeting signal (PTS1) conferring protein localization in peroxisomes (Lingard and Bartel, 2009). Computational analysis of Lon3 N-terminal domain identified a potential ambiguous presequence for dual-organellar localization to chloroplasts and mitochondria. The Lon1 dual-targeting is regulated both at the transcriptional and translational level (Daras et al., 2014).

THE SUBSTRATE RECOGNITION MOTIFS ARE HIGHLY VARIABLE AMONG THE *Arabidopsis* LON PROTEIN ISOFORMS

The AAA$^+$ Lon protease is a soluble single-ringed multimeric holoenzyme. Adjacent to the AAA$^+$ module is the sensor- and substrate-discrimination (SSD) domain mainly involved in modulating selective substrate recognition by Lon so as the target protein to be degraded. In line with its highly selective mode of action, the SSD domain exhibits substantial interspecies and within the same species diversity (Rigas et al., 2009b). The yeast *S. cerevisiae* Lon purified from mitochondria is a ring-shaped complex with seven flexible subunits as determined by analytic ultracentrifugation and cryoelectron microscopy (Stahlberg et al., 1999). Subunits of *E. coli* Lon are known to assemble into ring-shaped homohexamers that enclose an internal degradation chamber. These hexamers may also interact to form a dodecamer at physiological protein concentrations (Vieux et al., 2013). Insights may also be gained from the structure of an intact, assembled Lon protease from the hyperthermophilic archaea *Thermococcus onnurineus* (*Ton*Lon) that is ubiquitously present in various deep-sea hydrothermal vent systems. *Ton*Lon is a 635-residue protein belonging to the B-Lon family having the protease domains arranged with a near perfect sixfold symmetry relative to the axial pore (Cha et al., 2010). This crystal structure suggests that the P-domains of each subunit form a bowl-like chamber with a lid formed by the AAA$^+$ domains, such that substrates and degradation products may enter and exit the proteolytic chamber via opposing axial pores. Likewise, the homology model of human Lon suggests an hexameric complex formation that has an asymmetric, open-ring arrangement reminiscent of yeast Lon, albeit the yeast Lon fails to be modeled as a hexamer (Venkatesh et al., 2012). The structural features of Lon proteases in line with homology modeling provide conclusive

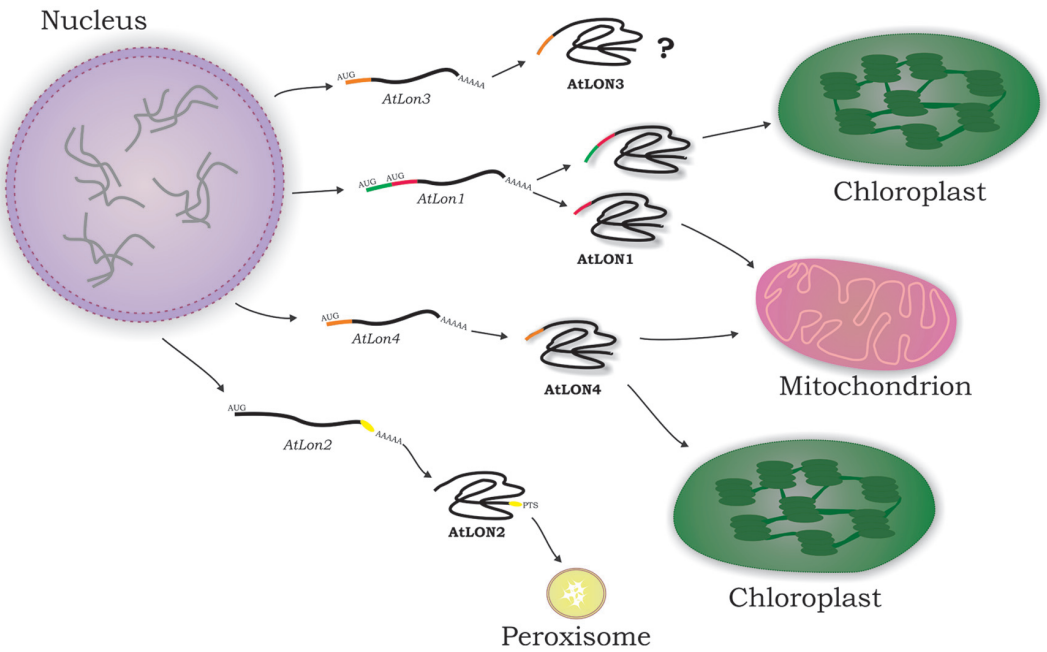


FIGURE 2 | *Arabidopsis* Lon protein isoforms are present in the subcellular compartments involved in energy metabolism. The majority of nuclear-encoded organellar proteins are translated on cytosolic ribosomes. In a distinct post-translational process, they have to be transported to their final destination in the organelle directed by specific targeting signals. Lon1 and Lon4 are dual-targeted to mitochondria and chloroplasts due to twin and ambiguous presequences, respectively, at the N-terminus of the encoded protein isoforms. Lon2 is imported into peroxisomes by a canonical C-terminal type 1 peroxisome-targeting signal (PTS1). Lon3 subcellular localization remains elusive as yet.

evidence that distinguishes bacterial and human Lon proteases as hexameric complexes from yeast Lon, which is uniquely heptameric.

As the SSD domain is the most variable domain among the *Arabidopsis* Lon proteases, the architectural features of protein monomers were analyzed by molecular modeling. These ribbon models were in turn compared with the hexameric complexes of bacterial (*Ec*Lon) and human (*Hs*Lon) Lon proteases and with the heptameric complex of yeast Pim1. Homology modeling confirmed that *Ec*Lon and *Hs*Lon share the same structural features but differ from the heptameric Pim1 complex (**Figure 3A**). As reported by Venkatesh et al. (2012), this is most likely explained by the primary amino acid sequences of *Ec*Lon and *Hs*Lon, which are significantly shorter than Pim1 and *Arabidopsis* Lon sequences (Rigas et al., 2009b). Interestingly, the analysis revealed that structurally the *Arabidopsis* Lon proteases deviate from the hexameric *Ec*Lon and *Hs*Lon proteases fitting best with the heptameric yeast structure and due to distinct structural features they are classified into two groups (**Figure 3A**). The first group includes *At*Lon1 and *At*Lon3 that share similar structural properties with Pim1, all preserving a single pair of parallel β-sheets (depicted in red color) that is typical of *Ec*Lon and *Hs*Lon. *At*Lon2 and *At*Lon4 belong in the second group bearing between the α-helices (depicted in gray color) an internal loop polypeptide configuration (depicted in yellow color) with different secondary structure (depicted in purple color) compared to the members of the first group. Additionally, a surprising core structure is discovered in Pim1 and *Arabidopsis* Lon proteases likely originating from the hexameric *Ec*Lon and *Hs*Lon complexes (**Figure 3B**). The alignment between the models of *At*Lon1 or *At*Lon4 with the core structure of *Hs*Lon show that *At*Lon1 internal geometry differs from *At*Lon4. The internal loop domain of *At*Lon4 shows a right-handed extension, whereas in *At*Lon1 is left-handed. This structural difference between the two major representative proteases of *Arabidopsis* suggests that *Lon1* and *Lon4* are gene paralogs performing specialized functions without being necessarily redundant. However, the possibility of recognizing similar protein targets under adverse environmental conditions that considerably modify the internal milieu of mitochondria and chloroplasts cannot be excluded. Future studies are required to assess the homology models of *Arabidopsis* Lon proteases and to solve the crystal structure of the holoenzyme, providing insights on Lon structural dynamics and functional versatility.

Lon1 AND *Lon4* PARALOGS EVOLVED DISTINCT STRUCTURAL AND FUNCTIONAL FEATURES

Ancient invasions by eubacteria gave rise through symbiosis to mitochondria and chloroplasts that have enormous impact on bioenergetic and metabolic homeostasis of plants (Dyall et al., 2004). Mitochondria originated first from an endosymbiotic event of α-proteobacterial fusion. A second cyanobacterial invasion supplied the plant cell with the present-day chloroplast capable for photosynthesis. During the endosymbiotic process, the symbionts lost their autonomy by massive transfer of their genetic information to the host nucleus resulting in genetic redundancy. The evolution and establishment of the protein translocation machinery caused bulk gene loss leading to organellar genome reduction. The translocation process involved N-terminal extensions of the nuclear-encoded precursor protein

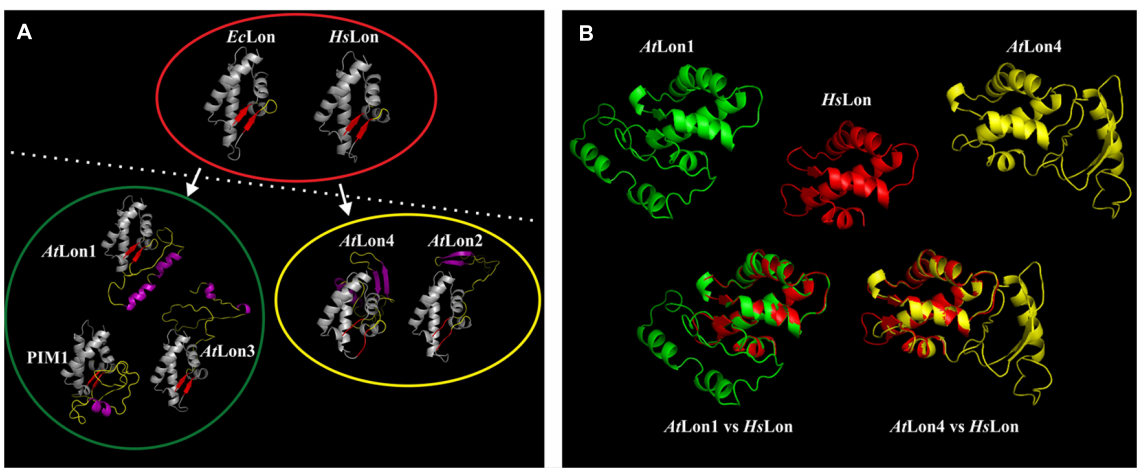


FIGURE 3 | Molecular modeling provides insights into the structural features of *Arabidopsis* Lon proteases. (A) Monomer structure comparison of the ribbon model of the sensor- and substrate-discrimination (SSD) domain discriminates the hexameric bacterial and human Lon complexes encompassed by red eclipse from possibly heptameric complexes of Pim1 and *Arabidopsis* Lon homologs. On the basis of discrete structural features the *Arabidopsis* proteases could be further categorized into two groups encompassed by green and yellow eclipses. The Lon protein accessions and the coordinates of the SSD domains given in parentheses are *At*Lon1: NP_568490 (603–739), *At*Lon2: NP_568675 (547–784), *At*Lon3: NP_566258 (586–726), and *At*Lon4: NP_566259 (585–733) from *Arabidopsis thaliana*, the *Homo sapiens* Lon: NP_004784 (662–747), Pim1: P36775 (772–911) from *Saccharomyces cerevisiae* and *Ec*Lon: AAC36871 (494–580) *from Escherichia coli*. Modeling of the SSD domain was performed on the basis of known crystallographic data mainly available from AAA^+ proteins and bacterial Lon proteases, which were automatically detected by the Phyre2 Protein Fold Recognition Server (www.sbg.bio.ic.ac.uk/phyre2). The ribbon model was generated in PyMol (www.pymol.org). **(B)** Homology modeling distinguishes *At*Lon1 (green) protease from *At*Lon4 (yellow), albeit both preserve the core structure of the hexameric human Lon (red) homolog.

synthesized on cytoplasmic ribosomes. Coordinated evolution of protein import machineries from ancient symbionts led to dual-targeting of nuclear-encoded proteins to both mitochondria and chloroplasts.

Contrary to *Lon1* dual-targeting that is attributed to twin presequences, an ambiguous presequence confers *Lon4* dual-targeting specificity (Sakamoto, 2006; Ostersetzer et al., 2007). Besides the annotated initiation codon, surprisingly an additional upstream AUG is present in *Lon4* at the same place as the first initiation codon of *Lon1*. However, the reading frame between the first and second initiation codons of *Lon4* is interrupted by a single thymine base insertion that results in a TGA stop codon (**Figure 4**). Upon removal of this base the reading frame becomes open encoding an N-terminal extension conferring Lon4 targeting specificity to chloroplasts similarly to the N-terminal extension of Lon1. Moreover, Lon1 and Lon4 are remarkably similar in terms of amino acid identity and similarity of the structural domains besides the SSD (Rigas et al., 2009b). As the structure of the SSD domain is tightly associated with Lon proteolytic activity (**Figure 3**), *Lon1* and *Lon4* gene duplicates were at the molecular level preserved through adaptive evolution with complementing sets of subfunctions (Lynch and Conery, 2000). The process of subfunctionalization provides an adaptive advantage by permitting a dynamic model of gene regulation so that each daughter protein performs a specialized function with greater precision than the ancestor. Taken together, these observations support the notion that *Lon1* and *Lon4* are gene paralogs that evolved distinct mechanisms for dual-targeting and subsets of function.

The features of the two paralogs support the existence of an archaic *Lon* gene originated from the first bacterial endosymbiotic event that duplicated leading to the ancestral *Lon1* and *Lon4* genes with twin-presequence structures for dual-organellar targeting (**Figure 4**). This duplication event most likely occurred upon land plant evolution over 400 million years ago. Both ancestral paralogs evolved through adaptive evolution discrete SSD domains and thereby specialized roles in plant development and stress-response. Moreover, the ancestral *Lon4* gene evolved the mitochondrial presequence into an ambiguous one. This evolutionary step was characterized by a single thymine insertion interrupting the reading frame of *Lon4* to prevent the synthesis of the chloroplast transit peptide. This evolutionary process drifts protein dual-targeting from twin presequences to the ambiguous one. Considering that the cases of protein dual-targeting by ambiguous presequence prevail over twin presequences (Carrie et al., 2009), the dual-targeting driven by twin presequences plausibly represents an evolutionary fossil. In line with this model, additional duplication events of the archaic or the ancestral *Lon* genes likely occurred generating the present-day *Lon3* gene that is in close proximity to *Lon4* in a head-to-tail orientation and *Lon2*. While *Lon2* acquired a peroxisome-targeting signal, the mitochondrial presequence deteriorated. This series of duplication events does not exclude other

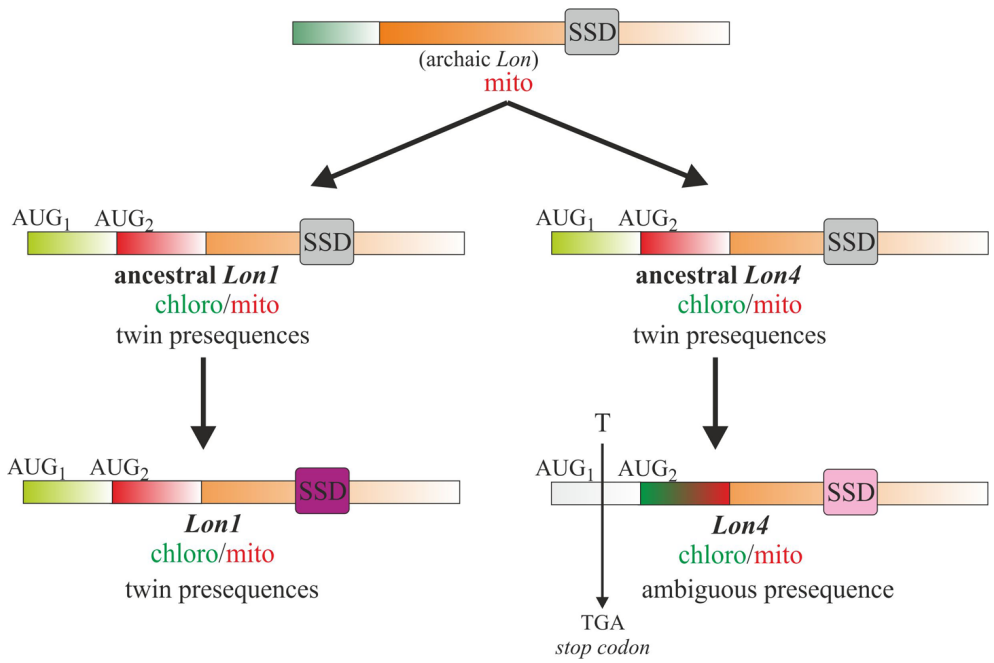


FIGURE 4 | Model for the evolution of *Arabidopsis Lon1* and *Lon4* gene paralogs. The *Lon* gene in plant organelles is of bacterial origin, most likely introduced upon the first endosymbiotic invasion. This archaic *Lon* gene was first duplicated to the ancestral *Lon1* and *Lon4* genes that evolved dual-organellar protein translocation properties by acquiring twin N-terminal presequences. The two ancestral paralogs were further diverged to the present-day *Lon1* and *Lon4* genes with discrete targeting mechanisms and SSD domains. A single base insertion between the two AUGs of the ancestral *Lon4* was the impetus to evolve an ambiguous presequence for dual-organellar targeting. The variation of the SSD domain between *Lon1* and *Lon4* is associated with complementing sets of subfunctions allowing, through adaptive evolution, the maintenance of both functional paralogs.

evolutionary pathways resulting in quadruple Lons in *Arabidopsis* genome. Nevertheless, the features of *Arabidopsis Lon* genes that determine protein isoform translocation in plant organelles together with the structure of the SSD domains argue in favor of the proposed model.

Lon1-DEPENDENT MITOCHONDRIAL BIOGENESIS IS ASSOCIATED WITH OXPHOS CAPACITY

Seed germination and seedling establishment depend on the assembly or biogenesis of mitochondria and the mobilization of storage reserves. In oilseed species like *Arabidopsis*, seedling establishment is supported by soluble sugars that are generated by storage oil mobilization. The mobilization of storage oil to sucrose involves main biochemical pathways compartmentalized into distinct organelles. The triacylglycerols contained in oil bodies are hydrolyzed to free fatty acids (FFAs). The FFAs are imported into the peroxisome entering the reactions of β-oxidation and the glyoxylate cycle. Seedling establishment additionally depends on the mitochondrial tricarboxylic acid (TCA) cycle and on gluconeogenesis that operates in the cytosol.

Molecular genetics revealed that *Lon1* is involved in the biogenesis and maintenance of mitochondrial function to ensure the proper operation of such biochemical network. Transmission electron microscopy studies of *lon1* mutants revealed the presence of mitochondria with abnormal morphology. The *lon1* mitochondria are swollen, having a poorly developed internal membrane system composed of few discernible cristae (Rigas et al., 2009a). These ultrastructural features of *lon1* mitochondria are reminiscent of the pro-mitochondrial morphology of dry seeds, supporting the role of *Arabidopsis* Lon1 protease in mitochondrial biogenesis during germination. Likewise, electron microscopy performed on Lon-deficient mitochondria of yeast (Suzuki et al., 1994) and human (Bota et al., 2005) cells revealed aberrant mitochondrial morphology with electron-dense inclusion bodies in the mitochondrial matrix most likely representing oxidatively modified and aggregated proteins. These severe phenotypes of Lon deficiency across eukaryotes demonstrate the importance of this proteolytic machine to maintain proper mitochondrial function. As the main mitochondrial electron transport chain consists of coupled respiratory chain complexes found in the inner mitochondrial membrane, the OXPHOS capacity of *lon1* mitochondria is expected to be impaired due to the abnormal mitochondrial morphology. Mitochondria isolated from *lon1* mutants showed reduced respiratory capacity when oxidizing succinate and cytochrome *c* via decreased activity of complexes II and IV, respectively (Rigas et al., 2009a). Additionally, in the absence of Lon1 the activities of at least five TCA cycle enzymes were significantly decreased. Analysis of the mitochondrial proteome revealed that complex I was additionally affected in *lon1* mutants (Solheim et al., 2012). Taken together, these results support the notion that Lon protease sustains the activity of major OXPHOS complexes during germination in *Arabidopsis*.

Despite primary metabolism and energy supply through OXPHOS, mitochondria also play a crucial role in cell signaling and communication. In mammalian cells, Lon protease under hypoxic conditions optimizes the activity of the electron transport chain by modulating the equilibrium between cytochrome *c* oxidase (COX; complex IV) subunits COX4-1 and COX4-2 (Fukuda et al., 2007). Under reduced O_2 availability, the hypoxia-inducible transcription factor HIF-1α binds to hypoxia response elements (HRE) of *Lon* gene promoter leading to the induction of *Lon* expression for the degradation of COX4-1. At the same time, HIF-1α up-regulates an alternate isoform-COX4-2, which is assembled into the COX complex replacing COX4-1. In hypoxic cells, the COX4-2 containing complexes are better optimized for transporting electrons and increasing the efficiency of respiration. Additionally, *Lon* expression is enhanced *in vitro* by hypoxia or under endoplasmic reticulum (ER) stress and *in vivo* by brain ischemia (Hori et al., 2002). Under hypoxia or ER stress, a novel signaling pathway from ER to mitochondria disturbs the expression and assembly of COX, whereas the expression of *Lon* protects the mitochondria from unassembled complexes. Intriguingly, Lon was recently reported to be implicated into the cellular homeostasis of the *bZip* transcription factor *ATFS-1* (activating transcription factor associated with stress-1) that is required for the mitochondrial unfolded protein response (UPR^{mt}) cascade (Nargund et al., 2012). During mitochondrial stress, ATFS-1 accumulates in the nucleus and activates the UPR^{mt} as ATFS-1 import in mitochondria is inhibited due to reduced mitochondrial import efficiency by the localized in the inner mitochondrial membrane ATP-binding cassette transporter HAF-1 [half transporter (P-glycoprotein related)]. In healthy cells, the UPR^{mt} is not activated as ATFS-1 is compartmentalized away from the nucleus efficiently imported in mitochondria matrix, where is rapidly degraded by the Lon protease. Consequently, mitochondrial homeostasis is maintained by the conditional-dependent translocation of a transcription activator between the nucleus, where it activates the stress response cascade, and mitochondria where it is removed by Lon protease.

CONCLUSIONS AND FUTURE PERSPECTIVES

Protein misfolding and degradation, especially in mitochondria which are the main source for oxidants in the cell, are processes that determine protein fate causing mitochondrial dysfunction. Mitochondrial dysfunction has now been implicated in aging, cancer and in a variety of age-related degenerative diseases. Lon, in association with other AAA^+ proteases, modulate protein quality control, constitutive metabolism and adaptive responses to cellular or environmental stress. Our understanding of the physiological role of Lon proteases in plants is still evolving, although great advancement is made in non-plant species. However, contrary to the bacterial, yeast and mammalian counterparts, *Arabidopsis* has a genetic pluralism in terms of *Lon* gene copies within the nuclear genome. This could be attributed to the presence of an additional organelle in plants, the chloroplast, and to the fact that plants are sessile organisms exposed to extreme environmental conditions. The *Arabidopsis Lon* genes could be considered paralogs that evolved distinct structural and functional features including gene regulation and expression, subcellular targeting localization and substrate recognition mechanisms. Moreover, *Arabidopsis* has the genetic and molecular tools to contribute toward better understanding of the functional role of Lon as key

controller of proteostasis in organelles and in response to intrinsic or environmental cues. These *Arabidopsis* paralogs could be proven valuable assets to unravel the substrate recognition mechanisms and organelle-to-nucleus communication circuits. This knowledge might be of use to precisely comprehend the role of Lon in non-plant species including humans and thereby to improve life quality and expectancy.

ACKNOWLEDGMENTS

We apologize to colleagues whose relevant work could not be included due to space restrictions. Polydefkis Hatzopoulos acknowledges support by GSRT ARISTEIA/1200 2012-2015 grant. Gerasimos Daras and Dikran Tsitsekian are indebted for funding to IKY Fellowships of Excellence for Postgraduate Studies in Greece-Siemens Program.

REFERENCES

Adam, Z., Adamska, I., Nakabayashi, K., Ostersetzer, O., Haussuhl, K., Manuell, A., et al. (2001). Chloroplast and mitochondrial proteases in *Arabidopsis*. A proposed nomenclature. *Plant Physiol.* 125, 1912–1918. doi: 10.1104/pp.125.4.1912

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399. doi: 10.1146/annurev.arplant.55.031903.141701

Baudisch, B., Langner, U., Garz, I., and Klösgen, R. B. (2014). The exception proves the rule? Dual targeting of nuclear-encoded proteins into endosymbiotic organelles. *New Phytol.* 201, 80–90. doi: 10.1111/nph.12482

Beckman, K. B., and Ames, B. N. (1998). The free radical theory of aging matures. *Physiol. Rev.* 78, 547–581.

Berglund, A. K., Pujol, C., Duchene, A. M., and Glaser, E. (2009a). Defining the determinants for dual targeting of amino acyl-tRNA synthetases to mitochondria and chloroplasts. *J. Mol. Biol.* 393, 803–814. doi: 10.1016/j.jmb.2009.08.072

Berglund, A. K., Spånning, E., Biverståhl, H., Maddalo, G., Tellgren-Roth, C., Mäler, L., et al. (2009b). Dual targeting to mitochondria and chloroplasts: characterization of Thr-tRNA synthetase targeting peptide. *Mol. Plant* 2, 1298–1309. doi: 10.1093/mp/ssp048

Bota, D. A., Ngo, J. K., and Davies, K. J. (2005). Downregulation of the human Lon protease impairs mitochondrial structure and function and causes cell death. *Free Radic. Biol. Med.* 38, 665–677. doi: 10.1016/j.freeradbiomed.2004.11.017

Botos, I., Melnikov, E. E., Cherry, S., Tropea, J. E., Khalatova, A. G., Rasulova, F., et al. (2004). The catalytic domain of *Escherichia coli* Lon protease has a unique fold and a Ser-Lys dyad in the active site. *J. Biol. Chem.* 279, 8140–8148. doi: 10.1074/jbc.M312243200

Carrie, C., Giraud, E., and Whelan, J. (2009). Protein transport in organelles: dual targeting of proteins to mitochondria and chloroplasts. *FEBS J.* 276, 1187–1195. doi: 10.1111/j.1742-4658.2009.06876.x

Carrie, C., and Small, I. (2013). A reevaluation of dual-targeting of proteins to mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1833, 253–259. doi: 10.1016/j.bbamcr.2012.05.029

Cha, S. S., An, Y. J., Lee, C. R., Lee, H. S., Kim, Y. G., Kim, S. J., et al. (2010). Crystal structure of Lon protease: molecular architecture of gated entry to a sequestered degradation chamber. *EMBO J.* 29, 3520–3530. doi: 10.1038/emboj.2010.226

Chung, C. H., and Goldberg, A. L. (1981). The product of the lon (capR) gene in *Escherichia coli* is the ATP-dependent protease, protease La. *Proc. Natl. Acad. Sci. U.S.A.* 78, 4931–4935. doi: 10.1073/pnas.78.8.4931

Coux, O., Tanaka, K., and Goldberg, A. L. (1996). Structure and functions of the 20S and 26S proteasomes. *Annu. Rev. Biochem.* 65, 801–847. doi: 10.1146/annurev.bi.65.070196.004101

Danpure, C. J. (1995). How can the products of a single gene be localized to more than one intracellular compartment? *Trends Cell Biol.* 5, 230–238. doi: 10.1016/S0962-8924(00)89016-9

Daras, G., Rigas, S., Tsitsekian, D., Zur, H., Tuller, T., and Hatzopoulos, P. (2014). Alternative transcription initiation and the AUG context configuration control dual organellar targeting and functional competence of *Arabidopsis* Lon1 protease. *Mol. Plant* doi: 10.1093/mp/ssu030 [Epub ahead of print].

Davies, K. J. (1995). Oxidative stress: the paradox of aerobic life. *Biochem. Soc. Symp.* 61, 1–31.

Dukan, S., Farewell, A., Ballesteros, M., Taddei, F., Radman, M., and Nyström, T. (2000). Protein oxidation in response to increased transcriptional or translational errors. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5746–5749. doi: 10.1073/pnas.100422497

Dyall, S. D., Brown, M. T., and Johnson, P. J. (2004). Ancient invasions: from endosymbionts to organelles. *Science* 304, 253–257. doi: 10.1126/science.1094884

Finkel, T., and Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239–247. doi: 10.1038/35041687

Friguet, B., Bulteau, A. L., and Petropoulos, I. (2008). Mitochondrial protein quality control: implications in ageing. *Biotechnol. J.* 3, 757–764. doi: 10.1002/biot.200800041

Fukuda, R., Zhang, H., Kim, J. W., Shimoda, L., Dang, C. V., and Semenza, G. L. (2007). HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* 129, 111–122. doi: 10.1016/j.cell.2007.01.047

Ge, C., Spånning, E., Glaser, E., and Wieslander, A. (2014). Import determinants of organelle-specific and dual targeting peptides of mitochondria and chloroplasts in *Arabidopsis thaliana*. *Mol. Plant* 7, 121–136. doi: 10.1093/mp/sst148

Gur, E., and Sauer, R. T. (2008). Recognition of misfolded proteins by Lon, a AAA^+ protease. *Genes Dev.* 22, 2267–2277. doi: 10.1101/gad.1670908

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* 11, 298–300. doi: 10.1093/geronj/11.3.298

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu. Rev. Biochem.* 67, 425–479. doi: 10.1146/annurev.biochem.67.1.425

Hori, O., Ichinoda, F., Tamatani, T., Yamaguchi, A., Sato, N., Ozawa, K., et al. (2002). Transmission of cell stress from endoplasmic reticulum to mitochondria: enhanced expression of Lon protease. *J. Cell Biol.* 157, 1151–1160. doi: 10.1083/jcb.200108103

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., et al. (2008). Genevestigator V3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics* 2008:420747. doi: 10.1155/2008/420747

Janska, H., Kwasniak, M., and Szczepanowska, J. (2013). Protein quality control in organelles – AAA/FtsH story. *Biochim. Biophys. Acta* 1833, 381–387. doi: 10.1016/j.bbamcr.2012.03.016

Janska, H., Piechota, J., and Kwasniak, M. (2010). ATP-dependent proteases in biogenesis and maintenance of plant mitochondria. *Biochim. Biophys. Acta* 1797, 1071–1075. doi: 10.1016/j.bbabio.2010.02.027

Lingard, M. J., and Bartel, B. (2009). *Arabidopsis* LON2 is necessary for peroxisomal function and sustained matrix protein import. *Plant Physiol.* 151, 1354–1365. doi: 10.1104/pp.109.142505

Lynch, M., and Conery, J. S. (2000). The evolutionary fate and consequences of duplicate genes. *Science* 290, 1151–1155. doi: 10.1126/science.290.5494.1151

Mackenzie, S. A. (2005). Plant organellar protein targeting: a traffic plan still under construction. *Trends Cell Biol.* 15, 548–554. doi: 10.1016/j.tcb.2005.08.007

Millar, A. H., Whelan, J., and Small, I. (2006). Recent surprises in protein targeting to mitochondria and plastids. *Curr. Opin. Plant Biol.* 9, 610–615. doi: 10.1016/j.pbi.2006.09.002

Møller, I. M., Jensen, P. E., and Hansson, A. (2007). Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* 58, 459–481. doi: 10.1146/annurev.arplant.58.032806.103946

Nargund, A. M., Pellegrino, M. W., Fiorese, C. J., Baker, B. M., and Haynes, C. M. (2012). Mitochondrial import efficiency of ATFS-1 regulates mitochondrial UPR activation. *Science* 337, 587–590. doi: 10.1126/science.1223560

Ondrovicová, G., Liu, T., Singh, K., Tian, B., Li, H., Gakh, O., et al. (2005). Cleavage site selection within a folded substrate by the ATP-dependent lon protease. *J. Biol. Chem.* 280, 25103–25110. doi: 10.1074/jbc.M502796200

Ostersetzer, O., Kato, Y., Adam, Z., and Sakamoto, W. (2007). Multiple intracellular locations of Lon protease in *Arabidopsis*: evidence for the localization of AtLon4 to chloroplasts. *Plant Cell Physiol.* 48, 881–885. doi: 10.1093/pcp/pcm052

Peeters, N., and Small, I. (2001). Dual targeting to mitochondria and chloroplasts. *Biochim. Biophys. Acta* 1541, 54–63. doi: 10.1016/S0167-4889(01)00146-X

Petropoulos, I., and Friguet, B. (2006). Maintenance of proteins and aging: the role of oxidized protein repair. *Free Radic. Res.* 40, 1269–1276. doi: 10.1080/10715760600917144

Rigas, S., Daras, G., Laxa, M., Marathias, N., Fasseas, C., Sweetlove, L. J., et al. (2009a). The role of Lon1 protease in post-germinative growth and maintenance of mitochondrial function in *Arabidopsis thaliana*. *New Phytol.* 181, 588–600. doi: 10.1111/j.1469-8137.2008.02701.x

Rigas, S., Daras, G., Sweetlove, L. J., and Hatzopoulos, P. (2009b). Mitochondria biogenesis via Lon1 selective proteolysis: who dares to live for ever? *Plant Signal. Behav.* 4, 221–224. doi: 10.4161/psb.4.3.7863

Rigas, S., Daras, G., Tsitsekian, D., and Hatzopoulos, P. (2012). The multifaceted role of Lon proteolysis in seedling establishment and maintenance of plant organelle function: living from protein destruction. *Physiol. Plant.* 145, 215–223. doi: 10.1111/j.1399-3054.2011.01537.x

Rotanova, T. V., Botos, I., Melnikov, E. E., Rasulova, F., Gustchina, A., Maurizi, M. R., et al. (2006). Slicing a protease: structural features of the ATP-dependent Lon proteases gleaned from investigations of isolated domains. *Protein Sci.* 15, 1815–1828. doi: 10.1110/ps.052069306

Sakamoto, W. (2006). Protein degradation machineries in plastids. *Annu. Rev. Plant Biol.* 57, 599–621. doi: 10.1146/annurev.arplant.57.032905.105401

Silva-Filho, M. C. (2003). One ticket for multiple destinations: dual targeting of proteins to distinct subcellular locations. *Curr. Opin. Plant Biol.* 6, 589–595. doi: 10.1016/j.pbi.2003.09.008

Sinvany-Villalobo, G., Davydov, O., Ben-Ari, G., Zaltsman, A., Raskind, A., and Adam, Z. (2004). Expression in multigene families. Analysis of chloroplast and mitochondrial proteases. *Plant Physiol.* 135, 1336–1345. doi: 10.1104/pp.104.043299

Smalle, J., and Vierstra, R. D. (2004). The ubiquitin 26S proteasome proteolytic pathway. *Annu. Rev. Plant Biol.* 55, 555–590. doi: 10.1146/annurev.arplant.55.031903.141801

Solheim, C., Li, L., Hatzopoulos, P., and Millar, A. H. (2012). Loss of Lon1 in *Arabidopsis* changes the mitochondrial proteome leading to altered metabolite profiles and growth retardation without an accumulation of oxidative damage. *Plant Physiol.* 160, 1187–1203. doi: 10.1104/pp.112.203711

Stahlberg, H., Kutejová, E., Suda, K., Wolpensinger, B., Lustig, A., Schatz, G., et al. (1999). Mitochondrial Lon of *Saccharomyces cerevisiae* is a ring-shaped protease with seven flexible subunits. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6787–6790. doi: 10.1073/pnas.96.12.6787

Suzuki, C. K., Suda, K., Wang, N., and Schatz, G. (1994). Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. *Science* 264, 273–276. doi: 10.1126/science.8146662

Swamy, K. H., and Goldberg, A. L. (1981). *E. coli* contains eight soluble proteolytic activities, one being ATP dependent. *Nature* 292, 652–654. doi: 10.1038/292652a0

Venkatesh, S., Lee, J., Singh, K., Lee, I., and Suzuki, C. K. (2012). Multitasking in the mitochondrion by the ATP-dependent Lon protease. *Biochim. Biophys. Acta* 1823, 56–66. doi: 10.1016/j.bbamcr.2011.11.003

Vieux, E. F., Wohlever, M. L., Chen, J. Z., Sauer, R. T., and Baker, T. A. (2013). Distinct quaternary structures of the AAA+ Lon protease control substrate degradation. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2002–E2008. doi: 10.1073/pnas.1307066110

Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* 68, 1015–1068. doi: 10.1146/annurev.biochem.68.1.1015

von Janowsky, B., Knapp, K., Major, T., Krayl, M., Guiard, B., and Voos, W. (2005). Structural properties of substrate proteins determine their proteolysis by the mitochondrial AAA+ protease Pim1. *Biol. Chem.* 386, 1307–1317. doi: 10.1515/BC.2005.149

Voos, W. (2013). Chaperone-protease networks in mitochondrial protein homeostasis. *Biochim. Biophys. Acta* 1833, 388–399. doi: 10.1016/j.bbamcr.2012.06.005

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 November 2013; accepted: 26 March 2014; published online: 11 April 2014.

Citation: Rigas S, Daras G, Tsitsekian D, Alatzas A and Hatzopoulos P (2014) Evolution and significance of the Lon gene family in Arabidopsis organelle biogenesis and energy metabolism. Front. Plant Sci. ***5****:145. doi: 10.3389/fpls.2014.00145*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

MINI REVIEW ARTICLE
published: 08 April 2014
doi: 10.3389/fpls.2014.00141

Mitochondrial Band-7 family proteins: scaffolds for respiratory chain assembly?

Bernadette Gehl and Lee J. Sweetlove*

Department of Plant Sciences, University of Oxford, Oxford, UK

Edited by:
Daniel H. Gonzalez, Universidad Nacional del Litoral, Argentina

Reviewed by:
Olivier Van Aken, University of Western Australia, Australia
Hanna Janska, University of Wroclaw, Poland

***Correspondence:**
Lee J. Sweetlove, Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK
e-mail: lee.sweetlove@plants.ox.ac.uk

The band-7 protein family comprises a diverse set of membrane-bound proteins characterized by the presence of a conserved domain. The exact function of this band-7 domain remains elusive, but examples from animal and bacterial stomatin-type proteins demonstrate binding to lipids and the ability to assemble into membrane-bound oligomers that form putative scaffolds. Some members, such as prohibitins (PHB) and human stomatin-like protein 2 (HsSLP2), localize to the mitochondrial inner membrane where they function in cristae formation and hyperfusion. In *Arabidopsis*, the band-7 protein family has diversified and includes plant-specific members. Mitochondrial-localized members include prohibitins (AtPHBs) and two stomatin-like proteins (AtSLP1 and -2). Studies into PHB function in plants have demonstrated an involvement in root meristem proliferation and putative scaffold formation for mAAA proteases, but it remains unknown how these roles are achieved at the molecular level. In this minireview we summarize the current status of band-7 protein functions in *Arabidopsis*, and speculate how the mitochondrial members might recruit specific lipids to form microdomains that could shape the organization and functioning of the respiratory chain.

Keywords: plant mitochondria, respiratory chain, protein complexes, band-7 protein family, cardiolipin, microdomains

INTRODUCTION

Biological membranes are highly organized proteolipid domains and there is increasing evidence of fine-scale organization into microdomains (Whitelegge, 2011; Holthuis and Ungermann, 2013). These comprise localized assemblies of specific proteins and lipids and are important in the spatial and temporal control of membrane protein complex assembly and regulation. Membrane microdomains are formed by specific protein–protein and protein–lipid interactions that take place within and in the vicinity of membranes, often guided by specialized proteins acting as scaffolds. Some of the most important membrane-integrated protein complexes occur in the inner mitochondrial membrane, which houses the respiratory chain. Despite our increasing knowledge about the composition of respiratory complexes, we have a much less detailed understanding about the dynamics, regulation, and assembly of these complexes at the molecular level within the membrane environment. It is also unclear to what extent scaffold proteins and interactions with specific lipids are involved. In this review we highlight the functions of the band-7 family of scaffold proteins in plants and speculate how a conserved mechanism of self assembly into oligomeric ring structures together with lipid interactions could contribute to the creation of microenvironments within the mitochondrial inner membrane.

BAND-7 FAMILY PROTEINS: MOLECULAR SCAFFOLD PROTEINS WITH DIVERSE FUNCTIONS

The band-7 protein family comprises a diverse set of prokaryotic and eukaryotic membrane proteins characterized by the presence of a conserved "band-7" domain in the central regions of the protein sequence. The domain name stems from the first identified member, human stomatin (or erythrocyte band 7.2b protein). The superfamily is also known as "SPFH" according to the initials of its members (stomatin, prohibitin, flotillin, HflC/K; Tavernarakis et al., 1999). Band-7 family proteins generally form oligomers and regulate the assembly and activity of super-molecular protein complexes in various cellular localizations, often linked to membrane microdomains (Browman et al., 2007). Despite its high degree of conservation, the precise function of the band-7 domain remains unknown in most organisms. Over the years numerous examples have emerged demonstrating regulation of various ion channels and transporters by stomatins (Price et al., 2004; Huber et al., 2006; Montel-Hagen et al., 2008). Prohibitins (PHB) function in mitochondrial cristae formation (Merkwirth et al., 2008), flotillins are lipid raft markers involved in trafficking events in animal cells (Glebov et al., 2006), and prokaryotic HflC/K type proteins regulate the activity of membrane-bound proteases (Kihara et al., 1996).

YEAST AND ANIMAL MITOCHONDRIAL BAND-7 PROTEINS ARE INVOLVED IN THE TURNOVER OF MEMBRANE PROTEINS AND AFFECT RESPIRATORY CHAIN ORGANIZATION AND MITOCHONDRIAL MORPHOLOGY

A small subset of eukaryotic band-7 proteins is localized to mitochondria. These include PHBs and mammalian stomatin-like protein 2 (SLP2), both of which have been implicated in regulating the activities of mitochondrial metalloproteases, thereby affecting processes such as cristae formation and respiratory chain assembly

(Steglich et al., 1999; Da Cruz et al., 2008; Merkwirth et al., 2008; Tondera et al., 2009).

The first native band-7 protein complex studied was the yeast PHB complex. This large complex (1.2 MDa) is composed of PHB1 and PHB2 units arranged as an oligomeric ring of 16–20 nm diameter and is associated with the mitochondrial inner membrane facing the intermembrane space (Nijtmans et al., 2000; Tatsuta et al., 2005). A second PHB complex was discovered in yeast that additionally contains a matrix-exposed AAA protease (Steglich et al., 1999). AAA-type proteases belong to the metalloprotease family and contain an additional ATP-hydrolysing domain. They are thought to function in membrane protein quality control (Langer, 2000). PHB in yeast was found to negatively regulate matrix-AAA (mAAA) activity, thereby influencing turnover rates of mitochondrial-encoded respiratory chain subunits (Steglich et al., 1999). Transient associations of PHBs with cytochrome c oxidase subunits were demonstrated in yeast, implying that the PHB complex has chaperone functions in complex IV assembly (Nijtmans et al., 2000). Mammalian PHBs were also shown to interact with complex IV subunits, as well as with subunits of complex I (NADH dehydrogenase; Bourges et al., 2004; Schleicher et al., 2008; Strub et al., 2010). Knockdown of PHBs in mouse cells also affects mitochondrial morphology because of altered proteolytic processing of the inner membrane GTPase OPA1 (optical atrophy 1) by metalloproteases (Merkwirth et al., 2008).

Detailed insight into band-7 protein complex formation was gained from a cystallography study of the conserved stomatin domain from mouse (Brand et al., 2012). The basic unit in the crystal was found to be a banana-shaped dimer capable of forming a ring-shaped structure required for stomatin function in ion channel modulation. A ring structure was also observed by single particle analysis of a purified stomatin complex from cyanobacteria (Boehm et al., 2009), making it likely that other band-7 family proteins might also adapt this shape as assembled complexes. A related stomatin-like protein from human and rodents, SLP2 (stomatin-like protein 2), also forms a large (1.8 MDa) complex in mitochondria (Reifschneider et al., 2006). Notably, no ortholog of mammalian SLP2 is present in the yeast *Saccharomyces cerevisiae*. Mammalian SLP2 is peripherally associated with the mitochondrial inner membrane on the side of the intermembrane space (Hajek et al., 2007; Da Cruz et al., 2008), where it forms a complex with mitofusin-2 (Mfn-2), a GTPase of the outer membrane mediating mitochondrial fusion. Mammalian SLP2 also interacts with PHBs in a smaller 250 kDa complex (Da Cruz et al., 2008). Knockdown of SLP2 in HeLa cells caused increased proteolysis of PHBs and respiratory chain subunits from complexes I and IV by metalloproteases (Da Cruz et al., 2008), as well as a reduction in membrane potential, but had no effect on mitochondrial morphology (Hajek et al., 2007). By contrast, SLP2 knockdown in mice was reported to be embryo lethal (Christie et al., 2012), the same as knockouts of PHBs in mice (Merkwirth et al., 2008). A T-cell specific knockdown of SLP2 caused a reduction of complex I (NADH dehydrogenase) subunits and reduced complex I activity (Christie et al., 2012). Interestingly, human recombinant SLP2 was demonstrated to bind preferentially to cardiolipin (CL) in an *in vitro* pull-down assay that utilized liposomes with varying phospholipid composition (Christie et al., 2011), although the specificity of this interaction is debatable because the assay lacked additional control proteins. Additionally, yeast genetic studies have revealed that enzymes involved in CL and phosphatidylethanolamine (PE) synthesis pathways are essential for survival in *phb* knockout strains, underlining a functional link between lipid synthesis and PHBs (Birner et al., 2003; Osman et al., 2009).

PLANT BAND-7 PROTEINS

The band-7 protein family is more diverse in higher plants than it is in yeast and animals, and includes a plant-specific group of proteins classified as HIR (hypersensitive response induced), as well as a larger number of PHBs and two stomatin-like proteins (Nadimpalli et al., 2000). This large diversity in higher plants is down to gene duplications and may be linked to the requirement to adapt to environmental stress conditions (Van Aken et al., 2010).

The *Arabidopsis* genome encodes 17 genes that contain the band-7 domain (InterPro IPR001107). The gene products fall into five distinct classes based on sequence homologies with animal and yeast orthologs: seven genes belong to the PHBs, of which five are expressed, two are stomatin-like (AtSLP1 and -2), four belong to the HIR proteins (HIRs1-4), (Qi et al., 2011), three resemble flotillins, and one protein has similarities to erlin proteins from animals (Browman et al., 2007; **Figure 1A**). *Arabidopsis* band-7 proteins are found in various subcellular membrane localizations according to the SUBA database (Heazlewood et al., 2007). Most of these locations are based on mass spectrometry data from various proteomics studies, but for PHBs, SLPs and HIRs, additional *in vivo* data from fluorescent tagging experiments are available (Marmagne et al., 2004; Van Aken et al., 2007, 2009; Qi et al., 2011; Gehl et al., 2014). According to these data, *Arabidopsis* PHBs are primarily localized to mitochondria (Van Aken et al., 2007), although localization to the cytoplasm and the nucleus was suggested in a separate study (Christians and Larsen, 2007). However, this result is controversial because cytoplasmic localization was not confirmed by any additional cytosolic markers. AtSLPs are found exclusively in mitochondria (Gehl et al., 2014), whereas HIRs are localized to the plasma membrane (Qi et al., 2011). Although nothing is known about the functions of the three flotillin-like proteins or the erlin-like protein, forward and reverse genetics studies have started to elucidate the molecular roles of HIRs, PHBs, and SLPs.

PLANT MITOCHONDRIAL BAND-7 MEMBERS

In plants, much less is known about the specific functions of SLPs and PHBs in mitochondria and how the complexes they form relate to each other. Research into plant PHBs and SLPs so far suggests that the two complexes are physically distinct and are not functionally redundant, yet they seem to share several functions with animal and yeast orthologs.

The best-studied plant band-7 family proteins are the PHBs. The seven *Arabidopsis* PHBs fall into two classes (type I or II) according to similarities with yeast and animal PHB1 and PHB2 (Van Aken et al., 2007, 2010). Reverse genetics has revealed diverse functions for plant PHBs (Van Aken et al., 2007). Knockout of *AtPHB3* resulted in retarded growth of roots and shoots which was linked to reduced cell division and expansion in apical meristems, as well as alterations in mitochondrial morphology, indicative of a

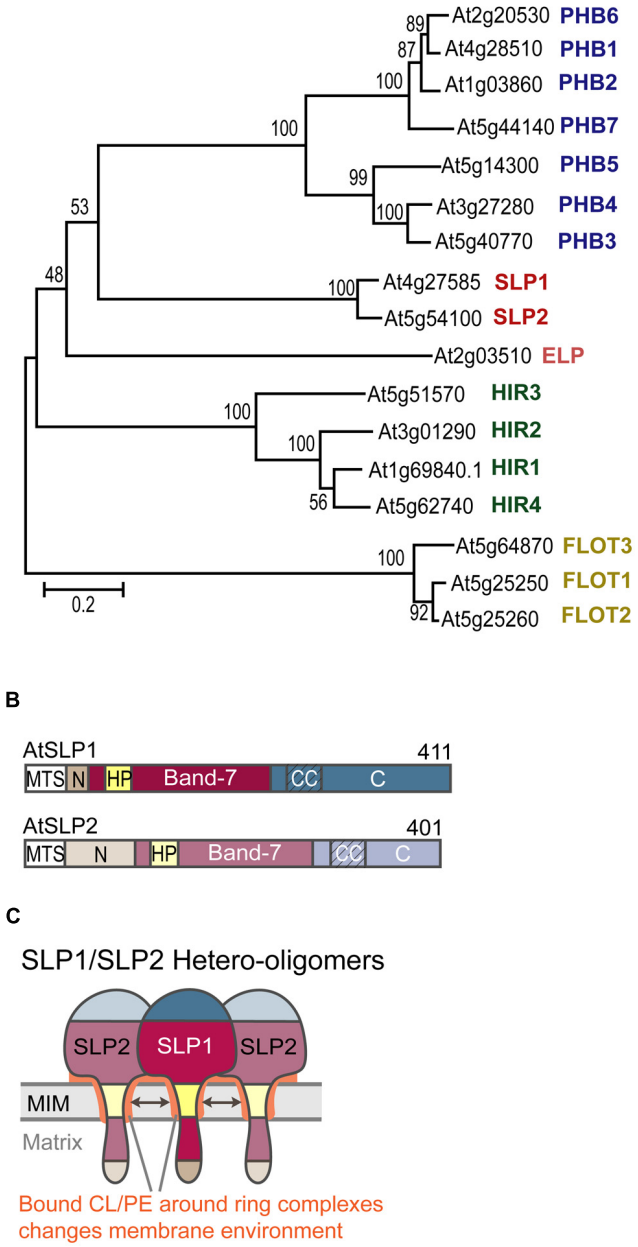


FIGURE 1 | *Arabidopsis thaliana* band-7 protein family members. **(A)** Phylogenetic bootstrap tree of the *Arabidopsis* band-7 protein family. PHBs (prohibitins) in blue, SLPs (stomatin-like proteins) in red, ELP (erlin-like protein) in pink, HIRs (hypersensitive response induced proteins) in green, FLOTs (flotillin-like proteins) in yellow. This tree was created by the neighborhood-joining method. Reprinted with permission by ASPB. **(B)** Schematic of AtSLP domains. MTS (mitochondrial targeting sequence), N (N-terminal domain), HP (hydrophobic patch), Band-7 (band-7 domain), CC (predicted coiled-coil regions), C (C-terminal domain). **(C)** Schematic of SLP1-SLP2 hetero-oligomers embedded within the mitochondrial inner membrane. Coloring corresponds to the scheme in **B**. Cardiolipin-binding to SLP oligomers is indicated by the orange color around the hydrophobic patches and between the inner membrane and the band-7 domains.

lack of cristae. By contrast, knockout of *AtPHB4* did not result in any obvious growth phenotypes, but a double *phb3/4* mutant was lethal. From this study it was concluded that AtPHBs are important to sustain increased metabolic demands related to cell division in meristems supporting differentiation in apical tissues.

A mutant in the *phb3* gene was also identified as *eer3-1*, a loss-of function conditional point mutation allele leading to an extreme constitutive ethylene response in etiolated seedlings (Christians and Larsen, 2007). An independent loss-of function allele, *phb3-3* was identified in a mutagenesis screen for deficiencies in hydrogen peroxide-induced nitric oxide (NO) accumulation (Wang et al., 2010). This point mutation was mapped to a glycine to aspartate change inside the conserved band-7 domain, but it remains unknown what effect this mutation has on PHB complex formation. Both *phb3-3* and an independent T-DNA knockout allele showed NO-related phenotypes and increased resistance to high salinity, pointing toward yet unknown functions of PHB3 in NO homeostasis, possibly via the respiratory chain.

Tandem-affinity purification of tagged PHB3 revealed that it interacts with all other expressed PHBs (1, 2, 3, 4, and 6), as well as some enzymes and proteins of unknown function (Van Aken et al., 2007). Some class II PHBs have also been found associated with subcomplexes of complex I, possibly as contaminants in mass spectrometry studies (Klodmann et al., 2010). The native PHB complex was later characterized by two-dimensional blue native and SDS-PAGE (Piechota et al., 2010). *Arabidopsis* PHBs form a hetero-oligomeric complex of 1 MDa, but they also participate in a 2 MDa complex together with the mAAA proteases FtsH3 and FtsH10. No complex containing a FtsH protease without PHBs could be identified, suggesting that the PHB complex acts as a scaffold to stabilize the FtsH oligomeric complexes. Other studies of PHBs in Petunia flowers and in tobacco leaves indicated links to cellular senescence, reactive oxygen species production and mitochondrial morphology (Chen et al., 2005; Ahn et al., 2006). Based on these findings, PHBs were suggested to act as universal scaffolds in the mitochondrial inner membrane, likely associated with lipid microdomains that affect a variety of mitochondrial processes (Van Aken et al., 2010).

The two other mitochondrial band-7 family members, stomatin-like proteins (SLPs) were established as mitochondrial proteins in various proteomics studies (Millar et al., 2001; Heazlewood et al., 2004; Dunkley et al., 2006). SLP1 has also been identified in detergent-resistant membrane fractions thought to be derived from the plasma membrane (Borner et al., 2005). The AtSLP1 protein was also shown to be capable of binding to Zn^{2+} (Tan et al., 2010) and is threonine-phosphorylated within its hydrophilic C-terminus (Ito et al., 2009). Both AtSLP transcripts were upregulated in the *phb3* mutant, and AtSLP2 has been identified as a stress-responsive gene in a number of microarray experiments (Van Aken et al., 2009).

Our own work has dealt with functionally characterizing AtSLP1 (At4g27585) and AtSLP2 (At5g54100). Both AtSLPs have one conserved band-7 domain, as well as one hydrophobic stretch located within this conserved domain (**Figure 1B**). We have identified a class II (-3R) mitochondrial targeting sequence (MTC) in both SLP sequences, which, upon cleavage, results in a short N-terminal sequence that is probably located in the mitochondrial

matrix. The bulk of the proteins likely reside in the intermembrane space. Mature SLP1 is slightly longer (368 amino acids) than SLP2 (360 amino acids), and it possesses a unique hydrophilic C-terminus not present in SLP2 that harbors the phosphorylation site (Ito et al., 2009).

We have localized SLP1 to a large protein complex (3 MDa) in the mitochondrial inner membrane where it most likely interacts with AtSLP2, possibly organized in a ring shape. Sequence homologies suggest that AtSLPs are the plant orthologs of animal SLP2. Knockout of AtSLP1, but not AtSLP2 affects the abundance of complex I and related supercomplexes, but not other respiratory complexes (Gehl et al., 2014). We interpret this specific effect on complex I either as a consequence of deficient complex I assembly, or an increased complex I turnover that is mediated by proteases (likely of the AAA-type) in the inner membrane.

We also hypothesize that complex I deficiency in the absence of SLP1 is related to changes that occur in the local membrane environment. The sequence homology of AtSLPs with human SLP2 may suggest that *Arabidopsis* SLPs can also bind to specific mitochondrial inner membrane lipids such as CL and PE. Lipid-binding could occur at residues located within the hydrophobic SLP membrane anchor, and may help stabilize the membrane anchorage of the SLP oligomers. Additionally, residues found within the band-7 domain could bind to lipids, possibly to keep the SLP complex in close proximity to the inner membrane (**Figure 1C**). This scenario resembles binding of cholesterol by the stomatin proteins podocin and *C. elegans* Mec-2 to the N-terminal hydrophobic domains and the band-7 domain (Huber et al., 2006). Cholesterol binding was mapped to, a conserved proline residue located just upstream of the band-7 domain that proved to be crucial for ion channel regulation by Mec-2.

In a similar manner, the plant PHB complex is likely to bind lipids (CL and/or PE) and assembles into a ring-shaped structure, with the C-termini facing the intermembrane space. Protein interaction data derived from PHBs so far suggest no physical associations between SLPs and PHBs in *Arabidopsis* (Van Aken et al., 2007; Piechota et al., 2010), and it is not clear how the two complexes relate to each other functionally. Because of their different gene expression patterns in *Arabidopsis* based on microarray data and on promoter-GUS fusion plants (Van Aken et al., 2007; Gehl et al., 2014) and the differing growth phenotypes of knockout mutants, we conclude that PHBs and SLPs are not functionally redundant. For example, class I PHBs are highly expressed in root meristem tissue and single *phb3* knockout mutants have a dwarfed growth phenotype with short roots, whereas class I double *phb* mutants are embryo lethal. By contrast, *SLP* genes are not highly expressed in root meristems, and *slp1/2* double knockout plants are viable and do not show abnormal growth morphology (Gehl et al., 2014). Currently nothing is known about respiratory chain function and the abundance of supercomplexes in *phb* mutants.

DO PLANT PROHIBITINS AND STOMATIN-LIKE PROTEINS COOPERATE IN RESPIRATORY CHAIN ASSEMBLY?

We suggest that a possible solution to the apparent functional specificity of SLPs and PHBs despite overlapping properties of the

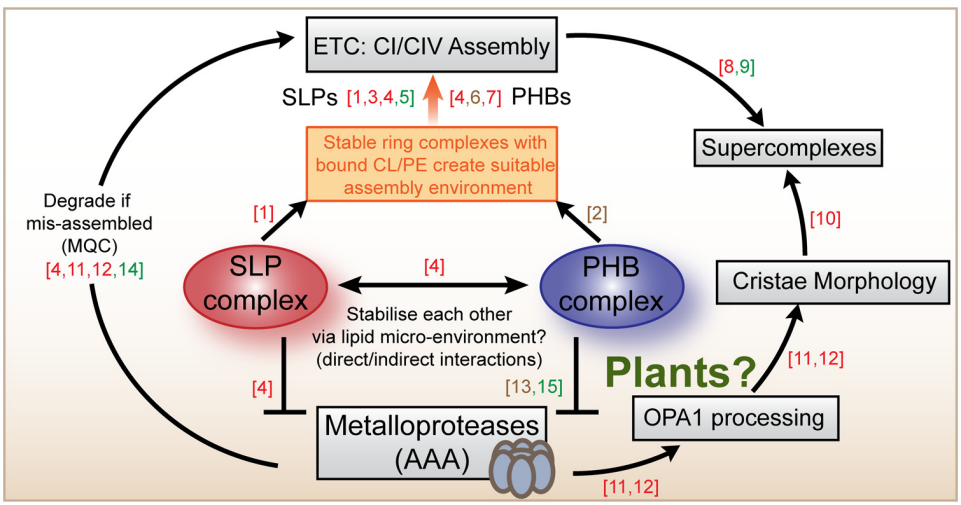


FIGURE 2 | Summary scheme of SLP and PHB functions across species. The SLP and the PHB complexes are localized to the mitochondrial inner membrane where they probably bind to cardiolipin and/or phosphatidylethanolamine and participate in the assembly of complexes I and complex IV. In animal cells, SLPs and PHBs have been located to the same complexes (250 kDa) and have been demonstrated to interact. Both are implied to inhibit the activities of chaperone-like proteases of the AAA type which are also embedded in the inner membrane. PHBs from plants and from yeast form a complex with mAAA proteases. AAA proteases are known to participate in mitochondrial quality control (MQC) mechanisms that ensure appropriate electron transport chain (ETC) assembly and functioning. Mammalian AAA proteases are also known to participate in the proteolytic processing of OPA-1 that determines cristae morphology in animal cells. Mitochondrial morphology itself was recently demonstrated to influence the assembly status of supercomplexes in mouse cells. Literature references are as follows: (1) Christie et al. (2011), (2) Osman et al. (2009), (3) Christie et al. (2012), (4) Da Cruz et al. (2008), (5) Gehl et al. (2014), (6) Nijtmans et al. (2000), (7) Bourges et al. (2004), (8) Acín-Pérez et al. (2008), (9) Eubel et al. (2003), (10) Cogliati et al. (2013), (11) Merkwirth et al. (2008), (12) Tondera et al. (2009), (13) Steglich et al. (1999), (14) Kolodziejczak et al. (2007), (15) Piechota et al. (2010). Coloring indicates which model system was studied: red (animals), brown (yeast), green (plants).

proteins could be that the two complexes cooperatively connect mitochondrial quality control (MQC) by proteases with respiratory chain assembly (**Figure 2**). Evidence from our work and from mice and HeLa cells points toward a specific function for the SLP complex in the assembly and/or turnover of complex I (Da Cruz et al., 2008; Christie et al., 2012; Gehl et al., 2014). Studies into yeast and mouse PHBs suggest that the PHB complex likely functions in the assembly or turnover of complex IV (Nijtmans et al., 2000; Strub et al., 2010), although other studies imply that it is also related to complex I (Bourges et al., 2004; Acín-Pérez et al., 2008). Cardiolipin-binding by SLP and PHB ring complexes could directly affect the formation of functional supercomplexes which are known to be dependent on the incorporation of multiple CL molecules internally and at the interphases between complexes (Bazan et al., 2013; Pineau et al., 2013). The SLP and PHB complexes likely change their local membrane environment by specifically sequestering CL and/or PE, possibly by forming a localized network of rings that helps respiratory chain assembly at specific sites. This could take place in a coordinated fashion between both complexes, such that both rings affect the membrane environment and each other by altering the tension, charge distribution and possibly even curvature of the inner membrane. Membrane-bound AAA-proteases likely contribute to turnover rates of respiratory chain components as part of MQC. In animal cells metalloproteases also help processing OPA1, thereby determining cristae ultrastructure and mitochondrial morphology (Merkwirth et al., 2008; Tondera et al., 2009). Recently, cristae morphology governed by OPA1 processing has also been linked to the assembly status of supercomplexes, although a direct connection between OPA1 and supercomplexes is so far missing (Cogliati et al., 2013). In plants, no OPA1-like protein exists and the mechanism determining cristae morphology remains unknown.

In summary, we place SLP and PHB complexes at the heart of a mechanism that incorporates AAA proteases and phospholipids, thereby affecting respiratory chain function at the point of assembly and turnover. We hypothesize that CL/PE binding by SLPs/PHBs creates defined areas of respiratory chain assembly and quality control. Currently this theory is speculative, but could be addressed experimentally. Firstly, a detailed inventory about defects in the respiratory chain in the respective *Arabidopsis* mutant backgrounds is needed, in combination with an analysis of the lipid-binding properties of AtPHBs and AtSLPs. These results, together with complementary structure-function approaches and high resolution imaging techniques will give new insights into the extent of cooperation between these protein complexes and will clarify where their specificities lie. This information will not only advance our understanding of inner membrane compartmentation, but also help to elucidate band-7 protein function throughout the kingdoms.

REFERENCES

Acín-Pérez, R., Fernandez-Silva, P., Peleato, M. L., Perez-Martos, A., and Enriquez, J. A. (2008). Respiratory active mitochondrial supercomplexes. *Mol. Cell* 32, 529–539. doi: 10.1016/j.molcel.2008.10.021

Ahn, C. S., Lee, J. H., Hwang, A. R., Kim, W. T., and Pai, H. S. (2006). Prohibitin is involved in mitochondrial biogenesis in plants. *Plant J.* 46, 658–667. doi: 10.1111/j.1365-313X.2006.02726.x

Bazan, S., Mileykovskaya, E., Mallampalli, V., Heacock, P., Sparagna, G. C., and Dowhan, W. (2013). Cardiolipin-dependent reconstitution of respiratory supercomplexes from purified *Saccharomyces cerevisiae* complexes III and IV. *J. Biol. Chem.* 288, 401–411. doi: 10.1074/jbc.M112.425876

Birner, R., Nebauer, R., Schneiter, R., and Daum, G. (2003). Synthetic lethal interaction of the mitochondrial phosphatidylethanolamine biosynthetic machinery with the prohibitin complex of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 14, 370–383. doi: 10.1091/mbc.E02-05-0263

Boehm, M., Nield, J., Zhang, P. P., Aro, E. M., Komenda, J., and Nixon, P. J. (2009). Structural and mutational analysis of band 7 proteins in the cyanobacterium *Synechocystis* sp strain PCC 6803. *J. Bacteriol.* 191, 6425–6435. doi: 10.1128/JB.00644-09

Borner, G. H. H., Sherrier, D. J., Weimar, T., Michaelson, L. V., Hawkins, N. D., MacAskill, A., et al. (2005). Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. *Plant Physiol.* 137, 104–116. doi: 10.1104/pp.104.053041

Bourges, I., Ramus, C., de Camaret, B. M., Beugnot, R., Remacle, C., Cardol, P., et al. (2004). Structural organization of mitochondrial human complex I: role of the ND4 and ND5 mitochondria-encoded subunits and interaction with prohibitin. *Biochem. J.* 383, 491–499. doi: 10.1042/BJ20040256

Brand, J., Smith, E. S., Schwefel, D., Lapatsina, L., Poole, K., Omerbasic, D., et al. (2012). A stomatin dimer modulates the activity of acid-sensing ion channels. *EMBO J.* 31, 3635–3646. doi: 10.1038/emboj.2012.203

Browman, D. T., Hoegg, M. B., and Robbins, S. M. (2007). The SPFH domain-containing proteins: more than lipid raft markers. *Trends Cell Biol.* 17, 394–402. doi: 10.1016/j.tcb.2007.06.005

Chen, J. C., Jiang, C. Z., and Reid, M. S. (2005). Silencing a prohibitin alters plant development and senescence. *Plant J.* 44, 16–24. doi: 10.1111/j.1365-313X.2005.02505.x

Christians, M. J., and Larsen, P. B. (2007). Mutational loss of the prohibitin AtPHB3 results in an extreme constitutive ethylene response phenotype coupled with partial loss of ethylene-inducible gene expression in *Arabidopsis* seedlings. *J. Exp. Bot.* 58, 2237–2248. doi: 10.1093/jxb/erm086

Christie, D. A., Lemke, C. D., Elias, I. M., Chau, L. A., Kirchhof, M. G., Li, B., et al. (2011). Stomatin-like protein 2 binds cardiolipin and regulates mitochondrial biogenesis and function. *Mol. Cell. Biol.* 31, 3845–3856. doi: 10.1128/MCB.05393-11

Christie, D. A., Mitsopoulos, P., Blagih, J., Dunn, S. D., St-Pierre, J., Jones, R. G., et al. (2012). Stomatin-like protein 2 deficiency in T cells is associated with altered mitochondrial respiration and defective CD4(+) T cell responses. *J. Immunol.* 189, 4349–4360. doi: 10.4049/jimmunol.1103829

Cogliati, S., Frezza, C., Soriano, M. E., Varanita, T., Quintana-Cabrera, R., Corrado, M., et al. (2013). Mitochondrial cristae shape determines respiratory chain supercomplexes assembly and respiratory efficiency. *Cell* 155, 160–171. doi: 10.1016/j.cell.2013.08.032

Da Cruz, S., Parone, P. A., Gonzalo, P., Bienvenut, W. V., Tondera, D., Jourdain, A., et al. (2008). SLP-2 interacts with prohibitins in the mitochondrial inner membrane and contributes to their stability. *Biochim. Biophys. Acta* 1783, 904–911. doi: 10.1016/j.bbamcr.2008.02.006

Dunkley, T. P. J., Hester, S., Shadforth, I. P., Runions, J., Weimar, T., Hanton, S. L., et al. (2006). Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6518–6523. doi: 10.1073/pnas.0506958103

Eubel, H., Jansch, L., and Braun, H. P. (2003). New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. *Plant Physiol.* 133, 274–286. doi: 10.1104/pp.103.024620

Gehl, B., Lee, C. P., Bota, P., Blatt, M. R., and Sweetlove, L. J. (2014). An *Arabidopsis* stomatin-like protein affects mitochondrial respiratory supercomplex organisation. *Plant Physiol.* 164, 1389–1400. doi: 10.1104/pp.113.230383

Glebov, O. O., Bright, N. A., and Nichols, B. J. (2006). Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat. Cell Biol.* 8, 46–54. doi: 10.1038/ncb1342

Hajek, P., Chomyn, A., and Attardi, G. (2007). Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2. *J. Biol. Chem.* 282, 5670–5681. doi: 10.1074/jbc.M608168200

Heazlewood, J. L., Tonti-Filippini, J. S., Gout, A. M., Day, D. A., Whelan, J., and Millar, A. H. (2004). Experimental analysis of the *Arabidopsis* mitochondrial proteome highlights signaling and regulatory components, provides assessment of targeting prediction programs, and indicates plant-specific mitochondrial proteins. *Plant Cell* 16, 241–256. doi: 10.1105/tpc.016055

Heazlewood, J. L., Verboom, R. E., Tonti-Filippini, J., Small, I., and Millar, A. H. (2007). SUBA: the *Arabidopsis* subcellular database. *Nucleic Acids Res.* 35, D213–D218. doi: 10.1093/nar/gkl863

Holthuis, J. C. M., and Ungermann, C. (2013). Cellular microcompartments constitute general suborganellar functional units in cells. *Biol. Chem.* 394, 151–161. doi: 10.1515/hsz-2012-0265

Huber, T. B., Schermer, B., Muller, R. U., Hohne, M., Bartram, M., Calixto, A., et al. (2006). Podocin and MEC-2 bind cholesterol to regulate the activity of associated ion channels. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17079–17086. doi: 10.1073/pnas.0607465103

Ito, J., Taylor, N. L., Castleden, I., Weckwerth, W., Millar, A. H., and Heazlewood, J. L. (2009). A survey of the *Arabidopsis thaliana* mitochondrial phosphoproteome. *Proteomics* 9, 4229–4240. doi: 10.1002/pmic.200900064

Kihara, A., Akiyama, Y., and Ito, K. (1996). A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO J.* 15, 6122–6131.

Klodmann, J., Sunderhaus, S., Nimtz, M., Jansch, L., and Braun, H. P. (2010). Internal Architecture of Mitochondrial Complex I from *Arabidopsis thaliana*. *Plant Cell* 22, 797–810. doi: 10.1105/tpc.109.073726

Kolodziejczak, M., Gibala, M., Urantowka, A., and Janska, H. (2007). The significance of *Arabidopsis* AAA proteases for activity and assembly/stability of mitochondrial OXPHOS complexes. *Physiol. Plant.* 129, 135–142. doi: 10.1111/j.1399-3054.2006.00835.x

Langer, T. (2000). AAA proteases: cellular machines for degrading membrane proteins. *Trends Biochem. Sci.* 25, 247–251. doi: 10.1016/S0968-0004(99)01541-8

Marmagne, A., Rouet, M. A., Ferro, M., Rolland, N., Alcon, C., Joyard, J., et al. (2004). Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. *Mol. Cell. Proteomics* 3, 675–691. doi: 10.1074/mcp.M400001-MCP200

Merkwirth, C., Dargazanli, S., Tatsuta, T., Geimer, S., Lower, B., Wunderlich, F. T., et al. (2008). Prohibitins control cell proliferation and apoptosis by regulating OPA1-dependent cristae morphogenesis in mitochondria. *Genes Dev.* 22, 476–488. doi: 10.1101/gad.460708

Millar, A. H., Sweetlove, L. J., Giege, P., and Leaver, C. J. (2001). Analysis of the *Arabidopsis* mitochondrial proteome. *Plant Physiol.* 127, 1711–1727. doi: 10.1104/pp.010387

Montel-Hagen, A., Kinet, S., Manel, N., Mongellaz, C., Prohaska, R., Battini, J. L., et al. (2008). Erythrocyte glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell* 132, 1039–1048. doi: 10.1016/j.cell.2008.01.042

Nadimpalli, R., Yalpani, N., Johal, G. S., and Simmons, C. R. (2000). Prohibitins, stomatins, and plant disease response genes compose a protein superfamily that controls cell proliferation, ion channel regulation, and death. *J. Biol. Chem.* 275, 29579–29586. doi: 10.1074/jbc.M002339200

Nijtmans, L. G. J., de Jong, L., Sanz, M. A., Coates, P. J., Berden, J. A., Back, J. W., et al. (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. *EMBO J.* 19, 2444–2451. doi: 10.1093/emboj/19.11.2444

Osman, C., Haag, M., Potting, C., Rodenfels, J., Dip, P. V., Wieland, F. T., et al. (2009). The genetic interactome of prohibitins: coordinated control of cardiolipin and phosphatidylethanolamine by conserved regulators in mitochondria. *J. Cell Biol.* 184, 583–596. doi: 10.1083/jcb.200810189

Piechota, J., Kolodziejczak, M., Juszczak, I., Sakamoto, W., and Janska, H. (2010). Identification and characterization of high molecular weight complexes formed by matrix AAA proteases and prohibitins in mitochondria of *Arabidopsis thaliana*. *J. Biol. Chem.* 285, 12512–12521. doi: 10.1074/jbc.M109.063644

Pineau, B., Bourge, M., Marion, J., Mauve, C., Gilard, F., Maneta-Peyret, L., et al. (2013). The importance of cardiolipin synthase for mitochondrial ultrastructure, respiratory function, plant development, and stress responses in *Arabidopsis*. *Plant Cell* 25, 4195–4208. doi: 10.1105/tpc.113.118018

Price, M. P., Thompson, R. J., Eshcol, J. O., Wemmie, J. A., and Benson, C. J. (2004). Stomatin modulates gating of acid-sensing ion channels. *J. Biol. Chem.* 279, 53886–53891. doi: 10.1074/jbc.M407708200

Qi, Y. P., Tsuda, K., Nguyen, L. V., Wang, X., Lin, J. S., Murphy, A. S., et al. (2011). Physical association of *Arabidopsis* hypersensitive induced reaction proteins (HIRs) with the immune receptor RPS2. *J. Biol. Chem.* 286, 31297–31307. doi: 10.1074/jbc.M110.211615

Reifschneider, N. H., Goto, S., Nakamoto, H., Takahashi, R., Sugawa, M., Dencher, N. A., et al. (2006). Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D blue-native/SDS-PAGE. *J. Proteome Res.* 5, 1117–1132. doi: 10.1021/pr0504440

Schleicher, M., Shepherd, B. R., Suarez, Y., Fernandez-Hernando, C., Yu, J., Pan, Y., et al. (2008). Prohibitin-1 maintains the angiogenic capacity of endothelial cells by regulating mitochondrial function and senescence. *J. Cell Biol.* 180, 101–112. doi: 10.1083/jcb.200706072

Steglich, G., Neupert, W., and Langer, T. (1999). Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. *Mol.Cell. Biol.* 19, 3435–3442.

Strub, G. M., Paillard, M., Liang, J., Gomez, L., Allegood, J. C., Hait, N. C., et al. (2010). Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. *FASEB J.* 25, 600–612. doi: 10.1096/fj.10-167502

Tan, Y. F., O'Toole, N., Taylor, N. L., and Millar, A. H. (2010). Divalent metal ions in plant mitochondria and their role in interactions with proteins and oxidative stress-induced damage to respiratory function. *Plant Physiol.* 152, 747–761. doi: 10.1104/pp.109.147942

Tatsuta, T., Model, K., and Langer, T. (2005). Formation of membrane-bound ring complexes by prohibitins in mitochondria. *Mol. Biol. Cell* 16, 248–259. doi: 10.1091/mbc.E04-09-0807

Tavernarakis, N., Driscoll, M., and Kyrpides, N. C. (1999). The SPFH domain: implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins. *Trends Biochem. Sci.* 24, 425–427. doi: 10.1016/S0968-0004(99)01467-X

Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., et al. (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. *EMBO J.* 28, 1589–1600. doi: 10.1038/emboj.2009.89

Van Aken, O., Pecenkova, T., van de Cotte, B., De Rycke, R., Eeckhout, D., Fromm, H., et al. (2007). Mitochondrial type-I prohibitins of *Arabidopsis thaliana* are required for supporting proficient meristem development. *Plant J.* 52, 850–864. doi: 10.1111/j.1365-313X.2007.03276.x

Van Aken, O., Whelan, J., and Van Breusegem, F. (2010). Prohibitins: mitochondrial partners in development and stress response. *Trends Plant Sci.* 15, 275–282. doi: 10.1016/j.tplants.2010.02.002

Van Aken, O., Zhang, B. T., Carrie, C., Uggalla, V., Paynter, E., Giraud, E., et al. (2009). Defining the mitochondrial stress response in *Arabidopsis thaliana*. *Mol. Plant* 2, 1310–1324. doi: 10.1093/mp/ssp053

Wang, Y., Ries, A., Wu, K. T., Yang, A., and Crawford, N. M. (2010). The *Arabidopsis* prohibitin gene PHB3 functions in nitric oxide-mediated responses and in hydrogen peroxide-induced nitric oxide accumulation. *Plant Cell* 22, 249–259. doi: 10.1105/tpc.109.072066

Whitelegge, J. (2011). Up close with membrane lipid-protein complexes. *Science* 334, 320–321. doi: 10.1126/science.1214084

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 31 January 2014; accepted: 24 March 2014; published online: 08 April 2014. Citation: Gehl B and Sweetlove LJ (2014) Mitochondrial Band-7 family proteins: scaffolds for respiratory chain assembly? Front. Plant Sci. **5**:141. doi: 10.3389/fpls.2014.00141*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.

frontiers in
PLANT SCIENCE

ORIGINAL RESEARCH ARTICLE
published: 25 March 2014
doi: 10.3389/fpls.2014.00087

Divergent functions of the Arabidopsis mitochondrial SCO proteins: HCC1 is essential for COX activity while HCC2 is involved in the UV-B stress response

Iris Steinebrunner[1]*, Uta Gey[1], Manuela Andres[1], Lucila Garcia[2] and Daniel H. Gonzalez[2]

[1] *Department of Biology, Technische Universität Dresden, Dresden, Germany*
[2] *Instituto de Agrobiotecnología del Litoral (CONICET-UNL), Universidad Nacional del Litoral, Santa Fe, Argentina*

Edited by:
Philippe Giegé, Centre National de la Recherche Scientifique, France

Reviewed by:
Xia Wu, University of Washington, USA
Monika Weronika Murcha, University of Western Australia, Australia

****Correspondence:***
Iris Steinebrunner, Department of Biology, Technische Universität Dresden, Helmholtzstr. 10, 01062 Dresden, Germany
e-mail: iris.steinebrunner@tu-dresden.de

The two related putative cytochrome *c* oxidase (COX) assembly factors HCC1 and HCC2 from *Arabidopsis thaliana* are Homologs of the yeast Copper Chaperones Sco1p and Sco2p. The *hcc1* null mutation was previously shown to be embryo lethal while the disruption of the *HCC2* gene function had no obvious effect on plant development, but increased the expression of stress-responsive genes. Both HCC1 and HCC2 contain a thioredoxin domain, but only HCC1 carries a Cu-binding motif also found in Sco1p and Sco2p. In order to investigate the physiological implications suggested by this difference, various *hcc1* and *hcc2* mutants were generated and analyzed. The lethality of the *hcc1* knockout mutation was rescued by complementation with the *HCC1* gene under the control of the embryo-specific promoter *ABSCISIC ACID INSENSITIVE 3*. However, the complemented seedlings did not grow into mature plants, underscoring the general importance of *HCC1* for plant growth. The HCC2 homolog was shown to localize to mitochondria like HCC1, yet the function of *HCC2* is evidently different, because two *hcc2* knockout lines developed normally and exhibited only mild growth suppression compared with the wild type (WT). However, *hcc2* knockouts were more sensitive to UV-B treatment than the WT. Complementation of the *hcc2* knockout with *HCC2* rescued the UV-B-sensitive phenotype. In agreement with this, exposure of wild-type plants to UV-B led to an increase of *HCC2* transcripts. In order to corroborate a function of HCC1 and HCC2 in COX biogenesis, COX activity of *hcc1* and *hcc2* mutants was compared. While the loss of *HCC2* function had no significant effect on COX activity, the disruption of one *HCC1* gene copy was enough to suppress respiration by more than half compared with the WT. Therefore, we conclude that HCC1 is essential for COX function, most likely by delivering Cu to the catalytic center. HCC2, on the other hand, seems to be involved directly or indirectly in UV-B-stress responses.

Keywords: SCO (synthesis of cytochrome *c* oxidase), mitochondria, copper chaperone, COX complex, UV-B stress, plant growth and development, BN-PAGE, *Arabidopsis thaliana*

INTRODUCTION

Mitochondrial (mt) biogenesis requires the assembly of proteins synthesized in two different compartments. In addition, due to the essential redox nature of many processes that take place in mitochondria, the formation of active components also requires the synthesis, transport and insertion of a set of cofactors (Herrmann and Funes, 2005; Barrientos et al., 2009; Kim et al., 2012). Among these, copper is one of the components which is required for the activity of cytochrome *c* oxidase (COX or complex IV; Cobine et al., 2006). COX contains two copper centers located in the subunits COX1 and COX2. Insertion of copper into COX is an intricate process that requires the participation of several mt proteins that function in either its delivery or redox reactions related with the assembly process (Herrmann and Funes, 2005; Cobine et al., 2006). The occurrence of COX assembly factors in prokaryotes suggests that some of them were already present in the endosymbiont that originated the mitochondrion while others are more recent acquisitions.

A family of proteins that has been related with copper insertion into COX, particularly into the COX2 subunit, is the SCO family. SCO proteins are of prokaryotic origin and usually contain a transmembrane domain and a soluble domain that contains redox-active cysteines and a histidine presumably involved in copper binding (Banci et al., 2011). The fact that the soluble domain contains a thioredoxin fold has prompted some authors to postulate that SCO proteins do not act in the direct transfer of copper to COX but rather in the reduction of the COX2 cysteines involved in copper binding (Balatri et al., 2003; Abriata et al., 2008). SCO proteins were first analyzed in *Saccharomyces (S.) cerevisiae* mutants defective in COX assembly (hence their name, Synthesis of Cytochrome *c* Oxidase; Schulze and Rödel, 1988). However, current evidence of the presence of SCO proteins in bacteria that do not contain COX-like proteins (Arnesano et al.,

2005; Banci et al., 2007) suggests that some members of the family may have different or additional functions. In support of this, *S. cerevisiae* contains two *SCO* genes, but only *SCO1* is essential for COX assembly, while mutations in *SCO2* do not have a significant effect (Glerum et al., 1996).

Also higher eukaryotes like humans and seed plants contain more than one *SCO* gene. However, the duplication events that led to this divergence seem to have occurred independently (Attallah et al., 2011). Accordingly, the functional consequences of duplication also seem to differ. Unlike the case in *S. cerevisiae* mentioned above, both human SCO proteins participate in COX assembly, but they are not redundant and fulfill different roles (Leary et al., 2004). In plants, knockout (KO) mutations of the *Arabidopsis (A.) thaliana HCC1* gene caused embryo lethality, possibly due to defects in COX assembly (Attallah et al., 2011; Steinebrunner et al., 2011). This hypothesis is supported by its localization in mitochondria (Steinebrunner et al., 2011) and the presence of a Cu-binding motif. A *HCC2* mutation altered the expression of genes related to copper homeostasis and stress responses, but contrary to the KO of *HCC1*, did not show a strong phenotype change compared with the wild type (WT) under normal growth conditions (Attallah et al., 2011).

In the present work, we reinforce the argument that the two proteins have divergent functions. Mutants with only one intact *HCC1* copy showed diminished COX activity, corroborating that HCC1 is indeed required for complex IV assembly. The *HCC2* loss-of-function, on the other hand, did not impair COX activity, but reduced the tolerance to UV-B stress. We summarize our data in a working model, showing how the two proteins might function in plant mitochondria. While HCC1 directly affects COX performance, HCC2 seems to be important for UV-B stress response, possibly by directly or indirectly participating in reactive oxygen (ROS) defense mechanisms.

MATERIALS AND METHODS

PLANT MATERIAL AND CULTURE CONDITIONS

Of the plant lines used in this work, the following were generated previously or obtained from public seed collections, GABI-Kat (German Plant Genomics Research Program—Kölner *Arabidopsis* T-DNA lines; Rosso et al., 2003) and NASC (Nottingham *Arabidopsis* Stock Center; (Scholl et al., 2000)), respectively: *hcc1* (GABI-Kat 923A11; termed *hcc1-2* in Steinebrunner et al., 2011), *hcc2-1* (GABI-Kat 843H01), *hcc2-2* (GABI-Kat 640A10), mt-gk (NASC ID N16263; Nelson et al., 2007), and *HCC1:GUS* line 1 (Steinebrunner et al., 2011). The ecotype Columbia was used as the WT which is also the background of all the mutants used in this study. Sterilized seeds were imbibed overnight at 4°C and then grown on 0.8% (w/v) agar plates (pH 5.7) with 1x Murashige and Skoog basal salts (MS) and 1% (w/v) sucrose. Antibiotics were added when applicable at 25 μg mL^{-1} (kanamycin, hygromycin) and 50 μg mL^{-1} (sulfadiazine). For experiments with adult plants or seed production, seedlings were transferred to soil (Einheitserde, type P, Pätzer, Sinntal-Jossa, Germany) mixed with sand 10:1. The plant growth chamber conditions were 16 h of photosynthetically active radiation (PAR) (100–150 μmol photons m^{-2} s^{-1}, determined with a quantum sensor LI-190SA from LI-COR) per day, 35% humidity and 24°C (light)/21°C (dark).

BIOINFORMATIC ANALYSES

Primers for cloning and genotyping were selected with the help of the SeqViewer tool on the *Arabidopsis* Information Resource database (Rhee et al., 2003). Protein sequences were obtained from the UNIPROT database (www.uniprot.org). Sequence alignment was done with the ClustalO 1.2.0 alignment tool (Sievers et al., 2011; www.ebi.ac.uk/Tools/mhsa/clustalo/). Prediction of transmembrane domains and disulfide bonds was performed with TMPred (Hofmann and Stoffel, 1993; www.ch.embnet.org/software/TMPRED_form.html) and DiANNA (Ferrè and Clote, 2006; clavius.bc.edu/~clotelab/DiANNA/), respectively. Sources for microarray data were the AtGenExpress visualization tool (www.weigelworld.org/expviz/expviz.jsp) and the *Arabidopsis* eFP Browser (Winter et al., 2007).

GENOTYPIC CHARACTERIZATION OF *hcc2-1* AND *hcc2-2*

The seeds for the lines *hcc2-1* and *hcc2-2* were germinated on MS agar containing sulfadiazine (sul). For genotyping of these and any other mutants used in this work, DNA was isolated as described elsewhere (Steinebrunner et al., 2011). DNA from resistant progeny was subjected to PCR analysis to identify homozygous mutants. The *hcc2-1* and *hcc2-2* alleles were detected with the primer pair 8409/Sco2E1F (330 bp) and 8409/Sco2E5R (570 bp), respectively. The presence of the intact *HCC2* gene was checked with the primer pair Sco2E1F/Sco2E5R. The primer sequences are listed in **Supplementary Table 1**.

GENERATION OF GUS REPORTER LINES

The *ABI3:GUS* and *HCC2:GUS* constructs were generated by Gateway cloning (Life Technologies). The *ABI3* (*ABSCISIC ACID INSENSITIVE 3*) promoter region (5 kb) comprised the sequence upstream of the *ABI3* start codon and was amplified with the primer pair ABI3F/ABI3R. The *HCC2* promoter region consisted of 1532 bp upstream of the start codon of *HCC2* and was amplified with the primer pair AtSco2PF/AtSco2PR. All primer sequences contained *attB* recombination sites and are listed in **Supplementary Table 1**. Each *attB*-flanked PCR product was recombined with the donor vector pDONR221 (Life Technologies) to generate entry clones. The pMDC163 vector (Curtis and Grossniklaus, 2003) which carries the *GUS* (glucuronidase) reporter gene, served as the destination vector for each promoter region present in the entry clone. Plant transformants resulting from *Agrobacterium*-mediated transformation were selected on MS agar plates containing hygromycin (hyg). In addition, the presence of the *ABI3:GUS* construct in the transgenic lines was confirmed by PCR (primers ABI3P-F/pMDC163_GUS-R; **Supplementary Table 1**).

GUS ACTIVITY STAINING

Plant material was immersed in 90% (v/v) acetone at −20°C for 1 h. After aspirating the acetone, three washes with 50 mM sodium phosphate buffer (pH 7.0) followed. Incubation in 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (x-gluc) solution [1 mM x-gluc; 50 mM phosphate buffer pH 7.0, 10 mM

potassium ferrocyanide, 10 mM potassium ferricyanide, 0.2% (v/v) triton X-100] was done overnight at 37°C. The plant material was transferred to 70% (v/v) ethanol and stored at 4°C.

COMPLEMENTATION OF *hcc1* WITH *ABI3:HCC1*

For the Gateway cloning (Life Technologies) of the *ABI3:HCC1* construct, PCR products of the *ABI3* promoter region and the *HCC1* sequence which both contained *attB* attachment sites for recombination with suitable donor vectors, were generated. The same *ABI3* promoter region as described for the *ABI3:GUS* construct was used and recombined into the plasmid pDONR221 P1-P5r (Life Technologies). The complete coding sequence of *HCC1* without the stop codon was amplified (primer pair HCC1F/HCC1R) and recombined into the plasmid pDONR221 P5-P2 (Life Technologies). The *HCC1* cDNA U19562 (Yamada et al., 2003) served as the PCR template. The promoterless destination vector pGWB516 (Nakagawa et al., 2007) for the two entry clones *ABI3* promoter and *HCC1* contained four consecutive myc epitope sequences. Therefore, the final *ABI3:HCC1* construct coded for a HCC1 fusion protein that was C-terminally labeled with a 4x myc-tag. Hemizygous *HCC1/hcc1* mutants were transformed with the construct. The progeny were selected on sul and hyg and then genotyped: the *HCC1* transgene was detected with the primers E5F/HCC1E5-6R or E5F/E7R, the genomic *HCC1* allele with the primers E5F/HCC1I6R or E5F/E7R and the *hcc1* allele with the primers E5F/8409. All primer sequences are listed in **Supplementary Table 1**.

RT-PCR ANALYSIS

Total RNA was extracted from leaves using the peqGOLD plant RNA kit (Peqlab). For reverse transcription, 2 μg of RNA were incubated in a total volume of 9 μL for 5 min at 65°C and then placed on ice for 2 min. The reverse transcriptase M-MuLV (New England Biolabs), oligo(dT) primers, buffer and dNTPs were added in a final volume of 20 μL according to the manufacturer's instructions. The reaction was incubated at 37°C for 60 min and stopped at 70°C for 10 min. The cDNA for the housekeeping gene *UBC21* (ubiquitin-conjugating enzyme 21) was amplified with the primers UBCF/UBCR and for the *HCC2* gene with the primers Sco2E1F/Sco2E5R.

COMPLEMENTATION OF *hcc2-2* WITH *HCC2:HCC2*

For the complementation of the *hcc2* KO mutant, the entire *HCC2* coding sequence was amplified from *A. thaliana* cDNA (primers Sco2F/Sco2R; **Supplementary Table 1**) and fused to the Snap-tag (Keppler et al., 2003). The fusion construct was under the control of the identical *HCC2* promoter region used for the *HCC2:GUS* construct. The Gateway technology (Life Technologies) was applied to clone the *HCC2* promoter region and the *HCC2* cDNA into the donor plasmid pDONR P1-P4 and pDONR P4r-P3r, respectively. The cloning of the Snap-tag entry clone is described elsewhere (Steinebrunner et al., 2011). The three entry clones were recombined into the destination vector pGWB516 (Nakagawa et al., 2007). The Snap-tag sequence included a stop codon to avoid fusion with the 4x myc-tag present in pGWB516. The final *HCC2-Snap* sequence coded for a HCC2 protein with a C-terminal 19.6-kDa Snap-tag. Homozygous *hcc2-2* mutants were transformed with agrobacteria containing the *HCC2:HCC2-Snap* construct, hereafter referred to as *HCC2:HCC2*. Transformed progeny were selected on sul and hyg. The presence of the *HCC2* cDNA was confirmed by the detection of a 569-bp amplicon with the primers Sco2E1F/Sco2E5R. The two lines 1–4 and 2–6 used in this work were homozygous for the *HCC2:HCC2* construct and are abbreviated as line 1 and line 2, respectively, hereafter.

GENERATION OF *HCC2-mRFP* OVEREXPRESSORS

A 1.5-kb *HCC2* gene fragment comprising the sequence from the start codon to 28 nt before the stop codon was amplified with the primers HCC2-BamHIF/HCC2-SalIR, introducing BamHI and SalI restriction sites. The PCR product was cloned into the pENTR 3C dual selection vector (Life Technologies) digested with BamHI and XhoI. Using this clone, the *HCC2* fragment plus the *att* recombination sites from pENTR 3C were amplified by PCR with the oligonucleotides AHL1F/AHL2R. Finally, the PCR product was transferred into the destination vector pGWB554 (Nakagawa et al., 2007) by the Gateway cloning system (Life Technologies). This binary vector contained the constitutive cauliflower mosaic virus *35S* promoter for the expression of the target protein C-terminally fused to monomeric RFP (mRFP). The sequence of the final construct in pGWB554 coded for a HCC2-mRFP fusion protein of 58.1 kDa (30.8 kDa plus the tag of 27.3 kDa). Transformed agrobacteria were used to transform the homozygous kanamycin-resistant *Arabidopsis* mt-gk line (Nelson et al., 2007). This line expresses GFP targeted to mitochondria (mt-GFP). Transformed plants were selected on MS agar containing kanamycin and hyg. The presence of the *HCC2-mRFP* construct was validated by PCR, using genomic DNA and the primer pair HCC2-BamHIF/mCherryR1. In addition, the elevated expression of *HCC2* transcripts in the individual lines was corroborated by qRT-PCR analysis of RNA from roots of 20-day-old seedlings (primer combination HCC2-RT-F/HCC2-RT-R).

CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

For CLSM, the upright Zeiss LSM 780 equipped with water immersion objectives (C-Apochromat 10x/1.20 W Korr M27 or C-Apochromat 63x/1.20 W Korr M27) was used. Roots of 8- to 9-day-old transgenic seedlings co-expressing *HCC2-mRFP* and *mt-GFP* which had been selected on hyg-containing MS agar plates were imaged in water. GFP and mRFP were excited with the 488-nm and 561-nm laser, respectively. Imaging was done in the channel mode choosing 491–552 nm for GFP detection and 587–631 nm for mRFP detection. GFP and mRFP were co-imaged by unidirectional scanning, switching tracks every line. The power of each laser was always kept below the saturation of pixels with the help of the range indicator. The pinhole was set to one airy unit. Bright field-type images were acquired with the transmitted light detector. Crosstalk between channels was ruled out with tissue sections in which individual cells in the same image showed either signals in the GFP or in the mRFP detection channel, but not in both. The images were analyzed with the Zeiss Zen 2010 software.

PREPARATION OF MITOCHONDRIA AND OTHER SUBCELLULAR FRACTIONS

Mitochondrial crude fractions (MCFs) as well as the fractions SII, PI, and PII were prepared from etioled 10- to 13-day-old seedlings as described previously (Steinebrunner et al., 2011) with slight modifications. The MS medium contained 1% (w/v) sucrose. Baffled flasks were used for better aeration and shaken at 40 rpm. Ethylenediaminetetraacetic acid (EDTA) was added to the grinding buffer to a final concentration of 2 mM. The MCFs were resuspended in 60 μL washing buffer per gram of fresh weight. The *HCC1/hcc1* hemizygotes and the *hcc2-2* KO mutants were grown in the presence of sul. Protein concentrations were determined in duplicates using the bicinchoninic acid protein assay kit from Pierce.

SDS-PAGE AND WESTERN BLOT ANALYSIS

Preparation of 10% SDS gels and protein electrophoresis was carried out according to Laemmli (1970). The "PageRuler Plus Prestained Protein Ladder" (Thermo Scientific) was applied as a molecular weight marker. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore), incubated with primary antibodies, and detected with horseradish peroxidase-conjugated secondary antibodies and the ECL Plus Western blotting detection reagents (GE Healthcare). Primary polyclonal antibodies were diluted in 5% (w/v) nonfat dry milk 1:5000 against mRFP (Rockland), 1:2000 against COX2 (cytochrome *c* oxidase subunit 2; Agrisera), 1:10,000 against RbcL (large subunit of Rubisco; Agrisera) and 1:10,000 against VDAC1 (voltage-dependent anion-selective channel protein; Agrisera). Membranes were stripped between consecutive primary antibody incubations with stripping buffer [62.5 mM Tris pH 6.7, 2% (w/v) SDS, 100 mM β-mercaptoethanol] for 30 min at 55°C.

MEASUREMENT OF COX ACTIVITY

As the purity of the different MCFs can vary, the citrate synthase activity (CSA) was chosen as an mt marker to calibrate the content of mitochondria in each MCF. The CSA was determined spectrophotometrically in a 96-well plate. Each well was successively filled with 130 μL TE-buffer (50 mM Tris/HCl, 2 mM EDTA, pH 8.0), 10 μL 10 mM oxaloacetate, 30 μL 5 mM acetyl-coenzyme A and 10 μL 10 mM 5,5′-dithiobis-2-nitrobenzoic acid. The reaction was started by the addition of 100 μg of MCF per well. Increase in the absorption at 412 nm was followed in a TECAN InfiniteM200 plate reader at 25°C. Measurements were performed in duplicates or triplicates. The slope of the linear phase of the reaction was used to determine the activity. The CSA in the WT MCF was used to recalculate the mt protein amounts in the other MCFs (WT CSA/sample CSA × sample protein amount = calibrated sample protein amount). These adjusted protein concentrations were used for the COX activity measurements and in-gel COX activity stainings.

Measurements of COX activity in isolated mitochondria were performed as described by Sweetlove et al. (2007), using a Clark electrode (STRATHKELVIN Oxygen Meter 782 connected to a Mitocell MT200 chamber). Briefly, 500 μL of mitochondria reaction buffer [0.3 mM sucrose, 10 mM TES/KOH pH 7.5, 10 mM KH_2PO_4, 3 mM $MgSO_4$, 10 mM NaCl, 0.1% (w/v) bovine serum albumin] were supplemented with 200 μg of MCF followed by addition of 10 μL 0.5 M sodium ascorbate and 25 μL 1 mM reduced cytochrome *c* [from horse heart; prepared without using trichloroacetic acid (TCA); Sigma-Aldrich]. The reaction was started by lysing the mitochondria with 2.5 μL 10% (v/v) Triton X-100. The linear decline of oxygen concentration in the chamber was used to calculate COX activity in comparison with the WT.

BLUE NATIVE GEL ELECTROPHORESIS (BN-PAGE) AND IN-GEL COX ACTIVITY STAINING

The method of BN-PAGE was performed as described (Schägger and von Jagow, 1991; Schägger, 2001). For the analysis, 150 μg mitochondria (MCF) from etiolated plants were lysed with digitonin (detergent:protein ratio of 4:1), and the lysate was applied to 3–13% BN gradient gels. The protein mixture of the "Gel Filtration Calibration Kit High Molecular Weight" (GE Healthcare) was used as a native molecular weight marker. In-gel COX activity staining was performed modified as described by Thomas et al. (1976). BN gels were equilibrated in COX buffer (75 mg/mL sucrose, 50 mM potassium phosphate buffer, pH 7.4) for 1 h followed by replacement with fresh COX buffer containing 1 mg/mL diaminobenzidine (Fluka) and 1 mg/mL cytochrome *c* (from horse heart; prepared without using TCA; Sigma-Aldrich). The formation of brown precipitates was followed over time and photographically documented.

QUANTITATIVE REVERSE TRANSCRIPTASE-PCR (qRT-PCR) ANALYSIS OF *HCC2* EXPRESSION

Aerial tissue (30 mg each) was frozen in liquid nitrogen at various time points after a 60-min UV-B irradiation. RNA was prepared with TRIzol reagent (Life Technologies) according to the instructions of the manufacturer. First strand cDNA synthesis was performed using oligo $dTV_{(20)}$ primers and M-MuLV (RNase H^-) reverse transcriptase (New England Biolabs) in a final volume of 20 μL according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with 2 μL of cDNA synthesis reaction, using primers specific for *HCC2* (HCC2-RT-F/HCC2-RT-R) or *PP2AA3* (protein phosphatase 2A subunit A3) (PP2AA3F/PP2AA3R) (**Supplementary Table 1**). Both primer pairs spanned two introns each. The qPCR was carried out in a 20 μL final volume, containing 1x of DyNAmo flash SYBR green qPCR kit (Thermo Scientific) and run in triplicates in a Mastercycler ep realplex apparatus (Eppendorf). Relative transcript levels were calculated by the comparative C_T method (Livak and Schmittgen, 2001). Expression values were calibrated to transcript levels of the reference gene *PP2AA3* as recommended by Czechowski et al. (2005).

UV-B TREATMENTS

For the *HCC2* promoter activity studies, the *HCC2:GUS* seedlings were selected on hyg-containing MS agar plates. The 7-day-old seedlings were exposed to a 1-h UV-B treatment (Sankyo Denki G15T8E lamps; 1.9 μmol $m^{-2}s^{-1}$, determined with a LightScout UV meter from Spectrum Technologies) supplemented with PAR [Osram L15W/840 (daywhite) fluorescent lamps; 28 μmol $m^{-2}s^{-1}$]. Then the plates with the seedlings were returned to the

plant growth chamber and stained for GUS activity 3 and 24 h later, respectively.

For UV-B stress tests on soil, seedlings were grown without antibiotics on MS agar plates for 7 days and then transferred to soil. For each genotype, 30 plants for the control and 30 plants for the treatment were planted. All plants were cultivated as described under "Plant material and culture conditions," except that after 10 days on soil the treated plants were irradiated daily for 1 h with UV-B light (1.9 μmol $m^{-2}s^{-1}$) supplemented with PAR (28 μmol $m^{-2}s^{-1}$). The daily UV-B treatment started 6 h after the beginning of the light period. Pictures were taken on day 1 (right before the first UV-B treatment) and weekly (right after the UV-B exposure) until day 21 (=final UV-B irradiation).

GENE IDENTIFIERS

The loci of the genes from this study are provided in parentheses: *ABI3* (At3g24650), *HCC1* (At3g08950), *HCC2* (At4g39740), *PP2AA3* (At1g13320), *UBC21* (At5g25760).

RESULTS

A. THALIANA CONTAINS TWO SCO PROTEINS, BUT ONLY ONE OF THEM CARRIES THE HIGHLY CONSERVED CU-BINDING MOTIF

The family of SCO-like proteins is highly conserved among prokaryotes and eukaryotes (Arnesano et al., 2005), in which eukaryotes frequently contain at least two of these proteins. **Figure 1** shows an alignment of the yeast *S. cerevisiae*, human and *Arabidopsis* SCO proteins. Experimental studies which were mainly performed in yeast unraveled the role of SCO1 in copper transport to the COX complex (Schulze and Rödel, 1988; Rentzsch et al., 1999) and structural analysis revealed the special importance of the CxxxC motif and one histidine residue for copper ligation (Nittis et al., 2001). The members of the protein family in different organisms almost perfectly meet the typical features such as a single transmembrane domain, a high conservation in the C-terminal part, and the Cu-binding motif. However, the latter one is not conserved in HCC2 (**Figure 1**). Interestingly, one SCO protein containing and one lacking this conserved Cu-binding domain can be found in other higher plants as well (**Supplementary Figure 1**).

These structural characteristics give hints that HCC2 is rather not involved in copper transport and might have a different function in plants. Therefore, we compared the effects of *HCC1* and *HCC2* loss-of-functions on COX and plant growth performance.

HCC1 IS ESSENTIAL FOR PLANT DEVELOPMENT

HCC1 was shown to be essential for embryo development, because T-DNA *hcc1* KO mutants (**Figure 2A**) died as embryos, mostly at the heart stage (Attallah et al., 2011; Steinebrunner et al., 2011). Previous *HCC1:GUS* analyses revealed *HCC1* promoter activity in seedlings and adult plants, indicating that HCC1 might have a function beyond the embryonic stage (Attallah et al., 2011; Steinebrunner et al., 2011). In order to find out if HCC1 is essential for later stages of plant development as well, a partial complementation strategy was employed. For this, a promoter was needed that would be active during the entire embryo development, but not at any other developmental stage. The *ABI3* promoter was described to meet these criteria (Parcy et al., 1994; Devic et al., 1996). To confirm this, transgenic reporter lines were generated, expressing *GUS* under the control of the *ABI3* promoter. The GUS activity was checked in two *ABI3:GUS* lines at different time points after seedling germination (**Figure 2B**). In 2-day-old *ABI3:GUS* seedlings, strong GUS staining was visible in cotyledons and in the vasculature of the hypocotyl and root. Eight days after germination of the seeds, the GUS staining was still very strong. After 21 days, however, the GUS activity had considerably faded and only weak staining of the vasculature in older leaves, the hypocotyl and roots remained. The activity of the *HCC1* promoter, on the other hand, was still highly active in the apex and young leaf tissue, substantiating a role of HCC1 in post-embryonic development.

The GUS analyses further demonstrated that the *ABI3* promoter was still active at early stages of seedling growth. The presence of the GUS protein in young seedlings had been reported before by Parcy et al. (1994), although in their analysis, the *GUS* transcript had disappeared by day 4, and the GUS protein, which is more stable, by day 7. For sure, the *ABI3* promoter activity was suppressed at day 21 after seed germination. Therefore, the *ABI3* promoter was selected for the partial complementation approach and fused with the *HCC1* cDNA. Of the two previously characterized T-DNA KO mutants *hcc1-1* (Attallah et al., 2011; Steinebrunner et al., 2011) and *hcc1-2* (Steinebrunner et al., 2011), the T-DNA allele *hcc1-2* (**Figure 2A**) was chosen for the transformation with *ABI3:HCC1*.

Since the embryo-specific complementation had to be performed in the hemizygous mutant background, nine genotypes were possible as the outcome of the selfing cross (**Table 1**), assuming that the loci of *HCC1/hcc1* and the transgene were not linked. However, as the *hcc1* KO is embryo lethal, the expected number of viable genotypes in the offspring was only eight.

In order to narrow down the search for the rescued *hcc1* KO mutant, the seeds from the described cross were germinated both on sul and hyg to eliminate all the seedlings that did not carry the *hcc1* mutation and the transgene (**Table 1**). Thus, 40% of the progeny should get purged. After 21 days of growth, indeed 36% of the 243 offspring plantlets displayed an antibiotic-sensitive phenotype (**Table 2**). The remaining 155 surviving seedlings could be divided into two phenotypes. One group (45%) had developed several sets of true leaves and a long root, while the other group (19%) had grown only two true leaves and very short roots (**Figure 2C**; **Table 2**). The observed percentages of the phenotypes matched the expected percentages (**Table 2**), indicating that the stunted phenotype represented the complemented *hcc1* KO mutation. As expected, the genotyping of the two phenotypes (**Figure 2C**) by PCR confirmed that the dwarf plantlets were in fact rescued KO mutants.

In order to rule out that the presence of the transgene exerted a negative effect on plant growth, seeds from an *ABI3:HCC1*-complemented hemizygous selfing cross were germinated on MS agar for 13 days without antibiotics. Thirty-four seedlings were photographed and subsequently genotyped. Now only two different phenotypes appeared: seedlings with long roots or with short roots (**Figure 2D**). Again, the short roots belonged to the rescued KO mutants. The long roots were grown by


```
ScSCO1(P23833)   ----------------------------MLKLSRSANLRL-----VQLPAARLSG--NG  24
ScSCO2(P38072)   ----------------------------MLNSSRKYACRS-LFRQANVSIKGLFY--NG  28
HsSCO1(O75880)   MAMLVLVPGRVMRPLGGQLWRFLPRG--------------------------LEFWGPAE  34
HsSCO2(O43819)   ------------------------------------------------------------   0
AtHCC1(Q8VYP0)   MASALCR--TASRLRSVQLFRRIRVSSDLLSASSPSPACISDALRHGDFSLPRSFFSLNC  58
AtHCC2(Q8LAL0)    ----------------------------------MLPCR------------R-------  6

ScSCO1(P23833)   A-KLL-------------------TQRGFFTVTRLWQSN--------GKKPLSRVPVGG-  55
ScSCO2(P38072)   G---A-------------------YRRGFSTGCCLRSDN--------KESPSARQPLDRL  58
HsSCO1(O75880)   GTARVLLRQFCARQAEAW-RASGR------PGYCLGTRPLSTARP--P-PPWS--QKG--  80
HsSCO2(O43819)   -------MLLLTRSPTAW-HRLSQLKPRVLPGT-LGGQA----LH--L-RSWLLSRQG--  42
AtHCC1(Q8VYP0)   GIEMLKMDQRCLLSTSAS-DTTSKH---------DSGKPETKSSEKNEKSGGSESSDGGS 108
AtHCC2(Q8LAL0)   ------LVLSCKNQAASFLRRCGPSKRIQSVNYCKSTRQGHEIP---DVKPL--FPTGG-  54

ScSCO1(P23833)   ---TPIKDNGKVREGSIEFSTGKAIALFLAVGGALSYFFNR--EKRRLETQKEA------ 104
ScSCO2(P38072)   QLGDEINEPEPIRTRFFQFSRWKATIALLLLSGGTYAYLSR--KRRLLETEKEA------ 110
HsSCO1(O75880)   -PGD---STRPSKP---GPVSWKSLAITF----AIGGALLA--GMKHVKKEKAE--KLEK 125
HsSCO2(O43819)   -PAETGGQGQPQGP---GLR--TRLLITGLFGAGLGGAWLA------LRAEKER--LQQQ  88
AtHCC1(Q8VYP0)   DHKNERASGKDVRG---GPVSWMSFFLLFATGAGLVYYYDT--QKKRHIEDINK--NSIA1 61
AtHCC2(Q8LAL0)   ------GTQAPSRS----RARYAVPAILLGFA-GFVGFLHYNDERRAVPRGQASSNSGCG 103

ScSCO1(P23833)   -EANRGYGKPSLGGPFHLEDMYGNEFTEKNLLGKFSIIYFGFSNCPDICPDELDKLGLWL 163
ScSCO2(P38072)   -DANRAYGSVALGGPFNLTDFNGKPFTEENLKGKFSILYFGFSHCPDICPEELDRLTYWI 169
HsSCO1(O75880)   ERQRH-IGKPLLGGPFSLTTHTGERKTDKDYLGQWLLIYFGFTHCPDVCPEELEKMIQVV 184
HsSCO2(O43819)   KRTEALRQAAVGQGDFHLLDHRGRARCKADFRGQWVLMYFGFTHCPDICPDELEKLVQVV 148
AtHCC1(Q8VYP0)   VKEGPSAGKAAIGGPFSLIRDDGKRVTEKNLMGKWTILYFGFTHCPDICPDELIKLAAAI 221
AtHCC2(Q8LAL0)   CGSNTTVKGPIIGGPFTLVSTENKIVTENDFCGKWVLLYFGYSFSPDVGPEQLKMMSKAV 163

ScSCO1(P23833)   NTLSSKYGI-TLQPLFITCDPARDSPAVLKEYLSDFHPSILGLTGTFDEVKNACKKYRVY 222
ScSCO2(P38072)   SELDDKDHI-KIQPLFISCDPARDTPDVLKEYLSDFHPAIIGLTGTYDQVKSVCKKYKVY 228
HsSCO1(O75880)   DEIDSITTLPDLTPLFISIDPERDTKEAIANYVKEFSPKLVGLTGTREEVDQVARAYRVY 244
HsSCO2(O43819)   RQLEAEPGLPPVQPVFITVDPERDDVEAMARYVQDFHPRLLGLTGSTKQVAQASHSYRVY 208
AtHCC1(Q8VYP0)   DKIKENSGV-DVVPVFISVDPERDTVQQVHEYVKEFHPKLIGLTGSPEEIKSVARSYRVY 280
AtHCC2(Q8LAL0)   DKLESKHNE-KILPVFVTLDPQRDTPSHLHAYLKEFDSRILGLTGTASAMRQMAQEYRVY 222

ScSCO1(P23833)   FSTPPNVKPGQDYLVDHSIFFYLMDPEGQFVDALGRNYDEKTGVDKIVEHVKSYVPAEQR 282
ScSCO2(P38072)   FSTPRDVKPNQDYLVDHSIFFYLIDPEGQFIDALGRNYDEQSGLEKIREQIQAYVPKEER 288
HsSCO1(O75880)   YSPGPK-DEDEDYIVDHTIIMYLIGPDGEFLDYFGQNKRKGEIAASIATHMRPYRKKS-- 301
HsSCO2(O43819)   YNAGPK-DEDQDYIVDHSIAIYLLNPDGLFTDYYGRSRSAEQISDSVRRHMAAFRSVLS- 266
AtHCC1(Q8VYP0)   YMK-TE-EEDSDYLVDHSIVMYLMSPEMNFVKFYGKNHDVDSLTDGVVKEIRQYRK---- 334
AtHCC2(Q8LAL0)   FKKVQ--EDGEDYLVDTSHNMYLINPKMEIVRCFGVEYNPDELSQELLKEVASVSQ---- 276

ScSCO1(P23833)   AKQKEAWYSFLFK      295
ScSCO2(P38072)   ERRSKKWYSFIFN      301
HsSCO1(O75880)   -------------      301
HsSCO2(O43819)   -------------      266
AtHCC1(Q8VYP0)   -------------      334
AtHCC2(Q8LAL0)   -------------      276
```

FIGURE 1 | Protein sequence alignment of SCO proteins from *S. cerevisiae* (ScSCO1 and ScSCO2), human (HsSCO1 and HsSCO2), and *A. thaliana* (AtHCC1 and AtHCC2). The alignment was performed with protein sequences from the UNIPROT database (accession codes are given) using the ClustalO 1.2.0 alignment tool (Sievers et al., 2011). The consensus sequence is shown below ("*" conserved in all sequences, "." conserved in some sequences, ":" homologous in all sequences). The predicted transmembrane segments (TMPred; Hofmann and Stoffel, 1993) are shown in blue. The amino acids involved in Cu-binding which includes the CxxxC motif as well as one histidine residue (Rentzsch et al., 1999; Nittis et al., 2001) are marked in red, if present.

all other genotypes, regardless of the presence of the transgene. The phenotype and genotype comparison also showed that the disruption of one *HCC1* gene copy did not impair plant growth.

The *hcc1* KO phenotype became visible as early as day 13 after germination (**Figure 2D**). The KO mutants did not develop beyond the development of two tiny true leaves and died after transfer to soil (data not shown).

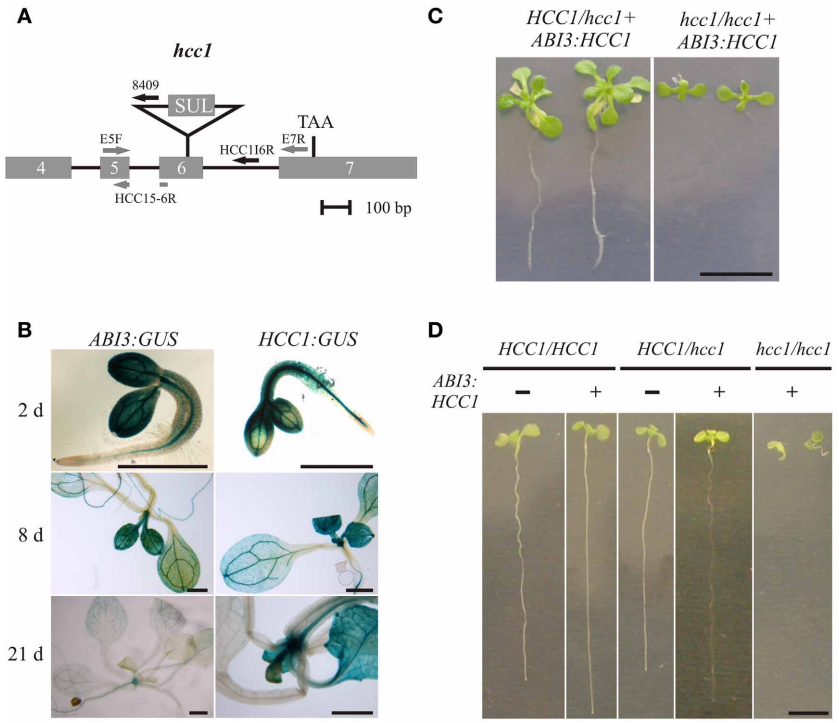


FIGURE 2 | Embryo-specific complementation of the *hcc1* knockout. (A) A schematic diagram of the *hcc1* allele is shown. The exons 4–7 and introns 4–6 of *HCC1* are denoted by gray boxes and solid lines, respectively. The labeled arrows represent the primers used for genotyping. The gray and black arrow colors denote a binding site in a coding and noncoding region, respectively. Primer HCC1E5-6R spans the exon5-exon6 boundary. SUL, sulfadiazine resistance cassette; TAA, stop codon. **(B)** Seedlings selected on hyg from two *ABI3:GUS* lines (3 and 6) and the *HCC1:GUS* line 1 were stained for GUS activity at the indicated days after germination. The staining pattern was similar in both *ABI3:GUS* lines and representative images of line 3 are shown. The scale bars equal 1 mm each. **(C)** Progeny from a selfing cross of the hemizygous *hcc1* mutant complemented with *ABI3:HCC1* (line "Nils") were grown on MS agar with hyg and sul for 21 days. The plants were photographed and then used for DNA isolation to determine their genotypes. The scale bar (=1 cm) is valid for both panels. **(D)** Progeny from a selfing cross of the hemizygous *hcc1* mutant complemented *with ABI3:HCC1* (line "Nils") were grown on an MS agar plate without antibiotics for 13 days. Before each seedling was sacrificed for DNA isolation, the seedlings (sample size = 34) were laid out on agar plates, numbered consecutively and photographed. With the genotyping results at hand, representative images of the WT and the hemizygous *HCC1/hcc1* mutant, both with or without the *ABI3:HCC1* transgene, and of the *ABI3:HCC1*-complemented *hcc1/hcc1* mutant were assembled for the overview. The scale bar equals 5 mm and is valid for all panels.

Table 1 | Punnett square of the selfing cross of the *ABI3:HCC1*-complemented hemizygous *hcc1* mutant.

	HA	Ha	ha	hA
HA	HHAA	HHAa	HhAa	HhAA
Ha	HHAa	HHaa	Hhaa	HhAa
ha	HhAa	Hhaa	hhaa	hhAa
hA	HhAA	HhAa	hhAa	hhAA

The genotypes of the offspring seedlings sensitive to the antibiotic selection of sul (HHAa, HHAA), hyg (Hhaa), or both (HHaa) are highlighted in blue. The genotypes of the double-resistant WT phenotypes are labeled in green. The genotypes of the double-resistant KO phenotypes are indicated in red. The embryo-lethal genotype is marked in black. H, HCC1; h, hcc1; A, ABI3:HCC1; a, no ABI3:HCC1.

The complementation of the *hcc1* KO mutation with the *ABI3:HCC1* construct demonstrated that *HCC1* is not only essential for embryonic development, but also for plant development in general.

THE DISRUPTION OF THE *HCC2* GENE HAS MILD ADVERSE EFFECTS ON PLANT GROWTH

The function of HCC1 is obviously crucial for plant development. GUS activity analyses of *HCC2:GUS* transgenic lines (**Supplementary Figure 2**; Attallah et al., 2011) show staining of mainly the same tissues as observed in *HCC1:GUS* lines (Attallah et al., 2011; Steinebrunner et al., 2011), suggesting similar functions. However, the structural differences (**Figure 1**) rather pointed to different functions. To resolve this issue, a KO mutant with a T-DNA insertion in intron 2 of the *HCC2* gene (Salk_008313) was studied previously (Attallah et al., 2011). Plants homozygous for the insertion behaved like WT, except for a delayed development of inflorescences (Attallah et al., 2011).

In this study, we analyzed two other T-DNA insertion mutants of the *HCC2* gene. One of the two new mutants, *hcc2-1*, bore a T-DNA insertion in intron 1, while the other one, *hcc2-2*, had the T-DNA inserted exactly between exon 3 and intron 3 (**Figure 3A**). RT-PCR analysis of cDNA from homozygous *hcc2-1* and *hcc2-2* mutants with primers spanning the T-DNA insertions yielded no *HCC2* transcripts, suggesting that both mutants

Table 2 | Segregation analysis of a selfing cross of the *ABI3:HCC1*-complemented hemizygous *hcc1* mutant.

Phenotype (Genotype)	Percentage expected (%)	Percentage observed (%)
Sensitive (HHAA, HHAa, HHaa, Hhaa)	40	36
Resistant, long root (HhAa, HhAA)	40	45
Resistant, short root (hhAa, hhAA)	20	19

*The seeds (T2 generation) from a selfing cross of the ABI3:HCC1-complemented hemizygous hcc1 mutant (line "Nils") were germinated on MS agar containing both sul and hyg. After 21 days the seedlings (n = 243) were sorted by phenotype that was either sensitive or resistant to the antibiotic double selection. The resistant seedlings were subdivided into two groups with long or short roots. The expected percentages for each phenotype group were calculated based on the assumptions that the transgene ABI3:HCC1 and the HCC1/hcc1 loci were not linked and that the transgene was only inserted once into the genome. Because of the embryo-lethality of the allele combination hhaa, the total number of seedling-viable allele combinations was 15 (see **Table 1**). The deviations of the expected from the observed values were statistically insignificant ($p > 0.05$; chi-square = 2.5; degrees of freedom = 2). The colors refer to the coding in **Table 1**. H, HCC1; h, hcc1; A, ABI3:HCC1; a, no ABI3:HCC1.*

were KOs (**Figure 3B**). The phenotypes of the homozygous *hcc2* seedlings were indistinguishable from the WT (data not shown). However, after 31 days of growth a slight, but statistically significant ($p < 0.01$) reduction in primary shoot lengths became apparent in comparison with the WT (**Figure 3C**). In order to confirm that this phenotype was caused by the disruption of the *HCC2* gene, the rescue of the shoot phenotype was pursued by complementing the KO with a functional *HCC2* cDNA fused to the native *HCC2* promoter region. In addition, the *HCC2* cDNA was fused to the Snap-tag (Keppler et al., 2003) to produce a C-terminally tagged HCC2 protein. Two *HCC2*-complemented lines were included in the shoot length analysis and both lines grew primary stems like the WT (**Figure 3C**), demonstrating that the shoot growth retardation was indeed caused by the *HCC2* loss-of-function.

The *hcc2-2* KO mutant was selected as the background for the rescue experiment for two reasons. First, segregation analysis results available on the website of the GABI-Kat T-DNA mutant collection (Rosso et al., 2003; Kleinboelting et al., 2012) implied that the *hcc2-1* mutant contained at least one more T-DNA insertion locus. Second, qRT-PCR analysis of the *hcc2-1* KO mutant with primers downstream of the known T-DNA insertion revealed about 20-fold higher *HCC2* transcript levels than WT (data not shown; for *HCC2* primer sequences see Attallah et al., 2011). The accumulation of the partial *HCC2* transcripts can be explained by the presence of the suitably oriented *35S* promoter in the T-DNA (**Figure 3A**). These partial transcripts probably do not give rise to functional HCC2 proteins, because the *hcc2-1* KO mutants are phenotypically identical to the *hcc2-2* KOs (**Figure 3C**). Nevertheless, all further studies were conducted solely with the *hcc2-2* KO mutant.

The analysis of the two *hcc2* KO mutants corroborated that the importance of HCC2 for plant development, unlike the function of HCC1, is limited under normal growth conditions.

HCC2 IS LOCALIZED IN MITOCHONDRIA

Several programs predict the localization of both plant SCO proteins in mitochondria (SUBA database; Heazlewood et al., 2007). Different experimental approaches confirmed the mt localization of HCC1. HCC1 was detected in mt fractions by mass spectrometry (Dunkley et al., 2006) and by Western blot analyses, using a Snap-tagged version of HCC1 (Steinebrunner et al., 2011). However, there were no experimental data available for HCC2.

Initially, the same Snap-tagging approach was attempted for HCC2 to prove its presence in mitochondria. However, it was impossible to detect the biotinylated HCC2-Snap protein in mitochondria (data not shown). One explanation for the detection failure could be that HCC2 is less abundant than HCC1. In fact, studies in the yeast *S. cerevisiae* revealed that there are 1.7 times more Sco1p than Sco2p molecules per cell (Ghaemmaghami et al., 2003).

As an alternative strategy to investigate the predicted localization, HCC2 was C-terminally fused to mRFP and expressed under the control of the *35S* promoter. The construct was transformed into a transgenic line which expressed GFP targeted to mitochondria (mt-GFP) (Nelson et al., 2007).

Roots of various transgenic lines co-expressing *HCC2-mRFP* and *mt-GFP* were imaged by confocal laser scanning microscopy (CSLM). Numerous dot-like and often fast-moving structures were visible in the cytosol, using the GFP detection channel (**Figure 4A**, top panel). The diameter of these GFP signals was approximately 0.5 μm, matching the size of *Arabidopsis* mitochondria (Nelson et al., 2007). When detecting the mRFP in the same cells, the fluorescence signal pattern was very similar (**Figure 4A**, middle panel), suggesting co-localization. Indeed, the GFP and mRFP signals nicely overlapped (**Figure 4A**, bottom panel), suggesting that HCC2 is localized in mitochondria. No mRFP signal was detectable in any other compartments inside or outside of cells.

In order to confirm the localization results, Western blot analyses of different fractions obtained during the preparation of mitochondria (M) from *HCC2-mRFP* expressing lines and the WT control were performed (**Figure 4B**). Identity of the different fractions was confirmed by the detection of COX2 and RbcL as mt and chloroplastic marker proteins, respectively. The COX2 and RbcL proteins were absent in the supernatant fraction II which is expected to contain soluble proteins and to be organelle-free. The chloroplast marker RbcL was present in all pellet fractions (PI, PII, M). The mt marker COX2, on the other hand, was faintly detectable in PII, but strongly enriched in the mt fraction (M).

Immunological detection using an mRFP antibody yielded a weak signal in the PII fraction and a strong signal in the mt fraction of the *HCC2-mRFP* overexpressing line, but not of the WT which served as a control for the specificity of the antibody. The molecular weight (~57 kDa) of the signal corresponded to the theoretical size of HCC2 (31 kDa minus 1 kDa signal peptide) fused to mRFP (27 kDa). The molecular weight of the signal also demonstrated that the HCC2-mRFP protein exists *in planta* and that the fluorescence detected by CLSM was not originating from free mRFP.

The signal pattern for COX2 and HCC2-mRFP matched, indicating the mt localization of HCC2-mRFP. The missing signal for

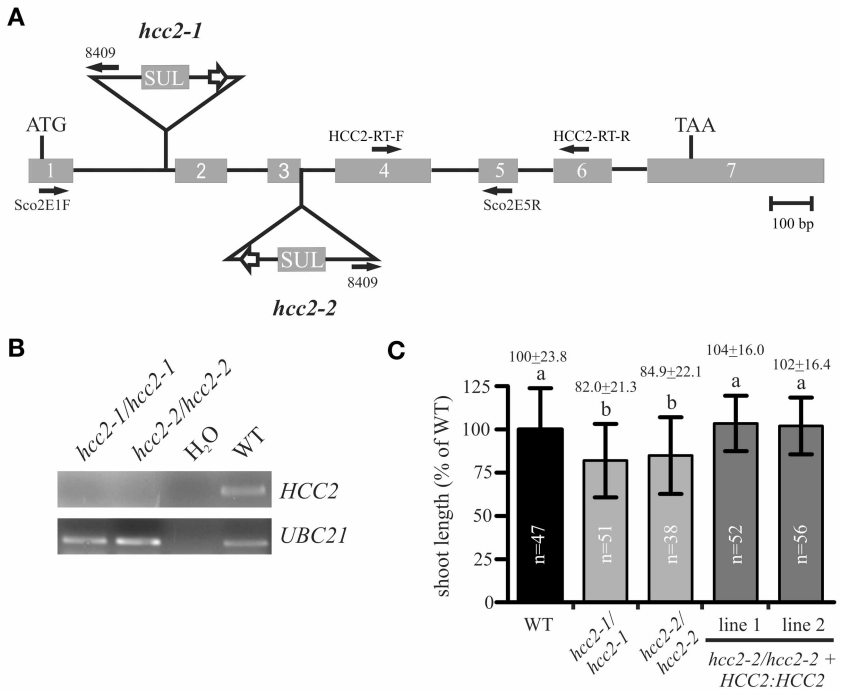


FIGURE 3 | Characterization of *hcc2* T-DNA mutants. (A) The schematic diagram of the *HCC2* gene shows the insertion sites of T-DNA for the *hcc2-1* and *hcc2-2* mutant, respectively, the orientation and location of primers (solid arrows), the start (ATG) and stop (TAA) codon. Exons (gray boxes) and introns (solid lines) are drawn to scale. The white arrows denote *35S* promoters. SUL, sulfadiazine resistance cassette. **(B)** RT-PCR analysis of *HCC2* expression in homozygous *hcc2* mutants and the WT. Water instead of cDNA was used as a negative control. The amplification of a *UBC21* DNA fragment served as evidence for the presence of cDNA in a reaction. **(C)** The primary stems of 38-day-old plants were measured. The average length (=24 cm) of WT stems was normalized to 100%. Values represent means ± standard deviations (SD). Columns with different letters are significantly different from each other ($p < 0.01$; one-way analysis of variance; Tukey test). The diagram combines the data from two independent experiments. The sample sizes (*n*) are indicated.

HCC2-mRFP in the fraction PI, which showed a strong signal for RbcL, argues against an additional localization of this protein in chloroplasts.

Our data obtained by two independent methods give experimental evidence for the localization of HCC2 in plant mitochondria.

COX ACTIVITY IS SEVERELY AFFECTED IN *HCC1/hcc1* HEMIZYGOTES, BUT NOT IN *hcc2* KO MUTANTS

SCO proteins are hypothesized to function in the copper transport and/or assembly of the COX complex in eukaryotes. Indeed, previous results using diaminobenzidine staining showed no COX activity in *hcc1-1/hcc1-1* embryos (Attallah et al., 2011). However, since these embryos do not develop into adult plants, it is impossible to further investigate the COX complex assembly in the KO background. Therefore, hemizygous *HCC1/hcc1* plants were chosen to see if the deletion of one *HCC1* gene copy was sufficient to cause COX alterations. In order to analyze a possible influence of HCC2 on COX function, the *hcc2-2* KO was included in the study. From these mutants as well as the WT, mt fractions were prepared and the amount of mitochondria in these fractions was equilibrated by measuring CSA (data not shown).

In *S. cerevisiae*, a lack of Sco1p results in not only missing COX activity, but also destabilization of the complex and degradation of the COX subunits (Paret et al., 2000). Therefore, the protein level of COX2 was previously investigated in the *hcc2* Salk KO mutant and found to be unaffected (Attallah et al., 2011). The Western blot analysis was also done with the *hcc2-2* KO to confirm that COX2 levels were independent of the presence of functional HCC2 proteins. Clearly, the amount of COX2 was not reduced in the *hcc2-2* KO compared with the WT (**Supplementary Figure 3**), providing a first hint that HCC2 is not directly involved in COX assembly.

For a more direct analysis of a link between the HCC proteins and COX, its activity was measured by two different approaches. First, blue native gel electrophoresis (BN-PAGE) allowed us to study COX activity in conjunction with the molecular organization of the complex (**Figure 5A**). The in-gel staining revealed COX activity in a broad molecular range of about 300–450 kDa (**Figure 5A**, COX), in which the strongest band was visible in the lower range of the stained area. The activity in the *hcc2-2* KO mutant was very similar to that in the WT, whereas the staining intensity seemed to be weaker in the hemizygous *HCC1/hcc1* plants. The molecular organization of the complex in this molecular weight range did not seem to be different among the three genotypes.

Interestingly, also two light-brownish bands in the range above 1 MDa could be detected in the WT and the *hcc2-2* KO, but not in the *HCC1/hcc1* mutant (**Figure 5A**; white arrowheads).

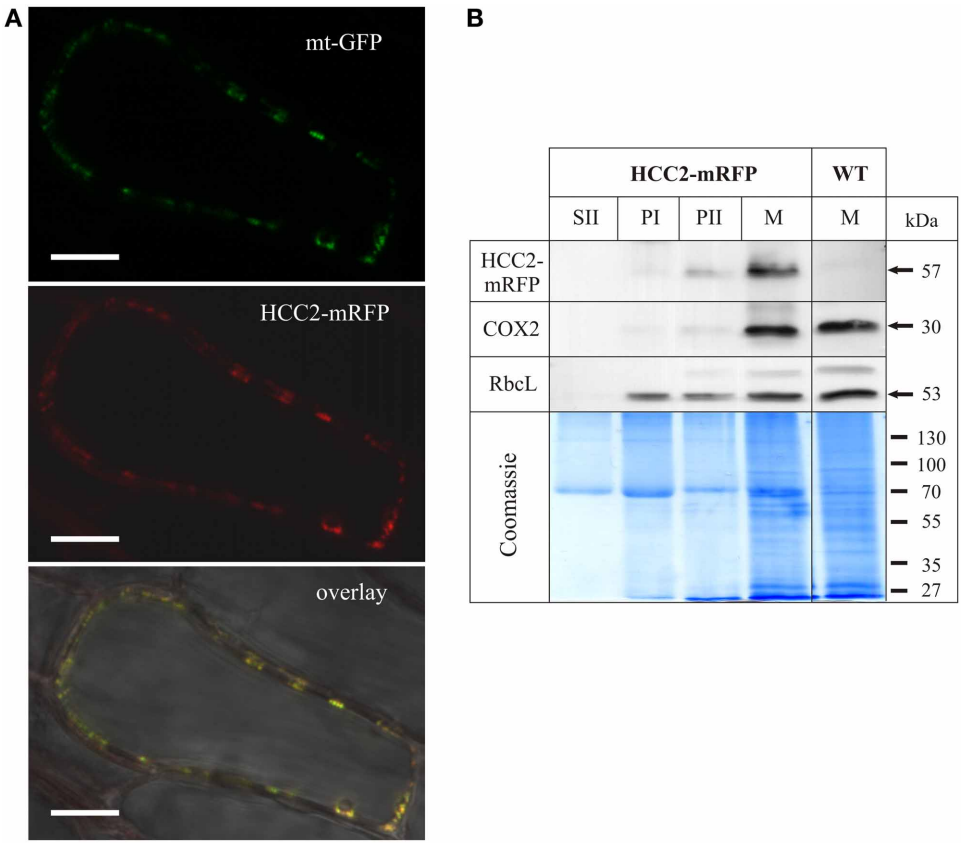


FIGURE 4 | Subcellular localization analysis of HCC2-mRFP. (A) Five different transgenic lines co-expressing *HCC2-mRFP* and GFP targeted to mitochondria (mt-GFP) were imaged by laser scanning microscopy, and co-localization of HCC2-mRFP and mt-GFP was found in each line. A representative cell (line 3) is shown, in which co-localization of the green mt-GFP (top panel) and the red HCC2-mRFP signals (middle panel) is depicted as yellow signals after merging the two images (overlay). The overlay also includes a bright-field image of the cell. The image was acquired with a resolution of 1024 × 1024 pixels and a pixel dwell of 3.15 μs. Scale bars correspond to 10 μm each. **(B)** Mitochondria (M), supernatant (SII) and pellet fractions (PI, PII) were prepared as described in Material and Methods from light-grown WT seedlings or *HCC2-mRFP* overexpressors (line 5) selected on hyg. The indicated fractions (50 μg each) obtained during the preparations were subjected to 10% SDS-PAGE. Western blot analyses of the same membrane were performed with antibodies directed against mRFP, cytochrome *c* oxidase subunit 2 (COX2) and the large subunit of Rubisco (RbcL). The total protein in the gel was visualized after the transfer by colloidal Coomassie staining.

These bands might represent COX associations with other complexes of the respiratory chain (so-called supercomplexes), which were described in several organisms (Schägger, 2002) including the plants potato, spinach and asparagus (reviewed in Dudkina et al., 2008). In *Arabidopsis*, only respiratory chain supercomplexes consisting of complex I and III have been described so far (Eubel et al., 2003; Klodmann et al., 2011). However, these previous studies differed strongly in their experimental design from our analysis, since they used suspension cell culture as starting material and harsher lysis conditions of the mitochondria. This might also explain why we detected activity deriving from monomeric—and possibly dimeric—COX at a slightly higher molecular weight range (up to 450 kDa) than in these publications (at 200–230 kDa).

Whether the higher molecular weight complexes we detected indeed contain COX and under which physiological conditions these supercomplexes are present, remains to be elucidated.

Although the BN results gave hints that the COX activity was reduced in the *HCC1*/*hcc1* mutant, but not in the *hcc2-2* KO, the staining was not suitable for quantification of the enzyme activity. Therefore, COX activity measurements were performed in isolated mitochondria, using a Clark electrode as the second approach (**Figure 5B**). Our results showed that the activity was only minimally reduced in the *hcc2-2* KO (∼14%) compared with the WT, and the difference was not statistically significant. In the hemizygous *HCC1*/*hcc1* mutant, however, it was suppressed by almost 60% relative to the WT.

These data validate the proposition that HCC1 is crucial for COX function, since the deletion of one *HCC1* gene copy already leads to a severe drop in COX activity. Furthermore, the data suggest that the homozygous knockout of *HCC2* does not affect COX activity and strengthen the hypothesis that this protein is involved in a different cellular pathway in *Arabidopsis*.

HCC2 KO MUTANTS ARE MORE SENSITIVE TO UV-B STRESS

Public databases of microarray data indicated that *HCC2* is induced by UV-B light. *HCC2* transcript levels were approximately two times higher 1 h and 3 h after UV-B irradiation than

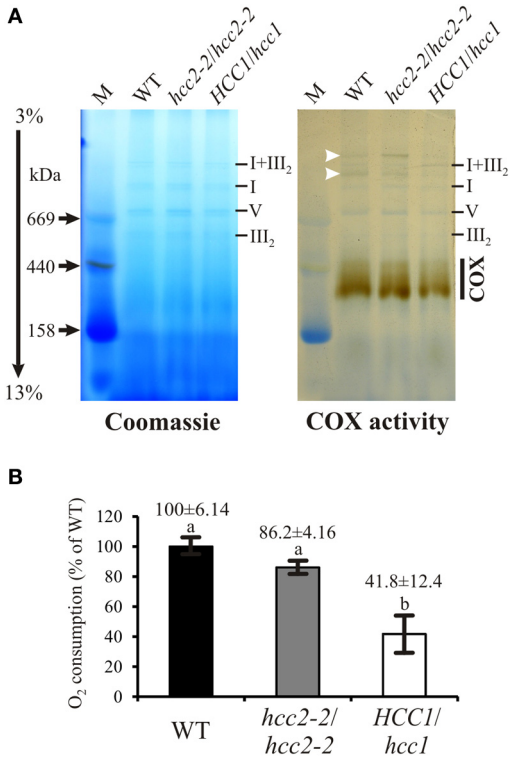


FIGURE 5 | COX activity analysis in the WT, *hcc2* KO and hemizygous *HCC1/hcc1* mutants. (A) Mitochondria (150 μg each) from WT and mutant etiolated seedlings were solubilized using digitonin. The protein complexes were separated in a 3–13% gradient gel by BN-PAGE (left panel). COX complex activity was visualized in the same gel (right panel) as described in Material and Methods. The positions of abundant respiratory chain complexes (III_2, V, I, and I+III_2) were determined by comparison to the BN electrophoretic mobility analysis of Klodmann et al. (2011). The white arrowheads mark putative supercomplexes explained in the Results section. M, molecular weight marker. **(B)** COX activity in mitochondria was determined by measuring oxygen consumption with a Clark electrode after addition of reduced cytochrome *c*. Mean values derive from triplicate measurements of two independent mt preparations. Error bars indicate standard deviations. The mean WT activity (=O_2 consumption of 24 μmol $L^{-1}min^{-1}$) was set to 100%. Different letters indicate means that are significantly different from each other ($p < 0.01$; one-way analysis of variance; Tukey test).

at time point zero (AtGenExpress visualization tool; *Arabidopsis* eFP Browser). For confirmation, *HCC2:GUS* seedlings were irradiated with UV-B light for 1 h and stained for GUS activity at subsequent time points. At 3 h as well as 24 h after the UV-B treatment, the GUS staining was noticeably stronger compared with the untreated control (**Figure 6A**), showing that UV-B light stimulated *HCC2* promoter activity.

Additionally, we wanted to confirm the increase of *HCC2* transcript amounts by UV-B, especially under our treatment conditions. The plants were started off on MS agar for 7 days and let continue to grow on soil for 10 days. For the *HCC2* transcript analysis, RNA samples were taken shortly after the UV-B treatment to minimize possible stimulation of *HCC2* expression by secondary effects such as tissue damage. *HCC2* transcript amounts increased about two-fold 1 h after the UV-B exposure ended and remained at this level 4 h later (**Figure 6B**). This increase lies within the same range observed after a 15-min UV-B treatment under similar conditions (AtGenExpress visualization tool; *Arabidopsis* eFP Browser; Kilian et al., 2007).

In order to see if the UV-B-induced activation of *HCC2* transcription had a beneficial effect, the performance of the *hcc2-2* KO and WT was compared during a 3-week-long UV-B stress test (see Material and Methods). The 17-day-old plants were treated daily with UV-B light. Four daily UV-B exposure times (15 min, 30 min, 45 min, 60 min) were tested. Of the different exposure times, 15 min turned out to cause no visible damage (data not shown). The longer exposures led to yellowing and stunting of rosette leaves and sometimes even plant death. Sixty minutes allowed the best distinction of phenotypic differences among the various genotypes compared.

All genotypes were negatively affected by the treatment as the comparison with the untreated plants demonstrated, but the WT was more tolerant to the stress than the *hcc2-2* KO (**Figure 6C**). Complementing the *hcc2-2* KO with a functional *HCC2* cDNA remedied the heightened UV-B sensitivity (**Figure 6C**).

Our data show that *HCC2* is upregulated by UV-B exposure and that a deletion of *HCC2* increases the UV-B-sensitivity of the plants.

DISCUSSION

The genomes of seed plants encode two proteins with sequence similarity to SCO proteins from other organisms. Even though it has been postulated that SCO proteins may have additional functions, little information is available about the involvement of SCO proteins in processes unrelated to COX assembly. In the present work, we investigated the functions of HCC1 and HCC2, the two SCO proteins from *A. thaliana*, through the analysis of a variety of mutants.

HCC1 IS ESSENTIAL FOR COX ACTIVITY

For HCC1, the presence of conserved amino acids (aa) which were demonstrated in *S. cerevisiae* to be required for copper binding (Rentzsch et al., 1999; Nittis et al., 2001) implied that HCC1 can bind Cu. qRT-PCR analyses showed that *HCC1* transcript levels increased after copper treatment, analogous to the rise of other copper chaperone transcript amounts by copper (Del Pozo et al., 2010). Genetic evidence for a role in Cu binding and delivery was provided by complementation studies of the yeast deletion mutant Δ*sco1* with a gene encoding a chimeric ScSco1p-HCC1 fusion protein. The rescue of the mutant was enhanced by Cu supplementation (Steinebrunner et al., 2011). Since the yeast Δ*sco1* mutant was respiratory-deficient (Schulze and Rödel, 1988), the copper chaperone function of HCC1 seemed to be essential for respiration. However, only indirect experimental evidence existed for this role in *Arabidopsis* (Attallah et al., 2011). This work unequivocally shows that the *HCC1* loss-of-function influences COX activity, as the disruption of one WT *HCC1* allele was enough to lower the COX activity to more than half of the WT level. It would be interesting to see if the HCC1 protein levels and COX activity directly correlate or if even a small reduction in HCC1 amounts is enough to compromise COX function.

A reduction of COX activity (∼20%) was observed in a *S. cerevisiae* mutant with a point mutation in *SCO1*, but despite the suppressed respiration capacity the yeast cells continued to grow

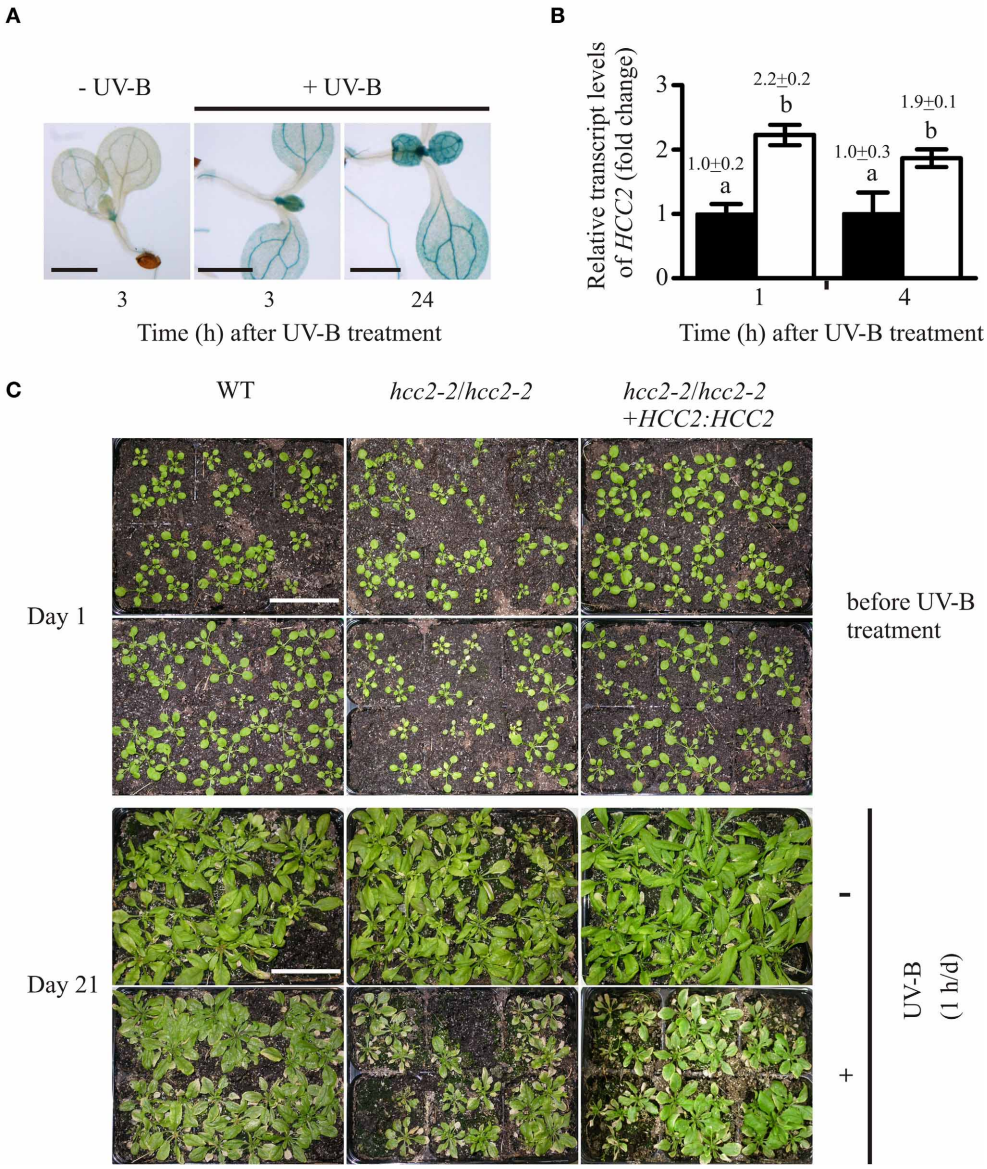


FIGURE 6 | Response of *hcc2* mutants and WT to UV-B treatment. (A) *HCC2:GUS* seedlings were stained for GUS activity 3 h or 24 h after a 1-h treatment with UV-B light. Untreated seedlings were stained in parallel to the 3-h time point. Two GUS lines (lines 3 and 7) were included in the experiment, and three seedlings were photographed per time point. Here seedlings of the line 3 are shown. The experiment was repeated once providing the same staining pattern. The scale bars equal 1 mm each. **(B)** The relative amounts of *HCC2* transcripts were analyzed in 14-day-old WT seedlings by qRT-PCR which were either treated with UV-B for 1 h (white bars) or left untreated (black bars). RNA was isolated from leaves harvested at the indicated time points. The amounts of *HCC2* transcripts at time point 1 h of the untreated control were set to 1. *PP2AA3* transcript levels were used as a reference for normalization. Error bars represent standard deviations calculated from the normalized *HCC2* expression values of two independent RNA preparations of the same UV-B assay. Different letters denote values which are statistically significantly different from each other ($p < 0.05$; one-way analysis of variance; Tukey test). *HCC2* levels shown in this graph are representative of two independent UV-B experiments. **(C)** Day 1 marks the beginning of a UV-B stress test in which 17-day-old WT plants, *hcc2-2* KOs and complemented *hcc2-2* KOs (line 2) either served as untreated controls (top row) or were irradiated daily with UV-B light for 1 h (bottom row). The phenotypes of the same plants are shown after they had grown for 21 days in the absence of any UV-B light (-UV-B) or exposed daily to UV-B (+UV-B). Any plants which died during the UV-B stress test were removed. At day 21, the shoots were cut for unobstructed view of the rosettes. The scale bars equal 5 cm each and are valid for all panels. This UV-B assay was conducted three times showing the same phenotypic responses.

on nonfermentable carbon sources like the WT (Lode, 2001). Similarly, the strong effect on COX activity did not seem to affect plant growth. Plants with only one intact *HCC1* copy looked phenotypically indistinguishable from the WT (**Figure 2D**). In agreement with this, COX-deprived mutants of the green alga *Chlamydomonas reinhardtii* continued to grow like the WT when kept in the light (Colin et al., 1995; Remacle et al., 2010). Apparently, the reduced production of ATP is compensated by

photosynthesis, or other metabolic pathways such as glycolysis may contribute some ATP. The latter process might be enhanced by the presence of sucrose in our growth media. In future studies it might therefore be interesting to compare the adenylate status of the different mutant lines under various growth conditions.

However, COX activity is linked to many important processes other than the provision of ATP. For example, it maintains the mt membrane potential necessary for the import of many mt proteins from the cytosol (reviewed in Chacinska et al., 2009). In addition, the synthesis of ascorbate is dependent on COX activity. The oxidation of L-galactono-γ-lactone to ascorbate by L-galactono-γ-lactone dehydrogenase uses cytochrome *c* as an electron acceptor. Therefore, a continuous electron flow from cytochrome *c* to complex IV is prerequisite for ascorbate biosynthesis (Bartoli et al., 2000). Ascorbate affects growth, because low levels correlate with low cell division rates (Kerk and Feldman, 1995). In addition, ascorbate is a co-factor for prolyl hydroxylases (Smirnoff, 2000) which produce hydroxyproline-rich glycoproteins relevant for cell wall structure (Showalter, 2001). This function might be the reason why *HCC1* promoter activity is not only high in metabolically active cell types, but also in trichome support cells (Steinebrunner et al., 2011) which need mechanical reinforcements. This function in ascorbate synthesis could also explain why *Arabidopsis* mutants without functional cytochrome *c* (Welchen et al., 2012) and *HCC1* (Attallah et al., 2011; Steinebrunner et al., 2011) die as embryos. For the *hcc1* KO embryos, the predominant time point of their developmental arrest was pinned to the heart stage (Steinebrunner et al., 2011). At this stage, the embryos start growing anisodiametrically, forming two symmetric protrusions as they transit into the torpedo stage. The cell wall plays a pivotal role for these local cell divisions and expansions as proposed for the cell wall mutant *cyt1*. The affected *CYT1* gene codes for the precursor of cell wall polysaccharides and ascorbate (reviewed in Smirnoff, 2000) and the knockout mutant also stops growing as a heart-shaped embryo (Lukowitz et al., 2001).

POSSIBLE REDOX ROLES OF HCC2

The homolog HCC2 does not contain the conserved Cu-binding motif, contradicting a function as a copper chaperone. However, its promoter activity overlapped with the *HCC1* promoter activity (Attallah et al., 2011; Steinebrunner et al., 2011; **Supplementary Figure 2**), hinting at similar functions. However, the determined WT levels of COX activity in the *hcc2-2* KO mutant provided compelling evidence that HCC2 is not required for COX function.

No effect on respiration efficiency was also documented for the deletion of *SCO2* in yeast (Glerum et al., 1996). Instead, an indirect involvement of Sco2p in COX assembly was postulated. The two cysteines of the Cu-binding motif in the thioredoxin domain of Sco2p could possibly maintain the proper redox state of Sco1p, allowing Sco1p to bind and release Cu (Banci et al., 2007). In human cells it could indeed be shown that SCO2 acts as thiol-disulfide oxidoreductase for SCO1 (Leary et al., 2009).

However, HCC2 strikingly lacks these cysteines as well as the histidine residue contributing to Cu binding. The general occurrence of a SCO homolog that bears no conserved amino acids relevant to Cu binding seems to be a common feature in plants (**Supplementary Figure 1**), arguing against its function in metal transfer. But what could be such a plant-specific function?

The results presented here suggest a role in the UV-B stress response, however, it is not clear if HCC2 specifically responds to UV-B or if it fulfills a general function as an antioxidant through its thioredoxin-like fold. In favor of a UV-B-specific function is the finding that (i) *HCC2* is upregulated by UV-B treatment and that (ii) HCC2 is not upregulated by UV-A or by other factors causing oxidative stress such as paraquat (Kilian et al., 2007). These data should be confirmed and complemented with phenotypic studies in the future. Preliminary studies indicate that *hcc2* KO plants contain higher lipid peroxidation levels under basal growth conditions, suggesting that HCC2 could serve as a regulator of ROS levels.

The homologous yeast proteins Sco1p and Sco2p were both shown to reside in the inner mt membrane and to protrude their catalytically active C-terminal domain into the intermembrane space (IMS) (Krummeck, 1992; Lode et al., 2002). The data of our work do not allow conclusions about the submitochondrial localization of HCC2, but nicely prove its presence in mitochondria.

Nevertheless, the analysis tool TMPred predicts for HCC2 one transmembrane helix from aa 66 to 82 with an inside-outside orientation, arguing for a localization of the active domain of HCC2 in the mt IMS space. This subcompartment represents a suitable site for a protein with an oxidoreductive function, because the IMS contains the highest abundance of cysteine-rich proteins in the cell (Herrmann and Funes, 2005).

MODEL FOR HCC1 AND HCC2 FUNCTION

Incorporating our experimental data, we propose the following model (**Figure 7**). HCC1 is essential for plant life, because it ensures COX function, most conceivably through delivery of the co-factor Cu to complex IV. HCC2, on the contrary, is not an essential plant protein, but important nonetheless. As a putative oxidoreductase, HCC2 could maintain the proper redox state of redox-sensitive proteins, such as HCC1, although COX activity was not significantly suppressed in the *hcc2-2* KO. However, the HCC2 activity is possibly compensated in the mutant by an mt oxidoreductase of redundant function, or HCC2 may only be important under certain conditions, such as UV-B stress.

High UV-B fluence rates—as used in our experiments—trigger the production of ROS (reviewed in Mackerness et al., 2001 and Hideg et al., 2013) which cause oxidative damage of cell components. The damage leads to the release of more ROS, amplifying the original ROS levels. HCC2 could protect against ROS damage by exerting the oxidoreductive function of its thioredoxin domain, protecting the plant indirectly against UV-B. However, as a caveat for this hypothesis it must be considered that despite the fact that HCC2 contains a thioredoxin fold, the typical thioredoxin motif CxxC directly involved in catalysis (reviewed in Meyer et al., 2012) is missing. Nevertheless, HCC2 is a very cysteine-rich protein with five cysteines remaining in the protein after the putative signal peptide (aa 1–29) is cleaved off. In addition, there is a CGC-motif (aa 102–104) which is predicted to form a disulfide bond with C_{253} by the DiANNA software.

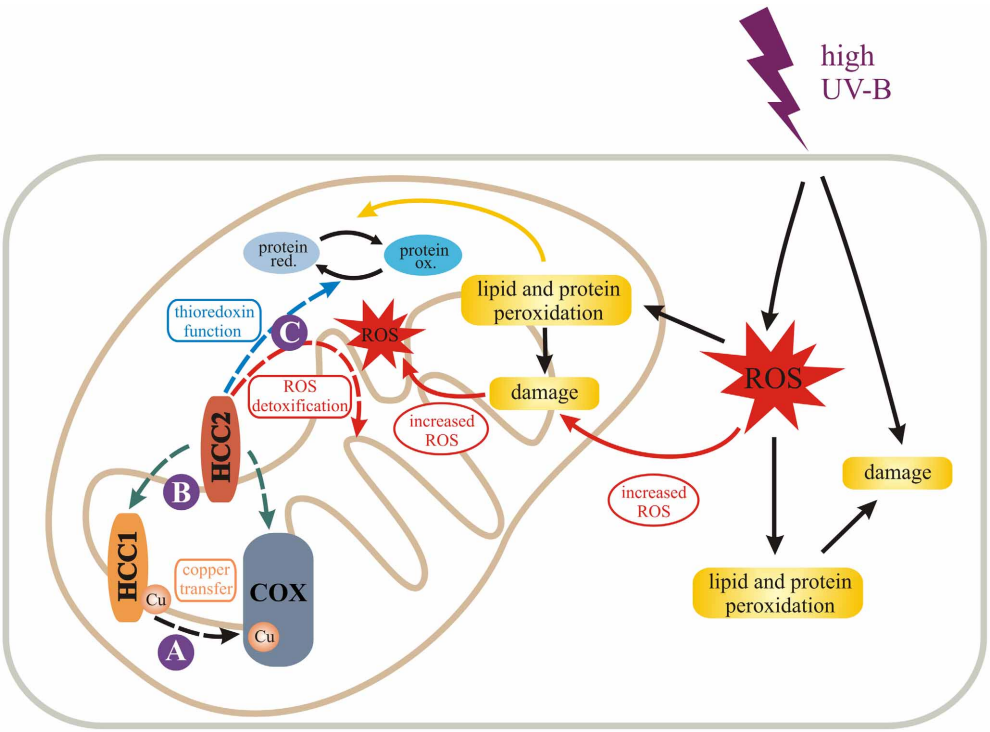


FIGURE 7 | Model for the role of HCC1 and HCC2 in plant mitochondria. We propose different functions (dashed arrows) for the two SCO proteins in mitochondria of *A. thaliana* indicated by solid purple circles. HCC1 is an essential plant protein and is most likely involved in copper transport to COX (complex IV) of the respiratory chain (A). HCC2 might have rather a slight influence on COX activity by either directly or indirectly supporting HCC1 function (B). In fact it is more likely that HCC2 functions as a thioredoxin, converting oxidized (ox.) proteins back to their reduced (red.) forms, or detoxifies reactive oxygen species (ROS) in mitochondria (C). This function is of special importance under UV-B stress which leads to increased ROS damage in cells and organelles, e.g., by lipid and protein peroxidation. The figure was partially adapted from Nawkar et al. (2013).

Additionally, any of the other cysteines could exert a redox-active function. Alternatively, HCC2 may not act directly as an oxidoreductase, but may modify the activity of other redox proteins present in the mitochondrial IMS.

In conclusion, plants have evolved two different SCO proteins with specific functions in COX assembly and stress responses. Even though our results indicate that HCC1 and HCC2 have completely separate roles, the presence of both proteins in mitochondria and the conservation in structure does not rule out that they exert their functions through partially overlapping pathways. The results highlight the role of mitochondria in many physiological responses and indicate that plants have adapted preexisting proteins to serve additional functions related to their specific needs.

AUTHOR CONTRIBUTIONS

Iris Steinebrunner conceived of the study, characterized mutant lines (*HCC2:HCC2*, *hcc2-1*, *hcc2-2*, *hcc1* lines complemented with *ABI3:HCC1* or *ABI3:GUS*), did the confocal microscopy and segregation analyses and drafted the manuscript. Uta Gey conducted the COX activity experiments, the BN-PAGE and Western blot analyses, performed the sequence alignments and participated in the writing of the manuscript. Manuela Andres did the UV-B experiments and analyzed the *GUS* expression controlled by the *HCC2* promoter in response to UV-B light and by the *ABI3* promoter. Lucila Garcia generated the *35S:HCC2-mRFP* lines and did the qRT-PCR analyses. Daniel H. Gonzalez advised the study and participated in the writing. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

We are very grateful for the help of the following students: René Morgenstern (cloning of *ABI3:GUS*, *ABI3:HCC1*, and *HCC2:HCC2* constructs), Annelie Müller (contributions to the characterization of *hcc1*-complemented lines and establishment of the UV-B assay) and Marlen Landschreiber (RT-PCR and *HCC2:GUS* analyses). We would like to thank Gerhard Rödel for his ongoing support of the project and helpful comments. Silke Hilbig is thanked for her technical assistance. The Light Microscopy Facility of the Biotechnology Center TU Dresden provided excellent training and technical assistance with the CSLM imaging. The T-DNA mutants were generated in the context of the GABI-Kat program and provided by Bernd Weisshaar (Max Planck Institute for Plant Breeding Research, Cologne, Germany). The cDNA clone U19562 was donated by Athanasios Theologis, Joseph Ecker, and Ronald Davis to the *Arabidopsis* Biological Resource Center who provided the clone to us. Many thanks to the following people for providing materials: Elena Taverna (mRFP antibody), Mark Curtis (pMDC163), Francois Parcy (*ABI3* promoter region), and Tsuyoshi Nakagawa (pGWB516, pGWB553, pGWB554).

FUNDING

The grant STE 1455/5-1 from the German Research Foundation (DFG) awarded to Iris Steinebrunner supported the research stays of Uta Gey and Lucila Garcia in Daniel H. Gonzalez's and Iris Steinebrunner's laboratory, respectively, which resulted in data presented in this study. This work was also funded by Daniel H. Gonzalez's grants PICT1035 and PICT1203 from the National Agency for the Promotion of Science of Argentina (ANPCyT). Daniel H. Gonzalez is a member of the National Research Council of Argentina (CONICET) which co-funded Uta Gey's stay at the Universidad Nacional del Litoral (UNL); Lucila Garcia is a CONICET fellow at UNL. The publication fee for this article was covered by the DFG and the Open Access Publication Funds of the Technische Universität Dresden.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00087/abstract

Supplementary Table 1 | Primer details. *The attachment site sequences necessary for recombination were omitted. Only the gene-specific sequences are listed.

Supplementary Figure 1 | Protein sequence alignment of SCO (HCC) proteins from plants: *Arabidopsis thaliana* (At), *Oryza sativa* subsp. *japonica* (Os), *Brassica rapa* subsp. *pekinensis* (Br), *Glycine max* (Gm), and *Solanum lycopersicum* (Sl). Protein sequences were retrieved from the UNIPROT database (UNIPROT numbers are given). The sequence alignment was performed using the ClustalO 1.2.0 alignment tool (Sievers et al., 2011). The consensus is quoted below the sequences ("*" conserved in all sequences, "." partially conserved, ":" homology in all sequences). The amino acids involved in Cu-binding which includes the CxxxC motif as well as one histidine residue (Rentzsch et al., 1999; Nittis et al., 2001) are marked in red, if present. Proteins containing these conserved residues are titled "HCC1," whereas those missing the motif are named "HCC2." If more than one sequence in the UNIPROT database met this criterion, they were distinguished by different letters (a, b, c).

Supplementary Figure 2 | *HCC2:GUS* studies of different developmental stages. Various tissues from *HCC2:GUS* transgenic lines were stained for GUS activity: Ovules **(A,B)**, embryo **(C)**, cotyledon **(D,E)**, roots **(F,G)**, leaves **(H,I)**, flower buds **(J)**, flower **(K)** and silique **(L)**. The tissues stemmed from 7-day-old seedlings **(D–G)** and 4-week-old plants **(A–C, H–L)**, respectively. Exemplary guard cells are indicated by arrows in **(E)**. Staining of guard and trichome support cells was only observed in younger leaves. ch, chalaza; f, funiculus; r, root; h, hypocotyl; tsc, trichome support cells; c, connective; az, abscission zone.

Supplementary Figure 3 | Analysis of steady-state COX2 levels in the WT and *hcc2-2* KO mutants. MCF (50 μg each, normalized to WT CSA levels) prepared from liquid cultures of etiolated seedlings (WT and *hcc2-2*/*hcc2-2* mutants) were separated in a 15% SDS gel for Western blot analysis. Immunological detection of COX2 and VDAC1 was performed successively on the same membrane. VDAC1 was detected as the loading control for equal amounts of mt protein. The Western blot analysis was performed three times with two independent sets of protein extracts, producing the same results.

REFERENCES

Abriata, L. A., Banci, L., Bertini, I., Ciofi-Baffoni, S., Gkazonis, P., Spyroulias, G. A., et al. (2008). Mechanism of Cu(A) assembly. *Nat. Chem. Biol.* 4, 599–601. doi: 10.1038/nchembio.110

A.-H.-Mackerness, S., John, C. F., Jordan, B., and Thomas, B. (2001). Early signaling components in ultraviolet-B responses: distinct roles for different reactive oxygen species and nitric oxide. *FEBS Lett.* 489, 237–242. doi: 10.1016/S0014-5793(01)02103-2

Arnesano, F., Banci, L., Bertini, I., and Martinelli, M. (2005). Ortholog search of proteins involved in copper delivery to cytochrome *c* oxidase and functional analysis of paralogs and gene neighbors by genomic context. *J. Proteome Res.* 4, 63–70. doi: 10.1021/pr049862f

Attallah, C. V., Welchen, E., Martin, A. P., Spinelli, S. V., Bonnard, G., Palatnik, J. F., et al. (2011). Plants contain two SCO proteins that are differentially involved in cytochrome *c* oxidase function and copper and redox homeostasis. *J. Exp. Bot.* 62, 4281–4294. doi: 10.1093/jxb/err138

Balatri, E., Banci, L., Bertini, I., Cantini, F., and Ciofi-Baffoni, S. (2003). Solution structure of Sco1: a thioredoxin-like protein involved in cytochrome *c* oxidase assembly. *Structure* 11, 1431–1443. doi: 10.1016/j.str.2003.10.004

Banci, L., Bertini, I., Cavallaro, G., and Ciofi-Baffoni, S. (2011). Seeking the determinants of the elusive functions of Sco proteins. *FEBS J.* 278, 2244–2262. doi: 10.1111/j.1742-4658.2011.08141.x

Banci, L., Bertini, I., Cavallaro, G., and Rosato, A. (2007). The functions of Sco proteins from genome-based analysis. *J. Proteome Res.* 6, 1568–1579. doi: 10.1021/pr060538p

Barrientos, A., Gouget, K., Horn, D., Soto, I. C., and Fontanesi, F. (2009). Suppression mechanisms of COX assembly defects in yeast and human: insights into the COX assembly process. *Biochim. Biophys. Acta* 1793, 97–107. doi: 10.1016/j.bbamcr.2008.05.003

Bartoli, C. G., Pastori, G. M., and Foyer, C. H. (2000). Ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV. *Plant Physiol.* 123, 335–344. doi: 10.1104/pp.123.1.335

Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138, 628–644. doi: 10.1016/j.cell.2009.08.005

Cobine, P. A., Pierrel, F., and Winge, D. R. (2006). Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. *Biochim. Biophys. Acta* 1763, 759–772. doi: 10.1016/j.bbamcr.2006.03.002

Colin, M., Dorthu, M. P., Duby, F., Remacle, C., Dinant, M., Wolwertz, M. R., et al. (1995). Mutations affecting the mitochondrial genes encoding the cytochrome oxidase subunit I and apocytochrome b of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* 249, 179–184. doi: 10.1007/BF00290364

Curtis, M. D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes *in planta*. *Plant Physiol.* 133, 462–469. doi: 10.1104/pp.103.027979

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M. K., and Scheible, W. R. (2005). Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiol.* 139, 5–17. doi: 10.1104/pp.105.063743

Del Pozo, T., Cambiazo, V., and Gonzalez, M. (2010). Gene expression profiling analysis of copper homeostasis in *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* 393, 248–252. doi: 10.1016/j.bbrc.2010.01.111

Devic, M., Albert, S., and Delseny, M. (1996). Induction and expression of seed-specific promoters in *Arabidopsis* embryo-defective mutants. *Plant J.* 9, 205–215. doi: 10.1046/j.1365-313X.1996.09020205.x

Dudkina, N. V., Sunderhaus, S., Boekema, E. J., and Braun, H. P. (2008). The higher level of organization of the oxidative phosphorylation system: mitochondrial supercomplexes. *J. Bioenerg. Biomembr.* 40, 419–424. doi: 10.1007/s10863-008-9167-5

Dunkley, T. P., Hester, S., Shadforth, I. P., Runions, J., Weimar, T., Hanton, S. L., et al. (2006). Mapping the *Arabidopsis* organelle proteome. *Proc. Natl. Acad. Sci. U.S.A.* 103, 6518–6523. doi: 10.1073/pnas.0506958103

Eubel, H., Jansch, L., and Braun, H. P. (2003). New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II. *Plant Physiol.* 133, 274–286. doi: 10.1104/pp.103.024620

Ferrè, F., and Clote, P. (2006). DiANNA 1.1: an extension of the DiANNA web server for ternary cysteine classification. *Nucleic Acids Res.* 34, W182–W185. doi: 10.1093/nar/gkl189

Ghaemmaghami, S., Huh, W. K., Bower, K., Howson, R. W., Belle, A., Dephoure, N., et al. (2003). Global analysis of protein expression in yeast. *Nature* 425, 737–741. doi: 10.1038/nature02046

Glerum, D. M., Shtanko, A., and Tzagoloff, A. (1996). SCO1 and SCO2 act as high copy suppressors of a mitochondrial copper recruitment defect in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 271, 20531–20535. doi: 10.1074/jbc.271.34.20531

Heazlewood, J. L., Verboom, R. E., Tonti-Filippini, J., Small, I., and Millar, A. H. (2007). SUBA: the *Arabidopsis* Subcellular Database. *Nucleic Acids Res.* 35, D213–D218. doi: 10.1093/nar/gkl863

Herrmann, J. M., and Funes, S. (2005). Biogenesis of cytochrome oxidase-sophisticated assembly lines in the mitochondrial inner membrane. *Gene* 354, 43–52. doi: 10.1016/j.gene.2005.03.017

Hideg, E., Jansen, M. A., and Strid, A. (2013). UV-B exposure, ROS, and stress: inseparable companions or loosely linked associates? *Trends Plant. Sci.* 18, 107–115. doi: 10.1016/j.tplants.2012.09.003

Hofmann, K., and Stoffel, W. (1993). TMbase: a database of membrane spanning protein segments. *Biol. Chem. Hoppe-Seyler* 374, 166.

Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003). A general method for the covalent labeling of fusion proteins with small molecules *in vivo*. *Nat. Biotechnol.* 21, 86–89. doi: 10.1038/nbt765

Kerk, N. M., and Feldman, N. J. (1995). A biochemical model for the initiation and maintenance of the quiescent center: implications for organization of root meristems. *Development* 121, 2825–2833.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., et al. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant J.* 50, 347–363. doi: 10.1111/j.1365-313X.2007.03052.x

Kim, H. J., Khalimonchuk, O., Smith, P. M., and Winge, D. R. (2012). Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. *Biochim. Biophys. Acta* 1823, 1604–1616. doi: 10.1016/j.bbamcr.2012.04.008

Kleinboelting, N., Huep, G., Kloetgen, A., Viehoever, P., and Weisshaar, B. (2012). GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Res.* 40, D1211–D1215. doi: 10.1093/nar/gkr1047

Klodmann, J., Senkler, M., Rode, C., and Braun, H. P. (2011). Defining the protein complex proteome of plant mitochondria. *Plant Physiol.* 157, 587–598. doi: 10.1104/pp.111.182352

Krummeck, G. (1992). *Das mitochondriale SCO1 Protein von Saccharomyces cerevisiae: Untersuchungen zur Expression, Topologie und Funktion*. Ph.D. thesis, Ludwig Maximilian University Munich.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

Leary, S. C., Kaufman, B. A., Pellecchia, G., Guercin, G. H., Mattman, A., Jaksch, M., et al. (2004). Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome *c* oxidase. *Hum. Mol. Genet.* 13, 1839–1848. doi: 10.1093/hmg/ddh197

Leary, S. C., Sasarman, F., Nishimura, T., and Shoubridge, E. A. (2009). Human SCO2 is required for the synthesis of CO II and as a thiol-disulphide oxidoreductase for SCO1. *Hum. Mol. Genet.* 18, 2230–2240. doi: 10.1093/hmg/ddp158

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262

Lode, A. (2001). *Assemblierung der Cytochrom c Oxidase: molekulare und biochemische Charakterisierung des mitochondrialen Sco1p aus Saccharomyces cerevisiae und homologer Proteine*. Ph.D. thesis, Technische Universität Dresden.

Lode, A., Paret, C., and Rödel, G. (2002). Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p. *Yeast* 19, 909–922. doi: 10.1002/yea.883

Lukowitz, W., Nickle, T. C., Meinke, D. W., Last, R. L., Conklin, P. L., and Somerville, C. R. (2001). *Arabidopsis cyt1* mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. *Proc. Natl. Acad. Sci. U.S.A* 98, 2262–2267. doi: 10.1073/pnas.051625798

Meyer, Y., Belin, C., Delorme-Hinoux, V., Reichheld, J. P., and Riondet, C. (2012). Thioredoxin and glutaredoxin systems in plants: molecular mechanisms, crosstalks, and functional significance. *Antioxid. Redox Signal.* 17, 1124–1160. doi: 10.1089/ars.2011.4327

Nakagawa, T., Suzuki, T., Murata, S., Nakamura, S., Hino, T., Maeo, K., et al. (2007). Improved Gateway binary vectors: high-performance vectors for creation of fusion constructs in transgenic analysis of plants. *Biosci. Biotechnol. Biochem.* 71, 2095–2100. doi: 10.1271/bbb.70216

Nawkar, G. M., Maibam, P., Park, J. H., Sahi, V. P., Lee, S. Y., and Kang, C. H. (2013). UV-induced cell death in plants. *Int. J. Mol. Sci.* 14, 1608–1628. doi: 10.3390/ijms14011608

Nelson, B. K., Cai, X., and Nebenführ, A. (2007). A multicolored set of *in vivo* organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J.* 51, 1126–1136. doi: 10.1111/j.1365-313X.2007.03212.x

Nittis, T., George, G. N., and Winge, D. R. (2001). Yeast Sco1, a protein essential for cytochrome *c* oxidase function is a Cu(I)-binding protein. *J. Biol. Chem.* 276, 42520–42526. doi: 10.1074/jbc.M107077200

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during *Arabidopsis* seed development: roles of the *ABI3* locus and of endogenous abscisic acid. *Plant Cell* 6, 1567–1582.

Paret, C., Lode, A., Krause-Buchholz, U., and Rödel, G. (2000). The P(174)L mutation in the human hSCO1 gene affects the assembly of cytochrome *c* oxidase. *Biochem. Biophys. Res. Commun.* 279, 341–347. doi: 10.1006/bbrc.2000.3949

Remacle, C., Coosemans, N., Jans, F., Hanikenne, M., Motte, P., and Cardol, P. (2010). Knock-down of the COX3 and COX17 gene expression of cytochrome *c* oxidase in the unicellular green alga *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* 74, 223–233. doi: 10.1007/s11103-010-9668-6

Rentzsch, A., Krummeck-Weiss, G., Hofer, A., Bartuschka, A., Ostermann, K., and Rödel, G. (1999). Mitochondrial copper metabolism in yeast: mutational analysis of Sco1p involved in the biogenesis of cytochrome *c* oxidase. *Curr. Genet.* 35, 103–108. doi: 10.1007/s002940050438

Rhee, S. Y., Beavis, W., Berardini, T. Z., Chen, G., Dixon, D., Doyle, A., et al. (2003). The *Arabidopsis* Information Resource (TAIR): a model organism database providing a centralized, curated gateway to *Arabidopsis* biology, research materials and community. *Nucleic Acids Res.* 31, 224–228. doi: 10.1093/nar/gkg076

Rosso, M. G., Li, Y., Strizhov, N., Reiss, B., Dekker, K., and Weisshaar, B. (2003). An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol. Biol.* 53, 247–259. doi: 10.1023/B:PLAN.0000009297.37235.4a

Schägger, H. (2001). Blue-native gels to isolate protein complexes from mitochondria. *Methods Cell Biol.* 65, 231–244. doi: 10.1016/S0091-679X(01)65014-3

Schägger, H. (2002). Respiratory chain supercomplexes of mitochondria and bacteria. *Biochim. Biophys. Acta* 1555, 154–159. doi: 10.1016/S0005-2728(02)00271-2

Schägger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. *Anal. Biochem.* 199, 223–231. doi: 10.1016/0003-2697(91)90094-A

Scholl, R. L., May, S. T., and Ware, D. H. (2000). Seed and molecular resources for *Arabidopsis*. *Plant Physiol.* 124, 1477–1480. doi: 10.1104/pp.124.4.1477

Schulze, M., and Rödel, G. (1988). SCO1, a yeast nuclear gene essential for accumulation of mitochondrial cytochrome *c* oxidase subunit II. *Mol. Gen. Genet.* 211, 492–498. doi: 10.1007/BF00425706

Showalter, A. M. (2001). Arabinogalactan-proteins: structure, expression and function. *Cell. Mol. Life Sci.* 58, 1399–1417. doi: 10.1007/PL00000784

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7, 539. doi: 10.1038/msb.2011.75

Smirnoff, N. (2000). Ascorbic acid: metabolism and functions of a multi-facetted molecule. *Curr. Opin. Plant Biol.* 3, 229–235. doi: 10.1016/S1369-5266(00)80070-9

Steinebrunner, I., Landschreiber, M., Krause-Buchholz, U., Teichmann, J., and Rödel, G. (2011). HCC1, the *Arabidopsis* homologue of the yeast mitochondrial copper chaperone SCO1, is essential for embryonic development. *J. Exp. Bot.* 62, 319–330. doi: 10.1093/jxb/erq269

Sweetlove, L. J., Taylor, N. L., and Leaver, C. J. (2007). Isolation of intact, functional mitochondria from the model plant *Arabidopsis thaliana*. *Methods Mol. Biol.* 372, 125–136. doi: 10.1007/978-1-59745-365-3_9

Thomas, P. E., Ryan, D., and Levin, W. (1976). An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal. Biochem.* 75, 168–176. doi: 10.1016/0003-2697(76)90067-1

Welchen, E., Hildebrandt, T. M., Lewejohann, D., Gonzalez, D. H., and Braun, H. P. (2012). Lack of cytochrome *c* in *Arabidopsis* decreases stability of Complex IV and modifies redox metabolism without affecting Complexes I and III. *Biochim. Biophys. Acta* 1817, 990–1001. doi: 10.1016/j.bbabio.2012.04.008

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G. V., and Provart, N. J. (2007). An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* 2:e718. doi: 10.1371/journal.pone.0000718

Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., et al. (2003). Empirical analysis of transcriptional activity in the *Arabidopsis* genome. *Science* 302, 842–846. doi: 10.1126/science.1088305

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 02 December 2013; paper pending published: 07 January 2014; accepted: 24 February 2014; published online: 25 March 2014.

*Citation: Steinebrunner I, Gey U, Andres M, Garcia L and Gonzalez DH (2014) Divergent functions of the Arabidopsis mitochondrial SCO proteins: HCC1 is essential for COX activity while HCC2 is involved in the UV-B stress response. Front. Plant Sci. **5**:87. doi: 10.3389/fpls.2014.00087*

This article was submitted to Plant Physiology, a section of the journal Frontiers in Plant Science.