The Plant Cell, Vol. 9, 627–640, April 1997 © 1997 American Society of Plant Physiologists

Photosynthetic Electron Transport Regulates the Expression of Cytosolic Ascorbate Peroxidase Genes in Arabidopsis during Excess Light Stress

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Exposure of Arabidopsis plants that were maintained under low light (200 μmol of photons m^{-2} sec^{-1}) to excess light (2000 μmol of photons m^{-2} sec^{-1}) for 1 hr caused reversible photoinhibition of photosynthesis. Measurements of photosynthetic parameters and the use of electron transport inhibitors indicated that a novel signal transduction pathway was initiated at plastoquinone and regulated, at least in part, by the redox status of the plastoquinone pool. This signal, which preceded the photooxidative burst of hydrogen peroxide (H_2O_2) associated with photoinhibition of photosynthesis, resulted in a rapid increase (within 15 min) in mRNA levels of two cytosolic ascorbate peroxidase genes (*APX1* and *APX2*). Treatment of leaves with exogenous reduced glutathione abolished this signal, suggesting that glutathione or the redox status of the glutathione pool has a regulatory impact on this signaling pathway. During recovery from photooxidative stress, transcripts for cytosolic glutathione reductase (*GOR2*) increased, emphasizing the role of glutathione in this stress.

INTRODUCTION

In plant cells, a consequence of most if not all environmental stresses is an increased rate of production of reactive oxygen intermediates (ROIs), such as superoxide ($O^{2\cdot-}$), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), and singlet oxygen ($^1O_2$), which have both positive and deleterious effects (Bowler et al., 1992; Chen et al., 1993; Asada, 1994; Levine et al., 1994; Prasad et al., 1994). ROIs are known to be involved in such diverse processes as the hypersensitive response (HR) and systemic acquired resistance (Dixon and Lamb, 1990; Chen et al., 1993; Levine et al., 1994), chilling responses (Prasad et al., 1994), cross tolerance to different abiotic stresses (Bowler et al., 1992), and regulation of photosynthesis (Hormann et al., 1993). The dual role of ROIs, acting both as toxic compounds in the cell and also mediating the induction of stress tolerance, indicates a high degree of complexity in the metabolic systems involved.

Under low-light (LL) conditions and a constant photoperiod, the photosystem II (PSII) light-harvesting complex (LHC) antenna and the electron carrier systems in chloroplasts are adapted to minimize uncontrolled and inappropriate electron transfer. Because light is continuously absorbed by chlorophyll, excess light (EL) leads to the immediate maximum excitation of the chlorophyll molecules, and this may lead to the overproduction of electrons by the water-splitting system. Excess electrons can damage the reaction center of PSII, in particular the D1 protein, and may lead to the perturbation and inhibition of photosynthetic electron transport (Krause, 1988; Andersson and Styring, 1991; Andersson et al., 1992; Öquist et al., 1992; Aro et al., 1993; Russell et al., 1995). This phenomenon is known as photoinhibition of photosynthesis (Andersson and Styring, 1991; Andersson et al., 1992). The mechanisms leading to an inhibition of electron transport through PSII are not known, but the multiple reduction/oxidation (redox) reactions in PSII have a potential to create ROIs, and it is known that photoinhibition may lead to D1 protein degradation, probably due to an excess of ROIs (Krause, 1988; Andersson and Styring, 1991; Andersson et al., 1992; Öquist et al., 1992; Russell et al., 1995). Photoinhibitory stress also has a great potential to damage the LHC. The expression of genes encoding the photosynthetic apparatus, such as *LHC* for PSII (*LHCB*) in plants, is controlled by phytochrome (e.g., Millar et al., 1995).

The most relevant functions of reduced glutathione (GSH) in the context of oxidative stress are those in which GSH participates in redox reactions, and therefore, the oxidized form of glutathione (GSSG) is generated. Glutathione reductase (GR; EC 1.6.4.2) is a flavoprotein that catalyzes the reduction of GSSG to GSH in the presence of NADPH. The proposed ascorbate–glutathione cycle of plants (Foyer and Halliwell, 1976) and the redox cycle involving GR and glutathione peroxidase (GPX) in mammals (Schirmer et al., 1989) are examples of such reactions. Recently, other important roles of

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glutathione in oxidative stress responses have been considered. Many regulators in bacterial and mammalian cells, for example, *oxyR*, *soxR*, NF-κB, and AP-1, are directly sensitive to redox reactions (Malbon et al., 1987; Storz et al., 1990; Meyer et al., 1993; Hidalgo and Demple, 1994; Kullik and Storz, 1994; Ginnpease and Whisler, 1996). For example, GSH and H_2O_2 can inactivate and activate, respectively, the nuclear factor NF-κB in mammalian cells (Meyer et al., 1993; Ginnpease and Whisler, 1996).

In plants, many compounds have been nominated as agents involved in signaling both in biotic and abiotic stress responses. These compounds include salicylic acid, H_2O_2 (Chen et al., 1993; Levine et al., 1994; Prasad et al., 1994; Bi et al., 1995; Neuenschwander et al., 1995), GSH and GSSG (Wingate et al., 1988; Hérouart et al., 1993; Wingsle and Karpinski, 1996), calcium (Ca^{2+}; Price et al., 1994; Monroy and Dhindsa, 1995), and photoreceptors with Ca^{2+} (Neuhaus et al., 1993; Millar et al., 1995). However, very little is known about the signaling cascades initiated by these responses (Neuhaus et al., 1993; Millar et al., 1995). Wingate et al. (1988) found that high concentrations of GSH but not GSSG enhanced expression of genes encoding enzymes involved in phytoalexin and lignin biosynthesis and suggested a general role for GSH in signaling systems in biological stress. Different thiols such as GSH, cysteine, and DTT increased the transcript level of a reporter gene coupled with the cytosolic copper-zinc superoxide dismutase (CuZn-*SOD*) gene promoter in transgenic tobacco protoplasts (Hérouart et al., 1993). Recently, it was reported that changes in the redox status of glutathione have a regulatory impact on cytosolic and chloroplastic isoforms of CuZn-*SOD* gene expression in Scots pine (Wingsle and Karpinski, 1996).

H_2O_2 is the most stable of the ROIs and can behave as both an oxidant and a reductant, although it has a low ability to react with most organic molecules if no metal catalysts are present (Salin, 1987). The electron transfer chain of the chloroplasts is the best-documented source of H_2O_2 (Mehler, 1951; Asada, 1994). Mitochondria and peroxisomes are also major sources of H_2O_2 (Cadenas, 1989; del Rio et al., 1992). In the light, the key enzyme involved in H_2O_2 scavenging is ascorbate peroxidase (APX; EC 1.11.1.11), which catalyzes the reaction 2 ascorbate + $H_2O_2 \rightarrow$ 2 monodehydroascorbate + $2H_2O$. This enzyme, together with SOD, monodehydroascorbate reductase (MDR), dehydroascorbate reductase (DHR), and GR, is thought to constitute the major defense system against ROIs in the chloroplast (Foyer and Halliwell, 1976; Asada, 1994). Similarly, the defense system against ROIs in the cytosol is proposed to consist of APX, SOD, catalase (CAT), GPX, GR, MDR, and DHR (Foyer and Halliwell, 1976; Asada, 1994; Creissen et al., 1994).

We set out to determine whether the levels of mRNAs encoding the enzymes of ROI metabolism could be altered in Arabidopsis plants subjected to photooxidative stress brought on by excess light. Surprisingly, these experiments have provided the first indication of a novel signal transduction pathway in higher plants.

RESULTS

Functions of PSII during Excess of Light and the Poststress Period

The meanings of the photosynthetic parameters used in this study are given in Methods. Exposure of 4-week-old Arabidopsis plants, grown in LL conditions (200 μmol of photons m^{-2} sec^{-1}) and an 18-hr photoperiod, to a 10-fold increased irradiance (2000 μmol of photons m^{-2} sec^{-1}) for 1 hr caused substantial photoinhibition of photosynthesis, as indicated by the decline in the maximum photochemical efficiency of photosynthesis. A significant drop in the variable fluorescence (F_v) and maximum fluorescence (F_m) ratio (F_v/F_m) was detected after 7 min and reached a minimum after 2 hr of the poststress period (Figure 1). The slow recovery of photosynthesis was confirmed by an increase in this parameter. After 24 hr of poststress, F_v/F_m reached almost the same value as in control plants (Figure 1). Zero fluorescence (F_0) increased during photoinhibition and declined during the poststress period (data not shown). The photochemical quenching parameter (q_p) decreased after 30 min of EL, reached a minimum after 2 hr of the poststress period, and increased after 24 hr of poststress (Figure 1). The nonphotochemical quenching parameter (q_n) increased after 30 min, achieved a maximum after 2 hr of recovery, and decreased after 24 hr of poststress. Neither q_p nor q_n recovered to the control value after 24 hr, indicating that the chloroplasts suffered severe photoinhibition (Figure 1). In addition, photosynthetic oxygen evolution decreased during 1 hr of EL and for 2 hr of the poststress period and had almost recovered after 24 hr (data not shown). As a result of this stress, PSII electron transport efficiency (ΦPSII) decreased twofold after 30 min of photoinhibition and reached a minimum (sevenfold reduction compared with the start of the experiments) after 2 hr of the poststress period. After 24 hr of the poststress period, ΦPSII increased significantly, indicating that photosynthesis had begun to recover. However, ΦPSII did not return to control levels.

In summary, these data were consistent with the decline in the concentration of functional PSII centers during the 1 hr of EL and 2 hr of the poststress period. The results presented above clearly indicate that in our experiments, we caused a reversible photoinhibition of photosynthesis in Arabidopsis chloroplasts.

Levels of H_2O_2, GSH, and GSSG and the Redox Status of Glutathione

In control plants, the foliar levels of H_2O_2 did not change significantly during the 24 hr of the experimental period (Figure 2). In contrast, after 15 min of stress, a transient reduction of H_2O_2 levels was observed followed by an increase in the

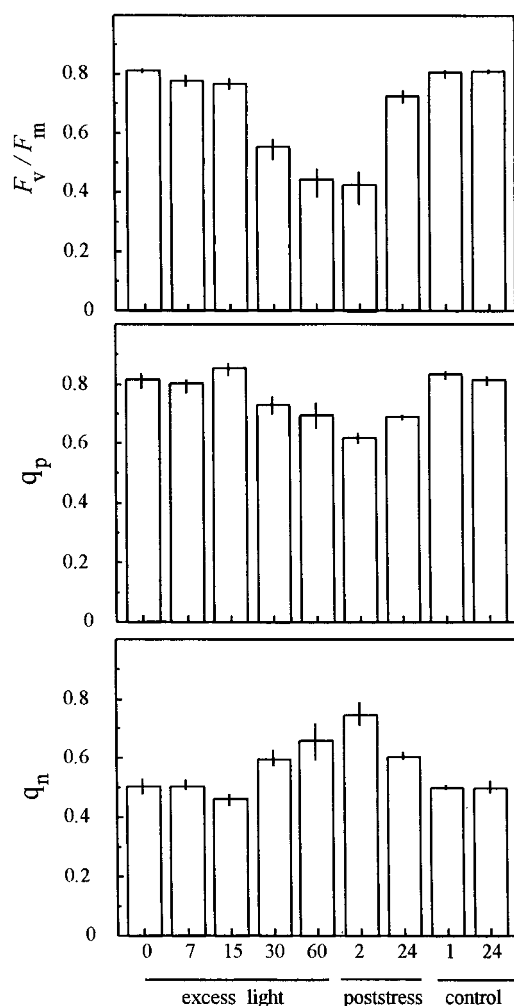

Figure 1. Photosynthetic Parameters Measured in Leaves Exposed to EL.

The seedlings were grown in low light (200 μmol of photons m^{-2} sec^{-1}) with an 18-hr photoperiod. In the middle of the photoperiod, 4-week-old plants were exposed to excess light (2000 ± 100 μmol of photons m^{-2} sec^{-1}) for 1 hr. After 1 hr, seedlings were reexposed to low light. Control plants were grown in the same phytochamber at low light. The maximum photochemical efficiency of PSII reaction centers (F_v/F_m), photochemical quenching (q_p), and nonphotochemical quenching (q_n) were determined. Measurements were made after 0, 7, 15, 30, and 60 min in EL and 2 and 24 hr poststress. Measurements for the controls were made 1 and 24 hr after the start of the experiments. Parameters were measured in four individual plants obtained from three independent experiments (n = 12) after 1 to 2 hr of dark adaptation. Vertical bars represent standard errors.

amount of H_2O_2, which reached a maximum after 1 hr (Figure 2). After 2 hr of the poststress period, the level of H_2O_2 was still higher than the level at the start of the stress; however, by 24 hr, it had fallen to a level twofold lower than that of the control plants (Figure 2).

The foliar concentration of GSH fluctuated during the EL stress and the poststress period; however, after 24 hr of poststress, it was higher than in control plants (Figure 2). In

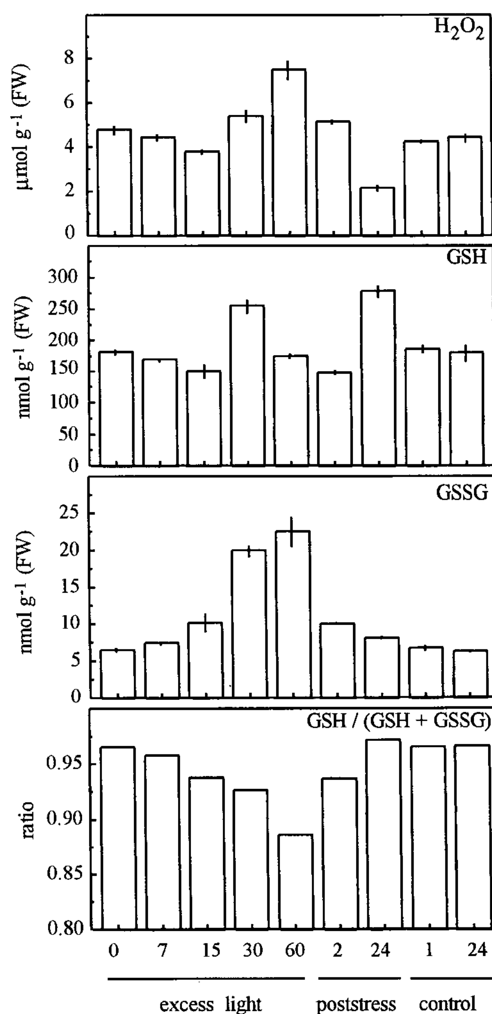

Figure 2. Biochemical Parameters Measured in Leaves Exposed to EL.

Levels of H_2O_2, GSH, and GSSG were determined, and the redox status of glutathione (GSH/[GSH + GSSG]) was calculated. These measurements were made at the same time points as given in Figure 1. H_2O_2 and glutathione levels were measured in pooled samples of leaves of four individual plants obtained from three independent experiments (n = 3). Vertical bars represent standard errors. FW, fresh weight.

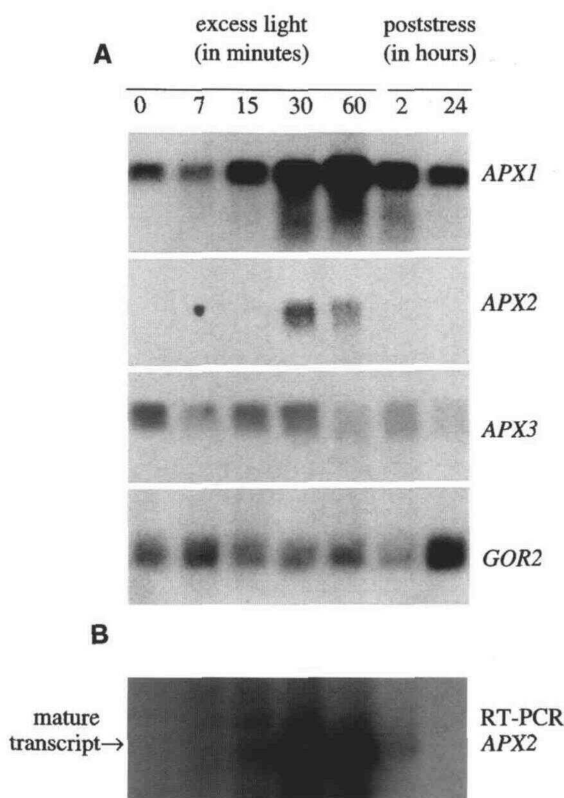

Figure 3. Gel Blot Analysis of mRNA Levels in Leaves Exposed to EL.

(A) Total RNA (10 μg per lane) was separated by gel electrophoresis, transferred to a filter, and hybridized with homologous *APX1*, *APX2*, and *APX3*, and heterologous (pea) cytosolic *GOR2* cDNA probes, as described in Methods.

(B) DNA gel blot hybridization analysis of *APX2* cDNA obtained by RT-PCR of *APX2* mRNA (see Methods). The cDNA obtained by reverse transcription of 2 μg of total RNA was subsequently amplified by PCR (30 cycles), separated by gel electrophoresis, transferred to a filter, and hybridized with a homologous *APX2* cDNA probe. The *APX2* cDNA was detected at 7, 15, 30, and 60 min EL (2000 μmol of photons m^{-2} sec^{-1}) and 2 hr poststress (200 μmol of photons m^{-2} sec^{-1}).

mRNA and cDNA levels were analyzed at the same time points (except control) as given in Figure 1. The data are representative for pooled samples of leaves of five individual plants obtained from two independent experiments (n = 2).

contrast to GSH, the foliar GSSG concentration increased steadily during the stress (from 7 to 60 min; Figure 2). In the poststress period, the GSSG concentration had fallen back to prestress levels by the end of the experiment. In the control plants, no significant changes in the concentrations of GSH and GSSG were observed during the 24-hr period. In spite of the variation in GSH levels, a continuous decrease in the redox status of glutathione (GSH/[GSH + GSSG]) was detected at 7 min and reached a minimum after 60 min. Two hours after EL stress, the redox status of glutathione increased, returning to a higher value than in the control plants after 24 hr (Figure 2). All observed changes in the redox status of glutathione (Figure 2) are significant because the largest standard deviation was <0.004 ($n = 3$).

mRNA Levels

Transcript levels were monitored at 7, 15, 30, and 60 min of exposure to EL and after 2 and 24 hr of the poststress period. The transcripts for cytosolic *APX1*, *APX2*, *APX3*, chloroplastic GR (*GOR1*), cytosolic *GOR2*, cytosolic and chloroplastic CuZn–*SOD*, chloroplastic iron (Fe)–*SOD*, mitochondrial manganese (Mn)–*SOD*, cytosolic *CAT1* and *CAT2*, cytosolic *MDR*, glutathione *S*-transferase (*GST*) with glutathione–peroxidase activity, *GPX*, phenylalanine ammonia-lyase (*PAL*), heat shock protein 70 (*HSP70*), *LHCB*, the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (*RBCL*), and the D1 protein (*PSBA*) genes were analyzed by RNA gel blot and slot blot hybridizations (Figure 3A and Table 1). In addition, *APX2* transcripts were also analyzed by DNA gel blot hybridization of amplified cDNAs obtained by the reverse transcriptase–polymerase chain reaction (RT-PCR; Figure 3B). The RT-PCR analysis was not used to obtain values for relative transcript levels. Only *APX1*, *APX2*, *CAT1*, *GOR2*, *LHCB*, and *PSBA* mRNAs levels changed markedly in these experiments.

APX1 mRNA was detected before, during, and after the EL stress. Higher (threefold) levels were observed after 15 min of EL and reached a maximum value after 60 min (18-fold; Figure 3A and Table 1). In the poststress period, the *APX1* transcript levels declined, although after 24 hr poststress, they were still higher (threefold) than in the control plants.

We could not detect transcripts for *APX2* in non-stress conditions by RNA gel blot hybridization or by RT-PCR methods (Figure 3 and Table 1). Using RNA gel blot hybridization, we were able to detect *APX2* transcripts after 30 and 60 min of EL (Figure 3A). However, by DNA blot hybridization of PCR-amplified *APX2* cDNA, transcripts were detected after 7 and 15 min of EL. After 30 min in EL stress, transcript levels for *APX2* reached a maximum. After 2 hr in the poststress period, *APX2* mRNA almost vanished and was again undetectable after 24 hr (Figure 3B).

After 1 hr in EL, we observed a 2.5-fold increase in mRNA levels for *CAT1*. In the poststress period, this transcript declined (Table 1) to a level similar to control plants. Compared with the control material, no differences for *GOR2*, *LHCB*, and *PSBA* transcript levels were found during 60 min of EL stress. However, after 2 and 24 hr of the poststress period, transcript levels for *PSBA* were higher than in control plants (Table 1). The mRNA levels for *LHCB* were halved 2 hr after EL stress and after 24 hr had increased to the level of the control (Table 1). *GOR2* transcript levels were fourfold higher 24 hr after EL stress. In contrast to the six transcripts men-

Table 1. Relative Transcript Levels for ROI Scavenging Enzymes and Other Stress-Related and Photosynthetic Proteins in Leaves Exposed to EL

		EL (min)				Poststress (hr)		Control (hr)			
Probe[a]	Experiment No.	7	15	30	60	2	24	1	24	Reference/Source	Origin
APX1	1	0.7[b]	3.8	9.4	18.7	7.3	2.9	1.4	1.7	Kubo et al. (1992)	Arabidopsis
	2	1.2	3.2	7.8	17.4	7.6	3.1	0.9	1.3		
APX2	1	1.0	5.2	40.0	40.0	1.4	ND[c]	ND	ND	Santos et al. (1996)	Arabidopsis
	2	1.0	4.9	40.0	40.0	1.9	ND	ND	ND		
APX3	1	0.7	0.9	1.3	1.1	1.5	1.2	0.8	1.2	EMBL accession number Z34316	Arabidopsis
	2	1.2	1.5	1.1	1.3	0.8	1.2	1.4	1.0		
GOR2	1	1.4	1.5	0.9	1.2	1.5	3.7	1.0	1.1	EMBL accession number X98274	Pea
	2	0.8	1.3	1.2	1.0	1.4	4.3	1.4	0.9		
CAT1	1	1.3	0.9	1.4	2.4	1.8	1.3	1.0	1.3	Willekens et al. (1994b)	Tobacco
	2	0.8	1.3	1.1	1.7	1.9	0.9	1.4	1.6		
LHCB	1	0.9	1.3	1.1	0.8	0.4	1.7	1.2	1.4	Jansson and Gustafsson (1990)	Scots pine
	2	1.2	1.0	0.8	1.0	0.3	1.9	1.3	0.9		
PSBA	1	1.4	1.6	1.5	1.7	2.6	2.7	1.4	1.6	Karpinska and Karpinski (1993)	Scots pine
	2	1.6	1.9	1.8	1.5	2.4	2.8	1.3	1.4		
Others											

[a] Small changes (less than twofold) were observed in transcripts levels for *GOR1*, cytosolic and chloroplastic CuZn–*SOD*, chloroplastic Fe–*SOD*, mitochondrial Mn–*SOD*, *CAT2*, *MDR1*, *GST*, *GPX*, *PAL*, *HSP70*, and *RBCL* genes. References for these probes and their origins are given in Methods.

[b] Values were obtained after the scanning of slot blot and RNA gel blot hybridization with cDNA and gene probes (see Methods). Value at time 0 is set to 1, except for *APX2*, where the value at 7 min is set to 1.

[c] ND, not determined.

tioned above, the transcript levels of *GOR1*, cytosolic and chloroplastic CuZn–*SOD*, chloroplastic Fe–*SOD*, mitochondrial Mn–*SOD*, *CAT2*, *MDR1*, *GST*, *GPX*, *PAL*, *HSP70*, and *RBCL* genes did not change markedly during the EL and the poststress period (Table 1).

Characterization of cDNAs for *APX2* and *APX3*

To ensure that the RT-PCR reactions were specific for *APX2* mRNA and to confirm the coding sequence of the *APX2* gene predicted by Santos et al. (1996), the amplified cDNA (Figure 3B) was cloned and sequenced. For this cloning, we used RNA isolated from leaves exposed for 30 min to EL. In addition, we have recently identified a third *APX* cDNA from Arabidopsis *APX3,* which was found as a partially sequenced cDNA clone in the Arabidopsis Biological Resource Center (Columbus, OH; ABRC accession number VBVEB09) and in the EMBL data base (accession number Z34614). *APX2* and *APX3* cDNA clones were completely sequenced. The sequence analysis of *APX2* confirmed the predicted coding sequence (Santos et al., 1996) and the specificity of the RT-PCR for this class of mRNA. Comparison of the derived amino acid sequences indicated 88% similarity between those encoded by *APX1* and *APX2*, 75% between *APX1* and *APX3*, and 73% between *APX2* and *APX3.* In addition, we have detected a putative membrane-spanning domain for the *APX3* polypeptide localized at the C-terminal end of the amino acid sequence (Bunkelmann and Trelease, 1996). These data suggest that the *APX3*-encoded enzyme has a membrane-associated subcellular localization.

Photosynthetic Electron Transport Inhibitors and Expression of *APX1* and *APX2* Genes

To obtain more information about regulatory mechanisms controlling the levels of *APX1* and *APX2* mRNA and to confirm the involvement of PSII in the EL stress responses, we performed additional experiments with the photosynthetic electron transport inhibitors 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). Initial pilot experiments were performed to determine the appropriate concentration of inhibitors to use (see Methods). Table 2 presents the values of photosynthetic parameters F_v/F_m, q_p, and oxygen evolution for all of these experiments. A concentration of 4 μM DCMU after a 3-hr treatment in LL at 200 μmol of photons m^{-2} sec^{-1} caused an ~50% inhibition of oxygen evolution and of F_v/F_m as well as a 30% drop in the proportion of open PSII reaction centers (q_p value) in comparison with control leaf discs. Additional treatment of DCMU-treated leaf discs for 30 min in EL at 2000 μmol of photons m^{-2} sec^{-1} caused a further drop in all of these parameters to a level well below those of control leaf discs. A 3-hr treatment with DBMIB (14 μM) in LL caused a 35% reduction in oxygen evolution, but

Table 2. Photosynthetic Parameters and Oxygen Evolution in Leaf Discs Treated with Photosynthetic Electron Transport Inhibitors and Glutathione

	LL[a]						LL + EL[b]		
	0 Hr			3 Hr			3 Hr + 30 Min		
Treatment	F_v/F_m[c]	q_p[c]	O_2 ev.[c,d]	F_v/F_m	q_p	O_2 ev.	F_v/F_m	q_p	O_2 ev.
DCMU	0.805 ±0.013	0.843 ±0.018	15.80 ±0.48	0.408 ±0.028	0.591 ±0.032	7.44 ±0.45	0.312 ±0.033	0.577 ±0.41	6.19 ±0.37
GSH	0.798 ±0.011	0.838 ±0.016	15.60 ±0.51	0.732 ±0.015	0.792 ±0.014	14.10 ±0.31	0.232 ±0.029	0.606 ±0.23	5.49 ±0.32
DBMIB	0.807 ±0.016	0.851 ±0.012	15.15 ±0.38	0.781 ±0.019	0.808 ±0.019	9.80 ±0.63	0.605 ±0.028	0.695 ±0.33	11.15 ±0.58
Water	0.812 ±0.017	0.850 ±0.013	15.45 ±0.41	0.801 ±0.013	0.845 ±0.011	15.30 ±0.38	0.626 ±0.022	0.714 ±0.021	12.30 ±0.43

[a] LL, low light (200 μmol of photons m^{-2} sec^{-1}).
[b] EL, excess light (2000 μmol of photons m^{-2} sec^{-1}).
[c] F_v/F_m, q_p, and O_2 ev. values were determined in leaf discs treated with 4 μM DCMU, 10 mM GSH, 14 μM DBMIB, or water (control). Parameters were measured in five different leaf discs obtained from two independent experiments ($n = 10 \pm$ SE).
[d] O_2 ev., oxygen evolution (μmol m^{-2} sec^{-1}).

>93% of PSII centers remained open and F_v/F_m values were reduced only by 2 to 6%. Additional treatment for 30 min in EL caused a drop in all of these parameters, but this drop was similar to the control plants for the same light condition. These two inhibitors thus provided treatments with similar inhibition of oxygen evolution (linear electron transport), but DBMIB has a completely different effect than does DCMU on PSII closure (q_p value). Similar effects of these inhibitors on PSII closure were observed in Synechococcus (Campbell et al., 1995).

APX1 mRNA levels were increased by DBMIB treatment in LL conditions (Figure 4). This increase was even greater after 30 min of EL treatment. In contrast, treatment with DCMU resulted in reduction in *APX1* mRNA levels in LL and no increase in EL. We could not detect any increase in *APX2* mRNA levels in LL after any treatments (Figure 4) by using slot blot hybridization procedures. However, in DBMIB-treated leaf discs, transcripts were detected by RT-PCR procedures (data not shown). In EL, an increase of *APX2* mRNA levels was evident in the DBMIB-treated leaf discs but not in DCMU-treated leaf discs. Thus, these treatments had a similar effect on *APX1* and *APX2* gene expression in LL and EL conditions. The mRNA levels for *APX3* were not changed in any light condition or by any treatment.

Exogenous GSH and Expression of *APX1* and *APX2* Genes

We have tested the effect of exogenous GSH on expression of the *APX1*, *APX2*, and *APX3* genes. A concentration of 10 mM GSH after a 3-hr treatment in LL did not cause a reduction in oxygen evolution, and a high proportion (95%) of PSII centers remained open (Table 2). The F_v/F_m values were slightly reduced by 7 to 9%. However, additional treatment of GSH-treated leaf discs for 30 min in EL caused a drop in all of these parameters (30 to 55% below those of control leaf discs for the same light condition). Thus, the application of exogenous GSH enhanced the photoinhibition of photosynthesis in EL conditions (Table 2).

Figure 4 shows that *APX1* mRNA levels were halved in GSH-treated leaf discs at LL in comparison with the level in control leaf discs treated with water. Additional treatment of GSH-treated leaf discs for 30 min at EL did not cause the considerable increase in mRNA levels for *APX1* and *APX2* genes observed after treatment with electron transport inhibitors. Thus, exogenous GSH prevented the increase in mRNA levels for *APX1* and *APX2* in EL. The exogenous GSH had no effect on *APX3* mRNA levels in any light condition.

DISCUSSION

The involvement of ROIs in key cellular processes has generated great interest in the regulatory and signaling systems mediating stress responses in plants (Wingate et al., 1988; Bowler et al., 1992; Chen et al., 1993; Hérouart et al., 1993; Levine et al., 1994; Prasad et al., 1994; Price et al., 1994; Bi et al., 1995; Monroy and Dhindsa, 1995; Neuenschwander et al., 1995; Wingsle and Karpinski, 1996). To understand and manipulate these processes, we need to determine the role of ROIs in the sensing of environmental stimuli that leads to stress responses and adaptation. The use of EL as a stress applied to low light–adapted plants is a highly controllable and reproducible approach (Russell et al., 1995). Such an experimental system should provide us with a better understanding of the interactions of photosynthesis,

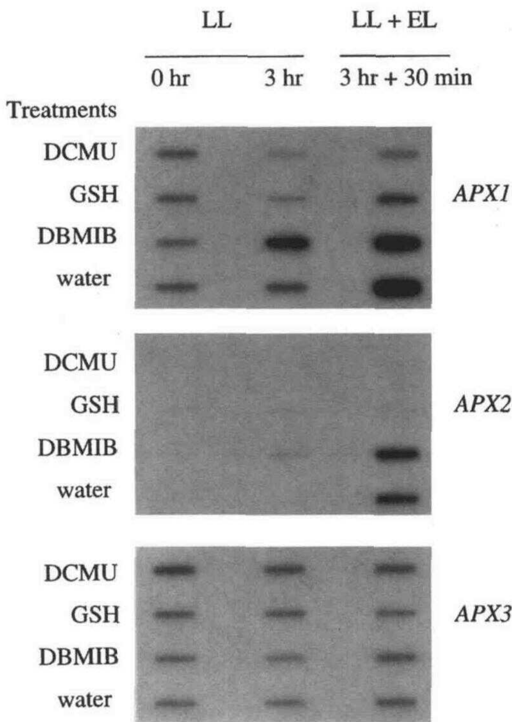

Figure 4. Slot Blot Hybridization Analysis of mRNA Levels in Leaf Discs Treated with Photosynthetic Electron Transport Inhibitors and Glutathione.

Total RNA was isolated at three different time periods: 0 and 3 hr after vacuum infiltration with 4 μM DCMU, 10 mM GSH, 14 μM DBMIB, or water (control) in LL (200 μmol of photons m^{-2} sec^{-1}) and after 3 hr at LL plus 30 min under EL (2000 μmol of photons m^{-2} sec^{-1}). Total RNA (20 μg per slot) was transferred to a filter and hybridized with homologous *APX1*, *APX2*, and *APX3* cDNA probes, as described in Methods. Pooled samples of 30 leaf discs obtained from 10 to 14 individuals were used for every treatment and time point. The data are representative for two independent experiments (n = 2).

photooxidative stress, photoinhibition (and recovery), and acclimation processes as well as the induction of a specific secondary messenger system(s) emanating from the chloroplast that regulates the expression of nuclear-encoded genes for ROI-scavenging enzymes.

Order of the Events

The changes in key parameters and timing of these changes in response to EL and poststress are presented in Figure 5. An increase in the *APX2* mRNA level was detected after 7 min of EL. In the case of *APX1*, a higher transcript level was detected after 15 min of the EL stress. In the first 7 and 15 min of EL, we observed a reduction in maximum photochemical efficiency of PSII, a decrease in GSH levels, and an increase in GSSG levels, bringing about a reduction in the redox status of glutathione that was associated with a transient drop in H_2O_2 levels. The subsequent increased levels of H_2O_2 were detected after 30 min of EL and achieved a maximum after 60 min, followed by a marked reduction in all photosynthetic parameters for PSII (Figure 5). The level of *CAT1* mRNA increased twofold after 60 min of EL.

After 2 hr of the poststress period, we observed a different situation: an increase in the redox status of glutathione; reduction of the GSSG and GSH levels; a slight reduction in H_2O_2 levels, although they were still higher than in control plants; and photoinhibition of photosynthesis was at its maximum. These changes were accompanied by a rapid and dramatic reduction of transcript levels for *APX2* and less so (twofold) for *APX1*. The *PSBA* transcript level increased, whereas that of *LHCB* and *CAT1* decreased.

After 24 hr of the poststress period, we observed higher transcript levels for *GOR2* and *PSBA*, slightly higher transcript levels for *LHCB*, no transcripts for *APX2*, and a further reduction (sixfold) of *APX1* mRNA levels, which were associated with a maximum value for the redox status of glutathione, minimum levels of H_2O_2, and substantial but incomplete recovery of photosynthesis.

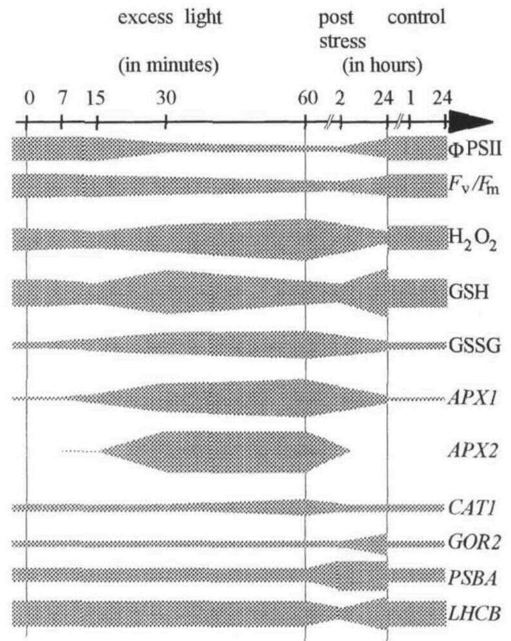

Figure 5. Order of the Events.

Timing of functional acclimation of PSII, and of biochemical and molecular responses to excess light and in the poststress period in comparison with control. The thickness of the bars is proportional to the values of the parameters at time zero.

Gene Regulation during and after Excess Light

There were four different responses among the genes encoding ROI scavengers: first, a rapid increase (within 7 min) of the *APX2* mRNA levels, giving a dramatic rise after 30 min (Figures 3 and 5, and Table 1). We could not detect *APX2* transcripts by RT-PCR procedures in nonstressed plants (Santos, 1995; Santos et al., 1996; Figure 3). The appearance of the *APX2* transcripts from an undetectable level (within 7 min) suggests that the rise of *APX2* mRNA could be at least in part due to de novo synthesis. To our knowledge, this is the fastest increase of mRNA levels for any ROI scavenging enzyme so far observed in plants' responses to biotic or abiotic stress (Bowler et al., 1992; Williamson and Scandalios, 1992; Hérouart et al., 1993; Karpinski et al., 1993; Levine et al., 1994; Mittler and Zilinskas, 1994; Willekens et al., 1994a; Conklin and Last, 1995; Kubo et al., 1995; Wingsle and Karpinski, 1996).

Second, after 15 min in EL, there was an increase in *APX1* mRNA levels, rising to 18-fold after 60 min. Although such a response may suggest that *APX1* expression could also be regulated at least in part at the level of de novo transcription, the response of *APX1* is neither as rapid nor as dramatic as for *APX2*. Increased levels of transcripts for *APX1* have also been observed in ozone-fumigated Arabidopsis (Conklin and Last, 1995; Kubo et al., 1995) and in drought-stressed peas (Mittler and Zilinskas, 1992, 1994). However, the changes observed by Conklin and Last (1995) could have been caused by a diurnal rhythm of *APX1* mRNA abundance (Kubo et al., 1995), and the timing of all of these responses was measured in hours rather than in minutes. Mittler and Zilinskas (1992) suggested that *APX1* in pea (which is a homolog of *APX1* in Arabidopsis) could be regulated by a putative *cis*-acting heat shock element residing in the gene's putative promoter region. The putative *cis*-acting heat shock elements have also been detected in the promoter regions of *APX1* and *APX2* (Santos, 1995; Santos et al., 1996). In this particular experimental system, we can exclude a heat shock type of regulation because HSP transcript levels were unaltered during the 24 hr of the experimental period (Table 1).

Third, after 60 min in EL, a 2.5-fold increase was observed for *CAT1* transcript levels. This increase was not as rapid or as dramatic as the response of *APX1* and *APX2*. Nevertheless, such a result suggests that the *CAT1* gene is regulated in a similar manner to *APX1* and *APX2*. Differential expression of *CAT* genes was observed in tobacco subjected to different oxidative stresses (Williamson and Scandalios, 1992; Willekens et al., 1994a). In maize, *CAT* genes were induced in response to the photoactivated fungal toxin cercosporin (Williamson and Scandalios, 1992).

Fourth, after 24 hr of the poststress period, a fourfold increase of mRNA level for *GOR2* was observed. In peas, *GOR2* mRNA levels were induced in the poststress periods after drought or chilling stresses (R. Stevens, G. Creissen, and P.M. Mullineaux, manuscript in preparation). Taken together, these data suggest that the poststress increase in *GOR2* transcript levels could be a common feature of different plant species in response to different environmental stresses. Additionally, this result emphasized the role of glutathione in the photooxidative stress response.

Surprisingly, no responses were observed for transcripts encoding other ROI scavenging enzymes, such as chloroplastic Fe–SOD, mitochondrial Mn–SOD, cytosolic and chloroplastic CuZn–SOD, chloroplastic GR, cytosolic MDR, a second isoform of catalase—CAT2, and GPX. Constant mRNA levels for *GOR1* have been observed in other plant species subjected to different oxidative stresses, including photooxidative stress (Karpinski et al., 1993; Edwards et al., 1994). In tobacco, Fe–*SOD* and CuZn–*SOD* transcript levels were upregulated in the response to light and the herbicide paraquat (Tsang et al., 1991); in Scots pine, transcript levels for cytosolic and chloroplastic isoforms of CuZn–*SOD* were higher at the beginning of the recovery process from winter photooxidative stress (Karpinski et al., 1993). All of these data indicate that it is becoming apparent that in different plant species, different gene responses have evolved to deal with photooxidative stress, although the signaling systems regulating these responses could still be similar. Another possible explanation for noninduction of the gene encoding the chloroplastic Fe–SOD isoform in our experiments could be that photosystem I (PSI) was not sufficiently stressed to trigger a signal regulating this gene. It has been shown in *Nicotiana plumbaginifolia* that increased mRNA levels for Fe–*SOD*, brought on by exposure of etiolated seedlings to white light, were triggered by $O_2^{\cdot-}$ generated in the proximity of PSI. However, this response was detected only after 2- and 24-hr exposures to light (Tsang et al., 1991), indicating that the rise in Fe–*SOD* mRNA levels in this species during photooxidative stress was not as rapid or as dramatic as the response of *APX1* and *APX2* mRNA levels in Arabidopsis to a similar type of stress.

Two hours after EL stress, 2.5-fold higher transcript levels were observed for *PSBA* (Table 1 and Figure 5). At the same time, there was a twofold reduction in *LHCB* transcript levels. Twenty-four hours after EL stress, we observed still higher transcript levels for *PSBA* and also an increased level for *LHCB*. Transcription of these genes in plants is controlled by phytochrome (Eskins and Alexander, 1993; Millar et al., 1995), and these results suggest that phytochrome-mediated responses in our experimental system were evident in the poststress period.

Another interesting observation in this study is the lack of induction of *PAL* and *GPX* transcript levels. These have been shown to increase during the plant hypersensitive disease resistance response, which is proposed to be regulated by an oxidative burst of H_2O_2 in localized areas surrounding sites of pathogen entry (Levine et al., 1994). The failure to induce transcripts, such as *PAL* and *GPX*, with the EL stress (Table 1) suggests that the increase in H_2O_2 levels was not sufficient, or was not generated in the correct subcellular compartment, to trigger the signaling of the HR (Chen et al., 1993; Levine et al., 1994), although there is some doubt that H_2O_2

alone can induce a plant HR (Bi et al., 1995; Neuenschwander et al., 1995). Therefore, it is unlikely that the signal in photooxidative stress shares common features with that involved in the HR. Supporting this idea is the observation that injection of H_2O_2 into maize leaves also did not induce the HR but did cause an increase in the levels of *CAT3* mRNA and triggered the mechanism of chilling acclimation (Prasad et al., 1994). This gene in maize is controlled by a circadian period regulation (Boldt and Scandalios, 1995).

Nature of the Signal(s) Regulating *APX1* and *APX2* Gene Expression

It has been suggested that cytosolic *APX* transcripts can be upregulated by increased levels of H_2O_2 in tobacco chloroplasts as a result of CuZn–*SOD* overexpression (Gupta et al., 1993). Our results suggest that in Arabidopsis, a photooxidative burst of H_2O_2 was not involved in the regulation of *APX1* or *APX2* gene expression because H_2O_2 levels were static or transiently lowered up to 15 min into the EL stress (Figure 2) when the expression of both genes was already rising (Figures 3 and 5). We have measured total foliar H_2O_2 levels; however, different subcellular activities of ROI-scavenging enzymes could interact in the cell to create local differences of H_2O_2 levels in different subcellular compartments, and therefore, we cannot exclude the involvement of H_2O_2 in this signaling pathway.

It has been reported that APX activity can be regulated by phytochrome (Thomsen et al., 1992), and a diurnal rhythm of *APX1* mRNA abundance has recently been shown in Arabidopsis (Kubo et al., 1995). An involvement of photoreceptors in the rapid regulation of *APX1* and *APX2* gene expression is less likely because the timing of the responses mediated by a photoreceptor during changes in light intensity is usually measured in hours (Millar et al., 1995). In addition, partial inhibition of photosynthetic electron transport suggests that the effect of a 10-fold increased irradiance can be simulated partly by the manipulation of the redox status of the plastoquinone pool in the chloroplast.

DCMU and DBMIB block the reduction and oxidation, respectively, of the plastoquinone pool. Partial inhibition of photosynthetic electron transport by DCMU mimics the effect of LL conditions. After this treatment, mRNA levels for *APX1* were reduced in LL. In EL, DCMU abolished the upregulation of mRNA levels for *APX1* and *APX2*. Whereas DCMU specifically inhibited the oxidation of the Q_A of PSII (q_p value reflects the extent of Q_A reduction; Table 2), DBMIB reduced the rate of oxidation of the plastoqinone pool by competitively binding to the cytochrome b_6/f complex (Jones and Whitmarsh, 1988). After DBMIB treatment, we observed an opposite effect to DCMU treatment. The partial inhibition of photosynthetic electron transport by DBMIB mimicked the effect of EL. In this treatment, we observed five- to sixfold higher transcript levels for *APX1* in LL conditions, and in contrast to the DCMU-treated leaf discs, the signal regulating the *APX2* mRNA levels by the EL was not blocked. These data suggest that expression of both genes can be partly controlled by the redox status of the plastoquinone pool.

Involvement of Glutathione in Regulation of *APX1* and *APX2*

We do not know the cause of the changes in glutathione levels and redox status of glutathione that occurred during 60 min of EL. It is also not possible to determine which subcellular compartment participated in the inhibition of *APX1* and *APX2* genes in GSH-treated leaf discs. However, the rapid changes in the redox status of PSII could suggest that it is the redox state of the chloroplast glutathione pool that was mostly affected. Nonenzymatic or GPX-catalyzed reactions involving LOOH·, generated in photooxidative stress (Elstner and Osswald, 1994), or H_2O_2 (Figure 2) with GSH could explain the sustained increase in GSSG and the decrease in the redox status of glutathione during EL stress. Changes in the redox state of the cytosolic glutathione pool may also have occurred during and/or after the photooxidative stress, as suggested by the increase in *GOR2* transcript levels.

The treatment of Arabidopsis leaf discs with GSH caused a reduction of *APX1* transcript levels under LL conditions and abolished the increase of mRNA levels for *APX1* and *APX2* in EL conditions (Figure 4). Uptake of exogenous GSH may have prevented a decline in the redox state of the endogenous glutathione pool when the leaf discs were subjected to EL stress. We suggest that under these conditions, the glutathione pool failed to transduce the signal from the plastoqinone pool and thereby inhibited *APX1* and *APX2* gene expression (Figure 4). This led to enhanced photooxidative stress as evidenced by the sharp decline of photosynthetic parameters compared with the control treatments (Table 2). Similar effects on *APX1* and *APX2* transcript levels were observed in the GSSG treatment of Arabidopsis leaf discs (results not shown). Similar effects of GSH and GSSG on gene expression in plants have been observed previously (Edwards et al., 1991; Wingsle and Karpinski, 1996).

The results presented here are consistent with the idea that levels of glutathione or the redox status of glutathione could be involved in rapid signaling from the chloroplast to the nucleus in Arabidopsis during EL stress. However, in these experiments we have not measured the levels and redox status of the foliar ascorbate pool. Ascorbate is an important reducing agent in plastids and theoretically could influence this signal transduction pathway during photooxidative stress.

Sources of H_2O_2 and Photoinhibition

Chloroplasts generate 150 to 250 μmol (mg chlorophyll)$^{-1}$ hr^{-1} of H_2O_2 during photosynthesis (Asada, 1994). Levels of H_2O_2 in chloroplasts during photosynthesis are a result of a

dynamic equilibrium between the rates of production and scavenging. Production of H_2O_2 could be due to reduction of $O_2^{\cdot-}$ by SOD, ascorbate, thiols, ferredoxin, Mn ions, and self-dismutation of $O_2^{\cdot-}$ (Mehler, 1951; Asada, 1994). Scavenging of H_2O_2 in the chloroplast is proposed to be performed by a thylakoid-bound and a stromal chloroplastic APX (Miyake et al., 1993; Ishikawa et al., 1996) and nonenzymatic reactions with ascorbate or GSH.

The increased H_2O_2 level was detected simultaneously with a decrease in ΦPSII and photoinhibition of photosynthesis (Figures 1, 2, and 5). This observation suggests that the photooxidative burst of H_2O_2 is associated with the damage and degradation of the D1 protein in PSII (Andersson and Styring, 1991; Andersson et al., 1992; Öquist et al., 1992; Aro et al., 1993; Russell et al., 1995). The possibility that other places in the chloroplast, such as PSI, could contribute to changes in H_2O_2 levels is less likely because PSI is associated with a powerful APX- and ascorbate-based H_2O_2 scavenging system (Asada, 1994). However, the burst of H_2O_2 could also have been enhanced by an increased rate of photorespiration, which in turn would have stimulated the photorespiratory cycle in peroxisomes, leading to an increased rate of H_2O_2 production in these organelles (Cadenas, 1989; del Rio et al., 1992). Theoretically, this provides another possible mechanism for signal transduction from the inside to the outside of the chloroplast during EL stress.

Conclusions

All of these data suggest that we have found a novel and rapid light-sensing mechanism in higher plants that regulates the expression of genes not directly involved in photosynthesis or protection of the photosynthetic apparatus but in the scavenging of H_2O_2 in the cytosol. This mechanism is controlled by the redox events in the proximity of PSII—probably the redox status of plastoquinone pool. Moreover, GSH levels or redox status of glutathione could have a regulatory impact on this signaling pathway. These results indicate that photooxidative stress in plants alters the levels of ROIs not only in the chloroplast but also in the cytosol. This rapid induction of the cytosolic ROI scavengers could provide a secondary line of defense that would have to be induced before the chloroplastic ROI scavenging system is saturated.

METHODS

Plant Material and Growth and Stress Conditions

Arabidopsis thaliana (ecotype Columbia) seedlings, individually planted in pots, were grown in a climate chamber under the following conditions: an 18-hr photoperiod, photon flux density (PFD) of 200 ± 25 μmol of photons m^{-2} sec^{-1}, 21 ± 2.5°C, and relative humidity of 75 ± 5%. In the middle of the photoperiod, 4-week-old plants were exposed to a white light pulse (the same quality as the low light [LL]) for 1 hr with PFD of 2000 ± 100 μmol of photons m^{-2} sec^{-1}. To eliminate heat effects and to disperse light evenly, light was reflected by a mirror and directed through a frosted glass filter filled with cold water. After this period, plants were reexposed to a low PFD of 200 ± 25 μmol of photons m^{-2} sec^{-1}, with environmental conditions being otherwise unchanged.

For all measurements, only fully exposed leaves were collected. Control plants were grown in the same phytochamber. The fast chlorophyll *a* induction kinetics and glutathione and hydrogen peroxide (H_2O_2) levels were analyzed from at least four individuals taken from three independent experiments. Pooled samples of leaves harvested from five different plants from two independent experiments were used in the analysis of transcript abundance and for the cloning of the *APX2* cDNA.

For 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), and reduced glutathione (GSH) experiments, we used Arabidopsis leaf discs after vacuum infiltration. The vacuum infiltration time was 3 min. The plants for these experiments were grown under the same conditions and in the same phytochamber as described above. The photosynthetic electron transport inhibitors were tested in concentration-dependent inhibition experiments after 3 hr of treatment. The concentrations tested were in the range between 0.5 and 15 μM at 200 (for 3 hr) and 2000 (for an extra 30 min) μmol of photons m^{-2} sec^{-1}. Control leaf discs were vacuum infiltrated with water and were treated in the same way as DCMU-, DBMIB- and GSH-treated leaf discs. Concentrations of DCMU, DBMIB, and GSH at 4 μM, 14 μM, and 10 mM, respectively, were used for leaf disc experiments.

Chlorophyll *a* Fluorescence Parameters Measured in These Experiments

The ratio of variable fluorescence and maximum fluorescence (F_v/F_m) of the dark-adapted chlorophyll *a* fluorescence parameters indicates the extent and functional consequences of changes in the maximum photochemical efficiency of photosystem II (PSII) reaction centers (Krause, 1988; Franklin et al., 1992; Öquist et al., 1992). An elevated zero fluorescence (F_0) has been considered to reflect thylakoid membrane disturbance (Krause and Weis, 1991). Slowly reversible, extensive reduction in ΦPSII is indicated by low values of dark-adapted F_v/F_m and elevated F_0; these reflect the functioning and turnover of the D1 protein (Osmond, 1994). The photochemical quenching parameter (q_p) reflects the extent of reduction of the primary electron acceptor plastoquinone (Q_A) that is associated with the PSII complex. It is suggested that Q_A has the potential to cause photooxidative damage during photoinhibition of photosynthesis (Krause, 1988; Krause and Weis, 1991; Aro et al., 1993). The nonphotochemical quenching parameter (q_n) indicates photoprotective processes that lead to a dissipation of excitation energy as heat (Demmig-Adams and Adams, 1992).

The fast chlorophyll *a* induction kinetics were measured using a modulated fluorescence system (PAM; Heinz Walz, Effeltrich, Germany) described by Schreiber et al. (1986). Chlorophyll *a* fluorescence measurements were made after 1 to 2 hr of dark adaptation of leaves. The actinic irradiance used was between 800 and 2000 μmol of photons m^{-2} sec^{-1}. One or two saturating flashes (0.8 sec, with 2000 μmol of photons m^{-2} sec^{-1} at 30-sec intervals) were applied before turning on the actinic beam. Fluorescence quenching parameters were determined by application of pulses (0.8 sec) of saturating white light at 30-sec intervals. The F_v, F_m, and F_0 were measured, and

the F_v/F_m ratio was calculated. The quenching parameters q_p and q_n were calculated according to Van Kooten and Snel (1990). The analysis of ΦPSII was performed according to Genty et al. (1989). Oxygen evolution in leaf discs was determined in an oxygen electrode by using an actinic lamp (Hansatech, Kings Lynn, UK), according to the manufacturer's instructions.

Quantitative Analysis of Hydrogen Peroxide, GSH, and Oxidized Glutathione

Leaves for H_2O_2 and glutathione measurements were immediately frozen in liquid nitrogen and then stored at −80°C until analysis was performed. A portion of the leaves (0.2 g) stored at −80°C was frozen again in liquid nitrogen and ground twice for 1 min in a dismembrator (Retsch, Haan, Germany). The frozen powder was suspended in 3 mL of 0.25 mM HCl containing 5 mM homocysteine (as an internal standard for HPLC analysis of glutathione) and homogenized for 1 min in the dismembrator. The extract was then sonicated for 30 sec (Soniprep 150; Measuring Scientific Equipment, Leicester, UK). This extract was used for quantitative H_2O_2 and glutathione analyses. In addition, for H_2O_2 analysis, separate extracts in 100 mM perchloric acid were used. Quantification of H_2O_2 in both extracts was determined by chemiluminescence with luminol, as described by Warm and Laties (1982). Extracts (1 to 5 mL) were used for each measurement. The concentration of H_2O_2 in extracts was measured with and without an internal H_2O_2 standard before and after incubation with catalase (CAT). Background luminescence was measured and eliminated. The external standard of H_2O_2 concentration was prepared in a range between 0.2 and 10 μM. Glutathione quantification was performed by HPLC analysis as described previously (Wingsle et al., 1989).

Preparation of RNA, cDNA, and DNA Probes

A LiCl-based extraction method was used for RNA preparations (Karpinski et al., 1992). Total RNA was incubated with 40 units of RNase-free DNase I for 1 hr at 37°C. After phenol extraction and ethanol precipitation, total RNA was used for RNA gel blot and slot blot hybridization experiments, cDNA synthesis, and polymerase chain reaction (PCR) analysis. We used the following cDNA and gene probes in these experiments: *APX1* and *APX2* from Arabidopsis encoding cytosolic isoforms of ascorbate peroxidase (Kubo et al., 1992, 1993; Santos et al., 1996) and *APX3* encoding a putative membrane–associated cytosolic APX isoform (Arabidopsis Biological Resource Center; accession number VBVEB09). Gene-specific probes were generated from these *APX* sequences by PCR amplification of the most variable coding regions.

The following were used in this study: Fe-, Mn- and CuZn-*SOD* cDNAs from Scots pine and *Nicotiana plumbaginafiolia* (Bowler et al., 1989; Van Camp et al., 1990; Karpinski et al., 1992); cytosolic *GOR2* and organellar *GOR1* from pea (Creissen et al., 1992; EMBL accession number X98274); *CAT1* and *CAT2* from tobacco (Willekens et al., 1994b); *MDR* from pea (P.M. Mullineaux, unpublished results); *GST* with glutathione–peroxidase activity from Arabidopsis (Bartling et al., 1993); *GPX* from pea (P. Mullineaux, unpublished results); *LHCB* (Jansson and Gustafsson, 1990); *PSBA* and large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (*RBCL*) from Scots pine (Karpinska and Karpinski, 1993); phenylalanine ammonialyase (*PAL*) from Antirrhinum (Martin et al., 1991); and heat shock protein 70 (*HSP70*) from pea (Domoney et al., 1991). Probes were labeled with α-^{32}P-dCTP by the random primer method (Sambrook et al., 1989) and were used accordingly in RNA and DNA gel blots and RNA slot blot hybridizations.

RNA and DNA Gel Blots and RNA Slot Blot Hybridization

For RNA gel blot hybridization analyses, total RNA samples (10 μg) were separated on 1.4% (w/v) denaturing (formaldehyde) agarose gels. For DNA gel blot hybridization analyses of the cDNA for *APX2*, 1 μL of the of total 50 μL of reverse transcriptase–PCR (RT-PCR) reaction mixture was separated on 1.0% (w/v) agarose gels. The RNAs and cDNAs were transferred to Hybond N membranes (Amersham, Aylesburgh, UK). For RNA slot blot hybridization, total RNA samples were prepared for blotting according to Sambrook et al. (1989). Blotting to Hybond N membranes was performed in a Minifold II apparatus (Schleicher & Schuell), according to the protocol of the manufacturer.

Filters were prehybridized at 65°C for 3 hr in 6 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 50 mM sodium phosphate, 0.1% SDS, and 100 μg/mL salmon sperm DNA. The filters were hybridized in the same solution with a radioactive probe for 24 hr and washed four times for 30 min in 0.1 × SSC and 0.1% SDS at 65°C for homologous probes and 2 × SSC and 0.1% SDS at 65°C for heterologous probes. At 0.1 × SSC and 0.1% SDS at 65°C, no cross-hybridization for *APX1, APX2,* and *APX3* probes was observed. The filters were exposed to x-ray film and/or were visualized on a BAS 1000 PhosphorImager analyzer (Fuji Photofilm Co., Kanagawa, Japan). Scanning values were calculated by BASIS software (Fuji Photofilm Co.) installed on BAS 1000.

RT-PCR Amplification of the *APX2* cDNA and Cloning

Each RT-PCR reaction was prepared from 2.0 μg of total RNA, and cDNAs were amplified in 30 cycles in a 50-μL volume. Two primer sequences were designed on the basis of the published sequence (Santos et al., 1996). One primer was homologous to the 5′ end of *APX2*, including the putative translation start codon (5′-GAAGGAAGCGAATTTGAAAAAAATGG-3′), and the second primer, in reverse orientation, was homologous to the 3′ end (5′-GGAGATGACACCAGATTCCAGATTAC-3′), including the putative translation stop codon. cDNA was obtained by RT-PCR. All RT-PCR parameters were according to standard procedures (Sambrook et al., 1989). The absence of DNA contamination in DNase I–treated RNA was confirmed by simultaneous PCR amplification of 2.0 μg of RNA samples that had not been treated with reverse transcriptase. The cloning of amplified cDNA fragments was performed according to standard procedures (Sambrook et al., 1989).

Sequencing of cDNAs Corresponding to *APX2* and *APX3*

All DNA sequencing was performed on a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA) with an ABI PRISM dyeterminator cycle sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA) and specific primers, according to the manufacturer's instructions. The DNA sequence output was edited and aligned using data base assembly programs of Staden for UNIX (Dear and Staden, 1991). Analysis of the completed cDNA sequences was performed using the Genetics Computer Group (Madison, WI) programs (Devereux et al., 1984).

ACKNOWLEDGMENTS

Special thanks are offered to Prof. Jan-Erik Hällgren and Dr. Gunnar Wingsle for help and for providing the laboratory facilities for the EL stress system and measurements of photosynthetic and biochemical parameters. We thank Prof. Jan-Erik Hällgren, Dr. Gunnar Wingsle, and Dr. Rod Casey for critical review of the manuscript. We are grateful to Drs. Dieter Bartling, Rod Casey, Dirk Inzé, Cathie Martin, Wim Van Camp, and Hilde Willekens as well as the Arabidopsis Research Center for kindly providing the above-listed cDNAs and gene probes and to Margareta Zetherström for excellent technical assistance. This work was supported by grants from the Swedish Council for Forestry and Agricultural Research and the Swedish Council for International Cooperation in Research and Higher Education to S.K., a category 20 AIR Fellowship from the Commission of the European Communities (CEC) to C.E., and grants from the Biotechnology and Biological Sciences Research Council and CEC to P.M.M.

Received November 25, 1996; accepted January 30, 1997.

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