

Increased *de novo* riboflavin synthesis and hydrolysis of FMN are involved in riboflavin secretion from *Hyoscyamus albus* hairy roots under iron deficiency

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ABSTRACT

Riboflavin secretion by *Hyoscyamus albus* hairy roots under Fe deficiency was examined to determine where riboflavin is produced and whether production occurs via an enhancement of riboflavin biosynthesis or a stimulation of flavin mononucleotide (FMN) hydrolysis. Confocal fluorescent microscopy showed that riboflavin was mainly localized in the epidermis and cortex of the root tip and, at the cellular level, in the apoplast. The expressions of three genes involved in the *de novo* biosynthesis of riboflavin (*GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase*; *6,7-dimethyl-8-ribityllumazine synthase*; *riboflavin synthase*) were compared between Fe-starved and Fe-replete roots over a time course of 7 days, using RT-PCR. All three genes were found to be highly expressed over the period 1-7 days in the roots cultured under Fe deficiency. Since riboflavin secretion began to be detected only from 3 days, there was a lag phase observed between the increased transcript accumulations and riboflavin secretion. To determine whether FMN hydrolysis might contribute to the riboflavin secretion in Fe-deficient root cultures, FMN hydrolase activity was determined and was found to be substantially increased after 3 days, when riboflavin secretion became detectable. These results suggested that not only *de novo* riboflavin synthesis but also the hydrolysis of FMN contributes to riboflavin secretion under conditions of Fe deficiency. Respiration activity was assayed during the time-course, and was also found to be enhanced after 3 days under Fe deficiency, suggesting a possible link with riboflavin secretion. On the other hand, several respiratory inhibitors were found not to affect *riboflavin synthase* transcript accumulation.

Key words: *Hyoscyamus albus*; hairy roots; iron deficiency; riboflavin secretion; riboflavin biosynthesis; FMN hydrolase; respiration.

Abbreviations

ADX: alternative NAD(P)H dehydrogenase · *AOX*: alternative oxidase · *DMSO*: dimethyl sulfoxide · *ETC*: electron transport chain · *FAD*: flavin adenine dinucleotide · *FHy*: FMN hydrolase · *FMN*: flavin mononucleotide · *mt*: mitochondria · *SHAM*: salicylhydroxamic acid · *TTC*: 2,3,5-triphenyltetrazolium ·

RibA: GTP cyclohydrolase II · *RibB*: 3,4-dihydroxy-2-butanone 4-phosphate synthase · *RibC*:
6,7-dimethyl-8-ribityllumazine synthase · *RibD*: riboflavin synthase

1. Introduction

Riboflavin (vitamin B₂) appears to be an essential chemical for all organisms, including humans, which are unable to biosynthesize it. Plants, in common with other organisms, can biosynthesize riboflavin *de novo* and then convert it into its nucleotide derivatives, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), both of which are usually incorporated into various proteins as co-factors. These flavins mediate electron transfers and are involved in various redox-requiring processes, such as mitochondrial electron transport, photosynthesis, fatty acid oxidation, photoreception, DNA repair and the biosynthesis of various secondary metabolites [1, 2]; accordingly, flavins in free form are not usually abundant in ordinary living plant tissues. Interestingly, however, free flavins, especially riboflavin, have been detected in the roots and/or root exudates of some taxonomically unrelated dicotyledonous plants, including tobacco and sunflower, growing under conditions of Fe deficiency [3, 4]. Recently, Vorwieger et al. [5] confirmed that the 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, although not in those of tomato, which do not excrete riboflavin under Fe-deficient conditions. Hence, riboflavin secretion under Fe deficiency is genetically regulated.

Iron restriction is potentially a serious problem for plants, just as it is for humans and other organisms. In particular, plants growing on calcareous soils are at risk of Fe deficiency because although ferric ions are often abundant in these soils they are largely unavailable to plant roots, owing to the extremely low solubility of ferric compounds. Therefore, mechanisms for the avoidance or correction of Fe deficiency in plants have been intensively studied [6-10]. However, it is not yet known why or how some plant species secrete flavins from their roots under conditions of Fe deficiency. Recent proteomic analyses of Fe deficiency with/without CaCO₃ in *Medicago truncatula* have revealed the *de novo* accumulation of 6,7-dimethyl-8-ribityllumazine synthase (DMRLs or RibC) and GTP cyclohydrolase II (GTPcII or RibA), along with increases in the corresponding mRNA levels, thereby confirming the up-regulation of *de novo* riboflavin biosynthesis under

Fe deficiency [11]; and very recently, the production of both riboflavin and novel flavins has been demonstrated [4]. The authors have speculated that flavins accumulated in the roots might act as electron donors or as cofactors for Fe (III) reductase [11], because the Fe (III) reductase protein contains an FAD-binding sequence motif [12].

In a previous paper, we demonstrated that both riboflavin secretion and increased respiration occur in response to Fe deficiency in *Hyoscyamus albus* hairy roots [13]. We focused on aerobic respiration and the involvement of the mitochondrial electron transport chains (mtETC). The plant mtETC in the mitochondrial membrane consists of several members, including four components found in all living cells, complex I to complex IV [14], in addition to a plant-specific alternative oxidase (AOX) and NAD(P)H dehydrogenases (ADX). The results we obtained from feeding experiments with respiratory component-specific inhibitors suggested that, in *H. albus* roots under Fe deficiency, proton pumping is associated mainly with electron flow from ADX to complex III and complex IV; neither complexes I and II, which contain flavins as cofactors, nor AOX, which has no ability to pump protons, appear to be fully functional. We hypothesized that flavins not required for incorporation into fully-functional complexes I and II might be transported outside the mitochondria, reaching the apoplasts and the rhizosphere [13].

In order to understand the mechanism of riboflavin secretion into the rhizosphere that occurs under Fe deficiency in our *H. albus* root system, it is important to establish whether the secreted riboflavin is produced by *de novo* synthesis, as reported in *M. truncatula* roots [11], or by the liberation and partial decomposition of protein-bound flavins, such as FMN and FAD, which are usually found as cofactors of redox proteins such as complex I and complex II [15] and Fe (III) reductase [12]. Although the existence of FMN hydrolase activity in plants has been confirmed based on gene expression and enzyme activity, its physiological relevance remains speculative [1, 16]. In the present paper, therefore, we have investigated whether secreted riboflavin originates from the *de novo* synthesis of riboflavin or, alternatively, from the partial decomposition of FMN. In addition, we have examined further the relationship between riboflavin production and respiration activity.

2. Results

2.1. Site of riboflavin secretion

As described previously [17], riboflavin secretion was detectable in the tips of *H. albus* roots cultured for more than 7 days in Fe-deficient medium without pre-culture. To confirm further the site of riboflavin secretion, 7-day old root tips, from which riboflavin was not yet visibly detectable, were subjected to confocal microscopy. Since riboflavin has intrinsic fluorescence, it is detectable without staining. The results showed that, at the organ level, riboflavin was essentially localized to the tip of Fe-deficient root (Fig. 1a, b) and, at the cellular level, mainly to the apoplast (Fig. 1c). Within the root tip, riboflavin was highly localized to the epidermis and cortex, rather than the stele, essentially as observed near the apex in sliced sections of *M. truncatula* roots [4].

2.2. Transcript accumulations involved in riboflavin biosynthesis

A priori, there are two possible ways to produce free riboflavin: by *de novo* synthesis and by liberation from conjugated flavins. Firstly, we examined whether or not the *de novo* synthesis of riboflavin was enhanced under Fe deficiency. It is known that riboflavin is biosynthesized starting from GTP and ribulose-5-phosphate, the further products of which are combined to produce 6,7-dimethyl-8-ribityllumazine, which is then converted to riboflavin [18]. The initial rearrangement of GTP and formation of 3,4-dihydroxy-2-butanone 4-phosphate from ribulose-5-phosphate are catalyzed by a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase (denoted as RibA/B); the final reactions are carried out by 6,7-dimethyl-8-ribityllumazine synthase (RibC) and riboflavin synthase (RibD), respectively [18]. Substrates for enzyme assays are not commercially available and instead we determined levels of gene expression for *RibA/B*, *RibC* and *RibD*. These genes were unreported in *H. albus*, however; therefore, we first undertook database searches in GenBank to generate consensus sequences between *Arabidopsis thaliana* and various solanaceous plants such as *Solanum*, *Nicotiana* and *Lycopersicon species* that could be used to design primer sets for PCR in *H. albus*. The products of RT-PCR were cloned and sequenced, and then their partial sequences were confirmed by comparative analysis based on nucleotide and

translated amino acid levels (Table 1). At the amino acid level, the putative *RibA/B*, *RibC* and *RibD* from *H. albus* displayed 87, 76 and 76% identity, respectively, to the corresponding genes from *A. thaliana*, and 93, 94 and 91% identity to those from the solanaceous plants. Based on these results, we assigned these sequences as *HaRibA/B*, *HaRibC* and *HaRibD* and have deposited them in the database (accession numbers are AB712368, AB712369 and AB712370, respectively).

We compared the gene expression of *HaRibA/B*, *HaRibC* and *HaRibD* between whole roots cultured under Fe-deficient and those cultured under Fe-replete conditions, using RT-PCR with the gene-specific primer sets given in Table 2. However, there were no notable differences between the two groups of roots (data not shown). Therefore, we separated the cultured roots into young roots, including the root tips, and the remaining, older parts of the roots; and for each type of root tissue we compared gene expression under Fe-deficient and Fe-replete conditions. Although no significant differences were observed in relation to Fe status in the case of the older root parts, which excluded root tips (data not shown), apparent differences were detected in the case of the younger roots, which included root tips. In this case, time-course analysis of gene expression showed that the riboflavin-biosynthetic genes *HaRibA/B*, *HaRibC* and *HaRibD* were all apparently up-regulated under Fe deficiency (Fig. 2a, b). Semiquantitative determination showed that, relative to 16-h, Fe-replete roots, transcript accumulations for *HaRibA/B*, *HaRibC* and *HaRibD*, Fe-deficient roots were increased by factors of 2.9 ± 0.4 , 5.9 ± 1.0 and 5.7 ± 0.4 , respectively (Fig. 2c). Up-regulation in response to Fe-deficiency was therefore observed within a single day and it continued until day 7.

Riboflavin secretion into the liquid culture medium was also analyzed throughout the time course; under Fe-deficient conditions, it became detectable from day 3 onwards and increased until day 7 (Fig. 2d). In contrast, no riboflavin secretion was detectable under Fe-replete conditions (data not shown), suggesting that under these conditions there was little (or no) riboflavin present in an uncombined form.

We next focused on the possibility of riboflavin liberation from FMN by the action of FMN hydrolase (FHy). We aimed to clone *H. albus*-specific FHy cDNA; however, a database search revealed that FHy was fused with the gene encoding riboflavin kinase, *RibK*. Cloning of the putative *HaFHy/RibK* was therefore carried out (Table 1), similarly as described above for the other riboflavin-biosynthetic genes, based on the known domain sequence from *A. thaliana* (NM_118267, AY878327) [16, 19] and the corresponding sequence of *Lycopersicon esculentum* (BT014255). The results of RT-PCR revealed that the expression of

HaFHy/RibK (Accession number, AB712371) appeared also to be enhanced under Fe deficiency (Fig. 2a, b).

2.3. Change in FMN hydrolase activity

Although an enhancement of *de novo* riboflavin synthesis occurred under Fe deficiency, there was a relatively long lag between the up-regulation of expression of riboflavin-biosynthetic genes (at 16 h) and the initiation of riboflavin secretion (at 3 days). This suggested that another mechanism might be involved in the observed secretion of riboflavin. We therefore postulated that a partial decomposition of conjugated riboflavin might be essential for the production of free riboflavin. To explore this possibility, FMN hydrolase (EC 3.1.3.2) activity was assayed at intervals during the 7-day time-course, in both Fe-deficient and Fe-replete roots. In Fe-deficient roots, the activity increased sharply after 3 days, whereas in Fe-replete roots the activity gradually declined during the same period (Fig. 3). Initially, FMN hydrolase activity in Fe-deficient roots was lower than that in Fe-replete roots, but after day 3 the activity in Fe-deficient roots surpassed that in Fe-replete roots: the activity was significantly higher at day 5 ($p < 0.05$) and day 7 ($p < 0.01$). Day 3 therefore represented a breakpoint in FMN hydrolase activity in roots cultured under Fe-deficient conditions.

2.4. Respiration activity

Previous studies with 7-day-old *H. albus* roots had shown, by assay of TTC-reducing activity, that respiration was enhanced by Fe deficiency [13], even though aerobic respiration by the mitochondrial electron transport chain (mtETC) requires the participation of many Fe ions. Alongside these studies, plant-mtETC-specific inhibitor-feeding experiments *in vivo* were performed and, in addition, mitochondrial Fe contents were determined in both Fe-replete and Fe-deficient roots. The results suggested that electron flow linked to proton pumping occurred mainly between ADX and complexes III and IV and that, in *H. albus* roots under Fe deficiency, neither complex I nor complex II was active. Since both complex I and complex II contain not only large numbers of Fe ions but also flavins as cofactors, it is conceivable that flavins normally incorporated into these complexes might, during a response to Fe-starvation stress, be transported outside the mitochondria, such as to the apoplasts and to the rhizosphere.

In order to consider further the possible relationship between respiration and riboflavin secretion and make a direct comparison with changes in FMN hydrolase activity, respiration activity was measured during a 7-day time-course in roots cultured with/without Fe (Fig. 4). Following the transfer of roots to either Fe-replete or Fe-deficient media at the start of the time-course, root respiration was little affected for the first 3 days, regardless of Fe status. Thereafter, however, differences in respiration activity with Fe status became apparent: under Fe deficiency, the activity was increased both at day 5 ($p < 0.05$) and at day 7 ($p < 0.01$). Hence, as with FMN hydrolase activity, day 3 was a breakpoint for respiration activity in Fe-deficient roots.

2.5. Effect of mtETC-specific inhibitors on gene expression of riboflavin synthase

In our previous paper we showed, for the first time to the best of our knowledge, that of several mtETC-specific inhibitors studied, the ADX inhibitor, dicoumarol, the complex III inhibitor, antimycin and the complex IV inhibitor, KCN, but not other inhibitors, each decreased riboflavin secretion under Fe deficiency [13]. However, it was unclear how respiratory inhibitors might reduce riboflavin secretion, although such inhibitors are known to affect respiratory proteins. We wondered whether a reduction in riboflavin secretion might be a secondary effect of the inhibition of respiratory proteins, perhaps through a feedback mechanism acting to inhibit riboflavin synthesis. Therefore, to determine whether riboflavin synthesis was being suppressed at the gene-expression level by those mtETC-specific inhibitors that decreased riboflavin secretion under Fe deficiency, and in the light of our discovery that the expression of the riboflavin synthase gene (*RibD*) was clearly up-regulated under Fe deficiency (Fig. 2a, b), we measured the effects of a range of mtETC-specific inhibitors upon the expression of *RibD* in both Fe-replete and Fe-deficient roots cultured for 7 days. (Since *RibD* was highly expressed under Fe deficiency, as shown in Fig. 2b, the PCR parameters were modified slightly in these experiments [Fig. 5 and legend].)

The results again confirmed that *RibD* expression was enhanced under Fe deficiency, in comparison to expression under Fe repletion, but there was no apparent effect of inhibitor treatments, regardless both of the type of inhibitor or of Fe status (Fig. 5). Although the analysis was confined to studying the expression of a single gene (*RibD*), there was therefore no evidence for any effect of mtETC inhibitors on the riboflavin biosynthetic pathway.

3. Discussion

Riboflavin secretion was detected only in root tips of *H. albus* roots cultured under Fe-deficient conditions (Fig. 1b); it was not detected in roots cultured under Fe-replete conditions (Fig. 1a) [13, 17]. In this study, therefore, we examined whether or not this secretion was associated with an up-regulation of the riboflavin biosynthetic pathway. Analysis of transcript accumulations involved in the *de novo* synthesis of riboflavin revealed up-regulation occurring in the younger roots, which included root tips, within 1 day of transfer of the roots to Fe-deficient conditions and continuing over the 7-day experimental period (Fig. 2b). A similar enhanced expression of riboflavin-biosynthetic genes (*RibA/B*, *C*, *D*, *E*) has been observed by qRT-PCR in *M. truncatula* roots subjected to Fe deficiency for 6 days [11].

It is likely that active metabolic changes occurred very rapidly in *H. albus* roots after the onset of Fe deficiency. In fact, up-regulation of H^+ -ATPase I transcript accumulation in *M. truncatula* roots [20] and of ferric chelate reductase (*FRO2*) and ferrous iron transporter (*IRT*) transcript accumulations in *A. thaliana* roots have been reported to occur within 1 day under Fe deficiency [21]. On the other hand, *H. albus* roots did not exhibit detectable riboflavin secretion until 3 days from transfer to Fe-deficient conditions (Fig. 2d); a relatively long lag therefore existed between the up-regulation of riboflavin-biosynthetic gene expression (16 h) and the initiation of riboflavin secretion (day 3). Furthermore, riboflavin secretion increased in a time-dependent manner (Fig. 2d), whereas elevated transcript levels of *RibA/B*, *RibC* and *RibD* were observed continually, up to day 7 (Fig. 2b). A similar mismatch between gene expression and riboflavin production has been reported in *M. truncatula* roots, where enhancement of *RibC* (or *DMRLs*) expression, occurring from day 3, preceded the appearance of riboflavin, detectable on day 7 [20]. The authors proposed the possibility that flavins accumulated in the roots under Fe deficiency could act as electron donors or as cofactors for Fe (III) reductase [11], because the Fe reductase protein contains an FAD-binding sequence motif [12]. However, the expression of both *RibC* and *FRO1* was enhanced well before riboflavin production became apparent [20]. Taking all these considerations into account, we considered the possibility of another mechanism to produce free riboflavin: the hydrolysis of FMN. We therefore assayed FMN hydrolase (FHy) activity and demonstrated an apparent increase in FHy activity in *H. albus* roots after 3 days of Fe deficiency (Fig. 3). These results supported our hypothesis that both *de novo* riboflavin synthesis and

hydrolysis of FMN are involved in riboflavin secretion from *H. albus* hairy roots under Fe deficiency. Although on this basis the total riboflavin production actually observed should be the sum of that generated by *de novo* synthesis and of that arising by hydrolysis of FMN, there is the possibility that some newly-synthesized riboflavin continues to be metabolized further to flavin nucleotides (FMN and FAD) and that *net* riboflavin produced by *de novo* synthesis is an underestimate of *gross* riboflavin produced *de novo*. If, alongside the production of flavin nucleotides, there is concurrent hydrolysis of these compounds, then there is the possibility of an (apparently) ‘futile cycle’ of flavin nucleotide production and hydrolysis. The conversion of riboflavin to FMN is catalyzed by riboflavin kinase; we have not yet detected the activity of this enzyme in *H. albus* roots, although Sandoval and Roje [16] have reported its assay using a recombinant *Escherichia coli*. These authors have found FMN hydrolase to be present both in the cytosol [16] and in chloroplasts [1]; and in the case of the cytosolic hydrolase, there is fusion of *FMN hydrolase (FHy)* and *riboflavin kinase (RibK)* genes (AY878327) [16]. In our present study, we have successfully cloned the corresponding fused cDNA from *H. albus* (Table 1); and therefore – if both genes are functional – confirmation of this gene fusion in *H. albus* relates directly to the possibility of an apparent ‘futile cycle’, as discussed above. Although, in common with the riboflavin-biosynthetic genes, *FHy/RibK* transcript accumulation seemed to be enhanced within one day under Fe deficiency (Fig. 2a, b), we could not distinguish *FHy* and *RibK* at the gene-expression level and it is therefore impossible to know whether the increase in riboflavin hydrolase activity is accompanied by a corresponding increase in riboflavin kinase activity. Further elucidation awaits the determination of RibK activity in *H. albus* roots.

Previously, we had showed that respiration (TTC-reducing) activity in 7-day-old *H. albus* roots was enhanced by Fe deficiency [13]. Measurement during a 7-day time-course in the present paper revealed that initially the activity was not significantly changed, but that after 3 days, significant increases in respiration were observed, in Fe-deficient roots only (Fig. 4). Similar enhancements of respiration in Fe-deficient roots have been described in cucumber [22, 23] and sugar beet [24] monitoring oxygen consumption, indicating an increase in aerobic respiration. In the case of *H. albus*, a change in the activity of the mtETC must be involved in this increase, as discussed below.

Changes in both FMN hydrolase activity (Fig. 3) and TTC-reducing activity (Fig. 4) occurred almost in parallel under Fe deficiency: that is, both activities were enhanced after 3 days in comparison to their activity under Fe-replete conditions. The length of this time period is suggestive of changes beyond simple metabolic

adjustments. As suggested previously by inhibitor-feeding experiments in our system [13], complex I of the mtETC, which requires a large number of Fe ions together with FMN, seemed not to function under Fe deficiency, in contrast to ADX, which was active. Vigani and Zocchi [25] have also presented data, in cucumber roots, suggesting that Fe deficiency induces ADX and enables an impaired complex I to be bypassed. In our previous findings [13], this functional change was detected in *H. albus* roots after 1 week of Fe deficiency, but it probably occurred after as little as 3 days, as suggested from the results described above. This change in the mtETC could be related to the hydrolysis of FMN, resulting in riboflavin secretion as discussed above, *i.e.* FMN no longer required for incorporation into complex I could be transported outside the mitochondria, in particular to the apoplasts and to the rhizosphere and, either before or after this process, FMN could be decomposed to riboflavin by FMN hydrolase. Imaging by confocal microscopy showed that riboflavin is restricted to the apoplasts (Fig. 1c), consistent with this suggestion.

Although inhibitors of ADX, complex III and complex IV severely reduced riboflavin production under Fe-deficient conditions [13], these inhibitors did not affect the accumulation of *RibD* transcripts involved in the *de novo* synthesis of riboflavin (Fig. 5). These inhibitors targeted mtETC proteins; the results we have presented here provide no evidence for feedback regulation by mtETC of riboflavin synthesis at the gene-expression level.

4. Materials and methods

4.1. Root materials and culture conditions

Hairy roots of *H. albus* L. (Solanaceae) used in these experiments were established as reported previously [17]. Roots were maintained on Murashige and Skoog basal medium [26] solidified with 0.2% gellan gum. A primary root tip with a few lateral roots (*ca.* 2 cm in length), isolated from *ca.* 2-week-old root cultures, was pre-propagated in liquid B5 medium [27] containing 1% sucrose for 2 weeks. The medium was then exchanged for either a Fe-deficient B5 medium or a Fe-containing B5 medium, each with 1% sucrose, followed in each case by further culture for up to 1 week. Only in the case of observation by confocal microscopy, a root tip was directly inoculated and cultured on solid Fe-deficient B5 medium [27] containing

1% sucrose. Fe-deficient B5 medium was prepared by omission of Fe-EDTA from B5 basal medium, prior to autoclaving at 121 °C for 15 min. All cultures were maintained in 100 ml conical flasks containing 25 ml of liquid medium and incubated at 25 °C with agitation at 80 rpm in the dark. The cultures were then harvested by vacuum filtration after the following periods: 16 h, 2 days, 3 days, 5 days and, finally, 7 days. Cultures were separated into roots and media, except when used for the TTC-reducing assay. Collected fresh roots were weighed and then stored at -80 °C, whereas media were stored at -20 °C until use. Feeding experiments with mtETC-specific inhibitors were performed as previously reported [13]. For the expression analysis of mtETC components, specific inhibitors were used as follows, at the concentrations indicated: complex I: barbiturate, 100 µM; ADX: dicoumarol, 10 µM; complex II: malonic acid, 800 µM; complex III: antimycin, 20 µM; complex IV: KCN, 10 µM; AOX: salicylhydroxamic acid (SHAM), 10 µM.

4.2. Confocal fluorescence microscopy

The tips of roots cultured on either Fe-deficient or Fe-replete medium, respectively, were imaged by confocal microscopy in order to determine the site of riboflavin localization. The intrinsic fluorescence of riboflavin was imaged with an Olympus FV-1000 confocal microscope, using excitation at 440 nm and detecting emission at 476 nm, and employing proprietary software (fluoreview FV-1000, Olympus, Japan).

4.3. RNA isolation and PCR cloning

Total RNA was isolated from frozen roots of *H. albus* (100 mg) using an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) and following the manufacturer's instructions. The concentrations of RNAs were determined using a NanoDrop ND-1000 spectrometer (USA). For the analysis of the expression of genes involved in riboflavin production, partial cDNA sequences corresponding to *RibA/B*, *RibC*, *RibD* and *FHy/RibK* were obtained from RNA of *H. albus* roots by RT-PCR amplification using a TaKaRa RNA PCR Kit (AMV) Ver.3.0 (Takara Bio, Japan). Because no genomic information on *H. albus* was available, initial primers were designed by the alignment of conserved cDNA sequences of these genes from *A. thaliana* and various plants of the Solanaceae, such as *Solanum*, *Nicotiana* and *Lycopersicon* species, previously deposited in the database (GenBank). Primers used were as follows: *RibA/B*, forward (5'-GGATAGTTTGTGTGAGCATG-3') and reverse (5'-CTAGCCATGGAACCATCATC-3'); *RibC*, forward

(5'-GCTTTTGGANGGAGC-3') and reverse (5'-ACACCAAATATGCA-3'); *RibD* forward (5'-CAGTAGCTGAGTTCGATACCCA-3') and reverse (5'-CAGCAATGAATCCTTTTGGCAC-3'); *FHy/RibK* forward (5'-GATTAACCATTTGAGCGGTCA-3') and reverse (5'-CTTCAACAACACAGAGAAGAC-3'). After PCR amplification, products were cloned using a TOPO TA Cloning kit (Invitrogen, Japan), according to the manufacturer's instructions. After being sequenced, the cDNA sequences and their translated amino acid sequences were subjected to BLAST search (NCBI); putative gene homologues from *H. albus* were confirmed by sequence comparison with those of *A. thaliana* and several species of the Solanaceae, as given in Table 1. The GenBank accession numbers of putative *HaRibA/B*, *HaRibC*, *HaRibD* and *HaFHy/RibK* genes are AB712368, AB712369, AB712370 and AB712371, respectively. Based on the sequence information, gene-specific primers for RT-PCR were designed (Table 2).

4.4. Gene expression by RT-PCR

RT-PCR was performed using an mRNA-selective PCR kit (Takara Bio, Japan) with the primers listed in Table 2. A sample aliquot containing 0.5 µg RNA was subjected to reverse transcription (30 min at 45°C). The PCR conditions were slightly varied, depending on the genes, as follows: *RibA/B* and *RibD* (1 min at 85°C, 1 min at 47°C, 1 min at 72°C, 21 cycles); *RibC* (1 min at 85°C, 1 min at 37°C, 1 min at 72°C, 22 cycles); and *RibK/FHy* (1 min at 85°C, 1 min at 47°C, 1 min at 72°C, 22 cycles). In the case of mtETC-inhibitor-feeding experiments, 19 cycles were employed for the amplification of *RibD*. RT-PCR products were loaded onto 3% (w/v) agarose gels and stained with ethidium bromide. Ribosomal RNA (rRNA) was used as a control, because the expression of α -tubulin in *H. albus* was affected severely under Fe deficiency (data not presented). A 100-bp DNA ladder (Takara) was used as a molecular marker. Pictures were taken with a grey-scale digital camera (Scion Corp., USA. Model CFW-1310M) and band intensities were measured using Image J software (NIH, USA).

4.5. Assay of FMN hydrolase activity

Crude enzyme extracts were prepared at 0~4°C as described previously (Sandoval and Roje, 2005), with some modifications. Frozen roots (0.5 g) were mixed with polyvinylpyrrolidone (0.05 g) and

homogenized in a mortar in 2.5 ml of 100 mM Tris-HCl buffer (pH 7.5), containing 1 mM DTT and 1 mM MgCl₂. After centrifugation at 20,000 x g for 20min, the supernatant was applied to a PD-10 column pre-equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM DTT, 1 mM MgCl₂ and 10% glycerol; the protein fraction was then eluted and used as a crude enzyme solution. Protein contents were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Japan), according to Bradford's method [28] and using bovine serum albumin as a standard.

Flavin mononucleotide (FMN) hydrolase activity was assayed as previously reported [16]. The reaction mixture comprised 0.2 ml of 50 mM TrisHCl buffer (pH 7.5), containing 100 µM FMN, 1 mM DTT, 10 mM MgCl₂, and 20 µg of crude enzyme solution. Following incubation at 30 °C for 30 min, the reaction was terminated by adding 10 µl of acetic acid. After incubation at 80 °C for 15 min followed by centrifugation (20,000 g, 15 min), the supernatant was collected and analysed by HPLC. Blanks were prepared using boiled enzyme (98 °C, 15min).

For the measurement of riboflavin, the FMN hydrolase reaction product, 20 µl of the stopped assay solution was applied to a HPLC system (Jasco LC-Net II/ADC, Japan) fitted with a Wakosil-II 5C8 RS column (Wako Corporation, Japan, 4.6 x 150 mm). The eluent conditions were as follows: flow rate, 0.8 ml/min; column temp, 40 °C; solvent A, MeOH; solvent B, 10 mM NaH₂PO₄ (pH 5.5, adjusted with 1 M NaOH); isocratic elution, with A:B = 35:65. Flavins were detected with a fluorescence detector (Jasco FP-2020 plus). Excitation and emission wavelengths were 444 and 530 nm, respectively. A UV detector (Jasco UV-2070 plus) was also used, for detection at 371 nm. Retention times were: FAD, 2.5 min; FMN 3.1 min; riboflavin, 4.0 min. Quantification was based on a standard curve using authentic riboflavin.

4.6. Assay of TTC-reducing activity

For the assay of respiration activity, we employed the 2,3,5-triphenyltetrazolium chloride (TTC)-reducing assay [29], with modifications as described previously [13, 29]. The medium was removed from the root culture using a pipette, the roots were washed with 5 ml of sterile water, and then 10 ml of filter-sterilized 0.5% (w/v) TTC reagent (Sigma) in 50 mM potassium phosphate buffer (pH 7.0) was added. Incubation with TTC reagent was performed under sterile conditions for 3 h and with shaking (80 rpm). 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. The reduction activity was calculated using a standard curve that was previously obtained using authentic 1,3,5-triphenylformazan (Tokyo Kasei Co., Japan) dissolved in 95% (v/v) ethanol.

4.7. Riboflavin content

Riboflavin secretion into the culture medium was confirmed and analyzed by HPLC, as mentioned above. The content was routinely determined using a UV-VIS spectrophotometer (Shimazu UV-1600) at 444 nm, as previously reported [13, 17].

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Figure Legends

Fig. 1 Confocal laser-scanning microscope images of *H. albus* roots cultured under Fe deficiency.

Images of riboflavin distribution were captured from 7-day-old root tips cultured on solid Fe-deficient and -replete B5 medium. Images a and b were captured from a root tip supplied with or without Fe, respectively; image c was around 100–200 μm from the root tip of b. The bars in (a, b) and (c) represent 100 μm and 10 μm , respectively.

Fig. 2 Expression of riboflavin-biosynthetic genes in cultured roots of *H. albus*.

Time-courses of gene expression for *HaRibA/B*, *HaRibC*, *HaRibD* and *HaFHy/RibK* under conditions of Fe repletion and Fe deficiency are shown in (a) and (b), respectively; (c) shows a semiquantitative analysis of the expression, at the 16 h time-point, of *HaRibA/B*, *HaRibC* and *HaRibD*. A time-course of riboflavin secretion under Fe deficiency, paralleling the gene-expression data in (b), is shown in (d).

RibA, GTP cyclohydrolase II; RibB, 3,4-dihydroxy-2-butanone 4-phosphate synthase; RibC, 6,7-dimethyl-8-ribityllumazine synthase; RibD, riboflavin synthase; FHy, FMN hydrolase; RibK, riboflavin kinase. RNA was extracted from 0.1 g of fresh roots, including root tips, and 500 ng RNA was used for RT-PCR. Using primers specific for *RibA/B*, *RibC*, *RibD* and *FHy/RibK*, corresponding fragments of 367, 244, 215 and 576 bp, respectively, were amplified. Ribosomal RNA (rRNA) was used as a control. –Fe, no addition of Fe; +Fe, with addition of 0.1 mM Fe(III)-EDTA. For the semiquantitative analysis (c), expressions of *HaRibA/B*, *HaRibC* and *HaRibD* in Fe-replete roots at 16 h were treated as control (1.0) based on their band intensities and those in Fe-deficient ones were given as relative intensities.

Fig. 3 Time-course of FMN hydrolase activity in *H. albus* roots cultured under Fe deficiency and Fe repletion.

Results are means of 3 independent experiments, and bars indicate standard deviations of means. Data are analyzed by ANOVA and Student t test. * and ** reveal significant differences at the levels of $p < 0.05$ and $p < 0.01$, respectively. FMN, flavin mononucleotide; –Fe, no addition of Fe; +Fe, with addition of 0.1 mM Fe(III)-EDTA.

Fig. 4 Time-course of TTC-reducing activity in *H. albus* roots cultured under Fe deficiency and Fe repletion.

Results are means of 3 independent experiments, and bars indicate standard deviations of means. Data are analyzed by ANOVA and Student t test. * and ** reveal significant difference at the level of $p < 0.05$ and $p < 0.01$, respectively. –Fe, no addition of Fe; +Fe, with addition of 0.1 mM Fe(III)-EDTA.

Fig. 5 Effects of mtETC-specific inhibitors on *riboflavin synthase* (*HaRibD*) expression in *H. albus* hairy roots under Fe deficiency and Fe repletion.

Profiles of RT-PCR products of *HaRibD* gene (279 bp) in hairy roots treated with various inhibitors for 7 days under Fe deficiency and Fe repletion are shown. Inhibitors and concentrations were as follows: Cont (control), no inhibitor; Bar (barbiturate), 100 μM ; Dic (dicoumarol), 10 μM ; Malo (malonic acid), 800 μM ; Anti (antimycin), 20 μM ; KCN, 10 μM ; SHAM (salicylhydroxamic acid), 10 μM . A RNA fraction corresponding to 500 ng was supplied to each lane and rRNA loadings are shown in the lower panel. –Fe, no addition of Fe; +Fe, with addition of 0.1 mM Fe(III)-EDTA. For this analysis, 19 cycles of amplification were used in RT-PCR, instead of the 21 cycles used in obtaining the data in Fig. 2.

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Fig.1

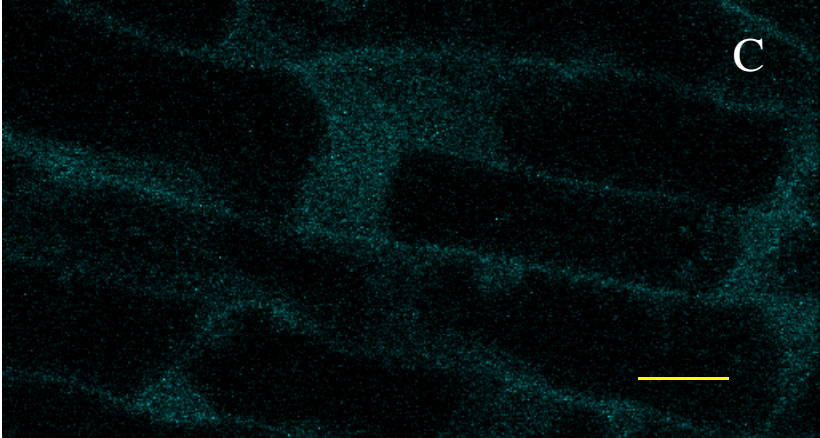
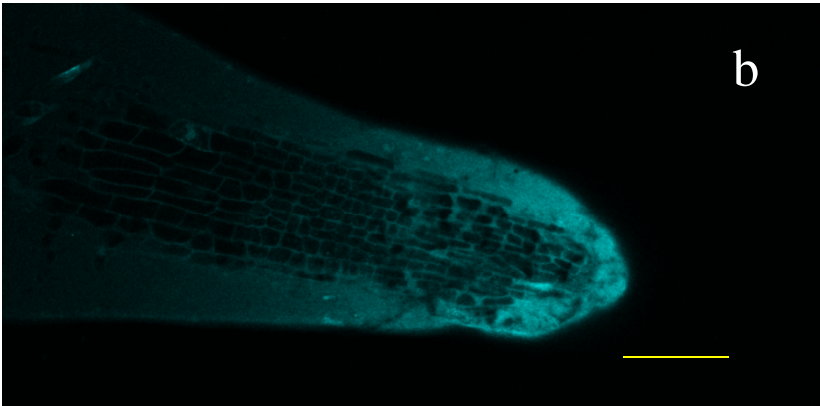


Fig.2

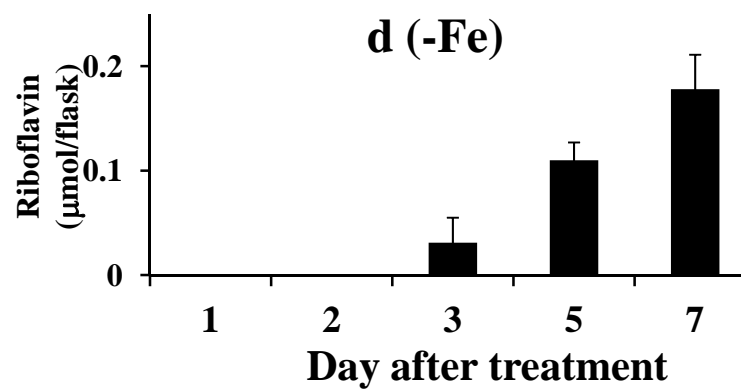
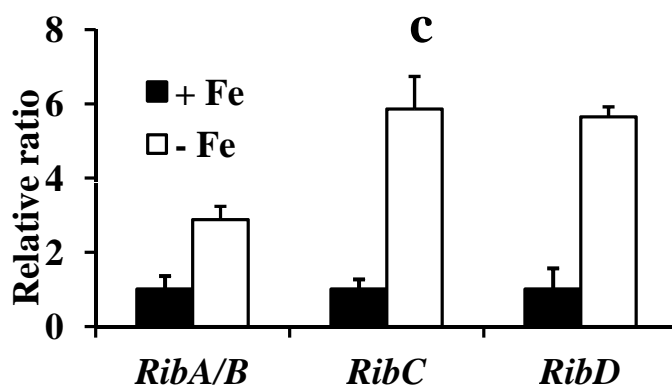
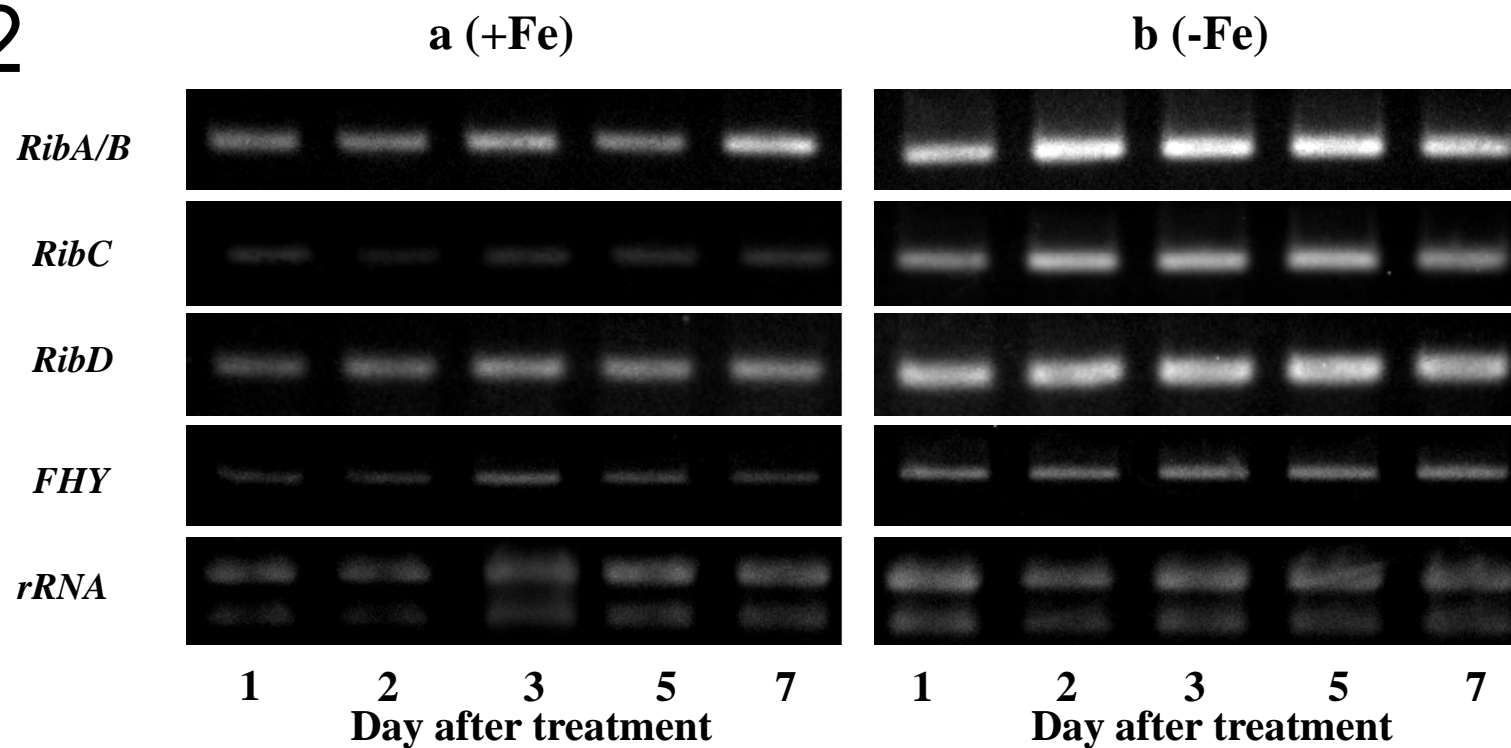


Fig.3

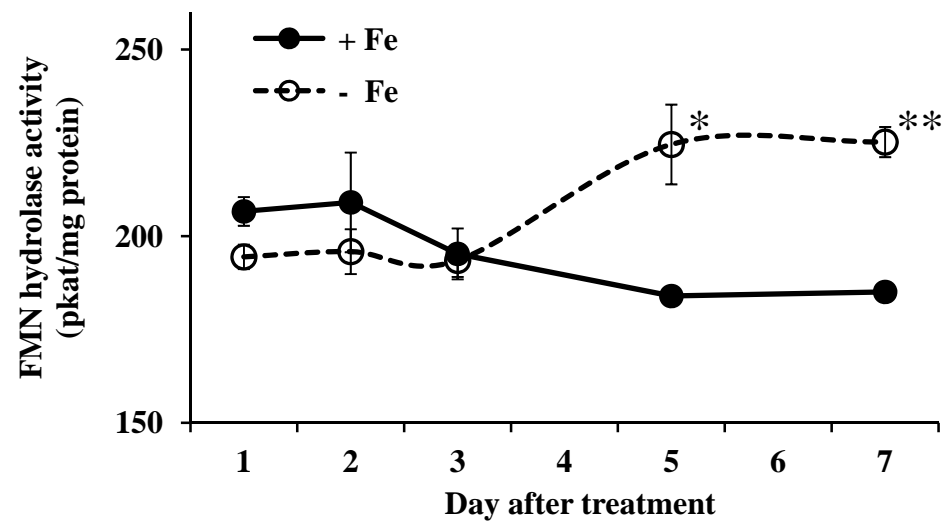


Fig.4

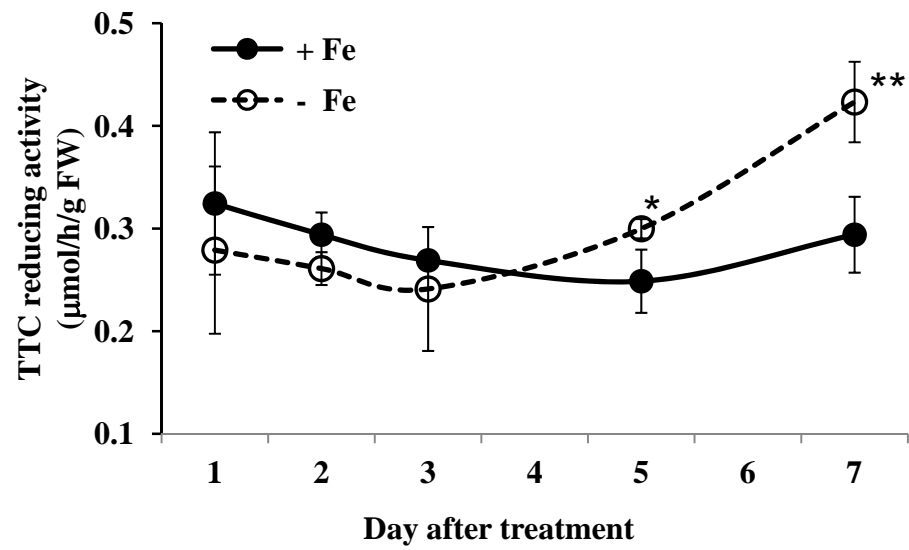


Table 1. Identification of putative *RibA/B*, *RibC*, *RibD* and *RibK/FHy* genes in *Hyoscyamus albus* roots by homology searches based on nucleotide and amino acid levels.

Putative gene in <i>H. albus</i>	Corresponding gene deposited in database		Identities (%)	
	Plant species	Accession number	nucleotide*	amino acid**
<i>RibA/B</i>	<i>Arabidopsis thaliana</i>	NM_125826	76	87
	<i>Lycopersicon esculentum</i>	AJ002298, NM_001247847	92	93
	<i>Nicotiana benthamiana</i>	AB538870	89	93
<i>RibC</i>	<i>Arabidopsis thaliana</i>	NM_129967	76	76
	<i>Nicotiana tabacum</i>	AF422802	91	94
	<i>Solanum chacoense</i>	EU526014	93	92
<i>RibD</i>	<i>Arabidopsis thaliana</i>	NM_127633	70	76
	<i>Solanum tuberosum</i>	BQ121327	89	91
<i>FHy/RibK</i>	<i>Arabidopsis thaliana</i>	NM_118267, AY878327	73	76
<i>FHY domain</i>	<i>Lycopersicon esculentum</i>	BT014255	93	96
<i>RibK domain</i>	<i>Lycopersicon esculentum</i>	BT014255	88	91

Identities were conducted using BLASTN* and TBLASTX/BLASTP** searches in GenBank. RibA, GTP cyclohydrolase II; RibB, 3,4-dihydroxy-2-butanone 4-phosphate synthase; RibC, 6,7-dimethyl-8-ribityllumazine synthase; RibD, riboflavin synthase; FHy, FMN hydrolase; RibK, riboflavin kinase.

Table 2. Gene-specific primers employed for RT-PCR

Gene	Forward primer	Reverse primer	Product (bp)
<i>HaRibA/B</i>	5'-GGATAGTTTGTGTGAGCATG-3'	5'-CTAGCCATGGAACCATCATCATC-3'	367
<i>HaRibC</i>	5'-GGAGCTTTGGACACTTTC-3'	5'-CAAATATGCAAGGAGTACC-3'	244
<i>HaRibD</i>	5'-GTTGTCGGAATTTAGTGTCCG-3'	5'-TCCCAGTCTTGACCTTCACC-3'	215
<i>HaFH_y/RibK</i>	5'-GGTGTACCCATGGCATTAGC-3'	5'-CCAACCAATGCTCATGAC-3'	576

RibA, GTP cyclohydrolase II; RibB, 3,4-dihydroxy-2-butanone 4-phosphate synthase; RibC, 6,7-dimethyl-8-ribityllumazine synthase; RibD, riboflavin synthase; FH_y, FMN hydrolase; RibK, riboflavin kinase.