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Involvement of Arabidopsis glutaredoxin S14 in the maintenance of chlorophyll content

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Abstract:	<p>Plant class-II glutaredoxins (GRXs) are oxidoreductases carrying a CGFS active site signature and are able to bind iron-sulfur clusters in vitro. In order to explore the physiological functions of the two plastidial class-II isoforms, GRXS14 and GRXS16, we generated knockdown and overexpression <i>Arabidopsis thaliana</i> lines and characterized their phenotypes using physiological and biochemical approaches. Plants deficient in one GRX did not display any growth defect, whereas the growth of plants lacking both was slowed. Plants overexpressing GRXS14 exhibited reduced chlorophyll content in control, high light and high salt conditions. However, when exposed to prolonged darkness, plants lacking GRXS14 showed accelerated chlorophyll loss compared to WT and overexpression lines. We observed that the GRXS14 abundance and the proportion of reduced form were modified in WT upon darkness and high salt. The dark treatment also resulted in decreased abundance of proteins involved in the maturation of iron-sulfur proteins. We propose that the phenotype of GRXS14-modified lines results from its participation in the control of chlorophyll content in relation with light and osmotic conditions, possibly through a dual action e.g. regulating the redox status of biosynthetic enzymes and contributing to the biogenesis of iron-sulfur clusters, which are essential cofactors in chlorophyll metabolism.</p>

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Involvement of Arabidopsis glutaredoxin S14 in the maintenance of chlorophyll content

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Short running title: Control of chlorophyll content by glutaredoxins

26 **Abstract**

27 Plant class-II glutaredoxins (GRXs) are oxidoreductases carrying a CGFS active site
 28 signature and are able to bind iron-sulfur clusters *in vitro*. In order to explore the
 29 physiological functions of the two plastidial class-II isoforms, GRXS14 and GRXS16, we
 30 generated knockdown and overexpression *Arabidopsis thaliana* lines and characterized their
 31 phenotypes using physiological and biochemical approaches. Plants deficient in one GRX did
 32 not display any growth defect, whereas the growth of plants lacking both was slowed. Plants
 33 overexpressing GRXS14 exhibited reduced chlorophyll content in control, high light and high
 34 salt conditions. However, when exposed to prolonged darkness, plants lacking GRXS14
 35 showed accelerated chlorophyll loss compared to WT and overexpression lines. We observed
 36 that the GRXS14 abundance and the proportion of reduced form were modified in WT upon
 37 darkness and high salt. The dark treatment also resulted in decreased abundance of proteins
 38 involved in the maturation of iron-sulfur proteins. We propose that the phenotype of
 39 GRXS14-modified lines results from its participation in the control of chlorophyll content in
 40 relation with light and osmotic conditions, possibly through a dual action *e.g.* regulating the
 41 redox status of biosynthetic enzymes and contributing to the biogenesis of iron-sulfur clusters,
 42 which are essential cofactors in chlorophyll metabolism.

43

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45 **Keywords:** *Arabidopsis thaliana*, chloroplast, chlorophyll, glutaredoxin, iron-sulfur cluster,
 46 redox, senescence.

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49 Introduction

50 Glutaredoxins (GRXs) are small and ubiquitous oxidoreductases belonging to the thioredoxin
51 (TRX) superfamily (Rouhier et al. 2008; Meyer et al. 2009). They display a 4-residue active-
52 site signature including one or two redox-active cysteines that allow the reduction of disulfide
53 bonds usually using glutathione (GSH) as an electron donor (Rouhier et al. 2006). Compared
54 to other organisms, higher plants are characterized by an elevated number of *GRX* genes, at
55 least 31 in *Arabidopsis thaliana*, which are subdivided into four classes (Couturier et al.
56 2009). Class-I and -II GRXs exhibit in most cases CPXC and CGFS active site motifs,
57 respectively, and are present in all photosynthetic organisms. GRXs from class III display a
58 CCXX active site sequence, are restricted to terrestrial plants and are the most represented
59 with 21 isoforms in *A. thaliana*. The last class (IV) is found in algae and terrestrial plants and
60 contains proteins with one N-terminal GRX domain carrying a CXXC/S motif and two other
61 domains of unknown function. Interestingly, several plant class-I and -II GRXs bind iron-
62 sulfur (Fe-S) clusters *in vitro* (Rouhier et al. 2007; Bandyopadhyay et al. 2008; Rouhier et al.
63 2010). Consistently, the yeast mitochondrial class-II GRX5 participates in Fe-S cluster
64 assembly by transferring preformed Fe-S clusters to other specific carrier proteins before final
65 insertion into acceptor proteins (Rodriguez-Manzanque et al. 2002). In other respects, the Fe-
66 S cluster in human class-I GRX2, which is dissociated in the presence of oxidized glutathione,
67 might play a redox sensor role by restoring the reductase activity that is lost upon Fe-S cluster
68 ligation (Lillig et al. 2005). The participation of class-II GRXs in Fe-S cluster assembly *in*
69 *vivo* in plants remained elusive until recent reports on *Arabidopsis* lines expressing
70 mitochondrial GRXS15 variant forms which displayed decreased activity of aconitase, an Fe-
71 S cluster-containing enzyme (Moseler et al. 2015), and on knockdown lines showing defects
72 in the maturation of the mitochondrial lipoate synthase, an Fe-S enzyme (Ströher et al. 2016).
73 Concerning the nucleo-cytosolic GRXS17, the evidence comes from its association with

74 known cytosolic Fe-S cluster assembly components of the cytosolic machinery and with
75 several putative Fe-S client proteins (Inigo et al. 2016).

76 In the last years, plant GRXs have been shown to fulfil key roles in developmental
77 processes. For instance, in Arabidopsis, the deficiency in both class-I GRXC1 and GRXC2
78 results in a lethal phenotype due to impaired embryo development (Riondet et al. 2012). Two
79 class-III GRXs participate in the development of floral organs via interaction with bZIP-type
80 TGA transcription factors (Xing et al. 2005; Xing and Zachgo 2008). Another evidence of the
81 involvement of class-III GRXs in plant reproduction is illustrated by the rice male sterile *mill*
82 mutant (Hong et al. 2012). OsMIL1 encodes a GRX involved in the meiotic entry of
83 sporogenous cells. Consistently, the fate of germ cells in maize anthers depends on a class-III
84 GRX, which also regulates meristem functioning, presumably via interaction with a TGA
85 transcription factor (Chaubal et al. 2003; Kelliher and Walbot, 2012; Yang et al. 2015). The
86 class-II GRXS17, which is composed of one TRX-like domain and three GRX domains
87 containing each a CGFS motif, plays a key role in the control of plant development in relation
88 with environment. Arabidopsis *grxs17* plants grown at 28°C exhibit reduced primary root
89 growth and impaired flowering (Cheng et al. 2011) and this mutant also displays a strongly
90 impaired vegetative and reproductive development under long-day conditions (Knuesting et
91 al. 2015). GRXS17, which interacts with the NF-YC11/NC2 α transcription factor, has been
92 proposed to relay a redox signal generated by environmental conditions and required for
93 proper apical meristem functioning. It is worth mentioning that the roles of class-II GRXs in
94 plant development could be fulfilled through interplays with signalling pathways involving
95 hormones like auxin (Cheng et al. 2011).

96 Besides their functions in development, plant class-III GRXs also participate in
97 responses to environmental constraints such as photooxidative stress (Laporte et al. 2012) and
98 to pathogens (Ndamukong et al. 2007; La Camera et al. 2011). With regard to class-II GRXs,

99 *Arabidopsis* plastidial GRXS14 and mitochondrial GRXS15, which consist of a unique GRX
100 domain, are presumed to play important roles upon oxidative stress, high temperature and
101 arsenic exposure (Cheng et al. 2006; Sundaram et al. 2009; Sundaram and Rathinasabapathi,
102 2010; Ströher et al. 2016). Regarding the other plastidial class-II GRX, Guo et al. (2010)
103 reported that tomato plants silenced for *GRXS16* expression are more sensitive to osmotic
104 constraints. Of note, GRXS16 is composed of two modules, an N-terminal domain which
105 possesses endonuclease activity fused to a C-terminal GRX domain (Liu et al. 2013). This
106 could suggest a link between redox regulation and plastidial DNA metabolism.

107 In the present work, we undertook a genetic approach to investigate the physiological
108 functions of the two plastidial class-II GRXs in the view of their dual *in vitro* biochemical
109 functions (reductase activity vs Fe-S cluster ligation). Our data revealed that GRXS14 and
110 GRXS16 play important roles in the control of vegetative growth, and that GRXS14 possesses
111 specific functions in the maintenance of chlorophyll content depending on environmental and
112 light conditions.

114 **Materials and methods**

115 **Plant material, growth conditions and environmental constraints**

116 In standard conditions, *Arabidopsis thaliana* Col-0 plants were grown in soil in phytotron
117 under an 8-h photoperiod and a photon flux density of 200 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ at 22°C.
118 Several environmental constraints were applied on soil-grown plants (Cerveau et al. 2016). A
119 gradually increasing water deficit was applied on 5-week-old plants by withholding watering
120 for *ca* 7 days. For high light and high temperature treatment, 4-week-old plants were
121 transferred at 825 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ under an 8-h photoperiod and 30°C/18°C (day/night)
122 for 1 week. Another high light condition was applied from sowing at 450 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$
123 and 22°C/18°C (day/night, 16-h photoperiod) for 3 weeks. Four-week old plants were

exposed to high salinity by watering with a solution of 150 mM NaCl for either 2 weeks or 1 month in the standard phytotron light conditions. For low temperature treatment, 3-week-old plants were transferred at 10°C in a growth chamber (200 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$, 8-h photoperiod) for 4 weeks. For photooxidative conditions, 5-week-old plants were exposed in a growth chamber to 1400 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ (8-h photoperiod) at 6°C for 7 days. For senescence experiments, well-expanded leaves of 6-week-old plants were covered with aluminum foil and plants were further cultivated in the phytotron in standard conditions for 8 days before analysis of covered leaves.

Osmotic and oxidative constraints were also investigated on leaf disks by performing incubation on water, 150 mM NaCl, 150 mM KCl, 300 mM mannitol or 2 μM methyl viologen with the leaf abaxial side directly in contact with the solution. Chemicals were purchased from Sigma. Discs (1.3-cm diameter) were excised from adult well-expanded leaves from 6- to 7-week old *Arabidopsis* plants grown in control conditions and floated for 48 h in the phytotron conditions (200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 8-h photoperiod) or in continuous dark for 2 to 5 days.

Transformation of *A. thaliana* plants and PCR analyses

The full-length GRXS14 and GRXS16 coding sequences were cloned into the pB2GW7 or pH2GW7 vectors (GATEWAY® Invitrogen) for overexpression and the partial GRXS16 coding sequence into the pK7GWIWG2(I) vector (GATEWAY® Invitrogen) for RNA interference using appropriate primers (Table S1). Following transformation using *Agrobacterium tumefaciens* C58 strain (Clough and Bent, 1998), homozygous lines (T2) were obtained from resistance segregation assays. Leaf genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) to perform PCR using appropriate primers (Table S1), Taq

DNA polymerase (Life Technologies) and the GeneAmp PCRSystem 2700 (Applied Biosystems).

Protein preparation, electrophoresis and Western blot analysis

Organs from Arabidopsis were ground in liquid nitrogen and the powder was suspended in 50 mM Tris-HCl pH 8, 1 mM PMSF and 50 mM β -mercaptoethanol to prepare soluble proteins as described in Rey et al. (2005). For extraction in non-reducing conditions, β -mercaptoethanol was omitted. Following vigorous shaking at 4°C for 20 min and centrifugation (20 min, 15,000 rpm, 4°C), the supernatant was precipitated by adding 2 volumes of acetone. Protein concentration was determined using the “Protein Quantification BCA Assay” kit (Interchim, France). Proteins were separated using SDS-PAGE and electroblotted onto 0.45 μ m nitrocellulose membranes (Pall Corporation, NY, USA) to carry out immunoblot analysis (Rey et al. 2005). The primary antibodies used were raised in rabbit (Table S2) and detected using the goat anti-rabbit “Alexa Fluor® 680” IgG (1:10,000) (Invitrogen, Carlsbad, USA). Revelation and quantification of band intensity were performed using the Odyssey Infrared Imager (Licor, Lincoln, NE, USA).

mPEG maleimide labelling

Leaf soluble proteins were prepared as described above in 50 mM Tris-HCl pH 8, 1 mM PMSF in the absence of reductant. Proteins were precipitated using 2 volumes of acetone at -20°C for 2 h and immediately used for labelling. Following centrifugation, an aliquot of 200 μ g protein was suspended in PBS, 1% SDS and 1.7 mg.mL⁻¹ mPEG-maleimide-2000 (Laysan Bio Arab, AL, USA) and incubated at room temperature for 3 h. Finally, the reaction mixture was added with SDS-PAGE loading buffer (X 4) devoid of reductant to perform SDS-PAGE and Western blot analyses as described above.

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Chlorophyll content determination

One-cm diameter leaf disks were taken from well-expanded adult leaves, stored at -80°C and crushed in 1 mL 80% acetone. Following vigorous shaking, overnight storage in the dark at 4°C and centrifugation (14,000 g, 10 min), the content in chlorophylls *a* and *b* was measured spectrophotometrically and calculated according to Lichtenthaler (1987).

Chlorophyll fluorescence measurements

Chlorophyll fluorescence emission from the upper leaf surface was measured at room temperature using a PAM-2000 modulated Walz fluorometer (Effeltrich, Germany; Havaux et al. 2000). After dark adaptation for 20 min, the initial level (F_0) of chlorophyll fluorescence was excited by a dim red light modulated at 600 Hz and determined after a 1 s illumination with far-red light. The maximal level of chlorophyll fluorescence (F_m) was induced by a pulse of intense white light. The maximal quantum yield of photosystem II (PSII) photochemistry was calculated as $(F_m - F_0)/F_m = F_v/F_m$ (F_v : variable fluorescence). PSI and PSII chlorophyll fluorescence emission spectra at low temperature were measured from leaf discs in liquid nitrogen using a PerkinElmer LS50B luminometer equipped with fiber optic light guides (Havaux et al. 2000).

Results

Expression of GRXS14 and GRXS16 during development and environmental constraints in Arabidopsis

The information about the plastidial GRXS14 and GRXS16 levels being poor, we first investigated their abundance using Western blot analysis. Soluble protein extracts were prepared from leaves at various developmental stages (young less than one 1 cm long, well-expanded and old non-senescent) of WT Arabidopsis plants grown in short-day conditions for six weeks (rosette stage) or from various organs of plants cultivated in long-day conditions for the same period (reproductive stage). Fig. 1a shows that GRXs have quite similar distribution patterns and are present in most organs. GRXS14 was found in all organs, although barely detectable in seeds, and displayed a higher abundance in photosynthetic organs particularly young leaves, as compared to roots, flower buds and flowers. In comparison, the GRXS16 protein was slightly detected in root extracts and its abundance was almost as high in buds and flowers as in leaves and stems.

Then we analyzed the abundance of GRXs in leaves of soil-grown plants exposed to high light in long day (HL-LD), high light and high temperature (HL-HT), photooxidative treatment (PT), low temperature (LT), high salinity (S) and water deficit (WD). Upon these constraints, no substantial change was observed in the abundance of RubisCO and 2-Cys peroxiredoxin (PRX) (Cerveau et al. 2016). Decreased GRXS14 protein levels were observed in high light conditions (HL-LD and HL-HT), whereas strongly increased amounts were noticed upon osmotic constraints, *i.e.* low temperature, salt and drought (+ *ca* 100, 150 and 200%, respectively, Fig. 1b). In contrast, no change was observed regarding the GRXS16 abundance except upon high light conditions (HL-LD and HL-HT), which led to protein levels reduced by *ca* 30% (Fig. 1b). These data reveal differential abundance of the two plastidial class-II GRXs in response to environmental constraints.

Generation of Arabidopsis lines modified in the expression of *GRXS14* and/or *GRXS16*

Homozygous Arabidopsis plants knockout for *GRXS14* expression at the protein level were selected from the SALK_125902 line termed K. Two independent lines, termed O1 and O2 (Table 1), overexpressing *GRXS14* in the WT background were selected based on a much higher protein level in leaf extracts compared to WT (Fig. 2a,c). Note that in these 3 lines, the *GRXS16* abundance was not altered. In parallel, we transformed WT plants with the *GRXS16* cDNA under the control of the CaMV-35S promoter. Two types of lines, either overexpressing *GRXS16* at a high level or lacking the protein, very likely due to a co-suppression phenomenon, were obtained (Table 1, Fig. S1a, b). In the 4 lines either up- or down-regulated for *GRXS16* expression selected for further studies, no change in leaf *GRXS14* abundance was observed (Fig. S1b). Finally, we generated Arabidopsis plants down-regulated for the expression of both *GRX* genes. Due to the lack of T-DNA insertion mutants for *GRXS16* and the instability of co-suppression mechanisms in the progeny, we developed a RNA-interference strategy in the *grxS14* genetic background. Two lines termed KI1 and KI2 (Table 1) displaying a leaf *GRXS16* abundance decreased by *ca* 50 and 80%, respectively, compared to WT and *grxS14* (K) plants, were selected (Fig. 2b,d).

Characterization of plants modified for *GRXS14* and *GRXS16* expression

We first compared the growth of modified lines by measuring the rosette weight of 6-week-old plants in control conditions. We noticed that germination was similar for all lines. Compared to WT, we observed no difference regarding lines knockout for *GRXS14* expression (K), down-regulated for *GRXS16* expression (C2 and C4) or overexpressing this gene (O4 and O5) (Fig. 3a). In contrast, at the same age, the rosette weight of lines overexpressing *GRXS14* (O1 and O2) was significantly increased by *ca* 20% (2.75 g)

compared to that of WT (2.3 g) whereas *grxS14* lines knockdown for *GRXS16* expression (KI1 and KI2) displayed a substantially reduced rosette weight (*ca* -25%, 1.75 g) (Fig. 3a, b). Of note, the growth of the 2 lines restored for *GRXS14* expression (KI1-R1 and KI1-R2, Table 1) was similar to that of WT (Fig. S2a, b). We then focused our study on the lines modified for *GRXS14* expression (K, O1 and O2) and those knockout for *GRXS14* and down-regulated for *GRXS16* (KI1 and KI2) to investigate some photosynthetic parameters. In control conditions, no difference in the maximal PSII photochemical efficiency, assessed by the F_v/F_m parameter, was noticed (Fig. S3a). In terms of leaf chlorophyll content, both lines overexpressing *GRXS14* displayed a significantly lower level compared to that in WT and no variation was noticed in other lines (Fig. 3c). Finally, as plastidial *GRXS14* and *S16* are presumed to participate in the maturation of Fe-S proteins, we examined parameters related to two major photosynthetic actors containing Fe-S clusters, *i.e.* photosystem I and ferredoxin (FDX). Low temperature fluorescence emission spectra measured in leaf disks, which reflect the organization of PSI and PSII complexes, and Western analysis of FDX2 abundance did not reveal any difference in GRX-modified lines (Fig. S4a, b). Looking at the content of some actors involved in the maturation of plastidial Fe-S proteins, we observed that the abundance of NFU2 and SUFE1 was decreased by *ca* 30% in lines overexpressing *GRXS14* or *GRXS16*, whereas no change was noticed for other proteins (NFU3, NFS2, SUFA1, SUFB) (Fig. S4b, c, d). Collectively, these results indicate that plants modified in the expression of plastidial class-II GRXs exhibit a growth phenotype associated with alterations in the chlorophyll content and in the abundance of some actors involved in the maturation of Fe-S proteins.

Response of plants modified for *GRXS14* and *GRXS16* expression to oxidative stress

Studies using plate-grown plantlets or leaf disks suggested a role of *GRXS14* and *GRXS16* in oxidative stress responses (Cheng et al. 2006; Guo et al. 2010). Therefore, we first exposed

soil-grown GRX-modified plants from sowing to high light and long photoperiod, conditions affecting redox homeostasis (Laugier et al. 2010) and impairing the development of *grxS17* plants (Knuesting et al. 2015). After 3 weeks, the rosette weights of plants overexpressing *GRXS14* or of *grxS14* plants knockdown for *GRXS16* were on average 25% higher and 22% lower, respectively, than that of WT (Fig. S5a), indicating that high light does not further affect the growth of modified lines compared to standard conditions (Fig. 3a). Again, both overexpressing lines displayed significantly lower chlorophyll contents (Fig. S5b). In another series of experiments, we exposed plants to acute photooxidative stress, very high light at 6°C, conditions leading to photobleaching of thylakoids (Rey et al. 2007). After 11 days, no difference was observed in modified lines compared to WT, none of them showing severe damage (Fig. S5c). Finally, we incubated leaf disks on methyl viologen and measured the maximal PSII activity. Disks from plants deficient in *GRXS14* displayed lower, but non-significantly different, F_v/F_m values compared to WT and *GRXS14*-overexpressing plants (Fig. S5d). Similarly, when incubating disks from plants modified for *GRXS16* expression on methyl viologen, no significant difference in F_v/F_m values was noticed (Fig. S6a). Taken collectively, these data indicate that *GRXS14* and *GRXS16* do not likely play essential roles *in vivo* in the protection of photosynthetic structures against oxidative damage.

Response of plants modified for *GRXS14* and *GRXS16* expression to osmotic stress

Overexpression of tomato *GRXS16* in *Arabidopsis* has been reported to confer tolerance to NaCl both in *in vitro* and *in vivo* conditions (Guo et al. 2010). We thus investigated the response of the lines generated in this work to high salinity by first watering soil-grown plants with a solution containing 0.15 M NaCl during one month. Plant growth was considerably reduced since the rosette weight at the end of the experiment was around 0.7 g (Fig. 3d) compared to 2.3 g in control conditions (Fig. 3a). Most interestingly, similar rosette weights

were measured for WT, GRXS14-overexpressing (O1 and O2) and *grxS14* plants knockdown or not for *GRXS16* (K, KI1 and KI2) in contrast to standard conditions (Fig. 3a, d). While no change in the maximal PSII activity was observed (Fig. S3b), higher chlorophyll contents were measured compared to control conditions, but consistent with the data acquired in other environmental conditions, lines overexpressing GRXS14 (O1 and O2) displayed substantially lower pigment levels in well-expanded leaves than other lines. (Fig. 3c, f). Strikingly, a loss of pigments was observed in very young leaves of O1 and O2 plants, which were also almost necrotic in contrast to what was observed in other genotypes (Fig. 3e). When performing a similar salt treatment on lines modified for *GRXS16* expression, plant growth and chlorophyll content were not impaired compared to WT while the PSII photosynthetic efficiency was significantly reduced in plants lacking GRXS16, but not in overexpressing ones (Fig. S6b-e).

To further investigate the effects of high salt, we carried out incubation experiments using leaf disks. On water, no difference with regard to maximal PSII activity and chlorophyll content was noticed between WT and lines modified for the expression of GRXS14 and/or GRXS16, except for the O2 line (Figs. 4a, S3c, S6a). In contrast, the F_v/F_m values (*ca* 0.30) of discs from *grxS14* plants knockdown or not for *GRXS16* (K, KI1 and KI2) were significantly lower than that of WT (0.40) upon incubation on 0.15 M NaCl in the light phytotron conditions for 48 h and higher (0.46) in disks from GRXS14-overexpressing O1 and O2 plants (Fig. S3c). Of note, all lines exhibited a similar chlorophyll content following the incubation on salt in the light for 2 days (Fig. 4a). To determine whether the PSII phenotype trait of GRXS14-modified plants was related to osmotic stress or to NaCl toxicity, we incubated discs on 0.3 M mannitol or 0.15 M KCl (Fig. S3c). Mannitol and KCl induced slight and strong, respectively, decreases in PSII activity, but to the same extent in GRXS14-modified lines and WT. These data reveal that the higher sensitivity to NaCl of leaf disks from GRXS14-deficient plants is very likely associated with Na^+ toxicity. We then performed

the NaCl treatment in the dark. The treatment on water strongly and similarly impaired the PSII activity in all lines (Fig S3d), but barely affected the chlorophyll content (Fig. 4b). In the presence of NaCl, *grxS14* plants knockdown or not for *GRXS16* (K, KI1 and KI2) exhibited F_v/F_m values decreased to 0.4 and chlorophyll contents reduced to $10 \mu\text{g}\cdot\text{cm}^{-2}$ (compared to 0.65 and $17 \mu\text{g}\cdot\text{cm}^{-2}$, respectively, in WT) (Figs. S3d, 4b). Altogether, these data reveal that GRXS14 plays important, but complex roles in the responses to NaCl, since contrasted results were obtained when using whole plants or leaf disks.

Response of plants modified for *GRXS14* and *GRXS16* expression to dark exposure

The strong decrease in chlorophyll content in *grxS14* leaf disks exposed to NaCl in the dark (Fig. 4b), coupled to the observed decrease in GRXS14 transcript level in senescent leaves (eFP Browser; <http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi>), prompted us to study the response to dark exposure of *GRXS14*-modified lines. First, we incubated disks on water in the dark for 3 to 5 days (Fig. 4c). Following 3 days, almost no decrease was noticed in the chlorophyll content of WT and GRXS14-overexpressing plants compared to light conditions whereas a marked reduction, from *ca* 25 to $20 \mu\text{g}\cdot\text{cm}^{-2}$, occurred in disks from GRXS14-deficient K, KI1 and KI2 lines (Figs. 4a, c). Thereafter, a dramatic decrease in pigment content was observed in all lines after 4 or 5 days in the dark. Of note, this decrease was much more pronounced in *grxS14* lines down-regulated or not for *GRXS16* expression (K, KI1 and KI2), since they showed a twice lower chlorophyll content than WT and GRXS14-overexpressing plants (Figs. S7a, 4c). We then covered leaves with aluminum foil for 8 days on entire plants in standard light conditions. Such a treatment has been reported to induce senescence (Weaver and Amasino, 2001). Most dark-exposed WT leaves remained pale green and some exhibited limited yellowing (Fig. 4d). In these leaves, a lower chlorophyll content was measured compared to control conditions (17.0 vs $26.5 \mu\text{g}\cdot\text{cm}^{-2}$, Figs. 3c, 4e). A very

similar pigment level was measured in covered leaves from GRXS14-overexpressing plants. In contrast, a high proportion (50%) of dark-treated leaves from *grxS14* plants down-regulated or not for *GRXS16* (K, KI1 and KI2) displayed pronounced yellowing (Fig. 4d). Consequently, their pigment content (8 to 10 $\mu\text{g}\cdot\text{cm}^{-2}$) was much lower than that in leaves from WT and from KI1 plants restored for *GRXS14* expression (KI1-R1 and -R2) subjected to the same treatment (*ca* 17 $\mu\text{g}\cdot\text{cm}^{-2}$) (Figs. 4e, S2c). Experiments on dark-exposed disks and leaves of lines modified for *GRXS16* expression did not reveal any difference in the pigment content compared to WT (Fig. S7b-e), clearly demonstrating that plants deficient in GRXS14, but not GRXS16, are sensitive to prolonged darkness.

Redox status of GRXS14 in leaf extracts

The redox status of GRXS14 *in planta*, here in leaves, is an important question to ask as this is likely directly connected to its reductase activity. This was first analyzed by preparing extracts in the absence of reductant and performing non-reducing SDS-PAGE followed by Western blot analysis. In this experiment, a much lower protein amount from the GRXS14-overexpressing line (5 μg) was loaded compared to that from *grxS14* (K) and WT lines (30 μg) to avoid signal saturation. In this case, the serum against GRXS14 revealed several specific bands in protein extracts from WT and GRXS14-overexpressing plants (Fig. 5a). Indeed, two bands very likely corresponding to monomers displaying different redox status were observed at *ca* 12 kDa. A band at *ca* 24 kDa that could correspond to a covalent GRXS14 dimer or a GRXS14-partner complex was clearly detected in overexpressing plants. Another band at *ca* 70 kDa, which might correspond to an oligomer containing GRXS14 alone or linked to partner(s), was specifically revealed in extracts from overexpressing plants (Fig. 5a). The latter band was also faintly revealed in WT protein extracts.

The GRXS14 redox status was then analyzed by carrying out alkylation experiments using mPEG-maleimide-2000 that forms stable thioether bonds with thiol groups and allows the detection of reduced cysteines due to a 2-kDa size increase per free alkylated thiol. Following extraction in non-reducing conditions, incubation with this compound and Western analysis, the 12-kDa forms were almost no more visible in WT and GRXS14-overexpression leaf samples (Fig. 5b). Instead, two bands at *ca* 14 and 18 kDa were clearly and specifically observed, these bands being absent in samples from *grxS14* (K) plants (Fig. 5b). Note that in this experiment, the loading per lane of extracts from overexpressing plants was also much lower (15 μ g) than that of WT plants (100 μ g) to avoid saturated signal levels for the specific 14- and 18-kDa bands. Considering that GRXS14 contains 3 cysteines, the 2 and 6 kDa mass increases likely correspond to the alkylation of one and three thiols, respectively, and allow distinguishing a monomeric form likely displaying one intramolecular disulfide and one reduced cysteine, from a fully reduced form. Based on the signal level of the 18-kDa band, we conclude that the completely reduced GRX form (3 free cysteines) is more abundant in WT extracts in control conditions. Note that, in extracts from overexpressing plants, this form was proportionally less abundant, possibly due to impaired reduction of the huge GRX amount.

This prompted us to investigate whether environmental constraints could affect the GRXS14 redox state in WT plants. In control conditions, the proportion of the 3-thiol form was in the range of 60% with the remaining corresponding to the oxidized 14-kDa form. Interestingly, the ratio was significantly lower in plants exposed to NaCl for 1 month (*ca* 40%) and in leaves covered with aluminum for 8 days (*ca* 50%) and no variation in this proportion was noticed in plants grown in high light (Fig. 5c-f). These data indicate that the *in planta* GRXS14 redox status is modified during specific environmental constraints such as high salinity or prolonged darkness.

Abundance of plastidial redoxins and of proteins involved in Fe-S cluster biogenesis in response to dark exposure

We previously showed that leaves from GRXS14-deficient lines displayed accelerated yellowing upon prolonged darkness (Fig. 4) and that this treatment led to GRX oxidation in WT (Fig. 5). We then analyzed the GRXS14 abundance in dark-exposed WT leaves and observed a reduced protein amount (- 25%) compared to control, no change being observed regarding GRXS16 abundance (Fig. 6a,b). In parallel, we investigated the amount of some plastidial thiol reductases involved in redox homeostasis maintenance, notably PRXs which detoxify H₂O₂ and organic peroxides and methionine sulfoxide reductases (MSRs) which repair oxidized proteins (Vieira dos Santos and Rey, 2006). Regarding PRXs, no change was observed for PRXIIIE and PRXQ (Fig. 6a), the abundance of which can vary in response to abiotic constraints (Gama et al. 2007). An increased amount (+ 20%) of 2-Cys PRX, the most abundant PRX, was noticed in dark-treated leaves and most interestingly, its overoxidized form was much more abundant (+ 100%) (Fig. 6a,b). The plastidial MSRA4 displays two redox forms, the proportion of which varies depending on light intensity (Vieira dos Santos et al. 2005). Upon darkness, no substantial variation was observed in both reductase amount and redox status. The analysis was then extended to proteins participating in the biogenesis of Fe-S proteins in plastids, a process in which GRXS14 could be involved (Couturier et al. 2013). Our data showed that the dark treatment led to strong decreases in the abundance of NFU2 (- 55%), NFU3 (- 60%), SUFA1 (- 33%) and SUFB (- 72%), but not of NFS2, SUFE1 and BOLA4 (Fig. 6a,b). Taken collectively, these data revealed that prolonged darkness results on one hand in substantial changes in the plastidial redox homeostasis, as illustrated by the high amount of overoxidized 2-Cys PRX form and the modified redox status of GRXS14, and on the other hand in strong decreases in the abundance of several key components of the chloroplastic Fe-S cluster assembly machinery.

Discussion

A single plastidial CGFS-GRX is sufficient for proper growth of *A. thaliana* plants

This study aimed at clarifying and comparing the physiological functions of the two plastidial class-II GRXs, GRXS14 and GRXS16, in *A. thaliana*. In standard conditions, plants lacking a single GRX did not show any obvious phenotype whereas the rosette weight of lines knockout for *GRXS14* and knockdown for *GRXS16* was reduced by more than 20%, indicating that a deficiency in both GRXs impairs growth (Fig. 3). Thus, in physiological conditions, the absence of one GRX is compensated by the other which is consistent with their quite similar protein expression patterns (Fig. 1) and the presence of a regular GRX domain in both. On the other hand, plants overexpressing GRXS14, but not GRXS16, displayed enhanced growth in all environmental conditions tested, except upon high salinity (Figs. 3, S5). Accordingly, overexpression of a fern GRXS14 orthologue in Arabidopsis led to either comparable or better growth in control conditions and also upon heat or arsenic treatments (Sundaram et al. 2009; Sundaram and Rathinasabapathi, 2010). On the other hand, no beneficial effect was reported upon overexpression of tomato GRXS16 in Arabidopsis (Guo et al. 2010). There are in fact numerous examples of thiol reductases being required for plant growth or development particularly upon environmental conditions that alter redox homeostasis (Rouhier et al. 2015). This is the case of plastidial TRXs y or methionine sulfoxide reductases B, the deficiency of which results in reduced growth under high light conditions (Laugier et al. 2010; Laugier et al. 2013). Regarding the GRX superfamily, several members, including the two other class-II GRXs (GRXS15 and GRXS17), are important for plant development (Xing and Zachgo, 2005; Riondet et al. 2012; Knuesting et al. 2015; Moseler et al. 2015; Yang et al. 2015) or involved in responses to environmental constraints (Laporte et al. 2012). A strongly reduced growth was noticed for plants knockdown for GRXS13 expression, but no change was

observed in overexpression lines (Laporte et al. 2012), in contrast to what was observed here for GRXS14. To conclude, these data indicate that plastidial class-II GRXs fulfil important roles in the control of growth in Arabidopsis, the overexpression of GRXS14 being even beneficial in various environmental conditions.

GRXS14 participates in chlorophyll maintenance

GRXS14 and GRXS16 have been proposed to participate in responses to oxidative stress based on data mostly gained either using leaf discs or *in vitro* plantlets (Cheng et al. 2006; Guo et al. 2010) or upon ectopic expression in Arabidopsis (Sundaram et al. 2009; Guo et al. 2010). Having noticed the substantial variation in GRXS14 abundance upon light or osmotic constraints (Fig. 1b), our goal was to explore the phenotype of GRXS14-modified lines in these conditions. In comparison with previously published reports, constraints have been mainly applied on soil-grown plants in relatively long-term experiments. When applying conditions generating severe oxidative stress, no susceptibility of lines deficient in GRXS14 and/or GRXS16 was observed (Figs. S5, S6). This might appear contradictory to the root growth reduction observed *in vitro* upon H₂O₂ treatment (Cheng et al. 2006), but it is likely that the heterotrophic metabolism *in vitro* results in H₂O₂ responses non representative of what happens in more physiological conditions.

When investigating the responses to salt of lines modified for *GRXS16*, no difference was noticed in short-term experiments performed on disks. But GRXS16-lacking plants watered with 150 mM NaCl showed reduced PSII activity although this had no impact on the rosette weight (Fig. S6). This is in agreement with the better tolerance of Arabidopsis plants overexpressing tomato GRXS16 exposed to 300 mM NaCl (Guo et al. 2010). Regarding lines modified for *GRXS14* expression, the response to salt greatly varied as a function of the type of material used. Using leaf discs, plants lacking GRXS14 exhibited a lower tolerance to

NaCl as illustrated by a decrease in PSII photosynthetic activity (Fig. S3c). Consistently, discs from plants overexpressing GRXS14 better tolerated the NaCl treatment (Fig. S3c). On the other hand, when analyzing salt-treated plants, GRXS14 overexpression was detrimental as inferred from the measurement of substantially reduced chlorophyll content and the presence of yellowish young developing leaves (Fig. 3e, f). These data clearly highlight the complex/subtle roles that GRXS14 could play in response to salt and the importance of the type of material used for investigating physiological responses of plants. The observed discrepancy might be explained by the fact that leaf discs and whole plants do undoubtedly respond in a distinct manner to the salt application, *i.e.* stomatal entry in the case of discs and root absorption in soil-grown plants. Hence, the first cells perceiving the high sodium concentration are distinct, probably leading to contrasted responses.

In experiments promoting dark-induced senescence, the responses of disks and covered leaves showed consistency. Indeed, in both cases, a strong decrease in chlorophyll content was observed in plants deficient in GRXS14 compared to WT and overexpressing lines (Fig. 4), while there was no effect in lines modified for *GRXS16* expression (Fig. S7). Of possible importance is that in disks this phenotype trait was exacerbated in the presence of salt (Fig. 4b). This additive effect could point to possible distinct and cumulating GRXS14 functions related to light and sodium stresses. Coupled with the observation that *GRXS14*-overexpressing lines display a significantly lower chlorophyll content in control, high salt or high light conditions (Figs. 3, S5), we conclude that GRXS14 plays critical and specific roles in the maintenance of chlorophyll content.

What is the molecular role of GRXS14 in chlorophyll maintenance?

In the last years, the involvement of several types of thiol reductases in the regulation of chlorophyll content has been reported. For instance, plants lacking plastidial methionine

sulfoxide reductases B exhibit a reduced pigment level in high light conditions (Laugier et al. 2010). MSRBs have been proposed to prevent oxidative damage in the system targeting proteins in chlorophyll antennae. In other respects, the stability and/or activity of enzymes involved in tetrapyrrole biogenesis is finely controlled at the post-translational level by thiol-based redox mechanisms (Richter and Grimm, 2013). Indeed, the NADPH-dependent TRX reductase C (NTRC), which has a TRX domain, regulates the redox status of magnesium protoporphyrin IX methyltransferase (Richter et al. 2013). Accordingly, plants deficient in NTRC show pale leaves and impaired growth depending on light and photoperiod conditions (Toivola et al. 2013). So far, a role of GRXs in the redox regulation of tetrapyrrole biosynthesis has not been demonstrated, but proteomic studies in *Chlamydomonas reinhardtii* and *Synechocystis* PCC6803 suggested that several enzymes of this pathway could be prone to glutathionylation, an oxidative post-translational modification quite specifically reversed by GRXs (Michelet et al. 2008; Zaffagnini et al. 2012; Chardonnet et al. 2015). Of note, the senescence process is accompanied by increased ROS formation that could promote glutathionylation (Zimmermann and Zentgraf, 2005). Here, the impaired redox homeostasis in dark-treated leaves is revealed by the increased level of overoxidized 2-Cys PRX (Fig. 6). Most importantly, the GRXS14 redox status varied toward a more oxidized intramolecular disulfide-containing form (Fig. 5) upon dark exposure and upon salt treatment, which also impairs redox homeostasis (Munns and Tester, 2008), arguing for a redox regulatory function. Indeed, the formation of an intramolecular disulfide could constitute a regular intermediate recycling state for class-II GRXs upon deglutathionylation or disulfide bond reduction in target proteins (Zaffagnini et al. 2008).

Alternatively, the GRXS14 function could be related to its participation in the maturation of Fe-S proteins based on its capacity to bind a labile $[\text{Fe}_2\text{S}_2]$ cluster and to transfer it to acceptor proteins (Bandyopadhyay et al. 2008). Evidence for such a

physiological role has been only reported for the mitochondrial GRXS15 isoform (Moseler et al. 2015; Ströher et al. 2016). Several key enzymes in chlorophyll metabolism incorporate an Fe-S center (Balk and Pilon, 2011): chlorophyllide *a* oxygenase (CAO) and 7-hydroxymethyl chlorophyll *a* reductase (HCAR), which are involved in the conversion between chlorophylls *a* and *b* (Oster et al. 2000), and pheophorbide *a* oxygenase (PAO), which is involved in chlorophyll breakdown (Pruzinska et al. 2003). Moreover, other proteins in this biosynthetic pathway require the [Fe₂S₂]-containing FDX for functioning (Richter & Grimm, 2013). Accordingly, Arabidopsis mutants deficient in subunits of the central SUFBC₂D scaffold complex of the plastidial Fe-S cluster assembly machinery show a pale phenotype, accompanied by lower PAO, HCAR and FDX amounts (Hu et al. 2017). Here, we obtained evidence that the protein levels of most SUF components, SUFB, NFU2, NFU3, SUFA1 and GRXS14, but not those of the very early acting NFS2 and SUFE1 proteins, are substantially reduced in WT leaves upon dark-induced senescence (Fig. 6). This could indicate a general down-regulation of the SUF machinery, in agreement with the rapid shut down of chloroplast metabolism in this condition (Chrobok et al. 2016). The findings that GRXS14 deficiency results in drastically reduced chlorophyll content in dark conditions and that its overexpression is associated with both modified chlorophyll content and reduced NFU2 and SUFE1 amounts, strongly suggest that GRXS14 acts within the SUF machinery to regulate chlorophyll metabolism in relation with environmental conditions, and that its deficiency or a non-physiological amount impair the functioning of this machinery. Which step(s) is(are) controlled by GRXS14 in the chlorophyll biosynthetic or degradation pathways and whether its redox status modifies the Fe-S cluster binding properties remain to be studied.

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Table 1 Characteristics and names of *Arabidopsis thaliana* lines modified for the expression of *GRXS14* and/or *GRXS16*

Name	Characteristics
WT	Col-0
K	<i>grxS14</i> , <u>k</u> nockout for <i>GRXS14</i> expression (SALK_125902),
O1 (OE-S14)	<i>GRXS14</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background
O2 (OE-S14)	<i>GRXS14</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background
KI1	<i>grxS14</i> <u>K</u> background, knockdown for <i>GRXS16</i> expression, RNA- <u>i</u> nterference
KI2	<i>grxS14</i> <u>K</u> background, knockdown for <i>GRXS16</i> expression, RNA- <u>i</u> nterference
C2 (C-S16)	<i>GRXS16</i> <u>c</u> o-suppression (CaMV-35S promoter) in WT background
C4 (C-S16)	<i>GRXS16</i> <u>c</u> o-suppression (CaMV-35S promoter) in WT background
O4 (OE-S16)	<i>GRXS16</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background
O5 (OE-S14)	<i>GRXS16</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background
KI1-R1	<u>KI1</u> background, <i>GRXS14</i> <u>r</u> estoration (CaMV-35S promoter)
KI1-R2	<u>KI1</u> background, <i>GRXS14</i> <u>r</u> estoration (CaMV-35S promoter)

Legends to figures

Figure 1. Abundance of GRXS14 and GRXS16 proteins in *Arabidopsis thaliana* organs and in leaves upon environmental constraints.

(a) Western analysis of RubisCO, GRXS14 and GRXS16 abundance in the organs of 6-week-old *Arabidopsis* plants grown in short-day conditions (YL, young leaf less than 1 cm long; AL, adult well-expanded leaf; OL, old non-senescent leaf) and of 6-week-old plants grown in long-day conditions (R, root; St, stem; CL, cauline leaf; FB, flower bud; F, flower; GP, green pod). Seeds (Se) were harvested from plants grown for 11 weeks in long-day conditions. Gels for Western analysis were loaded as in the Coomassie blue-stained gel. **(b)** Western analysis of RubisCO, 2-Cys PRX, GRXS14 and GRXS16 abundance in adult leaves of *Arabidopsis* plants grown for 6 weeks in control conditions (C) or exposed to various light, temperature and osmotic constraints: 450 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ under an 16-h photoperiod (HL-LD, high light, long day), 825 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ and 30°C under an 8-h photoperiod (HL-HT, high light, high temperature), 1400 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$, 6°C, 8-h photoperiod for 1 week (PT, photooxidative conditions), 10°C for 4 weeks (LT, low temperature), watering with 150 mM NaCl for 2 weeks (S, high salinity) and water deficit for 1 week (WD). Light intensity for C, LT, S and WD: 200 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$. Similar results were obtained when using extracts from two independent experiments.

Figure 2. Characterization of *Arabidopsis* lines modified in the expression of *GRXS14* and/or *GRXS16*.

(a) and **(b)**, PCR analysis of genomic DNA. **(c)** and **(d)**, Western analysis of GRXS14 and GRXS16 abundance in leaf extracts. **(a)** and **(c)**, lines modified for *GRXS14* expression. **(b)** and **(d)**, lines modified for *GRXS14* and *GRXS16* expression. WT, wild-type; K, SALK_125902 T-DNA-line knockout for *GRXS14* expression; OE-S14 (O1 and O2), two

independent lines overexpressing *GRXS14* in the WT background; K RNAi-S16 (KI1 and KI2), two independent RNAi lines for *GRXS16* expression generated in the SALK_125902 background.

Figure 3. Growth and chlorophyll content of plants modified for *GRXS14* and/or *GRXS16* expression in control and high salt conditions.

(a, b, c) Plants grown for 6 weeks in control conditions: moderate light ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . (a) Rosette weight. (b) Photograph of KI1, WT and O1 plants. (c) Leaf chlorophyll content. (c, d, e) Plants aged of 21 days grown in control conditions and then watered with 0.15 M NaCl for 31 days in the phytotron conditions (8-h photoperiod, $200 \mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ at 22°C). (d) Rosette weight. (e) Photograph of WT, K, KI1, KI2, O1 and O2 plants. Red arrows indicate young necrotic leaves. (f) Leaf chlorophyll content. KI1 and KI2: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*. Data in control conditions are means $\pm$ SD of six (rosette weight) and three (chlorophyll) average values originating from independent experiments (at least 5 plants and 5 pigment measurements for each genotype per experiment). In high salt conditions, growth data are means $\pm$ SD of values gained from seven plants per genotype (the experiment was performed twice and led to similar results) and chlorophyll data are means $\pm$ SD from 5 independent measurements. *, ** and ***, significantly different from the WT value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

Figure 4. Effect of high salt and prolonged dark exposure on the chlorophyll content of *Arabidopsis* plants modified in the expression of *GRXS14* and/or *GRXS16*.

(a, b), Chlorophyll content of leaf disks incubated for 2 days on water or 0.15 M NaCl in the light phytotron conditions (200 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$; 8-h photoperiod) (a) or in the dark (b). Data are means $\pm$ SD of at least eight values gained in two independent experiments (at least 4 plants for each genotype per experiment). (c), Chlorophyll content of leaf disks incubated on water in the dark for 3, 4 or 5 days. Means $\pm$ SD of 5 (3 and 4 days) and of 3 (5 days) independent measurements per genotype are presented. (d) Leaves covered with aluminum foil for 8 days on entire 6-week-old plants grown on soil in the light phytotron conditions (8-h photoperiod, 200 $\mu\text{moles photons.m}^{-2}.\text{s}^{-1}$). (e) Chlorophyll content in leaves covered with aluminum foil for 8 days. Means $\pm$ SD of 11 independent measurements per genotype. KI2, KI1: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; O1 and O2, lines overexpressing *GRXS14*. *, ** and ***, significantly different from the WT value with P $s < 0.05$, < 0.01 and < 0.001 , respectively (t -test).

Figure 5. Redox status of GRXS14 in leaves of plants grown in control conditions or exposed to environmental constraints.

(a, b) Western analysis of GRXS14 abundance in soluble leaf proteins from control K, WT and O1 plants extracted in non-reducing conditions either non-alkylated (a) or alkylated using mPEG-maleimide-2000 (b). (c, d, e) Western analysis of GRXS14 redox status in soluble leaf proteins from plants exposed to salt (c, d) or high light (d), or from dark-exposed leaves (e) extracted in non-reducing conditions and alkylated using mPEG-maleimide-2000. (f) Proportion of the 18-kDa GRXS14 form (*i.e.* completely reduced form) in extracts from WT plants grown in control, high salt and high light conditions and from WT leaves exposed to dark. Control: plants grown under 200 $\mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ (8-h photoperiod) for 6 weeks; Salt: plants watered with 0.15 M NaCl for 1 month; High light: plants grown under 450 $\mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ and a long photoperiod (16 h) for 3 weeks. Dark, leaves exposed to

dark on whole plants in control light conditions for 8 days. Means $\pm$ SD of 15 (control), 10 (salt), 3 (high light) and 6 (dark) independent experiments are presented. W, wild-type; K, *grxs14*; O1, line overexpressing *GRXS14*. NS, non-specific band. * and ***, significantly different from the control value with $P < 0.05$ and $P < 0.001$, respectively (*t*-test).

Figure 6. Effect of exposure to dark on the abundance of various plastidial thiol reductases and proteins participating in Fe-S cluster biogenesis.

(a) Western analysis of the abundance of RubisCO large subunit, plastidial thiol reductases (GRXS16, GRXS14, 2-Cys PRX, 2-Cys PRX Ox, PRXQ, PRXIIIE and MSRA4 either reduced (Red) or oxidized (Ox)) and various plastidial proteins involved in the maturation of Fe-S proteins (NFU2, NFU3, BOLA4, SUFA1, SUFB, SUFE1 and NFS2). **(b)** Histograms showing the abundance or redox status of several proteins shown in **(a)**. Light, control leaves; Dark, leaves covered with aluminum foil for 8 days on entire 6-week-old plants grown on soil in control phytotron conditions (8-h photoperiod, 200 μ moles photons.m⁻².s⁻¹; 22°C). Leaf proteins were separated using SDS-PAGE in the presence of reductant. Band intensity was quantified using the “Odyssey” software (A.U. Arbitrary Unit). Means $\pm$ SD from 6 independent protein extracts are presented. *, ** and ***, significantly different from the control value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

Supporting information**Table S1. List of primers used for PCR analysis and cloning.****Table S2. List and characteristics of antibodies used for Western blot analyses.****Figure S1. Characterization of Arabidopsis lines modified in the expression of *GRXS16*.**

PCR analysis of genomic DNA **(a)** and Western analysis of GRXS14 and GRXS16 abundance **(b)** in WT and four independent lines carrying the *GRXS16* cDNA under the CaMV-35S promoter. C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*.

Figure S2. Phenotype of Arabidopsis plants knockout for *GRXS14*, knockdown for *GRXS16* and restored with *GRXS14* expression.

Two independent KI1 lines restored for *GRXS14* expression, termed KI1-R1 and KI1-R2, were generated following transformation to integrate the GRXS14 cDNA under the CaMV-35S promoter in genomic DNA. **(a)** Western analysis of RubisCO and GRXS14 abundance in leaves of 6-week-old plants. **(b)** Rosette weight of plants grown for 6 weeks in control conditions, moderate light ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C. Data are means $\pm$ SD of ten to fifteen independent measurements per genotype. **(c)** Chlorophyll content in leaves covered with aluminum foil for 8 days. Means $\pm$ SD of 13 (WT), 3 (K and KI1) and 5 (KI1-R1 and KI1-R2) independent measurements. KI1: line KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; KI1-R1 and KI1-R2: lines KO for *GRXS14*, RNAi for *GRXS16* and restored for *GRXS14* expression. *, significantly different from the WT value with $P < 0.05$ (*t*-test).

Figure S3. Maximal photochemical efficiency of photosystem II (F_v/F_m) in plants modified for the expression of *GRXS14* and/or *GRXS16* in various environmental conditions.

(a) Plants grown for 6 weeks in control light conditions ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$; 8-h photoperiod) at 22°C. (b), Plants aged of 21 days grown in control conditions and then watered with 0.15 M NaCl for 31 days in the same light conditions. (c) Leaf disks incubated on water, 0.15 M NaCl, 0.15 M KCl or 0.3 M mannitol in control light conditions for 48 h. (d) Leaf disks incubated on water or 0.15 M NaCl in the dark for 48 h. Data in (a, b) are means $\pm$ SD of at least five average values originating from independent plants (at least 5 measurements per plant). Data in (c, Water and NaCl) are means $\pm$ SD of six average values originating from independent experiments (10 measurements per genotype per experiment) and in (c, KCl, mannitol) and (d), means $\pm$ SD of at least eight values gained in two independent experiments (at least 4 plants for each genotype per experiment). KI2, KI1: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *, ** and ***, significantly different from the WT value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (t-test). (t-test).

Figure S4. Characteristics related to plastidial Fe-S proteins in plants modified in the expression of *GRXS14* and/or *GRXS16*.

Ratio of the heights of the fluorescence peaks emitted in liquid nitrogen by photosystems I and II (a) and Western analysis of the abundance of components of the plastidial Fe-S machinery (b, c, d). Western analysis of RubisCO LSU, 2-Cys PRX, FDX2 (two isoforms), NFU2, SUFE1, NFU3, SUFA1, SUFB and NFS2 abundance (b). Quantification of the abundance of NFU2 (c) and SUFE1 (d) abundance. Data in (c, d) are means $\pm$ SD from four independent extracts per genotype. KI1 and KI2: lines KO for *GRXS14* and RNAi for

GRXS16; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*. Band intensity was quantified using the Odyssey software (A.U., arbitrary unit). *, ** and ***, significantly different from the WT value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

Figure S5. Responses to photooxidative conditions of Arabidopsis lines modified in the expression of *GRXS14* and/or *GRXS16*.

(a) Growth in moderate photooxidative stress conditions. Plants were grown from sowing for 3 weeks in high light conditions ($450 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and long photoperiod (16 h) at 22°C. **(a)** Rosette weight. **(b)** Leaf chlorophyll content. **(c,d)** Response to acute photooxidative stress conditions. **(c)** Five-week old plants exposed to high light ($1400 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) at low temperature (6°C) for 11 days. **(d)** Maximal photochemical efficiency of photosystem II (F_v/F_m) of leaf disks incubated on 2 μM methyl viologen for 48 h in the phytotron ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$; 8-h photoperiod). KI1 and KI2: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2, line co-suppressed for *GRXS16* expression; OE-S16 O4, line overexpressing *GRXS16*. Data are means $\pm$ SD of six (rosette weight) and three (other data) average values originating from independent experiments (at least 5 plants and 3 measurements for each genotype per experiment). *, ** and ***, significantly different from the WT value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

Figure S6. Responses of Arabidopsis plants modified in the expression of *GRXS16* to osmotic and oxidative constraints.

(a) Effect of NaCl and methyl viologen (MV) on the PSII activity of leaf discs. The maximal photochemical efficiency of photosystem II (F_v/F_m) was measured on disks incubated on water, 0.15 M NaCl or 2 μ M MV in the phytotron conditions (200 μ mol photons. $m^{-2}.s^{-1}$; 8-h photoperiod; 22°C) for 48 h. Mean $\pm$ SD of 10 independent measurements. (b-e) Growth and photosynthetic parameters of plants exposed to NaCl. Plants aged of 21 days grown in control conditions were then watered with 0.15 M NaCl in the phytotron conditions for 31 days (b) Rosette weight of salt-treated plants. Mean $\pm$ SD of 5 independent values. (c) Maximal photochemical efficiency of photosystem II (F_v/F_m) of salt-treated plants. Mean $\pm$ SD of 20 independent measurements performed on 4 plants. (d) Leaf chlorophyll content of salt-treated plants. Mean $\pm$ SD of 5 independent measurements. C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; WT, wild type; OE-S16 O4 and O5, lines overexpressing *GRXS16*. * and ***, significantly different from the WT value with $P < 0.05$ and < 0.001 , respectively (*t*-test).

Figure S7. Effect of exposure to dark on the chlorophyll content of Arabidopsis plants modified for *GRXS14* and/or *GRXS16* expression.

(a, b) Leaf disks incubated on water in the dark for 4 days. (c) Chlorophyll content of disks incubated in the dark for 4 days. Means $\pm$ SD of 6 independent values per genotype. (d) Leaves covered with aluminum foil for 8 days on entire 6-week old plants grown on soil in standard light conditions (8-h photoperiod, 200 μ moles photons. $m^{-2}.s^{-1}$; 22°C). (e) Chlorophyll content in leaves covered with aluminum foil for 8 days. Mean $\pm$ SD of 6 independent measurements per genotype. KI2, KI1: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*.

Figure 1

(a)

(b)

Figure 2

Figure 5

Figure 6

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(a) Effect of NaCl and methyl viologen (MV) on the PSII activity of leaf discs. The maximal photochemical efficiency of photosystem II (F_v/F_m) was measured on disks incubated on water, 0.15 M NaCl or 2 μ M MV in the phytotron conditions (200 μ mol photons.m⁻².s⁻¹; 8-h photoperiod; 22°C) for 48 h. Mean $\pm$ SD of 10 independent measurements. (b-e) Growth and photosynthetic parameters of plants exposed to NaCl. Plants aged of 21 days grown in control conditions were then watered with 0.15 M NaCl in the phytotron conditions for 31 days (b) Rosette weight of salt-treated plants. Mean $\pm$ SD of 5 independent values. (c) Maximal photochemical efficiency of photosystem II (F_v/F_m) of salt-treated plants. Mean $\pm$ SD of 20 independent measurements performed on 4 plants. (d) Leaf chlorophyll content of salt-treated plants. Mean $\pm$ SD of 5 independent measurements. C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; WT, wild type; OE-S16 O4 and O5, lines overexpressing *GRXS16*. * and ***, significantly different from the WT value with $P < 0.05$ and < 0.001 , respectively (t -test).

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(a, b) Leaf disks incubated on water in the dark for 4 days. **(c)** Chlorophyll content of disks incubated in the dark for 4 days. Means $\pm$ SD of 6 independent values per genotype. **(d)** Leaves covered with aluminum foil for 8 days on entire 6-week old plants grown on soil in standard light conditions (8-h photoperiod, 200 μ moles photons.m⁻².s⁻¹; 22°C). **(e)** Chlorophyll content in leaves covered with aluminum foil for 8 days. Mean $\pm$ SD of 6 independent measurements per genotype. KI2, KI1: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*.