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Expression of the barley psbA gene in *Escherichia coli* yields a functional in vitro photosystem II protein D1

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Abstract

The barley chloroplast psbA gene encoding D1 protein, one of the main photosystem II components, has been over-expressed in *E. coli* cells. The existance of two in vivo expression products, a protein with M_r about 33.5 kDa, corresponding to the full-length precursor of the 32 kDa D1 mature form, and a truncated 29 kDa polypeptide was revealed. A modified D1 protein containing six histidine residues at the carboxy-terminus was also obtained. After isolation and renaturation, the ability of the recombinant D1 protein to bind atrazine and pigments from barley thylakoids was demonstrated.

Key words: Photosystem II; Recombinant D1 protein; Histidine-binding domain; Protein–pigment complex

1. Introduction

Photosystem II (PSII) is a membrane pigment–protein complex present in all oxygenic photosynthetic organisms, which catalyzes the light-induced reduction of plastoquinone by water. The primary light-induced electron transfer reactions of PSII occur within the thylakoid membrane in a heterodimer of two polypeptides termed D1 and D2, which are the key elements of PSII [1]. In higher plants, D1 protein (or herbicide-binding protein) is coded by the psbA gene located in the chloroplast genome as a single copy [2]. In chloroplasts this protein first appears as a precursor form, which has an apparent M_r of 33.5 kDa, and is then processed to a mature form of M_r 32 kDa [3].

To investigate the structure–function relationship of the D1 protein within PSII, work on the creation of a microbiological source of this protein was started. The corresponding psbA gene was isolated from barley chloroplast DNA and sequenced, and its in vitro expression in the rabbit reticulocyte lysate system and then in vivo expression in *E. coli* cells was shown [4–6]. The production of the barley D1 protein by over-expression of its gene in bacteria provides a valuable approach towards an understanding of the complicated interactions at the protein complex level as well as with regard to single amino acid residues in the protein. Thus, it allows the study of assembly of pigment–protein complexes with a uniform D1 protein in the absence of other PSII components. In this paper we report the results on the isolation and renaturation of the recombinant D1 protein and the reconstitution of its complexes with pigments from barley thylakoids.

2. Experimental

The vectors pGT7A and pVTΩA were constructed as described earlier [5,6]. Plasmid pGT7H was obtained from pGT7A as shown in Fig. 1. Oligonucleotides were synthesized by the H-phosphonate method [7]. *E. coli* strain BL21(DE3) was used as host in the expression experiments [8].

Bacterial cultures containing expression plasmids were grown in LB medium containing ampicillin. After the addition of IPTG (1 mM) and incubation at 37°C for 5–7 h, the cells were collected and sonicated as described [6]. The lysate was centrifuged, and the pellet was suspended in 0.5% Triton X-100, 20 mM Tris-HCl (pH 7.5), 1 mM ME. The suspension was centrifuged (15,000 × *g*, 15 min). The pellet was solubilized in 8 M urea, 0.1 M Tris-HCl (pH 8.0), 2% ME. The recombinant D1 protein was renaturated by dialysis against 50 mM Tris-HCl, 0.1% Triton X-100, and then was applied to a Red–Sepharose column (Pharmacia). The column was washed with 50 mM Tris-HCl (pH 6.8), 0.1% OGP, and the purified D1 protein was eluted with 0.3 M NaCl. In the case of D1-His protein, the pellet obtained after sonication of cells was suspended in 6 M guanidine-HCl (pH 8.0) and loaded onto a Ni^{2+}-NTA-agarose column (Qiagen). The purification was performed using a protocol recommended by producer. The bound D1-His protein was renaturated using a linear gradient of 8 M urea, 0.05 M Tris-HCl (pH 8.0)–0.05 M Tris-HCl (pH 6.8), 0.1% OGP, and then was eluted from the column by imidazole. In a parallel experiment, 0.1 mM [^{14}C]atrazine in 0.05 M Tris-HCl, 0.1% OGP was passed through the column with the renaturated D1-His protein. Proteins were analysed by 12% SDS-PAGE [9] and by Western blotting [6] with rabbit antibodies against mature barley D1 protein isolated as described [10,11].

A PSII reaction center complex consisting of D1 and D2 proteins and cytochrome b_{559} was isolated from barley thylakoids [10]. Pigment extracts were obtained according to [12] and dried under nitrogen. The

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Abbreviations: SDS, sodium dodecylsulfate; ME, β-mercaptoethanol; OGP, octyl-β-D-glucopyranoside; MES, morpholinoethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

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solution of D1 protein (100–150 μg/ml) in buffer containing 50 mM MES (pH 8.0), 10% glycerol, 1 mM dodecyl-β-D-maltoside, 0.05% SDS and 15 μM DTT was added to the dry green film of pigments and lipids. After gentle shaking for 24 h at 5°C in darkness under nitrogen, and following centrifugation (10,000 × g, 15 min), the pigment–protein complex solution obtained was analysed by native PAGE in the presence of Deriphate-160 [13]. Analytical IEF was performed at 4°C using a polyacrylamide gel containing 0.1% ampholytes (LKB Ampholine, pH 4–6) and 0.1% maltoside.

3. Results and discussion

Initially our attempts to clone the barley psbA gene in to several vectors failed due to the toxicity of its expression product for bacteria. We overcame this problem with plasmid pGEM1 and vector pVEV (previously constructed by us [5]), which are suitable for both in vivo and in vitro expression [6]. The psbA gene was cloned into pGEM1 and pVEV plasmids under the control of the T7 promoter. The resulting plasmids pGT7A and pVTΩA provided the desired protein synthesis only when they were introduced into an *E. coli* strain carrying the cloned T7 RNA polymerase gene in the chromosome under the lac UV5 promoter, such as BL21(DE3) [8]. As a variant, a hybrid of D1 protein with a peptide containing the poly-histidine domain situated at the D1 C-terminus was obtained (plasmid pGT7H, Fig. 1).

The yield of the target protein in *E. coli* BL21(DE3) cells transformed with pGT7A, pVTΩA and pGT7H was estimated as 20–25% of the total cell protein content.

Two products which migrated at the positions of 33.5 kDa (corresponding to the unprocessed D1 barley protein) and about 29 kDa were observed on SDS-PAGE (Fig. 2). Confirmation that the bands corresponded to the desired products was provided by Western-blotting analysis. The presence of a lower molecular weight protein is in agreement with the existance in the psbA gene of two potential transcription start sites in the same reading frame, Met-1 and Met-37. Moreover, the psbA gene contains a sequence similar to a prokaryotic Shine-Dalgarno sequence just upstream of the Met-37 codon. Our results correlate with data obtained earlier on the expression of psbA genes of other higher plants in prokariotic in vitro translation systems [3,14]. At the same time, in our experiments on in vitro expression of the barley psbA gene in the rabbit reticulocyte lysate system, a major expression product of M_r ~33.5 kDa, and only a small amount of 29 kDa protein was observed [6].

The analysis of the distribution of target proteins between the pellet and the supernatant fractions of cell lysates showed that the recombinant D1 protein, being hydrophobic, was present in cell the lysate debris. Following washing procedures the majority of contaminating bacterial proteins were removed from the insoluble material, and the crude desired protein was solubilized from inclusion bodies by 6 M guanidine-HCl, or 8 M urea. For renaturation, samples of the partially purified recombinant D1 protein were dialyzed against a buffer containing Triton X-100. Further purification was

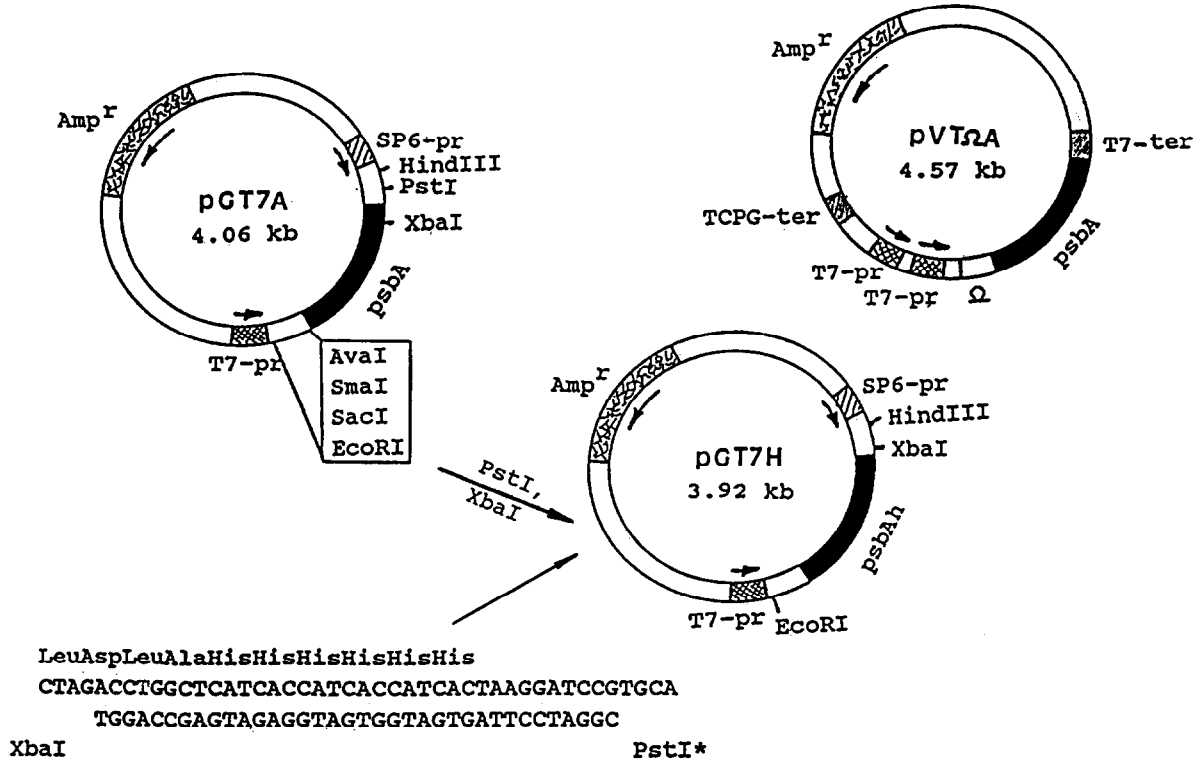

Fig. 1. The expression plasmids pGT7A, pVTΩA and pGT7H. Pr, promoter region; ter, sequence corresponding to terminator region; Ω, enhancer of mRNA translation.

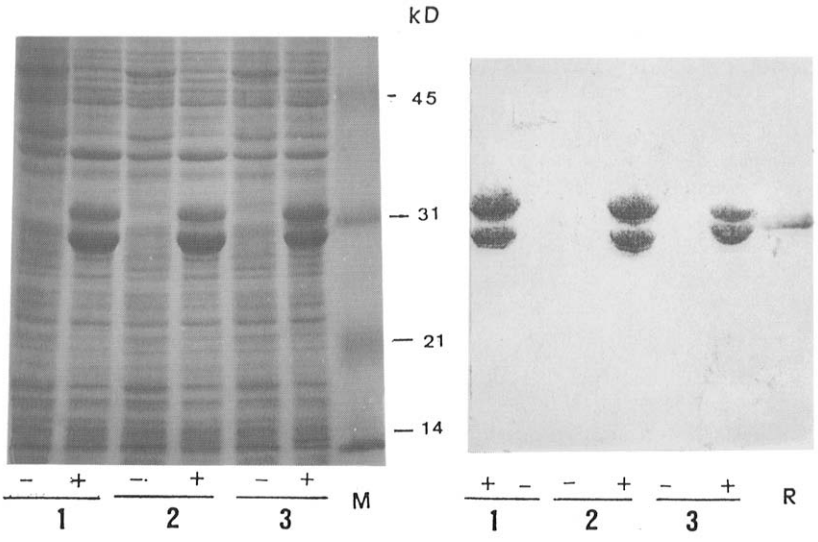

Fig. 2. Expression of D1 protein by *E. coli* BL21(DE3) containing recombinant plasmids pGT7A (1), pVTΩA (2) and pGT7H (3). Extracts from bacterial cells before (−), or after (+) induction by IPTG were analysed by SDS-PAGE with the following staining of the gel with Coomassie blue (A) and by Western blot analysis (B). The bands representing recombinant proteins are marked by arrows. Lane R, mature D1 protein. Lane M contains molecular weight size markers.

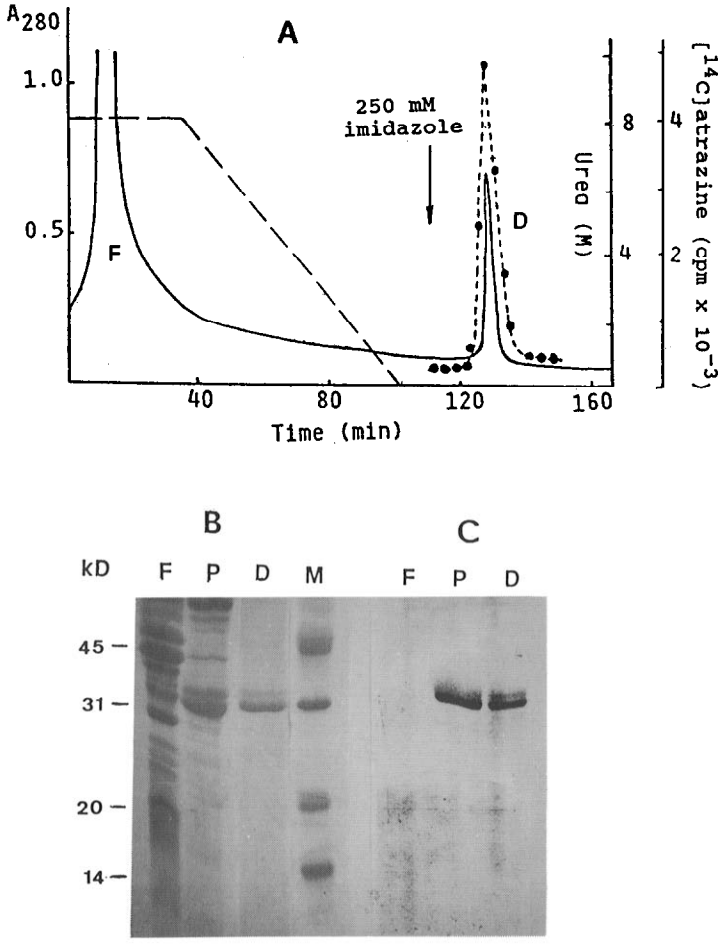

Fig. 3. Purification of the recombinant D1-His protein by affinity chromatography on Ni^{2+}-NTA-agarose. (A) UV absorbance profile of the guanidine-HCl solubilized material (P) after chromatography. Fraction (F) indicates the flowthrough, fraction (D) contained material eluted in the presence of imidazole. Binding of [^{14}C]atrazine to D1-His protein is shown by a dotted line. Fractions were analysed by SDS-PAGE (B) and Western blotting (C).

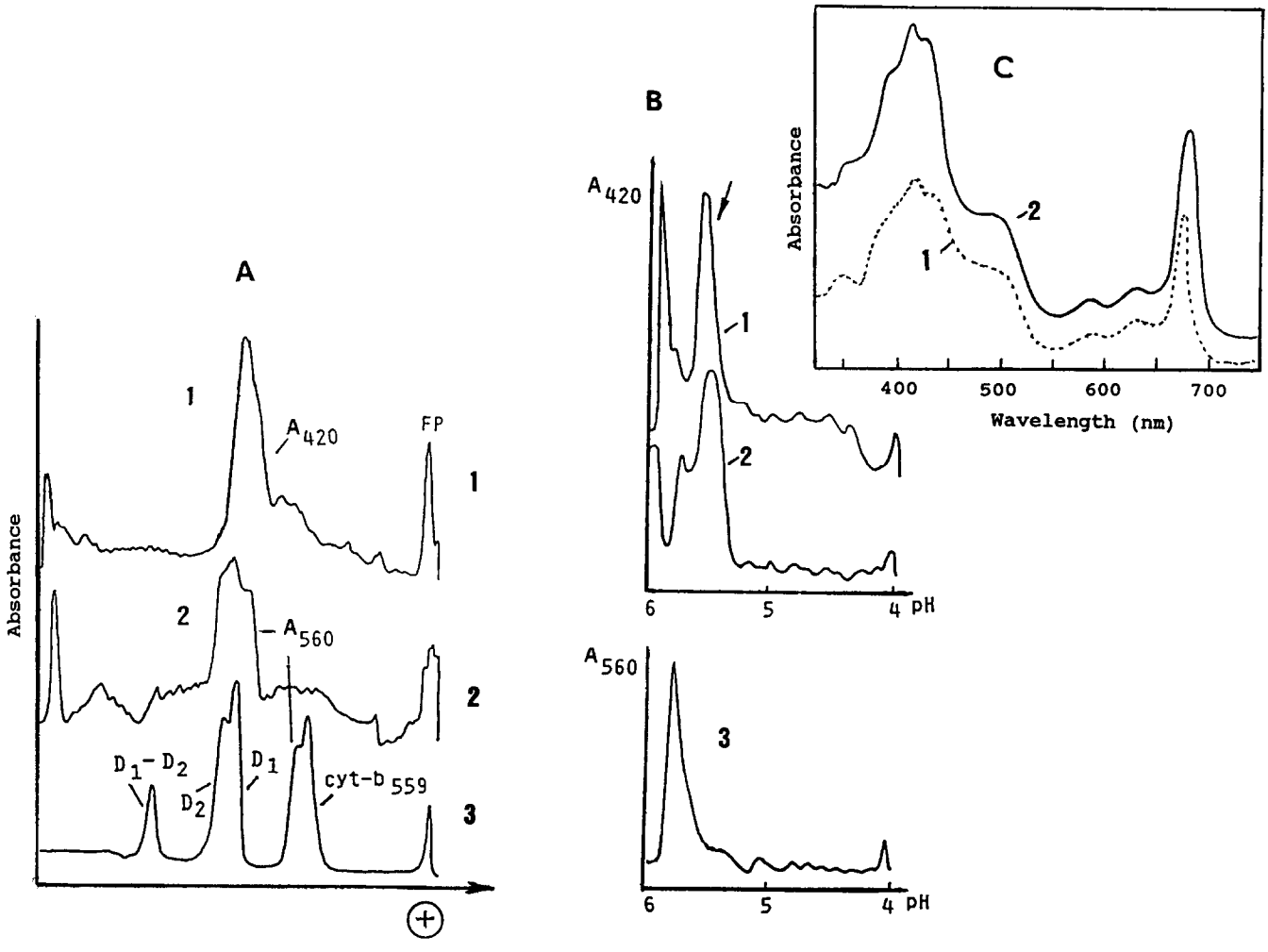

Fig. 4. Reconstitution of the recombiant D1 protein with a total pigment extract from barley thylakoids. (A) Densitometric evaluation of PAGE under native conditions ('Green gel') of the reaction mixture containing the partially purified recombinant D1 protein and pigment extracts at 420 nm (1). After the staining with Coomassie blue, the gels containing D1–pigment complex (2) and mature complex D1-D2-Cyt b_{559} (3) were scanned at 560 nm. (B) Densitometer tracing of the purified recombinant D1 protein–total pigment complex extract (1) resolved by IEF. Curve (2) represents mature D1-D2-Cyt b_{559} complex. Curve (3) represents densitometric evaluation of the Coomassie blue staining of the recombinant D1 protein by IEF analysis. (C) Absorption spectra of the recombinant D1 protein–pigment complex (1) isolated from an IEF gel band marked by an arrow and the mature complex D1-D2-Cyt b_{559} (2).

achieved by chromatography on a triazine dye column [15]. The D1-His protein was purifid to homogeneity by affinity chromatography using Ni^{2+} as a ligand [16]. It was renaturated directly on Ni-agarose by gradual dilution of the denaturing agent and eluted from the column by the addition of imidazole (Fig. 3).

In order to assess the affinity of the constructed proteins to herbicides, a solution of ^{14}C-labelled atrazine was passed through the column with the renaturated D1-His protein immobilized on Ni-agarose. After washing, the bind material was eluted from the column by imidazole solution, and the radioactivity of the fractions collected during elution was measured (Fig. 3A). It follows from the data obtained that the recombinant D1 protein is able to bind atrazine.

The recombinant D1 and D1-His proteins were used for in vitro reconstitution experiments with pigment extracts from barley thylakoids. The reconstitution products were analysed by PAGE in non-denaturing conditions (Fig. 4A). A complex was observed on the gel as a green band that had a somewhat higher mobility than the free protein. Samples of the protein–pigment complexes were also subjected to IEF. Densitometric evaluation of the gels showed that the recombiant D1 protein–pigment complexes migrate to a position in the gradient which indicated an isoelectric point (pI) close to the pI of one of the bands characteristic for the native D1-D2-Cyt b_{559} complex of the PSII reaction center. Moreover, the shift of the pI point of the D1 protein after binding with pigment extracts to a lower pH is consistent with binding of the D1 protein to the negatively charged lipids from pigment extracts (Fig. 4B).

To find out whether the reconstituted D1-pigment complexes are structurally related to the native D1-D2-Cyt b_{559} complex from barley thylakoids, we compared their spectroscopic properties after isolation from the polyacrylamide gel. The absorption spectra at room temperature of the recombinant D1 protein-pigment complexes are very similar to that of the D1-D2-Cyt b_{559} PSII complex (Fig. 4C). Thus, in both spectra the chlorophyll *a* absorption peak is observed near 670 nm. The presence of carotenoids is indicated by the absorption shoulders around 460–490 nm, and the presence of pheophytin is indicated by a strong absorption peak at about 415 nm [13]. The complexes of pigments with D1-His protein and apoprotein isolated from barley show similar characteristics. From these data we conclude that the pigment arrangement in the recombinant D1 protein–pigment complexes assembled in vitro closely resemble the ones in native complexes isolated from thylakoids, and that D1-pigment assembly in vitro is specific.

Thus, the results obtained have shown for the first time the construction of *E. coli* strains producing recombinant D1 protein of cereals at high levels, and its ability to bind atrazine. We have also shown that this protein can be reconstituted with pigments (chlorophyll *a*, β-carotene and pheophytin *a*) in the presence of lipids to yield specific pigment–protein complexes. Experiments on the reconstitution of the PSII core complex with the recombinant D1 protein are now in progress.

References

[1] Nanba, O. and Saton, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[2] Zurawski, G., Bohnert, H.J., Whitfield, P.R. and Bottomley, W. (1982) Proc. Natl. Acad. Sci. USA 79, 7699–7703.
[3] Eyal, Y., Goloubinoff, P. and Edelman, M. (1987) Plant Mol. Biol. 8, 337–343.
[4] Efimov, V.A., Andreeva, A.V., Reverdatto, S.V., Jung, R. and Chakhmakhcheva, O.G. (1988) Nucleic Acids Res. 16, 5685.
[5] Reverdatto, S.V., Beilinson, V.A., Fradkov, A.F., Polushin, N.N. and Efimov, V.A. (1991) Nucleic Acids Symp. Ser. No. 24, 306–307.
[6] Efimov, V.A., Reverdatto, S.V., Beilinson, V.A., Fradkov, A.F. and Chakhmakhcheva, O.G. (1994) Bioorgan. Khimia (Rus) 20, 524–535.
[7] Efimov, V.A., Kalinkina, A.L. and Chakhmakhcheva, O.G. (1993) Nucleic Acids Res. 21, 5337–5344.
[8] Sano, T. and Cantor, C. (1991) Biochem. Biophys. Res. Commun. 176, 571–577.
[9] Laemmli, U.K. (1970) Nature 227, 680–685.
[10] Nanha, O. and Saton, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[11] Adir, N. and Ohad, I. (1988) J. Biol. Chem. 263, 283–289.
[12] Paulsen, H., Rumler, U. and Rudiger, W. (1990) Planta 181, 204–211.
[13] Peter, C.F. and Thornber, J. (1991) Plant Cell Physiol. 32, 1237–1250.
[14] Cohen, B.N., Coleman, T.A., Schmitt, J.J. and Wiessbach, H. (1984) Nucleic Acids Res. 12, 6221–6230.
[15] Lowe, C.R., Small, D. and Atkinson, A. (1981) Int. J. Biochem. 13, 33–40.
[16] Smith, M.C., Furman, T.C., Ingolia, T.D. and Pidgeon, C. (1988) J. Biol. Chem. 263, 7211–7215.