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Nucleus-encoded plastid sigma factor SIG3 transcribes specifically the *psb*N gene in plastids

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ABSTRACT

We have investigated the function of one of the six plastid sigma-like transcription factors, sigma 3 (SIG3), by analysing two different *Arabidopsis* T-DNA insertion lines having disrupted *SIG3* genes. Hybridization of wild-type and *sig3* plant RNA to a plastid specific microarray revealed a strong reduction of the plastid *psb*N mRNA. The microarray result has been confirmed by northern blot analysis. The SIG3-specific promoter region has been localized on the DNA by primer extension and mRNA capping experiments. Results suggest tight regulation of *psb*N gene expression by a SIG3-PEP holoenzyme. The *psb*N gene is localized on the opposite strand of the *psb*B operon, between the *psb*T and *psb*H genes, and the SIG3-dependent *psb*N transcription produces antisense RNA to the *psb*T–*psb*H intergenic region. We show that this antisense RNA is not limited to the intergenic region, i.e. it does not terminate at the end of the *psb*N gene but extends as antisense transcript to cover the whole *psb*T coding region. Thus, by specific transcription initiation at the *psb*N gene promoter, SIG3-PEP holoenzyme could also influence the expression of the *psb*B operon by producing *psb*T antisense RNA.

INTRODUCTION

Plastids are semiautonomous plant organelles harbouring their own transcription system that originate from a cyanobacteria- and proteobacteria-like endosymbiote. The cyanobacteria-like ancestor of chloroplasts has contributed with a eubacteria-type RNA polymerase and the proteobacteria-like ancestor of mitochondria with a phage-type RNA polymerase to the transcriptional apparatus of higher plants (1–3). Transcription regulation of the plastid genome of higher plants is rather complex. In dicotyledon plants, two different phage type RNA polymerases (NEPs, nucleus encoded RNA polymerases, RPOTp and RPOTmp) and one eubacteria-type RNA polymerase (PEP, plastid encoded RNA polymerase) participate in the transcription of the ~120 genes that are encoded on the plastid genome. The PEP core enzymes is composed of four different subunits, α, β, β′ and β″, which are encoded on the plastid genome in two different transcription units. Genes coding the β, β′ and β″ subunits are arranged as operon analogous to the *rif* operon of *Escherichia coli*. The α subunit is encoded in a S10 or spe-like operon together with genes coding for ribosomal proteins [(4), reviewed in (5)].

The activity of the PEP core enzyme (α_2, β, β′, β″) is regulated by sigma-like transcription factors (SLFs), which have at first been characterized by *in vitro* transcription assays (6–8). The first cDNA sequences coding plant nucleus-encoded SLFs have been described much later (9–15) and finally six different sigma factors, SIG1–SIG6, have been described for *Arabidopsis thaliana* (16). The mRNAs of these SLFs are translated in the cytoplasm and corresponding proteins are subsequently imported as precursor proteins into the plastids. The functions of all these sigma factors are not yet completely elucidated. Besides of specificity in the recognition of different promoter regions, SLFs are also differentially expressed during plant development and plastid differentiation (17,18). Transcription of most of the sigma factor coding genes is under light control, but tissue/organ specific expression and regulation by circadian rhythm have also been described previously (11,13,19). In addition, regulation of PEP activity by phosphorylation either of SLFs or RNA polymerase subunits has been described (20,21).

In general, it seems that SLFs have overlapping as well as specific functions (15,22,23). Although overlapping functions have been demonstrated by *in vitro* transcription assays that are performed without competition by other sigma factors, the specific functions are more easily detected by analyses that reflect *in vivo* competition conditions, i.e. by characterization of specific sigma knock-out plants. Most of the results concerning the specific functions of plant sigma factors have been obtained by analyses of *Arabidopsis* T-DNA insertion mutants. From these results it can be concluded that a SIG2-PEP holoenzyme transcribes specifically some of the tRNA genes (24) and the *psa*J gene (25). SIG5 has been shown to play an important role in the recognition of the

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blue-light dependent promoter of the *psb*D gene (26–28) and might in addition have specific functions during embryogenesis (29). SIG6 plays a more general role during early plastid differentiation and plant development (30), and SIG4 is of specific importance for *ndh*F gene transcription (31). The functions of SIG1 and SIG3 *in vivo* have not yet been described.

From results obtained by *in vitro* transcription assays, it is suggested that the activity of SIG1 might be regulated by its interaction with additional protein(s) (32) and that the activity of SIG3 might be regulated by proteolytic cleavage (15,22). In the present paper, we have analysed the plastid gene expression pattern of an *Arabidopsis SIG3* T-DNA insertion mutant in order to characterize the function of SIG3 in plastid gene transcription.

MATERIALS AND METHODS

Isolation of SIG3 T-DNA insertion lines

Two different *A.thaliana* (ecotype Columbia, Co) *SIG3* T-DNA insertion lines have been obtained from the SALK collection (SALK_009166 and SALK_081321, named *sig3-2* and *sig3-4*, respectively). The T-DNA is inserted at the border of intron 1 and exon 2 in line *sig3-2* and within exon 4 in line *sig3-4*. None of the two lines was kanamycin resistant. The heterozygous *SIG3* insertion lines were at first backcrossed with wild-type (WT, Co.) plants two times in order to eliminate any other T-DNA insertion or mutations. Every generation resulting from self-pollination was analysed by PCR for the presence of the T-DNA insertion in the *SIG3* gene. Resulting homozygotes were isolated for both lines. The sequences of the primers that have been used for the characterization of the T-DNA lines are as follows:

1: 5′-GATGATACTGGTTGTGCCGCC-3′; 2: 5′-AACGGCAAGCACAAAGAGACG-3′; 3: 5′-TGCCAAAAGGTTCTTTGCCAG-3′; 4: 5′-GCGTGGACCGCTTGCTGCAACT-3′; 5: 5′-TTCAATTCGTTCCCCATTCCC-3′. PCRs have been performed as described previously (31).

Plant material and RNA isolation

Surface-sterilized *Arabidopsis* seeds were spread on MS agar plates, kept for 72 h at 4°C in darkness and then transferred into a growth chamber and grown for 6 days at 23°C under 16/8 h light/dark cycle at 110 μmol of photons m^{-2} s^{-1}. Total RNA was prepared from seedling as described in (23).

DNA microarray preparation

The *A.thaliana* plastid DNA microarray was constructed by spotting 60mer synthetic oligonucleotides that corresponded to sequences of 80 protein genes on nitrocellulose membranes. Oligonucleotides have been chosen within 200 nt sequences downstream of the ATG translation initiation codons. The spotting procedure was performed by Eurogentec (Belgium). Each DNA sample was spotted two times on a nitrocellulose membrane.

cDNA synthesis and array analyses

Total RNA was treated twice with DNase I (2 U/μg RNA) in order to remove traces of DNA. An aliquot of 4 μg of each RNA preparation have been labelled for microarray hybridization. RNA was reverse transcribed using specific primers corresponding to the 80 protein coding genes that we wanted to analyse on the microarray. Primers are localized as near as possible to the 3′ end of the 60mers that have been spotted onto the filters. The reaction was performed as described (31) in the presence of 100 μCi of [α-^{32}P]dATP (Amersham Bioscience) using Superscript II reverse transcriptase (Invitrogene). Samples were treated with RNase H at 37°C for 15 min and non-incorporated deoxyribonucleotides were removed by passage through Sephadex G50. An aliquot of each of the synthesized cDNAs was analyzed on a 6% denaturing polyacrylamide gel in order to verify the quality of the synthesized cDNA. Hybridization was performed under the same conditions as indicated for northern experiments, however, hybridization time was extended to 3 days. After 3 weeks exposure to Fujifilm Imaging Plates, the plates were analyzed using a Phosphoimager (Fujifilm FLA-8000) and the accompanying software. Background substraction was performed by application of the R Project for Statistical Computing (33) (http://www.R-project.org). Before calculating mean values and standard deviations, results from all independent experiments were normalized taking the highest-labelled experiment as reference.

Primer extension

Using isolated total DNA from *Arabidopsis* as template, the *clp*P, *atp*H, *psb*N and *psb*T promoter regions have been PCR amplified and cloned into pCR^{R}2.1-TOPO^{R} (Invitrogen) with the following primers: 5′-CCAATATGCAATGGGGG-3′ and 5′-GTATCTCCTTCTCCAGG-3′ (*clp*P), 5′-GGATAGGAACTACTATC-3′ and 5′-GTCCAATAGAAGCAAGC-3′ (*atp*H, −44), 5′-GTGAGTCTATGGAAGGTC-3′ and 5′-CGCTAAGATTAATCCAGCC-3′ (*atp*H, −413), 5′-CATCCAGTAGAACAGAAG-3′ and 5′-CTCTTAGTTGTTGAGAGG-3′ (*psb*N) and 5′-AGGTTGGTTTACGTTTG-3′ and 5′-TCATTTTTTAGTTGAAATTTT-3′ (*psb*T). Primer extension experiments have been performed as described (31) using 10 μg of total RNA. The following primers have been used for primer extension and to establish the accompanying sequence ladders: 5′-GTATCTCCTTCTCCAGG-3′ (*clp*P), 5′-GTCCAATAGAAGCAAGC-3′ (*atp*H), 5′-CAAAGGCAGTATATAGAGC-3′ (*psb*N), 5′-GAAATTTTAGGTGGTTC-3′ (*psb*T) and 5′-ATGGAAGCATTGGTTTATAC-3′ (*psb*N/*psb*T-antisense).

Capping

In vitro capping reactions were performed in a final volume of 30 μl, using 15 μg of total RNA and 10 U of guanylyltransferase (Ambion), in the presence of 100 μCi of [α-^{32}P]GTP (3000 Ci/mmol) and 20 U of RNase inhibitor. The reactions were incubated at 37°C for 1 h. RNAs were purified with phenol/chloroform extractions and precipitated with 3 vol of ethanol. Transcripts were then hybridized to 5 ng of complementary riboprobe and subjected to ribonuclease protection assay, using the RPA III^{TM} Ribonuclease Protection Assay Kit (Ambion), according to the manufacturer's protocol.

5′-RACE

The discrimination between transcription start sites and processing sites of precursor RNAs was done by RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE Kit; Ambion) without and with previous TAP treatment of RNAs. Reactions were performed according to the supplier's protocol but without removal of free 5′-phosphates by calf intestine alkaline phosphatase. PCR products were analysed on agarose gels after two successive PCR amplifications, the first using two outer primers and the second using two inner primers. Primers are as follows: *psb*T-as outer: 5′-ATGGAAGCATTGGTTTATACATTTC-3′, *psb*T-as inner: 5′-CGGGAACCACCTAAAATTTCAAC-3′, *psb*N outer: 5′-CTCTTAGTTGTTGAGAGG-3′ and *psb*N inner: 5′-CCC-AAAGGCAGTATATAGAC-3′. The inner and outer Adapter primers are those of the RLM-RACE Kit.

Northern

For northern blot hybridization, PCR fragments have been ^{32}P-labelled by random priming (*psb*H, *psb*N, *psb*B and *rbc*L) or riboprobes have been generated by T7 RNA polymerase (*psb*T). The gene-specific PCR fragments have been obtained with the following primers: 5′-GGCTACACAA-ACTGTTGAAG-3′ and 5′-CTAATTCACTGAAATTCCAT-CC-3′ (*psb*H), 5′-GGAAACAGCAACCCTAGTCGCC-3′ and 5′-CCCCGTGTTCCTCGAATGGATC-3′ (*psb*N), 5′-GGT-CCTGGAATATGGGTATCC-3′ and 5′-GCCCCGAATTC-CACTTGAGC-3′ (*psb*B), 5′-AACCAAGGATACTGATAT-CTTGGC-3′ and 5′-ATCGTCCTTTGTAACGATCAAGGC-3′ (*rbc*L). Prehybridization (1 h at 65°C) and hybridization (24 h at 65°C) were performed in 0.5 M $NaHPO_4$, pH 7. 2, 1 mM EDTA, 7% SDS and 1% BSA. After hybridization filters were washed in 40 mM $NaHPO_4$, pH 7. 2, 1 mM EDTA and 7% SDS at room temperature for 10 min followed by washing at 65°C for 5 min.

The *psb*T PCR fragment has been cloned into pCR2.1-TOPO vector (Invitrogen), the construct was linearized by BamHI and transcribed using T7 RNA polymerase in order to obtain a *psb*T antisense riboprobe. Hybridization of the *psb*T antisense RNA was performed overnight in 50% formamide, 5× SSC, 5× Denhardt and 1% SDS at 60°C. After hybridization, filters were washed twice in 0.2× SSC containing 0.1% SDS at 42°C for 10 min.

RESULTS

Isolation of SIG3 T-DNA insertion lines

Two different *A.thaliana* SIG3 T-DNA insertion lines have been obtained from the SALK collection (named here *sig3-2* for SALK_009166 and *sig3-4* for SALK_081321). The positions of the T-DNA insertions in the *SIG3* gene are indicated in Figure 1A. Homozygous plants were selected for each of the two lines by PCR analyses using one primer pair that amplifies the border between T-DNA and the *SIG3* gene (primers 2 and 5 for *sig3-2* and primers 3 and 5 for *sig3-4*) and one primer pair that amplifies part of the SIG3 gene (primers 1 and 2 for *sig3-2* and primers 3 and 4 for *sig3-4*). Figure 1B shows the PCR analyses obtained after cleaning of the mutants by two successive backcrosses (see Materials and Methods). Results reveal three homozygous plants for *sig3-2* (Figure 1B, lanes 1/7, 3/9 and 4/10) and one homozygous plant for *sig3-4* (Figure 1B, lanes 2/8). These plants have been propagated and their descendents have been analysed in the following experiments. Figure 1C shows the visible phenotypes of WTand mutant plants (*sig3-4*) when grown at 23°C under 16/8 h light/dark cycle at 110 μmol of photons m^{-2} s^{-1}, i.e. visible phenotypes are not different. We have also compared different light conditions, varying from 50 to 200 μmol of photons m^{-2} s^{-1}, using light/dark cycle or continuous illumination. Up to now, we could not detect visible differences between phenotypes of WT and *sig3* (*sig3-2* and *sig3-4*) plants (data not shown). If not otherwise indicated, in all following experiments *sig3-4* plants have been analysed.

Analyses of plastid gene expression in the SIG3 T-DNA insertion mutants

In order to characterize the function of SIG3 in plastid gene expression, we performed an overall transcript profiling by microarray hybridization. Transcript levels of 6-day-old *SIG3* insertion mutants corresponding to 80 ORFs of the *A.thaliana* plastid genome (see Materials and Methods) were compared to that of WT plants. Three independent experiments have been made. Normalized average values (*sig3*/WT) and standard deviations are summarized in Table 1 (Supplementary Table S1 is at available at *NAR* online). If the commonly used threshold value of 0.66 (1/1.5) is applied (34), only two mRNAs, *psb*N and *atp*H, are significantly reduced in the *sig3* plants thus representing valuable candidates for specific transcription by a SIG3-PEP holoenzyme. These two mRNAs have been analysed in more detail by primer extension in order to determine the promoter region for specific SIG3 dependent transcription (Figure 2A and B).

The determination of 5′ ends of precursor RNAs by primer extension shows that *psb*N mRNA is transcribed from a single PEP promoter (Figure 2A, lanes 5 and 6). Transcription initiation at position −32 was confirmed by *in vitro* capping of *psb*N mRNA (Figure 2A, lanes 7–9). The upstream DNA sequence of the transcription start site harbours −35 and −10 sequences reminiscent of prokaryotic-type promoter elements (Figure 2D, upper lane). The *psb*N transcript is strongly reduced in the *sig3* plants, thus indicating that the *psb*N transcription is under specific control of SIG3. This experiment was made with the two different *sig3* mutants, *sig3-2* and *sig3-4*, and in both cases we obtained the same result (data not shown). This shows that the lack of *psb*N mRNA in the two mutants is indeed caused by the lack of sigma factor 3 and not by an additional, undetected, mutation.

The *atp*H gene yields two different transcripts. One of them ends up at position −44 from the ATG translation initiation codon (Figure 2B, lanes 1 and 2). This mRNA should result from transcription by NEP, as its upstream sequence does not reveal recognizable prokaryotic-type promoter elements. The exact position of the longer mRNA in Figure 2B could not be determined since the accompanying sequence ladder could not be read up to this point. Therefore, we have cloned the DNA sequence upstream of the −44 RNA and repeated the primer extension analysis using a primer that is close enough to determine the 5′-end of the long *atp*H transcript (Figure 2B, lanes 11 and 12). The upstream DNA

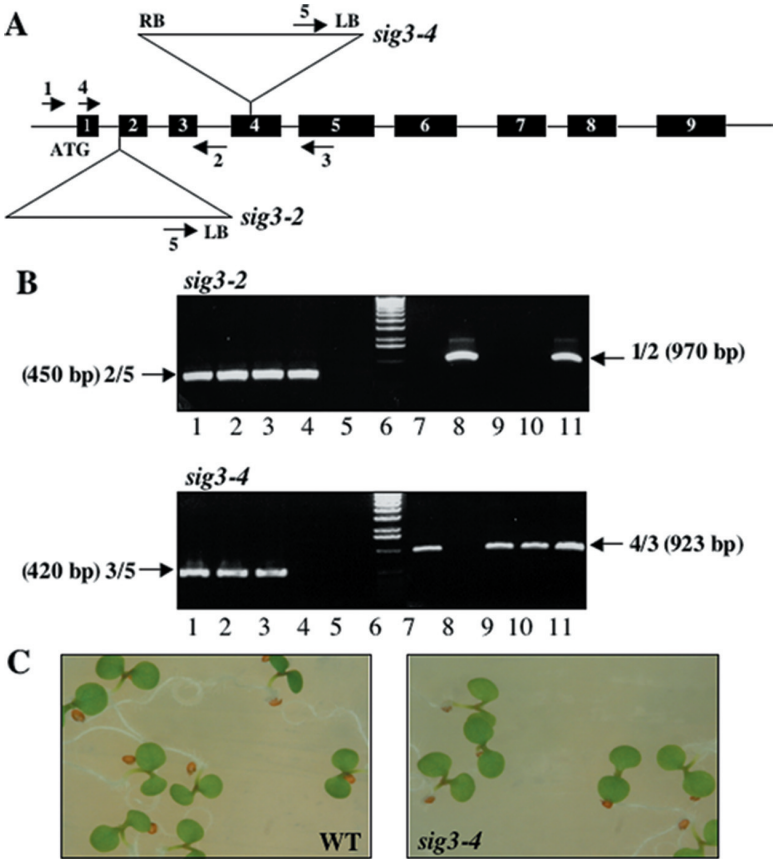

Figure 1. Characterization of two different *SIG3* T-DNA insertion mutants. (**A**) Schematic representation of the T-DNA insertions and the location of the primers that have been used for PCR analyses. (**B**) Selection of homozygous lines for *sig3-2* and *sig3-4* after the second backcross. Total DNA was prepared from five individual F_1 lines obtained by self-pollination of heterozygous plants after the second backcross. The presence of the T-DNA was verified by PCR using primers 2 and 5 (*sig3-2*, lanes 1–5) or primers 3 and 5 (*sig3-4*, lanes 1–5). The presence of WT DNA was analysed by PCR using primers 1 and 2 (*sig3-2*, lanes 7–11) or primers 4 and 3 (*sig3-4*, lanes 7–11). (**C**) WT and homozygous *SIG3* T-DNA insertion mutants grown on agar plates under 16 h light/8 h dark cycle for 1 week.

sequence of the long transcript starting at position −413 is reported in Figure 2D, lower lane. This long RNA should result from specific transcription by SIG3-PEP holoenzyme because this RNA is completely absent in *sig3* plants (Figure 2B, lane 12). Altogether, these results show that the *atp*H gene is under two-promoter/two-RNA polymerase control, and one of the two promoters is recognized specifically by a SIG3-PEP holoenzyme. As control for an mRNA whose expression does not change, i.e. it is not under control of SIG3, we have analysed also *clp*P mRNAs by primer extension. The predominant −53 precursor RNA that is produced by NEP (35) is present in equal amount in WT and *sig3* plants (Figure 2C, lanes 4 and 5).

The lack of *psb*N mRNA does not change the processing of the *psb*B operon

The *psb*N mRNA represents naturally occurring antisense RNA to the *psb*B polycistronic mRNA (36). Therefore, we wanted to know whether the strong reduction of the *psb*N mRNA in the *sig3* plants influences the processing of the polycistronic transcript of the *psb*B operon which consists of *psb*B, *psb*T, *psb*H, *pet*B and *pet*D mRNAs (37,38). The organization of the *psb*B operon is schematically represented in Figure 3A.

At first we have analysed the intermediary RNA species that result from processing of the long co-transcript by northern analyses (Figure 3B). By using probes that correspond to the *psb*B, *psb*T and *psb*H genes northern analysis do not show remarkable differences between WT and *sig3* RNAs (Figure 3B). The *rbc*L mRNA is analysed because the microarray analyses for this gene had a high average value and a high standard deviation (see Table 1). The *rbc*L probe had been spotted in one of the corners of the nitrocellulose membrane that had been utilized to handle the membrane. Background hybridization was very high in some experiments and the microarray results are not conclusive. Therefore, we have analysed the *rbc*L transcripts by northern hybridizations. As shown in Figure 3B, the *rbc*L mRNA level does not change significantly in *sig3* plants. The *psb*N mRNA has been analysed in order to verify the microarray result. The result confirms that *psb*N transcription is indeed very much reduced in the *sig3* mutant.

The *psb*T sense and antisense mRNAs were analysed by primer extension, a method that is more sensitive than northern analysis (Figure 4). The localizations of the primers that have been used in these experiments is schematically indicated in Figure 4A. Primer extension analysis using primer (1), i.e. analysing *psb*T-antisense RNA, yields two different RNAs (∼330 and ∼140 bases) that are both absent in *sig3*

Table 1. Transcript analysis of plastid genes in *sig3* compared with WT plants

Gene name	Ratio *sig4*/WT	Gene name	Ratio *sig4*/WT	Gene name	Ratio *sig4*/WT
psbN	0.49 ± 0.46	ndhE	0.96 ± 0.25	petN	1.08 ± 0.21
atpH	0.64 ± 0.20	psaJ	0.97 ± 0.09	rps12	1.09 ± 0.33
atpA	0.72 ± 0.17	psbI	0.97 ± 0.15	rpl16	1.09 ± 0.03
atpE	0.75 ± 0.20	petL	0.97 ± 0.18	rpl22	1.09 ± 0.13
atpF	0.77 ± 0.32	psbT	0.98 ± 0.25	ycf2	1.09 ± 0.24
rpoC1	0.79 ± 0.13	ndhF	0.98 ± 0.22	rps 8	1.11 ± 0.04
rps18	0.80 ± 0.26	rpoC2	1.00 ± 0.45	ycf5	1.11 ± 0.45
rpoB	0.80 ± 0.52	psbK	1.00 ± 0.12	ndhB	1.12 ± 0.05
ndhH	0.85 ± 0.28	psbB	1.02 ± 0.36	rps4	1.12 ± 0.26
petA	0.87 ± 0.09	Rps7	1.02 ± 0.30	psbM	1.12 ± 0.43
rpl36	0.88 ± 0.11	clpP	1.02 ± 0.37	ndhJ	1.13 ± 0.41
psaC	0.91 ± 0.17	psbJ	1.02 ± 0.27	psaA	1.14 ± 0.43
rps14	0.91 ± 0.12	ndhD	1.02 ± 0.27	ycf1	1.15 ± 0.47
petB	0.91 ± 0.15	matK	1.03 ± 0.22	rps19	1.15 ± 0.50
atpB	0.91 ± 0.11	accD	1.03 ± 0.17	psbZ	1.17 ± 0.13
psbC	0.92 ± 0.33	rps14	1.04 ± 0.15	rpl33	1.18 ± 0.09
petG	0.92 ± 0.10	ndhG	1.04 ± 0.20	ndhK	1.20 ± 0.26
petD	0.92 ± 0.09	rpl20	1.04 ± 0.23	psbL	1.21 ± 0.25
cemA	0.92 ± 0.10	rpL23	1.05 ± 0.04	rps16	1.24 ± 0.64
rps11	0.92 ± 0.33	Rps15	1.05 ± 0.30	rps3	1.24 ± 0.26
ndhC	0.93 ± 0.26	rps2	1.05 ± 0.10	psbH	1.31 ± 0.05
psbE	0.94 ± 0.05	Ycf4	1.05 ± 0.54	ycf3	1.33 ± 0.31
rpl32	0.94 ± 0.22	psaI	1.05 ± 0.49	psbF	1.45 ± 0.39
rpl2	0.94 ± 0.15	psaB	1.07 ± 0.09	psbD	1.50 ± 0.63
rpoA	0.94 ± 0.14	ndhA	1.07 ± 0.27	psbA	2.07 ± 1.03
ndhI	0.96 ± 0.03	atpI	1.08 ± 0.15	rbcL	2.07 ± 1.44

Values have been obtained from three independent experiments, each one performed in two replicates.

plants (Figure 4B, left-hand side). From this result we can conclude that both of these RNAs are under the control of SIG3. The shorter of the two transcripts results from processing and the longer one is a primary transcript, as shown by 5′-RACE without or after treatment of mRNAs with tobacco acid pyrophosphatase [TAP, (39)] (Figure 4B, left-hand side, lanes 7 and 8). This means that the longer RNA corresponds to a *psb*N/*psb*T-antisense co-transcript, and the shorter one (i.e. the *psb*T-antisense RNA) results from processing of the long *psb*N/*psb*T-antisense co-transcript. The shorter RNA ends up in the intergenic region, between *psb*T and *psb*N. The exact cleavage site is shown in Figure 4C (upper lane). The processing event separates the *psb*T-antisense RNA from the *psb*N mRNA. Altogether, this experiment shows that transcription from the *psb*N promoter produces antisense RNA that covers the whole *psb*T reading frame and whose production is dependent on sigma factor 3.

Primer extension using primer (2) reveals two different RNAs having their 5′ ends located either in the intergenic region of the *psb*B and the *psb*T genes (several transcripts ∼154 bases) or within the coding region of the *psb*B gene (∼345 bases). Both RNAs are present in WT as well as in *sig3* plants (Figure 4B, right-hand side, lanes 5 and 6). The ∼154 bases transcripts diverge between the last 6 nt at the 5′ end. These RNAs could be processing intermediates of the larger RNA. But the sequence upstream of these mRNAs reveals two possible NEP promoter consensus sequences, underlined in Figure 4C, that could also be responsible for transcription initiation of RNAs starting at different, closely situated, sites.

DISCUSSION

In the present paper, we have analysed plastid gene expression in two different SIG3 T-DNA insertion mutants that had been obtained from the SALK collection. Plastid mRNAs have been characterized by three different methods. At first, RNA levels of all plastid mRNA coding genes have been monitored by hybridization on a plastid specific microarray and in a second step, selected mRNAs have been analysed in more detail either by northern or by primer extension experiments. In each analysis, RNAs prepared from *sig3* mutants have been compared to RNAs obtained from WT plants.

Microarray analysis revealed only two significantly reduced mRNAs in *sig3* plants, i.e. *psb*N and *atp*H (Table 1). These two mRNAs have been further characterized by primer extension, a method that allows localization of the 5′ ends of the corresponding precursor RNAs (Figure 2A and B). This analysis shows that the *psb*N gene is under control of a single promoter that is specifically recognized by SIG3-PEP holoenzyme. The promoter sequence of the SIG3-specific PEP promoter of *psb*N is shown in Figure 3 (upper line). The −10 sequence is remarkably rich in adenine, while the −35 element harbours the consensus TTG triplet. On the other hand, the *atp*H gene is transcribed from two different promoters. The presence of two different promoters explains why the *atp*H mRNA is less reduced in *sig3* mutants than the *psb*N mRNA. Only the minor, longer, *atp*H RNA (−413) is under control of SIG3-PEP holoenzyme.

The comparison of the two different SIG3-specific promoter sequences reveals similarities in an extended −35 region, in the region between the −10 and −35 consensus elements and immediately upstream of the transcription start site. The −10 consensus element is less conserved (Figure 2D). The question of what makes the specificity of SIG3 to these promoters cannot be answered by simple sequence comparison and represents a challenge for future work. The same holds true for other sigma factor/promoter interactions such as SIG2/*psa*J (25), SIG2/*trn*E-V-M and Q (24), SIG4/*ndh*F (31) and SIG5/*psb*D BLRD (27). At a first glance, the specificity of SIG3 reported here for the *psb*N gene promoter seems to be in contradiction with previous publications showing recognition of the *psb*A and *rbc*L promoters by SIG3 (15,23). However, these experiments have been made using *in vitro* transcription conditions without sigma competition. Similar results showing sigma specificity *in vivo* under competitive conditions and apparently less specificity under non-competitive conditions are also reported for SIG2 with respect to the *psb*A promoter (15,23,25,27). In addition to redundant action of sigma factors under non-competitive conditions we can also not exclude that additional SIG3-specific promoters exist in the multiple promoter regions that precede many of the plastid transcription units. Some of these promoters might be more easily revealed in other developmental or environmental situations than used in our studies. On the other hand, the absence of one sigma factor might be compensated by overexpression of other sigma factors that might initiate from another PEP promoter within the same promoter region thus compensating the lack of one sigma factor for the overall transcription of a given gene. In such case, no change will be detectable by microarray analysis.

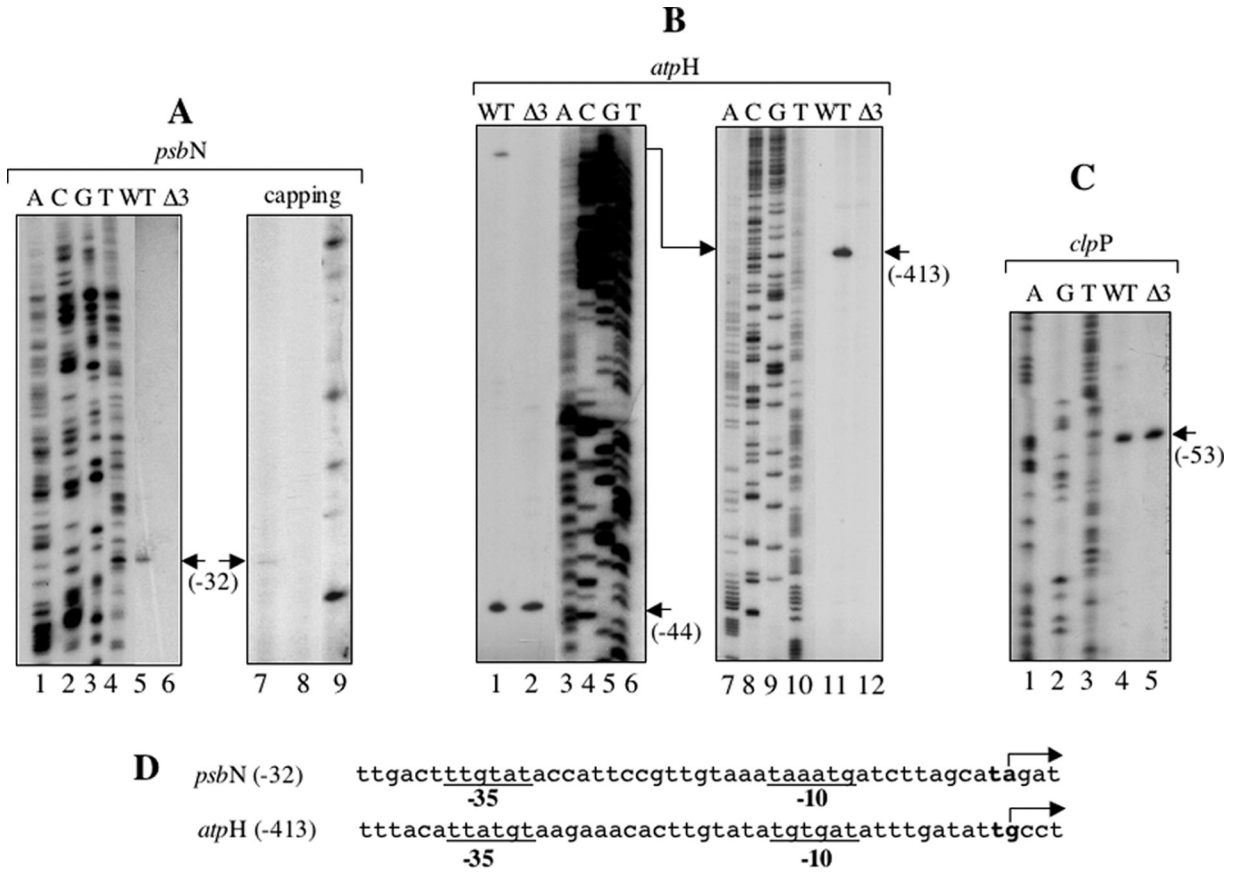

Figure 2. Analyses of *psb*N, *atp*H and *clp*P precursor RNAs. Total RNA was prepared from 6-day-old *Arabidopsis* WT and *sig3* (Δ3) plantlets, RNA was either reverse transcribed and cDNAs were separated on 6% denaturing polyacrylamide gels (*psb*N, *atp*H and *clp*P) or in addition 5′-labelled by guanylyltransferase and analysed after RNase protection (*psb*N). The accompanying sequence ladders have been prepared using the same primers as for primer extension. (**A**) Primer extension analysis of *psb*N mRNA from WT (lane 5) and *sig3* (lane 6) plantlets and analysis of the −32 transcript by *in vitro* capping (lanes 7–9). Capped RNA was analysed either directly (lane 9) or after RNase digestion without (lane 8) and after prior hybridization to *psb*N complementary riboprobe (lane 7). (**B**) Primer extension analyses of *atp*H mRNA prepared from WT and *sig3* (Δ3) plantlets using a primer that is located within the *atp*H coding region (lanes 1 and 2) or a primer located within the 5′-non-coding region (lanes 11 and 12). (**C**) Primer extension analysis of *clp*P mRNA from WT (lane 4) and *sig3* (lane 5) plantlets. (**D**) The nucleotide sequences upstream of the *psb*N (−32) and, *atp*H (−413) precursor RNAs are shown. Transcription start sites are indicated by arrows and by boldface in the sequences. The −10 and −35 consensus sequences of the SIG3-specific *psb*N and *atp*H promoters are underlined.

Under our experimental conditions, there is only one gene on the plastid genome whose expression is remarkably reduced when SIG3/PEP is lacking. This is *psb*N. The function of the PsbN protein is still unknown. On the basis of antibody experiments it had been shown that PsbN is localized on the thylakoid membranes (40). However, the suggestion that PsbN represents one of the small proteins of photosystem II (PSII) has been recently contradicted by systematic sequencing of all PSII centre proteins (41). We have grown *sig3* plants under various light conditions (50–200 μmol of photons m^{-2} s^{-1}, 8 h/16 h dark/light cycle or continuous light), but we did not observe changes in the visible phenotype when compared with WT plants. The question of why evolution has conserved one sigma factor to regulate specifically the transcription of the *psb*N gene cannot be answered at the moment. The *psb*N gene expression might serve a regulatory function for the expression of the *psb*B operon that is located on the opposite DNA strand (see Figure 3A). The expression of the *psb*B operon has been extensively studied in *Euglena gracilis* (42), *Chlamydomonas reinhardtii* (43,44) and *Arabidopsis* (45–47). In fact, two different 5′ leader segments have been described in *Arabidopsis* for processed *psb*H transcripts, one of them overlapping with the 5′ leader of the *psb*N transcript (45). The *psb*N mRNA might therefore influence the processing of the *psb*H mRNA. To verify this hypothesis we have analysed the expression pattern of the *psb*B operon in WT and *sig3* plants by northern hybridization and by primer extension (Figures 3B and 4B). Using these two methods, we could not detect changes in the processing pattern of the *psb*B operon in *sig3* plants. Equally, western blot analyses of protein extracts prepared from WT and *sig3* plants using commercially available antibodies against PsbB and PsbH proteins do not reveal remarkable differences (data not shown). Unfortunately, antibodies against the PsbT and PsbN proteins are not yet available in order to test also these two proteins.

Primer extension using a primer that starts at the ATG of the *psb*T gene to detect *psb*T antisense transcripts (primer 1 in Figure 4A and B) shows that the *psb*N mRNA extends at it 3′ end at least up to the beginning of *psb*T thus covering the entire *psb*T gene as antisense RNA. Both mRNAs, *psb*N and *psb*T, could therefore be regulated by antisense RNA.

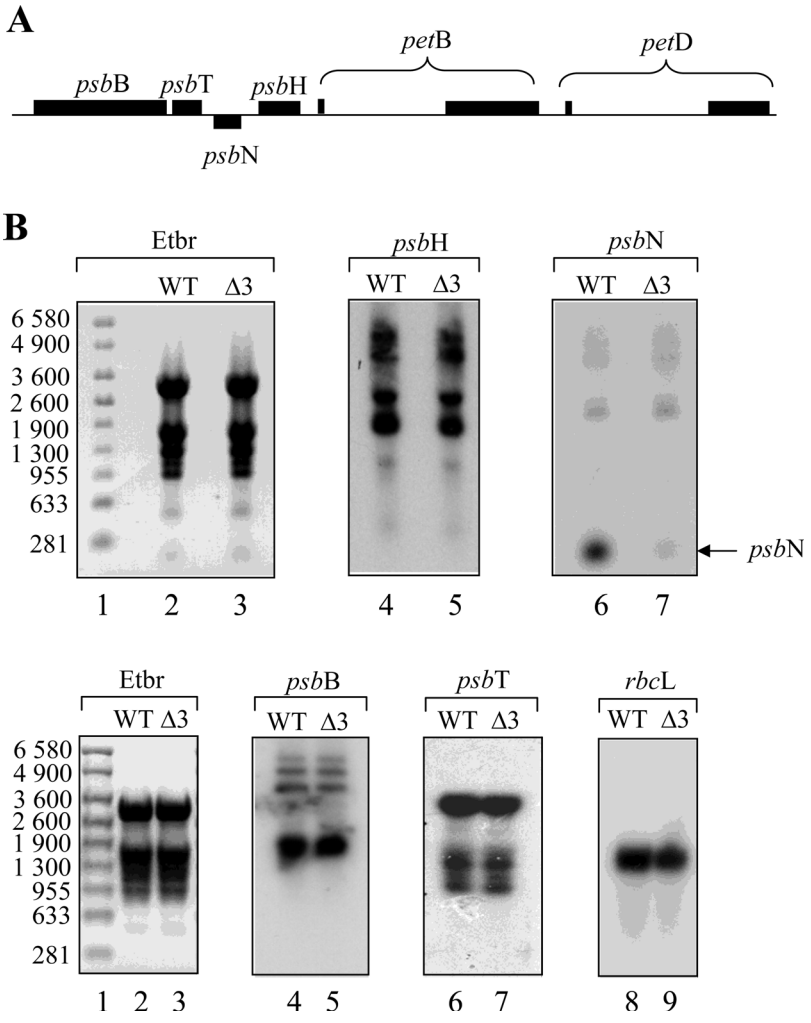

Figure 3. Analysis of the expression of the *psb*N gene and of the *psb*B operon by northern experiments. (**A**) Schematic representation of the *psb*B operon. (**B**) Gel blot hybridization of WT and *sig3* (Δ3) RNAs. Total RNA (10 μg) of 6-day-old plantlets have been separated on denaturing agarose gels and stained directly by ethidium bromide (lanes 2 and 3, upper and lower panels) or RNA has been transferred to nitrocellulose and hybridized with probes corresponding to *psb*H (lanes 4 and 5, upper panel), *psb*N (lanes 6 and 7, upper panel), *psb*B (lanes 4 and 5, lower panel), *psb*T (lanes 6 and 7, lower panel) and *rbc*L (lanes 8 and 9, lower panel). Hybridization probes correspond to ^{32}P-labelled PCR fragments (*psb*H, *psb*N, *psb*B and *rbc*L) or to complementary riboprobe (*psb*T). Molecular weight markers are shown on the left-hand side.

Noteworthy, by specific transcription of the *psb*N gene, SIG3 could also regulate the expression of the *psb*B operon by producing *psb*T antisense RNA.

Primer extension analysis of the *psb*T sense RNAs revealed a yet unknown *psb*T transcript whose 5′ end is located within the coding region of *psb*B (∼345 bases, Figure 4A and B). It would be interesting to investigate whether this RNA results from transcription initiation or from processing. Initiation of the *psb*T mRNA within the *psb*B coding region would mean that under certain conditions both genes, *psb*B and *psb*T, need to be transcribed separately. On the other hand, processing of the polycistronic mRNA within the *psb*B coding region means inactivation of the *psb*B mRNA. Thus, our results indicate an additional, so far unknown, mode of regulation of the *psb*B operon, concerning especially the two proximal genes, *psb*B and *psb*T. The shorter *psb*T sense transcript (∼154 bases, Figure 4A and B) ends up within the intergenic region between the *psb*B and *psb*T genes. The 5′ ends of these transcripts extend over six bases that are located downstream of two different potential NEP promoters. Thus, although cleavage of a dicistronic *psb*B-*psb*T mRNA is expected as part of processing of the polycistronic *psb*B precursor RNA we cannot exclude that the shorter *psb*T mRNA is produced by transcription initiation. It has recently been shown that antisense transcripts stabilize polyadenylated mRNA in chloroplasts (48). Therefore, it would be likely to assume that the absence of *psb*N/*psb*T-antisense RNA in *sig3* plants destabilized the *psb*T sense transcript. However, the quantity of the ∼154 bases *psb*T sense transcripts in *sig3* plants is not significantly different from WT plants. Thus, regulation by antisense stabilization is not applicable at least not under the growth conditions that have been routinely used in the present work (i.e. 110 μmol of photons m^{-2} s^{-1}). Experiments are actually in progress to analyse quantitative changes in *psb*N and *psb*T sense and antisense RNAs during plant growth and development and under specific stress conditions. We hope that these experiments will provide an idea under which conditions *psb*T antisense RNA regulation might be

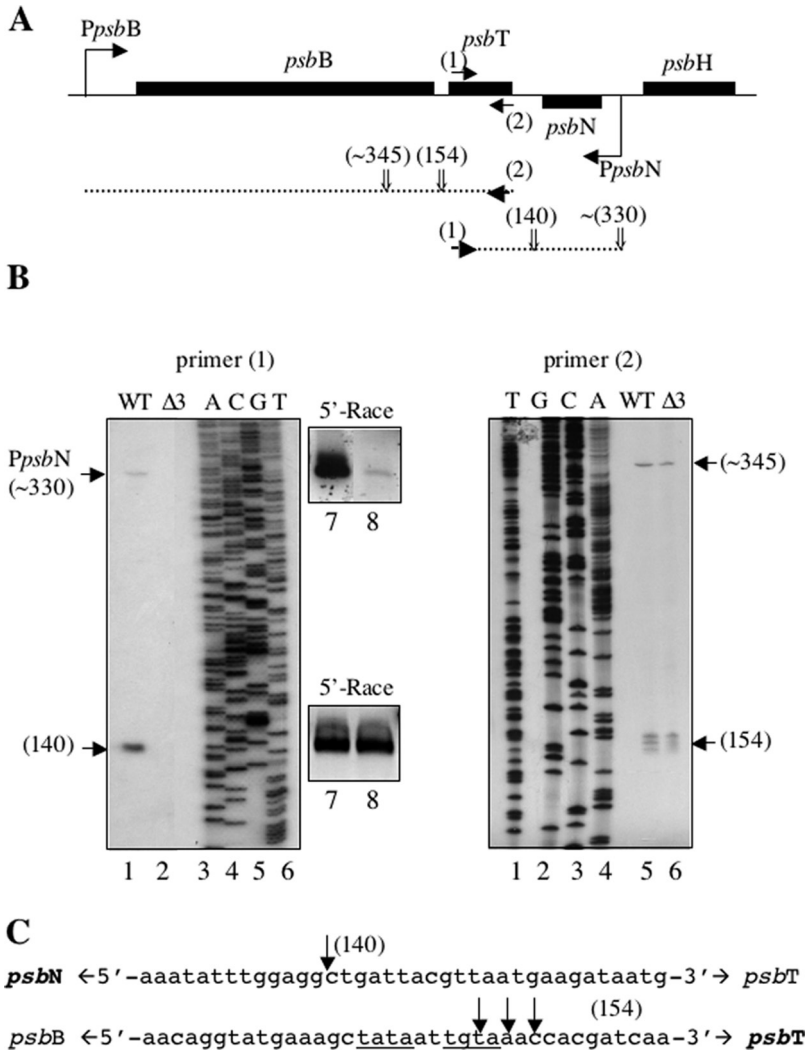

Figure 4. Transcript patterns of the *psb*T sense and antisense transcripts. (**A**) Schematic representation of part of the *psb*B operon. Primers that have been used for primer extension analyses are indicated by horizontal, closed, arrows and by numbers. RNAs that have been revealed by primer extension are indicated by dotted lines and processing sites are labelled by vertical, open, arrows. The unique *psb*B and *psb*N promoters are indicated as PpsbB and PpsbN. (**B**) Primer extension analyses of *psb*T sense and antisense RNAS. The *psb*T antisense RNA has been analysed using primer (1) and the *psb*T sense RNA (mRNA) has been analysed using primer (2). The sequence ladders have been established using the same primers as for primer extension. The 5′ ends of the 140 and ~330 bases *psb*N transcripts have been analysed by 5′-RACE after (lane 7) and without (lane 8) TAP treatment of mRNAs. (**C**) Nucleotide sequences surrounding the cleavage sites between *psb*N sense and *psb*T-antisense RNA and the 5′ end(s) of the shorter *psb*T mRNAs. Numbers indicate the distance from the 5′ end of the primers.

important in plastids. The *psb*T protein has been shown to play a role in dimerization of PSII (49) and to be required for efficient repair of photodamaged PSII reaction centre (50). Environmental conditions that lead to photodamage of PSII might therefore be connected to a specific induction of PsbT protein expression, regulated by transcription from independent promoter(s) and/or by antisense RNA. It would be interesting to test such a hypothesis in the future. The *sig3* plants described here represent a powerful means to investigate on such type of regulation of the *psb*B operon, but also to elucidate the function of the psbN protein.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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