

Proliferation-dependent pattern of expression of a dihydrofolate reductase-thymidylate synthase gene from *Daucus carota*

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The pattern of expression of a carrot *dhfr-ts* gene was evaluated in different plant organs, in somatic embryos, and in hypocotyl explants induced to dedifferentiate *in vitro* by the addition of the synthetic auxin 2,4 dichlorophenoxyacetic acid. The promoter of this gene was also placed upstream of a *uidA* (GUS) reporter gene and, using biolistic and protoplasts transient expression assays, was shown to drive a particularly high level of expression in actively growing suspension cells. The results from these expression analyses combined with the presence of putative cell cycle-related *cis*-acting elements in the *dhfr-ts* promoter, strongly point to a cell division-dependent expression of this gene.

Key words: *Daucus carota*, dihydrofolate reductase-thymidylate synthase, embryogenesis, gene expression, *in situ* hybridization, transient expression, promoter.

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Like protozoa, higher plants contain a bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) with the DHFR domain at the amino-terminus and the TS domain at the carboxyl-terminus (Cella and Parisi 1993). DHFR and TS are key enzymes in the pathway leading to the synthesis of DNA precursors. DHFR (EC 1.5.1.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate, the precursor of folic cofactors. TS (EC 2.1.1.45) catalyzes the synthesis of deoxythymidine-monophosphate from deoxyuridine-monophosphate, using 5,10-methylenetetrahydrofolate as both a carrier of a carbon unit and a reducing agent which is then reconverted to dihydrofolate. Thus, TS is dependent on DHFR for the regeneration of 5,10-methylenetetrahydrofolate and the presence of both enzymatic activities on a single molecule, a phenomenon known as metabolic channeling, clearly underlines the importance of a concerted regulation of these two enzymes. However, in organisms other than plants and protozoa, DHFR and TS occur as distinct mono-functional polypeptides and according to one report, plants may also contain mono-functional enzymes (Toth *et al.*, 1987).

Genomic and cDNA sequences coding for the bifunctional DHFR-TS have been cloned from *Arabidopsis thaliana*, *Daucus carota*, *Glycine max* and *Zea mays*. (Lazar *et al.*, 1993; Luo *et al.*, 1993; Wang *et al.*, 1995; Cox *et al.*, 1999). Mapping the 5' end of the carrot *dhfr-ts* gene by primer extension and by rapid amplification of 5' cDNA ends (RACE) has revealed the production of two major classes of transcripts derived from alternative promoters (Figure 1) (Luo *et al.*, 1997). Moreover, sequencing of the 5' flanking genomic region has confirmed the presence of two well

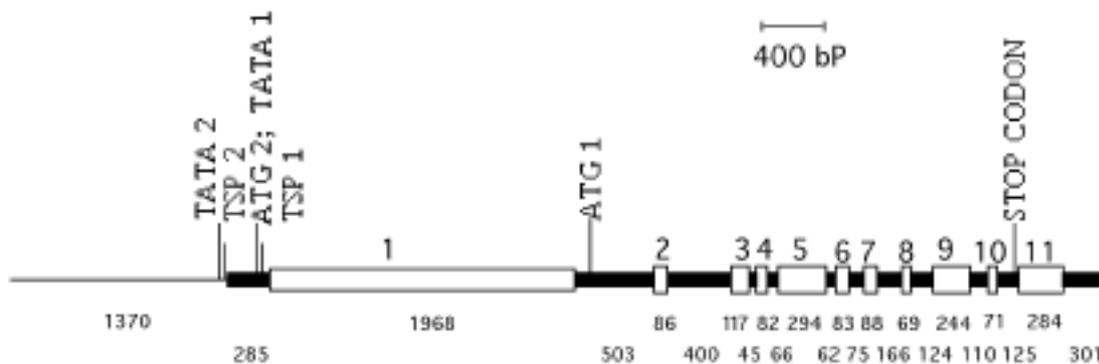


Figure 1. Structure of the carrot gene coding for dihydrofolate reductase-thymidylate synthase. Black and open boxes indicate exons and introns, respectively. Numbers indicate the length in bp. TSP and ATG indicate the position of transcription and translation start points, respectively. TATA shows the position of TATA boxes. The bar at the 5' end of the gene indicates position and length of the probe specific for the longer transcripts (LT) used in Northern experiments.

defined TATA box sequences located 25 to 27 bp upstream of the most proximal transcription start points (Luo and Cella 1998).

The use of alternative transcription start points appears to be linked to the production of two enzyme isoforms with different sub-cellular localization. In fact, the longer *dhfr-ts* transcripts contain an upstream ATG start codon that is in frame with the putative translational start point found in the shorter transcripts and, accordingly, give rise to a polypeptide with an amino-terminal extension of 58 residues showing the features of a transit peptide. Supporting this evidence, immunogold labelling and biochemical studies have indeed shown an organellar localization of DHFR-TS that appears to be plastidial in carrot suspension cells, but mitochondrial in pea leaves and potato tubers (Luo *et al.*, 1997; Neuburger *et al.*, 1996). The mitochondrial localization of this enzyme is consistent with the evidence that these organelles contain the major folate pool (over 50%) of pea leaf cells and appear to be the major site for thymidylate synthesis (Neuburger *et al.*, 1996).

In this study we have analyzed the pattern of expression of the carrot *dhfr-ts* gene in different plant organs, in somatic embryos and in dedifferentiating hypocotyl explants induced *in vitro* by the addition of 2,4 dichlorophenoxyacetic acid. Moreover, using transient expression assays, we have shown that the upstream promoter of this gene is able to drive the expression of the *uidA* (GUS) reporter gene in actively growing carrot suspension cells.

Materials and Methods

Plant material

Daucus carota (cv Lunga di Amsterdam) cell suspensions were maintained as described (Luo *et al.*, 1993). Embryogenic suspension cells of *Daucus carota* were maintained in Gamborg's B5 liquid medium (Sigma) supplemented with 0.5 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D). Somatic embryogenesis was induced by diluting size-selected pro-embryogenic masses (PEM) to a final concentration of 2-3 x 10³ PEM mL⁻¹ in hormone free Gamborg's B5 liquid medium as described (Vergara *et al.*, 1990).

The *Arabidopsis thaliana* T87 cell line (Axelos *et al.*, 1992) was maintained at 23°C under continuous dim light in B5 Gamborg's medium (Sigma) pH 5.8 supplemented with 30 g l⁻¹ sucrose and 1 mM naphthaleneacetic acid (NAA), and was sub-cultured weekly by transferring a 5 mL aliquot into 100 ml of fresh medium. To isolate protoplasts, 3-day old suspension cells were sub-cultured by transferring 30 mL into 100 mL of fresh medium and collected after two additional days. Cells were then washed in protoplast isolation buffer (27.2 mg/L KH₂PO₄, 101 mg/L KNO₃, 1.4 g/L CaCl₂, 246 mg/L MgSO₄, 0.16 mg/L KI, 0.025 mg/L CuSO₄, 10 mM MES and 0,7 M sorbitol, pH 5.5) and resuspended in approximately 5 volumes of this buffer containing the enzyme mixture 1% cellulase Onozuka R-10 (Yakult) and 0.5% Pectinase (Serva) as described previously (Albani *et al.*, 2000).

DNA extraction and PCR amplification

Carrot genomic DNA was extracted as described by Dellaporta *et al.*, (1983) and purified by cesium chloride gradient centrifugation (Sambrook *et al.*, 1989). To amplify the *dhfr-ts* promoter region, a PCR reaction was performed with the upstream primer of sequence 5'-CTCATGCACATGTTTTATGAG-3', that anneals at the 5' end of the known 5' flanking sequence (Luo *et al.*, 1997), along with the downstream primer of sequence 5'-TAGAAGAGGAAATGTGGATGA-3', that overlaps the transcription start point of the downstream promoter (TSP 1). PCR was performed in a volume of 50 μ L containing 1x PCR buffer (Amersham Biosciences Inc.), 0.2 mM dNTPs, 20 picomoles of each primer, and 180 ng of carrot genomic DNA. Following 5 min at 94°C, 2.5 units of Taq polymerase (Amersham Biosciences Inc.) were added and 35 cycles of 1 min at 94°C, 1 min at 58°C, 2 min at 72°C were performed and followed by a final extension of 7 min at 72°C. The resulting PCR fragment was digested at internal *EcoRV* and *SpeI* sites located respectively 1278 bp upstream and 147 bp downstream of the transcription start point of the upstream promoter (TSP 2) and inserted into the pBluescriptII KS⁺ plasmid (Stratagene) to yield the plasmid named pDHP1.4. This promoter fragment was sequenced to verify the fidelity of the PCR reaction and then inserted as a *HindIII/XbaI* fragment into a promoterless GUS cassette plasmid obtained by cloning into pUC19 the *BamHI/EcoRI* fragment from pBI121 (Jefferson 1987), thus generating the *dhfr-ts/GUS/nos* gene construct pDG1278. The 867 bp long promoter fragment of the pDG715 construct was obtained by digesting the pDHP1.4 plasmid at the *EcoRV* cloning site and at the internal *Csp45I* site (located 716 bp upstream of TSP2), blunt ending the *Csp45I* site using klenow and re-ligating the plasmid DNA, thus excising the distal fragment of the promoter and obtaining the pDHP0.8 plasmid. The shortest promoter fragment of the pDG348 construct was obtained by nested PCR using a primer of sequence 5'-TGAGACTAGTGTATCAAGATG-3' that anneals 334 bp upstream of TSP 2 and contains a *SpeI* site (underlined in the sequence) allowing cloning of the PCR product into the pBluescriptII KS⁺ plasmid (Stratagene) as a *Spe I* fragment to yield the pDHP0.5 plasmid.

RNA isolation and northern blot hybridization

Total RNA was extracted from 5 day old suspension cells and 40 day old carrot plantlets using the Extract-A-Plant RNA isolation kit (Clontech) according to the manufacturer's instructions. Poly(A)⁺ RNA was purified using the polyAtract mRNA isolation system II (Promega) following the manufacturer's instructions. For northern blot analysis, mRNAs were resolved on a 1% agarose/formaldehyde gel, transferred onto a Hybond N⁺ membrane (Amersham Biosciences Inc.) and hybridized overnight at 42°C with [³²P]-labeled probes. The membrane was washed (2 X SSC/0.1% SDS) twice at room temperature for 15 min, once at 65°C for 15 min and then autoradiographed.

In situ hybridization

In situ hybridizations were performed on paraffin-embedded tissue sections essentially as described by Smith *et al.*, (1987). Labeled [³⁵S] sense and antisense riboprobes were produced by transcription with the T7 or SP6 RNA polymerase using the plasmid containing the *pDHFR-TS* cDNA clone (Luo *et al.*, 1993) after it was linearized with *SmaI* or *HindIII*, respectively. Autoradiographs were developed following a 2 week exposure at 4°C.

Transient expression assays

For transient expression experiments with carrot cell cultures, the three pDG constructs were delivered separately into cells using a simple, inexpensive, home-made particle bombardment device (Finer *et al.*, 1992) that allows the pressurized helium acceleration of plasmid DNA-coated gold microprojectile particles (5 μ g DNA mg⁻¹ gold particles, NaCl/EtOH precipitated). For each experiment, 2 mL of a very fine carrot cell suspension grown to early exponential phase in liquid medium, were layered on filter paper, dried and bombarded within the vacuum chamber at a helium pressure setting of 6 bar. Five bombardments were applied for each construct. After bombardment, the filter papers with the cells were placed over solid medium containing 0.2 M mannitol and 0.2 M sorbitol, and incubated 24 h in the dark. The efficiency of transient expression of the different constructs was assessed by counting the blue spots on the filters following histochemical GUS detection (Jefferson 1987).

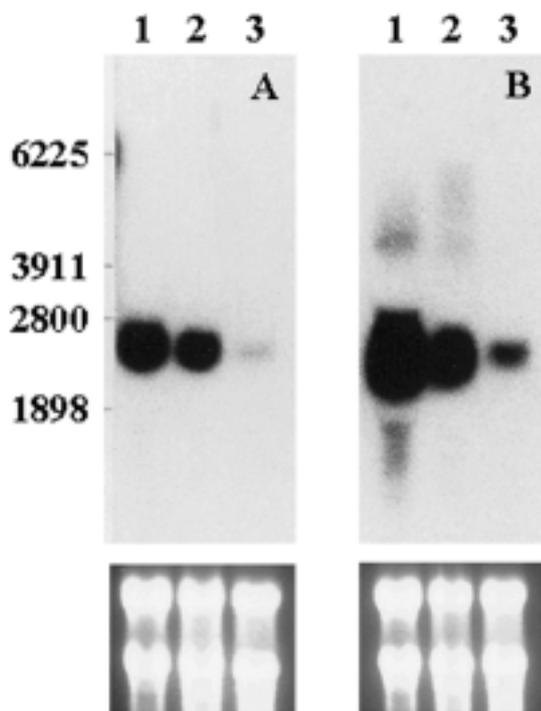


Figure 2. Northern blot analysis of poly (A)⁺ RNA extracted from 5 day old suspension cells (lane 1), as well as roots (lane 2) and leaves (lane 3) from 40 day old carrot plants. The probe used in A was specific for the longer transcripts (LT, 110 nt to 365 nt upstream of ATG 1), while that used in B was the full length cDNA (Luo *et al.*, 1993) that spanned the entire coding region. The amount of RNA loaded was checked by staining the gels with ethidium bromide as shown in the lower panels.

Quantitative transient expression assay experiments, normalized against chloramphenicol acetyltransferase (CAT) reporter activity, were performed using protoplasts isolated from actively dividing *Arabidopsis* cell suspension cultures, following the procedure described by Mariconti *et al.*, (2002). GUS activity was measured as described by Gallie *et al.*, (1989) with minor modifications. Protoplasts were collected by centrifugation, resuspended in 0.5 mL of buffer (50 mM sodium phosphate, pH 7.0, 10 mM β -mercaptoethanol, 1 mM EDTA) and then sonicated. After addition of 1 mM 4-methylumbelliferyl- β -D-glucuronide (MUG) (Sigma), aliquots were incubated at 37°C for 15 to 180 min. The reaction was terminated by adding 0.2 M sodium carbonate. Fluorescence was measured by excitation at 365 nm and emission at 455 nm using a Hoefer DyNA Quant 200 fluorometer (Amersham Pharmacia Biotech). GUS specific activity was expressed as picomoles of MU produced per minute per milligram of protein. CAT assays were performed using the CAT detection kit

(Roche Molecular Biochemicals) as described by the manufacturer.

Results

Expression of the carrot *dhfr-ts* gene

As shown in Figure 2, Northern analyses performed with poly (A)⁺ RNA indicate that the *dhfr-ts* gene is expressed highly in actively dividing suspension cells (lane 1), slightly less in roots (lane 2) and very little in leaves (lane 3). Essentially similar results were obtained with either a probe specific for the longer class of transcripts (Figure 2A) or for the entire coding region (Figure 2B).

In order to investigate in more detail the pattern of expression of this gene, *in situ* hybridization analyses were performed on sections of carrot somatic cells and embryos at various stages of development. As shown in Figure 3, pro-embryogenic masses show a very high level of accumulation of *dhfr-ts* mRNAs (Figure 3 A, E); similarly, the globular and heart stage somatic embryos also exhibit a strong hybridization signal that appears to be distributed throughout the embryo (Figure 3 B, F). No signal above background levels was detected after hybridization with the sense probe (data not shown). Starting from the torpedo stage, *dhfr-ts* mRNAs show an uneven pattern of distribution (Figure 3 C, G), and at later stages of development accumulate preferentially in shoot meristems and in cotyledons (Figure 3 D, H).

To further confirm these observations, *dhfr-ts* gene expression was followed during carrot hypocotyl de-differentiation in the presence of 2,4-D, a process that is characterized by resumption of cell proliferation. Figure 4A and 4C show bright- and dark-field photographs of a longitudinal section of a hypocotyl after 15 days of culture in liquid medium in the presence of auxin. In this section, the ring (or cylinder) of inter- and intra-vascular cambium generated by the proliferation of the parenchymatic cells very close to the xylem strands is well evident. This cambium activity no further develops provascular tissue. Instead it determines the continuous formation of dividing cells, which, after a gradual expansion process, are released in the medium generating new embryogenic cells (de Vries *et al.*, 1988; Nuti-Ronchi and Giorgetti 1995). The characteristics of this procambium make it comparable to a terminal meristem since the cells generating from it are capable of initiating

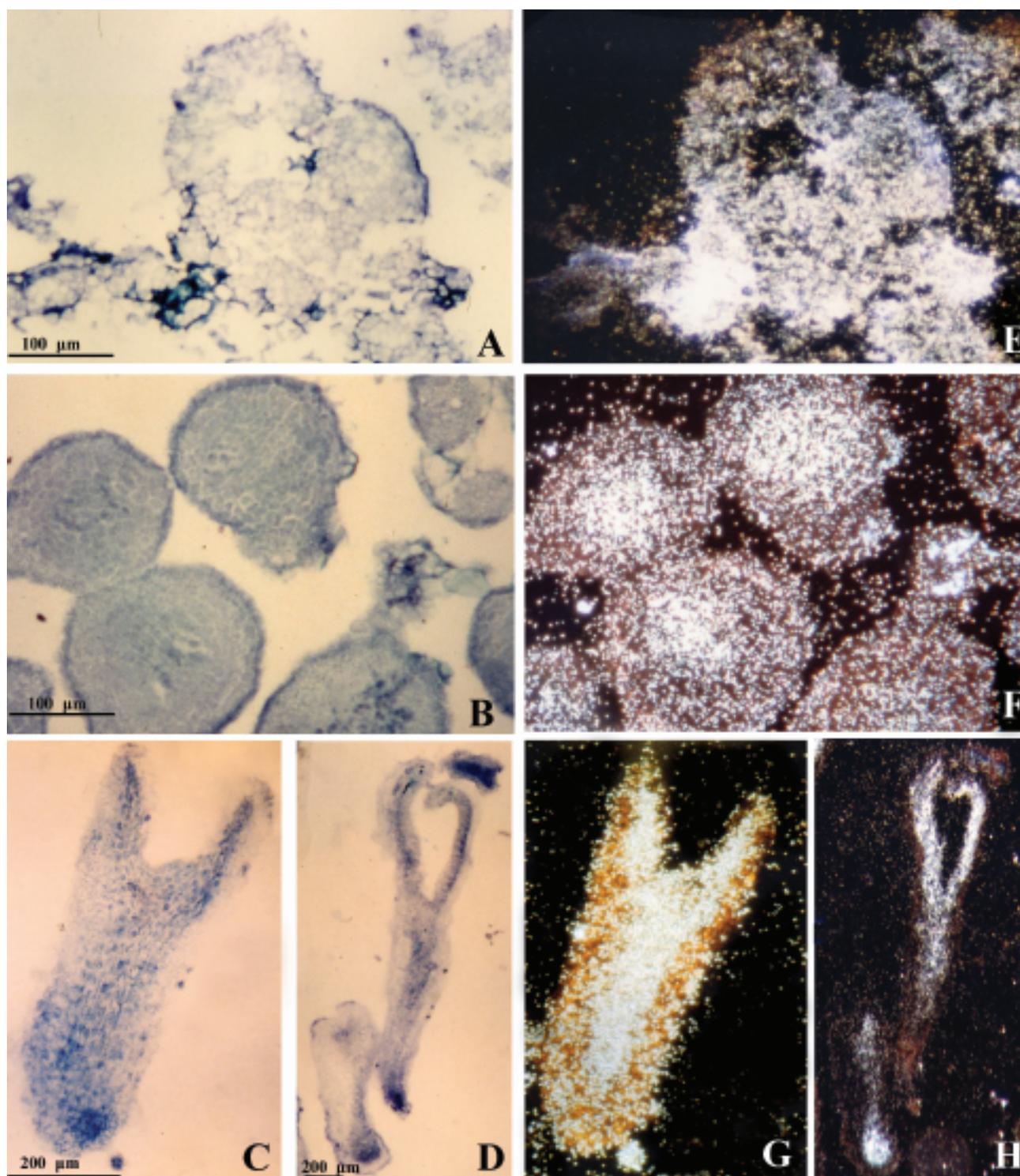


Figure 3. *In situ* localization of *dhfr-ts* mRNA in carrot cells and somatic embryos. Bright- (A-D) and dark- (E-H) field photographs of cross-sections from induced carrot cell cultures (pro-embryogenic masses) (A, E), globular- and heart-stage embryos (B, F), early torpedo (C, G) and mature torpedo (D, H) stages.

an entire plant. In this tissue the *dhfr-ts* mRNAs, which are almost absent from 0 to 7 days of culture, accumulate dramatically 15 days after the

induction of de-differentiation, as shown in Figures 4C and 4D. Gene expression is almost absent in the cortical cylinder and in the vascular strands.

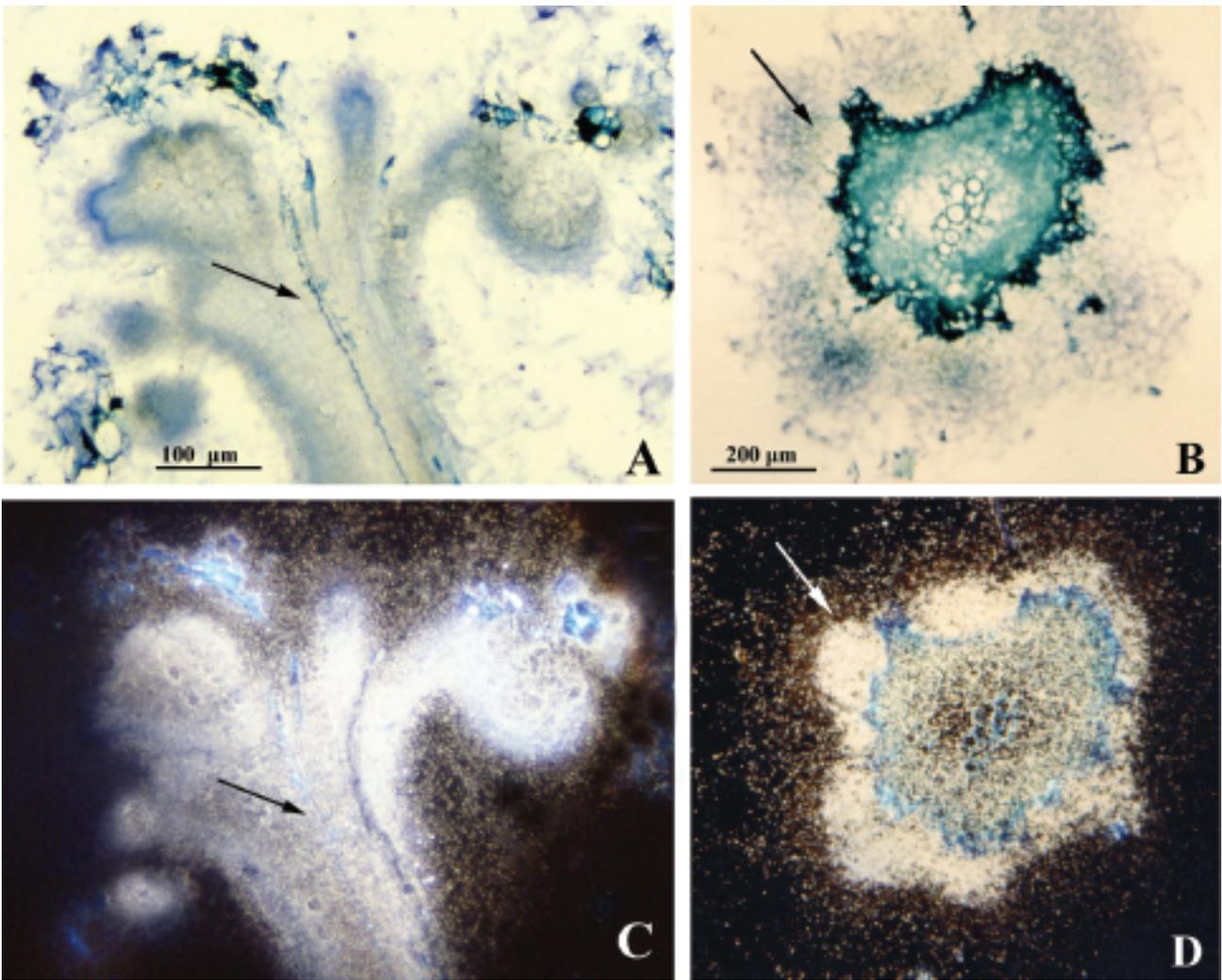


Figure 4. *In situ* localization of *dhfr-ts* mRNA in carrot hypocotyl explants cultured in the presence of 2,4-D. Bright-field photographs of longitudinal (A) and transversal (B) sections from hypocotyls after 15 days in liquid medium. (C) and (D) show photographs of the same sections under dark-field microscopy. Arrows indicate cambium activity.

Functional analysis of the carrot *dhfr-ts* promoter

To isolate the *dhfr-ts* promoter as a single DNA fragment, a PCR reaction with carrot genomic DNA was performed as described in Materials and Methods. The resulting DNA fragment, which included also the downstream promoter and the transcription start point for the short transcripts (TSP 1), was digested at the *EcoRV* and *SpeI* sites, located 1278 bp upstream and 147 bp downstream of TSP 2 respectively, and cloned upstream of the marker gene GUS to generate the plasmid pDG1278. The digestion at the *SpeI* site removed the translational start codon ATG 2 as well as the core of the downstream promoter. Two shorter GUS fusion constructs containing 715 bp (pDG715) or

348 bp (pDG348) of the region upstream of TSP were generated by removing distal portions of the *dhfr-ts* promoter. In preliminary experiments to verify the functionality of these *dhfr-ts* promoter fragments, transient expression assays were performed on carrot suspension cells transformed with the reporter plasmids using the biolistic method. As a negative control, transformations were also performed with a promoterless GUS construct. After transformation, the cells were subjected to histochemical analysis to detect GUS activity and, as expected, transformation with the promoterless GUS construct did not show any blue spots, whereas the three pDG constructs gave rise to several GUS foci per plate, thus confirming the functional-

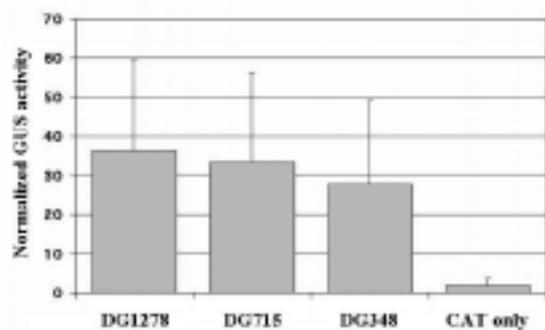


Figure 5. Activity of the carrot *dhfr-ts* promoter constructs in transiently transformed protoplasts of *Arabidopsis thaliana*. Co-transfection of a CAT reporter construct was included in all the experiments to normalize for transfection efficiency. The average normalized GUS activity obtained in six independent transfections are shown. Bars represent the standard deviations.

ity of the different *dhfr-ts* promoter fragments (data not shown). More precisely, out of five independent transient experiments with pDG1278, pDG715 and pDG348, a total of 319, 315 and 294 blue spots, respectively, were counted. The number of spots in replicas for each construct did not differ by more than 20%. In similar studies, it was suggested that the strength of a promoter construct is directly proportional to the number of GUS foci detected histochemically (Shiina *et al.*, 1997). According to this assumption, it would appear that the pDG348 construct displays nearly full promoter activity. To verify this possibility and to determine more precisely the relative strength of the three promoter constructs, quantitative GUS assays were conducted in transiently transformed protoplasts of *Arabidopsis*. Moreover, to account for variations in the efficiency of transfection, co-transformation with a CAT reporter construct was performed in each experiment and the fluorimetric measurement of GUS activity was normalized against the CAT activity. The results of these quantitative experiments, averaged from six replicate analyses, are reported in Figure 5 and show that although the three promoter constructs are all active in protoplasts, their relative levels of expression differ slightly. More precisely, the progressive deletion of distal promoter regions correlated with a decrease in the level of activity observed. In fact, the expression of the pDG715 construct was approximately 8% lower than that of pDG1278, whereas the activity of the shorter construct, pDG348, was about 24% lower. Although we cannot rule out the possibility that the *dhfr-ts* promoter constructs

might express differently in *Arabidopsis* protoplasts than in carrot cells, it is likely that the partially reduced expression of the shorter construct observed in *Arabidopsis* was not sufficient to yield relevant differences in the number of GUS foci in the preliminary biolistic experiments conducted in carrot cells.

In view of the relatively high activity observed for the smallest promoter fragment, it is worth noting that the 349 bp sequence upstream of TSP 2 is particularly AT rich and does not possess regions of homology to known putative cis-acting elements that could account for expression in proliferating cells. On the other hand, in silico analysis of the region downstream of TSP 2 reveals the presence of several sites showing similarity to well known cis-acting elements. As shown in Figure 1, it is worth noting that this 5' untranslated region of the longer *dhfr-ts* transcripts could also regulate the downstream *dhfr-ts* promoter distally. Most remarkably, 30 bp downstream of TSP 2, the sequence CTTG-GCGGC is highly similar to the TTT(C/G)(G/C)CG(C/G) consensus binding site for E2F transcription factors that are well known regulators of mammalian *dhfr* genes and of other mammalian cell cycle-regulated genes (Trimarchi and Lees 2002). Immediately downstream of this putative E2F site, the sequence ACTCCGCGTCAGGCGT contains two sites similar to the yeast cis-acting element ACGCGT (with the emphasis on the central CGCG), a *MluI* restriction site that has become known as the *MluI* cell cycle box (MCB) and that is observed in most of the yeast genes coding for proteins required for DNA synthesis (Wolfsberg *et al.*, 1999). Moreover, other similarities with well known plant cis-acting elements are found in this short DNA region. In fact, its 5' end is partially similar to the reverse complement GATCCGCG of the octamer motif, an element involved in the cell cycle-regulated expression of plant histone genes (Ohtsubo *et al.*, 1997), whereas its central sequence GCGTCA is exactly complementary to the known binding site of the TGA1 family of plant bZIP proteins (Miao *et al.*, 1994). The presence of many putative regulatory elements in this region of the *dhfr-ts* gene makes it particularly interesting, but whether some of these cis-elements are actually involved in regulating the activity of the two carrot *dhfr-ts* promoters remains to be verified.

In view of the very low expression of the *dhfr-ts* gene in leaves compared to proliferating tissues, we

analyzed whether DNA methylation could play a role in the silencing of the *dhfr-ts* promoter. To this end, DNA from both carrot leaves and actively proliferating carrot suspension cells was digested with several C⁵-methylation-sensitive restriction enzymes and hybridized to the promoter region. Results of this analysis failed to reveal differences in the restriction patterns obtained, thus suggesting that C5-methylation might not be involved in the regulation of the *dhfr-ts* promoter activity (data not shown).

Discussion

Results of northern and in situ hybridization analyses reported in this paper show that the carrot *dhfr-ts* gene corresponding to a cDNA previously isolated (Luo et al., 1993) is expressed preferentially in highly dividing meristematic tissues. In particular, a strong hybridization signal was evident in pro-embryogenic masses as well as in shoot and root meristems of somatic embryos at the torpedo stage. In addition, somatic embryos at the torpedo/plantlet stage showed a well defined expression in meristems and in expanding cotyledons. This pattern of expression is in full agreement with the role played by the bifunctional DHFR-TS in the biosynthesis of thymidylate: in fact, it is well known that genes coding for enzymes involved in the synthesis of DNA and its precursors are activated at the G1/S transition of the cell-cycle of proliferating or endoreduplicating cells (den Boer and Murray 2000; Stals and Inzé 2001; Rossi and Varotto 2002). Moreover, northern hybridization experiments with RNA extracted from *Arabidopsis* suspension cells, performed under low stringency using the carrot *dhfr-ts* cDNA as a probe, have confirmed a higher accumulation of *dhfr-ts* transcripts in proliferating suspension cells compared to cells in stationary phase or cells blocked with propyzamide (unpublished data).

Results of northern blot analyses have also revealed a very low expression of the *dhfr-ts* gene in carrot leaves. This observation is in contrast with the report of Neuburger et al., (1996) that describes a strong accumulation of DHFR-TS in the mitochondria of pea leaves. However, this discrepancy could derive from the presence of paralog genes that might be differentially expressed during development. Two genes coding for DHFR-TS have been cloned in *Arabidopsis* (Lazar et al., 1993)

and a putative third gene is present in the genome of this species. Thus, it is reasonable to assume that additional *dhfr-ts* genes could also exist in the carrot genome and that the *dhfr-ts* gene analyzed in this study is prevalently expressed in proliferating tissues.

This conclusion is also supported by the results of the carrot biolistic and *Arabidopsis* protoplast transient expression experiments which show that the upstream carrot *dhfr-ts* promoter is able to drive the expression of a reporter gene in actively growing suspension cells. Interestingly, several putative cis-elements, which in other species are involved in cell-cycle regulation of genes activated at or near to the G1/S phase, have been identified in the 5' untranslated region of the longer transcripts of this gene. The presence of a putative consensus binding site for E2F factors, also found in the 5' untranslated region of mammalian *dhfr* genes, appears particularly relevant. E2F cis-elements have been shown to bind efficiently plant E2F proteins (Albani et al., 2000; Ramirez-Parra and Gutierrez 2000; de Jager et al., 2001; Mariconti et al., 2002; Kosugi and Ohashi 2002) and to be functionally important in several cell cycle-regulated plant promoters (Chabouté et al., 2002; Egelkrout et al., 2002; Stevens et al., 2002). In this regard, it is worth noting that the 5' flanking region of one of the three *dhfr-ts* genes of *A. thaliana* contains a putative E2F binding site which, according to chromatin-immunoprecipitation analyses, is likely to be functional (unpublished data). Moreover, the fact that putative consensus E2F sites are not found in the two remaining *dhfr-ts* genes of *Arabidopsis* supports the hypothesis that different patterns of expression of the *dhfr-ts* paralogs could account for the observed differences in accumulation of DHFR-TS in proliferating and differentiated tissues.

Additional functional analyses of the carrot *dhfr-ts* promoters, presently underway in our laboratories, should reveal whether any of the putative cis-elements identified in this report are actually involved in the regulation of gene expression during cell proliferation, a topic of major importance for the study of plant growth and development.

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