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Proteomics-based sequence analysis of plant gene expression – the chloroplast transcription apparatus

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This paper is dedicated to Professor Gerhard Richter on the occasion of his 75th birthday

Abstract

The chloroplast transcription apparatus has turned out to be more complex than anticipated, with core polypeptides surrounded by multiple accessory proteins of diverse, and in part unexpected, functions. At least two different RNA-binding proteins and several redox-responsive proteins are components of the major chloroplast RNA polymerase termed PEP-A. One of the key-regulatory factors has been identified as a Ser/Thr-specific protein kinase that is sensitive to SH group modification by glutathione and by this means is able to modulate transcription. The cloned plastid transcription kinase from mustard (*Sinapis alba* L.) has been assigned as a member of the (mostly nucleo-cytosolic) CK2 family and hence has been termed cpCK2. Despite its apparent role in mustard chloroplast transcription, until recently no data have been available for other plant species. Using the web database resources, we find evidence for an evolutionarily conserved role of this redox-sensitive plastid transcription factor.

Keywords: Chloroplast transcription; Mass spectrometry; Protein kinase; RNA-binding proteins; Sequence database searches

1. Introduction

Chloroplasts and other plastid forms are an essential part of plant cell architecture and physiology. Moreover, it is well-accepted that life in general depends on the biochemical activities of these unique plant cell organelles, which have their own DNA and a complete machinery for gene expression (Bogorad, 1991). Like all subsequent steps along this pathway, the transcription of plastid DNA into primary RNA is catalyzed by a multi-component macromolecular complex (Hess and Börner, 1999; Cahoon and Stern, 2001). It consists of at least two different types of enzymes that, based on the intracellular coding site of their catalytic polypeptides, were termed Nuclear-Encoded Polymerase (NEP) and Plastid-Encoded Polymerase (PEP) (Maliga, 1998). PEP is a multi-subunit RNA polymerase resembling that of eubacteria, where the α, β and β′ core subunits assemble with σ (sigma) specificity factor(s) to form the initiation-competent holoenzyme (Bogorad, 1991; Sugita and Sugiura, 1996). The structural and functional similarity of the prokaryotic and plastid transcription apparatus is more obvious in the case of etioplasts (from dark-grown plants) than of photosynthetically active chloroplasts. PEP preparations from the latter plastid type are much

Abbreviations: CK2, casein kinase 2 (EC 2.7.1.37); CSP41, chloroplast stem-loop binding 3′-RNA-processing endonuclease of 41 kDa (EC 3.1.25.X); GSH, glutathione (reduced form); ESI-QTOF, electrospray ionization quadrupole time of flight; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; NEP, nuclear-encoded phage-type plastid RNA polymerase (EC 2.7.7.6); PEP, bacterial-type plastid RNA polymerase with core subunits encoded by organellar genes (EC 2.7.7.6); PTK, plastid transcription kinase (EC 2.7.1.37); RBP, RNA-binding protein; SAP, sequence motif of a group of DNA- and RNA-binding proteins involved in nuclear gene regulation (naming by first letters of representative members); SDR, short chain dehydrogenase/reductase superfamily.

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larger due to the presence of additional polypeptides and differ in biochemical characteristics from their etioplast counterparts. To accomodate these differences, the two polymerase forms have been termed PEP-A and PEP-B (Pfannschmidt and Link, 1994). The PEP-A form, which is the prevalent RNA polymerase in functional chloroplasts, is subject to regulation both by changes in phosphorylation and reduction/oxidation (redox) state (Link, 2003). In view of the physical proximity to the photosynthetic apparatus, and the need for rapid replenishment of reaction center proteins during photosynthesis (Aro and Ohad, 2003), this has stimulated efforts to characterize regulatory components of this multi-subunit transcription complex.

Pioneering work by Hu and Bogorad (1990) and Hu et al. (1991) using N-terminal sequencing of maize PEP preparations has led to the direct identification of the core polypeptides α, β, β' and β'' (the latter two together equivalent to the bacterial β' subunit). Using N-terminal sequencing and mass spectrometry, both the core subunits and a number of additional polymerase-associated polypeptides were identified in PEP-A preparations from mustard (*Sinapis alba* L.) (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002). Unlike the core subunits, these accessory components were all found to represent products of nuclear genes (Table 1). Moreover, the sequence data pointed to functions not previously thought to be part of chloroplast transcription, but rather as being related to functions such as post-transcriptional RNA-processing (36 kDa RNA-binding protein; RBP) or detoxification of reactive oxygen species as byproducts of photosynthesis (Fe-superoxide dismutase and annexin) (Link, 2003). Because in the initial analyses several PEP-A polypeptides gave high scores only with hypothetical proteins of unknown function (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002), we recently re-screened the databases. This not only broadened the basis for the previously identified polypeptides, but also provided initial clues as to the role of at least one additional component, i.e., a putative RNA-processing protein (Table 1 and Fig. 1).

Another PEP-associated polypeptide listed in Table 1 (at 38 kDa) was previously identified as a protein kinase that selectively phosphorylates σ-like plastid transcription factors (Baginsky et al., 1997; Homann and Link, 2003). This Ser/Thr-specific kinase termed Plastid transcription kinase (PTK) was shown to be sensitive to the SH-group reagent glutathione (GSH) in vitro (Baginsky et al., 1999), suggesting the possibility that it might have a function as a mediator of redox regulation in vivo. This view was strengthened by findings that the intensity of in-organello run-on transcription in isolated chloroplasts reflected changes in glutathione redox state following plant growth under standard light vs. high-intensity (photostress) conditions (Baena-González et al., 2001). The cloned PTK protein reveals high sequence similarity with the nucleo-cytosolic CK2 family (Pinna, 1997) and hence has been referred to as cpCK2 (Ogrzewalla et al., 2002). The results of database screening presented here support the view that this plastid transcription kinase, which has so far only been characterized from mustard, is likely to play a conserved role in a wide range of photosynthetic organisms.

2. Results and discussion

2.1. RNA-interacting polymerase-associated protein(s)

Recent (re-)screening of sequence databases not only revealed additional orthologs of the previously identified PEP-A components from mustard (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002) but also provided information on the possible identity of others. An example is given in Table 1 for an approx. 100–110 kDa polypeptide, which was initially found to be related to a hypothetical

Table 1
Assignment of PEP-A polypeptides from mustard (*Sinapis alba* L.) chloroplasts by mass spectrometry and database screening

Size (kDa)	Tentative protein	Coding site	Genbank identifier (gi)
140	β'' Core subunit	C	5725463
110/107	β Core subunit	C	563343
72–75	β' Core subunit	C	5457428
38	α Core subunit	C	7388101
38	Protein kinase (cpCK2α)	N	17977867
36	RNA-binding protein (CSP41-like)	N	2765080
29	Annexin-like protein	N	3785997
26	Fe-superoxide dismutase	N	15237281
100–110	Putative RNA-processing protein	N	22165107

PEP-A preparations from chloroplasts of 5-day old seedlings were fractionated by SDS–PAGE.

Gel segments were collected and proteins subjected to *in-gel* trypsin digestion, followed by mass spectrometry and peptide fragment identification (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Link, 2003; see also Table 2).

Sequence alignments were carried out using BlastP and Clustal W and chloroplast transit sequences were predicted by ChloroP, PSORT, and PCLR. C, chloroplast; N. nuclear-encoded. Among the proteins defined by the gi numbers, the top five are from *S. alba*, the next three from *A. thaliana*, and the last one from *O. sativa*.

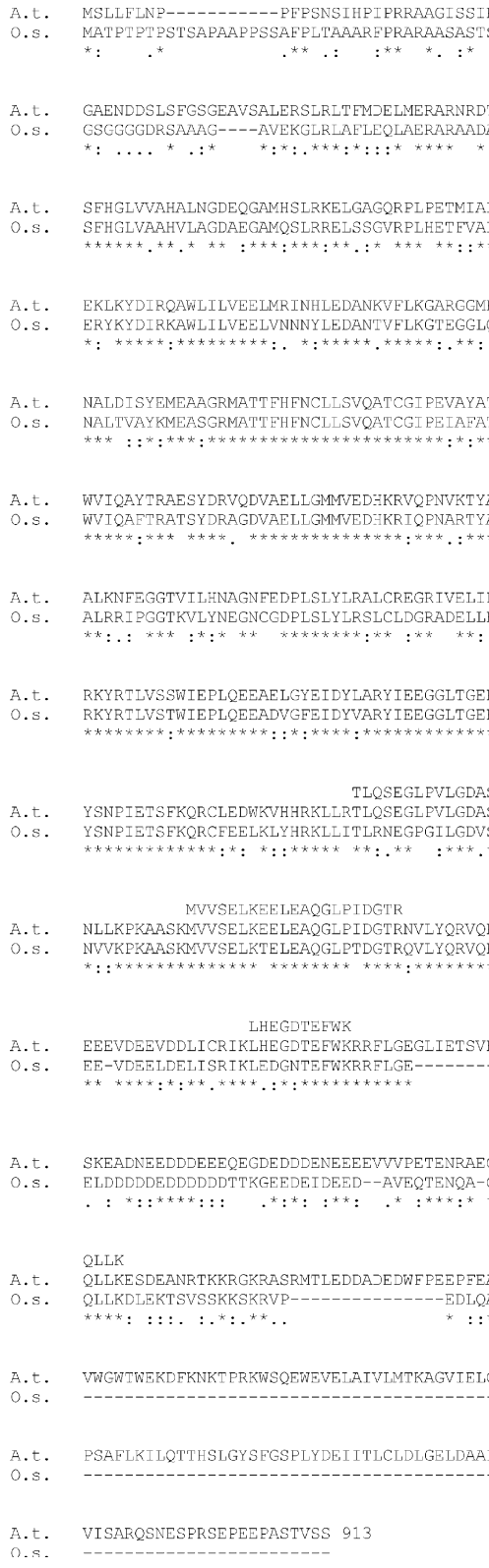

Fig. 1. Alignment of sequences identified by peptide fragments of the 100–110 kDa PEP-A associated protein from mustard (Tables 1 and 2). Shown is a pairwise Clustal W comparison of the hypothetical protein from *A. thaliana* (A.t.; gi 6721160) and the putative chloroplast RNA-processing protein from *O. sativa* (O.s.; gi 22165107). Also shown are four MS peptide fragments from the mustard protein (top line) (see Table 2). Asterisks: identical residues; colons: conservative exchanges.

Table 2
Peptide fragments from the 100–110 kDa component of PEP-A from mustard

Peptide	z	MH^+	dM	Ions	Sequence
1a	2	2169.2	0.2	28/38	(R) TLQSEGLPVLGDASESDYMR
1b	2	2184.5	0.9	24/38	(R) TLQSEGLPVLGDASESDYM*R
2	2	2315.2	0.5	24/40	(K) MVVSELKEELEAQGLPIDGTR
3	2	1296.3	0.3	17/20	(K) HLQM*IGVQLLK
4	2	1262.0	0.4	16/18	(K) LHEGDTEFWK

The MS/MS data of the 100–110 kDa region of SDS–PAGE purified PEP-A were interpreted using SEQUEST (Eng et al., 1994) in combination with the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov). The highest-scoring fragments included – in addition to those representing the β core subunit (not shown) – the five species listed on the right (Sequence), which match regions of the *A. thaliana* hypothetical protein gi 6721160 (Fig. 1). Additional columns shown (from left to right) include charge (z), peptide mass (MH^+), delta mass (dM), and the number of fragment ions matched out of the total number of fragment masses for each peptide (Ions). Residues in parenthesis (R) and (K) were those preceding the listed peptides in the database, indicating expected trypsin cleavage sites. Peptides 1a/1b were identical, except for methionine oxidation state (M*: oxidized Met).

protein from *Arabidopsis thaliana* (gi 6721160) (Table 2). As shown in Fig. 1, the latter has high overall sequence similarity with a rice (*Oryza sativa*) ORF assigned to a chloroplast RNA-processing protein (gi 22165107).

Another putative RBP, 36 kDa in size (Table 1), was previously identified among the PEP-A associated polypeptides (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002). The latter reveals similarity to the RNA-binding protein g5bf from *A. thaliana* (gi 276580), which is similar to two cyanobacterial proteins, from *Nostoc* (gi 17232323) and *Synechocystis* (gi 16330056), respectively. Furthermore, g5bf is identical to an entry termed CSP41b (gi 30681086) from *A. thaliana*, based on its partial similarity to spinach (*Spinacia oleracia*) CSP41 (gi 1532134). The latter is a functional chloroplast stem-loop RNA-binding protein of 41 kDa that acts as an endoribonuclease in the cleavage of 3′ untranslated sequences of plastid mRNAs (Yang et al., 1996; Bollenbach and Stern, 2003a). CSP41b shows regional similarity to another *Arabidopsis* protein CSP41a (gi 30695735), which, in view of its high (70%) similarity to spinach chloroplast CSP41, appears to be the direct ortholog of that protein (Bollenbach and Stern, 2003b). Based on the sequence comparisons summarized in Table 3 it is suggested that the 100–110 and 36 kDa RNA-interacting proteins identified on the basis of their peptide fragments by mass spectrometry (Tables 1 and 2) are distinct PEP-associated components from mustard. This is reflected by characteristic structural features of the two different RBPs: one (CSP41) is a member of the short chain dehydrogenase/reductase (SDR) superfamily containing a so-called Rossman fold (Bollenbach and Stern, 2003a,b), whereas the other (100–110 kDA PEP component) is a predicted SAP protein based on the modular architecture of the rice ortholog (gi 22165107). As recognized by SMART at http://smart.embl-heidelberg.de (Schultz et al., 1998), the latter reveals a SAP motif between residues 544 and 578 (data not shown). SAP proteins are a heterogeneous group of both DNA and RNA-binding nuclear proteins involved in various aspects of gene regulation (Aravind and Koonin, 2000).

Since none of the many other proteins of the chloroplast post-transcriptional machinery (Monde et al., 2000) were detected in the PEP-A preparations (not shown), the two (36 and 100–110 kDa) putative RBPs may play true functional roles in the multi-subunit transcription complex, perhaps by integrating RNA chain termination and/or processing with earlier steps in plastid gene expression. In this regard, it is interesting to

Table 3
Clustal W similarity scores (%) of pairwise comparisons of entries corresponding to the 100–110 and 36 kDa PEP-A associated proteins from mustard (Table 1)

Query sequence	Hyp. Prot. (A.t.) (100–110 kDa)	CSP41b (A.t.) (36 kDa)	Genbank identifier (gi)
Hyp. prot. (A.t.)	100	8	6721160
RNA proc. prot. (O.s.)	61	7	22165107
CSP41 (S.o.)	11	34	1532134
CSP41a (A.t.)	8	7	30695735
RNA-bind. prot. (*Nostoc* sp.)	5	55	17232323
Hyp. prot. (*Synechocystis* sp.)	9	50	16330056
CSP41b (A.t.)	8	100	30681086

Hyp. prot. (A.t.), hypothetical protein from *A. thaliana* (gi 6721160); RNA proc. (O.s.), putative chloroplast RNA-processing protein from *O. sativa* (gi 22165107); CSP41 (S.o.), chloroplast mRNA-binding protein CSP41 from *S. oleracea* (gi 1532134); CSP41a (A.t.), mRNA-binding protein precursor from *A. thaliana* (gi 30695735); Binding prot. (*Nostoc* sp.), mRNA-binding protein from *Nostoc* sp. (gi 17232323); Hyp. prot. (*Synechocystis* sp.), hypothetical protein from *Synechocystis* sp. (gi 16330056); CSP41b (A.t.), RNA-binding protein identical to g5bf from *A. thaliana* (gi 30681086).

note that antisense plants with reduced expression of CSP41 showed both down-regulated degradation rates of several chloroplast transcripts and a negative effect on their transcription (Bollenbach et al., 2003). This may point to a regulatory loop connecting these two levels of chloroplast gene expression, in which CSP41 is involved.

2.2. The chloroplast transcription kinase

Sequence databases were also screened for entries related to the catalytic α component of cpCK2 from *S. alba* (Ogrzewalla et al., 2002), i.e., the cloned plastid transcription kinase previously identified (Baginsky

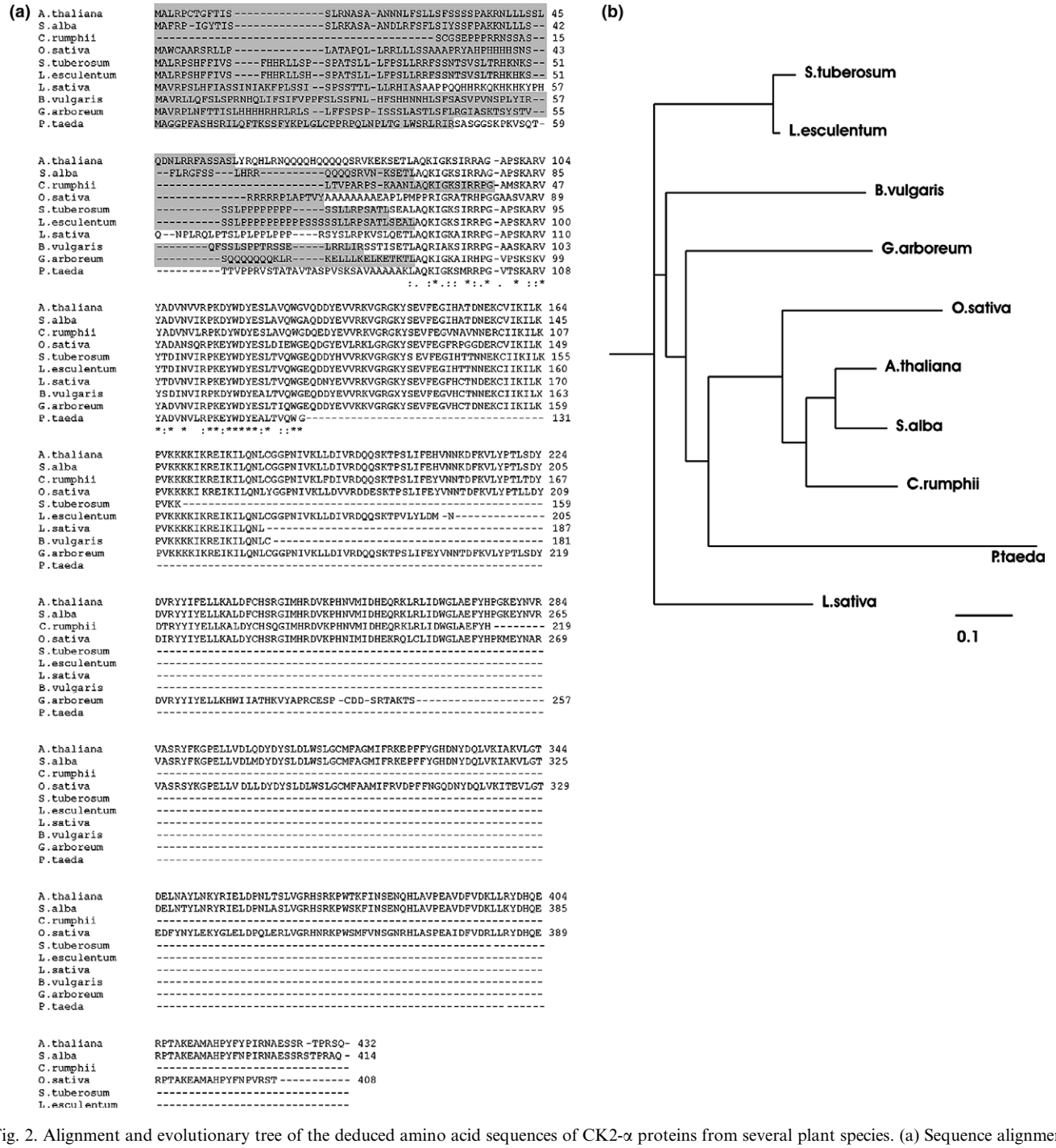

Fig. 2. Alignment and evolutionary tree of the deduced amino acid sequences of CK2-α proteins from several plant species. (a) Sequence alignment with entries from *Arabidopsis thaliana* (accession number NP_179889), *Sinapis alba* (AJ420786), *Cycas rumphii* (CB092846), *Oryza sativa* (AAL34126), *Solanum tuberosum* (BE343591), *Lycopersicon esculentum* (BI421585), *Lactuca sativa* (BU002086), *Beta vulgaris* (BQ060538), *Gossypium arboreum* (BG444944) and *Pinus taeda* (BQ702794). Shaded region: putative transit peptide. Asterisks: identical residues; colons: conservative exchanges. (b) Phylodendron view (http://iubio.bio.indiana.edu) of plant cpCK2-α illustrating the relationships of sequences aligned in (a). Scale bar: 10% sequence divergence.

et al., 1997, 1999). This led to the identification of putative orthologs from a number of other plant species that cover a broad range of phylogenetic lineages, including the full-length cDNA-derived polypeptides from *A. thaliana* and *O. sativa* as well as several expressed sequence tags (ESTs) of shorter size from various species (Fig. 2a). Given the grossly variable lengths of the aligned sequences, it was not meaningful to present the homology values calculated by Clustal W, but the overall sequence similarity was readily discernable by visual inspection. In all cases, the criteria of a conserved CK2-α region preceded by a putative transit peptide was fulfilled. Although only the sequences from *A. thaliana*, *S. alba* and *O. sativa* represented a full-length polypeptide, all ten entries showed the typical SKARVY CK2-specific motif (Pinna, 1997) followed by at least 70–90 highly conserved residues. Furthermore, in each case the SKARVY motif is preceded by a stretch of amino acids (shaded region in Fig. 2a) that is of sufficient length and sequence to qualify as a putative chloroplast transit sequence. The calculated ChloroP (Emanuelsson et al., 1999) values were within the range of 38 (for *L. sativa*) to 81 (for *L. esculentum*), i.e., they all were above the threshold for prediction of chloroplast import.

To show graphically similarities among the aligned sequences in Fig. 2a, a Phylodendron-based (http://iubio.bio.indiana.edu) representation was chosen (Fig. 2b). Due to the variable lengths of sequence regions that were compared, this analysis was not meant to reflect phylogenetic relationships of the various plant species. Nevertheless, it is notable that the close distance between the cpCK2-α seqences from *A. thaliana* and *S. alba* correctly reflects the relatedness of these two Brassica species and the same is true for *L. esculentum* and *Solanum tuberosum* (Solanaceae). It is anticipated that a much more precise picture can be drawn once the remaining full-length sequences become available.

In addition to the overall sequence similarity between the putative cpCK2-α polypeptides (Fig. 2), at least several database entries were of sufficient length to search for central functional regions known to be present in nucleo-cytosolic CK2 (Niefind et al., 1998, 2001). Nine out of the 10 sequences (except *Pinus taeda*) were long enough to be aligned within the expected nucleotide-binding site (Pinna, 1997). As shown in Fig. 3, they all are highly conserved within this region, matching the consensus for nucleo-cytosolic CK2s (Pinna, 1997). With the exception of the *S. tuberosum* sequence, the aligned plant sequences have also completely retained

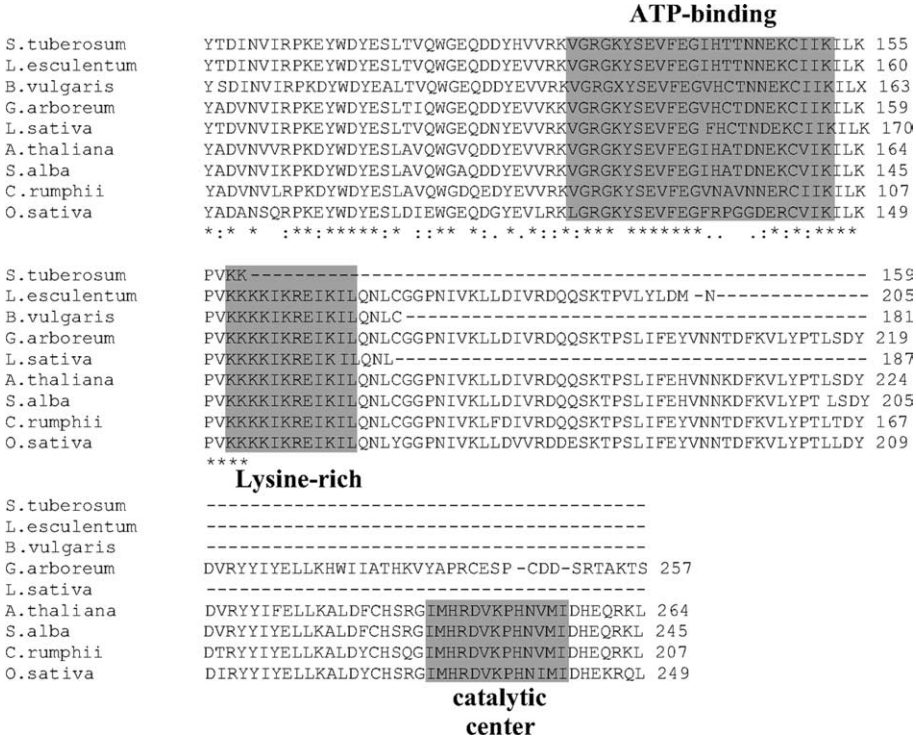

Fig. 3. Conservation of functional domains in cpCK2-α sequences. Functional elements that were identified in nucleo-cytosolic CK2 and are also visible in the cpCK2-sequences are indicated (shaded areas). The derived protein sequences shown, from top to bottom, were from *Solanum tuberosum* (accession number BE343591), *Lycopersicon esculentum* (BI421585), *Beta vulgaris* (BQ060538), *Gossypium arboreum* (BG444944), *Lactuca sativa* (BU002086), *Arabidopsis thaliana* (NP_179889), *Sinapis alba* (AJ420786), *Cycas rumphii* (CB092846), and *Oryza sativa* (AAL34126). Asterisks: identical residues; colons: conservative exchanges.

another typical region known as the (lysine-rich) basic region. The four longest sequences extend even further into the region that harbours the catalytic loop of CK2 (Niefind et al., 1998, 2001). It is evident from the alignment (Fig. 2) that all four sequences are highly conserved within the region known to contain the catalytic center (Pinna, 1997).

Our database searches do not provide any clues to the presence of more than a single cpCK2-α sequence for any plant species from which cDNA and/or EST information is available. On the other hand, it is well-established for nucleo-cytosolic CK2 that its specificity is highly dependent on the degree of pre-phosphorylation of substrate sites by "pathfinder" kinases (Roach et al., 1991; Stone and Walker, 1995). Photosynthesis as well as organellar transcription and post-transcriptional processes all involve complex multi-protein complexes. It is conceivable that one and the same cpCK2 catalytic component is responsible, if its activity and specificity is modified by different interaction partners.

Based on its in vitro properties, the plastid transcripion kinase from *S. alba* has been implicated in phosphorylation by so far unknown – "upstream" protein kinase(s) (Baginsky et al., 1997, 1999). NetPhos (Blom et al., 1999) analyses of the cloned mustard protein (Ogrzewalla et al., 2002) as well as the other putative cpCK2-α sequences in Fig. 2a revealed the presence of multiple potential phosphorylation sites for CK2 and other Ser/Thr-specific protein kinases (data not shown). Differential phosphorylation of these sites by "upstream" kinase(s) would be a simple and highly effective mechanism (Holmberg et al., 2002) to modulate the properties of this enzyme depending on context, i.e., not necessarily only transcription but also post-transcriptional steps in gene expression and photosynthetic electron flow. Candidates for such "upstream" kinases are likely to be among those that have been described as potential regulators of photosynthetic activity (Aro and Ohad, 2003).

3. Conclusions

Experimental strategies such as those exemplified here can be expected to help in a better understanding of the macromolecular complexes that play a role in the biogenesis and function of the chloroplast. It has become increasingly clear that there is functional interaction between the enzymatic machinery involved in photosynthesis and redox homeostasis of the organelle with that for plastid gene expression. For instance, both the RNA-binding protein(s) and the transcription kinase presented here may qualify as regulatory connectors between these distinct, but interdependent, processes with key importance for the entire plant as well as other living organisms.

4. Experimental

4.1. Plant material, organelle isolation

Mustard seedlings were grown for 5 days on soil at 25 °C under white light from mercury discharge lamps (photon fluence rate 250 $\mu mol\, m^{-2}\, s^{-1}$). The cotyledons were harvested on ice and immediately used for chloroplast isolation by differential centrifugation, followed by a sucrose gradient step (Pfannschmidt and Link, 1994).

4.2. Purification of chloroplast RNA polymerase (PEP-A)

Isolated chloroplasts were gently lysed and transcriptionally active material was then enriched by chromatography on a heparin–Sepharose column using $(NH_4)_2SO_4$ gradient elution. The transcriptionally active fractions were further purified by subsequent phosphocellulose chromatography, followed by glycerol gradient centrifugation. The final PEP-A preparations after this step were concentrated by precipitation in 80% (v/v) methanol (Pfannschmidt et al., 2000).

4.3. Protein sample preparation, mass spectrometry

Glycerol-gradient-fractionated and concentrated PEP-A was subjected either directly to (one-dimensional) SDS–PAGE or was first separated in a native first-dimension gel followed by SDS–PAGE (Pfannschmidt et al., 2000). Polypeptides were stained with Coomassie brilliant blue R-250, excised, and subjected to *in-gel* digestion with sequencing-grade modified trypsin (Promega). Eluted peptide fragments were concentrated using ZipTips C18 (Millipore). They were then analysed either by MALDI-MS on a Bruker Reflex III (Pfannschmidt et al., 2000) at the University of Bochum proteomics center, or by ESI-MS in a Q-TOF2 (Micromass) at the Plant Physiology MS facility (Ogrzewalla et al., 2002). Nanospray samples were introduced in positive mode and spectra recorded at m/z 400–1600 and 2.4 s integration time.

4.4. Data analyses

Spectra of charged molecules selected for fragmentation in MS/MS mode were obtained using the MAXENT3 algorithm and BIOLYNX software (Micromass). Interpretation of MS/MS data was by SEQUEST (Eng et al., 1994) in combination with the NCBI nonredundant protein database (http://www.ncbi.nlm.nih.gov). The NCBI entrance point was also used for database screening by BlastP (Altschul et al., 1997). Multiple sequence alignments were carried out using Clustal W (Thompson et al., 1994). Phylogenetic trees were

constructed using Phylodendron (http://iubio.bio.indiana.edu/soft) via the Clustal W interface at Embnet-EBI (www.ebi.ac.uk/clustalw). Chloroplast transit peptide signatures were identified based on the predictions by ChloroP (Emanuelsson et al., 1999), PSORT (Nakai and Kanehisa, 1992), and PCLR (Schein et al., 2001). Putative phosphorylation sites were located by NetPhos at www.cbs.dtu.dk (Blom et al., 1999). Motif screens were carried out using SMART (Schultz et al., 1998) at http://smart.embl-heidelberg.de.

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