

The Water-Water Cycle Is Essential for Chloroplast Protection in the Absence of Stress*[§]

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Maintaining electron flow through the photosynthetic apparatus, even in the absence of a sufficient amount of NADP⁺ as an electron acceptor, is essential for chloroplast protection from photooxidative stress. At least two different pathways are thought to participate in this process, i.e. cyclic electron flow and the water-water cycle. Although the function of the water-water cycle was inferred from a number of biochemical and physiological studies, genetic evidence for the function of this cycle is very limited. Here we show that knockdown *Arabidopsis* plants with suppressed expression of the key water-water cycle enzyme, thylakoid-attached copper/zinc superoxide dismutase (KD-SOD), are suppressed in their growth and development. Chloroplast size, chlorophyll content, and photosynthetic activity were also reduced in KD-SOD plants. Microarray analysis of KD-SOD plants, grown under controlled conditions, revealed changes in transcript expression consistent with an acclimation response to light stress. Although a number of transcripts involved in the defense of plants from oxidative stress were induced in KD-SOD plants, and seedlings of KD-SOD plants were more tolerant to oxidative stress, these mechanisms were unable to compensate for the suppression of the water-water cycle in mature leaves. Thus, the localization of copper/zinc superoxide dismutase at the vicinity of photosystem I may be essential for its function. Our studies provide genetic evidence for the importance of the water-water cycle in protecting the photosynthetic apparatus of higher plants from photooxidative damage.

Dissipation of excess energy absorbed by the photosynthetic apparatus is a fundamental process essential for the survival of almost all photosynthetic organisms. It prevents photooxidative damage that occurs when excited chlorophyll molecules improperly transfer their higher energy state to oxygen or neighboring molecules and convert them into reactive molecules or toxic radicals (1–3). This process is especially crucial when CO₂ fixation is limited because of environmental condi-

tions such as cold or drought. Under these conditions, the energy absorbed by the photosynthetic apparatus cannot be channeled into the reduction of CO₂, and photooxidative damage may occur (4). Maintaining electron flow through the photosynthetic membrane, even under stressful conditions, is therefore vital for preventing damage to plant cells (2). A number of different pathways are thought to cooperate in protecting the photosynthetic apparatus from photooxidative stress. These include the zeaxanthin cycle that directly protects the antenna molecules and the cyclic electron flow and the water-water cycle that shunt electrons through the photosynthetic apparatus and maintain the pH gradient in the chloroplast, which is essential for the function of the zeaxanthin cycle (1, 2).

The water-water cycle channels electrons obtained from the splitting of water molecules at photosystem II (PSII)¹ through the photosynthetic apparatus. These electrons are transferred to oxygen by photosystem I (PSI) and result in the formation of superoxide radicals (O₂⁻) (5). A membrane-attached copper/zinc superoxide dismutase (Cu/ZnSOD) converts the superoxide radicals into hydrogen peroxide, and a membrane-bound ascorbate peroxidase (thylakoid-APX) converts the hydrogen peroxide back into water. Ascorbic acid, used by the thylakoid-bound APX as a reductant, is converted during this process into ascorbic acid radical (monodehydroascorbate), and this radical is reduced back to ascorbic acid by ferredoxin using electrons from PSI. The water-water cycle, therefore, maintains electron flow through the photosynthetic apparatus even when CO₂ fixation is limited or inhibited. It completes the cycle of electrons from one water molecule at PSII to another water molecule, the product of a peroxidase reaction, at close proximity to PSI. Thus, it maintains proton pumping across the thylakoid membrane by electron flow through plastoquinone (1, 2).

Although the role of the water-water cycle was inferred from a number of different biochemical and physiological studies, genetic evidence supporting the function of this pathway in protecting chloroplasts is very limited. A number of studies have shown that enhancing the expression of the thylakoid-attached Cu/ZnSOD or the thylakoid-bound APX enhances the abiotic stress tolerance of transgenic plants (6, 7). However, loss of function studies for these two enzymes were not published, and the role of the water-water cycle in maintaining chloroplast functions in the absence of environmental stresses was not documented, possibly because the loss of function of these enzymes is lethal (7). The role of the water-water cycle in protecting the photosynthetic apparatus was also challenged by a number of studies claiming that the amount of electrons transferred through this pathway is very small and not sufficient to protect the chloroplast from photooxidative stress (2).

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplementary Figs. 1 and 2 and supplementary Tables I, II, and III.

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¹ The abbreviations used are: PSII, photosystem II; APX, ascorbate peroxidase; ch, chloroplastic; KD, knockdown; PSI, photosystem I; SOD, superoxide dismutase.

To study the function of the water-water cycle in plants, we characterized knockdown *Arabidopsis* plants with suppressed expression of thylakoid-attached Cu/ZnSOD. Our studies reveal that the water-water pathway is essential for the protection of chloroplasts even in the absence of environmental stress conditions that limit the availability of the electron acceptor NADP^+ in chloroplasts.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—*Arabidopsis thaliana* (cv Columbia) plants were grown in growth chambers (Percival E-30HB) under controlled conditions at 21–22 °C for 18 h or a constant light cycle at 100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ and a relative humidity of 70%. Knockdown *Arabidopsis* plants containing a T-DNA insert in the promoter of chloroplastic Cu/ZnSOD (KD-SOD) were outcrossed and selfed to check for segregation and obtain a pure homozygote line as recommended (8). Confirmation of chloroplastic Cu/ZnSOD suppression and segregation analysis were performed by PCR, genomic DNA blots, and RNA and activity gels. All experiments were performed in triplicate and repeated at least three times. Structural analysis of leaves with light and transmission electron microscopy was performed as described in (9).

Molecular, Physiological, and Biochemical Analysis—RNA and protein were isolated and analyzed by RNA blots and activity gels as described previously (10). A ribosomal 18 S rRNA probe was used to control for RNA loading. Coomassie Blue staining of protein gels was used to control for protein loading. Photosynthesis, stomatal conductance, and dark respiration were measured with a Li-Cor LI-6400 apparatus as described (11) using the *Arabidopsis* leaf chamber (Li-Cor, Lincoln, NE). Reduced glutathione was determined by high pressure liquid chromatography as described (11), and protein oxidation was assayed (12). Chloroplasts were isolated as described (13), and anthocyanin level was determined (14).

DNA Chip Analysis—In three independent experiments, RNA was isolated from 40–50 wild type or KD-SOD plants (a total of 120–150 plants per line) grown under controlled conditions as described above. This RNA was used to perform chip analyses (*Arabidopsis* ATH1 chips; Affymetrix, Santa Clara, CA) at the University of Iowa DNA facility (dna-9.int-med.uiowa.edu/microarrays.htm). Conditions for RNA isolation, labeling, hybridization, and data analysis are described (11). Comparative analysis of samples was performed with the GeneChip mining tool version 5.0 and the Silicon Genetics GeneSpring version 5.1. Some of the comparison results were confirmed by RNA blots.

Oxidative Stress Assay—Seeds of wild type and knockout/knockdown lines were surface-sterilized with bleach and placed in rows on 1.5% agar plates (0.5 \times Murashige and Skoog medium) containing different concentrations of paraquat (Sigma). Each row of seeds placed on a plate was divided into two parts, i.e. wild type and knockout or knockdown seeds. Thus, the different seeds were placed side-by-side on the same plate. Plates were maintained vertically in a growth room (21–22 °C, constant light, 80–100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$), and percentage of germination and root length were scored 5 days after seed sterilization and plating.

RESULTS

Chloroplastic, thylakoid-attached Cu/ZnSOD (chl-Cu/ZnSOD) is encoded by a single gene in *Arabidopsis* (At2g28190). We obtained a knockout line for this gene from the SIGnAL project (signal.salk.edu/about.html). As shown in Fig. 1A, the T-DNA insert in this line is positioned 51 bp upstream from the initiation of the transcription site. As shown in Fig. 1B, this insertion results in the suppression of chl-Cu/ZnSOD expression at the RNA and activity levels. This type of suppression is usually referred to as knockdown because it does not eliminate the transcript but rather suppresses its expression (8). As shown in Fig. 1C, compared with wild type plants, knockdown chl-Cu/ZnSOD plants grown under controlled conditions were suppressed in their growth. Developmental characterization of KD-SOD plants revealed that they were delayed by at least 3 days in their flowering; however, they produced fertile seeds (supplementary Table I, available in the on-line version of this article). The relative suppression of growth and development in KD-SOD plants was reversible when plants were grown under a very low light intensity (i.e. 20–25 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ rather than 55–100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$; supplementary Fig. 1, available

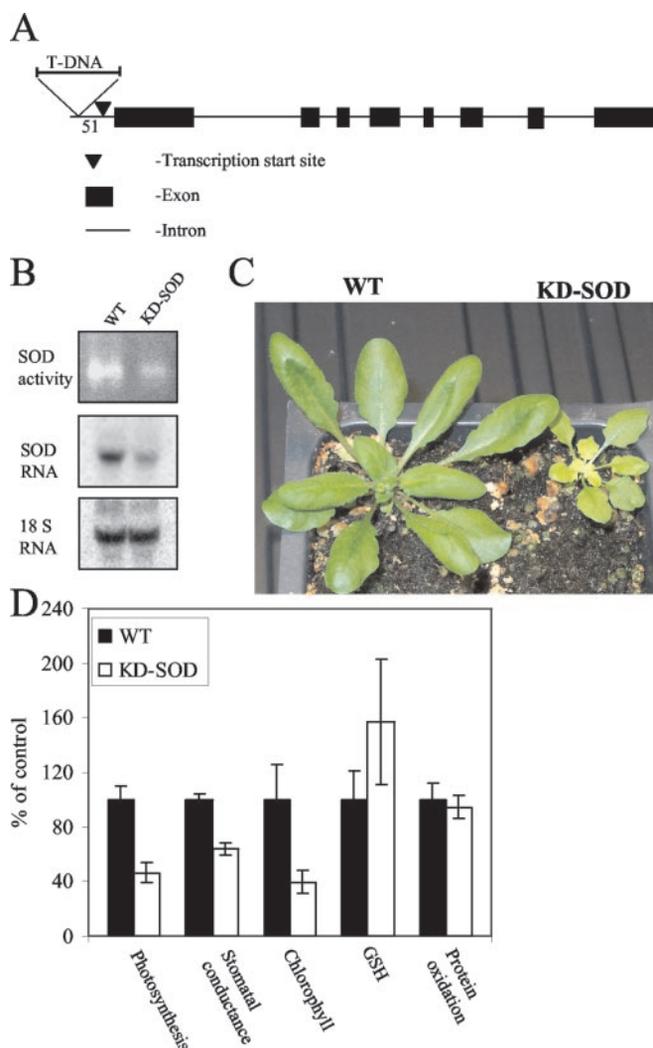


FIG. 1. Characterization of KD-SOD plants. A, map showing the T-DNA insertion site at the promoter of chl-Cu/ZnSOD. B, activity gel and RNA blots showing the suppression of chl-Cu/ZnSOD expression in KD-SOD plants. Activity gel was performed with extracts obtained from purified chloroplasts (10, 13). C, photograph showing a 4-week-old wild type (WT) and KD-SOD plants grown under controlled conditions. D, biochemical and physiological characterization of WT and KD-SOD plants showing the suppression of photosynthetic activity and chlorophyll content in KD-SOD plants. Analysis in panels B and D was performed with fully expanded leaves of 3-week-old plants grown under controlled conditions.

in the on-line version of this article) or when KD-SOD plants were transformed with a binary vector expressing chl-Cu/ZnSOD under the control of the CaMV35S promoter (supplementary Fig. 2, available in the on-line version of this article). To avoid complications resulting from developmental differences between plants, we conducted all of our comparisons between wild type and KD-SOD plants with 3-week-old plants that were developmentally indistinguishable (all growing under optimal growth conditions, i.e. 21–22 °C, constant light cycle, 100 $\mu\text{mol m}^{-2} \text{sec}^{-1}$, and a relative humidity of 70%).

Physiological and biochemical characterization of KD-SOD plants (Fig. 1D) revealed that they had a suppressed rate of photosynthesis and a lower level of chlorophyll. However, the content of oxidized proteins, a measure of oxidative stress (12), was not significantly different between KD-SOD plants and wild type plants, and the level of reduced GSH was only slightly elevated in KD-SOD plants. *In situ* staining for superoxide (15) did not reveal a difference between KD-SOD and wild type plants grown under controlled conditions (not

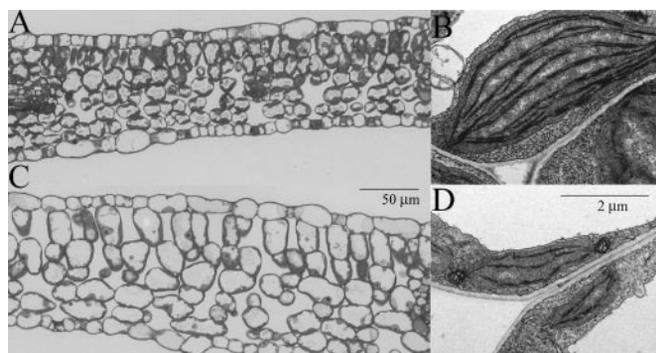


FIG. 2. **Structural analysis of KD-SOD plants.** A and C, cross-sections through young leaves of similar age and size obtained from 3-week-old wild type (A) and KD-SOD (C) plants. Magnification bar for panels A and C is shown in panel C. B and D, transmission electron microscopy of chloroplasts from wild type (B) and KD-SOD (D) leaves (shown in panels A and C). Magnification bar for panels B and D is shown in panel D.

shown), possibly because KD-SOD plants adjusted their metabolism to compensate for the suppression of chl-Cu/ZnSOD.

Structural analysis of KD-SOD and wild type plants using young leaves of similar ages and sizes revealed that, although the mesophyll cells of KD-SOD plants were larger than those of wild type, the chloroplasts of KD-SOD plants were smaller (Fig. 2). In addition, the granal thylakoids of KD-SOD chloroplasts were less organized and contained fewer stacks. We could not, however, find any additional differences in cell structure between KD-SOD plants and wild type plants, and the chloroplasts of KD-SOD contained starch grains (not shown).

Global analysis of transcript expression (*Arabidopsis* 22,000-gene ATH1 chips) in wild type and KD-SOD plants, grown under controlled conditions, revealed that the suppression of chl-Cu/ZnSOD resulted in the induction of two different transcripts encoding chloroplastic iron superoxide dismutase (FeSOD) and two transcripts encoding catalase (CAT1 and CAT3). In addition, a transcript encoding ferritin was up-regulated (Table I). In contrast, transcripts encoding thylakoid-APX, 2-Cys peroxiredoxin (a possible peroxidase involved in the water-water cycle) (16), chloroplastic and cytosolic glutathione reductase, and an NADPH oxidase were suppressed in KD-SOD plants (Table I).

The global expression profile of KD-SOD plants revealed a high degree of similarity to that of cyanobacterial cells exposed to high light stress (Table II) (17). Thus, transcripts encoding antenna proteins (chlorophyll *a/b* binding proteins in *Arabidopsis*) and the oxygen-evolving complex of PSII were suppressed, and transcripts encoding PSII core, NADH dehydrogenases, molecular chaperones, small ribosomal proteins, and the large subunit of ribulose-bisphosphate carboxylase/oxygenase (Rubisco) were elevated (some of the core subunits of the different photosystems might be up-regulated to compensate for the high turnover of their corresponding protein products; Ref. 17). In addition, at least three transcripts encoding enzymes involved in the biosynthesis of anthocyanins were induced, and the level of anthocyanins in KD-SOD plants was higher than that of wild type (Table II). Complete tables including all array results showing induction or suppression of transcripts in KD-SOD plants can be found in the supplementary material (supplementary Tables II and III) available in the on-line version of this article. Analysis of the global expression profiles of KD-SOD and wild type plants also revealed that many components of the water-water cycle are suppressed in KD-SOD plants. These include chl-Cu/ZnSOD, thylakoid-APX and 2-Cys peroxiredoxin (Table I), the oxygen-evolving complex (seven different transcripts), ferredoxin-NADP⁺ reductase, and PSI subunit II (Table II).

TABLE I

Changes in the expression pattern of transcripts involved in the defense of plants against reactive oxygen species in KD-SOD plants

Wild type and KD-SOD plants were grown under controlled conditions. Changes in transcript abundance were measured in the leaves of 3-week-old plants with Affymetrix chips. Complete tables of chip results can be found in the supplementary material in the on-line version of this article. All measurements were performed as described under "Experimental Procedures." GPX, glutathione peroxidase; GR, glutathione reductase.

Transcript	Gene number	Fold Change (log ₂)	
		Average	S.D.
Induced			
Peroxidase ATP7a	At3g21770	2.30	0.24
Ferritin 1	At5g01600	1.77	0.36
FeSOD	At4g25100	1.73	0.17
Putative peroxidase	At5g58390	1.43	0.26
NPK1-related protein kinase	At3g06030	1.37	0.28
Class 1 hemoglobin	At2g16060	1.37	0.20
Catalase (CAT1)	At1g20630	1.23	0.08
Catalase (CAT3)	At1g20620	1.14	0.26
Putative ferritin	At2g40300	1.07	0.12
FeSOD3	At5g23310	0.94	0.14
Phospholipid hydroperoxide	At4g11600	0.60	0.08
Suppressed			
Chloroplastic Cu/ZnSOD	At2g28190	-3.40	0.29
Putative peroxidase	At1g62250	-1.97	0.09
O ₂ ⁻ -generating NADPH oxidase	At1g23020	-1.40	0.16
Catalase	At4g35090	-1.23	0.17
Thylakoid-bound APX	At1g77490	-1.13	0.24
Cytosolic GR	At3g24170	-1.10	0.08
Chloroplastic GR	At3g54660	-1.01	0.08
Putative GPX	At2g25080	-1	0.08
Putative GPX	At2g25080	-0.93	0.04
Blue copper-binding protein	At3g27200	-0.84	0.33
Putative APX	At4g09010	-0.77	0.23
2-Cys peroxiredoxin	At5g06290	-0.77	0.04
Blue copper-binding protein	At4g12880	-0.70	0.08
Putative GPX	At2g31570	-0.51	0.08

TABLE II

Changes in transcript expression resembling light stress response in KD-SOD plants grown under controlled growth conditions

Increase (I) or decrease (D) in steady state transcript level was measured with Affymetrix chips. Fold change in transcript level (I or D) was at least 2–6 fold. All transcripts indicated in the table had a significant change in their expression (average and S.D. of three different measurements). Complete tables of chip results can be found in the supplementary material in the on-line version of this article. Anthocyanin levels were determined in leaf extracts. All measurements were performed as described under "Experimental Procedures" using the leaves of 3-week-old plants. Cyt, cytochrome; PS, photosystem; Rbc, ribulose-bisphosphate carboxylase/oxygenase (Rubisco); RbcL, Rubisco large subunit.

Transcript/function	No. of different transcripts affected	Increased/decreased
Chlorophyll <i>a/b</i> binding (antenna)	8	D
Oxygen-evolving complex (PSII)	7	D
Ferredoxin-NADP ⁺ reductase (FNR)	1	D
PSI subunit II (FNR-binding)	2	D
PSII core (D1, D2, CP47, CP43, Cytb559)	6	I
PSI core (A1, A2)	3	I
CytB6f complex	3	I
NADH dehydrogenase	8	I
Molecular chaperones	10	I
RbcL and Rbc chaperone	2	I
Ribosomal protein	21	I
Anthocyanin biosynthesis	3	I
Total anthocyanin		I ^a

^a 2.5 fold; S.D. = 0.2.

To test whether the suppression of chl-Cu/ZnSOD resulted in greater susceptibility of KD-SOD plants to oxidative stress not directly involving the water-water cycle, we subjected wild type plants, knockout plants deficient in cytosolic *Apx1* (KO-APX; a

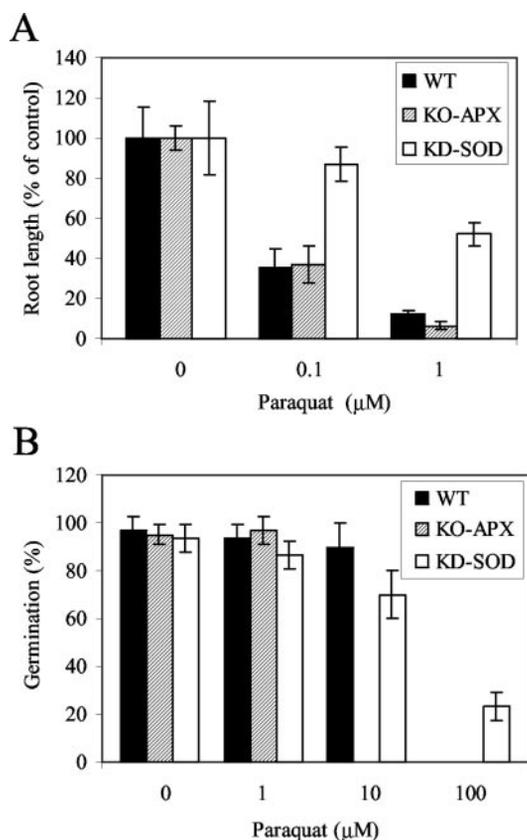


FIG. 3. Enhanced tolerance of KD-SOD seedlings to oxidative stress. A, root length of 5-day-old wild type (WT), knockout APX1 (*KO-APX*), and KD-SOD seedlings germinated on agar plates in the presence of the superoxide-generating agent paraquat. B, germination rates (%) of WT, KO-APX, and KD-SOD seeds on agar plates containing paraquat. Germination rates were scored 5 days post plating.

control for plants that are less tolerant to oxidative stress; Ref. 11), and KD-SOD plants to an oxidative stress assay based on measuring the percentage of germination and root length of seedlings germinated on agar plates in the presence of the superoxide-generating agent paraquat. As shown in Fig. 3, KD-SOD plants were more resistant to this treatment than wild type plants. Thus, compared with wild type plants, they germinated and were able to maintain their root growth at higher concentrations of paraquat. In contrast, knockout APX plants were retarded in their germination rate compared with wild type plants.

DISCUSSION

Our analyses of KD-SOD plants suggest that the water-water cycle is essential for the protection of chloroplasts. Thus, KD-SOD plants grown under controlled conditions were impaired in their growth and had a lower rate of photosynthetic activity (Fig. 1). Although the suppression of chl-Cu/ZnSOD did not alter chloroplast development, similar to some mutants deficient in the protection of chloroplasts from photooxidative damage (18), the chloroplasts of KD-SOD plants were smaller and contained fewer stacks of granal thylakoids (Fig. 3). Global analysis of gene expression in KD-SOD plants revealed that the response of plants to the suppression of chl-Cu/ZnSOD under controlled conditions was similar to that of photosynthetic cells subjected to light stress (Table II) (17, 19). Thus, changes in transcript expression in KD-SOD plants were consistent with alterations to the photosynthetic apparatus aimed at lowering the amount of energy absorbed by the thylakoids (e.g. suppression of antenna proteins). In addition, KD-SOD plants accumulated anthocyanins (Table II). However, not all

transcripts enhanced during light stress in plants were up-regulated in KD-SOD plants. Thus, APX1 and APX2 (3, 11, 19) were not significantly induced in KD-SOD plants, possibly because KD-SOD plants were acclimated to the absence of chl-Cu/ZnSOD (as opposed to wild type plants maintained at low light and subjected to a high light treatment). The similarities observed between the response of plants to chl-Cu/ZnSOD suppression (Table I and II) and light stress (17, 19) might suggest that the suppression of the water-water cycle in KD-SOD plants resulted in an enhanced photooxidative stress that, in turn, altered the expression pattern of transcripts encoding different components of the photosynthetic apparatus and enhanced the accumulation of anthocyanins (Table II).

The co-suppression of different components of the water-water cycle in KD-SOD plants (Tables I and II) suggest that different transcripts encoding different members of this cycle are regulated as a pathway. Interestingly, the induction of catalases (CAT1 and CAT2) and chloroplastic FeSODs (Table I) could not compensate for the suppression of chl-Cu/ZnSOD. This finding suggests that the localization of chl-Cu/ZnSOD, attached to the thylakoid membrane at the vicinity of PSI (1), is crucial for its function in the water-water cycle. This hypothesis is supported by the finding that the oxidative stress pathway(s) that could not compensate for chl-Cu/ZnSOD function in the water-water cycle (possibly involving FeSODs and catalases; Table I) appeared to enhance the resistance of KD-SOD seedlings to oxidative stress induced by paraquat (Fig. 3). Root growth and the percentage of germination used for this assay (Fig. 3) are not likely to be directly dependent upon the function of the water-water cycle, because germinating seedlings mainly rely on storage tissue for growth and development. The differential sensitivity of KD-SOD plants to the different conditions/treatments, *i.e.* light stress in the chloroplast (Fig. 1; Table II) *versus* paraquat stress in roots and germinating seedlings (Fig. 3), demonstrates the specificity of chl-Cu/ZnSOD and the water-water cycle for the defense of chloroplasts against over-reduction of the photosynthetic apparatus. Thus, the phenotype of KD-SOD that could be reversed by growth at low light (supplementary Fig. 1, available in the on-line version of this article) could not be reversed by the induction of chloroplastic FeSODs and catalases (Table I).

The suppression of chl-Cu/ZnSOD in KD-SOD plants was not complete (Fig. 1). It is possible that a complete suppression of this enzyme (*i.e.* a true knockout) is lethal to plants. In this respect it should be noted that unsuccessful attempts to suppress thylakoid-APX, an additional component of the water-water cycle, by antisense expression led researchers to speculate that this enzyme is crucial for chloroplast protection (7). Although we could not determine from our studies what fraction of electron flow through the thylakoid membrane is mediated by the water-water cycle, the cyclic electron flow pathway, or regular electron flow, it is clear that, in the absence of catalase in chloroplasts, the majority of electrons donated to oxygen at PSI and disproportioned by chl-Cu/ZnSOD to hydrogen peroxide would be turned into water by a peroxidase reaction (1, 16). Thus, the suppression of chl-Cu/ZnSOD would directly reduce the action of the water-water pathway, and the suppression of photosynthesis and growth in KD-SOD plants, under controlled conditions, could be directly attributed to the suppression of the water-water cycle. However, it is also possible that superoxide radicals not scavenged by chl-Cu/ZnSOD (and by the induced FeSODs in KD-SOD plants) damage the photosynthetic apparatus and result in the suppression of photosynthesis and growth (20). Nevertheless, the production of these superoxide radicals is a direct result of electrons flowing through the water-water cycle and not through the cyclic elec-

tron flow pathway. Moreover, the cyclic electron flow pathway could not compensate for the deficiency in chl-Cu/ZnSOD (Fig. 1), suggesting that the water-water pathway and the cyclic electron flow pathway are not fully redundant in their capability to protect the photosynthetic apparatus.

One of the transcripts suppressed in KD-SOD plants is a transcript encoding the superoxide-producing enzyme NADPH oxidase (Table I). It was recently shown that NADPH oxidases function in cells even in the absence of stress or pathogen attack (21, 22). Although it is not clear how the suppression of a thylakoid-attached SOD affects the expression of a plasma membrane-bound NADPH oxidase, the link between these two transcripts might suggest that a global cellular network in plants regulates the overall production and scavenging of reactive oxygen intermediates such as superoxide radicals. Such a network would have a central role in plants, controlling different processes including defense, development, and growth (4, 23, 24). We are currently studying knockout lines deficient in different reactive oxygen-scavenging enzymes from different cellular compartments (4, 11) in an attempt to determine whether this network exists and to study how it is regulated.

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