Hydrogen Peroxide Mediates the Induction of Chloroplastic Ndh Complex under Photooxidative Stress in Barley[1]

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Chloroplast-encoded NDH polypeptides (components of the plastid Ndh complex) and the NADH dehydrogenase activity of the Ndh complex (NADH-DH) increased under photooxidative stress. The possible involvement of H_2O_2-mediated signaling in the photooxidative induction of chloroplastic *ndh* genes was thoroughly studied. We have analyzed the changes in the NADH-DH and steady-state levels of NDH-F polypeptide and *ndhB* and *ndhF* transcripts in barley (*Hordeum vulgare* cv Hassan) leaves. Subapical leaf segments were incubated in growing light (GL), photooxidative light (PhL), GL and H_2O_2 (GL + H_2O_2), or PhL and 50 nM paraquat in the incubation medium. Treatments with H_2O_2 under GL mimicked the photooxidative stimulus, causing a dose-dependent increase of NADH-DH and NDH-F polypeptide. The kinetic of Ndh complex induction was further studied in leaves pre-incubated with or without the H_2O_2-scavenger dimethyltiourea. NADH-DH and NDH-F polypeptide rapidly increased up to 16 h in PhL, GL+ H_2O_2, and, at higher rate, in PhL and paraquat. The observed increases of NADH-DH and NDH-F after 4 h in PhL and GL + H_2O_2 were not accompanied by significant changes in *ndhB* and *ndhF* transcripts. However, at 16-h incubations NADH-DH and NDH-F changes closely correlated with higher *ndhB* and *ndhF* transcript levels. All these effects were prevented by dimethylthiourea. It is proposed that the induction of chloroplastic *ndh* genes under photooxidative stress is mediated by H_2O_2 through mechanisms that involve a rapid translation of pre-existing transcripts and the increase of the *ndh* transcript levels.

The plastid DNA contains 11 *ndh* genes (Maier et al., 1995) encoding polypeptides (NDH) that are components of the plastid Ndh complex, analogous to the NADH dehydrogenase or complex I (EC 1.6.5.3) of mitochondrial respiratory chain (Sazanov et al., 1998; Casano et al., 2000). The increases of NDH polypeptides and NADH dehydrogenase activity of the Ndh complex (NADH-DH) under photooxidative stress (Martín et al., 1996; Casano et al., 1999, 2000) suggest that the Ndh complex is involved in the protection against such stress. In fact, *ndh*-less mutants show increased sensitivity to photooxidative stress (Endo et al., 1999; Horvath et al., 2000). The purified Ndh complex catalyzes the transfer of electrons from NADH to plastoquinone and, in vivo a thylakoid plastoquinol peroxidase probably oxidizes the reduced plastoquinone with H_2O_2 (Casano et al., 2000). Ndh complex (providing electrons) plus plastoquinol peroxidase with Mehler reaction and superoxide dismutase (draining electrons) might poise the redox level of the electron carriers. This mechanism (chlororespiration) would most likely ensure the photosynthetic electron transport under a variety of environmental conditions that include rapid changes of light intensity associated with sunflecks and leaf movements. In addition, the chlororespiration may act as system scavenging reactive oxygen species generated under continuous photooxidative stress or by the successions of sunflecks and light gaps (Casano et al., 2000).

The increase in the levels of NDH polypeptides and Ndh complex activity (Martín et al., 1996; Casano et al., 1999, 2000) is the first described case of plastid DNA-encoded proteins that are stimulated by photooxidative stress. Thus, even though assuming an initial control of the plastid-targeted actions at the level of nucleus-cytoplasmic system, it is of interest to investigate whether or not H_2O_2 generated in the chloroplast could mediate the increase in the level of plastid-encoded proteins.

The photooxidative stress response shares strong similarities with the response of plants to pathogens (Levine, 1999), where a still poorly understood signal transduction pathway includes H_2O_2 and salicylic acid as components. Increasing evidence (for review, see Levine, 1999) suggests that the high concentrations of superoxide anion radical and H_2O_2 in the infection focus are high enough to kill not only the pathogen, but also the targeted plant cells (hypersensitive response). In these cells the concentration of H_2O_2 would trigger a programmed cell death. Meanwhile, the concentration of H_2O_2 in neighboring cells, at appropriate distance, would reach a lower level, which induces a succession of protecting genes that encode the pathogenesis-related proteins. The response of leaves to increasing photooxidative stress

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is very similar (Casano et al., 1999). At low photooxidative stress the battery of enzymes scavenging or preventing reactive oxygen species accumulation is induced. However, at higher photooxidative stress even these protective enzymes are more rapidly destroyed than induced (Casano et al., 1999). Moreover, H_2O_2 seems to be involved in the oxidative stress-mediated induction of nuclear-encoded defensive enzymes such as cytosolic ascorbate peroxidase (Karpinski et al., 1999; Morita et al., 1999), glutathione *S*-transferase, and catalase (Polidoros and Scandalios, 1999). Significant age-dependent differences in the response of protective enzymes to increasing photooxidative stress (Casano et al., 1999) also suggest a close relation among the mechanisms involved in photooxidative stress response, pathogen defense response, leaf cell senescence, and hypersensitive response.

Bearing in mind the above mentioned similarities we have investigated the possible involvement of H_2O_2 in the increase of plastid NDH polypeptides and NADH-DH under photooxidative stress. We have also investigated the possibility of whether these increases are related to the increases of the levels of the corresponding chloroplastic mRNAs.

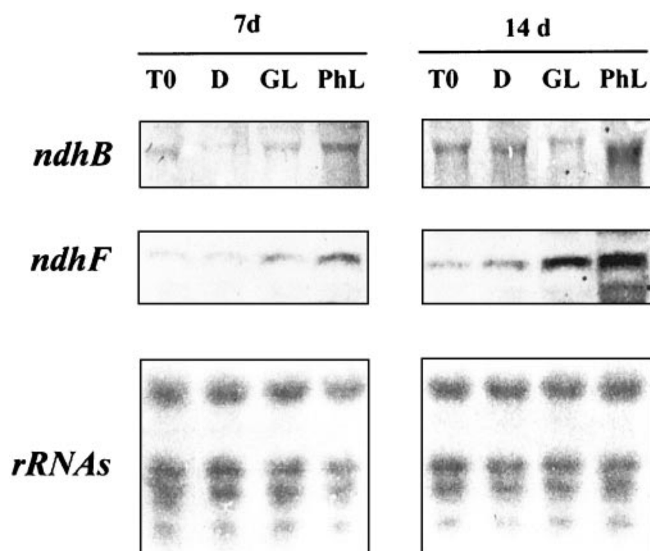

Figure 1. Changes in the steady-state transcript levels of the plastid *ndh*B and *ndhF* genes in response to different light treatments. Primary leaves from young (7-d-old) and mature-senescent (14-d-old) plants were incubated for 20 h at 23°C in darkness (D), GL (100 μmol photon m^{-2} s^{-1}), or PhL (300 μmol photon m^{-2} s^{-1}). Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with *ndhB* or *ndhF* probes or stained with methylene blue (for rRNAs) as described in "Materials and Methods."

RESULTS

Expression of Plastid *ndh* Genes in Response to Photooxidative Stress

Most of *ndh* genes are transcribed as policistrons in a fashion similar to a number of chloroplast-encoded genes (del Campo et al., 2000). However, *ndhB* and *ndhF* are transcribed monocistronically (Martínez et al., 1997) and their transcripts were detected at their predicted size, 1,650 and 2,400 b (data not shown), respectively, in freshly detached leaves of 7- and 14-d-old barley (*Hordeum vulgare* cv Hassan) plants (Fig. 1). The steady-state level of *ndhB* transcripts decreased or did not change after darkness or under growing light (GL) conditions, whereas *ndhF* transcripts slightly increased under GL in both types of leaves. In contrast, a marked increase of the steady-state level of both transcripts was observed in response to 20-h incubation under relatively excess light (photooxidative light [PhL]). However, the initial as well as the photooxidative-induced levels of both transcripts were significantly higher in 14-d-old leaves than in expanding 7-d-old leaves. This pattern of *ndh* transcripts correlates with parallel changes in the amount of NDH-F protein and the activity of the Ndh complex (Casano et al., 1999).

Effects of Hydrogen Peroxide on the Expression of *ndh* Genes

Generation of H_2O_2 during photooxidative stress has been proposed as a part of the signaling cascade leading to induction of nuclear-encoded protecting enzymes (Morita et al., 1999; Polidoros and Scandalios, 1999). It was interesting to investigate whether or not H_2O_2 is involved in the induction of Ndh complex, which participates in the protection of chloroplasts against photooxidative stress (Casano et al., 1999, 2000). As a consequence, a study was carried out on 14-d-old leaves due to their increased response to photooxidative treatments as stated above.

Changes in the Activity of the Ndh Complex and in the Level of NDH-F Protein

The NADH-DH of the thylakoid Ndh complex can be determined in crude extracts through zymogram analysis since it can be clearly distinguished from other pyridine nucleotide dehydrogenases (Casano et al., 2000). The incubation of leaf segments in the presence of H_2O_2 for 20 h under GL caused a dose-dependent increase of NADH-DH, reaching a 2-fold increase at 5 mM over the control incubated with water (Fig. 2). The incubation with H_2O_2 seemed to mimic photooxidative treatment, and its inductive effect was also observed in leaves maintained under darkness. In addition, H_2O_2-induced changes in NADH-DH closely correlated with variations in the level of one of the subunits of the Ndh complex, the NDH-F polypeptide (Fig. 2).

To investigate further the involvement of H_2O_2 in the photooxidative induction of Ndh complex, leaf segments were pre-incubated with dimethylthiourea

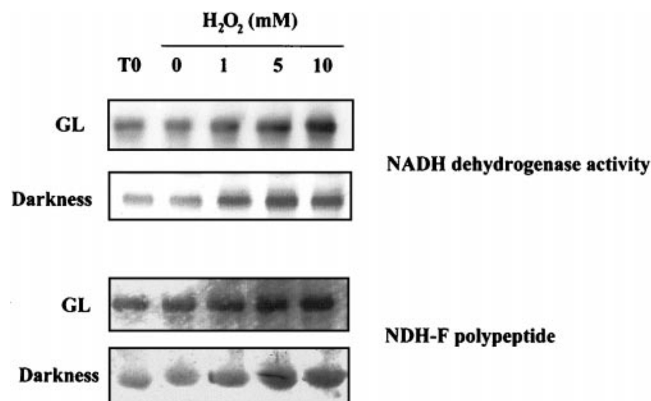

Figure 2. Effect of hydrogen peroxide on the Ndh complex activity and NDH-F protein. Typical zymogram of plastid NADH-DH and western blot with antibody against NDH-F of the Ndh complex from 14-d-old leaves, including activities of freshly detached leaves (T0) and of leaves incubated at 23°C for 20 h with indicated concentrations of H_2O_2 in GL or darkness. For zymograms, 50 μg of protein and for protein blots, 25 μg of protein of leaf crude extracts was loaded per lane. Detailed procedures are described in "Materials and Methods."

(DMTU), a trap for H_2O_2 (Levine et al., 1994), and then transferred to different treatments. The results shown in Figure 3 indicate that pre-incubation with or without DMTU did not have a direct effect on the NADH-DH. As expected, NADH-DH was not affected by a subsequent incubation for 20 h under GL. However, treatments that presumably increase the endogenous generation of H_2O_2 such as PhL and PhL with paraquat (PQ) in the incubation medium (PhL + PQ), or the exogenous addition of H_2O_2 to leaves under GL (GL + H_2O_2) increased NADH-DH by 2.5-, 5-, and 3-fold of the initial level, respectively. In accordance with this, when H_2O_2 was quenched by pre-incubation with DMTU, no inductive effect of photooxidative treatments was observed.

The kinetic of photooxidative induction of the Ndh complex was studied in leaves pre-incubated with or without DMTU and then incubated under GL, PhL, GL + H_2O_2, and PhL + PQ up to 30 h at 23°C. Typical zymograms are shown in Figure 4A. Incubation under GL did not change NADH-DH or NDH-F polypeptide levels in water- and DMTU-pretreated leaves (Fig. 4, B–E). However, NADH-DH rapidly increased up to 16 h and then continued to rise at a lower rate in PhL and GL+ H_2O_2 or it began to decrease in PhL + PQ (Fig. 4B). The pre-incubation with DMTU prevented the photooxidative induction of the enzyme up to 16 h (Fig. 4C). The amount of NDH-F polypeptide followed a pattern similar to that of NADH-DH during the course of incubations up to 16 h in both pretreated leaves (Fig. 4, D and E). Further incubation times produced complex effects. Thus, although PhL or GL + H_2O_2, but not PhL + PQ, reduced NDH-F level, PhL + PQ, but not PhL or GL + H_2O_2, reduced NADH dehydrogenase. At first glance this differential response seems contradictory and requires further investigation because many factors are likely to be involved, e.g. membrane disassembly, changing barriers to diffusion of externally added H_2O_2, and/or complex dose-dependent effects of H_2O_2. In summary, NADH-DH and NDH-F polypeptide of Ndh complex were strongly induced by H_2O_2 and conditions that increase the generation of active oxygen species.

Changes in the Level of ndhB and ndhF Transcripts

To study whether or not the photooxidative- and H_2O_2-mediated induction of Ndh complex correlates with variations in the expression of plastid *ndh* genes we have analyzed the changes in the steady-state levels of *ndhB* and *ndhF* transcripts up to the 16-h incubation with H_2O_2 or under photooxidative conditions in leaves pretreated with water or DMTU. Typical northern blots for *ndhB* and *ndhF* are shown in Figures 5A and 6A, respectively. A 4-h incubation under GL, GL + H_2O_2, or PhL did not modify the levels of both transcripts in water- and DMTU-pretreated leaves (Figs. 5B and 6B, respectively). However, the amount of *ndhB* and *ndhF* transcripts was increased 2-fold with respect to the initial level by PhL + PQ in water pretreated leaves. In general, after a 16-h incubation a strong increase in the level of both transcripts was observed, with changes more

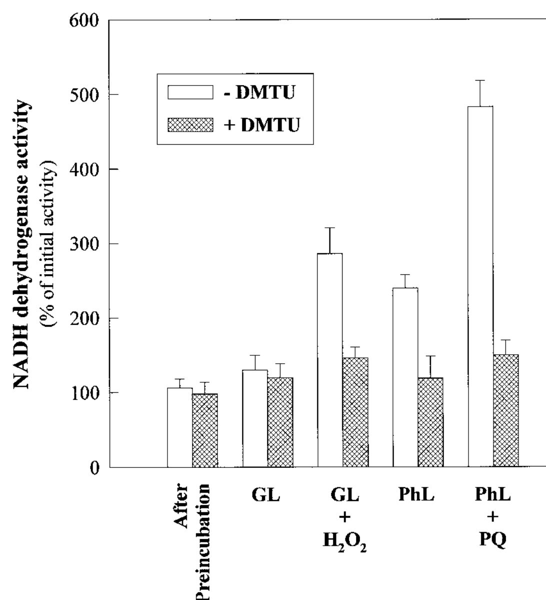

Figure 3. Effects of DMTU, hydrogen peroxide, and photooxidative stress on Ndh activity. NADH-DH of Ndh complex was deduced from zymograms (not shown) of crude extracts (50 μg of protein per lane) from 14-d-old leaves pre-incubated with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m^{-2} s^{-1}. Thereafter, leaves were transferred to GL, GL and 5 mM H_2O_2 in the incubation medium (GL + H_2O_2), PhL and PhL and 50 nM PQ in the incubation medium (PhL + PQ) for 20 h at 23°C. Activities were expressed as percentages of the values in freshly detached leaves (7 nmol NADH oxidized min^{-1} mg^{-1} protein). Values are means of four different experiments.

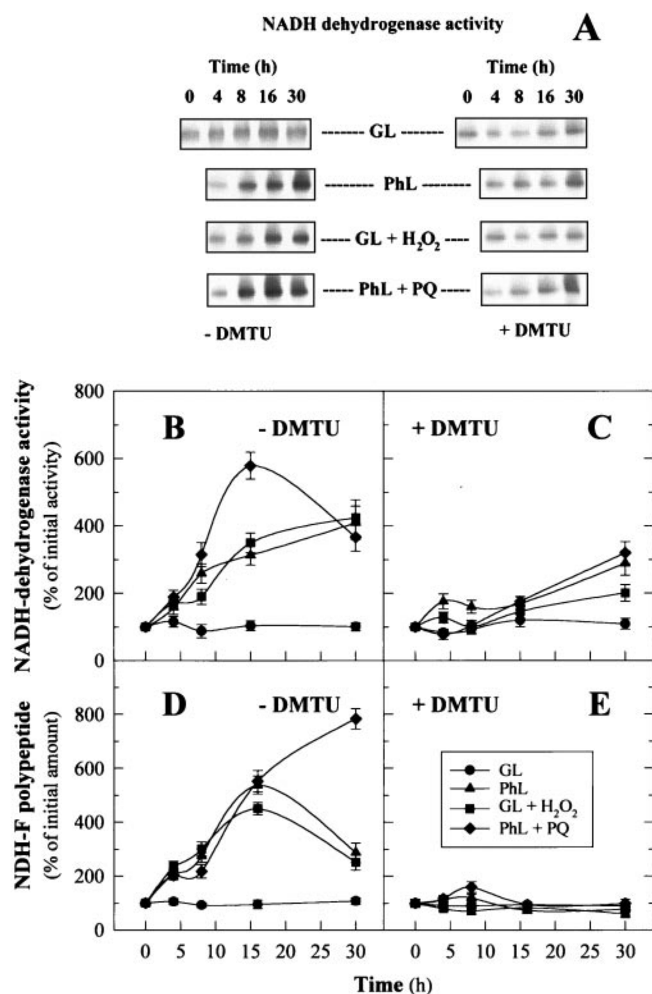

Figure 4. Time-dependent induction of plastid Ndh complex activity and NDH-F protein in photooxidative stressed leaves. A, Typical zymogram of NADH-DH from 14-d-old leaves, including activities of leaves immediately after pre-incubation with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m^{-2} s^{-1} (Time, 0), and after transfer to GL, PhL, GL and 5 mM H_2O_2 in the incubation medium (GL + H_2O_2), and PhL and 50 nM PQ in the incubation medium (PhL + PQ) up to 30 h at 23°C. B and C, Specific NADH dehydrogenase activities of Ndh complex were deduced from experiments as those of A. D and E, Level of NDH-F protein of Ndh complex relative to total soluble protein in leaves treated as in A and deduced from western blots with antibody against NDH-F. For zymograms, 50 μg of protein and for protein blots, 25 μg of protein of leaf crude extracts was loaded per lane. In B through E, activities and protein were expressed as percentages of the values in leaves immediately after pre-incubation (T0). Detailed procedures are described in "Materials and Methods." Values are means of at least three different experiments.

intense in *ndhF* (Figs. 5C and 6C). Under GL, only *ndhF* transcript of water-pretreated leaves increased 3-fold with respect to the initial level. Photooxidative conditions (PhL and PhL + PQ) and H_2O_2 had a very strong stimulating effect on the levels of both transcripts in water-pretreated leaves. However, quenching of H_2O_2 through pre-incubation with DMTU prevented partially, but significantly, this photooxidative induction.

Effects of Hydrogen Peroxide on Thylakoid Peroxidase

The thylakoid peroxidase can scavenge H_2O_2 by oxidizing the plastoquinol, which has been reduced by the action of Ndh complex (Casano et al., 2000). Like Ndh complex, plastoquinol peroxidase activity increases under photooxidative stress, especially in mature-senescent leaves (Casano et al., 1999). Therefore, it was interesting to study whether or not H_2O_2 is also involved in the induction of the thylakoid peroxidase activity. Time-dependent changes of peroxidase activity were studied in leaves pre-incubated with or without DMTU and then incubated under GL, PhL, and GL + H_2O_2 up to 30 h at 23°C. Typical peroxidase zymograms are shown in Figure 7A. Incubation under GL did not change peroxidase activity significantly in water- and DMTU-pretreated leaves (Fig. 7, B and C, respectively). However, in water-pretreated leaves PhL caused a sharp increase of the enzymatic activity after a 4-h incubation, reaching a 3-fold level of the initial value and it then stabilized (Fig. 7B). The early inductive effect was also triggered by incubation with H_2O_2 (GL + H_2O_2), but after a 16-h incubation peroxidase activity did not differ from that of control. When leaves were pretreated with DMTU, no inductive effect of PhL or GL + H_2O_2 on peroxidase activity was observed (Fig. 7C). In general, the obtained results are in agreement with previous observations (Casano et al., 1999, 2000) and suggest that photooxidative induction of thylakoid peroxidase precedes in time that of Ndh complex.

DISCUSSION

The *ndhB* and *ndhF* genes are transcribed from monocistronic units encoded in the inverted repeated and the small single-copy region of plastid DNA, respectively (Freyer et al., 1995; Maier et al., 1995; Martínez et al., 1997). On the other hand, six (*H*, *A*, *I*, *G*, *E*, and *D*) and three (*C*, *K*,, and *J*) *ndh* genes are respectively grouped within two polycistronic transcriptional units that produce multiple transcripts, probably by a complex processing of primary transcripts (Maier et al., 1995; del Campo et al., 2000). The first unit also includes the *psaC* gene encoding a polypeptide of the photosystem I between *ndhE* and *ndhD*. There are uncertainties about which transcripts of each of the two polycistronic units are translated. Preliminary assays in our laboratory indicate that photooxidative and hormone treatments affect the steady-state levels of the different transcripts of polycistronic units in a complex way, suggesting that post-transcriptional processing may be involved in the control of the NDH polypeptides synthesis encoded in polycistronic units. In contrast, the effects

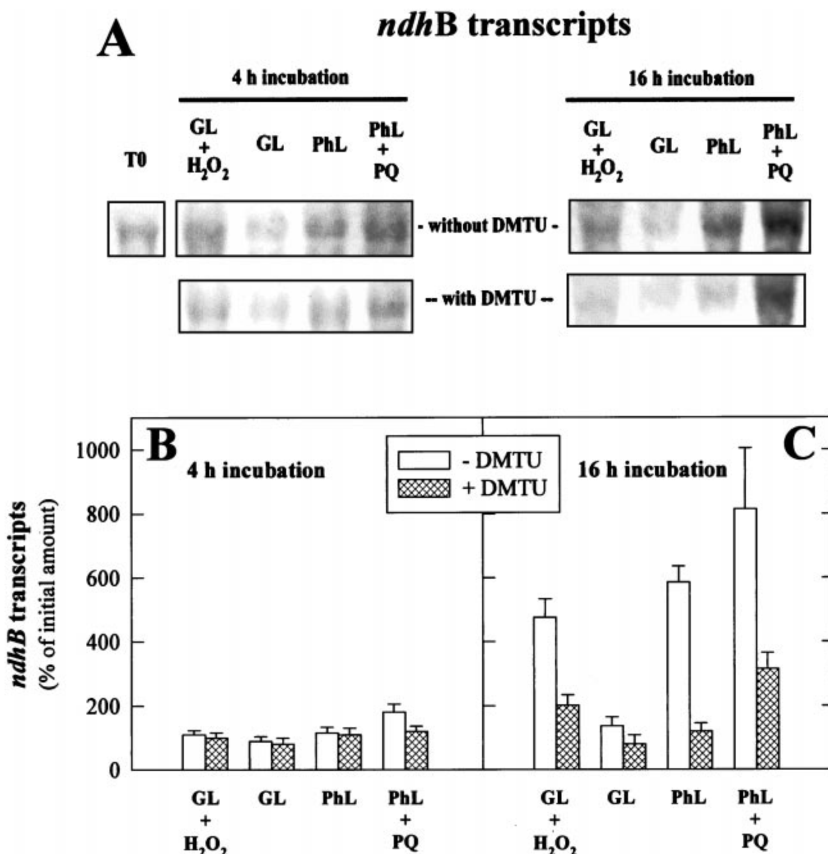

Figure 5. Effects of DMTU, hydrogen peroxide, and photooxidative stress on the steady-state levels of plastid *ndh*B transcripts. A, Northern blot of total RNA from 14-d-old leaves, including RNA of freshly detached leaves (T0) and RNA of leaves pre-incubated with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m^{-2} s^{-1}, and then transferred to GL and 5 mM H_2O_2 in the incubation medium (GL + H_2O_2), GL and PhL, and PhL and 50 nM PQ in the incubation medium (PhL + PQ) at 23°C for the indicated times. Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with *ndh*B probe as described in "Materials and Methods." B and C, Relative level of *ndh*B transcripts was deduced from experiments as those of A and normalized to the respective ribosomal RNA. Values were expressed as percentages of those in freshly detached leaves and represent the mean of three experiments.

of leaf treatments on the levels of the single transcripts of *ndhB* and *ndhF* genes can be more easily investigated. Figures 1, 5, and 6 show that *ndhB* and *ndhF* transcripts increased after treatments producing oxidative stress, especially in mature-senescent leaves. This correlates well with increases of NDH polypeptides and NADH-DH of Ndh complex under photooxidative conditions (Martín et al., 1996; Catalá et al., 1997; Casano et al., 1999, 2000). All together, the results indicate that the induction of plastid Ndh complex is mediated, at least in part, by increases of mRNA levels.

Even though photooxidative stress is initiated within the chloroplasts, the unscavenged excess of H_2O_2 can rapidly diffuse out of the plastid (directly or indirectly), generating a situation of high risk of oxidative damage for the entire cell. To orchestrate an effective cell protection, a number of nuclear- and chloroplast-encoded genes must be induced coordinately. It is possible that some common signaling intermediate is involved in the antioxidant response at chloroplastic and nuclear levels. Environmental stresses such as suboptimal or extreme temperatures (Prassad et al., 1994; Fadzillah et al., 1996; Dat et al., 1998) and excess light (Karpinski et al., 1997) are known to increase the steady-state levels of the H_2O_2. The photooxidative protecting *Cat* and *Gst1* nuclear-encoded genes (Polidoros and Scandalios, 1999) are induced by H_2O_2 in maize in a dose- and time-dependent fashion. Kovtun et al. (2000) recently demonstrated that a specific H_2O_2-responsive mitogen-activated protein kinase cascade mediates the H_2O_2 induction of *Gst* expression. A direct signaling action of H_2O_2 has also been described (Karpinski et al., 1999; Morita et al., 1999) for the nuclear-encoded cytosolic ascorbate peroxidase. In a similar manner, the incubation of barley leaf segments with H_2O_2 increased NADH-DH (Figs. 2 and 3), NDH-F polypeptide (Fig. 2), and *ndhB* and *ndhF* transcripts (Figs. 5 and 6). H_2O_2 applied under GL mimicked the effects of PhL and PhL + PQ, and all these effects were suppressed when leaf segments were pre-incubated with the H_2O_2 scavenger DMTU. These results strongly suggest a direct signaling of H_2O_2 in the induction of protective response against photooxidative stress in chloroplasts. Thus, H_2O_2, which is diffusible through membranes and, at least under photooxidative conditions, is mainly generated in the chloroplasts, induces the expression of specific nuclear and plastid genes. One may wonder whether its action on the expression of plastid *ndh* genes would depend on previous action(s) at the nucleus-cytoplasmic compartments.

Although increases of NADH-DH and NDH-F polypeptide after 10 to 30 h of H_2O_2 or photooxidative treatments (PhL and PhL + PQ; Fig. 4) may be mainly due to increases of transcript levels, the 100% increase of NDH-F and the 50% to 80% increase of

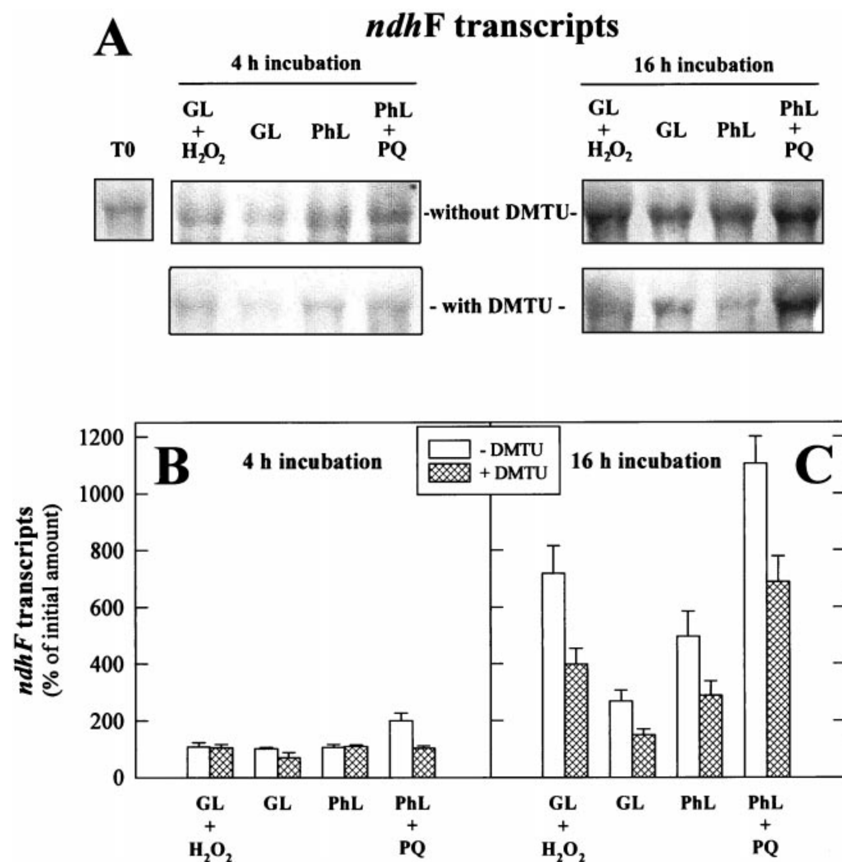

Figure 6. Effects of DMTU, hydrogen peroxide, and photooxidative stress on the steady-state levels of plastid *ndhF* transcripts. A, Northern blot of total RNA from 14-d-old leaves, including RNA of freshly detached leaves (T0) and RNA of leaves pre-incubated with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m^{-2} s^{-1}, and then transferred to GL and 5 mM H_2O_2 in the incubation medium (GL + H_2O_2), GL, PhL, and PhL and 50 nM PQ in the incubation medium (PhL + PQ) at 23°C for the indicated times. Total RNA was isolated from barley leaves, separated by agarose electrophoresis (12.5 μg each), blotted on a nylon membrane, and hybridized with *ndhF* probe as described in "Materials and Methods." B and C, Relative level of *ndhF* transcripts was deduced from experiments as those of A and normalized to the respective ribosomal RNA. Values were expressed as percentages of those in freshly detached leaves and represent the mean of three experiments.

NADH-DH after 4 h of treatment were not accounted for by increases of *ndhB* and *ndhF* transcripts (Figs. 5 and 6), except for PhL + PQ treatment. This suggests that in addition to the effect mediated by increasing mRNAs levels, photooxidative treatment and H_2O_2 (as its transduction signal) promote the translation of pre-existing transcripts of *ndh* genes. It is significant that an increase in NDH-F polypeptide and NADH-DH under oxidative treatment (Fig. 4, B and D) showed a two-phase behavior, the first one probably due to an effect on the translatability and the second one to an effect on mRNA level.

On the other hand, the relatively small, but significant increase of transcripts after 4 h of incubation with PhL + PQ (Figs. 5B and 6B) suggests that the intrachloroplastic H_2O_2 produced under such conditions could modulate transcript levels without the involvement of newly synthesized nucleus-cytoplasmic intermediates. However, the rapid increase of thylakoid peroxidase (Fig. 7, A and B), which is presumably encoded in the nucleus, also suggests a rapid action of H_2O_2 in the nucleus-cytoplasmic compartments, as reported for other H_2O_2-scavenging enzymes (Karpinski et al., 1999; Morita et al., 1999; Polidoros and Scandalios, 1999). An alternative explanation is that the thylakoid peroxidase could be activated directly by H_2O_2.

The enhanced expression of *ndh* genes reported in this paper is first described for plastid DNA genes under photooxidative stress. Plastid DNA from several species has been completely sequenced and although the control plastid gene expression includes different post-transcriptional steps (del Campo et al., 2000; Mayfield et al., 1995), its low size makes it a good candidate for investigating the induction of gene expression under photooxidative stress. The involvement of H_2O_2 as a key component of the signal transduction pathway in the responses of nucleus and chloroplast DNAs suggests that some similar steps or mechanisms coordinate the responses of the two genomes against photooxidative stress.

MATERIALS AND METHODS

Plant Material

Barley (*Hordeum vulgare* cv Hassan) was grown on vermiculite in a controlled growth chamber at 23°C under a 16-h photoperiod of 100 μmol photon m^{-2} s^{-1} white light as described by Casano et al. (1999). In the present work we have used primary leaves of 7- and 14-d-old plants as young expanding and aged-senescent leaves, respectively. Subapical leaf segments (3 cm in length) were cut 4 to 5 h after the beginning of photoperiod and were incubated at 23°C up to 30 h with different concentrations of H_2O_2 or 50 nM PQ in darkness, GL (100 μmol photon m^{-2} s^{-1}), or PhL (300 μmol photon m^{-2} s^{-1}). In some experiments the leaf

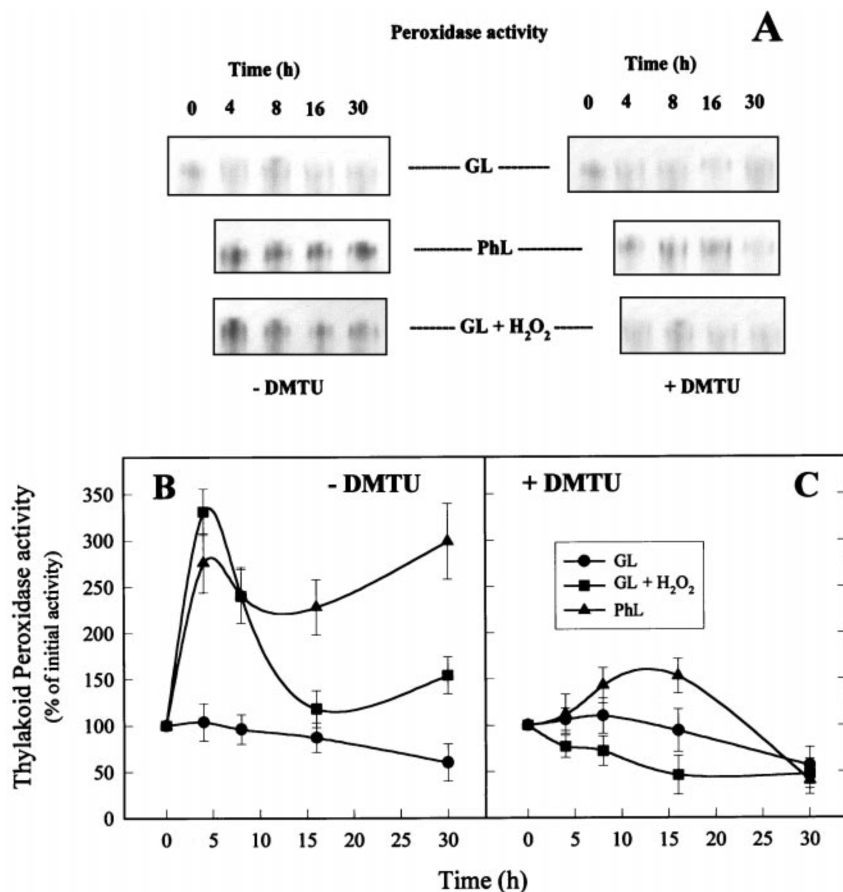

Figure 7. Time-dependent induction of thylakoid peroxidase activity in photooxidative stressed leaves. A, Typical zymogram of thylakoid peroxidase activity from 14-d-old leaves, including activities of leaves immediately after pre-incubation with 0 and 5 mM DMTU for 4 h at 23°C under 100 μmol photon m^{-2} s^{-1} (T0), and after transfer to GL, PhL, and GL and 5 mM H_2O_2 in the incubation medium (GL + H_2O_2) up to 30 h at 23°C. B and C, Specific thylakoid peroxidase activities were deduced from experiments as those of A. Fifty micrograms of protein of leaf crude extracts was loaded per lane. In B and C, activities were expressed as percentages of the values in leaves immediately after pre-incubation (T0; 70 nmol HQ oxidized min^{-1} mg^{-1} protein). Detailed procedures are described in "Materials and Methods." Values are means of at least three different experiments.

segments were treated with 0 or 5 mM DMTU for 4 h in GL at 23°C prior to treatment of leaves as described above.

RNA Isolation and Northern-Blot Analysis

Total RNA of leaf segments (2 g) was extracted by phenol-SDS treatment and selective precipitation with LiCl as described (Jones et al., 1985; Eker and Davies, 1987). It was typical that RNA yields were around 0.25 mg/g leaves. RNA samples (12.5 μg) were denatured in formaldehyde and run on 1.2% (w/v) agarose-18% (v/v) formaldehyde gels (Sambrook et al., 1989). After electrophoresis, denatured RNA was immobilized on nylon membranes (Zeta-Probe, Bio-Rad, Hercules, CA). Ribosomal RNAs and M_r markers (Boehringer Mannheim, Mannheim, Germany) were stained with methylene blue (Sambrook et al., 1989) and then scanned using an UVP Easy digital image analyzer (Ultra-Violet Limited, San Gabriel, CA), with automatic background correction. Thereafter, membranes were hybridized to digoxigenin-labeled PCR probes of *ndhB* and *ndhF*. Washings were performed under high stringency conditions. Transcript bands were scanned and mRNA levels were expressed on a total rRNA basis as described (Casano et al., 1994).

Homologous digoxigenin-labeled PCR probes of *ndhB* and *ndhF* genes were prepared (Lo et al., 1990) from barley plastid DNA as template. Barley plastid DNA was isolated as described by Heinhorst et al. (1988). The probes were enriched in the strand complementary to transcripts by using a 50:1 ratio of 3′ terminal:5′ terminal primer for each corresponding mRNA. In this order the used primers were: ATCGATTCAACCTCTGAT and AGCCTCATTAGACCG-TAG spanning a 396-bp of *ndhB* probe in barley (Freyer et al., 1995) and CCCACAGTAACTACCT and GCGTTT-TATATGTTTCGG spanning a 735-bp *ndhF* probe near the 3′ end in rice (Hiratsuka et al., 1989) and probably in barley.

Preparation of Leaf Crude Extracts

For zymographic and western-blot assays, activities and proteins were assayed in whole-leaf extracts obtained as follows: 10 leaf segments were homogenized with a mortar and pestle in 2 mL of 50 mM potassium phosphate, pH 7.0, 1 mM L-ascorbic acid, 1 mM EDTA, and 5% (w/v) polyvinylpirrolidone, and were centrifuged at 500*g* for 10 min. Triton X-100 was added to supernatant to make a final 2% (w/v) solution and gently stirred for 30 min. The suspension was centrifuged at 20,000*g* for 30 min. Supernatants contained 0.7 to 1.3 mg protein mL^{-1}. The entire procedure was carried out at 4°C.

Gel Electrophoresis, Zymograms, and Immunoassays

Native PAGE was carried out at 5°C (usually with 100 μg of protein samples) in a linear gradient gel of 3% to 10%

(w/v) polyacrylamide (2.5% [w/v] bis-acrylamide) in the same way as SDS-PAGE with the exception that gels contained 0.1% (w/v) Triton X-100 instead of SDS (Casano et al., 1999). For zymograms, NADH-DH of Ndh complex was detected by incubation of gel slices for 20 to 30 min at 30°C in darkness in 50 mM potassium phosphate (pH 8.0), 1 mM Na_2-EDTA, 0.2 mM NADH, and 0.5 mg mL^{-1} nitroblue tetrazolium. In controls without NADH, no stain developed. Staining for peroxidase was performed by following standard methods with 4-methoxy-α-naphthol, as described by Casano et al. (1999). For immunoblot analyses, after SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The immunocomplex with antibodies prepared against the NDH-F polypeptide encoded by the *ndhF* gene (Catalá et al., 1997) was detected with the alkaline phosphatase western-blotting analysis system (Boehringer Mannheim).

Bands from zymograms and immunoblots were scanned with a UVP Easy Digital Image analyzer to comparatively quantify activity and protein values, which were expressed as percentages of the reference (that of freshly detached primary leaves).

Other Determinations

The NADH:FeCN oxidoreductase activity, specific for Ndh complex, was assayed at 30°C by measuring the reduction of FeCN at 420 nm and the oxidation of NADH at 340 nm as described (Casano et al., 2000). The spectrophotometric assay of thylakoid peroxidase was performed at 30°C by measuring the oxidation rate of hydroquinone in the presence of H_2O_2, as described by Casano et al. (1999). Specific activities were expressed as micromoles of NADH or hydroquinone consumed per minute per milligram of protein.

Protein concentration was quantified by the Bradford method (1976) with a Protein Assay Kit (Bio-Rad) using bovine serum albumin as a standard.

All reported results were reproduced at least three times. When appropriate, standard deviations were indicated by bars in figures.

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