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► **To cite this version:**

Pascal Rey, Noëlle Becuwe, Sébastien Tourrette, Nicolas Rouhier. Involvement of *Arabidopsis* glutaredoxin S14 in the maintenance of chlorophyll content. *Plant, Cell and Environment*, 2017, 40 (10), pp.2319-2332. 10.1111/pce.13036 . hal-01686869

HAL Id: hal-01686869

<https://hal.science/hal-01686869>

Submitted on 17 May 2018

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Involvement of Arabidopsis glutaredoxin S14 in the maintenance of chlorophyll content

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|-------------------------------|---|
| Journal: | <i>Plant, Cell & Environment</i> |
| Manuscript ID | Draft |
| Wiley - Manuscript type: | Original Article |
| Date Submitted by the Author: | n/a |
| Complete List of Authors: | Rey, Pascal; CEA, UMR 7265 Bécuwe, Noëlle; CEA, UMR 7265 Tourrette, Sébastien; CEA, UMR 7265 Rouhier, Nicolas; Université de Lorraine, UMR Interactions Arbres-Microorganismes |
| Environment Keywords: | salinity, oxidative stress |
| Physiology Keywords: | growth, photosynthesis: electron transport |
| Other Keywords: | chlorophyll, glutaredoxin, redox, senescence |
| 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 Arabidopsis thaliana 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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1 **Involvement of Arabidopsis glutaredoxin S14 in the maintenance of chlorophyll content**

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23 **Short running title:** Control of chlorophyll content by glutaredoxins

24

25

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

44

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

47

48

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-Manzaneque 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.

113

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

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

132 Osmotic and oxidative constraints were also investigated on leaf disks by performing
133 incubation on water, 150 mM NaCl, 150 mM KCl, 300 mM mannitol or 2 μM methyl
134 viologen with the leaf abaxial side directly in contact with the solution. Chemicals were
135 purchased from Sigma. Discs (1.3-cm diameter) were excised from adult well-expanded
136 leaves from 6- to 7-week old *Arabidopsis* plants grown in control conditions and floated for
137 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
138 dark for 2 to 5 days.

139

140 **Transformation of *A. thaliana* plants and PCR analyses**

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

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

150

151 **Protein preparation, electrophoresis and Western blot analysis**

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

164

165 **mPEG maleimide labelling**

166 Leaf soluble proteins were prepared as described above in 50 mM Tris-HCl pH 8, 1 mM
167 PMSF in the absence of reductant. Proteins were precipitated using 2 volumes of acetone at -
168 20°C for 2 h and immediately used for labelling. Following centrifugation, an aliquot of 200
169 μ g protein was suspended in PBS, 1% SDS and 1.7 mg.mL⁻¹ mPEG-maleimide-2000 (Laysan
170 Bio Arab, AL, USA) and incubated at room temperature for 3 h. Finally, the reaction mixture
171 was added with SDS-PAGE loading buffer (X 4) devoid of reductant to perform SDS-PAGE
172 and Western blot analyses as described above.

173

174 Chlorophyll content determination

175 One-cm diameter leaf disks were taken from well-expanded adult leaves, stored at -80°C and
176 crushed in 1 mL 80% acetone. Following vigorous shaking, overnight storage in the dark at
177 4°C and centrifugation (14,000 g, 10 min), the content in chlorophylls *a* and *b* was measured
178 spectrophotometrically and calculated according to Lichtenthaler (1987).

179

180 Chlorophyll fluorescence measurements

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

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197

198 **Results**

199 **Expression of GRXS14 and GRXS16 during development and environmental** 200 **constraints in Arabidopsis**

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

212 Then we analyzed the abundance of GRXs in leaves of soil-grown plants exposed to
213 high light in long day (HL-LD), high light and high temperature (HL-HT), photooxidative
214 treatment (PT), low temperature (LT), high salinity (S) and water deficit (WD). Upon these
215 constraints, no substantial change was observed in the abundance of RubisCO and 2-Cys
216 peroxiredoxin (PRX) (Cerveau et al. 2016). Decreased GRXS14 protein levels were observed
217 in high light conditions (HL-LD and HL-HT), whereas strongly increased amounts were
218 noticed upon osmotic constraints, *i.e.* low temperature, salt and drought (+ *ca* 100, 150 and
219 200%, respectively, Fig. 1b). In contrast, no change was observed regarding the GRXS16
220 abundance except upon high light conditions (HL-LD and HL-HT), which led to protein
221 levels reduced by *ca* 30% (Fig. 1b). These data reveal differential abundance of the two
222 plastidial class-II GRXs in response to environmental constraints.

223

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

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

240

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

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

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

269

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

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

273 soil-grown GRX-modified plants from sowing to high light and long photoperiod, conditions
274 affecting redox homeostasis (Laugier et al. 2010) and impairing the development of *grxS17*
275 plants (Knuesting et al. 2015). After 3 weeks, the rosette weights of plants overexpressing
276 *GRXS14* or of *grxS14* plants knockdown for *GRXS16* were on average 25% higher and 22%
277 lower, respectively, than that of WT (Fig. S5a), indicating that high light does not further
278 affect the growth of modified lines compared to standard conditions (Fig. 3a). Again, both
279 overexpressing lines displayed significantly lower chlorophyll contents (Fig. S5b). In another
280 series of experiments, we exposed plants to acute photooxidative stress, very high light at
281 6°C, conditions leading to photobleaching of thylakoids (Rey et al. 2007). After 11 days, no
282 difference was observed in modified lines compared to WT, none of them showing severe
283 damage (Fig. S5c). Finally, we incubated leaf disks on methyl viologen and measured the
284 maximal PSII activity. Disks from plants deficient in *GRXS14* displayed lower, but non-
285 significantly different, F_v/F_m values compared to WT and *GRXS14*-overexpressing plants
286 (Fig. S5d). Similarly, when incubating disks from plants modified for *GRXS16* expression on
287 methyl viologen, no significant difference in F_v/F_m values was noticed (Fig. S6a). Taken
288 collectively, these data indicate that *GRXS14* and *GRXS16* do not likely play essential roles
289 *in vivo* in the protection of photosynthetic structures against oxidative damage.

290

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

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

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

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

323 the NaCl treatment in the dark. The treatment on water strongly and similarly impaired the
324 PSII activity in all lines (Fig S3d), but barely affected the chlorophyll content (Fig. 4b). In the
325 presence of NaCl, *grxS14* plants knockdown or not for *GRXS16* (K, KI1 and KI2) exhibited
326 F_v/F_m values decreased to 0.4 and chlorophyll contents reduced to $10 \mu\text{g}\cdot\text{cm}^{-2}$ (compared to
327 0.65 and $17 \mu\text{g}\cdot\text{cm}^{-2}$, respectively, in WT) (Figs. S3d, 4b). Altogether, these data reveal that
328 GRXS14 plays important, but complex roles in the responses to NaCl, since contrasted results
329 were obtained when using whole plants or leaf disks.

330

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

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

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

357

358 **Redox status of GRXS14 in leaf extracts**

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

372 The GRXS14 redox status was then analyzed by carrying out alkylation experiments
373 using mPEG-maleimide-2000 that forms stable thioether bonds with thiol groups and allows
374 the detection of reduced cysteines due to a 2-kDa size increase per free alkylated thiol.
375 Following extraction in non-reducing conditions, incubation with this compound and Western
376 analysis, the 12-kDa forms were almost no more visible in WT and GRXS14-overexpression
377 leaf samples (Fig. 5b). Instead, two bands at *ca* 14 and 18 kDa were clearly and specifically
378 observed, these bands being absent in samples from *grxS14* (K) plants (Fig. 5b). Note that in
379 this experiment, the loading per lane of extracts from overexpressing plants was also much
380 lower (15 μ g) than that of WT plants (100 μ g) to avoid saturated signal levels for the specific
381 14- and 18-kDa bands. Considering that GRXS14 contains 3 cysteines, the 2 and 6 kDa mass
382 increases likely correspond to the alkylation of one and three thiols, respectively, and allow
383 distinguishing a monomeric form likely displaying one intramolecular disulfide and one
384 reduced cysteine, from a fully reduced form. Based on the signal level of the 18-kDa band, we
385 conclude that the completely reduced GRX form (3 free cysteines) is more abundant in WT
386 extracts in control conditions. Note that, in extracts from overexpressing plants, this form was
387 proportionally less abundant, possibly due to impaired reduction of the huge GRX amount.

388 This prompted us to investigate whether environmental constraints could affect the
389 GRXS14 redox state in WT plants. In control conditions, the proportion of the 3-thiol form
390 was in the range of 60% with the remaining corresponding to the oxidized 14-kDa form.
391 Interestingly, the ratio was significantly lower in plants exposed to NaCl for 1 month (*ca*
392 40%) and in leaves covered with aluminum for 8 days (*ca* 50%) and no variation in this
393 proportion was noticed in plants grown in high light (Fig. 5c-f). These data indicate that the *in*
394 *planta* GRXS14 redox status is modified during specific environmental constraints such as
395 high salinity or prolonged darkness.

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

398 We previously showed that leaves from GRXS14-deficient lines displayed accelerated
399 yellowing upon prolonged darkness (Fig. 4) and that this treatment led to GRX oxidation in
400 WT (Fig. 5). We then analyzed the GRXS14 abundance in dark-exposed WT leaves and
401 observed a reduced protein amount (- 25%) compared to control, no change being observed
402 regarding GRXS16 abundance (Fig. 6a,b). In parallel, we investigated the amount of some
403 plastidial thiol reductases involved in redox homeostasis maintenance, notably PRXs which
404 detoxify H₂O₂ and organic peroxides and methionine sulfoxide reductases (MSRs) which
405 repair oxidized proteins (Vieira dos Santos and Rey, 2006). Regarding PRXs, no change was
406 observed for PRXIIIE and PRXQ (Fig. 6a), the abundance of which can vary in response to
407 abiotic constraints (Gama et al. 2007). An increased amount (+ 20%) of 2-Cys PRX, the most
408 abundant PRX, was noticed in dark-treated leaves and most interestingly, its overoxidized
409 form was much more abundant (+ 100%) (Fig. 6a,b). The plastidial MSRA4 displays two
410 redox forms, the proportion of which varies depending on light intensity (Vieira dos Santos et
411 al. 2005). Upon darkness, no substantial variation was observed in both reductase amount and
412 redox status. The analysis was then extended to proteins participating in the biogenesis of Fe-
413 S proteins in plastids, a process in which GRXS14 could be involved (Couturier et al. 2013).
414 Our data showed that the dark treatment led to strong decreases in the abundance of NFU2 (-
415 55%), NFU3 (- 60%), SUFA1 (- 33%) and SUFB (- 72%), but not of NFS2, SUFE1 and
416 BOLA4 (Fig. 6a,b). Taken collectively, these data revealed that prolonged darkness results on
417 one hand in substantial changes in the plastidial redox homeostasis, as illustrated by the high
418 amount of overoxidized 2-Cys PRX form and the modified redox status of GRXS14, and on
419 the other hand in strong decreases in the abundance of several key components of the
420 chloroplastic Fe-S cluster assembly machinery.

421

422 **Discussion**423 **A single plastidial CGFS-GRX is sufficient for proper growth of *A. thaliana* plants**

424 This study aimed at clarifying and comparing the physiological functions of the two plastidial
425 class-II GRXs, GRXS14 and GRXS16, in *A. thaliana*. In standard conditions, plants lacking a
426 single GRX did not show any obvious phenotype whereas the rosette weight of lines knockout
427 for *GRXS14* and knockdown for *GRXS16* was reduced by more than 20%, indicating that a
428 deficiency in both GRXs impairs growth (Fig. 3). Thus, in physiological conditions, the
429 absence of one GRX is compensated by the other which is consistent with their quite similar
430 protein expression patterns (Fig. 1) and the presence of a regular GRX domain in both. On the
431 other hand, plants overexpressing GRXS14, but not GRXS16, displayed enhanced growth in
432 all environmental conditions tested, except upon high salinity (Figs. 3, S5). Accordingly,
433 overexpression of a fern GRXS14 orthologue in Arabidopsis led to either comparable or
434 better growth in control conditions and also upon heat or arsenic treatments (Sundaram et al.
435 2009; Sundaram and Rathinasabapathi, 2010). On the other hand, no beneficial effect was
436 reported upon overexpression of tomato GRXS16 in Arabidopsis (Guo et al. 2010). There are
437 in fact numerous examples of thiol reductases being required for plant growth or development
438 particularly upon environmental conditions that alter redox homeostasis (Rouhier et al. 2015).
439 This is the case of plastidial TRXs y or methionine sulfoxide reductases B, the deficiency of
440 which results in reduced growth under high light conditions (Laugier et al. 2010; Laugier et
441 al. 2013). Regarding the GRX superfamily, several members, including the two other class-II
442 GRXs (GRXS15 and GRXS17), are important for plant development (Xing and Zachgo,
443 2005; Riondet et al. 2012; Knuesting et al. 2015; Moseler et al. 2015; Yang et al. 2015) or
444 involved in responses to environmental constraints (Laporte et al. 2012). A strongly reduced
445 growth was noticed for plants knockdown for GRXS13 expression, but no change was

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

450

451 **GRXS14 participates in chlorophyll maintenance**

452 GRXS14 and GRXS16 have been proposed to participate in responses to oxidative stress
453 based on data mostly gained either using leaf discs or *in vitro* plantlets (Cheng et al. 2006;
454 Guo et al. 2010) or upon ectopic expression in Arabidopsis (Sundaram et al. 2009; Guo et al.
455 2010). Having noticed the substantial variation in GRXS14 abundance upon light or osmotic
456 constraints (Fig. 1b), our goal was to explore the phenotype of GRXS14-modified lines in
457 these conditions. In comparison with previously published reports, constraints have been
458 mainly applied on soil-grown plants in relatively long-term experiments. When applying
459 conditions generating severe oxidative stress, no susceptibility of lines deficient in GRXS14
460 and/or GRXS16 was observed (Figs. S5, S6). This might appear contradictory to the root
461 growth reduction observed *in vitro* upon H₂O₂ treatment (Cheng et al. 2006), but it is likely
462 that the heterotrophic metabolism *in vitro* results in H₂O₂ responses non representative of
463 what happens in more physiological conditions.

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

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

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

492

493 **What is the molecular role of GRXS14 in chlorophyll maintenance?**

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

496 sulfoxide reductases B exhibit a reduced pigment level in high light conditions (Laugier et al.
497 2010). MSRBs have been proposed to prevent oxidative damage in the system targeting
498 proteins in chlorophyll antennae. In other respects, the stability and/or activity of enzymes
499 involved in tetrapyrrole biogenesis is finely controlled at the post-translational level by thiol-
500 based redox mechanisms (Richter and Grimm, 2013). Indeed, the NADPH-dependent TRX
501 reductase C (NTRC), which has a TRX domain, regulates the redox status of magnesium
502 protoporphyrin IX methyltransferase (Richter et al. 2013). Accordingly, plants deficient in
503 NTRC show pale leaves and impaired growth depending on light and photoperiod conditions
504 (Toivola et al. 2013). So far, a role of GRXs in the redox regulation of tetrapyrrole
505 biosynthesis has not been demonstrated, but proteomic studies in *Chlamydomonas reinhardtii*
506 and *Synechocystis* PCC6803 suggested that several enzymes of this pathway could be prone to
507 glutathionylation, an oxidative post-translational modification quite specifically reversed by
508 GRXs (Michelet et al. 2008; Zaffagnini et al. 2012; Chardonnet et al. 2015). Of note, the
509 senescence process is accompanied by increased ROS formation that could promote
510 glutathionylation (Zimmermann and Zentgraf, 2005). Here, the impaired redox homeostasis in
511 dark-treated leaves is revealed by the increased level of overoxidized 2-Cys PRX (Fig. 6).
512 Most importantly, the GRXS14 redox status varied toward a more oxidized intramolecular
513 disulfide-containing form (Fig. 5) upon dark exposure and upon salt treatment, which also
514 impairs redox homeostasis (Munns and Tester, 2008), arguing for a redox regulatory function.
515 Indeed, the formation of an intramolecular disulfide could constitute a regular intermediate
516 recycling state for class-II GRXs upon deglutathionylation or disulfide bond reduction in
517 target proteins (Zaffagnini et al. 2008).

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

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

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546 **Acknowledgements**

547 We are very grateful to the Groupe de Recherche Appliquée en Phytotechnologie, Patricia
548 Henri and Dr Delphine Cerveau (CEA, DRF, BIAM) for assistance with growth chambers and
549 preparation of plant extracts. We would like also to thank Dr Michel Havaux and Dr
550 Dominique Rumeau (UMR 7265, CEA-CNRS-AMU) for advice regarding photosynthetic
551 measurements and transformation procedures and Dr Olivier Keech (Umea University), Dr.
552 Jérémy Couturier and Prof Jean-Pierre Jacquot (University of Lorraine) for critical reading of
553 the manuscript. This work was supported by the Agence Nationale de la Recherche (ANR),
554 grant no. 2010 BLAN-1616. Moreover, the UMR 1136 is supported by a grant overseen by
555 ANR as part of the "Investissements d'Avenir" program (ANR-11-LABX-0002-01, Lab of
556 Excellence ARBRE).

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

| 747 | <hr/> | |
|-------|-------------|--|
| 748 | Name | Characteristics |
| <hr/> | | |
| 749 | WT | Col-0 |
| 750 | | |
| 751 | K | <i>grxS14</i> , <u>k</u> nockout for <i>GRXS14</i> expression (SALK_125902), |
| 752 | | |
| 753 | O1 (OE-S14) | <i>GRXS14</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background |
| 754 | O2 (OE-S14) | <i>GRXS14</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background |
| 755 | | |
| 756 | KI1 | <i>grxS14</i> <u>k</u> background, knockdown for <i>GRXS16</i> expression, RNA- <u>i</u> nterference |
| 757 | KI2 | <i>grxS14</i> <u>k</u> background, knockdown for <i>GRXS16</i> expression, RNA- <u>i</u> nterference |
| 758 | | |
| 759 | C2 (C-S16) | <i>GRXS16</i> <u>c</u> o-suppression (CaMV-35S promoter) in WT background |
| 760 | C4 (C-S16) | <i>GRXS16</i> <u>c</u> o-suppression (CaMV-35S promoter) in WT background |
| 761 | | |
| 762 | O4 (OE-S16) | <i>GRXS16</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background |
| 763 | O5 (OE-S14) | <i>GRXS16</i> <u>o</u> verexpression (CaMV-35S promoter) in WT background |
| 764 | | |
| 765 | KI1-R1 | <u>KI1</u> background, <i>GRXS14</i> <u>r</u> estoration (CaMV-35S promoter) |
| 766 | KI1-R2 | <u>KI1</u> background, <i>GRXS14</i> <u>r</u> estoration (CaMV-35S promoter) |
| 767 | <hr/> | |

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783 **Legends to figures**784 **Figure 1. Abundance of GRXS14 and GRXS16 proteins in *Arabidopsis thaliana* organs**
785 **and in leaves upon environmental constraints.**

786 **(a)** Western analysis of RubisCO, GRXS14 and GRXS16 abundance in the organs of 6-week-
787 old *Arabidopsis* plants grown in short-day conditions (YL, young leaf less than 1 cm long;
788 AL, adult well-expanded leaf; OL, old non-senescent leaf) and of 6-week-old plants grown in
789 long-day conditions (R, root; St, stem; CL, cauline leaf; FB, flower bud; F, flower; GP, green
790 pod). Seeds (Se) were harvested from plants grown for 11 weeks in long-day conditions. Gels
791 for Western analysis were loaded as in the Coomassie blue-stained gel. **(b)** Western analysis
792 of RubisCO, 2-Cys PRX, GRXS14 and GRXS16 abundance in adult leaves of *Arabidopsis*
793 plants grown for 6 weeks in control conditions (C) or exposed to various light, temperature
794 and osmotic constraints: 450 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ under an 16-h photoperiod (HL-LD, high
795 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
796 light, high temperature), 1400 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$, 6°C, 8-h photoperiod for 1 week (PT,
797 photooxidative conditions), 10°C for 4 weeks (LT, low temperature), watering with 150 mM
798 NaCl for 2 weeks (S, high salinity) and water deficit for 1 week (WD). Light intensity for C,
799 LT, S and WD: 200 $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$. Similar results were obtained when using extracts
800 from two independent experiments.

801

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

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

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

811

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

814 (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
815 O1 plants. (c) Leaf chlorophyll content. (c, d, e) Plants aged of 21 days grown in control
816 conditions and then watered with 0.15 M NaCl for 31 days in the phytotron conditions (8-h
817 photoperiod, 200 $\mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ at 22°C). (d) Rosette weight. (e) Photograph of WT,
818 K, KI1, KI2, O1 and O2 plants. Red arrows indicate young necrotic leaves. (f) Leaf
819 chlorophyll content. KI1 and KI2: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*;
820 WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-S16 C2 and C4, lines
821 co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines overexpressing *GRXS16*.
822 Data in control conditions are means \pm SD of six (rosette weight) and three (chlorophyll)
823 average values originating from independent experiments (at least 5 plants and 5 pigment
824 measurements for each genotype per experiment). In high salt conditions, growth data are
825 means \pm SD of values gained from seven plants per genotype (the experiment was performed
826 twice and led to similar results) and chlorophyll data are means \pm SD from 5 independent
827 measurements. *, ** and ***, significantly different from the WT value with $P < 0.05$, $P <$
828 0.01 and $P < 0.001$, respectively (*t*-test).

830

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

833 (a, b), Chlorophyll content of leaf disks incubated for 2 days on water or 0.15 M NaCl in the
834 light phytotron conditions ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$; 8-h photoperiod) (a) or in the dark (b).
835 Data are means \pm SD of at least eight values gained in two independent experiments (at least 4
836 plants for each genotype per experiment). (c), Chlorophyll content of leaf disks incubated on
837 water in the dark for 3, 4 or 5 days. Means \pm SD of 5 (3 and 4 days) and of 3 (5 days)
838 independent measurements per genotype are presented. (d) Leaves covered with aluminum
839 foil for 8 days on entire 6-week-old plants grown on soil in the light phytotron conditions (8-h
840 photoperiod, $200 \mu\text{moles photons.m}^{-2}.\text{s}^{-1}$). (e) Chlorophyll content in leaves covered with
841 aluminum foil for 8 days. Means \pm SD of 11 independent measurements per genotype. KI2,
842 KI1: lines KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; O1 and O2,
843 lines overexpressing *GRXS14*. *, ** and ***, significantly different from the WT value with P
844 $s < 0.05$, < 0.01 and < 0.001 , respectively (*t*-test).

845

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

848 (a, b) Western analysis of GRXS14 abundance in soluble leaf proteins from control K, WT
849 and O1 plants extracted in non-reducing conditions either non-alkylated (a) or alkylated using
850 mPEG-maleimide-2000 (b). (c, d, e) Western analysis of GRXS14 redox status in soluble leaf
851 proteins from plants exposed to salt (c, d) or high light (d), or from dark-exposed leaves (e)
852 extracted in non-reducing conditions and alkylated using mPEG-maleimide-2000. (f)
853 Proportion of the 18-kDa GRXS14 form (*i.e.* completely reduced form) in extracts from WT
854 plants grown in control, high salt and high light conditions and from WT leaves exposed to
855 dark. Control: plants grown under $200 \mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ (8-h photoperiod) for 6 weeks;
856 Salt: plants watered with 0.15 M NaCl for 1 month; High light: plants grown under 450
857 $\mu\text{moles photons.m}^{-2}.\text{s}^{-1}$ and a long photoperiod (16 h) for 3 weeks. Dark, leaves exposed to

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

862

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

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

876 **Supporting information**

877 **Table S1. List of primers used for PCR analysis and cloning.**

878 **Table S2. List and characteristics of antibodies used for Western blot analyses.**

879

880 **Figure S1. Characterization of Arabidopsis lines modified in the expression of *GRXS16*.**

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

885

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

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

899

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

903 (a) Plants grown for 6 weeks in control light conditions ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 8-h
904 photoperiod) at 22°C. (b), Plants aged of 21 days grown in control conditions and then
905 watered with 0.15 M NaCl for 31 days in the same light conditions. (c) Leaf disks incubated
906 on water, 0.15 M NaCl, 0.15 M KCl or 0.3 M mannitol in control light conditions for 48 h. (d)
907 Leaf disks incubated on water or 0.15 M NaCl in the dark for 48 h. Data in (a, b) are means \pm
908 SD of at least five average values originating from independent plants (at least 5
909 measurements per plant). Data in (c, Water and NaCl) are means \pm SD of six average values
910 originating from independent experiments (10 measurements per genotype per experiment)
911 and in (c, KCl, mannitol) and (d), means \pm SD of at least eight values gained in two
912 independent experiments (at least 4 plants for each genotype per experiment). KI2, KI1: lines
913 KO for *GRXS14* and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2,
914 lines overexpressing *, ** and ***, significantly different from the WT value with $P < 0.05$, P
915 < 0.01 and $P < 0.001$, respectively (t-test). (t-test).

916

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

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

925 *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing *GRXS14*; C-
926 S16 C2 and C4, lines co-suppressed for *GRXS16* expression; OE-S16 O4 and O5, lines
927 overexpressing *GRXS16*. Band intensity was quantified using the Odyssey software (A.U.,
928 arbitrary unit). *, ** and ***, significantly different from the WT value with $P < 0.05$, $P <$
929 0.01 and $P < 0.001$, respectively (*t*-test).

930

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

933 **(a)** Growth in moderate photooxidative stress conditions. Plants were grown from sowing for
934 3 weeks in high light conditions ($450 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and long photoperiod (16 h) at
935 22°C . **(a)** Rosette weight. **(b)** Leaf chlorophyll content. **(c,d)** Response to acute
936 photooxidative stress conditions. **(c)** Five-week old plants exposed to high light (1400
937 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) at low temperature (6°C) for 11 days. **(d)** Maximal photochemical efficiency of
938 photosystem II (F_v/F_m) of leaf disks incubated on $2 \mu\text{M}$ methyl viologen for 48 h in the
939 phytotron ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$; 8-h photoperiod). KI1 and KI2: lines KO for *GRXS14*
940 and RNAi for *GRXS16*; K, *grxS14*; WT, wild type; OE-S14 O1 and O2, lines overexpressing
941 *GRXS14*; C-S16 C2, line co-suppressed for *GRXS16* expression; OE-S16 O4, line
942 overexpressing *GRXS16*. Data are means \pm SD of six (rosette weight) and three (other data)
943 average values originating from independent experiments (at least 5 plants and 3
944 measurements for each genotype per experiment). *, ** and ***, significantly different from
945 the WT value with $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (*t*-test).

946

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

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

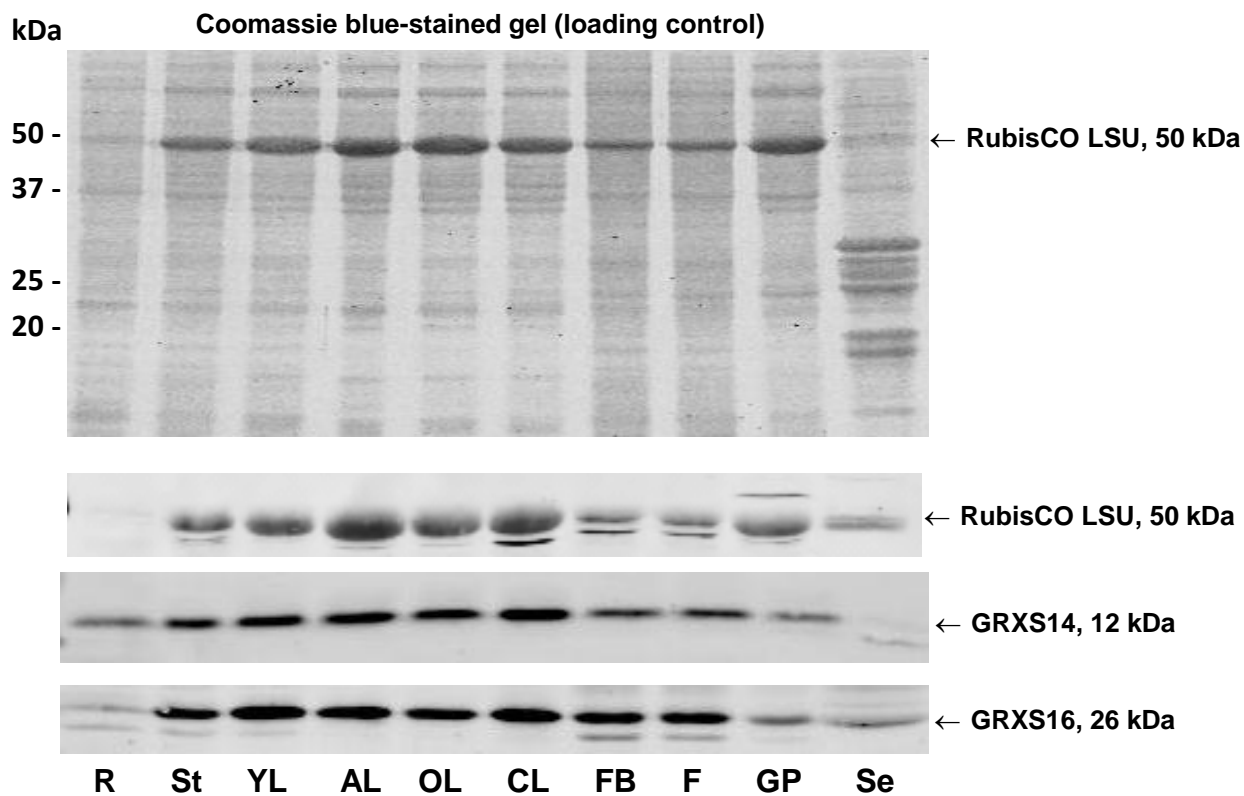
961

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

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

Figure 1

(a)



(b)

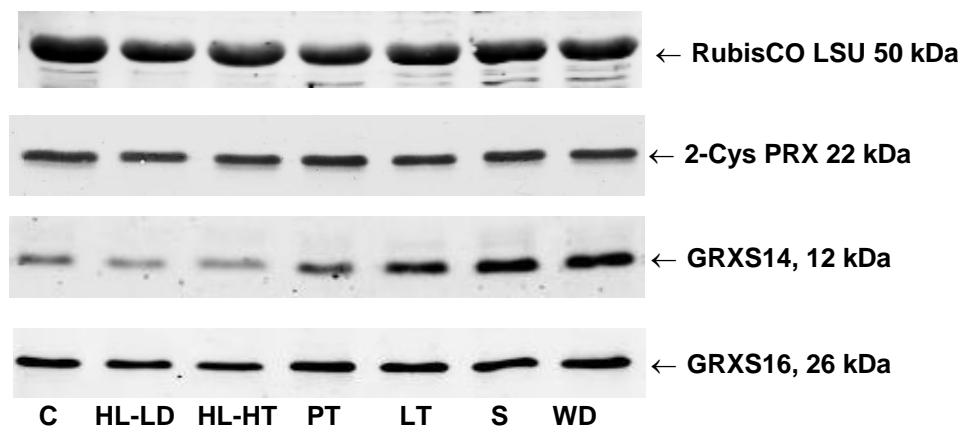
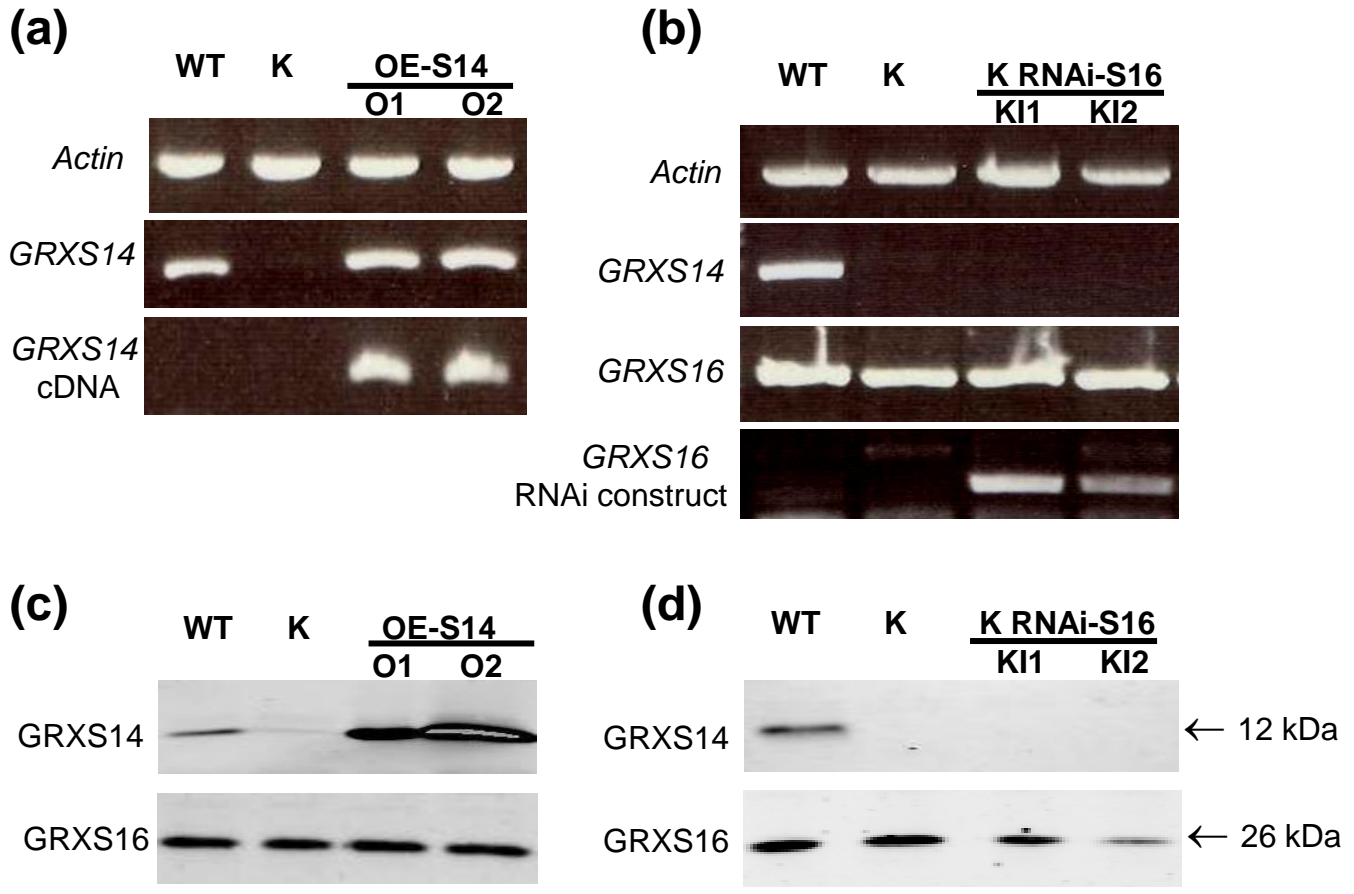
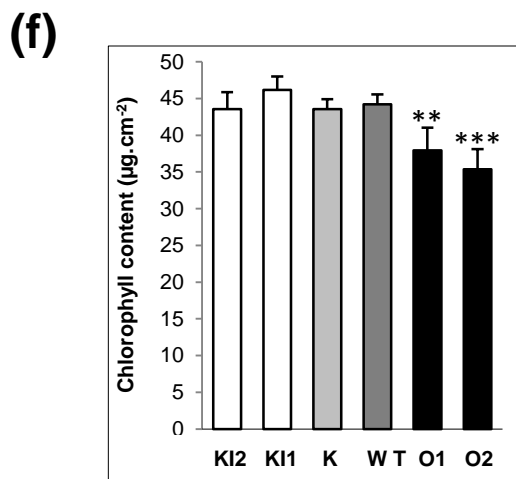
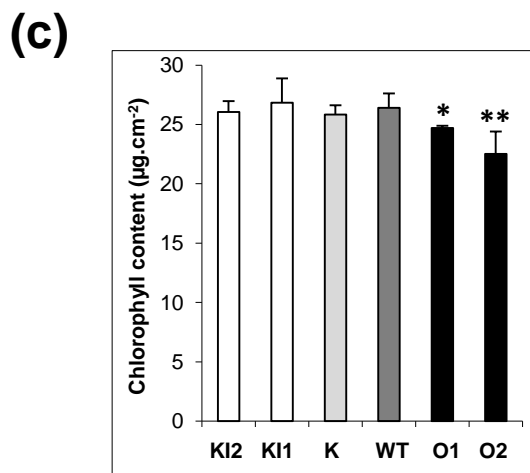
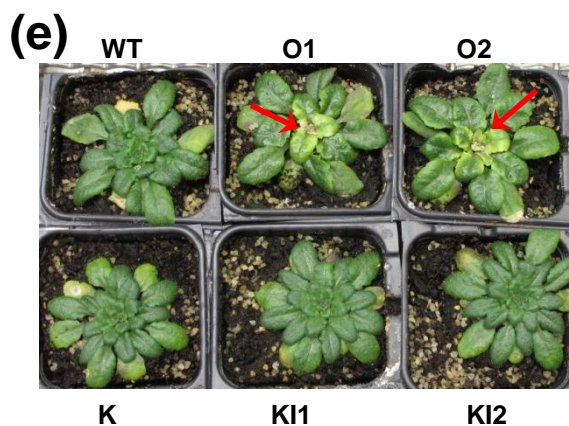
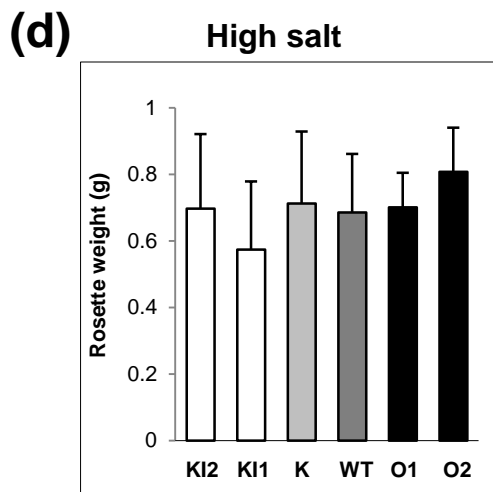
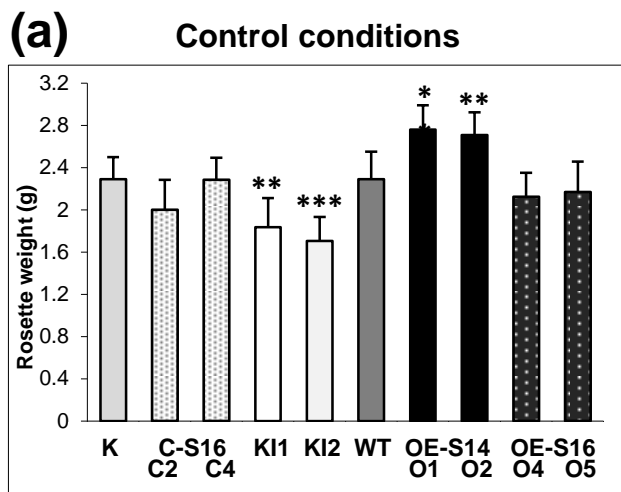
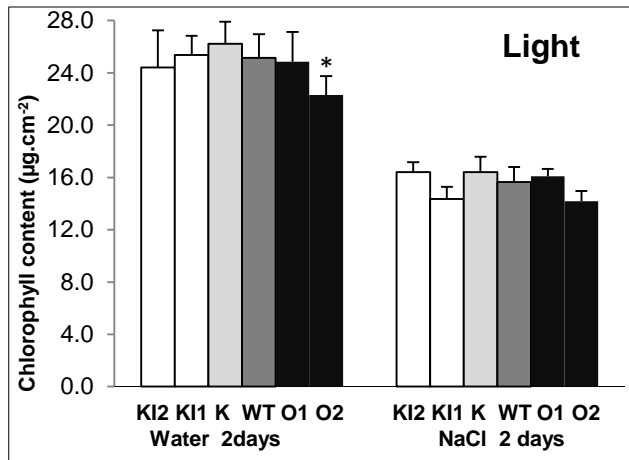


Figure 2

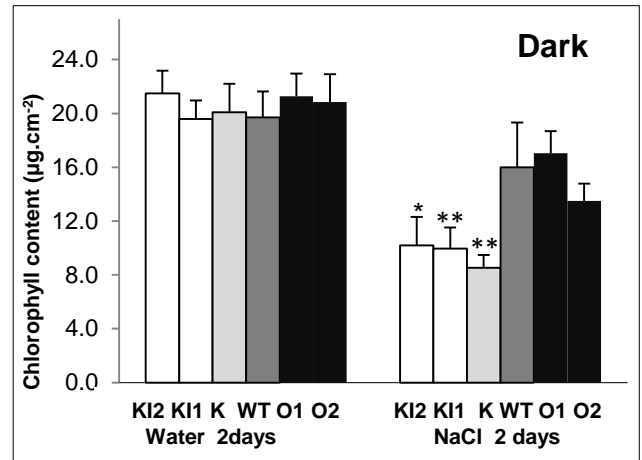




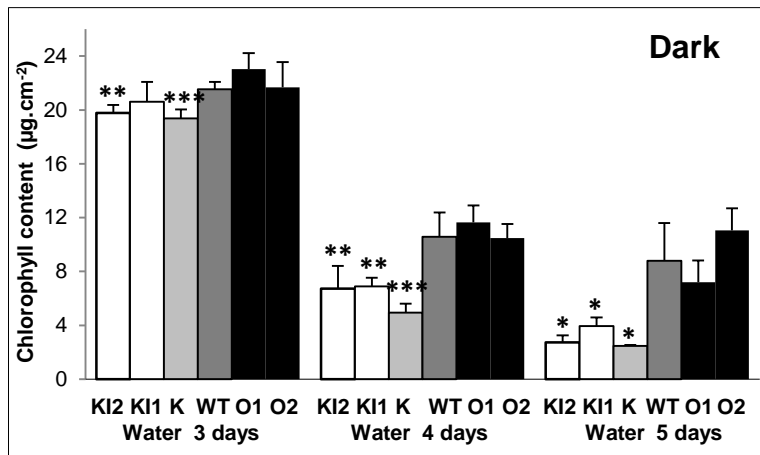
(a)



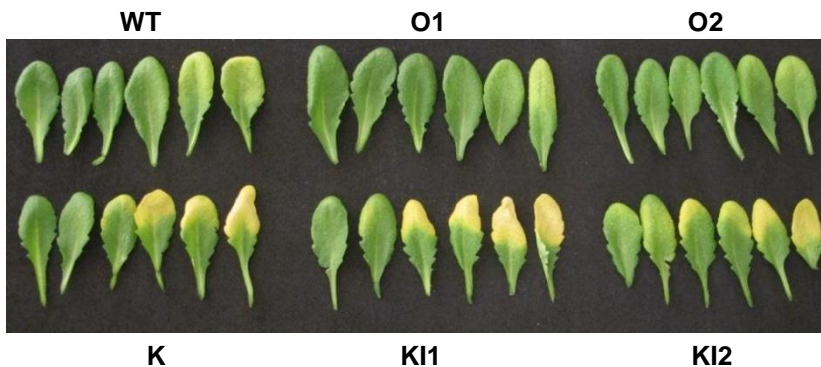
(b)



(c)



(d)



(e)

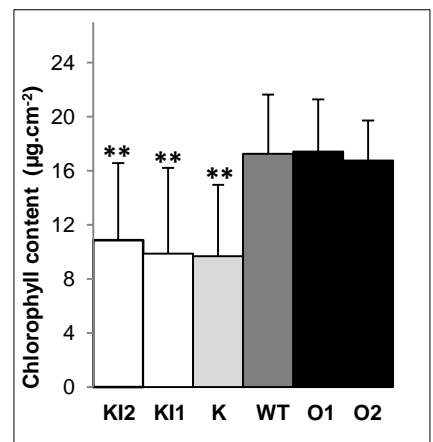


Figure 5

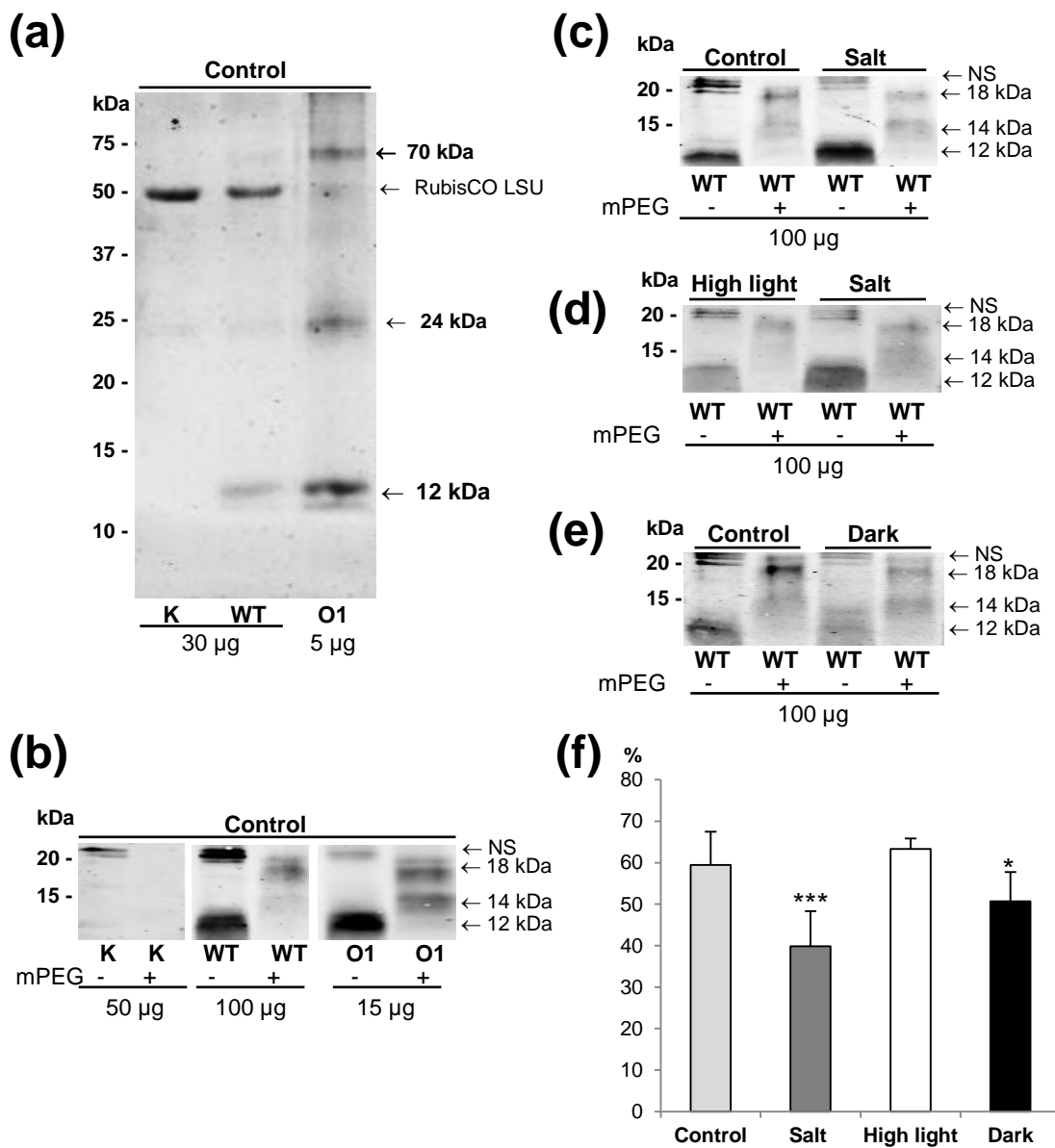
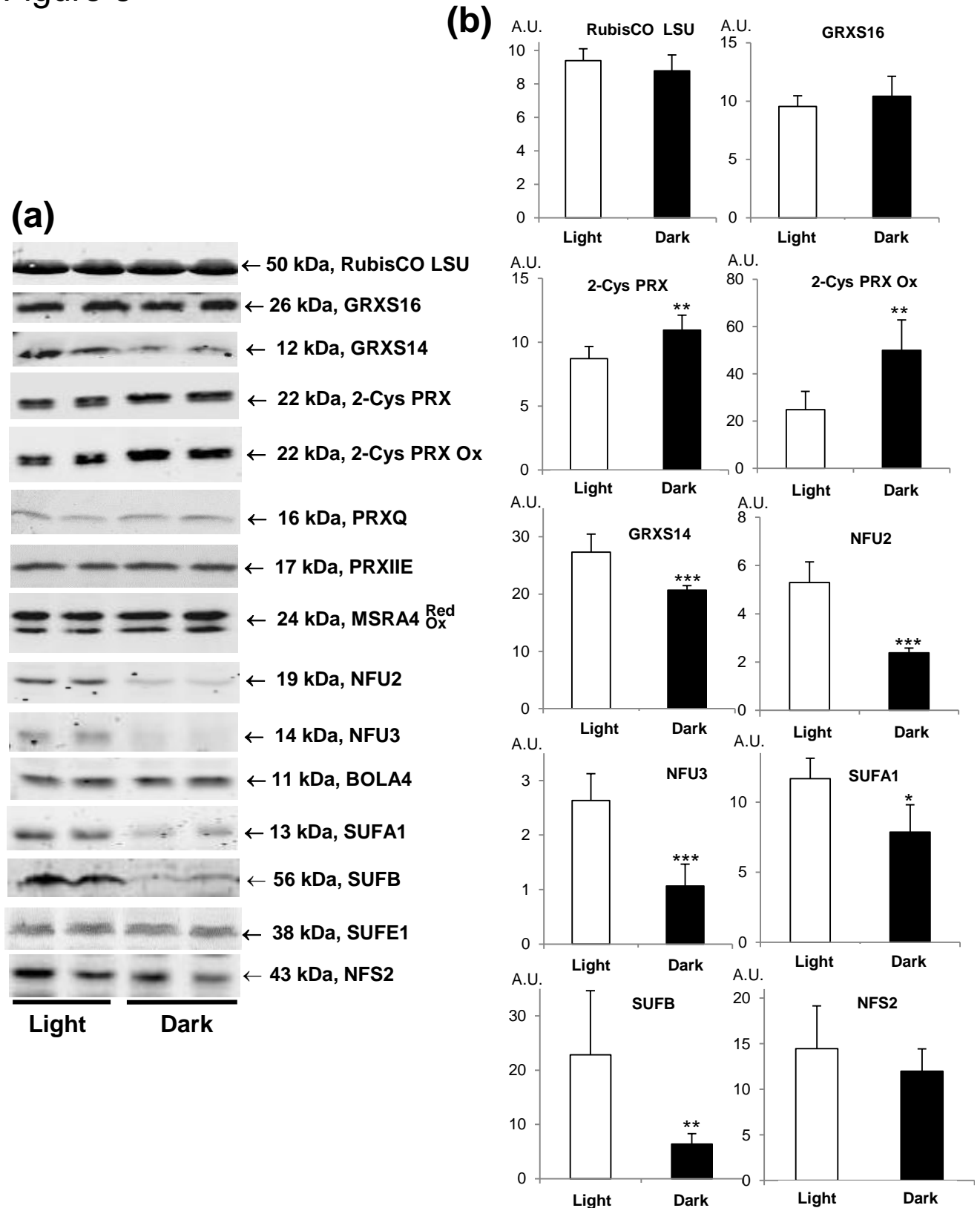


Figure 6



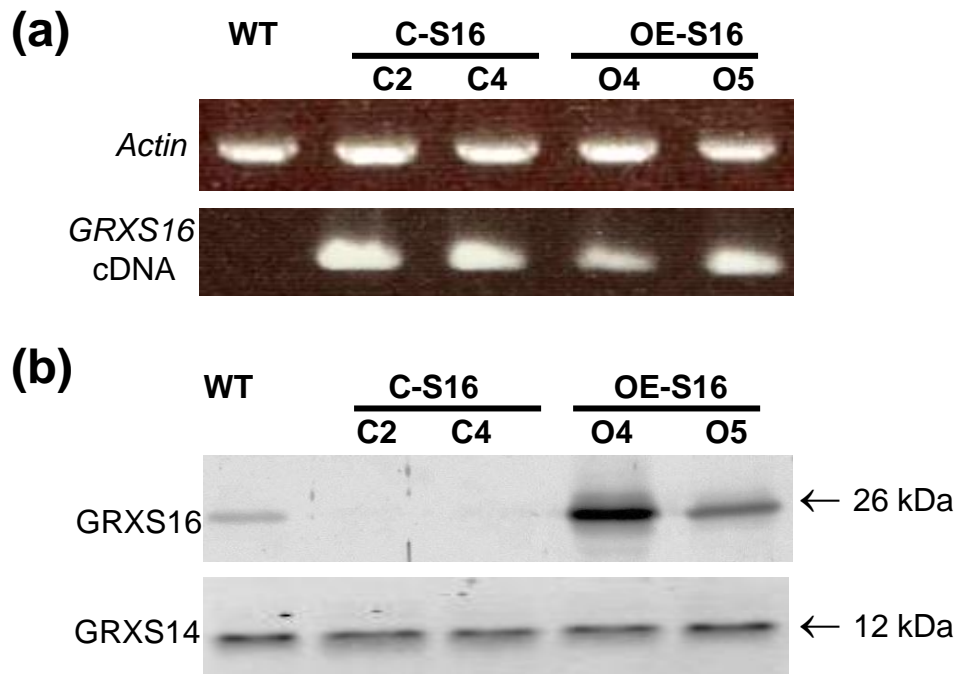


Figure S1. Characterization of Arabidopsis lines modified in the expression of *GRXS16*.

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*.

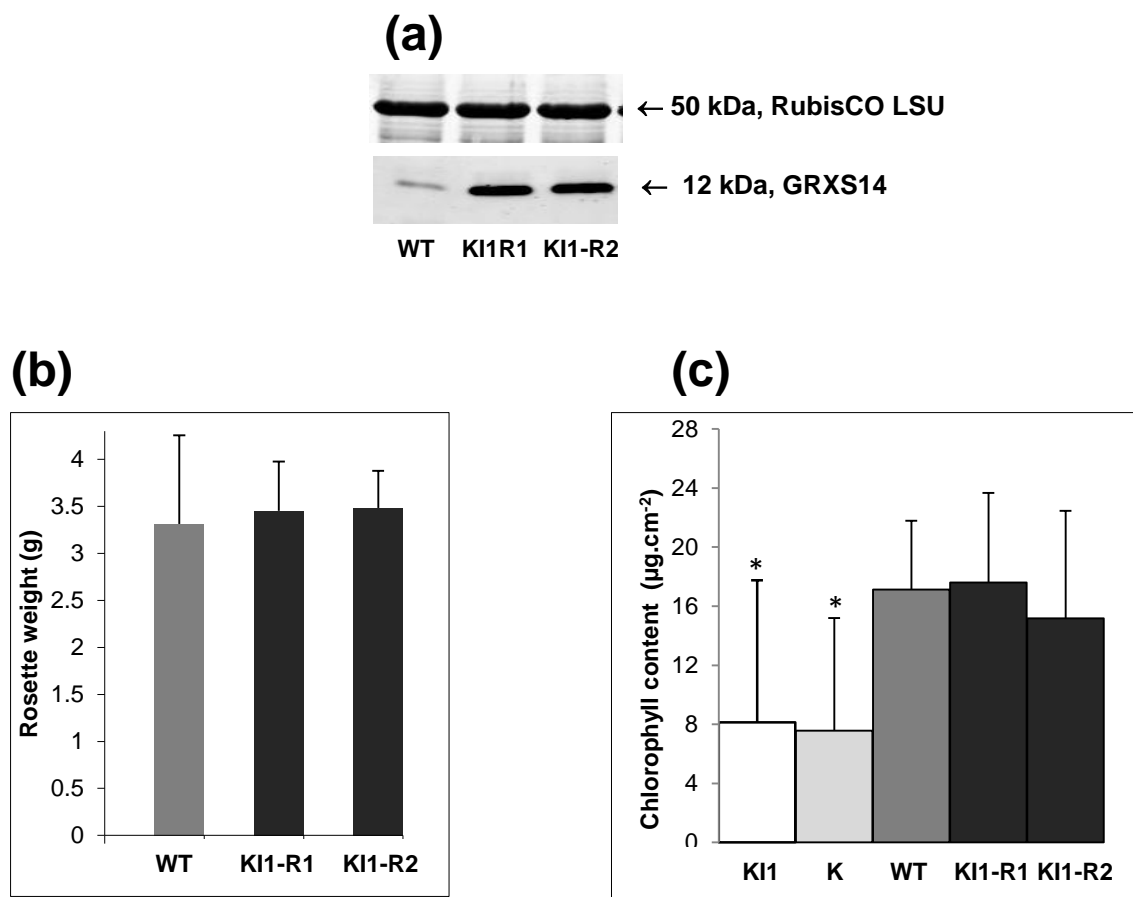


Figure S2. Phenotype of Arabidopsis plants knockout for *GRX14*, knockdown for *GRX16* and restored with *GRXS14* expression.

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).

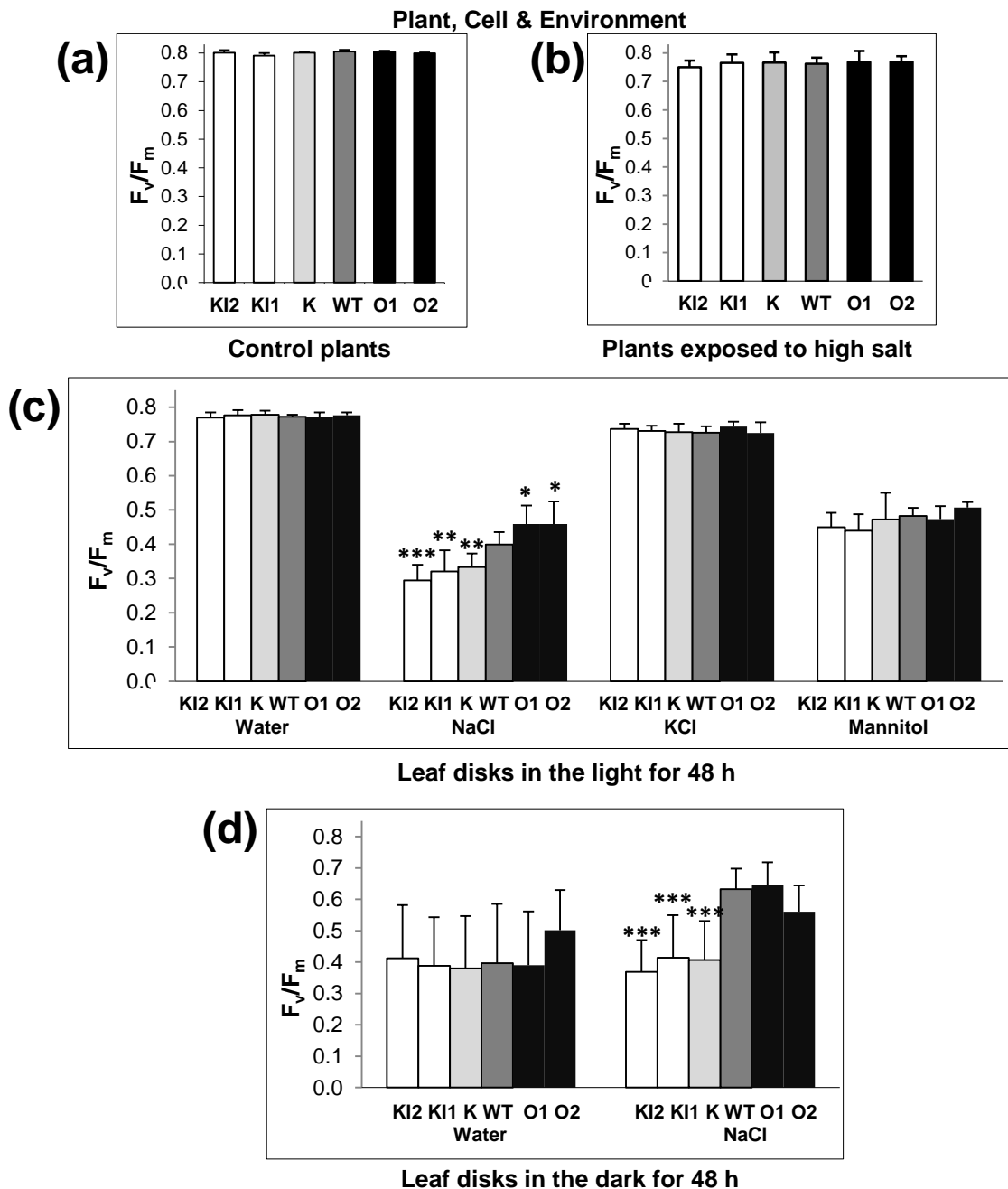


Figure S3. Maximal photochemical efficiency of photosystem II (F_v/F_m) in plants modified for the expression of *GRXS14* and/or *GRXS16* in various environmental conditions.

(a) Plants grown for 6 weeks in control light conditions ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 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).

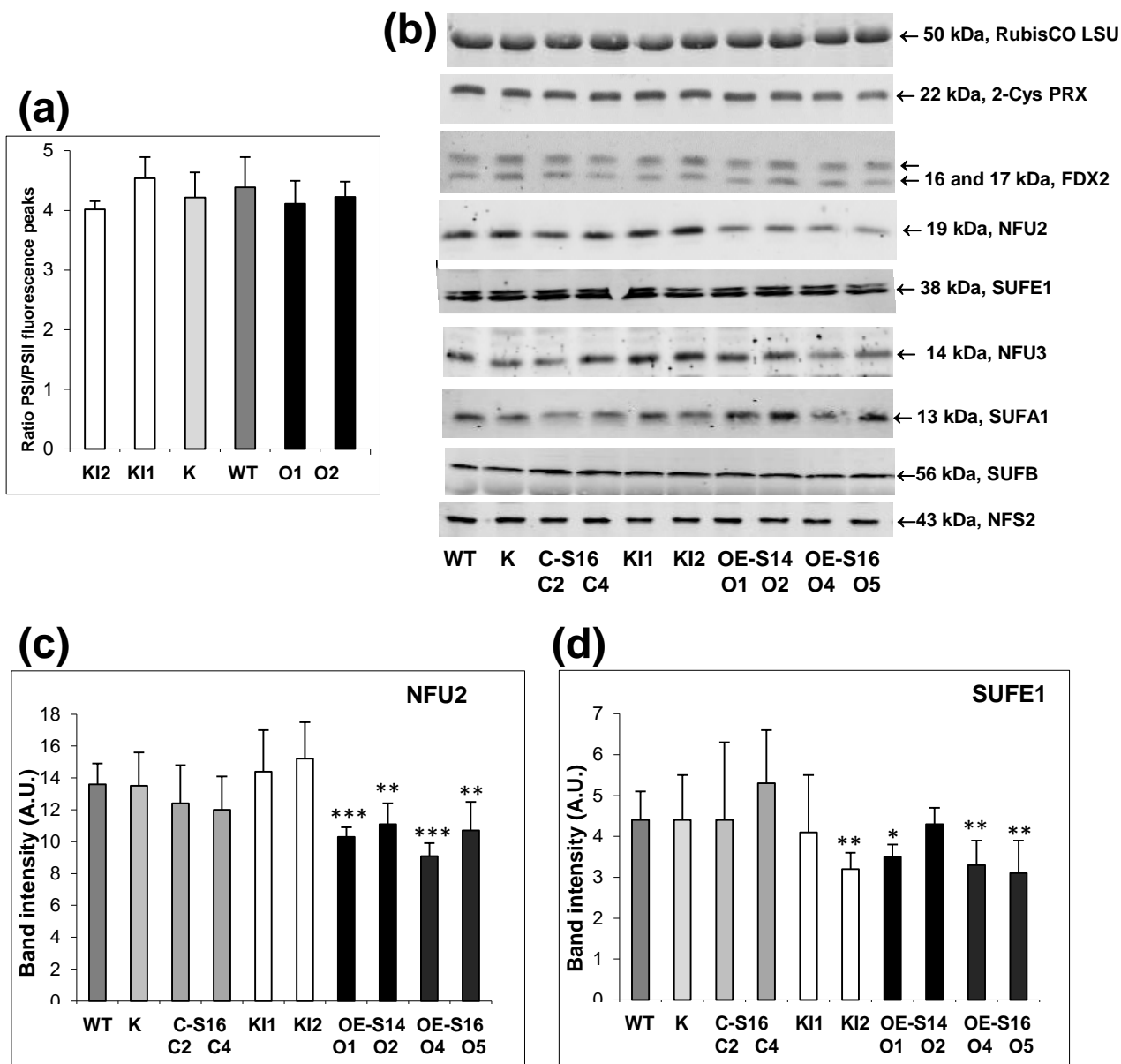


Figure S4. Characteristics related to plastidial Fe-S proteins in plants modified in the expression of *GRXS14* and/or *GRXS16*.

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).

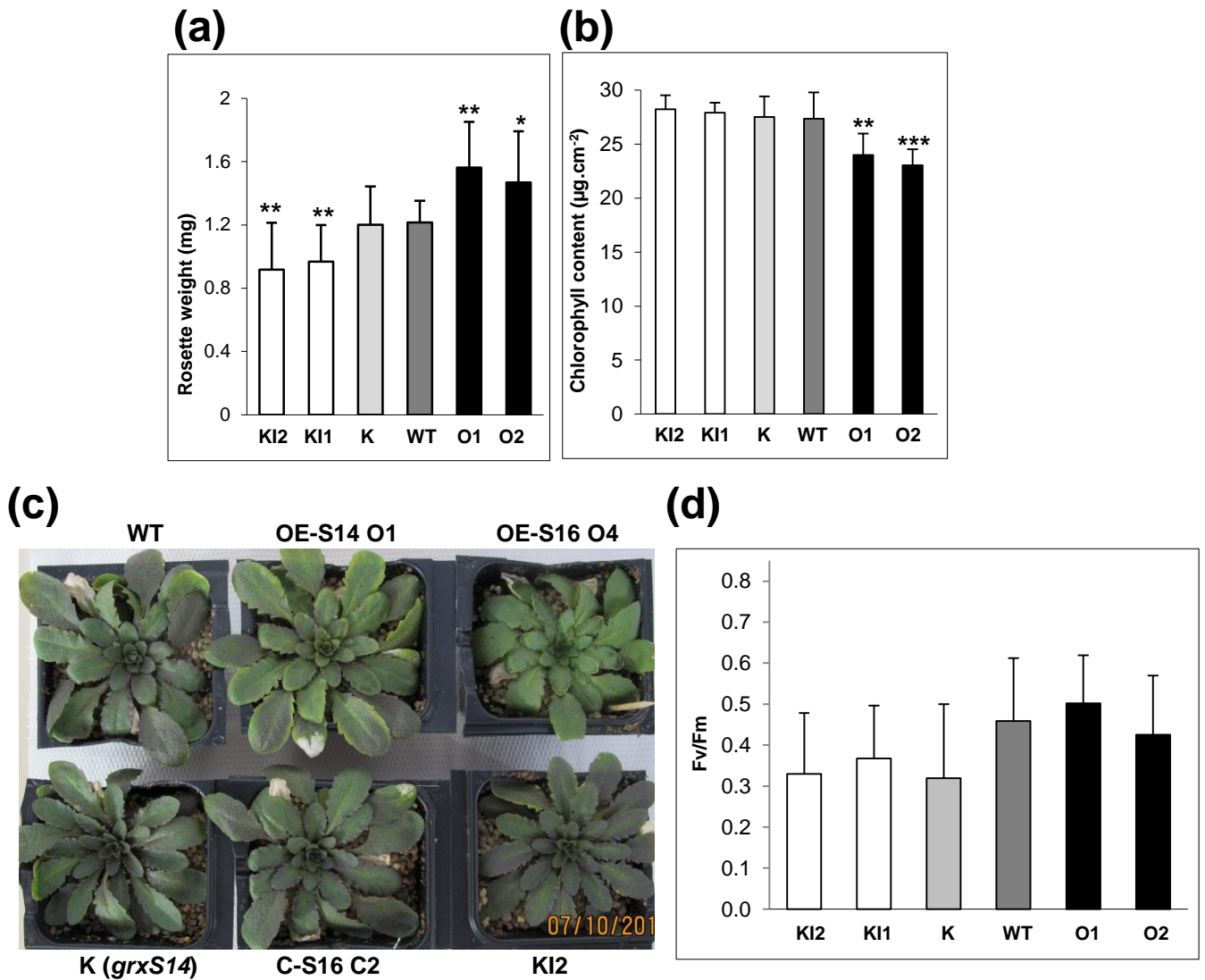


Figure S5. Responses to photooxidative conditions of Arabidopsis lines modified in the expression of *GRXS14* and/or *GRXS16*.

(a) Growth in moderate photooxidative stress conditions. Plants were grown from sowing for 3 weeks in high light conditions ($450 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) 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 ($1400 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at low temperature (6°C) for 11 days. (d) Maximal photochemical efficiency of photosystem II (F_v/F_m) of leaf disks incubated on $2 \mu\text{M}$ methyl viologen for 48 h in the phytotron ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; 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).

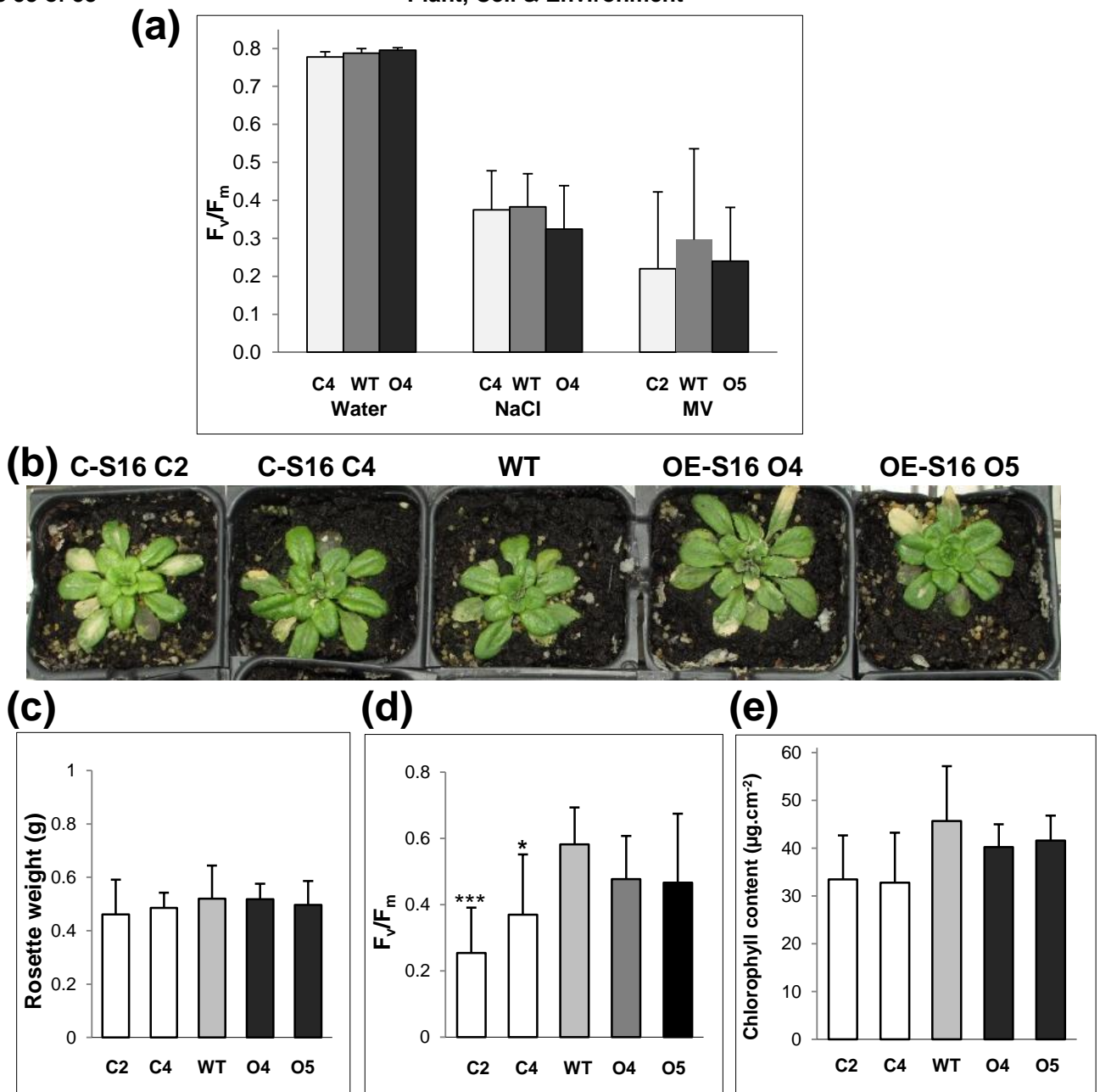


Figure S6. Responses of Arabidopsis plants modified in the expression of *GRXS16* to osmotic and oxidative constraints.

(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).

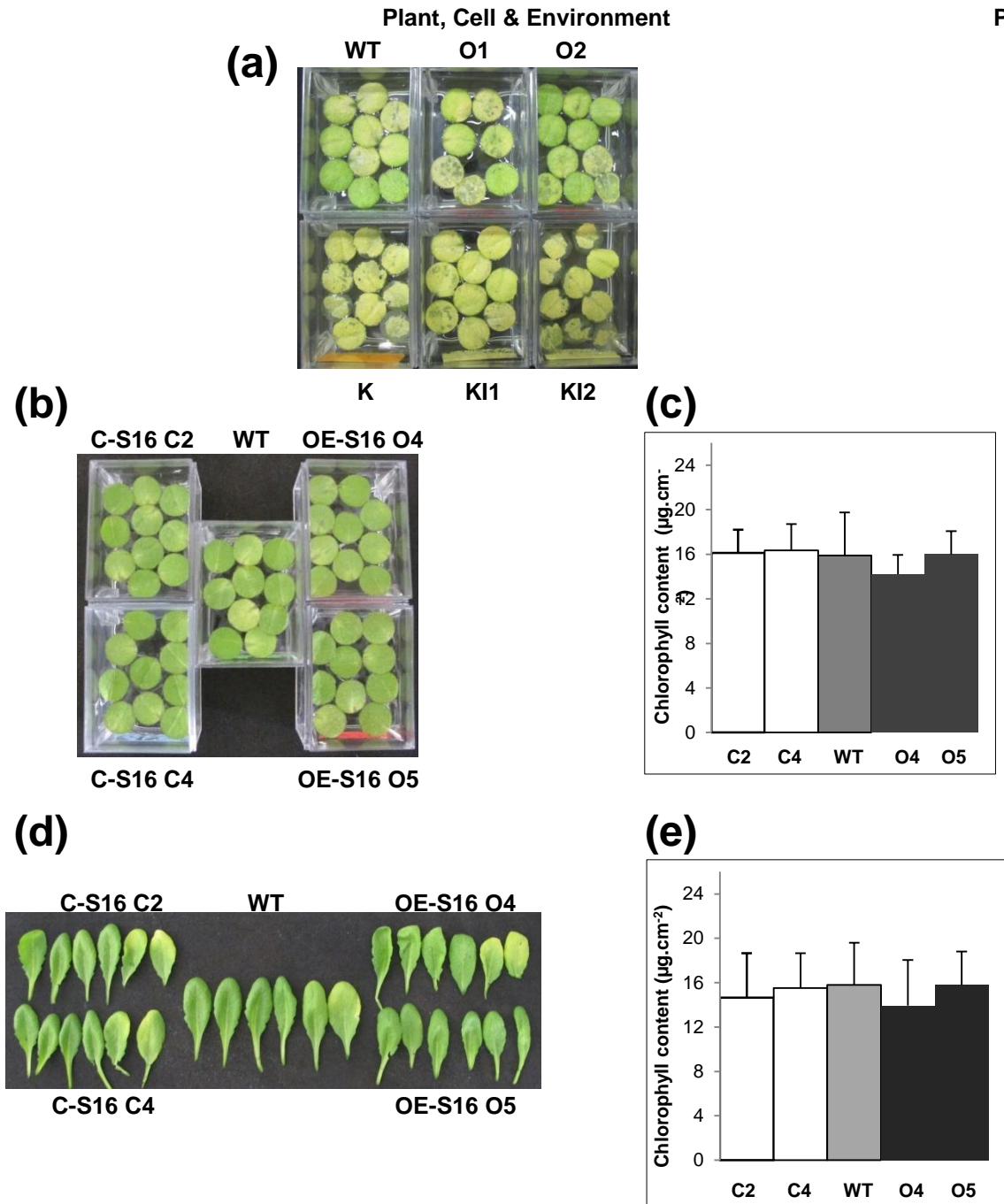


Figure S7. Effect of exposure to dark on the chlorophyll content of *Arabidopsis* plants modified for *GRXS14* and/or *GRXS16* expression.

(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*.