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## **Adenylates regulate Arabidopsis plastidial thioredoxin activities through the binding of a CBS domain protein.**

Kevin Baudry, Félix Barbut, Séverine Domenichini, Damien Guillaumot, Mai Pham Thy, Hélène Vanacker, Wojciech Majeran, Anja Krieger-Liszkay, Emmanuelle Issakidis-Bourguet, Claire Lurin

### ► To cite this version:

Kevin Baudry, Félix Barbut, Séverine Domenichini, Damien Guillaumot, Mai Pham Thy, et al.. Adenylates regulate Arabidopsis plastidial thioredoxin activities through the binding of a CBS domain protein.. *Plant Physiology*, 2022, 189 (4), pp.2298-2314. 10.1093/plphys/kiac199 . hal-03866164

**HAL Id: hal-03866164**

**<https://hal.science/hal-03866164v1>**

Submitted on 22 Nov 2022

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1 **Short title:** CBSX2 regulates Thioredoxins m in plastids

2 **Title:**

3 **Adenylates regulate Arabidopsis plastidial thioredoxin activities through the binding of**  
4 **a CBS domain protein**

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21 **Author contributions:** C. L., E. I-B., W. M., H. V. and A. K-L. designed the research and  
22 supervised the experiments; F. B., S. D., D. G. and M. P. T. performed experiments;. K. B.,  
23 A. K-L. and E. I-B performed experiments and analyzed the data; K. B., C. L., A. K-L. and E.  
24 I-B. wrote the manuscript.

25 **Fundings:** K.B. research was supported by a French Ph. D. fellowship from “Ministère de la  
26 Recherche et de l’Enseignement Supérieur”. D.G. research was supported by a post-doctoral  
27 fellowship from a French State grant (Saclay Plant Sciences, reference n° ANR-17-EUR-  
28 0007, EUR SPS-GSR) managed by the French National Research Agency under an  
29 Investments for the Future program (reference n° ANR-11-IDEX-0003-02). The IPS2 and the  
30 I2BC benefit from the support of the Labex Saclay Plant Sciences-SPS (ANR-17-EUR-0007).  
31 This work was supported by the French Infrastructure for Integrated Structural Biology  
32 (FRISBI; grant number ANR-10-INSB-05).

33 **One-sentence summary:** A cystathionine- $\beta$ -synthase protein inhibits thioredoxins m  
34 activities towards NADP-malate dehydrogenase and 2-Cys peroxiredoxin in an adenylate-  
35 dependent manner.  
36 .

37 **ABSTRACT**

38 Cystathionine- $\beta$ -synthase (CBS) domains are found in proteins of all living organisms and  
39 were proposed to play a role as energy sensors regulating protein activities through their  
40 adenosyl ligand binding capacity. In plants, members of the CBSX protein family carry a  
41 stand-alone pair of CBS domains. In Arabidopsis, CBSX1 and CBSX2 are targeted to plastids  
42 where they were proposed to regulate thioredoxins (TRX). TRX are ubiquitous cysteine thiol  
43 oxido-reductases involved in the redox-based regulation of numerous enzymatic activities as  
44 well as in the regeneration of thiol-dependent peroxidases. In Arabidopsis, 10 TRX isoforms  
45 were identified in plastids and divided in five sub-types.

46 Here, we show that CBSX2 specifically inhibits the activities of m-type TRXs towards two  
47 chloroplast TRX-related targets. By testing activation of NADP-malate dehydrogenase and  
48 reduction of 2-Cys peroxiredoxin, we found that TRX m1/2 inhibition by CBSX2 was  
49 alleviated in presence of AMP or ATP.

50 We also evidenced, by pull-down assays, a direct interaction of CBSX2 with reduced TRX  
51 m1 and m2 that was abolished in presence of adenosyl ligands. In addition, we report that, in  
52 comparison to wild-type plants, the Arabidopsis T-DNA double mutant *cbx1 cbx2* exhibits  
53 growth and chlorophyll accumulation defects in cold conditions, suggesting a function of  
54 plastidial CBSX proteins in plant stress adaptation. Altogether, our results show an energy  
55 sensing regulation of plastid TRX m activities by CBSX, possibly allowing a feedback  
56 regulation of ATP homeostasis via activation of cyclic electron flow in the chloroplast, to  
57 maintain a high energy level for an optimal growth.

## 58 INTRODUCTION

59 Plants depend on light for the generation of energy and reducing power in the forms of ATP  
60 and NADPH produced in the chloroplasts and consumed in assimilatory processes.

61 The major role played by chloroplasts as the source and target of reduction–oxidation (redox)  
62 regulation is now well established in plant cells (Baier and Dietz, 2005). Thioredoxins (TRX)  
63 are key actors of the underlying complex redox regulatory network. TRX are small (~12kDa)  
64 ubiquitous redox proteins found in all cellular compartments. Among the 20 isoforms encoded  
65 by the Arabidopsis (*Arabidopsis thaliana*) genome, 10 are targeted to plastids and were sub-  
66 divided in five groups (4 TRX m, 2 TRX f, 2 TRX y, 1 TRX x and 1 TRX z). In plastids,  
67 TRX have numerous targets involved in various metabolic and stress response functions  
68 (Gelhaye et al., 2005; Lemaire et al., 2007; Geigenberger et al., 2017). TRX can act either as  
69 regulators of the activity of their targets by post-translational modification or as reducing  
70 substrate for their target. TRX can activate their targets through direct disulfide bridge  
71 reduction, as for example, plastidial NADPH-dependent malate dehydrogenase (NADP-  
72 MDH). They can also provide reducing power to their targets as in case of the 2-cysteine  
73 peroxiredoxin (2-Cys PRX) (Collin et al., 2003; Collin et al., 2004).

74 While the number of functions attributed to TRX in plastids is very large (Montrichard et al.,  
75 2009), knowledge about their regulation is limited. In plastids, TRXs redox state mainly  
76 depends on the balance between the “electron pressure” light-fueled by the photosynthetic  
77 electron transport chain and the “oxidative pressure” of H<sub>2</sub>O<sub>2</sub> (Vaseghi et al., 2018; Yoshida  
78 and Hisabori, 2018). Data about additional factors directly regulating TRX redox state are  
79 very scarce. For instance, TRX f was found glutathionylated and consequently losing its  
80 capability to regulate its targets (Michelet et al., 2005). Yoo *et al.* (2011) suggested that the  
81 CBSX proteins are ubiquitous regulators of TRXs in plastids to control H<sub>2</sub>O<sub>2</sub> levels and  
82 regulate lignin polymerization in the anther endothecium.

83 Cystathionine-β-synthase (CBS) domain (Pfam: PF00571) was identified in proteins from  
84 bacteria, yeasts, animals and plants (Ignoul and Eggermont, 2005; Kushwaha et al., 2009;  
85 Crozet et al., 2014; Shahbaaz et al., 2015). This small domain of approximately 60 amino  
86 acids forms an antiparallel β-sheet flanked by helices on one side. It is generally found as a  
87 tandem repeat forming a CBS pair or the so-called Bateman domain (Bateman, 1997) that  
88 defines the CBS-Domain-Containing-Protein (CDCP) family (Ignoul and Eggermont, 2005).  
89 Homodimers of CBS pairs are generally involved in the binding of regulatory adenosyl  
90 ligands such as AMP, ATP or S-adenosylmethionine (Kemp, 2004; Ereño-Orbea et al., 2013).

91 Consequently, CBS domains were proposed to function as sensors of cell energy status (Scott  
92 et al., 2004; Kushwaha et al., 2009). Associated to several different functional domains, in a  
93 wide range of functionally different CDCP proteins, CBS domains act generally as regulatory  
94 domains of protein activity through adenosyl ligand binding (Hardie and Hawley, 2001; Scott  
95 et al., 2004; Ignoul and Eggermont, 2005; Buey et al., 2017). For examples, the highly  
96 conserved eukaryotic energy sensor AMPK/SNF1/SnRK1 is regulated by its  $\gamma$ -subunit  
97 composed of four CBS domains in tandem (Polge and Thomas, 2007; Ramon et al., 2013;  
98 Crozet et al., 2014). In mammals, AMP/ADP binding to the CBS domains of the AMP-  
99 activated protein kinase (AMPK) induces a conformational change that stabilizes the  
100 phosphorylated activation loop of the protein by upstream kinases (Hardie and Hawley, 2001;  
101 Ignoul and Eggermont, 2005; Xiao et al., 2011).

102 The Arabidopsis genome encodes 34 CDCPs each containing one or two pairs of CBS  
103 domains that are generally closely associated with other functional protein domain(s). In these  
104 proteins, CBS domains are expected to regulate the molecular function of other adjacent  
105 domains. In contrast, the members of the CBSX subfamily of CDCP proteins carry a stand-  
106 alone pair of CBS domains and are thus supposed to perform their regulatory function through  
107 protein interactions (Kushwaha et al., 2009). In Arabidopsis, the six members of the CBSX  
108 subfamily were proposed to be localized in several cellular compartments. Indeed, whereas  
109 CBSX1 and CBSX2 were shown to be plastid targeted (Yoo et al., 2011; Jung et al., 2013),  
110 CBSX3 was recently shown to be localized in mitochondria (Shin et al., 2020), CBSX4 is  
111 predicted to be cytosolic and CBSX5 and CBSX6 are expected to be located in the  
112 endoplasmic reticulum (Ok et al., 2012).

113 The two Arabidopsis plastidial CBSX, CBSX1 (AT4G36910) and CBSX2 (AT4G34120),  
114 share 91% similarity and 74% identity in their amino acid sequences. Crystal structures of  
115 Arabidopsis CBSX1 and CBSX2 were solved and showed that these two proteins form anti-  
116 parallel (*head-to-tail*) homodimers exhibiting similar quaternary structures (Jeong et al.,  
117 2013a; Jeong et al., 2013b).

118 To characterize CBSX1 and CBSX2 functions, Yoo *et al.* (2011) identified candidate protein  
119 interactors using yeast 2-hybrid. Among them, were several chloroplast thioredoxins (TRX)  
120 for which interactions were confirmed by *in vitro* pull-down assays and *in planta* bimolecular  
121 fluorescence complementation (BiFC) experiments (Yoo et al., 2011; Jung et al., 2013). Both  
122 CBSX1 and CBSX2 were shown to increase the activity of TRX m2, TRX f1, TRX x and  
123 TRX y1 in a standard TRX activity assay based on bovine insulin reduction. These activities  
124 were enhanced in presence of AMP (Yoo et al., 2011). Similarly, it was shown that CBSX3

125 enhances the *in vitro* activity of the mitochondrial TRX o1 and TRX o2 (Yoo et al., 2011;  
126 Shin et al., 2020). Physiological functions of plastidial CBSX proteins were investigated in  
127 transformed Arabidopsis over-expressing *CBSX1* and *CBSX2*. These plants exhibited a sterile  
128 phenotype related to anther indehiscence. A defect in endotecium secondary cell wall  
129 thickening during anther development was observed in over-expressors and linked to  
130 insufficient reactive oxygen species (ROS) accumulation (Yoo et al., 2011; Jung et al., 2013).  
131 *CBSX3* knockdown lines surprisingly presented a similar sterile phenotype related to an  
132 anther indehiscence defect (Yoo et al., 2011; Jung et al., 2013; Shin et al., 2020). Plant  
133 sterility of CBSX1 and CBSX2 overexpressors, on one hand, and CBSX3 knockdown, on the  
134 other hand, is supposedly linked to improper thioredoxin activities in plastids or in  
135 mitochondria (Yoo et al., 2011; Jung et al., 2013; Shin et al., 2020).  
136 Despite the previous in depth molecular characterization of the plastidial CBSX structures and  
137 the demonstration of their interaction with TRX, the functional and physiological relevance of  
138 CBSX regulatory role on TRX activities remains questioned. In this study, by evaluating  
139 plastid CBSX regulation of TRX-dependent activities *in vitro* using two physiologically-  
140 relevant targets of plastidial TRX, we show that CBSX2 specifically inhibits TRX m activities  
141 and that this effect is reversed by ATP and, to a lesser extent, by AMP. Adenylates impairing  
142 direct CBSX-TRX interaction, we propose a model in which CBSX2 specifically inhibits  
143 reduced TRX m to maintain chloroplast energy status (ATP) for optimal plant growth during  
144 acclimation to low temperature.

145

## 146 RESULTS

### 147 Phylogenetic Analysis of AtCBSX

148 To investigate relationships between the 6 Arabidopsis CBSX proteins, we constructed a  
149 maximum likelihood phylogenetic tree from their full-length amino acid sequences. Our  
150 analysis suggests that CBSX proteins that are localized in the same subcellular compartment  
151 are closer in the phylogenetic tree than CBSX targeted to different locations (Figure S1). In  
152 particular, the two closest Arabidopsis CBSX proteins, CBSX1 and CBSX2, share 74% of  
153 amino acid sequence identity (similarity 91%). They were both shown to be targeted to the  
154 chloroplasts (Yoo et al., 2011; Jung et al., 2013). The closest protein of the plastidial CBSX  
155 group, CBSX3, shares 26% of amino acid sequence identity with CBSX2 (similarity 44%)  
156 and is localized in mitochondria. CBSX4, (also known as AtPV42b) is a close homolog of  
157 AtPV42a, a  $\gamma$ -type subunit of SnRK1 containing two CBS pairs (Fang et al., 2011).  
158 Accordingly, it appears to be a non canonical CBSX standing apart in our phylogenetic tree.  
159 This tree focused on the Arabidopsis CBSX subfamily is consistent with the wide  
160 phylogenetic analysis made on the 34 CDCPs of *A. thaliana* and the 59 CDCPs of *Oryza*  
161 *sativa* (Kushwaha et al., 2009).

### 162 CBSX1 and CBSX2 are able to form homomers and heteromers in plastids

163 We first confirmed the subcellular location of the chloroplast and mitochondrial CBSX  
164 isoforms. Transiently transformed *Nicotiana benthamiana* leaves with CBSX-RFP fusions  
165 were observed by confocal microscopy. Consistently with previous studies and predictions  
166 (Yoo et al., 2011; Jung et al., 2013), fluorescence signals of full-length CBSX1-RFP and  
167 CBSX2-RFP co-localized with the chlorophyll fluorescence of chloroplasts (Figure S2). In  
168 contrast, the CBSX3-RFP fluorescence signal was observed as spots outside the chloroplasts  
169 consistently with the recent finding from Shin *et al.* (2020) showing a mitochondrial  
170 localization of CBSX3 (Figure S2).

171 Crystal structures of CBSX1 and CBSX2 previously showed that these two proteins form  
172 homodimers with highly similar structures. The high similarity of their amino-acid sequences  
173 suggests that they may also form heterodimers. We investigated this possibility in a yeast 2-  
174 hybrid (Y2H) assay. CBSX1, CBSX2 and CBSX3 lacking their predicted organelle target  
175 peptides were fused to Gal4 activation and DNA binding domains and expressed in relevant  
176 Y2H strains. In a mating-based Y2H matricial assay, we showed that CBSX1 and CBSX2 can  
177 form homomers as well as heteromers (Figure 1). In contrast, we did not identify any  
178 interaction involving CBSX3, neither homomeric nor heteromeric.



179 To confirm these interactions in plant cells, we performed bimolecular fluorescence  
180 complementation (BiFC) analysis. *Nicotiana benthamiana* leaves were transiently co-  
181 transformed with full-length CBSX1 and CBSX2 fused at their C-termini to either the N-ter  
182 moiety (nYFP) or the C-ter moiety (cYFP) of the yellow fluorescent protein. To enhance YFP  
183 signals, plants were also co-transformed with HC-Pro, an inhibitor of gene silencing (Stephan  
184 et al., 2011). Four days after transformation, specific chloroplastic YFP signals were observed  
185 in both homomeric combinations (Figure 2). To test heteromerisation of CBSX1 and CBSX2,  
186 two YFP combinations were tested. YFP signals were only observed when CBSX2-nYFP and  
187 CBSX1-cYFP fusion proteins were co-expressed. The reciprocal combination did not give  
188 any signal. Consistently with CBSX localization, signals were observed in plastids. For each  
189 interaction signal, a spectral analysis of at least two spots was performed to confirm the YFP  
190 fluorescence signature.

191 Altogether our results confirm that both CBSX1 and CBSX2 are targeted to plastid where  
192 they form homomers as well as heteromers.

### 193 **CBSX2 inhibits the ability of TRX m to activate NADP-MDH and AMP modulates this** 194 **effect**

195 CBSX1 and CBSX2 proteins were previously found to activate TRX-dependent insulin  
196 reduction (Yoo et al., 2011). Here, we investigated the ability of CBSX proteins to modulate  
197 physiologically-relevant plastid TRX-dependent enzyme activities. To this aim, we produced  
198 and purified recombinant his-tagged CBSX1 and CBSX2 in a bacterial expression system.  
199 Untagged TRXs and TRX-regulated NADP-MDH were also obtained as previously described  
200 (Collin et al., 2003). All proteins were produced without their target peptide. NADP-MDH  
201 was previously shown to be efficiently activated *in vitro* by the members of the TRX m and  
202 TRX f subfamilies (Collin et al., 2003) and its regulation by TRX f1, TRX m1 and TRX m2  
203 was validated *in planta* (Thormählen et al., 2015; Thormählen et al., 2017). The plastid TRX-  
204 regulated MDH uses NADPH as a cofactor to reduce oxaloacetate (OAA) into malate. Its  
205 activation is strictly TRX dependent and requires the reduction of two constitutive regulatory  
206 disulfide bonds, and a third transient disulfide (Issakidis et al., 1994; Ruelland et al., 1997).  
207 Here, we tested the ability of five TRX isoforms (TRX m1, TRX m2, TRX m4, TRX f1 and  
208 TRX f2) to activate MDH in presence or absence of CBSX. We incubated MDH with DTE-  
209 reduced TRX, with or without addition of CBSX for 20 min prior to measuring MDH activity.  
210 We checked that activation kinetics were linear and did not reach a plateau within 20 min of  
211 incubation (Figure S3). We defined the MDH activation rate as the value of the initial activity

212 slope calculated after activation treatment. Our results show that CBSX1 does not have any  
213 significant effect on the capacity of the five TRXs tested to activate NADP-MDH (Figure 3A-  
214 E). In contrast, CBSX2 inhibits the enzyme activation by TRX m1 or TRX m2 or TRX m4  
215 (Figure 3A-C), but not by TRX f1 or TRX f2 (Figure 3D,E). In presence of CBSX2 with TRX  
216 m1, TRX m2 or TRX m4, NADP-MDH activation was reduced to 30-60% of the maximum  
217 activation reached in absence of CBSX2 (Figure 3A-C). Because CBS domains were shown  
218 to bind several adenosyl ligands (Ignoul and Eggermont, 2005) and, specifically, CBSX2  
219 structure was shown to be strongly modified upon AMP binding (Jeong et al., 2013a), we  
220 tested the effect of this adenylate on the TRX limitation exerted by CBSX2. While TRX-  
221 dependent MDH activation was not significantly affected by AMP (Figures 3 and S4), we  
222 found that AMP limited the inhibitory effect of CBSX2 on TRX m (Figure 3A-C). Indeed,  
223 TRX m-dependent MDH activation could reach 65-80% of its maximum in presence of  
224 CBSX2 and AMP at a concentration of 1 mM, a concentration commonly used  
225 experimentally to test regulation of CDCPs by adenylate ligands (Yoo et al., 2011; Jung et al.,  
226 2013; Buey et al., 2017).

#### 227 **CBSX2 directly regulates TRX activities**

228 Our results showed that CBSX2 selectively inhibits TRXs m but not TRXs f in their  
229 capacities to activate NADP-MDH. They strongly suggested an inhibitory action of CBSX2  
230 on TRX instead of a direct inhibition of MDH activity. To confirm these results, we took  
231 advantage of a mutant form of NADP-MDH, the DMC (Double Mutation C-terminus)-MDH,  
232 in which the two cysteines that form the C-terminal regulatory disulfide bond are substituted  
233 by serines. The DMC-MDH has a basal enzyme activity without any activating treatment, but  
234 reduced TRX is required to reach full activity (Issakidis et al., 1994). Without treatment, or in  
235 presence of DTE alone, DMC-MDH exhibits *ca.* 25% of the activity reached after a 20 min  
236 activation treatment in presence of TRX m1 (Figure 4). When DMC-MDH was incubated  
237 with CBSX2 and DTE, its activity remained unchanged (Figure 4) confirming that CBSX2  
238 does not directly inhibit DMC-MDH activity. We also checked that AMP had no direct effect  
239 on DMC-MDH activity (Figure S5). By contrast, DMC-MDH activation by TRX m1 or TRX  
240 m2, like for the wild-type enzyme, was strongly impaired in presence of CBSX2, and AMP  
241 limited this effect (Figure 4). Taken together our results strongly support a specific role  
242 played by CBSX2 as a negative regulator of TRXs m, with AMP acting as a modulator.

### 243 **Adenosyl nucleotides differentially modulate TRX m inhibition by CBSX2**

244 Noticeably, we found that AMP alleviates the CBSX2 effect on TRXs m only partially in our  
245 experimental conditions. The AMP concentration (1 mM) commonly used in this kind of  
246 experiments corresponds to a concentration by far higher than reported AMP concentrations  
247 in the chloroplast that do not exceed 1  $\mu$ M (Stitt et al., 1982). Thus, we also tested AMP at  
248 lower concentrations, as well as other adenylate nucleotides, *e.g.* ADP and ATP prevailing in  
249 the chloroplast (Figure 5). At 1 mM, we found that all adenylates could suppress the  
250 inhibitory effect of CBSX2 (Figure 5), while they had no significant effect on TRX m1-  
251 dependent activation of NADP-MDH (without CBSX) (Figure S4). At 1 mM, a physiological  
252 concentration for ATP in the chloroplast, ATP could totally inhibit the effect of CBSX2  
253 (Figure S6). We also tested MgATP, since *in vivo* 95-99 % of ATP is predicted to be  
254 complexed with Mg<sup>2+</sup>, and we also found its strong effect on TRX m. Interestingly, by testing  
255 lower concentrations of adenylate nucleotides, we could evidence that they have differential  
256 effects on CBSX2 (Figure 5). Indeed, at 10  $\mu$ M, ATP, but neither AMP nor ADP, had an  
257 effect, and at 50  $\mu$ M, AMP and ATP, but not ADP, restricted CBSX2 inhibition. At 1 mM,  
258 ATP was significantly more efficient than ADP to limit CBSX2 inhibition. Thus, our data  
259 suggest that AMP may not exert any effect on CBSX2 at physiologically relevant  
260 concentrations. They also suggested that ADP may be much less efficient than ATP in  
261 limiting CBSX2 effect on TRX, estimated concentrations of ADP and ATP in the chloroplast  
262 ranging from 0.5 to 0.8 mM and 0.8 to 1.2 mM, respectively (Stitt et al., 1982; Voon et al.,  
263 2018). We also tested the effect of ATP together with ADP on the time course of NADP-  
264 MDH activation in presence of CBSX2, at adenylate concentrations and ratios corresponding  
265 to light and dark conditions prevailing in the chloroplast (Usuda, 1988) (Figure S7). Again,  
266 CBSX2 effect was attenuated in presence of adenylates and, clearly, the effect was more  
267 pronounced in the light mimicking condition compared to the dark mimicking condition.

### 268 **CBSX2 inhibits TRX m-dependent reductive regeneration of 2-Cys PRX**

269 TRXs can regulate the activity of their targets (like NADP-MDH) and they can also provide  
270 electrons to thiol-dependent peroxidases named peroxiredoxins (PRX). It was previously  
271 shown that plastidial TRXs can reduce 2-Cys PRX *in vitro* with various efficiencies (Collin et  
272 al., 2003; Collin et al., 2004; Bohrer et al., 2012). To extend CBSX function towards TRXs ,  
273 we also tested other TRX isoforms that do not regulate NADP-MDH, *e.g.* TRX y1 and TRX  
274 x, for their capacity to serve as reducing substrate for 2-Cys PRX (obtained as described in  
275 Collin et al., 2003) in presence of CBSX. We found that PRX activities measured without

276 TRX (DTT alone) in absence and in presence of CBSX were very similar, showing that  
277 neither CBSX1 nor CBSX2 has any direct effect on 2-Cys PRX reduction of tBOOH (an  
278 alkyl-hydroperoxide) (Figure 6A). When combining PRX, TRX and CBSX proteins, we  
279 found that the addition of CBSX1 had no effect on the peroxidase activity, and that TRX f1,  
280 TRX y1 or TRX x were not affected by CBSX2 (Figure 6B-D). Similarly to its effect when  
281 testing NADP-MDH activation, CBSX2 inhibited TRX m1-dependent reduction of the 2-Cys  
282 PRX (Figure 6E). We also tested NTRC, a physiological substrate for this target, and found  
283 that it was not significantly affected by CBSX2 (Figure 6F). As for NADP-MDH activation,  
284 the addition of AMP restricted the inhibitory effect of CBSX2 on TRX m1 (Figure 7). These  
285 results show that CBSX2 is able to specifically inhibit TRX m in serving as reducing  
286 substrate for 2-Cys PRX and that, like in NADP-MDH activation, an adenylate nucleotide can  
287 prevent this effect.

#### 288 **Direct interaction between CBSX2 and TRX m1 proteins is modulated by adenosyl** 289 **ligands**

290 It was previously reported that CBSXs directly interact with TRXs (Yoo et al., 2011; Jung et  
291 al., 2013; Shin et al., 2020). To validate these interactions and further investigate the effect of  
292 adenosyl ligands, we performed pull-down assays with 6His-CBSXs and TRXm1 in presence  
293 or absence of AMP or ATP. After incubation of TRX m1 and CBSX2 in reducing conditions  
294 (presence of DTE), proteins were mixed with nickel affinity resin. After washings, they co-  
295 eluted from the resin (Figure 8A). In contrast, interaction between CBSX2 and TRX m1 was  
296 not observed when the incubation was performed in absence of a chemical reductant. Notably,  
297 interaction was also abolished in presence of 1 mM AMP or ATP (Figure 8A,B). Similar  
298 experiments were performed using 6His-CBSX1 and TRX m1, and no interaction was  
299 detected (Figure 8B). Taken together these results show that CBSX2, but not CBSX1, is able  
300 to interact with reduced TRX m1 in absence of ATP or AMP. These observations are  
301 consistent with the specific inhibitory effect of CBSX2 on TRX m1 activities (NADP-MDH  
302 activation and 2-Cys PRX reduction) and suggest that adenylyl ligands can modulate TRX m  
303 inhibition by CBSX2 by preventing CBSX-TRX direct interaction. Notably, the absence of  
304 effect of CBSX1 on TRXs could be linked to the lack of interaction between these two  
305 proteins.

306 **The double mutant *cbx1 cbx2* is smaller and exhibits chlorophyll accumulation defects**  
307 **at low temperature**

308 To investigate the role of CBSX in connection with TRX related functions *in planta*, we  
309 characterized *cbx1* and *cbx2* Arabidopsis T-DNA insertion mutants. These mutants were  
310 previously described in Yoo *et al.* (2011) and Jung *et al.* (2013), respectively, and did not  
311 show any macroscopic phenotype at the rosette stage. Because we suspected functional  
312 redundancy between the two CBSX plastid isoforms and found that they can form heteromers  
313 *in vivo* (Figure 2), we crossed the corresponding single mutants to obtain the *cbx1 cbx2*  
314 double mutant. In standard long day growth conditions, single and double mutants showed no  
315 obvious phenotypes compared to wild-type (WT) plants (Figure 9A), as previously reported  
316 under short day or continuous light (Yoo *et al.* 2011; Jung *et al.* 2013; Murai *et al.* 2021).  
317 Because *CBSX1* and *CBSX2* genes were reported to exhibit an increased level of expression at  
318 low temperature (ePlant database; (Waese *et al.*, 2017)), the double mutant was germinated  
319 and grown at 12°C. In this condition, the *cbx1 cbx2* double mutant rosettes were slightly  
320 smaller and pale green in comparison with the WT (Col-0 ecotype) or single mutant plants  
321 (Figure 9B). These differences were confirmed by comparing rosette fresh-weights and  
322 chlorophyll contents of the double mutant, which were significantly decreased compared to  
323 single mutants and WT plants (Figure 9C,D). These results suggest that both CBSX1 and  
324 CBSX2 are required for normal growth and chloroplast functions at low temperature.

325 **The double mutant *cbx1 cbx2* shows altered P700 oxidation and reduction kinetics**

326 Since TRXs were proposed as specific regulators of the photosynthetic electron transport,  
327 we investigated whether it was altered in *cbx1 cbx2*. We followed oxidation and re-  
328 duction of P700, the primary electron donor of photosystem I, by absorption spectroscopy in  
329 the near infrared, in this double mutant, compared to WT and to TRX mutants (double mutant  
330 *trxm1 trxm2* and single mutant *trxm4*). As shown in figure 10A, upon onset of actinic light,  
331 the first fast transient oxidation level of P700 was higher in *cbx1 cbx2* than in WT and  
332 *trxm1 trxm2*. Then a drop of the signal below the baseline, showing ferredoxin reduction, was  
333 observed in all genotypes. This drop was followed by a multiphasic increase of the signal  
334 until a steady state level of P700<sup>+</sup> was reached. It is striking that in *cbx1 cbx2* this steady  
335 state level was reached much faster than in the other genotypes. Illumination with far-red  
336 light, exciting preferentially photosystem I, led to the same oxidation level in all genotypes,  
337 with *trxm1 trxm2* reaching the maximum level of P700<sup>+</sup> slower than WT and *cbx1 cbx2*.  
338 Then far-red light was turned off and a saturating flash was given to achieve the same  
339 maximum P700 oxidation level in all genotypes. We observed clear differences in the

340 oxidation kinetics between genotypes, showing that the pool of electron donors to  
341 photosystem I available in *cbsx1 cbsx2* is smaller than in WT and *trxm1 trxm2*. This may be  
342 interpreted as a sign of lower cyclic electron flow in *cbsx1 cbsx2*.

343 Next, we measured the rates of P700<sup>+</sup> re-reduction after illumination of dark-adapted leaves  
344 with far-red light. The re-reduction of P700<sup>+</sup> after 1 min far-red illumination was much slower  
345 in *cbsx1 cbsx2* than in WT, while in TRX mutants *trxm1 trxm2* and *trxm4* it was faster than in  
346 WT as previously found in the same mutant lines Thormählen et al., 2017; Courteille et al.,  
347 2013) (Figure 10B and S9). This indicates a lower reduction state of the plastoquinone pool  
348 after far-red illumination in *cbsx1 cbsx2* and can be interpreted as a lower cyclic flow in *cbsx1*  
349 *cbsx2* compared with the other genotypes.

350 Taken together, both the P700 oxidation in actinic light and its re-reduction after far-red light  
351 indicate that the electron transport chain is more oxidized in *cbsx1 cbsx2* and that in *cbsx1*  
352 *cbsx2* lower cyclic flow takes place, both under actinic and far-red illumination.

353

354 **DISCUSSION**

355 Arabidopsis CBSX proteins were previously shown as regulatory interactors of chloroplastic  
356 TRXs. However, their function was characterized using a non-physiological reduction assay.  
357 Here, in order to understand the physiological function of CBSX1 and CBSX2 towards TRXs,  
358 we tested the chloroplast TRX targets NADP-MDH and 2-Cys PRX. We show that CBSX2  
359 specifically inhibits the activity of m-type TRXs, but does not have any effect on other  
360 plastidial TRX isoforms (TRX f, TRX x and TRX y) and NTRC. In the same experimental  
361 conditions, CBSX1 had no effect on tested TRX activities suggesting a different function for  
362 this CBSX isoform in the chloroplast. Interestingly, despite its high sequence and structure  
363 similarity with CBSX2, CBSX1 was shown to present some differences especially in the  
364 relative angle between the monomers (Jeong et al., 2013b) that may be responsible for distinct  
365 functionalities between the two isoforms. However, we cannot totally exclude that our  
366 CBSX1 protein preparation, although obtained following exactly the same methodology as for  
367 CBSX2, could be inactive.

368

369 We found that both ADP and ATP are able to alleviate CBSX2 inhibition of TRX m, at  
370 physiologically relevant concentrations, with a stronger effect of ATP. In chloroplasts, ATP is  
371 produced by the thylakoid membrane ATP synthase complex from ADP and inorganic  
372 phosphate. Upon illumination, a rapid increase in the level of ATP accompanied by a marked  
373 decrease in AMP and a slight decrease in ADP were observed (Santarius and Heber, 1965;  
374 Kobayashi et al., 1979; Voon et al., 2018). This raise in ATP levels in chloroplasts in the light  
375 and the higher efficiency of ATP in modulating CBSX effect on TRX suggest that ATP is the  
376 major ligand of CBSX2 in plant cells. Generally, CDCPs, like AMPK $\gamma$ , that bind AMP or  
377 ATP are affected by both but exhibit differential affinity (Scott et al., 2004). Although crystal  
378 structure analysis suggested that CBSX2 cannot bind ATP (Jeong et al., 2013a), our data  
379 clearly show a modulating effect of ATP onto CBSX2 functions towards TRXs m. ATP  
380 probably binds to CBSX2 and ATP-bound CBSX2 crystal structure might validate this  
381 possibility in the future.

382 We found a relationship between protein-protein as well as ligand-protein interactions and  
383 functional inhibitory effects, allowing us to propose a model integrating molecular  
384 interactions in the mechanism of TRX m inhibition by CBSX2 (Figure 11). When ATP  
385 concentration is low, CBSX2 is able to interact with reduced TRX m. This interaction inhibits  
386 TRX m ability to reduce its targets. Since CBSX proteins are devoid of any Cys residue the  
387 hypothesis of a competition between CBSX2 and targets for electron transfer from TRX can

388 be ruled out. Instead, a competition between CBSX2 and a TRX target may occur at the  
389 surface of TRX, or interaction of CBSX2 with TRX m may induce a conformational change  
390 of the TRX that would be unfavorable for electron transfer and/or target interaction. The  
391 CBSX2 structure in complex with AMP, solved at 2.2Å resolution, appeared to be  
392 approximately flat, with a strong reshaping of the protein upon AMP binding (Jeong et al.,  
393 2013a). This could be also hypothesized for ATP binding. Thus, when ligand concentration is  
394 high, CBSX2 protein would undergo a conformational change that may prevent interaction  
395 with TRX. Our model based on direct protein-protein interaction would explain the specificity  
396 of the inhibition of TRX m by CBSX2.

397

398 *In planta*, by measuring leaf NADP-MDH activity (extractable and maximal) and 2-Cys PRX  
399 redox state (monomer/dimer status and over-oxidation), we found no clear difference in *cbsx*  
400 mutant leaves compared to wild-type plants cultivated at control or at low temperature (Figure  
401 S8). This result suggests that in our growth conditions these two targets might be principally  
402 regulated by TRX isoforms other than TRXs m, or NTRC, as already reported *in vitro* and *in*  
403 *vivo* (Collin et al., 2003; Pulido et al., 2010; Yoshida et al., 2015; Pérez-Ruiz et al., 2017).  
404 Recent genetic studies have allowed investigating specifically the roles of TRXs m *in vivo*  
405 and point to their role in regulating the proton motive force (pmf). pmf primarily drives ATP  
406 synthesis and is a prerequisite for light-dependent activation of ATP synthase (CF1- $\gamma$   
407 reduction) by TRXs m and f (Junesch and Gräber, 1987; Sekiguchi et al., 2020). TRX m1 and  
408 m2 would be necessary for full activation of photosynthesis during the high light periods  
409 where elevated levels of reduced photosystem I (PSI) acceptors favor cyclic electron transport  
410 (CET) (Thormählen et al., 2017). By recycling electrons from ferredoxin to the thylakoid  
411 plastoquinone (PQ) pool via PQ reductases, PSI-CET allows maintaining the appropriate pH  
412 range of the thylakoid lumen contributing to the  $\Delta$ pH causal to the pmf that drives ATP  
413 synthase. Since ATP synthesis occurs without production of NADPH, PSI-CET is important  
414 to maintain an optimal ATP/NADPH ratio to meet metabolic demand and thereby optimal  
415 plant growth (Yamori and Shikanai, 2016). Notably, when the *trxm1\_m2\_m4* triple mutant  
416 was compared to the wild-type, Okegawa and Motohashi (2015) found that the ATP level was  
417 higher in the light, and the initial rate of light-dependent CF1- $\gamma$  reduction was slowed down  
418 (Sekiguchi et al., 2020). Data obtained in Arabidopsis show that both PQ reductase pathways,  
419 namely the Proton Gradient Regulation 5 (PGR5)/PGRL1 complex and the NADH  
420 dehydrogenase-like complex (NDH), contribute to supply sufficient acceptors from PSI  
421 (Shikanai, 2016). While a predominant role is played by the PGR5/PGRL1 pathway in normal



422 growth conditions (Nakano et al., 2019), NDH is believed to protect the photosynthetic  
423 apparatus at chilling stress under low irradiance when  $\Delta\text{pH}$  is low (Yamori et al., 2011;  
424 Yamori et al., 2015). Courteille *et al.* (2013) reported that Tobacco TRX m overexpressing  
425 plants are characterized by a down regulation of PSI-CET via the NDH pathway while NDH-  
426 dependent PQ reduction was up-regulated in Arabidopsis mutant plants specifically lacking  
427 TRX m4. It was proposed that TRXs m may promote reduction of dimeric PGRL1 to its  
428 monomeric activated form (Hertle et al., 2013) and interaction of TRX m1 with PGRL1 has  
429 been shown *in planta* (Nikkanen et al., 2018). Recently, Okegawa and Motohashi, confirmed  
430 the regulation of the PGR5/PGRL1 complex by m-type TRXs and reported that TRX m4  
431 downregulates the PGR5/ PGRL1-dependent pathway via a direct interaction with PGRL1  
432 (2020). Here, we found that, compared to wild-type, TRX m lacking plants (*trxm1m2* double  
433 mutant and *trxm4* single mutant) exhibited a faster P700<sup>+</sup> reduction (Figure 10B and Figure  
434 S9), similarly to previously reported data (Courteille et al., 2013). On the contrary, the *cbsx1*  
435 *cbsx2* mutant showed a slower reduction indicative of decreased oxidation of the PQ pool  
436 favorable for PSI-CET. Thus, CBSX affects P700 photo-oxidation reduction cycle in PSI. By  
437 avoiding accumulation of electrons in the PQ pool, CBSX would allow maintaining a pH  
438 range in the luminal space of the thylakoid membrane appropriate for ATP synthesis (Miyake,  
439 2020).

440 At low temperature, transcriptomics databases indicate a higher expression level of *CBSX1*  
441 and *CBSX2* genes (Waese et al., 2017) while transcripts abundance for TRX m1, TRX m2 and  
442 TRX m4 are decreased (Dreyer and Dietz, 2018). When we investigated the *cbsx1 cbsx2*  
443 mutants growth at low temperature (12°C), and under a weak illumination (60  $\mu\text{mol}$   
444  $\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) to avoid photoinhibition, we found that the double mutant exhibited growth  
445 and chlorophyll accumulation defects in these conditions. These phenotypes may be caused  
446 by a suppression of PSI-CET. Noticeably, PSI-CET is enhanced in pea leaves by chilling  
447 (Cornic et al., 2000), as in rice, where NDH-dependent cyclic electron flow plays a significant  
448 role for photosynthesis and plant growth at low temperature (Yamori et al., 2011). Moreover,  
449 both pathways of PSI-CET protect tomato leaves against photoinhibition when exposed to  
450 chilling stress (Wang et al., 2020).

451 Notably, impaired growth and decreased chlorophyll content were found in tobacco over-  
452 expressing TRX m (Rey et al., 2013). Da *et al.* (2017) reported a high pmf in TRX m-  
453 deficient Arabidopsis plants and Thormählen *et al.* (2017) found that in *trxm1m2* mutants PSI  
454 yield was enhanced and accompanied by an increased oxidation of the PQ pool, indicative of  
455 PSI-CET stimulation. Thus, in our chilling growth conditions (low temperature and low light

456 intensity), we believe that no damage of photosynthetic membranes occurred (no  
457 photoinhibition) and that a high PSI-CET was necessary to sustain ATP homeostasis and  
458 plant growth. Based on previous studies on TRXs m, this would imply alleviation of their  
459 negative regulation exerted on PSI-CET, and, based on the present work, we hypothesize that  
460 this would implicate CBSX proteins.

461 Interestingly, a recent work reported the role played by adenylates to balance the “plastid  
462 energy budget”, supporting the view that PSI-CET is activated under conditions where  
463 stromal ATP is low, but is downregulated as ATP levels build up, allowing for effective ATP  
464 homeostasis (Fisher et al., 2019). Accordingly, we propose a model (Figure 12) in which  
465 CBSX2, acting as a sensor of the chloroplast energy status, would specifically decrease  
466 reduced TRX m activity when ATP concentration is low. This would consequently decrease  
467 TRX m-dependent negative regulation of PSI-CET to allow a higher pmf-driven ATP  
468 production, since TRXs f are not affected by CBSX2 and efficiently activate ATP synthase.

469 Overall, present findings reveal a new ATP sensing mechanism involving Cystathionine- $\beta$ -  
470 synthase containing domain protein and thioredoxin possibly acting for optimal ATP  
471 homeostasis in the chloroplast.

472

473

## 474 MATERIALS AND METHODS

### 475 Plants Materials

476 T-DNA insertion mutants *cbsx1* (Gabi\_050D12), *cbsx2* (Salk\_136934), *trxm1 trxm2*  
477 (Salk\_087118\_ and Salk\_123570), *trxm4* (Salk\_0 532538) were previously described in Yoo  
478 et al. (2011), Jung et al. (2013), Thormählen et al. (2017), and Courteille et al. (2013),  
479 respectively. They were obtained from the NASC (Nottingham Arabidopsis Stock Centre)  
480 (Scholl et al., 2000). Plants are sown and grown under long days conditions (16h light/day) at  
481 20°C or 12°C constant temperature. Light intensity was 60  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (tubular  
482 fluorescent lamps: Sylvania Lynx-LE 840, white light) at the level of the leaves.

### 483 Phylogenetic Analysis

484 We used TAIR10 models to determine the full-length amino acid sequences of the six *A.*  
485 *thaliana* CBSX: CBSX1 (AT4G36910), CBSX2 (AT4G34120), CBSX3 (AT5G10860),  
486 CBSX4 (AT1G80090), CBSX5 (AT4G27460) and CBSX6 (AT1G65320). The phylogenetic  
487 tree was inferred using the Maximum Likelihood method based on the Jones-Taylor-Thornton

488 (JTT) matrix-based model (Jones et al., 1992), bootstraps: 1000. Analysis were conducted on  
489 MEGA7 (Kumar et al., 2016).

#### 490 **Yeast 2-Hybrid**

491 *CBSX1* (72-236), *CBSX2* (72-238) and *CBSX3* (16-206) truncated ORF were amplified and  
492 cloned into the pDONR207 vector using Gateway BP Clonase enzyme (Invitrogen). Primers  
493 (ORF\_intern + ORF\_stop) were designed to amplify ORF encoding mature proteins lacking  
494 their putative target peptide (prediction by TargetP 1.1 (Nielsen et al., 1997; Emanuelsson et  
495 al., 2000). *CBSX* ORF were then subcloned into pDEST-AD-CYH2 and pDEST-DB  
496 destination vectors (Dreze et al., 2010) using Gateway LR Clonase enzyme (Invitrogen)  
497 according to manufacturer's instructions. Y8800 MAT $\alpha$  and Y8930 MAT $\alpha$  yeast strains  
498 (James et al., 1996) were transformed with AD-ORF and DB-ORF, respectively. Yeast  
499 mating and selection were performed according to the InterATOME platform pipeline as  
500 previously described in Monachello *et al.* (2019). As positive interacting protein control we  
501 used DB-mNUWA and AD-mDYW2 (Guillaumot et al., 2017).

#### 502 **Bimolecular Fluorescence Complementation and Subcellular localization**

503 *CBSX1* (1-236), *CBSX2* (1-238) and *CBSX3* (1-206) full length ORF were amplified and  
504 cloned into the pDONR207 vector using Gateway BP Clonase enzyme (Invitrogen). Primers  
505 (ORF\_start + ORF\_end) were designed to amplify ORF without stop codon allowing C-  
506 terminal fusion after Gateway LR transfert into pGreen-0229-RFP, pBiFC1 or pBiFC4  
507 destination vectors (Boussardon et al., 2012). For subcellular localization, C58C1 pSOUP  
508 *Agrobacterium tumefaciens* cells were transformed with pGreen-0229-RFP vectors. For  
509 Bimolecular Fluorescence Complementation, C58C1 pMP90 *Agrobacterium tumefaciens*  
510 cells were transformed with pBiFC1 and pBiFC4 vectors. For all experiments leaves of  
511 *Nicotiana benthamiana* were co-infiltrated with agrobacteria containing required vectors and  
512 the silencing inhibitor HC-Pro (Stephan et al., 2011). Leaves were observed 4 days after  
513 transformation using a confocal microscope (Zeiss LSM 880). Image acquisition and image  
514 analysis were performed using the IPS2 Imaging Facility.

#### 515 **Protein Production and Purification**

516 *CBSX1* (72-236) and *CBSX2* (72-238) ORF in pDONR207 were subcloned into the  
517 expression vector pDEST17 (Invitrogen) using Gateway LR Clonase enzyme (Invitrogen)  
518 according to manufacturer's instructions. Protein production in *Escherichia coli* BL21 cells  
519 was induced overnight at 30°C in LB medium containing 100  $\mu$ g/mL carbenicilline and 400

520  $\mu\text{M}$  IPTG. Bacteria lysis was performed in 30 mM Tris pH7.9 containing protease inhibitor  
521 (complete Mini EDTA-free Roche®) using a French press. Proteins were purified by affinity  
522 chromatography using a nickel column (GE healthcare® His-trap HP 1mL). Column washing  
523 was performed with 30 mM Tris pH 7.9 containing 20 mM imidazole. Proteins elution was  
524 performed with 30 mM Tris pH 7.9 containing 200 mM imidazole. Eluted proteins were  
525 dialysed and concentrated in 30 mM Tris pH 7.9 using Amicon columns (Sigma Amicon  
526 ultra-4 Z740191-8EA 30K). All TRX and 2-Cys PRX proteins were produced as previously  
527 described in Collin *et al.* (2003; 2004). NTRC was obtained as described in Bohrer *et al.*,  
528 2012. Fully activated wild-type MDH and mutant DMC-MDH recombinant enzymes,  
529 obtained as previously described in Issakidis *et al.* (1994), had a specific activity (u/mg) of  
530 368 +/- 42 and 386 +/- 25, respectively. 2-Cys PRX exhibited a  $K_{\text{cat}}$  of 179 +/- 17  $\text{s}^{-1}$  with  
531 TRXx as reducing substrate. All these values being very close to those previously reported  
532 (Issakidis *et al.*, 1994; Collin *et al.*,2003) certified that appropriate purified protein  
533 preparations were used for the present study.

#### 534 **MDH activity**

535 MDH (0.24 g/L) was activated by incubation with 10 mM DTE-reduced TRX with the  
536 eventual addition of 30  $\mu\text{M}$  CBSX and 1 mM AMP or ATP, as indicated, in 30 mM Tris  
537 pH7.9 at 21°C. TRX concentration was 10  $\mu\text{M}$  for all TRX excepted TRX f1 used at 1  $\mu\text{M}$ . 3  
538  $\mu\text{L}$  aliquots of reaction medium were periodically withdrawn and added to 200  $\mu\text{L}$  of 30°C  
539 pre-heated reaction medium (for wild-type MDH: 160  $\mu\text{M}$  NADPH, 750  $\mu\text{M}$  OAA, 30 mM  
540 Tris pH7.9 ; for DMC-MDH: 160  $\mu\text{M}$  NADPH, 1 mM OAA, 30 mM Tris pH7.9).  
541 Experiments with MgATP were performed by incubation with 1 mM ATP and 10 mM  
542  $\text{MgCl}_2$ . The MDH activity was measured by following the NADPH consumption at 340 nm  
543 during 1 min in a Tecan infinite m200 PRO plate reader. Each experiment has been repeated  
544 at least twice, with three to four technical replicates in each experiment. MDH activities  
545 (initial velocities) were estimated as the slopes calculated during 20 seconds. When the  
546 coefficient of variation of the estimated slope was higher than 15%, we discarded the data.  
547 We defined TRX activity as the MDH activity after 20 minutes of activation. Statistical  
548 analysis of MDH activity dataset was performed with a linear model taking into account TRX,  
549 CBSX and adenylate effects.

#### 550 **PRX activity**

551 0,4 mM of tBOOH (tert-butyl hydroperoxide) was reduced by 15  $\mu\text{M}$  2-Cys PRX A, 15  $\mu\text{M}$   
552 of 0,5 mM DTE-reduced TRX, 45  $\mu\text{M}$  CBSX and 1 mM AMP in 100 mM Tris pH7.9.

553 Unreduced tBOOH in 10  $\mu$ L aliquots was periodically quantified at 560 nm using the FOX  
554 coloration procedure described in Collin *et al.* (2004). CBSX direct effect on PRX was  
555 assayed in presence of 1 mM DTE.

#### 556 **Analysis of chlorophyll content**

557 Chlorophylls) quantification was performed by acetone extraction on 50 mg fresh rosette leaf  
558 as previously described in Sumanta *et al.* (2014).

#### 559 **P700 absorption**

560 The redox state of P<sub>700</sub> was monitored by following differences of the 875 nm and 830 nm  
561 transmittance signals using a DUAL-PAM-100 (Walz, Effeltrich, Germany). When P700  
562 oxidation was measured with actinic light, plants were kept prior to the measurements in the  
563 light in the growth chamber so that the Calvin-Benson cycle enzymes were active. When the  
564 pool of electron donors was measured by following P700<sup>+</sup> re-reduction after far-red  
565 illumination, plants were kept in the dark prior to the measurement to insure inactive Calvin-  
566 Benson cycle enzymes. The acquisition rate was set to 1 ms.

#### 567 **Statistical analysis**

568 All statistical analyses were conducted on R (version 3.2.5). To analyze each dataset, we used  
569 ANOVA with several factors and when necessary statistical interactions were considered. To  
570 control the family-wise error rate, p-values were adjusted using the Bonferroni procedure. A  
571 difference was considered significant when its adjusted p-value was lower than 0.05.

572 For the MDH activities dataset with the four TRXs, two CBSXs and AMP, the mean is  
573 defined as the single TRX, CBSX and AMP effects, their pairwise interactions and the third  
574 order interaction.

575 For the DMC-MDH activities dataset, the mean is defined as the single TRX, CBSX and  
576 AMP effects and the interaction between CBSX and TRX. This model is the most complete  
577 according to the experimental design.

578 For the MDH activities dataset with AMP, ATP and MgATP, the mean is defined as the  
579 single CBSX and ligand effects and their interaction.

580 For MDH activities dataset with AMP, ADP and ATP, the mean is defined as the single  
581 CBSX and ligand effects and their interaction.

582 For the MDH activities dataset with AMP and ATP ranging from 1 $\mu$ M to 1mM, the mean is  
583 defined as the single CBSX and ligand effects and their interaction.

584 For the PRX activities dataset with CBSX2 and AMP effects on TRX m1 (Figure 7), the  
585 mean is defined as the single CBSX and AMP effects and their interaction.

586 For the rosette fresh-weight and chlorophyll content datasets, the mean is defined as the  
587 genotype effect.

588 For *in planta* MDH initial and maximal activities dataset, the mean is defined as the genotype  
589 effect.

#### 590 **Pull-down**

591 Purified 6His-CBSX2 protein (500 µg) was incubated with TRX m1 (285 µg) in 350 µL assay  
592 buffer (1x PBS, ± 1 mM DTE ± 1 mM AMP ± 1 mM ATP) for 1h at room temperature. After  
593 adding 50 µL of Ni-NTA agarose beads (Qiagen) and incubation for 1h at room temperature,  
594 beads were washed 3 times with 500 µL of assay buffer. Proteins were eluted with 200 mM  
595 imidazole. Immunoblotting was performed with mouse anti-6HIS (Roche® #11922416001)  
596 and rabbit specific anti-TRXm1 (generated against AtTRXm1 recombinant protein,  
597 Genosphere Biotechnologies) as primary antibodies, and anti-mouse (Sigma A5906) and anti-  
598 rabbit (Sigma A6154) as HRP-conjugated secondary antibodies. Blot revelation was  
599 performed with the BIORAD Clarity™ Western ECL Substrate on a BIORAD® Chemidoc  
600 ISV0025.

601

#### 602 **ACCESSION NUMBERS**

603 *CBSX1* (AT4G36910), *CBSX2* (AT4G34120), *CBSX3* (AT5G10860), *CBSX4* (AT1G80090),  
604 *CBSX5* (AT4G27460), *CBSX6* (AT1G65320), *TRXM1* (AT1G03680), *TRXM2* (AT4G03520),  
605 *TRXF1* (AT3G02730), *TRXF2* (AT5G16400), *TRXX* (AT1G50320), *TRXY1* (AT1G76760),  
606 *NUWA* (AT3G49240) and *DYW2* (AT2G15690).

607 *2-CYS PRXA* (AT5G58330), *NADP-MDH* (AT3G11630).

#### 608 **SUPPLEMENTAL MATERIAL**

609 Table S1: Primer used

610 Figure S1: *Arabidopsis thaliana* CBSX phylogenetic tree.

611 Figure S2: Subcellular localization of CBSX1, CBSX2 and CBSX3.

612 Figure S3: NADP-MDH activation kinetics.

613 Figure S4: Test of direct effect of adenylates on TRXm1 dependent NADP-MDH activation.

614 Figure S5: Effect of AMP on mutant DMC-MDH activity.

615 Figure S6: Effect of ATP on CBSX2 towards TRXm1-dependent NADP-MDH activation.

616 Figure S7: Effects of ATP together with ADP at concentrations and ratios mimicking light

617 and night conditions, on CBSX2 towards TRXm1-dependent NADP-MDH activation.

618 Figure S8: TRX targets in the context of CBSX mutations.

619 Figure S9: P700+ reduction.

620

## 621 ACKNOWLEDGMENTS

622 We greatly thank Marie-Laure Martin-Magniette who helped us for statistical analysis. We

623 are grateful to Cécile Raynaud who provided us with the *Agrobacterium* strain containing the

624 HC-pro silencing inhibitor. We would also like to thank Myroslawa Miginiac-Maslow for

625 constructive criticism of the manuscript.

## 626 FIGURE LEGENDS

627 **Figure 1.** Protein interactions between Arabidopsis CBSX isoforms assayed by yeast 2-

628 hybrid.

629 Interactions were tested using a mating-based Y2H matricial assay with AD fusions on the

630 horizontal axis and the DB fusions on the vertical axis. In the “H<sub>2</sub>O” row and column, yeast

631 cells were replaced by water; in the “Ø” row and column, mating was performed with yeasts

632 transformed with an empty vector. As a positive interaction control, we used DB-mNUWA

633 and AD-mDYW2 (Guillaumot et al., 2017). Interactions were tested on selective medium

634 without histidine.

635 **Figure 2.** *In planta* protein interactions between CBSX isoforms assayed by BiFC.

636 BiFC of YFP in transiently transformed *Nicotiana benthamiana* leaves. Left column, YFP

637 fluorescence signal. Middle column, chlorophyll fluorescence signal. Right column, merge of

638 fluorescent signals. Fluorescence spots confirmed as YFP fluorescence signals by spectral

639 analysis are indicated by white arrows. Observations were performed four days after

640 transformations. Bars=10 µm.

641 **Figure 3.** Effect of CBSX1 and 2 on TRX capacity to activate wild-type NADP-MDH, and

642 effect of AMP.

643 NADP-MDH activation was measured after 20 min incubation at room temperature in

644 presence of 10 µM TRX (except TRXf1 at 1 µM) and 10 mM DTE. 100% activation rate

645 corresponds to the activation with DTE-reduced TRX (without CBSX and AMP). (A)

646 TRXm1 (mean SA = 372 u/mg), (B) TRXm2 (mean SA = 314 u/mg), (C) TRXm4 (mean SA

647 = 383 u/mg), (D) TRXf1 (mean SA = 432 u/mg) and (E) TRXf2 (mean SA = 340 u/mg).

648 CBSX1/2 and AMP final concentration were 30  $\mu$ M and 1 mM, respectively. Values are  
649 means  $\pm$  SD (n=4). Bars with different letters correspond to significantly different values.  $P <$   
650 0.05. SA: specific activity of NADP-MDH at maximal activation (100%) expressed in units  
651 (u:  $\mu$ mole NADPH oxidized per min) per mg of enzyme.

652 **Figure 4.** Effect of CBSX2 on mutant DMC-MDH activity and TRXm-dependent activation,  
653 and effect of AMP.

654 DMC-MDH activation was measured after 20 min incubation at room temperature in presence  
655 of DTE alone ( $\emptyset$  TRX) or DTE + TRXm1 or DTE + TRXm2. 100% activation rate  
656 corresponds to the maximal activation of DMC-MDH by DTE-reduced TRXm1/2 (without  
657 CBSX and AMP). Mean specific activity of DMC-MDH at maximal activation (100%) were  
658 391 u/mg and 382 u/mg, with TRXm1 and TRXm2, respectively. Values are means  $\pm$  SD  
659 (n=4). TRXm1/2, CBSX2 and AMP were at a final concentration of 10  $\mu$ M, 30  $\mu$ M and 1  
660 mM, respectively. Bars with different letters correspond to significantly different values.  $P <$   
661 0.05.

662 **Figure 5.** Effects of adenylates on CBSX2 inhibition of TRXm-dependent NADP-MDH  
663 activation.

664 Wild-type MDH activation was measured after 20 min incubation in presence of DTE-  
665 reduced TRXm1 (10  $\mu$ M) with CBSX2 (30  $\mu$ M) and 0, 1, 10, 50 or 1000  $\mu$ M of AMP, ADP  
666 or ATP. Values are means  $\pm$  SD (n=4-6). Bars with different letters correspond to  
667 significantly different values.  $P <$  0.05.

668 **Figure 6.** Effect of CBSX1 and 2 on 2-Cys PRX activity.

669 Time course of tBOOH reduction by 2-Cys PRXA was quantified by the FOX assay in  
670 presence of DTT alone (black curves), DTT-reduced TRX (grey curves), DTT-reduced TRX  
671 and CBSX1 (blue curves) or DTT-reduced TRX and CBSX2 (orange curves). (A) DTT alone;  
672 (B) DTT + TRX x; (C) DTT + TRX f1; (D) DTT + TRX y1; (E) DTT + TRX m1; (F) DTT +  
673 NTRC. The values are means  $\pm$  SD (n=3).

674 **Figure 7.** Effect of 1 mM AMP on CBSX2 towards TRXm1-dependent 2-Cys PRX activity.  
675 2-Cys PRX activities were defined as the tBOOH reduction kinetic slope between 2 and 10  
676 min and were normalized to the activity in the control condition (without CBSX and AMP)  
677 considered as 100%. The values are means  $\pm$  SD (n=3). Bars with different letters correspond  
678 to significantly different values.  $P <$  0.05.

679 **Figure 8.** Pull-down assays with CBSX and TRXm proteins.

680 Pull-down of His-CBSX and TRXm1 in absence or presence of DTE, AMP and ATP. (A)  
681 Impact of 1 mM AMP and DTE on CBSX2-TRXm1 interaction. (B) Impact of 1 mM ATP or



682 1 mM AMP on CBSX-TRXm1 interaction. Input corresponds to proteins initially mixed  
683 before incubation with His-affinity agarose beads. Output corresponds to proteins eluted (after  
684 washes) from the resin by 200 mM imidazole. The bracket indicates an unspecific low  
685 molecular weight signal obtained using anti-TRX m1 antibodies on CBSX preparations.

686 **Figure 9.** *cbsx* mutant phenotypes.

687 (A) Representative 5-week old plants grown in long day conditions at 20°C (bar= 1cm). (B)  
688 Representative 11-week old plants grown in long day conditions at 12°C (bar= 1cm). (C) and  
689 (D) Plants grown at control temperature. (E) and (F) Plants grown at low temperature. (C) and  
690 (E) Rosette fresh weight, values are means  $\pm$  SD (n=10), \*:  $P < 0.01$ . (D) and (F) Chlorophyll  
691 a and b contents, and Chla/Chlb ratios, values are means  $\pm$  SD (n=10). \*:  $P = 0.02$ .

692 **Figure 10.** P700 measurements on leaves of wild-type plants and Trx m and CBSX-deficient  
693 mutants.

694 The redox state of the PSI primary donor P700 was monitored through the changes in  
695 absorbance at 830 versus 875 nm. (A) Representative curves are shown of plants kept at  
696 growth light intensity; WT: black, *cbsx1cbsx2*: red, *trxm1trxm2*: green. Leaves were  
697 illuminated with actinic red light, AL ( $I=830 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) to oxidize P700 until a  
698 steady state was reached. Then far-red light, FR, was switched on. To achieve the maximum  
699 oxidation state a saturating flash, SF, was given at the end of the FR illumination. (B) Half-  
700 time of P700+ reduction after FR illumination of dark-adapted plants. The traces of the  
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703 **Figure 11.** Model of TRX activity regulation by CBSX2.

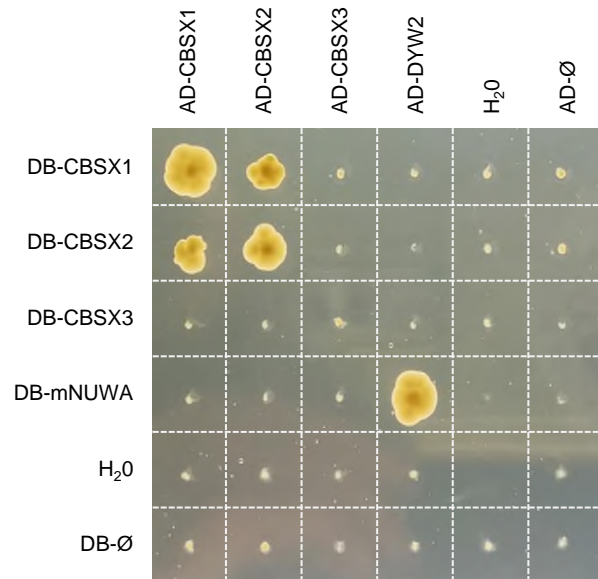
704 CBSX2 homodimer (yellow disc) is unable to interact with oxidized TRX m (green circle)  
705 (A), but is able to interact with reduced TRXm and inhibits TRX m capacity to reduce its  
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709 **Figure 12.** Model for functional relationship between CBSX and TRX in the context of ATP  
710 homeostasis in the chloroplast.

711 In the proposed model, when ATP level is low (A), CBSX can interact with TRX m impeding  
712 its negative regulation towards PSI-CET, primarily coupled to the generation of the proton  
713 motive force driving the synthesis of ATP by the thylakoid ATP synthase complex. When  
714 ATP level increases (B), ATP binds to CBSX which cannot interact with TRX m, allowing  
715 down-regulation of the PSI-CET and thereby lowering ATP synthesis.

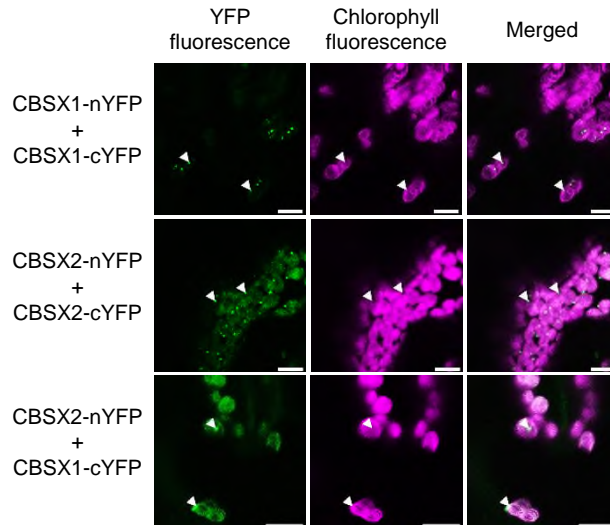
716 Electron and proton transfers are shown by red and blue arrows, respectively. Abbreviations  
717 are : PSI/II, photosystem I/II; Cyt b6/f, cytochrome b6/f complex; Fd, ferredoxin; FNR,  
718 ferredoxin-NADP reductase; FTR, ferredoxin/thioredoxin reductase; TRX, thioredoxin;  
719 CBSX, Cystathionine- $\beta$ -synthase containing domain protein; PQ, plastoquinone; PQH2,  
720 plastoquinol; PC, plastocyanin; PSI-CET, cyclic electron transport around PSI.

721



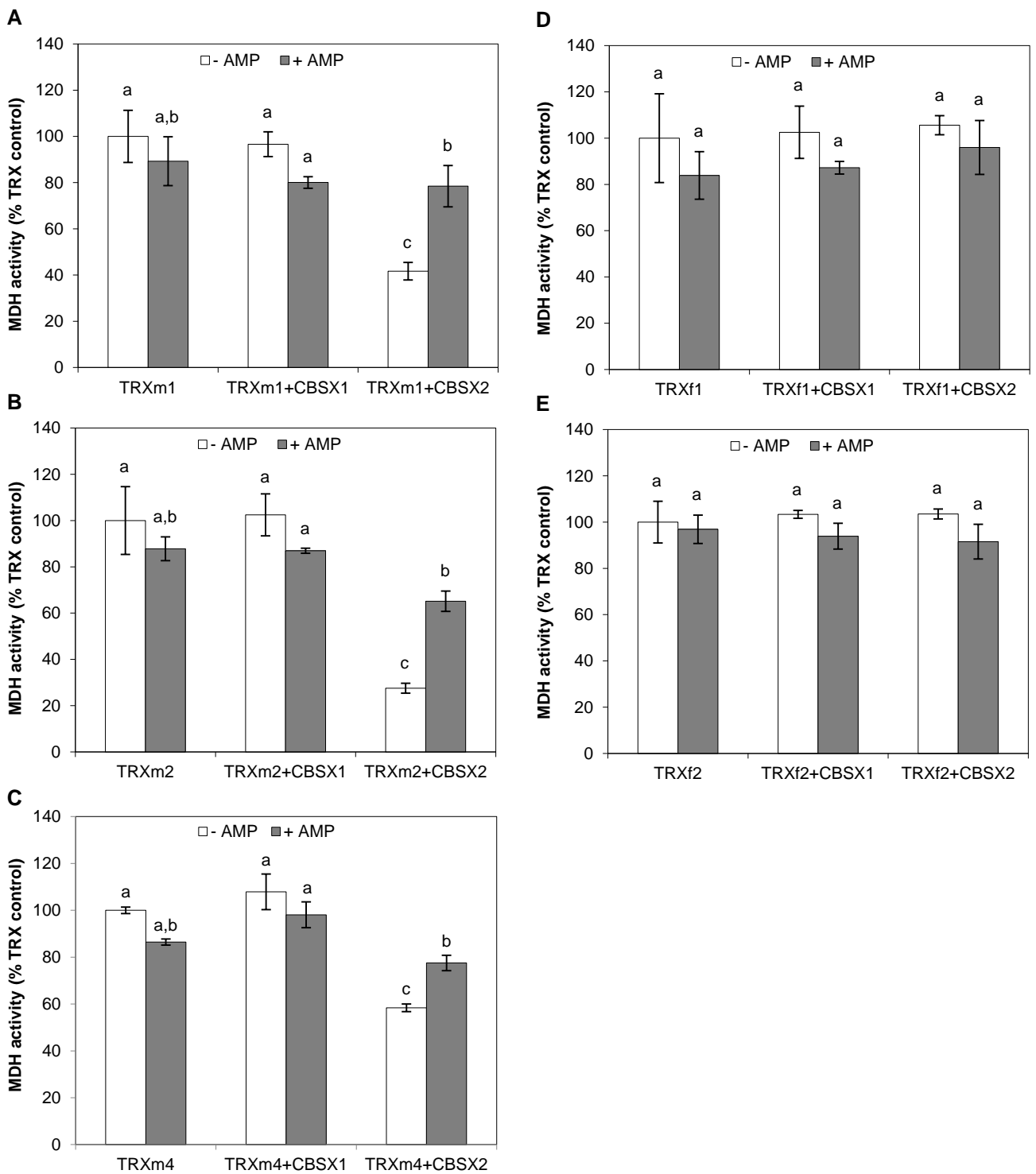
**Figure 1.** Protein interactions between Arabidopsis CBSX isoforms assayed by yeast 2-hybrid.

Interactions were tested using a mating-based Y2H matricial assay with AD fusions on the horizontal axis and the DB fusions on the vertical axis. In the “H<sub>2</sub>O” row and column, yeast cells were replaced by water; in the “Ø” row and column, mating was performed with yeasts transformed with an empty vector. As a positive interaction control, we used DB-mNUWA and AD-mDYW2 (Guillaumot et al., 2017). Interactions were tested on selective medium without histidine.



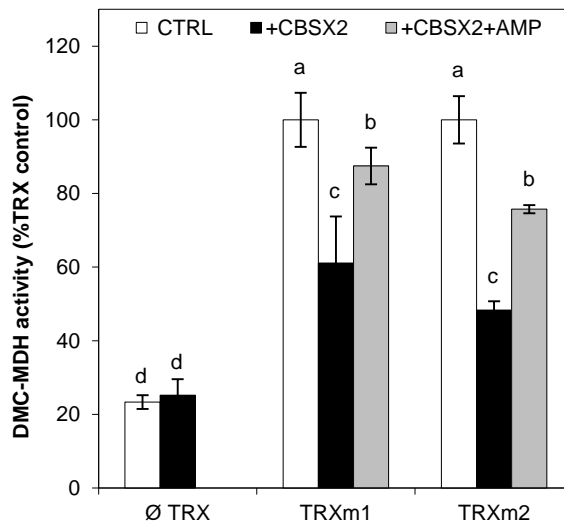
**Figure 2.** *In planta* protein interactions between CBSX isoforms assayed by BiFC.

BiFC of YFP in transiently transformed *Nicotiana benthamiana* leaves. Left column, YFP fluorescence signal. Middle column, chlorophyll fluorescence signal. Right column, merge of fluorescent signals. Fluorescence spots confirmed as YFP fluorescence signals by spectral analysis are indicated by white arrows. Observations were performed four days after transformations. Bars=10  $\mu$ m.



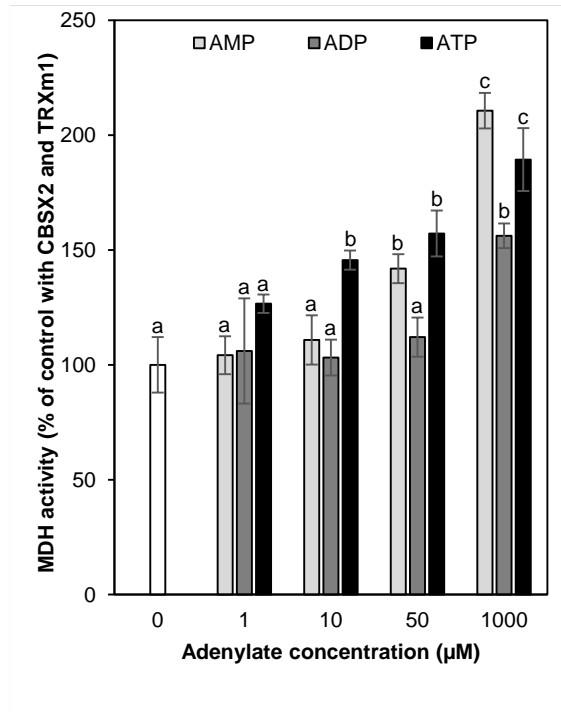
**Figure 3.** Effect of CBSX1 and 2 on TRX capacity to activate wild-type NADP-MDH, and effect of AMP.

NADP-MDH activation was measured after 20 min incubation at room temperature in presence of 10  $\mu$ M TRX (except TRXf1 at 1  $\mu$ M) and 10 mM DTE. 100% activation rate corresponds to the activation with DTE-reduced TRX (without CBSX and AMP). (A) TRXm1 (mean SA = 372 u/mg), (B) TRXm2 (mean SA = 314 u/mg), (C) TRXm4 (mean SA = 383 u/mg), (D) TRXf1 (mean SA = 432 u/mg) and (E) TRXf2 (mean SA = 340 u/mg). CBSX1/2 and AMP final concentration were 30  $\mu$ M and 1 mM, respectively. Values are means  $\pm$  SD (n=4). Bars with different letters correspond to significantly different values. P < 0.05. SA: specific activity of NADP-MDH at maximal activation (100%) expressed in units (u:  $\mu$ mole NADPH oxidized per min) per mg of enzyme.



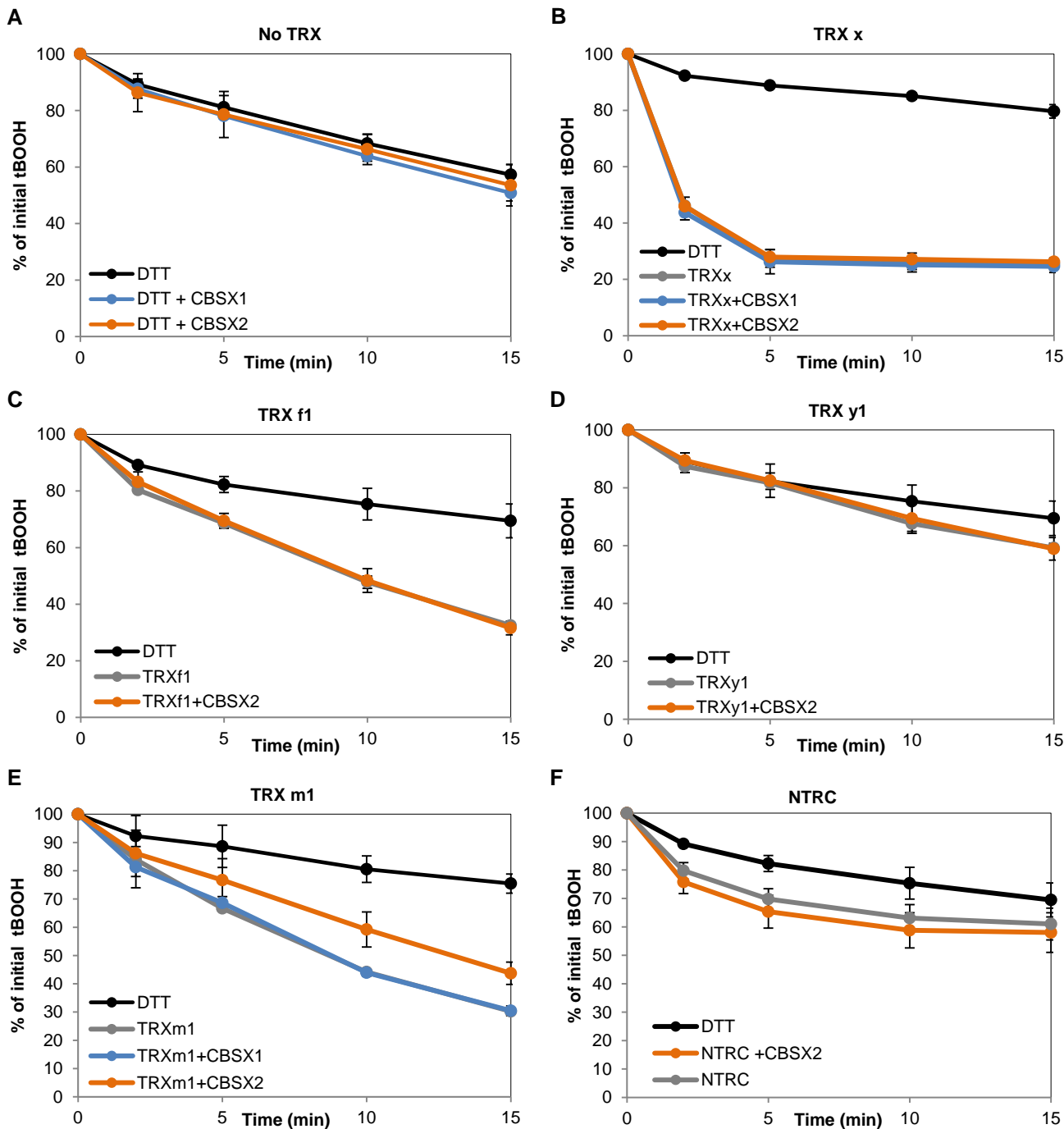
**Figure 4.** Effect of CBSX2 on mutant DMC-MDH activity and TRXm-dependent activation, and effect of AMP.

DMC-MDH activation was measured after 20 min incubation at room temperature in presence of DTE alone (Ø TRX) or DTE + TRXm1 or DTE + TRXm2. 100% activation rate corresponds to the maximal activation of DMC-MDH by DTE-reduced TRXm1/2 (without CBSX and AMP). Mean specific activity of DMC-MDH at maximal activation (100%) were 391 u/mg and 382 u/mg, with TRXm1 and TRXm2, respectively. Values are means  $\pm$  SD (n=4). TRXm1/2, CBSX2 and AMP were at a final concentration of 10  $\mu$ M, 30  $\mu$ M and 1 mM, respectively. Bars with different letters correspond to significantly different values.  $P < 0.05$ .



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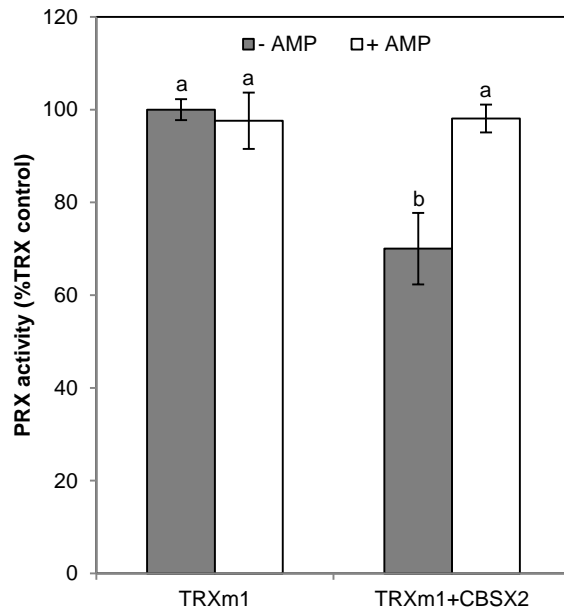
Wild-type MDH activation was measured after 20 min incubation in presence of DTE-reduced TRXm1 (10 µM) with CBSX2 (30 µM) and 0, 1, 10, 50 or 1000 µM of AMP, ADP or ATP. Values are means  $\pm$  SD (n=4-6). Bars with different letters correspond to significantly different values.  $P < 0.05$ .



**Figure 6.** Effect of CBSX1 and 2 on 2-Cys PRX activity.

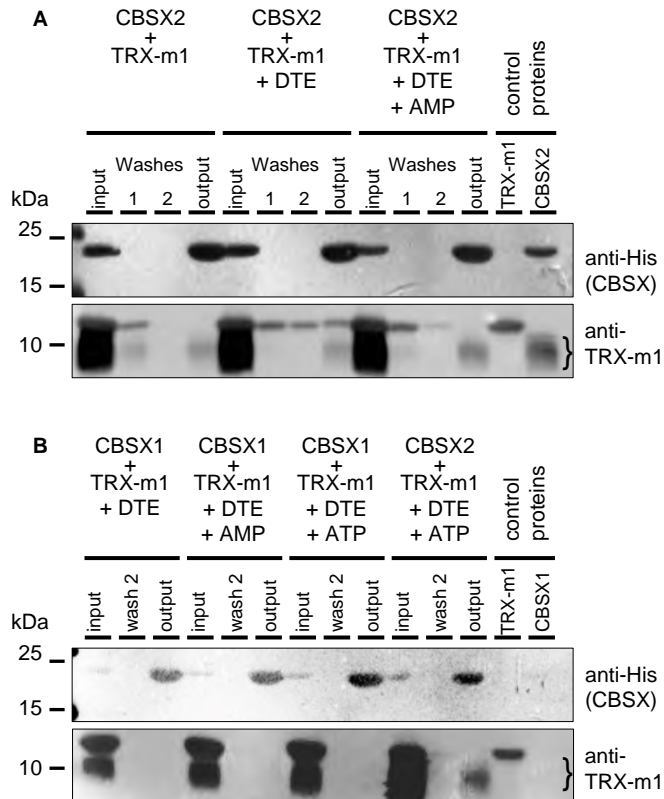
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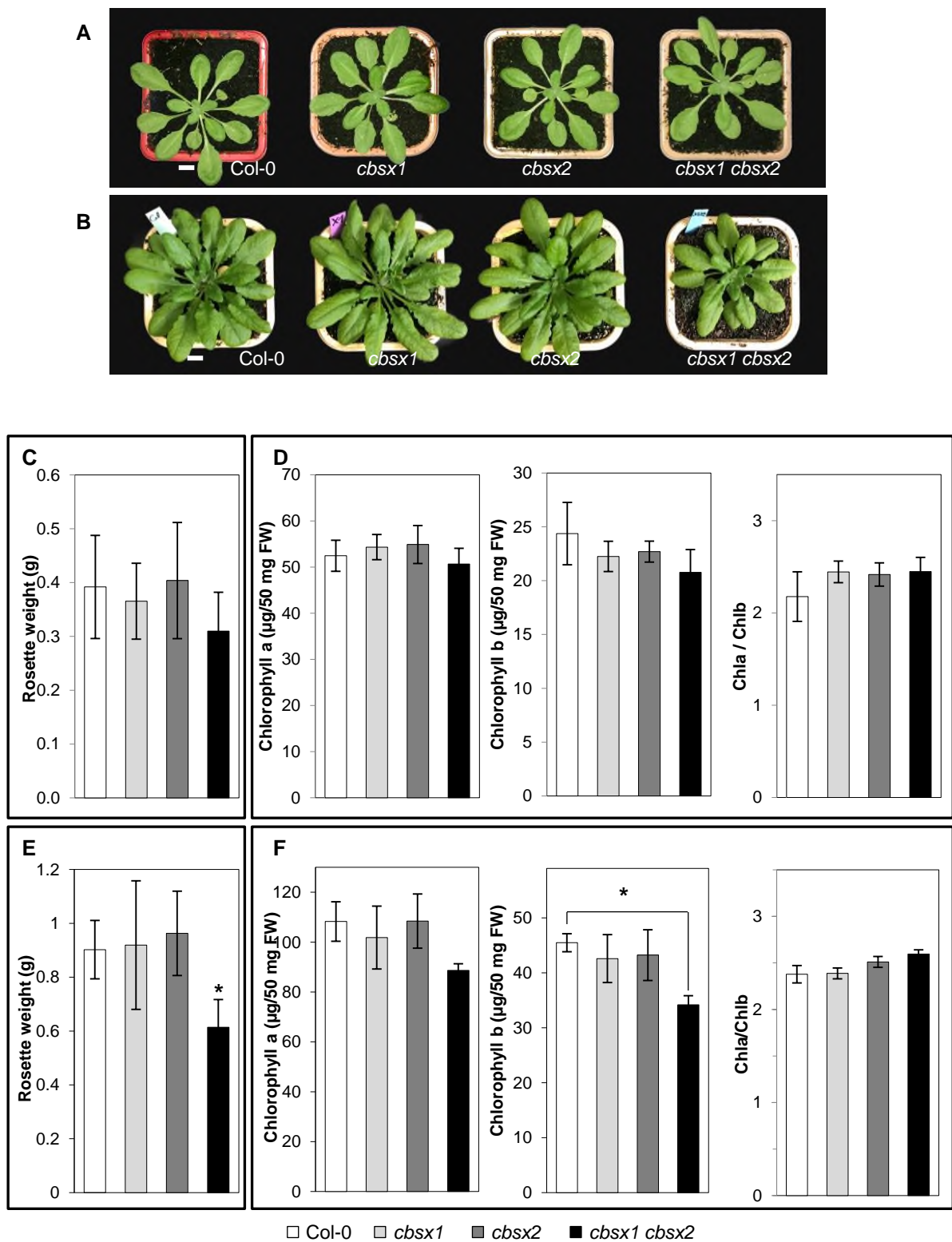
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2-Cys PRX activities were defined as the tBOOH reduction kinetic slope between 2 and 10 min and were normalized to the activity in the control condition (without CBSX and AMP) considered as 100%. The values are means  $\pm$  SD (n=3). Bars with different letters correspond to significantly different values.  $P < 0.05$ .



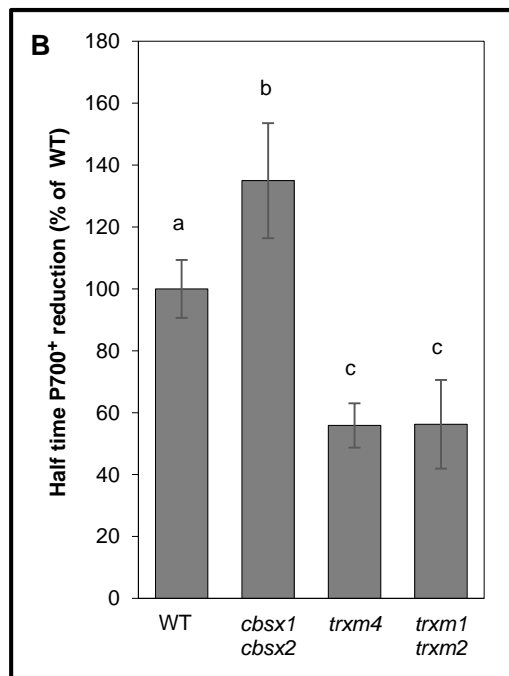
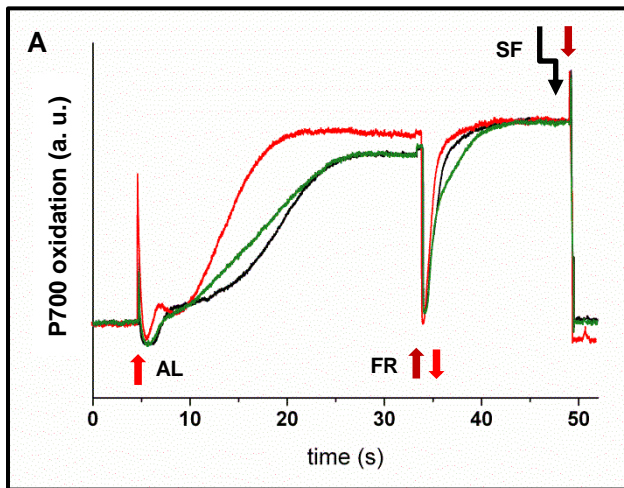
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Pull-down of His-CBSX and TRXm1 in absence or presence of DTE, AMP and ATP. (A) Impact of 1 mM AMP and DTE on CBSX2-TRXm1 interaction. (B) Impact of 1 mM ATP or 1 mM AMP on CBSX-TRXm1 interaction. Input corresponds to proteins initially mixed before incubation with His-affinity agarose beads. Output corresponds to proteins eluted (after washes) from the resin by 200 mM imidazole. The bracket indicates an unspecific low molecular weight signal obtained using anti-TRX m1 antibodies on CBSX preparations.



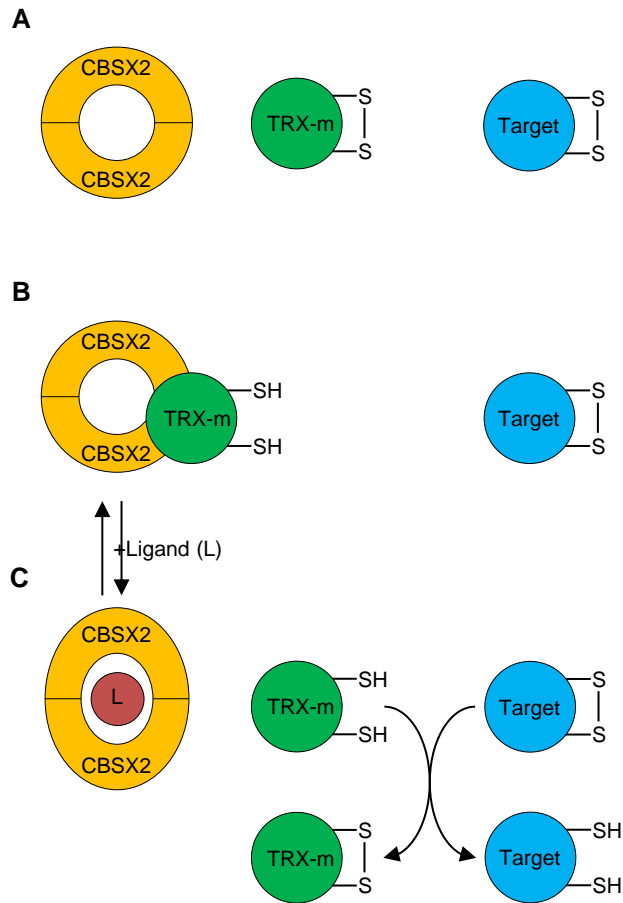
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**Figure 10.** P700 measurements on leaves of wild-type plants and Trx m and CBSX-deficient mutants.

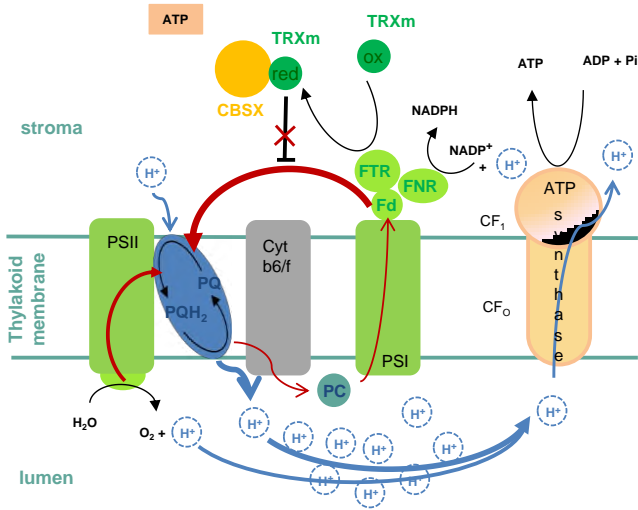
The redox state of the PSI primary donor P700 was monitored through the changes in absorbance at 830 versus 875 nm. (A) Representative curves are shown of plants kept at growth light intensity; WT: black, *cbsx1cbsx2*: red, *trxm1trxm2*: green. Leaves were illuminated with actinic red light, AL ( $I = 830 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$ ) to oxidize P700 until a steady state was reached. Then far-red light, FR, was switched on. To achieve the maximum oxidation state a saturating flash, SF, was given at the end of the FR illumination. (B) Half-time of P700<sup>+</sup> reduction after FR illumination of dark-adapted plants. The traces of the kinetics are shown in Figure S9. Bars with different letters correspond to significantly different values between genotypes.  $P < 0.05$ .



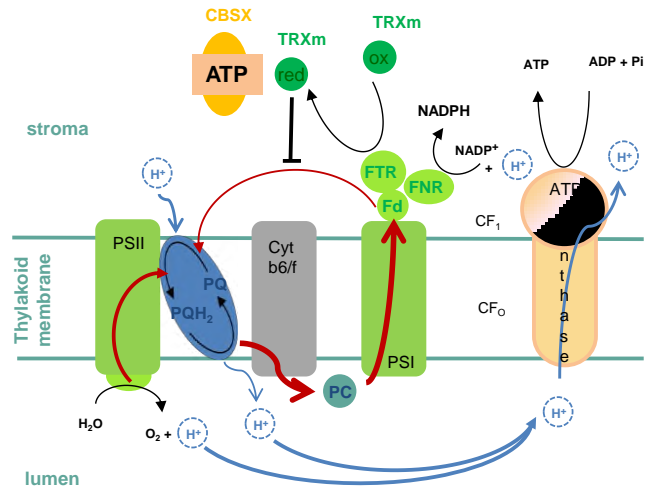
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(A) Low ATP



(B) High ATP



**Figure 12.** Model for functional relationship between CBSX and TRX in the context of ATP homeostasis in the chloroplast.

In the proposed model, when ATP level is low (A), CBSX can interact with TRX m impeding its negative regulation towards PSI-CET, primarily coupled to the generation of the proton motive force driving the synthesis of ATP by the thylakoid ATP synthase complex. When ATP level increases (B), ATP binds to CBSX which cannot interact with TRX m, allowing down-regulation of the PSI-CET and thereby lowering ATP synthesis. Electron and proton transfers are shown by red and blue arrows, respectively. Abbreviations are : PSII, photosystem I/II; Cyt b6/f, cytochrome b6/f complex; Fd, ferredoxin; FNR, ferredoxin-NADP reductase; FTR, ferredoxin/thioredoxin reductase; TRX, thioredoxin; CBSX, Cystathionine- $\beta$ -synthase containing domain protein; PQ, plastoquinone; PQH<sub>2</sub>, plastoquinol; PC, plastocyanin; PSI-CET, cyclic electron transport around PSI.

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