Effect of Fe deficiency on mitochondrial alternative NAD(P)H dehydrogenases in cucumber roots

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Abstract
Iron deficiency affects the function of the respiratory chain, primarily at the complex I and complex II levels. Because plant mitochondria possess alternative NAD(P)H dehydrogenases located in the inner membrane, oxidizing NAD(P)H from both cytosol and matrix, we investigated these activities in mitochondria of Fe-deficient roots. External and internal NAD(P)H dehydrogenase activity increased in Fe-deficient mitochondria. Accordingly, NDB1 protein strongly accumulated, while NDA1 did not show differences in Fe-deficient roots. The data presented support, for the first time, the hypothesis that Fe deficiency induces the alternative NAD(P)H dehydrogenases, bypassing the impaired complex I.

Keywords
Alternative NAD(P)H dehydrogenase*Cucumis sativus* L.Fe deficiencyMitochondriaRoot
Abbreviations
FCCPcarbonyl cyanide p-(trifluoromethoxy)phenylhydrazoneND-DHsalternative NAD(P)H dehydrogenasesNDexexternal NAD(P)H dehydrogenaseNDininternal NAD(P)H dehydrogenaseUQubiquinoneUQH2ubiquinol
Introduction
Strategy I plants (Römheld and Marschner, 1986) respond to Fe shortage in the soil by increasing three main types of activity at the root level: Fe(III)-chelate reductase (FC-R), the H+-ATPase and the iron-regulated transporters (IRT) (Curie and Briat, 2003). Their induction strongly increases the demand for NAD(P)H and ATP, leading to an overall adaptation of the metabolism. Indeed, in Fe-deficient plants, several metabolic changes occur (Zocchi, 2006 and references therein). Moreover, mitochondria are strongly affected by Fe deficiency, mainly at the respiratory chain level (Vigani et al., 2009). Notwithstanding these effects, they might still play a pivotal role in the metabolic changes that occur under these conditions (Vigani and Zocchi, 2009). In fact, plant mitochondria are characterized by the presence of several alternative pathways able to oxidize reducing equivalents, such as alternative NAD(P)H dehydrogenases (ND-DHs) (Møller and Lin, 1986; Rasmusson et al., 1998). Distinct external rotenone-insensitive enzymes oxidize cytoplasmic NADH and NADPH in a calcium-dependent manner (Roberts et al., 1995). Matrix NADH can be oxidized via two alternative enzymes. NADH oxidation, through the rotenone-sensitive complex I, mediates ATP production via proton pumping, whereas the activity of the rotenone-insensitive NADH dehydrogenases is not restricted by coupling to energy conservation (Rasmusson and Møller, 1991a). Additionally, a calcium-dependent rotenone-insensitive NADPH dehydrogenase is present at the matrix surface of the inner membrane (Rasmusson and Møller, 1991b; Melo et al., 1996).
As reported by Vigani et al. (2009), ND-DHs could be activated to bypass the dramatic loss of complex I and complex II activities occurring in Fe-deficient cucumber roots. The external and internal localization of these enzymes in the inner mitochondrial membrane allows them to oxidize the NAD(P)H from both the cytosolic and the matricial sides, but there is no evidence thus far to support their involvement in the response to Fe deficiency. In this work, we report results supporting this involvement.
Materials and methods
Plant material and isolation of mitochondria
Seed germination and growth of cucumber (*Cucumis sativus* L. cv. Marketer) plants were performed according to Vigani et al. (2009). Iron-deficient plants showed the morphological responses typical of Fe deficiency (development of leaf Fe deficiency chlorosis, stunted growth and

appearance of lateral roots) and the increases in specific activities (Fe3+-chelate reductase and H+-ATPase) typical of Strategy I plants (Supplementary Table 1). Mitochondria were isolated from Fe-sufficient and 10-day deficient roots according to Vigani et al. (2009). Mitochondrial integrity was calculated according to Wigge and Gardeström (1987).

Protein gel blot analysis

Protein gel blot analysis of purified mitochondria extracts was performed according to Vigani et al. (2009) using two different polyclonal antibodies corresponding to the NDA1 and NDB1 from potato (Svensson and Rasmusson, 2001) and the antibody corresponding to the porin from maize as control protein (Vigani et al., 2009). Protein content of purified mitochondria was determined according to Bradford (1976), using BSA as a standard.

Dehydrogenase activity

The alternative NAD(P)H dehydrogenase activities were measured on intact mitochondria according to Svensson and Rasmusson (2001) with a small modification in the buffer composition (0.4 M mannitol, 10 mM MOPS pH 7.2, 2.5 mM MgCl2, 0.1 mM EGTA, 0.4 mM carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP)).

Miscellaneous

Determination of FC-R and H+-ATPase activity was performed according to Rabotti and Zocchi (1994), and citrate synthase according to Vigani et al. (2009).

Results

Alternative NAD(P)H dehydrogenase activities in Fe-deficient mitochondria

In order to confirm the hypothesis postulating the induction of alternative NAD(P)H dehydrogenase activity to bypass the inhibition of complex I induced by Fe deficiency, mitochondria were purified from roots of cucumber plants grown in the presence or in the absence of Fe. Purified mitochondria from control and Fe-deficient roots showed high integrity (86% and 84%, respectively) (Table 1). It was possible to discriminate between external and internal NAD(P)H oxidation by an osmotic burst of mitochondria.

Table 1. Integrity of mitochondria purified from root of cucumber plant grown in the presence (+Fe) or in the absence (–Fe) of Fe.

Sample	**Cytochrome *c* oxidase activity (nmol min–1 mg–1)**		**Integrity (%)**
	–Triton	+Triton	
+Fe	93±7	665±51	86
–Fe	108±8	678±54	84

The integrity of mitochondria was determined as following: 100–(100×(cytochrome *c* oxidase activity without triton/cytochrome *c* oxidase activity with triton)). Data are the means±SE of three independent experiments in triplicate (n=9). SE never exceeded 8%.

Table 2a shows that, in Fe-deficient mitochondria, the internal rotenone-sensitive NADH oxidation (via complex I) strongly decreased, in agreement with the inhibition of complex I activity observed previously (Vigani et al., 2009). On the contrary, the internal rotenone-insensitive NADH oxidation activity increased in Fe-deficient mitochondria, while both the external NADH and NADPH oxidation activities increased three-fold compared to the control. In our previous work, we normalized mitochondrial activity by using citrate synthase activity, since under Fe deficiency there is an increase in the number of mitochondria (Vigani et al., 2009). When these results were normalized (Table 2b) a one-fold increase of the external NAD(P)H activity was still present, while for internal rotenone-insensitive NADH, only a small increase was evidenced. These results suggest a general increase in internal NAD(P)H dehydrogenase (NDin) and external NAD(P)H dehydrogenase (NDex) in Fe-deficient cucumber roots.

Table 2. NAD(P)H oxidation activities of mitochondria purified from root of cucumber plant grown in the presence (+Fe) or in the absence (–Fe) of Fe.

Enzymes	**a (nmol min–1 mg)**			**b (specific activity)**		
	+Fe	–Fe	%	+Fe	–Fe	%
CS				408±24 a	798±57 b	
NDin – Rot	940±65.8	113±4.5 b	–88	230.4±16.1 a	14.1±0.7 b	–95

sensitive	a					
NDin – Rot insensitive	60±2.4 b	142±8.5 a	+126	14.7±0.8 b	17.8±1.0 a	+21
NDex – NADH	160±6.4 b	660±52.8 a	+412	39.2±2.3 b	82.7±5.8 a	+111
NDex – NADPH	155±9.3 b	602±42.1 a	+406	37.9±1.9 b	75.4±5.3 a	+99

The activities were expressed as non-normalized (a) and as normalized values on CS activities (b) (Vigani et al., 2009). % is the variation of the activity of –Fe treatment with respect to the control. Data are the means±SE of three independent experiments in triplicate (n=9). SE never exceeded 7%. Data were analyzed by ANOVA (Student's t-test; p<0.01).

Protein levels of *NDA1* and *NDB1*

The plant homologues to rotenone-insensitive NADH dehydrogenases of yeast mitochondria and *E. coli* have been identified in potato. The gene products NDA1 and NDB1 are localized at the internal and external sides of the inner mitochondrial membrane, respectively (Rasmusson et al., 1999).

As reported by Svensson and Rasmusson (2001), the polyclonal antisera against peptides deduced from *nda1* and *ndb1* sequences of potato specifically recognize polypeptides with apparent masses of 48 and 61 kDa, respectively. In Fe-deficient mitochondria, a protein accumulation of the 61 kDa polypeptide recognized by the *NDB1* antibody was observed (Fig. 1), in agreement with the increased NDex activity (Table 2). In addition, a second immuno-decorated band of approximately 45 kDa was detected (Fig. 1). This is in agreement with the results previously observed with potato mitochondria (Svensson and Rasmusson, 2001). It is unclear whether this band derives from another ND-type homologue, or from a different protein. In any case, this band was increased under Fe deficiency, indicating a possible response to this condition. The *NDA1* antibody recognized an evident band of approximately 48 kDa without any appreciable difference in the intensity between +Fe and –Fe mitochondria (Fig. 1). This finding might be consistent with the lower enhancement in the internal NAD(P)H oxidation activities observed in –Fe mitochondria (Table 2). In any case, as discussed by Rasmusson and Agius (2001), *NDA1* and *NDB1* antisera specifically detect their respective target polypeptides in mitochondrial protein fractions, and are thus suitable for analysis of NDA- and NDB-type proteins in mitochondria.

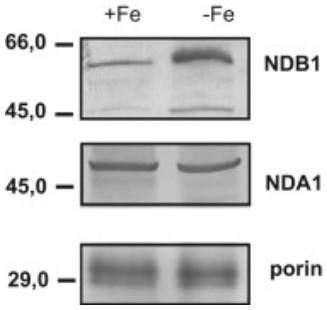

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Fig. 1. Protein gel blot analysis of alternative NAD(P)H dehydrogenase enzymes in mitochondria from roots of cucumber plants grown in the presence (+Fe) or in the absence (–Fe) of Fe. For each sample, 10 µg of protein were used. NDex protein levels were investigated using anti-*NDB1*, while NDin protein levels were investigated using anti-*NDA1* according to Svensson and Rasmusson (2001). The proteins analyzed are shown on the right and molecular mass markers (in kDa) are denoted on the left. The analysis was repeated in three independent experiments with the similar results.

Discussion

In Fe-deficient roots, the relationships between reducing and oxidizing processes are quite complex, since the strong activation of FC-R and H+-ATPase activities leads to a strong need for

energetic substrates. The lack of Fe strongly induces the NAD(P)H oxidizing activities over the NAD(P)+ reducing activities (Schmidt and Schuck, 1996; López-Millán et al., 2000, 2009).
We found (Vigani et al., 2009) that Fe deficiency strongly impaired the respiratory chain activity in cucumber roots. Since both the cytosolic and the mitochondrial NAD(P)H need to be re-oxidized to keep the catabolic pathways working, less efficient respiratory chain activity could impair this turnover, leading to a higher ratio of reduced over oxidized pyridine nucleotide compounds. We have previously hypothesized an involvement of the alternative ND-DH activities in NAD(P)H oxidation bypassing the strong decrease in the complex I activity occurring in Fe-deficient cucumber roots (Vigani et al., 2009). In this work, for the first time, we demonstrated that, in Fe-deficient mitochondria, the NDex are strongly induced with respect to the NDin, both at the enzymatic activity (Table 2) and the protein levels (Fig. 1). These findings suggest that, through the NDex, the respiratory chain can contribute to cytosolic NAD(P)H turnover. The lower induction of NDin activity with respect to the NDex could be related to a lower request level for NAD(P)H turnover from the matrix than from the cytosol (Fig. 2). Additionally, while the NDex are able to directly oxidize NAD(P)H from the cytosol, the NADH oxidation performed by the NDin should be regulated. In fact, the NDin show a lower affinity for the matricial NADH than complex I (Møller and Palmer, 1982), and so NDin should be active when the membrane potential and the matrix NADH level is higher (Svensson and Rasmusson, 2001). In Fe-deficient mitochondria, complex I activity is almost totally inhibited, so the NADH level tends to be higher, inducing NDin to catalyze NADH oxidation in the matrix.

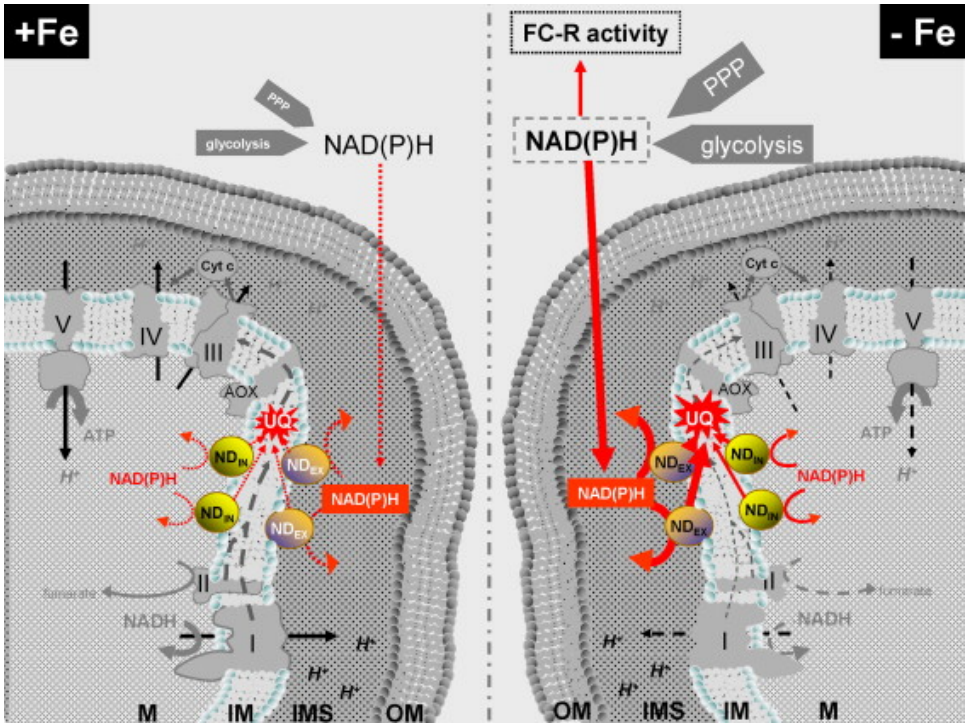

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Fig. 2. Schematic representation of the alternative NAD(P)H dehydrogenases in cucumber roots. In the left panel, control mitochondrial activities are reported. The right panel shows the effect of Fe deficiency on the alternative NAD(P)H dehydrogenases. The different arrow thickness in the –Fe condition indicates the changes in the electron flow and in the ND reducing activities. M, matrix; IM, inner membrane; IMS, inter membrane space; OM, outer membrane; PPP, pentose phosphate pathway.

It is important to note that the mtETC of Fe-deficient cucumber roots is not completely blocked and can still partially contribute to oxygen consumption and ATP synthesis. In this study, we showed that, in mitochondria of Fe-deficient cucumber roots, alternative NAD(P)H dehydrogenase activity is induced, and leading (i) to a bypass of the impairment of the ubiquinone (UQ) reduction process (complex I and II activities) allowing electrons to flow alongside the respiratory chain and,

consequently, the ATP synthesis; (ii) to allow the NAD(P)H turnover of the cell. Fig. 2 reports the changes that occurred in electron flow and reducing activities of the Fe-deficient mitochondria.
In conclusion, as sessile organisms, plants require flexible metabolism to adapt to changing environmental conditions. Plant mitochondria, having several alternative pathways, can properly regulate their own function in response to different stress conditions. In this report, we provide, for the first time, new evidence that the alternative ND-DHs could be an important target for the regulation of metabolic responses induced by Fe deficiency.
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Appendix A. Supplementary materials

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Supplementary Table.
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