TCP34, a Nuclear-encoded Response Regulator-like TPR Protein of Higher Plant Chloroplasts

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We describe the identification of a novel chloroplast protein, designated TCP34 (tetratricopeptide-containing chloroplast protein of 34 kDa) due to the presence of three tandemly arranged tetratricopeptide repeat (TPR) arrays. The presence of the genes encoding this protein only in the genomes of higher plants but not in photosynthetic cyanobacterial prokaryotes suggests that TCP34 evolved after the separation of the higher plant lineage. The in vitro translated precursor could be imported into intact spinach chloroplasts and the processed products showed stable association with thylakoid membranes. Using a specific polyclonal antiserum raised against TCP34, three protein variants were detected. Two forms, T1 and T2, were associated with the thylakoid membranes and one, S1, was found released in the stroma. TCP34 protein was not present in etioplasts and appeared only in developing chloroplasts. The ratio of membrane-bound and soluble forms was maximal at the onset of photosynthesis. The high molecular mass thylakoid TCP34 variant was found in association with a transcriptionally active protein/DNA complex (TAC) from chloroplasts and recombinant TCP34 showed specific binding to Spinacia oleracea chloroplast DNA. Two TCP34 forms, T1 and S1, were found to be phosphorylated. An as yet unidentified phosphorelay signal may modulate its capability for plastid DNA binding through the phosphorylation state of the putative response regulator-like domain. Based on the structural properties and biochemical analyses, we discuss the putative regulatory function of TCP34 in plastid gene expression.

Introduction

The expression of chloroplast genes coding for components of the photosynthetic machinery depends largely on nucleus-encoded factors that are synthesized in the cytosol and subsequently imported into the organelle.1-6 Both, genetic and biochemical data suggest that these factors act either as constituents of the organellar transcription/translation machinery or are involved in various post-transcriptional processes, such as RNA stabilization and processing.7-10 Recent work indicates that several chloroplast proteins involved in post-transcriptional steps of chloroplast gene expression contain tetratricopeptide repeat (TPR) or TPR-like motifs, which usually interact with large protein complexes.11,12 TPR arrays consist of a repeated, degenerated stretch of 34 amino acid residues and are presumed to form helix-turn structures acting as scaffolds to mediate protein–protein interactions and often in the assembly/disassembly of multiprotein complexes.13,14

The level of expression of chloroplast genes varies considerably during plastid development...
and differentiation, and is substantially influenced by changes in light intensity and quality. Protein phosphorylation and redox control are considered to provide regulatory feed-back connections between photosynthesis and organellar gene expression. Activation and coordination of these mechanisms require a precise sensing and regulation which can, in principle, be managed by "classical" two-component systems consisting of a sensor kinase and its cognate response regulator.\textsuperscript{15–17} The sensor component recognizes environmental stimuli through phosphorylation of a conserved histidine residue within its kinase domain. Subsequently, the phosphoryl group is transferred to a conserved aspartate residue in the receiver domain of the response regulator component. As a consequence of the phosphorylation-induced conformational change, the output activity of the response regulator is modulated. Several proteins similar to components involved in animal and fungal signal transduction pathways have been identified in various compartments of the plant cell.\textsuperscript{18–21} To elucidate an entire plant signal transduction pathway incorporating a two-component regulatory system, the output activity of plant response regulator proteins must be known. Whereas various bacterial response regulator proteins are known, and function as transcription factors in the regulation of gene expression, only a limited number of plant counterparts with DNA-binding activities have been isolated so far. Among 14 Arabidopsis thaliana response regulator homologues identified up to now,\textsuperscript{20,22–24} two, ARR1 and ARR2, have been found to bind double-stranded DNA in a sequence-specific manner. For plastids, only one response regulator-like protein has been reported, i.e. in rhodoplasts from the raphidophytic alga Heterosigma akashiwo.\textsuperscript{26}

Here, we report the identification and characterization of a novel nucleus-encoded, plastid-located polypeptide of 37.2 kDa, designated TCP34 (tetrapricopeptide-containing chloroplast protein of 34 kDa). The protein possesses remarkable structural features and specifically binds to plastid protein–DNA complexes, suggesting a regulatory role in plastid gene expression by means of protein–protein and/or protein–DNA interactions.

**Results**

**Isolation and characterization of a spinach cDNA encoding a novel chloroplast precursor protein**

During the course of studies aimed at isolating thylakoid protein kinases and molecular components involved in regulation of photosynthesis and chloroplast gene expression, a spinach cDNA encoding a novel tetrapricopeptide-containing polypeptide, designated TCP34, was identified. The full-length cDNA contained a single open reading frame of 1237 bp that coded for a polypeptide of 339 amino acid residues (Figure 1(a)) with a predicted molecular mass of 38.2 kDa. The protein-coding sequence starts from ATG at position 61, which is embedded in a typical plant translation initiation consensus sequence AACAATGGC.\textsuperscript{27} The ATG codon is preceded by an in-frame upstream stop codon, implying that it represents the codon for the potential initiator methionine. The 3' end of the cDNA carried a poly(A) sequence.

The most recent protein databases were searched with the deduced 339 amino acid sequence as the query using the BLAST and FASTA3 programs at

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**Figure 1.** (a) Schematic presentation of functional domains and amino acid sequence of spinach TCP34 precursor protein. The hydrophilic polypeptide contains a single predicted membrane-anchoring (MA) domain located at the C terminus (italics in the sequence). The putative response regulator-like domain (RR) is in boldface. Asp95, the putative phosphorylation site in that domain, is presented in italic boldface. The two serine-rich regions S56–S73, and S268–S298 are in grey italic boldface. The TPR domain is underlined. Two histidine residues within the second TPR unit are in grey boldface. A single cysteine residue is indicated with an asterisk, a putative processing site of the transit peptide (TP) by an arrow. HLHI, helix-loop-helix. The numbers below the protein scheme indicate the amino acid range of each domain.

(b) Predicted secondary structure of TCP34. The smaller cylinder depicts six TPR amphipatic helices forming a bulky moiety with exposed charged residues. Larger cylinders represent two helices forming a putative helix-loop-helix domain. The separating loop is highly negatively charged. Regions rich in serine are indicated with S; interchanging charged residues are represented by + or −. The protein is presumed to be anchored monolobically in the thylakoid membrane with its carboxy-terminal hydrophobic stretch.
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the NCBI and GenomeNet www-services. A search of the A. thaliana databases revealed only a single homologous gene (At3g26580) encoding a protein of unknown function with 67% identity. Northern analysis of total RNA isolated from spinach and A. thaliana seedlings revealed a single transcript of 2.0 kb (data not shown), which argues for an absence of alternative splicing of the TCP34 transcript. Two cyanobacterial proteins, encoded by the hypothetical open reading frame slr1052 and the stress-inducible gene ycS7 (slr0171) were identified with region-confined similarity scores of 36% and 30%, respectively, notably in the TPR array. The search also revealed a regional similarity of 49% to MOM72, a 72 kDa mitochondrial outer membrane import receptor for the ADP/ATP carrier from Neurospora crassa (P23231). The domain homology was found mainly in TPR regions. No homology in other domains, like response regulator or DNA-binding helix-loop-helix motifs, could be observed with cyanobacterial homologues. The sequence analysis demonstrated the presence of both a response regulator motive and an Asp residue, in the TCP34 molecule that seems to be unique for higher plant chloroplasts.

Sequence and structural analysis of TCP34, a TPR-containing protein with unique molecular features

Inspection of the aforementioned local similarities and application of algorithms for secondary structure predictions led to the identification of three tandemly arranged, imperfect 34 amino acid residue repeat motifs, known as TPR arrays (Figure 1(a)).28 Because of the presence of these structural motifs and the predicted chloroplast location, the novel protein was designated TCP34. The TPR arrays in TCP34 are centrally positioned, spanning residues A163 to S275 within the large, mostly hydrophilic segment (Figure 1(a) and (b)). Based on analogy with other TPR-containing proteins, each of the three units consists of a pair of amphipatic, antiparallel -helices of nearly equal length (domains A and B, respectively), punctuated by proline-induced turns (Figure 2(a)).

Another conspicuous feature of TCP34 is an N-terminal response regulator-like domain integrating a predicted helix-loop-helix motif. The large segment directly preceding the TPR array (from E83 to R160) contains 45 clustered charged residues and shows a substantial net negative charge (Figure 2(b)). The two amphipatic -helices (~E97 to ~I124, and ~E136 to ~T163, respectively), and the loop between them represented by a negatively charged spacer region with predicted -sheet features that can generate a structure known as the helix-loop-helix or EF-hand motif. Furthermore, two serine-rich domains with intervening charged residues (segments S56–S73 and S268–S298) are present in the TCP34 sequence. Similar serine-rich, but more positively charged, domains precede the leucine zipper motif in some bZip transcription factors29 and the catalytic domain of PPZ1 phosphatase.30 The charged region, integrating the helix-loop-helix motif, and the serine-rich segment together form an ~140 residue long domain similar to the CheY superfamily of two-component response regulators (Figure 2(b)). A crucial feature of these components is the conservation of D34, D95 and K147 (corresponding to D12, D57 and K109 of CheY), which are invariant in all response regulator proteins and indispensable for their phosphorylation-induced activation.31

Chloroplast localization and topological studies of TCP34

Hydropathy analysis of the deduced TCP34 amino acid sequence as described32 suggested a predominantly hydrophilic protein with a C-terminally located transmembrane domain of 17 amino acid residues (Figure 1). Its predicted pre-sequence displays all attributes of stroma targeting chloroplast transit peptide.33 This peptide contains a high percentage of leucine residues (~24%) but is not particularly abundant in serine and threonine residues. The processing site is ascribed to position P17 (Figure 1(a)) resulting in a mature protein of ~34 kDa.

In organello experiments were conducted to verify that the novel polypeptide resides indeed in the chloroplast. Following cDNA transcription and translation of the resulting mRNA in vitro in the presence of [35S]methionine, the labelled TCP34 polypeptide (Figure 3(a), lane TP) was incubated with isolated intact spinach chloroplasts. Translation of TCP34 in vitro resulted in a highly labelled band of 40 kDa, which slightly varied from the theoretically predicted size of 38.2 kDa, and other polypeptides of lower molecular masses. The latter ones could result from translation starting from each methionine in vitro. Since these products lack the transit peptide sequences they cannot be imported into chloroplasts. After washing and thermolysin treatment, chloroplasts were sub-fractionated into stroma (lane S) and thylakoids (lane T). Figure 3(a) illustrates that the in vitro-made, labelled precursor (arrowhead in Figure 3(a)) was efficiently imported after 30 min of reaction into the organelle, processed to the expected mature size of the protein and inserted into the thylakoid membrane in two forms that were designated T1 and T2 (lane T). A minute signal of unprocessed precursor has also been consistently noted in the thylakoid fraction (marked with an arrowhead). No signal was detected in the stroma fraction. The two TCP34 forms remained stably associated with thylakoid membranes even in the presence of chaotropic salts or alkaline solutions (lanes M). To analyse whether the C-terminal hydrophobic domain is responsible for the anchoring of the polypeptide to the thylakoid membrane the construct lacking the hydrophobic C terminus (the last 27 amino acid residues) was translated in vitro as well and imported into intact spinach
Figure 2. (a) Sequence alignment of TCP34 TPR arrays and (b) putative response regulator receiver domain in TCP34 with the similar motifs found in other sequence related proteins. (a) The TPR motif of TCP34 was compared with homologous motifs from other TPR-containing proteins. Asterisks indicate residues used by Sikorski et al.28 to define TPR motifs. The TPR consensus sequence usually comprises the highly conserved amino acid residues L7, G8, Y11, A20, F24, and A27, as well as P32.14 These residues have been proposed to interact with each other and thereby to mediate intra- or intermolecular protein–protein interactions. TPR consensus 1 and 2 are modified from Sikorski et al.,28 Becker et al.,71 and Chen et al.72 The sign § represents any residue with a large hydrophobic side-chain. Aligned are the TPRs of the following proteins: serine/threonine phosphatases PP5 from Rattus norvegicus (P53042) and PPT1 from TCP34, a Response Regulator-like TPR Protein.
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To understand the nature of these two TCP34 forms and to check whether one of them could be the result of processing of the higher molecular mass forms, import kinetics were performed with radiolabelled TCP34 precursor. Radiolabelled precursor was incubated with intact spinach chloroplasts for 10, 20, 30 and 60 min (Figure 3(b)). Three TCP34 forms including the protein precursor were found in approximately equal quantities after the first 10 min of incubation of the assay. After import, the precursor protein was quite stable, but processed with increasing incubation time and disappeared after 60 min of import. TCP34-T1 and -T2 were present as well after 10 min and increased slightly in amount after 60 min of import reaction. The T1 and T2 doublets were not interconverted within our experimental time-range and probably were processed within the first 10 min of precursor translocation into chloroplasts. Consistent with our observations above, no processing forms were found in the stroma. Treatment of thylakoid membranes with thermolysin (Figure 3(b), lane, +) completely removed all three thylakoid membrane-associated TCP34 variants. This confirmed that most part of the protein is exposed to the stroma face.

To further characterize the TCP34 protein, polyclonal, monospecific antisera were raised against the recombinant precursor molecule overexpressed in *Escherichia coli* cells. The overexpression of TCP34 in *E. coli* cells transformed with the plasmid was induced by addition of IPTG. Cells were fractionated into soluble and insoluble proteins, which could contain inclusion bodies as well, and the overexpressed TCP34 was found in the insoluble fraction of IPTG-induced cells (Figure 4(a)). This protein was used for immunisation of rabbits and antiserum specificity was tested on *E. coli* cells with overexpressed TCP34 (Figure 4(b)). The antisera reacted specifically with only one band, the overexpressed protein after IPTG induction.

The TCP34 antisera were then used for probing chloroplast subfractions. Unexpectedly, the protein, probed by immunological analysis of fractionated chloroplast proteins from 14 day-old pea plants, was found in the stroma fraction, but not in thylakoid or in the envelope membranes (Figure 5(a)). Comparison of TCP34 localisation in pea, chosen because of its standardized envelope preparations, and spinach chloroplast fractions did not reveal substantial differences. The translation product was approximately 3 kDa smaller than the entire precursor protein. Its import resulted in three TCP34 variants, which were targeted to the stroma (Figure 3(a)). This suggests that the predicted C-terminal hydrophobic domain is indeed involved in the association of TCP34 with the membrane and that the doublet TCP34-T1 and -T2 is not the result of processing the transmembrane domain.

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Saccharomyces cerevisiae (P53043), heat shock protein STII from *S. cerevisiae* (P15705), mitochondrial import receptor MOM72 from *Neurospona crassa* (P25231), FKBP56-binding immunophilin FKBP52 from *Oryctolagus cuniculus* (M84988), cyclophilin CYP40 from *bos taurus* (P26882), and protein kinase PKR inhibitor P58, also from *B. taurus* (A56534).

(b) Sequence alignment of the TCP34 N-terminal region with regulatory domains of selected CheY superfamily members, and the conserved regulatory domain of the ethylene response receptor, ETR1, from *Arabidopsis*. In consensus 1 conserved residues in both the CheY and the TCP34 are indicated in capital letters, asterisks represent residues which are conserved in at least one of the 78 other superfamily members listed by Volz. Consensus 2 according to Volz, in pairwise comparison individual members may show as little as 6% identity. The three residues that are highly conserved in the receiver domains are boxed. Aligned are response regulators; CheY from *E. coli* (NP_416396), RegA from *Rhodobacter capsulatus* (S41451), OmpR from *E. coli* (NP_417864), Spol0A from *Bacillus subtilis* (P06534), NRI from *E. coli* (NP_290493), ArcB from *E. coli* (NP_418818), and ETR1 from *A. thaliana* (NP_176808). D34, D95 and K147 indicate the amino acid residues in spinach TCP34 that are conserved in response regulator domains of other proteins.

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The purity of the isolated protein subfractions was checked immunologically with antisera raised against the large subunit of the stroma enzyme rubisco (LSU), the chlorophyll a-binding protein of photosystem II, CP47, located in thylakoid membranes, and the TOC34 protein of the outer envelope membrane import machinery (Figure 5(a)). The last mentioned component was enriched in envelope membranes, and of the small subunit of rubisco (SSU). On the contrary, chaperonin 60 protein (Cpn60), a protein not related to photosynthesis, decreased and could not be detected in chloroplasts after six weeks of light exposure. This is consistent with findings of a higher expression of Cpn60 protein in non-photosynthetic tissues.

To understand the cause for the different localisation and protein processing noted from the in organello approach and immunologically by in vivo experiments, the TCP34 protein was studied immunologically in etiolated spinach seedlings and after their exposure for 10 h, 24 h, 48 h and two or six weeks to light (Figure 5(c)). First, the amount of photosynthesis-related proteins was estimated in etioplasts and chloroplasts extracted from spinach material exposed for the aforementioned periods to light (Figure 5(c)). Exposure to light increased the accumulation of the β subunit of ATP synthase, associated with thylakoid membranes, and of the small subunit of rubisco (SSU). On the contrary, chaperonin 60 protein (Cpn60), a protein not related to photosynthesis, decreased and could not be detected in chloroplasts after six weeks of light exposure. This is consistent with findings of a higher expression of Cpn60 protein in non-photosynthetic tissues.

To analyse the localisation of TCP34 forms, etioplasts (E) and chloroplasts (C) were purified from etiolated and light-exposed material, respectively, and fractionated into prolamellar membranes (PM) and soluble proteins (PS) from etioplasts, or thylakoids (T) and stromal (S) proteins from chloroplasts (Figure 5(c)). No TCP34 signals were immunodetected in membrane and soluble fractions from the etiolated material. In contrast, the anti-TCP34 serum revealed three polypeptide species in developing chloroplasts that showed different accumulation patterns with the time of exposure to light: two forms, TCP34-T1 and -T2, were found in thylakoid membranes and a single product, TCP34-S1, in the soluble stroma fraction. The two thylakoid-bound forms accumulated in developing chloroplasts, i.e. between 48 h and two weeks of exposure to light, before decreasing drastically during the subsequent four weeks of illumination. The soluble form S1 appeared in small amounts at very early stages, i.e. 10 h after exposure to light. It then accumulated steadily for 48 h and then remained nearly constant, over six weeks of light exposure. This experiment demonstrated that there are at least three variants of TCP34 that can be distinguished both by their pattern of developmental regulation, apparent molecular masses and localisation. To verify co-migration of the TCP34 variants detected by in organello experiments and immunological analysis the samples were run on the same gel (data not shown). This analysis demonstrated the identity of three variants in both, the in organello assay and immunological analysis, respectively.

**Evidence for plastid DNA-binding activity of TCP34**

The presence of a helix-loop-helix domain, resembling the DNA-binding module of the
prokaryotic response regulator OmpR, raised the possibility that TCP34 possesses DNA-binding activity. The recombinant precursor molecule was therefore probed with plastid DNA (ptDNA) and used for South-Western analysis (Figure 6(a)). Significant binding to ptDNA (Figure 6(a), lane 2) was detected. The competition experiments with non-labelled ptDNA in amounts that were equal or higher than radioactively labelled plastid ptDNA demonstrated its specific binding to TCP34 protein. The signals reflecting the DNA-binding obtained with 32P-labelled DNA decreased when the higher amounts of non-labelled ptDNA (1 and 10 μg) were incubated together with the radioactive probes. To probe whether TCP34 binds specifically chloroplast DNA or can bind to any other DNA, competition experiments with different amounts of genomic E. coli DNA were performed (Figure 6(b)). No changes of DNA-binding by TCP34 were observed in the presence or absence of E. coli DNA. To pinpoint the region(s) of the plastid chromosome that is (are) recognized by TCP34, four chloroplast DNA fragments encoding genes for the photosynthetic machinery, notably psaA, psbC, psbD, or rbcL-atpB, were incubated with the recombinant precursor protein. All these chloroplast DNA fragments could bind the recombinant protein (Figure 6(c), lanes 2 and 3).

**Supercomplex localization of TCP34 component**

The presence of three tandemly arranged TPR arrays and the ability of TCP34 to bind specifically to ptDNA raised the possibility that TCP34 is associated with other proteins or with multimeric complexes. Therefore, protein–DNA complexes, the so-called TAC (transcriptionally active chromosomes), released from photosynthetic membranes by Triton X-100 solubilisation was checked for the presence of TCP34. Size-exclusion chromatography has shown that the TAC complex possesses a high molecular mass (~2 MDa) and contains 30–40 different polypeptide bands including subunits of the plastid-encoded RNA polymerase (PEP). The protein pattern of the TAC complex extracted from spinach chloroplasts was compared with...
chloroplast proteins by silver staining after SDS-PAGE (Figure 7(a)) and TCP34 was immunologically checked for in both fractions (Figure 7(b)). The TCP34 protein was clearly detectable in the TAC fraction immunologically but could not be visualized by silver staining. The presence of the α-subunit of PEP in the same complex provides additional evidence that this is a bona fide TAC complex. No reaction with the TAC fraction was observed when an antiserum against cytochrome f, which possesses a molecular mass similar to TCP34, and large subunit of rubisco complex were used as a control for TAC purity.

**Phosphorylation of TCP34 protein**

In most cases the regulation of response regulators and factors involved in gene expression is controlled by phosphorylation/dephosphorylation mechanisms. Phosphorylation of TCP34 was analysed in two ways, first immunologically with antisera raised against phosphothreonine residues in spinach chloroplast, stroma and thylakoid protein fractions (Figure 8(a)). The antisera recognized the TCP34-T1 and TCP34-S1 forms, but not TCP34-T2 among various phosphorylated proteins; the TCP34 variants separated in one gel system were identified immunologically by anti-TCP34 and phosphothreonine serum (data not shown).

To analyse, whether the serological reaction of these TCP34 forms was due to phosphorylation and not due to some non-specific binding of phosphothreonine antisera, fractionated chloroplast proteins were treated with alkaline phosphatase that hydrolyses orthophosphoric monoester groups (Figure 8(a), lanes C AP). Dephosphorylation was efficient for most phosphorylated components, although the CP43 protein of photosystem II and some stroma proteins were not completely dephosphorylated by this approach. The incomplete dephosphorylation could have resulted from an improper ratio of enzyme and substrate(s), usage of a heterologous enzyme–substrate system and/or from a lower accessibility of the phosphorylated sites masked due to secondary/tertiary structures of the protein(s) to the alkaline phosphatase.

Dephosphorylation of the stroma form resulted in the appearance of a second, slightly lower molecular mass band, designated TCP34-S2 (Figure 8(a), lower panel). The TCP34-T1 form was fully dephosphorylated in vitro in thylakoids, while TCP34-S1...
was dephosphorylated only partially. This could have a similar explanation as the uncompleted dephosphorylation of CP43, which was described above. The protein fractions treated or untreated with alkaline phosphatase were probed with TCP34 antisera. No visible shifts or redistribution of the TCP34-T1 and -T2 forms could be noted, although the bands often became slightly diffuse in the latter case (Figure 8(a), lower panel). This indicates that the TCP34-T2 variant is probably not the result of protein dephosphorylation. We conclude that the doublet of thylakoid TCP34 should result from some other protein modifications that may involve processing of the TCP34-T1 variant from the N terminus.

We also investigated the state of phosphorylation of TCP34 in vitro upon incubation of broken chloroplasts with [γ-32P]ATP and protein kinase (Figure 8(b)). The radiolabelling with [γ-32P]ATP confirmed the phosphorylation of TCP34-T1 and -S1 forms as found with phosphothreonine antisera. The purified TAC complex was deficient of autophosphorylation activity, probably due to the lack of associated protein kinase(s), and an extrinsic enzyme had to be added in order to phosphorylate TAC proteins. Phosphorylation of TAC proteins resulted in the detection of a phosphoprotein band comigrating with the TCP34-T1 variant. Immuno-precipitation of a radiolabelled TAC protein fraction with antisera against TCP34 picked up only one protein, corresponding to TCP34-T1. These data argue for a TAC complex containing at least the higher molecular mass form of TCP34, which can be reversibly phosphorylated.

Discussion

The TCP34 protein described here represents a novel response regulator-like component in chloroplasts with intriguing features. It provides a unique example of a chloroplast TPR protein that also displays a helix-loop-helix containing response regulator-like structure. TCP34 is both, associated with thylakoid membranes, specifically with the membrane-associated transcriptionally active chromosomes but also found in the stroma, depending on the developmental stage of the material. TCP34 homologues are found in other sequenced plant genomes (Arabidopsis and rice) but not in the chloroplast ancestors, photosynthetic cyanobacteria.

TCP34 mature forms are generated by multiple processing events

Two lines of evidence establish that the cDNA we isolated encodes the entire TCP34 polypeptide. First, the deduced translation initiation codon is embedded in a typical canonical plant consensus sequence and preceded by an in-frame stop codon implying that this ATG codon represents the actual initiation codon. Second, the in vitro synthesized translation product is competent for import into isolated chloroplasts. The presence of a plastid-targeting signal, in organello experiments and cellular subfractionation clearly demonstrate that TCP34 is a chloroplast protein. These experimental data were recently proven by proteomic analysis of thylakoid membrane proteins that found the
TCP34 Arabidopsis homologue in association with chloroplast thylakoids. The detection of two processed forms of that protein are that bound to thylakoid membranes in in organello experiments differs from the situation in vivo where only one soluble variant in the stroma was immunologically detectable in chloroplasts of mature spinach plants. The fact that the C-terminally truncated protein that lacks its hydrophobic domain accumulates in the stroma of intact spinach chloroplasts when translated and imported in organello suggests that the membrane interaction of TCP34 is controlled by a post-translational cleavage of the hydrophobic C terminus. As judged from the import experiments of full-size TCP34 protein, it is conceivable that the regulatory system responsible for this C-terminal conversion does not operate properly in the in organello import system, therefore preserving membrane attachment of two mature TCP34 variants that probably differ from each other by some additional N-terminal processing. Interestingly, the probably C-terminally processed form and the mature thylakoid membrane-associated form T1 possess similar molecular masses. This suggests that the C-terminally processed stromal S1 variant contains an unprocessed N terminus. It is therefore likely that after import and maturation of the precursor form there are two additional, perhaps alternative processing events that operate either from the N terminus that preserves membrane binding of TCP34, or from the C terminus that releases the product into the chloroplast stroma. Processing of various transcription factors has been reported in mammals and prokaryotes. Two candidate membrane proteases homologous to bacterial peptidases involved in the processing of transcription factors have recently been found in the Arabidopsis genome and were predicted to be targeted to chloroplasts.

Developmental control of TCP34 localisation

The membrane and soluble forms of TCP34 showed unique and differing accumulation patterns in developing chloroplasts. TCP34 was absent in etioplasts but significant protein levels were reached within two days of light exposure, a period during which etioplasts turn into photosynthetically active chloroplasts. Interestingly, whereas the membrane-bound forms reached a maximum level within two weeks of light exposure, before dropping in mature leaves, the soluble form was also light-induced but its amount remained highly stable in fully developed chloroplasts. This contrasting behaviour argues for a transient requirement of the membrane-bound forms at the time of leaf and photosynthetic development. In this respect, the presence of one membrane-bound form in the TAC complex (see below), together with its ability to bind specifically to ptDNA in vitro, suggests a role of TCP34 in promoting transcription of photosynthetic genes. At the present stage, the role of the soluble TCP34 form, if any, is not clear. Its release from the membrane could reflect an inactivation process, but the preservation of large amounts of the presumably C-terminally truncated product TCP34-S1 in chloroplasts from green mature spinach leaves suggests that it also serves some critical function. The processing of TCP34 with its release into the chloroplast stroma could as well be an additional control mechanism for the activity of TCP34; for instance, in regulating the efficiency of gene expression by replacing a highly active thylakoid-bound form for a poorly active stromal form and/or by modulating the association of nucleoids with thylakoid membranes.

TCP34 is a TPR protein that associates with plastid DNA

The presence of a TPR module in the central region with an N-terminally positioned potential response regulator-like domain that includes a putative helix-loop-helix motif could be responsible for DNA-binding and/or to some proteins as well. Prediction and biochemical evidence suggest that in TCP34 both modules are stroma-exposed, forming a large solvent-accessible moiety. Two highly conserved amino acid residues appear to be of central importance in TPR repeats: residue G8 in domain A has a small side-chain and forms a hole into which the bulky F24 residue, serving as a knob, fits. These residues are conserved in the TPR arrays of the TCP34 protein as well, whereas the hydrophobic residues Y11 and A20 are replaced by alanine, and serine, respectively. The replacement of Y11 appears to be a common feature of TPR-containing chloroplast proteins identified up to now. It clearly distinguishes them from members of the PPR family, which are characterized by tandemly arranged 35 amino acid repeats, and are supposed to mediate RNA binding rather than protein–protein interactions.

In plastids, DNA exists as large protein–DNA complexes, organised in nucleoids. DNA in nucleoids is generally bound to membranes of the inner envelope in etioplasts. During the initial steps of chloroplast development nucleoids relocate to thylakoid membranes of mature chloroplasts. Several DNA-binding chloroplast proteins identified up to now are associated with plastid nucleoids, in particular the tobacco CND41 protein, the pea PEND protein, a member of the bZip protein family, and the MFP1 DNA-binding protein. The MFP1 protein was found in thylakoid membranes of developed plastids and is supposed to represent an anchor component of nucleoids to the thylakoid membrane system. Similarly to MFP1 protein, TCP34 was also found in association with transcriptionally active chromosomes, which represent nucleoïd fractions and thylakoid membranes.

Three tandemly arranged TPR arrays are probably responsible for the association of TCP34 with a large transcriptionally active protein–DNA complex. The association of only one, the higher
molecular mass TCP34-T^1 form, with the TAC argues for its functionality in gene expression and/or cellular signalling. Since the TAC complex is extracted from thylakoid membranes that are washed to remove stroma proteins, we suggest that it contains the thylakoid-associated TCP34-T^1 form and not the soluble S^1 variant.

The association of TCP34 with the TAC complex and its DNA binding suggest involvement of TCP34 in the expression of plastid genes. If so, the question arises how this gene expression is controlled and whether this gene expression is under redox control. Although redox regulation has been reported for some plastid genes^54 the mechanism and components that are involved have not been identified.

Phosphorylation of TCP34 variants

We found that at least two of the TCP34 variants, T^1 and S^1, can be phosphorylated. Analysis of the TCP34 phosphorylation status both with phosphothreonine antisera and by radiolabelling with [γ-32P]ATP shows reversible phosphorylation of the T^1 variant. Absence of phosphorylation of the T^2 domain indicates that the phosphorylation site is located closer to the N terminus and probably cleaved off from the T^2 variant. Dephosphorylation of the stroma variant TCP34-S^1 in vitro resulted in an electrophoretic shift of the protein band corroborating the phosphorylation of the soluble TCP34-S^1 variant. The fact that it is seen only in vitro indicates that the dephosphorylated S^2 variant is rapidly degraded in vivo or can be reversibly phosphorylated and converted to form S^1.

The presence of an N-terminally positioned response regulator-like domain raises the possibility that TCP34 is part of a regulatory chloroplast two-component His-to-Asp phospho-relay system. The presence of an aspartate residue in this TCP34 domain raised the possibility of its phosphorylation. However, attempts to transfer the phosphorylated group of a histidine residue heterologously with overexpressed EnvZ histidine kinase from E. coli to an aspartate residue of overexpressed spinach TCP34 protein were not successful (data not shown). On one hand, this could mean that despite a high level of sequence homology with ancient response regulators, the mechanisms of signal transfer are modified in eukaryotic homologous components. On the other hand, one could suggest that the extracted protein fraction as well as overexpressed protein did not contain the corresponding histidine sensor kinase. Regarding the evolution of plant two-component systems, it is of particular interest that distinct cyanobacterial histidine kinases have been proposed as ancestors of plant phytochromes, which, despite their histidine kinase-like domains, show serine/threonine protein kinase activity.55,56 One could therefore speculate that spinach chloroplasts contain a modified two-component system in which the classical histidine kinase is replaced by a serine/threonine kinase. Another crucial question is whether “one-component” systems exist in which an “orphan” receiver or transmitter module operates without a partner.57 The phosphorylated amino acid residue(s) in TCP34 have not been identified. The reaction of TCP34 with anti-phosphothreonine serum and the stability of the of TCP34 phosphorylation against acid treatment argue for phosphorylation of threonine or serine residues. On the other hand, the limited dephosphorylation of TCP34 by alkaline phosphatase that is specific for O-phosphorylation of threonine or serine residues indicates possible phosphorylation of residues other than threonine or serine. The data suggest also that several phosphorylation sites in TCP34 could be involved in the case of a one-component system grouping receiving and transmitting modules.

Reversible protein phosphorylation has been shown to affect DNA binding and properties of various transcription factors in yeast and animals.58–61 Relatively little is known about phosphorylation of plant transcription factors.62 Furthermore, the absence of a phosphorylated TCP34 form in the TAC preparation, but its potential phosphorylation with externally added protein kinase, suggest a possible modulation of its affinity for ptDNA and/or a DNA-binding protein complex by post-translational modification of distinct threonine and/or serine residues.

In conclusion, the discovery of TCP34 provides first evidence for a possible involvement of a nucleus-encoded, TPR-containing response regulator-like protein in the regulation of plastid gene expression. It reinforces the importance of post-translational modification, such as processing and protein phosphorylation, in TPR-mediated protein–protein interactions in chloroplast signal transduction processes.

Materials and Methods

Plant material and growth conditions

Spinach (Spinacia oleracea, var. Monopa, Fa. Sperling) and pea plants were grown in a greenhouse at 100 μE m^{-2} s^{-1}. For all experiments, two month-old spinach plants were used for the isolation of chloroplasts, if not otherwise indicated. For kinetic experiments, spinach plants were germinated in darkness, grown for six to seven days and transferred to light (100 μE m^{-2} s^{-1}) for 10 h, 24 h, 48 h and two and six weeks.

Isolation of TCP34 cDNA

Heterologous serological screening of a λgt11-based green leaf cDNA library from spinach was performed using an antibody raised against Synecocystis sp. PCC 6803 histidine kinase slr0311. After three cycles of screening total DNA from positive phages was isolated using standard PEG/NaCl precipitation of lambda particles,63 followed by a phenolisation step and ethanol precipitation. After restriction with EcoRI the cDNA
inserts were purified and sub-cloned into pBluescript SK− vector for sequencing. Two inserts of different sizes (350 bp and 900 bp, respectively) contained identical sequences, which showed 68% homology to an unknown open reading frame from A. italiana. Expectedly, none of the selected cDNA fragments encoded a complete open reading frame. Therefore, a second round of screening was performed using radioactively labelled cDNA fragments as probes. Five recombinant phage were selected. The largest insert of 1237 bp represented a reading frame. Therefore, a second round of screening selected cDNA fragments encoded a complete open reading frame from Arabidopsis. Analyses were obtained from the TIGR databases

Sequence analysis

Analysis of protein and gene homologies were performed using a BLAST and FASTA search on NCBI and CLUSTAL W on the EMBL database. Sequence analyses were obtained from the Arabidopsis and rice genome TIGR databases. Predictions of chloroplast localisation of TCP34 were obtained by TargetP and Predotar. The position of transit peptide cleavage sites was predicted by Signal P software.

Protein gel electrophoresis and immunological analysis

Proteins were separated by Tris–glycine SDS-PAGE and denaturing 6 M urea SDS-PAGE. For Western analysis, proteins were transferred onto either nitrocellulose or polyvinylidene difluoride membranes (Biograde NT; PALL, Filtrol, Dreieich, Germany). Immunodetection was performed using the enhanced chemiluminiscence system. Chloroplast phosphoproteins were immunodetected by polyclonal anti-phosphothreonine antibodies obtained from Zymed Laboratories (San Francisco, CA).

For competition experiments with antisera raised against TCP34 protein with sub-chloroplast fractions the proteins transferred onto nitrocellulose membrane were incubated with anti-TCP34 non-treated and pre-treated proteins transferred onto nitrocellulose membrane were incubated with anti-TCP34 to assess heterologous specificity. In the latter case, anti-TCP34 diluted in milk was incubated for 1 h with 1 µg of overexpressed in E. coli TCP34 denatured for 10 min at 80 °C.

Protein import into isolated spinach chloroplasts

The DNA sequence encoding for full-length TCP34 was cloned into pQE30 plasmid (Qiagen GmbH, Hilden). Expression of the protein was performed in the E. coli M15 host. For fractionation of E. coli cells, they were sedimented and lysed for 30 min in a buffer containing 0.3 M NaCl, 0.05 M NaH2PO4 (pH 8.0), 0.01 M imidazole and 1 mg/ml of lysozyme, sonicated four times for 5 s each and centrifuged for 30 min at 10,000 g. The overexpressed protein was excised from the gel, eluted and injected into rabbits.

Fractionation of intact spinach chloroplasts and preparation of the plastid TAC complex

Etioplasts from spinach were isolated according to a described procedure. Intact chloroplasts were isolated from spinach leaves using discontinuous Percoll gradients and were subsequently osmotically shocked in lysis buffer (10 mM Hepes-KOH (pH 8.0), 5 mM MgCl2). Thylakoid membranes were separated from the stroma fraction by centrifugation for 10 min at 5000 g. The membranes were washed in buffer containing 10 mM Tricine–HCl (pH 8.0), 100 mM sucrose and 5 mM MgCl2. Fractions containing transcriptionally active chromosomes were prepared as described.

Expression and purification of recombinant TCP34

The E. coli expression plasmid was constructed by cloning of a 1269 bp HincI-clf fragment, obtained from SAMS2B4.1T7, into Smal-digested pQE30 (Qiagen GmbH, Hilden, Germany). An overnight culture of E. coli M15 carrying the His-tagged version of TCP34 precursor was used to inoculate 30 ml of LB containing 100 µg/ml of ampicillin. Four hours after induction of protein synthesis with 1 mM isopropyl-b-D-thiogalacto- pyranoside (IPTG) cells were lysed in 300 mM NaCl, 50 mM NaH2PO4 (pH 8.0), and 10 mM imidazole in the presence of lysozyme (1 mg/ml) followed by sonication. The pelleted insoluble protein fraction containing recombinant TCP34 was resuspended in lysis buffer without lysozyme and used for further analyses.
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Antibody preparation

Recombinant TCP34 precursor was expressed in *E. coli* M15 as described above and the insoluble protein fraction was separated by SDS-12% (w/v) PAGE. For further purification the recombinant protein was excised from the gel, subjected once more to SDS-PAGE, followed by electrotransfer onto nitrocellulose membrane. Prior to injection into rabbits a membrane strip (1 cm) containing recombinant TCP34 was dissolved in 200 µl of dimethyl sulfoxide.

Phosphorylation in vitro and immunoprecipitation of phosphorylated proteins

Phosphorylation of chloroplast, thylakoid and stroma proteins was performed for 30 min at 25 °C in buffer containing 10 mM Tricine-NaOH (pH 7.8), 100 mM sorbitol, 5 mM MgCl₂, 10 mM NaF and 0.2 mM [γ-³²P]ATP (0.05 µCi/µl; 1 µCi = 37 KBq). The reaction was stopped by addition of 6 mM ATP. Phosphorylation experiments with TAC fraction were performed in a 100 µl volume as described.³⁸ TAC proteins were phosphorylated by addition of protein kinase A (Sigma). Immunoprecipitation of proteins was performed by addition of one volume of buffer containing 100 mM Tris–HCl (pH 7.5), 600 mM NaCl, 10 mM EDTA, 2% (v/v) Triton X-100, 40 µl of the TCP34 antibody solution and 40 µl of protein A Sepharose. After 60 min of incubation and centrifugation the pellet was washed four times in buffer containing 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 2% Triton X-100, 0.1% (w/v) SDS and once in Tris–HCl (pH 6.8). Proteins were separated by denaturing 6 M urea SDS-PAGE and transferred onto nitrocellulose membranes.

Dephosphorylation of phosphorylated chloroplast polypeptides

In order to dephosphorylate thylakoid and stroma proteins the samples were incubated with bovine alkaline phosphatase (Sigma, Schnelldorf).³⁹ For dephosphorylation assays phosphorylation in vitro was performed as described above but without adding phosphatase inhibitor (NaF). The phosphorylated proteins were incubated with or without (control) alkaline phosphatase in 0.1 M glycine (pH 10.4), 1 mM MgCl₂, 1 mM ZnCl₂ and proteinase inhibitor cocktail (Sigma, Schnelldorf) for 30 min at room temperature. The reaction was stopped by addition of 120 mM EDTA.

In vitro DNA-binding assay

The insoluble protein fraction containing recombinant TCP34 precursor was separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. The membrane was probed with 100 ng of [³²P]-labelled DNA isolated from spinach chloroplasts as described ⁴⁰ in buffer containing 10 mM Tris–HCl (pH 7.5), 75 mM NaCl, 0.13% (w/v) BSA, 0.02% (w/v) NaN₃, 10 µg/ml of salmon sperm DNA and 1 mM DTE. For competition experiments 100 ng, 1 µg and 10 µg of non-labelled plastid DNA or 100 ng and 1 µg of genomic *E. coli* DNA were added during the hybridisation step with radiolabelled DNA probe to nitrocellulose membrane with overexpressed protein. To determine the binding specificity of TCP34 for chloroplast DNA fragments, inserts of spinach chloroplast DNA clones were isolated for recombinant plasmids.

Data Bank accession number

The TCP34 sequence data have been submitted to the DDBJ/EMBL/GenBank databank under the accession no. Y14198.

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References


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