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Tobacco Chloroplast Open Reading Frame Ycf10: Deletion and Consequences?

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Abstract: The complete nucleotide sequence of chloroplast genome is available for a number of organisms including lower and higher plants. From the plastome, a number of genes (-120) have been identified, however some of the open reading frames are remaining to assign their functions. One of the orfs is ycf10 whose protein product has been localized to the inner membrane of envelope with a proposed function, CO_2 transporter on the basis of sequence similarities with ycf10 of *Chlamydomonas reinhardtii*. The repLacement of the ycf10 resulted in a mixed population of wild-type and transgene containing plastomes. The lack of homoplasmicity for the transgene suggests that ycf10 gene product is very essential for cell survival, deletion of this protein appeared to be detrimental in highel plants. A number of phenotypic changes have been observed in transformants, these phenotypic changes appeared not to be gene related and must be ascribed to some other unknown reasons, such as somaclonal variation or due to spectinomycin effects.

Key words: Chloroplast, orf, ycf10, tobacco, *Chlamydomonas*, phenotype, plastome, heteroplasmy

Introduction

Originally, 10 open reading frames (ycf1-10) were identified as being conserved in a number of chloroplast genomes (Shimada and Sugiura, 1991). The open reading frame targeted for disruption is orf229 (ycf10) which lies upstream of the petA gene encoding cytochrome f. ycf10 was first identified in pea chloroplasts (Willey *et al*., 1984) and is conserved in higher green plants but is not present in *Epifagus* and *Euglena* plastid DNA. ycf10 was originally proposed to encode a heme-binding protein because of a short region of sequence similarity, around a conserved histidine residue, to the heme-binding domain of two cytochrome b polypeptides (Willey and Gray, 1990). The product of ycf10 has subsequently been localized immunochemically in the inner envelope membrane of pea chloroplasts (Sasaki *et al*., 1993; Craig, 1957). Recent sequence information for ycf10 from *Chlamydomonas reinhardtii* indicates the presence of an asparagine residue in place of the conserved histidine residue ycf10 is therefore probably not a heme protein.

The ycf10 protein was proposed to be acting as a facilitative transporter for CO_2 or as a component of a known HCO_3^- transporter (Katoh *et al*., 1996) as it can complement mutant lines of *Synechocystis* PCC6803. Alternatively, the mutation may be affecting the transport of sodium ions (Katoh *et al*., 1996) which is known to be important in the HCO_3^- transport mechanism (Volokita *et al*., 1984). Supporting evidence for a role in facilitative CO_2 diffusion has come from work on *Chlamydomonas reinhardtii* where ycf10 has been interrupted by introducing a chimeric *aadA* gene and mutant lines produced. These mutants show high light sensitivity but have a normal photosynthetic apparatus, carotenoid composition and ATP synthase activity. *Chlamydomonas reinhardtii* possesses a CO_2 concentrating mechanism by which CO_2 can be concentrated inside the chloroplast under low CO_2 conditions (Moroney *et al*., 1987). CO_2 is generated in the periplasm from HCO_3^-, particularly under low CO_2 conditions, by carbonic anhydrase (Moroney *et al*., 1987) and transported into the cytosol where uncatalysed hydration or an unidentified cytosolic carbonic anhydrase generates HCO_3. A high-affinity transporter in the chloroplast envelope results in the accumulation of HCO_3 in the stroma and stromal carbonic anhydrase generates CO_2 for photosynthesis. A membrane-permeable inhibitor of carbonic anhydrase, ethoxyzolamide (EZ) and a general inhibitor of envelope transporter proteins (including the HCO_3-transporter), 4,4'-diisothiocyanostilbene-2, 2-disulphonate (DIDS) were used to analyze inorganic carbon ($CO_2 + HCO_3$-) transport in these *Chlamydomonas* ycf10 mutants. With either EZ or DIDS present, blocking the known HCO_3-based CO_2 accumulation mechanism, the ycf10 mutant had strongly diminished photosynthetic 0_2 evolution rates relative to wild-type cells. However these studies have not shown directly that disruption of ycf10 results in decreased CO_2 uptake by chloroplasts, and the results obtained could have resulted from other indirect effects. The aim of the work described here is to delete ycf10 in the tobacco chloroplast genome and determine the consequences.

Materials and Methods

Generation of reporter gene constructs: Three constructs namely pMSK4, pMSK5 and pMSK6, for ycf10 replacement, were constructed. The details are given in result section.

Generation of reporter gene constructs: The transformation vectors pMSK4, pMSK5 and pMSK6 were introduced into leaves of seedling-derived tobacco plants by the biolistic process and selection of transformants was carried out on regeneration medium containing 500 mg/l spectinomycin (Svab and Maliga, 1993).

DNA analysis of the transplastomic lines by PCR: Total cellular DNA was extracted using the mini preparation method described by Spychalla and Bevan (1993) with some modifications. The total cellular DNA was used as template in PCR using Tag polymerase (Bioline, London, UK) to screen the transformants and to estimate the

proportion of transformed to non-transformed plastome copies. Detailed analysis is given in result section.

Analysis of transformants using Northern blots: Plant RNA was extracted using the RNeasy total RNA purification protocol (Qiagen Ltd. Dorking, Surrey, UK. RNA was electrophoresed on formaldehyde-agarose gel. RNA was blotted onto nylon membranes using the technique described by Southern (1975) and blots were probed with a^{32}P-labelled DNA fragments specific to ycf 10 as well as petA and aadA genes.

Immunochemical assay of the transplastomic lines: The total chloroplast membranes as well as fractions were solublized and proteins separated in 12% (w/v) polyacrylamide gels. After electrophoresis was complete, proteins were transferred to nitrocellulose membrane and detected with antibodies specific to ycf10 as well as antibodies against control proteins. Antibodies against ycf10 protein were raised using a hydrophilic stretch of DNA between first two hydrophilic regions.

Carbon isotope discrimination measurements using mass spectrometry: Samples for mass spectrometry were prepared by weighing 1.92 mg of fine leaf powder in small tin capsules (Elemental Microanalysis Ltd, Okehampton, Devon, UK) which were then folded to small balls. Samples prepared were used for carbon isotope analysis and carbon isotope readings were obtained and results were expressed relative to the Vienna PDB Standard (Coplen, 1995). Carbon isotope ratios of ,samples were measured using a mass spectrometer, by comparing the molar abundance ratio R_p, defined as $R_p = 13CO_2/12CO_2$, with the molar abundance ratio of a reference, R_{ref},. using the equation:

$$\delta^{13}C_p = (R_p\text{-}R_{ref})/R_{ref} \quad (1)$$

The reference material used was CO_2 extracted from a fossil belemtine from the Pee Dee formation in South Carolina and it is denoted as PDB, for which $R_{ref} = 0.01124$ (Craig, 1957). In our calculations, R_{re}f was VPDB which has the same values as for PDB (Coplen, 1995). These isotope ratios were used to calculate the discrimination ratio of the isotope using equation (2) if the ratios R are given in the 6 notation relative to VPDB (see eq. 1):

$$\Delta^{13}C_p = (\delta^{13}C_p\text{-}\delta^{13}C.)/(1+\delta^{13}C_a) \quad (2)$$

Where $\delta^{13}C_p$ is the stable carbon isotope ratio ($^{13}C/^{2}C$,) for the plant samples described above and $\delta^{13}C_a$ is the stable carbon isotope ratio ($^{13}C/^{12}C$) for the air surrounding the plants. Air samples were collected in 5 liter air sample flasks from the air surrounding the plants in greenhouse, and CO_2 was cryogenically trapped and other non-condensable gases were pumped away. The trapped CO_2 was used in mass spectrometer for carbon isotope analysis as done for leaf samples.

Photosynthetic rates measurements: Measurements of maximal photosynthetic rates in saturating CO_2 were made (Walker, 1987) using a leaf-disc O_2 electrode (Hansatech Instruments, Kings Lynn, Norfolk, UK) and O_2 evolution measurements were carried out at a photosynthetically active radiation of 350 mmol photons m^{-2} s^{-1} at 26°C.

Results

Generation of ycf10 replacement vector constructs: Three final constructs to replace completely the coding region of ycf10 with chimeric *aadA* genes that allow selection of transformants on spectinomycin were produced: one containing *trc-aadA-rrnB,* one containing rrn-aadA-psbA and one containing a *trc-aadA* gene lacking the terminator. The 1.2 kb BamHl-Sall fragment containing *petA* was cut out of the plasmid pTBal , which contains the 19.6 kb BamHl fragment of tobacco chloroplast DNA in pBR322 (Sugiura *et al.*, 1986). This fragment was inserted into pBCSK$^+$ at the *BamHl* and Sall sites after gel purification. The resulting plasmid was called pMSK2. The 736 by Spel-Xbal fragment containing ycf4 was isolated from pTB22, which contains a 4.8 kb insert from a partial BamHl digest of tobacco chloroplast DNA in pBR322 (Sugiura *et al.*, 1986) and was inserted into pMSK2 cut with Spel to give pMSK3. The orientation of the 73,6 by Spel-Xbal fragment in the Spel site was confirmed by double digestion with Spel and BamHl. If the fragment is in correct orientation, it produced a fragment of 740 by by double digestion, otherwise the plasmid is only linearised. pMSK3 contains tobacco chloroplast DNA flanking both sides of ycf10 and was used as the recipient of the chimeric aadA genes. The 1.6 kb Hincll fragment containing the trc-aadA-rrnB gene from pMSK1 (Maenpaa *et al.*, 2000) was inserted into pMSK3 after cutting with BamHl and filling in the ends with Klenow fragment of DNA polymerase I in the presence of dNTPs. This generated the pMSK4 transformation vector construct (Fig. 1). The rrnB terminator was removed from this construct by digestion with Hindi!! and BsrGI and religation of the ends after filling in the 5' overhangs with Klenow fragment of DNA polymerase I. This produced the transformation vector pMSK5 (Fig. 1). The transformation construct containing the rrn-aadA-psbA chimeric gene was produced by inserting the 1.3 kb BamHl fragment from pZS197 into pMSK3 cut with BamHl. This construct was called pMSK6 (Fig. 1). All transformations were carried out with *E. coif* DH5a and recombinant colonies were selected on solid LB medium contain chloramphenicol (34 pg/ml).

Transformation and regeneration of transgenic plants: The transformation vectors for the replacement of ycf 10 were introduced into leaves 3 to 4 cm long from 4 to 6 week-old seed-grown plants by the biolistic process (Svab and Maliga, 1993; Khan and Maliga, 1999). Bombarded leaves were placed on RMOP agar plates without antibiotic and after two days 5 x 5 mm leaf sections were placed on the same medium containing spectinomycin (500 mg/l). Leaf sections bleached within 4 to 5 weeks and green calli and shoots were observed on some of the bleached leaf sections within 4 to 9 weeks. Leaves 1 to 2 cm long from primary transformants were sectioned and placed on the same selective medium for further regeneration. Regenerated shoots were shifted to MS medium containing spectinomycin (500 mg/l) for leaf proliferation and rooting. Further rounds of regeneration were carried out to increase the transgene levels in transformants by excising leaf pieces and growing on RMOP medium containing spectinomycin (500 mg/l).

Genetic analysis of transformants: Putative transformants grown on spectinomycin medium after three rounds of selection and regeneration were subjected to DNA analysis. The analysis of a large number of transformants is more convenient using PCR techniques rather than the more time-

consuming Southern blotting. PCR using primers that anneal to DNA flanking the target gene should produce different patterns of bands for wild-type plants and for homoplastomic and heteroplastomic plants containing the pMSK4 and pMSK6 constructs because of the difference in size between the *aadA* gene cassettes and the target gene. However, identical size products would be produced for wild-type plants and plants containing the pMSK5 construct, moreover, it was difficult to analyze with Southern probing because there is no difference in size between the *aadA* gene and the target gene.

Analysis of transformants using PCR techniques was carried out on total cellular DNA extracted from leaves of tobacco plants using a miniprep method (Spychalla and Bevan, 1993). Two different set of primers, two flanking the *aadA* gene and one specifically from the *aadA* coding region were used to analyze the transformants. First of all, to screen plants for the presence of the aadA cassette in the plastome, a forward primer, 5'-CATTCGAAGTGAATTCT TAGC-3' (complementary to asequence of the ycf4 gene in the chloroplast genome (position 63230 to 63251; Shinozaki *et al*., 1986) which flanks the 5' end of the *aadA* gene in the plasmid construct), and a reverse primer, 5'-ACGGTCACCGTAACCAGC-3' (complementary to aadA gene sequence (position 552 to 570; Hollingshead and Vapnek, 1985) were used to carry out PCR reactions using an annealing temperature of 57°C. These primers should amplify the aadA coding region specifically from the transplastome.

A large number of transformants was analyzed for the presence of the *aadA* cassette and the results for some of the plants are shown in Fig. 2. Lanes 1 to 6 contain DNA amplified from wild-type, plasmid (pMSK5 DNA), 308, 236, 237 and 219 DNA, respectively. The numbers indicate transformants obtained with pMSK5. The first numeral indicates the number of rounds of regeneration used to produce the plants. Lane 1 shows that no PCR product has been amplified from DNA of a wild-type plant, which confirms the specificity of the PCR reactions and also indicates that the PCR products in lanes 2 to 6 are the result of amplification of the aadA gene. Lanes 3 to 6 show a PCR product of the expected size (330 bp), amplified from 4 transformants regenerated from leaves bombarded with pMSK5. The plants obtained from leaves bombarded with pMSK4 did not produce any fragment when PCR analysis was made after the third round of selection, however with plasmid pMSK6 did produce (Data not shown).

It was necessary to analyze the proportion of the plastomes in these plants that contained the *aadA* cassette in place of the ycf10 gene. Primers flank the ycf10 gene or the introduced *aadA* cassette were used. The forward primer was from ycf4 as described above and the reverse primer (5'-CCCTAGCCACGATTCTGC-3') was complementary to intergenic sequence between ycf 10 and petA (position 64350 to 64368; Shinozaki *et al*., 1986). Using these two primers, a PCR product of 900 by is expected from wild-type plants and transformants containing the pMSK5 construct, where the aadA cassette is about the same size as the replaced ycf10 region (Fig. 3). A PCR product of 900 by was amplified from transformants 308, 236, 237 and 219 obtained after shooting tobacco leaves with plasmid pMSK5 (lanes 1 to 4). A similar product was obtained from pMSK5 (lane 5). To distinguish between ycf10 and the aadA cassette, the PCR products were digested with Hind111, which cuts *ycf10* DNA once at position 63855 (Shinozaki *et al*., 1986), yielding two fragments of 589 and 318 bp, but does not cut the aadA cassette. Lanes 6 to 13 (Fig. 3) show the PCR products amplified from a wild-type plant, 236, 308, 237, pMSK5 DNA and 219 DNA digested with HindIII. The proportion of the chloroplast genome containing the *aadA* cassette was roughly estimated using densitometric analysis after photocopying the Polaroid photograph onto a transparency. The *aadA* cassette appeared to be present in about 30% of the chloroplast genomes in the plants shown.

The proportion of the chloroplast genomes containing the *aadA* cassette was examined in plants grown under various conditions. Plants transferred to soil for biochemical and physiological analyses were analyzed for *aadA,* it was found that most of the transformants lost the inserted aadA gene when grown without selection (Data not shown). Therefore another set of the same plants regenerated from nodal cuttings in tissue culture was transferred to soil and irrigated with a spectinomycin solution (500 mg/l). The reason for watering transformants with spectinomycin was to continue the selection pressure and prevent loss of the *aadA* gene. Plants were analyzed for the presence of the *aadA* gene by PCR. Transformants transferred to soil irrigated with spectinomycin had almost the same amount (30%) of the transgene as plants grown on antibiotic-containing MS medium (Data not shown).

The transformants were also grown under low light and under high CO_2 conditions because deletion of ycf 10 might result in plants sensitive to high-light conditions and normal CO_2 levels might not be sufficient for transformants to grow normally. Plants grown under low light of about 90 pmol photons m^{-2} s^{-1} by wrapping magenta pots in three layers of Whatman 3 MM paper and grown under elevated CO_2 (1%) conditions were analyzed for aadA gene levels. No difference in aadA gene levels was observed when plants were grown either under low light or elevated CO_2 conditions.

Northern blot analysis of transformants: RNA analysis of transformants and wild-type plants was carried out by extracting total RNA from leaves of plants growing on agar medium, with spectinomycin (500 mg/l) for transformants or without spectinomycin for wild-type, using the RNeasy total RNA purification protocol. RNA was quantified by spectrophotometer and equal amounts of RNA (10 pg per lane) were fractionated by electrophoresis on a 1.2% agarose gel containing formaldehyde. After electrophoresis was complete, the RNA was transferred onto GeneScreen Plus nylon membrane by salt transfer (Southern, 1975) and the membrane was hybridized with gene-specific probes.

To determine how much the ycf 10 transcripts were affected by the aadA gene insertion, blots were probed with ^{32}P-labelled ycf10 coding region. A fragment of 469 by from the ycf10 coding region was purified and labelled with [α^{32}P] dATP by random priming. The hybridization pattern of transcripts is shown in Fig. 4A and demonstrates that the probe hybridized strongly with a complex pattern of transcripts of 3.4, 2.7, 2.4, 1.8, 1.6 and 0.9 kb in each sample. However, the hybridizing bands were clearly less intense in lanes 3, 4 and 6 which represent transformants 308, 236 and 219, respectively, compared to the wild-type plant (lane 1). Transformants 237 (lane 5) gave intense bands comparable to those of the wild-type plant. To ensure that equal amounts of chloroplast RNA had been loaded on the gels, the same membrane was reprobed with the *psbA* gene from a region of the chloroplast genome

unaffected by the transformation events. The psbA coding region was prepared by digesting pTB28 (Sugiura *et al*., 1986) with EcoRV and HindIII and labelled with [α^{32}P] dATP by random priming. The deprobed membranes were reprobed with labelled *psbA* and the results are shown in Fig. 4B, which demonstrates that a single transcript of the expected size (1 kb) hybridized to the probe. Densitometric analysis showed that the amounts of RNA loaded for transformants 308 and 236 were similar to wild-type but higher amounts were loaded for 237 and lower amounts were loaded for 219. On the basis of the amounts of RNA loaded, ycf10 transcripts were reduced in transformants 308, 236 and 219 to about 50 to 60% of wild-type levels whereas transformant 237 were comparable to wild-type plant.

To determine whether the ycf 10 deletions affected the downstream *petA* gene, the deprobed blots were reprobed with ^{32}P-labelled *petA* coding region. Transcripts of 3.4, 2.7 and 1.8 kb hybridized strongly to the probe. From these results it is concluded that insertions of the aadA gene had the same effect on transcripts from petA as for ycf10 (Data not shown). The plants transferred to soil and grown under spectinomycin-selection pressure for biochemical and physiological analyses were also subjected to RNA analysis. Similar results to those described above were obtained when blots were probed with radiolabelled ycf10, *petA* and *psbA* (Data not shown). Decreased levels of transcripts for *petA* are probably because ycf10 is cotranscribed with *petA,* as found in pea chloroplasts (Willey and Gray, 1990).

Protein analysis of transformants: To determine whether there were any changes in the amount of the ycf10 protein, chloroplast envelope membranes were prepared from leaves of a wild-type plant and transformants using discontinuous sucrose-gradient centrifugation. Proteins were subjected to electrophoresis using SDS 12% polyacrylamide gel and were transferred to nitrocellulose membrane by semi-dry electroblotting. The membranes were incubated with ycf10specific antibodies (5 pl at 1:5000 dilution) prepared against hydrophilic stretch of DNA between third and fourth hydrophobic DNA sequences of ycf10 and the proteins which reacted with the antibodies were detected by ECL. The results shown in Fig. 5A clearly demonstrate that the antibodies recognized a protein of 34 kDa in membranes from wild-type, 308, 236 and 219 plants (lanes 1 to 4). The antibodies also recognized a protein of 25 kDa in membranes from transformants 308 and 236. Densitometry was carried out to quantify differences in the intensities of bands obtained from the western blots. To ensure that the differences in band intensity were not due to differences in the total amount of chloroplast envelope proteins loaded on the gels, identical gels were blotted onto nitrocellulose membranes and probed with antibodies to inner envelope membrane protein E37 (Joyard *et al*., 1982). The membranes were probed with antibodies against inner envelope protein Fig. 5B shows that the apparent differences in intensity of bands obtained with ycf10 antibodies were due to unequal loading of membrane proteins on to the gel.

A number of physiological and biochemical experiments were carried out as described in materials and methods to see if there is any effect of knock out on protein functioning but no considerable differences were found (Data not shown) as there was no apparent differences in protein amounts for ycf10 knock out were found. Therefore it appears to be very essential protein for the plant survival unlike in *Chlamydomonas.* However, a different strategy is required to find out ycf10 protein function.

Discussion

The deletion of ycf10 from the tobacco chloroplast genome by replacement with chimeric aadA cassettes was attempted. PCR analyses of the transformants were carried out because Southern hybridization gave no indication of the homoplasmicity of the transgene. PCR analyses of the transformants showed that they were all heteroplasmic, only 30% of the wild-type genome had been replaced. In an attempt to make the transformants homoplasmic, several more rounds of regeneration were carried out on RMOP medium containing spectinomycin (500 mg/l). However, no differences in transgene level were observed. Plants were also grown under low light conditions, obtained by wrapping the magenta pots in three layers of Whatman 3MM paper, because plants with ycf 10 deletions might be sensitive to high light intensities as had been reported for *Chlamydomonas* ycf10 mutants. However this did not help to improve transgene levels in the transformants. Transgene levels of the transformants were also not increased by growing them in 1% CO_2enriched air (Data not shown). These results are in line with those for ycf10 mutants of *Chlamydomonas,* where the high light sensitivity of the ycf10-deficient mutants can not be overcome by growing the cells with CO_2-enriched air. However, they are in contrast with results for all previously described mutants of *Chlamydomonas reinhardtii* affected in one of the components of the CO_2 concentrating mechanism which can be rescued by aeration with CO_2-enriched air (Spalding *et al*., 1983a, b; Moroney *et al*., 1987).

These results indicate that it was not possible to produce tobacco plants homoplasmic for the ycf10. This may be due to a lethal phenotype of plants with the ycf10 deletion, although this was not observed in *Chlamydomonas*. It may be due to inadequate selection pressure or due to the use of a different variety of *Nicotiana tabacum* to that previously used by Maliga and coworkers to produce homoplasmic lines. But such experiments have also been performed using variety Petit Havana and similar results, heteroplastomic plants for ycf 10 knock out and mutagenesis (Pal Maliga and Pryia Sarrirman, personal communication) were found. The selection pressure recommended to produce homoplasmic lines for gene insertions into the plastome (Svab *et al*., 1990; Staub and Maliga, 1992; Svab and Maliga, 1993; Zoubenko *et al*., 1994) and for gene deletions (Kanevski and Maliga, 1994; Allison *et al*., 1996) was 500 mg/l spectinomycin, and this was used for plants used in ycf10 deletions. However, it was difficult to maintain this selection pressure on plants grown in soil and loss of the transgene was observed in plants that were not constantly treated with spectinomycin (Data not shown). Maliga and co-workers have used *Nicotiana tabacum* var. Petit Havana to produce homoplasmic plants but in the ycf10 deletion studies a different variety Samsun was used. So far, none of the chloroplast transformants of this tobacco variety have been shown to be homoplasmic (Maenpaa *et al*., 1998, 2000). Therefore, it is concluded that either the selection pressure is inadequate or the tobacco variety Samsun is not suitable for production of homoplasmic transgenic plants or gene knock out is lethal.

After DNA analysis, plants were subjected to RNA analysis to examine how much the transcript levels for ycf10 had

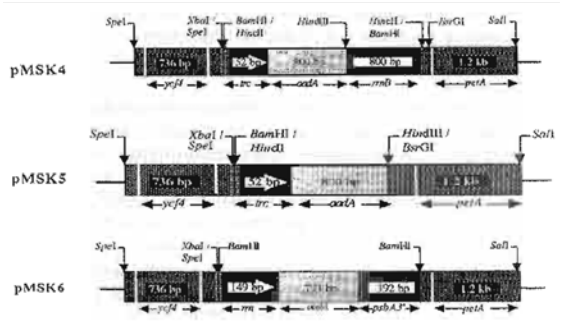

Fig. 1: ycf10 replacement vector constructs. Coding region of ycf10 was replaced with aadA gene cassettes, trc-aadA-rrn6, trc-aadA and rrn-aadApsbA3, Restrction sites used are discussed in results

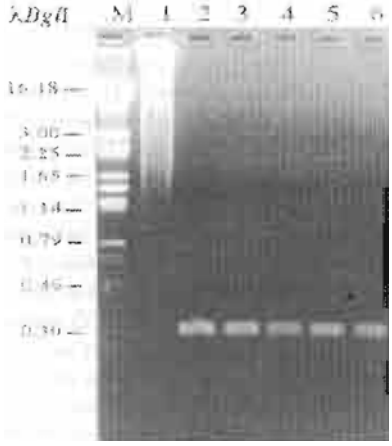

Fig. 2: PCR analysis of total cellular DNA from transformants. PCR using Taq polymerase was carried out and PCR product amplified from wild-type plant (lane 1), plasmid (pMSK5) DNA (lane 2), transformant 236 (lane 3), 308 (lane 4), 237 (lane 5) and 219 (lane 6) is shown

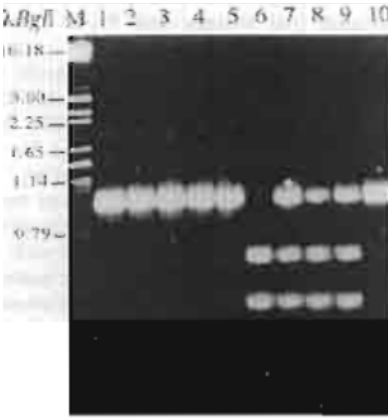

Fig. 3: Analysis of heteroplasmy for transgene in tranformants. PCR using primers flanking the aadA gene was carried out and products were loaded on a 0.8% agarose gel. Lanes 1-4 represent DNA amplified from four independent transformants 236, 308, 237 and 219 and disgested with HindIII enzyme

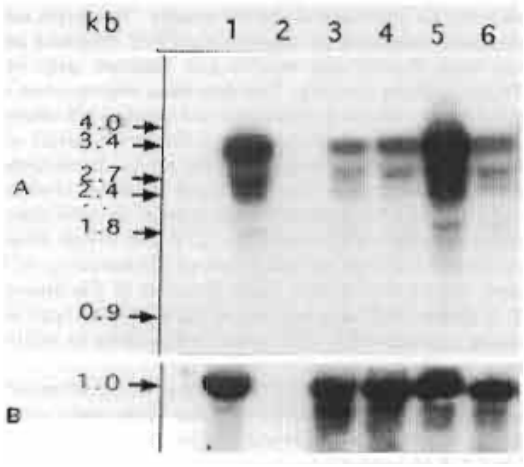

Fig. 4: Northern analysis of transcripts for ycfl 0 and psbA. Total cellular RNA was extracted and after electrophoresis transferred onto GeneScreen Plus nylon emebrane and blots were hybridized with probes A. Ycf10 coding region. B. PsbA gene. Lane 1 shows transcripts from wild-type plant. Lanes 3-6 show transformants 308, 236, 237 and 219, respectively, obtained with pMSK5

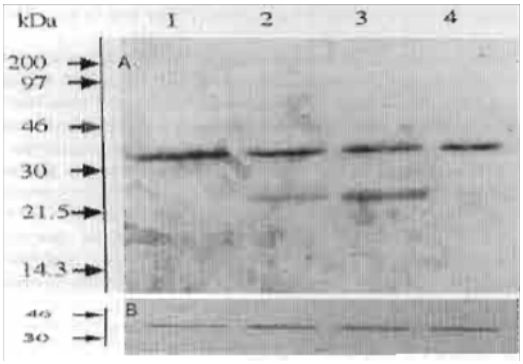

Fig. 5: Western blot analysis of chloroplast envelope protein from transformants. Crude preparation of chloroplast envelope proteins from leaves of equal weight (0.5 g) from transformant and wild-type plants were subjected to SOS-PAGE through a 12% polyacrylamide gel. And probed with A. Ycfl 0, B. E37-specific antibodies. Lane 1 shows wild-type and lanes 2-4 transformants 308, 236 and 219, respectively

been affected by targeted deletions of ycf10. Northern blot hybridization clearly indicated that the ycfl 0-specific probe hybridized with transcripts of 3.4, 2.7, 2.4, 1.8 and 0.9 kb in all the transformants and wild-type plants, but the ycf 10 transcript levels for transformants 308, 236 and 219 were about 50 to 60% lower than those of the wild-type. Transformant 237 was comparable to the wild-type plant. In plants grown in the greenhouse ycf10 transcripts were also affected but showed a decrease of only 25 to 30%. However, *petA* transcripts were also affected by ycf10 deletions to the extent as ycf10 transcripts in transformants 308, 236 and 219 compared to the wild-type

plant. Low levels of transcripts for *petA* are perhaps due to cotranscription of ycf10 with in chloroplast DNA (Willey and Gray, 1990). The northern blots have demonstrated a complex pattern of transcripts from the region of tobacco chloroplast DNA containing ycf10 and *petA,* similar to that described for the same region from pea chloroplast DNA (Willey and Gray, 1990). Gene-specific probes for the ycf10 and petA hybridized with three bands which are common (3.4, 2.7 and 1.8 kb) and two (2.4 and 0.9 kb) which are specific for the ycf10 probe. This pattern is different from that obtained with pea chloroplasts where Willey and Gray (1990) proposed the 3.4 kb transcript to be a polycistronic mRNA which was processed to produce smaller 'gene-specific' transcripts of 0.9 kb for ycf10 and 1.8 kb for *petA.*

Mass spectrometric analysis of samples prepared from homogenized dried leaves showed a decreased discrimination against ^{13}C by the transformants compared to a wild-type plant grown under the same conditions in the greenhouse Data not shown). All the plants analysed were grown under the same conditions, to eliminate differences within plants of each line which might be due to variations in atmospheric CO_2 levels that are known to affect the morphology as well as the physiology of plants.

Antibodies against ycf10 were raised against the hydrophilic region between the first two hydrophobic regions. Western blots on expressed ycf 10 proteins in *E. coil* cells and from wild-type plants confirmed the specificity of the antibodies. There are, however, ambiguities between the size of the proteins analysed by Willey and Gray (1990) and Sasaki *et al.* (1993) using SOS-PAGE. The estimated size of the pea ycf10 protein from transcription and translation in an *E. coil* cell-free system was 20 kDa (Willey and Gray, 1990), whereas western blotting of pea chloroplast envelopes showed a band of 34 kDa (Sasaki *et al*., 1993). A protein of 34 kDa was also detected in tobacco chloroplast envelope membranes (Fig. 5A). The expected size of the RbcS-ycf10 fusion protein examined by Craig (1957) 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 ycf 10 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 (1957). Dimerization of the ycf10 protein *in vivo* would give a protein of 34 kDa as observed in pea and tobacco (Sasaki et *al.,* 1993; Fig. 5A).

As deletions of ycf10 in heteroplasmic plants did not affect ycf10 protein levels, these phenotypic changes appear not to be gene related and must be ascribed to some other unknown reasons, such as somaclonal variation or due to spectinomycin effects (Fromm *et al*., 1987; Moll *et al*., 1990; Svab *et al*., 1990; Svab and Maliga, 1991). Therefore, we think a different strategy is required to find out the function of ycf 10 gene product.

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