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Protective Function of Chloroplast 2-Cysteine Peroxiredoxin in Photosynthesis. Evidence from Transgenic Arabidopsis[1]

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2-Cysteine peroxiredoxins (2-CPs) constitute a ubiquitous group of peroxidases that reduce cell-toxic alkyl hydroperoxides to their corresponding alcohols. Recently, we cloned 2-CP cDNAs from plants and characterized them as chloroplast proteins. To elucidate the physiological function of the 2-CP in plant metabolism, we generated antisense mutants in Arabidopsis. In the mutant lines a 2-CP deficiency developed during early leaf and plant development and eventually the protein accumulated to wild-type levels. In young mutants with reduced amounts of 2-CP, photosynthesis was impaired and the levels of D1 protein, the light-harvesting protein complex associated with photosystem II, chloroplast ATP synthase, and ribulose-1,5-bisphosphate carboxylase/oxygenase were decreased. Photoinhibition was particularly pronounced after the application of the protein synthesis inhibitor, lincomycin. We concluded that the photosynthetic machinery needs high levels of 2-CP during leaf development to protect it from oxidative damage and that the damage is reduced by the accumulation of 2-CP protein, by the de novo synthesis and replacement of damaged proteins, and by the induction of other antioxidant defenses in 2-CP mutants.

ROS are produced either from light-dependent energy conversion or by chemical electron transfer to molecular oxygen in the metabolism of aerobic organisms (Elstner, 1990). ROS in turn oxidize susceptible biomolecules, and, subsequently alkyl hydroperoxides are formed. In plants the chloroplast is particularly prone to oxidative damage by photosynthetic oxygen production and activation. Despite the presence of elaborate enzymatic and nonenzymatic antioxidative defense mechanisms, ROS escape from detoxification and oxidize organic compounds such as proteins, nucleic acids, terpenoids, and fatty acids to the respective peroxides (Baier and Dietz, 1998). In addition, alkyl hydroperoxides are formed by enzymatic reactions in chloroplasts, e.g. lipoxygenase catalyzes peroxidation of fatty acids and other desaturated organic biomolecules, such as carotenoids (Grosch and Laskawy, 1979; Canfield et al., 1992).

Detoxification of alkyl hydroperoxides is important because they can act as long-distance mediators of oxidative damage by oxidizing other biomolecules and initiating radical chain reactions (Elstner, 1990). For example, proteins and membrane lipids are oxidized, which results in degradation, loss of membrane function, and finally death of the organism (Jacobson et al., 1989; Poole and Ellis, 1996). Apparently detoxification of alkyl hydroperoxides is indispensable but yet not understood in plants.

Recently, we identified the first plant homologs of 2-CPs (Baier and Dietz, 1996a, 1996b) that are homodimeric enzymes reducing H_2O_2 and alkyl hydroperoxides (Chae et al., 1993; Poole and Ellis, 1996). Members of the 2-CP family of peroxidases have been identified in organisms from all systematic groups (Baier and Dietz, 1996c). It is interesting that the plant homolog is posttranslationally imported into the chloroplast stroma (Baier and Dietz, 1997). Inside the chloroplast it is assumed to detoxify alkyl hydroperoxide in the vicinity of the thylakoid membrane, the site of the most active oxygen metabolism in living plant cells.

To test the hypothesis that 2-CP in chloroplast metabolism has a protective function, Arabidopsis mutants were generated whose 2-CP amounts were reduced by antisense suppression of the transcript level. To establish the significance of 2-CPs in plants, we analyzed such physiological indicators as PSII and peroxidase activity and the stability of chloroplast proteins and related them to 2-CP expression.

MATERIALS AND METHODS

Plant Material

Arabidopsis plants were grown in a soil culture or on MSAR plates (Koncz et al., 1990), using a 25°C/20°C day/night cycle (10-h day at a PAR of 200–300 μmol m^{-2} s^{-1}). We induced flowering by increasing the light phase to 14 h d^{-1} for 7 d.

Transformation of Arabidopsis

The transformation was done by vacuum infiltration according to the protocol of Bechtold and Bouchez (1995), using the pPCV702 vector system (Koncz et al., 1990) and the *Agrobacterium tumefaciens* strain GV3101. The barley 2-CP cDNA fragment (accession no. Z34917) was cloned in antisense orientation into the *Bam*HI site of pPCV702, after the ligation of *Bam*HI linkers, so that its expression was

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Abbreviations: 2-CP, 2-Cys peroxiredoxin; CF1, coupling factor of thylakoid ATP synthase; F_m, maximum fluorescence; F_m', actual maximum fluorescence yield; LHCII, light-harvesting complex associated with PSII; MSAR, Murashige and Skoog medium optimized for Arabidopsis regeneration; PHGPx, phospholipid hydroperoxide glutathione peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SUE, subunit E of vacuolar ATPase.

under the control of the cauliflower mosaic virus 35S promoter. Transformed plants were initially selected on MSAR plates (Koncz et al., 1990) and supplemented with 50 μg mL^{-1} kanamycin. Consecutive generations were selected by spraying 2-week-old seedlings grown in soil culture daily for at least 5 d with a solution containing 0.5 to 1 mg mL^{-1} kanamycin and 0.05% (v/v) Triton X-100.

Extraction of Genomic DNA, PCR, Southern-Blot Hybridization, and Other Molecular Cloning Techniques

For extraction of genomic DNA, 1 g of destarched leaf material was ground in liquid nitrogen. Buffer (8.76 g of NaCl, 8.00 g of dodecyl trimethyl ammonium bromide, 1.21 g of Tris [pH 8.6], and 2.08 g of tetrasodium dihydrate EDTA, in a total volume of 100 mL) was added to the frozen powder at a volume-to-fresh-weight ratio of 2 mL g^{-1}. After the material was incubated at 68°C for 5 min, the homogenate was extracted with 3 mL of chloroform: isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated from the aqueous phase by adding 3 mL of cold isopropanol and resuspending them in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). To precipitate the RNA, one-third volume of 8 M LiCl was added. After at least 2 h on ice, the RNA was removed by centrifugation at 10,000*g* for 10 min. From the supernatant, DNA was precipitated by the addition of 2.5 volumes of ethanol. Ten micrograms of DNA was digested with 10 units *Eco*RI overnight, separated on a 0.9% (w/v) agarose gel, and blotted according to standard methods (Sambrook et al., 1988).

The genomic integration of the antisense construct was verified for the selected transformants by PCR, using barley 2-CP-specific primers (BAS-O1: CACCTTCGTCTGCCC; BAS-O4: CACACCCTCCTTGTC; 25 pmol each). The 50-μL reaction contained 1 pg of genomic DNA as a template, 0.2 mM dNTP (dATP, dGTP, dCTP, dTTP), primers, and 1.6 units of *Taq*-DNA polymerase (Promega) in 1× PCR buffer containing 1.5 mM $MgCl_2$ (Promega). PCR was performed in 35 cycles at a 42°C annealing temperature.

For Southern blotting, nucleic acids were separated in 1% agarose gel and blotted onto nylon membranes (Hybond-N, Amersham) by capillary transfer. The membranes were hybridized with 100 pg of digoxygenin-labeled probes at high stringency (42°C, 50% [v/v] formamide, 5× SSC, 3× Denhardt's solution, 0.5% [w/v] SDS, and 200 μg of salmon-sperm DNA). The nonspecifically bound probe was removed in three washing steps at 42°C, once in 2× SSC and 0.5% SDS for 15 min, and twice in 0.5× SSC and 0.5% SDS for 25 min. Hybridization signals were developed using the DIG luminescence detection kit (Boehringer Mannheim) according the protocol of Leroy (1997).

The probe was synthesized by PCR, using barley 2-CP cDNA (accession no. Z34917) or a genomic 2-CP fragment from Arabidopsis (accession no. X97910) as a template. The template was amplified with BAS-O1 and BAS-O4 at 45°C or 42°C, respectively, using the PCR DIG labeling mixture (Boehringer Mannheim). Extraction of RNA from plant tissues and northern-blot hybridization with radiolabeled Arabidopsis 2-CP cDNA (accession no. Y10478) and pea psbA were performed as described recently (Baier et al., 1996). Relative amounts of 2-CP mRNA were calculated from the signal intensities in northern blots using GELSCAN 3D software (BioSCITEC, Marburg, Germany).

PAGE, Western Blotting, and Antibody Preparation

PAGE, western blotting, and generation of the antiserum against heterologously expressed 2-CP were performed as described recently (Baier and Dietz, 1997). Dr. A. Radunz and Dr. G.H. Schmid (Universität Bielefeld, Germany) provided antibodies against other chloroplast proteins. They were raised in rabbits with purified proteins from tobacco as antigens. The generation and specificity of the antibody against SUE, purified from barley, was described by Betz and Dietz (1991). The relative protein amounts were calculated from the signal intensities in western blots using the GELSCAN 3D software.

Peroxidase and SOD Activity

Peroxidase activity was quantified by measuring the rate of guajacol tetramerization. The spectrophotometric assay contained 100 mM potassium phosphate buffer (pH 6.5), 2 mM guajacol, 1 mM H_2O_2, and the sample. Changes in absorption were monitored at 436 nm and rates were calculated using a molar extinction coefficient of 2550 M^{-1} cm^{-1}.

The determination of SOD activity was based on the inhibition of the photochemical reduction of nitroblue tetrazolium (Dhindsa et al., 1981). One relative unit corresponds to 1% inhibition of nitroblue tetrazolium reaction compared to a reaction mixture lacking the enzyme.

Chlorophyll *a* Fluorescence Measurements

We used chlorophyll *a* fluorescence as a nondestructive measure of photosynthetic activity with a PAM 101 (Walz, Effeltrich, Germany). Calculations of photosynthetic parameters were performed as described by Schreiber and Bilger (1993). For comparison of the mutants with the control plants, dark-adapted Arabidopsis seedlings were illuminated at a photon fluence rate of approximately 6000 μmol m^{-2} s^{-1}, which induced an emission of F_m by transient reduction of the primary quinone electron acceptor of PSII. During the following 1500 s of continuous actinic illumination at a photon fluence rate of 1100 μmol m^{-2} s^{-1}, the induction phase of photosynthetic CO_2 fixation was completed and a steady state of photosynthesis was reached. Concomitantly, the fluorescence yield decreased from the initial maximum to a lower value, F_s'. At intervals of 100 s, additional light pulses of 5000 μmol m^{-2} s^{-1} and 1-s duration were applied to transiently reduce the primary quinone electron acceptor of PSII and to determine the F_m' from which the effective quantum yield of PSII was calculated as $(F_m' - F_s')/F_m'$. Actinic light was turned off after 1500 s. The chlorophyll *a* fluorescence yield, excited by a weakly modulated and metabolically insignificant measuring beam of less than 0.05 μmol m^{-2} s^{-1}, was continuously monitored throughout the experiment. Lincomycin, an in-

hibitor of organellar protein synthesis, was applied by floating shoots of the seedlings on a 5 mM lincomycin solution in darkness for the time indicated.

RESULTS

Construction, Selection, and Verification of 2-CP Antisense Mutants

Transgenic mutants with reduced levels of 2-CP were generated in the genetic background of Arabidopsis. The cDNA fragment encoding the mature form of barley 2-CP (accession no. Z34917; Baier and Dietz, 1996a), with a 79.9% sequence homology to Arabidopsis cDNA, was chosen for antisense suppression of the endogenous 2-CP gene instead of the homologous Arabidopsis cDNA (accession no. Y10478; Baier and Dietz, 1996b). We expected the heterologous cDNA to be sufficient for mRNA suppression. To produce high-suppression intensities, the barley cDNA was fused to the constitutively expressed and highly active cauliflower mosaic virus 35S promoter (Holtorf et al., 1995). Transgenic plants were generated containing the T-DNA without the cDNA insert to serve as a control.

Transformed plants were selected on MSAR plates containing 50 μg mL^{-1} kanamycin. The insertion of the barley 2-CP cDNA fragment in the Arabidopsis genome was verified by PCR, using barley 2-CP-specific primers with genomic DNA as the template. The inserted heterologous barley cDNA can easily be distinguished from the endogenous Arabidopsis gene, which contains two short, intervening introns. It results in a PCR product of 585 bp, whereas the barley cDNA yields a 273-bp DNA product. This 273-bp product was detected in all antisense mutants and was absent in the control plants transformed with insertless pPCV702 (data not shown).

When we analyzed the transformed lines for phenotypic performance, the most conspicuous phenotypic distinction between wild-type plants and antisense lines appeared during the early rosette stage, when the plants were grown on MSAR plates. After sterilization and spreading, wild-type and antisense plants germinated with similar efficiency and kinetics. Further development of some antisense lines was delayed but became indistinguishable in the late rosette stage (data not shown). Even the various mutant lines exhibited a wide range of phenotypic variation; the mutant lines, bas-23 and bas-24, expressed the most striking phenotype of retardation, which we then chose for further analysis.

Bas-23 and bas-24 are independent mutants with T-DNA insertion at different positions of the genome. Southern-blot hybridization of genomic *Eco*RI digests with digoxygenin-labeled barley 2-CP cDNA revealed one signal in mutant bas-23 and two in mutant bas-24. *Eco*RI cut the T-DNA close to the 5′ end of the cauliflower mosaic virus 35S promoter. The barley 2-CP cDNA contained no *Eco*RI restriction site. As a consequence, each *Eco*RI fragment detected by hybridization represented one cDNA insertion. In the genomic Southern blot depicted in Figure 1, one *Eco*RI fragment of 3400 bp was labeled in lane bas-23 and two fragments of 1000 and 1400 bp were labeled in lane bas-24. We concluded that the mutant bas-23 contained one cDNA insertion, whereas two gene copies were inserted in the genome of mutant bas-24. The difference in the size of the cDNA-containing fragments demonstrated that the T-DNA was integrated at different positions of the genome in bas-23 and bas-24.

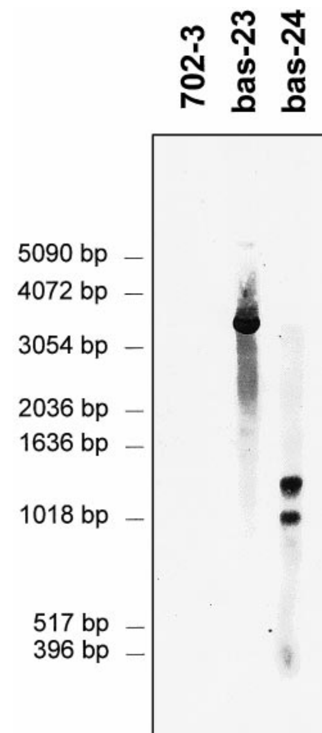

Figure 1. Detection of *Eco*RI fragments containing the barley 2-CP cDNA in genomic DNA extracted from mutants bas-23 and bas-24 and control (702-3) plants. Ten micrograms of genomic DNA was digested with *Eco*RI and separated on a 0.9% (w/v) agarose gel. The nucleic acids were blotted on a nylon membrane and hybridized with the 273-bp barley 2-CP cDNA fragment under high-stringency conditions.

Leaf Contents of 2-CP Transcript and Protein

During plant transformation, insertion of the T-DNA took place at various positions of the genome and in different copy numbers. Such positional and gene-dosage effects determined the expressional intensity that usually varied between mutant lines. To determine the degree of 2-CP suppression in transgenic plants, the transcript level was analyzed in mutant lines by northern-blot hybridization and compared with that of the control plants, 702-3. The autoradiogram (Fig. 2A) shows significantly decreased 2-CP mRNA levels in the mutants bas-23 and bas-24. From the signal intensities in the northern blot, a reduction of 2-CP in the amount of 64.4% was calculated for mutant bas-23 and 60.0% for mutant bas-24. Insignificant or no reduction was seen in bas-22 (91% of control) and bas-32 (108% of control), showing only weak (bas-22) or no (bas-32) phenotype when grown on MSAR plates (data not shown).

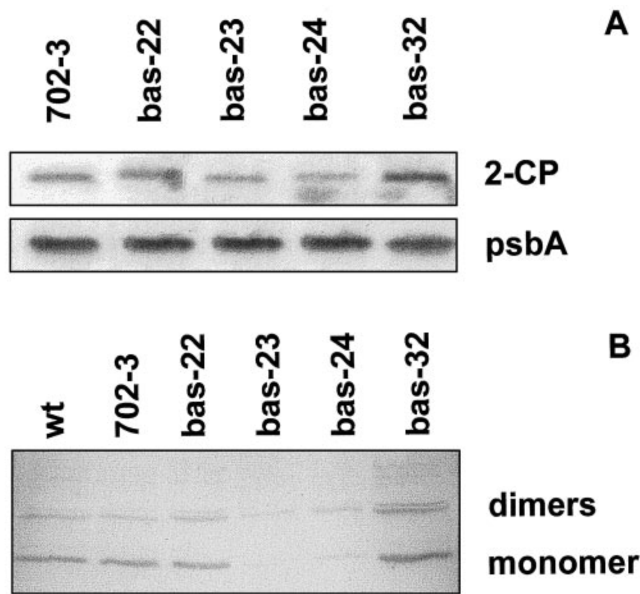

Figure 2. 2-CP expression in transgenic and control plants. A, Determination of 2-CP and psbA mRNA levels on northern blots with nucleic acids (20 μg per lane) from rosettes of 6-week-old mutants and control (702-3) plants grown on soil. The 2-CP mRNA was detected with radiolabeled 2-CP cDNA. Duplicate filters were hybridized with pea psbA. B, Detection of 2-CP protein in western blots. Protein extracts of 6-week-old mutants, wild-type (wt), and control (702-3) plants corresponding to 20 mg fresh weight were separated by PAGE and blotted on nitrocellulose membranes. The 2-CP was detected using an antibody against the mature form of barley 2-CP. Depending on the redox state, the protein was detected as monomer or dimer, respectively.

The 2-CP protein content followed the mRNA pattern and was strongly reduced in young, developing rosettes of mutants bas-23 and bas-24 (Fig. 2B). Quantification of western blots revealed a decrease in 2-CP protein in the amount of 53% to 88% in mutant bas-23 and 42% to 82% in mutant bas-24 (Fig. 2B). The protein content decreased only in young leaves and reached control levels in mature leaves (Fig. 3). This indicated the accumulation of 2-CP during leaf aging, even in mutants with decreased 2-CP expression. The assumed accumulation during leaf organogenesis corresponded to data published for barley (Baier and Dietz, 1996a). During leaf development, the 2-CP protein amount continued to increase, although the mRNA decreased to very low levels after the termination of leaf elongation.

Quantum Yield of PSII

The plant 2-CP is a nuclear-encoded chloroplast protein (Baier and Dietz, 1997). The sequence homology to yeast thioredoxin-dependent peroxide reductase (Baier and Dietz, 1996b, 1996c) and the increased tolerance to peroxide treatment of *Escherichia coli* expressing barley 2-CP (Baier and Dietz, 1997) implied a physiological function in the detoxification of alkyl hydroperoxides in photosynthetically active chloroplasts. Baier and Dietz (1997) then hypothesized that the 2-CP is part of the antioxidant defense protecting the photosynthetic machinery from oxidative damage.

The maximum quantum yield of PSII photochemistry (F_v/F_m) was similar in antisense and control plants and, therefore, independent of the expression of the antisense construct (Fig. 4). Conversely, the quantum yield of PSII electron transport was reduced during the steady-state photosynthesis of the young mutants with reduced 2-CP amounts. Figure 4 shows the quantum yield of PSII electron transport of the mutant line bas-23, as compared with the control plants transformed with "empty" pPCV702-T-DNA. Each point represents the average of five to seven determinations from at least 4 to 10 seedlings each.

In the light phase, differences in fluorescence parameters were small in 4-d-old seedlings and insignificant during dark relaxation (Fig. 4; first data point). In 9-d-old seedlings the quantum yield of PSII electron transport was reduced to 0.292 ± 0.073 in bas-23 during steady-state photosynthesis, compared with 0.325 ± 0.067 in the control plants. The difference in the fluorescence performance of the leaves was also evident in the phase of dark relaxation. In the mutants the quantum yield of PSII electron transport was 0.653 ± 0.010 after 1000 s in darkness, compared with 0.664 ± 0.037 in the controls. The quantum yield of the transgenic line bas-23 was even less efficient at an age of 2 weeks. When the rosettes reached their almost mature size at an age of approximately 6 weeks, the antisense line revealed improved photosynthetic performance (Fig. 4). Whereas the quantum yield of the control transformant was 0.265 ± 0.040 in the light and 0.643 ± 0.029 after 1500 s in the dark, the mutant line revealed values of 0.357 ± 0.058 in the light and 0.689 ± 0.021 in the dark.

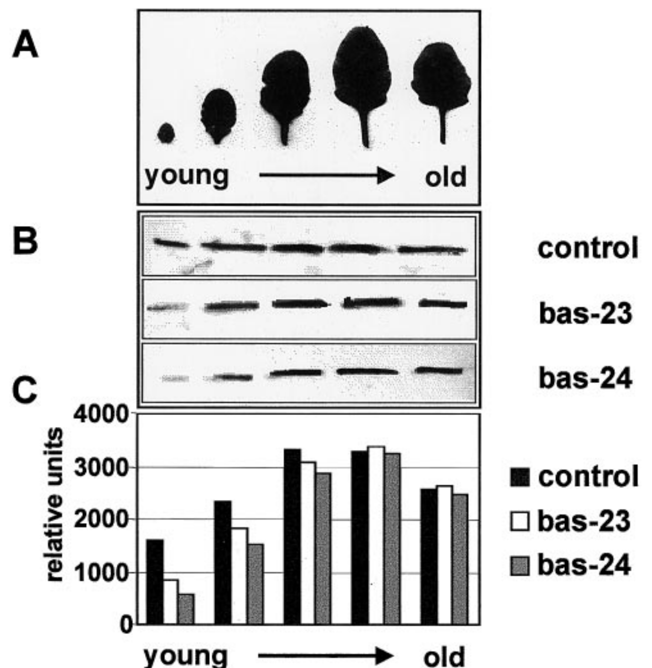

Figure 3. Western-blot analysis of 2-CP in 14-week-old plants (late rosette stage). 2-CP protein was detected in leaves of different ages (A) using an antibody against heterologously expressed 2-CP (B). Quantification of the protein amounts (C) was performed by evaluating band density.

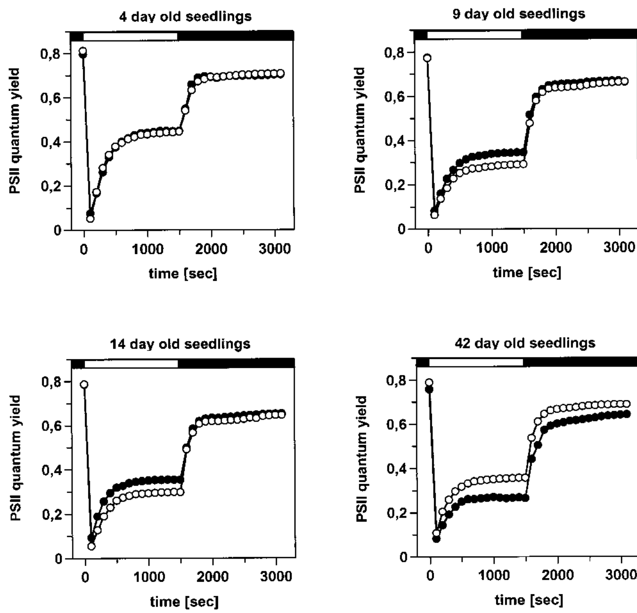

Figure 4. Quantum yield of PSII electron transport of the mutant bas-23 (○) and control 702-3 (●). Chlorophyll *a* fluorescence was measured in 4-, 9-, 14-, and 42-d-old, dark-adapted plants during a 30-min illumination period (white bars) followed by a subsequent 30-min dark period (black bars).

Importance of Chloroplast Protein Biosynthesis

After the inhibition of chloroplast protein synthesis by lincomycin administration, the phenotypic effect of young mutants was enhanced (Fig. 5). The quantum yield of PSII electron transport was slightly reduced in control leaves upon treatment with lincomycin for 4 and 8 h in the light and in the consecutive dark periods, respectively, as depicted in Figures 4 and 5. Compared with the controls, antisense mutants showed increased fluorescence quenching in the light, indicating a decreased quantum yield of PSII electron transport. The large difference in chlorophyll *a* fluorescence was maintained upon transfer to dark. The decreased F_v/F_m at steady state in the dark, after a light treatment, suggests that photodamage may have occurred.

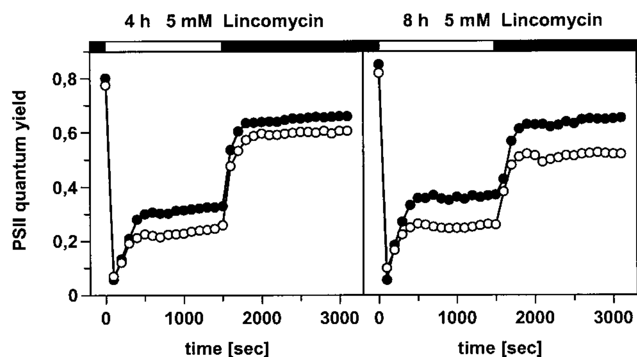

Figure 5. Quantum yield of PSII electron transport of the mutant bas-23 (○) and control line 702-3 (●) after feeding lincomycin for 4 and 8 h. Chlorophyll *a* fluorescence was measured in dark-adapted 8-d-old plants grown on soil. A 30-min illumination period (white bars) was followed by a 30-min dark period (black bars).

This result demonstrates that in 2-CP antisense mutants the recovery from photoinhibition depended on chloroplast-protein synthesis to a much greater extent than in the controls. The lincomycin treatment accentuated the metabolic regulation and/or the damage in the plants with a suppressed 2-CP level.

Protein Degradation in Chloroplasts

Well-known targets of oxidative stress are the D1 protein in the reaction center of PSII (Mattoo et al., 1984), Rubisco (Mehta et al., 1992), and LHCII (Rintamäki et al., 1997). We used western-blot analysis to determine the abundance of these proteins in the antisense mutants bas-23 and bas-24, and in the control plants. The D1 protein amount was strongly decreased in the mutants bas-23 and bas-24, as was the CFI, Rubisco, and the LHCII (Fig. 6). As indicated by the constant amount SUE, the damage to proteins was more likely to be restricted to chloroplasts. In comparison with the data on the psbA message level shown in Figure 2A, we concluded that the reduction of D1 protein abundance was not a consequence of decreased gene expression but of D1 degradation.

Peroxidase and SOD Activity

The age-dependent relief of the inhibition of photosynthesis shows the capacity of the antisense plants to com-

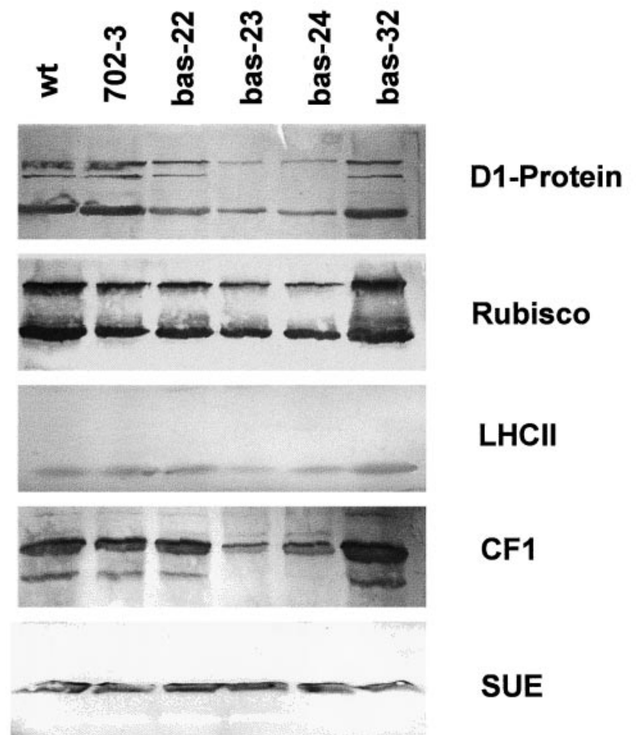

Figure 6. Western-blot analysis of D1, Rubisco, LHCII, CF1, and SUE protein amounts in extracts of 6-week-old mutants and control plants grown on soil. Protein extracts corresponding to 20 mg (fresh weight) were separated by PAGE, blotted on nitrocellulose membranes, and analyzed with the respective antibody.

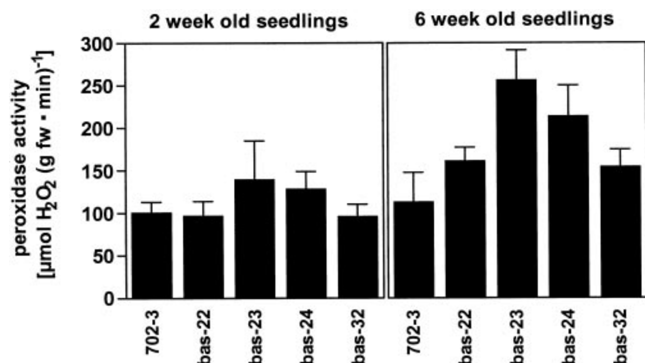

Figure 7. Total peroxidase activity in 2- and 6-week-old Arabidopsis mutant seedlings grown on soil. fw, Fresh weight.

pensate for the loss of 2-CP. In the mutants the 2-CP amount was up-regulated to control levels during leaf organogenesis (Fig. 3). However, the overcompensation in the 6-week-old seedlings, as indicated by the increase in the quantum yield of PSII electron transport (Fig. 4), cannot be explained by the accumulation of 2-CP protein alone. As depicted in Figure 2, the overall 2-CP content was still reduced in the rosettes of the 6-week-old seedlings. Therefore, we postulate that the imbalance in alkyl hydroperoxide metabolism in young leaves caused increased oxidative stress, which then induced other antioxidant defenses. We measured guajacol peroxidase activity as a first indicator. It constitutes a general stress response; Van Assche et al. (1988) has shown that in some cases it allows for quantitation of the stress dose.

In the 2-CP mutants bas-23 and bas-24, a slight and insignificant increase in peroxidase activity was observed in the early rosette stage (2-week-old seedlings). Four weeks later (6-week-old seedlings), the peroxidase activity had more than doubled in the line bas-23 (226% of control) and in mutant bas-24 (189% of control) plants (Fig. 7), indicating an induction of antioxidant defenses.

Total leaf SOD activity was measured in the 2, 4, and 6-week-old control (line 702) and in the mutant plants (bas-23 and -24). SOD activity increased in all developmental stages; for instance, in 2-week-old seedlings it rose from 26.5 $\pm$ 5.7 relative units in the controls to 37.7 $\pm$ 8.5 and 33.0 $\pm$ 5.7 in the antisense mutants (six determinations with three replicates each; significance in the paired t test >95%).

DISCUSSION

A series of in vitro and in vivo studies has established that 2-CPs reduce alkyl hydroperoxides to the corresponding alcohols and hydrogen peroxide to water (Chae et al., 1994; Poole and Ellis, 1996). After their first identification in plants (Baier and Dietz, 1996a), they were shown to be nuclear-encoded proteins that were posttranslationally imported into chloroplasts, and a physiological role in the antioxidative defense of chloroplasts was hypothesized (Baier and Dietz, 1997). The experiments described in this paper can be used to evaluate this hypothesis.

The 2-CP Is Part of the Antioxidant Network Protecting the Photosynthetic Apparatus

Among a number of Arabidopsis lines transformed with barley 2-CP cDNA, two independent antisense mutants were identified that expressed the 2-CP on a significantly reduced level, compared with appropriate control or wild-type plants. Reduced 2-CP expression resulted in decreased photosynthetic activity and increased degradation of soluble as well as membrane-bound chloroplast proteins (Figs. 4–6).

Light-stimulated degradation of chloroplast proteins is known to be tightly related to photooxidative processes (Mehta et al., 1992; Steiger and Feller, 1997). Conversely, the light-dependent increase in stromal ATP does not significantly affect the proteolytic degradation of chloroplast proteins, although the proteolytic reaction consumes ATP (Steiger and Feller, 1997). Damage followed by degradation can be caused directly by ROS derived from electron leakage of the photosynthetic light reaction or indirectly by oxidation, e.g. by organic hydroperoxides formed through uncontrolled oxidation in chloroplasts. An alkyl hydroperoxide reductase such as 2-CP may protect chloroplast proteins from indirect oxidation cascades. Their increased degradation in 2-CP mutants demonstrates that plant 2-CP is indeed involved in the protection of chloroplast structures.

The decrease in chloroplast protein levels (Fig. 6) revealed the polysymptomatic effect of the 2-CP suppression. A loss of D1 protein, LHCII, CF1, and Rubisco may reduce the quantum yield of photosynthesis by destruction of the D1 protein, by increased antenna quenching, by increased high-energy-state quenching (as a result of decreased activity of the gradient-relaxing ATP synthase), or by decreased energy consumption in the Calvin cycle (Schreiber and Bilger, 1993). The number of proteins affected may indicate that 2-CP is involved in an indirect protection mechanism of chloroplast metabolism. Inhibited detoxification of alkyl hydroperoxides that mediate oxidation of biomolecules over long distances may provide a sufficient physiological basis for the phenotype.

In this context it is interesting to note that Russell et al. (1995) reported a remarkably high stability of D1 protein in Arabidopsis. Loss of D1 protein occurred only when illumination at the high rate of 1350 μmol m^{-2} s^{-1} continued for more than 5 h (Russell et al., 1995). In contrast to the reported stability of D1 protein, the amount of D1 protein was markedly reduced in the transgenic Arabidopsis bas-23 and bas-24 plants grown at PAR of 200 to 300 μmol m^{-2} s^{-1} (Fig. 6). These data demonstrate the requirement of leaves for 2-CP in the maintenance of efficient photosynthesis. 2-CP knock-out mutants of the cyanobacterium *Synechocystis* PCC 6803 (Klughammer et al., 1998), which serves as a prokaryotic model for investigating photosynthetic metabolism, showed that 2-CPs are not essential for photooxidative metabolism. In the mutants that survived, however, doubling times were increased from approximately 8 to 14 h (Klughammer et al., 1998).

Compensation of the Antisense Suppression of 2-CPs

Chlorophyll *a* fluorescence analysis demonstrated that the phenotype developed only transiently; indications of metabolic adaptation were observed in 6-week-old mutants with reduced 2-CP mRNA amounts. By deduction, three processes appear to be involved in the adaptive response: (a) posttranslational accumulation of 2-CP, (b) de novo synthesis of proteins, and (c) induction of expression of other components of the antioxidant network.

Accumulation of 2-CP Protein during Leaf Organogenesis

The 2-CP protein accumulated up to control levels even in the leaves of mutants with the most suppressed transcript levels (Fig. 3). 2-CP amounts were reduced only in young developing leaves. It has to be concluded that translational and especially posttranslational regulation facilitates 2-CP accumulation in the mutant plants.

Gene expression was highest in young dicot leaves (M. Baier and K.-J. Dietz, unpublished data) and in developing parts of monocot leaves (Baier and Dietz, 1996a). Conversely, the amount of protein increased with aging. In the mature blade of barley primary leaves, 2-CP protein amounts continued to increase, although the endogenous mRNA had dropped to a low level (Baier and Dietz, 1996a). Such posttranslational accumulation can compensate for the suppression of 2-CP transcript in transgenic Arabidopsis.

The accumulation of 2-CP protein during leaf aging (Fig. 3) results in an increased metabolic potential to reduce alkyl hydroperoxides. However, it appears insufficient to account for the overcompensation observed in the experiments depicted in Figure 4. The chlorophyll *a* fluorescence data indicated an improved photosynthetic performance in high light in 6-week-old mutants (Fig. 4), although the 2-CP protein amount was still reduced (Fig. 2).

De Novo Protein Synthesis Compensates 2-CP Activity

Pretreatment of mutants with lincomycin enhanced the difference in light-dependent chlorophyll fluorescence quenching between controls and mutants, and it inhibited the recovery of the quantum yield of PSII electron transport in 2-CP antisense plants upon darkening (Fig. 5). Conversely, the lincomycin treatment had little effect on control plants.

Lincomycin selectively inhibits protein synthesis at 70S ribosomes but does not affect de novo synthesis of proteins at 80S ribosomes, which are the sites of 2-CP synthesis and of other chloroplast antioxidant enzymes, such as ascorbate peroxidase (Jespersen et al., 1997) and PHGPx (Mullineaux et al., 1998). Therefore, we conclude that the lincomycin experiments indicate the general effect of organellar protein synthesis.

Damage of D1 protein (Mattoo et al., 1984), Rubisco (Mehta et al., 1992), and LHCII (Rintamäki et al., 1997) is stimulated by photooxidative stress. Figure 6 shows that the levels of these proteins as well as those of CF1 decreased in 2-CP antisense mutants. In the case of the D1 protein, repair is well studied and known to depend on de novo protein synthesis and replacement (Hideg, 1997). Inhibition of the repair mechanism by lincomycin accentuated the photoinhibiton in 2-CP antisense mutants (Fig. 5). Thus, it can be concluded that de novo synthesis of chloroplast proteins greatly compensates for the damage in untreated mutant plants.

Induction of the Antioxidant Network

The accumulation of 2-CP and the replacement of damaged proteins are likely to increase the protective potential of the mutants, but these responses do not explain the overcompensation observed. A response of the antioxidant network must be assumed. However, all important chloroplast antioxidant enzymes, e.g. the stromal and thylakoid-bound ascorbate peroxidases (Jespersen et al., 1997) and enzymes involved in the biosynthesis and reduction of low-M_r antioxidants, e.g. γ-glutamylcysteine synthetase (May and Leaver, 1994) and glutathione reductase (Kubo et al., 1993), are nuclear-encoded enzymes. Signal transduction to the nucleus is necessary for induction. The nonspecific activation of antioxidant defense genes after pathogen attack, photooxidative stress, or application of other stressors indicates a common regulatory element linked to the cellular redox poise (Baier and Dietz, 1998). Leaf cells may sense the reduction of 2-CP activity as oxidative load. Consequently, expression of antioxidant defenses may be induced in the nucleus.

Peroxidase activity was measured as one well-characterized parameter in the response to oxidative stress (Macek et al., 1996). Its activity slightly increased in 2-week-old mutants with reduced 2-CP amounts and almost doubled in 6-week-old mutants. Its induction demonstrates a nuclear response to 2-CP suppression. Consequently, the induction of antioxidant defense genes could explain the overcompensation of 2-CP suppression on PSII activity. In addition to peroxidase, SOD activity also significantly increased in the 2-, 4-, and 6-week-old mutants. The induction of both enzymes supports the hypothesis that suppression of 2-CP causes a significant disturbance in the cellular redox state of photosynthesizing cells.

The chloroplast 2-CP is likely to detoxify alkyl hydroperoxides in the chloroplast (Baier and Dietz, 1997). In this context it is interesting to note that recently a nuclear-encoded PHGPx was shown to be targeted to the chloroplast stroma (Mullineaux et al., 1998). PHGPx also reduces alkyl hydroperoxides (Eshdat et al., 1997). However, the strong effect of 2-CP suppression on photosynthetic quantum yield, even under the low light conditions used in this communication, indicates that neither PHGPx nor the lipid hydroperoxide reductase associated with the envelope membrane (Bleé and Joyard, 1996) can substitute for the 2-CP. We suggest that, in addition to substrate specificity, suborganellar localization may define specific physiological functions of the enzymes. The lipid hydroperoxide reductase functions at the envelope membrane. The PHGPx is reported to be located in the stroma (Mullineaux et al., 1998). Conversely, the 2-CP appears to be preferen-

tially attached to stroma-exposed thylakoids (M. Baier, U. Kahmann, H.-G. Ruppel, and K.-J. Dietz, unpublished data). Thus, 2-CP may represent the important detoxification device in close vicinity of the thylakoids needed for safe reduction of highly reactive alkyl hydroperoxides.

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