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Specific function of a plastid sigma factor for *ndh*F gene transcription

Jean-Jacques Favory, Masanori Kobayshi[1], Kan Tanaka[1], Gilles Peltier[2], Martin Kreis[3], Jean-Gabriel Valay and Silva Lerbs-Mache*

Laboratoire Plastes et différenciation cellulaire, Université Joseph Fourier and Centre National de la Recherche Scientifique, B.P. 53, 38041 Grenoble, France, [1]Laboratory of Molecular Genetics, Department of Molecular Biology, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan, [2]CEA Cadarache, Direction des Sciences du Vivant, Laboratoire d'Ecophysiologie de la Photosynthèse, Département d'Ecophysiologie Végétale et de Microbiologie, Unité Mixte de Recherche 6191 CNRS-CEA-Université de la Méditerranée,13 108 Saint Paul lez Durance, France and [3]Institut de Biotechnologie des Plantes, Université de Paris Sud, 91405 Orsay, France

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ABSTRACT

The complexity of the plastid transcriptional apparatus (two or three different RNA polymerases and numerous regulatory proteins) makes it very difficult to attribute specific function(s) to its individual components. We have characterized an Arabidopsis T-DNA insertion line disrupting the nuclear gene coding for one of the six plastid sigma factors (SIG4) that regulate the activity of the plastid-encoded RNA polymerase PEP. This mutant shows a specific diminution of transcription of the plastid *ndhF* gene, coding for a subunit of the plastid NDH [NAD(P)H dehydrogenase] complex. The absence of another NDH subunit, i.e. NDHH, and the absence of a chlorophyll fluorescence transient previously attributed to the activity of the plastid NDH complex indicate a strong down-regulation of NDH activity in the mutant plants. Results suggest that plastid NDH activity is regulated on the transcriptional level by an *ndhF*-specific plastid sigma factor, SIG4.

INTRODUCTION

The plastid genome of higher plants encodes ~120 genes mostly organized into polycistronic transcription units forming altogether ~35 transcription units. These few transcription units are transcribed by two different RNA polymerases, named PEP (**P**lastidial-**E**ncoded RNA **P**olymerase) and NEP (**N**ucleus-**E**ncoded RNA **P**olymerases) [reviewed in (1,2)]. NEP enzymes are monomeric and of the phage-type (3–5). PEP represents a multimeric, prokaryotic-type enzyme. Its activity is regulated by nucleus-encoded sigma-type transcription initiation factors (6,7).

NEP enzymes are constitutively expressed (8) and perform overall transcription of the whole plastid genome (9). In photosynthetically active tissues this basic overall transcriptional activity is overlaid by PEP transcriptional activity that plays a predominant role in the transcription of photosynthesis-related genes (10). The switch from predominant NEP to predominant PEP activity during plant development might be regulated by glutamyl-tRNA (11). In *Arabidopsis*, cDNAs encoding six different sigma-like transcription factors that could be involved in the regulation of plastid transcription have been isolated and sequenced (12–14).

In adaptation to this multiple polymerase transcription system, many of the plastid genes or transcription units are preceded by NEP as well as PEP promoters thus allowing transcription by NEP as well as PEP enzymes or specific regulation of transcription by NEP/PEP competition for a promoter region containing overlapping promoters. Well-characterized examples for such multiple promoter regions are those directing the *psb*D-*psb*C, *rrn*, *atp*B, *atp*I and *clp*P genes (15–21). Single promoters are rare. Two of them, *rbc*L and *psb*A, are relatively well characterized. It has been shown that they can be recognized by several sigma factors having overlapping specificity (22–24) and by several RNA polymerase forms (25–28). In addition to production from heterogeneous promoters, quantitative differences in NEP and PEP transcripts are probably related to quantitative changes between NEP/PEP enzymes and/or enzyme activities (8,21). Differential turnover of NEP and PEP originating transcripts has also been described previously (29).

*To whom correspondence should be addressed. Tel: +33 04 76 63 57 44; Fax: +33 04 76 63 55 86; Email: Silva.Lerbs-Mache@ujf-grenoble.fr

This complexity of plastid gene expression makes it particularly difficult to recognize and to follow specific events on the transcriptional level and to determine the components that are engaged in specific gene expression on a molecular level. Pools of different transcripts corresponding to each gene have to be dissected and each promoter of a multiple promoter region should be characterized individually.

Break-through in revealing specificity of transcription in plastids came only recently from characterization of *Arabidopsis* sigma knock-out mutants. Based on these analyses, it could be shown that the transcription of several tRNA genes (*trn*V, *trn*M and *trn*E) is under predominant control of AtSIG2 (30,31) and that transcription initiation at the *psb*D light-responsive promoter (32,33) is mediated by AtSIG5 (34,35).

In the present paper, we report on the characterization of an *Arabidopsis* knock-out mutant of the *SIG4* gene. We show that AtSIG4 is specifically important for the transcription of the plastid *ndh*F gene encoding a subunit of the plastid NDH complex.

MATERIALS AND METHODS

Isolation of *SIG4* knock-out lines

Genomic DNA from 11 000 insertion lines [INRA collection, Versailles, ecotype Wassilewskija (WS) (36)], divided into 11 super pools (SP) of 1000 independent lines each, was screened by PCR. PCR was performed on 50 ng template DNA in a final volume of 50 µl using AdvanTaq Plus™ DNA Polymerase (Clontech) in the presence of 0.2 mM of all four dNTPs and 0.4 µM of each primer. To reduce unspecific hybridization, temperature was decreased for 1 degree/cycle (65–55°C) during the first 10 cycles (30″ denaturation, 30″ hybridization and 3 min elongation), followed by 35 cycles at 55°C and a final elongation of 5 min. Each of the two primers specific to the genomic sequence (1) 5′-AATCTAAGGTGTGGGAAGCTC-TGC-3′ and (4) 5′-ACTAAGTACCTGCAATGTCATGC-3′ was combined with each of the two primers specific to the left and right borders of the T-DNA (2) 5′-CTACAAATTGC-CTTTTCTTATCGAC-3′ and (3) 5′-CTGCCAGTTCAGTTC-GTTGTTCAC-3′, respectively. Amplification products were separated by electrophoresis on 1% agarose gels at 100 V. DNA bands were gel-extracted, purified using GeneClean II kit (Bio101™) and sequenced to determine the flanking sequence of the T-DNA borders and the position of the T-DNA in the *SIG4* gene.

Heterozygous *SIG4* insertion lines were backcrossed with WT (WS) plants in order to eliminate any other T-DNA insertion. Every generation resulting from self-pollination were analysed for 3:1 kanamycin-resistance segregation and by PCR for the presence of the T-DNA insertion in the *SIG4* gene. Five homozygote lines resulting from three successive backcrosses were isolated.

Array analyses

Plant materials and growth conditions. Imbibed seeds of *Arabidopsis thaliana* were sown on 0.4% gelrite (Wako Co. Ltd) plates of Murashige and Skoog (MS) medium (Wako Co. Ltd) without sucrose and were grown for 7 days at 23°C under continuous white light.

Nucleic acids preparation from plant materials. Plant materials were frozen in liquid nitrogen and ground with Multi-beads shocker (Yasui Kikai Co. Ltd). DNA was purified using DNeasy Plant Mini Kit (Qiagen). RNA was first prepared using RNeasy Plant Mini Kit (Qiagen) followed by DNase I (0.1 U/µl; NIPPON GENE) treatment at 37°C for 15 min. After phenol/chloroform/isoamyl alcohol (25:24:1) extraction, the RNA was precipitated with ethanol. *Q*-RNA was synthesized by Riboprobe System–SP6 (Promega CO. Ltd) and *Q*-DNA fragment containing a SP6 promoter and a poly(T) tract, which allows us to use this PCR product as templates for the transcription reaction and the reverse transcription reaction, respectively. *Q*-DNA fragment was prepared by PCR using lambda DNA and following primer sets, QS-plus 5′-TCATTTAGGTGACACTATAGGGCGCATGAGACTCGA-AAGCGTAGC-3′ with the SP6 transcription promoter at the 5′ end and QE-plus 5′-TTTTTTTTTTTTTTTTTTCATGCT-GCTAACGTGTGACCGCATTC-3′ with the poly(T) tract at the 5′ end.

DNA microarray preparation. The *A.thaliana* plastid DNA [ecotype Columbia (Col)] microarray was constructed from 81 PCR products that corresponded to 79 plastid-encoded protein genes, and the lambda phage *Q*-gene as a control as described previously (37). For three intron-containing genes (*petB*, *clpP* and *rpl2*), DNA was obtained by RT–PCR (RT–PCR kit, Toyobo) amplification of total RNA (Col). Each DNA sample was spotted four times on the glass slides.

Microarray analysis. For the fluorescent probe preparation, 20 µg of each RNA sample was mixed with 35 ng of *Q*-RNA as an external control. This RNA solution was added with the primer mixture containing 0.5 pmol each of gene specific primers [antisense (R) primers for each gene], and the fluorescent-labelling and the purification were performed using Atlas Glass Fluorescent Labelling Kit (Clontech) and Cy3 (wild-type plant) or Cy5 (*sig4* plant) Mono-Reactive Dye (Amersham Pharmacia) as described by the supplier. Finally, the purified labelled probe was precipitated with ethanol and dissolved in 5 µl water. For hybridization, 5 µl each of Cy3- and Cy5-labelled probes were mixed with 35 µl of ULTRA hyb hybridization buffer (Ambion), incubated at 95°C for 5 min, cooled at 65°C and applied on the microarray. The array was covered with Spaced Cover Glass L (TaKaRa) and incubated at 50°C for 16 h. Thereafter, the cover glass was slipped from the array in 2× SSC. The slide glass was washed twice with 0.1× SSC containing 0.1% SDS for 5 min, and twice in 0.1× SSC for 5 min. Finally, the slide glass was dipped in water and in 99.5% ethanol, and dried by centrifuging at 2500 *g* for 2 min at room temperature. Microarrays were scanned with two wavelengths for Cy3 (560 nm) and Cy5 (675 nm) by a laser fluorescent scanner (GeneTAC LS IV, Genomic Solutions). The lambda phage *Q*-gene spots were used to standardize the two channels with respect to the signal intensity, e.g. the fluorescence ratios of the four control DNA spots were set to 1.00 and the standardization coefficient (0.917) was applied to calculate the values for all other genes. Gene TAC Analyser software version 3.0.1 (Genomic Solutions) was used for the data analysis.

RT–PCR

RT–PCR was performed using 2 μg of total RNA as described previously (23). The cDNA synthesis was carried out using poly(dT)18 primer and Superscript RNAse H-Reverse Transcriptase (Life Technologies). For amplification (~500 bp) of the cDNAs corresponding to the six *SIG* genes, the following primers were used:

SIG1, 5′-GGCGAAGTATTTAGAAGCTTTAGC-3′ and 5′-ATCAACTTCCTGCGCAACAAGACG-3′; *SIG2*, 5′-TTGCTTCTACTGAGAGACCTGGC-3′ and 5′-CCGAGATATCTTCAAGATACTGC-3′; *SIG3*, 5′-TTAGTGCGATCGAGTTTAACATCG-3′ and 5′-TAAGCACGACGTGATTGAGGAACC-3′; *SIG4*, 5′-ACAATCTCTCCCTTACTCAGAACG-3′ and 5′-AACAACCAACCTACGGTAACAACG-3′; *SIG5*, 5′-CTGTTCTTTCTTCTACTGAACATGC-3′ and 5′-CTCAAACCATAGCTCAGTCTTTGC-3′; and *SIG6*, 5′-ACTAGCTCAGAAGGCTTTATCAGC-3′ and 5′-ATGGACTACCAGACGTAGGTTTGC-3′. PCR was performed in 50 μl reaction volumes using the same concentrations of primers and dNTPs as described previously. Thirty cycles of 30″ at 94°C, 30″ at 55°C and 45″ at 72°C were used for *SIG1*, *SIG3*, *SIG4*, *SIG5* and *SIG6* mRNAs. For *SIG2* mRNA, the hybridization temperature was lowered to 48°C. Amplification products were separated by electrophoresis on agarose (1.2%) gels.

For semi-quantitative amplification of *ndh*F and *psb*N mRNAs only 1 μg of DNase I-treated total RNA was used. After cDNA synthesis using specific 3′ primers instead of poly(dT)18, the sample was divided into two equal parts for PCR amplification (25 cycles) of either *ndh*F or *psb*N cDNA. The following primers have been used:

*Ndh*F, 5′-ATAATTCACTTTTTCTGTATTTGC-3′ and 5′-GCACGCGATTTCAAACC-3′; *Psb*N, 5′-ATGGAAACAGCAACCCTAGTCG-3′ and 5′-GTCCCCGTGTTCCTCGAATGG-3′.

Complementation of the *sig4-1* mutant

Full-length *SIG*4 cDNA was amplified by RT–PCR using total RNA from 7-day-old *Arabidopsis* plantlets. The following primers have been used: 5′-*cgggatcc*ATGGCGACGACGATTCC-3′ and 5′-*gcgtcgac*TTATTCACTAGAGGAATAG-3′. The translation start and termination codons are underlined. In order to facilitate cloning, BamHI (5′) and SalI (3′) restriction site (indicated in italics) have been added to the primers. The BamHI–SalI digested PCR product was ligated into BamHI–SalI digested pBluescript II KS(−) (Stratagene) and controlled by sequencing. Later, the *SIG4* cDNA was cloned in the binary vector pFP101 (http://www.isv.cnrs-gif.fr/jg/alligator/vectors.html) using the same restriction sites. Stable transformation of *Arabidopsis* was performed using *Agrobacterium tumefaciens*-based standard (38). Transformed lines were selected by means of the seed-specific GFP fluorescence.

Primer extension

Using isolated plastid DNA from *Arabidopsis* as template, the *ndh*F, *ndh*G and *ycf*4 promoter regions have been PCR amplified and cloned into pCR^{R}2.1–TOPO^{R} (Invitrogen) with the following primers:

*ndh*F, 5′-TGTCCAATATCCCTTCC-3′ and 5′-GAAAGGGATGATCCATH-3′; *ndh*G, 5′-CTCAAACAAAAAATGGG-3′ and 5′-CCCAGAAAAACTAAAAG-3′; *ycf*4, 5′-AGACCGAATCATATCAC-3′ and 5′-CCAGCAGAAATTACTTG-3′.

Primer extension experiments have been performed as described (39) using 10 μg of total RNA. The following primers have been used for primer extension as well to establish the accompanying sequence ladders: 5′-GCACGCGATTTCAAACC-3′ (*ndh*F); 5′-CATGTATTGGTCCAGGC-3′ (*ndh*G) and 5′-CCAGCAGAAATTACTTG -3′ (*ycf*4).

Chlorophyll fluorescence measurements

For chlorophyll fluorescence measurements, *Arabidopsis* plants were grown in soil in a growth chamber in short day conditions, with a 8-h-light period (150 μmol photons m^{-2} s^{-1}) at 22°C and a 16-h-dark period at 18°C. Post-illumination chlorophyll fluorescence rises were measured at room temperature on attached leaves using a PAM-2000 modulated fluorometer (Walz Effeltrich, Germany). The basal chlorophyll fluorescence level measured under low non-actinic light was recorded following a 5 min actinic illumination (220 μmol photons m^{-2} s^{-1}).

Chloroplast subfractionation and protein analyses

All operations were carried out at 0–5°C. Intact chloroplasts were obtained from 2-week-old *Arabidopsis* plantlets as described (40). Briefly, plantlets were homogenized (0.45 M sorbitol, 20 mM Tricine, 10 mM EDTA, 10 mM $NaHCO_3$, 0.1% BSA w/v, pH 8.4), the homogenate was filtered (50 μm), and crude chloroplasts were obtained by centrifugation at 5000 *g* for 3 min. Chloroplasts were further purified by short centrifugation (5 min) through a Percoll cushion [40% Percoll in BP (0.3 M sorbitol, 20 mM Tricine, 5 mM $MgCl_2$, 2.5 mM EDTA, pH 7.6)] and three successive washes in the same buffer. After osmotic shock (20 mM Tricine, pH 7.6) chloroplast membranes were pelleted by centrifugation at 5000 *g* for 5 min and membrane proteins were analysed by western immunoblotting as described previously (23).

RESULTS

Identification of *Arabidopsis* lines having a T-DNA insertion in the *SIG4* gene

To study the function of SIG4, we have isolated a T-DNA tagged line from the INRA collection (Versailles) by PCR-screening of DNA pools (see Materials and Methods). Sequencing of the right and left borders located the insertion to the first exon of the *SIG4* gene at position 210 downstream of the ATG initiation start codon (Figure 1A). After cleaning by three successive backcrosses, five homozygous insertion plants were selected. RT–PCR analyses of mRNAs corresponding to all six *Arabidopsis* sigma factors show complete absence of *SIG4* mRNA in all five plants (Figure 1B shows only one of these plants as example). No noticeable phenotype is visible between *sig4-1* plants and WT plants independent of plant age (Figure 1C and data not shown). The absence of obvious phenotypic changes in *sig4-1* plants might be due to overlapping functions of sigma factors, i.e. other sigma factors can replace SIG4 function(s) in the knock-out mutant (23,24) or to specific, but non-essential, SIG4 function. To learn something more on the function of SIG4, we have analysed

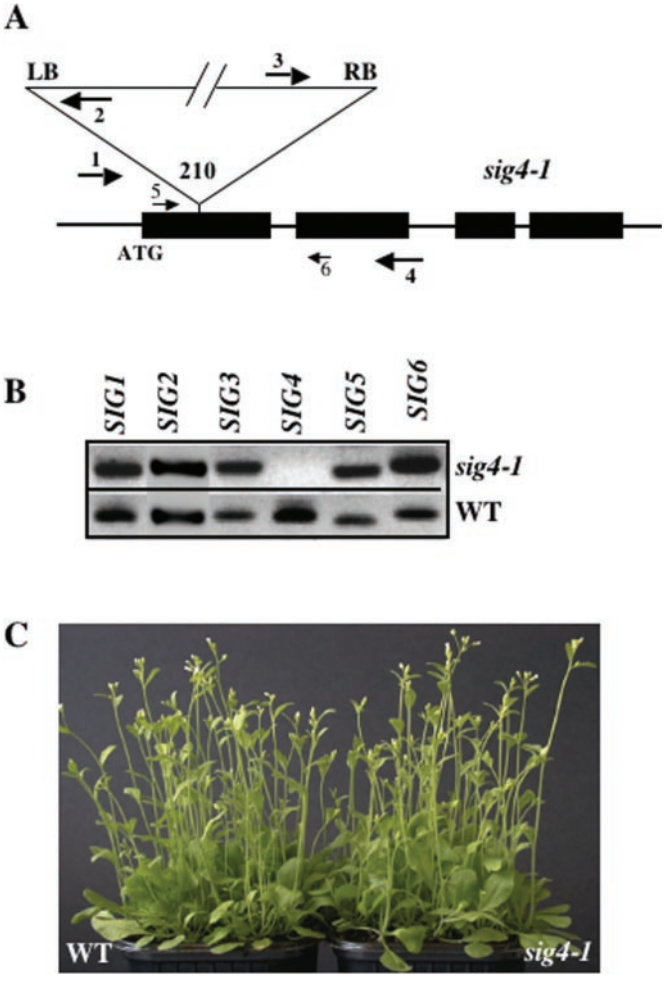

Figure 1. Characterization of the *sig4* knock-out mutant. (**A**) Schematic presentation of the T-DNA insertion and the location of the primers. The exact position of the T-DNA insertion into the *SIG4* gene (210) was determined by sequencing of the DNA fragments amplified by primer pairs 1/2 and 3/4. (**B**) The absence of *SIG4* mRNA in the T-DNA insertion line was verified by RT–PCR. Total RNA prepared from 7-day-old WT and *sig4* plantlets has been analysed in parallel for the presence of all six sigma factors. (**C**) WT and *sig4* knock-out plants grown for 4 weeks under 16 h light/8 h dark cycle.

the plastid transcriptome of *sig4-1* plants and compared it with WT plants.

Analyses of plastid gene expression in the *SIG4* knock-out mutant

To get a rough idea on whether there is replacement of function or specificity of gene transcription of a SIG4/PEP holoenzyme, we performed an overall transcript profiling by microarray hybridization. Transcript levels of 7-day-old *SIG4* insertion mutants corresponding to all 79 open reading frames of the *A.thaliana* plastid genome were compared with that of WT plants by DNA microarray analysis. Analysis was carried out according to Nagashima *et al*. (37). Two independent experiments have been made using either WS or Col as WT plants. For each analysis, values have been registered from four individual DNA spottings. Normalized average values (*sig4*/WT) and standard deviations are indicated in Table 1 (Supplementary Table S1). If the commonly used threshold value of 0.66 (1/1.5) is applied (41), only two mRNAs (*ndh*F and *ycf*4) are significantly reduced in *sig4* plants.

Table 1. Transcript analysis of plastid genes in *sig4* compared with WT plants

Gene name	Ratio *sig4*/WT	Gene name	Ratio *sig4*/WT	Gene name	Ratio *sig4*/WT
ndhF	0.43 ± 0.10	*psbC*	0.90 ± 0.25	*psbF*	1.10 ± 0.29
ycf4	0.58 ± 0.01	*psbL*	0.91 ± 0.38	*atpB*	1.11 ± 0.18
ndhG	0.67 ± 0.17	*ndhE*	0.92 ± 0.13	*rpl14*	1.11 ± 0.02
petA	0.68 ± 0.00	*psaA*	0.93 ± 0.00	*atpA*	1.12 ± 0.16
psbB	0.69 ± 0.19	*atpI*	0.94 ± 0.01	*rps15*	1.13 ± 0.49
rps16	0.69 ± 0.05	*psaI*	0.95 ± 0.39	*psbE*	1.14 ± 0.21
ndhI	0.72 ± 0.02	*rpoC1*	0.95 ± 0.24	*matK*	1.17 ± 0.30
clpP	0.73 ± 0.10	*rpl2*	0.95 ± 0.06	*rpl16*	1.18 ± 0.03
psbD	0.73 ± 0.17	*ndhK*	0.95 ± 0.07	*rpl33*	1.19 ± 0.04
ycf1	0.73 ± 0.04	*rps19*	0.96 ± 0.07	*rps2*	1.20 ± 0.31
rpoB	0.74 ± 0.16	*psbT*	0.96 ± 0.15	*rpl22*	1.20 ± 0.12
cemA	0.77 ± 0.02	*rps7*	0.97 ± 0.09	*atpF*	1.21 ± 0.28
ndhD	0.78 ± 0.00	*rps11*	0.98 ± 0.31	*psbK*	1.22 ± 0.19
rpoA	0.79 ± 0.11	*ccsA*	0.98 ± 0.11	*rps14*	1.22 ± 0.39
psbJ	0.79 ± 0.10	*psbA*	0.98 ± 0.37	*petG*	1.23 ± 0.19
rbcL	0.81 ± 0.15	*ndhB*	0.98 ± 0.01	*psbZ*	1.24 ± 0.00
petD	0.81 ± 0.21	*ycf3*	0.99 ± 0.14	*atpE*	1.25 ± 0.07
ndhA	0.81 ± 0.04	*3'rps12*	1.00 ± 0.12	*rps3*	1.25 ± 0.02
rps8	0.83 ± 0.15	*psbH*	1.01 ± 0.08	*rps18*	1.26 ± 0.20
psbI	0.83 ± 0.13	*petL*	1.01 ± 0.02	*psbM*	1.26 ± 0.59
ndhC	0.84 ± 0.21	*ycf15*	1.02 ± 0.17	*psaC*	1.31 ± 0.16
ndhH	0.85 ± 0.01	*psbN*	1.03 ± 0.02	*rpl23*	1.35 ± 0.33
ycf2	0.87 ± 0.04	*rps4*	1.03 ± 0.42	*petN*	1.42 ± 0.13
petB	0.87 ± 0.29	*rpl20*	1.03 ± 0.18	*rpl32*	1.52 ± 0.06
rpoC2	0.89 ± 0.11	*ndhJ*	1.05 ± 0.13	*rpl36*	1.54 ± 0.62
5'rps12	0.89 ± 0.31	*atpH*	1.07 ± 0.31	*psaJ*	1.83 ± 0.37
accD	0.90 ± 0.02	*psaB*	1.10 ± 0.30		

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

Next, we have analysed the *ndhF*, *ndhG* and *ycf4* mRNAs in more detail by primer extension. Upstream regions of *ndh*F, *ndh*G and *ycf*4 genes were cloned and WT and *sig4-1* total RNA preparations were analysed by primer extension in order to map the 5′ ends of the corresponding precursor RNAs (Figure 2). The analysis of *ndh*F mRNA reveals only a single RNA. It has its 5′ end located at position −320 upstream of the ATG translation start codon. This RNA is strongly reduced in *sig4-1* plants confirming SIG4-mediated transcription of *ndh*F (Figure 2A, upper part). The putative transcription start site is preceded by typical prokaryotic-type −35 and −10 consensus sequences (Figure 2D). The strong reduction of *ndh*F mRNA was verified in addition by semi-quantitative RT–PCR analysis using another RNA as control whose level is not modified in the knock-out mutant (*psb*N) (Figure 2A, lower part).

The analyses of the *ndh*G and *ycf*4 mRNAs show several transcripts (Figure 2B). However, neither of these transcripts is reduced in the *sig4-1* plants indicating that these two genes do not depend on SIG4 for transcription. DNA sequences preceding the 5′ ends of the two most abundant transcripts of the *ndh*G gene are shown in Figure 2D. They are located at positions −138 and −213 relative to the translation initiation codon, respectively. Upstream sequences do not reveal typical prokaryotic-type promoter elements. On the

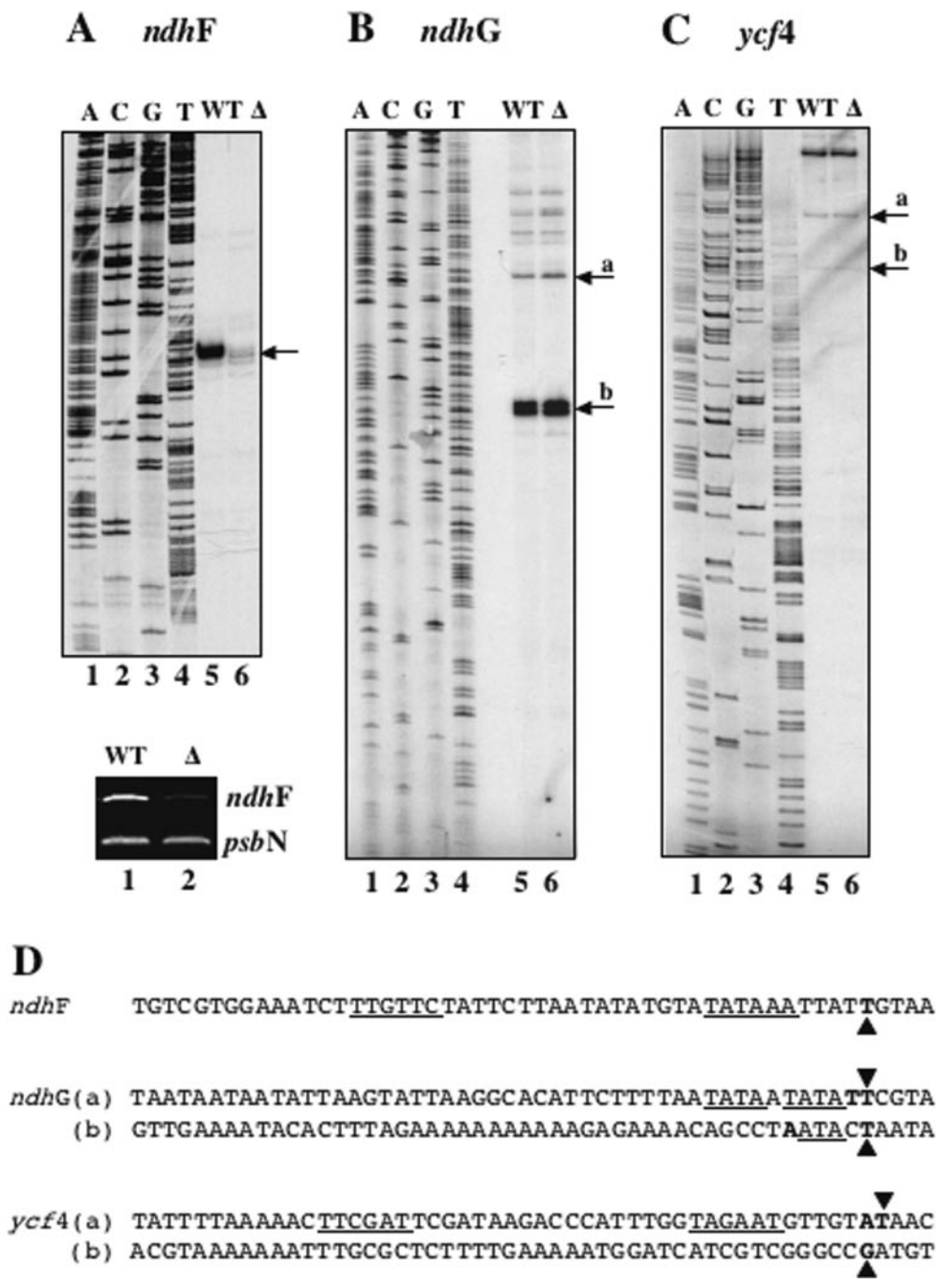

Figure 2. Analyses of *ndh*F, *ndh*G and *ycf*4 precursor RNAs. Total RNA was isolated from 7-day-old *Arabidopsis* plantlets that had been grown under 16 h light/8 h dark cycle. RNA was reverse transcribed and cDNAs were separated on 6% polyacrylamide gels under denaturing conditions. The accompanying sequence ladders are established with the same primer that was used for primer extension. (**A**) An aliquot of 10 µg (upper part) or 1 µg (lower part) of total RNA prepared from *Arabidopsis* WT (lanes 5 and 1, upper part and lower part, respectively) and *sig4* plantlets (lanes 6 and 2, upper part and lower part, respectively) have been analysed by primer extension using a primer specific to *ndh*F (upper part) or by RT–PCR using primers specific for *ndh*F and *psb*N. (**B**) An aliquot of 10 µg of total RNA prepared from *Arabidopsis* WT (lane 5) and *sig4* plantlets (lane 6) have been analysed by primer extension using a primer specific to *ndh*G. (**C**) An aliquot of 10 µg of total RNA prepared from *Arabidopsis* WT (lane 5) and *sig4* plants (lane 6) have been analysed by primer extension using a primer specific to *ycf*4. (**D**) The nucleotide sequences upstream of the *ndh*F, *ndh*G and *ycf*4 precursor RNAs are aligned. Putative promoter sequences are underlined, and 5′ ends of transcripts [positions −320 (*ndh*F), −213 and −138 (*ndh*G), −326 and −226 (*ycf*4)] are marked in bold letters and by vertical arrows.

other hand, NEP consensus YRTA sequences are discernable in both cases, suggesting transcription by NEP. The most important transcript of the *ycf*4 gene is located further than 400 bases upstream of the ATG codon and the sequence is not readable up to that point. The second transcript ends at position −326 relative to the translation start codon. The DNA sequence preceding this 5′ end contains a −35 and −10 consensus element reminiscent for PEP promoters. The minor transcript mapping at position −226 probably corresponds to a processing product since neither PEP nor NEP consensus promoter elements are detectable in the upstream DNA sequence (Figure 2C and D).

The strong decrease of *ndhF* precursor RNA is due to the lack of SIG4 in *sig4-1* plants and leads to a strong decrease of NDH complex amount and activity

To confirm that the strong decrease of *ndhF* precursor RNA in *sig4-1* plants is indeed due to the lack of SIG4 we complemented the mutant by agrobacterium-mediated transformation using the *SIG4* cDNA placed under control of the 35S promoter. Figure 3A shows the comparison of *ndhF* precursor RNAs in WT and *sig4-1* plants and after complementation of the *sig4-1* plants with *35S-SIG4*. Three different complementation lines have been analysed. The expression of *SIG4* cDNA under control of the 35S promoter completely restores the level of *ndhF* precursor RNA in all three lines showing even overexpression of the *ndhF* gene compared with WT plants. The mRNA levels of another *ndh* gene, *ndh*G (only transcript b is shown but both transcripts, a and b, show the same behaviour), are not changed in the three different complemented lines and in the *sig4-1* mutant.

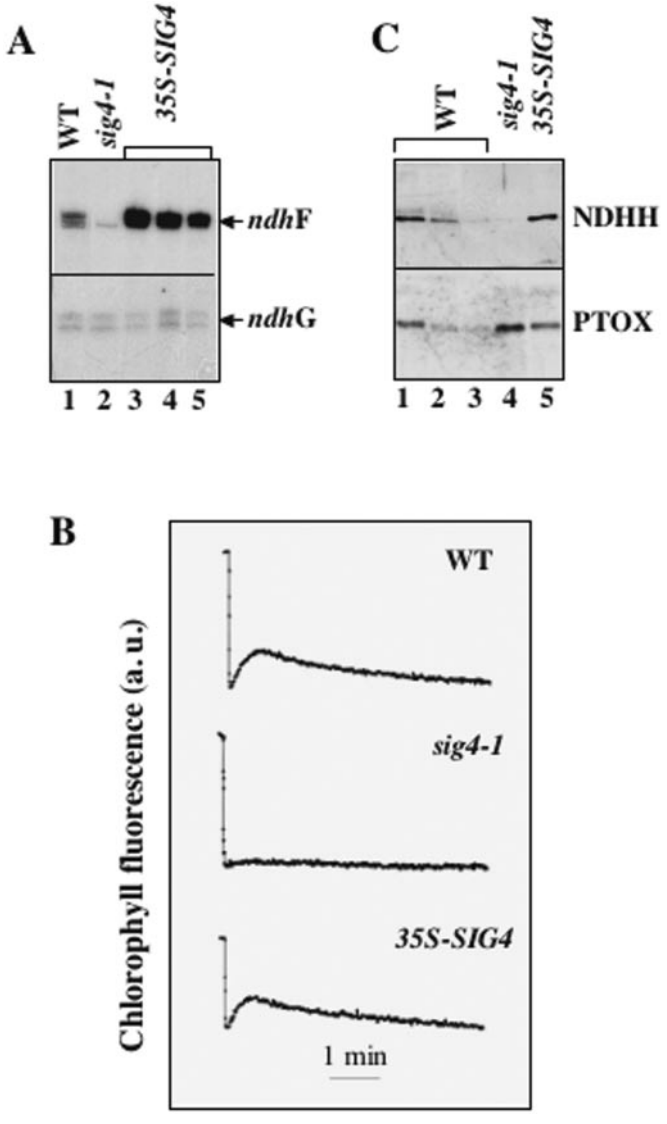

Figure 3. Characterization of the *sig4* knock-out mutant by complementation and fluorescence induction. (**A**) An aliquot of 10 µg of total RNA prepared from 7-day-old *Arabidopsis* WT (lane 1), *sig4* (lane 2) and three different *35S-SIG4* complemented lines (lanes 3–5) have been analysed by primer extension using the same primers as in Figure 2A (*ndh*F) and 2B (*ndh*G, only the b transcript is shown). (**B**) Rosette leaves of 6-week-old *Arabidopsis* plants have been used to measure transient chlorophyll fluorescence rise under non-actinic light following a 5 min illumination of WT, *sig4* and *35S-SIG4* plants. (**C**) Thylakoid membranes have been prepared from 2-week-old WT, *sig4* and *35S-SIG4* plants and 40 µg (lanes 1, 4 and 5), 10 µg (lane 2) and 4 µg (lane 3) of protein have been analysed by western immunoblotting using antibodies made against the NDHH subunit or the plastid terminal oxidase (PTOX).

To obtain information on the influence of the strong decrease in *ndhF* gene expression on NDH activity in general, we compared WT, *sig4-1* and *35S-SIG4* complemented plants by measuring chlorophyll fluorescence during a light to dark transition. Such an analysis was previously used to characterize plastid *ndh* knock-out mutants (42), a nuclear mutant affected in a factor that is essential for *ndhB* expression (43) and, more recently, nuclear knock-out mutants of novel NDH complex subunits (44). It was concluded from these studies that the lack of one subunit of the plastid NDH complex abolishes both, the assembly and the function, of the whole complex resulting in a suppression of the transient increase in chlorophyll fluorescence measured following actinic illumination. The fluorescence induction curve of *sig4-1* plants is comparable with that previously reported for *ndh* mutants (Figure 3B) (41,44), i.e. the transient increase in chlorophyll fluorescence observed in WT plants disappears in the mutants. On the other hand, the fluorescence induction curve of the *35S-SIG4* complemented plants resembles that of the WT. This demonstrates that the lack of SIG4 results in strong down-regulation of NDH activity.

Finally, we also analysed whether the lack of *ndhF* expression has consequences on the expression of another subunit of the NDH complex on the protein level. For this aim, we analysed the presence of the NDHH subunit (43) in the WT, *sig4-1* and *35S-SIG4* plants by western immunoblotting (Figure 3C). The result shows that in the *sig4-1* mutant the NDHH protein is not detectable any more, i.e. its level is at least >10-fold decreased compared with the WT dilution analysis (Figure 3C, compare lanes 1–3 with lane 4). On the other hand, in the *35S-SIG4* complemented plants, NDHH is present again in well detectable amount. The amount of plastid terminal oxidase [PTOX (45)], another putative chlororespiratory enzyme, is not affected in the mutants and can be used as a loading control.

DISCUSSION

We have analysed plastid gene expression in *Arabidopsis* plants deficient in SIG4 expression. Overall analysis of transcript levels of a 7-day-old *sig4* insertion mutant shows up-regulated and down-regulated mRNAs (Table 1). The most down-regulated mRNA corresponds to *ndh*F and the most up-regulated mRNA is *psa*J (Table 1). Up-regulation of mRNAs in a given sigma knock-out mutant could be due to changes in turnover rates or to overexpression of (an)other sigma factor(s). For instance, overexpression of SIG3 in *SIG2* antisense plants has recently been shown (23). In order to explain the overexpression of *psa*J in *sig4* plants, it would be interesting to analyse the expression of all other sigma factors on the protein level in the future.

As a first step towards determination of gene promoters that are specifically recognized by SIG4, we have analysed the three most reduced mRNAs (*ndh*F > *ndh*G, *ycf*4) by primer extension (Figure 2). In addition to quantitative aspects, primer extension also permits to localize 5′ ends of precursor RNAs, i.e. to determine transcription initiation and/or processing sites of mRNAs. These analyses as well as semi-quantitative RT–PCR analysis confirmed the strong underexpression of the *ndh*F gene in the *sig4* mutant (Figure 2A). However, for the two other genes, *ndh*G and *ycf*4, equal amounts of precursor RNAs are detected (Figure 2B and C and Figure 3A). The reason for these quantitative differences between primer extension and microarray results is not completely clear. However, *ndh*F represents a single gene while *ndh*G and *ycf*4 are localized within clusters of genes (46–48) that give rise to a mixture of monocistronic as well as long polycistronic mRNAs (see below). On double-stranded DNA microarrays, the whole population of monocistronic and polycistronic mRNAs will be analysed including antisense RNAs while primer extension reveals only the mRNAs corresponding to the gene of interest.

A previous transcript analysis of the *ndh*H-D operon showed a complex pattern of transcripts, including monocistronic transcripts for *psa*C and *ndh*D (46,47). *Ndh*G represents the forth *ndh* gene within the *ndh*H-D operon and had not been analysed for the presence of monocistronic transcripts. Our study shows the existence of two *ndh*G RNAs whose 5′ ends locate between *ndh*G and the preceding *ndh*I gene suggesting the existence of monocistronic RNAs also for *ndh*G. These RNAs might arise from processing of longer transcripts or from activation of internal promoters. The DNA sequences preceding the 5′ ends of the two major *ndh*G mRNAs do reveal YRTA NEP consensus sequences, i.e. the two observed transcripts could be produced by NEP. Prokaryotic-type promoter structures cannot be detected (Figure 2D). This indicates that the *ndh*G gene is not transcribed by PEP, i.e. precursor RNAs should not be influenced by the lack of a sigma factor that belongs to the PEP transcription system. This is in accordance with our primer extension result that does not show differences between WT and *sig4* plants. The *ndh*G mRNA level is also not influenced by overexpression of *ndh*F mRNA in *35S-SIG4* plants (Figure 3A), indicating that there is no coordination of expression of different *ndh* genes on the mRNA level.

The upstream sequence of the −326 *ycf*4 transcript harbours prokaryotic-type promoter elements (Figure 2D), i.e. it could be transcribed by PEP. However, the two consensus promoter sequences differ from those of the *ndh*F gene. In addition, all three detectable *ycf*4 transcripts are present in equal amounts in WT plants and *sig4* mutants. Therefore, it is unlikely that one of these transcripts is under control of SIG4. In conclusion, the strong reduction of *ndh*F RNA observed by microarray as well as primer extension results suggests specific transcription of the *ndh*F gene by a sigma4/PEP holoenzyme. Although plastid DNA sequences are highly conserved between different plant species, the question of whether this type of *ndh*F transcriptional regulation by sigma 4 is conserved in other plant species cannot be answered at the moment. Simple alignment of *ndh*F upstream sequences does not show real sequence conservation (data not shown), and knock-out mutants are not available for other species. Also, only few sigma factor coding genes are actually sequenced in other species.

Disruption of *ndh* genes in tobacco showed that chloroplast NDH complexes reduce the plastoquinone pool non-photochemically and mediate cyclic electron flow around photosystem I. However, overall electron transport is not affected in *ndh* knock-out mutants. This was explained by the existence of two parallel pathways of electrons around PSI, one involving the NDH complex and the other an antimycin A-sensitive component (49–51). *Ndh* knock-out plants do not show easily detectable phenotypes (42,49,52–55) and, in this respect, these mutants resemble well to our *sig4* mutant. In addition, the strong diminution of *ndhF* expression in *sig4* plants seems to provoke the same phenotype as it has been found in *ndh* knock-out mutants, i.e. it abolishes the transient increase in chlorophyll fluorescence after light to dark transition which is related to the activity of the plastid NDH complex (Figure 3B) (42–44). Finally, western blot analysis indicates the absence of other NDH subunits, e.g. NDHH, in *sig4* mutants (Figure 3C). This result fits well to previous findings showing that in the absence of one of the NDH proteins the whole NDH complex cannot be assembled (55) and the other subunits disappear (42–44,52,56). Our results show that the diminution of *ndh*F mRNA does not influence the quantity of another *ndh* mRNA (e.g. *ndh*G) but it influences the quantity of another NDH subunit on the protein level (e.g. NDHH). It seems therefore likely that NDH complex assembly is regulated on the post-transcriptional level in a way that the quantity of whole NDH complexes could be determined by the quantity of one of its subunits, e.g. NDHF. The PTOX, which might participate in a chloroplast respiratory chain (50) and has been localized to thylakoïd stromal lamellae (57), is not influenced in the different *sig4* mutants and is used as loading control (Figure 3C). This result shows that the regulation of the expression of the NDH complex and PTOX is not generally coupled although both components are enhanced in plants with severe defects in photosystem II (58).

NDHF is an important subunit of the NDH complex because the activity of NDH complexes is regulated by phosphorylation of the NDHF polypeptide (59). In addition, NDHF is encoded by a single gene that is not part of a multicistronic transcription unit, i.e. its transcription does not influence the transcription of other, unrelated, genes. Altogether, our results suggest that, via *ndh*F transcription, SIG4 regulates the overall quantity of NDH complexes and thus influences NDH activity. Future studies will focus on the question of whether SIG4 expression is modulated by environmental and/or developmental parameters, as the NDH complex has been proposed to be involved in plant stress responses [water stress, high light (55,60)] and in leaf senescence (56). Under our presently used growth conditions, we could not detect retardation of senescence of the *sig4* mutants as has been published recently for tobacco *ndh*F knock-out mutants (56).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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