pTAC2, -6, and -12 Are Components of the Transcriptionally Active Plastid Chromosome That Are Required for Plastid Gene Expression

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Transcription in plastids is mediated by a plastid-encoded multimeric (PEP) and a nuclear-encoded single-subunit RNA polymerase (NEP) and a still unknown number of nuclear-encoded factors. By combining gel filtration and affinity chromatography purification steps, we isolated transcriptionally active chromosomes from Arabidopsis thaliana and mustard (Sinapis alba) chloroplasts and identified 35 components by electrospray ionization ion trap tandem mass spectrometry. Eighteen components, called plastid transcriptionally active chromosome proteins (pTACs), have not yet been described. T-DNA insertions in three corresponding genes, ptac2, -6, and -12, are lethal without exogenous carbon sources. Expression patterns of the plastid-encoded genes in the corresponding knockout lines resemble those of ∆rpo mutants. For instance, expression of plastid genes with PEP promoters is downregulated, while expression of genes with NEP promoters is either not affected or upregulated in the mutants. All three components might also be involved in posttranscriptional processes, such as RNA processing and/or mRNA stability. Thus, pTAC2, -6, and -12 are clearly involved in plastid gene expression.

INTRODUCTION

Chloroplasts of higher plants possess at least two RNA polymerases with different biochemical properties and phylogenetic origins: a plastid-encoded multimeric RNA polymerase (PEP), which resembles eubacterial RNA polymerases, and a nucleus-encoded phage-type RNA polymerase (NEP) (Hu and Bogorad, 1990; Igloi and Kössel, 1992; Leibs-Mache, 1993; Pfannschmidt and Link, 1994; Liere and Maliga, 1999). In addition to PEP, the existence of NEP has already been suggested from studies with the parasite Epifagus virginiana, which lacks the plastid rpoBC operon (Morden et al., 1991). Similar results were obtained for the plastid ribosome-deficient barley (Hordeum vulgare) mutant albostrians (Hess et al., 1993) and tobacco (Nicotiana tabacum) rpo deletion mutants (Allison et al., 1996; Hajdukiewicz et al., 1997), both still synthesizing plastid transcripts. NEP genes have been identified in the genome of many plant species (Hedtke et al., 1997; Weihe et al., 1997; Young et al., 1998; Ikeda and Gray, 1999). Arabidopsis thaliana contains three NEPs: RpoTm is located in mitochondria, while RpoTmp and RpoTp are found in plastids (Hedtke et al., 1997, 2000; Liere et al., 2004). RpoTmp is also imported into mitochondria, suggesting a dual function in organellar transcription (Hedtke et al., 2000). NEP genes for plastid proteins might have derived from a gene duplication event of a mitochondrial phage-type RNA polymerase gene (Hedtke et al., 1997).

NEP preferentially transcribes housekeeping genes, while PEP is predominantly involved in the transcription of photosynthesis genes (Allison et al., 1996; Hajdukiewicz et al., 1997). However, in PEP-deficient mutants, spurious transcripts initiated by NEP cover the entire plastome. Besides selective promoter utilization, the transcript pattern of plastids is also determined by transcript stability (Krause et al., 2000; Legen et al., 2002), whereas the RNA stability itself seems to depend on the type of the generating RNA polymerase (Cahoon et al., 2004).

While the subunits αββ′ of the core complex of PEP are plastid encoded, the specificity of the holoenzyme (αββ′)′ is determined by nuclear-encoded sigma factors (Tiller et al., 1991; Homann and Link, 2003; Suzuki et al., 2004). They are expressed in a light-dependent, developmental, and tissue-specific manner (Allison, 2000; Kasai et al., 2004; Ishizaki et al., 2005). Based on the promoter structures, three classes of plastid genes can be distinguished: class I genes are preferentially transcribed by PEP, class II genes by NEP, and class III genes by both polymerases (Hajdukiewicz et al., 1997; Ishizaki et al., 2005).

Many attempts have been made to identify nuclear-encoded proteins involved in plastid transcription and translation. Subunits of the PEP core are present in two plastid protein preparations, the transcriptionally active chromosome (TAC) and the soluble RNA polymerase (sRNAP). The TAC is membrane attached and consists of several multimeric protein complexes. In vitro assays, the TAC can transcribe endogenously bound DNA, while the transcriptional activity of sRNAP requires the addition of DNA (Igloi and Kössel, 1992; Krause et al., 2000). TAC fractions have been isolated by gel filtration from different organisms (Briot et al., 1979; Rushlow and Hallick, 1982; Reiss...
and Link, 1985). The specific activity of the isolated complexes depends on the purification procedure (Suck et al., 1996; Krause et al., 2000). Initially, it was believed that sRNAP transcribes preferentially tRNA genes, while TAC is involved in rRNA transcription (Gruissem et al., 1983; Narita et al., 1985); however, run-on transcription assays uncovered that both highly purified fractions transcribed rRNA, tRNA, and plastid protein-coding genes (Reiss and Link, 1985; Rajashekar et al., 1991; Krupinska and Falk, 1994). Finally, TAC and sRNAP preparations from proplastids, chloroplasts, and etioplasts have different protein compositions, demonstrating their dynamics in response to environmental and developmental changes (Reiss and Link, 1985; Pfannschmidt and Link, 1994; Suck et al., 1996).

Forty to sixty polypeptides appear to be present in the TAC from chloroplasts, along with two core subunits of PEP and ETCHED1 (ET1) with high homology to the nuclear transcription elongation factor TFSII (Suck et al., 1996; Krause and Krupinska, 2000; da Costa e Silva et al., 2004). Together with the findings that the ET1-R mutants exhibit quantitative, but no qualitative, differences in TAC activity, it was suggested that ET1 may be involved in plastid mRNA elongation (da Costa e Silva et al., 2004). Furthermore, analyses of TAC preparations from ribosome-deficient mutants suggest that the fraction also contains a nuclear-encoded RNA polymerase activity (Suck et al., 1996). Analyses of highly purified sRNAP preparations as well as plastid DNA–bound proteins (nucleoids) led to the identification of additional nuclear-encoded components (Murakami et al., 2000; Pfannschmidt et al., 2000; Chi-Ham et al., 2002; Sekine et al., 2002; Jeong et al., 2003; Loschelder et al., 2004; Suzuki et al., 2004). One of the best-characterized components of sRNAP is the transcription kinase cpCK2, which plays a role in the regulation of plastid gene expression via phosphorylation and redox signaling (Baginsky et al., 1997, 1999; Ogrzewalla et al., 2002). The nucleoid-associated protein MFP1 is a substrate of cpCK2, which inhibits the DNA binding activity of MFP1 by phosphorylation (Jeong et al., 2004). In mature chloroplasts, MFP1 is attached to thylakoids; hence, it may possibly anchor the nucleoids to the thylakoid membranes (Jeong et al., 2003). Other well-characterized proteins in nucleoids are a sulfite reductase, which participates in organellar nucleoid organization (Cannon et al., 1999; Chi-Ham et al., 2002; Sekine et al., 2002), and a DNA binding protein CND41 with aspartic protease activity (Murakami et al., 2000), possibly involved in regulation of senescence (Kato et al., 2004).

We isolated highly purified TACs from Arabidopsis and mustard (Sinapis alba) and analyzed their protein composition by electrospray ionization ion trap tandem mass spectrometry. Out of 35 identified components, 18 have not yet been described. In this study, we focused on three TAC components: pTAC2, -6, and -12. Interestingly, pTAC2 and pTAC6 are also present in sRNAP fractions, which have eight additional components besides the four PEP core subunits (Suzuki et al., 2004). While pTAC2 encodes a pentatricopeptide repeat–containing protein, the other two components, pTAC6 and pTAC12, contain no known domain and exhibit no homologies that would allow prediction of their function in plastid gene expression. Analyses of the expression of plastid genes in knockout lines suggest that the three TAC components are required for proper function of...
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the PEP transcription machinery. Based on run on transcription assays, pTAC2 is required for proper transcription of psbA by PEP but not for transcription for atpB and clpP by NEP. All three components might also be involved in posttranscriptional processes, such as RNA processing and/or mRNA stability.

RESULTS

Isolation of TAC from Arabidopsis and Mustard

In order to identify new components involved in plastid gene expression, we purified TACs from Arabidopsis and mustard. Several previously published purification protocols were combined to obtain highly purified TAC preparations (Rushlow and Hallick, 1982; Suck et al., 1996; Krause and Krupinska, 2000). After lyses of chloroplasts obtained by sucrose gradient centrifugation, soluble proteins were applied onto a Sepharose 4B column. Transcriptional active protein fractions were combined, and the TAC in these fractions was concentrated by ultracentrifugation. The precipitated complexes were dissolved and applied onto a Sepharose 2B column. Transcriptional active protein fractions were combined, and the TAC in these fractions was concentrated by ultracentrifugation. The precipitated complexes were dissolved and applied to a second column (either Sepharose 2B, Q Sepharose, or Heparin Sepharose CL-6B). Figure 1A demonstrates that the second gel filtration column causes a substantial enrichment of the specific transcription activity, similar to results reported previously for other species (Krause and Krupinska, 2000; data shown for Sepharose 2B). After phenol/chloroform extraction and trypsin digestion, these three preparations were directly used for mass spectrometry. Table 1 contains a list of 35 proteins that were identified with cross-correlation factor (xcorr) values >3.5, 2.5, and 1.5 for triple-, double-, and single-charged peptide ions in independent TAC preparations and with at least two independent peptides. Also present in our preparations were light-capturing chlorophyll a/b binding proteins, D1, D2, and CP47 of photosystem II, α- and β-subunits of the ATP synthase, dihydrolipoamide S-acetyltransferase (At3g25860), and a putative ribulose-bisphosphate carboxylase activase (At1g73110), which were considered as contaminants.

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<th>ΔM 2</th>
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</table>

Analysis of tandem MS (MS/MS) spectra is based on the Arabidopsis database (ftp://ftp.arabidopsis.org/home/tair/sequences/blast_datasets/).

1 National Center for Biotechnology Information/Arabidopsis Genome Initiative accession numbers.

2 Position of identified peptides within the database protein sequence.

3 The charge state of the measured ion (z).

4 The calculated deviation of the experimentally determined mass from the theoretical average mass of the peptide. Ara, Arabidopsis; Sin, S. alba.

5 xcorr value calculated using the sequest algorithm.

Thirteen proteins identified in this preparation have also been identified in sRNAP preparations (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Loschelder et al., 2004; Suzuki et al., 2004). Seventeen of the polypeptides have been described earlier; however, only two of them were identified as components of the TAC. The other 18 proteins reported here have not yet been described. Computer analyses predicted plastid-directing trans-sit sequences for 33 of the identified proteins. No clear information is available for pTAC9 (At4g20010) and pTAC13 (At3g09210). Seventeen of the 35 polypeptides are involved in replication, transcription, translation, detoxification, protein modifications, or plastid metabolism. For the others, no function has been described. However, the domain structure of eight of them suggests that they might also be involved in replication, transcription, or translation. Besides the four subunits α, β, β’, and β” of PEP, we identified Fe-dependent superoxide dismutases (SODs) and phosphofructokinases (PFKs). One of the identified SODs (SOD3, At5g22310) and one of the PFK-B-type enzymes (PKFB2, At3g54090) are also present in highly purified sRNAP preparations from mustard and tobacco (Pfnanschmidt et al., 2000; Ogrzewalla et al., 2002; Loschelder et al., 2004; Suzuki et al., 2004). Among the known proteins, a putative DNA-polymerase A (At3g20540), two putative subunits of the DNA gyrase (At3g10690 and At3g10270), the elongation factor Tu (PFK-B, At3g54090) are also present in highly purified TAC preparations from mustard and tobacco (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Loschelder et al., 2004; Suzuki et al., 2004). Among the known proteins, a putative DNA-polymerase A (At3g20540), two putative subunits of the DNA gyrase (At3g10690 and At3g10270), the elongation factor Tu (At4g20360), three ribosomal proteins (L12-A [At3g27830], S3 [ArthCp061], and L29 [At5g65220]), the UDP-N-acetylmuramoyl-l-alanyl-d-glutamate-2,6-diaminopimelate ligase (At1g63680), and a putative thioredoxin (At3g06730) have not yet been described as TAC components. The 18 new components were named plastid TAC proteins 1 to 18 (pTAC1-18). Based on the analyses of their domain structures, some of them contain either DNA or RNA binding domains, domains involved in protein–protein interactions, or epitopes described for other cellular functions (Table 2). However, database searches for four proteins did not reveal known functional domains.

Twenty-six of the 35 polypeptides were identified in TAC preparations from Arabidopsis and mustard (Tables 1 and 3), four proteins only in Arabidopsis (At3g20540, At5g23310, At2g46820, and At5g54180), and additional five only in mustard preparations (At3g27830, At5g65220, ArthCp061, At1g65260, and At1g80480). We isolated large amounts of TAC from mustard seedlings and
solubilized the proteins in Laemmli buffer after sonication. Twenty major bands could be visualized on a silver-stained gel, and they were identified by mass spectrometry (Figure 1B). Seventeen of these bands correspond to proteins also identified in unresolved TAC samples (Table 3). One additional protein not detected by the original mass spectrometry (MS) was found by analysis of the PAGE-derived protein bands (At2g05430). However, the size of protein bands resolved by PAGE does not always correspond to the predicted molecular masses of the polypeptides. This is not surprising considering that complete solubilization of the final TAC in the gel loading buffer could only be achieved by sonication. It appears that the proteins are tightly associated with each other or with nucleic acids. Some identified polypeptides contain RNA or DNA binding motifs, such as pentatricopeptide repeat motif (PPR), small MutS-related motif (SMR), SAP (for SAF-A/B, Acinus, and PIAS), S1, oligonucleotide/oligosaccharide binding (OB) fold, KOW (for Kyrpides, Ouzounis, and Woese), NGN, or mitochondrial transcription termination factor motif (mTERF) (Table 2). Therefore, it seems likely that the proteins identified in this study are components involved in plastid transcription/translation.

**Identification and Characterization of Knockout Lines with Lesions in TAC Components**

To further substantiate that pTACs are involved in plastid transcription, *Arabidopsis* knockout lines for pTAC2, -6, and -12 were analyzed. pTAC2 (At1g74850) contains two nucleotide binding domains (PPR and SMR) and tetratricopeptide repeat (TPR) domains involved in protein–protein interaction. No obvious protein domains could be detected for pTAC6 (At1g21600) and pTAC12 (At2g34640). Mutations in homozygote knockout lines for these proteins are lethal when they were grown without exogenous carbon sources. DNA sequence analyses confirmed the positions of the T-DNA insertions provided by the Nottingham Arabidopsis Stock Centre (NASC) (Table 3). One additional protein not detected by the original mass spectrometry (MS) was found by analysis of the PAGE-derived protein bands (At2g05430). However, the size of protein bands resolved by PAGE does not always correspond to the predicted molecular masses of the polypeptides. This is not surprising considering that complete solubilization of the final TAC in the gel loading buffer could only be achieved by sonication. It appears that the proteins are tightly associated with each other or with nucleic acids. Some identified polypeptides contain RNA or DNA binding motifs, such as pentatricopeptide repeat motif (PPR), small MutS-related motif (SMR), SAP (for SAF-A/B, Acinus, and PIAS), S1, oligonucleotide/oligosaccharide binding (OB) fold, KOW (for Kyrpides, Ouzounis, and Woese), NGN, or mitochondrial transcription termination factor motif (mTERF) (Table 2). Therefore, it seems likely that the proteins identified in this study are components involved in plastid transcription/translation.

### Table 2. Analysis of pTAC Domains

<table>
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<td>SAP</td>
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<td>PspA/IM30</td>
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a Arabidopsis Genome Initiative accession number.
b ChloroP prediction of the presence (+) or absence (–) of a transit sequence (cTP). The putative maturation site is given (Emanuelsson et al., 1999).
c Protein domains predicted by InterProScan (Apweiler et al., 2000) and BLAST (Altschul et al., 1997).
Table 3. Identification of Bands from an SDS Polyacrylamide Gel with Mustard TAC Proteins

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(Continued)
remains to be determined whether the amounts of these proteins correlate with the number of plastids or nucleoids per cell.

The homozygote seedlings develop white cotyledons, fail to accumulate chlorophyll even under low light intensities, and do not produce primary leaves. On sucrose medium, the mutants reach the rosette stage, but they are much smaller and grow slower than the wild type (Figure 4). While \textit{ptac2} develops yellow cotyledons and greenish primary leaves on sucrose medium, the other two mutants stay more yellowish. By contrast, dark-grown \textit{ptac} plants show the phenotype of the etiolated wild type. A detailed analysis of pigments from low-light-grown \textit{ptac} plants revealed that chlorophylls and carotenoids accumulate in the mutants, although the overall amounts are quite low. The decrease of total chlorophyll (\textit{ptac2}, \textit{ptac6}, and \textit{ptac12}) was accompanied by a noticeable decrease in the chlorophyll \textit{a:b} ratio in \textit{ptac2} and \textit{ptac12} and increase in \textit{ptac6} plants (Table 4). These changes might be due to an increase in the photosystem II light-harvesting system and/or a decrease in the photosystem I:II ratio in case of \textit{ptac2} and \textit{ptac12} and the opposite for \textit{ptac6} plants, respectively. Furthermore, we observed a significant increase in the carotenoid:chlorophyll as well as in the xanthophyll:chlorophyll ratio (Table 4) in mutant seedlings. Interestingly, the antheraxanthin level is relatively high and the zeaxanthin level below detectability. Both xanthophyll pigments are not detectable in the wild type, which is known to be the case for plants grown under moderate conditions (Demmig-Adams and Adams, 2002). Fluorescence quenching analysis indicates effects on photochemical efficiency in the mutant lines. As shown in Table 4, the maximum quantum yield (Fv/Fm) and photochemical quenching ([Fm’ − Ft]/Fm’) are significantly lower than those of the wild type. The reduced ratio of variable-to-maximum fluorescence (Fv/Fm) indicates smaller

**Figure 2. Mutant Analyses.**

The knockout lines \textit{ptac2}, -6, and -12, which were determined to be homozygotes by PCR, fail to accumulate normal amounts of messages from the inactivated genes. Total RNA was isolated from wild-type and knockout seedlings and used for RT-PCR with gene-specific primers. \textit{ptac2} (34 cycles), \textit{ptac6} (30 cycles), and \textit{ptac12} (45 cycles). DNA: PCR with genomic DNA from wild-type seedlings (23 cycles). Size markers in kilobases are at left.
photosystem II antennae and/or fewer functional photosystem II centers (Meurer et al., 1998). In accordance with this observation, ptac2 reveals higher values of nonphotochemical quenching (NPQ; Krause and Weis, 1991). In case of ptac6 and -12, the NPQ values might be caused by the reduced chlorophyll contents.

Analysis of the plastid structure in the mutants showed that the organelle development is severely impaired (Figure 5). Compared with the wild type, grana structures in ptac2 plants are unequally expanded. Grana-interlacing stroma thylakoids are completely missing (Figure 5B). Chloroplasts of ptac6 and -12 plants do not contain grana thylakoids. They are replaced by oval-shaped vesicles (Figures 5C and 5D). Surprisingly, an accumulation of starch is only observed in the old leaves of ptac6 and ptac12 mutants. Frequently a multiplicity of plastoglobuli is found in close proximity to the above-mentioned vesicles (Figures 5B and 5C). Both plastid- and nuclear-encoded soluble polypeptides and polypeptides associated with the thylakoid membrane are either absent or strongly reduced in the mutants (Figure 6). Since plastid-encoded polypeptides, such as the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, subunit IV of cytochrome b6/f complex, and CF1, are still detectable in all three mutants, the mutations do not affect general processes in plastid transcription and/or translation.

Macroarray analyses demonstrated that all plastid-encoded genes are expressed in the mutants. In most cases, these data confirmed the results obtained by RNA gel blot analyses. Differences in the mRNA results might be caused by radiolabeled antisense RNA, which also hybridized to the probes (Krause et al., 2000; Legen et al., 2002). However, compared with the wild type, we observed remarkable alterations (Figure 7). The transcript levels for components of the two photosystems and for the cytochrome b6/f complex are significantly reduced. For instance, the psbA mRNA level (for the D1 protein) in ptac2 and ptac12 and the psbA, psbC, and psbE mRNA levels (for CP43 and cytochrome b6/f complex) in ptac6 plants are reduced by 80 and 50%, respectively. In all three mutants, petB (for a subunit of the cytochrome b6/f complex) and rbcL (for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase) transcripts accumulate to >50%. These mRNAs are probably transcribed from genes with PEP promoters. By contrast, rps, rpl (for ribosomal proteins), ndh (for subunits of the NADH dehydrogenase), atp (for subunits of the ATP synthase), rpo (for subunits of the RNA polymerase), and ycf (for hypothetical chloroplast open

Figure 3. Tissue-Specific Expression of ptac2, -6, and -12.

An equal amount of cDNA from the cotyledons (set as 1.0), rosette leaves (RS), stems (S), flowers (F), roots (RT), and cauline leaves (CL) from Arabidopsis was used for real-time PCR with gene-specific primers for the actin gene (Robinson et al., 1999), ptac2, -6, and -12. Fold induction values of genes were calculated with the ΔΔCT equation of Pfaffl (2001) and related to the mRNA level of the genes in cotyledons, which was defined as 1.0. This value is not shown in the graph. The data shown here were obtained from three independent experiments, and the error bars represent standard deviation.

Figure 4. Phenotype of ptac2, -6, and -12 Mutant Plants.
Ptac2, -6, and -12 and wild-type seedlings after 12 (A) and 18 (B) d grown on Murashige and Skoog medium under long-day conditions.
reading frames) genes are expressed at higher levels in all three mutants. These messages are transcribed from genes that may contain NEP promoter elements, either alone or in combination with PEP promoter elements. The expression pattern of the plastid genes in the mutants is identical to that described for Δrpo mutants and mutants that do not accumulate PEP. These mutants also contain reduced transcript levels for photosynthesis proteins, while ndh, atp, and rpo genes are expressed at higher levels (Hess et al., 1993; Allison et al., 1996; Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998; De Santis-Maciejsekk et al., 1999; Krause et al., 2000; Legen et al., 2002). However, for some plastid genes (e.g., for clpP), the expression level in the wild type was not significantly different from the background. Finally, the reduced psbA mRNA level in ptac2 plants is caused by a reduced rate of psbA transcription as shown by run-on transcription assays, while transcription of atpB and clpP appear to be not affected in the mutant (Figure 8). We could not obtain enough chloroplasts from the homozygote mutants to unambiguously confirm this for the other genes and mutants.

To further substantiate the findings derived by macroarray analyses, representative genes were chosen for RNA gel blot analyses (Figure 9). Figure 9A shows genes (psaAB, psaC, psaJ, psbA, rbcL, and rps14) that are downregulated in the mutants, Figures 9B and 9D shows genes (atpB, clpP, and the nuclear-encoded genes psaH, psaE, and psbO) that do not respond significantly to the mutations, and Figure 9C shows genes (accD, atpA, ndhF, ndhB, and ycf3) that are upregulated in the mutants. Genes shown in Figure 9A contain only PEP promoter sequences, and their expression is also downregulated in Δrpo mutants and other mutants impaired in PEP function (Hess et al., 1993; Allison et al., 1996; Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998; De Santis-Maciejsekk et al., 1999; Krause et al., 2000; Legen et al., 2002). By contrast, the Figures 9B and 9C show genes that contain NEP promoter elements either alone or in combination with PEP promoter elements. Again, these genes exhibit the same expression behavior in Δrpo mutants and other mutants impaired in PEP function (Hess et al., 1993; Allison et al., 1996; Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998; De Santis-Maciejsekk et al., 1999; Krause et al., 2000; Legen et al., 2002). Furthermore, we observed in all mutants defects in mRNA processing, since larger transcripts accumulate for accD, atpB, clpP, ndhB, ndhF, psaAB, rbcL, rps14, and ycf3. This is most obvious for the polycistronic transcript ycf3/psaA/psaB/rps14. Besides mRNAs with 3.2 and 5.2 kb, additional messages with 5.8 and 7.3 kb hybridize to the psaAB probe in the mutants. They represent unprocessed transcripts and include sequences for ycf3, as shown by the hybridization with the ycf3-specific probe (Figure 9C; Summer et al., 2000; Legen et al., 2002). Again, the very same lesions in the processing patterns were observed for Δrpo mutants and other PEP mutants. Taken together, these data suggest that the expression of genes encoding the three novel TAC proteins is required for proper function of PEP transcription machinery. Since messages for all genes studied are present in all three mutants and since at least some of them are also properly processed, the mutations do not completely inhibit crucial steps in plastid transcription or translation. pTAC2 and pTAC6 are also present in sRNAP preparations (Suzuki et al.,

Table 4. Pigment Analysis and Fluorescence Measurements of Wild-Type and Mutant Lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Chlorophyll a/b (mol/mol)</th>
<th>Carotenoids/Chlorophyll (mol/mol)</th>
<th>VAZ/Chlorophyll (mmol/mol)</th>
<th>Chlorophyll Fluorescence Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>3.2 ± 0.034</td>
<td>0.25 ± 0.008</td>
<td>40.7 ± 1.4</td>
<td>0.854 ± 0.012</td>
</tr>
<tr>
<td>tac2</td>
<td>2.8 ± 0.106</td>
<td>0.35 ± 0.012</td>
<td>99.1 ± 4.8</td>
<td>0.497 ± 0.055</td>
</tr>
<tr>
<td>tac6</td>
<td>4.4 ± 0.286</td>
<td>1.48 ± 0.071</td>
<td>467.9 ± 18.0</td>
<td>0.424 ± 0.089</td>
</tr>
<tr>
<td>tac12</td>
<td>2.8 ± 0.057</td>
<td>0.60 ± 0.042</td>
<td>216.7 ± 13.5</td>
<td>0.546 ± 0.013</td>
</tr>
</tbody>
</table>

Pigment data are the average of three independent samples. Fluorescence quenching analysis is averaged from nine plants. VAZ is the xanthophyll pool size (violaxanthin + antheraxanthin + zeaxanthin).
D1/2, cytochrome b559 and PsbO of photosystem II (PSII), against CF1,2. The immunological detection of the proteins occurred with anti-
ptac pyg NEP and PEP promoter elements (Figure 10). In these studies, we
matically downregulated in ptac2 mutants. Contrast, the signal at the P-515/-520 PEP side of
atpB signal intensities at the NEP promoter site P-57 of
clpP mutant (Figure 10, lanes 9 and 10), it appears that the PEP
signal at this PEP site is nearly equal in the wild type and in the
ptac mutants. Since signals at the promoter initiation site are de-
scription rather than translation.

To analyze the effect of the pTAC-2, -6, and -12 mutations on
PEP and NEP promoter activity in leaf tissue, we determined transcript levels by primer extension analyses mapping transcript
5'-ends for clpP and atpB, two representative plastid genes with
NEP and PEP promoter elements (Figure 10). In these studies, we
also included the pale-yellow-green (pyg) mutant 8 (lane 10; J.
Stöckel and R. Oelmüller, unpublished data) because it exhibits a
very similar phenotype and growth behavior as the ptac
mutants. Since signals at the promoter initiation site are de-
pendent on the mRNA level, we included into our investigations
only such genes whose mRNA levels are not affected by the
mutations. We did not observe any significant difference in the
signal intensities at the NEP promoter site P-57 of clpP (panel
clpP; Sirraman et al., 1998). This example indicates that the NEP
promoter usage is not altered in the ptac and pyg mutants. By
contrast, the signal at the P-515/-520 PEP side of atpB is dra-
matical downregulated in ptac2 and ptac12 mutants (panel
atpB, lanes 6 and 8) and below detectability in ptac6 plants (lane 7).
Sequence alignments of atpB promoter sequences from dif-
ferent species indicate that the conserved regions represent PEP
(-35/-10) elements (data not shown). Since the primer extension
signal at this PEP site is nearly equal in the wild type and in the
pyg8 mutant (Figure 10, lanes 9 and 10), it appears that the PEP
promoter usage is specifically affected in the ptac mutants.
The expression of ptac2 -6, and -12 in organs with different
types of plastids (Figure 3) suggests that the gene products are
required for general processes of the PEP activity and not
restricted to photosynthetically active plastids. To test this fur-
ther and to exclude the possibility that the phenotypes of the
mutants grown in low light (see Methods) are caused by
photooxidative damage due to light stress, we repeated the
RNA gel blot and primer extension analyses with etiolated
material. Segregating populations were germinated in low light
for 3 d to identify the homozygote seedlings. They were then
transferred to complete darkness for an additional 18 d before
harvest of the newly emerged etiolated leaves. RNA gel blot
analyses for representative genes of the three classes described
above (Figure 9) clearly indicate that the observed differences in
the transcript levels in the mutants are not light dependent
(Figure 9E). Furthermore, primer extension analysis for atpB also
gave comparable results as obtained for seedlings grown in low
light (data not shown; Figure 10). Finally, as described for
seedlings grown in low light, the atpB transcript levels in etiolated
material are similar or even higher in ptac2 and -12 plants (Figure
9E), although the primer extension signals at the PEP sites were
downregulated (data not shown). This clearly indicates that
ptac2 -6, and -12 function is not related to thylakoid biogenesis,
photosynthesis, or photooxidative stress.

**DISCUSSION**

We hypothesized that nuclear-encoded components required
for transcription and translation in plastids might be associated
with the TAC. TAC preparations from various organisms were
analyzed on SDS gels, and in this way, at least 40 polypeptides
associated with TAC have been detected. However, only the
α- and β-subunits of the PEP (Suck et al., 1996; Krause and
Krupinska, 2000), a homolog of the nuclear transcription elon-
gation factor TFIIS (da Costa e Silva et al., 2004), and few plastid
DNA-bound proteins, including PEND (Sato et al., 1993), sulfite
reductase (Cannon et al., 1999; Chi-Ham et al., 2002; Sekine
et al., 2002), CND41 (Murakami et al., 2000), and MFP1 (Jeong
et al., 2003), have been identified until now. We used different
gel filtration and affinity chromatography purification steps for the
isolation of TACs from mustard and *Arabidopsis*.

The available sequence information allowed us to identify the
*Arabidopsis* TAC components with high fidelity and to analyze
their role in knockout lines. Furthermore, this allowed us to
compare the TAC composition of two related organisms. Using
the above-mentioned criteria (at least two independent peptides
in independent preparations), we identified 35 polypeptides in
our TAC preparations, 18 of these components have not yet been
described so far (cf. also below). We could demonstrate that
three of them influence plastid transcription, RNA accumulation,
and processing and therefore are essential for plastid gene
expression.

Twenty-five polypeptides could be identified in TAC prepara-
tions from *Arabidopsis* and mustard (Tables 1 and 3). Four addi-
tional proteins (At3g20540, At5g23310, At2g46820, and At5g54180)
were only detected in *Arabidopsis* TAC preparations. Only five
polypeptides were identified in mustard but not in the *Arabidop-
sis* TAC (At3g27830, At5g65220, At10g06710, At1g65260, and
At10g0480). These differences might be caused by the different
developmental stages of the plastids, by the different abundance
of individual proteins in the two species, or by the fact that the
identification of mustard proteins with *Arabidopsis* databases is
not possible in all instances. Based on homology searches, the

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**Figure 6.** Immunoblot Analyses of Plastid Proteins.
Fifteen micrograms of plastid proteins of wild-type and mutant (ptac2, -6, and -12) seedlings were separated on polyacrylamide gels containing
SDS. The immunological detection of the proteins occurred with anti-
body against the subunits A, C, D, and H of photosystem I (PSI), against
D1/2, cytochrome b559 and PsbO of photosystem II (PSII), against CF1,2
and CF1,ε of the ATP synthase, against cytochrome b6 and the subunit IV
of the cytochrome b6/f complex, and against the large subunit of
ribulose-1,5-bis-phosphate carboxylase/oxygenase (LSU).

**Figure 9E.**
Figure 7. Changes in Transcript Abundance of Plastome-Encoded Genes.

A macroarray representing all protein-coding genes of the plastome was hybridized with probes synthesized from the mutants (ptac2, ptac6, and ptac12) and wild type. The diagram shows the log$_2$ induction factor (log$_2$ IF). IF was calculated from the ratio of the mutant and wild-type signal intensities. Log$_2$ IF is given where 3.32 corresponds to a 10-fold upregulation and −3.32 to a 10-fold downregulation. *** Indicates $P < 0.05$, ** indicates $0.05 < P < 0.10$, and * indicates $0.10 < P < 0.20$. 
TAC contains polypeptides involved in replication, transcription, translation, detoxification, protein modification, and plastid metabolism. Eleven identified proteins are DNA or RNA binding proteins or complexes (α-, β-, β'-, and β"-subunits of PEP, a putative DNA polymerase A, two putative subunits of the DNA gyrase, the elongation factor EF-Tu, and three ribosomal proteins, S3, L12-A, and L26). Eight additional proteins contain DNA/RNA binding domains, such as the PPR, SMR, SAP, OB fold, S1, KOW, mTERF, or single-stranded DNA (ssDNA) binding motifs (Table 2). These motifs are found in proteins involved in chromatin organization, DNA repair, transcription, RNA processing, or translation (Boni et al., 1991; Kyrpides et al., 1996; Fernandez-Silva et al., 1997; Draper and Reynaldo, 1999; Moreira and Philippe, 1999; Aravind and Koonin, 2000; Loschelder et al., 2004). Furthermore, pTAC1 and pTAC11 exhibit striking similarities to p24 proteins, which are members of the Whirly proteins of nuclear transcription factors (Desveaux et al., 2002, 2004). All Whirly proteins have target sequences for organelles (Krause et al., 2005); thus, they might be dually targeted to the nucleus and the plastid. The exact functions of the pTAC proteins remain to be determined; however, the striking similarities of the observed phenotypes of the knockout lines for three of these proteins with those of Δrpo mutants and other mutants impaired in PEP function suggests that they are also involved in plastid gene expression. Like Δrpo plants, ptac2, -6, and -12 mutants are defective in plastid gene expression as well as proper mRNA processing and/or stability, suggesting that they belong to a complex that is involved in different steps of plastid gene expression. This is particularly interesting for pTAC6 and -12, two plastid-localized proteins for which no domains or functions could be predicted. Only the presence of these proteins in our TAC preparations in combination with the observed phenotype of the knockout lines allows us to predict a role of these proteins in plastid gene expression.

In *Escherichia coli* and bacteriophage λ, different proteins, including subunits of the ribosomes, are components of the transcription and translation machinery, at least during some stages of the cell cycle (Zellars and Squires, 1999). Some RNAP subunits play an important role during transcription initiation, antitermination, elongation, and termination (Squires and Zaporozjko, 2000). Moreover, many ribosomal proteins participate in the transcription of their own genes or of RNA genes (Zengel et al., 1980; Squires and Zaporozjko, 2000). Oleskina et al. (1999) also identified ribosomal proteins that are associated with plastid DNA. Studies on phage Q replication demonstrated that S1 and the elongation factors EF-TU and EF-TS are also subunits of the β-RNA replicase (Brown and Gold, 1996). Likewise, EF-Tu, S3, L12, and L29 could have a dual function in plastid gene expression in that they coordinate replication, transcription, and translation. Furthermore, pTAC15 contains a KOW domain, which is characteristic for members of the NusG protein family (Kyrpides et al., 1996). NusG is an essential factor for coupled transcription/translation in *E. coli* and bacteriophage λ (Zellars and Squires, 1999).

While a putative DNA polymerase (Kimura et al., 2002) and two subunits of the topoisomerase II (Reece and Maxwell, 1991; Cho et al., 2004) are primarily involved in replication and the four plastid-encoded subunits α, β, β', and β" of PEP in transcription in the TAC, the exact role of the other components identified in this fraction is less clear. A Fe-SOD has also been detected in the PEP-A complex of a soluble plastid fraction from mustard (Pfannschmidt et al., 2000; Ogrzewalla et al., 2002; Loschelder et al., 2004), together with putative kinases, which we did not find in our preparations. The absence of the cpk2 kinase can be explained by the loose association with the sRNAP (Baginsky et al., 1997). Our data indicate that at least two Fe-SODs are present in our TAC preparations. They might function as protective agents against reactive oxygen species (Pfannschmidt et al., 2000). Hydrogen peroxides, for instance, can stimulate transcription of plastid *ndh* genes under photooxidative stress (Casano et al., 2001). We also identified two PFKs in the TAC, one of them was also found in sRNAP preparations (Suzuki et al., 2004). PFK might couple the expression of photosynthesis genes to sucrose signals in the plant cell (Oswald et al., 2001). Finally, thioredoxin, which was also identified in our preparations, regulates key enzymes in the plastids via redox signals (Schürmann and Jacquot, 2000). In *Rhodobacter sphaeroides*, expression of genes from the *puf* operon is controlled by thioredoxin (Pasternak et al., 1999). Furthermore, redox signals via thioredoxin are able to influence gene expression by affecting gyrase activity (Li et al., 2004). Thus, the association of these components to the TAC might ensure its regulation in response to various signals.

We could not identify the TAC component ET1, which was recently detected immunologically in maize (*Zea mays*) (da Costa e Silva et al., 2004). However, it cannot be excluded that this protein is present in the TAC fraction. It is conceivable that ET1 was not detected by MS because of simultaneous elution of other abundant peptide ions. Presumably, it is also the case with the nuclear-encoded RNA polymerase RpoTp (*RpoT*3; At2g24120), which we could only identify with scores slightly below our

![Figure 8. Analyses of Transcription Rates.](Image 74x512 to 272x693)
RNA gel blot analyses for plastid- and nuclear-encoded genes were performed with RNA from wild-type (lanes 1 to 3) and mutant (lane 4, ptac2; lane 5, ptac6; lane 6, ptac12) seedlings. RNA loaded per lane is as follows: 3.25 μg (lane 1), 7.5 μg (lane 2), and 15 μg RNA (lanes 3 to 6). The gene probes are indicated. The 18S rRNA accumulation is shown in the ethidium bromide–stained gels below each plot to confirm equal loading. Bars mark the position of cotranscripts.

(A) and (C) The transcript levels are reduced (enhanced) in the mutants.

(B) The amount of transcripts is similar in the wild type and mutants.

(D) RNA gel blot analyses of nuclear-encoded genes.

(E) RNA gel blot analyses of 18-d-old dark-grown material.
standard criteria ($\chi^2_{corr}$, 2.47 for the double-charged peptide ion $^{294}$[KNNAGDSQEELK]$_{305}$ and 1.59 for the single-charged peptide ion $^{149}$[IERKIDIDK]$_{156}$). In future experiments, multidimensional chromatography (e.g., strong cation exchange coupled with reversed-phase liquid chromatography) would probably enable the identification of more peptides by MS, especially low abundant ones. As mentioned above, Table 1 contains only those proteins that were identified by at least two independent peptides in independent preparations. At least 40 other candidate proteins that do not fulfill this criterion were also identified in our preparation, and four of them have been described before as putative candidates of the TAC. This includes PEND (Sato et al., 1993), sulfite reductase (Cannon et al., 1999; Chi-Ham et al., 2002; Sekine et al., 2002), CND41 (Murakami et al., 2000), and MFPI (Jeong et al., 2003). Some of them are clearly contamination of other plastid multiprotein complexes, such as the small subunit of ribulose bisphosphate carboxylase, PsbS (subunit S of the photosystem II), the $\alpha$-subunit of cytochrome b$_6$f, and a putative ferritin subunit (At3g11050). Thus, additional work is required to clearly demonstrate which proteins are tightly associated with TAC.

The role of the new components identified in our studies is more enigmatic. Arabidopsis knockout lines for three of these components were analyzed in this study. These components have been chosen because bioinformatic analysis would not allow any prediction of their association with TAC. The knockout lines have severe lesions in plastid transcription and RNA metabolism that lead to almost identical phenotypes reported for $\Delta$po mutants and mutants that do not accumulate PEP (Hess et al., 1993; Allison et al., 1996; Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998; De Santis-Maciossek et al., 1999; Krause et al., 2000; Legen et al., 2002). While transcripts for all plastid-encoded genes can be detected in the mutants, their expression profiles differ. For some genes, <10% of the wild-type transcript level can be detected. Interestingly, transcription of such genes is only driven from PEP promoters. Expression of genes with NEP promoters is either not altered or upregulated. Similar to the expression profiles in $\Delta$po mutants and mutants with lesions in PEP function (Hess et al., 1993; Allison et al., 1996; Hajdukiewicz et al., 1997; Silhavy and Maliga, 1998; De Santis-Maciossek et al., 1999; Krause et al., 2000; Legen et al., 2002), we observe the accumulation of larger amounts of not properly processed transcripts. Primer extension analyses indicate that either the transcriptional activity of the PEP enzyme and/or the mRNA stability of plastid genes are affected in the mutants. Since atpB mRNA levels and transcription rates are not downregulated in the ptac2 mutants (Figures 8 and 9), it is likely that the lower signal at the PEP promoter site of atpB is specific for the PEP activity. We have chosen the atpB gene for this study because the transcript level for this gene is comparable in mutant and wild-type seedlings. A more detailed analysis of NEP and PEP promoter site usages in the ptac mutants requires a better knowledge of the exact initiation sites of plastid genes in Arabidopsis.

Proper expression of plastid genes is essential for the differentiation of proplastids to chloroplasts (Baumgartner et al., 1993; Mache et al., 1997). Hence, defects in plastid gene expression lead to reduced chlorophyll levels and obviously counteract any adverse effects, such as the assembly of thylakoid structures. However, electron micrographs of chloroplasts from wild-type and ptac2, -6, and -12 plants (Figure 5) reveal abnormal membrane structure in mutant plants. This is in accordance with the observation of reduced photochemical efficiency (Table 4) in ptac2, -6, and -12 plants, allowing them to grow only under heterotrophic conditions. The ptac mutants investigated are phenotypically very similar to PEP-deficient tobacco mutants. Both contain undeveloped plastids that have vesiculated internal membranes instead of thylakoids, depending on the stage of the leaf (De Santis-Maciossek et al., 1999). Furthermore, several other mutants affected in plastid gene expression also have severe lesions in chloroplast development (Shirano et al., 2000; Ishizaki et al., 2005).
PTAC2, -6, and -12 exhibit no obvious sequence similarities to other known proteins from prokaryotic organisms, except that pTAC2 contains PPR and TPR motifs, which are characteristic for proteins involved in mRNA processing, stability, and/or translation (Fisk et al., 1999; Boudreau et al., 2000; Yamazaki et al., 2004). Thus, these genes must have newly developed after the establishment of eukaryotism. Several lines of evidence support the idea that these proteins might be involved in plastid transcription/translation. Since pta2, -6, and pta12 are expressed in cells with different types of plastids, the proteins should be involved in a more general plastid function. The PEP promoter activity of atpB is specifically downregulated in the mutants, while the NEP promoter in clpP is not (Figure 10). The same alterations in plastid gene expression are observed in etiolated and light-grown seedlings. Thus, neither a block in the establishment of eukaryotism. Several lines of evidence support the idea that these proteins might be involved in plastid transcription/translation. Since pta2, -6, and pta12 are expressed in cells with different types of plastids, the proteins should be involved in a more general plastid function. The PEP promoter activity of atpB is specifically downregulated in the mutants, while the NEP promoter in clpP is not (Figure 10). The same alterations in plastid gene expression are observed in etiolated and light-grown seedlings. Thus, neither a block in the establishment of eukaryotism. Several lines of evidence support the idea that these proteins might be involved in plastid transcription/translation. Since pta2, -6, and pta12 are expressed in cells with different types of plastids, the proteins should be involved in a more general plastid function. The PEP promoter activity of atpB is specifically downregulated in the mutants, while the NEP promoter in clpP is not (Figure 10). The same alterations in plastid gene expression are observed in etiolated and light-grown seedlings. Thus, neither a block in

METHODS

Plant Material and Growth

Sinapis alba var Albatros and Arabidopsis thaliana (ecotype Columbia) were used for all studies. The T-DNA insertion lines (Salk_075736, Salk_024431, and Salk_025098) were obtained from NASC. Arabidopsis was grown on Murashige and Skoog medium (Murashige and Skoog, 1962) at 21 °C with a day/night cycle of 16/8 h (50 to 60 μE m⁻² s⁻¹; Osrarn LW30; if not, other light intensities are mentioned. For propagation, Arabidopsis seedlings were transferred to soil after 18 d, and the seeds were harvested in Aracun tubes (Beta Tech). For analysis of tissue-specific gene expression and biochemical studies, Arabidopsis and mustard plants were grown on soil in a growth chamber (day/night rhythm of 16/8 h; 80 to 100 μE m⁻² s⁻¹; 21°C; Osrarn LW30).

Chlorophyll and Fluorescence Induction Measurements

Chlorophyll and fluorescence measurements were performed with 14-d-old Arabidopsis seedlings (5 to 10 μE m⁻² s⁻¹; Osrarn LW30) grown on Murashige and Skoog medium. For fluorescence measurements, a Fluorcam 700 MF (Photon System Instruments) was used. After dark acclimation (15 min), the fluorescence were measured according to Wagner et al. (2005). The nomenclature of the standard chlorophyll parameters refer to van Kooten and Snel (1990). Chlorophyll and carotenoid pigments were determined by HPLC according to Büh et al. (1994). Samples were ground in liquid nitrogen in Eppendorf tubes and extracted as described by Ensminger et al. (2001).

Isolation of TAC

Chloroplasts were isolated from 2 to 3 kg cotyledons of 5-d-old mustard seedlings or 4-week-old Arabidopsis rosette leaves by sucrose gradient centrifugation (18 gradients at 35 mL; Tiller et al., 1991) at 4 °C. After lyses of the chloroplasts in 200 mL buffer A (50 mM Tris-HCl, pH 7.6, 100 mM [NH₄]₂SO₄, 4 mM EDTA, 25% glycerol, 1% Triton X-100, 40 mM 2-mercaptoethanol, and 50 μg/mL phenylmethylsulfonyl fluoride [PMSF], 2-mercaptoethanol, and 50 μg/mL PMSF). After lyses of the chloroplasts in 200 mL buffer A (50 mM Tris-HCl, pH 7.6, 100 mM [NH₄]₂SO₄, 4 mM EDTA, 25% glycerol, 1% Triton X-100, 40 mM 2-mercaptoethanol, and 50 μg/mL PMSF), the insoluble material was removed by centrifugation (20,000g, 4°C, 30 min).

Thirty microliters of the soluble fraction was used for gel filtration on several Sepharose 4B columns (2 cm diameter, 100 cm length, column volume 380 mL) at 4 °C with buffer A. Fractions with transcriptional activity were combined and the TAC precipitated by ultracentrifugation (5 h, 200,000g, 4°C). The pellets were resuspended in 15 mL of buffer B (50 mM Tris-HCl, pH 7.6, 100 mM [NH₄]₂SO₄, 4 mM EDTA, 25% glycerol, 40 mM 2-mercaptoethanol, and 50 μg/mL PMSF), the insoluble material removed by centrifugation (10 min, 20,000g, 4°C), and the soluble proteins applied to a Sepharose 2B column (diameter 1.5 cm, length 170 cm, and volume 301 mL). After elusion of the transcriptional active fractions with buffer B, the TAC was obtained by ultracentrifugation as described above. Final resuspension occurred in 1 mL of buffer C (50 mM Tris-HCl, pH 7.6, 50 mM [NH₄]₂SO₄, 4 mM EDTA, 10% glycerol, 40 mM 2-mercaptoethanol, and 50 μg/mL PMSF). Alternatively, the eluate of the first column was subjected to either Heparin Sepharose CL-6B or Q Sepharose chromatography. After resuspension of the TAC in 50 mM Tris-HCl, pH 7.6, 50 mM [NH₄]₂SO₄, 5 mM MgCl₂, 10% glycerol, 40 mM 2-mercaptoethanol, and 50 μg/mL PMSF, and DNase/RNase treatment at 37°C for 1 h, it was loaded onto a Heparin Sepharose CL-6B column (diameter 1.0 cm, length 10 cm, and volume 7 mL). The proteins were eluted with 50 mM Tris-HCl, pH 7.6, 2 M [NH₄]₂SO₄, 10% glycerol, 4 mM EDTA, 40 mM 2-mercaptoethanol, and 50 μg/mL PMSF, dialyzed and concentrated against the buffer C, and used for MS. For Q Sepharose chromatography (diameter 1.0 cm, length 10 cm, and volume 7 mL), the DNase/RNase treatment was omitted.

Transcription Assay

In vitro transcription was analyzed by measuring [³H]UTP incorporation into RNA under conditions as described by Reiss and Link (1985). One unit was defined as incorporation of 1 fmol [³H] into UTP in 30 min at 30°C (Suck et al., 1996).

Preparation of Samples and MS

After phenol/chloroform extraction of the transcriptionally active fraction, proteins were precipitated with ethanol, dried, and resuspended in 50 μL 50 mM NH₄HCO₃ and 0.05% β-dodecylmaltoside. After denaturation (95°C for 10 min), proteins were digested with trypsin according to manufacturer’s instructions (Promega) at 37°C for 16 h. The peptides were then purified with POROS-R2 (Applied Biosystems) on a C18 Zip-Tip column (Millipore; Stauber et al., 2003). Silver-stained bands from protein gels were analyzed according to Stauber et al. (2003). MS and MS/MS spectra were recorded with a LDC Deca XP IONTRAP mass spectrometer (Thermo) equipped with a nano-HPLC system (Ultimate; Dionex) according to Stauber et al. (2003). Liquid chromatography was performed with a reverse-phase column (PepMap C18 column; LC-Packings, Dionex). Identification of the proteins occurred with the Finnigan Sequest/Turbo Sequest software (revision 2.0; ThermoQuest) by comparing the recorded masses with those calculated for trypsin digestion products of all Arabidopsis peptides available in the databases (ftp://ftp.arabidopsis.org/sequences/blast_databases/; The Arabidopsis Information Resource).

Array Hybridization and Quantification

Theme-specific plastome macroarrays were produced by spotting gene-specific PCR products of all 78 plastome-encoded proteins (100 to 500 bp in length, amplified from the 3’ end of the cDNA) in duplicates on Hybrid N⁺ membranes (Amersham Pharmacia Biotech). DIG-labeled cDNA of the wild type and mutants were synthesized from 5 μg of RNA and...
quantified as described by Kandlbinder et al. (2004). Each cDNA from three independent experiments was hybridized with an array filter of the plastome macroarrays. Array hybridization and data evaluation were performed as described in detail by Kandlbinder et al. (2004). The spot intensities were quantified with AIDA Array Vision software (Raytest) and normalized according to whole intensity of all spots on the array. Data management excluded all spots that gave signal intensities below 120% of the background or whose signals varied >50% in duplicates in at least one experiment. The normalized spot intensities were tested for each treatment against the wild type by the use of Student’s $t$-test analysis ($P < 0.05$, $P < 0.1$, and $P < 0.2$). To quantify differential expression of the mutant lines, IF was calculated by the ratio of the average normalized signal intensities of the mutant lines and the wild type.

**Real-Time PCR**

Real-time quantitative RT-PCR was performed using an iCycler iQ real-time PCR detection system and iCycler software version 2.2 (Bio-Rad). Total RNA was isolated from three independent replicates of seedlings. For the amplification of PCR products, iQ SYBR Green Supermix (Bio-Rad) was used according to the manufacturer’s protocol in a final volume of 25 μL. The iCycler was programmed to 95°C 2 min; 40 × (95°C 15 s, 60°C 15 s, 72°C 10 s) with gene-specific and T-DNA–specific primers. After cloning into the pBluescript II SK(-) vector, the sequencing of inserts was performed with T3 and T7 primers. PCR reactions were performed in triplicate. The mRNA levels for each cDNA probe were normalized with respect to the actin mRNA level. Fold induction values of target genes were calculated with the $\Delta\Delta$Ct equation of Pfaffl (2001) and related to the mRNA level of target genes in cotyledons, which was defined as 1.0.

**Primer Extension Analysis**

Seeds were either kept in light (5 to 10 μE m$^{-2}$ s$^{-1}$; Osrarn LW30) or 3-d-old light-grown seedlings were transferred to darkness for an additional 18 d. Primer extension reactions were performed with 10 μg of total leaf RNA according to standard protocols (Sambrook et al., 1989). Briefly, primer PE3AthatpB (5'-CGGTTATGGCGTGCTCCATC-3'; position 54,357) and PE104AtchpC (5'-GGTACTTTTGGAACGCCAAATCG-3'; position 71,857) were end-labeled with [γ-32P]-ATP and T4 polynucleotide kinase. Primer extensions were performed using Superscript III MMLV reverse transcriptase ( Gibco BRL) at 55°C and the resulting products analyzed on 5% sequencing gels. DNA sequences were generated by sequencing plasmid pMS2 and pMS3 using the same primer. Plasmid pMS2 harbors the Arabidopsis rbcL- atpB intergenic region (position 54,079 to 55,090), and pMS3 contains the Arabidopsis clpP upstream region (position 71,774 to 72,897; M. Swietecka and K. Liere, unpublished data).

**Run-On Transcription**

Run-on assays were performed with 2 × 10$^{-7}$ chloroplasts isolated from 12 g of leaves of 24-d-old plants in 100 μL of 50 mM Hepes-KOH, pH 8.0, 33 mM KCl, 10 mM MgCl$_2$, 125 μM ATP, 125 μM CTP, 125 μM GTP, 10 μM UTP, 50 μCi α-32P-UTP (110 TBq/mmol), and 55 μg heparin (Mullet and Klein, 1987). Incorporation of α-32P-UTP into RNA was determined according to Rushlow and Hallick (1982). Four picomoles plasmid DNA, denatured in 0.5 M NaOH, was immobilized on nylon membranes and hybridized to the radiolabeled transcripts. Analyses occurred with the Storm PhosphorImager (Molecular Dynamics).

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL database libraries under accession numbers listed in Table 2.

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