Mutants of Chloroplast Coupling Factor Reduction in Arabidopsis

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We have devised a two-step screening strategy for the selection of chloroplast coupling factor reduction mutants from an M2 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: ΔμH+γ, 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 photoactivated 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 ΔμH+ is normally required for the initiation and maintenance of catalytic activity. Although the size of the threshold ΔμH+ is smaller when the γ subunit disulfide has been reduced by thioredoxin to vicinal diithiols, it appears that even in intact leaves ΔμH+ depends upon the CF activity (e.g. 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 ΔμH+ values, thereby limiting the rate of photophosphorylation. In fact, CF reduction actually increases the efficiency of ATP formation as a function of ΔμH+ (e.g. Ketcham et al., 1984; Hangarter et al., 1987).

Abbreviations: CF, chloroplast coupling factor or chloroplast CF; CFγ, ATP synthase; cf, coupling factor intermediate recovery; cfγ, coupling factor quick recovery; cfs, coupling factor slow recovery; DCCD, dicyclohexylcarbodiimide; ΔμH+γ, flash-induced electrochromic absorbance change measured at 518 nm; ΔμH+, 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 diethiols 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 Δμₜ 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 is diminished 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 Δμₜ 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 M₁ and M₂ generations. Here we report on the selection and initial characterization of three CF activation mutants of Arabidopsis.
adapted overnight. (Kramer et al., 1990). Those plants showing abnormal CF activation were carefully isolated and allowed to self pollinate, and plants from the M₃ and, thereafter, the M₄ generations were rescreened to establish the heritability of the putative mutant phenotype.

Experiments with the M₃ and M₄ 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 ΔμH⁺ 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 absorbance 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 ΔΔA₁₈₁ 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 absorbance 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...
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systematic comparison of intact leaves presented here has been largely verified by

dopsis Vallejos et al., 1983; Kramer and Crofts, 1989; Ort et al., the large

distinctly altered vigor at low irradiance followed by recovery upon return to

normal irradiance levels, we selected the fully dark-adapted state.

train resulted in CF reduction as well as generation of a large \( \Delta u_{\text{H+}} \) (Kramer and Crofts, 1989; Kramer et al., 1990; Ort et al., 1990). When the \( \Delta u_{\text{H+}} \) 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, A). With CF prereduced, a single flash was capable of activating CF and initiating ATP formation (Fig. 2, cf. A to D), albeit to a lesser extent than when the flash was given in addition to the large \( \Delta u_{\text{H+}} \) generated during the preilluminating flash train (Fig. 2, cf. A to D). The interpretation of the \( \Delta A_{518} \) 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 \( \Delta A_{518} \) 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 M4 plants. Twenty-two of these exhibited distinctly altered \( \Delta A_{518} \) relaxation kinetics. Figure 3 shows an example of the behavior that we expected for a CF reduction mutant. In the dark-adapted plant, the \( \Delta A_{518} \) relaxation kinetics were indistinguishable from those of wild-type Arabidopsis leaves (cf. Fig. 2, C, and Fig. 3, C). However, in this case, light adaptation by 200 flashes (Fig. 3, D) resulted in much less acceleration of the \( \Delta A_{518} \) decay (i.e. less ATP formation) and, following a 2-min dark interval to allow dissipation of the \( \Delta u_{\text{H+}} \) formed during the preilluminating flash train, the relaxation kinetics fully returned to the dark-adapted profile (Fig. 3, A). 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 M3 and M4 generation plants on the basis of \( \Delta A_{518} \) relaxation kinetics, 3 mutants with reproducibly altered relaxation kinetics were selected for more detailed study. Figure 4 shows the characteristics of the \( \Delta A_{518} \) 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 \( \Delta A_{518} \) relaxation kinetics following 12 h of dark adaptation (Dad), immediately after 3 s of light adaptation with 65 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \) red light (L4), and after 2 min (2') or 4 min (4') of dark readaptation.

The most conspicuous differences in the \( \Delta A_{518} \) relaxation kinetics are visible for the mutant designated cfq, which behaved very similarly to the infertile plant in Figure 3. Once the \( \Delta u_{\text{H+}} \) that was formed during the 3-s preillumination had largely dissipated (Fig. 4, trace 2'), the \( \Delta A_{518} \) relaxation kinetics were identical to those recorded after 12 h of dark adaptation (Fig. 4, D4). 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 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \text{ppfd} \)), 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 \( \mu \text{mol quanta m}^{-2} \text{s}^{-1} \text{ppfd} \) did a slightly smaller leaf size make the cfq mutant visually distinguishable from the wild type. However, at the selection
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that was in equilibrium with the CF.

That is, the lowering of the 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 ΔA518 relaxation kinetics, indicates that light-adaptation treatment fails to induce CF reduction. That is, only immediately after light adaptation, when a significant ΔµH+ 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, 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 sulphydryl 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 ΔA518 relaxation that is sensitive to DCCD (Fig. 5). Furthermore, in the cfq 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 ΔA518 relaxation characteristics of the cfs mutant could be adequately explained by an un gated proton leak through CF as would occur, for example, if a portion of the CF1 population were disconnected or improperly connected to CF0.

In this paper we have demonstrated the usefulness of our screening strategy for the selection of CF reduction mutants from M2 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 M3 and M4 plants produced by self pollination. Backcrossing each of the mutants to the wild type and report on the initial characterization of several promising mutant phenotypes. We have demonstrated that these mutant phenotypes are heritable by examining M3 and M4 plants produced by self pollination. Backcrossing each of the mutants to the wild type backcrossing and genetic analysis of the cfq and cfi mutants documented here arise from single gene mutations. 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.

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