Plant Physiol. (1994) 104: 769–776

Mutants of Chloroplast Coupling Factor Reduction in *Arabidopsis*[1]

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We have devised a two-step screening strategy for the selection of chloroplast coupling factor reduction mutants from an M_2 population of *Arabidopsis thaliana*. The selection strategy relies on a lowered energetic threshold for catalytic activation of the enzyme that has been shown to accompany thioredoxin-mediated reduction of a cysteine bridge on the γ subunit of coupling factor. We selected first for plants that grew poorly under low irradiance but performed satisfactorily at high irradiance when the transmembrane electrochemical potential of hydrogen ions is large and competent to maintain a high level of coupling factor activation without γ subunit reduction. In the second step of the screen we monitored the flash-induced electrochromic change to select putative coupling factor reduction mutants from other sorts of mutations that shared the phenotype of poor growth and vigor when transferred from high to low irradiance. Among the mutants selected, one appears incapable of reducing coupling factor, whereas another behaves as though coupling factor is at least partially reduced even in dark-adapted plants.

The light-dependent regulation of key enzymes in photosynthesis is a central factor in ensuring efficient energy storage in the highly dynamic diurnal light environment of natural habitats. The thylakoid membrane CF is one of the enzymes of the chloroplast regulated by light, presumably to ensure efficient ATP synthesis under favorable energetic conditions and to prevent the wasteful hydrolysis of stromal ATP in the dark. Experimental evidence points to a regulatory hierarchy for CF involving three principal components: $\Delta\mu_{H^+}$, the oxidation state of the CF γ subunit Cys bridge, and the interactive binding of ATP, ADP, and Pi to the CF (e.g. Bichel-Sandkötter and Strotmann, 1981; Ketcham et al., 1984; Mills and Mitchell, 1984; Hangarter et al., 1987; Junesch and Gräber, 1987; Kramer and Crofts, 1989). The interrelationship among these three components of CF regulation has been extensively studied in numerous laboratories over the past decade and was recently reviewed by Ort and Oxborough (1992).

The process of CF reduction is mediated in vivo by thioredoxin, located in the chloroplast stroma and photoreduced by PSI, via Fd, in a reaction catalyzed by Fd-thioredoxin reductase (Wolosiuk and Buchanan, 1977). The thioredoxins are also involved in the modulation of several other chloroplast enzymes, including four that catalyze reactions of the photosynthetic carbon reduction cycle (Buchanan, 1991). Two different thioredoxins have been identified in chloroplasts of higher plants: thioredoxin f, so named for its ability to activate Fru-1,6-bisphosphatase, and thioredoxin m, for activating NADP-malate dehydrogenase; both appear to be capable of reducing the CF γ subunit regulatory sulfhydryl groups.

The role of thioredoxin-mediated reduction in the overall in situ regulatory mechanism of CF activity is not clearly defined. The transmembrane electrochemical potential exerts primary kinetic control of CF in that a large $\Delta\mu_{H^+}$ is normally required for the initiation and maintenance of catalytic activity. Although the size of the threshold $\Delta\mu_{H^+}$ is smaller when the γ subunit disulfide has been reduced by thioredoxin to vicinal dithiols, it appears that even in intact leaves $\Delta\mu_{H^+}$-dependent activation of CF catalytic activity precedes thioredoxin-dependent γ subunit reduction (e.g. Kramer et al., 1990). Reduction of the CF complex appears to have its main benefit to photosynthetic energy transduction under conditions of limiting irradiance. Whereas the maximum rate of light-driven ATP formation is unaffected, CF reduction permits higher rates of ATP formation at limiting $\Delta\mu_{H^+}$ values (Ketcham et al., 1984; Mills and Mitchell, 1984; Junesch and Gräber, 1985). Without reduction, the higher energetic threshold for CF activation along with the higher probability of deactivation conspire to lower the proportion of the CF pool that is activated at intermediate $\Delta\mu_{H^+}$ values, thereby limiting the rate of photophosphorylation. In fact, CF reduction actually increases the efficiency of ATP formation as a function of $\Delta\mu_{H^+}$ (e.g. Ketcham et al., 1984; Hangarter et al., 1987).

[1] This work was supported in part by the National Research Initiative Competitive Grants Program/U.S. Department of Agriculture (grant No. 91–37100–6620) to D.R.O. and by the Department of Energy (grant No. DE-FG02–86ER13594) to A.R.C.

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Abbreviations: CF, chloroplast coupling factor or chloroplast CF_1CF_o-ATP synthase; cfi, coupling factor intermediate recovery; cfq, coupling factor quick recovery; cfs, coupling factor slow recovery; DCCD, dicyclohexylcarbodiimide; ΔA_{518}, flash-induced electrochromic absorbance change measured at 518 nm; $\Delta\mu_{H^+}$, transmembrane electrochemical potential of hydrogen ions.

The physiological benefits of the oxidation of CF are more cryptic. Although the value of inactivating CF in the dark has apparent advantages, oxidation of CF is not required for deactivation. In intact leaves following a light-to-dark transition, deactivation normally occurs long before oxidation of the regulatory vicinal dithiols of the γ subunit (Kramer and Crofts, 1989; Ortiz-Lopez et al., 1991). From their work with *Dunaliella*, Noctor and Mills (1988) suggested that oxidation may stabilize the inactivated state of CF, thereby preventing "spontaneous" activation analogous to the stabilization of the activated state by reduction. It has also been suggested (Ort and Oxborough, 1992) that oxidation may serve as a mechanism that matches the energetics of CF activation with the prevailing Gibbs free energy change for ATP synthesis that is attained in the stroma in the dark. In chloroplasts, the value of the Gibbs free energy change for ATP synthesis is influenced by the action of adenylate kinase, which maintains the nucleotide ratio of the stroma in the dark near its mass action ratio, thereby poising the free energy difference for ATP near 42 kJ mol^{-1} (Giersch et al., 1980). Since this value is near or even slightly above the energetic threshold for the activation of reduced CF (Hangarter et al., 1987), the increase in the threshold associated with oxidation may delay activation until a $\Delta\mu_{H^+}$ is established that is large enough to ensure that net synthesis, rather than net hydrolysis, of ATP will occur.

Although substantial progress has been made, there are still central features about both the mechanism of CF reduction and reoxidation as well as the role of this redox modulation in the in situ regulation of CF activity that remain to be established. One approach that could yield further insights is to select mutants that have dysfunctions in the CF activation/reduction process. This paper reports on the development of a two-step screening strategy for the selection of CF activation mutants from *Arabidopsis thaliana*. The basis for the initial step of the screen was our expectation that, under very low-irradiance conditions, mutants that are unable to reduce CF would also be unable to attain full activation of the CF pool. In consequence, photophosphorylation should be restricted, causing these mutants to grow and develop poorly under low growth irradiance but to perform satisfactorily at high irradiance when the $\Delta\mu_{H^+}$ is large and able to maintain nearly full CF activation without γ subunit reduction.

The purpose of the second step of the screen was to select putative CF-reduction mutants from other sorts of mutations that shared the phenotype of poor growth and vigor when transferred from high- to low-irradiance growth conditions. We used a flash kinetic spectrophotometric assay to monitor CF activity in intact leaves and to assess the ability of the mutants to reduce CF (e.g. Kramer and Crofts, 1989; Wise and Ort, 1989; Kramer et al., 1990). The heritability of the phenotype was established using the kinetic spectroscopy assay to monitor CF reduction and activation in the M_3 and M_4 generations. Here we report on the selection and initial characterization of three CF activation mutants of *Arabidopsis*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Mutants were isolated from an M_2 population of *Arabidopsis thaliana* (ecotype Columbia). The M_2 seed, which was harvested from M_1 plants that had been grown from ethylmethanesulfonate-mutagenized seed, was obtained from Lehle Seeds (Tucson, AZ). Seeds were sown on commercial potting soil (Jiffy-Mix) at an average density of 5 seeds cm^{-2}. Plants were grown in an EGC (Chagrin Falls, OH) model M-31 growth chamber at 23 ± 2°C, 70 to 80% RH, on a day/night regime of 16 h light/8 h dark. The plants were watered as needed and fertilized twice weekly with nutrient solution (Somerville and Ogren, 1982). Illumination was supplied by fluorescent tubes (General Electric, F72T12-CW-1500) supplemented with incandescent bulbs. The standard growth light intensity was 270 ± 30 μmol quanta m^{-2} s^{-1} PPFD.

Mutant Isolation Strategy

After germination, M_2 plants were grown under a 16-h photoperiod at 270 ± 30 μmol quanta m^{-2} s^{-1} PPFD (standard illumination conditions) for 1 week. At this stage the first true leaves had expanded on the majority of plants. All noticeably stunted, deformed, or abnormally pigmented plants were removed and discarded at this stage. Subsequently, copper screens were used to reduce the illumination intensity to 12 ± 3 μmol quanta m^{-2} s^{-1} PPFD coincident with decreasing the photoperiod from 16 to 8 h. After 2 weeks under this low-irradiance regime, all plants demonstrating seemingly normal development and pigmentation were removed and discarded, whereas those exhibiting suspended growth, chlorosis, or any signs of leaf necrosis were returned stepwise over a 5-d period to standard illumination conditions. Plants that did not recover were discarded, whereas those that did improve were carried through the second step of the mutant isolation procedure. Control plants for the second step were grown from wild-type seed but otherwise treated identically in the first step as the M_2 plants.

A double-flash kinetic spectrophotometer (Kramer and Crofts, 1990) was used to screen the selected M_2 plants for aberrant electrochromic absorbance change relaxation kinetics to diagnosis anomalies in the CF activation process (Ort and Oxborough, 1992). The putative mutant plants were dark-adapted for 12 h prior to the electrochromic absorbance change measurement to ensure full oxidation of the CF regulatory sulfhydryl groups. Detached leaves were mounted and covered with a piece of transparent, gas-permeable plastic film and sealed within a thin frame to prevent dehydration during measurements. Since the leaves of some putative mutants were quite small, several leaves were sometimes necessary to cover the entire measuring window. Care was taken to minimize overlapping surfaces of leaves. All measurements were conducted at room temperature. The electrochromic change was induced by a single 2-μs (duration at half of peak intensity) red flash that was about 60% saturating. A train of 200 of these red flashes was delivered to the leaf prior to measuring the electrochromic change to drive the thioredoxin-dependent reduction of the γ subunit

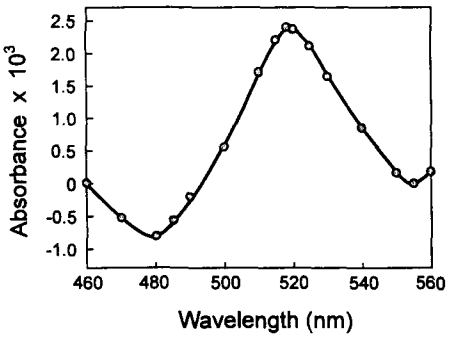

Figure 1. Wavelength dependence of the flash-induced absorbance change in intact *A. thaliana* leaves. Absorbance change amplitudes were measured 4 ms after a single-turnover (4 μs at half of peak height) flash of 0.38 J m^{-2}. Each data point is the average of the results obtained from four separate leaves that had been dark-adapted overnight.

(Kramer et al., 1990). Those plants showing abnormal CF activation were carefully isolated and allowed to self pollinate, and plants from the M_3 and, thereafter, the M_4 generations were rescreened to establish the heritability of the putative mutant phenotype.

Experiments with the M_3 and M_4 generation plants, as well as the determination of wavelength dependence, were performed with a conventional single-beam flash kinetic absorption spectrophotometer identical to that used previously by Wise and Ort (1989). In this case, thioredoxin-dependent reduction of CF γ subunit was induced by 3 s of preillumination with continuous red light (65 μmol quanta m^{-2} s^{-1} unless otherwise specified).

Treatment of *Arabidopsis* Leaves with DCCD

Detached leaves were submerged in a solution of 5 mM DCCD, 2% (v/v) methanol, and 1% (v/v) Tween 20 for 10 min in the dark. The control treatment consisted of submerging leaves in a solution containing methanol and Tween 20 but lacking DCCD (Wise and Ort, 1989).

RESULTS

The Flash-Induced Electrochromic Absorbance Change as a Monitor of CF Activity in Intact *Arabidopsis* Leaves

The in situ activity of CF can be monitored in intact leaves by taking advantage of the contribution that the electric potential of the $\Delta\mu_{H^+}$ makes to flash-driven ATP formation (e.g. Morita et al., 1983; Vallejos et al., 1983; Kramer and Crofts, 1989; Wise and Ort, 1989). In photosynthetic membranes, the fate of the electric potential can be conveniently monitored through the effect of the electric field on the absorption spectrum of a specialized group of pigments within the membrane. Membrane-depolarizing proton efflux through the CF complex associated with ATP synthesis results in an accelerated relaxation of the electric field-associated absorption change (Witt, 1979). Loss of the naturally low conductance of the thylakoid bilayer to protons and other ions would also cause an accelerated decay, but then the rate of decay would not respond to factors that control CF activity (such as treatment with the inhibitor DCCD), and the two possibilities can be distinguished on that basis. This approach can be extended to monitor the reduction of CF, since reduction of the CF γ subunit significantly lowers the energetic threshold, and therefore the number of actinic flashes, necessary to activate CF and initiate ATP formation (Hangarter et al., 1987).

The wavelength dependence of the flash-induced absorbance change measured in detached wild-type *Arabidopsis* leaves 20 ms after an actinic flash (Fig. 1) was indistinguishable from that reported for isolated thylakoids (e.g. Witt, 1979) or intact leaves of several different higher plant species (Garab et al., 1983; Chylla and Whitmarsh, 1989; Wise and Ort, 1989; Ortiz-Lopez, 1990) with a maximum difference near 518 nm.

Figure 2 illustrates the capability of the ΔA_{518} to monitor separately both the activation state and the redox state of CF in intact *Arabidopsis* leaves. After prolonged dark adaptation, the membrane was deenergized and the CF was in its oxidized state. In this situation (Fig. 2, ■), a single actinic flash is inadequate to activate CF and initiate ATP formation (e.g. Junesch and Gräber, 1985; Hangarter et al., 1987). As a result, the relaxation of the trans-thylakoid electric field, and therefore the electrochromic absorption change, was very slow (half-time > 400 ms). Following light adaptation (by two hundred 60%-saturating red flashes delivered at 5 Hz), a single actinic flash induced a pronounced acceleration of the decay attributable to proton efflux through CF associated with ATP formation (Fig. 2, ●). The preilluminating flash

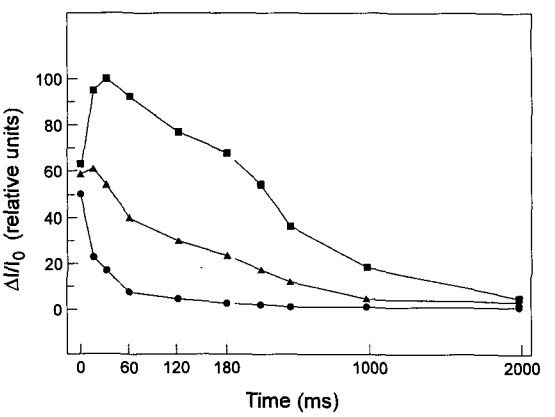

Figure 2. Effects of light and dark adaptation on the decay kinetics of the ΔA_{518} in intact *Arabidopsis* leaves. The ΔA_{518} relaxed slowly in leaves taken from plants that had been dark-adapted for 12 h (■). The rate of decay was stimulated more than 10-fold 10 s after light adaptation (●) by a train of two hundred 60%-saturating flashes delivered to the leaf at 5 Hz. Following 2 min in the dark to allow dissipation of the $\Delta\mu_{H^+}$ formed during the preilluminating flash train, the effect of CF reduction alone on the energetics of activation and ATP formation were visible (▲). The data points presented are the means of 15 independent measurements. SE values ranged between 2 and 11% of the mean value.

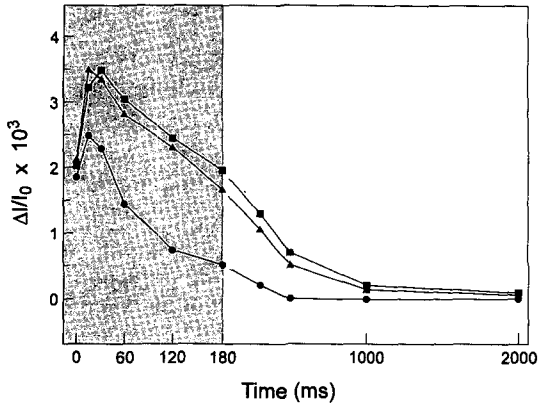

Figure 3. Effects of light and dark adaptation on the decay kinetics of the ΔA_{518} in a putative CF reduction mutant of *Arabidopsis*. The experimental protocol and the meaning of the symbols are as given in Figure 2. Note that after only 2 min of dark readaptation (▲) after the light-adaptation flash train, the ΔA_{518} relaxation kinetics returned to the fully dark-adapted state.

train resulted in CF reduction as well as generation of a large $\Delta\mu_{H^+}$ (Kramer and Crofts, 1989; Kramer et al., 1990; Ort et al., 1990). When the $\Delta\mu_{H^+}$ produced by the preilluminating flash train was given time to dissipate (2 min in the dark), the effects of CF reduction alone on the energetics of CF activation and ATP formation became visible (Fig. 2, ▲). With CF prereduced, a single flash was capable of activating CF and initiating ATP formation (Fig. 2, cf. ▲ to ■), albeit to a lesser extent than when the flash was given in addition to the large $\Delta\mu_{H^+}$ generated during the preilluminating flash train (Fig. 2, cf. ▲ to ●). The interpretation of the ΔA_{518} in intact leaves presented here has been largely verified by direct measurements of CF activity (Morita et al., 1983; Vallejos et al., 1983; Kramer and Crofts, 1989; Ort et al., 1990). Nevertheless, a priority of our ongoing work is a systematic comparison of ΔA_{518} kinetics in intact *Arabidopsis* leaves with direct biochemical measurements of CF activity in vitro.

Identification of Putative CF Reduction Mutants in *Arabidopsis*

Based on our initial phenotypic screen for poor growth and vigor at low irradiance followed by recovery upon return to normal irradiance levels, we selected 117 putative mutants from about 10^5 M_2 plants. Twenty-two of these exhibited distinctly altered ΔA_{518} relaxation kinetics. Figure 3 shows an example of the behavior that we expected for a CF reduction mutant. In the dark-adapted plant, the ΔA_{518} relaxation kinetics were indistinguishable from those of wild-type *Arabidopsis* leaves (cf. Fig. 2, ■, and Fig. 3, ■). However, in this case, light adaptation by 200 flashes (Fig. 3, ●) resulted in much less acceleration of the ΔA_{518} decay (i.e. less ATP formation) and, following a 2-min dark interval to allow dissipation of the $\Delta\mu_{H^+}$ formed during the preilluminating flash train, the relaxation kinetics fully returned to the dark-adapted profile (Fig. 3, ▲). This is exactly the behavior that is observed in isolated thylakoids where the reduction of CF does not occur due to the absence of thioredoxin and Fd, which are lost during the thylakoid isolation procedure.

The plant depicted in Figure 3 is an example of one of the 5 of 22 apparent CF mutants that did not form viable seed. Seed from the remaining 17 was collected and, following rescreening of the M_3 and M_4 generation plants on the basis of ΔA_{518} relaxation kinetics, 3 mutants with reproducibly altered relaxation kinetics were selected for more detailed study. Figure 4 shows the characteristics of the ΔA_{518} of the mutants in comparison to wild-type *Arabidopsis* leaves, in this case using a conventional kinetic spectrophotometer employing a continuous measuring beam instead of the double-flash spectrometer used in Figures 2 and 3. In experiments with this instrument, we measured the ΔA_{518} relaxation kinetics following 12 h of dark adaptation (D_{ad}), immediately after 3 s of light adaptation with 65 μmol quanta m^{-2} s^{-1} red light (L_{ad}), and after 2 min (2′) or 4 min (4′) of dark readaptation.

The most conspicuous differences in the ΔA_{518} relaxation kinetics are visible for the mutant designated cfq, which behaved very similarly to the infertile plant in Figure 3. Once the $\Delta\mu_{H^+}$ that was formed during the 3-s preillumination had largely dissipated (Fig. 4, trace 2′), the ΔA_{518} relaxation kinetics were identical to those recorded after 12 h of dark adaptation (Fig. 4, D_{ad}). We interpret this behavior as indicating that CF was not reduced during the 3-s preillumination period. When grown under standard light conditions (i.e. 16-h photoperiod at 270 ± 30 μmol quanta m^{-2} s^{-1} PPFD), the cfq mutant was vigorous and very similar in overall appearance to the wild-type plant. Not until the growth light intensity was lowered below 120 μmol quanta m^{-2} s^{-1} PPFD did a slightly smaller leaf size make the cfq mutant visually distinguishable from the wild type. However, at the selection

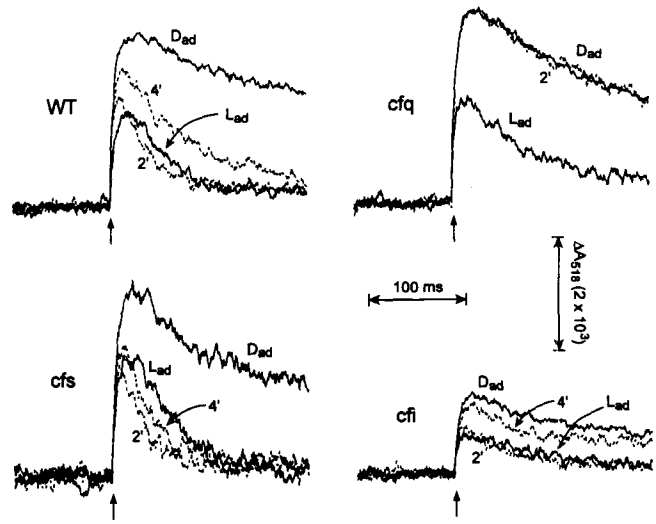

Figure 4. Anomalous characteristics of light adaptation and dark readaptation in M_3 *Arabidopsis* selections. The ΔA_{518} (↑) relaxation kinetics are shown for wild-type (WT) and three mutant phenotypes of *Arabidopsis*: cfq, cfs, and cfi. Plants were initially dark-adapted for 12 h (D_{ad}), then light-adapted by 3 s of preillumination with 65 μmol m^{-2} s^{-1} of red light (L_{ad}), and thereafter dark-readapted for 2 min (2′) or 4 min (4′).

Table I. *ΔA_{518} relaxation time constants for wild-type and mutant phenotype Arabidopsis*

The plants were initially dark-adapted for 12 h (D_{ad}) followed by 3 s of light adaptation at 65 μmol m^{-2} s^{-1} (L_{ad}). The plants were then re-dark-adapted for periods of 2 or 4 min. Between 5 and 11 individual traces were averaged (values given in parentheses) and the time constants were calculated from the averaged traces as described in the text.

Plant Phenotype	Relaxation Time Constant			
	D_{ad}	L_{ad}	2 min	4 min
	ms			
Wild type (11)	270	62	50	84
cfq mutant (5)	312	87	270	263
cfi mutant (6)	227	83	67	86
cfs mutant (5)	144	47	33	49

illumination condition (i.e. 8-h photoperiod at 12 ± 3 μmol quanta m^{-2} s^{-1} PPFD) the cfq mutant grew poorly and appeared to be unhealthy in comparison with wild-type, control plants.

Similar tendencies, however less pronounced, were observed in the ΔA_{518} relaxation kinetics in the mutant designated cfi. The smaller amplitude of the flash-induced absorbance change was associated with the moderately chlorotic leaves of this mutant because, throughout the experiments presented in this paper, an actinic flash strength that was 60% saturating for wild-type plants was employed. It might be thought that the slower relaxation kinetics of the ΔA_{518} were also caused by the chlorosis if the decrease in light absorption produced a $\Delta\mu_{H^+}$ too small to adequately activate the CF pool. However, five of five other chlorotic mutants that we examined also had strongly reduced ΔA_{518} amplitude but did not differ significantly in relaxation kinetics from control plants (data not shown).

The third mutant, denoted cfs, grew quite slowly and had to be rescued after only a few days under the weak light selection condition. Mature plants were small with heavy anthocyanin pigmentation of the leaves. The plants flowered late and produced a relatively small number of seeds of limited viability. Figure 4 shows that the ΔA_{518} relaxation kinetics after dark adaptation (D_{ad}) in cfs were substantially faster and, as a consequence, the acceleration by light adaptation (L_{ad}) was somewhat smaller compared with wild-type plants. The most interesting feature of this mutant is that dark readaptation (Fig. 4, cf. the 2′ and 4′ traces with D_{ad}) was very slow.

Table I summarizes the relaxation time constants for each of the plants and conditions presented in Figure 4. To improve the signal-to-noise ratio, the individual traces from multiple individual experiments identical to those in Figure 4 were summed for signal averaging. The relaxation time constants were calculated by fitting the initial 100 ms of the decay to a first-order exponential using an iterative, nonlinear least squares program. Comparison of the time constants shows a smaller acceleration in the ΔA_{518} relaxation kinetics by light in all three mutants. Dark readaptation is dramatically more rapid in the cfq phenotype than in the wild type. In the cfs mutant, relaxation of the ΔA_{518} is in all cases more rapid than in wild-type plants.

Effect of DCCD on the ΔA_{518} Relaxation Kinetics in Intact *Arabidopsis* Leaves

To confirm that the acceleration in ΔA_{518} relaxation kinetics following light adaptation is due to proton efflux through CF, we treated *Arabidopsis* leaves with the ATP synthesis inhibitor DCCD. DCCD is known to prevent the egress of protons through the integral membrane protein portion of CF, thereby inhibiting ATP formation. The effect of the inhibitor was to substantially slow the ΔA_{518} relaxation kinetics in light-adapted leaves (Fig. 5, L_{ad}), consistent with the interpretation that the major proton efflux accounting for the acceleration of ΔA_{518} relaxation kinetics is through CF. In dark-adapted leaves, in which CF was not reduced and consequently a single actinic flash was inadequate to initiate CF activation and ATP formation, the DCCD treatment had negligible effect except in the case of the cfs mutant. DCCD reversed the anomalously rapid relaxation of the electric field that occurs in dark-adapted leaves of this mutant.

The Effect of Light Adaptation Fluence Rate on the Time Course of CF Reduction and Reoxidation

The light-intensity dependence of CF redox behavior could provide clues concerning the origin of the dysfunction in the various mutant phenotypes. Thus, we extended our investigation by increasing and decreasing the light adaptation fluence rate by a factor of 10 and recording complete dark readaptation profiles for each. The dark readaptation profiles are presented in Figure 6 as the percentage of the ΔA_{518} amplitude remaining 180 ms after the actinic flash. Kramer and Crofts (1989) have demonstrated that the component of the ΔA_{518} relaxation associated with ATP formation in intact

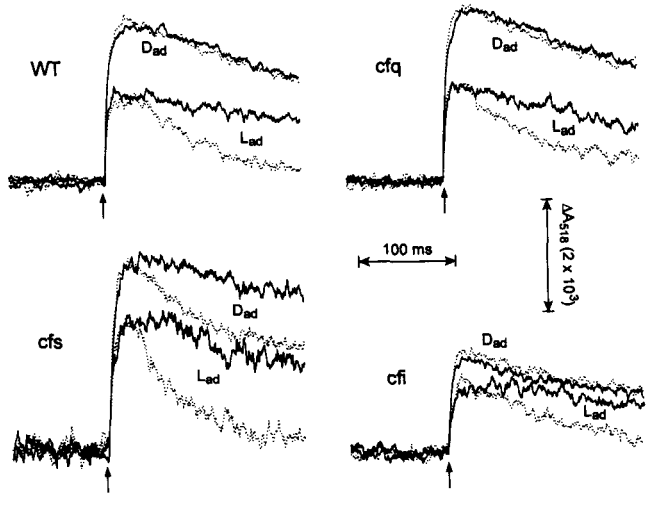

Figure 5. Effect of the CF inhibitor DCCD on the ΔA_{518} relaxation kinetics in wild-type and three mutant phenotypes of *Arabidopsis*. Detached leaves were treated with 5 mM DCCD for 10 min as described in "Materials and Methods." The experimental protocol and the meaning of the notations are as given in Figure 4.

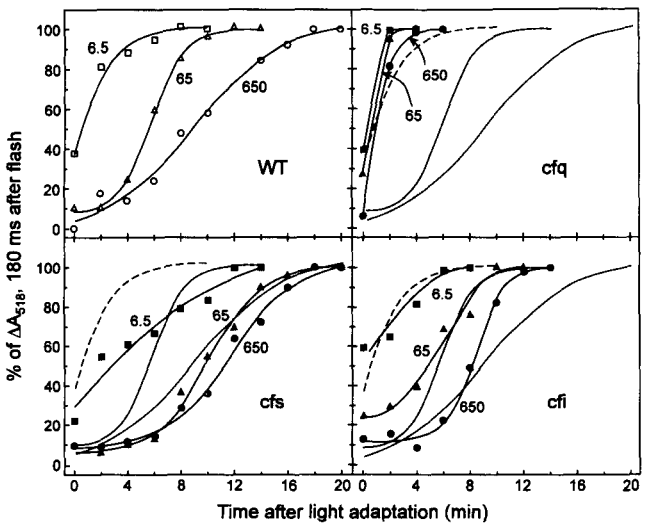

Figure 6. The influence of preillumination intensity on the dark readaptation time course in wild-type compared with mutant phenotypes of *Arabidopsis*. The plants were preilluminated for 3 s at 6.5, 65, or 650 μmol m^{-2} s^{-1} of red light and the ΔA_{518} amplitudes were measured 180 ms after the single-turnover actinic flash. For purposes of comparison, the dark readaptation profiles for the wild type (WT) are reproduced with the profiles of each mutant phenotype.

leaves is nearly complete within 180 ms and that, for a specified level of excitation, the size of the remaining, slowly decaying amplitude is negatively correlated with the reduction state of the CF pool. For example, the half-time for the reoxidation of the CF pool in wild-type *Arabidopsis* leaves is 5 to 6 min following 3 s of light adaptation at 65 μmol quanta m^{-2} s^{-1} of red light (Fig. 6, Δ). Following this preillumination regime, the CF of the wild type was judged to be fully reoxidized within 10 to 12 min, since a single actinic flash could no longer induce ATP formation. As reported previously for other species (Kramer and Crofts, 1989; Ort et al., 1990), higher intensity (Fig. 6, O) or longer periods of preillumination can significantly extend the CF reoxidation half-time. Kramer and Crofts (1989) demonstrated previously that the reoxidation kinetics show a lag that is dependent on the amount (intensity or duration) of light adaptation. They interpreted the lag as indicating the presence of a chloroplast redox buffer pool that was in equilibrium with the CF γ subunit.

Regardless of the preillumination intensity (Fig. 6) or duration (data not shown), a single actinic flash could induce CF activation in the cfq mutant only during the brief interval when the $\Delta\mu_{H^+}$ produced by the preillumination was still present. That is, the lowering of the energetic threshold for CF activation normally associated with reduction of the γ subunit appears not to have taken place in the cfq mutant. In the cfs mutant, although CF reoxidation appeared to be quite slow relative to the wild type following the 3-s, 65 μmol quanta m^{-2} s^{-1} preillumination regime (Figs. 4 and 6), this difference largely disappeared when the light adaptation fluence rate was increased 10-fold (Fig. 6).

DISCUSSION

We have devised a two-step selection procedure for the isolation of chloroplast CF reduction mutants that relies on a lowered energetic threshold for activation that accompanies thioredoxin-mediated reduction of a Cys bridge on the γ subunit. The role of reduction in controlling chloroplast CF catalytic activity differs in a fundamental way from other chloroplast enzymes regulated by thioredoxin. Whereas activation of CF does not require reduction, nor is reduction sufficient for activation, in the other thioredoxin-modulated enzymes (for example Fru bisphosphatase or phosphoribulokinase) reduction of the regulatory Cys bridge on the protein is an obligate requirement for catalytic activity under physiological conditions. In contrast, it is clear that the catalytic activation of CF is a prerequisite of thioredoxin-dependent reduction (Ort and Oxborough, 1992). Because of the obligate role of thioredoxin in the activation of these other crucial photosynthetic enzymes, we anticipated that any mutants lacking thioredoxin or severely restricted in their ability to reduce thioredoxin (e.g. mutations interfering with the function of Fd-thioredoxin oxidoreductase) would be lethal. Thus, we further anticipated that the mutations that we would select using this strategy would either be localized on CF itself or involve features of the reduction/oxidation pathway unique to CF.

The cfq mutant is our most promising selection to date because it displays a clean phenotype and is a vigorous and reproductively viable plant when grown under permissive, high-irradiance conditions. The initial characterization of this mutant, based on measurement of ΔA_{518} relaxation kinetics, indicates that light-adaptation treatment fails to induce CF reduction. That is, only immediately after light adaptation, when a significant $\Delta\mu_{H^+}$ lingers, can a single flash induce CF activation (Fig. 4) and DCCD-sensitive ATP formation (Fig. 5) in the cfq mutant. Even increasing the light-adaptation intensity by a factor of 10 failed to lower the energetic threshold for CF activation (Fig. 6), which is the expected result of CF reduction.

Although the reduction of CF normally occurs rapidly even in very weak light (Quick and Mills, 1986; Kramer and Crofts, 1989; Kramer et al., 1990), the thioredoxin modulation of other chloroplast enzymes such as Fru bisphosphatase or NADP-malate dehydrogenase is slower, and full reduction requires substantially higher light intensities (e.g. Leegood et al., 1982; Rebeille and Hatch, 1986a, 1986b; Sassenrath et al., 1990). Although a kinetic explanation has generally been given for this difference in behavior, citing slower reoxidation of CF (Shahak, 1985; Selman-Reimer and Selman, 1988) than of the carbon reduction cycle enzymes (Crawford et al., 1989), evidence is beginning to emerge that the redox midpoint potentials of the thiol groups of thioredoxin-modulated enzymes may play a central role in determining the regulatory behavior. Equilibrium redox titrations reveal that, although the regulatory sulfhydryl on CF is nearly equipotential with thioredoxin f at −270 mV (Ort and Oxborough, 1992; Hutchison, 1993), the chloroplast bisphosphatases are about 40 mV more reducing (Rebeille and Hatch, 1986a, 1986b; Hutchison, 1993). This large difference in midpoint potential was used as the basis of a model that emphasizes the possible impor-

tance of the thermodynamics of electron sharing between thioredoxin and its target enzymes. This model, elaborated as a computer simulation, provides a good accounting of the differential responses to light intensity and the kinetics of reduction and oxidation of the different thioredoxin-modulated enzymes (Kramer et al., 1990). It follows from this model that mutations that resulted in a significantly more negative midpoint potential for the regulatory sulfhydryl of CF could produce the characteristics of CF activation observed in the cfq mutant without significant impact on the thioredoxin-regulated enzymes of the photosynthetic carbon-reduction cycle. Although this is an interesting possibility, it will need to be investigated directly by equilibrium redox titrations of the γ subunit regulatory sulfhydryls.

In contrast to the cfq mutant, CF reduction in the cfs mutant appears to proceed more readily and to be more persistent than in the wild type. Even in fully dark-adapted plants there is a component of the ΔA_{518} relaxation that is sensitive to DCCD (Fig. 5). Furthermore, in the cfs mutant, reoxidation of CF following preillumination is very slow in comparison with the wild type, the difference being particularly accentuated at low preillumination intensities (Fig. 6). Perhaps most intriguing is that the lag in CF reoxidation develops at much lower preillumination fluence rates compared with the wild type (Fig. 6). Within the framework of the model presented above, this would be the expected result of a 20-mV or more increase (i.e. more positive) in the midpoint potential of the γ subunit regulatory sulfhydryl. It should also be noted, however, that the ΔA_{518} relaxation characteristics of the cfs mutant could be adequately explained by an ungated proton leak through CF as would occur, for example, if a portion of the CF_1 population were disconnected or improperly connected to CF_o.

In this paper we have demonstrated the usefulness of our screening strategy for the selection of CF reduction mutants from M_2 populations of *Arabidopsis* and report on the initial characterization of several promising mutant phenotypes. We have demonstrated that these mutant phenotypes are heritable by examining M_3 and M_4 plants produced by self pollination. Backcrossing each of the mutants to the wild type and segregation analysis is now underway to determine if the changes in CF reduction characteristics that we have documented here arise from single gene mutations. Although backcrossing and genetic analysis of the cfq and cfi mutants should be straightforward, the poor vigor and seed viability of the cfs mutant has already caused difficulties. Although we will continue to work with this mutant, we will also rescreen in an attempt to select the cfs phenotype in a more vigorous plant specimen. We anticipate that the combination of genetic and biochemical analyses of these putative CF mutations now underway will identify the bases of the dysfunctions and provide further insights into the regulation of CF activity in vivo.

Received October 18, 1993; accepted November 18, 1993.

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