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A plant basal *in vitro* system supporting accurate transcription of both RNA polymerase II- and III-dependent genes: supplement of green leaf component(s) drives accurate transcription of a light-responsive *rbcS* gene

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An *in vitro* transcription initiation system has been developed from nuclei of rapidly growing, non-green tobacco (*Nicotiana tabacum*) cultured (BY-2) cells. Conditions for nuclear extraction and *in vitro* transcription reaction have been optimized with a tobacco β-1,3-glucanase gene, a constitutively expressed gene in BY-2 cells. The *in vitro* system supports accurate transcription of RNA polymerase II-dependent promoters from not only plant genes (tobacco β-1,3-glucanase gene, cauliflower mosaic virus 35S promoter) but also animal genes (adenovirus 2 major late promoter, simian virus 40 early major promoter). In addition, this system drives accurate transcription of an RNA polymerase III-dependent *Arabidopsis thaliana* U6 snRNA gene. As BY-2 cells do not differentiate in response to light or any other stimuli, they would provide a basal transcription system which lacks tissue-specific and light-responsive nuclear signals as well as chloroplast-derived signals. Consequently, the BY-2 cell-free system is unable to transcribe the tomato gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS3C*) whose expression is tissue-specific and light-inducible. However, the transcription of *rbcS3C* was obtained by supplementing the BY-2 system with a nuclear extract of light-grown tomato seedlings. The promoter regions necessary for *rbcS* transcription was mapped *in vitro* using a series of 5′ deletion mutants. The 351 bp upstream sequence is essential and the further upstream region from −351 to −441 enhances its transcription. The *in vitro* basal system will be useful to identify specific signals from both the nucleus and chloroplast in green leaves and other organs/tissues.

Key words: *in vitro* transcription/RNA polymerase II (pol II)/RNA polymerase III (Pol III)/*rbcS*/U6 snRNA

Introduction

The growth and development of plants depend on the expression of tens of thousands of genes, which is regulated at the level of transcription, RNA processing, translation and post-translation. Transcription, being the first step, is the most important regulatory level that involves complex sets of DNA−protein and protein−protein interactions. To elucidate various regulatory components and their interactions, the necessity of versatile plant *in vitro* transcription systems that provide faithful reproduction of *in vivo* gene expression has always been acknowledged. Attempts have been made to develop RNA polymerase II (Pol II)-dependent *in vitro* transcription systems from wheat germ (Ackerman *et al.*, 1987; Flynn *et al.*, 1987; Yamazaki and Imamato 1987; Yamazaki *et al.*, 1990a,b) and plant cultured cells (Cooke and Penon, 1990; Roberts and Okita, 1991; Arias *et al.*, 1993; Yamaguchi *et al.*, 1993; Frohnmeyer *et al.*, 1994). However, these systems are either inefficient or of limited use (see Discussion).

We have analyzed tobacco plastid genes (Sugiura, 1992) and nuclear genes encoding plastid components (Li *et al.*, 1993; Sugita *et al.*, 1994) and are now studying transcriptional regulation of the nuclear genes during plastid development. The tobacco plant provides a powerful tool to analyse plant gene transcription *in vivo* because of the well-established transgenic technique. Therefore, we started to develop an *in vitro* nuclear transcription system from tobacco (*Nicotiana tabacum*) as a complement to transgenic tobacco technique. We selected tobacco non-green suspension BY-2 cells as a source, since this cell line has the fastest growth rate among the plant cell lines reported (Nagata *et al.*, 1992). In addition, BY-2 cells contain proplastids which do not differentiate, or in other words, many nuclear genes encoding plastid proteins and controlling plastid development are not expressed in BY-2 cells. This would give a basal *in vitro* transcription system. By combination of the basal transcription system and active fractions from organs and tissues in which the genes in question are specifically transcribed, *cis*-elements and *trans*-factors involved in their expression are expected to be detected *in vitro*. To test this rationale, the nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (*rbcS*), which is expressed in light- and organ-dependent manners (e.g. Herrera-Estrella *et al.*, 1984; Morelli *et al.*, 1985; Sugita and Gruissem, 1987) was practised in the system.

Here we describe the development of a basal *in vitro* transcription initiation system from tobacco BY-2 cell nuclei. This *in vitro* system supports accurate transcription of Pol II-dependent constitutively expressed genes and a Pol II-dependent *rbcS* gene only when supplemented with leaf nuclear extracts. Furthermore, we describe for the first time that an RNA polymerase III (Pol III)-dependent plant gene has also been accurately transcribed in the plant *in vitro* system.

Results

Development of a Pol II-dependent in vitro transcription initiation system

Tobacco BY-2 cell line derived from the seedlings of *N.tabacum* L. cv. Bright Yellow 2 grows fast and multiplies 80- to 100-fold in a week (the generation time is 13 h);

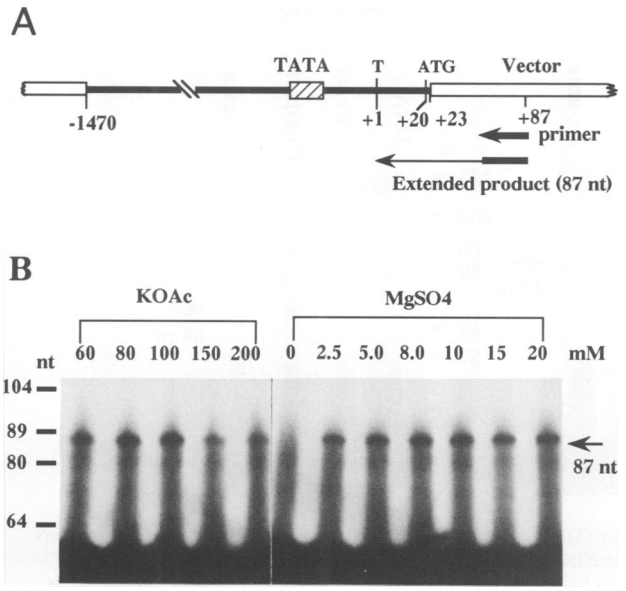

Fig. 1. *In vitro* transcription of tobacco β-1,3-glucanase gene (*GNPS*) with the BY-2 nuclear extract. (**A**) Schematic view of pGNPS DNA template and primer extension assay. A short arrow indicates the T7DKS primer. A long arrow shows the expected extended product (87 nt) calculated from the primer (+87) to the *in vivo* transcription start site (+1). (**B**) Optimization of potassium and magnesium concentrations. Reverse transcribed cDNAs (extended products, 87 nt) of the *in vitro* synthesized transcripts are indicated by an arrow. The size marker is the pBR322 *Hae*III digest.

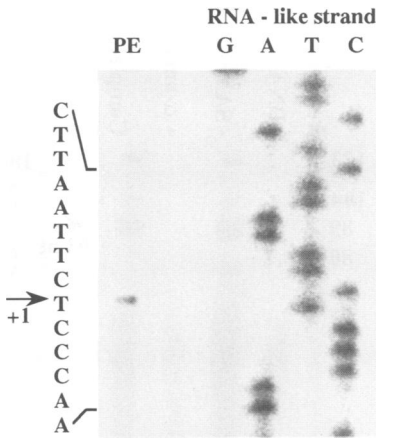

Fig. 2. Determination of the transcription initiation site *in vitro* from *GNPS*. The primer extension product from transcripts was compared with DNA sequence ladders generated by the same primer. The RNA start site is shown as +1.

thus it is suitable for molecular and biochemical studies of plant cells (Nagata *et al.*, 1992). Our preliminary experiments indicated that RNA synthesis activity ([^{3}H]UTP incorporation) is the highest and RNase activity is relatively low at the mid-log phase of BY-2 cells. Considering that nuclease and protease activities are much higher in whole cell extracts than in nuclear extracts, we used nuclei from mid-log phase cells for further experiments. Nuclear extracts were prepared by disrupting purified nuclei with a high concentration of salt followed by ultracentrifugation to remove endogenous DNA.

Experiments were conducted to determine optimal conditions for accurate *in vitro* transcription with the promoter of a tobacco β-1,3-glucanase gene (*GNPS*) which is expressed highly in BY-2 cells (Ohme-Takagi and Shinshi, 1990) and its expression is further stimulated during the preparation of protoplasts. We have measured transcription activity by primer extension analysis of transcripts synthesized *in vitro* as this assay yields transcriptional start sites at a nucleotide level and prevents pseudo results sometimes observed by using the G-free cassette assay (our unpublished observation). In addition, the use of primers complementary to vector sequences in circle DNA templates greatly reduces background due to non-specific transcription from endogenous DNA incompletely removed by centrifugation of the nuclear extracts. As shown in Figure 1A, the transcription initiation site of *GNPS in vivo* has been mapped at the initiation site (T) located 20 bp upstream from the coding region (Ohme-Takagi and Shinshi, 1990), and hence correct initiation of *GNPS* transcription *in vitro* is expected to generate an extended product of 87 nucleotides (nt). Our *in vitro* reaction with *GNPS* produced a single clear band of predicted size (Figure 1B), and T was verified by separating the extended product in parallel with the corresponding sequence ladders (Figure 2). The results indicate that our *in vitro* system from tobacco BY-2 cells supports accurate initiation of *GNPS* transcription.

It was found that the optimal salt concentration for KOAc was 80 to 100 mM (Figure 1B) and for KCl was 60 mM (data not shown). An optimal $MgSO_4$ concentration was broad, from 2.5 to 15 mM, with a peak at 5–8 mM (Figure 1B). Mn^{2+} could not be substituted for Mg^{2+}; rather, the addition of Mn^{2+} resulted in non-specific transcription (data not shown). Optimal reaction temperature and time were 28–30°C and 30 min, respectively. Incubation longer than 45 min caused product degradation. The amount of DNA template was optimal at 1 pmol in a 50 μl reaction mixture containing ~150 μg nuclear proteins, and saturated at ~1.5 pmol (data not shown). The addition of 1 mM *S*-adenosyl-L-methionine chloride apparently reduced the degradation of the transcripts (Figure 3, *ADML*).

To examine whether the products transcribed by the tobacco nuclear extract were Pol II dependent, *in vitro* reactions were performed in the presence or absence of α-amanitin at a low concentration (20 μg/ml), which specifically inhibited Pol II activity (Jendrisak and Guilfoyle, 1978). The results with *GNPS*, adenovirus 2 major late promoter (*ADML*), simian virus 40 early major promoter (*SV40*) and cauliflower mosaic virus 35S promoter (*CaMV35S*) showed that all the transcribed products were DNA template dependent, α-amanitin sensitive and initiated at the accurate start sites as determined *in vivo* (Figure 3 and some data not shown). These results suggest that at least basal factors for plant transcription are similar to those of animals and are functionally exchangeable. The maximum transcripts obtained from *GNPS* and *ADML* were ~1 fmol, which corresponded to 0.001 transcript per template. Transcription from *CaMV35S* was ~10-fold lower than that from *GNPS* in our reaction conditions.

Characterization of the rbcS3C promoter in vitro after supplementing a nuclear extract from light-grown seedlings

The tomato *rbcS3C* gene, one of the most extensively studied *rbcS* genes, is actively transcribed in cotyledons

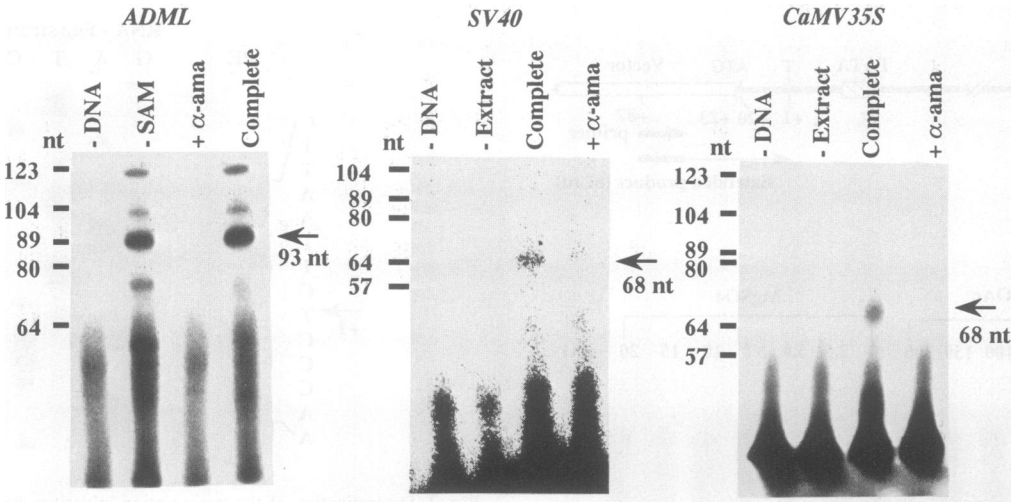

Fig. 3. *In vitro* transcription of adenovirus major late (*ADML*), SV40 early major (*SV40*) and cauliflower mosaic virus 35S (*CaMV35S*) promoters with the BY-2 nuclear extract. Standard transcription conditions were used. Where indicated, α-amanitin (α-ama) was added to 20 μg/ml (final concentration). Extended products from *in vitro* synthesized transcripts are shown by arrows (see Table I for their sizes). In *ADML*, minor bands above the 93 nt band were sometimes observed depending on extracts and are non-specific transcripts. The size marker is the pBR322 *Hae*III digest.

of light-grown seedlings and leaves, but not in dark-grown seedlings and other non-photosynthetic organs (Sugita and Gruissem, 1987; Sugita *et al.*, 1987; Manzara *et al.*, 1991; Carrasco *et al.*, 1993). We then carried out *in vitro* transcription with the *rbcS3C* promoter from tomato, considering that tomato is close to tobacco (both belong to the Solanaceae family). Initially we confirmed by Northern blot analysis that *rbcS* genes are absolutely inactive in tobacco BY-2 cells, but actively expressed in tobacco leaves (Figure 4).

Two promoter constructs from tomato *rbcS3C* were first tested *in vitro*; a full-length promoter (pRBC-1657) containing the 1657 bp upstream sequence, in which all regulatory sequences are considered to be present (see Figure 7), and a truncated promoter (pRBC-174) containing the 174 bp upstream region. As shown in Figure 5 (lane 7), transcription from the *rbcS3C* promoter pRBC-1657 could not be detected with the BY-2 nuclear extract as expected. We then prepared a nuclear extract from light-grown tomato leaves. After supplement of the tomato extract, the BY-2 system drove accurate transcription from the *rbcS* promoter, detected as a clear 100 nt band by primer extension assay (Figure 5, lanes 3 and 4). The transcription was α-amanitin sensitive (lanes 5 and 6) and hence was Pol II dependent. The transcription initiation site was confirmed to be exactly the same as *in vivo* (Carrasco *et al.*, 1993) (Figure 6). The tomato nuclear extract itself did not support *rbcS* transcription (Figure 5, lane 2), probably because of low Pol II activity compared with that of BY-2 cells. The truncated *rbcS3C* promoter pRBC-174 was not transcribed by the BY-2 extract with or without the tomato extracts (Figure 5, lanes 9 and 10), indicating that sequences upstream of −174 are necessary for green leaf extract-dependent transcription.

A series of deleted promoters from *rbcS3C* were prepared to detect additional *cis*-elements located further upstream from −174 (Figure 7). The promoter region up to −351 could permit gene transcription *in vitro* under the above condition although the signal was weak (Figure 7, lane 9). The efficiency of transcription remained unchanged with deletion promoters carrying 441 bp or

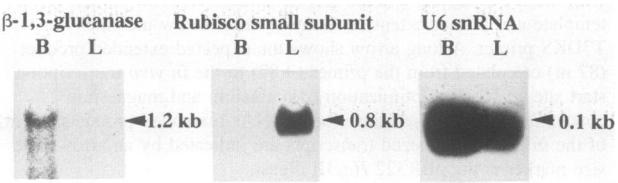

Fig. 4. Transcript levels of the genes encoding β-1,3-glucanase, Rubisco small subunit and U6 snRNA in tobacco BY-2 cells (B) and leaves (L). RNA samples (10 μg each) were analyzed by Northern blot hybridization using gene-specific probes. Arrows indicate transcript sizes.

longer (up to 1657) of the 5′-upstream sequence (lanes 2–8). Therefore, a region from −351 to −441 must function as an enhancer-like element, which activated the transcription nearly 10-fold as compared with the −351 promoter. Our results indicate that transcription of tomato *rbcS3C* is regulated by *cis*-elements located in the 5′ upstream region of up to position −441 and by *trans*-factor(s) present in green leaves but absent in BY-2 cells.

Pol III-dependent in vitro transcription of a U6 snRNA gene

In our extract, Pol III activity was estimated to be ~10% of total RNA polymerase activities based on [^{3}H]UTP incorporation experiments in the presence of a low level (4 μg/ml) of α-amanitin (data not shown). To investigate whether this low α-amanitin-resistant RNA synthesis is due to Pol III activity, we tested transcription *in vitro* of an *Arabidopsis* U6 snRNA gene, which has been reported to be transcribed by Pol III *in vivo* (Waibel and Filipowicz, 1990a,b). Northern blot analysis suggested that U6 snRNA genes are strongly expressed in tobacco BY-2 cells (Figure 4). *In vitro* reactions were conducted with the plasmid pBU6-28 DNA containing the promoter of U6 snRNA gene under the same conditions as in the reaction of Pol II. As shown in Figure 8, U6 gene transcription was initiated in both the absence and presence of a low concentration of α-amanitin (20 μg/ml) but not with a high concentration (400 μg/ml) which is known to inhibit

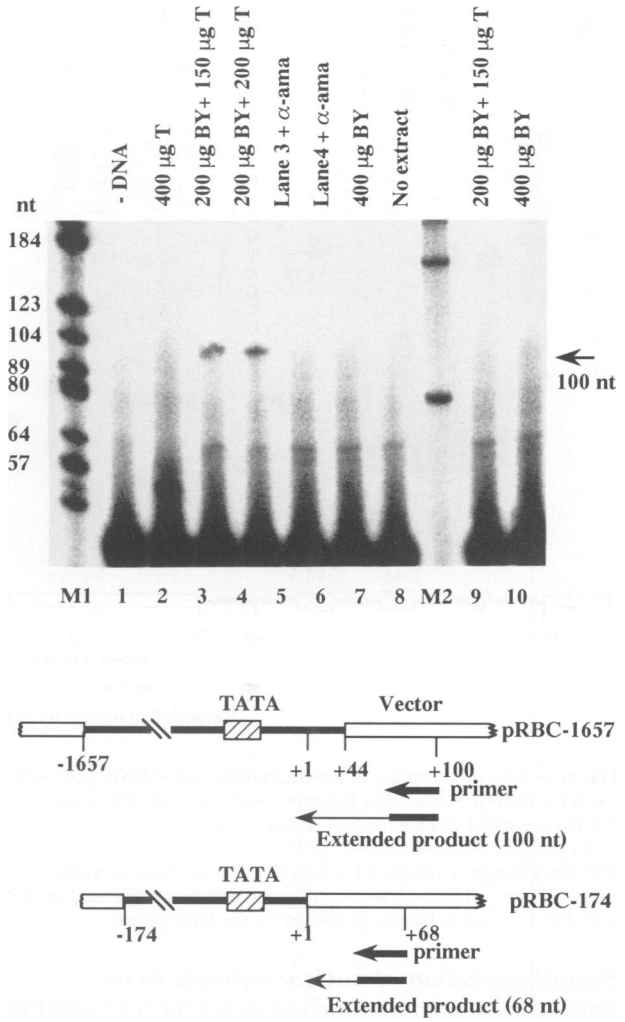

Fig. 5. *In vitro* transcription of the tomato *rbcS3C* gene. A full-length promoter (pRBC-1657) (lanes 1–8) and a truncated promoter (pRBC-174) (lanes 9 and 10) were transcribed by the tobacco BY-2 nuclear extract (BY) with or without the tomato leaf nuclear extract (T). Extended products (100 nt) from *in vitro* synthesized transcripts are shown by an arrow. Size markers are pBR322 *Hae*III digest (M1) and ØX174-RF-DNA *Hin*cII digest (M2). Structure of the *rbcS3C* promoter and the assay design are illustrated below.

both Pol II and Pol III activities. The *in vitro* transcription initiation site was determined by primer extension and found to be the same as that *in vivo* (Waibel and Filipowicz, 1990b). This indicates that the *in vitro* system supports accurate transcription of Pol III-dependent genes in addition to Pol II-dependent genes.

Discussion

Development of plant in vitro transcription systems

Development of *in vitro* transcription systems has been reported using whole cell and nuclear extracts from plant suspension cultured cells and wheat germ. Whole cell extracts generally contain an unacceptable amount of nucleases and unknown transcription inhibitors and it is necessary either to fractionate the extracts or to immobilize the templates for removing inhibitors and reducing the non-specific transcription background (Cooke and Penon,

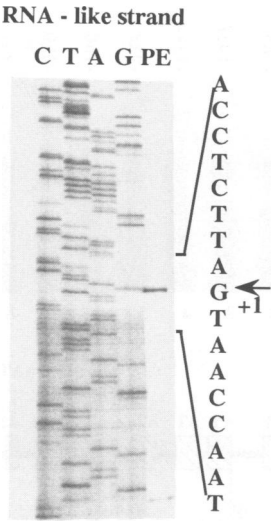

Fig. 6. Determination of the transcription initiation site *in vitro* from the tomato *rbcS3C* gene. The extended product from *in vitro* transcripts was compared with DNA sequence ladders generated by the same primer. The RNA start site is shown as +1.

1990; Arias *et al.*, 1993). Furthermore, most of the systems have been developed only for plant virus promoters (Ackerman *et al.*, 1987; Yamazaki and Imamoto, 1987; Yamazaki *et al.*, 1990a,b; Roberts and Okita, 1991; Yamaguchi *et al.*, 1993). Recently, a light-responsive *in vitro* transcription system derived from evacuolated parsley protoplasts has been reported (Frohnmeyer *et al.*, 1994). This system will be useful for elucidating signalling mechanisms but should be prepared freshly before use; in addition, it is also difficult to eliminate possible interference caused by endogenous DNA and RNA.

In vitro transcription systems from isolated nuclei of animals and yeast have provided powerful tools for studying gene expression *in vitro* (e.g. Gorski *et al.*, 1986; Lue and Kornberg, 1987; Kamakaka *et al.*, 1991). Purified nuclei simplify the biochemical fractionation and analyses of nuclear components. Therefore we selected nuclei from tobacco BY-2 cells as a source for developing an *in vitro* plant transcription system. As the nuclear envelope is permeable to small solutes and proteins, the leakage of necessary factors is inevitable during the isolation of nuclei. A high concentration of Ficoll seems to prevent this rapid loss of nuclear constituents (Lue and Kornberg, 1987). We used 18% Ficoll (final concentration) during nuclear isolation because we have obtained no active nuclear extracts with a Ficoll concentration <15%. Phosphorylation and dephosphorylation play a very important role in regulation of gene expression through modification of specific proteins (Sun *et al.*, 1993). Nuclear extracts inactivated by dephosphorylation could not be rescued by the addition of protein kinase C, suggesting that some specific phosphoproteins are critical in transcription steps (data not shown). Therefore, we routinely added 10 mM NaF in isolation buffers to inhibit phosphatase activities.

The present reaction conditions were optimized using a homologous (tobacco) gene *GNPS*, which has a strong promoter in BY-2 cells. The ion requirement was similar to those of some other reports (Roberts and Okita, 1991;

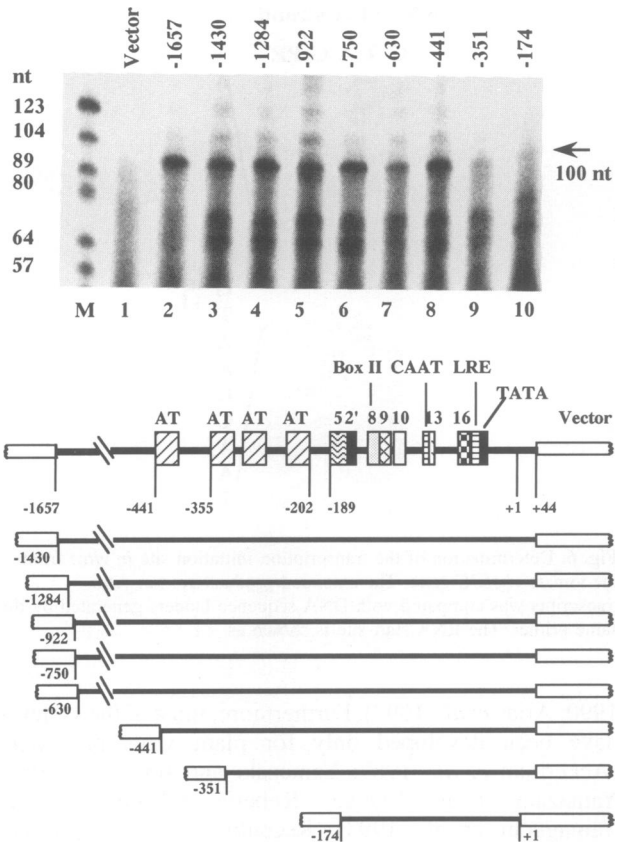

Fig. 7. *In vitro* transcription of a series of truncated promoters from the *rbcS3C* gene. Numbers above each lanes represent the size of 5′ upstream regions (see below). Each reaction contains ~150 μg protein of the tobacco BY-2 nuclear extract and 150 μg protein of the tomato leaf nuclear extract. An arrow (100 nt) indicates expected products. The assay design is shown as in Figure 5. Schematic representation of the promoter regions of *rbcS3C* and its deletion mutants are shown below. Boxes represent conserved sequence motifs and their designations are from Manzara *et al.* (1991). Numbers below the diagrams indicate the distance from the transcription start site (+1).

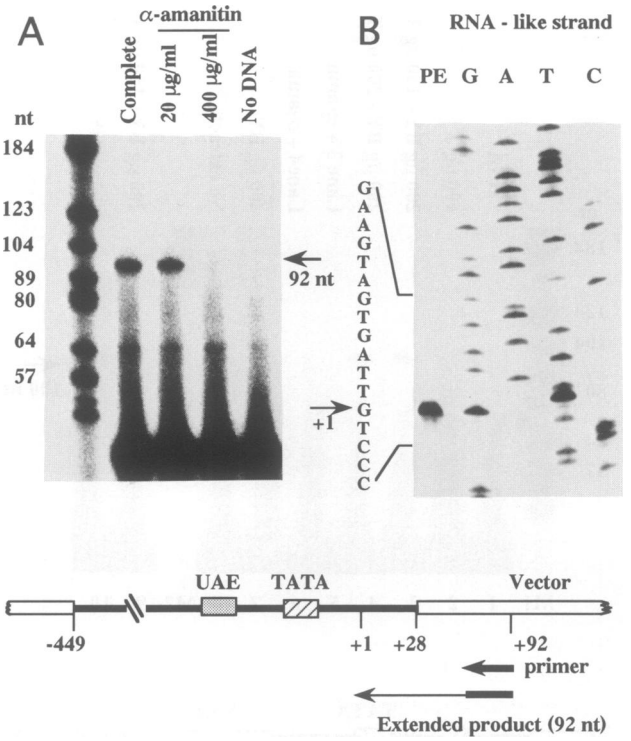

Fig. 8. *In vitro* transcription of the *Arabidopsis* U6 snRNA gene with the BY-2 nuclear extract. (**A**) Reaction conditions followed those of Pol II except that α-amanitin was added to the indicated concentrations. Extended products (92 nt) are indicated by an arrow. The assay design is illustrated below. (**B**) The *in vitro* transcription initiation site (+1) was determined by comparing the extended product with DNA sequence ladders generated by the same primer.

Arias *et al.*, 1993; Frohnmeyer *et al.*, 1994) but contrasted with the wheat germ chromatin extract which requires exclusively Mn^{2+} (Yamazaki and Imamoto, 1987; Yamazaki *et al.*, 1990a,b). Efficiency of our system was ~0.001 transcript per template, which was low compared with that attained by a soluble nuclear fraction (SNF) from *Drosophila* embryos (0.45; Kamakaka *et al.*, 1991) and that of HeLa cell extracts (1.4–4; Shapiro *et al.*, 1988), but similar to that (0.001–0.0001) of yeast nuclear extracts (Lue and Kornberg, 1987). Using Sarkosyl, which permits elongation by Pol II but inhibits assembly of new initiation complexes (Kadonaga, 1990), we estimated that our extract supports one-and-a-half rounds of transcription (data not shown) which is lower than the two rounds by SNF (Kamakaka *et al.*, 1991) and 10 rounds by a *Drosophila* extract (Kadonaga, 1990). One possibility for the lower efficiency is that the protein content of plant cell nuclei is much lower than that of animal cells. We could routinely obtain concentrations of only 6–8 mg/ml protein in BY-2 nuclear extracts, but of 30 mg/ml in SNF. Another possibility is that BY-2 nuclear extracts contain more inhibitors, including nucleases, phosphatases, proteases and non-specific DNA-binding proteins than do those from animal cells.

Supplementation of nuclear extracts from light-grown seedlings drives accurate transcription of rbcS3C

Transcription of plant genes *in vitro* has not been reported so far from plant developed tissues. Gel-shift and DNA footprint experiments have indicated the presence of specific regulatory factors in the nuclear extracts of specific plant tissues (e.g. Green *et al.*, 1987; Buzby *et al.*, 1990; Manzara *et al.*, 1991; Carrasco *et al.*, 1993). We have shown for the first time that the *rbcS* gene was accurately transcribed *in vitro* only by supplementing with the nuclear extract from the green tissues. The *rbcS3C* promoters with longer than 441 bp of its 5′ upstream sequence were fully active in our *in vitro* system, while the −174 truncated promoter, which contains TATA, LRE, CAAT and Box II (see Figure 7), was transcriptionally inactive. As the −351 truncated promoter was weakly transcribed, essential *cis*-element(s) must reside in positions −351 to −174 and enhancer like element(s) in positions −441 to −351. The sequence from −441 to −174 contains several AT-rich motifs which may serve as basic and enhancer elements.

AT-rich motifs have been identified in the promoter regions of other plant genes (Jofuku *et al.*, 1987; Jensen *et al.*, 1988; Bustos *et al.*, 1989; Datta and Cashmore, 1989; Jordano *et al.*, 1989; Jacobsen *et al.*, 1990; Laursen *et al.*, 1994). An AT-rich motif in the β-phaseolin promoter has been shown to enhance its expression in transgenic tobacco plants (Bustos *et al.*, 1989). Nuclear factor AT-1 from pea could be reversibly phosphorylated and only its non-phosphorylated form binds to the AT-rich motif of

Table I. DNA templates used in this work

Plasmid name	Segments of promoters[a]	Size of transcripts[b]	Genes
pGNPS	−1470 to +23	87	Tobacco β-1,3-glucanase
pB35S-440	−440 to +1	68	Cauliflower mosaic virus 35S
pADML[c]	−459 to +438	93	Adenovirus major late
pBSV40	−234 to +1	68	Simian virus 40 early major
pRBC-1657	−1657 to +44	100	Tomato *rbcS3C* full length
pRBC-1430	−1430 to +44	100	*rbcS3C* deletion
pRBC-1284	−1284 to +44	100	*rbcS3C* deletion
pRBC-922	−922 to +44	100	*rbcS3C* deletion
pRBC-750	−750 to +44	100	*rbcS3C* deletion
pRBC-630	−630 to +44	100	*rbcS3C* deletion
pRBC-441	−441 to +44	100	*rbcS3C* deletion
pRBC-351	−351 to +44	100	*rbcS3C* deletion
pRBC-174	−174 to +1	68	*rbcS3C* deletion
pBAU6-28	−449 to +28	92	*Arabidopsis* U6 snRNA
pRRNA	−122 to +15	84	*Vicia faba* rDNA

[a]+1 represents the initiation site determined *in vivo*.
[b]Calculated sizes (nt).
[c]In pUC19.

tomato *rbcS3A* gene *in vitro* (Datta and Cashmore, 1989). Recently, the AT-rich sequence of soybean leghaemoglobin c3 promoter, binding with a nodule nuclear factor (NAT2), was shown to stimulate transcription as a general *cis*-element (Laursen *et al.*, 1994). DNase I protection patterns in the three tomato AT-rich motifs (located between −355 and −202) of *rbcS3C* promoter showed no difference between the extracts of green leaves (transcriptionally active) and young fruits (inactive), while one AT-rich motif was unprotected by the extract from dark-grown cotyledons (inactive) (Carrasco *et al.*, 1993). Our preliminary gel-shift analysis indicated that the AT-rich regions were bound by proteins of nuclear extracts from green seedling and BY-2 cells (data not shown). It might be possible that some AT-rich binding factors interfere with some repressor-like binding proteins, since the light-induced transcription of *rbcS3C* is not directly controlled at the level of DNA−protein interaction but is apparently controlled by protein−protein interaction or modification of DNA-binding proteins (Manzara *et al.*, 1991). The *in vitro* system will facilitate functional analysis of protein−protein interaction and protein modification in transcriptional initiation.

Transcription of *rbcS* and *cab* genes has been reported to be controlled also by chloroplast signals (Taylor, 1989; Susek *et al.*, 1993). To date the molecular nature of chloroplast signals is unknown. As BY-2 cells do not differentiate in response to light or any other stimuli, our *in vitro* system is thought to lack tissue-specific and light-responsive nuclear signals as well as chloroplast-derived signals, indicating that the system is useful to identify specific signals from both the nucleus and chloroplast in green leaves.

Pol III-dependent transcription in vitro

Pol III synthesizes tRNAs, 5S rRNA and other types of cytoplasmic and nuclear small RNAs (snRNA) including U6 snRNA. U6 snRNA and their genes were isolated from several plant sources (Szkukalek *et al.*, 1990; Waibel and Filipowicz, 1990b; Solymosy and Pollák, 1993). The expression of an *Arabidopsis* U6 snRNA gene has been extensively studied in transfected mesophyll protoplasts of tobacco (*Nicotiana plumbaginifolia*). This gene contains an upstream sequence element (USE) and a −30 TATA box which are essential for transcription by Pol III (Waibel and Filipowicz, 1990a,b). Pol III and RNA polymerase I (Pol I) activities in the BY-2 extract were estimated to be ~10% and 4%, respectively, of total RNA polymerase activities. We demonstrated here that the *Arabidopsis* U6 snRNA gene is transcribed accurately *in vitro* by Pol III as that determined *in vivo* (Waibel and Filipowicz, 1990b). As the promoter construct used contains only the first 28 bp coding sequence, the synthesis of *Arabidopsis* U6 snRNA does not depend on the A box or other intragenic sequences, confirming the *in vivo* results (Waibel and Filipowicz 1990b; Connelly and Filipowicz, 1993). To our knowledge, this is the first report on a Pol III-dependent *in vitro* transcription system for plants. Our *in vitro* system provides a powerful tool for detailed biochemical analysis of Pol-III dependent transcription.

In addition to Pol II and Pol III activities, Pol I-specific transcription was tested with the rRNA gene promoter from *Vicia faba*, which is accurately transcribed *in vitro* with embryo extracts from the same species (Yamashita *et al.*, 1993). So far no specific transcripts were detected in our tobacco extract under the current conditions (data not shown). Pol I-specific rRNA transcription is known to be species specific (Grummt *et al.*, 1982), and this is a possible reason that our tobacco extract could not transcribe the rRNA gene of *Vicia faba*.

Materials and methods

Plant materials

Tobacco BY-2 cells were grown in modified MS medium as described (Nagata *et al.*, 1981) except that KH_2PO_4 and inositol were added to 540 mg/l and 100 mg/l, respectively. The culture was transferred to fresh medium (3:100) weekly and shaken at 130 r.p.m. in the dark at 27°C. Cells were harvested at middle log phase (86–96 h after inoculation) by centrifugation at 200 *g* for 5 min. Sterilized tomato seeds (*Lycopersicon esculentum*) were germinated on fibre glass soaked with 1:1000 diluted Hyponex in a growth chamber under a 16 h light/8 h dark cycle at 28°C. Green cotyledons (along with the one-quarter of the hypocotyl) and roots were collected respectively from 7-day-old seedlings and frozen in liquid nitrogen.

Preparation of nuclei and nuclear extracts

Protoplasts were prepared by the method of Nemoto *et al.* (1988). BY-2 cells (ca. 200 g) were washed twice with 0.4 M mannitol solution (pH 5.4) and digested in 1 l of enzyme solution (1% Onozuka-RS cellulase and 0.1% pectolyase Y-23 in MS medium containing 0.4 M mannitol, pH 5.4) at 30°C for 45 min. Protoplasts were harvested by centrifugation at 200 *g* for 5 min and washed three times with ice-cold 0.4 M mannitol solution. The pellet was suspended in 1 vol of ice-cold nuclear isolation buffer [30 mM HEPES−KOH, pH 7.9, 36% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, 20 mM NaF, 5 mM DTT, 2 mM EGTA, 1 mM EDTA, 0.3 mM spermine, 1 mM spermidine, 1 mM PMSF, 1 mM benzamide, 3 μg/ml pepstatin A and 2 μg/ml leupeptin] and 1/20 volume of 20% freshly prepared skim-milk solution. The mixture was vacuum-filtered through two layers of nylon mesh (20 μm pore size) and the filtrate centrifuged at 2500 *g* for 10 min at 2°C. The precipitated nuclei were gently resuspended in 80 ml of 0.5 × nuclear isolation buffer minus skim-milk with a paintbrush and centrifuged at 2500 *g* for 5 min at 2°C. This step was repeated twice more. The preparation of nuclei from tomato tissues was essentially as described by Manzara *et al.* (1991) except that the isolation buffer consists of 20 mM HEPES−KOH pH 7.0, 18% (w/v) sucrose, 1% (w/v) polyvinylpyrrolidone, 10 mM NaF, 5 mM DTT, 0.1% (v/v) Triton X-100, 0.5 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.5 spermidine, 3 μg/ml pepstatin A, 2 μg/ml leupeptin, 1 mM PMSF and 1 mM benzamide.

Nuclei were immediately suspended in 2 vol of nuclear extraction buffer (25 mM HEPES−KOH, pH 7.9, 25% glycerol, 4 mM $MgSO_4$, 0.2 mM EGTA, 10 mM NaF, 5 mM DTT, 3 μg/ml pepstatin A, 2 μg/ml leupeptin and 1 mM PMSF). Lysis was carried out by adding 4 M ammonium sulfate at a final concentration of 0.42 M and shaking gently at 4°C for 30 min. The lysate was centrifuged at 200 000 *g* for 1 h at 2°C and the supernatant was immediately dialyzed twice on ice for 2–3 h against 500 ml buffer containing 20 mM HEPES−KOH, pH 7.9, 20% glycerol, 100 mM KOAc, 0.2 mM EGTA, 0.1 mM EDTA, 2 mM DTT and 0.5 mM PMSF. The dialyzed extract was then centrifuged at 5000 *g* in a microcentrifuge at 2°C for 5 min. The supernatant was aliquot-frozen in liquid nitrogen and stored at −80°C for up to 3 months. Typically, 3–3.5 ml of nuclear extracts containing 6–8 mg/ml protein as determined by Bradford Dye binding assay were obtained from 200 g of BY-2 cells. Only 0.4 ml of a nuclear extract containing 5 mg/ml protein was obtained from 200 g of tomato tissue after concentration of the dialyzate with Centricut-10.

Construction of DNA templates

Unless otherwise indicated, all techniques used for manipulating DNA and RNA were as described by Sambrook *et al.* (1989). The tobacco β-1,3-glucanase gene (pX2) was a gift from H.Shinshi, 35S promoter (pCaMCN) was purchased from Pharmacia Co., *ADML* promoter from Stratagene Co., *SV40* promoter from Promega Co., the tomato *rbcS3C* gene (a 3.4 kb *Hin*dIII fragment) was a gift from M.Sugita and W.Gruissem, the *Arabidopsis* U6 snRNA gene (U6-26) was a gift from W.Filipowicz, and the *Vicia faba* rRNA gene (a 133 bp *Dar*I−*Eco*O109I fragment) was a gift from A.Kato. All DNA templates, except *ADML*, were subcloned into Bluescript KS^+ at suitable restriction enzyme sites and verified by sequence analysis (Table I). Plasmids were amplified in *Escherichia coli* XL1-Blue, isolated by alkaline lysis method and purified further by CsCl gradient centrifugation. Closed circle plasmid DNAs were used as templates.

In vitro transcription and analysis of transcripts

A typical transcription reaction was performed in a 50 μl volume containing 1 pmol of DNA template, 32 mM HEPES−KOH (pH 7.9), 12% glycerol, 80 mM KOAc, 5 mM $MgSO_4$, 2.5 mM DTT , 0.05 mM EDTA, 0.1 mM EGTA, 100 U of pancreatic ribonuclease inhibitor (TaKaRa Co.), 0.5 mM each of four NTPs, 1 mM SAM, 150 μg protein of the nuclear extract. All constituents except NTPs were mixed well and preincubated on ice for 10 min and transcription was initiated by the addition of 2 μl of 12.5 mM each of four NTPs. After incubation 30 min at 28°C, the reaction was stopped by adding 200 μl stop solution (20 mM EDTA, 0.2 M NaCl, 1% SDS, 50 mg/ml glycogen and 1 μl of 20 mg/ml proteinase K) and incubated at 37°C for 10 min. Then 200 μl of 0.5 M NaOAc was added and the solution was extracted twice with 500 μl of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) and once with chloroform:isoamyl alcohol (24:1). The resulting aqueous phase was recovered.

In vitro transcripts were assayed by the primer extension essentially as described (Kadonaga, 1990). Transcripts in the above aqueous phase were precipitated twice with ethanol after addition of 0.1 pmol of a 5′-^{32}P-labelled primer. The primer for pADML was 5′-CTGACAATCTTA-GCGCAGAAGTCATGCCC-3′, complementary to the coding sequence (positions + 64 to +93), and that for the other templates was T7DKS (5′-GGGCGAATTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGA-3′), complementary to the vector sequence (positions 644–683). The pellet was washed with 75% ethanol, dried at room temperature and dissolved in 10 μl of reverse transcriptase buffer (50 mM Tris−HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT). To anneal a primer, the solution was subsequently heated at 75°C for 2 min, then incubated at 60°C for 30 min and cooled to room temperature. Then 10 μl of solution containing 1 mM each of four dNTPs, 100 μg actinomycin D, 200 U of M-MLV reverse transcriptase (USB), 20 U of RNase inhibitor in reverse transcriptase buffer was added and incubated at 37°C for 1 h. The reaction was terminated by the addition of an equal volume of loading buffer (50 mM EDTA, 1% BPB, 1% xylene cyanol, 95% formamide). Samples were denatured at 95°C for 3 min and resolved on a denatured 8% polyacrylamide gel. After electrophoresis, ^{32}P-labelled products were assayed using a Bio-imaging Analyzer BAS-2000 (Fuji Photo Film, Japan). The size of expected extended products was calculated from the 5′-end of primer to the initiation site determined *in vivo*.

For tomato *rbcS3C* transcription, reaction mixtures (100 μl) were preincubated with the tomato nuclear extract on ice for 10 min, followed by the addition of the BY-2 nuclear extract and incubation for further 10 min on ice before the addition of NTPs to start the reaction. All DNA sequence ladders were generated by dsDNA PCR cycle sequencing kit (Gibco BRL) with ^{32}P-labelled T7DKS primer.

Northern blot analysis

The ssDNA probe for *GNPS* was a PCR fragment generated from the 3′ specific region (positions 3911–4432) and labelled by a PCR ssDNA labelling method (unpublished). The probe for *rbcS* was prepared from the 3′ region, a 271 bp *Hin*cII fragment of tomato *rbcS3C* (positions 761–1032) and labelled at its 5′ end using [γ^{32}P]ATP and T4 polynucleotide kinase. The antisense U6 snRNA probe was generated by transcription with SP6 RNA polymerase from plasmid B15 (gift from W.Filipowicz).

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