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ORIGINAL ARTICLE

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Electron flow to photosystem I from stromal reductants in vivo: the size of the pool of stromal reductants controls the rate of electron donation to both rapidly and slowly reducing photosystem I units

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Abstract Electron donation from stromal reductants to photosystem I (PSI) was studied using the kinetics of $P700^+$ (the oxidized primary donor of PSI) reduction in the dark after irradiation of barley (*Hordeum vulgare* L.) leaves. The leaves were treated with diuron and methyl viologen to abolish both the electron flow from PSII and PSI-driven cyclic electron transport. The redox state of P700 was monitored using the absorbance changes at 830 nm (ΔA_{830}). Two exponentially decaying components with half-times of about 3 s (the slow component) and about 0.6 s (the fast one) were distinguished in the kinetic curves of ΔA_{830} relaxation after a 1-s pulse of far-red light. The complex kinetics of $P700^+$ reduction thus manifested two types of PSI unit differing in the rate of electron input from stromal reductants. The rates of both kinetic components assayed after 1-s pulses were increased about 20-fold by a short (2–5 min) heat-pretreatment of leaves, indicating the accelerated input of electrons to both types of PSI unit. The increased rates of electron flow to $P700^+$ were even observed 1.5 h after the action of heat had been completed. Both kinetic components were dramatically slowed down upon irradiation of heat-treated leaves for 20–30 s. Their rates were restored after a short (20–30 s) period of darkness. A 5-min leaf exposure at 38 °C was sufficient to stimulate by severalfold the reduction of $P700^+$ pre-oxidized by a brief light pulse. In contrast, the acceleration of $P700^+$ reduction after a 1-min irradiation was observed only if leaves were subjected to temperatures above 40 °C. Neither heat treatment of leaves nor light–dark modulations in the rates of the fast and the slow components of $P700^+$ dark reduction influenced the relative magnitudes of the two kinetic components, providing strong additional evidence in favor of two distinct types of PSI existing per se in barley leaves. The key role in the control of the activity of electron donation to $P700^+$ in both rapidly and slowly reducing PSI units was attributed to the amount of stromal reductants available for $P700^+$ reduction. The latter was expected to be reduced under illumination in the presence of methyl viologen, while increased again in the dark. The regeneration of the pool of stromal reductants in the dark was likely provided by starch breakdown within the chloroplast stroma, but not by import of reducing equivalents from the cytosol. This was evidenced by much lower rates, compared with 1-h dark-adapted leaves, of dark reduction of both components of $P700^+$ in leaves stored for 24 h in the dark and thus depleted of starch but containing large amounts of glucose, the respiratory substrate.

Keywords Alternative electron transport · High temperature · *Hordeum*. · Photosystem I · Stromal reductants

Abbreviations FR: far-red · MV: methyl viologen · PSI and PSII: photosystem I and photosystem II · P700: primary electron donor of photosystem I

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Introduction

Linear photosynthetic electron transport, that involves photosystem II (PSII) and PSI operating in series, yields the energy-rich molecules and the reductants required for carbon fixation. In addition to that major electron flow, several alternative pathways driven by PSI alone have been found in chloroplasts. Proposed functions for these alternative electron transport routes are to adjust ATP to NADPH stoichiometry and to provide

additional control on PSII activity (Heber and Walker 1992). A significant contribution from alternative electron transport routes to the energetics of chloroplasts under light is questionable in healthy leaves, as the capacity of linear electron transport already exceeds that of carbon metabolism (Farquhar et al. 1980). This is not the case, however, in leaves experiencing unfavorable environmental conditions.

Numerous stresses weaken PSII, which is in most cases less tolerant than PSI (Baker 1991). As a consequence, the rate of linear electron transport, the main source for generation of the trans-thylakoid proton gradient, declines. This creates a deficit in energy supply for the dark enzymatic reactions in the chloroplast stroma. In addition, down-regulation of residual PSII activity mainly provided by the trans-thylakoid ΔpH (Horton et al. 1996) becomes weakened as well. The significance of alternative PSI-driven electron transport routes for the energetics and the control of metabolic activity is expected, therefore, to be increased in leaves exposed to stressful conditions.

Several entries for electrons are known or hypothesized at the level of the plastoquinone pool. The most studied path is the ferredoxin-mediated cyclic electron transport around PSI, which is accompanied by the formation of ΔpH across the thylakoid membrane and the production of ATP (Tagawa et al. 1963). Two dehydrogenases, the NAD(P)H dehydrogenase complex analogous to complex I of the mitochondrial respiratory chain (Guedeney et al. 1996; Sazanov et al. 1998a) and non-electrogenic NAD(P)H dehydrogenase (Corneille et al. 1998), have been found in thylakoids. They mediate the reduction of plastoquinone from reductants such as NADH or/and NADPH located in the chloroplast stroma.

At least two kinds of extreme environmental conditions, elevated temperatures (Havaux 1996; Bukhov et al. 2000) and high salinity (Endo et al. 1995), were shown to enhance the input of reducing equivalents from soluble stromal reductants to the chloroplast electron transport chain. Both stresses highly suppressed the activity of PSII. The alternative electron transport flows coupled at the level of the plastoquinone pool to the translocation of protons from the stroma to the intra-thylakoid lumen thus appear to functionally substitute for weakened linear electron transport in stressed leaves.

From the general point of view, it is clear that (i) the enzyme activity mediating the donation of electrons to plastoquinone from stromal reductants, (ii) the current size of the pool of stromal reductants, and (iii) the ability for that pool to be permanently regenerated from some external reservoir must determine all together the capacity of electron flow from stromal reductants to plastoquinone. As the rate of light-independent enzymatic re-oxidation of reduced plastoquinone is low relative to electron flow to PSI (Bennoun 1994; Cournac et al. 2000), the kinetics of reduction of $P700^+$, the oxidized primary donor of PSI, after irradiation characterizes the properties of electron donation to plastoquinone from stromal reductants. Very recently, we have reported the complex kinetics of $P700^+$ reduction in the dark under conditions completely preventing electron transfer from PSII to PSI and electron cyclization around PSI (Bukhov et al. 2001b). That finding evidenced the functional heterogeneity of PSI units with respect to their capacity to receive electrons from stromal reductants. The conditions under which the pool of stromal reductants can be exhausted were also evaluated (Bukhov et al. 2001a).

The goal of this study was to examine in detail the behavior of heat-stimulated electron flow from stromal reductants to PSI by using the complex kinetics of $P700^+$ reduction. We report here that the irradiation reversibly reduces the activity of heat-induced electron input from stromal reductants to PSI, which is brought about by decreased amounts of available reductants. Regeneration of the pool of stromal reductants is supplied by the hydrolysis of starch. Similar temperature profiles and similar behavior during light–dark cycles found for the fast and slow components that characterized $P700^+$ reduction show that both components are mediated by enzymes similar in nature, but differing in their accessibility to stromal reductants because of location in different chloroplast membranes.

Materials and methods

Primary leaves of 8- to 9-day-old barley (*Hordeum vulgare* L.) seedlings were used in the experiments. Plants were grown in a growth cabinet under light of 170–200 μmol photons m^{-2} s^{-1} provided by a xenon lamp or by a set of white fluorescence tubes. Treatment of leaves with methyl viologen (MV) plus diuron was done by floating the segments of dark-adapted leaves on a solution containing 200 μM diuron and 2 mM MV. The segments were kept in the above solutions in complete darkness for 30 min.

For starch extraction, liquid-nitrogen-frozen leaf tissue was pulverized in a cooled mortar. The powder was extracted with 60% (v/v) ethanol, filtered through Miracloth, and stored on ice for 10 min prior to centrifugation for 10 min at 10,000 *g* and 4 °C. The pellet was successively washed with 50% ethanol and twice with 80% ethanol to remove precipitated proteins and chlorophylls. For starch hydrolysis, the starch-containing pellet was suspended in 2 ml dimethyl sulfoxide to which 0.5 ml HCl was added. After heating for 90 min at 55 °C, the extract was neutralized with 6 N NaOH and centrifuged for 5 min at 10,000 *g* and 4 °C before dilution to 7 ml with water. Hydrolyzed fragments were cleaved to glucose by addition of 200 μl of 0.3 M citrate buffer (pH 4.6) to 100 μl of the extract and incubation for 60 min at 55 °C in the presence of amyloglucosidase. The concentration of glucose was determined with an F-kit (Boehringer-Mannheim, Mannheim, Germany).

Soluble sugars were extracted from pulverized frozen leaf tissue in a medium containing 3.5 ml methanol, 1.5 ml $CHCl_3$, and 0.6 ml of 1 M Tris–HCl buffer (pH 8.4). After 30-min storage on ice, 3 ml H_2O was added. The mixture was centrifuged for 10 min at 10,000 *g* and 4 °C. The supernatant was rapidly dried on ice under vacuum. The residue was dissolved in 800 μl of 0.5% PVP-40. Fructose and sucrose were measured spectrophotometrically according to Bergmeyer (1974).

The redox changes of P700 were monitored as the light induced changes in absorption at 830 nm (ΔA_{830}) with a PAM fluorometer (Schreiber et al. 1988). The ED-P700DW dual-wavelength unit was used for the measurements of absorbance changes. The absorbance changes obtained with that unit were solely due to P700 redox

changes, as the contribution of plastocyanin absorbance changes is abolished by the ED-P700DW unit. A ΔA_{830} signal was recorded with a computer using the PAM Data Acquisition System PDA-100. The sampling rate was 1 or 3 ms/point. Far-red (FR) light was obtained by passing the light from a Fiber-Lite light source (Microview, Canada) through an RG-9 filter (Schott, Mainz, Germany). The FR light was switched on and off by an electronic shutter. Multiple-turnover pulses (5–50 ms) were provided by a xenon lamp (XF-103; Walz, Effeltrich, Germany) using an XMT 103 power supply unit (Walz).

Results

The complex kinetics of $P700^+$ reduction after short irradiation of leaves with FR light

Figure 1A illustrates the kinetics of absorbance changes at 830 nm (ΔA_{830}) initiated in barley leaves treated with diuron and MV by a 1-s pulse of FR light. The ΔA_{830} signals measured in leaves either not exposed to elevated temperatures or treated for 5 min at 42 °C were compared. Irradiation caused the oxidation of P700, monitored as the absorbance increase. The ΔA_{830} relaxed after the FR light had been turned off, and the dark decay proceeded much faster in heat-treated leaves, thus demonstrating heat-induced acceleration of $P700^+$ reduction. As already noted the only source of electrons available for PSI under those conditions originated from soluble reductants located in the chloroplast stroma.

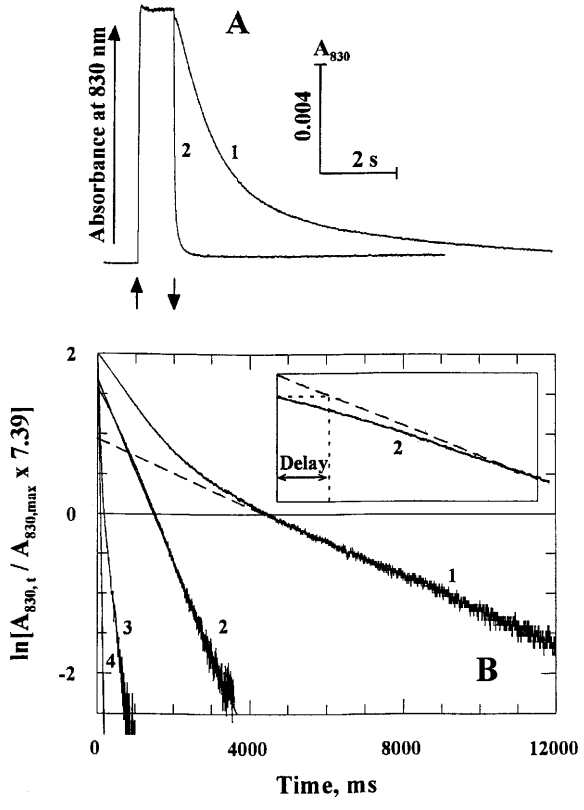

Fig. 1. **A** Original traces of the absorbance changes at 830 nm induced by a 1-s pulse of FR light in dark-adapted barley (*Hordeum vulgare*) leaves treated with diuron and MV. Leaves were either not exposed to elevated temperature (*1*) or exposed for 5 min to 42 °C (*2*). **B** Semi-logarithmic plots of the ΔA_{830} dark-decay kinetics (*1*, *3*) and deconvoluted fast component (*2*, *4*) for non-heated leaves (*1*, *2*) or leaves exposed for 5 min to 42 °C (*3*, *4*). *Upward arrows* FR light on, *downward arrows* FR light off. *Inset*: initial part of fast relaxing component with demonstration of the approach for determination of the delay in its development

Figure 1B shows semi-logarithmic plots of the kinetic curves of $P700^+$ dark reduction presented on Fig. 1A. Two components were clearly resolved in the kinetics. The slow one decayed exponentially with half-times of 3,200 and 165 ms in non-heated leaves and in leaves exposed for 5 min to 42 °C, respectively. The relative magnitudes of the components were barely affected by heat treatment: the slow component accounted for 35 and 29% of the total amplitude before and after heat treatment, respectively. The fast component also decayed exponentially, but only after a marked lag phase (inset to Fig. 1B). Thus, the fast component of $P700^+$ reduction in the dark was further characterized using two parameters. The first parameter is the half-time of the exponentially decaying part of that component, the values for which were found to be 610 and 26 ms for non-heated and heat-exposed leaves, respectively. The second parameter is the time gap determined at the level of maximum magnitude of the deconvoluted fast component between the end of FR illumination (zero point on *x*-axis) and the point at which the linear fit calculated for the exponentially proceeding part of that component crosses the level of maximum magnitude of the fast component (see inset to Fig. 1B). The latter parameter, which is denoted as the delay, characterizes the developing part of the fast component. Values of 110 and 25 ms were obtained for the delay in non-heated and heat-stressed leaves, respectively.

Figure 2 clarifies the conditions that brought about the delay in the development of the fast component. On that figure, semilogarithmic plots of the kinetics of $P700^+$ reduction in the dark are presented for leaves exposed for 5 min to 45 °C and then irradiated by multiple-turnover pulses of various durations. The pulse duration did not influence the rate of the slow component of $P700^+$ reduction (Fig. 2A). The maximum rates of the fast component, determined as the slopes of linear fits, were not sensitive to the duration of the pulse either (Fig. 2B). When P700 was oxidized by 5- or 10-ms pulses, its dark reduction was strictly exponential, with a half-time of 12–13 ms. However, as the pulse became more prolonged, the fast component started to develop only after some lag phase, i.e. the delay appeared.

From the half-times of the exponential decays, it is clear that heat treatment of leaves accelerated the input of electrons to the plastoquinone pool from stromal reductants. Importantly, the extent of acceleration was similar for the fast and the slow components. Further, heat treatment did not influence the relative magnitudes of the two kinetic components. In both cases the slow components accounted for about one-third of the total ΔA_{830} signal.

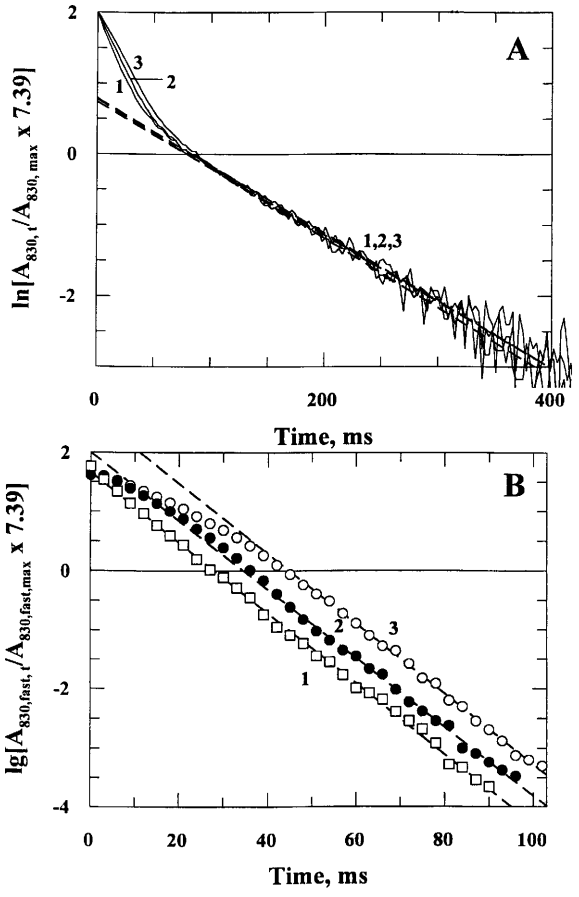

Fig. 2. Semilogarithmic plots of the dark decay of the increase in absorbance at 830 nm (**A**) and the deconvoluted fast component of the dark decay (**B**) in barley leaves exposed for 5 min to 45 °C and irradiated by 5 (*1*), 30 (*2*), or 50-ms (*3*) multiple-turnover pulses of FR light

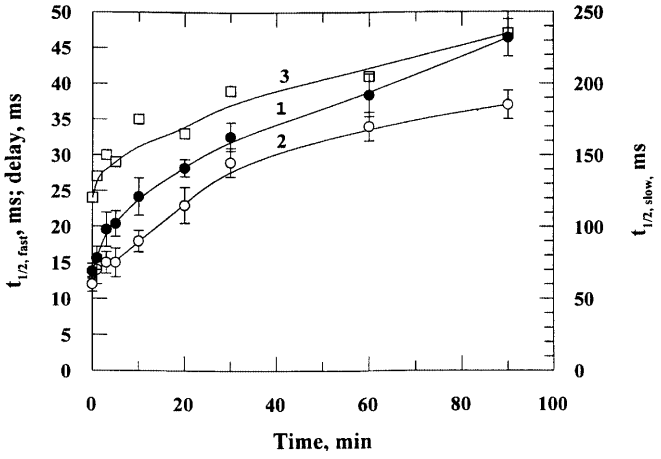

Fig. 3. Half-times of the slow (*1*) and fast (*2*) components of ΔA_{830} dark-decay kinetics and the delay in the development of the fast component (*3*) measured after given periods of time following a 5-min exposure of barley leaves to 45 °C. Absorbance changes at 830 nm were induced by 1-s pulses of FR light

The heat-induced acceleration of electron flow to PSI from stromal reductants was a persistent, but not irreversible phenomenon. Figure 3 demonstrates that the half-times of both components of $P700^+$ reduction gradually increased with time following heat treatment. However, they still greatly exceeded the corresponding values obtained in untreated leaves even 1.5 h after exposure to elevated temperature.

Modulation of the kinetics of $P700^+$ reduction by light–dark cycles

Despite the fact that the enhanced flow of electrons to $P700^+$ supported by stromal reductants persisted for a long time after heat treatment, its rate could easily be modulated by the irradiation and following dark adaptation of heat-exposed leaves. Figure 4 shows the semilogarithmic plots of the kinetics of $P700^+$ reduction measured in leaves preheated for 5 min at 45 °C and irradiated for various durations with FR light. Both fast and slow components of the kinetics slowed down with increasing time of leaf irradiation. As shown in Fig. 5A,

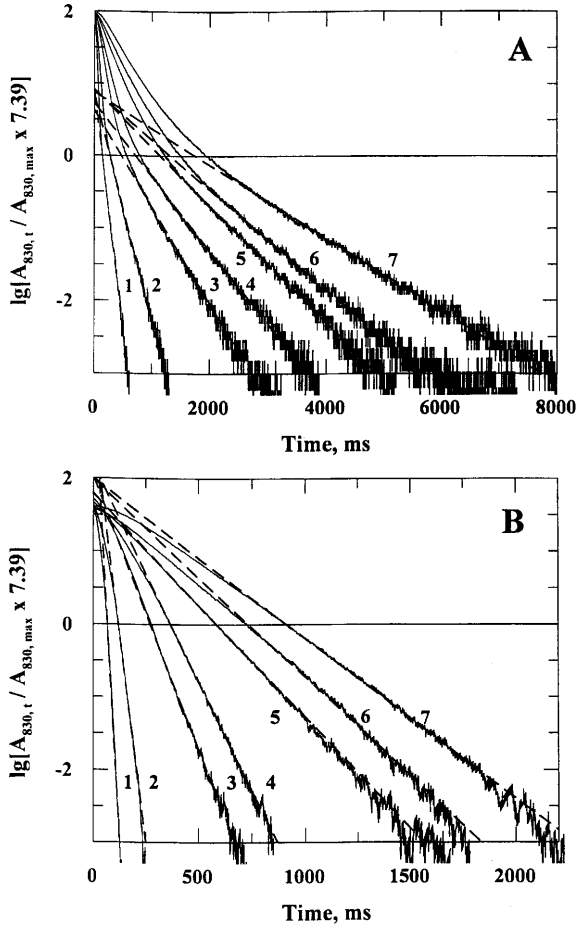

Fig. 4. Semilogarithmic plots of the dark decay of ΔA_{830} (**A**) and the deconvoluted fast component of the dark decay (**B**) in barley leaves exposed for 5 min to 45 °C and then irradiated with FR light for 1 (*1*), 3 (*2*), 5 (*3*), 10 (*4*), 15 (*5*), 60 (*6*), or 120 s (*7*). *Dashed lines* The linear fits calculated for corresponding kinetic components

the half-times of both kinetic components of $P700^+$ dark reduction increased by at least a factor of 10 after a 20-s exposure of heat-stressed leaves to FR light. Further irradiation caused a slow additional increase to values similar to those of non-heated leaves. The delay times increased in parallel with the rise in half-times of the two kinetic components (Fig. 5A).

The light-induced decrease in the rate of $P700^+$ reduction represented a reversible phenomenon. We examined the dark recovery of light-induced modulation of electron input to PSI using the following approach. Dark-adapted leaves treated for 5 min at 45 °C were irradiated for 1 min by FR light, and then a 1-s pulse of FR light was given after various times of darkness. The kinetics of $P700^+$ reduction after that pulse was analyzed to provide the magnitude and half-time of the components together with the delay time. Figure 5B shows the half-time of the slow and fast components of the reduction of $P700^+$ oxidized by the 1-s pulse of FR light as a function of the dark time period preceding that pulse. Relatively short (10–20 s) dark adaptation was already sufficient to restore the input of electrons from stromal reductants to $P700^+$ that was suppressed by the previous 1-min illumination. The responses were similar for both kinetic components. The exposure to FR light and the following dark adaptation did not influence the relative magnitudes of the fast and slow components of $P700^+$ reduction despite the fact that their half-times varied dramatically. Importantly, the modulation in the rate of $P700^+$ reduction by light–dark cycles could be initiated repeatedly manifesting the reversible character of the response (data not shown).

Figure 6 demonstrates that the stimulation of electron input to PSI from stromal reductants developed rapidly during heat treatment of leaves. Both fast and slow components reached their maximum rates after 2 min exposure to 45 °C.

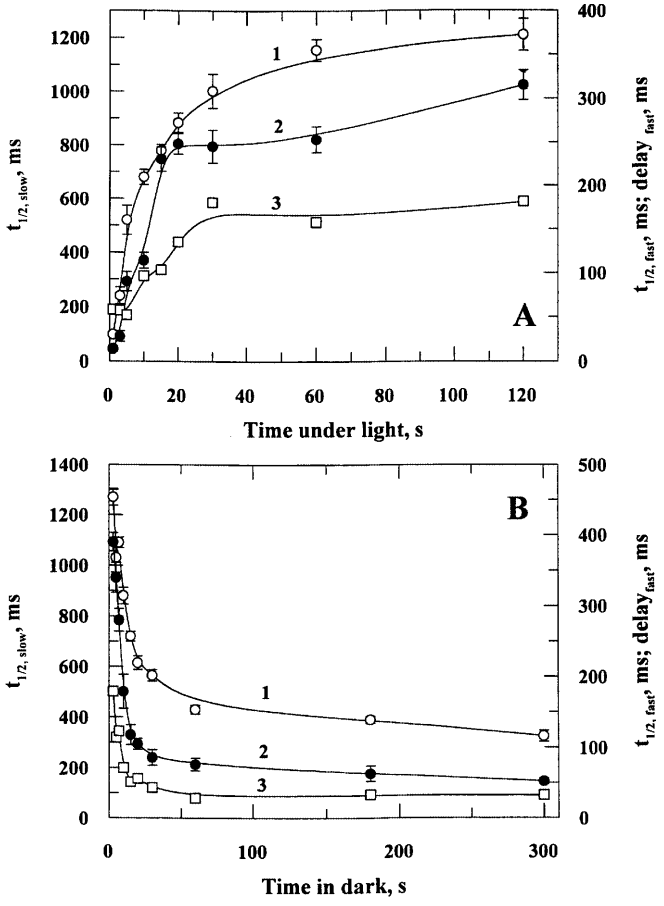

Fig. 5. Half-times of slow (*1*) and fast (*2*) components of ΔA_{830} dark-decay kinetics, and the values for the delay in development of the fast components (*3*) as a function of either time of irradiation of dark-adapted barley leaves with FR light (**A**) or of time of dark adaptation following irradiation for 1 min with FR light (**B**). Prior to the measurements, leaves were exposed for 5 min to 45 °C

The effect of various elevated temperatures on the kinetics of $P700^+$ dark reduction

The most plausible explanation for the light-dependent decrease in the rates of $P700^+$ reduction is that irradiation rapidly depleted the chloroplast stroma of reductants owing to electron transfer through PSI to oxygen. The action of MV was important for that response. First, it greatly enhanced the affinity of the acceptor side of PSI to oxygen. Second, being able to intercept electrons with high efficiency on the PSI acceptor side before soluble ferredoxin (Fujii et al. 1990), MV prevented both the light-induced regeneration of NADPH and electron cyclization around PSI. We examined the effect of various elevated temperatures on the activities of both routes of electron donation to $P700^+$ using two different modes of irradiation. One was the application of 1-s FR light pulses, which were not expected to severely exhaust the pool of stromal reductants. The second type of light exposure, during which the leaves were irradiated for 1 min with FR light and then the kinetics of $P700^+$ reduction was measured and analyzed, allowed the electron input to PSI to be

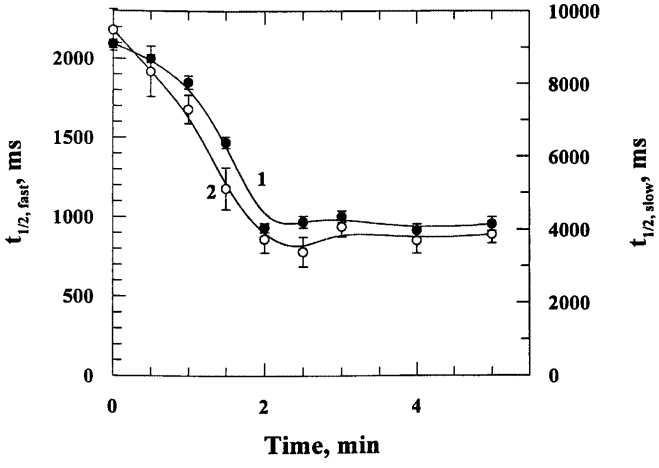

Fig. 6. Half-times of slow (*1*) and fast (*2*) components of the kinetics of ΔA_{830} decay measured after irradiation for 60 s with FR light, as a function of time of barley leaf exposure to 45 °C

examined under conditions in which the chloroplast stroma was depleted of reductants.

Figure 7 shows the original traces of $P700^+$ reduction after 1 s (upper set of traces) or 1 min (lower set of traces) irradiation of leaves exposed for 5 min to various elevated temperatures. It should be noted that the final amplitudes of absorbance changes are similar, 0.011 and 0.0105, after irradiation for 1 and 60 s, respectively. Even when the pool is largely depleted by a longer pre-illumination, the remaining reductants are still capable of fully reducing P700 in the dark but at a much slower rate. Thus the final amplitude of P700 dark reduction is not influenced by the size of the pool. The increase in the temperature of heat pre-treatment up to 46 °C caused the gradual acceleration of the dark reduction of $P700^+$ irrespective of which light exposure was used for P700 photooxidation. However, this effect of heat treatment was much more pronounced when a 1-s pulse had oxidized P700. Figure 8 provides quantitative evidence for that statement. A dramatic difference was found in the temperature profiles of half-times of the slow component of $P700^+$ reduction measured after the two different conditions of irradiation with FR light. Temperatures from 38 °C were able to greatly accelerate the slow component of the dark reduction of $P700^+$ oxidized by a 1-s pulse but were, however, absolutely ineffective if the reduction of $P700^+$ was measured after a 1-min light exposure. In the latter case, the stimulation of electron input to PSI was observed only if the temperature of heat-pretreatment was increased above 40 °C. A qualitatively similar difference was found between the temperature profiles obtained for the fast component of $P700^+$ reduction with 1-s or 1-min irradiation.

Long dark adaptation of leaves decreased the rate of electron donation to PSI

The rate of electron flow from stromal reductants to $P700^+$ decreased under illumination, but was rapidly restored in the dark. The recovery of its high capacity was evidently caused by the filling up of the chloroplast stroma with reductants. In this respect, the nature of the reservoir supporting the dark regeneration of the pool of stromal reductants is of a great interest. We compared the electron donation to PSI in barley leaves that were stored in the dark for either 1 or 24 h and then subjected to heat. Figure 9A shows that those leaves demonstrated similar rates of slow and fast components of $P700^+$ reduction after irradiation for 1 min with FR light. In large contrast, much lower rates of both components were found in leaves dark-adapted for 24 h compared to ones stored in darkness for only 1 h if a 1-s pulse of FR light was applied (Fig. 9B). Nevertheless, they

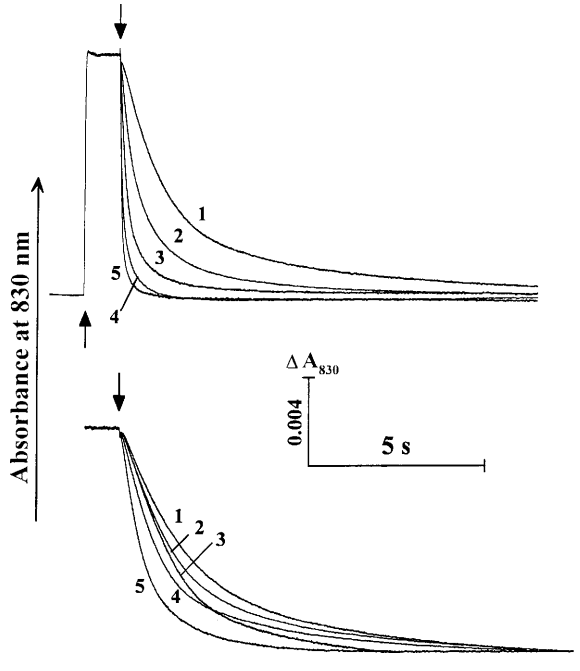

Fig. 7. Original traces of ΔA_{830} induced by a 1-s pulse of FR light (**A**) and kinetics of the dark decay of ΔA_{830} after irradiation for 1 min with FR light (**B**). Barley leaves were either not exposed to heat (*1*) or were exposed for 5 min at 36 (*2*), 38 (*3*), 40 (*4*), or 44 °C (*5*). *Upward arrows* FR light on, *downward arrows* FR light off

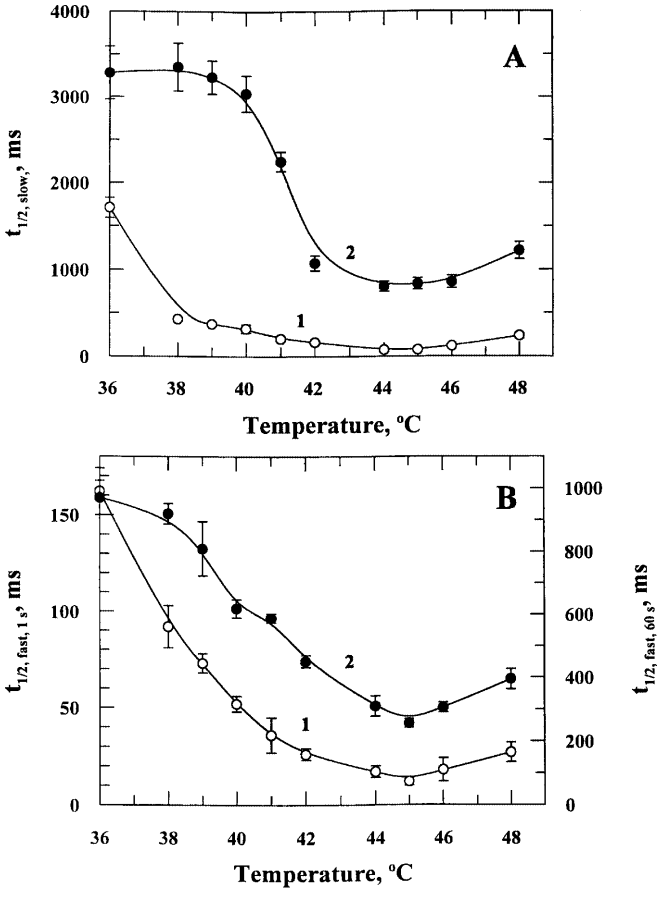

Fig. 8. Half-times of slow (**A**) and fast (**B**) components of the kinetics of ΔA_{830} decay, measured in previously dark-adapted barley leaves after irradiation for either 1 s (*1*) or 60 s (*2*) with FR light, as a function of temperature in leaves exposed for 5 min to the various stated temperatures

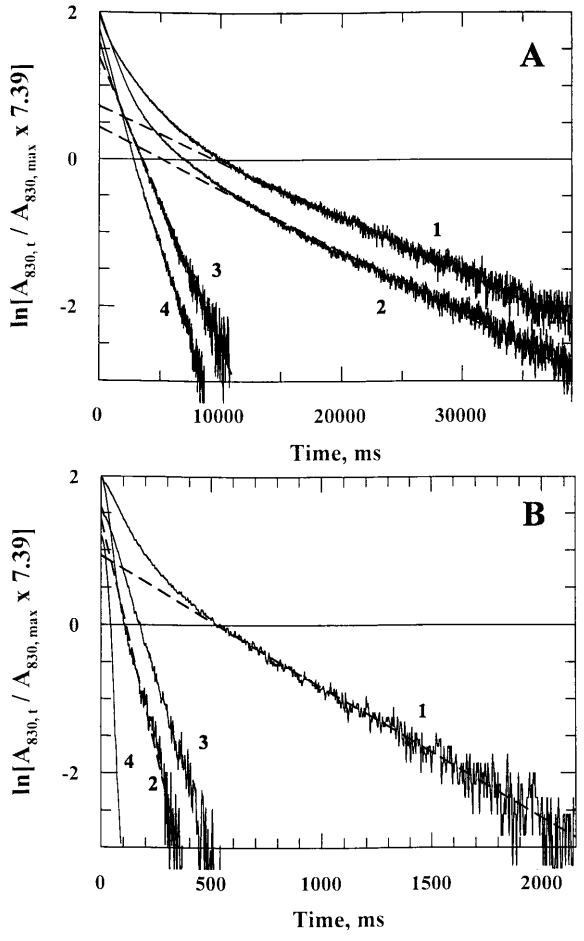

Fig. 9. Semilogarithmic plots of ΔA_{830} dark-decay kinetics (*1*, *2*) and their deconvoluted fast components (*3*, *4*), measured after irradiation for 60 s (**A**) or 1 s (**B**) with FR light in barley leaves stored in the dark for 24 h (*1*, *3*) or for 1 h (*2*, *4*)

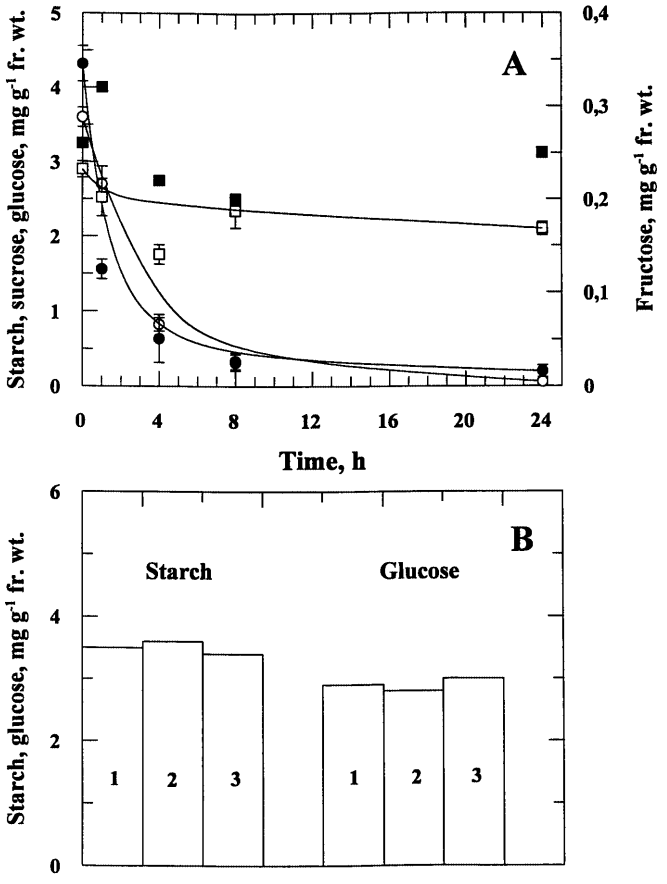

Fig. 10. **A** Changes in the contents of starch (*open circles*), sucrose (*filled circles*), glucose (*open squares*) or fructose (*filled squares*) during dark adaptation of barley leaves that were previously grown under continuous white light of 200 μmol photons m^{-2} s^{-1}. **B** Contents of starch and glucose before (*1*), and 5 min (*2*) or 30 min (*3*) after a 5-min leaf treatment at 45 °C

substantially exceeded the rates for corresponding components observed in 24-h dark-adapted leaves after 1 min irradiation.

The prolonged dark adaptation of leaves was accompanied by a gradual decline in the contents of leaf starch and sucrose, and the leaves were nearly completely depleted of those substances after 24 h storage in the dark (Fig. 10A). In contrast, only a small decrease in the amount of glucose and fructose was observed after prolonged dark adaptation of leaves (Fig. 10A).

A 5-min exposure of leaves to 45 °C did not initiate measurable acceleration of starch breakdown or glucose accumulation (Fig. 10B). The absence of an effect of high-temperature treatment on the amount of the above substances in leaf tissue was clear both 5 min and 30 min after leaves had been subjected to 45 °C.

Discussion

This study represents a continuation of our previous work on the electron donation to PSI from stromal reductants done largely using photoacoustic techniques. Our previous reports demonstrated the ability of that alternative route to support a relatively high turnover of PSI centers under steady-state illumination in heat-treated leaves (Bukhov et al. 2000). The especially high capacity of stromal reductants to donate electrons to PSI was observed during the first few seconds of irradiation of dark-adapted leaves (Bukhov et al. 2001a). Direct monitoring of the redox state of P700 by the absorbance changes at 830 nm allowed further insight into the mechanism of those processes. Hence, two kinetic components of $P700^+$ reduction, related to slowly and rapidly reducing PSI units, were distinguished under conditions that permitted PSI to receive electrons only from stromal reductants, i.e. in leaves treated with diuron and MV (Bukhov et al. 2001b).

Heat pretreatment dramatically stimulated electron flow supported by stromal reductants through both slowly and rapidly reducing PSI units. The stimulation was already completed after 2 min of heat treatment (see Fig. 6). This finding indicates the modification of pre-existing cell structures with no influence of polypeptides newly synthesized under elevated temperatures, such as

the so-called heat shock proteins (Vierling 1991). At the same time, those modifications do not involve some irreversible denaturation of chloroplast structures, as the heat-increased rates of both components of $P700^+$ dark decay were gradually returning to normal at the end of the thermal treatment (see Fig. 3). Importantly, the time courses of both the stimulation during heat treatment and the relaxation of the increased rates following termination of heat exposure occurred simultaneously in rapidly and slowly reducing PSI centers. The above suggests some similarities in the properties of the two types of PSI unit.

The data presented in Fig. 2 further evidence the availability of stromal reductants to keep the rate of electron donation to PSI under control. The reduction of $P700^+$ oxidized by 5- or 10-ms multiple-turnover pulses proceeded exponentially with a half-time of 12–13 ms, which corresponds to the characteristic time of in vivo electron transfer between PSII and PSI (Harbinson and Hedley 1989; Laisk and Oja 1994). The limiting step of that transfer is the oxidation of plastohydroquinone by the cytochrome b_6/f complex (Witt 1971). Thus, after 5- or 10-ms pulses, the plastoquinone pool was still filled with reducing equivalents and capable of transporting them to the cytochrome b_6/f complex to finally reduce $P700^+$ (see Fig. 2, curve 1). Obviously, an increase in duration of the multiple-turnover pulses had to stimulate the oxidation of plastoquinone pool. The same maximum rate of donation was observed, however, after multiple-turnover pulses as long as 30 or 50 ms (see Fig. 2, curves 2 and 3). Thus, even after long multiple-turnover pulses, the diffusion of reduced plastoquinone towards the cytochrome b_6/f complex, rather than electron donation to plastoquinone from stromal reductants, restricted the maximum rate of $P700^+$ reduction.

The important observation was made that $P700^+$ reduction did not proceed gradually, but developed some time after application of a relatively long multiple-turnover pulse (see Fig. 2). A delay was found prior to the kinetics of $P700^+$ reduction becoming exponential. Thus, during that period the input of reducing equivalents from stromal reductants was unable to operate at rates similar to maximum rates of electron transfer between PSII and PSI. The delay evidently reflected the movement of reductants in the stroma towards the enzymic entries into to the electron transport chain at the level of the plastoquinone pool. The delay observed in the development of the fast component of $P700^+$ reduction became longer as the half-time of that component increased (see Figs. 3 and 5). Likely, the variations in the half-time of the fast component and the delay in its development are related processes. Indeed, after long irradiation of heat-treated leaves, the half-time of the fast component exceeded by severalfold that observed after short multiple-turnover pulses. Obviously, under conditions of an exhausted pool of stromal reductants, the reduction of plastoquinone, but not their diffusion to the cytochrome b_6/f complex, becomes a limiting step in the electron transport to PSI. The low availability of reductants must both restrict the maximum rate of the fast component and slow down its development after termination of light.

The molecular entities of the enzymes that mediate the increased input of electrons to PSI at elevated temperatures are still unclear. Indeed, Sazanov et al. (1998b) reported that, unlike in untreated leaves, no difference in the kinetics of dark $P700^+$ reduction was observed between leaves of wild-type tobacco plants and those of a mutant lacking the Ndh complex if leaves were exposed to high temperatures. In untreated leaves, wild types demonstrated a higher capacity to reduce $P700^+$ than the mutant. This questioned whether the same enzymes that mediate the input of electrons from stromal reductants to plastoquinone in non-heated leaves operate in heat-treated leaves as well (Sazanov et al. 1998b). Our data provide several lines of evidence that the same entries for reducing equivalents derived from the stromal substances are functioning in untreated leaves and in leaves that have experienced heat. First, heat treatments barely altered the relative magnitudes of the two components of $P700^+$ dark reduction (Fig. 1B) thus indicating an unchanged ratio of the two types of PSI unit. Second, the rates of the two above components varied synchronously during either dark-to-light or light-to-dark transitions (see Fig. 5), or in the course of heat treatment (see Fig. 6). Third, the temperature profiles for the rates of the slow and the fast components of P700 dark reduction were found to be qualitatively similar (see Fig. 8).

Figure 5A shows that the pool of stromal reductants was exhausted under light and rapidly regenerated following dark adaptation of heat-treated leaves. This coincides well with our previous data obtained by photacoustic methods (Bukhov et al. 2001a). To be continuously regenerating, the pool of stromal reductants obviously requires some metabolic reservoir filled with storage substances. In principal, two compartments can support such regeneration, the chloroplast stroma itself by starch breakdown and the cytosol by exporting the reducing equivalents to chloroplasts through the malate–oxaloacetate shuttle (Krömer 1995). Several authors (Field et al. 1998; Sazanov et al. 1998b), based on data on variations of the initial (F_o) level of chlorophyll fluorescence in the dark, concluded that the hydrolysis of starch may supply the pool of stromal reductants. We re-examined that problem using the kinetics of $P700^+$ dark reduction, which was reported to provide results contrary to those obtained by chlorophyll fluorescence (Sazanov et al. 1998b). Even after 24 h dark adaptation, which was accompanied by a near-complete breakdown of leaf starch and sucrose (see Fig. 10), heat-treated leaves demonstrated increased rates of the two components of $P700^+$ dark reduction compared to non-heated leaves (see Fig. 9). The absolute rates of both kinetic components were, however, found to be severalfold smaller in leaves stored in darkness for 24 h than in leaves that had experienced only short dark adaptation (Fig. 9). This finding provides evidence that the reduced ability of the pool of

stromal reductants to support the electron flow to PSI in 24-h dark-adapted leaves is likely caused by a slowdown in its regeneration. This occurred despite the fact that the amount of glucose, the respiratory substrate, was maintained in such leaves at a high level similar to that in leaves stored in the dark for 1 h only (Fig. 10). We can conclude, therefore, that starch breakdown provides NADH for fast regeneration of the pool of stromal reductants, while the export of reducing equivalents from the cytosol is at least much less efficient in this respect.

In summary, the data reported here provide evidence that the rates of both fast and slow pathways of electron donation from stromal reductants to PSI are under the control of a pool of reductants. Under prolonged light, the regeneration of that pool restricts the rate of electron flow through PSI. The regeneration of that pool is supported mostly, if not exclusively, by starch breakdown. The rates of the two above pathways demonstrated similarity in the responses to various elevated temperatures and light–dark cycles. That similarity may indicate that the two kinetically distinct pathways are mediated by an enzyme of the same nature. The existence of the two kinetic components could be due to an unspecific response of one enzyme donating electrons to the plastoquinone pool from two different stromal reductants such as NADPH and NADH. This could be related to different affinities of the enzyme for NADPH and NADH. However, this suggestion seems unlikely because the two components were also resolved after a short irradiation when the pool(s) of stromal reductants had to be large. A better hypothesis is that the enzyme would be located in different regions of the chloroplast membrane system that differ in accessibility to stromal reductants (Albertsson et al. 1990). The rapidly operating pathway could be driven by enzymes located in the stroma lamellae, whereas enzymes mediating the slow pathway are probably inserted into the appressed thylakoid regions where the penetration of soluble substances from the stroma would be impeded.

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References

Albertsson PA, Andreasson E, Svensson P (1990) The domain organization of the plant thylakoid membrane. FEBS Lett 273:36–40

Baker NR (1991) A possible role for photosystem II in environmental perturbations of photosynthesis. Physiol Plant 81:563–570

Bennoun P (1994) Chlororespiration revisited: mitochondrial–plastid interactions in *Chlamydomonas*. Biochim Biophys Acta 1186:59–66

Bergmeyer HU (1974) Methods of enzymic analysis, vol 2. Verlag Chemie, Weinheim

Bukhov NG, Samson G, Carpentier R (2000) Nonphotosynthetic reduction of the intersystem electron transport chain of chloroplasts following heat stress. Steady-state rate. Photochem Photobiol 72:351–357

Bukhov NG, Samson G, Carpentier R (2001a) Nonphotosynthetic reduction of the intersystem electron transport chain of chloroplasts following heat stress. The pool size of stromal reductants. Photochem Photobiol 74:438–443

Bukhov NG, Carpentier R, Samson G (2001b) Heterogeneity of photosystem I reaction centers in barley leaves as related to the donation from stromal reductants. Photosynth Res 70:273–279

Corneille S, Cournac L, Guedeney G, Havaux M, Peltier G (1998) Reduction of the plastoquinone pool by exogenous NADH and NADPH in higher plant chloroplasts Characterization of a NAD(P)H-plastoquinone oxidoreductase activity. Biochim Biophys Acta 1363: 59–69

Cournac L, Josse E-M, Joët T, Rumeau D, Redding K, Kuntz M, Peltier J (2000) Flexibility in photosynthetic electron transport: a newly identified chloroplast oxidase involved in chlororespiration. Phil Trans R Soc Lond B 355:1447–1454

Endo T, Schreiber U, Asada, K (1995) Suppression of quantum yield of photosystem II by hyperosmotic stress in *Chlamydomonas reinhardtii*. Plant Cell Physiol 36:1253–1258

Farquhar GD, von Cammerer S, Berry JA (1980) A biochemical model of photosynthetic CO_2 fixation in leaves of C_3 species. Planta 149:78–90

Field TS, Nedbal L, Ort DR (1998) Non-photochemical reduction of the plastoquinone pool in sunflower leaves originated from chlororespiration. Plant Physiol 116:1209–1218

Fujii T, Yokoyama E, Inoue K, Sakurai H (1990) The sites of electron donation of photosystem I to methyl viologen. Biochim Biophys Acta 1015:41–48

Guedeney G, Corneille S, Cuine S, Peltier G (1996) Evidence for an association of *ndh* B, *ndh* J gene products and ferredoxin-NADP-reductase as components of a chloroplastic NAD(P)H dehydrogenase complex. FEBS Lett 378:277–280

Harbinson J, Hedley CL (1989) The kinetic of P_{700} reduction in leaves: a novel in situ probe of thylakoid functioning. Plant Cell Environ 12:357–369

Havaux M (1996) Short-term responses of photosystem I to heat stress. Induction of a PS II-independent electron transport through PS I fed by stromal components. Photosynth Res 47:85–97

Heber U, Walker DA (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol 100:1621–1626

Horton P, Ruban AV, Walters RG (1996) Regulation of light harvesting in green plants. Annu Rev Plant Physiol Planat Mol Biol 47:655–884

Krömer S (1995) Respiration during photosynthesis. Annu Rev Plant Physiol Plant Mol Biol 46:45–70

Laisk A, Oja V (1994) Range of photosynthetic control of post illumination $P700^+$ reduction side in sunflower leaves. Photosynth Res 39:39–50

Sazanov LA, Burrows PA, Nixon PJ (1998a) The plastid *ndh* genes code for an NADH-specific dehydrogenase: isolation of a complex I analogue from pea thylakoid membranes. Proc Natl Acad Sci USA 95:1319–1324

Sazanov LA, Burrows P, Nixon P (1998b) The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. FEBS Lett 429:115–118

Schreiber U, Klughammer C, Neubauer C (1988) Measuring P700 absorbance changes around 830 nm with a new type of pulse modulated system. Z Naturforsch C 43:686–698

Tagawa K, Tsujimoto HY, Arnon DI (1963) Role of chloroplast ferredoxin in the energy conversion process of photosynthesis. Proc Natl Acad Sci USA 49:567–572

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:549–562

Witt HT (1971) Coupling of quanta, electrons, fields, ions and phosphorylation in the functional membrane of photosynthesis. Results by pulse spectrophotometric methods. Quart Rev Biophys 4:365–477