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Igor Florez-Sarasa, Toshihiro Obata, Néstor Fernández Del-Saz, Jean-Philippe Reichheld, Etienne Meyer, et al.. The Lack of Mitochondrial Thioredoxin TRXo1 Affects In Vivo Alternative Oxidase Activity and Carbon Metabolism under Different Light Conditions. *Plant and Cell Physiology*, 2019, 60 (11), pp.2369-2381. 10.1093/pcp/pcz123 . hal-02290024

HAL Id: hal-02290024

<https://univ-perp.hal.science/hal-02290024v1>

Submitted on 16 Jan 2020

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The lack of mitochondrial thioredoxin TRXo1 affects in vivo alternative oxidase activity and carbon metabolism under different light conditions

Journal:	<i>Plant and Cell Physiology</i>
Manuscript ID	PCP-2019-E-00006.R1
Manuscript Type:	Regular Paper
Date Submitted by the Author:	n/a
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Keywords:	alternative oxidase, mitochondrial thioredoxin, oxygen isotope fractionation, carbon fluxes, high light, Arabidopsis thaliana

1 **Article type:** Regular paper

2

3 **Title:** The lack of mitochondrial thioredoxin TRXo1 affects *in vivo* alternative oxidase
4 activity and carbon metabolism under different light conditions

5

6 **Running head:** Thioredoxin regulation of (photo)respiratory metabolism

7

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13 **Subject Areas:**

14 - Photosynthesis, respiration and bioenergetics

15 - Proteins, enzymes and metabolism

16

17 **Black and white Figures:** 6

18 **Colour Figures:** 1

19 **Supplemental Figures:** 2

20 **Supplemental Tables:** 5

21 **Title: The lack of mitochondrial thioredoxin TRXo1 affects *in vivo***
22 **alternative oxidase activity and carbon metabolism under different light**
23 **conditions**

24

25 **Running head: Thioredoxin regulation of (photo)respiratory metabolism**

26

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44

45 **ABSTRACT**

46 The alternative oxidase (AOX) constitutes a non-phosphorylating pathway of electron
47 transport in the mitochondrial respiratory chain that provides flexibility to energy and
48 carbon primary metabolism. Its activity is regulated *in vitro* by the mitochondrial
49 thioredoxin (TRX) system which reduces conserved cysteines residues of AOX.
50 However, *in vivo* evidence for redox regulation of the AOX activity is still scarce. In the
51 present study, the redox state, protein levels and *in vivo* activity of the AOX in parallel to
52 photosynthetic parameters were determined in *Arabidopsis* knock out mutants lacking
53 mitochondrial *trxo1* under moderate (ML) and high light (HL) conditions, known to
54 induce *in vivo* AOX activity. In addition, ¹³C- and ¹⁴C-labeling experiments together with
55 metabolite profiling were performed to better understand the metabolic coordination
56 between energy and carbon metabolism in the *trxo1* mutants. Our results show that the *in*
57 *vivo* AOX activity is higher in the *trxo1* mutants at ML while the AOX redox state is
58 apparently unaltered. These results suggest that mitochondrial thiol redox systems are
59 responsible for maintaining AOX in its reduced form rather than regulating its activity *in*
60 *vivo*. Moreover, the negative regulation of the TCA cycle by the TRX system is
61 coordinated with the increased input of electrons into the AOX pathway. Under HL
62 conditions, while AOX and photosynthesis displayed similar patterns in the mutants,
63 photorespiration is restricted at the level of glycine decarboxylation most likely as a
64 consequence of redox imbalance.

65

66 **Key words:** alternative oxidase, mitochondrial thioredoxin, oxygen isotope fractionation,
67 carbon fluxes, high light, *Arabidopsis thaliana*.

68

69 INTRODUCTION

70 The alternative oxidase (AOX) is a terminal oxidase located in the plant mitochondrial
71 electron transport chain (mETC) which constitutes a pathway of electron transport
72 alternative to the cytochrome oxidase (COX) pathway that is mainly responsible for
73 mitochondrial ATP synthesis (Moore and Siedow, 1991). The non-phosphorylating
74 nature of AOX has raised many questions about its function in plants many of them which
75 are focused on the possibility that it balances cellular energy and carbon metabolism
76 under environmental perturbations (Rasmusson *et al.*, 2009; Vanlerberghe, 2013). In
77 order to gain insight into how this protein is regulated, early studies reported *in vitro*
78 properties of AOX such as post-translational modifications (Millar *et al.*, 1993; Umbach
79 and Siedow, 1993). Plant AOX protein exists in a dimeric state that can be redox regulated
80 through a disulfide/sulphydryl system (Umbach and Siedow, 1993). This system allows
81 the reversible formation of disulfide bonds between adjacent monomers of the AOX
82 dimer leading to a covalently-linked inactive dimer or a non-covalently linked active
83 AOX dimer. Once in its reduced (active) state, the AOX activity can be stimulated on
84 interaction with α -ketoacids including pyruvate (Millar *et al.*, 1993; Umbach and Siedow,
85 1994; Millar *et al.*, 1996; Selinski *et al.*, 2017). Studies in isolated mitochondria reported
86 a significant activity of the AOX after its reduction and addition of pyruvate even when
87 less than 30% of the ubiquinone (UQ) is in its reduced state (Millar *et al.*, 1993; Umbach
88 and Siedow, 1994; Millar *et al.*, 1996). At this UQ reduction state the COX pathway is
89 not fully saturated, therefore, these *in vitro* regulatory features indicated that the AOX
90 pathway can compete with the COX pathway for the electrons of the UQ pool (Hoefnagel
91 *et al.*, 1995, Ribas-Carbo *et al.*, 1995). Competition between the COX and AOX pathways
92 was definitively demonstrated with measurements of the individual activities of both
93 pathways by using the oxygen-isotope fractionation technique (Ribas-Carbo *et al.*, 1995;
94 1997). It is currently well accepted that the *in vivo* activities of both the AOX and COX
95 pathways can be determined by the oxygen-isotope fractionation technique while
96 inhibitors can only be used for measuring AOX and COX capacities (Ribas-Carbo *et al.*,
97 2005; Del-Saz *et al.*, 2018).

98 The two regulatory features described above are currently considered as the main post-
99 translational mechanisms responsible for plant AOX activation, although the extent to
100 which these mechanisms affect its activity *in vivo* still remains as a matter of debate
101 (Riemer *et al.*, 2015; Del-Saz *et al.*, 2018). The AOX has generally been only detected in

102 its reduced-active form in whole tissue extracts, a fact often interpreted to imply that
103 redox regulation is unlikely to occur *in vivo* (Millenaar and Lambers, 2003). However,
104 Noguchi *et al.* (2005) showed that the AOX activity can be regulated by its reduction state
105 in leaves of *Alocasia odora* (a shade species) under different light conditions. Moreover,
106 in different C3 species, post-translational regulation of AOX has recently been shown to
107 be responsible for inducing AOX activity *in vivo* after high light (HL) treatment (Florez-
108 Sarasa *et al.*, 2016). Previous observations suggest that an increase in the levels of
109 NADPH inside mitochondrial matrix would favor the reduction of AOX through the
110 action of a thioredoxin/thioredoxin (TRX) reductase system (Gray *et al.*, 2004). Indeed,
111 there is evidence that the TRX system can modulate AOX reduction state and enhance
112 AOX capacity in the mitochondria isolated from leaves of poplar (Gelhaye *et al.*, 2004),
113 pea (Marti *et al.*, 2009) and Arabidopsis (Yoshida *et al.*, 2013). However, aside from
114 studies in isolated mitochondria, **whether** AOX is indeed regulated by the TRX system *in*
115 *vivo* **is still unclear** (Geigenberger *et al.*, 2017).

116 The plant mitochondrial TRX system consists of NADPH-dependent TRX reductases and
117 mitochondrial TRXs (Laloi *et al.*, 2001; Reichheld *et al.*, 2005; Geigenberger *et al.*,
118 2017). The *TRXo1* gene has been confirmed to encode a mitochondrial TRX while its
119 paralog *TRXo2* is expressed at very low levels and its localization has not yet been
120 confirmed (Meyer *et al.*, 2012). Poplar *PtTrxh2* has been shown to be in mitochondria
121 while its ortholog in Arabidopsis, *AtTrxh2*, is found not only in the cytosol and
122 mitochondria (Meng *et al.*, 2009; Meyer *et al.*, 2012) but also in the endoplasmic
123 reticulum and Golgi (Traverso *et al.*, 2013). Moreover, addition and activation of
124 recombinant TRXo1 protein to isolated mitochondria induces the thiol redox switch of
125 the AOX protein in Arabidopsis, pea and thermogenic skunk cabbage (Marti *et al.*, 2009;
126 Yoshida *et al.*, 2013; Umekawa and Ito, 2018). Therefore, the use of genetically modified
127 *TRXo1* plants is a promising approach to test the *in vivo* function of the mitochondrial
128 TRX system. In this respect, biochemical analyses of *trxo1* T-DNA mutants in
129 Arabidopsis have already been proven highly useful in unraveling the regulatory
130 mechanisms by which the TRX-system regulates TCA cycle enzymes *in vivo* (Daloso *et*
131 *al.*, 2015; Yoshida and Hisabori, 2016).

132 In the present study, we examined the response of *trxo1* knock-out mutants on shifting
133 from **moderate** light (ML) to high-light (HL) which has previously been reported to
134 induce *in vivo* AOX activity. For this purpose, we measured *in vivo* AOX and COX leaf

135 activities, photosynthetic parameters, AOX capacity, protein levels and redox state,
136 relative rates of the TCA cycle and other respiratory fluxes, leaf metabolite profile and
137 ^{13}C label redistribution following incubation with ^{13}C -glucose and ^{13}C -malate. The aims
138 of these experiments were (i) to determine the impact of deficiency of the mitochondrial
139 TRX on the regulation of the *in vivo* AOX activity under ML and HL conditions and (ii)
140 to explore the influence of the mitochondrial TRX on respiratory fluxes and primary
141 metabolism under ML and HL conditions. The TRXo1 has been proposed as the
142 physiological candidate for redox activation of the AOX based on *in vitro* results (Marti
143 et al., 2009; Yoshida et al., 2013; Umekawa and Ito, 2018). In this respect, we report for
144 the first time the *in vivo* AOX activity in plants lacking of *trxo1* under different light
145 conditions. Furthermore, changes on photorespiration were observed under HL
146 conditions denoting the relevance of TRXo1 on the interaction between mitochondrial
147 redox and carbon metabolism under light stress conditions. All these observations are
148 discussed on the basis of the changes on central carbon metabolic fluxes.

149

150 RESULTS

151 *TRXo1* inactivation leads to slight changes in the expression of other TRX-related genes
152 and growth impairment

153 The T-DNA insertion within the *TRXo1* gene was confirmed in both mutant lines (*trxo1-1*
154 and *trxo1-2*) using genomic PCR with T-DNA-specific primers and seeds from
155 homozygous lines were used for subsequent experiments. The insertions in the *TRXo1*
156 gene in both *trxo1-1* and *trxo1-2* lines have previously been mapped and resulted in KO
157 of gene expression (Daloso et al., 2015; Yoshida and Hisabori, 2016). Here, we have
158 confirmed the suppression of *TRXo1* expression in both lines by quantitative PCR
159 analyses (Supplementary Figure S1A). The transcript levels of other genes related to the
160 mitochondrial TRX system were slightly but significantly increased in both mutants
161 (*NTRA*) or only in *trxo1-2* (*TRXo2* and *TRXh*) (Supplemental Figure S1A). In addition,
162 we analysed the transcript levels of *AOX1a* which encodes the most abundant and redox-
163 sensitive AOX isoform; similar levels were observed in all genotypes. After 6 weeks of
164 growth, the rosettes of mutant lines were smaller than wild-type *Col-0* rosettes
165 (Supplemental Figure S1B), thus displaying a significant ($P < 0.05$) 12% and 22%
166 reduction in total rosette fresh weight (Supplemental Figure S1C).

167

168 *Respiratory activities of AOX and COX pathways under moderate and high light*
169 *conditions*

170 Total respiration (V_t) and oxygen-isotope fractionation was measured in order to
171 determine the *in vivo* activities of AOX and COX pathways. V_t was significantly
172 increased after 2 and 4 hours of high light (HL) treatment in all lines (Figure 1A) and
173 reached similar activity in all genotypes. Under moderate light (ML) conditions, *trxo1-1*
174 line displayed significantly ($P < 0.05$) higher V_t than *Col-0* plants and *trxo1-2* line also
175 displayed a similar trend (Figure 1A). The *in vivo* activity of COX pathway (v_{cyt}) was
176 also increased after the HL treatment in all lines and no significant differences were
177 observed between *Col-0* and mutant lines at any light condition (Figure 1B). As for the
178 *in vivo* activity of AOX pathway (v_{alt}), a different HL induction pattern was observed
179 between mutant and *Col-0* plants (Figure 1C). The v_{alt} was significantly increased after
180 HL treatment in *Col-0* plants but not in the mutants (Figure 1C). While v_{alt} was similar in
181 all genotypes following HL treatment, both mutant lines displayed higher v_{alt} than *Col-0*
182 plants under ML conditions but does not further increase under HL conditions (Figure
183 1C).

184

185 *AOX capacity, protein levels and redox state*

186 We measured the AOX pathway capacity (V_{alt}), i.e. as respiration in the presence of
187 cyanide, as well as AOX protein levels and its redox state in order to further investigate
188 the causes of the altered *in vivo* AOX activities (v_{alt}) -i.e. measured in the absence of
189 inhibitors by oxygen-isotope fractionation. Unlike the v_{alt} , the V_{alt} was similar among
190 genotypes under ML conditions (Figure 2A). In order to evaluate the AOX protein levels
191 in the different genetic backgrounds, we performed immunoblots using anti-AOX
192 antibodies (Figure 2B). The AOX antibody used detects both AOX1 and 2-type isoforms
193 from Arabidopsis (see further information in **Material and Methods**). After porin levels
194 correction (see **Material and Methods** for details), the AOX protein levels were similar
195 among genotypes under ML conditions (Figure 2B). On the other hand, the AOX capacity
196 and protein levels were not significantly increased after HL treatment in any of the
197 genotypes (Figure 2). In general, results clearly show that the capacity to respire via AOX

198 was not altered in the mutants at any light condition and was not induced after HL
199 treatment. Moreover, the percentage of the ratio between activity and capacity of the AOX
200 (previously defined by others as AOX engagement and here calculated from Figure 1C
201 and Figure 2A data) indicates that AOX was engaged at $75 \pm 7\%$ (mean \pm SE) in *Col-0*
202 plants while it was fully engaged in the mutants ($107 \pm 12\%$ and $111 \pm 11\%$). These
203 results suggest that inactivation of *TRXo1* altered the AOX regulation.

204 In order to further study the AOX regulation, the redox state of the AOX protein was
205 analyzed in isolated leaf membranes with or without the addition of DTT or diamide
206 (Figure 3 and Supplemental Figure S2B, see Material and Methods for details). The AOX
207 monomeric (reduced) and dimeric (oxidized) forms were detected in the presence of DTT
208 and diamide, respectively (Figure 3 and Supplemental Figure S2B). After porin levels
209 correction, the total amount of AOX was similar between genotypes but significantly
210 different between redox treatments, being the mean of the total AOX amount in non-
211 treated, diamide-treated and DTT-treated samples of 0.57 ± 0.04 , 0.13 ± 0.02 and $1.28 \pm$
212 0.28 , respectively. Therefore, the redox treatments significantly affected the
213 mitochondrial protein extracted. The reason for these differences remain uncertain,
214 however, these results do not affect to the evaluation of the AOX redox state among
215 genotypes in the non-treated samples. In the absence of redox chemicals, the AOX was
216 mainly found in its monomeric (reduced) form, thus representing 89 to 93% of the total
217 AOX detected in all genotypes (Figure 3 and Supplemental Figure S2B). Signals of the
218 dimeric forms, one with a lower molecular weight perhaps indicating heterodimers from
219 different isoforms, were only between 7% and 11% of the total AOX detected in all
220 genotypes (Figure 3 and Supplemental Figure S2B). Porin levels were very similar among
221 genotypes thus indicating similar mitochondrial loading. Therefore, it is concluded that
222 inactivation of *TRXo1* does not modify the redox state of AOX (Figure 3 and
223 Supplemental Figure S2B).

224

225 *Respiratory CO₂ evolution for TCA cycle and other respiratory flux analysis under*
226 *moderate light conditions*

227 In order to determine whether changes on the *in vivo* AOX activity are linked to the other
228 respiratory fluxes the evolution of ¹⁴CO₂ was recorded following incubation of leaf discs
229 in positionally labeled [¹⁴C]glucose. Leaf discs were supplied with [1-¹⁴C]glucose and

230 [3:4-¹⁴C]glucose over the period of 4 h and evolved ¹⁴CO₂ was determined at hourly
231 interval. The radioactivity released from position C1 of glucose is attributed to
232 decarboxylation processes in the oxidative pentose phosphate pathway and the TCA
233 cycle. The CO₂ released from positions C3:4 is due to the action of pyruvate
234 dehydrogenase or malic enzyme and derived from mitochondrial respiration. The CO₂
235 evolution from the C1 position of glucose to that from C3:4 position indicates relative
236 respiratory flux through the TCA cycle in relation to other respiratory processes (Kruger
237 et al., 2017). Mutant lines displayed lower CO₂ emission from [1-¹⁴C] glucose, being
238 those differences significant in *trxo1-2* after 3 and 4 hours of incubation (Figure 4). On
239 the other hand, CO₂ emission from [3:4-¹⁴C] glucose was similar in all lines (Figure 4).
240 When ratios between both emissions are calculated, both mutant lines showed higher C3:4
241 to C1 ratios than *Col-0* plants, being significantly higher in *trxo1-2* and *trxo1-1* lines after
242 3 and 4 hours, respectively (Figure 4). These results suggest that mutants display higher
243 TCA cycle flux in relation to other respiratory pathways.

244

245 *Photosynthetic and photorespiratory parameters under moderate and high light*
246 *conditions*

247 Changes in photosynthesis and photorespiration could cause growth and respiratory
248 metabolism alterations in the *trxo1* mutants. Therefore, gas exchange and chlorophyll
249 fluorescence measurements were performed in fully expanded leaves of all three lines at
250 **ML** and **HL** conditions. Net photosynthesis (A_N), stomatal conductance (g_s) and
251 chloroplast electron transport rate (ETR) were higher at **HL** than at **ML** conditions in all
252 three lines (Figure 5). Photosynthetic parameters were similar in all lines at **HL**
253 conditions, with the exception of A_N at 4h of **HL** which was significantly lower in *trxo1-1*
254 (Figure 5). By contrast, ETR in both mutants and A_N in *trxo1-1* were significantly lower
255 than in *Col-0* plants under **ML** conditions (Figure 5), although differences were not
256 physiologically relevant. Finally, A_N was measured at saturating light conditions under
257 normal and low O₂ concentration in all genotypes and treatments. The percentage of O₂
258 inhibition of A_N was then calculated as an indication of photorespiration. Results indicate
259 a significantly lower photorespiration in both mutants under 2h of **HL** as compared to
260 *Col-0* plants, while similar values among lines were observed at the other light conditions
261 (Figure 5).

262

263 *Metabolite profiles and ¹³C enrichment analysis under moderate and high light*
264 *conditions*

265 In order to further investigate the metabolic changes underlying the altered responses of
266 (photo) respiratory pathways in the mutants, GC-TOF-MS metabolite profiling analysis
267 was performed on samples harvested under the different light conditions. A total of 36
268 metabolites were annotated after GC-TOF-MS analyses (Table S1) and their relative
269 levels were normalized to the mean levels of *Col-0* plants under ML conditions. Leaves
270 of *Col-0* plants displayed significant increases in 23 and 28 metabolites after 2 and 4 hours
271 of HL treatment, respectively (Figure 6 and Table S2). The greatest fold-change was
272 observed in glycine levels (20 to 37 fold increase) followed by glutamine (10 to 20 fold
273 increase). Metabolite levels in the mutants displayed very similar patterns after HL
274 treatment as compared to *Col-0* plants (Figure 6). Indeed, only trehalose displayed
275 significantly lower levels in both mutant lines after 2h of HL treatment as compared to
276 *Col-0* plants, while isoleucine and threonine levels were higher only in the *trxo1-2* line
277 (Figure 6 and Table S2). On the other hand, the levels of fumarate and *myo*-inositol were
278 consistently lower in both mutant lines under ML conditions (Figure 6), while threonate
279 and 1,4-lactone galactonate levels were lower only in *trxo1-2* mutants.

280 Changes on the relative levels of metabolites are not always linked to changes in
281 metabolic flux (Fernie and Stitt, 2012). In order to have a better estimation of metabolic
282 fluxes in the respiratory pathways, the total ¹³C label redistribution in different
283 metabolites was determined after 2 and 4 hours of ¹³C-Glucose and ¹³C-Malate labeling
284 under ML (100 μmol m⁻² s⁻¹ in this case) and HL (400 μmol m⁻² s⁻¹ in this case) conditions
285 (Figure 7 and Tables S3 and S4). Under ML conditions, most metabolites showed an
286 increase in ¹³C enrichment after 2 and 4 hours of ¹³C-Glc feeding (Tables S3 and S4) but
287 only fumarate (in both mutants) and glycine (in *trxo1-1*) displayed statistically higher ¹³C
288 redistribution in mutants as compared to *Col-0* plants (Figure 7A and Table S3). Higher
289 ¹³C label redistribution to glycine was also observed after ¹³C-Mal feeding in *trxo1-2*
290 while label redistribution to fumarate was similar in all genotypes (Figure 7A and Table
291 S4). In addition, increased label redistribution to GABA was observed in both mutants as
292 compared to *Col-0* plants after 4 h of ¹³C-Mal feeding (Figure 7A). By contrast, decreased
293 label redistribution to *myo*-inositol was observed in *trxo1-1* and *trxo1-2* mutants

294 following 2h and 4h of ^{13}C -Mal feeding, respectively (Figure 7A). Raffinose and alanine
295 displayed some lower label redistribution only in *trxol-1* after 2h of ^{13}C -Mal feeding
296 under **ML** conditions (Table S4). The ^{13}C label redistribution in some metabolites was
297 much higher after HL treatment than under **ML** conditions, however, this was not
298 consistent across the different mutant lines or time points (Tables S3 and S4). Fumarate
299 (^{13}C -Glc feeding), proline (^{13}C -Glc feeding), tryptophan (^{13}C -Mal feeding) and glucose
300 (^{13}C -Mal feeding) displayed significant changes in label redistribution after HL treatment
301 only in single mutant lines and time points (Tables S3 and S4). However, higher label
302 redistribution to glycine following ^{13}C -Mal feeding was consistently observed in both
303 mutant lines and time points (Figure 7B). Moreover, higher label redistribution to glycine
304 after ^{13}C -Glc feeding was also observed in both mutant lines, though at different time
305 points (Figure 7B). Similarly, higher label redistribution to serine was detected in both
306 lines following 2 and 4h of HL treatment of ^{13}C -Glc and ^{13}C -Mal feeding, respectively.
307 Higher label redistribution to alanine and erythritol was detected in both mutant lines
308 following ^{13}C -Glc (2h) and ^{13}C -Mal (4h) feeding, respectively (Figure 7B).

309

310 **DISCUSSION**

311 *The mitochondrial TRXo1 is not required for the AOX activation in vivo*

312 Here we investigated the *in vivo* role of the mitochondrial TRX in the regulation of AOX
313 by combining measurements of the *in vivo* AOX activity in leaves of Arabidopsis mutants
314 lacking mitochondrial *TRXo1* with a range of other physiological analysis. The TRX
315 system has been shown to regulate the AOX activity *in vitro* by reducing conserved Cys
316 thus yielding an active and non-covalently linked AOX homodimer (Gelhay *et al.*, 2004;
317 Marti *et al.*, 2009; Yoshida *et al.*, 2013). Therefore, the *in vivo* AOX activity was
318 anticipated to be down-regulated in the mutants as compared to *Col-0* plants but
319 surprisingly, our results clearly demonstrate that this is not the case (Figure 1). *In vivo*
320 AOX activity was detected in both mutant lines under **ML** and HL conditions (Figure 1)
321 at similar or higher levels than in *Col-0* plants. **While**, these observations suggest that
322 mitochondrial thioredoxin *TRXo1* is not required for the activation of the AOX *in vivo*,
323 we cannot rule out that redox activation of the AOX is being carried out by other
324 thioredoxins or by other thiol reductases (e.g. glutaredoxins) located in the mitochondria
325 (Meng *et al.*, 2009; Moseler *et al.*, 2015). The increased transcript levels of *NTRA*, *TRXh2*,

326 and *TRXo2* in the *trxo1* mutants may involve possible compensation effects (Figure S1A).
327 Candidate mitochondrial TRXs are *TRXh2* and *TRXo2* which were suggested to be
328 located in mitochondria (Meng *et al.*, 2009; Yoshida and Hisabori, 2016), although direct
329 evidence of their presence in the matrix is still scarce. Further studies involving genetic
330 approaches using multiple mutants of the different redox systems would be required to
331 confirm a compensation effect, which would explain why both reduced and oxidized
332 forms of the AOX were similar in all genotypes (Figure 3 and Supplemental Figure S2B).
333 Nevertheless, evidence against the *in vivo* relevance of the redox regulation of AOX
334 activity has been reported mainly based on the fact that the AOX protein has been detected
335 in its reduced state in different tissues, species and experimental conditions (Del Saz *et al.*,
336 2018 and references therein). Recently, the oxidized form of the AOX has not been
337 detected neither in *Col-0* nor in *trxo1* mutant *Arabidopsis* plants by using a different redox
338 approach in enriched mitochondrial fractions (Sanchez-Guerrero *et al.*, 2019). While
339 oxidized forms were detected here, the AOX was mainly detected in its reduced form in
340 both in *Col-0* and *trxo1* mutant leaves (Figure 3 and Supplemental Figure S2B). In
341 agreement with our hypothesis, Nietzel *et al.* (2017) have recently suggested that the thiol
342 redox switch of the AOX which operates *in vitro* does not necessarily imply any
343 regulatory function *in vivo* but may rather play an important maintenance function in the
344 avoidance of AOX oxidation.

345 Vanlerberghe *et al.* (1999) previously suggested that the presence of reduced AOX is
346 necessary for its activity but other factors such as α -ketoacids and the redox state of the
347 UQ pool are critical to ensure AOX activity *in vivo*. Changes in the level of reduced UQ
348 have subsequently been revealed as a key factor explaining AOX activity *in vivo* but
349 importantly not the only factor (Millenaar *et al.*, 2001). The alteration of the different
350 regulatory factors individually (i.e. by using genetically modified plants with altered
351 synthesis of UQ) in combination *in vivo* AOX activity measurements will be key to
352 precisely determine the impact of UQ levels on AOX regulation. The regulation of AOX
353 via interaction with α -ketoacids has also been questioned given that mitochondrial levels
354 of pyruvate have been estimated to be high enough to fully activate AOX (Millenaar and
355 Lambers, 2003). However, increased pyruvate levels and AOX *in vivo* activity were
356 observed in soybean roots following treatment with an inhibitor of branched-chain amino
357 acid biosynthesis (Gaston *et al.*, 2003). Additionally, evidence for pyruvate stimulation
358 of AOX capacity was reported in tubers of transgenic potato with decreased pyruvate

359 kinase activity (Oliver *et al.*, 2008) and in pea mesophyll protoplasts following high light
360 treatment (Dinakar *et al.*, 2010).

361

362 *AOX activity is upregulated in vivo by the lack of mitochondrial TRX*

363 Perhaps even more unexpected than the presence of AOX activity is that activity was
364 higher in the *trxo1* mutants than in *Col-0* plants (Figure 1). The higher *in vivo* AOX
365 activity in the mutants consequently reduced the energetic efficiency of respiration. This
366 finding, but not the minor changes observed in photosynthetic carbon assimilation (Figure
367 5), could explain the rosette growth retardation (Figure 1B). **A detailed analysis on**
368 **adenylate levels in different subcellular compartments (Krueger et al., 2009; Gardeström**
369 **and Igamberdiev, 2016) would be required at different growth stages to stablish the**
370 **importance of the mitochondrial ATP synthesis efficiency on the mutants growth. On the**
371 **other hand, myo-inositol levels were consistently reduced in both *trxo1* mutants as well**
372 **as displaying a lower label redistribution to this metabolite, which can indicate a different**
373 **use of raffinose family oligosacarides as carbon sources (Van den Ende, 2013). In**
374 **agreement, Sanchez-Guerrero et al. (2019) has recently found a very similar decrease in**
375 ***myo*-inositol levels in *trxo1* mutants, while also marked decreases in sugar levels were**
376 **observed. The metabolic differences observed in Sanchez-Guerrero et al. (2019) are**
377 **probably related to the growth of the plants in the presence of sucrose-containing media.**
378 **Nevertheless, both metabolic phenotypes are in line with an up-regulation of the**
379 **respiratory metabolism (Sanchez-Guerrero et al., 2019). In addition, the percentage of**
380 **germination was previously observed to be unaffected in the *trxo1* mutants in the absence**
381 **of sucrose and stress (Daloso et al., 2015; Ortiz-Espin et al., 2017), similar to the case in**
382 **the present study. An increased AOX expression in *trxo1* mutants could explain a higher**
383 ***in vivo* activity of the AOX under ML. However, the AOX capacity and protein levels**
384 **clearly show that the capacity to respire via AOX was not higher in the mutants than in**
385 ***Col-0* plants (Figure 2). The AOX capacity (i.e. the rate of oxygen uptake following**
386 **cyanide inhibition) is well-documented to correlate well with AOX protein abundance**
387 **but frequently does not correlate with the *in vivo* activity (i.e. as determined with the O₂**
388 **isotope technique in the absence of inhibitors, Del-Saz et al. (2018) and references**
389 **therein). Furthermore, the AOX activity and capacity were similar in the mutants under**
390 **ML conditions (Figure 1C and Figure 2A) indicating that the AOX was running at its full**
391 **capacity in the mutants. These observations suggest that lack of TRXo1 activates the**

392 AOX protein *in vivo*. A direct redox regulation of the *in vivo* AOX activity by the TRXo1
393 is, however, unlikely since we could not detect changes in the redox state of the AOX in
394 *trxo1* mutants as compared to *Col-0* plants (Figure 3 and Supplemental Figure S2B).

395 As a cause for the observed increased *in vivo* AOX activity we alternatively propose an
396 indirect effect of the lack of TRXo1. Our results indicate a higher ratio of TCA cycle to
397 other respiratory fluxes in both mutant lines as compared to the *Col-0* (Figure 4), thus
398 confirming the previously reported negative regulation of the TCA cycle by TRX system
399 (Daloso *et al.*, 2015). In particular, Daloso *et al.* (2015) reported that succinate
400 dehydrogenase (SDH) and fumarase (FUM) activities were higher in TRX mutants. These
401 previous observations could explain the lower levels of fumarate observed in both mutant
402 lines (Figure 6). Note that no change in fumarate but increased malate accumulation was
403 previously reported in TRX mutant leaves at the end of the day (Daloso *et al.*, 2015),
404 while the lower levels of fumarate observed here correspond to analyses of leaf samples
405 at the start of the day. Despite lower fumarate levels, higher ¹³C label redistribution to
406 fumarate was detected in both mutants after ¹³C-Glc labeling. In addition, the higher ¹³C
407 label redistribution to GABA after ¹³C-Mal labeling suggests an increased GABA shunt
408 activity likely in order to replenish succinate levels in the TRX mutants. Taken together,
409 these observations support an increased TCA cycle flux (i.e. from succinate to malate)
410 which provides extra electrons into UQ pool of the electron transport chain and explains
411 the higher AOX *in vivo* activity in *trxo1* mutants under ML conditions. In fact, the higher
412 *in vivo* AOX activity probably maintained the UQ reduction level stable in the *trxo1*
413 mutants, as it is the main general role of AOX (Millenaar and Lambers, 2003; Del-Saz *et al.*,
414 2018). The similar transcript levels of *AOX1a* observed in *Col-0* and mutant plants
415 supports this view, because *AOX1a* levels are very sensitive and responsive to UQ
416 reduction levels and its ROS associated signals (Rasmusson *et al.*, 2009).

417

418 *Metabolic adjustments observed in TRX mutants under HL treatment*

419 Lower levels of trehalose were consistently found in both *trxo1* mutants after 2h of HL
420 treatment as compared to *Col-0* plants (Figure 6). Similarly, trehalose levels were lower
421 in *trxo1* mutants after salinity treatment as compared to *Col-0* plants (Sanchez-Guerrero
422 *et al.*, 2019). These concomitant results denotes a specific altered response of trehalose
423 metabolism in the *trxo1* mutants under stress. Trehalose has protection roles against

424 several abiotic stresses by stabilizing membranes, proteins and other cellular components
425 as well as protecting against ROS (Lunn *et al.*, 2014). Nevertheless, understanding on the
426 trehalose changes observed in the *trxo1* mutants deserve further investigation given that
427 some of the protective roles attributed to trehalose under stress are questioned while
428 others emerge on sugar signaling (Lunn *et al.*, 2014). On the other hand, higher label
429 redistribution to glycine and serine was also consistently observed in both *trxo1* mutants
430 under HL treatment (Figure 7). These results can be explained by a direct effect of TRX
431 on glycine decarboxylase (GDC) activity. Indeed, GDC has been reported as a putative
432 target of the TRX system (Balmer *et al.*, 2004) and its activity in isolated mitochondria
433 can be regulated by the glutaredoxin system (Hoffmann *et al.*, 2013). However, *in vivo*
434 evidence in plants is still missing. In cyanobacteria, the P-protein of the GDC has been
435 proposed to be redox regulated through the formation of a disulfide bond between Cys972
436 in the C terminus and Cys353 located in the active site (Hasse *et al.*, 2013). Here, glycine
437 and serine ¹³C labeling data suggest a reduced photorespiratory flux *in vivo* in the *trxo1*
438 mutants under HL conditions. Moreover, the lower O₂ inhibition of photosynthesis
439 observed in the mutants after 2h of HL treatment supports this view (Figure 5). The fact
440 that decreased photorespiration was not observed after 4h of HL treatment might indicate
441 the activation of other mechanisms for the dissipation of the reductants at longer time
442 exposition to the stress. Nevertheless, short-term transitions to high light have been
443 recognized as frequent physiological transitions in which mitochondrial metabolism is
444 able to rapidly respond to support photosynthetic activity (Finkemeier and
445 Schwarzländer, 2018). Moreover, a restriction in photorespiration in *AOX1a* mutants has
446 recently been reported by similar decreases in O₂ inhibition of photosynthesis as observed
447 in here and also coinciding with high glycine to serine ratios (Zhang *et al.*, 2017).
448 Therefore, our gas exchange data together with our data on metabolite levels and ¹³C
449 redistribution strongly suggest a restriction on photorespiration in the *trxo1* mutants at the
450 level of glycine decarboxylation.

451 As discussed above, the higher AOX *in vivo* activity observed in *trxo1* mutants is
452 probably due to an increased activity of the TCA cycle. Photorespiratory activity
453 increases mitochondrial NAD(P)H/NAD(P) ratios in illuminated leaves which can inhibit
454 the activities of TCA cycle NAD(P)H dehydrogenases (Igamberdiev and Gardeström,
455 2003), thus altering the operation of the TCA cycle (Sweetlove *et al.*, 2010). Because of
456 these redox links between mitochondrial dehydrogenases, it is unsurprising that the

457 observed alterations in the TCA cycle reactions in the TRX mutants could affect glycine
458 decarboxylation. In contrast to **ML** conditions, total respiration and AOX activity *in vivo*
459 were similar in *Col-0* and mutants plants after HL treatment, and all genotypes displayed
460 a full engagement of AOX (i.e. activity was essentially equivalent to capacity). The HL-
461 induction of the *in vivo* AOX activity can be impeded if AOX capacity is limiting; this
462 was observed as a species-specific feature (Florez-Sarasa *et al.*, 2016) and also in
463 Arabidopsis plants with *AOX1a* suppression (Florez-Sarasa *et al.*, 2011). In the last case,
464 plants were exhibiting a full AOX engagement already under **ML** (Florez-Sarasa *et al.*,
465 2011) as was the case for the *trxo1* mutants in present study. As a consequence of this
466 capacity limitation, the induction of the *in vivo* AOX activity was prevented in the *trxo1*
467 mutants and thus the reoxidation of the extra reductants produced by the TCA cycle under
468 HL. The extra reducing power produced by *trxo1* mutants under HL conditions could then
469 be the cause for the glycine decarboxylation restriction.

470

471 *Concluding remarks*

472 The *in vivo* AOX activity was higher in *trxo1* mutants under **moderate** light conditions,
473 as compared to *Col-0* plants, in spite of lacking *TRXo1* which has been proposed to redox
474 activate the AOX (Marti *et al.*, 2009; Yoshida *et al.*, 2013; Umekawa and Ito, 2018).
475 Moreover, we did not detect differences on the redox state of the AOX protein between
476 the mutant and *Col-0* plants. These observations together with the increased transcript
477 levels of other TRX-related systems in the *trxo1* mutants suggest that the AOX is
478 maintained in its reduced state by different mitochondrial redox systems rather than being
479 actively regulated. In addition, the combined analysis of *in vivo* fluxes of the electron
480 transport chain and the TCA cycle has allowed us to better understand the regulation of
481 mitochondrial metabolism from a broader and unprecedented perspective under different
482 light conditions. The *in vivo* AOX activity was increased in *trxo1* mutants thus yielding
483 a fully engaged AOX which was linked to an up-regulation of the TCA flux. Such a higher
484 and less efficient respiration **might be** the cause of the lower biomass accumulation in
485 *trxo1* mutants, **although more detailed analysis of the energy status at different subcellular**
486 **compartments (Krueger *et al.*, 2009; Gardeström and Igamberdiev, 2016) would be**
487 **required to unravel this issue.** After high light treatment, the *trxo1* mutants displayed a
488 restricted photorespiration at the level of glycine decarboxylation possibly by the lack of
489 AOX response and excess of reductant power in the mitochondrial matrix.

490

491 **MATERIAL AND METHODS**492 *Plant material and growth conditions*

493 The *Arabidopsis* (*Arabidopsis thaliana*) mutants *trxol-1* and *trxol-2* were isolated from
494 T-DNA insertion lines SALK_042792 and SALK_143294, respectively, obtained from
495 the Arabidopsis Biological Resource Centre. Genotyping of *trxol* mutants was performed
496 by PCR using gene- and T-DNA-specific primers as previously described (Daloso *et al.*,
497 2015; Ortiz-Espín *et al.*, 2017). Selected homozygous seeds from both mutant lines were
498 used for all the experiments. Mutant and wild type Columbia-0 (*Col-0*) plants were grown
499 in soil under short photoperiod (8h light/16h dark) and controlled temperature
500 (22°C/18°C), relative humidity (above 60%) and light intensity (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), **which**
501 **was considered the moderate light (ML) treatment unless otherwise noted**. Plants were
502 grown for 6 to 7 weeks under ML and then transferred to high light conditions (600 μmol
503 $\text{m}^{-2} \text{s}^{-1}$) for 2 and 4h, **which was considered the high light (HL) treatment unless otherwise**
504 **noted**.

505

506 *Gene expression analysis*

507 RNA was isolated from lyophilized leaves by using Maxwell® RSC Plant RNA Kit
508 (Promega) and automated system Maxwell® RSC Instrument (Promega) according to the
509 manufacturer's instructions, and was quantified using a NanoDrop 1000
510 spectrophotometer (Thermo Scientific, <http://www.nanodrop.com/>). Afterwards cDNA
511 synthesis was performed following the recommendations of the Transcriptor First Strand
512 cDNA Synthesis Kit (Roche). Relative mRNA abundance was evaluated by quantitative
513 PCR using LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480
514 real-time PCR system (Roche). Primers used and the related information are detailed in
515 the Supplemental Table S5. Two technical replicates of each biological replicate were
516 performed, and the mean values were used for further calculations. *Arabidopsis* UBC
517 (*At5g25760*) was used as a reference gene to correct for differences in the total amount
518 of transcripts and the $2^{-\Delta\Delta\text{Ct}}$ method (Livak and Schmittgen 2001) was used to calculate
519 the fold change of gene expression. Finally, data were normalized to the mean value of

520 *Col-0* plants in moderate light conditions (i.e. the level of all transcripts for *Col-0* at
521 moderate light was set to 1).

522

523 *Measurements of respiratory oxygen consumption and isotope fractionation*

524 Measurements of oxygen consumption and isotope fractionation during respiration were
525 performed as described by Florez-Sarasa *et al.* (2007) in order to determine the *in vivo*
526 activities of the COX and AOX pathways in Arabidopsis leaves. As for the end-point
527 fractionation values, the oxygen isotope fractionation of the AOX pathway was
528 determined after incubation with 10 mM KCN as described previously (Florez-Sarasa *et*
529 *al.*, 2007) and a mean value of 31.1‰ was used from all the measurements performed
530 both in the mutant and *Col-0* plants because no differences were observed between
531 genotypes. On the other hand, an end-point value of 20.9‰ corresponding to the oxygen
532 isotope fractionation of the COX pathway was taken from previous measurements in
533 Arabidopsis leaves (Florez-Sarasa *et al.*, 2007). Calculations of the electron partitioning
534 and the activities of the AOX and COX pathways were performed following Guy *et al.*
535 (1989). In addition, the AOX capacity was determined with a Clark-type oxygen electrode
536 as described previously (Florez-Sarasa *et al.*, 2009). Five replicates (each representing a
537 different plant) per line and light treatment were performed for both *in vivo* activities and
538 AOX capacity.

539

540 *Western Blot analysis*

541 For determining the total amount of AOX, 20 mg of frozen leaf powder per genotype and
542 light condition were used for protein extraction directly in 100 µl SDS sample buffer [2%
543 (w/v) SDS, 62.5mM Tris-HCl (pH 6.8), 10% (v/v) glycerol and 0.007% (w/v)
544 bromophenol blue] including 50 mM DTT and protease inhibitor (Roche). Samples were
545 incubated 30 min at 4°C to allow full reduction of the AOX protein and then boiled (95°C)
546 for 5 min. For the analysis of the AOX redox state, leaf membranes were isolated as
547 described in Noguchi *et al.* (2005) with the following modifications. Five leaves (approx.
548 500 mg) per genotype under moderate light condition were ground with a mortar and
549 pestle in 1 mL of grinding medium (0.45 M mannitol, 50 mM TES, 0.5% (w/v) BSA,
550 0.5% (w/v) PVP-40, 2 mM EGTA, 20 mM ascorbate) containing either 50 mM DTT (for

551 AOX reduction), 50 mM diamide (for AOX oxidation) or nothing else. After approx. 1
552 min of grinding, samples were centrifuged for 5 min at 1200 g and supernatant was
553 transferred into a new tube and centrifuged at 10000g for another 5 min. Subsequently,
554 supernatant was discarded and the pellet was resuspended in SDS sample buffer
555 containing DTT or diamide at the same concentrations as those used during the extraction.
556 Both, extracts for total amount and redox state determinations of the AOX protein were
557 frozen at -20°C.

558 Twenty microliters were loaded and separated on 12% and 10% SDS-PAGE gels for the
559 total amount and redox state samples, respectively. Proteins were transferred to
560 nitrocellulose membranes using wet Mini-PROTEAN system of Bio-Rad for the total
561 amount samples; PVDF membranes and PeqLab mini gel system was used for the redox
562 samples. The following primary antibodies and dilutions were used for detecting
563 mitochondrial proteins: monoclonal anti-Porin, voltage-dependent anion channel porin
564 (PM035, from Dr Tom Elthon, Lincoln, NE) at 1:5000 dilution; polyclonal anti-AOX,
565 alternative oxidase 1 and 2 (AS04054, Agrisera, Sweden) at 1:500 dilution (used for the
566 total AOX amount); monoclonal anti-AOX (AOA, from Dr Tom Elthon, Lincoln, NE) at
567 a dilution 1:10000. Secondary antibodies linked to horseradish peroxidase were used
568 (Sigma-Aldrich Co.). The signals were detected by chemiluminescence using Pierce™
569 ECL Western Blotting Substrate (ThermoFischer) and a Luminescent Image Analyzer
570 (G-Box-Chemi XT4, Syngene). The protein band quantifications were performed with
571 GeneTools analysis software from the Luminescent Image Analyzer (G-Box-Chemi XT4,
572 Syngene) according to manufacturer's instructions. The obtained band intensities for
573 AOX were corrected for their corresponding porin band intensities and then normalized
574 to the levels of the *Col-0* plants under moderate light. Four different immunoblot
575 experiments per protein were performed with similar results.

576

577 *Measurements of respiratory CO₂ evolution*

578 Ten leaf discs with 10 mm diameter were incubated in a 100 ml flask containing 5 mL of
579 10 mM MES (pH 6.5) under moderate light intensity at room temperature for 30 min prior
580 to the addition of [1-¹⁴C]glucose or [3:4-¹⁴C]glucose (6.2 MBq mmol⁻¹) to a final
581 concentration of 1.0 mM. Each flask was then sealed with Parafilm and shaken at 100
582 rpm. Evolved ¹⁴CO₂ was collected in 0.5 ml of 10% (w/v) KOH in a 1.5 ml

583 microcentrifuge tube suspended in the flask. The KOH solution was replaced every hour
584 for 4 h. Radioactivity evolved as CO₂ was determined as the radioactivity in the KOH
585 solution mixed with 4 mL of scintillation cocktail (Rotizint Eco Plus, ROTH) measured
586 by a liquid scintillation counter (LS6500, Beckman Coulter; Kühn et al., 2015). The yield
587 of ¹⁴CO₂ was calculated by expressing the cumulative radioactivity released (Kruger et
588 al., 2017).

589

590 *Metabolite profiling*

591 Metabolite extractions were performed as described previously (Lisec *et al.*, 2006) using
592 approximately 50 mg of frozen-powdered leaf tissue. Derivatization and gas
593 chromatography-time of flight-mass spectrometry (GC-TOF-MS) analyses were carried
594 out as described previously (Lisec *et al.*, 2006). Metabolites were identified manually by
595 TagFinder software (Luedemann *et al.*, 2008) using the reference library mass spectra
596 and retention indices housed in the Golm Metabolome Database ([http://gmd.mpimp-
597 golm.mpg.de](http://gmd.mpimp-golm.mpg.de); Kopka *et al.*, 2005). The parameters used for the peak annotation of the 36
598 metabolites can be found in Table S1 which follows the reporting recommendations of
599 Fernie et al. (2011). Data were normalized to the mean value of *Col-0* plants in moderate
600 light conditions (i.e. the value of all metabolites for *Col-0* at moderate light was set to 1).
601 Values presented are means ± SE of seven replicates which were taken from same rosettes
602 used for photosynthesis and respiration analyses.

603

604 *Measurements of the ¹³C isotope redistribution*

605 Leaves of similar size from the *Col-0* and mutant plants were fed via the petiole by placing
606 in a solution containing 10 mM MES-KOH (pH 6.5) and either 15 mM [U-¹³C] Glucose
607 or 15mM [U-¹³C] Malate (from Cambridge Isotope Laboratories) for 4 h. Leaves under
608 control and high light treatments were subjected to 100 and 400 μmoles m⁻² s⁻¹,
609 respectively, so that an approx. the light intensities applied during the petiole feeding
610 were lower than in the other experiments in order to avoid leaf wilting induced by the
611 high transpiration occurring during the high light treatment. Four fold-increase in light
612 intensity during the high light treatment was maintained as in the other experiments. At
613 0, 2 and 4h of incubation and light treatment, leaves were snap-frozen in liquid nitrogen.

614 Thereafter, approx. 30 mg of frozen-powdered leaf tissue was extracted and derivatized
615 as described above and analyzed by GC-TOF-MS in parallel with a mixture of authentic
616 standards of several metabolites at four different concentrations. The peak intensity
617 matrix containing all available mass isotopomers of characteristic mass fragments
618 generated by TagFinder was then processed using the CORRECTOR software tool
619 ([http://www.mpimp-golm.mpg.de/10871/Supplementary Materials](http://www.mpimp-golm.mpg.de/10871/Supplementary_Materials)) in order to correct
620 for the natural abundance of the isotope and to calculate the fractional enrichment (Huege
621 *et al.*, 2014). Absolute concentrations were determined for all the metabolites detected
622 and present in the authentic standards mixture. The total ^{13}C label redistributed in a
623 metabolite pool was calculated by multiplying the fractional enrichment with the absolute
624 concentration of the corresponding metabolite.

625

626 *Leaf gas exchange and chlorophyll fluorescence measurements*

627 Net CO_2 assimilation (A_N), stomatal conductance (g_s) and chlorophyll fluorescence were
628 measured simultaneously with an open infrared gas-exchange analyser system (Li- 6400;
629 Li-Cor Inc., Lincoln, NE, USA) equipped with a leaf chamber fluorometer (Li-6400-40,
630 Li-Cor Inc.). Fully expanded leaves were clamped and leaf chamber conditions were set
631 to: $400 \mu\text{mol CO}_2 \text{ mol}^{-1}$ air (C_a), temperature of 23°C and photosynthetically active
632 photon flux density (PPFD) of 150 and $600 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (provided by the light source of
633 the Li-6400 with 10% blue light) at moderate light and after high light treatment,
634 respectively. After approx. 3 min, gas-exchange and chlorophyll fluorescence
635 measurements were performed in the light. The actual quantum efficiency of the
636 photosystem II (PSII)-driven electron transport (Φ_{PSII}) and the electron transport rate
637 (ETR) were determined as previously described in Florez-Sarasa *et al.* (2011) except that
638 0.5 and 0.84 values were used for leaf absorptance and the partitioning of absorbed quanta
639 between photosystems I and II. Six replicates per genotype and light treatment were
640 performed in leaves of different plants.

641 In addition, A_N was measured at saturating light conditions (PPFD of $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$)
642 under photorespiratory (21% O_2) and non-photorespiratory (approx. 2% O_2) conditions
643 in all genotypes and treatments. The percentage of O_2 inhibition of A_N was calculated in
644 order to obtain an estimation of photorespiratory activity as previously described (Ku and

645 Edwards, 1977): $\%(A_{N\ 2\%O_2} - A_{N\ 21\%O_2})/ A_{N\ 2\%O_2}$. Four replicates per genotype and light
646 treatment were performed in leaves of different plants.

647

648 *Statistical analyses*

649 Student's t-tests were used for statistical analyses in all the figures and tables in order to
650 compare *trxol* mutants to the *Col-0* plants in each light treatment and also to compare
651 different high light conditions to the moderate light condition in each genotype.

652

For Peer Review

653 **FUNDING**

654 This work was supported by the Alexander von Humboldt Foundation and by the Spanish
655 Ministry of Economy and Competitiveness, through the “Severo Ochoa Programme for
656 Centres of Excellence in R&D” 2016–2019 (SEV - 2015 - 0533)” (I.F.-S.). N.F.D-S.
657 and Miquel R-C. were supported by Spanish Ministries MICINN (BFU2011-23294) and
658 MINECO (CTM2014-53902-C2-1-P). J.-P.R. was supported by the Centre National de la
659 Recherche Scientifique and the Agence Nationale de la Recherche ANR-Blanc Cynthiol
660 12-BSV6-0011.

661

662 **DISCLOSURES**

663 The authors declare no conflict of interest.

664

665 **ACKNOWLEDGEMENTS**

666 We would like to thank Danilo M. Daloso for his helpful discussions and comments on
667 the manuscript. Also thanks to Yunuen Avalos Padilla for helping with Western Blot
668 analyses. And finally we are also very grateful to Dr Biel Martorell at the Serveis
669 Científico-Técnicos of the Universitat de les Illes Balears for his help while running IRMS
670 experiments.

671

672

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925 **FIGURE LEGENDS**

926 **Figure 1.** *In vivo* mitochondrial electron transport chain activities in *trxo1* mutants. (A)
927 Total respiration (V_t), (B) COX pathway activity (v_{cyt}) and (C) AOX pathway activity
928 (v_{alt}) in leaves of *Col-0*, *trxo1-1* and *trxo1-2* (see Experimental Procedures) *Arabidopsis*
929 *thaliana* plants at moderate light (ML) and after 2 and 4 hours of high light (HL)
930 treatment. Values are means \pm SE of five replicates. Asterisks denote significant
931 differences ($P < 0.05$) to the ML conditions in each genotype and number symbols
932 indicate significant difference ($P < 0.05$) to the *Col-0* in each light condition.

933

934 **Figure 2.** AOX capacity and protein levels in *trxo1* mutants. (A) AOX capacity (V_{alt}) and
935 (B) protein levels in leaves of *Col-0*, *trxo1-1* and *trxo1-2* plants at moderate light (ML)
936 and after 2 and 4 hours of high light (HL) treatment. Values in A are means \pm SE of five
937 replicates. In B, one representative blot is presented and the relative values shown are the
938 mean of the four immunoblot experiments performed with similar results (Supplemental
939 Figure S2). The relative values indicate the intensities of the signals from AOX
940 normalized to those from porin, and then expressed as fold-changes relative to values of
941 *Col-0* under ML intensity.

942

943 **Figure 3.** AOX protein redox state in *trxo1* mutants. AOX protein immunodetection in
944 leaf membranes fractions from *Col-0*, *trxo1-1* and *trxo1-2* plants grown under ML
945 conditions (see Material and Methods) and treated with 50 mM DTT (for reduced-
946 monomeric form detection) or 50 mM diamide (for oxidized-dimeric form detection) or
947 in absence of both (-). Porin immunodetection in the same samples shows a similar
948 mitochondrial loading among genotypes in each (redox) treatment. The table below the
949 image shows the mean values ($n=2$) of the percentages on the oxidized and reduced forms
950 in the different genotypes after band quantification of the blot in the current figure and in
951 Supplemental Figure S2.

952

953 **Figure 4.** TCA cycle and other respiratory fluxes in *trxo1* mutants. Radioactivity in the
954 CO_2 evolved from illuminated leaf discs was measured after incubation with glucose
955 (Glc) ^{14}C -labeled at different positions. The $^{14}\text{CO}_2$ evolution from Glc labeled at position

956 1 (C1) or positions 3 and 4 (C3-4) corresponds to the metabolic flux through glycolysis
957 and TCA cycle, respectively. The $^{14}\text{CO}_2$ evolution was measured every hour after the
958 addition of the labeled Glc and summed up to calculate the total ^{14}C evolved. Each point
959 represent the means \pm SE of three replicates. Asterisks denote significant differences (P
960 < 0.05) to the *Col-0* plants in each time point.

961

962 **Figure 5.** Photosynthetic and photorespiratory parameters under high light stress
963 conditions. Net photosynthesis (A_N), photosynthetic electron transport rate (ETR),
964 stomatal conductance (g_s) and the percentage of O_2 inhibition of photosynthesis in leaves
965 in *Col-0*, *trxo1-1* and *trxo1-2* plants grown under ML and after 2 and 4h of high light
966 (HL) treatment. Details on the gas exchange and chlorophyll fluorescence measurements
967 can be found in the **Material and Methods** section. Values are means \pm SE of six (A_N , g_s
968 and ETR) or four (% O_2 inhibition) replicates and asterisks denote significant differences
969 ($P < 0.05$) to the *Col-0* plants in each light condition.

970

971 **Figure 6.** Metabolite profiling in *trxo1* mutants. Heat map showing the relative levels of
972 the GC-MS-analyzed metabolites in *Col-0*, *trxo1-1* and *trxo1-2* plants grown under ML
973 and after 2 and 4h of high light (HL) treatment. Metabolites were clustered per class into
974 amino acids, organic acids, sugars and sugar alcohols, and other metabolites. Relative
975 metabolite levels in leaves of *Col-0*, *trxo1-1* and *trxo1-2* plants under all light conditions
976 were normalized to the mean level of the *Col-0* plants under ML conditions and fold-
977 change values were log2 transformed (i.e. the level of all metabolites of *Col-0* plants
978 under ML is 0). In this heat map, red and blue colors represent log2 fold-increased and -
979 decreased metabolites, respectively. Values are means \pm SE of six replicates and asterisks
980 denote significant differences ($P < 0.05$) to the *Col-0* plants in each light condition. The
981 statistical differences between ML and HL treatments in each genotype are presented in
982 Supplemental Table S2.

983

984 **Figure 7.** Total ^{13}C label redistribution into primary metabolites in *trxo1* mutants. The
985 total ^{13}C label redistribution in selected leaf metabolites of *Col-0*, *trxo1-1* and *trxo1-2*
986 plants was determined after 2 and 4 hours of ^{13}C -labeling under (A) moderate light and

987 (B) high light conditions. Values are means \pm SE of six replicates and asterisks denote
988 significant differences ($P < 0.05$) to the *Col-0* plants in each time point and light condition.
989 Only metabolites showing significant differences to the *Col-0* in both mutant lines in each
990 light treatment, considering all time points and labeling substrates, are shown. Data
991 including total ^{13}C label redistribution in all metabolites is presented in Supplemental
992 Table S3 and Table S4.

993

994

For Peer Review

995 **SUPPORTING INFORMATION**

996 **Supplemental Table S1.** Parameters used for peak annotation in GC-MS analysis.

997

998 **Supplemental Table S2.** Relative metabolite levels in leaves of *Col-0*, *trxo1-1* and *trxo1-*
999 *2* plants under moderate light (ML) and after 2 and 4h of high light (HL) treatment. Data
1000 is presented as means \pm SE for six biological replicates normalized to the mean level of
1001 the *Col-0* plants under ML. Bold numbers denote significant differences ($P < 0.05$) to the
1002 ML conditions in each genotype and asterisks indicate significant difference ($P < 0.05$)
1003 to the *Col-0* in each light condition.

1004

1005 **Supplemental Table S3.** Total ^{13}C label redistribution in leaf metabolites of *Col-0*, *trxo1-*
1006 *1* and *trxo1-2* plants after 2 and 4 hours of ^{13}C -Glucose labeling under moderate light and
1007 high light conditions. Values are means \pm SE of six replicates, and those values bold and
1008 underlined denote significant differences ($P < 0.05$) to the *Col-0* plants in each time point
1009 and light condition.

1010

1011 **Supplemental Table S4.** Total ^{13}C label redistribution in leaf metabolites of *Col-0*, *trxo1-*
1012 *1* and *trxo1-2* plants after 2 and 4 hours of ^{13}C -Malate labeling under moderate light and
1013 high light conditions. Values are means \pm SE of six replicates, and those values bold and
1014 underlined denote significant differences ($P < 0.05$) to the *Col-0* plants in each time point
1015 and light condition.

1016

1017 **Supplemental Table S5.** Primers used in the qPCR analyses performed in this study.

1018

1019 **Supplemental Figure S1.** Gene expression analysis and biomass accumulation of *trxo1*
1020 mutants. (A) qPCR analysis of transcript levels from genes related to mitochondrial TRX
1021 system and *AOX1a* in leaves *trxo1-1* and *trxo1-2* plants under moderate light conditions
1022 (ML). Primers used and gene information can be found in Supplemental Table S5. Values
1023 are means \pm SE of 4 replicates and asterisks denote significant differences ($P < 0.05$) to

1024 the *Col-0* plants; n.d. (not detected). (B) Photograph representative of *Col-0*, *trxo1-1* and
1025 *trxo1-2* plants after growing for 6 weeks under ML conditions. (C) Rosette biomass
1026 accumulation of plants grown as in B. Values are means \pm SE of twelve replicates and
1027 asterisks denote significant differences ($P < 0.05$) to the *Col-0* plants.

1028

1029 **Supplemental Figure S2.** AOX protein amount and redox analysis. (A) AOX and porin
1030 protein levels in leaves of *Col-0*, *trxo1-1* and *trxo1-2* plants at moderate light (ML) and
1031 after 2 and 4 hours of high light (HL) treatment. Three blots of each AOX and porin
1032 proteins are shown, which were used for the AOX amount quantifications, together with
1033 the blot shown in Figure 2B, as described in Material and Methods section. (B) AOX
1034 protein redox state in *trxo1* mutants. AOX protein immunodetection in leaf membranes
1035 fractions from *Col-0*, *trxo1-1* and *trxo1-2* plants grown under ML conditions (see
1036 Experimental Procedures) and treated with 50 mM DTT (for reduced-monomeric form
1037 detection) or 50 mM diamide (for oxidized-dimeric form detection) or in absence of both
1038 (-).

1039

Figure 1

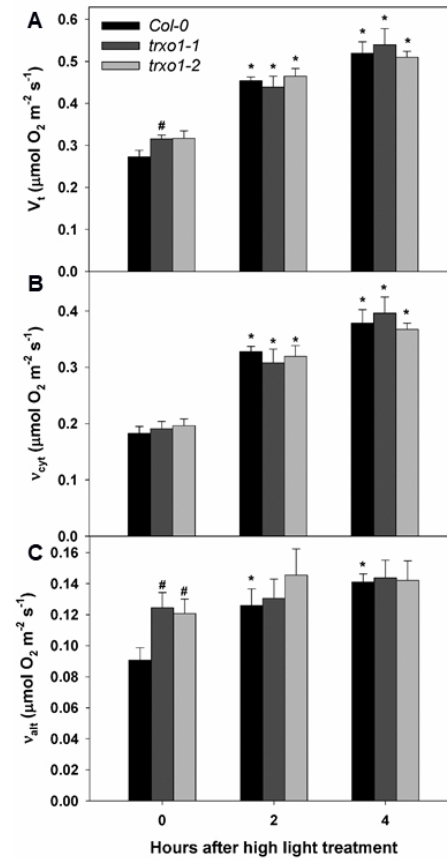


Figure 1. In vivo mitochondrial electron transport chain activities in *trxo1* mutants. (A) Total respiration (V_t), (B) COX pathway activity (V_{cyt}) and (C) AOX pathway activity (V_{alt}) in leaves of *Col-0*, *trxo1-1* and *trxo1-2* (see Experimental Procedures) *Arabidopsis thaliana* plants at growth light (GL) and after 2 and 4 hours of high light (HL) treatment. Values are means \pm SE of five replicates. Asterisks denote significant differences ($P < 0.05$) to the GL conditions in each genotype and number symbols indicate significant difference ($P < 0.05$) to the *Col-0* in each light condition.

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Figure 2

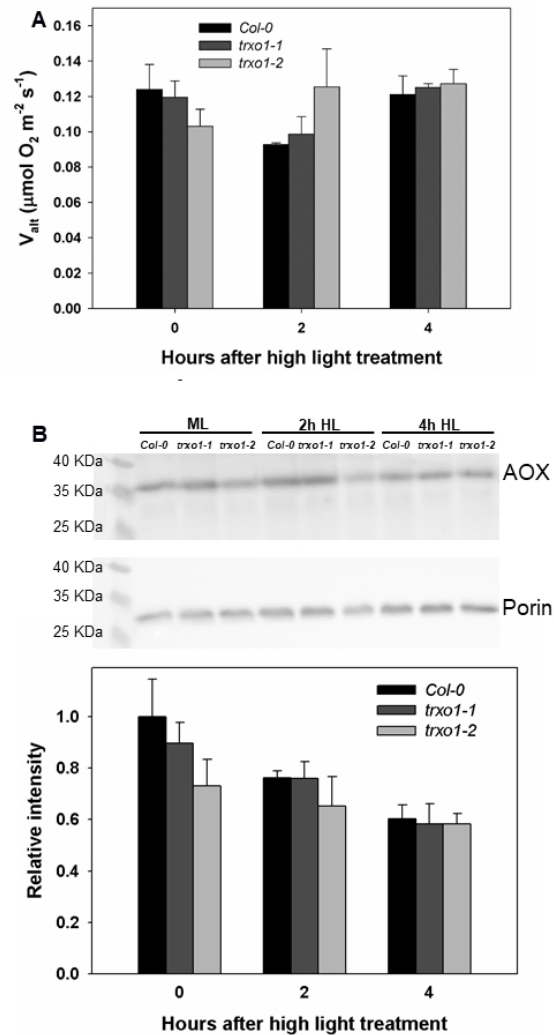


Figure 2. AOX capacity and protein levels in *trxo1* mutants. (A) AOX capacity (V_{ait}) and (B) protein levels in leaves of Col-0, *trxo1-1* and *trxo1-2* plants at moderate light (ML) and after 2 and 4 hours of high light (HL) treatment. Values in A are means \pm SE of five replicates. In B, one representative blot is presented and the relative values shown are the mean of the four immunoblot experiments performed with similar results (Supplemental Figure S2). The relative values indicate the intensities of the signals from AOX normalized to those from porin, and then expressed as fold-changes relative to values of Col-0 under ML intensity.

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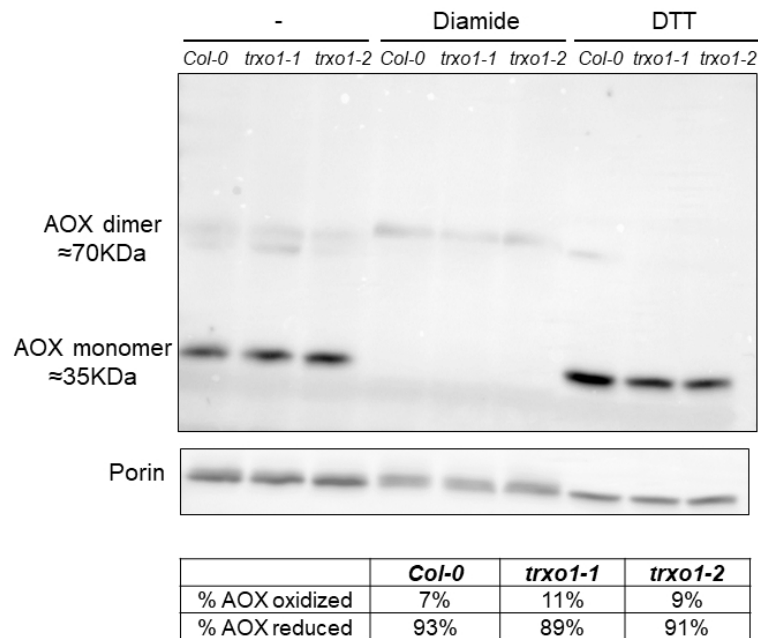
Figure 3

Figure 3. AOX protein redox state in *trxo1* mutants. AOX protein immunodetection in leaf membranes fractions from *Col-0*, *trxo1-1* and *trxo1-2* plants grown under ML conditions (see Material and Methods) and treated with 50 mM DTT (for reduced-monomeric form detection) or 50 mM diamide (for oxidized-dimeric form detection) or in absence of both (-). Porin immunodetection in the same samples shows a similar mitochondrial loading among genotypes in each (redox) treatment. The table below the image shows the mean values ($n=2$) of the percentages on the oxidized and reduced forms in the different genotypes after band quantification of the blot in the current figure and in Supplemental Figure S2.

190x275mm (96 x 96 DPI)

Figure 4

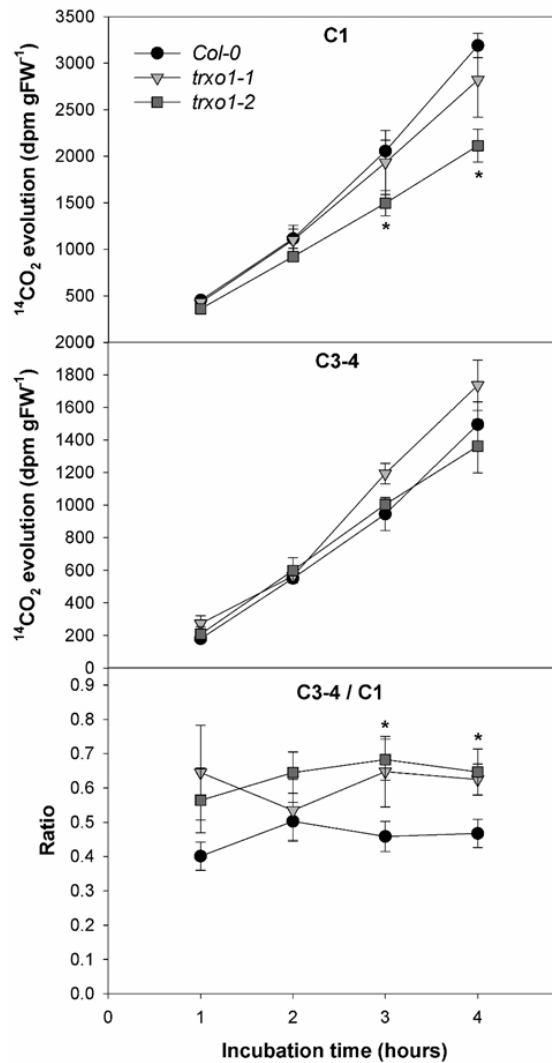


Figure 4. TCA cycle and other respiratory fluxes in *trxo1* mutants. Radioactivity in the CO_2 evolved from illuminated leaf discs was measured after incubation with glucose (Glc) ^{14}C -labeled at different positions. The $^{14}\text{CO}_2$ evolution from Glc labeled at position 1 (C1) or positions 3 and 4 (C3-4) corresponds to the metabolic flux through glycolysis and TCA cycle, respectively. The $^{14}\text{CO}_2$ evolution was measured every hour after the addition of the labeled Glc and summed up to calculate the total ^{14}C evolved. Each point represent the means \pm SE of three replicates. Asterisks denote significant differences ($P < 0.05$) to the Col-0 plants in each time point.

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Figure 5

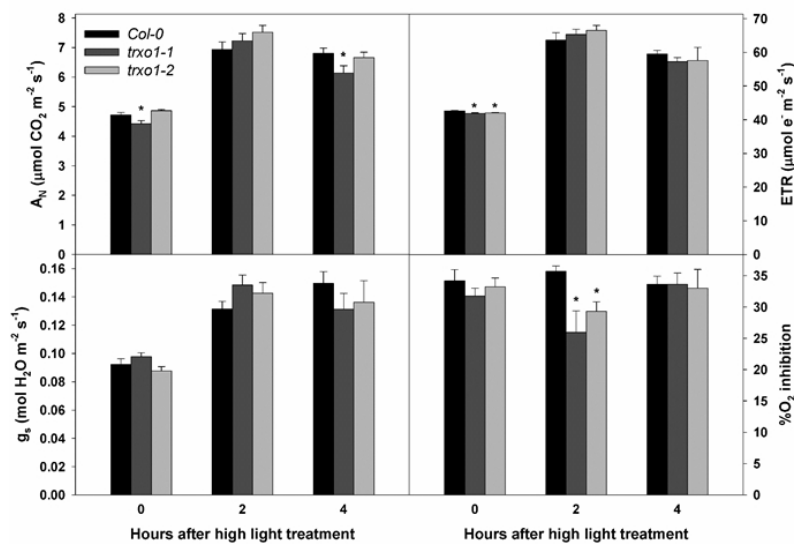


Figure 5. Photosynthetic and photorespiratory parameters under high light stress conditions. Net photosynthesis (AN), photosynthetic electron transport rate (ETR), stomatal conductance (gs) and the percentage of O₂ inhibition of photosynthesis in leaves in Col-0, *trx1-1* and *trx1-2* plants grown under GL and after 2 and 4h of high light (HL) treatment. Details on the gas exchange and chlorophyll fluorescence measurements can be found in the Experimental Procedures section. Values are means \pm SE of six (AN, gs and ETR) or four (% O₂ inhibition) replicates and asterisks denote significant differences ($P < 0.05$) to the Col-0 plants in each light condition.

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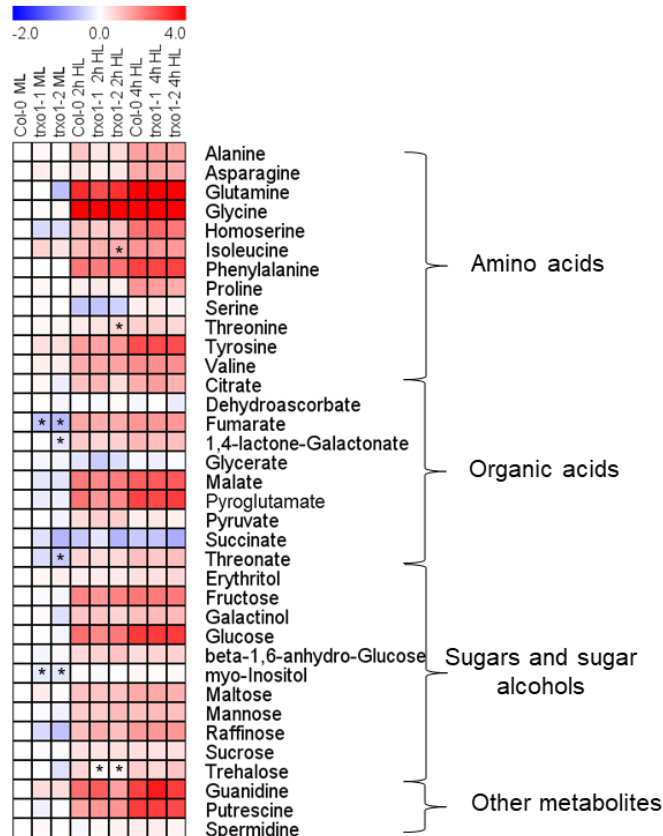
Figure 6

Figure 6. Metabolite profiling in trxo1 mutants. Heat map showing the relative levels of the GC-MS-analyzed metabolites in Col-0, trxo1-1 and trxo1-2 plants grown under ML and after 2 and 4h of high light (HL) treatment. Metabolites were clustered per class into amino acids, organic acids, sugars and sugar alcohols, and other metabolites. Relative metabolite levels in leaves of Col-0, trxo1-1 and trxo1-2 plants under all light conditions were normalized to the mean level of the Col-0 plants under ML conditions and fold-change values were log₂ transformed (i.e. the level of all metabolites of Col-0 plants under ML is 0). In this heat map, red and blue colors represent log₂ fold-increased and -decreased metabolites, respectively. Values are means \pm SE of six replicates and asterisks denote significant differences (P < 0.05) to the Col-0 plants in each light condition. The statistical differences between ML and HL treatments in each genotype are presented in Supplemental Table S2.

190x275mm (96 x 96 DPI)

Figure 7

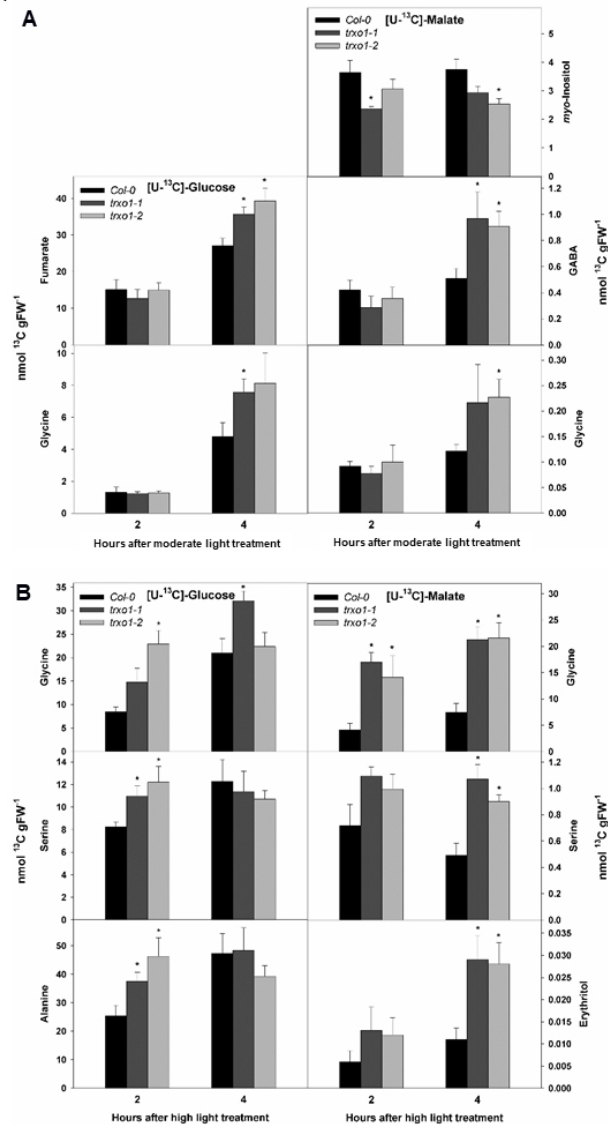


Figure 7. Total ^{13}C label redistribution into primary metabolites in *trxo1* mutants. The total ^{13}C label redistribution in selected leaf metabolites of Col-0, *trxo1-1* and *trxo1-2* plants was determined after 2 and 4 hours of ^{13}C -labeling under (A) moderate light and (B) high light conditions. Values are means \pm SE of six replicates and asterisks denote significant differences ($P < 0.05$) to the Col-0 plants in each time point and light condition. Only metabolites showing significant differences to the Col-0 in both mutant lines in each light treatment, considering all time points and labeling substrates, are shown. Data including total ^{13}C label redistribution in all metabolites is presented in Supplemental Table S3 and Table S4.

190x275mm (96 x 96 DPI)