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Research article

Identification of an *Arabidopsis* mitoferrinlike carrier protein involved in Fe metabolism

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ABSTRACT

Iron has a major role in mitochondrial as well as in chloroplast metabolism, however the processes involved in organelle iron transport in plants are only partially understood.

To identify mitochondrial iron transporters in *Arabidopsis*, we searched for proteins homologous to the *Danio rerio* (zebrafish) Mitoferrin2 MFRN2, a mitochondrial iron importer in non-erythroid cells. Among the identified putative *Arabidopsis* mitoferrinlike proteins, we focused on that one encoded by At5g42130, which we named *AtMfl1* (MitoFerrinLike1).

AtMfl1 expression strongly correlates with genes coding for proteins involved in chloroplast metabolism. Such an unexpected result is supported by the identification by different research groups, of the protein encoded by At5g42130 and of its homologs from various plant species in the inner chloroplastic envelope membrane proteome. Notably, neither the protein encoded by At5g42130 nor its homologs from other plant species have been identified in the mitochondrial proteome.

AtMfl1 gene expression is dependent on Fe supply: *AtMfl1* transcript strongly accumulates under Fe excess, moderately under Fe sufficiency and weakly under Fe deficiency.

In order to understand the physiological role of *AtMfl1*, we isolated and characterized two independent *AtMfl1* KO mutants, *atmfl1-1* and *atmfl1-2*: both show reduced vegetative growth. When grown under conditions of Fe excess, *atmfl1-1* and *atmfl1-2* mutants (seedlings, rosette leaves) contain less total Fe than wt and also reduced expression of the iron storage ferritin *AtFer1*.

Taken together, these results suggest that *Arabidopsis* mitoferrinlike gene *AtMfl1* is involved in Fe transport into chloroplasts, under different conditions of Fe supply and that suppression of its expression alters plant Fe accumulation in various developmental stages.

1. Introduction

Fe is an essential element for all living organisms [1]; the eukaryotic subcellular compartmentalization poses the question of the mechanisms facilitating Fe transport into such organelles, the regulation of their Fe homeostasis and Fe trafficking in such compartments.

In plant cells the synthesis of heme and [Fe–S] clusters, two different co-factors required for proper functionality of many different Fe-containing enzymes, takes place in both chloroplasts and mitochondria, though with different pathways for the [Fe–S] cluster [2]. Heme, like all tetrapyrroles, is synthesised in chloroplasts but evidence for the occurrence of the last three steps of heme biosynthesis in mitochondria has been reported [3–6].

Demand for Fe in both organelles is therefore high and indeed around 90% of Fe in leaf cells is located in chloroplasts, with 75–80% in the chloroplastic stroma [7]; however, mechanisms of Fe transport to chloroplasts and mitochondria have just begun to be elucidated.

At2g15290 encodes an *Arabidopsis* protein essential for plants; this protein, named either PIC1 (Permease in Chloroplasts) [8] or TIC21 (Translocon at the Inner envelope membrane of Chloroplasts) [9,10] is localized in the inner envelope of chloroplasts [8,9]. *Arabidopsis pic1* KO mutants grow only heterotrophically and show a dwarfish and chlorotic phenotype [8]. PIC1 and its homolog from *Synechocystis* mediate iron uptake in yeast double mutant *fet3fet4*, which is defective in Fe uptake [8]; also, accumulation of ferritin clusters occurs in *pic1* chloroplasts, leading to the hypothesis that PIC1 functions in Fe transport across that membrane [8]. However,

Abbreviations: AIS, Arabidopsis growth medium; Col, Columbia; DW, dry weight; EDTA, ethylenediaminetetracetic acid; KO, knock out; EDDHA, ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic acid); FW, fresh weight; SE, standard error; T-DNA, transferred DNA; WT, wild-type.

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conflicting results on the role of PIC1 have been reported: total iron content in *pic1* leaves is not altered, when compared to wt [8] and PIC1 expression is not regulated by Fe. In fact, other research groups showed that At2g15290 encodes a component of a translocation complex which mediates chloroplast protein import across the inner envelope membrane, which has been named TIC21 [9,10]. Such hypothesis is supported also by the evidence that *tic21* (alias *pic1*) mutants are defective in the import of photosynthetic proteins.

Dicots and non graminaceous monocots absorb Fe from the soil by a three-step strategy I: solubilization of Fe(III) through acidification by H^+-ATPase activity, reduction of Fe(III) to Fe(II) by Ferric chelate reductases and Fe(II) uptake by Iron Root Transporters [1,11–13]. Fe(III) has a very low solubility at neutral pH in the presence of oxygen, and an alternative to Fe(III) reduction to Fe(II) is direct chelation of Fe(III) from the soil though phytosiderophores, as it occurs in graminaceous monocots [1,12–14]. Ferric chelate reductase FRO2 is responsible for reduction of iron at the root surface [11,12] but also other members of the FRO family reduce ferric Fe (III) chelates to form soluble Fe(II) [13]. Some FROs isoforms are localized on organellar membranes, such as the chloroplast FRO7, which contributes to the chloroplast Fe acquisition [15].

FRO isoforms are also located in mitochondria, such as FRO3 and FRO8 [12]. FRO3 expression levels are much higher in *Arabidopsis atfer4* mutants whose mitochondrial Fe content is higher than wt [16]. FRO3 expression is induced by Fe deficiency [11] and exposure to Fe excess reduces its expression in *atfer4* cells [16]. FRO8 is not regulated by Fe availability [11] and it is not expressed in *atfer4* cells [16], thus suggesting that FRO3 and FRO8 play different roles in mitochondrial Fe transport.

AtATM3 (alias STA1), an ATP-binding cassette mitochondrial transporter of *Arabidopsis*, is essential for Fe homeostasis and it is implicated in heavy metal resistance [17,18]. Experimental evidence suggests that AtATM3 has a role in transporting [Fe–S] clusters and in regulating cellular GSH levels, possibly by transporting GS-conjugated Cd (II) and/or [Fe–S] clusters across the mitochondrial membrane [18].

Current knowledge of mitochondrial Fe uptake in vertebrate cells can assist the search for Fe transporters into plant mitochondria, in particular *Danio rerio* (zebrafish) is a model used to study different heritable and hematological disorders in humans. Cloning of the *D. rerio frs* locus, associated with severe anaemia and paucity of mature erythrocites cells, allowed the identification of a member of the SLC25 carrier family, expressed in the inner mitochondrial membrane of eukaryotes; this gene mutated in *frs* mutants was therefore named mitoferrin1 *Mfrn1* [19]. Subsequently, a second mitoferrin was also identified, named mitoferrin2 (*Mfrn2*) [19], also localized in mitochondria [20]. *Mfrn1* and *Mfrn2* expression pattern was investigated in different cell lines, as well as mitochondrial iron assimilation in different Mfrn-deficient cell lines (erythroid and non-erythroid) and the effects of *Mfrn1* and *Mfrn2* ectopic expression [19,20]. Results indicate that both MFRN1 and MFRN2 are involved in Fe (II) assimilation into mitochondria and that they contribute to mitochondrial iron metabolism, because heme synthesis is severely reduced when both MFRNs are absent in non-erythroid cells. Although MFRN1 and MFRN2 are functionally redundant in non-erythroid cells, MFRN2 transports more iron into mitochondria than MFRN1 in undifferentiated cells, whereas MFRN1 is the principal mitochondrial iron importer and therefore essential for heme biosynthesis in erythroid cells [19,20].

To identify candidate genes involved in iron transport into plant mitochondria, we searched for *Arabidopsis* proteins showing sequence homology with the *D. rerio* (zebrafish) MFRN2, with the assumption that the plant cell requirement for iron might be more similar to undifferentiated animal cells than to erythroid ones. Among the different mitoferrinlike candidates identified so far, we focused on the *Arabidopsis* MitoFerrinLike1 *AtMfl1* gene At5g42130. We report in the present paper the analysis of its physiological role through the characterization of two independent *atmfl1* KO mutants.

2. Results

2.1. Arabidopsis gene At5g42130, named AtMfl1 (Mitoferrinlike1), encodes a protein belonging to the mitochondrial carrier family and similar to animal mitoferrin

To identify putative plant mitoferrins involved in mitochondrial Fe transport, a search of *Arabidopsis* candidate proteins homologous to MFRN2 was performed with BlastP program. The four *Arabidopsis* genes coding for proteins with highest sequence similarity with MFRN2 are the following (in brackets percent aminoacid identity): At1g07030 (37%), At2g30160 (36%), At1g34065 (30%), At5g42130 (27%), all members of the *Arabidopsis* mitochondrial carriers family [21] (Fig. 1). At1g07030 and At2g30160 were found to be duplicates residing in recent segment duplications between chromosome 1 and 2 [22] whereas At1g34065 codes for a protein named SAMC2 [23] since it has 62% amino acid sequence similarity with a plastid S-adenosylmethionine transporter [23,24], although its real physiological function is still unclear [25]. An un-rooted similarity tree of the various mitochondrial carrier family members, including At1g07030, At2g30160 and At5g42130 has been reported in [22].

Members of this family of carrier proteins are presumed to be targeted to the mitochondrial inner membrane [22]. Aramemnon is a plant membrane protein database which takes advantage of up to 17 individual programs for predicting subcellular location of a given protein (at http//aramemnon.botanik.uni-koeln.de). Aramemnon suggests in fact a chloroplast localization for the proteins encoded by At5g42130 and At1g34065 genes. According to ChloroP 1.1 (at http://www.cbs.dtu.dk/services/ChloroP/) which is one of the prediction programs used by Aramemnon, the chloroplast transit peptide (cTP) of the protein encoded by At5g42130 gene consists of its N-terminal 98 aa whereas the cTP of the protein encoded by At1g34065 gene consists of its N-terminal 43 aa.

No comments on the possible localization are provided by Aramemnon for proteins encoded by At1g07030 and At2g30160 genes.

To verify whether the genes listed in Fig. 1 can be genuinely attributed to the class of carrier proteins, the prediction of their transmembrane regions was also performed: according to TMpred all four encoded proteins have transmembrane domains: five transmembrane helices for At1g07030, At2g30160 and six predicted transmembrane helices for At5g42130 and At1g34065 (Fig. S1A). This latter prediction is consistent with the primary structure of six transmembrane α-helices described in an early study on non-plant mitochondrial carriers [26]. On the other hand, TMHMM Server v. 2.0 suggests three transmembrane domains (Fig. S1B) for At5g42130 and At1g34065.

Among these last two genes, we focused our analysis on *Arabidopsis* gene At5g42130, which we named *AtMfl1* (*MitoFerrinLike1*).

2.2. AtMfl1 expression correlates with the expression of genes involved in chloroplast metabolism and AtMFL1 protein is part of the chloroplast inner envelope membrane proteome

Correlation analysis has been recently applied for investigating gene function but also for identifying new candidate genes for specific processes [27–29]. The extent of correlation can be measured by the Pearson's correlation coefficient [30] using expression values as such or after logarithmic transformation. We have recently applied correlation analysis of a cytochrome P450