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An assessment of the pathways of dark reduction and oxidation of the plastoquinone pool in thylakoid membranes of higher plants and green algae

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Introduction

The first suggestions that respiratory enzymes might exist in thylakoids arose from the recognition that the thylakoid plastoquinone pool could still undergo redox changes in the dark in response to changes of physiological conditions (Goedheer, 1963). The notion of respiratory-like activities in thylakoids was strengthened later in particular by the identification of 11 ORFs (*ndhA* - *ndhK*) in the higher plant chloroplast genome coding for homologues of protein components of mitochondrial complex I (Ohyama et al, 1988) and by the identification of a thylakoid homologue of the mitochondrial alternative quinol oxidase (Carol and Kuntz, 2001). Bennoun (1992) had earlier coined the term chlororespiration to distinguish algal thylakoid dehydrogenases and oxidases from their mitochondrial counterparts. Further evidence for various dehydrogenase and oxidase activities in both higher plant and algal thylakoids has since appeared, although a consistent view has yet to emerge.

In this report, possible pathways for non-photochemical control of the redox state of the thylakoid plastoquinone pool of *Chlamydomonas reinhardtii* and of higher plants are assessed, both by bioinformatic analysis of the *Arabidopsis* and *Chlamydomonas* genomes and by direct assay of plastoquinone pool redox state and its kinetics of change in the dark by analyses of fluorescence induction curves in spinach thylakoids, in a mutant strain of *Arabidopsis thaliana* in which the *immutans* gene was truncated and in a strain of *Nicotiana tabacum* in which the thylakoid *ndhC* and *ndhK* genes were inactivated by partial deletion.

Materials and methods

Spinach plants were grown in a growth chamber at 20-25 °C, in an 8/16 hour light/dark regime. The illuminance at leaf level was about 400 $\mu E.m^{-2}.s^{-1}$. *Arabidopsis thaliana* WT (Col-1, stock number CS3176) and the *immutans* mutant, CS3157, which is the result of a splicing defect at the sixth exon/sixth intron boundary (Wu et al, 1999), were obtained from the *Arabidopsis* Biological Resource Center. They were grown in compost under 10/14 h light/dark photoperiods at 20 °C in a temperature-controlled cabinet with 60% humidity and 60 $\mu E/m^2/s$ tungsten/fluorescent lighting. The tobacco *ndh* mutant, delta 1, which contains a partial deletion of *ndhK* and *ndhC* genes was constructed through biolistic

transformation of the chloroplast genome (Burrows et al, 1998). Plants were grown in compost at 25 °C in a greenhouse in spring/summer. Broken thylakoids were prepared as in (Moss and Bendall, 1984) and stored in liquid nitrogen in resuspension medium plus 5% dimethylsulphoxide.

Far red (>715 nm) fluorescence induction curves were measured on illumination with blue (BG39-filtered) actinic illumination either in whole leaves, or in thylakoid or cell suspensions at 10 μg/ml chlA+B. The plastoquinone pool was firstly reduced with blue preillumination of sufficient intensity and duration to cause fluorescence to rise to a maximum. After variable dark adaptation times, fluorescence induction curves were again recorded and the extent of plastoquinone pool reoxidation estimated from areas under the induction curves (Forbush and Kok, 1968).

Sequence data were retrieved from the MIPS *Arabidopsis thaliana* database (MAtDB) at www.mips.biochem.mpg.de/proj/thal/db/index.html. BLASTP (MIPS) and WU-Blast2 (www.ebi.ac.uk) were used to interrogate the MAtDB. Signal sequence prediction and analysis was performed using TargetP (www.cbs.dtu.dk/services/TargetP/). Non-*Arabidopsis* protein sequences were obtained from SWISS-PROT (release 39.23). Chlamydomonas ESTs from www.kazusa.or.jp/en/plant/chlamy/EST were searched using TBLASTN.

Results and discussion

(1) Pathways of plastoquinol oxidation

Analysis of the *A. thaliana* genome limits the types of plastoquinol oxidase that can be present in thylakoids. As expected, no homologues of the bacterial quinol oxidases, cytochrome *bo* and cytochrome *bd*, exist. A search for homologues of the core subunits I, II and III of cytochrome oxidase revealed only the mitochondrially-encoded versions. Hence, a chloroplast form of cytochrome oxidase can also be ruled out and casts doubt on the presence of a cyanide-sensitive thylakoid oxidase. This leaves only the well-documented *immutans* gene that encodes an alternative oxidase homologue and, unlike the other five alternative oxidase genes found in the *A. thaliana* genome, is chloroplast-, rather then mitochondrial-, targeted. Interestingly, a partial protein sequence of the C-terminal end of an *immutans* homologue was also found in a *Chlamydomonas* EST sequence. This aligns much more closely with the higher plant plastidic oxidases than with the two known *Chlamydomonas* mitochondrial alternative oxidases.

In order to assess the level to which the *immutans* protein might contribute to the dark rate of plastoquinol oxidation, the rate of plastoquinol oxidation in isolated spinach thylakoids was measured using fluorescence induction kinetics and its degree of inhibition by *n-propyl*-gallate or *octyl*-gallate was assessed. Both of these compounds increased the F_o value and slowed the risetime of the curve, indicative of an inhibition of photosystem II, a result confirmed by direct assay of PSII activity (not shown). However, the kinetics of recovery due to plastoquinol reoxidation were unaffected, suggesting that plastoquinol oxidation in isolated thylakoids in not due primarily to the *immutans* protein. In a second experiment, the rates of recovery of

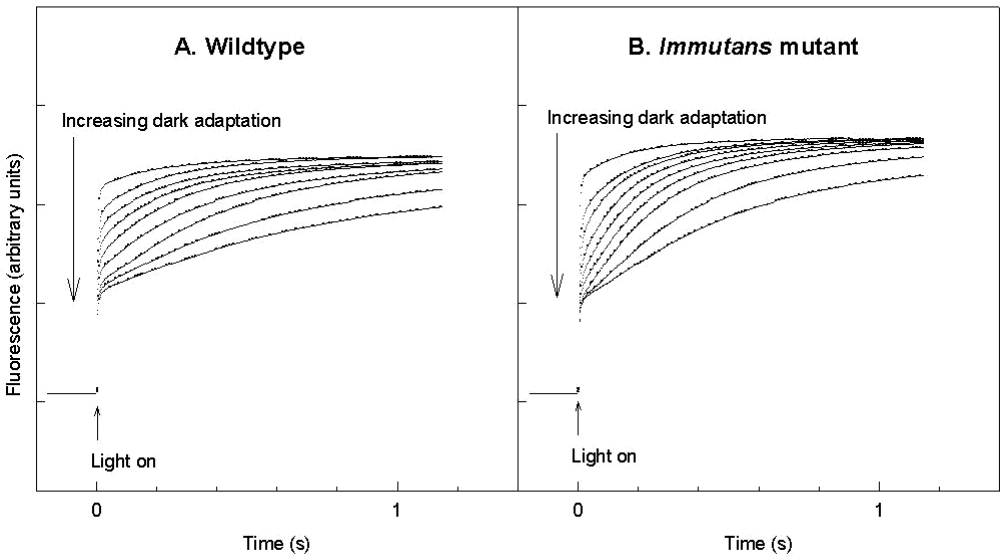

Fig. 1. Fluorescence induction kinetics of thylakoids derived from leaves of wildtype and the *immutans* mutant of *A. thaliana*. Fluorescence induction kinetics were monitored after different dark adaptation times following a pulse of light sufficient to reduce the plastoquinone pool. The dark times were 1, 5, 10, 20, 30, 60, 120-, 240 and 480 seconds in both cases.

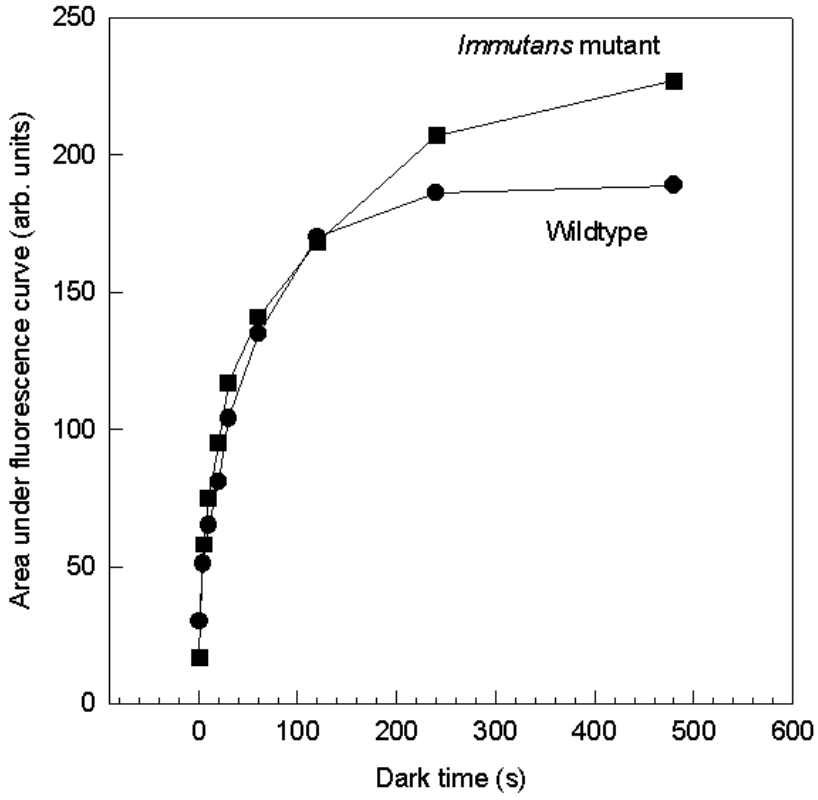

Fig. 2. Kinetics of plastoquinone pool oxidation derived from the areas under the fluorescence induction curves of fig. 1. See Methods for details.

fluorescence induction kinetics were measured in intact leaves of wildtype and the *immutans* mutant of *A. thaliana*. In this case, the mutant appeared to have a somewhat more reduced level of plastoquinone in the dark, but recovery rates of both were similar. When thylakoids were isolated from both plants, the rates of recovery of fluorescence induction kinetics were similar in both wildtype and mutant (figures 1 and 2).

Overall, it is concluded that at least three pathways for dark plastoquinol oxidation may be operative in intact systems: that mediated by *immutans*, which is not a dominant route in mature thylakoids, that due to backflow to the mitochondrial chain, presumably through mediation by NAD(P) and/or ferredoxin, and a third pathway that dominates in isolated thylakoids that does not have a clear enzymatic origin.

(2) Pathways of Plastoquinone Reduction

Although the chloroplast genome of higher plants generally contains the complex I homologues *ndhA-K*, no chloroplast-encoded or chloroplast-targeted nuclear subunits corresponding to the NADH oxidising domain of mitochondrial Complex I (the 51, 75 & 24 kD subunits) can be identified in the *A. thaliana* genome. It therefore seems likely that the chloroplast complex has a novel site for interaction with a reductant. This would most likely be a cassette for oxidation of NADPH or ferredoxin, perhaps even FNR (Guedeney et al, 1996). However, the finding of a chloroplast-targeted homologue of coenzyme $F_{420}H_2$ (Deppenmeier et al, 1999) in the *Arabidopsis* genome leaves open the possibility for more novel reductants. It is already known that the genes for complex I homologues are absent from the chloroplast genome of *Chlamydomonas*. A search of the *Chlamydomonas* EST databases for matches to the mitochondrially-encoded *Chlamydomonas* complex I subunits ND1, 2, 4, 5 and 6 or to the five chloroplast-encoded complex I homologues from the green alga *Mesostigma viride* (Lemieux et al, 2000) failed to produce any significant matches. Hence, at present there is no molecular evidence for a chloroplast complex I homologue in *Chlamydomonas*. Analysis of the *A. thaliana* genome also revealed that no genes were present that could provide a chloroplastic succinate dehydrogenase. However, of seven 'alternative' NDH-2 homologues in *A. thaliana*, four were mitochondrially-targeted, two were possibly directed targeted to the secretory pathway whereas one could not be assigned, leaving open the possibility of a chlororespiratory NDH-2 (Cornielle et al, 1998) that requires further investigation.

Fluorescence experiments were performed to determine the possible contributions of different pathways of plastoquinone reduction. In isolated spinach thylakoids, plastoquinone can be reduced slowly by added NADH and more rapidly by NADPH, especially in the presence of ferredoxin (Rich et al, 1998). None of a wide range of complex I antagonists showed any inhibitory effects. Further experiments were performed on whole leaves of the wildtype and *ndhK/C*$^-$ mutant of *Nicotiana tabacum*. Both the redox poise of the plastoquinone pool in the dark, and kinetics of recovery after photoreduction, were similar. It is concluded that the antimycin-sensitive ferredoxin-plastoquinone oxidoreductase and an undefined antimycin-insensitive route are the major pathways for plastoquinone reduction. In intact higher plant chloroplasts, the complex I homologue most likely provides an additional low capacity pathway that is able to alter the steady state level of ATP and the redox poise of key reductants during sustained illumination. This poise of components results in a burst of plastoquinone pool reduction on cessation of illumination but is altered when the complex is disrupted such that no burst is observed (Shikanai et al, 2001; Kofer et al, 1998; Burrows et al, 1998; Joet et al, 2001).

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References

Bennoun, P (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4352-4356.
Burrows, P, Sazanov, LA, Svab Z, Maliga P, Nixon, PJ (1998) *EMBO Journal* **17**, 868-876.
Carol, P, Kuntz M (2001) *Trends in Plant Sciences* **6**, 31-36.
Cornielle S, Cournac L, Guedeney G, Havaux M, Peltier G (1998) *Biochimica et Biophysica Acta* **1363**, 59-69.
Deppenmeier, U, Lienard, T, Gottschalk, G (1999) *FEBS Letters*, **457**, 291-297.
Forbush, B, Kok, B (1968) *Biochimica et Biophysica Acta* **162**, 243-253.
Goedheer JC (1963) *Biochimica et Biophysica Acta* **66**, 61-71.
Guedeney G, Corneille S, Cuiné S, Peltier G (1996) *FEBS Letters*, **378**, 277-280.
Joet T, Cournac L, Horvath EM, Medgyesy P, Peltier G (2001) *Plant Physiology* **125**, 1919-1929.
Kofer W, Koop H-U, Steinmüller K (1998) *Molecular and General Genetics* **258**, 166-173.
Lemieux C, Otis C, Turmel M (2000) *Nature* **403**, 649-652.
Moss, DA, Bendall DS (1984) *Biochimica et Biophysica Acta* **767**, 389-395.
Ohyama K, Kohchi T, Sano, Y, Yamada, Y (1988) *Trends in Biochemical Sciences* **13**, 19-22.
Rich PR, Hoefnagel, MHN, Wiskich, JT (1998) *Plant Mitochondria: From Gene to Function* (Möller et al, eds), pp. 17-23, Backhuys Publishers.
Shikanai, T, Endo, T, Hashimoto T, Yamada Y, Asada, K (2001) *Proc. Natl. Acad. Sci. USA* **95**, 9705-9709.
Wu DY, Wright DA, Wetzel C, Voytas DF, Rodermel S (1999) *Plant Cell* **11**, 43-55.