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Organ-dependent responses of the African rice to short-term iron toxicity: ferritin regulation and antioxidative responses

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Abstract

Hydroponically grown African rice (*Oryza glaberrima*) was exposed for 72 h to a high Fe^{2+} concentration (500 mg dm^{-3}) to identify the first steps of iron toxicity response in various organs. Iron accumulated in all plant parts analysed and had only a limited impact on absorption and translocation of other nutrients. The content of the iron-storage protein ferritin increased as a consequence of transcription stimulation or increase in mRNA stability and culminated after 48 h of treatment in laminae and to a lesser extent in sheaths but was not detected in roots. Although endogenous iron concentrations were similar in sheaths and laminae, superoxide dismutase activity was stimulated only in sheaths while ascorbate peroxidase activity increased mainly in laminae. It is concluded that both ferritin synthesis and antioxidative response may play a key role in the resistance of *Oryza glaberrima* to iron toxicity but that their relative importance are not the same in all organs.

Additional key words: ascorbate, glutathione, malondialdehyde, *Oryza glaberrima*, oxidative stress, superoxide dismutase.

Introduction

Iron is an essential element for plant metabolism but is extremely insoluble in oxygenated environments, being mainly present as oxihydrates. Plants therefore display a fascinating series of adaptation for iron acquisition (Briat and Lobréaux 1997, Hell and Stephan 2003). In contrast, anaerobiosis may prevail in waterlogged soils and large soluble Fe^{2+} concentrations may occur in soil solution because of strongly reducing conditions in the soil and low pH (Mandal and Haldar 1980). An excess of endogenous ferrous iron in plant tissues induces a wide range of metabolic disorders, mainly through the hastening of Fenton reaction leading to the production of toxic reactive oxygen species (ROS) such as the extremely reactive $OH^{\bullet}$ radical (Becana *et al.* 1998). Resistance to ferrous iron may involve oxidation by the roots and Fe^{3+} deposition at the root surface in an iron plaque. Protection against the harmful effects of endogenous Fe^{2+} also involves enhancement of antioxidative mechanisms (Vansuyt *et al.* 1997, Becana *et al*. 1998, Shainberg *et al.* 2000) and induction of ferritin, a class of multimeric proteins involved in the control of iron homeostasis because of their ability to sequester up to 4500 atoms of iron in a safe form as hydrous ferric oxide-phosphate inside a protein coat (Briat 1996, Murgia *et al.* 2002).

The Asian rice species *Oryza sativa* L. is rather sensitive to iron toxicity and stressed plants exhibit stunted growth, limited tillering, reduction in root oxidation power and leaf bronzing as a consequence of oxidative stress (Bode *et al.* 1995, Thongbai and Goodman 2000, Fang *et al.* 2001), ethylene over-production (Peng and Yamauchi 1993, Yamauchi and

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Abbreviations: APX - ascorbate peroxidase; ASA - ascorbate; BCIP - 5-bromo-4-chloro-3-indolyl phosphate; DTT - dithiotreitol; GSH - reduced glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; MPA - metaphosphoric acid; NBT - nitroblue tetrazolium; NEM - N-ethylmaleimide; OPA - *o*-phthalaldehyde; PMSF - phenazine methosulfate; PVP - polyvinylpyrrolidone; SOD - superoxide dismutase; TCA - trichloroacetic acid.
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Peng 1995) and nutritional disorders (Ottow *et al.* 1983, Genon *et al.* 1994). The African rice species, *Oryza glaberrima*, has lower yield potential than *Oryza sativa*. However, it is more resistant to several biotic and abiotic stresses including iron toxicity and is therefore an interesting pool of useful traits for rice improvement (Jones *et al.* 1997, Linares 2002). De Dorlodot *et al.* (2005) recently showed that an interspecific hybrid *Oryza sativa* × *Oryza glaberrima* exposed to iron stress (125 mg dm^{-3}) did not show iron toxicity symptoms despite a high endogenous iron concentration. The underlying biochemical and physiological properties sustaining the response to iron toxicity in *O. glaberrima*, however, remain elusive.

As far as rice is concerned, our knowledge on the kinetics of physiological responses to a sudden rise in soil Fe^{2+} remains elusive. Several studies reported the effect of iron toxicity on the aerial part (Bode *et al.* 1995, Zhang *et al.* 1999, Fang *et al.* 2001) but most of them focused on laminae, which are considered as the main contributors to photosynthesis as well as the site of expression of bronzing symptoms. In contrast, sheaths received little attention, although for other ion toxicities, such as salt stress, they are a site of toxic ions retention preserving the laminae from photosynthesis inhibition (Flowers *et al.* 1988, Hasegawa *et al.* 1995) as well as the main site for expression of genes involved in plant tolerance to NaCl (Claes *et al.* 1990). Most cultivars belonging to *O. glaberrima* exhibit a low laminae/sheath mass ratio but the importance of sheaths in the plant response to iron toxicity is unknown.

The present study was undertaken in order to assess the relative contribution of antioxidant mechanisms and ferritin regulation in the short term response of *O. glaberrima* to a high Fe^{2+} dose. Enzyme activities, concentration of antioxidants, kinetics of ferritin expression were analysed in relation to mineral nutrition and symptoms of oxidative stress separately in roots, sheaths and laminae of stressed plants.

Materials and methods

Plants and growth conditions: Seeds of *Oryza glaberrima* Steud. (accession No. IRGC 104047) were provided by the International Rice Research Institute (IRRI, Los Baños, The Philippines) and multiplied in our greenhouses. Seeds were germinated in glass flasks (10 cm diameter, 10 seeds per flask) on *Whatman No. 2* filter paper moistened with 10 cm^3 deionised water at 25 °C. A mean irradiance of 150 µmol m^{-2} s^{-1} was provided by *Sylvania* standard lamps (*F36W/133*) under a 12-h photoperiod. After 10 d, plants were acclimated on nutrient solution (Yoshida *et al*. 1976) in a phytotron, at a temperature of 25 °C and a mean irradiance of 360 µmol m^{-2} s^{-1} provided by *Philips HPLR 400W* lamps under a 12-h photoperiod. Relative humidity varied between 86 and 100 %. Plants were distributed among 8 tanks containing 25 dm^3 of nutrient solution and fixed on floating polystyrene plates (25 plants per tank). After 2 weeks of acclimation in the absence of stress, $FeSO_4 . 7 H_2O$ was applied to half of the tanks in order to obtain a final concentration of 500 mg dm^{-3} of ferrous iron. The pH was adjusted each day to 4.5 using KOH (3.5 M stock solution) in all tanks. Solutions were not aerated in order to avoid oxidation of Fe^{2+} to insoluble Fe^{3+}. Oxidoreduction potential of the nutritive solution was quantified using an *OR Sentix* probe (*WTW*, Weilheim, Germany). Soluble ferrous iron in the solution was quantified by the *Test Fer* kit (Merck, Darmstadt, Germany) using the spectrophotometric determination of an Fe-1,10-phenantroline complex according to the manufacturer's instructions.

Plants were harvested prior to stress imposition and after 24, 48 and 72 h of treatment. For each treatment and stress duration, 50 plants were harvested. Roots were rinsed during 1 min in $SrCl_2$ 1 mM in order to remove ions from the free spaces. For each plant, roots, sheaths and laminae were separated. Three randomly chosen groups of five plants were used for determination of growth parameters and ion content. The remaining organs were directly frozen in liquid nitrogen and stored at -80 °C until analysis.

Maize plants (cv. LG3267) used as control for ferritin induction and detection by Western blot (see below) were cultured under a 16-h photoperiod, a mean temperature of 25 °C and a mean irradiance of 360 µmol m^{-2} s^{-1} provided by *Philips HPLR 400W* lamps. These plants were grown in culture medium according to the procedure of Motta *et al.* (2001). After 11 - 15 d of culture, iron (500 µM Fe-EDTA, 150 µM trisodium citrate, 75 µM $FeSO_4$) was added to the culture medium.

Mineral analysis and malondialdehyde quantification: Organs of harvested pooled plants were weighed and incubated for 72 h in an oven at 70 °C (dry mass determination). Relative growth rate (RGR) was calculated according to the following formula $RGR = (\ln DM_i - \ln DM_f)/\Delta t$ where DM_i and DM_f are the initial mean dry mass of plants at the time of stress exposure and at the time of harvest, respectively. For each type of organ, samples of *ca.* 50 mg dry material were digested in the presence of 35 % HNO_3 at 80 °C. Minerals were solubilized in HCl 0.1 M and samples were filtred on *Macherey-Nagel Mn615* filters. Ion concentrations were then quantified by inductively coupled plasma emission spectroscopy (*ICP*, *Varian-Vista MPX*, Melbourne, Australia) using multi-elemental standards (*Reagecon*, Shannon, Ireland). Each measurement was performed in triplicates.

Malondialdehyde (MDA, a product of membrane lipid peroxidation commonly considered as an indicator of oxidative stress) was extracted from 250 mg of fresh

tissue homogenised in 5 cm^3 of 5 % trichloroacetic acid (TCA). The resulting mixture was centrifuged at 12 000 *g* and 4 °C for 10 min. MDA concentration was quantified in the supernatant using thiobarbituric acid according to Heath and Packer (1968).

Glutathione and ascorbate quantification: Reduced (GSH) and oxidized (GSSG) glutathione were quantified by high performance liquid chromatography after derivatization by *o*-phthalaldehyde (OPA) according to Cereser *et al.* (2001) after HPLC separation of GSH-OPA adducts on an *X Terra™ M5 C18* (*Waters*, Ireland) column maintained at 30 °C. Fluorimetric detection was performed with a spectra system *FL3000* fluorescence detector (*Thermoquest*, Wilrijk, Belgium) at 420 nm after excitation at 340 nm. Derivaties were eluted in an acetonitril gradient in a 50 mM sodium acetate buffer pH 6.2. Total glutathione was quantified after a reduction step of GSSG by dithiothreitol (DTT) according to Cereser *et al.* (2001). The amount of GSSG was obtained by substracting of the GSH from the total glutathione value.

For ascorbate (ASA) quantification, 250 mg (f.m.) were ground in a prechilled mortar and homogenised in 4 cm^3 trichloroacetic acid (TCA, 6 % m/v). After 15 min incubation on ice, samples were centrifuged at 12 000 *g* at 4 °C for 5 min. Ascorbate concentration in the supernatant was determined according to Okamura (1980) using α,α'-dipyridyl. After 1 h incubation at 37 °C, samples were read at 525 nm. Dehydroascorbate was reduced to ascorbate by incubation in the presence of DTT (10 mM) during 15 min at 42 °C. N-ethylmaleimide (NEM; 0.5 % m/v) was used to remove the excess of DTT and total ascorbate was determined. The amount of dehydroascorbate is then calculated by substracting ASA from the total ascorbate.

Protein extraction and enzyme activities: The homogenization solution for superoxide dismutase (SOD; EC 1.15.11) contained 50 mM phosphate buffer (KH_2PO_4, K_2HPO_4) pH 7.0 and 1 % (m/v) PVP. Samples of 250 mg (f.m.) were homogenized in 5 cm^3 of extraction buffer and centrifuged during 30 min at 12 000 *g* and 4 °C. Supernatants were filtered on Machery-Nagel Mn 615 membrane and directly used for total SOD assays according to Dhindsa *et al.* (1981) exploiting the ability of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). One unit of enzyme is defined as the amount required to inhibit the photoreduction of NBT by 50 % after 20 min under irradiance of 132.5 µmol m^{-2} s^{-1}.

For glutathione reductase (GR, EC 1.6.4.2), ascorbate peroxidase (APX, EC 1.11.1.11) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) tissue samples (500 mg f.m.) were homogenized in a mortar and pestle with liquid nitrogen, suspended in a 50 mM potassium phosphate buffer pH 7.0, 1 M NaCl, 0.1 mM EDTA, 1 % (m/v) PVP, glycerol 5 % (v/v) and 1 mM ascorbate and centrifuged for 10 min at 12 000 *g*. The assay for GR at 25 °C involved monitoring the oxidation of NADPH at 340 nm in 1 cm^3 reaction solutions that contained 0.1 M Tris-HCl (pH 7.8), 2 mM EDTA, 75 µM NADPH, 0.5 mM oxidized glutathione (GSSG) and 0.05 cm^3 of enzyme extracts. APX and DHAR activities were quantified on the same extracts at 25 °C following the procedure of Nakano and Asada (1981) and Kuźniak and Sklodowska (1999), respectively.

RNA extraction and Northern-blot analysis: Total RNA was isolated using the *RNeasy® Midi Kit* (*Qiagen*, Westburg, Belgium) according to the manufacturer's instructions. Equal RNA aliquots were resolved electrophoretically in 1.2 % (m/v) agarose gel containing 18 % (v/v) formaldehyde and 20 % (v/v) of 5 × MOPS solution. RNA was transferred to *Hybond-N* nylon membrane (*Hybond™-N+*, *Amersham,* Roosendaal, The Netherlands) and was cross-linked using an *UV-Cross Linkers* (Vilber Lourmat, France) during 4 min at 0.12 J.

The cDNA corresponding to the *Oryza sativa* ferritin gene (*OsFer*1; Gross *et al.* 2003) (EST clone - C10641) was kindly provided by the Maff DNA Bank-Rice Genome Project and was inserted in plamid pGEX-KT. A 531 bp fragment located downstream of the putative transit and extension peptides was amplified by PCR using the following primers (the BamHI restriction site is underlined): 5'-TAC<u>GGATCC</u>GACCAGTCTCTCGC-3' and 5'-CTGCTTGAGGAAGAAGCTCATCACCATC ACCATCAC<u>GGATCC</u>TCG-3' and inserted in pGEX-KT plasmid at the BamH1 site. The plasmid was amplified in *E. coli* (strain dh5α), recovered and digested by BAMHI. The fragment corresponding to the truncated ferritin cDNA was purified on gel and used to generate [α^{32}P]dCTP labelled probe *in vitro* using hexameric random primers according to standard procedures. Hybridization of the probe on the membrane was conducted at 42 °C. The blots were then washed three times (15 min each) in 2× SSC and 0.1 % m/v SDS at 42 °C and once during 10 min in 0.1× SSC and 0.1 (m/v) SDS at 60 °C.

Proteins extraction and Western-blot analysis: Fresh tissues (*ca*. 500 mg f.m.) were ground in liquid nitrogen and disrupted in 1 cm^3 of extraction buffer [SDS 4 % (m/v), Tris HCl 50 mM pH 7.0, PVP 1 % (m/v), PMSF 1 mM, DDT 50 mM, EDTA 20 mM, 1,10-phenanthroline 0.1 % (m/v), glycerol 20 % (v/v)] and centrifuged at 15 000 *g* and 4 °C during 30 min. The supernatant was recentrifuged for 15 min under similar conditions. The concentration of protein in the extract was quantified according to Bradford (1976). Equal amounts of proteins (5 µg) were loaded on two SDS-polyacrylamide gel and SDS-PAGE was performed according to Laemmli (1970). One of the gel was used for visual comparison after Coomassie blue staining and the other for immunodetection after protein transfer. The transfer of proteins from SDS-PAGE gels to nitrocellulose membrane (*Protran® de Schleicher and Schuell*, Dassel, Germany) was carried out using Tris 25 mM, glycine 192 mM and methanol 20 % (v/v) at 100 mA during 1 h

15 min. The membrane was then washed for 10 min in TBS 1× containing 0.1 % (v/v) *Tween 20* and then incubated for 30 min in TBS 1× containing 0.3 % (v/v) *Tween 20*, 5 % (m/v) of skimmed dry milk and 0.1 % goat serum (*Sigma-Aldrich*, Bornem, Belgium). The blot was incubated overnight with the primary antibody (a polyclonal antibody raised against maize seed ferritin (Lobréaux *et al.* 1992) at 1:2000 dilution) at 4 °C. The antigen was detected using alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (*Sigma-Aldrich*) and the *SigmaFast*^{TM} *BCIP/NBT* alkaline phosphatase substrate. The reaction was stopped by incubating the blot in 25 mM EDTA.

Statistical analysis: Two types of variance analysis (*ANOVA*) with crossed fixed factors were considered, depending on the analysed parameters: iron dose × duration of stress exposure and duration of stress exposure × iron dose × plant organs. The significance of differences between control and each treatment was analysed using the Student's *t*-test. The experiment was performed twice and exhibited similar trends. All statistical analyses were performed according to *SAS/STAT*® software (SAS Institute Inc. 1990).

Results

Nutrient solution changes: The pH value of control and iron-containing solutions remained closed to the adjusted value of 4.5 throughout the experiment. The redox potential varied between 208 and 319 mV for control solution and between 100 and 184 mV for iron-containing solution. After 3 d of treatment, there was no change in the total iron concentration of control solution, although 50 % shifted from the ferric to the ferrous form. In the iron-containing solution, the ferrous iron concentration decreased from 480 to 380 mg dm^{-3} while the ferric iron concentration increased from 15 to 60 mg dm^{-3}. There was a small but significant decrease in the total soluble iron pool in stressing solutions which may be partly related to absorption by the plants but also to root iron-coating or iron precipitation.

Dry mass, water and ion concentrations: Already after 48 h of exposure to iron, roots of treated plants turned light-brown, suggesting the formation of an iron plaque at the root surface. The dry mass of all plant organs increased with time ($P < 0.05$) but was not affected by iron stress (mean values of 0.82, 1.32 and 2.29 g for roots, sheaths and laminae, respectively at the end of the treatment). Iron treatment, however, decreased the mean

Table 1. Concentrations of mineral ions [μmol g^{-1} (d.m.)] in *Oryza glaberrima* cultured for 3 d in the absence (0) or in the presence of 500 mg dm^{-3} ferrous iron in nutrient solution (Yoshida *et al.* 1976) in roots, sheaths and laminae (means of 3 replicates ± SE). For a given element and a given organ, means followed by the same letter are not significantly different ($P > 0.05$).

Element	Roots		Sheaths		Laminae	
	0	500 mg dm^{-3}	0	500 mg dm^{-3}	0	500 mg dm^{-3}
Fe	123.2 ± 27.1a	1127.7 ± 100.6b	5.1 ± 0.3a	43.3 ± 0.5b	6.1 ± 1.3a	42.9 ± 7.0b
Ca	30.8 ± 3.4a	42.0 ± 13.2a	51.8 ± 9.2a	42.7 ± 6.6a	124.1 ± 9.9a	136.2 ± 11.7a
Cu	3.0 ± 0.7a	1.1 ± 0.1b	0.4 ± 0.0a	0.4 ± 0.0a	0.4 ± 0.0a	0.4 ± 0.0a
K	1797.0 ± 361.1a	958.3 ± 18.7a	1365.3 ± 107.6a	1258.1 ± 53.8a	604.7 ± 16.2a	547.2 ± 10.7b
Mg	143.1 ± 25.0a	58.4 ± 1.5b	176.3 ± 18.9a	149.7 ± 16.1a	234.4 ± 12.1a	196.7 ± 14.0a
Mn	1.0 ± 0.0a	0.5 ± 0.0b	8.1 ± 0.8a	4.2 ± 0.6b	14.6 ± 1.8a	10.3 ± 1.3a
Na	109.6 ± 9.9a	95.8 ± 4.2a	75.1 ± 2.7a	70.9 ± 6.3a	75.6 ± 12.2a	68.6 ± 6.3a
P	312.9 ± 16.1a	539.6 ± 24.8b	249.6 ± 13.6a	230.5 ± 5.7a	158.3 ± 11.0a	112.5 ± 6.8b
Zn	2.3 ± 0.7a	1.8 ± 0.3a	1.6 ± 0.0a	1.5 ± 0.0a	1.7 ± 0.1a	2.0 ± 0.3a

Table 2. Malondialdehyde concentration [nmol g^{-1} (f.m.)] of *Oryza glaberrima* cultured for 1, 2 and 3 d in the absence (0) or in the presence of 500 mg dm^{-3} ferrous iron, in nutrient solution (Yoshida *et al.* 1976) in roots, sheaths and laminae (means of 3 replicates ± SE). For a given organ, means followed by the same letter are not significantly different ($P > 0.05$).

Treatment	Roots		Sheaths		Laminae	
[d]	0	500 mg dm^{-3}	0	500 mg dm^{-3}	0	500 mg dm^{-3}
0	4.74 ± 0.07a	-	5.77 ± 0.70a	-	8.34 ± 0.23a	-
1	4.19 ± 0.11a	8.22 ± 0.32b	11.03 ± 0.98b	8.06 ± 0.29c	7.54 ± 0.34b	10.38 ± 0.20c
2	5.94 ± 0.28c	10.16 ± 0.67d	5.53 ± 0.71a	11.40 ± 0.61b	10.80 ± 0.61c	11.89 ± 0.44d
3	5.50 ± 0.47c	9.82 ± 0.42d	5.50 ± 0.47a	15.67 ± 0.31d	8.90 ± 0.14a	22.60 ± 0.14e

water content of laminae and sheaths ($P < 0.01$) already after two days of stress (90.4 and 76 % for sheaths and laminae in controls; 86.9 and 69.3 % in stressed plants). No obvious symptom of bronzing was recorded for the duration of the experiment.

The concentration of iron drastically increased with treatment in all plants parts (Table 1). Such an increase was already significant after 1 and 2 d of stress, respectively, in laminae and roots (data not shown). The concentration of mineral nutrients was most affected in roots: iron concentration in stressed plants was 20 times higher in roots than in shoots. Iron accumulated to a similar extent in sheaths and laminae and was 7 times higher after 72 h in stressed plants than in controls. In roots of plants exposed to iron stress, Cu, Mg, Mn concentrations decreased significantly whereas P increased ($P < 0.01$). In sheaths, Mn concentration also decreased but was the only element beside iron affected by iron treatment. Potassium and P concentration decreased in laminae of stressed plants compared to controls but no other nutrient appeared to be affected by iron toxicity.

Oxidative stress and antioxidant responses: The application of ferrous iron in the nutrient solution induced a significant increase in MDA concentration in all organs ($P < 0.01$) already after 24 h (Table 2). At the end of the stress period such an increase was clearly higher in sheaths (284 %) and in laminae (254 %) than in roots (144 %). Nevertheless MDA concentration was significantly higher in laminae than in sheaths.

The concentration of GSH in treated plants was higher in all plant organs under iron stress, mainly in sheaths and laminae (Fig. 1). The GSSG content was not affected in roots and sheaths by the iron treatment but increased markedly in laminae after 3 d of iron stress. Accumulation of GSSG in laminae occurred concomitantly with a slight but significant decrease in GSH concentration between 48 and 72 h, and might not be the consequence of GR inhibition since the activity of this enzyme at the end of exposure was higher in laminae of stressed plants than in controls (Fig. 1). GR activity also increased in sheaths of stressed plants but was not significantly different from controls in the roots.

In the roots of control plants, ASA was present only in very small amounts (Fig. 2). Ascorbate concentration was far higher in the aerial part and was ten times higher in laminae than in sheaths. Ascorbate concentration in stressed plants culminated after 48 h in roots and after 24 h in laminae. After 72 h of treatment, ASA

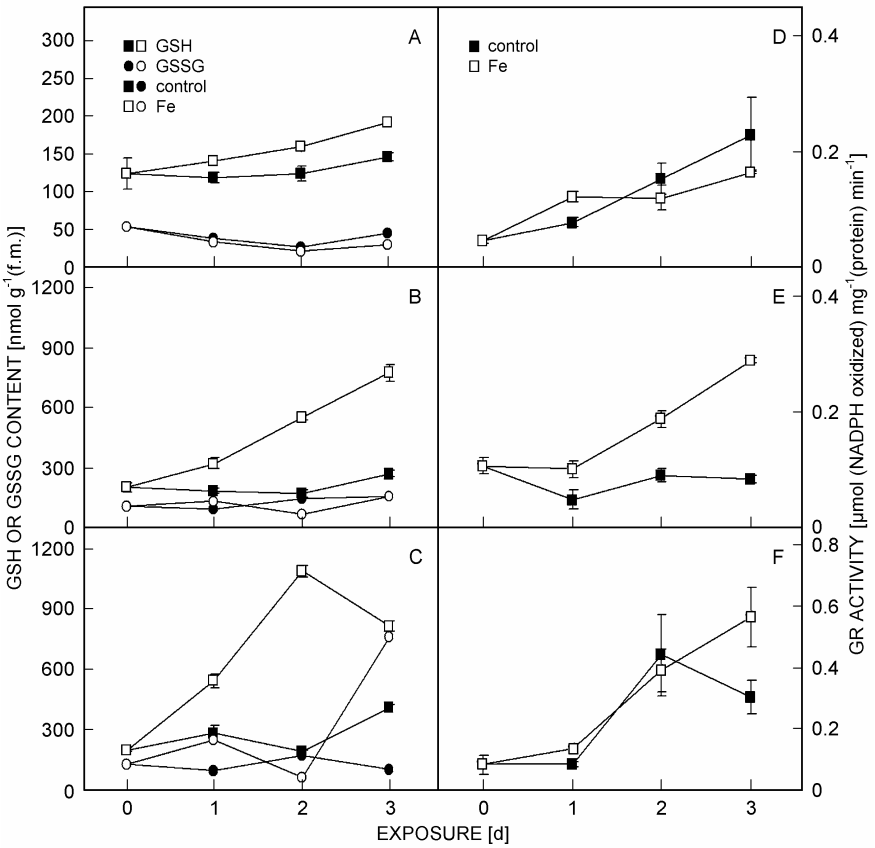

Fig. 1. Changes in glutathione (GSH-GSSG) concentrations (*A*,*B*,*C*) and glutathione reductase activity (*D*,*E*,*F*) in *O. glaberrima.* Plants were cultured for 1, 2 and 3 d in the absence (control) or in the presence of 500 mg dm^{-3} ferrous iron. Values are given separately for roots (*A*,*D*), sheaths (*B*,*E*) and laminae (*C*,*F*). Vertical bars represent SE ($n = 3$). Only those SE larger than symbol size are shown.

concentration was lower in laminae of treated plants than in control while an opposite trend was recorded in sheaths. Such a decrease was not due to the synthesis of dehydroascorbate since this compound remained at very low levels whatever the treatment or the plant organ (data not shown). Dehydroascorbate reductase activity was similar in stressed and controls, except after 48 h of exposure, where DHAR activity was significantly lower in roots and higher in sheaths and laminae of stressed plants than in control ones (Fig. 2). The activity of APX (Fig. 3) was higher in iron treated roots than in controls during the first two days of treatment but was similar to the activity recorded in controls at the end of exposure. Ferrous iron had no impact on APX activity in sheaths but it increased it in laminae after 48 and, to a lesser extent after 72 h of stress.

Iron stress slightly increased total SOD activity in the roots (Fig. 3). The iron-induced increase in total SOD activity in sheaths remained rather small, although significant, during the first 2 days of exposure to iron but then increased suddenly during the last day of exposure to 500 mg dm^{-3} Fe^{2+}. Such an effect was not recorded in laminae where total SOD activity transiently increased compared to controls after 24 h of stress exposure only.

Regulation of ferritin mRNA and protein: mRNA was slightly induced in sheaths and more strongly in laminae in response to iron stress as early as 24 h after the beginning of the treatment (Fig. 4). At the end of the treatment, the levels of ferritin mRNA induction was the highest in laminae. In contrast, no induction was recorded in root tissues although the level of iron was quite high in this organ, and this even after 72 h of exposure to iron (data not shown).

In control plants, ferritin protein was detected in all organs, albeit at relatively low level in roots. A marginal increase of the ferritin content could be observed after 24 h treatment in roots, sheaths and laminae (data not shown). The increase of the ferritin protein appeared to culminate approximately 48 h after the beginning of the iron treatment. The amount of the protein appeared slightly higher in laminae than in sheaths (Fig. 5*A*). The ferritin content then decreased in all organs during the last day of stress, although it remained still higher in treated plants than in control ones. At the end of the stress exposure, the maximal amount of ferritin detected on Western blot was still noticed for laminae (Fig. 5*B*).

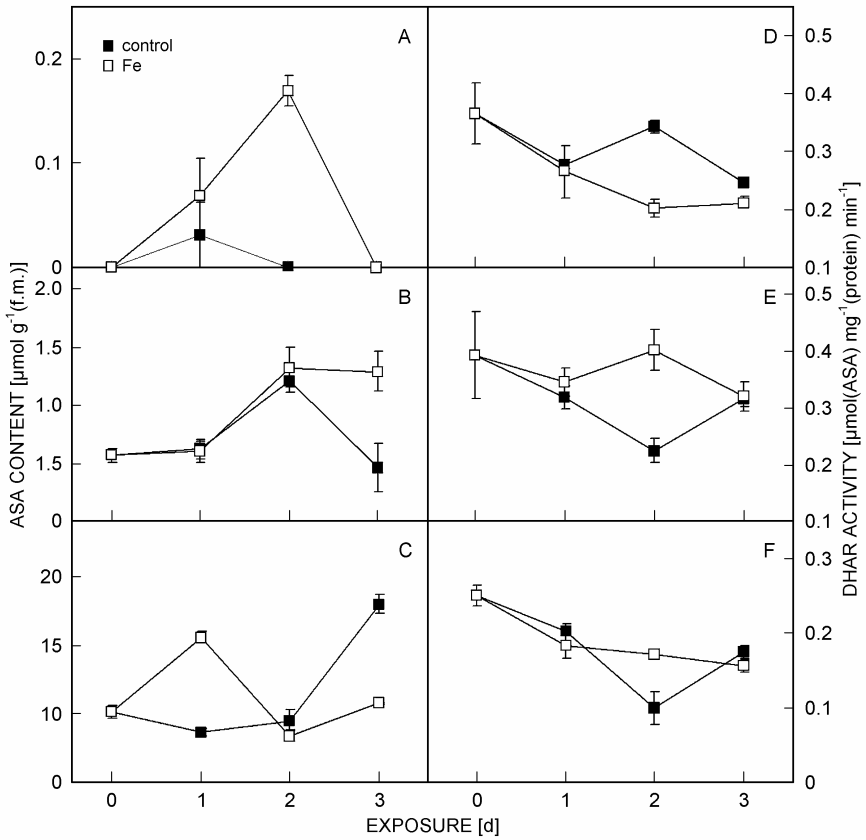

Fig. 2. Changes in ascorbate content (*A*,*B*,*C*) and dehydroascorbate reductase activity (*D*,*E*,*F*) in *O. glaberrima.* Plants were cultured for 1, 2 and 3 d in the absence (control) or in the presence of 500 mg dm^{-3} ferrous iron. Values are given separately for roots (*A*,*D*), sheaths (*B*,*E*) and laminae (*C*,*F*). Vertical bars represent SE (n = 3). Only those SE larger than symbol size are shown.

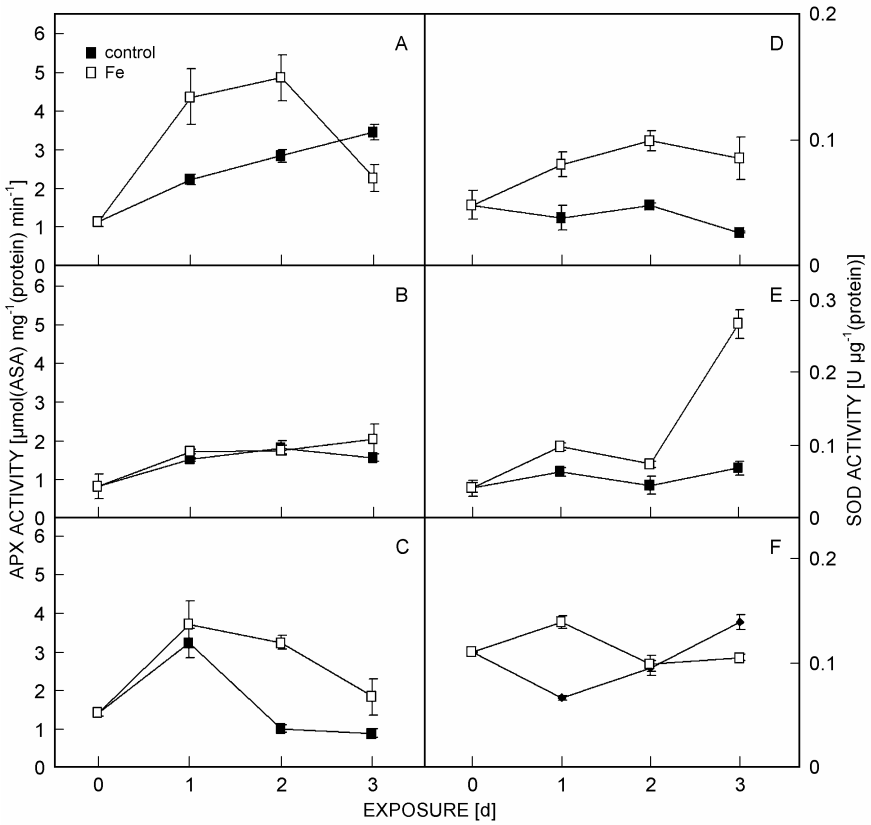

Fig. 3. Time course of the changes in ascorbate peroxidase activity (*A*,*B*,*C*) and superoxide dismutase activity (*D*,*E*,*F*) in *O. glaberrima.* Plants were cultured for 1, 2 and 3 d in the absence (control) or in the presence of 500 mg dm^{-3} ferrous iron. Values are given separately for roots (*A*,*D*), sheaths (*B*,*E*) and laminae (*C*,*F*). Vertical bars represent SE (n = 3). Only those SE larger than symbol size are shown.

Discussion

In the Asian rice *Oryza sativa,* growth can be reduced and bronzing may appear at external concentrations as low as 10 mg dm^{-3} Fe^{2+} in culture medium (Tadano and Yoshida 1978). In our work, when plants of the African rice were submitted to 500 mg dm^{-3} Fe^{2+} at pH 4.5 for 3 d, no obvious symptom of bronzing was recorded and the plant growth, measured on a dry mass basis, was not affected despite a slight decrease in the mean water content of laminae and sheaths. Various studies aiming at quantifying the impact of iron toxicity upon rice yield used longer exposure time than we did (Ottow *et al.*

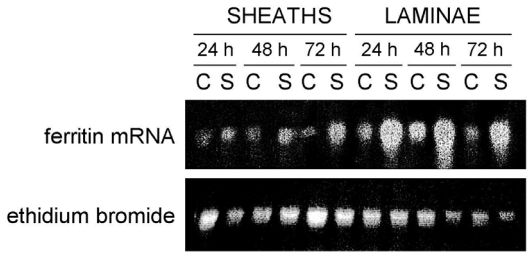

Fig. 4. Time-course of ferritin mRNA in roots, sheaths and laminae of *O. glaberrima*. Plants were cultured for 24, 48 and 72 h in the absence (C) and in the presence of 500 mg dm^{-3} ferrous iron (S). Ethidium bromide staining of the RNA gel is reported as a control of gel loading.

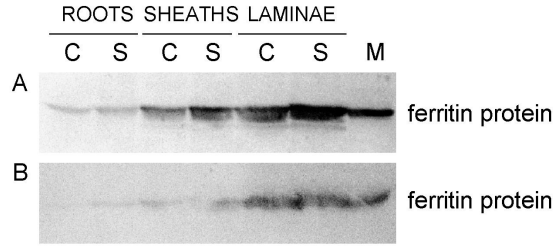

Fig. 5. Time-course of ferritin protein in roots, sheaths and laminae of *O. glaberrima*. Plants were cultured for 24, 48 and 72 h in the absence and in the presence of 500 mg dm^{-3} ferrous iron. Protein samples (5 µg in each lane) were electrophoresed through a 12 % SDS polyacrylamide gel, transferred to nitrocellulose membranes, and immunostained with rabbit antibodies raised against maize ferritin at a dilution of 1:2000. Relative ferritin protein abundance is shown in roots, sheaths and laminae of rice plants treated or not with iron for 48 h (*A*) or 72 h (*B*). *Lane C* - control plant, *lane S* - iron-treated plants, *lane M* - treated maize sample used as immudetection control.

1983, Genon *et al.* 1994, Sahrawat *et al.* 1996, De Dorlodot *et al.* 2005) but it is worth mentioning that for *Oryza sativa*, obvious symptoms of bronzing were reported after a few days of exposure to even lower Fe^{2+} concentration than the one used in this study (Peng and Yamauchi 1993, Bode *et al.* 1995). The oxidizing power of rice roots functions as a defence mechanism against toxic substances such as ferrous iron and hydrogen sulfite. In our experiments, iron plaque appeared already after 24 h of exposure, as reported earlier for *Oryza sativa* (Greipsson and Crowder 1992). According to Yamauchi and Peng (1995), roots may somewhat limit iron translocation through the transpiration stream in the Asian rice, at least for short terms. For this species, however, iron toxicity symptoms such as bronzing has been reported for leaf iron concentration as low as 108 µg g^{-1} (d.m.) (Montás-Ramírez *et al.* 2002), 170 µg g^{-1} (d.m.) (Ottow *et al.* 1983), 250 µg g^{-1} (d.m.) (Genon *et al.* 1994). Sharawat *et al.* (1996) consider that 300 µg g^{-1} (d.m.) is a critical endogenous iron concentration leading to metabolic disorders in *Oryza sativa*. When plants of *O. sativa* were partially de-rooted and exposed to high external Fe^{2+} concentration to allow massive translocation of iron to the leaves, the stress-induced ethylene production, which is responsible for several deleterious symptoms, already culminated after 24 h of stress exposure (Peng and Yamauchi 1993). It is therefore noteworthy that our intact plants of *O. glaberrima* exposed for 72 h to iron stress may accumulate more than 2 200 µg g^{-1} in the shoots without exhibiting any symptoms. This result suggests *1*) that translocation to the shoots occurred very quickly, *2*) that root retention, despite the appearance of the iron plaque, did not enable the plant to fully prevent iron accumulation in the shoots and *3*) *O. glaberrima* should possess tolerance mechanisms allowing the species to cope with such potential high concentration of endogenous iron.

It has been reported that iron concentration by itself does not fully reflect the plant physiological status since iron toxicity is related to multiple nutrional stress linked not only to excessive soil Fe^{2+} content, but also to high Mn as well as low K, P, Zn and sometimes Ca and Mg (Ottow *et al.* 1983, Sahrawat *et al.* 1996, Montás-Ramírez *et al.* 2002). According to Kirk and Bajita (1995) and to Zhang *et al.* (1998), iron excess may increase Zn absorption in roots. In contrast, both Mn and Mg were reduced in iron-treated plants (Genon *et al.* 1994). Some authors, however, reported opposite trends: in response to ferrous iron, Zn was found to decrease while Mn increased in *Oryza sativa* (Montás-Ramírez *et al.* 2002). Interactions between iron and phosphorus absorption were also suggested (Zhang *et al.* 1998, 1999).

As far as *O. glaberrima* is concerned, such interactions between iron and other nutrients should be limited: indeed, a strong and obvious modification of iron was found in the shoots, but it was not accompanied by any significant modifications in other nutrients, except Mn which slightly decreased in the sheaths and P which slightly decrease in the laminae only. At the root level, only a slight decrease in Mg and Mn concentration was noticed.

Iron-induced modification in mineral nutrition may also be the consequence of secondary oxidative stress leading to phospholipid peroxidation (Vansuyt *et al.* 1997, Becana *et al.* 1998, Shainberg *et al.* 2000) which induces an irreversible inhibition of plasma membrane H^+-ATPase as shown by Sousa-Santos *et al.* (2001) in maize roots. Oxidative breakdown products such as MDA may also have inhibitory effects on Fe uptake (Benderliev *et al.* 2003). However, even though iron concentration was high at the root level in *O. glaberrima,* oxidative damages quantified on the basis of MDA concentration were lower than in shoots. Stress-induced stimulation of APX might be involved in protection against H_2O_2. However, lower concentration of antioxidative compounds such as glutathione or ascorbate in roots comparatively to shoots, as well as the absence of iron stress effect on root glutathione reductase activity suggests that antioxidative mechanisms were not the only property involved in the prevention of iron induced damages in roots. Similarly, ferritin protein was present only in small amounts and thus did probably not contribute significantly to the resistance of root tissues. A consistent portion of the quantified iron in roots may be localised at the root surface and in intercellular spaces in the root cortex rather than within the cells. The washing step procedure probably allowed us to remove soluble Fe^{2+} from the free space but not Fe^{3+} precipitated at the cell wall level.

Within shoots, iron is evenly distributed between sheaths and laminae: after 72 h of exposure, iron concentration was the same in both organs. Our results suggest however that the physiological way to cope with accumulated iron was not the same in sheaths and laminae. Fang *et al.* (2001) reported that iron stress decreases SOD activities but increased both APX and GR activities in leaves of *O. sativa*. In contrast, Bode *et al.* (1995) reported that iron stress had no effect on leaf APX activities in this species. In both studies, however, the authors quantified the enzyme activities from the whole leaf system. Our results show that in *O. glaberrima*, total SOD stimulation occurred in response to iron stress in sheaths while APX mainly increased in laminae. In sheaths, H_2O_2, which is a product of SOD activity, thus probably also increased. However, an increase in the endogenous concentration of glutathione in sheaths may be a part of a H_2O_2-detoxication mechanism. The absence of concomitant increase in GSSG could be related to a significant increase in GR activity which occurred already after 48 h of treatment. Indeed, an iron-induced stimulation of GR activity has been reported in other experimental systems (Becana *et al.* 1998, Shainberg *et al.* 2000). In laminae, total SOD was not significantly modified (except a small increase after 24 h of exposure) while APX was significantly higher in treated plants than in controls especially after 48 and 72 h. A decrease of ASA concentration at this time followed an initial

increase and this suggests that ASA regeneration from oxidized forms was compromised in treated plants.

In plants, ferritin is mainly located in the stroma of chloroplasts and this specific localization may explain the lower amounts of ferritin detected in the roots of *O. glaberrima*. The presence of ferritin is under a tight developmental control: ferritin is mainly detectable in seeds where it constitutes the main source of iron for the young seedling, but is usually almost undetectable in vegetative organs such as roots and leaves of non-stressed plants (Lobréaux and Briat 1991). In *O. glaberrima*, however, we clearly detected the presence of ferritin in unstressed plants, thus suggesting that the species would be able to immediately manage a sudden rise in iron concentration, as it may occur in field conditions after a brutal submergence of acid soil (Mandal and Haldar 1980). Iron stress, however, also increased the amounts of ferritin as exemplified by the up-regulation of the transcripts levels which was already detectable 24 h after stress exposure. This induction was higher in laminae than in sheaths, although both organs accumulated similar concentrations of iron. It was not possible to quantify the proportion of accumulated iron which may be sequestred in ferritin in both types of organs. In Asian rice, two genes coding for ferritin and exhibiting a high level of homology for each other have been cloned (Gross *et al.* 2003). Unfortunately, data are not yet available on the putative *cis*-acting sequences in their respective promoter. Since *O. glaberrima* is sharing with *O. sativa* the A genome of the *sativa* complex, one may expect that these 2 genes may also be present in the African rice and that their identification and characterization may shed more light on the response of this species to iron stress.

Ferritin regulation should also be analysed in relation to oxidative stress since genes coding for ferritin have been reported to be activated by two different signal transductions pathways involving ABA and H_2O_2 (Briat 1996, Briat and Lobréaux 1997, Murgia *et al.* 2002), although a third pathway involving a direct interaction of iron with gene transcription cannot be ruled out (Lobréaux, unpublished results). The ascorbate concentration may also interfere with iron loading by ferritin: it was demonstrated *in vitro* that ASA concentrations lower than 2.5 mM favour iron uptake *in vitro* by pea seed ferritin, while higher concentrations promote iron release (Briat 1996). In our work, the ASA concentration may be as high as 10 mM and may thus generate unfavourable conditions for iron uptake by ferritin, even if the protein is highly expressed following a potential transcriptional up-regulation of the corresponding gene(s). From our experiment, however, there is no proof that *in planta*, ferritin and ascorbate are in the same cellular compartment and may exhibit such an interaction.

Further studies are thus required to precisely quantify iron uptake by ferritin in iron-stressed tissues of *O. glaberrima*.

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