

Involvement of sulphur nutrition in modulating iron deficiency responses in photosynthetic organelles of oilseed rape (*Brassica napus* L.)

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Abstract The aim of this study was to characterize the roles of sulphur (S) nutrition in modulating the responses to iron (Fe) deficiency in the photosynthetic organelles of oilseed rape. Eight-week-old plants grown hydroponically were fed with S-sufficient or S-deprived solution with or without Fe^{III}-EDTA. Responses to four S and Fe combined treatments were analysed after 5 and 10 days. Leaf chlorosis was generated by either S- or Fe-deprivation, with a decrease in chlorophyll and carotenoid content. These negative effects were more severe in the absence of S. The expression of Fe²⁺ transporter (*IRT1*) and Fe(III) chelate reductase (*FRO1*) gene was induced for the first 5 days and decreased after 10 days in the S-deprived roots, but largely improved by S supply even in the absence of Fe. Lack of ferric chelate reducing activity in the Fe-deprived roots in the absence of S was largely improved by S supply. The activity of photosynthesis, RuBisCO and sucrose synthase was closely related to S status in leaves. Electron microscopic observation showed that the Fe-deficiency in the absence of S greatly resulted in a severe disorganisation of thylakoid lamellae with loss of grana. However, these impacts of Fe-deficiency were largely restored in the presence of S. The present results indicate that S nutrition has

significant role in ameliorating the damages in photosynthetic apparatus caused by Fe-deficiency.

Keywords *Brassica napus* · Chloroplast structure · Iron deficiency · Photosynthetic activity · Sulphur deficiency

Abbreviations

DTNB 5,5'-Dithiobis-2-nitrobenzoic acid
RuBisCO Ribulose-1,5-bisphosphate carboxylase/oxygenase

Introduction

Iron (Fe) is an essential element in plants because it is required by proteins that carry electrons during photosynthesis and respiration, thus it is a cofactor for energy metabolism, oxygen transport and DNA synthesis (Vigani 2012). It also undergoes alternative redox reactivity between the Fe²⁺ and Fe³⁺ states (Aisen et al. 2001). Maintaining Fe homeostasis is an important physiological factor in building prosthetic groups such as haem and iron(Fe)-Sulphur(S) clusters, and in assembling them into apoproteins, which are major components of plant metabolism (Briat et al. 2010). Fe–S clusters are cofactors of proteins that function in vital processes such as photosynthesis, respiration, S and N metabolism, plant hormone and coenzyme synthesis (Balk and Pilon 2011). Fe-deficiency is a common abiotic stress, especially for many photosynthetic organisms, varying from high value crops in arid and semiarid regions (Muneer et al. 2011) to phytoplankton living in the sea. Fe-deficient leaves are initially characterized by interveinal chlorosis, and then the whole leaf

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becomes chlorotic. In severe cases, the leaves become white with necrotic lesions. Decreases in the apparent efficiency of photosynthetic energy conversion in response to Fe-deficiency, estimated by chlorophyll fluorescence level, have been reported in higher plants.

Oilseed rape (*Brassica napus* L.) has become a plant of major agro-economic importance for the production of animal feed, vegetable oil for human consumption and biodiesel. World production is growing rapidly, with FAO reporting that 47 million tonnes of oilseed rape was produced in 2007, and estimates of 58.4 million tonnes in the 2010–2011 season. As with many other *Brassicaceae*, oilseed rape is high sulphur (S)-demanding plant; for example the production of 1 ton of rape seeds requires about 16 kg S (Blake-Kalff et al. 2001), whereas only 2–3 kg of S is needed per ton of wheat grains (Zhao et al. 1999). Therefore, oilseed rape is particularly sensitive to S-deficiency or limitation, which reduces both seed quality (De Pascale et al. 2008) and yield by as much as 40 % (Scherer 2001).

On the other hand, the uptake of Fe and its mobilization in plant organ is often limited because it is mainly present as insoluble Fe(III) precipitates. To cope with low Fe availability, higher plants exert two major mechanisms: the excretion of phytosiderophores to complex Fe(III) and enable its uptake, or production of reductase capable of reduction of Fe(III) to Fe(II) that can be taken up by plants. There are several experimental evidence reported that S supply significantly affects Fe acquisition mechanism. Zuchi et al. (2012) recently demonstrated that high S supply increased the concentrations of Fe and S in the leaves of durum wheat plants. It has been also reported that the availability of sulphate could affect accumulation and release of high-affinity chelating compounds, phytosiderophores, in barley roots (Astolfi et al. 2010). These positive effects of S nutrition on Fe utilization were mainly explained by an increased production of phytosiderophores and nictianamine (NA) (Astolfi et al. 2012), possibly due to increased methionine level accompanied by the increase in ATPS and OASTL activity (Astolfi et al. 2010; Zuchi et al. 2012) in Strategy II plants (graminaceous monocotyledonous species). Similarly, Zuchi et al. (2009) showed that S-deficiency could limit the capacity to cope with Fe-shortage by preventing the induction of the Fe(III)-chelate reductase and limiting expression of the Fe²⁺ transporter in a Strategy I plant tomato. Despite extensive researches attempting to elucidate the interactions between external sulphate supply and plant Fe acquisition, to our knowledge, the responses of the photosynthetic mechanism to combined deficiency of S and Fe have not yet been fully investigated.

We hypothesize that interactions between S and Fe can alleviate the detrimental effects of Fe-deficiency on photosynthetic mechanism. To test this hypothesis, the

responses of photosynthetic parameters, Fe acquisition and chloroplast ultrastructure were compared at 5 and 10 days of four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control), sufficient S but deprived Fe (+S/–Fe), deprived S but sufficient Fe (–S/+Fe) and deprived S and Fe (–S/–Fe). Furthermore, expression of Fe²⁺ transporter (*IRT1*) and Fe(III) chelate reductase (*FRO1*) genes were also analysed.

Materials and methods

Plant culture and treatment

Surface-sterilized seeds of *Brassica napus* L. cv. Mosa were germinated in wet filter paper in Petri dishes at 30 °C in the dark for 3 days. Four seedlings were transplanted and then thinned to two after 2 weeks. The seedlings were grown in a 3-l plastic pots with hydroponic nutrient solution containing (mM for the macro elements): 1.0 NH₄NO₃; 0.4 KH₂PO₄; 3.0 CaCl₂; 1.5 MgSO₄; 0.15 K₂HPO₄; 0.2 Fe–Na EDTA; and (μM for the micro elements): 14 H₃BO₃; 5.0 MnSO₄·H₂O, 3.0 ZnSO₄·7H₂O; 0.7 CuSO₄·5H₂O; 0.7 (NH₄)₆Mo₇O₂₄; 0.1 CoCl₂. The nutrient solution was continuously aerated and renewed every 5 days. Natural light was supplemented with 200 μmol m^{–2} s^{–1} at the canopy height for 16 h day^{–1}. 8-week-old plants were divided in four groups to receive different treatments: sufficient in S and Fe (+S/+Fe, control), sufficient S but deprived Fe (+S/–Fe), deprived S but sufficient Fe (–S/+Fe) and deprived S and Fe (–S/–Fe) with four replicates. After 5 and 10 days of treatment, leaves were separated by the order of ontogenic appearance, which was designated as leaf number (i.e. giving leaf No. 1 for the oldest leaf). In this study, mature leaves which are the leaf ranks numbered 4 and 5 were considered. Entire root system excised on the root base was combined to minimize the variation caused by the different root tissues. Plant samples were immediately frozen in liquid N₂ and stored in deep-freezer for further analysis.

Photosynthetic pigments and chemical analysis

The content of chlorophyll and carotenoid was estimated by the method of Hiscox and Israclstam (1979). Fresh leaves were collected in glass vials to which 10 ml dimethyl sulfoxide were added and were kept in an oven at 65 °C for complete leeching of pigments for 1 h. Optical density was recorded at 480, 520, 645 and 663 nm. The content of total chlorophyll and carotenoid was calculated using the formulae given by Arnon and Israelstam (1949).

To determine S concentration, about 1 g of freeze-dried leaf sample was ash muffled in furnace at 600 °C. The

ashes were dissolved in 10 ml of 3 N HCl. After a reaction with BaCl₂, a BaSO₄ precipitate was determined turbidimetrically (Bardsley and Lancaster 1962).

For the determination of Fe concentration, about 1 g of freeze-dried leaf sample was digested with 50 % perchloric acid and concentrated H₂SO₄ at 350 °C for 5 h. The digested samples were then filtered and diluted to a proper volume. The elemental content was determined by an inductively coupled plasma optical emission spectrometry, (ICP-OES, Thermo Elemental—IRIS Advantage, USA).

Enzyme assays

Root Fe(III)-chelate reductase (FCR) was assayed by measuring the formation of Fe(II)-bathophenanthroline-disulfonate (BPDS) complex from Fe(III)-EDTA Welch et al. (1993). Roots were placed in deionized water for 5 min. They were then transferred to an aerated assay solution consisting of 0.5 mM CaSO₄, 0.25 mM Fe(III)-EDTA, 0.6 mM BPDS and 10 mM MES at pH 5.5 adjusted by 1 M KOH, and incubated in the dark at 25 °C for 30 min. Change in absorbance of the assay solution resulting from reduction of Fe(III) was determined spectrophotometrically at 535 nm. The concentration of Fe(II)-BPDS was calculated using a molar extinction coefficient of 22.1 mM⁻¹ cm⁻¹.

RuBisCO activity in leaves was estimated by the method given by Usuda (1985) with some modifications. One g of frozen leaf sample was homogenized with extraction buffer containing 250 mM Tris-HCl (pH 7.8), 50 mM MgCl₂, 2.5 mM EDTA and 0.25 mM DTT, and then centrifuged at 4 °C at 10,000×g for 10 min. The resulting supernatant immediately used for enzyme activity assay. The reaction mixture contained 100 mM Tris-HCl (pH 7.8), 40 mM NaHCO₃, 10 mM MgCl₂, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA and 5 mM DTT, 1 U of glyceraldehyde 3-phosphodehydrogenase and 1 U of 3-phosphoglycerate kinase and 0.2 mM RuBP. After the addition of enzyme extract to assay medium, the activity was determined by monitoring NADH oxidation at 340 nm.

Sucrose synthase in leaves was estimated spectrophotometrically by the method given by Xu et al. (1989). Sucrose synthase was assayed in a 1 ml reaction mixture containing 100 mM MES (pH 6.5), 3 mM Mg-acetate, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 0.02 mM glucose 1,6-diP, 0.5 mM NAD, 1 mM UDP, 1 mM PPI, 50 mM sucrose (for crude enzyme) or 200 mM sucrose (for purified enzyme), 1 unit phosphoglucosyltransferase, 2 units glucose-6-P dehydrogenase (from *Leuconostoc*) and 1 unit UDP-glucose pyrophosphorylase was added. Sucrose synthase activity was measured following the oxidation of pyrophosphates at 340 nm.

Total RNA extraction and RT-PCR analysis

Isolation of RNA from roots was performed by using RNA isolation kit according to manufacturer's instructions (Promega, USA). One µg of DNA as-treated RNA was reverse transcribed by reverse transcriptase kit (Promega, USA) to synthesize first strand cDNA. Real time PCR was performed with a C1000 thermal cycler (Bio-Rad), using ROX Passive Reference Dye provide by Real Helix qPCR kit (NanoHelix, Korea) for 5 min at 95 °C followed by 40 cycles consisting of 20 s at 95 °C, 30 s for 57 °C and 30 s at 72 °C then 10 min at 72 °C. Gene-specific primers—designed for tomato (Li et al. 2004) were used in this study: *IRT1*-F: 5'-TGGCTGTGGCTGAAATCATGTTC-3'; *IRT1*-R: 5'-ATTGATGAAAGCAAAGTGCAGCT-3'; (accession number AF136579.1) *FRO1*-F: 5'-ATTGGGTATTTCTACTTTGA-3'; *FRO1*-R: 5'-GAGTGCAATAAACATCACAAA AAA-3'; (accession number AY224079.1) *ACTIN*- F: 5'-GATTCCGTTGCCCTGAAGTA-3'; *ACTIN*- R: 5'-GCGACCACCTTGATCTTCAT-3'. All quantifications were normalized to ACTIN. The RT-PCR reactions were performed in four independent RNA preparations from independently grown plants were analysed with three technical replicates for the qPCR. To calculate relative expression (treatment/control), individual values for treated plants were divided by the mean value for control (+S/+Fe) plants at each sampling date.

Measurement of photosynthetic activity and stomatal observation

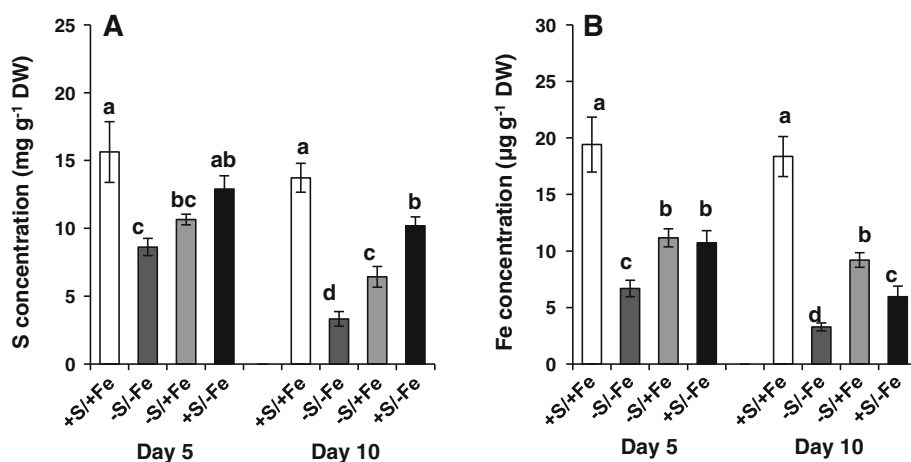
Photosynthesis rate, stomatal conductance and transpiration were measured using a portable photosynthesis measurement system (LI-6400. LI-COR, Inc., Lincoln, NE). One of fully expanded mature leaves from per plant was tagged with a small wire, and the measurement was followed on the same tagged leaf for 10 days of treatment. The measurement was done 4 h after the beginning of the photoperiod under greenhouse conditions.

For stomatal observation, thin layer of leaf tissues were carefully cut and were laid on glass slide, covered with cover slip by adding few drops of water and were observed under light microscope under 10× and 40× magnification. The number of stomata was observed by counting the number in present leaf area. The stomatal density was calculated by dividing the number of stomata counted by ten times the area of 1 grid square.

Ultrastructure of chloroplasts

Fresh leaves were cut into 1–3 mm² pieces; the ultrastructural organization of chloroplasts was examined on sections of leaf fragments. Fragments were cut out between

Fig. 1 Changes in the concentration of **a** sulphur and **b** Fe in leaves as affected by four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control *white bars*), deprived S and Fe (–S/–Fe *dark grey bars*), deprived S but sufficient Fe (–S/+Fe *light grey bars*) and sufficient S but deprived Fe (+S/–Fe *black bars*) for 10 days. *Vertical bars* indicate \pm SE of the means for $n = 4$. Means denoted by the *different letter* are significantly different at $P < 0.05$ according to the Tukey's studentized range test



leaf ribs and fixed in 1.25 % glutaryldehyde in phosphate buffer (pH 7.4) for 2 h. Then, specimens were post-fixed in 1 % OsO₄ at room temperature for 2 h. The dehydration, embedding in epoxy resin, thin sectioning using a diamond knife and specimen staining have been followed as previously described (Goodenough and Levine 1969). Specimens were examined with an electron microscope (JEOL JEM-1400, Jeol, Japan).

Statistical analysis

A complete randomized design was utilized with four replicates for two S supply levels and four sampling dates. An individual pot containing two plants represented a replicate. The Tukey's studentized range test was employed to compare the means of separate replicates. Unless stated otherwise, the conclusions are predicated on differences between the means, with a significance level set at $P < 0.05$.

Results

S and Fe concentration

S-deprivation reduced total S concentration by more than 31 % for the first 5 days irrespective of Fe supply, and further decreased by 53 % in the presence of Fe (–S/+Fe) and 75 % in the absence of Fe (–S/–Fe) after 10 days of treatment, whereas Fe-deprivation in the presence of S decreased total S only by 26 % compared with control (+S/+F) (Fig. 1a). Compared with the control, Fe-deprivation in the absence of S (–S/–Fe) decreased Fe concentration by 66 % for 5 days and further decreased by 82 % after 10 days of treatment, whereas the decrease in Fe concentration was significantly less in the presence of S (+S/–Fe) (Fig. 1b).

Photosynthetic pigments and leaf chlorosis

The total chlorophyll and carotenoid contents were significantly decreased by S- or Fe-deprivation, with no significant difference between –S/+Fe and +S/–Fe leaves. The negative effects on these photosynthetic pigments were more prominent in the –S/–Fe leaves (Fig. 2a, b).

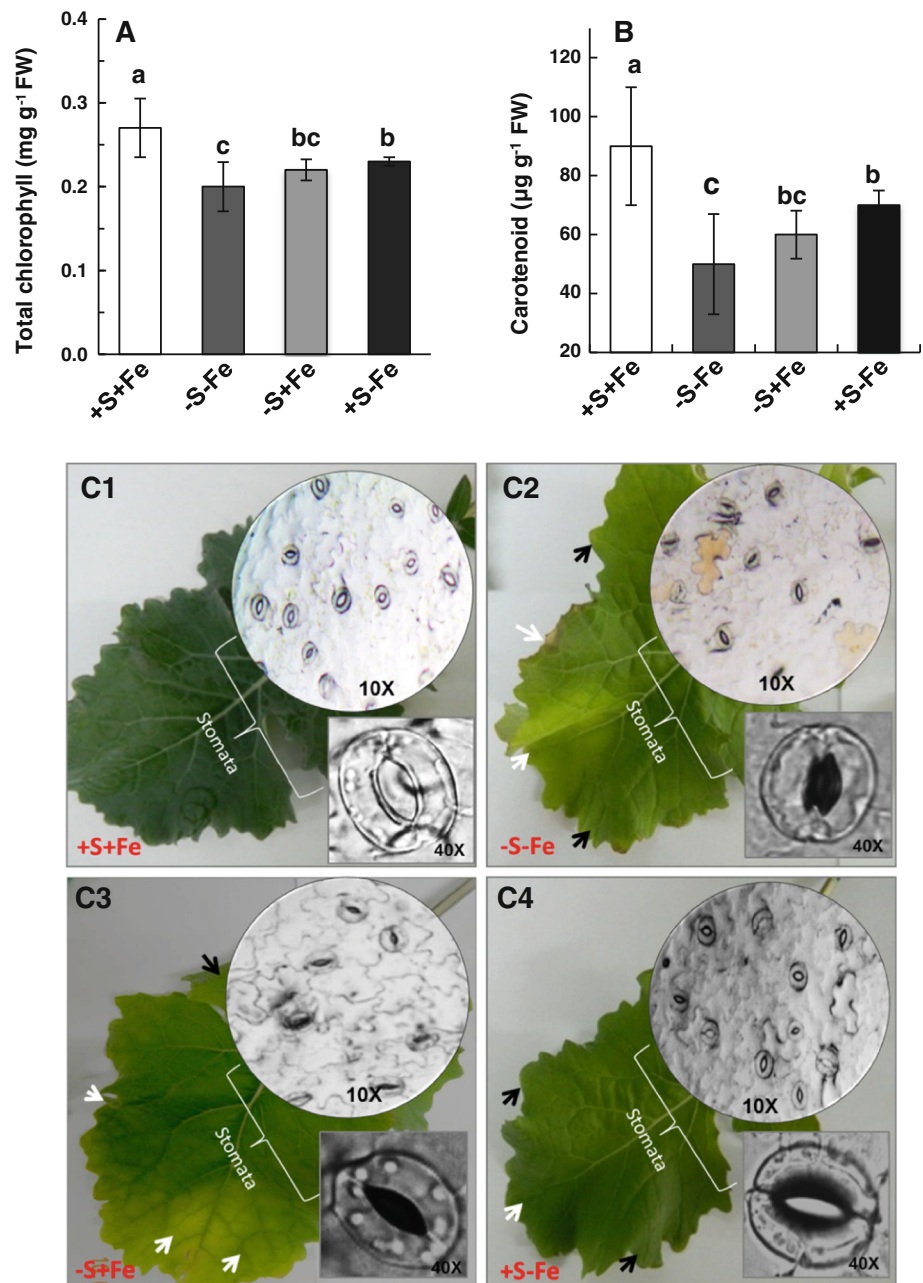
S- or Fe-deprived leaves exhibited visible chlorosis, characterized by the yellowish and unevenly expanded leaves (Fig. 2 C2) with bleached, chlorotic and sometimes necrotic spots (indicated by white arrows) as well as prominent wrinkles in the lamina and curling of the leaf margins (indicated by black arrows). S-deficiency in absence of Fe increased the severity of leaf chlorosis (Fig. 2 C3).

Microscopic observation (10 \times) showed that the number of stomata was also decreased in number by S- and/or Fe-deprivation (inset round micrographs in Fig. 2c). The most severe reduction in stomatal number was observed in –S/–Fe leaves and it was observed that number of stomata reduces to 9 from 12 in number compared with control at a particular magnification chosen under microscope. It was also observed that stomata in S-deprived leaves irrespective of Fe supply were to be closed, while maintained open in +S/–Fe leaves (inset square micrographs in Fig. 2c). Similarly, the density of stomata was 9.2 stomata per mm² to S- or –Fe-deprivation compared with control. However, in +S/–Fe leaves, the density of stomata was 11.1 stomata per mm².

Photosynthetic activity

The photosynthesis rate was decreased by S- and/or Fe-deprivation (Fig. 3a). The extent of decrease was most prominent in the –S/–Fe leaves, reaching 36.1 % of control after 10 days of treatment. The photosynthesis rate was improved to 66.4 % of control in the presence of Fe (–S/+Fe) or 77.2 % of control in the presence of

Fig. 2 Changes in the content of photosynthetic pigments. **a** Total chlorophyll and **b** carotenoid as affected by four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control *white bars*), deprived S and Fe (−S/−Fe *dark grey bars*), deprived S but sufficient Fe (−S/+Fe *light grey bars*) and sufficient S but deprived Fe (+S/−Fe *black bars*) for 10 days. Morphology of leaves (C1–C4) was observed visually showing chlorosis and necrosis (indicated with *white arrows*), curling of leaves (indicated with *black arrows*). Representative images of stomata observations with optical microscope ($\times 10$ and $\times 40$) are present on the inlet over leaves



S (+S/−Fe). The patterns of stomatal conductance (Fig. 3b) and transpiration (Fig. 3c) in response to S- and/or Fe-deprivation were similar with that of photosynthesis rate.

The activity of ferric chelate reductase, RuBisCO and sucrose synthase

Five days after treatment, S-deprivation resulted in a decrease of Fe(III) chelate reductase activity in roots under Fe-deprived (−23 %) and Fe-supplied (−15.9 %) condition, whereas the activity was significantly increased (+33 % compared with the control) by S supply. After 10 days of treatment, Fe-deprivation decreased ferric

chelate reductase activity by more than 86 % without positive impact of S nutrition, and S-deprivation in the presence of Fe decreased the activity by 39 % compared to the control (Fig. 4a).

The response of RuBisCO and sucrose synthase activity to S/Fe combined treatment was similar (Fig. 5a, b). S-and/or Fe-deprivation decreased the activity of RuBisCO and sucrose synthase slightly for the first 5 days, and decreased further by 60 and 55 %, respectively, compared with control after 10 days of treatment. The decreased activity of RuBisCO and sucrose synthase was alleviated to 70 and 55 % of control in presence of Fe (−S/+Fe) or 80 and 78 % of control in presence of Fe (+S/−Fe).

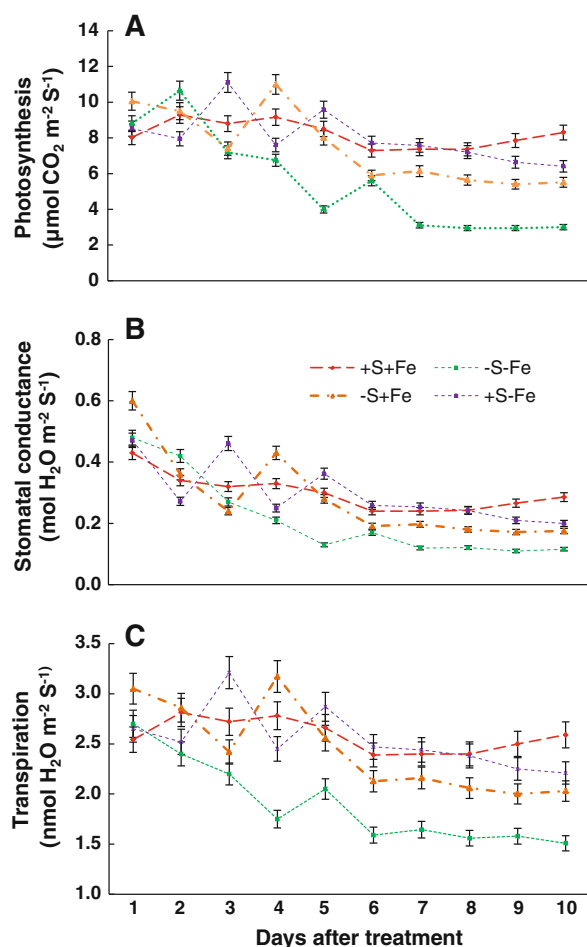


Fig. 3 Changes in photosynthetic parameters. **a** Photosynthesis rate, **b** stomatal conductance and **c** transpiration as affected by four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control red line), deprived S and Fe (-S/-Fe green line), deprived S but sufficient Fe (-S/+Fe orange line) and sufficient S but deprived Fe (+S/-Fe purple line) for 10 days. Vertical bars indicate \pm SE of the means for $n = 4$

Expression of *IRT1* and *FRO1* genes in roots

Expression of genes involved in Fe acquisition such as Fe^{+2} transport (*IRT1*) and Fe(III) chelate reductase (*FRO1*) was examined in the roots as affected by S and Fe nutrition (Fig. 4b, c). For the first 5 days after treatment, S-deprivation increased the expression of *IRT1* by 3.1-fold in the absence of Fe (-S/-Fe) and 4.2-fold in presence of Fe (-S/+Fe), while up to 10.9-fold in Fe-deprived in the presence of S (+S/-Fe). After 10 days of treatment, the expression of *IRT1* gene in the S-deprived roots was significantly depressed regardless of Fe supply. S supply even in the absence of Fe (+S/-Fe) maintained *IRT1* expression to the control level (Fig. 4b). Similarly, for the first 5 days of treatment, the expression of *FRO1* gene responded to S/Fe nutrition expression,

representing 2.4-fold increase in -S/-Fe, 3.2-fold in -S/+Fe and 8.3-fold in +S/-Fe roots. After 10 days of treatment, low expressions of *FRO1* gene were observed with significant decreases only in S-deprived roots (Fig. 4b).

Relationships among descriptive parameters of Fe uptake and photosynthetic activity

Linear correlations among descriptive parameters of Fe uptake and photosynthetic activity were assessed using the values as affected by S and Fe combined treatments (Table 1). S content in leaves was closely correlated ($P < 0.001$) with the descriptive parameters of Fe uptake and transport (the expression of *IRT1* and *FRO1* gene, the activity of ferric chelate reductase in roots), and with Fe content in leaves for first 5 days, while no more significant with the expression of *IRT1* and *FRO1* gene after 10 days of treatment. Each of these parameters was highly associated with Fe content in leaves ($P < 0.001$) for the first 5 days, whereas much lower correlations between the expression of *IRT1* and *FRO1* in roots, and Fe content in leaves were found after 10 days of treatment. The relationships of S and Fe content in leaves with RuBisCO, sucrose synthase and net photosynthesis rate were highly significant after 10 days of treatment.

Chloroplast ultrastructure

Electron micrographs provided significant clues about the impact of S- and/or Fe-deficiency on the ultrastructure of chloroplasts (Fig. 6). In control (+S/+Fe) plants, the number and shape of chloroplasts was found in the typical form, well organized grana/thylakoid, lamellar network and well developed outer cell wall (Fig. 6 A1–A3). Some osmophilic compounds were commonly observed because of using osmium tetroxide for preparing samples for image analysis. In Fe-deprived plants, the number of chloroplasts was reduced (Fig. 6 C1), and the chloroplast envelope was detached from the inner body with starch accumulation (Fig. 6 C2). Some degree of thylakoid disintegration was observed with starch accumulation (Fig. 6 C3). These deteriorating impacts of Fe-deficiency were more severe in the absence of S (Fig. 6b). In the -S/-Fe plants, the chloroplast envelope was severely disrupted with the increment of starch granule (Fig. 6 B2), and grana were blurred with severe disorganisation of lamellae (Fig. 6 B3). S supply even in the absence of Fe largely alleviated the Fe-deficiency responses (Fig. 6d). In +S/-Fe plants, the number of chloroplasts was increased (Fig. 6 D1), and their shape was well organized (Fig. 6 D2). An abundant amount of thylakoids with grana stacks

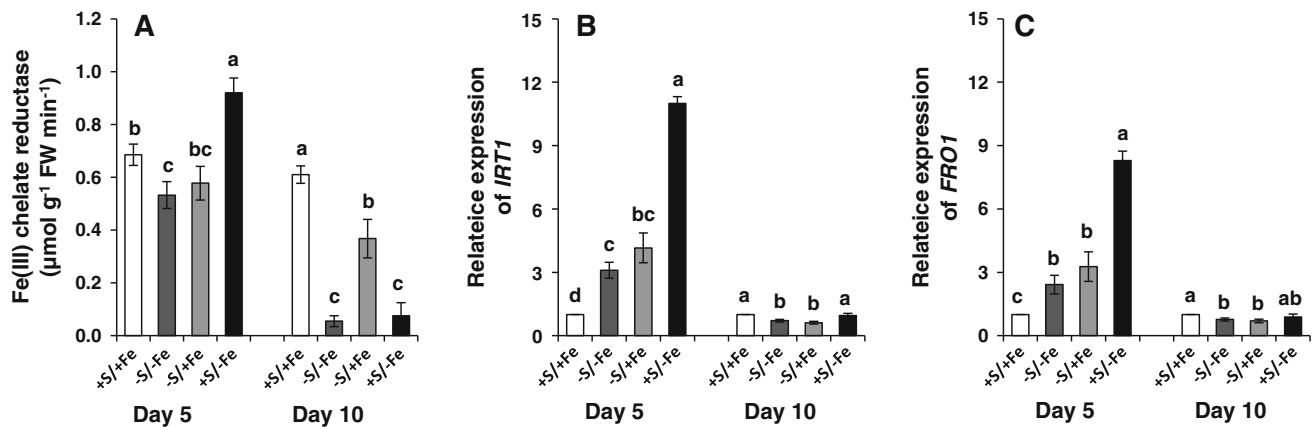


Fig. 4 Changes in **a** Fe-chelate reductase activity, and relative expression of **b** Fe transport gene, *BnIRT1* and **c** Fe(III) chelate reductase gene, *BnFRO1* in root as affected by four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control white bars), deprived S and Fe (-S/-Fe dark grey bars), deprived S but sufficient

Fe (-S/+Fe light grey bars) and sufficient S but deprived Fe (+S/-Fe black bars) for 10 days. The values in the control plants at each sampling date were set to 1. Vertical bars indicate \pm SE of the means for $n = 4$. Means denoted by the different letter are significantly different at $P < 0.05$ according to the Tukey's studentized range test

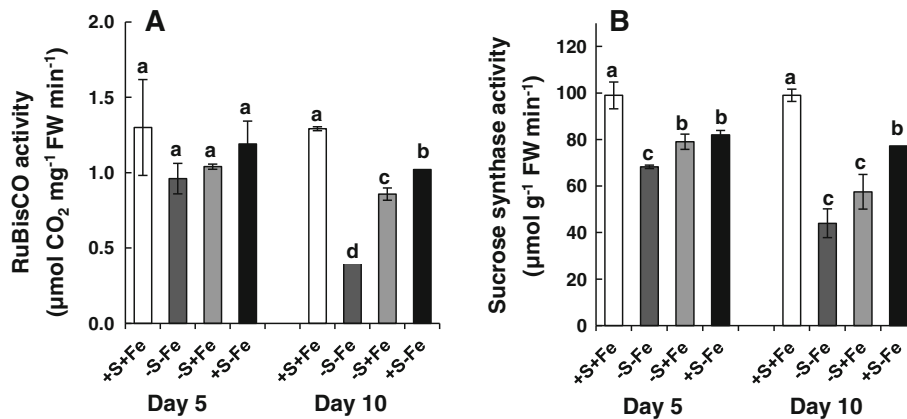


Fig. 5 Changes in enzyme activity of **a** RuBisCO and **b** Sucrose synthase as affected by four S/Fe combined treatments: sufficient in S and Fe (+S/+Fe, control white bars), deprived S and Fe (-S/-Fe dark grey bars), deprived S but sufficient Fe (-S/+Fe light grey

bars), and sufficient S but deprived Fe (+S/-Fe black bars) for 10 days. Vertical bars indicate \pm SE of the means for $n = 4$. Means denoted by the different letter are significantly different at $P < 0.05$ according to the Tukey's studentized range test

were found (Fig. 6 D2), but chloroplasts still showed a distorted shape and starch accumulation (Fig. 6 D3).

Discussion

The present study provides experimental evidences that significant interaction between S and Fe nutrition occurs in plants. The decrease in Fe content by Fe-deprivation was significantly alleviated by S supply (Fig. 1b), whereas total S concentration in S-deprived leaves was not affected by Fe availability for the first 5 days (Fig. 1a). In recent studies (Astolfi et al. 2010, 2012; Zuchi et al. 2012), maize and barley plants exposed to S starvation showed a lower Fe concentration than S-sufficient plants. The decrease in Fe accumulation in leaf tissues under S-deficient condition

was explained as an inhibition of Fe uptake as a consequence of the reduction of phyto siderophores (PS) release in the roots (Astolfi et al. 2010, 2012). In disagreement with our findings, Fe-deficiency increased S concentration in barely seedlings under S-deficient conditions (Astolfi et al. 2006). An enhanced S accumulation in Fe-deficient plants was suggested to be related to the needs of sustaining the PS biosynthetic pathway in Strategy II plants (Astolfi et al. 2006, 2010, 2012). However, Fe supply did not influence S concentration under S-deficient condition in tomato (Zuchi et al. 2009), a Strategy I plant, similar with our results with oilseed rape (Fig. 1a). In the Strategy I plants which do not release PS, ethylene and NA are main components involved in Fe uptake (Romera and Alcántara 2004). Given that S-deficiency reduced ethylene production and virtually abolished expression of the NA

Table 1 Linear correlations among the descriptive parameters of Fe uptake, photosynthetic activity and C assimilation

	days	S	Fe	<i>IRT1</i>	<i>FRO1</i>	FCR	RuBisCO	SSA	NPR
S	(5 days)	–	$r = 0.807^{***}$	$r = 0.758^{***}$	$r = 0.766^{***}$	$r = 0.610^{**}$	$r = 0.217$	$r = 0.796^{***}$	$r = 0.381$
	(10 days)		$r = 0.782^{***}$	$r = 0.377$	$r = 0.214$	$r = 0.648^{**}$	$r = 0.955^{***}$	$r = 0.926^{***}$	$r = 0.673^{**}$
Fe	(5 days)		–	$r = 0.760^{***}$	$r = 0.730^{***}$	$r = 0.784^{***}$	$r = 0.485^*$	$r = 0.936^{***}$	$r = 0.454$
	(10 days)			$r = 0.484^*$	$r = 0.166$	$r = 0.929^{***}$	$r = 0.829^{***}$	$r = 0.801^{***}$	$r = 0.567^*$
<i>IRT1</i>	(5 days)			–	$r = 0.973^{***}$	$r = 0.721^{**}$	$r = 0.457$	$r = 0.769^{***}$	$r = 0.646^{**}$
	(10 days)				$r = 0.136$	$r = 0.394$	$r = 0.274$	$r = 0.454$	$r = 0.052$
<i>FRO1</i>	(5 days)				–	$r = 0.647^{**}$	$r = 0.460$	$r = 0.782^{***}$	$r = 0.714^{**}$
	(10 days)					$r = 0.153$	$r = 0.222$	$r = 0.067$	$r = 0.283$
FCR	(5 days)					–	$r = 0.245$	$r = 0.663^{**}$	$r = 0.494^*$
	(10 days)						$r = 0.703^{**}$	$r = 0.632^{**}$	$r = 0.155$
RuBisCO	(5 days)						–	$r = 0.504^*$	$r = 0.732^{***}$
	(10 days)							$r = 0.917^{***}$	$r = 0.722^{**}$
SSA	(5 days)							–	$r = 0.530^*$
	(10 days)								$r = 0.598^*$
NPR	(5 days)								–
	(10 days)								–

The values measured at 5 and 10 days after S/Fe combined treatment were used for correlation analysis. The correlation coefficient (r) and significant differences are given; $n = 16$

S S concentration in leaves, Fe Fe concentration in leaves, *IRT1* *IRT1* gene expression in roots, *FRO1* *FRO1* gene expression in roots, FCR activity of ferric chelate reductase in roots, *RuBisCO* RuBisCO activity in leaves, *SSA* sucrose synthase activity in leaves, *NPR* net photosynthesis rate

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

synthetase gene (*LeNAS*) in the absence of Fe (Zuchi et al. 2009), the observed decrease in Fe concentration in S-deprived plants (Fig. 1b) may result from the inadequate supply of S to maintain the ethylene and NA biosynthesis because both components are synthesized from methionine.

To address the question of whether S nutritional status contributes in improving Fe uptake, the expression of Fe²⁺ transporter (*IRT1*) and Fe(III)-chelate reductase (*FRO1*) genes in the roots were examined by using the primers designed for tomato (Li et al. 2004) (Fig. 4). Fe-deprivation resulted in an increase in the expression level of *IRT1* and *FRO1* gene. Moreover, the extent of increase in the expression of these genes was remarkably higher in the presence of S (+S/–Fe) than in the absence of S (–S/–Fe), especially for the first 5 days of treatment (Fig. 4b, c), in accordance with a common response to Fe-deficiency (Vert et al. 2002; Zuchi et al. 2009). However, when Fe-deficiency prolonged up to 10 days, the expression of *IRT1* and *FRO1* was depressed, but maintained to the control level in the presence of S (+S/–Fe). Similarly, for the first 5 days, the decreased enzymatic activity of ferric chelate reductase in Fe-deprived roots in the absence of S was largely increased by S supply (Fig. 4a). These results indicate that S nutrition plays a significant role in root Fe uptake by up-regulating the expression of *IRT1* and *FRO1*, and by increasing ferric chelating activity for the early

period of Fe-deficiency. However, a prolonged S-deficiency up to 10 days limited the expression of these genes and ferric chelate reducing activity. This might be due to cell damage in leaf tissue, as shown by severe chlorosis and necrosis in some parts (Fig. 2), which knock-out the certain genes and in turn also results in reduction of photosynthetic activity such as stomatal conductance and transpiration (Henriques et al. 2002). Indeed, for the first 5 days, the relationships of S content in leaves with the expression of *IRT1* and *FRO1* in roots were highly significant, but not significant at day 10 (Table 1). It can be thus suggested that the nutritional status of S is a more crucial factor in determining Fe uptake and transport especially for the early period of Fe-deficiency.

In addition, Fe- and/or S-deficient leaves showed characteristic symptoms of chlorosis (Fig. 2c) as previously observed in barley and bean plants (Juszczuk and Ostaszewska 2011; Astolfi et al. 2012), and accompanied with a reduction of chlorophyll and carotenoid content (Fig. 2a, b). The loss of these photosynthetic pigments caused by Fe-deficiency was accelerated in absence of S (Fig. 2a, b). The loss of carotenoids may be responsible for the reduction of chlorophyll content because carotenoids absorb light energy for use in photosynthesis, and they protect chlorophyll from photodamage (Armstrong and Hearst 1996). Such decrease in chlorophyll content may be

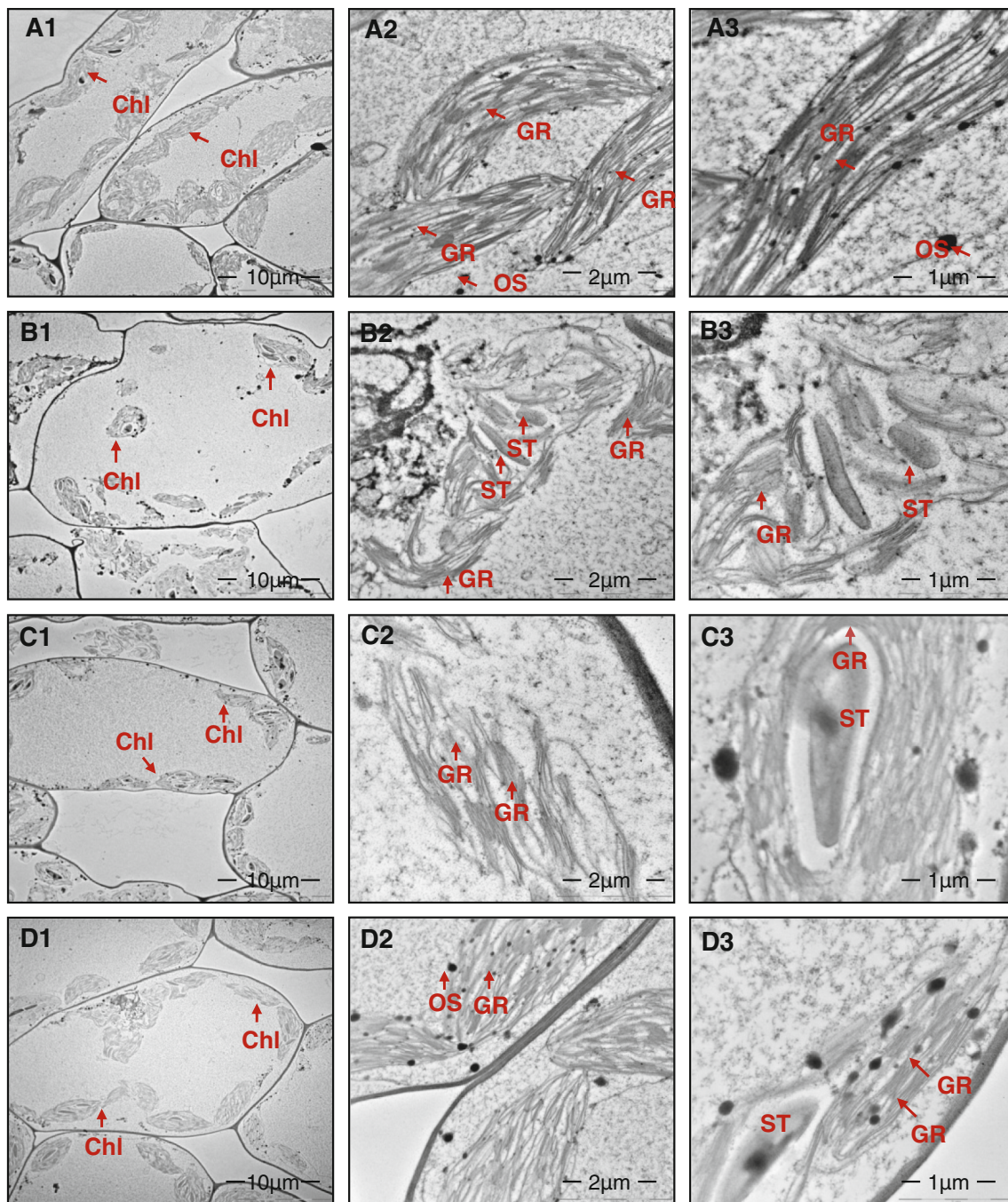


Fig. 6 The response of chloroplast ultrastructure to four S/Fe combined treatments: sufficient in S and Fe (A1–A3; +S/+Fe, control), deprived S and Fe (B1–B3; –S/–Fe), deprived S but sufficient Fe (C1–C3; –S/+Fe) and sufficient S but deprived Fe (D1–D3; +S/–Fe) for 10 days. Leaf tissues were cut into 1–3 mm² pieces,

treated with glutaraldehyde and post-fixed with 1 % OsO₄ embedded in epoxy resin, thin sectioning using a diamond knife and specimen were stained and examined under electron microscope at different magnifications. In figures *Chl* indicates chloroplasts, *GR* indicates grana, *Os* indicates osmium tetroxide, *ST* indicates starch grain

also attributed to Fe requirement for the formation of precursor molecules, δ -aminolevulinic acid and protochlorophyllide (Marschner 1986). An additional loss of photosynthetic pigments under S-deficient condition is certainly due to the decreased S availability (Fig. 2), since S is an integral constituent of two S-rich amino acids,

cysteine and methionine, which both act as structural and functional elements of proteins (Droux 2004). Juszczak and Ostaszewska (2011) reported that a strong decline in chlorophyll content in chlorotic bean leaves was accompanied by a decrease in S concentration. Fe- and/or S-deficient leaves appeared to have only about half of the

stomatal number and density of controls. Leaf stomatal density often rises under stress condition (Zhang et al. 2006; Xu and Zhou 2008). However, the present results showed that severe chlorosis or necrosis, caused by Fe- and S-deprivation for 10 days, might lead to a reduction in stomatal density. This response may be explained by a parabola-shaped pattern as shown by Xu and Zhou (2008) who reported that an increase in stomatal density under moderate drought stress was caused by a decrease in leaf area, but an inhibition of guard cell division in relation to senescence induced by severe drought can lead to a reduction in total stomatal number on a given leaf. The reduction of stomatal number and density led to poor gaseous exchange which resulted in a more severe loss of stomatal conductivity and transpiration in Fe-deprived in the absence of S (Fig. 3).

The decline of chloroplastic pigments in the leaves may be the result of an overall decrease of biosynthesis of chloroplast-targeted proteins. It has been well documented that both metabolism of photosynthetic pigments and chloroplast ultrastructure are dependent on Fe (Briat et al. 2007). However, S-dependent effects have been relatively less studied. It has been generally established that leaf chlorosis is accompanied by reduction of number of granal and stromal lamellae per chloroplast (Spiller and Terry 1980). The present electron micrographs of chloroplasts revealed that Fe-deficiency results in a reduction of chloroplast number, and the disintegration of the lamellar network inside the chloroplast, most severely in the absence of S (Fig. 6a, b, d). The classical features of ultra-structural changes in chloroplast morphology caused by major mineral (N, Ca, Mg, K, P, S) deficiencies in maize leaves (Hall et al. 1972) and by Fe-deficiency in Indian mustard (Qureshi et al. 2010) have been described. It has been shown that the loss of photosystem I (PSI) in response to Fe-deficiency modifies the polypeptide composition of light harvesting complex I (LHCI) (Hippler et al. 2001), which was correlated with the functional drop in excitation of energy transfer efficiency between LHCI and PSI (Naumann et al. 2005). The present data clearly showed that the symptoms of chlorosis in the –S/–Fe-deficient leaves, which were characterized by the accumulation of starch (Fig. 6 B2), the disruption of chloroplast membrane (Fig. 6 B3) were largely restored in the presence of S. Moreover, the decrease in the activity of RuBisCO, sucrose synthase and net photosynthesis in response to Fe-deficiency in the absence of S, including a large recovery in the presence of S, was paralleled with the responses of thylakoid protein complexes and chloroplast ultrastructure to Fe-deficiency in the absence or presence of S. Our results thus indicate that S nutrition plays significant roles in alleviating chloroplast damage caused by Fe-deficiency, and that the damages are inversely aggravated in the absence of S.

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