Immunological evidence for the presence of the D1 and D2 proteins in PS II cores of higher plants

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Received 11 August 1986; revised version received 25 September 1986

The *psb A* and *psb D* genes isolated from *Poa annua* and *Triticum aestivum*, respectively, have been expressed in *Escherichia coli* as β-galactosidase fusion proteins and used to raise antibodies. The antibodies to the *psb D* gene product cross-reacted with a lysine containing polypeptide with an apparent molecular mass of 31 kDa which was present in a PS II core preparation. In contrast, the antibody to the *psb A* gene product recognised predominantly, a 34 kDa protein in the PS II core which was insensitive to treatments with a lysine specific protease. It is concluded that the latter polypeptide is the lysine-free D1 protein and that the antibody raised to the *psb D* gene product monospecifically reacted with a component presumed to be the D2 protein. It is therefore suggested that the product of the *psb D* gene, as well as that of the *psb A* gene, is a component of the PS2 core complex of higher plants.

Photosynthesis *Photosystem II* *Antibody* *Gene fusion* *D1 protein* *D2 protein*

1. INTRODUCTION

Recently the chloroplast gene, *psb D* has been isolated and sequenced for a number of species [1–4]. It has been noticed that considerable homology exists between this gene and another chloroplast gene, *psb A*, which codes for the PS II polypeptide D1 (also known as the 32 kDa herbicide-Q_B binding protein) [4]. Initially it was suggested that this homology reflected a common property of the two gene products to bind quinones [4]. However, the *psb A* and *psb D* genes also show certain regions of high homology with the genes of the L and M subunits of the reaction centres from the purple non-sulphur photosynthetic bacteria, *Rhodopseudomonas capsulata* [5] and *Rhodopseudomonas sphaeroides* [6,7]. The significance of this has been emphasised following the recent crystallisation of the isolated reaction centre of *Rhodopseudomonas viridis* and the determination of its structure to a spatial resolution of 2.9 Å [8]. From this work it seems that those regions of high homology with the *psb A* and *psb D* gene products are responsible for the binding of bacteriochlorophyll, bacteriopheophytin, quinone and iron. This has led Deisenhofer et al. [8] to suggest that the *psb A* and *psb D* genes code for proteins which may be the reaction centre polypeptides of PS II in higher plants, a postulate also strongly advocated by Trebst [9]. Prior to this suggestion the PS II reaction centre had been assigned to a 47 kDa polypeptide [10] which is the product of the chloroplast *psb B* gene [11]. Indeed, as yet, there is no evidence that the *psb A* gene product, the D1 protein, binds chlorophyll or forms a heterodimer with another polypeptide as would be required according to the suggestion of Deisenhofer et al. [8].

Although the link between the D1 protein and the *psb A* gene is well established for a wide range of species [12] the experimental evidence for the *psb D* gene product being a part of the PS II complex is poorer and restricted to studies with algae, particularly *Chlamydomonas* [4]. In these algal systems it is thought that the *psb D* gene codes for the PS II component known as the D2 protein

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

[4,13], a concept also suggested by Rasmussen et al. [1]. In order to investigate this further we have expressed the *psb D* gene from wheat (*Triticum aestivum*) and the *psb A* gene from *Poa annua* in the bacterium *Escherichia coli* (strain JM101). Antibodies raised against the gene products were used to probe polypeptides of an isolated PS II core preparation.

2. MATERIALS AND METHODS

2.1. *Production of antibodies to the* psb D *gene product*

The cloning and expression of the *psb D* gene will be discussed in detail elsewhere. Briefly, a 1.2 kb fragment of DNA was isolated from wheat chloroplast DNA by heterologous hybridisation using a *psb D* probe from spinach, kindly given by Dr J. Gray. The 1.2 kb fragment was sequenced and shown by comparison with previously published sequences to contain 97% of the *psb D* gene [1–4]. Plasmid pPND2 was constructed so that the 1.2 kb *psb D* fragment was fused, in the same reading frame, to the 3′-end of the *lac Z* gene encoding β-galactosidase. *E. coli* (strain JM101) which carried pPND2 produced upon induction a β-galactosidase fusion protein consisting of potentially 97% of the *psb D* gene product fused to the carboxy-terminus of β-galactosidase. This hybrid protein was excised from preparative SDS-polyacrylamide gels and used in a course of injections to raise antibodies in rabbit.

Antiserum was stored at −20°C. Antibodies specific for the *lac Z/psb D* gene fusion product were purified by affinity chromatography using an antigen-Sepharose column. 5 mg of purified fusion protein was coupled to 1 g of dry CNBr activated Sepharose 4B according to manufacturer's instructions (Pharmacia). Chromatography of the crude antiserum was carried out according to Chua et al. [14].

2.2. *Production of antibodies to the* psb A *gene product*

A similar method to that used for expressing the *psb D* gene was used to express the *psb A* gene. A 1.2 kb fragment of DNA containing the entire *psb A* gene from *Poa annua* (kindly given by Dr D. Barros) was used to construct the plasmid pPND1. Upon induction the *psb A* gene product was produced in *E. coli* (JM101) as a fusion protein with β-galactosidase. This purified fusion protein was used to raise antibodies in rabbit. The crude antiserum was found to be suitable for immunoblotting experiments.

2.3. *Electrophoresis*

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [15] normally on 7–17% (w/v) gradient gels containing 2 M urea. Samples to be loaded were solubilised at room temperature. Molecular mass markers were purchased from Pharmacia.

2.4. *Immunoblotting*

Protein was transferred onto nitrocellulose paper of pore size 0.2 μm after electrophoresis using the method of Burnette [16]. Blots were probed with purified antibodies to the *lac Z/psb D* gene fusion product (equivalent to a 100-fold dilution of the original serum). Antiserum to the *lac Z/psb A* gene fusion product was also used at a 100-fold dilution. Bound antibody was visualised by first incubating with mouse anti-rabbit IgG conjugated to biotin (Sigma) followed by incubation with streptavidin conjugated to alkaline phosphatase (BRL). The substrates used in the phosphatase reaction were 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) with a positive reaction yielding a purple band [17].

2.5. *Preparation of plant material*

Thylakoid membranes were prepared from *Pisum sativum* (pea) using the method of Nakatani and Barber [18]. PS II enriched particles were made from pea using essentially the method of Berthold et al. [19]. PS II core preparations were made from pea using Triton X-100 by following the method of Gounaris and Barber [20]. This PS II core preparation is essentially free of the 33 kDa lysine-rich protein characterised by Murata and Kuwabara [21].

2.6. *Lys-C protease treatment*

PS II core fractions from pea were incubated with the endoproteinase Lys-C so as to cleave specifically at lysine residues. The protocol developed by Millner et al. [22] was followed with digestions carried out at room temperature in the

dark for 4 h. Lys-C was used at concentrations of 0.3 μg/ml and 3.0 μg/ml. Reactions were stopped by the addition of solubilisation buffer prior to electrophoresis on 12–25% (w/v) gradient polyacrylamide gels containing 2 M urea.

3. RESULTS

3.1. *Cross-reaction with thylakoid fractions*

Purified antibodies to the fusion protein derived from the *psb D* gene were used to probe thylakoid fractions as shown in fig.1. A positive cross-reaction was obtained against a diffuse band of apparent molecular mass 31 kDa. This band was present in unfractionated thylakoids in the Berthold et al. type PS II-enriched particles [19] and, most importantly, in the PS II core preparation. The pre-immune serum and antibodies directed against β-galactosidase showed no cross-reaction with PS II core proteins (not shown).

3.2. *Monospecificity of the fusion antibodies*

As mentioned the *psb D* gene shows a high degree of homology with the gene encoding the D1 protein. It is therefore possible that the cross-reaction obtained in fig.1B may arise from the cross-reaction between the antibody derived from the *psb D* gene and the D1 polypeptide since this component is known to be present in the PS II cores [12]. To show the monospecificity of the antibodies raised to the *psb D* gene product, an immunoblot of Lys-C treated cores was performed (fig.2). From sequence data of the D1 polypeptide [23] it is known that no lysine residues are present; hence D1 is predicted to be Lys-C resistant as shown in a recent study [22]. The *psb D* gene does, however, encode for a polypeptide which contains lysine [1,4] and therefore should be sensitive to Lys-C.

The results in fig.2B show that the antibodies against the *psb D* gene product recognise a species which, upon lysine digestion (3.0 μg/ml), is degraded from an apparent molecular mass of 31 kDa to that of about 29 kDa. In a parallel experiment, antibodies raised against the *psb A* gene product show no such digestion profile but instead cross-react predominantly with a protein species of apparent molecular mass 34 kDa resistant to Lys-

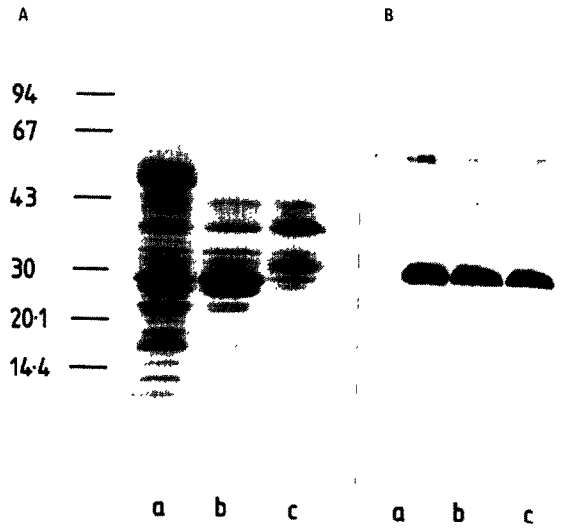

Fig.1. (A) 7–17% gradient SDS-PAGE of plant extracts visualised by Coomassie blue staining. Lanes: (a) thylakoid membranes (25 μg chlorophyll loaded); (b) PS II enriched particles (25 μg chlorophyll loaded); (c) PS II core preparation (15 μg chlorophyll loaded). (B) Equivalent profile immunoblotted with purified antibodies to the *psb D* gene product. Molecular mass standards are marked in kDa.

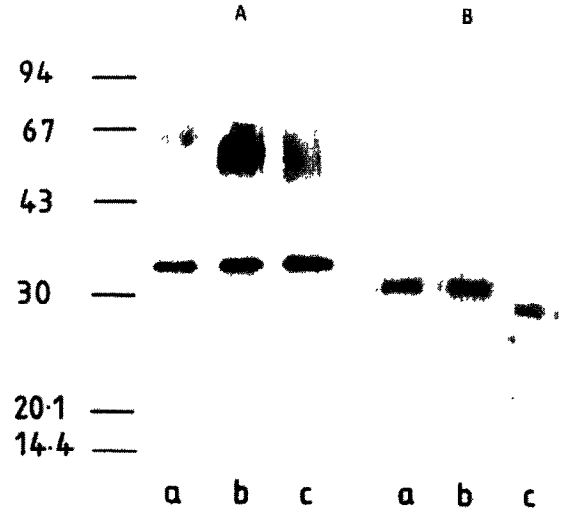

Fig.2. Immunoblots of Lys-C treated cores (1 μg of chlorophyll loaded per track) probed with antibodies raised to (A) *psb A* and (B) *psb D* gene products. Lanes: (a) incubated with no Lys-C; (b) incubated with 0.3 μg/ml Lys-C; (c) incubated with 3.0 μg/ml Lys-C. Molecular mass markers are shown in kDa.

C digestion, presumably the D1 protein (see fig.2A). Interestingly with this antibody a minor cross-reaction was also observed against a protein species of apparent molecular mass 30 kDa which also seems to be lysine-free and could be a breakdown product of D1. The origins of the cross-reactions with proteins of higher molecular masses are unclear. It is therefore concluded from this experiment that the two antibodies are monospecific for each gene product.

4. CONCLUSION

The results presented here provide evidence that antibodies raised against the *psb D* gene product cross-react with a protein present in the PS II core. Moreover, it seems likely that this protein is the D2 polypeptide which has been well characterised in *Chlamydomonas* [4] and which is of similar molecular mass. Therefore we conclude that the *psb D* gene of higher plant chloroplasts does code for a protein equivalent to the D2 polypeptide of the algal system. Our results with antibodies raised to the *psb A* gene product also confirm that the D1 protein is a part of the PS II core.

The antibodies, according to the experiments using Lys-C, seem to be monospecific. This monospecificity has been achieved by obtaining antisera to gene products and is valuable since it links particular genes with specific proteins in the natural membrane. The production of these antibodies should be important for further characterisation of the PS II organisation, especially for investigating the possibility that the D1 and D2 proteins form a heterodimer analogous with that of the L and M subunits of the bacterial reaction centre.

ACKNOWLEDGEMENTS

We wish to thank the Science and Engineering Research Council (SERC) and Agricultural and Food Research Council (AFRC) for financial support. One of us (P.N.) is a recipient of a SERC post-graduate studentship.

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