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Supplementary Information for

Oxidative regulation of chloroplast enzymes by thioredoxin and thioredoxin-like proteins in *Arabidopsis thaliana*

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SI appendix Figures. S1 to S8 and Table. S1

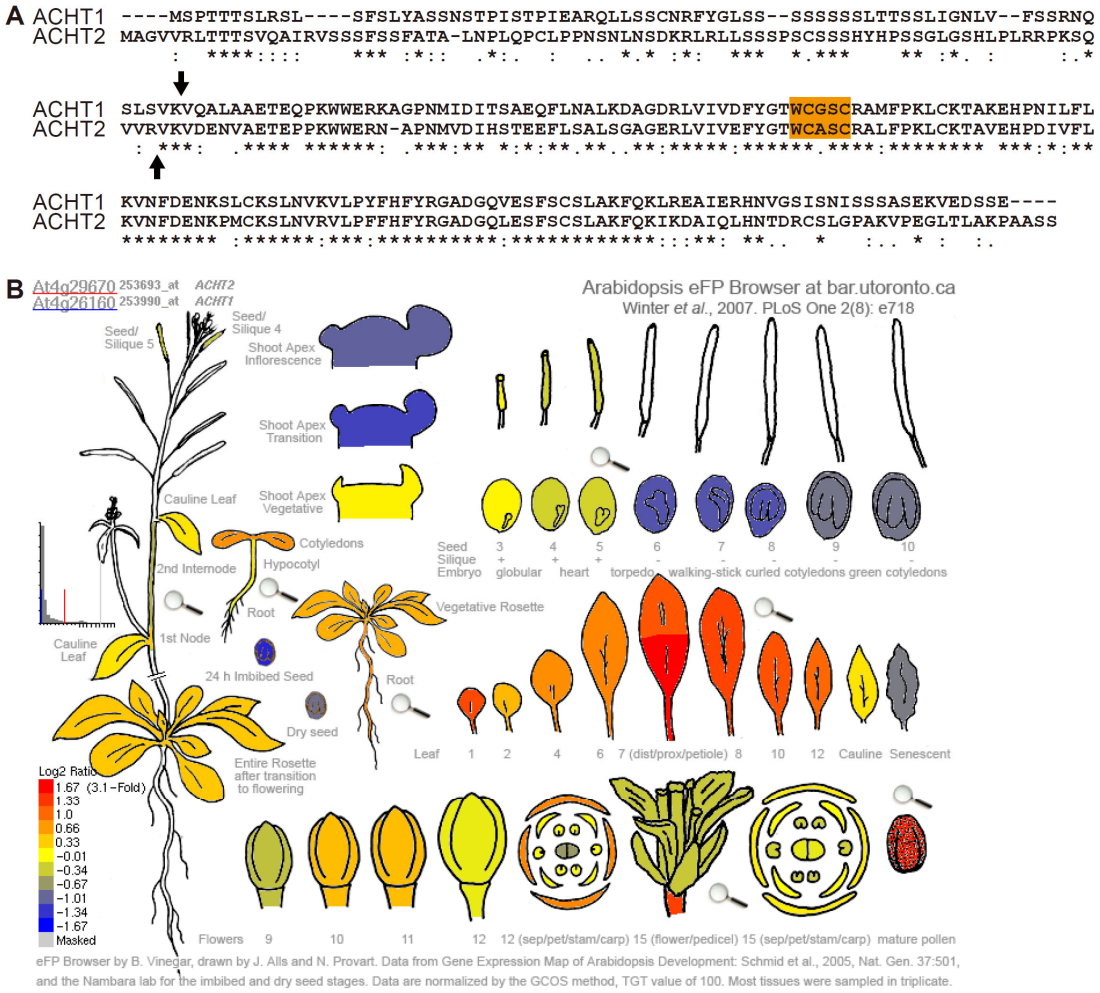

Fig. S1. Comparison of ACHT1 and ACHT2.

(A) Comparison of amino acid sequences of ACHT1 and ACHT2. Amino acid sequences are aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the consensus symbols (*, fully conserved; :, strongly conserved; and ., weakly conserved) are shown below the alignment result. The active site residues are highlighted in orange. An arrow indicates the putative cleavage site of the transit peptide of each protein (1). **(B)** Comparison of the messenger RNA (mRNA) expression patterns of *ACHT1* and *ACHT2* in each tissue. The mRNA expression pattern of *ACHT2* relative to *ACHT1* was investigated using the "Compare mode" of the Arabidopsis eFP Browser database (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

A

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TrxL2.1 MAFENLKISRHISSSTKNLLKAKKIMAALASNPQMLTRQAHGNKKEQLWRKESLNLEKKCGFCVSVYS------NEKLGR
TrxL2.2 -----MSEIVNLSSSLRSL------------NPKISP-------LVPPYRQTSSSFSRPRNFKYHSFTDKICLAAERIRA
              :.   ::*** :.*            **::              :*: * .:.:  .*    ::       *::

TrxL2.1 SHMEKEWRLKAFWSNIAQPTTLEMEPINNVEELDAVLSHARQLSQPIIIEWMASWCRKCIYLKPKLEKLAAEYNNRAKFY
TrxL2.2 VDIQK--QDGGLQELDDSPVSVELGPICGESHFDQVMEDAQKLGESVVIVWMAAWCRKCIYLKPKLEKLAAEFYPRLRFY
         .::*  :  .: .   .*.::*: ** . ..:* *:..*::*.: ::* ***:******************:  * :**

TrxL2.1 YVDVNKVPQTLVKRGNISKMPTIQLWKEDEMKEEVIGGHKGWLVIEEVRELINKFV--
TrxL2.2 HVDVNAVPYRLVSRAGVTKMPTIQLWRDGQKQAEVIGGHKAHFVVNEVREMIENDSIT
        :**** **  **.*..::********::.: : *******. :*::****:*::
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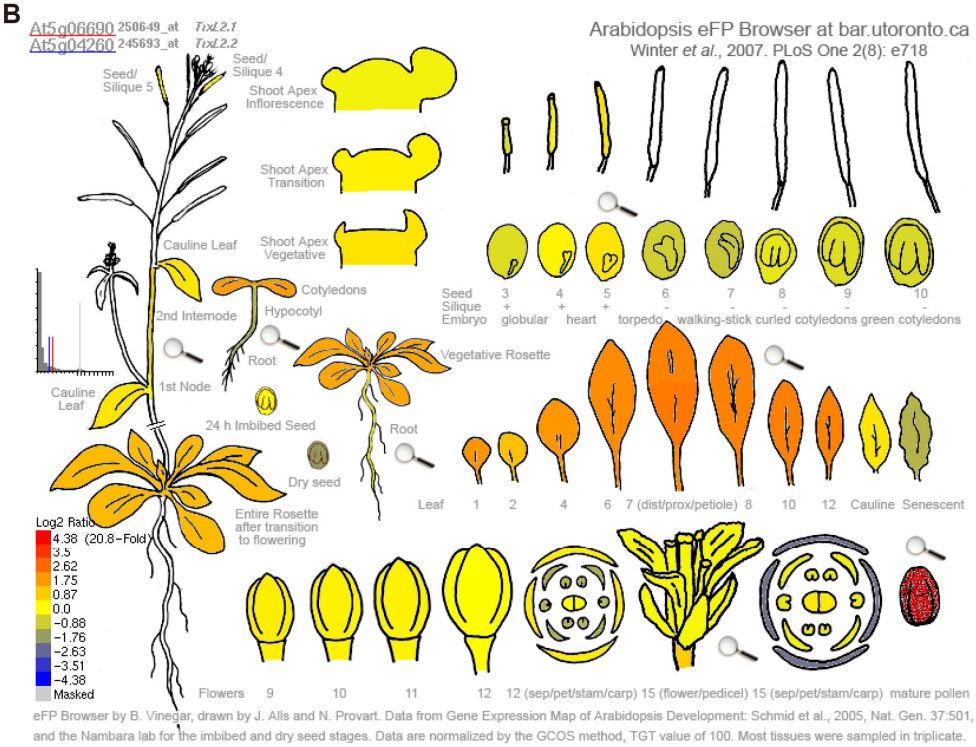
B



eFP Browser by B. Vinegar, drawn by J. Alls and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

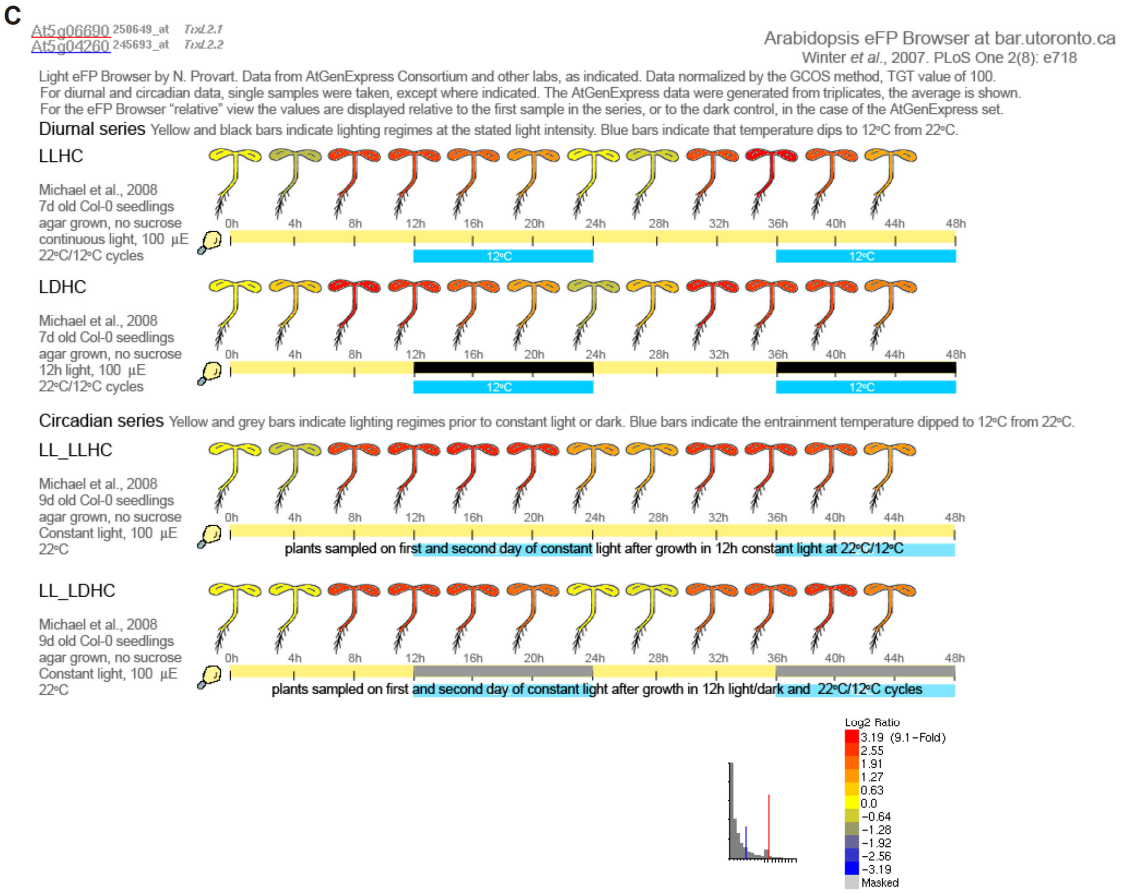


Fig. S2. Comparison of TrxL2.1 and TrxL2.2.

(A) Comparison of amino acid sequences of TrxL2.1 and TrxL2.2. Amino acid sequences are aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the consensus symbols (*, fully conserved; :, strongly conserved; and ., weakly conserved) are shown below the alignment result. The active site residues are highlighted in orange. An arrow indicates the putative cleavage site of the transit peptide of each protein (2). **(B and C)** Comparison of the mRNA expression patterns of *TrxL2.1* and *TrxL2.2*. The mRNA expression pattern of *TrxL2.1* relative to *TrxL2.2* **(B)** in each tissue or **(C)** during light/dark and/or temperature cycle was investigated using the "Compare mode" of the Arabidopsis eFP Browser database (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). **(C)** Data of diurnal series and circadian series obtained using Col-0 seedlings grown on agar without sucrose are shown.

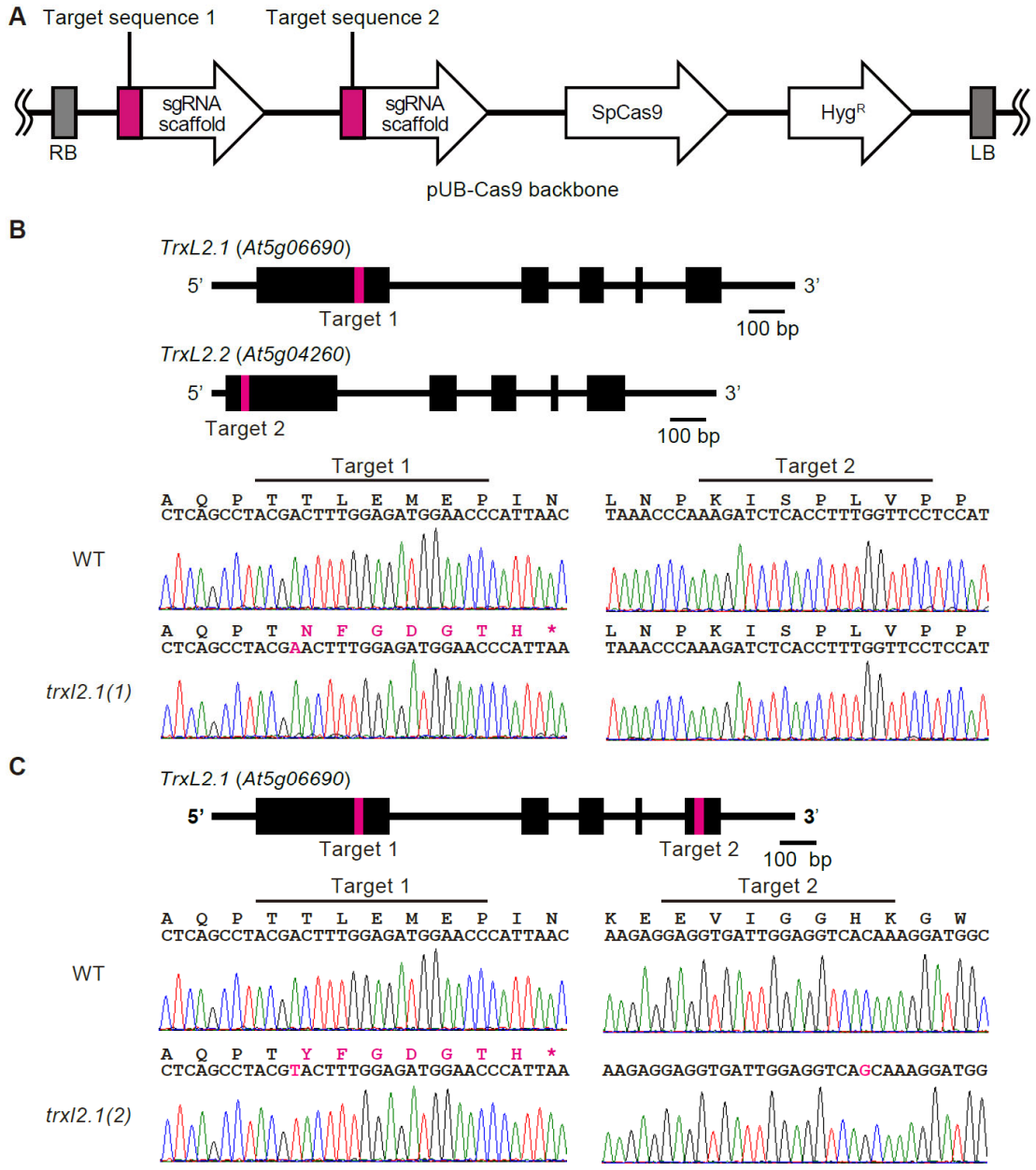


Fig. S3. DNA sequences of TrxL2.1-deficient plant lines.

(A) Schematic representation of the construct for CRISPR/Cas9-mediated genome editing. The pUB-Cas9 vector (3) was used as a backbone. Abbreviations: RB, right border; LB, left border; sgRNA, single guide RNA; SpCas9, *Streptococcus pyogenes*-derived Cas9; Hyg^R, hygromycin-resistance gene cassette. **(B and C)** DNA sequences around Cas9-targeted sites in the genome of the **(B)** *trxl2.1(1)* and **(C)** *trxl2.1(2)* lines. Cas9-targeted sites are indicated in magenta in each gene structure. Mutated sites are shown in magenta in DNA and protein sequences.

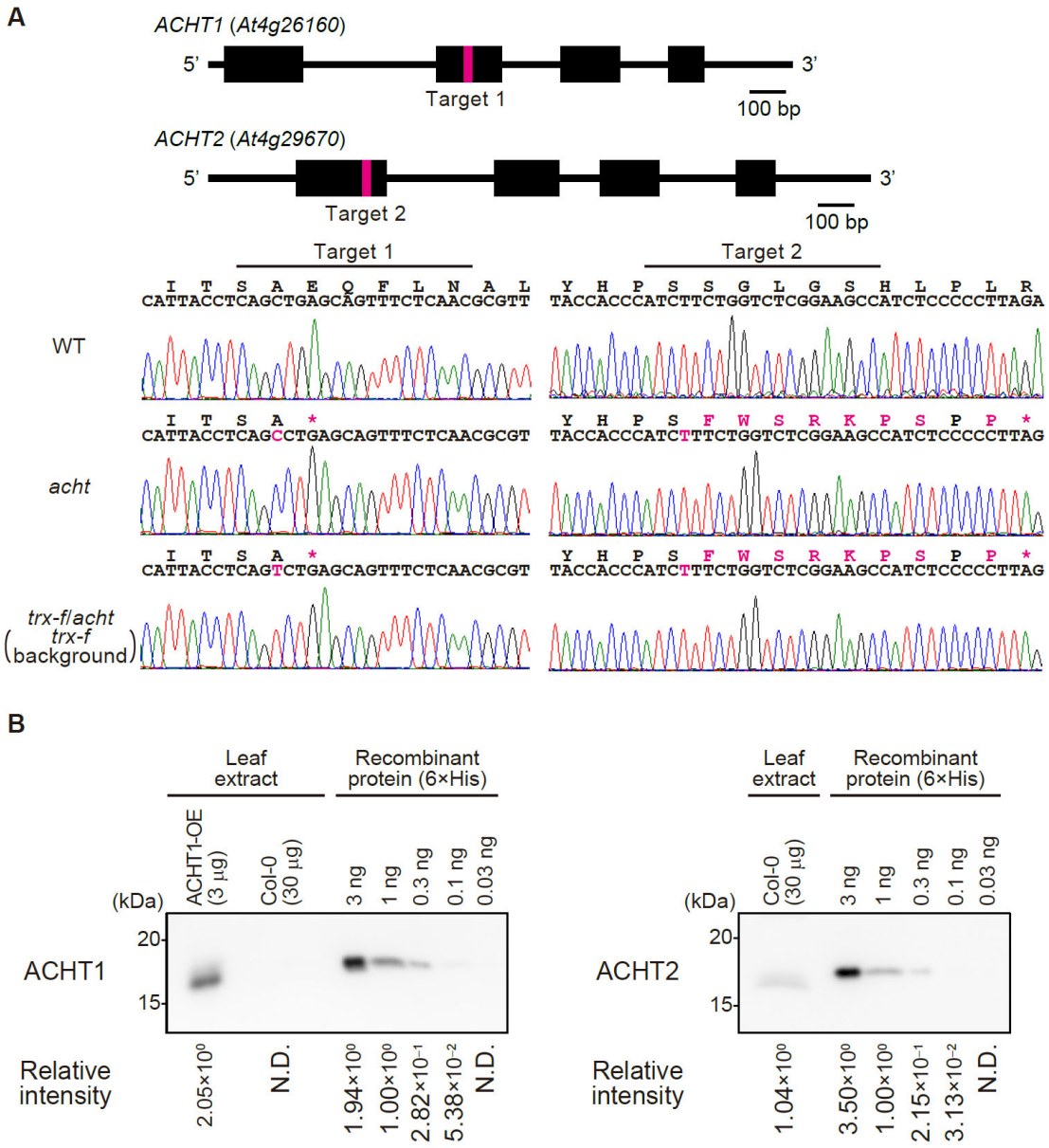


Fig. S4. Confirmation of ACHT1 and ACHT2 deficiency in mutant plant lines.

(A) DNA sequences around Cas9-targeted sites in the genome of the *acht* line. Cas9-targeted sites are indicated in magenta in each gene structure. Mutated sites are shown in magenta in DNA and protein sequences. **(B)** Quantification of the ACHT1 and ACHT2 proteins in leaf extract by western blotting. Recombinant ACHT1 or ACHT2 protein fused to 6×His tags were used as standards. Band intensity relative to that of 1-ng recombinant protein is indicated below the western blotting results. N.D.: not detected.

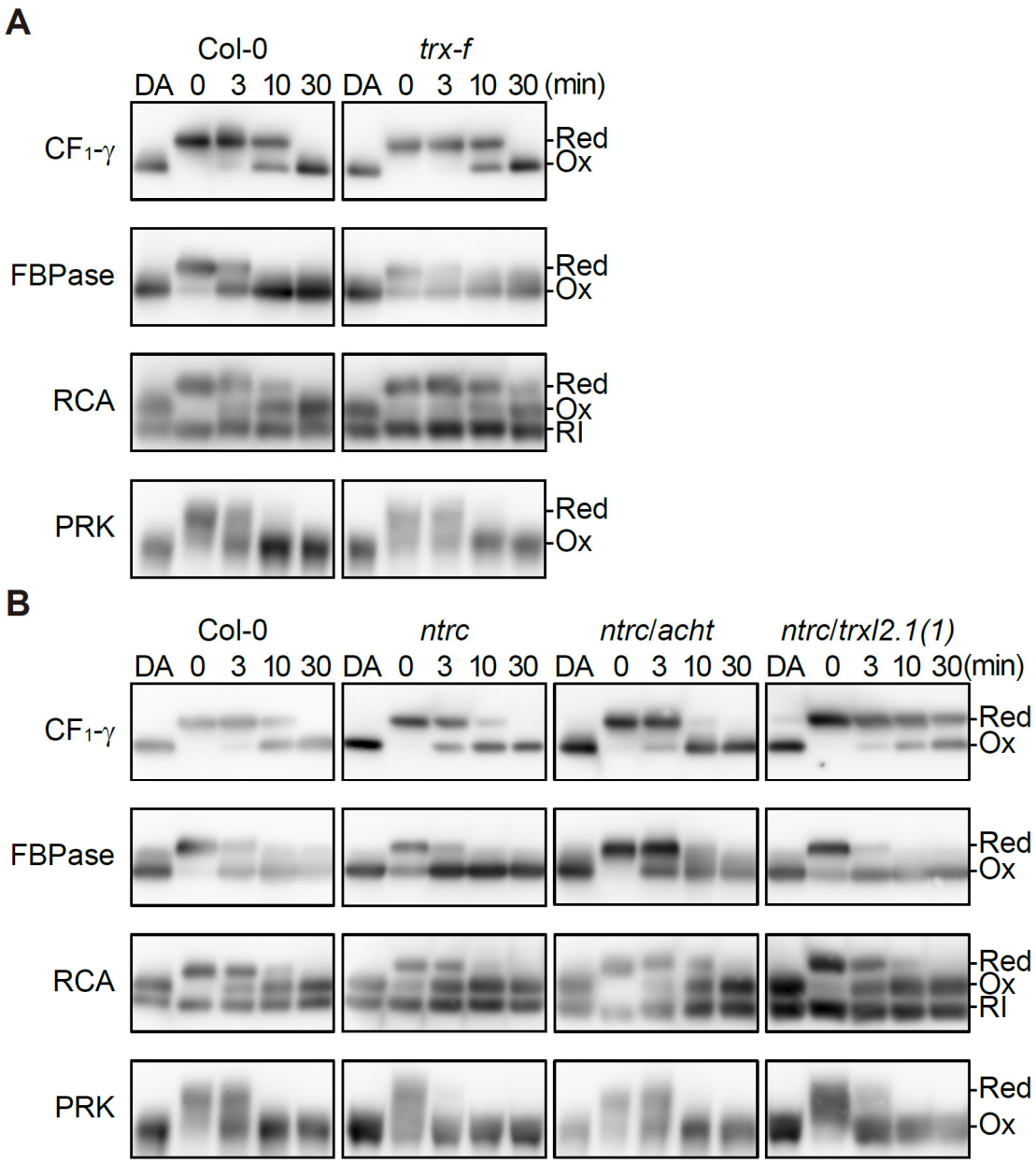


Fig. S5. Western blotting analyses of the oxidation of Trx-targeted proteins.

Oxidation of Trx-targeted proteins in plants deficient in **(A)** Trx-*f* or **(B)** NTRC and Trx-like proteins. Plants were preilluminated with 700-µmol photons $m^{-2} \cdot s^{-1}$ light intensity for **(A)** 60 min or **(B)** 30 min. The redox states of Trx-targeted proteins in dark-adapted (DA) plants or plants during light-to-dark transitions were monitored using a redox shift assay with the thiol-modifying reagent AMS. Abbreviations: Red, reduced; Ox, oxidized; RI, redox-insensitive splicing variant. **(B)** The same data shown in Fig. 2A are used for Col-0.

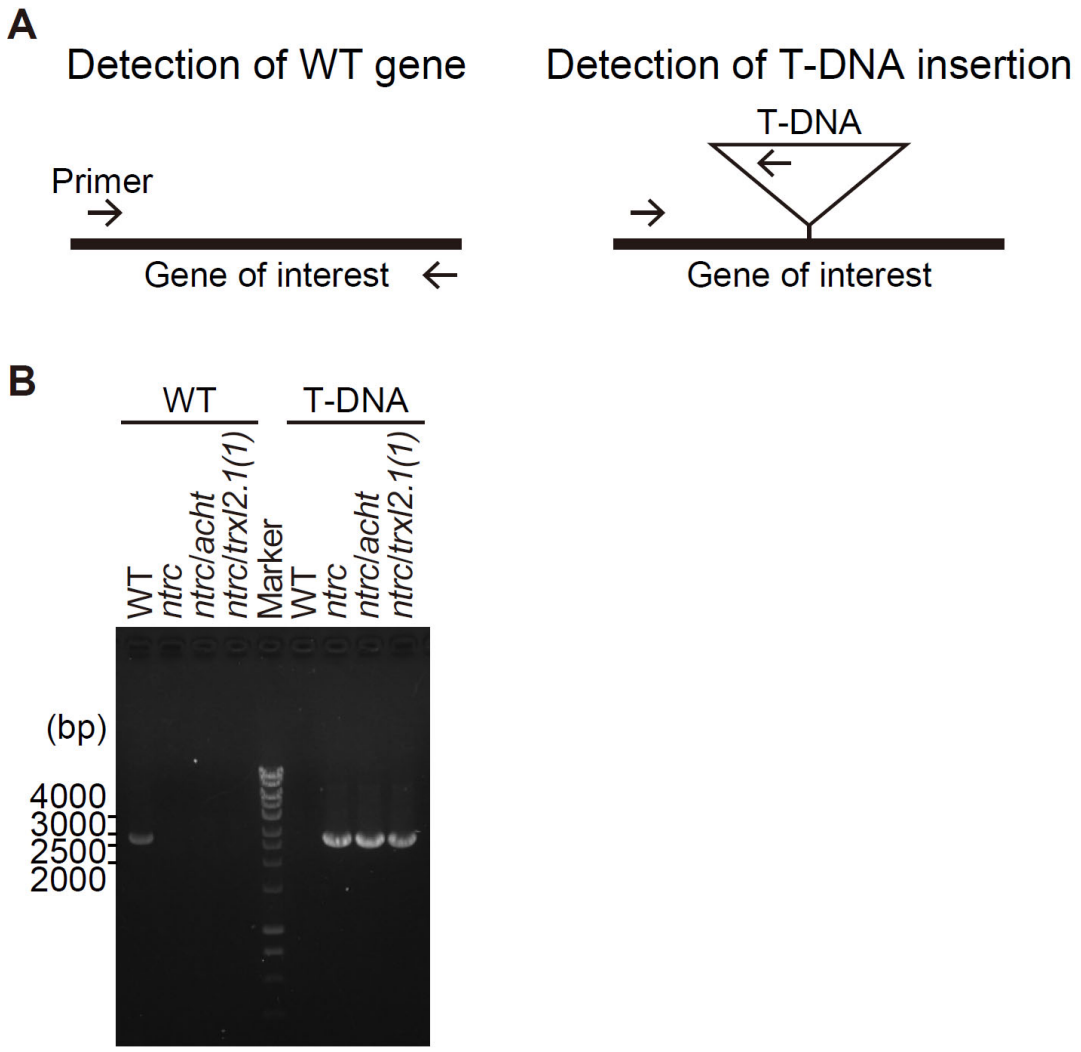


Fig. S6. Confirmation of NTRC deficiency by polymerase chain reaction (PCR).

(A) Schematic representation of the PCR-based method used to confirm T-DNA insertion. Arrows indicate the primer sets for the detection of the WT gene or T-DNA insertion. **(B)** Confirmation of the T-DNA insertion in the *NTRC* gene. Primers used for these experiments are shown in Table S1.

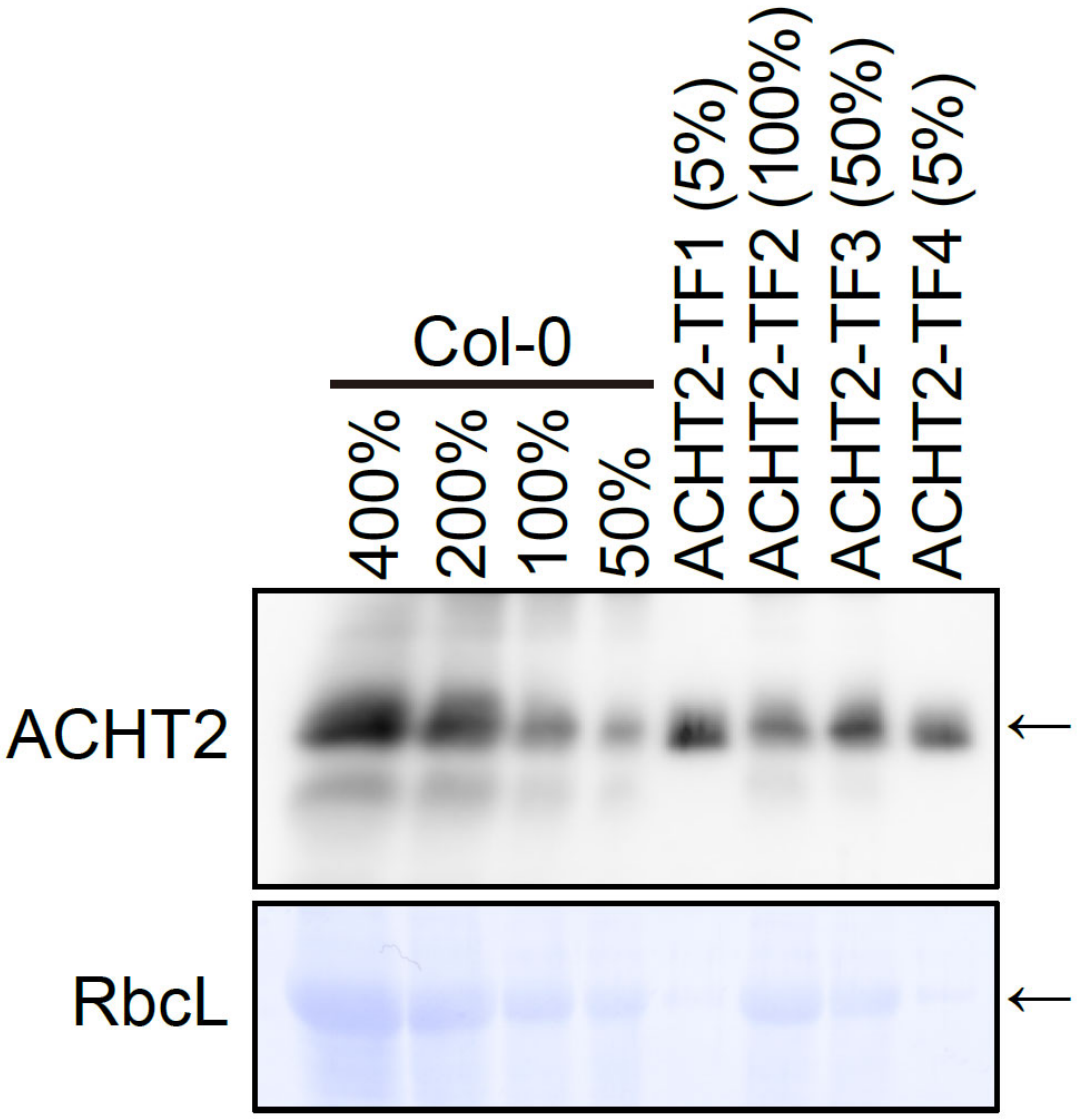


Fig. S7. Expression levels of ACHT2 in ACHT2-TF plants.

Results of western blotting with diluted leaf extracts and anti-ACHT2 antibody are shown. Values (%) indicate the loaded amounts of leaf extract proteins. Arrows indicate the proteins of interest. CBB-stained Rubisco large subunit (RbcL) is shown as a loading control.

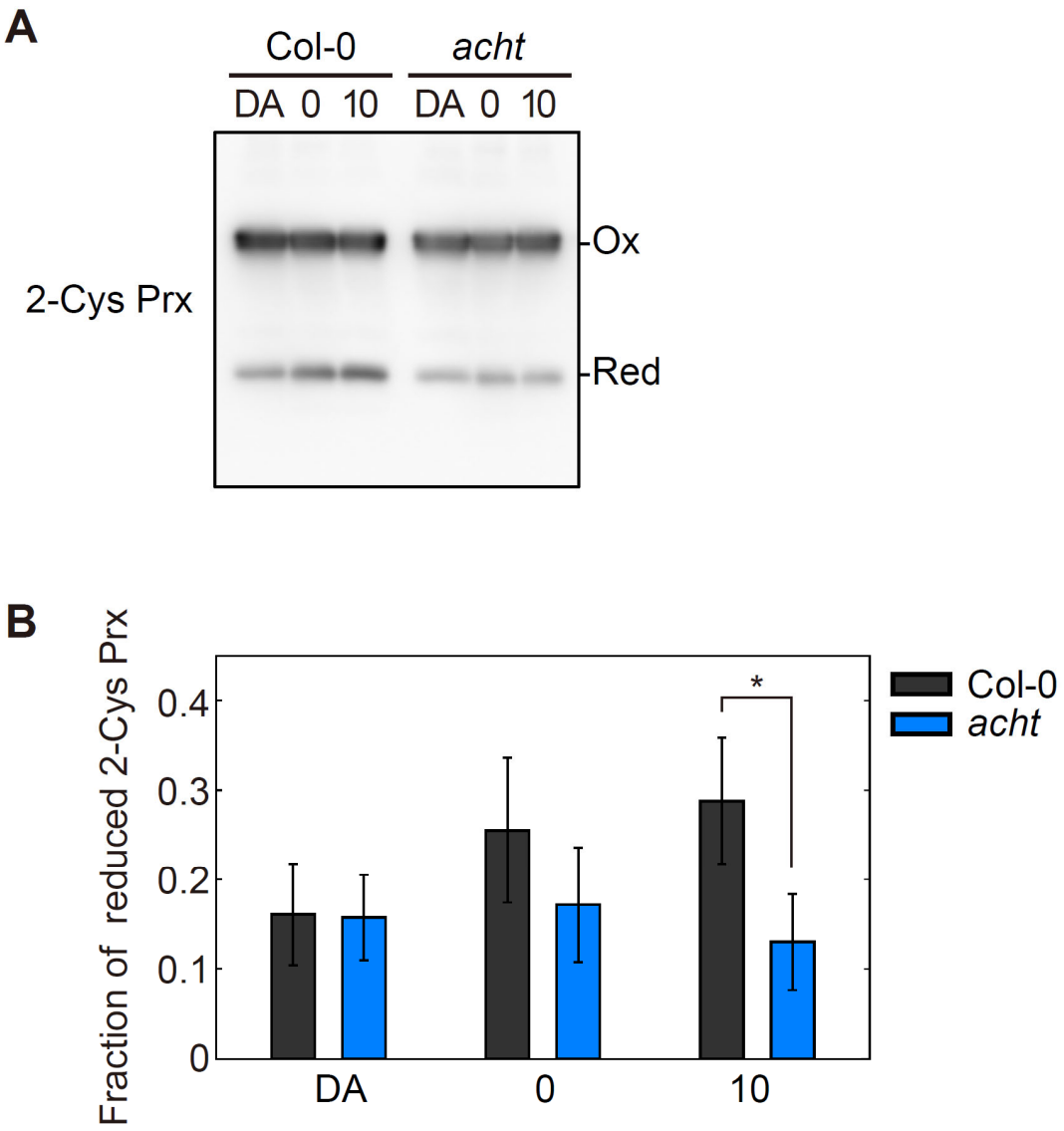


Fig. S8. Redox state of 2-Cys Prx in the *acht* line at each time point.

(A) Redox state determination of 2-Cys Prx by western blotting. The result of western blotting with anti-2-Cys Prx A antibody is shown. Abbreviations: Red, reduced; Ox, oxidized; dark-adapted, DA. **(B)** Comparison of the redox states of 2-Cys Prx in the Col-0 line and *acht* line. By measuring the band intensity of (A), the redox state was determined and is shown as the mean ± SD (n = 6). The asterisk indicates a significant difference between Col-0 and *acht* ($P < 0.05$, Student's *t*-test).

Table S1. Primers used in this study.

Name	Sequence (5′ to 3′)
TrxL2.1 sgRNA1 Fw	ATTGGTTCCATCTCCAAAGTCGT
TrxL2.1 sgRNA1 Rev	AAACACGACTTTGGAGATGGAAC
TrxL2.1 sgRNA2 Fw	ATTGAGGTGATTGGAGGTCACAA
TrxL2.1 sgRNA2 Rev	AAACTTGTGACCTCCAATCACCT
TrxL2.2 sgRNA Fw	ATTGGAACCAAAGGTGAGATCTT
TrxL2.2 sgRNA Rev	AAACAAGATCTCACCTTTGGTTC
ACHT1 sgRNA Fw	ATTGTTGAGAAACTGCTCAGCTG
ACHT1 sgRNA Rev	AAACCAGCTGAGCAGTTTCTCAA
ACHT2 sgRNA Fw	ATTGGCTTCCGAGACCAGAAGAT
ACHT2 sgRNA Rev	AAACATCTTCTGGTCTCGGAAGC
FH41[a]	AAACGACGGCCAGTGCCAGAATTGGGCCCGACGTCG
FH42[a]	TACTGACTCGTCGGGTACCAAGCTATGCATCCAACGCG
FH254[a]	GCCCAATTCCAAGCTATGCATCCAACGCG
FH255[a]	CATAGCTTGGAATTGGGCCCGACGTCG
NTRC WT/T-DNA Fw[b]	GCTGCGTCTCCCAAGATAGG
NTRC WT Rev[c]	GTTGTTTTGTAAAATCTTAAAGC
NTRC T-DNA Rev[d]	ATTTTGCCGATTTCGGAAC
ACHT1 ACHT1-TF Fw	GTTGATACATATGTCTCCGACGACGACATC
ACHT1 ACHT1-TF Rev	GAATTGTCGATTATTCACTTGAATCTTCAACTTTCTC
pRI201 ACHT1-TF Fw	AAGTGAATAATCGACAATTCTGAATCAACAAC
pRI201 ACHT1-TF Rev	TCGGAGACATATGTATCAACAGTGAAG
ACHT2 ACHT2-TF Fw	GTTGATACATATGGCTGGAGTTGTGCG
ACHT2 ACHT2-TF Rev	GAATTGTCGATCAACTTGATGCAGCTGGTTTG
pRI201 ACHT2-TF Fw	ATCAAGTTGATCGACAATTCTGAATCAACAAC
pRI201 ACHT2-TF Rev	CTCCAGCCATATGTATCAACAGTGAAG
PRK cloning Fw	TATCATATGGTGATCGGACTAGCTGCTGAC
PRK cloning Rev	TATCTCGAGGGCTTTAGCTTCTGCACGAGC

a: primers used by Hahn et al. (3).

b and c: primers used as NTRC Fw and NTRC Rev, respectively, by Yoshida and Hisabori (4).

d: a primer designed as LBb1.3 in the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/).

References

1. I. Dangoor, H. Peled-Zehavi, A. Levitan, O. Pasand, A. Danon, A small family of chloroplast atypical thioredoxins. *Plant Physiol* **149**, 1240-1250 (2009).
2. K. Yoshida, A. Hara, K. Sugiura, Y. Fukaya, T. Hisabori, Thioredoxin-like2/2-Cys peroxiredoxin redox cascade supports oxidative thiol modulation in chloroplasts. *Proc Natl Acad Sci U S A* **115**, E8296-E8304 (2018).
3. F. Hahn, M. Eisenhut, O. Mantegazza, A. P. M. Weber, Generation of Targeted Knockout Mutants in Arabidopsis thaliana Using CRISPR/Cas9. *Bio-Protocol* **7**, e2384 (2017).
4. K. Yoshida, T. Hisabori, Two distinct redox cascades cooperatively regulate chloroplast functions and sustain plant viability. *Proc Natl Acad Sci U S A* **113**, E3967-3976 (2016).