Characterization of a plant mitochondrial active chromosome

Julien Fey[1], Matthieu Vermel[1], Jean-Michel Grienenberger, Laurence Maréchal-Drouard,
José M. Gualberto*

*Institut de Biologie Moléculaire des Plantes du CNRS, UPRA0406, Université Louis Pasteur, 12 rue du Général Zimmer,
67084 Strasbourg Cedex, France*

Abstract **A method is presented for the partial purification of a plant mitochondrial active chromosome (MAC). This method is based on the presence of the mitochondrial chromosome in the insoluble mitochondrial fraction which allows for its rapid purification from the bulk of detergent-solubilized proteins by ultra-centrifugation. The resuspended MAC carrying DNA and RNA-binding proteins retains DNA synthesis and transcription activities comparable to the ones found in isolated mitochondria. In comparison, tRNA-nucleotidyl terminal transferase taken as an example of RNA modifying activities remains in the soluble fraction. MAC purification is proposed as a rapid and efficient first step in the purification of DNA-binding proteins involved in DNA replication and transcription.**
© 1999 Federation of European Biochemical Societies.

Key words: Plant mitochondrion; Active chromosome;
DNA-binding protein; tRNA-nucleotidyl terminal transferase

1. Introduction

Advances in the understanding of the sequences and protein factors involved in plant mitochondrial DNA (mtDNA) replication, recombination and transcription have been hampered by the difficulty in establishing corresponding in vitro systems. Therefore, isolation and identification of specific DNA and RNA-binding proteins should tell us much about the processes in which they are involved by their homologies to proteins whose biochemical roles are better characterized in other model organisms.

Conventional approaches have been tried to identify plant mtDNA or RNA-binding proteins, like gel shifts, UV-cross-link or affinity chromatography, but usually giving unclear results. In one such an attempt, Hatzack et al. [1] identified two proteins from pea mitochondria that can bind to a plant mitochondrial promoter and its upstream sequence, but their function has still not been characterized. Regarding plant mitochondrial RNA-binding proteins, a gel shift approach resulted in the identification of superoxide dismutase [2], but it is quite possible that its capability to bind to nucleic acids in vitro does not reflect any in vivo function. Thus, to purify and characterize plant mtDNA and RNA-binding proteins, fractions enriched for the relevant mitochondrial activities should be utilized.

Both in chloroplast and yeast mitochondria, DNA-protein complexes (nucleoids) have been characterized that are believed to be the segregating unit of the organellar DNA [3,4]. In chloroplasts, comparable structures can be purified as a transcriptionally active chromosome (TAC) [5,6] retaining the bulk of the transcription activity. Up to now, no similar complexes are reported in plant mitochondria.

We have developed a rapid protocol for the purification of a mitochondrial active chromosome (MAC). The association of the mitochondrial chromosome with the inner mitochondrial membrane [7] allows for rapid purification from the bulk of detergent-solubilized mitochondrial proteins by ultra-centrifugation. We have shown that the mtDNA fraction thus obtained retains DNA and RNA synthesis activities, indicating that DNA-binding proteins involved in replication and transcription co-purify with the mtDNA. To show that a soluble RNA-binding protein involved in RNA processing co-purifies with RNA, we have identified tRNA-nucleotidyl terminal transferase (CCase) activity in vitro.

2. Materials and methods

2.1. Mitochondria isolation

Potato (*Solanum tuberosum*) mitochondria were isolated from tubers mainly as described by Neuburger et al. [8]. For purification, the mitochondrial suspension was loaded on Percoll gradients (0.3 M sucrose, 10 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.1% (w/v) bovine serum albumin (BSA), 30% (v/v) Percoll) and centrifuged for 90 min at 18 000 rpm (JA20 rotor, Beckman). Maize (*Zea mays*) mitochondria were isolated from 8 day etiolated plants by differential centrifugations followed by mitochondria purification on discontinuous sucrose gradients [9].

2.2. Mitochondria fractionation

Based on protocols described for the purification of chloroplast TAC [5,6], isolated mitochondria were resuspended in 25% glycerol, 50 mM Tris-HCl, pH 8.0, lysed with 2.5% Triton X-100 and centrifuged for 20 min at $65\,000 \times g$ in order to obtain the pellet (P65) and soluble fraction (S65). The P65 fraction was resuspended at 10 mg/ml of mitochondrial protein in 10% glycerol, 50 mM Tris-HCl, pH 8.0, and 5 mM dithiothreitol (DTT). Protein dosage (BCA Protein Assay Reagent, Pierce) showed that 25% of the total mitochondrial proteins remain in P65.

To remove endogenous DNA, P65 and S65 fractions were treated with 200 U/ml of micrococcal nuclease (MCN, Pharmacia Biotech) in the presence of 2 mM $CaCl_2$, for 30 min at 37°C. The nuclease was then inhibited with 5 mM EGTA. The samples are then suitable for transcription and replication assays in the presence of 1 μg exogenous DNA template.

2.3. Run-on transcription assays

Run-on assays were performed according to Mulligan et al. [10]. Transcription assays were carried out for 30 min at room temperature in 100 μl reaction mixture containing 10 mM Tricine-NaOH, pH 7.2, 10 mM $MgCl_2$, 1.25 mM EDTA, 50 mM KCl, 2 mM DTT, 20 μg/ml BSA, 25 μCi [α-^{32}P]UTP or [α-^{32}P]CTP (800 Ci/mmol), 100 μM of

*Corresponding author. Fax: (33) (3) 88 61 44 42.
E-mail: jose.gualberto@ibmp-ulp.u-strasbg.fr

[1] Joint first authors.

each one of the other three ribonucleotides and 200 μg mitochondrial protein. The reaction was stopped by the addition of 25 mM EDTA and labelled RNA was analyzed on 8% denaturing polyacrylamide gels.

2.4. DNA synthesis assay

Replication assays were performed in a reaction volume of 100 μl containing 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 20 μCi [α-^{32}P]dCTP (800 Ci/mmol), 100 μM dATP, dTTP, dGTP and 200 μg mitochondrial protein. After a 30 min incubation at room temperature, the DNA was extracted with phenol/chloroform and ethanol-precipitated.

2.5. Editing assay

Editing activity was investigated according to the protocol described by Yu and Schuster [11]. Briefly, [α-^{32}P]CTP-labelled RNAs were digested with 10 U of P1 nuclease (Gibco BRL) at 37°C for 16 h. The digestion products were resolved by two dimensional thin layer chromatography (2D-TLC) on cellulose plates. The solvent systems were isobutyric acid:water:25% ammonia (66:33:1 v/v/v) for the first dimension and 2-propanol:HCl:water (70:15:15 v/v/v) for the second dimension. Plates were analyzed by autoradiography to detect the presence of [^{32}P]UMP.

2.6. In vitro CCase assay

A transcript corresponding to potato mitochondrial tRNA^{Cys}(GCA) (EMBL accession no. X93575) was utilized as substrate. The potato *trnC* was PCR-amplified using a 5′-primer (5′-*GAATTGTAATACGACTCACTATA*GGCTAGGTAACATAATGGAA-3′) containing the T7 RNA polymerase promoter sequence (in italics) just upstream of the *trnC* 5′-end and a 3′-primer (5′-AGGCCAAGGACGGGGTCGA-3′) complementary to the *trnC* 3′-end. The PCR product was suitable for in vitro transcription with T7 RNA polymerase (Biolabs), generating a transcript corresponding to the mature tRNA^{Cys} lacking the 3′-CCA and modifications.

To test for CCase activity, 100 μg protein is incubated for 20 min at room temperature in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ in the presence of 100 μM CTP and ATP to completely process the endogenous tRNA pool. CTP and ATP are then digested with alkaline phosphatase (20 U/ml for 30 min at 37°C) that is subsequently inhibited with sodium vanadate (100 μg/ml). The tRNA^{Cys} transcript (50 ng) is then added with 10 μCi [α-^{32}P]CTP (3000 Ci/mmol) and 50 μM ATP (except when noted) and incubated for 20 min at room temperature. After phenol/chloroform extraction, the transcript is analyzed on a 15%, 7 M urea polyacrylamide gel.
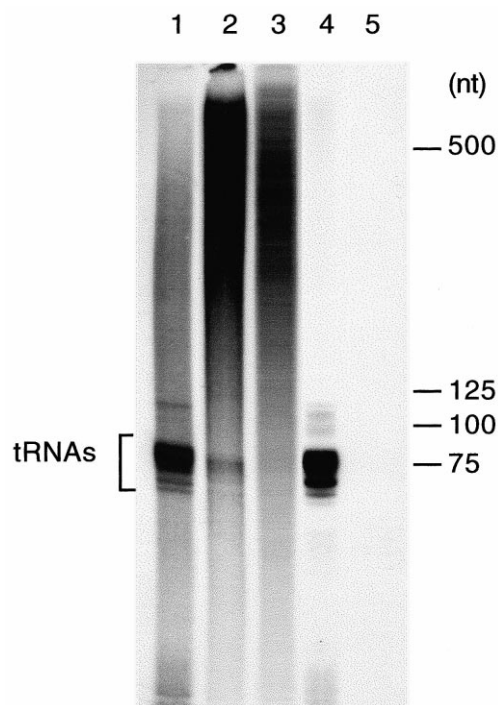
Fig. 2. Comparison between run-on transcription products obtained with intact mitochondria (lane 1) and with the P65 (lanes 2 and 3) and S65 (lanes 4 and 5) fractions. Samples were loaded on a 6% polyacrylamide, 7 M urea gel. The run-on assays were performed in the presence of radio-labelled CTP (lanes 2 and 4) or UTP (lanes 3 and 5). Size markers are indicated in nucleotides (nt).

2.7. Southern analysis

Potato mtDNA was digested with *Eco*RI or *Bam*HI, fractionated on a 0.8% agarose gel and transferred to a Hybond N$^+$ membrane (Amersham). Radio-labelled probes were hybridized for 12 h at 42°C in 50% formamide, 1 M NaCl and 1% sodium dodecyl sulfate (SDS). The membranes were washed at 60°C with 2×SSC, 0.1% SDS.

3. Results

3.1. Characterization of the P65 and S65 fractions

After mitochondria lysis and ultra-centrifugation, P65 and S65 fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Different protein patterns are revealed (not shown). To check that the mtDNA remains in the resuspended P65 fraction, nucleic acids were extracted from the two fractions and analyzed on agarose gels (Fig. 1). In P65, a high molecular weight band (lane 1) that is sensitive to DNAse I digestion (lane 2) is present that should correspond to mtDNA. In the S65 fraction, mtDNA is absent, while RNAse A sensitive bands corresponding to tRNAs and large RNAs are visible (lanes 3 and 4).

Maize mitochondrial P65 and S65 fractions were checked for the presence of transcription and DNA synthesis activities. Similar results were obtained when using potato mitochondria (not shown). Transcription run-on products labelled either with [α-^{32}P]CTP or [α-^{32}P]UTP were analyzed by PAGE (Fig. 2). With intact mitochondria, both tRNAs and higher molecular weight transcripts are labelled (lane 1), but labelling of tRNAs is reduced if run-on is performed with [α-^{32}P]UTP, indicating that tRNA labelling with [α-^{32}P]CTP results from
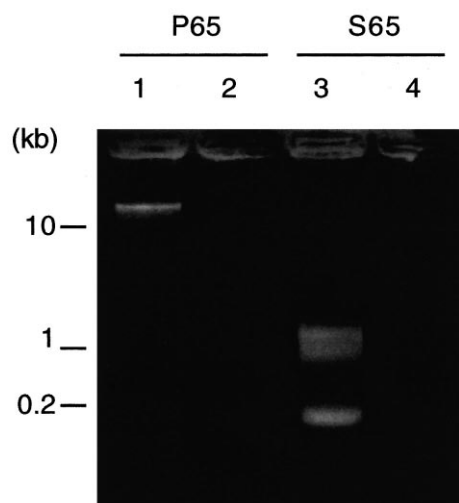

Fig. 1. Agarose gel analysis of nucleic acids present in the soluble (S65) and pellet (P65) potato fractions. Nucleic acids from the P65 fraction were loaded before (lane 1) and after (lane 2) DNAse I digestion. Nucleic acids from the S65 fraction were loaded before (lane 3) and after (lane 4) RNAse A digestion. Size markers are indicated in kb.

intrinsic CCase activity (data not shown). With the P65 fraction, strong labelling of high molecular weight RNA is observed, indicating that the isolated mtDNA is transcriptionally active (lanes 2 and 3). In this fraction, specific tRNA labelling with [α-^{32}P]CTP is weak (lane 2) and undetectable with [α-^{32}P]UTP (lane 3). On the contrary, with the S65 fraction, no labelling of high molecular weight RNA could be detected, because RNA polymerase activity and/or mtDNA are absent. However, CCase activity co-purifies with S65, as indicated by the strong labelling of tRNAs with [α-^{32}P]CTP (lane 4), but not with [α-^{32}P]UTP (lane 5).

Transcripts labelled by run-on transcription in intact potato mitochondria and in the P65 fraction were used as probes for Southern hybridization with potato mtDNA. Both probes hybridize with equivalent intensities to the same restriction fragments of the mitochondrial genome (Fig. 3a and b). Therefore, P65 contains the MAC that retains the transcription activity found in the intact organelle.

To test if RNA polymerase activity is still present in the S65 fraction and if the P65 fraction can re-initiate transcription on exogenous DNA, maize fractions were treated with MCN. No run-on transcription could be detected in MCN-treated P65 in the absence of exogenous DNA (Fig. 4, lane 1). After addition of total maize mtDNA, labelled transcripts were obtained with P65 (lane 2) but not with S65 (lane 5). This result indicates that most RNA polymerase activity remains in the pellet. However, the transcription activity of crude P65 is unable to initiate specific transcription: using plasmid pBH0.7 (which contains the promoter region of the maize mitochondrial *atp1* gene [12]) linearized with *Hin*dIII, the expected 298 nucleotides transcript is not present (lane 3). Unspecific tran-
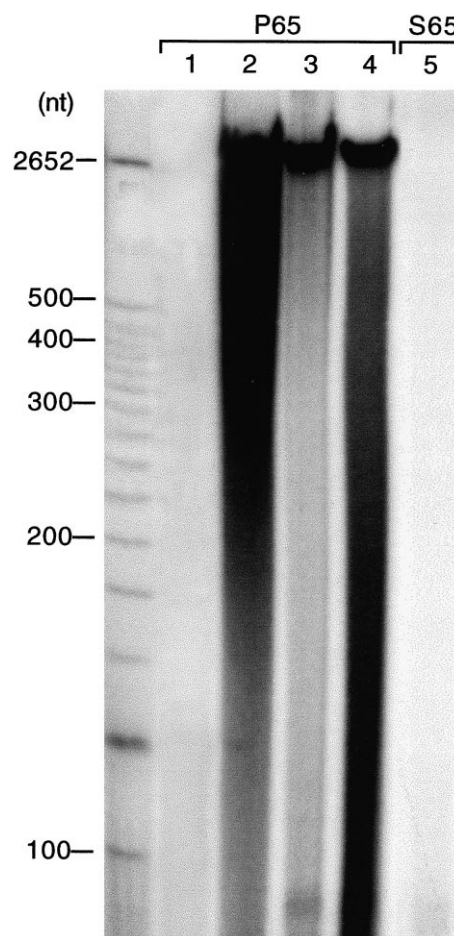

Fig. 4. Transcription assays of maize mitochondrial P65 (lanes 1 to 4) and S65 (lane 5) fractions in the presence of exogenous DNA templates. Lane 1 shows the assay after digestion of the endogenous mtDNA by MCN. Transcription assays by MCN-treated fractions were performed in the presence of added maize mtDNA (lanes 2 and 5), of plasmid pBH0.7 linearized with *Hin*dIII (lane 3) and of circular pBluescript SK$^-$ vector (lane 4).
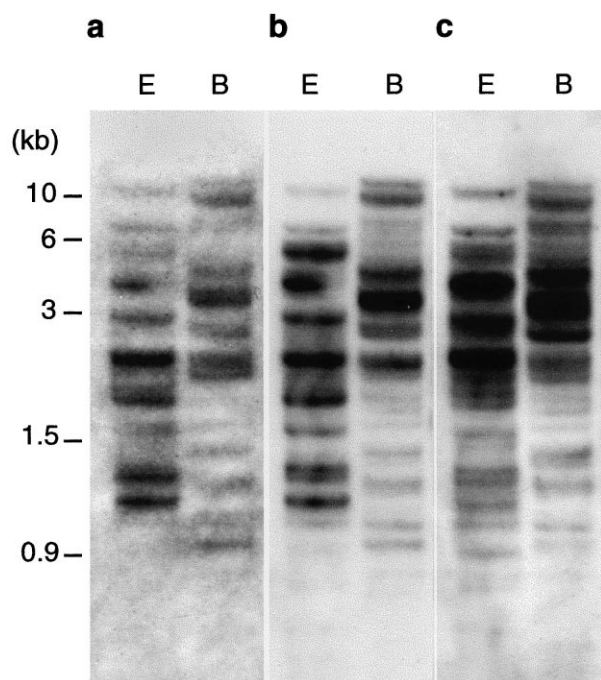

Fig. 3. Southern hybridization of potato mtDNA digested by *Eco*RI (E) and *Bam*HI (B) with probes generated by a run-on transcription assay with intact potato mitochondria (a), with the potato P65 fraction (b) and by DNA replication in P65 in the presence of [α-^{32}P]dCTP (c). Size markers are indicated in kb.
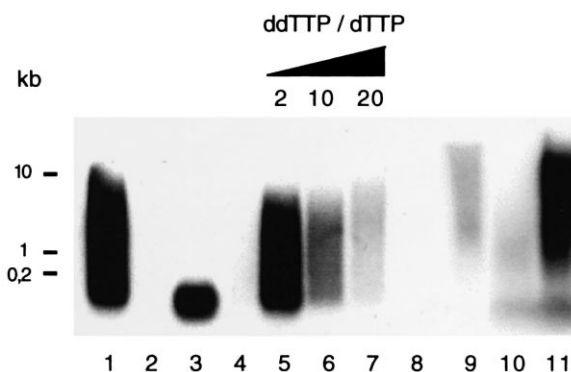

Fig. 5. Agarose gel analysis of DNA synthesis assays with maize P65 and S65 fractions. Lane 1 shows the control incorporation of [α-^{32}P]dCTP by P65 in the presence of endogenous mtDNA template. The same incubation was performed in the presence of 10 mM EDTA (lane 2), after DNAse I treatment (lane 3), in the absence of the other three dNTPs (lane 4) or in the presence of ddTTP as a competitive inhibitor (lanes 5–7). Lanes 8 and 10 show the absence of label incorporation by MCN-treated S65 and P65 fractions, respectively. Lanes 9 and 11 show DNA synthesis using exogenous mtDNA as template by the MCN-treated S65 and P65 fractions.
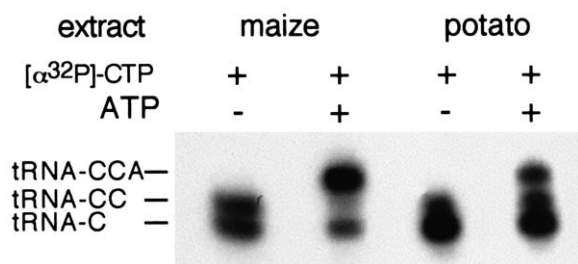
Fig. 6. Evidence for CCase activity in the soluble mitochondrial S65 fractions of maize and potato. A transcript corresponding to the potato mitochondrial tRNA[Cys] lacking 3′-CCA was incubated in the presence of S65 fractions and radio-labelled CTP (all lanes) plus or minus cold ATP. Samples were loaded on a 15% polyacrylamide, 7 M urea denaturing gel.

scription could also be obtained with plasmid pBluescript SK[−] (lane 4).

DNA synthesis activity present in the P65 and S65 maize fractions was monitored by incorporation of $[\alpha\text{-}^{32}P]dCTP$ in DNA (Fig. 5). Most of the activity remains in the P65 fraction (lane 1). Labelling is due to a DNA polymerase activity, as confirmed by EDTA inhibition (lane 2) and DNAse I digestion of the labelled products (lane 3). The activity relies on DNA elongation, since no label is incorporated in the absence of the other three dNTPs (lane 4) and is competitively inhibited by ddTTP (lanes 5–7). Southern hybridization with potato P65 DNA shows that synthesis products hybridize to potato mtDNA, confirming that the activity utilizes the endogenous DNA as template (Fig. 3c).

To evaluate how the DNA polymerase activity distributes between the P65 and S65 fractions, assays were performed with exogenously added DNA. With MCN-treated P65 and S65 fractions, virtually no DNA synthesis is detected (lanes 8 and 10). Addition of total maize mtDNA results in a strong DNA synthesis with P65 (lane 11), as compared to S65 (lane 9). Phosphor-Imager (Fuji Bas 1000, MacBas 2.2 software) quantification estimated that 85% of the DNA polymerase activity remains in the P65 fraction.

3.2. Separation of MAC from other RNA modifying activities

To investigate the presence of RNA modifying activities in the P65 and S65 fractions, CCase was taken as example, although additional activities should be tested to better assess the biochemical relevance of our fractionation procedure.

We have shown that most RNA is present in the soluble S65 fraction (Fig. 1) and, as discussed above, the CCase activity seems to co-purify with tRNA (Fig. 2). This hypothesis was checked by testing CCase activity in both fractions, using potato mitochondrial tRNA[Cys] as substrate (see Section 2). CCase activity was detected in the S65 fraction, both from potato and maize: the mature tRNA (+CCA) and blocked intermediates (tRNA+C/tRNA+CC) were obtained in the presence and absence of ATP, respectively (Fig. 6). In the P65 fraction, less than 5% of the total CCase activity was found (not shown).

RNA editing, a C to U post-transcriptional modification of plant mitochondrial transcripts, was previously shown to rely on a cytidine deaminase activity, but the enzyme has not yet been identified (reviewed in [13]). The deamination of α-phosphate radio-labelled C residues incorporated in a transcript results in the formation of radio-labelled U residues. To test for this activity in the P65 fraction, run-on transcripts labelled with $[\alpha\text{-}^{32}P]CTP$ were digested by P1 nuclease and the products analyzed by 2D-TLC. No radio-labelled 5′-pU residue could be detected, even after long exposure on Phosphor-Imager plates, showing that transcripts generated by the MAC are not edited (data no shown). Previously, it has been shown that editing occurs in organello in isolated mitochondria [14] but because editing seems to be uncoupled from transcription [15], the absence of this activity in the P65 fraction is not surprising.

4. Discussion

The experiments described were performed with mitochondria isolated both from potato and maize and can likely be applied to other plant species. The plant MAC fractionates in the P65 pellet obtained after 2.5% Triton X-100 solubilization followed by centrifugation at $65\,000 \times g$. The MAC retains DNA and RNA polymerase activities, thus indicating that DNA-binding proteins mostly co-purify with the mtDNA. As compared with the S100 soluble fraction used as a first purification step for in vitro transcription systems [12,16,17] and because of the separation from RNA and RNA-binding proteins present in the soluble fraction, the MAC appears to be an appropriate first step to purify plant mtDNA-binding proteins. No specific initiation of transcription could be observed with exogenous templates, raising the question of whether the initiation factors required are present in the MAC. For the preparation of in vitro transcription systems, S100 fractions obtained as first purification step cannot correctly initiate transcription, either because of insufficient enrichment of the required factors or because of the presence of inhibitors. The same seems to be true for MAC, but it is also possible that in vivo, these factors only bind transiently to the mtDNA and fractionate in the S65 soluble fraction. Thus, further analyses and purification steps will be necessary to characterize the proteins associated with the mtDNA in MAC.

References

[1] Hatzack, F., Dombrowski, S., Brennicke, A. and Binder, S. (1998) Plant Physiol. 116, 519–527.
[2] Fester, T. and Schuster, W. (1995) Biochem. Mol. Biol. Int. 36, 67–75.
[3] Miyakawa, I., Fumoto, S., Kuroiwa, T. and Sando, N. (1995) Plant Cell Physiol. 36, 1179–1188.
[4] Cannon, C.G., Ward, N.W., Case, C.I. and Heinhorst, S. (1999) Plant Mol. Biol. 39, 835–845.
[5] Tewari, K. and Goel, A. (1983) Biochemistry 22, 2142–2148.
[6] Lakhani, S., Khanna, N.C. and Tewari, K.K. (1993) Plant Mol. Biol. 23, 963–979.
[7] Echeverria, M., Robert, D., Carde, J.P. and Litvak, S. (1991) Plant Mol. Biol. 16, 301–315.
[8] Neuburger, M., Journet, E.P., Bligny, R., Carde, J.P. and Douce, R. (1982) Arch. Biochem. Biophys. 217, 312–323.
[9] Kemble, R.J. (1987) Theor. Appl. Genet. 73, 364–370.
[10] Mulligan, R.M., Leon, P. and Walbot, V. (1991) Mol. Cell. Biol. 11, 533–543.

[11] Yu, W., Sanchez, H. and Schuster, W. (1998) Methods: a Companion to Methods in Enzymology, pp. 63–74, Academic Press.

[12] Rapp, W.D. and Stern, D.B. (1992) EMBO J. 11, 1065–1073.

[13] Maier, R.M., Zeltz, P., Kössel, H., Bonnard, G., Gualberto, J.M. and Grienenberger, J.M. (1996) Plant Mol. Biol. 32, 343–365.

[14] Rajasekhar, V.K. and Mulligan, R.M. (1993) Plant Cell 5, 1843–1852.

[15] Gualberto, J.M., Bonnard, G., Lamattina, L. and Grienenberger, J.M. (1991) Plant Cell 3, 1109–1120.

[16] Hanic-Joyce, P.J. and Gray, M.W. (1991) Mol. Cell. Biol. 11, 2035–2039.

[17] Binder, S., Hatzack, F. and Brennicke, A. (1995) J. Biol. Chem. 270, 22182–22189.