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A Chloroplast-Targeted Heat Shock Protein 70 (HSP70) Contributes to the Photoprotection and Repair of Photosystem II during and after Photoinhibition

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Dark-grown *Chlamydomonas reinhardtii* cultures that were illuminated at low fluence rates before exposure to high-light conditions exhibited a faster rate of recovery from photoinhibition than did dark-grown cells that were directly exposed to photoinhibitory conditions. This pretreatment has been shown to induce the expression of several nuclear heat shock protein 70 (*HSP70*) genes, including *HSP70B*, encoding a chloroplast-localized chaperone. To investigate a possible role of plastidic HSP70B in photoprotection and repair of photosystem II, which is the major target of photoinhibition, we have constructed strains overexpressing or underexpressing *HSP70B*. The effect of light stress on photosystem II in nuclear transformants harboring *HSP70B* in the sense or antisense orientation was monitored by measuring variable fluorescence, flash-induced charge separation, and relative amounts of various photosystem II polypeptides. Underexpression of *HSP70B* caused an increased light sensitivity of photosystem II, whereas overexpression of *HSP70B* had a protective effect. Furthermore, the reactivation of photosystem II after photoinhibition was enhanced in the *HSP70B*-overexpressing strain when compared with the wild type, both in the presence or absence of synthesis of chloroplast-encoded proteins. Therefore, HSP70B may participate in vivo both in the molecular protection of the photosystem II reaction centers during photoinhibition and in the process of photosystem II repair.

INTRODUCTION

Members of the heat shock protein 70 (HSP70) family, also known as stress 70 molecular chaperones, have been found in almost all organisms, from archaebacteria to eukaryotes and eubacteria, and represent one of the most conserved protein families known to date (Boorstein et al., 1994). In eukaryotes, multiple members of the stress 70 chaperone family are known. They are located in the cytosol as well as in specific subcellular compartments, such as the endoplasmic reticulum lumen and organelles. Like all chaperones, their basic property is to bind unfolded polypeptides and to release them in an ATP-dependent reaction (Rüdiger et al., 1997).

HSP70s are involved in almost every step of protein biogenesis (reviewed in Hartl, 1996). In addition to other activities, they assist in the correct folding of nascent polypeptide chains as they emerge from the ribosome, they participate in transmembrane protein transport, and they prevent aggregation of denatured proteins and allow the disassembly of multimolecular complexes. The different isoforms appear to have little selectivity in terms of peptide binding, and thus, their functional specificities seem to arise mostly from their distinct subcellular locations and from their specific interactions with other proteins (Rassow et al., 1995). The best known of these cochaperones are the bacterial DnaJ and GrpE proteins and their eukaryotic homologs.

Our understanding of HSP70 function in organelles has come mainly from work with yeast mitochondria. A single, matrix-located HSP70 is present in *Saccharomyces cerevisiae* mitochondria and is encoded by an essential nuclear gene (Craig et al., 1989). It has been shown to associate with newly imported polypeptide chains both at an early stage of import (in a complex with GrpE and the inner membrane channel protein TIM44) and at a later stage (bound to GrpE and the mitochondrial DnaJ homolog) (Horst et al., 1997). The matrix-located HSP70 may act as a mechanochemical enzyme, pulling proteins through the import channel by cycles of ATP binding and hydrolysis (Horst et al., 1997).

In contrast, multiple HSP70 isoforms are found in higher plant chloroplasts, located either in the chloroplast outer envelope (Ko et al., 1992; Schnell et al., 1994) or in the stroma (Marshall et al., 1990). The stromal HSP70s are more closely related to the eubacterial and mitochondrial proteins,

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whereas the envelope-associated HSP70s exhibit higher similarity to eukaryotic stress 70 chaperones. To date, the best-documented function of plastidic HSP70s is the import of nuclear-encoded polypeptides: two HSP70 homologs of the chloroplast envelope, Iap70 and Com70, have been identified as components of the import machinery (Schnell et al., 1994; Kourtz and Ko, 1997). Stromal HSP70 has been found to interact with newly imported ferredoxin–$NADP^+$ reductase (Tsugeki and Nishimura, 1993) and the Rieske iron–sulfur protein (Madueño et al., 1993). It also was shown to associate with phytoene desaturase in *Narcissus pseudonarcissus* chromoplasts (Bonk et al., 1996). However, in contrast to HSP70 of the mitochondrial matrix, a specific function of stromal HSP70 in the import process has not been demonstrated thus far.

In the unicellular green alga *Chlamydomonas reinhardtii*, three HSP70 genes have been identified (von Gromoff et al., 1989). Of the two genes analyzed, *HSP70A* encodes a cytosolic HSP70, and *HSP70B* encodes a gene product that is located in the stroma of the chloroplast (Drzymalla et al., 1996). Interestingly, all three genes are induced not only by heat shock but also by light of low intensity after a period of dark incubation (von Gromoff et al., 1989; Kropat et al., 1995). The *HSP70B* gene is also regulated by a circadian rhythm, with maximum expression at subjective dawn (S. Jacobshagen, personal communication). The function of this expression pattern may be to prepare cells for a subsequent exposure to high light (Kropat et al., 1995). A role of HSPs in protection against photoinhibition has already been proposed (Schuster et al., 1988a), based on the finding that exposure of Chlamydomonas cells to a heat shock results in enhanced resistance to light stress. Similarly, heat pretreatment of barley plantlets significantly enhanced resistance against photoinhibition under heat shock conditions (Stapel et al., 1993).

Photoinhibition is a major limitation to photosynthesis under field conditions, and its molecular mechanism has received extensive attention (reviewed in Prasil et al., 1992; Aro et al., 1993; Ohad et al., 1994). The main target for light stress is the oxygen-evolving photosystem II (PSII) complex in the thylakoid membrane. Excessive irradiation results in an overreduction of the electron transfer chain, which eventually causes an impairment of electron transport and irreversible damage to the subunits of PSII reaction centers. Among the PSII core proteins, the Q_B binding protein D1 is the main target (Kyle et al., 1984), although degradation of other PSII components also has been observed (Schuster et al., 1988b; Andersson and Barber, 1994; Zer et al., 1994; Zer and Ohad, 1995).

Plants have evolved several strategies to keep photoinhibition at a minimum. One of these is the state transition process, whereby the major antenna protein of PSII, light-harvesting complex II, becomes phosphorylated and migrates from PSII to PSI-rich nonappressed thylakoid regions (reviewed in Allen, 1992). This antenna movement diverts part of the excess light energy away from PSII and thereby acts as a photoprotection mechanism (Keren and Ohad, 1998). In addition, nonradiative dissipation of excess excitation energy can be brought about by deepoxidation of specific xanthophylls, resulting in nonphotochemical quenching of chlorophyll fluorescence (Horton et al., 1994). Once the capacity of these protective mechanisms has been exceeded, maintaining photosynthetic activity rests with the repair of the photodamaged reaction centers and on the synthesis of new centers. When the rate of photodamage exceeds the rate of repair and replacement, photoinhibition of PSII takes place and the accumulation of damaged PSII centers occurs (Greer et al., 1986; Vasilikiotis and Melis, 1994).

Photoinhibition of PSII induces a series of sequential events leading to the proteolytic degradation of the D1 protein and its replacement by a de novo–synthesized protein (Ohad et al., 1984, 1994; Greenberg et al., 1987; Melis, 1991; Aro et al., 1993; van Wijk et al., 1994). The disappearance of D1 during photoinactivation of electron transport has been demonstrated both in vivo (Ohad et al., 1984; Schuster et al., 1988b) and in vitro (Ohad et al., 1985). Damaged PSII centers undergo lateral migration from appressed regions of thylakoid membranes to nonappressed stroma-exposed regions where repair takes place (Adir et al., 1990; Hundal et al., 1990; Melis, 1991). Partial disassembly of PSII complexes has been observed in these stroma-exposed thylakoid membranes (Hundal et al., 1990). The first step in the degradation of D1 appears to be mediated by a protease that is associated with the PSII complex, although its molecular identification remains controversial (reviewed in Andersson and Aro, 1997). Degradation of D1 induces several changes in the organization of the PSII complex, such as the release of the three oxygen-evolving enhancer (OEE) subunits located at the lumenal surface of the membrane (Eisenberg-Domovich et al., 1995) and the loss of manganese. The newly synthesized D1 protein is cotranslationally inserted into the same membrane regions; from here, repaired PSII centers migrate back to the appressed regions (Wettern, 1986; Mattoo and Edelman, 1987).

The repair of damaged PSII reaction centers is the most important means by which plant cells keep these centers functional during and after light stress. It should be noted that PSII recovery from photoinhibition in most photosynthetic organisms can be regarded as an amplification of a continuous repair process that also occurs in dim light, as has been demonstrated by Keren et al. (1995). Thus, the molecular machinery required for PSII recovery after photoinhibition is expected to be stimulated rather than specifically induced under light stress conditions.

In this study, we present direct evidence that the stromal HSP70B protein is involved in resistance to photoinhibition: overexpression of *HSP70B* reduces photoinactivation of PSII and enhances recovery, whereas underexpression causes the opposite effect. We propose that HSP70B acts by both preventing the destruction of inactivated reaction centers and promoting the synthesis of new centers.

RESULTS

Pretreatment of Dark-Adapted Cells with Low Light Improves Recovery of PSII from Photoinhibition

Transfer of dark-adapted Chlamydomonas cells to low light previously has been shown to result in an induction of several *HSP70* genes (von Gromoff et al., 1989). In particular, it was observed that such low-light treatment of dark-adapted cells increased the HSP70B levels in the chloroplast compartment (Drzymalla et al., 1996).

To test whether this low-light pretreatment would protect PSII activity from exposure to light stress, cells grown for 18 hr in the dark were either directly exposed to high light (photoinhibitory conditions of 2000 μE m^{-2} sec^{-1}) for 1 hr or preilluminated with low light (20 μE m^{-2} sec^{-1}) for 90 min before the high-light treatment. PSII activity was determined by fluorescence induction and is reflected by the F_v/F_{max} ratios ($F_v = F_{max} - F_0$, where F_v is variable fluorescence, and F_{max} and F_0 are the fluorescence emitted when all PSII reaction centers are closed and open, respectively) (reviewed in Krause and Weis, 1991) (Figure 1A): a value of ~0.75 is typical for a wild-type strain with fully active PSII centers, whereas mutants lacking PSII display no variable fluorescence and therefore would have a value of 0 in this index. Chlamydomonas cells, irrespective of their pretreatment, lost most of their PSII activity after 60 min of photoinhibition (F_v/F_{max} of ≈0.1). However, preilluminated cells recovered their PSII activity significantly faster than did cells that were kept in the dark: in the results from a typical experiment shown in Figure 1A, the half-time of recovery of the original F_v/F_{max} ratio was 55 min versus 80 min. Throughout the experiment, preilluminated cells exhibited 30 to 50% higher levels of HSP70B protein than did cells that had been incubated in the dark (Figure 1B). Also, both preilluminated and dark-adapted cells showed a 70 to 80% increase in HSP70B levels during the recovery phase.

Construction of Strains Overexpressing and Underexpressing *HSP70B*

To elucidate a possible function of elevated HSP70B levels in the recovery of PSII from photoinhibition, we constructed strains that exhibited either increased or decreased levels of HSP70B. The construct generated for overexpression (pCB740) is shown in Figure 2A. A genomic copy of *HSP70B* was cloned behind the fused Chlamydomonas *HSP70A* and ribulose bisphosphate carboxylase small subunit (*RBCS2*) promoters. This promoter fusion exhibits high basal-level expression as well as inducibility by heat shock and light (M. Schroda, D. Blöcker, and C.F. Beck, submitted manuscript). The *HSP70B* construct was cloned in a vector that harbors the argininosuccinate lyase (*ARG7*) gene of Chlamydomonas, used for the selection of transformants in *arg7* mutants. Two

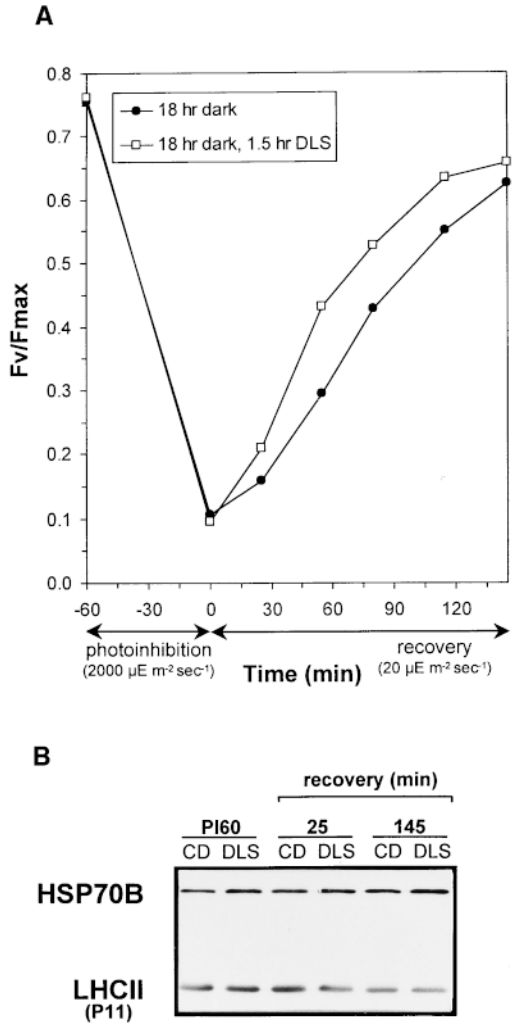

Figure 1. Analysis of PSII Recovery from Photoinhibition of Dark-Adapted Chlamydomonas Cultures.

(A) The results of a typical experiment showing the time course of PSII activity during photoinhibition and recovery measured as variable fluorescence ($F_v = F_{max} - F_0$) normalized to F_{max}. Cultures of the cell-walled strain were kept in the dark for 18 hr, one half of the culture was directly photoinhibited at a light intensity of 2000 μE m^{-2} sec^{-1} for 60 min, and the other half was exposed to a fluence rate of 20 μE m^{-2} sec^{-1} of white light for 1.5 hr before photoinhibition (DLS). Recovery of PSII activity took place in dim light (20 μE m^{-2} sec^{-1}).

(B) HSP70B protein levels from the same experiment as described in **(A)** after 60 min of photoinhibition (PI60) and during the recovery phase in dim light. Cells subjected to dark–light shift (DLS) were compared with those kept in the dark (CD). For each lane, 10 μg of whole-cell protein was separated on a 12% SDS–polyacrylamide gel, blotted, and immunodecorated with antibodies raised against HSP70B and light-harvesting complex II (LHCII; protein P11). The latter was used as a loading control.

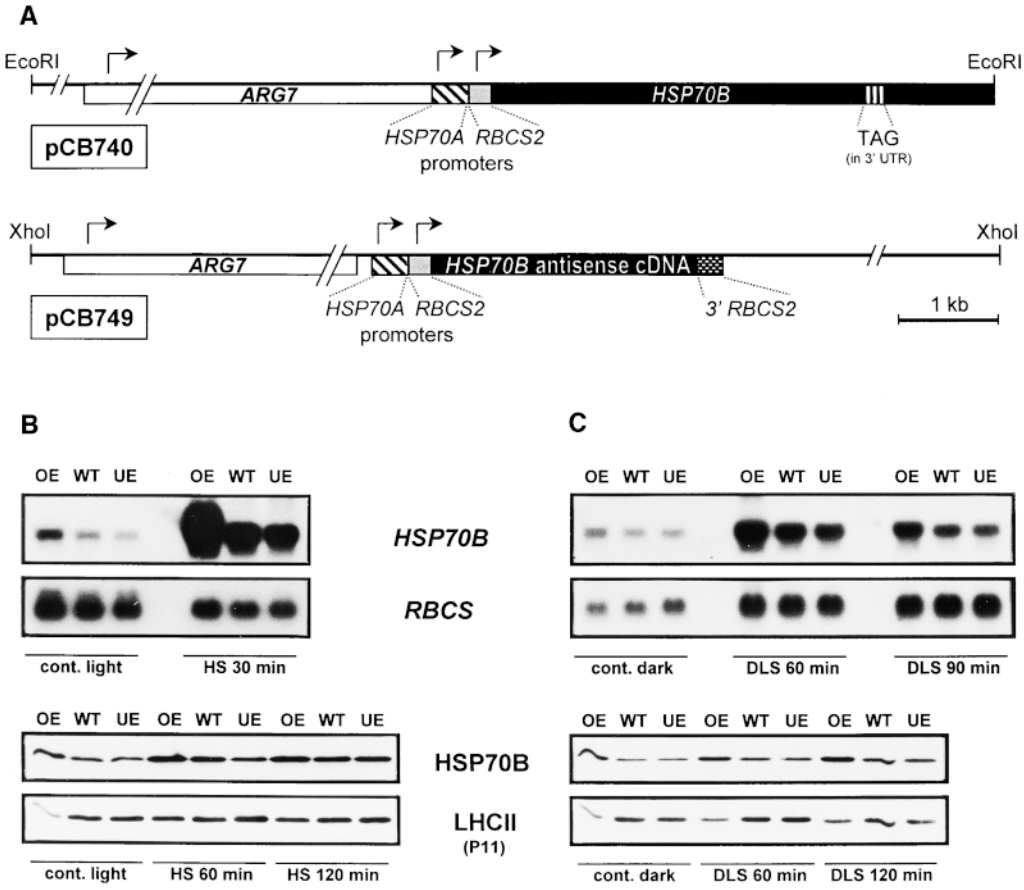

Figure 2. Gene Constructs and Expression Levels of Transformants Overexpressing and Underexpressing *HSP70B*.

(A) pCB740 consists of a promoterless 5-kb genomic fragment containing the *HSP70B* gene (black box) with a 213-bp bacterial sequence (TAG) inserted into the 3′ untranslated region (UTR; vertically striped box). pCB749 consists of an *HSP70B* cDNA in an antisense orientation (black box) lacking 370 bp of its 3′ untranslated region and trailed by a 230-bp 3′ *RBCS2* gene fragment (checkered box). Both genes are placed downstream of a fusion of the Δ196 *HSP70A* promoter (diagonally striped box) and the Δ183 *RBCS2* promoter (gray box). Both constructs also contain the *ARG7* gene as a selectable marker (white box). Bent arrows indicate the direction of transcription initiating at promoters *ARG7*, *HSP70A*, and *RBCS2*, respectively.

(B) *HSP70B* mRNA (top) and protein levels (bottom) of individual transformants in a cell wall–deficient background in continuous (cont.) light and after heat shock (HS) at 40°C.

(C) *HSP70B* mRNA (top) and protein levels (bottom) of transformants in continuous darkness (16 hr) and after shift to low light (DLS) (30 μE m^{-2} sec^{-1}). OE, overexpressor; UE, underexpressor; WT, control transformant. Loading controls were *RBCS* for RNA gel blot analysis, and light-harvesting complex II (LHCII; protein P11) was used for immunoblots.

different *arg7* strains were used. One is walled (CF30), and the other is cell wall deficient (cw15-302). Each was complemented by transformation with pCB740. One transformant of each recipient strain that exhibited elevated levels of HSP70B was analyzed in more detail. DNA gel blot analysis revealed a single insert in the cw15-302 transformant (CF184) and two inserts in the CF30 transformant (CF182). One of the two CF30 inserts was incomplete (data not shown).

To reduce expression of the endogenous *HSP70B*, we used an antisense strategy. For this purpose, a partial *HSP70B* cDNA copy was cloned in an inverted orientation behind the fused *HSP70A–RBCS2* promoter (Figure 2A). This construct, placed in a vector with the *ARG7* gene (pCB749), was introduced into cw15-302. DNA gel blot analysis of one of the transformants that exhibited reduced levels of HSP70B revealed two intact copies of the antisense construct (data not shown).

Figure 2B illustrates the degree of overexpression and underexpression achieved using these constructs in the cell wall–less background. In continuous light and after heat shock (Figure 2B, top gel), *HSP70B* mRNA levels were 60 to

65% higher in the *HSP70B*-overexpressing strain as compared with a control wild-type transformant (i.e., transformed with *ARG7* alone). In the underexpressing strain, mRNA levels were 20 to 40% lower than in the wild type. When cells were incubated in complete darkness (Figure 2C, top gel), the overexpressing strain showed an approximately twofold higher level of *HSP70B* mRNA as compared with the wild type, whereas in the underexpresser only a small reduction in mRNA level was seen. When transferred to light, mRNA abundance increased, with a maximum at ~60 min; however, this increase was less pronounced in the underexpresser. At this time, the *HSP70B* mRNA level was ~90% higher in the overexpresser than in the wild type and ~30% lower in the underexpresser.

The level of accumulation of the HSP70B protein (Figures 2B and 2C, gels at bottom) followed a pattern similar to that of the mRNA, except that differences were less marked. Compared with that in the wild type, the HSP70B level in the overexpresser was 20 to 30% higher in continuous light and after heat shock, whereas the underexpresser showed a normal accumulation in continuous light and a 30% reduction after a 60-min heat shock treatment. In continuous darkness, the HSP70B protein level in the overexpresser was more than twofold higher than in the wild type, but no difference was seen between the underexpresser and the wild-type strain. A dark–light shift induced a rise in HSP70B levels in all three strains, with increased accumulation most pronounced in the wild-type strain. This led to a decrease in the difference of HSP70B levels between the wild type and overexpresser (40% more in the overexpresser) and an increase between the wild type and underexpresser (15% more in the wild type). In conclusion, whereas the overexpressing strain consistently had a higher HSP70B protein level than did the wild type, the effect of the underexpression construct was best observed after induction by heat shock or a shift from dark to light.

For the cell-walled overexpressing strain, similar observations were made, except that the degree of overexpression was somewhat lower (data not shown). Similar effects were observed for heat shock and dark–light shift. The differences in *HSP70B* expression between the wild type and the overexpressing or underexpressing transformants have been reproducibly observed at the protein as well as the mRNA levels in several independent experiments. We noted, however, that the exact level of *HSP70B* expression is subject to fluctuations depending on physiological parameters, such as cell density or light intensity.

Strains Altered in Their HSP70B Levels Differ in the Extent of Photoinhibitory Damage to PSII and in Recovery Rate

The availability of strains differing from each other only in the absence or presence of sense or antisense *HSP70B* transgenes allowed us to test whether the improved recovery of PSII from photoinhibition observed after light induction (Figure 1) was due to an increase in HSP70B level. Strains were grown in continuous light and then photoinhibited at high light intensities for 1 hr and allowed to recover in dim light, as described in Methods. Figure 3 shows the values of F_v/F_{max}, averaged over several experiments with *HSP70B*-overexpressing and *HSP70B*-underexpressing strains and with a control transformant. All were in the cell wall–defective background.

Some differences in the extent of PSII inactivation were observed immediately after photoinhibition: the *HSP70B*-overexpressing strain CF184 on average retained 21% of its original F_v/F_{max} ratio when compared with ~10% for the control strain CF185. The *HSP70B*-underexpressing strain CF186 was most severely photoinhibited and retained only

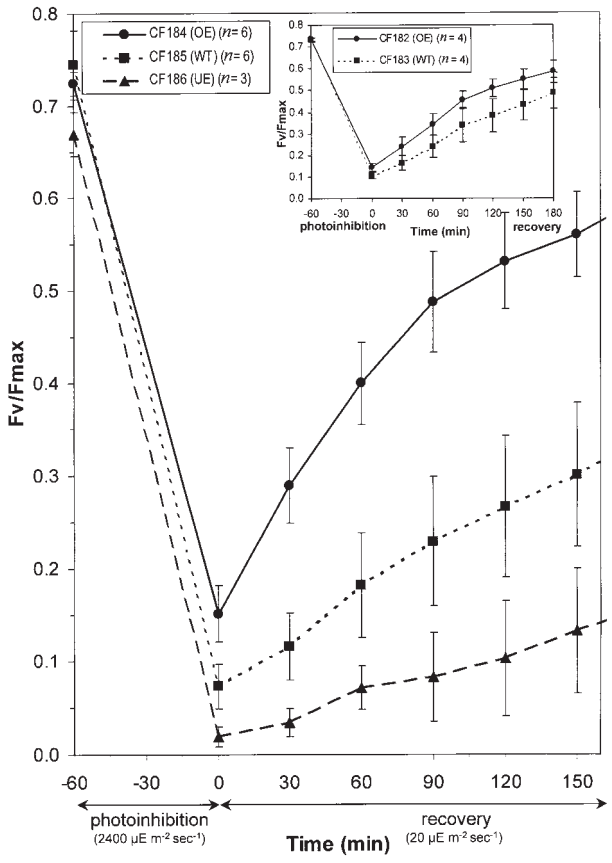

Figure 3. Effect of High-Light Treatment on PSII Activity.

The F_v/F_{max} values of the wild type (WT) and strains overexpressing (OE) or underexpressing (UE) *HSP70B* are compared. Data using cell wall–deficient strains are shown; in the inset, data of cell-walled strains are shown. Cells were photoinhibited for 60 min at 2400 µE m^{-2} sec^{-1} and then allowed to recover in low light (20 µE m^{-2} sec^{-1}). PSII activity was monitored by the F_v/F_{max} values. Bars indicate standard errors.

~3% of its initial F_v/F_{max} ratio. Similarly, the cell-walled *HSP70B*-overexpressing strain CF182 retained a higher F_v/F_{max} ratio after 60 min of high-intensity illumination when compared with the control strain CF183 (inset in Figure 3).

The major effect of the differential expression of *HSP70B* was observed in the recovery phase. Whereas the half-time in the recovery of the original F_v/F_{max} ratio was ~75 min in the *HSP70B*-overexpressing strain CF184, the control strain had recovered only ~20% of the original value at that time point. PSII recovery in the *HSP70B*-underexpressing strain was severely reduced, with only 8% recovery within 75 min. The same observation, although to a somewhat lesser degree, was made with the cell-walled strains, in which PSII activity recovered faster in the overexpresser CF182 than in the control CF183 (inset in Figure 3). In general, we found that our cell-walled strains (see Figure 1 and inset of Figure 3) recovered faster from photoinhibition than did the wall-less ones. It is important to note that the observed effects became significant only after extensive photoinhibition, that is, when strains had lost at least 70% of their initial PSII activity.

Analysis of HSP70B and PSII Protein Levels during Photoinhibition and Recovery

As noted above, photoinactivation of PSII is the first of a series of events leading to the degradation of PSII reaction center polypeptides, primarily the D1 protein. In the results from the two typical experiments shown in Figure 4, the protein levels in the control and overexpressing and underexpressing transformants were monitored at the same time as F_v/F_{max}. As can be seen in the gels in Figures 4A and 4B, the photoinhibition treatment itself affected HSP70B abundance differently in the three strains. The overexpresser had a 1.5- to twofold higher level than did the control strain before and immediately after photoinhibition. During recovery, the increase in HSP70B levels was more pronounced in the control strain, resulting in similar HSP70B concentrations after 220 min of recovery. In contrast, the level of HSP70B in the underexpresser did not increase after photoinhibition, so that it was 30 to 50% lower than in the wild type during most of the recovery phase.

PSII polypeptides behaved very differently in the three strains during and after photoinhibition. Clearly, the wild type lost more D1 polypeptide than did the overexpresser (70 versus 30%; Figure 4A). This correlated with a more pronounced decrease in PSII activity (90 versus 75%). The behavior of CP43—a PSII core antenna polypeptide—was similar to that of D1. For both proteins, recovery was faster in the overexpressing strain, again correlating with an improved recovery of PSII activity. Consistent with these results, the loss of D1 and CP43 in the underexpresser was more extensive than in the wild type (Figure 4B). Here, no recovery was seen during exposure to dim light, but instead further loss was observed. As expected, PSII activity also did not recover in the underexpressing strain.

Probably as a result of degradation of the transmembrane components of PSII, we noted the accumulation of a degradation product of the OEE2 protein at the end of the photoinhibition period in the underexpresser (Figure 4B). D1 degradation has been shown to lead to the release of OEEs into the lumenal space (Eisenberg-Domovich et al., 1995), where they could be subject to proteolysis. The putative OEE2 degradation product also was detected to a much lower extent in the wild type but was completely absent in the overexpressing strain (data not shown).

Effect of Inhibition of Organelle Protein Synthesis on Recovery of PSII Activity and PSII Protein Levels

Recovery of PSII activity after photoinhibition occurs through two distinct processes: functional reactivation of PSII centers, which does not require replacement of chloroplast-encoded subunits, and replacement of the damaged D1 protein, which requires chloroplast protein synthesis (Ohad et al., 1990). To distinguish between the two processes, chloramphenicol, an inhibitor of organellar translation, was added at the end of the photoinhibition period.

In this experiment, PSII activity was monitored by the F_v/F_{max} ratio and by the PSII contribution to the electrochromic shift at 515 nm that develops upon charge separation across the thylakoid membranes (Figure 5A). The fluorescence parameter is sensitive to changes in quenching processes other than PSII photochemistry, for instance, those occurring in the antenna. In addition, changes in fluorescence yield do not vary linearly with the percentage of PSII activity because of excitation energy transfer between inactive and active PSII units (Joliot et al., 1973): typically, ~90% inactivation of PSII leads to a decrease of F_v/F_{max} by only 70% (Wollman et al., 1980). In contrast, the electrochromic shift is a direct measure of the number of charge separations (i.e., with secondary electron donors Y_Z/Y_D and primary quinone type acceptor Q_A intact). However, we cannot presently exclude the possibility that photoinhibition per se may alter the response of the pigment sensors to charge separation. Thus, the two sets of data for the experiments described below should be taken as indicative for changes in PSII photochemistry rather than as absolute measurements of PSII activity.

After 60 min of photoinhibition, ~10 to 20% of PSII activity remained in the control and overexpressing strains, as shown both by fluorescence and absorption changes. In the absence of chloramphenicol, the overexpresser recovered most or all of its PSII activity within 180 min in dim light, whereas only a little PSII activity was regained in the wild type during that period of time. Interestingly, approximately half of PSII recovery was still observed in the presence of chloramphenicol in the overexpresser, whereas no significant recovery was seen in the wild type under these conditions.

Figure 5B shows a characterization of membrane-bound D1, OEE2, OEE3, and Rieske protein content in the thylakoid

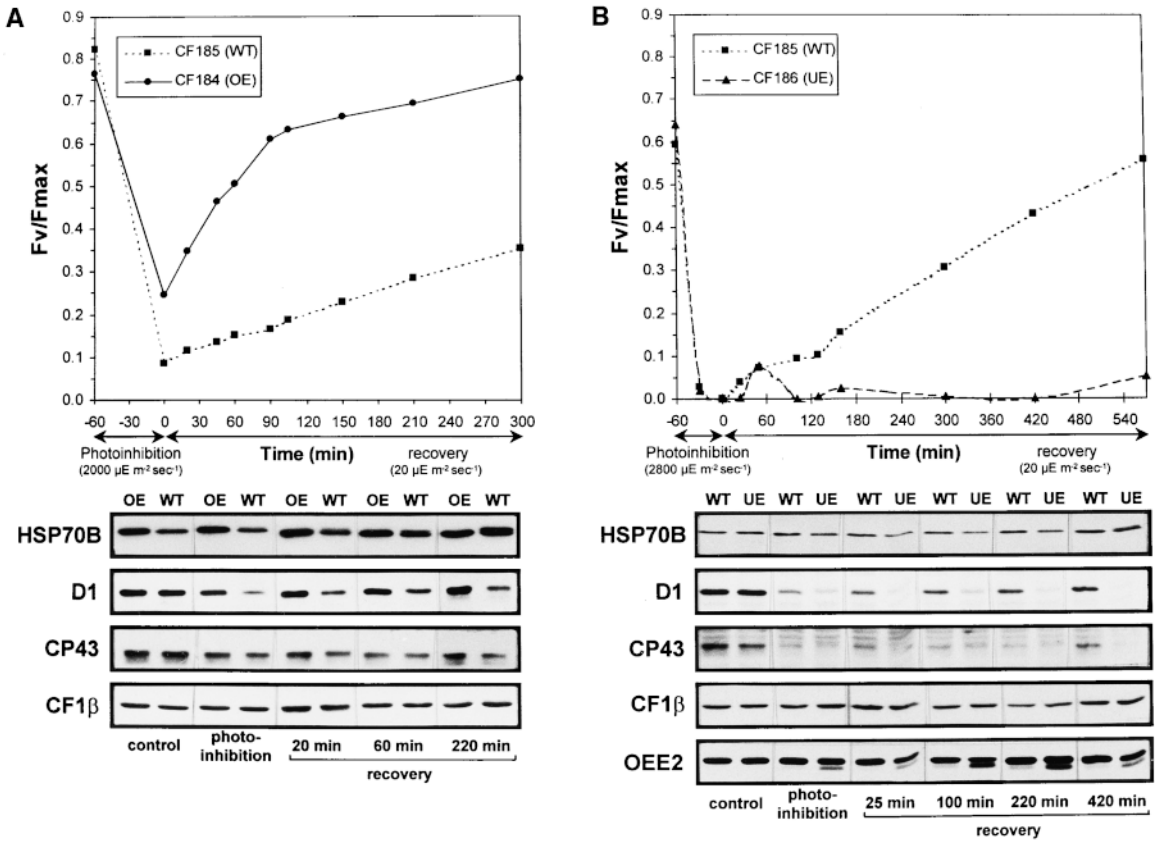

Figure 4. Time Course of Photoinhibition and Recovery as Monitored by PSII Activity and Levels of HSP70B and PSII Core Proteins.

(A) Wild type (WT) and the *HSP70B*-overexpressing strain (OE).
(B) Wild type (WT) and the *HSP70B*-underexpressing strain (UE). The fluence rate used for photoinhibition (2800 µE m^{-2} sec^{-1}) was higher than that in **(A)**.
At top in **(A)** and **(B)**, PSII activity is displayed as the F_v/F_{max} ratio. At bottom, gel blot analyses of whole-cell protein samples taken before photoinhibition (control), at the end of the photoinhibition period, and during the recovery phase are shown. For each lane, 10 µg of protein was separated on 12% SDS–polyacrylamide gels, blotted, and immunodecorated with antibodies raised against HSP70B, D1, CP43, OEE2, and CF1β as a loading control. Membranes were stripped after each incubation and reprobed.

membranes prepared at various time points of the experiment. The content in the Rieske protein, a nuclear-encoded subunit of the cytochrome b_6/f complex, was used here as a loading control and remained essentially unaltered. The extent of D1 degradation in the control strain (CF185) was similar to that observed by others under similar experimental conditions (van Wijk et al., 1994). As we have observed previously (Figure 4A), the D1 protein was less affected by photoinhibition in the overexpresser than in the control strain. Similarly, the levels of membrane-bound OEE2 and OEE3 proteins were almost unaffected by photoinhibition in the overexpresser, whereas a clear loss was observed in the wild type. As expected from the action of chloramphenicol as a chloroplast translation inhibitor, its addition prevented any increase in D1 content during recovery and even led to its further loss in both strains. Also, both strains exhibited slightly decreasing OEE2 and OEE3 levels in the presence of chloramphenicol, but all three proteins were better preserved in the overexpresser than in the control strain. It should be stressed that a substantial part of the recovery in PSII activity observed in the overexpresser (Figure 5A) actually occurred in the absence of D1 replacement (Figure 5B).

DISCUSSION

Light Induction of *HSP70* Genes as a Protection Mechanism against Photoinhibition

We have previously shown that *HSP70* genes are induced by a dark-to-light shift in Chlamydomonas (von Gromoff et

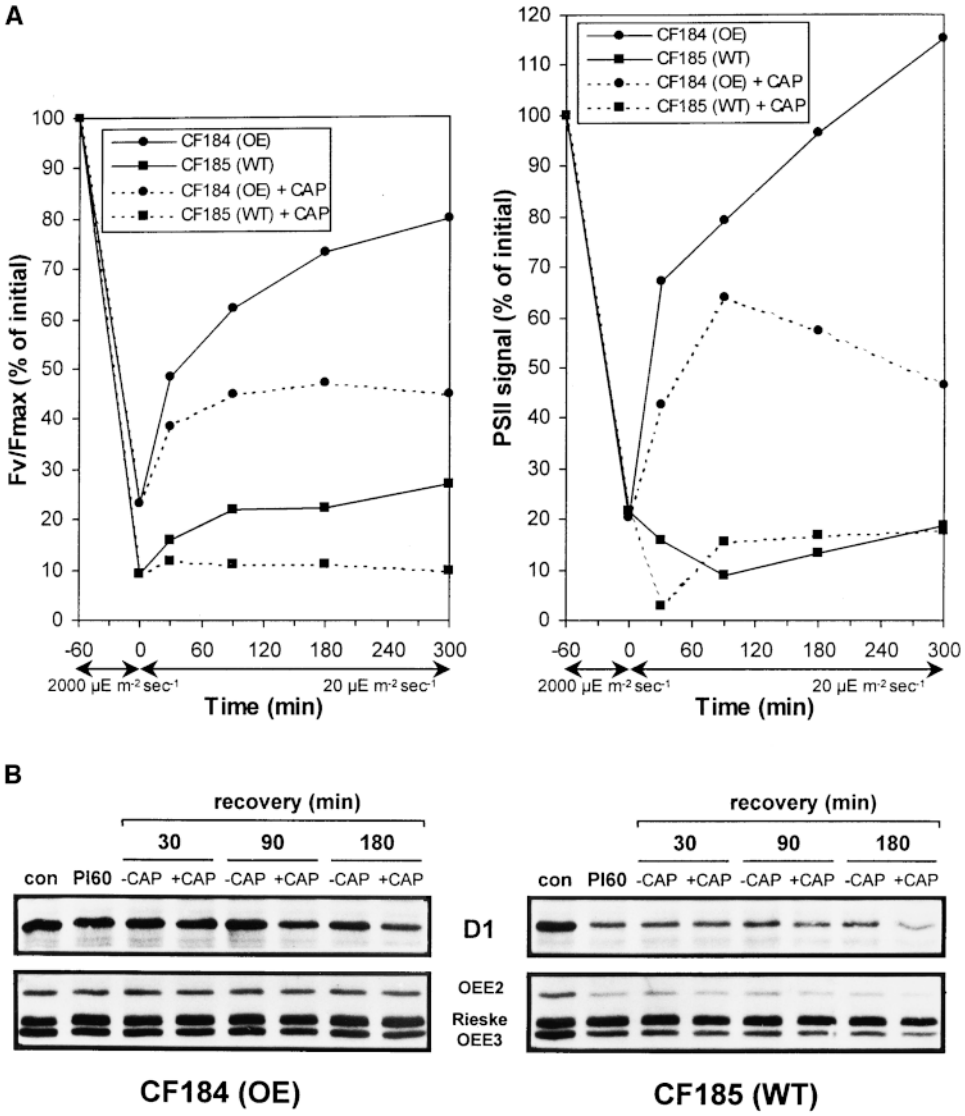

Figure 5. Recovery of PSII Activity and Changes in Membrane-Bound Thylakoid Proteins in the Presence and Absence of Organellar Protein Synthesis.

(A) PSII activity measured by fluorescence induction (left) or electrochromic shift (right) in a wild-type (WT) strain (CF185) and an *HSP70B*-overexpressing (OE) strain (CF184). Values are plotted as percentage of initial activity. Chloramphenicol (CAP) was added to a final concentration of 100 μg/mL to half of each culture before the recovery phase (+CAP).

(B) Time course of changes in membrane-bound D1, OEE2, OEE3, and Rieske protein levels. Samples were collected before photoinhibition (con), immediately after a 60-min high-light incubation (PI60), and during the recovery period from both the chloramphenicol-treated (+CAP) and the untreated (−CAP) halves of the culture. Crude membrane fractions were separated on 12% SDS–polyacrylamide gels, blotted, and immunodecorated with the respective antibodies. Even loading of the gels was confirmed by comparing the Rieske protein levels.

al., 1989). In nature, low light intensity at dawn announces the high-light intensities that may be experienced during the day. Therefore, we have examined the effect of a dark–light shift on the ability of cells to withstand photoinhibition. Indeed, cells that had been subjected to a dark–light shift before photoinhibition were able to restore PSII activity in a subsequent low-light incubation faster than did control cells (Figure 1A). At least part of this effect seems to be due to induction of the *HSP70B* gene, because its constitutive overproduction led to enhanced resistance to photoinhibition

and faster recovery, whereas underexpression had opposite effects.

Overexpression and Underexpression of *HSP70B*

The fusion of promoters *HSP70A* and *RBCS2*, which we have used to express the *HSP70B* sense and antisense constructs, resulted in a combination of the properties of each individual promoter. In addition, a synergistic effect of the individual promoters upon each other was observed, especially after heat shock and light induction (M. Schroda, D. Blöcker, and C.F. Beck, submitted manuscript). As expected, the level of the HSP70B protein is increased in transformants carrying the *HSP70B* sense construct both under basal and induced conditions. However, the reduction by the antisense construct is more pronounced when the *HSP70A–RBCS2* promoter fusion in front of the antisense gene is induced, that is, after heat shock, shift from dark to light, or high-light treatment (Figures 2 and 4). Here, we have successfully applied the antisense strategy in Chlamydomonas, probably made possible by the high level of inducible expression provided by this synthetic promoter. Clearly, changes at the protein level are less marked than at the mRNA level. In part, this may be accounted for by the intrinsic stability of the HSP70B protein but may also reflect a limited tolerance of the chloroplast to changes in HSP70B concentration. In support of this latter idea, we have observed slightly decreased mixotrophic growth rates for both the *HSP70B*-overexpressing and *HSP70B*-underexpressing strains when compared with that of the wild type (data not shown).

HSP70B May Protect PSII against Photoinhibition by Distinct Mechanisms

The comparison of wild-type and HSP70B-overexpressing and HSP70B-underexpressing strains under various illumination conditions clearly showed that the level of this protein in the chloroplast is a major determinant of the cell's ability to withstand photoinhibition and to recover after light stress. The effect of changes in HSP70B levels becomes significant only after severe photoinhibition, that is, when most of the PSII centers are irreversibly photodamaged.

Through what mechanism does HSP70B counteract photoinhibition? Despite extensive research on the subject, the molecular mechanisms of photodamage and repair are still poorly understood. Photoinhibition is thought to occur in two major steps: reversible inactivation of PSII is followed by an irreversible photodamage of the PSII protein complex (reviewed in Prasil et al., 1992; Aro et al., 1993; Ohad et al., 1994). After irreversible photodamage, one of the major PSII reaction center subunits, the D1 protein, is tagged for degradation, most likely involving a conformational change of the D1 protein (Ohad et al., 1988). The damaged PSII core complexes move to stroma-exposed thylakoid regions (Adir et al., 1990), where D1 is degraded. D1 degradation is accompanied by the formation of a 160-kD complex comprising D1, D2 (the other major subunit of the reaction center), and additional proteins (Kim et al., 1993). After its degradation, D1 is replaced by a de novo–synthesized protein, which integrates into a D1-less reaction center (Adir et al., 1990). Because D1-less centers appear to exhibit an increased susceptibility to proteolysis as compared with fully assembled PSII centers, an extended delay in D1 resynthesis will lead to a pleiotropic loss in all PSII subunits, resulting in a cataclysmic loss of the repair ability. Accordingly, degradation of CP47, another PSII core protein, under high-light conditions already has been observed in Chlamydomonas (Zer and Ohad, 1995) and in isolated spinach thylakoids (Andersson and Barber, 1994). Here, we observed a similar loss in CP43, another PSII core subunit.

Our finding that HSP70B participates in the photoprotection and repair of PSII sheds some new light on the molecular aspects of photoinhibition. On one hand, we observed that in contrast to that in the wild type, a significant proportion of the restoration of PSII activity was chloramphenicol insensitive in the overexpresser, that is, independent of de novo synthesis of D1. This observation argues for a role of HSP70B in the protection of PSII subunits against photodamage. We speculate that HSP70B interacts with polypeptide sequences of D1 or other PSII reaction center proteins that become exposed at the stromal surface of the photoinactivated centers. Interaction of HSP70B with these sequences may either facilitate reactivation of the acceptor side of PSII or limit the extent of inappropriate targeting of D1 for degradation. Both actions would account equally well for the observed preservation of photodamaged PSII centers that could eventually be reactivated.

On the other hand, HSP70B also plays a critical role in the assembly of new PSII cores. We observed that during the repair process, accumulation of the D1 and, to a lesser extent, CP43 proteins resumed in the wild-type strain but not in the underexpresser. Thus, HSP70B also appears to contribute to the biogenesis of chloroplast proteins required for the repair process, possibly by playing a role in coordinating D1 degradation with D1 de novo synthesis and PSII reaction center assembly or by improving the import in the chloroplast of nuclear-encoded products that are required for PSII repair. D1 phosphorylation has been proposed to induce a conformational change at the N terminus of D1 that prevents D1 degradation and subsequent PSII disassembly until they can proceed in a coordinated manner with de novo synthesis (Rintamäki et al., 1995). In vivo, this eventually results in rather constant D1 levels, even after severe photoinhibition in higher plants (Cleland et al., 1990). In contrast to that in higher plants, photoinactivated D1 protein is not phosphorylated in green algae (de Vitry et al., 1991), a distinction that may explain why the D1 pool can be lost almost completely in the underexpresser after severe photoinhibition (Figure 4B). Therefore, elevated HSP70B levels in Chlamydomonas

may mimic the effect of D1 phosphorylation observed in higher plants.

It is noteworthy that relatively minor alterations in HSP70B abundance (a 50 to 100% increase or a 30 to 50% decrease) lead to substantial changes in the ability of the cells to cope with photoinhibition. According to the possible mechanisms of HSP70B action discussed above, increased HSP70B concentrations would be expected to result in a larger number of reversibly inactivated PSII centers and an increased stabilization of irreversibly damaged PSII centers awaiting repair. In contrast, low HSP70B levels would lead to high numbers of damaged, insufficiently stabilized PSII centers. In the former case, PSII reactivation and D1 replacement might proceed rapidly, whereas in the latter case, PSII disassembly would predominate, making PSII recovery completely dependent on de novo synthesis of all PSII subunits. This sequence of events may account for the apparent disproportionality between small changes in HSP70B levels and large differences in recovery from photoinhibition.

In summary, our study points to a dual role of stromal HSP70 in the recovery from photoinhibition. Interestingly, we found evidence for an early role of HSP70B during the reversible phase of PSII photoinactivation that suggests a possible protective interaction with unfolding domains of PSII proteins. In addition, HSP70B participates in the repair process by promoting either synthesis or assembly of new reaction center components. More work is required to identify which other stromal and membrane-bound components act together with HSP70B in the assembly and repair of damaged thylakoid membrane protein complexes.

METHODS

Algal Strains and Culture Conditions

Chlamydomonas reinhardtii cw15-302 (cw_d *mt+ arg7*) was kindly provided by R. Matagne (University of Liège, Belgium) and strain CF30 (*mt−* lrg2 arg7) has been described by Glöckner and Beck (1997). Strains were grown photomixotrophically in TAP medium (Harris, 1989) on a rotary shaker at 23°C under continuous irradiation with white light (30 $\mu E\ m^{-2}\ sec^{-1}$) provided by fluorescent tubes (Osram L36W/25; Munich, Germany). Light induction was performed according to Kropat et al. (1997), and heat shock treatments were conducted by rapidly shifting cultures from growth conditions to a waterbath at 40°C.

Construction of the Heat Shock *HSP70B* Sense and Antisense Genes under the Control of the *HSP70A–RBCS2* Promoter Fusion

The *HSP70B* sense gene was constructed as a reporter gene overexpressing the HSP70B protein with a C-terminal hexahistidine tag. This type of modification was shown previously to be functional in vivo (Bolliger et al., 1994). This *HSP70B* gene was constructed in three steps.

(1) The *HSP70B* gene was subcloned and converted into a reporter gene. The 6-kb ApaI fragment from clone *hsp80-35* (von Gromoff et al., 1989), containing the complete *HSP70B* gene, was cloned into the ApaI site of vector pBluescript SK+ (Stratagene, La Jolla, CA), yielding pCB590. pCB590 was digested at its unique HpaI site in the 3′ untranslated region of the *HSP70B* gene, and the 213-bp EcoRI-SalI fragment originating from the *pucβcαc* gene of *Rhodopseudomonas palustris* (Kropat et al., 1995) was inserted after filling in the ends with the Klenow fragment of DNA polymerase I, giving rise to pCB593. The 6.2-kb KpnI-HindIII insert of pCB593 was then transferred to KpnI-HindIII–digested pBluescript SK+, from which a fragment between the SacI and SmaI sites had been deleted, resulting in pCB617.

(2) A C-terminal hexahistidine tag was inserted. With pCB617 as template, two independent polymerase chain reactions (PCR) were performed using Vent Polymerase (New England Biolabs, Schwalbach, Germany). First, a 998-bp fragment was amplified using primer hexahis-sense (5′-CACCATCACT**AAGCtt**CCTGCATTGAGGCTACTG-3′; the HindIII restriction site is in boldface, and altered bases are lowercase) and primer GSP14 (5′-GCCTTGCCCGCTTCTGTC-3′). After digestion with HindIII, the fragment was cloned into SmaI-HindIII–digested pBluescript SK+, generating pCB624. Second, a 361-bp fragment was amplified using primer P4330 (5′-CCAGGCGGAGACCATGGTG-3′) and primer hexahis-antisense (5′-GCAGG**aaGCTT**AGTGATGGTGATGGTGATGGTAACCCTTCTTGTCAGTGAACTCGGCG-39; the HindIII restriction site is in boldface, and altered bases are lowercase). This fragment as well as pBluescript SK+ were digested with SacI and HindIII and ligated together to give pCB632. Digestion of the latter with HindIII and SalI and insertion of the 551-bp HindIII-SalI fragment of pCB624 created pCB638. Finally, the 677-bp SacI-XbaI fragment of pCB617 was replaced by the 701-bp, hexahistidine codon–containing counterpart from pCB638, yielding pCB650. Correct cloning was confirmed by sequencing.

(3) The *HSP70B* promoter was replaced by a *HSP70A–RBCS2* promoter fusion. By using pCB536 (Kropat et al., 1995) as a template, a 336-bp PCR fragment was amplified with primers T7–BamHI (5′-AATA**gGAtcC**ACTATAGGGC-3′; the BamHI restriction site is in boldface, and altered bases are lowercase) and *HSP70A*–BglII-NheI (5′-CT**agaTCT**TAA**GCtAGc**TGAGTGG-3′; BglII and NheI restriction sites are in boldface, and altered bases are lowercase), containing 196 bp of *HSP70A* promoter sequence upstream from the transcriptional start site. After digestion with BamHI, the PCR fragment was cloned into BamHI-SmaI–digested pBluescript SK+, producing pCB710. Ligation of the 5-kb NheI-EcoRI fragment of pCB650 into NheI-EcoRI–digested pCB710 produced construct pCB714, which contains the *HSP70B* gene fused to the Δ196 *HSP70A* promoter (Kropat et al., 1995). The latter was digested with BamHI and EcoRI, releasing a 5.4-kb fragment that was cloned into BamHI-EcoRI–digested pCB412 (G. Glöckner and C.F. Beck, unpublished data) next to the argininosuccinate lyase *ARG7* gene, thereby replacing the cos sites of pCB412 and giving rise to pCB720. The ribulose bisphosphate carboxylase small subunit *RBCS2* promoter was amplified by PCR from pSP108 (Stevens et al., 1996) as a template, using primers *RBCS2*–BamHI (5′-CG**GgaTCC**CGGGCGCGCC-3′; the BamHI restriction site is in boldface, and altered bases are lowercase) and *RBCS2*–NheI (5′-TGA**gcTagC**TCTCTTGTAAA-3′; the NheI restriction site is in boldface, and altered bases are lowercase). The 213-bp PCR fragment obtained, containing 183 bp of *RBCS2* promoter sequences upstream from the transcriptional start site, was digested with BamHI and NheI and used to replace the *HSP70A* promoter of pCB714, yielding pCB738. Finally, the promoterless *HSP70B* gene was removed from pCB720 by digestion with NheI

and EcoRI, and the 5.2-kb SpeI-EcoRI fragment from pCB738, harboring the *RBCS2* promoter fused to the *HSP70B* gene, was placed in front of the *HSP70A* promoter that had remained in pCB720, giving rise to pCB740.

The *HSP70B* antisense gene was constructed using an *HSP70B* cDNA in antisense orientation fused to the strong *HSP70A–RBCS2* tandem promoter. The 216-bp PCR product containing the *RBCS2* promoter (described above) was digested with BamHI and ligated into BglII-EcoRV–digested pCB710. The resulting pCB745 contained the Δ196 *HSP70A* promoter and the Δ183 *RBCS2* promoter in tandem. *HSP70B* cDNA clones were obtained from a λ ZAP (Stratagene) cDNA library (kindly provided by V. Kurvari, University of Texas, Dallas) by hybridization with a radiolabeled 278-bp AatII-HpaI genomic DNA fragment. Phages from plaques giving rise to positive signals were subjected to the ExAssist helper phage system (Stratagene), which produced *HSP70B* cDNAs already subcloned into pBluescript SK+. One of these clones was digested with BamHI and SalI, generating a 2.5-kb fragment containing the entire *HSP70B* cDNA, except for 370 bp of the terminal 3′ end. Ligation of this fragment into BamHI-SalI–digested pSP108 (Stevens et al., 1996) generated pCB746, carrying the truncated *HSP70B* cDNA in an antisense orientation between the *ble* gene of *Streptoalloteichus hindustanus* and a 230-bp 3′ *RBCS2* fragment. The 2.8-kb SalI-KpnI fragment of pCB746 was ligated into SalI-KpnI–digested pCB745, giving rise to pCB747, which contained the *HSP70A–RBCS2* tandem promoter fused to the antisense *HSP70B* cDNA trailed by a 230-bp 3′ *RBCS2* fragment. In a final step, pCB747 was cleaved by KpnI, blunt ended by T4 DNA-polymerase treatment, and further digested by XbaI. The resulting 3.4-kb fragment, harboring the complete antisense gene, was then ligated into NheI-EcoRV–digested pCB412, replacing one cos site next to the *ARG7* gene and giving rise to pCB749.

Nuclear Transformation of Chlamydomonas

Chlamydomonas nuclear transformation was performed using the glass beads method (Kindle, 1990), modified as described by Kropat et al. (1995). Before transformation, construct pCB740 was linearized by digestion with EcoRI and construct pCB749 by digestion with XhoI. Strains used for transformation were cw15-302 and CF30. The cell wall of the latter was digested by autolysin before transformation. One-hundred nanograms of plasmid DNA was sufficient to routinely generate at least 100 to 200 transformants.

RNA Gel Blot Analysis

Total RNA was isolated from 50-mL cultures grown to 2 to 4 $\times$ 10^6 cells per mL. After centrifugation, the cell pellet was resuspended in 500 μL of AE buffer (50 mM sodium acetate, pH 5.3, and 10 mM EDTA). After the addition of SDS (2% final concentration) and 1 volume of AE-saturated phenol, samples were frozen in liquid nitrogen. After a 10-min incubation at 65°C, the aqueous phase was collected and subjected to further extractions with 1 volume of AE-saturated phenol–chloroform–isoamyl alcohol (25:24:1) until the interphase was clear. After precipitation with 2.5 volumes of ethanol, the RNA pellet was resuspended in diethylpyrocarbonate-treated water. Separation of RNAs on formaldehyde-containing 1.2% agarose gels (10 μg of RNA per lane) and transfer to nylon membranes (Hybond N^+; Amersham, Braunschweig, Germany) by capillary blotting was performed as described by Sambrook et al. (1989).

Radioactive DNA probes were prepared by the random priming technique (Feinberg and Vogelstein, 1983), using α-^{32}P-dCTP (Amersham). Blots were probed sequentially with a 1.4-kb NheI-PstI fragment from the *HSP70B* coding region and a 370-bp SstII-AlwNI fragment from the *RBCS2* coding region that hybridizes with both the *RBCS1* and *RBCS2* mRNAs. Prehybridization (6 hr) and hybridization (16 hr) were performed at 65°C in 50 mM Tris-HCl, pH 7.5, 55% formamide, 1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% sodium pyrophosphate, 10 $\times$ Denhardt's solution (1 $\times$ Denhardt's is 0.02% Ficoll 400, 0.02% PVP, and 0.02% BSA), 1% SDS, and 200 μg/mL sheared, denatured herring sperm DNA. After hybridization, membranes were washed twice in 2 $\times$ SSC at room temperature and once in 1 $\times$ SSC and 1% SDS at 65°C. Quantitation of RNA gel blots was conducted by exposure to BAS-MP imaging plates (Fuji, Tokyo) and evaluation by using the TINA program (Version 2.08d; Raytest, Straubenhardt, Germany). Signals obtained for the *HSP70B* probe were standardized to the respective *RBCS* signals present at basal or inducing conditions.

Photoinhibition

Strains to be compared were diluted from precultures to equal cell densities and cultured for an additional 15 hr before photoinhibition. Cells at 1.5 to 5 $\times$ 10^6 cells per mL were transferred to beakers, and these were placed onto equally illuminated spots of a rotary shaker below two strong light sources (Osram HLX 250 W 64663 Xenophot). Infrared radiation was cut-off by a translucent waterbath placed between light sources and beakers. Cells were photoinhibited at 23°C for 1 hr at 2 to 3 mE m^{-2} sec^{-1}. Beaker positions were swapped at regular intervals during photoinhibition to ensure equal illumination. During the light treatment, the temperature of the cultures remained constant. Recovery of photosynthesis was allowed to take place at an irradiation of 20 μE m^{-2} sec^{-1}. Light intensities were determined using a Luxmeter (Minolta illuminance meter T1; Carrièrer sur Seine, France). Conversion from lux to Einstein was performed by assuming that 1000 lux at photosynthetic active radiation between 400 and 700 nm equals 20 μE m^{-2} sec^{-1} (Harris, 1989).

Protein Gel Blot Analyses

Cells were centrifuged and resuspended in 20 mM Hepes-KOH, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM ε-aminocaproic acid, and 0.1 mM benzamidine. Cells were either subjected to three cycles of freeze thawing (membrane fraction, for cw15) or recentrifuged directly (whole cells), resuspended in 0.1 M Na_2CO_3 and 0.1 M DTT, and stored at −80°C until further processing. After adding 0.66 volumes of 5% SDS and 30% sucrose, proteins were solubilized by rapid shaking at room temperature for 20 min. Protein concentrations were determined by staining with amido black and using a BSA standard (Popov et al., 1975). SDS-PAGE (12% acrylamide) was performed according to Laemmli (1970). Proteins were transferred to nitrocellulose membranes (Hybond C-super; Amersham) by semidry blotting by using a discontinuous buffer system, according to the manufacturer's recommendations (Bio-Rad). Immunodetection was performed using enhanced chemiluminescence, according to the instructions given by the manufacturer (Amersham). To ensure reproducibility of the results obtained from immunoblots, multiple experiments with varying exposure times were performed. Quantitation of immunoblots was conducted using the AIDA software package (Version 2.0 beta; Raytest).

The D1 antibody was kindly provided by U. Johanningmeier (Universität Halle, Institut für Pflanzenphysiologie und Zellphysiologie, Halle, Germany). Antibodies raised against CP43, CF1β, oxygen-evolving enhancers OEE2 and OEE3, and the Rieske protein were described by de Vitry et al. (1989). The polyclonal HSP70B antibody was raised against the denatured HSP70B protein overexpressed in *Escherichia coli* via the pQE vector system and purified using Ni-affinity chromatography, according to the protocol of the manufacturer (Qiagen, Chatsworth, CA).

Fluorescence Induction and Spectroscopic Analyses

Fluorescence induction measurements were performed on whole cells at room temperature by using an HLMT CL00 fluorometer (built at the Institut de Biologie Physico-Chimique), as described by Bennoun (1994). Basal fluorescence (F_0) was determined after dark-adapting cells for 2 to 3 min. Maximum fluorescence (F_{max}) was determined in the presence of 10 μM dichlorophenyl dimethylurea. Transmembrane charge separation was measured according to Junge and Witt (1968) by following the flash-induced electrochromic shift at 515 nm 100 μsec after the flash. The spectrophotometer used has been described by Joliot and Joliot (1994). The photosystem II (PSII) contribution was measured by substracting the dichlorophenyl dimethylurea–insensitive signal attributed to PSI.

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