

1 **Arabidopsis glutathione reductase 2 is indispensable in**
2 **plastids, while mitochondrial glutathione is safeguarded by**
3 **additional reduction and transport systems**

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29

30 **Summary**

31 • A highly negative glutathione redox potential (E_{GSH}) is maintained in the cytosol, plastids and
32 mitochondria of plant cells to support fundamental processes, including antioxidant defence,
33 redox regulation and iron-sulfur cluster biogenesis. Out of two glutathione reductase (GR)
34 proteins in *Arabidopsis*, GR2 is predicted to be dual-targeted to plastids and mitochondria, but
35 its differential roles in these organelles remain unclear.

36 • We dissected the role of GR2 in organelle glutathione redox homeostasis and plant
37 development using a combination of genetic complementation and stacked mutants,
38 biochemical activity studies, immunogold labelling and *in vivo* biosensing.

39 • Our data demonstrate that GR2 is dual-targeted to plastids and mitochondria, but embryo
40 lethality of *gr2* null mutants is caused specifically in plastids. Whereas lack of mitochondrial
41 GR2 leads to a partially oxidised glutathione pool in the matrix, the ABC transporter ATM3 and
42 the mitochondrial thioredoxin system provide functional backup and maintain plant viability.

43 • We identify GR2 as essential in the plastid stroma, where it counters GSSG accumulation and
44 developmental arrest. By contrast a functional triad of GR2, ATM3 and the thioredoxin system
45 in the mitochondria provides resilience to excessive glutathione oxidation.

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47

48 **Key words**

49 ABCB25, dual-targeting, embryo lethality, glutathione redox status, glutathione reductase 2,
50 mitochondria, NTR, redox-sensitive GFP

51

52 Introduction

53 The use of oxygen by aerobic organisms allows them to obtain more energy from carbohydrates,
54 by accessing a larger reduction potential difference. Other metabolic reactions in the cell,
55 however, also lead to reduction of oxygen and give rise to reactive oxygen species (ROS), such as
56 superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals. Low levels of ROS have been
57 implicated in signalling between subcellular compartments as well as long-distance signalling
58 regulating plant development and metabolism (Foyer & Noctor, 2009; Gilroy *et al.*, 2016).
59 However, at higher concentrations ROS may oxidize lipids, proteins and DNA and render these
60 molecules non-functional. Therefore, ROS constitute a major threat to the cell and need to be
61 tightly controlled. Cellular compartments that can be particularly affected by oxidative stress
62 include the plastids, mitochondria and peroxisomes (Mittler *et al.*, 2004). In chloroplasts, the
63 Mehler reaction and antenna pigments are major sources of O_2^- formation (Asada, 1999). In
64 mitochondria, over-reduction of the electron transport chain results in O_2^- production at
65 complexes I and III (Moller, 2001). In both compartments, O_2^- quickly dismutates to H_2O_2 and O_2
66 in a reaction catalysed by superoxide dismutase (SOD).

67 In plants, H_2O_2 produced by SODs is further reduced to water by peroxidases such as ascorbate
68 peroxidases (APXs), glutathione peroxidase-like enzymes (GPXLs) and peroxiredoxins (PRXs).
69 While GPXLs and PRXs are dependent on thioredoxins (TRXs) as primary electron donor
70 (Finkemeier *et al.*, 2005; Navrot *et al.*, 2006; Attacha *et al.*, 2017), APXs use electrons from
71 ascorbate. The resulting dehydroascorbate is recycled through the ascorbate–glutathione cycle,
72 which links H_2O_2 detoxification to the redox dynamics of the glutathione redox couple.

73 Glutathione is the most abundant low-molecular weight thiol-redox buffer in all eukaryotic
74 organisms and most Gram-negative bacteria, including cyanobacteria and purple bacteria, and is
75 present at millimolar concentrations (Fahey, 2001; Meyer *et al.*, 2001). After synthesis in plastids
76 and the cytosol (Pasternak *et al.*, 2008; Maughan *et al.*, 2010), reduced glutathione (GSH) is
77 transported throughout the cell to fulfil a broad range of functions in metabolism and
78 detoxification of xenobiotics and H_2O_2 (Meyer, 2008). Upon oxidation, two molecules of GSH
79 convert to glutathione disulfide (GSSG). In the cytosol, mitochondria and plastids the glutathione
80 redox potential (E_{GSH}) is highly reduced with only nanomolar concentrations of GSSG present

81 under non-stress conditions (Meyer *et al.*, 2007; Schwarzländer *et al.*, 2008). Such a high
82 GSH/GSSG ratio is believed to be maintained by NADPH-dependent glutathione reductases (GRs).
83 In contrast to bacteria, animals and yeast, plant genomes encode two GRs (Xu *et al.*, 2013). In
84 Arabidopsis, GR1 is present in the cytosol, the nucleus and peroxisomes (Reumann *et al.*, 2007;
85 Marty *et al.*, 2009; Delorme-Hinoux *et al.*, 2016). The second isoform, GR2, is dual-targeted to
86 mitochondria and plastids (Creissen *et al.*, 1995; Chew *et al.*, 2003), as supported by proteomics
87 analyses of purified organelles (Ito *et al.*, 2006; Peltier *et al.*, 2006). However, Yu and colleagues
88 found no evidence for mitochondrial targeting of full-length GR2-YFP constructs and concluded
89 exclusive plastidic localization (Yu *et al.*, 2013). While we found that Arabidopsis mutants lacking
90 cytosolic GR1 are fully viable (Marty *et al.*, 2009), a deletion mutant lacking functional GR2 is
91 embryo lethal (Tzafrir *et al.*, 2004; Bryant *et al.*, 2011). These observations raise important
92 questions about the exact localization of GR2, the cause of lethality in *gr2* mutants, and about
93 the mechanisms maintaining a highly negative glutathione redox potential (E_{GSH}) in plastids and
94 mitochondria.

95 Viability of Arabidopsis mutants lacking cytosolic GR1 is maintained by the NADPH-dependent
96 TRX system (NTS) (Marty *et al.*, 2009). In this case, electrons supplied by NADPH are transferred
97 to GSSG via NADPH-dependent TRX reductase (NTR) and TRX. Arabidopsis contains two NTRs,
98 NTRA and NTRB, which are both targeted to the cytosol, the nucleus and mitochondria (Reichheld
99 *et al.*, 2005; Marchal *et al.*, 2014). In addition, plastids contain a remotely related bifunctional
100 NTR, NTRC, which contains its own TRX domain (Serrato *et al.*, 2004). NTRC has been shown to
101 be responsible for transferring electrons from NADPH to 2-Cys PRX for H_2O_2 detoxification (Perez-
102 Ruiz *et al.*, 2017) and for redox regulation of ADP-glucose pyrophosphorylase (Michalska *et al.*,
103 2009). While NTRC may be active in the dark and in heterotrophic tissues, classical non-fused
104 plastidic TRXs are reduced by electrons from the photosynthetic electron transport chain via
105 ferredoxin-dependent TRX reductase (FTR) which implies that they exercise their reductive
106 capacity only in the light (Buchanan & Balmer, 2005). Whether and to what extent the different
107 subcellular TRX systems contribute to organellar glutathione redox homeostasis is yet unknown.
108 Beyond maintenance of a highly negative E_{GSH} through continuous reduction of GSSG, ATP-driven
109 sequestration of GSSG to the vacuole by ATP-binding cassette (ABC)-transporters has been

110 shown to provide an overflow valve for high cytosolic GSSG amounts in yeast (Morgan *et al.*,
111 2013). Similarly, the ABC-transporter of the mitochondria Atm1 in yeast and its functional
112 homologue ATM3 in Arabidopsis (systematic name ABCB25) transport GSSG, but not GSH, driven
113 by ATP hydrolysis (Schaeidler *et al.*, 2014). The ATPase domain of Atm1 is orientated towards the
114 mitochondrial matrix (Leighton & Schatz, 1995), indicating that these proteins export GSSG out
115 of the mitochondria. In support of this, Arabidopsis *atm3* mutants were shown to have a more
116 oxidized E_{GSH} in the mitochondrial matrix compared to wild type (Schaeidler *et al* 2014). However,
117 the primary substrate of Atm1/ATM3 is thought to be GSH-bound persulfide for the biosynthesis
118 of cytosolic iron-sulfur clusters (Kispal *et al.*, 1999; Srinivasan *et al.*, 2014). This raises the question
119 of the ATM3's relative contribution to other systems in GSSG clearance of the mitochondrial
120 matrix.

121 Here, we address the role of GR2 in glutathione redox homeostasis in mitochondria and plastids.
122 We localize GR2 by immunogold labelling and complement the lethal *gr2* null mutant in a
123 compartment-specific manner. Those analyses provide clear evidence for dual localization of GR2
124 and for an essential role in plastids only. To address the question of why mitochondria are less
125 affected than plastids by the lack of GR2, we focussed our attention on other possible
126 mechanisms involved in the maintaining the E_{GSH} in the matrix. Using a series of physiological and
127 genetic analyses we identify the involvement of alternative GSSG reduction systems and of GSSG
128 export in E_{GSH} maintenance in the mitochondrial matrix.

129

130

131 **Materials and Methods**

132 The following procedures are described in **Supporting Information Methods S1 and S2**:
133 Antibody production and gel blot analysis; Immunogold labelling and electron microscopy.
134 Primers used in this work are given in **Supporting Information Table S1**.

135

136 **Plant material and growth conditions**

137 The study was conducted with *Arabidopsis thaliana* ecotype Columbia-0 ([L.] Heynh.) as the wild-
138 type (WT) control and the mutants *gr2-1* (SALK_040170, (Alonso *et al.*, 2003), *emb2360-1* (Tzafrir

139 *et al.*, 2004), *ntra ntrb* (Reichheld *et al.*, 2007), *rml1* (Vernoux *et al.*, 2000) and *gsh1-1* (Cairns *et*
140 *al.*, 2006), *gr1-1* (Marty *et al.*, 2009), and *atm3-4* (Bernard *et al.*, 2009), all generated in the Col-
141 0 background. Plants were grown under short day conditions: 8 h light at 22 °C and 16 h dark at
142 19 °C and light intensity was set to 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. To induce flowering, plants were
143 transferred to long day conditions: 16 h light at 22 °C and 8 h dark at 19 °C, light intensity was
144 160 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

145 To genotype WT and mutant plants, genomic DNA was extracted from the leaf tissue according
146 to Edwards *et al.* (1991).

147 Seeds produced by a double heterozygous *gr2-1 rml1* plant were plated on phytigel as described
148 before (Meyer & Fricker, 2000). For rescue experiments, the growth medium was supplemented
149 with filter-sterilized GSH (Sigma-Aldrich) to a final concentration of 1 mM before gelling.
150 Seedlings exhibiting a characteristic *rml1* phenotype after germination were transferred to GSH
151 plates and further development was monitored for 12 d.

152

153 **Cloning and plant transformation**

154 Standard molecular biology technologies like growth of bacteria, plasmid isolation and
155 polymerase chain reaction (PCR) were applied according to (Sambrook *et al.*, 1989). For cloning,
156 all DNA fragments were amplified by PCR and blunt-end subcloned into pCAP (Roche Applied
157 Science, Mannheim, Germany) with primers listed in Table S1. Accuracy of the cloned fragment
158 was verified by sequencing by SeqLab (Göttingen, Germany). Different signal peptides were
159 ligated into the pBinAR vector (Höfgen & Willmitzer, 1990) to achieve compartment-specific
160 complementation. The transketolase targeting peptide (TK_{TP}) sequence was cloned into pBinAR
161 as described before (Wirtz & Hell, 2003). Similarly, the serine hydroxymethyltransferase target
162 peptide (SHMT_{TP}) was amplified and cloned behind the 35S promoter of pBinAR using *Kpn*I and
163 *Bam*HI. To verify targeting, both signal peptides were also fused to the N-terminus of roGFP. For
164 compartment-specific complementation the GR2 sequence without its endogenous target
165 peptide of 77 amino acids (aa) consistently determined by ChloroP 1.1
166 (<http://www.cbs.dtu.dk/services/ChloroP/>) and SignalP 3.0
167 (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen *et al.*, 2004) was amplified from cDNA.

168 Since the *gr2-1* mutants carried kanamycin resistance the constructs were transferred to the
169 Basta® resistance vector pBarA (a gift from Sabine Zachgo) using *Hind*III and *Eco*RI restriction
170 sites. For constructs with the endogenous GR2 promoter, 2.1 kb were amplified from genomic
171 DNA and used to replace the 35S promoter in pBinAR with *Eco*RI and *Kpn*I.
172 Plant transformation was carried out by floral dip (Clough and Bent, 1998). For selection of
173 positive transformants two-week-old plants were sprayed with Basta® (200 mg l⁻¹ glufosinate
174 ammonium, Bayer Crop Science).
175 Arabidopsis plants were transformed with the vector pBinAR-SHMT-roGFP2-Grx1 as described
176 earlier (Albrecht *et al.*, 2014). For both WT and plastid-complemented *gr2* mutants, lines with
177 incomplete mitochondrial targeting of roGFP2-Grx1 were selected.

178

179 **Protein purification and mitochondrial isolation**

180 For recombinant protein expression, the pET28a and pETG10a constructs were transformed in
181 *E. coli* HMS174 cells. Cells were grown at 37 °C to an OD600 nm of 0.8 in selective media
182 containing 50 µg ml⁻¹ kanamycin for pET28a or 100 µg ml⁻¹ ampicillin for pETG10a. Protein
183 purification was performed as reported before (Marty *et al.*, 2009). Mitochondria were isolated
184 from 13- to 16-day-old hydroponic Arabidopsis seedling cultures as described previously
185 (Sweetlove *et al.*, 2007).

186

187 **HPLC measurement of low-molecular weight thiols**

188 Leaf material was harvested from six-week-old plants grown on soil under short-day conditions.
189 Leaf tissue was snap frozen in liquid nitrogen, ground to fine powder and extracted. Extraction,
190 derivatisation and quantification of low-molecular weight thiols by HPLC were done as described
191 before (Meyer *et al.*, 2007).

192

193 **Analysis of embryo development and pollen viability**

194 Embryos, ovules or whole siliques were destained with Hoyer's solution (7.5 g gum arabic, 100 g
195 chloral hydrate, 5 ml glycerol, 60 ml water) for at least 4 h. The embryos were analysed by
196 differential interference contrast (DIC) microscopy with a Leica DM/RB microscope equipped

197 with a DFC 320 camera (Leica Microsystems). Pollen from mature flowers were stained according
198 to Alexander (1969) and analysed by bright field microscopy.

199

200 **Chlorophyll fluorescence measurement**

201 Chlorophyll fluorescence was recorded after dark adaptation for at least 30 min with a pulse-
202 amplitude modulated (PAM) fluorimeter (Junior PAM, Walz, Effeltrich, Germany). F_v/F_m was
203 calculated as a measure of the maximum potential quantum yield of photosystem II.

204

205 **Confocal laser scanning microscopy**

206 Whole leaves of 3-week old *Arabidopsis* plants were placed in water on a slide and covered with
207 a coverslip. Leaves were imaged on Zeiss confocal microscopes LSM510META or LSM780 (Carl
208 Zeiss MicroImaging, Jena, Germany) equipped with lasers for 405- and 488-nm excitation. Images
209 were collected with either a 25x lens (Plan-Neofluar 25x/0.8 Imm corr, Zeiss) or a 63x lens (C
210 Apochromat 63x/1.2 W corr, Zeiss). For localization studies GFP was excited at 488 nm and
211 emission was collected with a 505-530 nm band-pass filter. Chlorophyll autofluorescence was
212 excited at 405 nm and recorded above 630 nm. For counterstaining mitochondria leaf tissue was
213 incubated with 1 μ M tetramethylrhodamine methyl ester (TMRM) for 30-60 min. TMRM was
214 excited at 543 nm and recorded at 582-646 nm. For glutathione labelling in roots of *Arabidopsis*
215 seedlings intact seedlings were incubated with 100 μ M monochlorobimane (MCB) and 50 μ M
216 propidium iodide (PI) for 30 min and imaged as reported earlier (Meyer *et al.*, 2001). For
217 ratiometric imaging of E_{GSH} roGFP2-Grx1 was excited in multi-track mode with line switching
218 between 488 nm illumination and 405 nm illumination. The roGFP2 fluorescence was collected
219 with a 505–550 nm emission band-pass filter. Ratiometric image analysis was done as reported
220 previously (Schwarzländer *et al.*, 2008) using a custom Matlab programme (Fricker, 2016).

221

222

223 **Results**

224 ***gr2* T-DNA insertion allele causes early embryonic lethality**

225 To investigate the role of GR2 for glutathione redox homeostasis, T-DNA insertion lines for *GR2*
226 were identified and characterized. The T-DNA insertion lines *emb2360-1*, *emb2360-2*, *emb2360-*
227 3 (Tzafrir *et al.*, 2004), and *gr2-1* (SALK_040170) were obtained from NASC. The T-DNA insertion
228 in *gr2-1* was localized in the 8th intron (Fig. 1a) and the insertion site was confirmed by sequencing
229 using a T-DNA left border primer. A population of 133 plants derived from a heterozygous *gr2-1*
230 plant segregated with 85 kanamycin-resistant (Kan^R) : 48 kanamycin-sensitive plants. This
231 segregation of the antibiotic resistance in a 2:1 ratio ($\chi^2 = 0.45$, $P = 0.79$) indicates that
232 homozygous *gr2-1* segregates as a single locus and is sporophytic lethal. Genotyping of soil-
233 grown Kan^R plants confirmed that all Kan^R plants were heterozygous *gr2-1*, demonstrating that
234 Kan^R is caused by the T-DNA in the *GR2* locus. Microscopic examination of immature siliques of
235 heterozygous *gr2-1* mutants showed that 25 % (31:97, $\chi^2 = 0.04$ for a 1:3 segregation, $P = 0.98$)
236 seeds were white with embryos arrested at globular stage of development (Fig. 1d,e). Consistent
237 segregation patterns and phenotypes were found for the three *emb2360* alleles
238 (http://seedgenes.org/SeedGeneProfile_geneSymbol_EMB_2360.html), providing independent
239 evidence for a link between GR2 function and embryonic lethality. All further experiments were
240 done with *gr2-1*, which is from now referred to as *gr2*.

241

242 **GR2 solely targeted to plastids rescues the lethal *gr2* phenotype**

243 With the precedence of contradicting results regarding the subcellular localization of GR2 (Chew
244 *et al.*, 2003; Yu *et al.*, 2013) we attempted an orthogonal method to explore localization and
245 raised polyclonal antibodies against GR2 (Fig. S2b). Immunogold labelling of GR2 in leaf tissue of
246 WT plants independently demonstrates its dual localization (Fig. 2a,d). The early embryonic lethal
247 phenotype of *gr2* may thus be caused by mitochondrial or plastidic defects, or both. To resolve
248 this, *gr2* mutants were complemented with organelle-specific *GR2* constructs. The endogenous
249 signal peptide of 77 aa was replaced by the signal peptides SHMT for mitochondria or TK_{TP} for
250 plastids (Schwarzländer *et al.*, 2008; Albrecht *et al.*, 2014) (Fig. S1).
251 Compartment-specific complementation of *gr2* was initially done with GR2 constructs driven by
252 the *CaMV 35S* promoter (*35S_{pro}*) (Fig. S1). T2 transformants (named *pc* for plastid
253 complementation) were selected with Basta® and genotyped for the *gr2* locus to screen for

254 successful complementation of the lethal phenotype. Transformation with $35S_{pro}:TK_{TP}-\Delta_{1-77}GR2$
255 resulted in 22 % of the progeny being homozygous for *gr2* consistent with the theoretical value
256 of 25 % rescued homozygous mutants (Table S2). From these complemented mutants three
257 independent lines, named *pc-1*, *pc-2*, and *pc-3*, were selected and characterized in more detail
258 (Fig. S2). Protein gel blots of WT leaf extracts consistently revealed two distinct protein bands of
259 ~53 and ~110 kDa (Fig. S2b). The 110 kDa protein band was less intense than the 53 kDa band
260 and most likely represents a GR2 homodimer. The unprocessed WT GR2 protein including the
261 endogenous signal peptide of 77 aa has a predicted size of 61 kDa. The 53 kDa band detected in
262 the protein gel blot likely corresponds to the mature processed protein with a predicted size of
263 52.7 kDa. This result indicates that cleavage of the endogenous GR2 signal peptide is the same
264 for plastids and mitochondria. In *pc* lines over-expressing TK_{TP} -GR2, a very strong increase in both
265 bands was observed without significant change in the relative distribution between the two
266 bands. Moreover, in all $35S_{pro}:GR2$ over-expression lines several additional protein bands with
267 masses below that of GR2 may indicate partial degradation of GR2. Total GR activity in leaf tissue
268 extracts was 49 nmol min⁻¹ mg⁻¹ for WT and about 700 nmol min⁻¹ mg⁻¹ for the *pc* lines (Fig. S2c).
269 This 15-fold increase is consistent with the pronounced increase in GR2 abundance observed in
270 the *pc* lines. All *pc* lines showed WT-like GSH and GSSG levels with no changes in the GSH/GSSG
271 ratio detectable by HPLC-based thiol analysis (Fig. S2d). At random time points in their
272 development several T1 plants complemented with $35S_{pro}:TK_{TP}-\Delta_{1-77}GR2$ unexpectedly showed
273 wilted, dwarfed and purple coloured phenotypes, ultimately resulting in plant death before seed
274 setting (Fig. S2a). This phenotype occurred irrespective of whether the genetic background of the
275 respective plants was WT, *gr2*^{+/−} or *gr2*^{−/−}. The occurrence of plant death in WT and *gr2*^{+/−} plants
276 strongly suggests that the phenotype was caused by co-suppression of the endogenous *GR2* gene
277 together with the transgene.
278 To avoid potential artefacts resulting from extremely high levels of GR2 protein and also to
279 minimize the risk of silencing, *gr2* mutants were complemented with *GR2* driven by its
280 endogenous promoter (Fig. S1). In this case, however, only 10 % of the progeny, named *epc*, were
281 found to be homozygous for *gr2* (Table S2). The lower frequency of the complementation with
282 *GR2* driven by the endogenous promoter is most likely due to the much lower activity of this

283 promoter compared to the $35S_{pro}$. In some cases, the activity of the used $GR2_{pro}$ may not be
284 sufficient to achieve full complementation and thus cause premature abortion of homozygous
285 *gr2* embryos.

286 In contrast, none of the transgenic lines transformed with either $35S_{pro}:SHMT_{TP}-\Delta_{1-77}GR2$ or
287 $GR2_{pro}:SHMT_{TP}-\Delta_{1-77}GR2$ for mitochondrial targeting of GR2 was found to be a homozygous
288 knockout for *gr2* (Table S2). Thus, only $TK_{TP}-\Delta_{1-77}GR2$ constructs controlled by either the
289 endogenous $GR2_{pro}$ or $35S_{pro}$ rescued the lethal *gr2* mutant, indicating that only plastid-localized
290 GR2, but not the mitochondria-localized GR2, is essential for development. Correct targeting of
291 both GR2 plastid complementation constructs was confirmed by immunogold labelling (Fig. 2b-
292 d).

293 Three independent homozygous *gr2* mutants complemented with $GR2_{pro}:TK_{TP}-\Delta_{1-77}GR2$
294 constructs were isolated and named *epc-1*, *epc-2*, and *epc-3*, respectively. When grown on soil
295 under short day conditions, all three *epc* lines showed WT-like phenotypes in growth and
296 development (Fig. 3a). GR2 protein levels in T1 plants with different genotypes with respect to
297 the *gr2* locus were determined by protein gel blot analysis (Fig. 3b). Similar to WT and the $35S_{pro}$
298 complemented lines, protein gel blot analysis of total protein extracts consistently revealed two
299 bands of ~110 kDa and ~53 kDa, respectively, with the 53 kDa band being much more intense. In
300 addition to the 53 kDa and 110 kDa bands, protein extracts of $TK_{TP}-\Delta_{1-77}GR2$ transformants
301 showed a third band with a size of ~55 kDa. The appearance of this band suggests that import
302 processing of the $TK_{TP}-\Delta_{1-77}GR2$ protein differed from WT GR2. Indeed, ChloroP predicts $TK_{TP}-\Delta_{1-77}GR2$
303 processing to result in a mature protein with a mass of 54.2 kDa. In all three homozygous
304 *gr2* complementation lines only the 55 kDa protein was identified while the 53 kDa band was
305 absent, further confirming at biochemical level that all three selected *epc* lines were indeed *gr2*
306 null mutants successfully complemented with $TK_{TP}-\Delta_{1-77}GR2$. Furthermore, the absence of a
307 larger band of 61 kDa indicates that plastid import of GR2 was highly efficient without detectable
308 traces of non-processed cytosolic protein. Protein gel blot analysis of plants transformed with
309 $GR2_{pro}:SHMT_{TP}-\Delta_{1-77}GR2$ showed the same two-band pattern found in WT (Fig. 3b, lane d). Lack
310 of additional bands suggests that the processed WT GR2 and processed $SHMT_{TP}-\Delta_{1-77}GR2$
311 proteins have similar sizes, which is also supported by bioinformatics prediction by SignalP 3.0.

312

313 **Minute amounts of functional GR2 are sufficient for growth**

314 GR activity of total protein extracts of the *epc* lines was 60 nmol min⁻¹ mg⁻¹ and thus ~30 % lower
315 than WT GR activity of 90 nmol min⁻¹ mg⁻¹ (Fig. 3c). Both, protein gel blot analysis and GR activity
316 measurements of all three independent *epc* lines, showed that the amount of GR2 protein was
317 significantly lower than in WT. This indicates that either promoter and/or enhancer elements
318 other than the 2.1 kb fragment used here, may contribute to control the GR2 expression in WT
319 plants, or that positional effects may have led to partial repression of GR2 in *epc* lines. The
320 content of GSH and GSSG was comparable to WT plants (Fig. 3d).

321 The GR activity in *epc* lines is due to the *GR2* transgene and the endogenous GR1 activity, which
322 had been reported to account for 40 to 60 % of the total GR activity in leaves (Marty *et al.*, 2009;
323 Mhamdi *et al.*, 2010). To further separate these activities and to better assess the requirement
324 for GR2 necessary for normal growth, we generated a double heterozygous *gr1^{+/−} gr2^{+/−}* plant and
325 complemented this with the same *GR2_{pro}:TK_{TP}-Δ₁₋₇₇GR2* construct used before. From the F2
326 progeny we selected two homozygous *gr1 gr2* double knockouts that expressed GR2 exclusively
327 in the plastids (lines *epc-9* and *epc-27*). These *gr1 gr2* plants did not show any obvious phenotype
328 in their growth and development during the vegetative phase under standard growth conditions
329 (Fig. 4a). The total GR activity in leaf extracts was only between 10 ± 2.6 and 1.5 ± 0.1 nmol mg⁻¹
330 min⁻¹ compared to 53.2 ± 3.5 nmol mg⁻¹ min⁻¹ in WT leaves (Fig. 4b). For comparison, *gr1* mutants
331 and the plastid-complemented *gr2* mutant (*epc-2*) showed intermediate GR-activities of
332 37.5 ± 2.6 and 24.2 ± 2.6 nmol mg⁻¹ min⁻¹, respectively (Fig. 4b). Lack of GR1 in *gr1-1* and in *gr1*
333 *gr2* double deletion mutants consistently resulted in an increase in total glutathione (Fig. 4c).
334 Further analysis of the glutathione pool revealed that this increase was largely due to an increase
335 in GSSG, which was about six-fold higher compared to WT controls (Fig. 4d). If at all, plastid-
336 specific complementation of *gr2* resulted in only a minor increase in glutathione (Figs. 3d and 4c).
337 To further test for mitochondrial GR activity, we first isolated mitochondria from the plastid
338 complemented line *epc-2* and determined the specific GR activity in mitochondrial extracts.
339 While mitochondria isolated from WT plants had a specific GR activity of 47.9 nmol min⁻¹ mg⁻¹,
340 mitochondria from *epc-2* contained an activity of 2.4 nmol min⁻¹ mg⁻¹ (Fig. 5a). Identity of

341 mitochondrial preparations was confirmed by detection of the mitochondrial marker
342 peroxiredoxin II F (PRXII F; (Finkemeier *et al.*, 2005)). Further analysis of the respective extracts
343 indicated that GR2 was absent in *epc-2* mitochondria (Fig. 5c). Hybridization of the respective
344 protein blots with a GR1 antibody (Marty *et al.*, 2009) revealed that the mitochondrial fraction
345 also contained GR1 (Fig. 5c) which can be accounted for by the presence of peroxisomes that
346 contain GR1, in mitochondrial preparations from Arabidopsis seedlings (Sweetlove *et al.*, 2007).
347 No GR activity was found in mitochondria isolated from line *epc-27* (Fig. 5b). Protein gel blots
348 confirmed absence of both GR1 and GR2 from *epc-27* mitochondria (Fig. 5d).

349

350 **Glutathione deficiency partially suppresses embryo lethality of *gr2***

351 Early embryonic lethality of *gr2* null mutants may be explained by lack of appropriate backup
352 systems for GSSG reduction, or an additional, yet unknown, moonlighting function (Jeffery, 2009)
353 of plastidic GR2. To elucidate whether the early embryonic lethal phenotype is caused solely by
354 accumulation of GSSG in plastids, we reasoned that GSH-deficiency would necessarily go along
355 with deficiency of GSSG and thus might suppress the early embryonic lethal phenotype of *gr2*.
356 To test this hypothesis, *gr2* was crossed with the two GSH-deficient mutants *rml1* and *gsh1-1*,
357 which are both compromised in the first step of GSH biosynthesis catalysed by glutamate-
358 cysteine ligase (GSH1) and contain strongly decreased glutathione levels (Vernoux *et al.*, 2000;
359 Cairns *et al.*, 2006). While *rml1* completes embryogenesis and germinates, the null mutant *gsh1-1*
360 reaches U-turn stage before it dies (Vernoux *et al.*, 2000; Cairns *et al.*, 2006). While no double
361 homozygous *rml1 gr2* could be found (Fig. S4) the *gr2 gsh1* cross and selfing of a double
362 heterozygous plant resulted in approximately 24 % aborted seeds albeit with a distinct
363 phenotypic variation (Fig. 6c). Closer inspection of the aborted seeds revealed that about 1/4 of
364 seeds were aborted later than the typical *gr2* embryo, which were seen as white turgescent
365 ovules (Fig. 6c). Indeed developing seeds collected from a selfed double heterozygous
366 *gr2⁺⁻ gsh1⁺⁻* plant showed a segregation of their embryos in 334 U-turn : 92 globular : 30 torpedo
367 (12:3:1, $\chi^2 = 0.76$, $P = 0.34$) (Fig. 6d-g). This result indicates that GSH-deficiency caused by *gsh1*
368 partially suppressed the early embryo-lethal *gr2* phenotype extending development until the

369 torpedo stage. This points to GSSG toxicity, as opposed to an oxidative shift in E_{GSH} , as a cause of
370 the embryo lethality in the absence of GR2 from the plastid stroma.

371

372 **The mitochondrial glutathione pool of plastid-complemented *gr2* mutants shows increased**
373 **oxidation**

374 Full viability of plastid complemented *gr2* plants suggests that mitochondria can maintain their
375 function in the absence of GR2. This suggests that they are either able to cope with a fully oxidized
376 glutathione pool made up of GSSG, or other mechanisms of maintaining reduction exist.
377 Candidate mechanisms include GSSG export from the matrix for reduction in the cytosol as well
378 as an alternative enzymatic system for efficient internal GSSG reduction. To address this
379 question, we tested whether E_{GSH} in the mitochondrial matrix of mutants lacking GR2 in
380 mitochondria is more oxidized than in WT controls. Plastid complemented *gr2*^{-/-} mutants (*epc-2*)
381 were transformed with the E_{GSH} biosensor roGFP2-Grx1 targeted to the mitochondrial matrix
382 (Albrecht *et al.*, 2014). roGFP2 constructs were expressed from $35S_{pro}$ and we deliberately
383 selected strongly expressing lines in which part of the sensor proteins remained in the cytosol
384 and the nucleus (Fig. 7 and Fig. S5). The resulting dual localization of roGFP2-Grx1 allowed
385 simultaneous ratiometric imaging of roGFP2 in both compartments by confocal microscopy.
386 Because the roGFP2-Grx1 in the cytosol and the nucleus provides an internal control for close-
387 to-complete sensor reduction (Meyer *et al.*, 2007; Schwarzländer *et al.*, 2008) the ratiometric
388 images are informative without any further treatment with reducing and oxidizing compounds.
389 While the merge of the raw images collected after excitation at 405 nm and 488 nm in WT
390 seedlings show similar green colour in cytosol and mitochondria (Fig. 7a) the same merge for the
391 plastid complemented *gr2*^{-/-} mutant shows a pronounced difference: Cytosol and nucleus appear
392 in green like in the WT control, while the mitochondria appear in yellow because the fluorescence
393 in the 488 nm channel decreased and the fluorescence in the 405 nm channel increased (Fig. 7b).
394 The differential behaviour of the signals in mitochondria and the cytosol is even more obvious
395 after ratiometric analysis, which shows a partial oxidation of roGFP2-Grx1 in the GR2-deficient
396 mitochondria. The degree of sensor oxidation can be estimated to approximately the midpoint

397 of the sensor, i.e. around 50 %. This corresponds to a pronounced shift in mitochondrial matrix
398 E_{GSH} of about 30 mV, but is remains far from a complete oxidation of the matrix glutathione pool.
399

400 **The mitochondrial ABC-transporter ATM3 has a critical function in GR2-deficient
401 mitochondria**

402 Export of GSSG from the mitochondrial matrix and subsequent reduction by cytosolic GR1 activity
403 would provide an alternative mechanism to adjust the matrix E_{GSH} . To elucidate whether the GSSG
404 export ability of ATM3 could help to maintain low GSSG levels and support the mitochondrial
405 GR2 activity, we crossed the mutant *gr2 epc-2* deficient in mitochondrial GR2 with *atm3-4*, and
406 isolated the double mutant. *atm3-4* is a relatively weak mutant allele, with low expression of
407 functional *ATM3* due to a 39-nucleotide deletion in the promoter (Bernard *et al.*, 2009). The
408 *atm3-4 gr2 epc2* double mutant showed an enhanced phenotype compared to both the *gr2 epc2*
409 and *atm3-4* parents, with short roots at seedling stage and smaller rosettes when grown on soil
410 (Fig. 8). Leaves appeared chlorotic and displayed a significantly lower F_v/F_m (Fig. 8d). These
411 observations suggest synergistic action of the two mutations with lack of mitochondrial GR2
412 causing increased GSSG levels in the matrix that cannot be efficiently decreased when ATM3
413 protein levels are strongly depleted.

414

415 **NADPH-dependent thioredoxin reductases backup mitochondrial GR2**

416 Both, NTRA and NTRB, are known to be dual-targeted to the cytosol and mitochondria (Reichheld
417 *et al.*, 2005). Based on the observation that the NTR/TRX system efficiently reduces GSSG in the
418 cytosol (Marty *et al.*, 2009) we hypothesized that NTRA and NTRB together with mitochondrial
419 TRXo1 and o2 (Yoshida & Hisabori, 2016), or TRXh2 (Meng *et al.*, 2010) may provide a functional
420 backup system for mitochondrial GR2. To further support this hypothesis, we also tested
421 mitochondrial TRXs for their ability to reduce GSSG *in vitro* and determined steady-state kinetic
422 parameters for GSSG for TRXh2, TRXo1 and GR2. In conjunction with NTRA recombinant TRXh2
423 and TRXo1 are both capable of reducing GSSG, albeit with K_m values of only 2 to $4 \times 10^3 \mu\text{M}$, which
424 is nearly 100-fold higher than the K_m of GR2 ($13 \mu\text{M}$) (Fig. 9). While the efficiency k_{cat}/K_m of GR2
425 approximates the diffusion limit, k_{cat}/K_m for both TRXs is about 1000-fold lower. The GSSG

426 reduction activity of both TRXs is independent of which NTR isoform is available for TRX reduction
427 (Fig. S6).

428 To further test the hypothesis that mitochondrial NTRs provide the rescue for lack of
429 mitochondrial GR2, we generated a cross between an *ntra ntrb* double null mutant and a
430 homozygous *gr2* complemented with plastid-targeted GR2 (Fig. S7). From this cross, *F*₃ plants
431 expressing GR2 were preselected by Basta® to ensure only GR2 complemented plants to be
432 maintained. Next, plants segregating for *ntrb* only were selected through genotyping, and selfed.
433 71 tested *F*₄ progeny segregated for the mutant allele *ntrb* 23 *NTRB/NTRB* : 48 *NTRB/ntrb* : 0
434 *ntrb/ntrb* ($\chi^2 = 0.03$, $P > 0.95$) indicating that homozygous *ntra ntrb gr2* triple mutants are not
435 viable. The 1:2 segregation of viable plants suggested that the lethal effect of *ntra ntrb gr2* is not
436 gametophytic but rather manifests itself only after fertilization. Consistent with this, viability
437 staining of pollen resulted in nearly 100 % viable pollen (Fig. S8). To further confirm full viability
438 of *ntra ntrb gr2* pollen during pollen tube growth, we backcrossed the *ntra/ntra NTRB/ntrb*
439 *gr2/gr2 pIGR2* plant to WT. This cross resulted in a 1:1 segregation for *ntrb* ($\chi^2 = 0.7$, $P = 0.34$;
440 Table S3a). Similarly, the reverse cross of WT pollen to an *ntra/ntra NTRB/ntrb gr2/gr2 pIGR2*
441 triple mutant resulted in a 1:1 segregation for *ntrb* ($\chi^2 = 0.34$, $P = 0.58$; Table S3b). These results
442 showed that both pollen and oocytes carrying all three mutant alleles are fully viable.
443 Developing siliques from self-fertilized *ntra/ntra NTRB/ntrb gr2/gr2 pIGR2* plants in contrast
444 contained many aborted seeds (Fig. 10a,b) supporting that the lethal phenotype was established
445 at the diploid stage. However, the overall abortion rate of 13.4 % in siliques collected from ten
446 randomly selected plants was significantly below the expected 25 % (114 aborted seeds out of
447 852 seeds; $\chi^2 = 61.35$, $P < 0.001$). A closer inspection of seedlings germinated from these seeds
448 indicated some barely viable seedlings that ceased growth about three weeks after germination
449 and died (Fig. 10c). PCR genotyping confirmed that these seedlings were homozygous for the
450 segregating allele *ntrb* (Fig. 10d,e).

451

452

453 **Discussion**

454 **GR2 is indispensable in plastids**

455 Appropriate detoxification of H_2O_2 produced during normal metabolism or in response to
456 environmental stress is mandatory to ensure maintenance of sufficiently reducing conditions for
457 normal metabolism (Moller *et al.*, 2007; Waszczak *et al.*, 2018). The glutathione-ascorbate cycle
458 has attained much attention and connects H_2O_2 detoxification via ascorbate peroxidases
459 ultimately to the local glutathione pool with GSH providing the required electrons (Foyer &
460 Noctor, 2011). The generated GSSG is subsequently reduced back to GSH by GRs with electrons
461 provided by NADPH. While in *Arabidopsis* GR1 activity in the cytosol and peroxisomes can be
462 substituted to a sufficient extent by the cytosolic NTS to avoid lethality of null mutants (Marty *et*
463 *al.*, 2009), *gr2* null mutants are early embryonic lethal (Bryant *et al.*, 2011). Our results
464 unanimously show that GR2 is dual-targeted to plastids and mitochondria but is essential only in
465 plastids.

466 A shift of E_{GSH} towards less negative values can be caused either by depletion of GSH or by
467 increased amounts of GSSG. The GSH-deficient mutant *gsh1* completes embryogenesis up to the
468 seed maturation phase because maternal tissues provide a minimum amount of GSH to the
469 embryo (Cairns *et al.*, 2006; Lim *et al.*, 2014). In *rm1*, the very low GSH content results in an E_{GSH}
470 of about -260 mV (Aller *et al.*, 2013). Despite the pronounced shift in E_{GSH} the situation is not
471 deleterious *per se*. Partial suppression of the early embryo arrest in *gr2 gsh1* double mutants
472 rather points at the accumulation of GSSG being toxic. The severely restricted ability to build up
473 normal GSH levels initially helps keeping the GSSG concentration low and enables embryos to
474 develop beyond globular stage. However, the arrest at torpedo stage still occurs much earlier
475 than the arrest in *gsh1* during seed maturation. This strongly suggests that even without normal
476 levels of GSH, GSSG quickly accumulates to toxic levels in plastids if no GR2 is present. Plastids
477 apparently have no backup system for GSSG reduction and no (or insufficient) ability to export
478 GSSG to the cytosol for reduction. In developing *Arabidopsis* embryos, the first chloroplasts start
479 differentiating and greening with the initiation of cotyledons at heart stage (Mansfield & Briarty,
480 1991). Silencing of GR2 in mature plants indicates that light-dependent reduction of GSSG via
481 plastidic TRXs may not be sufficient to maintain growth. This suggests that, in contrast to the
482 cytosolic NTS, the plastidic FTR/TRX system cannot compensate for the lack of GR activity. This
483 conclusion is consistent with current findings in *Physcomitrella patens* plants lacking organellar

484 GR, where stromal E_{GSH} is oxidised but not rescued by active photosynthetic electron transport
485 (Müller-Schüssle *et al.*, 2019). Developmental arrest of *gr2* mutants at globular stage before
486 chloroplast differentiation indicates that the accumulation of GSSG is not related to
487 photosynthesis as a possible source of ROS (Foyer & Noctor, 2009), but rather to other basic
488 metabolic processes. Possible processes include the formation of GSSG through reduction of
489 oxidised GRX by GSH (Begas *et al.*, 2017) and the reduction of adenosine 5'-phosphosulfate by
490 GSH (Bick *et al.*, 1998). In addition, several metabolic pathways in plastids involve oxidation steps
491 in which molecular oxygen is reduced to H_2O_2 . The reactions include the pyridoxal 5'-phosphate
492 salvage pathway in which the plastidic pyridoxine/pyridoxamine 5'-phosphate oxidase produces
493 one molecule H_2O_2 (Sang *et al.*, 2011), the oxidation of L-aspartate by L-aspartate oxidase in the
494 early steps of NAD biosynthesis (Katoh *et al.*, 2006), and the three-step oxidation of
495 protoporphyrinogen IX to protoporphyrin IX by the enzyme protoporphyrinogen oxidase PPOX
496 (Koch *et al.*, 2004; Mochizuki *et al.*, 2010).

497 Minute amounts of GR2 activity are necessary to avoid accumulation of GSSG to toxic levels. GR,
498 in general, has been reported to be highly active with a very low K_m for GSSG leaving only low
499 nanomolar traces of GSSG (Veech *et al.*, 1969). This activity dominates E_{GSH} *in vivo* keeping it
500 highly reduced as confirmed by *in vivo* imaging with roGFP-based probes (Marty *et al.*, 2009;
501 Schwarzländer *et al.*, 2016). Accumulating GSSG may interfere with fundamental plastidic
502 processes, such as transcription, translation and enzymatic functions. For instance, fatty acid
503 biosynthesis is vital in developing oilseed embryos and involves the redox-regulated heteromeric
504 plastidic acetyl-CoA-carboxylase (ACCase) (Ke *et al.*, 2000; Sasaki & Nagano, 2004; Bryant *et al.*,
505 2011). The extraordinary importance of plastidic ACCase for embryo development is further
506 supported by the early embryo lethal phenotype of null mutants for BCCP1 (Li *et al.*, 2011). One
507 of four ACCase subunits, CT β (AtCg00500, AccD), is plastid encoded. Thus, interfering with thiol
508 switches regulating the plastidic gene expression machinery (Dietz & Pfannschmidt, 2011) or vital
509 enzyme activities would constitute one possible scenario to account for our observations.
510 Another possible toxic effect of GSSG is the disruption of Fe-S cluster transfer by monothiol GRXs
511 which involves binding of GSH as a cofactor on its backbone (Moseler *et al.*, 2015). If present in
512 high concentrations GSSG may interfere with GSH binding and disrupt Fe-S coordination (Berndt

513 *et al.*, 2007). Plastids contain two monothiol GRXs of which GRXS16 carries an additional
514 regulatory disulfide that is responsive to GSSG (Zannini *et al.*, 2019). GSSG-mediated oxidation of
515 GRXS16 modulates its oxidoreductase function and enables protein glutathionylation. Dissecting
516 the mechanistic cause of embryo lethality is beyond the scope of this work, but promises
517 intriguing novel insights into the role of redox dynamics in organelle biogenesis and early plant
518 development.

519

520 **ATM3 and the mitochondrial TRX system safeguard the matrix E_{GSH}**

521 Direct comparison of cytosol and mitochondrial matrix in plastid complemented *gr2* mutants
522 clearly showed that the readout of roGFP2-Grx1 was shifted to higher ratio values indicating a
523 shift in the local E_{GSH} towards less negative values. While roGFP2 in the cytosol of plastid-
524 complemented *gr2* lines is almost fully reduced indicating an E_{GSH} of -310 mV or even more
525 negative, roGFP2 in the mitochondrial matrix is clearly partially oxidised. The observed degree of
526 sensor oxidation of ~50 % would indicate an E_{GSH} of -280 mV assuming a matrix pH of 7.0 (-310
527 mV at pH 7.5). In a solution with estimated 2 mM GSH this point would be reached with
528 approximately 200 nM GSSG. This calculation shows that the amount of GSSG determined by
529 HPLC in plants extracts is a significant overestimation of the amount of GSSG really present in the
530 respective subcellular compartment. This is in line with earlier conclusions on sensor-based
531 subcellular E_{GSH} measurements (Meyer & Dick, 2010; Schwarzländer *et al.*, 2016).

532 Although we showed that GSSG accumulation in plastids is causing embryo lethality, plants
533 lacking GR2 in mitochondria have no obvious phenotype under control conditions. Two possible
534 scenarios can be drawn to explain this observation: (i) GSSG can be exported to the cytosol to get
535 reduced by GR1, or (ii) GSSG is reduced in the matrix by other enzymes. Knock-down mutants
536 with a severely limited ATM3 capacity have been shown earlier to have a less negative E_{GSH} in the
537 mitochondrial matrix than WT (Schaedler *et al.*, 2014). The pronounced chlorotic phenotype of
538 *gr2 atm3-4* supports the interpretation that ATM3 export GSSG from mitochondria under
539 physiological conditions. This mechanism may be important when NADPH is low, and GR2 activity
540 limited. It should be noted, however, that toxic effects of GSSG in this case may also result from
541 competition with other substrates of ATM3, such as persulfides as required for Fe-S cluster

542 biosynthesis in the cytosol. In the presence of high concentrations of GSSG and with limited
543 transport capacity in *atm3-4* these metabolites may not be exported efficiently.
544 Dithiol GRXs are capable of catalysing the reduction of GSSG by dihydrolipoamide (Porras *et al.*,
545 2002). In contrast to several non-plant species, *Arabidopsis* mitochondria contain only the
546 monothiol GRX15 which was found inactive as an oxidoreductase (Moseler *et al.*, 2015). Non-
547 catalysed reduction of GSSG by dihydrolipoamide would be extremely inefficient and unlikely to
548 contribute efficiently to GSSG removal. In contrast, the GSSG reduction activity of NTRs together
549 with mitochondrial TRXs appears high enough to reduce significant amounts of GSSG. Lethality
550 of *gr2 epc-2 ntra ntrb* shows that the situation in the mitochondrial matrix resembles the NTS-
551 based backup of cytosolic GR1 (Marty *et al.*, 2009). The presence of multiple backup systems may
552 explain why the lethal effect becomes apparent only after germination. In contrast to *gr2* plastids
553 where direct detection of glutathionylated proteins, protein disulfides and further thiol
554 modifications is not possible due to the minute amount of material in early embryos, proteomic
555 studies in mitochondria lacking GR2 seem feasible. In the future, such measurements may reveal
556 novel metabolic bottlenecks generated from oxidative modification of protein thiols and
557 resulting imbalances in thiol switching.

558

559

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570

571

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573 AJM, RH and JPR designed research; LM, DB, CM, SAKB, AM, MS, BZ, CR, and MW performed
574 research; JB contributed plant lines; LM, DB, CM, MS, SJMS, JPR and AJM analyzed data and
575 SJMS, MS and AJM wrote the manuscript with assistance from all co-authors.

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822 **Supporting Information**

823 **Fig. S1** Schematic representation of constructs used to confirm signal peptide functionality and
824 for compartment-specific complementation of *gr2* mutants.

825 **Fig. S2** Characterization of transgenic *Arabidopsis* lines over-expressing plastid-targeted GR2.

826 **Fig. S3** Protein gel blot analysis of GR1 and GR2 in mitochondrial preparations of WT and
827 plastid-complemented *gr2* (*epc-2*).

828 **Fig. S4** Characterization of *gr2 rml1* double mutants.

829 **Fig. S5** High expression of SHMT_{TP}-roGFP2-Grx1 results in incomplete mitochondrial targeting.

830 **Fig. S6** Mitochondrial thioredoxins reduce GSSG *in vitro* with electrons provided by NTRA and
831 NTRB with similar efficiencies.

832 **Fig. S7** Crossing scheme for generation of *gr2 ntra ntrb*.

833 **Fig. S8** Viability stain of pollen from *ntra*^{-/-} *gr2*^{-/-} *NTRB/ntrb pIGR2* plants.

834 **Table S1** Oligonucleotides used in this study.

835 **Table S2** Genetic complementation of the *gr2* mutant with compartment-specific *GR2*
836 constructs.

837 **Table S3** Reciprocal cross between *ntra/ntra* *NTRB/ntrb* *gr2/gr2 pIGR2* and WT.

838 **Methods S1** Antibody production and gel blot analysis.

839 **Methods S2** Immunogold labelling and electron microscopy

840

841 **Figure legends**

842 **Fig. 1** Isolation and phenotypic characterization of *gr2* null mutants. (a) Exon/intron structure of
843 the Arabidopsis *GR2* gene (At3g54660). Exons are represented by large arrows and the triangles
844 show the T-DNA insertions for three *emb2360* alleles
845 (http://seedgenes.org/SeedGeneProfile_geneSymbol_EMB_2360.html) and the allele *gr2-1*.
846 Primer binding sites for genotyping of *gr2-1* are indicated by black arrows. (b) Genotype analysis
847 of different *gr2-1* lines. Upper panel: PCR performed with *GR2* gene specific primers. Lower
848 panel: PCR with a gene-specific primer and a primer for the left T-DNA border. Lane a: DNA
849 marker: size is indicated in kb; Lane b: WT; Lanes c-e: heterozygous *gr2-1* mutants. (c) Embryo-
850 lethal phenotype associated with *gr2-1*. Immature siliques from self-fertilized WT (upper panel)
851 and heterozygous *gr2-1* mutants (lower panel) were opened to observe segregation of seed
852 phenotypes. Bar, 500 μ m. 25 % of the seeds in the *gr2* siliques show a lethal phenotype visible as
853 white seeds. (d-e) Developing seeds of heterozygous *gr2-1* mutants were cleared using Hoyer's
854 solution and analysed by bright field microscopy (d) and DIC microscopy (e). Arrows indicate
855 white ovules containing embryos arrested at globular stage. Bars, 50 μ m.

856

857 **Fig. 2** Immunogold localization of GR2 in plastid complemented *gr2* deletion lines. Fixed and
858 dissected mature leaves were probed with a primary antibody raised against Arabidopsis GR2. C:
859 chloroplast; M: mitochondrion; Px: peroxisome; V: vacuole; N: nucleus; St: starch grain. Bars,
860 1 μ m. (a) WT. (b) Line *pc-2* ('plastid complemented') with $TK_{TP}-\Delta_{1-77}GR2$ expressed from the 35S
861 promoter. (c) Line *epc-2* with $TK_{TP}-\Delta_{1-77}GR2$ expressed from the endogenous GR2 promoter. (d)
862 Quantitative analysis of gold particles observed in electron microscopy micrographs. Values are
863 means \pm SE and document the amount of gold particles per μ m² in the respective organelle. Data
864 were analysed by the Kruskal-Wallis test, followed by post hoc comparison according to Conover.
865 Different lowercase letters indicate significant differences ($P < 0.05$). $n > 60$. nd = not detected.

866

867 **Fig. 3** Characterization of lines expressing plastid-targeted GR2 controlled by its endogenous
868 promoter. (a) Growth phenotypes of WT (a) and three independent homozygous *gr2* deletion
869 mutants complemented with $GR2_{pro}:TK_{TP}-\Delta_{1-77}GR2$: *epc-1* (b), *epc-2* (c), *epc-3* (d). All transgenic

870 plants were from the Basta[®]-selected T1 generation. (b) Protein gel blot analysis of transgenic
871 Basta[®]-selected T1 lines transformed with complementation constructs driven by the
872 endogenous *GR2* promoter and targeted to either mitochondria or plastids. Loading was as
873 follows: a: Pre-stained molecular mass standard, b: WT, c: WT transformed with *GR2_{pro}:SHMT_{TP}-*
874 $\Delta_{1-77}GR2$, d: *gr2^{+/−}* transformed with *SHMT_{TP}-* $\Delta_{1-77}GR2$, e: WT transformed with *GR2_{pro}:TK_{TP}-* $\Delta_{1-77}GR2$,
875 f: *gr2^{+/−}* transformed with *GR2_{pro}:TK_{TP}-* $\Delta_{1-77}GR2$, g-i: three independent homozygous *gr2*
876 deletion lines transformed with *GR2_{pro}:TK_{TP}-* $\Delta_{1-77}GR2$ (*epc-1*, *epc-2* and *epc-3*). Arrows indicate
877 protein bands with the size of 110 kDa (black), 55 kDa (dark grey) and 53 kDa (light grey). (c) GR
878 activity in total protein extract from leaves. Proteins were extracted from WT and T2 plants of
879 the homozygous *gr2* lines *epc-1*, *epc-2* and *epc-3*. Means \pm SD of three independent plants of
880 each line are shown. (d) Contents of oxidized and reduced glutathione. After extraction of leaf
881 tissue of 5-week-old plants reduced glutathione (GSH) and glutathione disulfide (GSSG) were
882 determined by HPLC. Means \pm SD of 6 independent plants of each line are shown.

883

884 **Fig. 4** Glutathione reductase activity and low molecular weight thiols of *gr1 gr2* double
885 homozygous mutants complemented with plastid-targeted GR2. (a) Rosette phenotypes of plants
886 grown on soil for six weeks under short day conditions (8 h : 16 h, light : dark). Bar, 1 cm. (b) GR
887 activity in total protein extracts from leaves. Proteins were extracted from WT, *gr1*, plastid
888 complemented *gr2* (*epc-2*) and two *gr1 gr2* double mutants (*epc-9* and *epc-27*) that were
889 complemented with *GR2_{pro}:TK_{TP}-* $\Delta_{1-77}GR2$. Means \pm SD of three independent plants of each
890 complemented line are shown. (c) Low-molecular weight thiols analysed by HPLC from leaf
891 extracts. GSH in this case refers to total glutathione. Means \pm SD, $n = 3$. (d) Reduced glutathione
892 (GSH) and glutathione disulfide (GSSG) determined by HPLC in leaf extracts. Means \pm SD, $n = 3$.

893

894 **Fig. 5** Glutathione reductase activity in isolated mitochondria. Intact mitochondria were isolated
895 from two-week old hydroponically grown *Arabidopsis* plants (WT and *epc-2* lacking endogenous
896 *GR2*, or *epc-27* lacking both endogenous GRs, respectively). Activities represent the means from
897 four independent preparations with six technical replicates. Error bars represent SD. (a, b) GR
898 activity. n.d. = not detected; * indicates $P < 5 \times 10^{-4}$. (c, d) Protein gel blots of PRXII F, GR2 and GR1

899 in mitochondrial preparations. Loading controls were stained with amido black. In addition to
900 GR1 (54 kDa), the GR1 antibody detected also a smaller protein of about 50 kDa (Fig. 5d). This
901 band, however, appears to result from a side activity against another mitochondrial protein that
902 is detectable in concentrated isolated mitochondria, but not in whole leaf extracts (Fig. S3b).
903 Mitochondria contain two lipoamide dehydrogenases (MTLPD1 and MTLPD2) that are closely
904 related to GR and are both predicted with a molecular mass of 49.9 kDa after cleavage of the
905 mitochondrial target peptide (Lutziger & Oliver, 2001). Thus, it is likely that the additional band
906 results from a cross reaction of the antibody with MTLPDs.

907
908 **Fig. 6** Characterization of *gr2 gsh1* double mutants. (a) WT siliques at 14 d after fertilization (DAF).
909 (b) Siliques of self-fertilized heterozygous *gr2* plant displaying 25 % aborted seeds. (c) Siliques of
910 self-fertilized double heterozygous *gr2^{+/−} gsh1^{+/−}* plant displaying segregation of green WT seeds,
911 partially bleached *gsh1* seeds (▲), brownish early aborted *gr2* seeds (*), and transparent seeds
912 (⊗) that remain fully turgescent significantly longer than *gr2* seeds. (d-g) DIC images of ovules
913 developed in a siliques of a self-fertilized double heterozygous *gr2^{+/−} gsh1^{+/−}* plant 14 DAF. Bars, 50
914 µm.

915
916 **Fig. 7** Simultaneous imaging of the local glutathione redox status in mitochondria and the cytosol.
917 The ratiometric redox probe roGFP2-Grx1 was expressed with a SHMT_{TP} mitochondrial targeting
918 sequence driven by a 35S_{pro}. After transformation lines with residual roGFP2-Grx1 in the cytosol
919 were selected and used for simultaneous redox imaging in mitochondria and the cytosol. Images
920 show hypocotyl cells in WT seedlings (a) or plastid complemented *gr2^{−/−}* seedlings (b). Images
921 taken after excitation at 405 nm are false colour coded in red and images taken after excitation
922 at 488 nm are coded in green in order to visualize a pronounced colour change in the merge
923 image from green to yellow in the mitochondria. Bars, 10 µm.

924
925 **Fig. 8** *atm3-4 gr2 epc2* double mutants show a semi-dwarf and chlorotic phenotype. (a) 8-d-old
926 seedlings of the double mutant compared to WT and single mutants. Bar, 0.5 cm. (b) Root length
927 of the mutants compared to WT. Plants were grown on ½ MS medium containing 0.5 % sucrose

928 and 0.8 % phytagel for 5-8 days under long-day conditions after stratification for 2 d (means \pm
929 SD, $n = 5-12$). (c) Plants were grown under long-day conditions for four weeks. (d) Chlorophyll
930 fluorescence of 4-week-old plants grown on soil under long-day conditions (means \pm SD, $n = 7$).
931 The statistical analysis (one way ANOVA with post hoc Holm-Sidak comparisons for WT vs.
932 mutant) indicates significant changes; $*P \leq 0.05$; $***P \leq 0.001$.

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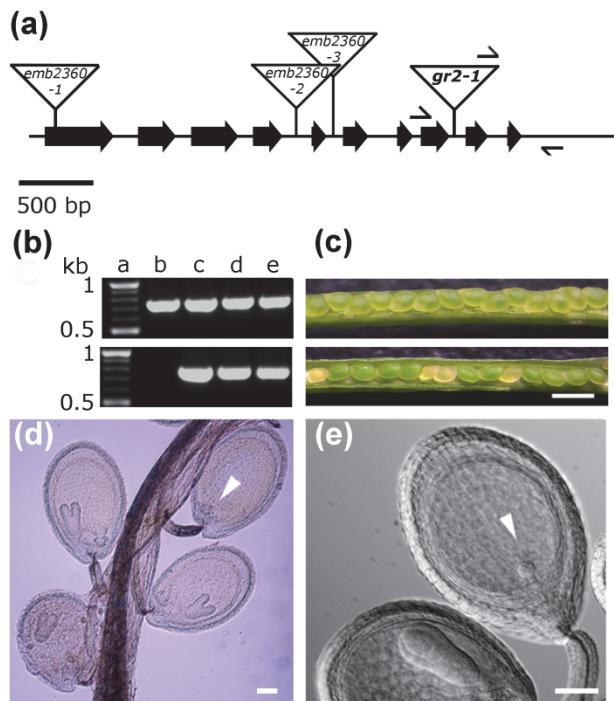
934 **Fig. 9** Mitochondrial TRXs in conjunction with NTRA can reduce GSSG *in vitro*. (a) Activity is
935 monitored as NADPH oxidation. Enzymes and substrates were used at the following
936 concentrations: NADPH, 250 μ M; GSSG, 1 mM; GR2, 0.01 μ M; TRXh2 and TRXo1, 2 μ M; NTRA, 1
937 μ M; Means \pm SD ($n = 3$). Note that the amount of disulfide reductase protein varied between the
938 assays. (b) Steady-state kinetic parameters of GR2 and TRX-dependent GSSG reduction systems.
939

940 **Fig. 10** Characterization of *ntra ntrb gr2* triple mutants expressing plastid-targeted GR2 under
941 control of its endogenous promoter. (a) WT siliques 20 d after fertilization. (b) Siliques from a plant
942 homozygous for both *ntra* and *gr2*, complemented with *p1GR2* and heterozygous for *ntrb*. (c)
943 Phenotype of mutant seedlings 18 d after germination. (d, e) Genotyping of seedlings shown in
944 panel c for *NTRB* (d) and the *ntrb* T-DNA insertion (e).

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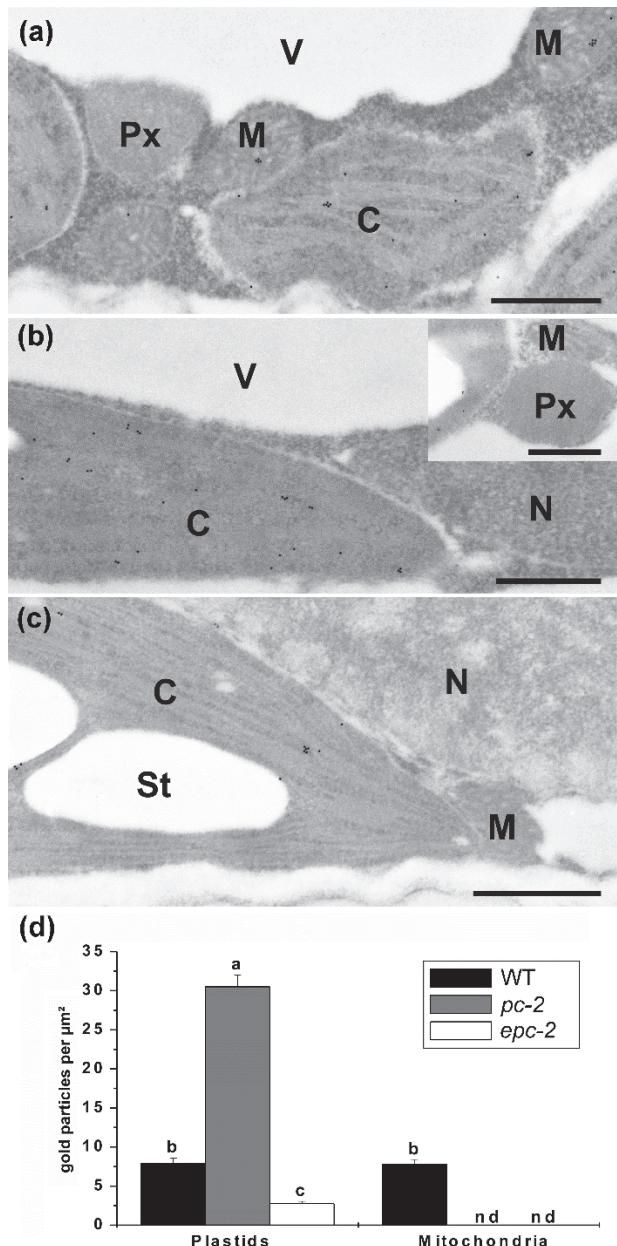
947 **Figure 1**



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949

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951 the *Arabidopsis* *GR2* gene (At3g54660). Exons are represented by large arrows and the triangles
952 show the T-DNA insertions for three *emb2360* alleles (http://seedgenes.org/SeedGeneProfile_geneSymbol_EMB_2360.html) and the allele *gr2-1*.
953 Primer binding sites for genotyping of *gr2-1* are indicated by black arrows. (b) Genotype analysis
954 of different *gr2-1* lines. Upper panel: PCR performed with *GR2* gene specific primers. Lower
955 panel: PCR with a gene-specific primer and a primer for the left T-DNA border. Lane a: DNA
956 marker: size is indicated in kb; Lane b: WT; Lanes c-e: heterozygous *gr2-1* mutants. (c) Embryo-
957 lethal phenotype associated with *gr2-1*. Immature siliques from self-fertilized WT (upper panel)
958 and heterozygous *gr2-1* mutants (lower panel) were opened to observe segregation of seed
959 phenotypes. Bar, 500 μ m. 25 % of the seeds in the *gr2* siliques show a lethal phenotype visible as
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961 solution and analysed by bright field microscopy (d) and DIC microscopy (e). Arrows indicate
962 white ovules containing embryos arrested at globular stage. Bars, 50 μ m.

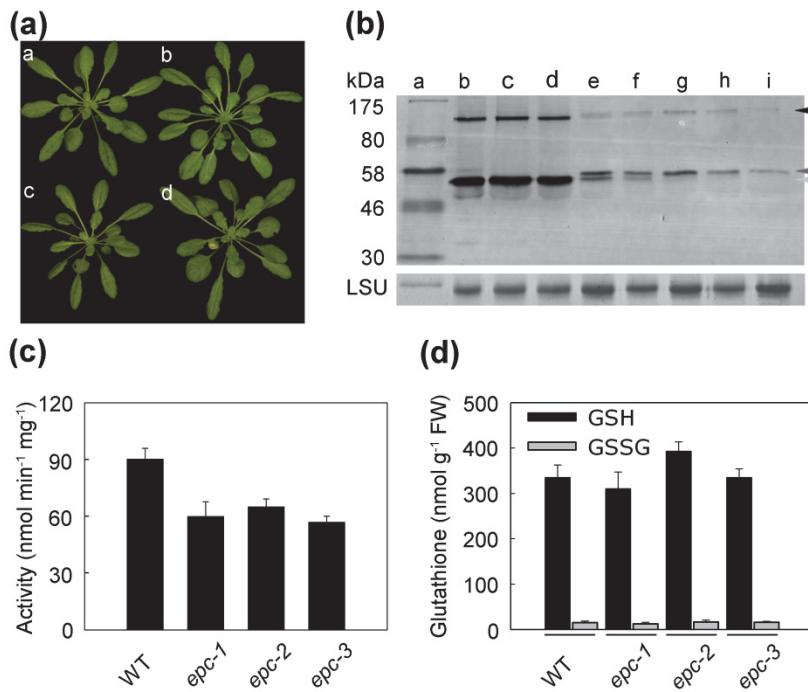
964 **Figure 2**



965

966 **Fig. 2** Immunogold localization of GR2 in plastid complemented *gr2* deletion lines. Fixed and
967 dissected mature leaves were probed with a primary antibody raised against *Arabidopsis* GR2. C:
968 chloroplast; M: mitochondrion; Px: peroxisome; V: vacuole; N: nucleus; St: starch grain. Bars,
969 1 μm . (a) WT. (b) Line *pc-2* ('plastid complemented') with $\text{TK}_{\text{TP}}\text{-}\Delta_{1-77}\text{GR2}$ expressed from the 35S
970 promoter. (c) Line *epc-2* with $\text{TK}_{\text{TP}}\text{-}\Delta_{1-77}\text{GR2}$ expressed from the endogenous GR2 promoter. (d)
971 Quantitative analysis of gold particles observed in electron microscopy micrographs. Values are
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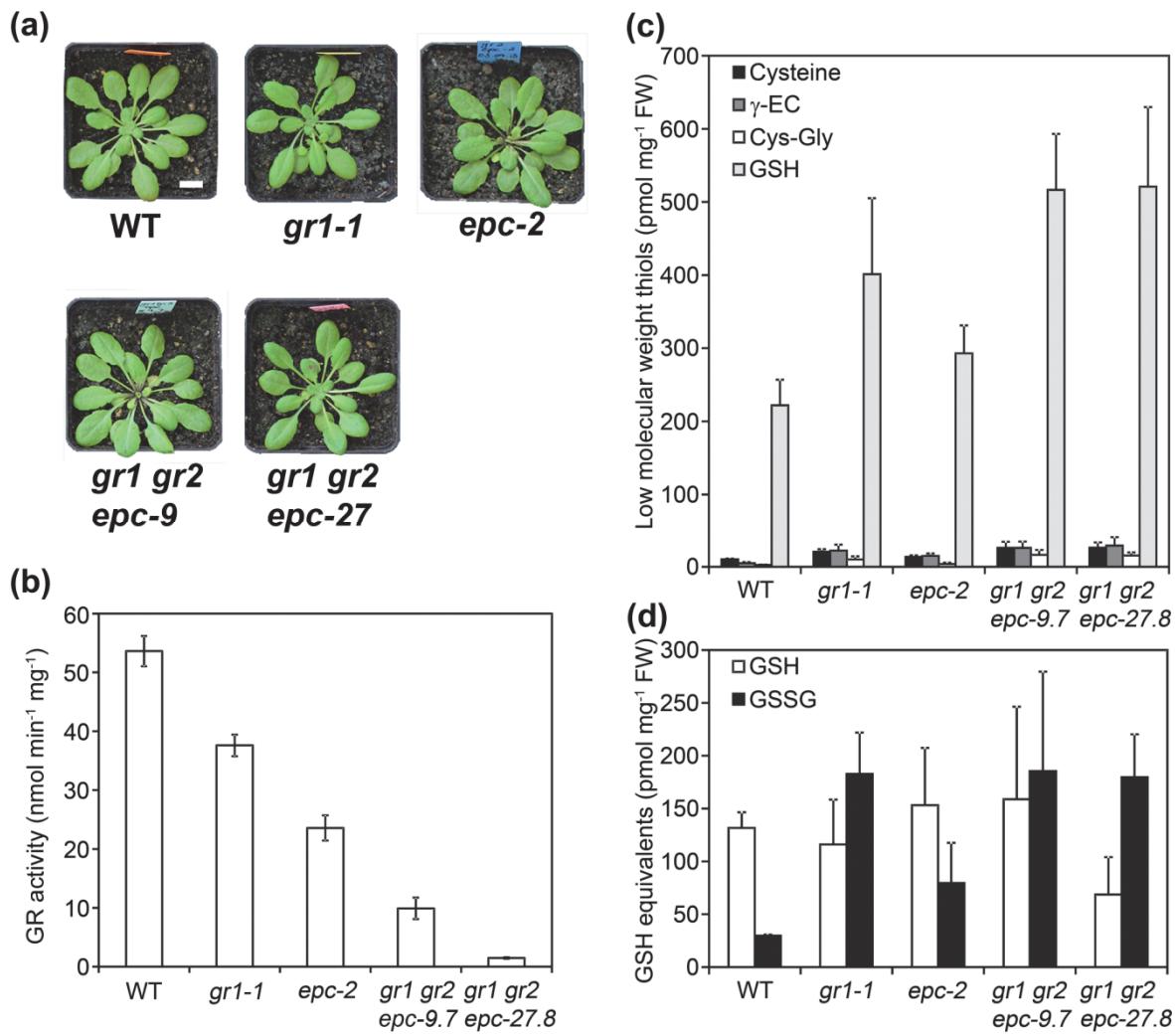
975 **Figure 3**



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977

978 **Fig. 3** Characterization of lines expressing plastid-targeted GR2 controlled by its endogenous
979 promoter. (a) Growth phenotypes of WT (a) and three independent homozygous *gr2* deletion
980 mutants complemented with *GR2_{pro}:TK_{TP}-Δ₁₋₇₇GR2*: *epc-1* (b), *epc-2* (c), *epc-3* (d). All transgenic
981 plants were from the Basta®-selected T1 generation. (b) Protein gel blot analysis of transgenic
982 Basta®-selected T1 lines transformed with complementation constructs driven by the
983 endogenous *GR2* promoter and targeted to either mitochondria or plastids. Loading was as
984 follows: a: Pre-stained molecular mass standard, b: WT, c: WT transformed with *GR2_{pro}:SHMT_{TP}-*
985 *Δ₁₋₇₇GR2*, d: *gr2^{+/−}* transformed with *SHMT_{TP}-Δ₁₋₇₇GR2*, e: WT transformed with *GR2_{pro}:TK_{TP}-Δ₁₋*
986 *77**GR2*, f: *gr2^{+/−}* transformed with *GR2_{pro}:TK_{TP}-Δ₁₋₇₇GR2*, g-i: three independent homozygous *gr2*
987 deletion lines transformed with *GR2_{pro}:TK_{TP}-Δ₁₋₇₇GR2* (*epc-1*, *epc-2* and *epc-3*). Arrows indicate
988 protein bands with the size of 110 kDa (black), 55 kDa (dark grey) and 53 kDa (light grey). (c) GR
989 activity in total protein extract from leaves. Proteins were extracted from WT and T2 plants of
990 the homozygous *gr2* lines *epc-1*, *epc-2* and *epc-3*. Means ± SD of three independent plants of
991 each line are shown. (d) Contents of oxidized and reduced glutathione. After extraction of leaf
992 tissue of 5-week-old plants reduced glutathione (GSH) and glutathione disulfide (GSSG) were
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994 **Figure 4**

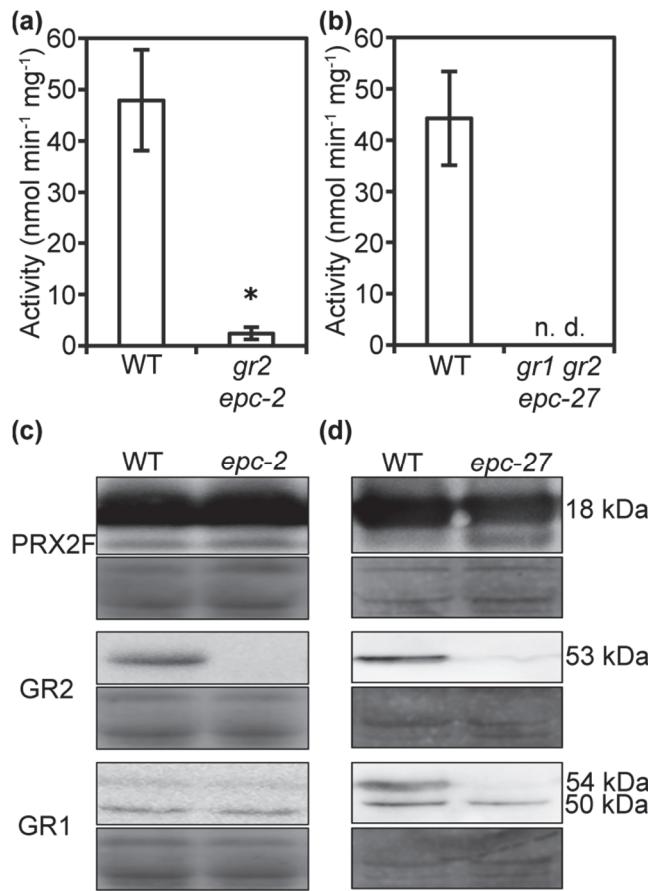


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997 **Fig. 4** Glutathione reductase activity and low molecular weight thiols of *gr1 gr2* double
998 homozygous mutants complemented with plastid-targeted GR2. (a) Rosette phenotypes of plants
999 grown on soil for six weeks under short day conditions (8 h : 16 h, light : dark). Bar, 1 cm. (b) GR
1000 activity in total protein extracts from leaves. Proteins were extracted from WT, *gr1*, plastid
1001 complemented *gr2* (*epc-2*) and two *gr1 gr2* double mutants (*epc-9* and *epc-27*) that were
1002 complemented with *GR2_{pro}:TK_{TP}-Δ₁₋₇₇GR2*. Means ± SD of three independent plants of each
1003 complemented line are shown. (c) Low-molecular weight thiols analysed by HPLC from leaf
1004 extracts. GSH in this case refers to total glutathione. Means ± SD, *n* = 3. (d) Reduced glutathione
1005 (GSH) and glutathione disulfide (GSSG) determined by HPLC in leaf extracts. Means ± SD, *n* = 3.
1006

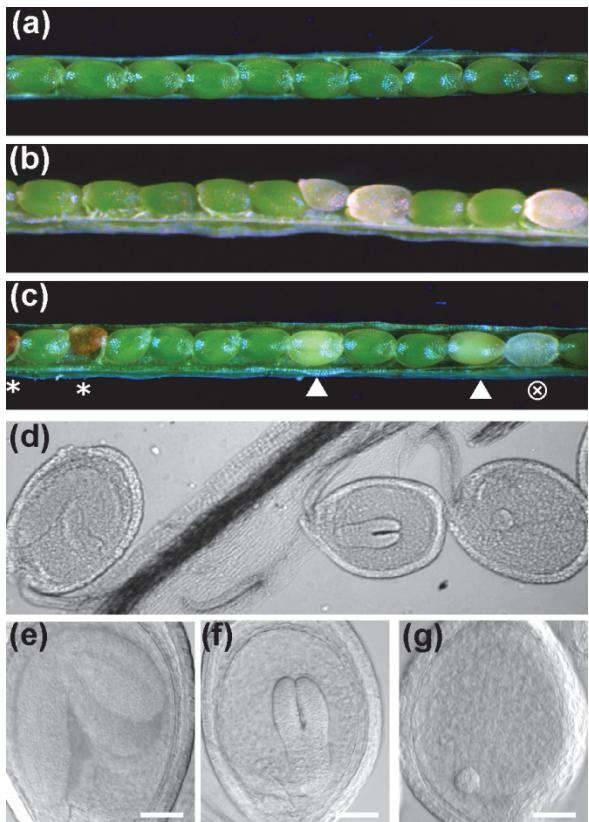
1007 **Figure 5**



1008

1009 **Fig. 5** Glutathione reductase activity in isolated mitochondria. Intact mitochondria were
1010 isolated from two-week old hydroponically grown *Arabidopsis* plants (WT and *epc-2* lacking
1011 endogenous GR2, or *epc-27* lacking both endogenous GRs, respectively). Activities represent
1012 the means from four independent preparations with six technical replicates. Error bars
1013 represent SD. (a, b) GR activity. n.d. = not detected; * indicates $P < 5 \times 10^{-4}$. (c, d) Protein gel
1014 blots of PRXII F, GR2 and GR1 in mitochondrial preparations. Loading controls were stained with
1015 amido black. In addition to GR1 (54 kDa), the GR1 antibody detected also a smaller protein of
1016 about 50 kDa (Fig. 5d). This band, however, appears to result from a side activity against
1017 another mitochondrial protein that is detectable in concentrated isolated mitochondria, but not
1018 in whole leaf extracts (Fig. S3b). Mitochondria contain two lipoamide dehydrogenases (MTLPD1
1019 and MTLPD2) that are closely related to GR and are both predicted with a molecular mass of
1020 49.9 kDa after cleavage of the mitochondrial target peptide (Lutziger & Oliver, 2001). Thus, it is
1021 likely that the additional band results from a cross reaction of the antibody with MTLPDs.

1022 **Figure 6**

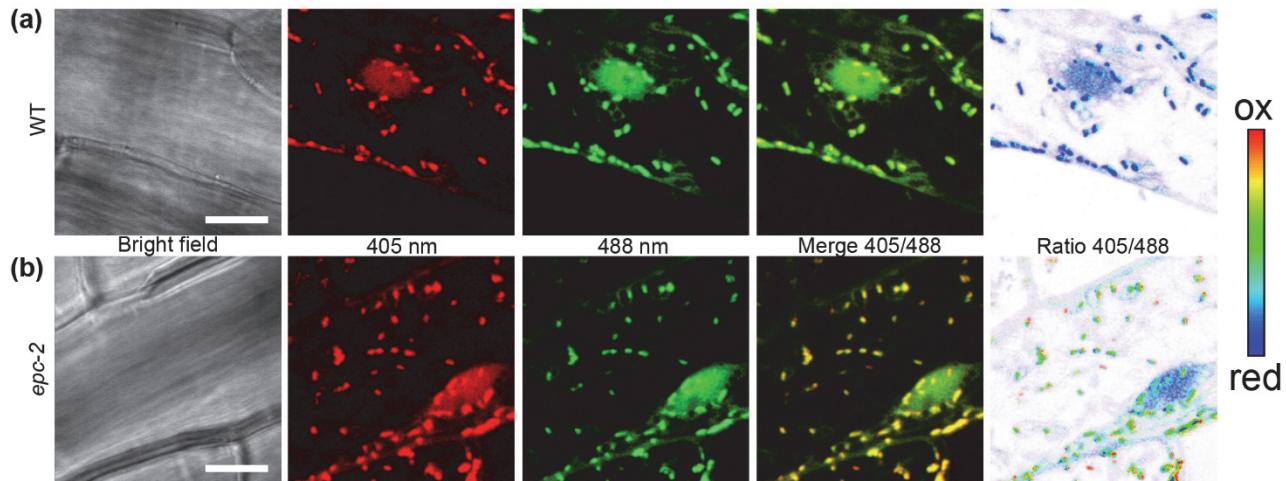


1023

1024 **Fig. 6** Characterization of *gr2 gsh1* double mutants. (a) WT siliques at 14 d after fertilization (DAF).
1025 (b) Siliques of self-fertilized heterozygous *gr2* plant displaying 25 % aborted seeds. (c) Siliques of
1026 self-fertilized double heterozygous *gr2^{+/−} gsh1^{+/−}* plant displaying segregation of green WT seeds,
1027 partially bleached *gsh1* seeds (▲), brownish early aborted *gr2* seeds (*), and transparent seeds
1028 (⊗) that remain fully turgescent significantly longer than *gr2* seeds. (d-g) DIC images of ovules
1029 developed in a siliques of a self-fertilized double heterozygous *gr2^{+/−} gsh1^{+/−}* plant 14 DAF. Bars, 50
1030 μm.

1031

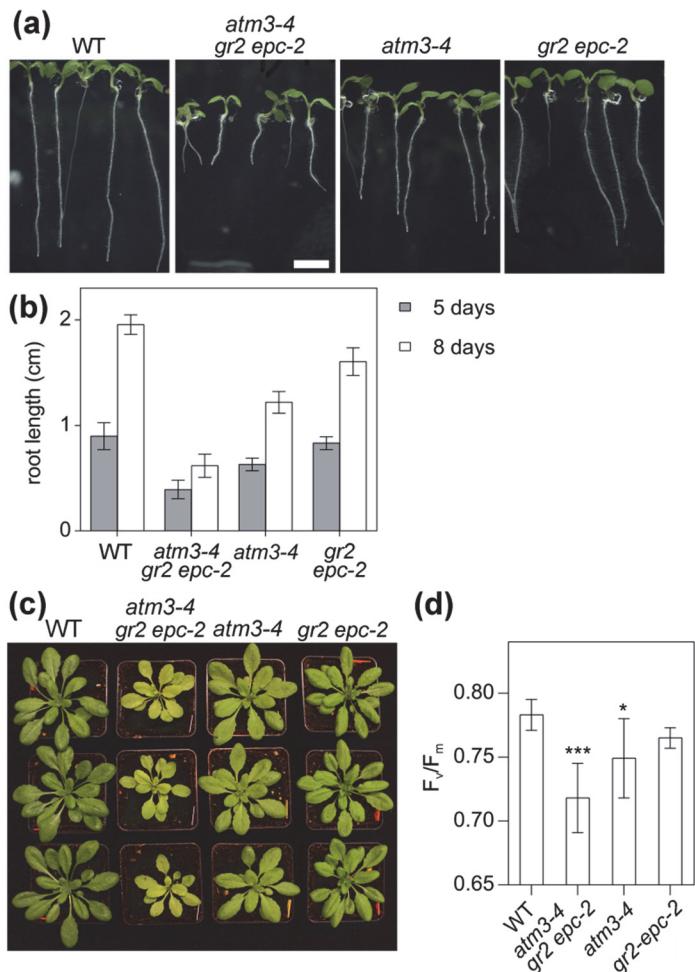
1032 **Figure 7**



1033

1034 **Fig. 7** Simultaneous imaging of the local glutathione redox status in mitochondria and the cytosol.
1035 The ratiometric redox probe roGFP2-Grx1 was expressed with a SHMT_{TP} mitochondrial targeting
1036 sequence driven by a 35S_{pro}. After transformation lines with residual roGFP2-Grx1 in the cytosol
1037 were selected and used for simultaneous redox imaging in mitochondria and the cytosol. Images
1038 show hypocotyl cells in WT seedlings (a) or plastid complemented *gr2*^{-/-} seedlings (b). Images
1039 taken after excitation at 405 nm are false colour coded in red and images taken after excitation
1040 at 488 nm are coded in green in order to visualize a pronounced colour change in the merge
1041 image from green to yellow in the mitochondria. Bars, 10 μ m.
1042

1043 **Figure 8**

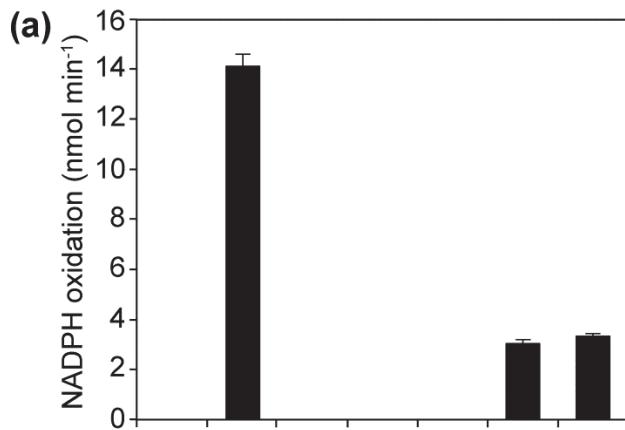


1044

1045 **Fig. 8** *atm3-4 gr2 epc2* double mutants show a semi-dwarf and chlorotic phenotype. (a) 8-d-old
1046 seedlings of the double mutant compared to WT and single mutants. Bar, 0.5 cm. (b) Root length
1047 of the mutants compared to WT. Plants were grown on $\frac{1}{2}$ MS medium containing 0.5 % sucrose
1048 and 0.8 % phytagel for 5-8 days under long-day conditions after stratification for 2 d (means \pm
1049 SD, $n = 5-12$). (c) Plants were grown under long-day conditions for four weeks. (d) Chlorophyll
1050 fluorescence of 4-week-old plants grown on soil under long-day conditions (means \pm SD, $n = 7$).
1051 The statistical analysis (one way ANOVA with post hoc Holm-Sidak comparisons for WT vs.
1052 mutant) indicates significant changes; * $P \leq 0.05$; *** $P \leq 0.001$.

1053

1054 **Figure 9**



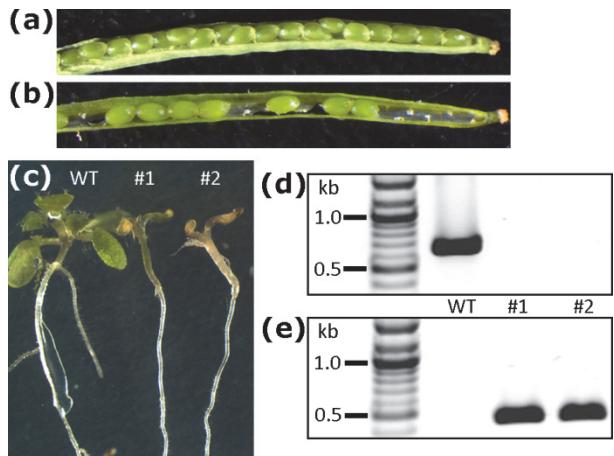
(b)

	GR2	TRXh2	TRXo1
K_m (μM)	12.8 ± 0.5	2.1x10 ³ ± 0.1x10 ³	4.3x10 ³ ± 0.3x10 ³
k_{cat} (s ⁻¹)	6.34x10 ⁴ ± 0.79x10 ⁴	0.96x10 ⁴ ± 0.02x10 ⁴	1.63x10 ⁴ ± 0.07x10 ⁴
k_{cat}/K_m (μM ⁻¹ s ⁻¹)	4953 ± 810.7	4.6 ± 0.3	3.9 ± 0.4

1055

1056 **Fig. 9** Mitochondrial TRXs in conjunction with NTRA can reduce GSSG *in vitro*. (a) Activity is
1057 monitored as NADPH oxidation. Enzymes and substrates were used at the following
1058 concentrations: NADPH, 250 μM; GSSG, 1 mM; GR2, 0.01 μM; TRXh2 and TRXo1, 2 μM; NTRA, 1
1059 μM; Means ± SD ($n = 3$). Note that the amount of disulfide reductase protein varied between the
1060 assays. (b) Steady-state kinetic parameters of GR2 and TRX-dependent GSSG reduction systems.

1061 **Figure 10**



1062

1063 **Fig. 10** Characterization of *ntra ntrb gr2* triple mutants expressing plastid-targeted GR2 under
1064 control of its endogenous promoter. (a) WT siliques 20 d after fertilization. (b) Siliques from a plant
1065 homozygous for both *ntra* and *gr2*, complemented with *p/GR2* and heterozygous for *ntrb*. (c)
1066 Phenotype of mutant seedlings 18 d after germination. (d, e) Genotyping of seedlings shown in
1067 panel c for *NTRB* (d) and the *ntrb* T-DNA insertion (e).
1068