Antisense Suppression of 2-Cysteine Peroxiredoxin in Arabidopsis Specifically Enhances the Activities and Expression of Enzymes Associated with Ascorbate Metabolism But Not Glutathione Metabolism[1]

Margarete Baier[2]*, Graham Noctor, Christine H. Foyer, and Karl-Josef Dietz

Stoffwechselphysiologie und Biochemie der Pflanzen, Universität Bielefeld, Universitätsstraβe 25, 33615 Bielefeld, Germany (M.B., K.-J.D.); and Biochemistry and Physiology Department, IACR Rothamsted, Harpenden, Hertfordshire AL5 2JQ, United Kingdom (G.N., C.H.F.)

The aim of this study was to characterize the effect of decreased 2-cysteine peroxiredoxin (2-CP) on the leaf anti-oxidative system in Arabidopsis. At three stages of leaf development, two lines of transgenic Arabidopsis mutants with decreased contents of chloroplast 2-CP were compared with wild type and a control line transformed with an empty vector. Glutathione contents and redox state were similar in all plants, and no changes in transcript levels for enzymes involved in glutathione metabolism were observed. Transcript levels for chloroplastic glutathione peroxidase were much lower than those for 2-CP, and both cytosolic and chloroplastic glutathione peroxidase were not increased in the mutants. In contrast, the foliar ascorbate pool was more oxidized in the mutants, although the difference decreased with plant age. The activities of thylakoid and stromal ascorbate peroxidase and particularly monodehydroascorbate reductase were increased as were transcripts for these enzymes. No change in dehydroascorbate reductase activity was observed, and effects on transcript abundance for glutathione reductase, catalase, and superoxide dismutase were slight or absent. The results demonstrate that 2-CP forms an integral part of the anti-oxidant network of chloroplasts and is functionally interconnected with other defense systems. Suppression of 2-CP leads to increased expression of other anti-oxidative genes possibly mediated by increased oxidation state of the leaf ascorbate pool.

Many metabolic reactions, such as photosynthesis, photorespiration, and respiration involve the formation of reactive oxygen species, particularly under extreme environmental conditions (Baier and Dietz, 1998a; Noctor and Foyer, 1998a; Foyer and Noctor, 2000). The anti-oxidant network hence plays a key role in survival. Various genes involved in anti-oxidant protection mechanisms are up-regulated when metabolic disorder occurs (Willekens et al., 1994; Kubo et al., 1995; Kliebenstein et al., 1998). 2-Cys peroxiredoxins (2-CP), which are known to be anti-oxidative enzymes in other organisms, have recently been described in plants (Baier and Dietz, 1999b). The nuclear-encoded plant homolog of animal thioredoxin peroxidases and bacterial alkyl hydroperoxide reductases (Baier and Dietz, 1996) was shown to be post-translationally targeted to chloroplasts (Baier and Dietz, 1997) where it protects the photosynthetic membrane from oxidative damage (Baier and Dietz, 1999a).

Complementation analysis demonstrated that the plant homolog rescues *Escherichia coli* 2-CP deletion mutants. 2-CP provided protection from otherwise lethal treatment with the alkyl hydroperoxide, cumene hydroperoxide (Baier and Dietz, 1998b). To evaluate its significance in the protection of photosynthetic function, the 2-CP gene was deleted in the blue alga *Synechocystis PCC 6803* (Klughammer et al., 1998). In the mutants, doubling time increased from 8 to 14 h, demonstrating the severe effect of 2-CP deletion on growth. The quantum yield of photosystem II was strongly decreased in the light, and photoinhibition occurred at quantum fluence rates of 200 $\mu mol\ m^{-2}\ s^{-1}$, whereas electron transport rates saturated at much higher light intensities in wild-type cells.

In transgenic Arabidopsis with decreased 2-CP, photosynthesis was impaired and degradation of chloroplast proteins like D1 protein, LHCP II, and chloroplast ATP synthase was accelerated (Baier and Dietz, 1999a). The antisense effect was lost during leaf development due to post-transcriptional accumulation of 2-CP protein to wild-type levels, apparently attributable to a long half-life of the 2-CP protein, and due to increased de novo synthesis of damaged proteins (Baier and Dietz, 1999a). Furthermore, assay of un-specific peroxidase activity pro-

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[2] Present address: Department of Molecular Genetics, John Innes Centre, Norwich, Norfolk NR4 7UH, UK.

* Corresponding author; e-mail margarete.baier@biologie.uni-bielefeld.de; fax 49–521–106–6039.

vided an indication that the anti-oxidant protection system was up-regulated to compensate for 2-CP deficiency (Baier and Dietz, 1999a). The transient phenotype was strongest in plants of 4 weeks of age. At 6 weeks, adaptation reactions had fully compensated the antisense effect, and analysis of chlorophyll *a* fluorescence revealed that the mutants had an even higher level of stress tolerance than control plants (Baier and Dietz, 1999a).

In addition to the un-specific peroxidase already measured (Baier and Dietz, 1999a), there are two types of peroxidases in leaves that might compensate for decreased 2-CP. These are glutathione peroxidases (GPx) and ascorbate peroxidases (APx). In chloroplasts, APx is the major H_2O_2-reducing peroxidase: Two distinct isoforms are present in the stroma and at the thylakoid membrane (Groden and Beck, 1979; Kelly and Latzko, 1979). However, APx does not reduce alkyl hydroperoxides that are putative substrates of 2-CP in addition to H_2O_2 (Asada, 1999). Therefore, GPx might be the more likely candidate to compensate for low 2-CP activity. Recently, a GPx isoform was shown to be post-translationally imported into chloroplasts where it is suggested to reduce alkyl hydroperoxides and H_2O_2 (Mullineaux et al., 1998).

In this paper we explore effects of 2-CP suppression on the anti-oxidant system in Arabidopsis. We report that 2-CP suppression does not affect foliar glutathione contents and redox state or GPx expression. However, the reduced ascorbate (Asc) pool was significantly modified as were associated enzymes. The data would suggest ascorbate-mediated control of gene expression.

RESULTS

Recent analyses of transgenic plants with decreased 2-CP indicate that this protein is crucial in protecting photosynthesis in stress conditions (Baier and Dietz, 1999a). Our aim here was to identify the role of 2-CP in leaf metabolism and the interaction of 2-CP with the anti-oxidative system. Accordingly, foliar contents of ascorbate and glutathione as well as anti-oxidative enzyme activities and transcript abundance were analyzed in 2-, 4-, and 6-week-old antisense, wild-type, and control plants (see "Materials and Methods"). Antisense plants were from two independent lines: bas-23, containing the T-DNA inserted at a single site, and bas-24, which contains two copies of the T-DNA (Baier and Dietz, 1999a). All tests were performed 60 min after onset of illumination in the morning to ensure standardized metabolic conditions.

The Ascorbate Pool Is More Oxidized, Whereas the Glutathione Pool Is Stabilized

At all leaf ages analyzed, the glutathione pool of 2-CP suppression mutants was similar to control plants, both in respect of contents (Fig. 1A) and redox state (data not shown). The glutathione pool was approximately 99% reduced in all plants at each time point of analysis. The mean concentration of glutathione calculated from all measurements was 0.765 ± 0.147 μmol mg^{-1} pheophytin. This corresponds to an average concentration of 0.455 mM in whole shoot tissue.

Total Asc contents were approximately 5 to 8 times higher than the glutathione contents in all plants. Although some variability was observed, total ascorbate contents (reduced + oxidized) were significantly increased in the 2-CP mutants by factors of 1.4 in 2-week-old seedlings (calculated from the mean values of mutant 23 and 24 compared with the means of wild-type and control plants), and 1.2 in 4- and 6-week-old seedlings (calculated from the mean val-

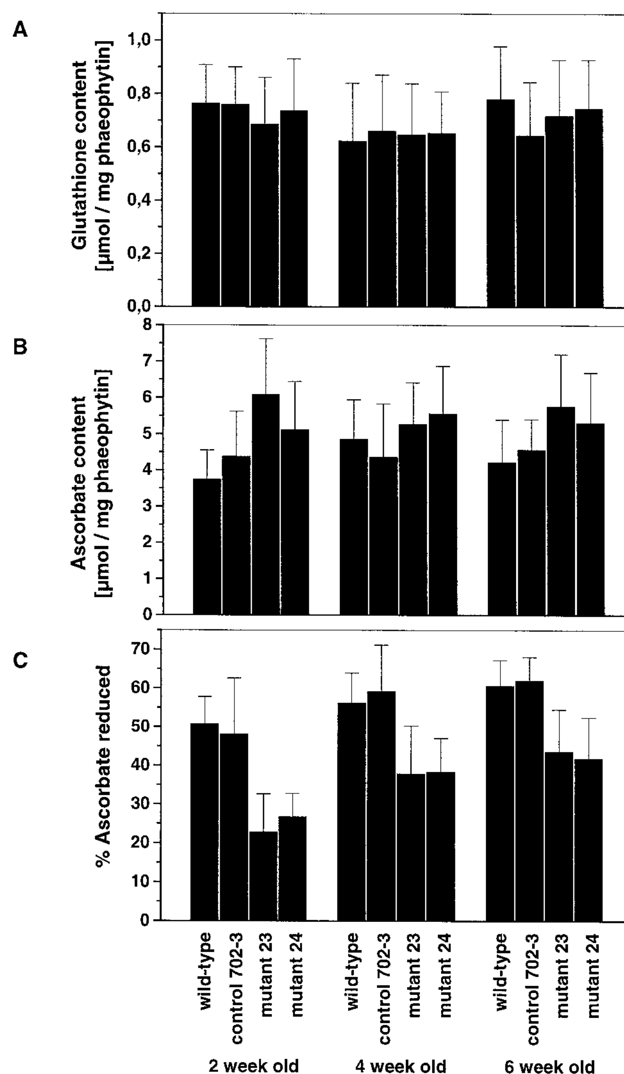

Figure 1. Total contents of glutathione (A), ascorbate (B), and redox poise of ascorbate (C) in 2-CP antisense mutants, wild-type, and control plants of Arabidopsis 60 min after onset of illumination (mean ± SD; *n* = 7–8 [ascorbate]; *n* = 6–8 [glutathione]).

ues of mutant 23 and 24 compared with the means of wild-type and control plants) (Fig. 1B).

In contrast to glutathione whose reduction state was similar in all plants, ascorbate was significantly ($P < 0.001$) more oxidized in the antisense plants compared with control plants and wild type. The difference in the redox state was strongest at 2 weeks, when only 24.8% ± 7.9% of the ascorbate pool was reduced in the mutants compared with 61.3% ± 6.4% in plants with wild-type 2-CP expression (Fig. 1C) and least at 6 weeks (Fig. 1C).

Transcript Abundance of Anti-Oxidative Enzymes

Only cDNAs and genomic DNA sequences from Arabidopsis var Columbia were used for primer design to ensure similar amplification conditions by maintaining identity of primer and target sequences. The linearity between signal intensity and transcript amount was established by comparing PCR signals from various dilutions of the cDNA probe and in the range of 20 to 25 cycles of amplification (data not shown).

The cDNA content was standardized according to the abundance of actin transcripts (Fig. 2), which provides a valid basis for comparison of mutants and controls at the same developmental stage. With aging of the plants the amount of actin transcripts decreased slightly. From reverse transcriptase (RT)-PCR probes standardized on the same amount of RNA the age-dependent decrease of actin transcript amount is estimated to be close to 10% (data not shown).

To analyze expression of the transgene, 2-CP transcripts were amplified with primers that do not distinguish between the endogenous Arabidopsis transcript and barley antisense RNA. The high transcript abundance in the antisense plants demonstrate that the transgene is active (Fig. 2). As shown previously by high stringency northern-blot analysis, overexpression of the transgene decreases the amount of Arabidopsis 2-CP mRNA (Baier and Dietz, 1999a). The reliability of RT-PCR analysis is supported by the age-related decrease in the signal intensity in control and wild-type plants, an observation previously reported using northern-blot analysis (Baier and Dietz, 1999a).

GPx1 is the only other cloned enzyme considered likely to be important in the reduction of alkyl hydroperoxides inside chloroplasts (Mullineaux et al., 1998). Its similar substrate spectrum suggests that it could potentially act as a partial substitute for 2-CP in the antisense mutants (Baier and Dietz, 1999b). It is important to note that GPx1 transcripts had a much lower abundance than those of 2-CP. The weak GPx1 signals shown in Figure 2 were obtained with 50 μL of PCR sample after 28 cycles and were detected at a 3.2 times higher light intensity than the 2-CP signals, which corresponded to a 20-μL reaction mixture after 25 cycles. Since annealing and elongation conditions were almost identical for all RT-PCRs, it is concluded that the 2-CP transcript amount exceeds that of GPx1

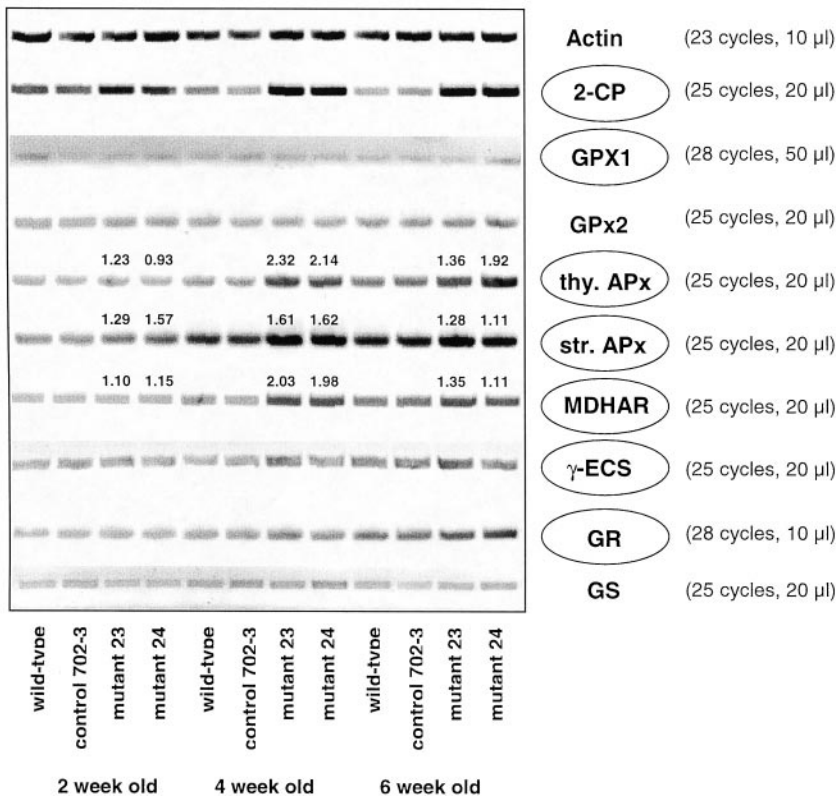

Figure 2. Transcript amounts of chloroplast and cytosolic enzymes involved in the anti-oxidant system of leaves in 2-, 4-, and 6-week-old 2-CP mutants, controls, and wild-type plants. Transcript level of cytosolic actin, chloroplast GPx1, cytosolic GPx2, thylakoid and stromal APx, MDHAR, chloroplast γ-glutamylcysteine synthetase, chloroplast GR, and cytosolic glutathione synthetase were detected by RT-PCR with gene-specific primers on agarose gels by ethidium bromide staining. The numbers above the bands show the induction level of thylakoid and stromal APx and MDHAR calculated from the differential integrated density of bands. Cycle numbers and amount of probe used for analysis are given in brackets. Chloroplast localization of the gene products is indicated by oval circles.

by approximately 100 times. The difference decreased with age because GPx1 transcript amount remained constant, whereas the 2-CP transcripts decreased. No difference was observed in the abundance of transcripts for either chloroplastic GPx1 or the putative cytosolic GPx2 between control and antisense mutants (Fig. 2).

In contrast to the cytosolic and chloroplastic GPx, the transcripts of thylakoid and stromal APx and chloroplast MDHAR were enhanced in the 2-CP mutants at 4 and 6 weeks (Fig. 2). The strongest relative increases were observed at 4 weeks. Thylakoid APx transcript abundance was increased by mean factors of 2.24 at 4 weeks and 1.64 at 6 weeks in the antisense mutants compared with the mean of control and wild-type plants. The concentrations of stromal APx and MDHA(R) transcripts were 1.61-fold and 2.01-fold higher at 4 weeks and 1.19-fold and 1.23-fold higher at 6 weeks in the mutants, respectively (Fig. 2). These numbers demonstrate that trends are similar for all three genes.

Transcript amounts for enzymes involved in glutathione biosynthesis (γ-glutamylcysteine synthetase, glutathione synthetase) were similar in all plants. Those for chloroplastic glutathione reductase (GR) were slightly increased in the mutants at 6 weeks in one experiment (Fig. 2) but not with two other cDNA probes. Transcripts for Fe-superoxide dismutase (SOD) (FSD1), Mn-SOD (MSD1), Cu/Zn-SOD (CSD3), and catalase (data not shown) showed no difference in abundance between the plants.

In summary, transcripts for two components of the ascorbate-dependent peroxide removal system in chloroplasts were induced in the mutants. In contrast, transcripts for glutathione-linked enzymes were largely unchanged. It is unfortunate that no cDNA sequences for dehydroascorbate (reductase) (DHAR) have been identified from Arabidopsis var Columbia to date. However, as shown in Table I, the extractable activity of this enzyme was similar in all plants.

Table I. *DHAR-, GR-, and catalase activity in leaves of 2-, 4-, and 6-week-old Arabidopsis plants. The 2-CP antisense mutants 23 and 24 are compared with wild-type and control plants transformed with pPCV 702 without 2-CP-cDNA*

	DHAR	GR	Catalase
	μmol DHA min × mg protein	*nmol GSSG min × mg protein*	*mmol H_2O_2 min × mg protein*
2-Week-old			
Wild-type	0.084 ± 0.068	39.55 ± 9.49	25.34 ± 7.04
Control	0.084 ± 0.050	39.19 ± 6.90	13.93 ± 8.97
Mutant 23	0.093 ± 0.077	37.32 ± 7.30	23.32 ± 6.02
Mutant 24	0.095 ± 0.060	39.57 ± 17.73	28.84 ± 12.16
4-Week-old			
Wild-type	0.140 ± 0.040	32.31 ± 5.19	76.12 ± 24.24
Control	0.137 ± 0.043	38.68 ± 8.54	70.04 ± 38.50
Mutant 23	0.151 ± 0.053	35.64 ± 9.60	64.90 ± 22.30
Mutant 24	0.130 ± 0.033	33.05 ± 10.28	68.77 ± 32.15
6-Week-old			
Wild-type	0.148 ± 0.035	41.00 ± 11.93	94.02 ± 25.09
Control	0.157 ± 0.053	42.81 ± 14.62	78.79 ± 22.02
Mutant 23	0.151 ± 0.053	37.92 ± 11.06	95.47 ± 35.18
Mutant 24	0.130 ± 0.033	38.00 ± 9.53	89.07 ± 22.88

Activities of Enzymes Involved in Anti-Oxidant Defense

Like DHAR, GR and catalase activities were similar in controls and mutants at all developmental stages analyzed (Table I), although DHAR and catalase did show a strong age-dependent increase in activity in all plants. Both chlorophyll and total soluble protein also increased with age but at all ages were similar between the plants (Fig. 3).

The activities of total and soluble SOD were slightly increased in 2-CP antisense plants (Fig. 4) and reached significantly ($P < 0.05$) higher levels in 6-week-old mutants and, in the case of total SOD activity, also in 4-week-old mutants. The increase was, however, very low (mean increase of between 1.17- and 1.38-fold). Similar to the slight induction of SOD, activities of total and soluble APx were increased by 1.2- to 1.3-fold (Fig. 5, A and B). These increases were significant ($P < 0.05$) only for soluble APx at 2 weeks and for total and soluble APx at 4 and 6 weeks. Similar relative increases were observed in soluble and membrane-bound APx (Fig. 5C). Much more striking was the marked increase in the activities of both NADH- and NADPH-monodehydroascorbate reductase (MDHAR) in the 2-CP mutants (Fig. 6). In all cases, NADH-MDHAR activity was only approximately 25% of the total MDHAR activity. This activity was enhanced by a factor of approximately 2, whereas the predominant NADPH-MDHAR activity was increased up to 8-fold in 4-week-old mutants.

DISCUSSION

The function of the chloroplastic protein 2-CP remains unknown, but the role of homologs in other organisms suggests that it acts to remove peroxides (Baier and Dietz, 1999b). A clue to the physiological significance of 2-CP comes from the observation that plants with decreased amounts of the enzyme display increased sensitivity to oxidative stress and photo-inhibition (Baier and Dietz, 1999a). The present work demonstrates that 2-CP interacts directly with the anti-oxidant network in leaves, most particularly the ascorbate pool, and that other anti-oxidant enzymes (MDHAR, APx) are increased when this protein is decreased.

Response of Chloroplast GPx to 2-CP Suppression

It is of interest to note that 2-CP transcripts were 100 times more abundant than GPx1, suggesting that 2-CP makes a major contribution to the detoxification

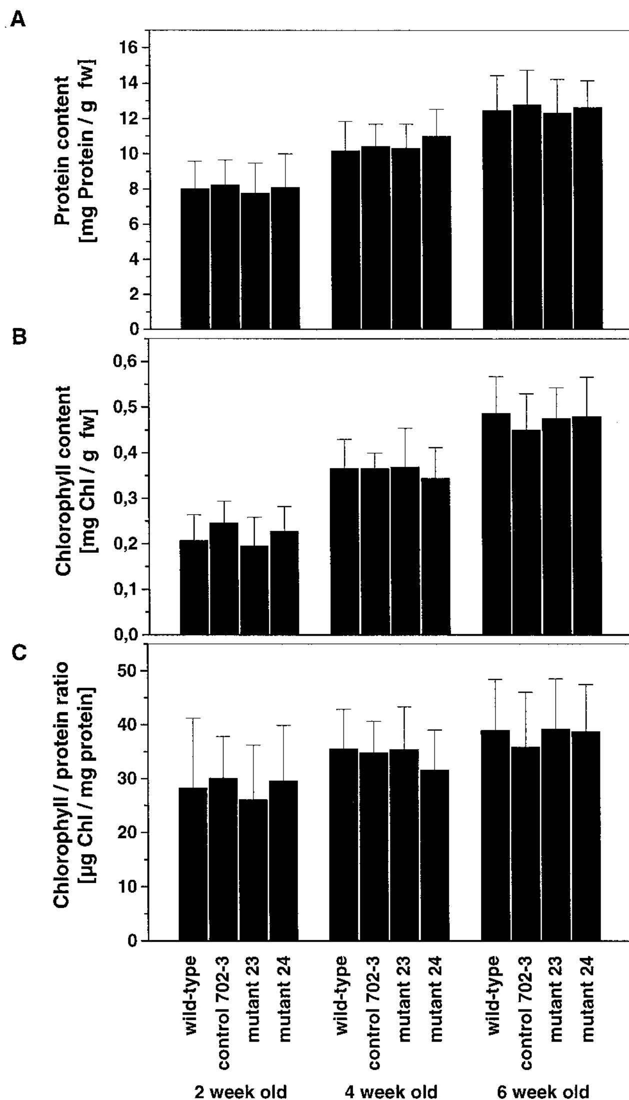

Figure 3. Total protein (A) and chlorophyll contents (B) and chlorophyll/protein ratio (C) of 2-, 4-, and 6-week-old 2-CP mutant, control, and wild-type plants (mean ± SD; $n = 23$).

of organic peroxides in the chloroplast. Like 2-CP, the GPx1 is a nuclear-encoded chloroplast protein (Mullineaux et al., 1998) thought to reduce both alkyl hydroperoxides and H_2O_2. In 2-CP antisense mutants, the unchanged transcript levels of chloroplastic GPx1 and cytosolic GPx2 demonstrate that there is no direct effect of decreased 2-CP on GPx transcripts.

Previous investigations indicated that other chloroplast anti-oxidant mechanisms cannot substitute for the function of 2-CP completely (Baier and Dietz, 1999a). The necessity for 2-CP and its irreplaceability by GPx may reflect a specific localization for the two enzymes, as suggested previously (Baier and Dietz, 1999b). Whereas GPx appears to be found in the stroma (Mullineaux et al., 1998), 2-CP is probably attached to the stroma-exposed thylakoid membrane (J. König, F. Horling, U. Kahmann, K.-J. Dietz, and M. Baier, unpublished data). It remains to be established whether the enzymes show distinctive substrate specificities.

Ascorbate Cycling in 2-CP Mutants

The most striking effects of decreased 2-CP are on the redox state of Asc and the expression and activity of MDHAR. The decreased Asc/DHA ratio indicates increased oxidative stress. Ascorbate, which is an anti-oxidant found in all aqueous phases of the plant cell, must be rapidly cycled in order to sustain H_2O_2 removal (Noctor and Foyer, 1998a; Asada, 1999). The question arises: How does a decrease in a membrane-associated anti-oxidative component lead to the specific oxidation of ascorbate? It should be noted that ascorbate, although water-soluble, participates in

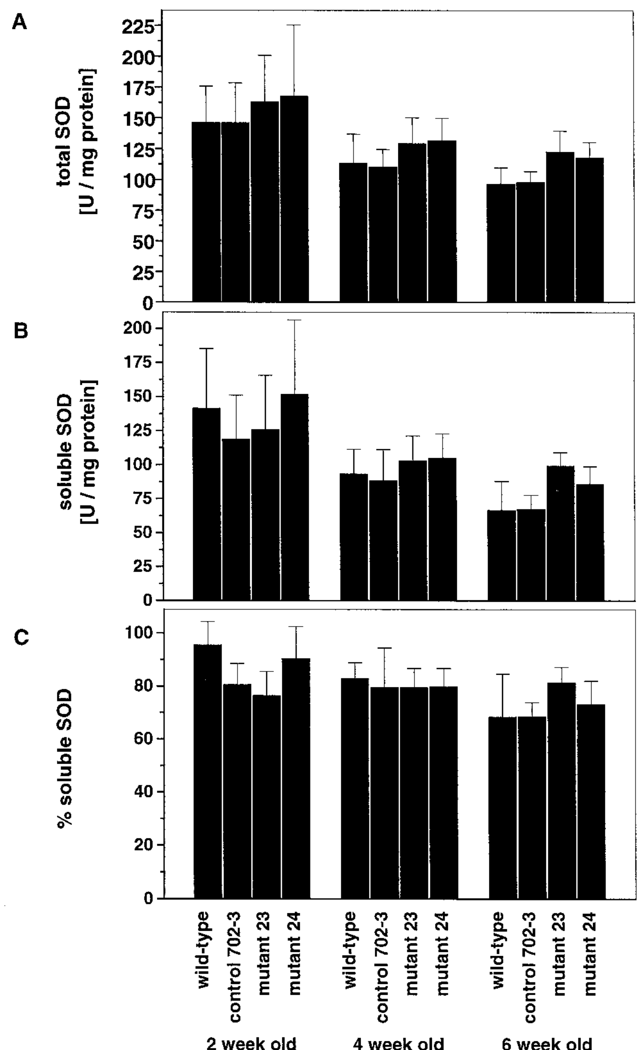

Figure 4. Total SOD (A), soluble SOD activity (B), and percent fraction of soluble SOD (C) of 2-, 4-, and 6-week-old 2-CP mutant, control, and wild-type plants calculated from inhibition of formazan formation by the xanthine/xanthine oxidase system with 1 relative unit corresponding to 50% inhibition (mean ± SD; $n = 6$).

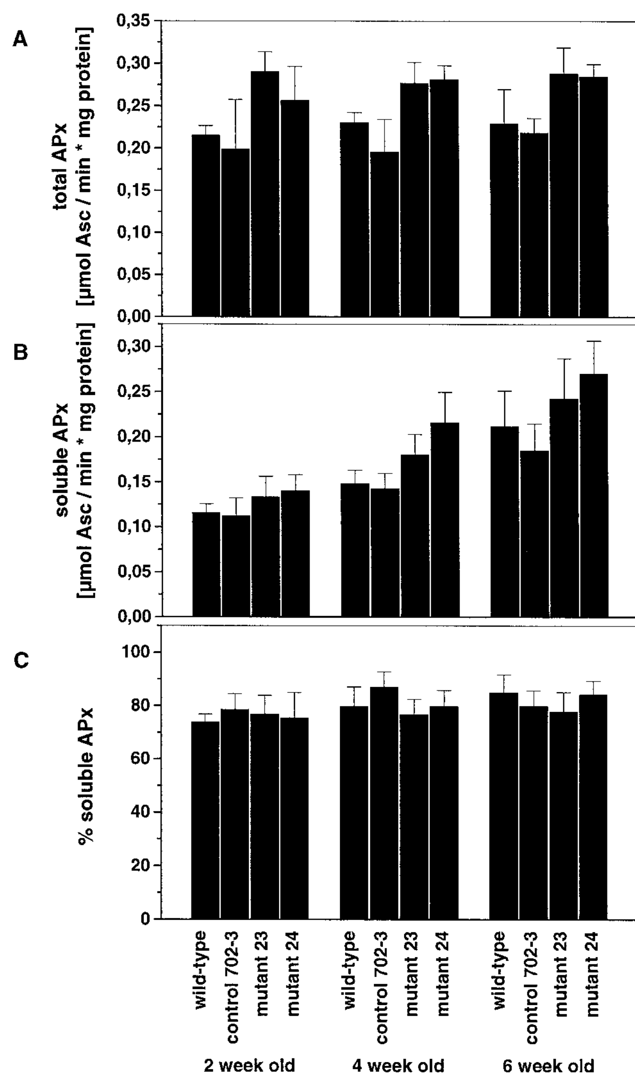

Figure 5. Total (A) and soluble APx activities (B) and percent fraction of soluble APx (C) of 2-, 4-, and 6-week-old 2-CP antisense mutant, control, and wild-type plants in aqueous extracts stabilized with 5 mM ascorbate (mean ± SD; $n = 6$).

many thylakoid reactions, most notably as a substrate for violaxanthin de-epoxidase and in the regeneration of α-tocopherol (Noctor and Foyer, 1998a). Ascorbate is also able to reduce components of the thylakoid electron transport chain. It is therefore possible that insufficient 2-CP activity shifts the burden of removing lipid peroxides to the ascorbate pool. This could occur via direct oxidation of ascorbate (acting as a primary anti-oxidant) or via accelerated oxidation of α-tocopherol (ascorbate acting as a secondary anti-oxidant). Additionally, if hydroperoxide detoxification capacity is decreased due to 2-CP suppression, organic hydroperoxides can accumulate, leading to acceleration of chain reactions and general accumulation of reactive oxygen species including H_2O_2 (Elstner, 1990). Finally, increased rates of Asc oxidation may be linked to increased flux through the reaction catalyzed by APx. If 2-CP is involved in direct H_2O_2 reduction in higher plants as previously shown for the cyanobacterium *Synechocystis* PCC 6803 (Yamamoto et al., 1999), the decrease of this activity in the mutants may result in increased Asc oxidation even though the extractable APx activity of this enzyme was not appreciably increased.

Redox Decoupling of the Ascorbate and Glutathione Pools

Although this study does not distinguish between anti-oxidant pools in the different intracellular compartments, the differential response of the ascorbate and glutathione couples in the mutants is worthy of comment. Maintenance of the glutathione redox state and oxidation of the ascorbate pool cannot be explained on purely thermodynamic grounds. The glutathione redox couple has a lower redox potential than that of ascorbate (Foyer and Noctor, 2000) and so should be preferentially oxidized. The present data clearly demonstrate that this does not occur in the 2-CP mutants, and therefore the two pools cannot be perfectly redox-coupled. The observations are also in contrast to those observed in catalase-deficient plants where the glutathione pool is indeed preferentially oxidized, whereas the ascorbate redox state does not change (Smith et al., 1984; Willekens et al.,

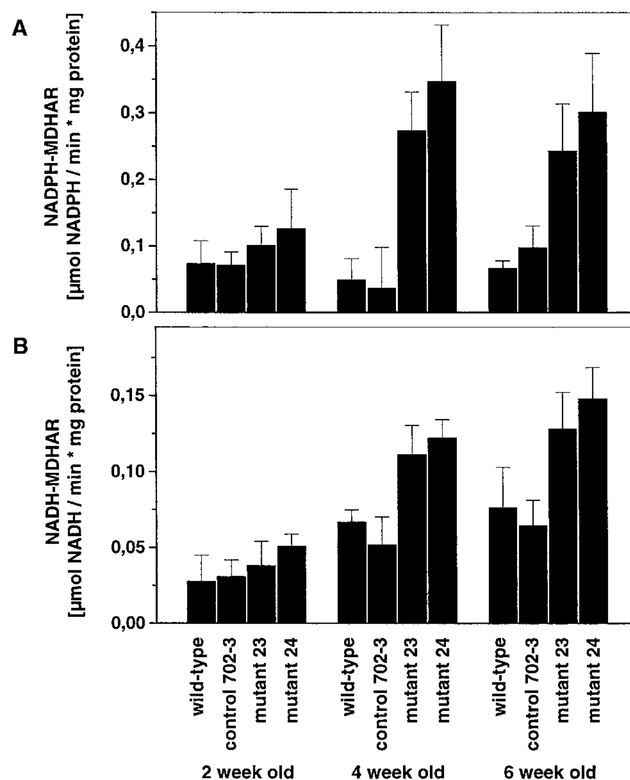

Figure 6. Soluble NADPH-MDHAR (A) and NADH-MDHAR activities (B) of 2-, 4-, and 6-week-old 2-CP mutant, control, and wild-type plants in aqueous extracts (mean ± SD; $n = 5$–6).

1997). One explanation of the present observations may be that the change in ascorbate redox state is entirely due to chloroplastic events and that redox-coupling of ascorbate and glutathione is less robust (or indeed less necessary) in this compartment than in others such as the cytosol. This may be because chloroplastic DHAR activity is low in Arabidopsis, as it is in some other species (Foyer and Mullineaux, 1998; Foyer and Noctor, 2000). Indeed, unlike APx and MDHAR, DHAR activity was not increased in the mutants. It should be noted that the chloroplast contains two other mechanisms of Asc regeneration. The first, nonenzymic reduction of MDHA by ferredoxin may have the largest capacity. The second, enzymic reduction by MDHAR was observed to increase markedly in the mutants. This increase, however, was not sufficient to maintain the same ascorbate redox state as that found in the controls (Fig. 1C).

Expression of Enzymes Closely Linked to the Ascorbate Pool Is Differentially Regulated

The results obtained with the 2-CP antisense mutants also provide insight into the regulation of gene expression of anti-oxidant enzymes. Stromal and thylakoid-bound APx, 2-CP, and all other chloroplast anti-oxidant enzymes are encoded in the nucleus and post-translationally imported by the chloroplast. It is well established that gene expression of anti-oxidant enzymes is regulated in response to the anti-oxidant demand. However, the nature of the redox signal and the signal transduction pathway(s) from the chloroplast to the nucleus is not yet understood (Escoubas et al., 1995; Allen and Nilsson, 1997; Baier and Dietz, 1998a; Karpinski et al., 1999).

The comparative analysis of 2-CP antisense and control plants yields information on the regulation of genes for ascorbate-linked enzymes. The unchanged redox state of the glutathione pool correlated with virtually unchanged transcripts or activities of enzymes involved in glutathione biosynthesis, glutathione regeneration, or glutathione-dependent DHA reduction. Similarly, the abundance of transcripts for Mn-SOD (MSD1; data not shown), Cu/Zn-SOD (CSD3; data not shown), Fe-SOD (FSD1; data not shown) were unchanged. In contrast, the increased oxidation state of ascorbate was accompanied by increased transcript abundance of stromal and thylakoid-bound APx as well as choroplast MDHAR.

Only transcripts for chloroplastic enzymes directly involved in ascorbate metabolism were significantly enhanced in response to 2-CP suppression. This points to a specific signal transduction pathway linked to or correlated with the ascorbate redox state. Whereas several previous studies have provided evidence of a role for glutathione in the regulation of gene expression (Wingate et al., 1988; Hérouart et al., 1993; Wingsle and Karpinski, 1996; Liere and Link, 1997), very few have considered the possibility of similar control by ascorbate. One study has shown specific changes in gene expression related to ascorbate concentration or redox state (Allen et al., 1995). Many investigations have emphasized the role of H_2O_2 in signal transduction, but it is becoming increasingly clear from studies such as our own that key components that interact with H_2O_2 are monitored as indicators of redox homeostasis and elicit changes in gene expression.

MATERIALS AND METHODS

Plant Material

This study used three types of Arabidopsis var Columbia (Baier and Dietz, 1999a). These were: (a) 2-CP antisense mutants (bas-23 and bas-24) expressing barley 2-CP cDNA in antisense orientation under control of the CaMV35S promoter; (b) control plants (line 702-3) containing the same T-DNA as bas-23 and bas-24 but no barley 2-CP cDNA; and (c) wild-type plants. All were grown in soil culture in a growth room at 25°C and a relative humidity of 50% to 60%. Plants were watered every 2 d and illuminated for 10 h at a photosynthetically active radiation of 75 μmol m^{-2} s^{-1} with FLUORA neon strips (Osram, Germany). Shoots of 2-, 4-, and 6-week-old seedlings were harvested in the morning 1 h after onset of illumination, immediately immersed in liquid nitrogen, and stored at −80°C until analysis. During harvest shading of the plant material was avoided.

Determination of Ascorbate and Glutathione

Asc was measured directly via the decrease of absorption at 265 nm after addition of ascorbate oxidase (Foyer et al., 1983). Total ascorbate was measured by the same assay after pre-incubation of extracts with dithiothreitol. Total glutathione and oxidized glutathione (GSSG) contents were analyzed using GR and vinylpyridine (Griffiths, 1980) with minor modifications as described by Noctor and Foyer (1998b). Recovery was checked by analyzing standards of 5 nmol (reduced glutathione [GSH] + GSSG) or (Asc + DHA) at a reduction state of 25%, 50%, 75%, and 100%, respectively, which were added to 100 mg of plant material before extraction. The results were corrected for metabolite losses using the recovery rates of 91% for total glutathione, 85% for GSSG, 83% for total ascorbate, and 94% for Asc.

Determination of Protein Contents and Biochemical Analysis of Enzyme Activities

The protein contents of aqueous extracts were quantified spectrophotometrically using the Bio-Rad Protein Assay according to the instructions of the supplier (Bio-Rad Laboratories, Munich). For determination of enzyme activities the frozen plant material was ground to a fine powder in liquid N_2 and extracted in buffer as indicated. For all enzyme measurements, except for APx where 5 mM ascorbate was included in the extraction medium, 100 mg of plant

material was extracted in 1 mL of 100 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid)-NaOH, pH 7.5. DHAR was always assayed first on account of its low stability by the following protocol. Twenty microliters of extract was added to 1 mL of reaction mixture containing 50 mM potassium phosphate (pH 6.5), 1 mM EDTA, and 2 mM DHA. The reaction was started by addition of 2.5 mM GSH. GSH-dependent DHA reduction was monitored at 265 nm (ϵ = 14 mM^{-1} cm^{-1}). For analysis of GR, GSSG-dependent NADPH oxidation was monitored at 340 nm (Foyer and Halliwell, 1976) in a 1-mL reaction volume containing 50 mM HEPES-NaOH (pH 7.8), 1 mM EDTA, 3 mM $MgCl_2$, 20 to 50 μL of plant extract, and 0.1 mM NADPH. The reaction was started by addition of 1 μmol GSSG. Catalase activity was analyzed polarographically (Rank oxygen electrode, Rank Brothers, Cambridge, UK) by determination of O_2 evolution upon addition of 0.05 M H_2O_2 final concentration to the reaction medium containing 100 mM HEPES-KOH (pH 7.4) and 5 to 20 μL of 1:100 diluted plant extract at 20°C (Clairborne, 1985). NADPH- and NADH-dependent MDHAR activities were assayed spectrophotometrically at 340 nm as MDHA-dependent NADPH oxidation by a modification of the method of Miyake and Asada (1992).

The assay mixture contained 50 mM HEPES-KOH (pH 7.6), 100 μM NADPH or NADH, 2.5 mM Asc, 20 to 100 μL of extract, and 0.1 U ascorbate oxidase. The linearity of MDHA formation by ascorbate oxidase was checked at 265 nm prior to analysis. SOD activity was measured at 560 nm by inhibition of formazan formation according to McCord and Fridovich (1969) in 50 mM potassium phosphate buffer (pH 7.5) supplemented with 0.5 mM nitroblue tetrazolium. For superoxide generation, 4 mM xanthine and 0.025 U xanthine oxidase were added. Because initial experiments with varying volumes indicated that SOD activity was linear with extract volume from 10 to 60 μL of extracts, this enzyme was routinely measured with 20 μL of undiluted extract. One unit is the activity that inhibits color formation by 50% (McCord and Fridovich, 1969). APx was assayed by a modification of the method of Hossain and Asada (1984). Total APx activity was measured in the supernatant produced by centrifugation of leaf extracts at 500*g* for 2 min. Soluble APx was measured in the supernatant produced by centrifugation at 10,000*g* for 5 min. In both cases, activity was monitored at 290 nm as H_2O_2-dependent oxidation of ascorbate (ϵ = 2.8 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM HEPES-NaOH (pH 7.6), 250 μM ascorbate, 10 to 20 μL of extract, and 0.3 mM H_2O_2.

Chlorophyll and Pheophytin Content

Prior to centrifugation, aliquots (50 μL) of crude extracts were transferred to 950 μL of 80% (v/v) acetone, mixed, and the pigments left to extract in the dark at −20°C. Pigments were assayed in the clear supernatant obtained after centrifugation for 5 min at 10,000*g*. Chlorophyll content was quantified according to Arnon (1949). For comparative expression of metabolite contents, phaeophytin was assayed according to Vernon (1960).

Statistical Analysis

For statistical analysis data of the 2-CP mutants 23 and 24 were combined and data of wild-type and control plants. From the calculated means and standard deviations the significance was determined by Student's *t* test. Data sets were designated significantly different, if the *P* value was below 0.05.

Nucleic Acid Extraction and RT-PCR

Nucleic acids were extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with RLT extraction buffer according to the supplier's recommendation. Residual DNA was removed with RNase-free DNase (GIBCO-BRL, Karlsruhe, Germany). To inactivate the DNase prior to cDNA synthesis, EDTA was added to a final concentration of 2.5 mM, and the samples were incubated at 70°C for 20 min. RNA yield was quantified spectrophotometrically, and the samples were checked for DNA contamination by a PCR of 35 cycles using the primers BAS-O1 and BAS-O4 (Baier and Dietz, 1999a), which give a 585-bp product with genomic DNA and a 273-bp product with cDNA. Reverse transcription was performed with 1 μg of total RNA and 0.2 μg of oligo(dT) (T_{15}) in 50 mM Tris(tris[hydroxymethyl]-aminomethane)-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM deoxynucleotides (0.5 mM dATP, dCTP, dGTP, and dTTP, each) with 200 U Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL for 1 h) at 37°C after denaturing the RNA for 10 min at 70°C and 5 min primer annealing phase at room temperature. The cDNA was diluted 100-fold in distilled water and standardized by PCR for actin contents using the gene-specific primers Atact-S (GAGAAGATGACTCAGATC) and Atact-A (ATCCTTCCTGATATCGAC). For all transcripts of interest gene-specific primers were derived from database entries (EMBL Aj000469, Ab001568, Y10478, X98926, X98925, Z29490, U22359, U37697, Af061520, Af061518, M55910, D88417, X64271).

To ensure similar amplification conditions, only 18- to 19-bp-long oligonucleotides were used as primers, which had a uniform GC content of approximately 45% and yielded products of 500 to 600 bp. The PCR conditions were optimized empirically by testing various annealing temperatures. The identity of the PCR products was verified by single-strand sequencing (IIT Bielefeld, Germany; MWG, Ebersberg, Germany) and sequence comparison with EMBL data bases using the Fasta3 service at EBI (Hinxton, UK). For quantitative PCR the cycle number was reduced until the reaction was in the linear range. Depending on the product concentration, 20- to 50-μL probes were loaded on 1% (w/v) agarose gels containing 0.5 μg/mL ethidium bromide after addition of loading buffer (final concentration: 40 mM Tris-acetate, pH 7.5, 10 mM EDTA, 3% [w/v] Ficoll, 0.1% [w/v] SDS, 0.01% [w/v] bromphenol blue, and 0.01% [w/v] xylene cyanol) and separated electrophoretically in a buffer containing 40 mM Tris-acetate (pH 7.5) and 1 mM EDTA at a constant voltage of 80 V. The bands were detected on a UV lamp with a CCD camera system (INTAS, Göttingen, Germany). For publication the gray scale was

inverted electronically without further modification to give black bands on a white background in view of a better printing quality. Band intensities were quantified with the GELSCAN software (BIOSCITECH, Marburg, Germany).

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