Evidence for Transcriptional Regulation of Plastid Photosynthesis Genes in *Arabidopsis thaliana* Roots[1]

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Mechanisms underlying suppressed levels of transcripts for plastid photosynthesis genes in nongreen tissues such as roots and calli were analyzed in *Arabidopsis thaliana*, a plant suitable for further genetic dissection. A region encoding promoters of *rbcL*, the gene encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, and the *atpB/E* operon for the β and ϵ subunits of coupling factor one were cloned and sequenced. Transcripts for *rbcL*, *atpB/E*, and *psbA*, the gene for the D1 protein in the photosystem II reaction center, were barely detectable in roots of *A. thaliana*, whereas 16S rRNA was detected at a low level. The run-on transcription experiment revealed that expression of *rbcL*, *atpB/E*, and *psbA* was regulated at transcription. The copy number of plastid DNA in roots was one-fifth that in green leaves on the basis of total cellular DNA, suggesting that in the latter the DNA copy-number regulation also exists in plastid gene expression. Digestion of DNA with methyl-sensitive and -insensitive isoschizomeric endonucleases and subsequent polymerase chain reaction, as well as in vitro transcription of plastid DNAs with *Escherichia coli* RNA polymerase, resulted in no evidence of regulation by DNA modification. In spite of predominant suppression of expression of *rbcL*, *atpB/E*, and *psbA* at transcription in roots and calli, 16S rRNA levels were decreased because of low RNA stability.

The expression of most photosynthesis genes encoded in plastid DNA is closely related to the developmental state of plastids; it is promoted during chloroplast development (Rodermel and Bogorad, 1985; Deng and Gruissem, 1987; Mullet and Klein, 1987; Klein and Mullet, 1990) but suppressed during formation of amyloplasts or chromoplasts (Piechulla et al., 1985, 1986; Deng and Gruissem, 1988; Kobayashi et al., 1990; Kobayashi, 1991). Gene expression in plastids has been proposed to be regulated at multiple levels such as by DNA copy number (Aguettaz et al., 1987), DNA superhelicity (Stirdivant et al., 1985; Lam and Chua, 1987; Thompson and Mosig, 1987), DNA methylation (Ngernprasirtsiri et al., 1988; Kobayashi et al., 1990), and DNA transcription (Schrubar et al., 1990), and RNA stability (Deng

and Gruissem, 1987, 1988; Mullet and Klein, 1987; Stern and Gruissem, 1987; Gruissem et al., 1988; Stern et al., 1989) and RNA translation (Deng and Gruissem, 1988; Berry et al., 1990) in respect to involvement of the 5' and 3' untranslated regions therein (Mayfield et al., 1995). This variety may result from differences in tissues or plant species.

The copy number of plastid DNA is known to play a role in the regulation of the plastid transcript level during conversion of amyloplasts to chloroplasts in mixotrophic cell suspensions of spinach (Aguettaz et al., 1987). Although data available about gene expression in nongreen plastids are limited, we have proposed that DNA methylation is involved in the selective suppression of photosynthetic genes at the transcriptional level in chromoplasts of tomato fruits (Kobayashi et al., 1990) and in amyloplasts of heterotrophically cultured cells of sycamore (Ngernprasirtsiri et al., 1988). The expression of most plastid genes in spinach root amyloplasts is reported to be controlled not only posttranscriptionally but also translationally (Deng and Gruissem, 1988). It has been proposed that proteins binding to stem-loop structures at the 3' end of mRNAs could control the stability of mRNAs (Stern and Gruissem, 1987; Hayes et al., 1996; Yang et al., 1996). However, RNA stability does not seem to regulate transcript levels in heterotrophically cultured sycamore cells (Ngernprasirtsiri et al., 1990), and it has been shown by *Chlamydomonas* plastid transformation that the stem-loop structures are involved in transcriptional termination but not in RNA stability (Blowers et al., 1993); the 5' region of mRNA is responsible for RNA stability (Salvador et al., 1993).

Mechanisms underlying the regulation of tissue-specific expression of plastid genes cannot be understood without a knowledge of the participation of nuclear genes in its regulation. *Arabidopsis thaliana* is recognized as a plant suitable for the analysis of nuclear genes. However, investigations of plastid gene expression in *A. thaliana* have been limited to cases of photomorphogenesis mutants such as the pleiotropic *det* (Chory and Peto, 1990) and *cop* (Kwok et al., 1996). We have tried to reveal mechanisms of regulation of expression of photosynthesis genes in nongreen plastids of *A. thaliana*, focusing on the expression of the well-known plastid genes *rbcL* and *psbA*, which encode the large subunit of Rubisco and the D1 protein in the PSII reaction center, respectively, as well as *atpB/E*, the operon for the β

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Abbreviation: SLF, sigma-like transcription factors.

and ϵ subunits of coupling factor one (*atpB* and *atpE* are overlapped and cotranscribed).

MATERIALS AND METHODS

Arabidopsis thaliana ecotype Columbia was grown for 6 weeks on soil composed of vermiculite, perlite, and peat moss at 22°C under 16-h light/8-h dark conditions, and its leaves and roots were harvested. Calli were generated on 0.5/0.05 callus-inducing medium (Valvekens et al., 1988) under the same conditions.

Preparation of Nucleic Acids

Total cellular RNA was prepared from leaves, roots, and calli with an RNA separator kit (Total RNA Separator Kit, Clontech, Palo Alto, CA). Total cellular DNA was prepared from leaves, roots, and calli using cetyltriethylammonium bromide (Rogers and Bendich, 1985). Amounts of nucleic acids were determined by A_{260} (Sambrook et al., 1989). The contents of plastid DNAs were determined by DNA-DNA hybridization (Sambrook et al., 1989).

DNA and RNA Probes

The following plasmids were used for RNA-DNA hybridization, DNA-DNA hybridization, and determination of in vitro transcription activity with *Escherichia coli* RNA polymerase: pZmc532, pZmc427, pZR4876 (Kobayashi et al., 1990), and pTCB29 (Shinozaki et al., 1986), containing a 3.1-kb *Bam*HI fragment of 16S rDNA from maize, a 2.2-kbp *Bam*HI/*Eco*RI fragment of *psbA* from maize, a 2.0-kb *Bam*HI fragment of *atpB/E* from maize, and a 1.2-kb *Bam*HI fragment of *rbcL* from tobacco, respectively. The 2.2-kbp insert in pZmc427 has recently been revealed to also encode *matK*, probably for intron maturase (Maier et al., 1995), but *matK* transcript was barely detected (less than 0.37% of *psbA* transcript) in RNA-DNA hybridization with RNA from leaves of *A. thaliana*. To synthesize antisense RNA probes for the run-on transcription assay, 16S rDNA (1.7-kb *Acc*I/*Bam*HI fragment of pZmc532), *psbA* (2.2-kb *Bam*HI/*Eco*RI fragment of pZmc427), *atpB/E* (2.0-kb *Bam*HI fragment of pZR4876), and *rbcL* (1.2-kb *Bam*HI fragment of pTCB29) were cloned into pBluescript II KS− (Stratagene). Antisense RNA probes were generated from these plasmids with T3 (Stratagene) and T7 (Takara, Otsu, Japan) RNA polymerases, and then the template plasmids were digested with 4 units of RNase-free DNase I (RQ1, Promega).

Blotting and Hybridization of Nucleic Acids

Total cellular RNA was electrophoresed as described previously (Sambrook et al., 1989), except for the use of a 1.2% agarose gel containing 0.66 M formaldehyde, and transferred to a nylon membrane (PhotoGene, GIBCO-BRL) using a pressure blotter (PosiBlot, Stratagene). RNA solution was also blotted (Sambrook et al., 1989; Dot Plate, Advantec, Tokyo, Japan). DNA was denatured by boiling and dot-blotted in the same way as for RNA. Both prehy-

bridization and hybridization were performed at 65°C (Sambrook et al., 1989). Membranes were washed with 2× SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0) containing 0.1% (w/v) SDS for 30 min at 65°C, and in 0.5× SSC with 0.1% SDS under the same conditions. Radioactivity on membranes was detected by an imaging analyzer (Fujix BAS 2000, Fuji, Tokyo, Japan).

Cloning and Sequencing of the *rbcL* Promoter Region

In plants *rbcL* is known to be encoded in the opposite direction of *atpB/E*, but shares the upstream sequence. Oligonucleotide primers complementary to conserved sequences in the coding strands of *rbcL* and *atpB/E* in spinach, pea, and tobacco were synthesized following the analysis of their nucleotide sequence data taken from GenBank: 5'GTAGCACTCATAGCTA3' (no. 1), 229- to 245-bp downstream of the ATG initiation codon of *atpB*; and 5'GGAACTCCAGGTTGAGGA3' (no. 2), and 5'ACAGTT-GTCCATGTACCAGT3' (no. 3), 129- to 147-bp and 187- to 207-bp downstream of the initiation codon of *rbcL*, respectively. The region between *atpB/E* and *rbcL* was amplified by PCR with total cellular DNA from *A. thaliana* as the templates and the above primers employing Vent DNA polymerase (New England Biolabs) with 3' → 5' proofreading exonuclease activity. The products amplified with primers nos. 1 and 2 or nos. 1 and 3 were cloned into pCR1000 (Invitrogen) and sequenced by a DNA sequencer (373A, Applied Biosystems) following the manufacturer's instructions. The nucleotide sequence data are currently being submitted to the DNA Data Bank of Japan.

Run-On Transcription

Plastids were isolated at 4°C from 10 g each of leaves, roots, and calli of *A. thaliana* according to the procedures of Deng and Gruissem (1988) and Ngernprasirtsiri et al. (1990). Plastids were suspended with 60 µL of buffer consisting of 1 mM sodium pyrophosphate, 50 mM Hepes-NaOH (pH 6.8), 0.33 M sorbitol, 10 mM DTT, 1 mM $MgCl_2$, and 2 mM EDTA. The plastid run-on transcription assay was carried out with 30 µL of the suspended plastids in 100 µL (final volume) of the reaction mixture composed of 66 mM sorbitol, 22 mM Hepes-NaOH (pH 7.9), 0.2 mM sodium pyrophosphate, 10 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 0.5 mM each of ATP, CTP, and GTP, 50 µM [α-^{32}P]UTP (3.7 MBq, Amersham), 2 units of the RNase inhibitor Inhibit-ACE (5 Prime→3 Prime, Inc., Boulder, CO) and 0.5 mg mL^{-1} heparin at 25°C for 20 min. After incubation at 85°C for 5 min, the reaction mixture was treated with RQ1 DNase (Promega) for 30 min, followed by phenol-chloroform-isoamyl alcohol extraction (Sambrook et al., 1989). Unincorporated nucleotides were removed using a NICK column (Pharmacia). Radioactive transcripts were subjected to hybridization with antisense RNA probes blotted onto membranes. To determine the contents of endogenous plastid DNA in the preparation, a lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, and 2.5% (w/v) sarcosinate was added to the plastid fraction, followed by incubation at 65°C for 30 min and subsequent treatment with RNase A preheated for inactivat-

ing contaminated DNases (Sambrook et al., 1989). After extraction with phenol-chloroform-isoamyl alcohol (Sambrook et al., 1989), plastid DNA was precipitated with ethanol in the presence of Ethachinmate (NipponGene, Toyama, Japan), which is made of a polyacrylamide for promoting precipitation.

In Vitro Transcription with *E. coli* RNA Polymerase

Total cellular DNA (1 μg) from leaves, roots, and calli of *A. thaliana* was transcribed with *E. coli* RNA polymerase (Epicentre Technologies, Madison, WI) at 37°C for 60 min in 20 μL of reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 30 mM KCl, 150 μg mL^{-1} BSA, 0.1 mM DTT, and 0.5 mM each of ATP, CTP, UTP, and GTP in the presence of 1 unit of Inhibit-ACE, followed by termination by heating at 80°C for 5 min. Each DNA template was digested with 4 units of RQ1 DNase for 30 min. These samples were subjected to a resin treatment (StrataClean Resin, Stratagene), blotted onto a membrane (Zeta-Probe, Bio-Rad), and hybridized with gene-specific probes.

RESULTS

Low Transcript Levels in Roots and Calli

The amounts of individual RNA species for the plastid genes 16S rRNA, *rbcL*, *psbA*, and *atpB/E* in several mature tissues of *A. thaliana* grown for 6 weeks were determined by RNA-DNA hybridization (Fig. 1). The results showed that RNA levels for all of the genes in leaves were the highest among the tissues. The 16S rRNA level in nonphotosynthetic tissues such as roots and calli decreased in comparison with that in leaves. The transcripts for *rbcL*, *psbA*, and *atpB/E* were diminished in calli and less detectable in roots.

Transcriptional Activities of Plastid Genes

Transcript levels are regulated by RNA synthesis and its degradation. In the former, transcriptional activity and the gene-dose effect are involved. To determine the role of RNA synthesis, we performed plastid run-on transcription
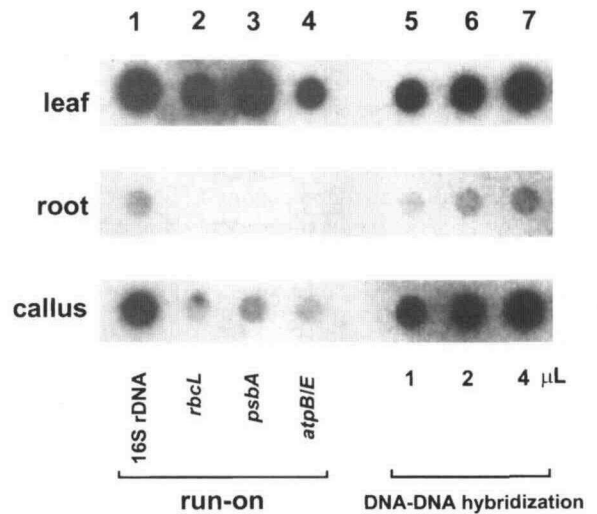

Figure 2. Run-on transcription of plastids prepared from leaves, roots, and calli of *A. thaliana*. The same amount of suspended plastids (30 μL) was employed for each reaction. The antisense RNAs for 16S rDNA, *rbcL*, *psbA*, and *atpB/E* (0.5 μg each) were blotted onto membranes, subjected to hybridization with run-on transcription products from chloroplasts (leaves) and amyloplasts (roots and calli), and detected by an image analyzer (rows 1–4). Contents of endogenous plastid DNA in each plastid preparation (1, 2, and 4 μL) were determined by DNA-DNA hybridization with the 260-bp *Sau*3AI/*Nde*I fragment (see Fig. 4A).

using leaves, roots, and calli focusing on the four plastid genes (Fig. 2). Antisense RNAs for 16S rDNA, *rbcL*, *psbA*, and *atpB/E* were blotted onto membranes and hybridized with radioactive run-on transcription products. The intensities of spots may reflect the in vivo transcriptional activities. The activities hybridized with antisense RNAs for *rbcL* (row 2), *psbA* (row 3), and *atpB/E* (row 4) were remarkably decreased in calli and roots compared with leaves, whereas the signal for 16S rDNA (row 1) was high in leaves and calli. Although the same volumes of plastid preparations derived from the same fresh weights of harvested tissues were used, plastid DNA contents in each preparation must be different because of variable DNA contents per plastid and different plastid numbers per cell. There-
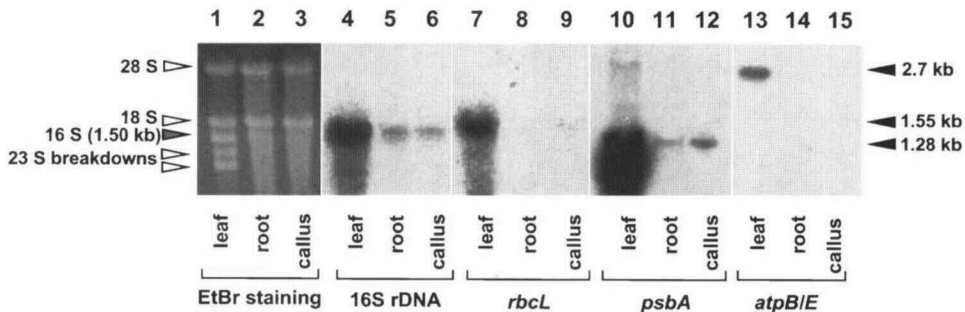

Figure 1. Transcripts for plastid genes from *A. thaliana*. Seventeen micrograms of total RNA from leaves and roots grown on soil for 6 weeks and from calli cultured for 6 weeks was electrophoresed in an agarose gel and transferred to membranes. RNA-DNA hybridization was performed with the 1.2-kbp fragment of tobacco *rbcL*, the 2.2-kbp fragment of maize *psbA*, the 2.0-kbp fragment of maize *atpB/E*, and the 3.1-kbp fragment of maize 16S rDNA as probes. Radioactive bands on the membrane were detected by an image analyzer. Black arrowheads, 1.50 kb, 1.55 kb, 1.28 kb, and 2.70 kb, are estimated sizes of transcripts for 16S rDNA, *rbcL*, *psbA*, and *atpB/E*, respectively.

fore, the endogenous plastid DNA contents were determined by DNA-DNA hybridization with a probe of an intergenic region between *rbcL* and *atpB/E* (rows 5–7), and the run-on activities were standardized as arbitrary units per plastid DNA (Table I). Run-on signals of *rbcL*, *psbA*, and *atpB/E* in calli were 7 to 9% of those in leaves after normalization to endogenous plastid DNAs in reaction mixtures, whereas those in roots were undetectable.

Reduced Plastid DNA Copy Numbers in Roots and Calli

Copy numbers of plastid DNA were examined to determine whether they influenced the rates of RNA synthesis in cells. The amount of plastid DNA in the total cellular DNA from leaves, roots, or calli was determined by DNA-DNA hybridization with the intergenic region between *rbcL* and *atpB/E* as a probe. We have confirmed no hybridization of this probe to nuclear DNA or the other parts of plastid DNA (data not shown). The DNA copy numbers were calculated on the basis of nuclear DNA content determined by hybridization with a gene for the small subunit of Rubisco (*RBCS-3B*; data not shown). The copy number of plastid DNA per nuclear DNA decreased in the order of leaves, calli, and roots (Fig. 3; leaves:roots:calli = 100:20±2:50±5). The results suggest that the alteration of the DNA copy number may be involved in the plastid gene expression.

Nucleotide Sequence of the Region Covering *rbcL* and *atpB/E* Promoters

The region covering *rbcL* and *atpB/E* promoters was cloned and characterized for further analysis to clarify steps regulating the transcript levels. The region including *rbcL* and *atpB/E* promoters was cloned through PCR with thermophilic DNA polymerase with proofreading activity and sequenced (Fig. 4A). The nucleotide sequences of two DNA fragments amplified with primers nos. 1 and 2 and
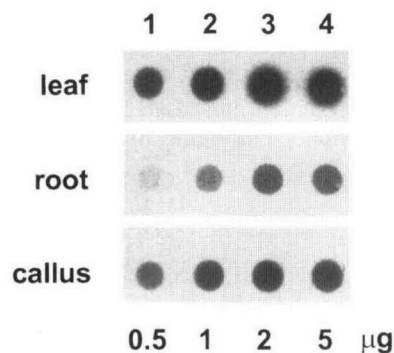

Figure 3. Plastid DNA contents in different tissues of *A. thaliana*. Total cellular DNAs (0.5, 1, 2, and 5 μg each) from leaves, roots, and calli were blotted on membranes and hybridized with the *Sau*3AI/*Nde*I fragment (see Fig. 4A).

nos. 1 and 3 were cloned to obtain pABL11 and pABL12, respectively. These two nucleotide sequences were completely consistent with each other at the overlapping region. Conserved sequences at −35 and −10 in promoters were present in *rbcL*.

No Significant Regulation of DNA Template Activity at Transcription

We previously reported that the expression of some photosynthesis genes was regulated by DNA methylation at transcription (Ngernprasirtsiri et al., 1988; Kobayashi et al., 1990). Therefore, we have examined differences in DNA methylation status of the upstream region of *rbcL* in each tissue using methyl-sensitive and -insensitive endonucleases (*Hpa*II and *Msp*I recognizing the 5′CCGG3′ sequence and *Dpn*I, *Mbo*I, and *Sau*3AI for 5′GATC3′), followed by the subsequent PCR (see Fig. 4A for sites recognized by these enzymes and the PCR primers employed). However,

Table I. *A summary of results of steps regulating expression of plastid genes in A. thaliana*

Copy numbers of plastid DNA, activities of in vitro transcription with *E. coli* RNA polymerase and run-on transcription, and transcript levels were calculated on the basis of amounts of hybridized radioactivities in repeated experiments (three or more times) as presented in Figures 3, 5, 2, and 1, respectively, and the activities and transcript levels were normalized to plastid DNAs. The values are shown as percentages of those obtained with materials derived from leaves.

Plastid Gene	Tissue	Plastid DNA per Cell	Arbitrary Units per Plastid DNA		
			In vitro Transcriptional Activity	Run-on Transcriptional Activity	In vivo RNA Level
16S rDNA	Leaf	100	100	100	100
	Root	20 ± 2	100 ± 5	97 ± 4	23 ± 13
	Callus	50 ± 5	110 ± 4	90 ± 15	8 ± 4
rbcL	Leaf	100	100	100	100
	Root	20 ± 2	110 ± 5	nd[a]	nd
	Callus	50 ± 5	104 ± 4	7 ± 2	6 ± 2
psbA	Leaf	100	100	100	100
	Root	20 ± 2	—[b]	nd	1 ± 0.5
	Callus	50 ± 5	—	7 ± 2	6 ± 2
atpB/E	Leaf	100	100	100	100
	Root	20 ± 2	—	nd	nd
	Callus	50 ± 5	—	9 ± 1	nd

[a] nd, Not detectable (<0.1).   [b] —, Not determined.
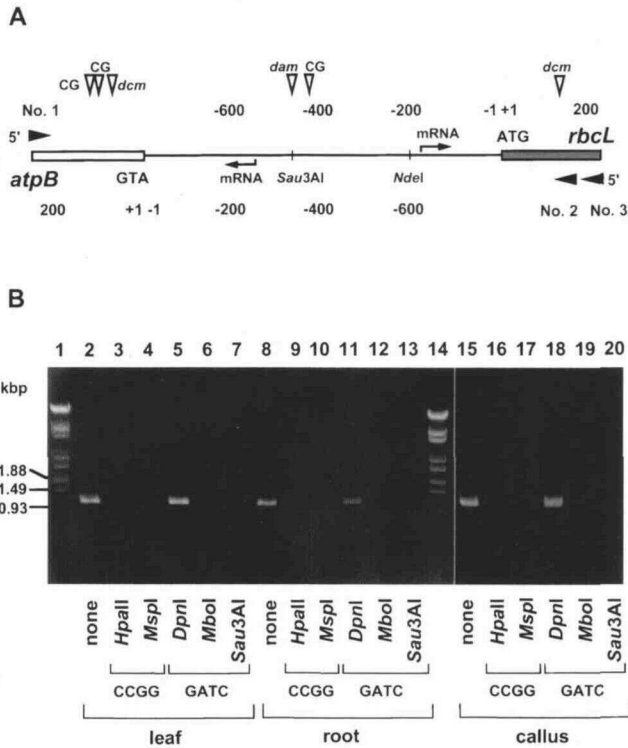
A

B

Figure 4. A, Schematic representation of the region covering promoters of *rbcL* and *atpB/E* of *A. thaliana* based on DNA sequencing of cloned DNA fragments. Open and shaded bars, *atpB* and *rbcL* coding regions, respectively; CG, dcm, and dam, 5'CCGG3', 5'CCA/TGG3', and 5'GATC3' sequences, respectively. B, DNA methylation status at 5'CCGG3' and 5'GATC3' sequences in the region covering *rbcL* and *atpB/E* promoters from *A. thaliana*. Total cellular DNAs (0.1 μg each) from leaves, roots, and calli were digested with the restriction endonucleases: HpaII and MspI recognizing 5'CCGG3', and DpnI, MboI, and Sau3AI for 5'GATC3', in which HpaII and MboI cannot cut the sequences containing 5-methylcytosine and an internal N^6-methyladenine, respectively, while MspI and Sau3AI can cleave the sequences independently of the methylation status, and DpnI can cut only the sequence containing an internal N^6-methyladenine. The digested DNA was subsequently amplified by PCR and electrophoresed in a 0.8% agarose gel. Lanes 1 and 14, Molecular markers; lanes 2, 8, and 15, products with nondigested DNAs. The positions recognized by these endonucleases and a set of primers (nos. 1 and 2) are shown in A.

we could not detect any methylation at these endonuclease sites in any of the tissues examined (Fig. 4B).

To further investigate transcriptional template activities, total cellular DNA from the three tissues was subjected to in vitro transcription for *rbcL* and 16S rDNA using *E. coli* RNA polymerase, which is known to transcribe most plastid genes (Ngernprasirtsiri et al., 1988; Kobayashi et al., 1990). The relevance of this kind of in vitro transcription was proven in our previous work on plastid DNA from tomato on the criterion of the generation of in vitro, full-sized transcripts (Kobayashi et al., 1990). Transcriptional template activities of DNAs from roots, calli, and leaves were 20±1:55±2:100 for 16S rDNA, and 22±1:52±2:100 for *rbcL* (Fig. 5). The proportions of template activities among tissues were almost equivalent to the proportion of the

plastid DNA copy number, as shown in Figure 3 (a summary after standardization by plastid DNA contents is presented in Table I).

To evaluate the quality of total cellular DNA as the templates used in Figure 5, the DNA was electrophoresed in a 0.3% agarose gel and subjected to DNA-DNA hybridization with an *rbcL* probe. The results showed that the sizes of prepared DNA were higher than 100 kb (data not shown), indicating equally high-quality DNA templates from preparations from leaves, roots, and calli. Ngernprasirtsiri et al. (1988) and Kobayashi et al. (1990) demonstrated that plastid DNAs derived from nongreen tissues showed less activity as the templates in contrast to detectable activities of plastid DNA from green tissues. However, in *A. thaliana* three kinds of DNA prepared from three different tissues, i.e. leaves, roots, and calli, have shown similar intensities after normalization to plastid DNA in the in vitro transcription assay.

DISCUSSION

Posttranscriptional (Gruissem et al., 1988; Stern et al., 1989) or transcriptional (Ngernprasirtsiri et al., 1988; Kobayashi et al., 1990; Schrubar et al., 1990) regulation is recognized as a predominant step in the regulation of expression of plastid genes for photosynthesis proteins in nongreen tissues. Therefore, we analyzed the regulatory steps in roots and calli of *A. thaliana*, which is a potentially very attractive plant for further genetic dissection. In this investigation we tried to harvest a mass of *A. thaliana* roots and analyze the key steps of expression of *rbcL*, *psbA*, *atpB/E*, and 16S rDNA. Expression of *rbcL*, *psbA*, and *atpB/E* is concluded to be regulated mainly by activities of transcriptional machinery, and 16S rRNA levels are controlled by
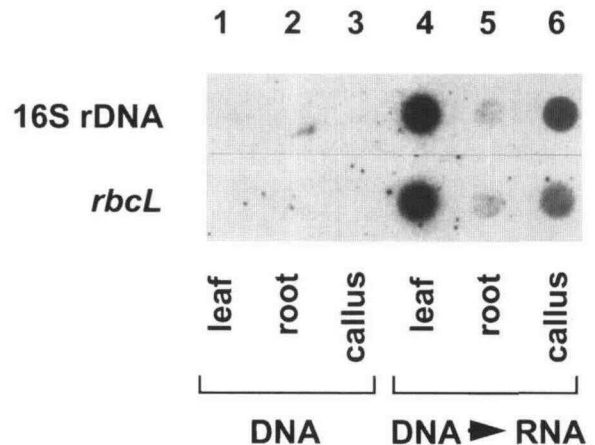

Figure 5. In vitro transcription of plastid DNAs from *A. thaliana*. RNAs generated with *E. coli* RNA polymerase (indicated as "DNA ➤ RNA") from total cellular DNAs (1.0 μg each) were blotted and subjected to hybridization with the radioactive 3.1-kb fragment of 16S rDNA and 1.2-kb fragment of *rbcL* as probes. Each sample without *E. coli* RNA polymerase (indicated as "DNA") was analyzed as a control to confirm the lack of endogenous RNA contaminated in the DNA preparation.

RNA stability, in addition to plastid DNA copy number regulation (see Table I).

Transcriptional activity in plastids may depend on the plastid DNA copy number (Aguettaz et al., 1987), DNA modification (Ngernprasirtsiri et al., 1988; Kobayashi et al., 1990), DNA conformation (Stirdivant et al., 1985; Thompson and Mosig, 1987), or plastid RNA polymerase (Schrubar et al., 1990) and its associated factors. In this investigation we have demonstrated that the plastid DNA content per cell is variable among leaves, roots, and calli, approximately 100:20:50 in proportion (Fig. 3; Table I). Although the decrease of the DNA copy number in amyloplasts in roots and calli results in the decline of plastid gene expression, this decrease is insufficient to entirely account for scarcely detectable levels of RNA of 16S rDNA, rbcL, psbA, and atpB/E (Fig. 1). Therefore, plastid copy number regulation does not play the complete role in the control of plastid gene expression in A. thaliana. The incomplete correlation between DNA copy number and plastid transcriptional activity has also been reported in the chloroplast development of spinach (Deng and Gruissem, 1987) and barley (Baumgartner et al., 1989), and in a study with a barley pigment-deficient mutant (Rapp and Mullet, 1991).

DNA methylation is reported as a mechanism of transcriptional suppression in nonphotosynthetic tissues such as tomato fruits (Kobayashi et al., 1990) and a white-culture cell line of sycamore (Ngernprasirtsiri et al., 1988), in which the in vitro transcription with E. coli RNA polymerase or soluble plastid RNA polymerase was suppressed by methylated DNA templates. Influence of DNA superhelicity on transcriptional activities of plastid genes has also been proposed (Stirdivant et al., 1985; Lam and Chua, 1987; Thompson and Mosig, 1987). To investigate the altered activity of DNA templates in A. thaliana, we have performed the in vitro transcription with E. coli RNA polymerase. Results of the in vitro transcription have revealed that all transcriptional activities depend on the amounts of plastid DNA but not on the sources of DNA (Figs. 3 and 5; Table I). No methylation at 5'CCGG3' and 5'GATC3' sequences in the upstream region of the rbcL reading frame (Fig. 4) supports the lack of regulation by DNA template activity at transcription.

There are some contradictory results reported concerning DNA methylation status revealed with methylsensitive and -insensitive endonucleases (Kobayashi et al., 1990; Kobayashi, 1991; Marano and Carrillo, 1991). Careful arguments about DNA methylation status are desired, because the endonucleases provide information of nucleotide sequences not necessarily involved in transcriptional regulation. In addition to precise analysis of methylation by HPLC of nucleosides and bases derived from hydrolyzed DNA and by direct genomic sequencing, evaluation of DNA template activities by in vitro transcription with prepared RNA polymerases is needed. The significant difference in the involvement of DNA methylation between results obtained here and others observed in tomato fruits (Kobayashi et al., 1990) and the white-culture cell line of sycamore (Ngernprasirtsiri et al., 1988) may be due to differences in the tissues; roots and calli cultured for short periods, as employed here, have the potency to differentiate into photosynthetic mature plants in the presence of plant hormones (data not shown). This is in contrast to tomato fruits and the white-culture cell line of sycamore, which never turn green. DNA methylation is recognized as an irreversible process, as no enzymic demethylation activities have been reported in any organisms. Therefore, DNA methylation may be involved in the suppression of expression of photosynthesis genes in irreversibly degreened tissues.

The relative transcriptional activity of 16S rDNA per plastid DNA determined by the run-on assay is leaves:roots:calli = 100:97:90 (Table I). The results show that 16S rDNA is transcribed at the identical rate in each tissue on the basis of the same amounts of plastid DNA, leading to the speculation that the instability of the transcribed RNA (16S rRNA) must be the cause for the low levels of 16S rRNA in roots and calli (Table I), whereas expression of rbcL, psbA, and atpB/E was transcriptionally suppressed (Table I). We suppose that association of 16S rRNA with ribosomal proteins may stabilize 16S rRNA. This situation has been observed in E. coli at low growth rates (Gausing, 1977). It is supposed that ribosomal proteins directed by plastid and nuclear genes are deficient in nongreen plastids so that rRNAs could not assemble ribosomes. By contrast, the 16S rRNA level does not significantly decrease in nongreen plastids in tomato fruits (Kobayashi et al., 1990) and the white-culture cell line of sycamore (Ngernprasirtsiri et al., 1988). The regulation by RNA stability for plastid genes is also proposed in hydroponic roots (Deng and Gruissem, 1988) and developing chloroplasts of spinach (Deng and Gruissem, 1987) and barley (Mullet and Klein, 1987).

Transcriptional activities of rbcL, psbA, and atpB/E in A. thaliana calli as judged by the run-on assay were 10 to 14 times lower than those in leaves on the basis of the same amounts of plastid DNA (Table I). Therefore, the low level of transcripts for rbcL, psbA, and atpB/E is likely ascribed to the lower transcriptional activity. Since there is little possibility of transcriptional regulation by methylation of template DNA, as described above, plastid RNA polymerase and its possible associated regulatory factors may play distinctive roles in transcription of rbcL, psbA, and atpB/E. It has been reported that the increase of plastid transcriptional activity in greening sorghum seedlings is correlated with the increasing level of the β subunit of plastid RNA polymerase (Schrubar et al., 1990). Although it has been reported that the transcriptional activities of most genes in amyloplasts do not significantly differ from those in chloroplasts (Deng and Gruissem, 1987, 1988), the net transcriptional activities might be mimicked by artifacts, as pointed out by Ngernprasirtsiri et al. (1990). A portion of $[\alpha^{-32}P]UTP$ is supposed to be converted into $[\alpha^{-32}P]dCTP$ via catalysis by CTP synthetase, nucleoside diphosphate kinase, and ribonucleotide reductase possibly present in prepared crude plastids for run-on transcription, and it may be incorporated into small sizes of DNA by enzyme activities associated with the plastid fraction. Therefore, complete DNase digestion after

removing phenol by diethylether extraction or without phenol treatment is needed to eliminate the newly synthesized radioactive DNA species.

Present knowledge is that plastids have at least two types of RNA polymerases, one tightly bound to plastid DNA and involved in the transcription of rRNA, and the other present as a soluble form and responsible for transcription of both tRNA and mRNA (Greenberg et al., 1984, 1985). Most recently, Allison et al. (1996) demonstrated the presence of plastid transcription system that does not require E. coli-type promoters and preferentially transcribes housekeeping genes rather than photosynthesis genes such as rbcL and psbA. The participation of two distinct RNA polymerases for rRNA and mRNA might explain our results showing different transcriptional regulation for 16S rDNA and the other genes, rbcL, psbA, and atpB/E. On the other hand, Link and colleagues revealed evidence that sigma-like transcription factors from mustard (Bülow and Link, 1988) were phosphorylated in etioplasts but not in chloroplasts (Tiller and Link, 1993), and proposed that the phosphorylation of the sigma-like transcription factors might suppress transcriptional elongation. Therefore, a decrease in the transcriptional activity of rbcL, psbA, and atpB/E in amyloplasts observed in this investigation may be caused by: (a) phosphorylation of the sigma-like transcription factors; (b) a low level of the active soluble form of RNA polymerase; and/or (c) conversion of RNA polymerase to an inactive status by other factors such as the changed concentration of K^+ (Greenberg et al., 1985). Plastid RNA polymerases in chloroplasts and nonphotosynthetic plastids should be further analyzed to reveal their regulatory mechanisms.

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