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Plastidial and cytosolic thiol reductases participate in the control of stomatal functioning

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Abstract:	<p>Stomatal movements via the control of gas exchanges condition plant growth in relation to environmental stimuli through a complex signaling network involving reactive oxygen species that lead to post-translational modifications of Cys and Met residues, and alter protein activity and/or conformation. Thiol-reductases (TRs), which include thioredoxins, glutaredoxins (GRXs) and peroxiredoxins (PRXs), participate in signaling pathways through the control of Cys redox status in client proteins. Their involvement in stomatal functioning remains poorly characterized. By performing a mass spectrometry-based proteomic analysis, we show that numerous thiol reductases, like PRXs, are highly abundant in guard cells. When investigating various Arabidopsis mutants impaired in the expression of TR genes, no change in stomatal density and index was noticed. In optimal growth conditions, a line deficient in cytosolic NADPH-thioredoxin reductases displayed higher stomatal conductance and lower leaf temperature evaluated by thermal infrared imaging. In contrast, lines deficient in plastidial 2-CysPRXs or type-II GRXs exhibited compared to WT reduced conductance and warmer leaves in optimal conditions, and enhanced stomatal closure in epidermal peels treated with abscisic acid or hydrogen peroxide. Altogether, these data strongly support the contribution of thiol redox switches within the signaling network regulating guard cell movements and stomatal functioning.</p>

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1 **Plastidial and cytosolic thiol reductases participate in the control of stomatal functioning**

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30 **Abstract**

31 Stomatal movements *via* the control of gas exchanges condition plant growth in relation to
32 environmental stimuli through a complex signaling network involving reactive oxygen species
33 that lead to post-translational modifications of Cys and Met residues, and alter protein activity
34 and/or conformation. Thiol-reductases (TRs), which include thioredoxins, glutaredoxins
35 (GRXs) and peroxiredoxins (PRXs), participate in signaling pathways through the control of
36 Cys redox status in client proteins. Their involvement in stomatal functioning remains poorly
37 characterized. By performing a mass spectrometry-based proteomic analysis, we show that
38 numerous thiol reductases, like PRXs, are highly abundant in guard cells. When investigating
39 various *Arabidopsis* mutants impaired in the expression of *TR* genes, no change in stomatal
40 density and index was noticed. In optimal growth conditions, a line deficient in cytosolic
41 NADPH-thioredoxin reductases displayed higher stomatal conductance and lower leaf
42 temperature evaluated by thermal infrared imaging. In contrast, lines deficient in plastidial 2-
43 CysPRXs or type-II GRXs exhibited compared to WT reduced conductance and warmer leaves
44 in optimal conditions, and enhanced stomatal closure in epidermal peels treated with abscisic
45 acid or hydrogen peroxide. Altogether, these data strongly support the contribution of thiol
46 redox switches within the signaling network regulating guard cell movements and stomatal
47 functioning.

48 **Summary statement:** The contribution of thiol reductases to the signaling network
49 governing stomatal functioning remains poorly known. Using proteomics, we show that
50 numerous thiol reductases are abundant in a guard cell-enriched fraction, and we unveil a
51 stomatal phenotype in *Arabidopsis* mutants for thiol reductases.

52 **Keywords:** *Arabidopsis thaliana*, glutaredoxin, guard cell, peroxiredoxin, proteomics,
53 thiol reductase, signaling, stomata, thioredoxin.

54 **Introduction**

55 The movements of stomata, pores delimited by two guard cells, govern gas exchange and
56 transpiration at the leaf surface. Plant growth is thus conditioned by stomatal opening, which
57 allows CO₂ supply for photosynthetic assimilation, and ensures appropriate hydration and
58 temperature in leaf tissues (Hetherington and Woodward, 2003; Berry et al, 2010). During
59 stomatal movements, the turgor and volume of vacuole in guard cells change due to osmolyte
60 fluxes thanks to the fine regulation of channels and carriers in plasma and vacuole membranes
61 (Kim et al, 2010; Roux and Leonhardt, 2018). These movements result from the sensing of
62 environmental stimuli (primary input signals), the production of secondary signals such as
63 hormones and the signal transduction *via* numerous messengers including kinases, calcium ions
64 and reactive oxygen species (ROS) (Murata et al, 2015; Kollist et al, 2014).

65 Stomatal opening is mainly caused by blue light and photosynthetic active radiations
66 (Shimazaki et al., 2007; Kollist et al, 2014). In comparison, multiple primary stimuli promote
67 stomatal closure to limit water loss in response to the varying environment that plants face (Kim
68 et al, 2010; Murata et al, 2015). Abscisic acid (ABA) plays a central role in this process notably
69 *via* the production of ROS, which are recognized signaling molecules in stomatal movements
70 (Kwak et al, 2003; Song et al, 2014; Sierla et al, 2016). ROS fulfill signaling functions in
71 development and responses to environment by reacting with antioxidant molecules, lipids and
72 proteins (Foyer and Noctor, 2016). Oxidation of sulfur-containing amino acids, Cys and Met,
73 leads to redox post-translational modifications and switches altering enzymatic activity, protein
74 conformation or subcellular distribution (Delaunay et al, 2002; Tada et al, 2008; Geigenberger
75 et al., 2017).

76 Plant thiol reductases (TRs), which display catalytic Cys, accomplish key biological
77 functions including signaling by controlling Cys and Met redox status in partner proteins
78 (Rouhier et al, 2015; Geigenberger et al, 2017; Rey and Tarrago, 2018). They belong to

79 multigene families (Tarrago et al, 2009; Meyer et al, 2012; Liebthal et al, 2018), and those
80 considered here include thioredoxins (TRXs), glutaredoxins (GRXs), methionine sulfoxide
81 reductases (MSRs) and the related thiol peroxidases: peroxiredoxins (PRXs) and glutathione
82 peroxidases (GPXs) named by analogy to mammalian counterparts albeit using preferentially
83 TRXs as reductants (Navrot et al, 2006). Plant TRXs are well-characterized small disulfide
84 reductases carrying a Cys-Xxx-Xxx-Cys active site. Plastidial isoforms regulate photosynthetic
85 metabolism in relation with light and redox pressure (Schürmann and Buchanan, 2008;
86 Courteille et al, 2013; Naranjo et al, 2016). Cytosolic h-type TRXs are supplied in reducing
87 power by NADPH-TRX reductases (NTRs), and participate in the mobilization of seed reserves
88 upon germination or in responses to biotic constraints (Sweat and Wolpert, 2007; Hägglund et
89 al, 2016). GRXs are oxidoreductases closely related to TRXs using glutathione (GSH) as an
90 electron donor (Rouhier et al, 2008). Among TRX and GRX targets, thiol peroxidases play
91 essential signaling roles *via* the control of peroxide concentration or direct thiol oxidation in
92 protein partners (Day et al., 2012; Rhee and Woo, 2011; Stöcker et al., 2017). Thus, plastidial
93 2-CysPRXs participate in photosynthesis deactivation during the light-dark transition (Yoshida
94 et al., 2018). Their signaling function is further supported by their ability to interact with more
95 than 150 proteins (Cerveau et al, 2016a). Plant TRXs and GRXs, by modifying the Cys redox
96 status in a very broad set of proteins (Rouhier et al, 2005; Montrichard et al, 2009), are involved
97 in multiple metabolic, developmental and stress-related processes (Rey et al., 2005; Vieira Dos
98 Santos and Rey, 2006; Bashandy et al., 2010; Knesting et al, 2015; Rouhier et al, 2015;
99 Liebthal et al, 2018). However, their involvement in stomatal functioning remains elusive. In a
100 pioneer work, Jahan et al. (2008) reported enhanced ABA-induced stomatal closure in
101 Arabidopsis GSH-deficient plants. Proteomic analyses showed high abundance of several TRs
102 in Arabidopsis and *B. napus* guard cells (Zhao et al., 2008; Zhu et al., 2009; 2010), and
103 identified potential TR partners in these cells (Zhu et al., 2014; Zhang et al., 2016). Of note,

104 GPX3 based on the phenotype of Arabidopsis mutants and on its ability to interact with ABA-
105 related signaling actors was presumed to act as both a ROS scavenger and a redox transducer
106 in guard cells (Miao et al, 2006). Changes in stomatal functioning have been also reported in
107 plants modified for the abundance of plastidial 2-CysPRXs (Mao et al., 2018), mitochondrial
108 TRXs o1 and h2 (Calderon et al., 2018; da Fonseca et al., 2019), or cytosolic GRXS17 (Hu et
109 al., 2017).

110 Altogether, these data suggest that thiol redox switch, *via* the action of TRs, is a major
111 component of the cell guard signaling network. However, the knowledge on this process is still
112 very poor due to the limited data regarding the presence and abundance of TRs in guard cells.
113 In this work, we characterized the proteome of a fraction highly enriched in guard cells and we
114 present a detailed analysis of the expression of Arabidopsis *TR* genes. We found that numerous
115 TRs are abundant in this fraction, and uncovered a stomatal phenotype for mutants deficient in
116 NTRs, PRXs or GRXs. Finally, we discuss the potential functions of TRs in the signaling
117 pathways governing stomatal movements.

118

119 **Material and methods**

120 **Plant material**

121 Arabidopsis plants were grown in the “Phytotec” platform (CEA, DRF, BIAM) from
122 sowing in soil in standard conditions under an 8-h photoperiod and a photon flux density of 200
123 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a temperature regime of 22/18°C (day/night) and a relative humidity
124 of 55% as reported in Cerveau et al. (2016b). The genotypes used were wild-type Col-0, two
125 T-DNA mutant lines for *TRXh3* (SAIL_314_G04 and SALK_111160), two T-DNA mutant
126 lines for *TRXo1* (SALK_143294C and SALK_042792), one T-DNA mutant line for *PRXQ*
127 (SAIL_742_G10), one T-DNA mutant line for *GRXS17* (SALK_021301), one double T-DNA
128 mutant line for *2-CysPRXA* and *2-CysPRXB* (Cerveau et al., 2016b), one double T-DNA mutant

129 line for *NTRA* and *NTRB* (Reichheld et al., 2007), several lines either impaired for or over-
130 expressing *GRXS14* and/or *GRXS16* (Rey et al., 2017), and the *ost2-2D* mutant (Merlot et al.,
131 2007). Arabidopsis leaf genomic DNA from *trxh3* and *prxq* mutants was extracted using the
132 Phire plant direct kit (ThermoFisher) to perform PCR using appropriate primers corresponding
133 to 5' and 3' ends of coding sequences, Taq DNA polymerase (Life Technologies), and the
134 GeneAmp PCRSystem 2700 (Applied Biosystems). The absence of expression in homozygous
135 plants was checked at the protein level. Rosette weight measurements were performed
136 following 5 or 6 weeks of growth in standard conditions. Plants expressing promoter-GUS
137 reporter constructs were previously described (Reichheld et al., 2002; Reichheld et al., 2007).
138 GUS histochemical staining was performed according to Reichheld et al. (2007).

139

140 **Preparation of fractions enriched in guard cells from leaf epidermis**

141 Epidermis were prepared from six week-old plants grown in standard conditions. Leaves
142 (35 g) were ground at max speed for 3 min in 1 L bowl of a Waring Blender™ containing about
143 200 ml deionised water and a handful of crushed ice. The suspension was filtered through 100-
144 µm nylon sieve, and then rinsed off thoroughly with deionised water. The obtained retentate
145 was rapidly dried on paper towels, suspended in 10 mM Tris-Mes buffer pH 6.0 containing 30
146 mM KCl 0.5 M NaCl and 0.5 mM SDS (1 g material in 5 mL buffer). The suspension was
147 gently stirred at room temperature for 15 min before filtration (100 µm), rinsed with deionised
148 water and dried on paper towels. After rapid freezing in liquid N₂, samples were stored at -20°C
149 before protein extraction and analyses.

150

151 **Protein extraction from leaf and epidermis fractions**

152 Protein extracts, prepared according to Wang et al., (2006) from 0.5 g of leaf or epidermis,
153 and sampled from three independent plant cultures were mixed to gain a representative sample

154 of each type of material. Protein concentration was determined using the “Protein
155 Quantification BCA Assay” kit (Interchim). The resulting protein pellets were resuspended in
156 sodium phosphate buffer 0.1 M pH 7.2 containing 0.15M NaCl and 1% SDS, diluted (1:4) in 4
157 X Laemmli buffer and heat-denatured 5 min at 95°C.

158

159 **Mass spectrometry-based proteomic analyses**

160 Twenty-five micrograms of proteins were stacked in a single band in the top of a SDS-
161 PAGE gel (4-12% NuPAGE, Life Technologies) and stained with Coomassie blue R-250 before
162 in-gel digestion using modified trypsin (Promega, sequencing grade) as previously described
163 (Salveti et al., 2016). Resulting peptides were fractionated by tip-based strong cation exchange
164 and analysed by nanoliquid chromatography coupled to tandem mass spectrometry as
165 previously described (Farhat et al. 2020).

166 RAW files were processed using MaxQuant (Tyanova et al., 2016) version 1.5.8.3. Spectra
167 were searched against the Uniprot database (Arabidopsis thaliana reference proteome, February
168 2017 version), the frequently observed contaminants database embedded in MaxQuant, and the
169 corresponding reversed databases. Trypsin was chosen as the enzyme and two missed cleavages
170 were allowed. Precursor and fragment mass error tolerances were set at their default values.
171 Peptide modifications allowed during the search were: Carbamidomethyl (C, fixed), Acetyl
172 (Protein N-term, variable) and Oxidation (M, variable). Minimum number of peptides, razor +
173 unique peptides and unique peptides were set to one. Maximum false discovery rates were set
174 to 0.01 at PSM and protein levels. The match between runs option was activated. Quantification
175 of proteins was based on unique + razor peptides. For sample comparison, intensity-based
176 absolute quantification (iBAQ) values were loaded into ProStaR (Wieczorek et al., 2017).
177 Peptides and proteins identified in the reverse and contaminant databases were discarded. After
178 log₂ transformation, iBAQ values were median normalized, and their log₂(fold change)

179 between conditions calculated, after missing value imputation (replacing missing value by the
180 mean first percentile of both samples).

181

182 **Bioinformatics and data processing**

183 Minimum number of peptides, razor + unique peptides and unique peptides were set to one.
184 Maximum false discovery rates were set to 0.01 at PSM and protein levels. The match between
185 runs option was activated. Quantification of proteins was based on unique + razor peptides. For
186 comparison of samples, intensity-based absolute quantification (iBAQ) values were loaded into
187 ProStaR (Wieczorek et al., 2017). Peptides and proteins identified in the reverse and
188 contaminant databases were discarded. After log₂ transformation, iBAQ values were median
189 normalized and their log₂ (fold change) between conditions calculated.

190

191 **Electrophoresis and immunoblot analysis**

192 Proteins were SDS-PAGE separated in reducing conditions, and either stained using
193 Coomassie blue (Imperial™ Protein Stain, Thermo Scientific) or electroblotted onto 0.45 μm
194 nitrocellulose (Pall Corporation) for immunoblot analysis. Primary antibodies raised against
195 At2-CysPRX (Broin *et al.*, 2002), poplar PRXQ (Rouhier *et al.*, 2004), AtPRXII-B (Bréhelin
196 *et al.*, 2003), AtTRX h3 (Reichheld *et al.*, 2007) and AtGRXS14 (Rey *et al.*, 2017) were used
197 diluted 1:10,000, 1:1000, 1:10,000, 1:10,000 and 1:800, respectively. Antibodies against
198 hyperoxidized 2-CysPRX (LF-PA0004; AbFrontier, Seoul, Korea) were diluted 1:3,000. The
199 serum against spinach RubisCO (kindly provided by Dr. GH Schmid, University of Bielefeld,
200 Germany) was diluted 1:10,000. Bound antibodies were detected using either a goat anti-rabbit
201 secondary antibody coupled to a fluorescent molecule at a dilution of 1:10,000 (Alexa Fluor
202 680, Invitrogen) using the 'Odyssey Infrared Imager' at 680 nm (Licor, Lincoln, NE, USA) or
203 an anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Sigma).

204

205 Chlorophyll content determination

206 One-cm diameter leaf disks were taken from well-expanded adult leaves and crushed in
207 1 mL 80% acetone. Following vigorous shaking, overnight storage in the dark at 4°C and
208 centrifugation (14,000 g, 10 min), the chlorophyll content was measured
209 spectrophotometrically, and calculated according to Lichtenthaler (1987).

210

211 Preparation of adaxial and abaxial leaf epidermis for stomatal counting.

212 Adaxial (upper) and abaxial (lower) epidermis were prepared according to a protocol
213 derived from the “Tape-Arabidopsis Sandwich” reported by Wu et al., (2009). The upper or
214 lower epidermal surface was stuck on a strip of Time tape (Time Med, IL., USA) while the
215 opposite surface was affixed to a strip of Scotch Crystal tape (3M, Minn., USA). The Scotch
216 Crystal tape was then sharply removed, and put in a Petri dish containing 10 mM Tris-Mes
217 buffer pH 6.0, 30 mM KCl to avoid tissue desiccation. Then with the help of a microscope cover
218 slip, the strip has been scraped off to get rid, the best as possible, of mesophyll tissues. Stomata
219 were counted with the help of a Leica LMD6000 microscope. Stomatal index was calculated
220 using the formula $S/(E + S)$, S being the number of stomata per area unit and E the number of
221 ordinary epidermal cells in the same area.

222

223 Measurements of stomatal aperture

224 The abaxial side of half leaf blades from two 4–5-week-old plants of the same genotype
225 grown in standard conditions was stuck on cover slips and peeled. Samples were then placed in
226 Petri dishes containing 20 mL of buffer containing 10 mM MES/Tris pH 6.0, 30 mM KCl. After
227 30 min in the dark, epidermal peels (except controls) were transferred under light (300 μmol
228 $\text{photons.m}^{-2}.\text{s}^{-1}$) at 22°C for 2.5 h in order to ensure that most stomata were open before

229 treatments. For promotion of closure, 20 μL of stock solutions of ABA in ethanol and of H_2O_2
230 in water were directly diluted in the buffer in contact with peels. Thereafter, samples were
231 incubated in the light for 2.5 h before aperture measurements according to Merlot et al., (2007).
232 To avoid bias results, double blind experiments were performed, so that, experimenters in
233 charge of measurements were not informed of genotype or treatment. Values reported are the
234 means \pm SD of two independent experiments, for which 60 aperture widths were measured each
235 time. Error bars represent standard deviations of the means.

236

237 **Porometry measurements**

238 Stomatal conductance of fully expanded leaves was measured on 6-week old well
239 drained plants grown in standard conditions. Measurements were carried out using a hand-held
240 AP4 porometer (Delta-T Devices, Cambridge, UK) on the abaxial leaf side in the middle of the
241 light period, and two to four hours after watering by flooding. Usually, eight measurements per
242 plant were made on 6 to 12 independent plants per genotype. Due to variations in calibration
243 resulting from slight differences in the moisture of the paper used as a reference, differences
244 were noticed in the level of conductance in WT plants between experiments despite very similar
245 growth and measurement conditions. All experiments and repetitions for characterizing one
246 genotype and corresponding WT plants were carried out using the same calibration reference.

247

248 **Infrared determination of leaf temperature**

249 Plants were cultivated on soil as described above for up to 6 weeks in a dedicated
250 phytotron, in which low relative humidity ($45\% \pm 5\%$) and low wind speed were applied the
251 day before infrared thermographic imaging to ensure optimal contrast between lines. Images
252 were acquired using a Thermacam PM250 infrared camera as described by Costa et al., (2015).

253

254 **Results**

255 **Analysis of *TR* gene expression in guard cells by transcriptomics**

256 As a first step, using transcriptomic data from guard and mesophyll cell protoplasts
257 representing a large part of Arabidopsis genes (Yang et al., 2008), we extracted those available
258 for *TR* genes. Based on relative expression values, we noticed that most TR transcripts are
259 present in both cell types (data not shown), and observed that a number of them displayed a
260 ratio between the two values higher than 2, indicating preferential expression in one cell type.
261 This was noticed for *NADPH-Thioredoxin Reductase A (NTRA)*, which encodes the reducer for
262 cytosolic TRXs and exhibits higher transcript level in guard cells (Fig. S1A). Regarding *TRX*
263 genes (Fig. S1A), we observed that *TRXh3* exhibits compared to others the highest expression
264 in guard cells, and a substantially higher transcript level compared to mesophyll cells. Most
265 interestingly, nine other *TRX* genes (*h2*, *h4*, *m3*, *o1*, *o2*, *TDX*, *CLOT*, *NRX1* and *LILIUM-1*),
266 coding for various typical or unusual isoforms located in the main subcellular compartments,
267 exhibited higher expression in guard cells, while two (*h5* and *CDSP32*) were preferentially
268 expressed in mesophyll cells. Regarding other *TR* genes (Fig. S1B), we noticed that *GRXs S15*
269 and *ROXY10*, *PRXII-B* and *GPX2*, and *MSRs A1* and *A4* displayed a transcript abundance higher
270 in guard cells compared to mesophyll cells. To deepen some of these microarray data, we
271 analyzed gene expression by promoter-GUS histochemistry (Fig S2). GUS staining was clearly
272 observed in guard cells of *PrTRXh2-*, *PrTRXh3-*, *PrPRXII-B-* and *PrNTRB-GUS* lines,
273 confirming the expression of these *TR* genes in guard cells.

274

275 **Preparation of a guard cell-enriched fraction**

276 We then investigated *TR* gene expression in guard cells by implementing a proteomic
277 approach. We did not used protoplasts since during preparation, redox homeostasis is
278 dramatically impaired (Papadakis and Roubelakis-Angelakis, 1999), leading to changes in the

279 expression, abundance, redox status and conformation of TRs. To obtain a fraction enriched in
280 guard cells, we treated epidermis from Arabidopsis plants as described in Experimental
281 Procedures. Microscope observations of this fraction revealed the presence of intact guard cells,
282 predominantly broken and open pavement and trichome cells, and some pieces of xylem vessels
283 (data not shown). The presence of chloroplasts served as an indication of cell integrity.
284 Vigorous shearing of leaf tissues and extensive washing of epidermis kept intact only guard
285 cells, which are protected from mechanical constraints due to their cell wall thickness. We
286 therefore assumed that a significant part of proteins in this extract, notably the soluble pool,
287 originates from guard cells. To estimate the enrichment, we counted guard cells in both
288 epidermal sides of all leaves of 6-week old plants, measured the sizes of representative guard
289 cells to estimate their average volume, and determined the protein concentrations in whole
290 leaves and in broken epidermis. Based on these measurements (Table S1), the total GC volume
291 has been rated at 0.13% of the leaf volume, and assuming that protein concentration ($2 \text{ mg}\cdot\text{g}^{-1}$
292 leaf FW) is identical regardless of cell type, we estimated this content in GC to $0.0026 \text{ mg}\cdot\text{g}^{-1}$.
293 Given that the protein concentration in grinded epidermis was $0.028 \text{ mg}\cdot\text{g}^{-1}$ FW, the inference
294 is that 9.3 % come from guard cells. Therefore, we calculated an enrichment factor of 71
295 ($9.3:0.13$) in broken epidermis that was thus termed guard cell-enriched (GCE) fraction. Finally,
296 we compared the protein patterns of leaf and GCE fractions by SDS-PAGE (Fig. 1A), and
297 observed clear differences, such as dramatically decreased abundance of RubisCO subunits at
298 50 and 13 kDa in the GCE fraction. This was confirmed in immunoblots, where a much higher
299 band intensity was observed for both subunits in the leaf extract (Fig. 1B). In contrast, the
300 intensity of two bands at 22 and 75 kDa was more elevated in the GCE fraction (Fig. 1A).

301

302

303

304 **Proteomic analysis of the GCE fraction and extracted abundance of guard cell actors**

305 The GCE fraction, along with a whole leaf extract prepared in a similar manner, were
306 analyzed by mass spectrometry-based proteomics, leading to the characterization of 4842
307 proteins identified with two peptides and quantified in at least in one of the two samples,
308 representing 4694 and 4248 proteins in GCE and leaf fractions, respectively (Table S2). To
309 estimate the relative abundance in each sample, we ranked proteins according to their computed
310 intensity-based absolute quantification (iBAQ, Schwanhäusser et al., 2011) values. The iBAQ
311 values were also used to compare the abundance of each protein between GCE and leaf
312 fractions. The functional categorization of the genes encoding the 25 % most abundant proteins
313 in each fraction has been determined using the Gene Ontology annotation retrieval tool
314 available on the TAIR site (https://www.arabidopsis.org/tools/go_term_enrichment.jsp). The
315 comparison uncovered some striking differences (Fig. 2), such as under-representation (- 9 to -
316 16%) of proteins related to plastid, chloroplast and thylakoid functioning in the GCE fraction,
317 consistent with the elimination of photosynthetic mesophyll cells during the preparation.

318 We also noticed enrichment (+ 9%) in the GCE fraction of extracellular and cell wall
319 proteins (Fig. 2), partly due to the presence of many cell phantoms originating from the
320 pavement. With the help of the reports of Irshad et al., (2008), Duruflé et al., (2019) and
321 Showalter et al., (2010) that characterized Arabidopsis cell wall proteome using bioinformatic
322 and proteomic approaches, we reckoned 322 proteins contributing to cell wall structure or
323 function (Table S3). A majority (85%) is more abundant in the GCE fraction, 16% being not
324 detected in leaf. Of note, based on microarray data (Yang et al., 2008), more than one third of
325 the genes encoding these proteins were found preferentially expressed in GC (ratio of
326 expression GC/MC higher than 2). Regarding extracellular proteins (Table S3), seven germins
327 and germin-like proteins (GLPs) are more abundant in the GCE fraction, two (GLP1-6 and
328 GLP5-1) being the first and the fifth most abundant proteins, respectively. GLPS are

329 glycoproteins associated with the extracellular matrix, encoded by 32 genes in Arabidopsis, and
330 involved in responses to osmotic constraints (Li et al., 2016). Among cell wall proteins more
331 abundant in the GCE fraction (Table S3), we noticed the presence of a large number of GDSSL-
332 type esterases/lipases, 18 out of 45 putative ones, which are notably involved in biotic stress
333 responses (Lai et al., 2017), and of 17 expansins out of the 36 genes identified in Arabidopsis
334 (Lee et al., 2001). Expansins interact with cell wall carbohydrates, cellulose and hemicellulose.
335 Most interestingly, expansin A1, which plays a critical role in stomatal movements (Wei et al.,
336 2011; Zhang et al., 2011), is one of the most abundant protein (rank 29) in the GCE fraction.

337 In other respects, Leonhardt et al. (2004) identified 63 genes preferentially expressed in
338 guard cells, 25 being present in our analysis. Table S4 shows that only one protein encoded by
339 this set of genes is more represented in the leaf fraction, and that 20 are more abundant in the
340 GCE fraction, eight being specific of this sample. Finally, to further figure out the validity of
341 the GCE fraction regarding stomatal functions, we scrutinized the protein list to find out
342 candidates previously described as key players in stomatal movements or development (Table
343 1). Hence, 56 of these proteins were pinpointed, 43 being more abundant (R value > 1.5) in the
344 GCE fraction than in leaf. Consistently, a large majority of genes encoding these proteins are
345 more expressed in guard cells than in mesophyll cells (Table S4) (Yang et al., 2008), and 22
346 were identified in Arabidopsis GC protoplasts (Zhao et al., 2008, 2010). Among the 56 proteins,
347 we identified 12 kinases and phosphatases, 14 ion channels, pumps and transporters, 3 ABA
348 receptors, and 19 enzymes and other proteins acting in osmoregulation in guard cells, in
349 addition to eight effectors involved in stomatal development and patterning. We also identified
350 GRF6 (14-3-3 λ) that is involved in blue light-mediated stomatal opening (Tseng et al., 2012),
351 and present in the GCE fraction at a high level (top 5%). 14-3-3 proteins (GRFs) are regulators
352 of stomatal movements (Cotelle and Leonhardt, 2016), through the binding of phosphorylated
353 client proteins such as phototropins, plasma membrane H⁺-ATPases or ion channels. In our

354 analysis, we detected 10 out of the 13 members of this family, all being more abundant in the
355 GCE fraction (Table S5). Taken together, these data indicate that grinding and extensively
356 washing epidermis is as an easy way to concentrate guard cell proteins as evidenced by the high
357 enrichment factor and the exclusive presence in the GCE fraction of numerous proteins known
358 to function in stomatal movements. This approach can be thus used for identifying new proteins,
359 the activity of which could be determinant for guard cell functioning.

360

361 **Abundance of TRs in the GCE fraction**

362 We thus analyzed the protein list from the GCE fraction to retrieve all TRs, and ranked
363 them according to extracted abundance (Table 2). Thirty-nine TRs, out of the approximately
364 100 genes belonging to these enzyme families in *Arabidopsis thaliana*, were identified. Most
365 interestingly, we observed the presence of various PRXs (PRXQ, 2-CysPRXA, PRXII-B,
366 PRXII-F) in the first decile, and of other TR types (NTRA, PRXII-E, TRX h3, GPX6, 2-
367 CysPRXB, MSRA2, NRX1) in the first quartile. Numerous TRs (NTRB, MSRA4, GPX1,
368 GPX2, TRX z, TRX h2, TRX m2, NTRC, TRX f1) were present in the first half of the ranking
369 list. Then, we compared the relative abundance of TRs in GCE and leaf fractions using the R
370 parameter (Table 2). When selecting those displaying a R value superior to 1.5, we pointed 19
371 TRs, many being much more abundant in the GCE fraction than in leaf, notably PRXII-B,
372 NTRA, TRX h3, MSRA2, NRX1, NTRB, GPX2, TRX h2, GPX3 and TRX CxxS1. Of note,
373 five TRs were only detected in the GCE fraction, namely TRX z, TRXh9, GRXS15, TDX and
374 TRX Liliun 6. In other respects, we also searched for the TRs relatively more abundant in the
375 leaf fraction. We found that all those having a very low R value (< 0.1) are involved in the
376 regulation of photosynthesis or in the maintenance of plastidial redox homeostasis. This is the
377 case of TRXs m1, m2, m4, f1, CDSP32, HCF164, and MSRB2. By crossing transcriptomic and
378 proteomic data (Fig. S1, Table 2), we observed good consistency between the two approaches.

379 Indeed, among the 17 *TR* genes displaying preferential expression in GC, 11 were identified by
380 proteomics and nine encode proteins more abundant in the GCE fraction. Finally, by comparing
381 with the pioneer proteomic works on guard cell protoplasts, in which a much lower number of
382 proteins (*ca* 1000) was identified, we noticed that all TRs detected in these reports (Zhao et al.,
383 2008; 2010) are present in the GCE fraction (Table 2). Altogether, these findings lead us to
384 conclude that numerous TRs are present in guard cells, several of them being very abundant.

385

386 **Western blot analysis of TR abundance in the GCE fraction**

387 We then assessed the abundance of some TRs in the GCE and leaf fractions by Western
388 blot analysis (Fig. 1B). We noticed a much higher amount of TRX h3 in the GCE fraction than
389 in leaf, and observed relative abundances of 2-CysPRXs, PRXQ, PRX-IIIE and PRXII-B in the
390 two fractions consistent with proteomic data. Further, we noticed comparable abundance in the
391 two fractions of GRXS14, which was not detected in the proteomic analysis. Most interestingly,
392 substantial changes in the redox status of 2-CysPRXs and GRXS14 were detected in the GCE
393 fraction. Indeed, while the level of hyperoxidized 2-CysPRXs was relatively low in leaf, as
394 already observed (Cerveau et al. 2016b; Cerveau et al. 2019), this form was much more
395 abundant in the GCE fraction. Regarding GRXS14, we previously reported that the GRX has
396 two redox forms showing differential migration (Rey et al., 2017). Whereas only one redox
397 GRXS14 form was detected in leaf proteins, two specific bands were revealed in the GCE
398 fraction. These data reveal that TRs could display specific redox features in guard cells
399 compared to photosynthetic cells.

400

401 **Phenotype of mutants impaired in the expression of *TR* genes**

402 To gain further insights about TR functions in stomatal biology, we investigated the
403 phenotype of mutants modified for the expression of *PRXQ*, *2-CysPRXs*, *NTRA* and *NTRB*,

404 *TRXh3*, *TRXo1*, *GRXS17*, *GRXS14* or *GRXS16*. These lines were selected based on protein
405 abundance in guard cells, mutant availability (Reichheld et al., 2007; Knuesting et al., 2015;
406 Cerveau et al., 2016b; Rey et al., 2017; Calderon et al., 2018), bibliographical data (Hu et al,
407 2017; Calderon et al, 2018; Mao et al, 2018), and preliminary experiments. Regarding *prxq* and
408 *trxh3* mutants, gene disruption was validated at the protein level (Fig. S3).

409 First, we investigated the growth of mutants in standard short-day, light, temperature and
410 watering conditions, in which stomata function in an optimal manner. Growth was characterized
411 by measuring the rosette fresh weight of 5- to 6-week old plants (Fig. S4). We previously
412 reported that Arabidopsis plants impaired for both *GRXS14* and *GRXS16* expression displayed
413 reduced growth (Fig. S5), whereas those over-expressing *GRXS14* exhibited higher rosette
414 weight (Rey et al., 2017). Plants deficient in *GRXS17* expression did not exhibit any growth
415 phenotype in short photoperiod (Fig. S4C) as already observed (Knuesting et al., 2015).
416 Regarding lines affected in plastidial PRXs, we did not observe any difference for *prxq* as
417 reported for another mutant (Lamkemeyer et al. 2006), but noticed that *2-cysprxa 2-cysprxb*
418 plants displayed substantially reduced growth (Figs. S4A, S5), consistently with the reports of
419 Pulido et al., (2010) and Awad et al., (2015). A somewhat reduced growth was noticed for *ntra*
420 *ntrb* plants (Figs. S4B, S5) as in Reichheld et al., (2007), and *trxh3* and *trxo1* plants displayed
421 slightly higher, but non-significantly different, rosette weights in agreement with Park et al.
422 (2009) on one *trxh3* line, and Ortiz-Espin et al. (2017) on two *trxo1* mutants (Fig. S4D-E).

423 In addition, we measured the chlorophyll content and observed that most lines displayed a
424 level similar to that in WT (Fig. S6), except the *2-cysprxa 2-cysprxb* mutant, which exhibited
425 pale green leaf phenotype (Fig. S5) and chlorophyll content reduced by *ca* 20% (Fig. S6) as
426 observed by Pulido et al., (2010). In other respects, the two lines over-expressing *GRXS14* were
427 previously reported to display a somewhat reduced pigment content (Rey et al., 2017).

428

429 **Stomatal phenotype of mutants impaired in the cytosolic NTR/TRX system**

430 We then characterized TR-modified lines with regard to stomatal functioning, and first
431 investigated the phenotype of those deficient in cytosolic NADPH-thioredoxin reductases A
432 and B, TRXs h3 or o1. Beforehand, we determined the density and number of stomata in relation
433 with the number of epidermal cells (stomatal index), since variations in these parameters can
434 lead to differences in leaf capacity for gas exchange. We observed very close values of density
435 (ca 270 per mm²) and index (0.16) in WT, *ntra ntrb* plants, *trxh3* and *trxo1* mutants (Fig. 3A-
436 B; Fig S7A-B), indicating no change in stomatal differentiation and development in the absence
437 of cytosolic NTRs, TRXs h3 or o1.

438 Porometry measurements were conducted to evaluate the stomatal conductance by
439 measuring the rate of water efflux from the leaf abaxial side of plants grown in optimal
440 conditions. We observed that the mutant lacking both cytosolic NTRA and NTRB displayed a
441 significantly higher conductance compared to WT (182.4 ± 10.5 mmol H₂O.m⁻².s⁻¹ vs 162.7
442 ± 16.9) (Fig. 3C). No variation in stomatal conductance was detected in *trxh3* and *trx o1* plants
443 (Fig S8A-C). We then approached the stomatal functioning in 6-week old plants cultivated in
444 standard conditions by evaluating leaf temperature using infrared (IR) imaging, since leaf
445 cooling is ensured by opening of stomata. To validate the approach, we used the *ost2-2D* (*open*
446 *stomata 2*) dominant mutant that displays stomatal closure defects due to constitutive H⁺-
447 ATPase activity (Merlot et al., 2007). IR imaging revealed a strong difference in the leaf
448 temperature of this mutant compared to WT. In the *ost2-2D* mutant, most leaves appear
449 purple/blue corresponding to a temperature in the range of 16 to 16.5°C, whereas in the WT the
450 leaf color (green to yellow) reflected a temperature in the range of 17.2 to 17.8°C (Fig. 3D).
451 This is consistent with constitutive wide stomatal opening and constant cooling in *ost2-2D*
452 plants. Regarding mutants lacking either TRX h3 or TRX o1, no difference in leaf temperature
453 was observed (data not shown). When examining *ntra ntrb* plants, we observed that their

454 temperature was lower than that of WT, as evidenced by the much higher number of red to
455 yellow leaves in the latter (Fig. 3E). Based on the color scale, the difference could be evaluated
456 to *ca* 0.5°C. Taken collectively, these data reveal that cytosolic NTRs that are highly abundant
457 in the GCE fraction (Table 2) participate in stomatal functioning.

458

459 **Stomatal phenotype of lines deficient in plastidial PRXs**

460 As previously shown (Table 2), plastidial PRXs are well represented in the GCE fraction,
461 PRXQ being the most abundant TR and 2-CysPRXA and B present in the top-ten proteins. We
462 thus investigated the phenotype of lines lacking these PRXs. Compared to WT, no difference
463 was observed in either stomatal density or index in both *2cysprxa 2cysprxb* and *prxq* plants
464 (Fig. 4A-B). In standard watering conditions, a significantly lower stomatal conductance was
465 measured in those deficient in 2-Cys-PRXs (*ca* 220 mmol H₂O.m⁻².s⁻¹ vs 260 in WT), while
466 a slight non-significant decrease was noticed in *prxq* plants (Fig. 4C). When using thermal
467 infrared imaging, we noticed that many leaves of the mutant lacking both 2-CysPRXs displayed
468 red to yellow color corresponding to a temperature between 17 and 18°C, whereas most WT
469 leaves were green to red with a temperature between 16.5 and 17.5°C (Fig. 4D). With regard to
470 *prxq* plants, we noticed no substantial temperature difference compared to WT (Fig. 4E).

471

472 **Stomatal phenotype of lines deficient in type-II GRXs**

473 We then investigated the phenotype of Arabidopsis lines affected in the expression of type-
474 II GRXs based on the presence of cytosolic GRXS17 and plastidial GRXS16 in the GCE
475 fraction (Table 2), and on the report of Hu et al. (2017) showing a stomatal phenotype in
476 *GRXS17*-silenced rice plants. The Arabidopsis line deficient in GRXS17 did not exhibit any
477 variation in stomatal density and index (Fig. S7A-B) in short-day conditions, in which the

478 mutant has no obvious phenotype (Knuesting et al., 2015). No noticeable change in either
479 stomatal conductance (Fig. S8D) or leaf temperature (data not shown) was observed.

480 We performed the analysis of lines modified for the expression of the two genes encoding
481 plastidial type-II GRXs, GRXS14 and GRXS16 (Rey et al., 2017). First, we examined knockout
482 mutants for *GRXS14*, lines co-suppressed for *GRXS16* (Rey et al., 2017), and lines
483 overexpressing one of the two GRXs. No change was observed in the density and stomatal
484 index of any of these lines (Fig. 5A-B; Fig. S7C-D), nor in the stomatal conductance (Fig. 5C;
485 Fig. S8E-G) and leaf temperature (Fig. 5D, data not shown).

486 Finally, we analyzed the phenotype in standard growth conditions of two independent lines,
487 termed KI1 and KI2, knockout for *GRXS14* and impaired for *GRXS16* expression due to RNA-
488 interference (Rey et al., 2017). Compared to WT, both lines did not exhibit any significant
489 variation in stomatal density and index (Fig. 5A-B). Interestingly, substantially reduced
490 stomatal conductance was recorded in the two KI1 and KI2 lines compared to WT (*ca* 230 and
491 240 $\text{mmoles H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, respectively, *vs* 280 in WT) (Fig. 5C). Consistently, when imaging
492 leaf temperature, we observed higher temperature in both lines (Fig. 5D-E). This was
493 particularly obvious in the KI1 line, where all leaves, particularly the youngest ones, displayed
494 a red to yellow color corresponding to a temperature in the range of or higher than 18°C,
495 whereas most WT leaves exhibited green to red color with a temperature lower than 17.5°C.

496

497 **Stomatal responses to ABA and H₂O₂ in lines deficient in thiol reductases**

498 Considering that ABA and H₂O₂ are major actors promoting stomatal closure, we
499 investigated whether mutants deficient in thiol reductases showed altered sensitivity to these
500 compounds. Stomatal apertures were measured in light-pretreated epidermal peels, incubated
501 for 2.5 h on buffer containing 0.5 to 100 μM ABA or 0.01 to 1 mM H₂O₂ (Fig. 6). We observed
502 that at the lowest ABA concentration (0.5 μM), *2cysprxa 2cysprxb*, KI1 and KI2 plants were

503 much more reactive compared to WT and *ntra ntrb* plants. Indeed, whereas the latter displayed
504 no change compared to light treatment alone, stomatal aperture was significantly decreased by
505 *ca* 24% in *2cysprxa 2cysprxb*, KI1 and KI2 (Fig. 6A-B). At 1 μ M, this differential behavior
506 tended to diminish since a significant difference was observed only between WT and *2cysprxa*
507 *2cysprxb*, with aperture decreases of 20 and 40%, respectively. No difference among all lines
508 was noticed at higher hormone concentrations. When epidermal peels were exposed to H₂O₂,
509 substantial differences were observed for *2cysprxa 2cysprxb*, KI1 and KI2 lines, which clearly
510 displayed enhanced sensitivity. In the three lines, stomatal aperture at 10 μ M H₂O₂ was reduced
511 by *ca* 35% and significantly lower compared to that recorded in WT and *ntra ntrb* plants (-
512 10%) (Fig. 6 C-D). Higher susceptibility was also recorded at 100 μ M H₂O₂ for the three mutant
513 lines, but not at the highest concentration (1 mM). Altogether, these data indicate that plastidial
514 thiol reductases modulate ABA- and H₂O₂-dependent signaling pathways controlling stomatal
515 closure.

516

517 **Discussion**

518 **Presence and abundance of TRs in guard cells**

519 So far, the knowledge about the abundance of TRs in guard cells is limited and based on
520 the proteome of guard cell protoplasts in *Arabidopsis thaliana* and *Brassica napus* (Zhao et al.,
521 2008; 2010; Zhu et al, 2009; 2010). We attempted to strengthen this knowledge by taking
522 advantage of the great advance in mass spectrometry-based proteomics made since. To avoid
523 redox biases and changes due to protoplast preparation, we implemented another approach
524 using broken and extensively washed epidermis to get a fraction highly enriched in guard cells.
525 The enrichment of this fraction was validated by the relatively high abundance of numerous
526 key actors involved in stomatal functioning (Table 1). With regard to TRs, the proteomic
527 analysis of the GCE fraction highlighted several findings: i) among about 4700 proteins

528 identified, 39 TRs belonging to the TRX, GRX, PRX, GPX and MSR families are present; ii)
529 *ca* half of them are relatively more abundant in the GCE fraction than in leaf. iii) NADPH-
530 thioredoxin reductases A and B, the electron source for extra-plastidial TRXs, are present in
531 the first quartile when ranking proteins as a function of abundance within the GCE fraction
532 (Table 2); iv) six PRXs (PRXQ the most abundant TR, the two 2-CysPRXs and three PRX-II:
533 B, E and F) out of the nine in *Arabidopsis* are also present in the first quartile of the list. Most
534 importantly, these proteomic data are in accordance with transcriptomics of guard cell
535 protoplasts, that revealed higher transcript levels for various TR types compared to mesophyll
536 cells (Fig. S1). Further, all the TRs (13) identified in the first proteomic analyses of guard cell
537 protoplasts in *Arabidopsis thaliana* and *Brassica napus* (Zhao et al., 2008; 2010; Zhu et al.,
538 2009) are present in the GCE fraction, notably four PRXs (2-CysPRXA and B, PRXII-E and
539 PRXII-F). Thereby, these reports and the present work strongly support a key contribution of
540 TRs in the functioning of guard cells. Numerous TRs are present in both GCE and leaf fractions
541 (Table S2), indicating that they might also participate in long-distance signaling pathways
542 originating from mesophyll and controlling stomatal movements in relation with photosynthesis
543 level (Lawson, 2009). Nonetheless, our data revealing high abundance of many TRs in guard
544 cells and phenotypes for mutants impaired for several of them, give strong reliability to essential
545 roles of these reductases within the stomatal signaling network and machinery.

546

547 **Participation of cytosolic TRs in stomatal functioning**

548 We observed that plants deficient in NTRA and NTRB display increased stomatal opening
549 in standard growth conditions as evidenced by porometry measurements and thermal infrared
550 imaging (Fig. 3). In other respects, bioassay experiments carried out on epidermal peels did not
551 reveal any substantial aperture change in this line compared to WT in response to ABA or H₂O₂
552 treatments applied over a relatively short period of time (2.5 h) (Fig. 6). Both NTRs ensure the

553 reduction of cytosolic and mitochondrial TRXs, such as h and o isoforms. Nonetheless,
554 Arabidopsis mutant plants lacking either cytosolic TRX h3 or mitochondrial TRX o1 did not
555 show any noticeable change in stomatal functioning (Fig. S8). Interestingly, da Fonseca-Pereira
556 et al., (2019) reported that *ntra ntrb* and *trxo1* plants displayed faster recovery after two cycles
557 of drought episodes in relation with higher stomatal conductance. This is consistent with our
558 data unveiling higher conductance in the mutant lacking cytosolic and mitochondrial NTR
559 systems. Therefore, the role of the NTR/TRX system in stomata might be of importance
560 following drought stress. In other respects, Calderon et al. (2018) reported higher stomatal
561 density and index in the two *trxo1* mutant lines compared to WT, revealing a possible role of
562 these TRXs in stomatal development. When characterizing these mutants, we did not notice
563 such a phenotype. (Fig. S7). It is worth mentioning that in this study, the density and index
564 ranges (*ca* 120.mm⁻² and 0.45) are very different from ours. This might originate from different
565 culture conditions and measurements performed at distinct developmental stages in the two
566 works. Of note, the *trxo1* lines also displayed reduced water loss upon high salinity, possibly
567 due to altered stomatal functioning (Calderon et al., 2018). Finally, da Fonseca-Pereira et al.,
568 (2020) reported that Arabidopsis mutants lacking TRX h2, which is located in both cytosol and
569 mitochondria, do not exhibit any growth phenotype in short day and low light intensity, but
570 decreased stomatal conductance and higher water use efficiency. These data indicate that the
571 cytosolic NTR/TRX system could fine tune guard cell functioning in relation with light
572 parameters via pathways remaining to be unveiled (Fig. 7).

573 This system is likely to fulfill several functions through redox interaction with known
574 actors of the signaling network regulating stomatal movements. Thus, cytosolic TRs might
575 control the redox status of OST1/SnRK2.6, BAK1, SnRK2.4 and CPK21, which are likely
576 prone to thiol-based modifications as they display cysteine thiol redox switches modifying their
577 activity *in vitro*, or interact *in vitro* with TRs (Bender et al. 2015; Wang et al. 2015; Zhu et al.

2017; Ma et al. 2018). This hypothesis is further supported by the identification of *ca* 80 potential TRX targets in a fraction enriched in guard cells (Zhang et al., 2016), and of numerous proteins harboring redox sensitive Cys in *Brassica napus* guard cells (Zhu et al., 2014). One major challenge will consist of identifying their reductants among TRs and determining their physiological functions in stomatal movements (Fig. 7).

583

584 **Participation of plastidial TRs in stomatal functioning**

585 Plants deficient in type-II GRXs or 2-Cys PRXs exhibited decreased stomatal opening in
586 optimal growth conditions as shown by porometry and thermal infrared imaging (Figs. 4-5),
587 and enhanced stomatal closure in epidermal peels treated with ABA or H₂O₂ (Fig. 6). H₂O₂ is
588 an essential redox signaling messenger promoting closure, and likely enters the cytoplasm of
589 guard cells (Rodrigues et al., 2017) following the ABA-induced ROS burst in apoplast (Kwak
590 et al., 2003). Our data clearly reveal that GRXS14, GRXS16 and 2-CysPRXs limit ABA- and
591 ROS-induced stomatal closure (Fig. 6). Strikingly, these reductases are located in plastids,
592 highlighting the involvement of this compartment in stomatal functioning. This is fully
593 consistent with the hypothesis of redox signals originating from chloroplasts in the network
594 controlling stomatal movements (Chen and Gallie, 2004; Song et al, 2014), H₂O₂ accumulation
595 in chloroplasts of guard cells being even considered as one of the earliest hallmarks of stomatal
596 closure (Sierla et al, 2016). The involvement of subcellular compartments is further supported
597 by the participation of mitochondrial TRX o1 in guard cell functioning (Calderon et al., 2018),
598 and the presence of numerous organelle TRs in the GCE fraction (Table 2). Plastidial and
599 mitochondrial TRs play master roles in energy generation and consumption (Schurmann and
600 Buchanan, 2008; Daloso et al., 2015), and are thus probably needed in the regulation and
601 coordination of the highly active metabolism in guard cells during stomatal movements.

602 Plastidial GRXS14 and S16 might modulate stomatal movements *via* the control of Cys
603 redox status in partner proteins that participate in ROS-related transduction pathways inducing
604 closure (Fig. 7). Consistent with this hypothesis, another GRX is able to inhibit *in vitro* the
605 activity of BAK1 *via* glutathionylation (Bender et al., 2015). So far, very little is known *in vivo*
606 about the participation of GRXs in stomatal functioning, except for GRXS17, which also
607 belongs to class-II and is located in cytosol and nucleus. Silencing of its expression in rice is
608 associated with increased stomatal closure (Hu et al., 2017). Moreover, GRXS17-deficient lines
609 are more tolerant to water deficit (Hu et al., 2017). However, when investigating the stomatal
610 phenotype of an Arabidopsis *grxs17* line using porometry and thermal imaging, we did not
611 notice any difference with WT in standard conditions (Fig. S8 and data not shown), indicating
612 environmental and/or species influence on the role of the GRX.

613 The Arabidopsis line lacking plastidial 2-CysPRXs exhibited a marked stomatal phenotype
614 (Figs. 4, 6), which might underlie, at least partially, its decreased growth (Fig. S4) due to
615 reduced CO₂ supply to photosynthetic cells. It is worth mentioning that this pleiotropic mutant
616 also displays less chlorophyll (Figs. S5-S6) and increased susceptibility to photooxidative stress
617 (Awad et al., 2015). Note that deficiency and overexpression of 2-CysPRX in rice lead to
618 enhanced and decreased stomatal opening, respectively (Mao et al., 2017), suggesting that this
619 peroxidase plays distinct roles in stomata of monocotyledons and dicotyledons. 2-Cys PRXs
620 are considered as a central redox buffer in plastid since they are efficient scavengers of
621 hydrogen and organic peroxides (Liebthal et al., 2018; Cerveau et al., 2019). Thus, they are
622 prime candidates to sense local changes in H₂O₂ content in guard cells. This function could take
623 place in the transduction pathway involving H₂O₂ and promoting stomatal closure. Since H₂O₂-
624 induced closure is strongly enhanced in the mutant lacking both 2-Cys PRXs (Fig. 6), we can
625 hypothesize that these peroxidases directly mitigate, *via* scavenging, the peroxide signal
626 intensity leading to closure (Fig. 7). Further, many recent lines of evidence revealed that these

enzymes achieve essential roles in redox signaling processes through the control of thiol switch in partner proteins (Rhee and Woo, 2011; Liebthal et al., 2018). Accordingly, Stöcker et al. (2017) reported that 2-CysPRXs enable rapid and sensitive thiol oxidation in various protein types at the expense of H₂O₂. In plants, 2-CysPRXs play a key role in Calvin cycle deactivation at night *via* oxidation of TRX partners (Yoshida et al., 2018). Further, 2-Cys PRXs interact with a large number of potentially redox-regulated proteins (Cerveau et al., 2016a; Liebthal et al., 2020). Some such as β -amylases, which are redox-regulated enzymes involved in starch degradation, very likely participate in osmotic adjustment during stomata opening (Valerio et al., 2011; Horrер et al., 2016). Thus, we can presume that similarly to their role in photosynthesis regulation, 2-Cys PRXs participate in the daytime control of starch degradation, which drives light-induced stomatal opening (Horrер et al., 2016). Such a function might underlie the phenotype observed in whole plants (Fig. 4). To conclude, plastidial 2-Cys PRXs are likely to accomplish several signaling functions in relation with H₂O₂ level in guard cells (Fig. 7). This hypothesis is further supported by the much higher level of protein hyperoxidation in the GCE fraction compared to leaf (Fig. 1B). Cys hyperoxidation to a sulfinic acid form is reversible thanks to the action of sulfiredoxin (Rey et al., 2007), and leads based on the floodgate theory to local H₂O₂ accumulation and initiation of signaling pathways (Poole et al., 2011). Finally, 2-CysPRXs possess several phosphorylation sites (Liebthal et al., 2018) susceptible to modulate peroxidase activity as shown in its human counterpart (Woo et al., 2010) and possibly underlying interplays between redox and phosphorylation transduction pathways, which are essential in stomatal movements (Kim et al., 2010).

648

649 **Conclusions**

650 The present work provides evidence of altered stomatal functioning in plants deficient for
651 several TR types. In Arabidopsis lines lacking class-II GRXs or 2-CysPRXs, the lower stomatal

652 conductance is associated with reduced growth. However, this cannot be regarded as a general
653 rule since plants deficient in NTRs A and B concomitantly exhibit reduced growth (Fig. S4)
654 and more open stomata (Fig. 3). Taking collectively, the few works reporting a stomatal
655 phenotype resulting from TR deficiency (Hu et al, 2017; Calderon et al, 2018; Mao et al, 2018)
656 and our data highlight the complexity of TR roles in stomata that very likely depend on
657 environmental conditions, developmental stage, and species. This work clearly unveils the
658 participation of plastidial TRs in signaling transduction pathways controlling stomatal closure,
659 and opens new avenues of research to investigate the roles of thiol redox switch in stomatal
660 functioning. In particular, it will be essential to identify TR physiological partners in guard
661 cells, and unravel their integration within the hormone- and ROS-related transduction pathways
662 regulating stomatal movements in response to abiotic environmental stimuli, but also biotic
663 ones, since stomata participate in immune defense mechanisms (Melotto et al., 2006; Montillet
664 and Hirt, 2013). Consistently Pang et al., (2020) recently reported changes in the expression of
665 several *TRX* and *PRX* genes in a GCE fraction following treatment with a bacterial pathogen.

666

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- 975

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AGI Number	Protein ID	Description	GCE	L	GCE-L	R	GC proteomics	GC/M transcript ratio > 2	Reference
AT5G26000	P37702	Myrosinase 1 *	31,43	29,21	2,22	4,66	X	●	[54]
AT1G69530	Q9C554	Expansin-A1 *	29,30	24,39	4,91	30,1		●	[48]; [53]
AT3G53420	P43286	Aquaporin PIP2-1 #	29,05	29,17	-0,12	0,92	X		[43]
AT2G18960	P20649	ATPase 1 #	27,77	24,99	2,78	6,87	X	●	[21]
AT5G56030	P55737	Heat shock protein 90-2 *	27,46	27,15	0,32	1,25	X	●	[6]
AT5G10450	F4KGV2	G-box regulating factor 6 *	27,36	23,98	3,38	10,4			[39]
AT5G67500	Q9FJX3	Voltage-dependent anion channel 2 #	27,23	24,61	2,62	6,15	X	●	[27]
AT1G70410	Q94CE4	Beta carbonic anhydrase 4 *	27,08	25,87	1,21	2,31	X		[15]
AT3G15730	Q38882	Phospholipase D alpha 1 *	26,60	25,83	0,77	1,71	X	●	[41]
AT5G02500	F4KCE5	Heat shock cognate 70 kDa protein 1 *	26,53	27,09	-0,57	0,68	X	●	[6]
AT1G12840	Q9SDS7	V-type proton ATPase subunit C #	26,17	24,86	1,31	2,48		●	[1]; [18]
AT1G15690	P31414	Vacuolar membrane proton pump 1 #	25,96	26,85	-0,89	0,54	X		[3]
AT1G11260	P23586	Sugar transport protein 1 #	25,70	21,16	4,54	23,3	X	●	[35]; [31]
AT4G29130	Q42525	Hexokinase-1 §	25,00	24,43	0,57	1,48		●	[24]
AT3G18490	Q9LS40	Aspartic protease in guard cell 1 *	24,98	23,42	1,57	2,96		●	[51]
AT1G04110	O64495	Subtilisin-like protease SBT1.2 □	24,83	15,90	8,93	489			[42]
AT4G05590	Q8L7H8	Mitochondrial pyruvate carrier 3 *	24,73	15,18	9,55	GCE			[52]; [35]
AT5G57110	Q9LF79	Calcium-transporting ATPase 8 #	24,37	22,47	1,89	3,72	X		[50]
AT4G03560	Q94KI8	Two pore calcium channel protein 1 #	24,31	20,57	3,74	13,4		●	[34]
AT3G02880	Q9M8T0	Probable inactive receptor kinase §	23,81	23,47	0,33	1,26	X		[18]
AT5G46790	Q8VZS8	Abscisic acid receptor PYL1 !	23,59	22,21	1,38	2,60		●	[45]
AT1G13320	Q38951	Protein phosphatase 2A subunit A3 §	23,57	20,06	3,51	11,4			[26]
AT4G23650	Q42479	Calcium-dependent protein kinase 3 §	23,32	24,62	-1,30	0,41	X		[30]
AT4G30190	P19456	ATPase 2, plasma membrane-type #	23,25	20,95	2,30	4,92	X	●	[46]
AT2G26040	O80992	Abscisic acid receptor PYL2 !	22,92	15,18	7,74	GCE	X	●	[45]
AT1G55020	Q06327	Linoleate 9S-lipoxygenase 1 *	22,91	20,35	2,56	5,90		●	[29]
AT1G52340	Q9C826	Xanthoxin dehydrogenase *	22,89	21,21	1,68	3,20		●	[49]
AT2G46070	Q8GYQ5	Mitogen-activated protein kinase 12 §	22,81	17,65	5,16	35,8	X	●	[20]
AT5G50000	Q9FGB1	Protein kinase superfamily protein §	22,80	21,03	1,76	3,39	X	●	[14]
AT4G17870	O49686	Abscisic acid receptor PYR1 !	22,73	21,86	0,87	1,83		●	[33]
AT4G35790	Q9C5Y0	Phospholipase D delta *	22,46	20,88	1,58	2,99	X	●	[41]
AT5G13630	Q9FNB0	Magnesium-chelatase subunit ChlH *	21,77	24,15	-2,38	0,19			[40]
AT2G43350	O22850	Glutathione peroxidase 3 *	21,56	15,18	6,38	GCE		●	[28]
AT2G43790	Q39026	Mitogen-activated protein kinase 6 §	21,29	19,99	1,30	2,46		●	[29]
AT2G13540	Q9SIU2	Nuclear cap-binding protein subunit 1 *	21,21	18,32	2,89	7,41			[17]
AT3G53720	Q9M353	Cation/H(+) antiporter 20 #	21,21	15,18	6,03	GCE	X	●	[32]
AT1G32080	Q9FVQ4	Plastidal glycolate/glycerate translocator#	21,19	23,68	-2,48	0,18			[7]
AT5G40890	P92941	Chloride channel protein CLC-a #	20,92	19,53	1,40	2,63	X	●	[47]
AT5G24520	Q9XGN1	TRANSPARENT TESTA GLABRA 1 □	20,81	18,93	1,87	3,66		●	[9]
AT1G28490	Q946Y7	Syntaxin-61 *	20,64	18,52	2,12	4,35		●	[55]
AT4G14480	O23304	Serine/threonine-protein kinase BLUS1 §	20,56	15,18	5,38	41,6	X	●	[38]
AT5G49890	Q96282	Chloride channel protein CLC-c #	20,22	15,18	5,04	GCE		●	[22]
AT4G04720	Q9ZSA2	Calcium-dependent protein kinase 21 §	20,15	20,73	-0,57	0,67			[10]
AT1G23200	O49298	Pectinesterase/pectinesterase inhibitor 6 *	19,84	15,18	4,66	GCE		●	[2]
AT3G08550	Q9C9Z9	Glycosyltransferase-like KOBITO 1 *	19,46	19,62	-0,15	0,90		●	[25]
AT4G08920	Q43125	Cryptochrome-1 □	19,35	18,73	0,63	1,55			[23]
AT4G17615	Q9LTB8	Calcineurin B-like protein 1 *	19,31	15,18	4,13	GCE			[5]
AT5G63870	Q9FN02	Serine/threonine-protein phosphatase 7 §	19,12	19,04	0,07	1,05		●	[37]

AT1G04400	Q96524	Cryptochrome-2 \square	19,01	15,18	3,83	GCE		●	[4]
AT1G12110	Q05085	Nitrate transporter 1.1 #	18,76	<u>17,50</u>	1,26	2,39		●	[11]
AT2G36850	Q9SJM0	Callose synthase 10 \square	18,77	16,02	2,75	6,72		●	[12]
AT1G49040	Q8RXA7	DENN-domain WD-repeat protein \square	18,69	17,87	0,82	1,77	X		[8]
AT3G45640	Q39023	Mitogen-activated protein kinase 3 §	17,93	<u>17,01</u>	0,92	1,89			[44]
AT1G35670	Q39016	Calcium-dependent protein kinase 2 §	17,83	<u>16,81</u>	1,03	2,04			[16]
AT1G62400	Q2MHE4	Serine/threonine-protein kinase HT1 §	17,44	15,18	2,26	GCE		●	[13]
AT2G18790	P42497	Phytochrome \square	17,43	19,21	-1,78	0,29		●	[23]

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977 **Table 1. List of proteins present in the guard cell-enriched fraction and playing a role in**
 978 **stomatal functioning and development.**

979 Proteins are ranked from the most abundant to the less in the GCE fraction. Bold lines separate
 980 quartiles. In GCE and L columns are reported \log_2 of imputed normiBAQ that reflect relative
 981 abundance of each protein in guard cell-enriched and leaf fractions, respectively. R represents
 982 protein abundance ratio in the GC-enriched fraction epidermis relative to leaf fraction and is
 983 equal to $2^{(E-L)}$. Bold R value, protein having a value > 1.5 and thus more abundant in the GCE
 984 fraction than in the L fraction. **GCE**, protein found only in the guard cell-enriched fraction
 985 Underlined L value, only 1 peptide detected; bold L value, no peptide detected. **X**, Protein
 986 identified in Arabidopsis guard cell proteomics by Zhao et al., (2008; 2010). ●, Transcript ratio,
 987 GC protoplast level/mesophyll protoplast level, higher than 2 (Yang et al., 2008). Classification
 988 of proteins: §, kinases and phosphatases involved in stomatal movements; #, Ion channels,
 989 transporters and pumps involved in stomatal movements;!, ABA receptors; \square , Proteins involved
 990 in regulation of stomatal development and patterning ; *, Enzymes and other protein functions.
 991 References are listed in Table S6.

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AGI Number	Protein ID	Description	Name	GCE	L	GCE-L	R	GC proteomics
AT3G26060	Q9LU86	Peroxiredoxin Q	PRXQ	28,20	29,82	-1,62	0,33	
AT3G11630	Q96291	2-Cys peroxiredoxin A	2-CysPrxA	27,71	27,09	0,63	1,55	X
AT1G65980	Q9XEX2	Peroxiredoxin IIB	Prx IIB	26,14	23,27	2,87	7,29	
AT3G06050	Q9M7T0	Peroxiredoxin IIF	Prx IIF	25,99	25,28	0,71	1,64	X
AT2G17420	Q39242	NADPH-thioredoxin reductase A	NTRA	25,94	24,01	1,92	3,79	
AT3G52960	Q949U7	Peroxiredoxin IIE	Prx IIE	25,61	27,37	-1,76	0,30	X
AT5G42980	Q42403	Thioredoxin h3	TRX h3	25,00	22,90	2,10	4,29	X
AT4G11600	O48646	Glutathione peroxidase 6	GPX6	24,44	23,79	0,65	1,56	X
AT5G06290	Q9C5R8	2-Cys peroxiredoxin B	2-CysPrxB	24,19	25,08	-0,88	0,54	X
AT5G07460	Q9LY15	Peptide methionine sulfoxide reductase A2	MSRA2	24,17	22,55	1,62	3,08	X
AT1G60420	O80763	Nucleoredoxin 1	NRX1	23,95	21,82	2,13	4,38	
AT4G35460	Q39243	NADPH-thioredoxin reductase B	NTRB	23,93	20,25	3,69	12,9	
AT4G25130	P54150	Peptide methionine sulfoxide reductase A4	MSRA4	23,87	26,50	-2,63	0,16	X
AT2G25080	P52032	Glutathione peroxidase 1	GPX1	23,20	23,81	-0,61	0,65	
AT2G31570	O04922	Glutathione peroxidase 2	GPX2	22,76	20,98	1,78	3,43	X
AT3G06730	Q9M7X9	Thioredoxin z	TRX z	22,71	15,18	7,53	GCE	
AT5G39950	Q38879	Thioredoxin h2	TRX h2	22,43	19,63	2,80	6,96	
AT4G03520	Q9SEU8	Thioredoxin m2	TRX m2	22,17	26,87	-4,70	0,04	X
AT2G38270	Q8H7F6	Glutaredoxin S16	GRXS16	22,14	24,32	-2,18	0,22	X
AT2G41680	O22229	NADPH-thioredoxin reductase C	NTRC	22,09	26,15	-4,06	0,06	
AT3G02730	Q9XFH8	Thioredoxin f1	TRX f1	21,96	25,88	-3,93	0,07	
AT3G08710	Q9C9Y6	Thioredoxin h9	TRX h9	21,81	15,18	6,63	GCE	
AT3G15660	Q8LBK6	Glutaredoxin S15,	GRXS15	21,80	15,18	6,62	GCE	
AT4G04950	Q9ZPH2	Glutaredoxin S17	GRXS17	21,72	20,97	0,75	1,68	
AT3G15360	Q9SEU6	Thioredoxin m4	TRX m4	21,72	26,37	-4,66	0,04	X
AT1G03680	O48737	Thioredoxin m1	TRX m1	21,65	25,86	-4,21	0,05	
AT3G63080	Q9LYB4	Glutathione peroxidase 5	AtGPX5	21,59	22,49	-0,89	0,54	
AT2G43350	O22850	Glutathione peroxidase 3	AtGPX3	21,56	15,18	6,38	355	
AT1G76080	Q9SGS4	Chloroplastic Drought Stress 32-kDa Protein	TRX CDSP32	21,41	25,66	-4,25	0,05	
AT4G29670	Q8LCT3	Atypical cysteine/histidine-rich thioredoxin 2	ACHT2	21,39	23,63	-2,24	0,21	
AT4G28730	Q8GWS0	Glutaredoxin C5	GRXC5	21,26	23,56	-2,30	0,20	
AT2G35010	O64764	Thioredoxin o1	TRX o1	20,99	20,43	0,55	1,47	X
AT1G11530	Q8LDI5	Thioredoxin-like, Mono-cysteine 1	TRX CxxS1	20,89	18,83	2,06	4,21	
AT4G21860	Q9C5C8	Methionine sulfoxide reductase B2	MSRB2	20,78	24,26	-3,48	0,09	
AT3G17880	Q8VWG7	Tetratricoredoxin	TDX	20,61	15,18	5,43	GCE	
AT5G08410	Q8LBP6	Ferredoxin/thioredoxin reductase subunit A2	FTRA2	20,27	21,52	-1,25	0,42	
AT1G07700	Q9C5C5	Thioredoxin-like, Lilium-type thioredoxin 6	Lilium6	20,08	15,18	4,90	GCE	
AT2G18030	Q9SL43	Methionine sulfoxide reductase A5	MSRA5	20,07	20,31	-0,24	0,84	
AT4G37200	O23166	Thioredoxin High Chlorophyll Fluorescence 164	TRX HCF164	19,72	23,43	-3,71	0,08	

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1010

1011 **Table 2. List of thiol reductases identified in the guard cell-enriched fraction**
1012 Proteins are ranked from the most abundant to the less in the GCE fraction. Bold lines separate
1013 quartiles. In GCE and L columns are reported \log_2 of imputed normiBAQ that reflect relative
1014 abundance of each protein in guard cell-enriched and leaf fractions, respectively. R represents
1015 protein abundance ratio in the GC-enriched fraction epidermis relative to leaf fraction and is
1016 equal to $2^{(E-L)}$. Bold R value, protein having a value > 1.5 and thus more abundant in the GCE
1017 fraction than in the L fraction. **GCE**, protein found only in the guard cell enriched-fraction.
1018 Bold L value, no peptide detected. **X**, Protein identified in Arabidopsis guard cell proteomics
1019 by Zhao et al., (2008; 2010).

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1038 **Figure legends**

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1040 **Figure 1. Western blot analysis of the abundance of thiol reductases in extracts from a guard cell-**
1041 **enriched fraction**

1042 **A.** Coomassie Blue-stained gel of SDS-PAGE separated proteins from leaf blades (L) and GCE
1043 fractions. The arrows indicate proteins noticeably more abundant in the GCE fraction. LSU and SSU,
1044 large and small RubisCO subunits, respectively. **B.** Western blot analysis of RubisCO subunits and of
1045 various types of thiol reductases in L and GCE fractions. Blots (20 to 30 μg proteins per lane, depending
1046 on the serum used, for both fractions) were probed using antibodies raised against RubisCO (1:10,000),
1047 TRX h3 (1:20,000), 2-CysPRX (1:10,000) 2-CysPRX-Ox, hyperoxidized 2-CysPRX (1:4000), PRXQ
1048 (1:1000), PRX-IIIE (1:10,000) PRXII-B (1:20,000) and GRXS14 (1:800). For the latter, the asterisk
1049 points to a non-specific band and the two arrows indicate two GRX redox forms.

1050

1051 **Figure 2. Functional categorization (GO Cellular Component) of proteins identified in guard cell-**
1052 **enriched and leaf fractions.**

1053 Functional categorization was performed using of the GO annotation retrieval tool available on the site
1054 TAIR: <https://www.arabidopsis.org/tools/bulk/go/index.jsp>. Analysis was performed on genes encoding
1055 the 25% most abundant proteins of each sample (in GCE fraction, 1102 proteins having a $\text{Log}_2(\text{iBAQ})$
1056 ≥ 24.06 and in leaf fraction 913 proteins with $\text{Log}_2(\text{iBAQ}) \geq 23.97$).

1057

1058 **Figure 3. Stomatal phenotype of the Arabidopsis *ntra ntrb* mutant**

1059 **A.** Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values
1060 using peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control
1061 conditions: moderate light ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . **C.** Stomatal
1062 conductance measurements using porometry. Measurements of the water efflux from leaf abaxial side
1063 were performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD
1064 of twelve average values per genotype originating from independent plants (8 measurements per plant
1065 for each genotype per experiment). ***, significantly different from the WT value with $P < 0.001$ (*t*-
1066 test). **D.** and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-
1067 week-old plants grown in control conditions. The color scale on the right shows increasing temperature
1068 from purple to yellow. Pictures were taken at the end of the night period two hours before the light
1069 period. As a control, the *ost2-2D* mutant was used and shown to exhibit lower leaf temperature due to
1070 open stomata in the dark (Merlot et al., 2007). *ntra ntrb*, double *ntra ntrb* mutant plants.

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1074 **Figure 4. Stomatal phenotype of Arabidopsis mutants deficient in plastidial PRXs**

1075 **A.** Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values
 1076 using peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control
 1077 conditions: moderate light ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . **C.** Stomatal
 1078 conductance measurements using porometry. Measurements of the water efflux from leaf abaxial side
 1079 were performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD
 1080 of eight average values per genotype originating from independent plants (8 measurements per plant for
 1081 each genotype per experiment). **, significantly different from the WT value with $P < 0.01$ (*t*-test). **D.**
 1082 and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-week-
 1083 old plants grown in control conditions. The color scale on the right shows increasing temperature from
 1084 purple to yellow. Pictures were taken at the end of the night period two hours before the light period.
 1085 *2cprx*, double *2cysprxa 2cysprxb* mutant plants; *prxq*, knockout *PRXQ* plants.

1086

1087 **Figure 5. Stomatal phenotype of Arabidopsis lines modified for plastidial type-II GRXs. A.**

1088 Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values using
 1089 peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control conditions:
 1090 moderate light ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . **C.** Stomatal conductance
 1091 measurements using porometry. Measurements of the water efflux from leaf abaxial side were
 1092 performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD of at
 1093 least 17 average values per genotype originating from independent plants (8 measurements per plant for
 1094 each genotype per experiment). ***, significantly different from the WT value with $P < 0.001$ (*t*-test).
 1095 **D.** and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-week-
 1096 old plants grown in control conditions. Pictures were taken at the end of the night period two hours
 1097 before the light period. The color scale on the right shows increasing temperature from purple to yellow.
 1098 As a control, the *ost2-2D* mutant was used and shown to exhibit lower leaf temperature due to open
 1099 stomata in the dark (Merlot et al., 2007). K11 and K12, independent *grxS14* GRXS16-RNAi lines; OE1
 1100 and OE2, independent *GRXS14*-overexpressing lines.

1101

1102 **Figure 6. Effect of ABA and H₂O₂ on stomatal aperture in Arabidopsis mutants deficient in**
 1103 **cytosolic NTRs, plastidial 2Cys-PRXs or plastidial type-II GRXs.**

1104 Stomatal aperture measurements in response to ABA concentrations of 0.5, 1, 10 and 100 μM
 1105 (**A-B**) or to H₂O₂ concentrations of 10, 100 and 1000 μM (**C-D**) in light-treated epidermis.
 1106 Data in panels **B** and **D** were calculated from the original raw data reported in panels **A** and **C**,
 1107 respectively, and represent the inhibitions of stomatal aperture due to the treatments expressed
 1108 in percents of that measured in light alone. Data represent means \pm SD of 2 independent
 1109 experiments with 60 measurements per genotype and treatment. Means were compared with

1110 the ANOVA test, and letters represent groups of means not statistically different ($P < 0.05$).
1111 *2cprx*, double *2cysprxa 2cysprxb* mutant plants; KI1 and KI2, independent *grxS14* GRXS16-
1112 RNAi lines; *ntra ntrb*, double *ntra ntrb* mutant plants.

1113

1114 **Figure 7. Model for positioning putative roles of plastidial 2-CysPRXs and type-II GRXs**
1115 **in the signaling network governing stomatal closure.** Based on the results gained on
1116 epidermal peels showing modified responses of mutants to ABA and H_2O_2 and the known
1117 biochemical functions of these enzymes, we propose that they participate in H_2O_2 -related
1118 transduction pathways. 2-CysPRXs might scavenge H_2O_2 within plastid or promote oxidation
1119 of partners that remain to be identified and are involved, when oxidized, in a pathway mitigating
1120 stomatal closure. Type II-GRXs might prevent oxidation of partners, the oxidized form of
1121 which could trigger closure. The roles of cytosolic NTRs and other TRs in guard cells remain
1122 to be determined.

1123

1124 **Supporting information**

1125 **Table S1. Leaf characteristics of 6 week-old plants and guard cell characteristics for evaluating**
1126 **the enrichment in guard cells in the broken and washed epidermal fraction.**

1127

1128 **Table S2: Proteins identified and quantified in guard cell-enriched and leaf fractions by mass**
1129 **spectrometry-based proteomics.**

1130

1131 **Table S3. List of cell wall and extracellular proteins identified in the guard cell-enriched fraction.**

1132

1133 **Table S4. List of proteins having transcript preferentially expressed in guard cell protoplasts**
1134 **(Leonhard et al., 2004) identified in the guard cell-enriched fraction.**

1135

1136 **Table S5. List of 14-3-3 proteins identified in the guard cell-enriched fraction.**

1137

1138 **Table S6. List of references cited in Table 1.**

1139

1140 **Figure S1: Transcriptomic analysis of *TR* expression in guard and mesophyll cell protoplasts.** Data
1141 were retrieved from the microarray analyses described by Yang et al., (2008). RNA was isolated from
1142 protoplasts prepared from guard or mesophyll cells and hybridized to the ATH1 GeneChip. Two
1143 independent hybridization experiments per cell type were performed. Means and SD of expression levels

1144 were retrieved using the Arabidopsis eFP Browser at bar-utoronto.ca (Winter et al., 2007). **A**, NADPH-
 1145 Thioredoxin reductase A (NTRA) and thioredoxins h2, h3, h4, h5, h9, TDX, o1, o2, Clot, m3, Lillium-
 1146 1 and CDSP32; **B**, Glutaredoxins S15 and ROXY10, peroxiredoxin II-B (II-B), glutathione peroxidase
 1147 2 (GPX2), and methionine sulfoxide reductases A1 and A4.

1148
 1149 **Figure S2. Gene expression in guard cells.** *TRXh2* (**A**), *TRXh3* (**B**), *NTRB* (**C**) and *PRX-IIB* (**D**) gene
 1150 promoters were fused to β -glucuronidase (*GUS*) as a reporter gene and gene expression was monitored
 1151 in 15 day-old plants by histochemical analysis. Inset: focus on one single stomata.

1152
 1153 **Figure S3. Characterization of mutants deficient in the expression of *PRXQ* and *TRXh3***
 1154 **genes.** **A.** Genotyping of SAIL_742_G10 plants (*prxq*) using primers corresponding to 5' and 3' ends
 1155 of *PRXQ* coding sequence. **B.** Genotyping of SAIL_314_G04 (*trxh3-1*) using primers corresponding to
 1156 5' and 3' ends of *TRXh3* coding sequence. WT: wild type. **C.** Western blot analysis of PRXQ abundance
 1157 in WT and *prxq* plants. **D.** Western blot analysis of TRX h3 abundance in WT, *trxh3-1* (homozygous
 1158 SAIL_742_G10) and *trxh3-2* (homozygous SALK_111160) plants.

1159
 1160 **Figure S4. Growth of plants modified for the expression of various *TR* genes.** Plants were grown
 1161 for 5 or 6 weeks in control conditions: moderate light (200 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod
 1162 (8 h) at 22°C. Rosette weight values are means \pm SD of four to six average values originating from
 1163 independent experiments (at least 6 plants for each genotype per experiment). * and ***, significantly
 1164 different from the WT value with $P < 0.05$ and $P < 0.001$, respectively (*t*-test). *2cprx*, double *2cysprxa*
 1165 *2cysprxb* mutant plants (**A**); *ntra ntrb*, double *ntra ntrb* mutant plants (**B**); *prxq* (**A**), *grxs17* (**C**), *trxh3-*
 1166 *1*, *trxh3-2* (**D**) and *trxo1-1*, *trxo1-2* (**E**) mutants lacking PRXQ, GRXS17, TRXh3 and TRXo1,
 1167 respectively.

1168
 1169 **Figure S5. Photograph of wild type (WT) plants and of plants modified for the expression of**
 1170 **various *TR* genes.** Plants were grown for 5 weeks in control conditions: moderate light (200 μmol
 1171 $\text{photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C. *2cprx*, double *2cysprxa 2cysprxb* mutant plant; *ntra*
 1172 *ntrb*, double *ntra ntrb* mutant plant; KI1 and KI2, independent *grxS14* GRXS16-RNAi lines.

1173
 1174 **Figure S6. Leaf chlorophyll content of plants modified for the expression of various *TR* genes.**
 1175 Plants grown for 6 weeks in control conditions: moderate light (200 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short
 1176 photoperiod (8 h) at 22°C. Data are means \pm SD of at least 5 values originating from adult leaves from
 1177 independent plants. ***, significantly different from the WT value with $P < 0.001$, (*t*-test). *2cprx*, double
 1178 *2cysprxa 2cysprxb* mutant plants; *ntra ntrb*, double *ntra ntrb* mutant plants; *prxq*, *grxs17*, *trxh3* and
 1179 *trxo1*, mutants lacking PRXQ, GRXS17, TRXh3 and TRXo1, respectively.

1180

1181 **Figure S7. Stomatal density (A and C) and stomatal index (B and D) of Arabidopsis lines affected**
1182 **in the expression of genes coding for various types of thiol reductases.** Data are means \pm SD of 12
1183 independent values using peeled epidermis from three leaves per genotype. Plants were grown for 4
1184 weeks in control conditions: moderate light ($200 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and short photoperiod (8 h) at
1185 22°C . *grxs17*, *trxh3*, *trxo1* and *grxs14*, mutants lacking GRXS17, TRXh3, TRXo1 and GRXS14,
1186 respectively; C-GRXS16 C2 and C4, two independent *GRXS16*-cosuppressed lines; OE-GRXS16 O4
1187 and O5, two independent *GRXS16*-overexpressing lines.

1188

1189 **Figure S8. Stomatal conductance of Arabidopsis lines affected in the expression of genes coding**
1190 **for various types of thiol reductases.** Measurements of the water efflux from leaf abaxial side were
1191 performed using porometry on plants grown for 6 weeks in control conditions. Conductance values are
1192 means \pm SD of six average values originating from independent plants (8 measurements per plant for
1193 each genotype per experiment); *trxh3* (A-B), *trxo1* (C) and *grxs17* (D), mutants lacking TRXh3, TRXo1
1194 and GRXS17, respectively; *grxs14* and OE-GRXS14 OE1 (E), lines lacking *GRXS14* expression and
1195 overexpressing *GRXS14*, respectively; C-GRXS16 C2 and C4 (F), two independent *GRXS16*-
1196 cosuppressed lines; OE-GRXS16 O4 and O5 (G), two independent *GRXS16*-overexpressing lines.

Summary statement: The contribution of thiol reductases to the signaling network governing stomatal functioning remains poorly known. Using proteomics, we show that numerous thiol reductases are abundant in a guard cell-enriched fraction, and we unveil a stomatal phenotype in *Arabidopsis* mutants for thiol reductases.

Do not distribute

Figure 1

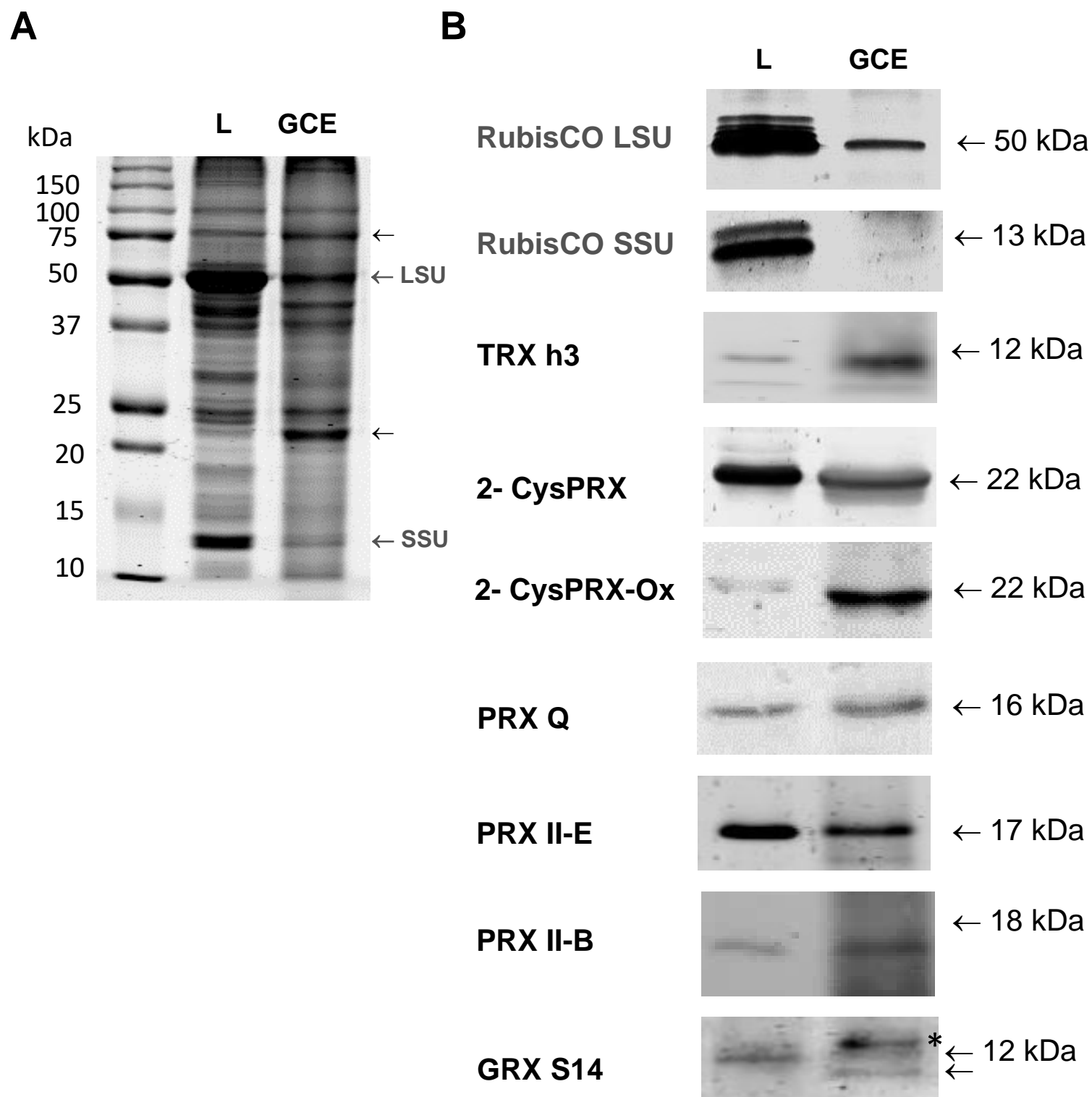
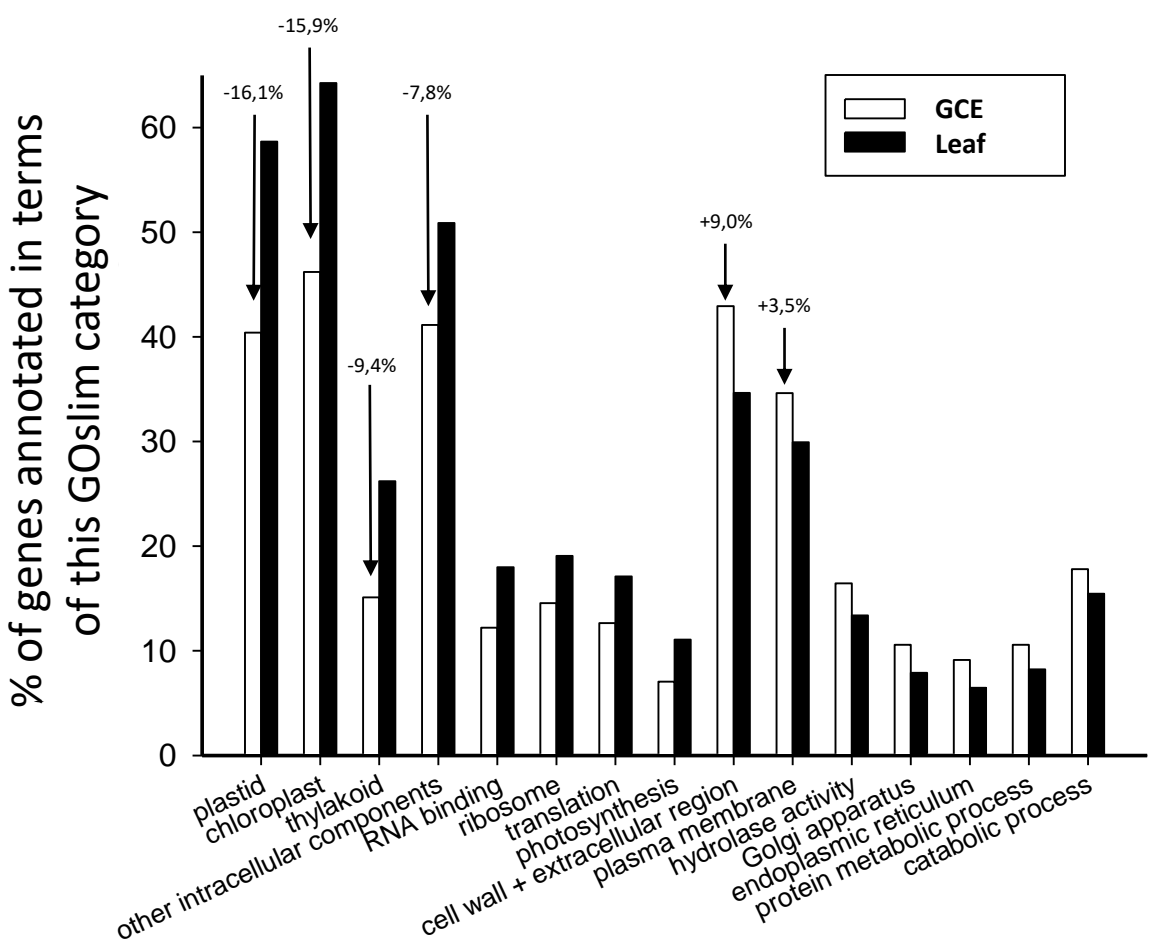


Figure 1 Western blot analysis of the abundance of thiol reductases in extracts from a guard cell-enriched fraction

A. Coomassie Blue-stained gel of SDS-PAGE separated proteins from leaf blades (L) and GCE fractions. The arrows indicate proteins more abundant in the GCE fraction. LSU and SSU, large and small RubisCO subunits, respectively. **B.** Western blot analysis of RubisCO subunits and of various types of thiol reductases in L and GCE fractions. Blots (20 to 30 μ g proteins per lane, depending on the serum used, for both fractions) were probed using antibodies raised against RubisCO (1:10,000), TRX h3 (1:20,000), 2-CysPRX (1:10,000) 2-CysPRX-Ox, hyperoxidized 2-CysPRX (1:4000), PRXQ (1:1000), PRX-II-E (1:10,000) PRXII-B (1:20,000) and GRXS14 (1:800). For the latter, the asterisk points to a non-specific band and the two arrows indicate two GRX redox forms.

Figure 2

Functional Categorization (GO Cellular Component)

Figure 2: Functional categorization was performed by making use of the GO annotation retrieval tool available on the site TAIR: <https://www.arabidopsis.org/tools/bulk/go/index.jsp>. Analysis was retrieved on genes encoding the 25% most abundant proteins of each sample (in broken epidermis, 1174 proteins having a Log_2 of imputed normiBAQ $\geq 23,93$ and in leaf, 1063 proteins $\geq 23,66$)

Figure 3

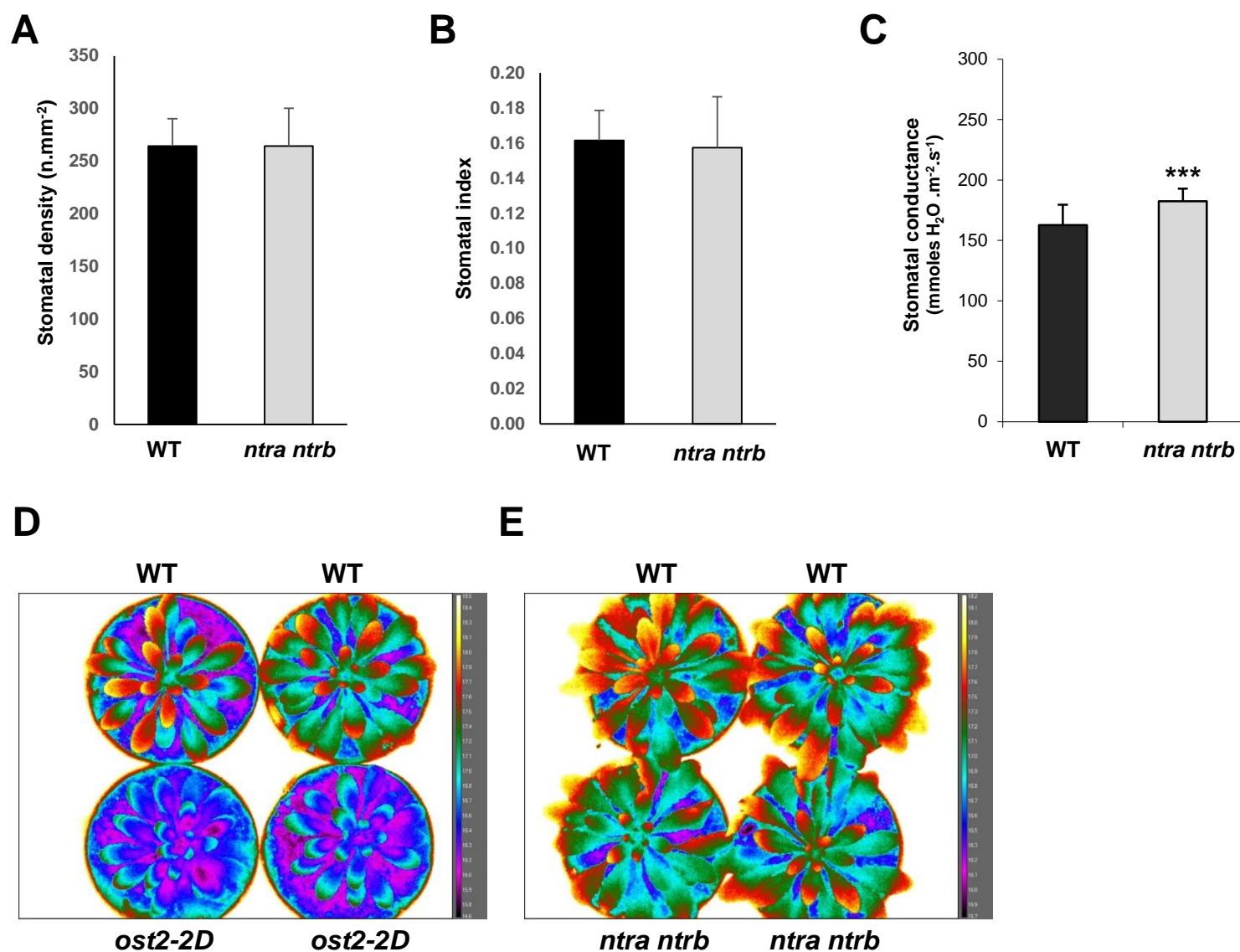
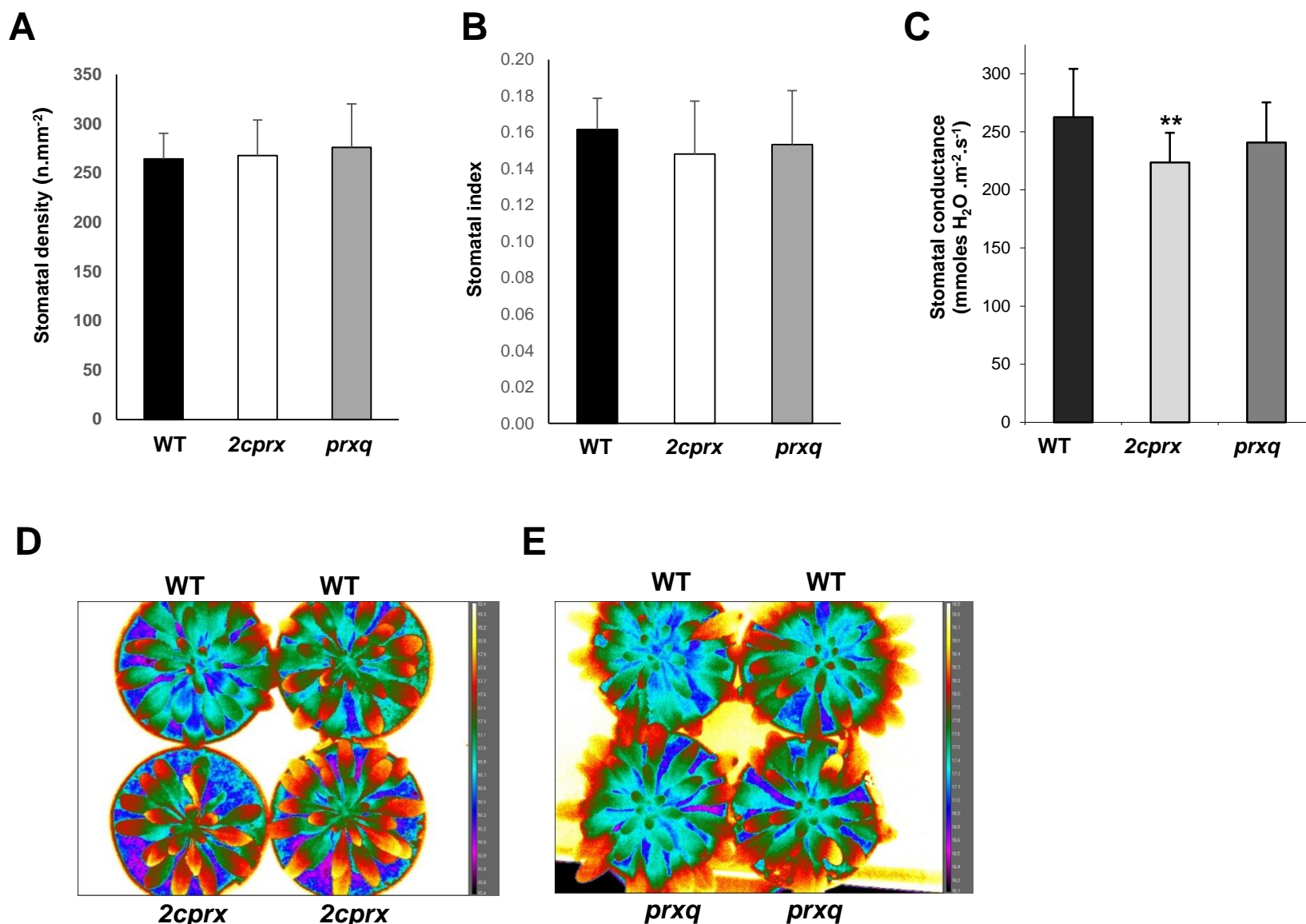


Figure 3. Stomatal phenotype of the Arabidopsis *ntra ntrb* mutant

A. Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values using peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control conditions: moderate light ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . **C.** Stomatal conductance measurements using porometry. Measurements of the water efflux from leaf abaxial side were performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD of twelve average values per genotype originating from independent plants (8 measurements per plant for each genotype per experiment). ***, significantly different from the WT value with $P < 0.001$ (*t*-test). **D.** and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-week-old plants grown in control conditions. The color scale on the right shows increasing temperature from purple to yellow. Pictures were taken at the end of the night period two hours before the light period. As a control, the *ost2-2D* mutant was used and shown to exhibit lower leaf temperature due to open stomata in the dark (Merlot et al., 2007). *ntra ntrb*, double *ntra ntrb* mutant plants.

Figure 4

**Figure 4. Stomatal phenotype of Arabidopsis mutants deficient in plastidial PRXs**

A. Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values using peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control conditions: moderate light (200 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C. **C.** Stomatal conductance measurements using porometry. Measurements of the water efflux from leaf abaxial side were performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD of eight average values per genotype originating from independent plants (8 measurements per plant for each genotype per experiment). **, significantly different from the WT value with $P < 0.01$ (t -test). **D.** and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-week-old plants grown in control conditions. The color scale on the right shows increasing temperature from purple to yellow. Pictures were taken at the end of the night period two hours before the light period. *2cprx*, double *2cysprxa 2cysprxb* mutant plants; *prxq*, knockout *PRXQ* plants.

Figure 5

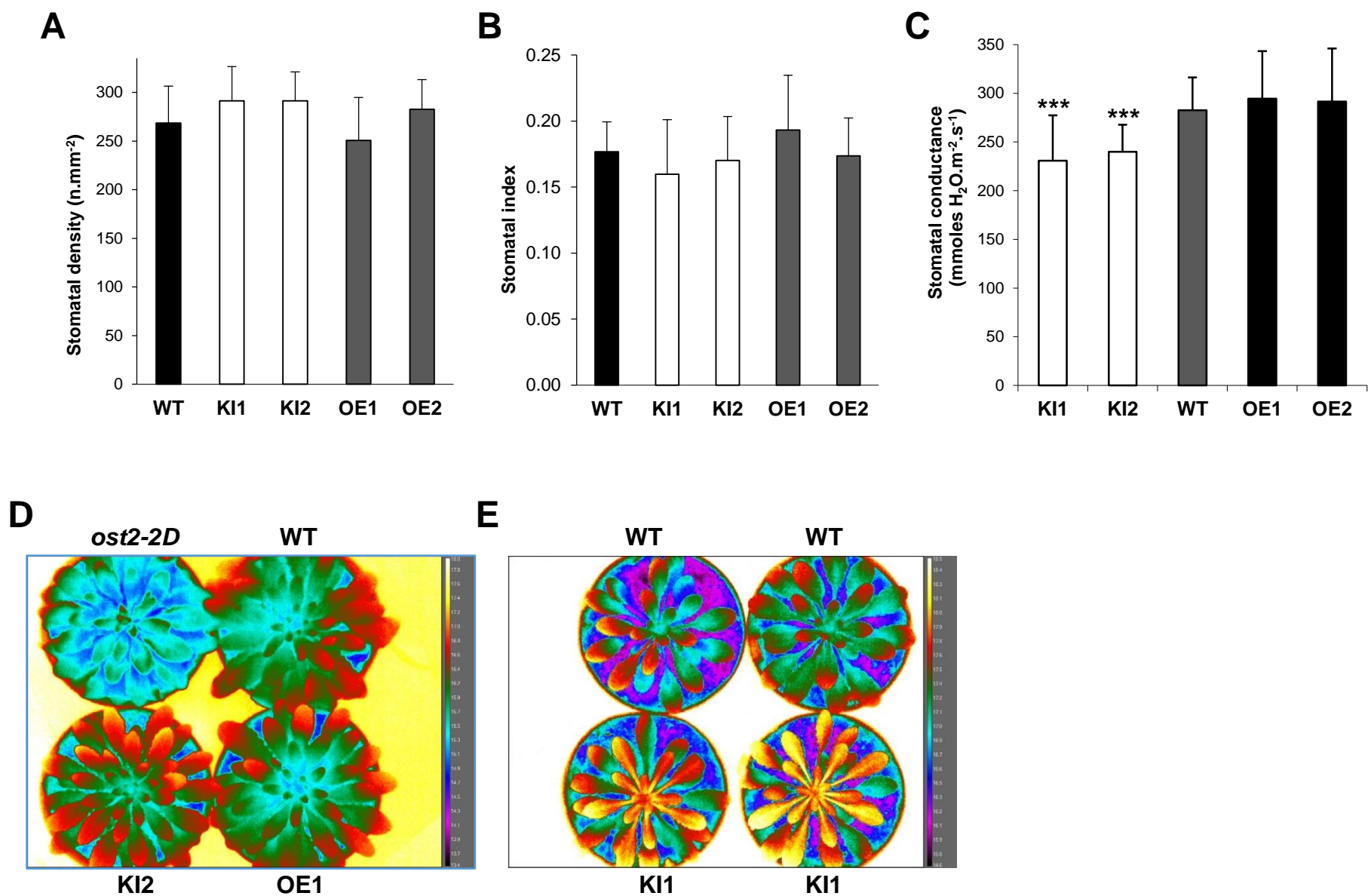


Figure 5. Stomatal phenotype of Arabidopsis lines modified for plastidial type-II GRXs. **A.** Stomatal density. **B.** Stomatal index. Data for **A** and **B** are means \pm SD of 12 independent values using peeled epidermis from three leaves per genotype. Plants were grown for 4 weeks in control conditions: moderate light ($200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and short photoperiod (8 h) at 22°C . **C.** Stomatal conductance measurements using porometry. Measurements of the water efflux from leaf abaxial side were performed on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD of at least 17 average values per genotype originating from independent plants (8 measurements per plant for each genotype per experiment). ***, significantly different from the WT value with $P < 0.001$ (*t*-test). **D.** and **E.** Infrared thermal imaging. Plants were dark-adapted. False colored infrared images of 6-week-old plants grown in control conditions. Pictures were taken at the end of the night period two hours before the light period. The color scale on the right shows increasing temperature from purple to yellow. As a control, the *ost2-2D* mutant was used and shown to exhibit lower leaf temperature due to open stomata in the dark (Merlot et al., 2007). KI1 and KI2, independent *grxS14* GRXS16-RNAi lines; OE1 and OE2, independent *GRXS14*-overexpressing lines.

Figure 6

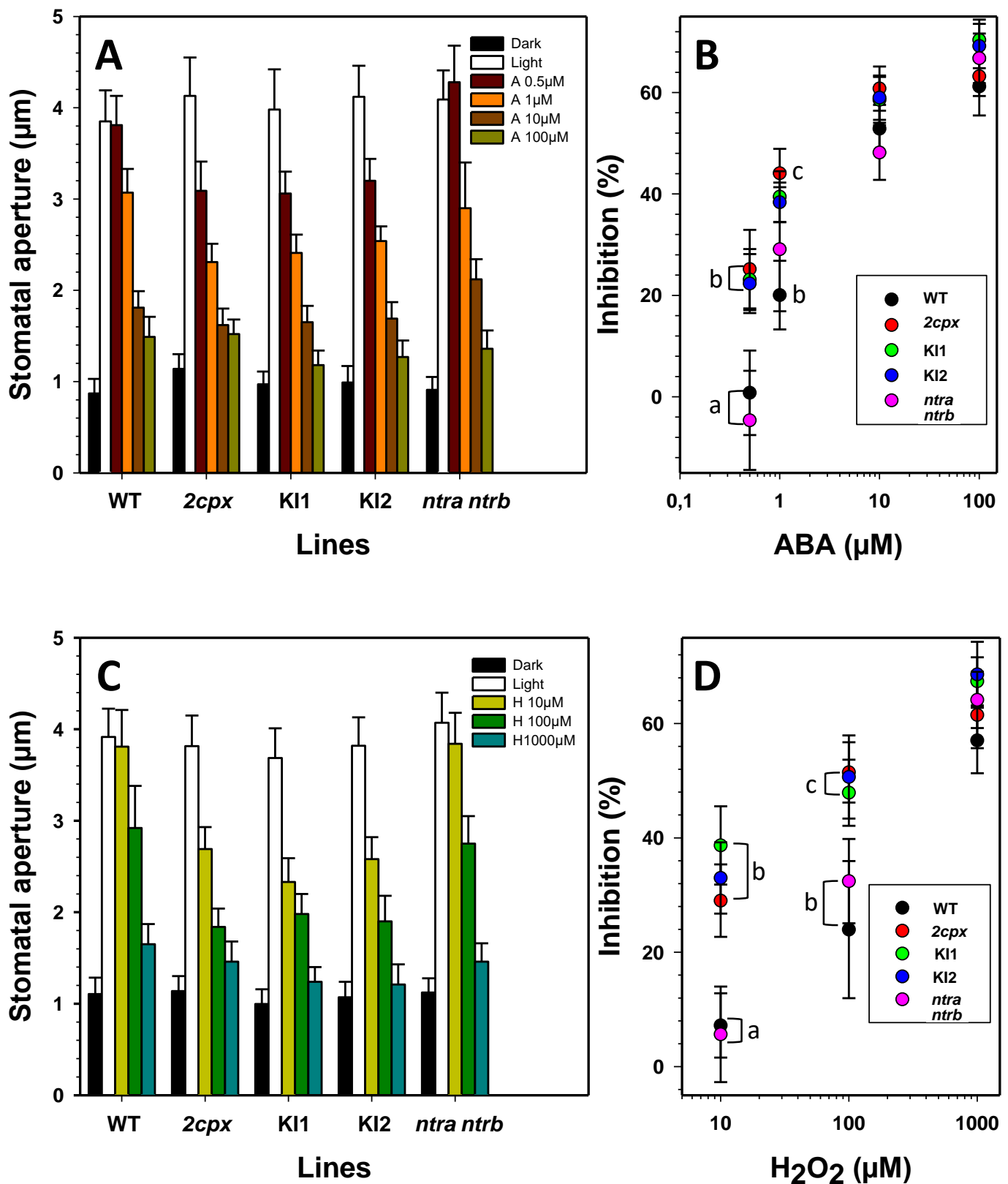


Figure 6. Effect of ABA and H₂O₂ on stomatal aperture in Arabidopsis mutants deficient in cytosolic NTRs, plastidial 2Cys-PRXs or plastidial type-II GRXs.

Stomatal aperture measurements in response to ABA concentrations of 0.5, 1, 10 and 100 µM (A-B) or to H₂O₂ concentrations of 10, 100 and 1000 µM (C-D) in light-treated epidermis. Data in panels B and D were calculated from the original raw data reported in panels A and C, respectively, and represent the inhibitions of stomatal aperture due to the treatments expressed in percents of that measured in light alone. Data represent means ± SD of 2 independent experiments with 60 measurements per genotype and treatment. Means were compared with the ANOVA test, and letters represent groups of means not statistically different (P<0.05). *2cpx*, double *2cysprxa 2cysprxb* mutant plants; KI1 and KI2, independent *grxS14* GRXS16-RNAi lines; *ntra ntrb*, double *ntra ntrb* mutant plants.

Figure 7

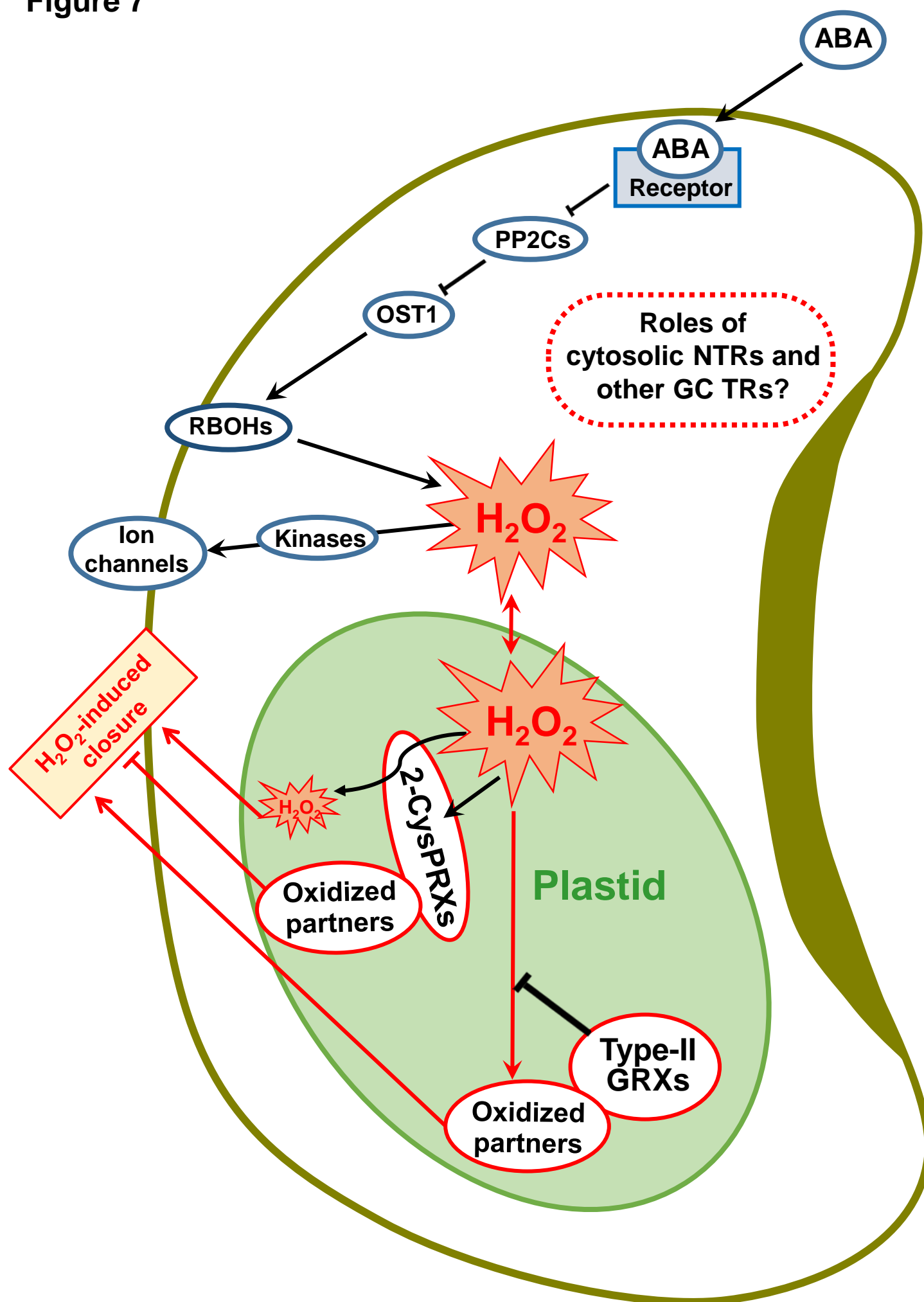


Figure 7. Model for positioning putative roles of plastidial 2-CysPRXs and type-II GRXs in the signaling network governing stomatal closure. Based on the results gained on epidermal peels showing modified responses of mutants to ABA and H₂O₂ and the known biochemical functions of these enzymes, we propose that they participate in H₂O₂-related transduction pathways. 2-CysPRXs might scavenge H₂O₂ within plastid or promote oxidation of partners that remain to be identified and are involved, when oxidized, in a pathway mitigating stomatal closure. Type II-GRXs might prevent oxidation of partners, the oxidized form of which could trigger closure. The roles of cytosolic NTRs and other TRs in guard cells remain to be determined.

Figure S1

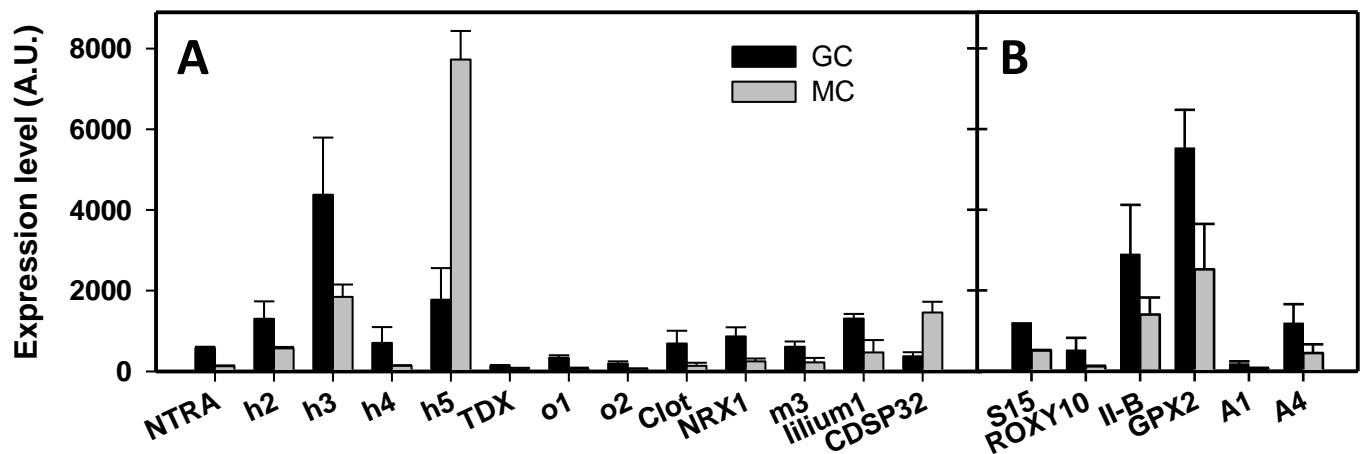


Figure S1: Transcriptomic analysis of *TR* expression in guard and mesophyll cell protoplasts. Data were retrieved from the microarray analyses described by Yang et al., (2008). RNA was isolated from protoplasts prepared from guard or mesophyll cells and hybridized to the ATH1 GeneChip. Two independent hybridization experiments per cell type were performed. Means and SD of expression levels were retrieved using the Arabidopsis eFP Browser at bar-utoronto.ca (Winter et al., 2007). Only *TR* genes displaying GC/MC or MC/GC ratios of expression ≥ 2 were considered for this figure. **A**, NADPH-Thioredoxin reductase A (NTRA) and thioredoxins h2, h3, h4, h5, TDX, o1, o2, Clot, m3, Lillium-1 and CDSP32; **B**, Glutaredoxins S15 and ROXY10, peroxiredoxin II-B (II-B), glutathione peroxidase 2 (GPX2), and methionine sulfoxide reductases A1 and A4.

Figure S2

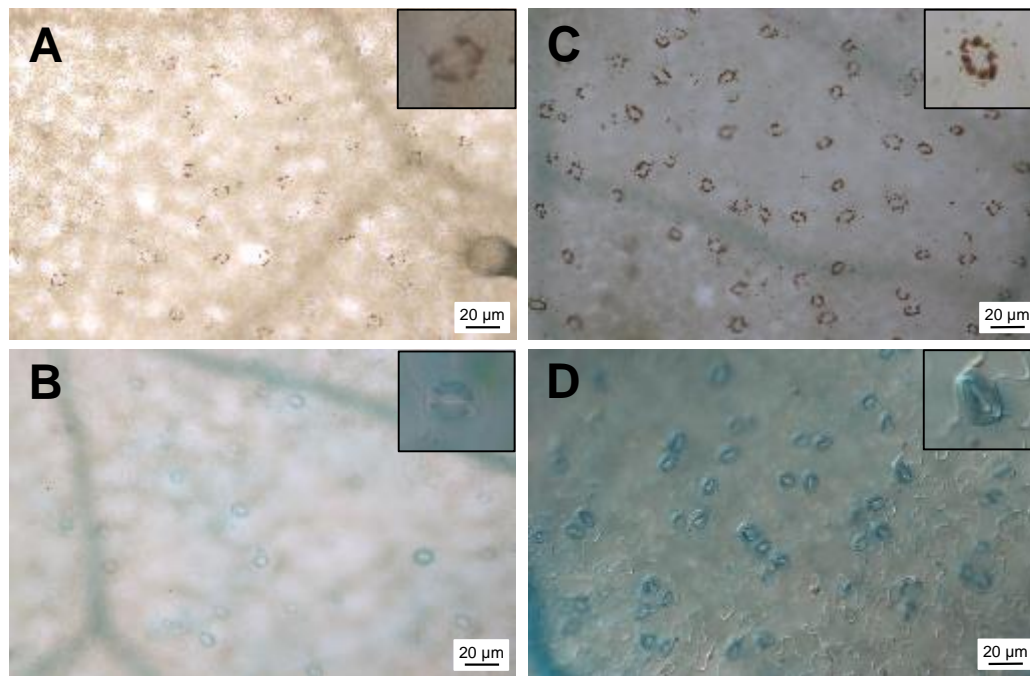


Figure S2. Gene expression in guard cells. *TRXh2* (A), *TRXh3* (B), *NTRB* (C) and *PRX-IIB* (D) gene promoters were fused to β -glucuronidase (*GUS*) as a reporter gene and gene expression was monitored in 15 day-old plants by histochemical analysis. Inset: focus on one single stomata.

Figure S3

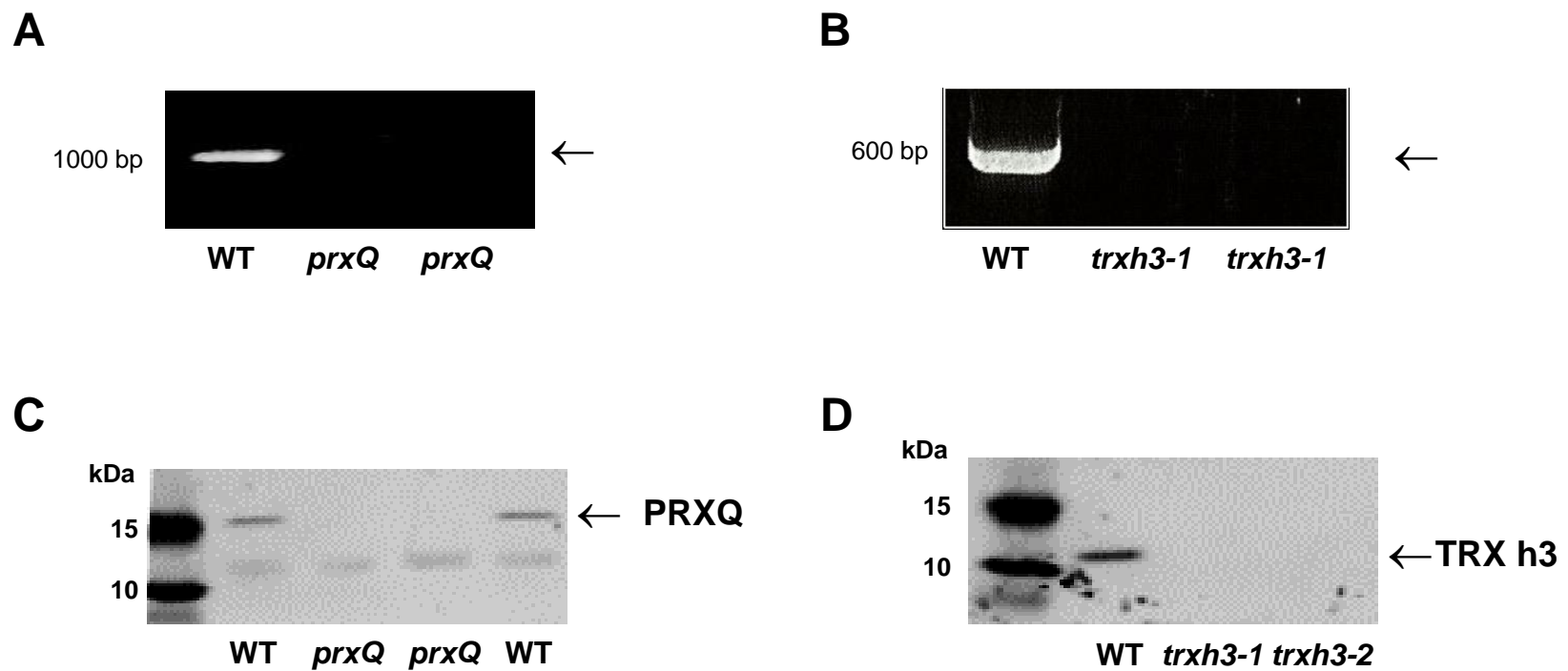


Figure S3. Characterization of mutants deficient in the expression of *PRXQ* and *TRXh3* genes.

A. Genotyping of SAIL_742_G10 plants (*prxq*) using primers corresponding to 5' and 3' ends of *PRXQ* coding sequence. **B.** Genotyping of SAIL_314_G04 (*trxh3-1*) using primers corresponding to 5' and 3' ends of *TRXh3* coding sequence. WT: wild type. **C.** Western blot analysis of PRXQ abundance in WT and *prxq* plants. **D.** Western blot analysis of TRX h3 abundance in WT, *trxh3-1* (homozygous SAIL_742_G10) and *trxh3-2* (homozygous SALK_111160) plants.

Figure S4

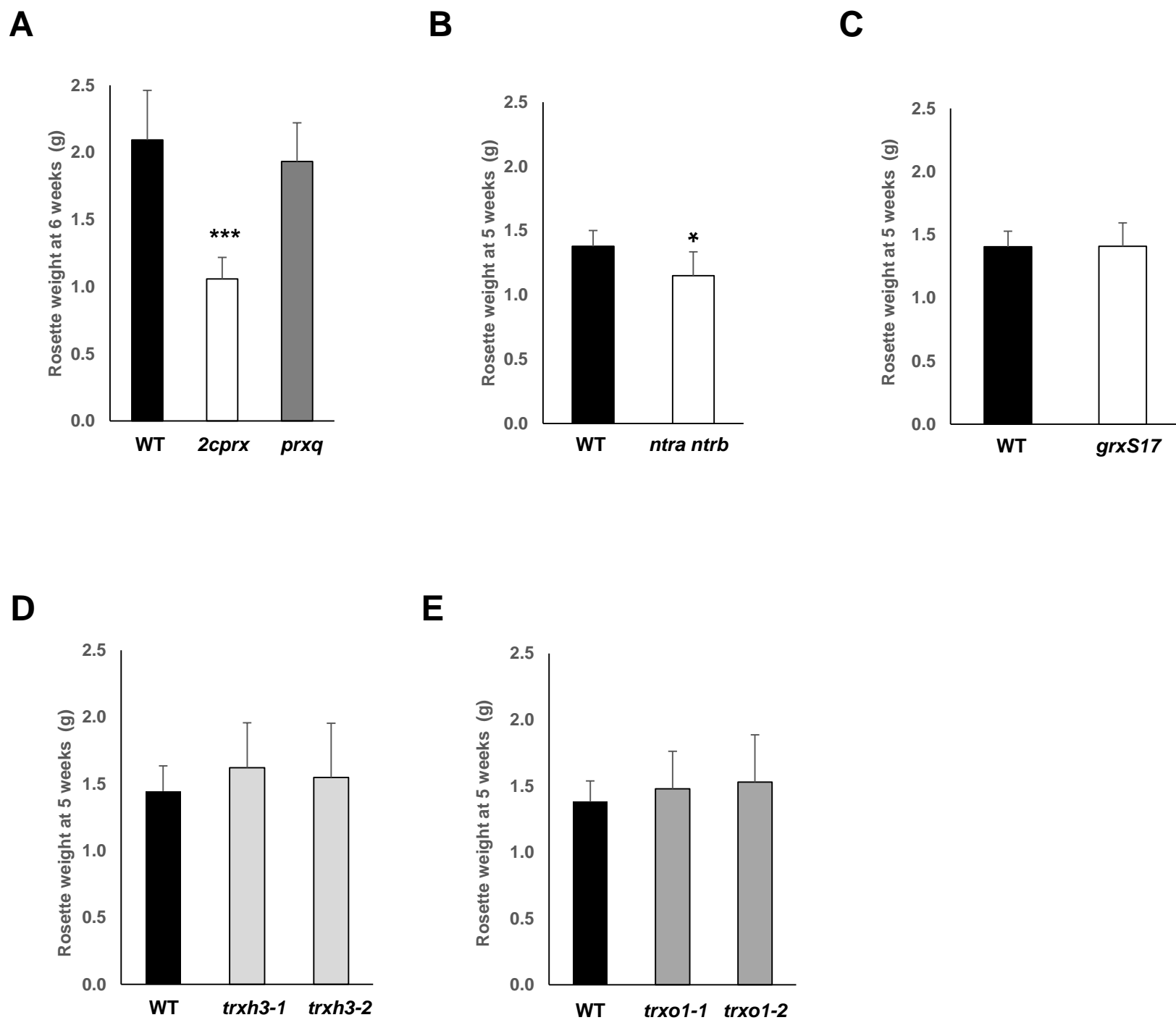


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Figure S5

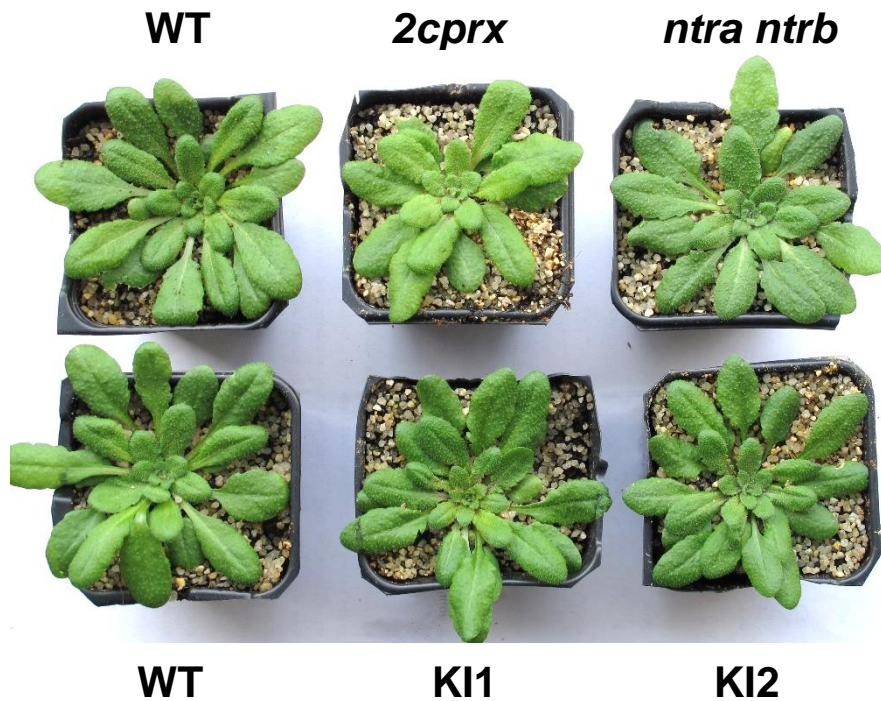


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Figure S6

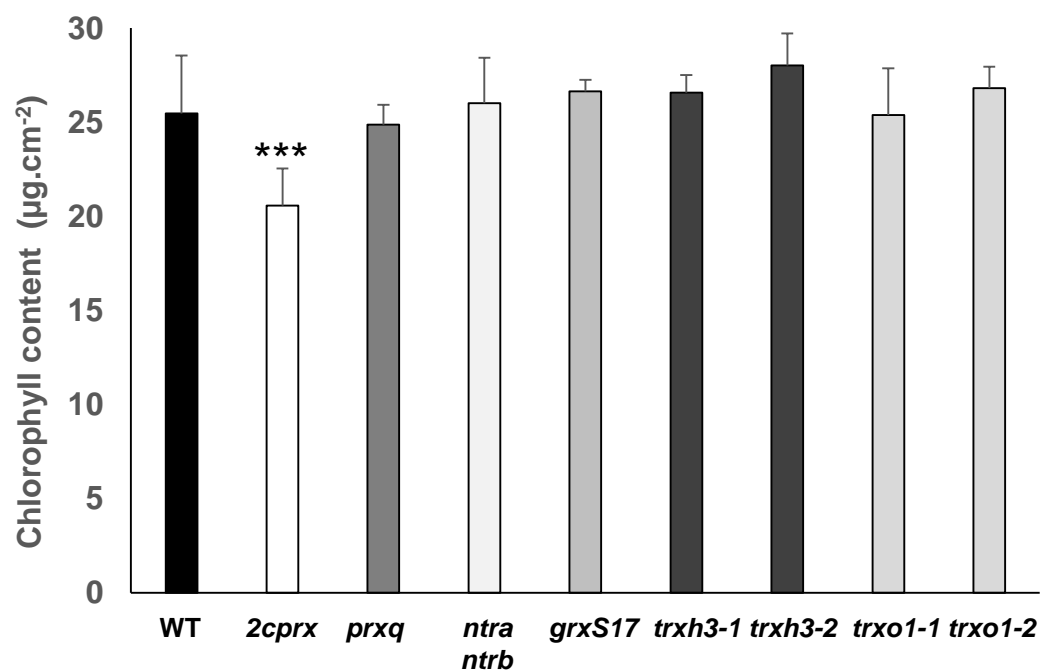


Figure S6. Leaf chlorophyll content of plants modified for the expression of various *TR* genes.

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 at least 5 values originating from adult leaves taken from independent plants. ***, significantly different from the WT value with $P < 0.001$, (*t*-test). *2cprx*, double *2cysprxa 2cysprxb* mutant plants; *ntra ntrb*, double *ntra ntrb* mutant plants; *prxq*, *grxs17*, *trxh3* and *trxo1*, mutants lacking PRXQ, GRXS17, TRXh3 and TRXo1, respectively.

Figure S7

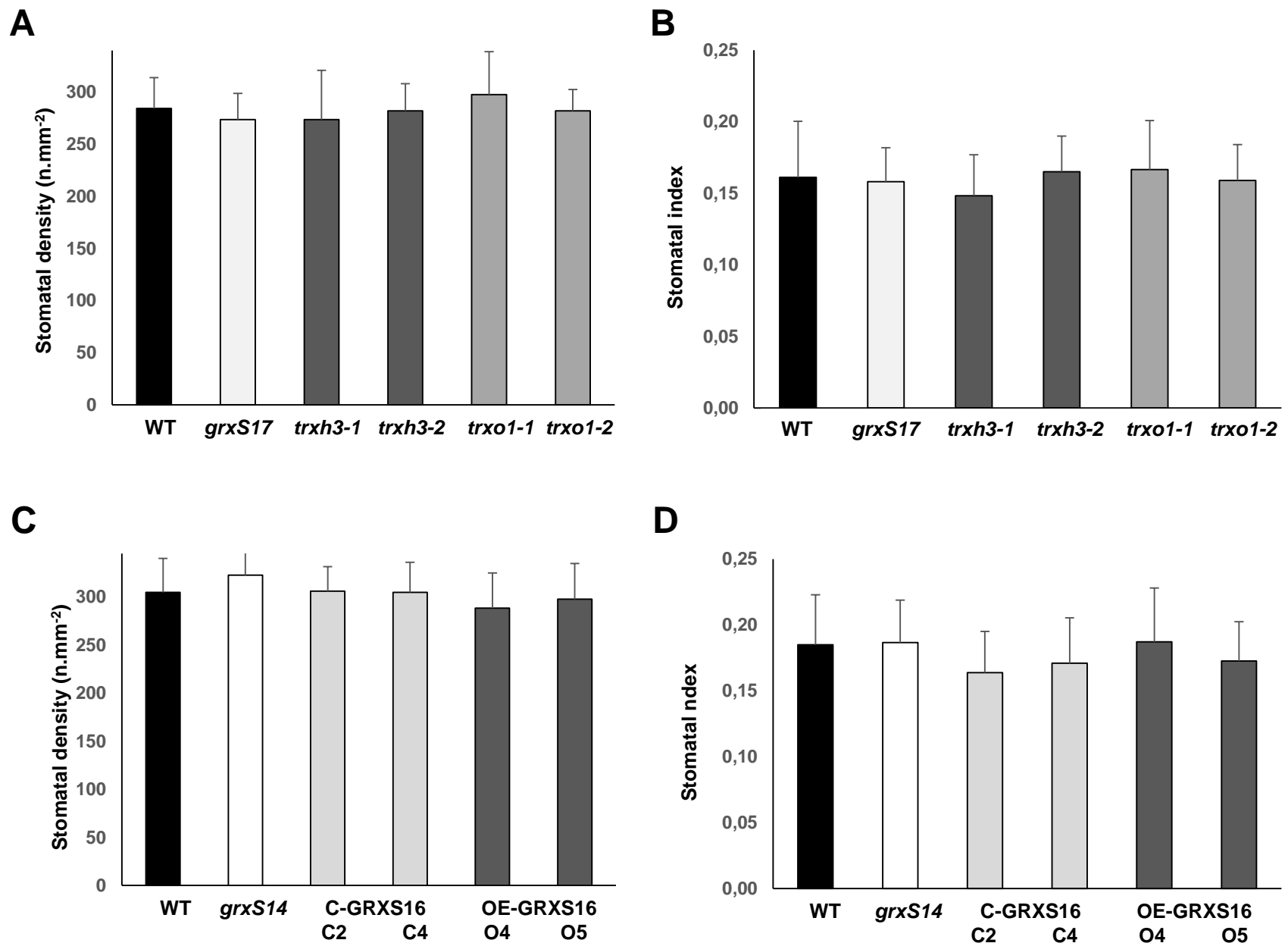


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Figure S8

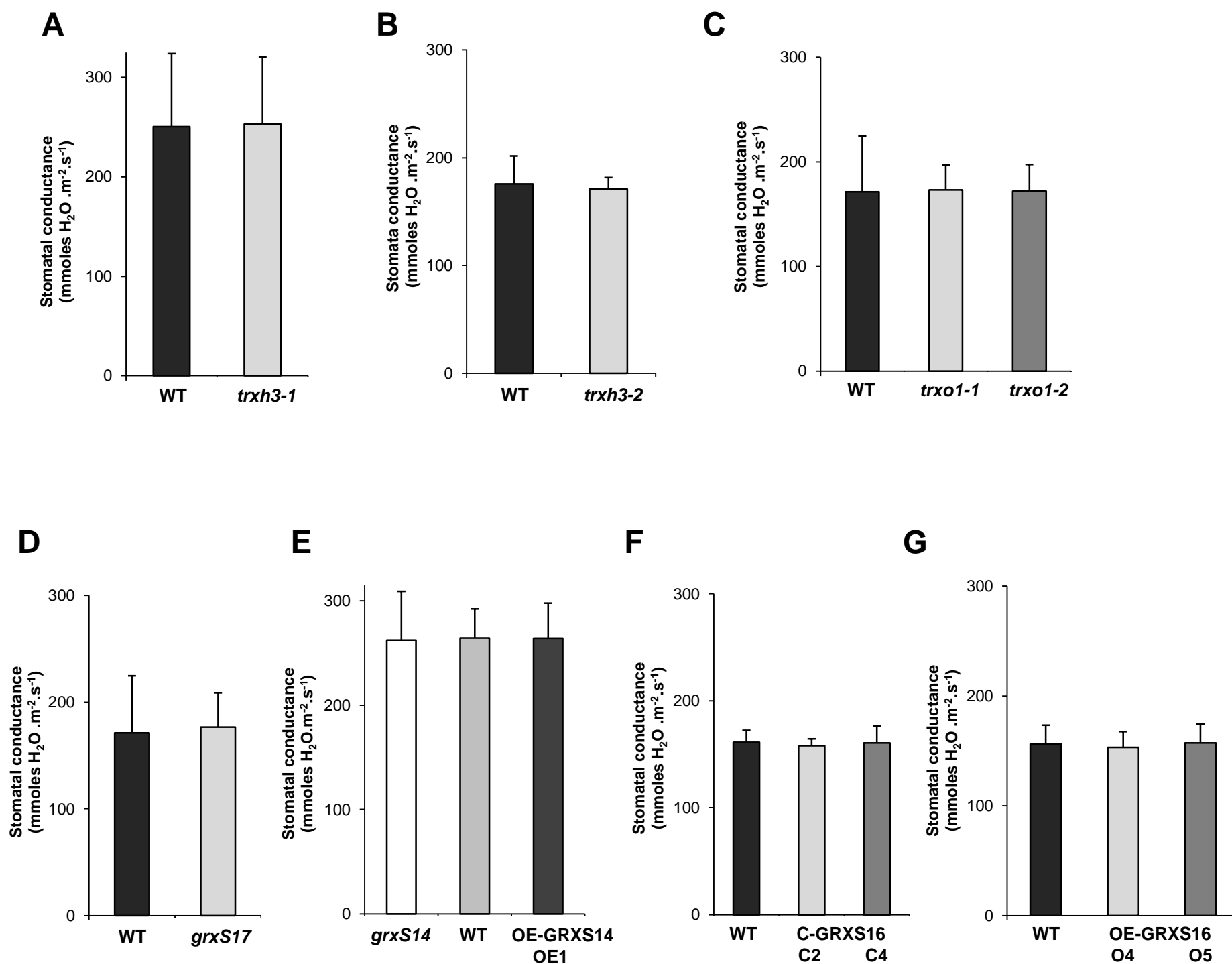


Figure S8. Stomatal conductance of Arabidopsis lines affected in the expression of genes coding for various types of thiol reductases. Measurements of the water efflux from the leaf abaxial side were performed using porometry on plants grown for 6 weeks in control conditions. Conductance values are means \pm SD of six average values originating from independent plants (8 measurements per plant for each genotype per experiment); *trxh3* (A-B), *trxo1* (C) and *grxs17* (D), mutants lacking TRXh3, TRXo1 and GRXS17, respectively; *grxs14* and OE-GRXS14 OE1 (E), lines lacking *GRXS14* expression and overexpressing *GRXS14*, respectively; C-GRXS16 C2 and C4 (F), two independent *GRXS16*-cosuppressed lines; OE-GRXS16 O4 and O5 (G), two independent *GRXS16*-overexpressing lines.