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

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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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27 supervised the experiments; F. B., S. D., D. G. and M. P. T. performed experiments;. K. B.,  
28 A. K-L. and E. I-B performed experiments and analyzed the data; K. B., C. L., A. K-L. and E.  
29 I-B. wrote the manuscript.

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

34 **ABSTRACT**

35 Cystathionine- $\beta$ -synthase (CBS) domains are found in proteins of all living organisms and  
36 have been proposed to play a role as energy sensors regulating protein activities through their  
37 adenosyl ligand binding capacity. In plants, members of the CBSX protein family carry a  
38 stand-alone pair of CBS domains. In *Arabidopsis thaliana*, CBSX1 and CBSX2  
39 are targeted to plastids where they have been proposed to regulate thioredoxins (TRX). TRX  
40 are ubiquitous cysteine thiol oxido-reductases involved in the redox-based regulation of  
41 numerous enzymatic activities as well as in the regeneration of thiol-dependent peroxidases.  
42 In *Arabidopsis*, 10 TRX isoforms have been identified in plastids and divided into five sub-  
43 types. Here, we show that CBSX2 specifically inhibits the activities of m-type TRXs towards  
44 two chloroplast TRX-related targets. By testing activation of NADP-malate dehydrogenase  
45 and reduction of 2-Cys peroxiredoxin, we found that TRXm1/2 inhibition by CBSX2 was  
46 alleviated in presence of AMP or ATP. We also evidenced, by pull-down assays, a direct  
47 interaction of CBSX2 with reduced TRXm1 and m2 that was abolished in presence of  
48 adenosyl ligands. In addition, we report that, in comparison to wild-type plants, the  
49 *Arabidopsis* T-DNA double mutant *cbx1 cbx2* exhibits growth and chlorophyll  
50 accumulation defects in cold conditions, suggesting a function of plastidial CBSX proteins in  
51 plant stress adaptation. Altogether, our results show an energy sensing regulation of plastid  
52 TRX m activities by CBSX, possibly allowing a feedback regulation of ATP homeostasis via  
53 activation of cyclic electron flow in the chloroplast, to maintain a high energy level for  
54 optimal growth.

## 55 INTRODUCTION

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

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

71 While the number of functions attributed to TRX in plastids is very large (Montrichard et al.,  
72 2009), knowledge about their regulation is limited. In plastids, TRXs redox state mainly  
73 depends on the balance between the “electron pressure” light-fueled by the photosynthetic  
74 electron transport chain and the “oxidative pressure” of H<sub>2</sub>O<sub>2</sub> (Vaseghi et al., 2018; Yoshida  
75 and Hisabori, 2018). Data about additional factors directly regulating TRX redox state are  
76 very scarce. For instance, TRX f was found glutathionylated and consequently losing its  
77 capability to regulate its targets (Michelet et al., 2005). Yoo *et al.* (2011) suggested that  
78 cystathionine-β-synthase proteins are ubiquitous regulators of TRXs in plastids to control  
79 H<sub>2</sub>O<sub>2</sub> levels and regulate lignin polymerization in the anther endothecium.

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

88 Consequently, CBS domains were proposed to function as sensors of cell energy status (Scott  
89 et al., 2004; Kushwaha et al., 2009). Associated to several different functional domains, in a  
90 wide range of functionally different CDCP proteins, CBS domains act generally as regulatory  
91 domains of protein activity through adenosyl ligand binding (Hardie and Hawley, 2001; Scott  
92 et al., 2004; Ignoul and Eggermont, 2005; Buey et al., 2017). For examples, the highly  
93 conserved eukaryotic energy sensor AMPK (AMP-activated protein kinase)/SNF1 (Sucrose  
94 Non-Fermenting kinase 1)/SnRK1 (SNF1 related kinase 1) is regulated by its  $\gamma$ -subunit  
95 composed of four CBS domains in tandem (Polge and Thomas, 2007; Ramon et al., 2013;  
96 Crozet et al., 2014). In mammals, AMP/ADP binding to the CBS domains of AMPK induces  
97 a conformational change that stabilizes the phosphorylated activation loop of the protein by  
98 upstream kinases (Hardie and Hawley, 2001; Ignoul and Eggermont, 2005; Xiao et al., 2011).  
99 The Arabidopsis genome encodes 34 CDCPs each containing one or two pairs of CBS  
100 domains that are generally closely associated with other functional protein domain(s). In these  
101 proteins, CBS domains are expected to regulate the molecular function of other adjacent  
102 domains. In contrast, the members of the CBSX subfamily of CDCP proteins carry a stand-  
103 alone pair of CBS domains and are thus supposed to perform their regulatory function through  
104 protein interactions (Kushwaha et al., 2009). In Arabidopsis, the six members of the CBSX  
105 subfamily were proposed to be localized in several cellular compartments. Indeed, whereas  
106 CBSX1 and CBSX2 were shown to be plastid targeted (Yoo et al., 2011; Jung et al., 2013),  
107 CBSX3 was recently shown to be localized in mitochondria (Shin et al., 2020), CBSX4 is  
108 predicted to be cytosolic and CBSX5 and CBSX6 are expected to be located in the  
109 endoplasmic reticulum (Ok et al., 2012).

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

115 To characterize CBSX1 and CBSX2 functions, Yoo *et al.* (2011) identified candidate protein  
116 interactors using yeast 2-hybrid. Among them, were several chloroplast thioredoxins (TRX)  
117 for which interactions were confirmed by *in vitro* pull-down assays and *in planta* bimolecular  
118 fluorescence complementation (BiFC) experiments (Yoo et al., 2011; Jung et al., 2013). Both  
119 CBSX1 and CBSX2 were shown to increase the activity of TRXm2, TRXf1, TRXx and  
120 TRXy1 in a standard TRX activity assay based on bovine insulin reduction. These activities  
121 were enhanced in presence of AMP (Yoo et al., 2011). Similarly, it was shown that CBSX3

122 enhances the *in vitro* activity of the mitochondrial TRXo1 and TRXo2 (Yoo et al., 2011; Shin  
123 et al., 2020). Physiological functions of plastidial CBSX proteins were investigated in  
124 transformed Arabidopsis over-expressing *CBSX1* and *CBSX2*. These plants exhibited a sterile  
125 phenotype related to anther indehiscence. A defect in endothecium secondary cell wall  
126 thickening during anther development was observed in over-expressors and linked to  
127 insufficient reactive oxygen species (ROS) accumulation (Yoo et al., 2011; Jung et al., 2013).  
128 *CBSX3* knockdown lines surprisingly presented a similar sterile phenotype related to an  
129 anther indehiscence defect (Yoo et al., 2011; Jung et al., 2013; Shin et al., 2020). Plant  
130 sterility of *CBSX1* and *CBSX2* over-expressors, on one hand, and *CBSX3* knockdown, on the  
131 other hand, is supposedly linked to improper thioredoxin activities in plastids or in  
132 mitochondria (Yoo et al., 2011; Jung et al., 2013; Shin et al., 2020).

133 Despite the previous in depth molecular characterization of the plastidial CBSX structures and  
134 the demonstration of their interaction with TRX, the functional and physiological relevance of  
135 CBSX regulatory role on TRX activities remains questioned. In this study, by evaluating  
136 plastid CBSX regulation of TRX-dependent activities *in vitro* using two physiologically-  
137 relevant targets of plastidial TRX, we show that CBSX2 specifically inhibits TRX m activities  
138 and that this effect is reversed by ATP and, to a lesser extent, by AMP. Adenylates impairing  
139 direct CBSX-TRX interaction, we propose a model in which CBSX2 specifically inhibits  
140 reduced TRX m to maintain chloroplast energy status (ATP) for optimal plant growth during  
141 acclimation to low temperature.

## 142 **RESULTS**

### 143 **Phylogenetic Analysis of AtCBSX**

144 To investigate relationships between the 6 Arabidopsis CBSX proteins, we constructed a  
145 maximum likelihood phylogenetic tree from their full-length amino acid sequences. Our  
146 analysis suggests that CBSX proteins that are localized in the same subcellular compartment  
147 are closer in the phylogenetic tree than CBSX targeted to different locations (Figure S1). In  
148 particular, the two closest Arabidopsis CBSX proteins, *CBSX1* and *CBSX2*, share 74% of  
149 amino acid sequence identity (similarity 91%). They were both shown to be targeted to the  
150 chloroplasts (Yoo et al., 2011; Jung et al., 2013). The closest protein of the plastidial CBSX  
151 group, *CBSX3*, shares 26% of amino acid sequence identity with *CBSX2* (similarity 44%)  
152 and is localized in mitochondria. *CBSX4*, (also known as AtPV42b) is homologous with  
153 AtPV42a, a  $\gamma$ -type subunit of SnRK1 containing two CBS pairs (Fang et al., 2011).

154 Accordingly, it appears to be a non canonical CBSX standing apart in our phylogenetic tree.  
155 This tree focused on the Arabidopsis CBSX subfamily is consistent with the wide  
156 phylogenetic analysis made on the 34 CDCPs of *A. thaliana* and the 59 CDCPs of rice (*Oryza*  
157 *sativa*) (Kushwaha et al., 2009).

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

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

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

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



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

189 **CBSX2 inhibits the ability of TRX m to activate NADP-MDH and AMP modulates this**  
190 **effect**

191 CBSX1 and CBSX2 proteins were previously found to activate TRX-dependent insulin  
192 reduction (Yoo et al., 2011). Here, we investigated the ability of CBSX proteins to modulate  
193 physiologically-relevant plastid TRX-dependent enzyme activities. To this aim, we produced  
194 and purified recombinant his-tagged CBSX1 and CBSX2 in a bacterial expression system.  
195 Untagged TRXs and TRX-regulated NADP-MDH were also obtained as previously described  
196 (Collin et al., 2003). All proteins were produced without their target peptide. NADP-MDH  
197 was previously shown to be efficiently activated *in vitro* by the members of the TRX m and  
198 TRX f subfamilies (Collin et al., 2003) and its regulation by TRXf1, TRXm1 and TRXm2  
199 was validated *in planta* (Thormählen et al., 2015; Thormählen et al., 2017). The plastid TRX-  
200 regulated MDH uses NADPH as a cofactor to reduce oxaloacetate (OAA) into malate. Its  
201 activation is strictly TRX dependent and requires the reduction of two constitutive regulatory  
202 disulfide bonds, and a third transient disulfide (Issakidis et al., 1994; Ruelland et al., 1997).  
203 Here, we tested the ability of five TRX isoforms (TRXm1, TRXm2, TRXm4, TRXf1 and  
204 TRXf2) to activate MDH in presence or absence of CBSX. We incubated MDH with DTE-  
205 reduced TRX, with or without addition of CBSX for 20 min prior to measuring MDH activity.  
206 We checked that activation kinetics were linear and did not reach a plateau within 20 min of  
207 incubation (Figure S3). We defined the MDH activation rate as the value of the initial activity  
208 slope calculated after activation treatment. Our results show that CBSX1 does not have any  
209 significant effect on the capacity of the five TRXs tested to activate NADP-MDH (Figure 3A-  
210 E). In contrast, CBSX2 inhibits the enzyme activation by TRXm1 or TRXm2 or TRXm4  
211 (Figure 3A-C), but not by TRXf1 or TRXf2 (Figure 3D,E). In presence of CBSX2 with  
212 TRXm1, TRXm2 or TRXm4, NADP-MDH activation was reduced to 30-60% of the  
213 maximum activation reached in absence of CBSX2 (Figure 3A-C). Because CBS domains  
214 were shown to bind several adenosyl ligands (Ignoul and Eggermont, 2005) and, specifically,  
215 CBSX2 structure was shown to be strongly modified upon AMP binding (Jeong et al., 2013a),  
216 we tested the effect of this adenylate on the TRX limitation exerted by CBSX2. While TRX-  
217 dependent MDH activation was not significantly affected by AMP (Figures 3 and S4), we  
218 found that AMP limited the inhibitory effect of CBSX2 on TRX m (Figure 3A-C). Indeed,  
219 TRX m-dependent MDH activation could reach 65-80% of its maximum in presence of

220 CBSX2 and AMP at a concentration of 1 mM, a concentration commonly used  
221 experimentally to test regulation of CDCPs by adenylate ligands (Yoo et al., 2011; Jung et al.,  
222 2013; Buey et al., 2017).

### 223 **CBSX2 directly regulates TRX activities**

224 Our results showed that CBSX2 selectively inhibits TRXs m but not TRXs f in their  
225 capacities to activate NADP-MDH. They strongly suggested an inhibitory action of CBSX2  
226 on TRX instead of a direct inhibition of MDH activity. To confirm these results, we took  
227 advantage of a mutant form of NADP-MDH, the DMC (Double Mutation C-terminus)-MDH,  
228 in which the two cysteines that form the C-terminal regulatory disulfide bond are substituted  
229 by serines. The DMC-MDH has a basal enzyme activity without any activating treatment, but  
230 reduced TRX is required to reach full activity (Issakidis et al., 1994). Without treatment, or in  
231 presence of DTE alone, DMC-MDH exhibits *ca.* 25% of the activity reached after a 20 min  
232 activation treatment in presence of TRXm1 (Figure 4). When DMC-MDH was incubated with  
233 CBSX2 and DTE, its activity remained unchanged (Figure 4) confirming that CBSX2 does  
234 not directly inhibit DMC-MDH activity. We also checked that AMP had no direct effect on  
235 DMC-MDH activity (Figure S5). By contrast, DMC-MDH activation by TRXm1 or TRXm2,  
236 like for the wild-type enzyme, was strongly impaired in presence of CBSX2, and AMP  
237 limited this effect (Figure 4). Taken together our results strongly support a specific role  
238 played by CBSX2 as a negative regulator of TRXs m, with AMP acting as a modulator.

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

240 Noticeably, we found that AMP alleviates the CBSX2 effect on TRXs m only partially in our  
241 experimental conditions. The AMP concentration (1 mM) commonly used in this kind of  
242 experiments corresponds to a concentration by far higher than reported AMP concentrations  
243 in the chloroplast that do not exceed 1  $\mu$ M (Stitt et al., 1982). Thus, we also tested AMP at  
244 lower concentrations, as well as other adenylate nucleotides, *e.g.* ADP and ATP prevailing in  
245 the chloroplast (Figure 5). At 1 mM, we found that all adenylates could suppress the  
246 inhibitory effect of CBSX2 (Figure 5), while they had no significant effect on TRXm1-  
247 dependent activation of NADP-MDH (without CBSX) (Figure S4). At 1 mM, a physiological  
248 concentration for ATP in the chloroplast, ATP could totally inhibit the effect of CBSX2  
249 (Figure S6). We also tested MgATP, since *in vivo* 95-99 % of ATP is predicted to be  
250 complexed with Mg<sup>2+</sup>, and we also found its strong effect on TRX m. Interestingly, by testing  
251 lower concentrations of adenylate nucleotides, we could evidence that they have differential  
252 effects on CBSX2 (Figure 5). Indeed, at 10  $\mu$ M, ATP, but neither AMP nor ADP, had an

253 effect, and at 50  $\mu$ M, AMP and ATP, but not ADP, restricted CBSX2 inhibition. At 1 mM,  
254 ATP was significantly more efficient than ADP to limit CBSX2 inhibition. Thus, our data  
255 suggest that AMP may not exert any effect on CBSX2 at physiologically relevant  
256 concentrations. They also suggested that ADP may be much less efficient than ATP in  
257 limiting CBSX2 effect on TRX, with estimated concentrations of ADP and ATP in the  
258 chloroplast ranging from 0.5 to 0.8 mM and 0.8 to 1.2 mM, respectively (Stitt et al., 1982;  
259 Voon et al., 2018). We also tested the effect of ATP together with ADP on the time course of  
260 NADP-MDH activation in presence of CBSX2, at adenylate concentrations and ratios  
261 corresponding to light and dark conditions prevailing in the chloroplast (Usuda, 1988) (Figure  
262 S7). Again, CBSX2 effect was attenuated in presence of adenylates and, clearly, the effect  
263 was more pronounced in the light mimicking condition compared to the dark mimicking  
264 condition.

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

266 TRXs can regulate the activity of their targets (like NADP-MDH) and they can also provide  
267 electrons to thiol-dependent peroxidases named peroxiredoxins (PRX). It was previously  
268 shown that plastidial TRXs can reduce 2-Cys PRX *in vitro* with various efficiencies (Collin et  
269 al., 2003; Collin et al., 2004; Bohrer et al., 2012). To extend CBSX function towards TRXs ,  
270 we also tested other TRX isoforms that do not regulate NADP-MDH, *e.g.* TRXy1 and TRXx,  
271 for their capacity to serve as reducing substrate for 2-Cys PRX (obtained as described in  
272 Collin et al., 2003) in presence of CBSX. We found that PRX activities measured without  
273 TRX (DTT alone) in absence and in presence of CBSX were very similar, showing that  
274 neither CBSX1 nor CBSX2 has any direct effect on 2-Cys PRX reduction of tBOOH (an  
275 alkyl-hydroperoxide) (Figure 6A). When combining PRX, TRX and CBSX proteins, we  
276 found that the addition of CBSX1 had no effect on the peroxidase activity, and that TRXf1,  
277 TRXy1 or TRXx were not affected by CBSX2 (Figure 6B-D). Similarly to its effect when  
278 testing NADP-MDH activation, CBSX2 inhibited TRXm1-dependent reduction of the 2-Cys  
279 PRX (Figure 6E). We also tested NTRC, a physiological substrate for this target, and found  
280 that it was not significantly affected by CBSX2 (Figure 6F). As for NADP-MDH activation,  
281 the addition of AMP restricted the inhibitory effect of CBSX2 on TRXm1 (Figure 7). These  
282 results show that CBSX2 is able to specifically inhibit TRX m in serving as reducing  
283 substrate for 2-Cys PRX and that, like in NADP-MDH activation, an adenylate nucleotide can  
284 prevent this effect.

285 **Direct interaction between CBSX2 and TRXm1 proteins is modulated by adenosyl**  
286 **ligands**

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

303 **The double mutant *cbsx1 cbsx2* is smaller and exhibits chlorophyll accumulation defects**  
304 **at low temperature**

305 To investigate the role of CBSX in connection with TRX related functions *in planta*, we  
306 characterized *cbsx1* and *cbsx2* Arabidopsis T-DNA insertion mutants. These mutants were  
307 previously described in Yoo *et al.* (2011) and Jung *et al.* (2013), respectively, and did not  
308 show any macroscopic phenotype at the rosette stage. Because we suspected functional  
309 redundancy between the two CBSX plastid isoforms and found that they can form heteromers  
310 *in vivo* (Figure 2), we crossed the corresponding single mutants to obtain the *cbsx1 cbsx2*  
311 double mutant. In standard long day growth conditions, single and double mutants showed no  
312 obvious phenotypes compared to wild-type (WT) plants (Figure 9A), as previously reported  
313 under short day or continuous light (Yoo et al 2011; Jung et al 2013; Murai et al. 2021).  
314 Because *CBSX1* and *CBSX2* genes were reported to exhibit an increased level of expression at  
315 low temperature (ePlant database; (Waese et al., 2017)), the double mutant was germinated  
316 and grown at 12°C. In this condition, the *cbsx1 cbsx2* double mutant rosettes were slightly  
317 smaller and pale green in comparison with the WT (Col-0 ecotype) or single mutant plants

318 (Figure 9B). These differences were confirmed by comparing rosette fresh-weights and  
319 chlorophyll contents of the double mutant, which were significantly decreased compared to  
320 single mutants and WT plants (Figure 9E,F). These results suggest that both CBSX1 and  
321 CBSX2 are required for normal growth and chloroplast functions at low temperature.

### 322 **The double mutant *cbsx1 cbsx2* shows altered P700 oxidation and reduction kinetics**

323 Since TRXs were proposed as specific regulators of the photosynthetic electron transport  
324 (PET), we investigated whether PET was altered in *cbsx1 cbsx2*. We followed oxidation and  
325 re-reduction of P700, the primary electron donor of photosystem I, by absorption  
326 spectroscopy in the near infrared, in this double mutant, compared to WT and to TRX mutants  
327 (double mutant *trxm1 trxm2* and single mutant *trxm4*). As shown in figure 10A, upon onset of  
328 actinic light, the first fast transient oxidation level of P700 was higher in *cbsx1 cbsx2* than in  
329 WT and *trxm1 trxm2*. Then a drop of the signal below the baseline, showing ferredoxin  
330 reduction, was observed in all genotypes. This drop was followed by a multiphasic increase of  
331 the signal until a steady state level of P700<sup>+</sup> was reached. It is striking that in *cbsx1 cbsx2* this  
332 steady state level was reached much faster than in the other genotypes. Illumination with far-  
333 red light, exciting preferentially photosystem I, led to the same oxidation level in all  
334 genotypes, with *trxm1 trxm2* reaching the maximum level of P700<sup>+</sup> slower than WT and  
335 *cbsx1 cbsx2*. Then far-red light was turned off and a saturating flash was given to achieve the  
336 same maximum P700 oxidation level in all genotypes. We observed clear differences in the  
337 oxidation kinetics between genotypes, showing that the pool of electron donors to  
338 photosystem I available in *cbsx1 cbsx2* is smaller than in WT and *trxm1 trxm2*. This may be  
339 interpreted as a sign of lower cyclic electron flow in *cbsx1 cbsx2*.

340 Next, we measured the rates of P700<sup>+</sup> re-reduction after illumination of dark-adapted leaves  
341 with far-red light. The re-reduction of P700<sup>+</sup> after 1 min far-red illumination was much slower  
342 in *cbsx1 cbsx2* than in WT, while in TRX mutants *trxm1 trxm2* and *trxm4* it was faster than in  
343 WT as previously found in the same mutant lines (Thormählen et al., 2017; Courteille et al.,  
344 2013) (Figure 10B and S8). This indicates a lower reduction state of the plastoquinone pool  
345 after far-red illumination in *cbsx1 cbsx2* and can be interpreted as a lower cyclic flow in *cbsx1*  
346 *cbsx2* compared with the other genotypes.

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

350 **DISCUSSION**

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

364

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

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

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

393  
394 *In planta*, by measuring leaf NADP-MDH activity (extractable and maximal) and 2-Cys PRX  
395 redox state (monomer/dimer status and over-oxidation), we found no clear difference in *cbx*  
396 mutant leaves compared to wild-type plants cultivated at control or at low temperature (Figure  
397 S9). This result suggests that in our growth conditions these two targets might be principally  
398 regulated by TRX isoforms other than TRXs m, or NTRC, as already reported *in vitro* and *in*  
399 *vivo* (Collin et al., 2003; Pulido et al., 2010; Yoshida et al., 2015; Pérez-Ruiz et al., 2017).  
400 Recent genetic studies have allowed investigating specifically the roles of TRXs m *in vivo*  
401 and point to their role in regulating the proton motive force (pmf). pmf primarily drives ATP  
402 synthesis and is a prerequisite for light-dependent activation of ATP synthase (CF1- $\gamma$   
403 reduction) by TRXs m and f (Junesch and Gräber, 1987; Sekiguchi et al., 2020). TRX m1 and  
404 m2 would be necessary for full activation of photosynthesis during the high light periods  
405 where elevated levels of reduced photosystem I (PSI) acceptors favor cyclic electron transport  
406 (CET) (Thormählen et al., 2017). By recycling electrons from ferredoxin to the thylakoid  
407 plastoquinone (PQ) pool via PQ reductases, PSI-CET allows maintaining the appropriate pH  
408 range of the thylakoid lumen contributing to the  $\Delta$ pH causal to the pmf that drives ATP  
409 synthase. Since ATP synthesis occurs without production of NADPH, PSI-CET is important  
410 to maintain an optimal ATP/NADPH ratio to meet metabolic demand and thereby optimal  
411 plant growth (Yamori and Shikanai, 2016). Notably, when the *trxm1\_m2\_m4* triple mutant  
412 was compared to the wild-type, Okegawa and Motohashi (2015) found that the ATP level was  
413 higher in the light, and the initial rate of light-dependent CF1- $\gamma$  reduction was slowed down  
414 (Sekiguchi et al., 2020). Data obtained in Arabidopsis show that both PQ reductase pathways,  
415 namely the Proton Gradient Regulation 5 (PGR5)/PGRL1 (PGR-like 1) complex and the  
416 NADH dehydrogenase-like complex (NDH), contribute to supply sufficient acceptors from  
417 PSI (Shikanai, 2016). While a predominant role is played by the PGR5/PGRL1 pathway in

418 normal growth conditions (Nakano et al., 2019), NDH is believed to protect the  
419 photosynthetic apparatus at chilling stress under low irradiance when  $\Delta\text{pH}$  is low (Yamori et  
420 al., 2011; Yamori et al., 2015). Courteille *et al.* (2013) reported that Tobacco TRX m  
421 overexpressing plants are characterized by a down regulation of PSI-CET via the NDH  
422 pathway while NDH-dependent PQ reduction was up-regulated in Arabidopsis mutant plants  
423 specifically lacking TRXm4. It was proposed that TRXs m may promote reduction of dimeric  
424 PGRL1 to its monomeric activated form (Hertle et al., 2013) and interaction of TRXm1 with  
425 PGRL1 has been shown *in planta* (Nikkanen et al., 2018). Recently, Okegawa and Motohashi,  
426 confirmed the regulation of the PGR5/PGRL1 complex by m-type TRXs and reported that  
427 TRXm4 downregulates the PGR5/ PGRL1-dependent pathway via a direct interaction with  
428 PGRL1 (2020). Here, we found that, compared to wild-type, TRX m lacking plants (*trxm1m2*  
429 double mutant and *trxm4* single mutant) exhibited a faster P700<sup>+</sup> reduction (Figure 10B and  
430 Figure S9), similarly to previously reported data (Courteille et al., 2013). On the contrary, the  
431 *cbx1 cbx2* mutant showed a slower reduction indicative of decreased oxidation of the PQ  
432 pool favorable for PSI-CET. Thus, CBSX affects P700 photo-oxidation reduction cycle in  
433 PSI. By avoiding accumulation of electrons in the PQ pool, CBSX would allow maintaining a  
434 pH range in the luminal space of the thylakoid membrane appropriate for ATP synthesis  
435 (Miyake, 2020).

436 At low temperature, transcriptomics databases indicate a higher expression level of *CBSX1*  
437 and *CBSX2* genes (Waese et al., 2017) while transcripts abundance for TRXm1, TRXm2 and  
438 TRXm4 are decreased (Dreyer and Dietz, 2018). When we investigated the *cbx1 cbx2*  
439 mutants growth at low temperature (12°C), and under a weak illumination (60  $\mu\text{mol}$   
440  $\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) to avoid photoinhibition, we found that the double mutant exhibited growth  
441 and chlorophyll accumulation defects in these conditions. These phenotypes may be caused  
442 by a suppression of PSI-CET. Noticeably, PSI-CET is enhanced in pea leaves by chilling  
443 (Cornic et al., 2000), as in rice, where NDH-dependent cyclic electron flow plays a  
444 substantial role for photosynthesis and plant growth at low temperature (Yamori et al., 2011).  
445 Moreover, both pathways of PSI-CET protect tomato leaves against photoinhibition when  
446 exposed to chilling stress (Wang et al., 2020).

447 Notably, impaired growth and decreased chlorophyll content were found in Tobacco plants  
448 over-expressing TRX m (Rey et al., 2013). Da *et al.* (2017) reported a high pmf in TRX m-  
449 deficient Arabidopsis plants and Thormälen *et al.* (2017) found that in *trx m1m2* mutants PSI  
450 yield was enhanced and accompanied by an increased oxidation of the PQ pool, indicative of  
451 PSI-CET stimulation. Thus, in our chilling growth conditions (low temperature and low light



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

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

465 Overall, present findings reveal an ATP sensing mechanism involving cystathionine- $\beta$ -  
466 synthase domain protein and thioredoxin possibly acting for optimal ATP homeostasis in the  
467 chloroplast.

468

469

## 470 MATERIALS AND METHODS

### 471 Plants Materials

472 *Arabidopsis (Arabidopsis thaliana)* T-DNA insertion mutants *cbsx1* (Gabi\_050D12), *cbsx2*  
473 (Salk\_136934), *trxm1 trxm2* (Salk\_087118\_ and Salk\_123570), *trxm4* (Salk\_0 532538) were  
474 previously described in Yoo *et al.* (2011), Jung *et al.* (2013), Thormählen *et al.* (2017), and  
475 Courteille *et al.* (2013), respectively. They were obtained from the NASC (Nottingham  
476 *Arabidopsis* Stock Centre) (Scholl et al., 2000), and controlled by PCR genotyping (sequence  
477 of primers used can be found in Table S1). Plants are sown and grown under long days  
478 conditions (16h light/day) at 20°C or 12°C constant temperature. Light intensity was 60  $\mu\text{mol}$   
479  $\text{photons.m}^{-2}.\text{s}^{-1}$  (tubular fluorescent lamps: Sylvania Lynx-LE 840, white light) at the level of  
480 the leaves.

### 481 Phylogenetic Analysis

482 We used TAIR10 models to determine the full-length amino acid sequences of the six  
483 *Arabidopsis* CBSX: CBSX1 (AT4G36910), CBSX2 (AT4G34120), CBSX3 (AT5G10860),

484 CBSX4 (AT1G80090), CBSX5 (AT4G27460) and CBSX6 (AT1G65320). The phylogenetic  
485 tree was inferred using the Maximum Likelihood method based on the Jones-Taylor-Thornton  
486 (JTT) matrix-based model (Jones et al., 1992), bootstraps: 1000. Analysis were conducted on  
487 MEGA7 (Kumar et al., 2016).

#### 488 **Yeast 2-Hybrid**

489 *CBSX1* (72-236), *CBSX2* (72-238) and *CBSX3* (16-206) truncated ORF were amplified and  
490 cloned into the pDONR207 vector using Gateway BP Clonase enzyme (Invitrogen). Primers  
491 (ORF\_intern + ORF\_stop, sequences can be found in Table S1) were designed to amplify  
492 ORF encoding mature proteins lacking their putative target peptide (prediction by TargetP 1.1  
493 (Nielsen et al., 1997; Emanuelsson et al., 2000)). *CBSX* ORF were then subcloned into  
494 pDEST-AD-CYH2 and pDEST-DB destination vectors (Dreze et al., 2010) using Gateway  
495 LR Clonase enzyme (Invitrogen) according to manufacturer's instructions. Y8800 MAT $\alpha$  and  
496 Y8930 MAT $\alpha$  yeast strains (James et al., 1996) were transformed with AD-ORF and DB-  
497 ORF, respectively. Yeast mating and selection were performed according to the InterATOME  
498 platform pipeline as previously described in Monachello *et al.* (2019). As positive interacting  
499 protein control we used DB-mNUWA and AD-mDYW2 (Guillaumot et al., 2017).

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

501 *CBSX1* (1-236), *CBSX2* (1-238) and *CBSX3* (1-206) full length ORF were amplified and  
502 cloned into the pDONR207 vector using Gateway BP Clonase enzyme (Invitrogen). Primers  
503 (ORF\_start + ORF\_end, sequences can be found in Table S1) were designed to amplify ORF  
504 without stop codon allowing C-terminal fusion after Gateway LR transfert into pGreen-0229-  
505 RFP, pBiFC1 or pBiFC4 destination vectors (Boussardon et al., 2012). For subcellular  
506 localization, C58C1 pSOUP *Agrobacterium tumefaciens* cells were transformed with pGreen-  
507 0229-RFP vectors. For Bimolecular Fluorescence Complementation, C58C1 pMP90  
508 *Agrobacterium tumefaciens* cells were transformed with pBiFC1 and pBiFC4 vectors. For all  
509 experiments leaves of *Nicotiana benthamiana* were co-infiltrated with agrobacteria containing  
510 required vectors and the silencing inhibitor HC-Pro (Stephan et al., 2011). Leaves were  
511 observed 4 days after transformation using a Zeiss LSM880 confocal scanning microscope  
512 (Zeiss, Germany) with a Plan-APO 20x/0.8 objective (and a zoom 2x). The microscope was  
513 equipped with a 514nm laser Argon set to 5% power for YFP and chlorophyll  
514 autofluorescence excitation in BiFC experiments and a 561nm laser line set to 3% or 15%  
515 power depending on the observed CBSX protein for RFP and chlorophyll autofluorescence  
516 excitation in subcellular localization experiments. Green (YFP or RFP) and magenta

517 (chlorophyll) channels fluorescence emissions were collected using a sequential mode with  
518 variable beam splitter between 526-615 nm, 562-624 nm and 674-735 nm respectively, and  
519 with a 32-PMTs GaAsP detector. The pinhole was set to 0.72 airy unit. The detectors offset  
520 and gain were set to a small number of zero-value pixels and to optimize the dynamic range  
521 while ensuring minimal saturated pixels and were kept for all acquisitions of the same  
522 experiment. Images were acquired using ZEN Dark software (Zeiss, Germany) and processed  
523 using ZEN lite (Zeiss, Germany). Image acquisition and image analysis were performed using  
524 the IPS2 imaging facility platform.

### 525 **Protein Production and Purification**

526 *CBSX1* (72-236) and *CBSX2* (72-238) ORF in pDONR207 were subcloned into the  
527 expression vector pDEST17 (Invitrogen) using Gateway LR Clonase enzyme (Invitrogen)  
528 according to manufacturer's instructions. Protein production in *Escherichia coli* BL21 cells  
529 was induced overnight at 30°C in LB medium containing 100 µg/mL carbenicilline and 400  
530 µM IPTG. Bacteria lysis was performed in 30 mM Tris pH7.9 containing protease inhibitor  
531 (complete Mini EDTA-free Roche®) using a French press. Proteins were purified by affinity  
532 chromatography using a nickel column (GE healthcare® His-trap HP 1mL). Column washing  
533 was performed with 30 mM Tris pH 7.9 containing 20 mM imidazole. Proteins elution was  
534 performed with 30 mM Tris pH 7.9 containing 200 mM imidazole. Eluted proteins were  
535 dialysed and concentrated in 30 mM Tris pH 7.9 using Amicon columns (Sigma Amicon  
536 ultra-4 Z740191-8EA 30K). All TRX and 2-Cys PRX proteins were produced as previously  
537 described in Collin *et al.* (2003; 2004). NTRC was obtained as described in Bohrer *et al.*,  
538 2012. Fully activated wild-type MDH and mutant DMC-MDH recombinant enzymes,  
539 obtained as previously described in Issakidis *et al.* (1994), had a specific activity (u/mg) of  
540 368 +/- 42 and 386 +/- 25, respectively. 2-Cys PRX exhibited a  $K_{cat}$  of 179 +/- 17 s<sup>-1</sup> with  
541 TRXx as reducing substrate. All these values being very close to those previously reported  
542 (Issakidis *et al.*, 1994; Collin *et al.*,2003) certified that appropriate purified protein  
543 preparations were used for the present study.

### 544 **MDH activity**

545 MDH (0.24 g/L) was activated by incubation with 10 mM DTE-reduced TRX with the  
546 eventual addition of 30 µM CBSX and 1 mM AMP or ATP, as indicated, in 30 mM Tris  
547 pH7.9 at 21°C. TRX concentration was 10 µM for all TRX excepted TRX f1 used at 1 µM. 3  
548 µL aliquots of reaction medium were periodically withdrawn and added to 200 µL of 30°C  
549 pre-heated reaction medium (for wild-type MDH: 160 µM NADPH, 750 µM OAA, 30 mM

550 Tris pH7.9 ; for DMC-MDH: 160  $\mu$ M NADPH, 1 mM OAA, 30 mM Tris pH7.9).  
551 Experiments with MgATP were performed by incubation with 1 mM ATP and 10 mM  
552 MgCl<sub>2</sub>. The MDH activity was measured by following the NADPH consumption at 340 nm  
553 during 1 min in a Tecan infinite m200 PRO plate reader. Each experiment has been repeated  
554 at least twice, with three to four technical replicates in each experiment. MDH activities  
555 (initial velocities) were estimated as the slopes calculated during 20 seconds. When the  
556 coefficient of variation of the estimated slope was higher than 15%, we discarded the data.  
557 We defined TRX activity as the MDH activity after 20 minutes of activation. Statistical  
558 analysis of MDH activity dataset was performed with a linear model taking into account TRX,  
559 CBSX and adenylate effects.

#### 560 **PRX activity**

561 0.4 mM of tBOOH (tert-butyl hydroperoxide) was reduced by 15  $\mu$ M 2-Cys PRX A, 15  $\mu$ M  
562 of 0.5 mM DTE-reduced TRX, 45  $\mu$ M CBSX and 1 mM AMP in 100 mM Tris pH7.9.  
563 Unreduced tBOOH in 10  $\mu$ L aliquots was periodically quantified at 560 nm using the FOX  
564 coloration procedure described in Collin *et al.* (2004). CBSX direct effect on PRX was  
565 assayed in presence of 1 mM DTE.

#### 566 **Analysis of chlorophyll content**

567 Chlorophyll quantification was performed by acetone extraction on 50 mg fresh rosette leaf as  
568 previously described in Sumanta et al. (2014).

#### 569 **P700 absorption**

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

#### 577 **Statistical analysis**

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

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

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

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

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

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

594 For the PRX activities dataset with CBSX2 and AMP effects on TRX m1, the mean is defined  
595 as the single CBSX and AMP effects and their interaction.

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

598 For P700<sup>+</sup> datasets, the mean of half-time reduction is defined as the genotype effect.

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

#### 601 **Pull-down**

602 Purified 6His-CBSX2 protein (500  $\mu$ g) was incubated with TRX m1 (285  $\mu$ g) in 350  $\mu$ L assay  
603 buffer (1x PBS,  $\pm$  1 mM DTE  $\pm$  1 mM AMP  $\pm$  1 mM ATP) for 1h at room temperature. After  
604 adding 50  $\mu$ L of Ni-NTA agarose beads (Qiagen) and incubation for 1h at room temperature,  
605 beads were washed 3 times with 500  $\mu$ L of assay buffer. Proteins were eluted with 200 mM  
606 imidazole. Immunoblotting was performed with mouse anti-6HIS (Roche® #11922416001)  
607 and rabbit specific anti-TRXm1 (generated against AtTRXm1 recombinant protein,  
608 Genosphere Biotechnologies) as primary antibodies, and anti-mouse (Sigma A5906) and anti-  
609 rabbit (Sigma A6154) as HRP-conjugated secondary antibodies. Blot revelation was  
610 performed with the BIORAD Clarity™ Western ECL Substrate on a BIORAD® Chemidoc  
611 ISV0025.

612

#### 613 **ACCESSION NUMBERS**

614 *CBSX1* (AT4G36910), *CBSX2* (AT4G34120), *CBSX3* (AT5G10860), *CBSX4* (AT1G80090),  
615 *CBSX5* (AT4G27460), *CBSX6* (AT1G65320), *TRXM1* (AT1G03680), *TRXM2* (AT4G03520),  
616 *TRXF1* (AT3G02730), *TRXF2* (AT5G16400), *TRXX* (AT1G50320), *TRXY1* (AT1G76760),  
617 *NUWA* (AT3G49240) and *DYW2* (AT2G15690).  
618 *2-CYS PRXA* (AT5G58330), *NADP-MDH* (AT3G11630).

## 619 SUPPLEMENTAL DATA

620 Table S1: Primers used in the study.

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

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

623 Figure S3: NADP-MDH activation kinetics.

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

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

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

627 Figure S7: Effects of ATP together with ADP at concentrations and ratios mimicking light  
628 and night conditions on CBSX2 towards TRXm1-dependent NADP-MDH activation.

629 Figure S8: P700<sup>+</sup> reduction.

630 Figure S9: TRX targets in the context of CBSX mutations.

631

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645 **FIGURE LEGENDS**

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

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

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

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

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

662 NADP-MDH activation was measured after 20 min incubation at room temperature in  
663 presence of 10 µM TRX (except TRXf1 at 1 µM) and 10 mM DTE. 100% activation rate  
664 corresponds to the activation with DTE-reduced TRX (without CBSX and AMP). (A)  
665 TRXm1 (mean SA = 372 u/mg), (B) TRXm2 (mean SA = 314 u/mg), (C) TRXm4 (mean SA  
666 = 383 u/mg), (D) TRXf1 (mean SA = 432 u/mg) and (E) TRXf2 (mean SA = 340 u/mg).  
667 CBSX1/2 and AMP final concentration were 30 µM and 1 mM, respectively. Values are  
668 means ± SD (n=4). Bars with different letters correspond to significantly different values. *P* <  
669 0.05. SA: specific activity of NADP-MDH at maximal activation (100%) expressed in units  
670 (u: µmole NADPH oxidized per min) per mg of enzyme.

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

673 DMC-MDH activation was measured after 20 min incubation at room temperature in presence  
674 of DTE alone (Ø TRX), or DTE + TRXm1, or DTE + TRXm2. 100% activation rate  
675 corresponds to the maximal activation of DMC-MDH by DTE-reduced TRXm1/2 (without  
676 CBSX and AMP). Mean specific activity of DMC-MDH at maximal activation (100%) were  
677 391 u/mg and 382 u/mg, with TRXm1 and TRXm2, respectively. Values are means ± SD

678 (n=4). TRXm1/2, CBSX2 and AMP were at a final concentration of 10  $\mu$ M, 30  $\mu$ M and 1  
679 mM, respectively. Bars with different letters correspond to significantly different values.  $P <$   
680 0.05.

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

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

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

688 Time course of tBOOH reduction by 2-Cys PRXA in presence of DTT alone (black curves),  
689 DTT-reduced TRX (grey curves), DTT-reduced TRX and CBSX1 (blue curves) or CBSX2  
690 (orange curves). (A) DTT alone; (B) DTT + TRXx; (C) DTT + TRXf1; (D) DTT + TRXy1;  
691 (E) DTT + TRXm1; (F) DTT + NTRC. The values are means  $\pm$  SD (n=3).

692 **Figure 7.** Effect of 1 mM AMP on CBSX2 towards TRXm1-dependent 2-Cys PRX activity.  
693 2-Cys PRX activities were defined as the tBOOH reduction kinetic slope between 2 and 10  
694 min and were normalized to the activity in the control condition (without CBSX and AMP)  
695 considered as 100%. The values are means  $\pm$  SD (n=3). Bars with different letters correspond  
696 to significantly different values.  $P <$  0.05.

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

698 Pull-down of His-CBSX and TRXm1 in absence or presence of DTE, AMP and ATP. (A)  
699 Impact of 1 mM AMP and DTE on CBSX2-TRXm1 interaction. (B) Impact of 1 mM ATP or  
700 1 mM AMP on CBSX-TRXm1 interaction. Input corresponds to proteins initially mixed  
701 before incubation with His-affinity agarose beads. Output corresponds to proteins eluted (after  
702 washes) from the resin by 200 mM imidazole. The bracket indicates an unspecific low  
703 molecular weight signal obtained using anti-TRXm1 antibodies on CBSX preparations.

704 **Figure 9.** *cbx* mutant phenotypes.

705 (A) Representative 5-week old plants grown in long day conditions at 20°C (bar= 1cm). (B)  
706 Representative 11-week old plants grown in long day conditions at 12°C (bar= 1cm). Plant  
707 images were digitally extracted for comparison. (C) and (D) Plants grown at control  
708 temperature. (E) and (F) Plants grown at low temperature. (C) and (E) Rosette fresh weight,  
709 values are means  $\pm$  SD (n=10), \*:  $P <$  0.01. (D) and (F) Chlorophyll (Chl) a and b contents,  
710 and Chla/Chlb ratios, values are means  $\pm$  SD (n=10). \*:  $P =$  0.02.



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

713 The redox state of the PSI primary donor P700 was monitored through the changes in  
714 absorbance at 830 versus 875 nm. (A) Representative curves are shown of plants kept at  
715 growth light intensity; WT: black, *cbsx1cbsx2*: red, *trxm1trxm2*: green. Leaves were  
716 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  
717 steady state was reached. Then far-red light, FR, was switched on. To achieve the maximum  
718 oxidation state a saturating flash, SF, was given at the end of the FR illumination. (B) Half-  
719 time of P700<sup>+</sup> reduction after FR illumination of dark-adapted plants. The traces of the  
720 kinetics are shown in Figure S8. Bars with different letters correspond to significantly  
721 different values between genotypes.  $P < 0.05$ .

722 **Figure 11.** Model of TRX activity regulation by CBSX2.

723 CBSX2 homodimer (yellow disc) is unable to interact with oxidized TRX m (green circle)  
724 (A), but is able to interact with reduced TRXm and inhibits TRX m capacity to reduce its  
725 target (blue circle) (B). A conformational change of CBSX2 induced by adenosyl ligand (red  
726 circle) binding provokes the release of TRXm-CBSX2 interaction allowing TRXm to act on  
727 its target (C).

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

730 In the proposed model, when ATP level is low (A), CBSX can interact with TRX m,  
731 impeding its negative regulation of PSI-CET, which is primarily coupled to the generation of  
732 the proton motive force driving the synthesis of ATP by the thylakoid ATP synthase complex.  
733 When ATP level increases (B), ATP binds to CBSX, preventing interaction with TRX m and  
734 allowing down-regulation of the PSI-CET and thereby lowering ATP synthesis.  
735 Electron and proton transfers are shown by red and blue arrows, respectively. Abbreviations  
736 are : PSI/II, photosystem I/II; Cyt b6/f, cytochrome b6/f complex; Fd, ferredoxin; FNR,  
737 ferredoxin-NADP reductase; FTR, ferredoxin/thioredoxin reductase; TRX, thioredoxin;  
738 CBSX, cystathionine- $\beta$ -synthase domain protein; PQ, plastoquinone; PQH2, plastoquinol; PC,  
739 plastocyanin; PSI-CET, cyclic electron transport around PSI; CF0 and CF1, coupling factor 0  
740 and 1 of chloroplast ATP synthase, respectively.

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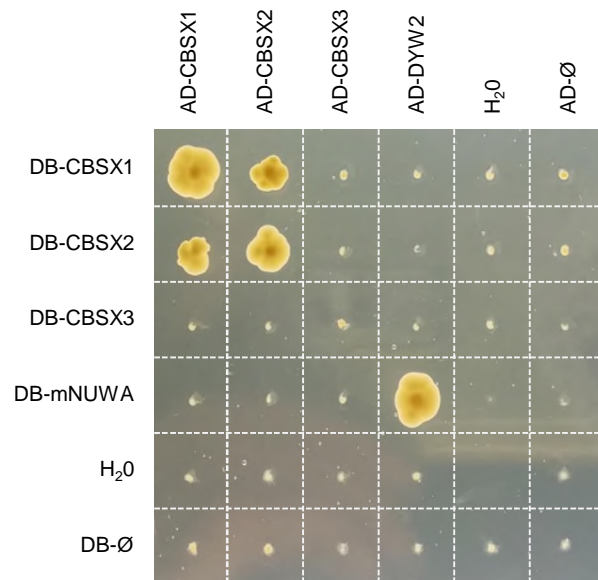
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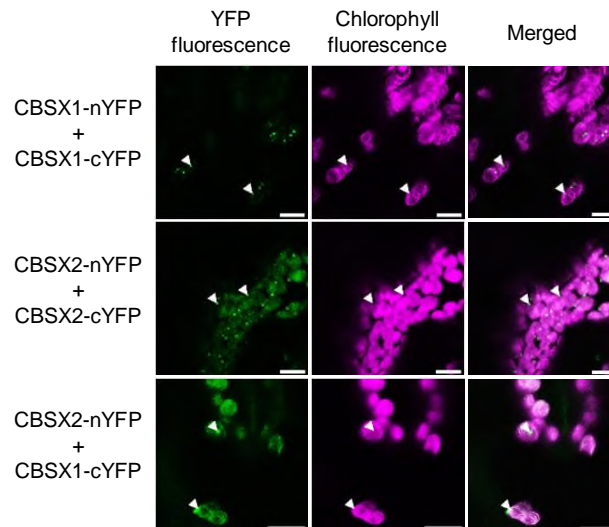
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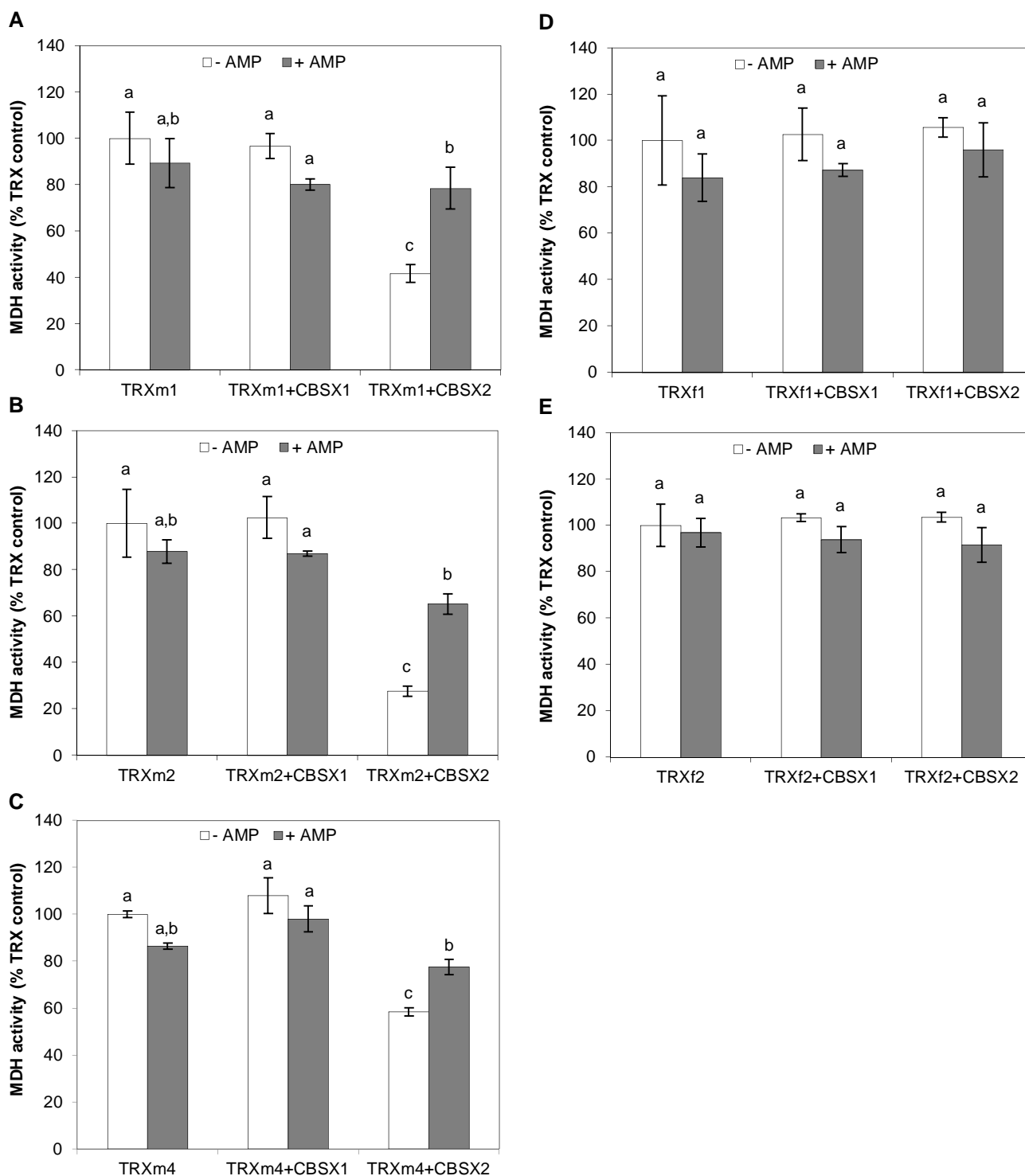
**Figure 1.** Protein interactions between Arabidopsis CBSX isoforms assayed by yeast 2-hybrid.

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.



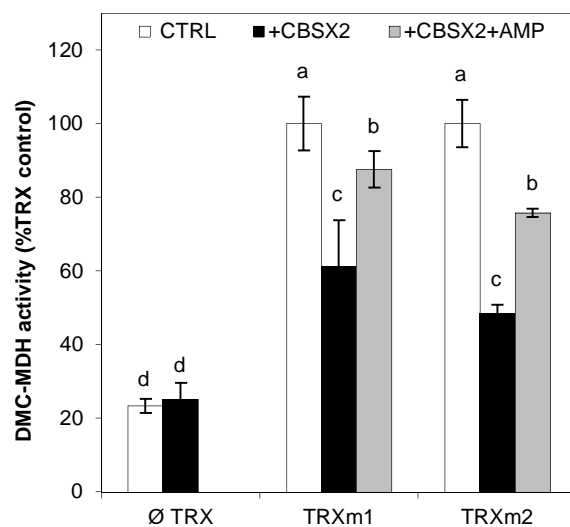
**Figure 2.** *In planta* protein interactions between CBSX isoforms assayed by BiFC.

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.



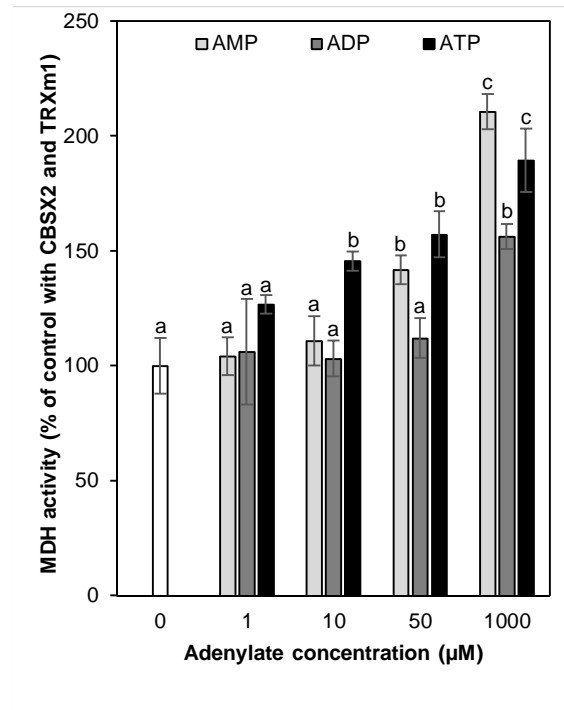
**Figure 3.** Effect of CBSX1 and 2 on TRX capacity to activate wild-type NADP-MDH, and effect of AMP.

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.



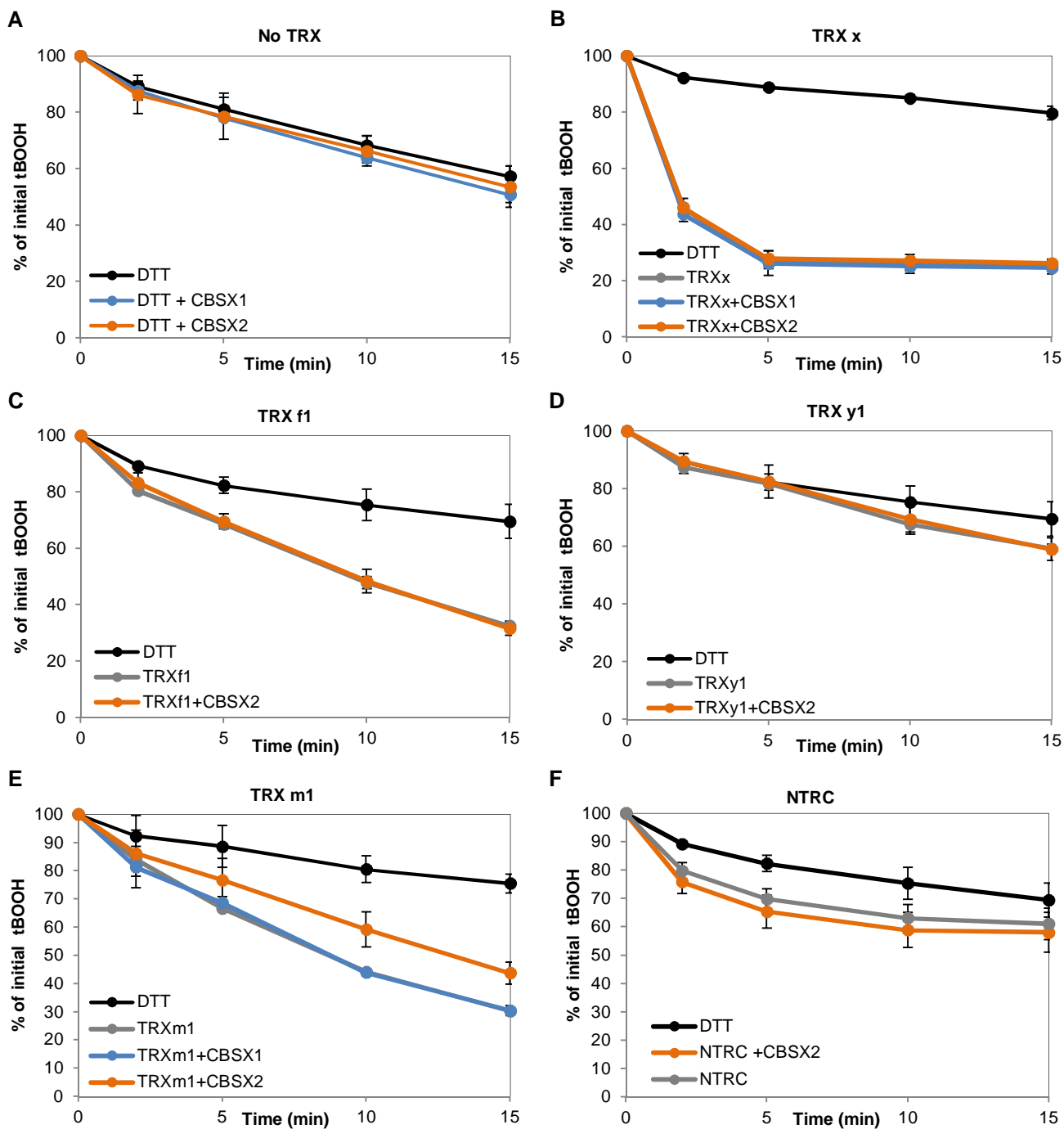
**Figure 4.** Effect of CBSX2 on mutant DMC-MDH activity and TRXm-dependent activation, and effect of AMP.

DMC-MDH activation was measured after 20 min incubation at room temperature in presence of DTE alone ( $\emptyset$  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.  $P < 0.05$ .



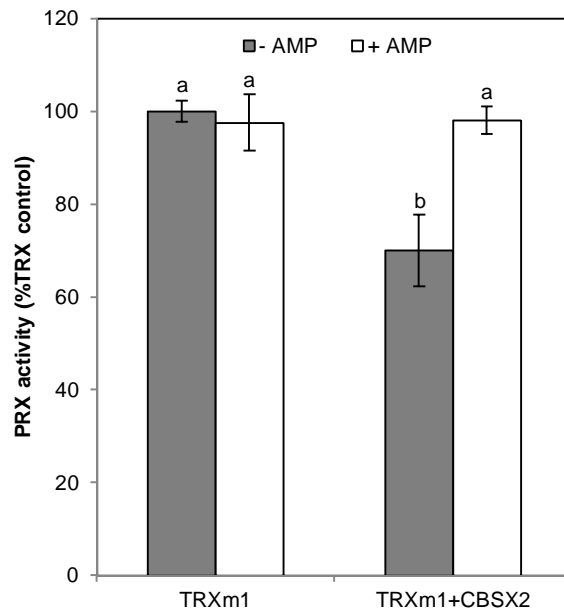
**Figure 5.** Effects of adenylytes on CBSX2 inhibition of TRXm-dependent NADP-MDH activation.

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.  $P < 0.05$ .



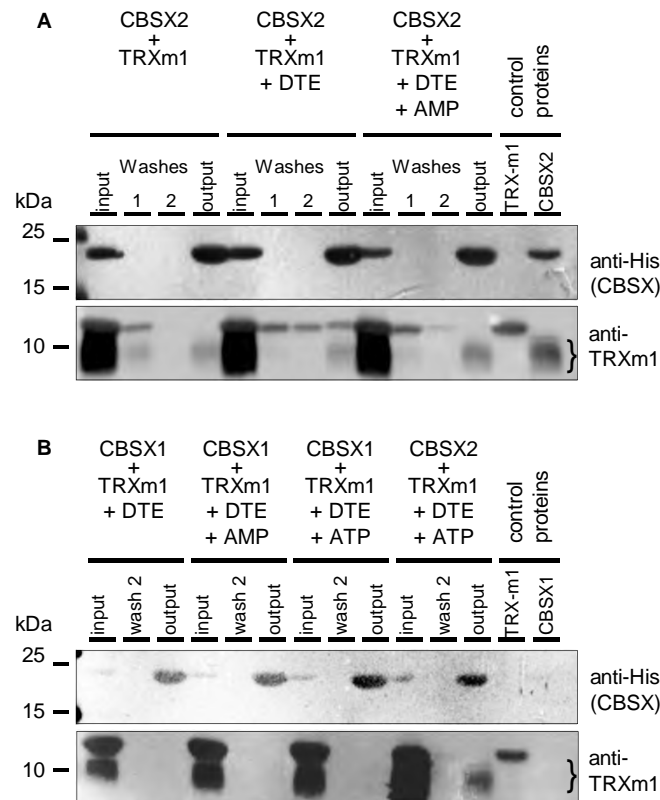
**Figure 6.** Effect of CBSX1 and 2 on 2-Cys PRX activity.

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



**Figure 7.** Effect of 1 mM AMP on CBSX2 towards TRXm1-dependent 2-Cys PRX activity.

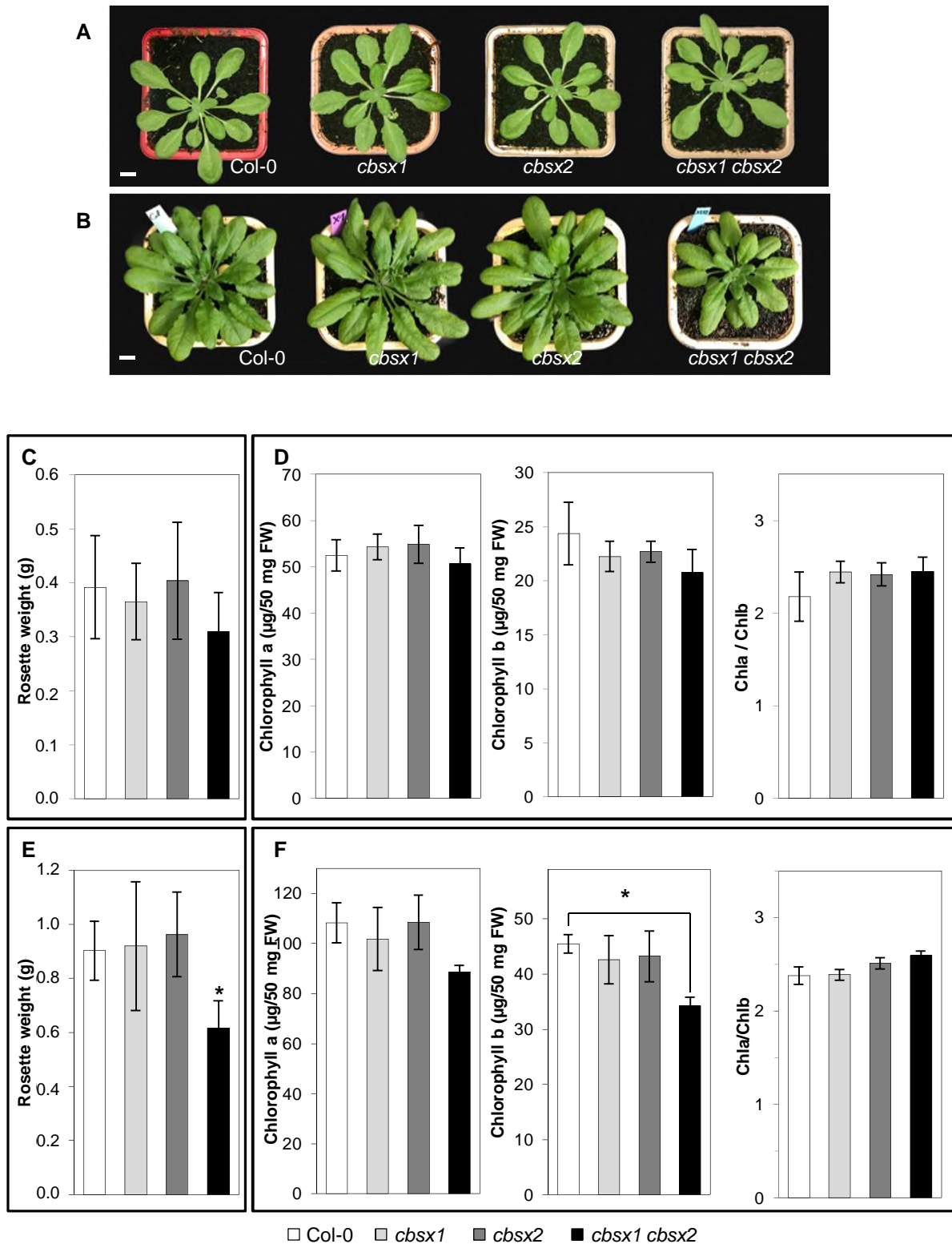
2-Cys PRX activities were defined as the tBOOH reduction kinetic slope between 2 and 10 min and were normalized to the activity in the control condition (without CBSX and AMP) considered as 100%. The values are means  $\pm$  SD (n=3). Bars with different letters correspond to significantly different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



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

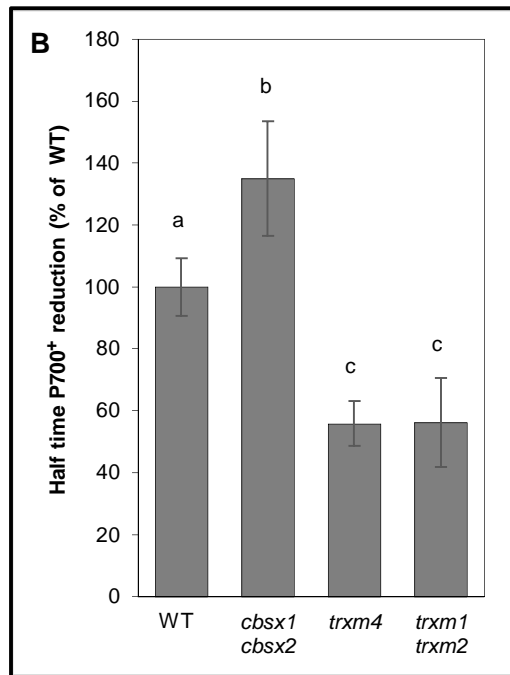
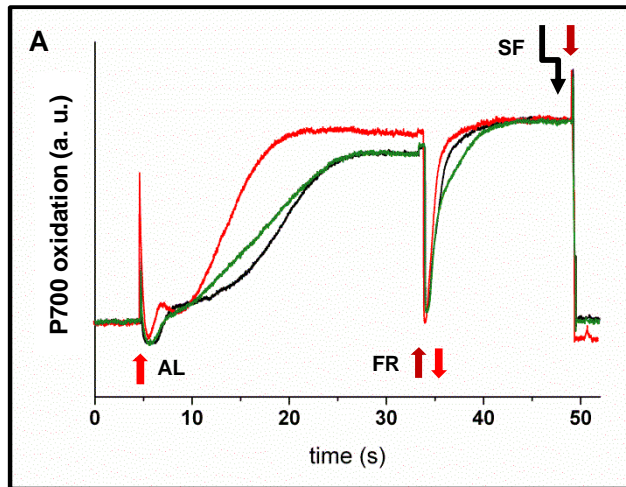
Pull-down of His-CBSX and TRXm1 in absence or presence of DTE, AMP and ATP. (A) Impact of 1 mM AMP and DTE on CBSX2-TRXm1 interaction. (B) Impact of 1 mM ATP or 1 mM AMP on CBSX-TRXm1 interaction. Input corresponds to proteins initially mixed before incubation with His-affinity agarose beads. Output 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-TRXm1 antibodies on CBSX preparations.





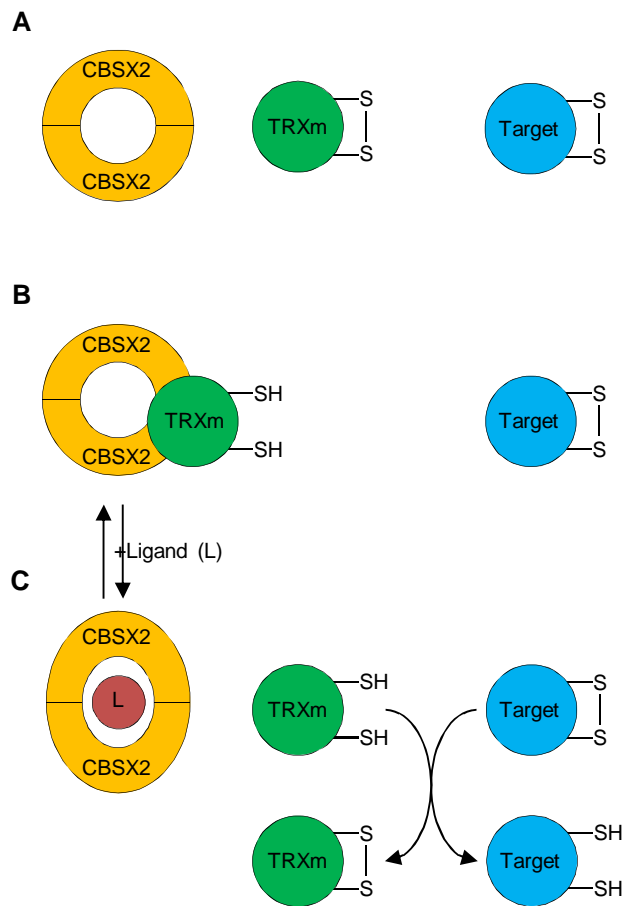
**Figure 9.** *cbsx* mutant phenotypes.

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



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

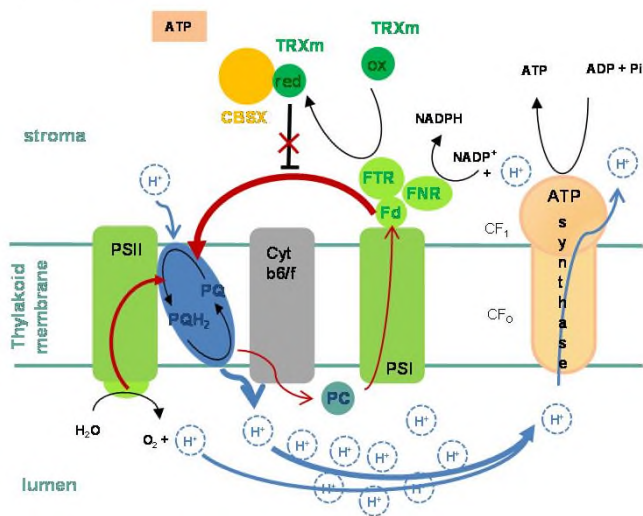
The redox state of the PSI primary donor P700 was monitored through the changes in absorbance at 830 versus 875 nm. (A) Representative curves are shown of plants kept at growth light intensity; WT: black, *cbsx1cbsx2*: red, *trxm1trxm2*: green. Leaves were illuminated with actinic red light, AL ( $I = 830 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) 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. Values are means  $\pm$  SD ( $n=4-6$ ). Bars with different letters correspond to significantly different values between genotypes.  $P < 0.05$ .



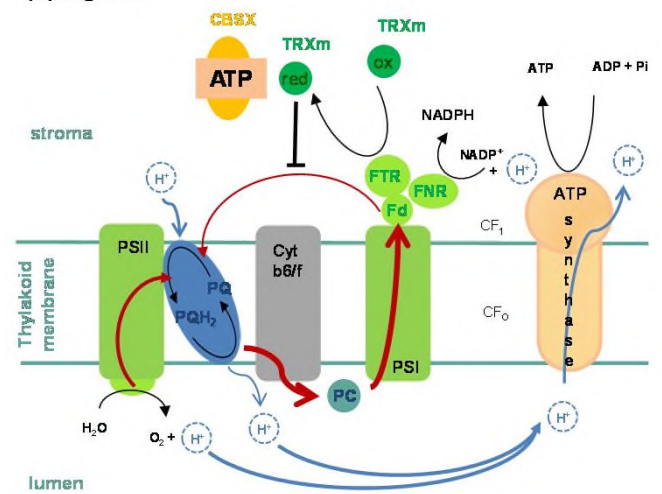
**Figure 11.** Model of TRX activity regulation by CBSX2.

CBSX2 homodimer (yellow disc) is unable to interact with oxidized TRX m (green circle) (A), but is able to interact with reduced TRXm and inhibits TRX m capacity to reduce its target (blue circle) (B). A conformational change of CBSX2 induced by adenosyl ligand (red circle) binding provokes the release of TRXm-CBSX2 interaction allowing TRXm to act on its target (C).

(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 of PSI-CET, which is 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, preventing interaction with TRX m and 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 domain protein; PQ, plastoquinone; PQH<sub>2</sub>, plastoquinol; PC, plastocyanin; PSI-CET, cyclic electron transport around PSI ; CF<sub>0</sub> and CF<sub>1</sub>, coupling factor 0 and 1 of chloroplast ATP synthase, respectively.

## **Supplemental data**

Baudry et al., Plant Physiol 2022

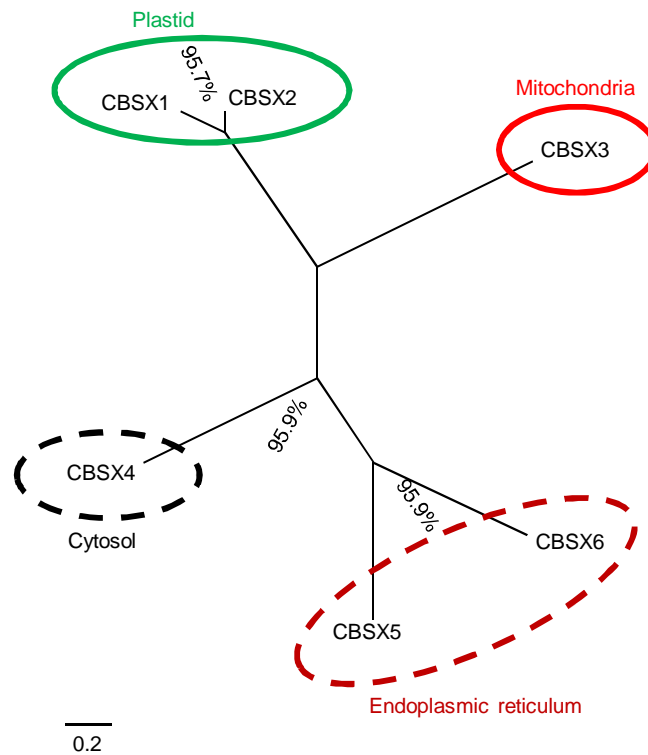
**Table S1.** Primers used in the study.

Cloning:

CBSX1\_start: GGAGATAGAACCATGGACGCCGTCCTTTACTC  
CBSX1\_intern: GGAGATAGAACCATGAGTGGAGTGACTACTGTTGG  
CBSX1\_end: TCCACCTCCGGATCCAGCATTCTATCACCAGAGC  
CBSX1\_stop: TCCACCTCCGGATCAAGCATTCTATCACCAGAGC  
CBSX2\_start: GGAGATAGAACCATGGGTTCAATCTCTTTATCC  
CBSX2\_intern: GGAGATAGAACCATGGCAAAAATGGAGGTTACACAG  
CBSX2\_end: TCCACCTCCGGATCCTGTAGAGTTCTCGGTTTCCC  
CBSX2\_stop: TCCACCTCCGGATCATGTAGAGTTCTCGGTTTCCC  
CBSX3\_start: GGAGATAGAACCATGCAAGGTGTGATTCGATCC  
CBSX3\_intern: GGAGATAGAACCATGAAAGGCTCTGTGCTGCAACA  
CBSX3\_end: TCCACCTCCGGATCCGTAACCTCCCTGAATATACGC  
CBSX3\_stop: TCCACCTCCGGATCAGTAACCTCCCTGAATATACGC

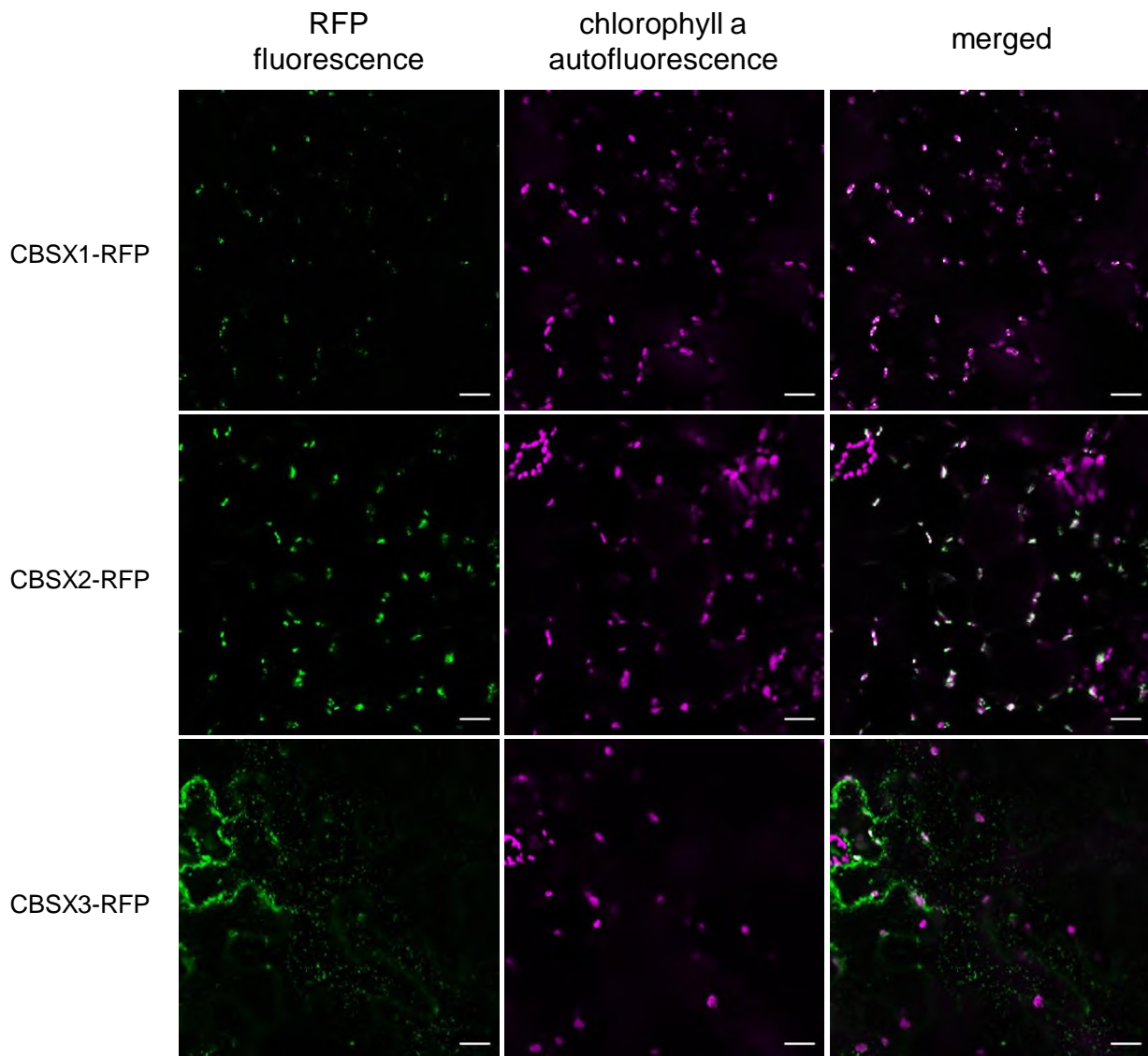
Genotyping:

SALK\_LBa1: TGGTTCACGTAGTGGGCCATCG  
Gabi\_o8474: ATAATAACGCTGCGGACATCTACATTTT  
cbsx1\_LP: CGGGATTCGATCTTAGCCTAG  
cbsx1\_RP: TTATTGTGCATGGTAATTGCC  
cbsx2\_LP: TTTCCAGTACATTGCGTCATG  
cbsx2\_RP: CAGAAGTTCCAACGCTGAAAG



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

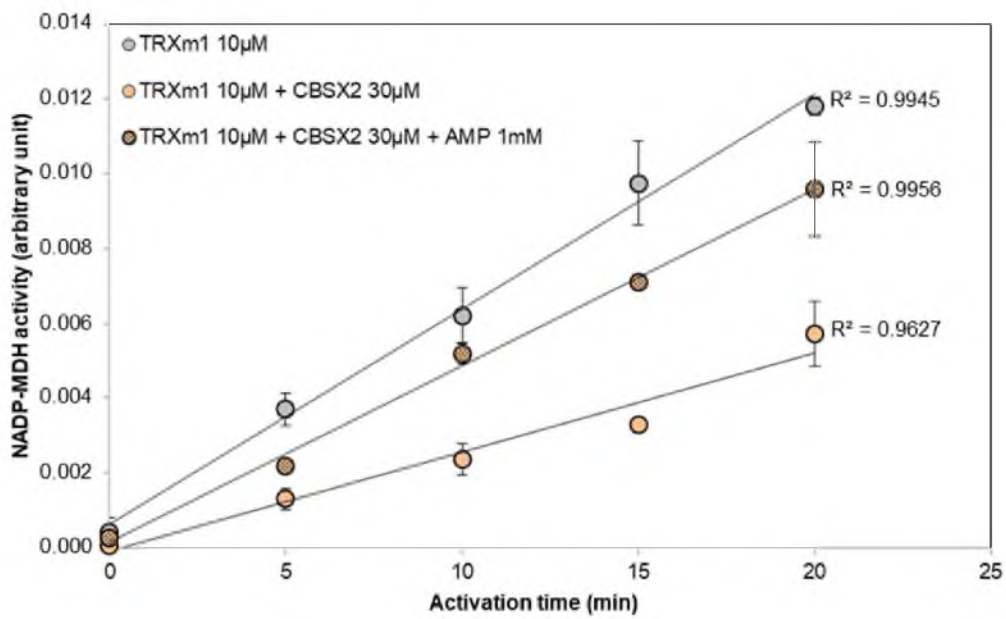
Phylogenetic tree of the 6 *A. thaliana* CBSX, obtained by Maximum Likelihood algorithm and the Jones-Taylor-Thornton (JTT) model, bootstrap: 1000 using MEGA7.0.2. The scale bar represents 0.2 substitutions per position. Dashed lines indicate the predicted location according to Ok et al (2012). Continued lines indicate experimental location according to Yoo et al (2011), Jung et al (2013) and this work.



**Figure S2.** Subcellular localization of CBSX1, CBSX2 and CBSX3.

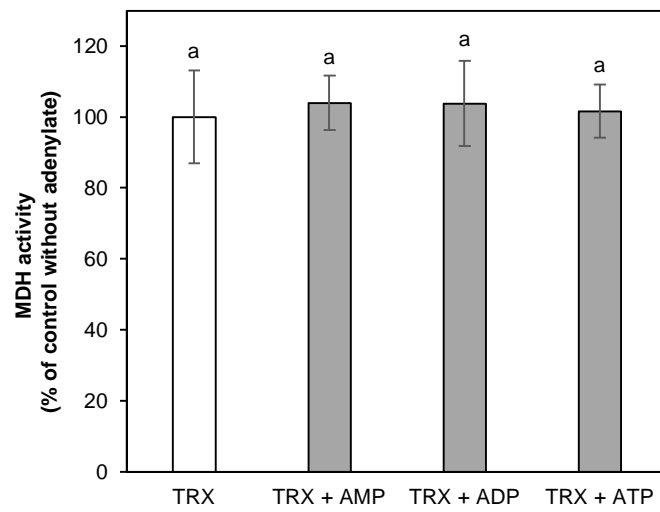
RFP signal from transiently transformed *Nicotiana benthamiana* leaves. All proteins were expressed as C-terminal fusions with RFP. Bars = 20µm.





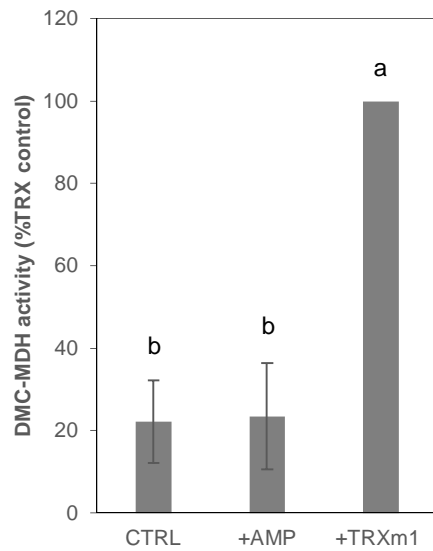
**Figure S3.** NADP-MDH activation kinetics.

Time course activation of wild-type NADP-MDH by DTE-reduced TRXm1 alone, with CBSX2, or with CBSX2 and AMP. The values are means  $\pm$  SD (n=3).



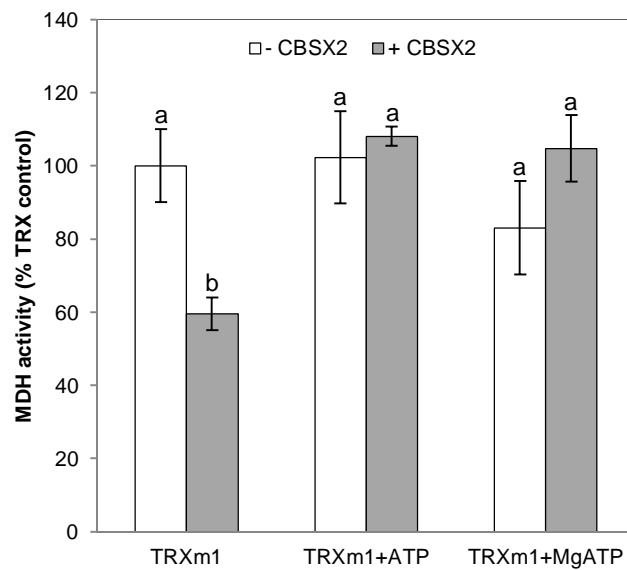
**Figure S4.** Test of direct effect of adenylylates on TRXm1-dependent NADP-MDH activation.

MDH activation by DTE-reduced TRXm1 without or with 1 mM AMP, ADP or ATP. The values are means  $\pm$  SD (n=3).  $P < 0.05$



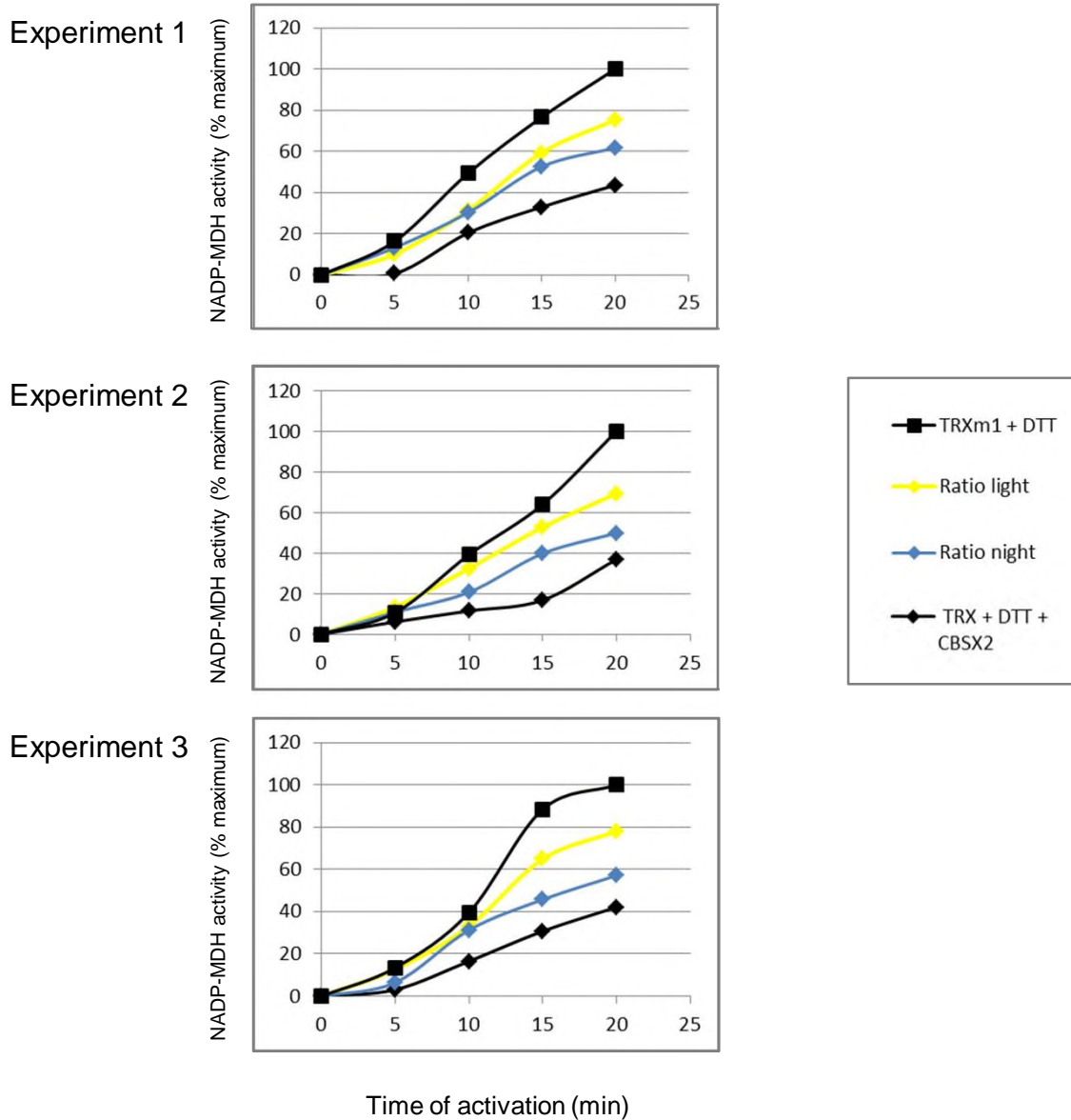
**Figure S5.** Effect of AMP on mutant DMC-MDH activity.

DMC-MDH activation was measured after 20 min incubation at room temperature in presence of DTE alone, with DTE and 1 mM AMP or with DTE and 30  $\mu$ M TRXm1. 100% activation rate corresponds to the maximal activation of DMC-MDH by DTE-reduced TRXm1. Values are means  $\pm$  SD (n=5). Bars with different letters correspond to significantly different values.  $P < 0.05$ .



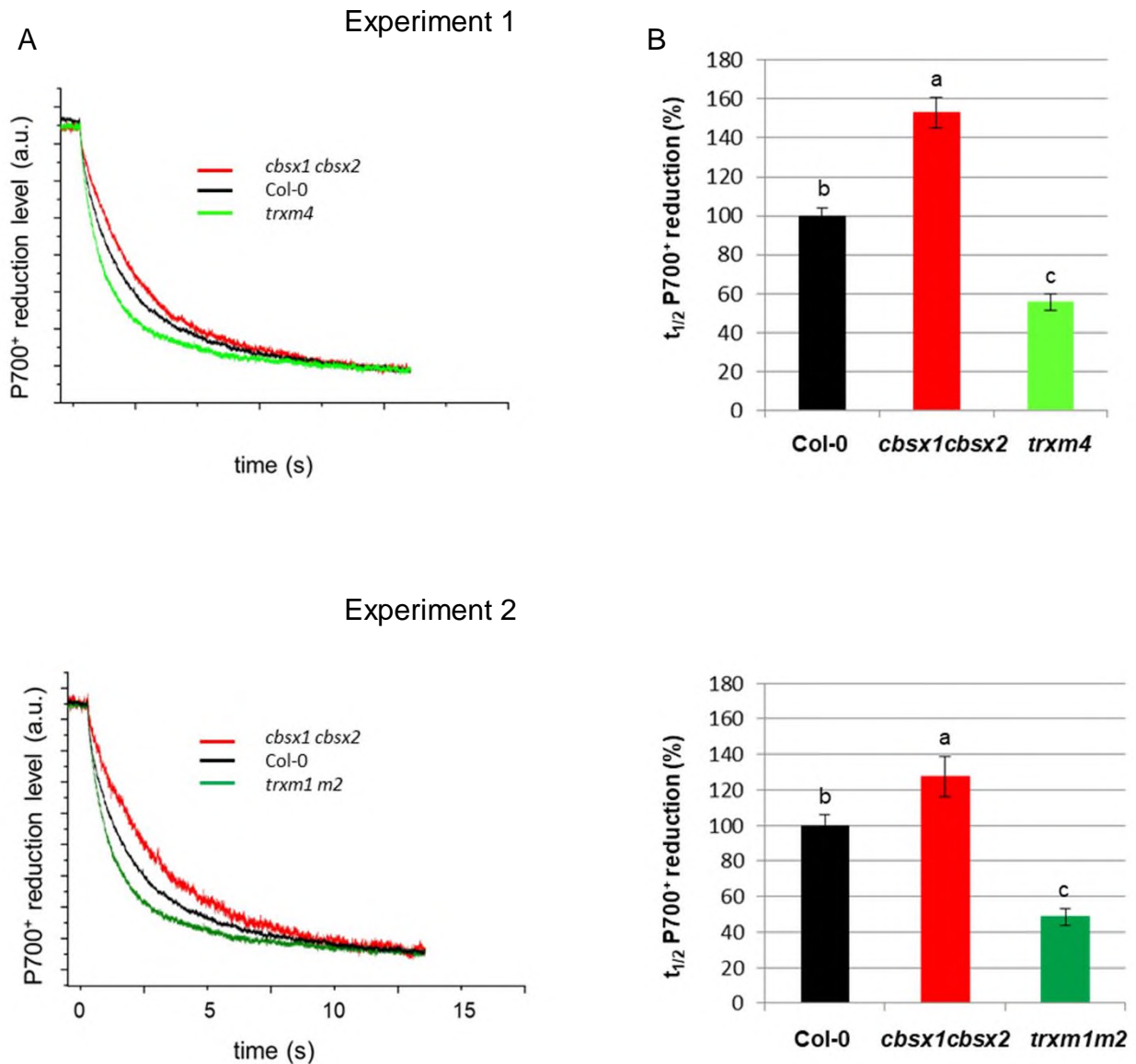
**Figure S6.** Effect of ATP on CBSX2 towards TRXm1-dependent NADP-MDH activation.

MDH activation by DTE-reduced TRX m1 in absence or in presence of CBSX2 and 1 mM ATP or MgATP. The values are means  $\pm$  SD (n=3). Bars with different letters correspond to significantly different values.  $P < 0.05$ .



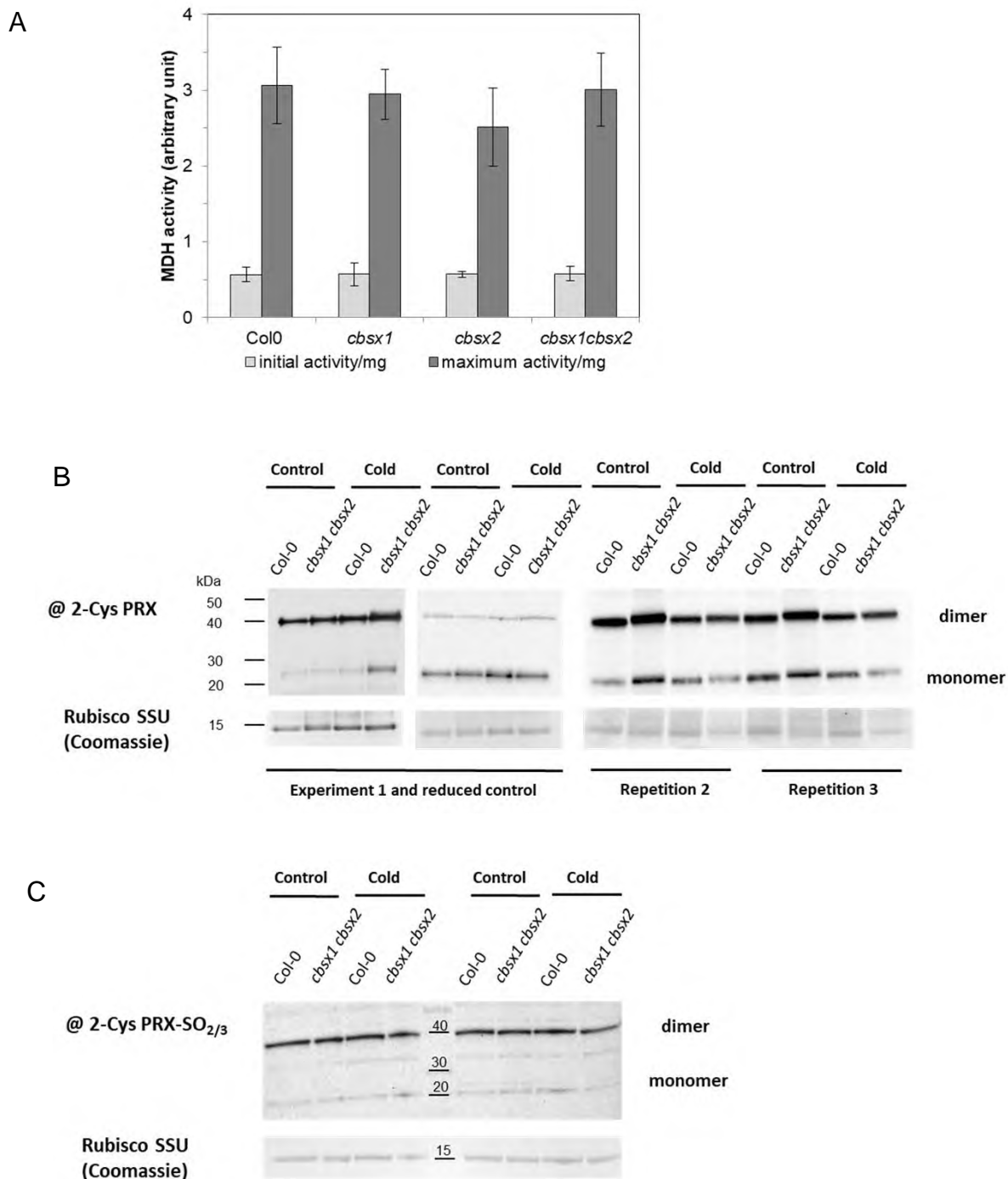
**Figure S7.** Effects of ATP together with ADP at concentrations and ratios mimicking light and night conditions on CBSX2 towards TRXm1-dependent NADP-MDH activation.

Time course of NADP-MDH activation by DTT-reduced TRX m1 in presence of CBSX2 without, and with adenylates at an ATP/ADP ratio of 2.5 (corresponding to light condition, yellow curve) or 0.3 (corresponding to night condition, blue curve). Adenylates total concentration was 2.0 and 1.3 mM in light and night mimicking conditions, respectively. Data from 3 independent experiments are shown.



**Figure S8. P700<sup>+</sup> reduction.**

P700<sup>+</sup> reduction was monitored after 1 min FR illumination of dark-adapted plants of Col-0, TRX m single and double mutants, and in *cbsx1 cbsx2*. (A) Representative curves for the different genotypes. (B) Differences in the half-times of P700<sup>+</sup> reduction normalized to Col-0. Values (in sec) were:  $1.21 \pm 0.10$ ,  $1.84 \pm 0.24$ ,  $0.67 \pm 0.09$  for Col-0, *cbsx1cbsx2* and *trxm4*, respectively in experiment 1; and  $0.83 \pm 0.10$ ,  $1.05 \pm 0.13$ ,  $0.40 \pm 0.08$  for Col-0, *cbsx1cbsx2* and *trxm1m2*, respectively in experiment 2. The values are means  $\pm$  SD (n=3-4). Bars with different letters correspond to significantly different values.  $P < 0.01$ .



**Figure S9.** TRX targets in the context of CBSX mutations.

- (A) NADP-MDH leaf extractable activity in Col-0, CBSX1/2 single and double Arabidopsis mutant plants grown in control conditions. Activities were measured without (initial activity of the enzyme) or after pretreatment (reductive activation giving the maximal activity *i.e.* the enzyme capacity) of extracts with 10 mM DTE and 10  $\mu$ M TRX m1 20 min at room temperature. The values are means  $\pm$  SD (n=3).
- (B) 2-Cys PRX oligomeric state in Col-0, and CBSX1/2 single and double Arabidopsis mutant plants grown in control or cold conditions. Total leaf protein extracts (50  $\mu$ g) were electrophoresed in non-reducing conditions and transferred onto a PVDF membrane for 2-Cys PRX immuno-detection. Reduction treatment (DTT 50 mM 30 min at RT prior SDS-PAGE) was performed as control to validate dimer and monomer corresponding to the oxidized and reduced form, respectively.
- (C) Detection of over-oxidized 2-Cys PRX (2-Cys PRX-SO<sub>2/3</sub>).