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Transgenic Tobacco Lines Expressing Sense or Antisense *FERROCHELATASE 1* RNA Show Modified Ferrochelatase Activity in Roots and Provide Experimental Evidence for Dual Localization of Ferrochelatase 1

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In plants, two genes encode ferrochelatase (FC), which catalyzes iron chelation into protoporphyrin IX at the final step of heme biosynthesis. *FERROCHELATASE1* (*FC1*) is continuously, but weakly expressed in roots and leaves, while *FC2* is dominantly active in leaves. As a continuation of previous studies on the physiological consequences of *FC2* inactivation in tobacco, we aimed to assign *FC1* function in plant organs. While reduced FC2 expression leads to protoporphyrin IX accumulation in leaves, *FC1* down-regulation and overproduction caused reduced and elevated FC activity in root tissue, respectively, but were not associated with changes in macroscopic phenotype, plant development or leaf pigmentation. In contrast to the lower heme content resulting from a deficiency of the dominant *FC2* expression in leaves, a reduction of FC1 in roots and leaves does not significantly disturb heme accumulation. The FC1 overexpression was used for an additional approach to re-examine FC activity in mitochondria. Transgenic FC1 protein was immunologically shown to be present in mitochondria. Although matching only a small portion of total cellular FC activity, the mitochondrial FC activity in a FC1 overexpressor line increased 5-fold in comparison with wild-type mitochondria. Thus, it is suggested that FC1 contributes to mitochondrial heme synthesis.

Keywords: ALA synthesis • Heme biosynthesis • Mitochondria • Transgenic tobacco plants • Tetrapyrroles.

Abbreviations: ALA, 5-aminolevulinic acid; BSA, bovine serum albumin; CaMV, *Cauliflower mosaic virus*, CPX, coproporphyrinogen oxidase; cyt, cytochrome; DMSO, dimethylsulfoxide; DTT, dithiothreitol; FC, ferrochelatase; GFP, green fluorescent protein; HA, hemagglutinin; LHC, light-harvesting complex; LHCP, light-harvesting chlorophyll-binding protein; MgProto IX, Mg protoporphyrin; Proto IX, protoporphyrin; PPOX, protoporphyrinogen oxidase; SNN, Samsun NN; TP, transit peptide; VDAC, voltage-dependent anion-carrier protein; ZnProto, Zn protoporphyrin.

Introduction

Among the tetrapyrroles, heme is required for almost all organisms, although exceptions are known to date, such as the plant-parasitic flagellate *Phytomonas serpens* (Koreny et al. 2012). The heme biosynthetic pathway begins with the synthesis of 5-aminolevulinic acid (ALA), the unique precursor of all tetrapyrrole end-products. Eight molecules of ALA are converted into the first tetrapyrroles. Heme synthesis in animals and fungi proceeds within a linear metabolic pathway (Ajioka et al. 2006, Franken et al. 2011, Bonkovsky et al. 2013). In photosynthetic organisms, tetrapyrrole biosynthesis branches to form different cyclic tetrapyrroles, namely Chl, heme and siroheme (Tanaka and Tanaka 2007, Brzezowski et al. 2015). The linear tetrapyrroles phytochromobilin and phycobilines are derived from protoheme, indicating that heme does not only act as an indispensable cofactor of proteins contributing to primary metabolism (redox reactions of electron transport chains), detoxification of xenobiotics (P450 enzymes) and oxidants (catalase, peroxidase). In addition, heme has been previously proposed to be a regulatory factor in control of transcription and intercellular signaling in yeast and animals (Mense and Zhang 2006, Tsiftsoglou et al. 2006), and more recently in higher plants and *Chlamydomonas* (Moulin and Smith 2005, von Gromoff et al. 2008, Woodson et al. 2011).

The tetrapyrrole biosynthesis pathway branches into Chl or protoheme synthesis, where the metabolite protoporphyrin IX (Proto IX) is the substrate of two differently structured metal chelatases. The Mg chelatase converts Proto IX to Mg protoporphyrin IX (MgProto IX) and ferrochelatase (FC) inserts ferrous iron into Proto IX to form protoheme. Although the biochemistry of the FC reaction is elucidated and X-ray crystallography revealed FC structures and highlighted essential amino acid residues for catalysis (Al-Karadaghi et al. 1997, Sellers et al. 2001), the control of the FC reaction within the tetrapyrrole biosynthetic pathway and the spatial as well as the temporal organization of heme synthesis remain undetermined in plants. All higher plants analyzed so far possess two genes

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encoding FC, which follow a differential tissue-specific and development-dependent expression pattern. It is proposed that *FERROCHELATASE 1* (*FC1*) is constitutively expressed at a low level, but dominantly in root tissue (Chow et al. 1998, Singh et al. 2002, Nagai et al. 2007). *FC1* is induced upon stress and in adverse environmental conditions, while *FERROCHELATASE 2* (*FC2*) is the dominant gene in leaves and shoots and serves predominantly for heme synthesis in photosynthetically active cells (Singh et al. 2002, Nagai et al. 2007).

The diverse expression pattern of the two *FC* genes favored the idea of subcellular localization and diversified functions of the two isoforms for the heme supply to heme-dependent proteins during plant development and in response to endogenous and environmental factors. Initial results obtained by in vitro uptake experiments with plant organelles favored translocation of FC2 to chloroplasts and revealed dual targeting of FC1 to both mitochondria and chloroplasts (Chow et al. 1997, Chow et al. 1998). Later reports brought these observations into question (Lister et al. 2001, Cleary et al. 2002). In chloroplasts, FC is associated with thylakoid and envelope membranes (Roper and Smith 1997, Suzuki et al. 2002). Biochemical separation of envelope and thylakoid membranes from cucumber showed a differential distribution of FC2 with a preferential accumulation in thylakoid membranes (Suzuki et al. 2002). Ultimately, this leads to the conclusion that both FC isoforms presumably serve in heme synthesis for different sets of proteins, which could be located in different cellular compartments. In this line, FC2 possesses a C-terminal extension, which resembles a transmembrane domain of light-harvesting chlorophyll-binding proteins (LHCPs) and is designated as the light-harvesting complex (LHC) motif (Jansson 1999). This peptide domain is proposed to function in Chl binding (Sobotka et al. 2008, Sobotka et al. 2011).

Subcellular localization of FC activity has been previously examined by several research groups. Cornah et al. (2002), Papenbrock et al. (2001) and Masuda et al. (2003) reported the main FC activity in chloroplasts and rather low FC activity in mitochondria. This distribution of subcellular heme synthesis suggests that the dominant plastid FC activity supplies plastidic and cytoplasmic proteins with heme. In *Chlamydomonas reinhardtii*, a single *FC* gene encodes a plastid-localized FC protein (van Lis et al. 2005), while Watanabe et al. (2013) reported a red algal FC isoform, which is found in mitochondrial extracts only.

To date, the organellar enzyme activity measurements could not be backed up by immunological proof of isoform-specific FC antibodies. Previous studies using FC antibodies revealed FC2 in chloroplast membranes (Suzuki et al. 2002, Masuda et al. 2003). However, the immunological confirmation of the subcellular localization of FC1 failed. Several efforts with stable or transient expression of *FC-GFP* (*green fluorescent protein*) gene constructs revealed FC1 as well as FC2 fusion products in plastids only. Also FC1–GFP fusion proteins in planta were apparently exclusively translocated into plastids (Masuda et al. 2003, the authors' own work). However, the assignment of both isoforms to the two organelles and/or the suborganellar membranes of chloroplasts still remains ambiguous in photosynthetic eukaryotes.

Tobacco (*Nicotiana tabacum*) *FC2* antisense lines corroborated the view of a dominant role for FC2 for leaf heme synthesis. These transgenic lines showed a necrotic leaf phenotype as result of accumulating Proto IX (Papenbrock et al. 2001). Apparently, *FC2* deficiency could not be compensated in leaves by *FC1* expression. Furthermore, the non-metabolized Proto IX destined for the Fe branch cannot be sufficiently metabolized by Mg chelatase, which is the initial enzyme of the Mg branch in leaf tissues. This is surprising since Mg^{2+}-chelated tetrapyrroles are by far the dominant end-products of leaf-specific tetrapyrrole biosynthesis, and this hints at a spatial separation of heme and Chl synthesis. Arabidopsis mutants with inactivated expression of the two FC-encoding genes revealed that an *FC1* knock-out mutation is embryo-lethal, while a complete inactivation of *FC2* generates growth-retarded, pale green and photosensitive seedlings (Woodson et al. 2011, Scharfenberg et al. 2015, the authors' own unpublished work).

The data presented to date are all in favor of differential functions of both FC isoforms. Despite the diversified expression of the two FC isoforms, the elucidation of the regulatory mechanisms for distinct heme synthesis still remains challenging. As a continuation of our previous studies on transgenic tobacco plants expressing antisense FC2 (Papenbrock et al. 2001) in photosynthetic leaf tissue, we were interested in examining the specific function of *FC1* in a transgenic tobacco line expressing *FC1* antisense or sense genes. The expression of *FC1* and *FC2* was determined in the transformants, and FC activity was assayed in roots and leaves. Finally, we attempted to re-assess heme synthesis in mitochondria and demonstrated FC activity in mitochondria of an FC1 overexpressor line and the wild type.

Results

Expression of the two FC genes in leaves and roots

A complete cDNA sequence of tobacco (*N. tabacum*) *FC1* was identified (JF428143.1, Alawady, A.). It encodes a protein with a molecular mass of 47.8 kDa. Sequence comparison and bioinformatics tools predicted that FC1 is synthesized as a precursor protein and possesses a cleavable transit peptide (TP) for organellar targeting, e.g. prediction with TargetP (Emanuelsson et al. 2000) gives rise to a value of 0.87 for chloroplastic TP and 0.19 for mitochondrial TP. The TP length is given as 60 amino acid residues. By means of the cDNA sequence, we analyzed the transcript levels of the two *FC* genes in the tobacco variety Samsun NN (SNN). The *FC2* transcript is 13-fold more abundant in leaves compared with roots. *FC1* mRNA accumulates in roots more strongly than in leaves. It is evident that *FC2* is much more highly expressed in leaves than *FC1*, while the roots contain more transcripts of *FC1* than those of *FC2* (**Fig. 1**). The tissue-specific expression pattern of the two *FC* genes results in a >2-fold higher FC activity in chloroplasts of leaves in comparison with plastids in roots (**Fig. 2C**).

However, based on dry weight, the heme content in leaf and root tissue is similar (Fig. 3A).

FC1 inactivation reduces FC enzyme activity in roots

The *FC1* cDNA sequence ultimately enabled us to study the physiological effects of deregulated *FC1* expression. We generated >30 transgenic tobacco lines which synthesize *FC1* antisense RNA under the control of the 35S *Cauliflower mosaic virus* (CaMV) promoter. Since none of the lines displayed a visibly modified phenotype under various light regimes, we selected two lines exhibiting the most pronounced reduction of *FC1* transcript levels for detailed analyses (designated AS#1 and AS#2 in Fig. 2).

We compared *FC1* and *FC2* transcript amounts of the transformants with wild-type expression (Fig. 2A). Due to inactivation by antisense RNA, the *FC1* transcript levels were reduced in root and leaf tissue to a maximum of 30% of the amounts detected in wild-type control plants. *FC2* expression was not significantly modified by *FC1* inactivation. Fig. 2B confirms the phenotypic similarity of transgenic and wild-type seedlings. The plastid FC activity in roots was reduced by 50% in AS#1 and by 30% in AS#2 (Fig. 2C). In contrast, FC activity in chloroplasts of leaves of antisense lines was not diminished by *FC1* inactivation. This is explained by the dominant *FC2* expression in photosynthetic tissue. As a result of partial inactivation of the *FC1* gene, the heme content in root and leaf tissue of *FC* antisense lines was not significantly reduced (Fig. 3A). Also, the Chl content did not differ in leaves of wild-type and antisense lines (Fig. 3B). The latter observation indicates that modified FC1 activitiy does not affect ALA biosynthesis and the Chl-synthesizing pathway in tobacco leaves.

Tobacco lines with decreased *FC2* expression were characterized by elevated steady-state levels of Proto in leaves (Papenbrock et al. 2001). However, the content of Proto IX did not differ significantly in leaf tissue of the two *FC1* antisense lines relative to control seedlings (Fig. 3C). The Proto IX levels in roots were below the level of detection. It can be concluded that the *FC1* inactivation did not reduce the plastid FC activity in photosynthetic tissue.

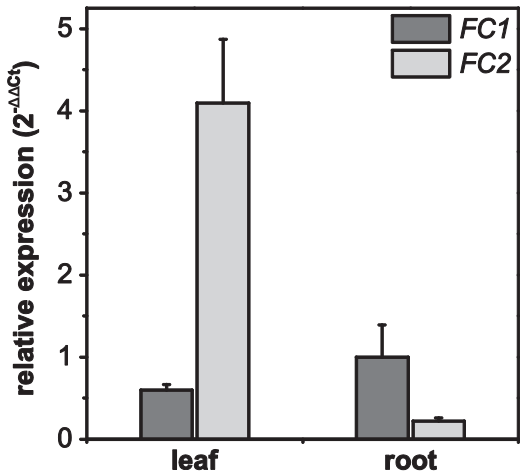

Fig. 1 Expression of tobacco *FC* genes in leaf and root tissue of *N. tabacum* wild-type plants. Data were analyzed by the $2^{-}\Delta\Delta^{Ct}$ method (reference is the *UBC2* gene) and normalized to the *FC1* expression in root tissue.

FC1 overexpression in tobacco causes elevated FC activity in roots

Transgenic tobacco lines were generated and selected for overexpression of *N. tabacum FC1* under control of the 35S CaMV promoter (p35S::FC1). Two lines exhibiting a moderate and high overexpression of *FC1* in leaf and root tissue were selected (Fig. 4A). Upon p35S-driven overexpression, *FC1* transcripts accumulated in the transgenic lines more strongly in leaves than in roots. *FC2* expression was not modified in response to *FC1* overexpression. The impact of the accumulation of *FC1* transcripts was determined using an FC activity assay. In roots, where *FC1* is dominantly expressed in comparison with *FC2*, the enzyme activity is elevated 4-fold in line #14 and 10-fold in line #19 (Fig. 4C). Even in leaves, an increase in FC activity could be determined in line #19, although *FC2*

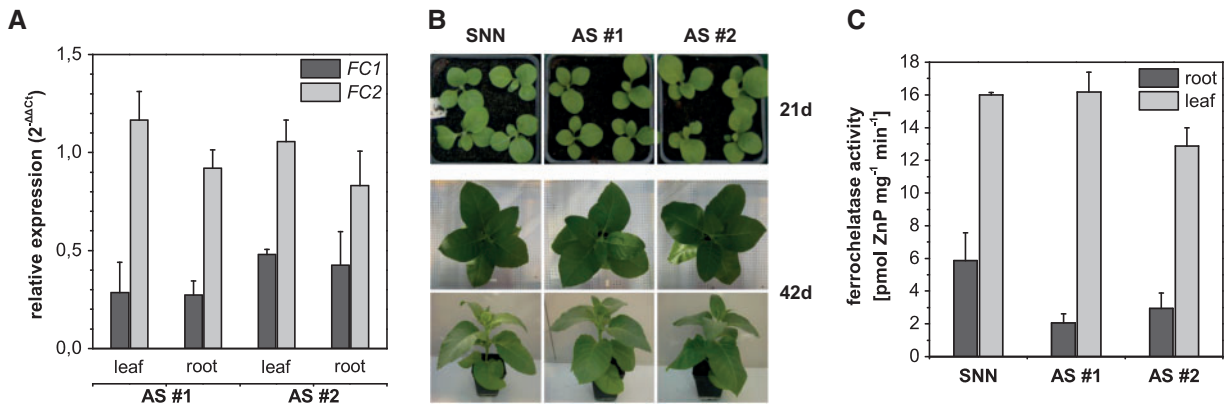

Fig. 2 (A) Confirmation of the selective down-regulation of *FC1* by qRT-PCR analysis of the *FC* genes in leaf and root tissue. Data were analyzed by the $2^{-}\Delta\Delta^{Ct}$ method (reference gene is *UBC2*) and normalized to the expression in wild-type plants. (B) Phenotype of 21- and 42-day-old plants from two independent *N. tabacum* lines expressing *FC1* antisense mRNA. (C) FC enzyme activity. FC activity was measured as the enzymatic production of zinc-protoporphyrin (ZnP) of isolated chloroplasts or crude root plastids, respectively.

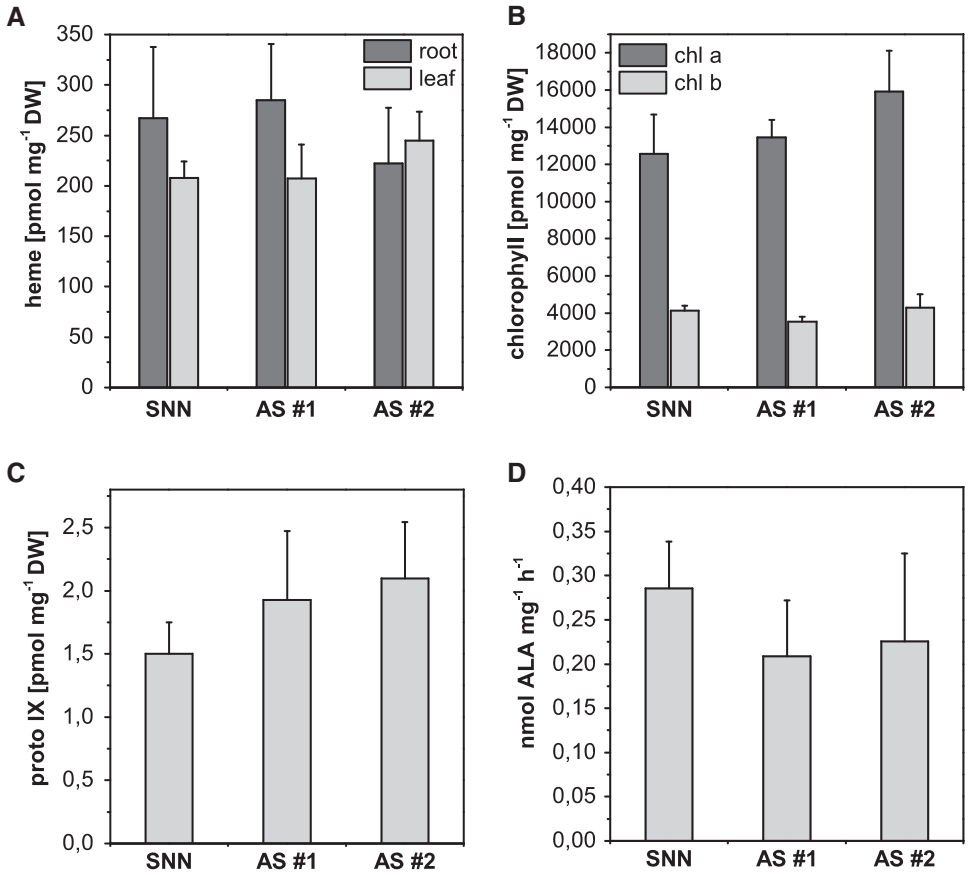

Fig. 3 (A) Heme content of leaf and root tissue from *FC1* antisense lines and wild-type plants. (B) Chl content of leaf tissue. (C) Protoporphyrin IX (Proto IX) contents of leaf tissue. (D) ALA synthesis rate of leaf tissue.

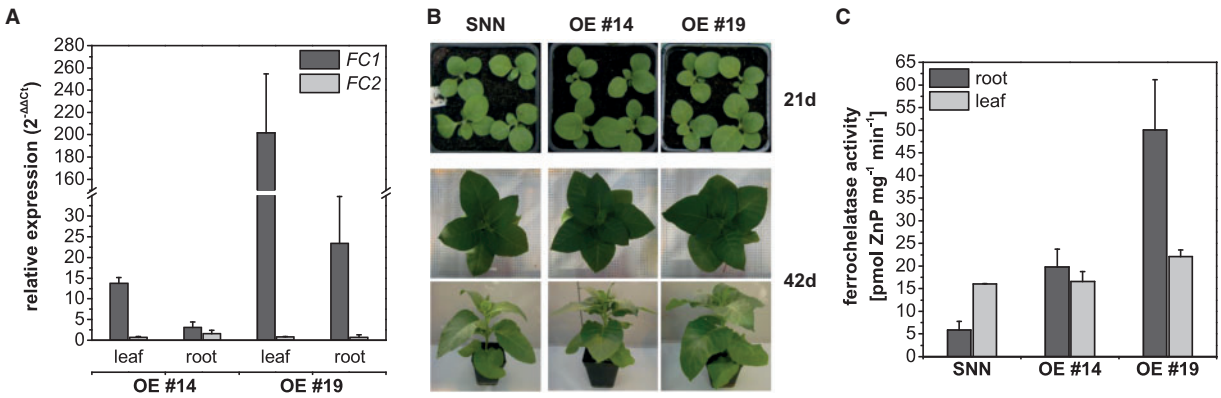

Fig. 4 (A) Confirmation of the selective overexpression of *FC1* by qRT-PCR analysis of the *FC* genes in leaf and root tissue. Data were analyzed by the $2^{-}\Delta\Delta^{Ct}$ method (reference gene *UBC2*) and normalized to the expression in wild-type plants. (B) Phenotype of 21- and 42-day-old plants from two independent *N. tabacum* lines overexpressing an *FC1* sense mRNA. (C) FC enzyme activity. FC activity was measured as the enzymatic production of zinc-protoporphyrin (ZnP) of isolated leaf chloroplasts or crude root plastids, respectively.

expression predominantly contributes to FC enzyme activity in this tissue. However, *FC1* overexpression did not cause any phenotypic alterations in growth and development of transgenic progeny in comparison with the wild type (**Fig. 4B**). These observations were supported by heme and Chl measurements (**Fig. 5A**, **B**) as well as by analyses of Proto IX steady-state levels and ALA synthesis rates (**Fig. 5C**, **D**). The amounts of end-products of tetrapyrrole biosynthesis were not elevated in *FC1* overexpressor lines. The foliar Proto IX content in transformants resembled the wild-type level. ALA synthesis rates were also not modified in response to *FC1* overexpression in leaves. It is concluded that tobacco *FC1* overexpressors display an increased overall FC activity that is more pronounced in root than in leaf tissue. The additional FC1 activity had no obvious impact on the tetrapyrrole biosynthesis pathway, including ALA synthesis and heme levels. Consequently, no changes of

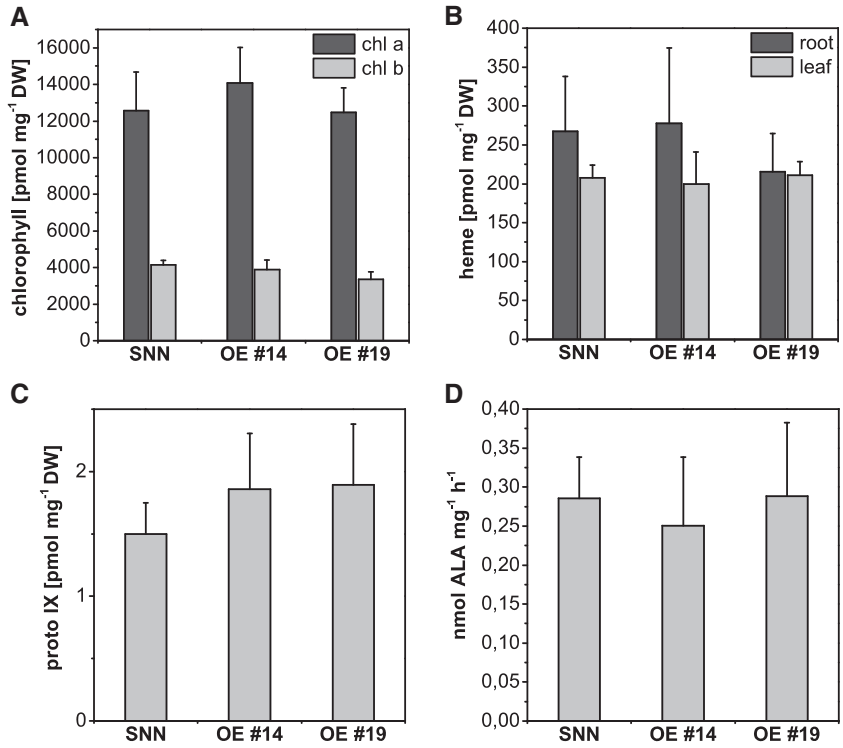

Fig. 5 (A) Chl content of leaf tissue from *FC1* overexpression lines and wild-type plants. (B) Heme content of leaf and root tissue. (C) Protoporphyrin IX (Proto IX) contents of leaf tissue. (D) ALA synthesis rate of leaf tissue.

the macroscopic phenotype of the *FC1* overexpressor lines were observed and it is expected that at the molecular and metabolic level no modification of the heme-dependent metabolic feedback can be determined.

FC activity in mitochondria of tobacco leaves

It was concluded in previous reports that the mitochondrial activity of FC was rather low (Cornah et al. 2002, Masuda et al. 2003). Overproduction of *FC1* was proposed to be appropriate to track down potential FC activity in mitochondria. Leaf mitochondria could be separated well from remnants of plastid membranes, as immune studies indicated for the thylakoid membrane-localized light-harvesting chlorophyll-binding protein LHCA and the mitochondrial voltage-dependent anion-carrier protein (VDAC) (**Fig. 6A**). The lack of an immune response for the anti-LHCA antibody in the mitochondrial fraction illustrates that the plastidic contamination is below the detection level (**Fig. 6A**).

The mitochondrial fraction of the *FC1*-overexpressor line #19 shows a >5-fold increase of FC activity in comparison with the wild-type control (**Fig. 6B**). Further studies proved the intactness of mitochondria of the wild type and *FC1* overexpressor employing Cyt *c* oxidase activity measurements (**Fig. 6C**). However, despite the elevated FC activity, the heme content did not differ in mitochondria of the *FC1* overexpressor and wild-type plants (**Fig. 6D**). It is proposed that either the elevated FC activity did not stimulate heme synthesis in mitochondria due to a limited supply of its substrate for heme synthesis from ALA synthesis or the heme turnover is enhanced as a result of an elevated heme production.

As an FC1-specific antibody is currently not available to assess the translocation of FC1 to mitochondria, we ectopically expressed FC1 harboring a hemagglutinin (HA)-tag in tobacco plants (see also the Materials and Methods). The tobacco line FC1-tag #6 showed similar *FC1* expression to the FC-1OE line #14. The wild-type-like phenotype also resembled that of the *FC1* overexpressor lines described above (data not shown). The immune analysis of the total and mitochondrial protein extracts revealed a significant fraction of FC1-HA to be localized in mitochondria (**Fig. 6E**). The purity of the mitochondrial fraction could be verified with plastidal and mitochondrial marker proteins. The anti-LHCB1 serum did not show an immune-reacting signal in the mitochondrial fraction. Comparing the intensity of the immune signal of the anti-VDAC antibody in the total fraction vs. the mitochondrial fraction, the signal was about 5-fold increased in mitochondria. The HA immune signal in mitochondria of the FC1-HA-expressing line was weaker than in the total protein extract, indicating that the FC1-HA protein is predominantly localized outside the mitochondria. We estimate that at least 10% of FC1-HA is detectable in mitochondria.

Discussion

The FC isoforms contribute to different heme pools

During phylogeny, angiosperms evolved two *FC* genes, and the encoded proteins differ in their primary structure (Chow et al. 1998, Obornik and Green 2005). An alignment of the two

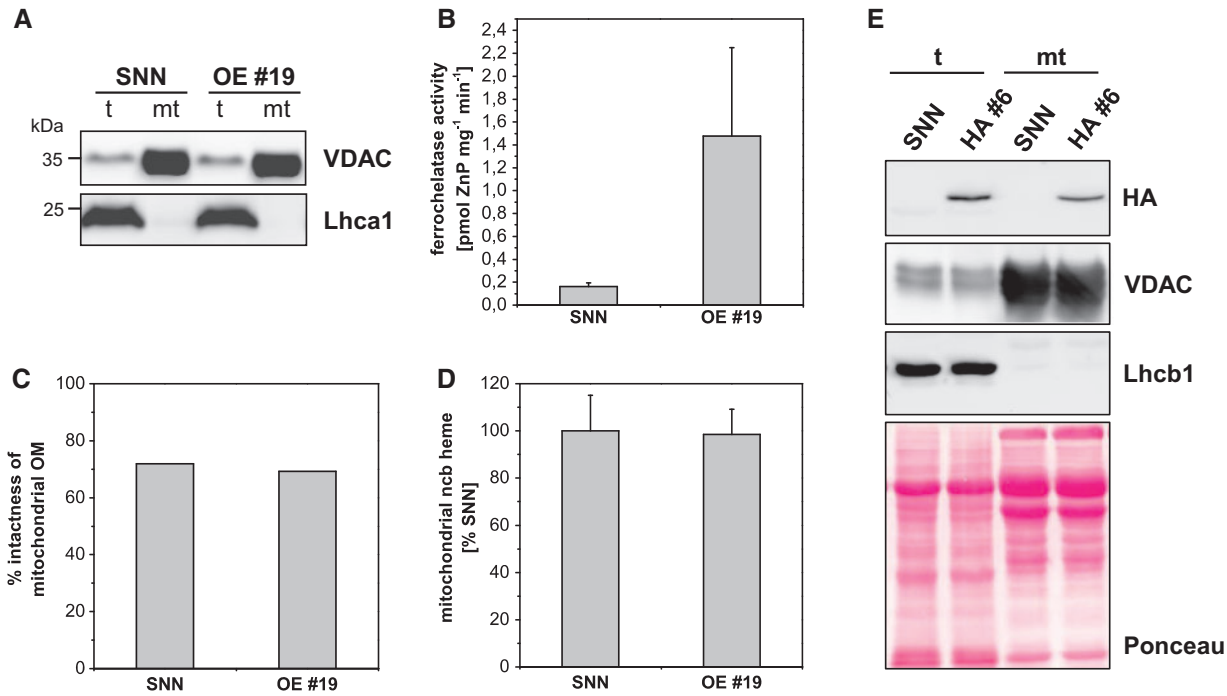

Fig. 6 (A) Depletion of plastidic membranes in the isolated mitochondria. Total leaf proteins (t) and mitochondrial proteins (mt) were separated by SDS–PAGE and, after membrane transfer, the membranes were probed with specific antibodies against VDAC (voltage-dependent anion carrier, porin) and Lhca1 (light-harvesting chlorophyll-binding protein of PSI), respectively. (B) FC enzyme activity measured with mitochondria isolated from wild-type plants and plants of the *FC1*-overexpressing line #19, respectively. (C) Intactness of the outer membrane of isolated mitochondria was determined by measuring the Cyt *c* oxidase activity with/without dodecyl maltoside. (D) Relative heme content of mitochondria in SNN wild type and the line #19. (E) Presence of FC1 protein in mitochondria. Total leaf proteins (t) and mitochondrial proteins (mt) of wild-type plants (SNN) and transgenic line #6 expressing FC1-HA (HA #6) were separated by SDS–PAGE and, after membrane transfer, the membranes were probed with specific antibodies against VDAC, Lhca1 and HA-tag, respectively.

mature FC sequences of tobacco reveals 62% identity. The identity of both isoforms increases up to 76% when the C-terminal extension of FC2 is not included in the alignment. However, due to the high similarity between the two plant isoforms and the structural diversity of these proteins in comparison with the animal FC, it was proposed that the two plant isogenes are not derived from two phylogenetically distant genomes (Obornik and Green 2005). When the two plant *FC* genes evolved from gene duplication, distinct expression profiles developed in different organs and under different growth conditions (**Fig. 1**), and the *FC* gene products took on different functions in heme synthesis.

Derived from the divergent transcript levels of both genes, previous suggestions support that *FC2* provides heme for heme-dependent proteins of photosynthetic processes, while the expression of tobacco *FC1* is stronger in non-photosynthetic tissue. Our result is consistent with findings in Arabidopsis, pea and cucumber (Chow et al. 1998, Singh et al. 2002, Masuda et al. 2003, Nagai et al. 2007, Scharfenberg et al. 2015). Considering that both FCs serve for different heme pools in different plant organs, it is not excluded that both FC isoforms are associated with different suborganellar compartments in plastids as specific membrane distribution of the two FC isoforms was reported (Suzuki et al. 2002).

Modified *FC1* expression is predominantly displayed in roots

The generation of *FC1* antisense tobacco plants enabled biochemical and physiological analyses of deregulated heme synthesis. As in previous studies employing *FC2* antisense transformants (Papenbrock et al. 2001), it was expected to obtain physiological indications for the functional and regulatory importance of *FC1* expression. While *FC2* gene silencing led to severe leaf necrosis and photodynamic cell death as a result of Proto IX accumulation, reduction of the *FC1* transcript levels did not cause macroscopic modifications of leaves and shoots of the transgenic lines. Although the *FC1* mRNA content was similarly reduced in roots and shoots, the FC activity in leaf tissue was not diminished compared with the wild type.

Interestingly, among all the investigated *FC1* antisense transformants, no lines were found with an almost complete *FC1* gene silencing. In this context, it is worth mentioning that the *Arabidopsis thaliana fc1-2* null mutant is lethal (Woodson et al. 2011, Scharfenberg et al. 2015, the authors' own unpublished observation). It seems that this isoform is indispensable during embryogenesis or seed development (Woodson et al. 2011). Another recently reported allelic *fc1-1* mutant possessing the T-DNA insertion in the 5′-untranslated region of the *FC1* gene remained wild-type-like during photoperiodic growth (Scharfenberg et al. 2015). In analogy to the observations of

the allelic *fc1* mutants in Arabidopsis, it is proposed that those tobacco *FC1* antisense lines which display a completely inactivated *FC1* expression would not survive because of an arrest in embryo or seed development.

Due to the fact that FC2 is the dominantly expressed isoform in leaf tissue, it is conceivable that FC activity will not be altered by the reduction of *FC1* expression obtained in the *FC1* antisense lines described here. In agreement with this, the FC activity, the ALA synthesis rate and the Proto IX steady state-levels in leaf tissue did not differ between control and FC1 antisense lines (**Figs. 2, 3**).

In contrast, root FC activity was reduced by 50–60% in both representative lines compared with the wild type. However, the reduced activity did not affect the heme content in roots. Proto IX levels as well as ALA synthesis rates could not be determined in root tissue due to low metabolic activities of tetrapyrrole biosynthesis (**Figs. 2, 3**). It is consistent that neither root formation and growth nor the root metabolic activities were visibly compromised. We assume that the observed partial deficiency of FC1 in roots of adult plants is sufficiently compensated for adequate heme formation by residual FC1 as well as FC2 activity. In leaves, the partial loss of FC1 activity is functionally substituted by FC2.

FC1 overexpression was examined in transformants displaying either moderate or strong *FC1* mRNA overproduction in lines #14 and #19. The *FC1* overexpression is more pronounced in transgenic leaves than in root tissue (**Fig. 4**). However, root FC activity increased 8-fold in comparison with wild-type seedlings, while the leaves of the *FC1* overexpressor line displayed only a 20–30% increase. The modified FC activities did not stimulate the overall pathway, as revealed by unaffected ALA synthesis rates and constant heme levels (**Fig. 5**). We conclude that partial inactivation as well as an overexpression of *FC1* leads to modified FC activity in roots, but the morphology and metabolism of roots do not seem to be altered.

The transgenic lines did not show obvious variations in the overall tetrapyrrole biosynthesis pathway upon *FC1* deregulation, as Chl and heme contents were not modified. It is striking that FC1 inactivation and overproduction did not lead to diminished and elevated heme levels, respectively. Plants seem to have a regulatory mechanism that secures heme homeostasis. It is suggested that moderately decreased as well as elevated *FC1* RNA levels can be compensated in a concerted control of synthesis and activity of both FC isoforms during plant growth under normal growth conditions providing an adequate heme synthesis. However, the balanced heme content was compromised either when *FC2* expression was silenced in tobacco or when *FC1* was overexpressed in 3- to 5-day-old Arabidopsis seedlings treated with norflurazon (Papenbrock et al. 2001, Woodson et al. 2015).

In spite of stable heme contents determined in plastids and mitochondria of transgenic tobacco lines with deregulated *FC1* expression, it is not excluded that a heme-mediated feedback control affects transcriptional or post-translational control of tetrapyrrole biosynthesis, as was found in yeast and animal heme synthesis (Ajioka et al. 2006, Franken et al. 2011). Alternatively, deregulated *FC1* expression is less relevant during normal growth of tobacco plants, once early embryo development has succeeded.

FC1 displays activity in mitochondria

Previous results have demonstrated that FC2 is exclusively located in chloroplasts. While cucumber FC2 is mainly localized in thylakoid membranes, FC1 was also found in the plastidic envelope membrane (Suzuki et al. 2002). FC1 was previously reported to be translocated into mitochondria as well as into plastids (Chow et al. 1997, Suzuki et al. 2002). However, clear-cut in planta evidence was not reported for a dual targeting of FC1. Several attempts employing FC1–GFP fusions as well as proteomic approaches failed to detect any (partial) mitochondrial localization of FC1 (i.e. in tobacco, the authors' own unpublished experiments; in cucumber, Masuda et al. 2003).

Interestingly, the two successive enzymes in plant tetrapyrrole biosynthesis, protoporphyrinogen oxidase (PPOX) and FC, are known to be present in two isoforms. This inter-relationship prompted the assumption that one of the two isoforms of each enzyme may be functioning in plastids and mitochondria, respectively. However, this hypothesis is still difficult to verify. PPOXI and PPOXII were located either in the envelope or in the thylakoid membrane of chloroplasts, respectively (Watanabe et al. 2001). In vitro organellar import as well as immunoblot studies of plant organelles hinted at PPOXII being targeted to mitochondria (Lermontova et al. 1997). To combine previous findings, it is proposed that PPOXII is dually targeted to plastids and mitochondria, although an enzyme assay for PPOX activity in plant mitochondria has not been reported to date. In this context, it is worth mentioning that Williams et al. (2006) reported two *CPX* genes in maize encoding coproporphyrinogen oxidase and showed exclusive targeting of CPX2–GFP to mitochondria, while CPX1–GFP was translocated to plastids. As a *CPX1* null mutant survives in tissue cultures, the authors proposed that the mutant is capable of heme synthesis through a CPX2-exploiting mitochondrial pathway (Williams et al. 2006).

We aimed in our experiments to distinguish mitochondrial FC activity from the plastid activity and to verify FC1 localization in mitochondria. We made use of isolated mitochondria from wild-type and *FC1*-overexpressing plants and determined a >5-fold increase of mitochondrial FC activity in line #19 in comparison with the wild-type control. Because the FC activity in chloroplasts of line #19 increased approximately 20% in comparison with the wild type, both results are indicative that *FC1* overexpression leads to elevated FC activity in both organs. The immune blot analyses (**Fig. 6A, E**) indicate that the determined FC activity of the mitochondrial fraction is not the result of contamination by plastids.

Low FC activity in mitochondria was also previously determined in cucumber (Masuda et al. 2003). Cornah et al. (2002) and Papenbrock et al. (2001) reported 30% mitochondrial FC activity compared with plastid activity in pea and tobacco. respectively, on the basis of similar protein amounts in isolated chloroplasts and mitochondria used for activity measurements. Taking into account that the protein content of mitochondria is low compared with the chloroplastic and overall cellular

protein amounts in tobacco leaf cells, the mitochondrial FC activity measured here represents a rather low proportion of total FC activity. Consistent with the mitochondrial FC activity of *FC1* overexpressor lines, the translocation of FC1 to mitochondria was confirmed by immune analysis using a HA-tagged FC1 protein which was expressed in tobacco. A comparison of the total and mitochondrial protein fractions confirmed the dual targeting of FC1. A minor but significant fraction was detected in mitochondria, while FC1 was predominantly targeted to plastids (**Fig. 6E**).

Distinct intracellular functions of FC1 and FC2 have been proposed in chloroplasts (Woodson et al. 2011). On the basis of the current data, it is assumed that due to its distinct expression, FC1 serves a role dependent on its organellar location for separate heme pools. Considering the FC activity in two subcellular compartments, it is proposed that FC1 directly supplies heme for mitochondrial proteins while the plastid-localized portion of FC1 serves for heme formation, which is required for plastid and cytoplasmic hemoproteins.

Final remarks

The subcellular distribution of the two FC isoforms in plants has been primarily investigated (Singh et al. 2002, Suzuki et al. 2002, Nagai et al. 2007). To date, additional experimental verifications have not been presented. We investigated the physiological relevance of FC1 in tobacco. It is evident that additional clarification of the subcellular heme synthesis is required. It will be a challenging task to verify mitochondrial function and localization of FC1 when a new series of elaborated and purified specific antibodies against FC are available to confirm the subcellular distinction of FC1. Then, the initial in vitro results on mitochondria-translocated FC1 can be verified and further research will be promoted with the perspective of addressing the assumed transfer of Proto(gen) and heme between the cellular compartments and the proposed heme pools generated by FC1 and FC2.

Materials and Methods

Plant material and growth conditions

Tobacco wild-type plants (cultivar SNN) and transgenic lines expressing *NtFC1* cDNA in sense or antisense orientation under control of the 35S CaMV promoter were grown on soil in a greenhouse at 200 µmol photons s^{-1} m^{-2} under cycling light conditions (16 h light, 8 h dark). For isolation of root plastids, 3-week-old plants were transferred from soil into hydroponic culture and grown under similar conditions for a further 2 weeks. As nutrient solution, 0.5× Hoagland medium was used (Hoagland and Arnon 1938, Hoagland et al. 1938) supplemented with 45 µM Fe-EDTA (Jacobson 1951). Generally, experiments were performed with 5- to 6-week-old plants.

Generation of transgic tobacco plants

To generate FC1 antisense lines (abbreviated as 'AS'), a 1.05 kb fragment of the coding region of tobacco *FC1* cDNA was amplified using TCCCGGGCATTGGA TCATTGTCAACTTC and TGTCGACGAAGAAAAGATTGGGGTGCTA, and cloned into vector pJET1.2 (Thermo Scientific). The *FC1* sequence was cut out using restriction endonucleases *Sma*I and *Sal*I (New England Biolabs) and cloned into the binary vector pBinAR (Hofgen and Willmitzer 1988) which was linearized using the same enzymes. For generation of FC1 overexpressor lines ('OE'), oligonucleotides GAGCTCACTATTTGATCCAAATCTTCAC and GAGC TCCTAAAGGATGTTCTTCCTAAA were employed. The cloned cDNA fragment was transferred from pJET1.2 to the binary vector pGL1 (Apitz et al. 2014) using *Sac*I restrictions. Sense orientation of the insert was checked by PCR. A C-terminally tagged tobacco FC1 protein ('HA') was created using GAG CTCACTATTTGATCCAAATCTTCAC and GAGCTCCTGCAGGAAGGATGTT CTTCCTAAAAGC. Following insertion into pJET1.2, the plasmid was opened with *Sbf*I. A linker carrying a HA as well as a FLAG tag was created by annealing the oligonucleotides GGATACCCATACGATGTTCCAGATTACGCTGACTACA AGGACGACGACGACAAGTAACCTGCA and GGTTACTTGTCGTCGTCGTCC TTGTAGTCAGCGTAATCTGGAACATCGTATGGGTATCCTGCA and subsequently ligated into the *Sbf*I site of the pJET clone. The fusion product was transferred to vector pGL1 with the help of *Sac*I. Transformation of tobacco plants (*N. tabacum* cv. SNN) was performed using *Agrobacterium tumefaciens* strain C58C1:pGV2260 as reported by Rosahl et al. (1987) .

Extraction of tetrapyrrole and HPLC analysis

Freeze-dried plant material (leaf and root tissue) was homogenized in liquid nitrogen. Chl and Proto IX were extracted in ice-cold basic acetone (acetone : 0.2 N NH_4OH, 9 : 1), whereas non-covalently bound (ncb) heme was extracted in acidic acetone [acetone : dimethylsulfoxide (DMSO) : 37% HCl, 100 : 20 : 5] at room temperature. In leaf material, Chl was initially removed by basic acetone extraction prior to heme extraction. The extracts were separated by HPLC, and tetrapyrroles were quantified using pure standards following the protocol described in Czarnecki et al. (2011).

Gene expression analyses

Total RNA from leaf and root tissue was extracted using the TriSure reagent (Bioline) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of RNA was treated with DNase I (Thermo), and cDNA was generated using RevertAid reverse transcriptase (Thermo). Quantitative real-time PCR was performed with SensiMixSYBR (Bioline) using a CFX 96 real-time system (Bio-Rad). Data were analyzed by the $2^{-}\Delta\Delta^{Ct}$ method (Pfaffl 2001) and normalized to wild-type expression using *UBC2* as the reference gene, unless otherwise stated. Primers used for qPCR had comparable primer efficiencies and the following sequences: UBC2 CTGGACAGCAGACTGACATC (F), CAGGATAATTTGCTGT AACAGATTA (R); FC1 TCGGCTTCTTCAACCAATGA (F), ATGGCAAGCAGG CATTAACC (R); FC2 TGGAAGAGCTATTGGCAGCA (F) and GCTCTTCCGTT CCACGTTTC (R).

SDS–PAGE, Western blot and immune detection

Total protein extracts were prepared by grinding leaf material in liquid nitrogen, followed by resuspension into 2-fold Laemmli buffer [100 mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 2 mM dithiothreitol (DTT), 0.01% bromophenol blue]. Before loading onto SDS–polyacrylamide gels, samples were incubated at 95°C for 10 min. Organellar protein extracts were prepared by pelleting organelles from wash buffer in a tabletop centrifuge followed by resuspension in Laemmli buffer (instead of FC assay buffer) and incubation at 95°C for 10 min. SDS–PAGE was performed in 12% acrylamide mini-gels (Laemmli 1970). Subsequently, proteins were transferred onto nitrocellulose membranes by semi-dry Western blotting, and membranes were probed using specific antibodies. Clarity™ Western ECL Blotting Substrate (Bio-Rad) was used for immune signal detection. Protein concentration was determined by the Bradford assay using RotiQuant solution (Roth) according to the manufacturer's instructions. As a standard, bovine serum albumin (BSA; Applichem) was used.

Isolation of chloroplasts, root plastids and mitochondria

Chloroplasts were isolated from young leaves. Briefly, 20 g of leaves were homogenized twice for 3 s in a Waring blender equipped with razor blades in extraction buffer (450 mM sorbitol, 20 mM Tricine-KOH pH 8.4, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1% BSA). After filtering through one layer of miracloth, crude chloroplasts were pelleted by centrifugation (20 min at 800×*g*). Chloroplasts were resuspended, loaded onto a Percoll step-gradient (20–80% Percoll in 300 mM sorbitol, 20 mM Tricine-KOH, pH 8.4) and separated by centrifugation

for 30 min at 6,500×*g*. Intact chloroplasts were taken from the interphase of both layers, washed once with wash buffer (300 mM sorbitol, 20 mM Tricine-KOH pH 8.4) by centrifugation (5 min at 3,800×*g*) and finally resuspended into a small volume of FC assay buffer.

Crude root plastids were isolated according to Emes and England (1986). In brief, roots (5–10 g) were cut into small slices with a razor blade followed by manual homogenization in extraction buffer (330 mM sorbitol, 50 mM Tricine-KOH pH 7.9, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA) using a mortar. After a short centrifugation step to remove insoluble material (2.5 min at 200×*g*), the supernatant was underlaid with a 10% Percoll solution in wash buffer (330 mM sorbitol, 50 mM Tricine-KOH, pH 7.9) and crude root plastids were enriched by centrifugation through this Percoll cushion (10 min at 4,000×*g*). The pellet was washed once with wash buffer by centrifugation (5 min at 4,000×*g*) and finally resuspended into a small volume of FC assay buffer.

Mitochondria were extracted from leaves according to Sabar et al. (2000). Leaves (50 g) were homogenized twice for 8 s in a Waring blender equipped with new razor blades in extraction buffer [600 mM sorbitol, 5 mM EDTA, 50 mM MOPS-KOH pH 7.5, 0.5% polyvinylpyrrolidone (PVP)-40, 0.4% BSA, 8 mM cysteine]. The homogenate was filtered through one layer of miracloth supported by a nylon mesh, and crude mitochondria were enriched by two successive low-speed and high-speed centrifugation steps (1,000×*g* for 10 min and 10,000×*g* for 20 min). After each low-speed step, the supernatant was used for the following high-speed step, whereas after the high-speed steps the pellet was carefully solubilized by using a Potter homogenizer. The enriched mitochondria were subsequently loaded onto a Percoll step gradient for further purification (18%–25%–50% in wash buffer: 600 mM sorbitol, 10 mM MOPS-KOH pH 7.5). After centrifugation (45,000×*g* for 45 min) pure mitochondria were isolated from the 25–50% interphase. The remaining Percoll was removed by washing the mitochondria three times in wash buffer and centrifugation (20,000×*g* for 10 min). Finally the mitochondria were resuspended in a small volume of FC assay buffer or stored in wash buffer at −80°C, respectively.

FC activity assay

FC enzyme activity was measured as the enzymatic formation of zinc-protoporphyrin IX (ZnProto IX) according to Papenbrock et al. (1999) with modifications: As total extracts showed unspecific formation of ZnProto IX, isolated organelles (500–1,000 μg of protein) were used. After isolation, the organelles were resuspended in assay buffer containing 100 mM Tris–HCl, pH 7.6, 1 mM DTT, 0.25% Triton X-100 and 1.75 mM palmitic acid. Samples were incubated at 30°C and the reaction was started by adding Proto IX and zinc sulfate to 10 and 100 μM, respectively. Aliquots were taken at the designated time points. ZnProto IX was extracted by adding 7 vols. of acetone : methanol : 0.1 N NH_4OH, 50 : 49 : 1) on ice and quantified by HPLC. Specific enzyme activity was defined as ZnProto IX formed per time and protein amount and expressed in pmol mg^{-1} protein min^{-1}.

ALA synthesis rate

The ALA synthesis rate was measured according to Mauzerall and Granick (1956) with minor changes. Briefly, leaf discs (50 mg FW) were incubated in 40 mM levulinic acid, 50 mM Tris–HCl, pH 7.2 for 3–4 h under growth light conditions. Then, the leaf material was ground in liquid nitrogen and resuspended into 50 mM KPO_4 buffer, pH 6.8. After centrifugation, the supernatant was mixed 4 + 1 with ethyl acetoacetate, boiled for 10 min and mixed 1 : 1 with modified Ehrlich reagent. ALA concentration was determined spectrophotometrically and compared with a standard.

Cyt *c* oxidase assay

Intactness of mitochondrial outer membranes was assayed with a Cyt *c* oxidase assay kit (Sigma) according to the manufacturer's instructions.

Statistical analysis and validation

Experiments were performed in general in at least three biological replicates. Mean values and SDs were calculated, and curve fittings of the enzyme activity measurements were performed with Microsoft Excel.

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Disclosures

The authors have no conflicts of interest to declare.

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