Running title: Change in mtETC and riboflavin secretion in Fe-starved *H. albus* roots

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Iron deficiency induce change in mitochondrial electron transport chain and riboflavin secretion in *Hyoscyamus albus* hairy roots

(Effect of iron deficiency on mitochondrial electron transport chain and riboflavin secretion in *Hyoscyamus albus* hairy roots)

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Summary

Hyoscyamus albus hairy roots secrete riboflavin under Fe-deficient conditions. To determine whether this secretion was linked to an enhancement of respiration, both riboflavin secretion and the reduction of 2,3,5-triphenyltetrazolium (TTC), as a measure of respiration activity, were determined in hairy roots cultured under Fe-deficient and Fe-replete conditions, with or without aeration. Appreciable TTC-reducing activity was detected at the root tips, at the bases of lateral roots and in internal tissues, notably the vascular system. TTC-reducing activity increased under Fe deficiency and this increase occurred in concert with riboflavin secretion and was more apparent under aeration. Riboflavin secretion was not apparent under Fe-replete conditions.

In order to examine which elements of the mitochondrial electron transport chain (mtETC) might be involved, the effects of the respiratory inhibitors, barbiturate, dicoumarol, malonic acid, antimycin, KCN and salicylhydroxamic acid (SHAM) were investigated. Under Fe-deficient conditions, malonic acid affected neither root growth, TTC-reducing activity nor riboflavin secretion, whereas barbiturate and SHAM inhibited only root growth and TTC-reducing activity, respectively, and the other compounds variously inhibited growth and TTC-reducing activity. Riboflavin secretion was decreased, in concert with TTC-reducing activity, by dicoumarol, antimycin and KCN, but not by SHAM. In Fe-replete roots, all inhibitors which reduced riboflavin secretion in Fe-deficient roots showed somewhat different effects: notably, antimycin and KCN did not significantly inhibit TTC-reducing activity and the inhibition by dicoumarol was much weaker in Fe-replete roots. Combined treatment with KCN and SHAM also revealed that Fe-deficient and Fe-replete roots reduced TTC in different ways. A decrease in the Fe content of mitochondria in Fe-deficient roots was confirmed. Overall, the results suggest that, under conditions of Fe deficiency in *H. albus* hairy roots, the alternative NAD(P)H dehydrogenases, complex III and complex IV, but not the alternative oxidase, are actively involved both in respiration and in riboflavin secretion.

Key words Electron transport chain · hairy roots · *Hyoscyamus albus* · respiratory inhibitor · riboflavin secretion

Abbreviations

TTC: 2,3,5-triphenyltetrazolium · *SHAM*: salicylhydroxamic acid ·*DMSO*: dimethyl sulfoxide · *PEPC*: phosphoenolpyruvate carboxylase · *mt*: mitochondrion · *ETC*: electron transport chain · *AOX*: alternative oxidase · *ADX*: alternative NAD(P)H dehydrogenase · *FW*: fresh weight· *Q*: ubiquinone · *Cytc*: cytochrome C

Introduction

Plants growing on calcareous soils commonly face Fe deficiency, because ferric irons abundant in the surface of the earth's crust are scarcely dissolved at neutral or alkaline pH and therefore they are not available to plant roots. It is well known that Fe-starved roots of dicotyledonous plants change morphologically, showing dense root hair development and typical subapical swellings. In parallel with this change, biochemical changes have been observed, including proton extrusion by the activation of a proton-pump H^+-ATPase, thereby reducing soil pH and increasing the solubility of Fe^{3+} (Dell'Orto et al., 2000; 2002; Waters et al., 2002; Hell and Stephan, 2003; Schmidt, 2003; Santi et al., 2005). Concomitantly, organic acids such as citric and malic acids that act as Fe^{3+}-chelators (Ryan et al., 2001; Dakora and Phillips, 2002) are released, chelated Fe^{3+} is reduced to Fe^{2+} by the action of a membrane-bound Fe^{3+} chelate reductase, and then Fe^{2+} is transported into cells by a Fe-regulated transporter (Waters et al., 2002; Connolly et al., 2003). To support all these processes directly connected with the enhancement of Fe uptake, a supply of ATP as well as of

reducing agents such as NAD(P)H is essential (Zocchi, 2007).

This in turn implies an up-regulation of energy consumption, requiring an enhancement of respiration: plant respiration comprises both the anaerobic glycolytic and pentose phosphate pathways in the cytosol and the aerobic TCA cycle and electron transport chain in the mitochondria. An increase in oxygen consumption under Fe starvation has indeed been reported in roots of cucumber (Espen et al., 2000; Vigani et al., 2009) and sugar beet (López- Millán et al., 2000), although not in tomato roots (López- Millán et al., 2009); and enhancements of glycolytic enzyme activities, cytosolic dehydrogenase activity, phosphoenolpyruvate carboxylase (PEPCase) activity and organic acid synthesis have all been observed in roots in response to Fe deficiency (Rabotti, 1995; De Nisi and Zocchi, 2000; Andaluz et al., 2002: López- Millán et al., 2009).

Roots under Fe deficiency have also been observed to accumulate and/or excrete flavins. Some taxonomically unrelated plants of strategy I (non-graminaceous plants), including cucumber, tobacco, and sunflower, secrete riboflavin into the rhizosphere under Fe-deficient conditions (Shinmachi, 1997; Welkie, 2000; Vardja et al., 2004); and in the case of sugar beet, unique riboflavin sulfates are accumulated in the roots or are secreted (Susín et al., 1993; 1994). Recently, Vorwieger et al. (2007) confirmed that ectopic expression of *Arabidopsis* basic helix-loop-helix transcription factors, which are induced under Fe deficiency, induced riboflavin excretion in transgenic hairy roots of tobacco and sunflower, but not in those of tomato. Since tomato roots neither increase their oxygen consumption nor excrete riboflavin under Fe starvation, riboflavin secretion seems to be associated with respiration, although study on the direct relationship between them has been very limited.

Recently, we have also reported that *Hyoscyamus albus* hairy roots cultured in Fe-deficient medium secrete riboflavin into the medium (Higa et al., 2008). Interestingly, these roots can grow under Fe-deficient condition. In order to grow, energy generation via respiration is essential; and the plant mitochondrial electron transport chain (mtETC) is well known to require a diversity of Fe-sulfur- and heme-containing proteins and flavoproteins (Browse, 2002; Balk and Lobre´aux,

2005; Rouault and Tong, 2005; Vigani and Zocchi, 2009; Vigani et al., 2009). Survival in an Fe-deficiency state is likely to require adaptive mechanisms such that respiration is carried out in a different manner under Fe-deficient conditions compared to under Fe-replete conditions (Vigani and Zocchi, 2009; Vigani et al., 2009). Previously, rhizodermal transfer cell formation has been observed in swollen root tips under Fe deficiency, where an increase in the number of mitochondria has been found (Landsberg, 1986). Similar results have been obtained with sycamore cells (Pascal and Douce, 1993) and cucumber roots (Dell'Orto et al., 2002). This increase in the number of mitochondria is likely to affect the respiratory activity, including the mtETC. The plant mtETC in the mitochondrial membrane consists of several members, including 4 components, complex I to complex IV (Dudkina et al., 2006), found in all living cells, in addition to a plant-specific alternative oxidase (AOX) and NAD(P)H dehydrogenases (ADX). Therefore, we decided to examine here whether the enhancement of respiration and riboflavin secretion are interlocked in *H. albus* roots under Fe deficiency, and if so, whether all components may be involved in respiratory activity and riboflavin secretion in *H. albus* roots under Fe deficiency, using experiments with plant-ETC-specific inhibitors.

Materials and methods

Chemicals

Antimycin A, malonic acid, pentobarbital sodium, rotenone, salicylhydroxamic acid (SHAM) and 2,3,5-triphenyltetrazolium chloride (TTC) were purchased from Sigma. Dicoumarol and 1,3,5-triphenylformazan were from Tokyo Kasei Co. (Japan), and KCN was from Katayama Chemical (Kyoto, Japan), respectively. Other chemicals were of the highest quality commercially available.

Root materials and culture conditions

Hairy roots of *Hyoscyamus albus* L. (Solanaceae) used in these experiments were established as reported in the previous paper (Higa et al., 2008). Roots were maintained on Murashige and Skoog (1962) basal medium solidified with 0.2% gellan gum. A primary root tip with a few lateral roots (ca. 2 cm in length) isolated from about 2-week-old root cultures was pre-propagated in the normal liquid B5 medium (Gamborg et al., 1968) for 2 weeks and then the medium was exchanged for either a Fe-deficient B5 medium or a Fe-containing B5 medium, followed in each case by further culture for 1 week. Fe-deficient B5 medium was prepared by elimination of Fe-EDTA from B5 basal medium, before autoclaving at 121 ˚C for 15 min. All cultures were performed in a 100 mL conical flask containing 25 mL of liquid medium and incubated at 25 ºC with/without agitation at 80 rpm in the dark. The cultures were harvested by vacuum filtration, except when used for the TTC-reducing assay, and separated into roots and media. Collected fresh roots were weighed and then stored at -70 ºC until use. Media were analyzed for pH and for riboflavin content.

Supply of inhibitors

Plant-ETC-specific inhibitors, their concentrations and the solvents used for administering them are listed in Table 1. Pentobarbital sodium, malonic acid, KCN and SHAM, each dissolved in distilled water, and rotenone, antimycin A and dicoumarol, each dissolved in dimethyl sulfoxide (DMSO), were filter-sterilized, respectively, before being supplied to the cultures. They were independently added to 2-week-old root cultures, just after changing the medium for fresh B5 medium containing 1% sucrose with/without Fe. Since root growth varied more or less depending on the initial state of the roots, a separate control was established for each experimental condition: water or DMSO, according to the inhibitors applied, was always added as a control. Cultures were incubated under the above-mentioned conditions for 1 week. In the case of combined treatments, both KCN and SHAM were always added at the same concentrations (Table 1).

Assay of TTC-reducing activity

For the assay of respiration activity, we employed the TTC-reducing assay, instead of monitoring the decrease in O_2 concentration in the aqueous phase (Espen et al., 2000; Lopez-Millan et al., 2000; 2009; Vigani et al., 2009). The TTC method was originally described by Lindstrőm and Nystrőm (1987) and was first applied to the investigation of respiratory activity by Comas et al. (2000). Colorless TTC is able to pass through the plasma membrane and reach the mitochondria, where it is converted into reddish formazan by reducing equivalents and where the colored products then remain immobilized, clearly indicating which parts of roots actively reduce TTC. The TTC method used here was modified from Comas et al. (2000) as follows: The medium was removed from the root culture using a pipette, the roots were washed with 5 mL of sterile water, and 10 mL of filter-sterilized TTC reagent was then added. The TTC reagent was prepared to be 0.5% TTC (w/v) in 50 mM potassium phosphate buffer (pH 7.0). Incubation with TTC reagent was performed under sterile conditions, usually for 3 h and without vacuum-infiltration but with shaking (80 rpm), to allow the reagent to permeate uniformly.

After incubation, the roots (50-150 mg) were ground into powder in a mortar with liquid nitrogen and a pestle. The powder was transferred to a centrifuge tube and then extracted with 3 mL of 95% (v/v) ethanol for 15 min in a water bath at 60 °C. After centrifugation (3,000 rpm, 15 min), the absorbance of the supernatant was recorded at 520 nm. Usually the absorbance is recorded at 480 nm; however, due to the risk of interference from endogenous plant pigments such as riboflavin at this wavelength, 520 nm was substituted (Steponkus and Lanphear 1967; Clemensson-Lindell, 1994). As a control, denatured roots boiled for 10 min were used initially. Since the absorbance was always 0.020 ± 0.001 at 520 nm, 95% (v/v) ethanol was used as a control and 0.02 was subtracted from the absorbance. The reduction activity was calculated using a standard curve that was previously obtained using authentic 1,3,5-triphenylformazan dissolved in 95% ethanol.

TTC-reducing activity was detected in a time-dependent manner for up to 4 h, both with and without vacuum-infiltration of the roots (Fig. 1). Although the activity recorded with

vacuum-infiltration was nearly twice that observed without, we assayed the activity without this treatment since measurements could be made more rapidly and were more consistent. The activity was expressed routinely on a fresh weight (FW) basis since there was found to be no significant difference in water content between roots cultured with and without Fe/inhibitor.

Assay of riboflavin content

Riboflavin secretion into the culture medium was confirmed by HPLC, which revealed a single peak in comparison to standard flavins (Fig. 2). Since the content of riboflavin in the roots themselves was negligible in the case of liquid cultures (less than 6% of total riboflavin) (Higa et al., 2008) when determined by HPLC analysis, only the medium was analyzed. The content was routinely determined by a UV-VIS spectrophotometer (Shimazu UV-1600) at 444 nm, as previously reported (Higa et al., 2008), because there was no difference in quantitation between HPLC and spectrophotometric analysis.

Analysis of Fe content

Mitochondrial fractions were prepared from *H. albus* roots according to the method of Wang et al. (2008) with some modifications. Harvested fresh roots were homogenized in pre-cold extraction buffer containing 50 mM Tri-HCl (pH 7.5), 250 mM sucrose, and 10 mM DTT. The cell wall was removed by nylon mesh filtration (82 μm, in vacuum) and then the filtrate was centrifuged at 3000 g for 15 min to remove cell wall debris, nucleus and plastids. Mitochondria were precipitated from the resulting supernatant fluid by centrifugation at 21,880 g for 30 min. TTC-reducing assay revealed that only this fraction was stained in red color. The mitochondrial fractions were digested with c-HNO$_3$ in a microwave oven (PerkinElmer, Multiwave) at 160°C for 20 min. Fe contents were determined by Atomic absorption spectroscopy (Hitachi Z-2000).

Results

I. Effects of aeration on fresh weight, pH, TTC-reducing activity and riboflavin secretion

Previous study with intact plants of *H. albus* had shown that in Fe-deficient liquid medium, riboflavin secretion into the medium from the plant roots was higher when plants were kept under shaking conditions, compared to under static conditions (unpublished data). Here the effect of shaking was therefore tested also in hairy roots of *H. albus*. The hairy roots were incubated in Fe-deficient and Fe-containing media with/without agitation at 80 rpm (4 different conditions); the incubations were carried out in the dark in order to prevent riboflavin decomposition by light. The fresh weight (FW) of the roots and their respiration activity based on the TTC-reducing assay, together with the pH and the riboflavin content of the medium, were then all measured (Fig. 3, Table 2).

The results on root FW showed that statistically significant differences ($p<0.05$) were found among 4 different conditions; the FW of roots incubated with agitation increased to around double that observed without agitation, both under Fe-replete (increasing from 0.34 g to 0.72 g) and Fe-deficient (increasing from 0.28 g to 0.54 g) conditions (Table 2). Thus, roots grew well with agitation even under Fe deficiency (Fig. 3c), although the root FW was only about 75% of that obtained under Fe-replete conditions (Fig. 3d). A pH decline was detected in the culture medium when roots were cultured in the absence of Fe (from 5.3 to 4.7 with agitation and from 5.5 to 5.0 without agitation); agitation did not affect pH significantly (Table 2).

In the TTC-reducing assay (Fig. 3e), both the root tips and the bases of growing lateral roots were obviously stained in roots cultured either with or without Fe (Figs. 3f, g), but they were more deeply stained, and the lateral roots themselves were also stained, under conditions of Fe deficiency (Fig. 3g). Within the roots, the internal tissues were more deeply stained than the peripheral: notably, no color was detected in the root hairs, but the vascular bundle system became more reddish (Fig.

3h). Roots incubated under Fe deficiency and with agitation exhibited the strongest staining amongst the roots treated under the 4 different conditions. Quantitative analysis of TTC-reducing activity indicated that the activity under Fe-deficient conditions with agitation was 0.171 μmolg^{-1} FWh^{-1} and this value was about double that under Fe-replete conditions with agitation (0.083 μmolg^{-1} FWh^{-1}), although apparent difference between with/without agitation was not found (Table 2).

Riboflavin was only detected under Fe-deficient conditions (Fig. 3a) and not under Fe-replete conditions (Fig. 3b), regardless of agitation (Table 2). Riboflavin secretion observed with agitation (0.287 μmolg^{-1}FW) was 32% up than that observed without (0.218 μmolg^{-1}FW). Under Fe deficiency, changes in both TTC-reducing activity and in riboflavin secretion occurred in parallel.

II. Effects upon FW, TTC-reducing activity and riboflavin secretion of supplying mtETC-specific inhibitors to Fe-deficient roots

As described above, TTC-reducing activity was enhanced by Fe deficiency in *H. albus* roots, along with an increase in riboflavin secretion. The plant mtETC consists of complex I to complex IV (Dudkina et al., 2006), and the alternative oxidase (AOX) and NAD(P)H dehydrogenases (ADX). Here, we examined whether all components may be involved in respiration activity in *H. albus* roots cultured under Fe deficiency with agitation, using plant-mtETC-specific inhibitors (Table 1) (taking into account that inhibitory effects are strongly dependent both upon the specific inhibitors and their concentrations). To determine the effects of these inhibitors upon riboflavin secretion, low-dose (μM level), long-term (1 week) treatments were employed. An individual control was established for each experimental condition by the addition of water or DMSO, respectively, in place of the inhibitor and data were recorded relative to these controls (set at 100%) (Fig. 4); all data were presented in this way.

A statistically analysis, based on ANOVA and Student t test, showed that apart from malonic

acid and SHAM, all the inhibitors significantly diminished FW (p<0.05) with the concentrations applied (except for 100 μM barbiturate) (Fig. 4A). Dicoumarol, antimycin and KCN with 20 μM inhibited growth to 38%, 47% and 51% of the controls, respectively, and barbiturate with 200 μM did to 49% of the control. On the other hand, TTC-reducing activity was suppressed by all the inhibitors (dicoumarol, antimycin, KCN and SHAM), except for barbiturate and malonic acid, depending upon their concentrations; dicoumarol and antimycin were especially effective and TTC-reducing activity was decreased to 16% and 36% of the controls, respectively, at 20 μM (Fig 5A). KCN and SHAM exhibited similar inhibitory effects (74% and 70% of the controls, respectively, at 20 μM). Barbiturate did not inhibit TTC-reducing activity and this was not in agreement with its inhibitory effect on FW. This opposite case was for SHAM, which inhibited TTC-reducing activity, but not FW.

Regarding riboflavin secretion, neither barbiturate, nor malonic acid nor SHAM showed clear effects (Fig. 6); for SHAM, therefore, this contrasted with its effect on TTC-reducing activity. In contrast, dicoumarol, KCN and antimycin severely reduced riboflavin secretion in this order, to 24%, 39% and 65% of the controls, respectively, at 20 μM (Fig. 6), in agreement with their inhibitory effect upon TTC-reducing activity (Fig. 5). Antimycin and KCN diminished riboflavin secretion in a dose-dependent manner, though this was not the case for dicoumarol (Fig. 6).

III. Effects upon FW and TTC-reducing activity of supplying mtETC-specific inhibitors to Fe-replete roots

Fe-replete roots were affected by mtETC-specific inhibitors, although not always in the same way as Fe-deficient roots. A statistical analysis revealed that neither malonic acid nor KCN decreased FW significantly (Fig. 4B). On the other hand, dicoumarol reduced FW to the greatest extent (33% of the control, with 20 μM), followed by antimycin and SHAM (FW diminished in each case to 53% and 57% of the controls, respectively, with 20 μM), and then barbiturate (59% of the control,

with 200 μM) (Fig. 4B).

Discriminatory differences between Fe-deficient and Fe-replete roots were observed when KCN or SHAM was applied. The two inhibitors acted conversely: Their effects applied with 20 μM showed that KCN inhibited the propagation of Fe-deficient roots apparently (51% of the control), but not apparently that of Fe-replete roots; on the other hand, SHAM reduced FW of Fe-replete roots (57% of the control), but not that of Fe-deficient roots. Fe-replete and Fe-deficient roots were also affected somewhat differently by antimycin, since FW of Fe-replete roots were reduced to 53%, 46% and 50% of the controls at 20, 40 and 80 μM, respectively, and appeared dose-independent, whereas these concentrations applied to Fe-deficient roots gave 47%, 28% and 19%, respectively, in a dose-dependent manner (Fig. 4).

A statistically significant difference ($p < 0.05$) in TTC-reducing activity was found only with dicoumarol and SHAM (Fig. 5B). Neither KCN (except at 40 μM) nor antimycin inhibit the TTC-reducing activity of Fe-replete roots significantly (Fig. 5B), although they suppressed the activity of Fe-deficient roots (Fig. 5A). Dicoumarol inhibited the TTC-reducing activity of Fe-replete roots to a lesser extent than that of Fe-deficient roots (46% of the control compared to 16%, at 20 μM), whereas SHAM inhibited it to a greater extent (53% of the control compared to 70%, at 20 μM).

Riboflavin secretion was not observed at all with Fe-replete roots (data not shown) and this was not affected by mtETC-specific inhibitors.

IV. Effects upon FW, TTC-reducing activity and riboflavin secretion of Fe-deficient and Fe-replete roots of combined treatment with KCN and SHAM

Complex IV and AOX are the terminal oxidases and bring about oxygen reduction to yield water. When both of them are totally inhibited, oxygen consumption in respiration must be abolished. The effect of combined treatment with KCN and SHAM on FW was not very different between

Fe-deficient and Fe-replete roots (66% of control compared to 64% , at 10 μM).

On the other hand, combined treatment with KCN and SHAM inhibited the TTC-reducing activity of Fe-deficient roots more severely. Whilst the inhibitory effects of KCN and SHAM, administered separately, upon TTC-reducing activity in Fe-deficient roots were similar (76% and 71% of controls, respectively, at 10 μM), in combination they were synergistic, resulting in a substantial inhibition even at only 10 μM concentration of each (36% of the control). Thus, the inhibition became a plateau more than 10 μM. Even after reaching a constant, oxygen consumption-independent TTC-reducing activity remained high (28% of control, at 40 μM). As indicated above, KCN had no inhibitory effect in Fe-replete roots and this was reflected in the lesser degree of inhibition produced by KCN and SHAM administered together to Fe-replete roots, where the activity was not apparently reduced (88% of the control, at 10 μM). However, the inhibition to Fe-replete roots was increased in a dose-dependent manner and became apparent at 40 μM (41% of the control).

Riboflavin secretion treated with KCN and SHAM together was similar to that with KCN solely (33% of the control compared to 39%, at 20 μM) (Fig. 6).

V. Fe contents in mitochondria

Many components of the plant-mtETC contain Fe atoms in the form of Fe-S clusters or heme. To determine the Fe status in mitochondria under Fe-deficient and Fe-replete conditions, mitochondrial fractions were isolated and their Fe contents were analyzed. In Fe-replete and Fe-deficient mitochondria, the content was 2.93 and 0.82 μg g^{-1}FW, respectively (Fig. 7). Thus, the Fe content under Fe-deficient conditions was found to be decreased to 28% of that under Fe-replete conditions.

Discussion

Here we showed that an enhancement of respiration and riboflavin secretion occurred in parallel under Fe deficiency, using the *H. albus* root system established previously (Higa et al., 2008) and for the first time, to the best of our knowledge, by determination of TTC-reducing activity. TTC is mainly reduced by dehydrogenases, most of which are associated with mitochondrial function (Comas et al., 2000). The sites actively producing electrons or NAD(P)H can be visually detected by virtue of staining. Interestingly, the most stained parts were the root tips and bases of original roots from which lateral roots were branching (Fig. 3f-h). In a previous report, we found that riboflavin productivity depended on the number and size of root tips (Higa et al., 2008). Concomitantly with the increase in root tip numbers, the number of root bases is also increased, suggesting that riboflavin secretion must be related to increased respiration (TTC-reducing activity).

Enhancement of respiration in roots under Fe starvation was supported by the observation of an increase in the numbers of mitochondria in rhizodermal cells of sugar beet and cucumber (Landsberg, 1986; Dell'Orto et al., 2002). However, most proteins involved in respiration in mitochondria contain Fe or Fe-sulfur centers to transmit electrons (Balk and Lobre´aux, 2005; Rouault and Tong, 2005). This means that there should be some discernible differences in the respiration mechanism of mitochondria between Fe-deficient and Fe-replete roots. To uncover these differences and to determine the relationship between respiratory activity and riboflavin secretion, inhibitor-feeding experiments *in vivo* were performed. In comparison to the previous studies (Lopez-Millan et al., 2000; 2009; Vigani et al., 2009), here much lower concentrations (μM against mM) were used and treatment was greatly prolonged (1 week compared to <1 h), since riboflavin secretion began at 3 days and, under Fe deficiency without inhibitor, amounts of riboflavin became clearly detectable at 1 week. We need to consider the fact that this long term treatment with the inhibitors affected root FW and some of the changes in TTC-reducing activity may be related to differences in growth more rather to the specificity of the inhibitor.

The largest difference in effects on growth between Fe-deficient and Fe-replete roots were observed with SHAM and KCN: KCN decreased the FW of Fe-deficient roots, but had no apparent effect upon Fe-replete roots, whereas SHAM showed converse effects (Fig. 4). The results for TTC-reducing activity also indicated that these inhibitors affected Fe-deficient and Fe-replete roots differently: notably, KCN inhibited the TTC-reducing activity of Fe-deficient roots, but not at all that of Fe-replete roots. SHAM inhibited the activity of both classes of roots, but more severely that of Fe-replete roots (Fig. 5). These results imply that AOX must play an important role in aerobic respiration in *H. albus* roots under Fe-replete conditions, since it is known that most plants possess cyanide resistance and AOX can catalyze the reduction of oxygen instead of complex IV. However, AOX does not pump protons and therefore complex IV rather than AOX seems to be very important in Fe-deficient roots. In fact, riboflavin secretion was significantly inhibited by KCN, but not by SHAM (Fig. 6). It seems to be inconsistent that SHAM exhibited an inhibition of TTC-reducing activity in Fe-deficient roots. This may occur because Fe-deficient roots consist of mixed types of cells, SHAM insensitive/KCN sensitive (adapted to Fe deficiency) cells and SHAM sensitive/KCN insensitive (not adapted) cells, although the cells in Fe-replete roots consist of same type (not adapted) of cells. Tomato roots possessed strong cyanide resistance under Fe-replete condition (López- Millán et al., 2009), but sugar beet and cucumber have very weak cyanide resistance (López- Millán et al., 2000; Vigani et al., 2009). Tomato does not secrete/accumulate flavins under Fe deficiency, but other plants can do so. From this aspect, *H. albus* roots seemed not to belong to any of these plant groups, although plant materials (intact plants or roots), culture methods (systematic or nonsystematic regulation), the treatments, notably inhibitor concentration and term of incubation, as well as the assay methods (oxygen consumption or TTC-reducing assay), are quite different from previous reports. Nevertheless, *H. albus* roots seem to provide valuable insights into adaptation mechanisms to Fe deficiency.

Whereas the respiratory activity of Fe-deficient roots was strongly inhibited by dicoumarol and

antimycin, these inhibitors suppressed relatively less or apparently not at all the TTC-reducing activity of Fe-replete roots (Fig. 5). This means that ADX and complex III were operating differently under the different conditions of Fe availability. Since complex III passes electrons to complex IV *via* cytochrome c, complexes III and IV must work together. That is, ADX rather than complex I, and complexes III and IV rather than AOX, seem to play major roles in the respiration of Fe-deficient roots (Fig. 8). Riboflavin secretion was again suppressed with dicoumarol, antimycin, and KCN, but not with SHAM (Fig. 8), indicating that ADX and complexes III and IV must play a key role in riboflavin secretion, as in respiration. Since dicoumarol inhibits rotenone-insensitive alternative mitochondrial NAD(P)H dehydrogenases (ADX), including the external dehydrogenases and internal dehydrogenases (Rasmusson et al., 2004), but is not strictly specific to only one dehydrogenase (Day and Wiskich, 1975), precisely which dehydrogenases were actually suppressed is unclear from *in vivo* experiments. To clarify, a study using isolated mitochondria membrane is necessary for further determination.

Neither barbiturate nor malonic acid affected TTC reducing activity (or riboflavin excretion), regardless of Fe status, indicating that the effects of these inhibitors upon respiration is not certain from our study. Complexes I, III and IV, but not II, are coupled to proton transport and therefore contribute to the generation of ATP. Since complex I proton pumping is more energy-demanding than complex III, complex I (as well as complex II) seems to be less involved in the respiration of *H. albus* roots under Fe deficiency. Very recently, Vigani et al. (2009) revealed that enzyme activities of complexes I and II, but not of complexes III and IV, were severely suppressed (90% and 52%, respectively), measured on a protein basis, in cucumber roots under Fe deficiency. They pointed out that the requirement for Fe ions in mtETC components is strongly correlated with their enzyme activity. Complexes 1 and II require at least 20 and 10 Fe ions, respectively, in contrast to 5 and 2 Fe ions in complexes III and IV, respectively. In fact, under Fe deficiency, the Fe content of cucumber-root mitochondria decreased to less than 4% of the control value. Similarly, we found a decrease in Fe content in Fe-deficient mitochondria of *H.albus* roots, but here the content was

diminished to 28% of the control value (Fig. 7), although roots were pre-cultured with Fe-containing medium in our experiment.

Complex I and complex II require not only large numbers of Fe ions but also flavins as cofactors. If these proteins could not function, unused flavins might be transported outside the mitochondria, such as to the apoplasts and to the rhizosphere. Of course, at present stage this is a highly speculative hypothesis to explain why H. albus roots may secrete riboflavin into the rhizosphere under Fe deficiency. In accordance with this hypothesis, we suggested previously that active riboflavin secretion occurs as a response to an internal requirement under Fe deficiency, since an external supply of riboflavin to the rhizosphere did not affect riboflavin secretion by the roots at all (Higa et al., 2008).

Combined treatment of *H. albus* roots with KCN and SHAM revealed that TTC-reducing activity reached a plateau at more than 10 μM of each inhibitor in Fe-deficient roots, indicating that aerobic respiration-independent reactions contribute to the rest of activity and the activity is appreciably high (Fig. 5). Therefore, NAD(P)H produced via anaerobic processes such as glycolysis and the pentose phosphate pathway in cytosol must contribute to this activity. The existence of mtETC-independent O_2 consumptions, especially under Fe-starved stress conditions, was reported (Lopez-Millan et al., 2000; Vigani et al., 2009). Since enrichments of ferric reductase were found in root tips of sugar beet (Lopez-Millan et al., 2000) and cucumber (Shinmachi, 1997), this enzyme must be involved in enhancement of TTC-reducing activity in *H. albus* roots, too. It is also suggested that reactive oxygen species (ROS) produced under stress conditions such as Fe deficiency (Vigani et al., 2009) also contribute to the increase of TTC-reducing activity. High accumulations of ascorbic acid found in the root tips of cucumber (Shinmachi, 1997) and sugar beet (Zaharieva and Abadía, 2003) may be another candidate for an increase of TTC-reducing activity. Further study is required to know what are actually involved in these residual activities and how they are important in Fe-deficient as well as Fe-replete roots.

Here we confirmed increased respiration based on TTC-reducing activity together with riboflavin secretion by *H. albus* roots under Fe deficiency, and these reactions were apparently inhibited by the ADX inhibitor, dicoumarol, by the complex III inhibitor, antimycin, and by the complex IV inhibitor, KCN. These results suggested that electrons mainly flow among ADX, complex III and complex IV to pump protons in *H. albus* roots under Fe deficiency (Fig. 8). Complex I and complex II require large numbers of Fe ions and flavins. Disuse of these complexes accompanied with riboflavin secretion in the rhizosphere is a speculative hypothesis to adapt to Fe-starved stress in *H. albus* roots. The H. albus roots exhibit different response to oxidase inhibitors, KCN and SHAM, from previously reported flavin-excreting/accumulating roots of sugar beet and cucumber (Lopez-Millan et al., 2000; Vigani et al., 2009). Using this unique root system, adaptation mechanisms including riboflavin excretion under Fe starvation should now be explored in further studies.

Acknowledgements

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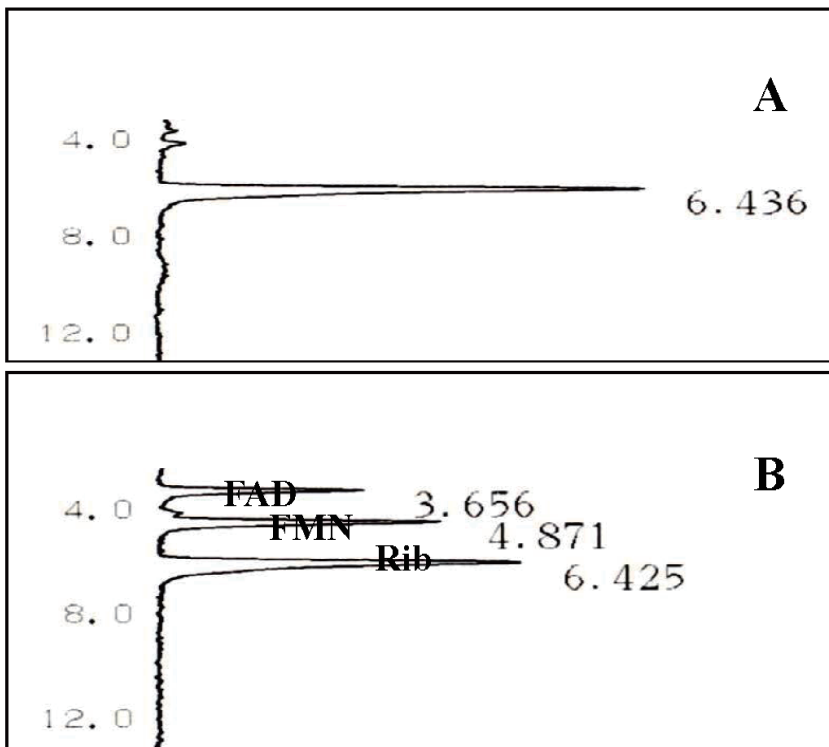
Figure Legends

Fig. 1 Time course of TTC-reducing activity with or without vacuum-infiltration treatment.
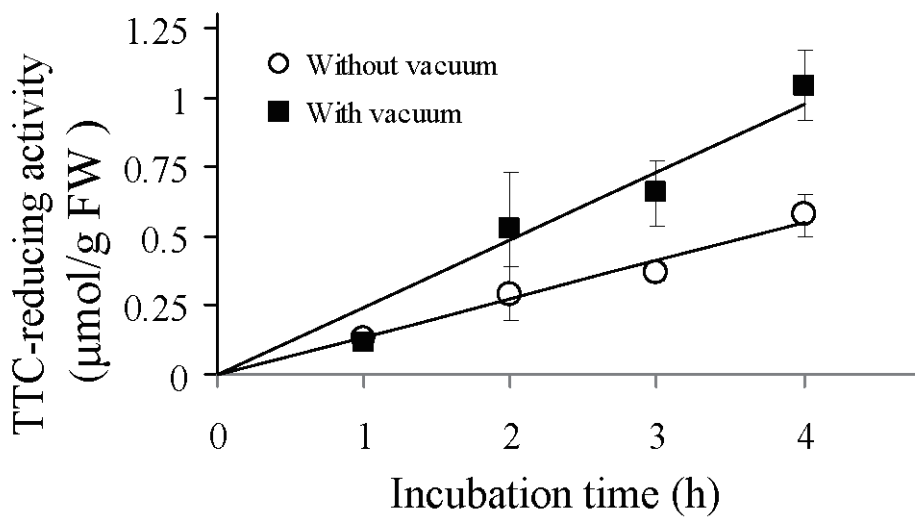
The activity was calculated on a fresh weight (FW) basis.

Fig. 2 HPLC chromatograms of cultured media (A) and authentic flavins (B).

Media were sucked from hairy root cultures of *H. albus* incubated without Fe and directly applied

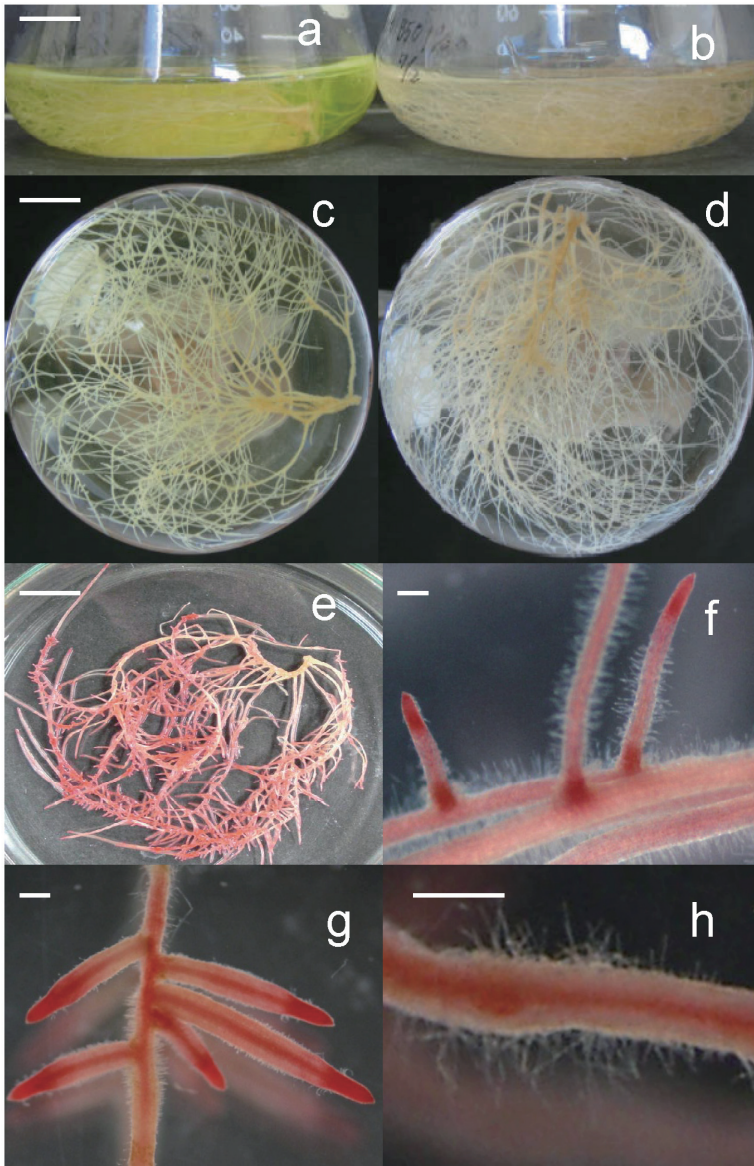to HPLC. Rib, Riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

Fig. 3 Riboflavin secretion and TTC staining. *H. albus* hairy roots were pre-cultured in liquid medium with Fe for 2 weeks and then incubated under Fe-deficient or Fe-replete conditions for a further 1 week with agitation at 80 rpm. Photos: a) and b), Medium with Fe and without Fe; c) and d), Roots with Fe and without Fe; e–h), Stained roots with TTC for 3 h; e), Whole roots without Fe; f), Root tips and bases of branches with Fe; g), Root branches characteristic of Fe deficiency; h), Main roots with Fe. White bars indicate 10 mm in length.
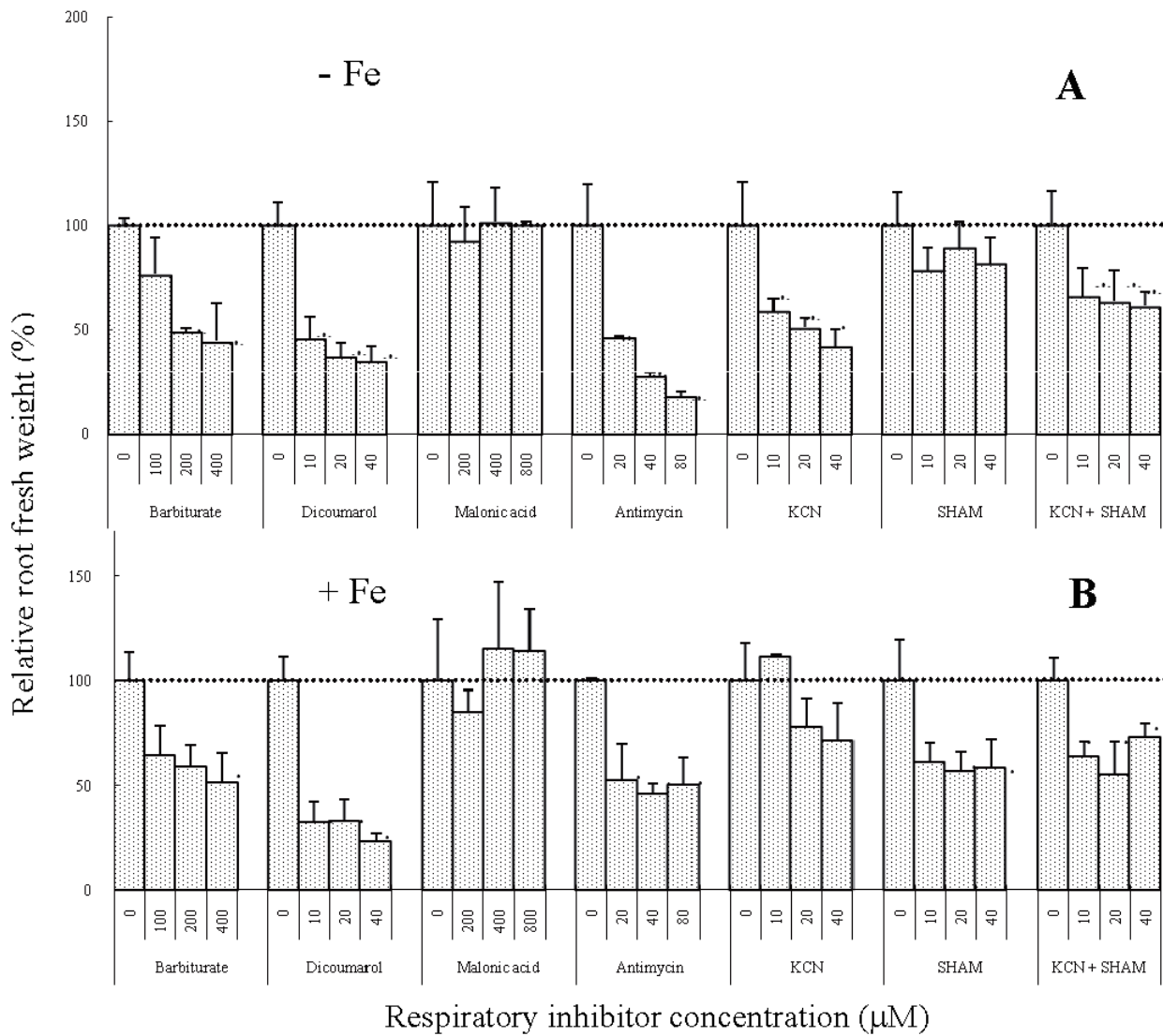
Fig. 4 Effects of mtETC-specific inhibitors on root FW of *H. albus* hairy roots under Fe deficiency.

Control is expressed as 100%. FW, fresh weight. Results are means of 3 independent experiments, and bars indicate standard deviations of means. Data are analyzed by ANOVA and Student t test. * reveal significant difference at the level of $p < 0.05$ compared with control.
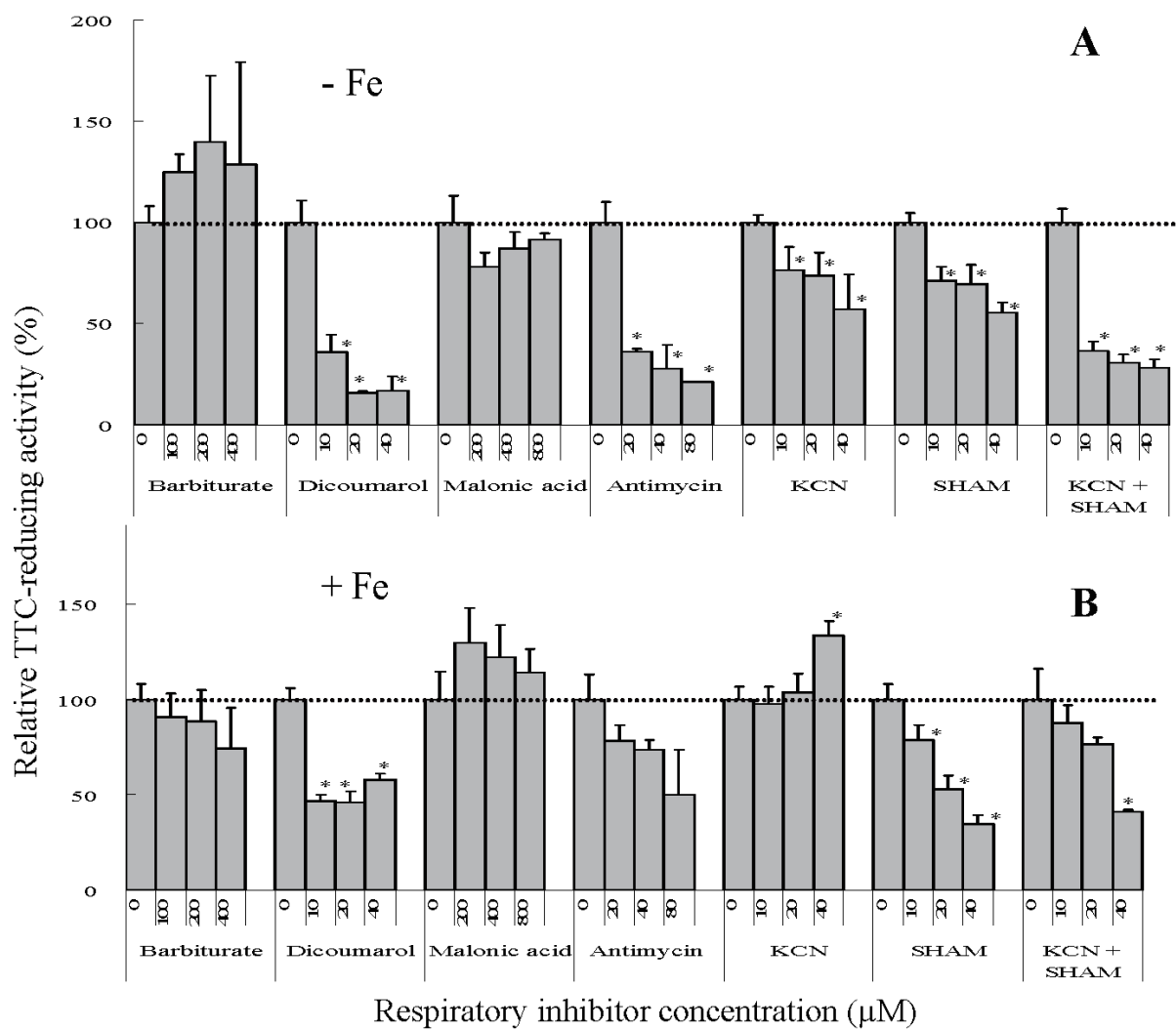
Fig. 5 Effects of mtETC specific-inhibitors on TTC-reducing activity of *H. albus* hairy roots under Fe deficiency and Fe sufficiency.
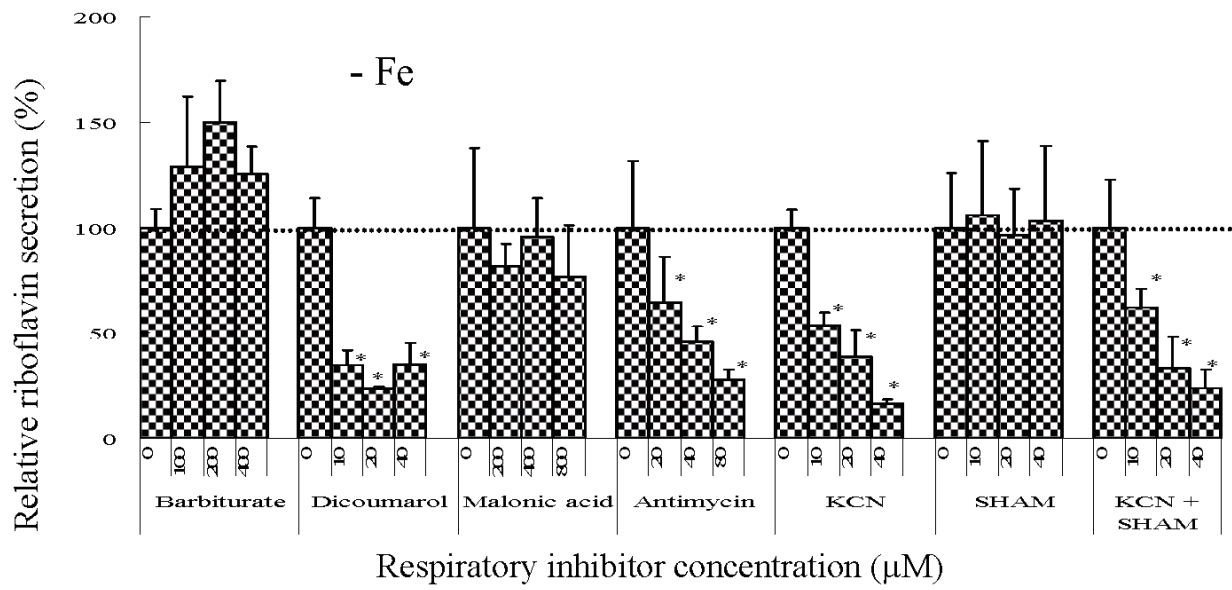
Legend as for Fig. 4.

Fig. 6 Effects of mtETC specific-inhibitors on riboflavin secretion of *H. albus* hairy roots under Fe deficiency.
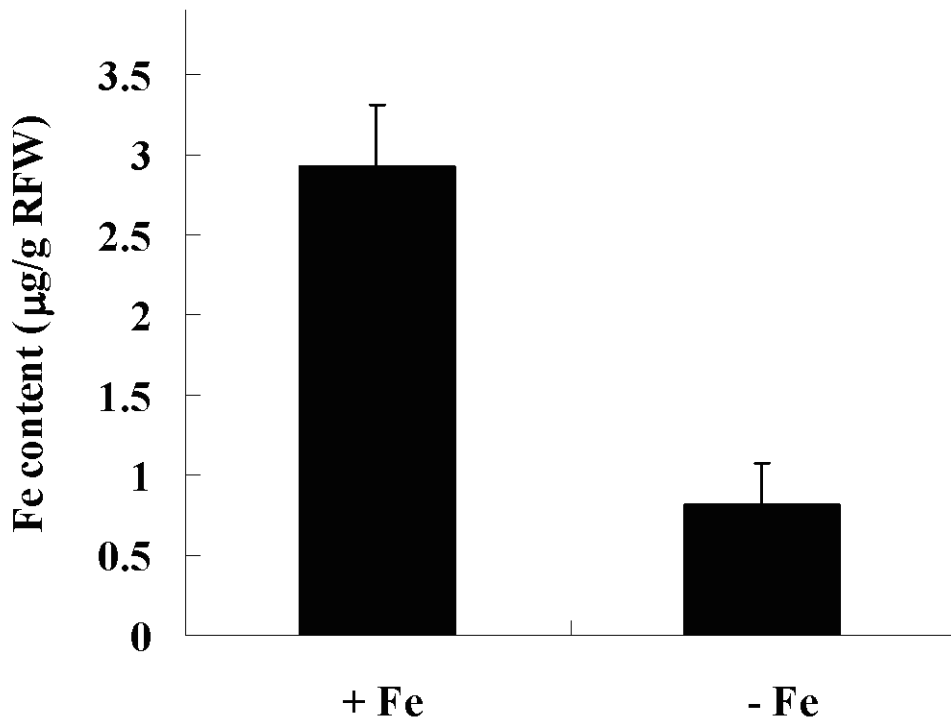
Legend as for Fig. 4.

Fig. 7 Fe contents in mitochondrial fractions of *H. albus* hairy roots under Fe sufficiency and Fe deficiency.

FW, fresh weight. Results are means of 3 independent experiments, and bars indicate standard deviations of means. Data are analyzed by Student t test. * reveal significant difference at the level of p<0.05 compared with Fe sufficiency.
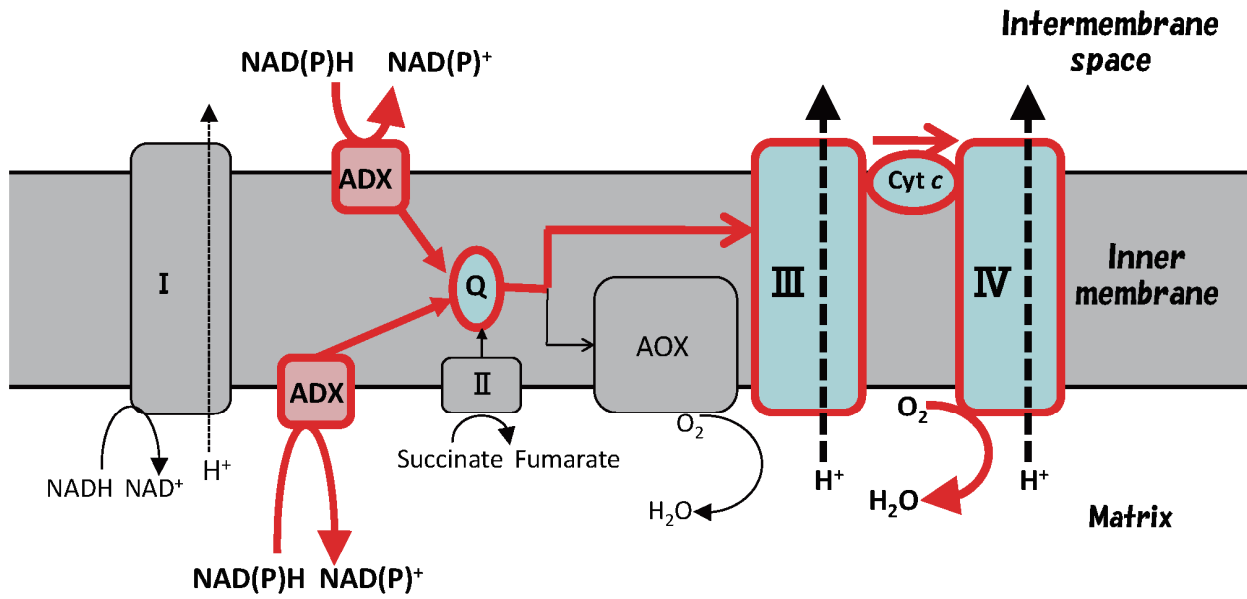
Fig. 8 Possible major mitochondrial electron transport chain (mtETC) operating under conditions in which riboflavin secretion is occurring.

Possible major proteins involved and electron flows are shown in bold squares/ellipsoids and bold arrows, respectively. Q, ubiquinone; Cyt c, cytochrome c.

Table 1. Mitochondrial electron transport chain (mtETC)-specific inhibitors used in the experiments

Component of mtETC	Inhibitor	Solvent	Concentration applied (μM)
Complex I	Barbiturate	H_2O	100 - 400
ADX	Dicoumarol	DMSO	10 - 40
Complex II	Malonic acid	H_2O	200 - 800
Complex III	Antimycin	DMSO	20 - 80
Complex IV	KCN	H_2O	10 - 40
AOX	SHAM	H_2O	10 - 40

ADX, alternative NAD(P)H dehydrogenase; AOX, alternative oxidase.

Table 2. Effects of iron-deficiency and -sufficiency on the root fresh weight (RFW), medium pH, TTC-reducing activity and riboflavin excretion in *H. albus* roots cultured with/without aeration

Culture condition		RFW (g/flask)	Final medium pH	TTC activity (μmol/g FW/h)	Riboflavin excretion (μmol/flask)	(μmol/g FW)
+ Fe	+ Aeration	0.72 ± 0.06 a	5.3 ± 0.1 a	0.083 ± 0.007 a	-	-
	- Aeration	0.34 ± 0.01 b	5.5 ± 0.1 a	0.069 ± 0.008 a	-	-
- Fe	+ Aeration	0.54 ± 0.06 c	4.7 ± 0.3 b	0.171 ± 0.020 b	0.153 ± 0.016 a	0.287 ± 0.022 a
	- Aeration	0.28 ± 0.02 d	5.0 ± 0.1 b	0.109 ± 0.032 a	0.061 ± 0.012 b	0.218 ± 0.032 b

Data are means and sd of triplicate. Significant differences (Student's t-test, $p < 0.05$) between different culture conditions are marked with different letters. -, not detected.