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The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves

Leonid A. Sazanov[1], Paul A. Burrows[2], Peter J. Nixon*

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, UK

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Abstract We have examined the effects of heat stress on electron transfer in the thylakoid membrane of an engineered plastid *ndh* deletion mutant, Δ1, incapable of performing the Ndh-mediated reduction of the plastoquinone pool in the chloroplast. Upon heat stress in the dark, the rate of PSII-independent reduction of PSI after subsequent illumination by far-red light is dramatically enhanced in both Δ1 and a wild-type control plant (WT). In contrast, in the dark, only the WT shows an increase in the reduction state of the plastoquinone pool. We conclude that the heat stress-induced reduction of the intersystem electron transport chain can be mediated by Ndh-independent pathways in the light but that in the dark the dominant pathway for reduction of the plastoquinone pool is catalysed by the Ndh complex. Our results therefore demonstrate a functional role for the Ndh complex in the dark.

Key words: *ndh*; Chloroplast; Plastoquinone; Chlororespiration; Heat stress; Cyclic electron flow

1. Introduction

The plastid genome of several plants contain *ndh* genes whose products show strong sequence similarites to subunits of complex I from mitochondria and eubacteria [1,2]. Recently we have provided biochemical evidence to show that the Ndh proteins form a large protein complex of low abundance within the thylakoid membrane [3,4]. From the analysis of tobacco mutants containing disrupted plastid *ndh* genes we have also shown that the Ndh complex mediates the post-illumination reduction of the plastoquinone pool and so acts as a novel respiratory complex within the thylakoid membrane [4].

The physiological function of the plastid Ndh complex is unknown although it may contribute to cyclic electron transfer around the photosystem I (PSI) complex in the light [2,4,5] and is possibly involved in a respiratory electron transfer chain in the dark [6]. Although an intact Ndh complex is dispensible for growth of tobacco under 'unstressed' growth conditions it is possible that the activity of the Ndh complex becomes more important under stress conditions that require enhanced ATP synthesis possibly driven by cyclic electron flow around PSI [5]. In this paper we investigate whether the Ndh complex plays a role in the response of plants to heat stress, a treatment that induces significant changes to electron flow within the chloroplast [7,8].

2. Materials and methods

2.1. Tobacco strains and growth conditions

The Δ1 mutant which lacks part of the plastid *ndhC* and *ndhK* genes was obtained by plastid transformation of *Nicotiana tabacum* cv. Petit Havana using a spectinomycin-resistance cassette as a selectable marker [4]. The WT control plant used in these studies contains the same spectinomycin-resistance cassette inserted into the *rbcL-accD* intergenic region of the chloroplast genome and its construction is described in [9]. Mature tobacco leaves from 10–12 week old plants grown in the greenhouse at 24°C on peat compost were used.

2.2. Measurements of chlorophyll fluorescence and re-reduction of $P700^+$

Leaves of similar size were detached from WT and Δ1 plants at about 1 pm and dark adapted for 1 h (or for 4 h where indicated). 10-cm^2 disks were cut out from the leaves and subjected to heat stress in the dark at 50°C for 10 min by placing the disks on a wet filter paper heated by temperature-controlled metal plate. Fluorescence induction curves and F_o levels were measured on the disks prior to heat stress and 10 min after heat stress, after allowing samples to adapt to room temperature in the dark. Only in Fig. 3A were measurements done immediately after stress to show the kinetics of the fluorescence changes. Chlorophyll fluorescence transients were measured according to [10] using a PAM chlorophyll fluorometer (Walz, Effeltrich, Germany) during illumination by white actinic light at 400 $\mu E\ m^{-2}\ s^{-1}$. Maximal fluorescence was determined using flashes of saturating white light (1000 $\mu E\ m^{-2}\ s^{-1}$, 400 ms). The rate of re-reduction of $P700^+$ in leaves was followed by measuring absorbance changes at 803 nm using an ED 800 T unit attached to the PAM fluorometer [11] after 2 min illumination by far-red light (5 W m^{-2}), provided by a 730-nm interference filter, to oxidise P700. Experiments were repeated 3–5 times to obtain mean ± S.D. values.

3. Results

3.1. Effect of heat stress on the rate of re-reduction of $P700^+$ after far-red illumination in the ndh mutant Δ1

Previous work has shown that mild heat stress of pea [7] and potato [8] leaves induces a dramatic increase in the rate of re-reduction of $P700^+$ in the dark after a period of far-red illumination. This type of assay gives a measure of the rate of PSII-independent reduction of PSI and is comprised of both cyclic electron transfer around PSI and electron donation to the intersystem electron transfer chain by stromal reductant [8,12]. Fig. 1 shows that a similar phenomenon occurs in WT tobacco leaves. Upon 10 min of heat stress at 50°C the rate of re-reduction of $P700^+$ is enhanced about 7-fold compared with unstressed leaves. For the *ndh* mutant, Δ1, under un-

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*Corresponding author. Fax: (44) (171) 594-5267.
E-mail: p.nixon@ic.ac.uk

[1] Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK.

[2] Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

Abbreviations: F_o, minimum level of chlorophyll fluorescence when PSII centres are open; F_m, maximum level of chlorophyll fluorescence when PSII centres are closed

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stressed conditions the rate of re-reduction of P700$^+$ was about half that of the WT ($t_{1/2}$ values of 1.27 ± 0.26 s (WT) and 2.48 ± 0.65 s (Δ1)) but upon heat stress a similar rate to the WT was achieved ($t_{1/2}$ values of 0.17 ± 0.04 s (WT) and 0.23 ± 0.06 s (Δ1)).

3.2. Chlorophyll fluorescence induction characteristics

Chlorophyll fluorescence induction experiments conducted in the presence of white light, shown in Fig. 2, are also consistent with enhanced donation of electrons to the intersystem electron transport chain in both WT and Δ1 upon heat stress. In such experiments a weak measuring beam of modulated red light is used to determine the level of chlorophyll fluorescence [10]. Before heat stress, actinic illumination produces a characteristic fluorescence induction profile. Initially the dark level of fluorescence is low (F_o) because the PSII centres are open. Upon illumination fluorescence rises rapidly to a maximum value (F_m), when all PSII reaction centres are closed, and is then quenched by photochemical and non-photochemical processes until a steady state level is achieved [13]. Once the actinic light is switched off, the WT but not the Δ1 mutant shows a transient increase in the 'dark' level of fluorescence (or 'apparent' F_o) which is indicative of Ndh-mediated reduction of the plastoquinone pool by stromal reductant [4]. By superimposing saturating flashes of light, the contribution of photochemical (qP), roughly proportional to the value of the spikes in Fig. 2, and non-photochemical quenching (qN) to the overall quenching can be estimated [14]. The major component of qN is qE which is generated by the pH gradient across the thylakoid membrane [13].

After heat stress, a number of differences in the fluorescence induction profile can be seen (Fig. 2). First the F_o value measured at the start of the experiment increases dramatically, more so in the WT than Δ1. Second, the rate of fluorescence quenching increases and is mainly non-photochemical. Third, after the actinic light is turned off a transient increase in the 'dark' fluorescence is now observed in Δ1 as well as the WT. The latter two observations are consistent with the heat stress-induced increase in the rates of cyclic electron transport and concomitant proton pumping in both WT and Δ1, leading to enhanced qE.

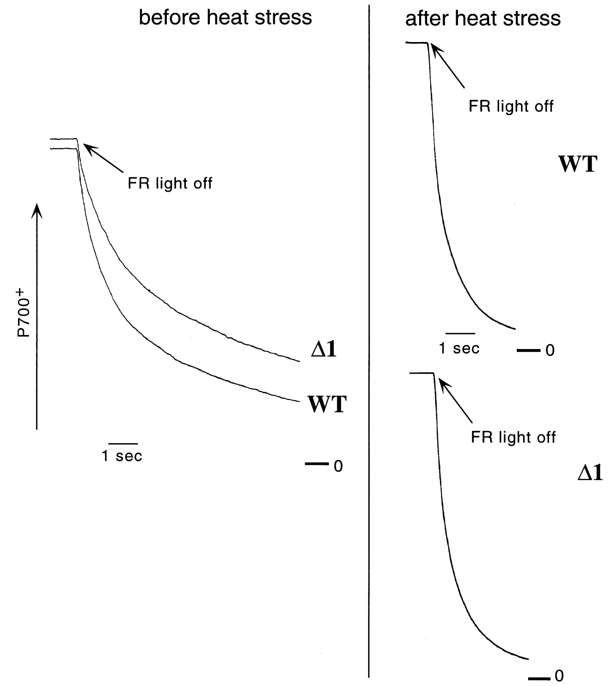

Fig. 1. Kinetics of the dark re-reduction of P700$^+$ in leaves of Δ1 and WT either before (left-hand panel) or after (right-hand panel) heat stress. Oxidation of P700 was mediated by far-red light (FR). 0 indicates reduction state of P700 prior to illumination.

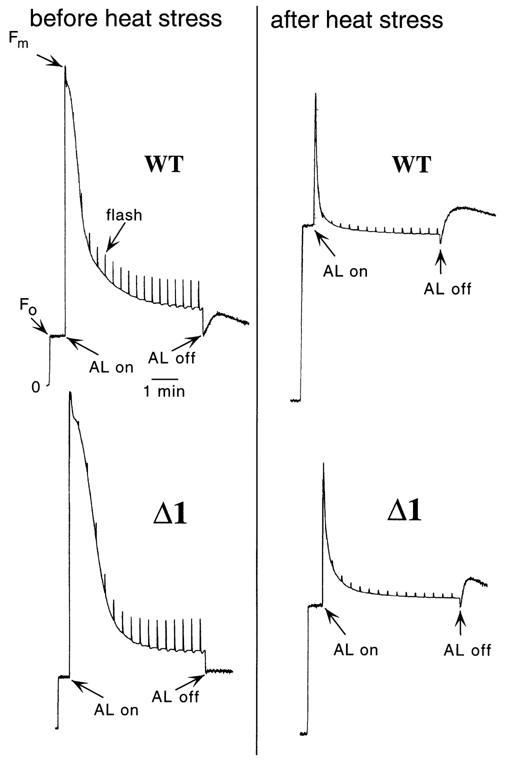

Fig. 2. Fluorescence induction analysis of dark-adapted leaves of WT or Δ1 either before (left-hand panel) or after (right-hand panel) heat stress. Flashes of saturating white were used to determine degree of non-photochemical quenching (see text). AL, actinic light; F_o, dark fluorescence value; F_m, maximum fluorescence value.

From Figs. 1 and 2 we can conclude that without stress the 'dormant' capacity for cyclic transport is low in the WT and even lower in the Δ1 mutant. However, after heat stress in the dark the capacity for cyclic transport under subsequent far-red or actinic light increases many-fold to similar levels in both the WT and the Δ1 mutant.

3.3. Reduction state of the plastoquinone pool in the dark

Fig. 3 shows that after heat stress the 'dark' F_o level is significantly higher in the WT as compared with the Δ1 mutant (Fig. 3A), although F_o increased in both plants. Previous work has suggested that this increase in F_o results from a combination of the detachment of LHC from PSII and an enhanced reduction state of the plastoquinone pool which leads to closure of PSII centres by back electron flow [8]. Reduction of the plastoquinone pool can be conveniently monitored by the rapid quenching of fluorescence by far-red light which promotes PSI-induced oxidation of the photosynthetic electron transport chain. Only in the WT could the increased fluorescence be partially quenched by far-red light

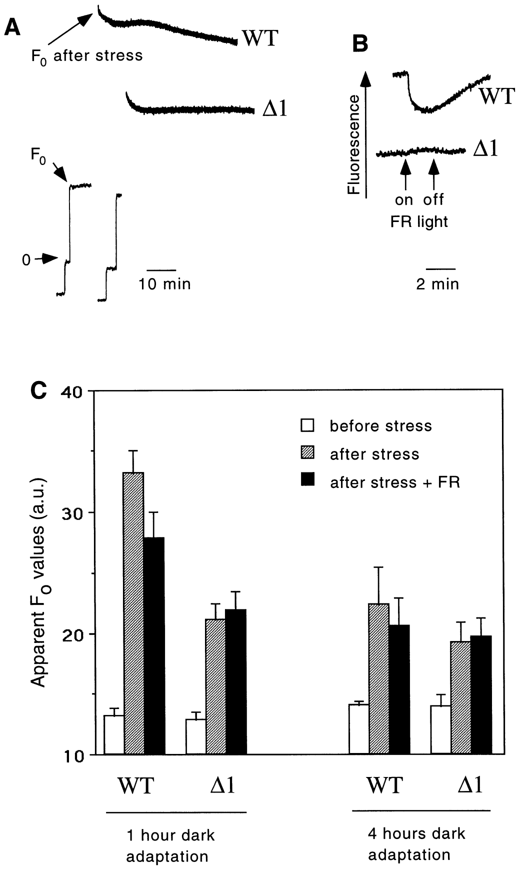

Fig. 3. Increase in 'apparent' F_0 levels after heat stress. Panel A: Relative 'apparent' F_0 values in a leaf of WT and Δ1 following heat stress. Panel B: Quenching of dark fluorescence by far-red light (FR) in WT but not Δ1. Panel C: Effect of time of dark adaptation prior to heat stress on 'apparent' F_0 values obtained with WT and Δ1.

(Fig. 3B). Repeated experiments showed that this particular component of F_0, which corresponds to approximately 25% of the total increase in F_0 in the WT, was pronounced in plants that had been dark-adapted, as usual, for 1 h prior to heat stress (Fig. 3C) but became very small when plants were dark-adapted for four or more hours.

4. Discussion

The aim of this work was to test the possible role of the chloroplast Ndh complex in electron flow within the thylakoid membrane following mild heat stress. Previous work using potato leaves has shown that heat stress induces the inactivation of PSII and an enhancement in the rate at which stromal reductant reduces the plastoquinone pool [8]. In higher plants and cyanobacteria a number of different avenues have been described for the non-photochemical reduction of the plastoquinone pool: a ferredoxin pathway, possibly involving a ferredoxin:plastoquinone oxidoreductase [5] or the PsaE protein of PSI [15] as well as the Ndh complex [4,16].

The Δ1 mutant contains a partial deletion of the *ndhC* and *ndhK* genes and lacks a functional Ndh complex in the thylakoid [4]. The data in Figs. 1 and 2 support the notion that in the light the Ndh-independent pathways are able to compensate for the loss of the Ndh complex. Presumably reductant produced by PSI such as reduced ferredoxin plays a role in this process with cyclic electron flow stimulated by heat-induced inhibition of CO_2 fixation [17]. One possible physiological reason for enhanced PSII-independent re-reduction of PSI may be to protect PSI from photoinhibition [8] and to generate ATP.

In the dark, heat stress induces a dramatic increase in 'dark' fluorescence (Fig. 3). Part of this increase is insensitive to far-red light treatment and is probably due to structural changes within the membrane such as dissociation of LHC from PSII [18,19]. Non-photochemical reduction of the plastoquinone pool also contributes to the increase in F_0 and can be identified on the basis of quenching by far-red light [8]. The lack of this component in the *ndh* mutant, Δ1, argues strongly in favour of the Ndh complex mediating the dark reduction of the pool in response to heat stress. Previous work had shown that the Ndh complex participated in the post-illumination reduction of the pool [4].

The physiological reason for enhanced reduction of the plastoquinone pool in the dark may be to induce a state transition to state II which would favour cyclic electron flow upon re-illumination [20]. Alternatively a reduced pool may reflect the heat-induced inhibition of plastoquinol oxidation in a possible respiratory electron transfer chain in the chloroplast.

Biochemical studies on the isolated Ndh complex indicate a substrate specificity for NADH and thus in the dark the Ndh complex may possibly use NADH produced by starch degradation to reduce the plastoquinone pool [3]. This proposal is consistent with the observation here that the Ndh-mediated increase in F_0 disappears after about 4 h of dark adaptation – a similar time scale to the decrease in starch concentration in tobacco plants in the dark [21].

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