Dual temporal Role of Plastid Sigma Factor 6 in Arabidopsis Development

Heike Loschelder, Jennifer Schweer, Brigitte Link, and Gerhard Link

Plant Cell Physiology, University of Bochum, D-44780 Bochum, Germany
Footnotes:

1 This work was supported by the Deutsche Forschungsgemeinschaft (SFB 480).

* Corresponding author; e-mail Gerhard.Link@ruhr-uni-bochum.de; fax +49-234-3214-188.
Abstract

Plants contain nuclear-coded sigma factors for initiation of chloroplast transcription. The *in vivo* function of individual members of the sigma gene family has become increasingly accessible by knockout and complementation strategies. Here we have investigated plastid gene expression in an *Arabidopsis thaliana* mutant with a defective gene for sigma factor 6. RNA gel blot hybridization and real-time RT-PCR together indicate that this factor has a dual developmental role, with both "early" and "persistent" (long-term) activities. The early role is evident from the sharp decrease of certain plastid transcripts only in young mutant seedlings. The second (persistent) role is reflected by the up- and down-regulation of other transcripts at the time of primary leaf formation and subsequent vegetative development. We conclude that sigma 6 does not represent a general factor but seems to have specialized roles in developmental stage- and gene-specific plastid transcription. The possibility that plastid DNA copy number might be responsible for the altered transcript patterns in mutant vs. wildtype was excluded by the results of DNA gel blot hybridization. Re-transformation of the knockout line with the full-length sigma 6 cDNA further established a causal relationship between the functional sigma gene and the resulting phenotype.
Chloroplasts and other plastid types contain their own genetic system consisting of DNA and a full set of proteins for gene expression. Transcription, the first step leading to (primary) RNA molecules, involves at least two different RNA polymerases. PEP (plastid-encoded polymerase) is the multi-subunit bacterial-type enzyme, while NEP (nucleus-encoded polymerase) is of the single-subunit type shared with the phage T3/T7 and mitochondrial enzymes (Hedtke et al., 1997; Maliga, 1998; Cahoon and Stern, 2001). The promoters recognized by each polymerase differ, with -35/-10 elements in the case of PEP and GAA/YRTA motifs in the case of NEP (Liere and Maliga, 2001; Shiina et al., 2005). PEP has major roles in functional chloroplasts, which is reflected by the large number of regulatory proteins surrounding the catalytic core (Pfannschmidt et al., 2000; Loschelder et al., 2004; Suzuki et al., 2005; Pfalz et al., 2006), including those that associate transiently such as the sigma factors (Tiller and Link, 1993a, b).

Sigma factors are the principal regulators of transcription initiation in bacteria (Gruber and Gross, 2003; Borukhov and Nudler, 2003), and - in view of phylogenetic relationships (Martin et al., 2005) - it has come as no surprise that chloroplasts contain such factor(s). Unlike the core subunits of the bacterial-type plastid RNA polymerase, the organellar sigma proteins are encoded by nuclear genes - in higher plants usually as a small gene family (Allison, 2000; Toyoshima et al., 2005). Arabidopsis contains 6 of these genes, AtSig1 to 6, all of which are unlinked and most of them on different chromosomes (Isono et al., 1997; Tanaka et al., 1997; Fujiwara et al., 2000; Hakimi et al., 2000; The Arabidopsis Genome Initiative, 2000). A common feature of these (highly split) genes is their coding region for the conserved C-terminal half of the derived protein (Hakimi et al., 2000), which contains the typical regions 1.2 to 4.2 for basic sigma functions (Gruber and Gross, 2003). In contrast, the N-terminal half consisting of the short transit peptide followed by considerable extrasequence of variable length is unconserved, suggesting that the latter might contain important determinants for specific properties of individual factors e.g. in development and stress response (Kanamaru and Tanaka, 2004).

While basic sigma functions have been tested to a large part using heterologous in vitro systems with authentic plastid or bacterially expressed sigma proteins and E. coli RNA polymerase (Hakimi et al., 2000; Hanaoka et al., 2003; Homann and Link, 2003), the availability of Arabidopsis sigma mutant lines has facilitated functional studies in vivo (Hanaoka et al., 2003, Privat et al, 2003; Nagashima et al., 2004; Tsunoyama et al., 2004; Favory et al., 2005; Ishizaki et al., 2005 ). Here we investigate an A. thaliana knockout line
with a Sig6 mutant allele (Rosso et al., 2003) that reveals a strong developmental stage-specific (albino) phenotype and characteristic changes in plastid gene expression.
RESULTS

Characterization of the Arabidopsis Sig6-2 Mutant Allele

We have chosen the knockout mutant line sig6-2 that had been generated in the GABI-Kat program at the Max-Planck-Institute fuer Zuechtungsforschung, Cologne (242G06; Rosso et al., 2003). Sequencing of T-DNA borders identified the insertion site within exon 5 of the genomic Sig6 sequence on chromosome II (At2g36990) 1,022 nt downstream of the ATG initiation start codon, which would correspond to a derived protein that lacks all functional domains for sigma factor activity (Fig. 1A). Genomic PCR and Southern blot analysis of selfed progeny lines together verified the selection of a stable homozygous mutant line with a single-copy T-DNA insertion (data not shown).

Using RT-PCR and gene-specific primers (see Materials and Methods), transcripts from individual members of the Arabidopsis sigma gene family were assessed. As shown in Fig. 1B, the signals for Sig1 to Sig6 were all clearly visible with wildtype RNA, whereas the Sig6 transcript was absent with mutant RNA.

The phenotype of sig6-2 differs from that of wildtype in a developmental stage-specific way. Homozygous mutant seedlings develop normally-shaped cotyledons, yet with increasing chlorophyll deficiency. Younger stages until approximately 4 d after sowing have pale green cotyledons, which then become yellowish and finally white during the next six to eight days (Fig. 1C). In contrast, the primary leaves and subsequent rosette leaves are seemingly unaffected. Except for the remainder of the white cotyledons, mutant plants are green and have normal morphological appearance (Fig. 1C). They tend, however, to be slightly smaller in size than wildtype of the same age, which could be related to delayed germination and/or seedling development.

Plastid Transcript Patterns in Wildtype vs. Sig6-2

Transcript levels of representative chloroplast genes at different times in development were assessed by northern blot hybridization. RNA samples from cotyledons (seedlings 4 to 10 days after sowing) or rosette leaves (plants 21 and 28 days after sowing) were fractionated
and hybridized with gene-specific RNA probes (Fig. 2) as described in Materials and Methods. The results were grouped according to the observed expression mode in the mutant, taking into account the classification of plastid genes based on their transcription by PEP vs. NEP (Hajdukiewicz et al., 1997; Shiina et al., 2005). Data for genes with an expression mode of typical (PEP-dependent) class I genes are presented in panel A and those for (PEP and NEP-dependent) class II genes in B. In panel C, patterns are shown for accD (an exclusively NEP-dependent) class III gene and clpP, a class II gene with a similar expression mode as accD.

The 1.3 kb psbA transcript (Fig. 2A, top row) is a prominent band in all wildtype lanes from 4 to 28 d after sowing. In the mutant, this transcript is dramatically downregulated at 4 d, i.e. the youngest seedling stage analyzed, and is then rapidly restored to wildtype levels. A similar time-course was noticeable both for the 1.9 kb rbcL transcript (second row) and the 0.6 kb precursor of the intron-containing trnV(UAC) gene (third row), again with a decrease only in the 4 d mutant sample. In addition, the trnV probe revealed a high-molecular (3.0 kb) signal in the mutant but not in wildtype. Unlike all other transcripts in Fig. 2A, this RNA species was not detectable before the 8 d stage.

In contrast to the genes in Fig. 2A, those in Fig. 2B did not give rise to transcripts with an early decrease at the 4 d mutant stage. The dicistronic (Sugita and Sugiura, 1996) atpB/E transcript at 2.0 kb (first and second row), the monocistronic atpE mRNA (0.7 kb; second row), and the tricistronic ndhC/K/J transcript (1.8 kb; third row) all were visible at almost constant intensity over the entire time span from 4 to 28 d. On the other hand, differences between wildtype and mutant patterns were evident in Fig. 2B, which were not noticeable for psbA and rbcL (Fig. 2A). These included the gradual weakening of the 2.6 kb atpB/E band beginning with the first (4 d) mutant stage until complete loss after day 10, and the transient appearance of a large 4.8 kb species (8 and 10 d lanes). The latter, mutant-specific, RNA spans the entire atpB/E coding region and ends in a short distance downstream but has considerable extra-sequence on the 5' side (data not shown). The ndhC probe detected a 3.0 kb transcript at day 8 and later. Both the time-course and size are reminiscent of the large band detected by the trnV probe (Fig. 2A, third row), suggesting that it is the same transcript spanning these two adjacent genes (Sato et al., 1999).

Finally, as shown in Fig. 2C (first row), the 2.5 kb transcript of the accD gene was present in the mutant in amounts that were equal to (21 and 28 d) or higher (4 to 10 d), but never lower than those in wildtype. A similar pattern was also observed for the 1.2 kb clpP transcript (second row).
To assess steady-state transcript levels of selected (monocistronic) genes more rigorously, quantitative real time PCR experiments were carried out (Fig. 3). Again, \textit{psbA} and \textit{rbcL} were found to give decreased transcript levels in 4, but not 10, day-old \textit{sig6-2} seedlings (Figs. 3A and B). For \textit{psbA} (Fig. 3, panel A), the down-regulation compared to wildtype exceeded a factor four at the 4 d-stage and was less than 0.5 at 10 d, while for \textit{rbcL} (panel B) the factors were larger than two (4 d) vs. less than 0.5 (10 d). In contrast, real time RT-PCR showed greater than threefold up-regulation for the \textit{clpP} transcript at day 4, and twofold at day 10 (Fig. 3, panel C). This pattern is in agreement with that observed for \textit{accD} and \textit{clpP} in the northern blot experiments (Fig. 2C). Because of their multiple overlapping transcripts, the polycistronic transcription units studied in Fig. 2B (\textit{atpB-E, ndhC-K-J}) were not investigated by real time quantification.

**Plastid/nuclear DNA Ratio is not responsible for altered Expression Patterns in \textit{Sig6-2}**

As changes in copy number of plastid DNA might contribute to the altered RNA patterns in the mutant, we tested this possibility by Southern hybridization with both plastid and nuclear probes. Total DNA was prepared from wildtype (WT) and mutant (\textit{sig6-2}), either at the 4 d seedling stage (Fig. 4A) or from 28 d plants (Fig. 4B). After digestion of equal amounts of DNA with \textit{HindIII}, followed by gel-fractionation and hybridization, a single signal at 7.5 kb was generated with the plastid \textit{psbA} probe (left panels), and a 9.0 kb band with the nuclear 18S rDNA probe (right panels). The WT and \textit{sig6-2} lanes always revealed bands of equal intensity, suggesting that DNA copy number was not responsible for the different \textit{psbA} transcript levels in mutant vs. wildtype that were observed at 4 but not 28 d (Fig. 2).

**Rescue of SIG6 Gene Function by Complementation**

To further confirm that insertional inactivation of the \textit{AtSig6} gene in the \textit{sig6-2} mutant is directly responsible for its phenotype, complementation experiments using the full-length cDNA were carried out. Following reverse transcription and amplification, the cloned \textit{AtSig6} cDNA was fused to the cauliflower mosaic virus 35S promoter of the binary vector pBINAR Höfgen and Willmitzer, 1990). Following floral dip transformation of \textit{sig6-2} (Clough and Bent, 1998), T2 plants and selfed progeny were analyzed for visible phenotype, DNA (Fig.
5A) and RNA patterns (Fig. 5B). Of the four different complementation lines that were tested, the representative results obtained with one line are shown.

We first examined the integration of the pBINAR T-DNA into the sig6-2 mutant line using genomic Southern Blot analyses (Fig. 5A). The sulf probe (left panel) established the absence of the (primary) T-DNA from wildtype (WT), and its presence in both the sig6-2 knockout (MT) and the complemented mutant line (C). The nptII probe (central panel) specifically detected the T-DNA insertion resulting from the secondary transformation, with a signal visible only in the complemented mutant (C). Using the sig6 probe (right panel), a single 5.3 kb band was generated in the wildtype (WT), whereas a 3.5 kb band was noticeable in both the knockout (MT) and re-transformed plants (C). The latter also showed two additional bands at approx. 6.0 kb and 4.5 kb. As these two bands were consistently observed under a variety of experimental conditions, they probably indicate the presence of an additional EcoRI site adjacent to the insertion rather than partial digestion (not shown). In any case, none of them is visible in the WT and MT lanes, suggesting that they mark a single secondary insertion at a unique site.

We next analyzed the gene expression patterns of the complemented line (C) in comparison with those from wildtype (WT) and the sig6-2 knockout (MT). As shown in Fig. 5B (upper left), RT-PCR amplification from total RNA of 4 d seedlings established that the AtSig6 transcript(s) are absent in MT but are present both in WT and C, and similar results were obtained with RNA from 10 d seedlings (not shown). Using RNA gel blot hybridization with a psbA probe (Fig. 5B, left panel), the intensity of the 1.3 kb transcript was decreased in MT compared to WT (see also Fig. 2A) but was restored to at least WT levels in the complemented line (C). These quantitative differences in signal strength among lines were much more pronounced for the 4 d (left) than for the 10 d seedlings (right). The hybridization results with the atpB and atpE probes (Fig. 5B, right panels) again showed the typical transcript patterns for WT and MT (compare Fig. 2B), and the complete restoration of the re-transformed mutant (C) to the wildtype situation. While the 2.0 kb (and 0.7 kb) transcripts are visible in all lanes, the 2.6 kb transcript is present only in WT and C, and the 4.8 kb band only in MT. Together these data provide evidence that the re-transformed line (C) has acquired wildtype properties with regard to AtSig6-dependent plastid gene expression. This notion is further supported by the visible phenotype, which is indistinguishable from wildtype (not shown).
DISCUSSION

In the present work, we have characterized a new AtSig6 mutant allele, sig6-2, both at DNA and RNA level as well as by complementation with the intact cDNA. This reverse genetics strategy established a causal link between the introduced gene and the visual and molecular phenotype of the rescued transformants, both of which resembled that of the wildtype (Fig. 5). PCR and Southern blot analysis together established the gene-specific (single) T-DNA insertion both in the knockout mutant and in the complemented line (Fig. 1). Using the same techniques, evidence was obtained that plastid DNA copy number does not seem to be a significant factor responsible for distinct plastid RNA patterns of wildtype vs. mutant (Fig. 4).

RT-PCR and northern blot analysis together suggested that gene expression patterns in both the wildtype and complemented line are similar if not identical, and those in the knockout mutant are clearly different (Figs. 2, 3 and 5). The plastid RNA patterns were thus of diagnostic value, both in the comparison of different Arabidopsis (wildtype, mutant, and complemented) lines, and different developmental stages of one single line. Furthermore, the expression patterns helped integrate the picture obtained for genes of different classes. For instance, as is evident from Fig. 2, both the class I genes (psbA and rbcL) and the split trnV(UAC) gene gave rise to transcripts of similar expression mode. We feel that the term "expression mode" can be particularly useful if (multiple) transcripts are considered, as is the case for the genes presented in Fig. 2B (atpB/E, trnV, ndhC) (Sato et al., 1999).

Unlike most mutants described for other Arabidopsis sigma factors (reviewed by Shiina et al., 2005; Toyoshima et al., 2005), those for sigma 6 reveal a developmental stage-specific phenotype. This was first shown for the mutant allele sig6-1 (Ishizaki et al., 2005), which has a pale green (chlorophyll-deficient) phenotype in 3- to 4-d seedlings and then regreens to wildtype levels until day 8. In addition, plastid gene expression at RNA level was affected in that mutant only in young (4 d) but not older (8 d) seedlings, which led the authors to conclude that AtSIG6 might have a function restricted to early seedling development (Ishizaki et al., 2005). The sig6-2 mutant allele analyzed in our present work has an even stronger phenotype than sig6-1, with cotyledons that are pale-green at day three to four and then become yellowish and finally white (day 10 to 12).

The transcript patterns (Figs. 2 and 3) of sig6-2 were in agreement with those obtained for sig6-1 in at least some cases. This is evident for transcripts of the class I genes psbA and rbcL, each of which showed a sharp decrease in steady-state concentration at day 4 but not
day 8 in \textit{sig6-1} (Ishizaki et al., 2005). In the \textit{sig6-2} line studied here by northern blot hybridization (Fig. 2) and quantitative real-time RT-PCR (Fig. 3), the \textit{psbA} and \textit{rbcL} transcript levels were strongly reduced at the earliest time-point (4 d) and rapidly recovered to almost wildtype levels by day 10. Hence, from the data obtained with class I genes, both the \textit{sig6-1} and \textit{sig6-2} mutant alleles are defective in a SIG6 function that plays a stage-specific critical role in early seedling development. Similar conclusions can be reached if the transcripts of the \textit{clpP} (class II) and \textit{accD} (class III) genes (Fig. 2C) are considered, although in these cases increased, rather than decreased, levels were found in the mutant as compared to wildtype.

A notable difference, however, is evident from the \textit{trnV(UAC)} transcript pattern (Fig. 2A, third row), consisting of two RNA species with different time-course during development. The smaller (0.6 kb) band shows the early decrease (4 d) as was seen for the class I transcripts \textit{psbA} and \textit{rbcL} (first and second row). The large 3.0 kb signal is visible only in the mutant, and only later throughout day 8 to 28. Neither effect was previously described for \textit{trnV} in \textit{sig6-1} (Ishizaki et al., 2005). The presence and differential time-course of these two RNAs thus distinguishes the two mutant alleles and, furthermore, points to a role of SIG6 not only in seedlings but also in rosette-stage plants.

This view is strengthened by the data obtained with the polycistronic \textit{ndhC} transcription unit (Fig. 2B, third row), which also results in two RNAs of different time-course. The smaller (1.8 kb) species appears to be present in relatively constant amounts without a decrease at the 4 d-seedling stage. The (mutant-specific) 3.0 kb RNA is first visible at day 8 and then remains at constant level, i.e. both its size and time-course match those of the large \textit{trnV} transcript (Fig. 2A, row 3). As \textit{trnV} and \textit{ndhC} are immediately adjacent (Sato et al., 1999), it is likely that the 3.0 kb RNA detected in both cases is identical.

The \textit{atpB-E} operon (Fig. 2B, first and second row) gives rise to several transcripts, none of which shows an early decrease comparable to that of the class I RNAs (Fig. 2A): \textit{(i)} The major 2.0 kb (\textit{atpB-E}) and the 0.7 kb (monocistronic \textit{atpE}) RNAs were both present in roughly constant amounts throughout development. \textit{(ii)} The 2.6 kb RNA species was visible both in wildtype and \textit{sig6-2} at 4 d but was absent in the mutant at all subsequent stages. \textit{(iii)} The mutant-specific 4.8 kb species accumulated transiently between 4 and 10 d and then completely disappeared (Fig. 2B, first and second row).

Together, the data presented in Fig. 2 indicate an unexpected complexity of SIG6-dependent responses in Arabidopsis development. The model depicted in Fig. 6 suggests a dual role consisting of both an "early" and "persistent" (long-term) activity of the factor. An
early decrease was seen for the transcripts of class I genes (Fig. 2A), but also for the 0.6 kb trnV transcript ("expression mode I"). The opposite effect, i.e. the early increase of the accD and clpP transcripts (Fig. 2C), may be functionally related, although it could be due to efficient NEP transcription (Allison et al., 1996; Legen et al., 2002) of these genes in this situation in the mutant ("expression mode III"). Perhaps most notable, none of the mature transcripts in Fig. 2B revealed an early effect, indicating that a different gene-specific mechanism might be involved ("expression mode II"). At this early time point, another sigma factor might be able to substitute for SIG6 in the transcription of the mode II genes (Fig. 2B), but less efficiently, if at all, in the transcription of the mode I genes (Fig. 2A). Likewise, the loss of the 2.6 kb atpB-E transcripts in the mutant is consistent with a second (long-term) role of SIG6, implying that it cannot fully be replaced by other factor(s) during late seedling development and rosette leaf formation. It is notable that none of the (monocistronic) class I genes psbA and rbcL showed any "persistent" effect such as mutant-specific transcripts of distinguishable size (Fig. 2A and data not shown). Together, this would mean that, at least during the developmental stages and at the genes (promoters) investigated here, SIG6 seems to act as a specialized rather than general factor.

The transient 4.8 kb RNA of the atpB/E region (Fig. 2) may be a consequence of the fact that both the early and persistent (long-term) functions of SIG6 are absent in the mutant. If not generated by an alternative sigma factor and PEP, this mutant-specific RNA could be the result of NEP-dependent transcription. A similar mechanism, i.e. formation of a large (polycistronic) transcript by usage of a NEP promoter in the absence of SIG6, could explain the 3.0 kb trnV (and ndhC) transcript. Furthermore, it was previously established that trnV is a PEP-dependent gene preferentially transcribed in the presence of SIG2 (Kanamaru et al., 2001; Hanaoka et al., 2003; Privat et al., 2003). The early decrease of the 0.6 kb RNA at day 4 (Fig. 2A) suggests that SIG6, in addition to SIG2, may have a - temporally restricted - role in the transcription of this tRNA gene.

A question that emerges relates to the mechanism(s) involved in the functional overlap of plastid sigma factors, throughout development or only at certain times (Kanamaru and Tanaka, 2004: Shiina et al., 2005). From in vitro studies using purified authentic (Tiller and Link, 1993a, b) or recombinant sigma proteins (Homann and Link, 2003) it appears that the phosphorylation state of these factors might be a critical determinant in transcription initiation activity. The protein kinase responsible for sigma phosphorylation (Baginsky et al., 1997, 1999) has been cloned and characterized (Ogrzewalla et al., 2002). This plastid transcription kinase (PTK), a known CK2-type enzyme also termed cpCK2 (Loschelder et al.,
2004), is regulated by phosphorylation itself and, moreover, is subject to redox control by glutathion (for review, see Baginsky and Link, 2005). It will be interesting to investigate whether AtSIG6 is a functional substrate for PTK, and possible consequences for plastid gene regulation.

In addition to phosphorylation and redox control, a number of other mechanisms could be envisaged for (time- and promoter-specific) usage of individual plastid sigma factors, including proteolytic cleavage (Hakimi et al., 2000; Homann and Link, 2003), splice variants (Fujiwara et al., 2000; Yao et al., 2003), interacting proteins (Morikawa et al., 2002), and other compositional changes of the (core) plastid transcription machinery (Pfannschmidt and Link, 1994; Pfalz et al., 2006). Studies using transgenic plants with functional and/or defective sigma genes (Suzuki et al., 2005) can be expected to provide further insights into the underlying mechanisms.
MATERIALS AND METHODS

Plant Material, Growth Conditions, Developmental Stages

The sig6-2 mutant of A. thaliana (ecotype Columbia) was identified in a collection of T-DNA insertion lines of the GABI-Kat project at the MPI fuer Zuechtungsforschung, Cologne (Rosso et al., 2003). Surface-sterilized seeds of wildtype and sig6-2 mutant were sown on Murashige and Skoog (MS) medium containing 0.4% (w/v) gelrite and 1% (w/v) sucrose. They were stratified at 4°C for two to three days and then transferred to 24°C for germination and growth under short-day conditions (8 h light and 16 h dark, 60 mol m⁻² s⁻¹). Seedlings were harvested 4, 8, or 10 d after sowing or growth was continued until day 14, at which time plantlets were transferred to sterile soil for another one or two weeks under the same environmental conditions. Rosette leaves were then harvested from the 21 or 28 d soil-grown plants. All samples were immediately frozen in liquid nitrogen and stored at -85°C until use.

Characterization of the Sig6 Knockout

For PCR analysis of the AtSig6 mutant, total DNA samples were isolated from rosette leaves of either wildtype or progeny of the GABI-Kat line (T3 or later) by using the plant mini kit (Qiagen, Hilden, Germany). The primer pair for the sulfonamide resistance gene of the T-DNA plasmid pAC161 (Rosso et al., 2003) allowed detection of the (single-copy) insertion. The Sig6-specific primers Sig6-HO1 (5’-CCACTCGCCTATTGTTGGTT-3’) and Sig6-HO2 (5’-GGAGAGGAGGCAGTTTGATG-3’), in combination with the left border-specific primer Sig6-LB2 (5’-TTTTTCTTGTGGCGTCTTT-3’), together verified the existence of homozygous progeny lines. SUL1a/b were also used for synthesis of probes to be used in gel blot hybridization (see below).
RT-PCR Detection of Sigma Factor Transcripts

Total RNA (2 g) from 6 day-old Arabidopsis seedlings was mixed with random primers (10 pM; Promega, Madison, WI), incubated at 70°C for 10 min, and chilled on ice for 1 min. After addition of 6 1 AMV-RT buffer (Promega), 1 RNasin (40 U/ l; Promega), 3 dNTPs (0.25 mM each), and 3 AMV reverse transcriptase (10 U/ l) to a final volume of 30 l, the reaction was incubated at 37°C for 90 min. Following heating to 95°C for 10 min, the mixture was chilled on ice for 1 min. One RNase A (10 g/ l, Sigma) was then added and incubation continued at 37°C for 15 min. The cDNAs corresponding to each Arabidopsis sigma factor were amplified using Taq DNA polymerase (Promega). Primers were RTSIG1-1 (5′-TTTTCTGCATGGTGTTTGA-3′) and RTSIG1-2 (5′-ACCGCTCTCTATGGCTCTGA-3′) for Sig1, RTSIG2-2 (5′-GAAAGAGGCC ACGAAAGCAAC-3′) and RTSIG2-3 (5′-CCAACGAATCCCATTACCAC-3′) for Sig2, RTSIG3-1 (5′-GAAAGCAAGGAGTGCGAGTG-3′) and RTSIG3-2 (5′-TCCATCGTTGTG TCTGGTGT-3′) for Sig3, RTSIG4-1 (5′-ACGACGATTCCCACACAGC-3′) and RTSIG4-2 (5′-CTCGAAGCGTTCAGCACCT-3′) for Sig4, RTSIG5-2 (5′-CTCTCCTCGT GAGCAAGT-3′) and RTSIG5-3 (5′-CATACCGCTGTACACATAGG-3′) for Sig5, and RTSIG6-1 (5′-GCCTGCTCAGCCAGACCC-3′) and RTSIG6-2 (5′-CTAGACAA GCAATCCAGA-3′) for Sig6. PCR reactions consisted of an initial heating step at 95°C for 2 min, followed by 35 cycles each at 95°C for 30 s, 57.5 to 60°C for 30 s, and 72°C for 2 min.

RNA Isolation, Northern Blot Analysis

Cotyledon or rosette leave samples (100 mg) were frozen in liquid nitrogen and ground to powder. RNA was isolated by the acid guanidinium-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Briefly, the powder was resuspended in 1.5 ml lysis buffer containing 4 M guanidine thiocyanate, 25 mM potassium citrate pH 7.0, 0.5% (w/v) N-lauroylsarcosine, 100 mM mercaptoethanol. After addition of 150 1 2 M potassium acetate pH 4.0, RNA was extracted with phenol / chloroform / isoamylalcohol (25:24:1) and precipitated with ethanol. Total RNA (1 g per lane) was separated on a 1.2% (w/v) agarose–formaldehyde gel, blotted to positively charged nylon membrane (Roche), and
hybridized with DIG-labeled RNA probes according to the Roche manual. Probes were generated by RT-PCR amplification and cloning of corresponding cDNAs into pGEM-T Easy (Promega), followed by *in vitro* transcription using phage RNA polymerases. The following primer sets were used: NorpsbA1 (5´-TTACCCAATCTGGGAAGCTG-3´), NorpsbA2 (5´-GCCTCAACAGCAGCTAGGTC-3´), RT7 (5´-GACAACTGTGTGGACCCCA TG-3´), RT8 (5´-TTCACCTGTTTCAGCCTCTG-3´), NorAccD-1 (5´-TCGCAATTTCATA TCGGATG-3´), NorAccD-2 (5´-CTTCTTGTCTGTCTGCTCCT-3´), atpB1nor (5´-GGGGA ACCCGTTGATAATTT-3´), atpB2nor (5´-AACGCTCAATTTTCGTGCT-3´), NorAtpEa (5´-GACTCGGAATCGAATTGTT-3´), NorAtpEb (5´-GTGTCCGAGCTCGT CTGAG-3´), NorndhC-1 (5´-TGCTATTTCTCTGGCATATT-3´), NorndhC-2 (5´-CCATT CCAATGCTCTTTTTG-3´), NortrnV-1 (5´-CTCGAACCGTAGACCTGCTC-3´), NortrnV-2 (5´-GAGTCCATCAGCAATCAA-3´).

**Real-Time PCR**

Real-time one-step RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen). The 50 µl PCR reaction contained gene-specific primer sets (0.5 M each) to yield amplicons of 150 to 200 bp, QuantiTect SYBR Green plus RT Mix (Qiagen), and 0.02 to 20 ng template RNA. Primers were QpsbA-1 (5´-TTTCCGGTGCCATTATTCCT-3´), QpsbA-2 (5´-TCATAAGGACCGCCGTTGTA-3´), QrbcL-1 (5´-TCGCTGAGGAACCTTT AGGC-3´), QrbcL-2 (5´-TGCAAGATCAGCTCCCTCAT-3´), QelP-1 (5´-ATCCATGAG CTTGGCTTC-3´), QelP-2 (5´-ACTTCCGGAACCATCACA-3´). Experiments were carried out in an Opticon 2 DNA engine (MJ Research) using cycling conditions as follows: 50°C for 30 min, 95°C for 15 min, 94 °C for 15 s, 50°C for 30 s, 72°C for 30 s, followed by 40 cycles at 94°C for 15 s and 55°C or 60°C for 1 min. To check for absence of dimer formation, the primers were subjected to melting curve analysis with incremental steps from 60°C to 95°C every 0.3°C for 3 s. Amplicon size for each primer pair was verified by gel electrophoresis and each reaction was carried out in triplicate. Primer pair efficiency was calculated using LinRegPCR (Ramakers et al., 2003). Mean Ct values (threshold cycles) normalized with *actin2* primers (Sigma, St Louis) as a reference were used for determination of expression ratios (Pfaffl, 2001). All real-time experiments were performed at least in duplicate with RNA samples that had been independently isolated.
Knockout Complementation by SIG6 cDNA

Full-length AtSIG6 cDNA including the transit peptide was PCR amplified from wildtype RNA using the primer pair SigF1 (5’-ATGGAAGCTACGAGGAACTTGG-3’) and SigF2 (5’-CTAGACAAGCAAATCAGCATA-3’) and cloned into the EcoRV site of vector pBSKS(-) (Stratagene). The insert of the resulting intermediate plasmid was controlled by sequencing, cut out, and ligated into the BamHI and SalI sites downstream from the CaMV 35S promoter of the binary vector pBINAR (Höfgen and Willmitzer, 1990). The fused (35S::SIG6) construct was introduced into Rhizobium radiobacter (Agrobacterium tumefaciens) strain GV3101 and then transformed into the sig6-2 mutant by floral dip (Clough and Bent, 1998). T1 plants were selected by resistance to kanamycin. The presence and copy number of the transgene in these plants was tested by PCR and Southern blot analyses using primers npt1 (5’-CGAAGAACTCCAGCATGAGA-3’) and npt2 (5’-GCTATGACTGGGCAGAACAG-3’). Sig6-specific primers were UKSIG6-RP (5’-GAAGAGCTAAACCAACATCCA-3’) and UKSIG6-LP (5’-TTAATGCGATTGGGTTCCTT-3’).

Genomic Southern Blot Hybridization

Genomic DNA for Southern blot analysis was prepared from cotyledons and rosette leaves by using the CTAB (cetyltrimethylammonium bromide) procedure (Doyle and Doyle, 1987). Two to five g of total genomic DNA were electrophoresed through an 0.7% (w/v) agarose gel and blotted to positively charged nylon membrane (Roche). The membrane was then hybridized with either DIG-labeled DNA or RNA probes at 42°C or 50°C, respectively, according to the Roche manual. The DNA probes were generated using the PCR DIG probe synthesis kit (Roche). RNA probes were obtained by cloning of PCR amplified regions in pGEM-T Easy (Promega), followed by in vitro transcription using the DIG RNA labeling mix (Roche). PCR Primers were SUL1a (5’-ATGGCTTTCTATGATATCC-3’) and SUL1b (5’-CTAGGCATGATCTAACCCTCGG-3’) for the sulfadiazine gene, Sig6-LB1 (5’-TGTAGATGTCCGCAGCGTTA-3’) and Sig6-LB2 (5’-TGTAGATGTCCGCAGCGTTA-3’) for T-DNA left-border sequences. To investigate plastid DNA copy number,
hybridization was carried out with probes that selectively detected either a chloroplast \((\text{psbA})\) or nuclear gene region \((18S\ r\text{DNA})\). The primer pairs for amplification were NorpsbA1 \(5´-\text{TTACCCAATCTGGGAAGCTG-3´}\), NorpsbA2 \(5´-\text{GCCTCAACAGCAGC\ TAGGTC-3´}\) as well as AT-18S-1 \(5´-\text{AAACGGCTACCACATCCAAG-3´}\) and AT-18S-2 \(5´-\text{GTACAAAGGCGAGGACGTA-3´}\).

**ACKNOWLEDGEMENTS**

We gratefully acknowledge the generous supply of the \(\text{sig6-2}\) mutant line by Prof. B. Weissshaar, University of Bielefeld, and the GABI-Kat team at the Max-Planck-Institute fuer Zuechtungsforschung, Cologne. We also like to thank Drs. M. Nowrousian and I. Kubigsteltig for their guidance and helpful discussion of the real-time RT-PCR and transgenic work, respectively.
LITERATURE CITED

Allison LA (2000) The role of sigma factors in plastid transcription. Biochimie 82: 537-548

Allison LA, Simon LD, Maliga P (1996) Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants. EMBO J 15: 2802-2809

Baginsky S, Link G (2005) Redox regulation of chloroplast gene expression. In B Demmig-Adams, WI Adams, AK Mattoo, eds, Photoprotection, photoinhibition, gene regulation, and environment. Springer, Dordrecht, The Netherlands, pp 269-287

Baginsky S, Tiller K, Link G (1997) Transcription factor phosphorylation by a protein kinase associated with chloroplast RNA polymerase from mustard (Sinapis alba). Plant Mol Biol 34: 181-189

Baginsky S, Tiller K, Pfannschmidt T, Link G (1999) PTK, the chloroplast RNA polymerase-associated protein kinase from mustard (Sinapis alba), mediates redox control of plastid in vitro transcription. Plant Mol Biol 39: 1013-1023

Borukhov S, Nudler E (2003) RNA polymerase holoenzyme: structure, function and biological implications. Curr Opin Microbiol 6: 93-100

Cahoon AB, Stern DB (2001) Plastid transcription: a menage à trois. Trends Plant Sci 6: 45-46

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156-159

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-744

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11-15

Favory J-J, Kobayashi M, Tanaka K, Peltier G, Kreis M, Valay J-G, Lerbs-Mache S (2005) Specific function of a plastid sigma factor for ndhF gene transcription. Nucleic Acids Res 33: 5991-5999

Fujiwara M, Nagashima A, Kanamaru K, Tanaka K, Takahashi H (2000) Three new nuclear genes, sigD, sigE and sigF, encoding putative plastid RNA polymerase sigma factors in Arabidopsis thaliana. FEBS Lett 481: 47-52

Gruber TM, Gross CA (2003) Multiple sigma subunits and the partitioning of bacterial
transcription space. Annu Rev Microbiol 57: 441-466

Hajdukiewicz PTJ, Allison LA, Maliga P (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. EMBO J 16: 4041-4048

Hakimi M-A, Privat I, Valay J-G, Lerbs-Mache S (2000) Evolutionary conservation of C-terminal domains of primary sigma 70-type transcription factors between plants and bacteria. J Biol Chem 275: 9215-9221

Hanaoka M, Kanamaru K, Takahashi H, Tanaka K (2003) Molecular genetic analysis of chloroplast gene promoters dependent on SIG2, a nucleus-encoded sigma factor for the plastid-encoded RNA polymerase, in Arabidopsis thaliana. Nucleic Acids Res 31: 7090-7098

Hedtke B, Börner T, Weihe A (1997) Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis. Science 277: 809-811

Homann A, Link G (2003) DNA-binding and transcription characteristics of three cloned sigma factors from mustard (Sinapis alba L.) suggest overlapping and distinct roles in plastid gene expression. Eur J Biochem 270: 1288-1300

Höffgen R, Willmitzer L (1990) Biochemical and genetic analysis of different patatin isoforms expressed in various organs of potato (Solanum tuberosum). Plant Sci 66: 221-230

Ishizaki Y, Tsunoyama Y, Hatano K, Ando K, Kato K, Shinmyo A, Kobori M, Takeba G, Nakahira Y, Shiina T (2005) A nuclear-encoded sigma factor, Arabidopsis SIG6, recognizes sigma-70 type chloroplast promoters and regulates early chloroplast development in cotyledons. Plant J 42: 133-144

Isono K, Shimizu M, Yoshimoto K, Niwa Y, Satoh K, Yokota A, Kobayashi H (1997) Leaf-specifically expressed genes for polypeptides destined for chloroplasts with domains of sigma 70 factors of bacterial RNA polymerases in Arabidopsis thaliana. Proc Natl Acad Sci USA 94: 14948-14953

Kanamaru K, Nagashima A, Fujiwara M, Shimada H, Shirano Y, Nakabayashi K, Shibata D, Tanaka K, Takahashi H (2001) An Arabidopsis sigma factor (SIG2)-dependent expression of plastid-encoded tRNAs in chloroplasts. Plant Cell Physiol 42: 1034-1043

Kanamaru K, Tanaka K (2004) Roles of chloroplast RNA polymerase sigma factors in chloroplast development and stress response in higher plants. Biosci Biotechnol Biochem 68: 2215-2223
Legen J, Kemp S, Krause K, Profanter B, Herrmann RG, Maier RM (2002) Comparative analysis of plastid transcription profiles of entire plastid chromosomes from tobacco attributed to wild-type and PEP-deficient transcription machineries. Plant J 31: 171-188

Liere K, Maliga P (2001) Plastid RNA polymerases in higher plants. In E-M Aro, B Andersson, eds, Regulation of photosynthesis. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 29-49

Loschelder H, Homann A, Ogrzewalla K, Link G (2004) Proteomics-based sequence analysis of plant gene expression - the chloroplast transcription apparatus. Phytochem 65: 1785-1793

Maliga P (1998) Two plastid RNA polymerases of higher plants: an evolving story. Trends Plant Sci 3: 4-6

Martin W, Deusch O, Stawski N, Grünheit N, Goremykin V (2005) Chloroplast genome phylogenetics: why we need independent approaches to plant molecular evolution. Trends Plant Sci 10: 203-209

Morikawa K, Shiina T, Murakami S, Toyoshima Y (2002) Novel nuclear-encoded proteins interacting with a plastid sigma factor, Sig1, in Arabidopsis thaliana. FEBS Lett 514: 300-304

Nagashima A, Hanaoka M, Shikanai T, Fujiwara M, Kanamaru K, Takahashi H, Tanaka K (2004) The multiple-stress responsive plastid sigma factor, SIG5, directs activation of the psbD blue light-responsive promoter (BLRP) in Arabidopsis thaliana. Plant Cell Physiol 45: 357-368

Ogrzewalla K, Piotrowski M, Reinbothe S, Link G (2002) The plastid transcription kinase from mustard (Sinapis alba L.) - A nuclear-encoded CK2-type chloroplast enzyme with redox-sensitive function. Eur J Biochem 269: 3329-3337

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45

Pfalz J, Liere K, Kandlbinder A, Dietz KJ, Oelmüller R (2006) PTAC2,-6, and-12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. Plant Cell 18: 176-197

Pfannschmidt T, Link G (1994) Separation of two classes of plastid DNA-dependent RNA polymerases that are differentially expressed in mustard (Sinapis alba L.) seedlings. Plant Mol Biol 25: 69-81
Pfannschmidt T, Ogrzewalla K, Baginsky S, Sickmann A, Meyer HE, Link G (2000) The multisubunit chloroplast RNA polymerase A from mustard (*Sinapis alba* L.): Integration of a prokaryotic core into a larger complex with organelle-specific functions. Eur J Biochem 267: 253-261

Privat I, Hakimi MA, Buhot L, Favory J-J, Lerbs-Mache S (2003) Characterization of *Arabidopsis* plastid sigma-like transcription factors SIG1, SIG2 and SIG3. Plant Mol Biol 51: 385-399

Ramakers C, Ruijter JM, Lekanne Deprez RH, Moormann AFM (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett 339: 62-66

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol 53: 247-259

Sato S, Nakamura Y, Kaneko T, Asamizu E, Tabata S (1999) Complete structure of the chloroplast genome of *Arabidopsis thaliana*. DNA Res 6: 283-290

Shiina T, Tsunoyama Y, Nakahira Y, Khan MS (2005) Plastid RNA polymerases, promoters, and transcription regulators in higher plants. Int Rev Cytol 244: 1-68

Sugita M, Sugiura M (1996) Regulation of gene expression in chloroplasts of higher plants. Plant Mol Biol 32: 315-326

Suzuki JY, Ytterberg AJ, Beardslee TA, Allison LA, Wijk KJ, Maliga P (2005) Affinity purification of the tobacco plastid RNA polymerase and *in vitro* reconstitution of the holoenzyme. Plant J 40: 164-172

Tanaka K, Tozawa Y, Mochizuki N, Shinozaki K, Nagatani A, Wakasa K, Takahashi H (1997) Characterization of three cDNA species encoding plastid RNA polymerase sigma factors in *Arabidopsis thaliana*: Evidence for the sigma factor heterogeneity in higher plant plastids. FEBS Lett 413: 309-313

The Arabidopsis Genome Initiative, Kaul S, Koo HL, Jenkins J, Rizzo M, Rooney T, Tallon LJ, Feldblyum T, Nierman W, Benito MI, Lin XY, Town CD, Venter JC, Fraser CM, Tabata S, Nakamura Y, Kaneko T, Sato S, Asamizu E, Kato T, Kotani H, Sasamoto S, Ecker JR, Theologis A, Federspiel NA (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815
Tiller K, Link G (1993a) Sigma-like transcription factors from mustard (*Sinapis alba* L.) etioplast are similar in size to, but functionally distinct from, their chloroplast counterparts. *Plant Mol Biol* 21: 503-513

Tiller K, Link G (1993b) Phosphorylation and dephosphorylation affect functional characteristics of chloroplast and etioplast transcription systems from mustard (*Sinapis alba* L.). *EMBO J* 12: 1745-1753

Toyoshima Y, Onda Y, Shiina T, Nakahira Y (2005) Plastid transcription in higher plants. *Crit Rev Plant Sci* 24: 59-81

Tsunoyama Y, Ishizaki Y, Morikawa K, Kobori M, Nakahira Y, Takeba G, Toyoshima Y, Shiina T (2004) Blue light-induced transcription of plastid-encoded *psbD* gene is mediated by a nuclear-encoded transcription initiation factor, *AtSig5*. *Proc Natl Acad Sci USA* 101: 3304-3309

Yao JL, Roy-Chowdhury S, Allison LA (2003) *AtSig5* is an essential nucleus-encoded Arabidopsis sigma-like factor. *Plant Physiol* 132: 739-747
FIGURE LEGENDS

**Figure 1.** Characterization of the Arabidopsis sig6-2 mutant. A, Genomic Sig6 region (At2g36990) showing exon/intron structure and T-DNA insertion site in exon 5, 1,022 nt downstream from the ATG start codon (upper line). The resulting cDNA with the fused exons but without the 5′ and 3′ UTRs is depicted below. Also given are features of the derived protein (TP, transit peptide; UR, unconserved region; 1 to 4, conserved regions for sigma activity). Genomic sequence and cDNA, but not T-DNA, are drawn to scale (scale bar on top). LB, left border; RB, right border; sul, sulfonamide (sulfadiazine) resistance gene. C, Wildtype (WT) and sig6-2 mutant phenotype during development (4, 8, 10, 12, 21 and 28 days after sowing). B, RT-PCR detection of sigma factor transcripts in wildtype (WT) and sig6-2 mutant. Total RNA was prepared from 6-d seedlings, reverse-transcribed, and cDNA was amplified using the gene-specific primer pairs as described in Materials and Methods.

**Figure 2.** Northern Blot Analysis. Total RNA (1 g per lane) from 4, 8, 10, 21 and 28-d stages of wildtype (WT) and mutant (MT) was gel-fractionated, blotted, and hybridized with DIG-labeled RNA probes. A, psbA, rbcL, trnV(UAC). B, atpB, atpE, ndhC. C, accD and clpP. Ethidium bromide-stained loading controls (25S rRNA) are shown at the bottom of each panel. Northern blot experiments were carried out at least three times with RNAs from independent preparations.

**Figure 3.** Real-time RT-PCR quantification of plastid RNAs. A, B, C, Transcript levels of the psbA, rbcL, and clpP genes, respectively, were determined in WT and sig6-2 by reverse transcription followed by quantitative PCR. Data are given as log2 of mutant/wildtype ratios with a mean from at least 3 independent experiments.

**Figure 4.** Plastid vs. nuclear DNA ratio in wildtype (WT) and mutant (sig6-2). A, young seedling (4 d). B, mature plant (28 d). Equal amounts of total DNA were digested with HindIII, electrophoretically separated, and hybridized with DIG-labeled probes for psbA (left panels) or nuclear 18S rDNA (right panels). DNA size markers (kb) are given in the left margins.
**Figure 5.** Complementation of the sig6-2 knockout mutant. A, T-DNA and AtSig6 detection by Southern blot hybridization of total genomic DNA from WT, MT, and C plants. Left panel: primary T-DNA insertion. DNA digested with EcoRV and hybridized with a sulf probe (see Figure 1). Central panel: secondary T-DNA insertion after re-transformation of sig6-2 knockout line. DNA digested with HindIII and hybridized with nptII (neomycin phosphotransferase II gene) probe. Right panel: AtSig6 detection. DNA digested with EcoRI and hybridized with Sig6-specific probe. Fragment sizes (kb) are given in the left margin of each panel. B, Transcript analysis using total RNA from wildtype (WT), sig6-2 (MT), and the sig6-2 complementation line (C). Upper left: RT-PCR products with AtSig6-specific primers. Left and right panel: RNA gel blot hybridization. The probes and transcript sizes (kb) are indicated in the left margins, and ethidium bromide-stained 25S rRNA is shown below each panel.

**Figure 6.** Model depicting the proposed dual role of AtSig6 in Arabidopsis development. Two distinct components of SIG6 activity (indicated by perpendicular bars separated by dashed lines) together determine its total activity (heavy-lined curve): "Early" role in young seedlings and "persistent" (long-term) role during subsequent development of seedlings and rosette-stage plants. The suggested early role is based on the observation that transcripts of "expression mode 1" such as those of psbA, rbcL, and the 0.6 kb trnV(UAC) transcript are strongly downregulated in young mutant seedlings at 4 days. Thereafter, they recover to almost wildtype amounts in 8- to 10-day mutant seedlings (Figure 2A). The persistent role relates to the continuous presence or absence of mutant-specific transcripts of "expression mode 2", including those from the atpB/E (4.8 and 2.6 kb) and trnV/ndhC region (3.0 kb) (Figure 2B). The trailing edge of the solid curve is thought to indicate overlap of functions. The region above the curve reflects (sigma-dependent) transcription activity mediated by SIG1 to 5, but also (sigma-independent) transcription by NEP.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6