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Two distinct translocation intermediates can be distinguished during protein transport by the TAT (Δph) pathway across the thylakoid membrane

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Abstract During thylakoid transport of the chimeric precursor protein 16/23 which takes place by the twin arginine translocation (TAT) (Δph)-dependent pathway, two distinct translocation intermediates can be identified which represent successive steps in the translocation process. Both intermediates are partially inserted into the thylakoid membrane and can be distinguished by specific degradation fragments occurring after thermolysin treatment of the thylakoids. While the formation of the early translocation intermediate does not depend on a functional TAT translocation machinery, the appearance of the late intermediate is strictly coupled to the Δph-dependent transport of the 16/23 chimera. Accordingly, this translocation intermediate is found associated with two distinct complexes in the thylakoid membrane having apparent molecular masses of approximately 560 and 620 kDa, respectively.

Key words: Protein transport; Thylakoid membrane; Δph-dependent pathway; TAT pathway; Twin-arginine motif; Transport intermediate

1. Introduction

Protein transport across the thylakoid membrane of higher plant chloroplasts can take place by at least four independent translocation pathways which are characterised as SecA-dependent, SRP-dependent, Δph-dependent or spontaneous (summarised in [1,2]). Among these, the Δph-dependent pathway has received particular attention in the past years, since a mechanism which operates independently of soluble factors and nucleoside triphosphates and requires solely a transthylakoidal proton gradient was unknown from other membrane systems. Proteins are targeted to this pathway by signal peptides which are characterised by a pair of arginine residues (twin-R motif) upstream of the hydrophobic core segment [3].

Recently, a phylogenetically related transport route was discovered in bacteria, which appears to be specifically utilised by periplasmic proteins carrying complex redox cofactors [4]. Like the thylakoidal Δph pathway, it operates with signal peptides carrying a twin-R motif and it is therefore termed twin arginine translocation (TAT) pathway. In general, the twin-R signal peptides of chloroplasts and bacteria are TAT-specific also in the heterologous situation, i.e. a bacterial twin-R signal peptide is capable of interacting with the thylakoidal Δph transport apparatus and vice versa [5–7], although there are exceptions from this rule [8]. The bacterial and thylakoidal TAT pathways resemble each other also in mechanistic terms, because they are both capable of translocating folded proteins [9–12]. However, while in bacteria folding is discussed to be a prerequisite for transport by the TAT pathway, thylakoids accept both folded and unfolded polypeptides as substrates [13].

The analysis of the thylakoidal TAT (Δph) translocation apparatus was initiated by the identification of hcf106, a high chlorophyll fluorescence mutant in maize which is specifically affected in the Δph-dependent protein transport across the thylakoid membrane [14,15]. Molecular characterisation of the mutant showed that the *hcf106* gene encodes a putative receptor component of the thylakoidal TAT machinery [16]. Genes with homology to *hcf106* have subsequently been identified in bacterial genomes sequenced to date, indicating that the TAT pathway may be a general translocation route also in prokaryotes. In *Escherichia coli*, three genes with homology to *hcf106* have been described (*tat*A, *tat*B, *tat*E). Two of them, *tat*A and *tat*B, are organised in an operon together with *tat*C and *tat*D which encode a putative translocation pore and a soluble factor of unknown function, respectively [17,18]. Except for TatD, all these Tat proteins are apparently essential for the function of the TAT apparatus [17–20]. Since homologs to *tat*C and *tat*D can be found also in the genomic sequence from *Arabidopsis thaliana*, a similar composition of the bacterial and thylakoidal TAT translocases appears to be possible.

One approach to characterise the thylakoidal TAT translocase rests on the biochemical isolation of the entire translocation complex from the membrane. As a first step into this direction, we have aimed to isolate transport intermediates, i.e. polypeptides which are arrested within the TAT translocation pore during membrane transfer. Analysing a chimeric protein consisting of a transit peptide and mature protein from different precursor polypeptides, this approach was successful and led to the identification of two distinct translocation steps during TAT (Δph)-dependent protein transport across the thylakoid membrane.

2. Materials and methods

2.1. Materials

Chloroplasts and thylakoids were isolated from seedlings of *Pisum sativum*, var. Feltham First, which were grown for 8–9 days under a 12 h photoperiod before harvesting.

2.2. Import into isolated chloroplasts and thylakoids

Precursor proteins were synthesised by in vitro transcription of the corresponding cDNA clones [21] and subsequent in vitro translation in cell-free rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Intact chloroplasts were isolated from young pea leaves by Percoll gradient centrifugation essentially as described [22].

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They were used in transport experiments either as intact organelles (in organello assays) [21] or after lysis and subsequent preparation of thylakoid vesicles (in thylakoido assays) [22]. In both assays, inhibitor studies as well as competition experiments were performed following the protocols of Michl et al. [23] and Karnauchov et al. [8]. Samples were subsequently analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24] and visualised after exposure to screens for a Fuji BAS-1500 phosphorimager using the software package TINA, vs. 2.0 (Raytest, Straubenhardt, Germany).

2.3. Blue native gel electrophoresis

Thylakoid membranes corresponding to 30 μg chlorophyll were resuspended in 15 μl lysis buffer (50 mM Bis-Tris pH 7.0, 1 M ε-aminocaproic acid, 5 mM EDTA, 0.5 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT) and supplemented with 7.5 μl 5% digitonin. Solubilisation was carried out on a rotating mixer at 4°C for 30–60 min. After centrifugation for 1 h at 40 000 × *g*, the supernatant was supplemented with 1.5 μl 5% Coomassie G-250, incubated for 10 min on ice and separated overnight at 100 V on a 5–13.5% blue native polyacrylamide gel containing 0.03% digitonin [25,26].

3. Results

3.1. The chimeric 16/23 protein is retarded at two distinct steps during thylakoid translocation

Among the numerous chimeric proteins which have been analysed in chloroplast import experiments (e.g. [21]), the chimera 16/23 shows particularly interesting transport characteristics. It consists of the transit peptide from the 16 kDa subunit and the mature part of the 23 kDa subunit of the oxygen evolving complex, which are both translocated by the TAT (Δph)-dependent pathway across the thylakoid membrane [27–29]. Unlike the corresponding authentic precursor polypeptides which accumulate quantitatively as the mature proteins in the thylakoid lumen when incubated with isolated intact chloroplasts, only a small fraction of the chimera is found terminally processed in the interior of the thylakoid system (Fig. 1). Instead, most of the 16/23 polypeptide is associated with the thylakoid membrane as a processing intermediate of about 27 kDa, indicating that the envelope transfer domain of its transit peptide was removed by the

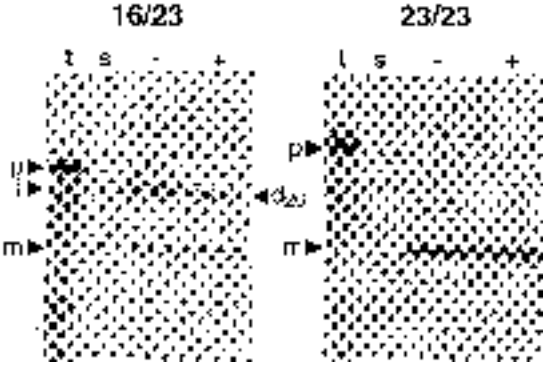

Fig. 1. Transport of the chimeric 16/23 and the authentic 23/23 precursor proteins into intact pea chloroplasts. Radiolabelled precursor proteins generated by in vitro transcription/translation were incubated for 30 min at 25°C with isolated, intact pea chloroplasts. After the import reaction, the chloroplasts were re-isolated and fractionated into stroma (s) and thylakoid membranes which were either mock-treated (lanes −) or treated with thermolysin (lanes +). The fractions were analysed by SDS-PAGE followed by autoradiography of the gel. The positions of the precursor (p), intermediate (i) and mature proteins (m) as well as of a degradation product of approximately 26 kDa (d_{26}) are indicated.

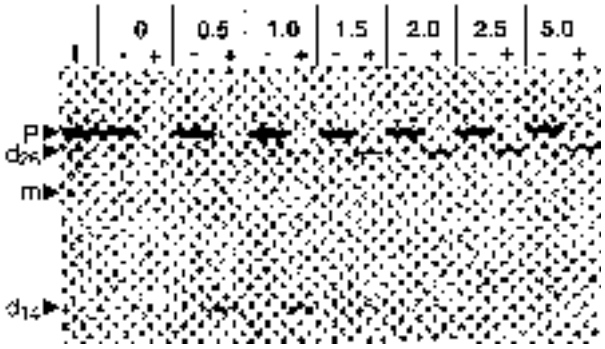

Fig. 2. Thylakoid transport of 16/23 is retarded at two distinct steps of the translocation process. An in thylakoido time-course experiment performed with the 16/23 chimera is shown. Prior to the actual import reaction, the assays were pre-incubated for 2 min on ice. The reaction was started by transferring the incubation mixtures to 25°C in the light. At the time-points indicated above the lanes (given in minutes), the reaction was terminated by washing the membranes with an excess amount of ice-cold import buffer followed by re-isolation of the thylakoid vesicles by centrifugation. One aliquot of the washed thylakoids was mock-treated (lanes −), another aliquot was incubated with thermolysin prior to separation by SDS-PAGE (lanes +). d_{14} indicates the position of a proteolytic fragment of approximately 14 kDa. For further details, see the legend to Fig. 1.

stromal processing activity, while the thylakoid transport signal is still present in the polypeptide. Incubation of the thylakoids after import with thermolysin showed that only few terminal residues of the membrane-bound chimera are exposed to the stromal phase leading to the formation of a proteolytic fragment of approximately 26 kDa (d_{26}, Fig. 1).

This phenomenon was studied in more detail in time-course experiments performed with isolated thylakoid vesicles from pea chloroplasts. As in the in organello assay described above, only a minor fraction of the 16/23 chimera is fully translocated into the lumen of these vesicles and terminally processed to the size of the mature 23 kDa protein (Fig. 2), confirming the observation that the thylakoid transfer of this protein is a relatively slow process. On the other hand, the affinity of the chimera for the thylakoid membrane is remarkably high. Already during the pre-incubation on ice, essentially all of the 16/23 precursor associates with the thylakoid vesicles (Fig. 2, lanes 0). This association is sufficiently firm to withstand repeated washing steps (Fig. 2 and data not shown). Thermolysin treatment of these thylakoids yields a degradation product of approximately 14 kDa (d_{14}, Fig. 2), indicating that the protein was already partially inserted into the membrane.

Immediately after starting the actual import reaction by transferring the assays to 25°C into the light, the amount of membrane-bound 16/23 chimera that is represented by the d_{14} degradation product increases further (Fig. 2). However, already after short incubation periods (>0.5 min), it begins to disappear and is replaced by the membrane-inserted form represented by the d_{26} degradation product that was observed already in the in organello experiments (Fig. 1). Thus, two distinct transport steps can be distinguished for the 16/23 chimera in the course of its thylakoid translocation, an initial membrane association which can take place also under conditions of limited membrane fluidity and a subsequent step in which the protein is almost completely translocated but not yet terminally processed and released into the lumen.

The appearance of similar degradation products (d_{26}) in

both the in organello and in thylakoido assays allows us to predict the orientation of the 16/23 translocation intermediate within the thylakoid membrane. In the in organello assay, a polypeptide of intermediate size which lacks the envelope transfer signal of the transit peptide is inserted into the membrane (Fig. 1), whereas in the thylakoid import assay, the uncleaved precursor protein integrates into the thylakoid vesicles, due to the incapability of isolated stromal extract to process the 16 kDa transit peptide. Since the corresponding degradation products have the same size though, it must be concluded that the N-terminus of the protein is exposed to the stroma and is thus accessible to the protease. This conclusion is confirmed by the finding that a derivative of the 16/23 chimera which carries six additional histidine residues at its C-terminus yields a slightly larger proteolytic fragment under these conditions (data not shown), proving that the C-terminal end of this translocation intermediate is not accessible to the protease.

3.2. The formation of the proteolytic d_{26} fragment depends on a functional TAT pathway

In order to examine whether the membrane-bound forms of the 16/23 chimera represented by the proteolytic fragments d_{14} and d_{26} are true translocation intermediates of the thylakoidal TAT (Δph) pathway, thylakoid import experiments were carried out in the presence of translocation inhibitors which are indicative for different thylakoid translocation routes. It turned out that the appearance of the d_{26} fragment is strictly coupled to a functional TAT (Δph)-dependent transport system, since no such product can be observed if the transthylakoidal proton gradient is dissipated by the addition of nigericin (Fig. 3A). On the other hand, neither apyrase which hydrolyses nucleoside triphosphates nor the SecA inhibitor sodium azide are able to prevent the appearance of the d_{26} product (Fig. 3A and data not shown), indicating that neither SecA nor other NTP-binding proteins like cpSRP54 are involved in membrane integration of the chimera. Furthermore, saturation of the thylakoidal TAT machinery by excess amounts of unlabelled precursor of the 23 kDa subunit of the oxygen evolving complex inhibits the formation of the d_{26} product by thermolysin treatment (Fig. 3B), proving that the corresponding integral form of the 16/23 chimera is an authentic translocation intermediate of this transport pathway.

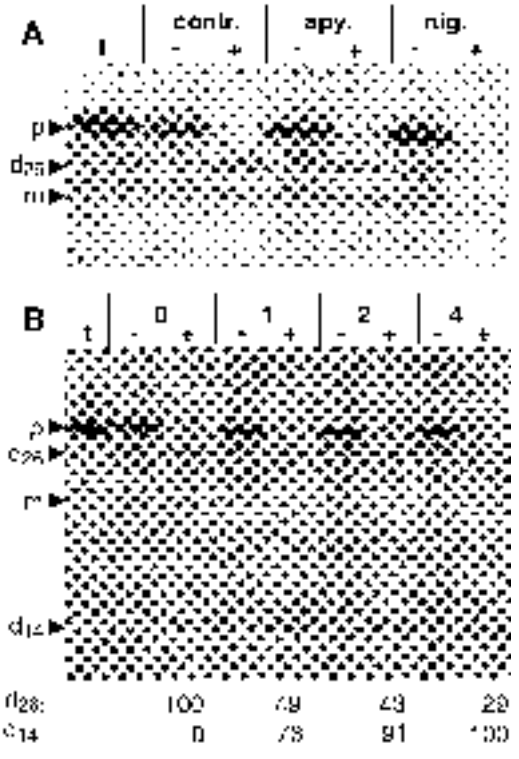

Fig. 3. The appearance of the proteolytic fragment d_{26} depends on the transport of the 16/23 chimera by the Δph-dependent pathway. (A) In thylakoido import reactions were performed for 20 min with the 16/23 chimera either under standard conditions (contr.) or after pre-incubation of the incubation mixtures with 1 U apyrase (apy.) or in the presence of 2 μM nigericin (nig.). (B) In thylakoido import of radiolabelled 16/23 protein in the presence of increasing concentrations (0–4 μM) of unlabelled precursor of the 23 kDa subunit of the oxygen evolving complex. The relative amounts of the two specific degradation products (d_{14} and d_{26}, respectively) are given below the lanes. For further details, see the legends to Figs. 1 and 2.

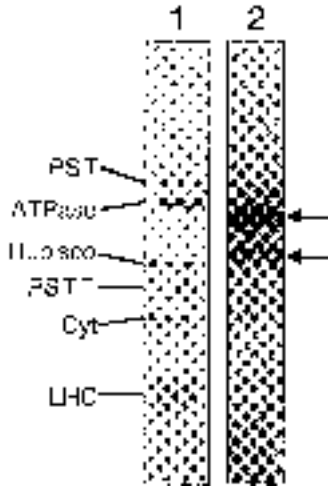

Fig. 4. The chimeric 16/23 protein associates with two complexes of high molecular mass in the thylakoid membrane. In vitro synthesised 16/23 chimera was incubated for 20 min with isolated thylakoids under standard conditions. After the import reaction, the membranes were washed twice and separated, after mild solubilisation, on a 5–13.5% blue native polyacrylamide gel. In 1, the untreated gel showing the stained protein complexes is depicted. In 2, an autoradiograph of this gel is shown. The positions of photosystem I (PS I, ca. 700 kDa), ATP synthase (ATPase, ca. 640 kDa), Ribulose-1,5-bisphosphate-carboxylase/oxygenase (Rubisco, ca. 540 kDa), photosystem II (PS II, ca. 480 kDa), the cytochrome b_6/f complex (Cyt, ca. 430 kDa) and LHC (ca. 280 kDa) are indicated. The arrows point to the positions of two putative TAT complexes of approximately 560 and 620 kDa to which the radiolabelled translocation intermediate of the 16/23 chimera has bound.

In contrast, the membrane-associated form of the chimera leading to the proteolytic fragment d_{14} represents probably an early step in the translocation process which does not depend on a functional TAT translocase. Instead, its formation is even supported by conditions which prevent protein transport by the Δph-dependent pathway. In the presence of nigericin or the 23 kDa competitor protein, an increase in the amount of d_{14} product can be observed after thermolysin treatment of the thylakoids (Fig. 3B and data not shown), suggesting that at this stage of the translocation process, the protein does not yet interact with components of the TAT-specific translocase. Possibly, the protein is associated with the membrane at a

position different from that of the translocation apparatus and needs to diffuse laterally within the plane of the membrane to reach the TAT translocase and continue its membrane transport.

3.3. The TAT-specific translocation intermediate of the 16/23 protein is associated in the thylakoid membrane with two complexes of high molecular mass

Since the formation of the d_{26} fragment depends on a functional TAT translocase in the thylakoid membrane, it can be assumed that at this stage of the transport process, the chimera is associated with components of the TAT machinery. We have therefore recovered the thylakoid vesicles after in thylakoido import of the 16/23 chimera and solubilised the membranes under conditions which should prevent the dissociation of membrane complexes. The fractions obtained were analysed by blue native gel electrophoresis using a method that was adapted from the protocol developed by Schägger and coworkers [25,26]. Under these conditions, the major photosynthetic protein complexes of the thylakoid membrane (photosystems I and II, cytochrome b_6/f complex, ATP synthase and light harvesting complex (LHC)) remain intact to a large extent and are therefore visible as blue or green (i.e. chlorophyll-containing) bands (Fig. 4A). Autoradiography of such gels showed that the newly imported, radiolabelled 16/23 protein is not associated with either of these major complexes. Instead, it accumulates at two positions on the gel which correspond to protein complexes of approximately 560 and 620 kDa, as deduced from the molecular masses of the neighbouring complexes (Fig. 4B). In the stained gel, no distinct bands can be detected at either of these positions, which suggests that the putative translocation complexes are present in the thylakoid membrane only in minor amounts.

4. Discussion

It was the initial goal of this work to examine why the chimeric 16/23 precursor protein is so inefficiently translocated across the thylakoid membrane. Although the mature 23 kDa polypeptide is known since long to be a particularly difficult passenger protein for foreign transit peptides [21], possibly due to the fact that it assumes a folded structure in the chloroplast stroma [30], inefficient thylakoid transport was unexpected in this case, since both parts of the chimera originated from precursor proteins that are targeted by the same pathway into the thylakoid lumen.

Indeed, the analysis shows that the translocation of the chimera across the thylakoid membrane is retarded only at a late step of the entire process. The initial association of the protein with the thylakoid membrane takes place very efficiently, even at low temperatures (Fig. 2), although it should be noted that this step does apparently not yet involve a specific interaction of the targeting signal with the translocation apparatus (Fig. 3). But also the subsequent interaction with the translocase and the actual membrane translocation of the mature polypeptide chain are efficient processes (Fig. 2) and can thus not be responsible for the extraordinarily slow accumulation of mature 23 kDa protein in the thylakoid lumen. Instead, it seems that the terminal processing step which is performed by the thylakoidal processing peptidase on the lumenal side of the membrane [31] and the release of the polypeptide into the lumen are the rate-limiting steps in the membrane transport of the chimera.

The reason for this retardation in terminal processing is unknown. Probably, the cleavage site is not well accessible to the processing activity, which might be due to the increased hydrophobicity of the cleavage site in the chimera as compared to the authentic 23 kDa precursor protein (data not shown). More important, however, the unprocessed chimera assumes a transmembrane orientation with the N-terminus being still exposed to the stromal side of the membrane (Figs. 1 and 2). This strongly suggests that the terminal processing step is a prerequisite for the release of the polypeptide from the membrane and/or translocase and might explain why mutant polypeptides that cannot be terminally cleaved have not yet been detected in the thylakoid lumen.

Finally, the integral transport intermediate of the 16/23 chimera might provide an excellent tool for the isolation of the TAT (Δph)-dependent translocation apparatus from the thylakoid membrane. Although we have no proof so far that either of the two membrane complexes which can be identified by blue native gel electrophoresis (Fig. 4) represents indeed a protein transport machinery of the thylakoid membrane, we have obtained preliminary data from competition experiments suggesting that the 16/23 chimera is retained for a relatively long time within the TAT translocation pore (data not shown). Experiments are underway to examine this observation in more detail.

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References

[1] Klösgen, R.B. (1997) J. Photochem. Photobiol. B Biol. 38, 1–9.
[2] Robinson, C., Hynds, P.J., Robinson, D. and Mant, A. (1998) Plant Mol. Biol. 38, 209–221.
[3] Chaddock, A.M., Mant, A., Karnauchov, I., Brink, S., Herrmann, R.G., Klösgen, R.B. and Robinson, C. (1995) EMBO J. 14, 2715–2722.
[4] Berks, B.C. (1996) Mol. Microbiol. 22, 393–404.
[5] Mori, H. and Cline, K. (1998) J. Biol. Chem. 273, 11405–11408.
[6] Wexler, M., Bogsch, E.G., Klösgen, R.B., Palmer, T., Robinson, C. and Berks, B.C. (1998) FEBS Lett. 431, 339–342.
[7] Halbig, D., Hou, B., Freudl, R., Sprenger, G.A. and Klösgen, R.B. (1999a) FEBS Lett. 447, 95–98.
[8] Karnauchov, I., Herrmann, R.G., Pakrasi, H.B. and Klösgen, R.B. (1997) Eur. J. Biochem. 249, 497–504.
[9] Clark, S.A. and Theg, S.M. (1997) Mol. Biol. Cell 8, 923–934.
[10] Santini, C.-L., Ize, B., Chanal, A., Müller, M., Giordano, G. and Wu, L.-F. (1998) EMBO J. 17, 101–112.
[11] Rodrigue, A., Chanal, A., Beck, K., Müller, M. and Wu, L.F. (1999) J. Biol. Chem. 274, 13223–13228.
[12] Halbig, D., Wiegert, T., Blaudeck, N., Freudl, R. and Sprenger, G.A. (1999b) Eur. J. Biochem. 263, 543–551.
[13] Hynds, P.J., Robinson, D. and Robinson, C. (1998) J. Biol. Chem. 273, 34868–34878.
[14] Barkan, A., Miles, D. and Taylor, W.C. (1986) EMBO J. 5, 1421–1427.
[15] Voelker, R. and Barkan, A. (1995) EMBO J. 14, 3905–3914.
[16] Settles, A.M., Yonetani, A., Baron, A., Bush, D.R., Cline, K. and Martienssen, R. (1997) Science 278, 1467–1470.
[17] Weiner, J.H., Bilous, P.T., Shaw, G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A. and Turner, R.J. (1998) Cell 93, 93–101.
[18] Sargent, F., Bogsch, E.G., Stanley, N.R., Wexler, M., Robinson, C., Berks, B.C. and Palmer, T. (1998) EMBO J. 17, 3640–3650.

[19] Bogsch, E.G., Sargent, F., Stanley, N.R., Berks, B.C., Robinson, C. and Palmer, T. (1998) J. Biol. Chem. 273, 18003–18006.
[20] Chanal, A., Santini, C. and Wu, L. (1998) Mol. Microbiol. 30, 674–676.
[21] Clausmeyer, S., Klösgen, R.B. and Herrmann, R.G. (1993) J. Biol. Chem. 268, 13869–13876.
[22] Brock, I.W., Hazell, L., Michl, D., Skovgaard Nielsen, V., Lindberg Møller, B., Herrmann, R.G., Klösgen, R.B. and Robinson, C. (1993) Plant Mol. Biol. 23, 717–725.
[23] Michl, D., Robinson, C., Shackleton, J.B., Herrmann, R.G. and Klösgen, R.B. (1994) EMBO J. 13, 1310–1317.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Schägger, H. and von Jagow, G. (1991) Anal. Biochem. 199, 223–231.
[26] Schägger, H., Cramer, W.A. and von Jagow, G. (1994) Anal. Biochem. 217, 220–230.
[27] Mould, R.M., Shackleton, J.B. and Robinson, C. (1991) J. Biol. Chem. 266, 17286–17289.
[28] Mould, R.M. and Robinson, C. (1991) J. Biol. Chem. 266, 12189–12193.
[29] Klösgen, R.B., Brock, I.A., Herrmann, R.G. and Robinson, C. (1992) Plant Mol. Biol. 18, 1031–1034.
[30] Creighton, A.M., Hulford, A., Mant, A., Robinson, D. and Robinson, C. (1995) J. Biol. Chem. 270, 1663–1669.
[31] Kirwin, P.M., Elderfield, P.D., Williams, R.S. and Robinson, C. (1988) J. Biol. Chem. 263, 18128–18132.