The chlorophyll *a*/*b*-proteins of PS I and PS II are immunologically related

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Polyclonal antisera were raised against three apoproteins of the light-harvesting chlorophyll *a*/*b*-protein complex of photosystem I (LHC I) from spinach. These antisera were found to cross-react with all four LHC I and both LHC II apoproteins on immunoblots. Affinity purification of the cross-reactive antibodies on either the 'native' LHC II complex or on the 28 kDa apoprotein of LHC II immobilized on nitrocellulose yielded antibodies which recognize the same apoproteins as the crude sera. Partial peptide mapping of LHC I and LHC II apoproteins with *Staphylococcus aureus* V8 protease and *N*-chlorosuccinimide showed different patterns for each polypeptide. These data demonstrate that the four polypeptides of LHC I are distinct from each other and from the two major polypeptides of LHC II but all six are immunologically related.

Chlorophyll *Light-harvesting complex* *Immunological cross-reactivity* *Immunological relationship* *Photosynthesis*

1. INTRODUCTION

Both PS I and PS II in higher plants and green algae are associated with Chl *a*/*b*-proteins which function as light-harvesting antennae. The light-harvesting complex of PS II (LHC II) that contains two major apoproteins has been well characterized [1]. The gene for one of the major LHC II apoproteins has been cloned and sequenced from several sources and the amino acid sequences deduced [2–4]. The proposed sequence for one of the *Lemna* LHC II polypeptides has been used to generate a hydropathy plot to predict the structure of the protein in the membrane [4]. The gene for the other LHC II polypeptide has not been isolated and its mRNA apparently does not hybridize to the gene encoding the major apoprotein [5,6]. Even though there may be a lack of homology at the nucleic acid level, the two proteins are antigenically very similar [7–9]. Further, at least in *Chlamydomonas* [10] and in *Acetabularia* [11], the LHC II polypeptides have similar amino acid compositions and some protein sequence homology. In higher plants, both polypeptides possess a 2 kDa, trypsin-sensitive, NH_2-terminal sequence required for thylakoid stacking [12,13]. The light-harvesting complex of PS I (LHC I) is less well characterized. LHC I has been isolated by detergent solubilization and sucrose gradient fractionation [14], and by mildly denaturing SDS-PAGE [15–19]. Lam et al. [19] and Bassi et al. [20] have further fractionated LHC I into two chlorophyll-containing complexes fluorescing at 680 and 730 nm at 77 K. However, the characterization of the two complexes by these groups differed with respect to Chl *a*/*b* ratios, circular dichroism spectra and polypeptide content [19,20]. The polypeptide content of LHC I preparations varies from 1 [17,18] or 2 [16] to as many as 3 [19] or 4 [14,20] putative Chl-binding proteins. We report here that all four of the putative Chl *a*/*b*-proteins of PS I and both of the

Abbreviations: PS, photosystem; Chl, chlorophyll; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin

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Chl *a/b*-proteins of PS II show some degree of antigenic similarity, but that all six are distinct gene products.

2. MATERIALS AND METHODS

Spinach PS I complex and LHC II complex were isolated by Triton X-100 solubilization of the thylakoids followed by fractionation on a sucrose gradient [21]. The 'native' LHC II complex was further purified by Mg^{2+}-induced aggregation and centrifugation through a sucrose cushion [22]. The apoproteins of LHC I were isolated from preparative SDS-PAGE of PS I complex. The fractionated proteins were detected as clear bands on an opaque background with 4 M Na acetate [23]. The bands of interest were excised, and the proteins were eluted electrophoretically and concentrated by lyophilization. Protein concentration was determined as in [24].

Each polypeptide (~50–100 μg) was emulsified with Freund's complete adjuvant and injected into rabbits at multiple sites intradermally. After 1 month a booster injection was given, and the rabbit was bled the following week. Additional booster injections were given 1 week before each bleeding.

Immunoblotting was performed essentially as described in [25]. Protein A labelled with ^{125}I or with horseradish peroxidase was used as probe. Peroxidase activity was detected using 4-chloro-1-naphthol [26]. Antibodies recognizing the 28 kDa LHC II polypeptide were obtained using electrophoretically purified 28 kDa protein blotted to nitrocellulose as an affinity matrix. Non-specific binding was eliminated by using 5% BSA as a blocking agent. After reaction with the various antisera, the nitrocellulose strips were thoroughly washed with 50 mM Tris, 0.15 M NaCl, pH 8.0. The bound antibodies were eluted with 0.2 M glycine, 0.15 M NaCl, pH 8.0. The bound antibodies were eluted with 0.2 M glycine, 0.15 M NaCl, pH 2.2, and neutralized with Tris to a final pH of 7.4. BSA was added (5% final concentration) as a stabilizer. Antibodies recognizing the native LHC II complex were allowed to bind to the Chl-protein and then recovered by pelleting the LHC II complex at 10000 × *g*, and eluting the bound antibodies using glycine, pH 2.2, as described above.

Digestion of polypeptides in gel slices with 200 ng of *Staphylococcus aureus* V8 protease was done as in [27]. Digestion products were analyzed on a 16% acrylamide gel containing 3.6 M urea [28]. After electrophoresis the resolved digest was blotted onto nitrocellulose and the peptides stained with acidic gold-sol [29]. Digestion of polypeptides in gel slices with *N*-chlorosuccinimide was performed as in [30]. The digest was analyzed by 10–18% SDS-PAGE containing 8 M urea with the Laemmli buffer system [31]; digestion products were stained with silver [32].

3. RESULTS

Fig.1 shows the polypeptide profiles of the isolated spinach PS I and LHC II complexes on a 10–18% acrylamide gel. The arrows in the non-

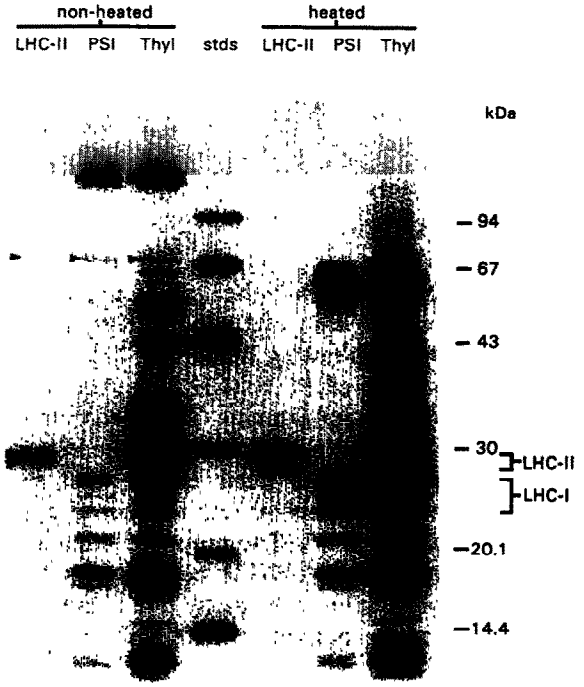


Fig.1. SDS-PAGE of spinach thylakoids, PS I complex and LHC II complex. The polypeptides were resolved on a 10–18% acrylamide gel and stained with Coomassie blue. Arrows indicate bands which were green before staining in the non-heated samples. Heated samples were treated at 70°C for 5 min. Molecular mass markers (kDa) were phosphorylase *b* (94), BSA (67), ovalbumin (43), carbonic anhydrase (30), soybean-trypsin inhibitor (20.1) and α-lactalbumin (14.4).

heated sample lanes mark the bands which were green before staining with Coomassie blue. The heated samples were treated at 70°C for 5 min. The 4 LHC I apoproteins were of M_r 25800, 25000, 24000 and 23000. The major apoproteins of LHC II were of M_r 28000 and 26500 with two faint bands of intermediate M_r.

Antisera were raised against each of the isolated LHC I apoproteins and the specificity of the sera was checked by immunoblot analysis. The resolved polypeptides of spinach thylakoids, PS I complex and LHC II complex were reacted with the different antisera obtained from the first bleeding (fig.2). The antiserum against the 25.8 kDa polypeptides reacted also with the 28 and 26.5 kDa peptides of LHC II and the 24 and 23 kDa proteins of LHC I. The rabbit injected with the 25 kDa protein died before antiserum could be obtained. The antiserum against the 24 kDa protein recognized the 24 and 25.8 kDa proteins of LHC I, and the 26.5 and 28 kDa proteins of LHC II. The antiserum against the 23 kDa polypeptide reacted with each of the LHC I and LHC II apoproteins to varying extents.

To check each of the gel-purified LHC I apoproteins for contamination by the other Chl *a/b*-apoproteins, approx. 5% of the total protein to be injected into the rabbit was electrophoresed and blotted onto nitrocellulose. Antisera to the 25.8, 24 and 23 kDa apoproteins were mixed and used to probe the blot. In this analysis, each of the

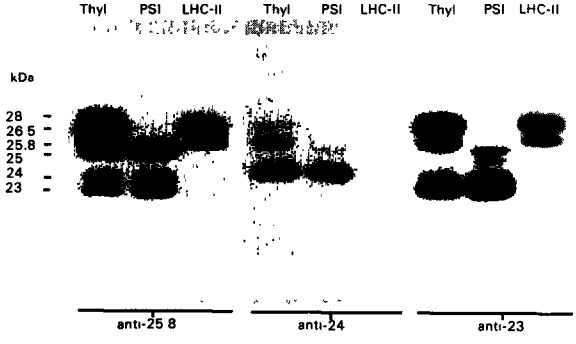


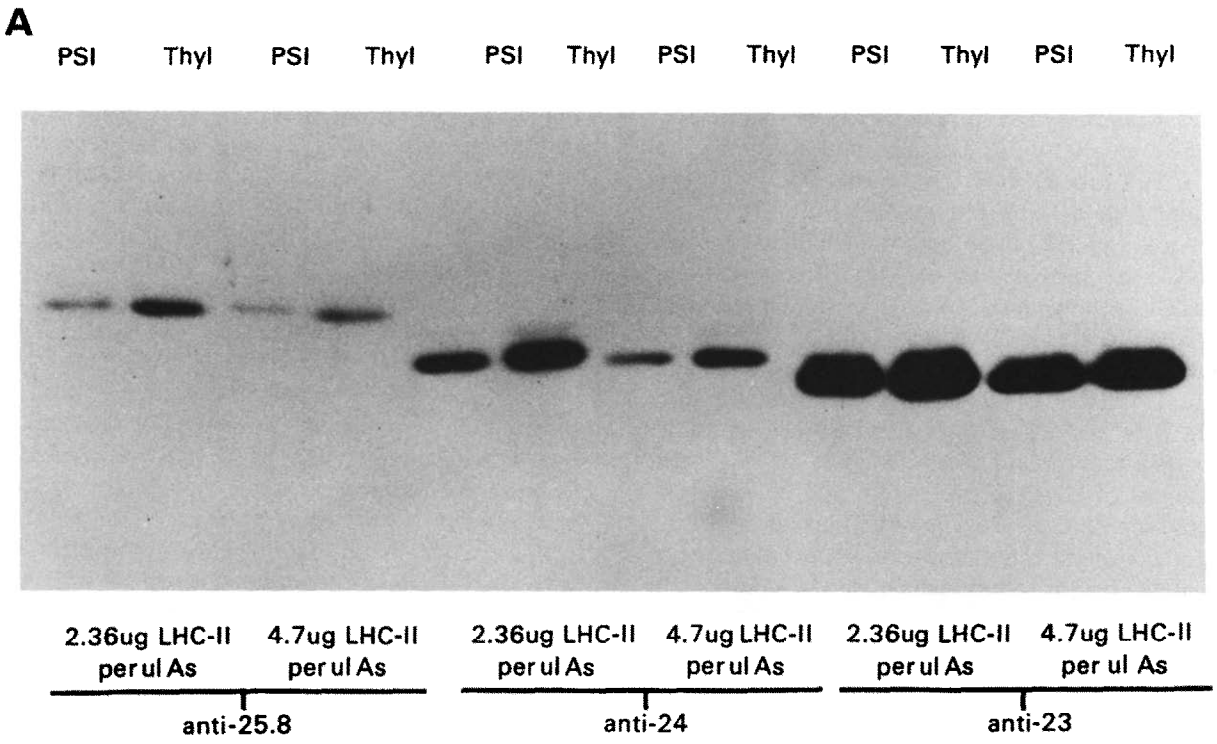
Fig.2. Autoradiogram of immunoblots of spinach thylakoids, PS I complex and LHC II complex. The polypeptides were resolved on an SDS gel, blotted to nitrocellulose and reacted with the individual antisera to the LHC I apoproteins. ^{125}I-labelled protein A was used to probe the blot. Autoradiography was done at −70°C with an intensifying screen.

purified polypeptides appeared as a single band with no contamination by the other LHC I and LHC II apoproteins (not shown).

Fig.3A shows an immunoblot using antisera absorbed with native LHC II complex. It can be seen that absorbing with an increasing amount of LHC II protein results in a depletion of the antibodies binding the immunizing antigen as well as the loss of antibodies recognizing the other LHC I and LHC II apoproteins. These results suggest that the cross-reactive antigenic site(s) is accessible in the native LHC II complex. Fig.3B shows an immunoblot using antibodies to the 25.8 kDa polypeptide eluted from the native LHC II complex with glycine, pH 2.2. The affinity purified antibodies still bind the same polypeptides as the crude sera.

Since a small amount of LHC I co-purifying with native LHC II could produce this result, we investigated this possibility by using electrophoretically purified LHC II 28 kDa protein as an affinity matrix for antibody purification. A preparative SDS-PAGE gel of LHC II was blotted to nitrocellulose and stained with Amido black (not shown). The 28 kDa polypeptide was excised and used to affinity purify antibodies from each of the LHC I apoprotein antisera. The eluted antibodies were used in the immunoblot shown in fig.4 and in each case antibodies that specifically bound to the 28 kDa also bind the same polypeptides as crude antisera. As a control for non-specific binding, a blank strip of nitrocellulose was treated in the same manner and no non-specific antibody binding could be detected using protein A-peroxidase staining (not shown). These results with the antibodies affinity purified on the 28 kDa LHC II polypeptide provide the strongest evidence that the light-harvesting proteins of PS I and PS II are indeed antigenically related.

Partial peptide maps were generated to compare the LHC I and LHC II apoproteins. Fig.5A shows an analysis of the apoproteins digested with *N*-chlorosuccinimide which cleaves at tryptophan residues [30]. Each of the Chl *a/b*-apoproteins showed a distinct digestion pattern. *S. aureus* V8 protease cleaves at the carboxyl-terminal side of either aspartate or glutamate residues depending upon buffer conditions [33]. As can be seen in fig.5B, each of the apoproteins showed a different cleavage pattern when treated with the V8 pro-



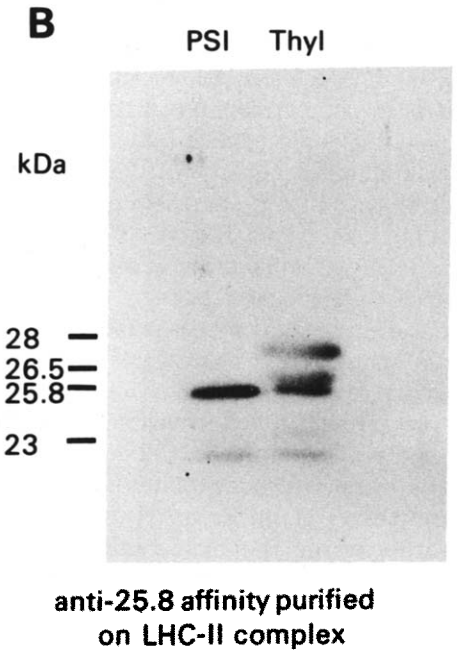


Fig.3. (A) Autoradiogram of immunoblots of spinach thylakoids and PS I complex reacted with LHC I polypeptide antisera absorbed with native LHC II complex. The concentration of LHC II is in μg protein. (B) Autoradiogram of an immunoblot of spinach PS I and thylakoids reacted with antibodies to the 25.8 kDa LHC I polypeptide affinity purified on native LHC II complex. Both blots were probed with ^{125}I-labelled protein A and autoradiographed as in fig.2.

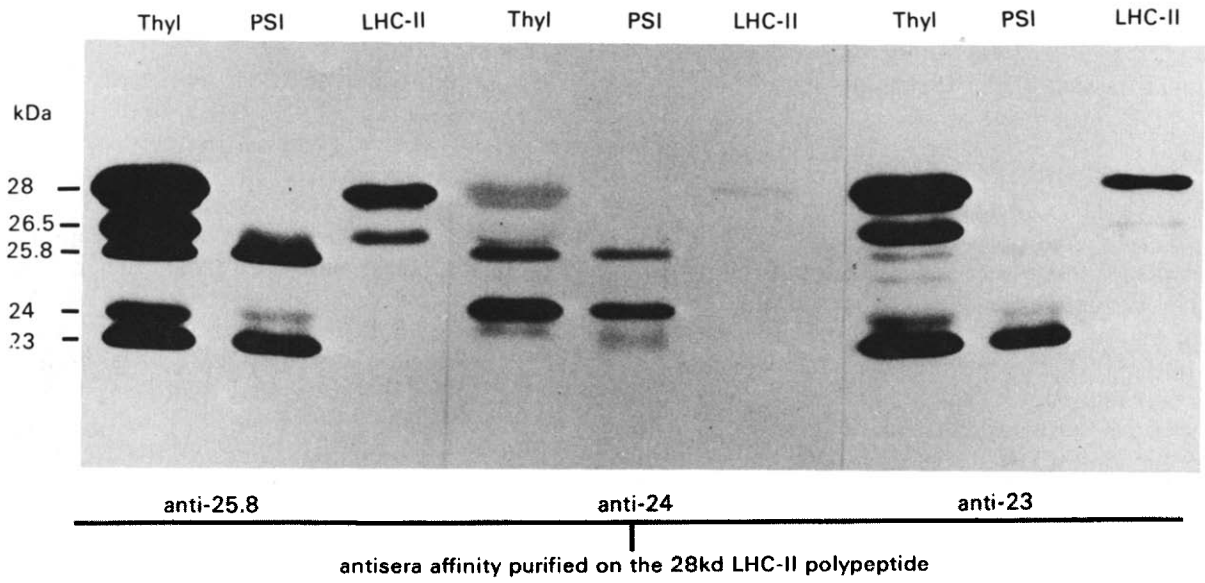


Fig.4. Immunoblots of spinach thylakoids, PS I complex and LHC II complex reacted with antibodies to the LHC I apoproteins affinity purified on the 28 kDa LHC II polypeptide. Protein A-peroxidase was used to probe the blots.

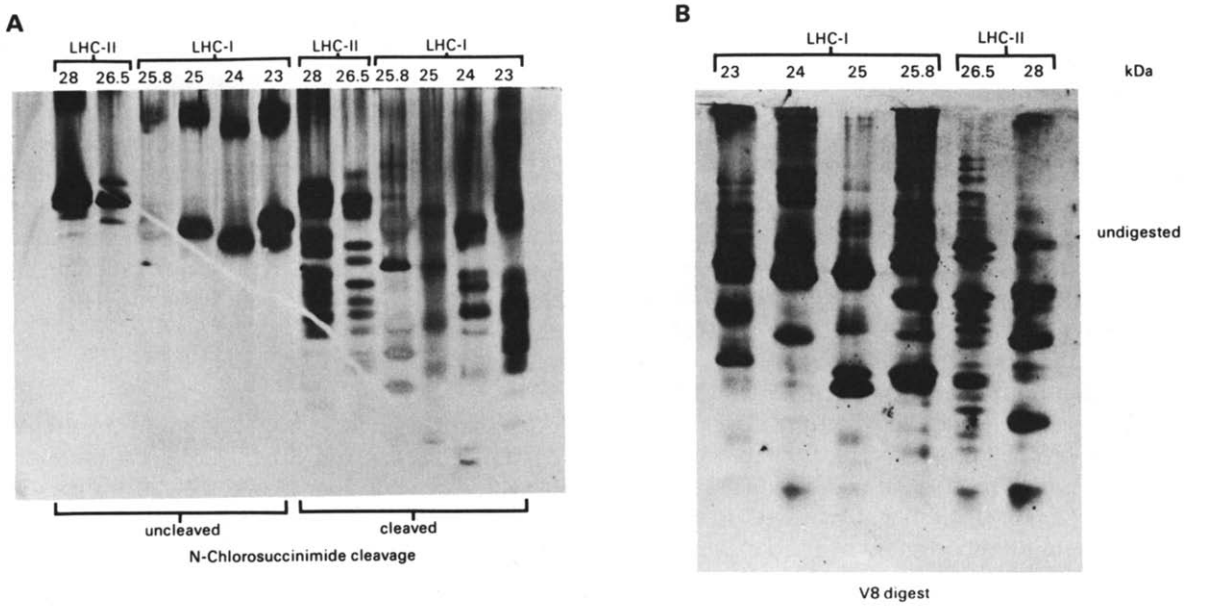


Fig.5. Partial digests of LHC I and LHC II apoproteins with (A) *N*-chlorosuccinimide and (B) *S. aureus* V8 protease. The digests were analyzed as described in section 2. Note that in (B) no reducing agent was used resulting in aggregated protein bands appearing at M_r above that of the undigested polypeptides.

tease. It should be noted that in the presence of urea, the 23 kDa polypeptide runs with a mobility similar to that of the 28.8 kDa polypeptide.

4. DISCUSSION

We prepared polyclonal antisera to 3 of the 4 putative Chl *a/b*-proteins of PS I and found that each antiserum cross-reacted to varying degrees not only with other LHC I apoproteins, but also with LHC II apoproteins. Our results are not due to contamination of the immunizing antigen with the other proteins since antibodies to LHC I apoproteins which had been affinity purified on the 28 kDa LHC II apoprotein recognized the same polypeptides as did crude antisera (fig.4). We made a similar observation with antibodies against LHC I apoproteins isolated on purified LHC I polypeptides immobilized to nitrocellulose (not shown). These results suggest some similarity in the amino acid sequences of the Chl *a/b*-proteins of PS I and PS II since the antibodies were raised to totally denatured apoproteins with presumably little tertiary structure.

The immunological relatedness of the apoproteins of LHC I and LHC II has not been reported before. Polyclonal antisera [12,15] and monoclonal antibodies [8] directed against LHC II did not cross-react with the LHC I apoproteins on immunoblots, although a different monoclonal antibody against LHC II recognized an unidentified protein of M_r 25000 in addition to the two LHC II polypeptides [9]. Also, Ortiz et al. [34] raised an antiserum against a LHC I fraction fluorescing at 730 nm which only recognized the LHC I 20 kDa polypeptide on immunoblots. We do not know why these antisera should have different specificities than those we have prepared, but factors such as the amount of antigen injected, the immunization schedule and the structural state of the antigen can affect the specificity of the antiserum. It is also possible that there are additional epitopes present on LHC II and native LHC I which dominate the antibody response to these antigens.

The nature of the cross-reacting sites is unknown, but apparently they are surface-exposed since the antibodies can be absorbed out with native LHC II (fig.3). Interestingly, it has been predicted that as much as 48% of the 28 kDa LHC II protein will protrude from the stromal side of the membrane [4].

A more intense reaction was seen on immunoblots with the immunizing antigen than with the cross-reactive polypeptides even with antibodies affinity purified on the 28 kDa LHC II apoprotein. This is especially noticeable with the antiserum against the 24 kDa LHC I apoprotein (fig.4), but higher dilutions of the other purified antibodies also gave similar results (not shown). This suggests that the cross-reacting site(s) on the Chl *a/b*-proteins is probably not identical or, alternatively, the antibody binding is influenced by different surrounding sequences.

We compared partial digests of the 6 Chl *a/b*-proteins, and our data suggest that the apoproteins are distinct gene products. However, this type of analysis is best suited to demonstrating that proteins have a precursor/product relationship or for simply determining whether proteins are identical or not. It is not always possible to determine intermediate degrees of sequence homology by this method as has been noted for the α and β-tubulins [27] and for the *Chlamydomonas* LHC II polypeptides [10]. For this reason, we do not exclude some sequence homology among the PS I and PS II Chl *a/b*-proteins.

Our data provide evidence for some degree of antigenic similarity among the Chl *a/b*-proteins of PS I and PS II. The simplest interpretation of these data would suggest some amino acid sequence homology among these polypeptides. A better understanding of the relationships among the light-harvesting proteins will be obtained when more of the genes encoding them have been cloned and analyzed. To date, only genes encoding the 28 kDa of LHC II have been isolated [2–6].

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REFERENCES

[1] Thornber, J.P. (1975) Annu. Rev. Plant Physiol. 26, 127–158.

[2] Coruzzi, G., Broglie, R., Cashmore, A. and Chua, N.-H. (1983) J. Biol. Chem. 258, 1399–1403.
[3] Dunsmuir, P. (1985) Nucleic Acid Res. 13, 2503–2518.
[4] Karlin-Neumann, G.A., Kohorn, B.D., Thornber, J.P. and Tobin, E.M. (1985) J. Mol. Appl. Genet. 3, 45–61.
[5] Broglie, R., Bellemare, G., Bartlett, S.G., Chua, N.-H. and Cashmore, A.R. (1981) Proc. Natl. Acad. Sci. USA 78, 7304–7308.
[6] Dunsmuir, P., Smith, S.M. and Bedbrook, J. (1983) J. Mol. Appl. Genet. 2, 285–300.
[7] Schmidt, G.W., Bartlett, S.G., Grossman, A.R., Cashmore, A.R. and Chua, N.-H. (1981) J. Cell Biol. 91, 468–478.
[8] Thaler, T. and Jay, F.A. (1985) FEBS Lett. 188, 21–26.
[9] Slovacek, R.E. and Harvey, M.A. (1984) Biochem. Biophys. Res. Commun. 123, 995–1001.
[10] Hoober, J.K., Millington, R.H. and D'Angelo, L.P. (1980) Arch. Biochem. Biophys. 202, 221–234.
[11] Apel, K. (1977) Biochim. Biophys. Acta 462, 390–402.
[12] Andersson, B., Anderson, J.M. and Ryrie, I.J. (1982) Eur. J. Biochem. 123, 465–472.
[13] Mullet, J.E. (1983) J. Biol. Chem. 258, 9941–9948.
[14] Haworth, P., Watson, J.L. and Arntzen, C.J. (1983) Biochim. Biophys. Acta 724, 151–158.
[15] Lam, E., Oritz, W., Mayfield, S. and Malkin, R. (1984) Plant Physiol. 74, 650–655.
[16] Anderson, J.M. (1984) Photobiochem. Photobiophys. 8, 221–228.
[17] Remy, R. and Ambard-Bretteville, F. (1984) FEBS Lett. 170, 174–180.
[18] Argyroudi-Akoyunoglou, J. (1984) FEBS Lett. 171, 47–53.
[19] Lam, E., Ortiz, W. and Malkin, R. (1984) FEBS Lett. 168, 10–14.
[20] Bassi, R., Machold, O. and Simpson, D. (1985) Carlsberg Res. Commun. 50, 145–162.
[21] Burke, J.J., Ditto, C.L. and Arntzen, C.J. (1978) Arch. Biochem. Biophys. 187, 252–263.
[22] Ryrie, I.J., Anderson, J.M. and Goodchild, D.J. (1980) Eur. J. Biochem. 107, 345–354.
[23] Higgins, R.C. and Dahmus, M.E. (1979) Anal. Biochem. 93, 257–260.
[24] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[25] Ryrie, I.J., Anderson, J.M. and Glare, T. (1985) Photobiochem. Photobiophys. 9, 145–157.
[26] Hawkes, R., Niday, E. and Gordon, J. (1982) Anal. Biochem. 119, 142–147.
[27] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[28] Merle, D. and Kadenbach, B. (1980) Eur. J. Biochem. 105, 499–507.
[29] Moeremans, M., Daneels, G. and DeMey, J. (1985) Anal. Biochem. 145, 315–321.
[30] Lischew, M.A. and Ochs, D. (1982) Anal. Biochem. 127, 453–457.
[31] Laemmli, U.K. (1970) Nature 227, 680–685.
[32] Sammons, D.W., Adams, L.D. and Nishizawa, E.E. (1981) Electrophoresis 2, 135–141.
[33] Houmard, J. and Drapeau, G.R. (1972) Proc. Natl. Acad. Sci. USA 69, 3507–3509.
[34] Oritz, W., Lam, E., Chollar, S., Munt, D. and Malkin, R. (1985) Plant Physiol. 77, 389–397.