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RESEARCH PAPER

Glutathione and ascorbic acid protect *Arabidopsis* plants against detrimental effects of iron deficiency

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Abstract

Iron is an essential micronutrient required for a wide variety of cellular functions in plant growth and development. Chlorosis is the first visible symptom in iron-deficient plants. Glutathione (GSH) and ascorbic acid (ASC) are multifunctional metabolites playing important roles in redox balancing. In this work, it was shown that GSH and ASC treatment prevented chlorosis and the accumulation of reactive oxygen species induced by iron deficiency in *Arabidopsis* leaves. In iron deficiency, GSH and ASC increased the activity of the heme protein ascorbate peroxidase at a similar level to that found in iron-sufficient seedlings. GSH was also able to preserve the levels of the iron–sulfur protein ferredoxin 2. GSH content decreased 25% in iron-deficient *Arabidopsis* seedlings, whereas the ASC levels were not affected. Taken together, these results showed that GSH and ASC supplementation protects *Arabidopsis* seedlings from iron deficiency, preserving cell redox homeostasis and improving internal iron availability.

Key words: *Arabidopsis thaliana*, ascorbic acid, chlorosis, glutathione, iron deficiency, reactive oxygen species.

Introduction

Abiotic stresses generally result in an imbalance of cell redox status due to the overproduction of oxidative radicals. This leads, in turn, to an increase in the synthesis of antioxidants such as glutathione (GSH) and ascorbic acid (ASC) and to an increase in the activity of antioxidant enzymes (Noctor and Foyer, 1998). Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot -}$), and hydroxyl radical ($OH^{\cdot}$) can be detoxified by oxidizing GSH and ASC.

The ASC–GSH cycle is a mechanism for removing H_2O_2. It consists of the enzymes ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), and glutathione reductase (Foyer and Noctor, 2011). APX reduces H_2O_2 to water through the oxidation of ASC, which is subsequently reduced by sequential reactions carried out by the enzymes monodehydroascorbate reductase and DHAR using GSH. Finally, glutathione reductase regenerates GSH from its oxidized form, GSSG, allowing the cycle to continue.

Because of its high cell concentrations, GSH and ASC act as ROS scavengers, keeping cell redox homeostasis under control. Many other primary and secondary metabolites may play a similar role; however, GSH and ASC differ from most because of the following characteristics: (i) there are specific enzymes that link GSH and ASC with H_2O_2 metabolism, (ii) the stability of the corresponding oxidized forms, and (iii) the ability to be recycled to reduced forms through a powerful enzymatic system that depends on the electron transport molecule NAD(P)H (Foyer and Noctor, 2011).

Although it has been determined that GSH and ASC can respond in a compensatory way to the cell redox imbalance, it has also been demonstrated that these two compounds have

Abbreviations: A, absorbance; ANOVA, analysis of variance; APX, ascorbate peroxidase; ASC, ascorbic acid; CAT, catalase; DAB, 3,3′-diaminobenzidine; DHAR, dehydroascorbate reductase; Fd, ferredoxin; GRX, glutaredoxin; GSH, glutathione; H_2DCF DA, 2′,7′-dichloro-dihydrofluorescein; ROS, reactive oxygen species; SE, standard error; SOD, superoxide dismutase.

other specific functions, highlighting that they should not be considered merely as antioxidants. *Arabidopsis* mutants deficient in GSH have shown that GSH plays a critical role in embryo and meristem development (Vernoux *et al*., 2000; Cairns *et al*., 2006; Bashandy *et al*., 2010), while complete deficiency of ASC causes lethality at the seedling stage (Dowdle *et al*., 2007).

GSH is involved in detoxification of xenobiotics and heavy metals, storage and transport of reduced sulfur, regulation of nuclear and plastid gene expression, and pathogen resistance, among others (Mullineaux and Rausch, 2005; Meyer, 2008; Rouhier *et al*., 2008; Foyer and Noctor, 2009). It has been established that GSH is closely related to glutaredoxins (GRX), enzymes that have a role in protein regulation through glutathiolation. Glutathiolation can protect the protein thiol groups of irreversible inactivation by oxidation and can also regulate the activity in a positive or negative way (Rouhier *et al*., 2008). It has been determined by *in vitro* and *in vivo* experiments that GRX14 and GRX16 are involved in the incorporation of [2Fe-2S] groups into proteins (Bandyopadhyay *et al*., 2008).

ASC participates in the regulation of programmed cell death, flower senescence, and response against pathogen attack, and in the protection of plants exposed to UV, heat, and high light intensity (Linster and Clarke, 2008; Foyer and Noctor, 2011).

There are few studies that have evaluated the relationship between nutrient deficiency and antioxidant defence mechanisms in plants. In sunflower, the level of H_2O_2 increases in the leaves and this correlates with a decrease in the activity of APX, peroxidases, and superoxide dismutase (SOD) as a consequence of low iron availability (Ranieri *et al*., 2001). In other studies, it was found that GSH and ASC levels were increased in cucumber and sugar beet exposed to conditions of iron deficiency (Zaharieva *et al*., 1999; Zaharieva and Abadía, 2003).

In this work, we have presented the results of studies directed to understand the effects of GSH and ASC in *Arabidopsis* seedlings grown under conditions of iron deficiency. Our results indicated that supplementation with GSH and ASC protect *Arabidopsis* against the detrimental effect of iron deficiency. GSH and ASC were able to preserve chlorophyll content without increasing internal iron concentration. The activity of GSH and ASC appeared to be mediated through their antioxidant capacity, as the protective effect correlated with decreased levels of ROS and higher activity of APX. Therefore, it is postulated that GSH and ASC treatments contribute towards keeping cell redox homeostasis in plants growing under iron-deficient conditions.

Material and methods

Plant material, growth conditions and treatments

Seedlings from *Arabidopsis thaliana* ecotype Columbia (Col-0) were used for these experiments. The seeds were surface sterilized in a solution containing 30% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 for 15 min, washed, and kept for 3 d at 4 °C in darkness for seed stratification. The seeds were then sown on plates containing nutrient solution composed of 5 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $MgSO_4.7H_2O$, 2.5 mM KPO_4, 70 mM H_3BO_3, 0.01 μM $MnCl_2$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 10 μM NaCl, 0.01 μM $CoCl_2$, and 50 μM FeNaEDTA. The nutrient solution was supplemented with 1% (w/v) sucrose and 0.8% (w/v) agar. The seedlings were grown for 7 d and then transferred to plates containing nutrient solution with either iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) or iron-deficient conditions (–Fe, 0.1 μM FeNaEDTA), with or without reduced L-glutathione (Sigma-Aldrich) or L (+)-ascorbic acid (Mallinckrodt Baker) for the time and concentrations indicated in the legends of the corresponding figures.

Chlorophyll quantification

One hundred milligrams of leaves was powdered with liquid N_2 and pigments were extracted with 4 vols of 80% (v/v) acetone. The extraction was performed in the dark for 2 h at 4 °C with agitation. The extracts were centrifuged at 10 000*g* for 15 min at 4 °C. Total chlorophyll content was quantified by measuring the absorbance at 652 nm (A_{652}) and its concentration was calculated as described by Arnon (1949).

GSH and ASC quantification

Leaves and roots were harvested (approx. 200 mg of each tissue), powdered with liquid N_2, and GSH and ASC were extracted as detailed by Bartoli *et al*. (2006). GSH and GSSG were determined as described by Griffith (1980), and reduced and oxidized ascorbic acid were determined as described by Bartoli *et al*. (2006).

Total iron quantification

Leaves and roots were harvested, washed in a solution containing 5 mM $CaSO_4$ and 10 mM EDTA, and dried at 70 °C overnight. Approximately 100 mg was incubated with 2 M HCl at 120 °C overnight. Total iron was quantified using the ferrozine method (Stookey, 1970). The samples were treated with 10% (w/v) hydroxylamine hydrochloride in 0.1 M HCl for 30 min, and then acetate buffer (pH 5.5) was added until the solution reached pH 4. Finally, the samples were treated with 0.5 mM ferrozine and the absorbance at 562 nm was measured using a spectrophotometer. The values were compared against a calibration curve made with a standard solution of ammonium iron sulfate treated in the same manner.

ROS detection

For fluorescent ROS detection, leaves were vacuum infiltrated with 10 μM of 2′,7′-dichloro-dihydrofluorescein (H_2DCF DA; Sigma-Aldrich) prepared in 5 mM MES buffer (pH 5.7) for 10 min, incubated for 20 min in darkness, and washed with fresh MES buffer for 30 min. The leaves were visualized by confocal fluorescence microscopy and bright-field microscopy in an Eclipse E 200 microscope (Nikon). Roots were incubated with 10 μM H_2DCF DA in 5 mM MES buffer (pH 5.7) for 30 min in the dark and washed with fresh MES buffer for 30 min. The roots were visualized by fluorescence microscopy and bright-field microscopy in an Eclipse T*i*-S microscope (Nikon).

For H_2O_2 detection with 3,3′-diaminobenzidine (DAB), leaves were incubated in a solution containing 1 mg ml^{-1} of DAB in HCl (pH 3.6) for 18 h and bleached at 90 °C in 95% (v/v) ethanol. The leaves were placed on a slide and observed under a stereomicroscope. The presence of H_2O_2 was indicated by the formation of a brown precipitate produced by the oxidation of DAB due to the presence of H_2O_2.

Ferredoxin 2 (Fd2) and ferredoxin 3 (Fd3) immunodetection

Leaves and roots were collected and powdered with liquid N_2. The samples were homogenized in 50 mM phosphate buffer containing

50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% (w/v) polyvinylpyrrolidone-40 (PVP-40), and 1% (v/v) Protease Inhibitor Cocktail (Sigma). The homogenates were centrifuged at 12 000*g* for 20 min at 4 °C and the soluble fraction was recovered. Samples for electrophoretic separation were denatured and separated on a 15% (w/v) SDS-PAGE gel followed by staining with Coomassie Brilliant Blue. The proteins were blotted onto PVDF membrane and immunodetected according to the method of Hanke *et al*. (2004). Polyclonal anti-Fd2 and anti-Fd3 antibodies were kindly provided by Professor Toshiharu Hase (Division of Enzymology, Osaka University, Japan).

Measurement of APX and catalase (CAT) activities

Leaves were powdered with liquid N_2. Soluble proteins were extracted by homogenizing the powder in 1 vol. of 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1% (w/v) PVP-40, and 1% (v/v) Protease Inhibitor Cocktail. The extraction buffer for APX activity additionally contained 5 mM ascorbate. The homogenates were centrifuged at 12 000*g* for 20 min at 4 °C and the supernatant was recovered for enzymatic assays.

APX activity was determined following the oxidation rate of ascorbate (extinction coefficient 2.8 mM^{-1} cm^{-1}) at 290 nm for 2 min using a spectrophotometer (Ultrospec 1100 *pro*; Amersham Biosciences). The reaction solution contained 50 mM phosphate buffer (pH 7.4), 0.5 mM ascorbate, 0.2 mM H_2O_2, and 50 µl of enzyme extract in a final volume of 1 ml.

CAT activity was determined by following the consumption of H_2O_2 (extinction coefficient of 39.4 mM^{-1} cm^{-1}) at 240 nm for 1 min using a spectrophotometer. The reaction solution contained 50 mM phosphate buffer (pH 7.4), 10 mM H_2O_2, and 50 µl of enzyme extract in a final volume of 1 ml.

Statistical analysis

For the evaluation of significant differences between treatments, statistical tests were performed using the program SigmaPlot, version 11.0.

Results

GSH treatment protects iron-deficient Arabidopsis *seedlings from chlorosis and alleviates the nutritional stress*

Chlorosis is one of the earliest symptoms observed in leaves of plants growing in soils with low iron availability. Fig. 1A shows that *Arabidopsis* seedlings exposed to iron deficiency for 7 d presented threefold less chlorophyll than seedlings growing under iron-sufficient conditions. Treatments with 0.25, 0.5, and 1 mM GSH protected seedlings from the decreased chlorophyll content generated by iron deficiency. In particular, treatments with 0.5 and 1 mM GSH were the most effective, presenting twofold more chlorophyll than the untreated seedlings (Fig. 1A).

The GSH effect was analysed in *Arabidopsis* seedlings growing at different iron concentrations. Fig. 1B shows that 1 µM FeNaEDTA caused a decrease in chlorophyll content relative to an iron-sufficient concentration (50 µM FeNaEDTA). Interestingly, GSH treatment attenuated the chlorophyll loss in seedlings growing at very low iron concentrations of 0.1 µM FeNaEDTA but had no effect at 1 µM FeNaEDTA, suggesting that GSH is able to prevent chlorophyll loss induced by iron deficiency when the damage is severe (Fig. 1B).

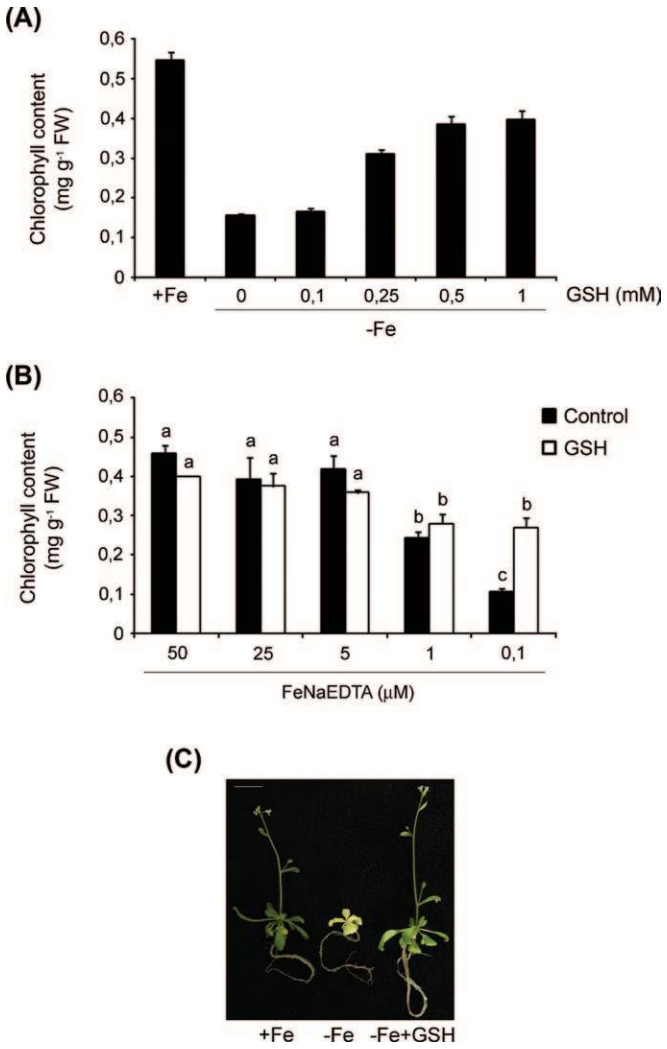

Fig. 1. GSH prevents chlorophyll loss and allows iron-deficient *Arabidopsis* seedlings to grow like iron-sufficient plants. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 µM FeNaEDTA) and then transferred to the treatments indicated. (A) Chlorophyll content in *Arabidopsis* seedlings grown under iron-sufficient (+Fe) or deficient (–Fe, 0.1 µM FeNaEDTA) conditions treated with or without 0.1, 0.25, 0.5, and 1 mM GSH for 7 d. The results show the mean ±standard error (SE) calculated from two independent experiments. (B) Chlorophyll content in *Arabidopsis* seedlings grown in 50, 25, 5, 1, or 0.1 µM FeNaEDTA treated with or without 1 mM GSH for 7 d. The results show the mean ±SE calculated from three independent experiments. Different letters indicate significant differences (one-way ANOVA, $P < 0.05$). FW: fresh weight. (C) *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 µM FeNaEDTA) and then transferred to iron-sufficient or deficient (–Fe, 0.1 µM FeNaEDTA) conditions with or without 1 mM GSH for 2 weeks. The pictures show the phenotype of representative *Arabidopsis* plants grown in Magenta boxes. Bar, 1 cm.

The effect of GSH treatment was assessed in *Arabidopsis* plants growing in iron-deficient conditions for 2 weeks. Fig. 1C shows that GSH reversed the effects of iron deficiency

allowing *Arabidopsis* plants grow and develop like plants grown in iron sufficiency.

GSH content in leaves and roots of iron-deficient Arabidopsis seedlings

Total GSH and the percentage of its oxidized form GSSG was quantified in leaves and roots of *Arabidopsis* seedlings exposed to iron deficiency. Table 1 shows that total GSH content decreased in leaves after 7 d of iron deficiency. The roots were shown to decrease their GSH content after 3 d and this persisted after 7 d of iron deficiency (Table 1). No significant differences in the percentage of GSSG were found in leaves and roots of iron-deficient *Arabidopsis* seedlings (Table 1).

ASC and GSH treatments prevent iron deficiency-induced chlorosis and restore the level of chlorophyll in already-established iron-deficient Arabidopsis seedlings

We hypothesized that GSH protects iron-deficient *Arabidopsis* seedlings due to its ability to arrest oxidation processes. Thus, we tested the effect of another important antioxidant in plants, ASC. Both GSH and ASC have been recognized as important cellular antioxidants; however, new and varied roles have been described for each (Foyer and Noctor, 2011). Fig. 2A shows that ASC treatment preserved the chlorophyll levels, as did GSH, when *Arabidopsis* seedlings were grown in iron deficiency. Fig. 2B shows the phenotype of *Arabidopsis* grown in iron deficiency and the protective effect of GSH and ASC.

The ASC content in iron-deficient *Arabidopsis* seedlings was evaluated. As shown in Table 2, total ASC and the percentage of oxidized ASC were not affected in leaves and roots of *Arabidopsis* seedlings during iron deficiency.

Table 1. Effect of iron deficiency on total GSH content and GSSG percentage in leaves and roots of *Arabidopsis*. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred (t_0) to iron-sufficient or iron-deficient (–Fe, 0.1 μM FeNaEDTA) conditions for 3 or 7 d. Asterisks indicate values that are significantly different relative to iron-sufficient conditions (one-way ANOVA, P <0.05). GSSG, oxidized glutathione; FW, fresh weight.

	Total GSH (nmol g^{-1} FW)	GSSG (%)
Leaf		
t_0	94±5	14±1
+Fe, 3 d	70±6	18±3
–Fe, 3 d	57±10	13±2
+Fe, 7 d	86±5	7±0.1
–Fe, 7 d	60±4*	6±1
Root		
t_0	71±6	7±3
+Fe, 3 d	76±5	13±5
–Fe, 3 d	58±7*	18±0.3
+Fe, 7 d	60±1	8±0.4
–Fe, 7 d	46±2*	8±0.3

We hypothesized a possible effect of GSH and ASC in increasing the iron availability or the iron content inside the plant. Thus, total iron content in iron-sufficient and iron-deficient *Arabidopsis* seedlings was measured using the ferrozine method. Fig. 3 shows that iron deficiency caused an iron content decrease of 30% in leaves and 20% in roots compared with the values under iron-sufficient conditions. GSH and ASC treatment did not restore the total iron content, indicating that the protection mediated by such treatments was not due to increased iron uptake and content in *Arabidopsis* seedlings.

As stated above, treatments with GSH and ASC were able to prevent the loss of chlorophyll in seedlings grown in iron deficiency. We were also interested in knowing whether GSH and ASC could promote the synthesis of chlorophyll in already-chlorotic *Arabidopsis* seedlings that were growing in iron deficiency. Thus, *Arabidopsis* seedlings were transferred from iron-sufficient conditions (t_0) to iron-deficient conditions for 3 d (–Fe, 3 d) (Fig. 4A). These seedlings were transferred to the following conditions for a further 4 d: iron sufficiency (+Fe, 4 d), iron deficiency (–Fe, 4 d), and iron deficiency with the addition of either 1 mM GSH (–Fe+GSH, 4 d) or 1 mM ASC (–Fe+ASC, 4 d) (Fig. 4A). Fig. 4B shows that chlorophyll content decreased in leaves of seedlings grown for 3 d in iron deficiency (–Fe, 3 d). The effect of iron deficiency on chlorophyll content was more evident after a further 4 d of iron deficiency (total of 7 d in iron deficiency) (Fig. 4B; –Fe, 4 d). Seedlings transferred to iron sufficiency were able to recover the chlorophyll content (Fig. 4B; +Fe, 4 d). Fig. 4B also shows that seedlings transferred to iron deficiency with the addition of 1 mM GSH or ASC increased their chlorophyll content close to that found in iron sufficiency (Fig. 4B; +Fe, 4 d). Overall, these results indicated that GSH and ASC not only protect *Arabidopsis* seedlings from a chlorophyll decrease induced by iron deficiency but also revert an already-established chlorotic phenotype in iron-deficient *Arabidopsis*.

GSH and ASC treatments reduce ROS content in leaves of iron-deficient Arabidopsis seedlings

Iron is an important component of proteins involved in the electron transport chain in mitochondria and chloroplasts, where iron deficiency can affect the transport of electrons leading to the overproduction of ROS (Graziano and Lamattina, 2005). As stated above, GSH and ASC are part of the complex and intricate antioxidative system in plants (Foyer and Noctor, 2011). To advance our study of the biological effects of GSH and ASC, we analysed ROS accumulation during iron deficiency in *Arabidopsis*. The fluorescent probe H_2DCF DA was used to visualize the ROS content in leaves and roots. As shown in Fig. 5A, leaves from *Arabidopsis* seedlings grown in iron deficiency presented higher ROS accumulation than leaves from iron-sufficient seedlings, which could be seen as an intense green fluorescence. In agreement with the results described above, leaves from seedlings grown in iron deficiency exhibited low levels of chlorophyll, which was visualized as red autofluorescence (Fig. 5A). The merged images of

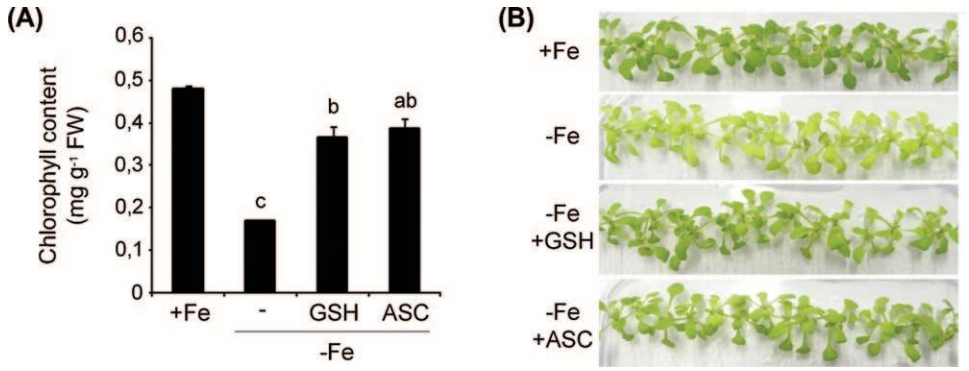

Fig. 2. ASC treatment prevents the chlorophyll loss in iron-deficient *Arabidopsis* seedlings. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred to iron-sufficient or deficient (–Fe, 0.1 μM FeNaEDTA) conditions with or without 1 mM GSH or ASC. (A) Chlorophyll content in *Arabidopsis* leaves. The results show the mean ±SE calculated from three independent experiments. Different letters indicate significant differences [one-way analysis of variance (ANOVA), P <0.05]. FW, fresh weight. (B) Representative pictures showing the phenotype of *Arabidopsis* after the treatments indicated.

red and green fluorescence in the iron-deficient condition suggested that ROS production would co-localize with chlorophyll. The green fluorescence was drastically reduced in leaves of iron-deficient seedlings treated with GSH or ASC, indicating lower levels of ROS compared with untreated plants (Fig. 5A). Fig. 5A also shows that the red fluorescence corresponding to chlorophyll was similar in iron-deficient leaves treated with GSH or ASC and in seedlings grown under iron-sufficient conditions.

Fig. 5B shows that H_2O_2 production detected with DAB was homogeneously located in leaves of seedlings grown in iron deficiency. Consistent with the results obtained with the fluorescent probe H_2DCF DA, DAB staining indicated that GSH and ASC reduce ROS accumulation in leaves (Fig. 5B).

The effect of GSH and ASC treatment in decreasing the basal level of ROS accumulation was also detected in roots (Fig. 5C).

Table 2. Effect of iron deficiency on total ASC content and oxidized ASC percentage in leaves and roots of *Arabidopsis*. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred (t_0) to either iron-sufficient or iron-deficient (–Fe, 0.1 μM FeNaEDTA) conditions for 3 or 7 d. FW, fresh weight.

	Total ASC (μmol g^{-1} FW)	Oxidized ASC (%)
Leaf		
t_0	0.67±0.02	13±0.5
+Fe, 3 d	0.57±0.05	14±1.5
–Fe, 3 d	0.54±0.05	15±0.5
+Fe, 7 d	0.77±0.1	8±0.25
–Fe, 7 d	0.74±0.06	9±0.5
Root		
t_0	0.40±0.05	12±0.25
+Fe, 3 d	0.43±0.09	8±0.25
–Fe, 3 d	0.48±0.07	7±0.5
+Fe, 7 d	0.50±0.05	6±0.25
–Fe, 7 d	0.61±0.09	5±0.25

GSH and ASC treatments protect proteins containing [Fe-S] clusters or heme groups

It has been shown that there is a decrease in the levels of proteins containing iron in plants grown in iron deficiency (Thimm *et al.*, 2001). Ferredoxin (Fd) has a redox active centre with a [2Fe-2S]. In chloroplasts, Fd accepts electrons from photosystem I and transfers it to the enzyme Fd:$NADP^+$ oxidoreductase for the photoreduction of $NADP^+$ (Arnon, 1988). In *Arabidopsis*, four genes encoding Fd have been identified. Fd2 is the most abundant in leaves, comprising approximately 90% of the Fd content. Fd3 is the more representative isoform in roots (Hanke *et al.*, 2004). Evidence suggests that iron availability in the growth medium regulates

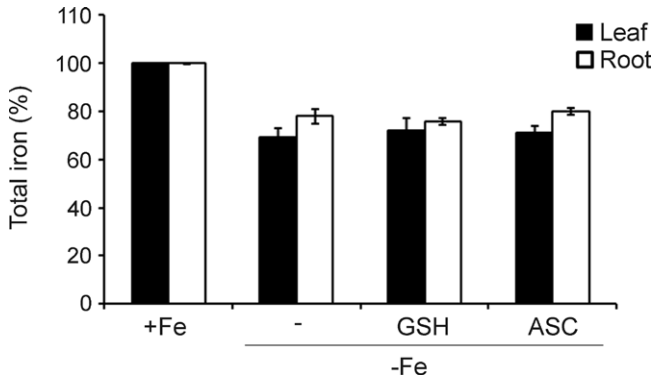

Fig. 3. Effect of GSH and ASC on total iron content in leaves and roots of iron-deficient *Arabidopsis* seedlings. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred to iron-sufficient or deficient (–Fe, 0.1 μM FeNaEDTA) conditions with or without 1 mM GSH or ASC for 7 d. Leaves and roots were harvested, and total iron was extracted and quantified using a ferrozine assay. The value obtained for +Fe was considered as 100% (leaves, 100%=20.2 μg Fe g^{-1} of dry weight; in roots, 100%=76.8 μg Fe g^{-1} of dry weight). The results show the mean ±SE calculated from two independent experiments.

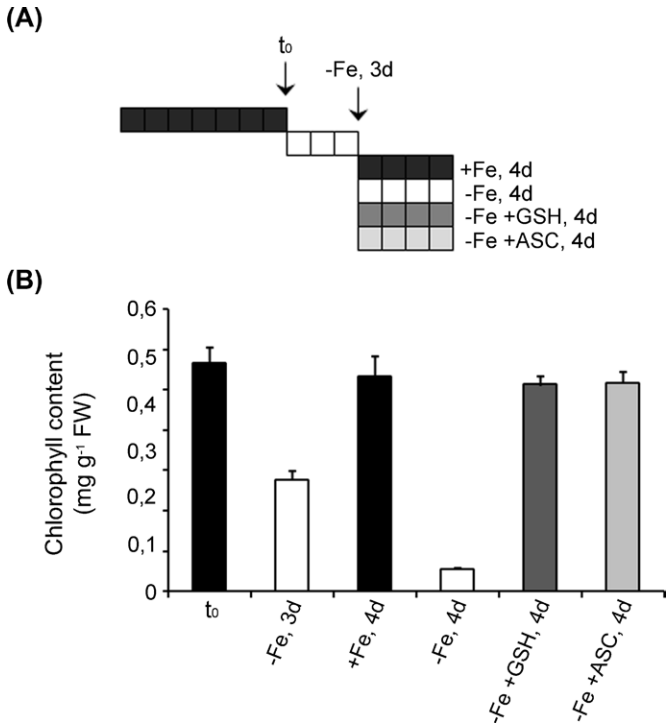

Fig. 4. GSH and ASC treatments revert the chlorosis of iron-deficient *Arabidopsis* seedlings. (A) Experimental design: *Arabidopsis* seedlings were grown for 7 d in iron-sufficient conditions (t_0; 50 μM FeNaEDTA) and then transferred to iron-deficient conditions (0.1 μM FeNaEDTA) for 3 d (–Fe, 3 d). Subsequently, the seedlings were transferred to the following conditions for 4 d: iron sufficiency (+Fe, 4 d), iron deficiency (–Fe, 4 d), and iron deficiency treated with 1 mM GSH (–Fe+GSH, 4 d) or 1 mM ASC (–Fe+ASC, 4 d). (B) Leaves were harvested and the chlorophyll levels quantified. The results show the mean ±SE calculated from two independent experiments. FW, fresh weight.

Fd levels, as *Arabidopsis* plants exposed to iron deficiency show a decrease in Fd transcripts levels (Thimm *et al.*, 2001), and in iron-deficient tobacco plants both transcripts and Fd protein were decreased (Tognetti *et al.*, 2007). As GSH protects iron-deficient *Arabidopsis* seedlings from chlorosis, we evaluated whether GSH treatment could also maintain Fd2 content. Fig. 6 shows that Fd2 content decreased in leaves of iron-deficient *Arabidopsis*, and GSH treatment restored the Fd2 levels to those observed under iron-sufficient condition. In roots, Fd3 content was not affected by iron deficiency (Fig. 6), consistent with the unchanged ROS concentration detected in iron-deficient *Arabidopsis* roots (Fig. 5C).

The enzymes APX and CAT have a heme group in their structure and play important roles in ROS detoxification. In several plant species such as sunflower, sugar beet, and maize, it has been shown that APX activity decreases in iron deficiency (Ranieri *et al.*, 2001; Zaharieva *et al.*, 2004; Sun *et al.*, 2007). Furthermore, maize plants also present a decrease in CAT activity under iron deficiency (Sun *et al.*, 2007). Because APX and CAT are involved in keeping the cellular redox homeostasis, the increased ROS content observed in leaves of iron-deficient seedlings may be due, in part, to a reduced activity of these enzymes. Based on this background, we examined the effect of GSH and ASC treatments on the activity of APX and CAT during iron deficiency. As shown in Fig. 7A, APX activity declined in leaves from seedlings grown in iron deficiency, but GSH and ASC treatment partially reversed that reduction. In contrast, CAT activity was not significantly modified by iron deficiency in *Arabidopsis* leaves (Fig. 7B) in contrast to that reported in maize plants (Sun *et al.*, 2007).

Discussion

This work showed that GSH and ASC protect *Arabidopsis* seedlings from the detrimental effects generated by iron deficiency. GSH treatment increased chlorophyll content and allowed iron-deficient *Arabidopsis* seedlings to grow as iron-sufficient plants. Accordingly, it has been demonstrated previously that GSH can confer an increased tolerance to environmental stresses like drought and salt stress in *Arabidopsis* plants (Chen *et al.*, 2012). Here, we also reported that treatment with another antioxidant, ASC, prevented chlorosis generated by iron deficiency in *Arabidopsis* seedlings. In addition, the chlorotic phenotype established by iron deficiency could be reversed applying GSH or ASC, highlighting the plant versatility and/or plasticity to recover from iron deficiency-mediated chlorosis.

Iron is a constituent of several components of the electron transport chain in mitochondria and chloroplasts, and thus iron deficiency disrupts normal electron transfer resulting in the overproduction of ROS. Under these conditions, the high levels of ROS generated exceed the possibility of being controlled by the antioxidant system, causing cell oxidative damage (Allen, 1995). Here, it was demonstrated that iron deficiency-induced ROS accumulation in *Arabidopsis* leaves was restored to basal levels through GSH or ASC treatment. These results suggest an antioxidant effect mediated by GSH and ASC treatment during the exposure of *Arabidopsis* seedlings to iron deficiency preventing oxidative processes, cell damage, and detrimental effects on growth processes.

It has been shown that GSH and ASC levels increase with the progression of iron deficiency in sugar beet roots, while chlorophyll levels and APX activity decrease (Zaharieva *et al.*, 2004). In *Arabidopsis*, GSH content decreased after 3 d in roots and after 7 d in leaves and roots of iron-deficient *Arabidopsis* seedlings. The decrease in GSH content during iron deficiency could negatively affect the cell redox homeostasis. In this sense, the exogenous application of GSH could maintain a critical threshold of GSH concentration required for controlling the redox imbalance generated from an excess of ROS production under iron-deficient conditions. In contrast, iron deficiency does not affect the level of ASC after 7 d. Recently, it has been proposed that environmental stresses induce different responses in each subcellular compartment, triggering changes in the GSH and ASC content that could not be detected by measurements in whole tissues or organs (Zechmann, 2011). In this work, ASC and GSH contents were measured in leaves and roots. It remains for future studies to

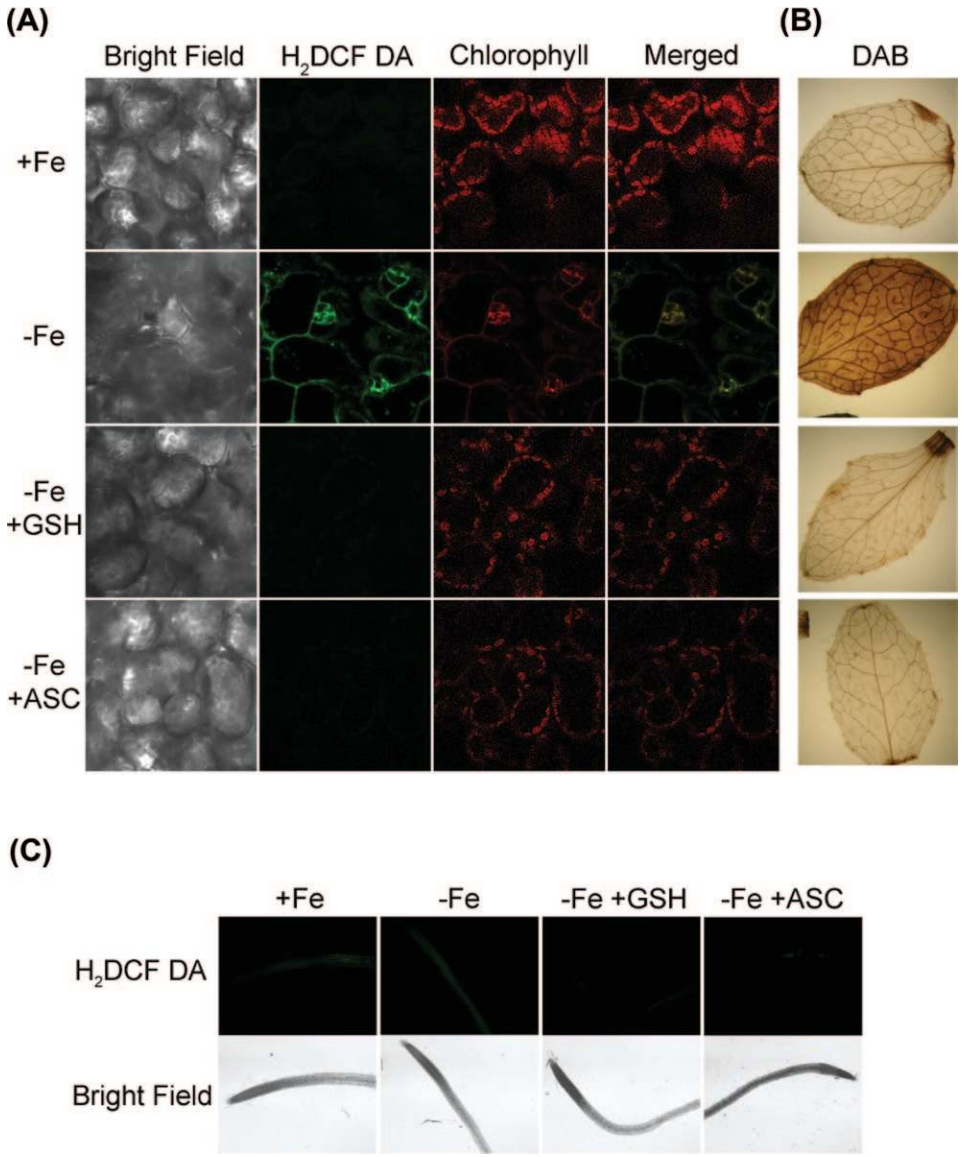

Fig. 5. Effect of GSH and ASC on ROS accumulation in leaves and roots of iron-deficient *Arabidopsis* seedlings. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred for 7 d to iron-sufficient or deficient (–Fe, 0.1 μM FeNaEDTA) conditions with or without 1 mM GSH or ASC. (A) Confocal microscopy images of leaves showing ROS accumulation as green fluorescence using an H_2DCF DA specific probe. Chloroplasts are indicated by the red chlorophyll autofluorescence. (B) *In situ* detection of H_2O_2 in leaves using DAB. (C) Representative microscopy images of roots showing ROS accumulation as green fluorescence with the probe H_2DCF DA.

analyse changes in GSH and ASC induced by iron deficiency at cellular/subcellular levels.

A complex regulatory system modulates ROS synthesis and degradation rates in different subcellular compartments, resulting in the control of ROS required for signalling and for physiological and defence purposes. In our studies, ROS overproduction due to iron deficiency appeared to be co-localized with chlorophyll. The fact that ROS mainly localized in chloroplasts of iron-deficient *Arabidopsis* seedlings indicates an important role for this organelle as a target for GSH and ASC effects. The antioxidant system of chloroplasts consist at least of three isoforms of APX, four different isoforms of SOD (one CuZnSOD and three FeSOD), and enzymes of the ASC–GSH cycle capable of reducing the oxidized forms of ASC and GSH. In addition, compared to other subcellular compartments, chloroplasts contain high levels of GSH and ASC (up to 5 and 25 mM, respectively; Noctor and Foyer, 1998). In contrast, several isoforms of CAT are localized in the cytosol, mitochondria, and peroxisomes, but are absent in chloroplasts (Foyer and Allen, 2003).

It has been suggested that plants exposed to iron deficiency may be more sensitive to oxidative stress because iron is a constituent of enzymes associated with the cellular antioxidant system such as APX, CAT, peroxidase, and FeSOD (Kumar *et al.*, 2010). In mulberry, maize, and cauliflower plants it has been shown that iron deficiency causes a decrease

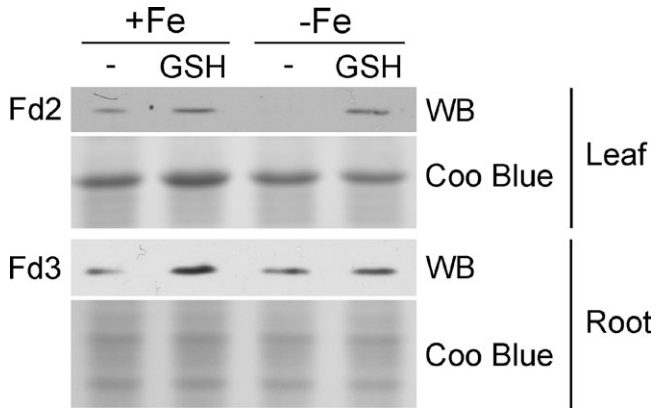

Fig. 6. Effect of GSH on the Fd2 andFd3 content in *Arabidopsis* seedlings grown under iron-deficient conditions. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred to iron-sufficient or deficient (–Fe, 0.1 μM FeNaEDTA) conditions with or without 1 mM GSH for a further 7 d. Western blot (WB) using polyclonal antibodies specific for Fd2 and Fd3 in leaves and root. Coomassie Blue (Coo Blue) staining represents a charge control.

in CAT, peroxidase, and APX activities with consequent accumulation of H_2O_2 (Tewari *et al*., 2005). Here, it was demonstrated that Fd2 levels and APX activity decreased due to the exposure of *Arabidopsis* to iron deficiency, and that GSH treatment recovered Fd2 levels as well as APX activity under conditions of nutritional stress. It was also demonstrated that ASC treatment increased APX activity in leaves under iron deficiency. Therefore, it is suggested that ASC could be preventing the loss of Fd2 in the same way that GSH does, as GSH and ASC seem to alleviate the iron-deficiency syndrome in an analogous manner. Nevertheless, further studies are needed to determine whether specific molecular mechanisms triggered by GSH and ASC could be occurring to preserve cellular homeostasis in iron deficiency.

Page *et al*. (2012) proposed the existence of a hierarchy for iron assembly for proteins in *Chlamydomonas reinhardtii*. They showed that oxidative stress was increased in iron-deficient *C. reinhardtii* and, as a consequence, SOD activity was augmented in the chloroplast. Interestingly, it was demonstrated that FeSOD *de novo* synthesis was preserved over that of other plastid proteins, which contributes to $O_2^{\cdot-}$ dissipation during iron deficiency (Page *et al*., 2012). The protective effect of GSH maintaining the level of Fd2 and APX activity in iron-deficient *Arabidopsis* could be due to a GSH-mediated change in iron allocation, allowing more iron to be available for assembly in proteins localized in chloroplasts.

An interesting point is that both GSH and ASC protected *Arabidopsis* seedlings exposed to iron deficiency without modifying the total iron content in the treated seedlings. It is therefore suggested that GSH and ASC mediate *Arabidopsis* protection by modulating iron availability without changing total iron content. It has been determined that GSH has a role in stabilizing the labile iron pool (Hider and Kong, 2011). The labile iron pool is composed of weakly chelated iron low-molecular-weight compounds. Most of this iron is Fe^{2+} and Fe^{3+} associated with ligands with a low affinity for iron. Protein synthesis during plant growth processes generates a constant flow of iron from the extracellular environment to the cytoplasm (Kruszewski, 2003). The cytoplasmic labile iron pool supplies iron for the synthesis of heme or [Fe-S] clusters, and is therefore essential in controlling numerous metabolic reactions. Some evidence indicates that GSH–Fe^{+2} complexes are the dominant form of this pool (Hider and Kong, 2011). Interesting, ASC has been demonstrated to increase iron solubilization in mammals (Atanassova and Tzatchev, 2008). Accordingly, *in vitro* experiments showed an increase in the iron remobilization rate associated with ferritin in the presence of ASC (Jackson and Kodanko, 2010).

The effect of GSH could also be related to the assembly of [Fe-S] groups. GRXs have been involved in the assembly of [Fe-S] groups and heme biosynthesis (Rouhier *et al*., 2010). Biochemical, spectroscopic, and analytical approaches have shown that the chloroplast GRXS14 and GRXS16 participate in the incorporation of [2Fe-2S] groups to Fd both *in vivo* and *in vitro*, indicating their role in maturation of proteins containing [Fe -S] groups. Evidence shows that these GRXs have the potential to function as scaffold proteins for the assembly of [2Fe-2S] and that their activity requires GSH,

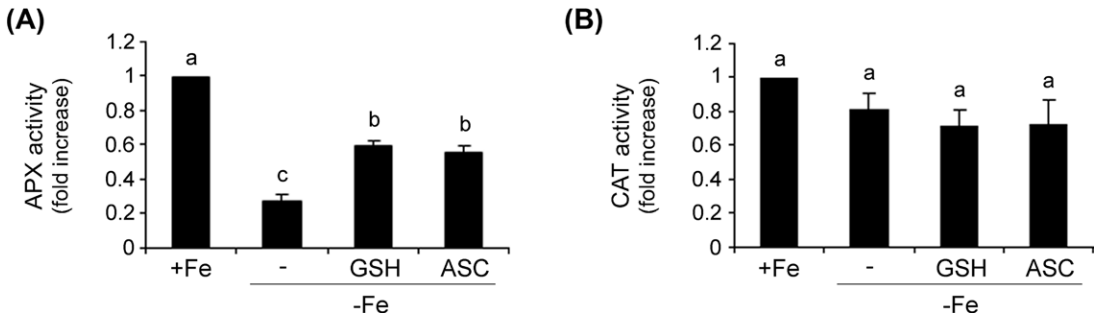

Fig. 7. Effect of GSH and ASC on APX and CAT activities in leaves of iron-deficient *Arabidopsis*. *Arabidopsis* seedlings were grown for 7 d under iron-sufficient conditions (+Fe, 50 μM FeNaEDTA) and then transferred to iron-sufficient or deficient (–Fe, 0.1 μM FeNaEDTA) conditions with or without 1 mM GSH or ASC for 7 d. (A) APX activity relative to the value obtained under the +Fe condition (1=30.97 nmol H_2O_2 mg^{-1} of protein min^{-1}). (B) CAT activity relative to the value obtained under the +Fe condition (1=4.62 nmol H_2O_2 mg^{-1} of protein min^{-1}). The results show the mean ±SE calculated from four independent experiments. Different letters indicate significant differences (one-way ANOVA, P <0.05).

which would link and stabilize the [2Fe-2S] groups (Rouhier *et al*., 2007; Bandyopadhyay *et al*., 2008; Qi *et al*., 2012).

On the other hand, GRXs can modulate protein activity by glutathiolation, a reversible post-translational modification disulfide formation between a protein thiol and GSH. This modification occurs more frequently in response to increased ROS or GSSG production (Rouhier *et al*., 2008). Glutathiolation may also protect the proteins from irreversible oxidation (Rouhier *et al*., 2008).

To summarize, the results presented in this work support the suggestion that the increase in chlorophyll content, the increased levels of APX activity, and the dramatic growth reactivation in response to GSH treatment in iron-deficient *Arabidopsis* plants could be mediated by the reduction of oxidative stress generated by ROS accumulation. ASC was demonstrated to be equally important in controlling the oxidative stress generated by the iron deficiency. It remains to be investigated whether an increase in iron availability for assembly into proteins and the protection of some key proteins from oxidation by glutathiolation are also operating as GSH-mediated protective effects.

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