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Biochemical analysis of IMMUTANS, a potential plastoquinol oxidase of the thylakoid membrane

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Introduction

In plants a number of variegated mutants exist, in which the cells in the green sectors of the leaves have morphologically normal chloroplasts, whereas cells in the white sectors have abnormal plastids deficient in both an organised lamellar structure and pigments (Wu *et al*., 1999). An example of such a variegated phenotype is provided by the *immutans* mutant of *Arabidopsis thaliana*. This mutant shows a light-dependent variegated phenotype in which light affects the *immutans* phenotype by increasing the amount of white tissue in the mutant (Carol *et al*., 1999). Because phytoene accumulates in chloroplasts from the white regions of the leaves, it has been proposed that the *immutans* gene product functions in phytoene desaturation, a step in carotenoid biosynthesis. It has been postulated that the absence of carotenoids results in chlorophyll bleaching under high light conditions in the early stages of chloroplast development, resulting in the observed variegation (Carol *et al*., 1999).

The recent cloning of the nuclear *immutans* gene has shown that the gene encodes a protein with about 29% identity to the plant mitochondrial alternative oxidase (Carol *et al*., 1999; Wu *et al*., 1999). Based on this sequence analysis and activity measurements following expression of IMMUTANS in *E. coli* (Josse *et al.*, 2000), it has been suggested that the *immutans* gene product may act as a plastid terminal oxidase (Wu *et al*., 1999; Carol *et al*., 1999). As the desaturation of phytoene in the carotenoid biosynthetic pathway requires oxidised plastoquinone as an electron acceptor (Wu *et al*., 1999), the role of the IMMUTANS protein may be to oxidise the plastoquinone pool by transferring electrons to oxygen. Because of its proposed function as a plastoquinol oxidase, it has been suggested that the IMMUTANS protein may also play a role in the phenomenon of chlororespiration (Carol *et al*., 1999; Wu *et al*., 1999; Nixon, 2000) and although this has not been demonstrated in higher plants, a protein in *Chlamydomonas* with immunological and pharmacological similarities to the IMMUTANS protein has been shown to play a role in this process (Cournac *et al*., 2000).

The location and orientation of IMMUTANS within the chloroplast is still unclear. Import experiments have indicated that IMMUTANS is membrane-bound (Carol et al., 1999) but it is unclear which membrane. Determining the orientation of IMMUTANS within the membrane is also an important test of whether IMMUTANS activity, like that of the alternative oxidases of mitochondria, is uncoupled from proton pumping . This study has addressed these issues, with a combination of chloroplast fractionation and protease protection assays.

Materials and methods

Chloroplast isolation and fractionation

Spinach was purchased from the local supermarket. Intact, purified chloroplasts were isolated from the spinach leaves according to the method of Siegenthaler and Dumont (1990). Thylakoids were prepared by washing the intact chloroplasts twice in 10 mM Na-Tricine pH 7.8, 10 mM NaCl and 10 mM $MgCl_2$. The pellet was resuspended to 0.4 mg chl/ml in the washing buffer with the addition of 0.1 M sorbitol and 0.5% digitonin. Stroma and grana lamellae were then isolated according to the method of Leto *et al.* (1985).

Protease treatment of thylakoid membranes

Intact spinach thylakoids were prepared by resuspending intact chloroplasts in 5 mM $MgCl_2$ and leaving for 20 seconds before the addition of an equal volume of 0.66 M sorbitol, 20 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.5. Thylakoids were collected by centrifugation at 3,500 g and the pellet resuspended in 50 mM HEPES pH 8.0 and 330 mM sorbitol to a chlorophyll concentration of 1mg/ml. Half of the thylakoids were broken with 3 cycles of freeze/ thawing. Both the unbroken and broken fractions were treated with a range of trypsin concentrations for 30 min at 4^{o}C.

SDS-PAGE and Immunoblotting

SDS-PAGE was performed using 12% gels essentially according to Laemmli (1970). For immunodetections proteins were transferred to nitrocellulose membranes and detected using an ECL immunoblotting kit (Amersham) according to the manufacturer's instructions. Antibodies to IMMUTANS were raised to protein overexpressed in *E. coli*. Either the crude antiserum (crude) or affinity-purified antibodies (purified) were used. The FtsH antibodies were a kind gift from Dr. T. Ogura (Kumamoto University).

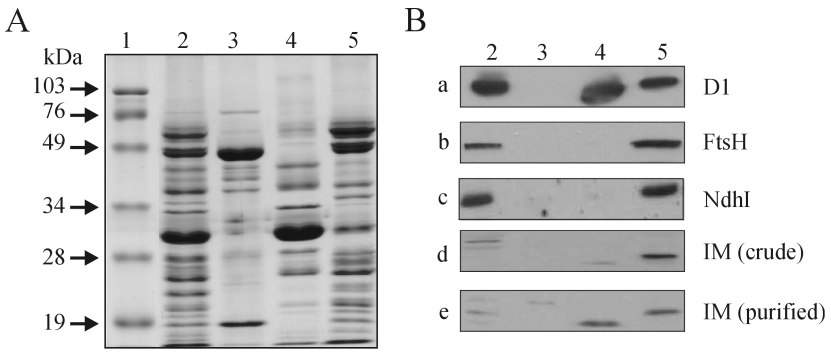

Figure 1. The protein profiles of spinach samples in a Coomassie-blue stained gel (Figure 1A) and the corresponding immunoblots (Figure 1B). The protein samples are thylakoids (lanes 2), stroma (lanes 3), grana lamellae (lanes 4) and stroma lamellae (lanes 5). Figure 1B shows the immunodetection of the sample with antibodies raised against D1, FtsH, NdhI, IMMUTANS (IM) antibody both crude extract and the affinity-purified antibody.

Results

Localisation of the IMMUTANS protein within the chloroplast

Spinach chloroplasts were fractionated into four fractions, thylakoids, stroma, grana lamellae and stroma lamellae. SDS-PAGE followed by Coomassie-blue staining showed a clear difference in the protein profile of each fraction (Fig. 1A). Following SDS-PAGE the proteins were transferred to nitrocellulose and probed with a number of different antibodies. In order to assess the degree of cross-contamination between the chloroplast fractions, antibodies raised against the D1, FtsH and NdhI proteins were used to probe the fractions. All of these proteins are found within the thylakoid membranes, but they are not present in the stromal fraction (Fig. 1B a-c). The antibodies raised against the NdhI subunit of the plastid Ndh complex detected protein within the stroma lamellae and not the grana lamallae, which is consistent with what has been observed in previous work on the Ndh complex (Nixon *et al*., 1989; Berger *et al*., 1993). A similar result was obtained when the chloroplast fractions were probed with antibodies directed towards the FtsH protein, again consistent with previous findings (Lindahl *et al*., 1996). When the fractions were probed with antibodies raised against the D1 protein a signal was seen in both the grana and stroma lamallae. Overall these results suggest that the fractions do not have any major cross-contamination.

The chloroplast fractions were also probed with antibodies raised against the *Arabidopsis* IMMUTANS protein using either crude or affinity-purified antibodies. In both cases the antibodies detected a protein in the stoma lamellae of the appropriate size for IMMUTANS of 35 kDa. The affinity-purified antibodies also detected a smaller protein within the grana lamellae (Fig. 1B d-e).

The orientation of the IMMUTANS protein within the thylakoid membrane

With the IMMUTANS protein localised to the stroma lamellae, it is important to determine on which side of the membrane the protein located. Current structural models for IMMUTANS indicate that it is an interfacial protein, but it remains unclear on which side of the thylakoid membrane it is exposed (Berthold *et al*., 2000). Both intact and broken thylakoids were treated with trypsin. SDS-PAGE was performed and the proteins transferred to nitrocellulose. The membranes were probed with antibodies raised against, PsbO, FtsH, NdhI and the IMMUTANS proteins (Fig. 2). Following treatment with protease it was observed that the PsbO protein, which is known to be located on the lumen side of the thylakoid membrane, was not affected by the protease in the intact thylakoids, but disappeared when broken thylakoids are treated with trypsin (Fig. 2a). This result confirmed the integrity of the thylakoid membranes.

As expected from previous work (Lindahl *et al*. 1996), the FtsH protein disappeared upon treatment with protease in both the broken and unbroken thylakoids (Fig. 2b), consistent with the location of the epitope on the stromal side of the thylakoid membrane.

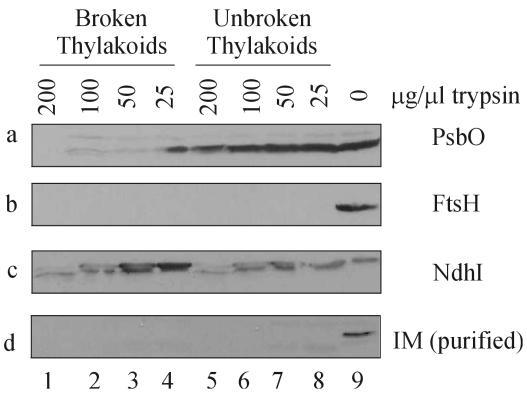

Figure 2. The protease treatment analysis of the spinach thylakoid membranes. Lane 1 to 4 are the broken thylakoids while lane 5-8 are the intact thylakoids. The amount of trypsin was indicated in the figure (µg/µl). The antibodies used were raised against PsbO, FtsH, NdhI and the affinity-purified IM protein (1:200).

The degradation profile of the NdhI protein was similar in both the broken and unbroken fractions (Fig.2c). This is consistent with the NdhI protein, which is in the putative interconnecting domain of the Ndh complex, lying on the stromal side of the membrane. However unlike the FtsH protein, the NdhI protein is not degraded fully but is cut to produce a second smaller protein, but at higher concentrations of protease both of these bands are reduced in intensity, with the larger intact NdhI protein being almost fully replaced with the smaller form. The IMMUTANS protein behaves in a similar manner to that of the FtsH protein, in that it disappears upon treatment with protease in both the broken and unbroken thylakoid membranes (Fig. 2d), consistent with its accessibility to the stromal phase of the membrane.

Discussion

The data presented in Fig. 1 demonstrates that the IMMUTANS protein is primarily located with the stromal lamallae. However using affinity-purified antibodies, a second smaller cross-reaction was observed in the granal lamallae fraction. It is possible that this represents a degradation product of the IMMUTANS protein. Of interest is the fact that both the Ndh complex and the IMMUTANS protein are localised to the same thylakoid region which is important if they are to participate in chlororespiration within the chloroplasts of higher plants (Nixon, 2000). It is, however, at present unclear as to whether these two components do indeed act as a functional respiratory chain.

The orientation of the IMMUTANS protein appears from this study to be towards the stromal side of the thylakoid membrane. Previous modelling of the plant mitochondrial alternative oxidase, to which the IMMUTANS protein shows homology, has suggested that the alternative oxidase is an interfacial membrane protein located on the matrix side of the inner membrane (Andersson and Nordlund, 1999). The IMMUTANS protein contains many of the structural motifs found in the alternative oxidase proteins, and it has been proposed to possess a similar structure. Therefore with no membrane-spanning helices most of the protein is located on one side of the membrane, in this case the stromal side of the thylakoid membrane, and is susceptible to protease treatment. This orientation of IMMUTANS further indicates that it is unlikely to have a role in proton pumping across the thylakoid membrane.

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