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Development of Polyclonal Antibodies against Membrane Protein of Higher Plant Plastids

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ABSTRACT

Over-expression of a highly hydrophobic protein in *E. coli* cells is difficult due to the development of inclusion bodies or cyto-toxic effects of the protein. Therefore, it is believed to be a pre-requisite to develop antigen by expression of hydrophilic region of the protein flanked by hydrophobic spans, provided that region carries antigen properties. In these studies, antibodies against the hydrophilic stretch between the first two hydrophobic regions spanned by 76 amino acid residues of *ycf10*, a membrane protein from tobacco chloroplasts, were raised. Western blots on expressed *ycf10* protein in *E. coli* cells and on membrane fractions of the chloroplasts as well as crude extracts from wild-type tobacco plants confirmed the specificity of the antibodies.

Key Words: *ycf10; E. coli*; Hydrophobic; Hydrophilic; Plastid; Polyclonal antibodies

INTRODUCTION

The open reading frame *orf229* known as *ycf10* lies upstream of the *petA* gene (Willey & Gray, 1989, 1990). *ycf10* was first identified in pea chloroplasts, conserved in higher green plants, and co-transcribed with cytochrome *f* (Willey *et al*., 1984a, b; Willey & Gray, 1989), but is not present in *Epifagus* and *Euglena* plastid DNA. The product of *ycf10* has been localized in the inner envelope membrane of pea chloroplasts (Sasaki *et al*., 1993; Craig, 1993).

The amino acid sequences of *ycf10* from tobacco, pea, rice and maize are highly conserved. The protein sequence shows four extended hydrophobic regions and a putative processing site for a processing peptidase (Willey & Gray, 1990). The putative processing site is located at the *C*-terminal side of the first hydrophobic region and cleavage would result in the removal of the *N*-terminal 24 amino acid residues, which have proposed to be a signal peptide responsible for the targeting of *ycf10* (Willey & Gray, 1990). The second and the third hydrophobic stretches are long enough to be α–helical trans-membrane spans, although the fourth hydrophobic domain is shorter than the previous three and no function was assigned. From the sequence, a fragment of 76 amino acid was PCR amplified and antibodies were raised against this peptide. These antibodies recognized *ycf10* gene product as a protein with the mobility of a 34 kDa protein on SDS-PAGE. In this paper, highly pure and specific antibodies has been raised that recognized *ycf10* protein expressed in *E. coli* cells as well as of tobacco chloroplasts.

MATERIALS AND METHODS

Generation of plasmid construct to over-express *YCF10*. The pMSK27 plasmid was constructed, as described in the result section, using pET-16B-kan expression system. A fragment of 228 bp of *ycf10* (nucleotides 63489-63716; Shinozaki *et al*., 1986) was amplified by PCR and cloned at *Nde*I and *Bam*HI sites in the pET-16B-kan vector.

Transcription and translation *in vitro*. The transcription of a cloned fragment in pET-16B-kan (Webster *et al*., 1997) was carried out after linearsing the vector with *Bam*HI. After digestion the linearised plasmid was purified using phenol: chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in de-ionized water at a concentration of 1 μg/μL. The transcription reactions were set up as follows: 2 μL of 1 mM CAP [m^7 G (5') ppp (5') G] dilithium salt, 4 μL of nucleotide mix consisting of 5 mM ATP, 5 mM CTP, 5 mM UTP and 1 mM GTP, 2 μL of 10 x transcription buffer (Boehringer Mannheim), 1 μL of RNase inhibitor (RNasin, Promega, Southampton, UK), 7 μL demonized autoclaved water, 2 μg (1 μg/μL) linearised plasmid DNA and 2 μL (40 U) of T7 RNA polymerase. The transcription reactions were incubated for 30 min at 37˚C then 2 μL of 8 mM GTP was added and the reactions were incubated for a further 30 min at the same temperature.

To carry out translation of the mRNA produced *in vitro*, three different volumes (1, 2 and 3 μL) of transcription-assay mix were used and for each volume of mRNA, three different volumes (0.3, 0.6 and 0.9

µL) of 1 M potassium acetate, were used to determine the conditions most favorable for translation. All reactions contained 5 µL wheat germ extract (Promega), 0.2 µL RNasin (8 U), 0.8 µL 1 mM amino acids without methionine and cysteine and 0.5 µL (7.5 µCi) [^{35}S]-methionine and [^{35}S]-cysteine (specific activity >1000 Ci/mmol; Amersham). Each set of reactions containing transcription-assay mix and potassium acetate in variable amounts was mixed on ice and incubated at 25˚C for 1 h. Five µL of each reaction with 2 µL of 2 x loading buffer (80 mM Tris-HCl pH 8.8, 10% (v/v) glycerol, 10% SDS (w/v), 0.002% (w/v) bromophenol blue 5% (v/v) 2-mercaptoethanol) were boiled for 3 min followed by electrophoresis of the translation products on a SDS-20%-polyacrylamide gel.

Expression of *ycf10* protein in *E. coli.* *E. coli* BL21 cells were transformed by electroporation with the pET-16B-kan expression vector containing *ycf10*, plated onto LB agar plates containing kanamycin (10 µg/mL), ampicillin (50 µg/mL) and chloramphenicol (34 µg/mL) and incubated overnight at 37˚C in an incubator. A single colony was picked and used to inoculate 5 mL LB liquid culture containing the same antibiotics. The inoculated culture was incubated overnight at 37˚C in a shaking incubator (200 rpm) and was used in expression cultures. Three sets of 10 mL cultures were grown to an OD_{600} of 0.5, 0.6 and 0.7 (measured with a Perkin Elmer Lambda 9 spectrophotometer) and induced with 2 mM IPTG. Time points were taken at 0, 30, 60, 90, 120 min and 16 h, and cells were collected by centrifugation. The pellets were resuspended in protein loading buffer, boiled for 3-5 min and loaded onto SDS-18% polyacrylamide gel to resolve the protein fragments. After electrophoresis was complete, the gel was stained with Coomassie blue.

Determination of the solubility of expressed protein. A 500 ml culture was used to scale up the production of expressed protein, and 100 ml from this was used to determine the solubility of the expressed protein. *E. coli* cells were harvested at OD_{600} of 0.60 by centrifugation at 500 *g* for 5 min. They were resuspended in 3 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) for each gram of cells followed by the addition of 8 µL of 50 mM PMSF and 80 µL of lysozyme (10 mg/mL) for each gram of cells. About 2 g of cells were lysed and the mixture was stirred with a glass rod for 20 min at 4˚C. Deoxycholic acid (4 mg per gram of *E. coli* cells) was added to the mixture while stirring continuously. After this, the lysate was placed at 37˚C in a water bath and stirred until it became viscous. DNAase I (1 mg/mL 20 µL per gram of *E. coli* cells) was added and the lysate was placed at room temperature for about 30 min until it was no longer viscous. The lysate was centrifuged for 30 min at 15,000 *g* to separate the soluble and insoluble fractions. The supernatant was poured off into a separate tube and the pellet was washed three times with lysis buffer to remove soluble protein. A small fraction of the pellet was resuspended in 1 x loading buffer (80 mM Tris-HCl pH 8.8, 10% (v/v) glycerol, 10% SDS (w/v), 0.002% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol) and 5 µL of the supernatant was added to 5 µL of 2 x loading buffer and these samples were boiled for 2-3 min at 100˚C. The samples were centrifuged at 11,500 *g* for 5 min and loaded onto an SDS-20% polyacrylamide gel. After electrophoresis was complete, the gel was stained with Coomassie brilliant blue.

Protein purification under denaturing conditions. Purification of insoluble proteins was carried out according to the methods described in the QIAGEN plasmid handbook (QIAGEN, Hilden, Germany). A 500 ml culture was grown and induced for protein expression. The cells were harvested by centrifugation at 4,000 *g* for 20 min and stored at -80˚C. The cells were placed on ice to thaw and were resuspended in buffer A (6 M guanidinium-HCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 8.0) at 5 mL per gram wet weight of cells (~ 2 g of cells were used) and stirred for 1 h at room temperature. The lysate was centrifuged for 15 min at 4˚C at 10,000 *g* and the supernatant was removed into a fresh 30 ml tube. A 50% (2 mL) slurry of Ni-NTA resin, previously equilibrated in buffer A, was added to the supernatant, mixed by shaking for 45 min and then poured into the barrel of a 5 ml syringe which had been blocked with porous polyethylene sheet (Omnifit, Cambridge, UK). The column was washed under gravity flow with 10 column volumes of buffer A, 5 volumes of buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 8.0), and 5 volumes of buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 6.3). After washing the column, the recombinant proteins were sequentially eluted with 3 ml volumes of buffer C, containing 20, 100, 200, 300 and 500 mM imidazole. The eluate was collected in separate tubes.

Rabbit's immune system response to antigen. The antigen was prepared by mixing 0.5 mL (1 mg/mL) of protein in 10% (w/v) SDS with 0.5 mL of Freund's complete adjuvant (Sigma) in a syringe until it formed a stable milky emulsion. Rabbits were injected at 4 subcutaneous sites with 0.25 mL of the emulsion per injection. Subsequent injections were given at two-week intervals using 0.5 mL of protein solution with 0.5 mL of incomplete Freund's adjuvant (Sigma). Before giving each injection, the rabbit was bled (5 mL) from a

marginal ear vein. Serum was prepared by incubating the blood at 37˚C for one hour and then subsequently storing it at 4˚C overnight for the clot to retract. The yellowish serum was removed from outside the clot with a Pasteur pipette and centrifuged at 10,000 *g* for 5 min to remove red blood cells and other particulate matter. Aliquots (0.8 mL) of serum were stored at -80˚C.

Immunochemical detection of proteins. Proteins separated by SDS-PAGE were transferred to nitrocellulose by a method based on that of Towbin *et al.* (1979). The SDS-polyacrylamide gel was equilibrated in transfer buffer (25 mM Tris pH 8.3, 192 mM glycine, 20% (v/v) methanol, 0.1% (w/v) SDS) for 30 min with gentle agitation. The proteins were electrotransferred using an AE-6670 Horizblot semi-dry electroblotting apparatus (ATTO Corporation, Tokyo, Japan). The lower carbon anode was wet with transfer buffer over which four Whatman 3MM paper sheets, which had been immersed in transfer, buffer were stacked. A sheet of 0.45 µm nitrocellulose (BA 85 membrane filters from Schleicher and Schüll, Dassel, Germany) was pre-wet with transfer buffer and placed on top of the Whatman 3MM paper. The equilibrated gel was placed on top of the nitrocellulose, making sure no air bubbles were trapped. Four more pre-wet Whatman 3MM sheets were placed on top of the gel before clamping the membrane/paper sandwich between the two carbon electrodes. Transfer was carried out at room temperature with a current of 1.5 mA/cm^2 of the gel area for 75 min. Once the transfer was complete, the membrane was blocked with 30 ml TBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) containing 3% (w/v) BSA. After incubation for 1 h, the membrane was washed twice for 5 min in TTBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.05% (v/v) Tween-20). The membrane was then incubated for 2 h with 25 mL of TTBS containing 1% (w/v) BSA and 25 µL of immune serum. The filter was washed twice with TTBS at room temperature with gentle agitation. It was then incubated for another 1 h with the second antibody, a biotinylated-anti-rabbit antibody (Amersham). The antibody was diluted 1:1000 in TTBS with 1% (w/v) BSA. The membrane was washed twice for 5 min with TTBS and then incubated with streptavidin-biotinylated horseradish peroxidase (Amersham) diluted 1:5000 in TTBS containing 1% (w/v) BSA at room temperature for ~1 h. The membrane was washed three times with TTBS. After the antibody incubations and washings were complete, the membrane was placed on a piece of Whatman 3MM paper and 4 mL of detection reagent (2 ml ECL1 mixed with 2 mL ECL2, Amersham) was poured onto the membrane and incubated for few minutes. Excess reagent was drained from the membrane and the membrane wrapped in Saran Wrap. After placing in a film cassette, the membrane was exposed with a sheet of high performance luminescence detection film (Hyper film ECL, Amersham) for a few seconds to several minutes depending on the antibodies used and the background. The film was then processed in a Fuji automatic X-ray processor.

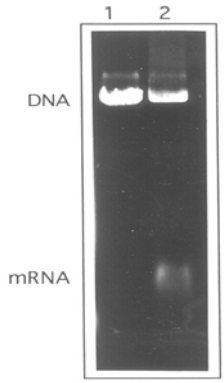
RESULTS AND DISCUSSION

Generation of plasmid construct to over-express *YCF10*. The hydrophilic region of 228 bp, amino acid residues 28-103, between the first two hydrophobic regions was PCR amplified using forward primer, 5'-GGGAATTCCATATGAATAAATGTCTGGAATCT-3' (*Nde*I site underlined) complementary to a sequence in the chloroplast genome (position 63489-63506; Shinozaki *et al*., 1986) and reverse primer, 5'-CGCGGATCCACGATTCTCATTTTGTTAGAA-3' (*Bam*HI site underlined, position 63700-63716; Shinozaki et *al*., 1986) and cloned into pET-16B-kan (Webster *et al*., 1997) into *Nde*I and *Bam*HI sites. The presence of the inserted fragment was confirmed by digestion with *Nde*I and *Bam*HI. The resulting plasmid was called pMSK27 (Fig. 1). Nucleotide sequence analysis of the plasmid confirmed that the insert was in frame with the His-tag of expression cassette pET-16B-kan.

Fig. 1. Generation of *ycf10* expression construct. A DNA fragment of 228 bp was PCR amplified and cloned into pET-16B-kan into *Nde*I and *Bam*HI sites to generate pMSK27

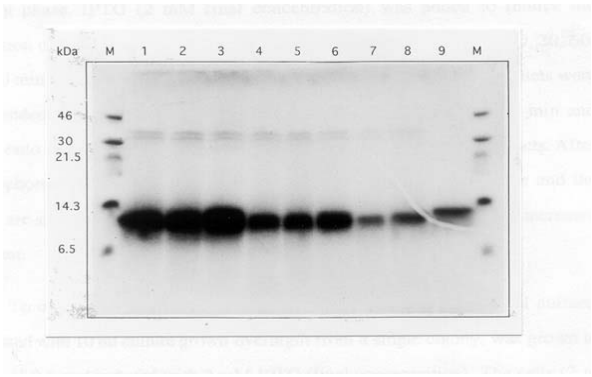
Confirmation of *ycf10* gene expression in *E. coli*. The plasmid pMSK27 was used for transcription and translation *in vitro* to ensure that the plasmid gives the correct protein product before using the construct for express in *E. coli.* The plasmid was linearised by digestion with *Bam*HI at the 3' end of the inserted fragment. The linearised plasmid was purified by phenol: chloroform extraction and ethanol precipitation and transcribed with T7 RNA polymerase. In Fig. 2, lane 1 contains the linearised plasmid DNA template, and lane 2 contains the transcription reaction mixture showing RNA running at the bottom. It is difficult to estimate the size of the RNA in the ordinary agarose gel. The transcription reaction was used in translation reactions with wheatgerm extract and [^{35}S]-labelled methionine and cysteine. The translation products were

Fig. 2. Transcription of cloned *ycf10* region *in vitro*. The plasmid pMSK27 was linearised by digestion with *Bam*HI and linearised DNA was incubated for transcription *in vitro*. DNA and RNA was stained with ethidium bromide and visualized under UV light. Lane 1 shows linearised pMSK27 and lane 2 transcription reaction product



analysed by SDS-PAGE and fluorography. A single product of the expected size (11.5 kDa) was obtained (Fig. 3). Several sets of translation reactions were carried out to optimize the translation reactions for maximum yield. Three amounts of RNA (1,2 and 3 µg) were used and for each amount of RNA, three different concentrations of added potassium acetate (30, 60 and 90 mM) were used. Fig. 3 shows that in the first three reactions where 1 µg RNA was used, the maximum

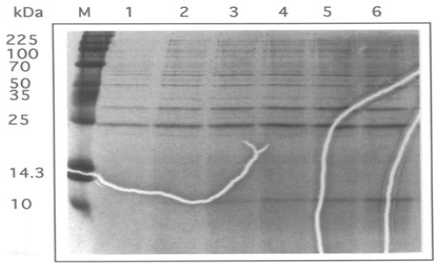
Fig. 3. Translation of RNA transcribed from cloned *ycf10* region was carried out and the reaction products were subjected to SDS-PAGE through 18% polyacrylamide gel. M is [^{14}C]-methylated protein marker; lanes 1, 2 and 3 show protein products obtained using RNA product (1 µg) and 30, 60 and 90 mM potassium acetate, respectively. Lanes 4, 5 and 6 show protein products for 2 µg RNA product and lanes 7, 8 and for RNA products of 3 µg, respectively



amounts of the translation product was obtained in lane 3 where 90 mM potassium acetate was used. In the second and third sets of reactions, where 2 µg and 3 µg RNA per reaction were used, similar results were obtained. From these results it is concluded that 1 µg RNA and 90 mM potassium acetate was optimum to obtain the maximum amount of translation product.

Purification of *ycf10* gene product under denaturing conditions. The expression of the *ycf10* fragment in pET-16B-kan was carried out in *E. coli* strain BL21 (Studier & Moffat, 1986) under the control of T7 promoter. *E. coli* cells harboring pMSK27 were grown in 10 mL LB medium containing ampicillin (50 µg/mL), kanamycin (10 µg/mL) and chloramphenicol (34 µg/mL) at 37˚C with constant shaking until reached mid-log phase. IPTG (2 mM final concentration) was added to induce the expression of the T7 polymerase and aliquots of 10 µL were taken after 0, 30, 60, 90, 120 min and 16 h and cells were collected by centrifugation. The pellets were resuspended in protein loading buffer, boiled for 3-5 min and loaded onto SDS-18% polyacrylamide gel to resolve the protein products. After the electrophoresis was complete, the gel was stained with coomassie blue and the results are shown in Fig. 4. The expression of a protein of 11.5 kDa increased with time.

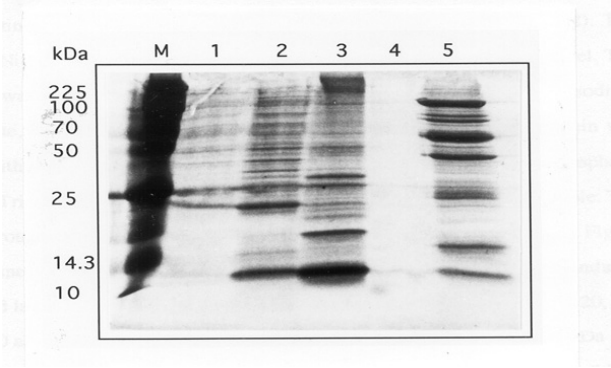
Fig. 4. Expression of *ycf10* protein in *E. coli* cells. Cells from samples (10 µL) were subjected to SDS-PAGE and proteins were visualized by coomassie blue staining. M is molecular marker. Lanes 1, 2, 3, 4, 5 and 6 show time points at 0, 30, 60, 90, 120 min and 16 h after incubation



To examine whether the induced protein was soluble, a 500 mL culture, inoculated with 10 mL culture grown overnight from a single colony, was grown to OD600 of 0.6 and induced with 2 mM IPTG (final concentration). The cells (2 g) harvested 3 h after induction were lysed using 6 mL lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) followed by the addition of 16 µL of 50 mM PMSF and 160 µL of lysozyme (10 mg/mL) and stirred continuously at 4˚C for 20 min followed by 30 min incubation at 37˚C. Dnase I (40 µL) was added and the

lysate was placed at room temperature for about 30 min until it was no longer viscous. The mixture was centrifuged and small fractions of the supernatant (5 µL) and pellet (~5 µg) protein were boiled with protein loading buffer and subjected to SDS-PAGE. The results

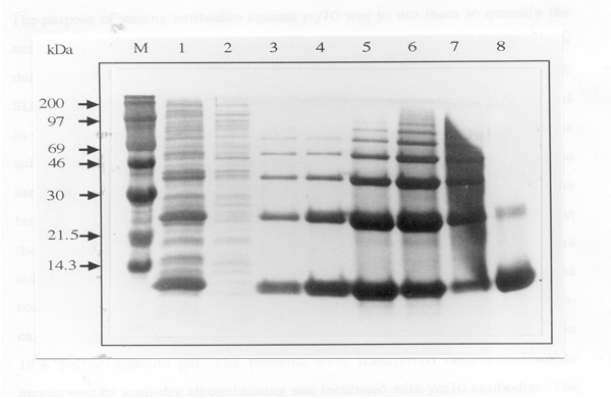
Fig. 5. Solubility of expressed *ycf10* protein in *E. coli* cells. M is molecular weight marker from sigma. Lane 1 is uninduced cells, lane 2 induced cells and lanes 3 and 5 insoluble (pellet) and soluble (supernatant) fractions, respectively



are shown in Fig. 5, where lane 1 shows proteins from uninduced cells, lane 2 from induced cells, lane 3 pellet and lane 5 supernatant fractions from induced cells. Lane 3 shows that most of the 11.5 kDa protein is insoluble although a small amount is found in the supernatant (lane 5).

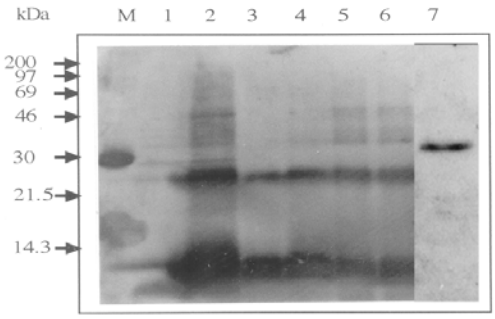
The harvested cells from the 500 mL culture described above were solublized under denaturing

Fig. 6. Purification of expression *ycf10* protein on Ni-NTA resin under denaturing conditions. Samples were subjected to SDS-PAGE and proteins were visualized by coomassie blue. M is marker, lanes 2 and 3 show proteins from induced and uninduced cells, respectively. Lanes 3, 4, 5, 6 and 7 show protein eluted with elution buffer containing 20, 50, 100, 200 and 500 mM imidazole, respectively. Lane 8 shows protein precipitated with 80% acetone



conditions using 10 mL buffer A (6 M guanidium-HCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl pH 8.0) and the soluble fraction was incubated with 2 ml of a 50% slurry of Ni-NTA resin, previously equilibrated in buffer A, with continuous slow shaking at room temperature for 1-2 h. The swollen Ni-NTA resin was used to make a column using a 5 ml syringe barrel. The column was washed with washing buffers B and C (8 M urea, 0.1 % sodium phosphate, 0.01 M Tris-HCl) of different pH (8.0 and 6.3) and the protein was eluted with 3 ml aliquots of elusion buffer C containing 20, 50, 100, 200 and 500 mM imidazole. The eluted proteins were subjected to electrophoresis and the results are shown in Fig. 6. Lane 1 shows proteins from induced cells; lane from uninduced cells, lane 3-7 show the proteins eluted with elution buffer containing 20, 50, 100, 200 and 500

Fig. 7. Protein *ycf10* expressed in *E. coli* cells and from tobacco chloroplasts probed with *ycf10* antibodies. M is molecular weight marker. Lanes 1 and 2 show proteins from uninduced and induced cells, respectively. Lanes 3-7 show eluted protein obtained as Figure 6. Lane 8 shows the protein from tobacco chloroplasts



mM imidazole. Lanes 3-7 show bands of 11.5, 22, 44 kDa and of higher molecular weight which are probably polymers of the expressed 11.5 kDa protein. These polymeric forms disappeared on precipitating the protein with 80 % acetone and solublization of the protein pellet in 10% SDS by boiling (lane 8).

The protein fraction eluted with buffer containing 100 mM imidazole was precipitated with 80% acetone and redisolved in 10% SDS, electrophoresed on an 18% polyacrylamide gel and electroblotted onto PVDF membrane. Both 11.5 and 44 kDa proteins were subjected to *N*-terminal sequencing. Both were found containing histidine residues. On the basis of these observations, it was likely that the expressed proteins were *ycf10* with an *N*-terminal His-tag.

Preparation and characterization of rabbit polyclonal antibodies. To prepare antibodies, 0.5 mg (in 0.5 mL of 10% SDS) of the purified expressed protein,

precipitated with 80% acetone and dissolved in 10% SDS, was mixed with 0.5 mL of Freund's complete adjuvant and injected subcutaneously into the rabbit at four different sites. Subsequent injections used the same amount of protein mixed with Freund's incomplete adjuvant. The rabbit was bled before each injection. The blood clotted on incubation at 37˚C for 1 h and was then placed at 4˚C overnight for the clot to retract. The yellowish serum was collected by centrifugation and stored at –80˚C. In order to examine if the antibodies recognize the *ycf10* protein, western blot analysis was carried out on E. coli expressed protein. *E. Coli*-expressed proteins were subjected to SDS-PAGE on an 18 % polyacrylamide gel. The proteins were transferred onto nitrocellulose membranes by semi-dry electroblotter and incubated with *ycf10* antibodies. The results are shown in Fig. 7 that demonstrates that the antibodies reacted with 11.5 and 22 kDa proteins (lanes 2-7). Lane 8 shows that antibodies recognized a protein of 34 kDa that is *YCF10* from tobacco chloroplast. The absence of band in lane 1 containing uninduced *E. coli* cells confirms the specificity of the antibodies produced. From Western blots, it is concluded that the antibodies recognized the *E. coli*-expressed as well tobacco chloroplast *ycf10* protein.

Antibodies against *ycf10* were raised against the hydrophilic region between the first two hydrophobic regions. Western blots on expressed *ycf10* proteins in *E. coli* cells and from wild-type plants confirmed the specificity of the antibodies. There are, however, ambiguities between size of the proteins analyzed by Willey and Gray (1990) and Sasaki *et al*. (1993) using SDS-PAGE. The estimated size of the pea *ycf10* protein from transcription and translation in *E. coli* cell-free system was 20 kDa (Willey & Gray, 1990), whereas western blotting of pea chloroplast envelopes showed band of 34 kDa (Sasaki *et al*., 1993). A protein of 34 kDa was also detected in tobacco chloroplast envelope membrane (Fig. 7). The expected size of the *rbcS-ycf10* fusion protein examined by Craig (1993) was 31.7 kDa and a protein of this size was observed, although forms of the protein with higher electrophoretic mobility were also present. After import by isolated chloroplasts, a protein of 17 kDa was detected in the envelope fraction. The differences in the estimated sizes of the *ycf10* protein may be reconciled if the protein observed by Willey and Gray (1990) is a full-length protein including the putative targeting sequence and the protein has a higher electrophoretic mobility than expected because of its hydrophobic nature. Removal of the targeting sequence would give a size reduction of 3 kDa to produce a protein of about 17 kDa as observed by Craig (1993). Determination of the *ycf10* protein in vivo would give a protein of 34 kDa as observed in pea and tobacco (Sasaki *et al.*, 1993, Fig. 7).

CONCLUSIONS

From these experiments it is concluded that antibodies raised against *ycf10* protein fragment of 76 amino acid residues are highly pure and specific to recognize chloroplasts inner envelope membrane protein, *YCF10*.

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